

**Investigating the micro-RNA and
metabolic signature of human post-
operative atrial fibrillation**

A thesis by

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For my parents, Susan and Kevin, and my sister Laura.

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Abstract

Atrial fibrillation (AF) is the commonest disorder of cardiac rhythm. Following coronary artery surgery approximately 1 in 3 patients will develop *de novo* post-operative AF (POAF) leading not only to prolonged hospital stay but also to increased morbidity and mortality. The pathophysiology of this arrhythmia is highly complex, and combines factors such as ion channel dysfunction, inflammation, oxidative stress, fibrosis and autonomic dysfunction that through electrical and structural remodelling promote both triggering and maintenance of this arrhythmia. For many years POAF has been regarded as a reactive phenomenon, occurring in response to post surgical inflammatory stressors and electrolyte imbalance. However, it is also possible that in a proportion of patients, prior cardiomyocyte remodelling predisposes to atrial arrhythmogenesis when exposed to surgical stress.

Understanding the genomic and metabolic mechanisms that underlie this substrate may not only provide novel diagnostic biomarkers to identify at risk patients, but also isolate previously unrecognised therapeutic targets for prevention and treatment. In this work, a clinical observational study was utilised to obtain microRNA, gene expression and metabolic profiles of patients developing POAF after coronary artery bypass graft (CABG) surgery. Based on these results, a network of genomic and subsequent downstream pathway interactions was established to characterise the atrial substrate of post-operative AF. Furthermore, analysis of pre-operative blood was performed in order to identify novel microRNA that may provide a platform for biomarker development. Finally, the metabolic signature of the atrial myocardium and its relationship with the surrounding epicardial adipose were investigated to complete a comprehensive overview of the central mechanisms thought to underlie POAF pathogenesis.

This work demonstrates that prior to surgery and the onset of arrhythmia, several distinct genomic and post-translational characteristics are evident in the both the myocardium and

circulating blood of patients going on to develop POAF. Analysis of right atrial biopsies highlights a characteristic microRNA profile associated with POAF, and identifies target genes regulating intracellular signalling pathways, leukocyte recruitment, and ion channel remodelling. Furthermore, selected gene expression analysis demonstrates a de-differentiated phenotype similar to that seen in chronic AF, whilst at the same time establishing that disordered cardiomyocyte calcium handling is apparent at the time of surgery. Finally, analysis of the pre-operative circulating blood serum highlights microRNA selectively upregulated in POAF and establishes the potential for future biomarker development.

In summary, the results presented here support the presence of a pre-existing atrial substrate in POAF, suggesting the potential exists for high-risk patients to be identified prior to surgery and the onset of arrhythmia. Furthermore, for the first time a number of similarities have been made apparent between post-operative and chronic AF, implying a common mechanistic spectrum of structural and electrical remodelling. As a consequence, the results presented in this thesis have not only improved our understanding of the complex interplay of factors leading to the pathogenesis of AF, but also provide a platform for both the development of a unique clinical biomarker and the identification of novel therapeutic targets.

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Statement of Originality

I hereby acknowledge that this thesis and the research it describes are the product of my own work, and that where it has been the result of collaboration with others, this is fully described. Quotations from the work of other researchers are fully acknowledged in accordance with standard referencing practice.

Leanne Harling

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Chapter 1

Introduction

1.1 Atrial Fibrillation: Definition

Atrial fibrillation is the commonest sustained disorder of cardiac rhythm.(1) It is characterised by fast, irregular and uncoordinated atrial depolarization and a loss of atrial mechanical function. Electrocardiography (ECG) typically demonstrates loss of 'P' waves with a fibrillatory baseline, and where atrioventricular conduction is intact there is an irregular, often fast, ventricular response ('QRS' depolarization). Clinically, AF may be asymptomatic, however acute paroxysms often present as palpitations and may be associated with dizziness, chest pain or even loss of consciousness in severe cases. Atrial fibrillation also leads to pooling and stasis of blood within the atria, which may subsequently lead to thrombo-embolism and stroke and associated increases in morbidity and mortality.(2)

1.2 Classification

Atrial fibrillation may be classified into 3 subtypes according to its duration and pattern of recurrence. Atrial fibrillation is classified as 'recurrent' when 2 or more episodes have occurred. Recurrent AF self-terminating within 7 days is classified as 'paroxysmal AF', however the frequency and symptomatology of these paroxysms may be variable. Multiple paroxysms over a short time period may degenerate into persistent AF when episodes no longer self terminate. Persistent AF is defined as recurrent AF lasting for more than 7 days which does not self terminate but requires medical or electrical cardioversion to terminate within 1 year. Permanent established AF is reserved for those cases where AF remains for longer than 1 year and medical or chemical cardioversion have failed or are not attempted. Previously terminated AF that has relapsed may also be considered in this category. It is

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notable that ‘permanent AF’ may be reversed in some cases whereby surgical or catheter ablation techniques are used to alter the conduction properties of the myocardium and restore sinus rhythm.(1) A summary of atrial fibrillation subtypes is shown in **Table 1-1**.(1, 3)

Classification	Definition
Recurrent AF	2 or more episodes. Includes paroxysmal and persistent AF
Paroxysmal AF	2 or more episodes which are self-terminating and last less than 7 days in duration
Persistent AF	Atrial fibrillation lasting more than 7 days but terminating within 1 year either spontaneously or by means of electrical or chemical cardioversion
Permanent AF	Atrial fibrillation lasting more than 1 year where electrical or chemical cardioversion have been unsuccessful or not attempted.

Table 1-1: Classification of Atrial Fibrillation

1.3 The Burden of Atrial Fibrillation

Based on 38-year follow up from the Framingham Heart Study, the lifetime risk of atrial fibrillation is estimated at 1 in 4 individuals.(4) In their 1998 study of 211 general practice (GP) surgeries (1.4 Million patients), Majeed et al. found a prevalence of AF of 12.1/1000 men and 12.7/1000 women across England and Wales.(5) A further UK-wide assessment in 1995 estimated the total number of patients living with AF to be approximately 534 000.(6) In the United States, results from the Framingham study identified 2.2 Million adults suffering from AF, with the prevalence doubling with every decade of age (**Figure 1-1 (a)**).(4) Furthermore, Atrial fibrillation was also more common in men than women, even when adjusting for AF risk factors and co-morbidities (**Figure 1-1 (b)**).

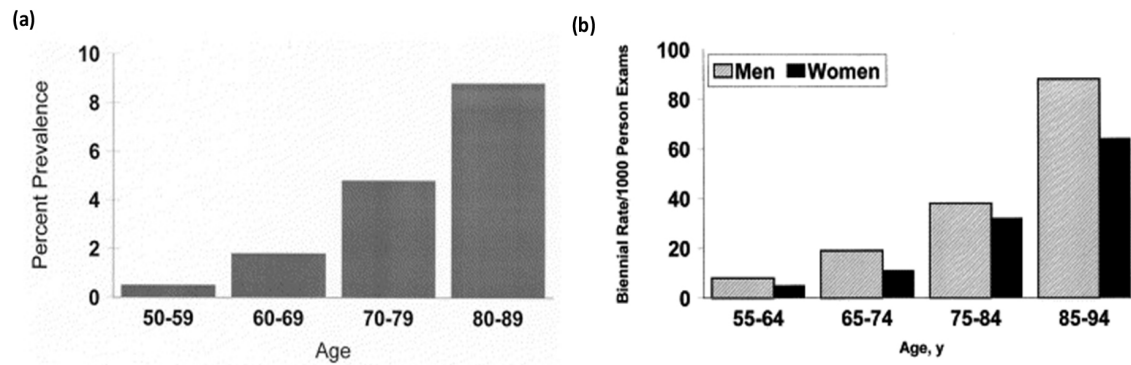


Figure 1-1: (a) Age related increase in prevalence of AF in men and women; (b) Sex related differences in AF with increasing age. Data from Framingham heart study database. Images taken from Kannel et al. 1998.(4)

The global burden of AF is increasing, and it is likely that the current prevalence is now substantially higher than these figures taken from large historical epidemiological studies. In their aforementioned study, Majeed et al. were able to demonstrate an increase in AF prevalence of 22% between the start of their study in 1994 and its conclusion in 1998.(5) This echoes earlier findings from the US Framingham report whereby retrospective analysis of national hospital discharge survey data demonstrated an increase in the age-standardized prevalence of AF from 3.2% to 9.1% in men and 2.8% to 4.7% in women between 1968 and 1989.(4) In 2006 NICE estimated that when applied to the 2006 national population, the work by Majeed et al would give an estimate of 639,000 cases of AF in England, resulting in a national prevalence rate of 1.28%.(7)

The US retrospective Medicare Beneficiary Study gives a recent estimate of AF prevalence in 433, 123 patients between 1993 and 2007. Whilst AF incidence was 1.94% and 7.3% in 1993 in 66-69 year olds and 85-89 year olds respectively, this increased to 3.61 and 16.25% in 2007 (**Figure 1-2**). (8) In Europe, one recent Spanish multicenter observational study of 1043 patients >60 years suggested an overall prevalence of AF of 10.9%, 20.1% of which was undiagnosed.(9) Furthermore, 14% of patients 75-79 years of age suffered from the condition, increasing to 19.6% and 24.4% at 80-84 and ≥ 85 years respectively.

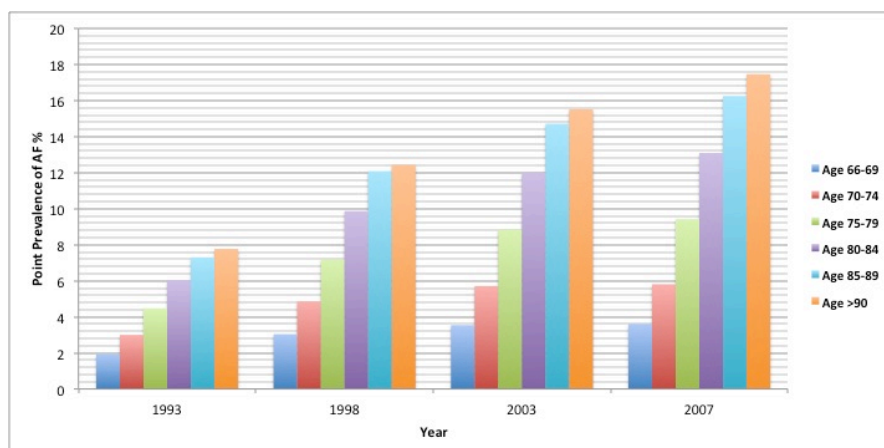


Figure 1-2: The point prevalence of AF with age demonstrating increases from 1993 to 2007. Data taken from the Medicare Beneficiary Study, Piccini et al. 2012. (8)

A number of risk factors and co-morbidities pre-dispose to an increased risk of atrial fibrillation within the general population (**Table 1-2**).

Risk Factor	Relative Risk	Odds Ratio	95% Confidence Interval
Data taken from meta-analysis by Ball et al. 2013 (10)			
Hypertension	73%	1.73	1.31, 2.28
Male sex	64%	1.64	1.45, 1.86
Acute Coronary Syndrome	77%	1.77	1.44, 2.19
Diabetes	44%	1.44	1.07, 1.94
Hyperthyroidism	68%	1.68	1.29, 2.18
Obesity (BMI$\geq$30)	22%	1.22	1.14, 1.30
Smoking	47%	1.47	1.22, 1.78
Data taken from Kannel et al. 1998 (4)			
Heart Failure	Men: 4.5x		Women: 5.9x
Valvular heart disease	Men: 1.8x		Women: 5.9x
LA enlargement	39% per 5 mm increase in diameter		
LV fractional shortening	34% per 5% reduction in FS		
LV wall thickness	28% per 4 mm increase in thickness		

Table 1-2: Relative risk of factors contributing to the incidence of Atrial Fibrillation. LA – Left Atrium, LV – Left Ventricle, FS – Fractional Shortening.

The most significant of these are Valvular heart disease, heart failure, acute coronary syndrome and hypertension.(4, 10) Studies have also indicated an association of chronic

conditions such as pulmonary disease, renal dysfunction, rheumatoid arthritis and coeliac disease with an increase in the incidence of AF. Furthermore, both obesity (BMI $\geq$ 30) and sleep apnoea may also increase AF prevalence. Bearing in mind the emerging global epidemic of obesity and given that overall more than 10% of the world's population are now obese,(11) it is possible that this association may contribute to rises in AF prevalence over forthcoming decades, particularly in younger age groups.

1.3.1 The economic cost of AF

In their economic analysis of atrial fibrillation in the general population, Stuart et al. estimate the "direct" cost of health care for patients suffering from AF to be £244 Million, which accounted for 0.62% of total NHS expenditure in 1995. Furthermore, due to the aging population and increasing prevalence of AF, they predicted an increase in this figure to £459 Million by 2000, accounting for 0.97% of total NHS expenditure. In 2006 the National Institute for Clinical Excellence (NICE) produced a detailed cost analysis of for all AF across England based upon the number of cases of AF, the cost of diagnosis and treatment and taking into account savings made by avoidance of stroke due to therapeutic anticoagulation. (7)The total cost of fully implementing clinical guidelines on the diagnosis and management of AF was calculated at £21.86 Million per annum (**table 1-3**).

	Resource impact, £million
All cases of suspected AF confirmed by ECG	4.10
Change in cost of anti-coagulant provision	63.54
Total direct cost	67.64
Costs avoided due to reduction in strokes	-54.33
Cost incurred due to major bleeds	6.27
Cost incurred due to minor bleeds	2.29
Total indirect costs	-45.78
Net resource impact	21.86

Table 1-3: Total cost analysis of significant resource impact recommendations for Atrial Fibrillation in England (Taken from NICE CG036 costings report, 2006).(7)

The specific cost of post-operative AF (POAF) has been estimated to range from \$2574 - \$6356 per patient.(12, 13) As described in the above analysis of all cause AF this expenditure reflects increased resource utilisation in several areas but also includes increased hospital stay, use of ward and ITU consumables, nursing care, laboratory fees, imaging, pharmacotherapy and anaesthetic support.(13)

1.4 Atrial Fibrillation Pathophysiology

Atrial fibrillation is a multi-system disease with a complex underlying pathophysiology. Established AF requires a combination of triggering factors acting to initiate rhythm disturbance, and maintaining factors, which allow the arrhythmia to become sustained. Broadly there are two distinct concepts as to the propagation of electrical activity in atrial fibrillation: the single and multiple wavelet theories. The former suggests that atrial fibrillation involves a single macro re-entrant circuit that may originate from either a fixed or variable site of rapidly firing atrial myocardium. As this single re-entrant circuit propagates, it meets areas of refractory myocardium thus leading to fibrillatory contraction. The latter multiple wavelet theory describes AF as a number of irregular, dysynchronous re-entrant circuits which may either self propagate or initiate each other. As with the single wavelet theory, these circuits are disrupted by areas of refractoriness and conduction block within the myocardium producing fibrillatory contraction.(14-16)

In their seminal 1998 work, Haissaguerre and colleagues mapped the site of electrical triggers for atrial fibrillation to predominate from the region of the left atrium surrounding the pulmonary veins.(17) However, it is also recognised that non-pulmonary vein triggers may initiate AF, including the autonomic ganglionic plexi and other regions of atrial myocardium exhibiting complex fractionated atrial electrograms.(18) Triggering of AF from within these regions relies on a number of factors that produce changes in the cellular properties of the myocardium enhancing excitability, increasing automaticity and reducing the refractory

period. Once initiated, maintenance of atrial fibrillation requires changes to the atrial substrate such that multiple re-entrant circuits may be set up. This atrial re-modelling may be a combination between electrical, mechanical and structural changes that vary in contribution according to the underlying cause and duration of AF.

1.4.1 Electrical Remodelling: 'AF begets AF'

Once AF is established, tachycardia produces further atrial electrical remodelling perpetuating the maintenance of the arrhythmia. Increased inducibility of depolarization and shortening of the effective refractory period (ERP) leads to increased susceptibility to repeated depolarization. In this way 'AF begets AF' and progression from an initial triggering insult may result in sustained arrhythmia.(19, 20)

A number of ion flux changes associated with electrical remodelling both in the triggering and maintenance of AF. These are summarised as follows:

Calcium

Changes in intracellular calcium handling play a role in both the initiation and maintenance of AF. The commonest cause of electrical triggering (focal ectopic discharge) is afterdepolarizations which may occur before (early) or subsequent to (late) full repolarization.(21) Early afterdepolarization occurs secondary to prolongation of the action potential duration and recovery of the L-type calcium current (I_{CaL}) allowing Ca^{2+} influx into the cell. This then depolarizes surrounding myocardium leading to ectopic discharge. Conversely, late afterdepolarization and subsequent spontaneous action potential generation occurs due to a transient rise in intracellular Ca^{2+} generated by the sodium-calcium exchange current (NCX, **figure 1-4**) during diastole.(21)

Furthermore, calcium handling is also implicated in the maintenance of AF. Atrial stretch occurring secondary to impaired diastolic function increases intracellular Ca^{2+} via stimulation of stretch activated calcium channels, producing Ca^{2+} mediated Ca^{2+} release from the sarcoplasmic reticulum.(22) Animal models of AF utilising rapid atrial pacing demonstrate a reduction in transient outward (I_{to}) and L-type calcium currents (I_{CaL}), with a subsequent shortening of action potential duration (APD). In studies of human atrial cardiomyocytes, this reduction in I_{CaL} has been shown to be as much as 73% in chronic AF, with slowed I_{CaL} inactivation, decreasing Ca^{2+} influx during tachycardia.(23) This shortening of APD allows a larger number of re-entrant circuits to be set up and sustained, increasing the likelihood of fibrillatory contraction.

Sodium

Pacing induced tachycardia has been associated with a reduction in the sodium current (I_{Na}) in animal models. This slows conduction velocity and shortens the action potential duration in turn facilitating re-entry as previously described. Furthermore, patch clamp studies of isolated human right atrial myocytes demonstrate a shift in voltage-dependent inactivation of I_{Na} towards more positive potentials in chronic AF thus potentially increasing AP amplitude and facilitating its propagation.(23) However, despite these findings the role of I_{Na} in human atrial fibrillation remains uncertain. Recent evidence suggests that a loss of function mutation in the voltage gated sodium channel SCN5A may contribute to lone familial AF although the mechanism by which this occurs is yet to be determined.(24)

Potassium

Increases in the inward rectifying potassium current (I_{K1}) have been demonstrated in patients with chronic AF when compared to those in sinus rhythm.(25-27) This results in heightened repolarization and thus reduces the ERP and prolongs the APD allowing for the setting up of re-entrant circuits. Changes in I_{KACH} have also been associated with AF and may be in the

form of a protective down-regulation of $I_{K_{ACh}}$ in an attempt to balance other ion flux changes producing a reduction in ERP,(26) or as a primary cause of ERP shortening secondary to increased autonomic activity. In the latter instance, vagal stimulation releases Acetylcholine and activates the atrial ACh regulated potassium current leading to a reduction in the duration of phase 3 (repolarization) of the action potential and thus ERP shortening (**Figure 1-4**).(28) Finally, a reduction in the transient outward potassium current (I_{to}) is also demonstrated during AF electrical remodelling. This is thought to subsequently encourage AP propagation by increasing the amplitude of the action potential.(29)

Defects in ion channels (or ‘channelopathies’) can therefore predispose to AF through the alteration of ion channel activation and atrial conduction properties. In familial AF, single nucleotide polymorphisms at the chromosome 4q25 locus (notably the rs2200733 and rs10033464 SNPs) have been associated with dysfunction of a number of ion channels including the I_{Ks} potassium channel (α -subunit encoded for by the KCNQ1 gene), the I_{Kur} potassium channel (encoded for by KCNA5) and the sodium I_{Na} channel (encoded for by SCN5A). These alterations in ion channel expression have been found to be mediated through the paired-like homeodomain transcription factor 2 (PITX2) gene located adjacent to the 4q25 locus.(30) Furthermore, other genewide linkage studies have also demonstrated SNPs at the 1q21 locus (rs13376333) affecting transcription of the KCNN3 gene encoding for the voltage-independent calcium-activated channel. In addition to these SNPs affecting promoter regions of genes encoding the ion channels mentioned above, familial AF has been associated with a number of mutations directly affecting particular ion channel genes. These include missense mutations in the KCNJ2 (encoding the Kir2.1 protein forming the α -subunit of the I_{K1} channel), KCNE 1-5 (encoding for the β -subunit of the I_{Ks} channel), and KCNH2 (encoding for the α -subunit of the cardiac I_{Kr} channel) genes as well as non-sense mutations in the KCNA5 gene.(31)

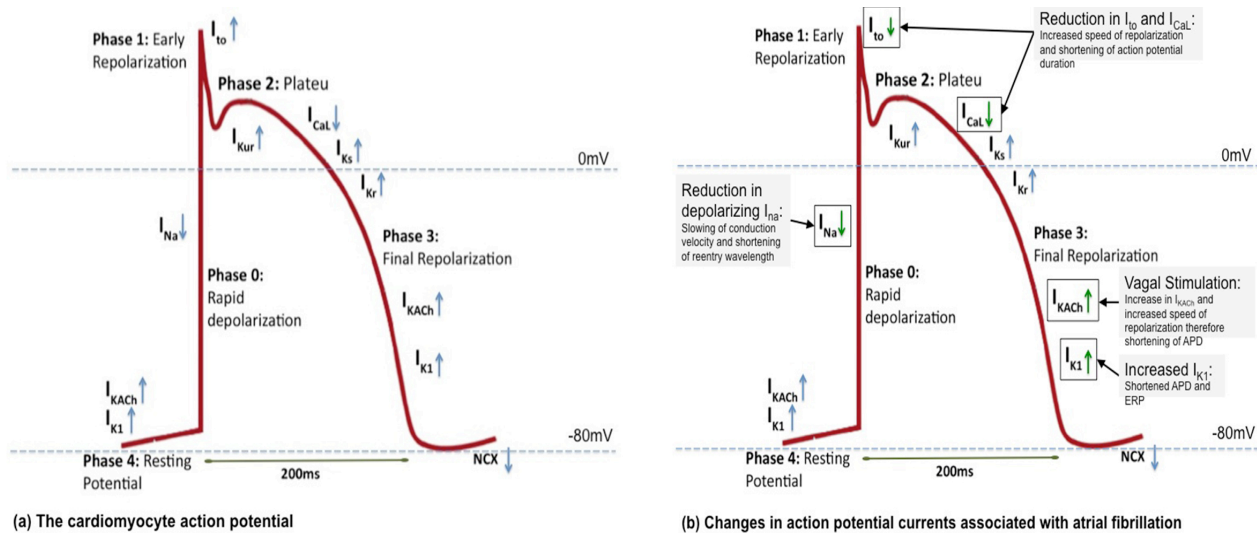


Figure 1-3: The cardiomyocyte action potential and ion flux changes associated with atrial fibrillation

1.4.2 Structural Re-modelling

Macroscopically, loss of coordinated atrial contraction in AF impairs diastolic filling and reduces stroke volume leading to pooling of blood in the atria, increased atrial stretch and an enlargement in atrial dimensions. These changes stimulate the release of natriuretic peptides, alter intracellular calcium handling, activate the renin-angiotensin system, and promote the release of inflammatory cytokines and proteins such as Endothelin-1 and heat shock proteins. On a cellular level, this gives rise to a number of changes globally termed ‘atrial structural remodeling’ which include: (i) loss of perinuclear sarcoplasmic contractile structures and their replacement with glycogen resulting in a gradual loss of contractile function; (ii) Interstitial fibrosis with an increase in extracellular matrix, fibroblasts and collagen deposition; (iii) de-differentiation of cells to a more fetal phenotype and (iv) cellular apoptosis.(22, 32) Furthermore, impairment of gap junction function may also result in localized conduction block thus promoting re-entry and fibrillatory contraction.

The renin-angiotensin system

The renin-angiotensin (RAA) system may be activated in AF both by atrial stretch and secondary to atrial fibrosis. Interstitial fibrosis promotes atrial fibrillation by producing areas of conduction block that prevent action potential propagation and promote re-entry. Atrial stretch may foster fibrillation by several mechanisms, and has been shown to both directly activate the angiotensin receptor 1 (AT1R) and to promote transcription of genes encoding angiotensinogen and angiotensin converting enzyme (ACE) in turn increasing angiotensin-II (ATII) production.(22, 29) Through binding with the AT-1 receptor, AT-II may stimulate fibrosis and cellular apoptosis via the Ras signaling cascade, as well as promoting intracellular calcium release by activating protein kinase C (via inositol 1,4,5-trisphosphate (IP3)).(29)

Angiotensin II also activates membrane associated protein kinases (MAPKs), which consequently stimulate the production of pro-fibrotic mediators such as TGF- β , and connective tissue derived growth factor (CTGF) via signaling pathways involving tumor necrosis factor receptor associated factor 6 (TRAF6) and TGF- β -associated kinase 1 (TAK1).(33) Furthermore, TGF- β itself activates angiotensin II producing a positive feedback mechanism leading to fibrosis, collagen deposition, and the production of extracellular proteins including fibronectin, and proteoglycans.(34)

Atrial overload and Platelet Derived Growth Factor (PDGF)

Atrial fibrillation is associated with a pro-inflammatory state as demonstrated by histological evidence of inflammatory cell infiltrate and the prevention of atrial structural remodeling in mast cell deficient C57BL/6 mice.(35, 36) Recently, Liao et al. have highlighted the link between mast cell activity and upregulation of platelet derived growth factor-A (PDGF-A), demonstrating abolition of atrial fibrosis with antibody blockade of the PDGF receptor PDGFR- α . PDGF itself promotes atrial fibrosis by stimulating tyrosine kinase receptors

which themselves autophosphorylate, initiating several intracellular signaling cascades including the MAPK and phospholipase C pathways. PDGF then exerts both autocrine and paracrine effects on a number of other growth factors and cytokines to promote fibroblast proliferation and extracellular matrix protein deposition as described above. Interestingly, DNA microarray and subsequent qPCR validation experiments have demonstrated significant increases in PDGF and PDGFR- α expression in canine atria when compared ventricular myocardium, suggesting that PDGF may play a role in the atrial specificity of fibrosis in AF.(37)

Gap junctions and Connexins

Connexins are transmembrane gap junction proteins that regulate cell-cell communication, forming low resistance pores that allow transmission of small molecules or ions from one cell to the next. Gap junctions form the key mechanism by which action potentials may be conducted between cardiomyocytes and disruption of these tightly regulated channels may produce conduction block, variable refractoriness and potentiate arrhythmogenesis.(38) Whilst there are 3 connexins present in atrial cardiomyocytes, Cx40, Cx43 and Cx45, it is Cx40 and Cx43 that have been identified to play a role in the pathophysiology of AF. Increased expression of these two connexins (Cx40 and Cx43), has been shown to reduce susceptibility to atrial fibrillation in animal models, however whether this is predominantly due to increased refractoriness or slowed cell-cell conduction remains to be determined.(39, 40)

Connexins also play a fundamental role in the unique anisotropic conduction properties of cardiac myocytes. A shift in the localization of these proteins from the intercalated disc to the lateral cell membrane may result in a reduction in anisotropic conduction. Indeed, analysis of atrial tissue biopsies from chronic AF patients has demonstrated lateralization of Cx43,

without changes in the absolute number of Cx43 proteins, is associated with an increase in action potential propagation velocity transverse to the myocyte fibers.(41)

The function of connexins may also be regulated by phosphorylation of their cytoplasmic C-terminal domains, with connexin activity being increased in the phosphorylated state.(42) Hypophosphorylation of connexin 43 has been associated with chronic AF in both animal models and human patients, and increased phosphorylation of connexin 40 has been demonstrated in the right atrial appendage of mitral valve disease patients with chronic AF.(41, 43) It has also been recognized that AF associated increases in oxidative stress and inflammation play a part in the mechanism of this re-distribution of connexins by reducing microtubule trafficking of connexins to the intercalated discs.(44)

In summary, variation in the number, localization and phosphorylation state of connexins may all contribute to the creation of a susceptible substrate in which AF may be set up and maintained. However, the exact interplay between these factors in different subgroups of human AF remains unclear and is the subject of extensive ongoing research.

1.4.3 Inflammation

Atrial fibrillation has been associated with a generalized pro-inflammatory state, however whether this inflammatory environment is produced as a consequence of or predisposes to AF continues to remain a subject of debate. Inflammatory mediators implicated in AF pathogenesis include the interleukins- 1, 2, 6, and 8 (IL-1, IL-2, IL-6 and IL-8), tumour necrosis factor- α (TNF- α), C-reactive protein (CRP), heat shock proteins, and matrix metalloproteinases.

Interleukins

Interleukin-1 is the most recent of the IL family to be implicated in AF pathogenesis and consists of a group of 11 cytokines that have a range of pro- and anti-inflammatory properties. Interleukin-1 receptor antagonist (IL-1RN) is a pro-inflammatory gene located at the IL-1 locus that has recently been implicated in lone AF. In their prospective study of 140 patients (70 lone AF, 70 non-AF), Gungor et al. demonstrated a significantly higher frequency of the allele 2 VNTR (variable number of the tandem repeat) polymorphism of IL-1RN in lone AF when compared to controls. Furthermore, the presence of this allele 2 polymorphism was found along with elevations in CRP to be an independent predictor of lone atrial fibrillation in multivariate regression analysis.(45)

Interleukin-2 is a pro-inflammatory cytokine whose primary role is in T-cell growth and development. High admission serum levels of IL-2 have been shown to correlate with failure of medical conversion (amiodarone) in new onset AF, highlighting a role of acute inflammation in AF maintenance.(46) Furthermore, IL-2 has also been implicated in the pathogenesis of post-operative atrial fibrillation (POAF). Increased levels of Interleukin-2 have been demonstrated pre- and at 3 hours after coronary artery bypass grafting in patients developing early de novo POAF when compared to those who maintain sinus rhythm. However, this did not remain true for those patients developing POAF between 2 and 5 days after surgery.(47)

Interleukin-6 is an acute phase cytokine with both pro- and anti-inflammatory properties. Increased levels of IL-6 have been associated with both chronic and post-operative AF.(48-51) Furthermore, high IL-6 levels may predict a worse prognosis and increased risk of adverse cardiovascular events in anticoagulated patients with chronic AF.(52) Increased levels of IL-6 at 6 months follow up were also associated with recurrence of AF in patients receiving valsartan pharmacotherapy in one prospective, multicentre randomised controlled

trial of 382 patients, although no association was found between pre-treatment IL-6 levels and the risk of AF recurrence.(53) Genewide association studies have demonstrated several single nucleotide polymorphisms in the IL-6 receptor gene that are associated with AF. Recently, the IL-6 -634C/G polymorphism has also been correlated with AF in a hypertensive Han Chinese population.(54)

Interleukin-8 is a pro-inflammatory cytokine produced by macrophages, monocytes, endothelial cells and fibroblasts that predominantly acts to induce neutrophil chemotaxis and promote subsequent phagocytosis. In their study of 113 patients undergoing CABG, Wu and colleagues found significantly higher levels of IL-8 on post operative days 1 and 2 in patients developing POAF compared to those who did not. Interestingly, this group did not demonstrate significant differences in IL-6 between POAF and non-POAF patients.(55) Conversely however, Ishida et al. did not demonstrate any difference in IL-8 levels between patients developing POAF after off pump surgery compared to those maintaining sinus rhythm, whereas IL-6 levels at 3 and 6 hours were significantly higher in the POAF group.(56) Increased IL-8 levels have also been demonstrated in chronic AF when compared to controls. Furthermore, in their study of 29 patients undergoing radiofrequency catheter ablation, Liuba et al suggested plasma IL-8 levels were also higher in patients with permanent AF when compared to paroxysmal AF when taken from sites including the femoral vein, right atrium, and coronary sinus.(57)

Tumour Necrosis Factor- α

Tumor necrosis factor- α is a multifunctional pro-inflammatory cytokine secreted by macrophages, monocytes, natural killer and T-cells in response to stimulation by bacterial lipopolysaccharide. By acting on 2 receptors, TNFR1 and TNFR2, TNF- α activates a number of signaling cascades involved in inflammation, cell proliferation and apoptosis.(58) In their study of 67 patients undergoing cardiac surgery, Deng et al observed a significant increase in

TNF- α in the blood plasma and left atrial tissue of patients with chronic atrial fibrillation compared to those in sinus rhythm. Furthermore, the levels of soluble TNFR1 were also significantly higher in AF vs. non-AF patients.(59) In a mixed cohort of permanent, persistent and paroxysmal AF, Cheng et al. similarly demonstrated a rise in TNF- α in AF vs. non-AF and found TNF- α to be an independent predictor of AF multivariate analysis (OR 1.80; 95% CI, 1.03-3.20).(60) It is thought that TNF- α may increase susceptibility to AF by producing structural remodeling and atrial fibrosis. In a recent mouse model, systemic TNF- α infusion produced significantly more atrial fibrotic changes when compared to control animals, with a corresponding reduction in connexin 40 and borderline decrease in connexin 43 expression. Furthermore, TNF- α infusion produced up regulation of Smad3 suggesting involvement of the TGF- β /Smad signaling pathway which stimulates fibroblast proliferation and collagen deposition.(61)

C-Reactive Protein

A number of studies have correlated increased levels of CRP with an increase in the risk of both all cause and post-operative atrial fibrillation. However, two recent large population studies using a Mendelian randomization design have questioned the causative nature of this relationship. The Copenhagen General Population Study (CGPS) began in 2003 and is a cross-sectional study including a total of 36 000 individuals. In their 2010 analysis of this dataset, Marott et al. examined the potential causal association between elevated CRP levels and increased risk of AF by examining whether CRP genotype combinations correlated with increased atrial fibrillation risk in line with their long-term elevated plasma CRP levels.(62) Increased high sensitivity C-reactive protein (hsCRP) was associated with a significant increase in unadjusted AF ($p < 0.001$) as well as AF after multifactorial adjustment for age, gender, statin use and genotype. Further adjustment for heart failure and fibrinogen reduced the risk of AF however this remained significant ($p = 0.02$ and 0.03 respectively). However, CRP genotypes/polymorphisms known correlate with life-long increases (rs1130864 and

rs3091244) and decreases (rs3093077) in CRP levels did not correlate with a respective increased or decreased risk of AF. Furthermore, these observations were echoed in a similar study by Alegret et al. (n=375 controls, 249 AF) where increases in plasma CRP were found in patients with permanent AF when compared to controls but no difference in distribution of genotypes associated with elevated CRP was seen between AF and non-AF groups.(63) Interestingly, this study did not demonstrate a difference in plasma CRP between paroxysmal AF and controls and plasma CRP was not an independent predictor of AF in their risk adjusted multivariate model.(63)

1.4.4 Oxidative Stress

The potential role of oxidative stress in the initiation and maintenance of AF is becoming increasingly recognised. Not only has AF been associated with a shift in gene expression, promoting genes coding for reactive oxygen species (ROS),(64) but peroxynitrite and hydroxyl radicals have been identified in the atrial appendages of patients with chronic AF.(65) In their canine model of AF, Carnes and colleagues go on to link this oxidative stress to electrical atrial remodelling. Using rapid *in vivo* pacing of canine atria they observed an increase in markers of peroxynitrite and a reduction in effective refractory period (ERP).(66)

Although the mechanisms by which ROS affect electrophysiological remodelling remain unclear, several factors are likely to contribute to this process. Firstly, ROS may cause modulation of intracellular Ca^{2+} , resulting in cytosolic Ca^{2+} overload, a reduction in the L-type calcium current (I_{Ca}), and alterations in excitation-contraction coupling. In an initial attempt to counteract this Ca^{2+} overload, a compensatory reduction in action potential duration (APD) and ERP is observed, promoting re-entry and development of tachyarrhythmia.(67) Secondly, post- translational modifications caused by peroxynitrite and hydroxyl radicals may generate both structural and functional changes in myofibrillar proteins, potentiating arrhythmogenesis by impairment of myofibrillar contractile

function.(65) In particular, irreversible peroxynitrite- induced inhibition and nitration of monomeric myofibrillar creatinine kinase (MM-CK) may reduce the supply of high energy phosphate to myosin ATPase, negatively affecting myofibrillar contractile function.(68) Finally, oxidative stress may lead to a derangement in ion channel regulatory mechanisms in turn leading to the generation and perpetuation of AF. ROS may both accelerate the activation kinetics and reduce the inactivation of voltage gated K^+ channels, resulting in a reduction in APD and ERP and promoting re-entry.(69) Furthermore, oxidative stress may enhance slow inactivating Na^+ current (I_{Na}) and slow inactivation of voltage gated Na^+ channels,(70, 71) promoting diastolic depolarisation, action potential generation and arrhythmogenesis.

As mounting evidence for the role of oxidative stress in AF pathogenesis has become apparent, antioxidant vitamins have been explored as both preventative and therapeutic agents. Vitamins C and E are chain- breaking antioxidants that act on water- and lipid-soluble zones respectively, to scavenge free radicals and terminate the propagation of free radical reactions. Their differing solubility not only enables synergistic ROS scavenging but also regeneration of vitamin E by vitamin C at the lipid-water interface.(72, 73) Carnes and colleagues demonstrated a reduction in atrial tissue levels of vitamin C reflecting increased consumption following rapid atrial pacing. Moreover, exogenous administration of vitamin C both attenuated this pacing induced reduction in ERP and blunted the production of peroxynitrite.(66) Aside from this ROS scavenging, several other mechanisms exist by which vitamins C and E reduce oxidative stress, including down-regulation of NAD(P)H oxidase and up-regulation of endothelial nitric oxide synthase. This results in a reduction in superoxide formation and increase in nitric oxide (NO) generation, and in turn has beneficial effects on both endothelial cell function and vascular tone regulation.(72)

1.5 Post-operative Atrial Fibrillation

Post-operative Atrial fibrillation (AF) affects approximately 30-60% of patients undergoing cardiac surgery despite no prior history of this arrhythmia.(74, 75) With appropriate medical therapy POAF is often self- limiting, however it is associated with significant post- operative morbidity and mortality, resulting in triple the risk of cardiac related death and quadruple the risk of disabling embolic events.(74) Recent evidence also demonstrates a significantly worse long- term survival in patients developing POAF following both valvular and coronary surgery,(76, 77) with a 10- year mortality up to 48% higher in patients developing POAF compared to those that do not.(78) In addition, POAF results in a significantly longer intensive care unit (ITU) and hospital stay, an increase in resource utilisation, and an increase in the financial cost of healthcare.(79-81)

Post- operative AF is thought to result from a number of ‘triggering’ and ‘sustaining’ factors acting on a pre-existing pro-arrhythmogenic substrate. However, the balance of these factors differs from chronic atrial fibrillation with acute changes such as local and systemic inflammation, oxidative stress and autonomic stimulation being relatively more influential in the pathophysiology of post operative AF.

The role of inflammation

As previously discussed, the surge of inflammatory cytokines produced intraoperatively and in the immediate post operative period undoubtedly plays a role in AF pathogenesis and is likely to act as a key triggering factor particularly in the early post-operative days. Intraoperatively, contact of blood with the bypass circuit results in activation of the alternative complement pathway and release of TNF-alpha.(82)

The role of oxidative stress

Several mechanisms of reactive oxygen species (ROS) generation within the atrial myocyte have been described.(65, 79, 83) Whilst a number of cellular mechanisms protect against oxidant damage in normal myocardium, persistent oxidative stress depletes endogenous antioxidants and ultimately results in oxidative damage. Ischaemia-reperfusion injury following coronary surgery, aortic cross clamping and cardiopulmonary bypass stimulate the production of nitrogen and oxygen free radicals including superoxide, peroxynitrite, hydroxyl and hydrogen peroxide and result in depletion of endogenous antioxidants such as glutathione.(79) Furthermore, oxidative damage, as demonstrated by the presence of glutathione disulphide, may be seen for up to 24 hours following aortic cross clamp removal.(79)

Management and preventative strategies for post operative AF

Guidelines currently exist in both Europe and the United States regarding the pharmacological prophylaxis and management of POAF following cardiac surgery.(84, 85) At present β blockers are recommended as first line pharmacotherapy (84, 85) with sotalol and amiodarone used as alternative agents.(84) However, these pharmacological therapies are subject to a number of limitations including treatment failure, toxicity and pro-arrhythmogenicity. Significant effort has therefore been placed in the development of alternative preventative strategies, and the identification of oxidative stress as a mechanism for AF development has made way for antioxidant vitamins as novel therapeutic agents. Vitamins C and E are chain breaking antioxidants which scavenge free radicals and terminate the propagation of free radical reactions.(73) Vitamin E is pivotal to maintenance of membrane stability, acting to prevent lipid peroxidation, whereas Vitamin C both independently scavenges water soluble free radicals and acts synergistically with Vitamin E, reducing vitamin E radicals and allowing its regeneration.(73)

1.6 Thesis Aims

The aim of this thesis is to determine whether *de novo* post-operative atrial fibrillation (POAF) is associated with changes in the metabolic and microRNA profiles of patients undergoing coronary artery bypass grafting (CABG).

Chapter 2

MicroRNA: A novel biomarker for post-operative atrial fibrillation

2.1 Background

2.1.1 The role of microRNA

First discovered in *C. Elegans* and published in 1993 by Victor Ambros, Rosalind Lee and Rhonda Feinbaum,(86) microRNA are small non-coding RNA molecules approximately 19 to 25 nucleotides in length acting to suppress their target mRNA by inhibiting its translation or promoting its degradation. They may therefore be considered as post-transcriptional regulators of mRNA expression. The miRBASE database (Release 21) currently lists a total of 35828 mature miRNA products in 223 species, with 2588 mature sequence human microRNAs. Each of these mature sequence miRNAs may in turn have several hundred potential mRNA targets.(87-89)

In addition to miRNA found in cell and tissue lysates, these small non-coding RNAs have also been identified in a number of biofluids including blood serum, plasma, urine, saliva, semen and cerebrospinal fluid.(90-93) These circulating miRNA are most commonly derived from platelets, mononuclear phagocytes and endothelial cells, although low levels of tissue specific miRNA detected in biofluids has led to the suggestion that miRNA can also be actively secreted into the plasma and may even play a role in exerting paracrine effects on distant targets. Furthermore, miRNA may be released in the circulation by dead or dying cells, either contained within apoptotic bodies or as free miRNA.(94, 95) Both apoptotic bodies and microvesicles enhance the stability of miRNA in the peripheral blood by

providing resistance to endogenous RNases. Moreover, under strict regulation microvesicles may act to specifically deliver miRNA to their target cells contributing to a potential paracrine function.(94) Free circulating miRNA identified outside microvesicles are thought to be shed into the circulation by apoptotic or necrotic cells. Although such free miRNA would be expected to be relatively unstable and undergo degradation by endogenous RNases, by binding to the component of the RISC complex, Argonaute 2 protein (Ago2), free miRNA also resist degradation and have been shown to be stable in cell lysate for at least 2 months.(95)

Although these results suggest the potential for circulating miRNA to exert paracrine effects on target cells, the exact regulatory role played by these miRNA remains unclear. The low concentration of paracellular miRNA detectable in human plasma and other biofluids argues against a substantial paracrine function particularly given the one to one nature of microRNA-mRNA interaction which would require high levels of intracellular microRNA (>1000 copies/cell) in order to produce significant paracrine effects on their target.(96) Furthermore, common miRNA expression between blood cells and the organ/disease of interest may lead to difficulty in interpreting the significance of circulating miRNA particularly in the context of biomarker discovery.(97)

2.1.2 MicroRNA Biosynthesis

MicroRNA biosynthesis can broadly be described to follow one of two routes, the 'Canonical' pathway, describing the synthesis of miRNA transcribed by non-coding regions of the genome; and the 'intronic' or 'mirtronic' pathway, describing the synthesis of miRNA coded for within the introns of their host genes.

The Canonical pathway

MicroRNA biosynthesis begins in the nucleus with transcription of the primary miRNA transcript (or pri-miRNA) by either RNA polymerase II (the most common) or RNA polymerase III (where the miRNA is associated with Alu repeats). The pri-miRNA is a hairpin structure, made up of a terminal loop attached to a stem region of approximately 33 base pairs with 2 single stranded flanking regions at each end (**Figure 2-1**). This pri-miRNA is cleaved by the microprocessor RNase III enzyme complex of Drosha (RNASEN) and Pasha (DGCR8 or DiGeorge critical region 8), to form pre-miRNA,(98) a precursor hairpin structure that can subsequently be exported from the nucleus by the Exportin-5-Ran-GTP complex. Once in the cytoplasm, the pre-miRNA is bound by a group of proteins including Dicer RNase, Tar RNA binding protein (TRBT), protein activator of PKR (PACT) and Argonaute 2 protein (Ago2), which form the RISC loading complex and later facilitate binding to RISC. During binding of the RISC loading complex, the pre-miRNA hairpin is cleaved by Dicer to form a double stranded miRNA duplex. This duplex contains both a functional strand, which acts to guide the RISC (RNA silencing complex) to its complementary mRNA target, and an antisense passenger strand (denoted by * in RNA nomenclature), which is then degraded. It is thought that the unwinding of the duplex and degradation of the passenger strand is mediated by either a specific helicase or helicase complex associated with RISC formation, or by the Ago2 protein associated with the RISC complex, which may itself promote cleavage of the passenger strand.(98) The guide strand itself is thought to be selected due to a difference in thermodynamic stability of the base pairs at the 5' end of the helix. The guide strand exhibits less stable base pairing at the 5' end.(99) The final step of the canonical pathway is the binding of the functional strand to the RISC complex which may then in turn regulate the target mRNA. This functions to guide the RISC complex to its target by imperfect base pairing within the 5-8 nucleotide 'seed' region at the 5' end of the strand. The target RNA is then inhibited, either by destabilization of the mRNA-

target interaction, cleavage and degradation of the mRNA strand or by deadenylation and translational inhibition.

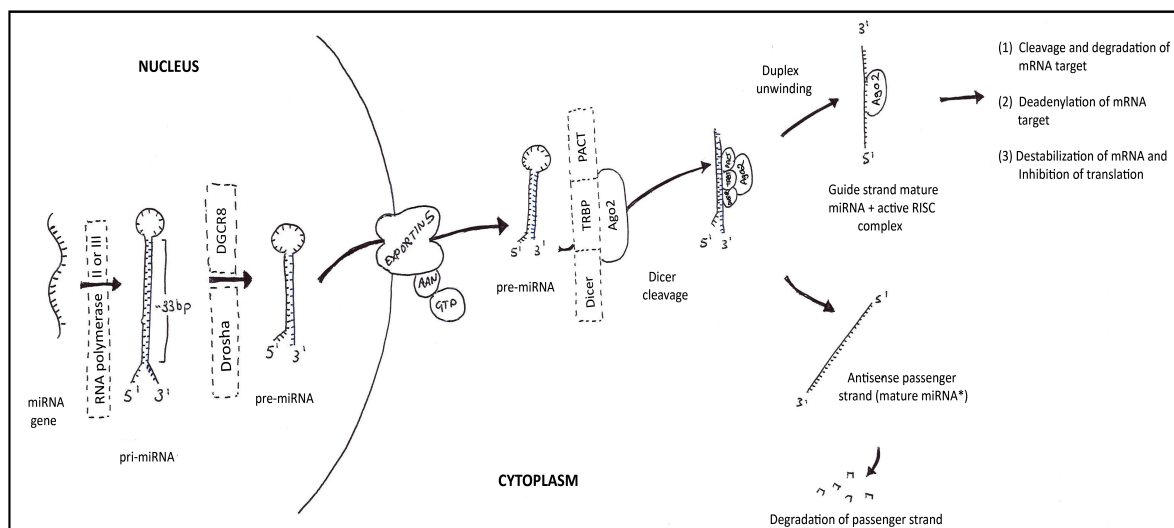


Figure 2-1. The canonical pathway of miRNA biosynthesis

Non-canonical (intronic) miRNA synthesis

Although the majority of human miRNA genes are found in non-coding regions of the genome, approximately 37% of miRNA are ‘intronic’ coded for within the introns of pre-mRNA.(100) Such miRNA are regulated by the same promoter regions as their corresponding protein coding genes and are therefore often co-expressed. Under the regulation of the promoter region of the associated gene, intronic pri-miRNA is transcribed in the cell nucleus by RNA polymerase II. In the majority of instances, intron splicing then occurs, mediated by the spliceosome, which excises the pri-miRNA from its associated exons and simultaneously releases the spliced continuous exon product. The resultant pri-miRNA then enters the same canonical pathway as described above, following the same processing steps to form mature miRNA which may then go on to regulate mRNA translation (**Figure 1-6**). (101) Interestingly, there is also evidence to suggest that splicing of the pri-miRNA from its attached exons is not always necessary for Drosha processing of intronic miRNA to take place. In fact, it seems that drosha-DGCR8 may cleave the pri-miRNA directly from the unspliced intron to form pre-miRNA and its flanking exons connected by a discontinuous intron. The 5’ and 3’ splice

sites on the resultant mRNA are linked by a group of proteins known as the ‘splicing commitment complex’ which tethers together the exons allowing for accurate splicing by the spliceosome even after cleavage of the pri-miRNA. In this way the total mRNA product is not affected, even if Drosha cleavage occurs prior to splicing.(102)

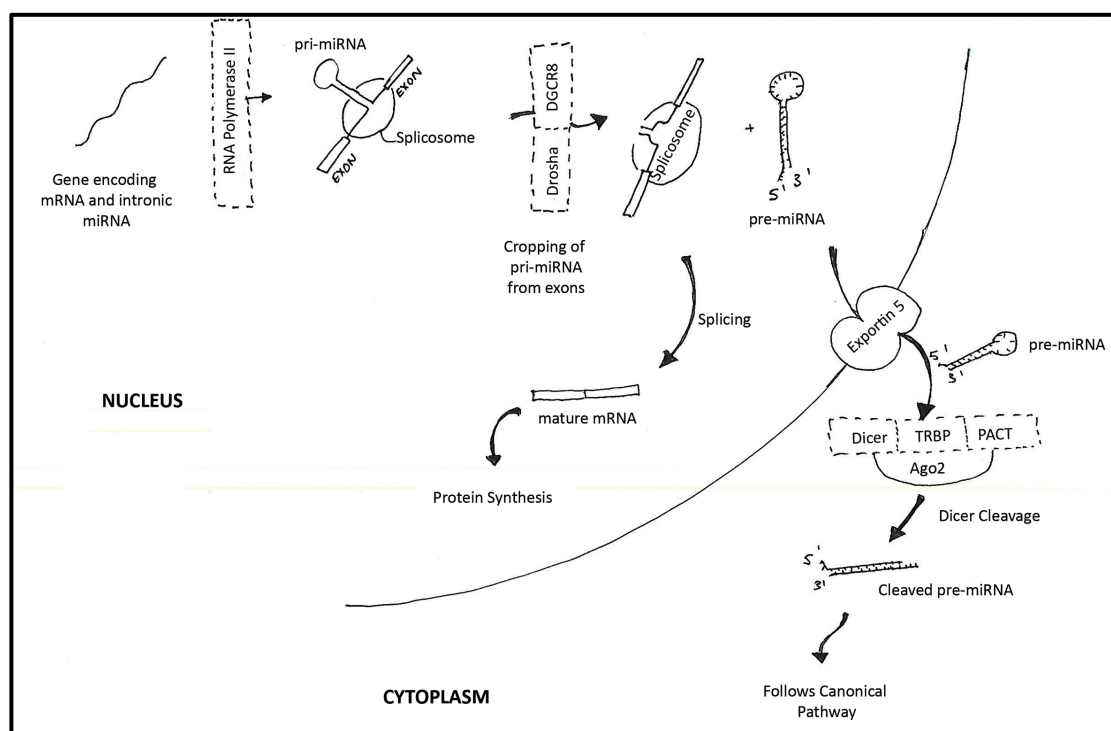


Figure 2-2. The non-canonical ‘intronic’ pathway of miRNA biosynthesis

MicroRNA clustering

A number of microRNA genes may exist on close proximity within a single chromosome forming a functional ‘cluster’ that may share the same promoter resulting in a polycistronic pri-miRNA transcript.(103) These clusters may also be highly conserved between species, suggesting evolutionary significance.(104) Although the exact functional implications of miRNA clusters is unknown, it is thought that the common transcription of these miRNA may improve the efficiency of complex cell signalling and have a role in coordinated expression of multiple related miRNAs targeting a common pathway.(103, 105) It is therefore important to consider functional miRNA clustering when determining the miRNA signature of any given

disease process and to determine whether in fact a polycistronic cluster rather than a single miRNA may be regulating pathogenesis.

MicroRNA Nomenclature

The microRNA naming system follows a specific nomenclature allowing the reader to distinguish between pre-miRNA, mature miRNA and its subtypes and miRNA originating from different species. A summary of the miRNA nomenclature is shown in **Table 1-2**.

MiRNA Type	Nomenclature	Example
Pre-miRNA		
– Two pre-miRNA at different distinct genomic loci leading to identical mature miRNA	Prefix ‘mir’ – number of miRNA	mir-223
Mature miRNA	Prefix ‘miR’ – number of miRNA	miR-223
– Two miRNA with nearly identical sequences	Denoted by lowercase letter after number	miR-208a
– Two miRNAs originating from opposite arms of the same pre-miRNA (<i>known</i> relative expression)	The principal miRNA is written as above, the lesser miRNA is marked with an asterisk	miR-24-1 miR-24-1*
– Two miRNAs originating from opposite arms of the same pre-miRNA (<i>unknown</i> relative expression)	The suffixes ‘5p’ and ‘3p’ are used to denote the miRNA derived from the 5’ and 3’ ends respectively	miR-483-3p miR-483-5p
Species subtypes	The organism species is denoted by a three letter prefix	hsa-miR-223 (homo-sapiens)

***The numbering of miR is sequential in accordance with their discovery. Before designation the new gene must be verified by cloning or with evidence of expression and processing.**

2.1.3 MicroRNA Target Interactions

The microRNA-mRNA interaction is mediated by a 6-8 nucleotide 'seed' region located at the 5' end of the mRNA, and stabilized by several nucleotides outside the seed region also binding in a complementary fashion.(86) In plants microRNA usually bind with complete complementarity to their target mRNA resulting in degradation, however, in eukaryotic cells microRNA-mRNA pairing exhibits partial complementarity in approximately 60% of interactions, resulting in bulging or mismatching of nucleotides.(87)

The aforementioned description of miRNA-mRNA interaction may be described as following the conventional canonical rules. More recently however, miRNA binding has been shown to be increasingly complex, with evidence to suggest binding at the 3' end with little interaction in the 5' region in up to 18% of pairings.(87)

2.1.4 The role of microRNA in Post Operative Atrial Fibrillation

A number of miRNAs have been implicated in cardiovascular disease, particularly in cardiac hypertrophy (miR-23, miR-208a, miR-133), myocardial ischaemia (miR-214),(106) and fibrosis (miR-133).(107) The role of miRNA in arrhythmogenesis is now also becoming increasingly apparent, with miRNA targets regulating the energy substrate, structural and electrical make-up of the myocardium. MiRNA associated with regulation of cardiomyocyte metabolism (miR-208a and miR-223) are likely to regulate the provision of energy substrate required to maintain AF,(108-110) whereas miR-328, miR-1, and miR-26 are thought to play a central role in electrical remodelling, modulating calcium and potassium currents respectively. Likewise, miR-133, miR-590, miR-29b, miR-208, miR-638, have been shown to regulate structural changes such as myocardial fibrosis which may promote arrhythmogenicity (**figure 2-3**).(111)

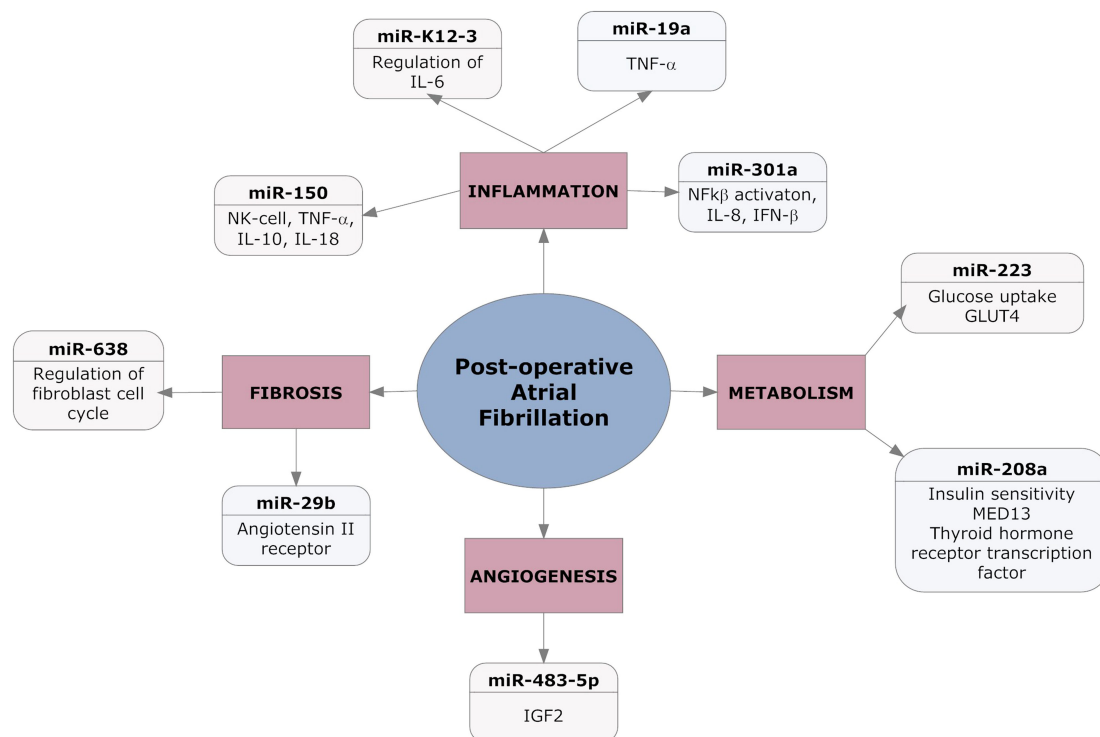


Figure 2-3. Potential roles of microRNA in the regulation of post-operative atrial fibrillation

2.1.5 Aims

In light of the ever-increasing evidence supporting a role for microRNA in the regulation of cardiac arrhythmogenesis, this study aims to:

1. Ascertain whether microRNA regulation is altered in the atrial tissue of AF naive patients developing *de novo* post-operative AF.
2. Identify whether post-operative atrial fibrillation is associated with any changes in circulating or urinary microRNA expression.
3. Determine whether any changes in microRNA expression may be detected pre-operatively and subsequently hold potential to act as a biomarker in post-operative AF.

2.2. Clinical Methods

2.2.1. Regional Ethics approval

The study was approved by the local and regional research ethics committee on 07/05/2009 (Ref: 09/H0711/23). Following this, substantive amendments were approved to allow for intraoperative recording of atrial electrograms, assessment of susceptibility to AF on epicardial stimulation (08/10/2010) and the additional intra-operative sampling of epicardial adipose tissue (09/11/2010).

2.2.2. Study Population, Inclusion and Exclusion Criteria

Between November 2010 and September 2011 thirty-four patients undergoing non-emergent, on-pump coronary artery bypass grafting (CABG) at The Hammersmith Hospital, Imperial College NHS Trust, were prospectively selected to participate in this study. All patients fulfilling these inclusion criteria were approached on arrival for pre-operative assessment. Patients were given a full written information pack and allowed a minimum of 24 hours to reach a decision regarding participation in the study. Informed consent was obtained on arrival the day before surgery. At this time a full medical history and examination was carried out and patients were again screened against study exclusion criteria.

Inclusion and Exclusion Criteria

Emergency cases, those requiring adjunctive procedures (e.g. valve repair or replacement), patients with a prior history of atrial or ventricular arrhythmia, thyroid disease, patients taking anti-arrhythmic agents, or those undergoing surgery with mini-cardiopulmonary bypass (CPB) systems were excluded.

2.2.3. Power Calculation

It is not possible to calculate a required sample size using traditional methods, as there are no studies currently describing the microRNA profile of post-operative AF. Similarly, only one study currently exists reporting the metabolic profile of post-operative AF. Therefore, our sample size calculations are based on the prevalence of POAF and the number of on-pump, elective CABG cases performed annually at Hammersmith Hospital. With an annual prevalence of 18% POAF and a total of 470 consecutive cases per year this equates to 85 cases. Therefore, the final study sample size will be 160 patients (85+85). Following this initial calculation, a pilot study of the first 24 patients will be performed in order to estimate the effect size and thus determine the sample size required to adequately power the remainder of the study.

2.2.4. Patient Groups

Patients were grouped retrospectively (on discharge following Holter analysis) according to the absence (Group 1) or presence (Group 2) of AF during the post-operative period.

	Group 1	Group 2
Patient Cohort	Surgical patients (CABG) not developing POAF	Surgical (CABG) patients developing POAF
Sampling protocol	Group 1 & 2: Blood & Urine at baseline, intra-operative, 48 and 96hrs. Right atrium & epicardial adipose sampled intra-operatively pre bypass. Group 2 only: Blood & Urine at AF onset	

Table 2-2. Patient grouping and sampling protocol

2.2.5. Assessment of Cardiac Rhythm

In accordance with recommendations made by the Heart Rhythm Society,(112) all patients underwent continuous Holter (Novocor Vista 5 lead system, 2 channel recording) monitoring

from the time of admission to the time of surgery (12-24hrs). Post-operatively, all episodes of AF, atrial flutter, or tachycardia of at least 30 seconds duration were documented. Only Atrial Fibrillation episodes >30s were categorized as AF positive (group 2) and patients were grouped as in **Table 2-2**.

2.2.6. Sample collection protocol

All patients were subjected to a uniform sampling protocol as outlined in **Table 2-2**.

Blood Samples

10mls of whole blood was taken into venous blood collection vacutainers (BD™) and at the following timepoints: (i) Pre-operatively on arrival in hospital, (ii) Intra-operatively at the point of discontinuation of cardiopulmonary bypass (fully re-warmed), (iii) Post-operative day 2, (iv) Post-operative day 4. Blood samples were immediately taken to the laboratory and serum and plasma obtained from whole blood.

Serum: Whole blood collected into clot activator, silicon coated vacutainers (BD™) was left to clot for 30 minutes at room temperature. Samples were then centrifuged for 8 minutes at 4500g to separate serum. The supernatant (serum) was removed under Class II conditions and aliquoted into three cryovials. These were stored at -80°C until use.

Plasma: Whole blood collected into lithium heparin vacutainers (BD™) and immediately taken to the laboratory where it was centrifuged for 8 minutes at 4500g to separate plasma. The supernatant (plasma) was removed and stored as above.

Urine

Mid-stream (MSU) or catheter urine samples were taken: (i) Pre-operatively, (ii) Intra-operatively (CSU), (iii) Day 2 (MSU or CSU), and (iv) Day 4 (MSU)

Note: Catheter samples were obtained from tubing rather than bag to ensure sterility and timing of sample.

Atrial tissue biopsy: Atrial samples were obtained prior to cardiopulmonary bypass at the point of insertion of the right atrial cannula on the free wall of the right atrium (**Figure 2-4**). Immediately following sampling biopsy specimens were divided into 4 and snap frozen in liquid nitrogen (-196 degrees Celsius). These were then stored at -80 degrees until use.

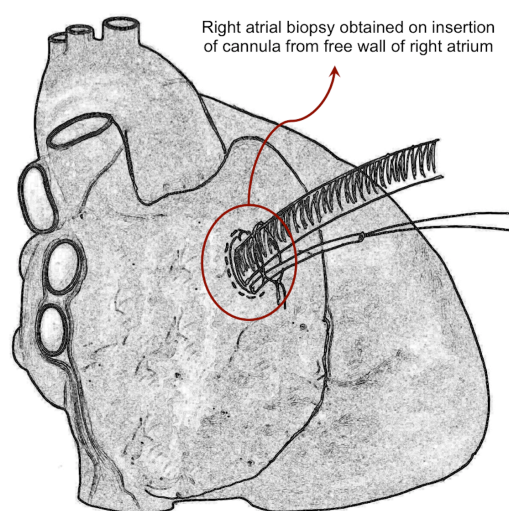


Figure 2-4. Site of Right Atrial Tissue Biopsy

2.3. Laboratory Methods

2.3.1. RNA Extraction

Atrium

Whole RNA was extracted from atrial tissue using TRIzol[®] Reagent as described in the manufacturer protocol (113). Briefly, tissue was homogenised with 1ml of TRIzol[®] per 50mg of tissue. Samples were incubated for 5 minutes at 20°C and 0.2mls of chloroform added per 1 ml of TRIzol[®]. Samples were then shaken and incubated for a further 3 minutes at 20°C and centrifuged at 12 000g for 10 minutes at 4 degrees centigrade to allow phase

separation. The aqueous phase was subsequently transferred to a separate tube. 0.5ml Isopropyl alcohol was added per 1ml of TRIzol[®] and samples were incubated again at 20°C for a further 10 minutes, followed by centrifugation at 12 000g for 10 minutes at 4 degrees. The supernatant was removed and the pellet washed with 75% ethanol at a ratio of 1ml EtOH to 1ml TRIzol[®] used for extraction. Pellets were washed by vortexing and then centrifuged at 7500g for 5 mins at 4 degrees centigrade. The washing step was repeated 3 times to minimize TRIzol[®] contamination of samples. The supernatant was removed and pellets left open to air to dry for 5-10 minutes. The pellet was then re-dissolved in 5µl of RNAase free water (Gibco[®]) and RNA quality and concentrations were checked with the Nanodrop 1000 spectrophotometer and Agilent 2100 Bioanalyser. All RNA integrity numbers (RIN) were >7.5. Where RNA quality or concentrations did not meet required standards, samples were re-extracted and quality control repeated to ensure improvement.

Serum

Serum samples were thawed at room temperature and stored on ice. RNA was extracted according to manufacturer protocol using the MirVana[™] PARIS[™] kit (Applied Biosystems). Briefly, 250µl of cell disruption buffer and 500µl of denaturing solution was added to an aliquot of 250µl serum. Samples were mixed and stored on ice for 10 minutes. 6 µl of synthetic *C. Elegans* miRNA (cel-miR-39) was added at a concentration of 5 fmol/µl to each sample to act as a control and enable normalisation of C_t values following qPCR. 1ml of Acid:Phenol Chloroform was added to each sample and samples were vortexed for 30 seconds to mix. They were then centrifuged at 10 000g for 5 minutes at room temperature to separate the mixture into aqueous and organic phases. The upper (aqueous) layer was then transferred to a new tube (recording the volume) without disturbing the interphase and lower phases, which were subsequently discarded. A corresponding volume of Acid:Phenol Chloroform was then added to the newly isolated aqueous layer and the process repeated, again recording the volume of the aqueous phase removed. A volume of Ethanol was then

added corresponding to 1.25x the volume of the aqueous phase and samples were then mixed and briefly centrifuged. Supplied filter cartridges were added to new collection tubes and 700ml of sample transferred onto the filter. The tubes were then centrifuged at 8000 g for 30 seconds until all liquid had passed through the filter and the filtrate was subsequently discarded. 700µl of Wash Solution 1 was added to the filter, the cartridge centrifuged at 8000g for 20 seconds and the filtrate discarded. 500µl of Wash Solution 2 was added to the filter and centrifuged again at 8000g for 20s. After discarding the filtrate this step was repeated a further 2 times to reduce contamination and the cartridge-filter assembly spun at 8000g for 1 minute to remove excess fluid from the filter. Finally, the filter was transferred into a fresh collection tube and 100µl of elution solution (pre-heated to 95°C) added to the filter. The assembly was spun at 8000g for 30s and the filtrate containing the extracted RNA collected and stored at -80°C until use.

2.3.2. MicroArray Analysis

Extracted RNA was diluted to a total concentration of 50ng/µl. 2µl of RNA was added to 2µl of prepared Calf Intestinal Phosphatase (CIP) master mix (0.4µl 10xCIP Buffer, 1.1µl labeling spike in solution, 0.5µl CIP) per reaction. After mixing, samples were incubated at 37°C for 30 minutes to allow dephosphorylation to occur. 2.8µl of Dimethyl Sulphoxide (DMSO) buffer was then added to each sample, and samples were denatured by incubation at 100°C for 5 minutes before being immediately transferred into an ice water bath. MiRNA was ligated by adding 4.5µl of ligation master mix (1.0 µl 10x Ligase Buffer, 3.0µl cyanine 3-pCp, 0.5µl T4 RNA ligase) to reach a total sample volume of 11.3µl. Samples were then mixed and incubated at 16°C for 2 hours to allow the labeling reaction to occur. This reaction labels the miRNA with cyanine dye (pCp-Cy3) at its 3' end to create miRNA with an additional 3' cytidine and one cyanine dye on the 3' end. Labeled miRNA was dried in the Speed Vacuum volume concentrator for 2-3 hours until pellets were dry.

To prepare for hybridization each dried RNA sample was re-suspended in 17µl of RNase free water (Gibco®). 1µl of pre-prepared hybridization spike in solution was added to each sample along with 4.5µl of GE blocking agent and 22.5µl of hi-RPM hybridization buffer. Samples were then incubated at 100°C for 5 minutes on the vortex mixer and immediately transferred to an ice water bath for 15 minutes to prevent re-annealing.

A total sample volume of 45µl was loaded into the corresponding gasket wells and Agilent MicroArray slides (8x60k) placed on top to prepare the final gasket assembly. Wells were checked for air bubbles and were then hybridized using the Agilent Sure-hyb hybridization chamber on for 20hrs at 55 degrees centigrade.

Following completion of hybridization slides were washed 3 times according to manufacturer protocol. Slides were immediately scanned using the Agilent G2505C MicroArray Scanner and feature extraction performed using the Agilent Feature Extraction Software version 10.7.3.1. Analysis was performed using Agilent GeneSpring software GX11.0.

2.3.3. Real Time- Quantitative Polymerase Chain Reaction (RT-qPCR)

Atrium

MiRNA levels were quantified using specific TaqManTM assays based upon the results of atrial microarray analysis (Applied Biosystems, Foster City, CA, USA). Briefly, 4 miRNAs of interest were selected from the 16 differentially expressed miRNAs identified by microarray analysis. These included hsa-miR-483-5p (most up-regulated), hsa-miR-208a (most down-regulated), hsa-miR-1202 and hsa-miR-223 (clinically interesting miRNA based upon previous literature).

Extracted RNA samples were thawed at room temperature, briefly mixed by pipetting and centrifuged at 2000 rpm for 30 seconds. RNA was subsequently diluted to a concentration of 2 ng/ μ l and 5 μ l aliquots of each sample transferred into pre-labeled PCR tubes.

Reverse Transcription (RT): Reverse transcription (RT) master mix was made up according to the number of samples to be analysed. This comprised of: 0.15 μ l dNTP (2'-deoxynucleoside 5'-triphosphate), 1.0 μ l RT Enzyme, 1.5 μ l 10x RT Buffer, 0.19 μ l RNase inhibitor, 4.16 μ l water) and 3 μ l of TaqMan RT primer per sample. A total of 10 μ l of RT master mix was then added to each sample. For each study primer (e.g. hsa-miRNA-483-5p) an identical RT reaction was prepared for hsa-miR-186, which acted as a control for analysis. This control microRNA was chosen based upon the results of previous microarray analysis, which demonstrated miR-186 to be the most conserved miRNA between the AF and non-AF groups.

After addition of the RT master and primer mix, samples were mixed by pipetting and centrifuged at 2000 rpm for 30 seconds. Samples were then transferred to the thermocycler and incubated according to the manufacturer's protocol (HOLD 30 mins at 16 degrees, HOLD 30 minutes at 42 degrees, HOLD 5 mins at 85 degrees, HOLD ∞ at 4 degrees). After incubation, resultant cDNA was either used immediately for PCR reaction or stored at -80°C until use.

PCR Reaction: Into new PCR tubes 2.4 μ l of sample cDNA (RT product) was added to 32 μ l of PCR Master Mix (17.25 μ l of TaqMan fast universal PCR master mix, 1.725 μ l of qPCR probe, 13.23 μ l of RNase free water). For each study probe an identical control PCR reaction was also carried out.

Samples were mixed by pipetting and centrifuged at 2000g for 30 seconds. 10 μ l of qPCR sample mixture was then added to each well of a 96-well PCR plate. Three technical

50

replicates were performed for each of the samples and controls. Plates were then centrifuged at 500g for 30s prior to being placed into the Applied Biosystems 7500 fast Real-Time PCR System for qPCR reaction. The PCR reaction was carried out in accordance with the manufacturers protocol (114): Hold at 50°C for 2 minutes, Hold at 95°C for 20s, Cycle (40 cycles) 95°C for 3s, Hold at 60°C for 30 seconds. Amplification plots were examined for adequate amplification and successful PCR reaction. Baseline settings were checked to ensure appropriateness according to the amplification curve. A delta R_n threshold value of 0.2 was set to ensure comparability across plates, however this was checked to ensure it was within the exponential phase of amplification prior to analysis. C_t values were recorded for both samples and controls and compared using the $\Delta\Delta C_t$ method.

Serum

RT-qPCR of serum samples was performed according to the protocol outlined above. Analysis of serum samples differed from atrial tissue RNA only in that synthetic cel-miR-39 was spiked in at equal concentrations during serum miRNA extraction to enable its use as a control for these experiments. This therefore replaced the has-miR-186 control described above.

2.4. Results

2.4.1. Clinical Study

Between November 2010 and April 2011 34 patients undergoing non-emergent, on-pump coronary artery bypass grafting (CABG) at The Hammersmith Hospital, Imperial College NHS Trust were recruited to participate in this study. Of these 21 patients did not develop POAF and were classified into Group 1. The remaining 13 patients developed POAF and were classified into Group 2. The mean time to onset of AF was 2.5 days.

Pre-operative Demographics

A summary of the pre-operative demographics is shown in **Table 2-3**. Patients in Group 2 were significantly older than those in Group 1. However no differences were observed in gender, pre-operative risk stratification score (logEuroSCORE) or other recognised pre-operative cardiovascular risk factors such as diabetes, hypertension, previous MI/TIA/Stroke, renal dysfunction, peripheral vascular disease, hypercholesterolaemia or family history.

	AF	Non-AF	
n	13	21	
Age	67.18±9.21	58.77±12.26	p=0.037*
%male	64%	77%	p=0.453
Smoking	1/11 (10)	6/24 (26.1)	p=0.252
DM	6/11 (60)	10/24 (43.5)	p=0.408
Insulin	2/11 (20)	6/24 (26.1)	p=0.713
Weight	79.16±12.57	78.30±11.38	p=0.864
Height	1.60±0.18	1.69±0.09	p=0.204
BMI	30.65±6.39	27.31±3.26	p=0.196
Log Euroscore (%)	3.94±4.56	1.91±1.03	p=0.197
Alcohol (Y/N)	4/10 (40%)	16/22 (73%)	p=0.008*
FH	5/11 (55.6)	14/24 (63.6)	p=0.848
CHOL	11/11 (100)	19/24 (82.6)	p=0.083
HBP	8/11 (80)	18/24 (78.3)	p=0.713
PCI	0/11 (0)	0/24 (0)	
PVD	2/11 (20)	1/24 (4.3)	p=0.288
CVA	0/11 (0)	1/24 (4.3)	p=0.328
TIA	1/11 (10)	3/24 (13.0)	p=0.807
MI	2/11 (20)	7/24 (30.4)	p=0.536

Table 2-3. Pre-operative Demographics

2.4.2. MiRNA microarray

24 samples (12 AF, 12nAF) determined to have an RNA integrity number (RIN) >7 were selected for miRNA microarray analysis. Results were analysed using Agilent's GeneSpring software. Initial analysis parameters were set to detect miRNA with a >2 fold change difference in signal intensity between AF and non-AF groups at a level of significance <0.05.

This did not reveal any significant differences in miRNA profile between the disease and control groups. Repeat analysis was therefore performed using a fold change significance of >1.5, again at the $p < 0.05$ significance level. 16 miRNA were subsequently found to be differentially expressed between the group 1 and 2 (**Figure 2-5; Table 2-4.**). Of these, hsa-miR-483-5p was found to be the most up-regulated (FC = 1.804) and hsa-miR-208a to be the most down-regulated (FC = 2.458).

Targeted analysis was selected based on microarray results. In addition to the 2 aforementioned miRNA, literature review highlighted hsa-miR-223, hsa-miR-1202 and hsa-miR-29b to be of particular clinical interest in the study of atrial fibrillation and as such these miRNA were selected for further targeted analysis.(109, 115, 116)

systematic_name	p-value	FCAbsolute	regulation	[nAF](raw)	[AF](raw)	[nAF](normalized)	[AF](normalized)	active_sequence
hsa-miR-1202	0.044782475	1.676717	up	622.3137	1051.706	1.7895061	2.5351453	CTCCCCACTGCA
hsa-miR-150	0.010922834	1.6919414	up	347.45084	585.4175	1.1396161	1.8982958	CACTGGTACAAGGGTTGG
hsa-miR-19a	0.03999829	1.6058515	down	311.4344	229.47179	0.8447729	0.16143441	TCAGTTTGCATAGATTGCA
hsa-miR-208a	0.020505523	2.4579535	down	545.5674	265.15802	1.3534186	0.055961013	ACAAGCTTTTGTCTCGTCTT
hsa-miR-223	0.046394624	1.6013817	up	823.5629	1566.4321	2.240393	2.9197102	TGGGGTATTTGACAACTGAC
hsa-miR-24-1*	0.01340312	1.80848	down	110.973076	63.722378	-0.60796523	-1.4627429	ACTGATATCAGCTCAGTAGGC
hsa-miR-2861	0.034302548	1.5233945	up	305.8123	470.8317	0.8293955	1.4366851	CCGCCACCGC
hsa-miR-29b	0.029768478	1.7645543	down	2694.43	1717.7926	3.9076028	3.088299	AACACTGATTTCAAATGGTGC
hsa-miR-301a	0.031906698	1.6050799	down	141.4016	93.78885	-0.30352333	-0.9861684	GCTTTGACAATACTATTGCAC
hsa-miR-3141	0.017763672	1.52383	up	29.93974	43.903683	-2.5096686	-1.9019666	TCCTCTCCACCCG
hsa-miR-3679-5p	0.03279372	1.7947133	up	67.447914	130.74852	-1.468605	-0.6248517	TCCCTTCCCTGCC
hsa-miR-4298	0.024814375	1.6312326	up	37.059013	60.376938	-2.2610044	-1.5550419	CTGCCTCCTCTCC
hsa-miR-483-5p	0.019342098	1.8036091	up	32.81519	65.55056	-2.4515853	-1.6006986	CTCCCTCTTTCTC
hsa-miR-572	0.019736553	1.686777	up	39.835545	70.39784	-2.1238549	-1.3695856	TGGGCCACCGCCG
hsa-miR-638	0.035336934	1.5245833	up	340.88522	531.9271	0.98846626	1.5968813	AGGCCGCCACCCGC
kshv-miR-K12-3	0.025042778	1.5513463	up	82.026985	126.07374	-1.0791585	-0.44563782	TCGCTGCCGTCC

Table 2-4: Significance analysis set to a fold change cut-off of 1.5 and a p-value cut off of < 0.05 demonstrating 16 differentially expressed miRNA between AF and nAF groups.

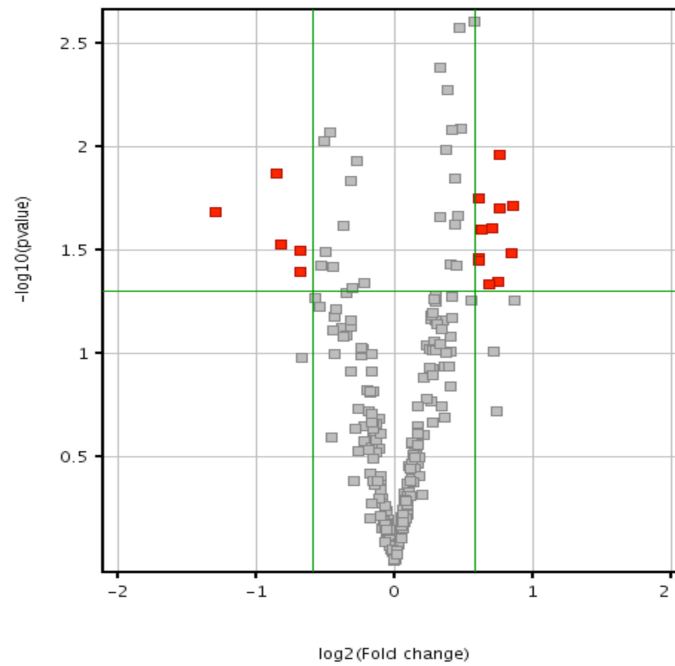


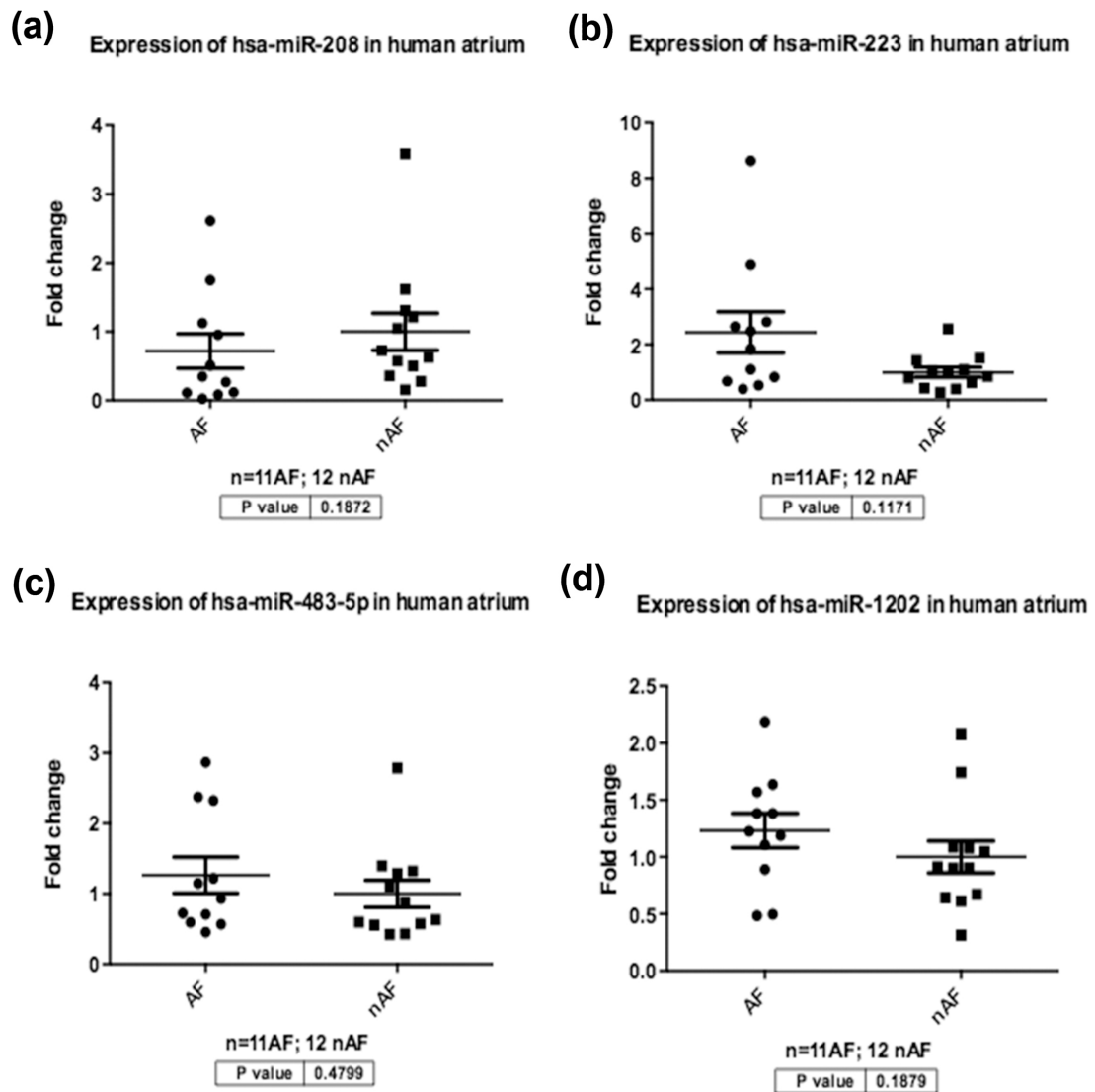
Figure 2-5. Volcano Plot demonstrating 16 differentially expressed miRNA (red) between AF and nAF groups

2.4.3. Atrial tissue RT-qPCR

In order to validate the microarray findings described above, atrial tissue RNA extract was also examined using targeted RT-qPCR for the following miRNA: hsa-miR-208, hsa-miR-483-5p, hsa-miR-1202 and hsa-miR-223. Although results demonstrated the same general trend in expression between AF and non-AF groups as observed by microarray these results did not reach statistical significance (**Figure 2-6**).

2.4.4. Targeted serum RT-qPCR

Based on the results of the atrial tissue microarray findings, targeted RT-qPCR of serum samples was performed for miR-223, miR-483-5p and miR-1202. miR-208a is a cardiomyocyte specific miRNA that is known not to be readily detectable in human serum unless in the presence of concomitant myocardial injury.(117) RT-qPCR confirmed that miR-208a was undetectable in the serum of both AF and non-AF groups.



MicroRNA	n AF	n non-AF	Median fold change AF	Median fold change nAF
hsa-miR-208a	11	12	0.3493	0.6774
hsa-miR-1202	11	12	1.225	0.9083
hsa-miR-223	11	12	1.841	0.9101
hsa-miR-483-5p	11	12	0.9318	0.7547

Figure 2-6. Atrial RT-qPCR validation of microarray findings. Targeted atrial RT-qPCR was unable to show significant differences between AF and non-AF patients when atrial tissue biopsies were analysed. Statistical analysis was performed using non-parametric independent samples Mann-Whitney testing at a level of significance of $p < 0.05$.

Pre-operative Serum: No differences were observed in the relative levels of miR-223 or miR-1202 between AF and non-AF groups. However, hsa-miR-483-5p was found to be significantly higher in the pre-operative serum of patients subsequently developing post-operative AF (Group 2) as shown in **Figure 2-7**.

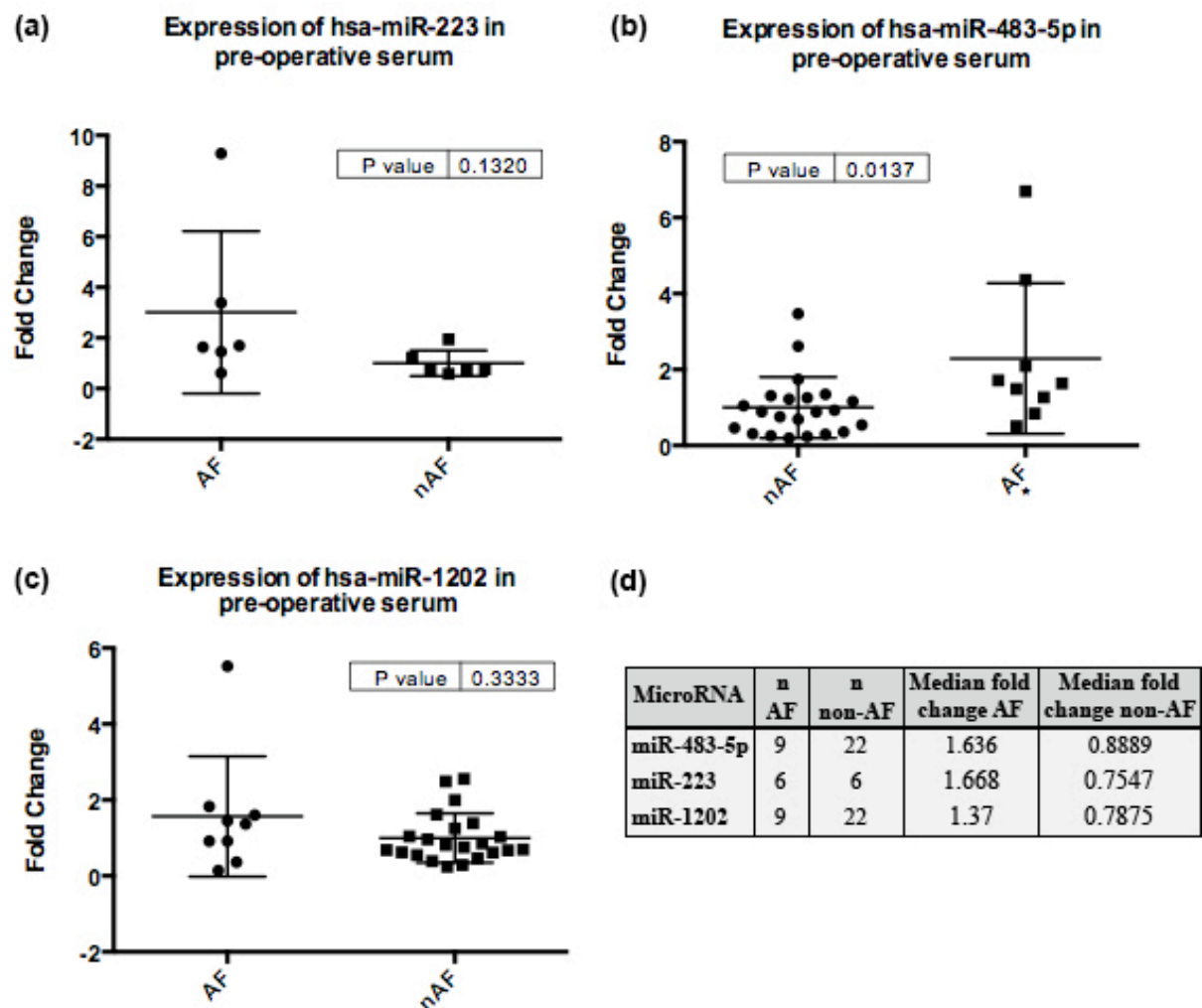


Figure 2-7: RT-qPCR of pre-operative serum. No significant difference was observed in miR-223 or miR-1202 between Groups 1 and 2. However, miR-483-5p was expressed at significantly higher levels in Group 2 when compared to Group 1. Statistical analysis was performed using non-parametric independent samples Mann-Whitney testing at a level of significance of $p < 0.05$

2.4.5. Temporal changes in serum miR-483-5p after surgery

In light of the observed pre-operative differences in miR-483-5p between the AF and non-AF groups, the expression of miR-483-5p was determined at 48 and 96hrs post operatively by means of RT-qPCR. No significant difference in miR-483-5p expression was seen between the AF and nAF groups at either time point (**Figure 2-8**). However, a trend was observed towards a more significant reduction in miR-483-5p expression in AF than non-AF patients between pre-operative and 96-hour time points (**Figure 2-9**).

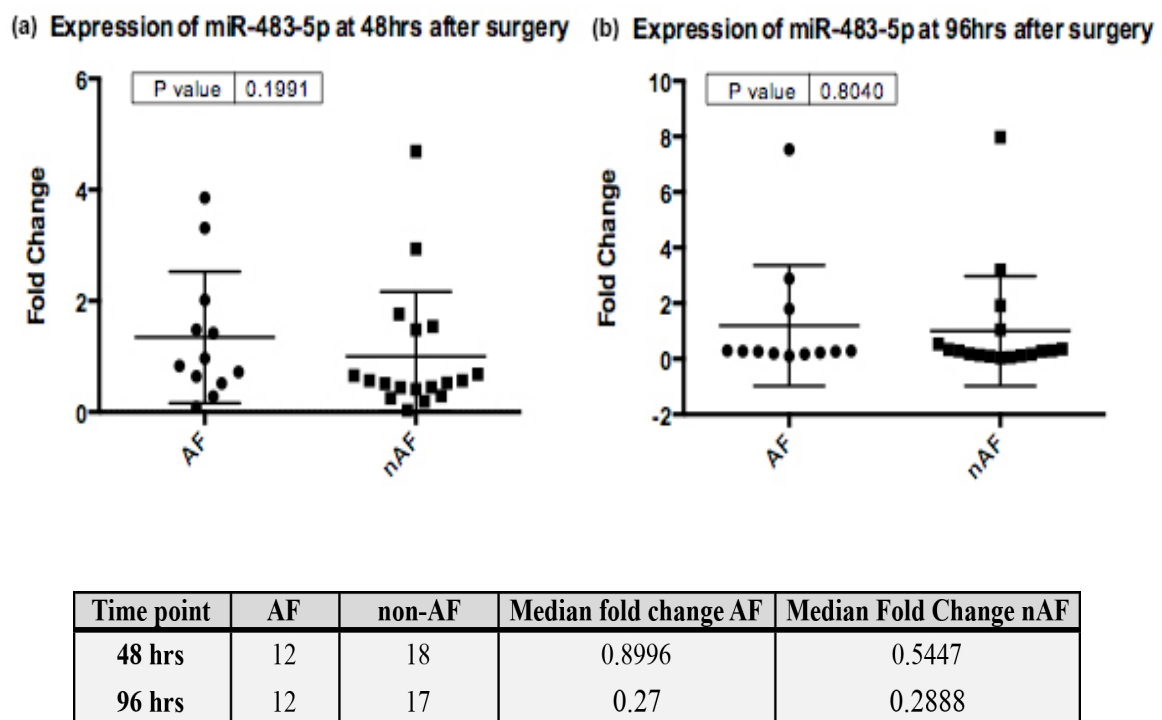


Figure 2-8. Expression of miR-483-5p at: (a) 48hrs after surgery, and (b) 96hrs after surgery.

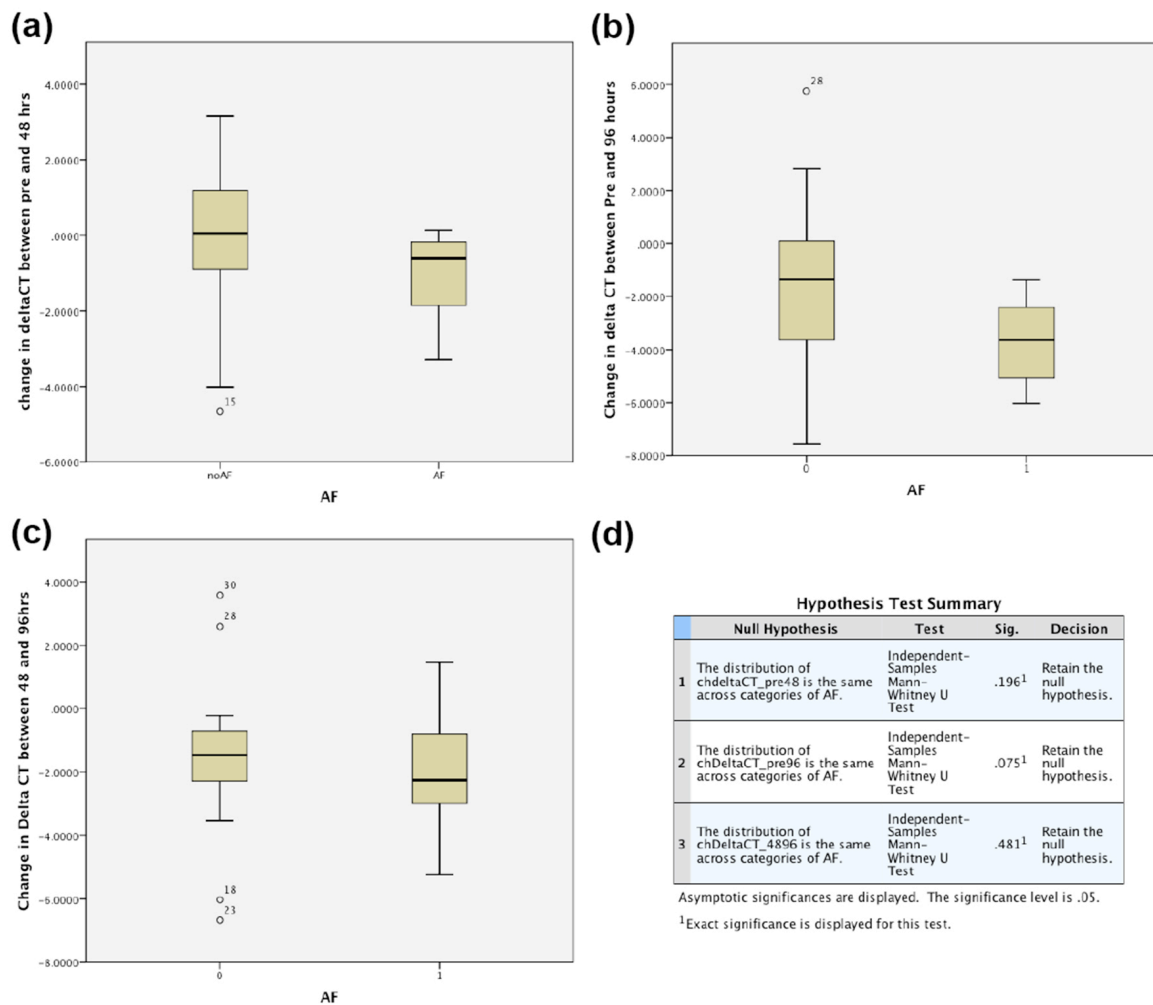


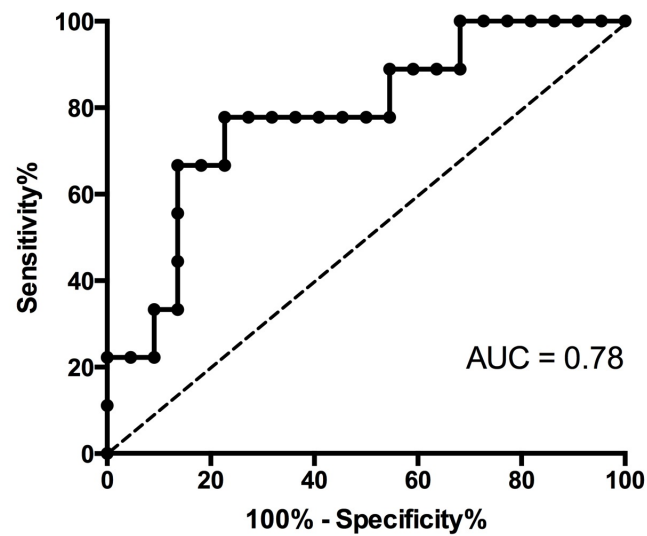
Figure 2-9. (a) Box plot demonstrating the change in ΔCt between pre-operative and 48hr time points ($p=0.196$); (b) Box plot demonstrating a trend towards a greater reduction in ΔCt between pre-operative and 96hr samples in AF vs. non-AF patients ($p=0.075$); (c) Change in ΔCt between 48 and 96hr time points ($p=0.481$); (d) Results on independent samples non-parametric Mann-Whitney U significance testing.

2.4.6. Developing a serum test for post-operative AF

Receiver operating characteristics (ROC) analysis was used to determine the diagnostic accuracy of pre-operative miRNA-483-5p as a diagnostic biomarker for post-operative AF. Comparing patients developing POAF to those that did not, the area under the curve (AUC) (i.e. the probability that a randomly selected positive has a higher test value than a randomly selected negative) was 0.78. Using 1.262 as a relative fold change cut off for the expression

level of miRNA-483-5p, the sensitivity and specificity were 77.78 and 77.27 respectively with a likelihood ratio of 3.422 (Figure 2-10).

ROC Curve of pre-operative serum miR-483-5p in AF vs. non-AF patients



	Results
Area under the ROC curve	
Area	0.7828
Std. Error	0.09191
95% confidence interval	0.6026 to 0.9630
P value	0.01484

Figure 2-10. Receiver operating characteristics curve of pre-operative has-miR-483-5p as a biomarker for post-operative AF.

2.5. Discussion

The results of this study provide the first documented insights into the microRNA signature of *de novo* human post-operative atrial fibrillation. Sixteen microRNA exhibit significant differential expression in the atria of POAF of which miR-208a is the most down-regulated and miR-483-5p is the most up-regulated. Beyond this, I have shown that not only is miR-483-5p detectable in the serum of human patients, but also that pre-operatively it is expressed at significantly higher levels in those patients going on to develop POAF at a mean of 2.5 days after surgery. Furthermore, the expression of this miRNA appears to become more similar in all patients in the post-operative period, with POAF patients demonstrating a trend towards a significant reduction in miR-483-5p expression by 96 hours after surgery.

2.5.1 The atrial microRNA signature

This work has identified pre-existing microRNA dysregulation in patients subsequently developing POAF, particularly demonstrating under-expression of miR-208a in the right atrium and over-expression of miR-483-5p in both the right atrium and pre-operative serum.

As previously described, atrial fibrillation is a multi-system disease with a complex underlying pathophysiology. A number of structural changes have been proposed to take place in the myocardium which when combined with changes in the extracellular environment, intracellular ion flux, and the systemic circulation provide a pro-arrhythmogenic substrate.⁽¹⁹⁾ Many of the structural and electrical changes associated with chronic atrial fibrillation have been characterised by changes in gene expression. For example, suppression of CACNA1C gene expression results in reduced L-type calcium channel production, giving rise to a fall in the L-type calcium current, resulting in decreased action potential duration (APD) and increased re-entry. Similarly, increased expression of the genes KCNN1-3 (SK1, SK2 and SK3) encoding calcium activated potassium channels may reduce APD by

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shortening atrial repolarization.(118) Furthermore, changes in intracellular conductance may be altered by modified gap junction function through a reduction in connexin 40 and 43 encoded by the GJA5 and GJA1 genes respectively.

As previously described, microRNA are short, non-coding RNA molecules which act to inhibit gene expression. This may occur either through inhibition of translation (through incomplete complementarity in miRNA-mRNA binding) or through mRNA degradation (complete complementarity of miRNA-mRNA interaction). As such, this post-transcriptional regulation forms a novel mechanism through which both cellular homeostasis and disease processes may be regulated, giving rise to an exciting and rapidly evolving field of molecular research.

miR-208

MicroRNA-208 is an intronic, cardiomyocyte specific microRNA encoded for and co-expressed with the α -Myosin Heavy Chain (α -MHC/Myh6) gene. It shares near sequence identity with miR-208b, similarly encoded for and co-expressed with the β -MHC (Myh7) gene, and together these miRs are thought to be involved in the development of cardiac hypertrophy and fibrosis.(116) Over-expression of miR-208a in a transgenic mouse model has been shown to result in hypertrophic growth along with up-regulation of the 'slower' isoform, β -MHC, subsequently resulting in loss of myocardial contractility and fibrosis. Similarly, loss of β -MHC transcript and protein expression has been noted in a miR-208^{-/-} knockout mouse model suggesting its regulation by miR-208a.(116)

Both the miR-208a over-expression transgenic mouse and the miR-208^{-/-} knockout mouse demonstrate cardiac conduction defects despite not displaying any macroscopic morphological anomalies. However, in their 2009 work, Callis et al. demonstrated an increased PR interval and heart block in mice over-expressing miR-208a, and detected atrial

fibrillation in 80% of miR-208a^{-/-} knockout mice.(116) The mechanistic role through which miRNA-208 may modulate atrial fibrillation is likely to be multifactorial, as this microRNA is likely to contribute to both the regulation of cardiomyocyte energy homeostasis and connexin expression.

miR-483-5p

MicroRNA 483-5p is a 22-nucleotide (AAGACGGGAGGAAAGAAGGGAG) mature microRNA formed from cleavage of the 76-nucleotide stem-loop pre-mir-483 by the enzyme Dicer. Its counterpart miR-483-3p is a distinct mature microRNA originating from the opposite arm of the pre-mir-483 stem-loop sequence. Although the majority of human microRNA are intergenic, miR-483-5p forms one of the 37% (n=408) of 'intronic' microRNAs coded within the intron of its host gene, *IGF2*, located on chromosome 11p15.5.(100) Situated within intron 2 of *IGF2*, hsa-miR-483-5p is co-expressed with *IGF2* and has also been demonstrated to play a feedback role, itself regulating *IGF2* expression.(100) MiR-483-5p has been isolated in several human tissue types including myocardium, hepatic and brain tissue as well as in blood serum.(100, 119) At present however, miR-483-5p remains relatively poorly studied. Its role is much less well characterised than that of miR-208 and the mechanism by which it regulates or is modulated by the clinical phenotype of POAF remains undetermined. A detailed discussion as to the potential target genes affected by both miRNA-483-5p and miRNA-208 can be found in chapter 3 where these cellular pathways will be assessed in more detail through formal pathway analysis and target validation.

miR-1202

MicroRNA 1202 is a 21-nucleotide (GUGCCAGCUGCAGUGGGGGAG) mature micro RNA. In their 2011 study, Xiao et al. examined the miRNA signature of right atrial biopsies taken from mitral stenosis patients undergoing surgical valve replacement (n=9AF; 4 non-

AF). When comparing AF to non-AF, microRNA-1202 was found to be the most differentially up-regulated microRNA, however no further mechanistic information was available from this patient cohort and this miRNA was not included in subsequent pathway analysis.(115) More recently, in their study of end-stage heart failure receiving left ventricular assist device (LVAD) support, Morley-Smith et al. have identified a correlation between baseline (prior to LVAD) miRNA-1202 expression and response to LVAD therapy at 3 months as measured by NT-proBNP levels. Furthermore, measurement of baseline miR-1202 allowed for accurate prediction of patient response to LVAD therapy.(120) Interestingly, this study also identifies that LVAD implantation is associated with a significant and sustained up-regulation of miR-483-3p (derived from the opposite arm of the same pre-miRNA (stem-loop) sequence as miR-483-5p) in both the myocardium and in the plasma, a finding that appears to mirror the observed concomitant reduction in NT-proBNP levels. Combined with the recognised relationship between elevated pre-operative plasma proBNP levels and *de novo* post operative AF,(121) these findings suggest a link between circulating miRNA-483, pro-BNP and AF which requires further investigation to ascertain its biological significance and any potential connective mechanism.

mir-223

mir-223 is a 21 nucleotide microRNA (TGTCAGTTTGTCAAATACCCC) found on the X chromosome at location Xq12. Although mir-223 has been reported to be involved in a number of cellular processes from the endothelial cell activation(122) to variations in individual patient responses to antiplatelet agents,(123) of significance in cardiac arrhythmogenesis is its role in control of cellular glucose uptake. Over-expression of mir-223 has been observed in the myocardium of patients with type 2 diabetes mellitus and *in vitro*, increases in mir-223 are associated with cellular glucose uptake in the absence of insulin stimulation, via increased glucose transporter (Glut4) expression.(109) Indeed, via directly targeting the Glut4 gene, miR-223 plays a homeostatic role to reduce Glut4 and thus glucose

uptake. Reduced glucose transporter (Glut3) expression has also been associated with *de novo* POAF.(124) As a result, subsequent impaired glucose availability can induce electrical instability during hypoxic states such as myocardial re-perfusion after coronary bypass grafting and thus pre-dispose to AF.(125) Therefore in our patients, the elevated miR-223 levels observed in AF may result in reduced Glut4 expression, impaired glucose uptake and a subsequent increased risk of post-operative arrhythmogenesis.

miR-29

Recently there has been increasing evidence surrounding the role of miR-29 in human AF. My microarray results demonstrated a significant reduction in miR-29 expression (fold change -1.76, p=0.0298) in patients developing POAF. This is similar to that observed in the work by Dawson et al.(126), who noted an approximately 50% reduction in miR-29b expression in chronic AF patients when compared to controls. MiR-29 has been shown to regulate the expression of extracellular matrix proteins including collagen 1 and fibrillin 1, and down regulation of miR-29 has been associated with the pro-fibrotic atrial substrate associated with heart failure in canines.(127, 128) Furthermore, miR-29 itself is downregulated by increases in the cytokine TGF- β , which in turn has been shown to be upregulated in chronic AF. Interestingly, increases in TGF- β have also been observed in patients with longstanding AF (>6months) who developed AF recurrence after surgical radiofrequency maze procedures when compared to those patients who maintained sinus rhythm(129). These observations were coupled with increases in the collagen type 1 volume fraction and although miR-29 levels were not measured in this study, these findings suggest that AF recurrence following surgical ablation may be mediated through miR-29.

2.5.2. MicroRNA as circulating biomarkers in POAF

The potential for MicroRNA to act as non-invasive molecular biomarkers of systemic disease is of great interest owing to their high level of stability in a many different biofluids including serum, plasma and urine.(130) As such, by means of a simple blood test performed at the time of routine patient assessment, quantitation of a single or cluster of microRNAs may provide a novel avenue for detection and risk prediction of systemic disease with minimal disturbance to the patient.

Several microRNA have been implicated in the regulation of cardiovascular disease and microRNA expression in chronic atrial fibrillation is an ever-expanding area of research (**Table 2-5**). Nevertheless, much of the work to date examines miRNA expression in right or left atrial tissue and there remains a paucity of evidence surrounding circulating microRNA in human AF. Recent work by 2 groups has however begun to address this question.(126, 131) Liu et al. identified circulating microRNA-150 to be significantly under-expressed in the serum of 60 prospectively collected patients with both paroxysmal (n=30; FC~17) and persistent AF (n=30; FC~20) when compared to healthy controls (n=30), demonstrating miR-150 to be an independent predictor of AF (OR 1.96; 95% CI [1.5, 3.57]; $P<0.001$). It is notable however, that in this study miRNA-150 levels were not able to differentiate paroxysmal from persistent AF (OR 1.02; 95% CI [0.82, 1.13]; $P = 0.61$). (131)

miRNA	Type of AF	Tissue/ Biofluid (Species)	Target Gene(s)	Direction of regulation	Signalling Pathways/channels Implicated
Inflammation/Structural Remodeling					
miR-133 miR-590 (132)	Tachypacing + nicotine animal model persistent	Left and right atria (Canine)	TGF- β 1, TGF- β 2	Down-regulated (FC dependent on nicotine dose: miR-133~0.8-0.4; miR-590 ~0.7-0.5)	Removal of inhibition of TGF- β 1, TGF- β 2 is pro-fibrotic. Stimulates fibroblast proliferation and adhesion.
miR-150 (131)	Paroxysmal Persistent	Serum (Human)	EIF4E, EIF4B, ARRB2, MAPK9, MAPK2K4, PAK1, PRKAR1A, SP1, CAMK2G, PRKCA, IRAK1, CAST, KCNIP1, TMP3.	Down regulated (paroxysmal: FC~17; persistent: FC~20)	Inflammation: mTOR, MAPK, Insulin, TGF- β , ErbB, Wnt and apoptosis signalling pathways. Calcium channels Fibrosis
miR-29 (126)	Mixed AF $\pm$ CHF (Serum: n=30SR, 33AF) (RAA: n=19SR, 9 pAF, 8 chronic AF) Tachypacing animal model (n=32 cont.; 5 sham; 19 VTP 24hr; 16 VTP 1wk, 22 VTP 2wk)	Serum and Right Atrial Appendage (Human) Left Atrium (Canine)	COL1A1, COL3A1, FBN	Canine: Down-regulation of miR-29b (87% at 24hrs). Down-regulation of miR- 133a and 133b Human: Down-regulation of miR-29b in plasma ~50%; also down- regulation of plasma miR- 21 in AF vs. control. Down-regulation 29b in RAA 40% in pAF and 54% chronic AF	Increased atrial fibrosis due to increased collagen production.
Ion Channel Remodelling					
miR-499 (133)	Permanent (n=4 SR; 4AF)	Right atrial appendage (Human)	KCNN3	Up-regulated (FC=2.33)	Repression of SK3 ion channel expression. Effects on APD and repolarization.
miR-26 (134)	Persistent (n=5AF; 5nAF)	Right atrial appendage (Human)	KCNJ2	Down-regulated; (FC~0.4 miR-26a)	Subunit of acetylcholine-sensitive potassium channel. Regulates APD and resting membrane potential.
miR-328 (135)	Tachypacing animal model persistent AF (n=7 AF; 7nAF)	Left atrial appendage (Canine)	CACNA1C CACNB1	Up-regulated (FC=3.5- 3.9)	Inhibits genes encoding α 1c and β 1 subunits of L-type calcium channel. Leads to reduction of I _{CaL} and increase in APD
miR-1 (136)	Persistent (n=31 AF; 31 SR for target analysis, n=7AF; 8SR miRNA analysis)	LA (AF patients with MVD); RA from SR patients (Human)	KCNJ2 GJA1	Down-regulated (FC~7; relative expression 100 $\pm$ 29 nAF vs. 14 $\pm$ 7 AF)	Subunit of acetylcholine-sensitive potassium channel. Regulates APD and resting membrane potential. GJA1 encodes connexin 43, altering cell-cell conduction

Table 2-5: MicroRNAs implicated in human and animal models of atrial fibrillation. AF- atrial fibrillation; pAF – paroxysmal atrial fibrillation; nAF – non-AF; VTP – ventricular tachypacing; RAA – right atrial appendage; LAA – left atrial appendage; SR – sinus rhythm; FC – fold change; APD – action potential duration.

More recently, Dawson et al. have also demonstrated a significant down-regulation of miR-29b in both the plasma (n=30SR, 33 AF) and right atrial appendage tissue of patients with paroxysmal (n=9) and chronic atrial fibrillation (n=8). The subsequent reduction in suppression of fibrillin (FBN) and the collagen (COL1A1 and COL3A1) mRNA transcripts is thought to promote atrial fibrosis thus promoting structural remodelling and increasing susceptibility to re-entry and atrial fibrillation.(126)

The results of this study demonstrate significant increase in miR-483-5p in the pre-operative serum of POAF patients. Furthermore, ROC analysis reveals an area under the curve of 0.78 when this biomarker is used to predict patients developing POAF. At present, there is no further evidence surrounding the use of circulating miR-483-5p as a serum biomarker. However, as previously discussed, an acute and sustained increase in circulating mir-483-3p has recently been isolated in heart failure patients after LVAD insertion, not only highlighting the ability for microRNA cleaved from the mir-483 stem loop sequence to be detected in the plasma, but also how this serum miR may vary alongside changes in cardiac stress.

2.5.3 Limitations

When interpreting the results of this study, it is also important to consider a number of limitations that may impact on these findings. Firstly, although the rigorous nature of this trial provides a robust definition of POAF with prospectively collected data, the numbers of patients recruited remain relatively small. Secondly, AF is a complex, multifactorial arrhythmia and microRNA dysregulation alone is unlikely to be the sole regulatory mechanism underlying the pathogenesis of post-operative atrial fibrillation. Third, although every attempt was made to match for environmental disparities, confounding factors such as electrolyte imbalance, post-operative sepsis (clinical or sub-clinical) and pharmacological therapies may all influence the relative expression of microRNA and the regulation of subsequent biological pathways.

2.6 Conclusions

This work has identified pre-existing microRNA dysregulation in patients subsequently developing POAF, particularly demonstrating under-expression of miR-208a and over-expression of miR-483-5p in the right atrium of POAF patients. In order to understand the significance of these changes it is now pertinent to clarify the potential mechanisms by which these miRNA may regulate cardiac conduction.

In addition, this study has provided the first documented description of circulating microRNA profile in POAF patients. Indeed, the observed differential expression of miRNA-483-5p in both the right atrial tissue and circulating plasma of these patients supports the hypothesis of a pre-existing atrial or systemic inflammatory substrate to this condition, highlighting the potential for biomarker development. Given these findings, it is now necessary to address the potential mechanisms behind these changes, and validate these findings as will be addressed in Chapter 3. Finally, prospective, targeted validation of these results in a larger patient cohort is required in order to determine the reliability of circulating microRNA-483-5p as a biomarker for POAF.

Chapter 3

Genomic mechanisms in POAF pathogenesis

3.1 Background

Following the recent advances in sequencing technology, there has been an exponential expansion in the number of microRNAs recorded in the literature from only 218 in 2002 to over 35 000 today (**Figure 3-1**). At publication in June 2014, the comprehensive microRNA database miRBase (Release 21, 26 June 2014; <http://www.mirbase.org>) listed 35828 mature miRNA and 28645 hairpin precursor miRNAs, across 223 species.(137)

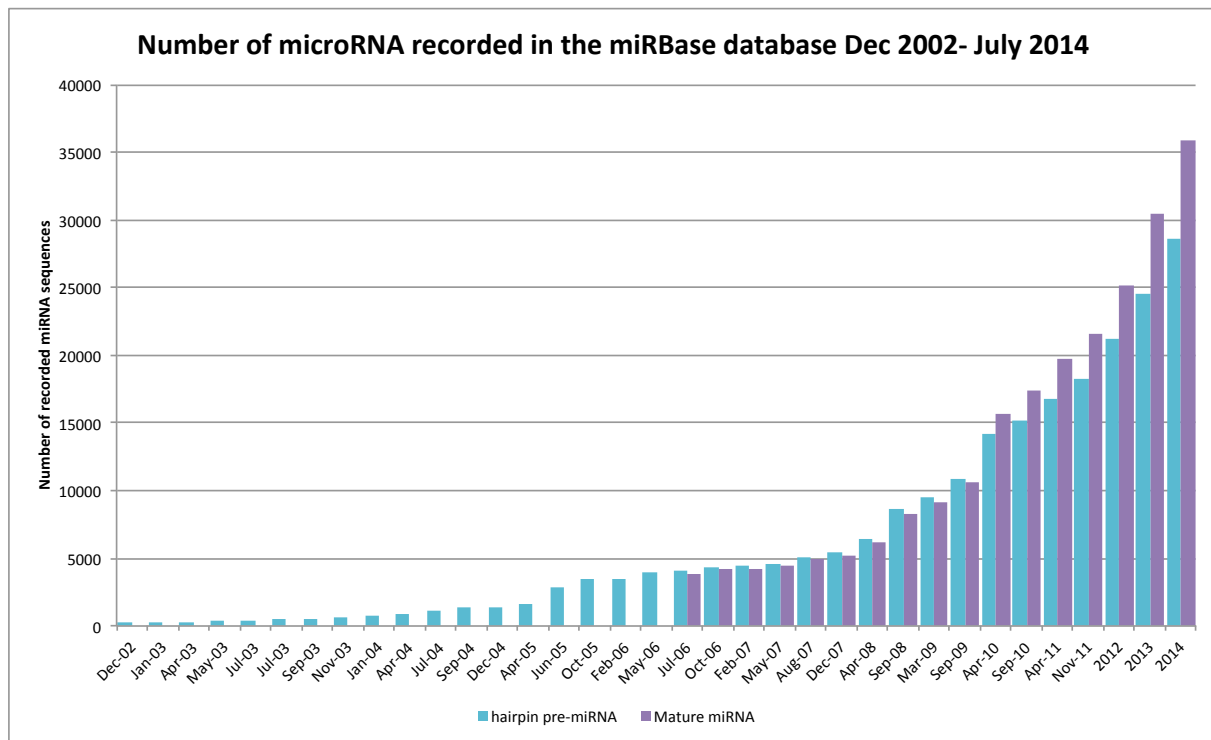


Figure 3-1. The exponential growth in recorded miRNA sequences as documented in the miRBase database. This is particularly notable after 2009 where the advent of more universally available sequencing technologies allowed for the more rapid discovery of novel miRNA species.

In order to determine the biological and clinical significance of these microRNAs it is vital to understand how they interact with their target genes. In light of the ever expanding numbers of miRNA and the fact each miRNA may target several hundred genes it is not possible to experimentally validate every potential miRNA-mRNA interaction and a robust method of target prediction is therefore required in order to guide researchers to mRNA targets likely to be relevant to the experiment or disease process being studied. In order to go about this a number of online target prediction packages have been developed. Each of these differs slightly in its layout and the way in which potentially functional targets are scored/ weighted, however for all, the initial basis for target prediction begins with determining target sites whereby there is complete or partial seed pairing of nucleotides 2-8 at the 5' end of the miRNA.

Until 2007, complete complementarity of these first 8 nucleotides was thought to be vital for miRNA-mRNA binding. However, it is now acknowledged that target mRNA knockdown requires more than simply seed region pairing. miRNA may bind with incomplete seed complementarity, and some miRNA-mRNA interactions may even be mediated outside of the seed region.(138) Furthermore, a given microRNA may have multiple binding sites on a particular gene, some of which may bind with perfect seed complementarity and others that bind only partially. Conversely, it has also been observed that where there is complete complementarity of 7mer or 8mer sites with the miRNA seed region, complete knockdown of the target mRNA does not always occur.(138) As a result, different target prediction algorithms rely on different degrees of seed pair binding with some even allowing a single base pair mismatch or bulging in the miRNA-mRNA duplex.

In fact, outside of the seed region, notably at nucleotides 12-17 (138), the remaining miRNA may bind with variable degrees of complementarity. The strength of this outside seed region binding may act to stabilize the miRNA-mRNA interaction and may even compensate for imperfect seed pairing.(138, 139) Furthermore, target genes may contain multiple binding

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sites for a single miRNA, particularly where there is a biological advantage to being able to achieve close to complete knockdown a given gene. In this case, miRNA may act at multiple binding sites to produce more marked suppression of the target. In fact, where sites are close together, there appears to be a synergistic effect resulting in even more marked suppression than that of combining individual site binding.(139)

In order to determine whether a given mRNA target has biological significance and warrants further experimental validation in the disease or cellular process being studied, it is pertinent to determine whether the miRNA-target interaction is likely to be functional or not. There are a number of observations accounted for in the common prediction algorithms that enable us to score the likelihood of target functionality (**Figure 3-2**). Described in detail by Grimson et al(138) these include: (1) *Position in relation to the 3'UTR of the target gene*: Binding sites are more likely to be effective if they reside close to the ends of the 3'UTR of the target gene, however they become less effective if they fall within 3'UTR sites that are within 15 nucleotides of the stop codon; (2) *AU enrichment*: Functional binding sites are more likely to be those surrounded by nucleotides enriched in AU bases; (3) *Degree of interspecies conservation*: 3'UTR targets sequences that are highly conserved between species are more likely to be functionally active; (4) *Proximity to target sites for co-expressed miRNAs*: Efficacious sites are more likely to be those in close proximity to co-expressed miRNAs. Indeed, in order to maximise the suppressive effect of a miRNA there may be multiple target sites within the same 3'UTR of a given mRNA.

Each of these factors may act independently to increase the likelihood of a target being a functional site. Therefore, instead of solely relying on seed region complementarity, target prediction algorithms now use a number of other methods to identify microRNA response elements (functional target sites) in target genes.

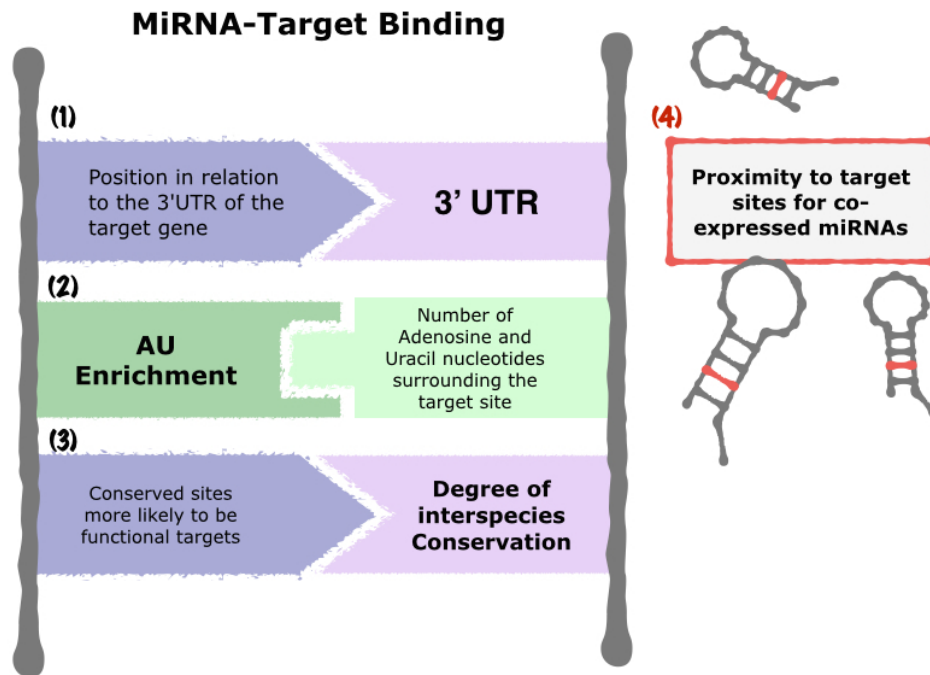


Figure 3-2: Mechanisms for determining the likelihood of microRNA target functionality beyond simple seed region pairing

Target Prediction Algorithms

There is a wide range of miRNA-mRNA target prediction algorithms available. These include: TargetScan, miRanda, PITA, RNA22, miRDB, RNAhybrid, PICTAR and Diana-microT. Of these, TargetScan, miRanda and PITA incorporate a cross section of the different features described above and are discussed in more detail below.

TargetScan

The TargetScan algorithm works by searching for seed pairing regions in 3'UTR alignments that are conserved between 28 vertebrate species.(140) Seed matching is based on 7mer binding (sites that match nucleotides 2-7 in the seed region as well as nucleotide 8 outside the seed region), and 8mer binding (sites that match the nucleotides 2-8 coupled with an A at position 1). In addition, potential binding sites are also reported where there is only partial complementarity in the seed region but 3' pairing is conserved. The efficacy of miRNA-mRNA pairing is given by the context+ score. This is designed to predict the accessibility of a

target site to a given miRNA by analysing both the sequence complementarity and the surrounding 3'UTR environment.

miRanda

MiRanda uses the aforementioned methods of target prediction and also provides a score to denote the likelihood of mRNA down-regulation when bound by a given miRNA. This score is known as the miRSVR score and is based upon a regression model incorporating both sequence complementarity and other local features that affect the likelihood of miRNA binding, including match and mismatch scores for base pairs, gap penalties and a secondary measure of the free energy of formation of the miRNA:mRNA duplex.(141) In addition to the miRSVR score, miRanda also provides a measure of conservation across mammalian genomes known as the PhastCons score. A score above 0.57 confers high degree of interspecies conservation.(141)

PITA

PITA (Segal Lab; http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html) uses an algorithm based on seed sequences from nucleotides 2-8 without allowing for mismatches or loops. For non-annotated genes, approximation is made of the 3'UTR. As with miRanda, the site conservation between mammalian species is also noted. In addition to these features, PITA focuses on the energetics of the microRNA-mRNA target interaction including the hybridization energy, the energy required to make the target accessible and the combined interaction energy.(142)

Although TargetScan and miRanda are the most commonly used in isolation, there is now an increasing trend towards a pooled approach to target prediction, with web-based programs such as miRWalk allowing the results of the main prediction algorithms to be viewed side by side and target genes selected for experimental validation based on their prediction by 2 or

more individual algorithms. However, although this approach is widely used within the published literature,(143-145) there is no protocol as to how the results of these algorithms should be weighted, and this approach may still yield several hundred to thousands of potential targets. Furthermore, the process of target prediction is made more complex by the functional nature of miRNA-mRNA interaction. It should also be remembered that the degree of binding may be related to the functional state of the cell, the expression of other miRNA, and the concentration of the miRNA in the cytoplasm.(140) Furthermore, the availability of regulatory proteins such as Argonaute may add even more complexity to the extent by which miRNA may knockdown their gene targets.(140)

Given the larger number of potential targets for a single miRNA the aim of target prediction in this study is to generate a clinically relevant list of potential genes for further experimental validation. The miRWalk program will be used to examine the aforementioned prediction algorithms in combination in an attempt to reduce the number of false positives. Given that miRNA do not result in 100% knockdown of their target mRNA, the degree of mRNA expression will also be experimentally validated. Finally, the effect this modulation of messenger RNA has on protein expression will ultimately be determined in order to provide a mechanistic understanding of the downstream impact miRNA changes may have on cellular function and ultimately disease pathogenesis.

3.1.1 Pathway Analysis

The role of pathway analysis subsequent to miRNA target prediction is to provide an overview of how changes in gene and protein expression may affect cellular function. Given a list of differentially expressed miRNA, we are able to establish a likely list of gene expression changes as previously mentioned. Subsequent to this, these genes may be inputted into a number of pathway prediction tools that examine the degree of enrichment of these genes in known pathways. These findings may then be correlated with the disease process of interest

and a mechanistic model of disease pathogenesis may be proposed. As with miRNA target prediction, a number of pathway tools are available, each with its advantages and disadvantages. These include Ingenuity Pathway Analysis (IPA) (<http://www.ingenuity.com>), DIANA-miRPath (<http://diana.imis.athena-innovation.gr>), (146) Kyoto Encyclopedia of Genes and Genomes (KEGG) (<http://www.genome.jp/kegg/pathway.html>) and Reactome (<http://www.reactome.org>) which provide a combination of commercial and free databases with independently curated pathways for a wide range of cellular processes and disease states. This study will use a combination of these methods in order to examine upstream and downstream regulation in post-operative atrial fibrillation as well as providing a visual representation of estimated perturbations in cellular signaling.

3.1.2 Aims

The aim of this chapter is to establish how microRNA changes may play a mechanistic role in altering gene and subsequent protein expression in post-operative AF. Online target enrichment resources and comprehensive bioinformatics databases will be used to predict potentially clinically relevant mechanisms for the microRNA changes described in Chapter 2. These pathways will then be systematically investigated from the transcriptome to protein level in order to establish whether they are likely to be involved in the pathogenesis of de novo post-operative atrial fibrillation.

3.2 Methods

3.2.1. RT-qPCR of target mRNA

Reverse Transcription (RT): Universal reverse transcription (RT) master mix (No AmpErase® UNG (Uracil N-Glycosylase); Applied Biosystems) was made up according to the number of samples to be analysed. This comprised of: 2.0µl 10x Reverse Transcription Buffer, 2.0µl 10x Random Primers, 1.0µl MultiScribe Reverse Transcriptase (50U/µl), 0.8µl 25x dNTP Mix (100mM), 1.0µl Nuclease free water, and 3.2µl RNase free water per sample. A total of 10µl of RT master mix was then added to each sample.

After addition of the RT master and primer mix, samples were mixed by pipetting and centrifuged at 2000 rpm for 30 seconds. Samples were then transferred to the thermocycler and reverse transcription was carried out according to the manufacturer's protocol (HOLD 10 mins at 25 degrees, HOLD 120 minutes at 37 degrees, HOLD 5 minutes at 85 degrees, HOLD ∞ at 4 degrees). After incubation, resultant cDNA was either used immediately for PCR reaction or stored at -80°C until use.

PCR Reaction: Into new PCR tubes 2.4µl of universal sample cDNA (RT product) was added to 32µl of PCR Master Mix (17.25µl of TaqMan fast universal PCR master mix, 1.725µl of TaqMan gene expression assay, 13.23µl of RNase free water). For each study probe an identical control PCR reaction (U6) was also carried out.

Samples were mixed by pipetting and centrifuged at 2000g for 30 seconds. 10µl of qPCR sample mixture was then added to each well of a 96-well PCR plate. Three technical replicates were performed for each of the samples and controls. Plates were then centrifuged at 500g for 30s prior to being placed into the Applied Biosystems 7500 fast Real-Time PCR System for qPCR reaction. The PCR reaction was carried out in accordance with the manufacturers protocol (114): Hold at 50°C for 2 minutes, Hold at 95°C for 20s, Cycle (40
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cycles) 95°C for 3s, Hold at 60°C for 30 seconds. Amplification plots were examined for adequate amplification and successful PCR reaction. Baseline settings were checked to ensure appropriateness according to the amplification curve. A delta R_n threshold value of 0.2 was set to ensure comparability across plates, however this was checked to ensure it was within the exponential phase of amplification prior to analysis. C_t values were recorded for both samples and controls and compared using the $\Delta\Delta C_t$ method.

3.2.2 Protein Extraction

Protein was extracted from atrial tissue using RIPA buffer (SIGMA) without the addition of phosphatase inhibitor as the phosphorylation states of target proteins were not to be examined in this experiment. 30-40µg of tissue was homogenised in a solution of 500µl cell disruption buffer and 5µl protease inhibitor and kept cool on ice to ensure maximal homogenisation. The homogenised samples were then centrifuged at 12 000g for 5 minutes and the supernatant discarded. 400µl of RIPA buffer was then added to the pellet containing the protein extract and samples were vortexed to ensure mixing. Samples were then placed in a sonicator at 4°C for 30 minutes to maximise tissue disruption. The samples were then spun at 12 000g for 20 minutes to pellet out the cell debris. The supernatant containing soluble protein was then transferred to a fresh sample tube and concentration of extracted protein assessed using the bicinchoninic acid (BCA) assay.

3.2.3 Protein Bicinchoninic Acid (BCA) quantification

The concentration of extracted protein was determined using the Pierce[®] protein assay kit and absorbance measured using the BMG FluoStar Galaxy microplate reader. This principle of this method is that the Cu^+ product of the Biuret reaction ($Cu^{2+} \rightarrow Cu^+$ by protein in the presence of alkali) is chelated with two molecules of BCA forming a purple-coloured end product. Experiments were carried out in accordance with the manufacturers protocol.

Standard stock solutions were first prepared producing a range from 25-2000 µg/mL. The BCA working reagent was then prepared by mixing BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) with BCA reagent B (4% cupric sulfate) at a ratio of 50:1. The microplate was prepared by adding 25µl of each standard and study sample to separate wells. All standards and samples were repeated in duplicate. 200µl of working reagent was then added to each well, samples mixed on a plate shaker for 30s and the plate incubated at 37°C for 30 minutes. After cooling to room temperature, the absorbance of each sample was recorded at a wavelength of 562nm. By measuring the absorbance of known standard assays, Microsoft Excel was used to create a standard graph from which the protein concentration of test samples could then be extrapolated.

3.2.4 Western Blotting

Prior to commencing western blotting, protein concentrations were determined by BCA assay as above. The volume of protein required to load 30mg of protein was calculated and aliquoted accordingly. RNase free water was then added to protein aliquots sufficient to reach a uniform loading volume of 20µl. 5µl of loading buffer mixed with β-mercaptoethanol (1:20 mix) was then added to each sample, samples were denatured for 5 minutes at 95°C and centrifuged at 10 000 rpm for 5 minutes. Samples and ladder markers were then loaded onto a pre-prepared, 10 or 15 well, 8% SDS-PAGE gel. For analysis of smaller proteins (<30kDa) 12% SDS-PAGE was used in order to obtain better separation at this range of molecular weight. This was run at 60V for 15-20 minutes (until stacked) followed by 100V for 1.5-2 hrs or until the protein had passed three quarters of the way through the gel. Once complete, the gels were carefully lifted out of the apparatus and removed from the slides.

Proteins were transferred onto a 0.45µm Protran[®] membrane by running in a transfer tank at 150V and 400mA for 1.5 hours. Successful protein transfer was confirmed with Ponceau S

stain and the membrane subsequently de-stained by washing three times in PBS-T (Phosphate Buffered Saline/Tween at a ratio of 1000ml:1ml) buffer for 5 minutes. The membrane was then blocked for 1 hour with 20ml of 5% milk protein blocking buffer and subsequently washed with PBS-T as previously described. The membrane and primary antibody were finally mixed with milk protein blocking buffer (1:10) and incubated overnight at 4°C.

The following day, the primary antibody was removed and the membrane washed again in PBS-T. The membrane was then incubated with the secondary antibody (anti- β -Actin) (with added anti-biotin (1:1000) to detect the biotinylated ladder) for 2 hours at room temperature. Following repeat wash the membrane was stained using 2mls of Luminata™ Forte Western HRP Substrate (Millipore) and scanned using the ChemiDoc™ MP imaging system (BioRad technologies) Chemiluminescence scanner to detect the presence of protein bands.

In order to detect β -Actin as a loading control the membrane was then re-blocked for 1 hour in blocking buffer. After repeat washing, the membrane was incubated in primary mouse β -Actin for 1 hr followed by washing and incubation in goat-anti-mouse secondary antibody for 1 hour. Finally, the membrane was washed, stained with chemiluminescence stain, and control bands measured as described above. Where required for repeat analysis membranes were stripped using ReBlot Plus Strong Antibody Stripping Solution, 10x (Millipore) (diluted 1:10) for 10 minutes prior to repeat blocking and incubation with the required antibodies. Quantitation of protein concentrations was preformed using Kodak Image Station 2000M analysis software.

3.2.5 Enzyme Linked Immuno-Sorbant Assay (ELISA)

A solid phase sandwich ELISA technique was used to quantify the concentration of VCAM1 target protein (Abcam®). ELISA was carried out according to the manufacturers protocol. Serum samples were first diluted 1:50 and reagents prepared as per protocol. All samples, blanks, controls and standards were run in duplicate. Standard dilutions were made by

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pipetting 200µl of standard into wells A1 and A2 and 100µl standard diluent into wells B1-F1 and B2 to F2. 100µl of liquid was transferred from A1 to B1 and mixed by pipetting. This was repeated from B1 to C1, C1 to D1, etc... until a series dilution from 50-1.56 ng/ml was formed (**Table 3-1**). Control and blank samples were added to H1/H2 and G1/G2 respectively and 100µl of sample was added into all remaining sample wells. 50µl of biotinylated monoclonal antibody specific for VCAM1 was added to each well and the plate incubated for 1hr at room temperature (RT). All wells were then emptied and washed 3 times with 300µl of wash buffer. After washing, 100µl of streptavidin-peroxidase enzyme was added to each well and the plate incubated again for 30 minutes at RT. After incubation, the plate was again washed 3 times with 300µl wash buffer to remove all unbound enzyme. 100µl of chromogen TMB substrate solution was then added to all wells, which then acts on the bound enzyme resulting in a coloured reaction proportional to the concentration of VCAM1 present in the well. After a dark incubation period of 12-15 mins at RT the reaction was stopped with 100µl sulphuric acid (H₂SO₄) and the absorbance read immediately using a spectrophotometer set to a primary wavelength of 450nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	50	50	Sample	S	S	S	S	S	S	S	S	S
B	25	25										
C	12.5	12.5										
D	6.25	6.25										
E	3.125	3.125										
F	1.56	1.56										
G	Blank	Blank										
H	Control	Control										

Table 3-1: ELISA well plate layout

3.2.6. Target Identification and Verification

Gene Ontology pathway enrichment

In order to determine the pathways potentially affected by the dysregulated miRNA profile associated with POAF, the 16 differentially expressed miRNA were input into the DIANA-miRPath web-server. DIANA-miRPath is a miRNA pathway analysis tool which utilises targets either experimentally validated in the TarBase database or predicted by the DIANA-microT-CDS algorithm based on their 3'UTR sequence homology to the miR seed region.(146) Resultant pathways were displayed where $p < 0.05$.

Selection of clinically relevant target proteins

Based on the results of the above RT-qPCR analysis potentially clinically relevant gene targets were assessed for hsa-miR-483-5p and hsa-miR-208. The miRWalk (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html>) database was used in order to determine the list of potential and validated target genes.(147) As previously discussed, the miRWalk database examines miRNA-target interactions as produced by 8 miRNA prediction programs based upon the 3' UTRs of all known human genes. This includes the following databases: RNA22, miRanda, miRDB, TargetScan, RNAhybrid, PICTAR and Diana-microT. All targets identified for hsa-miR-483-5p (**Appendix 1**) and hsa-mir-208 (**Appendix 2**) by more than 2 of the 8 databases were manually examined for clinical significance. A literature search was then performed of all potentially clinically relevant targets to determine any previous association with atrial fibrillation. For non-validated 7mer and 8mer seed matched targets, the likelihood of functionality was determined according to the degree of conservation of the target (PhastCons score), the miRSVR score and the context score using the and miRanda (microrna.org) and TargetScan algorithms respectively (see **Chapter 3.1** for a detailed description of the basis of these target prediction programs). As a

result, 4 clinically relevant targets were selected for further investigation and potential significance.

Targeted pathway analysis was secondarily performed using Ingenuity Pathway Analysis (IPA) software (<http://www.ingenuity.com/products/ipa>) to detect relevant target genes associated with human Atrial Fibrillation. In addition, canonical pathways relevant to the genes of interest were examined using the IPA, KEGG and Reactome databases. Finally, a systematic literature search was performed using the PubMed (<http://www.ncbi.nlm.nih.gov/pubmed>) and NCBI Gene (<http://www.ncbi.nlm.nih.gov/gene/>) databases in order to detect any further clinically relevant targets not previously identified. These were then cross-referenced with my dataset and subsequent pathway maps.

3.3 Results

3.3.1. Gene Ontology Pathway Enrichment

Gene ontology (GO) analysis predicted a number of enriched pathways as shown in **Figure 3-3**. A full summary of enriched pathways along with the miRNA and gene targets implicated is shown in **Table 3-2**. Of particular interest included the calcium signaling pathway (4 miRNA: miR-19a-3p; miR-19a-5p; miR-3679-5p; miR-4298), the TGF β signaling pathway (4 miRNA: miR-301a-3p, miR-150-3p, miR-150-5p, miR-638), gap junction function (4 miRNA: miR-483-5p, miR-301a-3p, miR-19a-3p, miR-3679-5p), insulin signaling pathway (4 miRNA: miR-301a-3p, miR-19a-3p, miR-150-5p, miR-3679-5p), phosphatidylinositol signaling system (3 miRNA: miR-301a-3p, miR-19a-3p, miR-4928) mTOR signaling pathway (2 miRNA: miR-301a-3p, miR-19a-3p) and the PI3K-Akt signaling pathway (miR-150-5p, miR-29b-3p, miR-29b-1-5p, miR-483-5p). Other pathways potentially clinically relevant to AF pathogenesis include extracellular matrix signaling, Ubiquitin mediated proteolysis, protein digestion and absorption and focal adhesion. Notably however, although all differentially expressed miRNA were input, the resultant DIANA-mirPath pathway analysis miR-did not include any of the predicted target sites from miR-208a.

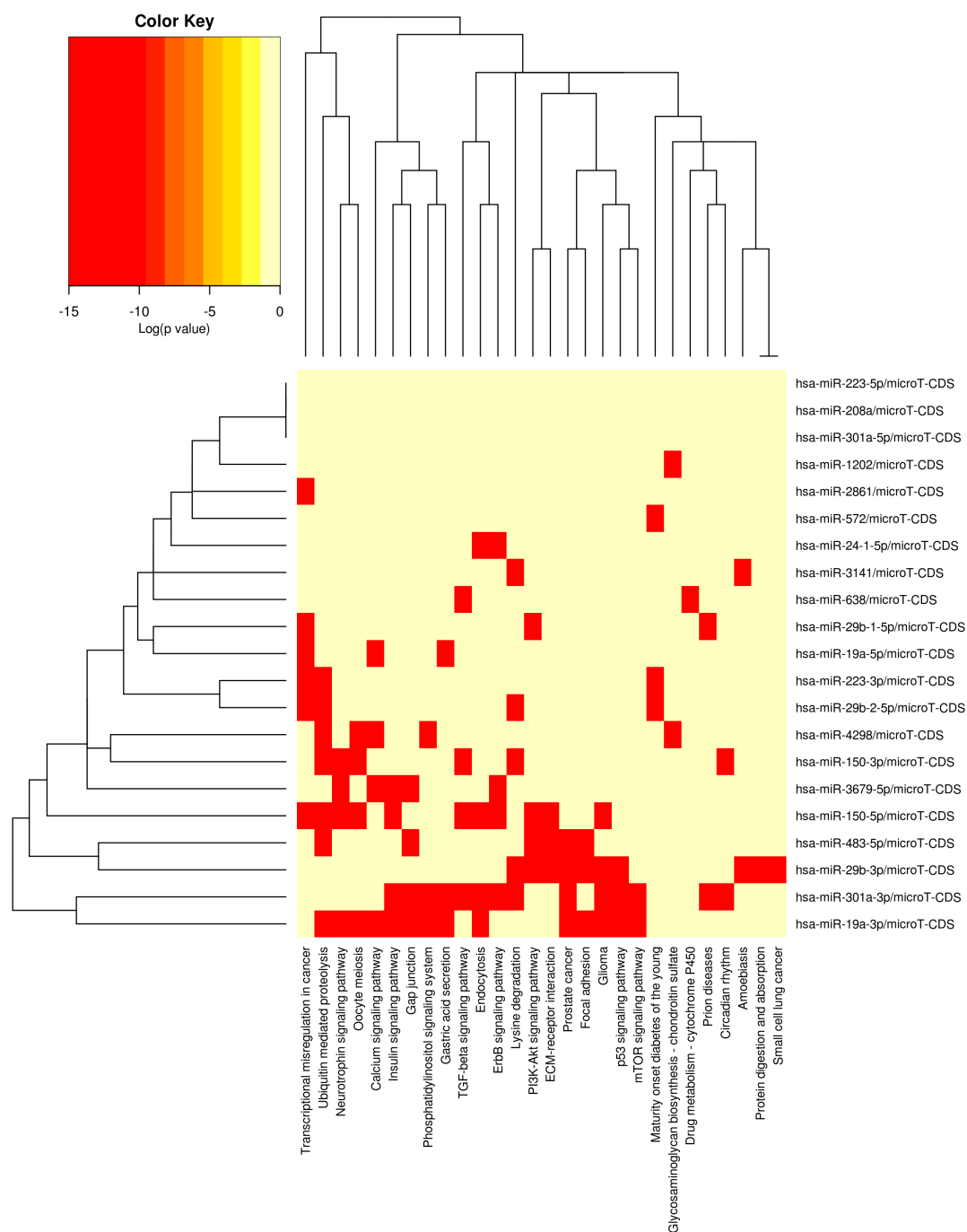


Figure 3-3: Results from DIANA-mirPath pathway analysis: Enriched pathways incorporating target genes of the 16 differentially expressed miRNA between the atrial tissue of POAF vs. non-POAF patients.

KEGG pathway	p-value	miRNAs regulating pathway	Genes targeted in pathway
Prion diseases	0	hsa-miR-29b-1-5p, hsa-miR-301a-3p	PRNP
ECM-receptor interaction	0	hsa-miR-483-5p, hsa-miR-150-5p, hsa-miR-29b-3p	SV2B, COL4A5, COL27A1, COL3A1, COL2A1, COL4A2, COL5A1, COL1A1, COL4A3, COL4A4, COL1A2, LAMC1, COL11A1, COL6A3, COL4A6, LAMA2, TNN, COL5A3, COL5A2, COL4A1, ITGB3
Drug metabolism - cytochrome P450	3.66E-14	hsa-miR-638	CYP3A43
Amoebiasis	1.07E-13	hsa-miR-29b-3p, hsa-miR-3141	COL4A5, COL27A1, SERPINB9, COL3A1, COL2A1, COL4A2, COL5A1, COL1A1, COL4A3, PIK3R1, COL4A4, COL1A2, LAMC1, COL11A1, COL4A6, LAMA2, COL5A3, ITGB2, COL5A2, COL4A1
Protein digestion and absorption	9.89E-13	hsa-miR-29b-3p	COL4A5, ELN, COL27A1, COL7A1, COL22A1, COL3A1, COL9A1, COL21A1, COL2A1, COL15A1, COL4A2, COL5A1, COL1A1, COL4A3, COL4A4, SLC36A1, COL1A2, COL11A1, COL6A3, COL4A6, COL5A3, COL5A2, COL4A1
Focal adhesion	5.49E-11	miR-483-5p, hsa-miR-19a-3p, hsa-miR-29b-3p	FLNC, COL4A5, RAP1A, CCND2, COL27A1, CAV2, ITGA1, COL3A1, AKT2, COL2A1, PPP1R12A, PIK3R3, CCND1, COL4A2, MAPK8, COL5A1, PDGFB, COL1A1, COL4A3, ITGA2, PIK3R1, COL4A4, PAK6, COL1A2, AMC1, IGF1, PDGFC, COL11A1, COL6A3, PDGFD, PIK3CA, COL4A6, LAMA2, ITGA6, TNN, COL5A3, PTEN, MAPK1, RAP1B, COL5A2, COL4A1, ITGB3, PDGFA
PI3K-Akt signaling pathway	6.02E-09	hsa-miR-483-5p, hsa-miR-150-5p, hsa-miR-29b-1-5p, hsa-miR-29b-3p	GSK3B, MYB, SOS2, PPP2CA, CREB5, COL4A5, COL24A1, MCL1, COL27A1, GNG12, PIK3AP1, CDK6, COL3A1, EFNA5, TP53, AKT2, PTK2, COL2A1, EIF4E, COL4A2, PPP2R2A, COL5A1, PPP2R1A, PDGFB, COL1A1, EIF4B, COL4A3, PIK3R1, COL4A4, KITLG, INSR, FGF18, COL1A2, LAMC1, IGF1, PDGFC, COL11A1, COL6A3, PDGFD, COL4A6, FOXO3, TNC, LAMA2, TNN, COL5A3, PTEN, CSF1R, KDR, COL5A2, TEK, RPS6KB1, COL4A1, ITGB3, PDGFA
mTOR signaling pathway	3.01E-07	hsa-miR-19a-3p, hsa-miR-301a-3p	TSC1, RRAAGD, PRKAA2, RPS6KA2, RICTOR, PIK3R3, PDK1, PRKAA1, IGF1, EIF4E2, PIK3CA, PTEN, CAB39, MAPK1, ULK2, CAB39L
Transcriptional misregulation in cancer	1.14E-06	hsa-miR-223-3p, hsa-miR-150-5p, hsa-miR-19a-5p, hsa-miR-2861, hsa-miR-29b-2-5p, hsa-miR-29b-1-5p	CCNT2, GOLPH3L, BAIAP3, LMO2, ELK4, HMG2A, AFF1, PAX5, PBX1, MLLT3, IGF1R, ZEB1, PAX8, TP53, CCNT1, PTK2, RUNX1T1, CDK9, NR4A3, NTRK1, WT1, NCOR1, JMJD1C, DD5, SP1, IGF1, MLL, TFE3, RELA, EWSR1, FOXO1, CSF1R, HHEX, SMAD1, NGFR, MEIS1
Lysine degradation	0.000209195	hsa-miR-150-3p, hsa-miR-29b-2-5p, hsa-miR-29b-3p, hsa-miR-301a-3p, hsa-miR-3141	SETMAR, OGDHL, WHSC1L1, SETD7, ALDH3A2, SETDB2, SETD2, NSD1, SETDB1, MLL2, DOT1L, WHSC1, SUV420H2, MLL, MLL3
Insulin signaling pathway	0.000724489	hsa-miR-150-5p, hsa-miR-19a-3p, hsa-miR-301a-3p, hsa-miR-3679-5p	BRAF, GSK3B, TSC1, SOS2, CBL, PRKAA2, CALM1, CALM2, SOCS3, CBLB, PPP1R3A, PIK3R3, EIF4E, SOCS1, MAPK8, PRKAG1, PDK1, PIK3R1, PRKAR2A, PRKX, IRS1, PRKAA1, PRKAR1A, SHC4, EIF4E2, PPARGC1A, PIK3CA, FLOT2, MAPK1, PRKACB, PPP1C8
Small cell lung cancer	0.000842802	hsa-miR-29b-3p	COL4A5, TRAF4, CDK6, AKT2, COL4A2, COL4A3, PIK3R1, COL4A4, LAMC1, COL4A6, LAMA2, CYCS, PTEN, COL4A1
Gap junction	0.002655966	hsa-miR-483-5p, hsa-miR-19a-3p, hsa-miR-301a-3p, hsa-miR-3679-5p	ADCY1, SOS2, ADCY7, PLCB1, ITPR1, PRKX, GNAQ, PDGFD, GJA1, MAP3K2, HTR2C, MAPK1, ADCY9, ADRB1, PLCB4, PRKACB
Maturity onset diabetes of the young	0.002973924	hsa-miR-223-3p, hsa-miR-29b-2-5p, hsa-miR-572	PAX6, SLC2A2, NEUROD1, ONECUT1, HHEX
Glioma	0.003787136	hsa-miR-150-5p, hsa-miR-19a-3p, hsa-miR-29b-3p, hsa-miR-301a-3p,	BRAF, SOS2, CAMK2G, E2F2, CALM3, CALM1, TGFA, CDK6, CALM2, TP53, AKT2, PIK3R3, CCND1, PDGFB, PIK3R1, IGF1, SHC4, PIK3CA, CDKN1A, PTEN, MAPK1, PDGFA
Glycosaminoglycan biosynthesis - chondroitin sulfate	0.004075271	hsa-miR-1202, hsa-miR-4298	CHSY3, CSGALNACT2
Ubiquitin mediated proteolysis	0.005075113	hsa-miR-223-3p, hsa-miR-483-5p, hsa-miR-150-3p, hsa-miR-150-5p, hsa-miR-19a-3p, hsa-miR-29b-2-5p, hsa-miR-4298	UBE2R2, CBL, BTRC, FBXW7, TRIM37, UBR5, WWP1, SMURF2, ITCH, UBE2J1, HERC1, TCEB1, MID1, SKP1, SOCS3, CBLB, BRCA1, CUL5, ANAPC4, UBE2A, SOCS1, UBE2N, KIAH1, NEDD4, HERC4, UBE2D1, UBE2D2, UBE2G1, CUL3, UBE2W, PPII2, FBXW11, UBE4B, XIAP
Prostate cancer	0.005626752	hsa-miR-483-5p, hsa-miR-19a-3p, hsa-miR-29b-3p, hsa-miR-301a-3p,	SOS2, E2F2, TCF4, CREB5, TGFA, AKT2, PIK3R3, CCND1, PDGFB, PDK1, PIK3R1, IGF1, PDGFC, CREB3L2, PDGFD, PIK3CA, CDKN1A, PTEN, MAPK1, PDGFA
TGF-beta signaling pathway	0.007508118	hsa-miR-150-3p, hsa-miR-150-5p, hsa-miR-301a-3p, hsa-miR-638	ROCK1, INHBB, SMURF2, INHBA, RHOA, ACVR1, SKP1, PPP2R1A, ZFYVE9, SMAD5, GDF6, SP1, TGF2B, LTBP1, TGFB2, INHBE, BMPR2
p53 signaling pathway	0.01127493	hsa-miR-19a-3p, hsa-miR-29b-3p, hsa-miR-301a-3p	ZMAT3, CDS1, CCND2, GADD45A, CDK6, ATM, CCND1, MDM4, IGF1, CASP8, IGF1BP3, CDKN1A, SESN3, CYCS, PTEN, CCNG2, PPM1D
Phosphatidylinositol signaling system	0.01156915	hsa-miR-19a-3p, hsa-miR-301a-3p, hsa-miR-4298	DGKE, CALM3, CALM1, ITPKB, PLCZ1, DGKG, CALM2, PLCB1, PIKFYVE, PIK3R3, ITPR1, PIP4K2A, PIK3C2A, PIK3CA, PTEN, ITPK1, PLCB4, PIK3C2G
Endocytosis	0.01590118	hsa-miR-19a-3p, hsa-miR-301a-3p, hsa-miR-150-5p, hsa-miR-24-1-5p	RNF41, ARAP2, CBL, VTA1, WWP1, PRKCI, SMURF2, EHD4, ITCH, PSD, EPN2, CLTC, CHMP4B, TSG101, ASAP1, RAB5A, VPS4B, CBLB, NTRK1, EPS15, ZFYVE9, RAB11A, TGF2B, GRK6, LDLR, DNMT3, CSF1R, CLTB, TGF2R2, ADRB1, ERBB4, ASAP2
Calcium signaling pathway	0.02317845	hsa-miR-19a-3p, hsa-miR-19a-5p, hsa-miR-3679-5p, hsa-miR-4298	PDE1C, ADCY1, ADCY7, ORAI2, CALM3, CALM1, ITPKB, CHRM3, ADRB3, PLCZ1, ATP2B1, CACNA1B, EDNRB, ITPR1, PRKX, STIM1, SLC25A6, PHKA1, PLCG1, HTR5A, GNAQ, EDNRA, HTR2C, P2RX7, GRIN2A, ADCY9, ADRB1, ERBB4, PRKACB
Neurotrophin signaling pathway	0.02796873	hsa-miR-150-3p, hsa-miR-150-5p, hsa-miR-19a-3p, hsa-miR-3679-5p	BRAF, GSK3B, SOS2, CAMK2G, CALM1, RAP1A, MAPK14, NTRK3, RPS6KA2, RHOA, CALM2, RPS6KA5, TP53, PIK3R3, MAPK8, NTRK1, PIK3R1, PTPN11, IRS1, PLCG1, GAB1, SHC4, PIK3CA, IRAK2, IRAK1, MAPK1, RAP1B, MAP3K5
ErbB signaling pathway	0.02865279	hsa-miR-150-5p, hsa-miR-24-1-5p, hsa-miR-301a-3p, hsa-miR-3679-5p	BRAF, GSK3B, SOS2, CBL, CAMK2G, TGFA, PTK2, CBLB, PIK3R1, PAK6, BTC, NRG1, PLCG1, GAB1, SHC4, CDKN1A, ABL2, ERBB4, EREG,,
Oocyte meiosis	0.03103499	hsa-miR-150-3p, hsa-miR-150-5p, hsa-miR-19a-3p, hsa-miR-4298	PPP2R5E, ADCY1, PGR, CAMK2G, ADCY7, BTRC, CALM3, CALM1, RPS6KA2, CPEB1, SMC3, PLCZ1, PPP2R5C, CALM2, SKP1, ANAPC4, PPP2R1A, ITPR1, YWHAZ, PRKX, RPS6KA3, IGF1, MAPK1, FBXW11, ADCY9
Circadian rhythm	0.0390915	hsa-miR-150-3p, hsa-miR-301a-3p	PRKAA2, BTRC, BHLHE41, SKP1, PRKAA1, FBXW11, CLOCK
Gastric acid secretion	0.04465674	hsa-miR-19a-3p, hsa-miR-19a-5p, hsa-miR-301a-3p	ADCY1, ADCY7, CALM1, ATP1A2, KCNK2, CALM2, SLC26A7, PLCB1, KCNK10, ITPR1, PRKX, KCNJ10, KCNJ2, GNAQ, ADCY9, PLCB4

Table 3-2: A summary of significantly enriched pathways in POAF vs. non-POAF patients. The microRNA with and their gene targets in each pathway are shown. Highlighted columns denote pathways with clinical relevance in AF pathogenesis.

3.3.2 Specific Target Identification and Verification

Generalised pathway analysis provides an overview of the signaling cascades potentially affected as a result of the dysregulated miRNA profile observed in our patient cohort. These findings represent a measure of the over-representation (or enrichment) of the gene targets for my differentially expressed miRNAs within each signaling cascade. However, these tools have a number of limitations not only in the algorithms used to predict potential miR targets, but also in the number of signaling cascades represented in the database, and the completeness of our understanding of each of the individual genes and proteins involved in each of these pathways.

As a result, for a more complete study of the mechanism by which these miRNA regulate AF pathogenesis, I combined this with a targeted approach of individual clinically relevant genes identified using the miRWalk database. Two clinically relevant targets were studied in detail for the most under-expressed and over-expressed miRNA in POAF patients when compared to those maintaining sinus rhythm.

(a) hsa-miR-483-5p:

The miRWalk database lists 17 validated targets for hsa-miR-483-5p (**Appendix 1a**). Based on a search restricted to miRNA binding at the 3'UTR, producing a list of all gene transcripts with a minimum seed length of 7 bp and a probability of distribution of random matches of a subsequence in the given sequence of $p=0.05$, miRWalk lists 4985 predicted targets of miR-483-5p of which 999 were predicted by 3 or more databases. (**Appendix 1b and 1c**)

MAPK3 (ERK1/2): This extracellular signaling kinase is well recognised to play a key role in a number of cell signaling processes including cell differentiation and proliferation. Increased MAPK3 expression has been previously identified in patients with chronic AF and

atrial fibrosis.(148) MAPK3 was found to be a predicted target of hsa-miR-483-5p with a 7 bp seed length (sequence: AGACGGG) at a p value of 0.0495. The MirSVR score for the miR-483-5p/MAPK interaction was found to be ‘good’ (>-0.1) at -1.0996,(149) with a PhastCons score of 0.5875 (>0.57) demonstrating good conservation across mammalian genomes.(141, 150)

KCNE5 (KCNE1L): KCNE5 forms part of a group of regulators (KCNE1-KCNE5) of the voltage gated potassium channel responsible for the slow inward rectifying potassium current (I_{Ks}). Its gene product, MiRP4, is a membrane spanning protein thought act as a down-regulator to the α -subunit KCNQ1 resulting in suppression of I_{Ks} . Reduced I_{Ks} within the atrial myocardium shortens the refractory period giving rise to the potential for macro re-entrant circuits to develop leading to atrial fibrillation.(151) KCNE5 was found to be a potential target for miR-483-5p by 3 of the 8 prediction programs (miRanda, TargetScan and PITA), however this target is yet to be validated. The MirSVR score for the miR-483-5p/KCNE1L interaction was found to be -0.0408, with a PhastCons score of 0.6923 again demonstrating good conservation across mammalian genomes.

(b) hsa-miR-208a:

The miRWalk database lists 15 validated targets (**Appendix 1d**) and 4688 predicted targets for miR-208a, of which 1994 were predicted by 3 or more databases (search criteria as described above).

MED13: MED13 is a validated target of hsa-MiR-208a with significant potential clinical relevance in the study of AF. It has been identified to both be involved in the cardiac control of systemic energy metabolism (108) and in stress-dependent cardiac growth and fibrosis.(152). Target analysis of the miR-208a/MED13 interaction reveals 4 potential binding sites on the MED13 mRNA beginning at the following sites from the 5’end: (1) Base 499 (2)

(1)	<pre> 3' ugUUCGAAAA---ACG-----AGCAGAAUa 5' hsa-miR-208a : : : 449:5' guAAGUUUUUAAAUGUAAAAUUGUCUUAu 3' MED13 </pre>	<p>mirSVR score: -0.1391</p> <p>PhastCons score: 0.7920</p>
(2)	<pre> 3' ugUUCGAAAAACGAGCAGAAUa 5' hsa-miR-208a 551:5' aaAAUAUAUGUAAUCGUCUUAa 3' MED13 </pre>	<p>mirSVR score: -1.2116</p> <p>PhastCons score: 0.7255</p>
(3)	<pre> 3' uguucgaaaaacgagCAGAAUa 5' hsa-miR-208a 775:5' uugaaaagguaccagGUCUUAc 3' MED13 </pre>	<p>mirSVR score: -0.0220</p> <p>PhastCons score: 0.7126</p>
(4)	<pre> 3' ugUUCGAAAAACG--AGCAGAAua 5' hsa-miR-208a : 2981:5' uaAAAUUAUUCUCCUUUCGUCUuca 3' MED13 </pre>	<p>mirSVR score: -0.1495</p> <p>PhastCons score: 0.7594</p>

3.3.3 Assessment of target mRNA

Expression of target atrial mRNA was assessed by RT-qPCR using universal RT primers. As discussed above, the following targets were evaluated:

- **miR-483-5p:** KCNE5 and MAPK3
- **miR-208a:** GATA4, MED13

No significant difference was observed in atrial mRNA expression of GATA4, MED13, KCNE5, or MAPK3 between AF and non-AF groups (**Table 3-3**). However, despite not reaching statistical significance, the expression of KCNE5 mRNA tended to be lower in the AF group suggesting that the presence of increased levels of miR-483-5p may be producing a modest degree of knockdown of this predicted target (**Figure 3-5**). Furthermore, the standard deviation appeared much greater in the AF group than in patients maintaining sinus rhythm suggesting that the degree of variation in target knockdown may be high between individuals. This finding would in fact be expected in a complex, multifactorial disease process such as post-operative atrial fibrillation and the expression of KCNE5 protein will therefore be examined in more detail (**Section 3.3.4**).

miRNA	Target mRNA	AF	non-AF	Median FC AF	Median FC nAF	P
miR-208a	GATA4	7	8	1.182	1.048	0.5265
	MED13	10	11	0.7768	0.9265	0.9515
miR-483-5p	KCNE5	10	11	1.348	0.9635	0.1506
	MAPK3	10	11	1.024	1.062	0.7712

Table 3-3: Mann-Whitney U test to assess the significance of differences in fold change of target mRNA expression between AF and non-AF groups. No significant differences were observed in any of the target mRNA identified.

Messenger RNA expression of the KCNE5 gene in the atrium of POAF vs. non-POAF patients

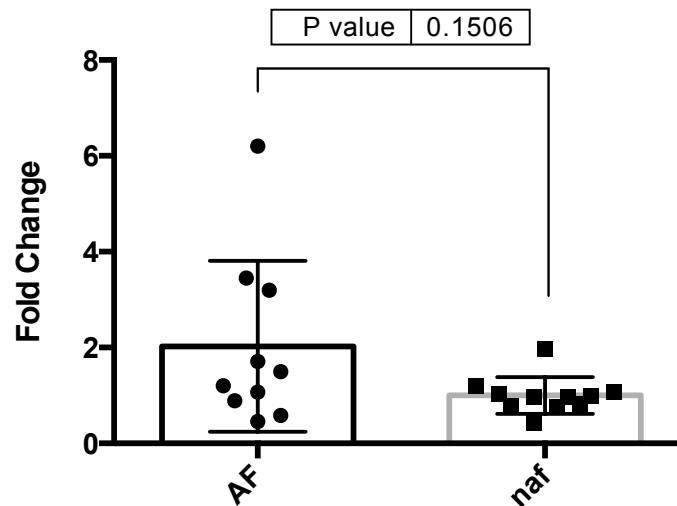


Figure 3-5: Differential expression of KCNE5 mRNA in AF vs. non-AF groups.

3.3.4 KCNE5 protein expression

Missense mutations in KCNE5 (KCNE1L) have been associated with non-familial or acquired AF. Mutant KCNE5 protein is thought to exert a lesser suppressive effect on the KCNQ1 subunit, resulting in a gain of function of the inward rectifier potassium channel, shortening of action potential duration and increased susceptibility to re-entrant tachyarrhythmia.(151)

KCNE5 (KCNE1L) protein levels were evaluated in the atrial tissue of a subgroup of 4 POAF and 5 non-POAF patients. Patients developing POAF were identified to have a significantly higher level of KCNE5 protein in atrial tissue samples taken at the time of surgery when compared to non-POAF patients ($p=0.0317$) (**Figure 3-6**).

(a) Difference in KCNE1L protein expression between a cohort of 4 AF and 5 non AF patients

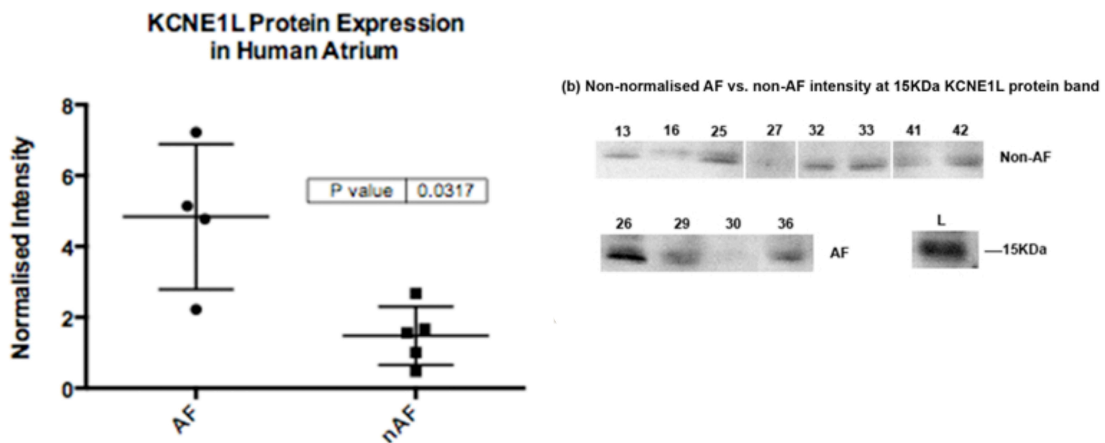


Figure 3-6: Western blot analysis of KCNE5 protein levels in atrial tissue biopsies of POAF vs. non-POAF patients

3.3.5 MiR-483-5p, systemic inflammation and cellular adhesion in POAF

Based on the significant miR-483-5p upregulation in the pre-operative serum and intra-operative atrial tissue of POAF patients, further pathway analysis was carried out on the predicted targets of this miRNA. The DIANA-mirPath and DAVID bioinformatics databases (<http://david.abcc.ncifcrf.gov>) (154, 155) were used to predict KEGG pathways significantly enriched by targets predicted using the miRWalk program as previously described. These results highlighted significant enrichment of 'focal adhesion' and 'cellular adhesion molecules (CAMs)' pathways (**Figure 3-7**) ($p=0.00055$ and $p=0.0037$ respectively).

The diagram illustrates the molecular mechanisms of leukocyte transendothelial migration (TEM) across the endothelium, categorized into four main pathways:

- Tight junction:** Shows the interaction between Endothelial cells and Leukocyte. Key molecules involved are CLDN (Cldn-1, Cldn-2), OCLN, JAM1, JAM2, JAM3, ESAM, CDH5, PECAM1, and CD99. The diagram highlights the role of CLDN and CDH5 in maintaining the tight junction barrier.
- Leukocyte transendothelial migration:** Shows the interaction between Leukocyte and Endothelial cell. Key molecules involved are JAM1, ICAM3, ICAM1, ICAM2, JAM3, JAM2, ITGA4, ITGB1, ITGA9, ITGB1, VCAM1, MAdCAM1, PECAM1, CD34, SELP, SELF, and CD2. The diagram highlights the role of ITGA4, ITGB1, and VCAM1 in leukocyte transendothelial migration.
- Complement and coagulation cascade:** Shows the interaction between Platelet and Endothelial cell. Key molecules involved are JAM1, CD40L, CD40, PECAM1, and PVR. The diagram highlights the role of CD40L and CD40 in the complement and coagulation cascade.
- Leukocyte transendothelial migration (P-selectin mediated):** Shows the interaction between Platelet and Endothelial cell. Key molecules involved are JAM1, CD40L, CD40, PECAM1, PVR, CD226, and PVR12. The diagram highlights the role of PVR and PVR12 in leukocyte transendothelial migration.

(b)

FOCAL ADHESION

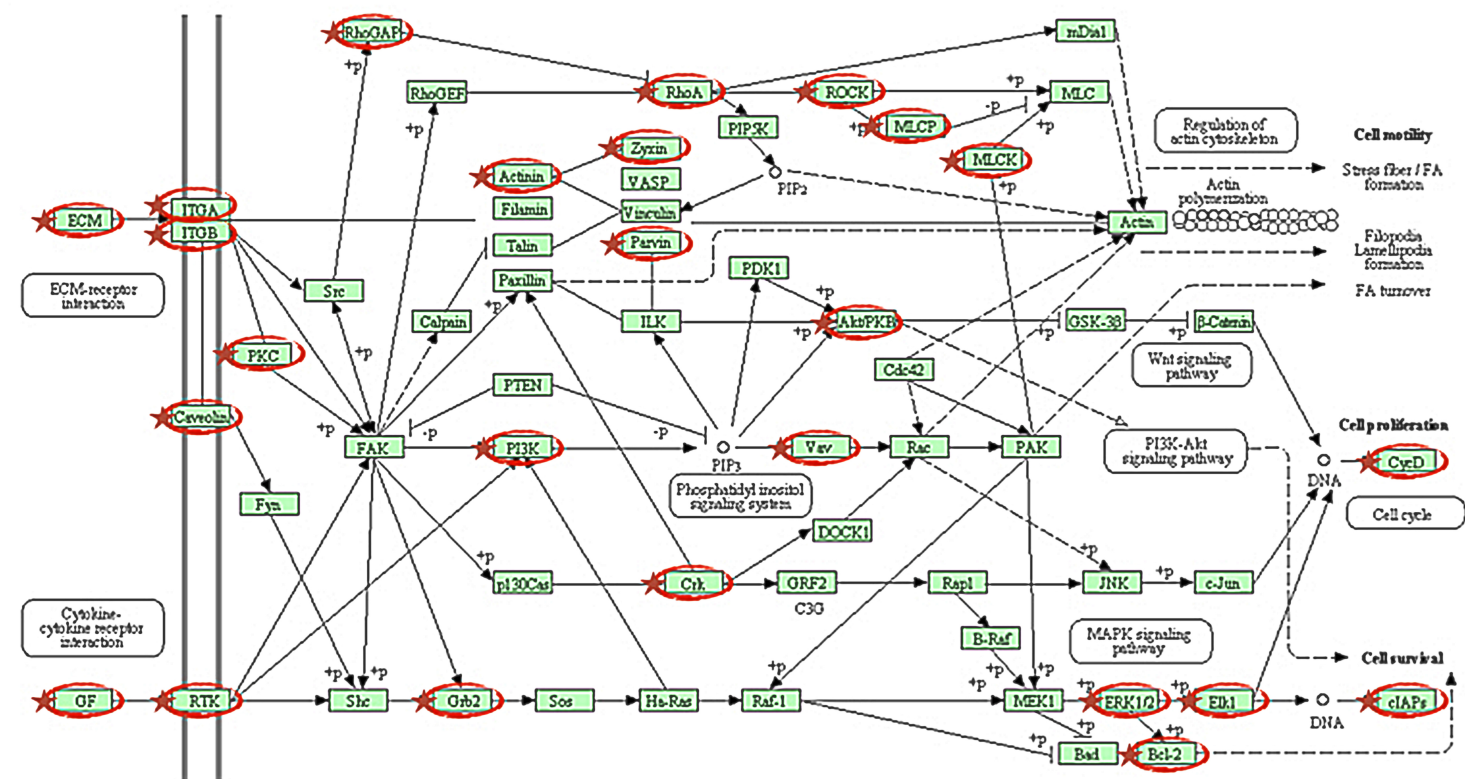


Figure 3-7: KEGG pathway for 'focal adhesion' and 'cellular adhesion molecules (CAMs)' showing potential targets of miR-483-5p highlighted in red.

Local and systemic inflammation is well recognised to play a role in both the triggering and maintenance of AF. Furthermore, recent evidence suggests a role for vascular cell adhesion protein VCAM1 as a potential pre-operative serum biomarker of post-operative AF.

As shown in **Figure 3-8**, through detailed exploration of the current literature, two potential regulatory mechanisms linking the derangement in miRNA profile seen in this POAF patient cohort and perturbation in the vascular cell adhesion molecule VCAM-1 can be observed:

- (1) MiR-483-5p may bind with target mRNA thus negatively regulating RhoA gene expression. RhoA protein is noted to decrease the expression of VCAM1 protein as mediated by the P2Y2R protein. It may therefore follow that increased pre-operative expression of mir-483-5p may reduce expression of RhoA thus giving rise to an increase in serum VCAM1 protein expression.
- (2) MiR-483-5p may bind with its MAPK3 gene target and subsequently reduce MAPK3 gene expression. MAPK3 can be observed to have a negative effect on VCAM1 gene expression, therefore a reduction in MAPK3 may result in increased VCAM1 protein levels.

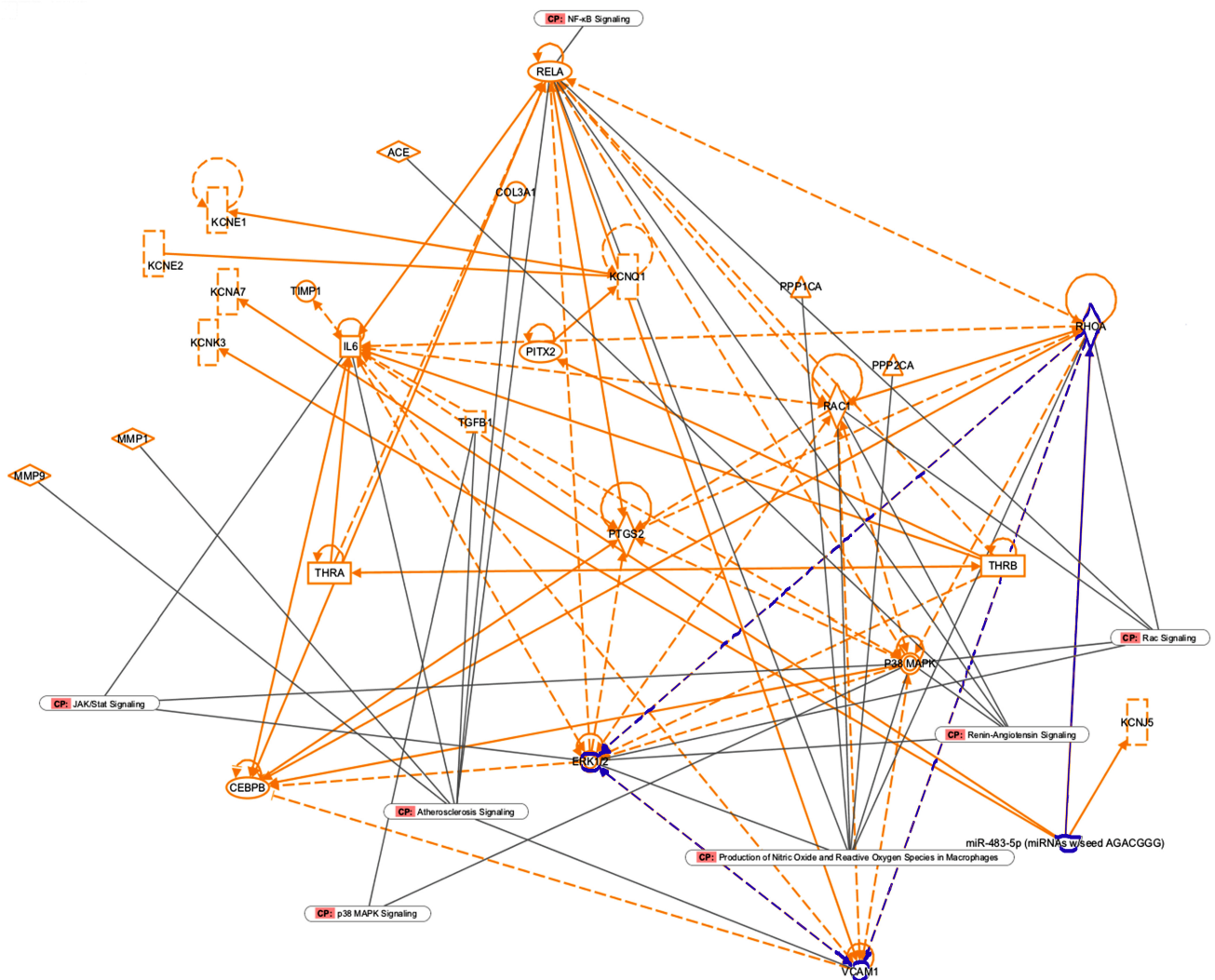


Figure 3-8: Pathway analysis of possible links between hsa-miR-483-5p and cellular pathways identified to play a role in human atrial fibrillation. Possible pathway links between VCAM1 in human serum and miR-483-5p gene expression are shown in blue.

3.3.6 Serum VCAM-1 expression is increased in POAF patients

Following cardiac surgery, both surgical trauma and cardiopulmonary bypass result in the activation of cytokines and leukocyte trafficking producing a local and systemic inflammatory response. The link between inflammation and post-operative atrial fibrillation has been confirmed by a number of epidemiological studies, and increased levels of IL-6 and IL-2 have been associated with an increased incidence of post-operative AF.(156) More recently, the soluble vascular endothelial cell adhesion molecule VCAM-1 has been found to

be significantly higher in the pre-operative plasma of patients developing post-operative atrial fibrillation compared to those that do not, suggesting the existence of a pre-existing pro-inflammatory state. (157)

Serum VCAM-1 was significantly increased in the pre-operative serum of patients developing post-operative atrial fibrillation when compared to controls ($p=0.022$) (**Figure 3-9**). This confirms the results observed by Verdejo et al. and highlights the potential role of serum VCAM-1 as a pre-operative biomarker of POAF.(157)

ELISA evaluation of serum VCAM-1 concentration at 48 hours (**Figure 3-10(a)**) and 96 hours (**Figure 3-10(b)**) post-operatively did not reveal any difference between the POAF and non-POAF groups, however a significant reduction in serum VCAM-1 concentration was observed between the pre-operative and 48hr post-operative time points in the AF group when compared to non-AF (**Figure 3-11**).

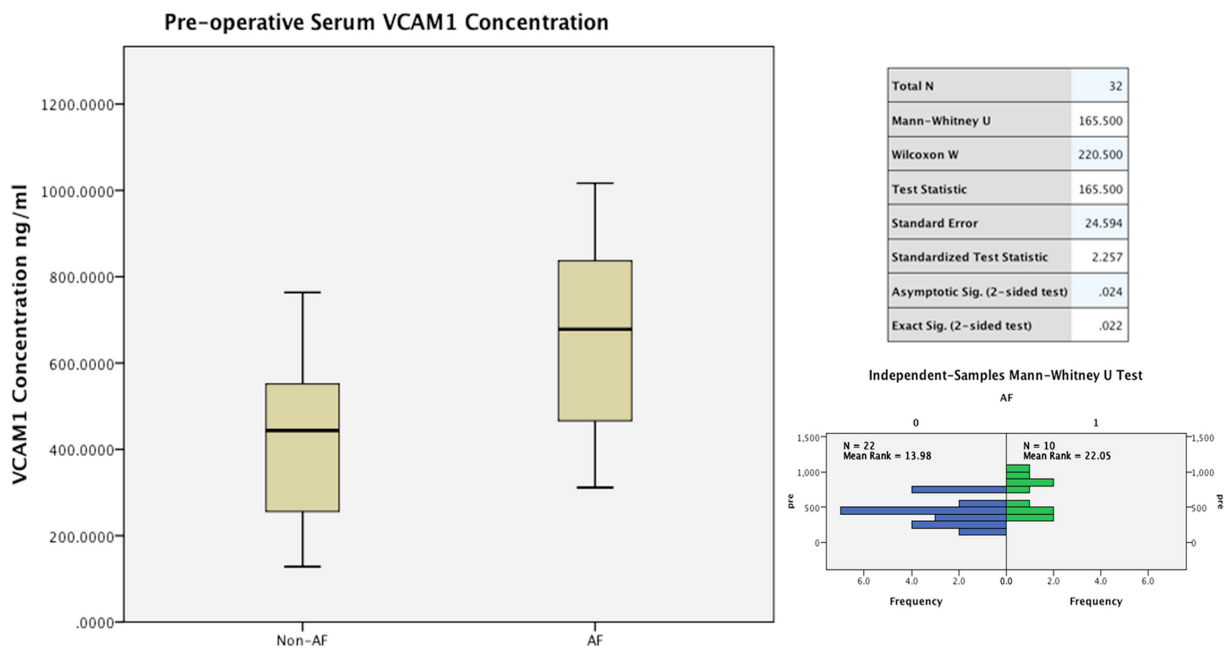


Figure 3-9: Concentration of pre-operative serum soluble VCAM-1 in AF vs. non-AF patients

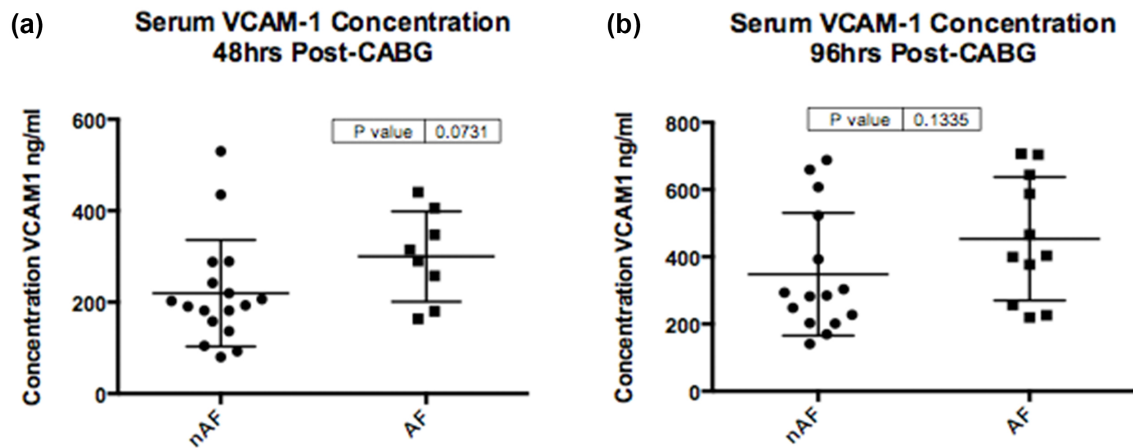


Figure 3-10: Concentration of serum soluble VCAM-1 in AF vs. non-AF patients at: (a) 48hrs post-operatively; (b) 96hrs post-operatively.

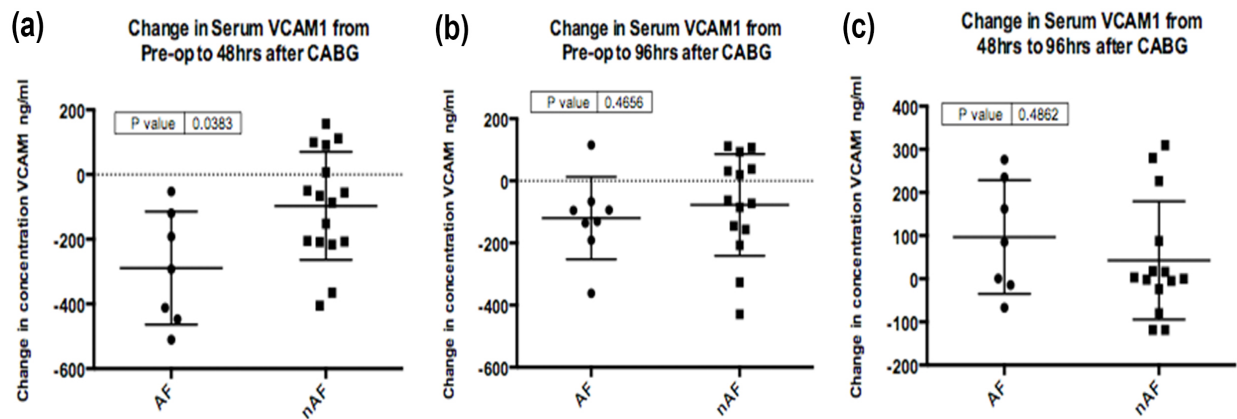


Figure 3-11: Change in concentration of serum soluble VCAM-1 in AF vs. non-AF patients between: (a) Pre-operative and 48hrs after surgery; (b) Pre-operative and 96hrs after surgery and (c) 48hrs and 96hrs after surgery.

3.4. Discussion

Based on the results of microarray and real-time quantitative PCR experiments discussed in **Chapter 2**, systematic bioinformatic target prediction and subsequent experimental validation has identified a potential interaction between miR-483-5p and the voltage gated potassium channel regulator subunit KCNE5, which in turn may play a role in the pathogenesis of *de novo* post-operative atrial fibrillation. However, similar analysis was not able to identify any changes in mRNA expression of the predicted miR-208a targets selected for experimental validation, and further investigation is required to validate the mechanistic role of this microRNA.

Second, this work has identified significantly higher levels of VCAM-1 in the pre-operative serum of AF patients and protein/gene network analysis has revealed a potential link between serum VCAM-1 and microRNA-483-5p. As such, I have identified not only two potential pre-operative serum biomarkers for POAF, but also a second potential mechanism whereby miRNA-483-5p may be involved in the pathogenesis of this arrhythmia.

3.4.1 MicroRNA dysregulation: A pre-existing atrial substrate for POAF?

As previously discussed, a wide variety of electrical and structural factors are likely to contribute to the triggering and maintenance of atrial fibrillation in the human heart. In patients undergoing cardiac surgery with no prior history or recognisable risk factors for the condition, approximately 1 in 3 will develop *de novo* post-operative atrial fibrillation, and although a proportion of these may be in part accounted for by aberrations in electrolyte balance or post-operative infection, others display no discernable predictors for the arrhythmia. Similarly, not all patients with temporary electrolyte disturbances or post-

operative infections will go on to develop POAF and thus it is hypothesised that a sub-clinical substrate for AF propagation and maintenance exists in these individuals.

In order to understand the significance of microRNA dysregulation in the complex pathogenesis of this arrhythmia this chapter has utilised several database and bioinformatics tools to identify potential mechanisms and pathways through which these miRNA may regulate cardiac conduction. For miR-483-5p a total of 4985 predicted and 17 validated targets were identified, with 4688 predicted and 15 validated target genes found for miR-208a.

3.4.2 MiR-208a

MicroRNA-208 is an intronic, cardiomyocyte specific microRNA encoded for and co-expressed with the α -Myosin Heavy Chain (α -MHC/Myh6) gene. Over the past 5-10 years this microRNA has received particular attention in field of cardiovascular research, especially in the regulation of cardiac energy homeostasis,(108, 152) hypertrophy and fibrosis.(116, 158, 159) In animal models, both the miR-208a over-expression transgenic mouse and the miR-208^{-/-} knockout mouse demonstrate cardiac conduction defects despite not displaying any macroscopic morphological anomalies. In their 2009 work, Callis et al. demonstrated an increased PR interval and heart block in mice over-expressing miR-208a, and detected atrial fibrillation in 80% (n=10) of miR-208a^{-/-} knockout mice.(116) Further analysis identified a significant reduction in the protein levels of the miR-208a target GATA4, itself a transcriptional regulator of the homeodomain-only protein (Hop) transcription factor that when suppressed results in a loss of Connexin 40 expression. By verifying this sequence of regulation from miR to connexin expression, this detailed study identified a likely mechanism by which suppression of miR-208a may result in atrial fibrillation.(116) It is also notable that GATA4 transcript levels remained unchanged despite suppression of GATA4 protein, a finding which may correlate with the results seen in my human study. These observations are

therefore consistent with post-transcriptional suppression of GATA4 activity by miR-208a, and further experimental validation of GATA4 protein suppression in human atrial tissue is now required to verify this pathway in human POAF pathogenesis.

In addition to its confirmed actions on GATA4, miR-208a has also been shown to directly regulate the thyroid hormone receptor protein-1 (THRAP1 or MED13), a component of the mediator complex which itself regulates thyroid hormone related transcription.(108) The mediator complex (MED) is a transcriptional co-activator complex recruited by transcriptional activators or nuclear receptors to induce gene expression.(160) It is thought that MED interacts with RNA polymerase II and thus promotes the formation of a transcriptional pre-initiation complex. The role of thyroid hormone receptor protein-1 in this complex is in forming the kinase sub-module, a sub-complex with MED12, cyclin dependent kinase-8 (CDK8) and Cyclin C, which in turn negatively regulates MED transactivation.(108, 160) Several studies have verified the role of miR-208a in regulating THRAP1 expression. Indeed, transgenic miR-208 overexpression mice express significantly lower levels of THRAP1 protein, and miR-208^{-/-} knockout mice express significantly higher levels of THRAP1 protein than wild-type littermates, without associated changes in THRAP1 transcript levels, again suggesting post-transcriptional regulation by miR-208a.(116)

At birth, thyroid hormone signaling regulates cardiac contractility by controlling a switch from the slow myosin heavy chain (β -MHC) to the fast twitch α -MHC isoform. Conversely, β -MHC is up-regulated in response to cardiac stress such as pressure overload. MicroRNA-208a has been shown to regulate this β -MHC stress response, and by binding to THRAP1 may thus increase β -MHC expression (**Figure 3-12**). Indeed, cardiac expression of both THRAP1 and miR-208a have been found to be involved in the control of systemic energy expenditure, diet induced obesity and metabolic syndrome,(108) and the hearts of miR-208^{-/-} mice demonstrate similarities to hyperthyroid hearts, with up-regulation of stress response genes and increases in fast-twitch skeletal muscle expression.(152) THRAP1 therefore

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represents a potential target whereby miRNA-208a may regulate human atrial fibrillation. Decreased expression of mir-208a removes translational inhibition on THRAP1 and thus reduces β -MHC expression, particularly in response to stress. It is therefore possible that the reduction in miR-208a observed in the right atrial biopsies of POAF patients produces an increased susceptibility to AF due to a relative up-regulation of fast-twitch α -MHC isoforms, particularly in response to stressful stimuli such as cardiopulmonary bypass, as a result of an under-expression of β -MHC and a hyperthyroid phenotype.

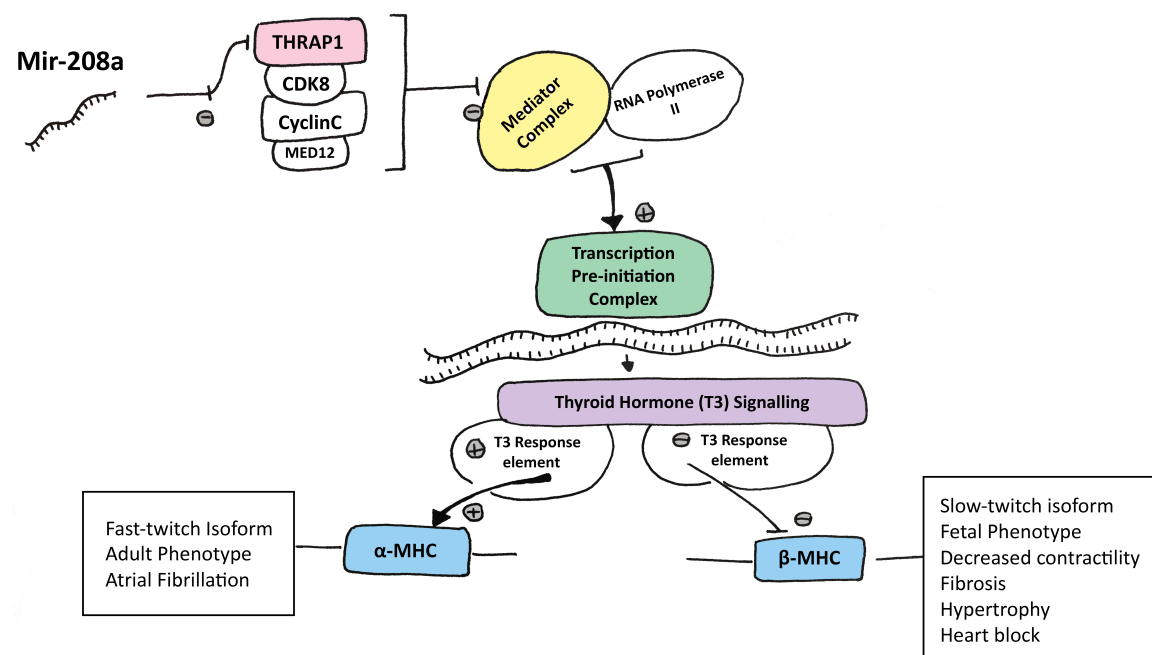


Figure 3-12: The role of miR-208 in regulating THRAP1 and thyroid hormone signaling pathways. Down-regulation of miR-208a results in an increase in THRAP1 protein and inhibition of the mediator complex. This inhibits T3 signaling, producing a relative increase in α -MHC and decrease in β -MHC. This effect is particularly notable in response to cardiac stress where β -MHC would usually be up-regulated.

Most recently, microRNA-208a has also been found to be down-regulated in the right atrium of human patients with chronic and paroxysmal atrial fibrillation undergoing CABG and or valvular surgery.(161) This study also highlighted an increased maximal mitochondrial respiration rate in AF vs. non-AF samples, suggesting that AF correlates with a perturbation of mitochondrial function; however the relationship between miR-208a dysregulation and mitochondrial function is yet to be investigated.

MiR-208 is usually undetectable in the circulating blood. However, its release into blood plasma/serum has been associated with ischaemic injury, both in the form of angina pectoris and in acute myocardial infarction.(162) MiR-208 was un-detectable in the pre-operative serum of both sinus rhythm (SR) and post-operative AF (POAF) patients included in this study (by RT-qPCR, $C_t > 34$; results not shown) suggesting that although all patients had confirmed diagnosis of significant coronary artery disease requiring surgical revascularization, acute ischaemia and cardiomyocyte damage prior to surgery was equivalent in both groups.

3.4.3 MicroRNA 483-5p (hsa-miR-483-5p)

Hsa-miR-483-5p has received relatively little attention when compared to other more widely studied microRNA; however, over recent years research into this miR is gaining momentum. In 2011, Qiao et al. demonstrated over-expression of mir-483-5p to be associated with an inhibition of wound healing and angiogenesis in a mouse model, and Soon et al. observed a poorer prognosis in patients with adrenocortical adenocarcinoma overexpressing miR-483-5p.(119, 163) Conversely, Wang and colleagues observed suppression of glioma cell proliferation and induced G0/G1 arrest in patients overexpressing hsa-miR-483-5p, and by identifying ERK1 as a direct target of this microRNA, provided evidence for its role as a glioma tumor suppressor.(164) More recently, hsa-miR-483-5p has been shown to play a role in regulation of human fetal neuronal development via modulation of methyl-CPG binding protein 2 (MeCP2) and the MeCP2-interacting corepressor complexes HDAC4 and TBL1X.(165) Of particular interest is the work by Wang et al, which demonstrated overexpression of serum hsa-miR-483-5p to be an independent predictor of 28-day mortality in their cohort of 214 sepsis patients admitted to the intensive care unit (univariate OR 1.08

95%CI (1.01, 1.15), $p=0.007$; multivariate OR1.29 (95% CI: 1.08–1.55) $p=0.006$), suggesting a potential role for this microRNA as a predictive biomarker of poor outcome.

The role of hsa-miR-483 in the heart currently remains largely undetermined. Recent Illumina Sequencing results published by Hsu et al (166) identified increased expression of hsa-miR-483-5p in left atrial biopsies when compared to samples taken from the right atrium of human patients (Atrial concentration (based on the average fraction of total mapped miRNA reads in the left and right atria)= $1.10E-03$; Absolute FC =1.56; $p=0.0092$; False discovery rate (FDR)= $7.92E-02$). However, this study was not designed to correlate these differential expression profiles with any particular pathology. Ward et al. identified a significant reduction in hsa-mir-483-5p expression in the blood plasma of patients with evolving NSTEMI as determined by increased plasma troponin-I 6 hours after arrival in the emergency department.(167)

3.4.3.1 A mechanistic role for Mir-483-5p?

Analysis of potential targets for miR-483-5p implicates both electrical and structural remodeling pathways. This work has demonstrated a significant increase in the expression of atrial KCNE5 protein in the right atrial tissue of patients subsequently developing POAF when compared to those maintaining sinus rhythm ($p=0.0317$). These results therefore highlight a mechanism for miRNA-483-5p in atrial electrical remodeling present prior to the onset of the clinical arrhythmia.

KCNE5 forms part of a group of regulators (KCNE1-KCNE5) of the voltage gated potassium channel responsible for the slow inward rectifying potassium current (I_{Ks}). Its gene product, MiRP4, is a membrane spanning protein thought act as a down-regulator to the α -subunit KCNQ1. Down-regulation of KCNQ1 subsequently results in the suppression of I_{Ks} , which may in turn shorten action potential duration.

Missense mutations leading to a loss of function of KCNE5 have been associated with non-familial or acquired AF. Mutant KCNE5 protein is thought to no longer exert the same suppressive effect on the KCNQ1 subunit, resulting in a gain of function of the inward rectifier potassium channel, shortening the action potential duration and increased susceptibility to re-entrant tachyarrhythmia.(151)

Upregulation of miR-483-5p may therefore also act to increase I_{Ks} through suppression of KCNE5, reduced MiRP4 and a loss of inhibition of KCNQ1. This up-regulation of I_{Ks} within the atrial myocardium would subsequently lead to shortening of the refractory period giving rise to the potential for macro re-entrant circuits to develop leading to increased susceptibility to atrial fibrillation.(151) As such, these findings now require further investigation with larger sample numbers to validate the level of KCNE1L protein expression and *in vitro* studies to confirm the KCNE1L/miR-483-5p interaction.

3.4.3.2 miR-483-5p and MAPK signaling

In addition to its role in regulating cardiac conduction through voltage gated potassium channel function, bioinformatic target prediction has highlighted the MAPK signaling pathway as a target site for miRNA-483-5p. Extracellular-signal-regulated kinases (ERK1/2) otherwise known as mitogen-activated protein kinases (MAPK3) are part of a family of intracellular signaling protein kinases that regulate a wide range of cellular processes including cell growth, proliferation, differentiation and apoptosis.(168) In the atrial myocardium, MAPK3 may be activated (phosphorylated) as a result of angiotensin II binding with AT1 receptors, resulting in stimulation of transcription factor activation and induction of expression of pro-fibrotic gene expression resulting in fibroblast differentiation, extracellular matrix accumulation and subsequent increased susceptibility to arrhythmia.(148) Indeed, both MAPK3 mRNA and protein expression has been found to be up-regulated in the right atrial appendage of patients with rheumatic heart disease and either chronic or paroxysmal AF

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when compared to sinus rhythm.(148) MAPK3 has however also been implicated in the systemic inflammatory response, with leucocyte adhesion and transendothelial migration also being mediated by MAPK3 signaling pathways. Up-regulation of MAPK3 may therefore stimulate endothelial activation thus contributing to inflammatory pathways underlying AF pathogenesis.(169)

MiR-483-5p demonstrates good interaction with its predicted MAPK3 target with a MirSVR score of -1.0996. It is therefore possible that binding of miR-483-5p with the MAPK3 target results in a reduction in MAPK3 protein expression altering the myocardial response to ATII signaling as well as acting on systemic inflammatory pathways and endothelial leukocyte recruitment. Given this multifaceted network of MAPK3 signaling, it is likely that the miR-483-5p/MAPK3 interaction mediates POAF pathogenesis by a number of mechanisms, and detailed further study both *in vitro* and *in vivo* are now required to elucidate its role in the pathogenesis of this multifactorial arrhythmia.

3.4.3.3 MicroRNA 483-5p and cellular adhesion in POAF

Vascular cell adhesion molecule 1 (VCAM-1) is an 81 KDa sialoglycoprotein expressed by cytokine activated vascular endothelium, dendritic and macrophage-like cells. It is a member of the immunoglobulin superfamily, with 6 or 7 extracellular Ig domains, capable of interacting with the integrins $\alpha_4\beta_1$ (VLA4), $\alpha_4\beta_7$, and $\alpha_d\beta_2$ present on eosinophils, basophils, mast cells, monocytes and other lymphocytes in order to mediate signal transduction and/or cell adhesion and transendothelial migration (**Figure 3-13**).(170) This binding also relies on the activation state of the integrins and co-expression of other factors such as CD24 and Galectin-3.

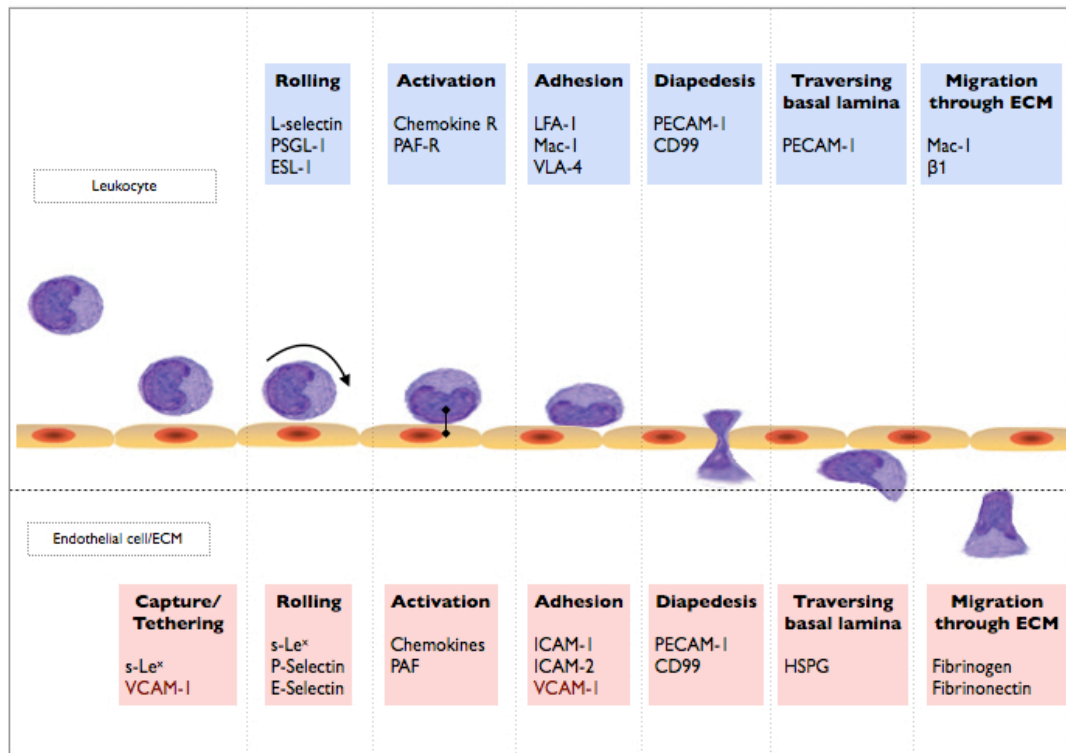


Figure 3-13: Leukocyte adhesion: The role of VCAM-1 and other mediators of transendothelial migration.

VCAM-1 expression is regulated by a number of factors including inflammatory cytokines such as $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and stimulation of toll like receptors on endothelial cells, fibroblasts and dendritic cells. Furthermore, VCAM-1 gene expression is regulated by the $\text{NF}\kappa\text{B}$ transcription factor, which may be activated by $\text{TNF-}\alpha$ and reactive oxygen species such as superoxide radicals generated by shear stress and microbial stimulation. Soluble VCAM-1 (sVCAM-1) may be released into the plasma by cleavage involving metalloprotease and disintegrin. Release of sVCAM-1 has not only been associated with a number of cardiovascular disease processes including human post-operative atrial fibrillation,⁽¹⁵⁷⁾ but is also observed as early as 3 hours after routine cardiopulmonary bypass alongside increased endothelial cell E-selectin expression.⁽¹⁷¹⁾ It has been shown that this soluble VCAM-1 may have a direct chemotactic effect on leucocytes as well as stimulating inflammation and angiogenesis in nearby endothelial cells in a paracrine manner.⁽¹⁷²⁾ However, it has also

been postulated that such soluble VCAM-1 may actually act to dampen down leukocyte adhesion by binding to cell surface receptors of circulating leukocytes and preventing adhesion.(173)

Binding of leucocyte integrins to endothelial VCAM-1 not only results in leucocyte adhesion but also stimulates a number of downstream processes mediated through Rac1 and reactive oxygen species (ROS). Upon binding to VCAM-1, Rac1 signaling cascades are activated and there is a rapid release of intracellular calcium through verapamil sensitive calcium channels. This in turn leads to the activation of NADPH oxidase and generation of the reactive oxygen species superoxide ($O^{\cdot-}$) and hydrogen peroxide (H_2O_2), which subsequently activate p38-membrane associated protein kinase (p38MAPK), matrix metalloproteases (MMPs) and protein kinase C ($PKC\alpha$). This 'outside-in' signaling is not only crucial for trans-endothelial leucocyte migration (mediated by ROS) but also produces a conformational change in endothelial cell structure by altering intracellular actin structure allowing the formation of gaps between cells of the endothelial monolayer (**figure 3-14**).(174)

As previously discussed, inflammation plays a key role in the pathogenesis of both de novo and post-operative atrial fibrillation. Interestingly, my results have verified findings by Verdejo et al. demonstrating that increased levels of circulating serum VCAM-1 predict POAF in patients with sinus rhythm undergoing CABG where there is no prior history of the arrhythmia.(157) These findings support the suggestion that POAF is associated with a pre-existing atrial substrate or systemic inflammatory state whereby this arrhythmia may be predicted prior to cardiac surgical intervention. Interestingly, pre-operative serum VCAM-1 appears to be a more reliable predictor of POAF than other markers of systemic inflammation such as hsCRP and IL-6, which are often confounded by age and other medical comorbidities.(36) As VCAM-1 more specifically reflects endothelial cell activation, it has been

suggested that endothelial dysfunction and thus susceptibility to CPB related ROS generation, may be a key factor underlying the pathogenesis of POAF.(157)

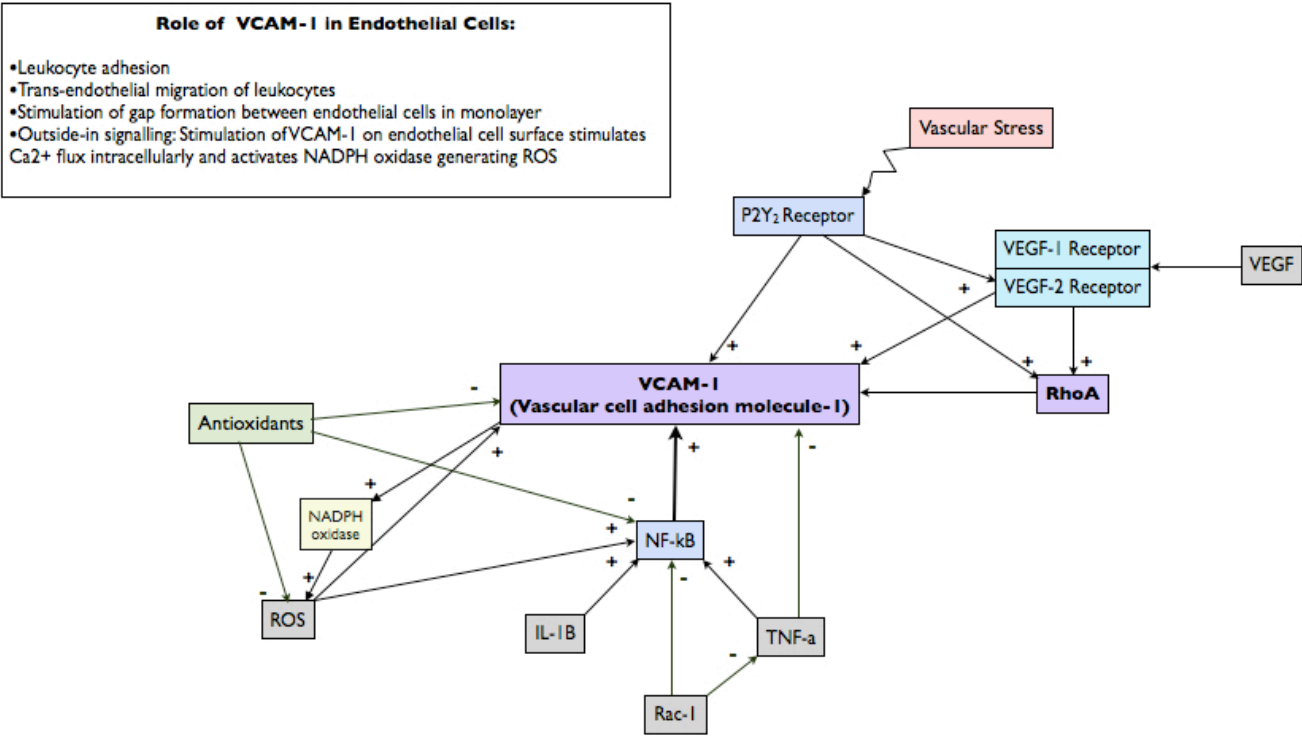


Figure 3-14: VCAM-1 signaling in endothelial cells and its regulation in inflammation

In light of my observed concomitant serum changes in the expression profile of microRNA-483-5p in this POAF, and the potential for this microRNA to regulate a number of inflammatory signaling cascades, I proceeded to investigate whether miRNA-483-5p may in fact regulate VCAM-1 expression and thus play a role in endothelial cell dysfunction.

Pathway analysis using Ingenuity Pathway Analysis (IPA) software (<http://www.ingenuity.com/products/ipa>) (Figure 3-8) identified two potential regulatory mechanisms linking hsa-miR-483-5p and VCAM-1 expression. As previously discussed, VCAM-1 signaling is in part mediated by the Rac signaling pathway with involves a number of small GTPase proteins including Ras homologue gene family, member A (RhoA) (Figure

3-15). RhoA is identified as a potential target of hsa-miR-483-5p with its binding site at base 158 from the 5' end of the RhoA mRNA. This binding site is well conserved with a PhastCons score of 0.7483 and shows moderate complementarity with a miRSVR score of -0.5121 (**Figure 3-16**).

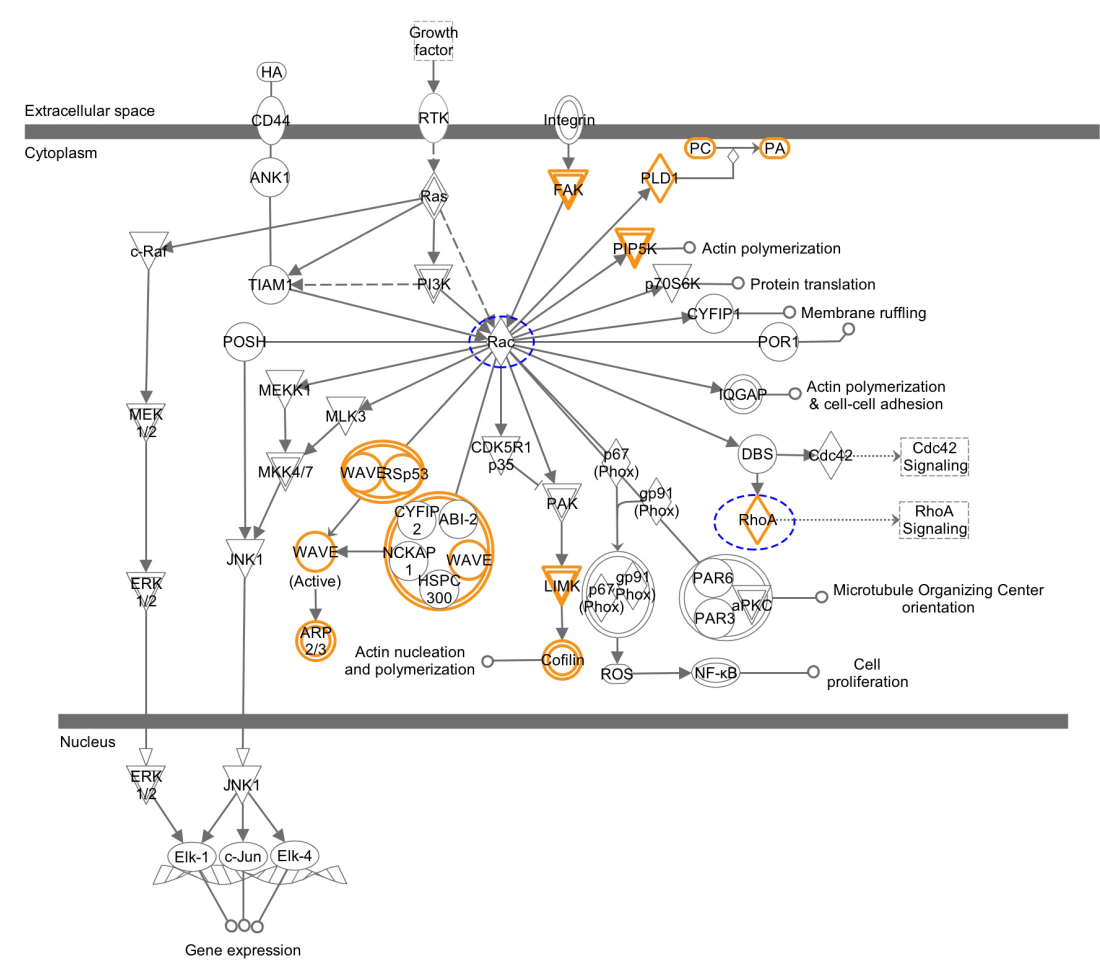


Figure 3-15: The Rac signaling pathway (courtesy of Ingenuity Systems inc.)

3' gaggggaagAAAGG-AGGGCAGAA 5' hsa-miR-483-5p 158:5' auuaacgaUGUCCAACCCGUCUG 3' RHOA	mirSVR score: -0.5121 PhastCons score: 0.7483
--	--

Figure 3-16: Binding of hsa-miR-483-5p with RhoA mRNA.

Inhibition of RhoA by miRNA-483-5p may increase activity or expression of the nuclear receptor ligand-activated transcription factor peroxisome proliferator-activated receptors (PPAR), which may in turn suppress NF- κ B and AP-1-mediated gene activation, down-regulating VCAM-1.(175) Interestingly, this mechanism of action may also be shared by HMG-CoA Reductase inhibitors (or ‘Statins’), a class of drug which although originally identified for their lipid lowering effects and subsequent role in cardiovascular disease prevention, also possess a number of “pleiotropic” properties, and are associated with a reduction in post operative atrial fibrillation.(176, 177) By inhibiting cholesterol synthesis, statins may themselves also inhibit RhoA activity by depleting isoprenoid production, preventing RhoA anchoring to the plasma membrane and thus rendering it inactive.(178) Through this and other mechanisms, statins increase endothelial nitric oxide synthesis, reduce paracellular permeability and reduce ROS generation thus improving endothelial function and reducing vascular inflammation.(179)

A second potential mechanism by which hsa-miR-483-5p may regulate VCAM-1 expression is via inhibition of the membrane associated protein kinase complex MAPK3 (or ERK1/2). This extracellular signaling kinase is involved in a number of cell signaling processes including cell differentiation and proliferation, and increased MAPK3 expression has been previously identified in patients with chronic AF and atrial fibrosis.(148) Furthermore, MAPK3 has been previously validated by luciferase assay to be a direct target of hsa-miR-483-5p.(164) There are several proposed mechanisms by which expression of VCAM-1 may be altered by changes in MAPK3 (or ERK1/2) signaling. Firstly, as previously discussed, binding to VCAM-1 stimulates a signaling cascade involving NADPH oxidase, protein kinase C α and protein tyrosine phosphatase 1B (PTP1B). VCAM-1 associated production of PTP1B has subsequently been shown to result in activation of MAPK3, which in turn acts to regulate leucocyte adhesion and transendothelial migration. In fact, VCAM-1 mediated transendothelial migration may be blocked by inhibition of MAPK3.(180) It is therefore possible that these observed increases in circulating hsa-miR-483-5p levels form part of a

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systemic response to inflammation, acting to inhibit MAPK3 and thus dampen the cellular response to increased VCAM-1 expression. Conversely however, phosphorylation of MAPK3 (ERK1/2) stimulates binding of the transcription factors NFkB, AP-1 and SP-1, all of which regulate cytokine dependent induction of VCAM-1 expression.(174, 181, 182) Binding of hsa-miR-483-5p to MAPK3 may therefore reduce transcription factor activity, thus resulting in a down-regulation of transcription factor related VCAM-1 expression and a fall in soluble VCAM-1 shed from circulating endothelial cells.

In order to investigate these theoretical mechanisms correlating hsa-miR-483-5p expression with soluble VCAM-1 levels detected in human serum mechanistic studies into the relative changes in MAPK3 and RhoA protein expression are now required. Unfortunately, it was not possible to evaluate RhoA expression in the atrial tissue owing to insufficient sample available for protein extraction, and, as an intracellular signaling protein, RhoA is not present in the circulating serum at sufficient levels to allow for quantitative analysis. Furthermore, the interaction between hsa-miR-483-5p and its predicted target RhoA must be validated and its functional significance tested.

3.4.3.4 MiRNA-483-5p and RhoA signaling

RhoA is a small GTPase protein belonging to the Ras superfamily involved in cell migration, transcription regulation and organization of the actin cytoskeleton (**Figure 3-17**). In the cardiovascular system, increased levels of activated RhoA (GTP bound) have been associated with protection against ischaemia-reperfusion injury,(183) and RhoA signaling has been identified to play a key role in cardiac hypertrophy, particularly in response to stretch.(184, 185) Furthermore, several studies utilizing C3 exoenzyme blockade of RhoA signaling have also demonstrated the importance of this pathway in promoting integrin dependent leukocyte adhesion (186) and de-adhesion.(187) By acting on the actin cytoskeleton, activation of the RhoA pathway prevents neutrophil spreading on the endothelial cell membrane, reducing the

affinity of integrin binding and thus promoting de-adhesion and preventing transendothelial migration.(187)

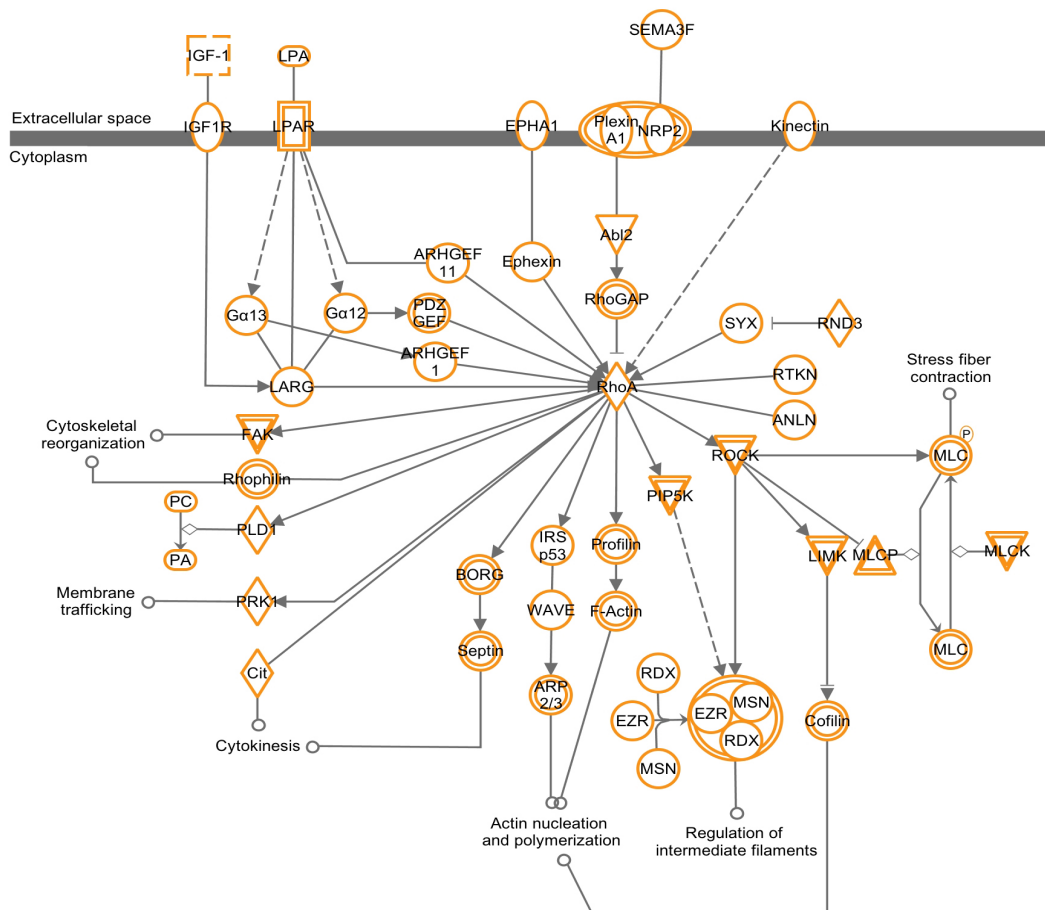


Figure 3-16: The RhoA signaling pathway (courtesy of Ingenuity Systems inc.)

It therefore follows that miR-483-5p post-transcriptional regulation of RHOA gene expression may play a key role in endothelial cell function and regulation of the systemic inflammatory response. Furthermore, as suppression of RhoA may also suppress NF- κ B and AP-1-mediated gene activation, this small GTPase may also explain the observed changes in VCAM-1 expression.

3.4.4 Limitations

This work demonstrates exciting novel findings in the field of atrial fibrillation biomarker research. However, several limitations should also be considered when interpreting these results. Firstly, the proposed interactions between microRNA-208 and microRNA-483-5p and their respective clinically relevant targets are based on previously published data and have not yet been validated either in this patient cohort or within the wider literature. Secondly, no single target prediction tool is perfect in determining the functional target sites for a given miRNA. Although most tools now incorporate a combination of seed pairing, target conservation and thermodynamic favourability into their prediction algorithms, in some cases functional sites may still be missed, and non-functional sites may be over represented. For example, the reliability of thermodynamic energy calculations remains under debate, and there is evidence to suggest that non-conserved binding sites in the 3'UTR may indeed be functional and thus missed by conventional prediction algorithms.(188, 189) Third, although prediction tools provide species specificity, there is little focus on tissue specificity and the functional nature of a mRNA:miRNA interaction may vary according to tissue type, and subtype as well as the functional state of the cell.(189) Finally, pathway enrichment analysis is reliant on the availability of published gene and protein interactions, and is thus limited by the degree of experimental data currently available. As such, subsequent experimental work may in future alter the way in which these cellular signaling pathways are understood and represented, thus changing the way in which the results of this study may be interpreted.

3.5 Conclusions

This work has utilised a comprehensive bioinformatic target prediction and network analysis to identify the potential mechanisms by which miRNA dysregulation may produce the downstream cellular changes that result in a post-operative atrial fibrillation.

Firstly, the predicted targets for the 16 differentially expressed miRNA evaluated in Chapter 2 were used to determine the cellular pathways that may be involved in AF pathogenesis. This

exposed the significant enrichment of cellular adhesion molecule pathways in patients over-expressing hsa-miR-483-5p, and for the first time revealed a potential link between this miRNA and the vascular endothelial adhesion molecule VCAM-1. Evaluation of serum VCAM-1 in this patient cohort corroborated previous findings that patients developing POAF express higher levels of pre-operative VCAM-1 than those maintaining sinus rhythm, and in addition showed a significant fall in VCAM-1 expression within 48hrs post-operatively. These findings together support the hypothesis of a systemic inflammatory substrate for this arrhythmia and highlight the potential for biomarker development both in miR-483-5p and VCAM-1.

Secondly, a focused assessment of clinically relevant miRNA target sites identified a potential interaction between miR-483-5p and KCNE5, which in turn may result in a loss of inhibition on the voltage gated potassium channel regulator subunit KCNQ1, resulting in a gain of function of the inward rectifier potassium channel, shortening of action potential duration and increased susceptibility to atrial fibrillation. This is the first study to evaluate this pathway in atrial fibrillation patients and subsequent investigation now requires validation of this finding in a larger patient cohort. In addition, the miR-483-5p:KCNE5 target interaction must now also be validated both in vitro and in subsequent small animal models in order to confirm the predicted functional disruption of the downstream cellular pathways that ultimately regulate cardiac conduction.

Chapter 4

Structural and Electrical remodeling: Dedifferentiation and aberrant calcium handling

4.1 Background

4.1.1 Gene expression in atrial fibrillation

Chapters 2 and 3 highlight a number of potential microRNA, gene targets and subsequent pathways that may be perturbed in the atrial myocardium of patients developing post-operative atrial fibrillation. Together these findings add weight to the hypothesis that prior atrial substrate changes pre-dispose to POAF, which encompasses aspects of both structural and electrical remodeling. These findings appear to mirror many observations in both paroxysmal and chronic AF, further suggesting that rather than ensuing as a result of post-surgical inflammation and disordered electrolyte balance, POAF forms part of a common spectrum of pro-arrhythmogenic modifications also observed in paroxysmal and persistent forms of this condition.

Over the past decade, there has been growing evidence to suggest that both structural and electrical remodeling occur at a transcriptional level. In their study of 30 patients (10 permanent AF, 20 SR), Barth et al.(190) identified 1434 genes differentially expressed between the atria of AF and SR patients. GO analysis of these genes highlighted a general downregulation of transcription factor activity particularly implicating calcium-dependent signaling pathways through reduced expression of calcineurin $A\alpha$, NFAT_{c3}, MAPK and CaMK.

In fact, a number of gene expression studies now implicate intracellular calcium signaling as a key mediator of chronic AF pathogenesis, both directly through reduced SR reuptake and/or increased Ca^{2+} release, and indirectly through inflammatory and oxidative pathways. Firstly, reduced SERCA2A gene expression may produce cytosolic calcium overload through decreased SR reuptake.(191-193) Second, upregulation of genes encoding a splice variant of the ryanodine receptor (RyR3) channel may promote calcium loading by increasing release from the SR.(190) Conversely however, AF associated gene expression changes may also act to increase SR calcium re-uptake and restore calcium homeostasis. Sarcolipin (SLN), an inhibitory regulator of SERCA, may be downregulated in chronic AF, thus leading to increased SERCA activity.(194) Furthermore, reduced RyR gene expression and RyR receptor activity (measured through the maximal number of RyR ^3H binding sites) has also been observed in chronic AF associated with mitral valve disease, likely at least in part as a consequence of mechanical volume overload.(193) Indirectly, dysregulation of RhoC signaling may promote AF pathogenesis through both perturbation of ROCK signaling pathways as discussed in Chapter 3, and through MAPK and PI3K signaling which in turn may alter sarcoplasmic reticulum calcium uptake.(195) Similarly, increased expression of the pro-oxidant monoamine oxidase B may produce intracellular Ca^{2+} overload through generation of H_2O_2 , mitochondrial damage and increased mitochondrial Ca^{2+} release.(64)

Further to changes in genes regulating myocyte calcium homeostasis, a number of studies have demonstrated dysregulation of inflammatory, oxidative and cell signaling pathways in chronic AF.(64, 190, 191) Upregulation of NF- κ B, SMAD3, SMAD5, VEGF, NF-IL-6, and macrophage migration inhibitory factor (MIF) emphasise the pro-inflammatory transcriptional changes associated with chronic AF.(190) Increased expression of pro-oxidative proteins such as monoamine oxidase B, in combination with a concomitant downregulation of anti-oxidant genes such as glutathione peroxidase, also highlight a state of increased oxidative stress in the atria of AF patients.(64) Furthermore, significant increases in reactive oxygen species and dysregulation of genes encoding glutathione-S-transferase,

thioredoxin, haeme oxidase, peroxiredoxin, cytochrome p-450, and xanthine dehydrogenase have been observed in post-CABG atrial fibrillation, implicating increases in mitochondrial derived oxidative stress as a central mediator in POAF pathogenesis.(64) Finally, idiopathic AF has also been associated with dysregulation of GJA5 and GJA1 genes encoding connexins 40 and 43 respectively.(127, 196) As such, in addition to disrupted calcium signaling, oxidative stress and activation of pro-inflammatory mediators, gene expression mediated alterations in cell-cell coupling may add additional contribution to electrical remodeling through production of a conduction block that promotes both the setting-up and maintenance of re-entrant circuits.

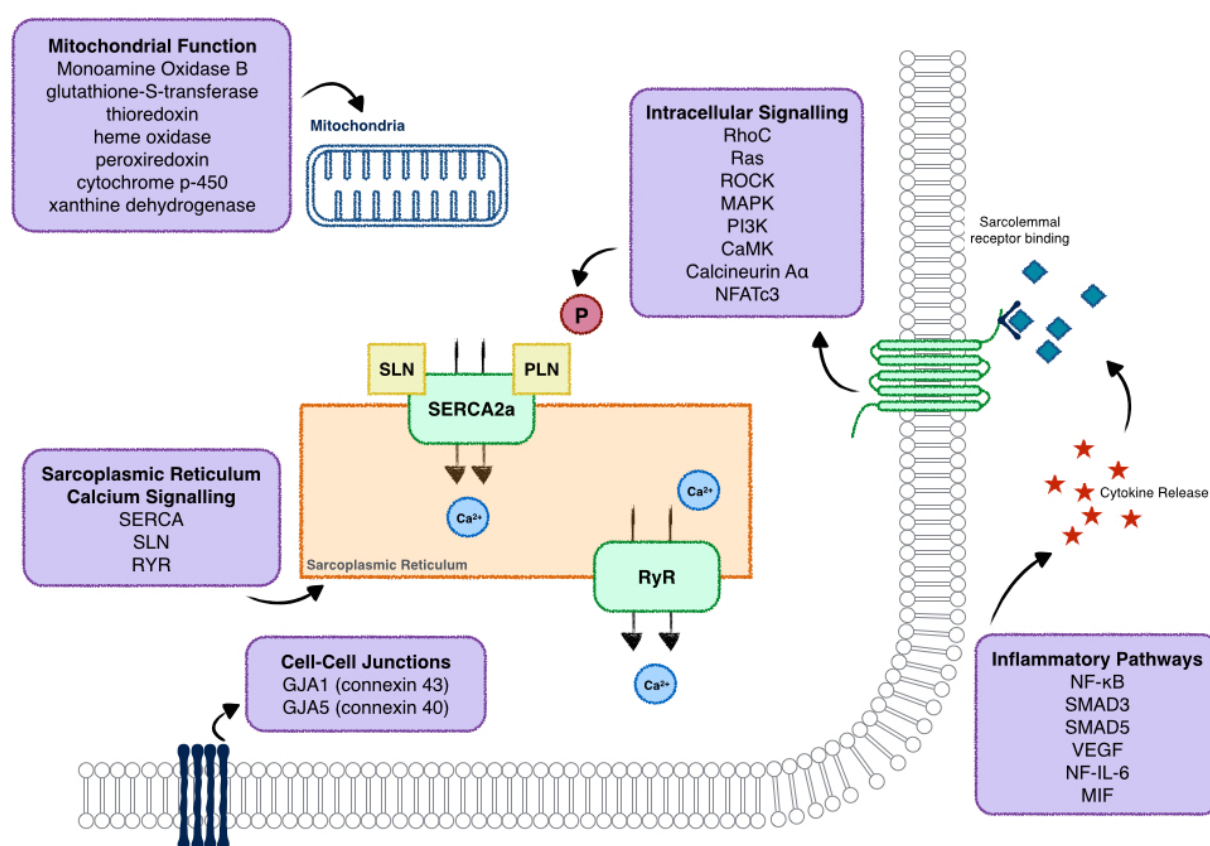


Figure 4-1: A summary of the gene expression changes observed in human atrial fibrillation. This incorporates upregulation of inflammatory pathways, mitochondrial oxidative stress, sarcoplasmic calcium channel remodeling, dysregulation of intracellular signaling and conduction block through dysregulation of gap junction connexins.

Together these gene expression changes demonstrate the complexity of signaling pathways, intra- and extra- cellular stimuli regulating AF pathogenesis through both electrical and structural remodeling (**Figure 4-1**). When combined with the network of post-transcriptional regulation through microRNA discussed in chapters 2 and 3, these findings highlight the intricacy of the genomic control of cardiac conduction, both in causation and in response to arrhythmia.

4.1.2 The role of epicardial adipose in atrial fibrillation pathogenesis

Epicardial adipose tissue (EAT) can be defined as the layer of fat between the epicardium and the visceral pericardium. It is embryologically derived from the splanchnopleuric mesoderm and retrieves its blood supply directly from the coronary arteries. It differs distinctly from pericardial adipose (PAT), which is located outside of the visceral pericardium and is not in direct continuity with the myocardium.(197) The role of the epicardial fat in post-operative atrial fibrillation was first evaluated in a 2004 study by Cummings et al, in which it was shown that preservation of the anterior fat pad between the pulmonary artery and the aorta may reduce the incidence of AF after coronary bypass surgery.(198) Since this time, there has been increasing focus on epicardial fat in both chronic and POAF pathogenesis with suggestion that adipose tissue may have a regulatory effect on cardiomyocytes through local paracrine signaling (**Figure 4-2**). Furthermore, neural plexi have since been identified within specific regions of EAT that provide direct autonomic regulation of cardiac conduction. In addition, increases in the volume of EAT have been shown to correlate positively with the severity of AF (199) and are associated with higher rates of AF recurrence after catheter ablation.(200)

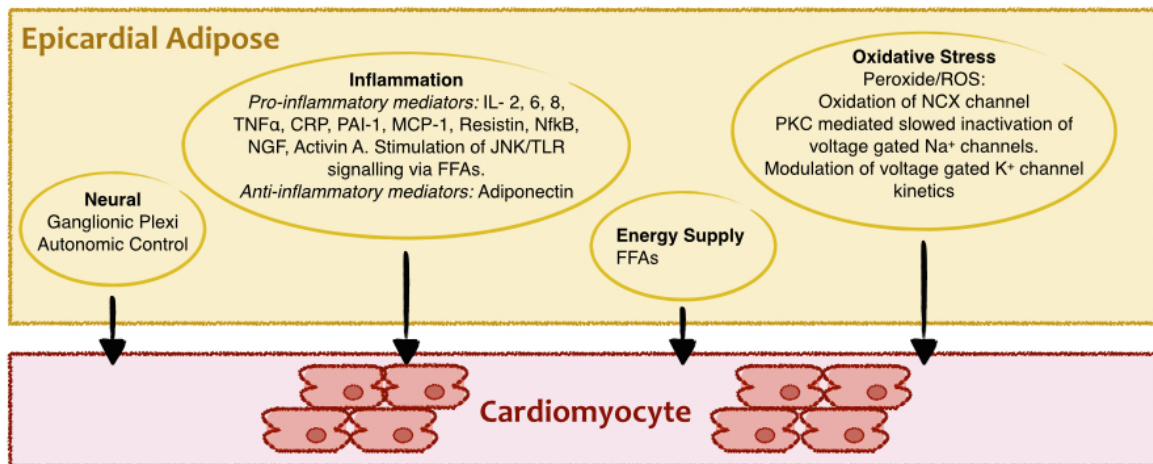


Figure 4-2: A summary of the mechanisms by which EAT may modulate cardiomyocyte conduction and the onset and maintenance of atrial fibrillation.

EAT and inflammation

Epicardial adipose cells exist in close proximity to cardiomyocytes, and the lack of any defined fascial layer gives rise to a direct continuity between these two tissue types. This allows for paracrine mediators secreted by adipose cells to alter the cellular environment of the cardiomyocyte. One of the key factors thought to influence the pathogenesis of both lone and post-operative atrial fibrillation is the presence of a pro-inflammatory substrate that is evident through increases in the interleukins (IL)- 2, 6, 8, as well as TNF α and CRP. This in turn may stimulate electrical and structural remodeling that promotes re-entry and the triggering and maintenance of AF. Furthermore, it has recently been suggested that pro-inflammatory mediators from the EAT may pre-dispose to increased incidence of POAF through promoting right ventricular dysfunction.(201, 202)

The EAT is well recognised to secrete both pro- and anti-inflammatory cytokines and thus alteration in the balance of these factors may allow changes in the EAT secretome to predispose to AF. Indeed, upregulation in the secretion of pro-inflammatory cytokines such as Interleukin-6 (IL-6), tumor necrosis factor- α (TNF α), plasminogen activator inhibitor-1 (PAI-1), monocyte chemoattractive protein-1 (MCP-1), nuclear factor Nf κ B, resistin and nerve

growth factor (NGF) have all been shown to be associated with AF.(203, 204) Through breakdown of triglycerides, the EAT is also able to release free fatty acids (FFAs) that act as an energy reserve for the myocardium in states of fasting. However, these FFAs present within the secretome may also activate JNK (c-Jun N-terminal kinase) and TLR (toll-like receptor)-2 and TLR-4 signaling pathways promoting inflammation and cellular insulin resistance.(205)

Activin A, a member of the TGF β superfamily has also been shown to be present in the secretome of EAT.(206) Through increases in collagen synthesis and upregulation of inflammatory genes encoding matrix metalloproteases (MMPs), vascular endothelial growth factor (VEGF) and hypoxia inducing factor (HIF1 α),(206) Activin A produced by the EAT may induce fibrotic changes within the atrial myocardium that in turn promote regions of conduction block and set up a substrate for re-entry.

Similarly, the EAT may also induce an anti-inflammatory substrate through release of cytokines such as adiponectin. Adiponectin is a collagen like protein encoded by the APM1 gene that is produced during adipocyte differentiation. It has a fundamental role in lipid and glucose metabolism where it has been shown not only to increase fatty acid oxidation and improve muscle uptake and utilisation of FFAs, but also to improve insulin sensitisation and reduce insulin resistance.(207) Through a loss of these protective effects, suppression of macrophage foam cell formation and inhibition of vascular smooth muscle cell proliferation, reduced levels of adiponectin have been associated with type 2 diabetes, metabolic syndrome, and atherosclerosis.(207, 208) Similarly, a fall in adiponectin levels may also promote atrial remodeling and fibrosis leading to an increased incidence of POAF after CABG surgery.(209) Furthermore, lower levels of adiponectin have recently been associated with worse cardiovascular outcomes (including stroke, pulmonary odema and acute coronary syndrome) in anticoagulated female patients with chronic AF,(210) suggesting that modulation of the

EAT secretome may not only affect the presence or absence of AF but also the subsequent long term cardiovascular risk profile associated with this arrhythmia.

EAT and Oxidative Stress

In addition to release of inflammatory mediators, the EAT secretome has also been shown to be a source of reactive oxidative species (ROS) such as superoxide and peroxynitrite and as such may promote atrial fibrillation through increases in oxidative stress.(211) Oxidation induced calcium entry through the NCX channel and reduction in the L- type calcium current (I_{Ca}) may result in intracellular calcium overload, producing a compensatory reduction in action potential duration (APD) and ERP thus promoting re-entry.(67, 212) Peroxide has also been shown to both accelerate activation kinetics and reduce inactivation of voltage gated potassium channels, resulting in a reduction in APD and ERP and promoting re-entry.(69) Exposure to oxidative stress may also influence cellular Na^+ currents both by directly slowing inactivation of voltage gated Na^+ channels and via secondary messengers such as protein kinase C (PKC).(71) Furthermore, peroxide may also enhance the slow inactivating Na^+ current (I_{Na}) (70, 71), promoting diastolic depolarisation, action potential generation and arrhythmogenesis.

In the long term, changes in gene expression to restore sarcoplasmic reticulum calcium storage and reduce intracellular overload as well as alterations in contractile protein expression (for example re-expression of β -myosin heavy chain and reversion to a fetal phenotype) may also promote the maintenance of AF.(67) Furthermore, peroxynitrite and hydroxyl radicals may also give rise to post-translational modification of myofibrillar proteins within atrial myocytes, leading to a reduction in high-energy phosphate supply to myosin ATPase.(65, 68).

EAT and neural control of cardiac conduction

In addition to paracrine signaling the EAT may have direct autonomic effects on cardiac conduction. The ganglionic plexi (GP) located within the epicardial fat pads form a key component of the autonomic nervous supply to the heart. GP stimulation results in increased parasympathetic effects, slowing heart rate and reducing atrial conduction time and refractory period. GP ablation is now common practice in the radiofrequency ablation of persistent AF.

4.1.3 Aims

The aim of this chapter was to investigate the relationship between the epicardial adipose tissue (EAT) and human post-operative atrial fibrillation. Through evaluation of the gene and subsequent protein expression, I aim to test the hypothesis that changes in make-up of the epicardial adipose modulate AF pathogenesis. Gene expression microarray techniques will be used to identify any differential expression between POAF and non-POAF samples from the same patient cohort studied in previous chapters. Any changes will be validated by RT-qPCR with subsequent downstream analysis of protein expression. Where possible I then aim to correlate these findings with my previous analysis of atrial tissue in order to provide a more comprehensive understanding of the interplay between these two tissue types in POAF pathogenesis.

4.2 Methods

4.2.1 Tissue Sampling

Epicardial adipose tissue (EAT) was defined as adipose located between the myocardium and the visceral pericardium. All EAT samples were taken prior to onset of cardiopulmonary bypass from the right atrio-ventricular groove. Where possible, diathermy was avoided in order to minimise thermal cellular damage that may impact on RNA and metabolite extractions. All samples were immediately snap frozen in liquid nitrogen and stored at -80°C until further use.

4.2.2 Lipid RNA extraction

Epicardial adipose samples were kept at -80 degrees until RNA extraction. Extraction was performed using the RNeasy Lipid extraction kit (Quiagen) according to the manufacturers protocol. Briefly, samples were homogenised in 1ml of QIAzol Lysis Reagent using a pestle and mortar and the homogenate left to incubate at room temperature for 5 minutes to promote the dissociation of nucleoprotein complexes. 200µl of chloroform was added, the mixture vortexed for 15s and left to incubate for a further 3 minutes at room temperature. The tubes were then centrifuged at 12 000g for 15 minutes at 4°C to ensure phase separation. The aqueous phase was transferred into a new tube, the volume recorded, and the same volume of 70% ethanol added to the tube prior to vortexing for 15 seconds. 700µl of the mixture was then transferred into a tube containing the RNeasy spin column and centrifuged for 15s at 8000g. The flow through was discarded and this step repeated for any remaining sample. 700µl of RW1 wash buffer was added to the column and the column centrifuged at 8000g to wash the membrane. The column was then transferred to a new tube and 500µl of RPE buffer added prior to centrifugation at 8000g. This step was subsequently repeated to ensure no ethanol contamination of the final RNA extract. After transferring the RNeasy spin column into a final collection tube, 30µl of RNase-free water was added and the tube centrifuged at 122

8000g for 1 minute to elute the RNA contained within the flow-through. The RNA quality and concentrations of the eluted RNA were assessed using the Nanodrop 1000 spectrophotometer and Agilent 2100 Bioanalyser as previously described. All samples with RNA integrity numbers (RIN) <6 were re-extracted in an attempt to improve RNA quality. Final RNA extractions were subsequently labeled and stored at -80 degrees until further use.

4.2.3 Gene expression microarray of Lipid mRNA

Extracted RNA was diluted to a total concentration of 100ng/μl. Agilent gene expression two-colour microarrays were performed according to the manufacturer protocol. Briefly, 1.5μl of RNA was added to 2μl of prepared two-colour diluted spike-in mix (A: Cyanine 3-CTP; B:Cyanine 5-CTP). 1.8μl of prepared T7 primer was added to each of the samples and the mixture denatured at 65°C for 10 minutes before being placed on ice. 4.7μl of prepared cDNA master mix (Per reaction: 2μl 5x first strand buffer, 1μl 0.1M DTT, 0.5μl 10mM dNTP mix, 1.2μl affinity script RNase block mix) was then added to each tube and the samples incubated for 2 hours at 40°C followed by 15 minutes at 70°C to arrest enzyme activity. The transcription master mix was then prepared (per reaction: 0.75μl nuclease free water, 3.2μl 5x transcription buffer, 0.6μl 0.1M DTT, 1μl NTP mix, 0.21μl T7 RNA polymerase blend 0.24μl Cy 3-CTP or Cy 5-CTP) and 6μl of Cy 3-CTP or Cy 5-CTP transcription preparation added to the Spike-in A and Spike-in B mixes respectively. The sample mix was then incubated in a water bath at 40°C for 2 hours to complete the labeling and amplification reaction.

The labeled and amplified RNA was then purified using the RNeasy mini kit (Quiagen) according to manufacturer protocol and the concentration and quality of the resultant cRNA assessed using the NanoDrop spectrophotometer as previously described.

To prepare for hybridization, for each microarray a fragmentation mix containing the labeled cRNA sample (containing: 300ng Cy3 amplified cRNA, 300ng Cy5 amplified cRNA, 5µl 10x gene expression blocking agent, 1µl 25x fragmentation buffer, nuclease free water to reach a volume of 24µl) was prepared and incubated at 60°C for 30 minutes to fragment the RNA before being cooled on ice. 25µl of 2x Hi-RPM Hybridization buffer was then added to 25µl of cRNA fragmentation mix and the mixture centrifuged at 13 000g for 1 minute before being immediately loaded onto the array.

A total sample volume of 40µl was loaded into the corresponding gasket wells and Agilent MicroArray slides (8x60k) placed on top to prepare the final gasket assembly. Five blank samples were included as hybridisation controls. Wells were checked for air bubbles before hybridization using the Agilent Sure-hyb hybridization chamber at 65°C for 17hrs.

Following completion of hybridization slides were washed according to manufacturer protocol. Slides were immediately scanned using the Agilent G2505C MicroArray Scanner and feature extraction performed using the Agilent Feature Extraction Software version 10.7.3.1. Analysis was performed using Agilent GeneSpring software GX11.0.

4.2.4 RT-qPCR of atrial mRNA

Real time- quantitative polymerase chain reaction and subsequent analysis was carried out in accordance with the protocol described in **Chapter 3** (3.2.1). Universal reverse transcription was performed as previously described. TaqMan qPCR gene expression assays were used for the following gene targets: SLN, PLN, TRDN, SERCA2A. For each study probe an identical PCR reaction was also carried out using ribosomal U6 as a control gene.

4.2.5 Western Blotting

Western blotting was performed according to the methods described in **Chapter 3** (Section 3.2.4) for the following target proteins: Sarcolipin (Anti-SLN, Rabbit polyclonal; #ABT13, Millipore UK), Phospholamban (Anti-PLN, Mouse monoclonal 2D12; #ab2865, Abcam), and SERCA2A (Anti-SERCA2A ATPase antibody 2A7-A1, Mouse monoclonal; #ab2861, Abcam). In order to improve resolution for the small molecular weight proteins SLN and PLN, 10-well 10% NuPAGE® Bis-Tris Precast Gels (Life Technologies) were used with MES running buffer, made up according to manufacturer recommendations.

4.3 Results

4.3.1 Gene expression microarray

Quality control highlighted poor integrity of 9 samples with evidence of RNA degradation and fragmentation. These samples were therefore excluded from further analysis. 19 samples (8 AF, 11nAF) determined to have an RNA integrity number (RIN) >4 were selected for miRNA microarray analysis. Results were analysed using Agilent's GeneSpring software. Following normalisation, principle component analysis (**figure 4-3**) did not identify significant clustering of AF and non-AF groups.

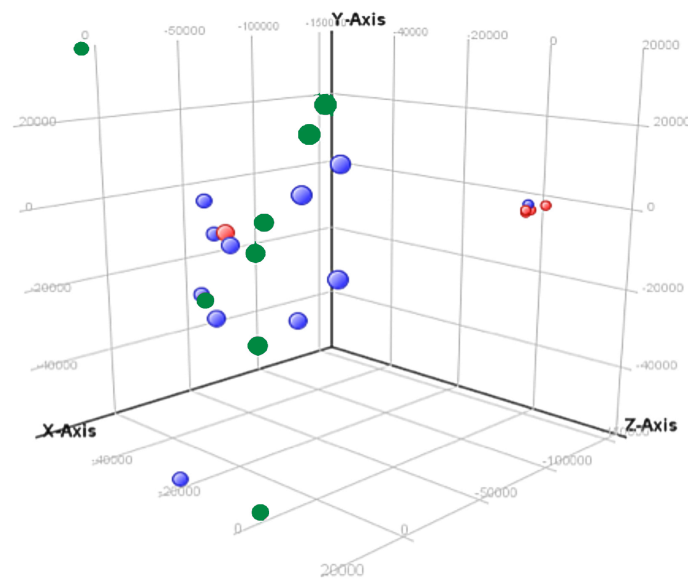


Figure 4-3: 3D PCA Scores plot showing no clear separation between AF samples (Green) and non-AF samples (blue) based on the gene expression profile of the epicardial fat. Blank samples are shown in red.

Similarly, analysis parameters set to detect genes with a >2 fold change difference in signal intensity at a level of significance <0.05 did not reveal any significant differences between AF and non-AF groups.

Following these initial results, secondary analysis was performed to detect gene ontology (GO) pathways enriched in the complete gene list categorised in order of differential expression between AF and non-AF groups. GO analysis (GORilla: <http://cbl-gorilla.cs.technion.ac.il>) revealed significant enrichment of pathways involved in (i) negative regulation of calcium ion transmembrane transporter activity and (ii) regulation of ryanodine-sensitive calcium-release channel activity (**figure 4-4**). A complete list of these genes and their GO pathways is shown in **table 4-2**.

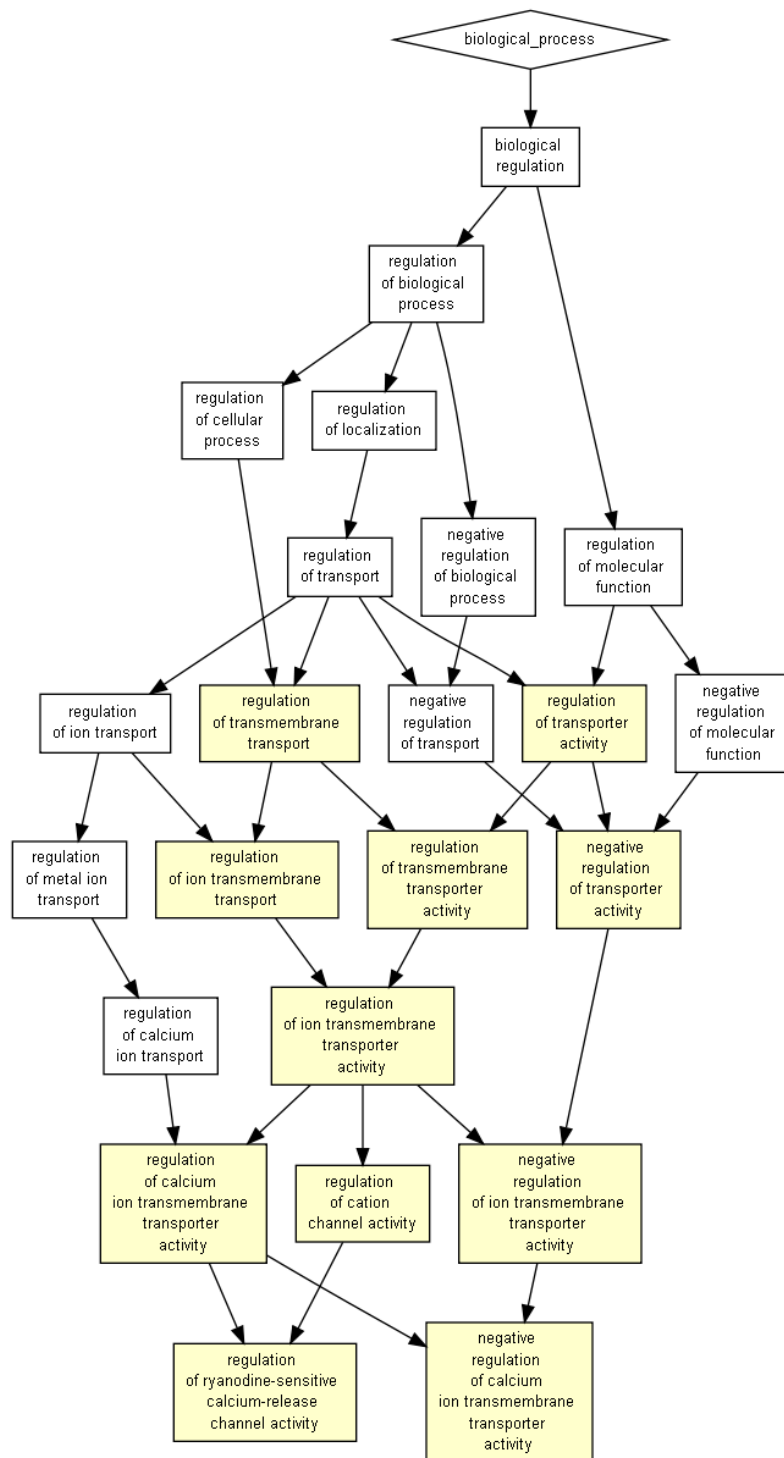


Figure 4-4: Gene ontology (GO) pathway enrichment of genes ordered by degree of differential expression between POAF and non-POAF groups.

Description	P-value	FDR q-value	Enrichment (N, B, n, b)	Genes
negative regulation of transporter activity	0.0000136	0.0502	10.37 (446,5,43,5)	CASQ, PLN, SLN, ACTN2, TRDN
negative regulation of ion transmembrane transporter activity	0.0000136	0.0251	10.37 (446,5,43,5)	CASQ, PLN, SLN, ACTN2, TRDN
negative regulation of calcium ion transmembrane transporter activity	0.0000198	0.0244	15.93 (446,4,28,4)	CASQ, PLN, SLN, TRDN
regulation of calcium ion transmembrane transporter activity	0.0000198	0.0183	15.93 (446,4,28,4)	CASQ, PLN, SLN, TRDN
regulation of ion transmembrane transporter	0.000119	0.0877	8.64 (446,6,43,5)	CASQ, PLN, SLN, ACTN2, TRDN
regulation of transporter activity	0.000119	0.0731	8.64 (446,6,43,5)	CASQ, PLN, SLN, ACTN2, TRDN
regulation of transmembrane transporter activity	0.000119	0.0626	8.64 (446,6,43,5)	CASQ, PLN, SLN, ACTN2, TRDN
regulation of cation channel activity	0.00037	0.171	16.52 (446,3,27,3)	CASQ, PLN, TRDN
regulation of ryanodine-sensitive calcium-release channel activity	0.00037	0.152	16.52 (446,3,27,3)	CASQ, PLN, TRDN
regulation of transmembrane transport	0.000387	0.143	5.58 (446,13,43,7)	CASQ, KCNA4, PLN, SLN, ACTN2, KCNK17, TRDN
regulation of ion transmembrane transport	0.000387	0.13	5.58 (446,13,43,7)	CASQ, KCNA4, PLN, SLN, ACTN2, KCNK17, TRDN

Table 4-1: Enriched GO terms based on gene list ranked in order of differential expression between POAF and non-POAF epicardial fat samples. 'P-value' is the enrichment p-value computed according to the mHG or HG model. This p-value is not corrected for multiple testing of 3697 GO terms. 'FDR q-value' is the correction of the above p-value for multiple testing using the Benjamini and Hochberg (1995) method. Namely, for the *i*th term (ranked according to p-value) the FDR q-value is (p-value * number of GO terms) / *i*. Enrichment (N, B, n, b) is defined as follows: N - is the total number of genes; B - is the total number of genes associated with a specific GO term; n - is the number of genes in the top of the user's input list or in the target set when appropriate b - is the number of genes in the intersection; Enrichment = (b/n) / (B/N). Genes: The list of associated genes that appear in the optimal top of the list for each GO term.(213)

4.3.2 RT-qPCR of Target Genes

Based on the above findings of the lipid microarray, a number of target genes were highlighted that may be clinically relevant to the pathogenesis of POAF. However, it was noted that these genes were all muscle specific, and included targets encoding myosin isoforms and regulators of sarcoplasmic reticulum proteins (**Table 4-1**). Although not evident on a macroscopic level, it was therefore most likely that these findings represented contamination of epicardial fat samples with atrial muscle given the close continuity of epicardial fat with the atrial myocardium.

As a result of this contamination and the poor quality of RNA extracted from the lipid tissue no further evaluation of the transcriptomic signature of epicardial fat was performed. However, given the involvement of these muscle specific genes in key arrhythmogenesis pathways, RT-qPCR was performed to evaluate the concentration of these mRNA in the atrial biopsies of my patient cohort. The following targets were evaluated: Sarcolipin (SLN), Phospholamban (PLN), Myosin Heavy Chain 6 (MYH6), Sarcoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2A/ ATP2A2) and Triadin (TRDN).

POAF was found to be associated with a significant reduction in the expression of SLN mRNA ($p=0.0080$) as well as a less significant reduction in MYH6 ($p=0.0297$) and SERCA2A ($p=0.0343$) gene expression (**Figure 4-5**).

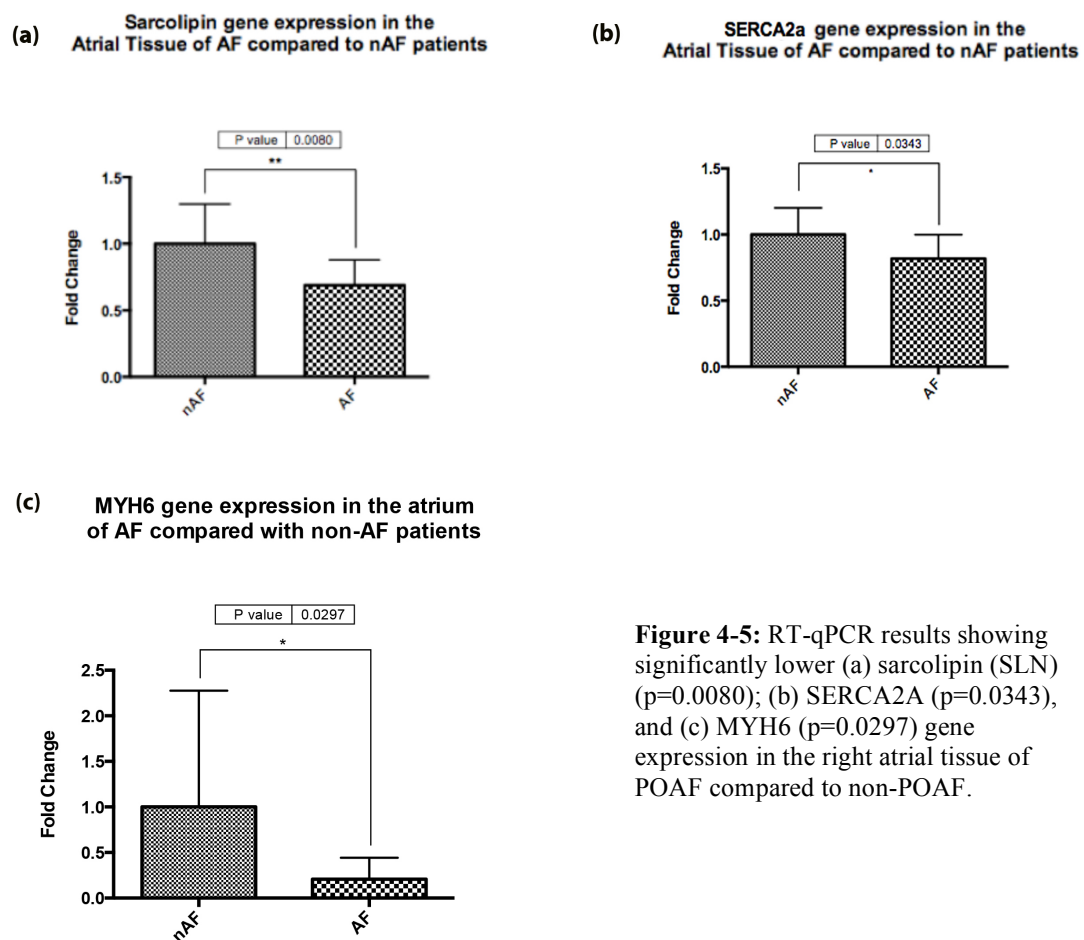


Figure 4-5: RT-qPCR results showing significantly lower (a) sarcolipin (SLN) ($p=0.0080$); (b) SERCA2A ($p=0.0343$), and (c) MYH6 ($p=0.0297$) gene expression in the right atrial tissue of POAF compared to non-POAF.

However, no significant difference was observed in the expression of either PLN ($p=0.7963$) or Triadin ($p=0.7963$) mRNA (**Figure 4-6**).

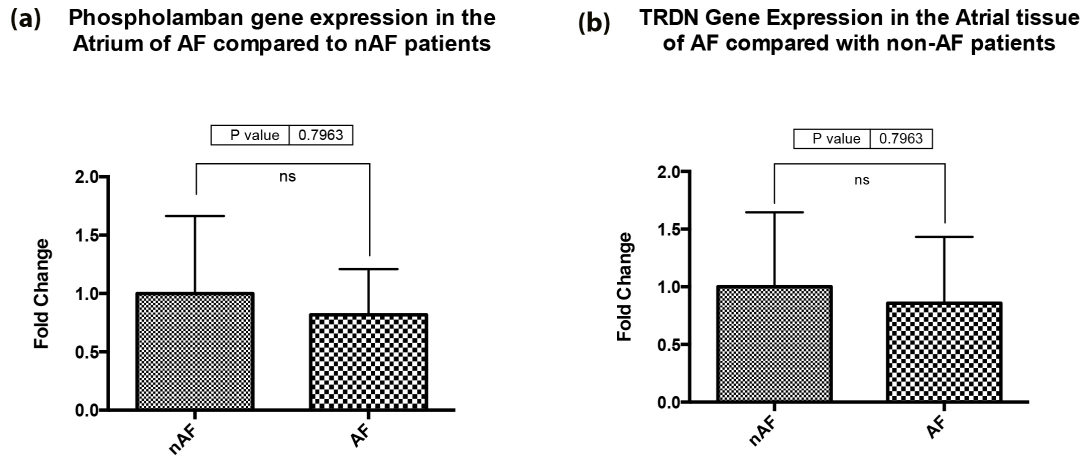
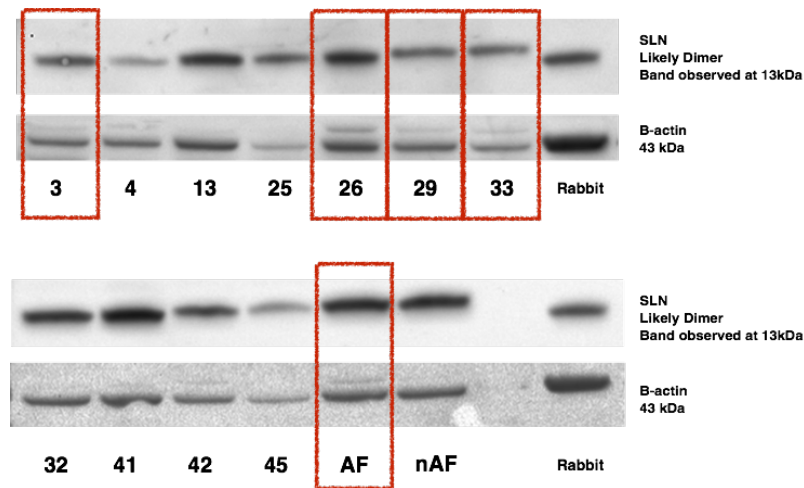


Figure 4-6: RT-qPCR results showing no significant difference in (a) Phospholamban (PLN) ($p=0.7963$) or (b) Triadin (TRDN) ($p=0.7963$) gene expression in the right atrial tissue of POAF compared to non-POAF.

4.3.3 Western Blotting

Sarcolipin (SLN) protein levels were evaluated in the atrial tissue of a subgroup of 4 POAF and 4 non-POAF patients matched to samples used in the aforementioned RT-qPCR experiment. Patients developing POAF had a significantly lower level of SLN protein expression in atrial tissue samples taken at the time of surgery when compared to non-POAF patients ($p=0.0286$) (**Figure 4-7**).

No significant difference was seen in the expression of the regulator protein phospholamban (PLN) or in the overall expression of SERCA2a (**Figure 4-8 and 4-9**). Insufficient atrial tissue sample was available to allow for evaluation of atrial myosin heavy chain 6 (cardiac muscle, alpha isoform) protein expression in the current study.



SLN Protein Expression

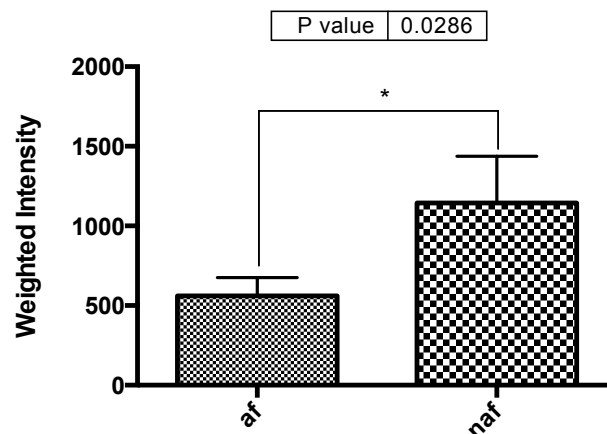
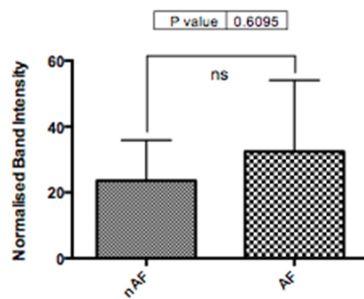


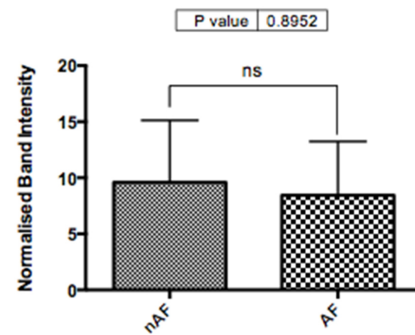
Figure 4-7: Western blot demonstrating significantly lower sarcolipin (SLN) expression ($p=0.0286$) in the right atrial tissue of patients developing post operative atrial fibrillation (POAF) compared to those maintaining sinus rhythm. Red boxes denote samples from POAF patients.

Note: uneven loading secondary to very small quantities of protein available for western blotting. Loading based on 10ug of protein per well but estimated as insufficient volume to quantitate with BCA or Bradford assay. Recognised band at 13kDa as reported by Millipore. Band at 6kDa not detected possibly due to low starting concentration of protein

(a) Monomeric Phospholamban Protein Expression in the Atrium of AF compared to nAF patients



(b) Pentameric Phospholamban Protein Expression in the Atrium of AF compared to nAF patients



(c)

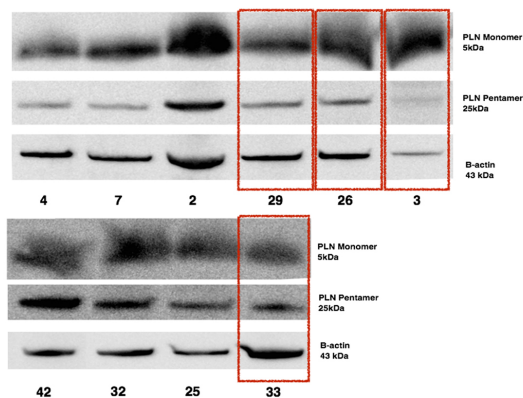
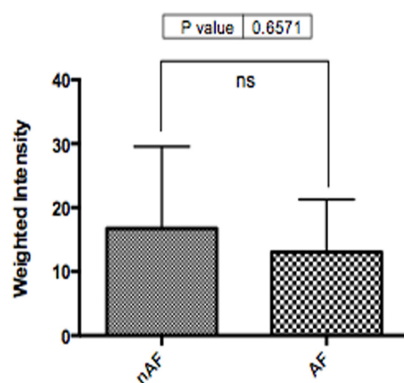


Figure 4-8: Western blot demonstrating no significant difference in (a) monomeric phospholamban (PLN) ($p=0.6095$) or (b) pentameric PLN ($p=0.8952$) expression in the right atrial tissue of patients developing post operative atrial fibrillation (POAF) compared to those maintaining sinus rhythm. Red boxes denote samples from POAF

(a) SERCA2a Protein Expression in the Atrial Tissue of Patients with AF compared to non-AF



(b)

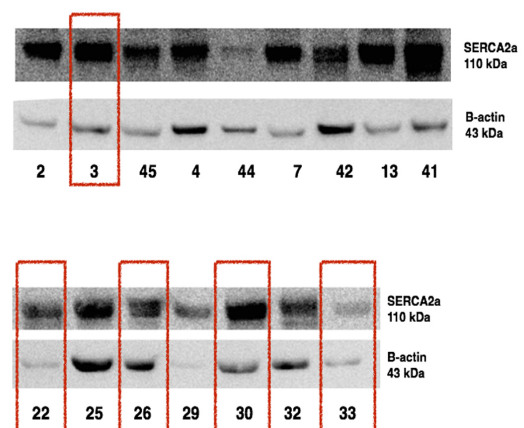


Figure 4-9: Western blot demonstrating no significant difference in the expression of SERCA2A protein ($p=0.6571$) in the right atrial tissue. Red boxes denote samples from POAF patients.

4.4 Discussion

4.4.1. Structural remodelling: MYH6 and cardiac mechanotransduction

Muscle myosins are motor proteins that enable muscle contraction through ATP-dependent binding with actin. Their structure comprises two heavy chains (MHC), a regulatory light chain and an essential light chain. Together the combined heavy chain N-terminals make up the motor domain of myosin, that contains both the actin and nucleotide binding sites (**Figure 4-10**).⁽²¹⁴⁾

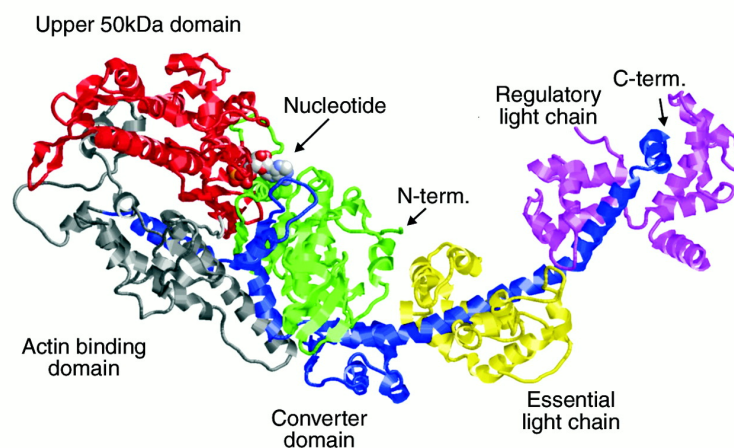


Figure 4-10: The myosin motor domain: The purple strand represents the regulatory light chain, the yellow the essential light chain. The red, grey and green segments represent the myosin heavy chain subfragments at 50kDa, 20kDa and 25kDa respectively. Image taken from Rüegg et al. 2002 *Molecular Motors: Force and Movement Generated by Single Myosin II Molecules*.⁽²¹⁵⁾

Two isoforms of myosin exist in cardiac muscle, α -myosin and β -myosin, determined by the structure of the globular motor domain made up of the myosin heavy chain proteins.⁽²¹⁴⁾ The MYH6 gene encodes for the alpha cardiac isoform of the myosin heavy chain 6 protein (α -MHC), and is found on chromosome 14 at location 14q12. Out of a total of 39 exons contained within the MYH6 gene, 37 contain protein coding sequences.⁽²¹⁶⁾ In human atrial myocardium there is an approximately equal breakdown between α -MHC and β -MHC (encoded by the MYH7 gene located 4kb upstream of MYH6 on intron 31 of chromosome

14; **figure 4-11**), however in ventricular tissue the β -MHC isoform is predominant with little α -MHC present (notably, the opposite is true in small animals such as mice and rats in which ventricular tissue is made up almost exclusively of the α -MHC isoform).(216) The key difference between the α - and β -MHC isoforms is their speed of contraction. α -MHC is the fast-twitch isoform, and exhibits a myosin cycling time approximately 10 times faster than β -MHC. This occurs as a consequence of a number of features including a 5-10 fold weaker affinity for actin, a 7- to 15-times weaker affinity for ADP, and faster ATP hydrolysis.(214) This faster cycle time is also reflected in the difference in energy (ATP) requirement for contraction between the two isoforms, and α -MHC demonstrates an approximately 3x higher actin activated ATPase activity than β -MHC.(217)

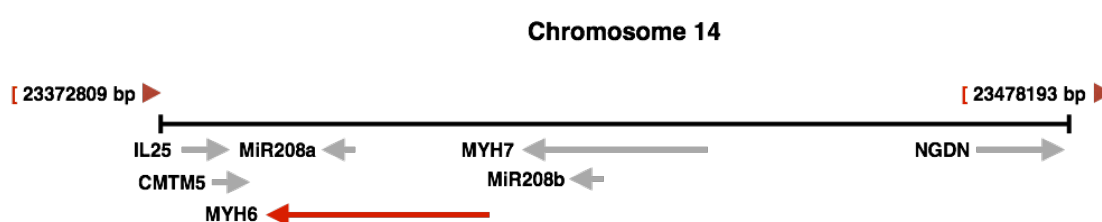


Figure 4-11: Schematic representation of the locations of the MYH6 gene and its intronic miRNA hsa-miR-208a

Several myocardial disease states including cardiac failure,(218) hypertrophy, pressure/volume overload(219) and chronic arrhythmia,(220) result in an MHC class switch within the atrial myocardium such that the α -MHC component is replaced with an increased proportion of β -MHC slow-twitch muscle.(221) Indeed, in their 2005 study comparing right atrial appendage biopsies of chronic AF (>3 months) patients undergoing cardiac surgery to those in sinus rhythm, Narolska et al. showed significant switch from the α - to the β -MHC isoform in their AF group.(221) Furthermore, a missense arginine to tryptophan mutation at position 22,936,019 bp within exon 18 of the MYH6 gene (c.2161C>T), has recently been identified in patients with sick sinus syndrome, a condition characterised by brady- and tachy-

arrhythmias including atrial fibrillation.(222) Furthermore, although atrial fibrillation was not a pre-defined outcome in this study, post-hoc analysis highlighted that individuals carrying this mutation also conveyed a significantly greater likelihood of atrial fibrillation (OR 2.03 95%CI [1.15, 3.58], p=0.015).(222)

These findings are echoed in the results from my patient cohort, whereby a significant reduction in MYH6 gene expression was observed in POAF patients when compared to those in sinus rhythm. Beyond this, my work is the first to suggest this relative class switch may occur prior to the onset of atrial fibrillation rather than as a consequence of it, implying a primary role for muscle myosins in the mechanism of structural remodeling in AF pathogenesis.

A number of factors may affect the relative concentrations of α - and β - isoforms present in cardiac tissue including age, circulating thyroid hormone (triiodothyronine (T3) increases α -MHC), and adrenergic activity (cAMP mediated increase in expression of α -MHC),(223) as well as the expression of the transcription factors GATA4,(224) Nfat3 and FoxP1.(225) However, at present the reason for this functional class switch in response to cardiac stress is not fully understood and it may indeed occur through a combination of the aforementioned mechanisms. It has been postulated that this relative reduction in fast-twitch muscle forms part of an adaptive mechanism designed to minimise energy requirement in failing myocardium; or in the case of atrial tachyarrhythmias, prevent energy depletion and contractile failure through economy of ATP. However, ultimately this class switch is likely to prove to be disadvantageous as reduced contractility subsequent to increased β -MHC expression may ultimately result in both contractile failure and diastolic dysfunction.(221)

MYH6 and miR-208a

Interestingly, this fall in MYH6 gene expression in POAF also correlates with the reduced expression of miR-208a observed in my patient cohort. MicroRNA-208a is encoded within intron 27 of the MYH6 gene (Figure 4-11 and 4-12)(152) and thus reduced transcription of the entire MYH6 gene would also be expected to reduce the expression of miRNA-208a as is seen in my POAF patient cohort.

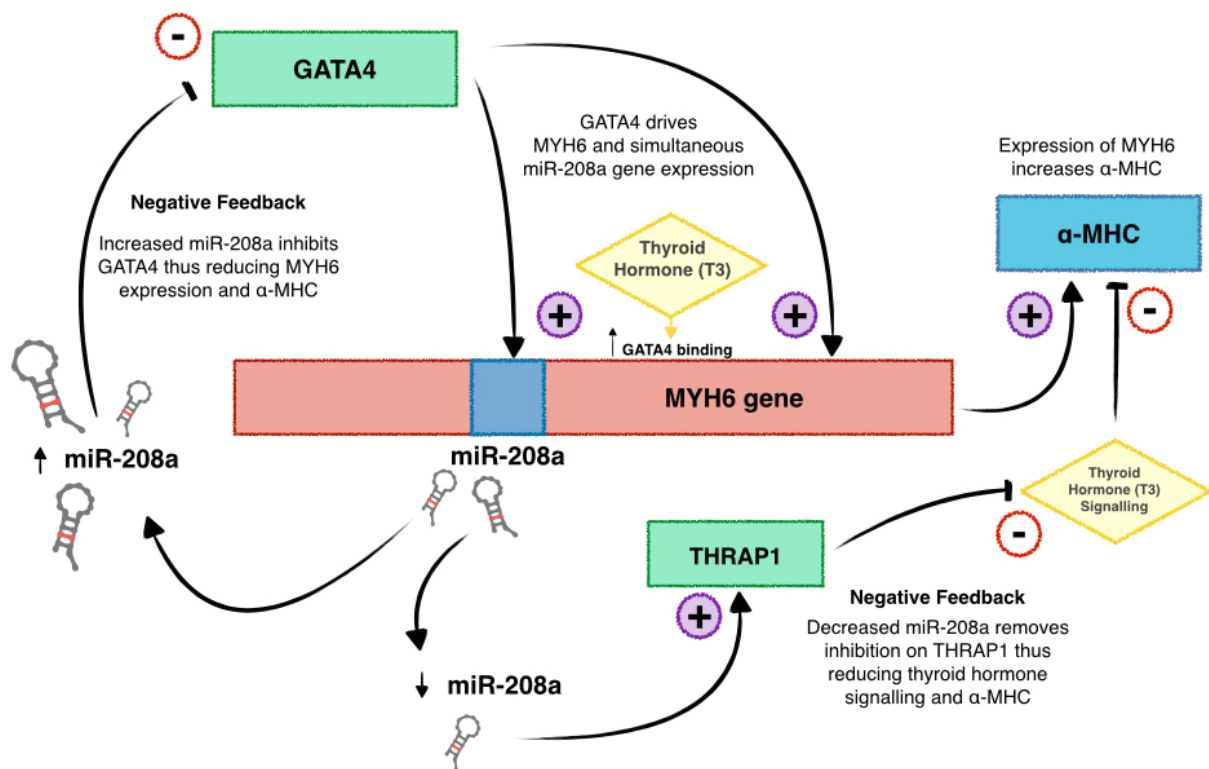


Figure 4-12: Regulation of MYH6 by GATA4 and the potential role of miR-208a as a negative feedback mechanism in the regulation of MYH6 transcription.

It is therefore possible that this reduced miR-208a expression is in fact a consequence of reduced MYH6 expression which, through changes in the relative expression of slow- and fast- twitch MHC isoforms, pre-disposes to subsequent atrial fibrillation in the post operative period. Indeed, this is supported by the findings of Montgomery and Van Rooj et al. who did not observe any electrophysiological changes in the surface ECGs of either rats treated with the antagomiR antimir-208a, or in miR-208^{+/+}/miR-208^{-/-} knockout mice.(108, 226)

Furthermore, although not demonstrated in my patient cohort (as discussed in chapter 3), it is possible that through inhibition of the transcription factor GATA4 which itself promotes α -MHC expression, miR-208a may produce a negative feedback mechanism acting to maintain a balance in MYH6 gene expression (**Figure 4-12**).

Of note, these findings add further insight into the discussion presented in Chapter 3 in which it was postulated that a fall in miR-208a might increase the overall expression of α -MHC through removal of translational inhibition on the thyroid hormone receptor protein-1 (THRAP) and inhibition of triiodothyronine (T3) signaling. In fact, these experimental results reveal that miR-208a acts as part of a complex regulatory network that acts to feedback on the transcription of both its co-expressed intron MYH6 and its translated protein α -MHC. A summary of this network is shown in **figure 4-12**.

4.4.2 Electrical Remodelling: Dysregulation of cardiomyocyte calcium handling in POAF atria

Another finding observed in my patient cohort was a significant reduction in sarcolipin (SLN) gene and subsequent protein expression. Sarcolipin is a small 4kDa, 31 amino acid protein (encoded by the SLN gene, location 11q22, chromosome 11), expressed predominantly in atria, that acts to inhibit the sarcoplasmic reticulum (SR) ATPase (SERCA) independent to its primary regulatory protein phospholamban (PLN). This reduction in SLN protein expression correlates with findings in patients with chronic AF as evidenced by Shanmugam et al. who observed a similar significant decrease in sarcolipin protein expression.(194, 227) Furthermore, my results also corroborate previous findings that this mechanism of SERCA regulation in AF occurs independent to the activity of the regulatory protein phospholamban, the expression of which remains unchanged.(194)

The sarcoplasmic reticulum Ca^{2+} ATPase, and consequently its regulatory proteins, plays a fundamental role in determining the availability of intracellular calcium within the cardiomyocyte. During normal contraction, on arrival of an action potential, L-type calcium channels are activated resulting in cytosolic calcium influx. This subsequently triggers calcium dependent calcium release from the SR through the ryanodine receptor channel (RyR2). Through binding with this available free cytosolic Ca^{2+} Troponin C (TnC) binding with Troponin I (TnI) is enabled, removing inhibition of tropomyosin and actin and thus triggering myofilament contraction.(228, 229) SR calcium reuptake through SERCA triggers dissociation of Ca^{2+} from TnC and thus results in relaxation (**Figure 4-13**). One such example of reduced SERCA activity can be seen in disease states including diabetic cardiomyopathy whereby there is failure of relaxation and subsequent diastolic dysfunction.(230)

Through inhibition of SERCA activity, SLN acts to reduce cellular reuptake of Ca^{2+} into the SR. Knockout of SLN removes this inhibition of SERCA2a in cardiac muscle, enhances SR Ca^{2+} reuptake and promotes SR Ca^{2+} overload.(21, 225) This in turn shortens of the effective refractory period and thus may increase susceptibility to AF. Furthermore, increased SR calcium load may also lead to diastolic calcium leak giving rise to the potential for delayed after depolarizations and thus the triggering of AF(29). Beyond this, SLN may also have wider effects on cardiomyocyte calcium homeostasis. In addition to increases in SERCA activity, SLN knockout mice also display increases in cardiomyocyte L-type Ca^{2+} channel activity thus increasing Ca^{2+} influx in response to an action potential. Furthermore, it is possible that increases in basal L-type calcium currents may also give rise to prolongation of the action potential, stimulation of NCX sodium influx (in exchange for Ca^{2+}) and thus early after depolarizations that trigger the initiation of AF.(21)

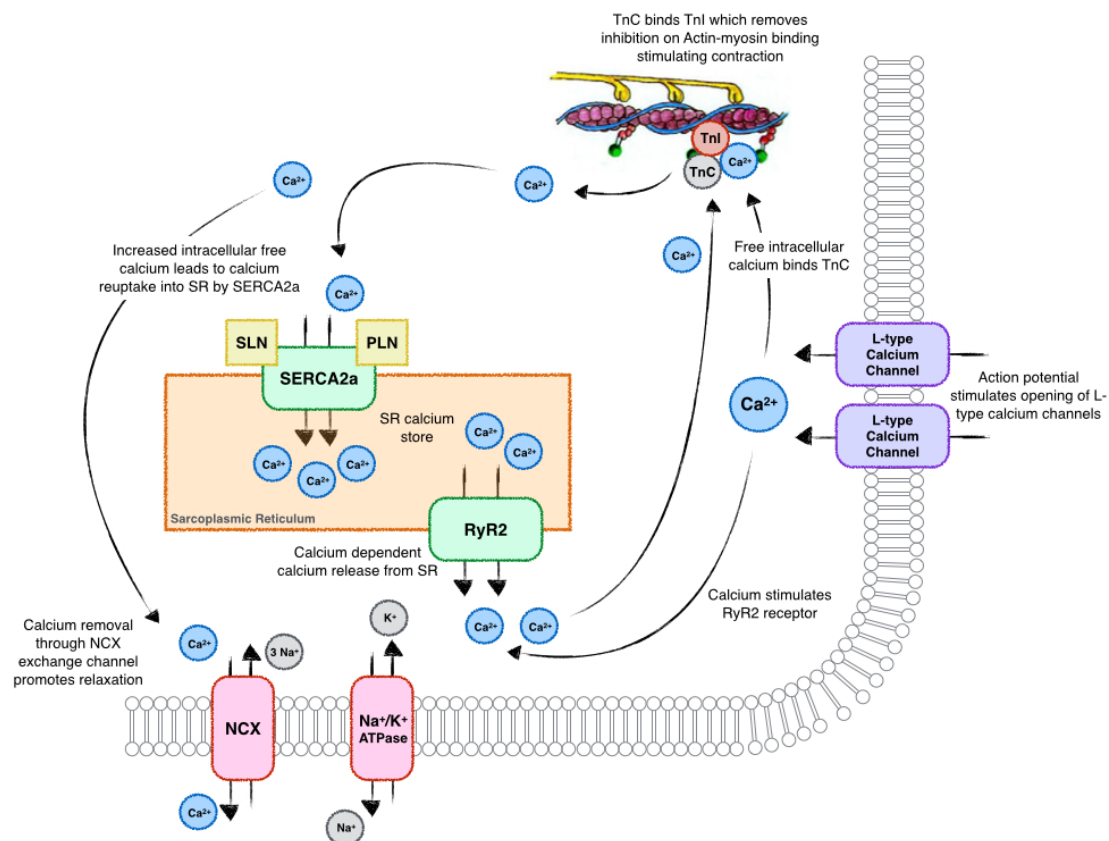


Figure 4-13: A simplified model of cardiomyocyte calcium handling. SERCA2a- sarcoplasmic reticulum Ca^{2+} ATPase; RyR2- ryanodine receptor; TnI- troponin I; TnC- troponin C; SR- sarcoplasmic reticulum; NCX- Sodium/calcium exchange transporter.

Sarcolipin gene expression has been shown to be modulated by a number of factors including: TGF β ,⁽²²⁵⁾ Thyroid hormone,⁽²³¹⁾ and GREM2 (Gremlin 2)⁽²³²⁾. The role of thyroid hormone is of particular interest given the common clinical association between hyperthyroidism and atrial fibrillation. Furthermore, as mentioned previously free-T3 has the ability to increase α -MHC transcription through enhancing binding of the transcription factor GATA4 with the MYH6 gene. In the hyperthyroid state, increased T3 has been shown to produce post-transcriptional inhibition of SLN mRNA thus subsequently increasing SR calcium reuptake through SERCA.⁽²³¹⁾ Notably, thyroid hormone exerts a similar suppressive effect on the other key SERCA inhibitory regulator phospholamban (PLN) further increasing SR calcium reuptake. As such, shortening of action potential duration and

SR calcium overload is likely to be one mechanism whereby hyperthyroidism is associated with tachyarrhythmias and ultimately atrial fibrillation.

Structural and electrical remodeling consistent with an AF phenotype prior to POAF onset

MHC isoform switch to increase β -MHC is a recognised sign of cardiomyocyte dedifferentiation and structural remodeling in chronic lone AF.(21, 233) Along with changes in cell size, glycogen storage, connexin expression and mitochondrial size and shape, this alteration in MHC composition represents a hallmark of cellular reversion to a fetal phenotype.(233) In the setting of chronic lone AF, it has been suggested that this phenomenon of dedifferentiation may occur in response to chronic calcium overload and metabolic stress, which in turn stimulates structural and electrical remodeling.(233) Indeed, β -MHC isoforms have been are less sensitive to intracellular calcium, and require an approximately 50% higher intracellular free calcium concentration to achieve half-maximal tension than α -MHC.(234) Therefore this MHC class switch may dampen the contractile response to intracellular calcium overload and ultimately prevent contractile failure.

In addition, up-regulation of other factors associated with a dedifferentiated fetal atrial phenotype such as the heat shock proteins (HSP70 and GRP94), may form part of a mechanism to prevent depletion of SR Ca^{2+} stores through sequestration of Ca^{2+} within the SR.(235) Similarly, the observed reduction in SLN expression in my POAF patient cohort may also represent an adaptive mechanism to counter increased intracellular calcium and preserve SR calcium stores through removal of inhibition of SERCA2a. Together these changes suggest that POAF may be associated with a basal state of cellular calcium overload prior to the onset of the arrhythmia. Although the exact mechanism for this remains unclear, a number of factors previously associated with AF pathogenesis may be responsible for this increase in intracellular Ca^{2+} including increased oxidative stress, mitochondrial dysfunction

and increased cellular glucose concentration.(236) As such, these stimuli may in fact represent the underlying causative mechanism behind the atrial remodeling observed in this POAF patient cohort.

4.4.3 Limitations

It is important to consider a number of limitations when interpreting the results of this study. Firstly, as previously described the study number remains small and further validation of these findings is required in a larger patient cohort. Second, as this study was not originally designed to examine cellular calcium regulation, although no difference was observed in the expression of the SERCA regulator PLN, these results represent only absolute PLN expression and the phosphorylation state of PLN was not investigated. Phosphorylation of PLN acts to inhibit its binding with SERCA thus removing its inhibitory effect and promoting Ca^{2+} reuptake into the SR. As a result, in order to isolate the effect of SLN down-regulation on calcium transport, future studies must ensure both PLN and phosphorylated-PLN levels are independently determined. Similarly, a restriction in sample availability did not allow for quantification of myosin heavy chain protein or gene expression and therefore relative changes in MYH6 (and likewise MYH7) gene expression must now be validated at the protein level.(218) Third, it is important to consider other regulatory mechanisms that may modulate intracellular calcium such as adrenergic stimulation of protein kinase A (PKA) which may not only lead to phosphorylation of phospholamban but also of L-type calcium channels, which in turn increase calcium cycling and speeding up SR calcium release.(237) Similarly PKA phosphorylation of Troponin I may also act to lower myofilament sensitivity to Ca^{2+} thus reducing the contractile response to intracellular calcium load.(238)

4.5 Conclusions

The results of this study highlight a number of exciting novel mechanisms for both electrical and structural remodeling in the pathogenesis of atrial fibrillation. Firstly, this work provides the first evidence to suggest that a relative class switch in myosin heavy chain protein may occur prior to the onset of atrial fibrillation rather than as a consequence of it, implying a primary role for muscle myosins in the structural remodeling associated with AF pathogenesis. Secondly, this study also provides evidence of pre-existing electrical remodeling in POAF atria, as a consequence of increases in sarcoplasmic reticulum calcium reuptake through a reduction in inhibitory regulation of the sarcoplasmic reticulum Ca^{2+} ATPase. Together the results from this study suggest that electrical and structural changes similar to those observed in chronic AF may also occur in POAF. However, rather than being a consequence of chronic atrial tachyarrhythmia, these results suggest that this remodeling may actually be a response to increased intracellular calcium concentrations occurring secondary to other stimuli such as oxidative stress, mitochondrial dysfunction and cellular hyperglycaemia. As such, POAF atrial remodeling may form part of the adaptive response to both preserve electromechanical function and restore intracellular Ca^{2+} homeostasis.

Future work must now focus on validating the findings of this study in a larger patient cohort. This must ensure the availability of sufficient atrial tissue to perform a detailed evaluation of both MYH6 and MYH7 gene expression along with quantification of α - and β -MHC protein levels. Simultaneous assessment of GATA4, miR-208a, miR-208b and the myoMiR miR-499 may also allow us to better understand the role of microRNA in the regulation and feedback control of MHC class switch in POAF atrial myocardium. Secondly, the role of sarcoplasmic reticulum calcium reuptake in POAF pathogenesis also requires further experimental validation. This requires quantification of not only SERCA and its regulators at both a gene and protein level, but also detailed study of cellular calcium signaling and investigation into calcium balance within cells cultured from the atrial myocardium of POAF patients.

Furthermore, investigation into other key regulators of calcium transport including the ryanodine (RyR2) and sodium calcium exchange transporters, is now also required in order to isolate the impact changes in SLN expression may have on cellular calcium availability *in vivo*.

Chapter 5

Downstream: The metabolic signature

5.1 Background

5.1.1 The metabolic profile of human atrial fibrillation

The results presented in previous chapters have identified a distinct microRNA and gene expression signature in patients developing *de novo* post-operative atrial fibrillation. However, in order to achieve a holistic understanding of AF pathogenesis, it is also pertinent to consider the metabolic processes that may contribute to the initiation and maintenance of this arrhythmia. In their gene expression analysis, Barth et al. demonstrated four of the twelve GO classes dysregulated in chronic AF to be related to upregulation of metabolic functions, in addition to downregulation of fatty acid and glycoprotein metabolism.(190) Furthermore, through increases in expression of genes encoding inner membrane formation, their results also suggest an upregulation of cellular mitochondrial production,(190) supporting the hypothesis that electrical and structural remodeling is coupled with an adaptive metabolic shift in response to the increasing energy demand of chronic AF.

Under normal conditions, energy provision for cardiomyocyte contraction is usually via oxidative phosphorylation of NADH and FADH₂ produced by the TCA cycle.(239) The mainstay of TCA cycle substrate provision arises from the β -oxidation of fatty acids, with glycolysis of glucose contributing between 10 and 40%.(239) The relative proportion of energy provision from glucose is variable and is responsive to extracellular glucose concentrations. Furthermore, glucose uptake is also sensitive to insulin via its control of the GLUT4 transmembrane glucose transporter. Amino acids, ketone bodies and lactate all provide additional means of substrate provision for the TCA cycle, however increases in

energy provision via these mechanisms are usually in response to starvation, high-energy requirement or hypoxia (**Figure 5-1**).

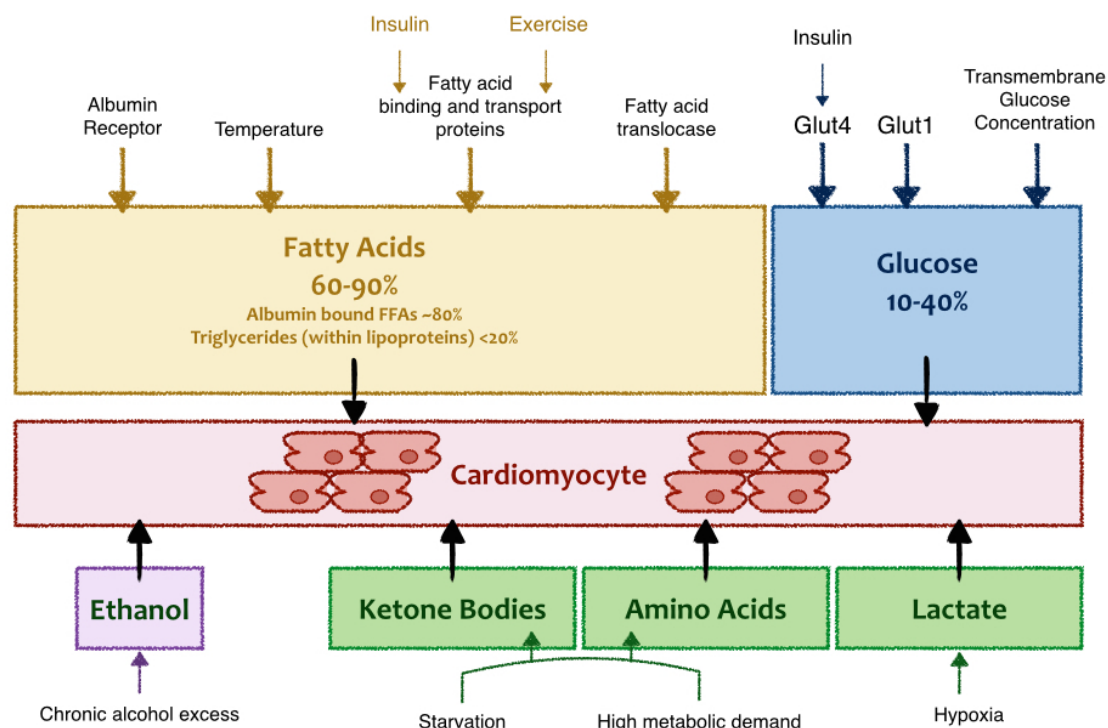


Figure 5-1: A summary of the energy substrates for TCA cycle metabolism in cardiomyocytes under normal conditions.

In chronic AF, results from NMR spectroscopy suggest a change in energy substrate provision so as to accommodate for the increasing energetic demand of the fibrillating myocardium. Significant increases in β -hydroxybutyrate along with the ketogenic amino acids tyrosine, leucine and glycine in the right atria of chronic AF patients suggests an increase in ketone body metabolism.(110) This metabolic shift is further supported by evidence demonstrating increased 3-Oxoacid transferase, an enzyme acting to catalyse ketone body utilisation.(240) The ketone body β -hydroxybutyrate is converted to acetoacetate and subsequently acetoacetyl-CoA, in turn providing a substrate for the TCA cycle upon cleavage into acetyl CoA. This delivers a direct source of acetyl CoA for the TCA cycle and provides approximately 30% greater energy yield per unit than pyruvate.(239) Whilst they usually

contribute only a minor proportion of myocardial energy supply, up-regulation of ketone body metabolism in the setting of AF is in keeping with an adaptive response to cellular energy deprivation, likely as a result of the increasing metabolic demand. This upregulation of ketone body metabolism can also be seen to be coupled with a reduction in glucose metabolism as demonstrated by inhibition of a number of intermediaries in the glycolysis pathway (Figure 5-2).

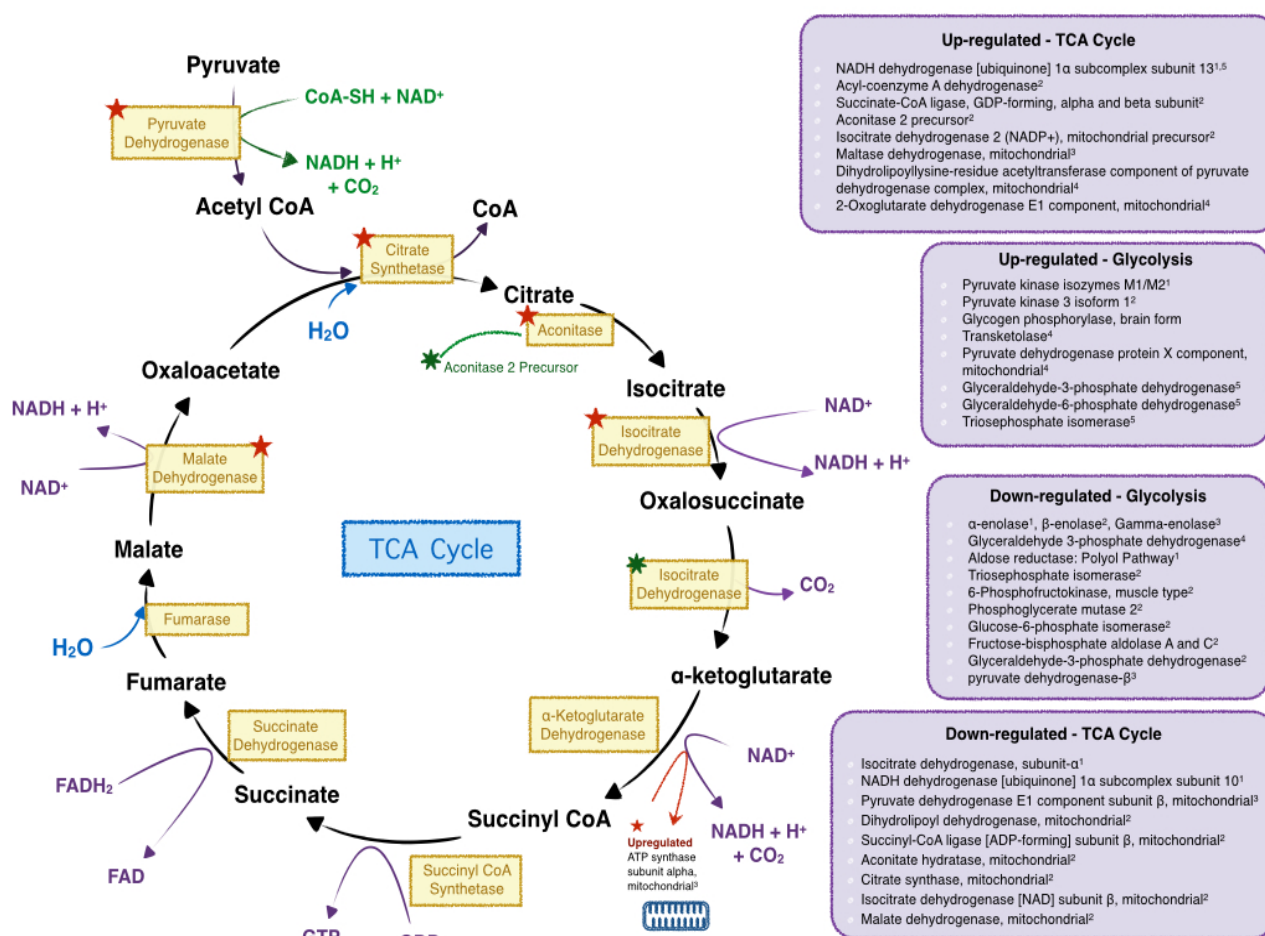


Figure 5-2: A summary of the metabolite changes associated with atrial fibrillation involved in glycolysis and the tricarboxylic acid (TCA) cycle. This includes studies of human atrium (right and left) along with both chronic and post-operative atrial fibrillation. Pooled results from 5 studies are given. References: ¹Huang et al.(241); ²Tu et al.(242); ³Kourliouros et al.(243); ⁴Gourdarzi et al.(240); ⁵Mayr et al.(110). Red star indicates upregulation; Green star indicates downregulation.

There is also evidence to suggest that post-operative AF is associated with a state of energy depletion and inhibition of glycolysis.(243) In their study of 20 patients (10 SR; 10 POAF) undergoing CABG surgery, Kourliouros et al. demonstrated a reduction in both pyruvate dehydrogenase- β and enolase activity signifying a fall in glucose metabolism.(243) These findings suggest that changes in energy provision precede, and may in fact give rise to the onset of arrhythmia in post-operative AF. As a result of energy depletion through reduced glucose utilisation, the cardiomyocyte may become more susceptible to triggers such as inflammation and oxidative stress, leading to degradation and fibrosis.(243) In turn, this may result in conduction block and ion channel dysregulation, allowing for both the triggering and maintenance of reentrant circuits.

Similar to chronic AF, post-operative AF has also been associated with an upregulation of ketone body metabolism as evidenced by increases in β -hydroxybutyrate. Furthermore, this appears to be coupled to decreases in the availability of glucose and inhibition of glycolysis pathways.(110) Indeed, it is important to consider that whilst these processes may occur simultaneously through different mechanisms, upregulation of one pathway may in turn directly inhibit another. Increases in the concentration of acetyl CoA produced via ketone body metabolism may inhibit pyruvate dehydrogenase and reduce conversion of pyruvate to acetyl CoA.(110) As such, these metabolite changes should be reviewed together as a global shift in cardiomyocyte energy provision rather than as an isolated upward or downward shift of a single pathway.

In addition to down-regulation of glycolysis, post-operative AF has also been found to be associated with a fall in NADPH-dependent conversion of glucose to sorbitol as demonstrated by a reduction in the expression of aldose reductase,(243) the enzyme catalysing the first step in polyol pathway of glucose metabolism. Conversely however, alternative energy sources such as lipid metabolism show signs of up-regulation as evidenced by increases in the high-density lipoprotein Apolipoprotein A-I.(243)

5.1.2 Lipid metabolism

In addition to changes in glucose and ketone body metabolism, atrial fibrillation is also associated with dysregulation of a number of enzymes and metabolites involved in lipid metabolism (**figure 5-3**). Myocardial energy provision through lipids is mainly via albumin bound fatty acids with a smaller proportion from blood lipoprotein triacylglycerols (**Figure 5-1**). Albumin bound fatty acids are taken up into the cardiomyocyte either through binding with the albumin receptor or via the fatty acid transmembrane transport system, which subsequently allow FA to be released directly or indirectly via the cell membrane into the cytoplasm.(239) Fatty acids are then transformed to long-chain acyl-CoA by acyl-CoA synthase on the mitochondrial membrane before uptake into the mitochondrion via the carnitine palmitoyltransferase system, which is regulated by malonyl-CoA. Once inside the mitochondrion, acyl-CoA enters β -oxidation.(239)

Chronic atrial fibrillation has been associated with decreases in the expression of fatty acid binding proteins and fatty acid synthase suggesting a decrease in fatty acid uptake.(242) Furthermore, decreases in mitochondrial very-long-chain (VLC) specific acyl-CoA dehydrogenase and the auxiliary enzyme $\delta(3,5)$ - $\delta(2,4)$ -dienoyl-CoA isomerase are likely to limit FA β -oxidation,(241, 242) also reducing the utilisation of those FA that are available for energy provision. Conversely however, the final ability for FA to be utilised for energy provision appeared to be upregulated in the right atria of patients with chronic AF undergoing maze procedures, through increased expression of Acetyl-CoA acyltransferase 2 (ACAA2), the mitochondrial enzyme catalyzing the final step in fatty acid oxidation to release acetyl CoA.(240) Similarly, other intermediaries of β -oxidation were also increased in this patient group including 2,4-Dienoyl CoA reductase-1 precursor, an enzyme that catalyzes the NADP-dependent reduction of 2,4-dienoyl-CoA to yield trans-3-enoyl-CoA.

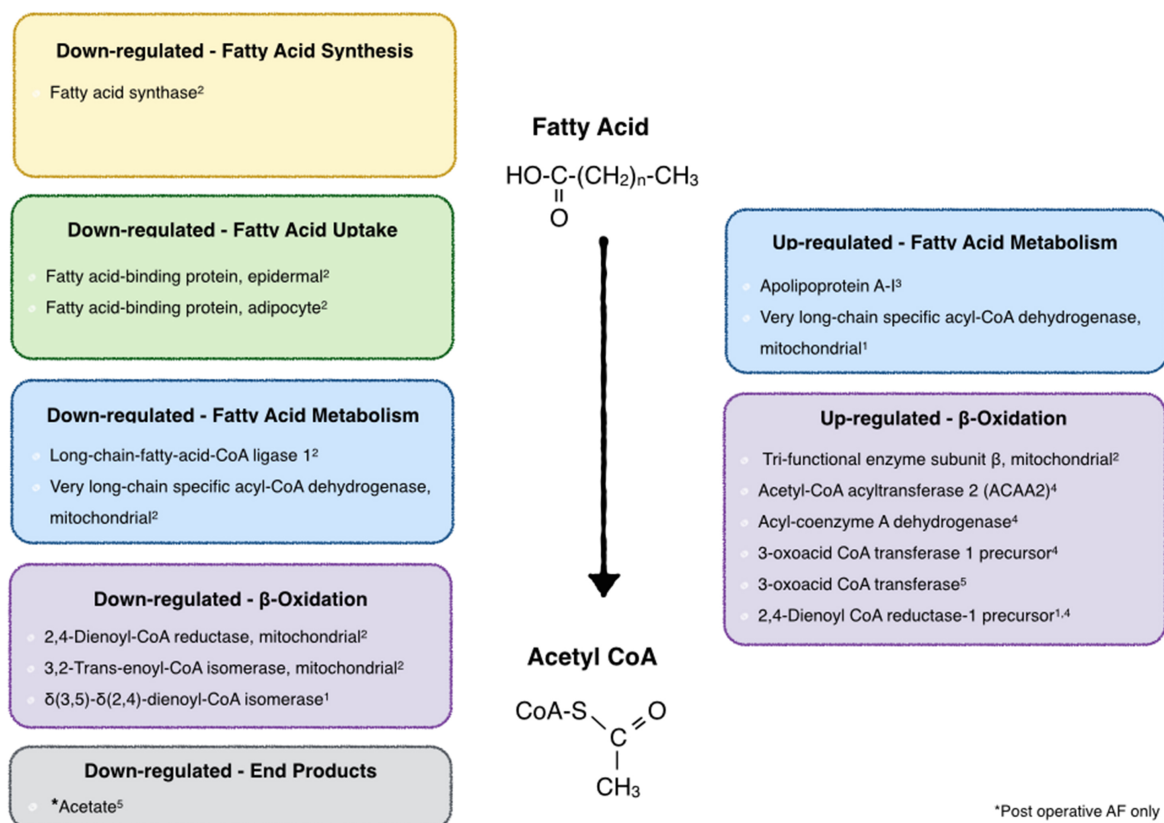


Figure 5-3: A summary of the lipid metabolite changes associated with Atrial Fibrillation. This includes studies of human atrium (right and left) along with both chronic and post-operative atrial fibrillation. Pooled results from 5 studies are given. References: ¹Huang et al.(241); ²Tu et al.(242); ³Kourliouros et al.(243); ⁴Gourdarzi et al.(240); ⁵Mayr et al.(110)

Interestingly, in their subgroup of patients developing *de novo* post-operative AF, Mayer et al.(110) observed decreased atrial levels of acetate when compared to those maintaining sinus rhythm. A fall in this end product of lipid metabolism may suggest overall down-regulation of lipid as an energy substrate; however, this finding was also coupled with even more significant decreases in glucose and the end products of its metabolism alanine and lactate. As a result, despite global decreases in both fatty acid and glucose metabolism, a fall in the ratio of glucose end products to lipid end products showed good correlation with the development of POAF ($r=0.66$), and in fact was able to correctly classify those patients going on to develop POAF in approximately 80% of cases.(110) These findings therefore suggest that fundamental metabolite changes not only predate the onset of AF and but play a key role in

its pathogenesis. Whilst it is likely that this is primarily a result of impaired glucose metabolism, the role of fatty acids remains significant and may indeed play a part in the progression to more sustained arrhythmia.

5.1.3 Aims

The aim of this chapter is to determine the metabolic signature of post-operative AF in my patient cohort. Utilising nuclear magnetic resonance (NMR) and mass spectrometry (MS) techniques, the results of metabonomic analysis will be compared to the genomic profile outlined in chapters 3 and 4 with the goal of establishing the relationship between gene expression and metabolite response. Finally, by combining the results from epicardial adipose (EAT) and myocardial tissues, this work also aims to characterise the metabolic interplay between these two tissue types.

5.2 Methods

5.2.1 Sample Preparation: Lipid metabolite extraction for Mass Spectrometry

Metabolites were extracted from human epicardial fat using a two-phase tissue extract protocol developed on site (Dr. Panagiotis Vorkis).

Aqueous extraction. Tissue samples were homogenised in pre-chilled methanol/water solution (1:1) (methanol HPLC gradient grade, Fisher; water LC-MS grade, Fluka). Beginning with a maximum of 1.5mL, and reducing proportionally to sample weight, the volume of solution was corrected to the weight of the sample. Samples (frozen on dry ice) were loaded onto a bead beater (zirconium beads; Bertin Technologies) vibrating at 6500Hz, for 40 seconds. After 2 cycles the samples were re-frozen on dry ice before a further 2 cycles were performed. Samples were centrifuged (Eppendorf, Centrifuge 5417R, Germany) at 13 000 rcf for 20 minutes at 4°C. 250µL of supernatant was aliquoted into Eppendorf tubes. Samples were spun on a vacuum concentrator for 3 hours at 45°C (Eppendorf Concentrator Plus, V-AQ mode) and then stored at -40°C until analysis.

Organic extraction. A solution of pre-chilled dichloromethane (MTBE/methanol (3:1) (methanol HPLC gradient grade, Fisher; MTBE LCMS grade, Sigma-Aldrich) was added to the supernatant from centrifuged aqueous samples, The volume of the solution added was proportional to the sample weight as described above. Samples were frozen on dry ice and re-loaded into the bead beater for 2 cycles of 40 seconds at 6500Hz. Following this, samples were centrifuged at 13,000 rcf for 20 minutes, and the organic phase (supernatant) aliquoted into glass vials. Samples were allowed to evaporate at room temperature in a fume hood overnight and then stored at -40°C for analysis.

5.2.2 Sample Processing: Magic Angle Spinning (MAS) Nuclear Magnetic Resonance (NMR) Spectroscopy

An aliquot of 20-40mg of atrial tissue sample taken as previously described was prepared for high resolution ^1H Magic Angle Spinning (MAS) NMR spectroscopy. Samples were packed into a zirconium oxide rotor (Bruker Analytische GmbH; Rheinstetten, Germany) measuring 4mm diameter. Saline D_2O was used as a field lock and ^1H NMR spectra were acquired on the 600 MHz Bruker DRX spectrometer (Rheinstetten, Germany) operating at 600.13MHz using a triple resonance MAS probe. The temperature was maintained at 283 K using dry ice and spin rate regulated at 5000Hz. Three ^1H spectra were acquired for each sample using a 1-D standard, Carr-Purcell-Meiboom-Gill (CPMG) and diffusion edited pulse sequences. A spin relaxation delay ($2\pi\tau$) of 200ms was used for the CPMG experiment. The 90-degree pulse length was set to 10 μs . The spectral width was 20ppm, and 128 transients were collected into 32k data points. An exponential function was applied to the FID prior to Fourier transformation with a line-broadening factor of 0.3Hz. 2-D ^1H - ^1H COSY (COrelated SpectroscopY) and ^1H - ^1H TOCSY (TOtal Correlated SpectroscopY) NMR spectra were acquired for all samples for structural assessment of metabolites. A total of 80 transients and 256 increments were collected into 2k data points with a spectral width of 10ppm. The ^1H - ^1H TOCSY NMR spectra were acquired using a MLEV-17 sequence for the spin lock with a spin-lock power of 6kHz. 2-D ^1H - ^1H COSY and ^1H - ^1H TOCSY NMR spectra were multiplied by an unshifted sine bell and shifted sine bell apodisation function respectively prior to Fourier transform.

5.2.3 Sample Processing: UPLC-MS of Lipid metabolite extracts

Hydrophilic interaction ultra performance liquid chromatography (HILIC-UPLC-MS) analysis of Aqueous Extracts

Samples were reconstituted in 200µL of solvent mixture of acetonitrile/water (95:5) (LC-MS grade, Fisher Scientific, USA), centrifuged for 20min at 13000 rcf, 4°C and then transferred into total recovery vials (Waters, USA).

UPLC (Ultra-Performance Liquid Chromatography) separation was conducted with the Acquity UPLC System (Waters Corp, USA). An Acquity UPLC BEH HILIC 2.1x100mm, 1.8µm, column (Waters Corp, USA) was used. The column temperature was set at 35°C, and a 10µL injection volume was used for both positive and negative ionization polarity modes. The auto-sampler was set at 4°C. Mobile phase A consisted of acetonitrile/water (95:5) and mobile phase B acetonitrile/water (50:50). Ammonium formate (98%, Fluka, USA) was diluted to 10mM, and formic acid (MS grade, Fluka, USA) diluted to 0.1% in both solutions. A summary of the chromatographic gradient program is shown in **Table 5-1**.

Eluting UPLC fractions were detected using a Synapt G2S (Waters MS Technologies Ltd., UK). A scan time of 0.20 sec and interscan time of 0.02 sec were used for acquisition. Leucine enkephalin was used for lock mass correction. Lock mass data were collected with scan time of 0.20 sec and scan frequency of 30 sec. Positive mode conditions were as follows: m/z range: 50-1200, cone voltage 30V, capillary voltage 3KV, Source Temperature 120°C, Desolvation Temperature 400°C, Desolvation Gas Flow 800L/Hr. Negative mode conditions were as follows: m/z range: 50-1200, cone voltage 30V, capillary voltage 2.5KV, Source Temperature 120°C, Desolvation Temperature 350°C, Desolvation Gas 800L/Hr.

UPLC-MS analysis was performed using a Quality Control (QC) format. This involved preparation of a pooled QC sample of the reconstituted extracts. This sample was then injected

10 times to condition the column before beginning the run. Once the run had begun, the sample was re-injected 7 times.

Time (min)	Flow rate (ml/min)	%A	%B	Curve
HILIC-UPLC-MS method				
0.0	0.4	99	1	-
2.0	0.4	99	1	-
8.0	0.4	45	55	6
9.0	0.4	1	99	6
9.1	0.8	1	99	6
11.0	0.8	1	99	-
11.1	0.8	99	1	6
19.0	0.8	99	1	-
19.1	0.4	99	1	6
23.0	0.4	99	1	-
Lipid profiling RP-UPLC-MS				
0.0	0.4	60	40	-
2.0	0.4	57	43	6
2.1	0.4	50	50	1
12.0	0.4	46	54	6
12.1	0.4	30	70	1
18.0	0.4	1	99	6
18.1	0.4	60	40	6
20.0	0.4	60	40	-

Table 5-1: Gradient programs of chromatography of the HILIC-UPLC-MS and the lipid profiling UPLC-MS methods.

Data processing

Data were processed using XCMS (<http://metlin.scripps.edu/xcms/>). Analysis incorporated peak-picking, grouping, and retention time correction. Results were considered statistically significant at a p value of less than 0.05 and a relative fold change above 2. Features were accepted where the co-efficient of variation of the control was less than 30%, indicating that the feature was reproducible throughout the run.

Data was uploaded to MATLAB (Version R2014a) for further processing. Normalisation was performed using median fold change and peak positions were adjusted by recursive segment wise peak alignment.(244) Large variability of high intensity peaks was compensated for across samples by log-based transformation of mass spectra. This also ensured a consistent noise structure for application of multivariate statistical techniques.(245)

Unsupervised analysis of the dataset was performed using principal component analysis (PCA). In addition, a recursive maximum margin criterion algorithm (RMMC) and linear discriminant analysis (LDA) were used for supervised classification.

Lipid Profiling

UPLC-MS analysis. Samples were reconstituted in 200 μ L of a solvent mixture consisting of H₂O/isopropanol/acetonitrile (1:2:1) (Optima, LC-MS grade, Fisher Scientific, USA), and centrifuged for 20 min at 14 000 rcf, 4°C transferred into Total Recovery vials (Waters, USA).

UPLC separation was conducted using an Acquity UPLC System (Waters Corp, USA). An Acquity UPLC CSH C18 2.1x100mm, 1.7 μ m, column (Waters Corp, USA) was used. Column temperature was set at 35°C. A 3 μ L injection volume was used for positive ion mode and a 7 μ L injection volume used for negative ionization mode. The auto-sampler was set at 4°C. Mobile phase A consisted of acetonitrile/water (60:40) and mobile phase B Isopropanol/acetonitrile (90:10). Ammonium formate (98%, Fluka, USA) was diluted to 10mM, and formic acid (MS 155

grade, Fluka, USA) diluted to 0.1% in both solutions. A summary of the chromatographic gradient program is shown in **Table 5-1**.

Eluting UPLC fractions were detected using a Premier Q-TOF (Waters MS Technologies, UK). A scan time of 0.200 sec and interscan time of 0.214 seconds were used for both MS and MS^E data acquisition. MS^E data were collected after ramping of the collision energy from 30 to 40V. Leucine enkephalin was used for lock mass correction. Lock mass data were collected with scan time of 0.20 sec and scan frequency of 30 sec. Positive mode conditions were as follows: m/z range:150-1200, cone voltage 30V, capillary voltage 2KV, Source Temperature 120°C, Desolvation Temperature 550°C, Desolvation Gas 900L/Hr. Negative mode conditions were as follows: m/z range: 50-1200, cone voltage 30V, capillary voltage 1KV, Source Temperature 120°C, Desolvation Temperature 550°C, Desolvation Gas 900L/Hr.

UPLC-MS analysis was performed using a Quality Control (QC) format as described for HILIC-UPLC-MS above.

Data processing

Data processing was performed as described for HILIC-UPLC-MS above. Results were considered statistically significant at a p value of less than 0.05 and a relative fold change above 2. Features were accepted where the co-efficient of variation of the control was less than 30%, indicating that the feature was reproducible throughout the run.

Data was uploaded to MATLAB (Version R2014a) for further processing. Normalisation, supervised and unsupervised analysis was performed as described for HILIC-UPLC-MS above.

5.3 Results

5.3.1 Magic Angle Spinning Nuclear Magnetic Resonance Spectroscopy

Standard proton spectra were acquired to determine the global profile of metabolites across the sample set (**figure 5-6**). 1D standard proton NMR spectra were also obtained from atrial myocardial tissue. The spectral data revealed dominant peaks at 0.83, 0.87, 1.22, 1.57, 2.0, 2.22 and 5.3 ppm, corresponding to lipid fractions (**table 5-2**), which, due to their high concentrations, prevented further analysis of other metabolites.

Lipid Fraction	Chemical Shift (ppm)
$\text{CH}_2\text{C}=\text{C}$	2.0
$\text{CH}_2\text{C}=\text{O}$	2.22
$\text{C}=\text{CCH}_2\text{C}=\text{C}$	2.75
$\text{CH}=\text{CH}$	5.30
$\text{CH}_3(\text{CH}_2)_n$	0.83
$(\text{CH}_2)_n$	1.22
$\text{CH}_3\text{CH}_2\text{CH}_2\text{C}$	0.87
$\text{CH}_2\text{CH}_2\text{CO}$	1.57

Table 5-2: Chemical shifts corresponding to different lipid fractions identifiable in standard proton mass spectra.

CPMG pulse sequence was used to filter out the signals from macromolecules. OPLS-DA coefficients plot derived from 1H MAS CPMG spectra of atrial myocardial tissue did not reveal any significant metabolite differences between POAF vs. non-POAF patients (A, B ($Q^2Y < 0$)) (**Figure 5-4**). Similarly, combined CPMG spectra did not reveal any significant metabolic discrimination between the groups (**Figure 5-5**; Blue=POAF (n=10); red=non-POAF (n=16)).

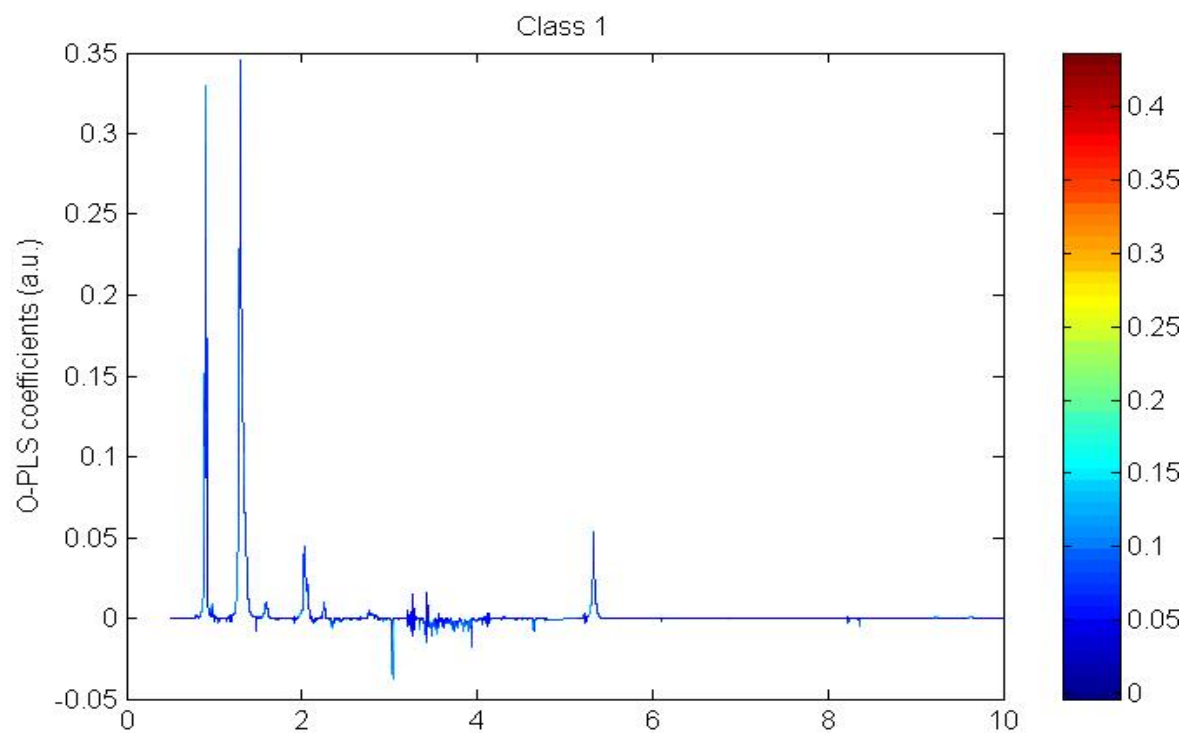


Figure 5-4: OPLS-DA loadings plot derived from ^1H MAS CPMG spectra of atrial tissue biopsies. The predictability $Q^2_Y < 0$.

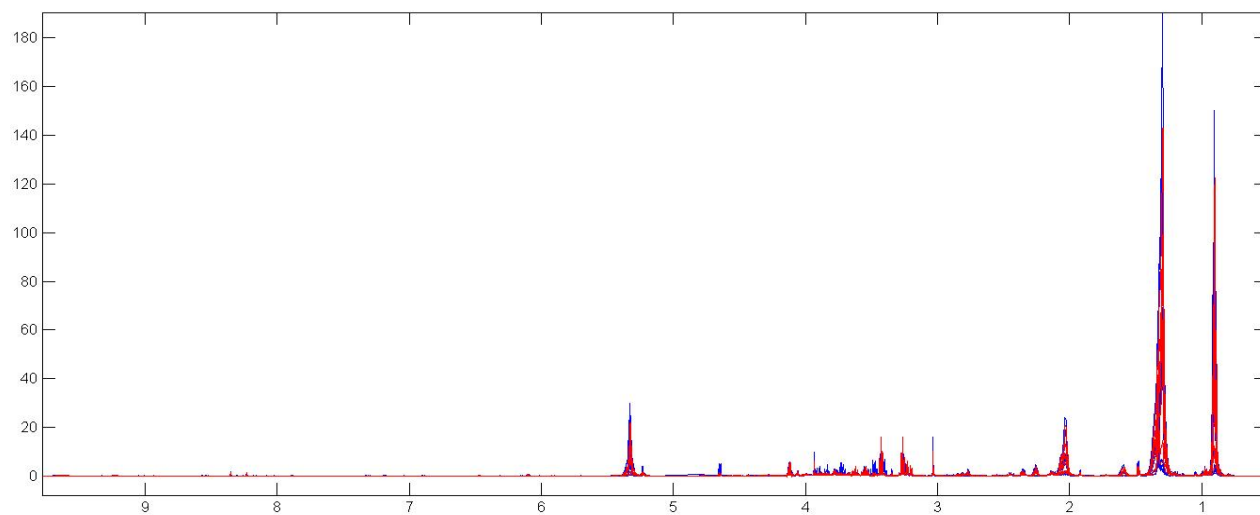


Figure 5-5: Combined CPMG spectra obtained from the atrial tissue of AF and non-AF patients. (Blue - POAF (n=10); red - non-POAF (n=16)).

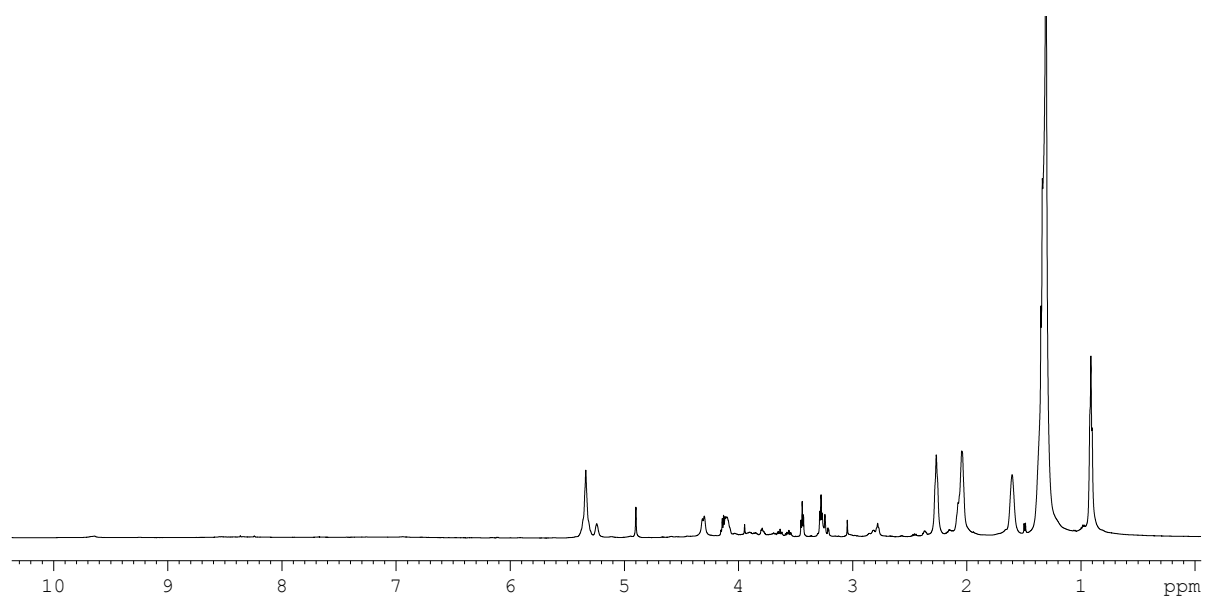


Figure 5-6: 1D standard proton NMR spectra of heart tissue from an AF patient. Dominant peaks at 1-2 ppm represent high fat content of atrial tissue samples which precluded further metabolic analysis.

5.3.2 UPLC-MS of Lipid metabolite extracts

In light of the close anatomical relationship between atrial myocardium and epicardial adipose and the high intensity peaks corresponding to lipid fractions within standard proton spectra derived from the atrial myocardium, further analysis focused on determining the metabolite profiles of epicardial adipose between POAF and non-POAF groups.

UPLC-MS revealed a significant difference in 10 species in positive ion mode and 2 species in negative ion mode between POAF and AF groups. In addition, HILIC UPLC-MS analysis revealed a significant difference in 21 species in positive ion mode and 6 species in negative ion mode between POAF and AF groups. Using published data, a correlation heatmap was drawn between these metabolites in order to determine any clusters of metabolites with high correlation (**Figure 5-7**).

Preliminary metabolite annotation was then performed according to mass using the METLIN metabolite database (http://metlin.scripps.edu/metabo_search_alt2.php). Positive and negative mode IDs were searched using a tolerance of ± 30 ppm and charge adducts of M+H/M+Na and M-H/M+Cl respectively. Of the 39 metabolites identified, 11 were later excluded where either no identification could be found or closer analysis revealed them to be artifact. A further 6 metabolites were also excluded due to very low peak intensities. In total 22 metabolites were positively identified and included in further analyses (**Table 5-3**).

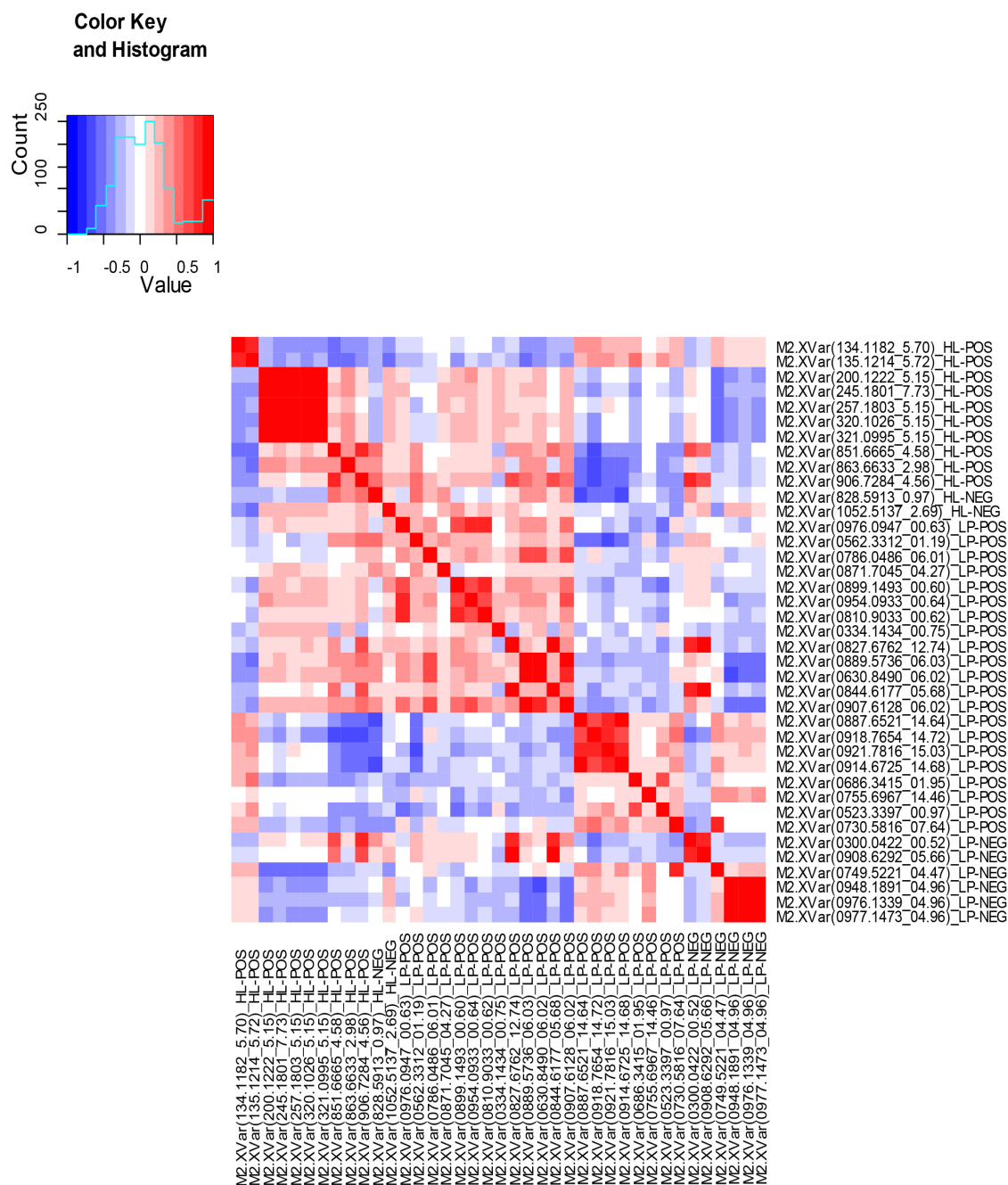


Figure 5-7: Heatmap of correlation matrix between the 39 metabolites differentially expressed between POAF and non-POAF patients.

Lipid Subtype	Lipid ID	Mass	Mean POAF	Mean SR	p	Direction
Glycerophosphoglycerols	PG(20:0/22:0)	863.6633	12.96	13.8	0.045585*	Decrease
	PG(22:0/22:1(11Z))	906.7284	12.37	13.37	0.019886*	Decrease
Glycerophosphoserine	PS(22:0/17:2(9Z,12Z))	828.5913	10.93	11.66	0.070132	Decrease
	PS(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/22:1(11Z))	907.6128	7.105	7.236	0.045585*	Decrease
	PS(20:3(8Z,11Z,14Z)/22:0)	887.6521	7.637	7.236	0.028715*	Increase
	PS(22:0/20:1(11Z))	908.6292	7.412	7.83	0.1384	Decrease
Glycerophosphoinositols	PI(17:0/19:0)	889.5736	8.579	9.022	0.045585*	Decrease
Triacylglycerols	TG(14:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z)/22:3(10Z,13Z,16Z))[iso6]	918.7654	9.237	8.677	0.036323*	Increase
	TG(16:0/17:1(9Z)/22:2(13Z,16Z))[iso6]	921.7816	10.91	10.37	0.045585*	Increase
Sterols	18:0-Glc-Campesterol	851.6665	14.02	15.18	0.0090346*	Decrease
Glycerophosphoethanolamines	PE(22:2(13Z,16Z)/22:1(13Z))	871.7045	7.207	7.724	0.15136	Decrease
	PE(O-20:0/22:4(7Z,10Z,13Z,16Z))	827.676	7.557	8.088	0.07773	Decrease
Sphingolipids	PE-Cer(d16:2(4E,6E)/22:1(13Z))	730.5816	7.917	7.455	0.032328*	Increase
Phosphatidylcholine	PC(22:4(7Z,10Z,13Z,16Z)/P-18:0)	844.6177	7.379	7.791	0.1384	Increase
	PC(24:0/20:5(5Z,8Z,11Z,14Z,17Z))	914.6725	7.236	6.956	0.45585	Increase
Glycerophosphates	PA(P-16:0/22:1(11Z))	749.5221	7.454	6.872	0.045585*	Increase
Long-chain Molecules	3-Isopropenylpimelyl-CoA	948.1891	6.985	6.591	0.077567	Increase
	(3S)-Citryl-CoA	976.1339	6.857	6.597	0.085941	Increase
Peptides	Lys Cys Lys Lys	523.3397	6.806	6.669	0.010356*	Increase
	Ile Arg Glu Gln	562.3312	6.888	7.169	0.032328*	Decrease
	Arg Trp Arg Phe	686.3415	6.806	6.653	0.0032819*	Increase
	Ser Cys Gly	300.0422	6.737	6.899	0.1384	Decrease

Table 5-3: Identification of 22 metabolites identified to be significantly differentially expressed in the epicardial adipose tissue of AF patients ($p < 0.05$ and $FC > 2$). Mean intensities POAF and sinus rhythm (SR) groups and results of unsupervised model (p values) are also shown.

Unsupervised principal component analysis (PCA) revealed visible clustering of POAF and sinus rhythm groups (**Figure 5-8**). Linear discriminant analysis (LDA) supervised classification revealed similar clustering (**Figure 5-9**) however in this more stringent model only 12 of the 22 metabolites previously identified demonstrated statistical significance (**Table 5-3**).

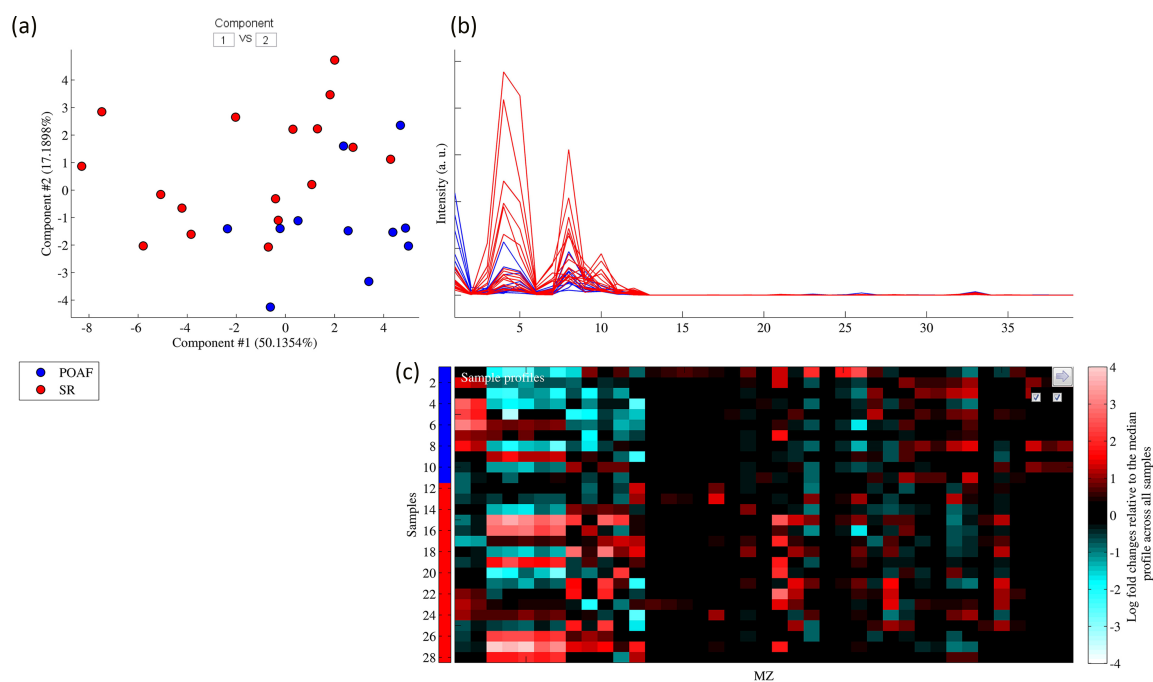


Figure 5-8: Unsupervised model: (a) PCA plot demonstrating clustering of POAF and non-POAF (sinus rhythm (SR)) groups. (b) Combined mass spectra of all samples (blue-POAF, red-SR); (c) heat map of $\log_{10}(\text{intensities})$ of each metabolite in POAF and SR groups.

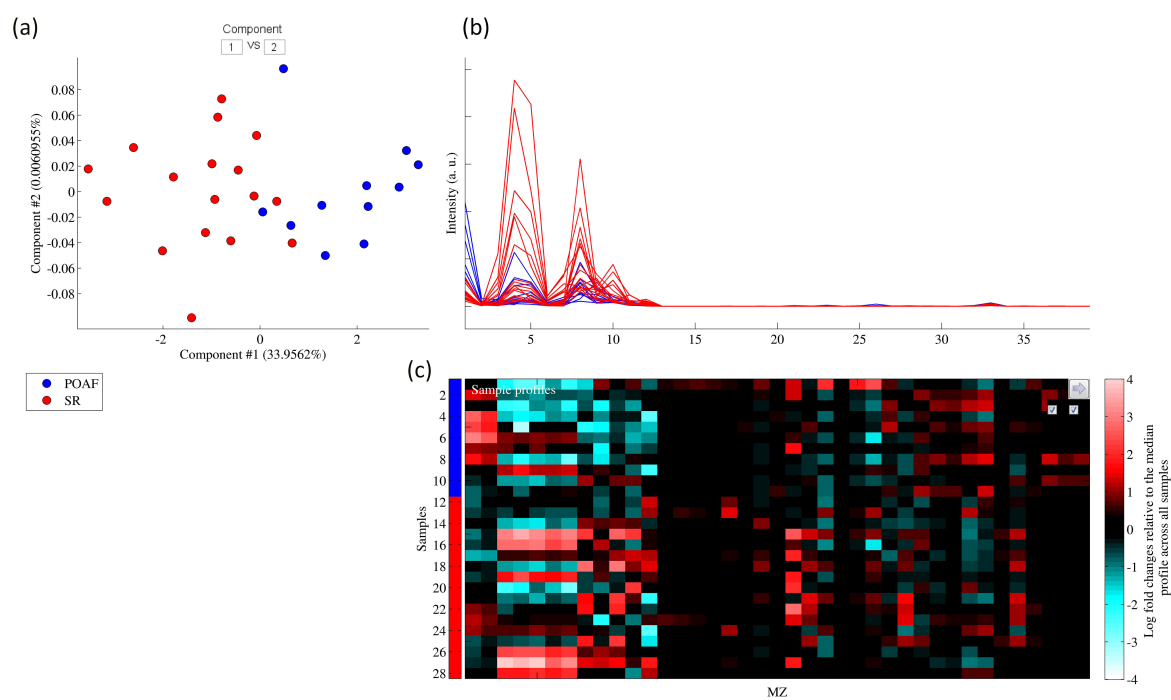


Figure 5-9: Supervised linear discriminant analysis: (a) PCA plot demonstrating clustering of POAF and non-POAF (sinus rhythm (SR)) groups. (b) Combined mass spectra of all samples (blue-POAF, red-SR); (c) heat map of $\log_{10}(\text{intensities})$ of each metabolite in POAF and SR groups.

Finally, leave one out cross validation analysis revealed a 78.6% overall predictability of the model to determine the class of a sample given its metabolite profile. Notably, predictability of the sinus rhythm class (88.2%) was greater than that for the POAF class (63.6%) most likely as a result of (a) the larger variance of POAF samples and (b) the larger number of samples in the SR group.

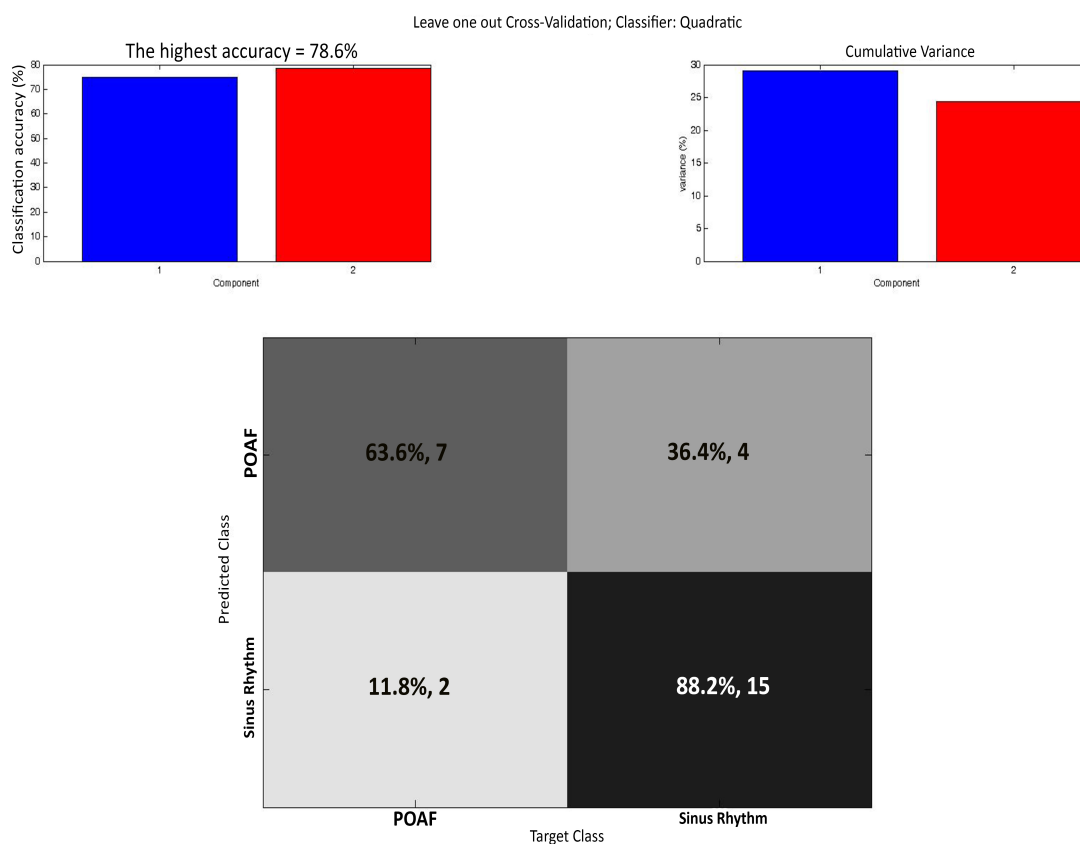
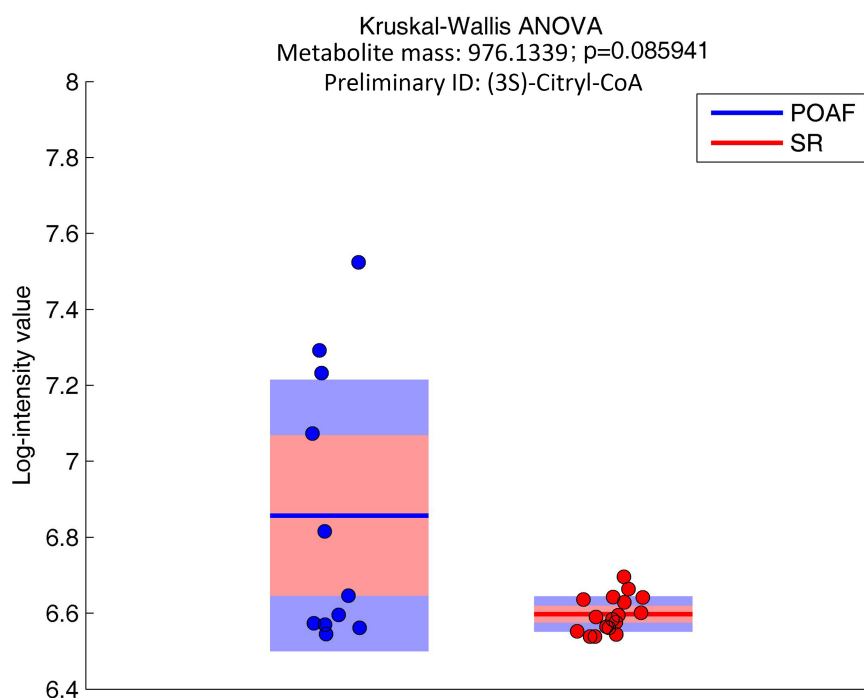


Figure 5-10: Leave one out cross validation analysis demonstrating an overall 78.6% ability of the model to correctly predict the class of the sample based upon its metabolite profile.

Of note, review of box plots for metabolites likely corresponding to the long chain molecules 3-Isopropenylpimelyl-CoA and (3S)-Citryl-CoA revealed two clear clusters within the POAF group with very tight clustering of the SR group (**Figure 5-11**).

(a)



(b)

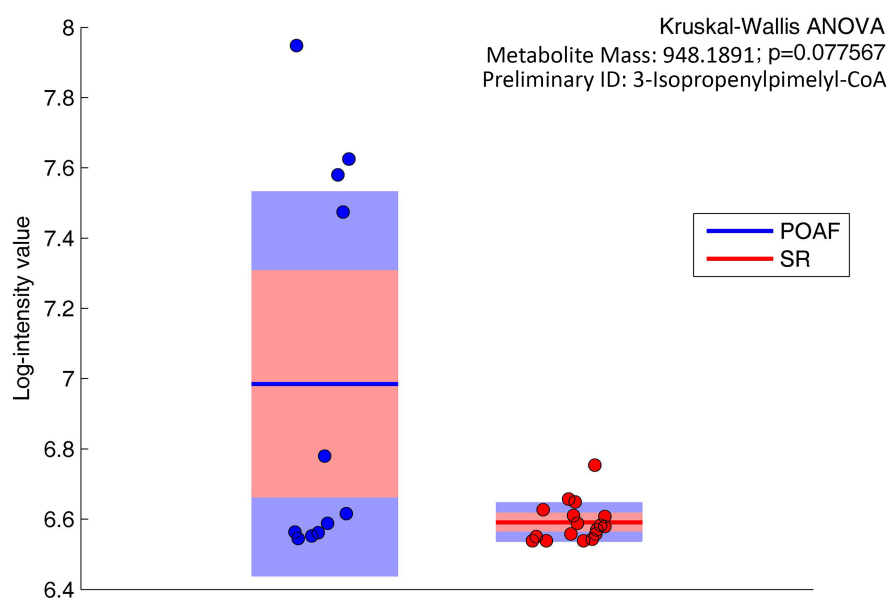


Figure 5-11: Box plots of specific long chain metabolites identified as: (a) (3S)-Citryl-CoA; and (b) 3-Isopropenylpimelyl-CoA; demonstrating two clear clusters within the POAF group.

5.4 Discussion

This chapter has established the metabolic signature of both atrial myocardium and epicardial adipose tissue in patients developing AF after coronary artery bypass grafting. Whilst MAS NMR metabolomic analysis was not able to discriminate POAF from non-POAF in the atrial tissue of my patient cohort, patients could be correctly classified in 78.6% of cases based on the metabolic profile obtained from analysis of their epicardial adipose tissue.

5.4.1 The epicardial lipidome of post-operative AF

These results demonstrated significant decreases in glycerophosphoglycerols, glycerophosphoinositols, 18:0-Glc-campesterol, the peptide Isoleucine-Arginine-Glutamate-Glutamine and the glycerophosphoserine PS(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/22:1(11Z)). Significant increases were seen in triacylglycerols, sphingolipids, glycerophosphates, the glycerophosphoserine PS(20:3(8Z,11Z,14Z)/22:0), and the peptides Lysine-Cysteine-Lysine-Lysine and Arginine-Tryptophan-Arginine-Phenylalanine. Notably two long chain molecules, 3-Isopropenylpimelyl-CoA and (3S)-Citryl-CoA, were also increased in the POAF group but split into two distinct subgroups, one corresponding to that of SR patients and another showing a much greater increase in these two molecules (**figure 5-11**). Although no clear reason for this was evident from further analysis of individual patient data, this finding demonstrates the spectrum of metabolic derangement in POAF, with evidence of a shift towards an 'AF phenotype'.

The condensation reaction of acetyl-CoA and oxaloacetate to form citrate within the TCA cycle is catalysed by the enzyme citrate synthase (**figure 5-2**). In fact, this occurs as part of a 2-step reaction first characterised by the formation of (3s)-citryl-CoA, which is subsequently hydrolysed to citrate and CoA. Through the production of citrate, citrate synthase acts to

restock the cytoplasmic pool of acetyl-CoA for lipogenic pathways during surplus energy availability. Similarly, a fall in citrate synthase activity is observed in situations of increased energy requirement and energy depletion.(246) The increase in (3s)-citryl-CoA observed in the epicardial fat of my POAF patient cohort would correlate with an increase in citrate production in these patients. This mitochondrial-derived citrate can then subsequently be exported into the cytoplasm and converted to Acetyl-CoA for fatty acid synthesis. Interestingly, this finding is mirrored in the right atrial myocardium of a porcine model of AF presented by Lai et al. whereby citrate synthase gene expression was increased 2.5 fold.(247)

Campesterol is a plant-derived phytosterol that is not synthesised in human cells but is naturally present in the diet. Its structure is similar to that of cholesterol and increased circulating levels are thought to have a cholesterol lowering effect through competitive binding with cholesterol receptors.(248) As such, moderate increases in campesterol are thought to lower cardiovascular risk through reducing cholesterol absorption and plaque burden. Furthermore, campesterol may also act to increase vascular smooth muscle prostacyclin synthesis and subsequently also have a protective effect on the vascular endothelium.(249, 250) It is therefore possible that the significantly reduced campesterol in my POAF group may signify increased cholesterol storage within the epicardial adipocyte for energy provision.(251) Similarly however, a fall in campesterol stored within the EAT may also be coupled with a fall in adipocyte and/or the coronary vascular endothelial cell prostacyclin synthesis. Reduced prostacyclin may in turn remove its protective effect and promote vascular endothelial cell dysfunction, which may in turn increase susceptibility to oxidative stress and inflammation thus predisposing to atrial fibrillation.

Adipocytes act as a storage pool for plasma triglycerides (TG), which form a fundamental energy reserve in times of fasting or increased metabolic demand. Under these conditions, stored TG may be hydrolysed to release glycerol and free fatty acids (FFA) that can in turn be taken up by the cardiomyocyte.(252) UPLC-MS analysis revealed a significant increase in

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both triglyceride (triacylglycerol) classes differentially observed in POAF compared to non-POAF patients (**table 5-2**), suggesting either increased storage or decreased metabolism of TG in the EAT of these patients. Whilst reduced triglyceride levels may signify a depletion of the adipocyte TG store, increased levels may also signify impaired TG utilisation and, as a result, reduced FFA availability. Furthermore, breakdown of TG into fatty acids is tightly governed by the action of the enzyme hormone sensitive lipase (HSL) which itself is regulated (inhibited) by insulin and/or adrenergic stimulation.(253)As both insulin resistance and increased adrenergic activity have been associated with increased susceptibility to AF,(82, 254) it is possible that these factors may also contribute to impaired TG utilisation and cardiomyocyte energy depletion, further enhancing the substrate for arrhythmogenesis.

In addition to triglycerides and cholesterol compounds, the complex lipid profile of EAT also contains a number of other glycerophospholipids including glycerophosphoglycerols, glycerophosphoserines, glycerophosphinositols and glycerophosphates. These compounds form part of the fundamental structure of the cell membrane lipid bilayer as well as serving both metabolic and signaling functions. This complex functionality leads to difficulty in determining the exact role of these lipids in the pathogenesis of post-operative atrial fibrillation. However, it is notable that five out of the six lipids identified in my patient cohort showed decreased abundance in POAF patients. It is therefore possible that this reflects an increase in the utilisation of glycerolipids as an energy substrate for fatty acid production, rather than the consumption of triglycerides as described above. In addition, it is notable that glycerophosphinositols may act as precursors to inositol phosphates. Variations in the availability of these lipids may therefore impair the activity of membrane derived intracellular signaling pathways which in turn may play a role in a number of mechanisms leading to the pathogenesis of cardiac arrhythmia.

5.4.2 The right atrial metabolome of POAF

The results of this study did not identify any significant difference in the right atrial metabolite profile of patients developing POAF when compared to those maintaining SR after coronary artery bypass grafting. These findings differ from those of Mayr et al. who demonstrated significantly lower concentrations of glucose, beta-hydroxybutyrate, and acetate in their cohort of 22 patients (n=11 POAF) developing de novo post-operative AF.(110) Similarly, in their proteomic study, Kourliouros et al. demonstrated a reduction in enolase and pyruvate dehydrogenase in the atrial tissue of POAF patients, further suggesting a downregulation of glycolysis and pyruvate metabolism.(243) Together, these studies add weight to the hypothesis that inhibition of glycolysis may pre-dispose to POAF through both a reduction in energy provision, which in turn promotes a pro-inflammatory substrate that may be more susceptible to oxidative stress.(243)

The exact reason as to why no similar finding was observed in this patient cohort is unknown. However, it is pertinent to note that the experimental methods used differ between this and previous metabonomic studies of atrial fibrillation. This study utilised magic angle spinning (MAS) nuclear magnetic resonance to obtain a solid-state, non-destructive metabolic profile of whole atrial tissue biopsies. The advantages of this approach are that it is able to give a high sensitivity metabolite profile more representative of *in vivo* tissue, as well as retaining information specific to the tissue compartment.(255) However, at the same time, this technique requires precise dissection of the tissue of interest in order to provide a homogeneous sample for analysis, and contamination with other cell types results in high heterogeneity. The results from this study revealed very intense lipid peaks in the standard proton spectra derived from all atrial samples, suggesting a significant degree of lipid contamination. Retrospectively, this is unsurprising given the close relationship between the atrial myocardium and the epicardial adipose, particularly in the region of the right atrial appendage. It is therefore likely that the lack of significant spectral differences between

POAF and non-POAF groups is a consequence of the high cellular heterogeneity present within the samples studied. The results of this analysis therefore highlight the need for future studies to focus on careful sample dissection whilst at the same time minimising the time taken for tissue preparation. Where this is not possible, future work should focus on a technique of metabolite extraction and 2D NMR analysis (110) in order to reliably determine the atrial metabolite profile of these patients.

5.4.3 Limitations

The untargeted methodology utilised in this study confers the benefit of both an unbiased and all-inclusive approach to metabolite detection when compared to targeted analysis. However, when considering the findings presented here a number of limitations should also be taken into account. Firstly, as described above, the high sensitivity and compartmental information provided by MAS NMR relies on the provision of a homogenous tissue type for analysis. Similarly, whole tissue analysis requires careful tissue handling, minimising the time spent at room temperature so as to prevent enzymatic degradation or changes in metabolite profile prior to freezing.(255)

These tissue handling requirements also exist for the use of epicardial adipose tissue for UPLC-MS analysis. All samples were maintained at room temperature for the shortest possible time period, however the exact impact of small variations in tissue handling on the EAT metabolite profile is impossible to quantify. Second, the lipid identification presented here is the result of preliminary peak annotation performed utilising the Metabolite and Tandem MS (METLIN) database (<http://metlin.scripps.edu/index.php>)(256). This approach utilised a 30 ppm tolerance for each peak analysed to maximise metabolite identification whilst minimising false positives. However, absolute verification of these metabolites requires further MS/MS analysis with comparison to known standards for the metabolites of interest and is the focus of ongoing work. Furthermore, although the METLIN database

includes over 64 000 structures in its most recent release, this continues to grow and as such is not entirely comprehensive. It is therefore possible that future analysis may uncover physiologically relevant metabolites that are not identifiable at the present time. Finally, whilst variations in chromatography performance were minimised in this analysis through the use of QC and internal standards and as a result of the relatively small sample size studied, the impact of these factors becomes more significant in larger, multicenter studies and should be carefully considered when planning multiple sample runs or when comparing metabolite profiles between institutions and instruments.

5.5 Conclusions

This study is the first of its kind to determine the metabolite profile of epicardial adipose tissue (EAT) in post-operative atrial fibrillation. Using ultra-performance liquid chromatography integrated with XCMS and online database analysis, this study has identified a derangement in glycerophospholipids, triglycerides, campesterol and the acyl donors (3s)-citryl-CoA and Isopropenylpimelyl-CoA. Furthermore, this distinct lipidomic profile was able to correctly categorize 78.6% of patients at risk of post-operative atrial fibrillation at the time of coronary artery revascularization.

Accounting for 60-90% of energy provision under normal conditions, free fatty acids (FFA) are particularly essential to maintain adequate energy supply to contracting cardiomyocytes in times of increased demand or systemic starvation. The altered metabolite profile of the epicardial adipose tissue characterised in this study highlights the plasticity of EAT metabolism, and identifies a number of changes which together may play an important role in the pathogenesis of AF both through alterations in FFA availability and through increases in susceptibility to other triggering factors such as inflammation and oxidative stress.

The findings presented here also highlight a number of areas for ongoing work. Firstly, where possible it is now necessary to compare this mass spectrometry data to that of known authentic standards, in order to validate these preliminary metabolite identifications. Second, the finding of two distinct subgroups within the metabolite profiles of both acyl donors requires further investigation in a larger patient cohort. Whilst it is possible that these results merely represent opposite ends of a spectrum of change in POAF, it is also plausible that there are distinct characteristics present in this subgroup of POAF patients that may be of clinical relevance. Finally, 2D NMR analysis of extracted atrial metabolites is now required to provide a holistic analysis of the atrial tissue in my patient cohort. Together these results will complete the most comprehensive analysis of microRNA, gene expression, metabolite and lipidomic analysis studied in a single cohort of POAF patients, and offer novel insights into the pathogenesis of this arrhythmia not only for ongoing research but also for future clinical risk reduction.

Chapter 6

Conclusions and further work

6.1 Summary and discussion of results

Atrial fibrillation (AF) is the commonest sustained disorder of cardiac rhythm, leading to increased risk of thromboembolism, stroke and overall mortality.(2) Post-operative AF affects approximately one third of people undergoing coronary artery bypass grafting despite no previous history of the disease and is a major cause of increased post-operative hospital stay, morbidity and even mortality.(74) From genome to metabolome, this research has highlighted a number of novel and exciting findings in the pathogenesis of human postoperative atrial fibrillation (POAF). Through the application of a diversity of laboratory techniques to both tissue and biofluids, the results presented in this thesis have provided a unique overview of the transcriptional, post-transcriptional and metabolite changes associated with this common arrhythmia. Furthermore, these observations characterise a number of structural, electrical and metabolic derangements that together produce an atrial substrate both susceptible to external triggers and amenable to the initiation and maintenance of electrical re-entry.

6.1.1 The microRNA signature of POAF and regulation of gene expression

For the first time, this research has identified a distinct microRNA profile in POAF, characterized by the differential expression of 16 microRNAs. The most over expressed of these is hsa-miR-483-5p and the most under expressed is the cardiomyocyte specific hsa-miR-208a. Furthermore, microRNA 483-5p was also found to be significantly upregulated in the pre-operative serum of patients with POAF, indicating a potential role for this microRNA as a clinical biomarker for arrhythmia susceptibility. Based on this microRNA profile,

downstream target analysis was performed in order to determine any potential mechanistic role for these microRNA in POAF pathogenesis. This established a possible link between miR-483-5p and the vascular endothelial adhesion molecule VCAM-1 via the action of the intracellular GTPase RhoA. Subsequent analysis confirmed a significant increase in serum VCAM-1 in my POAF patient cohort, signifying upregulation of leukocyte adhesion and suggesting a systemic pro-inflammatory phenotype in these patients.

Furthermore, based on this microRNA profile, detailed gene ontology and interaction network analysis has identified a number of novel gene expression changes in POAF, the diversity of which supports the presence of both electrical and structural atrial remodeling prior to the onset of arrhythmia. In this patient cohort, it was identified that disrupted potassium and calcium handling may play a critical role in cardiomyocyte electrical remodeling increasing the risk of arrhythmogenesis. Indeed, target prediction analysis identified KCNE5 (KCNE1L), a regulator of the KCNQ1 inhibitory subunit of the inward rectifier potassium channel, to contain a target site for miR-483-5p. Further analysis demonstrated up-regulation of KCNE5 in POAF, suggesting a tendency towards gain of function of the inward rectifier channel, which may subsequently contribute to shortening of the action potential duration and increased susceptibility to re-entry. Gene ontology analysis also highlighted an enrichment of cardiomyocyte calcium signaling pathways, and further gene expression and proteomic analysis revealed significant downregulation of the SERCA inhibitor sarcolipin (SLN) in my POAF patient cohort.

When considering structural remodeling, this study has provided the first evidence of pre-existing cardiomyocyte de-differentiation in the atria of POAF patients, characterised by a significant decrease in the adult myosin heavy chain subtype MYH6 alongside suppression of its intronic miR, miR-208a. Reversion to a slow-twitch, fetal/ventricular phenotype mirrors observations documented in chronic lone AF,(21, 233) however this has never before been characterised prior to the onset of arrhythmogenesis. In the setting of chronic lone AF, it is

suggested that dedifferentiation and MHC class switch results from chronic calcium overload and metabolic stress, owing to the reduced sensitivity of β -MHC to intracellular calcium.(234) Through dampening the contractile response to intracellular calcium it is therefore possible that in both chronic and post-operative AF, down-regulation of MYH6 expression may represent a protective mechanism that ultimately strives to prevent contractile failure where there is cytosolic calcium overload.

Similarly, reduced SLN expression observed in my POAF patient cohort may also represent an adaptive mechanism to counter increased intracellular calcium and preserve SR calcium stores through removal of inhibition of SERCA2a. Together these changes suggest that POAF may be associated with a basal state of cellular calcium overload prior to the onset of the arrhythmia, giving rise to a novel hypothesis that requires further targeted scientific investigation. At present, the exact mechanism for this remains unclear, however factors such as increased oxidative stress, mitochondrial dysfunction and dysregulation of cellular glucose metabolism may all promote cytosolic calcium overload and as such are likely to be fundamental to a mechanism that incorporates both structural and electrical remodeling.(236)

6.1.2 The role of the epicardial adipose lipidome in POAF

Beta-oxidation of free fatty acids (FFA) delivers the primary source of acetyl-CoA to the cardiomyocyte TCA cycle, accounting for approximately 60-90% of total energy substrate provision.(239) Glucose and ketone bodies contribute much smaller proportions of this energy supply, however inhibition of glycolysis and upregulation of ketone body metabolism have been shown to underlie the metabolic substrate that promotes both chronic and post-operative AF.(110, 243) Although this study was not able to characterise the metabolic profile of atrial myocardium in this POAF cohort, a number of novel findings were identified surrounding the role of lipid metabolism within the epicardial adipose tissue (EAT) of these patients.

Previous studies have indicated that chronic AF may be associated with a fall in cardiomyocyte fatty acid uptake and utilisation that contributes to a state of energy depletion.(110, 240-243) However, these studies did not examine the impact of FFA availability from the surrounding EAT. In this POAF patient cohort, lipidomic analysis of epicardial adipose tissue was able to identify a characteristic derangement in glycerophospholipids, triglycerides, campesterol and the acyl donors (3s)-citryl-CoA and Isopropenylpimelyl-CoA. Furthermore, this distinct lipidomic profile was able correctly categorize 78.6% of patients at risk of post-operative atrial fibrillation at the time of coronary artery revascularization.

The exact impact that each of these lipids has on AF pathogenesis is difficult to quantify given the complexity of the EAT lipidome and the vast array of lipid subtypes present. However, it is possible that observed increases in (3s)-citryl-CoA, indicating increased citrate production, signify upregulation of fatty acid synthesis within the epicardial adipose of POAF patients. Furthermore, impaired storage of the cardioprotective plant sterol campesterol may reduce its protective effects on the vascular endothelial cell, increasing susceptibility to oxidative stress and inflammation. In addition, dysregulation of triglyceride storage and glycerophospholipid metabolism may not only alter fatty acid availability but also modify the activity of membrane bound secondary messengers and subsequent intracellular signaling pathways. As such, the altered EAT lipidomic profile characterised in this study demonstrates a number of novel changes which together may play an important role in the pathogenesis of AF. Furthermore, these findings once again add to the increasing volume of evidence to support the dynamic role of the EAT which extends far beyond free fatty acid provision, to encompass paracrine control of the local cardiomyocyte environment, and regulate its susceptibility to key triggering factors such as inflammation and oxidative stress.

6.2 Unanswered questions and future research

Alongside the novel insights this study has provided into the mechanisms of POAF, it has also given rise to a number of equally novel questions. It is therefore paramount that these findings are considered as a platform on which to continue to address the many unanswered questions remaining in the pathogenesis of this complex and multifactorial arrhythmia.

1. Validating a novel serum biomarker

In order to reliably determine the role of circulating microRNA-483-5p as a biomarker for POAF, prospective and targeted validation of these results is now required in a larger patient cohort. Based on this work, post-hoc analysis estimates that at an effect size of 0.75, a total of 62 patients would be required to yield 80% power with a Type 1 error rate (α) of 0.05. In order to validate these findings it is therefore vital to first ensure reproducibility in a larger patient cohort. Beyond this, should results yield a suitably high sensitivity and specificity, the next stage in biomarker development would require formal feasibility assessment with estimation of both the practicality and cost of upscaling to clinical practice.

2. Investigating the role of cardiomyocyte de-differentiation

Characterisation of the relationship between MHC isoform switch and POAF, requires a detailed evaluation of both MYH6 and MYH7 gene expression along with quantification of α - and β -MHC protein levels in a larger patient cohort. Furthermore, simultaneous assessment of the co-expressed intronic microRNAs miR-208a and miR-208b, should be included to provide a holistic understanding of the role of microRNA in the regulation and feedback control of myosin heavy chain expression in the atrial myocardium.

3. Determining the impact of cytosolic calcium overload in POAF pathogenesis

The results of this work highlight a novel role for intracellular calcium signaling in the pathogenesis of post-operative AF through impaired sarcolipin (SLN) regulation of SERCA2A sarcoplasmic reticulum calcium reuptake. In order to identify the impact of SLN downregulation *in vivo*, future research must combine the study of SLN gene and protein expression with analysis of intracellular calcium balance within cells cultured from the atrial myocardium of POAF patients. Furthermore, in order to be fully comprehensive, this analysis must also incorporate other key regulators of SR calcium transport including SERCA, PLN, TRDN and the ryanodine (RyR2) transporter.

4. Combining the epicardial adipose lipidome with the myocardial metabolome in POAF

Another area for ongoing research highlighted here is the requirement for a united study of the metabolic substrate for atrial fibrillation. Adding to the results provided in previously published metabolomic and proteomic studies of atrial tissue, this research provides the first insight into the epicardial adipose lipidome in post-operative AF. Further work firstly necessitates authentication of this lipidomic profile against known standards, in order to validate these preliminary metabolite identifications. Beyond this, future direct integration of EAT lipidomic and atrial metabolite analysis is required to provide a holistic metabolic map of the atrial tissue and the impact of its local environment in POAF.

6.3 Conclusion

In conclusion, the results presented in this thesis have provided a comprehensive analysis of the microRNA, gene expression, metabolic and lipidomic signature of a single cohort of patients developing new onset post-CABG atrial fibrillation. These findings provide a body of evidence to support the existence of a pre-existing atrial substrate in these patients,

characterised by structural, electrical and metabolic remodeling. This not only offers novel insights into the pathogenesis of atrial fibrillation but also poses many new questions surrounding biomarker development and factors such as cardiomyocyte de-differentiation and calcium signaling. These findings therefore form a platform on which to build our understanding of the pathogenesis of this complex and multifactorial arrhythmia, with the ultimate aim to not only reduce clinical risk but also develop novel therapeutic strategies.

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Appendix

Appendix

Appendix 1a

Validated targets for miR-483-5p according to the miRWalk Database (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html>) Last accessed July 2014, last release March 2011.

MicroRNA Name	Stem Loop Name	miRNA Chromosome	Gene Name	EntrezID	Pubmed ID
hsa-miR-483-5p	hsa-mir-483	11	NOS3	4846	19213952
hsa-miR-483-5p	hsa-mir-483	11	MAPK3	5595	22465663
hsa-miR-483-5p	hsa-mir-483	11	MCF2L	23263	19958507
hsa-miR-483-5p	hsa-mir-483	11	SOAT1	6646	23199328
hsa-miR-483-5p	hsa-mir-483	11	MYC	4609	19958507
hsa-miR-483-5p	hsa-mir-483	11	STAT1	6772	23199328
hsa-miR-483-5p	hsa-mir-483	11	MZF1	7593	19958507
hsa-miR-483-5p	hsa-mir-483	11	API5	8539	23199328
hsa-miR-483-5p	hsa-mir-483	11	RUNX1	861	19958507
hsa-miR-483-5p	hsa-mir-483	11	ARCN1	372	23338559
hsa-miR-483-5p	hsa-mir-483	11	BBC3	27113	20388800
hsa-miR-483-5p	hsa-mir-483	11	CCND1	595	20621999
hsa-miR-483-5p	hsa-mir-483	11	SOCS3	9021	21146586
hsa-miR-483-5p	hsa-mir-483	11	WDR20	91833	21367285
hsa-miR-483-5p	hsa-mir-483	11	SRF	6722	21893058
hsa-miR-483-5p	hsa-mir-483	11	NOS2A	4843	19213952
hsa-miR-483-5p	hsa-mir-483	11	CCND1	595	22362728

Appendix 1b

miRWalk search criteria for predicted miRNA targets (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html>) 11 March 2013.

miRNA predicted target search				
Species	Human	Select your specie of interest		
Identifiers	MIMAT0004761	*miRNA putative targets can be searched by providing Stem loop or Mature Sequence IDs or by their names For eg. MI0000290 or MIMAT0000231 or miR-30a or hsa-mir-34a <input type="button" value="Example"/>		
Upload a file	<input type="button" value="Choose File"/> no file selected	OR Choose from a File (example list)		
Gene Region				
☐ Promoter	2000 Upstream flanked	Upstream flanking region (assumed promoter) Max. 10kb is allowed		
☐ 5' UTR ☐ CDS ☒ 3'UTR	Restrict your search of miRNA binding sites within specific region(s)			
Options				
Transcript	☒ All ☐ Longest	Select either all or the longest transcript of gene(s)		
Min Seed Length	7	Minimum number of nucleotides of miRNA sequence through which they can make one or more interactios with their possible targets		
pValue	0.05	A probability distribution of random matches of a subsequence in the given sequence		
Comparative analysis by 8 prediction programs				
☒ DIANA-mT	☒ miRanda	☒ miRDB	☒ miRWalk	☒ RNAhybrid
☒ PICTAR4	☒ PICTAR5	☒ PITA	☒ RNA22	☒ TargetScan
<input type="button" value="SEARCH"/>				

Appendix 1c

Predicted Targets of hsa-miR-483-5p as listed by the miRWalk database (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html>) 11 March 2013.

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
ABCF2	1	1	1	1	0	0	1	0	0	1	6
CLCN3	1	1	1	1	0	0	1	0	0	1	6
UGT3A1	1	1	1	1	0	0	1	0	0	1	6
A2ML1	1	1	1	1	0	0	1	0	0	1	6
ZNF417	1	1	1	1	0	0	1	0	0	1	6
ALCAM	1	1	1	1	0	0	1	0	0	1	6
F2R	1	1	1	1	0	0	1	0	0	1	6
ANKRD6	1	1	1	1	0	0	1	0	0	1	6
RUSC1	1	1	1	1	0	0	1	0	0	1	6
RNF165	1	1	1	1	0	0	1	0	0	1	6
MRTO4	1	1	1	1	0	0	1	0	0	1	6
PLA2G5	1	1	1	1	0	0	1	0	0	1	6
MAPK3	1	1	1	1	0	0	1	0	0	1	6
MRP63	1	1	1	1	0	0	1	0	0	1	6
SLC7A3	1	1	1	1	0	0	1	0	0	1	6
SLC35B4	1	1	1	1	0	0	1	0	0	1	6
STC2	1	1	1	1	0	0	1	0	0	1	6
MYOM2	1	1	1	1	0	0	1	0	0	1	6
M6PRBP1	1	1	0	1	0	0	1	0	0	1	5
COX4NB	1	1	0	1	0	0	1	0	0	1	5
PCGF3	1	1	0	1	0	0	1	0	0	1	5
KLF2	1	1	0	1	0	0	1	0	0	1	5
SPON2	1	1	0	1	0	0	1	0	0	1	5
PPIH	1	1	0	1	0	0	1	0	0	1	5
CRTAP	1	1	0	1	0	0	1	0	0	1	5
VAT1	1	1	0	1	0	0	1	0	0	1	5
SEMA6C	1	1	0	1	0	0	1	0	0	1	5
SORBS1	1	1	0	1	0	0	1	0	0	1	5
TGOLN2	1	1	0	1	0	0	1	0	0	1	5
IGF2BP1	1	1	0	1	0	0	1	0	0	1	5
ADCY1	1	1	0	1	0	0	1	0	0	1	5
C1QL1	1	1	0	1	0	0	1	0	0	1	5
LILRB3	1	1	0	1	0	0	1	0	0	1	5
TRIOBP	1	1	0	1	0	0	1	0	0	1	5
BTN3A2	1	1	0	1	0	0	1	0	0	1	5
POLR3A	1	1	0	1	0	0	1	0	0	1	5
SLC2A6	1	1	0	1	0	0	1	0	0	1	5
PROSC	1	1	0	1	0	0	1	0	0	1	5
CA5B	1	1	0	1	0	0	1	0	0	1	5
GPR44	1	1	0	1	0	0	1	0	0	1	5
TEX261	1	1	0	1	0	0	1	0	0	1	5
CHRNA4	1	1	0	1	0	0	1	0	0	1	5

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
CHRNA5	1	1	0	1	0	0	1	0	0	1	5
GPRIN1	1	1	0	1	0	0	1	0	0	1	5
SMYD4	1	1	0	1	0	0	1	0	0	1	5
SLAMF6	1	1	0	1	0	0	1	0	0	1	5
OSBPL7	1	1	0	1	0	0	1	0	0	1	5
OSBPL10	1	1	0	1	0	0	1	0	0	1	5
KLHDC3	1	1	0	1	0	0	1	0	0	1	5
WDR92	0	1	1	1	0	0	1	0	0	1	5
HSPA12B	1	1	0	1	0	0	1	0	0	1	5
SFXN4	1	1	0	1	0	0	1	0	0	1	5
FOXN4	1	1	0	1	0	0	1	0	0	1	5
PAQR4	1	1	0	1	0	0	1	0	0	1	5
RAVER1	1	1	0	1	0	0	1	0	0	1	5
ZNF440	1	1	0	1	0	0	1	0	0	1	5
C19orf39	1	1	0	1	0	0	1	0	0	1	5
FBXO27	1	1	0	1	0	0	1	0	0	1	5
PUSL1	1	1	0	1	0	0	1	0	0	1	5
B3GALT6	1	1	0	1	0	0	1	0	0	1	5
TPRG1L	1	1	0	1	0	0	1	0	0	1	5
TEDDM1	1	1	0	1	0	0	1	0	0	1	5
COL4A4	1	1	0	1	0	0	1	0	0	1	5
TBC1D20	1	1	0	1	0	0	1	0	0	1	5
COL6A1	1	1	0	1	0	0	1	0	0	1	5
C2orf60	1	1	0	1	0	0	1	0	0	1	5
MBOAT2	1	1	0	1	0	0	1	0	0	1	5
TAF8	1	1	0	1	0	0	1	0	0	1	5
ICA1L	1	1	0	1	0	0	1	0	0	1	5
GALM	1	1	0	1	0	0	1	0	0	1	5
COPA	1	1	0	1	0	0	1	0	0	1	5
SLC31A1	1	1	0	1	0	0	1	0	0	1	5
SHROOM1	1	1	0	1	0	0	1	0	0	1	5
CPT1A	1	1	0	1	0	0	1	0	0	1	5
HGSNAT	1	1	0	1	0	0	1	0	0	1	5
RIMS4	1	1	0	1	0	0	1	0	0	1	5
KRT72	1	1	0	1	0	0	1	0	0	1	5
STK35	1	1	0	1	0	0	1	0	0	1	5
KDELC2	1	1	0	1	0	0	1	0	0	1	5
SPTY2D1	1	1	0	1	0	0	1	0	0	1	5
NOTUM	1	1	0	1	0	0	1	0	0	1	5
WIPF2	1	1	0	1	0	0	1	0	0	1	5
HIPK4	1	1	0	1	0	0	1	0	0	1	5
C19orf55	1	1	0	1	0	0	1	0	0	1	5
PHF13	1	1	0	1	0	0	1	0	0	1	5
MFSD4	1	1	0	1	0	0	1	0	0	1	5
AANAT	1	1	0	1	0	0	1	0	0	1	5
HSCB	1	1	0	1	0	0	1	0	0	1	5
PNPLA5	1	1	0	1	0	0	1	0	0	1	5
CYB561	1	1	0	1	0	0	1	0	0	1	5
PLEKHG4B	1	1	0	1	0	0	1	0	0	1	5

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
C6orf151	1	1	0	1	0	0	1	0	0	1	5
C6orf129	1	1	0	1	0	0	1	0	0	1	5
ADRBK1	1	1	0	1	0	0	1	0	0	1	5
FAM84B	1	1	0	1	0	0	1	0	0	1	5
C9orf66	1	1	0	1	0	0	1	0	0	1	5
CYP7A1	1	1	0	1	0	0	1	0	0	1	5
USP54	1	1	0	1	0	0	1	0	0	1	5
MGC24039	1	1	0	1	0	0	1	0	0	1	5
DAPK1	1	1	0	1	0	0	1	0	0	1	5
ZNF114	1	1	0	1	0	0	1	0	0	1	5
PAQR7	1	1	0	1	0	0	1	0	0	1	5
DHX8	1	1	0	1	0	0	1	0	0	1	5
KLHDC6	1	1	0	1	0	0	1	0	0	1	5
DFFA	1	1	0	1	0	0	1	0	0	1	5
ADAMTS17	1	1	0	1	0	0	1	0	0	1	5
DLX1	1	1	0	1	0	0	1	0	0	1	5
DPYSL2	1	1	0	1	0	0	1	0	0	1	5
SLC26A2	1	1	0	1	0	0	1	0	0	1	5
E2F2	1	1	0	1	0	0	1	0	0	1	5
EDA	1	1	0	1	0	0	1	0	0	1	5
CELSR2	1	1	0	1	0	0	1	0	0	1	5
MEGF9	1	1	0	1	0	0	1	0	0	1	5
EIF4EBP2	1	1	0	1	0	0	1	0	0	1	5
CC2D1B	1	1	0	1	0	0	1	0	0	1	5
C1orf69	1	1	0	1	0	0	1	0	0	1	5
APOBEC3F	1	1	0	1	0	0	1	0	0	1	5
TRIM65	1	1	0	1	0	0	1	0	0	1	5
FAM116A	1	1	0	1	0	0	1	0	0	1	5
TMEM184A	1	1	0	1	0	0	1	0	0	1	5
LGI3	1	1	0	1	0	0	1	0	0	1	5
C9orf90	1	1	0	1	0	0	1	0	0	1	5
EVC	1	1	0	1	0	0	1	0	0	1	5
EXTL3	1	1	0	1	0	0	1	0	0	1	5
C10orf56	1	1	0	1	0	0	1	0	0	1	5
ORAOV1	1	1	0	1	0	0	1	0	0	1	5
DOK6	1	1	0	1	0	0	1	0	0	1	5
MS4A2	1	1	0	1	0	0	1	0	0	1	5
LOC221091	1	1	0	1	0	0	1	0	0	1	5
KCTD20	1	1	0	1	0	0	1	0	0	1	5
SLC29A4	1	1	0	1	0	0	1	0	0	1	5
RASA3	1	1	0	1	0	0	1	0	0	1	5
MLXIP	1	1	0	1	0	0	1	0	0	1	5
NCBP2	1	1	0	1	0	0	1	0	0	1	5
FOXC1	1	1	0	1	0	0	1	0	0	1	5
IGSF9B	0	1	1	1	0	0	1	0	0	1	5
FAIM2	1	1	0	1	0	0	1	0	0	1	5
UNC13A	1	1	0	1	0	0	1	0	0	1	5
KIF21B	1	1	0	1	0	0	1	0	0	1	5
KIAA0082	1	1	0	1	0	0	1	0	0	1	5

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
ARHGAP26	1	1	0	1	0	0	1	0	0	1	5
KIF1B	1	1	0	1	0	0	1	0	0	1	5
HIC2	1	1	0	1	0	0	1	0	0	1	5
CAMTA2	1	1	0	1	0	0	1	0	0	1	5
PLXND1	1	1	0	1	0	0	1	0	0	1	5
IQCE	0	1	1	1	0	0	1	0	0	1	5
SMG6	1	1	0	1	0	0	1	0	0	1	5
ANKS1A	1	1	0	1	0	0	1	0	0	1	5
WSCD1	1	1	0	1	0	0	1	0	0	1	5
C22orf9	1	1	0	1	0	0	1	0	0	1	5
SATB2	1	1	0	1	0	0	1	0	0	1	5
KIAA0323	1	1	0	1	0	0	1	0	0	1	5
PPP1R13B	1	1	0	1	0	0	1	0	0	1	5
SRGAP2	1	1	0	1	0	0	1	0	0	1	5
PIP5K1C	1	1	0	1	0	0	1	0	0	1	5
ICMT	1	1	0	1	0	0	1	0	0	1	5
TTC9	1	1	0	1	0	0	1	0	0	1	5
SEC14L2	1	1	0	1	0	0	1	0	0	1	5
CCRK	1	1	0	1	0	0	1	0	0	1	5
LDOC1	1	1	0	1	0	0	1	0	0	1	5
SGK3	1	1	0	1	0	0	1	0	0	1	5
MTCH1	1	1	0	1	0	0	1	0	0	1	5
FXN	1	1	0	1	0	0	1	0	0	1	5
FUT6	1	0	1	1	0	0	1	0	0	1	5
ZNF620	1	1	0	1	0	0	1	0	0	1	5
EBF3	1	1	0	1	0	0	1	0	0	1	5
MSRB3	1	1	0	1	0	0	1	0	0	1	5
GABRB2	1	1	0	1	0	0	1	0	0	1	5
RIPK5	1	1	0	1	0	0	1	0	0	1	5
TMEM184B	1	1	0	1	0	0	1	0	0	1	5
DKFZP434B0335	1	1	0	1	0	0	1	0	0	1	5
TRIM58	1	1	0	1	0	0	1	0	0	1	5
INTS7	1	1	0	1	0	0	1	0	0	1	5
C20orf194	1	1	0	1	0	0	1	0	0	1	5
GPR124	1	1	0	1	0	0	1	0	0	1	5
CHD5	1	1	0	1	0	0	1	0	0	1	5
UBXD7	1	1	0	1	0	0	1	0	0	1	5
TOR1AIP1	1	1	0	1	0	0	1	0	0	1	5
CCDC9	1	1	0	1	0	0	1	0	0	1	5
GTPBP5	1	1	0	1	0	0	1	0	0	1	5
GMEB2	1	1	0	1	0	0	1	0	0	1	5
PLDN	1	1	0	1	0	0	1	0	0	1	5
TSPAN17	1	1	0	1	0	0	1	0	0	1	5
MDGA1	1	1	0	1	0	0	1	0	0	1	5
AMFR	1	1	0	1	0	0	1	0	0	1	5
GFAP	1	1	0	1	0	0	1	0	0	1	5
CACYBP	1	1	0	1	0	0	1	0	0	1	5
PDE7B	1	1	0	1	0	0	1	0	0	1	5
ZNF364	1	1	0	1	0	0	1	0	0	1	5

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
GOLGA3	1	1	0	1	0	0	1	0	0	1	5
GPR3	1	1	0	1	0	0	1	0	0	1	5
LOC283331	1	1	0	1	0	0	1	0	0	1	5
LOC283514	1	1	0	1	0	0	1	0	0	1	5
C19orf63	1	1	0	1	0	0	1	0	0	1	5
KLK9	1	1	0	1	0	0	1	0	0	1	5
FAM83H	1	1	0	1	0	0	1	0	0	1	5
ZFP41	1	1	0	1	0	0	1	0	0	1	5
ILDR1	1	1	0	1	0	0	1	0	0	1	5
MKNK2	1	1	0	1	0	0	1	0	0	1	5
ACAD9	1	1	0	1	0	0	1	0	0	1	5
GRIN1	1	1	0	1	0	0	1	0	0	1	5
GRM4	1	1	0	1	0	0	1	0	0	1	5
GSK3A	1	1	0	1	0	0	1	0	0	1	5
OLA1	1	1	0	1	0	0	1	0	0	1	5
GUCA1B	1	1	0	1	0	0	1	0	0	1	5
REPIN1	1	0	1	1	0	0	1	0	0	1	5
CCDC106	1	1	0	1	0	0	1	0	0	1	5
HDAC2	1	1	0	1	0	0	1	0	0	1	5
HUNK	1	1	0	1	0	0	1	0	0	1	5
ST6GALNAC6	1	1	0	1	0	0	1	0	0	1	5
CDR2L	1	1	0	1	0	0	1	0	0	1	5
HIP1	1	1	0	1	0	0	1	0	0	1	5
HNFB4A	1	1	0	1	0	0	1	0	0	1	5
DDX51	1	1	0	1	0	0	1	0	0	1	5
APOF	1	1	0	1	0	0	1	0	0	1	5
XIAP	1	1	0	1	0	0	1	0	0	1	5
CCDC137	1	1	0	1	0	0	1	0	0	1	5
IRF8	1	1	0	1	0	0	1	0	0	1	5
IDS	1	1	0	1	0	0	1	0	0	1	5
STAC2	1	1	0	1	0	0	1	0	0	1	5
IFNAR1	1	1	0	1	0	0	1	0	0	1	5
PDDC1	1	1	0	1	0	0	1	0	0	1	5
NUP43	1	1	0	1	0	0	1	0	0	1	5
SHROOM2	1	1	0	1	0	0	1	0	0	1	5
IL10	1	1	0	1	0	0	1	0	0	1	5
AQP2	1	1	0	1	0	0	1	0	0	1	5
IL12B	1	1	0	1	0	0	1	0	0	1	5
FOXK2	1	1	0	1	0	0	1	0	0	1	5
ILF3	1	1	0	1	0	0	1	0	0	1	5
IVL	1	1	0	1	0	0	1	0	0	1	5
CD82	1	1	0	1	0	0	1	0	0	1	5
KCNA7	1	1	0	1	0	0	1	0	0	1	5
FAM73A	1	1	0	1	0	0	1	0	0	1	5
KCNJ5	1	1	0	1	0	0	1	0	0	1	5
KCNQ1	1	1	0	1	0	0	1	0	0	1	5
LOC388969	1	1	0	1	0	0	1	0	0	1	5
IER5L	1	1	0	1	0	0	1	0	0	1	5
LAD1	1	1	0	1	0	0	1	0	0	1	5

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
LOC391356	1	1	0	1	0	0	1	0	0	1	5
LDLR	1	1	0	1	0	0	1	0	0	1	5
LEP	1	1	0	1	0	0	1	0	0	1	5
ARHGDIA	1	1	0	1	0	0	1	0	0	1	5
LIM2	1	1	0	1	0	0	1	0	0	1	5
LMO1	1	1	0	1	0	0	1	0	0	1	5
PHOX2A	1	1	0	1	0	0	1	0	0	1	5
C10orf132	1	1	0	1	0	0	1	0	0	1	5
MCM4	1	1	0	1	0	0	1	0	0	1	5
MEA1	1	1	0	1	0	0	1	0	0	1	5
RAB8A	1	1	0	1	0	0	1	0	0	1	5
MLLT1	1	1	0	1	0	0	1	0	0	1	5
ACHE	1	1	0	1	0	0	1	0	0	1	5
MPZ	1	1	0	1	0	0	1	0	0	1	5
MTHFR	1	1	0	1	0	0	1	0	0	1	5
NFIX	1	1	0	1	0	0	1	0	0	1	5
NFKBIL2	1	1	0	1	0	0	1	0	0	1	5
NFYB	1	1	0	1	0	0	1	0	0	1	5
NKX2-2	1	1	0	1	0	0	1	0	0	1	5
NPTX2	1	1	0	1	0	0	1	0	0	1	5
NTSR1	1	1	0	1	0	0	1	0	0	1	5
ACO2	1	1	0	1	0	0	1	0	0	1	5
PAFAH2	1	1	0	1	0	0	1	0	0	1	5
SERPINE1	1	1	0	1	0	0	1	0	0	1	5
PAX1	1	1	0	1	0	0	1	0	0	1	5
ACOX1	1	1	0	1	0	0	1	0	0	1	5
MRPS16	1	1	0	1	0	0	1	0	0	1	5
FIS1	1	1	0	1	0	0	1	0	0	1	5
YARS2	1	1	0	1	0	0	1	0	0	1	5
PHF20	1	1	0	1	0	0	1	0	0	1	5
TMEM69	1	1	0	1	0	0	1	0	0	1	5
PCSK2	1	1	0	1	0	0	1	0	0	1	5
RBJ	1	1	0	1	0	0	1	0	0	1	5
TLR7	1	1	0	1	0	0	1	0	0	1	5
CD320	1	1	0	1	0	0	1	0	0	1	5
CDC40	1	1	0	1	0	0	1	0	0	1	5
GALNAC4S-6ST	1	1	0	1	0	0	1	0	0	1	5
SNX9	1	1	0	1	0	0	1	0	0	1	5
PRRX2	1	1	0	1	0	0	1	0	0	1	5
PDE6G	1	1	0	1	0	0	1	0	0	1	5
CTDSPL2	1	1	0	1	0	0	1	0	0	1	5
LARS	1	1	0	1	0	0	1	0	0	1	5
CXXC5	1	1	0	1	0	0	1	0	0	1	5
PDGFRA	1	1	0	1	0	0	1	0	0	1	5
TRIM33	1	1	0	1	0	0	1	0	0	1	5
TMBIM4	1	1	0	1	0	0	1	0	0	1	5
SLC26A4	1	1	0	1	0	0	1	0	0	1	5
PIK3R2	1	1	0	1	0	0	1	0	0	1	5
PLAGL2	1	1	0	1	0	0	1	0	0	1	5

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
PLEC1	1	1	0	1	0	0	1	0	0	1	5
PNOC	1	1	0	1	0	0	1	0	0	1	5
C11orf24	1	1	0	1	0	0	1	0	0	1	5
CSNK1G1	1	1	0	1	0	0	1	0	0	1	5
PCBP3	1	1	0	1	0	0	1	0	0	1	5
SMCR7L	1	1	0	1	0	0	1	0	0	1	5
RNF216	1	1	0	1	0	0	1	0	0	1	5
DGCR8	1	1	0	1	0	0	1	0	0	1	5
CCDC93	1	1	0	1	0	0	1	0	0	1	5
ANKRD16	1	1	0	1	0	0	1	0	0	1	5
GNL3L	1	1	0	1	0	0	1	0	0	1	5
HES2	1	1	0	1	0	0	1	0	0	1	5
IL17RD	1	1	0	1	0	0	1	0	0	1	5
ALKBH4	1	1	0	1	0	0	1	0	0	1	5
KLHL28	1	1	0	1	0	0	1	0	0	1	5
ASPN	1	1	0	1	0	0	1	0	0	1	5
C2orf18	1	1	0	1	0	0	1	0	0	1	5
PIWIL2	1	1	0	1	0	0	1	0	0	1	5
C20orf12	1	1	0	1	0	0	1	0	0	1	5
TMEM143	1	1	0	1	0	0	1	0	0	1	5
QRSL1	1	1	0	1	0	0	1	0	0	1	5
CWF19L1	1	1	0	1	0	0	1	0	0	1	5
RNF121	1	1	0	1	0	0	1	0	0	1	5
LSG1	1	1	0	1	0	0	1	0	0	1	5
DHTKD1	1	1	0	1	0	0	1	0	0	1	5
ELMOD1	1	1	0	1	0	0	1	0	0	1	5
PLXNA3	1	1	0	1	0	0	1	0	0	1	5
YEATS2	1	1	0	1	0	0	1	0	0	1	5
IPO9	1	1	0	1	0	0	1	0	0	1	5
N4BP2	1	1	0	1	0	0	1	0	0	1	5
40787	1	1	0	1	0	0	1	0	0	1	5
PRKCA	1	1	0	1	0	0	1	0	0	1	5
LRP2BP	1	1	0	1	0	0	1	0	0	1	5
HR	1	1	0	1	0	0	1	0	0	1	5
FOXJ2	1	1	0	1	0	0	1	0	0	1	5
WWC3	1	1	0	1	0	0	1	0	0	1	5
C1orf183	1	1	0	1	0	0	1	0	0	1	5
MAPK1	1	1	0	1	0	0	1	0	0	1	5
BARX1	1	1	0	1	0	0	1	0	0	1	5
MAP2K3	1	1	0	1	0	0	1	0	0	1	5
CRTAM	1	1	0	1	0	0	1	0	0	1	5
WDR45L	1	1	0	1	0	0	1	0	0	1	5
PRMT8	1	1	0	1	0	0	1	0	0	1	5
CYP26B1	1	1	0	1	0	0	1	0	0	1	5
INPP5E	1	1	0	1	0	0	1	0	0	1	5
BARHL1	1	1	0	1	0	0	1	0	0	1	5
SPHK2	1	1	0	1	0	0	1	0	0	1	5
THAP10	1	1	0	1	0	0	1	0	0	1	5
NCLN	1	1	0	1	0	0	1	0	0	1	5

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
RGMA	1	1	0	1	0	0	1	0	0	1	5
JPH2	1	1	0	1	0	0	1	0	0	1	5
ZNFX1	1	1	0	1	0	0	1	0	0	1	5
SLC45A4	1	1	0	1	0	0	1	0	0	1	5
JPH3	1	1	0	1	0	0	1	0	0	1	5
AICDA	1	1	0	1	0	0	1	0	0	1	5
PTGIS	1	1	0	1	0	0	1	0	0	1	5
CNOT6	1	1	0	1	0	0	1	0	0	1	5
KIAA1303	1	1	0	1	0	0	1	0	0	1	5
PTK6	1	1	0	1	0	0	1	0	0	1	5
SORCS2	1	1	0	1	0	0	1	0	0	1	5
TMEM181	1	1	0	1	0	0	1	0	0	1	5
PITPNM2	1	1	0	1	0	0	1	0	0	1	5
PTPN1	1	1	0	1	0	0	1	0	0	1	5
GATAD1	1	1	0	1	0	0	1	0	0	1	5
SLC4A5	1	1	0	1	0	0	1	0	0	1	5
PTPRS	1	1	0	1	0	0	1	0	0	1	5
PVRL1	1	1	0	1	0	0	1	0	0	1	5
ZNF250	0	1	1	1	0	0	1	0	0	1	5
RAB3B	1	1	0	1	0	0	1	0	0	1	5
RAB3IL1	1	1	0	1	0	0	1	0	0	1	5
RAD23B	1	1	0	1	0	0	1	0	0	1	5
MESDC1	1	1	0	1	0	0	1	0	0	1	5
REL	1	1	0	1	0	0	1	0	0	1	5
RPS6KA3	1	1	0	1	0	0	1	0	0	1	5
RSU1	1	1	0	1	0	0	1	0	0	1	5
SALL2	1	1	0	1	0	0	1	0	0	1	5
SCN2B	1	1	0	1	0	0	1	0	0	1	5
SCN4A	1	1	0	1	0	0	1	0	0	1	5
SCN4B	1	1	0	1	0	0	1	0	0	1	5
CCL25	1	1	0	1	0	0	1	0	0	1	5
NAPB	1	1	0	1	0	0	1	0	0	1	5
MUTED	1	1	0	1	0	0	1	0	0	1	5
TNN	1	1	0	1	0	0	1	0	0	1	5
XPNPEP3	1	1	0	1	0	0	1	0	0	1	5
ZNF667	1	1	0	1	0	0	1	0	0	1	5
PRDM16	1	1	0	1	0	0	1	0	0	1	5
FN3K	1	1	0	1	0	0	1	0	0	1	5
NOD2	1	1	0	1	0	0	1	0	0	1	5
ZFYVE20	1	1	0	1	0	0	1	0	0	1	5
PDLIM2	1	1	0	1	0	0	1	0	0	1	5
XPO4	1	1	0	1	0	0	1	0	0	1	5
ZFP106	1	1	0	1	0	0	1	0	0	1	5
C5orf28	1	1	0	1	0	0	1	0	0	1	5
NOM1	1	1	0	1	0	0	1	0	0	1	5
SGTA	1	1	0	1	0	0	1	0	0	1	5
UNQ338	1	1	0	1	0	0	1	0	0	1	5
RAI16	1	1	0	1	0	0	1	0	0	1	5
IPPK	1	1	0	1	0	0	1	0	0	1	5

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
C1orf176	1	1	0	1	0	0	1	0	0	1	5
MARCKSL1	1	1	0	1	0	0	1	0	0	1	5
ZBTB8	1	1	0	1	0	0	1	0	0	1	5
SLC7A1	1	1	0	1	0	0	1	0	0	1	5
BMPR2	1	1	0	1	0	0	1	0	0	1	5
LRRC61	1	1	0	1	0	0	1	0	0	1	5
CHID1	1	1	0	1	0	0	1	0	0	1	5
SMO	1	1	0	1	0	0	1	0	0	1	5
SNAPC1	1	1	0	1	0	0	1	0	0	1	5
SOX4	1	1	0	1	0	0	1	0	0	1	5
SOX11	1	1	0	1	0	0	1	0	0	1	5
AKR1D1	1	1	0	1	0	0	1	0	0	1	5
SSR3	1	1	0	1	0	0	1	0	0	1	5
STAT3	1	1	0	1	0	0	1	0	0	1	5
STAT5B	1	1	0	1	0	0	1	0	0	1	5
STK4	1	1	0	1	0	0	1	0	0	1	5
STXBP1	1	1	0	1	0	0	1	0	0	1	5
BSG	1	1	0	1	0	0	1	0	0	1	5
KLF9	1	1	0	1	0	0	1	0	0	1	5
TBCD	1	1	0	1	0	0	1	0	0	1	5
TEAD1	1	1	0	1	0	0	1	0	0	1	5
TERT	1	1	0	1	0	0	1	0	0	1	5
TFAP2B	1	1	0	1	0	0	1	0	0	1	5
TIMP2	1	1	0	1	0	0	1	0	0	1	5
TPMT	1	1	0	1	0	0	1	0	0	1	5
C5AR1	1	1	0	1	0	0	1	0	0	1	5
TYRO3	1	1	0	1	0	0	1	0	0	1	5
UBE2G1	1	1	0	1	0	0	1	0	0	1	5
UPK1B	1	1	0	1	0	0	1	0	0	1	5
UGDH	1	1	0	1	0	0	1	0	0	1	5
UVRAG	1	1	0	1	0	0	1	0	0	1	5
XPC	1	1	0	1	0	0	1	0	0	1	5
ZKSCAN1	1	1	0	1	0	0	1	0	0	1	5
ZNF70	1	1	0	1	0	0	1	0	0	1	5
PRRG4	1	1	0	1	0	0	1	0	0	1	5
FTO	1	1	0	1	0	0	1	0	0	1	5
FKRP	1	1	0	1	0	0	1	0	0	1	5
ALDH5A1	1	1	0	1	0	0	1	0	0	1	5
GLB1L	1	1	0	1	0	0	1	0	0	1	5
FN3KRP	1	1	0	1	0	0	1	0	0	1	5
C22orf29	1	1	0	1	0	0	1	0	0	1	5
ZYG11B	1	1	0	1	0	0	1	0	0	1	5
LIN28	1	1	0	1	0	0	1	0	0	1	5
CLIP4	1	1	0	1	0	0	1	0	0	1	5
MAFK	1	1	0	1	0	0	1	0	0	1	5
TMC5	1	1	0	1	0	0	1	0	0	1	5
SNX22	1	1	0	1	0	0	1	0	0	1	5
ADM2	1	1	0	1	0	0	1	0	0	1	5
ADCK4	1	1	0	1	0	0	1	0	0	1	5

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
ZNF556	1	1	0	1	0	0	1	0	0	1	5
ZC3H12A	1	1	0	1	0	0	1	0	0	1	5
C4orf29	1	1	0	1	0	0	1	0	0	1	5
SPSB1	1	1	0	1	0	0	1	0	0	1	5
MUS81	1	1	0	1	0	0	1	0	0	1	5
OPA3	0	1	1	1	0	0	1	0	0	1	5
RAB11FIP1	1	1	0	1	0	0	1	0	0	1	5
NUBPL	1	1	0	1	0	0	1	0	0	1	5
CXorf21	1	1	0	1	0	0	1	0	0	1	5
PDGFD	1	1	0	1	0	0	1	0	0	1	5
LRRC27	1	1	0	1	0	0	1	0	0	1	5
DNAJC5	1	1	0	1	0	0	1	0	0	1	5
TTYH3	1	1	0	1	0	0	1	0	0	1	5
ZBP1	1	1	0	1	0	0	1	0	0	1	5
NDEL1	1	1	0	1	0	0	1	0	0	1	5
SNX27	1	1	0	1	0	0	1	0	0	1	5
TMEM163	1	1	0	1	0	0	1	0	0	1	5
TRIM7	1	0	1	1	0	0	1	0	0	1	5
RNF170	1	1	0	1	0	0	1	0	0	1	5
TRIM56	1	1	0	1	0	0	1	0	0	1	5
FAHD1	1	1	0	1	0	0	1	0	0	1	5
GDF5	1	1	0	1	0	0	1	0	0	1	5
RP3-402G11.5	1	1	0	1	0	0	1	0	0	1	5
CASP6	1	1	0	1	0	0	1	0	0	1	5
STK40	1	1	0	1	0	0	1	0	0	1	5
KREMEN1	1	1	0	1	0	0	1	0	0	1	5
MAGT1	1	1	0	1	0	0	1	0	0	1	5
CASP8	1	1	0	1	0	0	1	0	0	1	5
NICN1	1	1	0	1	0	0	1	0	0	1	5
ING5	1	1	0	1	0	0	1	0	0	1	5
C10orf58	1	1	0	1	0	0	1	0	0	1	5
ACBD6	1	1	0	1	0	0	1	0	0	1	5
C16orf14	1	1	0	1	0	0	1	0	0	1	5
ARFGAP2	1	1	0	1	0	0	1	0	0	1	5
ZNF594	1	1	0	1	0	0	1	0	0	1	5
GLIS2	1	1	0	1	0	0	1	0	0	1	5
GPT2	1	1	0	1	0	0	1	0	0	1	5
PIGO	1	1	0	1	0	0	1	0	0	1	5
CBX2	1	1	0	1	0	0	1	0	0	1	5
CDC42BPA	1	1	0	1	0	0	1	0	0	1	5
MFSD9	1	1	0	1	0	0	1	0	0	1	5
RAX2	1	1	0	1	0	0	1	0	0	1	5
C1orf198	1	1	0	1	0	0	1	0	0	1	5
NFATC2IP	1	1	0	1	0	0	1	0	0	1	5
ZNF587	1	1	0	1	0	0	1	0	0	1	5
ZNRF1	1	1	0	1	0	0	1	0	0	1	5
MUM1	1	1	0	1	0	0	1	0	0	1	5
HSH2D	1	1	0	1	0	0	1	0	0	1	5
RANBP3	1	1	0	1	0	0	1	0	0	1	5

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
C18orf45	1	1	0	1	0	0	1	0	0	1	5
GCM1	1	1	0	1	0	0	1	0	0	1	5
GAS7	1	1	0	1	0	0	1	0	0	1	5
BCAS1	1	1	0	1	0	0	1	0	0	1	5
BARX2	1	1	0	1	0	0	1	0	0	1	5
PRIC285	1	1	0	1	0	0	1	0	0	1	5
FHDC1	1	1	0	1	0	0	1	0	0	1	5
SELI	1	1	0	1	0	0	1	0	0	1	5
MKNK1	1	1	0	1	0	0	1	0	0	1	5
CAV2	1	1	0	1	0	0	1	0	0	1	5
GTPBP10	1	1	0	1	0	0	1	0	0	1	5
OASL	1	1	0	1	0	0	1	0	0	1	5
PNPT1	1	1	0	1	0	0	1	0	0	1	5
MBTPS1	1	1	0	1	0	0	1	0	0	1	5
CBS	1	1	0	1	0	0	1	0	0	1	5
NAPA	1	1	0	1	0	0	1	0	0	1	5
PRPF4B	1	1	0	1	0	0	1	0	0	1	5
BSN	1	1	0	1	0	0	1	0	0	1	5
CCND2	1	1	0	1	0	0	1	0	0	1	5
CDK5R2	1	1	0	1	0	0	1	0	0	1	5
AP3D1	1	1	0	1	0	0	1	0	0	1	5
SIGLEC10	1	1	0	1	0	0	1	0	0	1	5
NAV1	1	1	0	1	0	0	1	0	0	1	5
NAV2	1	1	0	1	0	0	1	0	0	1	5
KLHL6	1	1	0	1	0	0	1	0	0	1	5
KBTBD6	1	1	0	1	0	0	1	0	0	1	5
C16orf45	1	1	0	1	0	0	1	0	0	1	5
FAM105B	1	1	0	1	0	0	1	0	0	1	5
C19orf52	1	1	0	1	0	0	1	0	0	1	5
LYRM7	1	1	0	1	0	0	1	0	0	1	5
SYT7	1	1	0	1	0	0	1	0	0	1	5
DPP9	1	1	0	1	0	0	1	0	0	1	5
INA	1	1	0	1	0	0	1	0	0	1	5
KRT75	1	1	0	1	0	0	1	0	0	1	5
NEURL	1	1	0	1	0	0	1	0	0	1	5
ANKRD44	1	1	0	1	0	0	1	0	0	1	5
SLC28A2	1	1	0	1	0	0	1	0	0	1	5
RSAD2	1	1	0	1	0	0	1	0	0	1	5
RASL10B	1	1	0	1	0	0	1	0	0	1	5
SFRS2IP	1	1	0	1	0	0	1	0	0	1	5
SNX29	1	1	0	1	0	0	1	0	0	1	5
RIPPLY1	1	1	0	1	0	0	1	0	0	1	5
DLG5	1	1	0	1	0	0	1	0	0	1	5
MSC	1	1	0	1	0	0	1	0	0	1	5
SOC6	1	1	0	1	0	0	1	0	0	1	5
CCDC114	1	1	0	1	0	0	1	0	0	1	5
CIAO1	1	1	0	1	0	0	1	0	0	1	5
ZNF264	1	1	0	1	0	0	1	0	0	1	5
KCNK6	1	1	0	1	0	0	1	0	0	1	5

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
MAP4K4	1	1	0	1	0	0	1	0	0	1	5
HAND2	1	1	0	1	0	0	1	0	0	1	5
PCYT1B	1	1	0	1	0	0	1	0	0	1	5
CHST3	1	1	0	1	0	0	1	0	0	1	5
ONECUT2	1	1	0	1	0	0	1	0	0	1	5
SPTLC2	1	1	0	1	0	0	1	0	0	1	5
RAB36	1	1	0	1	0	0	1	0	0	1	5
SH3PXD2A	1	1	0	1	0	0	1	0	0	1	5
FAM131B	1	1	0	1	0	0	1	0	0	1	5
SEMA3E	1	1	0	1	0	0	1	0	0	1	5
KIAA0562	1	1	0	1	0	0	1	0	0	1	5
IFT140	1	1	0	1	0	0	1	0	0	1	5
HDAC4	1	1	0	1	0	0	1	0	0	1	5
KIAA0513	1	1	0	1	0	0	1	0	0	1	5
KIAA0101	1	1	0	1	0	0	1	0	0	1	5
PJA2	1	1	0	1	0	0	1	0	0	1	5
RBM19	1	1	0	1	0	0	1	0	0	1	5
CDC34	1	1	0	1	0	0	1	0	0	1	5
GNE	1	1	0	1	0	0	0	0	0	1	4
GNE	1	0	0	1	0	0	1	0	0	1	4
GJC1	1	1	0	1	0	0	0	0	0	1	4
GJC1	1	0	0	1	0	0	1	0	0	1	4
IL18BP	1	1	0	1	0	0	0	0	0	1	4
IL18BP	1	0	0	1	0	0	1	0	0	1	4
CDH12	1	1	0	1	0	0	1	0	0	0	4
WASF2	1	1	0	1	0	0	1	0	0	0	4
ZNF197	1	1	0	1	0	0	1	0	0	0	4
CD96	1	1	0	1	0	0	0	0	0	1	4
CD96	1	0	0	1	0	0	1	0	0	1	4
CDKN1A	1	1	0	1	0	0	0	0	0	1	4
CDKN1A	1	0	0	1	0	0	1	0	0	1	4
ADARB1	1	0	0	1	0	0	1	0	0	1	4
ADARB1	1	1	0	1	0	0	0	0	0	1	4
RBM14	0	1	0	1	0	0	1	0	0	1	4
TACC3	1	0	0	1	0	0	1	0	0	1	4
TRIM38	1	1	0	1	0	0	1	0	0	0	4
CEACAM5	1	1	0	1	0	0	1	0	0	0	4
NXF1	0	1	0	1	0	0	1	0	0	1	4
SLC34A2	1	1	0	1	0	0	1	0	0	0	4
ZNF275	1	1	0	1	0	0	1	0	0	0	4
RALBP1	1	1	0	1	0	0	1	0	0	0	4
SFRS2B	0	1	0	1	0	0	1	0	0	1	4
SLC27A4	1	1	0	1	0	0	1	0	0	0	4
ABHD2	1	0	0	1	0	0	1	0	0	1	4
ABHD2	1	1	0	1	0	0	0	0	0	1	4
KLF12	1	1	0	1	0	0	1	0	0	0	4
TMEM106A	1	1	0	1	0	0	0	0	0	1	4
GBP4	1	1	0	1	0	0	1	0	0	0	4
CISH	1	1	0	1	0	0	0	0	0	1	4

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
LRRC58	0	1	0	1	0	0	1	0	0	1	4
C20orf108	1	1	0	1	0	0	1	0	0	0	4
OLIG1	1	1	0	1	0	0	1	0	0	0	4
PRAP1	1	0	0	1	0	0	1	0	0	1	4
CLCN6	1	1	0	1	0	0	1	0	0	0	4
TMEM132D	1	1	0	1	0	0	1	0	0	0	4
C15orf27	1	1	0	1	0	0	1	0	0	0	4
ZC3H18	1	1	0	1	0	0	1	0	0	0	4
CD300LB	1	1	0	1	0	0	1	0	0	0	4
ZFP3	1	1	0	1	0	0	1	0	0	0	4
GGT6	1	1	0	1	0	0	0	0	0	1	4
GJD3	0	1	0	1	0	0	1	0	0	1	4
SHE	1	1	0	1	0	0	1	0	0	0	4
DMBX1	0	1	0	1	0	0	1	0	0	1	4
COL4A6	1	1	0	1	0	0	1	0	0	0	4
COL6A2	1	1	0	1	0	0	1	0	0	0	4
BBS5	1	1	0	1	0	0	1	0	0	0	4
ADAT2	1	1	0	1	0	0	1	0	0	0	4
SRrp35	1	1	0	1	0	0	1	0	0	0	4
CREBBP	1	1	0	1	0	0	1	0	0	0	4
MYO3B	1	1	0	1	0	0	1	0	0	0	4
ZBTB46	1	1	0	1	0	0	1	0	0	0	4
SMCR8	0	1	0	1	0	0	1	0	0	1	4
CRYAA	1	1	0	1	0	0	1	0	0	0	4
CSK	1	1	0	1	0	0	0	0	0	1	4
CSK	1	0	0	1	0	0	1	0	0	1	4
CSN2	1	1	0	1	0	0	1	0	0	0	4
PRIMA1	1	1	0	1	0	0	1	0	0	0	4
C14orf145	1	1	0	1	0	0	1	0	0	0	4
NCAN	1	1	0	1	0	0	1	0	0	0	4
C18orf25	1	1	0	1	0	0	1	0	0	0	4
SLC35F3	1	1	0	1	0	0	1	0	0	0	4
SLC30A7	1	1	0	1	0	0	1	0	0	0	4
NFAM1	0	1	0	1	0	0	1	0	0	1	4
GPR155	1	0	0	1	0	0	1	0	0	1	4
GPR155	1	1	0	1	0	0	0	0	0	1	4
ADRA2C	0	1	0	1	0	0	1	0	0	1	4
PRELID2	1	0	0	1	0	0	1	0	0	1	4
PRELID2	1	1	0	1	0	0	0	0	0	1	4
ADRBK2	0	1	0	1	0	0	1	0	0	1	4
DBN1	1	1	0	1	0	0	0	0	0	1	4
DBN1	1	0	0	1	0	0	1	0	0	1	4
KANK4	0	1	0	1	0	0	1	0	0	1	4
SPATA5	0	1	0	1	0	0	1	0	0	1	4
MIER3	1	1	0	1	0	0	1	0	0	0	4
CYB5R3	1	1	0	1	0	0	0	0	0	1	4
DLX1	1	1	0	1	0	0	0	0	0	1	4
DVL3	1	1	0	1	0	0	1	0	0	0	4
B3GNT6	0	1	0	1	0	0	1	0	0	1	4

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
LOC196993	0	1	0	1	0	0	1	0	0	1	4
GRAMD2	1	1	0	1	0	0	1	0	0	0	4
ELK1	1	1	0	1	0	0	0	0	0	1	4
RNF215	1	1	0	1	0	0	1	0	0	0	4
ERBB3	1	1	0	1	0	0	1	0	0	0	4
ESRRG	1	1	0	1	0	0	0	0	0	1	4
ESRRG	1	0	0	1	0	0	1	0	0	1	4
C10orf27	1	1	0	1	0	0	1	0	0	0	4
FKTN	1	1	0	1	0	0	0	0	0	1	4
SDK1	1	1	0	1	0	0	1	0	0	0	4
FOXK1	1	1	0	1	0	0	1	0	0	0	4
NAPEPLD	1	1	0	1	0	0	0	0	0	1	4
TMEM130	1	1	0	1	0	0	0	0	0	1	4
FEN1	1	1	0	1	0	0	1	0	0	0	4
FGF2	1	1	0	1	0	0	1	0	0	0	4
AAK1	0	1	0	1	0	0	1	0	0	1	4
FOXF1	1	1	0	1	0	0	1	0	0	0	4
TPX2	1	1	0	1	0	0	1	0	0	0	4
PCNX	0	1	0	1	0	0	1	0	0	1	4
KIAA0265	1	1	0	1	0	0	1	0	0	0	4
FOXO3	0	1	0	1	0	0	1	0	0	1	4
KIF1B	1	1	0	1	0	0	0	0	0	1	4
IQSEC2	1	1	0	1	0	0	0	0	0	1	4
IQSEC2	1	1	0	1	0	0	0	0	0	1	4
CLASP2	1	1	0	1	0	0	1	0	0	0	4
TTLL12	1	1	0	1	0	0	1	0	0	0	4
DIP2A	1	1	0	1	0	0	1	0	0	0	4
FBXL7	1	1	0	1	0	0	1	0	0	0	4
JMJD6	0	1	0	1	0	0	1	0	0	1	4
RRS1	0	1	0	1	0	0	1	0	0	1	4
ZFR2	1	1	0	1	0	0	1	0	0	0	4
ANKRD12	0	1	0	1	0	0	1	0	0	1	4
VPS39	1	1	0	1	0	0	1	0	0	0	4
PSD3	1	1	0	1	0	0	1	0	0	0	4
POFUT1	1	1	0	1	0	0	1	0	0	0	4
NUP62	1	1	0	1	0	0	0	0	0	1	4
NUP62	1	0	0	1	0	0	1	0	0	1	4
HSPBP1	1	1	0	1	0	0	0	0	0	1	4
HSPBP1	1	0	0	1	0	0	1	0	0	1	4
FXN	1	1	0	1	0	0	0	0	0	1	4
ALPI	1	1	0	1	0	0	1	0	0	0	4
FUT6	1	1	0	1	0	0	0	0	0	1	4
KDSR	0	1	0	1	0	0	1	0	0	1	4
GABRB3	1	1	0	1	0	0	1	0	0	0	4
HYPK	0	1	0	1	0	0	1	0	0	1	4
BACE2	1	1	0	1	0	0	0	0	0	1	4
MMACHC	0	1	0	1	0	0	1	0	0	1	4
FBXW2	1	1	0	1	0	0	1	0	0	0	4
TSPAN17	1	1	0	1	0	0	0	0	0	1	4

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
HS6ST3	1	1	0	1	0	0	1	0	0	0	4
GNGT2	1	1	0	1	0	0	1	0	0	0	4
GPR1	1	1	0	1	0	0	0	0	0	1	4
GPR1	1	0	0	1	0	0	1	0	0	1	4
SPRYD4	1	1	0	1	0	0	0	0	0	1	4
C12orf61	0	1	0	1	0	0	1	0	0	1	4
KSR2	0	1	0	1	0	0	1	0	0	1	4
C14orf24	1	0	0	1	0	0	1	0	0	1	4
C14orf24	1	1	0	1	0	0	0	0	0	1	4
C19orf63	1	1	0	1	0	0	0	0	0	1	4
LOC285382	1	1	0	1	0	0	1	0	0	0	4
GPX3	0	1	0	1	0	0	1	0	0	1	4
GRB2	1	1	0	1	0	0	1	0	0	0	4
GRLF1	0	1	0	1	0	0	1	0	0	1	4
RBM15B	1	1	0	1	0	0	1	0	0	0	4
SEC61A1	0	1	0	1	0	0	1	0	0	1	4
HDLBP	1	0	0	1	0	0	1	0	0	1	4
HDLBP	1	1	0	1	0	0	0	0	0	1	4
HLA-E	1	1	0	1	0	0	0	0	0	1	4
ACACB	0	1	0	1	0	0	1	0	0	1	4
HOXC11	1	1	0	1	0	0	1	0	0	0	4
TMEM16J	1	1	0	1	0	0	1	0	0	0	4
ICAM4	1	1	0	1	0	0	0	0	0	1	4
ARL16	1	1	0	1	0	0	1	0	0	0	4
KY	0	1	0	1	0	0	1	0	0	1	4
MYLK4	1	1	0	1	0	0	1	0	0	0	4
NRBP2	1	0	0	1	0	0	1	0	0	1	4
NANOS1	0	1	0	1	0	0	1	0	0	1	4
IFIT3	1	0	0	1	0	0	1	0	0	1	4
INPP5B	0	1	0	1	0	0	1	0	0	1	4
ITGA6	1	0	0	1	0	0	1	0	0	1	4
KIFC3	1	1	0	1	0	0	0	0	0	1	4
VGLL3	1	1	0	1	0	0	1	0	0	0	4
SAMD5	1	1	0	1	0	0	1	0	0	0	4
LAIR2	0	1	0	1	0	0	1	0	0	1	4
ZNF793	0	1	0	1	0	0	1	0	0	1	4
FAM102A	1	1	0	1	0	0	1	0	0	0	4
SNX30	1	1	0	1	0	0	1	0	0	0	4
ZBTB34	1	1	0	1	0	0	1	0	0	0	4
HERV-FRD	0	1	0	1	0	0	1	0	0	1	4
ARR3	0	1	0	1	0	0	1	0	0	1	4
ARSD	1	1	0	1	0	0	1	0	0	0	4
MARK3	1	1	0	1	0	0	0	0	0	1	4
MECP2	1	0	0	1	0	0	1	0	0	1	4
MEF2D	1	1	0	1	0	0	1	0	0	0	4
MKLN1	1	1	0	1	0	0	1	0	0	0	4
TRIM67	0	1	0	1	0	0	1	0	0	1	4
MTM1	1	1	0	1	0	0	1	0	0	0	4
MYO1C	1	1	0	1	0	0	1	0	0	0	4

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
MYO9B	1	1	0	1	0	0	0	0	0	1	4
MYO9B	1	1	0	1	0	0	0	0	0	1	4
NDUF52	1	1	0	1	0	0	1	0	0	0	4
NDUFV3	1	1	0	1	0	0	1	0	0	0	4
NGFR	1	1	0	1	0	0	1	0	0	0	4
NHS	1	1	0	1	0	0	1	0	0	0	4
P4HB	1	1	0	1	0	0	0	0	0	1	4
NEUROG3	1	1	0	1	0	0	1	0	0	0	4
PARK2	1	1	0	1	0	0	0	0	0	1	4
PAX2	1	1	0	1	0	0	1	0	0	0	4
AK3	1	1	0	1	0	0	1	0	0	0	4
PCM1	0	1	0	1	0	0	1	0	0	1	4
KLHL5	1	1	0	1	0	0	1	0	0	0	4
IRAK4	1	1	0	1	0	0	0	0	0	1	4
ZNF580	1	1	0	1	0	0	0	0	0	1	4
CHMP1A	1	1	0	1	0	0	1	0	0	0	4
MGC29506	0	1	0	1	0	0	1	0	0	1	4
NRN1	1	1	0	1	0	0	1	0	0	0	4
MEX3C	1	1	0	1	0	0	1	0	0	0	4
CUTA	1	1	0	1	0	0	0	0	0	1	4
C7orf20	0	1	0	1	0	0	1	0	0	1	4
ATP6V1A	1	1	0	1	0	0	1	0	0	0	4
PHB	1	1	0	1	0	0	1	0	0	0	4
PHF2	1	1	0	1	0	0	1	0	0	0	4
PIK3C2A	0	1	0	1	0	0	1	0	0	1	4
PANK1	1	1	0	1	0	0	1	0	0	0	4
PLXNA2	1	1	0	1	0	0	1	0	0	0	4
38596	1	1	0	1	0	0	1	0	0	0	4
POLA1	1	1	0	1	0	0	1	0	0	0	4
EPB41L4B	1	1	0	1	0	0	1	0	0	0	4
L1TD1	0	1	0	1	0	0	1	0	0	1	4
PPARA	1	1	0	1	0	0	0	0	0	1	4
PPARA	1	0	0	1	0	0	1	0	0	1	4
SLC6A20	1	1	0	1	0	0	1	0	0	0	4
DNAJB12	1	1	0	1	0	0	1	0	0	0	4
FAM29A	1	1	0	1	0	0	1	0	0	0	4
BIVM	1	0	0	1	0	0	1	0	0	1	4
YTHDF1	1	1	0	1	0	0	1	0	0	0	4
C12orf41	1	1	0	1	0	0	1	0	0	0	4
PLEKHB2	0	1	0	1	0	0	1	0	0	1	4
PDPR	1	1	0	1	0	0	1	0	0	0	4
SRBD1	1	1	0	1	0	0	1	0	0	0	4
DDX19A	1	0	0	1	0	0	1	0	0	1	4
WDR33	1	1	0	1	0	0	0	0	0	1	4
PRELP	1	0	0	1	0	0	1	0	0	1	4
PRELP	1	1	0	1	0	0	0	0	0	1	4
STAB2	1	1	0	1	0	0	1	0	0	0	4
LUC7L	1	1	0	1	0	0	1	0	0	0	4
TSR1	1	1	0	1	0	0	1	0	0	0	4

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
FAM63A	1	0	0	1	0	0	1	0	0	1	4
MAPK3	1	1	0	1	0	0	0	0	0	1	4
UBFD1	1	1	0	1	0	0	1	0	0	0	4
PCDHB9	0	1	0	1	0	0	1	0	0	1	4
ANKH	0	1	0	1	0	0	1	0	0	1	4
LMOD3	0	1	0	1	0	0	1	0	0	1	4
RP5-1022P6.2	1	1	0	1	0	0	1	0	0	0	4
TMPRSS4	1	1	0	1	0	0	1	0	0	0	4
KIAA1191	1	1	0	1	0	0	0	0	0	1	4
KIAA1191	1	0	0	1	0	0	1	0	0	1	4
WDR18	1	1	0	1	0	0	1	0	0	0	4
NDRG2	1	0	0	1	0	0	1	0	0	1	4
NDRG2	1	1	0	1	0	0	0	0	0	1	4
SLC12A5	1	0	0	1	0	0	1	0	0	1	4
HECW2	1	1	0	1	0	0	1	0	0	0	4
KIAA1549	0	1	0	1	0	0	1	0	0	1	4
PCDHB16	1	1	0	1	0	0	1	0	0	0	4
PURB	0	1	0	1	0	0	1	0	0	1	4
PLEKHA2	0	1	0	1	0	0	1	0	0	1	4
RGS12	1	1	0	1	0	0	1	0	0	0	4
RPS6KA2	1	0	0	1	0	0	1	0	0	1	4
RYR3	1	1	0	1	0	0	1	0	0	0	4
SC5DL	1	0	0	1	0	0	1	0	0	1	4
SC5DL	1	1	0	1	0	0	0	0	0	1	4
ATXN1	1	0	0	1	0	0	1	0	0	1	4
ATXN1	1	1	0	1	0	0	0	0	0	1	4
LOC63920	0	1	0	1	0	0	1	0	0	1	4
GFRA4	1	1	0	1	0	0	0	0	0	1	4
GFRA4	1	0	0	1	0	0	1	0	0	1	4
ERAP2	1	1	0	1	0	0	0	0	0	1	4
ERAP2	1	0	0	1	0	0	1	0	0	1	4
LOC643641	0	1	0	1	0	0	1	0	0	1	4
hCG_2040376	0	1	0	1	0	0	1	0	0	1	4
RABL5	1	1	0	1	0	0	0	0	0	1	4
BMP3	0	1	0	1	0	0	1	0	0	1	4
MRPS34	1	1	0	1	0	0	1	0	0	0	4
SNAP25	1	1	0	1	0	0	1	0	0	0	4
SP4	1	1	0	1	0	0	1	0	0	0	4
SPAST	1	1	0	1	0	0	1	0	0	0	4
SPN	1	1	0	1	0	0	1	0	0	0	4
STAR	1	0	0	1	0	0	1	0	0	1	4
STXBP1	1	1	0	1	0	0	0	0	0	1	4
TMPO	1	0	0	1	0	0	1	0	0	1	4
TMPO	1	1	0	1	0	0	0	0	0	1	4
UBA1	1	1	0	1	0	0	0	0	0	1	4
SLC35A2	1	1	0	1	0	0	1	0	0	0	4
UTRN	1	1	0	1	0	0	1	0	0	0	4

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
VDR	1	1	0	1	0	0	0	0	0	1	4
WNT7B	1	1	0	1	0	0	1	0	0	0	4
ZNF19	1	1	0	1	0	0	1	0	0	0	4
ZNF215	1	1	0	1	0	0	1	0	0	0	4
ZYX	1	1	0	1	0	0	1	0	0	0	4
DNALI1	1	1	0	1	0	0	1	0	0	0	4
EVI5	1	1	0	1	0	0	1	0	0	0	4
CACNB2	1	0	0	1	0	0	1	0	0	1	4
CACNB2	1	1	0	1	0	0	0	0	0	1	4
ZNF655	1	0	0	1	0	0	1	0	0	1	4
ZNF655	1	1	0	1	0	0	0	0	0	1	4
C7orf26	1	1	0	1	0	0	1	0	0	0	4
ATG9A	1	0	0	1	0	0	1	0	0	1	4
ATG9A	1	1	0	1	0	0	0	0	0	1	4
METT10D	0	1	0	1	0	0	1	0	0	1	4
C20orf121	1	1	0	1	0	0	0	0	0	1	4
C20orf121	1	0	0	1	0	0	1	0	0	1	4
PPP1R3B	0	1	0	1	0	0	1	0	0	1	4
ZNF669	0	1	0	1	0	0	1	0	0	1	4
DCAKD	1	1	0	1	0	0	0	0	0	1	4
DCAKD	1	0	0	1	0	0	1	0	0	1	4
ZNF212	0	1	0	1	0	0	1	0	0	1	4
FOXRED2	1	1	0	1	0	0	0	0	0	1	4
FOXRED2	1	0	0	1	0	0	1	0	0	1	4
FBXL18	0	1	0	1	0	0	1	0	0	1	4
ITIH5	1	1	0	1	0	0	1	0	0	0	4
KIAA1715	1	1	0	1	0	0	1	0	0	0	4
HM13	0	1	0	1	0	0	1	0	0	1	4
SLC38A1	1	0	0	1	0	0	1	0	0	1	4
NDEL1	1	1	0	1	0	0	0	0	0	1	4
KAZALD1	1	1	0	1	0	0	1	0	0	0	4
TRIM7	1	1	0	1	0	0	0	0	0	1	4
VANGL1	1	1	0	1	0	0	1	0	0	0	4
SCRT1	1	1	0	1	0	0	0	0	0	1	4
TMUB1	1	0	0	1	0	0	1	0	0	1	4
FRMD8	1	1	0	1	0	0	1	0	0	0	4
PIP5K1A	1	0	0	1	0	0	1	0	0	1	4
TMPRSS13	1	1	0	1	0	0	1	0	0	0	4
KATNAL1	1	1	0	1	0	0	0	0	0	1	4
KATNAL1	1	0	0	1	0	0	1	0	0	1	4
GPR103	0	1	0	1	0	0	1	0	0	1	4
RNASEH2C	1	1	0	1	0	0	1	0	0	0	4
SETD3	0	1	0	1	0	0	1	0	0	1	4
GPR123	1	0	0	1	0	0	1	0	0	1	4
L3MBTL3	1	1	0	1	0	0	1	0	0	0	4
ZNF527	0	1	0	1	0	0	1	0	0	1	4
SLC12A8	0	1	0	1	0	0	1	0	0	1	4
FBXL10	1	1	0	1	0	0	1	0	0	0	4
ATCAY	0	1	0	1	0	0	1	0	0	1	4

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
DNAJC14	1	1	0	1	0	0	1	0	0	0	4
TSPYL5	0	1	0	1	0	0	1	0	0	1	4
KHSRP	0	1	0	1	0	0	1	0	0	1	4
CAV2	1	1	0	1	0	0	0	0	0	1	4
JRK	0	1	0	1	0	0	1	0	0	1	4
OASL	1	1	0	1	0	0	0	0	0	1	4
CBL	1	1	0	1	0	0	1	0	0	0	4
STX11	1	1	0	1	0	0	1	0	0	0	4
SERPINB7	1	1	0	1	0	0	0	0	0	1	4
SERPINB7	1	0	0	1	0	0	1	0	0	1	4
RGS11	1	0	0	1	0	0	1	0	0	1	4
RGS11	1	1	0	1	0	0	0	0	0	1	4
TNFRSF10A	1	1	0	1	0	0	1	0	0	0	4
DPM2	1	1	0	1	0	0	1	0	0	0	4
CACNA1I	1	1	0	1	0	0	0	0	0	1	4
CACNA1I	1	0	0	1	0	0	1	0	0	1	4
RAB7L1	1	0	0	1	0	0	1	0	0	1	4
UBE3B	1	0	0	1	0	0	1	0	0	1	4
UBE3B	1	1	0	1	0	0	0	0	0	1	4
LIMD1	1	1	0	1	0	0	1	0	0	0	4
ZSWIM1	0	1	0	1	0	0	1	0	0	1	4
PSTPIP2	1	1	0	1	0	0	1	0	0	0	4
COX19	0	1	0	1	0	0	1	0	0	1	4
HN1L	1	1	0	1	0	0	1	0	0	0	4
KIFC2	0	1	0	1	0	0	1	0	0	1	4
ACVR1B	1	1	0	1	0	0	1	0	0	0	4
CDKN2AIPNL	0	1	0	1	0	0	1	0	0	1	4
DCLK1	1	1	0	1	0	0	1	0	0	0	4
ZMYM6	0	1	0	1	0	0	1	0	0	1	4
C4orf42	0	1	0	1	0	0	1	0	0	1	4
DLGAP2	1	1	0	1	0	0	1	0	0	0	4
ZNF461	0	1	0	1	0	0	1	0	0	1	4
LYK5	1	1	0	1	0	0	1	0	0	0	4
MAPKAPK2	1	1	0	1	0	0	1	0	0	0	4
MGC16385	0	1	0	1	0	0	1	0	0	1	4
EDG5	0	1	0	1	0	0	1	0	0	1	4
39873	1	1	0	1	0	0	1	0	0	0	4
ACCN3	1	1	0	1	0	0	1	0	0	0	4
C9orf61	1	1	0	1	0	0	0	0	0	1	4
C9orf61	1	0	0	1	0	0	1	0	0	1	4
HAND1	1	1	0	1	0	0	1	0	0	0	4
H2AFV	0	1	0	1	0	0	1	0	0	1	4
MED23	1	1	0	1	0	0	1	0	0	0	4
GOSR1	1	0	0	1	0	0	1	0	0	1	4
GOSR1	1	1	0	1	0	0	0	0	0	1	4
PUNC	0	1	0	1	0	0	1	0	0	1	4
SOX13	0	1	0	1	0	0	1	0	0	1	4
ENTPD4	1	1	0	1	0	0	0	0	0	1	4

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
KIAA0226	1	1	0	1	0	0	1	0	0	0	4
NOS1AP	1	1	0	1	0	0	0	0	0	1	4
KIAA0101	1	1	0	1	0	0	0	0	0	1	4
MAML1	0	1	0	1	0	0	1	0	0	1	4
TATDN2	1	1	0	1	0	0	1	0	0	0	4
PLEKHM1	0	1	0	1	0	0	1	0	0	1	4
NUAK1	1	1	0	1	0	0	1	0	0	0	4
SRGAP3	1	1	0	1	0	0	1	0	0	0	4
MED6	1	1	0	0	0	0	1	0	0	1	4
SFRS14	1	1	0	0	0	0	1	0	0	1	4
LRRC56	1	1	0	0	0	0	1	0	0	1	4
ZNF526	1	1	0	0	0	0	1	0	0	1	4
TMEM45B	1	1	0	0	0	0	1	0	0	1	4
MOBK2A	1	1	0	0	0	0	1	0	0	1	4
SRCRB4D	1	1	0	0	0	0	1	0	0	1	4
ASB8	1	1	0	0	0	0	1	0	0	1	4
C20orf151	1	1	0	0	0	0	1	0	0	1	4
TTL	1	1	0	0	0	0	1	0	0	1	4
C5orf41	1	1	0	0	0	0	1	0	0	1	4
DLX2	1	1	0	0	0	0	1	0	0	1	4
DSG3	1	1	0	0	0	0	1	0	0	1	4
RHOBTB3	1	1	0	0	0	0	1	0	0	1	4
SMG1	1	1	0	0	0	0	1	0	0	1	4
ZC3H4	1	1	0	0	0	0	1	0	0	1	4
ARHGEF9	1	1	0	0	0	0	1	0	0	1	4
OTP	1	1	0	0	0	0	1	0	0	1	4
TRIM29	1	1	0	0	0	0	1	0	0	1	4
SHPK	1	1	0	0	0	0	1	0	0	1	4
KIF4A	1	1	0	0	0	0	1	0	0	1	4
BACE2	1	1	0	0	0	0	1	0	0	1	4
PNKD	1	1	0	0	0	0	1	0	0	1	4
KLHL3	1	1	0	0	0	0	1	0	0	1	4
C1orf107	1	1	0	0	0	0	1	0	0	1	4
SCARA5	1	1	0	0	0	0	1	0	0	1	4
TUSC5	1	1	0	0	0	0	1	0	0	1	4
PNMA3	1	1	0	0	0	0	1	0	0	1	4
C16orf5	1	1	0	0	0	0	1	0	0	1	4
PACSLN1	1	1	0	0	0	0	1	0	0	1	4
ICAM4	1	1	0	0	0	0	1	0	0	1	4
IL1R1	1	1	0	0	0	0	1	0	0	1	4
IL13	1	1	0	0	0	0	1	0	0	1	4
RHOA	1	1	0	0	0	0	1	0	0	1	4
FLG2	1	1	0	0	0	0	1	0	0	1	4
SH2D5	1	1	0	0	0	0	1	0	0	1	4
MLLT6	1	1	0	0	0	0	1	0	0	1	4
OSM	1	1	0	0	0	0	1	0	0	1	4
C21orf58	1	1	0	0	0	0	1	0	0	1	4
MRPS10	1	1	0	0	0	0	1	0	0	1	4
VPS13D	1	1	0	0	0	0	1	0	0	1	4

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
PARL	1	1	0	0	0	0	1	0	0	1	4
CCBL2	1	1	0	0	0	0	1	0	0	1	4
C12orf5	1	1	0	0	0	0	1	0	0	1	4
TBC1D24	1	1	0	0	0	0	1	0	0	1	4
CPNE5	1	1	0	0	0	0	1	0	0	1	4
PEX19	1	1	0	0	0	0	1	0	0	1	4
WIZ	1	1	0	0	0	0	1	0	0	1	4
SORT1	1	1	0	0	0	0	1	0	0	1	4
UNKL	1	1	0	0	0	0	1	0	0	1	4
SMARCD1	1	1	0	0	0	0	1	0	0	1	4
TPM3	1	1	0	0	0	0	1	0	0	1	4
TREML2	1	1	0	0	0	0	1	0	0	1	4
ASRGL1	1	1	0	0	0	0	1	0	0	1	4
CAPZB	1	1	0	0	0	0	1	0	0	1	4
FAM83A	1	1	0	0	0	0	1	0	0	1	4
KCNAB2	1	1	0	0	0	0	1	0	0	1	4
STX16	1	1	0	0	0	0	1	0	0	1	4
C12orf65	1	1	0	0	0	0	1	0	0	1	4
C22orf32	1	1	0	0	0	0	1	0	0	1	4
DSEL	1	1	0	0	0	0	1	0	0	1	4
SNPH	1	1	0	0	0	0	1	0	0	1	4
RUSC2	1	1	0	0	0	0	1	0	0	1	4
KIF14	1	1	0	0	0	0	1	0	0	1	4
IL18BP	1	0	0	1	0	0	0	0	0	1	3
B3GALT5	0	1	0	1	0	0	0	0	0	1	3
B3GALT5	1	0	0	1	0	0	0	0	0	1	3
B3GALT5	0	0	0	1	0	0	1	0	0	1	3
ADARB1	1	0	0	1	0	0	0	0	0	1	3
SPON2	1	0	0	1	0	0	0	0	0	1	3
SORBS1	1	0	0	1	0	0	0	0	0	1	3
SORBS1	1	0	0	1	0	0	0	0	0	1	3
SORBS1	1	0	0	1	0	0	0	0	0	1	3
SORBS1	1	0	0	1	0	0	0	0	0	1	3
SORBS1	1	0	0	1	0	0	0	0	0	1	3
SORBS1	1	0	0	1	0	0	0	0	0	1	3
ERLIN1	1	1	0	1	0	0	0	0	0	0	3
ERLIN1	1	0	0	1	0	0	1	0	0	0	3
FUSIP1	0	1	0	1	0	0	0	0	0	1	3
LILRB3	1	0	0	1	0	0	0	0	0	1	3
STON1	0	0	0	1	0	0	1	0	0	1	3
TRIOBP	1	0	0	1	0	0	0	0	0	1	3
DIDO1	1	0	0	1	0	0	0	0	0	1	3
ABCB8	0	1	0	1	0	0	1	0	0	0	3
PHF21B	1	0	0	1	0	0	1	0	0	0	3
DTX2	1	0	0	1	0	0	0	0	0	1	3
PALM2	0	1	0	1	0	0	0	0	0	1	3
PALM2	0	0	0	1	0	0	1	0	0	1	3
CISH	1	0	0	1	0	0	0	0	0	1	3
CLCN3	1	0	0	1	0	0	0	0	0	1	3

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
GGT6	1	0	0	1	0	0	0	0	0	1	3
DMBX1	1	0	0	1	0	0	0	0	0	1	3
COL6A2	1	1	0	1	0	0	0	0	0	0	3
COPA	1	0	0	1	0	0	0	0	0	1	3
DNAJC21	1	1	0	1	0	0	0	0	0	0	3
DNAJC21	1	0	0	1	0	0	1	0	0	0	3
HINT3	1	1	0	1	0	0	0	0	0	0	3
DNHD1	0	1	0	1	0	0	0	0	0	1	3
PRICKLE1	1	0	0	1	0	0	1	0	0	0	3
CYB561	1	0	0	1	0	0	0	0	0	1	3
CYB561	1	0	0	1	0	0	0	0	0	1	3
PRELID2	1	0	0	1	0	0	0	0	0	1	3
C16orf82	0	0	0	1	0	0	1	0	0	1	3
CYB5R3	1	0	0	1	0	0	0	0	0	1	3
CYB5R3	1	0	0	1	0	0	0	0	0	1	3
DIO2	0	1	0	1	0	0	0	0	0	1	3
DIO2	0	0	0	1	0	0	1	0	0	1	3
EDA	1	0	0	1	0	0	0	0	0	1	3
P76	0	1	0	1	0	0	1	0	0	0	3
ELK1	1	0	0	1	0	0	0	0	0	1	3
MUC20	0	0	0	1	0	0	1	0	0	1	3
MUC20	0	1	0	1	0	0	0	0	0	1	3
EMP2	0	1	0	1	0	0	1	0	0	0	3
TUBB	1	1	0	1	0	0	0	0	0	0	3
ESRRG	1	0	0	1	0	0	0	0	0	1	3
ETV1	1	0	0	1	0	0	1	0	0	0	3
EXT2	1	1	0	1	0	0	0	0	0	0	3
CYBASC3	1	0	0	1	0	0	1	0	0	0	3
ALDH3B1	0	0	0	1	0	0	1	0	0	1	3
ALDH3B1	0	1	0	1	0	0	0	0	0	1	3
FKTN	0	0	0	1	0	0	1	0	0	1	3
TMEM130	1	0	0	1	0	0	0	0	0	1	3
SLC29A4	1	0	0	1	0	0	0	0	0	1	3
FHL1	1	0	0	1	0	0	1	0	0	0	3
ZNF365	1	0	0	1	0	0	1	0	0	0	3
NCBP2	1	0	0	1	0	0	0	0	0	1	3
SPEN	0	1	0	1	0	0	1	0	0	0	3
TNRC6B	1	0	0	1	0	0	0	0	0	1	3
SULF1	0	1	0	1	0	0	0	0	0	1	3
FBXO28	0	1	0	1	0	0	0	0	0	1	3
C22orf9	1	0	0	1	0	0	0	0	0	1	3
CRTC1	0	1	0	1	0	0	1	0	0	0	3
BRD4	0	1	0	1	0	0	0	0	0	1	3
SLC39A14	1	0	0	1	0	0	0	0	0	1	3
SLC39A14	1	0	0	1	0	0	0	0	0	1	3
CCRK	1	0	0	1	0	0	0	0	0	1	3
CCRK	1	0	0	1	0	0	0	0	0	1	3
RUSC1	1	0	0	1	0	0	0	0	0	1	3
RUSC1	1	0	0	1	0	0	0	0	0	1	3

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
RUSC1	1	0	0	1	0	0	0	0	0	1	3
NUP62	1	0	0	1	0	0	0	0	0	1	3
SGK3	1	0	0	1	0	0	0	0	0	1	3
SGK3	1	0	0	1	0	0	0	0	0	1	3
ALOX12B	0	1	0	1	0	0	0	0	0	1	3
MSRB3	1	0	0	1	0	0	0	0	0	1	3
TMCO4	1	0	0	1	0	0	1	0	0	0	3
GABRB2	1	0	0	1	0	0	0	0	0	1	3
RIPK5	1	0	0	1	0	0	0	0	0	1	3
BACE2	1	0	0	1	0	0	0	0	0	1	3
THUMPD3	0	1	0	1	0	0	1	0	0	0	3
PNKD	1	0	0	1	0	0	0	0	0	1	3
TSPAN17	1	0	0	1	0	0	0	0	0	1	3
ABL2	0	1	0	1	0	0	0	0	0	1	3
ABL2	0	0	0	1	0	0	1	0	0	1	3
CACYBP	1	0	0	1	0	0	0	0	0	1	3
GNAO1	0	1	0	1	0	0	0	0	0	1	3
FLJ35848	0	0	0	1	0	0	1	0	0	1	3
FAM80A	0	1	0	1	0	0	0	0	0	1	3
RNF212	1	1	0	1	0	0	0	0	0	0	3
LOC285908	0	0	0	1	0	0	1	0	0	1	3
DPY19L4	0	1	0	1	0	0	0	0	0	1	3
HIPK2	0	1	0	1	0	0	0	0	0	1	3
GRIN1	1	0	0	1	0	0	0	0	0	1	3
GRIN1	1	0	0	1	0	0	0	0	0	1	3
OLA1	1	0	0	1	0	0	0	0	0	1	3
REPIN1	1	0	0	1	0	0	0	0	0	1	3
HNF4A	1	0	0	1	0	0	0	0	0	1	3
7A5	0	1	0	1	0	0	0	0	0	1	3
ITGA6	1	0	0	1	0	0	0	0	0	1	3
CD82	1	0	0	1	0	0	0	0	0	1	3
C1orf95	0	1	0	1	0	0	1	0	0	0	3
KCNJ16	1	1	0	1	0	0	0	0	0	0	3
KCNJ16	1	0	0	1	0	0	1	0	0	0	3
KCNK3	0	1	0	1	0	0	1	0	0	0	3
KCNQ1	1	0	0	1	0	0	0	0	0	1	3
KIFC3	1	0	0	1	0	0	0	0	0	1	3
KIFC3	1	0	0	1	0	0	0	0	0	1	3
SH2D5	1	0	0	1	0	0	0	0	0	1	3
LRP3	0	1	0	1	0	0	0	0	0	1	3
MAFG	1	0	0	1	0	0	1	0	0	0	3
MAFG	1	1	0	1	0	0	0	0	0	0	3
MARK3	1	0	0	1	0	0	0	0	0	1	3
MARK3	1	0	0	1	0	0	0	0	0	1	3
MARK3	1	0	0	1	0	0	0	0	0	1	3
MCM4	1	0	0	1	0	0	0	0	0	1	3
MEF2A	1	1	0	1	0	0	0	0	0	0	3
NEDD9	0	1	0	1	0	0	1	0	0	0	3
NPM1	0	1	0	1	0	0	1	0	0	0	3

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
OAS3	0	1	0	1	0	0	1	0	0	0	3
PARK2	1	0	0	1	0	0	0	0	0	1	3
PARK2	1	0	0	1	0	0	0	0	0	1	3
PAX2	1	1	0	1	0	0	0	0	0	0	3
ACOX1	1	0	0	1	0	0	0	0	0	1	3
ZNF580	0	0	0	1	0	0	1	0	0	1	3
ANKFY1	1	1	0	1	0	0	0	0	0	0	3
TRIM33	1	0	0	1	0	0	0	0	0	1	3
CUTA	1	0	0	1	0	0	0	0	0	1	3
CUTA	1	0	0	1	0	0	0	0	0	1	3
CUTA	1	0	0	1	0	0	0	0	0	1	3
CUTA	0	0	0	1	0	0	1	0	0	1	3
PGR	0	1	0	1	0	0	0	0	0	1	3
PLEC1	1	0	0	1	0	0	0	0	0	1	3
PLEC1	1	0	0	1	0	0	0	0	0	1	3
PLEC1	1	0	0	1	0	0	0	0	0	1	3
PLEC1	1	0	0	1	0	0	0	0	0	1	3
PLEC1	1	0	0	1	0	0	0	0	0	1	3
PCBP3	1	0	0	1	0	0	0	0	0	1	3
POLH	0	1	0	1	0	0	0	0	0	1	3
RNF216	1	0	0	1	0	0	0	0	0	1	3
POU6F1	0	1	0	1	0	0	1	0	0	0	3
DNAJB12	1	1	0	1	0	0	0	0	0	0	3
FLJ20184	0	1	0	1	0	0	1	0	0	0	3
FLJ20309	0	1	0	1	0	0	1	0	0	0	3
PPP2R5C	1	0	0	1	0	0	1	0	0	0	3
C17orf85	0	0	0	1	0	0	1	0	0	1	3
ZNF331	1	1	0	1	0	0	0	0	0	0	3
ZNF331	1	0	0	1	0	0	1	0	0	0	3
PARL	1	0	0	1	0	0	0	0	0	1	3
ELMOD1	1	0	0	1	0	0	0	0	0	1	3
LUC7L	1	1	0	1	0	0	0	0	0	0	3
H2AFJ	0	1	0	1	0	0	1	0	0	0	3
FAM63A	1	0	0	1	0	0	0	0	0	1	3
HR	1	0	0	1	0	0	0	0	0	1	3
C1orf183	1	0	0	1	0	0	0	0	0	1	3
KIAA1191	1	0	0	1	0	0	0	0	0	1	3
NDRG2	1	0	0	1	0	0	0	0	0	1	3
NDRG2	1	0	0	1	0	0	0	0	0	1	3
NDRG2	1	0	0	1	0	0	0	0	0	1	3
NDRG2	1	0	0	1	0	0	0	0	0	1	3
NDRG2	1	0	0	1	0	0	0	0	0	1	3
TMCC3	0	1	0	1	0	0	1	0	0	0	3
PPM1H	0	1	0	1	0	0	1	0	0	0	3
BEGAIN	1	0	0	1	0	0	1	0	0	0	3
C8orf79	0	1	0	1	0	0	1	0	0	0	3
PTPRS	1	0	0	1	0	0	0	0	0	1	3
PTPRS	1	0	0	1	0	0	0	0	0	1	3
GNB4	0	1	0	1	0	0	1	0	0	0	3

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
RSU1	1	0	0	1	0	0	0	0	0	1	3
SFRS4	1	1	0	1	0	0	0	0	0	0	3
C10orf141	0	1	0	1	0	0	1	0	0	0	3
HIF3A	1	0	0	1	0	0	1	0	0	0	3
CTAGE1	0	1	0	1	0	0	1	0	0	0	3
RABL5	1	0	0	1	0	0	0	0	0	1	3
RABL5	1	0	0	1	0	0	0	0	0	1	3
PLEKHG2	0	1	0	1	0	0	0	0	0	1	3
BMPR1B	0	1	0	1	0	0	0	0	0	1	3
SMARCD1	1	0	0	1	0	0	0	0	0	1	3
FSCN1	1	0	0	1	0	0	1	0	0	0	3
SP100	0	1	0	1	0	0	1	0	0	0	3
SSR1	0	1	0	1	0	0	0	0	0	1	3
STAR	0	1	0	1	0	0	0	0	0	1	3
STAT3	1	0	0	1	0	0	0	0	0	1	3
STAT3	0	1	0	1	0	0	0	0	0	1	3
BSG	1	0	0	1	0	0	0	0	0	1	3
BSG	1	0	0	1	0	0	0	0	0	1	3
SUPT5H	1	1	0	1	0	0	0	0	0	0	3
SUPT5H	1	0	0	1	0	0	1	0	0	0	3
SURF6	0	1	0	1	0	0	1	0	0	0	3
C6orf35	0	1	0	1	0	0	0	0	0	1	3
UBA1	1	0	0	1	0	0	0	0	0	1	3
SLC35A2	1	1	0	1	0	0	0	0	0	0	3
UCK2	0	1	0	1	0	0	0	0	0	1	3
CACNB2	1	0	0	1	0	0	0	0	0	1	3
CACNB2	1	0	0	1	0	0	0	0	0	1	3
CACNB2	1	0	0	1	0	0	0	0	0	1	3
CACNB2	1	0	0	1	0	0	0	0	0	1	3
ZNF655	1	0	0	1	0	0	0	0	0	1	3
FKRP	1	0	0	1	0	0	0	0	0	1	3
ALDH5A1	1	0	0	1	0	0	0	0	0	1	3
HMBX1	1	0	0	1	0	0	1	0	0	0	3
SH3TC2	0	1	0	1	0	0	1	0	0	0	3
CXorf36	1	1	0	1	0	0	0	0	0	0	3
GSTCD	1	1	0	1	0	0	0	0	0	0	3
GSTCD	1	0	0	1	0	0	1	0	0	0	3
ST7	1	0	0	1	0	0	1	0	0	0	3
TMC5	1	0	0	1	0	0	0	0	0	1	3
TMC5	1	0	0	1	0	0	0	0	0	1	3
WWC2	0	1	0	1	0	0	1	0	0	0	3
ASRGL1	1	0	0	1	0	0	0	0	0	1	3
PDGFD	1	0	0	1	0	0	0	0	0	1	3
PABPN1	0	1	0	1	0	0	1	0	0	0	3
TRIM7	1	0	0	1	0	0	0	0	0	1	3
TRIM7	1	0	0	1	0	0	0	0	0	1	3
COLQ	1	1	0	1	0	0	0	0	0	0	3
COLQ	1	0	0	1	0	0	1	0	0	0	3
DNAL1	0	1	0	1	0	0	0	0	0	1	3

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
TM2D2	1	1	0	1	0	0	0	0	0	0	3
CASP6	1	0	0	1	0	0	0	0	0	1	3
CASP8	1	0	0	1	0	0	0	0	0	1	3
CASP8	1	0	0	1	0	0	0	0	0	1	3
CASP8	1	0	0	1	0	0	0	0	0	1	3
CASP8	1	0	0	1	0	0	0	0	0	1	3
FBXL10	1	1	0	1	0	0	0	0	0	0	3
PIGO	1	0	0	1	0	0	0	0	0	1	3
CDC42BPA	1	0	0	1	0	0	0	0	0	1	3
ZDHC12	0	1	0	1	0	0	1	0	0	0	3
SLC7A3	1	0	0	1	0	0	0	0	0	1	3
RANBP3	1	0	0	1	0	0	0	0	0	1	3
RANBP3	1	0	0	1	0	0	0	0	0	1	3
KCNAB2	1	0	0	1	0	0	0	0	0	1	3
GAS7	1	0	0	1	0	0	0	0	0	1	3
GAS7	1	0	0	1	0	0	0	0	0	1	3
GAS7	1	0	0	1	0	0	0	0	0	1	3
APOL1	1	0	0	1	0	0	1	0	0	0	3
PRIC285	1	0	0	1	0	0	0	0	0	1	3
MKNK1	1	0	0	1	0	0	0	0	0	1	3
GTPBP10	1	0	0	1	0	0	0	0	0	1	3
STX16	1	0	0	1	0	0	0	0	0	1	3
AP3D1	1	0	0	1	0	0	0	0	0	1	3
NAV2	1	0	0	1	0	0	0	0	0	1	3
NAV2	1	0	0	1	0	0	0	0	0	1	3
CCDC149	1	1	0	1	0	0	0	0	0	0	3
C19orf6	1	1	0	1	0	0	0	0	0	0	3
ARHGEF2	1	0	0	1	0	0	1	0	0	0	3
GPR56	1	0	0	1	0	0	0	0	0	1	3
GPR56	0	0	0	1	0	0	1	0	0	1	3
ACCN3	1	1	0	1	0	0	0	0	0	0	3
SNAP29	0	1	0	1	0	0	1	0	0	0	3
EFTUD2	1	1	0	1	0	0	0	0	0	0	3
TAOK2	1	1	0	1	0	0	0	0	0	0	3
RECQL5	1	1	0	1	0	0	0	0	0	0	3
RECQL5	1	1	0	1	0	0	0	0	0	0	3
MAP4K4	1	0	0	1	0	0	0	0	0	1	3
GOSR1	1	0	0	1	0	0	0	0	0	1	3
ENTPD4	1	0	0	1	0	0	0	0	0	1	3
CLCA3	0	0	0	1	0	0	1	0	0	1	3
FAM131B	1	0	0	1	0	0	0	0	0	1	3
STARD8	1	0	0	1	0	0	1	0	0	0	3
APPBP2	1	1	0	0	0	0	1	0	0	0	3
PAICS	1	1	0	0	0	0	1	0	0	0	3
DIDO1	1	1	0	0	0	0	0	0	0	1	3
ADAMTS6	1	1	0	0	0	0	1	0	0	0	3
DTX2	1	1	0	0	0	0	0	0	0	1	3
DTX2	1	0	0	0	0	0	1	0	0	1	3
KLHDC7A	1	1	0	0	0	0	1	0	0	0	3

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
C7orf55	1	0	0	0	0	0	1	0	0	1	3
DFFA	1	1	0	0	0	0	0	0	0	1	3
EFNB3	1	1	0	0	0	0	1	0	0	0	3
CELSR3	1	0	0	0	0	0	1	0	0	1	3
TMEM16F	1	0	0	0	0	0	1	0	0	1	3
AKT2	1	0	0	0	0	0	1	0	0	1	3
NAPEPLD	1	1	0	0	0	0	0	0	0	1	3
ZHX2	1	1	0	0	0	0	1	0	0	0	3
TNRC6B	1	0	0	0	0	0	1	0	0	1	3
OTUD3	1	0	0	0	0	0	1	0	0	1	3
KIF13B	1	1	0	0	0	0	1	0	0	0	3
ACSL6	1	0	0	0	0	0	1	0	0	1	3
NPHP4	1	0	0	0	0	0	1	0	0	1	3
FRAG1	1	0	0	0	0	0	1	0	0	1	3
BCL9L	1	0	0	0	0	0	1	0	0	1	3
C11orf77	1	0	0	0	0	0	1	0	0	1	3
REPIN1	1	1	0	0	0	0	0	0	0	1	3
ANXA11	1	0	0	0	0	0	1	0	0	1	3
HTR4	1	1	0	0	0	0	1	0	0	0	3
ICAM4	1	1	0	0	0	0	0	0	0	1	3
IFIT3	1	1	0	0	0	0	0	0	0	1	3
LCE1B	1	1	0	0	0	0	1	0	0	0	3
SMAD3	1	1	0	0	0	0	1	0	0	0	3
MECP2	1	1	0	0	0	0	0	0	0	1	3
NOTCH2	1	0	0	0	0	0	1	0	0	1	3
PDE11A	1	1	0	0	0	0	1	0	0	0	3
FZR1	1	0	0	0	0	0	1	0	0	1	3
SLC25A39	1	0	0	0	0	0	1	0	0	1	3
PKD1	1	1	0	0	0	0	1	0	0	0	3
MTMR12	1	1	0	0	0	0	1	0	0	0	3
RBM35A	1	1	0	0	0	0	1	0	0	0	3
UQCC	1	0	0	0	0	0	1	0	0	1	3
PPP3CB	1	0	0	0	0	0	1	0	0	1	3
DPYSL5	1	1	0	0	0	0	1	0	0	0	3
SLC44A2	1	1	0	0	0	0	1	0	0	0	3
RPS6KA2	1	1	0	0	0	0	0	0	0	1	3
SP1	1	1	0	0	0	0	1	0	0	0	3
VAMP1	1	1	0	0	0	0	1	0	0	0	3
TBL1X	1	1	0	0	0	0	1	0	0	0	3
TMEM1	1	1	0	0	0	0	1	0	0	0	3
VDR	1	0	0	0	0	0	1	0	0	1	3
DERL1	1	1	0	0	0	0	1	0	0	0	3
C14orf45	1	1	0	0	0	0	1	0	0	0	3
SLC7A5	1	1	0	0	0	0	1	0	0	0	3
SLC38A1	1	1	0	0	0	0	0	0	0	1	3
ELL	1	0	0	0	0	0	1	0	0	1	3
ARID5B	1	0	0	0	0	0	1	0	0	1	3
LL22NC03-75B3.6	1	0	0	0	0	0	1	0	0	1	3

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
KIAA1754	1	1	0	0	0	0	1	0	0	0	3
AOC3	1	1	0	0	0	0	1	0	0	0	3
BRSK2	1	1	0	0	0	0	1	0	0	0	3
RICS	1	0	0	0	0	0	1	0	0	1	3
RNF144A	1	1	0	0	0	0	1	0	0	0	3
HS3ST3B1	1	1	0	0	0	0	1	0	0	0	3
CDH5	0	1	0	0	0	0	1	0	0	1	3
CTDSP2	0	1	0	0	0	0	1	0	0	1	3
STX6	0	1	0	0	0	0	1	0	0	1	3
WDR68	0	1	0	0	0	0	1	0	0	1	3
ABCC4	0	1	0	0	0	0	1	0	0	1	3
TRDN	0	1	0	0	0	0	1	0	0	1	3
CORO2B	0	1	0	0	0	0	1	0	0	1	3
CDIPT	0	1	0	0	0	0	1	0	0	1	3
MXD4	0	1	0	0	0	0	1	0	0	1	3
ZBTB6	0	1	0	0	0	0	1	0	0	1	3
CPLX1	0	1	0	0	0	0	1	0	0	1	3
METAP2	0	1	0	0	0	0	1	0	0	1	3
AKAP13	0	1	0	0	0	0	1	0	0	1	3
MAN1B1	0	1	0	0	0	0	1	0	0	1	3
MGAT4A	0	1	0	0	0	0	1	0	0	1	3
FOXP4	0	1	0	0	0	0	1	0	0	1	3
LOC116349	0	1	0	0	0	0	1	0	0	1	3
OSCAR	0	1	0	0	0	0	1	0	0	1	3
DHRS13	0	1	0	0	0	0	1	0	0	1	3
CSTB	0	1	0	0	0	0	1	0	0	1	3
SYT6	0	1	0	0	0	0	1	0	0	1	3
CYP2B6	0	1	0	0	0	0	1	0	0	1	3
C9orf97	0	1	0	0	0	0	1	0	0	1	3
REM2	0	1	0	0	0	0	1	0	0	1	3
DBT	0	1	0	0	0	0	1	0	0	1	3
HBEGF	0	1	0	0	0	0	1	0	0	1	3
DUSP5	0	1	0	0	0	0	1	0	0	1	3
AHR	0	1	0	0	0	0	1	0	0	1	3
TET3	0	1	0	0	0	0	1	0	0	1	3
ANKRD23	0	1	0	0	0	0	1	0	0	1	3
AK1	0	1	0	0	0	0	1	0	0	1	3
EPHA4	0	1	0	0	0	0	1	0	0	1	3
LEMD2	0	1	0	0	0	0	1	0	0	1	3
TFEC	0	1	0	0	0	0	1	0	0	1	3
VASH1	0	1	0	0	0	0	1	0	0	1	3
NMNAT2	0	1	0	0	0	0	1	0	0	1	3
GARNL4	0	1	0	0	0	0	1	0	0	1	3
FOSB	0	1	0	0	0	0	1	0	0	1	3
KCNE1L	0	1	0	0	0	0	1	0	0	1	3
STXBP4	0	1	0	0	0	0	1	0	0	1	3
FLJ25778	0	1	0	0	0	0	1	0	0	1	3
GABRA3	0	1	0	0	0	0	1	0	0	1	3
USP49	0	1	0	0	0	0	1	0	0	1	3

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
WSB1	0	1	0	0	0	0	1	0	0	1	3
LRRC32	0	1	0	0	0	0	1	0	0	1	3
PHGDH	0	1	0	0	0	0	1	0	0	1	3
TNRC6A	0	1	0	0	0	0	1	0	0	1	3
GNAT1	0	1	0	0	0	0	1	0	0	1	3
FLJ39743	0	1	0	0	0	0	1	0	0	1	3
GSTM5	0	1	0	0	0	0	1	0	0	1	3
HOXB2	0	1	0	0	0	0	1	0	0	1	3
AQP6	0	1	0	0	0	0	1	0	0	1	3
KPNA1	0	1	0	0	0	0	1	0	0	1	3
ZNF321	0	1	0	0	0	0	1	0	0	1	3
PIM3	0	1	0	0	0	0	1	0	0	1	3
MCAM	0	1	0	0	0	0	1	0	0	1	3
PPP1R12B	0	1	0	0	0	0	1	0	0	1	3
NCL	0	1	0	0	0	0	1	0	0	1	3
ORC2L	0	1	0	0	0	0	1	0	0	1	3
P2RX5	0	1	0	0	0	0	1	0	0	1	3
PAFAH1B1	0	1	0	0	0	0	1	0	0	1	3
PALM	0	1	0	0	0	0	1	0	0	1	3
MRPL30	0	1	0	0	0	0	1	0	0	1	3
PHF21A	0	1	0	0	0	0	1	0	0	1	3
KLF13	0	1	0	0	0	0	1	0	0	1	3
FXVD7	0	1	0	0	0	0	1	0	0	1	3
DLL4	0	1	0	0	0	0	1	0	0	1	3
POU4F2	0	1	0	0	0	0	1	0	0	1	3
C1orf181	0	1	0	0	0	0	1	0	0	1	3
KIF1A	0	1	0	0	0	0	1	0	0	1	3
C9orf167	0	1	0	0	0	0	1	0	0	1	3
TMEM104	0	1	0	0	0	0	1	0	0	1	3
MAGOHB	0	1	0	0	0	0	1	0	0	1	3
CDCA8	0	1	0	0	0	0	1	0	0	1	3
P15RS	0	1	0	0	0	0	1	0	0	1	3
LGI2	0	1	0	0	0	0	1	0	0	1	3
SMPD3	0	1	0	0	0	0	1	0	0	1	3
HIF1AN	0	1	0	0	0	0	1	0	0	1	3
CENPN	0	1	0	0	0	0	1	0	0	1	3
AGPAT3	0	1	0	0	0	0	1	0	0	1	3
CAMK1D	0	1	0	0	0	0	1	0	0	1	3
MAN1C1	0	1	0	0	0	0	1	0	0	1	3
PBXIP1	0	1	0	0	0	0	1	0	0	1	3
CYP20A1	0	1	0	0	0	0	1	0	0	1	3
REXO1	0	1	0	0	0	0	1	0	0	1	3
KIAA1377	0	1	0	0	0	0	1	0	0	1	3
C20orf77	0	1	0	0	0	0	1	0	0	1	3
RFC3	0	1	0	0	0	0	1	0	0	1	3
CX3CL1	0	1	0	0	0	0	1	0	0	1	3
SFRP1	0	1	0	0	0	0	1	0	0	1	3
SLC1A2	0	1	0	0	0	0	1	0	0	1	3
SMTN	0	1	0	0	0	0	1	0	0	1	3

Gene	DIANAmT	miRanda	miRDB	miRWalk	RNA hybrid	PICTAR4	PICTAR5	PITA	RNA22	Targetscan	Total programs predicting target
WNK2	0	1	0	0	0	0	1	0	0	1	3
SLC8A2	0	1	0	0	0	0	1	0	0	1	3
PHACTR4	0	1	0	0	0	0	1	0	0	1	3
TROVE2	0	1	0	0	0	0	1	0	0	1	3
TEF	0	1	0	0	0	0	1	0	0	1	3
TGFA	0	1	0	0	0	0	1	0	0	1	3
TIMP3	0	1	0	0	0	0	1	0	0	1	3
TNFSF4	0	1	0	0	0	0	1	0	0	1	3
CLIP2	0	1	0	0	0	0	1	0	0	1	3
MOBK12B	0	1	0	0	0	0	1	0	0	1	3
MAP6D1	0	1	0	0	0	0	1	0	0	1	3
TRIM45	0	1	0	0	0	0	1	0	0	1	3
CCDC6	0	1	0	0	0	0	1	0	0	1	3
EFHD1	0	1	0	0	0	0	1	0	0	1	3
TRABD	0	1	0	0	0	0	1	0	0	1	3
ANKRD13C	0	1	0	0	0	0	1	0	0	1	3
USP48	0	1	0	0	0	0	1	0	0	1	3
RAB11FIP4	0	1	0	0	0	0	1	0	0	1	3
PPP1R9B	0	1	0	0	0	0	1	0	0	1	3
ARHGAP19	0	1	0	0	0	0	1	0	0	1	3
REPS1	0	1	0	0	0	0	1	0	0	1	3
SSH2	0	1	0	0	0	0	1	0	0	1	3
BHLHB2	0	1	0	0	0	0	1	0	0	1	3
FAM125B	0	1	0	0	0	0	1	0	0	1	3
CLDN2	0	1	0	0	0	0	1	0	0	1	3
BCL7B	0	1	0	0	0	0	1	0	0	1	3
TRIP13	0	1	0	0	0	0	1	0	0	1	3
CD28	0	1	0	0	0	0	1	0	0	1	3
TMEM203	0	1	0	0	0	0	1	0	0	1	3
IER2	0	1	0	0	0	0	1	0	0	1	3
SPOCK2	0	1	0	0	0	0	1	0	0	1	3
FARP2	0	1	0	0	0	0	1	0	0	1	3
TOMM70A	0	1	0	0	0	0	1	0	0	1	3
TMCC2	0	1	0	0	0	0	1	0	0	1	3
KBTBD11	0	1	0	0	0	0	1	0	0	1	3