MOLECULAR MECHANISMS OF THORACIC AORTIC ANEURYSM IN MARFAN SYNDROME

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Abstract

Background: Marfan Syndrome (MFS) is a monogenic disorder resulting from mutations in fibrillin-1 (*FBN1*), that encompasses a range of multisystemic manifestations, including skeletal, ocular, and cardiovascular conditions, of which the most serious is thoracic aortic aneurysm and dissection (TAAD). Currently, effective treatments and a clear understanding of the underlying pathogenic mechanisms remain uncertain. Current thinking implicates TGFβ signalling dysregulation in the pathogenesis of TAA, however, the evidence is not conclusive, with emerging evidence that multiple signalling pathways contribute to TAA development. This study sought evidence of such pathway involvement and explored potential regulatory mechanisms of these pathways.

Methods: Comprehensive RNA-Seq, proteomics, miRNA, and DNA methylation studies were undertaken, using human aortic tissues from 15 MFS patients and 11 healthy controls. Subsequent pathway analysis and interrogation of related biological functions were performed.

Results: The transcriptomics and proteomics studies identified significant differential expression (DE) of multiple mRNA (n = 987) and proteins (n = 109) within the MFS aortic wall, allowing identification of potential pathogenic biological drivers such as inflammatory and oxidative stress responses. Furthermore, novel potential drivers, including mitochondrial dysfunction and mechanical stress responses, were also identified as likely contributors to the cellular alterations within the aortic wall associated with dysregulation in vascular smooth muscle cells (VSMC), endothelial cells (EC), and the extracellular matrix (ECM) in MFS pathogenesis. Moreover, the study investigated the interplay between these four factors and demonstrated their comprehensive interactions that ultimately lead to TAA development.

A number of 24 signalling pathways were identified in this study, although the relative importance of these pathways is unclear, most of them have been identified in TAA. In addition to altered TGFβ signalling, 6 of the pathways were identified by 3 separate omics techniques, which are HIF-1, JAK-STAT, cGMP-PKG, sphingolipid, Rap1, and PI3K/AKT pathways. Importantly, PI3K/AKT has been identified as a common intermediate pathway that demonstrated comprehensive crosstalk with all significant pathways. These PI3K/AKT-related interactions were shown to contribute to the proposed main pathogenic biological processes involved

in the development of MFS TAAD, highlighting the crucial role of PI3K/AKT signalling in MFS pathogenesis.

Furthermore, multi-omics analysis showed that the regulatory mechanisms controlling the DE of many RNA and proteins are regulated through both transcriptional and translational modulation, thus, suggesting the involvement of epigenetic regulation contributing to the pathogenesis of MFS TAAD. The investigation of DE miRNA and DNA methylation, two forms of epigenetic regulation, reinforced the signalling pathway findings of the driver mechanisms and identified 16 miRNAs and 2,367 DNA methylation CpG sites that were significantly DE between MFS patients and controls. Specifically, DE of two miRNAs, miR-154-5p and miR-424-5p, targeting MINDY2 and KIF5C, respectively, were shown to correspond to the DE protein changes in the aortic wall. Additionally, two DNA methylation CpG sites, cg06099173 and cg20365170, targeting MH7, and four CpG sites, cg02407342, cg24797574, cg16343401, and cg20911989, targeting GFAP, were implicated in the DNA methylation regulatory mechanisms involved in modulating the RNA and protein expression of these two proteins.

Taken together, these data highlight the additional epigenetic regulatory mechanisms involved in the pathogenesis of MFS and align with the findings from RNA and protein analyses, revealing the potential role of epigenetic factors alongside transcriptional and translational mechanisms in regulating MFS TAAD pathogenesis.

Interestingly, consistent abnormal expression of cardiomyocyte contractile genes and proteins throughout the multi-omics study paradoxically highlights the possible presence and involvement of cardiomyocyte-related cells within the ascending aortic wall, and the possible involvement of these cells in MFS pathogenesis. Finally, the detection of apparent haemoglobin infiltration in the aortic wall introduces a novel aspect of potential EC dysregulation.

Conclusion: This project has identified multiple biological processes involved in MFS pathogenesis, including inflammatory and oxidative stress responses, mitochondrial dysfunction, and mechanical stress responses, as well as alterations in signaling pathways, such as TGFβ, HIF-1, JAK-STAT, cGMP-PKG, sphingolipid, and Rap1. Furthermore, the epigenetic study identified two novel miRNAs and six DNA methylation CpG sites that could potentially contribute to regulatory mechanism in the pathogenesis of MFS. These findings provide new insights into

underlying disease regulatory mechanisms and provide potential novel targets for therapeutic interventions in MFS.

Relevant Publications & Awards

Research Article

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International Conference Abstract

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2020	Post Graduate Research Support Scheme (PRSS) Sydney Medical School, University of Sydney
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List of Abbreviations

TAAD	Thoracic aortic aneurysm and dissection
TAA	Thoracic aortic aneurysm
ECM	Extracellular matrix
EC	Endothelial cells
VSMC	Vascular smooth muscle cells
SMC	Smooth muscle cells
MFS	Marfan Syndrome
BAV	Bicuspid aortic valves
ROS	Reactive oxygen species
LTBP	Latent TGFβ binding protein
LTGF	Latent TGFβ
NO	Nitric Oxide
Ang II	Angiotensin II
PI3K/AKT	Phosphoinositide 3-kinase/Akt
mTOR	Mammalian target of rapamycin
ERK1/2	Extracellular signal-regulated kinase ½
RNA-seq	RNA-Sequencing
ARB	Angiotensin receptor blocker
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
MMP	Matrix metalloproteinase
WT	Wild type
TAV	Tricuspid aortic valves
TGFβ	Transforming growth factor-β
BMP	Bone morphogenetic protein
LAP	Latency-associated peptide
LLC	Large latent complex
SLC	Small latent complex
MAPK	Mitogen-activated protein kinase
MEK1	MAPK kinase 1
RAS	Renin-angiotensin system
RAAS	Renin-angiotensin-aldosterone system
ACE	Angiotensin-converting enzyme
AT1R	Angiotensin II receptor type 1
AT2R	Angiotensin II receptor type 2
ACE II	Angiotensin-converting enzyme II
NADPH	Nicotinamide adenine dinucleotide phosphate
FoxO	Forkhead box O
NFAT	Nuclear factor of activated T cells
JNK	JUN-N terminal kinase
TNF	Tumour necrosis factor
PDGF-BB	Platelet-derived growth factor BB
NOS	Nitric oxide synthases
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
CAD	Cardiovascular disease
SmgGDS	Small GTP-binding protein GDP dissociation stimulator
YAP	Yes-associated protein
AAA	Abdominal aortic aneurysms

TNFR1/2	TNF receptor 1 and 2
RNA	Ribonucleic acids
mRNA	Messenger RNA
iPSC	Induced Pluripotent Stem Cells
TIMP	Metallopeptidase inhibitor
miRNA(miR)	MicroRNA
3'UTRs	3' untranslated regions
5'UTRs	5' untranslated regions
Pri-miRNA	Primary miRNA
RISC	RNA-induced silencing complex
DNMT	DNA methyltransferases
CGI	CpG island
TET	Ten eleven translocation
RRAH	Royal Prince Alfred Hospital
BMI	Body mass index
GTEx	Genotype-Tissue Expression
KEGG	Kyoto Encyclopedia of Genes
GO	Gene Ontology
SVM	Support vector machine
RIN	RNA integrity number
AGRF	Australian Genome Research Facility
DE	Differentially expressed
FC	Fold change
FDR	False discovery rate
TPM	Transcript per million
PCA	Principal component analysis
Ca^{2+}	Calcium ion
scRNA-Seq	Single-cell RNA Sequencing
HDL	High-density lipoprotein
PTM	post-translational modification
CT	Threshold cycle
FDA	Food and Drug administration

Statement of Original Authorship

In accordance with the regulations of the University of Sydney, I hereby declare that this thesis represents original research conducted at the University of Sydney. All the results reported herein were the work of the author.

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This work has not been submitted for any other degree at this or any other institution.

Signature: Charlotte Xue Dong

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Chapter 1: Introduction

1.1 BACKGROUND

Thoracic aortic aneurysm and/or dissection (TAAD) can affect people of all ages. Acute rupture or dissection of a thoracic aortic aneurysm (TAA) can lead to fatal outcomes. The estimated prevalence of TAA is 5.3 cases per 100,000, with an incidence of rupture at 1.6 per 100,000 (Gouveia, Silva Duarte et al. 2022). Thus, early identification and treatment of TAA are crucial to prevent adverse outcomes. It is now recognized that TAAD may result from mutations in genes encoding key proteins in the extracellular matrix (ECM), endothelial cells (EC), and vascular smooth muscle cells (VSMC/SMC) (Gillis, Van Laer et al. 2013). These genetic aortopathies include Marfan and Loeys-Dietz syndromes, bicuspid aortic valve (BAV) aortopathy, and non-syndromal familial aneurysms. Some key VSMC contractile genes, such as ACTA2, MYLK, MYH11, and PRKG1, are often associated with non-syndromic TAAD, leading to the loss of VSMC contractility, ECM degradation, loss of mechanical stabilization, and ATP-binding abnormalities (Ostberg, Zafar et al. 2020). An essential question is how mutations in different genes result in aortic aneurysms and dissections (Jeremy, Robertson et al. 2013).

Marfan syndrome (MFS) (OMIM 154700) is commonly regarded as the paradigm of genetic aortopathies and is an autosomal dominant heritable disorder, resulting from mutations in the gene encoding fibrillin-1 (*FBN1*) (OMIM 134797). The estimated birth incidence is approximately 2-3 in 10,000 individuals (Judge and Dietz 2005). The multisystemic manifestations include skeletal, ocular, and cardiovascular features (Dietz 1996). Over 80% of individuals with MFS exhibit aortic dilatation, with TAA formation in the sinuses of Valsalva and the proximal aorta, although in some cases, it may extend more distally (Dietz 1996, Robertson, Dilworth et al. 2015).

This thesis aims to advance our comprehension of human TAAD pathogenesis, focusing on MFS to investigate signal transduction pathways linked to TAAD-related biological processes and cellular functions like inflammation, oxidative stress responses, ECM degradation, and VSMC apoptosis (Quintana and Taylor 2019, Zhang, Qiu et al. 2022). The choice of MFS as the focal point of our investigation is rooted in several considerations. Firstly, MFS is caused by mutations in a single gene, making it a monogenic disorder. This genetic specificity enables us to draw

broader conclusions about TAAD disease mechanisms, because monogenic disease often shares similar underlying molecular mechanisms and modifier genes with the common disease, offering insights into the systematic genome-wide exploration of novel molecular pathways and gene/protein regulation (Peltonen, Perola et al. 2006, Gallati 2014, Seibler and Klein 2019). Moreover, MFS represents one of the most prevalent subsets of TAAD cases, alongside BAV cases. However, BAV is more complex at the genetic level and is associated with multiple genes, which increases the complexity of investigating disease mechanisms (Otto 2002, Bravo-Jaimes and Prakash 2020, Tessler, Albuisson et al. 2021). Lastly, the paucity of effective therapeutic interventions underscores the importance of comprehending the fundamental mechanisms associate with TAAD within the context of MFS. Examining the signal transduction in cellular activity and related biological functions in MFS can be the first step toward understanding disease mechanisms, thus providing insight into possible new treatments and medical interventions.

Overall, the basic pathophysiology in TAAD includes ECM degradation, including elastin fragmentation and collagen deposition, VSMC apoptosis and phenotypic switch from contractile to synthetic phenotypic, and EC dysregulation (Tsamis, Krawiec et al. 2013, Quintana and Taylor 2019). Furthermore, increased inflammation and reactive oxygen species (ROS) have been observed in TAA (El-Hamamsy and Yacoub 2009, Quintana and Taylor 2019). Moreover, even though MFS is a monogenic disease, phenotypic heterogeneity is observed in MFS patients, where the severity varies between individuals, even within families, suggesting additional genetic and epigenetic regulatory mechanisms may both play a role in TAAD pathogenesis (Seo, Kim et al. 2018). However, the mechanism of involved signalling and biological processes and associated risk factors are unclear.

The most extensively investigated signaling pathway in MFS is the TGFβ pathway, which is modulated by *FBN1* expression (Loeys, Schwarze et al. 2006, Benke, Agg et al. 2013). FBN1 anchors to the ECM through latent TGFβ binding proteins (LTBPs) and latent TGFβ (LTGF), maintaining control over TGFβ signaling bioavailability by limiting LTGF secretion in a healthy aorta (Bhushan, Altinbas et al. 2019, Rifkin, Sachan et al. 2022). Although this mechanism is widely recognised by several studies, a recent study suggested that TGFβ is not required in FBN1-associated MFS aortopathy, and SMC TGFβ signalling may show a protective role in MFS pathogenesis (Wei, Hu et al. 2017). This suggests a controversial role of TGFβ signalling in mediating MFS TAAD pathogenesis. Nevertheless,

dysregulation of the TGFβ pathway has been implicated in the pathogenesis of aneurysms in both MFS and Loeys-Dietz syndrome. While elevated TGFβ levels are observed in the plasma of MFS patients, definitive evidence remains largely confined to animal models (Inamoto, Kwartler et al. 2010, Franken, Radonic et al. 2015).

In addition to TGFβ, several studies have suggested that various other cell signaling systems play crucial roles in maintaining aortic tissue architecture and cellular homeostasis, often interacting with the TGFβ pathway (Guo and Wang 2009, Luo 2017). Several pathways, including angiotensin II (Ang II), Wnt, PI3K-Akt (phosphoinositide 3-kinase), mTOR (mammalian target of rapamycin), and ERK1/2 (extracellular signal-regulated kinase 1/2) pathways, may contribute to adverse aortic remodelling (Kostina, Bjork et al. 2018, Bhushan, Altinbas et al. 2019). Evidence of crosstalk and pathway interactions in aortic disease points to complexity in the pathogenesis of TAA in genetic aortopathies (Nagashima, Sakomura et al. 2001, Guo and Wang 2009, Attisano and Wrana 2013, Luo 2017, Yu and Jeremy 2018, Bhushan, Altinbas et al. 2019).

Further work is required to decipher the contributions of different pathways in the mechanism of TAA formation, which facilitates the understanding of MFS and therapeutic development. This research program focuses on identification of key signalling systems involved in MFS aortic disease and examining the regulatory influences on these systems through genetic and epigenetic factors. Utilizing an MFS TAAD human aortic model, we aim to advance our understanding of genetic and epigenetic regulators in TAAD development, ultimately leading to more precise and effective treatment approaches.

1.2 HYPOTHESIS & PURPOSES

This study hypothesizes the underlying pathogenic biological processes of MFS TAAD are regulated by multiple signalling pathways through transcriptional, translational and epigenetic factors. Through this PhD project, the goal is to document changes in transcriptional, translational, and epigenetic regulation in MFS patients. The study will investigate their associations with key cell signalling pathways and biological functions linked to TAAD, aiming to advance our understanding of aneurysm formation mechanisms.

The project aims are:

1) Investigate transcriptional and translational regulation of genes and proteins in the human aorta in MFS.
2) Document changes in epigenetic regulation of gene transcription and translation in MFS using human aortic tissue.
3) Explore the changes in signal transduction pathways and related biological processes through comprehensive omics studies.

1.3 SIGNIFICANCE, SCOPE AND DEFINITIONS

This study is the largest MFS human omics study to date, using aortic tissue, and presents the findings of a broad multi-omics investigation. It unveils novel insights into genetic, epigenetic, and potential disease drivers within regulatory signalling pathways in human TAAD. This research offers potentially valuable new findings in the mechanisms underlying MFS-associated TAAD. By systematically exploring genetic and epigenetic expressions and their pathogenic pathways, this work lays the foundation for future studies aimed at developing innovative diagnostic and therapeutic interventions.

1.4 THESIS OUTLINE

Chapter 2 Literature Review – Firstly, it reviews the current state of knowledge regarding key clinical features of MFS, the genetics of *FBN1* mutations, and potential epigenetic regulators of gene expression and phenotypic severity. Secondly, it examines the evidence for the contribution of abnormal TGFβ signaling in the pathogenesis of TAA in MFS. Thirdly, it reviews the evidence for the involvement of other cell signaling pathways in aortic disease.

Chapter 3 Methods – describes the complementary omics research techniques employed, including RNA-Seq, proteomics, DNA methylation and miRNA expression studies.

Chapter 4 Transcriptomics in MFS – documents RNA expression patterns and the key signalling pathways and related biological functions within the aortic wall, by comparing tissue from normal controls and MFS.

Chapter 5 Proteomics in MFS – document protein expression and the key signalling pathways in the aortic wall and related biological functions, by comparison between normal controls and MFS.

Chapter 6 Multi-omics in MFS – integration of RNA and protein expression patterns to identify the key signal transduction pathways that may be regulated from different regulatory system layers, such as epigenetics.

Chapter 7 Epigenetic regulation in MFS – documents miRNA and DNA methylation expression patterns in MFS pathology and the signalling pathways that may be regulated by miRNA post-transcriptional and DNA methylation pre-transcriptional regulation in the aortic wall, by comparison between normal controls and MFS. Discover the potential key epigenetic regulator miRNAs and DNA methylation CpG sites and the involvement of related signalling pathways.

Chapter 8 – Conclusions.

Chapter 2: Literature Review

2.1 MARFAN SYNDROME

The first description of MFS in 1896 is commonly ascribed to Antoine Bernard-Jean Marfan, however, more recently it has been proposed that Marfan actually described a case of congenital contractural arachnodactyly (Ryuta, Mayuko et al. 2021). MFS is a multisystem disorder, affecting the cardiovascular, ocular and musculoskeletal systems (Dean 2007). Considerable phenotypic variability exists, even between family members harbouring the same underlying gene variant. One of the cardinal features of the cardiovascular pathologies is progressive aortic dilation which is commonly found initially in the sinus of Valsalva. Aorta dilation subsequently leads to TAA and, without appropriate medical treatment, aneurysm can lead to acute aortic dissection and rupture, with a high morbidity and mortality rate (Dean 2007, Milewicz, Braverman et al. 2021). Other associated cardiovascular features include pulmonary artery dilation, redundancy and prolapse of mitral and tricuspid valves, and aortic valve regurgitation (Dietz 2001, Bitterman and Sponseller 2017).

A breakthrough in understanding the pathogenesis of MFS was the discovery of the association with mutations in the gene on chromosome 15 encoding fibrillin-1 (*FBN1*) (Robinson, Arteaga-Solis et al. 2006). Fibrillin-1 is a key component of the extracellular microfibrils and disorganization of these microfibrils has been observed in MFS (Mannucci, Luciano et al. 2020). Subsequently, hundreds of different *FBN1* variants have been associated with MFS (Xiao, Liu et al. 2017). It has been proposed that *FBN1* variants resulting in haploinsufficiency are more deleterious than are those exerting a dominant negative effect (Milewicz, Braverman et al. 2021). Approximately 25% of *FBN1* variants appear to arise *de novo*. No racial, geographical, or gender specificity has been observed for *FBN1* variants (Aguirre and Borgeat 2011, Bollero, Arcuri et al. 2017, Salik and Rawla 2023). The *FBN1* gene is a large gene, with 65 exons, encoding a protein of 350 kDa (Sakai, Keene et al. 2016, Mannucci, Luciano et al. 2020).

2.2 DIAGNOSIS

The clinical diagnosis of MFS currently relies on the revised Ghent nosology (2010), which involves assessing clinical criteria, including major manifestations such as

aortic root enlargement, ectopia lentis, and other previously mentioned clinical features (Bart, Harry et al. 2010). The table below shows the weighting of each major or minor manifestation of MFS diagnostic facilitating the diagnosis process for physicians (Table 2.1) (Bart, Harry et al. 2010).

Table 2.1 Revised Ghent nosology for MFS diagnosis (Bart, Harry et al. 2010).

Points	**Systematic involvement (>7 point)**		**Diagnostic scenarios**	
3	OR	Wrist AND thumb sign	I	Aortic involvement AND Ectopia lentis
1		Wrist OR thumb sign	II	Aortic involvement AND FBN1 mutation
2	OR	Pectus carinatum	III	Aortic involvement AND Systemic involvement
1		Pectus excavatum OR chest asymmetry	IV	Aortic involvement AND Family history
2	OR	Hindfoot deformity	V	Ectopia lentis AND FBN1 mutation
1		Plain pes planus	VI	Ectopia lentis AND family history
2	Pneumothorax		VII	Systemic involvement AND Family history
2	Dural ectasia			
2	Protusio acetabuli			
1	USLS↓ AND ASHR↑ AND no scoliosis			
1	Scoliosis OR thoracolumbar kyphosis			
1	Reduced elbow extension			
1	Facial abnormalities (see above)			
1	Striae atrophicae			
1	Myopia > 3 diopters			
1	Mitral valve prolapse			

Note: Points: Systematic score point. ASHR: arm span to height ratio. USLS: upper segment to lower segment ratio.

2.3 MANAGEMENT

For individuals diagnosed with MFS, regular clinic visits, including examination and imaging with echocardiography or CT/MRI, are recommended for surveillance of cardiovascular features. Regular ophthalmological examination is also recommended (Isselbacher, Preventza et al. 2022). Beta-adrenergic blocker therapy and/or angiotensin receptor blockers (ARBs), which help reduce haemodynamic pressure in the proximal aorta, should be offered once aortic enlargement is observed in patients of any age (Isselbacher, Preventza et al. 2022). For patients with aortic root enlargement exceeding 50 mm or a mean growth rate of the aorta exceeding 1 mm to 1.5 mm over 3 years, or 4 mm to 5 mm over 5 years, prophylactic aortic root surgery should be recommended to prevent adverse outcomes (Keane and Pyeritz 2008, Wang, Deng et al. 2022).

The most recent meta-analysis available recommends combination blood pressure reduction therapy (ARBs + beta-blocker), which demonstrated superior outcomes with a significantly lower aortic dilation rate (Wang, Deng et al. 2022). However, the existing clinical trials have shown considerable discrepancies and contrasting results regarding the effects of ARBs and beta-blockers have emerged, suggesting limited benefits from these therapeutic interventions and emphasizing the urgent need for more efficient treatments (Yu and Jeremy 2018, Deleeuw, De Clercq et al. 2021, Wang, Deng et al. 2022).

Patients with a rapid rate of aorta dilation should undergo more frequent imaging, and if the aortic root or ascending aorta cannot be visualized by echocardiography, CT/MRI should be considered. Those who undergo prophylactic surgery should be carefully monitored for graft infection, pseudoaneurysm, and aneurysm or dissection in the distal aorta and distal to the graft (Isselbacher, Preventza et al. 2022). In the case of children, pregnancy and patients with high-risk factors (family history, rapid dissection and other locations of aortic enlargement), increased visits and close monitoring should be offered to track disease progression (Dietz 2001, Dean 2007, Keane and Pyeritz 2008, Milewicz, Braverman et al. 2021).

2.4 THORACIC AORTIC ANEURYSM IN MARFAN SYNDROME

Thoracic aortic aneurysm is one of the most important manifestations of MFS due to the high risk of fatal aortic dissection. Its morphology is characterized by progressive dilation of the aortic root or ascending aorta, and it is often asymptomatic until dissection or rupture occurs. Microscopically, TAA in MFS is

associated with ECM remodelling and degradation, VSMC apoptosis and dedifferentiation, as well as EC dysregulation associated with abnormal NO (nitric oxide) availability (Asano, Cantalupo et al. 2022).

Biological processes associated with MFS-related TAAD include the inflammatory response and oxidative stress, as well as impaired mechanical properties (Quintana and Taylor 2019, Asano, Cantalupo et al. 2022). It is proposed that mutation of *FBN1* leads to abnormal fibrillin-1 structure, reduced availability and/or altered function. This results in abnormal signalling within the aortic wall. A likely mechanism driving abnormal signalling is abnormal mechanotransducition within the aortic wall, affecting the normal homeostasis within the cells of the aortic wall, including EC, VSMC and fibroblasts (Jeremy, Robertson et al. 2013). The signalling mechanism that has been most extensively investigated is dysregulation of TGFβ signalling (which remains controversial) and loss of ECM integrity, subsequently affecting VSMC regulation and their interaction with the ECM and EC anchoring (Dietz 2001, Wei, Hu et al. 2017, Sun, Asano et al. 2023). The mechanism of TAA formation is likely driven by disrupted cellular signalling (e.g., TGFβ signalling), which is associated with dysregulated biological processes that could alter the structure and function of the aortic wall, however available studies on key regulatory mechanisms in MFS TAAD are limited (Asano, Cantalupo et al. 2022).

Our group has recently reviewed five key signalling pathways (TGFβ, Wnt, PI3K/AKT, Ang II and Notch signalling) that demonstrate altered regulatory function and extensive crosstalk in TAAD, which is associated with an underlying disease mechanism involving VSMC and EC dysregulation (Dong, Malecki et al. 2023). It remains to be determined whether or not these other pathways are also involved in the pathogenesis of TAAD in MFS. Understanding the signal transduction and related biological functions in MFS TAAD is key to understanding the complex disease pathogenesis causing MFS and for developing efficient therapeutic targets.

2.4.1 Healthy aorta structure and function

The aorta consists of three basic anatomical layers: the tunica intima, tunica media and tunica adventitia, with the innermost layer being the tunica intima (Figure 2.1). The tunica intima consists of a thin, monocellular layer of EC, composed of a basement membrane, subendothelium (contains loose connective tissue and fibroblasts) and an internal elastic membrane that separates the tunica intima from the tunica media (Komutrattananont, Mahakkanukrauh et al. 2019). Positioned on

the surface of the tunica intima, EC play a vital role in regulating hemodynamic signalling and ion and solute exchange between the bloodstream and the aortic wall (Robertson, Dilworth et al. 2015). Normal EC promote vasodilation through the production of NO, which includes three synthase isomers: eNOS (endothelial), iNOS (inducible) and nNOS (neuronal). NO extensively interacts with VSMC, controlling VSMC relaxation/ contraction, inhibiting VSMC proliferation and migration, and reducing platelet aggregation and adhesion while regulating the inflammatory response. However, NO availability is influenced by oxidative stress due to increased levels of ROS, which can lead to cellular damage (Asano, Cantalupo et al. 2022).

The tunica media, situated between the tunica intima and adventitia, is the anatomically thickest layer of the aortic wall. It comprises elastin laminae, type I and III collagen, FBN1 microfibrils, and proteoglycans, which together form the ECM surrounding VSMC (Komutrattananont, Mahakkanukrauh et al. 2019, Mozafari, Zhou et al. 2019). Healthy VSMC in this layer typically exhibit a quiescent, contractile phenotype with minimal synthetic activity or proliferation (Cao, Xuan et al. 2022). The tunica media plays a crucial role in regulating responses to pulsatile hemodynamic loads and the interaction between the ECM and VSMC; perturbations of load sensing increase the risk of TAA formation (Zieman, Melenovsky et al. 2005, Mozafari, Zhou et al. 2019, Boczar, Boodhwani et al. 2021, Zeigler, Sloan et al. 2021).

The outermost layer, the tunica adventitia, primarily contains type I and II collagen fibres, elastic fibres, fibroblasts, perivascular nerves and a variable number of mast cells. Small vessels known as vasa vasorum penetrate into the tunica media to supply blood throughout the aortic wall (Majesky, Dong et al. 2011, Komutrattananont, Mahakkanukrauh et al. 2019). This layer bears static loads, facilitates signal and cell exchange between surrounding tissues and the aortic wall, and actively participates in wall growth and repair (Majesky, Dong et al. 2011).

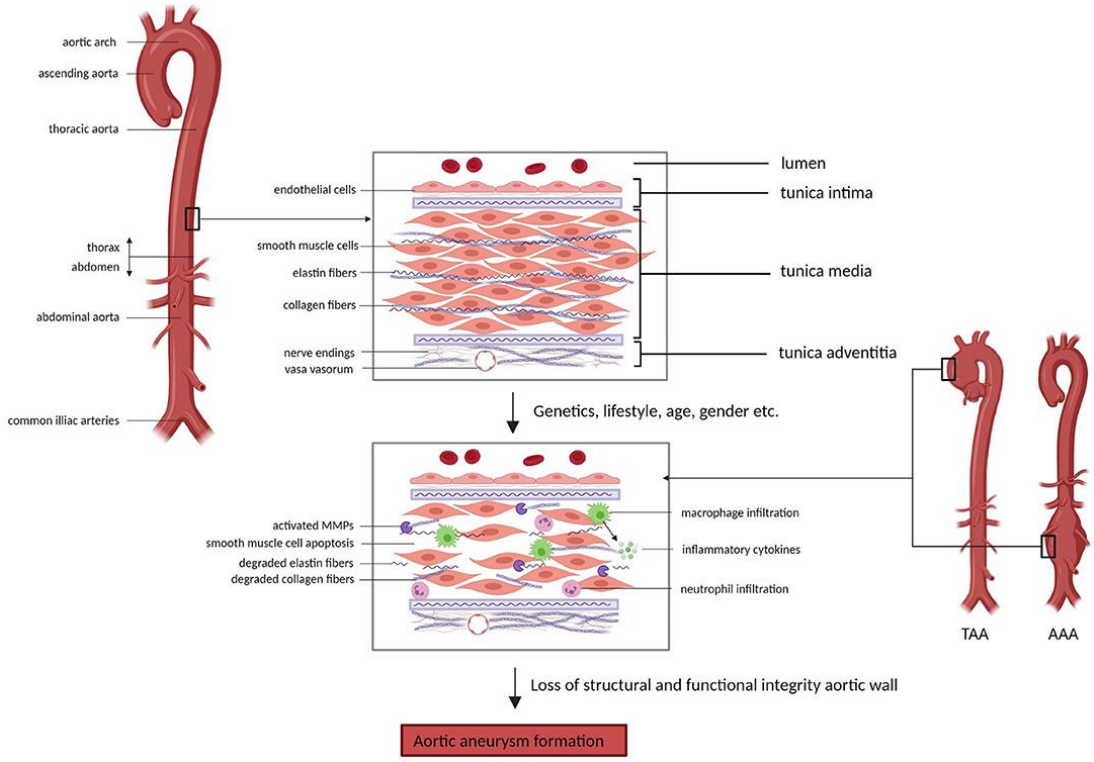


Figure 2.1 Normal and aneurysmal aorta anatomy (Rastogi, Stefens et al. 2022).

2.4.2 TAA aorta structure and function

In MFS, pathological features primarily manifest in the medial layer, a condition termed 'medial degeneration.' This process involves ECM degradation, VSMC apoptosis, and elastin laminae fragmentation (Quintana and Taylor 2019, Malecki, Hambly et al. 2020). The lower half of Figure 2.2 illustrates this pathological process in aneurysmal aorta, marked by the loss of ECM structure through elastin and collagen fibre degradation (Fernandez-Moure 2011, Blunder, Messner et al. 2012, Rastogi, Stefens et al. 2022). Figure 2.2 D and 2.2 E show elastic fibre fragmentation in a MFS murine model, leading to breaks and crosslinking between lamellae, and lamellae dysfunction, resulting in a loss of aortic wall integrity (López-Guimet, Andilla et al. 2017).

When stressed VSMC exhibit phenotypic switching from a quiescent contractile (differentiated) state to a synthetic (dedifferentiated) phenotype. This phenotypic plasticity enables VSMC to repair and remodel the aortic wall in response to injury. In the presence of injury, VSMC adopt a proliferative phenotype characterized by reduced apoptosis, adaptive ECM remodelling, cell proliferation, and migration to repair the aortic wall (Iyemere, Proudfoot et al. 2006, Kundi, Hollenbeck et al. 2009, Dong, Malecki et al. 2023). If vascular distress persists (e.g., during the aortic

enlargement process), VSMC eventually switch fully to a synthetic phenotype, losing their contractile properties. This transition promotes elastin degradation, inflammation, vascular fibrosis, and the expression of proteolytic enzymes, such as matrix metalloproteinases (MMPs), and ultimately leads to arterial stiffening (Rensen 2007, Rzucidlo, Martin et al. 2007, Lu, Du et al. 2021).

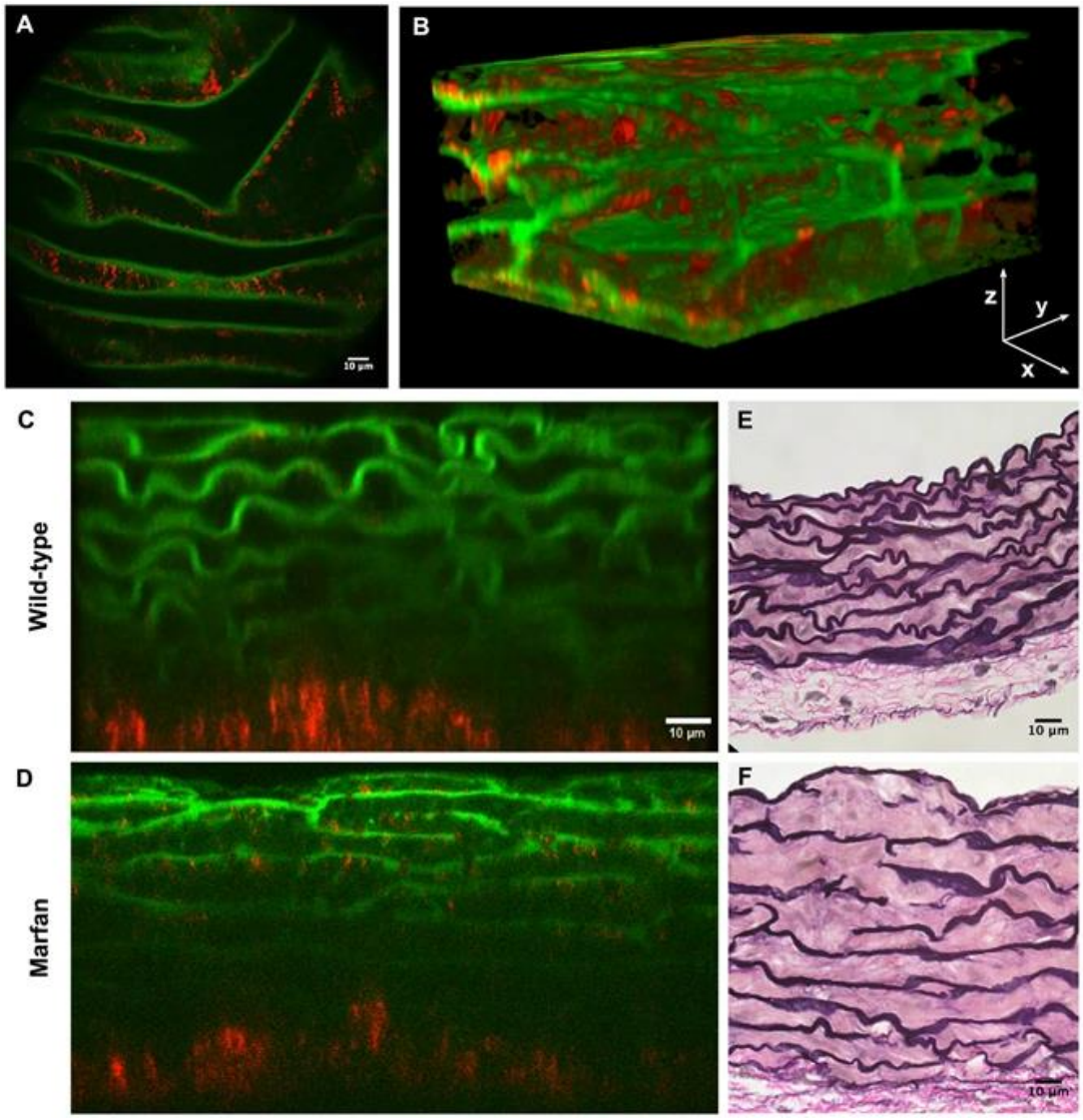


Figure 2.2 3D image of MFS murine model aorta. Elastin in green and collagen in Red. A-B) Wild-type (WT) murine ascending aorta image of tunica media represents healthy aortic structure. C-D) Transverse view under fluorescent illumination of tunica media of WT and MFS murine model showing thinning and fragmentation of elastin fibres (green). E-F) Verhoeff-van Gieson staining of elastic fibres (black) in WT and MFS murine model, also showing thinning and fragmentation. (López-Guimet, Andilla et al. 2017).

In MFS-related TAA, in addition to medial degeneration, dysregulation of EC is observed, and the interaction of dysregulated EC with VSMC plays a crucial role in TAA formation (Malashicheva, Kostina et al. 2016). When encountering vascular injury, quiescent EC are activated, transitioning into a pro-thrombotic and adhesive state. This EC activation subsequently affects VSMC phenotype and exacerbates TAA formation due to dysregulation of NO and pro-inflammatory signalling from EC (Malashicheva, Kostina et al. 2016). The decreased availability of NO impairs the regulatory interaction between EC and VSMC, and may also lead to mitochondrial dysfunction, which has been observed in animal models (Sandoo, van Zanten et al. 2010, Ali, Chapman et al. 2014, Irace, Cammisotto et al. 2021, Asano, Cantalupo et al. 2022). EC dysregulation in TAA is likely driven by altered signalling pathways such as TGFβ and Ang II, as well as the presence of ROS in MFS (Asano, Cantalupo et al. 2022).

2.4.3 Fibrillin-1 mutation

In humans, fibrillin is encoded by three fibrillin isomer genes: *FBN1*, *FBN2* and *FBN3*. Fibrillin is an ECM glycoprotein and a major component of microfibrils, serving as structural macromolecules that play a crucial role in maintaining the integrity of tissues and serves as a template for elastin deposition (Thomson, Singh et al. 2019). Among these isomers, FBN1 is the most abundant in adult tissue. It consists of 2,871 amino acids and 59 domains, including an N-terminal domain, several epidermal growth factor-like repeats (EGF), and multiple calcium-binding, TGFβ-binding-like, and hybrid domains (which harbor both calcium-binding EGF and TGFβ-binding-like domains) (Figure 2.3) (Thomson, Singh et al. 2019, Milewicz, Braverman et al. 2021).

Missense variants are the most commonly observed type of *FBN1* mutation, often affecting EGF repeats. Additionally, frameshift mutations, small insertions, deletions, nonsense mutations, and premature stop codons have been reported. Around 25% MFS patients exhibit *de novo FBN1* mutations. Approximately 10-15% of MFS individuals exhibit *FBN1* haploinsufficiency, and around 7% of MFS cases involve large mutations that result in the complete deletion of the *FBN1* gene (Milewicz, Braverman et al. 2021).

2.4.4 Fibrillin microfibrils

Fibrillin microfibrils are intricate protein structures found in the ECM and connective tissues. They typically exhibit a 'beads-on-a-string' appearance and have a uniform length, measuring approximately 10-12 nm (Kielty, Sherratt et al. 2005). Fibrillin proteins are arranged in a head-to-tail configuration within these microfibrils (Adamo, Zuk et al. 2021). When observed at high magnification by electron microscopy (Figure 2.3), these microfibrils display distinctive light and dark bands, which correspond to the head-to-tail junctions of fibrillin molecules and their association with other proteins (Sakai, Keene et al. 2016).

Fibrillin microfibrils primarily consist of fibrillin proteins, including fibrillin-1 and fibrillin-2 (Adamo, Zuk et al. 2021). These microfibrils are associated with elastic fibres and form concentric lamellae that provide support for elasticity and separate VSMC layers (Ramirez, Sakai et al. 2004). Additionally, microfibrils serve as structural support for the aorta by connecting lamellar rings to each other and to VSMC, as well as linking them to the subendothelial basement membrane (Ramirez, Sakai et al. 2004). A deficiency in fibrillin-1 could result in the disruption of the fibrillin microfibril connection between elastic lamellae and VSMC, subsequently leading to tissue remodelling and elastolysis, followed by an increase in inflammatory signalling, likely due to altered mechanosensensing by the VSMC (Jeremy, Robertson et al. 2013). Ultimately, this process contributes to the formation of TAAD (Ramirez, Sakai et al. 2004).

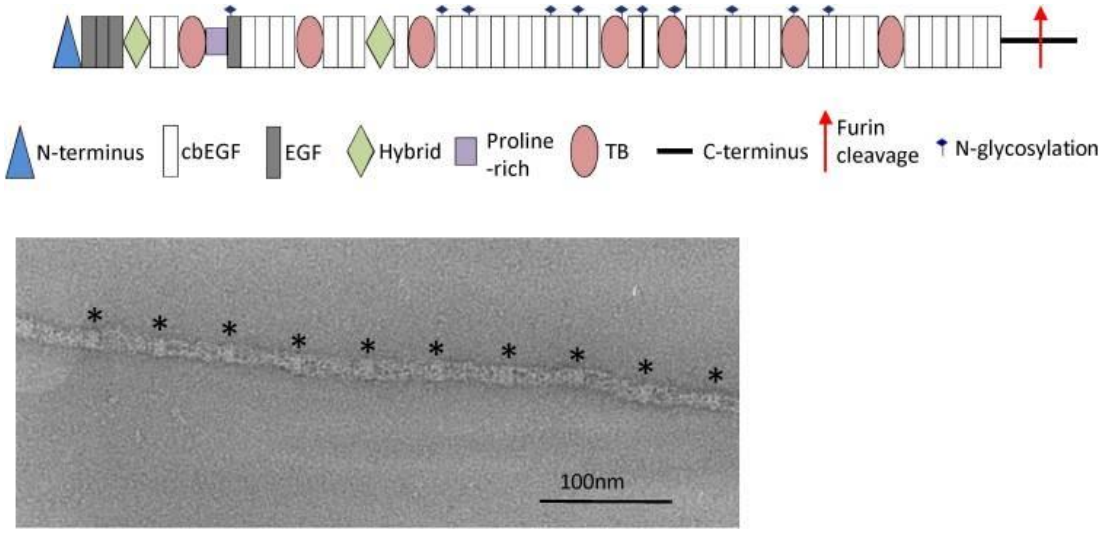


Figure 2.3 Fibrillin-1 structure and binding domains. cbEGF - calcium binding domain – EGF, TB - TGFβ-binding like domain. A single strand of microfibril is imaged by

negative stain TEM and * indicates the light and dark structure of the microfibril (Thomson, Singh et al. 2019).

2.4.5 LTBPs

Microfibrils interact with several binding proteins, primarily growth factors, for example, transforming growth factor-β (TGFβ) and bone morphogenetic proteins (BMPs) through specific binding domains. Key players in this interaction are latent TGFβ binding proteins (LTBPs) 1-4, which share structural similarities with fibrillin and bind to the N-terminus of the FBN1 binding domain. These growth factors play a critical role in TGFβ regulation. LTBPs are components of the ECM and engage in interaction with fibrillin microfibrils, as well as binding with other ECM components like fibronectin, ADAMTSL proteins and fibulins (Robertson, Horiguchi et al. 2015). Moreover, they have been demonstrated to facilitate the connection between fibrillin microfibrils and other ECM components (Robertson, Horiguchi et al. 2015, Milewicz, Braverman et al. 2021).

The LTBP family can covalently bind to the TGFβ latency-associated peptide (LAP), forming a large latent complex (LLC) that tightly regulates constitutively expressed TGFβ bioavailability via sequestration and helps maintain ECM integrity by anchoring it to FBN1 (Thomson, Singh et al. 2019, Rifkin, Sachan et al. 2022). Additionally, mature TGFβ homodimers and LAP can form a small latent complex (SLC) that remains inactive. The SLC can then bind to LTBP. Following release, TGFβ can interact with TGFβ receptors thus activating TGFβ signalling (Zeigler, Sloan et al. 2021). Regulation of TGFβ by LTBPs involves the secretion of LLC and interaction with integrins (ECM receptor), ultimately leading to the activation of the latent form of TGFβ and TGFβ signalling (Robertson, Horiguchi et al. 2015, Rifkin, Sachan et al. 2022). In MFS, mutations in FBN1, whether leading to abnormal protein or leading to deficiency, have been proposed to ultimately result in the failure to sequester inactive TGFβ in the ECM. Although this hypothesis has been disputed, this TGFβ dysregulation may lead to the over-activity of TGFβ and its downstream signaling cascades, contributing to the pathology of MFS (Wei, Hu et al. 2017, Takeda, Hara et al. 2018). Thus, much research over the last two decades has focussed on abnormalities in TGFβ signalling as the primary cause of MFS, although more recent studies have revealed that multiple signalling pathways, exhibiting extensive crosstalk, contribute to the pathogenesis of MFS (Dong, Malecki et al. 2023).

2.4.6 TGFβ signalling

2.4.6.1 Introduction to TGFβ signalling

TGFβ signalling is an important cellular signal transduction pathway that includes multiple ligands and receptor subtypes from the transforming growth factor superfamily. Although attention has been focused on TGFβ dysregulation in MFS pathology, the key role of TGFβ in MFS in controversial, as discussed previously (Wei, Hu et al. 2017). The TGFβ hypothesis proposes that mutations in FBN1 and consequent dysregulation in TGFβ signalling and its related genes can lead to malfunction of VSMC and EC, ultimately resulting in TAA formation in MFS (Takeda, Hara et al. 2018). TGFβ signalling operates through two main cascades: the canonical pathway, which involves SMAD genes and the non-canonical pathway, which signals through downstream PI3K/AKT and mitogen-activated protein kinase (MAPK) pathways (Derynck and Zhang 2003, Shi and Massagué 2003, Dong, Malecki et al. 2023).

TGFβ signalling involves a highly conserved group of ligands and cell receptors that regulate essential cellular processes, including cell growth, differentiation, homeostasis, and apoptosis (Massagué 2012). The two main ligands in the TGFβ family are TGFβ and BMPs, both of which extensively interact with fibrillin microfibrils to maintain tissue homeostasis (Sengle and Sakai 2015, Thomson, Singh et al. 2019).

The receptors for TGFβ signalling are divided into two main types: Type II receptors (for TGFβ and BMP subtypes) and Type I receptors (Activin-like kinases 1-7 subtypes). Each ALK subtype receptor binds to different proteins and activates distinct downstream cascades (Massagué 2012). Notably, the ALK5 receptor transduces TGFβ signals to activate SMAD2/3, while ALK1/2/3/6 transduces BMP signals to activate SMAD1/5/8. TGFβ and BMP co-signalling can activate multiple downstream pathways, including the previously mentioned PI3K/AKT, MAPK family (including ERK1/2), and mTORC, as well as other pathways (Gordon and Blobe 2008, Massagué 2012).

2.4.6.2 TGFβ signalling in VSMC

TGFβ signalling in VSMC is a bidirectional process. In a normal, homeostatically stable environment, TGFβ promotes a contractile VSMC phenotype. However, in the context of injury, it promotes a proliferative phenotype, leading to increased cell

proliferation and migration and anti-apoptosis effects (Owens 1995, Goumans, Liu et al. 2009, Pardali, Goumans et al. 2010, Guo and Chen 2012, Shi and Chen 2014). In VSMC, TGFβ is essential for normal differentiation and promotes the expression of contractile gene markers such as smooth muscle α-actin (SM α-actin), SM-22α and smooth muscle-myosin heavy chain (SM-MHC) (Deaton, Su et al. 2005, Tang, Urs et al. 2010). This signalling pathway involves the ALK5-SMAD2/3 cascade and may function synergistically with parallel Notch signalling to promote the expression of contractile markers and maintain the contractile phenotype of VSMC (Seay, Sedding et al. 2005).

In response to vascular injury, TGFβ signalling through SMAD3 and other downstream pathways initiates repair responses, leading to a VSMC proliferative phenotype. This includes activation of pathways like ERK1/2/MAPK and Wnt, which promote VSMC proliferation, migration and inhibit apoptosis, and facilitates ECM proteolysis and remodelling (Kundi, Hollenbeck et al. 2009, Tsai, Hollenbeck et al. 2009, DiRenzo, Chaudhary et al. 2016).

2.4.6.3 TGFβ signalling in EC

TGFβ also has a bidirectional effect on EC through different Type 1 TGFβ receptors: ALK1 (also known as ACVRL1) and ALK5 (also known as TGFBR1) (Goumans, Valdimarsdottir et al. 2002). As previously introduced in Chapter 2.4.6.1, ALK1 primarily activates SMAD1/5/8 (in EC only), while ALK5 activates SMAD2/3 and partially activates the downstream PI3K/AKT pathway (in both VSMC and EC). These distinct downstream pathways initiate opposite cellular processes, and the balance between ALK1 and ALK5 determines EC behaviour (quiescent or active) (Goumans, Valdimarsdottir et al. 2002, Ten Dijke, Goumans et al. 2002, Goumans 2003, Balsara, Castellino et al. 2006, van de Pol, Kurakula et al. 2017). In simpler terms, ALK1 promotes EC survival, while ALK5 stimulates EC activation, proliferation and migration (Goumans, Valdimarsdottir et al. 2002).

2.4.6.4 TGFβ signalling in MFS TAA

Thoracic aortic aneurysm in MFS is hypothesized to be linked to *FBN1* mutations, which potentially disrupt the homeostasis of TGFβ (Gillis, Van Laer et al. 2013). Hypothetically, these mutations cause a dysregulation in the sequestration and release of latent TGFβ within the ECM, likely resulting in increased TGFβ levels in

MFS individuals. However, contradictory results have been presented (Wei, Hu et al. 2017, Takeda, Hara et al. 2018). Thus, this hypothesis proposes that an elevated TGFβ level leads to an overactivation of the ERK/MAPK pathway and potentially involves PI3K/AKT signaling activity, ultimately contributing to the development of TAA. (Takeda, Hara et al. 2018)..

Interestingly, animal models have shown that inactivating mutations in TGFβ receptors can also lead to TAA formation. This has been shown to reduce TGFβ signaling through the SMAD3 cascade, leading to downstream ERK/MAPK activation and causing a switch in VSMC phenotype (Morello, Perino et al. 2009, Holm, Habashi et al. 2011, Li, Li et al. 2014, Yang, Schmit et al. 2016).

Precise inhibition of TGFβ signalling in a mouse model, in a controlled manner, with attention to timing and dosage, can reduce ERK/MAPK signaling through MAPK kinase-1 (MEK1), thereby attenuating aortic growth and TAA formation (Cook, Clayton et al. 2015, Chen, Rateri et al. 2016). Furthermore, TGFβ over-stimulation activates the downstream PI3K/AKT pathway, leading to a phenotypic switch in VSMC. Inhibition of both the PI3K/AKT and TGFβ pathways has shown greater effectiveness in preventing VSMC from losing their contractile phenotype, potentially contributing to the prevention of aneurysm and dissection formation (Shui-Bo Zhu 2015).

Taken together, these findings suggest that TGFβ signaling is tightly regulated in a healthy aorta, and both overexpression and underexpression of TGFβ can disrupt the balance of ERK/MAPK and PI3K/AKT activation, promoting VSMC proliferation, migration, and EC dysregulation (Wei, Hu et al. 2017, Takeda, Hara et al. 2018). Furthermore, these data suggest that TAA formation may be aggravated by either excessive or insufficient bioavailability of active TGFβ within the aortic wall, suggesting that the “excessive TFGβ signalling due to failure of sequestration by FBN1” hypothesis in MFS pathogenesis may be an oversimplification of the complex abnormal signaling that occurs within the aortic wall in MFS.

2.5 OTHER MOLECULAR MECHANISMS INVOLVED IN TAA-ASSOCIATED VSMC AND EC DYSREGULATION

In the recent review from my laboratory, it is now experimentally clear that multiple signalling pathways involving extensive crosstalk are involved in the pathogenesis of TAA (Dong, Malecki et al. 2023). While this thesis is focused on MFS, many, if not all signalling pathways are likely to be common to both MFS and other forms of TAA, hence, the analysis in this review is relevant to our understanding of the

pathogenesis of MFS. A summary figure from this publication helps to visualize the complex relationships between the signalling pathways within VSMC and EC (Figure 2.4). This section will review the range of signalling pathways, in addition to TGFβ signalling, that are likely to be implicated in MFS pathogenesis.

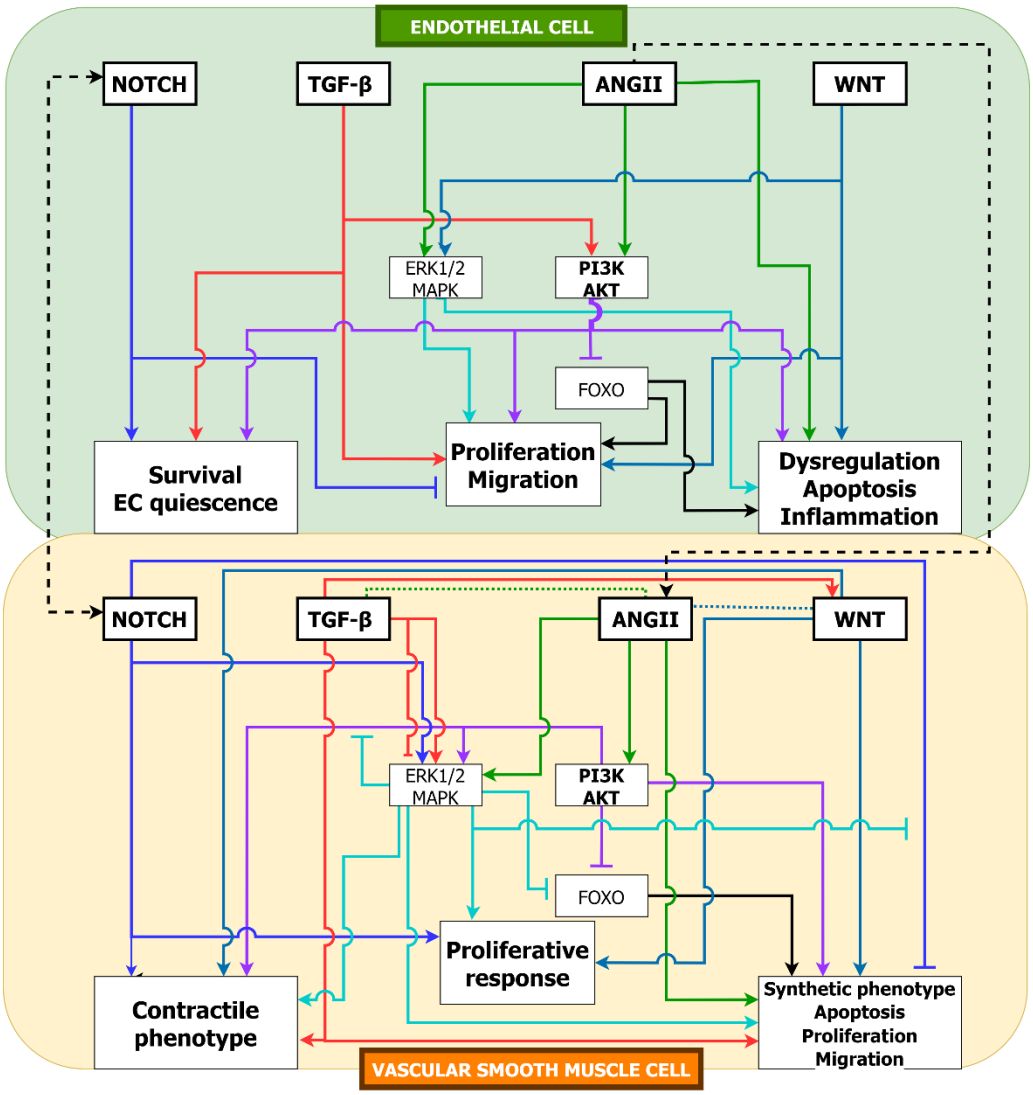


Figure 2.4 Pathways that are involved in VSMC and EC regulation associated with TAA (Dong, Malecki et al. 2023). Activation is shown by Arrows/ Stops show inhibition of the pathway/process. Coloured dashed lines show binding and/or co-signalling. Black dashed lines: VSMC-EC interactions. Solid lines show individual signalling pathways: Red, TGFβ; Teal, Wnt; Purple, PI3K/AKT; Blue, Notch; Green, Ang II; Light blue, ERK1/2/MAPK and Black, FoxO. Extensive signalling redundancy is illustrated. Notably, the MAPK and PI3K/AKT pathways are frequent intermediaries.

2.5.1 Renin-angiotensin system & Ang II signalling pathway

2.5.1.1 Renin-angiotensin system

The renin-angiotensin system (RAS), also known as the renin-angiotensin-aldosterone system (RAAS), is a complex biological process network that plays a role in various signal transduction processes and regulates the function of multiple organs and tissues through a set of hormones (Carey and Padia 2018). In particular, within the cardiovascular system, it acts as an upstream stimulator for downstream pathways such as PI3K/AKT/mTOR and MAPK, while also co-regulating TGFβ and Wnt pathways (Mehta and Griendling 2007).

The RAS pathway operates through two main ligands. The first is angiotensin I (Ang I), which is initially generated in the liver. It is then converted into angiotensin II (Ang II) by angiotensin-converting enzyme (ACE) (Carey and Padia 2018). Ang II is considered the primary effector of the system, as it activates two G-protein-coupled receptors, angiotensin II receptor type 1 (AT1R) and angiotensin II receptor type 2 (AT2R) (Figure 2.5) (D'Ardes, Boccatonda et al. 2020).

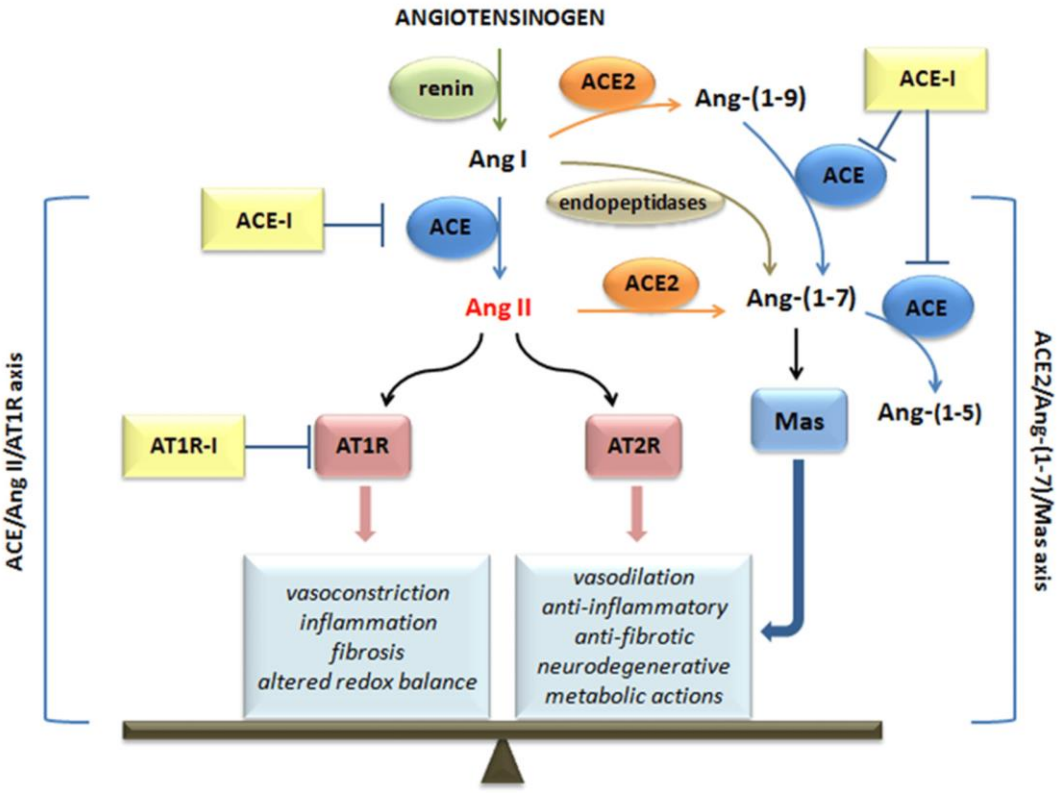


Figure 2.5 RAS system illustration (D'Ardes, Boccatonda et al. 2020).

The non-canonical or counter-regulatory RAS is derived from the action of angiotensin-converting enzyme II (ACE II), which converts Ang I into metabolites known as angiotensin 1-9 and subsequently angiotensin 1-7, originating from Ang II as well through the action of ACE II. Later, angiotensin 1-7 binds to the Mas receptor (MasR) and activates the Mas pathway, functioning similarly to Ang II by promoting vasodilation (D'Ardes, Boccatonda et al. 2020, Paz Ocaranza, Riquelme et al. 2020).

2.5.1.2 Introduction to Ang II signalling pathway

Angiotensin II (Ang II) plays a crucial role in regulating electrolyte homeostasis, vasoconstriction, and blood pressure (Carey and Padia 2018). Chronic stimulation by Ang II leads to hyperplasia and hypertrophy of VSMC, dysregulation of EC, and remodelling of the ECM (Mehta and Griendling 2007). The two receptors, AT1R and AT2R, exhibit contrasting functions. Activation of AT1R promotes arterial vasoconstriction, fibrosis, inflammation, and ECM remodelling, and it also contributes to EC dysregulation (Greindling, Lassegue et al. 1996, D'Ardes, Boccatonda et al. 2020). On the other hand, AT2R promotes vasodilation, reduces proliferation and fibrosis in VSMC and EC, and mitigates inflammation (Greindling, Lassegue et al. 1996, Forrester, Booz et al. 2018).

2.5.1.3 Ang II in VSMC

In VSMC pathology, Ang II primarily signals through AT1R and activates downstream MAPK/ERK signalling (Wang, Chang et al. 2015). This activation promotes the synthesis of TGFβ, resulting in increased VSMC contractility, hypertrophy, migration, and fibrosis (Gao, Stuart et al. 2008). Additionally, it is accompanied by ECM degradation, leading to stiffness, hypertension, and exacerbating the progression of vascular diseases (Gao, Stuart et al. 2008, Forrester, Booz et al. 2018).

The ROS also play a significant role in VSMC hypertrophy by elevating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, which leads to ROS generation, hypertension, and vascular remodelling (Dikalova, Clempus et al. 2005). Increased ROS levels activate the MAPK/ERK1/2 pathway, inducing VSMC growth (Zhang, Kimura et al. 2004, Yaghini, Song et al. 2010).

Inflammation in VSMC caused by Ang II is due in part to excessive MMP production, triggered by ROS generation and the activation of NF-κB signalling (St Paul, Corbett

et al. 2020). This inflammation is also observed in EC (Kranzhöfer, Schmidt et al. 1999). The MMPs are known to degrade the ECM, provoke an inflammatory response, promote cell migration, differentiation, proliferation, and apoptosis (Nagase, Visse et al. 2006). In VSMC, the overexpression of MMP contributes to vascular stiffness, increased collagen production, and the degradation of ECM proteoglycans, which ultimately leads to the formation of TAA (Mahmud and Feely 2004, Rabkin 2017).

2.5.1.4 Ang II in EC

Pathological effects on EC are closely linked to NO availability, inflammatory and oxidative stress responses (Watanabe, Barker et al. 2005). Ang II acting through AT1R leads to the generation of ROS, resulting in reduced eNOS production and decreased NO availability in EC, impairing NO synthesis (Nguyen Dinh Cat, Montezano et al. 2012). Furthermore, the Ang II pathway also induces EC apoptosis. Ang II, via AT1R, triggers apoptosis, which can be counterbalanced by Ang II acting through AT2R (Watanabe, Barker et al. 2005). Studies have demonstrated that ROS can reduce the levels of the anti-apoptotic protein Bcl-2 and increase the levels of the pro-apoptotic protein Bax through the MAPK/ERK1/2 pathway, leading to EC apoptosis (Watanabe, Barker et al. 2005). Specifically, in a MFS animal model, deletion of Ang II AT1R receptor attenuates aneurysm progression and decreases downstream ERK1/2 signalling in EC (Galatioto, Caescu et al. 2018).

2.5.1.5 Ang II in TAA

Ang II signalling plays a significant role in the formation of TAA, leading to VSMC phenotypic switching, aortic enlargement, fibrosis, inflammatory response, ECM remodelling and EC dysregulation (Jadli, Ballasy et al. 2022). Treatment with Ang 1-7 to antagonise Ang II signalling has been shown to attenuate mitochondrial fission, decrease ROS generation and inhibit VSMC proliferation (Jadli, Ballasy et al. 2022). The use of an AT1R inhibitor, such as losartan (commonly used to lower blood pressure), can prevent TAA formation and reduce aortic dilation in mouse and human non-MFS tissue studies by lowering ERK/MAPK expression (Habashi, Doyle et al. 2011, Chen, Lu et al. 2013, Nagasawa, Yoshimura et al. 2013). Additionally, ROS generation via Ang II infusion decreases contractile markers such as SM α-actin and ACTA2 in VSMC, contributing to TAA formation (Chen, Peters et al. 2017).

The crosstalk between TGFβ and Ang II has a significant impact on TAA formation (Chen, Lu et al. 2013). Treatment with a TGFβ neutralizing antibody in mouse models alongside Ang II infusion exacerbates aortic dilation and increases the risk of aortic rupture (Chen, Lu et al. 2013, Cook, Clayton et al. 2015). Although TGFβ and Ang II share a common downstream pathway, MAPK/ERK, inhibition of TGFβ in human *ex vivo* culture does not negate the inflammation response and elevated MMP expression induced by Ang II. This suggests that Ang II may be the dominant pathway in MMP regulation (Nagasawa, Yoshimura et al. 2013). Animal models with Ang II infusion impair TGFβ/SMAD2/3 signaling and LTBP1, further contributing to TAA formation (Shen, Lee et al. 2015).

Downstream of Ang II, the PI3K/AKT pathway plays a protective role in TAA formation (Shen, Zhang et al. 2013). Deficiency in PI3K/AKT leads to TAA formation, upregulation of MMPs, and downregulation of MMP inhibitors via the PI3K/AKT downstream Forkhead box O (FoxO) pathway (Shen, Zhang et al. 2013).

2.5.2 Wnt signalling pathway

2.5.2.1 Introduction to Wnt pathway

The Wnt pathway plays a significant role in organ development and is also found in adult cells, where it regulates cell homeostasis, survival, proliferation, and migration (Mill and George 2012, Foulquier, Daskalopoulos et al. 2018). Wnt signalling involves a number of receptors, including Frizzled receptors (Frizzled 1, 2, and 5) for ligands such as Wnt1, 2, 3, 3a, 5a, 7a, 8, and 11, which are expressed in VSMC or EC. The most well-known co-receptors in this pathway are low-density lipoprotein receptor-related proteins 5/6 (LRP5/6) (Niehrs 2012).

Different ligands in the Wnt pathway can activate either canonical or non-canonical signalling, promoting various cellular processes. Canonical Wnt signalling involves β-catenin and TCF/LEF transcription factors, resulting in altered expression of cell-cycle-related proteins (e.g., cyclin D1 and p21) in VSMC and other potential gene targets, including MMPs (Quasnichka, Slater et al. 2006). Non-canonical Wnt signalling operates in a β-catenin-independent manner, relying on Ca^{2+} or small GTPases to activate nuclear factor of activated T cells (NFAT) and JUN-N terminal kinase (JKN), which modulate cellular polarity and migration (Akoumianakis, Polkinghorne et al. 2022).

2.5.2.2 Wnt in VSMC

Wnt signalling in VSMC primarily responds to vascular injury, e.g., hypoxic conditions (Mill, Monk et al. 2014). Individual ligands within the Wnt family may promote either VSMC survival, attenuate VSMC apoptosis induced by ROS, or induce a VSMC proliferative phenotype, thereby increasing VSMC proliferation and migration (Mill and George 2012, Williams, Mill et al. 2016, Liu, Neogi et al. 2020).

The pro-survival characteristics of Wnt are observed in Wnt5a (both canonical and non-canonical) and Wnt3a (canonical) (Mill, Monk et al. 2014). The Wnt5a treatment in animal VSMC demonstrated attenuated VSMC apoptosis associated with oxidative stress (Mill, Jeremy et al. 2011, Mill and George 2012). In hypoxic conditions, VSMC apoptosis is decreased by either Wnt5a or Wnt3a stimulation, but they act through non-identical downstream pathways. However, the anti-apoptotic effect of Wnt5a diminishes with age, while Wnt3a remains unaffected (Mill, Monk et al. 2014, Brown, Connolly et al. 2019).

A proliferative phenotype in VSMC can be induced through non-canonical Wnt signalling by Wnt4, promoting VSMC proliferation (Reddy, Valente et al. 2011, Tsaousi, Williams et al. 2011, Marinou, Christodoulides et al. 2012, Mill and George 2012, Chen, Zhuang et al. 2016). Canonical Wnt signalling can also be activated in response to vascular injury through β-catenin, promoting a proliferative phenotype and assisting in VSMC survival (Wang, Xiao et al. 2002). This induction leads to venous SMC proliferation and migration, while also inhibiting apoptosis (Wang, Xiao et al. 2002, Wang, Adhikari et al. 2004, Mill, Jeremy et al. 2011, Williams, Mill et al. 2016).

2.5.2.3 Wnt in EC

Similar to VSMC, Wnt signalling in EC is primarily activated in response to vascular injury (Masckauchan, Agalliu et al. 2006, DiRenzo, Chaudhary et al. 2016). This signaling pathway promotes EC survival, migration, proliferation, and triggers inflammatory responses (Dejana and Kühl 2010). Wnt5a, through non-canonical Wnt signaling, activates ERK and suppresses canonical Wnt signaling to enhance EC proliferation and survival (Masckauchan, Agalliu et al. 2006, Cheng, Yeh et al. 2008). Inhibition of Wnt5a in cell culture leads to a decreased rate of EC proliferation (Masckauchan, Agalliu et al. 2006). Furthermore, Wnt5a induces the production of inflammatory cytokines such as IL-8, IL-6, and NF-κB (Kim, Kim et al.

2010). In conjunction with tumour necrosis factor (TNF), Wnt5a is capable of inducing inflammation in EC (Kim, Kim et al. 2010).

2.5.2.4 Wnt in TAA

In TAA, Wnt signalling has demonstrated increased activity in cultured EC from TAA patients, which is associated with impaired crosstalk with Notch signalling (Kostina, Bjork et al. 2018). In an *ApoE*$^{-/-}$ mouse model, Wnt signalling has shown potential roles in promoting aortic aneurysm formation. Inhibition of Wnt signalling decreases the expression of pro-aneurysmal genes, thus attenuating aneurysm formation (Krishna, Seto et al. 2017). Although no direct association has been demonstrated between Wnt and MFS-associated TAAD, crosstalk between Wnt and TGFβ promotes fibrillin assembly and deposition, leading to cardiac fibrosis in human cell studies (Dzialo, Czepiel et al. 2021). This suggests that the Wnt pathway could potentially contribute to TAA formation and demonstrates likely crosstalk between TGFβ and Notch signalling. However, the underlying mechanism remains to be elucidated.

2.5.3 PI3K/AKT signalling pathway

2.5.3.1 Introduction to PI3K/AKT pathway

The PI3K/AKT/mTOR signalling pathway functions potentially as an intermediate pathway that regulates cell survival, proliferation, growth, differentiation, and migration (Hemmings and Restuccia 2012). It primarily achieves this by inhibiting its downstream FoxO pathway (Guertin, Stevens et al. 2006). As discussed in Chapters 2.4.6 and 2.5.1, PI3K/AKT/mTOR receives upstream stimulation, such as from TGFβ and Ang II, and modulates cell behaviour accordingly (Dong, Malecki et al. 2023).

2.5.3.2 PI3K/AKT in VSMC

In a healthy aorta, PI3K/AKT inhibits the downstream pro-apoptotic FoxO pathway, promoting a contractile VSMC phenotype (Yang, Gong et al. 2013). This effect can be inhibited by the MAPK/ERK pathway under growth factor stimulation (Yang, Gong et al. 2013). However, in the context of vascular injury, PI3K/AKT promotes a proliferative phenotype to support VSMC survival by enhancing VSMC proliferation and inhibiting apoptosis (Allard, Figg et al. 2008, Tucka, Yu et al. 2014). There is

crosstalk between PI3K/AKT and MAPK/ERK, often displaying antagonistic effects, in promoting the proliferative phenotype (Abid, Yano et al. 2005, Yang, Gong et al. 2013). Growth factor stimulation is typically required for MAPK/ERK activation (Abid, Yano et al. 2005, Yang, Gong et al. 2013).

2.5.3.3 PI3K/AKT in EC

In healthy EC, the PI3K/AKT pathway engages in crosstalk with the Wnt signalling pathway to maintain EC integrity by stabilizing EC cell-cell contacts (Taddei, Giampietro et al. 2008). However, in response to oxidative stress, the PI3K/AKT signalling pathway plays a vital role in promoting EC survival by increasing NO availability, promoting eNOS generation (Zhao, Qian et al. 2021). Additionally, it inhibits EC apoptosis induced by the FoxO pathway (Abid, Guo et al. 2004, Skurk, Maatz et al. 2004, Malekmohammad, Sewell et al. 2020, Zhao, Qian et al. 2021).

2.5.3.4 PI3K/AKT in TAA

Impaired PI3K/AKT signalling is observed in TAA, and is associating with TGFβ and Ang II, and growth factor stimulation (Chung, Au Yeung et al. 2007). This reduction in PI3K/AKT signaling is linked to increased VSMC apoptosis, ECM degradation, and inflammation mediated by MMPs (Estrada, Irons et al. 2021). In mouse MFS model, decreased activation of PI3K/AKT and expression of eNOS is observed in the thoracic aorta, accompanied by EC dysregulation (Chung, Au Yeung et al. 2007). While treatment with an Ang II/AT1R blocker in the MFS mouse model can partially restore some endothelial function, it does not fully reverse the impairment of PI3K/AKT and eNOS (Chung, Au Yeung et al. 2007, Yang, Kim et al. 2009). Despite the apparent importance of PI3K/AKT as a key player and intermediary in signaling transduction, limited research has been conducted on PI3K/AKT in TAA. Further studies are needed to comprehensively understand PI3K/AKT signaling in TAA and the mechanisms of upstream stimulation of PI3K/AKT.

2.5.4 Notch signalling pathway

2.5.4.1 Introduction to Notch signalling

In the aorta, the Notch signalling pathway operates through Notch 1, 2, and 3 in VSMC, while Notch 4, Jagged 1, and the delta-like ligand (Dll4) are involved in EC (Baeten and Lilly 2017). This pathway interacts with several others, including TGFβ,

PI3K/AKT, MAPK/ERK, and Wnt pathways, to regulate VSMC phenotypic transitions and maintain an EC quiescent phenotype (Andersen, Uosaki et al. 2012, Baeten and Lilly 2015). Notch signalling demonstrates extensive crosstalk between EC and VSMC, where ECM proteins can influence Notch signalling by upregulating Notch expression or through integrin binding in EC (Miyamoto, Lau et al. 2006, Deford, Brown et al. 2016). Conversely, EC Notch signalling can regulate VSMC migration induced by growth factors and promote EC adhesion (Pedrosa, Trindade et al. 2015, Deford, Brown et al. 2016). Furthermore, EC Notch signalling regulates VSMC proliferation, and vice versa, as VSMC Notch is required for EC Notch activation and EC proliferation (Yang and Proweller 2011, Lin and Lilly 2014).

2.5.4.2 Notch in VSMC

In a healthy aorta, Notch promotes a contractile VSMC phenotype primarily through the Notch3 receptor (Sweeney, Morrow et al. 2004, Liu, Kennard et al. 2009, Tang, Urs et al. 2010, Lin and Lilly 2014). However, under the stimulation of upstream growth factors or in response to vascular injury, Notch promotes VSMC survival by inducing a shift towards a proliferative phenotype (Proweller, Pear et al. 2005, Li, Takeshita et al. 2009). This involves reducing VSMC differentiation and apoptosis while enhancing the expression of genes related to cell survival through the MAPK/ERK pathway (Miele and Osborne 1999, Proweller, Pear et al. 2005, Li, Takeshita et al. 2009, Baeten and Lilly 2015, Liu 2015). Notably, Notch2 can activate an anti-survival response in VSMC when responding to vascular injury (Boucher, Harrington et al. 2013). This leads to increased VSMC apoptosis and reduced proliferation, although these effects can be inhibited by MAPK/ERK signalling under the influence of growth factor stimulation (Boucher, Harrington et al. 2013). These pieces of evidence suggest that MAPK/ERK might serve as the intermediate pathway that regulates Notch signalling in VSMC, ultimately activating different cellular responses (Baeten and Lilly 2015). Inhibition of Notch signalling in VSMC also promotes the expression of MMPs, contributing to ECM degradation (Baeten and Lilly 2017).

2.5.4.3 Notch in EC

Notch signalling in EC plays a crucial role in maintaining an EC quiescent phenotype primarily through the involvement of Notch1 and Notch4 (Liu 2006). This signalling pathway helps preserve EC homeostasis, junction integrity, barrier function, and

prevents EC proliferation (Liu 2006, Benedito, Trindade et al. 2008). When subjected to shear stress stimulation, Notch signalling prevents EC activation in response to shear stress and helps maintain the quiescent cellular state (Mack, Mosqueiro et al. 2017, Mack and Iruela-Arispe 2018).

2.5.4.4 Notch in TAA

Notch activity in TAA is often observed in cases associated with BAV-TAA. Deficiencies and dysregulation in Notch signalling are frequently linked to VSMC apoptosis, which contributes to the formation of TAA (Bonderman, Gharehbaghi-Schnell et al. 1999, Garg, Muth et al. 2005, Sciacca, Pilato et al. 2013, Koenig, Bosse et al. 2016). However, in non-BAV TAA models, Notch signalling appears to be upregulated in the ascending aorta (Balistreri, Crapanzano et al. 2018). Notably, Jespersen et al. (2022) observed in an MFS animal model that inhibiting Notch reduces aorta dilation (Jespersen, Li et al. 2022). Conversely, VSMC in descending TAA showed decreased Notch expression (Zou, Ren et al. 2012). This suggests some uncertainty in the role of Notch signalling in TAA, and further studies are required to investigate the specific mechanisms of Notch signalling in TAA (Balistreri, Crapanzano et al. 2018, Kostina, Bjork et al. 2018, Jespersen, Li et al. 2022).

2.5.5 MAPK signalling pathway

2.5.5.1 Introduction to MAPK pathway

MAPK signalling consists of the ERK1/2, JNK1/2/3, and p38 pathways, generally promoting cell growth, differentiation, and survival (Morrison 2012). As a potential key intermediate pathway, MAPK demonstrates extensive crosstalk with other pathways and activates upon upstream stimuli, signalling through its downstream targets (Dong, Malecki et al. 2023). Under conditions of vascular injury, MAPK induces cell proliferation, migration, inflammatory responses, and apoptosis (Morrison 2012, Dong, Malecki et al. 2023).

2.5.5.2 MAPK in VSMC

In VSMC, MAPK promotes proliferation and migration while also contributing to the inflammatory response, which can lead to ECM degradation (Velarde 1999, Pantan, Tocharus et al. 2016). Various upstream stimulators such as TGFβ, Notch, Ang II,

and interactions with PI3K/AKT have been discussed in previous sections. In the context of inflammation, MAPK receives signals from upstream Ang II/AT1R and initiates the inflammatory process (Wang, Chang et al. 2015). Inhibition of MAPK/ERK has been shown to attenuate VSMC migration, proliferation, and inflammation, highlighting the crucial role of MAPK in regulating VSMC synthesis, ECM homeostasis, and inflammation (Goetze 1998, Hattori, Matsumura et al. 2003, Lee, Wu et al. 2016, Zhang, Ren et al. 2016).

2.5.5.3 MAPK in EC

Similarly to VSMC, MAPK serves as an intermediate pathway that responds to upstream stimuli in EC. The main effect of MAPK in EC is the promotion of proliferation (Watanabe, Barker et al. 2005, Schmidt 2015). MAPK has been shown to promote EC proliferation in response to growth factor stimuli (Nakagami, Morishita et al. 2001). However, under Ang II treatment, MAPK activation can lead to EC dysfunction (Zhang 2002, Schmidt 2015, Wang, Zhang et al. 2020).

2.5.5.4 MAPK in TAA

MAPK signalling has been found to play a significant role in promoting TAA formation (Holm, Habashi et al. 2011, Wisler, Harris et al. 2015). MAPK signalling can induce various processes in the aortic wall that contribute to TAA, including VSMC apoptosis, proliferation, ECM degradation, and inflammation, all of which are initiated by upstream stimuli (Holm, Habashi et al. 2011, Wang, Chang et al. 2015). As mentioned in Chapter 2.4.6, in MFS animal models, dysregulation of TGFβ can activate downstream MAPK/ERK1/2 signalling, leading to aortic dilation, phenotypic changes in VSMC, and the development of TAA. Inhibition of MAPK/ERK has been shown to effectively attenuate TAA progression and reduce elastic fibre destruction (Holm, Habashi et al. 2011, Liu, Li et al. 2019). Furthermore, MAPK signalling can trigger an inflammatory response through MMP production, contributing to TAA formation (Wang, Chang et al. 2015). This inflammatory response can be induced by treatments such as Ang II/AT1R infusion (Wang, Chang et al. 2015, Liu, Li et al. 2019). Inhibiting MAPK signalling in MFS animal model has demonstrated potential benefits in attenuating TAA formation (Holm, Habashi et al. 2011, Liu, Li et al. 2019).

2.5.6 FoxO signalling pathway

The FoxO signalling pathway is composed of a group of Forkhead box proteins (FoxO3a, FKHRL1, FKHR, AFX), which play important roles as downstream effectors of the PI3K/AKT pathway, promoting VSMC proliferation, migration and apoptosis (Abid, Yano et al. 2005, Tucka, Yu et al. 2014, Zhao, Qian et al. 2021). In healthy aorta, FoxO signalling is typically inhibited by PI3K/AKT, resulting in increased cell survival, as PI3K/AKT induces the nuclear exclusion of FoxO proteins and inhibits their expression (Abid, Guo et al. 2004, Abid, Yano et al. 2005). Furthermore, FoxO proteins can induce the production of MMPs in the ECM, which can result in ECM degradation and inflammation. Inhibition of FoxO signalling in animal model is crucial for preventing VSMC apoptosis, ECM degradation, and the formation of TAA (Tucka, Yu et al. 2014, Zhao, Qian et al. 2021).

FoxO signaling also plays a role in EC and has been associated with EC apoptosis. The activation of PI3K/AKT signalling can have a pro-survival effect on EC by inhibiting FoxO-induced EC apoptosis and stabilizing endothelial cell-cell contacts (Abid, Guo et al. 2004, Skurk, Maatz et al. 2004, Schmidt 2015, Zhao, Qian et al. 2021). FoxO signalling promotes TAAD by downregulating VSMC contractile genes and inducing apoptosis (Abid, Guo et al. 2004). Additionally, FoxO signaling can stimulate the production of MMPs, leading to ECM degradation which ultimately contributes to the formation of aneurysms (Wang, Gao et al. 2022, Du, Sun et al. 2023).

2.5.7 Hedgehog signalling pathway

Hedgehog signalling can be induced by upstream growth factors (e.g., Platelet-derived growth factor BB (PDGF-BB)) and promotes phenotypic switch and proliferation of VSMC via Sonic Hedgehog homologues (Shh) (Li, Duman-Scheel et al. 2010). Inhibition of Shh by its inhibitor (e.g., Cullin 3 (CUL3)) or Shh alliance Paired box 9 (Pax 9) in an *ApoE*$^{-/-}$ mouse model inhibits VSMC proliferation, migration and phenotypic switch (Xu, Zhang et al. 2020, Xiang, Li et al. 2021). In EC, Hedgehog signalling demonstrated crosstalk with PI3K/AKT by elevating PI3K expression and inducing EC proliferation and migration (Kanda, Mochizuki et al. 2003). Moreover, Shh interacts with the Rho/ROCK pathway and then subsequently activates downstream genes including MMP9 and osteopontin (OPN), resulting in EC proliferation, migration and angiogenesis (Renault, Roncalli et al. 2010). However, under Ang II stimulation, Shh increases NO production and decreases

oxidative stress via the PI3K/AKT pathway, attenuating hypertension and EC dysfunction (Marrachelli, Mastronardi et al. 2013).

2.5.8 HIF-1 signalling pathway

HIF-1 is a heterodimeric transcription factor that responds to hypoxia through its two subunits, HIF-1α and HIF-1β. The HIF system regulates over 100 downstream genes, and dysregulation in the HIF-1 pathway exhibits both beneficial and detrimental effects on the aortic wall, depending on the response to hypoxia (Yang, Li et al. 2020). HIF-1 signaling primarily responds to EC injury caused by hypoxic and anoxic states, promoting EC survival, and inducing EC proliferation and migration. It also promotes nitric oxide synthases (NOS) gene expression, increasing NO production to support EC survival (Palmer 1998, Manalo 2005, Jiang, Tian et al. 2019). Studies have demonstrated that the HIF-1 pathway can promote VSMC proliferation via PI3K/AKT signaling and induce an injury response from EC dysregulation (Lambert, Roy et al. 2010, Sun, Zhao et al. 2020). In a mouse model, under β-aminopropionitrile monofumarate (BAPN – an inhibitor of LOX collagen and elastin crosslinking) administration-induced TAAD, by inducing an intermediate hypoxia environment, HIF-1α shows elevated expression in response to the hypoxic conditions, which could attenuate ECM degradation, VSMC apoptosis, and inflammation infiltration. Thus HIF-1α elevation is able to alleviate BAPN-induced TAAD in a mouse model (Yang, Li et al. 2020). However, deficiency of VSMC-specific HIF-1α suppresses Ang II-induced VSMC hypertrophy, resulting in medial thickening in animal models (Imanishi, Tomita et al. 2014). Furthermore, VSMC HIF-1α deficiency suppresses Ang II-induced macrophage infiltration, indicating a relationship between VSMC inflammation and Ang II/HIF-1α activation (Imanishi, Tomita et al. 2014). Therefore, HIF-1 regulation in TAAD is potentially tightly linking to the hypoxia response and the regulation of ECM, EC and VSMC cellular responses.

2.5.9 Ras & Rap1 signalling pathway

Ras protein signaling remains relatively unexplored in the context of cardiovascular disease (CVD) and aneurysms. Its primary regulation involves a binary switch between the inactive guanosine diphosphate (GDP)-bound state and the active guanosine triphosphate (GTP)-bound state (Simanshu, Nissley et al. 2017). Ras signaling is initiated by upstream signals such as cytokine receptors, tyrosine kinase

receptors, G-protein-coupled receptors, and Ang II/AT1R, and subsequently activates downstream pathways like MAPK/ERK and PI3K/AKT (Simanshu, Nissley et al. 2017, Shah, Brock et al. 2019, Dillon, Lopez et al. 2021). In CVD, Ras has been shown to upregulate downstream MAPK/ERK, modulating cardiomyocyte proliferation and size, and contributing to hypertrophic cardiomyopathy, arrhythmias, and heart failure (Chien and Hoshijima 2004). The interaction between Ras and PI3K/AKT/mTOR exhibits bidirectional effects, depending on the context (Ramos-Kuri, Meka et al. 2021). Activation of PI3K/AKT/mTOR demonstrates both cardioprotective properties by promoting cell survival and anti-apoptosis effects, as well as pro-hypertrophic characteristics in a co-regulatory manner with downstream NF-kB signaling in cardiomyocytes (Gelb and Tartaglia 2011, Ramos-Kuri, Meka et al. 2021).

Rap1 belongs to the Ras-related protein family and demonstrates crosstalk with MAPK (ERK1/2/JNK) in VSMC (Li, Teng et al. 2018). As an upstream regulator for MAPK signaling, Rap1 induces VSMC proliferation and migration via the phosphorylation of MAPK genes (Li, Teng et al. 2018). In EC, Rap1 promotes cell-cell junction stabilization and VE-cadherin-dependent cell adhesion, thus maintaining EC homeostasis (Schmidt 2015). Inhibition of Rap1 leads to decreased junction stability and may result in endothelial dysfunction (Fukuhara, Sakurai et al. 2005, Schmidt 2015). A study by Nogi et al. (2018) found that Rap1 may be involved through small GTP-binding protein GDP dissociation stimulator (SmgGDS) activity in TAAD patients, which exhibit decreased expression in the ascending aorta and VSMC (Nogi, Satoh et al. 2018). SmgGDS deletion in Ang II-induced mice promotes TAA formation. Moreover, SmgGDS and FBN1 show lower expression in non-MFS TAA patients, and the expression of FBN1 and VSMC marker genes (e.g., ACTA2) drastically decreases in *Apoe*$^{-/-}$ *SmgGDS*$^{+/-}$ mice, suggesting potential crosstalk between FBN1 expression and Rap1 pathways in TAA development (Nogi, Satoh et al. 2018).

2.5.10 Hippo signalling pathway

The activation of the Hippo pathway, through its effectors Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (WWTR1, also known as TAZ), induces VSMC proliferation and migration while also suppressing VSMC contractile genes (Kimura, Duggirala et al. 2016, Chen and Liu 2018, He, Bao et al. 2018). This can lead to VSMC overgrowth and promote a phenotypic switch,

ultimately contributing to aortopathy formation (He, Bao et al. 2018, Xie, Wang et al. 2021). In EC, the Hippo pathway promotes an inflammatory response through the YAP/TAZ ligand, which is regulated by Ang II signalling. Inhibition of YAP/TAZ attenuates inflammation and the infiltration of monocytes in EC (Xie, Wang et al. 2021).

2.5.11 TNF signalling pathway

The TNF pathway is a downstream signalling pathway of PI3K/AKT, which interacts extensively with the NF-κB and MAPK pathways and is involved in inflammatory responses (Kalliolias and Ivashkiv 2016). TNF induces an inflammatory response in rat aortic VSMC via the NF-κB, PI3K/AKT and MAPK/JNK pathways (Chen, Hsu et al. 2014). This study suggests that both PI3K/AKT and MAPK signalling are involved in vascular injury responses, possibly triggered by inflammation (Chen, Hsu et al. 2014). In TAA, TNF receptor 1 and 2 (TNFR1/TNFR2) have been found to be positively correlated with MMP9 and TIMP, which are related to ECM degradation and inflammation (Rabajdová, Urban et al. 2017). Furthermore, in a calcium chloride-induced animal model of TAA, increased mTOR signalling resulted in the downregulation of TNF-α, MMP2, and MMP9 (Cao, Wu et al. 2017). This suggests that overexpressing mTOR may attenuate the inflammatory response via TNF signalling and associated ECM degradation in TAA (Cao, Wu et al. 2017).

2.6 GENETIC AND EPIGENETIC CHANGES IN TAA

2.6.1 RNA-Seq

Ribonucleic acids (RNA) are transcribed from DNA, and the study of RNA is known as transcriptomics. This field of study offers a deeper understanding of biological processes and cellular functions in diseases. Messenger RNA (mRNA) is the mature form of RNA that is eventually translated into proteins. The DNA-RNA-protein axis provides an overview of disease pathogenesis, but the relationship between transcription and translation is not always straightforward in transcriptomic studies. This is because transcriptomics encompasses coding and non-coding proteins, as well as other RNA-edited transcripts such as alternatively spliced, alternatively polyadenylated, alternatively initiated, sense, and antisense transcripts (Liang 2013).

In contrast to DNA, RNA exhibits greater dynamism, including amplitude of expression, in living organisms and shows high cell and tissue specificity. RNA chemistry is subject to regulation through direct promotion/ enhancement of transcription as well as epigenetic mechanisms including histone modification, DNA methylation, and non-coding RNA such as microRNA (Mattick, Amaral et al. 2009). Therefore, the regulation of transcriptomics has the potential to impact pathogenic processes and an understanding of such regulation is likely to provide valuable insights into understanding disease progression, may identify potential biomarkers, and unravel relevant aspects of molecular mechanisms that contribute to our understanding of disease aetiology, particularly in cancer studies (Cheng, He et al. 2019).

2.6.1.1 Transcriptomics in MFS

Many studies have aimed to identify potential RNA markers that can detect TAA formation or its progression. RNA expression profiles can differ among different types of TAA. For instance, familial TAA may show altered expression of mRNA coding for smooth muscle cell contractile gene markers like ACTA2, MYH11, PRKG1, and MYLK, while patients with BAV may exhibit changes in the expression of NOTCH1 and ROBO4 (Mizrak, Feng et al. 2022). Therefore, the transcriptomic study of TAA is highly dependent on the specific type of TAA and the underlying genetic factors (Mizrak, Feng et al. 2022). It is worth noting that, due to the hereditary nature of TAA caused by a single gene mutation in MFS, MFS cohorts are often excluded from genome-wide transcriptomic studies of TAA. However, some studies have suggested that long non-coding RNA HIF1α-AS1 could be a valuable marker for general TAA, which has been shown to promote VSMC proliferation and apoptosis, and ECM degradation, and is likely regulated by microRNA let-7g (Wang, Zhang et al. 2015, Zhang, Li et al. 2020).

In an iPSC (Induced Pluripotent Stem Cells)-derived MFS vascular model, decreased FBN1 expression was observed and an increased expression of a TGFβ signalling target, plasminogen activator inhibitor-1 (PAI-1/SERPINE1), along with elevated MMP1/2/9/10 expression (Granata, Serrano et al. 2017, Pedroza, Tashima et al. 2020). Some VSMC markers, including CNN1, ACTA2, TAGLN, and MYOCD, exhibited increased expression in MFS compared to the WT, accompanied by an abnormal calcium response, heightened levels of phosphorylated SMAD2, and MAPK/ERK/p38 signalling. However, it is noteworthy that the expression of CNN1

and ACTA2 yielded contrasting results in MFS single-cell analysis, suggesting that CNN1 and ACTA2 expression may vary depending on the stage of disease progression (Pedroza, Tashima et al. 2020).

ECM degradation has been correlated with increased MMP expression and decreased expression of the MMP inhibitor family of TIMP (metallopeptidase inhibitor) in MFS, both in humans and animal models (Jana, Hu et al. 2019). Notably, TIMP expression appeared to be upregulated in the early stage of TAA then downregulated later. Collagen expression (COL-1/ COLA1A) has been found to be higher in MFS compared to WT (Pedroza, Tashima et al. 2020). Importantly, the inhibition of the MAPK/ERK/p38 pathway and Krüppel-like factor 4 (KLF4) has been demonstrated to play a crucial role in ECM regulation. Inhibition of KLF4/ MAPK/ERK/p38 increases FBN1 deposition, increases the proliferation marker CCND1, and decreases cell cycle arrest promoting gene TP53 and CDKN1A in MFS (Pedroza, Tashima et al. 2020). This suggests that precise regulation of these pathways can restore SMC proliferation rates and inhibit apoptosis (Granata, Serrano et al. 2017).

Furthermore, two single-cell transcriptomics studies (Pedroza, Tashima et al. 2020, Zhang, Qiu et al. 2022) have been conducted on MFS, revealing the presence of inflammatory RNA transcripts of proteins associated with inflammatory cell types such as T cells, B cells, neutrophils, macrophages, monocytes, mast cells, along with VSMC, EC, and fibroblasts (Zhang, Qiu et al. 2022). In a MFS dissection cohort, genes like CCL3L3, CXCL2, FCER1G, CXCL8 and IL1B were significantly elevated, indicating an increased inflammatory response during dissection (Zhang, Qiu et al. 2022). Heat shock protein gene mRNA, including *DNAJA1, HSPH1*, *HSPA6* and *SOD2* were observed to be increased in MFS, suggesting a link between inflammatory and oxidative stress responses. Genes associated with immune activation and cell apoptosis such as *C5AR1, PLAUR* and *TIMP1*, were also observed to be regulated (Zhang, Qiu et al. 2022). Compared to control groups, MFS exhibited a significantly elevated inflammatory response and activated cytokine pathways, which could promote ECM degradation. However, when compare with a TAA hypertension cohort, an aortic dissection (AD) in MFS cohort demonstrates a weaker inflammatory response but a higher activation of the TGFβ pathway. Pedroza et al. (2020) conducted a single cell RNA study in a MFS mouse model that also reported significant elevations of *TNFRSF11B, VCAM1*, and *TGFB1* transcripts in MFS VSMC modulation. In contrast, transcription of *CNN1, ACTA2, MYL9* and *MYH11* were downregulated, consistent with a switch of VSMC into a

different phenotype, likely a fibroblast-like state. This transition was also observed in another MFS single-cell study in MFS-associated dissection, consistent with likely VSMC plasticity in MFS (Zhang, Qiu et al. 2022).

Fibroblasts play a crucial role in maintaining ECM homeostasis and are believed to be capable of differentiate into VSMC or EC to assist in ECM remodelling during disease states (Mackay, Jadli et al. 2022). In comparison to control groups, MFS human dissection tissue displayed increased expression of THBD, S100A16, and BMP2, promoting inflammatory reactions and epithelial-mesenchymal transition, as well as regulating angiogenesis (Zhang, Qiu et al. 2022). Pro-inflammatory responses regulated by IL1B, CXCL8, CCL2, and CXCL2 were also observed in fibroblasts, indicating the activation of an inflammatory response and conversion to a VSMC-like phenotype. The expression of MYH11, MYLK and RGS5 were elevated in MFS, consistent with promotion of cell migration and angiogenesis. Moreover, elastic fibre, cell junction and matrix maintenance genes like *ELN, DCN, LUM* and *FBLN1/2* were downregulated in MFS (Zhang, Qiu et al. 2022). Additionally, in advanced MFS, aneurysmal VSMC exhibited elevated expression of *IGFBP2, CTGF*, and *FN1*, but no changes were identified in *ACTA2, CNN1,* or *MYH11* (Pedroza, Tashima et al. 2020). Overall, elevated BMP2 leads to MMP9 secretion, promoting ECM degradation in MFS, and elevated HMGA1 and FOSL1 promotes an inflammatory response (Zhang, Qiu et al. 2022).

Regarding VSMC changes in MFS, inflammatory genes *CCL3L3, CLMP, CTSL*, *NFKBIA* and *PLAUR* were significantly upregulated in human tissue samples from AD MFS and hypertension cohorts (Zhang, Qiu et al. 2022). Genes associated with maintaining vascular integrity, like *CYR61, LMOD1, TNS1*, and *ITGA8*, displayed decreased expression in disease states. Furthermore, *CXCL8, CXCL2, TGM2* and *MT2A* were elevated in disease states, indicating an activated inflammatory response, leading to collagen degradation and elastin fragmentation (Zhang, Qiu et al. 2022). The transcriptomic changes in MFS from previous studies are summarized in Figure 2.6.

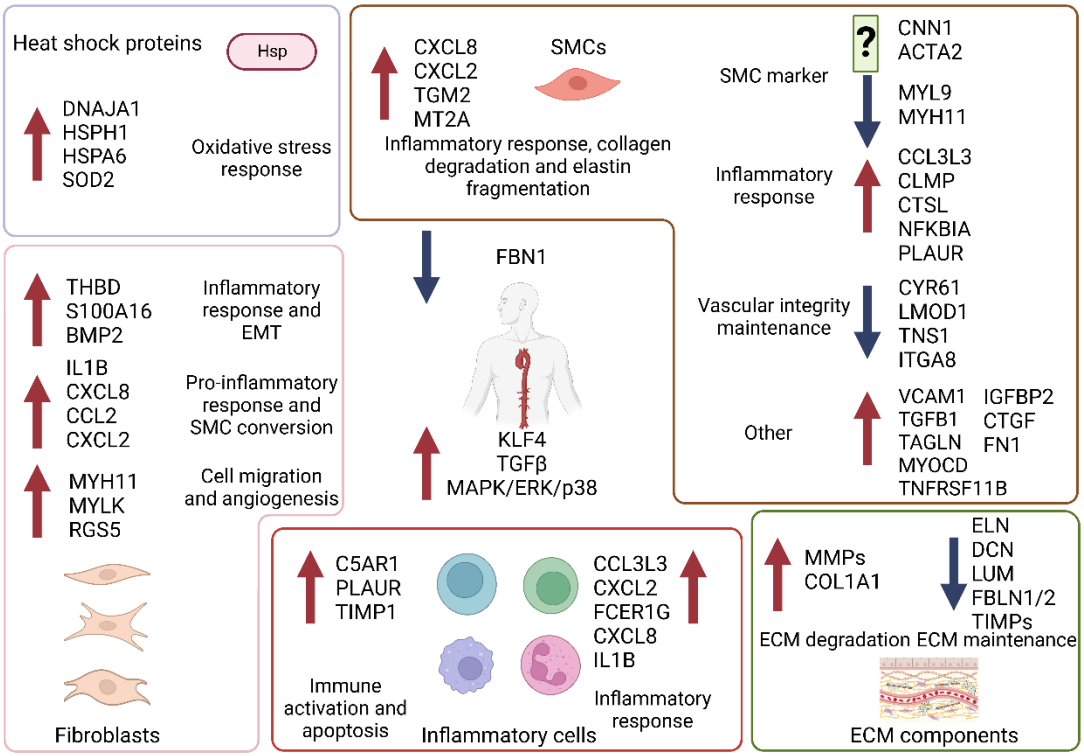


Figure 2.6 Summary of evidence of transcriptomic regulation in MFS pathology.

2.6.2 Proteomics

Proteomics is the study of proteins, encompassing the examination of their structure, biological functions, and regulation of expression (Gebauer and Hentze 2004). Proteomics encompasses three major areas: protein expression, protein-protein interactions, and protein structure. These areas allow researchers to explore protein activity, quantify protein expression, and gain insights into the structural aspects of proteins (Al-Amrani, Al-Jabri et al. 2021).

2.6.2.1 Proteomics in MFS

Two previous proteomics studies have been undertaken in MFS, investigating MFS human aortic tissue compared to cohorts without connective tissue disorders and an iPSC-derived MFS human study. These studies found a consistent change in the translational mechanisms in MFS, leading to phenotypic changes in VSMC and alternations in ECM components (Pilop, Aregger et al. 2009, Iosef, Pedroza et al. 2020).

For instance, the expression of the contractile VSMC markers transgelin (TAGLN) was decreased in MFS and synthetic VSMC markers like MMP2 and COL1A1 were increased in MFS compared to controls (Iosef, Pedroza et al. 2020). This finding suggests that MFS VSMC display phenotypic changes in the disease state. Furthermore, in the iPSC MFS model, ECM dysregulation and alternation of ECM adhesion were observed (Iosef, Pedroza et al. 2020). This is caused by upregulated ITGAV leading to decreased VSMC binding to the ECM. Additionally, the study showed that ECM remodelling and collagen degradation were associated with MFS by the downregulation of mannose receptor C type 2 (MRC2) and through membrane type-1 MMP (MT1-MMP) regulation (Iosef, Pedroza et al. 2020).

Proteins associated with aneurysm formation and aortic dilation were observed in MFS medial tissue with significant upregulated of expression. These proteins include filamin A (FLNA), vinculin (VCL), calponin1 (CNN1), microfibril-associated glycoprotein 4 (MFAP4) and myosin-10 (MYO10) (Pilop, Aregger et al. 2009). FLNA is involved in regulating filament arrangements and TGFβ signaling, while VCL and CNN1 respond to mechanical stretch of VSMC and play roles in focal adhesion stabilization and cell migration regulation (Pilop, Aregger et al. 2009). MFAP4 is associated with aortic dilation, binds to elastin and regulates elastogenesis, and MYO10 is involved in cytoskeleton linkage and integrin-cytoskeleton connections and may be associated with VSMC migration (Pilop, Aregger et al. 2009). These proteins may contribute to the pathogenesis of MFS-associated aneurysms (Pilop, Aregger et al. 2009).

Several proteins have been found to be downregulated in MFS, including Vitamin-D binding protein (DBP), fructose-bisphosphate aldolase C (ALDOC), apolipoprotein A-I (ApoA-I), serum amyloid P (SAP), TAGLN, and haemoglobin beta/alpha chain (HBB/HBA) (Pilop, Aregger et al. 2009). Previous studies have shown that HBA1 can be expressed by EC, and it is involved in NO release and diffusion within the vessel wall (Straub, Lohman et al. 2012, Butcher, Johnson et al. 2014). Notably, TAGLN demonstrates contrasting protein expression patterns within different proteomics studies and transcriptomics studies, which may be due to the distinct experimental models, and requires further investigation (Pilop, Aregger et al. 2009, Granata, Serrano et al. 2017, Iosef, Pedroza et al. 2020).

MFAP4 has been shown to be associated with ECM remodelling and is significantly elevated in MFS compared to non-MFS individuals. Its upregulation is associated with excessive TGFβ signaling and can be reversed by ALK receptor inhibitors, as

demonstrated in a glycoproteomic study comparing MFS human aortic tissue to non-MFS cohorts (Yin, Wanga et al. 2019). This underscores the role of TGFβ in regulating MFAP4 expression in MFS. Additionally, proteoglycans such as aggrecan (PGCA/ACAN) and versican (CSPG2/VCAN) have been found to be elevated in MFS (Yin, Wanga et al. 2019). These proteoglycans can be cleaved by ADAMTS enzymes, demonstrated by MFAP4 knockdown (Yin, Wanga et al. 2019). ADAMTS1 was significantly downregulated after MFAP4 knockdown, as well as VCAN and FBN1, but an increased level of mRNA for *ELN* was observed (Yin, Wanga et al. 2019). Overall, MFAP4 appears to assist in FBN1 binding and colocalization, but excessive expression of MFAP4 can impact ECM integrity and elastin fibre formation, which may in turn contribute to MFS TAAD formation (Yin, Wanga et al. 2019).

Proteomics studies have highlighted the dysregulation of proteins associated with TGFβ signaling in MFS. Proteins such as PAI-1, connective tissue growth factor (CTGF), TAGLN, MYH11, and smoothelin (SMTN) were found to have higher expression in both young and aged MFS animals compared to controls (Parker, Stotland et al. 2018). This aligns with observations from transcriptomics studies. Moreover, proteins like Rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR) and integrin-linked kinase (ILK) were elevated in MFS and associated with increased TGFβ and its downstream MAPK signaling by phosphorylation (Parker, Stotland et al. 2018). Pharmacological inhibition of integrin-linked kinase has been demonstrated to inhibit RICTOR and PI3K/AKT signalling (Parker, Stotland et al. 2018). Crosstalk between ILK and integrin was demonstrated in MFS with elevated expression of ITGAV/ITGB3, corresponding with the results from Iosef et.al. (2020) (Parker, Stotland et al. 2018, Iosef, Pedroza et al. 2020). These findings suggest that TGFβ signaling plays an important role in MFS pathogenesis. The proteomic changes in MFS from previous studies are summarized in Figure 2.7.

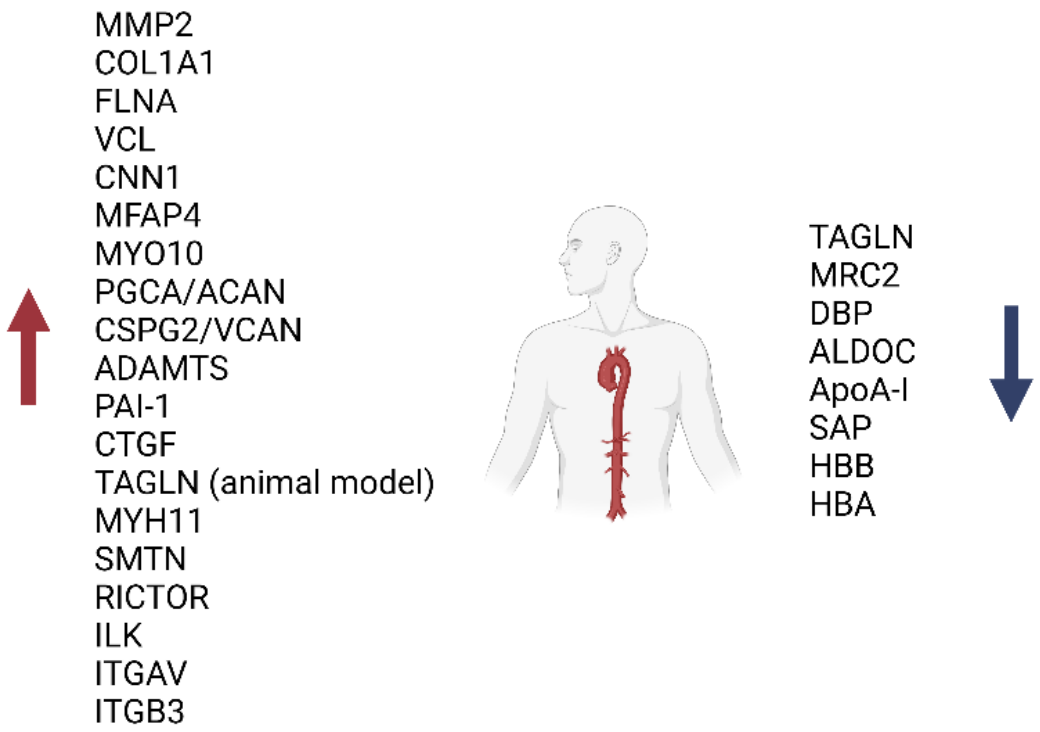


Figure 2.7 Evidence of proteomic regulation in MFS pathology.

2.6.3 microRNA

2.6.3.1 MicroRNA biogenesis and function

MicroRNAs (miRNAs/miRs) are small, noncoding RNA approximately 22 nucleotides in length that control post-transcriptional activities in biological processes (O'Brien, Hayder et al. 2018). The mechanism of miRNA regulation involves binding to complementary mRNA 3'-untranslated regions (UTRs) to repress or degrade the target mRNA, preventing protein translation (Macfarlane and Murphy 2010, Zhang and Wang 2017).

Canonical human miRNA maturation can be described as a two-step process (Figure 2.8). First, a hairpin-shaped primary miRNA (pri-miRNA) is generated from the miRNA gene in the nucleus, which is later exported to the cytoplasm to form precursor miRNA (pre-miRNA). Secondly, the pre-miRNA binds to Dicer in the cytoplasm to form the miRNA/miRNA* duplex. Generally, one of the miRNA strands is incorporated into an RNA-induced silencing complex (RISC), and the mature miRNA will then bind to its target mRNA (Macfarlane and Murphy 2010, O'Brien, Hayder et al. 2018). Once the miRNA-mRNA interaction occurs, mRNA degradation or translation inhibition is initiated (Zhang and Wang 2017).

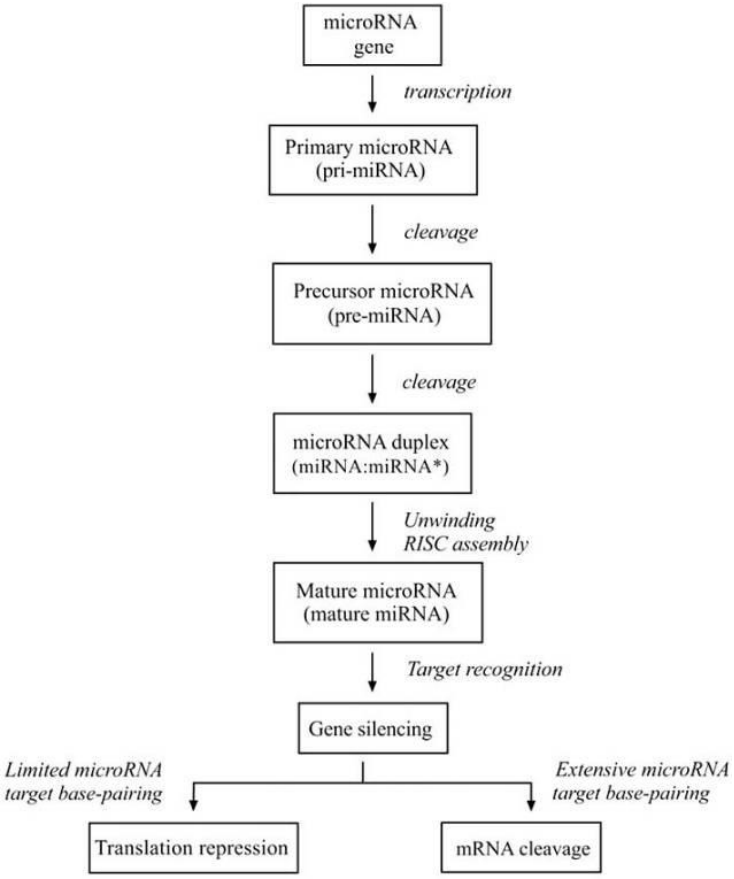


Figure 2.8 miRNA maturation process (Macfarlane and Murphy 2010).

2.6.3.2 miRNA in MFS

The post-transcriptional regulation characteristics of miRNA allows epigenetic studies to search for MFS TAA biomarkers that may regulate gene expression and alter signalling pathways. In past studies, several miRNAs have been implicated in the MFS mechanism, regulating the inflammatory response, VSMC turnover, ECM homeostasis, and TGFβ signalling regulation (Merk, Chin et al. 2012, Abu-Halima, Kahraman et al. 2018, D'Amico, Doldo et al. 2020).

In a peripheral blood study in MFS, 11 miRNAs have been identified in MFS patients, namely miR-1234-3p, miR-151-5p, miR-200c, miR-024, miR-30e, miR-324-5p, miR-362-5p, miR-500b, miR-502-3p, miR-627, and miR-331-3p, which are believed to target *DYSF, GBP2, LIMK2, MMP9, MX1*, and *POT1* mRNA. Pathway analysis demonstrated that the target genes of these altered miRNAs were involved in JAK-STAT, integrin, apoptosis, and angiogenesis pathways (Abu-Halima, Kahraman et al. 2018). These results demonstrate that cellular responses and inflammatory pathways are likely targeted by miRNA-regulation in MFS pathogenesis.

Another study investigated the differences in miRNA expression in TAA between MFS and non-MFS patients using human aortic tissue samples, which identified 25 miRNAs that are dysregulated in MFS patients compared to non-MFS TAA patients, namely, let-7f-5p, miR-101-3p, miR-125a-3p, miR-199b-5p, miR-217, miR-25-3p, miR-27a-3p, miR-27b-3p, miR-28-3p, miR-29c-3p, miR-302d-3p, miR-30d-5p, miR-338-3p, miR-378a-3p, miR-378i, miR-4461, miR-525-3p, miR-592, miR-614, miR-26a-5p, miR-29b-3p, miR-145-5p, and miR-632. These deregulated miRNAs demonstrate correlations with TGFβ, ECM-receptor interaction, Hippo, adherens junction, and focal adhesion signalling, targeting genes that regulate the inflammatory response, cellular response, ECM regulation, and activation of TGFβ (D'Amico, Doldo et al. 2020). These findings further illustrate the regulatory power of miRNAs and the potential biological processes regulated by miRNAs in MFS. However, since the comparison is between MFS and non-MFS TAA, the baseline expression of these miRNAs might differ in healthy controls. Nevertheless, this provides insight into miRNA regulation in MFS TAA pathogenesis compared to non-MFS TAA.

Finally, Merk et al. (2012) identified that miR-29b plays an essential role in early aneurysm formation in a MFS mouse model, where increased miR-29b expression is associated with increased cellular apoptosis and decreased ECM modulation (elevated MMPs and elastin fragments) in an animal model. miR-29b was shown to promote TAA formation through the TGFβ pathway. Moreover, miR-29b inhibitors NF-κB and c-Myc demonstrated concordantly downregulated expression in MFS. This study confirmed a likely regulatory role of miR-29b in TAA development. Overexpression of miR-29b has been observed in MFS animals, and early inhibition of miR-29b attenuates TAA formation, suggesting a potential therapeutic target for TAA intervention (Merk, Chin et al. 2012).

2.6.4 DNA methylation

2.6.4.1 DNA methylation and regulation

DNA methylation is an epigenetic mechanism that transfers a methyl group to the C5 position of the DNA cytosine ring, which is linked by a phosphate to a guanine nucleotide (CpG dinucleotide). This process is mediated by DNA methyltransferases (DNMTs) (Lanata, Chung et al. 2018). DNA methylation plays a pivotal role in development, genomic imprinting, X-chromosome inactivation, and transcriptional suppression, depending on the methylation position (CpG site) within the gene (Jin, Li et al. 2011). Generally, hypomethylation (reduced methylation)

within the gene's promoter region activates gene expression and *vice versa*, while hypermethylation (increased methylation) within the gene's body region promotes gene expression and *vice versa*. The pattern of DNA methylation occurs unevenly across the genome and has been shown to be cell and tissue-specific (Lanata, Chung et al. 2018).

Regions within the genome that have a high frequency of CpG sites are defined as CpG islands (CGIs) (Muñoz, Ares et al. 2022). Approximately 70% of CGIs are associated with gene promoter regions; however, CGIs can also be found within intragenic (inside gene bodies) or intergenic (between genes) regions (Deaton and Bird 2011). Furthermore, CpG sites can be further divided into different regions related to the position of the CGI, such as shelves, shores, islands, and opensea regions. The impact on regulation of genes varies depending on the position of each CpG site, although the current understanding of how each position is associated with different cell or tissue types is not fully elucidated (Figure 2.9) (Rechache, Wang et al. 2012, Bibikova 2016).

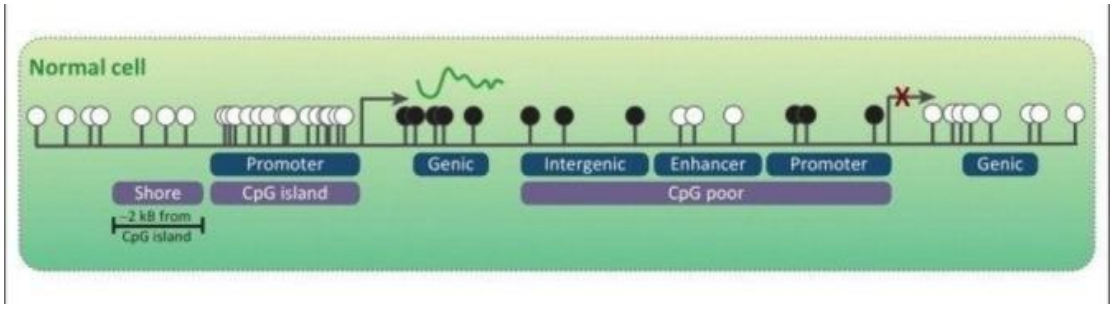


Figure 2.9 DNA methylation regulation mechanism. White CpG sites indicate hypomethylation, black CpG sites indicate hypermethylation. The plot demonstrates that hypomethylation in the promoter region activates gene expression however, hypermethylation inhibits such a process (Lanata, Chung et al. 2018).

Key DNA methylation enzymes include DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L. Each enzyme plays a single or dual role in methylation maintenance and *de novo* methylation during DNA synthesis (Jin, Li et al. 2011). This methylation process can be reversed to an unmethylated state by ten eleven translocation (TET) proteins, which are also cell and tissue-specific (Lanata, Chung et al. 2018). The majority of CpG sites remain relatively stable throughout a lifetime; however, 8-18% of CpG sites may change over time, potentially contributing to disease formation. During disease formation, DNMTs may lose their ability to maintain a methylation

mark, or TETs may remove such a methylation mark, resulting in demethylation at specific CpG sites, which, in turn, affects gene expression. Dysregulation of DNMTs is linked to environmental factors, oxidative stress, as well as interactions with histone modification (another epigenetic regulation factor) and miRNA regulation (Figure 2.8) (Lanata, Chung et al. 2018). For example, the knockdown of miR-290 indirectly regulates the expression of DNMT3A and DNMT3B, which, in turn, decreases histone methylation at H3K9, indicating crosstalk between these epigenetic regulatory mechanisms (Moore, Le et al. 2013).

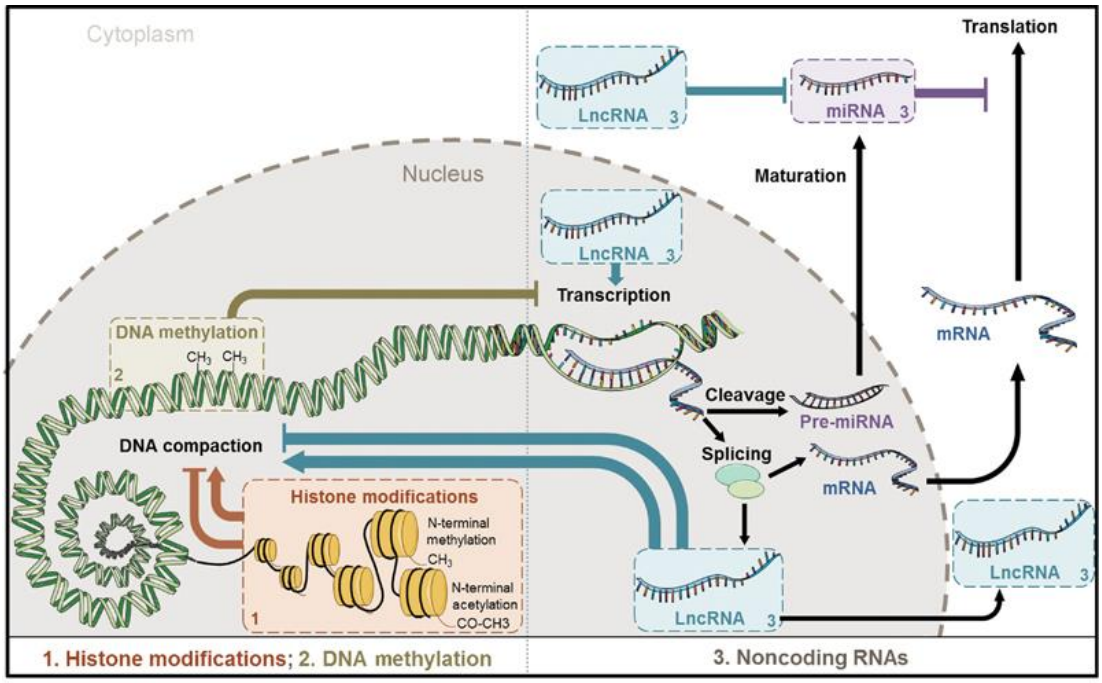


Figure 2.10 Epigenetic regulation mechanism overview (Boileau, Lindsay et al. 2018).

2.6.4.2 DNA methylation in MFS

Due to the complex regulatory mechanism of DNA methylation, the investigation of DNA methylation in MFS is not well-documented or well-defined. Nevertheless, some studies have examined DNA methylation in MFS, revealing alterations in CpG sites or CGIs, indicating a potential regulatory role of DNA methylation as an epigenetic regulator in the progression of MFS.

In an MFS TAA study of DNA methylation patterns in peripheral blood leucocytes, a total of 28 CpG sites have been identified, showing a significant association with aortic diameter. Among the CpG sites identified were *HDAC4* (cg10045864), *IGF2BP3* (cg05852760), *CASZ1* (cg24073777), *SDK1* (cg07829265), *PCDHGA1* (cg05149776), *DIO3* (cg05265042), and *PTPRN2* (cg21375490), all of which had

previously been associated with cardiovascular disease (van Andel, Groenink et al. 2021). However, the specific positions of these CpG sites were not defined in the study, making the regulatory mechanism unclear (van Andel, Groenink et al. 2021).

Furthermore, *FBN1*-specific DNA methylation in MFS indicated that the CGI shore position was altered in MFS compared to controls, leading to the suppression of *FBN1* mRNA expression in human iPSCs. This suggests a role for DNA methylation regulation in the *FBN1* gene in MFS (Arai, Umeyama et al. 2020). Additionally, in an MFS TAA animal model, the methyltransferase EZH2 was found within the promoter and body regions of the *TAGLN* gene, suppressing SM22α (also known as TAGLN) expression associated with TAAD. Inhibition of EZH2 restored SM22α function and contractile protein expression, attenuating TAA in MFS mice. This suggests that EZH2 might be a potential therapeutic target for TAA (Lino Cardenas, Kessinger et al. 2018).

In conclusion, DNA methylation has much to reveal in MFS TAA. Profiling the DNA methylation landscape is a crucial first step in understanding the involvement of DNA methylation mechanisms in MFS.

Chapter 3: Research Design

3.1 METHODOLOGY AND RESEARCH DESIGN

3.1.1 Materials

3.1.1.1 Tissue samples

All tissues were collected and stored at the Sydney Heart Bank (Charles Perkins Centre) in cryovaults at temperatures between -170°C and -180°C. Samples were collected within approximately 10 minutes after surgical removal of the aorta segment or snap-frozen within 10 minutes after the donor's death only when formal consent was obtained from the patient. Then samples were subsequently stored in 5x5mm segments. The handling of all tissues was conducted professionally and uniformly.

3.1.1.2 MFS patient samples and collection

Tissue samples were collected from patients who were diagnosed with MFS according to the revised Ghent nosology, who had undergone aortic replacement surgery from the Aortic Disease Clinic a at Royal Prince Alfred Hospital (RPAH), Sydney. All ascending aortic tissue samples from MFS patients were obtained immediately in the operating theatre and subsequently treated with RNA-later solution for 24 hours before long-term storage.

3.1.1.3 Control patient samples and collection

Control samples were obtained by donors with no known CAD or CAD risk factors, no family history of CAD, and were not deceased from cardiovascular complications. One of the criteria for tissue donation was a BMI of less than 30. The donor hearts were collected due to unsuccessful transplantation arrangements, which included reasons such as transportation logistics, immune incompatibility, and donor-recipient size mismatch. Subsequently, these hearts and aortic tissues were snap-frozen after collection in liquid nitrogen at -196°C and then stored at the Sydney Heart Bank.

3.1.2 Online open dataset

3.1.2.1 Cell and tissue expression

Cell expression was obtained from The Human Protein Atlas, which provides an overview of gene/protein expression in various cell types (https://www.proteinatlas.org/) (Karlsson, Zhang et al. 2021). The Human Protein Atlas Project is based in Sweden and initiated in 2003, offers a comprehensive reference for gene and protein expression in cells and tissues. It also provides a brief introduction to the implied biological processes and functions of the genes/proteins and is funded by the Knut & Alice Wallenberg Foundation.

Aortic expression data were obtained from the Genotype-Tissue Expression (GTEx) open database, which serves as a reference for tissue-specific gene expression in 432 human aorta samples, offering a comparative understanding of gene expression in healthy aortic tissue (https://gtexportal.org/home/). The GTEx project received support from the Common Fund of the Office of the Director of the National Institutes of Health, as well as from NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. The data used for the analyses described in this thesis were obtained from the GTEx Portal in the year 2023.

3.1.2.2 Signalling pathway and biological process and function

Pathway enrichment analysis was conducted using Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.kegg.jp/). The KEGG project is developed by Kanehisa Laboratories in 1995 and funded by Institute for Bioinformatics Research and Development (BIRD) of the Japan Science and Technology Agency (JST), is a publicly available online resource database that contains genomic information for various organisms. It provides information on molecular biological processes, such as signalling pathways and chemical substance networks, as well as drug and disease information. Additionally, KEGG facilitates the network mapping of disease mechanisms (Kanehisa and Goto 2000). KEGG pathway analysis aids in understanding molecular functions and cellular signal transduction mechanisms based on sequencing data, offering valuable insights into gene/protein regulatory processes (Kanehisa and Sato 2020).

For the analysis of biological processes, cellular components, and molecular functions in omics data, Gene Ontology (GO) analysis was applied. GO, developed in 1999, is funded by the National Human Genome Research Institute (US National

Institutes of Health) and co-funded by NIGMS. It provides a comprehensive overview of molecular functions for gene/protein regulation (https://geneontology.org/) (Ashburner, Ball et al. 2000, The Gene Ontology, Aleksander et al. 2023).

All KEGG pathway and GO functional analysis were performed using RStudio with the ClusterProfiler package (Yu, Wang et al. 2012, Wu, Hu et al. 2021).

3.1.2.3 microRNA target prediction

miRDB was utilized for miRNA-mRNA target prediction, and it is an online database designed to facilitate target prediction. The database employs computational machine learning algorithms, specifically the support vector machine (SVM), to map miRNA-mRNA targets. This mapping is based on the analysis of a vast dataset consisting of 1.5 billion reads from 54 RNA samples, which allows for the examination of downregulated transcription activities caused by overexpressed miRNAs. Additionally, the algorithm in miRDB utilizes publicly available CLIP-seq data to validate whether these miRNA-mRNA pairs belong to the same RISC complex (Liu and Wang 2019, Chen and Wang 2020). The algorithm also categorizes miRNA target pairs into five species, with the human database being used in this study.

3.2 RESEARCH DESIGN

The samples were stored in a -80°C freezer before RNA, protein, and miRNA extraction.

3.2.1 RNA and miRNA Extraction

One 5x5 segment per tissue sample was used for RNA and miRNA experiments, and the extraction was performed using the RNeasy Plus Universal Mini Kit from Qiagen (Cat. No. 73404). Tissues were homogenized using the TissueLyser LT (Qiagen), and the extraction process followed the manufacturer's manual. All extracted RNA and miRNA were eluted in nuclease-free water and stored in a -80°C freezer until subsequent experiments.

For microRNA extraction, the microRNA purification protocol from the same RNeasy Plus Universal Mini Kit was followed (Appendix C in the manufacturer's manual).

The initial quality examination of the RNA and miRNA for subsequent experiments was assessed using a Nanodrop™ 2000 Spectrophotometer (ThermoFisher) to examine the 260/280 nm and 260/230 nm ratios.

3.2.2 Protein Extraction

Tissue samples were initially powdered on dry ice, and 10 mg of aortic tissue was weighed and stored in a -80°C freezer prior to protein extraction. Firstly, 100 µL of SDC Lysis buffer from Wako (Cat. No. 194-08311) was added to each tissue sample, followed by heating to 95°C at 1000 rpm using the Eppendorf Thermomixer C with a heated lid for 10 minutes. The samples were then cooled to room temperature and sonicated in 2 mL Eppendorf tubes at 80% amplitude and 20°C setting, with 15 seconds ON and 15 seconds OFF cycles for 5 minutes using the QSonica sonicator (Bioke). Subsequently, the samples were centrifuged at 14,000 rpm for 10 minutes at room temperature. A 90 µL supernatant was collected and transferred to pre-labelled 1.5 mL Eppendorf tubes. A 1:10 dilution in phosphate-buffered saline (PBS) pH 7.0 was performed in a 0.5 mL Eppendorf tube.

For protein quantification, the BCA assay was used with the PierceTM BCA Protein Assay kit (ThermoFisher, Cat. No. 23225). Standard protein and a blank consisting of 6 µL SDC in 54 µL PBS were used for the aorta protein samples. Specifically, 25 µL of each standard and sample, along with 200 µL of buffer A and B, were added in duplicate to 96-well 1 mL deepwell plates (Eppendorf) and incubated for 30 minutes at 37°C. The protein concentration was measured in a plate reader at 562 nm, and the results were calculated and recorded in a Microsoft Excel sheet. The extracted protein was then stored in a -80°C freezer and is ready for profiling at the Sydney Mass Spectrometry facility.

3.2.3 RNA-Seq Experiment

RNA extraction and initial quality examination were carried out as described in Chapter 3.2.1. Further quality control of the RNA was assessed using a 2100 Bioanalyzer instrument (Agilent), which provided information such as the RNA Integrity Number (RIN), electropherogram, and concentration. Data were collected

using the 2100 Expert Software (Agilent). The criteria for acceptable sample quality were RIN > 6 for control snap-frozen samples and RIN > 8 for RNA-later treated samples. Electrophoretic assessment was performed to examine the RNA peaks (Puchta, Boczkowska et al. 2020).

Aliquots of each RNA sample were then sent to the Ramaciotti Centre for Genomics, University of New South Wales, Sydney, Australia, for RNA sequencing. The experiment was conducted using the Illumina Stranded Total RNA Prep with Ribo-Zero Plus kit (Illumina) and the NovaSeq 6000 S4 platform (Illumina), following the manufacturer's protocol.

3.2.4 Proteomics Experiment

Extracted protein (as detailed in Chapter 3.2.2) was thawed at 95°C with occasional vortexing. To prepare the samples for mass spectrometry, each sample was brought to a volume of 50 µL, containing 20 µg of protein. This was achieved by adding 1 µL of 1M CAA (Sigma-Aldrich, Cat. No. C0267), 1 µL of 0.5M TCEP (ThermoFisher, Cat. No. 77720), and SDC buffer to each lysate in 1.5 mL Eppendorf tubes. The protein concentration was determined using the BCA method.

The samples were then heated to 95°C for 10 minutes at 1000 rpm using the Thermomixer C with the heated lid. This step served to denature, reduce, and alkylate the proteins. After heating, the samples were allowed to cool to room temperature, at which point 150 µL of MilliQ water was added to each sample, followed by vortexing. Trypsin (Sigma-Aldrich, Cat. No. T6567) was added at a 1:50 ratio, and the samples were vortexed again. They were then incubated at 37°C for 16 hours at 1000 rpm using the Thermomixer C.

Following incubation, the tubes were placed on a room temperature rack, and 250 µL of a mixture containing 99% ethyl acetate (Millipore, Cat. No. 1.03649) and 1% TFA (Sigma-Aldrich, Cat. No. T6508) was added to each tube. The samples were vortexed and then spun in a centrifuge for 1 minute at 18,000g at room temperature. The bottom layer of each sample was collected for mass spectrometry preparation.

Eppendorf 200 µL tips were loaded with punched SDB-RPS discs (Sigma-Aldrich, Cat. No. 66886-U) and mounted into a 3D-printed holder. The empty tips were washed three times separately with 100 µL of 100% acetonitrile (Fisher Chemical, Cat. No. A955), 30% methanol (Ajax Finechem, Cat. No. AJA2314) with 1% TFA,

and 0.2% TFA, with each wash followed by centrifugation at 1,000g for 1 minute, 3 minutes, and 3 minutes, respectively. Waste was discarded after each wash.

Next, 100 µL of the bottom layer of the samples was loaded into the tips and spun at 1,000g for 3 minutes. This process was repeated for another 100 µL of peptides. After loading, the samples were washed three times separately with 100 µL of 99% ethyl acetate with 1% TFA, 99% ethyl acetate with 1% TFA, and 0.2% TFA, with each wash followed by centrifugation at 1,000g for 3 minutes. Waste was discarded after each wash.

The samples were collected after the washes by placing a clean 96-well plate under the tips. Then, 100 µL of a solution containing 5% ammonium hydroxide (Fluka, Cat. No. 44273) with 80% acetonitrile was added to the tip. The samples were subjected to a 1,000g spin for 5 minutes to elute the peptides.

The eluted samples were dried using Speedvac Vacuum concentrators (ThermoFisher) at a maximum temperature of 45°C, using the V-AP mode for 1 hour until the peptides were completely dry. The dried peptides were then resuspended in 60 µL of 5% formic acid at room temperature. They were vortexed for 5 minutes, sealed with a thermos silicon PCR mat, and stored at 4°C. These samples were subsequently sent to the Sydney Mass Spectrometry facility for proteomic profiling.

3.2.5 miRNA Experiment

MiRNA profiling was conducted using fixed-content Taqman® OpenArray™ Plates (ThermoFisher). First, cDNA was synthesized following the manufacturer's manual using the TaqMan® Advanced miRNA cDNA Synthesis Kit (Cat. No. A28007). Then, the TaqMan® OpenArray™ Human Advanced MicroRNA Panel (QuantStudio 12K Flex) (Cat. No. A32710) was loaded onto the plates using the OpenArray™ AccuFill™ instrument.

Real-time PCR was performed on a QuantStudio™ 12K Flex instrument, and the data were collected using QuantStudio™ 12K Flex software (ThermoFisher). All steps were executed following the manufacturer's protocol (Pub. No. MAN0016125).

3.2.6 DNA methylation Extraction & Experiment

DNA extraction was previously conducted in our laboratory by other researchers, and the extracted DNA samples were subsequently sent to The Australian Genome

Research Facility (AGRF) for DNA methylation profiling. The DNA CpG sites in aortic tissue samples were profiled using the Infinium MethylationEPIC BeadChip array (Illumina, Cat. No. WG-317-1001). Raw data were obtained in the form of idat files and stored in our laboratory and later analysed for this study.

3.3 ANALYSIS

All analysis was conducted in Microsoft Excel and RStudio (version 2023.03.1+446, R version 4.3.0).

3.3.1 RNA-Seq data

The RNA-Seq raw data were initially processed by bioinformaticians from the Precision Cardiovascular Laboratory from the Charles Perkins Centre, The University of Sydney, Sydney, NSW, Australia. They assessed initial data quality, converted the RNA-Seq FASTQ files into a readable, unnormalized counts matrix datasheet using the RStudio package Rsubread, and aligned them with the human genome h38 (Liao, Smyth et al. 2019). The resulting data were saved in a Microsoft Excel spreadsheet. Subsequently, data quality control, normalization, log transformation and analysis were performed using RStudio with the DESeq2 package (version 1.40.1) (Love, Huber et al. 2014).

3.3.2 Proteomics data

The proteomics raw data were obtained as .raw files from the mass spectrometry facility, utilizing a Q-Exactive Fusion Lumos mass spectrometer with HCD fragmentation (ThermoFisher). Subsequently, the data were processed in a manner similar to RNA-Seq data by bioinformaticians from the Precision Cardiovascular Laboratory, employing the integrated quantitative proteomics software DIA-NN104 in a Data-Independent Acquisition (DIA) mode (version 1.7) (Li, Parker et al. 2020).

Default filters were established, requiring all peptides to be between 7 and 30 residues long, with a precursor charge range set between 1 and 4. The precursor m/z range was defined as 350-1650, while the fragment ion m/z range was set to 300-2000. Carbamidomethylating of cysteine was set as a modification, and N-terminal acetylation, as well as oxidation of methionine, were designated as variable modifications. One missed cleavage was permitted (Harney, Cielesh et al. 2023).

Peptide quantification was determined by the intensities of fragment ions associated with each peptide, employing both peptide-centric and spectrum-centric strategies utilizing a deep learning neuron network method (DIA-NN) (Demichev, Messner et al. 2020). Spectra were matched using 20 variable isolation width search windows and comparing the actual fragments to in silico prediction fragmentation patterns (Demichev, Messner et al. 2020, Harney, Cielesh et al. 2023). Protein inference was conducted according to the corresponding gene. (Harney, Cielesh et al. 2023) A pooled sample served as the quality control standard for comparing peptide abundance across samples, with RT-dependent normalization applied for cross-run normalization. Finally, the DIA-NN output .tsv file of proteomics raw data was transformed into a readable expression matrix datasheet saved in a Microsoft Excel spreadsheet.

The criteria for acceptable quality encompassed filtering with a 1% false discovery rate (FDR) at a unique protein level for each sample, ensuring an average unique protein reading exceeding 4,000 proteins, and precluding any sample from significantly falling below the average reading (Demichev, Messner et al. 2020). Moreover, proteins with abundances examined in over 75% of readings per protein across the dataset were included (Hamid, Zimmerman et al. 2022). Duplicate protein values were amalgamated as one, employing mean values across the readings.

Subsequently, the proteomics dataset was median normalized and analysed utilizing the RStudio package limma (version 3.56.2). Log transformation was applied within the limma package (Ritchie, Phipson et al. 2015).

3.3.3 miRNA data

The miRNA raw data were obtained and analysed using ThermoFisher Connect™. Global normalization was applied during the analysis. Additionally, filtering criteria were set, including AMP<1.24 and Cq confidence<0.6, to refine the raw data. Any miRNA with a reading that fell below the specified threshold was excluded from the analysis. Other analytical settings followed the default ThermoFisher Connect™ configuration.

3.3.3.1 Data imputation

The raw data obtained from ThermoFisher ConnectTM underwent post-global normalization. For miRNAs marked as 'Undetermined' due to missing data in ThermoFisher ConnectTM, computational imputation was applied for two purposes: 1) to further filter out uninformative miRNAs and 2) to impute values for the 'Undetermined' data points to facilitate subsequent analysis.

Handling undetermined miRNA values can indeed be challenging due to various factors influencing the outcome, including technical issues, low or null miRNA expression, and other variables. One commonly used approach is assigning the 'Undetermined' value to threshold cycle (CT) = 40. However, this approach can introduce significant discrepancies, especially when the average reading is around the midpoint (e.g., CT = 25). Setting the undetermined reading to CT = 40 can artificially inflate the average reading of the samples, potentially leading to inaccurate results (de Ronde, Pinto et al. 2016).

To address this issue and enhance the quality of the raw data, imputation was performed using the following criteria in Python:

Imputation was carried out via Python in the following steps:

1. Separated the data into two groups and processed them individually.
2. Calculated the percentage of missing values for each miRNA in each group.
3. Excluded miRNAs with more than 75% missing values in each group.
4. Imputed missing values with the mean value of the group if there were less than 25% missing values for a given miRNA.
5. Merge the two datasets, retaining only miRNAs with readings in both groups for further analysis.

3.3.3.2 miRNA analysis

Statistical analysis for miRNA was carried out using p-values and $\Delta\Delta$CT to determine significant miRNAs. Although no housekeeping miRNA was utilized in this study, $\Delta\Delta$CT was calculated by subtracting the mean CT value of MFS patients from the mean CT value of the controls. The reason for not using a housekeeping miRNA is that no valid miRNA has been discovered in cardiovascular tissue research that can serve as a suitable housekeeping miRNA (Fichtlscherer, De Rosa

et al. 2010, Felekkis and Papaneophytou 2020). Therefore, ΔΔCT was calculated as described above and represents the fold change in this study. Microsoft Excel was employed to calculate the p-values using the Student's t-test with ΔΔCT, and a cutoff value of raw p-value=0.05 was applied.

3.3.3.3 Target prediction

Significant miRNAs were then input into online target prediction tools to obtain gene targets for each significant miRNA. miRDB was used with the following filters: 1) Human species only, 2) Excluding miRNAs with more than 2000 targets in the genome, and 3) Excluding miRNAs with prediction scores below 90.

3.3.3.4 miRNA family analysis

MiRNA family information for each miRNA was obtained from RStudio using Package miRBaseConverter (version 4.8.1) (Xu, Su et al. 2018), and miRNA family data were sourced from miRBase Version 21 (Griffiths-Jones 2006).

3.3.4 DNA methylation

The DNA methylation raw data folders were obtained as idat files from The Australian Genome Research Facility (AGRF). These raw files were processed in RStudio using ChAMP package (version 2.8.3) for quality control and differential CpG site analysis (Tian, Morris et al. 2017). The initial filtering, performed with the built-in function of the ChAMP package, excluded CpG probes with a detection p-value >0.01, those with less than 5% of samples per probe, and non-CpG probes (Tian, Morris et al. 2017). Data quality control and Beta mixture quantile dilation (BMIQ) normalization method were conducted using built-in ChAMP functions, and the analysed data was then exported to a Microsoft Excel spreadsheet for further analysis (Teschendorff, Marabita et al. 2013).

3.4 ETHICS AND LIMITATIONS

All samples in this study were collected and analysed with participant's written consent. The University approval number for tissue collection is 2021/122 and the Sydney Local Health District approval number is X-17-0353.

Chapter 4: Transcriptomics in Marfan Syndrome

4.1 INTRODUCTION

As RNA is the key element in gene transcription, study of RNA expression has the capacity to provide a dynamic view of biological processes and signal transduction within disease pathogenesis. As summarised in Chapter 2.5.1, previous transcriptomics studies in MFS have implicated the possible involvement of several biological processes that may contribute to disease progression, including:

1. Inflammatory response (including increased inflammatory gene expression in VSMC, inflammatory cells and fibroblasts)
2. Oxidative stress response (including upregulated heat shock proteins)
3. VSMC phenotype switching (VSMC marker genes alteration)
4. ECM degradation and remodelling (downregulation of ECM integrity maintenance genes)

Transcriptomics studies may help elucidate disease mechanisms by examining the signalling pathways involved in disease pathogenesis. As outlined in Chapter 2.6, there is evidence, of varying strength, for the involvement of multiple cell signalling pathways in the pathogenesis of TAA. To date, the most extensively investigated pathway in MFS has been the TGFβ pathway. Recently, our group has reviewed evidence that, in addition to TGFβ, the Wnt, PI3K/Akt, Ang II, and Notch pathways may have a role in TAA pathogenesis, particularly in relation to VSMC and EC behaviour (Dong, Malecki et al. 2023). An important conclusion drawn from this review is that there is likely to be significant crosstalk between these pathways.

An initial step in understanding the key regulatory mechanisms in any disease is to identify changes in gene expression. This allows us to identify the signal transduction pathways and related biological processes that may be involved in disease development, as suggested by Sebastian-Leon, Vidal et al. (2014). Subsequently, KEGG enrichment pathway analysis and GO cell process analysis can support investigation of the biological functions related to disease development (Sebastian-Leon, Vidal et al. 2014). The research described in this chapter aims to

provide evidence of changes in gene transcription affecting different signalling pathways and biological processes in individuals with MFS TAAD.

This chapter provides the results of a genome-wide RNA expression analysis conducted on aortic tissue samples obtained from both normal controls and individuals diagnosed with MFS TAAD.

The specific aims were:

1. Compare RNA expression in surgically resected human MFS ascending aortic tissue against control ascending aortic tissue samples.
2. Correlate the observed changes in RNA expression with cell signalling pathways.
3. Identify the likely cellular and biological processes altered in TAA in MFS.

4.2 METHODS

4.2.1 RNA extraction and RNA expression profiling

The extraction of RNA from aortic tissue was performed as described in Method Section 3.2.1. The RNA expression profiles were measured by RNA-Seq, performed according to in-house protocols at the Ramaciotti Centre for Genomics, University of New South Wales, Sydney, Australia (detailed in Method Section 3.2.3).

4.2.2 Data analysis

Data analysis and quality control for RNA-Seq data are described in Method Section 3.3.1.

Multiple testing corrections were performed in the transcriptomics study. Uncorrected p-values were employed for primary analysis of significant differentially expressed (DE) genes and downstream pathway and biological analyses, with subsequent correction for the false discovery rate (FDR) according to Benjamini-Hochberg correction, to describe the confidence level associated with significant DE genes. The reason behind this dual approach is that the FDR procedure can be overly conservative when applied to large datasets, which may increase the likelihood of false negatives (Yamada, Okada et al. 2021). By using a dual

approach, this study aims to mitigate potential type 2 errors and retain valuable data, whilst still describing the FDR (Aguinis, Vassar et al. 2021).

Enrichment KEGG and GO analyses were performed on significant DE genes before and after multiple testing correction, following the method described in Method Section 3.1.2. Initially, all significant 2 fold change (FC) DE genes were submitted to enrichment analysis. Subsequently, up and downregulated genes were analysed separately in distinct enrichment KEGG analyses. Only signal transduction and few selected relevant pathways were mapped and presented, e.g., infectious human diseases and drug pathways were excluded.

4.2.3 Statistical analysis

All statistical analyses and plots were generated using RStudio as described in Method Section 3.3.1. Significant changes in RNA expression were determined based on a raw p-value < 0.05 (Wald test correction) and an over 2FC in expression (McDermaid, Monier et al. 2019). Multiple testing correction was using the Benjamini-Hochberg correction.

4.3 RESULTS

4.3.1 Patient characteristics

The demographics of tissue donors are summarised in Table 4-1. All MFS patients and control donors were used for both transcriptomic and proteomic studies. Echocardiography data were not available for control donors. All three MFS patients who experienced chronic dissection had a history of type B dissection (involving the descending aorta), with no impact on the ascending aorta.

Table 4.1 Patient characteristics for transcriptomics studies.

	Marfan	Control	p
n	15	11	
Age (years)	34 ± 11	39 ± 15	NS
Male:Female (n)	11 : 4	5 : 6	NS
BMI (kg/m^2)	27.9 ± 6.3	<30	

Aorta (mm)	50 ± 8		
LVEF (%)	61 ± 5		
Aneurysm (n)	12		
Chronic Dissection (n)	3		
Aortic Regurgitation (n)			
Nil	7		
Mild	6		
Moderate/Severe	2		

Note: aortic diameter was only available for 10 MFS donors.
BMI = body mass index; LVEF = left ventricular ejection fraction.

4.3.2 Comparative RNA expression in Marfan syndrome

A total of 43,799 genes were initially mapped from the RNA-Seq output, including all RNA, such as non-coding RNA and RNA antisense transcripts. After quality control, 28,197 genes were included in the subsequent analysis. Using the DEseq2 package, we identified 511 upregulated and 476 downregulated DE genes ($p < 0.05$, > 2FC). These findings are visualized in the volcano plot (Figure 4.1 A), where upregulated genes are shown in red and downregulated genes in blue. The top 10 most significant DE genes in MFS compared to controls, ranked by ascending p-value, are labelled in the plot.

The top 10 most significant DE genes remained significant after the FDR procedure according to Benjamini-Hochberg correction, presented in Figure 4.1 B. After applying the FDR, a total of 566 genes remained as significant DE genes (p. adjust 0.05, > 2FC), with 274 upregulated and 292 downregulated (Figure 4.1B).

Two heatmaps were generated to visualize the expression of significant DE genes (before and after FDR procedure) across the two groups, both results displayed a clear distinction between the MFS and control groups (Figure 4.1 B-C) with concordance of gene expression within groups.

Principal component analysis (PCA) was conducted using significant DE genes, both before and after the FDR procedure. A PCA plot is a dimensional reducing vector that illustrates the relationships between samples. It utilizes two perpendicular vectors, PC1 and PC2, to visualize the data in two dimensions. The presence of the groups in different panels does not significantly contribute to the interpretation but rather reflects the dimensionality reduction techniques employed.

The crucial insight obtained from the PCA plots is whether the significant DE genes possess the statistical power to distinguish and separate the samples based on these input variables. The PCA plots (Figure 4.1 E-F) reveal a clear and non-overlapping distinction between the two groups, which aligns with the heatmap results. The data markers on the PCA plots indicate individual patients in the MFS and control groups.

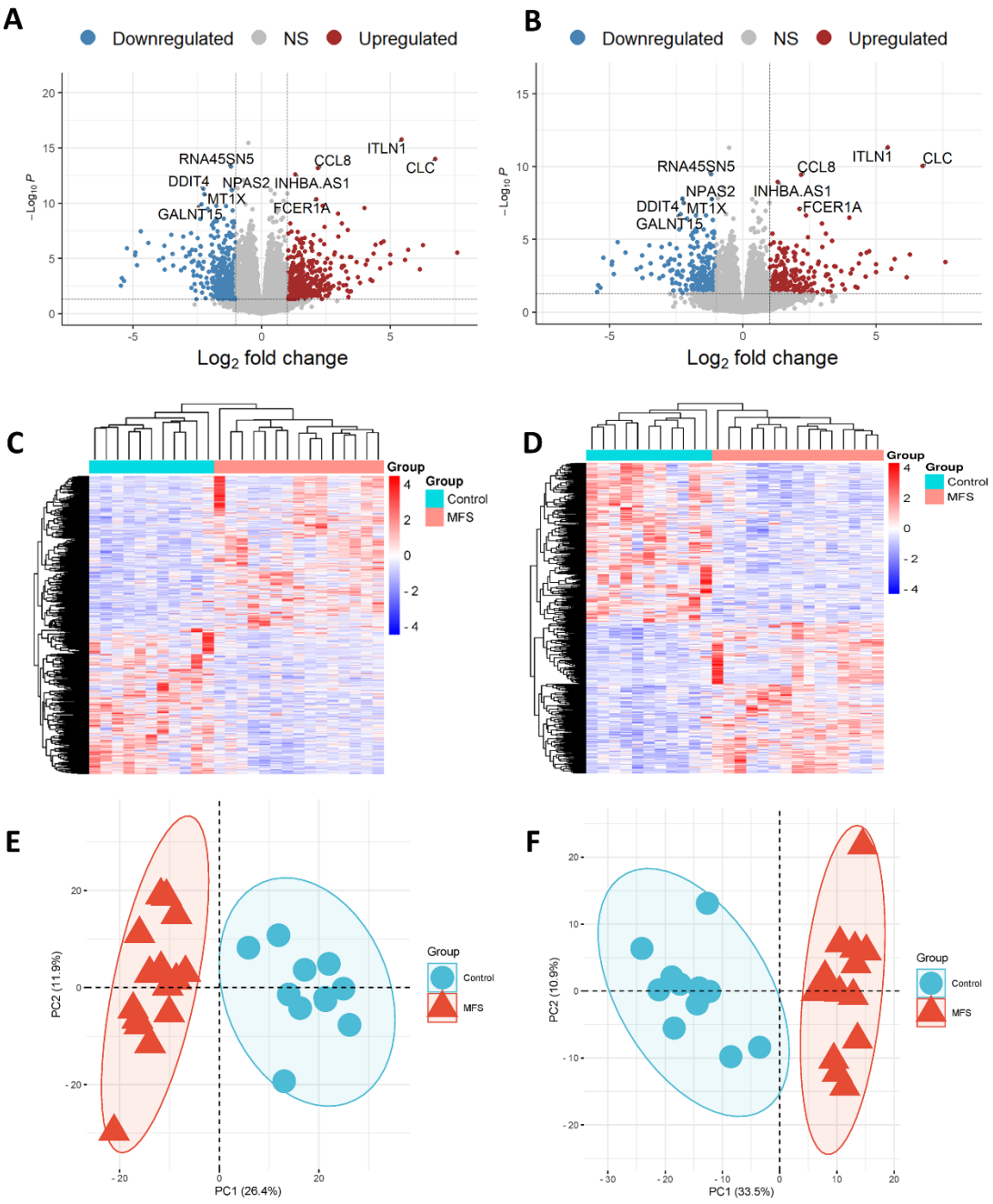


Figure 4.1 Visualization of significant DE genes between MFS patients and controls. A) Volcano plot of DE significant RNA by raw p-value, dots in blue indicate downregulated genes and dots in red indicate upregulated genes. B) Volcano plot of DE significant RNA by FDR. C) Heatmap of DE significant RNA by raw p-value, groups are coloured with aqua representing controls and salmon representing MFS patients. Blue indicates graduated downregulation and red indicates graduated upregulation of the gene. Data were scaled for the heatmap. D) Heatmap of DE significant RNA by FDR. E) PCA plot of DE significant RNA by raw p-value, aqua circles indicate controls and red triangles indicate MFS patients. F) PCA plot of DE significant RNA by FDR.

To initially assess the RNA dataset, the study compared the results with the 20 known highly transcribed genes within healthy human aortic tissue, according to the GTEx online database. GTEx is an open online database that documents RNA expression in 432 healthy aortic samples (See Method Section 3.1.2). In addition, FBN1 was also included given its role in the pathogenesis of MFS. Only one of the highly expressed genes approached a fold change cutoff of +/- 2FC (Log2FC<-1 or Log2FC >1). ELN had a log2FC = -0.97. Furthermore, widely recognized genes in TAA such as ECM gene MFAP4, and SMC contractile gene markers MYH11, and ACTA2, as well as IFITM3, VIM, GSN were significantly altered, even though the fold change did not reach the preset threshold of 2FC cutoff. This could be due to the baseline expression of these genes being high in the human aorta. Nonetheless, although below the 2FC cutoff, these changes could possibly still be functionally significant. Notably, the transcript level of FBN1 in the healthy aorta is relatively low compared to those of other highly transcribed genes, implying a lower transcription rate for FBN1 in this context.

Table 4.2 Highly transcribed gene in the aortic tissue from GTEx database compared to global RNA expression in MFS.

Entrez ID	Gene	Protein	Aorta Expression (TPM)	Log_2FC	p-value	adjust p
1191	CLU	Clusterin	1604	-0.12	0.52	0.77
2006	ELN	Elastin	1681	-0.97	0.0001	0.005
2335	FN1	Fibronectin1	3726	-0.51	0.06	0.26
4239	MFAP4	Microfibril Associated Protein 4	1778	0.75	2.9E-08	1.3E-05
1490	CCN2/ CTGF	Cellular Communication Network Factor 2	2043	-0.49	0.15	0.43
4629	MYH11	Myosin Heavy Chain 11	2357	0.44	0.0004	0.011
4256	MGP	Matrix Gla Protein	11940	-0.50	0.04	0.19
7169	TPM2	Tropomyosin 2	3155	-0.17	0.20	0.50
1292	COL6A2	Collagen Type VI Alpha 2 Chain	1878	-0.12	0.25	0.55
2316	FLNA	Filamin A	3732	0.15	0.17	0.45

59	ACTA2	Actin Alpha 2, Smooth Muscle	5736	0.52	0.0001	0.005
6876	TAGLN	Transgelin	6099	0.05	0.69	0.87
10398	MYL9	Myosin Light Chain 9	4275	0.24	0.01	0.10
4627	MYH9	Myosin Heavy Chain 9	1592	0.06	0.58	0.81
7431	VIM	Vimentin	3091	0.48	3.9E-07	0.0001
2934	GSN	Gelsolin	1620	-0.36	0.0007	0.016
1843	DUSP1	Dual Specificity Phosphatase 1	1984	0.02	0.96	0.98
3315	HSPB1	Heat Shock Protein Family B (Small) Member 1	3780	-0.32	0.037	0.19
6227	S100A6	S100 Calcium Binding Protein A6	2897	0.17	0.09	0.33
10410	IFITM3	Interferon Induced Transmembrane Protein 3	2274	-0.51	2.7E-05	0.002
2200	FBN1	Fibrillin 1	63	-0.14	0.39	0.67

Note: logFC represents log-transformed fold change, p-value indicates the raw p-value and adjust p indicates confidence level after FDR. Aorta expression data is obtained from the GTEx online dataset and is represented in transcripts per million (TPM). Significant differentially expressed genes identified as over 2FC and $p<0.05$.

The next step was to examine the 20 most significant upregulated and downregulated genes, ranked by both raw p-value and log-transformed fold change. These are listed in Tables 4.3 to 4.6, respectively. Non-coding RNA, RNA with location names only, tRNA, and antisense RNA were excluded due to their complexity or the current impossibility of interpreting their biological significance.

The top 20 significantly upregulated DE genes ranked by p-value, are primarily expressed by immune cells (15 out of 20) and have roles in regulating the inflammatory response (13 out of 20 are pro-inflammatory, and 2 out of 20 are anti-inflammatory). These pro-inflammatory genes include CLC, CCL8, CXCL8, and anti-inflammatory genes like ITLN1 and NRG1. This suggests the involvement and dysregulation of an inflammatory response within the MFS aortic wall (see Table 4.3). Additionally, genes related to oxidative stress and mitochondrial function were identified, including PER3 for oxidative stress and NRG1 for oxidative stress and

mitochondrial function. All of the top 20 significant upregulated DE genes, ranked by raw p-value, remained significant after Benjamini-Hochberg correction.

The top 20 upregulated significant DE genes, ranked by log-transformed fold change, included six genes promoting an inflammatory response (CLC, ANXA8, POU4F, MSLN, ALOX15, and CXCL8) and three genes associated with an anti-inflammatory response (ITLN1, KLK7, and KLK10) (Table 4.4). Additionally, genes primarily involved in cell death (e.g., SYT4, KRT1, MUC16) and cell proliferation (e.g., KLK1, ANXA8, POU4F1, ALOX15, LRP2, KRT1, FGF5, LPR2, BNC1) were also identified. Two (MUC16, p-adjust = 0.09 and KRT5, p-adjust = 0.18) out of the top 20 significant upregulated DE genes ranked by log-transformed fold change became insignificant after multiple testing correction, as indicated by underlined adjusted p-values in Table 4.4.

The top 20 downregulated significant DE genes, ranked by p-value, indicate the involvement of inflammation, with two pro-inflammatory genes (DDIT4 and SERPINA3) and seven anti-inflammatory genes (NPAS2, MT1X1, HIP1R, FKBP5, CD163, CSMD2, and GPX3) (Table 4.5). Additionally, these genes include those encoding proteins involved in ECM maintenance (7 genes: MT1X, COL7A1, SERPINA3, NRAP, GPX3, MMP19, and ARHGEF4) and those with protective roles against oxidative stress (5 genes: MT1X, MT2A, FKBP5, CBS, and GPX3). Furthermore, genes related to regulating oxidative stress related-mitochondrial regulation, mitochondrial metabolism, as well as ATP-ADP conversion were identified (3 genes: DDIT4, ARRDC2, ALDH1L1), suggesting the involvement of oxidative stress and abnormal mitochondrial function in MFS pathology. All top 20 significant downregulated DE genes ranked by raw p-value, remained significant after Benjamini-Hochberg correction.

The top 20 downregulated significant DE genes ranked by log-transformed fold change, reveal similar results in relation to mitochondrial regulation (3 genes: TECRL, SMYD1, and CKM), oxidative stress (3 genes: MB, SLC5A1, and ALOX15B), inflammatory response (3 genes: ALOX15B, MAP1LC3C, and CHIT1), and ECM modulation (3 genes: MMP3, ARHGEF4, and NRAP). Additionally, some ion regulatory genes (2 genes: TECRL, and XIRP1) and, surprisingly, genes involved in modulating cardiomyocytes contractility (5 genes: MYH6, MYH7, TNNT2, MYL2 and TNNI3) were identified among the downregulated DE genes, suggesting their potential involvement in MFS pathogenesis (Table 4.6). Only one gene (CHIT1, p-adjusted = 0.057) from the top 20 downregulated genes ranked by log-

transformed fold change did not remain significant after Benjamini-Hochberg correction.

Table 4.3 Top 20 significant (ranked by p-value) upregulated DE gene from transcriptomics study and their function.

Gene name	Protein	Log_2FC	p-value (adj.p)	Expressed cell type	Aorta expression (TPM)	Function	Category
ITLN1	Intelectin 1	5.44	1.8E-16 (4.9E-12)	Adipose tissue Monocytes Neutrophils	<10	Reduce inflammatory response through TNF signalling in EC. Activates PI3K/AKT pathway and NO production, inhibits apoptosis (Saddic, Nicoloro et al. 2017, Paval, Di Virgilio et al. 2022)	Anti-inflammation
CLC	Charcot-Leyden crystal galectin	6.74	9.8E-15 (9.2E-11)	Basophil Eosinophil	<10	Induce inflammatory response (Rodríguez-Alcázar, Ataide et al. 2019)	Pro-inflammation
CCL8	C-C motif chemokine ligand 8	2.19	6.5E-14 (3.7E-10)	Macrophages Monocytes SMC Adipocytes Fibroblasts	<10	Regulated by inflammatory response and TGFβ pathways (Rabin 2003) Activates immune cells and promote inflammatory response (Klupp, Schuler et al. 2020)	Pro-inflammation
FCER1A	Fc epsilon receptor Ia	2.13	4.4E-11 (8.4E-08)	Basophils	<10	Induce inflammatory response and fibrosis (Chen, Song et al. 2022) Promotes epithelial cell growth and differentiation (Hayes, Ward et al. 2020)	Pro-inflammation
PTGS2	Prostaglandin-endoperoxide synthase 2	2.38	1.7E-10 (2.3E-07)	Fibroblasts Granulocytes Monocytes Macrophages Neutrophils	<10	(COX2) Activated in inflammation, induced by IL-1β and signal through NF-κB cascade (Wu and DeMayo 2017)	Pro-inflammation
CXCL8	C-X-C motif chemokine ligand 8	4.00	2.7E-10 (3.3E-07)	Monocytes Macrophages Neutrophils	<10	(IL8) Pro-inflammation, activated by cytokines (interleukins, TNFα etc), ROS, hypoxia, stress and signal through NF-κB signalling (Ha, Debnath et al. 2017)	Pro-inflammation

TNFSF18	TNF superfamily member 18	2.97	9.0E-10 (8.4E-07)	Macrophages	<10	Activated by endothelial inflammation, and signal through JAK-STAT pathway in atherosclerosis (Gao, Wang et al. 2021)	Pro-inflammation
PER3	Period circadian regulator 3	1.11	6.8E-09 (4.1E-06)	Other	13.58	Regulates circadian rhythms, associates with obstructive sleep apnea/ hyponea, which altered circadian clock could be linked to oxidative stress and inflammatory responses (Yang, Lin et al. 2019, Li, Liu et al. 2022)	Oxidative stress related
ADGRE3	Adhesion G protein-coupled receptor E3	3.11	7.0E-09 (4.1E-06)	Monocytes Macrophages Eosinophils Neutrophils Basophils	<10	Activated in inflammatory response along with cytokines like TNFα, CXCL8 (Nijmeijer, Vischer et al. 2016)	Pro-inflammation
PRSS35	Serine protease 35	1.16	3.8E-08 (1.6E-05)	T-cell lymphocytes	12.31	Implicated in CXCL2-mediated extracellular neutrophil recruitment in cancer study (Wang, Zhou et al. 2023)	Pro-inflammation
LHFPL1	LHFPL tetraspan subfamily member 1	1.40	4.1E-08 (1.7E-05)	NK-cells	<10	Unclear	Unclear
EGR2	Early growth response 2	2.01	7.8E-08 (2.8E-05)	Macrophages Fibroblast Monocytes	<10	Implicated in myocardial infarction, promote inflammation and apoptosis induced by hypoxia (Bo, Huang et al. 2022)	Pro-inflammation
CCR3	C-C motif chemokine receptor 3	2.98	1.1E-07 (3.4E-05)	Neutrophil Basophil Eosinophil	<10	Regulated by intracellular calcium level, regulates chemokines, activate eosinophilic inflammation (Song, Shim et al. 2017)	Pro-inflammation
GCSAML	Germinal center associated signalling and motility like	1.67	1.4E-07 (3.9E-05)	Granulocytes Mast cells Basophils	<10	Positively associated with mast cell activation, basophil percentage, immune cell proliferation and maturation (Kristjansson, Oskarsson et al. 2023)	Pro-inflammation
KCTD16	Potassium	1.37	1.6E-07	Other	<10	Implicated in neuro- and cancer related	Ion regulation

	channel tetramerization domain containing 16		(4.2E-05)			disorders (Angrisani, Di Fiore et al. 2021) Regulates potassium conduction (Turecek, Schwenk et al. 2014)	
MNDA	Myeloid cell nuclear differentiation antigen	1.55	1.7E-07 (4.6E-05)	Macrophages Monocytes Neutrophils	<10	Positively regulates inflammation response, positively correlate to atherosclerosis progression (Briggs, Atkinson et al. 2005)	Pro-inflammation
F5	Coagulation factor V	1.47	2.4E-07 (5.9E-05)	Eosinophils Neutrophils T-cells Monocytes	<10	Blood coagulation, haemostasis, thrombosis, act as a prothrombotic factor (Humphries, Ridker et al. 2004)	Blood coagulation/ haemostasis
MSLN	Mesothelin	4.73	2.9E-07 (6.7E-05)	Monocytes SMC EC	<10	Activates AKT/PI3K/NF-κB signalling, induces apoptosis resistance and promotes inflammation and cell survival and proliferation, as well as MMP7 and MMP9 expression (Lv and Li 2019, Wen, Huang et al. 2022) Implicated in cell adhesion in cancer study (Rump, Morikawa et al. 2004)	Pro-inflammation
KCNB2	Potassium voltage-gated channel subfamily B member 2	1.91	3.6E-07 (7.8E-05)	Other	<10	Regulate potassium channels, associated with atrial fibrillation and miR-1 expression (Lozano-Velasco, Aranega et al. 2021)	Ion regulation
NRG1	Neuregulin 1	1.50	4.0E-07 (8.3E-05)	Monocytes Neutrophils EC Cardiomyocytes	<10	Signalling through ErbB pathway, can be regulated by NO availability, shows protection against cardiac injury. May regulate Hippo signalling (increase proliferation and hypertrophy) and activates downstream PI3K/AKT (upregulates NO and contractility, decrease apoptosis and	Anti-inflammation Anti-oxidative stress Mitochondrial function

						remodelling) from upstream ErbB stimulation. Interact with MAPK(decrease inflammatory response, oxidative stress), mTOR, Wnt pathways (Wang, Wei et al. 2022). Positively regulates mitochondrial function, regenerates EC and shows anti-inflammation and anti-oxidative effects through TNF signalling (Kang, Cheng et al. 2020)	

Note: Expressed cell type was obtained from Human Protein Atlas, only heart-related cell and immune cells were included, other indicates no documented heart or immune-related cell. Expression in aorta is obtained from GTex, expressions within healthy aorta less than 10 were presented as <10, otherwise with the actual expression. Red highlighted genes are identified in both rankings (p-values and log transformed FC). Adj. p refers to the Benjamini-Hochberg critical value.

Table 4.4 Top 20 significant (ranked by logFC) upregulated DE gene from transcriptomics study and their function.

Gene name	**Protein**	**Log_2FC**	**p-value (adj.p)**	**Expressed cell type**	**Aorta expression (TPM)**	**Function**	**Category**
KLK11	Kallikrein related peptidase 11	7.60	3.1E-06 (3.7E-04)	Other	<10	Activates mTOR signalling and promotes cardiac hypertrophy (Wang, Liao et al. 2021)	Proliferation
CLC	Charcot-Leyden crystal galectin	6.74	9.8E-15 (9.2E-11)	Basophil Eosinophil	<10	Induce inflammatory response (Rodríguez-Alcázar, Ataide et al. 2019)	Pro-inflammation
ANXA8	Annexin A8	6.26	6.2E-07 (1.1E-04)	Other	<10	Promotes cell proliferation, migration and endothelial-mesenchymal transition, regulates phenotypic plasticity in retinal EC; promotes inflammation (Mendez-Barbero, San Sebastian-Jaraba et al. 2022) Positively associates with lymphocytes and inflammatory response, induce cell migration, adhesion and regulates PI3K/AKT, focal adhesion and	Proliferation Pro-inflammation

						proteoglycans (Gou, Zhu et al. 2019)	
SYT4	Synaptotagmin 4	5.58	1.4E-05 (0.001)	Other	<10	Interact with ACTA1, ACTC1, ACTN2, MYH7, NRAP, TNNC2 and TNNI1 and promotes ferroptosis (Wo, Liu et al. 2022)	Cell death
ITLN1	Intelectin 1	5.44	1.7E-16 (4.9E-12)	Monocytes Neutrophils	<10	Reduce inflammatory response through TNF signalling in EC. Activates PI3K/AKT pathway and NO production, inhibits apoptosis (Saddic, Nicoloro et al. 2017, Paval, Di Virgilio et al. 2022)Activates PI3K/AKT pathway (Paval, Di Virgilio et al. 2022)	Anti-inflammation
POU4F1	POU class 4 homeobox 1	5.00	4.7E-06 (5.1E-04)	Other	<10	Increased in macrophage-myofibroblast transition under chronic inflammatory response, activates TGFβ pathway and promotes fibrosis (Tang, Zhang et al. 2020)	Pro-inflammation Fibrosis
ANXA8L1	Annexin A8 like 1	4.99	2.7E-05 (0.002)	Monocytes	<10	Paralog of ANXA8, correlated with worse survival rate and clinical outcomes in cancer (Wang, Cao et al. 2023)	Unclear
MSLN	Mesothelin	4.73	2.9E-07 (6.7E-05)	Monocytes SMC EC	<10	Overexpression of MSLN activates AKT/PI3K/NF-κB signalling, induce apoptosis resistance and promote inflammation and cell survival and proliferation, as well as MMP7 and MMP9 expression (Lv and Li 2019, Wen, Huang et al. 2022) Implicated in cell adhesion in cancer study (Rump, Morikawa et al. 2004)	Pro-inflammation
KRT1	Keratin 1	4.64	4.1E-07 (8.3E-05)	Memory CD4 T-cell	<10	Positively associate myocardial cell apoptosis, and inhibition increases proliferation, activates Notch signalling (Fang, Wu et al. 2019) Negatively regulates NO release in EC (Chu and Huang 2015)	Cell death
AP1M2	Adaptor related	4.61	7.9E-05	Neutrophils	<10	Associated with thrombosis (Hinds, Buil et al. 2016)	Blood

	protein complex 1 subunit mu 2		(0.004)	Basophils			coagulation
ALOX15	Arachidonate 15-lipoxygenase	4.42	5.9E-07 (0.0001)	Eosinophils	<10	Increases inflammatory response by elevating macrophage infiltration, also associated with fibrosis, systolic dysfunction and heart failure (Kayama, Minamino et al. 2009) Implicated in mitochondrial abnormal morphology and function and induce cardiomyocytes ferroptosis (Cai, Liu et al. 2023)	Pro-inflammation Fibrosis
FGF5	Fibroblast Growth Factor 5	4.30	1.1E-03 (0.021)	Other	<10	Demonstrates protective role under injury response, attenuates pyroptosis by inhibition of NF-κB pathway, overexpression alleviates oxidative stress and ROS (Cui, Li et al. 2022)	Anti-oxidative stress Anti-cell death
LRP2	LDL receptor related protein 2	4.11	1.5E-05 (0.001)	Other	<10	Regulates cardiomyocyte proliferation and differentiation, signal through Wnt signalling and possibly Hedgehog signalling (Theis, Vogler et al. 2020)	Proliferation
BNC1	Basonuclin 1	4.04	3.7E-05 (0.002)	Fibroblasts	<10	Exhibits co-expression with MSLN, and regulate cell proliferation and survival (Gambardella, McManus et al. 2019, Knight-Schrijver, Davaapil et al. 2022)	Proliferation
CXCL8	C-X-C motif chemokine ligand 8	4.00	2.7E-10 (3.3E-07)	Monocytes Macrophages Neutrophils	<10	(IL8) Pro-inflammation, activated by cytokines (interleukins, TNFα etc), ROS, hypoxia, stress and signal through NF-κB signalling (Ha, Debnath et al. 2017)	Pro-inflammation
KLK7	Kallikrein Related Peptidase 7	3.82	1.6E-03 (0.03)	Other	<10	Protect cardiac injury and inflammation, reduce inflammatory cells and apoptosis (Yin, Pan et al. 2022)	Anti-inflammation
MUC16	Mucin 16, cell surface	3.41	0.011 (0.09)	Other	<10	Regulates JAK-STAT signalling, promotes cell apoptosis (Das, Rachagani et al. 2015)	Cell death

	associated					Overexpression associated with heart failure (Núñez, de la Espriella et al. 2021)	
KRT5	Keratin 5	3.37	0.031 (0.18)	Other	<10	Regulates actin-cytoskeleton system through BMP signalling (Wang, Guo et al. 2022)	Contractility
KLK10	Kallikrein related peptidase 10	3.36	0.0007 (0.015)	EC	<10	Protect disrupted blood flow induced inflammation and permeability dysfunction in EC through NF-κB signalling, stabilize cell adhesion and protect against atherosclerosis (Williams, Mahmoud et al. 2022)	Haemostasis Anti-inflammation
VWA5B1	Von Willebrand factor A domain containing 5B1	3.28	0.003 (0.039)	Granulocytes	<10	ECM glycoprotein (Gouarderes, Ober et al. 2022, Samaržija and Konjevoda 2023)	ECM related

Note: Expressed cell type was obtained from Human Protein Atlas, only heart-related cell and immune cells were included, other indicates no documented heart or immune-related cell. Expression in aorta is obtained from GTex, expressions within healthy aorta less than 10 were presented as <10, otherwise with the actual expression. Red highlighted genes are identified in both rankings (p-values and log transformed FC). Adj. p refers to the Benjamini-Hochberg critical value, underlined critical value indicates p>0.05.

Table 4.5 Top 20 significant (ranked by p-value) downregulated DE gene from transcriptomics study and their function.

Gene name	**Protein**	**Log_2FC**	**p-value (adj.p)**	**Expressed cell type**	**Aorta expression (TPM)**	**Function**	**Category**
DDIT4	DNA damage inducible transcript 4	-2.26	5.0E-12 (1.6E-08)	NK-cells T-cell Lymphocytes	217.4	Regulates mitochondrial function and reduce oxidative stress, decreased DDIT4 shows pro-oxidative and abnormal mitochondrial function. Downstream of PI3K and HIF-1, promotes proliferation, migration, cell survival. Activates NF-κB to promote inflammation (Ding, Gao et al. 2021)	Mitochondrial function Proliferation Pro-inflammation
NPAS2	Neuronal PAS domain	-1.16	6.7E-12 (1.7E-08)	Other	26.65	Negatively regulate inflammatory response, prevent cell apoptosis through AKT/mTOR	Anti-inflammation

	protein 2					signalling (Huang, Qing et al. 2021)	
MT1X	Metallothionein 1X	-2.22	1.5E-11 (3.1E-08)	T-cell Lymphocytes	450.6	Protect cell stress caused by ROS, inflammation and damage, downregulated in BAC-TAA associated with ECM degradation (Klyachko, Kenny et al. 2006, Phillippi, Klyachko et al. 2009, Nishi, Ogata et al. 2019, Dai, Wang et al. 2021)	Anti-oxidative stress Anti-inflammation ECM related
GALNT15	Polypeptide N-acetylgalactosaminyltransferase 15	-2.34	1.2E-10 (1.9E-07)	Adipocytes, EC Fibroblasts Basophils	59.32	Unclear	Unclear
HIP1R	Huntingtin interacting protein 1 related	-1.38	1.8E-10 (2.3E-07)	B-cell Lymphocytes	60.37	Low expression associates with inflammatory response, activates interleukins and JAK-STAT signalling (Koh, Han et al. 2020)	Anti-inflammation
COL7A1	Collagen type VII alpha 1 chain	-1.46	2.6E-09 (2.1E-06)	Cardiomyocytes	15.79	Regulates fibril anchoring and epithelial basement membrane/ ECM organization and adherence, decreased expression may lead to ECM degradation and collage abnormality (Horev, Waran Lalin et al. 2003, Chung and Uitto 2010)	ECM related
MT2A	Metallothionein 2A	-1.77	2.7E-09 (2.1E-06)	Monocytes Adipocytes T-cell Lymphocytes	555.8	Protect cell against oxidative stress (Liu, Zou et al. 2022)	Anti-oxidative stress
FKBP5	FKBP prolyl isomerase 5	-2.39	2.7E-09 (2.1E-06)	Monocytes NK-cells T-cell Lymphocytes	68.59	Negatively regulate HIF-1 signalling, involves in inflammatory response (NF-κB-driven), hypoxia, calcium homeostasis and stress response and hypertrophy/ fibrosis (Zannas, Jia et al. 2019, Fatkin, Ohanian et al. 2023, Wang, Song et al. 2023)	Anti-oxidative stress Ani-inflammation Anti-stress

ARRDC2	Arrestin domain containing 2	-1.09	4.2E-09 (2.9E-06)	Adipocytes Monocytes B-cell Lymphocytes	76.25	Implicated in regulating skeletal muscle anabolic or catabolic regulation (Gordon, Rossetti et al. 2019)	Mitochondrial function
ALDH1L1	Aldehyde dehydrogenase 1 family member L1	-1.73	4.3E-09 (2.9E-06)	Other	31.83	Regulates mitochondrial metabolism that converts NADP+ to NADOH and CO2, loss of function leads to decreased apoptosis and increased cell migration (Sasaki, Yamamoto et al. 2023)	Mitochondrial function
CD163	CD163 molecule	-1.86	5.1E-09 (3.3E-06)	Macrophages Monocytes	115.2	Anti-inflammatory response (Durda, Raffield et al. 2022)	Anti-inflammation
CBS	Cystathionine beta-synthase	-1.94	9.4E-09 (5.4E-06)	Neutrophils	<10	Implicated in congenital heart disease, negatively regulates homocysteine which inhibits NO and produce oxidative stress in EC and cell apoptosis (Wang, Jhee et al. 2004, Zhao, Yang et al. 2013)	Anti-oxidative stress
ARHGEF19	Rho guanine nucleotide exchange factor 19	-1.33	1.0E-08 (5.6E-06)	Other	<10	Implicated in cardiac hypertrophy (van Berlo, Aronow et al. 2013)	Proliferation
SLC26A6	Solute carrier family 26 member 6	-1.10	1.5E-08 (8.0E-06)	Other	66.63	Regulates ion homeostasis, pH level, cell osmoregulation, regulates heart contraction (Wang, Wang et al. 2020)	Contractility
SERPINA3	Serpin family A member 3	-1.87	1.8E-08 (9.1E-06)	Fibroblasts	61.27	Anti-apoptotic role promotes cell survival and inhibit division and proliferation through MAPK and PI3K/AKT pathways, positively associates with inflammation response and EC, ECM dysregulation and remodelling (Zhou, Cheng et al. 2017, de Mezer, Rogaliński et al. 2023)	Pro-inflammation ECM related
NRAP	Nebulin related anchoring	-4.69	3.4E-08 (1.6E-05)	Cardiomyocytes	<10	Involved in myocardial contraction, muscle α-actin binding and cell adhesion, decreased expression associates with myofibril assembly and actin	Contractility ECM related

	protein					filament anchoring on the cell membrane, as well as myofibril to ECM interaction (Zhang, Xu et al. 2023)	
CSMD2	CUB and Sushi multiple domains 2	-1.71	3.6E-08 (1.6E-05)	Other	<10	Positively correlated with anti-tumour immune cells and negatively regulates immune evasion cells (Zhang, Huang et al. 2022)	Anti-inflammation
GPX3	Glutathione peroxidase 3	-1.24	4.8E-08 (2.0E-05)	Neutrophils	803.8	Cooperates with metallothioneins, decreased expression observed with AAA under hypoxia with increased aneurysm ruptures and angiogenesis (Liljeqvist, Hultgren et al. 2020) Negatively regulated by oxidative stress to modulate ECM expression and promote pro-fibrotic and pro-inflammatory microenvironment (Li, He et al. 2023)	Anti-oxidative stress ECM related Anti-inflammation
MMP19	Matrix metallopeptidase 19	-1.20	6.5E-08 (2.4E-05)	ECM, Macrophages Fibroblasts	52.7	Promotes ECM remodelling, wound healing, EC migration and angiogenesis. Elevated expression associated with TAA aorta dilation and MMP19 deficiency in lung fibroblasts promotes pro-fibrotic and collagen production, also increased SM-α actin expression, regulates fibroblasts and myofibroblast differentiation, migration and proliferation(Jackson, Olsson et al. 2012). Animal model shows MMP19 deficiency increases fibrosis and EC hyperplasia (Cui, Hu et al. 2017).	ECM related
ARHGEF4	Rho guanine nucleotide exchange factor 4	-3.51	7.1E-08 (2.6E-05)	Naïve CD8 T-cell Lymphocytes	<10	Promotes cell-cell adhesion and stabilizes endothelial cell-cell contacts, maintains adherens junction and tight junction, loss of ARHGEF4 shows decreased cell-cell adhesion (Kitt-Hopper 2020).	ECM related

Note: Expressed cell type was obtained from Human Protein Atlas, only heart-related cell and immune cells were included, other indicates no documented heart or immune-related cell. Expression in aorta is obtained from GTex, expressions within healthy aorta less than 10 were presented as <10, otherwise with the actual expression. Red highlighted genes are identified in both rankings (p-values and log transformed FC). Orange highlighted genes are identified in both rankings and also in protein top 20 tables. Adj. p refers to the Benjamini-Hochberg critical value.

Table 4.6 Top 20 significant (ranked by logFC) downregulated DE gene from transcriptomics study and their function.

Gene name	Protein	Log_2FC	p-value (adj.p)	Expressed cell type	Aorta expression (TPM)	Function	Category
TECRL	Trans-2,3-enoyl-CoA reductase like	-5.46	2.9E-03 (0.041)	Cardiomyocytes	<10	Regulates calcium level, and mitochondrial function. Decreased TCERL associates with mitochondrial dysfunction and increased ROS as well as impaired cardiac functions (Hou, Jiang et al. 2022)	Ion regulation Mitochondrial function
TNNI3	Troponin I3, cardiac type	-5.42	6.0E-04 (0.014)	Cardiomyocytes	<10	Implicated in hypertrophic cardiomyopathy, mutation causes PKA phosphorylation dysregulation, increase calcium sensitivity and leads to diastolic dysfunction (Fahed, Nemer et al. 2020)	Contractility
SMYD1	SET and MYND domain containing 1	-5.34	1.1E-03 (0.021)	Cardiomyocyte	<10	SMYD1 deficiency result in mitochondrial protein involved in oxidative phosphorylation downregulation, with decreased mitochondrial respiration (Warren, Tracy et al. 2018)	Mitochondrial function
MYH7	Myosin heavy chain 7	-5.23	1.2E-06 (0.0002)	Cardiomyocytes	<10	Associates with ATP-binding domain of the myosin protein, alternation in MYH7 modulates ATP-binding rates, ATP hydrolysis, ADP release and abnormal ATP, ADP within the cell (Yousaf, Khan et al. 2022)	Contractility
XIRP2	Xin actin binding	-4.91	5.1E-06	Cardiomyocytes	<10	Interacts with actin filaments and sensors	Mechanical

	repeat containing 2		(0.0005)			mechanical stress in intercalated disk and costamere, reduced expression associates with cardiac hypertrophy (McCalmon, Desjardins et al. 2010)	stress
MB	Myoglobin	-4.91	2.9E-06 (0.0004)	Cardiomyocytes NK-cells	<10	Regulates NO homeostasis, removes ROS, and promotes cell survival in hypoxia condition (Koch, Lüdemann et al. 2016)	Anti-oxidative stress
SLC5A1	Solute carrier family 5 member 1	-4.84	4.3E-05 (0.0024)	Other	<10	Regulated by AMPK signalling and upregulation associates with ERK pathway, growth factor and increased ROS production (Li, Agrawal et al. 2019)	Pro-oxidative stress
NRAP	Nebulin related anchoring protein	-4.69	3.4E-08 (1.6E-05)	Cardiomyocytes	<10	Involved in myocardial contraction, muscle α-actin binding and cell adhesion, decreased expression associates with myofibril assembly and actin filament anchoring on the cell membrane, as well as myofibril to ECM interaction (Zhang, Xu et al. 2023)	Contractility ECM related
ALOX15B	Arachidonate 15-lipoxygenase type B	-4.40	4.0E-07 (8.3E-05)	Monocytes Macrophages	<10	Induced by hypoxia via HIF-1 signalling and inflammatory responses, regulates cholesterol homeostasis (Benatzy, Palmer et al. 2022)	Oxidative stress / inflammation induced
MYH6	Myosin heavy chain 6	-3.99	1.4E-05 (0.001)	Cardiomyocytes	<10	Involved in force generation and heart contraction, decreased expression in heart failure, implicated in cardiomyopathy with MYH7 and cardiac fibrosis (Theis, Zimmermann et al. 2015)	Contractility
TNNT2	Troponin T2, cardiac type	-3.82	9.1E-06 (0.0008)	Cardiomyocytes	<10	Involved in heart contractility, signalling through MAPK, implicated in cardiomyopathy (Pettinato, Ladha et al. 2020)	Contractility
MYL2	Myosin light chain	-3.77	7.1E-07	Cardiomyocytes	<10	Involved in cardiac muscle contraction, driven by	Contractility

	2		(0.0001)	Naïve B-cells Memory B-cells		ATP conversion to mechanical movement. Associated with cardiomyopathy, decreased cardiac function, hypertrophy, cardiac torsion and fibrosis (Sheikh, Lyon et al. 2015)	
ARHGEF4	Rho guanine nucleotide exchange factor 4	-3.51	7.1E-08 (2.6E-05)	Naïve CD8 T-cell Lymphocytes	<10	Promotes cell-cell adhesion and stabilizes endothelial cell-cell contacts, maintains adherens junction and tight junction, loss of ARHGEF4 shows decreased cell-cell adhesion (Kitt-Hopper 2020).	ECM related
MMP3	Matrix metallopeptidase 3	-3.46	4.0E-06 (0.0005)	Fibroblasts SMC	<10	Activates other MMPs like MMP1, 7, 8, 9, 13 and can be activated by MMP12, MMP3 can degrades ECM components like collagen, fibronectin, laminin and proteoglycans, overexpression associates with inflammation and haemostasis, also VSMC proliferation and migration (Magdalena, Magdalena et al. 2006)	ECM related
LPAR3	Lysophosphatidic acid receptor 3	-3.13	1.2E-04 (0.005)	Cardiomyocytes	<10	Regulates Ras, MEK/ERK and PI3K/AKT pathways, associated with cell proliferation and apoptosis inhibition (Hwang, Kim et al. 2022)	Proliferation
XIRP1	Xin actin binding repeat containing 1	-3.06	1.2E-06 (0.0001)	Cardiomyocytes	<10	Involved in electrophysiology function in the heart and potassium level and transmission, may implicated in cardiac arrhythmia with conduction defect (Huang, Wu et al. 2018)	Ion regulation
CKM	Creatine kinase, M-type	-3.04	1.1E-07 (3.4E-05)	Cardiomyocytes	<10	(MCK) Regulates ATP-ADP conversion, implicated in myocardial infraction (Aydin, Ugur et al. 2019). Downregulation implicates mitochondrial dysfunction, deficient mitochondria leading to excessive ROS and calcium production, which in turn leads to cell death (Carreira, Lee et al. 2011)	Mitochondrial function

TDGF1	Teratocarcinoma-derived growth factor 1	-3.00	7.0E-05 (0.003)	Other	<10	Activates PI3K/ATK pathway and inhibits apoptosis (Williams, Monticone et al. 2010)	Anti-cell death
MAP1LC3C	Microtubule associated protein 1 light chain 3 gamma	-2.79	4.6E-06 (0.0005)	Fibroblasts	<10	Correlated to immune cells and immune modulators (Zhang, Li et al. 2022)	Pro-inflammation
CHIT1	Chitinase 1	-2.73	4.8E-03 (0.057)	Macrophages	<10	Increases TGFβ signalling and downstream MAPK/ERK, induces fibrosis (Lee, Herzog et al. 2012) Protective role against atherosclerosis by inhibiting inflammatory response (Kitamoto, Egashira et al. 2013)	Anti-inflammation

Note: Expressed cell type was obtained from Human Protein Atlas, only heart-related cell and immune cells were included, other indicates no documented heart or immune-related cell. Expression in aorta is obtained from GTex, expressions within healthy aorta less than 10 were presented as <10, otherwise with the actual expression. Red highlighted genes are identified in both rankings (p-values and log transformed FC). Orange highlighted genes are identified in both rankings and also in protein top 20 tables. Blue highlighted genes are identified in protein top 20 tables. Adj. p refers to the Benjamini-Hochberg critical value.

4.3.3 Signalling and biological function analysis

Current usual KEGG enrichment analysis encompasses various types of pathway analysis, which fall under seven main categories, namely metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases, and drug development. The additional disease or drug pathways are typically constructed based on the dysregulation of fundamental subcategories under environmental information processing-signal transduction pathways such as TGFβ, Wnt, PI3K/AKT, and MAPK pathways.

However, the inclusion of all disease and drug-related pathways introduces a significant amount of noise and distraction into the analysis. This noise can divert attention from the primary focus on altered signal transduction pathways and potentially lead to misinterpretation due to the inclusion of irrelevant disease pathways. Therefore, only pathways related to heart diseases and cellular metabolism were included in this study, with the intention of conducting a more detailed analysis of the signal transduction pathways to evaluate the fundamental signalling process.

A total of 38 significant pathways were identified through KEGG analysis using all significant DE RNA (n = 987). Among these, six out of the 38 significant pathways belong to the subcategory of signal transduction pathways within the environmental information processing category from the KEGG online database (Figure 4.2).

Figure 4.2 displays selected relevant significant pathways and their subcategories from the KEGG online dataset. Among these, six significant signal transduction pathways stand out: NF-κB ($p = 0.0005$), calcium ($p = 0.017$), PI3K/AKT ($p = 0.023$), cAMP ($p = 0.03$), TNF ($p = 0.03$), and Ras ($p = 0.04$) signalling. Furthermore, KEGG analysis also identified cardiac muscle contraction, cardiomyopathy, and atherosclerosis as relevant pathways, raised an intriguing question of the potential role and presence of cardiomyocytes in the ascending thoracic aortic wall.

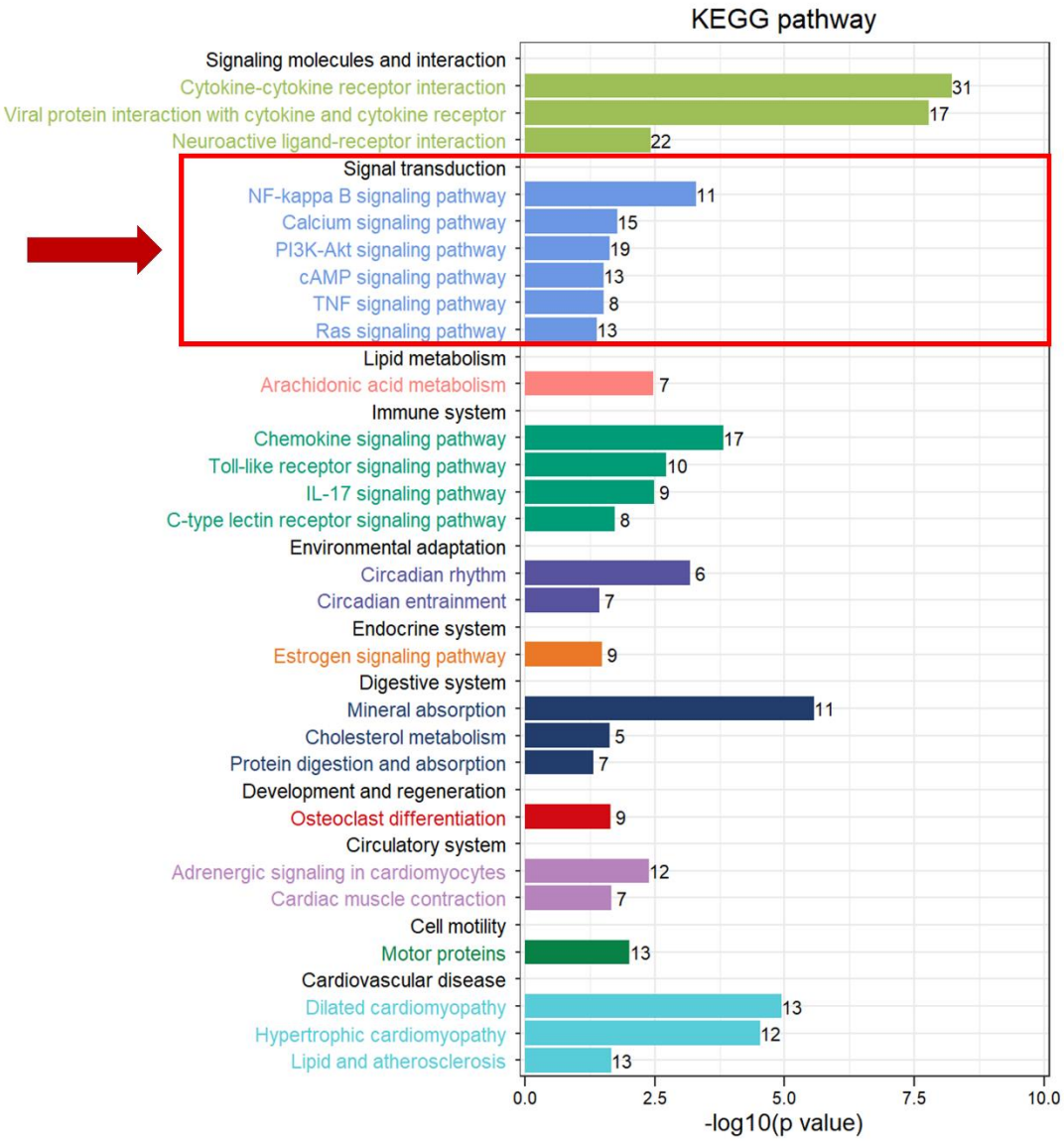


Figure 4.2 KEGG enrichment barplot for significant DE RNA. Significant pathways are illustrated under each KEGG category. Enrichment analysis with p<0.05 were included. Y-axis title indicates each pathway identified and the category (coloured in black) it belongs to, and each colour represent one KEGG category. X-axis indicates the -log10(p value) of each pathway, and the number after the bar represent the number of genes in each pathway that are identified by differential expression. The red box and arrow indicate that these pathways belong to the subcategory of signal transduction pathway.

Additionally, significant DE genes detected after the FDR procedure (n = 566) were used to conduct a downstream analysis with an increased confidence level compared to the raw p-value analyses (Figure 4.3). The result has identified 32 significant KEGG pathways, with five being signal transcription pathways.

Figure 4.3 displays the selected relevant significant KEGG pathways and their subcategories from the KEGG online dataset after Benjamini-Hochberg correction. Five significant signal transduction pathways emerge: NF-κB ($p = 0.00001$), TNF ($p = 0.003$), PI3K-AKT ($p = 0.02$), sphingolipid ($p = 0.047$), and calcium ($p = 0.048$) signalling. The significant genes identified after applying the FDR procedure using a different input gene set highlight the importance of the sphingolipid signal transduction pathway. Interestingly, this pathway was previously found to be insignificant when using the raw p-value input dataset. This could be due to reduced noise within the dataset. In addition, consistent with the raw p-value results, cardiomyopathy and cardiac muscle contraction signalling pathways were also identified through KEGG analysis.

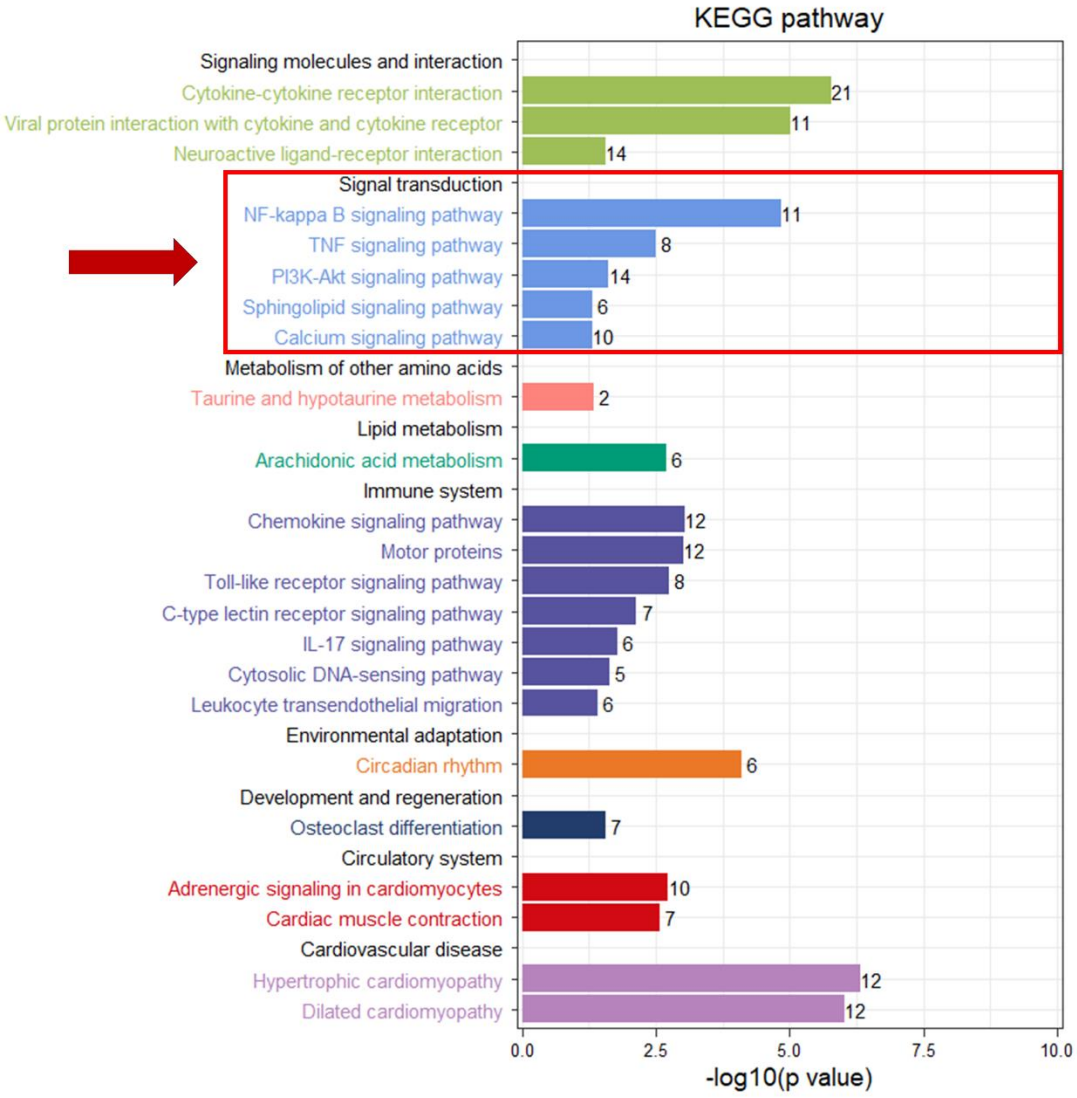


Figure 4.3 KEGG enrichment barplot for significant DE RNA after Benjamini-Hochberg correction. Significant pathways are illustrated under each KEGG category. Enrichment analysis with p<0.05 were included. Y-axis title indicates each pathway identified and the category (coloured in black) it belongs to, and each colour represent one KEGG category. X-axis indicates the -log10(p value) of each pathway, and the number after the bar represent the number of genes in each pathway that are identified by differential expression. The red box and arrow indicate that these pathways belong to the subcategory of signal transduction pathway.

In Figure 4.4, a KEGG circle plot is shown, which exclusively focuses on signal transduction pathways. Figure 4.4 displays the top 10 pathways, irrespective of their enrichment significance, ranked by ascending p-values and labelled on the right-hand side. Notably, insignificantly pathways may not be relevant to the pathogenesis of MFS, however, their failure to reach significance is likely to be due

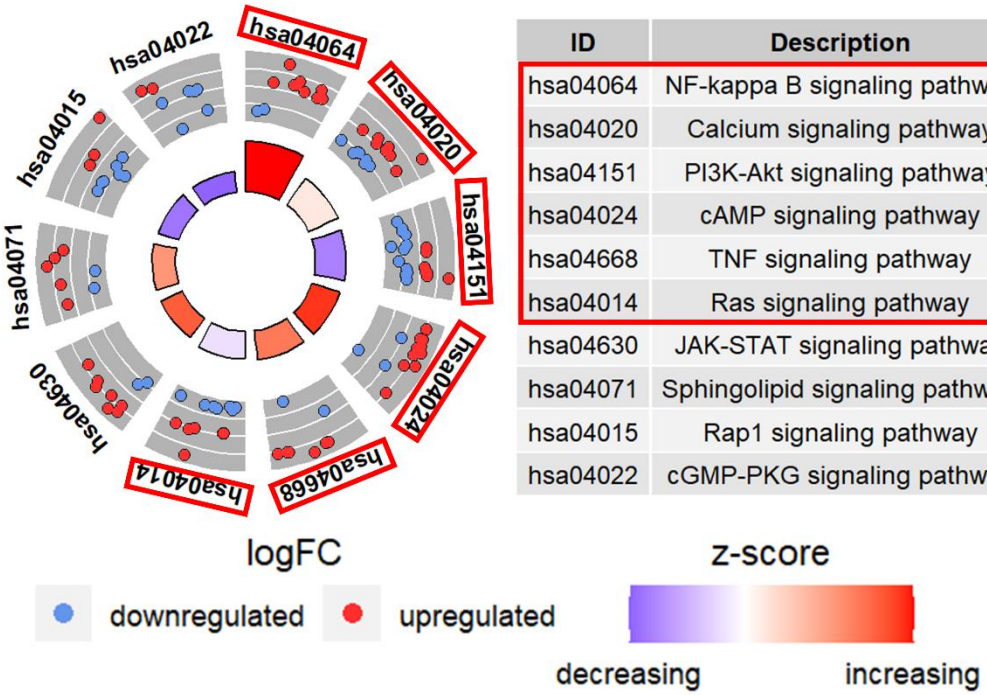
to insufficient number of significantly DE mRNA transcripts within that pathway being identified, due to the study being underpowered. However, including the insignificant pathways, that contain at least some significant DE genes, highlights the possibility of these pathways being significant in a larger study. Indeed, subsequently other omics methods identified some of these pathways as being significant (see later chapters). Notably, the changes in these significant DE genes within these pathways may represent a longer-term adaptive alteration within the pathway, indicating a chronic adaptive change in MFS rather than a transient alteration.

A Z-score, calculated as the ratio of upregulated genes to downregulated genes, is represented by colour. A decreasing Z-score (purple) indicates a higher number of downregulated genes, while an ascending Z-score suggests more upregulated genes within each pathway. This can potentially provide hints about the direction of involvement in that pathway, whether signalling is increased or decreased. However, it does not provide an accurate and definitive representation of signalling direction within each pathway, as each protein product may function to either enhance or inhibit signalling in that pathway. Nevertheless, changes in gene expression within pathway components strongly implicate the involvement of that pathway in the pathogenesis of MFS.

Within the signal transduction pathway category, two pathways, NF-κB and TNF, are related to the inflammatory response and exhibit a large number of upregulated genes. The Ras signalling pathway is also associated with the inflammatory response, receiving signals from upstream cytokine activities and transmitting them through downstream MAPK and PI3K/ AKT pathways. The involvement of cAMP may also indicate implications for the inflammatory response via the downstream PI3K/AKT pathway. Calcium pathway identification suggests that calcium regulation may serve as second messengers in MFS pathogenesis.

Some pathways did not reach the significance threshold, such as JAK-STAT ($p = 0.89$), sphingolipid ($p = 0.1$), Rap1 ($p = 0.14$) and cGMP-PKG ($p = 0.17$), which potentially could suggest a role for the inflammatory response, calcium and lipid regulation, EC and VSMC cellular regulation in MFS, however not with a high level of confidence.

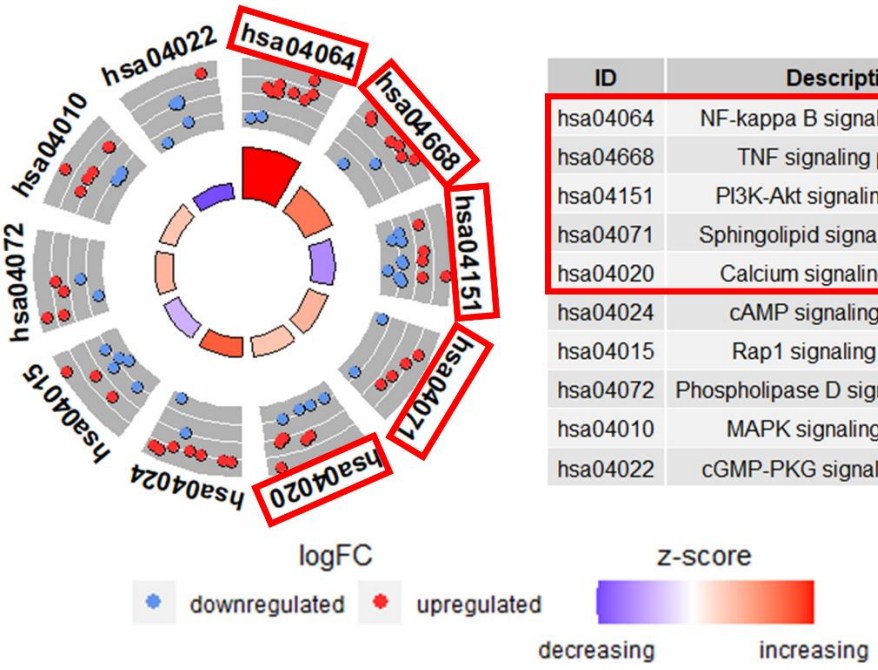
In summary, KEGG analysis, by identifying inflammatory and cellular response pathways, highlights the involvement of the inflammatory response and ECM regulation in MFS pathogenesis.



ID	Description
hsa04064	NF-kappa B signaling pathway
hsa04020	Calcium signaling pathway
hsa04151	PI3K-Akt signaling pathway
hsa04024	cAMP signaling pathway
hsa04668	TNF signaling pathway
hsa04014	Ras signaling pathway
hsa04630	JAK-STAT signaling pathway
hsa04071	Sphingolipid signaling pathway
hsa04015	Rap1 signaling pathway
hsa04022	cGMP-PKG signaling pathway

Figure 4.4 KEGG circle plot (signal transduction pathways only) for all significant DE genes from transcriptomics study. Pathways in red frame indicate the enrichment analysis p<0. 05. Z-score is calculated by the ratio of upregulated gene to downregulated gene.

In Figure 4.5, significant DE genes after Benjamini-Hochberg correction were presented to compare the downstream analysis to the raw p-value dataset. This figure demonstrates that the majority of the downstream pathways remained consistent with the raw p-value analysis (Figure 4.4). Notably, the sphingolipid pathway became significant despite the reduced number of DE genes after FDR. However, phospholipase D (though not significant, p = 0.1) and MAPK signalling (though not significant, p = 0.12) entered the top 10 signal transduction pathways, while Ras and JAK-STAT signalling became less significant, which is likely to be due to loss of significance of DE genes after the FDR procedure. This suggests that the main message from downstream KEGG pathway analysis remains relatively consistent, emphasizing the involvement of inflammatory pathways observed in RNA regulation.



ID	Description
hsa04064	NF-kappa B signaling pathway
hsa04668	TNF signaling pathway
hsa04151	PI3K-Akt signaling pathway
hsa04071	Sphingolipid signaling pathway
hsa04020	Calcium signaling pathway
hsa04024	cAMP signaling pathway
hsa04015	Rap1 signaling pathway
hsa04072	Phospholipase D signaling pathway
hsa04010	MAPK signaling pathway
hsa04022	cGMP-PKG signaling pathway

Figure 4.5 KEGG circle plot (signal transduction pathways only) for all significant DE genes after Benjamini-Hochberg correction from transcriptomics study.

The overall significant DE gene enrichment analysis provides information about transcriptional changes affecting candidate signalling pathways. To further investigate these changes, up- and down significant DE genes were examined separately.

In total, 37 significant KEGG pathways associated with upregulated genes were identified, with 4 being signal transduction pathways (Figure 4.6 A). These significant signal transduction pathways from upregulated genes included NF-κB ($p = 0.00008$), cAMP ($p = 0.006$), TNF ($p = 0.01$), and JAK-STAT ($p = 0.03$) pathways. The presence of these inflammatory signal transduction pathways, particularly NF-κB, TNF, and JAK-STAT, further underscores the observation of inflammatory regulation stemming from upregulated significant DE genes in MFS. To highlight the key genes and pathways contributing to the disease pathology, a Sankey plot was presented in Figure 4.7, emphasizing that interleukins, C-C motif chemokines (CCLs), and C-X-C motif chemokines (CXCLs) were upregulated in MFS patients, consistent with inflammation activation.

Among downregulated genes, 17 significant KEGG pathways were identified, with 3 being signal transduction pathways: PI3K/AKT ($p = 0.007$), Rap1 ($p = 0.04$), and cGMP-PKG ($p = 0.04$) signalling pathways (Figure 4.6 B). A Sankey plot in Figure 4.8 illustrates the genes targeting these three signal transduction pathways. Figures

4.6 B and 4.8 collectively suggest that PI3K/AKT is the most targeted pathway by significant DE genes and shares several common genes with Rap1, implying potential interactions between these two pathways and suggesting that PI3K/AKT may play a pivotal role in MFS pathogenesis.

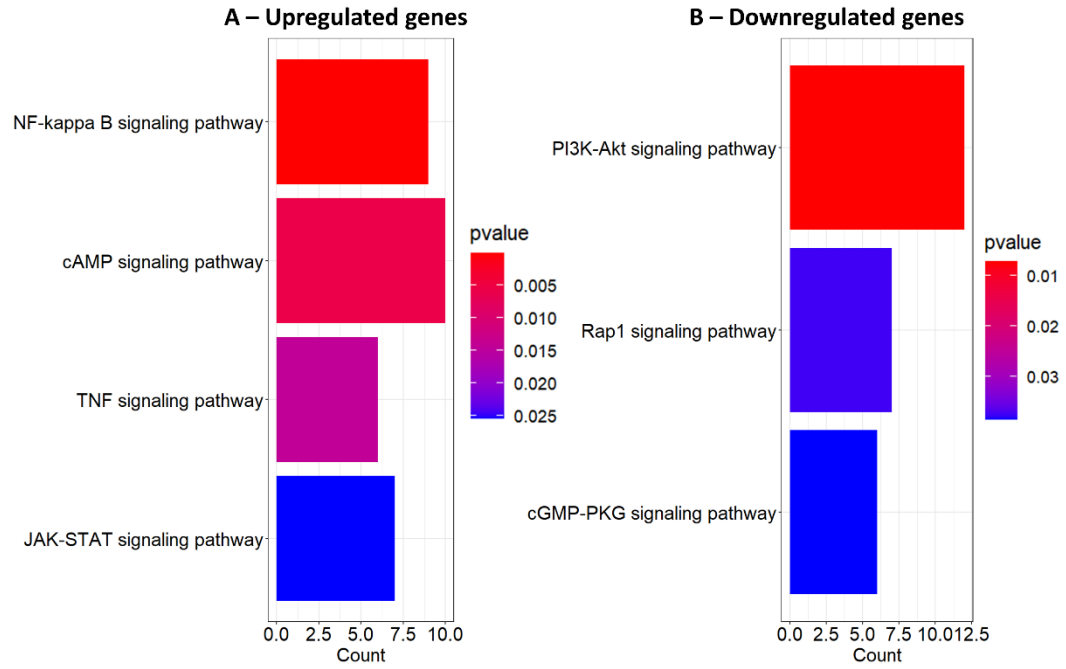


Figure 4.6 KEGG signal transduction pathway analysis for significant up and downregulated DE RNA. A) KEGG analysis for upregulated significant DE genes B) KEGG analysis for downregulated significant DE genes. Count referred to the number of genes that identified within the pathway.

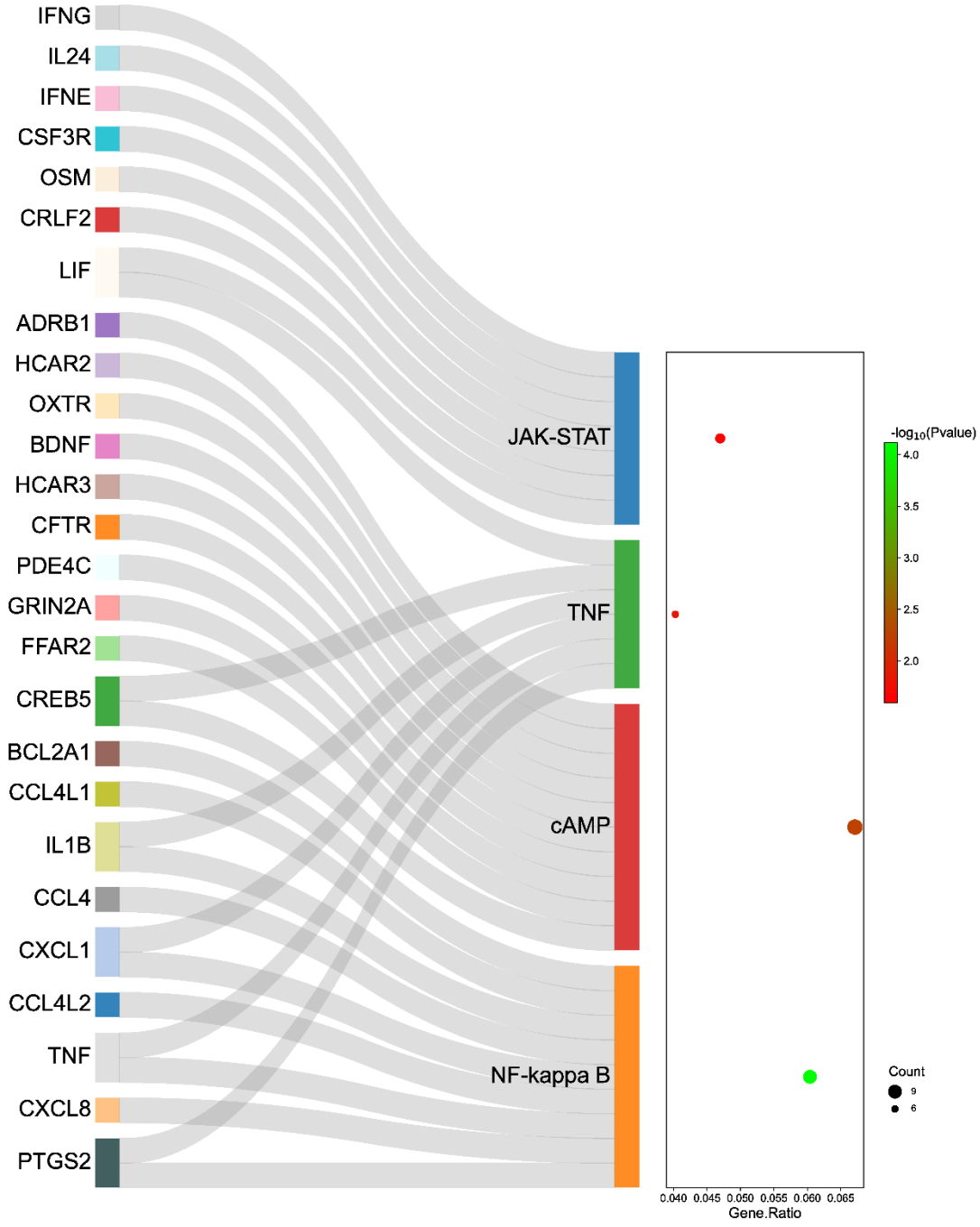


Figure 4.7 Sankey plot of significant signal transduction pathways for upregulated significant DE genes. Genes displayed on the left-hand side, connect to target pathway on the right-hand side. The side bubble plot indicates the gene ratio (percentage of genes identified within the pathway to the amount of gene input), green dots in the side bubble plot indicate smaller p value and red dots indicate larger p value. The size of the bubble indicates the sum of the genes.

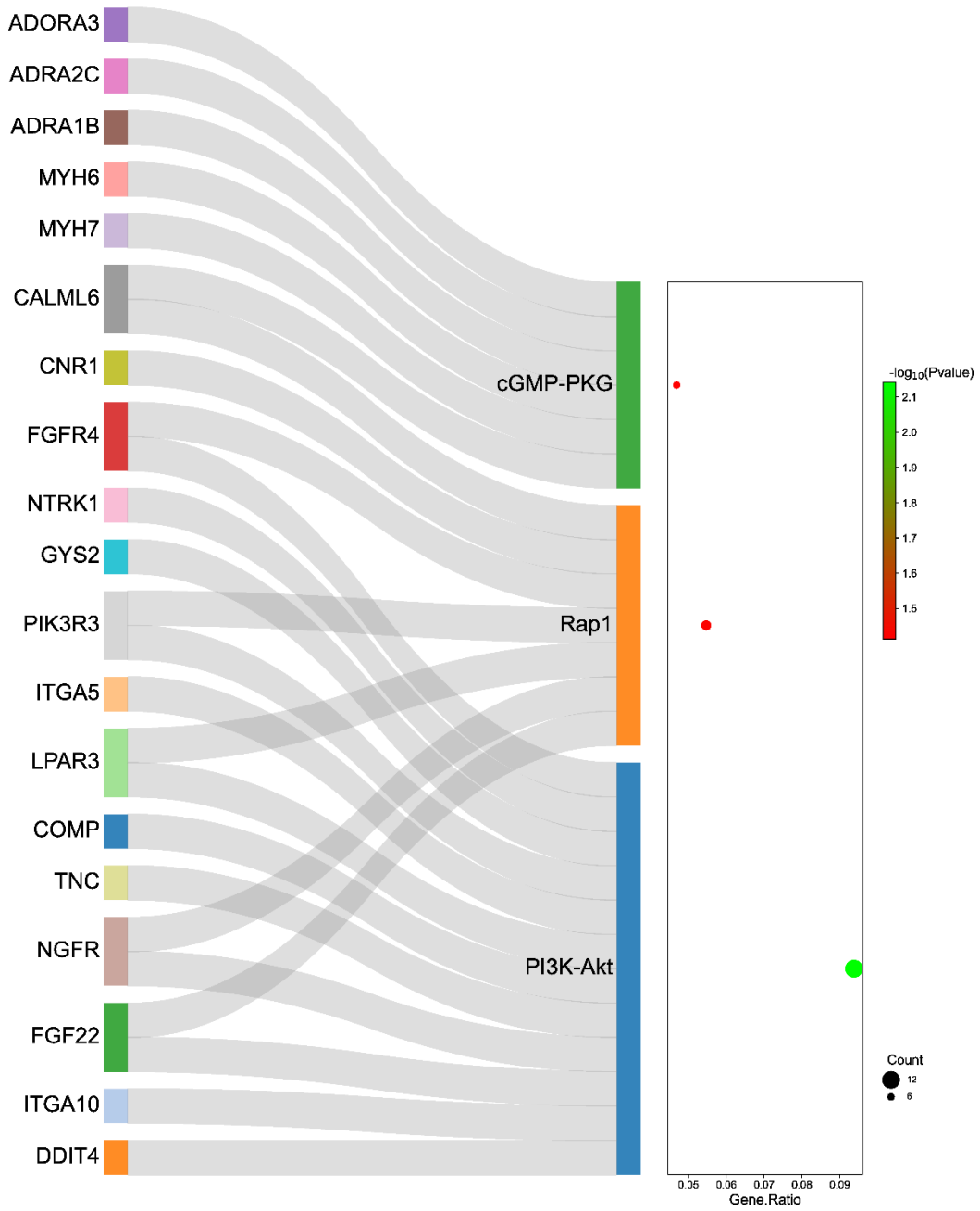


Figure 4.8 Sankey plot of significant signal transduction pathways for downregulated significant DE genes.

In Figure 4.9, significant up- and downregulated DE genes that identified pathways were presented after FDR procedure, and, as with the overall analysis, the majority of the pathways remained consistent. The findings indicate that upregulated significant DE genes continue to place a strong emphasis on the inflammatory pathway. The identification of the sphingolipid pathway by upregulated genes further underscores the significance of the inflammatory response in MFS pathogenesis. The Sankey plot for upregulated significant DE genes, as shown in Figure 4.10, suggests the presence of the same interleukins, CCLs, and CXCLs

genes in MFS patients compared to controls, further confirming the upregulation of inflammation in the MFS aortic wall. Similar to downregulated significant DE genes from raw p-value analysis, significant downregulated genes continue to target cGMP-PKG and PI3K/AKT pathways, highlighting their crucial role in MFS pathogenesis (Figure 4.11).

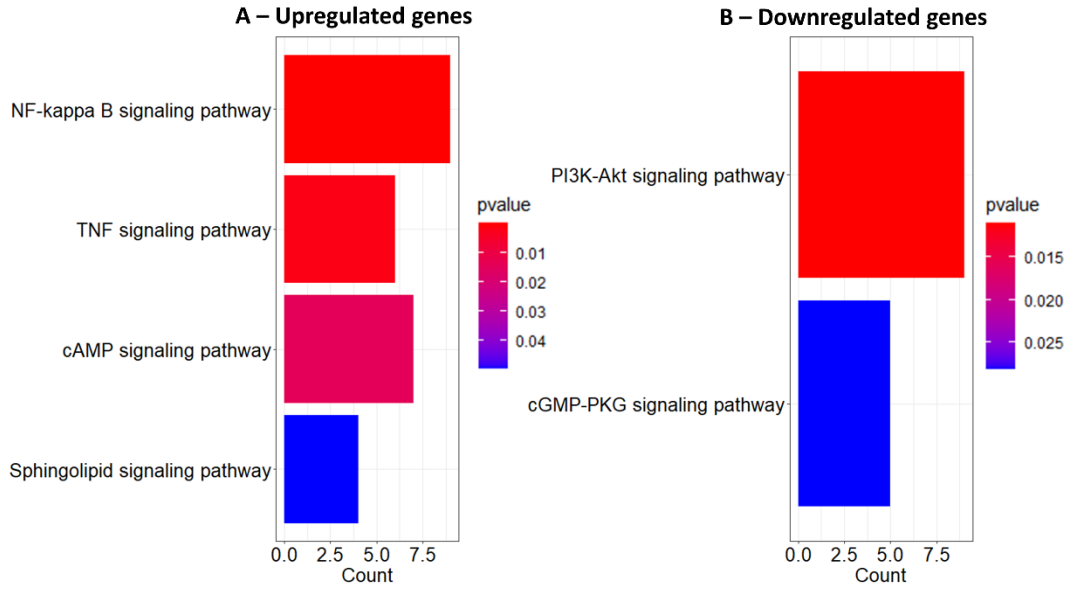


Figure 4.9 KEGG signal transduction pathway analysis for significant up and downregulated DE RNA after Benjamini-Hochberg correction.

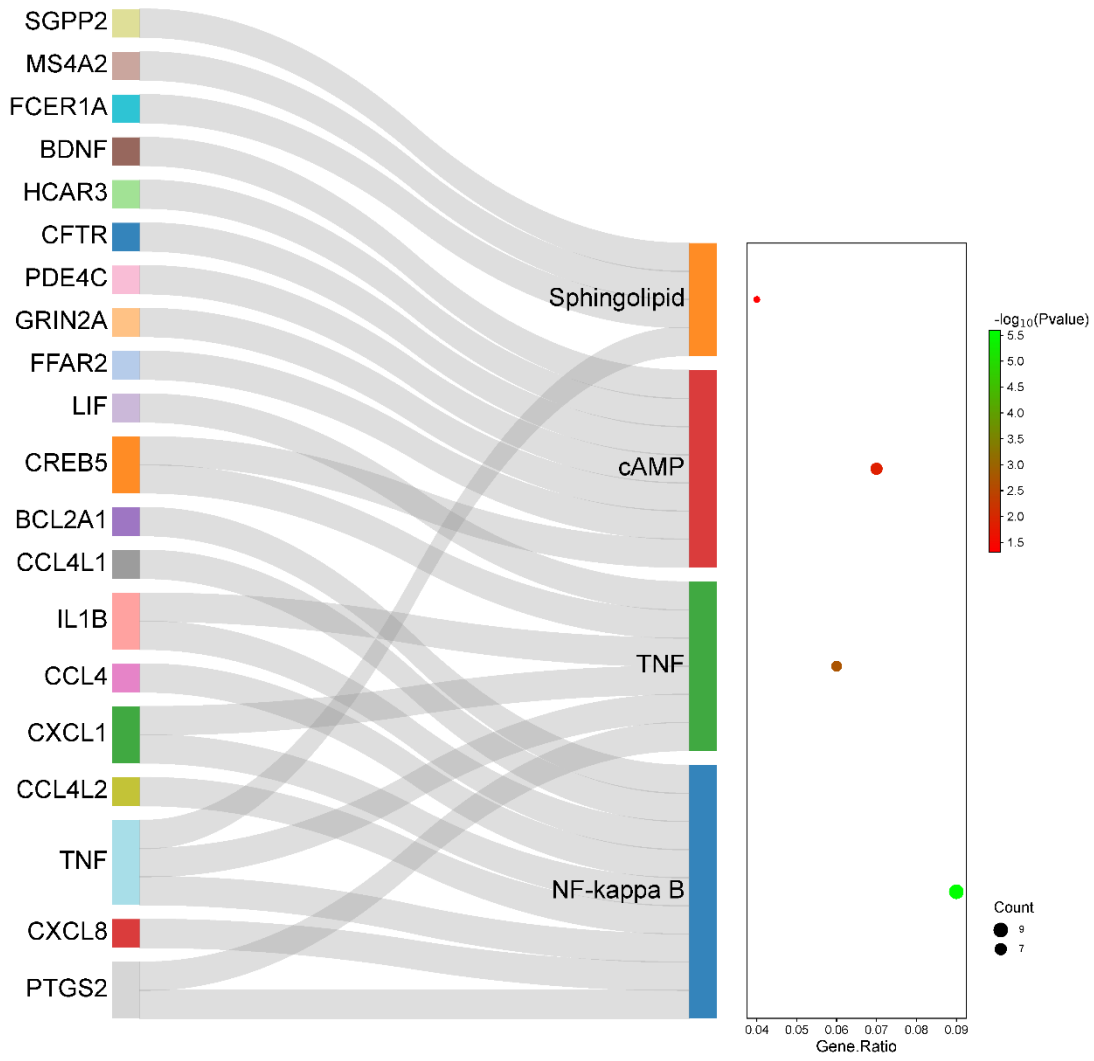


Figure 4.10 Sankey plot of significant signal transduction pathways for upregulated significant DE genes after Benjamini-Hochberg correction.

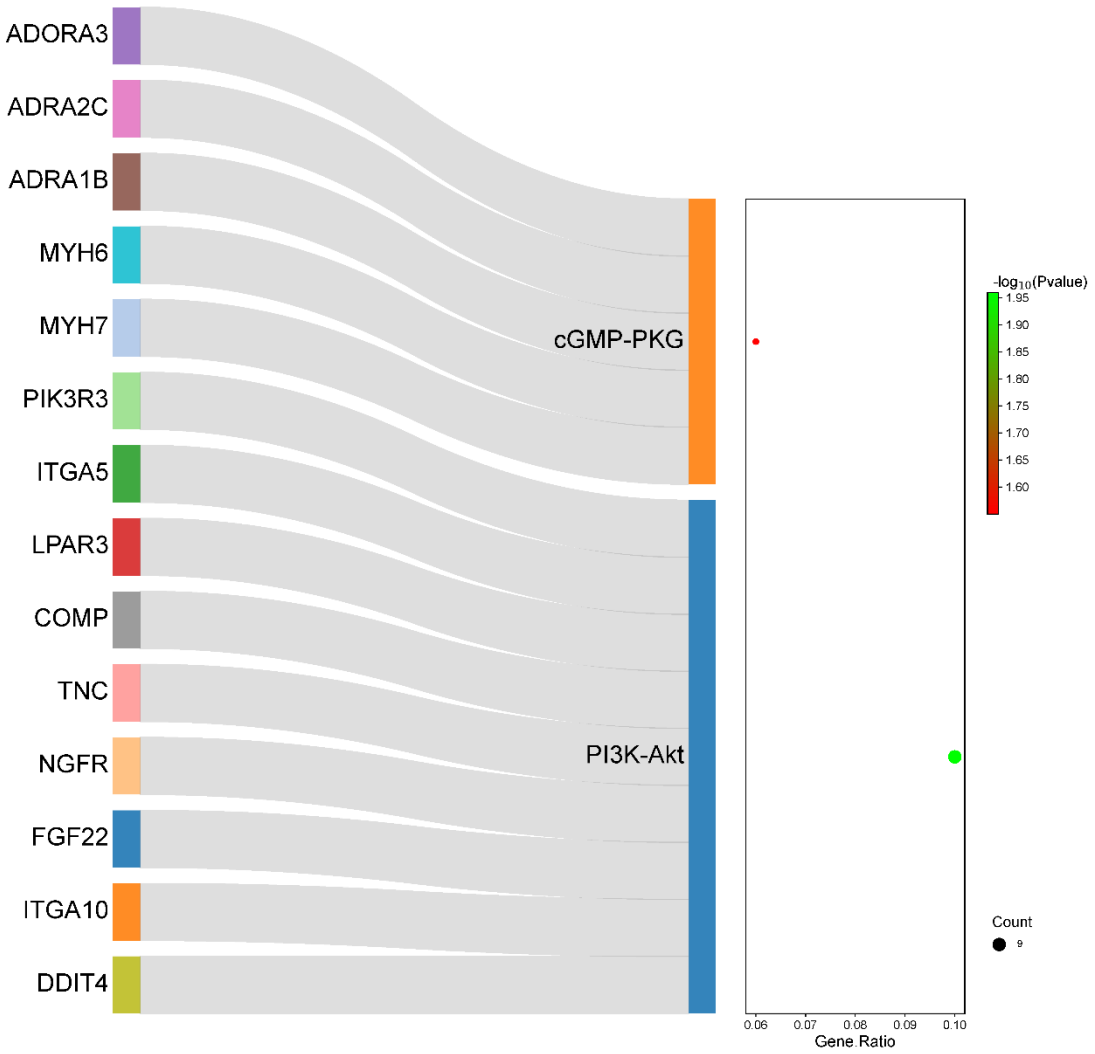


Figure 4.11 Sankey plot of significant signal transduction pathways for downregulated significant DE genes after Benjamini-Hochberg correction.

A GO analysis was conducted on DE genes between MFS patients and controls to gain further insights into dysregulated processes in MFS pathogenesis. GO circle plots were generated for BP (biological process), CC (cellular component), and MF (molecular function) categories, presenting the top 10 ontology terms in Figure 4.12. Similar to the KEGG analysis, Z-score is calculated as the ratio of upregulated genes to downregulated genes. Most of the GO analysis results indicate involvement in the inflammatory response (coloured in blue) and dysregulation of ECM components (coloured in orange). The findings emphasized the involvement of inflammatory response and heart muscle components regulation.

In Figure 4.13, the top 5 enrichments for each ontology along with the involved genes were illustrated. By evaluating the fold change of key genes in each GO ontology term, the results further illustrate the key genes in those pathways. The GO cnetplot displays the genes that target a particular ontology term, the result demonstrates that inflammatory regulation is mainly governed by overexpressed CCLs, CXCLs, and interleukins, while VSMC and cardiomyocytes constituents are primarily modulated by downregulated muscle contraction genes, including myosin heavy chain (MYH) genes, myosin light chain (MYL) genes, and troponin (TNNT2/TNNI3/TNNC1) genes, suggesting possible ECM dysregulation associated with inflammatory response and, paradoxically, loss of contractility of cardiomyocytes.

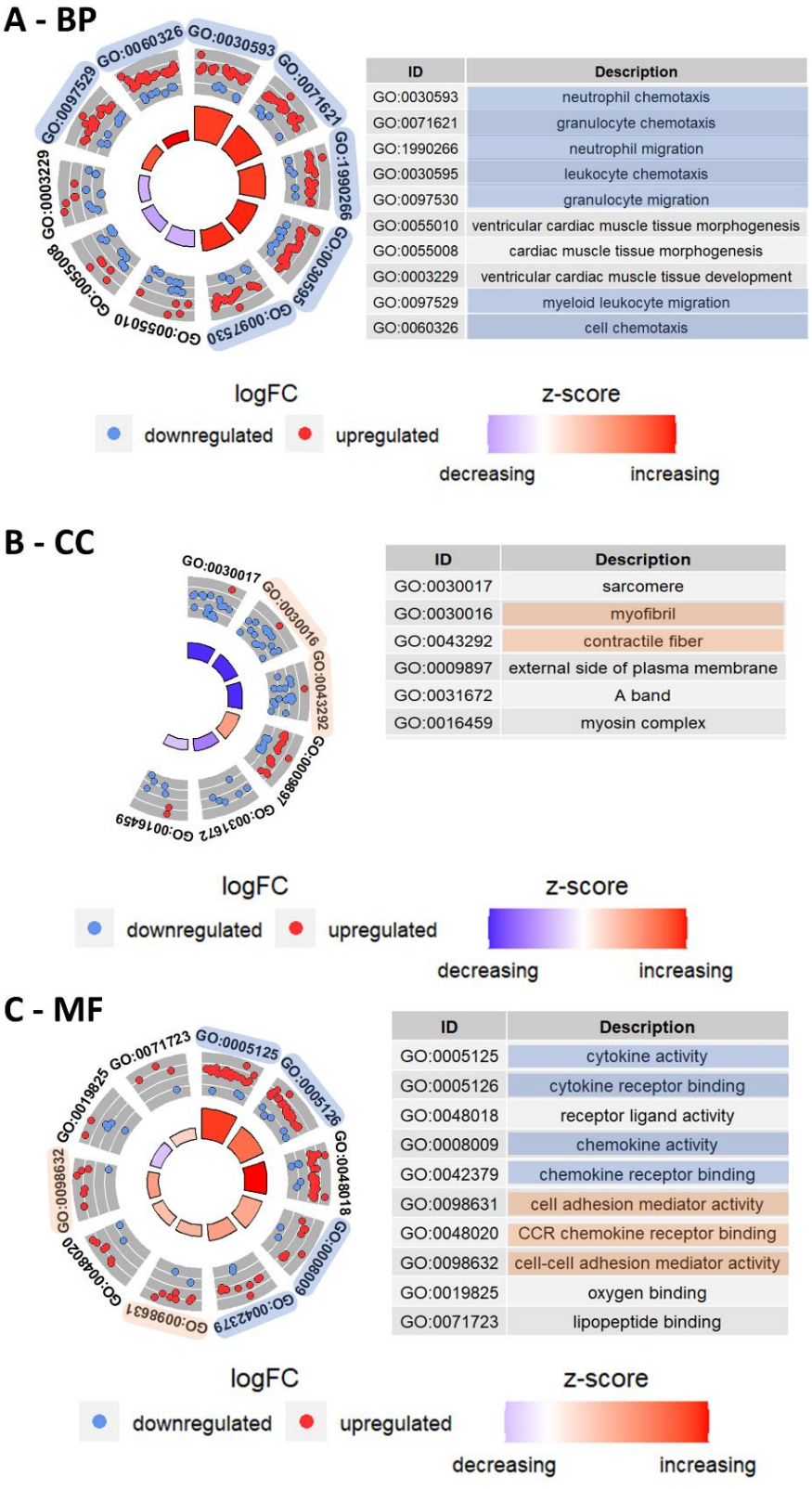


Figure 4.12 GO analysis for significant DE RNA. A) Biological process. B) Cellular component. C) Molecular function. Orange highlight indicates the ontology term is ECM related, blue highlight indicates the ontology term is immune related.

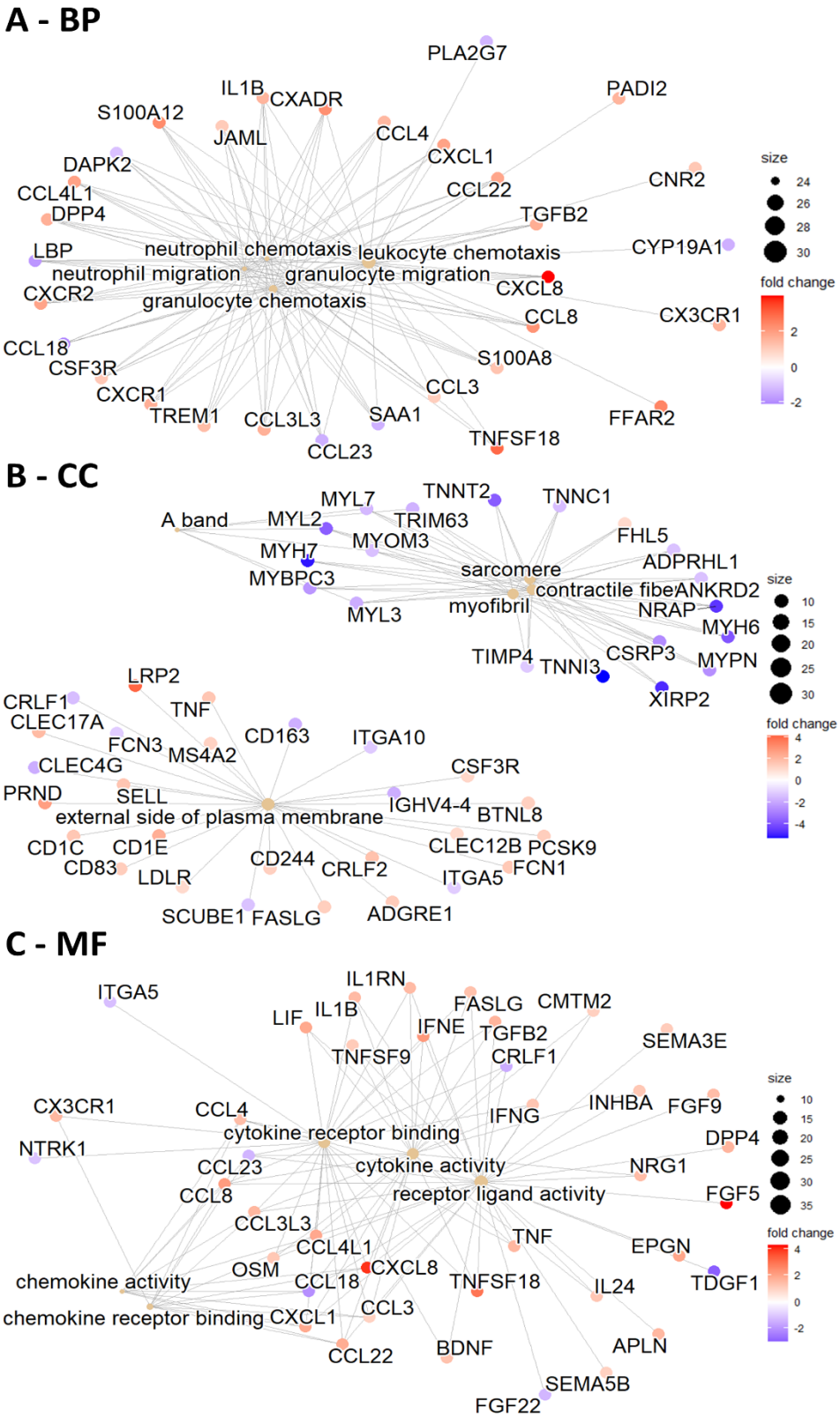
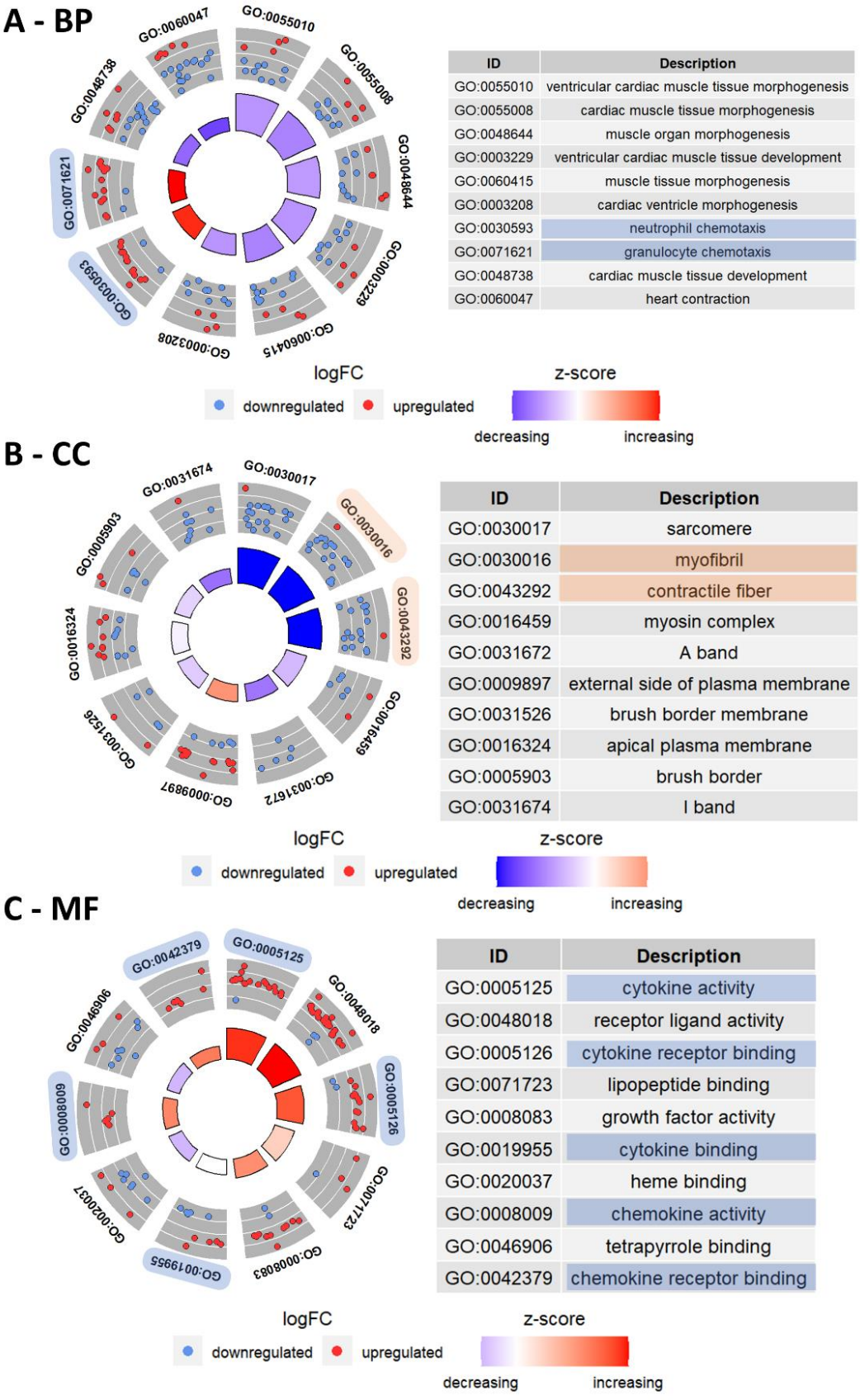


Figure 4.13 GO cnetplot for significant DE RNA. A) Biological process. B) Cellular component. C) Molecular function. Fold change of the genes were displayed from red (upregulated) to purple (downregulated).

Significant DE genes after Benjamini-Hochberg correction were utilized for conducting GO analysis as a comparison to the raw p-value GO analysis. In Figure 4.14 and Figure 4.15 (GO circle plot and cnetplot), similar inflammatory regulation and heart muscle components were identified, reinforcing that raw p-values yield similar downstream analysis results for both KEGG and GO. This also underscores the paradoxical involvement of cardiac muscle contractility, as mentioned above. After applying the FDR procedure, the significance of DE genes demonstrates a more pronounced emphasis on heart muscle contractility over the inflammatory response when compared to the raw p-value results. This suggests that the loss of muscle contractility genes may play a role in MFS pathogenesis. Nevertheless, the highlight of the inflammatory response still holds great significance due to the upregulation of inflammatory genes like interleukins, CCLs and CXCLs.



ID	Description
GO:0055010	ventricular cardiac muscle tissue morphogenesis
GO:0055008	cardiac muscle tissue morphogenesis
GO:0048644	muscle organ morphogenesis
GO:0003229	ventricular cardiac muscle tissue development
GO:0060415	muscle tissue morphogenesis
GO:0003208	cardiac ventricle morphogenesis
GO:0030593	neutrophil chemotaxis
GO:0071621	granulocyte chemotaxis
GO:0048738	cardiac muscle tissue development
GO:0060047	heart contraction

ID	Description
GO:0030017	sarcomere
GO:0030016	myofibril
GO:0043292	contractile fiber
GO:0016459	myosin complex
GO:0031672	A band
GO:0009897	external side of plasma membrane
GO:0031526	brush border membrane
GO:0016324	apical plasma membrane
GO:0005903	brush border
GO:0031674	I band

ID	Description
GO:0005125	cytokine activity
GO:0048018	receptor ligand activity
GO:0005126	cytokine receptor binding
GO:0071723	lipopeptide binding
GO:0008083	growth factor activity
GO:0019955	cytokine binding
GO:0020037	heme binding
GO:0008009	chemokine activity
GO:0046906	tetrapyrrole binding
GO:0042379	chemokine receptor binding

Figure 4.14 GO analysis for significant DE RNA after Benjamini-Hochberg correction.

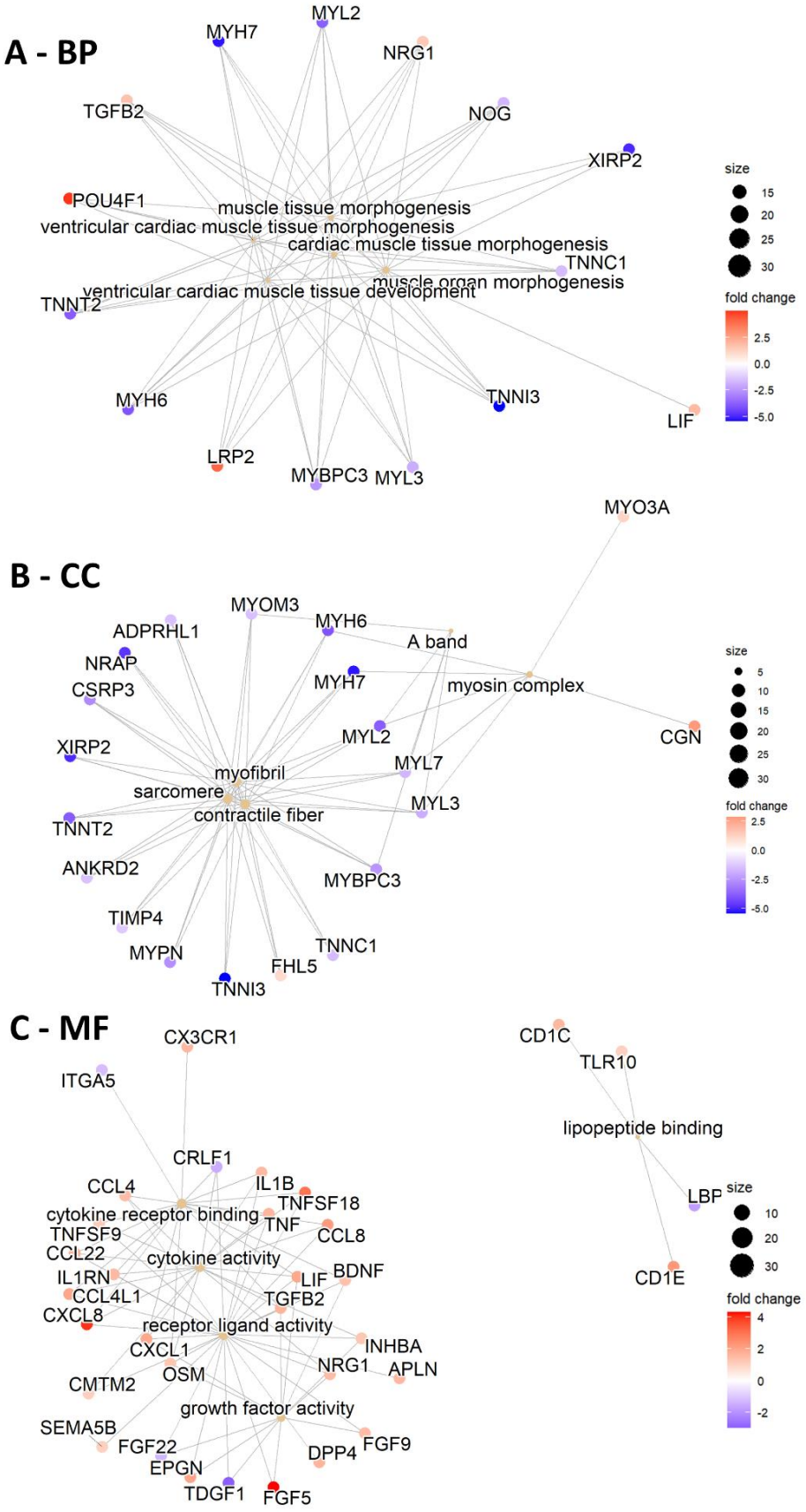


Figure 4.15 GO cnetplot for significant DE RNA after Benjamini-Hochberg correction.

4.4 DISCUSSION

This study investigated MFS and identified 511 upregulated and 476 downregulated RNA in MFS patients compared to controls. Notably, an upregulation of genes regulating inflammation and a concurrent downregulation of genes crucial for ECM homeostasis were observed. Furthermore, alterations in genes related to oxidative stress, mitochondrial function, and muscle contractility within the vessel wall have been identified. These findings strongly suggest that inflammation, oxidative stress, and mitochondrial dysfunction are contributors to MFS-associated TAA.

These findings are consistent with previous reports in MFS of an elevated inflammatory response, suggesting an increased cell plasticity under a vascular injury model and elevated oxidative stress observed in MFS pathology (Zhang, Qiu et al. 2022) (Chapter 2.5.2). Furthermore, the identified changes in genes associated with mitochondrial function and downregulation of the heart muscle contractility are a new observation in the pathogenesis of MFS.

4.4.1 Differential expression analysis of transcriptomics in MFS

By comparing the result to previous MFS transcriptomics studies (summarized in Figure 2.5), among genes previously identified, TIMP4, IL1B, CSCL8, CCL3L3, and MT2A were also identified in the present study, regulating ECM homeostasis, oxidative stress, and the inflammatory response (Pedroza, Tashima et al. 2020, Zhang, Qiu et al. 2022). However, MT2A exhibited contrasting results in this study compared to previous research, with the results indicating decreased expression in MFS tissue, while the other study showed upregulated expression (Zhang, Qiu et al. 2022). It is worth noting that the single-cell study was conducted on acute type A dissection MFS patients, whereas this study deliberately excluded acute dissection patients to eliminate potential dissection-induced cellular responses such as ROS and inflammatory response-induced VSMC apoptosis, vascular remodelling, and myofibroblast senescence (Cheng, Hu et al. 2022). Furthermore, the previous study involved three MFS dissection aortic tissue samples and three control patients, two of whom had cardiovascular-related diseases. Therefore, the increased expression observed in the other study could potentially be attributed to dissection-induced ROS and consequent inflammatory response, as well as an imperfect selection of controls. Consequently, MT2A expression is likely to be downregulated in MFS cohorts based on this current study. Overall, the downregulation of ECM genes and upregulation of inflammatory genes were consistent between studies. Nevertheless,

this study has identified mitochondrial dysfunction as a novel biological driver of MFS, providing new insights into the transcriptional regulation of MFS-associated TAAD pathogenesis.

When comparing genome-wide RNA expression to highly transcribed protein-coding genes in the GTEx database (Table 4.2), of the genes that were examined in this table, the analysis did not reveal any significant differences in RNA expression above the 2FC cutoff. Specifically, in MFS patients, FBN1 transcription level exhibited no significant change compared to controls (less than 10%). Similarly, the fold change in RNA expression of VSMC marker genes such as ACTA2, TAGLN, MYL9 and MYH11, or ECM regulatory genes like ELN and MFAP4 did not reach a 2FC cutoff, although ACTA2, MYH11, ELN and MFAP4 were significantly altered between MFS and controls (Rombouts, van Merrienboer et al. 2022). Notably, ELN transcript expression showed almost an approximate 2-fold decrease, almost reaching the cutoff. It is important to note that *de novo* ELN RNA transcription and protein synthesis are believed to not occur in adult life; they exhibit low basal levels of expression and slowly break down in adult cells and tissues over 70 years (Wang, Meng et al. 2021). However, the observed close to 2FC downregulation in ELN expression in MFS patients compared to controls suggests that at least some elastin transcription occurs in the normal adult aorta and that it is reduced in MFS. A previous study suggests that elastin transcription can be inhibited by growth factors, FoxO1 and interleukins that bind to different regions of the elastin gene promoter and inhibits the transcription process (Procknow and Kozel 2022). However, the key regulatory mechanisms that affect ELN transcription activities in adults remain unclear.

Furthermore, some VSMC markers like MYH11, ACTA2 and ECM component MFAP4 was significantly different in the MFS cohort, although the fold change remains relatively low. This could be due to the lateness of the disease stage (advanced stage requiring surgery) and high basal expression level. Notably, as this is the largest MFS human tissue study conducted with matching qualified and healthy controls, these results may indicate a significant difference between MFS human tissue studies and animal or cell-based research. For example, in this study ACTA2 was found to be upregulated 1.4FC, but has been reported to be downregulated in advanced MFS VSMC in an animal model (Chen, Peters et al. 2017). However for human studies, disease progression in MFS patients can be challenging to determine (Pedroza, Tashima et al. 2020). The MFS patients included in this study could be described as mostly having advanced-stage disease,

but the limited demographic data available was unclear whether each of them was closer to the end-stage or early stage of the disease. This uncertainty may yield different results compared to animal studies, where aneurysm progression can be tightly controlled.

An inflammatory response has been increasingly characterized in MFS TAA, with evidence of increased inflammatory cell infiltration leading to oxidative metabolism activation, ECM degradation through elevated MMPs production, and decreased TIMPs (Chapter 2.4.2) (He, Guo et al. 2008, Pedroza, Tashima et al. 2020). This process promotes VSMC phenotypic switching and EC dysfunction via dysregulated NO levels, ultimately contributing to TAA formation (Malecki, Hambly et al. 2020). Furthermore, by categorizing the infiltrating inflammatory cells (T-lymphocytes and macrophages) in MFS, CD3/ 4/ 8/ 20 /68 have been shown to be significantly elevated in MFS aorta adventitia and media, along with increased ICAM-1 and VCAM-1 around the vasa vasorum (He, Guo et al. 2008). Interestingly, the infiltration of T-lymphocytes and macrophages showed a negative correlation with the age of patients undergoing prophylactic surgery, suggesting that inflammation may play a pivotal role in aging and aging associated TAA disease progression (He, Guo et al. 2008, Margrethe Flesvig, Bjørn et al. 2019). However, He and Guo et. al. (2008) found that the infiltration of the immune cells is unrelated to the aortic size, which may infer that disrupted tissue structure correlates poorly with the inflammatory response (He, Guo et al. 2008). Thus, other factors could play a role in evoking an inflammatory response, for example, oxidative stress.

Oxidative stress may occur with or without an inflammatory response, however, oxidative stress can promote inflammation and an active immune response, which can in turn generate more ROS, demonstrating an interplay between these factors (Sena, Leandro et al. 2018). Oxidative stress is another significant biological process identified through significant DE RNA in MFS. Increased ROS production and oxidative stress have been observed in MFS TAA, leading to decreased NO production and subsequent EC dysregulation (Jiménez-Altayó, Meirelles et al. 2018, Irace, Cammisotto et al. 2021). Furthermore, oxidative stress is evident in MFS and contributes to mechanosignaling dysregulation, MMPs production, VSMC phenotypic switching, and apoptosis (Jiménez-Altayó, Meirelles et al. 2018). Additionally, the total antioxidant capacity in MFS plasma has been shown to be associated with the disease severity (Fiorillo, Becatti et al. 2010). An animal model of MFS has shown upregulated pro-oxidant enzyme expression and decreased ROS-scavenging antioxidants within the aortic wall, suggesting potential ROS

accumulation in the MFS aortic wall (Yang, van Breemen et al. 2010). The mechanism of increased oxidative stress could potentially involve an oxidant and antioxidant imbalance within the vessel wall with reduced superoxide dismutase (SOD) and elevated NADPH, iNOS and xanthine oxidase activity or may just simply be from an elevated inflammatory response (Rysz, Gluba-Brzózka et al. 2021).

Furthermore, this study has identified mitochondrial dysfunction may play a role in MFS pathogenesis. Mitochondrial dysfunction can initiate an inflammatory response by activating the NF-κB pathway, which, in turn, stimulates monocytes and lymphocytes, leading to chronic inflammation. This dysfunction can also impair oxidative metabolism due to reduced oxidative capacity and disrupted antioxidant regulation, thus creating a vicious cycle of inflammation (López-Armada, Riveiro-Naveira et al. 2013, Suslov, Afanasyev et al. 2021). This leads to ECM dysregulation with immune infiltration and oxidative stress accumulation (Bhatti, Bhatti et al. 2017, Suslov, Afanasyev et al. 2021). Elevated mitochondrial ROS and cytokine production have a synergistic effect on the vessel wall, activating fibroblasts and myofibroblasts, ultimately leading to fibrosis, excessive synthesis of ECM components, vessel dilation, and aneurysm progression (Suslov, Afanasyev et al. 2021). In MFS animal models, *Fbn1*$^{c1039g/+}$ mice exhibited mitochondrial dysfunction, accompanied by a loss of VSMC contractility and aneurysm formation (Oller, Gabandé-Rodríguez et al. 2021). Restoration of mitochondrial function was associated with reduction in aneurysm progression (Oller, Gabandé-Rodríguez et al. 2021). Hence, the interplay among mitochondrial dysfunction, inflammatory responses, and oxidative stress could potentially synergistically activate each other, ultimately promoting TAA formation.

In summary, the interplay between the inflammatory response, oxidative metabolism, and mitochondrial dysfunction appears to be contributing factors in the pathogenesis of MFS-associated TAAD. These processes contribute to ECM/EC dysregulation, VSMC phenotypic switching, and apoptosis.

Intriguingly, genes and biological processes that regulate heart muscle contractility were identified through RNA profiling in MFS patients, such as MYH6, MYH7, MYL2, and TNNT2. The expression of these genes is most likely limited to cardiomyocytes, which were traditionally thought not to exist within the aortic wall. However, recent evidence has shown the paradoxical presence of a small population of cardiomyocytes within the aortic root and wall, derived embryologically from the coronary artery stem (Chen, Poduri et al. 2014). The muscle contractility transcript changes observed in this study may arise from this possible population of

cardiomyocyte -like cells within the ascending aorta. This hypothesis will be further discussed in Chapter 8.

In contrast to previous studies, the present study did not find significantly altered expression of MMPs and TIMPs (Jana, Hu et al. 2019). The regulatory mechanism of MMPs involves recruitment as part of the injury response, induced by inflammation, with MMPs initiating the subsequent wound healing process. Firstly, MMPs eliminate all damaged matrix proteins from ECM, then allowing the ECM to promote the wound healing process, involving biological processes like cell adhesion, migration and remodelling (Kandhwal, Behl et al. 2022). A possible explanation for the lack of DE amongst MMPs and TIMPs observed in this study is that the progression of TAA has passed the initial stage of MMP-initiated ECM degradation and progressed into the wound healing process. However, studies have demonstrated that MMPs are required in all stage of wound healing and play an important role in chronic wound healing (Caley, Martins et al. 2015). Further studies are warranted to resolve the role of MMPs in the human aortic ECM remodelling process related to TAA.

The majority of the top 20 up- and downregulated RNA in MFS patients detected in this study are expressed in immune cells, however, only a limited immune cell infiltration is observed in MFS histology. Thus, it is possible that a potential morphological or functional change in VSMC or fibroblast cells may contribute to immune signalling (Grewal and Gittenberger-de Groot 2018, Sorokin, Vickneson et al. 2020), including macrophage-like signalling. While classic VSMC phenotypical changes in MFS TAA involve the transition from contractile to synthetic VSMC (detailed in Chapter 2.4.1-2.4.2), recent studies have revealed a broader heterogeneity of VSMC within the aorta wall. This includes proliferative VSMC, secreting VSMC, immune-related VSMC, proinflammatory VSMC, mesenchymal stem cell-like VSMC, stressed VSMC, and more (Sorokin, Vickneson et al. 2020). This indicates that the shift from contractile to synthetic VSMC may not be a simple binary switch but rather a gradual adaptation in response to various pathological factors such as shear stress and oxidative stress (Sorokin, Vickneson et al. 2020, Hu, Cai et al. 2023). Notably, under the category of immune-related VSMC, previous data suggest that contractile VSMC can transform into macrophage-like VSMC and T-cell-like VSMC, which can elevate macrophage and T lymphocyte markers and regulate inflammatory responses (Hu, Cai et al. 2023). These data indicate elevated inflammatory and immune gene expression may primarily arise in

immune cells, but further suggesting a potential heterogeneity of VSMC that may undergo morphological or phenotypical changes toward immune-related VSMC.

4.4.2 Signal transduction and biological processes in MFS transcriptomics

The signal transduction study identified important pathways that appear to be involved in MFS pathology. Notably, inflammatory pathways, such as NF-κB and TNF, were identified. NF-κB, known for its role in inflammatory responses, is activated and regulated by TNF signalling, interleukins, Toll-like receptors (TLRs), and mitochondrial dysfunction-induced ROS. Upon activation, NF-κB has been shown to recruit cytotoxic immune cells, promote cell survival, and inhibit apoptosis within the cancer environment (Hoesel and Schmid 2013). However, prolonged NF-κB activation can lead to chronic inflammation. NF-κB exhibits extensive crosstalk with various pathways, including JAK-STAT, TNF, MAPK, PI3K/AKT, where this signalling is associated with mitochondrial induced apoptosis, regulating inflammatory responses and subsequent cellular processes such as proliferation, apoptosis, or migration (Hoesel and Schmid 2013, Albensi 2019). Besides interacting with NF-κB, TNF is also associated with TAAD progression by increasing TNF-α levels and inducing a pro-inflammatory response (as detailed in Chapter 2.5.11). This results in the infiltration of inflammatory cells and MMP production, leading to elastic fibre destruction and medial degeneration through PI3K/AKT, mTOR, and MAPK pathways (Du, Liu et al. 2016, Bai, Gu et al. 2018).

Furthermore, cAMP, Ras signalling, and PI3K/AKT signalling appear to be involved in inflammatory-regulated responses as well. The roles of Ras and PI3K/AKT have been previously summarised in the Literature Review (detailed in Chapter 2.5.9 and 2.5.3, respectively). PI3K/AKT serves as a common downstream pathway for NF-κB, TNF, and Ras (which activates NF-κB). Since all three pathways elicit pro-inflammatory responses in TAA, PI3K/AKT may serve as an intermediate downstream pathway, regulating cellular responses in accordance with upstream inflammatory stimuli. The cAMP signalling pathway, along with cGMP-PKG signalling, both cyclic nucleotide pathways, regulate VSMC contractile function and ECM maintenance and integrity. Activation of cAMP/cGMP-PKG signalling through cilostazol reduces aortic inflammation, MMP activation, and aneurysm formation (Shen, LeMaire et al. 2020, Zhang, Zhao et al. 2021).

The KEGG results after Benjamini-Hochberg correction confirm our initial findings in relation to signalling pathways involved in MFS pathogenesis, but also shed light on the involvement of the sphingolipid pathway. Sphingolipid signalling can be activated by changes in calcium ion (Ca^{2+}) concentration and plays a significant role in cardiomyopathy by directly influencing cardiomyocyte contractility. Furthermore, this pathway is known to interact with TGFβ, indicating its involvement in myocardial fibrosis, which can eventually lead to heart failure. TGFβ can elevate sphingolipid expression and stimulate collagen production in cardiac tissue. In conditions such as hypertension and atherosclerosis, sphingolipids are known to reduce NO production in EC and induce inflammation. Moreover, sphingolipid signalling also promotes VSMC proliferation and migration in cardiovascular disease (Borodzicz-Jazdzyk, Jazdzyk et al. 2022). The identification of the sphingolipid pathway in this study provides an additional potential link between inflammation and the dysregulation of VSMC, ECM, and EC in MFS. This finding is consistent with the DE RNA analysis results.

By examining the upregulated and downregulated gene targeting pathways, it is clearer that upregulated significant DE genes are regulating inflammatory pathways (NF-κB, TNF, JAK-STAT, and cAMP). JAK-STAT is another classical inflammatory pathway that displays crosstalk with NF-κB and TNF, evokes an inflammatory response, increases MMPs production and activation, and then leads to ECM degradation and aneurysm formation (Rabkin 2017).

The downregulated significant DE genes correlated with PI3K/AKT, Rap1, and cGMP-PKG pathways. The Rap1 pathway was previously reviewed in Chapter 2.5.9, interacting with MAPK and assisting in EC stabilization and cell adhesion. Low expression is associated with TAAD formation. The cGMP-PKG pathway appeared in downregulated gene KEGG analysis with PI3K/AKT and Rap1. PKG plays a role in cardioprotection in ischemia-reperfusion by upstream NO production via PI3K/AKT stimulation to release ROS. This occurs through the opening of the mitochondrial ATP-sensitive K^+ ($mitoK_{ATP}$) channel, which then generates ROS, decreases ROS formation, decreases mitochondrial calcium levels, preserves ATP, and inhibits apoptosis by elevating anti-apoptotic protein BCL-XL expression (Oldenburg, Qin et al. 2004, Carreira, Lee et al. 2011). In contrast, chronic activation of cGMP-PKG in a MFS animal model demonstrated detrimental effects. Overproduction of NO in mice showed aortic dilation and aortopathy formation, suggesting the regulation of the cGMP-PKG pathway may be tightly regulated. Under- or overexpression of cGMP-PKG signalling may lead to TAAD formation (de

la Fuente-Alonso, Toral et al. 2021). Therefore, the downregulated significant DE RNA correlated pathways are likely associated with impaired mitochondrial function, EC regulation, and ROS modulation.

Overall, the signal transduction study has advanced our comprehension of MFS-related transcriptional signalling. It has underscored the findings of alternations in inflammatory responses, oxidative stress, mitochondrial function, and ECM/EC dysregulation linked to MFS pathogenesis.

The GO enrichment analysis has yielded valuable insights into the molecular processes involved in MFS. The analysis successfully identified multiple enrichments related to inflammation and heart muscle contractility. Specifically, the study observed that the upregulated inflammatory-related genes significantly targeted inflammatory activities (interleukins, CCLs, CXCLs, etc.), whereas the downregulated DE genes were associated with heart muscle-related ontologies. The GOBP plot indicated that downregulation of MYHs and MYLs were targeting cardiac muscle tissue-related enrichment, however, as previously discussed, the presence of cardiac muscle cells within the aorta at the moment is speculative. The GOMF plot highlighted that cytokine binding and activity are the most important enrichments targeted by the transcriptomics study. The GOMF cnetplot further confirmed that most significant DE genes targeting cytokine-related enrichment terms were upregulated, providing strong evidence supporting the involvement of an inflammatory response in MFS.

Overall, the GO enrichment analysis has provided valuable information regarding the regulatory changes in MFS, facilitating the understanding of complex molecular processes. These findings contribute to a deeper understanding of transcriptomics in MFS pathology and its potential links to inflammatory and heart muscle-related pathways.

4.4.3 Multiple testing corrections in large omics study

In this study, raw p-values were utilized for all primary analyses and result interpretations. Additionally, the Benjamini-Hochberg correction for multiple testing was calculated for the significance of DE transcripts and compared with the results of the raw p-value significant DE gene analysis. Overall, apart from a significant reduction in the primary DE dataset, downstream analyses exhibited considerable overlap and similarities. Multiple testing correction methods have been extensively

employed in medical research, including Bonferroni, Benjamini-Hochberg, permutation, and others, to enhance result reliability and reproducibility (Jafari and Ansari-Pour 2019, Shuken and McNerney 2023). Implementing multiple testing corrections in medical research boosts the statistical power of the analysis while mitigating type 1 errors by controlling for false discoveries. However, multiple testing corrections, whilst reducing type 1 errors, may increase type 2 errors, causing the loss of valuable data (Aguinis, Vassar et al. 2021). The application of multiple corrections is not arbitrary and should be tailored to the hypothesis and circumstances of each individual study to select the most appropriate method for downstream analyses (Armstrong 2014).

This study presents the largest MFS human aortic sample set analysed by transcriptomics to date, yet it is still constrained by a small sample size due to low disease prevalence and collection difficulties. It was utilized as a discovery study to identify significant DE genes and other regulatory omics in MFS patients compared to controls, followed by downstream pathway and related biological function analyses. From the downstream analysis, it is evident that there are limited differences between the results obtained from raw p-values and those after Benjamini-Hochberg correction. This suggests that multiple testing correction may be underpowered for such a discovery study and downstream pathway research (Lindquist and Mejia 2015). Furthermore, this study provides exact raw p-values (with significance < 0.05) to avoid ambiguity of simply stating significance, and applies a strict fold change (>2FC) threshold, ensuring relatively high data reliability and significance (Buas, Li et al. 2018, Di Leo and Sardanelli 2020). Additionally, genes were ranked using two different criteria (raw p-value and log-transformed fold change), providing a comprehensive view of changes in transcriptional regulation in MFS pathogenesis (Di Leo and Sardanelli 2020).

4.5 LIMITATIONS

4.5.1 Sample limitations

One of the limitations of this study is the sample size (MFS n = 15 and control n =11). A small sample size can lead to challenges in accurate statistical calculations, particularly because a small sample may not follow a normal distribution. Moreover, the statistical power and variability can be compromised due to the limited sample size, which may not be representative of the general population (Faber and Fonseca 2014, Vasileiou, Barnett et al. 2018). The difficulty in obtaining a larger sample size

can be attributed to several factors: 1) the relatively low prevalence of MFS, 2) the fact that only largely advanced MFS patients undergo elective surgery for aorta replacement, and 3) the strict criteria applied to select both MFS and control samples to ensure data quality, such as ensuring no acute dissection for MFS patients and no family history of cardiovascular disease in control patients. However, this sample size is relatively substantial. In fact, the sample size in this human study exceeds that of most similar experimental studies.

Another limitation related to the sample size issue concerns the representation of MFS disease progression. The study obtained MFS aortic tissue samples from patients undergoing aortic replacement surgery, indicating that these patients are in an advanced stage of TAA and/or aortic valve disease. Due to the surgical risk, early-stage MFS patients would not typically need to undergo this surgery. Therefore, the application of the data is limited when considering the differential gene profile between different disease stages.

4.5.2 Handling limitation

Another potential limitation in this study pertains to tissue handling. Many of the control sample tissues were obtained from donor hearts initially deemed suitable for heart transplantation but ultimately deemed unsuitable due to reasons including transportation logistics, immune incompatibility, and donor-recipient size mismatch (Lal, Li et al. 2015). However, some of the samples were collect during aortic trimming to allow surgical implantation. All these tissues were subsequently collected as snap-frozen samples, in most cases after a variable period of perfusion. In contrast, fresh tissues from MFS patients were collected during elective surgery and immediately (10 minutes) treated in RNAlater solution for long-term storage. Notably, previous studies have suggested that there are no significant differences in gene expression profiling between snap-frozen and RNAlater-treated tissues, provided they are handled professionally and cautiously (Mutter, Zahrieh et al. 2004).. This study acknowledges the varying tissue treatments among the samples, but has followed the practices recommended in the previous literature.

4.5.3 Experimental limitation

An experimental limitation of this study is the use of bulk RNA harvested from the entire aortic wall to investigate MFS TAAD pathogenesis in human tissue. This

approach makes it challenging to pinpoint which specific cell types may be expressing the differentially expressed genes. This limitation could be addressed and improved by employing the single-cell RNA sequencing (scRNA-Seq) method, which would enable us to investigate gene expression at the individual cell level. This would enhance our interpretation of the data and provide deeper insights into the cellular functions and regulatory mechanisms involved in MFS TAAD (Angerer, Simon et al. 2017). However, the relative yield of mRNA transcripts within individual cell populations will be confounded by the relative efficiency of harvesting individual single cell populations, for example, inflammatory cells are generally enriched compared to VSMC, which are fixed within the aortic wall. Previous scRNA-Seq studies in MFS have demonstrated that certain inflammatory gene expressions originate from fibroblasts and VSMC, indicating cellular plasticity within the aortic wall (Pedroza, Tashima et al. 2020, Zhang, Qiu et al. 2022). Given the observation of increased inflammatory gene expression in this study, conducting a single-cell study in the future could help identify the precise cell types responsible for these signalling pathways. This would be invaluable for gaining a deeper understanding of the mechanisms and cellular regulation involved in MFS TAAD.

4.5.4 Pathway analysis limitation

A limitation of the pathway analysis stems from the unavailability of phospho-proteomics data, which would allow determination of the directionality of signalling pathways. Phosphorylation plays a crucial role in post-translational regulation and signalling, and phospho-proteomics analysis would enable us to identify the activation of specific phosphopeptides. This information could provide valuable biological and clinical insights into the direction of signalling in the context of the disease (Savage and Zhang 2020). Further experimentation should incorporate phospho-proteomics to validate the directionality of signalling involvement in MFS.

4.6 CONCLUSION

The genome-wide transcriptomics study of MFS aortic tissue has yielded valuable insights into gene regulation, signal transduction, and biological processes implicated in MFS pathogenesis. Our analysis unveiled dysregulation of inflammation, oxidative stress, mitochondrial dysfunction, and ECM homeostasis as key factors driving TAAD progression in MFS patients. Additionally, increased expression of inflammatory genes, typically associated with immune cells, suggests

potential enhanced cell plasticity among cells within the aortic wall in response to vascular injury.

The results from signalling and biological process analyses confirm the involvement of the previously mentioned molecular functions and underscore the potential disruption of heart muscle contractility in MFS. These findings provide a comprehensive view of potential drivers in MFS pathogenesis and emphasize their interconnectedness. Furthermore, pathway and biological process analyses have furthered our findings by identifying novel signal transduction pathways that may contribute to the mechanisms of MFS TAAD.

Chapter 5: Proteomics in Marfan Syndrome

5.1 INTRODUCTION

The previous RNA analysis in MFS patients suggested potential drivers in MFS pathogenesis, including elevated inflammatory and oxidative stress responses, impaired mitochondrial function, and disruption of ECM homeostasis. Prior proteomics studies in MFS have highlighted the biological processes involvement in ECM and collagen degradation, VSMC regulation, and abnormal TGFβ signalling (detailed in Chapter 2.6.2). However, the current knowledge of protein regulation in MFS remains limited since there have been no large studies that have been performed in MFS human tissues compared to the healthy controls.

This chapter outlines the results of protein profiling in MFS patients compared to controls, aiming to investigate the alignment of proteomics data with the earlier findings in transcriptional activity. A specific objective was to assess whether the proteomics data substantiate a series of observations of an inflammatory response, oxidative stress, and mitochondrial dysfunction in MFS pathology. Furthermore, this study explored the potential for identifying additional factors contributing to MFS pathogenesis.

The specific aims of this work were:

1. Compare protein expression in human MFS aortic tissue with control aortic tissue samples.
2. Correlate observed changes in protein expression with cell signalling pathways.
3. Compare the identified cell signalling pathways with the current evidence regarding their roles in MFS TAAD.
4. Identify likely altered biological processes in TAA in MFS.

5.2 METHODS

5.2.1 Protein extraction and protein expression profiling

Protein extraction from aortic tissue was performed as described in detail in the Method Section 3.2.2. The proteomic measurements were performed at the Sydney Mass Spectrometry Centre at Charles Perkins Centre (Detailed in Method Section 3.2.4).

5.2.2 Data analysis

Data analysis for proteomics data is described in Method Section 3.3.2.

Similar to the transcriptomics study, significant DE protein analysis was conducted using raw p-values and subsequently provided with FDR to increase the confidence level. Multiple testing corrections were performed and analysed for downstream KEGG and GO analyses, serving as a comparison to the results obtained with raw p-values.

Enrichment KEGG and GO analysis was only performed on significant 1.5FC threshold proteins (Matta, Boocock et al. 2019). This approach was chosen because proteins may display more subtle changes compared to RNA, by applying a smaller fold change threshold, it can increase the data density and yields more informative results (Karve and Cheema 2011, Schork, Podwojski et al. 2021). Enrichment analyses were performed using all significant DE proteins before and after multiple testing correction, followed by enrichment analysis as detailed in Method Section 3.1.2. Firstly, all significant >1.5FC proteins were submitted to the enrichment analysis, followed by up and downregulated proteins being assessed and analysed separately. Only signal transduction and a few selected relevant pathways were mapped and presented, in the same manner as the RNA analysis.

5.2.3 Statistical analysis

All statistical analysis was performed in Microsoft Excel and RStudio as described in Method Section 3.3.2. Changes in protein expression were considered significant if the raw p-values were <0.05 (Moderated t-Test), with a change in expression of 2FC and 1.5FC (Roxas and Li 2008, Matta, Boocock et al. 2019, Cao, Zhu et al. 2020, Yuan, Sun et al. 2020). The Benjamini-Hochberg correction method was used for multiple testing correction. Notably, the proteomics raw data included null readings,

most likely due to insufficient readings or technical issues. Imputation of null reading with the group mean value was performed for proteins with missing values after quality control (detailed in Method Section 3.3.2) and used only for heatmap and PCA plot analyses (Jin, Bi et al. 2021).

5.3 RESULTS

5.3.1 Patient characteristics

The basic demographics of tissue donors are summarised in Table 5.1 for the proteomic study. Within sixteen MFS patients included in this study, the same fifteen MFS patients were used for both transcriptomic and proteomic studies and the same controls were used in both studies. The proteomics study included an extra sample from one additional MFS patient that was collected after RNA-Seq was performed. Echocardiography data were not available for control donors. All three MFS patients who experienced chronic dissection had a history of type B dissection, with no impact on the ascending aorta.

Table 5.1 Clinical parameters of two groups included in the proteomics study.

	Marfan	Control	p
n	16	11	
Age (years)	33 ± 11	39 ± 15	NS
Male:Female (n)	12 : 4	5 : 6	NS
BMI (kg/m^2)	27.3 ± 6.6	<30	
Aorta (mm)	50 ± 8		
LVEF (%)	61 ± 5		
Aneurysm (n)	13		
Chronic Dissection (n)	3		
Aortic Regurgitation (n)			
Nil	7		
Mild	7		
Moderate/Severe	2		

Note: aortic diameter was only available for 10 MFS donors

BMI = body mass index; LVEF = left ventricular ejection fraction.

5.3.2 Comparative protein expression in Marfan syndrome

A total of 6,701 proteins were initially identified using high-throughput mass spectrometry. After quality control (detailed in Method Section 3.3.2) which filtered out proteins with less than 75% readings across the dataset, 4,882 proteins were retained for further analysis. Given the challenges of distinguishing individual proteins, two different fold change thresholds were employed, namely 2FC and 1.5FC, along with a fixed p-value cutoff of 0.05.

With the 2FC threshold, 73 upregulated proteins and 36 downregulated proteins were identified. Using the 1.5FC threshold, a total of 182 upregulated proteins and 109 downregulated proteins were found. Volcano plots were used to visualize these proteins, with the top 10 significant DE proteins labelled in the plots (Figure 4.9 A-B). The proteins labelled in these plots differ between the 2FC and 1.5FC thresholds due to the ascending p-value ranking; proteins under the 1.5FC threshold may have a smaller FC but a more significant p-value between MFS patients and controls.

Similar to the RNA analysis, the FDR procedure was applied to the proteomics data. After the Benjamini-Hochberg correction, a total of 20 proteins were identified as significant, with 11 being upregulated and 9 downregulated, surpassing a 2FC threshold. When considering a 1.5FC threshold, 43 proteins remained statistically significant, consisting of 22 upregulated and 21 downregulated proteins. It is important to note that the FDR procedure, as discussed in Chapter 4.2.2, can be overly conservative for omics data, which limits the detection of proteins. As a result, this section will not include separate visualizations of significant DE proteins after multiple testing correction. Instead, the FDR values are shown alongside the raw p-value analysis in the tables to indicate the likely FDR associated with the significant DE proteins.

Heatmaps were generated to visualize the significant DE proteins under both the >2FC and >1.5FC thresholds (Figure 4.9 C-D). These heatmaps show distinct clusters for MFS patients and controls. While controls and MFS groups displayed similar expressions across the group, some proteins within individual groups showed variability.

Two PCA plots were conducted under different thresholds to further examine the separation between MFS patients and controls (Figure 4.9 E-F). These plots revealed a slight overlap between the two groups, consistent with the heatmap results, suggesting that significant DE proteins were not as effective as significant DE RNA in distinguishing MFS patients and controls. Interestingly, a protein

expression cutoff of >1.5FC demonstrated better separation power, indicating that a higher FC cutoff may not always be indicative of a true difference.

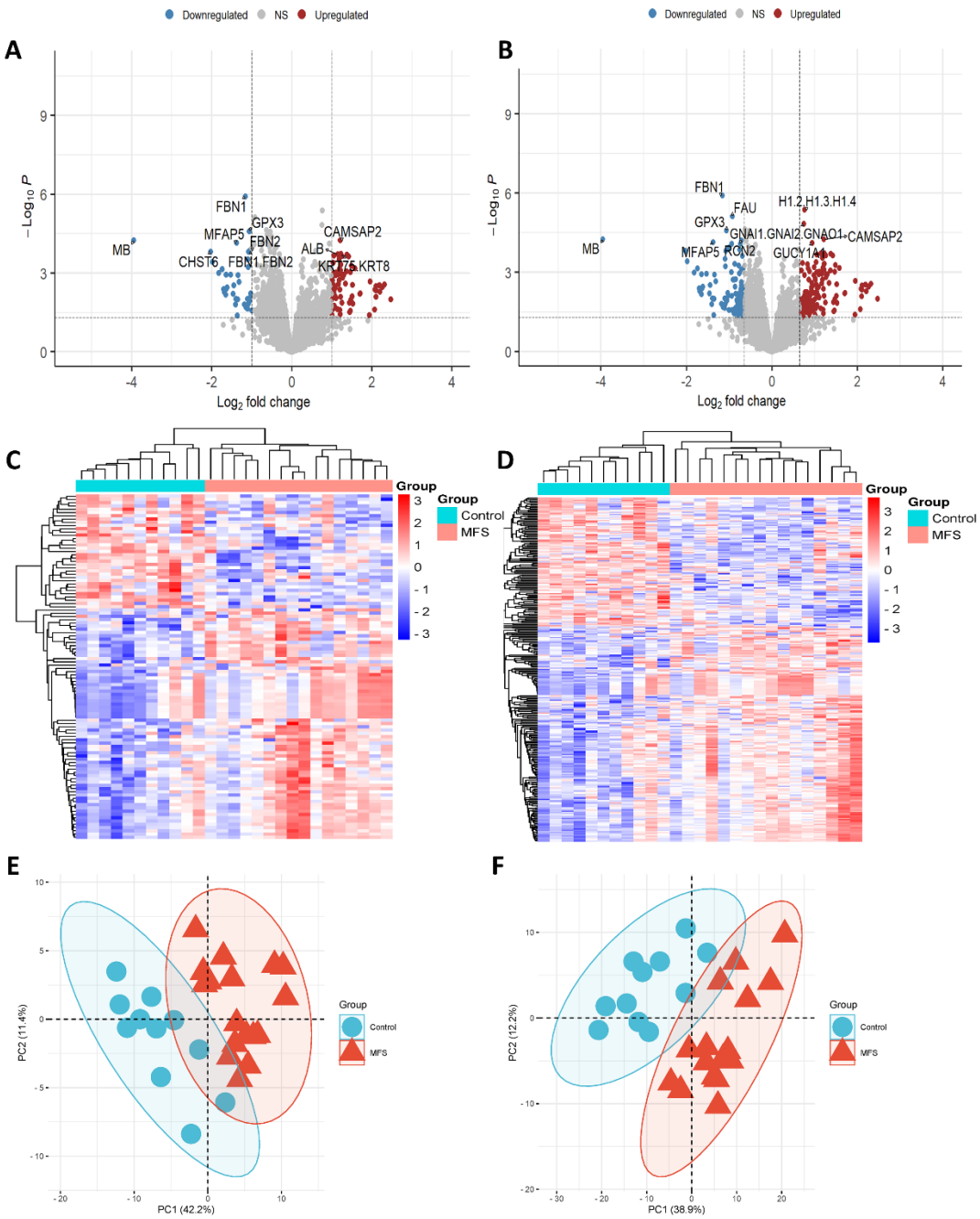


Figure 5.1 Visualization of significant DE proteins between MFS patients and controls. A) Volcano plot for DE proteins with significance cutoff of 2FC and p<0.05, top 10 proteins with the most significant p-values were labelled in the plots. B) Volcano plot for DE proteins with significance cutoff of 1.5FC and p<0.05. C) Heatmap for DE proteins with significance cutoff of 2FC and p<0.05. D) Heatmap for DE proteins with significance cutoff

of 1.5FC and p<0.05. E) PCA plot for DE proteins with significance cutoff of 2FC and p<0.05. F) PCA plot for DE proteins with significance cutoff of 1.5FC and p<0.05.

Similar to the RNA analysis, initial examination was conducted of the same 20 highly expressed aortic mRNA, including FBN1, in aortic tissue from healthy controls as documented in the GTEx online database. The proteins listed in Table 5.2 are identical to those in Table 4.2.

From the result, two proteins (GSN and FBN1) exhibited significant DE with fold changes over 2 (highlighted in red), and three proteins (ELN, MGP, and IFITM3) showed significant DE with fold change over 1.5 (highlighted in orange) using the raw p-value readings. This result demonstrated the fibrillin-1 protein has halved while as shown in the previous chapter, the *FBN1* transcript was not significantly altered. This finding aligns with MFS pathology, in which it has been reported previously that the *FBN1* mutation could lead to fibrillin-1 protein misfolding and destruction but no difference seen in the RNA level (Whiteman, Hutchinson et al. 2006, Tjeldhorn, Amundsen et al. 2015). Additionally, elastin and gelsolin proteins demonstrated alterations with fold change over 1.5 (log2FC > 0.65 or log2FC <-0.65) and 2, respectively, suggesting potential elastin degradation and fragmentation as in the RNA analysis, as well as actin filament dysregulation.

Table 5.2 Highly transcribed genes in the aortic tissue from GTEx database compared to protein expression in MFS.

Entrez ID	**Gene**	**Protein**	**Aorta Expression (TPM)**	**Log_2FC**	**p-value**	**adjust p**
1191	CLU	Clusterin	1604	-0.38	0.23	0.60
2006	ELN	Elastin	1681	-0.67	0.04	0.29
2335	FN1	Fibronectin1	3726	0.49	0.10	0.42
4239	MFAP4	Microfibril Associated Protein 4	1778	-0.13	0.46	0.77
1490	CCN2/ CTGF	Cellular Communication Network Factor 2	2043	-0.52	0.11	0.43
4629	MYH11	Myosin Heavy Chain 11	2357	-0.23	0.35	0.70
4256	MGP	Matrix Gla Protein	11940	-0.81	0.03	0.26

7169	TPM2	Tropomyosin 2	3155	-0.39	0.14	0.48
1292	COL6A2	Collagen Type VI Alpha 2 Chain	1878	-0.18	0.31	0.67
2316	FLNA	Filamin A	3732	0.20	0.22	0.58
59	ACTA2	Actin Alpha 2, Smooth Muscle	5736	N/A	N/A	N/A
6876	TAGLN	Transgelin	6099	0.01	0.96	0.99
10398	MYL9	Myosin Light Chain 9	4275	-0.31	0.08	0.37
4627	MYH9	Myosin Heavy Chain 9	1592	0.12	0.46	0.77
7431	VIM	Vimentin	3091	-0.19	0.17	0.53
2934	GSN	Gelsolin	1620	1.03	4.1E-04	0.04
1843	DUSP1	Dual Specificity Phosphatase 1	1984	N/A	N/A	N/A
3315	HSPB1	Heat Shock Protein Family B (Small) Member 1	3780	-0.50	0.001	0.06
6227	S100A6	S100 Calcium Binding Protein A6	2897	-0.01	0.95	0.99
10410	IFITM3	Interferon Induced Transmembrane Protein 3	2274	-0.66	0.01	0.14
2200	FBN1	Fibrillin 1	63	-1.16	1.2E-06	0.01

Note: logFC represents log-transformed fold change, p-value indicates the raw p-value and adjust p indicates confidence level after FDR. Aorta expression data is obtained from the GTEx online dataset and is represented in TPM. Significant differentially expressed proteins were identified over 2FC and 1.5FC and $p<0.05$.

Subsequently, the results present four tables listing significantly up- and downregulated DE proteins ranked by p-value and log-transformed fold change (Table 5.3-5.6). All proteins listed are from the 2FC threshold without FDR correction.

The top 20 significantly upregulated DE proteins ranked by p-value, suggest the involvement of intermediate filament and inflammatory proteins, hinting at a potential regulatory response to mechanical stress (n = 5, e.g., Keratin proteins such as KRT8, KRT18, KRT6A/B/C), inflammation (2 pro-inflammatory proteins and 4 anti-inflammatory proteins, e.g., HTRA4, APOP2), anti-oxidative stress (n = 5, e.g.,

SUPT6H, AFM), and some ECM and mitochondrial function-related proteins (e.g., PON3, ITIH) (Table 5.3). It is worth noting that after applying the Benjamini-Hochberg correction for multiple testing, five of these initially significant DE proteins were deemed only borderline significant, namely AFM (p. adjust = 0.051), PON1 (p. adjust = 0.052), GPLD (p. adjust = 0.053), PON3, and IGHG1/IGHG3/IGHG4 (p. adjust = 0.054).

The top 20 upregulated proteins ranked by log-transformed FC demonstrate similar results to those ranked by p-value. These proteins illustrate the involvement of the inflammatory response (8 pro-inflammatory proteins, 3 anti-inflammatory proteins, e.g., CA1, HBA1, KRT18), oxidative stress response proteins (8 pro-oxidative proteins and 2 anti-oxidative proteins, e.g., HBB, SLC16A3, SUPT6H), ECM-related proteins (n = 4, e.g., HDAC7, FHL5), and a substantial number of haemoglobin and coagulation-regulating proteins (n = 10, e.g., Haemoglobins HBB, HBA1, HBG) (Table 5.4). These findings align with the results from the top 20 significant DE RNA analysis (Table 4.3-4.4), emphasizing the role of inflammation, oxidative stress, ECM, and mitochondrial regulation. Notably, the presence of haemoglobin regulation is a novel finding in this proteomics study, suggesting it may contribute to MFS pathogenesis. Significantly, only two proteins, SUPT6H (p. adjust = 0.048) and APOA2 (p. adjust = 0.043), retained their significance after applying the Benjamini-Hochberg correction. Interestingly, these two proteins also appeared in Table 5.3, emphasizing their regulatory role in anti-oxidative stress and anti-inflammatory responses.

The top 20 significantly downregulated DE proteins ranked by p-value identified proteins involved in ECM regulation (n = 8), regulation of the inflammatory response (2 pro-inflammatory proteins and 2 anti-inflammatory proteins, PLA2G2A, CYTL1, GPX3, and TNFRSF11B), mitochondrial function (n = 4, e.g., ALDH1L1, GLUL, CKM), and anti-oxidative stress (n = 2, GPX3 and MB) (Table 5.5). Similar to the RNA analysis, proteins regulating cardiomyocyte contractility were identified among the significantly downregulated DE proteins (n = 3, MYH7, MYH6/7, and MYL2), paradoxically suggesting contractility dysfunction in MFS pathology. Nine out of the top 20 significantly downregulated DE proteins were borderline non-significant after multiple testing correction, namely MYL2 (p. adjust = 0.055), GLUL (p. adjust = 0.057), MYH7 (p. adjust = 0.057), CKB/CKM (p. adjust = 0.057), GXYLT2 (p. adjust = 0.075), ALDH1L1 (p. adjust = 0.088), PLA2G2A (p. adjust = 0.097), TNFRSF11B (p. adjust = 0.10), and CYTL1 (p. adjust = 0.11).

Due to the small number of significantly downregulated DE proteins (n = 36 over 2FC cutoff), 13 out of the top 20 proteins ranked by log-transformed fold change

overlapped with the p-value-ranked proteins. Overall, the results highlight the involvement of proteins in regulating anti-oxidative stress response (n = 2), mitochondrial function (n = 4), inflammation (4 pro-inflammatory proteins and 3 anti-inflammatory proteins), ECM maintenance (n = 5), and contractility (n = 3) (Table 5.6). These findings align with our RNA analysis, supporting the hypothesis that these biological processes play a role in MFS pathogenesis. Only five proteins retained their significance after Benjamini-Hochberg correction: MB (p. adjust = 0.024), CHST6 (p. adjust = 0.027), CKM (p. adjust = 0.04), MYH6/MYH7 (p. adjust = 0.048), and MFAP5 (p. adjust = 0.024). These proteins also appeared in Table 5.5 and were consistent with RNA studies (MB and CKM). This underscores the significance of the oxidative stress response and mitochondrial function regulation in MFS pathogenesis.

Table 5.3 Top 20 significant (ranked by p-value) upregulated DE proteins (2FC) from proteomics study and their function.

Gene	Protein	Uniprot ID	Log_2FC	P-value (adj. p)	Expressed cell type	Aorta expression (TPM)	Function and Pathway	Category
CAMSAP2	Calmodulin – regulated spectrin – associated protein 2	Q08AD1	1.21	5.6E-05 (0.024)	Other	32.88	Inhibits calcium accumulation, interacts with miR-125a-3p and miR-483-5p, may involve in vascular calcification (Cao, Guo et al. 2020). Risk factor of CAD (van der Harst and Verweij 2018)	Ion regulation
KRT75. KRT8	Keratin 75/ 8	O95678 P05787	1.23	1.9E-04 (0.029)	Other	<10/33.32	Type II intermediate filament. Absorbs mechanical stress, acts as a mechanical buffer to protect cells against mechanical stress and stabilizing cells and ECM, holds cell-cell and cell-matrix contact. Also involved in wound healing, inflammatory response and cell migration (Herrmann, Bär et al. 2007)	Mechanical stress ECM related
ALB	Albumin	P02768	1.15	1.9E-04 (0.029)	Other	<10	Regulation of colloidal osmotic pressure, low expression associates with inflammation, coronary artery disease severity (Suzuki, Hashizume et al. 2019)	Anti-inflammation
KRT6A/ 6B/ 6C/ 8	Keratin 6A/ 6B/6C/ 8	P02538 P04259 P48668 P05787	1.38	2.0E-04 (0.029)	Other	<10/<10/<10/33.32	Type II intermediate filament. Absorbs mechanical stress, acts as a mechanical buffer to protect cells against mechanical stress and stabilizing cells and ECM, holds cell-cell and cell-matrix contact. Also involved in wound healing, inflammatory response and cell migration (Herrmann, Bär	Mechanical stress ECM related

							et al. 2007) Proliferative response in epithelium and wound healing process (Karmouch, Zhou et al. 2017)	
KRT8	Keratin 8	P05787	1.17	2.1E-04 (0.030)	Other	33.32	Type II intermediate filament. KRT8/18 protects EC from inflammatory induced apoptosis signal from TNFα, promotes cell survival (Jacob, Coulombe et al. 2018)	Anti-inflammation Mechanical stress
KRT2/ 4/ 5/ 6A/ 6B/ 6C/ 7/ 75/ 77/ 79/ 8/ 84	Keratin 2/ 4/ 5/ 6A/ 6B/ 6C/ 7/ 75/ 77/ 79/ 8/ 84	P35908 P19013 P13647 P02538 P04259 P48668 P08729 O95678 Q7Z794 Q5XKE5 P05787 Q9NSB2	1.05	2.1E-04 (0.030)	T-cell Granulocytes Macrophages	<10/<10/<10/<10<10/<10/16.04/<10/<10/<10/33.32/<10	Type II intermediate filament. Absorbs mechanical stress, acts as a mechanical buffer to protect cells against mechanical stress and stabilizing cells and ECM, holds cell-cell and cell-matrix contact. Also involved in wound healing, inflammatory response and cell migration (Herrmann, Bär et al. 2007)	Mechanical stress ECM related
BCHE	Butyrylcholin esterase	P06276	1.26	2.3E-04 (0.031)	Other	<10	Interacts with albumin and apolipoproteins, high expression associates with lower cardiac mortality, associates with lipid metabolism and hypertension (Sulzgruber, Koller et al. 2015)	Lipid metabolism
KRT18. KRT19	Keratin 18/ 19	P05783 P08727	1.42	2.3E-04 (0.031)	Basophils	10.4/13.59	Type I intermediate filament KRT8/18 protects EC from inflammatory	Anti-inflammation

							induced apoptosis signal from TNFα, promotes cell survival (Jacob, Coulombe et al. 2018)	Mechanical stress
AHSG	Alpha-2-HS glycoprotein	P02765	1.18	2.9E-04 (0.036)	Other	<10	Regulated by calcium-regulatory glycoprotein, low AHSG is associated in atherosclerosis, chronic low-grade infection and vascular calcification (Stenvinkel, Wang et al. 2005, Szeberin, Fehérvári et al. 2011)	Ion regulation
GSN	Gelsolin	P06396	1.03	4.1E-04 (0.042)	Fibroblasts Eosinophil Macrophages	1620	Regulated by calcium level, involved in actin filament assembly and disassembly. Implicated in myocardial fibrosis and activates downstream TGFβ signalling and AMPK as well as mTOR (Jana, Aujla et al. 2021)	Ion regulation
HTRA4	HtrA serine peptidase 4	P83105	1.23	4.7E-04 (0.043)	Other	<10	Inhibit EC proliferation and EC repair, induce inflammation, leads to EC dysfunction (Singh, Zhao et al. 2015, Wang and Nie 2016, Wang, Lim et al. 2019)	Pro-inflammation
ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2	P19823	1.22	4.8E-04 (0.043)	Other	<10	Associates with ECM component hyaluronic acid, stabilizes ECM and involved in calcium regulation (Hamm, Veeck et al. 2008)	ECM related Ion regulation
APOA2	Apolipoprotein A2	P02652	1.49	5.3E-04 (0.043)	Monocytes Neutrophils	<10	HDL metabolism, lipid transport, anti-inflammatory and anti-oxidative stress, demonstrates protective role in atherosclerosis (Kihara, Yamagishi et al.	Anti-inflammation Anti-oxidative

							2021)	stress
SUPT6H	SPT6 homolog, histone chaperone	Q7KZ85	1.58	6.7E-04 (0.048)	Other	64.05	Negatively regulating oxidative stress, deficiency led to cardiomyopathy and increased oxidative stress, interacts with miR-423-5p (Xu, Liu et al. 2023)	Anti-oxidative stress
SERPIND1	Heparin cofactor 2	P05546	1.19	6.9E-04 (0.048)	Other	<10	Regulate blood coagulation (Bianchini, Auditeau et al. 2020) Inhibits thrombin and interacts with plasminogen during injury, promotes cell survival (Valiente, Obenauf et al. 2014)	Blood coagulation
AFM	Afamin	P43652	1.22	7.8E-04 (0.051)	Other	<10	Protect cells from oxidative stress-induced apoptosis, associated with insulin resistance and other metabolic issues (e.g., lipid metabolism) (Nowicki, Ślusarska et al. 2021, Juhász, Ujfalusi et al. 2022)	Anti-oxidative stress Lipid metabolism
PON1	Paraoxonase 1	P27169	1.37	8.5E-04 (0.052)	Other	<10	Protect against oxidative stress, associates with lipid metabolism, protects against atherosclerosis and AAA (Shih and Lusis 2009)	Anti-oxidative stress Lipid metabolism
GPLD1	Glycosylphosphatidylinositol specific phospholipase D1	P80108	1.19	8.9E-04 (0.053)	Other	<10	Interact with phosphatidylinositol glycans, lipid metabolism, glucose metabolism and promotes cell apoptosis by inhibiting phosphatidylinositol pathway (Cao, Zhou et al. 2023)	Lipid metabolism Cell death
PON3	Paraoxonase 3	Q15166	1.47	9.3E-04	Fibroblasts	<10	Involved in lipid metabolism can reduce early oxidative product and inhibits	Anti-oxidative stress

				(0.054)			oxidative, overexpression associates with less lipid hydroxides and monocyte chemotactic activities. Protects against mitochondrial oxidative stress, involved in mitochondrial function. Athero-protective role through anti-oxidative stress and inflammatory response initiation (Priyanka, Singh et al. 2019)	Mitochondrial function
IGHG1. IGHG3. IGHG4	immunoglobulin heavy constant gamma 1/ 3/ 4	P01857 P01860 P01861	1.11	9.5E-04 (0.054)	Memory B-cells Native B-cells	29.17/16.38 /<10	IgG shows increased expression in accompanied with immune response infiltration in heart failure, associates with cardiac remodelling. Inhibition of IgGs attenuates cardiomyopathy under chronic inflammation (van den Hoogen, de Jager et al. 2019)	Pro-inflammation

Note: Expressed cell type was obtained from Human Protein Atlas, only heart-related cell and immune cells were included, other indicates no documented heart or immune-related cell. Protein UniProt ID was obtained from UniProt, human species. Expression in aorta is obtained from GTex, expressions within healthy aorta less than 10 were presented as <10, otherwise with the actual expression. Red highlighted proteins are identified in both rankings (p-values and log-transformed FC). Adj. p refers to multiple testing correction adjusted FDR.

Table 5.4 Top 20 significant (ranked by logFC) upregulated DE proteins (2FC) from proteomics study and their function.

Gene	**Protein**	**Uniprot ID**	**Log_2FC**	**P-value (adj. p)**	**Expressed cell type**	**Aorta expression (TPM)**	**Function and Pathway**	**Category**
SLC4A1	Solute carrier	P02730	2.47	0.010	Other	<10	Regulates biosynthesis and mechanical property maintenance of erythrocytes,	Red blood cell related

	family 4 member 1			(0.15)			deficiency associates with collagen deposition and cardiac hypertrophy (Alvarez, Kieller et al. 2007)	ECM related
HBD	Haemoglobin subunit delta	P02042	2.32	0.003 (0.084)	Neutrophils Eosinophils	<10	Elevated haemoglobins associate with increased oxidative stress and inflammatory response, can be induced by mitochondrial stress. Haemoglobin activates pro-oxidative activities and removes NO under injury (Derakhshani, Safarpour et al. 2021, Manzoor, Kane et al. 2023) Correlates to TAA aorta diameter (Harrison, Cagampang et al. 2018)	Red blood cell related Pro-oxidative stress
HBB.HBD	Haemoglobin subunit beta/ delta	P68871 P02042	2.26	0.003 (0.097)	Neutrophils Eosinophils	252.4/<10	Elevated haemoglobins associate with increased oxidative stress and inflammatory response, can be induced by mitochondrial stress. Haemoglobin activates pro-oxidative activities and removes NO under injury (Derakhshani, Safarpour et al. 2021, Manzoor, Kane et al. 2023) Correlates to TAA aorta diameter (Harrison, Cagampang et al. 2018)	Red blood cell related Pro-oxidative stress Pro-inflammation
CA1	Carbonic anhydrase 1	P00915	2.21	0.005 (0.11)	Neutrophils	<10	Induces vascular calcification and promotes inflammatory response, proliferation, migration and apoptosis. Regulates lipid metabolism. Implicated in aneurysm and atherosclerosis (Yuan, Wang et al. 2019)	Pro-inflammation
HBB.HBD.	Haemoglobin subunit beta/ delta/	P68871	2.19	0.003	Neutrophils	252.4/<10/ <10/<10/<1	Elevated haemoglobins associate with increased oxidative stress and inflammatory response, can be induced by	Red blood cell related

HBE1.HBG1/ 2	epsilon 1/ gamma 1/ gamma 2	P02042 P02100 P69891 P69892		(0.088)	Eosinophils	0	mitochondrial stress. Haemoglobin activates pro-oxidative activities and removes NO under injury (Derakhshani, Safarpour et al. 2021, Manzoor, Kane et al. 2023) Correlates to TAA aorta diameter (Harrison, Cagampang et al. 2018)	Pro-oxidative stress Pro-inflammation
HBA1	Haemoglobin subunit alpha 1	P69905	2.19	0.003 (0.088)	Neutrophils	<10	Elevated haemoglobins associate with increased oxidative stress and inflammatory response, can be induced by mitochondrial stress. Haemoglobin activates pro-oxidative activities and removes NO under injury (Derakhshani, Safarpour et al. 2021, Manzoor, Kane et al. 2023) Correlates to TAA aorta diameter (Harrison, Cagampang et al. 2018)	Red blood cell related Pro-oxidative stress Pro-inflammation
HBA1.HBZ	Haemoglobin subunit alpha 1/ zeta	P69905 P02008	2.11	0.005 (0.11)	Neutrophils	<10/<10	Elevated haemoglobins associate with increased oxidative stress and inflammatory response, can be induced by mitochondrial stress. Haemoglobin activates pro-oxidative activities and removes NO under injury (Derakhshani, Safarpour et al. 2021, Manzoor, Kane et al. 2023) Correlates to TAA aorta diameter (Harrison, Cagampang et al. 2018)	Red blood cell related Pro-oxidative stress Pro-inflammation
HBB	Haemoglobin subunit beta	P68871	2.11	0.003 (0.091)	Neutrophils	252.4	Elevated haemoglobins associate with increased oxidative stress and inflammatory response, can be induced by	Red blood cell related

							mitochondrial stress. Haemoglobin activates pro-oxidative activities and removes NO under injury (Derakhshani, Safarpour et al. 2021, Manzoor, Kane et al. 2023) Correlates to TAA aorta diameter (Harrison, Cagampang et al. 2018)	Pro-oxidative stress Pro-inflammation
CA2	Carbonic anhydrase 2	P00918	2.10	0.008 (0.14)	Basophils	<10	Similar function to CA1, induces vascular calcification and associates with atherosclerosis (Argan, Çıkrıkçı et al. 2020)	Pro-inflammation
KRT7	Keratin 7	Q3KNV1	2.08	0.004 (0.11)	Other	16.04	Overexpression associates with proliferation, migration and regulates epithelial mesenchymal transition and cell-matrix adhesion though TGFβ pathway (Jones, Barbour et al. 2008, An, Liu et al. 2021)	ECM related
SPTA1	Spectrin alpha, erythrocytic 1	P02549	2.07	0.025 (0.23)	Neutrophils	<10	Mutation leads to red blood cell abnormalities (Chonat, Risinger et al. 2019)	Red blood cell related
KRT18	Keratin 18	P05783	1.96	0.002 (0.075)	Basophils	10.4	KRT8/18 protects EC from inflammatory induced apoptosis signal from TNFα, promotes cell survival (Jacob, Coulombe et al. 2018)	Anti-inflammation
HBG1.HBG2	Haemoglobin subunit gamma 1/ gamma 2	P69891 P69892	1.94	0.039 (0.28)	Other	<10/<10	Elevated haemoglobins associate with increased oxidative stress and inflammatory response, can be induced by mitochondrial stress. Haemoglobin activates pro-oxidative activities and	Red blood cell related Pro-oxidative

							removes NO under injury (Derakhshani, Safarpour et al. 2021, Manzoor, Kane et al. 2023) Correlates to TAA aorta diameter (Harrison, Cagampang et al. 2018)	stress Pro-inflammation
BPGM	Bisphosphoglycerate mutase	P07738	1.71	0.006 (0.12)	EC	19.07	Regulates haemoglobin oxygen affinity (Wei, Zhao et al. 2015)	Red blood cell related
SLC16A3	Solute carrier family 16 member 3	O15427	1.70	0.006 (0.12)	Neutrophils	24.6	Activate by HIF-1 pathway and increases expression under hypoxia condition (Choi, Kim et al. 2019)	Pro-oxidative stress
SUPT6H	SPT6 homolog, histone chaperone and transcription elongation factor	Q7KZ85	1.58	0.001 (0.048)	Other	64.05	Negatively regulating oxidative stress, deficiency led to cardiomyopathy and increased oxidative stress, interacts with miR-423-5p (Xu, Liu et al. 2023)	Anti-oxidative stress
HDAC7	Histone deacetylase 7	Q8WUI4	1.52	0.014 (0.18)	Neutrophils	100.2	Maintains ECM integrity, inhibits MMP10 (Chang, Young et al. 2006)	ECM related
PROC	Protein C, inactivator of coagulation factors Va	P04070	1.52	0.003 (0.087)	Other	<10	Demonstrates anti-inflammatory and cell protective roles, inhibits clot formation (Reiner, Carty et al. 2008)	Anti-inflammation Blood

	and VIIIa							coagulation
FHL5	Four and a half LIM domains 5	Q5TD97	1.51	0.008 (0.14)	EC	41.35	Overexpression promotes vascular calcification, abnormal calcium regulation and ECM dysregulation (Wong, Auguste et al. 2023)	ECM related
APOA2	Apolipoprotein A2	P02652	1.49	0.001 (0.043)	Other	<10	HDL metabolism, lipid transport, anti-inflammatory and anti-oxidative stress, demonstrates protective role in atherosclerosis (Kihara, Yamagishi et al. 2021)	Anti-inflammation Anti-oxidative stress

Note: Expressed cell type was obtained from Human Protein Atlas, only heart-related cell and immune cells were included, other indicates no documented heart or immune-related cell. Protein UniProt ID was obtained from UniProt, human species. Expression in aorta is obtained from GTex, expressions within healthy aorta less than 10 were presented as <10, otherwise with the actual expression. Red highlighted proteins are identified in both rankings (p-values and log-transformed FC). Adj. p refers to multiple testing correction adjusted FDR.

Table 5.5 Top 20 significant (ranked by p-value) downregulated DE proteins (2FC) from proteomics study and their function.

Protein	**Protein**	**Uniprot ID**	**Log_2FC**	**p-value (adj. p)**	**Cell type/ Tissue**	**Aorta expression (TPM)**	**Function**	**Category**
FBN1	Fibrillin 1	P35555	-1.16	1.2E-06 (0.006)	ECM SMC Fibroblasts	63.06	MFS mutation, regulates TGFβ pathway	ECM related
GPX3	Glutathione peroxidase 3	P22352	-1.06	2.6E-05 (0.019)	Neutrophils	803.8	Protect cells from oxidative damage and low expression associates with AAA with increased inflammatory response and pro-fibrosis response in ECM (Kaneko, Anzai et al. 2011, Lindquist Liljeqvist, Hultgren et al. 2020)	Anti-oxidative stress Anti-inflammation ECM related

MB	Myoglobin	P02144	-3.95	5.6E-05 (0.024)	Cardiomyocytes NK-cell	<10	Regulates NO homeostasis, removes ROS and promotes cell survival in hypoxia condition (Koch, Lüdemann et al. 2016)	Anti-oxidative stress
MFAP5	Microfibril associated protein 5	Q13361	-1.37	7.2E-05 (0.024)	ECM Fibroblasts Basophils Neutrophils	48.03	Deficiency in MFAP5 leads to familial TAA and dissections through TGFβ signalling, involved in ECM-FBN1 interaction (Barbier, Gross et al. 2014)	ECM related
FBN2	Fibrillin 2	P35556	-1.03	1.3E-04 (0.027)	ECM Monocytes	<10	Mutation causes aorta root dilation and regulates TGFβ pathway, also implicated in MFS (Gupta, Putnam et al. 2002, Gupta, Wallis et al. 2004)	ECM related
CHST6	Carbohydrate sulfotransferase 6	Q9GZX3	-2.02	1.5E-04 (0.027)	Other	<10	Mutation in CHST6 in corneal endothelium causes corneal stromal dystrophy (Bi Ning, Benxiang et al. 2021)	ECM related
FBN1.FBN2	Fibrillin 1/ 2	P35555 P35556	-1.08	1.6E-04 (0.027)	ECM SMC Fibroblasts Monocytes	63.03/<10	See details in FBN1 and FBN2	ECM related
LEFTY2	Left-right determination factor 2	O00292	-1.10	3.1E-04 (0.036)	Fibroblasts	23.77	Negatively regulates TGFβ pathway, inhibits proliferation and differentiation, implicated with congenital heart disease (Deng, Zhou et al. 2014)	Anti-proliferation and differentiation
CKM	Creatine kinase, M-type	P06732	-1.98	3.8E-04 (0.040)	Cardiomyocytes	<10	(MCK) Regulates ATP-ADP conversion, implicated in myocardial infraction (Aydin, Ugur et al. 2019). Downregulation implicates mitochondrial dysfunction, deficient mitochondria leading to excessive ROS and calcium	Mitochondrial function

							production, which in turn leads to cell death (Carreira, Lee et al. 2011)	
MMP23B	Matrix metallopeptidase 23B	O75900	-1.09	6.0E-04 (0.045)	ECM SMC Fibroblasts	<10	ECM degradation, downregulation may stabilize ECM and attenuates ECM dysregulation (Löffek, Schilling et al. 2011)	ECM related
MYH6.MYH7	Myosin heavy chain 6/ 7	P13533 P12883	-1.74	7.1E-04 (0.048)	Cardiomyocytes	<10/<10	Regulates cardiac muscle contractility and implicated in cardiomyopathy (Alpendurada, Wong et al. 2010, Holm, Gudbjartsson et al. 2011, Ferradini, Parca et al. 2021)	Contractility
MYL2	Myosin light chain 2	P10916	-1.82	1.0E-03 (0.055)	Cardiomyocytes Native B-cell Memory B-cell	<10	Involved in cardiac muscle contraction, driven by ATP conversion to mechanical movement. Associated with cardiomyopathy, decreased cardiac function, hypertrophy, cardiac torsion and fibrosis (Sheikh, Lyon et al. 2015)	Contractility
GLUL	Glutamate-ammonia ligase	P15104	-1.53	1.1E-03 (0.057)	Neutrophils	162.6	ATP-binding, angiogenesis Regulates mitochondrial and oxidative stress, catalyses ATP-dependent conversion (Wang, Kudoh et al. 1996, Wang, Qian et al. 2020)	Mitochondrial function
MYH7	Myosin heavy chain 7	P12883	-1.64	1.2E-03 (0.057)	Cardiomyocytes	<10	Associates with ATP-binding domain of the myosin protein, alternation in MYH7 modulates ATP-binding rates, ATP hydrolysis, ADP release and abnormal ATP, ADP within the cell (Yousaf, Khan et al. 2022)	Contractility
CKB.CKM	Creatine kinase B/	G3V461 P06732	-1.34	1.2E-03 (0.057)	Cardiomyocytes Monocytes	100.2/<10	CKB regulates mitochondrial ATP turnover, regulated by cAMP signalling,	Mitochondrial function

	Creatine kinase, M-type				Neutrophils		modulates inflammatory response and maintains ECM function, decreased CKB may implicate decreased mitochondrial abundance and basal oxygen consumption (Rahbani, Roesler et al. 2021) CKM – see CKM function above	ECM related
GXYLT2	Glucoside xylosyltransferase 2	A0PJZ3	-1.31	2.2E-03 (0.075)	ECM Fibroblasts Dendritic cells	14.52	Regulates EC function and cell proliferation and migration through Notch and MAPK signalling, inhibition of GXYLT2 shows inhibition of cell proliferation and migration (Bhushan, Altinbas et al. 2019, Cui, Xing et al. 2019, Kan, Zhang et al. 2021, Wu and Liao 2022).	Proliferation and migration
ALDH1L1	Aldehyde dehydrogenase 1 family member L1	O75891	-1.44	3.0E-03 (0.088)	Other	31.83	Regulates mitochondrial metabolism that converts NADP+ to NADOH and CO2, loss of function leads to decreased apoptosis and increased cell migration (Sasaki, Yamamoto et al. 2023)	Mitochondrial function
PLA2G2A	Phospholipase A2 group IIA	P14555	-1.61	3.5E-03 (0.097)	Fibroblasts	124.8	Induces inflammatory response, regulates phospholipase and Ras signal activities, induce cell proliferation via integrin binding (Hui 2012)	Pro-inflammation
TNFRSF11B	TNF receptor superfamily member 11b	O00300	-1.63	4.0E-03 (0.10)	VSMC EC	289.1	Promotes vascular calcification and atherogenesis, regulated by inflammatory response through NF-κN and TNF pathways, promotes VSMC accumulation and collagen production to	ECM related Anti-inflammation

							increase the vessel wall from rupture (Vorkapic, Kunath et al. 2018)	
CYTL1	Cytokine like 1	Q9NRR1	-1.70	4.1E-03 (0.11)	EC NK-cells	198.3	Signalling through TGFβ and MAPK/ERK signalling, demonstrates pro-fibrotic characteristics and pro-angiogenesis via VEGF signalling, also promotes inflammatory response (Zhu, Kuek et al. 2019)	Pro-inflammation

Note: Expressed cell type was obtained from Human Protein Atlas, only heart-related cell and immune cells were included, other indicates no documented heart or immune-related cell. Protein UniProt ID was obtained from UniProt, human species. Expression in aorta is obtained from GTex, expressions within healthy aorta less than 10 were presented as <10, otherwise with the actual expression. Red highlighted proteins are identified in both rankings (p-values and log-transformed FC). Orange highlighted proteins are identified in both rankings and also in RNA top 20 tables. Adj. p refers to multiple testing correction adjusted FDR.

Table 5.6 Top 20 significant (ranked by logFC) downregulated DE proteins (2FC) from proteomics study and their function.

Gene	**Protein**	**Uniprot ID**	**Log_2FC**	**P-value (adj. p)**	**Expressed cell type**	**Aorta Expression (TPM)**	**Function and Pathway**	**Category**
MB	Myoglobin	P02144	-3.95	0.0001 (0.024)	Cardiomyocytes NK-cell	<10	Regulates NO homeostasis, removes ROS and promotes cell survival in hypoxia condition (Koch, Lüdemann et al. 2016)	Anti-oxidative stress
CHST6	Carbohydrate sulfotransferase 6	Q9GZX3	-2.02	1.5E-04 (0.027)	Other	<10	Mutation in CHST6 in corneal endothelium causes corneal stromal dystrophy (Bi Ning, Benxiang et al. 2021)	ECM related
CKM	Creatine kinase, M-type	P06732	-1.98	3.8E-04 (0.040)	Cardiomyocytes	<10	(MCK) Regulates ATP-ADP conversion, implicated in myocardial infraction (Aydin, Ugur et al. 2019).	Mitochondrial function

							Downregulation implicates mitochondrial dysfunction, deficient mitochondria leading to excessive ROS and calcium production, which in turn leads to cell death (Carreira, Lee et al. 2011)	
MYL2	Myosin light chain 2	P10916	-1.82	1.0E-03 (0.055)	Cardiomyocytes Native B-cell Memory B-cell	<10	Involved in cardiac muscle contraction, driven by ATP conversion to mechanical movement. Associated with cardiomyopathy, decreased cardiac function, hypertrophy, cardiac torsion and fibrosis (Sheikh, Lyon et al. 2015)	Contractility
MYH6.MYH7	Myosin heavy chain 6/ 7	P13533 P12883	-1.74	7.1E-04 (0.048)	Cardiomyocytes	<10/<10	Regulates cardiac muscle contractility and implicated in cardiomyopathy (Alpendurada, Wong et al. 2010, Holm, Gudbjartsson et al. 2011, Ferradini, Parca et al. 2021)	Contractility
CYTL1	Cytokine like 1	Q9NRR 1	-1.70	4.1E-03 (0.105)	EC NK-cells	198.3	Signalling through TGFβ and MAPK/ERK signalling, demonstrates pro-fibrotic characteristics and pro-angiogenesis via VEGF signalling, also promotes inflammatory response (Zhu, Kuek et al. 2019)	Pro-inflammation
CCL18	C-C motif chemokine ligand 18	P55774	-1.68	0.0052 (0.12)	Macrophages	<10	Increase inflammatory response, decrease cardiac function, exercise capability and associates with coronary heart disease (De Sutter, Struyf et al. 2010)	Pro-inflammation
SFRP1	Secreted frizzled related	Q8N474	-1.65	0.0066 (0.13)	Cardiomyocytes Fibroblasts	113.8	Signal through Wnt, NF-κB and TNF signalling, inhibits inflammation, promotes cell survival and repair, also	Anti-inflammation

	protein 1						angiogenesis (Huang and Huang 2020)	
MYH7	Myosin heavy chain 7	P12883	-1.64	1.2E-03 (0.057)	Cardiomyocytes	<10	Associates with ATP-binding domain of the myosin protein, alternation in MYH7 modulates ATP-binding rates, ATP hydrolysis, ADP release and abnormal ATP, ADP within the cell (Yousaf, Khan et al. 2022)	Contractility
TNFRSF11B	TNF receptor superfamily member 11b	O00300	-1.63	4.0E-03 (0.103)	Other	289.1	Promotes vascular calcification and atherogenesis, regulated by inflammatory response through NF-κN and TNF pathways, promotes VSMC accumulation and collagen production to increase the vessel wall from rupture (Vorkapic, Kunath et al. 2018)	ECM related Anti-inflammation
PLA2G2A	Phospholipase A2 group IIA	P14555	-1.61	3.5E-03 (0.097)	Fibroblasts	124.8	Induces inflammatory response, regulates phospholipase and Ras signal activities, induce cell proliferation via integrin binding (Hui 2012)	Pro-inflammation
GLUL	Glutamate-ammonia ligase	P15104	-1.53	1.1E-03 (0.057)	Neutrophils	162.6	ATP-binding, angiogenesis Regulates mitochondrial and oxidative stress, catalyses ATP-dependent conversion (Wang, Kudoh et al. 1996, Wang, Qian et al. 2020)	Mitochondrial function
MYOZ2	Myozenin 2	Q9NPC6	-1.46	0.0062 (0.125)	Cardiomyocytes	44.6	Deficiency associates with hypertrophic cardiomyopathy, possibly affects mechanosensing in cardiac muscle (Osio, Tan et al. 2007)	Proliferation
ALDH1L1	Aldehyde dehydrogenase 1 family	O75891	-1.44	3.0E-03 (0.088)	Other	31.83	Regulates mitochondrial metabolism that converts NADP+ to NADOH and CO2, loss of function leads to	Mitochondrial function

	member L1						decreased apoptosis and increased cell migration (Sasaki, Yamamoto et al. 2023)	
SSC5D	Scavenger receptor cysteine rich family member with 5 domains	A1L4H1	-1.40	0.0159 (0.19)	Fibroblasts Monocytes Macrophages T-lymphocytes	15.04	Promotes inflammation and overexpression is implicated in heart failure, may signal through NF-κB, PI3K/AKT/mTOR signalling (Ge, Liu et al. 2023)	Pro-inflammation
GFAP	Glial fibrillary acidic protein	P14136	-1.39	0.0096 (0.15)	Neutrophil	<10	Type III intermediate filament Affects VSMC and EC development, may relate to hypothermia under cardiopulmonary bypass surgical setting (Vedovelli, Padalino et al. 2017, Osman, Wang et al. 2020) Maintain cell integrity, cell shape and mechanical stretch (Middeldorp and Hol 2011)	Mechanical stress
MFAP5	Microfibril associated protein 5	Q13361	-1.37	7.2E-05 (0.024)	ECM Fibroblasts Basophils Neutrophils	48.03	Deficiency in MFAP5 leads to familial TAA and dissections through TGFβ signalling, involved in ECM-FBN1 interaction (Barbier, Gross et al. 2014)	ECM related
CD55	CD55 molecule (Cromer blood group)	P08174	-1.36	0.0409 (0.28)	Monocytes Neutrophils EC	67.39	Regulated by VEGF signalling and inflammatory genes like TNFα, interleukins, protects EC from inflammation and coagulation event, promotes cell survival and inhibits apoptosis; signal through JAK-STAT, MAPK, NF-κB pathways (Morgan,	Anti-inflammatory

							Spendlove et al. 2002, Bharti, Dey et al. 2022)	
HAPLN1	Hyaluronan and proteoglycan link protein 1	P10915	-1.35	0.0101 (0.156)	SMC Fibroblasts	<10	Deficiency leads to ECM component hyaluronic acid and proteoglycans deposition, abnormal cardiomyopathy proliferation and regeneration, decreased expression associates with EC senescence via interleukin and CCL genes, protect cells from oxidative stress by reducing ROS and promote cell survival (Sun, Peterson et al. 2022, Zhou, Jang et al. 2023)	ECM-related Anti-oxidative stress
CKB.CKM	Creatine kinase B/ Creatine kinase, M-type	G3V461 P06732	-1.34	1.2E-03 (0.057)	Cardiomyocytes Monocytes Neutrophils	100.2/<10	CKB regulates mitochondrial ATP turnover, regulated by cAMP signalling, modulates inflammatory response and maintains ECM function, decreased CKB may implicate decreased mitochondrial abundance and basal oxygen consumption (Rahbani, Roesler et al. 2021) CKM – see CKM function above	Mitochondrial function ECM related

Note: Expressed cell type was obtained from Human Protein Atlas, only heart-related cell and immune cells were included, other indicates no documented heart or immune-related cell. Protein UniProt ID was obtained from UniProt, human species. Expression in aorta is obtained from GTex, expressions within healthy aorta less than 10 were presented as <10, otherwise with the actual expression. Red highlighted proteins are identified in both rankings (p-values and log-transformed FC). Orange highlighted proteins are identified in both rankings and also in RNA top 20 tables. Adj. p refers to multiple testing correction adjusted FDR.

5.3.3 Signalling and biological function analysis

Proteins over the 1.5FC threshold were used in KEGG and GO analyses since it gives slightly larger protein input (n = 291). Given the small input protein density from mass spectrometry output and considering some integrated proteins could not feed into KEGG analysis, adjusted KEGG enrichment p-values <0.1 were considered significant. Initial examination of the overall KEGG enrichment pathways related to significant DE proteins was conducted to gain a general understanding of the involved pathways followed by investigation of the involvement of separate up- and downregulated proteins between MFS patients and controls.

Firstly, using all significant DE proteins, two signal transduction pathways were identified under $p < 0.1$: the TGFβ and HIF-1 pathways ($p = 0.06$) (Figure 5.2). Interestingly, the complement and coagulation cascade pathways were also significantly identified in the KEGG analysis, which complements the top 20 significant DE protein analysis. Additionally, pathways related to cardiomyopathy, lipid metabolism, and cellular processes such as proliferation and cell death were also identified, aligning with the top 20 significant DE protein table results (Figure 5. 2). Notably, complement and coagulation cascade was identified with high significance and with the most DE proteins involved, highlighting its likely role in MFS pathogenesis.

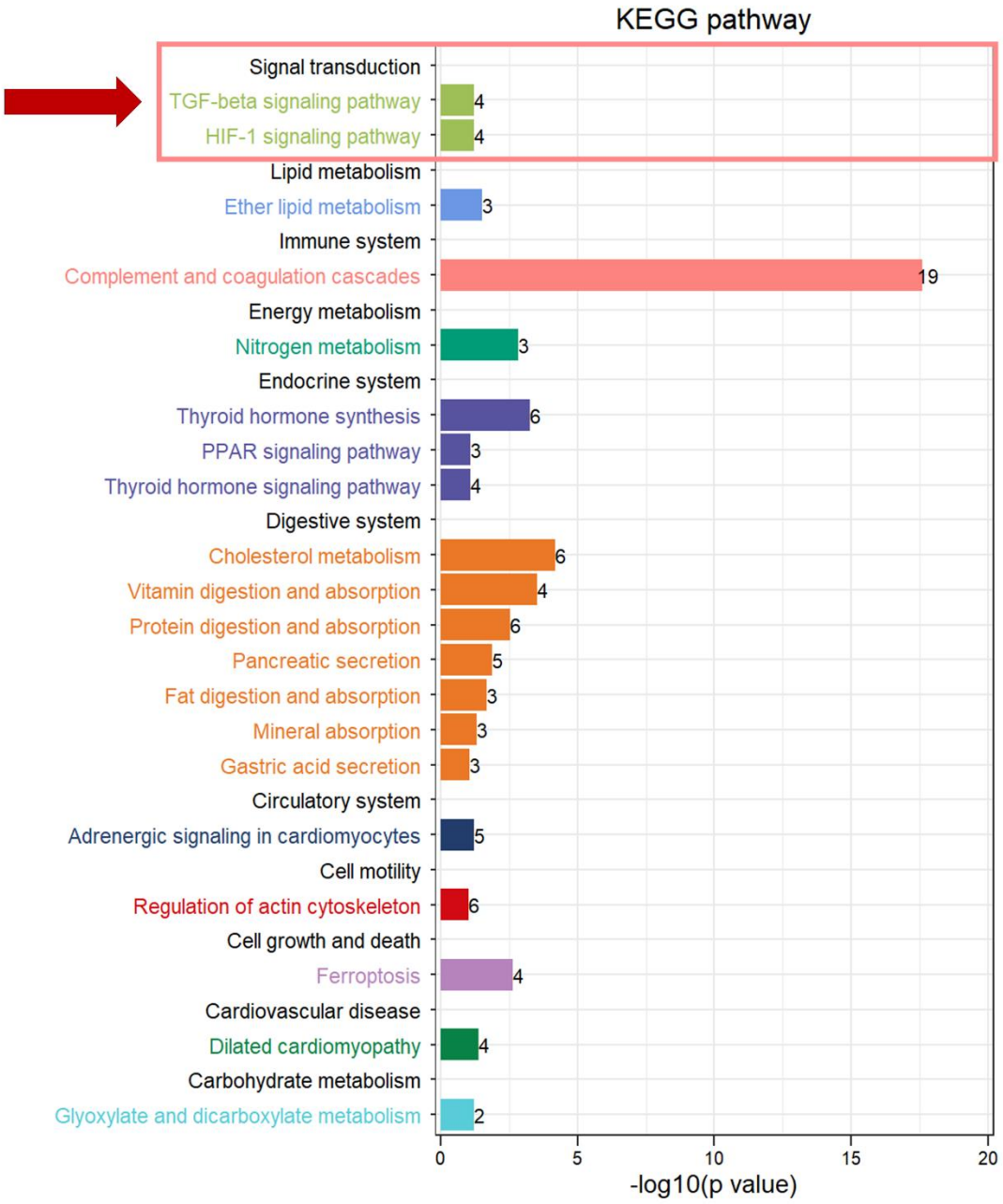
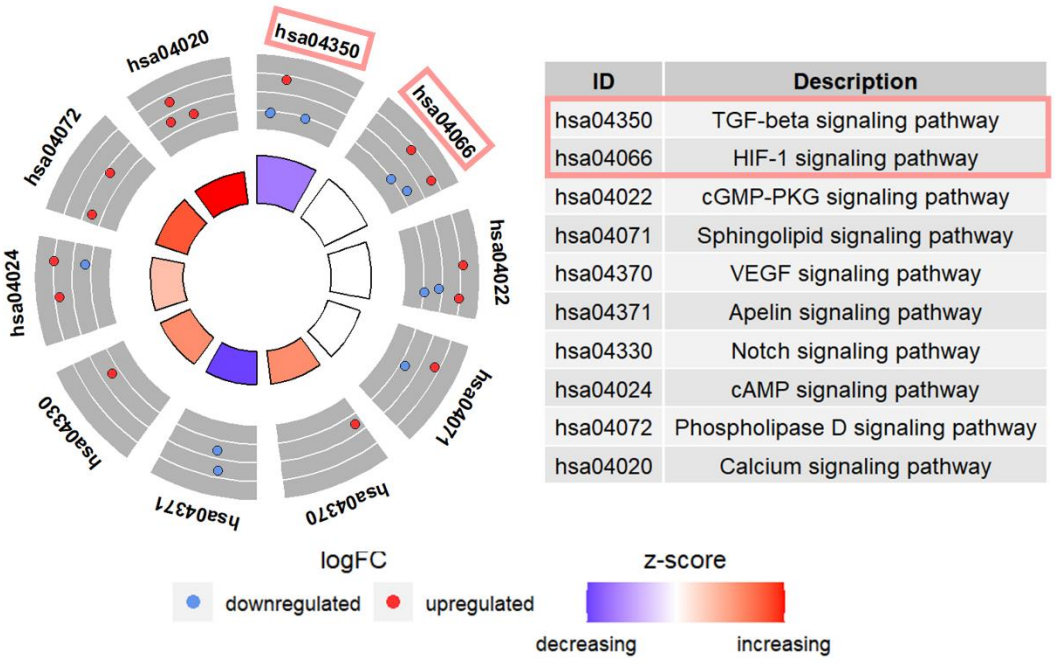


Figure 5.2 KEGG enrichment barplot for significant DE proteins (1.5FC). Significant pathways were illustrated under each KEGG category, enrichment analysis with p<0.1 were included. Y-axis title indicates each pathway identified and the category (coloured in black) it belongs to, and each colour represent one KEGG category. X-axis indicates the -log10(p-value) of each pathway, and the number after the bar represent the number of genes that detected in each pathway. The red box and arrow indicate that these pathways belong to subcategory of signal transduction pathway.

The signal transduction pathways identified by significant DE proteins were then examined. Out of the 22 signal transduction pathways identified, only HIF-1 and TGFβ pathways remained significant with a p-value <0.1 (Figure 5.3). This finding

emphasizes the likely involvement of oxidative stress regulation in MFS pathology at the translation level. The remaining pathways were targeted by very few genes, making it challenging to assess their significance in MFS pathology. Notably, cGMP-PKG, cAMP, and calcium signalling pathways each involved three proteins, suggesting a potential role in the inflammatory response, mitochondrial function, and calcium-modulated VSMC phenotypic switching.



ID	Description
hsa04350	TGF-beta signaling pathway
hsa04066	HIF-1 signaling pathway
hsa04022	cGMP-PKG signaling pathway
hsa04071	Sphingolipid signaling pathway
hsa04370	VEGF signaling pathway
hsa04371	Apelin signaling pathway
hsa04330	Notch signaling pathway
hsa04024	cAMP signaling pathway
hsa04072	Phospholipase D signaling pathway
hsa04020	Calcium signaling pathway

Figure 5.3 KEGG analysis for significant DE proteins from proteomics study (1.5FC). Pathways in pink frame indicate the p<0.1.

Among the upregulated proteins, 18 signal transduction pathways were identified, although none of them reached significance due to the limited protein input (data not shown). Notably, the HIF-1 signalling pathway showed the most promising trend with a p-value of 0.2 and two targeted proteins (PRKCA and TF). PRKCA, a key regulator within the HIF-1 pathway, may suggest increased ROS levels in MFS pathology, subsequently activating the HIF-1 signalling pathway.

In contrast, the downregulated proteins identified 12 signal transduction pathways, with TGFβ being the sole significant pathway (p = 0.03) (data not shown). This pathway was identified by downregulated FBN1, LEFTY2, and HAMP proteins. These findings align with the overall protein KEGG analysis, emphasizing the involvement of the TGFβ and HIF-1 pathways, thereby helping to validate the role of oxidative stress and TGFβ regulation at the translational level.

As mentioned earlier, the proteomics data yielded a very small number of significant DE proteins after Benjamini-Hochberg correction. To provide an overview of the pathway results, a KEGG enrichment plot was created. A KEGG enrichment p-value <0.1 was considered significant, and only seven pathways met this criterion (Figure 5.4). Interestingly, the only signal transduction pathway identified from the corrected significant DE proteins was the TGFβ signalling pathway, with a p-value of 0.017. This pathway was identified by only two proteins, FBN1 and LEFTY2, consistent with the KEGG results obtained from significant DE proteins using raw p-values. However, due to stringent and conservative correction from the FDR procedure, the analysis is significantly underpowered.

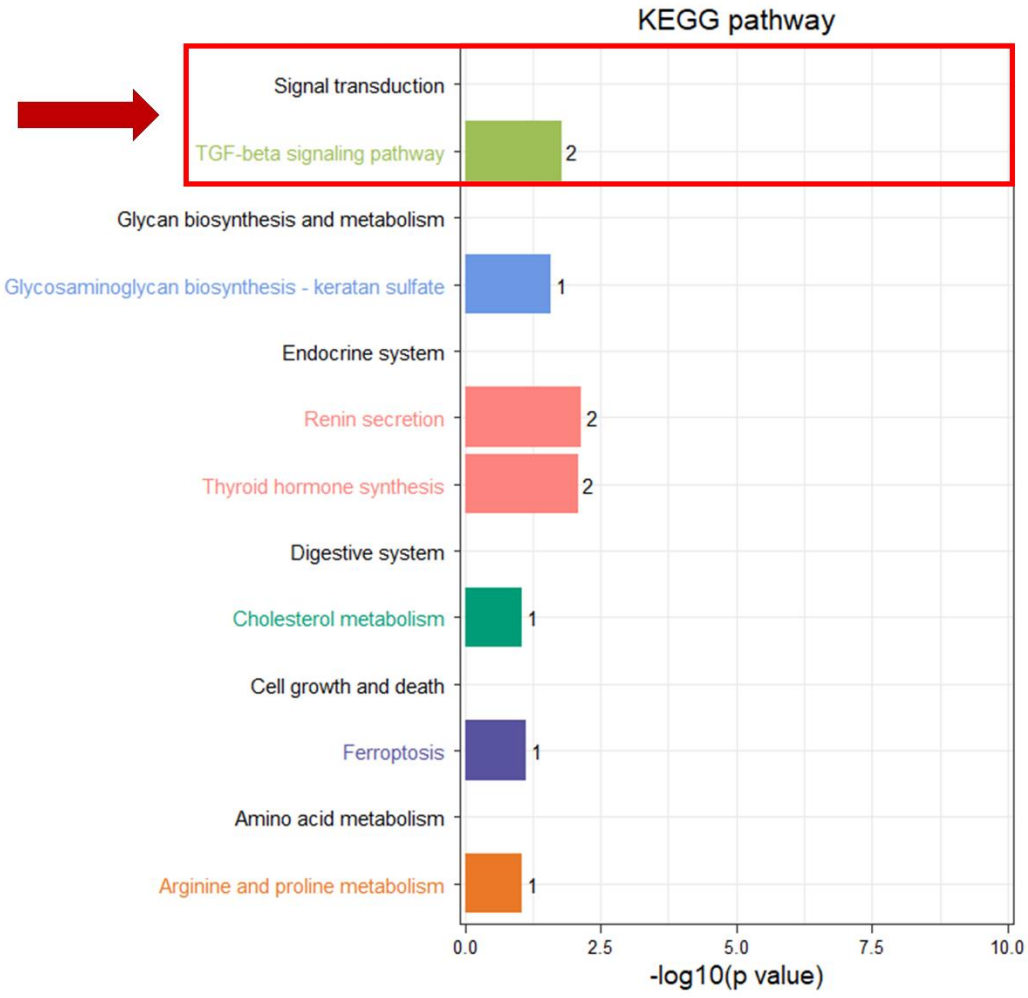
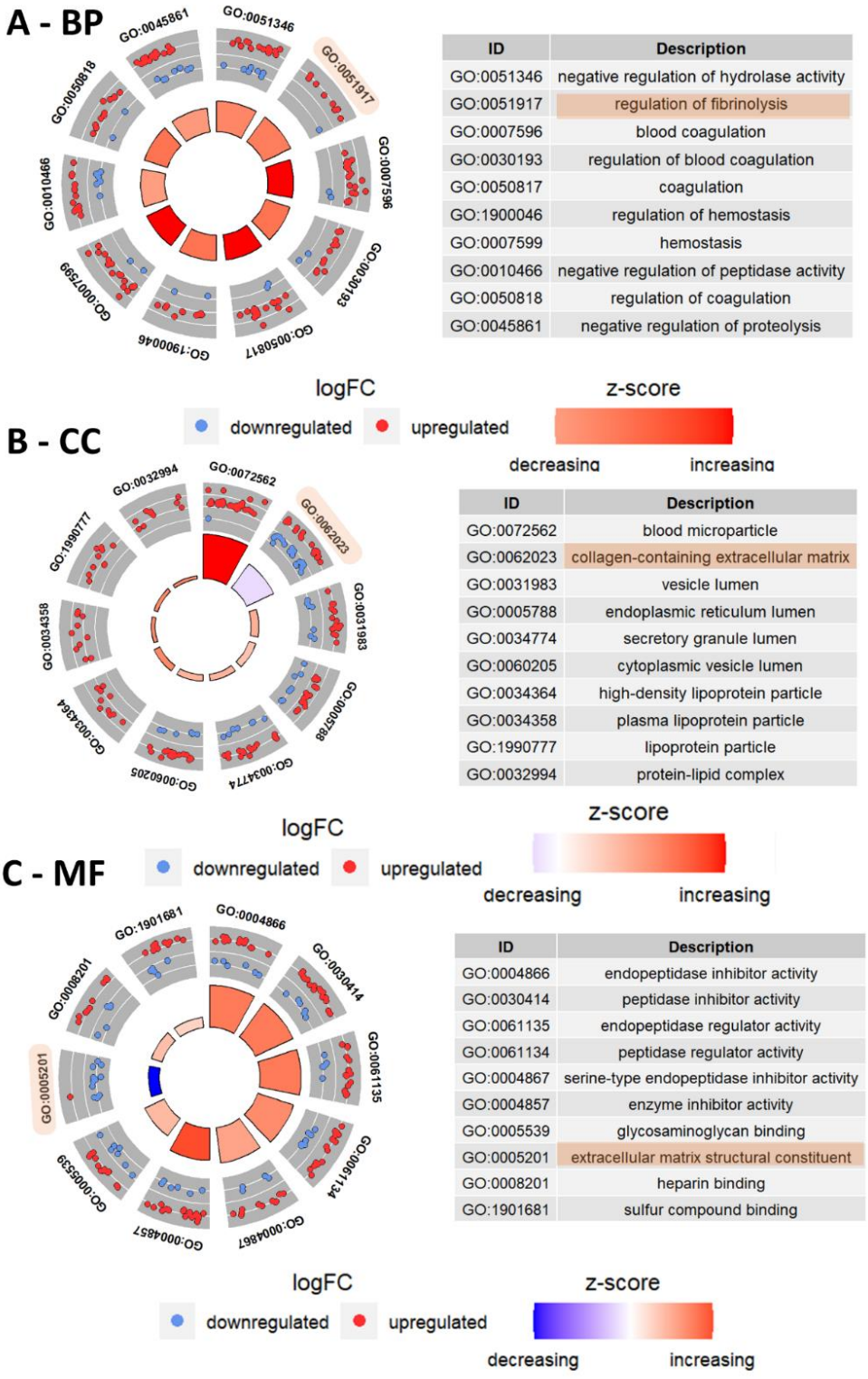


Figure 5.4 KEGG enrichment barplot for significant DE proteins (1.5FC) after Benjamini-Hochberg correction.

A GO analysis was conducted to investigate the biological processes affected by altered proteins in MFS. The circle plots generated from significant DE proteins revealed that coagulation and haemostasis were prominently involved in MFS protein regulation (Figure 5.5). Moreover, cellular component and molecular function analyses indicated a larger number of downregulated proteins associated with ECM-related terms, highlighting the impact on ECM components and functions in MFS. These findings confirmed that ECM dysregulation contributes to MFS pathology at both transcription and translation levels. Additionally, molecular function analysis identified various enzyme activities, with heparin binding being related to blood coagulation as an anti-coagulant. This analysis suggested a slight predominance of upregulated proteins in heparin binding, possibly indicative of an anti-coagulation process at the translational level.

To further illustrate the proteins involved in each term, GO cnetplots were generated for biological process, cellular component, and molecular function (Figure 5.6). These plots revealed that coagulation and haemostasis-involved proteins were mainly upregulated including several coagulation factors (coloured in red), which could indicate potential EC dysregulation while ECM-related proteins were predominantly downregulated, including FBN1, ELN, and MFAP proteins (coloured in purple). The upregulation of coagulation and haemostasis processes in MFS is consistent with our KEGG analyses, suggesting their potential significance. However, further investigation is needed to rule out potential surgical artifacts related to coagulation findings.



ID	Description
GO:0051346	negative regulation of hydrolase activity
GO:0051917	regulation of fibrinolysis
GO:0007596	blood coagulation
GO:0030193	regulation of blood coagulation
GO:0050817	coagulation
GO:1900046	regulation of hemostasis
GO:0007599	hemostasis
GO:0010466	negative regulation of peptidase activity
GO:0050818	regulation of coagulation
GO:0045861	negative regulation of proteolysis

ID	Description
GO:0072562	blood microparticle
GO:0062023	collagen-containing extracellular matrix
GO:0031983	vesicle lumen
GO:0005788	endoplasmic reticulum lumen
GO:0034774	secretory granule lumen
GO:0060205	cytoplasmic vesicle lumen
GO:0034364	high-density lipoprotein particle
GO:0034358	plasma lipoprotein particle
GO:1990777	lipoprotein particle
GO:0032994	protein-lipid complex

ID	Description
GO:0004866	endopeptidase inhibitor activity
GO:0030414	peptidase inhibitor activity
GO:0061135	endopeptidase regulator activity
GO:0061134	peptidase regulator activity
GO:0004867	serine-type endopeptidase inhibitor activity
GO:0004857	enzyme inhibitor activity
GO:0005539	glycosaminoglycan binding
GO:0005201	extracellular matrix structural constituent
GO:0008201	heparin binding
GO:1901681	sulfur compound binding

Figure 5.5 GO analysis for significant DE protein (1.5FC). A) Biological process. B) Cellular component. C) Molecular function. Orange highlight indicates the ontology term is ECM related.

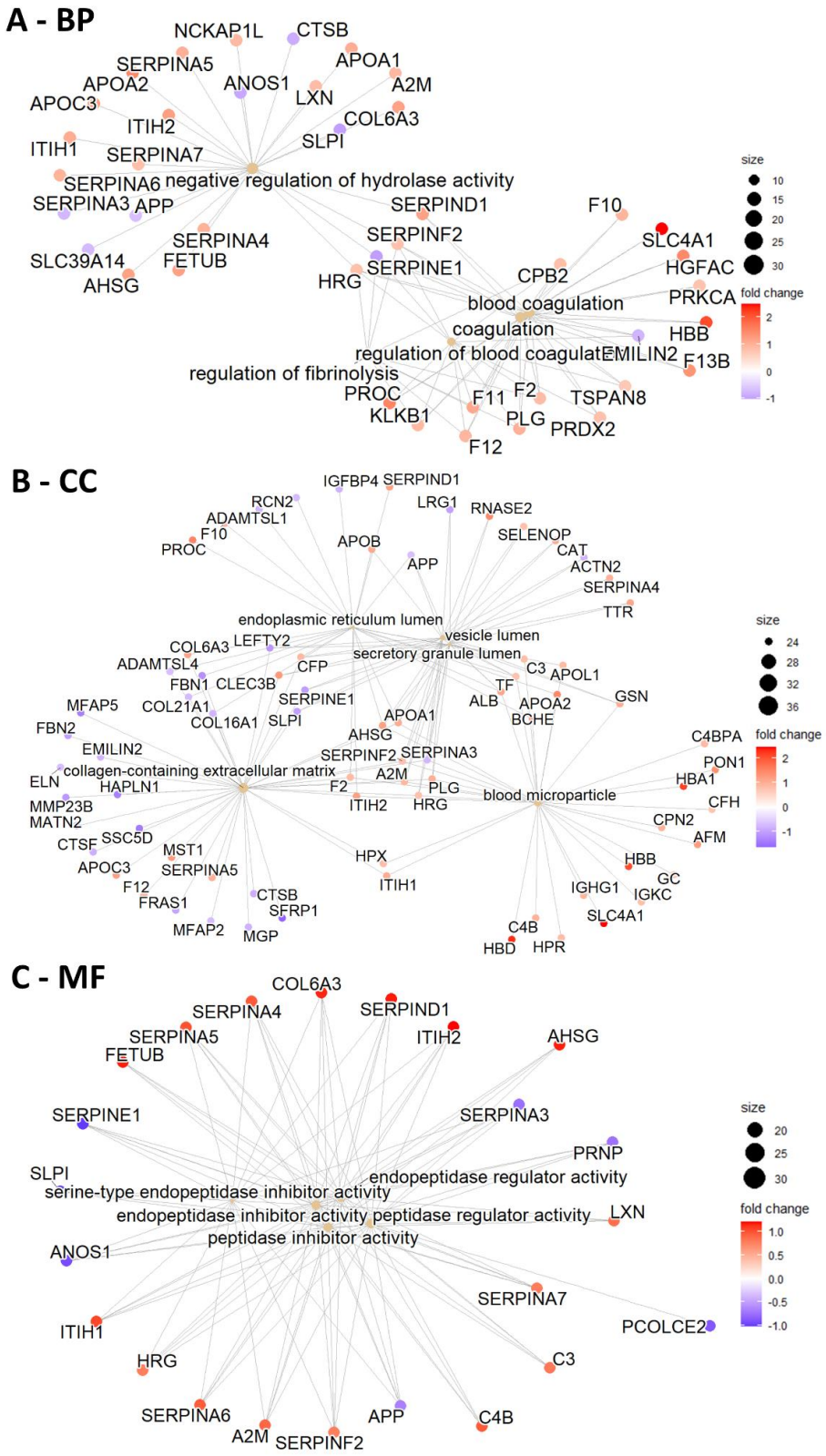


Figure 5.6 GO analysis for significant DE protein (1.5FC). A) Biological process. B) Cellular component. C) Molecular function. Fold change of the genes were displayed from red (upregulated) to purple (downregulated).

When the Benjamini-Hochberg correction was applied to the significant DE proteins for GO analysis, the results remained consistent with the raw p-value analysis, particularly regarding ECM regulation (Figure 5.7 and 5.8). These data, despite their limited input, provide a clear indication that ECM involvement is primarily modulated by downregulated fibrillins (FBN1/2) and microfibril- associated proteins (MFAPs), which were previously identified in other studies, regulating microfibril and elastin in the ECM, and being associated with aortic dilation (Figure 5.8). Notably, blood coagulation and haemostasis processes were not identified after the FDR procedure, suggesting the need for additional validation of the regulation of coagulation factors and haemoglobin proteins in MFS.

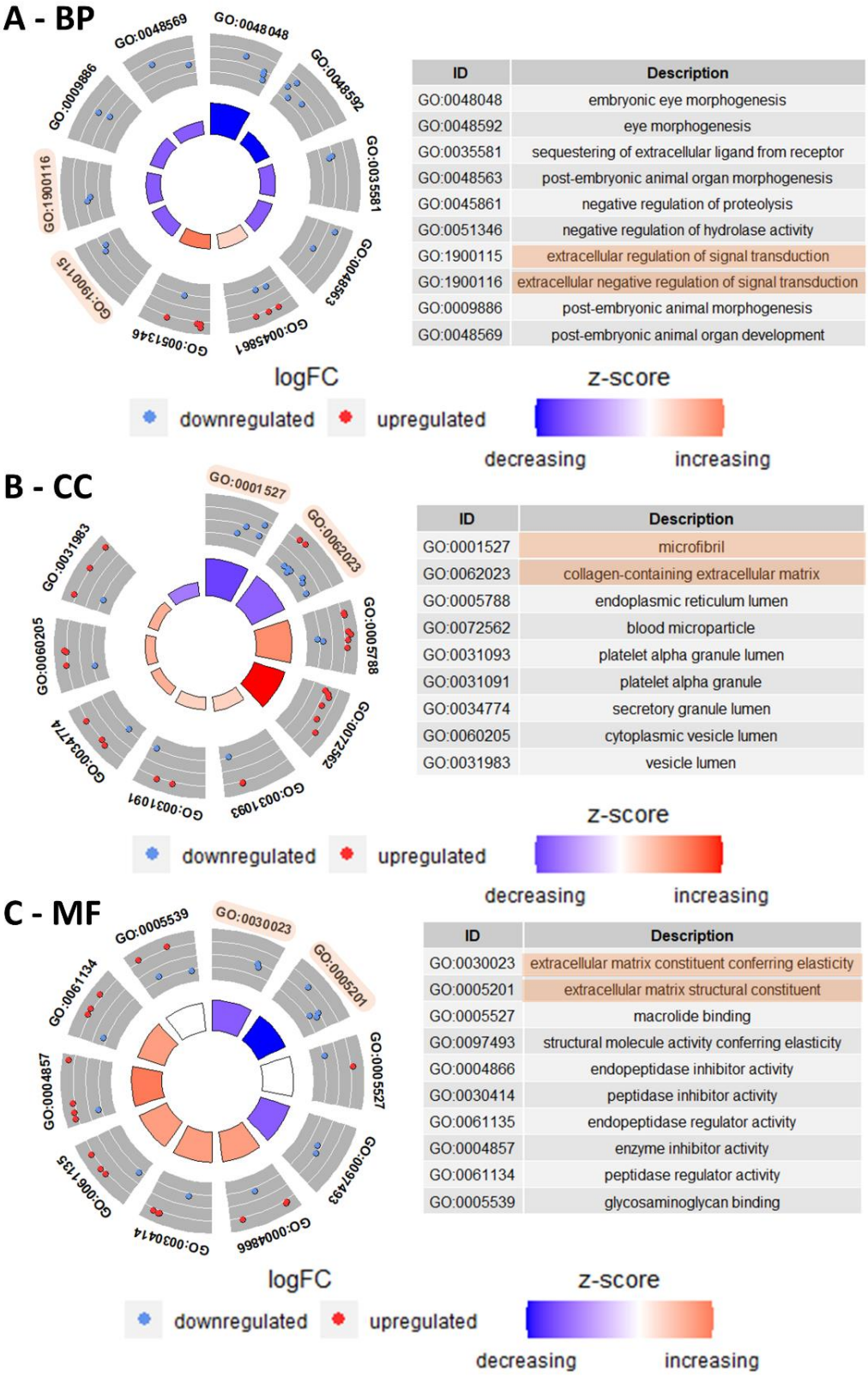


Figure 5.7 GO analysis for significant DE protein (1.5FC) after Benjamini-Hochberg correction.

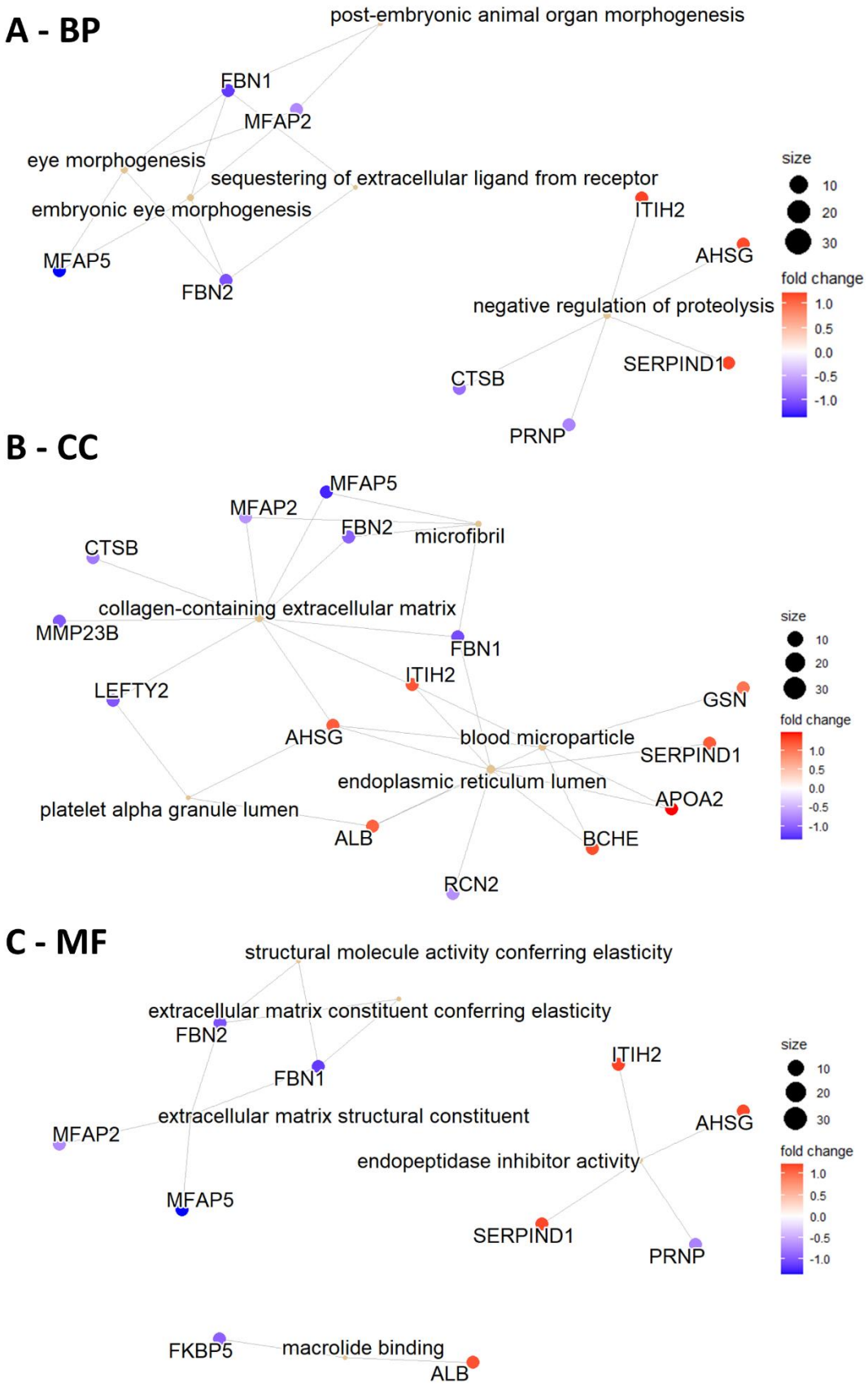


Figure 5.8 GO analysis for significant DE protein (1.5FC) after Benjamini-Hochberg correction.

5.4 DISCUSSION

The proteomics study identified 73 upregulated proteins and 36 downregulated significant proteins with more than a 2FC, and 182 upregulated and 109 downregulated significant proteins with more than a 1.5FC. The proteomics analysis confirmed observations from the RNA analysis, emphasizing the involvement of inflammation, oxidative stress, and mitochondrial dysfunction as key drivers in MFS pathogenesis. However, the proteomics analysis highlighted the involvement of mechanical stress, haemoglobin regulation, the coagulation cascade and also emphasised heart muscle contractility proteins as potentially contributing factors to MFS pathogenesis. Overall, the study aligns with the RNA findings, while also introducing new insights into mechanical stress, haemoglobin activities and abnormal coagulation as potential regulatory mechanisms in MFS TAAD.

5.4.1 Differential expression analysis of proteomics in MFS

Initially, highly expressed proteins were examined in the aortic wall, and fibrillin-1 stood out as downregulated by 2FC in MFS patients. Interestingly, as observed in the RNA analysis, FBN1 transcripts were not significantly different between the two groups, which has been reported previously, suggest a relatively normal transcription of FBN1 regardless of the mutation (Tjeldhorn, Amundsen et al. 2015). The halved fibrillin-1 protein expression could be attributed to protein misfolding and degradation resulting from mutations in the FBN1 gene. A past study has suggested if it is a nonsense variant FBN1 mutation, the misfolding in fibrillin-1 protein could be due to the premature translational termination codon located within the mRNA, resulting in nonsense-mediated mRNA decay, which subsequently decreases protein synthesis (Niu, Huang et al. 2020). In addition, another study suggested that missense FBN1 mutations could lead to fibrillin-1 protein misfolding by removed disulphide bonds that stabilize the folding of the protein (Whiteman and Handford 2003). This observation indicates that MFS patients with FBN1 mutations exhibit lower fibrillin-1 abundance in the aortic wall, potentially destabilising microfilament formation and contributing to aneurysm formation (Whiteman and Handford 2003, Whiteman, Hutchinson et al. 2006).

Furthermore, gelsolin (GSN) demonstrated elevated expression exceeding 2FC when compared to controls. Gelsolin is ubiquitously expressed and has been shown to play a role in actin-filament regulation, the mechanical stress response, cardiac remodelling, and fibrosis, and is also implicated in inhibiting apoptosis

(Koya, Fujita et al. 2000). Increased gelsolin levels may indicate actin cytoskeleton disruption and initiation of tissue remodelling in response to mechanical stress, possibly through the phosphatidylinositol and PI3K pathways by inhibiting the actin-serving and capping activities of gelsolins in the actin cytoskeleton (Patel, Zhabyeyev et al. 2018, Jana, Aujla et al. 2021). The elevation of gelsolin levels may suggest the presence of elevated mechanical stress in the aortic wall.

Three proteins: elastin (ELN), matrix Gla protein (MGP), and IFITM3 (Interferon-Induced Transmembrane Protein 3)—were significantly DE by over 1.5FC in MFS. ELN protein expression was downregulated in this study. Previous studies have shown that ELN expression is regulated at the transcriptional and post-transcriptional levels, with several regulatory mechanisms identified that can lead to ELN mRNA degradation (Morimoto, Wang et al. 2015). Additionally, ELN protein may be reduced by ECM degradation due to increased MMP production due to inflammation, and miRNA regulation, specifically involving miR-29a, miR-29b, and miR-195 (Morimoto, Wang et al. 2015, Wang, Meng et al. 2021). The low elastin level in the MFS patients potentially indicating decreased elastin composition and possible elastin degradation in the MFS aortic wall. This could result in the loss of ECM integrity and contribute to TAAD formation (Karimi and Milewicz 2016).

Matrix Gla protein (MGP) is produced by VSMC and play an important role in vitamin K regulation and associated arterial stiffness, which is specifically regulated by post-translational modifications (PTMs) and is implicated in AAA and other CVDs (Dalmeijer, van der Schouw et al. 2013, Kristensen, Melholt et al. 2021). The low MGP protein level observed in MFS patients with no significant RNA alteration may indicate PTM modulation, specifically associated with vitamin K bioavailability related carboxylation, and increased aortic stiffness (Dalmeijer, van der Schouw et al. 2013, Kristensen, Melholt et al. 2021). The last protein IFITM3 is associated with the inflammatory response induced by interferon and is expressed in macrophages and aneurysmal VSMC (Li, Ren et al. 2020). In summary, these findings suggest that ECM dysregulation and inflammatory responses play a significant role in MFS, as indicated by the altered expression of highly expressed proteins in the aorta. These protein expression changes without corresponding RNA expression alteration, may be attributed to factors such as mutation-induced misfolding, unsuccessful translation, post-transcriptional regulation, PTMs, and other epigenetic regulatory mechanisms (Karve and Cheema 2011).

By comparing the protein data from MFS tissue samples to previous proteomics studies on MFS, significant differences were identified, particularly in APOA1 and

haemoglobin levels, in contrast to previous research (Pilop, Aregger et al. 2009). APOA1, known for its role in lipid metabolism and high-density lipoprotein (HDL) regulation in plasma, has been extensively studied in AAA and atherosclerosis. Elevated APOA1 levels are observed in AAA and atherosclerosis and are associated with oxidative stress, inflammation, and HDL dysfunction (DiDonato, Huang et al. 2013, Martínez lópez, Cedo et al. 2018). Moreover, in cancer study, elevated APOA1 were identified and correlated with the inflammatory response, which suggests that APOA1 upregulated expression observed in this study may be involved in oxidative stress and inflammation in MFS pathogenesis (Wei, Gao et al. 2022). The elevated APOA1 protein level in this study could potentially be induced by oxidative stress and inflammatory response as discussed in Chapter 4. A previous study conducted by Pilop et al. (2009) utilized non-connective tissue disorder/tricuspid aortic valve stenosis patients as controls found APOA1 was downregulated in MFS. However, the choice of controls could potentially alter the base/control level of APOA1 due to the lipid deposition and inflammation involved in stenosis, thus elevating the base APOA1 level, hence when compared to MFS, APOA1 would be predicted to be lower in MFS patients compared to patients with aortic valve stenosis (Pilop, Aregger et al. 2009, Goody, Hosen et al. 2020).

It is noteworthy that the protein data appeared less powerful in comparison to the RNA data, potentially attributable in part to the technical complexities involved in protein identification. The presence of proteins with subtle structural differences, often referred to as isomers, poses a challenge in their differentiation, resulting in amalgamated names containing multiple protein identifications (Claes, Takeo et al. 2019). This underscores the importance of recognizing potential limitations in protein data, which may not support robust analysis compared to RNA data. Therefore, it is crucial to consider data underrepresentation when conducting significant DE gene analysis, as well as downstream pathway and biological process analyses. Additionally, it is worth mentioning that integrated proteins are discussed as groups and have been excluded from the enrichment analysis (Kim, Zhong et al. 2016).

From the DE protein study, the results revealed that among the top 20 significantly upregulated DE proteins (by both ranking approaches), the majority were, firstly, keratins that were likely to be associated with the mechanical stress response, secondly, proteins associated with inflammatory and oxidative stress response, and, thirdly, elevated haemoglobin abundance was also identified. Additionally, the top 20 significantly downregulated DE proteins included various proteins responsible for

ECM regulation, mitochondrial function, inflammatory responses, and muscle contractility. Overall, these findings closely align with the RNA analysis in MFS patients, except for keratins proteins and elevated haemoglobin abundance, providing strong support for the hypothesis that driver factors, such as increased inflammation and oxidative stress associated with mitochondrial dysfunction, contribute to the pathogenesis of MFS TAAD. However, the novel identification of keratins is consistent with a potential mechanical stress response in MFS pathogenesis.

Keratins are intermediate filament proteins, and play a critical role in maintaining cellular structure, signal transduction, and cell junction adherence (Laly, Sliogeryte et al. 2021, Ho, Thompson et al. 2022). They act as mechanical buffers, protecting cells from mechanical stress and stabilizing both cells and the ECM (Herrmann, Bär et al. 2007). In the context of epidermal skin cells, keratins help endure physical and mechanical stress while maintaining flexibility, similar to the mechanical requirements within the aortic wall. Studies on epidermal cells indicate that keratins serve as mechanosensors, maintaining cellular integrity, altering cell shape, and modulating cell proliferation and apoptosis, possibly through the MAPK pathway (Russell, Andrews et al. 2004). Furthermore, a study has reported the presence of keratin distribution in human blood vessel EC, specifically keratin 7 and 18. It has been suggested that keratin 7 and keratin 18 may have a role in focal reactivity in normal EC, which has implications in certain epithelioid vascular tumours. (Miettinen and Fetsch 2000). However, no study has investigated the role that keratin plays in the aortic wall. Further research is warranted to better understand the mechanism of keratin activity in the pathogenesis of TAA.

In the development of TAAD, impaired mechanotransduction in TAA results from EC dysregulation due to failures in mechanosensors and EC adhesion molecules, leading to pro-inflammatory responses (Martin-Blazquez, Heredero et al. 2021). Moreover, uneven aortic wall mechanical stress is observed in TAA patients, which may contribute to increased inflammation, macrophage infiltration, and MMPs production, further implicating mechanical stress as an activator of inflammatory responses and a contributor to TAAD pathogenesis (Celi and Berti 2014). Furthermore, abnormal blood flow patterns have been observed in the aorta of MFS patients, suggesting potential hemodynamic changes (Geiger, Hirtler et al. 2017). This could potentially contribute to our findings in relation to mechanical stress. In summary, upregulated keratins suggest the presence of mechanical stress in MFS,

with elevated keratin expression possibly representing an adaptive mechanism against mechanical stress.

This study has observed upregulated haemoglobins (HBD/ HBB/ HBE1/ HBG1/ HBG2/ HBA1/ HBZ) in MFS patients compared to controls. Notably, these findings have been reported in a previous MFS tissue proteomics study, although with contrasting results (Pilop, Aregger et al. 2009). While haemoglobin in the aortic wall is rare, previous studies have reported its presence. HBA1/2 has been shown to play a role in NO regulation between EC and VSMC within the blood vessel wall (Butcher, Johnson et al. 2014). Research indicates that HBA can be found at myoendothelial junctions in EC, regulating NO dioxygenase and peroxidase activities. α-haemoglobin stabilizing protein (AHSP), a chaperone protein for HBA, reduces ROS and stabilizes the ferric state (Straub, Lohman et al. 2012, Butcher, Johnson et al. 2014). Knockdown of HBA results in increased NO diffusion into the vessel wall, balancing ferrous and ferric iron states, demonstrating that HBA regulates NO modulation and vascular tone in the vessel wall (Straub, Lohman et al. 2012).

These results indicate high HBA2 expression in the aortic wall, suggesting that MFS patients may exhibit low NO bioavailability and increased ROS production. This could be linked to hypoxic conditions and altered vessel tone, in the context of MFS pathogenesis. However, HBB was not observed in these studies, suggesting a need for further research on haemoglobin presence, distribution and function in aortic tissue. Furthermore, studies involving the administration of haemoglobin-based oxygen carriers (HBOC) in animal models have demonstrated that haemoglobin under the influence of HBOC can penetrate EC by endocytosis, preventing NO diffusion into VSMC. This finding further supports the idea that haemoglobin may regulate NO bioavailability in EC and vascular tone through interactions with VSMC (Faivre-Fiorina, Caron et al. 1999).

Furthermore, free haemoglobin or heme can chelate NO, increasing VSMC contractility, leading to hypertension (Michel and Martin-Ventura 2020). Heme from free haemoglobin can also scavenge NO and convert ferrous iron to ferric iron, inhibiting oxygen binding (Michel and Martin-Ventura 2020). Under hypoxic conditions, NOS is not readily available, and NO production relies on nitrite reductases, including myoglobin (MB) and mitochondrial-driven nitrite reductases (Dungel, Penzenstadler et al. 2017). Both can convert nitrite to NO by binding to red blood cells/ haemoglobin under hypoxia. MB-derived NO is produced at low oxygen

tension, while mitochondrial-derived NO can be generated under both hypoxia and normoxia conditions, signalling through the cGMP-PKG pathway to regulate muscle contractility in the heart (Dungel, Penzenstadler et al. 2017).

This study indicates a significant decrease in MB protein in MFS patients, by almost 16-fold change (log2FC = -3.95); MB is regulated by oxygen level, assisting oxygen diffusion in cells, thus suggesting potential hypoxia and low oxygen tension within the MFS vessel wall (Gros, Wittenberg et al. 2010). Therefore, low MB levels may be associated with unsuccessful oxygen delivery and also imply unsuccessful cell rescue in removing ROS and producing NO, leading to EC dysregulation (Gros, Wittenberg et al. 2010, Dungel, Penzenstadler et al. 2017). Considering the previous data on mitochondrial dysfunction and cGMP-PKG signalling dysregulation in MFS patients, impaired mitochondrial function may affect NO production, resulting in lower NO availability in the aortic wall, causing EC dysregulation and loss of vascular tone.

In addition, heart muscle contractility proteins were also identified in this chapter, echoing the changes from the RNA analysis. As briefly discussed in Chapter 4.4.1, the possible presence of cardiomyocyte-like cells encompassing contractile protein expression, within the ascending aorta, may explain these findings. This will be discussed further in Chapter 8.

In summary, the increased abundance of keratins and haemoglobin in MFS patients suggests elevated wall stress, potential EC dysfunction, and their association with inflammatory responses, oxidative stress, and disrupted ECM homeostasis. The alignment of protein and RNA analysis validates the increased presence of inflammation, oxidative stress, and ECM/EC dysregulation in MFS pathogenesis, and indicates the involvement of mechanical stress.

5.4.2 Signal transduction and biological processes in MFS proteomics

For KEGG enrichment analysis, this study identified the two significant signal transduction pathways namely, TGFβ and HIF-1, with a significance threshold set at an enrichment p-value < 0.1. It is important to note that not all 291 proteins could be mapped in the KEGG analysis due to integrated gene names, as explained in Section 5.4.1. The limited number of identified signal transduction pathways can be attributed to the smaller sample input and the lower detectability of protein by the mass spectrometry technique (Perl, Ushakov et al. 2017). Nevertheless, the KEGG

analysis results support the overall findings of ECM dysregulation and an oxidative stress response.

The implication of oxidative stress in MFS pathogenesis is supported by the observation of altered HIF-1 signalling through significant DE proteins. As detailed in Chapter 2.5.8, the HIF-1 pathway is related to the hypoxia response. HIF-1 signalling can induce an inflammatory response and lead to aneurysm formation or rescue EC by increasing NO production under hypoxic conditions, depending on upstream stimuli. This result strengthens our conclusions regarding the involvement of oxidative stress in MFS pathogenesis, which is associated with EC dysregulation and a potential increase in the inflammatory response.

Although not statistically significant, cGMP-PKG, cAMP, and calcium signalling exhibited correlations with some of the significant DE proteins. As discussed previously in Chapter 4.4.2, cGMP-PKG and cAMP play roles in regulating mitochondrial function, oxidative stress, inflammatory responses, and are also implicated in ECM degradation. The third pathway, calcium signalling, is associated with VSMC elasticity, adhesion, and promotes VSMC phenotypical switching, contractility, and intracellular signal conduction. Additionally, it is involved in TAA formation (House, Potier et al. 2008, Zhu, Qu et al. 2019, Yang, Xie et al. 2023). These findings align with the results from DE RNA and proteins, further supporting the factors previously mentioned in MFS pathogenesis and suggesting that VSMC may indeed undergo phenotypic switching and a loss of contractility.

Furthermore, the identification of alterations in TGFβ signalling aligns with previous proteomics research in MFS, supporting the involvement of abnormal TGFβ abundance at the translational level (Pilop, Aregger et al. 2009, Parker, Stotland et al. 2018). However, the directionality of TGFβ signalling should be further investigated through a phospho-proteomics study.

Overall, data from various studies suggest that protein regulation in MFS involves multiple pathways contributing to the disease pathology. These pathways are linked to oxidative stress, inflammatory response, mitochondrial function, and ECM/EC/VSMC regulation. These implicated pathways and their functions represent potential targets for therapeutic interventions and further investigation to better understand the complex molecular mechanisms underlying MFS pathogenesis.

Additionally, GO analysis has provided valuable insights into the molecular changes in MFS. The identification of coagulation and haemostasis signalling pathways, revealed by the upregulation of coagulation proteins (such as coagulation factors F2, 7, 9, 10, 11, 12, 13) and anti-coagulation factors like serine protease inhibitors (SERPINs), is intriguing. This is especially noteworthy considering that previous studies have indicated lower coagulation activity in MFS patients compared to controls (von Willebrand factor, coagulation factor, and antigen) (Kornhuber, Seidel et al. 2019). The haemostatic system functions to prevent blood loss from vascular injuries. Coagulation factors initiate blood clotting and fibrinolysis in response to vascular injury. The homeostasis of haemostasis within the vessel wall is balanced by anti-coagulation factors such as SERPINs, activated protein C (APC), heparins, and coagulation factors (De Pablo-Moreno, Serrano et al. 2022). The upregulation of both coagulation and anti-coagulation factors observed in this study may be related to endothelial dysfunction within the aortic wall, potentially due to, amongst other things, increased wall shear stress due to turbulent blood flow from both the aneurysm and a possibly altered aortic valve (Kornhuber, Seidel et al. 2019), and also from underlying inflammation within the wall.

A sepsis study demonstrated that disrupted haemostatic dynamics can be induced due to inflammatory and oxidative stress responses, shear stress-induced mitochondrial dysregulation involving ATP generation and release, as well as EC dysfunction. Normal shear stress should provide intact mechanotransduction of the glycocalyx, which consists of proteoglycans that protect against coagulation and inflammation. However, under shear stress influence, dysregulation of the glycocalyx by pro-inflammatory cytokines and ROS induces mal-mechanosensing in the vessel wall, subsequently leading to inflammation, coagulation, and complement activation (Johnson, Choi et al. 1998, Lupu, Kinasewitz et al. 2020). In addition, glycocalyx dysregulation exacerbates EC dysregulation through shear-induced eNOS and NO phosphorylation, damaging mechanosensing and disrupting NO production by ECs. The parallel increase in anti-coagulant and anti-inflammatory proteins may also contribute to mitigating vessel damage and the coagulation cascade, as observed in early sepsis (Lupu, Kinasewitz et al. 2020). In this study, the increased levels of coagulation factors and anti-coagulation factors may indicate a potential feedback loop aimed at mitigating vessel damage in the aortic wall caused by inflammation, oxidative damage, and possibly mitochondrial dysfunction, as well as EC dysregulation. However, this finding could also potentially be attributed to procedural artifacts, such as the surgical harvesting of the aorta.

Further investigation is needed to understand the underlying reasons for the observed increase in coagulation and hemodynamic activity in MFS pathogenesis.

On the other hand, GO analysis of CC and MF has revealed downregulation in ECM proteins and constituents. This finding is consistent with the dysregulation of ECM components observed in MFS. The downregulation of key ECM proteins like FBN and MFAP proteins and collagens suggests a potential disruption in ECM maintenance and integrity, further supporting the notion of ECM dysregulation in MFS.

The overall findings before and after multiple testing correction did not show substantial differences. However, the FDR corrected data suffered from a significant reduction in the number of identified proteins. While the downstream analyses remained largely consistent, the analysis after correction lost the identification of the HIF-1 pathway and regulation of coagulation and haemoglobin. Notably, the regulation of haemoglobin, which has the potential to contribute to TAAD formation by influencing EC regulation (as detailed in Section 5.4.1), was not captured after applying the Benjamini-Hochberg correction. Nevertheless, this study maintains its adherence to the choice and rationale outlined in Chapter 4.4.3 of utilizing both raw and corrected p-values for this discovery study, to maximise identification of potentially important pathogenic mechanisms in MFS.

These results shed light on the complex molecular changes that occur in MFS, particularly concerning coagulation, hemodynamic, and ECM dysregulation. Understanding these molecular alterations can aid in identifying potential therapeutic targets and developing strategies to manage the disease more effectively. Further research is required to validate these findings and unravel the intricate mechanisms underlying MFS pathogenesis.

5.4.3 Fold change selection and significance in DE protein analysis

In this proteomics study, two different fold change cutoff values were selected for analysing significant DE proteins: 2FC and 1.5FC. From the observations in the PCA plots, it is evident that the smaller fold change cutoff (1.5FC) has resulted in slightly better separation in the PCA plot compared to the higher fold change cutoff of 2FC. Fold change reflects the biological relevance of the data, to some extent independently of statistical significance. One could argue that a protein with high statistical significance may not have biological significance if the change between

the groups is very small (Schork, Podwojski et al. 2021). However, it is important to note that the choice of fold change cutoff should not be arbitrary. Instead, it should be carefully and critically considered for each individual dataset.

The selection of the fold change cutoff should be adjusted based on the type of experiment and the biological relevance of the difference in mean abundance. Minor alterations in protein composition or the cellular environment can have significant implications for systemic homeostasis, particularly because of the protein regulatory mechanisms involving PTMs (Karve and Cheema 2011). Even small changes in these PTMs can exert a substantial impact on downstream protein activity, stability, and interactions with other molecules (Karve and Cheema 2011). Consequently, a 2FC cutoff threshold might overlook these small but biologically significant changes in protein expression, potentially reducing the statistical power and the ability to detect genuine alterations. Lowering the fold change cutoff threshold has the potential to reduce noise and variability within the data, potentially resulting in clearer separation in the PCA plot (Mutch, Berger et al. 2002). This effect is particularly pronounced with proteins that naturally have a higher level of abundance, where alterations are less likely to be large (Mutch, Berger et al. 2002). A high fold change cutoff could overshadow changes in these abundant proteins and place more emphasis on proteins with initially lower abundance. Therefore, a 1.5FC cutoff threshold was chosen in the proteomics study for downstream analysis to prevent potential data loss and maintain robust analysis.

5.5 LIMITATIONS

This study has certain limitations that are akin to those encountered in RNA analysis. These include a small sample size (n = 16 MFS, 11 controls), although it is still significantly larger compared to a number of other experimental studies, and ambiguities in pathway directionalities. A specific constraint in the protein study arises from the limitations of mass spectrometry, as it is unable to distinguish very similar isomers, as previously mentioned in this chapter. Furthermore, only proteins with known and correct sequence could be captured by the mass spectrometry technique. Therefore, if the proteins were modified or unknown, the information would be lost (Wang and Wilson 2013).

Specifically, diseases can influence protein folding, leading to abnormal binding and resulting in nonspecific protein aggregation (van Duijn 2010). For example, amyloid

is a histopathological hallmark in Alzheimer's disease and type 2 diabetes. A recent study has uncovered that amyloid abundance is significantly lower in TAAD, furthermore, medin, as a small fragment of vascular amyloid protein, could produce prefibrillar oligomeric aggregates instead of amyloid fibrils (Wagner, Degenhardt et al. 2022). This aggregated and misfold of medin could induce VSMC death and increase MMP2 production and potentially damage the aortic wall (Peng, Larsson et al. 2007). Subsequently, this affects the protein expression and global profiling.

Furthermore, current mass spectrometry technology may encounter challenges in distinguishing certain similar isomers, as previously mentioned. To identify specific protein structures, additional experimental approaches are necessary to address this issue. These approaches may involve enhancing molecular resolving power by further imaging techniques or implementing ion-specific mobility-based separation techniques (Claes, Takeo et al. 2019). Relying solely on mass spectrometry could impose limitations on the detection of isomers. Consequently, this study has opted for an alternative approach to interpret integrated protein names by discussing them as a group (Kim, Zhong et al. 2016). Nevertheless, further studies will be necessary to validate these results.

5.6 CONCLUSION

In conclusion, the comparison of genome-wide protein profiles between MFS patients and controls has enhanced our understanding of the biological processes and cellular functions involved in MFS pathogenesis. In general, the proteomics study corroborated the findings from RNA analysis in MFS, underscoring the significance of the inflammatory and oxidative stress responses, abnormalities in mitochondrial function, and may have implications for heart muscle contractility.

Furthermore, the proteomics study has unveiled fresh insights into the mechanical stress response, regulation of red blood cells, and coagulation activities observed in MFS patients. This suggests that the upregulation of keratin and haemoglobin proteins may play a role in mitigating increased mechanical stress and abnormal hemodynamic in MFS. These findings are further supported by pathway and biological process analyses, introducing novel aspects to our understanding of MFS-related TAAD formation.

Chapter 6: Multi-omics in MFS

6.1 INTRODUCTION

In the previous two chapters, the investigation into genome-wide transcriptomics and proteomics studies has provided valuable insights into potential signal transduction pathways and biological processes involved in MFS disease mechanisms. In this chapter, the study aims to conduct a multi-omics analysis of the previous findings to further our understanding of signal transduction mechanisms through transcriptional-translational regulation.

The term 'omics' encompasses a wide range of studies, including genomics, epigenomics, transcriptomics, proteomics, metabolomics, lipidomics, and more, each focusing on different biological processes within the human body (Micheel, Nass et al. 2012). However, when focus on genomics, transcriptomics and proteomics, it is essential to acknowledge that not all DNA is transcribed into RNA (approximately 80%), both coding and non-coding RNA, and an even smaller fraction of DNA is translated into proteins (approximately 3%) (Hasin, Seldin et al. 2017). This raises a crucial question: what “messages” are actually relayed from the genetic code to influence signalling pathways, cellular functions and biological processes? Examining only one layer of omics data can limit our understanding of disease mechanisms due to the influence of intricate and multi-level regulatory mechanisms. Therefore, integrated omics studies have the capacity to create a more comprehensive picture of biological regulation, particularly in relation to diseases.

Multi-omics, which involves integrating various omics studies and examining different layers of the regulatory system, has the capacity to reveal more accurate underlying information about a disease and provide further insights into its mechanisms (Hasin, Seldin et al. 2017). In Chapters 4 and 5, the study analysed data from transcriptomics and proteomics separately, conducting genome-wide qualitative and quantitative analyses of RNA and protein data, which offered a broad understanding of gene and protein regulation within MFS. However, this understanding has limitations due to the complex regulatory interactions between RNA and proteins.

From the nature of transcription-translational mechanism, many RNA nucleotide triplets are redundant or untranslatable into proteins (Telser 2002). Moreover, RNA-Seq data identifies more than protein-coding transcriptomes, including non-coding RNA (ncRNA), long non-coding RNA, and short RNA like microRNAs (Kukurba and Montgomery 2015). This results in information asymmetry, making it challenging to interpret and compare with downstream protein data. Furthermore, proteomic studies are even more complex, as proteins can be regulated by PTMs, including glycosylation, phosphorylation, acetylation and ubiquitylation, and other mechanisms like epigenetics (miRNAs), in addition to the transcription-translation mechanism (Karve and Cheema 2011, Micheel, Nass et al. 2012). Therefore, in multi-omics analysis, data harmonization is crucial to understand both the classic regulatory system and alternative regulation mechanisms influencing RNA or protein regulation, enhancing our understanding of the underlying disease aetiology (Krassowski, Das et al. 2020).

In summary, genomic studies open up possibilities for understanding disease aetiology, transcriptomic studies further investigate these possibilities, and proteomic studies reveal the actual final functional protein product that results from these regulation mechanisms within the disease. By examining only one layer of the regulatory mechanism, the information from the genetic transcriptional level can easily overshadow the downstream protein analysis, potentially causing us to miss informative and actionable discoveries related to proteins that illuminate fundamental changes in MFS (Krassowski, Das et al. 2020). Therefore, this study aims to unravel the regulatory mechanisms of RNA and protein and the associated signalling pathways in MFS, aiding in our understanding of RNA and protein regulation as well as the signal transduction pathways involved in these processes.

The aims of this study were to:

1. Examine the genes and proteins involved in classic transcription-translation regulation and targeted signal transduction pathways in MFS.
2. Investigate differentially expressed genes or proteins involved in MFS, illustrate targeted pathways at the transcriptional or translational level.
3. Identify key pathways altered in MFS through multi-omics studies.

6.2 METHODS

6.2.1 Data harmonization

Transcriptomics and proteomics data were gene symbol-matched and subsequently transformed into a datasheet with logFC and p-values for further analysis.

6.2.2 Statistical analysis

The significant p-value cutoff was set to 0. 05 as calculated for the previous transcriptomics and proteomics studies (Chapters 4.2.3 and 5.2.3), with a fold change threshold of 2FC for RNA and 1.5FC for protein applied to the scatter plot and KEGG/GO analysis. Microsoft Excel and RStudio were used to generate plots and tables. No multiple testing corrections were applied in this study.

6.3 RESULTS

First, RNA and protein expression were extracted from transcriptomics and proteomics data for all RNA-protein pairs, resulting in the identification of 4,025 pairs. A scatter plot was generated to visualize the expression patterns across transcription and translation levels, using specific thresholds (2FC for RNA and 1.5FC for protein) (Figure 6.1). The dots on the plot were color-coded to represent different scenarios: red dots indicated consistent changes observed in both transcriptomics and proteomics, blue dots represented contrasting expression results between RNA and protein, suggesting non-concordant regulation between transcription and translation mechanisms, almond-coloured dots indicated that RNA/protein pairs that were altered at the transcription level but not in the translation level, and green dots indicated the opposite. The data corresponding to each coloured dot was further analysed in separate sections.

Overall, the scatter plot shows that alterations at the transcription level were more pronounced than at the translation level, suggesting that protein regulation is more subtle. In summary, the most significantly changed corresponding RNA-protein pairs included elevated keratins and haemoglobins, as well as decreased genes/ proteins related to the ECM and EC regulation and muscle contractility.

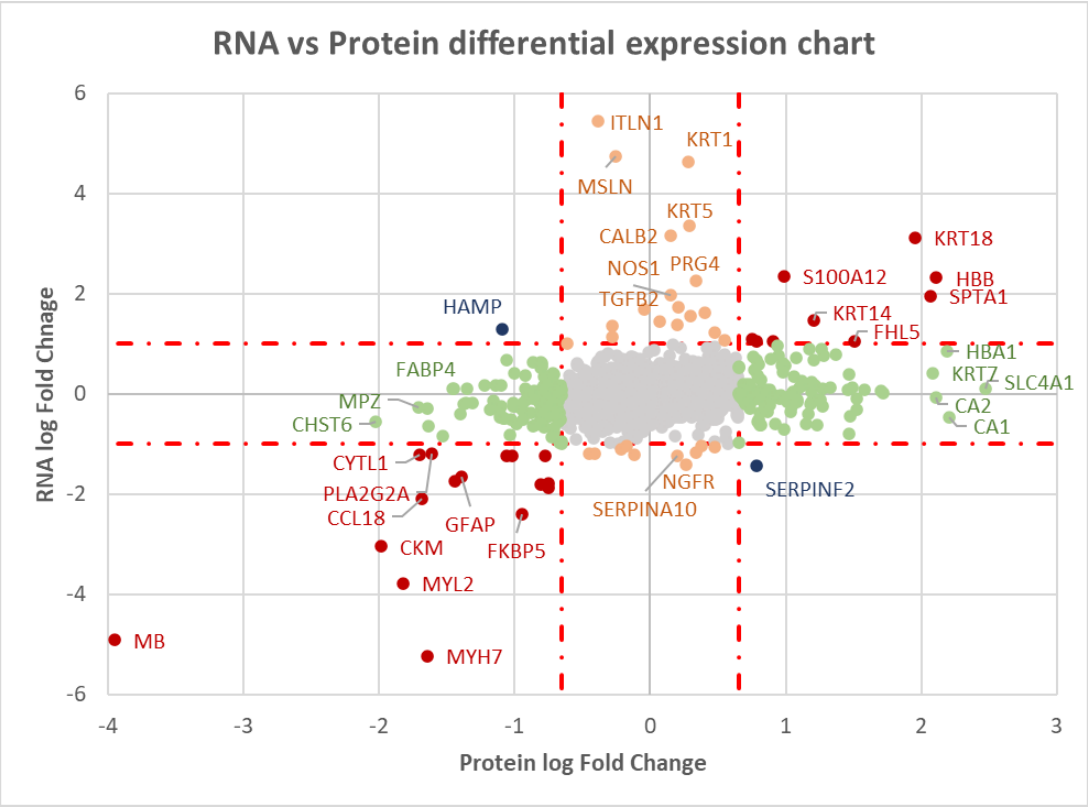


Figure 6.1 RNA vs protein differential expression scatter plot. Dark red dots indicate that both RNA and protein were differentially expressed in the same direction, dark blue dots indicate both RNA and protein were differentially expressed but in opposite directions. Almond dots indicate that only RNA was differentially expressed but not the corresponding protein, and light green dots indicate that only protein was differentially expressed but not the corresponding RNA. The middle area with grey dots indicates that both RNA and protein were not differentially expressed over the 2FC and 1.5FC threshold for RNA and protein, respectively. The scatter plot only demonstrates the fold change for RNA and protein, regardless of the significance.

6.3.1 Corresponding DE RNA and protein – red dots

Among the genes and proteins identified as DE in MFS, 25 pairs demonstrated corresponding expression changes, meaning both transcriptomics and proteomics either increased or decreased. Out of these, 19 gene-protein pairs reached statistical significance (Table 6.1). The average RNA-to-protein logFC ratio was approximately 1.6, indicating that changes in the corresponding RNA-protein pairs occurred at a ratio of roughly 1.5:1. This suggests that protein changes are more subtle compared to RNA changes. Overall, these corresponding genes and proteins highlight their involvement in processes such as inflammation, oxidative stress, mechanical stress response, ECM dysregulation, mitochondrial function, muscle

contractility, and lipid metabolism. These findings indicate that previous identified biological processes may be regulated by transcriptional and translational mechanisms.

Table 6.1 Corresponding transcriptomics – proteomics pair.

Gene name	RNA logFC	RNA p-value	Change	Protein logFC	Protein p-value	Change	Ratio	Brief function
GPX3	-1.24	4.8E-08	Down	-1.06	2.6E-05	Down	1.2	Anti-oxidative stress Anti-inflammation ECM related
MB	-4.91	2.9E-06	Down	-3.95	5.6E-05	Down	1.2	Anti-oxidative stress
FKBP5	-2.39	2.7E-09	Down	-0.94	8.2E-05	Down	2.5	Anti-oxidative stress Ani-inflammation Anti-mechanical stress
CKM	-3.04	1.1E-07	Down	-1.98	0.0004	Down	1.5	Mitochondrial function
MYL2	-3.77	7.1E-07	Down	-1.82	0.001	Down	2.1	Mitochondrial function Contractility
MYH7	-5.23	1.2E-06	Down	-1.64	0.001	Down	3.2	Mitochondrial function Contractility
KRT18	3.12	7.4E-06	Up	1.96	0.002	Up	1.6	Anti-inflammation Mechanical stress
ALDH1L1	-1.73	4.3E-09	Down	-1.44	0.003	Down	1.2	Mitochondrial function
CYTL1	-1.21	0.011	Down	-1.70	0.004	Down	0.7	Pro-inflammation
CCL18	-2.08	0.005	Down	-1.68	0.005	Down	1.2	Pro-inflammation
TPPP3	1.06	0.001	Up	0.78	0.006	Up	1.4	Pro-oxidative stress
FHL5	1.05	0.003	Up	1.51	0.008	Up	0.7	ECM related

GFAP	-1.66	0.0004	Down	-1.39	0.0096	Down	1.2	Mechanical stress
HPGDS	1.10	0.0001	Up	0.75	0.0156	Up	1.5	Lipid metabolism Pro-inflammation
ITGA10	-1.24	9.5E-08	Down	-0.78	0.0224	Down	1.6	Pro-inflammation
SPTA1	1.95	3.3E-05	Up	2.07	0.0245	Up	0.9	Red blood cell related
LBP	-1.80	0.0017	Down	-0.81	0.0313	Down	2.2	Lipid metabolism
KRT14	1.48	0.0386	Up	1.21	0.0365	Up	1.2	Mechanical stress
SERPINA3	-1.87	1.8E-08	Down	-0.75	0.0391	Down	2.5	Pro-inflammation ECM related

Notes: Almond colour encoded for transcriptomic study and green colour encoded for proteomics study. Pink colour indicates upregulation and blue indicates downregulation. Transcriptomics – proteomics ratio is calculated by logFC RNA divided by logFC protein. P-values have not been corrected for multiple comparisons.

Due to the limited number of genes and proteins analysed, GO and KEGG analyses yielded relatively low-quality results, but were performed primarily to investigate whether the concordant RNA-protein pairs were associated with specific biological processes or pathways similar to those already identified in the preceding chapters. The GO analysis revealed the significance of alterations in intermediate filaments in MFS pathology, regulated by intermediate filament proteins such as keratins and GFAP. These data suggest that the regulation of mechanical stress may be modulated by transcriptional-translational mechanisms.

Additionally, terms related to muscle contraction and ECM components were identified, with MYH7 and MYL2 genes playing a role, indicating a potential loss of contractility in MFS pathology (Figure 6.2).

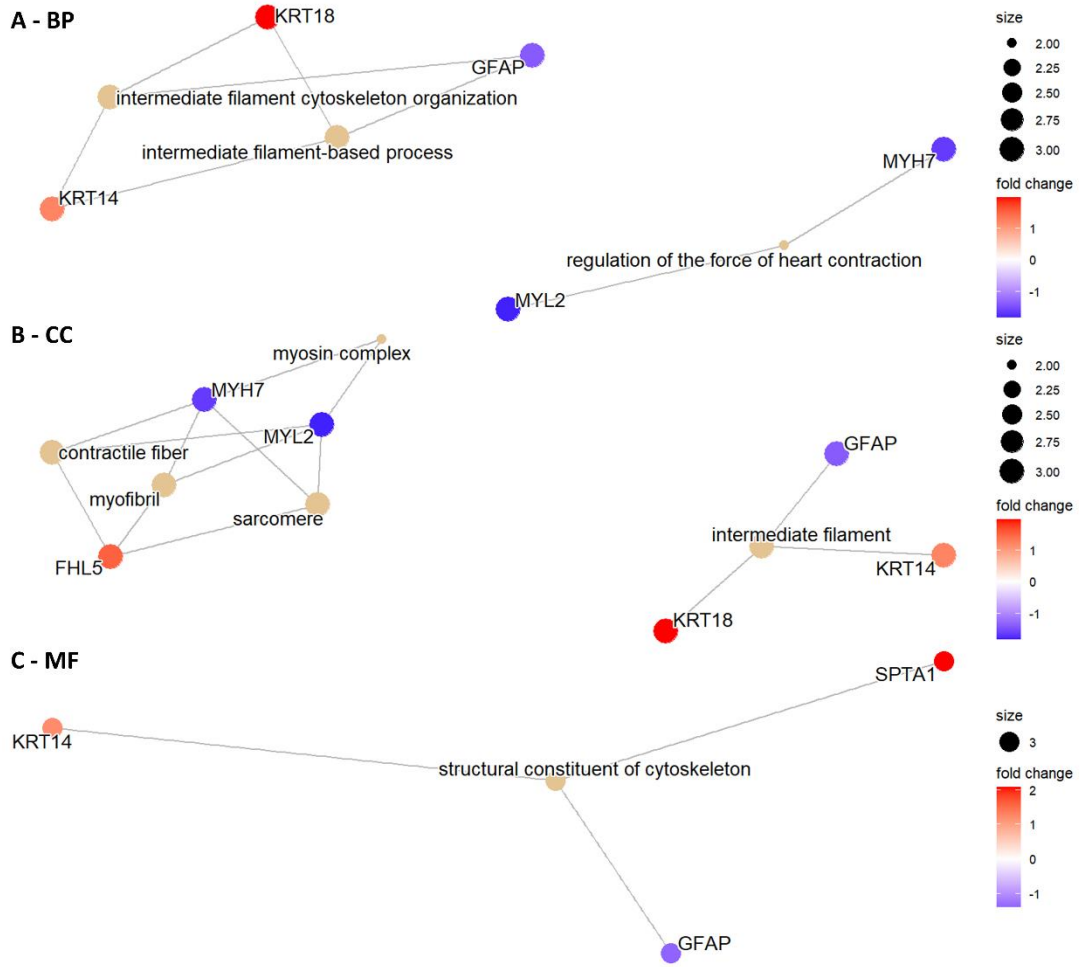


Figure 6.2 GO cnetplot for correspondence significant DE genes and protein. Protein logFC was used to illustrate the alteration. A) Biological process. B) Cellular components. C) Molecular function. Red indicates upregulation and purple/blue indicates downregulation.

The KEGG analysis identified correlations with only five RNA-protein pairs, regardless of their enrichment significance. These results reinforce the earlier findings related to the inflammatory response and suggest potential associations with muscle contractility and mitochondrial function through pathways such as NF-κB, JAK-STAT, cGMP-PKG, and PI3K/AKT. Although the quality of result is relatively low due to very small gene/protein input, the findings suggested potential alignment with the previous results, further highlighting the role of contractility genes/proteins and intermediate filament genes/ proteins in MFS.

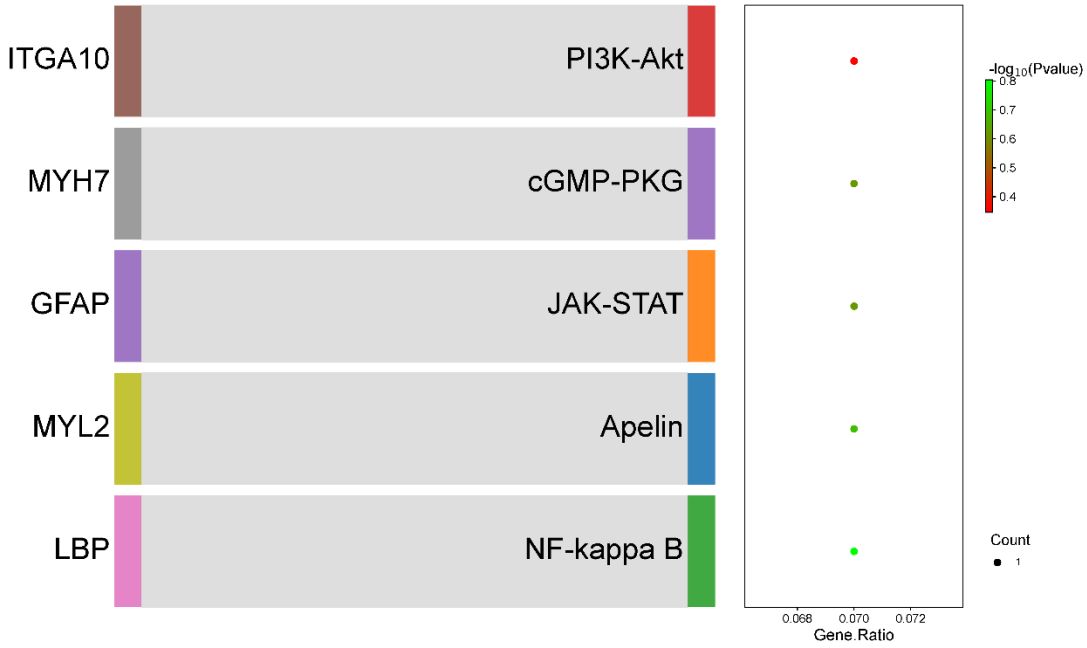


Figure 6.3 Sankey plot of KEGG analysis from concordance RNA-protein pairs that are regulated by the transcription-translation mechanism in MFS. None of the pathways reached significant level by enrichment analysis.

6.3.2 Contrasting DE RNA and protein – blue dots

Two gene/protein pairs were identified as DE in MFS with contrasting expression patterns, where either transcriptomics was upregulated and proteomics downregulated, or *vice versa*. Among these, one gene-protein pair, SERPINF2, was found to be significant (Table 6.2). The observed differences in RNA-protein regulation may be attributed to several factors, including post-transcriptional regulation, mRNA decay, protein degradation, or translation efficiency, among others. These findings suggest that these particular genes and proteins in MFS pathogenesis are not primarily regulated by the transcription-translation mechanism, indicating the presence of alternative regulatory systems. However, among these two contrasting RNA-protein pairs, HAMP did not meet the significant cutoff value, while SERPINF2 just exceeded the log-transformed fold change and significant cutoff for protein levels. This raises the possibility of a potential statistical error rather than a genuine pathophysiological observation.

Table 6.2 Contrasting transcriptomics – proteomics pair.

Gene name	RNA logFC	RNA p-value	Change	Protein logFC	Protein p-value	Change
SERPINF2	-1.42	0.00002	Down	0.79	0.023	Up

Notes: Almond colour encoded for transcriptomic study and green colour encoded for proteomics study. Pink colour indicates upregulation and blue indicates downregulation.

6.3.3 Differentially expressed RNA but no change at the translation level – almond dots

Twenty-eight significant DE genes were identified in MFS with no corresponding DE protein expression. Among these, nine genes were upregulated, and sixteen genes were downregulated without concurrent protein expression changes. This suggests that these significant DE RNA molecules did not result in corresponding protein alterations and may have limited contributions to MFS pathogenesis.

Similar to the results discussed in section 6.3.1, the small sample size (n = 25) led to KEGG and GO analyses being used primarily as a reference to explore the potential roles of these altered genes, providing insights into their potential functions in the context of MFS. A Sankey plot was generated using the altered RNA without corresponding protein changes. Among the pathways identified, PI3K/AKT was the only significant pathway associated with transcriptional regulation ($p = 0.02$), while MAPK showed significance at $p < 0.1$. The remaining pathways depicted in Figure 6.4 did not reach significance.

Despite the lack of statistical significance in some pathways, the Sankey plot offers an overview of the altered RNA and their potential involvement in various pathways. Notably, PI3K/AKT and MAPK signalling pathways were targeted by multiple genes, suggesting a role in MFS transcriptional regulation. Additionally, NGFR and TGFB2 appeared to influence multiple pathways, regardless of the significance level, indicating potential implications for PI3K/AKT and MAPK signalling and abnormal TGFβ signalling in MFS, respectively.

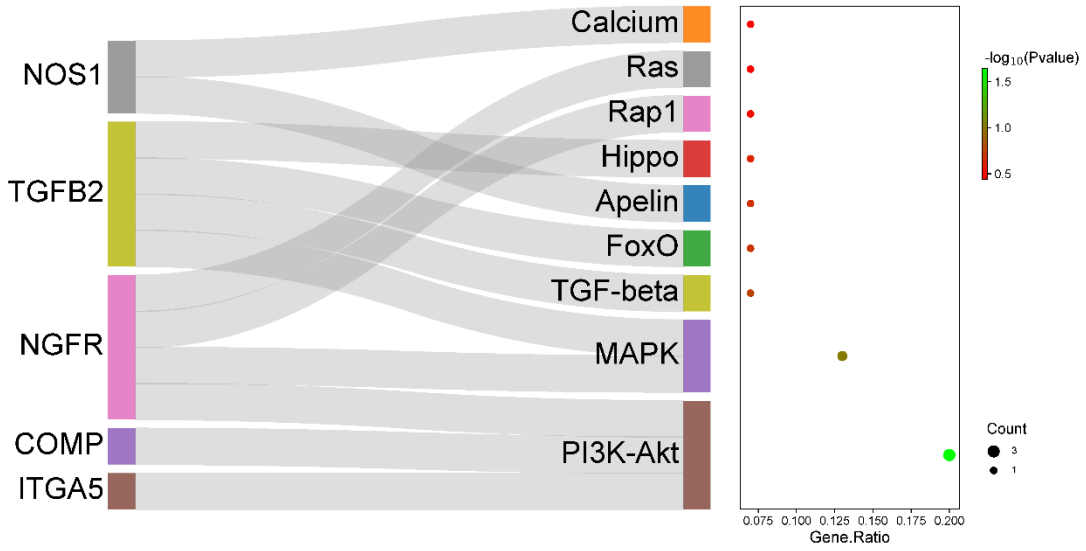


Figure 6.4 KEGG Sankey plot for transcriptomic – proteomic pair which RNA has altered but no change in protein expression.

GO analysis was performed to illustrate the biological processes, cellular components, and molecular functions associated with significant genes that did not exhibit corresponding protein changes. A GO bubble plot (Figure 6.5) was generated to visualize the significance of each ontology term, and a GO cnetplot (Figure 6.6) was created to illustrate the genes involved in these processes.

The GO analysis results revealed that processes such as wound healing, homeostasis, and artery development were identified through altered transcriptional regulation. Specifically, the wound healing process was associated with the upregulation of TGFB2, indicating potential involvement of TGFβ signalling in MFS-related wound healing. Additionally, both TGFB2 and KRT1 were linked to collagen-containing ECM components, suggesting that these genes may play a role in MFS pathogenesis, but only significantly at the transcriptional level.

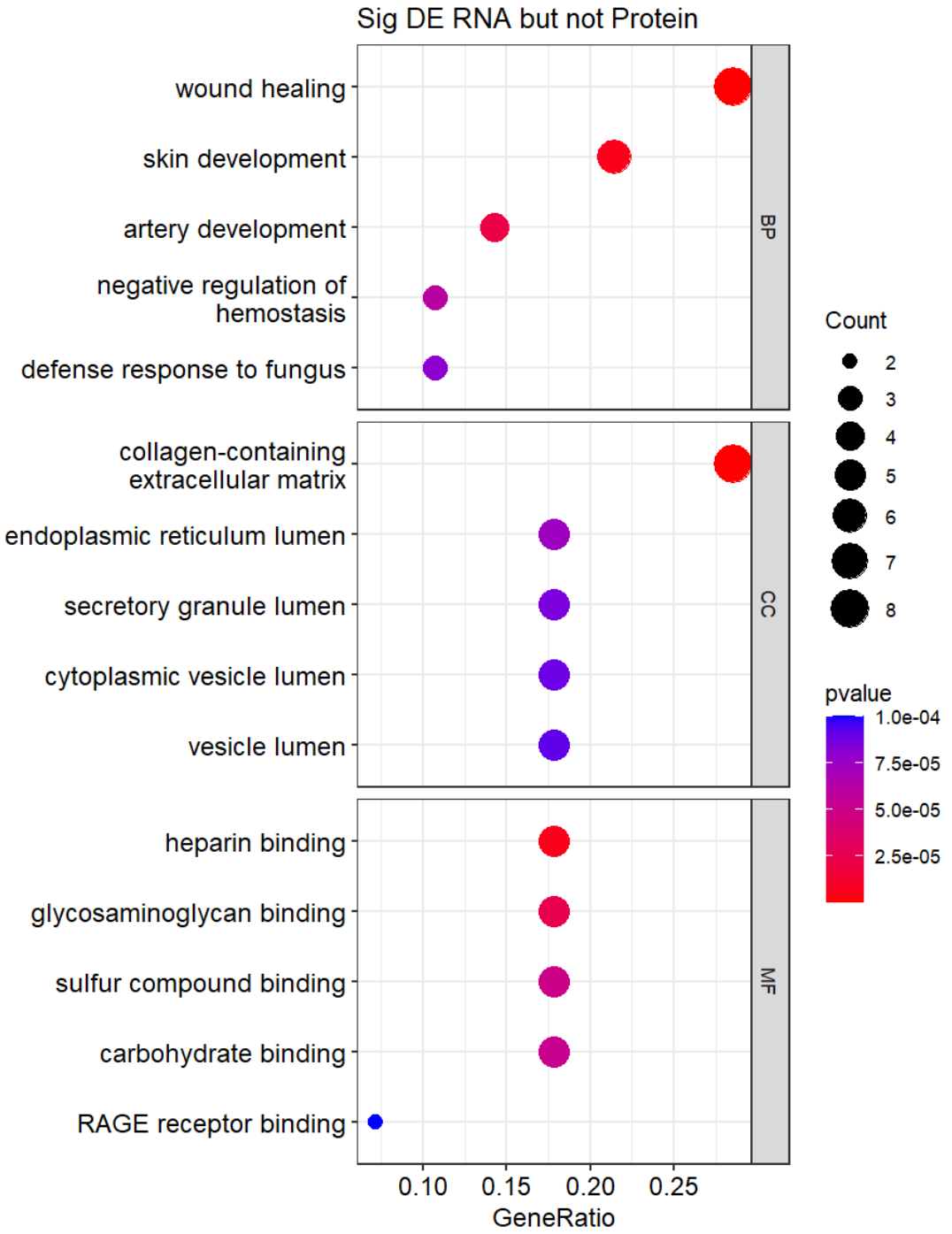


Figure 6.5 GO bubble plot for transcriptomic – proteomic pair which RNA has altered but no change in protein expression. BP – Biological process, CC – Cellular component, MF – Molecular function.

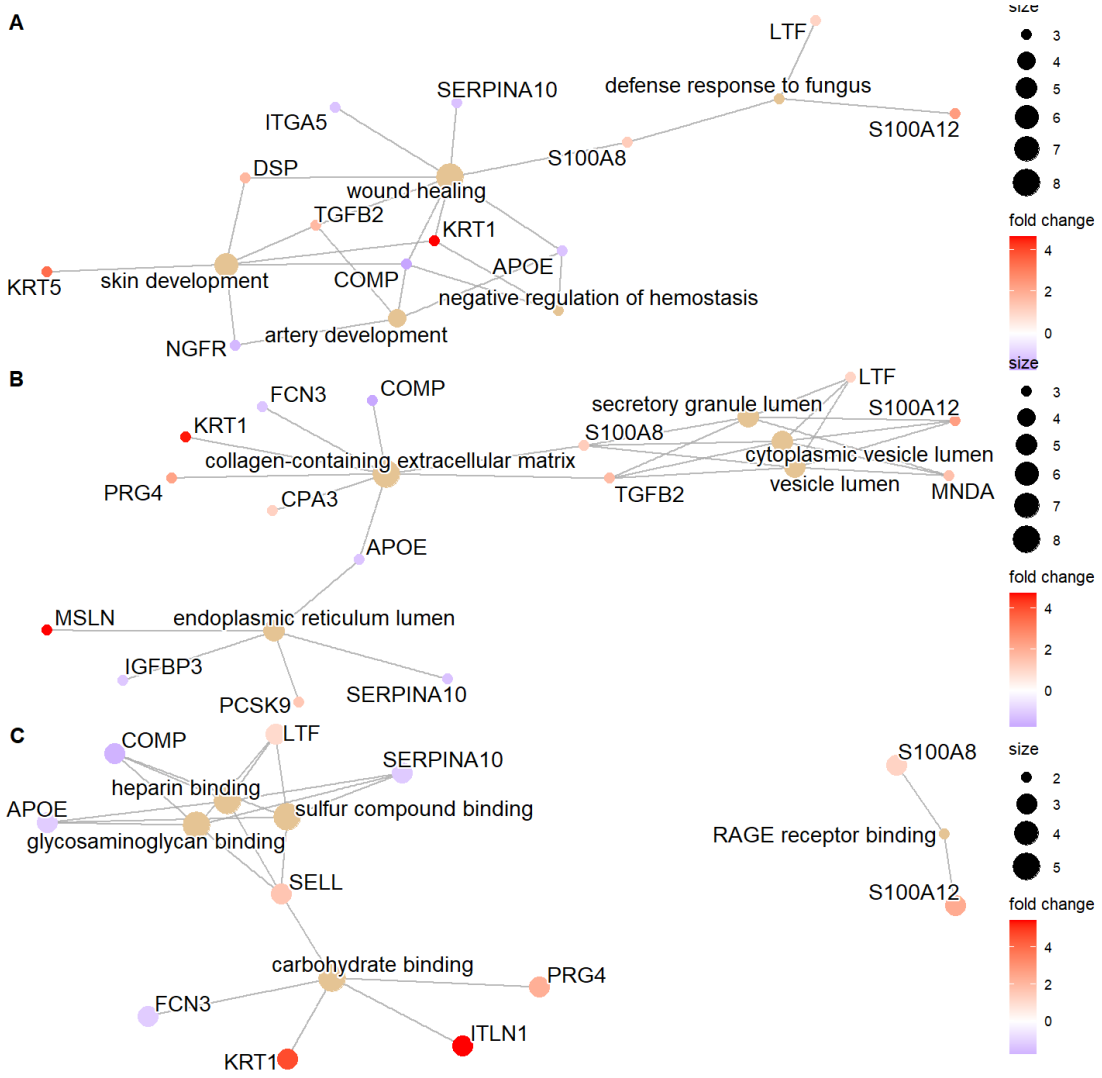


Figure 6.6 GO cnetplot for transcriptomic - proteomic pairs in which RNA has altered but there is no significant change in protein expression. A) Biological process, B) Cellular component, C) Molecular function.

6.3.4 Differentially expressed protein but no change at the transcription level – green dots

A total of 226 proteins without corresponding RNA changes have been identified as DE in MFS (e.g., no matching RNA expression due to integrated isomers or RNA expression is not differentially expressed, as discussed in Chapter 5.2.2). Overall, 174 proteins were significant, with 91 proteins showing upregulation and 83 proteins displaying downregulation in MFS patients compared to controls. Significant protein DE without corresponding RNA expression suggest the involvement of post-transcriptional regulation mechanisms or alternative regulatory processes independent of RNA expression or RNA degradation. Given that proteins occupy a more downstream position within the regulatory hierarchy and show a close interrelationship with metabolites activities, they are more predisposed to influence the signal transduction pathways underlying disease pathogenesis.

To gain insights into the signal transduction pathways affected by these altered proteins, KEGG analysis was performed. The TGFβ and HIF-1 pathways were identified as significant ($p = 0.03$), while the remaining pathways did not reach the enrichment significance cutoff of $p = 0.05$ (Figure 6.7). These findings align with previous protein analysis and highlight the involvement of HIF-1 in the oxidative stress response and dysregulation of the TGFβ pathway in MFS. Furthermore, PRKCA was found to target 13 signal transduction pathways, underscoring its importance and suggesting potential interactions between these pathways.

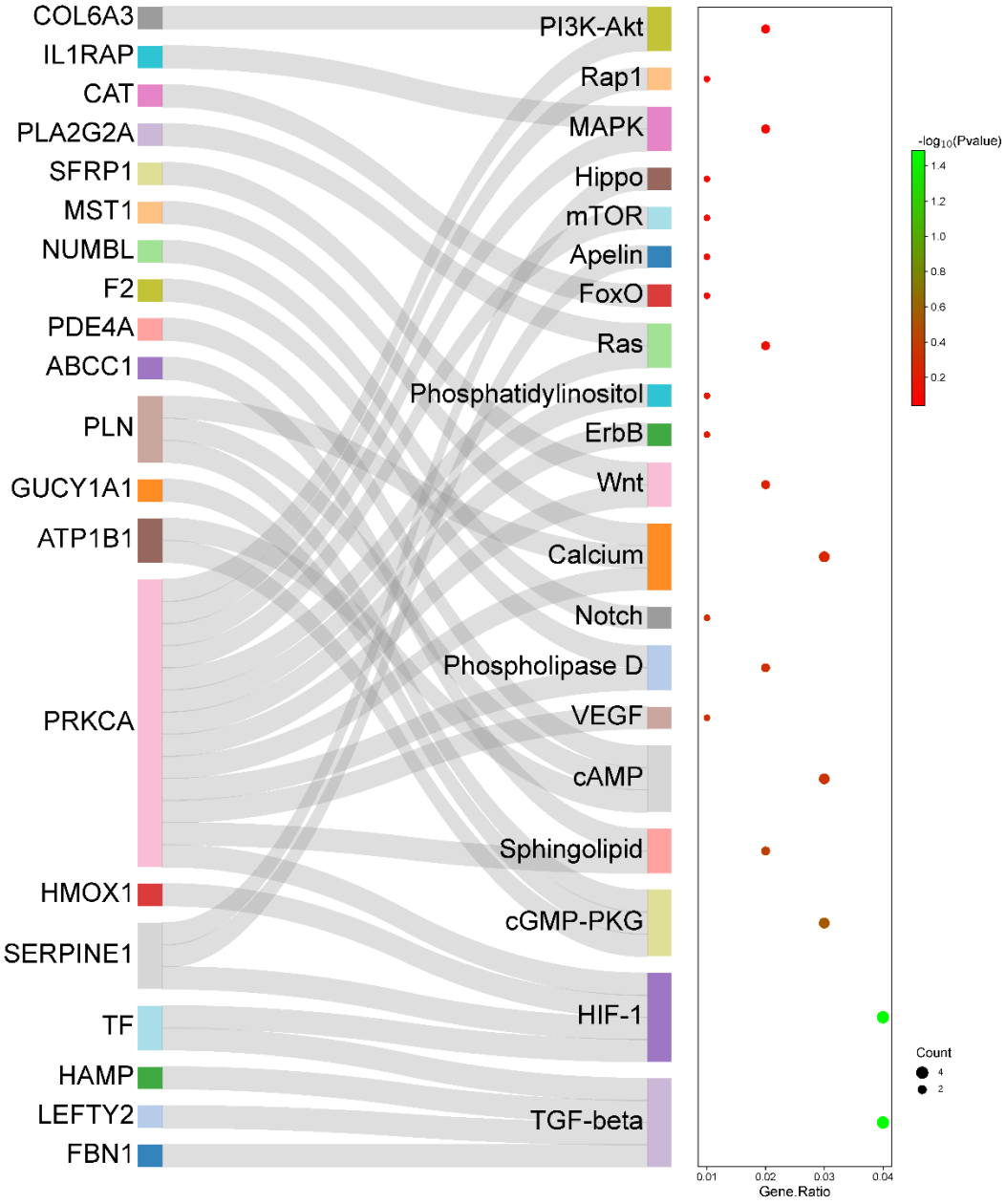


Figure 6.7 KEGG Sankey plot for transcriptomic – proteomic pairs in which protein has altered but there is no change in RNA expression. Only TGFβ and HIF-1 signalling pathways were significant (enrichment p-value <0.05), the remaining pathways were not significant.

A GO bubble plot (Figure 6.8) and cnetplot (Figure 6.9) were generated to visualize the significance of each ontology term and the proteins involved in these processes. The results echoed our findings from the proteomics study GO analysis. Notably, processes related to coagulation and haemostasis were identified as regulated by coagulation factors, which were not observed at the RNA level. This suggests that

the involvement of these processes is likely more pronounced at the translational level rather than the transcriptional level. Additionally, processes related to the ECM and related enzymatic activities, such as glycosaminoglycan binding, were identified.

Although the involvement of ECM regulation was identified independently in both RNA and protein analyses without concordant expression, it underscores the significance of ECM regulation in MFS pathogenesis.

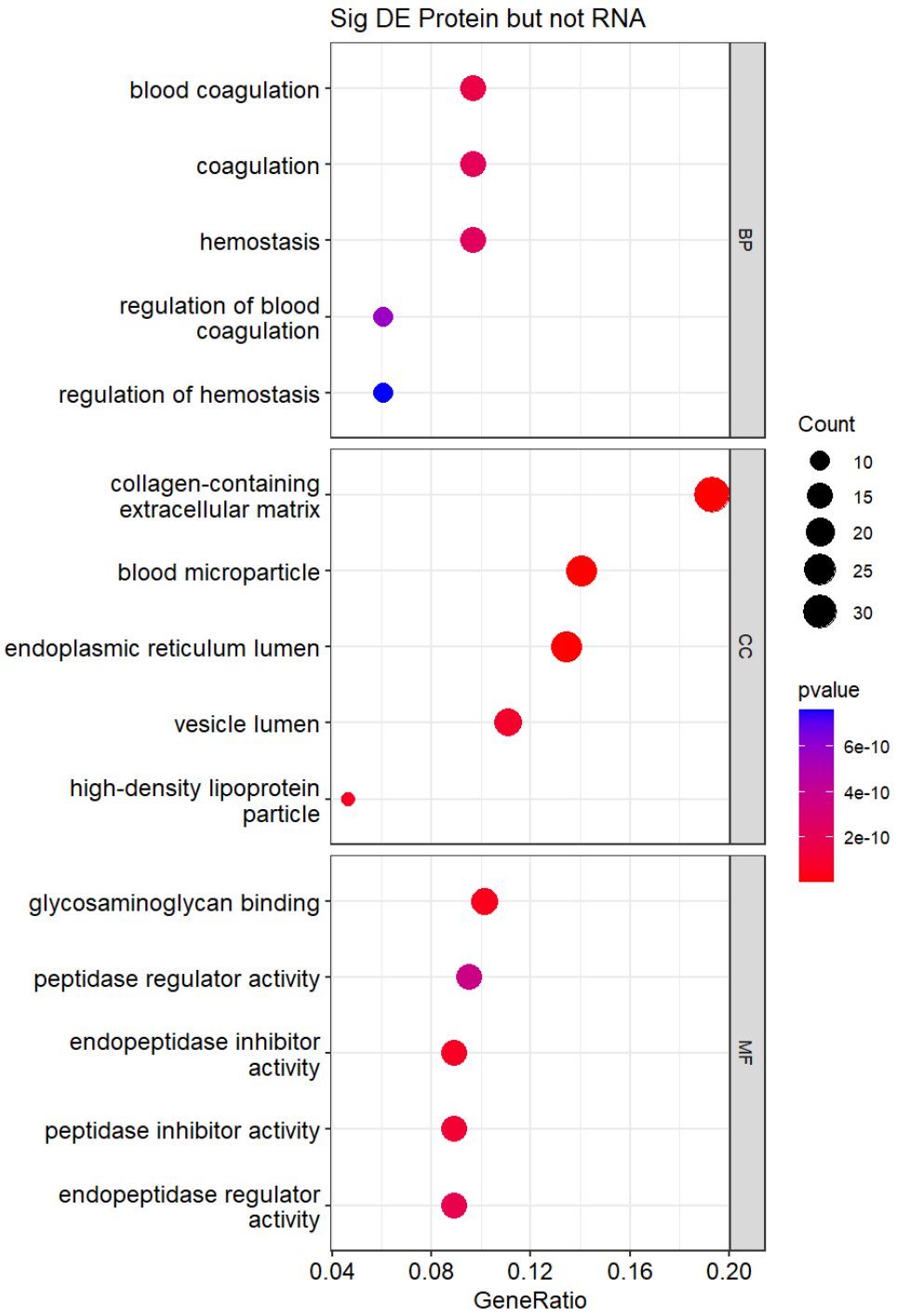


Figure 6.8 GO bubble plot for transcriptomic – proteomic pair which protein has altered but no change in RNA expression. BP – Biological process, CC – cellular component MF- Molecular function.

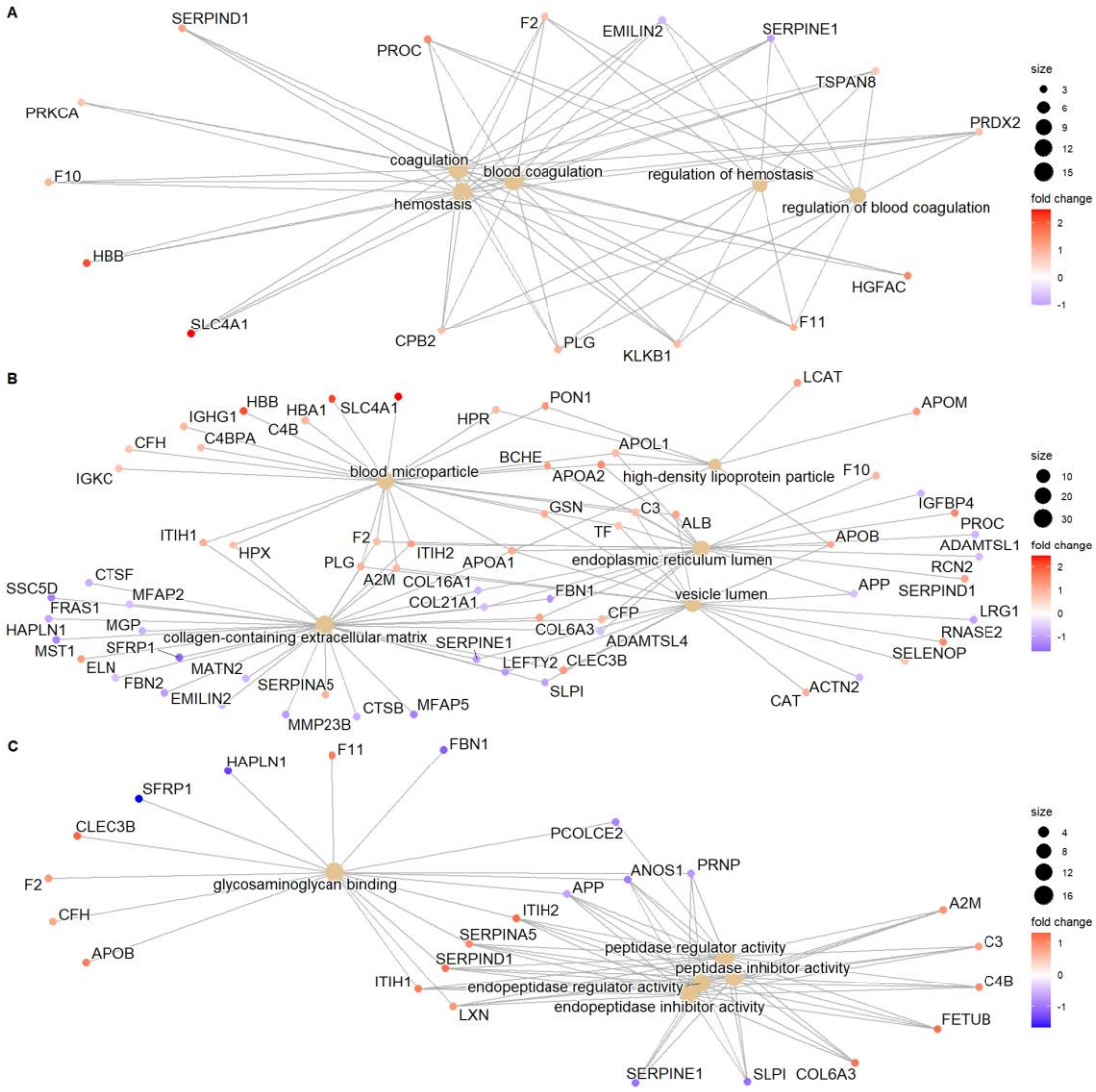


Figure 6.9 GO cnetplot for transcriptomic – proteomic pair which protein has altered but no change in RNA expression. A) Biological process, B) Molecular function, C) Cellular component.

6.4 DISCUSSION

The findings from the multi-omics analysis reveal a complex relationship between RNA and protein expression, which is not always consistent. The presence of changes exclusively at either the RNA or protein levels, without corresponding significant alterations in the other, suggests the involvement of additional regulatory mechanisms, such as post-transcriptional regulations. Understanding these intricate interactions between transcription, translation, and additional regulatory mechanisms is crucial for investigating the aetiology of MFS and gaining insights into the associated cellular functions and signalling pathways.

6.4.1 Corresponding transcription – translation

Out of the 4,025 protein – RNA pairs, only 25 pairs demonstrated concordant change in expression that met the fold change cutoff threshold. Of these, 19 RNA-protein pairs were significant. This result highlights the complexity of the transcription-translational mechanism in disease pathogenesis, indicating that only a small fraction of altered mRNA translates into protein and affects its expression. It also suggests a relatively weak correlation between RNA and protein, a phenomenon observed in other studies (Dumitriu, Golji et al. 2016). Additionally, within the corresponding RNA-protein pairs, the average RNA-protein fold change ratio is 1.6, indicating that RNA signalling and expression changes are more substantial compared to protein changes. This could be attributed to factors such as protein turnover rates, RNA buffering effect raising the apparent level of RNA detected with the RNA-Seq technique or technical limitations of mass spectrometry (Dumitriu, Golji et al. 2016, Perl, Ushakov et al. 2017). Furthermore, previous studies have shown that specific biological processes can be transcription- or translation-specific. For instance, immune-related processes were found to be more pronounced in the mRNA domain, while regulation related to hypoxia, heat shock proteins, and ion concentrations were more prominent in the translational domain (Perl, Ushakov et al. 2017). These findings align with the separate transcriptomics and proteomics studies. The RNA analysis emphasizes the inflammatory response, while the protein analysis highlights the oxidative stress response. This contributes to a deeper understanding of transcriptional and translational regulation.

To further investigate potential correlated pathways and biological processes, GO and KEGG analyses were conducted. The findings support the understanding of the inflammatory response related to NF-κB and JAK-STAT signalling, abnormalities in

mitochondrial function related to cGMP-PKG signalling, and the role of PI3K/AKT as an intermediate signalling pathway that transduces signals from upstream stimuli. Apelin appears to cooperate with Ang II, playing opposing roles to balance blood pressure. Apelin signalling lowers blood pressure by promoting VSMC relaxation and NO production, while Ang II demonstrates the opposite effect (Tatemoto, Takayama et al. 2001). Additionally, the Apelin pathway demonstrates complex crosstalk with several pathways, such as AMPK, PI3K/AKT/mTOR, and RAF/ERK1/2, regulating vasoconstriction, heart muscle contractility, vascular inflammatory and hypoxia response and energy metabolism (Dagamajalu, Rex et al. 2022). Furthermore, the Apelin pathway promotes angiogenesis via the ERK and AKT pathways, enhancing EC proliferation and angiogenesis (Masri, Lahlou et al. 2002).

The GO analysis further highlights the involvement of intermediate filament regulation and cardiomyocyte-related contractility processes in the transcription-translational mechanism, emphasizing the potential presence of mechanical stress in the vessel wall. Intermediate filaments may be involved in an adaptive response to this mechanical stress (as elucidated in Chapter 5). Additionally, downregulated heart contractility genes/proteins identified contractile fibre, myofibril, and sarcomere related ontology terms in the GO analysis suggest a consistent finding of potential loss of contractility, presumably in the paradoxically located cardiomyocyte-like cells that may occur within the ascending aorta (see Chapter 8 for a more detailed discussion).

6.4.2 Contrasting transcription – translation

Contrasting results between transcriptomics and proteomics were observed in this study, and this discrepancy is not a new phenomenon in our understanding of omics data (Wang 2008). It suggests that certain RNA and proteins are not regulated by the classic transcription-translation mechanism but instead by alternative mechanisms. There are several potential reasons for such discrepancies. Firstly, both RNA and protein byproducts can degrade after transcription and translation, resulting in null or minimal readings at each step while still maintaining stable readings in the other omics layer. This can lead to contrasting results when comparing RNA and protein levels (Wang 2008). Identifying the degradation rate of individual RNA and protein would require time-course experiments beyond the scope of this thesis. Furthermore, as mentioned in the introduction, post-

transcriptional and post-translational regulation can occur. Proteins can undergo various modifications, such as phosphorylation, that can impact their abundance and activity. Therefore, contrasting RNA-protein results may indicate either omics degradation or post-transcriptional regulation has occurred (Wang 2008).

6.4.3 Transcription regulation

In addition to the classic transcription-translation mechanism, post-transcriptional regulation can significantly influence protein levels, even in the absence of RNA degradation. In addition to protein degradation, as discussed in the previous section, which affects a protein's half-life independently of transcription, PTMs could potentially influence protein levels independently of RNA (Karve and Cheema 2011). Moreover, as discussed previously, RNA may exhibit a buffering effect, which could potentially be linked to the RNA-Seq method (Perl, Ushakov et al. 2017). The transcript buffering effect is described as changes in mRNA abundance that are not manifesting at protein levels. More specifically, the expression of mRNA is 'buffered' or amplified when downstream mRNA synthesis or degradation is affected by inactive mRNA or the transcription complex (Timmers and Tora 2018). This leads to increased mRNA expression without a one-to-one increase in protein expression (Perl, Ushakov et al. 2017). While these mechanisms are potential explanations, the underlying mechanism of the phenomenon remains unclear.

miRNA can be an important regulator for protein expression without RNA degradation (Liu, Beyer et al. 2016). As discussed in Chapter 2.5.3, miRNAs (microRNAs) play a crucial role in post-transcriptional regulation. MiRNAs are small non-coding RNA that can bind to target RNA, leading to repression, destabilization, or degradation of RNA expression (Zhang 2009, Barrett, Fletcher et al. 2012). A single miRNA can potentially regulate a large number of mRNAs through post-transcriptional modifications, thereby reducing their protein expression levels. While RNA expression data can provide valuable insights into disease pathology, it is more likely to be correlative rather than causative. Ultimately, protein expression and its interactions with other molecules and pathways provide more informative perspectives and a more accurate representation of true regulation for understanding complex disease mechanisms (Greenbaum, Colangelo et al. 2003).

However, understanding regulation at the transcriptional level, even in the absence of corresponding protein-level changes, can still shed light on the transcriptional regulation involved in MFS. In the KEGG analysis, the PI3K/AKT pathway was shown to be significantly involved in transcriptional regulation. This study identified

a reduction in ITGA5 mRNA in MFS, contributing to the identification of the PI3K/AKT pathway in the pathogenesis of MFS. ITGA5, a member of the integrin alpha chain family, dimerises with an integrin beta chain to form a fibronectin receptor involved in cell surface adhesion and increased signalling, including the AKT pathway. Increased expression of ITGA5 is involved in a number of disease processes, including tumour invasion (Wang, Chen et al. 2022) and VSMC proliferation (Song, Qin et al. 2016). Impaired PI3K/AKT signalling is observed in TAA (Chung, Au Yeung et al. 2007), consistent with the observed reduction in ITGA5 transcription observed in this study.

The MAPK and TGFβ pathways were also identified, although they did not reach statistical significance in the enrichment analysis. Notably, the significant DE RNA TGFB2 was found to target both the TGFβ and MAPK pathways. Given that MAPK is a classic downstream pathway of TGFβ, this finding suggests a potential transcriptional regulation of MAPK by TGFβ stimulation (as detailed in Chapter 2.6.5) (Lindsay, Schepers et al. 2012, Dong, Malecki et al. 2023). However, it is worth noting that previous studies in MFS have observed TGFB2 haploinsufficiency, which leads to TGFB2 loss of function and increased TGFβ signalling (Boileau, Guo et al. 2012, Lindsay, Schepers et al. 2012). In contrast, our data show a more than 4.6-fold increase in TGFB2 expression in MFS patients compared to the controls. This contrasting finding in this study requires further investigation in the future.

GO analysis further highlights essential processes and their target genes. Processes related to haemostasis/coagulation and wound healing were targeted by TGFB2 and KRT1, indicating the involvement of the TGFβ pathway and keratin regulation from the transcriptional level in these biological processes. Additionally, KRT1 was found to be involved in collagen-containing ECM regulation, which is essential for maintaining skin integrity, suggesting a similar role for other keratins in maintaining cellular structure and resilience against mechanical stress (Ho, Thompson et al. 2022). Moreover, the emphasis on heparin binding and glycosaminoglycan binding relates to blood coagulation and ECM regulation, suggesting the potential involvement of the same coagulation cascade at the transcriptional level, albeit regulated differently compared to the protein data (Shi, Sheng et al. 2021).

6.4.4 Translation regulation

The significant differential expression of proteins, even without the presence of RNA expression, is crucial for pathway analysis to gain a deeper understanding of MFS aetiology. As mentioned earlier, instances where protein levels are altered without

corresponding RNA changes can be attributed to various factors, including RNA degradation, miRNA-mediated regulation leading to the degradation of target RNA, and alternative mechanisms such as phosphorylation (Vogel and Marcotte 2012). While RNA represents only half of the story, assessing protein abundance provides a more comprehensive understanding of MFS pathology. However, further investigation into post-transcriptional regulation is needed to fully comprehend the alterations in proteins that lack corresponding transcriptomic data.

The KEGG enrichment analysis identified two significant pathways, TGFβ and HIF-1, which corresponding to the previous the findings, emphasizing the involvement of TGFβ dysregulation and oxidative stress in MFS. Of particular interest is the upregulation of PRKCA protein, which is involved in multiple pathways and has the potential to be a key mediator in signal regulation. PRKCA is a protein kinase C (PKC) alpha isoform. It targets mTOR and PI3K/AKT signalling, which are regulated by ROS, phospholipids, and calcium levels. PRKCA plays an essential role in cardiac contractility and blood pressure regulation and is expressed in various cell types, including EC, SMC, and cardiac myocytes (Turner, Boerwinkle et al. 2013). PRKCA upregulation has been associated with the inhibition of VSMC apoptosis in diabetic mice vasculature (Hall, Matter et al. 2000). Moreover, inhibiting PRKCA has been shown to decrease ROS accumulation, suggesting that PRKCA may play a role in oxidative stress (Xu, Miao et al. 2022). Therefore, the upregulation of PRKCA in the context of HIF-1 and other pathways could indicate a response to oxidative stress within the aortic wall. It may act protectively in MFS by reducing VSMC apoptosis and controlling VSMC contractility. However, further studies are required to investigate the precise role of PRKCA in MFS pathology.

The GO analysis supports the previous findings regarding the involvement of haemostasis/coagulation processes in MFS (Chapter 5 and Section 6.4.3). However, these haemostasis/coagulation processes are targeted by different RNA and proteins when comparing data involving one-sided alterations in either RNA or protein. As discussed in Chapter 5.4.2, coagulation activity has been shown to be lower in MFS patients. Therefore, the increase in coagulation activity due to elevated expression of coagulation factors could be due to EC dysregulation, but the definite answer remains unclear, and further studies are needed to elucidate this phenomenon. Finally, terms related to ECM regulation were identified primarily by downregulated proteins, suggesting potential ECM degradation and dysregulation in ECM structural constituents, which supports the previous findings.

6.5 LIMITATIONS

6.5.1 Technical limitation

This study has certain limitations primarily related to data harmonization and integration, especially when comparing transcriptomics and proteomics data obtained and analysed using different methods (Manzoni, Kia et al. 2018). Firstly, RNA expression data were acquired from RNA-Seq, while protein expression data were obtained from mass spectrometry. Due to platform differences, technical limitations, and the presence of non-coding RNA, RNA-Seq provides over 10 thousand readings, whereas proteomics yields only a few thousand or a few hundred valid readings. This results in data imbalance and challenges in data integration, particularly because proteomics data have significantly fewer valid readings compared to RNA data. However, previous studies have also indicated a low concordance rate of changes between RNA and protein expression, suggesting a generally weak correlation between the two (Dumitriu, Golji et al. 2016).

Secondly, as discussed in Chapter 5.5, the proteomics data consist of integrated protein isomers due to the technical limitations of mass spectrometry (Claes, Takeo et al. 2019). Proteins with integrated names were excluded from data integration with RNA, as it was not possible to identify individual proteins. This further leads to a loss of data.

Furthermore, during the identifier matching process, gene IDs may not always correspond one-to-one, as genes may have multiple aliases or different names. This complicates the integration of transcription-translation expression data and KEGG/GO analysis because genes may appear under different names (Krassowski, Das et al. 2020).

6.6 CONCLUSION

In conclusion, the multi-omics analysis of transcriptomics and proteomics data in the context of MFS has revealed several key findings. Firstly, a low correlation was observed between RNA and protein expression, suggesting that the traditional transcription-translational mechanism may not be the primary regulatory process in MFS pathogenesis. Secondly, common biological processes were identified that were affected in both transcriptional and translational analyses, whether they exhibited concordant or non-concordant changes. This reinforced the understanding of the involvement of inflammatory responses, oxidative stress,

mechanical stress, ECM dysregulation, and the implications for mitochondrial function and muscle contractility, which were influenced by each regulatory mechanism. Thirdly, several instances of non-concordant changes were noted, particularly in protein expression, indicating the potential involvement of alternative post-transcriptional regulatory mechanisms that may play a more significant role in the development and progression of MFS-associated TAA. Lastly, from the concordant RNA-protein pairs, a more pronounced fluctuation in RNA expression compared to protein expression was observed, suggesting either a natural variation in signal intensity at different regulatory levels or potential technical limitations that requires further investigation.

Overall, the analysis identified that TGFβ, HIF-1, and PI3K/AKT may play central roles in MFS pathogenesis, with implications for the regulation of ECM, EC, VSMC, and responses to oxidative stress and inflammatory stimuli. The highlighting of intermediate filament and muscle contractility genes/proteins opens up new avenues for research in the study of MFS-associated TAA.

Chapter 7: Epigenetic regulation in Marfan Syndrome

7.1 INTRODUCTION

In this chapter, the focus shifts towards investigating the potential epigenetic regulatory mechanisms in MFS associated TAAD using microRNA and DNA methylation analyses. These epigenetic processes can shed light on the regulation observed in RNA and protein expression in MFS patients and provide valuable insights into disease mechanism. Notably, epigenetics encompasses additional biological processes beyond the expression of miRNA and DNA methylation, for example, histone modification and several other non-coding RNA (see Literature Review section 2.6.3-2.6.4). The analysis undertaken in this study was limited to microRNA and DNA methylation changes since these techniques were readily available to us.

To date, the evidence base for the roles of miRNA and DNA methylation in pathogenesis of TAA in MFS is limited. Understanding miRNA and DNA methylation could be a valuable contribution to comprehending MFS disease mechanisms. Moreover, the investigation of miRNA and DNA methylation could potentially partly explain the regulation observed in concordant or non-concordant RNA and protein expression in MFS patients described in the last chapter. MiRNA regulation involves binding complementary mRNA, which represses RNA expression (Chapter 2.6.3) (Zhang and Wang 2017). Previous studies have identified several biological processes implicated in miRNA regulation in MFS, such as the inflammatory response, ECM dysregulation and cell apoptosis (Detailed in Chapter 2.6.3).

DNA methylation modulates gene expression by hyper- or hypomethylation of CpG sites, which may lead to activation or inhibition of gene expression in a location-, time-, cell-, and/or tissue-specific manner (described in more detail in Chapter 2.6.4) (Ehrlich and Lacey 2013). As a generalisation, under most circumstances, hypermethylation within the gene promoter is likely to inhibit gene expression, whereas hypermethylation within the gene body is more likely to associate with such expression (Lanata, Chung et al. 2018). The methylation state of a gene is usually considered to be a relatively stable process; however, altered methylation has been

observed in MFS, indicating its involvement to at least some extent in disease progression, which may specifically be a consequence of by environmental factors or oxidative stress (Donkena, Young et al. 2010, Arai, Umeyama et al. 2020).

Most importantly, both miRNA and DNA methylation can be potential diagnostic markers and are therapeutically targetable in disease. For example, therapeutically targeting overexpressed miRNAs that inhibit tumour-suppressing genes in cancer, that can help restore their function and attenuate cancer progression (Hanna, Hossain et al. 2019, Menon, Abd-Aziz et al. 2022). Furthermore, therapeutic targets for DNA methylation regulatory mechanisms, such as DNMTs (detailed in Chapter 2.6.4), can either inhibit or activate the methylation process, which in turn can suppress or restore gene expression in inflammatory disease (Ciechomska, Roszkowski et al. 2019). Hence, the exploration of miRNA and DNA methylation in the pathogenesis of MFS may reveal prospective targetable markers, thereby fostering advancements in therapeutic development and interventions.

Many studies only investigate alterations within miRNA and DNA methylation expression or compare them to transcriptomics data to further validate the results. The limitation of comparing single omics or even two layers of omics data is insufficient for understanding the downstream translational expression that is altered by miRNA or methylation modulation. Especially in miRNA studies, the mRNA-miRNA association is often obtained from computational prediction, which then requires further experiments to validate and investigate downstream protein expression (Hammell 2010, Thomson, Bracken et al. 2011). Therefore, in this study, a comprehensive comparative analysis was performed for RNA-miRNA-protein and DNA methylation-RNA-protein expression to investigate the potential changes throughout the regulatory mechanism in MFS pathogenesis.

In this study, a genome-wide miRNA and DNA methylation analysis between MFS patients and controls was performed, as well as examine their targeted signalling pathways and biological processes. Furthermore, a comprehensive analysis of multi-omics was conducted to investigate any interactions between miRNA/DNA methylation and the transcription-translation mechanism in MFS.

Specifically, the aims of this study were to:

1. Compare miRNA expression between MFS patients and controls to identify significant DE miRNA.

2. Correlate the observed changes in miRNA expression with predicted cell signalling and biological processes.
3. Compare mRNA-miRNA-protein expression to identify likely post-transcriptional regulations in MFS.
4. Compare DNA methylation expression between MFS patients and control to identify significant DE CpG sites.
5. Correlate the observed changes in DNA methylation expression with predicted transcriptional/translational outcomes, and correlate these outcomes with cell signalling and biological processes.
6. Compare DNA methylation-mRNA-protein expression to identify likely pre-transcriptional regulation mechanisms in MFS.

7.2 METHODS

7.2.1 Sample extraction and profiling

MiRNA sample extraction and profiling were conducted locally, as described in Method Sections 3.2.1 and 3.2.5, respectively.

DNA extraction was performed locally and subsequently sent to the Australian Genome Research Facility for DNA methylation profiling (as detailed in Method Section 3.2.6). As detailed in Chapter 3.2.6, all DNA methylation experiment data had previously been generated by a post-doctoral fellow Dr. Cassandra Malecki from our laboratory, subsequently, the raw data were obtained and analysed by the author for the purposes of this study.

7.2.2 miRNA imputation and quality control

Imputation and quality control were carried out on the miRNA data, as detailed in Method Section 3.3.3, to remove miRNAs with insufficient readings. Additionally, imputed miRNAs with less than 25% undetermined readings were replaced with the mean value of the group.

7.2.3 DNA methylation data processing and quality control

The DNA methylation data processing and quality control were described in Method Section 3.3.4.

7.2.4 Statistical analysis

All statistical analyses and plots were generated using Microsoft Excel and RStudio. A raw p-value <0.05 was considered significant for miRNA analysis using the Student's t Test (Abu-Halima, Kahraman et al. 2018). ΔΔCT was used to calculate the fold change of miRNA between MFS patients and controls.

For DNA methylation analysis, significance was defined as a raw p-value less than 0.05 (Moderated t-Test) and an absolute Δβ >0.1(Martino, Joo et al. 2014).

No multiple testing correction was applied to the miRNA and DNA methylation analysis since no significant miRNAs or CpG sites were detected after correction.

For KEGG analysis, significance was defined as a p-value < 0.05.

7.3 RESULTS

7.3.1 Patient characteristics

The basic demographics of tissue donors are summarised in Table 7.1 and 7.2 for the miRNA and DNA methylation studies, respectively. The same fourteen MFS patients were utilized for all miRNA, transcriptomic, and proteomic studies, and the same controls were used across all of these studies.

However, as the DNA methylation experiment was conducted earlier than the other omics studies, only seven MFS patients were available. The same seven MFS patients were used in the other omics studies to pair with the DNA methylation study. It is worth noting that one control donor was different from the other studies and had missing clinical information.

Echocardiography data were not available for control donors. All MFS patients who experienced chronic dissection had a history of type B dissection, with no impact on the ascending aorta.

Table 7.1 Clinical parameter of two groups included in the miRNA study.

	Marfan	Control	p
n	14	11	
Age (years)	*34 ± 12*	*39 ± 15*	NS
Male:Female (n)	10 : 4	5 : 6	NS
BMI (kg/m^2)	*27.8 ± 6.6*	<30	
Aorta (mm)	*50 ± 8*		
LVEF (%)	61 ± 5		
Aneurysm (n)	11		
Chronic Dissection (n)	3		
Aortic Regurgitation (n)			
Nil	6		
Mild	6		
Moderate/Severe	2		

Note: aortic diameter was only available for 10 MFS donors.

BMI = body mass index; LVEF = left ventricular ejection fraction.

Table 7.2 Clinical parameter of two groups included in the DNA methylation study.

	Marfan	Control	p
n	7	7	
Age (years)	*33 ± 13*	*39 ± 15*	NS
Male:Female (n)	5 : 2	4 : 2	NS
BMI (kg/m^2)	*28.5± 7.8*	<30	
Aorta (mm)	*46 ± 6*		
LVEF (%)	61 ± 5		
Aneurysm (n)	4		
Chronic Dissection (n)	3		
Aortic Regurgitation (n)			
Nil	4		
Mild	2		

Moderate/Severe	1		

Note: aortic diameter was only available for 5 MFS donors. One control donor does not have clinical information.
BMI = body mass index; LVEF = left ventricular ejection fraction.

7.3.2 Differentially expressed miRNAs in MFS

A total of 468 miRNAs had detectable readings from the Taqman OpenArray panels while 166 miRNAs remained after quality control (as detailed in Method Section 3.3.3). After DE analysis, 16 miRNAs were identified as significantly different between controls and MFS, with 8 miRNAs being upregulated and 8 downregulated. The significant miRNAs are labelled on the volcano plot (Figure 7.1 A). Notably, some of the most significant miRNAs with high ΔΔCT values included miR-376c-3p ($p = 0.006$, $\Delta\Delta CT = 1.4$), miR -451a ($p = 0.01$, $\Delta\Delta CT = -2.5$), miR-486-5p ($p = 0.02$, $\Delta\Delta CT = 1.7$), and miR-31-5p ($p = 0.03$, $\Delta\Delta CT = -1.6$).

A heatmap was generated to visualize the significant DE miRNAs between MFS and controls (Figure 7.1 B). While most MFS patients and controls were clustered together, the expression across the group was not very consistent. This inconsistency is further illustrated by the PCA plot (Figure 7.1 C), where some separation between the two groups is observed, but was not complete. The overlap between the two groups indicates that significant miRNAs could not effectively completely differentiate between the two groups.

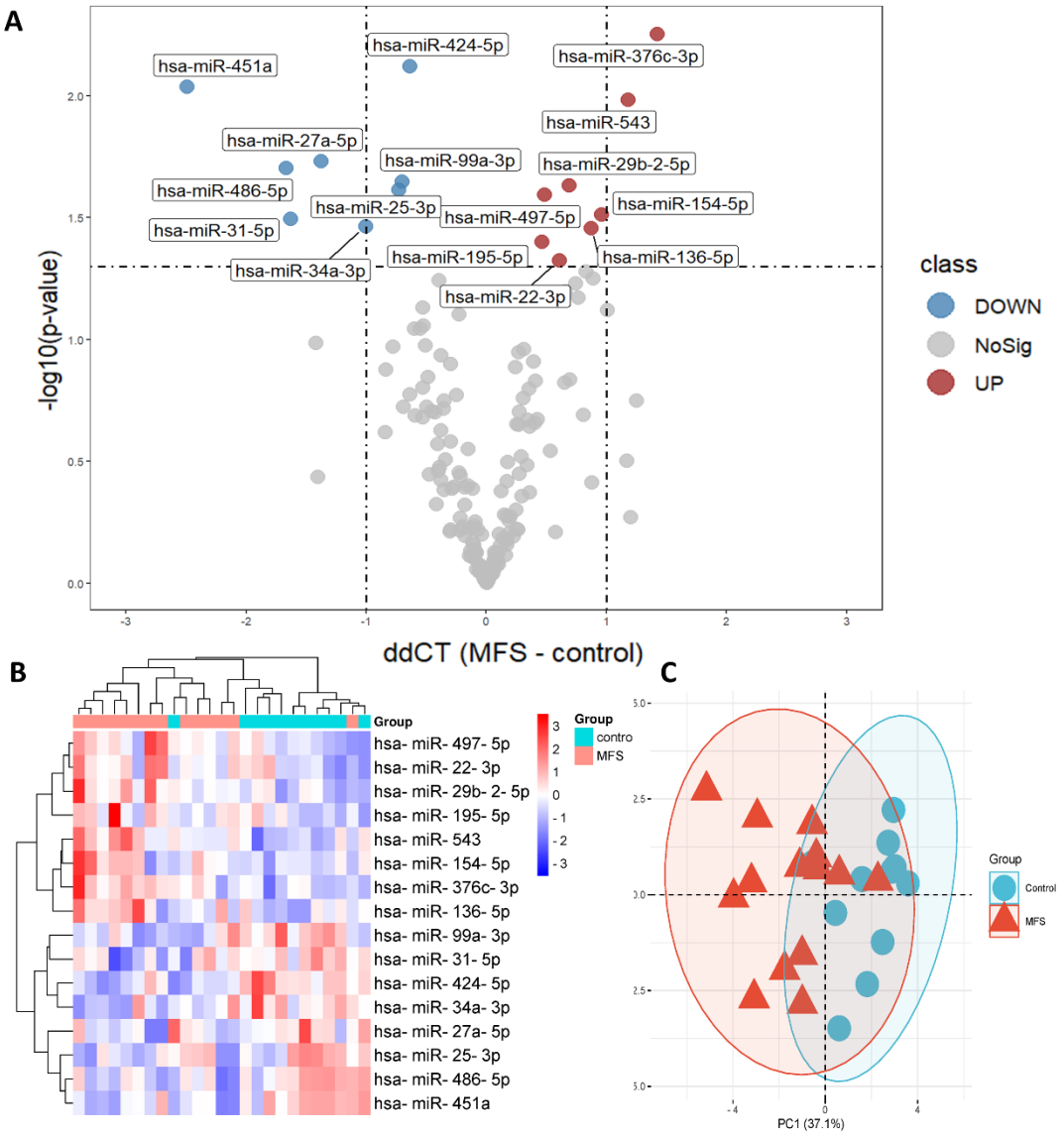


Figure 7.1 Visualization of significant miRNAs between MFS patients and controls. A) Volcano plot for all miRNA, red dots indicate upregulated significant miRNAs and blue dots indicate downregulated significant miRNAs. B) Heatmap for significant miRNAs (n = 16). C) PCA plot for significant miRNAs (n = 16).

The study then compared the findings to the previous literature on CVD involving significant miRNAs. The table reveals that all of the significant miRNAs have been implicated in various CVD (Table 7.3). Notably, three miRNAs (miR-451a, miR-29b-2-5p, and miR-25-3p) were previously identified in MFS pathogenesis, two miRNAs in TAA (miR-27a-5p and miR-485-5p), two miRNAs (miR-31-5p and miR-22-3p) were identified in aortic dissection, and three miRNAs (miR-27a-5p, miR-25-3p, and miR-497-5p) have been linked to the TGFβ pathway. These findings provide some validation for the significance of miRNAs detected in our results and demonstrate

their involvement in CVD, particularly in TGFβ dysregulation and MFS/TAAD pathology.

Table 7.3 Significant miRNAs and their previous literatures on CVD.

miRNA	p-value	ΔΔCT	miR family	Implication in CVD
hsa-miR-376c-3p	0.006	1.42	mir-368	CAD (Du 2020, Zhang, Qian et al. 2022)
hsa-miR-424-5p	0.008	-0.64	mir-322	Heart failure (Marques, Vizi et al. 2016)
hsa-miR-451a	0.009	-2.50	mir-451	MFS (Abu-Halima, Kahraman et al. 2018) Heart failure(Marques, Vizi et al. 2016) CAAD (Iwańczyk, Lehmann et al. 2023)
hsa-miR-543	0.010	1.18	mir-329	Hypertension (Wang, Zhang et al. 2022)
hsa-miR-27a-5p	0.019	-1.38	mir-27	TAA (Magouliotis, Fergadi et al. 2022) Anti-cardiac fibrosis/ TGFβ signalling (Teng, Huang et al. 2021)
hsa-miR-486-5p	0.020	-1.67	mir-486	TAA (Patuzzo, Pasquali et al. 2012) Atherosclerosis (Berkan, Arslan et al. 2019) CAD (Niculescu, Simionescu et al. 2015)
hsa-miR-99a-3p	0.022	-0.71	mir-10	PAH (Rothman, Restrepo et al. 2017)
hsa-miR-29b-2-5p	0.023	0.69	mir-29	MFS (Merk, Chin et al. 2012) PAH (Jin, Xu et al. 2021, Li, Liang et al. 2023) AF (Hu, Wei et al. 2019)
hsa-miR-25-3p	0.024	-0.73	mir-25	MFS (D'Amico, Doldo et al. 2020) CAD (Ji, Yan et al. 2021) HCM (Lombardi, Lazzeroni et al. 2021) TGFβ signalling/valve calcification (Yu, Wu et al. 2021)
hsa-miR-497-5p	0.025	0.48	mir-497	TGFβ signalling (Jafarzadeh, Mohammad Soltani et al. 2018) HCM/ TGFβ signalling (Osmak, Baulina et al. 2021)
hsa-miR-154-5p	0.031	0.96	mir-154	Heart remodelling (Wang, Yu et al. 2019)
hsa-miR-31-5p	0.032	-1.63	mir-31	AD (Zhang, Bian et al. 2021)
hsa-miR-34a-3p	0.034	-1.01	mir-34	AS (Shi, Liu et al. 2016) PAH (Chen, Dasgupta et al. 2018)
hsa-miR-136-5p	0.035	0.87	mir-136	CAD (Zhang, Qian et al. 2022)
hsa-miR-195-5p	0.040	0.46	mir-15	VSMC dysregulation (Xu, Dai et al. 2021) Heart failure (Marques, Vizi et al. 2016)
hsa-miR-22-3p	0.047	0.61	mir-22	AD (Xiao, Sun et al. 2020) Hypertension (Marketou, Kontaraki et al. 2019) Heart failure (Lu, Liu et al. 2023)

Note: CAD – coronary artery disease. CAAD – coronary artery aneurysmal disease. HCM – hypertrophic cardiomyopathy. PAH – pulmonary arterial hypertension. AF – atrial fibrillation. AS – aortic stenosis. AD – aortic dissection.

7.3.2.1 Gene target prediction

By inputting the 16 significant miRNAs into miRDB (detailed in Method Section 3.3.3), 581 genes were predicted that could be targeted by the 8 upregulated significant DE miRNAs. These genes were subsequently used for KEGG and GO analyses. Additionally, 576 genes were predicted to be targeted by the 8 downregulated significant miRNAs. Notably, miR-29b-2-5p did not have a predicted targeted gene, which could be attributed to the applied thresholds in miRDB.

7.3.3 Signal transduction and biological processes in MFS miRNA regulation

Upregulated miRNA target genes were found to be involved in 34 significant KEGG pathways, out of which 13 were related to signal transduction pathways, as illustrated in Figure 7.2A. However, due to the large number of input genes, only the five most significant pathways targeted by upregulated miRNA target genes were presented in the Sankey plot (Figure 7.3). These pathways included Hippo ($p = 0.0001$), MAPK ($p = 0.0005$), VEGF ($p = 0.002$), Wnt ($p = 0.003$), and mTOR ($p = 0.004$) pathways.

On the other hand, downregulated miRNA target genes were associated with 103 significant pathways, with 20 of them related to signal transduction pathways, as listed in Figure 7.2B. The five most significant signal transduction pathways targeted by downregulated miRNA target genes were presented in Figure 7.4. These pathways included FoxO ($p = 4.5E-06$), PI3K/AKT ($p = 1.3E-05$), mTOR ($p = 4.1E-05$), sphingolipid ($p = 0.0001$), and AMPK ($p = 0.0005$) pathways.

Overall, despite some overlap between the two KEGG analyses, there were unique pathways identified. Wnt signalling was the only unique pathway identified by upregulated miRNA targets, while FoxO, sphingolipid, phosphatidylinositol, Rap1, TNF, calcium, JAK-STAT, and TGFβ were exclusively identified from downregulated miRNA targets.

In summary, the investigation of unique pathways associated with up- or downregulated miRNA target genes revealed that Wnt signalling plays a role in inflammatory response and ECM maintenance, as detailed in Chapter 2.5.2. Additionally, pathways related to inflammation (TNF, JAK-STAT, sphingolipid),

mechanical stress (phosphatidylinositol), ECM, EC and VSMC responses (Rap1, calcium, FoxO, sphingolipid), and TGFβ dysregulation were implicated by downregulated miRNA targeting genes. Furthermore, the identification of the AMPK pathway suggests a potential regulatory mechanism of miRNA in mitochondrial function modulation.

Comparing these results to the RNA KEGG analyses, pathways such as PI3K/AKT and cGMP-PKG were previously identified by downregulated RNA and were also targets of upregulated miRNAs. This corresponds to post-transcriptional mechanisms affecting cellular processes and mitochondrial function. Additionally, gene targets of downregulated miRNAs identified inflammatory pathways (TNF and JAK-STAT) that were previously identified in upregulated RNA analyses.

These findings suggest that miRNAs may play a role in inflammatory and cellular responses, mitochondrial dysfunction, as well as TGFβ dysregulation, supporting the previous results regarding the biological processes involved in MFS pathogenesis.

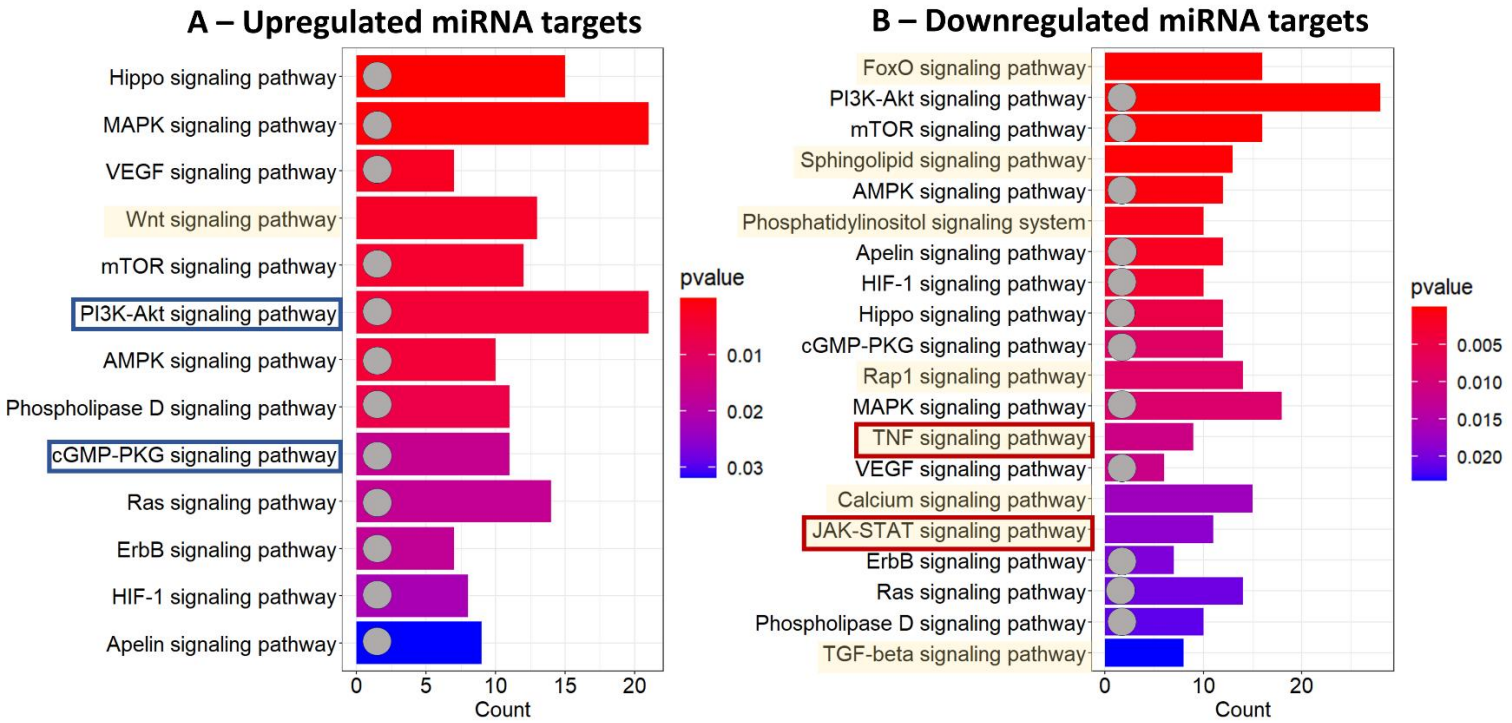


Figure 7.2 KEGG analysis for significant up- and downregulated miRNA predicted targets. Pathways with grey dots indicate such pathways that show overlap between up- and downregulated miRNA KEGG enrichment, yellow highlighted pathways indicate that such pathways were identified only in up/downregulated miRNA KEGG analysis. The **blue frame** over the pathway indicates that such pathways were previously identified from the downregulated RNA KEGG analysis and the **red frame** indicates such pathways that were previously identified from the upregulated RNA KEGG analysis.

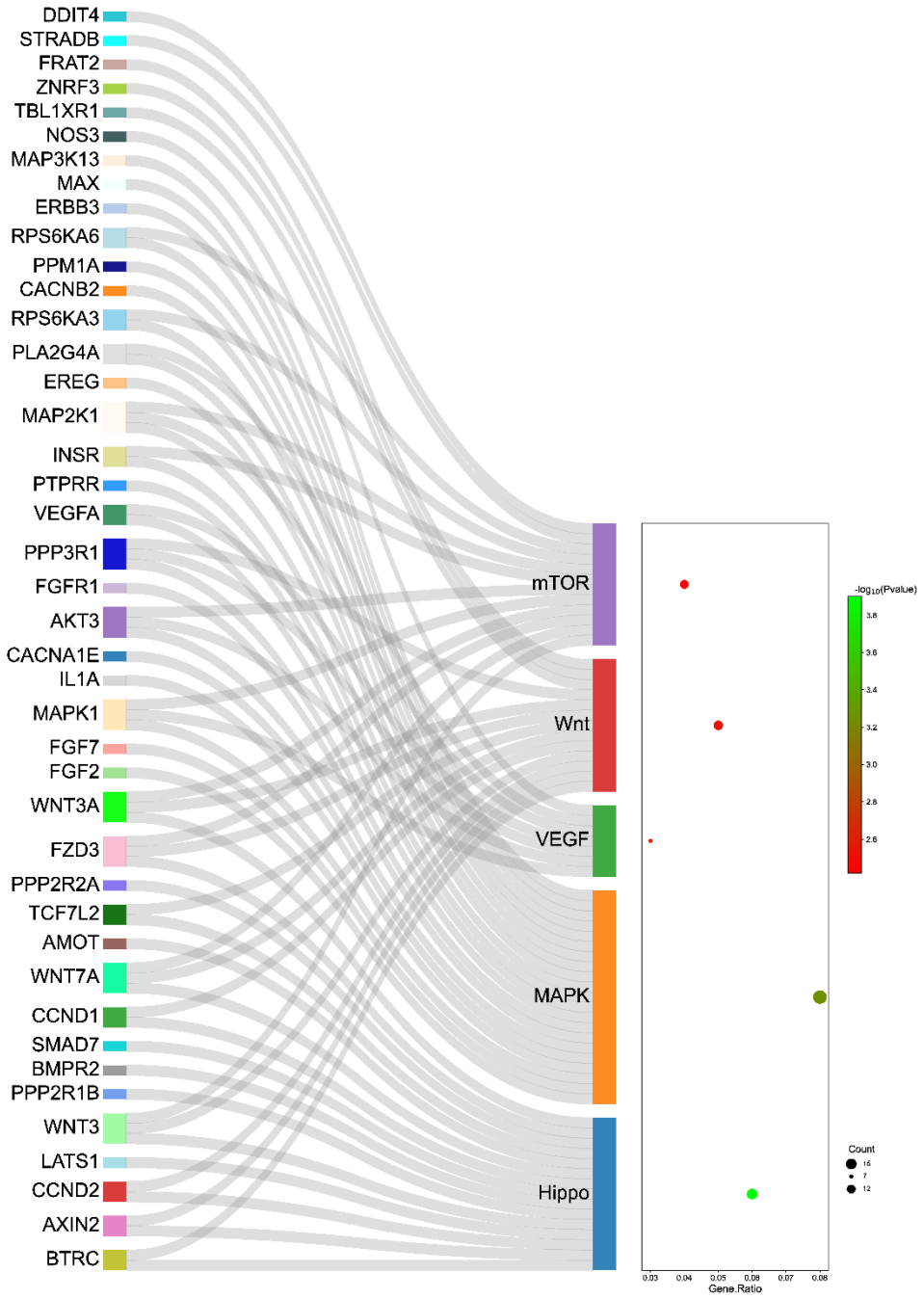


Figure 7.3 Sankey plot of significant signal transduction pathways for RNA targets of upregulated miRNA.

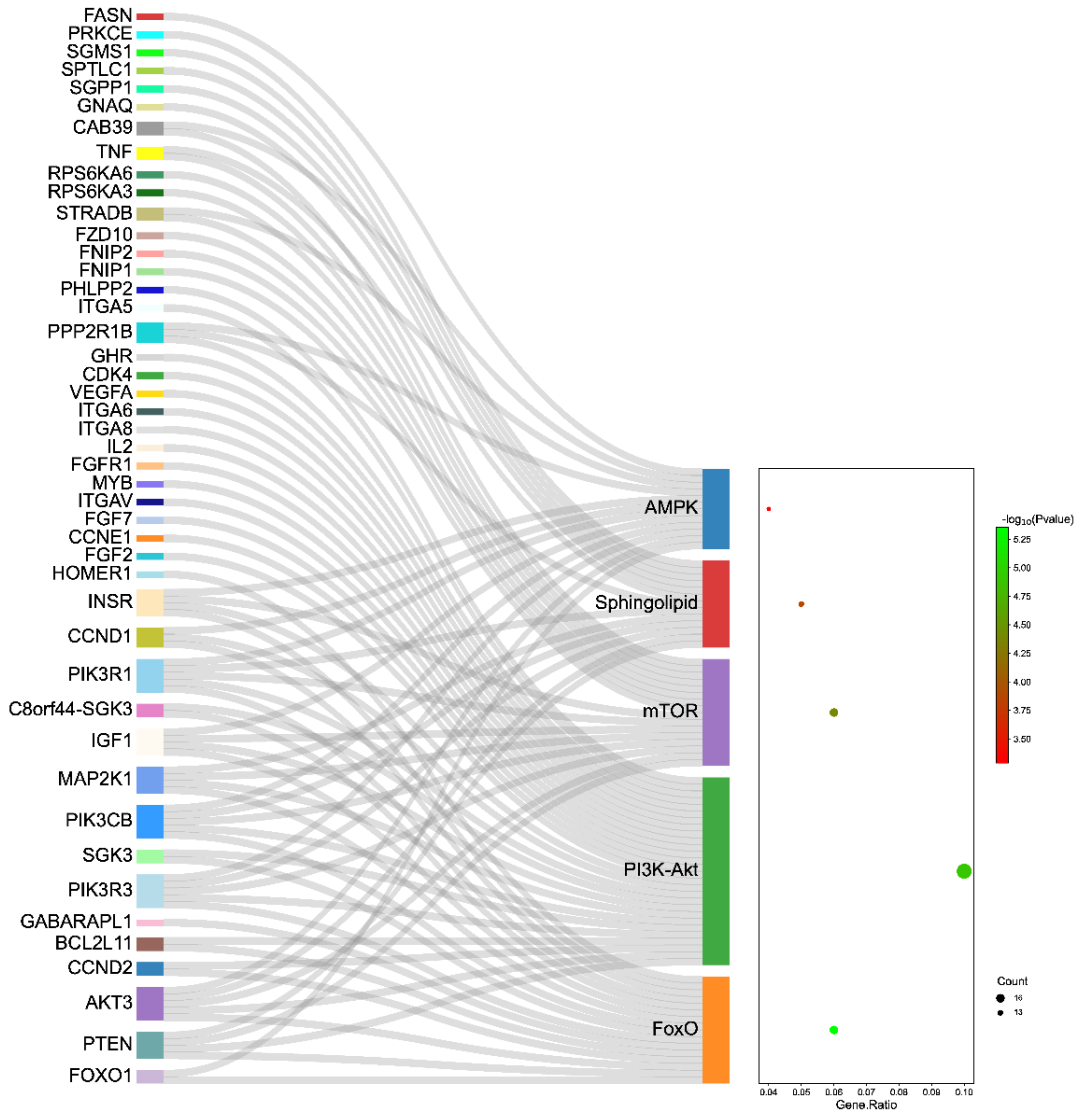


Figure 7.4 Sankey plot of significant signal transduction pathways for RNA targets of downregulated miRNA.

A GO ontology plot was generated for investigating the biological processes of targets of significant miRNAs, displaying the top 5 enrichments for each ontology in Figure 7.5. The GO analysis identified that upregulated miRNA target gene proteins were involved in kinase activity and β-catenin (a key canonical Wnt gene) binding. On the other hand, downregulated miRNA targets mainly targeted cell growth and focal adhesion, suggesting involvement in proliferation and growth pathways, as well as SMAD-binding function, indicating altered TGFβ pathway activities.

These findings align with the KEGG analysis, highlighting the regulatory mechanisms of Wnt, TGFβ dysregulation, abnormal cellular response, and ECM modulation in MFS pathogenesis. It is important to note that the GO enrichment also revealed several neurology-related processes. This could be attributed to

potential biases within the GO database, as neurology research is more advanced and better documented with the KEGG database compared to cardiovascular studies.

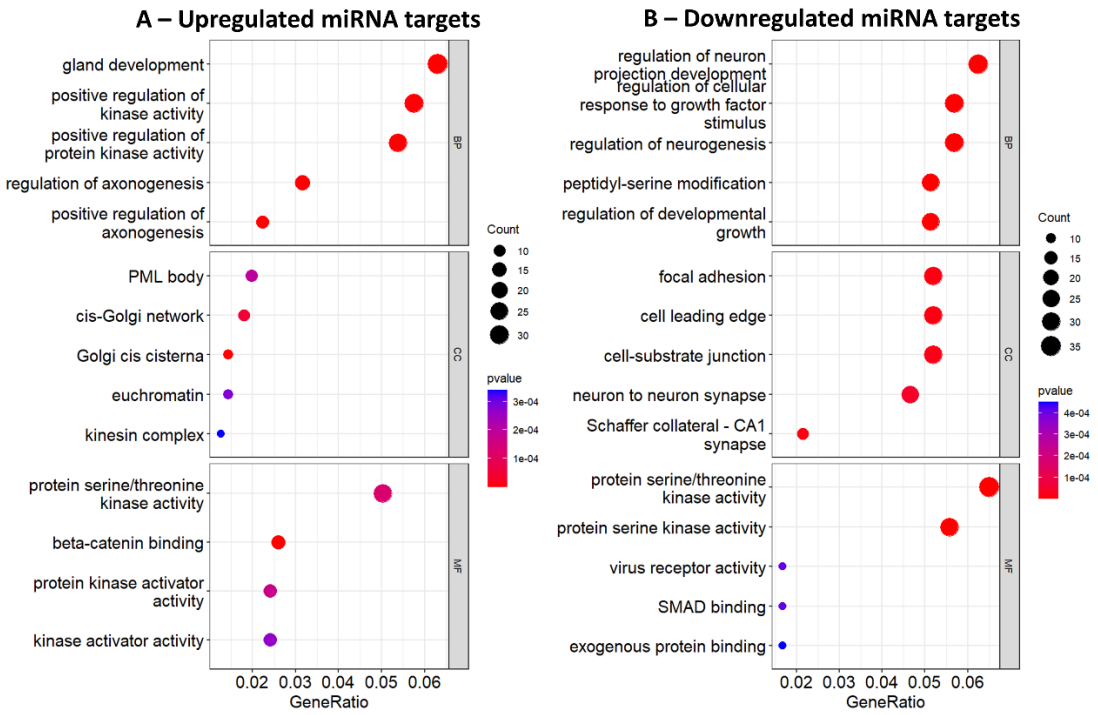


Figure 7.5 GO plot for targets of significant up- and downregulated miRNA predicated targets. BP – biological process. CC – cellular component. MF – molecular function.

7.3.4 miRNA-RNA-protein regulation

Eight miRNA-protein pairs from miRDB, along with their expression levels and corresponding RNA expression were identified. Additionally, only 2 out of the 8 miRNA-protein pairs demonstrated corresponding expression changes, where the miRNA upregulation led to protein downregulation or vice versa (as shown in Table 7.4). This intriguing result also suggests that all the targeted miRNA-protein pairs did not change significantly at the RNA level, indicating probable successful miRNA translational inhibition rather than degradation of the target mRNA. This correspondence between miRNA and protein expression highlights the potential post-transcriptional regulatory mechanisms at play in MFS.

Furthermore, FBN1 was predicted to be targeted by miR-25-3p. Although the miRNA was downregulated and the protein expression of FBN1 was also lower in MFS, this could imply a countereffect of the miRNA in attempting to “rescuing” FBN1 levels through a post-transcriptional mechanism. However, the downregulated protein level of FBN1 indicates that the initial attempt to upregulate it by miR-25-3p may have been unsuccessful, possibly due to the underlying FBN1 mutation causing lower initial FBN1 protein expression.

Moreover, the results revealed that the same RNA/protein could be targeted by multiple miRNAs, and *vice versa*, suggesting a more intricate regulatory system within the miRNAs. This system involves interactions, such as miRNA synergy or antagonism, between miRNAs, and may also incorporate a failproof mechanism for miRNA post-transcriptional regulation (as shown in Table 7.4).

In summary, the corresponding regulation between miRNA and protein expression supports the previous study findings and illustrates the potential role of post-transcriptional regulation in processes such as inflammatory response, VSMC phenotypic switching, and ECM regulation in MFS.

Table 7.4 RNA-miRNA-protein multi-omics comparison.

RNA	Change	miRNA	Change	protein	Change	Gene function
MINDY2	NS	miR-154-5p	Up	MINDY Lysine 48 Deubiquitinase 2	Down	Pro-inflammatory response (Liu, Liu et al. 2023)
KIF5C	NS	miR-195-5p	Up	Kinesin Family Member 5C	Up	VSMC phenotypic switch (Lee, Garvey et al. 2011)
FBN1	NS	miR-25-3p	Down	Fibrillin 1	Down	MFS mutation, ECM function and organization, regulates TGFβ signalling (Chapter 2.4.3)
ADAMTSL1	NS			ADAMTS Like 1		Modulated ADAMTS function - ECM regulation (implicated in fibrosis & heart failure) (Kelwick, Desanlis et al. 2015, Rypdal, Erusappan et al. 2021)
ADAMTSL3	NS			ADAMTS Like 3		Modulated ADAMTS function - ECM regulation (implicated in fibrosis & heart failure) (Kelwick, Desanlis et al. 2015, Rypdal, Erusappan et al. 2021)
PCOLCE2	NS			Procollagen C-Endopeptidase Enhancer 2		Collagen regulation, enhance BMP signalling (Baicu, Zhang et al. 2012)
KIF5C	NS	miR-424-5p	Down	Kinesin Family Member 5C	Up	VSMC phenotypic switch (Lee, Garvey et al. 2011)

RNA	Change	miRNA	Change	protein	Change	Gene function
KIF5C	NS	miR-497-5p	Up	Kinesin Family Member 5C	Up	VSMC phenotypic switch (Lee, Garvey et al. 2011)

Note: Red proteins indicate that these miRNA-protein pairs were changed concordantly by post-transcriptional regulatory mechanisms.

7.3.5 Differentially expressed DNA methylation in MFS

Following the quality control process, as described in Chapter 7.2.3, a total of 757,315 CpG sites were identified and included in the subsequent analysis. Out of these, 2,367 CpG sites were found to be significantly differentially expressed in MFS compared to the control group. Among the significant sites, 1,085 CpG sites were downregulated, while 1,282 sites were upregulated. To visualize these significant DE CpG sites, a volcano plot was generated (Figure 7.6 A).

To further explore the differential methylation patterns, a heatmap (Figure 7.6 B) was created to display all the significant DE DNA methylation CpG sites. The MFS and Control groups were well-clustered together, with no overlap between groups, indicating a high degree of similarity in methylation expression across the two groups. Nonetheless, a small proportion of the sites exhibited contrasting expression within each group, seen amongst the MFS patients and controls adjacent to the interface between the two groups. This observation is consistent with the findings from the PCA plots (Figure 7.6 C), which showed a very small overlap. These data show that the significant DE methylation CpG sites could effectively separate the two groups.

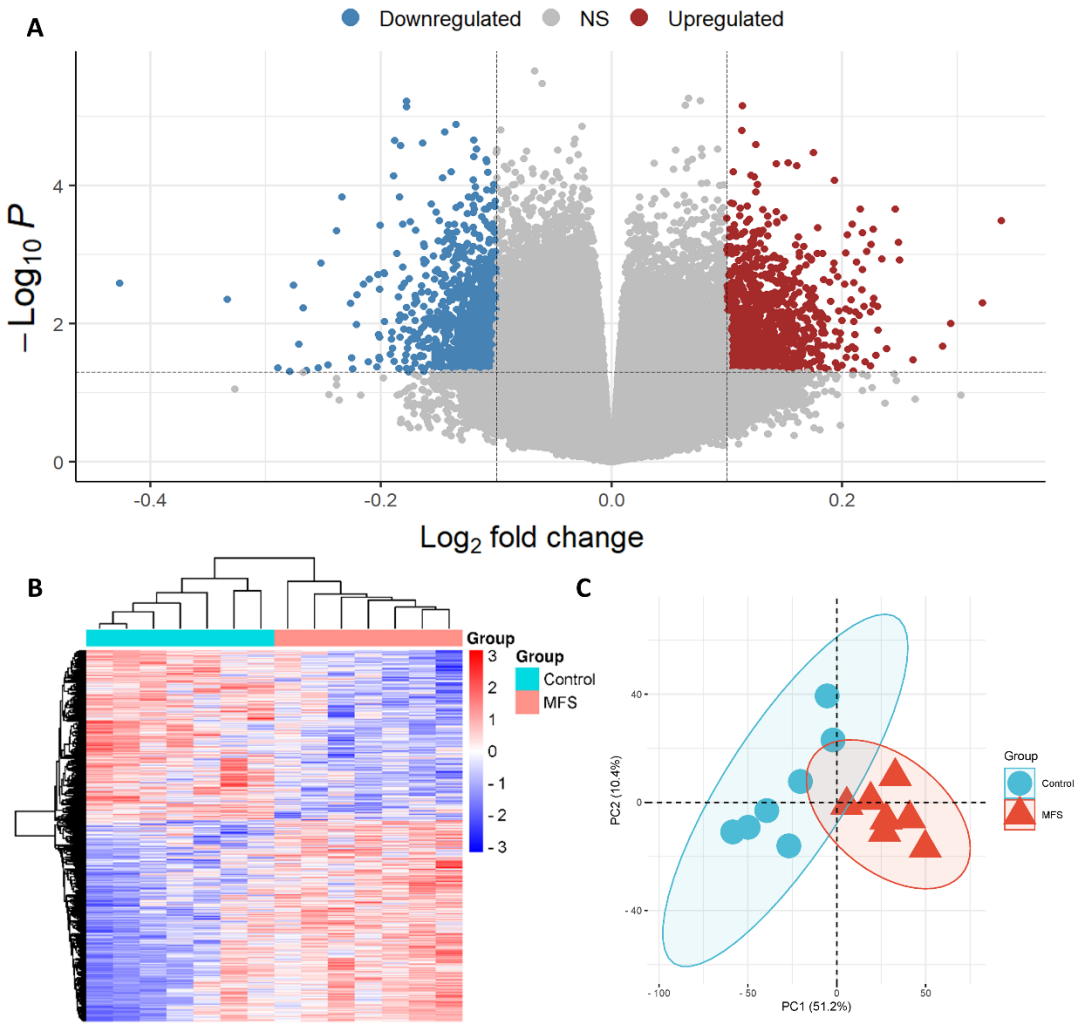


Figure 7.6 Overview of DE significant DNA methylation CpG sites. A) Volcano plot of all DNA methylation CpG sites, red dots indicate upregulated CpG sites (hypermethylation), and blue dots indicate downregulated CpG sites (hypomethylation). B) Heatmap of significant DE DNA methylation CpG sites. C) PCA plot of significant DE DNA methylation CpG sites.

In Table 7.5, the top 20 most significant DE CpG sites along with their corresponding genes and proteins were listed. Among these CpG sites, it is noteworthy that the top 20 significant sites primarily target the ECM and EC activities. This includes functions related to ECM organization, cell-cell interactions, and cell-matrix contact functions. These findings suggest that DNA methylation may play a role in regulating these essential cellular functions.

Additionally, inflammation emerges as another important pathological process within the top 20 significant DE CpG sites, indicating a potential role of DNA methylation in promoting inflammatory responses in the context of MFS pathogenesis.

Table 7.5 also details potential multi-omics correlations, showing the corresponding changes that have occurred in mRNA and protein expression. Broadly speaking, for most of the 20 top DE CpG sites, there was a poor correlation between DE DNA methylation and corresponding transcription and translation. This observation highlights the complexity and multiplicity of regulation of gene expression prior to the final protein product. Interpretation of these DE DNA methylation data is complicated by the uncertainty associated with the effect of the specific CpG site being methylated, for example, making the assumption that methylation within the promoter region will result in reduced transcription, while methylation within the body of a gene is associated with increased transcription. Interestingly, only 4 of the 20 top DE DNA methylation sites were located within promoter regions, while 14 of 20 sites were located within the body of the associated gene. 2 sites were located in the 3'UTR (3' untranslated region).

Specifically, 2 significant DE RNA that are targeted by CpG sites (INHBA and CPA3; Table 7.5) were identified. These RNA are expressed in various cell types, including fibroblasts, EC, adipocytes, and immune cells. They are involved in regulating ECM function, cellular development, and activation.

While none of the DE CpG sites corresponded to significant DE protein, the study has observed 6 DNA methylation-targeted proteins (TNXB, CRTAC1, POLR2A, SMOC1, TPM1 and CPA3) in the top 20 CpG sites table are expressed in fibroblasts, SMC, and immune cells. This suggests their potential involvement in ECM organization, inflammation, and muscle contraction.

In summary, the DNA methylation data support the previous findings related to inflammatory responses, ECM/EC dysregulation, and muscle contractility. It also provides additional insights into the potential regulatory mechanisms underlying these biological processes in the context of MFS pathogenesis.

Table 7.5 Absolute top 20 CpG sites and their corresponding genes correlated to RNA and protein expression.

Cpg Sites	logFC	pvalue	gene	feature	RNA logFC	RNA pvalue	protein logFC	protein pvalue	Cell	Gene function
cg02161417	-0.18	6.0E-06	INHBA	TSS1500	1.24	2.3E-06	NA	NA	Fibroblasts	ECM organization, regulating inhibins and activins, regulates TGFβ signalling
cg02857726	-0.13	1.3E-05	TM4SF1	Body	0.45	0.02	NA	NA	EC	Cell development, activation, and motility
cg01770799	-0.14	1.7E-05	IRF8	Body	0.08	0.80	NA	NA	Macrophages monocytes B-cells	Inflammation
cg21642103	-0.12	3.0E-05	TNXB	Body	-0.07	0.79	0.19	0.32	Fibroblasts	ECM organization, ECM-cell interaction, inhibit cell migration and promotes collagen fibril formation, wound healing
cg03048432	0.18	3.3E-05	NIN	5'UTR	0.17	3.7E-03	NA	NA	Monocytes Basophil eosinophils	Microtubule regulation
cg03201620	-0.12	3.8E-05	CRTAC1	Body	-0.63	0.09	-0.27	0.44	Other	Cell-cell and cell-matrix interaction
cg13583411	-0.11	4.2E-05	STON2	Body	0.29	0.43	NA	NA	Monocytes	Regulates endocytotic complexes
cg21011913	-0.11	4.5E-05	POLR2A	Body	-0.08	0.20	0.02	0.85	Monocytes Neutrophils	Synthesis mRNA
cg13725590	0.15	4.7E-05	DUSP2	3'UTR	0.25	0.50	NA	NA	NK-cells Monocytes T-cells	Regulates MAPK signalling
cg22328298	-0.14	6.3E-05	SMOC1	Body	-0.29	0.42	-0.14	0.73	Other	Osteoblast differentiation, eye and limb development
cg06040093	0.12	7.1E-05	MCF2L	Body	-0.72	2.2E-03	NA	NA	Adipocytes. EC	Regulates Rho/Rac signalling pathways
cg04256180	-0.19	7.3E-05	MCC	Body	0.08	0.57	NA	NA	Other	Regulates Wnt pathway, inhibits cell proliferation

cg16818286	-0.12	8.2E-05	TP63	Body	-0.19	0.51	NA	NA	B-cells	Regulates epithelial morphogenesis
cg02539846	0.19	8.4E-05	FGF7	Body	-0.40	0.13	NA	NA	Fibroblasts SMC Cardiomyocytes	ECM organization, regulates Ras signalling
cg13226072	0.13	9.5E-05	MECOM	Body	-0.06	0.71	NA	NA	Adipocytes, dendritic cells	Regulates JNK and TGFβ signalling, and haematopoiesis, apoptosis, development, cell differentiation and proliferation
cg21095660	-0.10	9.6E-05	MTSS1	3'UTR	-0.06	0.82	NA	NA	Monocytes	Response to fluid shear stress, negatively regulate EC proliferation, regulates actin filament polymerization, maintain adherens junction
cg11936410	-0.12	1.0E-04	TPM1	Body	0.23	0.06	-0.19	0.37	Cardiomyocytes SMC Fibroblasts Basophils	Regulates calcium dependent muscle contraction
cg13055288	-0.11	1E-04	SLC7A2	TSS200	0.04	0.87	NA	NA	SMC	Activates macrophages
cg13424229	-0.12	1E-04	CPA3	TSS1500	1.13	7.0E-06	-0.28	0.72	Mast cells Macrophages Fibroblasts Basophils	Degrades endogenous proteins by mast cell released protease
cg17109552	-0.23	1E-04	STON2	Body	0.29	0.43	NA	NA	Monocytes	Regulates endocytotic complexes

Note: Only CpG sites coding within a gene location are included, expressed cell type and gene function were obtained from The Human Protein Atlas. RNA p-value and fold change in red indicates such RNA was found significantly differentially expressed ($p<0.05$ and fold change >2) in our study. Other cell type indicates there was not enough evidence for heart- or immune-related cell documented in The Human Protein Atlas.

NA indicates there is no identified RNA/protein found in the transcriptomics or proteomics dataset.

7.3.6 Signal transduction and biological function analysis in DNA methylation

A KEGG enrichment analysis using the up and down-regulated genes corresponding to the differentially methylated CpG sites was conducted. This analysis revealed 38 significant pathways associated with upregulated genes (hypermethylated CpG sites), with 4 of them being significant signal transduction pathways (as shown in Figure 7.7 A). These significant pathways include phospholipase D ($p = 0.001$), cGMP-PKG ($p = 0.003$), phosphatidylinositol ($p = 0.02$), and PI3K/AKT ($p = 0.05$). However, due to the complexity of methylation regulation, it is challenging to pinpoint the specific regulatory role of hyper/hypomethylated CpG sites in these pathways. Nevertheless, these data suggest the potential involvement of significant signal transduction pathways that may be regulated by DNA methylation in MFS pathogenesis.

Among the hypermethylated CpG sites, we observed the involvement of processes related to oxidative stress (phospholipase D, cGMP-PKG), mechanical stress (phosphatidylinositol), and cell survival/proliferation (PI3K/AKT). This suggests that these processes could potentially be at least partially regulated by DNA methylation.

On the other hand, the analysis of hypomethylated CpG sites corresponding to genes revealed 42 significant pathways, with 11 of them being significant signal transduction pathways (as shown in Figure 7.7 B). These pathways include mTOR ($p = 7.4E-05$), PI3K/AKT ($p = 0.0006$), Hippo ($p = 0.004$), Rap1 ($p = 0.004$), TGFβ ($p = 0.01$), HIF-1 ($p = 0.01$), Wnt ($p = 0.02$), phospholipase D ($p = 0.02$), sphingolipid ($p = 0.02$), ErbB ($p = 0.05$), and VEGF ($p = 0.05$). These signal transduction pathways suggest that epigenetic regulation may be involved in processes such as cell survival, proliferation, apoptosis, inflammatory response, oxidative stress, and mechanical stress.

From the KEGG Sankey plots (Figures 7.8 & 7.9) and the barplot (Figure 7.7), it is evident that the PI3K/AKT pathway harbors the most genes corresponding to significant differentially methylated CpG sites. This highlights the importance of the PI3K/AKT pathway in MFS pathology and suggests that it might serve as a common intermediate downstream pathway among the majority of fundamental signal transduction pathways implicated in the disease.

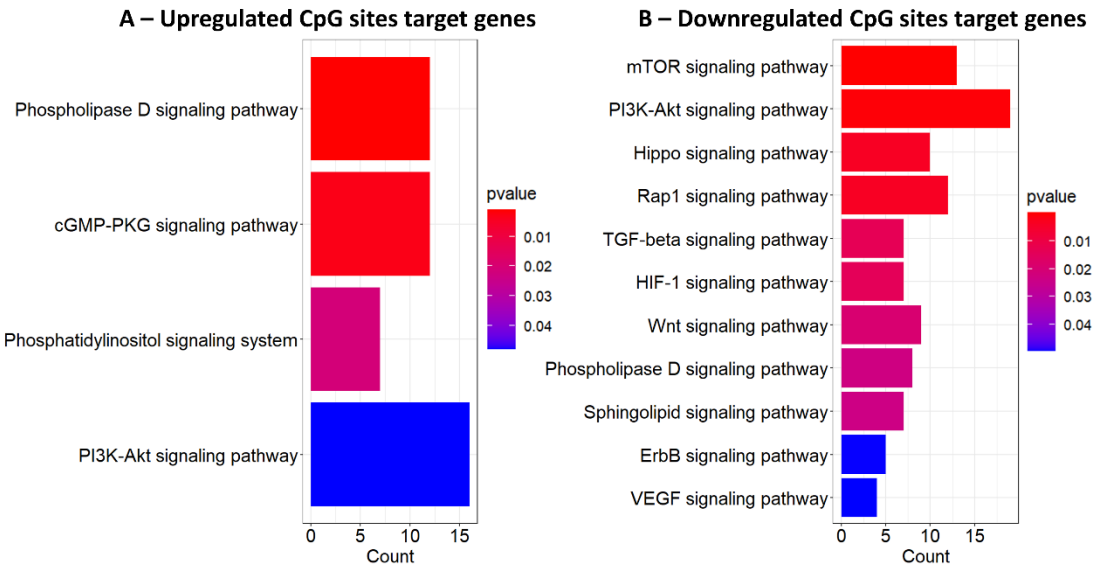


Figure 7.7 KEGG analysis for significant hyper/hypomethylated CpG site corresponding genes.

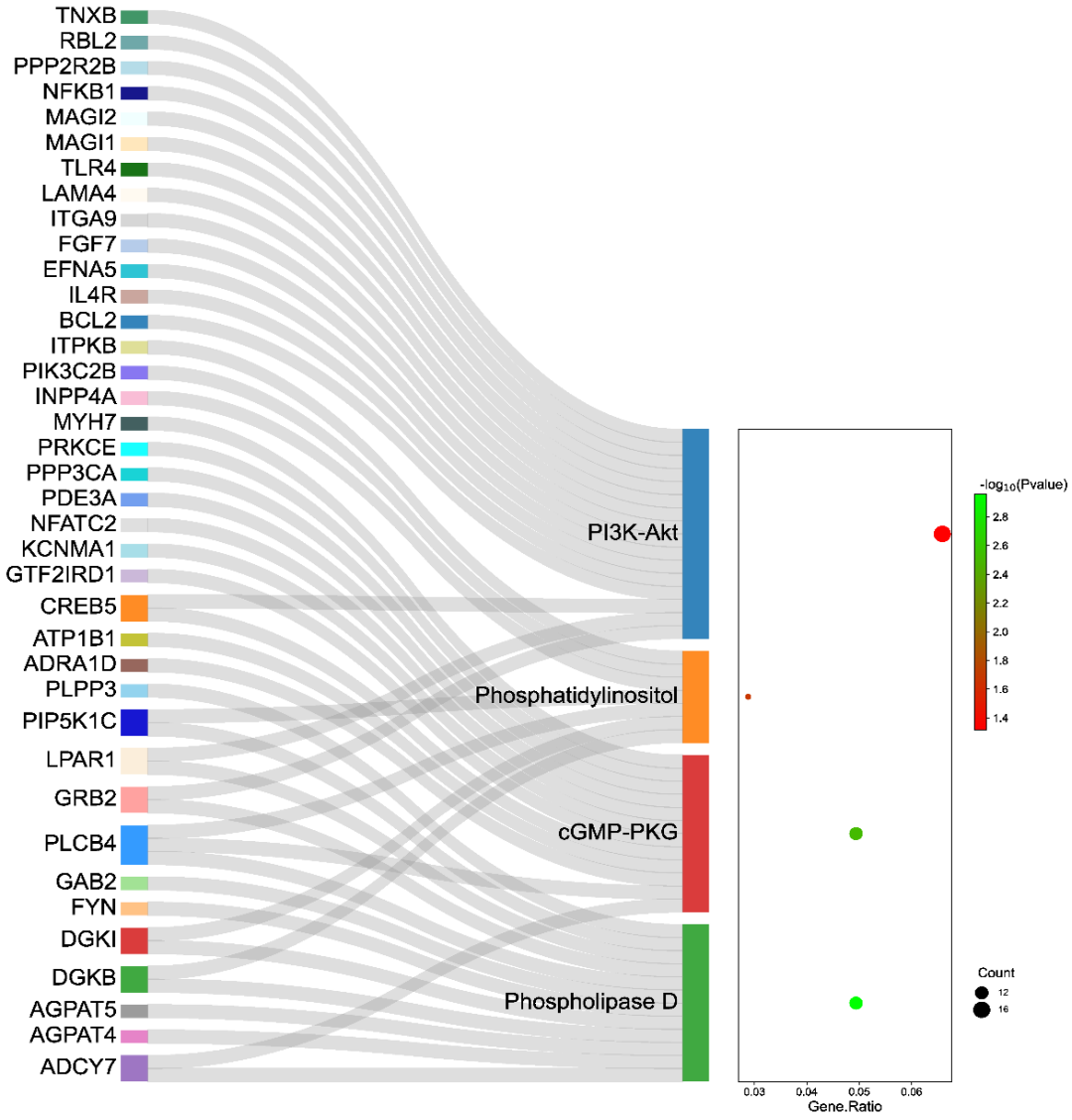


Figure 7.8 Sankey plot of significant signal transduction pathways for hypermethylated CpG site corresponding genes.

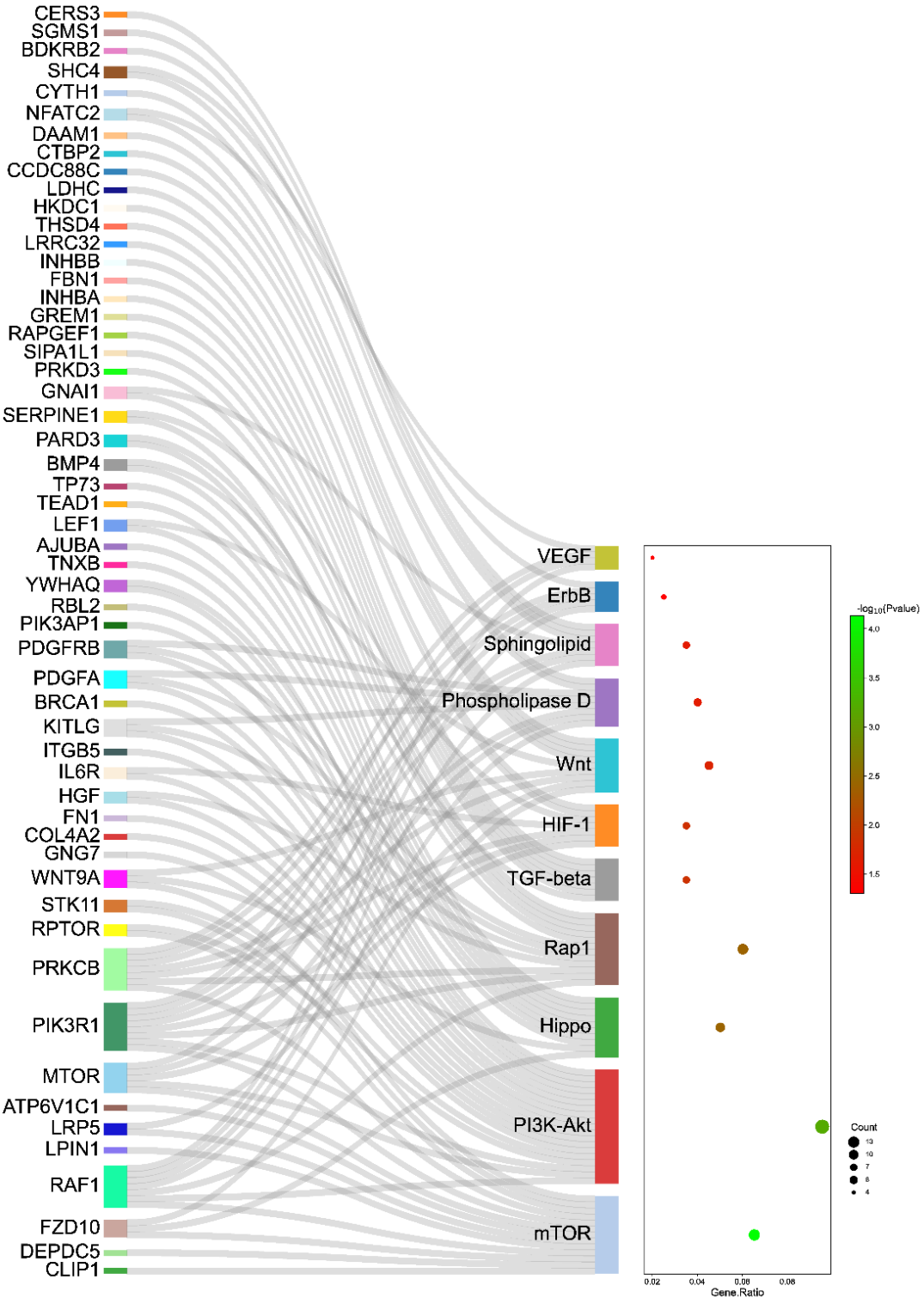


Figure 7.9 Sankey plot of significant signal transduction pathways for hypomethylated CpG site corresponding genes.

GO analysis was generated for hyper- and hypomethylated CpG site corresponding genes. The result indicates a focus on ontology terms related to muscle contractility (Figure 7.10). Specifically, terms like "actin binding" suggest the involvement of muscle contraction, implying that the regulation of contractility is altered in MFS. This finding aligns with the previous results from RNA and protein analysis.

Furthermore, the analysis revealed terms related to phosphatidylinositol and phospholipid binding, which correspond to the KEGG analysis findings involving phospholipase D and phosphatidylinositol signalling. This suggests a potential regulatory mechanism for oxidative and mechanical stress regulation mediated by altered methylation sites.

The GO analysis results mirror those of miRNA enrichment analysis, with an emphasis on several neurology-related processes. This similarity may be attributed to potential biases within the GO database, as it is more extensively documented for neurological biological processes compared to cardiovascular processes.

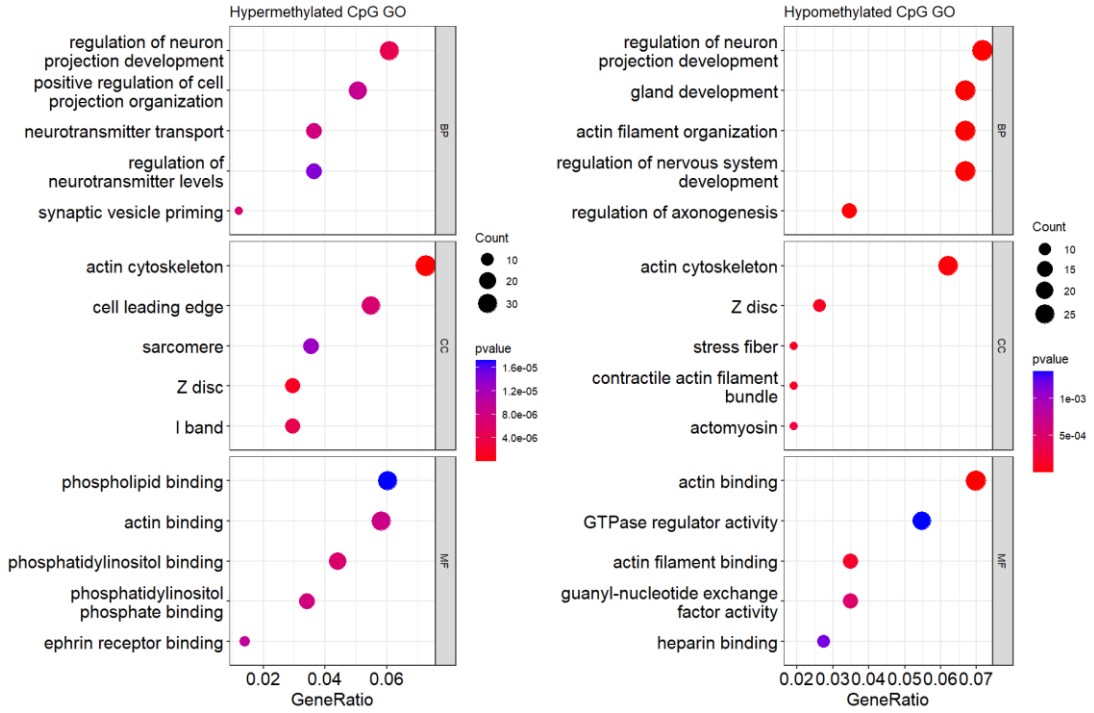


Figure 7.10 GO plot for targets of hyper/hypomethylated CpG sites corresponding genes.

7.3.7 DNA methylation – RNA – protein regulation

Then the study integrated DNA methylation, RNA, and protein data to explore the role of DNA methylation in MFS pathogenesis. In total, 2 DNA methylation – RNA – protein pairs were identified that consistently demonstrated significant DE across the multi-omics analysis (Table 7.6). Out of these pairs, 7 out of 8 CpG sites followed the expected corresponding changes in DNA methylation mechanisms, such as DNA methylation in a promoter region suppressing gene expression and DNA methylation in a gene body region activating the gene.

These corresponding CpG sites targeted genes, including MYH7 and GFAP, which are associated with muscle contraction proteins and intermediate filament proteins, respectively. The observed consistent changes in intermediate filament genes/proteins suggest that DNA methylation may regulate cellular structure and muscle contraction in the context of MFS.

Table 7.6 Corresponding methylation CpG sites - RNA - protein pairs.

Gene ID	CpG site	Gene location	Δβ	p-value	RNA	Protein
MYH7	cg06099173*	TSS1500	*0.153*	0.032	Down***	Down**
	cg20365170**		*0.129*	0.003		
GFAP	cg02407342*	Body	*-0.142*	0.012	Down***	Down***
	cg24797574**		*-0.118*	0.003		
	cg16343401*		*-0.116*	0.042		
	cg20911989*		*-0.104*	0.012		
	cg22100937*		*0.172*	0.017		

P-value was indicated by *, where * indicates p-value < 0.05, ** indicates p-value <0.01 and *** indicates p-value < 0.001. CpG sites labelled in red indicate that such CpG sites meet the basic mechanism (hypermethylation in the promoter region suppresses gene expression but the body region activates gene expression). Red indicating that such gene follows the most likely mechanism of DNA methylation rules. Pink colour indicates upregulation and blue indicates downregulation.

7.4 DISCUSSION

In the miRNA discovery study of MFS aortic tissue samples, 16 miRNAs were found to be significantly altered between MFS patients and controls. These miRNAs had previously been documented in several CVD, supporting the hypothesis that these miRNAs are active are likely to play an active role in post-transcriptional regulatory mechanisms in MFS TAAD development. Further investigation through KEGG and GO analysis illustrated that post-transcriptional mechanisms may be involved in the

inflammatory response, oxidative stress, mechanical stress, VSMC and ECM dysregulation, muscle contractility, and potentially mitochondrial function. The study has identified 8 miRNA-protein pairs that are potentially regulated by post-transcriptional mechanisms, and 2 out of the 8 miRNA-protein pairs demonstrated concordant changes.

The genome-wide DNA methylation profile in MFS tissue samples identified 2,367 significant DE CpG sites. Functional analysis demonstrated that these CpG sites potentially regulate oxidative stress, inflammatory response, mechanical stress, and ECM functions in MFS pathogenesis. Eight CpG sites were found to target significant DE RNA-protein pairs MYH7 and GFAP, and 7 out of 8 CpG sites demonstrated concordant changes according to the usual DNA methylation mechanism.

This study is the first to investigate the DNA methylation profile in the human aortic wall in both control and MFS tissue, and compare it across multiple omics data. The results from epigenetic regulators support the previous findings and further demonstrate that changes in inflammatory response, oxidative and mechanical stress, as well as muscle contractility and ECM functions can be regulated via pre- or post-transcriptional regulatory mechanisms in MFS.

7.4.1 Significant miRNA

In the DE analysis, 16 significant miRNAs were identified. The changes in the miRNA profile in MFS indicate the involvement of post-transcriptional regulation in MFS pathogenesis. Upon initial examination, all significant miRNAs were implicated in CVD. Three miRNAs (miR-451a, miR-29b-2-5p, and miR-25-3p) had been found in previous MFS studies, and two miRNAs (miR-27a-5p and miR-486-5p) were identified in previous TAA studies.

Functional analysis of miRNA effects yielded results complementary to the RNA analysis. Downregulated miRNAs targeting inflammatory pathways were previously targeted by upregulated DE RNA, while upregulated miRNAs targeting cellular response, mitochondrial function, and oxidative stress-related pathways were previously targeted by downregulated DE RNA. This concordant change indicates that miRNA regulation is likely involved in these biological processes.

Moreover, unique pathways identified by up- and downregulated miRNA targets suggest that, in addition to inflammatory response, oxidative stress, mitochondrial

function, TGFβ dysregulation, and cellular/ECM response, miRNAs could also regulate mechanical stress in the aortic wall.

The phosphatidylinositol and ErbB pathways are implicated in regulating mechanical stress. Phosphatidylinositol signalling contains membrane-bound phospholipids that respond to mechanical stress (shear stress and ECM stiffness, etc.). It interacts with key genes in the PI3K/AKT, phospholipase D, and cGMP-PKG/cAMP signalling pathways, as well as intracellular calcium signalling, playing a role in mechanotransduction in MFS (Krajnik, Brazzo et al. 2020). Additionally, in cardiomyocytes, ErbB signalling increases in the early disease stage in response to mechanical wall strain to stimulate a protective response then later decreases accompanied by an increased Ang II level, suggesting the initiation of the remodelling process in response to haemodynamic changes (Lemmens, Doggen et al. 2007). The functions of the ErbB pathway include cell survival, ECM and VSMC homeostasis and maintenance, ERK1/2 activation, and calcium regulation (Schreier, Dohler et al. 2011). The identification of both the phosphatidylinositol and ErbB pathways may indicate miRNA regulation of mechanical stress.

Furthermore, the AMPK pathway was also identified in the miRNA study. The AMPK signalling pathway is an energy-generating/sensory pathway. Activation of AMPK increases ATP generation (catabolic rate) and decreases ATP utilization (anabolic rate). Most importantly, AMPK can regulate mitochondrial function by replacing defective mitochondria with functional ones (Mihaylova and Shaw 2011). In the findings, AMPK targeted by significant DE miRNA may suggest mitochondrial function abnormalities occur in MFS, which has also been reported in ascending TAA (Li, Ren et al. 2020).

The GO analysis further illustrated these findings in the miRNA study, demonstrating changes in significant miRNA targets in Wnt, TGFβ, focal adhesion, and cellular responses such as growth and proliferation. This concordant result shows altered modulation in the above pathways, as well as ECM and cellular regulation.

7.4.2 miRNA as a post-transcriptional regulator

In the analysis comparing miRNAs to their target mRNAs and proteins, 8 mRNA-miRNA-protein pairs were identified. Interestingly, none of the mRNA level of expression were significantly altered, suggesting that mRNA translation might be suppressed, or mRNA may be degraded, by their target miRNAs. This observation

has led to the discovery of two novel miRNAs that are likely involved in post-transcriptional regulation in MFS: miR-154-5p and miR-424-5p. These miRNAs have not been previously identified in TAA but have been implicated in heart remodelling and heart failure.

Notably, miR-154-5p is associated with inflammatory responses induced by ROS and signalling through the ERK and NF-κB pathways (Kim, Jo et al. 2021). Furthermore, ROS-regulated miR-154-5p-dependent regulation has been observed in vascular dementia, where miR-154-5p inhibits angiogenesis and cellular responses such as proliferation, migration, and adhesion via the VEGF signalling pathway. It is also implicated in AMPK and Wnt pathways (Chen, Ma et al. 2020, Han, Zhou et al. 2022). The target gene of miR-154, MINDY2 (MINDY lysine 48 deubiquitinase 2), has been shown to play a role in inflammation, EMT, and angiogenesis and activates PI3K/AKT/mTOR pathways. Elevated MINDY2 level has been associated with inflammatory cells like macrophages, neutrophils, and T-cells, indicating activation of the inflammatory response (Liu, Liu et al. 2023). This data demonstrates that MINDY2 protein expression is downregulated, likely due at least in part to miRNA-154-5p-mediated inhibition. This suggests that miRNA-154-5p may play a role in managing the inflammatory response in MFS patients, attempting to attenuate inflammation within the vessel wall.

In addition, miR-424-5p has been shown to regulate cellular proliferation and migration in cancer studies, where its expression efficiently inhibits cancer cell proliferation and migration (Wang, Wang et al. 2018, Zhao, Zhu et al. 2020). Multiple pathways have been implicated in miR-424-5p's regulatory mechanisms, including VEGF, AKT, MAPK/ERK1/2, and TGFβ pathways (Xuan, Liu et al. 2022). In this study, the target gene of miR-424-5p is KIF5C (kinesin family member 5C), which has been previously investigated in brain malformation and neuron-related diseases (Michels, Foss et al. 2017). Other studies have investigated calcineurin (Cn) and NFAT-related VSMC pathology, showing that under growth factor stimulation, KIF5C expression was significantly upregulated, and closely correlated with Cn/NFAT inhibitors, leading to over a 90% decrease in expression. This suggests that KIF5C expression is correlated with Cn/NFAT in VSMC, potentially promoting SMC phenotype modulation, which is consistent with KIF5C overexpression observed in our study (Lee, Garvey et al. 2011). Another study in TAA identified KIF5C as a target of miR-376a-3p, although this miRNA was not significantly altered in this study. This suggests the possibility of the involvement of KIF5C in TAA pathology (Chen, Ji et al. 2021).

This study identified three significant miRNAs targeting KIF5C (miR-195-5p, miR-424-5p, and miR-497-5p), with only miR-424-5p expression corresponding to the miRNA regulatory mechanism. This suggests that miRNAs could exhibit synergistic or antagonistic effects with each other, and miR-424-5p may be, on balance, the only one successfully targeting KIF5C mRNA, leading to the suppression of its protein production. MiRNA synergy refers to multiple miRNAs targeting the same gene, enhancing post-transcriptional regulatory power and resulting in strong inhibition (Lutter, Marr et al. 2010, Gam, Babb et al. 2018). While miRNA synergy and antagonism have been observed in previous studies, further investigation is needed to confirm whether this data demonstrates miRNA synergistic or antagonistic effects.

Some mRNA-miRNA-protein pairs did not show alterations according to post-transcriptional mechanisms. This suggest that alternative mechanisms could be involved in regulating the expression of those proteins. Nevertheless, the targeting of FBN1 by miR-25-3p may indicate a survival role, where miR-25-3p attempts to upregulate low FBN1 expression in MFS patients. However, due to the already low FBN1 levels in MFS patients to begin with, this rescue mission appears to be unsuccessful. Further studies are required to clarify the interaction between miR-25-3p and FBN1 expression.

In conclusion, the interactions between miR-154-5p-MINDY2 and miR-424-5p-KIF5C suggest that oxidative stress-related inflammation and VSMC regulation/cellular response in MFS TAAD may be regulated by miRNA mechanisms. Additional experiments should be performed to validate these interactions and determine their biological functions.

7.4.3 Significant DE methylation CpG sites

As previously mentioned, the DNA methylation mechanism in MFS is not well understood. Initially, the study examined the global DNA methylation profile to identify significant DE CpG sites. Then close investigation was conducted on the top 20 gene-associated CpG sites. Most of these sites were found within the gene body, with a smaller portion in promoter regions (TSS1500, TSS200, and 5'UTR) and the 3'UTR region. The 5' and 3'UTR regions are untranslated regions that play roles in post-transcriptional regulation, modulating mRNA translational control and stability (Mignone, Gissi et al. 2002, McGuire, Herbrich et al. 2019).

Among the top 20 gene-associated CpG sites, many are likely involved in ECM organization, cell-cell/cell-matrix interactions, inflammation, and the regulation of various signal transduction pathways. Within these top 20 CpG sites, two were associated with significant RNA changes (INHBA – cg02161417; CPA3 – cg13424229). Upon closer examination, the study found that INHBA and CPA3 corresponded to the primary mechanism, with hypomethylation in the promoter region leading to downregulation of their target RNA. Both INHBA and CPA3 are expressed in fibroblasts, and CPA3 is also found in inflammatory cells.

INHBA is a member of the TGFβ superfamily, activating TGFβ signalling receptors and increasing TGFβ expression. In a TAA murine model, INHBA expression increased along with elevated TGFβ signalling through SMAD2/3 activation, leading to ECM degradation and TAA development (Jones, Barbour et al. 2008). Additionally, TAAD in a mouse model study showed compromised INHBA-associated TGFβ signalling expression in a YAP (key Hippo pathway protein) deficient environment, which protected against aortic wall degeneration, highlighting the involvement of the Hippo pathway in TAAD pathogenesis (Zhang, Li et al. 2023). The current data reveal that cg02161417 was hypomethylated in MFS at the promoter site of INHBA, leading to INHBA overexpression at the transcriptional level. This may be associated with elevated TGFβ signalling in MFS.

CPA3, or carboxypeptidase A3, has been found to be expressed in mast cells in TAA (Tian, Zhang et al. 2023). Generally, CPA3 is present within inflammatory cells, and studies have shown that it is downregulated in advanced coronary artery disease with blood vessel damage. CPA3 degrades apolipoprotein B (LDL particles) and Ang II, inhibits endothelin in the vessel wall, and protects against hypertension (Lewicki, Siebert et al. 2019, Dahlin, Maurer et al. 2022). In MFS, CPA3 was upregulated with hypomethylation of cg13424229 in the promoter region, suggesting that increased CPA3 may play an anti-hypertensive role in MFS.

The study then further examined hypermethylated and hypomethylated CpG sites targeting genes. Hypermethylated CpG sites were associated with genes involved in phospholipase D, cGMP-PKG, phosphatidylinositol, and PI3K/AKT signalling. As previously discussed (Chapter 4 and Section 7.4.1), these pathways exhibit extensive crosstalk with each other. Studies have suggested that the phospholipase D signalling is involved in oxidative stress and may play an essential role in Ang II-induced phospholipid D-related ROS production. Inhibition of phospholipid D and NADH/NADPH oxidase attenuates superoxide production induced by Ang II

administration (Touyz and Schiffrin 1999). Thus, the implication of phospholipid D may indicate a role in ROS and oxidative stress in MFS.

The crosstalk between these pathways can be summarized as cGMP-PKG being a downstream signalling effector of PI3K/AKT, thus playing a role in mitochondrial function and ROS regulation, as well as interacting with the phosphatidylinositol signal system and phospholipase D signalling associated with mechanical stress response and calcium signalling regulation. The results of hypermethylated genes suggest that DNA methylation may contribute to the regulation of these functions.

In addition to phospholipase D and PI3K/AKT signalling, hypomethylated CpG sites targeted mTOR, Hippo, Rap1, TGFβ, HIF-1, Wnt, sphingolipid, ErbB, and VEGF pathways. The VEGF pathway controls angiogenesis, vascular development, EC survival and proliferation (Zhou, Zha et al. 2023). A miRNA study in TAA human aortic tissue has shown that VEGF plays a critical role in TAA formation related to cell proliferation. VEGF was found to be increased in TAA due to miRNA suppression of VEGF inhibitors (Licholai, Blaż et al. 2016). Furthermore, in an AAA model with Ang II infusion, VEGF accelerated aneurysm formation by increasing aortic wall diameter and cross-section of the aneurysm site, along with increased MMP2 production (Choke, Cockerill et al. 2010). VEGF overexpression also promoted apoptosis, inflammation, and oxidative stress in SMC, contributing to TAA formation (Zhou, Zha et al. 2023).

In summary, these pathways implicate inflammatory responses (Hippo, HIF-1, Wnt, sphingolipid, VEGF), oxidative stress (phospholipase D, HIF-1, Wnt, VEGF), mechanical stress (ErbB), cellular response (PI3K/ATK, mTOR, Rap1, phospholipase D), as well as TGFβ dysregulation in MFS pathogenesis, potentially regulated by DNA methylation mechanisms.

GO analyses of the differentially methylated CpG sites targeting specific genes have identified the involvement of actin-binding related processes, including actin binding, actin filament organization, actin cytoskeleton, contractile actin bundle, and actin filament binding, primarily identified by hypomethylated CpG sites. This suggests that DNA methylation may play a vital role in regulating actin function and structure in MFS, especially through hypomethylated sites, which implies potential VSMC phenotypic switching events (Malloy, Wen et al. 2012). Furthermore, hypermethylated CpG sites were associated with phospholipid and phosphatidylinositol binding, reflecting the involvement of phospholipid and phosphatidylinositol pathways, which complements the KEGG analysis.

7.4.4 DNA methylation as an epigenetic regulator

After identifying significant DNA methylation sites, the study investigated the regulatory mechanism between DNA methylation, RNA, and protein in MFS pathogenesis. The results indicated that 8 CpG sites were targeting corresponding RNA and protein with significant alterations in their expression, while 6 CpG sites were aligned with the DNA methylation mechanism.

MYH7 was targeted by hypermethylation of cg06099173 and cg20365170, both of which lie in the promoter region of this gene, and correspondingly was associated with downregulated RNA and protein expression. Additionally, 4 hypomethylated CpG sites (cg02407342, cg24797574, cg16343401, and cg20911989) of GFAP, targeting the body region of the gene, showed corresponding changes in downregulated RNA and protein expression in MFS.

The changes observed in DNA methylation may be induced by chronic inflammation or oxidative stress, as discussed in Chapter 2.5.4. DNA methylation can be predominantly regulated by environmental factors. Studies have demonstrated that chronic low-grade inflammation may induce hypo- or hypermethylation and override transcriptional regulation (Wielscher, Mandaviya et al. 2022). For example, the elevation of levels of immune cells like leukocytes and monocytes can promote TNF and NOS2 signalling, which in turn induces methylation (Yamashita, Nanjo et al. 2019). Furthermore, oxidative stress might also be a significant inducer of methylation changes in MFS, where high oxidative stress inhibits TET activity, leading to reduced DNA demethylation and increased methylation levels (Hedman, Zilmer et al. 2016, García-Guede, Vera et al. 2020). These observations suggest a potential hypothesis for the findings, aligning with the previous RNA and protein expression results, where high immune gene/protein expression and oxidative stress-related gene/protein and pathways were observed. However, whether altered DNA methylation is a causative or consequent event is unclear; future studies are required to investigate the role and regulatory mechanism of DNA methylation in MFS.

MYH7 was previously investigated in Chapters 4, 5, and 6. MYH7 assists with muscle contractility, and the loss of MYH7 expression leads to cardiomyopathy (Holm, Habashi et al. 2011). Downregulated MYH7 may indicate an early sign of losing cardiac muscle contractility in MFS. However, the association of cardiomyocytes contractile gene expression within the aortic wall may or may not have an association with the cardiomyocytes, further study should investigate the

correlation between the expression of these altered heart muscle contractile genes. This result suggests that the alteration in MYH7 in MFS patients may be regulated by DNA methylation, making it a potential therapeutic target.

GFAP is also an intermediate filament protein, type III. Numerous studies have investigated GFAP in neurological disorders since GFAP is mainly found in astrocytes, Schwann cells, and enteric glial cells, associated with central nervous system injury and regeneration (Yang and Wang 2015). A more recent study found that GFAP-expressing progenitor cells are involved in VSMC and EC development in the thoracic aorta; however, the influence of GFAP on VSMC and EC in the adult stage is unclear (Osman, Wang et al. 2020). Another study found that during congenital heart defect operations, patients on cardiopulmonary bypass exhibited elevated GFAP expression which correlated with hypothermia; however, the cause and effect of the GFAP elevation in hypothermia during the operation is unclear (Vedovelli, Padalino et al. 2017). Although the patients in this study did not exhibit hypothermia during the operation, the elevation of GFAP levels may or may not be a surgical artifact. Further investigation is required to explore the function of GFAP and its DNA methylation regulation in MFS.

Both the roles of MYH7 and GFAP in transcription-translation regulatory mechanisms were underscored in Chapter 6.3.1. The concordance in changes of RNA and protein expression highlights their known biological functions in heart muscle contractility and maintaining cellular structure and function. This DNA methylation study further illustrates that these changes could potentially be regulated by DNA methylation. Further studies should validate the link between these CpG sites and genes, potentially shedding light on therapeutical target investigation and development.

7.5 LIMITATIONS

7.5.1 Sample limitation

A significant limitation of this study pertains to the sample size, which is a consistent observation across the transcriptomic, proteomics, and miRNA analyses. While the current study represents the largest investigation of human MFS aortic tissue to date, it is important to acknowledge that the sample numbers remain statistically small resulting in study being underpowered.

Specifically, for the miRNA study, 14 MFS aortic tissue samples and 11 control aortic tissue samples were utilized, it is essential to recognize that this dataset suffers from biases similar to those that discussed in Chapter 4.5.1.

Furthermore, the methylation study faces an even smaller sample size, with only 7 samples available for both MFS and control groups. It is worth noting that the DNA methylation experiment was conducted at a time when our sample availability was limited. The study anticipates that future improvements can be made as the continuous collection of MFS aortic tissues from the Sydney Heart Bank.

7.5.2 Statistical limitation

In addition to not utilizing a housekeeping miRNA, this study faces several statistical limitations. One such limitation is the absence of an established ΔΔCT cutoff value. Typically, a 2FC is commonly employed in statistical analysis, as seen in the RNA and protein data. However, it is important to note that the FC cutoff value is not universally fixed but rather depends on the data type and context (Love, Huber et al. 2014). MiRNA expression exhibits significant biological variability, especially in complex biological systems. Relying solely on a fold change cutoff might lead to the overlooking of potentially significant miRNAs that display small yet biologically relevant changes in expression. Particularly in the context of miRNA's role in target gene and protein interactions, miRNA expression levels can provide a more comprehensive understanding of pathway analysis and enrichment functions. Establishing an FC threshold could limit this perspective (Abu-Halima, Kahraman et al. 2018). Furthermore, the p-value from the Student's t-test holds considerable importance, as it provides statistical confidence in assessing differences between groups (Sarver 2010). By utilizing a p-value cutoff at 0.05 and implementing complex omics integration and comparison, this data should offer a certain level of confidence, even in the absence of a fold change threshold. Nevertheless, further analysis is required to validate these data.

The second statistical limitation revolves around the fact that all miRNA-mRNA pairs were identified solely through the miRDB computational database. Although the study applied stringent threshold criteria for prediction scores, it is crucial to acknowledge that these interactions may not be definitive. Additional validation is necessary to thoroughly examine the interactions between miRNA and its potential target mRNA.

Another notable limitation in this study is the absence of multiple testing correction for the miRNA and DNA methylation analyses. This exclusion could potentially lead to type 1 errors, as we previously discussed in Chapter 4.4.3. However, after correction for multiple testing there were no significant miRNA nor CpG sites identified from the studies. It is important to acknowledge that it may encounter false positive discoveries but as emphasized previously, this study was primarily designed as a discovery study intended to, amongst other outcomes, identify potential novel diagnostic and therapeutic targets. The goal was to uncover as much potentially informative information as possible and explore the potential biological functions and pathways associated with the identified miRNAs and CpG sites. In this context, this study has chosen not to implement multiple testing correction to optimize the capacity to identify novel insights and trends in the data.

In addition to the multiple testing correction, no miRNA or CpG sites passed the Benjamini-Hochberg correction significantly. This could be attributed to the miRNA study's heavy reliance on imputation to address undetermined values, potentially contributing to compromised statistical validity. This issue should be addressed in future studies by incorporating multiple replicates for each sample, which could possibly improve the CT readings and consequently yield better results (Daniels, Sillé et al. 2014).

Furthermore, in the context of DNA methylation CpG sites, the lack of significant CpG sites identified after applying the Benjamini-Hochberg correction may be due to the extremely large number of CpG sites detected. This is influenced by the nature of the Benjamini-Hochberg correction calculation; as the number of genes (in this case, CpG sites) increases, the FDR threshold becomes more stringent (Benjamini and Hochberg 1995). Additionally, the smaller sample size may contribute to the multiple testing issue in the DNA methylation study, as smaller sample sizes lead to subtle differences between groups, making it more challenging to detect true differences (Higdon, van Belle et al. 2008). Therefore, future studies should consider addressing these issues by either relaxing the FDR threshold or increasing the sample size to enhance statistical power and increase confidence in the data.

7.5.3 Experimental limitation

Interpreting DNA methylation is a complex and challenging task, and its role in disease is not yet well-established or fully understood, as extensively discussed in Chapter 2.5.4 and throughout this chapter. While it is widely recognized that CpG

sites in gene promoter regions can inhibit gene expression, and those in the body region can activate it, recent studies have shed light on the nuanced nature of DNA methylation regulation. It has become evident that methylation can vary based on location, timing, and cell or tissue specificity, adding layers of complexity to its interpretation.

This study has adhered to the most widely accepted general rules governing DNA methylation mechanisms. However, it is essential to acknowledge that the actual results may not always align with these established principles. Furthermore, there is no clear indication whether changes in methylation profiles are causative or merely consequences of other underlying processes. This ambiguity increases the challenge of interpreting DNA methylation results.

Therefore, it becomes imperative to conduct further studies aimed at investigating and validating the findings regarding these significant CpG sites and their corresponding genes and proteins, along with their roles in the pathogenesis of MFS. Such research will contribute to a deeper understanding of the intricate relationship between DNA methylation and MFS and help clarify its mechanistic complexities.

7.6 CONCLUSION

From the miRNA study, 16 miRNAs were identified that exhibited significant expression differences in MFS patients compared to controls. These miRNAs have previously been implicated in various CVD, including MFS, TAAD, and TGFβ dysregulation. The KEGG analysis revealed significant pathways that complemented the RNA pathway analysis, providing further insights into the inflammatory response, ECM maintenance, oxidative and mechanical stress, VSMC function, and TGFβ dysregulation. This suggests that these processes may be regulated by post-transcriptional mechanisms.

Through a more in-depth investigation that involved comparing RNA, miRNA, and protein data, two potential regulatory mRNA-miRNA pairs were identified: MINDY2-miR-154-5p and KIF5C-miR-424-5p. These pairs may play a role in post-transcriptional regulation within MFS pathogenesis, influencing inflammation and cellular responses such as proliferation, migration, and VSMC phenotypic switching.

The genome-wide DNA methylation analysis in MFS patients aligns with the previous findings, emphasizing the role of inflammation and abnormal cell-cell and cell-matrix interactions in MFS. KEGG pathway analysis further supports the discoveries in oxidative stress, mechanical stress, inflammation, VSMC and ECM dysregulation, as well as TGFβ abnormalities. These results suggest that DNA methylation plays a significant role in regulating these biological processes and may be a key factor in MFS pathogenesis.

Furthermore, when compared DNA methylation with RNA and protein expression, two potential genes/proteins were identified, MYH7 and GFAP, regulated by six CpG sites in MFS pathology, modulated by DNA methylation. These genes/proteins have previously been implicated in the regulation of muscle contractility and mechanical stress, although their possible role within the aorta is very unclear. These findings may be supportive of the hypotheses regarding the drivers of MFS pathogenesis and provide valuable insights into the role of DNA methylation in the development of TAAD, potentially offering therapeutic targets for MFS. Considerable further investigation of the roles of these genes is required.

In conclusion, the epigenetics study has significantly enhanced our understanding of the biological functions involved in MFS pathogenesis and has identified potential therapeutic targets. This study represents a significant step forward in unravelling the complex regulatory systems and aetiology of MFS, illuminating new perspectives for future research to advance our understanding of this condition.

Chapter 8: Conclusions and future directions

8.1 THESIS OVERVIEW

Marfan syndrome, as the paradigm of genetic TAA, exhibits significant phenotypic heterogeneity among individuals and is associated with aortic dilation, aneurysm formation, and dissection. Thus far, although beta-blockers and ARBs may attenuate the rate of aortic dilation in younger patients, no definitive effective treatment for MFS TAAD formation has been identified, necessitating prophylactic surgery for severe TAA (Chen, Chan et al. 2021). Consequently, improved understanding of the pathogenesis of MFS aortic disease is crucial for comprehending the mechanisms of the disease and identifying efficient interventions.

This thesis presents the largest human tissue “omics” study in MFS to date, utilizing multiple regulatory layers (multi-omics) to identify disease drivers and signal pathways involved in MFS. We conducted four genome-wide omics studies, exploring alterations in transcriptional, translational, post-transcriptional, and pre-transcriptional regulation, along with potential pathways contributing to MFS-TAAD development.

As a first step, RNA and protein were profiled to investigate abnormal expression patterns in the aorta of MFS patients compared to controls (Chapters 4 and 5). These studies identifying potential dysregulated genes and proteins involved in the inflammatory response, oxidative stress, mitochondrial dysfunction, mechanical stress, and ECM maintenance. Additionally, changes in expression of proteins regulating cardiomyocyte contractility and haemoglobin expression were also observed in MFS patients. The multi-omics comparison between transcriptomics and proteomics provides a more comprehensive understanding of complex disease regulation by transcription-translational modulation of aforementioned mechanisms in MFS (Chapter 6). However, the low correlation between RNA and protein expression that was observed suggests the involvement of alternative regulatory mechanisms, such as epigenetics, in MFS pathogenesis, beyond transcriptional-translational regulation.

Documentation of altered epigenetic regulation, as revealed by miRNA and DNA methylation profiles, advances our understanding of the regulatory mechanisms in MFS pathogenesis (Chapter 7). These results demonstrate that previously identified biological processes, such as the inflammatory response, oxidative stress, ECM dysregulation, mitochondrial dysfunction, and mechanical stress, are not solely controlled by transcriptional and translational regulation but can also be influenced by miRNA and DNA methylation. This work has identified miRNA and methylation CpG sites associated with altered RNA and protein expression, providing insights into potential novel therapeutic targets in MFS.

In summary, the study described in this thesis encompassed genome-wide expression profiles from four distinct omics datasets, identifying and exploring the signalling pathways and biological processes that contribute to MFS pathogenesis, thus advancing our understanding of MFS TAAD mechanisms and offering insights for future potential therapeutic development. Figure 8.1 provides an overview of the key findings elucidated in this thesis.

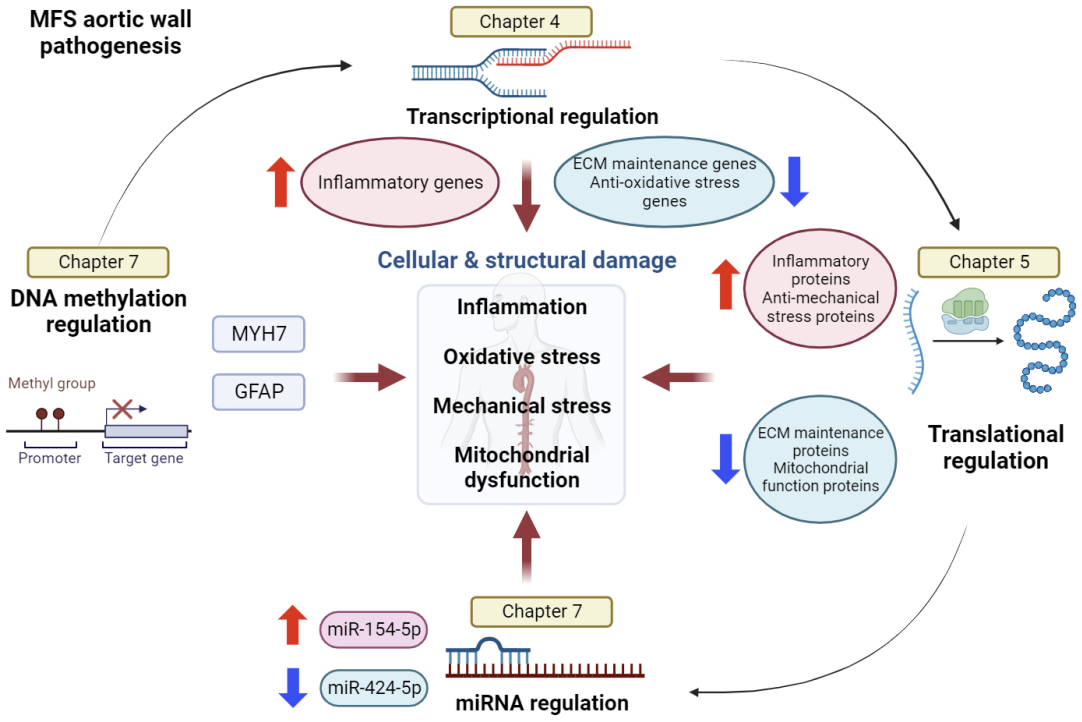


Figure 8.1 Overview of the key findings. Chapters 4 and 5 focussed on alterations in transcription and translation in MFS compared to controls, identifying four key pathogenic mechanisms: inflammation, oxidative and mechanical stress, and mitochondrial dysfunction. The multi-omics analysis undertaken in Chapter 6 suggested that the lack of

strong correlation between transcription and translation is likely due the involvement of other regulatory mechanisms, particularly epigenetic regulation. Chapter 7 investigated the involvement of epigenetics in MFS, determining differences in miRNA expression and DNA methylation profiles in MFS, correlating these changes with their likely gene targets and altered signalling pathways. These epigenetic alterations were also found to correspond to the four key pathogenic mechanisms listed above.

8.2 POSSIBLE BIOLOGICAL PROCESSES AND CELLULAR FUNCTIONS INVOLVED IN MFS

The findings from all four omics datasets corroborate and highlight the involvement of an inflammatory response, increased oxidative and mechanical stress, ECM dysregulation, and mitochondrial dysfunction. Chapters 4.4 and 5.4 detail alterations in gene and protein expression that illustrate how these biological processes disrupt aortic homeostasis and interact with each other. Throughout the results, the presence of an inflammatory and oxidative stress response stands out as having the most strongly supported evidence. These responses have been shown to be interconnected and mutually reinforcing in the pathogenesis of MFS (Radonic, de Witte et al. 2012). In addition to inflammatory and oxidative stress responses, this study has identified novel contributing factors: mitochondrial dysfunction and mechanical stress, which are believed to be involved in the pathogenesis of MFS-associated TAAD (Celi and Berti 2014, Oller, Gabandé-Rodríguez et al. 2021). Furthermore, these two factors demonstrate crosstalk and interactions with the inflammatory response and oxidative stress, contributing to a vicious cycle that leads to ECM degradation and dysregulation of EC and VSMC (Figure 8.2) (Li and Xu 2007, Vecchione, Carnevale et al. 2009).

The involvement of the inflammatory response in MFS pathogenesis has been an emerging concept over the last decade. Initially, inflammation was thought to play no part in MFS pathogenesis, primarily because of the absence of obvious inflammatory cells (Collins, Dev et al. 2008). However, emerging evidence has progressively implicated inflammation in the pathogenesis of MFS (Malecki, Hambly et al. 2020). An increased inflammatory response can be triggered by elevated ROS levels, and vice versa (Chatterjee 2016). Additionally, an increased inflammatory response and oxidative stress contribute to EC dysregulation, VSMC proliferation, migration, apoptosis, increased mechanical stress due to abnormal haemodynamics, and ECM degradation (Papaharalambus and Griendling 2007, Celi and Berti 2014, Kornhuber, Seidel et al. 2019). Interestingly, mechanical stress, the

inflammatory response, and oxidative stress also exhibit reciprocal interactions; increased mechanical stress can induce oxidative stress and an inflammatory response, resulting in vascular dysfunction, abnormal cellular responses (including VSMC and EC proliferation, migration, apoptosis, differentiation), cell cytoskeleton rearrangement, and signalling dysregulation (Li and Xu 2007, Vecchione, Carnevale et al. 2009). These abnormal biological processes have been shown to induce inflammatory cell infiltration (Radonic, de Witte et al. 2012, Rysz, Gluba-Brzózka et al. 2021). Inflammation also leads to ECM degradation with or without ROS stimulation which could further exacerbate the inflammatory reaction, promoting ECM remodelling and EC dysregulation in MFS.

Recent studies have implicated mitochondrial dysfunction in the pathogenesis of MFS (Oller, Gabandé-Rodríguez et al. 2021, Verhagen, Burger et al. 2022). This study also identified abnormalities in gene expression associated with mitochondrial dysfunction in MFS. Importantly, mitochondrial dysfunction is likely to have extensive connections with the inflammatory and oxidative stress responses. An inflammatory response can be induced both by mitochondrial dysfunction itself and by mitochondrial dysfunction-induced ROS (Ferrucci and Fabbri 2018, Marchi, Guilbaud et al. 2023). Additionally, oxidative stress and/or inflammation-induced oxidative stress can lead to DNA damage, further exacerbating mitochondrial dysfunction (Rosca and Hoppel 2013). Consequently, all of these biological processes are intricately linked, forming strong associations within a complex regulatory system. Thus, a slight change within one of the individual factors could impact other potential drivers to exacerbate the overall dysregulation within the aortic wall, resulting in abnormal cellular responses (ECM, EC, and VSMC dysregulation), ultimately contributing to TAAD formation (see Figure 8.2).

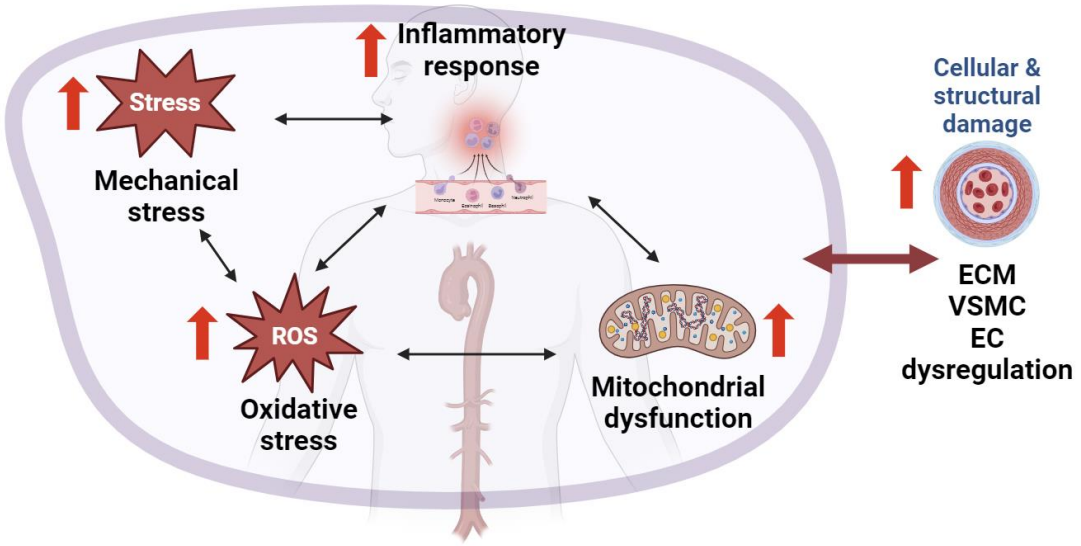


Figure 8.2 Identified biological processes involved in MFS pathogenesis in this study. The black arrow indicates the interaction between these potential drivers, and red up arrow indicates the increased responses observed in this study. The dark red arrow indicates an association between these four potential drivers and ECM/EC/VSMC dysregulation.

8.2.1 Muscle contractility markers

Consistent changes in heart muscle contractility genes and proteins emerge across our omics studies, particularly with respect to transcriptional-translational regulatory mechanisms. This raises the intriguing question of why heart muscle contractility genes and proteins are prominently featured in the aortic wall. In previous research, reduced VSMC contractility has been noted in MFS TAA. Vasoconstriction and contractile genes specific for VSMC (ACTA2, MYL9, MYH11, TPM2) are diminished in an MFS animal model, indicating an association between MFS and decreased VSMC contractility (Chung, Au Yeung et al. 2007, Pedroza, Tashima et al. 2020). However, in the current study, changes in classic VSMC contractile gene markers were not statistically altered. However, heart muscle contractile genes primarily expressed in cardiomyocytes exhibited significant downregulation (e.g., MYH6, MYH7, TNNT2, TPM1, MYL2, MYOZ2). These genes have been associated with cardiomyopathy, characterized by abnormalities in contractile mechanics of the myofibrils, such as myofibril disarrangements and low density (Kim, Devereux et al. 2011, Vikhorev and Vikhoreva 2018). Moreover, there is increasing evidence of primary and secondary cardiomyopathies in MFS, possibly due to abnormal

hemodynamic and mechanosignaling (Cook, Carta et al. 2014, Hetzer, Siegel et al. 2016).

Studies have revealed a subpopulation of cardiomyocytes derived from vessel stem sites (e.g., origins of the coronary arteries) within the aorta during heart development (Chen, Poduri et al. 2014, Buijtendijk, Barnett et al. 2020). An intriguing study using a chicken embryo model demonstrated that coronary artery stems arise via endothelial budding and that peritoneal vessels grow inward and fuse with the aorta through angiogenesis. Cardiomyocytes have been observed in the surrounding vessel stems within the aortic wall in both human and mouse hearts (Chen, Poduri et al. 2014). This embryological study supports the idea of a potential population of aortic cardiomyocytes arising during cardiac development. Furthermore, a stem-cell study utilizing cells derived from the aortic wall has demonstrated that adventitia cells possess the capability to generate aortic cardiomyocytes with both morphological and functional properties. These data suggest that the expression of cardiomyocyte-related genes may be manifested by these aortic wall stem or progenitor cells, possibly with cardiomyocyte functional capacity (Mekala, Wörsdörfer et al. 2018).

However, the exact population of cardiomyocytes or progenitor cells differentially expressing cardiomyocyte-related genes within the normal and MFS aortic wall remains to be elucidated. Understanding the mechanisms underlying changes in muscle contractility genes and their potential impacts on the aortic wall in MFS pathogenesis is a field for future research. This also raises the possibility of whether muscle contractility gene expression in the MFS aortic wall mirrors that in the MFS heart cardiomyocytes, potentially leading to MFS cardiomyopathy.

8.2.2 Haemoglobin expression

From the protein analysis of MFS patients, the top 20 elevated proteins, ranked by fold change, included several haemoglobin proteins, specifically HBD, HBB, HBE1, HBG1, HBG2, HBA1, and HBZ. Some of the identifications of haemoglobin isomers could be a result of sequence homology, as discussed in the Chapter 5. Previous studies have shown that HBA1 can be expressed by EC, and it is involved in NO release and diffusion within the vessel wall (Straub, Lohman et al. 2012, Butcher, Johnson et al. 2014). This study observed an increase in several haemoglobins in the aortic wall of MFS patients, Thus, elevated levels of haemoglobin within the aortic wall could signify EC dysregulation and disrupted vascular tone. Furthermore,

the results from transcriptional and translational studies have revealed that potential hypoxia responses and abnormal mitochondrial regulation in the MFS aortic wall may exacerbate NO release and be related to alterations in aortic haemoglobin levels. The low availability of NO may lead to EC dysregulation and subsequently promote the formation of TAA (Dungel, Penzenstadler et al. 2017).

In addition, microhemorrhages within the vessel wall could provide another potential explanation for the detection of haemoglobin expression within the aortic wall. In an Ang II infusion animal model of TAAD, studies have demonstrated the presence of interlaminar microhemorrhages and hematomas in the mouse aorta in the early stages of dissection (Rateri, Davis et al. 2014, Trachet, Piersigilli et al. 2016). This phenomenon might be attributed to increased vessel permeability and loss of EC (Trachet, Piersigilli et al. 2016). The results from the animal model may be reflected in the human aortic wall, suggesting EC dysregulation, which could potentially contribute to TAA formation. This is a novel finding in MFS human aortic vasculature; however, corresponding mRNA transcripts did not demonstrate corresponding increased expression, which may suggest alternative regulatory mechanisms for haemoglobin regulation or that the haemoglobin protein has been synthesised elsewhere in the body, e.g., in red blood cells, that have been involved in microhaemorrhages within the aortic wall. Studies demonstrated that haemoglobin human expression is demonstrated to be regulated by transcriptional and post-transcriptional processes (Peixeiro, Silva et al. 2011). The discrepancy between minimal mRNA haemoglobin expression and increased protein haemoglobin expression may be regulated by post-transcriptional mechanisms, requiring further study to investigate abnormal haemoglobin expression and its role in MFS TAAD formation.

8.3 SIGNAL TRANSDUCTION PATHWAYS IN MFS

Signal transduction plays a pivotal role in disease formation, and dysregulated TGFβ signalling has been proposed as a major contributor to MFS pathogenesis through various studies (Gillis, Van Laer et al. 2013, Takeda, Hara et al. 2018). However, the review paper on TAA signalling recently published by our group tabulates evidence that signalling pathways exhibit comprehensive interactions and crosstalk, with multiple signalling pathways dysregulated in TAA (Dong, Malecki et al. 2023). Using four different omics techniques, all 25 signal transduction pathways

related to environmental information processing that are listed in the KEGG online database under the signal transduction subcategory were examined (Figure 8.3).

From Figure 8.3, the results show that, apart from TGFβ, the JAK-STAT, HIF-1, Rap1, cGMP-PKG, PI3K/AKT, and sphingolipid pathways are correlated with omics changes in three out of four of the omics studies. The colours of the dots in the Upset plot indicate the significance of the pathways and the outer ring of the dots illustrates the identified pathways that were involved either by up- or down-regulated significant DE genes/ proteins. Due to the small protein input, only two overall significant pathways identified from the proteomics data (HIF-1 and TGFβ pathways) were included in the Figure 8.3. These findings align with the observations of the biological functions involved in MFS TAAD (Dong, Malecki et al. 2023), which implicate the role of the inflammatory response, oxidative stress, mitochondrial dysfunction, and VSMC/EC/ECM dysregulation in MFS pathogenesis.

Beyond TGFβ, this study illustrated that other pathways could be involved in MFS TAAD pathogenesis, such as inflammatory response (HIF-1, cGMP-PKG. JAK-STAT, sphingolipid), oxidative stress response (HIF-1, cGMP-PKG), cellular response and stabilization (Rap1). Additionally, the previous review suggested that the PI3K/AKT pathway is likely to be a common downstream pathway interacting with other signalling pathways, as was also demonstrated in this study. PI3K/AKT serves as a downstream pathway of non-canonical TGFβ signalling, regulating VSMC contractility, ECM maintenance and inflammatory responses (Estrada, Irons et al. 2021, Dong, Malecki et al. 2023). PI3K/AKT also exhibits crosstalk with JAK-STAT, inhibiting cell apoptosis induced by oxidative stress (Lu, Zhou et al. 2008). The interaction between the PI3K/AKT-HIF-1 signalling pathways promotes VSMC proliferation under hypoxic conditions, and PI3K/AKT likely assists HIF-1 in adapting to mechanical stress (Chun, Kim et al. 2009). Additionally, cGMP-PKG, Rap1, and PI3K/AKT regulate EC by modulating NO production and are implicated in ROS and mitochondrial function (Oldenburg, Qin et al. 2004, Carreira, Lee et al. 2011). Therefore, these significant pathways demonstrate comprehensive crosstalk and commonly interacted with the PI3K/AKT pathway and implicate these pathways in the proposed driver biological processes, such as inflammation, the oxidative and mechanical stress response, and mitochondrial dysfunction, provided further insights into additional potential pathway regulators of MFS TAAD, in addition to TGFβ signalling. To date, many drugs have been developed for the inhibition of PI3K/AKT and/or mTOR and corresponding downstream signalling (Yang, Nie et al. 2019). Several second-generation highly-selective PI3K inhibitors (idelalisib,

copanlisib and duvelisib) have received FDA approval for use in cancer and are currently undergoing testing in a range of cardiovascular diseases (atherosclerosis, myocardial infarction and thrombosis) (Eisenreich and Rauch 2011, Yang, Nie et al. 2019). These observations support a role for PI3K/AKT signalling in TAAD pathogenesis and highlights its potential as a therapeutic target for MFS and other forms of TAAD.

An additional compelling finding pertains to the involvement of the HIF-1 pathway, as previously elucidated in Chapter 2.5.8. Subsequent omics analysis underscores HIF-1 as a pivotal regulator of oxidative stress signalling. It intricately interacts with the PI3K/AKT pathway, modulating EC response in altering NO production under stressful conditions such as hypoxia. Moreover, it instigates inflammatory responses within the hypoxic environment of the aortic wall and fosters VSMC proliferation (Lambert, Roy et al. 2010, Sun, Zhao et al. 2020). Furthermore, emerging as the sole significant pathway besides TGFβ from proteomics analysis, the significant involvement of HIF-1 signalling underscores the role of oxidative stress in TAA pathogenesis. This corresponds with the inflammatory pathways identified through other omics techniques.

The role of HIF-1 signalling in cardiovascular pathology has been extensively reviewed in ischemic heart disease, heart failure, cardiac remodelling, blood pressure abnormalities, arterial fibrillation, and other conditions (Sato and Takeda 2023). Clinical trials investigating HIF-1 pathway inhibitors such as roxadustat, vadadustat, and molidustat are underway to enhance cardiac function. While molidustat treatment has shown promising results in animal models, further studies are warranted to assess the long-term benefits of HIF-1 inhibitor treatments (Sousa Fialho, Purnama et al. 2021, Sato and Takeda 2023).

Moreover, in studies concerning AAA, HIF-1α inhibitors such as digoxin and 2ME (2-methoxyestradiol) have successfully attenuated aortic enlargement, medial degradation, VSMC loss, and inflammatory responses in murine AAA models (Wang, Xu et al. 2018). This underscores the significance of aberrant HIF-1 signalling in aortic dilation and its association with inflammatory responses in the aortic wall, thus presenting a potential avenue for implementing HIF-1 inhibitors in MFS TAAD patients to mitigate disease progression.

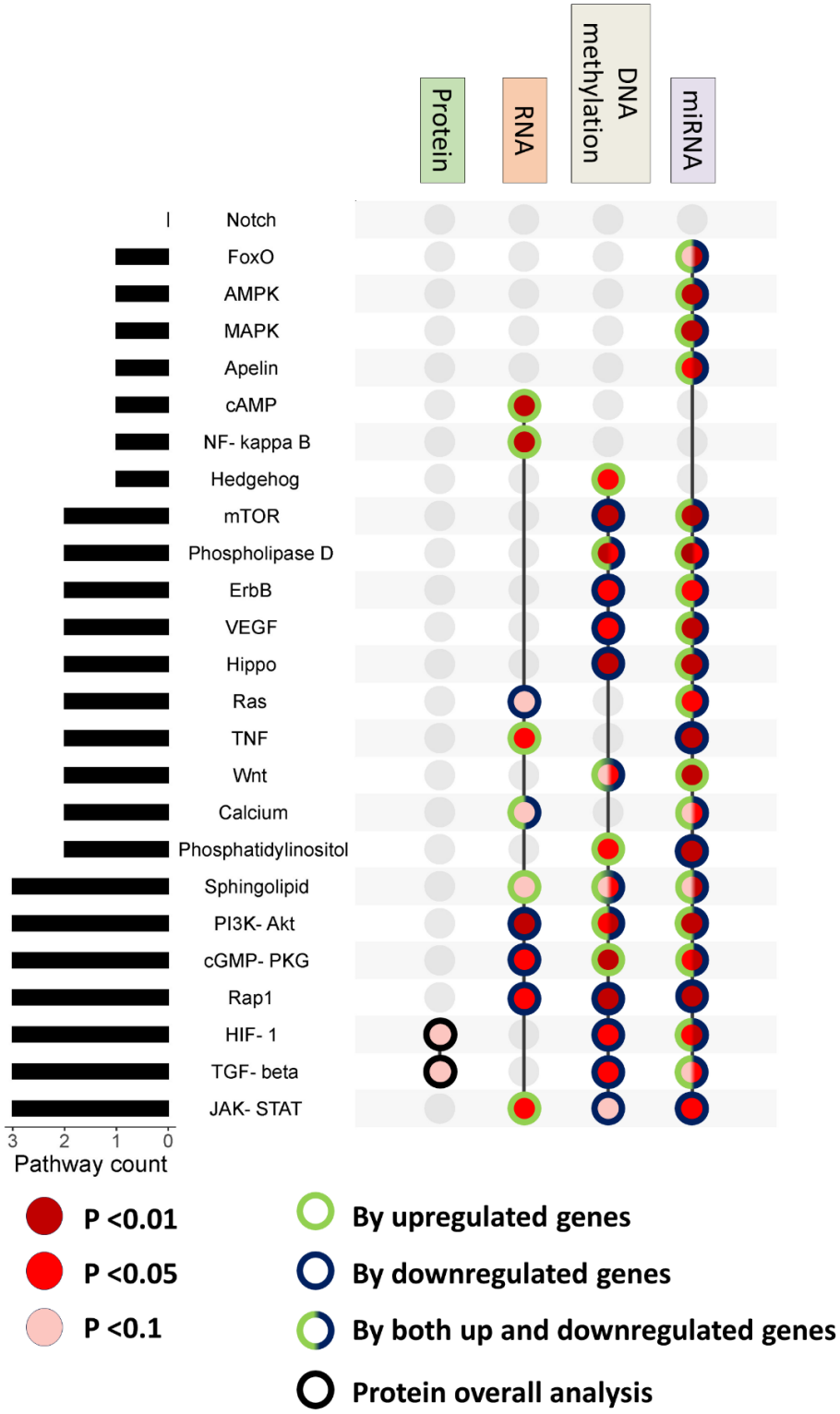


Figure 8.3 Upset plot of 4 omics analyses for KEGG pathways identification. The colours of the dots presented in the Upset plot indicate the significance level from the enrichment analysis, the colours of the outer rings of the dots indicate such pathway was identified by up-/ downregulated genes/proteins. The vertical bar on the left-hand side

indicates the number of times such pathway has been identified from different omics analysis. The labels at the top indicate the method of omics study.

8.3.1 Ang II signalling

In recent decades, extensive research has focused on Ang II signalling (part of the renin-angiotensin system) in the context of MFS, with several clinical trials and therapeutic drugs aimed at treating TAA (Wang, Deng et al. 2022). In Chapter 2.5.1, the mechanisms of Ang II and its interactions with other signalling pathways are summarized. Ang II signalling is not categorized as a signal transduction pathway in the KEGG database; instead, it belongs to the renin-angiotensin system, a component of endocrine system regulation. Interestingly, our omics analyses in MFS human aortic tissue did not reveal any significant association between the renin-angiotensin system or Ang II proteins. This raises the question of whether Ang II signalling actually contributes substantially to TAA formation in MFS. While Ang II can induce TAA formation in murine models, as discussed in Chapter 2.5.1, the situation may differ in MFS human tissues.

In MFS murine models, angiotensin receptor blockers (e.g., losartan) and beta-blockers are able to attenuate TAA formation (Nagasawa, Yoshimura et al. 2013, van Dorst, de Wagenaar et al. 2021). However, the results in human clinical trials with losartan and beta-blockers have been less clear cut (Detailed in Literature Review 2.3) (Yu and Jeremy 2018, Muiño-Mosquera and De Backer 2019). The present study of MFS human tissues did not identify a role of the renin-angiotensin system or Ang II signalling in MFS aortic tissue. This suggests that there may be fundamental differences in the aetiology of aortopathy between MFS TAA and murine models of TAA, stemming from factors such as size, metabolic rate, diets, microbiomes, pathogens, and breeding practices (e.g., the $Fbn1^{C1039G/+}$ mouse) and disease-inducing methods (e.g., Ang II infusion or other methods applied to induce TAA formation) (Perlman 2016, Cavanaugh, Qian et al. 2017). Therefore, despite animal studies providing invaluable insight into human disease and therapeutic development, we should recognise that there are limitations in the data obtained from animal studies.

8.4 CLINICAL SIGNIFICANCE, LIMITATIONS AND FUTURE DIRECTIONS

8.4.1 Clinical significance

Overall, the clinical significance of this study is that it has advanced our understanding of potential drivers in MFS TAAD pathogenesis. Through multi-omics analysis in MFS patients, we observed the involvement of previously identified factors like the inflammatory response and oxidative stress, as well as novel biological processes such as mitochondrial dysfunction and mechanical stress. Furthermore, through a close examination of the molecular functions involved, we identified extensive interactions between and synergistic effects of these biological processes that lead to VSMC, EC, and ECM dysregulation, subsequently contributing to TAAD formation.

Moreover, by examining epigenetic regulation in MFS, we identified two miRNAs (miR-154-5p and miR-424-5p) and six DNA methylation CpG sites (cg06099173, cg20365170, cg02407342, cg24797574, cg16343401, and cg20911989) that potentially play a role in MFS TAAD pathogenesis, regulating the inflammatory response, VSMC cellular response, heart muscle contractility genes, and mechanical stress. These regulatory mechanisms utilising epigenetic modifiers provide new insights into potential therapeutic targets in MFS TAAD.

Finally, the investigation of signal transduction pathways in MFS pathogenesis identified several pathways that correlate with different regulatory mechanisms, including transcriptional, translational, and epigenetic modulation. Our data demonstrate that the PI3K/AKT signalling pathway is ubiquitously involved in almost all omics regulatory processes and extensively interacts with other pathways, offering a potential option for pathway-based therapeutic intervention in MFS.

8.4.2 Limitations

The limitations of this study have been addressed in each chapter, and include sample size, experimental technical constraints inherent in omic technologies that interrogate the entire genome/proteome, pathway limitations, technical challenges, and statistical analysis. Despite being the largest human MFS tissue study to date, the sample size remains relatively small, posing a challenge in terms of its representation of the general population and patients with MFS. Notably, the accumulation of the tissue samples in this study has taken many years, and we continue to prospectively collect tissue samples for future studies.

The primary aim of this thesis has been to utilise four different omics techniques to investigate DE of genes/proteins, either directly (RNA-Seq and proteomics) or by inference (differential miRNA expression and DNA methylation). These data have then been used to predict likely changes in cellular function, particularly cell signalling pathways, that may underpin the pathogenesis of MFS. However, there are significant limitations to the use of DE patterns to clearly identify changes in signalling. An assumption that underpins the identification of signalling pathways is that DE of genes/proteins within that pathway implicates that pathway in pathogenesis. While this is a reasonable general assumption, there are significant limitations to this interpretation:

1. It is difficult to predict whether signalling is increased or decreased within a given pathway, based on DE alone. While abundance changes of particular proteins within a pathway suggest its possible involvement in the pathogenesis of MFS, additional more definitive evidence is required to support such a hypothesis, for example, measurement of signaling-relevant post-translational modifications (PTMs) (discussed in Chapter 5.4.1), for example, phospho-proteomics data (discussed in Chapter 4.5.4) (Savage and Zhang 2020). Thus, simple DE of abundance measurements have limitations for identifying alterations of signalling pathways, and further experimentation, beyond the scope of this thesis, should take place to validate such conclusions.

2. Changes in signalling activity during the pathogenesis of MFS may be subtle, which may correspond to minimal DE. This is especially the case for MFS, which is a chronic disease that takes up to decades to develop. Hence, the involvement of a particular pathway in MFS pathogenesis could be interpreted as a long-term (sustained) change in biological function rather than a large amplitude transient signal.

3. Following from point 2, only a small number of genes may undergo DE sufficient to observe above background noise, during activation or deactivation in a signalling pathway. Consequently, only a small number of DE genes may be identified within a particular signalling pathway, leading to uncertainty as to the involvement of that pathway in MFS pathogenesis. Additionally, this study utilized advanced disease-stage MFS aortic tissue, which represents an end-stage molecular change in the aortic aneurysm environment. The DE genes/proteins may signify sustained changes in biological function rather than transient alterations at the molecular level (or the phosphorylation state level), leading to long-term alterations in biological

outcomes (Fowler, Sen et al. 2011). Consequently, this results in a steady and long-term change in aortic wall signal transduction, providing insights into functional interpretation in the mechanisms of MFS.

4. Specific changes in particular genes (or patterns of genes) may relate to multiple pathways, not all of which may be involved in MFS pathogenesis, for example, GO analysis suggesting involvement of the neurological system. Consequently, as explained in Chapter 4.3.3, this study has focused specifically on KEGG signal transduction pathways to investigate the relevant alternations in MFS pathophysiology. Although some of the observed changes may seem peripheral among the pathway components, the study opens the possibility of pathway involvement regarding investigating the functional characteristics of underlying systematic change.

5. RNA alterations during a disease process are generally considered to be larger than the corresponding proteomic changes, making correlation between RNA-Seq and proteomic data more difficult (Dumitriu, Golji et al. 2016). This concept has been elaborated on in Chapter 6.5.1.

A further limitation that arises to the data presented in this thesis relates to the relative importance or weighting that can be given to DE genes/proteins identified in this study.

Within signalling pathways, certain proteins can be considered as central and highly relevant to that pathway, for example, ligands/receptors and downstream kinases, while other proteins may have only peripheral or tissue-specific involvement, e.g., protein products induced by the pathway or tissue-specific ligands. Thus, gene/protein weighting, in terms of pathway relevance, could provide additional evidence to support the importance of a particular pathway in the pathogenesis of MFS.

This thesis has not attempted to weight the relevance of DE genes/proteins identified, consistent with the input parameter (DE gene list) for the program (RStudio enrichKEGG package) used to identify pathways likely to be involved in the pathogenesis of MFS. However, an alternative approach to assessing the relevance of signalling pathways has been adopted in this thesis. To gain an insight into likely relevance of identified pathways, Figure 8.3 tabulates the number of omics techniques that have identified each specific pathway. Notably, the likely involvement of 7 pathways was identified by three different omics techniques,

including TGFβ signalling, 10 pathways were identified by two omics techniques and 7 pathways were identified by only 1 omics technique. Although some of the identified DE genes or proteins may play a peripheral role in the overview of the pathways, the fact that independent omics datasets identify common pathways may demonstrate the significance of the role of those particular pathways in MFS pathology.

Additionally, the experimental limitations highlight the need for further validation and a deeper understanding of our data. Techniques such as single-cell RNA sequencing and phospho-proteomics could provide more insights into signal expression at the individual cell level and the directionality of pathways in MFS TAAD. Furthermore, validation of miRNA and DNA methylation interactions should be performed to investigate and confirm the relationships between transcriptional, translational, and epigenetic regulation.

The presence of statistical issues has been acknowledged consistently throughout this study. While correction for multiple comparisons was applied for the RNA and protein analysis, the small sample size may have impacted the statistical analysis when using raw p-values. Although multiple testing correction were conducted and presented as a comparison to the raw p-value data, this discovery study was conducted based on raw p-values in all analyses. This decision was made to prevent the potential loss of informative data while exploring MFS TAAD. Similar considerations apply to the miRNA study, where the absence of a fold change threshold may introduce type 1 errors. In future research with larger sample sizes, we plan to implement both multiple testing corrections and fold-change criteria to enhance the statistical power and confidence in our investigations into MFS TAAD.

8.4.3 Future directions

As described in Chapter 8.4.1, in light of the limitations of this study, future research endeavours could address these shortcomings by increasing the sample size and conducting additional experiments and advanced statistical analyses to further advancing our understandings in MFS TAAD mechanism. This study provided a starting point for understanding signalling pathways in MFS TAAD. Some subsequential experiments can be applied to investigate the directionality of the pathways and verify some of the interesting finding like haemoglobin and cardiomyocytes contractile genes. Specifically, in vivo/ in vitro models could be

employed in future studies to explore the PI3K/AKT signalling mechanism in MFS TAAD, as well as the potential clinical application of PI3K/AKT modulators.

In addition, the significance of muscle contractility and abnormal haemoglobin regulation in the context of MFS TAAD should be investigated in the future studies. This investigation could potentially be accomplished through immunohistochemistry and single-cell RNA-Seq techniques to compare the expression of cardiomyocytes in the MFS heart to the potential small population within the aortic wall. This approach would allow for a deeper understanding of cardiomyocyte or cardiomyocyte progenitor/stem cell signalling and whether it mirrors the expression of cardiomyocytes within the heart. Additionally, we should aim to further validate alterations in epigenetic regulation to elucidate their mechanisms in MFS pathogenesis, potentially uncovering novel therapeutic targets for MFS aortopathies.

This study opens the possibility for further investigation of gene variants affecting key signalling proteins which may influence MFS phenotype. For instance, a linkage study investigated polymorphic mutations, such as DSC2 deletion (TCC/-), FBN1 nonsense (G/T), and LRP1 missense (G/A), in MFS patients. The results demonstrated that these variants could be associated with an increased risk of aortic dissection, dilation, and cardiomyopathy (Li, Yu et al. 2014). Subsequent investigations involving large-scale clinical genotype-phenotype comparisons in MFS manifestations associated with single nucleotide polymorphisms (SNPs) are warranted to further elucidate the signaling pathways impacted in MFS mechanisms. Therefore, this study proposes the exploration of genome-wide SNP screening to investigate MFS heterogeneity and its correlation with clinical parameters, e.g., aorta diameters. This approach could help determine whether specific SNPs impact disease outcomes and facilitate the implementation of polygenic risk scores, ultimately enhancing clinical assessment and disease intervention (Chagné, Crowhurst et al. 2012, Lewis and Vassos 2020).

8.5 FINAL CONCLUSION

In conclusion, the multi-omics analysis revealed several potential drivers in MFS TAAD pathogenesis. Inflammatory response, oxidative and mechanical stress, and mitochondrial dysfunction were identified as contributors to aortic wall VSMC, EC, and ECM dysregulation, ultimately leading to TAAD formation and progression. The combination of these biological processes appears to be mediated by

transcriptional-translational mechanisms and epigenetic regulation. These findings significantly advance our understanding of MFS TAAD pathogenesis and offer novel therapeutic targets for interventions in MFS TAAD.

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