THROMBOGENESIS IN SUBSTRATES OF ATRIAL FIBRILLATION

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This thesis is submitted to the University of Adelaide in fulfilment of the requirements of the degree of

Doctor of Philosophy

July 2014

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ABSTRACT

Background: Atrial Fibrillation (AF) is the most common atrial arrhythmia affecting Australia and the world, with patients with AF known to be at a 5times higher risk of stroke than that of the normal population. The substrates of AF are also known to significantly impact of this risk of stroke. Mitral stenosis (MS) is one of the leading causes of valvular AF in the developing world. Enlargement of the LA is one of the most common structural changes that occurs in MS and is known to lead to fibrosis and oxidative stress. These alterations can also cause atrial electrical remodelling leading to the development of AF. Patients with MS have been shown to have an increase in thrombogenic properties which include platelet reactivity, inflammation and endothelial dysfunction.

The precise mechanisms which underlie this phenomenon of atrial thrombus formation in AF are still unknown, furthermore it is also unknown if the substrate (cause) of AF influences the thromboembolic profile in AF patients. This thesis aims to evaluate the peripheral and atrial thrombogenic profile of both AF and the major substrate MS and their differing disease states alter the thrombus potential.

Methods: A total of 166 patients were collected for this study, 55 patients undergoing a radiofrequency ablation as a curative procedure for paroxysmal AF, at the Royal Adelaide Hospital, Adelaide, 59 patients with mitral stenosis (MS)undergoing a balloon valvuloplasty at

the Christian Medical Centre in Vellore, India, and 52 with aged matched control subjects, diagnosed with left sided accessory pathway supraventricular tachycardia (SVT) undergoing a routine elective electrophysiological study. Blood samples were collected from the peripheral, RA and LA circulation, during each of these procedures, for further analysis through flow cytometry, platelet aggregation and ELISA tests. Echocardiographic studies were used for atrial structure measurements.

Results: We found that within the AF population there is increase in thrombogenic markers within the heart compared to the peripheral circulation. More interestingly when comparing the MS and AF populations each of the different factors involved in thrombogenesis is altered differently, with AF having an increase in platelet reactivity and endothelial function (ADMA and ET-1) and inflammation through VCAM-1 and ICAM-1. However of inflammation through MPO, CD40L and IL-6 and structural remodelling (MMP-9 and TIMP-1) were more pronounced within the MS population.

Conclusion: This study has shown that AF and the valvular AF substrate mitral stenosis (MS) have two distinctly different mechanisms leading to atrial thrombus formation. This shows that MS as a substrate for valvular AF impacts on atrial thrombus formation through remodelling and inflammation whereas non valvular AF affects endothelial function and tissue inflammation. This illustrates that the

pathophysiology of each of the diseases states is different when comparing it to the normal haemostatic properties of the heart within a control (SVT) population to determine if these factors are in fact altered from the norm.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Carlee D Schultz

ACKNOWLEDGEMENTS

I wish to use this opportunity to thank those people who have been involved, supported and encouraged me through my studies over the last four years.

I would like to thank all my supervisors, Dr Anthony Brooks, Dr Bobby John, Dr Anand Gasean and Prof Prash Sanders for their guidance, supervision, help and support for the duration of my PhD.

In particular I would like to thank my primary supervisor Dr Scott Willoughby for his guidance, encouragement and mentorship throughout this time which has been a blessing. Thank you for believing in me, giving me confidence and courage to begin and the support to complete a PhD.

I am grateful for the postgraduate scholarship support I received from the Australian Government and University of Adelaide for the Australian Postgraduate Award. I am also grateful for the patients who volunteered their additional time for this research.

This thesis research was done over two continents as a part of the Australia India Strategic Research Fund (BF030018). A collaboration with the Royal Adelaide Hospital in Adelaide, South Australia, and the Christian Medical College in Vellore, India.

I am very thankful to all of the staff at the Cardiovascular Investigation Unit, Royal Adelaide Hospital and Centre for Heart Rhythm Disorders, University of Adelaide, In particular Ms Judith Hunt, Ms Lauren Wilson, Ms Melissa Middeldorp and Ms Aimie Paukner for your help with sorting, rearranging, finding patients and Information which has been a tremendous help.

Thank you to my family for providing support and enthusiasm during the course of my studies. My Parents, whose constant encouragement, support and love has helped tremendously; they always together with my brothers know how to bring me back down to earth and have always been a huge base of support.

Finally I would like to thank my fiancé Tom for his love, support, understanding and encouragement not only during the past few years but in all that I strive to achieve. This roller coaster ride of a PhD has seen our relationship grow and become something I could only have wished for, and will be forever grateful that he was right there next to me for the whole journey.

Thanks,

Carlee

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PRESENTATIONS AT NATIONALAND INTERNATIONAL SOCIETYS

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PUBLICATIONS ARRISING FROM THESIS

Chapter 3

Carlee D Schultz, Geetanjali Rangenker, Han S Lim; Angelique Fraudeau, Glenn Young, Kurt Roberts-Thomson, Bobby John, Matthew Worthley, Prashanthan Sanders, Scott R Willoughby. Characterization of Thrombogenic, Endothelial and Inflammatory Markers within the Normal Human Hearts. *Clinical and Experimental Pharmacology and Physiology, 2014*

Chapter 4

Schultz CD, Willoughby S, Lim H, Kumar S, Nair S, Srivastava A, Chandy ST, Lau D, John B, Sanders P. Influence of Ethnic Background on Inflammation, endothelial function and Thrombogenic Factors in Supraventricular Tachycardia.(To be submitted)

Chapter 5

Schultz CD, Willoughby S, Lim H, Kumar S, Nair S, Srivastava A, Chandy ST, Lau D, John B, Sanders P. Atrial Platelet Reactivity, Endothelial Function and Inflammation in Mitral Stenosis: Potential Thrombotic Risk. (To be submitted)

Chapter 6

Schultz CD, Willoughby S, Kumar S, Nair S, Srivastava A, Chandy ST, Lau D, John B, Sanders P. The Reversal of Chronic Atrial Stretch by Balloon Valvuloplasty Alters the Thrombogenic Profile of Patients with Mitral Stenosis. (To be submitted)

Chapter7

Schultz CD, Willoughby S, Lim H, Lau D, Sanders P. Atrial Endothelial Function, Inflammation and tissue remodelling in non-valvular Atrial Fibrillation: Potential Thrombotic Risk. (To be submitted)

Chapter 8

Schultz CD, Willoughby S, Lim H, Kumar S, Nair S, Srivastava A, Chandy ST, Lau D, John B, Sanders P. Atrial Endothelial Function and Inflammation between Atrial Fibrillation and its Substrate Mitral Stenosis (To be submitted)

PRIZES AND AWARDS DURING CANDIDTURE

Young Investigator Award

- Clinical

  5th Annual Asia Pacific Heart Rhythm Society Scientific Sessions, Taipei, Taiwan, October 3-6, 2012.

Travel Awards

- EO Myers Trust Fund, Travel Grant2013

  Awarded in October 2013.To aid in travel to the 6th Annual Asia Pacific Heart Rhythm Society Scientific Sessions, Hong Kong, October 3-6, 2013.

- Discipline of Medicine Travel Grants Round 1, 2013.

  Awarded in May 2013, in support for travel to the 2013 Heart Rhythm Scientific Sessions, Denver, USA.

- EO Myers Trust Fund, Travel Grant

  Awarded in October 2012.To aid in travel to the 5th Annual Asia Pacific Heart Rhythm Society Scientific Sessions, Taipei, Taiwan, October 3-6, 2012.

- Heart Foundation Travel Grant

  Awarded in March 2010. To aid in travel to the 31st Annual Heart Rhythm Society, Scientific Sessions, Denver, Colorado, May12-15th,2010.

Scholarships

- My PhD was supported by an Australian Postgraduate Award.

1 CHAPTER ONE

Review of the Literature

1.1 Introduction

Atrial fibrillation (AF) is the most common atrial arrhythmia affecting the global population.[2] Currently 1 in 5 people over the age of 80 has AF and the incidence is increasing with predictions that by 2050 over 15 million people in the United Stated alone will be affected by AF [Figure 1].[3] Furthermore, a recent Australian study reported that AF hospitalisations have steadily become one of the leading cardiovascular reasons for hospitalization [Figure 2].[4] With more recent studies showing in the US hospitalization rates for AF have increased exponentially from 2000 to 2010, despite an overall decline in hospital mortality.[5] AF is also an associated risk for thrombus formation within the heart that can lead to stroke.[6] Approximately 60,000 Australians suffered from a stroke in 2009, costing Australia $2.14 billion in health care costs.[7] It is estimated that up to 40% of all strokes are related to AF. Understanding and reducing the risk of stroke in these patients is key. The risk of stroke within the AF population is 5-fold higher than the normal population;[8] this risk further increase increases as patients get older and their disease profile becomes complicated with multiple risk factors.[9] There is now strong evidence that there is a progressive

increase in overall burden, incidence, prevalence, and AF-associated mortality across the world.[10]

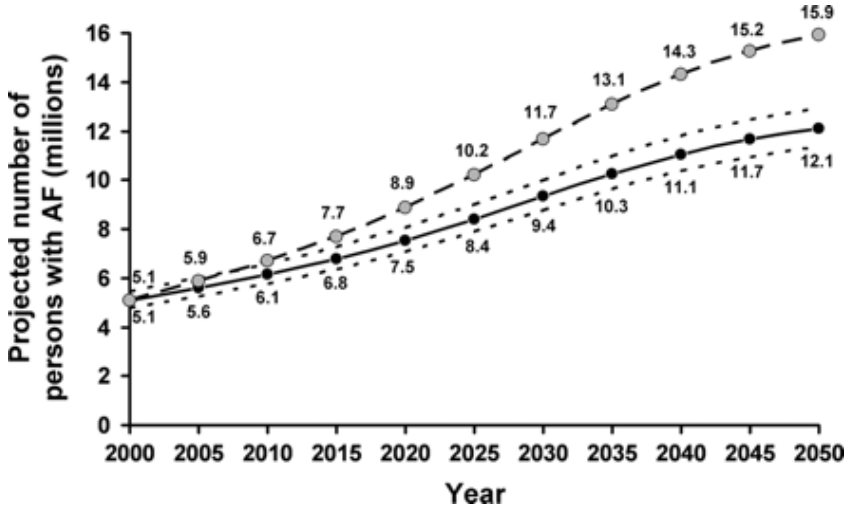


Figure 1 : Projected number of persons with AF in the United States between 2000 and 2050, assuming no further increase in age-adjusted AF incidence (solid curve) and assuming a continued increase in incidence rate as evident in 1980 to 2000 (dotted curve). Reproduced with permission.[3]

It is well recognised that AF is associated with an increase in thrombus formation, particularly occurring within the left atrium (LA)and left atrial appendage (LAA).[8] In patients with AF up to 70% of strokes are thought to be cardio-embolic in origin[11] with AF contributing to up to 40% of all strokes.[12] The abnormal beating of the atria during a burst of AF promotes for the formation of LA thrombus. As the atrial tissue has irregular contraction the blood flow becomes turbulent, and a pro-thrombotic environment is established.[13] This thrombotic risk is usually treated through initiation and continuation of anti-platelet or antithrombotic medication. Despite their medications, patients with AF

still have a substantial (30-40%) risk of stroke. The precise mechanisms which underlie this phenomenon of atrial thrombus formation in AF are still largely unknown; furthermore, whether the substrates (predisposing factors / causes) for AF influence the thromboembolic profile in AF patients is unknown.

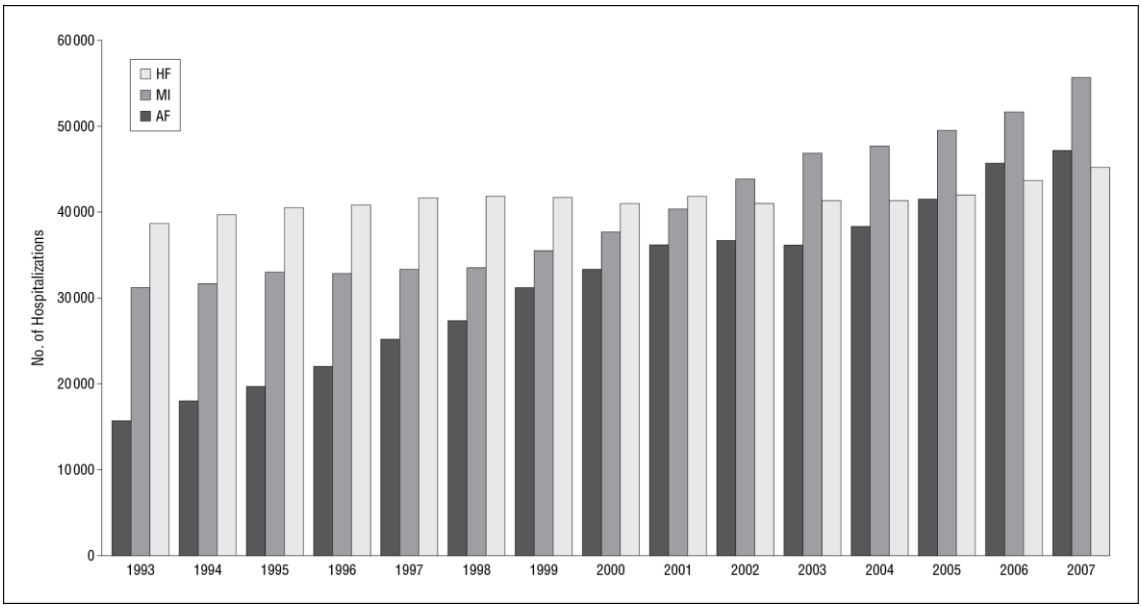


Figure 2 : Number of hospitalizations for atrial fibrillation (AF), myocardial infarction (MI), and heart failure (HF), from 1993 through 2007, inclusive. Reproduced with permission[14]

1.2 Atrial Fibrillation

Atrial fibrillation (AF) is the most common cardiac arrhythmia[15] and is characterised by disordered electrical activity of the atria, which leads to desynchronised beating of the heart and elevated heart rate. In AF, the normal electrical impulses that are generated by the Sino-atrial node are overwhelmed by disorganized electrical impulses that originate in the atria and pulmonary veins, leading to conduction of

irregular impulses to the ventricles that generate the heartbeat.[16] This disordered electrical activity in the atria leads to the desynchronised atrio-ventricular contractions. The incidence of AF is around 1% of the general population, effecting almost 2 million Americans and up to 6 million Europeans.[2,17] Within the Australian population is it estimated that over 165,000 persons have AF;[7] this number is expected to substantially rise due to the aging of the population. The prevalence of AF is known to increase with increased age, once over 60 years the risk of developing AF steadily increases, with persons 80 years or older having a 36% chance of developing AF.[17]

In the last decade admissions to Australian hospitals for AF have become one of the most common of cardiac related complications. AF now accounts for more admissions than heart failure [Fig 2].[18] The incidence of AF has a significant effect on the health care system, as AF is known to increase the chance of re-admissions to hospital as well as increase the amount of time spent in care.[19]

1.3 Types of Atrial Fibrillation

Atrial fibrillation (AF) is classified into two subcategories; valvular or non-valvular. The term valvular AF is used if the any of the valves within the heart are affected as part of the primary disease leading to AF.[20] For example, AF related to rheumatic valvular disease (predominantly mitral stenosis) or prosthetic heart valves.[21] Non-valvular refers to all other AF.

Non-valvular AF is the most common form of AF and is caused by progression of atrial remodelling by substrates such as hypertension, diabetes and obesity. There are four main categories of non-valvular AF; lone, paroxysmal, persistent and permanent (or chronic) AF.[22] Lone AF occurs in the absence of any substrate or without the presence of structural heart disease. It is believed that lifestyle factors may be the major cause of the arrhythmia development in this population.[23-25] If a first detected episode self-terminates in less than 7 days and then another episode begins at a later time, the AF has moved into the category of paroxysmal AF.[26] Although patients in this category have episodes lasting up to 7 days, in most cases of paroxysmal AF the episodes will self-terminate within 24 hours. Where the episode lasts for more than 7 days, it is unlikely to self-terminate and it is called persistent AF. [27,28] In this case, the episode may be still terminated by cardioversion. If cardioversion is unsuccessful or it is not attempted, and the episode is ongoing for a prolonged duration (a year or more), the patient's AF is called permanent or chronic.

1.3.1 Valvular AF

One of the primary cause of valvular AF is rheumatic mitral stenosis (MS), in addition MS is one of the most common forms of structural heart disease within Indigenous populations and in the developing world.[29] MS is a progressive disease, where rheumatic heart disease a chronic manifestation of rheumatic carditis, which occurs in 60% to

90% of cases of rheumatic fever.[30] This is primarily caused by the alteration in the wall of the atria caused by MS which leads to stretch and scar formation disrupting the normal conduction therefore leading to AF.[31] There is a significant relationship between the incidence of AF and type of MS, being that rheumatic MS is associated with AF whereas non-rheumatic is not.[32] A previous study has suggested that the onset of LA dilatation in MS is the result of an early increase in LA pressure, and that AF will develop irrespective of the severity of the MS. [33] AF is known to occur in 40-75% of patients who have MS, further reflecting how these two disease states are intertwined.[34] Unfortunately there are limited studies which involve patients with valvular AF specifically; many studies have determined changes in MS and AF separately or in progression.

It is known that there are significant structural changes particularly within the LA in patients with MS and valvular AF. This is mainly through a rise in LA area and volume, as well as a rise in LA pressure. [35-37] Previous studies suggested that the onset of LA dilatation in MS is the result of an early increase in LA pressure, leading to the development of AF, which develops irrespective of the severity of the MS, and further contributes to an enlargement of the left and right atria, and thrombus formation.[33] Current mapping technologies provide the ability to view the atrial remodelling or substrate in MS, leading to AF. As seen in Figure 3 MS is characterized by LA enlargement, loss of myocardium, and scarring associated with widespread and site-specific

conduction abnormalities, but without changes or an increase in effective refractory period. These abnormalities were associated with a heightened inducibility of AF.[31]

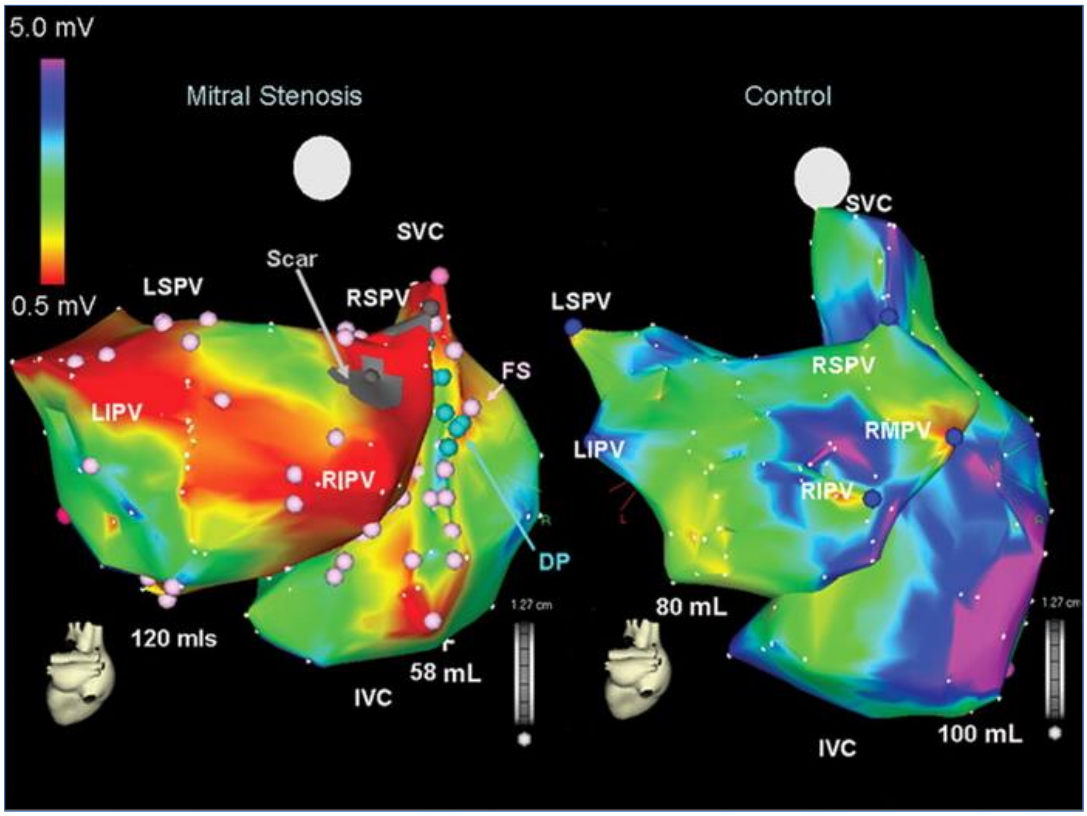


Figure 3: Electroanatomic bipolar voltage map of a patient with MS (left) and a representative control (right).reproduced with permission[31]

Mitral stenosis (MS) is a disease known to have prothrombotic properties, leading to an increase risk of stroke,[38] and in the presence of AF the risk of thromboembolic complications and stroke, are further increased.[34] Patients with MS may have visualised clot formation within the atria, as evidenced by spontaneous echo contrast, even when free of AF.[39] Finally, pro-thrombotic abnormalities are commonly reported seen in patients with MS including increased platelet aggregation and hypercoagulation (increased thrombin-antithrombin III complex and

fibrinogen).[29,40,41] It is thought that as in AF this increase in coagulation factors originates from within the left atrium.[29,35,40]

Previous studies have investigated alterations in coagulation within the peripheral circulation, LA and RA of patients of MS. It has also been reported that MS causes an increase in platelet activation, significantly increased with the LA.[35] These results are not reported consistently with a study by Yamamoto *et al* showing mixed results. A further study has found that some coagulation factors such as thrombin-antithrombin III complex were increased in the LA compared to the RA and peripheral circulation, however this was not consistent for all markers, or across the patients groups, with Plasma levels of platelet factor 4, beta-thromboglobulin, D-dimer and plasmin-alpha 2-plasmin inhibitor complex not altering. Furthermore, Chandrasekhar *et al*, 2009showed that although coagulation factors were increased in patients with MS compared to a control population, this was not consistent for all the markers measured. More recently a study by Chen *et al* showed that in MS patients, LA platelet activation was significantly increased compared to the RA and in the peripheral circulation.[35]

In addition to coagulation and platelet markers it is possible that other factors which are involved in both the progression of disease as well as the risk of thrombus formation are also been shown to be altered in MS. A study by Chang *et al* showed that circulating levels of TNF-α and IL-6 were elevated in both patients with isolated MS (without

cardiovascular risk factors) and chronic heart failure of rheumatic origin. [42] Additionally MS studies have previously found that the venous plasma soluble VCAM-1 levels were significantly higher than that of healthy volunteers or patients with lone AF.[43,44]

These studies all suggest that there are major thrombogenic changes occurring in MS antecedent to AF diagnosis. Despite this, variation in markers and results show there is still a large gap in the knowledge of how thrombus forms with the MS population, and if it is LA specific, as in AF. The altered thrombogenic properties in MS and the progression into AF, and how this may be affecting the risk of stroke in these patients is yet to be determined. There are very limited studies which have determined alteration in valvular AF specifically as patients are often too ill to be involved in studies. In patients with rheumatic MS and AF, there is some evidence of platelet activation detected by high platelet aggregation levels which have a significant direct relationship with the severity of MS. [45]

However it is still unknown if it is the disease state of MS which alters the thrombotic risk in a patient's pre AF diagnosis; or if it is the progression to AF which produces the thrombotic state in these patients. Atrial remodelling is known to induce endothelial dysfunction. Previous studies have suggested that there are significant variations in the indices of endothelial dysfunction between the LA and peripheral venous blood samples of patients with MS.[41] Other studies showed that

in patients with MS and sinus rhythm, if left atrial spontaneous echo contrast (LASEC) is present, endothelial dysfunction was similar to that of AF patients with AF.[46] A further study shown that there increased von Willebrand Factor (vWF) levels at 24 hours after balloon mitral valvuloplasty (BMV); however these patients also have AF.[47] The increase in LA size in MS patients is known to be associated with AF. MS patients with a LA size > or = 45 mm, were more likely to have LASEC.[39] Furthermore the frequency of LA and LA appendage clots on trans-oesophageal echocardiography in patients with severe MS is common, however it is more frequent in patients with MS and AF.[39]

MS is one of the most common forms of structural heart disease associated with AF (valvular AF) within the developing world, whilst in developed countries heart failure and hypertension (non-valvular AF) are much more aetiologically common. There is limited data in the comparison of thrombotic properties between valvular and non-valvular AF patients. The risk of stroke in the valvular AF population shown to be 12 times higher than that of the non-valvular AF population.[48] There has been one study which found that there is no difference in thrombotic rate between the two groups.[49] It is currently unknown how much the cause of the AF, whether valvular or non-valvular can affect thrombogenic mechanisms and potential stroke risk within these two very different populations.

1.3.2 Non – Valvular AF

Non-valvular AF is the most common type of AF, with2.3 million people in the US alone and up to 2% of the general population being affected by non-valvular AF.[3,50] The growing epidemic of non-valvular AF is associated with a high morbidity and mortality; it intersects with a number of conditions including aging, thromboembolism, stroke, congestive heart failure, hypertension, and perhaps the metabolic syndrome and inflammation. Non- valvular AF is one of the most highly researched types of AF, mainly through mechanisms of remodelling and thrombus formation, two of the biggest and potentially revisable or treatable areas of the condition.[50] However, beginning to know and understand the underlying mechanism of AF has taken decades of research. Hypertension and heart failure are seen as the two most common diseases which can perpetuate non-valvular AF.[15]

Two key areas of research in non-valvular AF are that of anticoagulation medication, and the restoration of sinus rhythm. With stroke prevention and bleeding risk being one of the major factors when treating AF, sometimes more so than the arrhythmia itself. These risk factors along with major symptomatic complications of AF area crucial component of patients care.

The development of LA thrombus and the potential for stroke in AF is a major consequence of the disease. The risk of stroke occurring in patients with AF is five times higher than that of a person without AF. Anti-thrombotic medications are consistently being regenerated to

attempt provide better risk stratification[51], along with risk scoring systems for AF patients.[52,53] There is now new research into the precise mechanisms of thrombus formation and alteration in haemostatic properties in AF patients and in particular within the LA. Much of this research is focused around the Virchow's triad, and in recognising how these mechanisms work together to increase risk.[54,55] Virchow's triad (Fig 7) for thrombogenesis includes abnormalities in the endothelium/endocardium (abnormal vessel wall), abnormalities of haemodynamic and turbulence at bifurcations, atheroma at vessel wall (abnormal blood flow) and abnormalities in platelets as well as the coagulation and fibrinolytic pathways (abnormal blood constituents).[54]

Treatment of the AF generally is for the treatment of symptoms of the disease. This can be very different for each person, with some people not being able to feel or tell they have AF at all, known as silent AF this can be the most dangerous as often treatment is not sought. Silent AF is associated with morbidity and mortality rates similar to those of symptomatic AF.[56] Treatment for AF is with a goal for permanent restoration of sinus "normal" rhythm. This can be achieved through many interventions dependent on the patient's symptoms, confounding disease states and preference for treatment. Restoration of sinus rhythm in AF can be achieved via electrical (DC) cardioversion,[57,58] or more aggressively treated through surgery such as radiofrequency or Cyro-ablation[21,59] or a more invasive surgical procedure Cox-maze procedure.[59,60] Medications such as rate or rhythm control, such as

antiarrhythmic drugs are used to reduce the severity and duration of AF. [61,62] However rate versus rhythm control as the "best" treatment strategy remains an issue of considerable, ongoing debate.[63]

AF progression from a lone or paroxysmal type, where there is period of sinus rhythm to a more persistent or permanent is often quite a chaotic process.[64] AF is responsible for atrial electrophysiological, contractile and structural remodelling, which in turn shorten the wavelength, accelerating AF cycle length and sustain and progress the arrhythmia.[16] In AF progression there is also a further increase in the process involved in thrombus formation as well as furthering the disease. Many studies showing how factors such as inflammation are increased according to the burden on AF, being high in lone, then higher again in persistent and permanent AF compared to aged match controls.[65,66] Progression of AF not only increases mortality rates, it also leads to more frequent hospitalizations, and diminished quality of life impairment.

As previously mentioned hypertension and heart failure are two of the most common disease states leading to non-valvular AF. Hypertension has been shown to cause AF mainly due to the increased pressure of the venous blood coming back into the right atrium from the systemic circulation and the left ventricle. The atria are unable to compensate for the pressure load, and the atria become enlarged.[67,68] This enlargement is able to alter the electrical pathways in the heart leading

to AF. [69]Atrial enlargement is well recorded in patients with long-term high blood pressure. Short-duration hypertension also is associated with significant atrial remodelling and a greater propensity for AF.[70] The Framingham study placed people with AF and hypertension at a three times higher risk of stroke than subjects free of high blood pressure.[71] Additionally, it is now undisputed that hypertension is associated with increased platelet reactivity.[72-76] Due to these complications and the thrombotic nature of both hypertension and AF it is expected that a combination of these two disease states would lead to a further alteration of platelet and endothelial factors leading to a higher rate of thromboembolism and stroke. The presence of AF and heart failure can create a vicious circle, with heart failure promoting the development of AF and vice versa.[77,78] Heart failure can be caused by various systemic diseases, which result in a reduced ventricular ejection fraction and overload damage. Chronic heart failure has previously been shown to increase many factors that lead to an increase risk of thrombus formation.[78] Treating the combination of these disease states can be difficult as it is now known specifically how each substrate disease effects AF progression or thrombotic risk.

1.3.3 Lone Atrial Fibrillation

Lone AF is defined where AF develops without the presence of any known substrate or without any structural heart disease. Previous findings suggest that subjects with lone AF, despite similar cardiovascular risk profiles to normal controls, have a distinct

preponderance of pre-existing electrocardiographic abnormalities.[79] Furthermore, contrary to general belief, lone AF is not a benign condition; it has a serious prognosis, indicating a greater need for detection and treatment. Not all medical professionals believe that lone AF exists; they believe in these cases that the cause is undiagnosed in these patients.[80,81] Some of the most explored causes or groups of persons in which lone AF occurs, range from the extremely fit endurance athletes to patients believed to be genetically susceptible, and also lifestyle factors such as smoking and excessive alcohol intake.[23] Other factors that were not originally recognised include: obesity, sleep apnea and increased aortic stiffness.

Endurance sports practice has been shown to have an impact on atrial ectopic beats, inflammatory changes, and atrial size and therefore may act as a promoter of these arrhythmias.[82] Heavy physical activity may also increase the thrombotic risk in these patients, with increases in platelet reactivity and von willebrand factor (endothelial function) levels during extreme exercise, whereas moderate exercise has no procoagulatory effect.[83,84] It has also been shown that warfarin therapy prevents exercise-induced thrombin generation only.[83] In an animal model, long-term intensive exercise training induced fibrosis in both atria and increased susceptibility to AF.[85,86]

Other life style factors such as smoking and alcohol intake have been current smokers (25.9% vs 15.6%, $P < 0.01$), and alcohol drinkers

(55.3% vs 31.2%, P<0.01).[23] In a prospective cohort study, it has been shown that the association between self-reported alcohol use and incident AF, with a further study showing that among both women and men, alcohol consumption throughout the moderate range was not associated with risk of AF. However, consumption of 35 or more drinks per week among men was associated with a hazard ratio of 1.45 (95% CI 1.02 to 2.04) although few women consumed this amount of alcohol.[87] However these factors are seen as modifiable and under all guidelines, reduction or cessation can decrease risk of AF development and ischemic stroke.[88,89]

1.4 Substrates for Atrial Fibrillation

AF is often associated with disease states which progressively dilate and remodel the atria; these constitute a substrate of the arrhythmia.[90]AF progression from its substrate is most often caused through complex prolonged structural and functional alterations of the LA myocardium such as: short action potentials, heterogeneous refractory periods, dystrophic myocyte and interstitial fibrosis which act together to favour local conduction block, activation of ectopic activity and the formation of micro-re-entries of the electrical excitation.[90] However, the underlying mechanisms by which substrates for AF lead to AF are not yet fully understood. Furthermore, the development of AF can also occur with no known substrate (lone AF), where there is no

pre-existing disease state, this suggest that atrial remodelling antedates the AF.

The most common substrates for AF are generally some of the common cardiac risk factors and diseases effecting the population. These include hypertension, heart failure, diabetes mellitus, age and more recently obesity, and obstructive sleep apnoea (OSA).[50,91-93]

1.4.1 Hypertension

Hypertension is one of the most common disease states to affect the general population. The World Health Organisation estimates that raised blood pressure will to cause 7.5 million deaths, which is approximately 12.8% of the total of all deaths globally.[94] The overall prevalence of raised blood pressure in adults aged 25 and over was around 40% in 2008. However, because of population growth and ageing, the number of people with uncontrolled hypertension rose from 600 million in 1980 to nearly 1 billion in 2008. Hypertension is commonly defined as a systolic blood pressure (SBP) over 140 mm Hg or a diastolic blood pressure (DBP) above 90 mm Hg, although there is not universal consensus with these specific measurements.[67]

Hypertension has been shown to cause AF mainly due to the increased pressure of the venous blood coming back into the heart from the systemic circulation and the left ventricle, which the atria is unable to compensate for. This leads to the RA and LA becoming the

enlarged.[68] This enlargement alters the electrical pathways in the heart leading to AF. The Framingham Heart Study found that hypertension is an independent predictor of the development for AF in both sexes with an odds ratio or association of 1.5 for men and 1.4 for women. A further study by Stewart *et al* found that an increase in blood pressure (systolic ≥169mmHg) was associated with a 2.1 fold risk of developing AF.[95] Hypertension acts as a substrate for the development of AF through the increase in pressure leading to atrial remodelling as compensation. A further study found that hypertension contributes to the prothrombotic state in AF. Increased stroke and systemic embolic events in hypertensive AF patients. Interesting the event rates markedly increase at SBP levels of ≥ 140 mmHg.[96] An association between hypertension and thrombotic events in patients with AF has previously been suggested. Healey *et al* found that endothelial dysfunction (vWF) and activation of the coagulation cascade(soluble thrombomodulin) were increased when hypertensive AF patients were compared to normotensive patients.[97] Due to the thrombotic nature of both hypertension and AF it can be anticipated that the combination of these two disease states will lead to a further alteration of platelet and endothelial factors leading to a higher rate of thromboembolism and stroke.

1.4.2 Heart Failure

Heart failure and AF are major cardiovascular diseases that often coexist. The presence of these two conditions can create a vicious

circle, with heart failure promoting the development of AF and vice versa.[77,78] Heart failure can be caused by various systemic diseases, which result in a decrease in myocardial efficiency and overload damage. In a healthy heart, increased filling of the ventricle result in increased contractility (by the Frank-Starling law of the heart) and therefore a rise in cardiac output. This mechanism fails in heart failure, as the ventricle is loaded with blood to the point where heart muscle contraction becomes less efficient. Due to this overload there is an enlargement of the LA which causes remodelling of the structural and contractile elements of the atria, promoting disordered electrical conduction and contraction of AF.[98,99] This is demonstrated in a previous study by Li *et al* in a canine model of heart failure.[100] The authors showed that in dogs with heart failure had a higher induction rate of AF and greatly sustained AF compared to control dogs.[100] Furthermore, it demonstrated that the increase in AF susceptibility was due to structural atrial remodelling (interstitial fibrosis in the atria), causing interference of local atrial conduction properties (electrical remodelling leading to AF.[100]

Heart failure is also associated with a prothrombotic state. Patients with heart failure have a four times greater risk of stroke compared to age matched control subjects.[48] Chronic heart failure has previously been shown to increase many factors that lead to an increase risk of thrombus formation.[78] When compared to patients with no structural heart disease patients with heart failure have been shown to have

increased plasma viscosity, increases in fibrinogen, potentially leading to clot formation, along with impaired endothelial dependent vasodilation and decreased release of nitric oxide, demonstrating the presence of endothelial damage and dysfunction.[78,101] Approximately 15% of patients with mild heart failure have co-existent AF; as it is common that these two disease states coincide. Whether there is any synergism between these diseases in a patient's risk of stroke. May be critical to guiding treatment and early prevention of thromboembolic events in this population.[78]

1.4.3 Diabetes Mellitus

Diabetes Mellitus (DM) has become one of the most critical health care issues for modern medicine and society. As lifestyle factors change, many more adults and children are becoming diabetic. It has been shown that prevalence rates commonly exceeded 10% in those over the age of 60. Men and women in the Framingham Study who were overweight by more than 40% had twice the prevalence of DM compared with those of normal weight.[102] Diabetes is most often associated with many other life style factors such as obesity which are also known to increase the risk of AF. The link between AF and diabetes has been shown to be strong, with a study by Benjamin *et al* finding that Diabetic has an odds ratio of developing AF of 1.4 for men and 1.6 for women.[9] This shows that after adjusting for age and other predisposing conditions diabetes is a significant independent risk factor for AF. AF in known to occur in (14.9%) DM patient's vs (10.3%) in the

control group ($p < 0.0001$). Atrial flutter occurred in (4%) of DM patients vs. (2.5%) of the control group ($p < 0.0001$)This was the first large-scale study finding DM as a strong, independent risk for the occurrence of AF and flutter and other cardiovascular diseases.[103] Diabetes not only affects risk of AF, but also increases the risk of cardiovascular disease, which may further increase the risk of development of AF and therefore stroke.[104]

1.4.4 Age

Age is one of the key risk factors for AF, as with advancing age it has been shown that the chances of developing AF increases.[105] It is estimated that 70% of patients with AF are between 65 and 80 years old.[1] AF is quite uncommon in persons under 60, with a person under 55 years having only a 0.1% chance of having AF.[17] The odds ratio (OR) of AF for each decade of advancing age has been shown to be 2.1 for men and 2.2 for women ($P<0.0001$).[9] Age-related fibrosis, intracellular age-related changes, and accumulation of accompanying disorders that occur with increasing frequency with older age may explain this association.[106] On the contrary, if AF occurs at young age, genetic factors are likely play a leading role.[107] Progression to permanent AF is a slow process, aging has been shown to be independently associated with progression of AF to permanent AF.[108]

1.4.5 Obesity

Obesity has become one of the most publicised risk factors for many common diseases. Obesity can also affect the likelihood of the development of AF mainly through the dilatation of the LA.[93] If the Body Mass Index (BMI) is between 25 and 29.9 a diagnosis of overweight is made. If the BMI is 30 or over, a diagnosis of obesity is made. Obesity-related risk factors, such as hypertension, vascular disease, obstructive sleep apnoea and increased pericardial fat, are thought to result in atrial electro-structural dysfunction. In addition, insulin resistance and associated abnormality in nutrient utilisation and intermediary metabolic by-products is associated with structural and functional abnormalities, ultimately promoting AF.[109] Previous studies also found that BMI was associated with short and long-term increases in AF risk, accounting for a large proportion of incident AF independent of traditional risk factors. Strategies for weight control have been shown to potentially reduce the increasing incidence of AF.[110] More recently a study by Abed *etal* showed that weight reduction with intensive risk factor management resulted in a reduction in AF symptoms, burden and severity.[89]

1.4.6 Obstructive Sleep Apnoea

It has recently been shown that Obstructive Sleep Apnoea (OSA) can increase the risk of developing AF. OSA is associated with significant atrial remodelling characterized by atrial enlargement, reduction in voltage, site-specific and widespread conduction abnormalities, and longer sinus node recovery. These features may in part explain the

association between OSA and AF.[92] With the odds of an arrhythmia after a respiratory disturbance were nearly 18 times (odds ratio: 17.5; 95% confidence interval: 5.3 to 58.4) the odds of an arrhythmia occurring after normal breathing.[111]

1.4.7 Substrates of Valvular Atrial Fibrillation

This thesis however focuses on the leading substrates of valvular AF through MS.[112,113] Rheumatic MS, the most common substrate for valvular AF begins from rheumatic fever usually contracted as a child. A recent study by Islam *et al* found that chronic rheumatic heart disease is one of the major causes of AF in hospital admitted patients and risk stratification revealed that most of the patients were at risk of future stroke.[114] Another study by Zhao *et al* found that the mitral regurgitation severity due to rheumatic MS correlated with the rate of recurrent atrial tachyarrhythmia and more severe grade of mitral regurgitation indicated more recurrent atrial tachyarrhythmia. [115] A previous study by Keren *et al* has suggested that the onset of LA dilatation in MS is the result of an early increase in LA pressure, and that AF, which develops irrespective of the severity of the MS, contributes to a further enlargement of the left and right atria, and thrombus formation.[33] The risk of stroke in patients who have MS and AF is approximately 10 folds higher than that of AF alone.[116]

1.5 Stroke Risk in Atrial Fibrillation

The risk of stroke in patients with AF is a major focus in the management of AF. AF confers a 5 fold increased risk of stroke increasing to 17.5 fold with valvular AF, one of the highest disease stroke risks. AF contributes to 15-40% of all strokes, with more that 70% of AF related strokes being deadly. It has been established that three out of four AF-related strokes can be prevented if the disease is known.[116,117] However, this does not seem to be the case. It has been estimated that one-third of Americans who have AF are still undiagnosed. Further to this 25% to 30% of stroke patients, the stroke mechanism cannot be determined (cryptogenic stroke).[118]AF is known to increase the risk of stroke through various methods; mainly through the irregular rhythm caused by the fluttering of the atrial during an episode of AF. This also can allow the blood to become stagnant or pool in areas of the heart, such as the atrial appendage. When normal blood flow is altered, it has the propensity to form clots which can then be carried to the brain, causing a stroke. It is also known that this irregular rhythm creates turbulent blood flow within the atria which can further influence the likelihood of the blood coagulating. The disease of AF itself it known to alter the structure of the atria, thereby creating endothelial dysfunction and a further hyper-coagulable state in the blood. In the long-term if left untreated AF reduces cardiac output by 15%, leading to a propensity to develop heart failure, and further increasing the risk of stroke.[78]

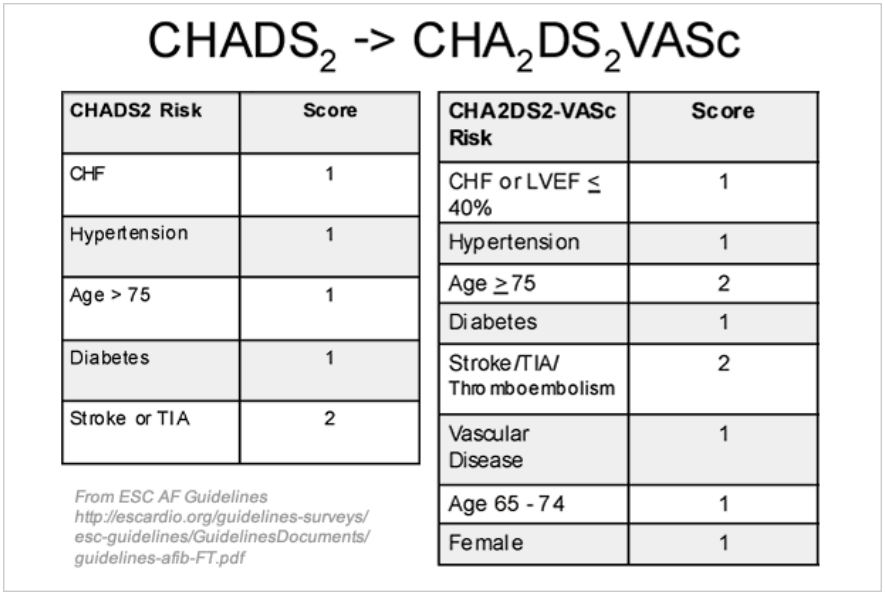
1.5.1 Stroke Risk Scores

Ischaemic stroke is managed in patients with AF largely through preventative oral anti-platelet and anticoagulation medications.[67] The benefits of these therapies in preventing strokes in AF are well established, some patients still remain at risk of thrombotic events as well as bleeding events from oral anticoagulation therapy.[119] The use of improved instruments, such as the progression in risk stratification scores from the CHADS, $CHADS_2$, to the CHA_2DS_2VASc [refer to Fig 4]and HAS-BLED [Figure 5], and interventional techniques for occlusion of the LA appendage, that are new promising options for stroke prevention.[120]

The CHADS, CHADS2, to the CHA_2DS_2VASc are various scores given to AF patients concerning their risk of stroke. They primarily involve a patient's disease history, along with age and more recently gender. The first of these the CHADS score, considering chronic heart failure, Hypertension, Age≤ 75years, Diabetes melinites and previous stroke (or transient ischaemic attach (TIA). A patient who had any of these conditions was given 1 point in the score scale, and their risk of having a stroke was assessed [Fig 4]. This was soon after converted to the $CHADS_2$ [Fig: 4], where by a patient with previous stroke or transient ischaemic attach was automatically given 2 points, instead of the previous 1 point. The $CHADS_2$ score was validated in a study published by Henriksson *et al* in 2001 using 1,733 AF patients tracked through Medicare claims, and become a turning point giving physicians a better

systematic approach to treat stroke risk in AF.[121] In 2012 Lip and colleagues created the CHA_2DS_2VASC score [Fig: 4], this further involved vascular disease, Age 65-74 and female sex as risk factors, as well as giving age over the age of 75 a score of 2.[53] The $CHADS_2$ and CHA_2DS_2cVASc scores have been shown to correlate with LASEC, showing that they are related to LA clot formation in AF.[122]

$CHAD_2$ / CHA_2DS_2VASc Scoring Guidelines

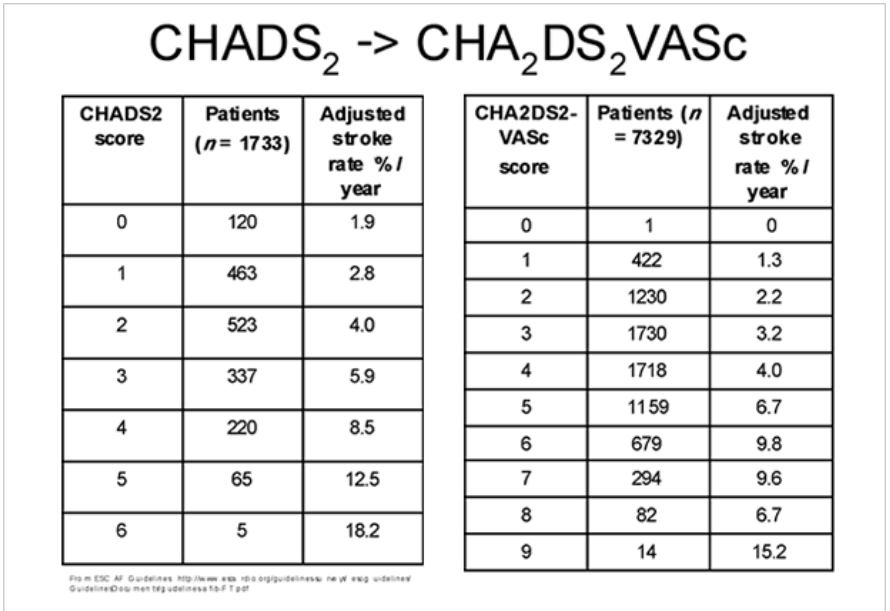


$CHADS_2$ -> CHA_2DS_2VASc

CHADS2 Risk	Score
CHF	1
Hypertension	1
Age > 75	1
Diabetes	1
Stroke or TIA	2

From ESC AF Guidelines http://escardio.org/guidelines-surveys/esc-guidelines/GuidelinesDocuments/guidelines-afib-FT.pdf

CHA2DS2-VASc Risk	Score
CHF or LVEF ≤ 40%	1
Hypertension	1
Age ≥ 75	2
Diabetes	1
Stroke/TIA/ Thromboembolism	2
Vascular Disease	1
Age 65 - 74	1
Female	1



$CHADS_2$ -> CHA_2DS_2VASc

CHADS2 score	Patients (n = 1733)	Adjusted stroke rate % / year
0	120	1.9
1	463	2.8
2	523	4.0
3	337	5.9
4	220	8.5
5	65	12.5
6	5	18.2

CHA2DS2-VASc score	Patients (n = 7329)	Adjusted stroke rate % / year
0	1	0
1	422	1.3
2	1230	2.2
3	1730	3.2
4	1718	4.0
5	1159	6.7
6	679	9.8
7	294	9.6
8	82	6.7
9	14	15.2

Figure 4: CHADS and CHADSVASC scoring system for AF patients, adapted from ECS guidelines. Reproduced with permission[24]

Where these score are associated with the risk of stroke in AF patients the HAS-BLED score (Fig 5) is relevant to a patient risk of having a major bleed or haemorrhagic stroke. The risk of bleeding in AF patients when on anti-coagulation has been shown to be approximately 2.8% per patient-year, and up to 4.4% per patient-year.[123,124] The HAS-BLED score stands for Hypertension, Abnormal renal and liver function, Stroke, Bleeding, Labile INR's, Elderly (if a person is aged > 65 years) and takes Drugs or alcohol. 1 and/or 2 points are given if a patient has abnormal renal and liver function, and takes drugs and alcohol. Consequently, a crucial part of AF management requires the appropriate use of thromboprophyl axis, and the assessment of stroke and bleeding risk to enable inform management decisions by clinicians.[125]

HAS-BLED Scoring Guidelines

Letter	Clinical characteristic[a]	Points awarded
H	Hypertension	1
A	Abnormal renal and liver function (1 point each)	1 or 2
S	Stroke	1
B	Bleeding	1
L	Labile INRs	1
E	Elderly (e.g. age >65 years)	1
D	Drugs or alcohol (1 point each)	1 or 2
		Maximum 9 points

Figure 5: HAS-BLED scoring system, Adapted from the guidelines for the management of atrial fibrillation.

1.6 Stroke Prevention

As previously mentioned due to the increase risk of stroke in AF patients, many of these patients are on an anti-platelet or anticoagulation therapy, to decrease this risk. Some strokes are fatal while others cause significant permanent or temporary disability. It is important to understand that stroke can be prevented, along with how and what is the best therapy.

There have been a number of new therapies that have become available within the last few years, however with differing efficacy. These medications potentially could lead to major progress in thrombus prevention management, with the call for individualised medicine is becoming more prevalent. The underlying cause of the ischaemic stroke in AF is not well known, therefore more in-depth research into the mechanisms are needed before a holistic approach can be taken in the treatment of these patients.[125] It is possible that more potent anti-platelet strategies or different acting pathways of anti-thrombotic medications may ultimately optimize prevention of cerebrovascular accidents while minimizing bleeding risk.[126]

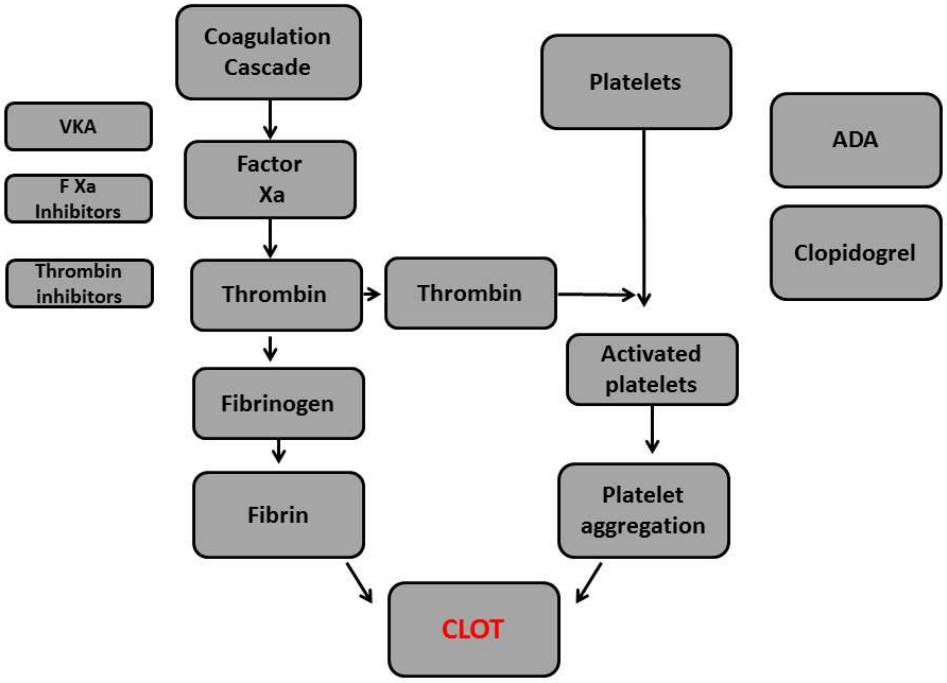


Figure 6 : Representation of how antiplatelet and antithrombotic therapies effect coagulation.

1.6.1 Anti-platelet Therapies

Anti-platelet therapies have been shown to reduce the risk of stroke by 22% (95% CI, 6% to 35%) compared with placebo, and based on meta-analysis of all randomized data from comparisons of all anti-platelet agents and controls.[127] Aspirin is the most common anti-platelet therapies, and has historically been shown to be effective in reducing the risk of stroke. However it is now known that it is not as effective as newer agents. The comparison of aspirin alone versus placebo, in meta-analysis of 7 trials and 3990 patients showed that aspirin reduced the risk of stroke by 19% with an absolute risk reduction of 0.8% per year,[127] and was associated with a greater reduction in non-disabling strokes.

Despite the improvement that aspirin alone gave patients with AF in reducing stroke, the Atrial fibrillation Clopidogrel Trial with Irbesartan for prevention Vascular Events (ACTIVE) compared Clopidogrel plus aspirin (dual therapy) with aspirin alone in patients with AF, this study showed that the addition of Clopidogrel to aspirin resulted in a further reduction of major vascular events.[126] The combined end point of stroke, myocardial infarction, systemic embolism and vascular death was reduced from 7.6% per year to 6.8% per year with dual anti-platelet therapy.[126] However, when these values were compared to warfarin in the ACTIVE-W study. Anti-platelet therapy was inferior to warfarin therapy.[128] The ACTIVE-A trial found the use of Clopidogrel in addition to Aspirin confers a further additional benefit in stroke prevention in patients with AF.[126,129] Ultimately, the enhanced understanding of the role of platelets in AF will contribute to better classification of patients at risk of thrombotic events.

1.6.2 Anticoagulation Therapy

Warfarin therapy is the corner stone of anticoagulation therapies, and has been shown to be effective in both primary and secondary prevention of stroke in patients with AF, and is one of the most commonly used anticoagulation therapies used in AF. Warfarin is a Vitamin-K antagonist and, inhibits the vitamin K-dependent synthesis of biologically active forms of the calcium-dependent clotting factors II, VII, IX and X, as well as other proteins not involved in blood clotting. A meta-analysis of twenty nine trials including 28,044 patients concluded

that adjusted-dose warfarin reduced stroke by 64% (95% CI, 49% to 74%) compared to controls.[127] Absolute risk reduction in all strokes with warfarin was 2.7% per year for primary prevention and 8.4% per year for secondary prevention.[127] However the anticoagulant effect of warfarin is accompanied by increased risk of bleeding. The risk for intracranial haemorrhage was doubled with adjusted-dose warfarin compared to aspirin, with an absolute risk increase of 0.2% per year.[130] Adjusted-dose warfarin was observed to be more efficacious than antiplatelet therapy with a relative risk reduction of 39% (CI 22% to 52%) on the basis of meta-analysis from 12 trials.[127] However, even with almost two-thirds relative risk reduction of stroke on warfarin, one-third of strokes were still being unaccounted for despite apparent optimal therapy. This may be due to limitations in the efficacy of the drug or a gap in our present understanding in the mechanisms of thromboembolism in these patients.

1.6.2.1 **Limitations of Warfarin Therapy**

Despite warfarin being the mainstay therapy for stroke prevention in AF patients, it has many limitations. Warfarin therapy carries with it the increased risk of minor and major bleeding. In particular, intracranial haemorrhage has been reported as high as 0.6% per year for patients on warfarin.[131-134]

It is important to note that intracranial haemorrhage accounts for ninety percent of fatal haemorrhages in patients treated with anticoagulant

therapy.[131] Perceived risk of bleeding from both patient and physician has been found to heavily influence the implementation of warfarin.[135] Vitamin-K antagonist therapy is complicated by numerous drug to drug interactions, especially in elderly patients, with a 13% increase in patients over 80 years.[135,136] Bleeding risk is magnified in patients with high international normalized ratio (INR), and clinical outcome and efficacy is compromised or similar to patients on aspirin if the INR is less than 2.0. Rates of discontinuation and poor adherence, particularly in the elderly are high, with up to 26% of elderly patients stopped warfarin in their first year.[137] The warfarin prescription rate for eligible patients is reported to be as low as 15% to 44% in various studies.[135,138] The numerous limitations associated with vitamin-K antagonist therapy, such as drug-drug and food-drug interactions, narrow therapeutic margin, necessity for frequent blood test monitoring and the side effect of bleeding has led to the development of the newer oral anticoagulation pharmacological therapies. [139]

1.6.2.2 **New Anticoagulation Therapy**

The recent approval by the Pharmaceutical Benefit Scheme (PBS) and introduction of many new anti-thrombotic medications, including Dabigatran [140], Rivaroxaban[141] and Apixaban[142] all currently show non-inferiority compared with Warfarin treatment. Warfarin is still the corner stone of anti-thrombotic medicine for AF patients at heightened risk of thrombus formation. These new strategies will need focused evaluation in the most challenging AF patients, those with a high risk of

bleeding, prior thromboembolism, or with thrombosis-prone surfaces such as mechanical heart valve prostheses or drug-eluting coronary stents, in whom the currently available treatment options are inadequate.[120] These new agents fall into two main groups, oral therapies which directly inhibit activated thrombin (DTI) and Factor Xa (FXa) two key serine proteases in the coagulation cascade.[143] The key advantages being proposed by these new oral anticoagulants is the linear pharmacokinetic characteristics, which allows for fixed dosing of the drugs without the need for coagulation monitoring.[144] However, there has not been a direct trial of these new anticoagulants compared with warfarin directly, and despite their significant reduction in side effects, they are all classed as non-inferior, not superior to warfarin, making warfarin the currently main stay therapy for thrombotic risk in AF. [139,144]

Direct thrombin inhibitors (DTI) are small, synthetic molecules that directly interact with thrombin, both clots bound and free.[145,146] Thrombin is pivotal in the coagulation cascade, activating other clotting substrates (Factor V, VIII, XI and XIII), converting fibrinogen to fibrin and causing the activation of platelet protease activated receptors.[145] The advantage of DTIs over vitamin K agonists (VKAs) such as warfarin is that by being selective for the active site of thrombin and not for other plasma proteins such as tissue plasminogen activator and plasmin, DTIs have quite a predictable anticoagulant response and

thus do not require frequent anticoagulant monitoring and may lessen bleeding risk in these patients.[100,146]

Dabigatran is currently the only DTI in the market. In clinical trials where Dabigatran has been compared to warfarin its was found that in patients with AF Dabigatran given at a dose of 110 mg was associated with rates of stroke and systemic embolism that were similar to those associated with warfarin, as well as lower rates of major haemorrhage. Dabigatran administered at a dose of 150 mg, as compared with warfarin, was associated with lower rates of stroke and systemic embolism but similar rates of major haemorrhage.[147]

Factor Xa is a final common pathway both the intrinsic and extrinsic pathways, and represents an opportune target for anticoagulation therapy.[146] Oral FXa inhibitors directly bind to the active site of FXa, blocking interaction with its substrate.[145,146] Direct FXa inhibitors inactivate both FXa within prothrombinase complexes and free FXa equally.[145,146] Several newer factor Xa inhibitors have since been developed; the two which are currently in use are Apixaban and Rivaroxaban.

Apixaban is an oral selective inhibitor of FXa, and is metabolised through the CYP3A4 pathway to several metabolites but is not a CYP450 inducer or inductor; because of this it has a low drug interaction profile. [145,146] In two clinical trials, the Apixaban versus

Acetylsalicylic Acid to Prevent Strokes (AVERROES) trial,[120,148] and the Apixaban for Reduction in Stroke and Other Thromboembolic Events in AF (ARISTOTLE) study. The rate of major bleeding was significantly lower in the Apixaban group at 2.13% per year compared to the warfarin group at 3.09% per year (HR, 0.69; 95% CI, 0.60 to 0.80; p<0.001).[149] The rate of haemorrhagic stroke was nearly halved in the Apixaban group at 0.24% per year compared to warfarin at 0.47% per year (HR, 0.51; 95% CI, 0.35 to 0.75; p<0.001).[149]Rivaroxaban is another oral selective, competitive, reversible FXa inhibitor. Like Apixaban, it has a high specificity for FXa and does not inhibit other serine proteases in the coagulation cascade. The Rivaroxaban Once Daily Oral Direct Factor Xa Inhibition Compared with Vitamin K Antagonism for Prevention of Stroke and Embolism Trial in AF (ROCKET AF) trial randomized 14,264 patients with non-valvular AF at moderate-to-high risk for stroke to receive either Rivaroxaban (20mg daily) or dose-adjusted warfarin, with the study achieving non-inferiority (HR 0.79; 95% CI, 0.66 to 0.96, p<0.001 for non-inferiority).[150] Rates of major and non-major bleeding were similar in the two groups (14.9% per year on Rivaroxaban versus 14.5% per year on warfarin, HR 1.03; 95% CI 0.96 to 1.11; p=0.44).

1.6.3 Limitations of Anticoagulation Therapies

None of the above mentioned therapies are optimally effective, with anti-platelet therapies seen as inadequate and anticoagulation therapies having major side effects and with poor patient

compliance.[151,152] There are also many newer oral anticoagulants which are now being compared with the efficacy of Warfarin which is the current leading anticoagulant therapy, and novel non pharmaceutical therapies such as LAA closure devices. Despite the gains in knowledge in the process of thrombus formation, and the development of new medications, 20-30% patient with AF are still at a risk of having disabling and fatal strokes. There is an urgent need to better understand the pathological mechanisms controlling thrombus formation in AF and its substrates. This thesis addresses some of the current deficiencies in our knowledge base.

1.6.4 Non Pharmaceutical Therapies

Left atrial appendage (LAA) removal or occlusion devices were designed in the belief that most if not all clots that from within the LA of patients with AF are actually are formed solely within the LAA.[153,154] These devises such as the Watchman[155] Amplatzer[156] are all designed to permanently occlude the LAA therefore eliminating the risk of thrombus formation and the associated need for anticoagulation. However, this device does not eliminate AF nor its symptoms. It has been shown that along with radiofrequency (RF) ablation, this can reduce the need for anticoagulants, without being a risk during repeat RF ablation procedures,[157] despite the risk of clot formation occurring on the occlusion device itself, albeit that this is rare.[158] Few long-term studies, have determined how effective these devices in comparison with anticoagulation therapies.

1.7 Mechanisms of Thrombus Formation

The underlying processes which are involved in thrombus formation are multifaceted and complex.[159] Thrombi are able to form in many environments and rely on key components to gain and maintain their structure. It is also known that the underlying disease states in patients can alter how these thrombi are formed.[160] Historically, in patients with AF it was believed that the primary cause of thrombus formation was due to atrial mechanical dysfunction which leads to blood stasis in the atria appendage and therefore thrombus formation.[45] More recently it is has been established that thrombus formation in AF is much more complex and that the diseases which perpetuate AF can also alter the processed leading to thrombus. [22]

Thrombotic risk is usually assessed via the components of the Virchow's triad [Fig 7]. The triad consists of three components which initiate thrombus formation; these are blood stasis, abnormal blood constituents and vessel wall injury. These three factors incorporate, structural abnormalities such as dilated atria, heart valve disease (mitral stenosis) and congestive heart failure and can contribute to two of the sides of the triad, with abnormalities in coagulation, haemostasis and the damage and dysfunctional endothelium fulfilling the third.[54,161] There are many factors which can affect these parameters, mainly the underlying disease states which either causes the AF or the patient's co-morbidities along with the AF. The two main categories which AF are classified are valvular and non-valvular AF, determined if the mitral

valve is affected as part of the primary disease leading to AF. The most common disease states which are known to lead to AF being, hypertension (non-valvular), mitral stenosis (valvular) and heart failure (non-valvular),[9] each of which carry their own risk of stroke, which may further alter platelet reactivity, endothelial function and inflammation leading to increased thrombotic tendencies for patients with a combination of these disease states.[162]

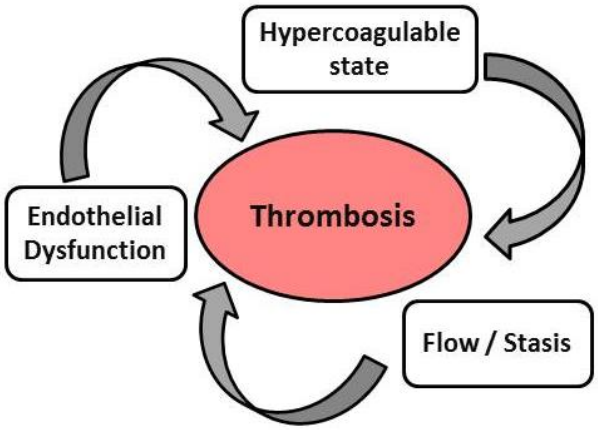


Figure 7: Representation of the Virchow's Triad of thrombosis.

There have been many previous studies that have investigated the prothrombotic state via haemostatic markers associated with AF. With abnormalities in haemostasis, platelets activation and aggregation, endothelial function and inflammation have all being described in the setting of AF.[55,73,163-165] A review of genetic polymorphisms and AF has also opened the door to many possible links to genetic influences on thromboembolic risk, as well as genetic influences on anticoagulant drug responsiveness.[166] Alterations within the atrial wall function have

been shown to play a major role in thrombogenesis[13], however it is now known to aid rather than be the single cause of thrombus formation, as was historically thought.[159] Despite all of the current treatment for reducing thrombus formation and stroke risk, patients with AF are still having catastrophic, debilitating and fatal strokes.

Mechanisms of Atrial Thrombus Formation

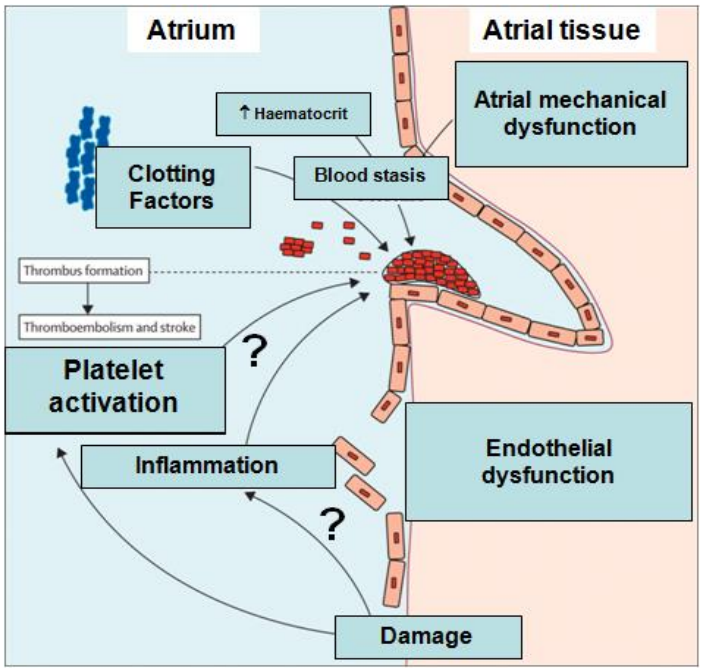


Figure 8: Schematic of possible contributors to thrombus formation in the left atria in AF. Adapted from Feinberg*et al*.[1]

1.7.1 Abnormalities in Blood Flow

Abnormalities of the blood flow, particularly disturbances of flow within the left atria (LA) are known to be associated with AF. Most commonly this is due to dilatation of the LA and the absence of atrial systole.[167] AF is known to lead to progressive enlargement of the atria which is also associated with endothelial dysfunction.[168] Stasis of slowing and

pooling of the blood allows for inappropriate activation and clotting, causing thrombus.

1.7.1.1 **Blood Stasis**

Blood stasis occurs within the atria during AF due to the disordered contractility. The abnormal and fibrillating atrial wall allows for pockets blood to become static, as compared with normal atrial contraction. This is also shown in the left atrial appendage (LAA), were LAA thrombus was found to be associated with both dilatation and poor LAA contraction.[169] Atrial dilatation if seen as one of the critical factors that can effect blood stasis in AF, with atrial size found to be an independent predictor of stroke in non-valvular AF.[170] Dilatation of the LA is more pronounced in valvular AF, and thrombosis due to stasis being highly likely within these patients.[33] A more recent article by Ohara *et al* study found that the severity of blood stasis in the LA correlates with an accumulation of clinical risk factors for thromboembolism in non-valvular AF patients. Additionally, the severity of blood stasis in the LA was greater in chronic AF patients than in paroxysmal AF patients at the comparable risk level.[171] Blood stasis and abnormalities in blood flow are usually assessed via echocardiography, and evidence of left atrial spontaneous echo contrast (LASEC) is apparent by the appearance of "smoke" or swirling density within the atria.[172] LASEC has been found to be associated with LA dilatation, left ventricular dysfunction and decreased

LAA flow.[173] This has also strongly been associated with valvular AF and is a strong clinical predictor of stroke.[174]

1.7.2 Abnormalities in the Blood Constituents

There are many factors in blood constituents which affect coagulation and thrombus generation; these mainly consist of alterations in the coagulation cascade, and the activation of platelets and inflammation. These and many other factors have been shown to be abnormally altered within AF patients, leading to an increase in thrombogenesis and stroke risk.

1.7.2.1 **Coagulation Cascade**

Alterations to the entire coagulation cascade in AF can be varied and all elements are usually co-dependent of many other cascade factors. Altered or abnormal activation of the coagulation cascade has been shown to be one of the main pathways of thrombus formation in the AF population.[55,175] The importance of this is also shown in the institution treatments to reduce the thrombotic risk in AF; including warfarin and newer anticoagulant therapies, which all target different properties of the coagulation cascade.[127,147,176] The main factors of the coagulation cascade which have been heavily researched in AF population are thrombin generation, fibrinogen and plasma d-dimer concentrations. These factors are all key markers of activation of the coagulation cascade, and are a part of the intrinsic pathways which lead to fibrin generation which is critical structure of the clot itself.

Thrombin generation is involved in the coagulation cascade as it acts as a serine protease that converts soluble fibrinogen into insoluble strands of fibrin, as well as catalysing many other coagulation-related reactions.[177] Thrombin generation through measurement of Thrombin anti thrombin complex (TAT) have been found to be increased with in the LA of patients with valvular AF.[40] This was also seen in a study by Akar *et al*, which analysed cardiac samples from patients who were in AF and found both thrombin generation and platelet reactivity were increased within the coronary sinus compared to a paced control.[75]

Further to this fibrinogen is a fundamental component of the coagulation cascade, and is a key part of the structure of the thrombus. Fibrinogen when converted becomes fibrin, and is involved in the crucial linking system between platelets and therefore is responsible for the maintenance of the clot formation. Fibrinogen has been shown to be increased in association with LASEC within the LA,[178] and that this LASEC could be associated with increases in fibrino-peptide.[179] However recently it has been shown that the levels of soluble fibrin in patients with AF on warfarin treatment are not able to predict thromboembolic events in AF patients.[180]

Another part of the coagulation cascade which has been shown to be predictive of thrombus formation in AF is D-Dimer. D-Dimer is a fibrin degradation product; a small protein fragment present in the blood after

a blood clot is degraded by fibrinolysis, and is commonly used to determine if a patient has thrombosis. D-Dimer levels have previously been used to rule out atrial thrombi in AF patients, where a high D-Dimer level would be predictive of LA thrombi.[181,182] D-dimer levels in combination with clinical risk factors have been shown to be effective in predicting subsequent thromboembolic events in patients with AF, even when treated with warfarin.[183] With a low D-Dimer concentration in patients with chronic AF that are receiving anticoagulant therapy,[184] and has been further shown that D-Dimer levels did not alter with age.[117] In addition, is has been shown that in AF patients treated with warfarin, not only the duration outside the target international normalised ratio(INR) range, but also the fluctuation in INR level may influence the prothrombotic state.[185]

1.7.2.2 **Platelet Function**

Platelets are small anucleated cells contained within the blood. The main function of platelets is to participate in primary homeostasis through four distinct steps: adhesion, activation, secretion, and aggregation.[186] Platelet granules contain different bioactive chemical mediators, many of which have a fundamental role in homeostasis and/or tissue healing. The sub-membrane region contains microfilaments of actin and myosin that mediate morphologic alterations characteristic of shape change. Resting platelets remain in the circulation for an average of approximately 10 days before being removed by macrophages of the endothelial system.[186] A wide variety

of transmembrane receptors cover the platelet membrane, all of these factors enable platelet to become activated, and therefore group together, ultimately causing a clot (thrombus). This process can be up and down regulated by the release of many circulating factors and by the platelet itself.[187] Platelets are also able to rapidly respond to small environmental changes. However, if this is insufficient to sustain activation and aggregation the platelet can revert to normal in active resting state. [188]

Under normal physiological conditions platelets play a key role in reducing blood loss when a vessel is damaged. This process involves the adhesion of platelets to the vessel wall (exposed sub-endothelium) and platelet-to-platelet adherence, which ultimately seals off the damaged vessel wall by the formation of a platelet plug.[189] In contrast, in disease states such atherosclerosis, diabetes mellitus and heart failure there is inappropriate platelet activation, leading to thrombus formation, which may contribute to myocardial infarction or stroke by provoking vessel closure and local ischemia.[190] This occurs as the underlying disease state can further attenuate this physiological process in AF leading to further inappropriate platelet aggregation.[159,191]

Inappropriate platelet activation has been rigorously shown in AF, whereby patients with AF, despite having no vessel damage, have increased platelet activation and aggregation.[55,74,192-195] This known to

be a leading contributing factor to thrombus formation within the AF population. This has been shown through increases in the increased expression of P-selectin in patients with chronic AF as well as permanent and paroxysmal AF, when compared to health controls,[76,168] A study by Kamath *et al* also found that the permanent AF resulted in a significant higher level of platelet activation then that of paroxysmal, suggesting that the time in AF can also affect thrombus formation.[168] A more recent study has also found that both the rapid atrial rates and disorder rhythm in AF in humans result in increased platelet activation and thrombin generation.[196] Prothrombotic activation occurs to a greater extent in the human LA compared with systemic circulation.[196] Platelet adhesion has also been demonstrated to be increased in AF patients not taking antithrombotic medications, with adhesion shown to be reduced upon starting an anti-platelet or antithrombotic therapy.[197]

Platelet mircoparticles, which are small cellular components released at the time of complete platelet activation, have been shown to be increased with the AF population compared to controls and cardiac disease controls.[73,193,198] Other factors where platelets can play a role in thrombus formation include ischemia leading to arrhythmogenesis. A recent study by Dhanj *al et al* found that platelets when infiltrating the ischemic zone facilitate the induction of ventricular fibrillation. Moreover, this was independent of their ability to participate in occlusive thrombosis, and the effect was unresponsive to antiplatelet

drugs,[199] Implying that platelets potentially have a role in the induction of the atrial arrhythmia also.

1.7.2.3 **Inflammation**

Inflammation had become one of the newest focuses for the establishment and perpetuation of AF and AF-related thrombosis and stroke.[200] Inflammation is able to be incorporated into two factors of the Virchow's triad in abnormalities of blood constituents and vessel wall injury. Inflammation is one of the largest underlying conditions associated with disease progression onto AF.[163] Inflammatory processes have long been associated with the development of thrombus and the risk of stroke.[201] Inflammation as a normal physiological process is involved in a myriad of pathological states and its primary role is as a protective attempt remove the injurious stimuli and to initiate the healing process. This can initiate clot formation through the activation of platelet and other thrombotic molecules. Patients with known major caused for AF such as hypertension, heart failure and MS have significantly higher levels of inflammation.[202,203] It has also been shown that other inflammation orientated diseases and disorders, such as alcoholism and obesity can increase the risk of a person having AF.[91,204,205] It has been shown that after first diagnosis of AF, high c-reactive protein (CRP) was associated with recurrence[206] of AF at one year follow up. Furthermore, increased inflammation levels have been associated with the early recurrence of AF post curative ablation procedure, and the inflammatory is predictive of AF recurrence

post ablation.[207] We have recently shown that the extent of inflammatory response predicts early AF recurrence but not late recurrence. Prothrombotic markers are elevated at one week after ablation and may contribute to increased risk of early thrombotic events after AF ablation.[208] Further to this there has been targeted research into markers of inflammation in AF and AF disease progression, where it has been shown that c-reactive protein(CRP) was significantly increased as AF progresses from paroxysmal to persistent.[209] The recruitment of leukocytes with the release of reactive oxygen species, cytokines and growth factors, leads to adverse atrial remodelling and suggests that inflammatory pathways are a prerequisite for AF,[164] with leukocyte activation considered an important inflammatory pathway underlying AF. A study by Gungor *et al* shows that genetic polymorphism of IL-1RN gene, as well as increased CRP may cause increased risk for lone AF.[210] These and several other studies further develop the association between increased inflammation and the increased prothrombotic environment in patients with AF. However, similar to endothelial dysfunction this has not been explored with the in the intra-cardiac environment of AF patients where the pro-thrombotic environment is known to be more prolific.

1.7.3 Vessel Wall Injury

Injury to the vessel wall and the atrial wall, are known to occur in AF, mainly through atrial stretch and increased blood pressures to and from the heart. This can be seen through increased LA size, and a decrease

in function, progressively leading to heart failure if left untreated.[162] Endothelium, the lining of blood vessels and the heart, is involved in many functions, and disruption to these can result in decreased contraction, reduction in nutrient supply to the muscles as well as fibrosis and clot formation.[211]

1.7.3.1 **Endothelial Dysfunction**

Platelets and the endothelium interact closely to play a major role in the maintenance of cardiovascular homeostasis. The endothelium is the thin cellular lining of the blood vessels that creates a semi-permeable barrier between the lumen and the underlining tissue.[209,212] This tissue also lines the inner surface of the heart (endocardium).[213] The cells which make up the endothelial layer have the capacity to release regulatory factors (i.e. nitric oxide), giving them the ability to modulate their own and thrombotic activity.[209] In addition the endothelium is responsible for maintenance of vascular tone, the release of inflammation markers, and movement of electrolytes and waste in and out of the blood.[214,215] These cells can also play an important role in both the progression to and the maintenance of AF, whereby damage to the endothelium by scar, caused by fibrosis from atrial stretch,[36] or electrical abnormalities , such as alterations in the cells, can alter the contractility of the atrial tissue ultimately causing AF. [216]A recent study by Greiser *et al* has shown that AF is associated with alteration in intracellular calcium current, showing that intracellular Ca^{2}+ homeostasis plays an important role in the development of the

contractile dysfunction and the changes in atrial electrophysiology, and begins to give some insight into the intracellular mechanisms of the alteration to endothelial function in AF patients.[217]

Under pathological conditions, such as AF, the function of the endothelium is compromised (endothelial dysfunction),[218,219] which can potentially lead to an increased thrombotic tendency. The irregular contraction of the atria in AF can be caused by endothelial dysfunction and increased thrombosis, and vice versa. In many cardiovascular disease states such as, hypertension and atherosclerosis, endothelial function is known to be altered or dysfunctional.[97,191] These disease states are also known to alter the thrombotic risk in AF patients, and have long been used as risk co-morbidities when assessing stroke risk in AF patients.[9] Endothelial dysfunction has also been shown to be associated with the risk of thrombus formation that is associated with AF, [211,220] with damage to the endothelium creating an environment for platelet activation, inflammation thus further fulfilling the Virchow's triad of mechanistic risk factors for clot formation.

It has been shown that in AF, von Willebrand factor (VWf), a common marker of endothelial dysfunction is associated with both an increased incidence of AF, as well as increasing the likelihood of thrombus formation.[202,220] vWF levels are increased in AF patients and this was independent of the underlying cause of AF or the presence of structural heart disease.[220,221] In addition, asymmetric dimethylarginine (ADMA,

an endogenous inhibitor of nitric oxide synthesis and thus a marker of endothelial dysfunction) is increased in patients with both paroxysmal and persistent AF.[222] Nitric oxide (NO) concentrations and cyclic GMP levels in platelets (as an indicator of NO activity) have also been shown to be reduced in patients with AF.[223] Minamion *et al* also showed that the plasma NO concentration decreases, whilst expression of P-selectin on platelets increase, shortly after the onset of AF in a canine model.[223]AF is associated with a marked decrease in endocardial NOS expression and NO* bioavailability and an increase in PAI-1 expression in the left atrium. Freestone *at el* showed that an external measure of endothelial function flow mediated dilatation was significantly impaired in AF patients, Similarly Wong *et al* showed that peripheral arterial tonometry measured endothelial response (reactive hyperaemia) were significantly de creased (indicating endothelial dysfunction) in patients in AF compared with patients in sinus rhythm.[165] Finally, we have recently shown that short duration (15 minutes) of AF has been shown to increase ADMA ($p<0.01$) and sCD40L ($p<0.001$) levels significantly.[196]

Collectively these studies suggest that AF is associated with impairment of endothelial function, and that this impairment in endothelial function is reversed by restoration of sinus rhythm.[224]

1.7.3.2 **Remodelling of the Atria**

Atrial remodelling can effect both the structure and the mechanical function of the heart, and is one of the major factors that is involved the initiation and persistence of AF. These can be normal changes with age or due to subsequent changes from damage such as scar formation from myocardial infarction. The functional remodelling of the atria then produced electrical remodelling that further progresses AF. The electrical remodelling (shortening of atrial refractoriness) has been shown to develop within the first days of AF and contributes to an increase in stability of AF.[117]AF is known to produce structural changes within the atria and the famous term by Dr Allessie[178] “AF begets AF” gives further explanation of the progressive remodelling and degeneration in the disease.

Mechanical remodelling generally involves the impairment of the atrial contractile function which is induced by AF. However, these changes are largely due to the structural remodelling that occur within the atria. LA dilatation increase leads to a loss of contractile function of the atria; this is mainly caused through an increase in the compliance of the atrial wall tissue.[117] Dilatation and further structural changes seen in the atria occur at the myocyte level through cell hypertrophy, glycogen accumulation, a breakdown of the contractile element of the cells, a loss of sarcoplasmic reticulum, changes in mitochondrial shape and size, and a change in the distribution of nuclear chromatin.[117,225] All of these factors can affect the contractility and normal function of the myocardium and the whole atria.

Markers of remodelling of the atria have been investigated in the setting of AF, mainly through structural alterations and the propensity of AF reoccurrence. Tissue Factor has recently been localised to the atria of patients with AF,[226] and its association with LA thrombus could indicate a key role of tissue factor in the generation of a hypercoagulable state in AF as well as endothelial damage.[226] Matrix metalloproteinase (MMP's) and tissue inhibitor of metalloproteinase (TIMP's) are involved in extracellular matrix remodelling and the potential to increase the vulnerability to AF. Previously it has been found that in AF patients, the level of MMP-9 in LAA was increased ($P<0.001$), while integrin β1 level was decreased ($P<0.05$) compared with those expressed in right atrial appendage (RAA) tissue.[227] Increased MMP-2 has been associated with paroxysmal AF, whereas increased MMP-9 with permanent AF. Additionally, lower levels of TIMP-1 had a strong association with AF incidence.[228] Another study by Mukherjee *et al* found that pre-cardioversion MMP-9 was higher and TIMP-4 lower, this study also found that circulating levels of MMPs and TIMPs predict AF recurrence.[229]

After atrial enlargement, fibrosis of atrial tissue has been shown to be prolific in patients with chronic AF.[185] Furthermore, the occurrence of gap junction remodelling and the subsequent conduction slowing in the fibrotic lesions was a necessary but not sufficient condition for AF development.[184]

The structural changes that occur in the atria in AF are commonly the cause of the mechanical remodelling, however they can be caused by other factors. Mechanical remodelling affects the atria through the impairment of the atrial contractive function, (or dysfunction) which is induced by atrial arrhythmogenesis. It is known that the mechanical remodelling and therefore atrial mechanical dysfunction leading to LA thrombus can occur in very short time periods.[180] Previous studies in humans, have found that within 15 min of AF there is altered contractile function. Daoud *et al* showing15 min of induced AF was sufficient to result in contractile dysfunction.[179] Further to this Sparks *et al* showed that 30 seconds of AF resulted in the presence of LASEC.[180] Chronic AF has been shown to alter the L-type calcium channels and increased calcium excretion from the cell, resulting in a reduction in atrial contractility.[230,231] Mechanical dysfunction has also been shown to be attenuated by verapamil, suggesting a role for calcium overload in AF.[179]

1.7.3.3 **Restoration of Sinus Rhythm: Cardioversion**

Electric cardioversion of the arrhythmia is responsible for responsible mechanical abnormalities and also increasing the risk of stroke.[26] This phenomenon called atrial mechanical “stunning”, where there is transient mechanical dysfunction which leads to blood stasis and an increased risk of thrombotic event.[57,183] Cardioversion was also believed to increase the risk of clot dislodgement from the atria,

however it has also been observed in patients without pre cardioversion clot who surprisingly have thrombogenic complications.[183] It has also been shown that that the restoration of sinus rhythm through cardioversion and electrophysiological ablation techniques are able to produce reverse remodelling of the atria.[181] Further to this that the maintenance of sinus rhythm may be able to reverse the long standing effect AF has on the heart.

1.8 Peripheral vs. Atrial Sampling Sites

Several studies have begun key investigations into the further areas of interest for the prothrombotic state in AF. A study by Conway *et al* has shown that there is an increase in platelet activation through raised platelet p-selectin in patients with AF over and above that of both other cardiovascular diseases and the control subjects. This is further emphasised by further studies that have shown increased levels of platelet activation, and increased platelet mircoparticles levels in patients with AF.[45,192] Endothelial function has become a growing area of investigation, a study by Li-Saw-Hee *et al* showed that AF patients have significantly elevated levels of plasma von Willebrand factor, a marker of endothelial dysfunction, compared to healthy controls.[232] Furthermore, inflammation which has long been regarded an issue in AF patients, is now becoming an area of interest for its influence in the thrombogenic processes.[200] It has been found that inflammation not

only contributes to the progression of AF it also promotes the thrombotic state.[164,233]

A seminal question to the thrombotic profile of AF patients is the peripheral circulation that same as the LA (origin of thrombus formation), With thrombogenesis in AF is now known to originate in the LA, With our recent study finding of increased platelet activation and thrombin generation in bothrapid atrial pacing and after the induction of AF, with the thrombotic activation occurring to a greater extent in the LA compared with systemic circulation.[72,196] These results suggest that platelet reactivity within the LA may be an important contributor to the prothrombotic state in atrial AF. Further work to expend these observations in AF and its substrates are clearly warranted.

It is well known that stroke in AF is overwhelmingly of cardioembolic nature,[234] with AF contributing to 50% of all cardioembolic strokes.[234] Thrombus formation is primarily derived with the LA and the left atrial appendage (LAA) in patients with AF.[235-237] However there have been very limited studies which have investigated how thrombogenic processes are altered directly within the LA. A study by Akar *et al* was one of the first that examined platelet reactivity in cardiac and peripheral samples taken from AF patients.[75] This study showed that following the induction of AF, cardiac (coronary sinus) platelet p-selectin levels were increased.[75] In contrast, no change in the peripheral blood samples were found.[75] More recently studies have

begun to investigate the effect AF has on thrombus formation specifically within the left atria (LA). In 2010 Willoughby *et al* showed platelet activation in the LA of patients with AF was increased compared to the RA and the peripheral circulation, suggesting chamber-specific alterations which may begin to explain the increased rate of LA thrombus formation in AF patients.[238] More recently, we have shown that the acute induction of AF alone is associated with endothelial dysfunction and inflammation,[196] when compared to a sinus rhythm group, with a history of AF. This further show that the changes in thrombogenesis in AF are both acute and chronic, and furthermore that previous studies implying a correlation between peripheral and atrial results have underestimated the importance of local atrial blood flow to thrombus formation. These studies further demonstrate that AF per se, is associated with cardiac specific changes in platelet activation and endothelial dysfunction, potentially leading to thrombogenesis.

These studies are limited in their ability to elucidate the precise mechanisms of thrombogenesis in AF as they have not included tissue sampling. A porcine model of AF has previously been used to investigate atrial tissue factors of endothelial function and thrombogenesis. Cai *et al*[239] compared tissue samples collected from the RA, LA, LAA and ascending aorta after a pacing AF induction protocol. Samples were analysed for the detection of endocardial nitric oxide (NO) and nitric oxide synthase (NOS) and Plasminogen activator inhibitor-1 (PAI-1) protein levels, markers of endothelial function and

thrombogenesis respectively. This study showed that the induction of AF led to a down regulation of endocardial NOS expression along with decreased basal NO production and increased expression of PAI-1 within the LA compared the RA and to controls. Demonstrating that AF specifically affects NO formation (synthesis) within the LA, and suggests that this could be a further mechanism of thrombus formation and stroke in AF.

1.8.1 Cardiac Controls

Unlike other cardiovascular disease, the pathology of AF is restricted to the heart.[240] In comparative studies LA thrombotic markers have been shown to be significantly higher than the peripheral circulation.[35,75] Giving a physiological basis for elevated clinical reports of LA thrombus formation within the AF population. However it is unknown if there are predisposing factors in the normal human heart which may lead to the development of thrombus formation. The limiting factor is obtaining direct access to the heart and atrium in healthy controls. Historically patients with AF were routinely compared to age-matched control subjects for peripheral measurement of platelet and endothelial function.[194,195] However, as mentioned previously AF is a disease state specific to the atria of the heart, it is essential to utilise a population which allows for intracardiac blood sampling, who have normal hearts. Supraventricular tachycardia (SVT) patients have previously been utilised as an arrhythmic cardiac control cohort[192] whereby it was found that peripherally platelet activation was not elevated in SVT patients in

sinus rhythm or during the period of SVT, with very few cases ever being found of emboli in these patients.[241]

SVT patients are known to have no structural alterations of the heart, leading to normal cardiac contractility.[242] However they do have a focal tachycardia described by a defined focal electrical signal. Accessory pathway SVT or Wolff Parkinson White[243] are types of SVT where this extra electrical signal originates from the LA tissue leading to an accelerated heart rate, requiring an electrophysiology study and radiofrequency ablation procedure.[244,245] Sampling blood from the atrial circulation limits the ability for a complete control matched group for cardiac disease studies. As a particular group of SVT patients undergo a radiofrequency ablation where a transeptal puncture if necessary to access the left atrium, we are able to gain access to blood samples from both the left and right atria, similar to in and AF procedure, something which is not normally possible in a control group.[246]

1.9 Scope of Current Projects

Patients with atrial fibrillation are known to be a significantly higher risk of stroke. This thesis aimed to define the thrombogenic mechanism in atrial fibrillation and its major substrate mitral stenosis.

Aims of this thesis include:

1) To characterise markers of thrombogenesis within the human atria and compare them to the peripheral circulation in subjects with structurally normal hearts.

2) To determine if there is variation in atrial and peripheral thrombogenesis through endothelial function, Inflammation and atrial remodelling between Indian and Caucasian population.

3) To determine if chronic atrial stretch due to mitral stenosis is associated with elevated LA markers of thrombogenesis when compared to the RA and peripheral sampling sites.

4) To investigated the immediate and short term (24 hour) effect of the reversal of chronic atrial stretch following a balloon valvuloplasty procedure on the thrombogenic profile of patients with mitral stenosis.

5) To determine the atrial specific alteration of haemostatic mechanisms (platelet activation, endothelial dysfunction and inflammation), as well as tissue remodelling in a population of AF patients who would be considered low risk with a history of paroxysmal AF, but currently sinus rhythm.

6) To determine if markers of thrombogenesis, through endothelial function and inflammation, are consistent between AF, and its valvular substrate MS. In addition, regional differences in the expression profile of these markers in each group were also examined.

In order to achieve the above aims, mechanisms of thrombogenesis through platelet reactivity, endothelial dysfunction, inflammation and tissue remodelling were measured peripherally and within the right and left atrial of control (SVT) patients, MS patients and a cohort of paroxysmal AF patients.

2 CHAPTER TWO

General Methods

This chapter describes the general methods used throughout this thesis. These methods include blood collection, blood preparation, platelet activation and aggregation techniques, as well as ELISA assays for the measurements of endothelial function, inflammation and remodelling markers. If additional methods were used these are described in the relevant subsequent project chapters.

2.1 Blood collection

Blood was collected during each of the procedures, either through the sheaths that are placed within a femoral vein and the atrial chambers as a part of the procedure further details for each of the specific procedure are included within the specific chapters (Electrophysiology study and ablation, or Balloon Valvuloplasty).

Blood was collected utilising a slow withdrawal technique (approximately 1 ml per second) and transferred into tubes containing 3.8% sodium citrate (ratio 1:9). Markers of platelet activation, endothelial function, inflammation, thrombogenesis and tissue remodelling were subsequently analysed in both whole blood and plasma preparations.

2.2 Whole Blood Preparation

For experiments which required whole blood the shortest possible time was taken to get blood from the electrophysiology operating theatre to the thrombosis laboratory for testing. A minimum of 30 minutes was left between blood collection and analysis in whole blood studies. The experiments which utilised whole blood were flow cytometry and aggregation studies.

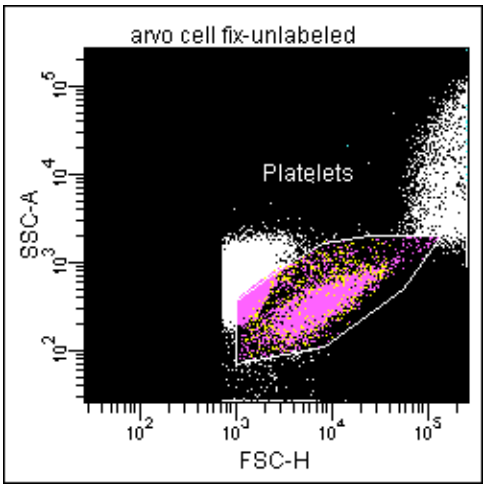
2.3 Plasma Preparation

Plasma from each sample site was obtained by centrifuging citrated blood samples at 2500g (3000rpm) for 15 minutes at 4 degrees. Aliquots of plasma were stored at -80 degrees for batch analysis. All tests were carried out through enzyme-linked Immunosorbent assay (ELISA). The ELISA tests were carried out as per specific company protocol.

2.4 Whole Blood Flow Cytometry

Whole blood flow cytometry was used to analyse surface expression of platelet activation receptor for p-selectin (CD62P, BD Biosciences) and procaspase activating compound -1 (PAC-1, BD Biosciences). Citrated whole blood samples were diluted 1:9 in 450µl of 10mmol TRIS-buffer before 5µl of FITC+ labelled monoclonal antibody was added. The sample was incubated at room temperature for 30 minutes out of sunlight. It was fixed using 400µl of CellFIX (BD biosciences) and stored at 4^{o}C. Using flow cytometry we were able to test for the

presence of platelet expressing specific labelled antibodies (FACScanto, Becton Dickinson, Oxford, UK). All antibodies were obtained from BD Bioscience. Platelets were identified using platelet specific CD42b antibody, and IgG FITC isotope control was used to detect non-specific binding and to define the threshold for activation dependent binding. Binding detection was done using forward scatter (size-dependent) and 90^{o} sideways scatter (density dependent), and was set at logarithmic gain for platelet identification on the basis of size using a platelet immunoglobulin bead suspension.



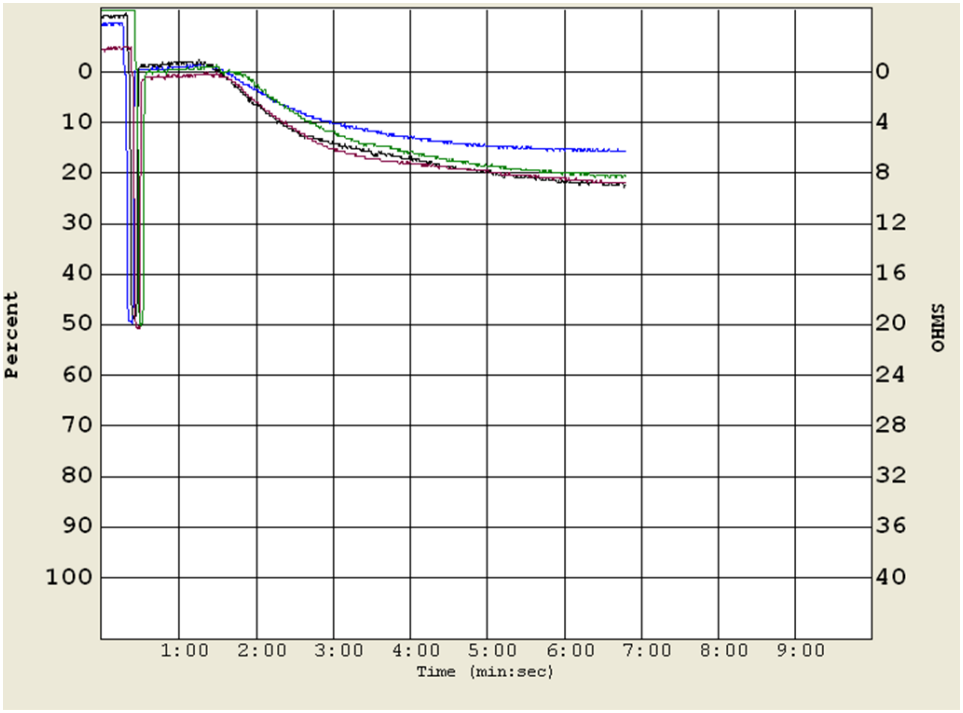
Representation of the platelet specific flow cytometry gate

The threshold for non-specific binding (defined with IgG FITC) was set at 1% of 10,000 platelet events recorded for each sample. To determine levels of platelet activation the percentage of platelets exhibiting specific binding of the antibody p-selectin (CD62p) and Glycoprotein IIb/IIIa expression (PAC-1) expressions were measured.

2.5 Aggregometry

2.5.1 Whole blood Impedance Aggregation

Aggregation studies were performed as previously described using a four channel impedance lumi-aggregometer (model 700, Chrono-Log, Havertown, PA, USA),[8] Aggregation was induced with adenosine 5'-diphosphate (ADP, 2.5µmol and 5µmol) with maximal response recorded for electrical impedance (in ohms), using a computer interface system (Aggrolink, chronology). This was utilised in chapter 3.



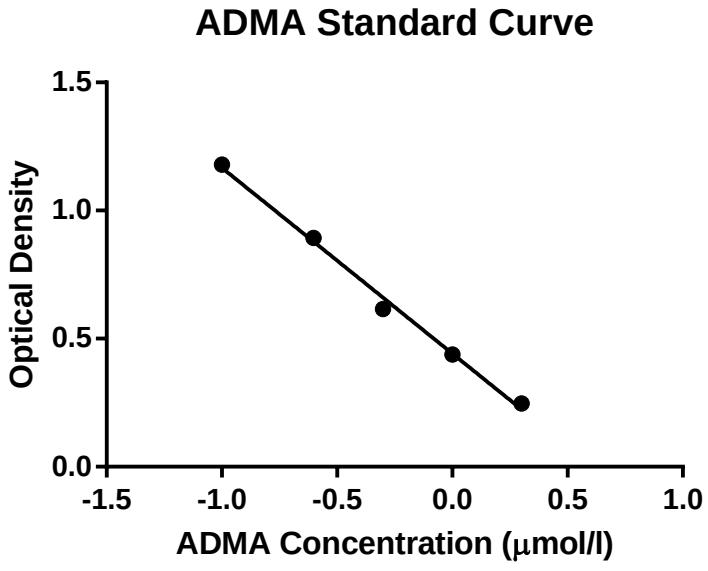
Representation of aggregation response using impedance aggregation.

2.6 Enzyme-Linked Immunosorbent Assay (ELISA)

2.6.1 Endothelial Function

2.6.1.1 **Asymmetric Dimethylarginine (ADMA)**

Asymmetric Dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide (NO) synthesis, and an enzyme that catalyses the production of NO from the amino acid arginine. NO is one of the major endothelium-derived vasoactive mediators and is involved in the modulation of the blood flow and the blood pressure. Even small changes of the ADMA concentration alter vascular NO production, vascular tone, and systemic vascular resistance.[247] Elevated ADMA concentrations are found in patients with diabetes mellitus, hypercholesterolemia, hypertension and peripheral arterial disease. ADMA levels predict cardiovascular risk independently of other variables, and it has been concluded that ADMA is a novel cardiovascular risk factor. This ELISA test is available commercially from Immundiagnostik®.

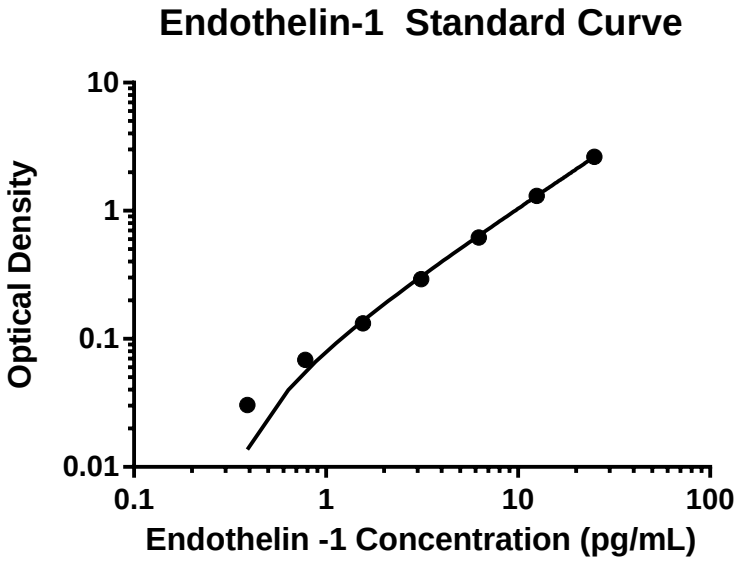


Reprehensive ADMA standard curve

2.6.1.2 **Endothelin-1**

Endothein -1 (ET-1) is produced by many tissues however its most prominent source is vascular endothelium. ET-1 is best known as a potent vasoconstrictor. Elevated blood ET-1 levels are associated with a variety of diseases, possibly as a function of a stress response.

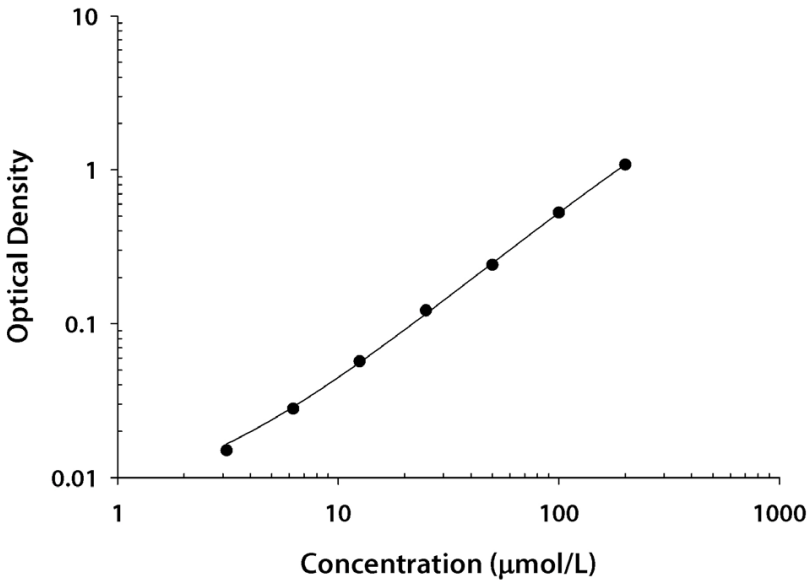
ET-1 also stimulates cardiac contraction, the growth of cardiac myocyte, regulates the release of vaso-active substances, and stimulates smooth muscle cell mutagenesis.[248] It also acts as a pro-survival factor for endothelial cells and regulates secretion by hypothalamic and pituitary cells.[249] ET-1 is known to control inflammatory responses by promoting the adhesion and migration of neutrophils and stimulating the production of pro-inflammatory cytokines. This ELISA test is available commercially through Quantikine ® R&D Systems.



Representation of ET-1 standard curve

2.6.1.3 **Nitrate/Nitrite Ratio**

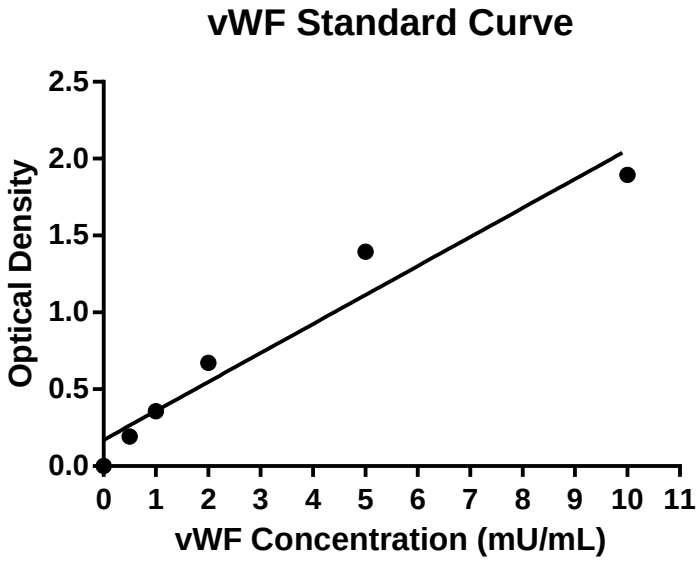
This ELIZA measures nitrate and nitrite levels as a measurement of nitric oxide (NO) metabolites. The biological activities of NO were first widely appreciated when it was identified as the endothelial-derived relaxing factor responsible for the potent vasodilation properties of stimulated endothelia. NO has the potential to mediate its effects on target cells via several different mechanisms. For instance, NO-mediated activation of the enzyme guanylyl cyclase catalyses the formation of the second messenger guanosine 3',5'-cyclic Monophosphate (cGMP). cGMP is implicated with a range of biological functions such as regulating smooth muscle contractility, cell survival, proliferation, axon guidance, synaptic plasticity, inflammation, and angiogenesis. This ELISA is commercially available through Cayman chemical.



Representation of nitrate/ nitrite standard curve

2.6.1.4 **von Willebrand Factor**

von Willebrand factor (vWF) is synthesised by endothelial cells and megakaryocytic, and is present in the sub-endothelium as well as in plasma and platelets. Its major function is to maintain vessel wall integrity. vWF does this through the promotion of the adhesion of platelets to the damaged endothelium and participates in platelet to platelet cohesion necessary for thrombus formation. vWF is used as a marker for endothelial cell function and integrity and have been shown to be increased in patients who have thrombosis. This ELISA test is available commercially through Imubind ® American Diagnostica Inc.

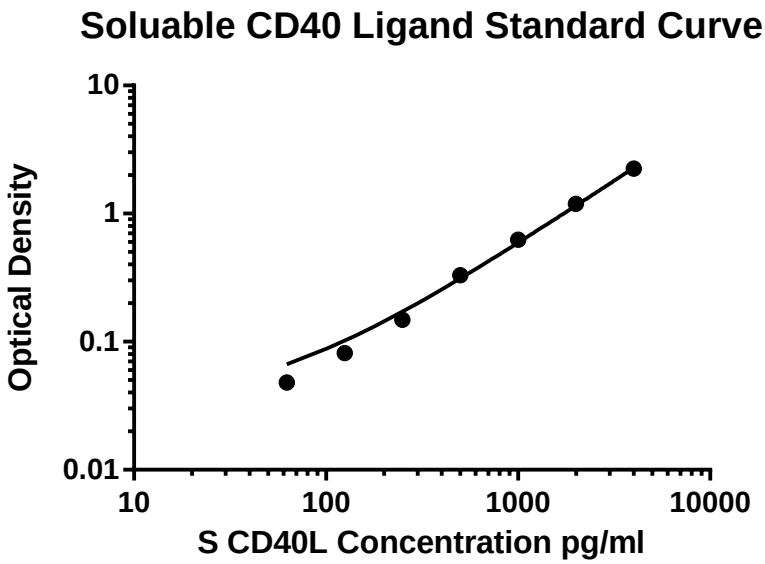


Representation of vWF standard curve

2.6.2 Inflammation

2.6.2.1 **Soluble CD40 Ligand**

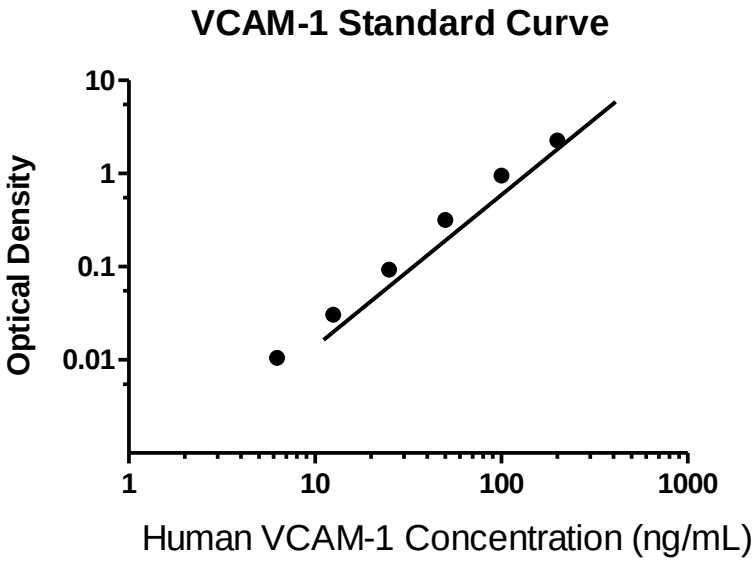
Soluble CD40 ligand (CD40L) is a member of the tumour necrosis factor superfamily and is produced in a variety of cells, including, inflammatory cells such as monocytes and basophils, vascular endothelial cells and platelets. CD40L is a mediator of both inflammatory and homeostasis processes and has been implicated in the pathogenesis of chronic inflammatory diseases such as atherosclerosis. This ELISA test is available commercially through Quantikine ® R&D Systems.



Representation of CD40 Ligand standard curve

2.6.2.2 **Vascular Cell Adhesion Molecule-1**

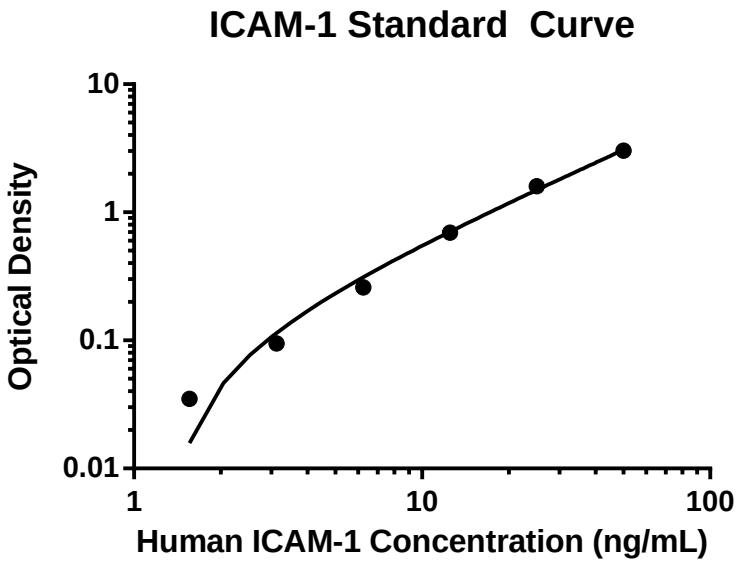
Vascular cell adhesion molecule (VACM-1) is a transmembrane glycoprotein which is typically expressed by the endothelium after stimulation from inflammatory cytokines. The VCAM-1 protein mediates the adhesion of lymphocytes, monocytes, eosinophil's, and basophils to vascular endothelium. It also functions in leukocyte-endothelial cell signal transduction, and it may play a role in the development of atherosclerosis and rheumatoid arthritis. This ELISA test is available commercially through Quantikine ® R&D Systems.



Representative if VCAM-1 standard curve

2.6.2.3 **Intracellular Adhesion Molecule-1**

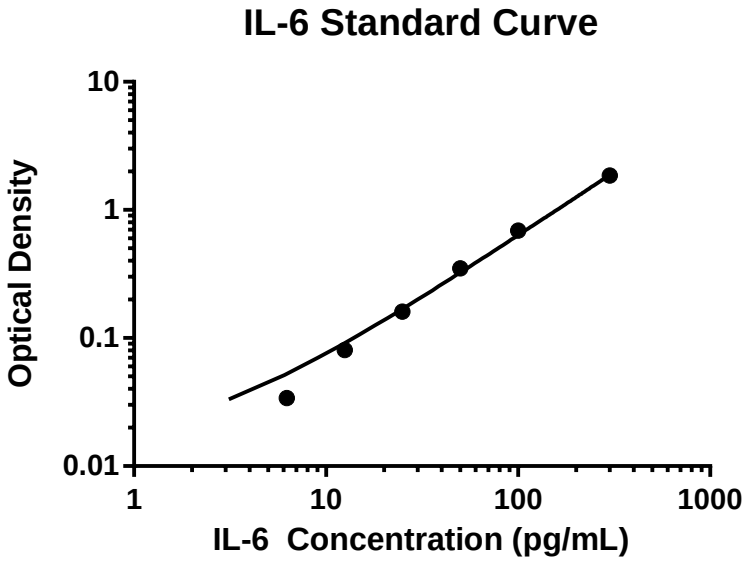
Intracellular adhesion molecule (ICAM-1) is a member of the immunoglobulin superfamily, the superfamily of proteins including antibodies and T-cell receptors. ICAM-1 is a transmembrane glycoprotein that plays a key role in leukocyte migration and activation. When activated, leukocytes bind to endothelial cells via ICAM-1/LFA-1 and then transmigrate into tissues. ICAM-1 ligation produces a pro-inflammatory effect, and has been associated with Subarachnoid haemorrhage, and cellular entry for the human rhinovirus. This ELISA test is available commercially through Quantikine ® R&D Systems.



Representative ICAM-1 standard curve

2.6.2.4 **Interleukin-6**

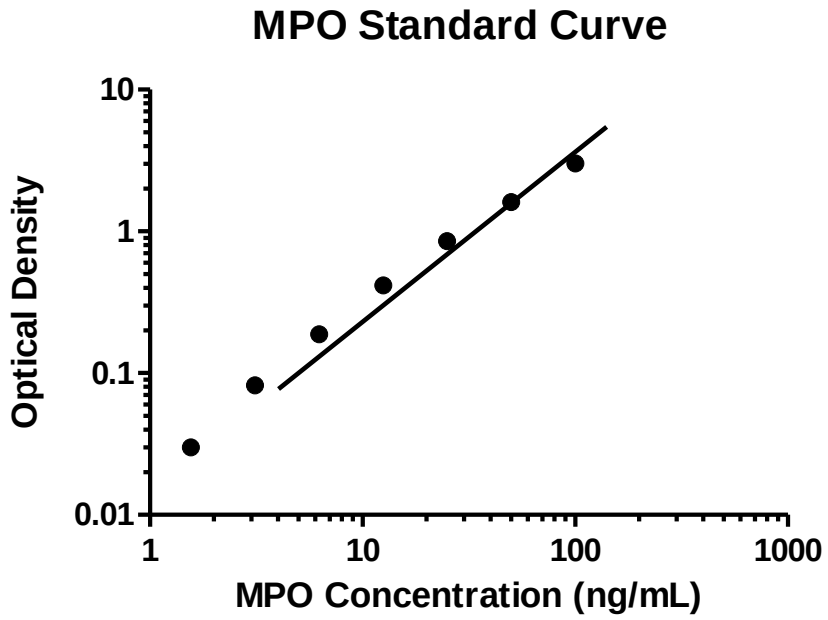
Interleukin-6 (IL-6) is a cytokine that plays important roles in inflammation associated reactions. IL-1 along with TNF-α and IL-1, drive the acute inflammatory response, and are almost solely responsible for fever and the acute phase response in the liver. It is also important in the transition from acute inflammation to either acquired immunity or chronic inflammatory disease. IL-6 also plays a role in chronic inflammation and the differentiation of naive T cells to inflammatory cells. It is able to modulate bone resumption and atherosclerotic plaque development. This ELISA test is available commercially through Quantikine ® R&D Systems.



Representitive of a IL-6 standard curve

2.6.2.5 **Myeloperoxidase**

Myeloperoxidase (MPO) is abundant in neutrophils and monocytes glycoproteins. MPO is involved in binding albumin, cytokeratin-1 and the macrophage receptor on the vascular endothelium to the integrin's in neutrophils. These interactions promote MPO clearance, a reduction of nitric oxide and bradykinin levels which results in reduced vasoconstriction and continued neutrophil activation. Elevated MPO levels have been associated with various clinical conditions such as inflammation, increased risk of cardiovascular events, vascular endothelial dysfunction and the severity of multiple sclerosis. This ELISA test is available commercially through Quantikine ® R&D Systems.

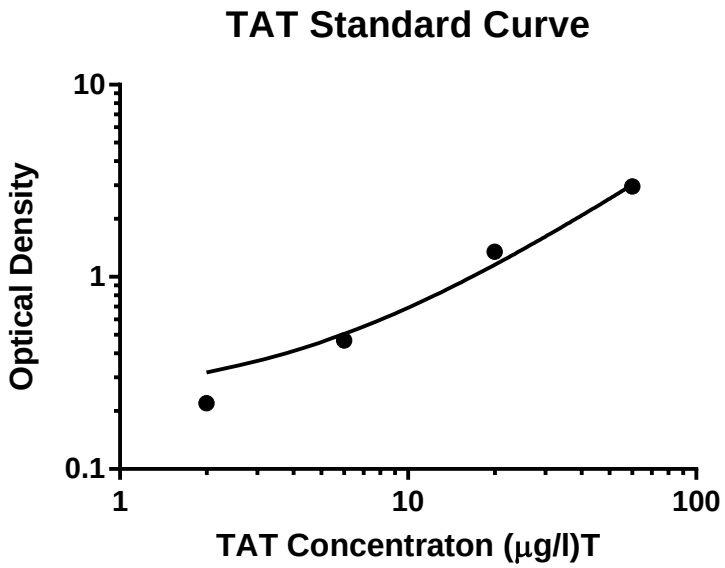


Representation of a MPO standard curve

2.6.3 Thrombin Generation

2.6.3.1 **Thrombin Anti-Thrombin**

The conversion of pro-thrombin into active thrombin is a key event within the coagulation cascade. Thrombin acts on various physiological substrates and is inhibited by anti-thrombin. The clinical significance of the altered levels of thrombin anti-thrombin complex (TAT) is that it predisposes to the occurrence of thrombotic events. These individuals have been found to be predisposed to thrombosis and disseminated intravascular coagulation, and have been found to have elevated levels of TAT. Raised levels of TAT have been observed in patients even when on a course of heparin or fibrinolysis therapy. This ELISA test is available commercially through Enzygnost ® Siemens.

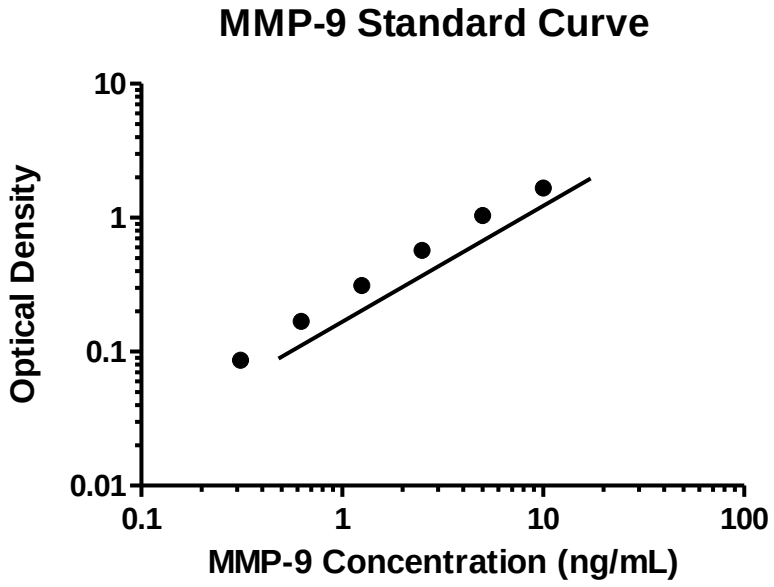


Representation of a TAT standard curve

2.6.4 Tissue Remodelling

2.6.4.1 **Matrix Metalloproteinase-9**

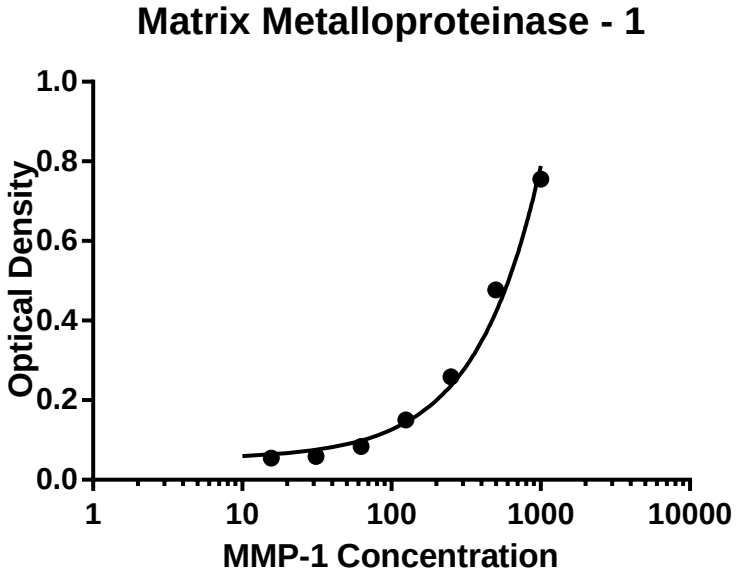
Human Matrix Metalloproteinase-9 (MMP-9) is secreted by monocytes, macrophages, fibroblasts, skeletal muscle satellite cells and endothelial cells. MMP-9's main role is tissue remodelling and wound healing through the mobilization of matrix-bound growth factors. This is primarily through the breakdown of extracellular matrix, and occurs in many normal physiological processes, such as embryonic development, reproduction, and tissue remodelling, as well as in disease processes, such as arthritis, intracerebral haemorrhage and metastasis. This ELISA test is available commercially through Quantikine ® R&D Systems.



Representation of MMP-9 standard curve

2.6.4.2 **Matrix Metalloproteinase-1**

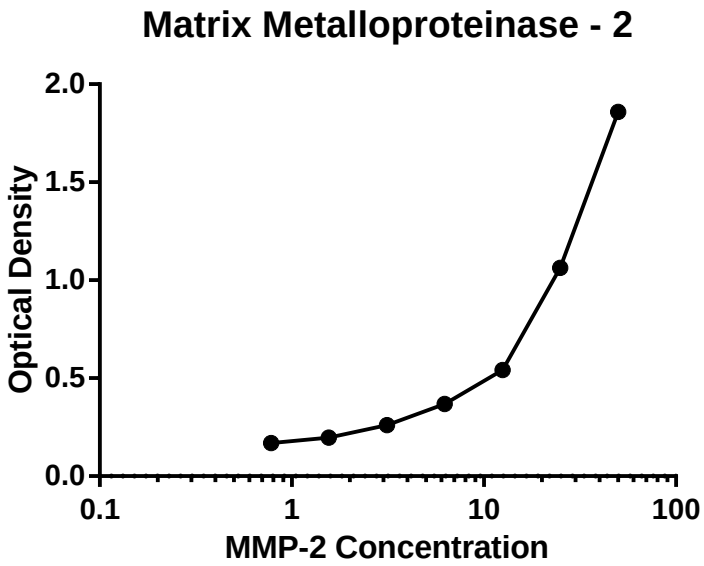
Human Matrix Metalloproteinase-1 (MMP-1) is also known as interstitial collagenase and fibroblast collagenase are enzymes involved in the breakdown of extracellular matrix in normal physiological processes, including embryonic development, reproduction, and tissue remodelling, as well as in disease processes, such as arthritis and metastasis. Specifically, MMP-1 breaks down the interstitial collagens, and has been shown to have increased expression with mechanical trauma. This ELISA test is available commercially through Quantikine ® R&D Systems.



Representation of MMP-1 Standard curve

2.6.4.3 **Matrix Metalloproteinase-2**

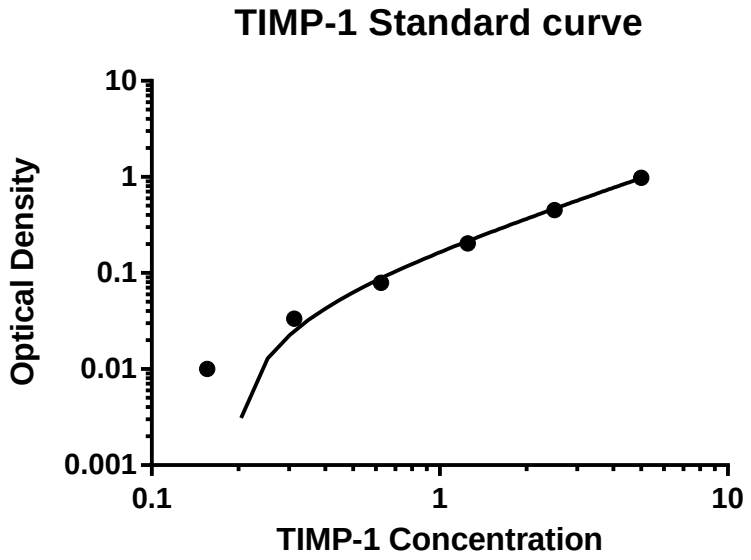
Human Matrix Metalloproteinase-2 (MMP-2) is a protein involved in the breakdown of extracellular matrix in many normal physiological processes. Activation of MMP-2 requires proteolytic processing, which is a complex of membrane type 1 MMP and the tissue inhibitor of metalloproteinase-2 (TIMP-2) which recruits pro-MMP-2 from the extracellular milieu to the cell surface. Mutation of the MMP-2 gene is associated with multi-centricosteolysis, arthritis syndrome, and possibly keloid scaring. This ELISA test is available commercially through Quantikine ® R&D Systems.



Representation of MMP-2 Standard curve

2.6.4.4 **Tissue Inhibitor of Metalloproteinase-1**

Tissue Inhibitor of Metalloproteinase -1 (TIMP-1) is widely synthesised by many cells, and its direct function is to block access of the substrate to the MMP catalytic site. TIMP's are highly specific to MMP generally, but not for any particular type. Many of the physiological functions of TIMP-1 are tied to the function of MMP-1, and an improper balance of TIMP and MMP production correlates with many pathological conditions. TIMP-1 also independently functions in multiple ways to support survival and growth of cells, in contrast to its function of inhibition of MMP. This ELISA test is available commercially through Quantikine ® R&D Systems.



Representation of TIMP-1 standard curve

3 CHAPTER THREE

Characterization of Thrombogenic, Endothelial and Inflammatory Markers in Supraventricular Tachycardia: A Study of Patients with Structurally Normal Hearts

Published in Clinical and Experimental Pharmacology and Physiology (CEPP), 2014

3.1 Summary

***Introduction*:** Patients with atrial fibrillation (AF) are at an increased risk of thromboembolism and stroke primarily from the development of thrombi within the left atria (LA). Pathological changes in blood constituents and atrial endothelial damage promote the LA thrombus formation. It is not known whether factors predisposing to LA thrombus formation in AF are disease specific, or also evident within the normal heart. The present study examined whether there are differences in platelet reactivity, endothelial function, inflammation and tissue remodelling in blood samples obtained from intra-cardiac and peripheral sites in subjects with normal hearts.

Methods: Twenty eight patients with diagnosed left sided supraventricular tachycardia (SVT) undergoing a routine elective electrophysiological study and ablation were studied. Blood samples were taken simultaneously from the femoral vein, right atrium and left

atrium, immediately following trans-septal puncture and prior to heparin bolus administration. All patients underwent echocardiograph to determine atrial sizes were within normal ranges. Blood samples were analysed for markers of thrombogenesis via ELISA technique.

Results: Patients with SVT showed no change in platelet reactivity or activation (CD62P P=0.91, sCD40L P=0.9), aggregation (P= 0.78), thrombus formation (TAT P=0.55), endothelial function (vWF P= 0.75, ADMA P=0.97 and NOx P=0.61), inflammation (VCAM-1 p=0.59 and ICAM-1 p=0.69) or tissue remodelling (MMP-9 p=0.79, MMP-1 p=0.40 and TIMP-1 p=0.12. between peripheral and atrial sample sites.

Conclusion**:** This study shows that subjects with normal hearts have evidence of consistent haemostatic function between atrial and peripheral sites. This suggests that structurally normal hearts with no remodelling do not contain predisposing thrombogenic, endothelial of inflammatory factors that promote and/or initiate thrombus formation. In contrast, abnormalities in the milieu of the LA of patients with AF promote thrombotic tendencies.

3.2 Background

Non-valvular atrial fibrillation (AF) is known to be associated with an increased risk of thrombus formation and stroke. The pathology of thrombus formation in patients with AF is restricted to the heart, which makes this disease unique in its thrombotic risk.[75,250] Previous studies have shown that in patients with a history of non-valvular AF there is an increase in platelet activation[74,238], coagulation[232], inflammation[163,251] and endothelial dysfunction[8,239] measured peripherally, all of which significantly contribute to thrombus formation. These comparisons were conducted based on the assumption that peripheral sampling correlated with intracardiac sampling. However, recent studies demonstrate that there is a difference between the atria and peripheral circulation in patients with a history of AF[75,238] and following AF induction.[196]

The ability to obtain atrial blood samples from healthy controls is extremely difficult. Historically, cardiovascular disease and AF have been compared with age matched control subjects for peripheral measurements of platelet and endothelial function. However, as mentioned in a disease state such as AF the pathology is restricted to the heart and specifically the atria. Therefore it is essential to utilise a population which allows intra-cardiac blood sampling to accurately determine the levels of platelet reactivity, endothelial function and inflammation in the structurally normal human heart, which does not exhibit AF. In the current study we chose to use patients with

supraventricular tachycardia (SVT) as a control group. SVT patients have a focal arrhythmia described by a defined focal electrical signal from the LA tissue leading to an accelerated heart rate.[242] Importantly, SVT patients have normal atrial structure and mechanical contraction, and no reported increase in thromboembolic events.[242] Furthermore, SVT patients undergo the same curative electrophysiological ablation procedure as AF patients thus making is possible to obtain blood samples directly from the left and right atria.

The purpose of this study is to characterise markers of thrombogenesis within the human atria and compare them to the peripheral circulation in subjects with structurally normal hearts. We hypothesized that there was no difference in platelet reactivity, thrombin generation, endothelial function, inflammation or marker of tissue remodelling between the peripheral and the human atria.

3.3 Methods

3.3.1 Patients Characteristics

This study consisted of 28 patients with structurally normal hearts, diagnosed with left sided accessory pathway supraventricular tachycardia (SVT) undergoing a routine elective electrophysiological study and ablation; all patients were in sinus rhythm a minimum of 48 hours prior to and at the time of the procedure. Left sided accessory pathway SVT is a focal tachycardia defined as a heart rate over 100beats per minute and described by a defined abnormal electrical re-

entry circuit from within the LA tissue. This extra electrical signal is the causes of the rapid heart rate in SVT patients. Patients with SVT have an increased risk of sudden cardiac death from this defined abnormal electrical activity; however they do not have an increased risk of thromboembolism or stroke.[242] Patients were excluded if they were younger than 18 years, had a clinical diagnosis of right sided disease, such as atrio-ventricular nodal re-entry tachycardia, if they had any previous clinical evidence of AF, or had structural heart disease. All patients underwent an echocardiography prior to the procedure to determine left and right atrial and ventricular dimensions, and to verify normal parameters of atrial size and function; any patients who had abnormal cardiac dimensions via echocardiography were also excluded. All patients were not taking antiarrhythmic or anticoagulant/platelet medication at the time of procedure. A baseline heart rate was recorded for all patients at the beginning of the procedure prior to catheter insertion.

All patients provided written informed consent to the study protocol that was approved by the Human Research Ethics Committees at the Royal Adelaide Hospital, Adelaide, Australia; University of Adelaide, Adelaide Australia.

3.3.2 Protocol

At the beginning of the electrophysiology procedure, blood samples were obtained from the femoral vein (peripheral) after puncture through

the inducer sheath after the patients were in the supine position for at least 20 min. Immediately following transseptal puncture and before the administration of heparin simultaneous blood samples (20mls) were collected through the sheaths in the left atrium (LA) and the right atrium (RA). Blood was collected utilizing a slow withdrawal technique (approximately 1 ml per second) and transferred into tubes containing 3.8% sodium citrate (ratio 1: 9). Markers of platelet activation, endothelial function, inflammation and thrombogenesis were subsequently analysed. (as described in Chapter 2)

3.3.3 Electrophysiology Study

The electrophysiological study was performed while patients were in a fasted and sedated state. Patients were administered local anaesthetic and were given sedation. Access to the right femoral vein was achieved using conventional (Seldinger) technique. A conventional transseptal puncture was performed to access the left atrium with a SLO sheath and BRK-1 needle (St Jude Medical, St Paul, MN). Following transseptal puncture blood samples were collected immediately from the LA, RA and femoral vein (Peripheral, P). The SVT ablation procedure is described in detail elsewhere. [192]

3.3.4 Statistical Analysis

Data is shown as mean ± standard deviation. Comparisons between the three sample sites were performed using a one-way ANOVA. Patient characteristics were compared using students T-test for

continuous data or a Fisher's exact test for categorical data. All data was tested for normality by a D'Agostino-Pearsons normality test, and log-transformed as appropriate Statistical analysis was performed in GraphPad Prism Version 6.0 (GraphPad Software). Statistical significance was defined as $p<0.05$.

3.4 Results

3.4.1 Subject Characteristics

Subjects with SVT were utilized as a control group allowing intra-cardiac sampling during curative electrophysiological procedure. Subject characteristics are shown in Table 1. All subjects had structurally normal hearts, defined by echocardiographic measurements. All had normal left and right atrial sizes, left ventricular dimensions and ejection fractions within predefined normal limits [Table 2].[252] No subject had significant risk factors for or concomitant coronary artery disease.

3.4.2 Platelet Activation

Platelet activation was measured as the percentage of platelets expressing p-selectin by whole blood flow cytometry, and platelet derived soluble CD40 ligand, in blood samples from the LA, RA and peripheral circulation, immediately following transseptal puncture. As demonstrated in figure 1A/B there was no significant difference in p-selectin, and Glycoprotein IIB/IIIA (PAC -1) expression levels across the three sample sites in the SVT patients ($p=0.31$ and $p=0.91$

respectively), this is also seen in figure 1C where neither the atrial nor peripheral circulation had altered sCD40L levels (p=0.97).

3.4.3 Platelet Aggregation

Platelet aggregation was measured with ADP (2.5μM and 5μM) induced impedance aggregation, in whole blood samples from the LA, RA and peripheral circulation. There was no significant difference in platelet aggregation found at low (2.5μM) dose ADP induced aggregation in SVT patients (Fig2A; p=0.18), and this was consistent for the high dose ADP (5μM), seen in (Fig 2B; P=0.78).

3.4.4 Endothelial Function

Asymmetric dimethylarginine (ADMA), von Willebrand Factor (vWF) and nitrate/nitrite (NOx) levels are all markers of endothelial dysfunction that have been shown to be elevated in many cardiovascular diseases and arrhythmias. As seen in figure 3A there is no alteration of ADMA levels in SVT patients across any of the sample sites (p=0.97), and furthermore, figure 3B shows there is no difference in vWF levels between the peripheral and atrial sites (p=0.75). Consistent with the results of ADMA, ET-1 and NOx there was no change in nitrate/nitrite (Fig 3C; p=0.61) and ET-1 (Fig 3D; p=0.61) levels across any of the sample sites.

3.4.5 Inflammation

Inflammatory markers were analysed in blood samples from the LA, RA and peripheral sites. Vascular cell adhesion molecule (VCAM-1, Fig 4A), intracellular adhesion molecule (ICAM-1, Fig 4B) and Interleukin-6 (IL-6 Fig 4C) are recognised as key factors in the human immune system with levels determined by ELISA test. No significant difference in the VCAM-1 or ICAM-1 levels were found between sample sites in the SVT patients ($p=0.73$, $p=0.69$ and $p=0.84$ respectively).

3.4.6 Thrombin Generation

Thrombin generation was analysed in plasma samples from the LA, RA and peripheral circulation. Thrombin anti-thrombin concentration (Fig5) is a key marker of thrombin generation, an essential component of the clotting cascade, and was measured through ELISA technique. There was no change in thrombin generation between the sample sites in the SVT ($p=0.55$).

3.4.7 Tissue Remodelling

There was no change found in the markers of tissue remodelling within the peripheral or atrial sites in SVT patients. This was consistent for both remodelling makers MMP-9 (Fig 6A; $p=0.79$) and MMP-1 (Fig 6B $p=0.40$), as well as the marker for reverse remodelling through TIMP-1 (Fig 6C $p=0.12$).

3.5 Discussion

Numerous studies into the prothrombotic state in non-valvular AF have shown that markers of haemostasis, platelet reactivity, endothelial function and inflammation measured in peripheral blood do not accurately reflect the intracardiac milieu.[35,75,238] Furthermore, there is marked difference between these parameters measured from the left and right sides of the heart in patients with AF. While these observations are consistent with the pattern of thrombus formation observed in non-valvular AF it is unknown if this thrombogenic profile exists in the normal heart.

The current study demonstrates that there are no differences in thrombogenic profile of the LA and RA, or between the central and peripheral circulation in normal hearts. This information provides a confirmatory basis that abnormalities in haemostasis, platelet reactivity, endothelial function and inflammation in AF are disease specific and not physiological in origin.

In order to understand the pathophysiology of increased thrombotic risk in a disease that is restricted to the heart, as in AF, it is essential to utilise a control group that allows for access to intracardiac blood sampling. Patients with SVT, have a focal tachycardia, with regular cardiac rhythm, described by a defined focal electrical signal from the atrial tissue leading to an accelerated heart rate.[253] As SVT patients with a left atrial accessory pathway undergo the same

electrophysiological procedure as AF patients; a radiofrequency ablation, with a transseptal puncture, access is gained to blood samples from the LA and LA. Importantly, SVT patients have no structural alterations of the heart and normal cardiac contractility.[242] The SVT patients enrolled in the current study were in sinus rhythm, with a normal heart rate (73 ± 16 bpm), and had normal heart dimensions and importantly have no documented increase in thrombotic risk. This was further verified with patients having no changes in the markers of atrial remodelling through MMP-9, MMP-1 or tissue reverse remodelling through TIMP-1(Fig6A, B and C) which is consistent with the normal atrial dimensions (Table 2). These patients were not on any anti-coagulation drug therapy nor have any other cardiac disease, thereby limiting any factors which could alter thrombotic measurements. We showed that there is no difference in platelet reactivity (Fig 1A&B, 2A&B), endothelial function (Fig 3A and B) and inflammation (Fig 5A, B and C) between the LA and RA, and between the intra-atrial and peripheral circulation in SVT patients. Our results are supported by a single study that has assessed platelet reactivity in patients with SVT.[192] Atalar *et al* showed that SVT patients had no change inbeta-thromboglobulinor platelet factor 4 within peripheral circulation when compared to age-matched controls; this remained consistent when SVT patents were in tachycardia.[192]

Peripheral blood sampling is routinely used to assess platelet function due to its convenience and ease of access. Patients with AF are

routinely compared to age-matched subjects by the peripheral measurement of platelet and endothelial function.[8,239] However, as the pathology of AF is restricted to the heart, it is expected that in AF patients haemostatic abnormalities occur at the cardiac level before manifesting in peripheral circulation. Recent data supports differences between the atrial and peripheral circulation of markers of thrombogenesis.[15,251,254] This was shown in our previous study that platelet reactivity was significantly increased within the LA of patients with non-valvular AF when compared to the RA.[238] In another study Akar *et al* has examined platelet reactivity in cardiac samples within AF patients.[75] This study showed following the induction of AF cardiac (coronary sinus) platelet p-selectin levels were increased, but there was no change in platelet p-selectin levels when measured in peripheral blood samples.[75] This has also been shown in patients with rheumatic mitral stenosis (a substrate of AF) where Chen *et al* found that p-selectin expression was significantly increased within the LA, this was not reflected within the RA or peripheral circulation.[35] However these finding are not consistently reported with other studies showing peripheral levels of coagulation are reflective of intracardiac levels.[232]

Patients with AF are known to be associated with a prothrombotic state and thrombin generation, with AF being one of the most important causes of ischemic stroke.[8,48,255] Previous studies have shown that AF is associated with alterations in platelet activation,[194,254] endothelial function,[129,194] and inflammation[65] within the peripheral circulation; all

important contributors to thrombus formation. Furthermore several studies have documented differences between the peripheral and cardiac sampling sites.[75,238,256] We have also demonstrated that platelet reactivity is significantly higher in the LA compared to the RA in patients with a history of AF when sampled in sinus rhythm.[238] Little is known about platelet reactivity within the normal human heart; the current study found no difference in platelet reactivity (Fig9A and 9B) or aggregation (Fig 10A and 10B) between the LA, RA and peripheral circulation in patients with SVT in sinus rhythm.

There was no change in thrombin generation between the LA, RA and peripheral samples, similar to results for platelet activation. This is one of the first studies to look specifically at thrombin generation as a precursor to thrombus formation in this group of patients, giving an insight into the haemostatic behaviour blood when the atrium is contracting normally.

Plasma vWF has been recognized as a key component in the response to endothelial dysfunction.[129] ADMA and Nitrate/Nitrite (nitric oxide (N)) have been demonstrated to be associated with several epidemiological risk factors for stroke; these factors are widely accepted markers of endothelial dysfunction in AF studies.[129,222] Endothelial function measured in SVT patients have shown that ADMA, vWF and NO levels were not alteration across any of the sample sites (fig 11A, B,C,D). Endothelial function has not previously been analysed within the intra-

cardiac circulation of SVT patients. This data gives a new insight into atrial endothelial function in a normal functioning heart, showing no indication of endothelial dysfunction. This provides further evidence that SVT patients are not at an increased risk of thrombotic events.

There is growing evidence that inflammation may be associated with thrombogenesis in AF, and has previously been used as a predictor for AF, and shown to be significantly elevated in AF patients.[163] We found no increase in inflammatory markers at any of the sample sites in SVT patients. This was consistent for all markers of inflammation VCAM-1 (Fig 4A), ICAM-1 (Fig 4B) and IL-6 (Fig 4C). Previous reports in SVT patients have shown results for inflammation markers that are consistent with the findings of this study. Where a study by Marcus *et al* found that neither C-reactive protein (CRP) nor Interleukin-6 (IL-6) differed between the femoral vein to the coronary sinus of SVT patients[251] These results further demonstrate that patients with structurally normal hearts have no signs of inflammation within the atrial and peripheral circulation, indicating no increase in thrombotic risk.

3.6 Limitations

The major limitation of this study is obtaining a normal cohort; where access to blood sampling of the atria is available. Patient with SVT have echographically and structural normal hearts and sinus rhythm

with a normal resting heart rate, but still have a defined electrical abnormality.

3.7 Conclusion

In conclusion, the results of the current study demonstrate that there are no regional differences in markers of thrombogenesis in structurally normal hearts. This demonstrates that the normal heart has stable haemostatic function between the atrial and peripheral circulations and hence no predisposing thrombogenic properties.

Table 1: Characteristics of Patients with SVT (n=28)

Characteristics	**Value**
Age (range), years	40 ± 13
Males : Females	14 : 14
BMI (±SD), kg/m^2	25.6 ± 4.7
Heart rate (±SD), BPM	73 ± 16
Left atrial diameter (±SD), mm	31.0 ± 10.5
Left atrial area (±SD), cm^2	19.1 ± 2.4
Right atrial area (±SD), cm^2	17.9 ± 4.3
Left ventricular end-diastolic diameter (±SD), mm	53.0 ± 5.4
Left ventricular end-systolic diameter (±SD), mm	36.6 ± 6.2
Ejection Fraction (%)	67.3 ± 6.7

Table 2: Normal LA Echocardiographic Dimensions

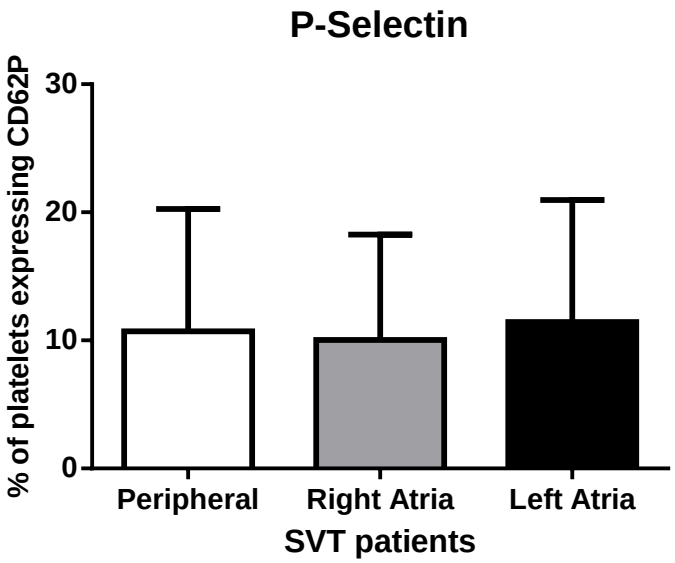
LA dimensions	Normal	Mildly dilated	Moderately dilated	Severely dilated
Diameter (mm)	28-40	41-46	47-52	>52
Major axis (mm)	41-61	62-67	68-76	>77
Area (cm2)	<20	20-30	30-40	>40
Volume (ml)	22-58	59-68	69-78	>79

Reference range for LA dimensions.[252]

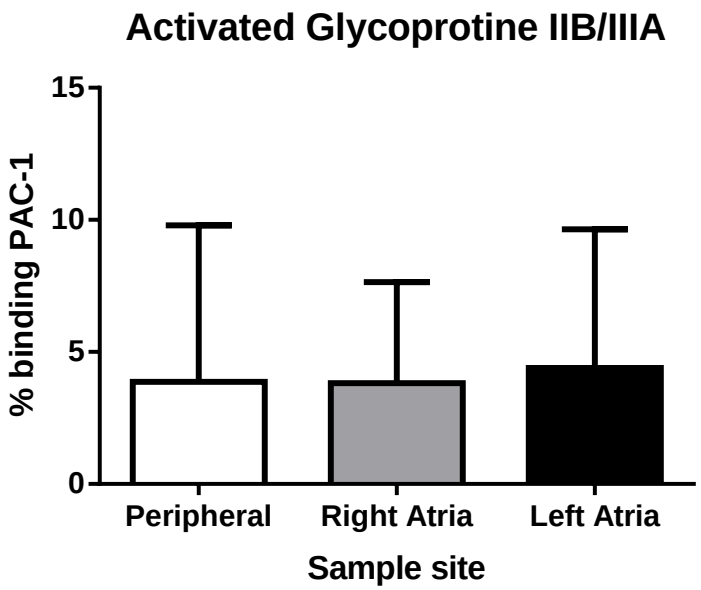
Platelet Activation

Figure 1: Platelet expression of P-selectin (A), glycoprotein IIB/IIIA(B) and plasma levels of sCD40L (c) in blood samples from the LA, RA and femoral vein (peripheral) in patients with SVT. There was no difference found in P-selectin levels between each of the sampling sites (peripheral vs. RA; p=0.48, LA vs. RA; p=0.19 LA vs. peripheral; p=0.41), and in glycoprotein IIB/IIA (peripheral vs. RA; p=0.8, LA vs. RA; p=0.6 and LA vs. peripheral; p=0.9). Similarly no difference in the levels of sCD40L was found between the sampling sites (peripheral vs. RA; p=0.95, LA vs. RA; p=0.88, LA vs. peripheral; p=0.83

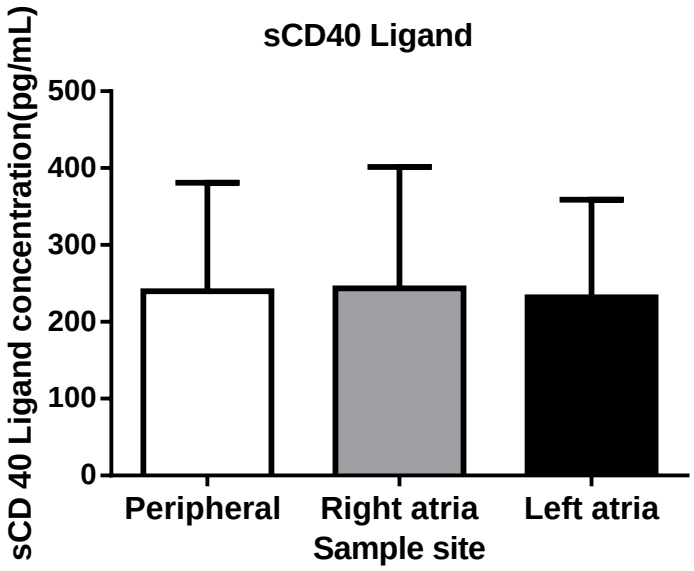
A.



B.


Activated Glycoprotine IIB/IIIA
% binding PAC-1
15
10
5
0
Peripheral
Right Atria
Left Atria
Sample site

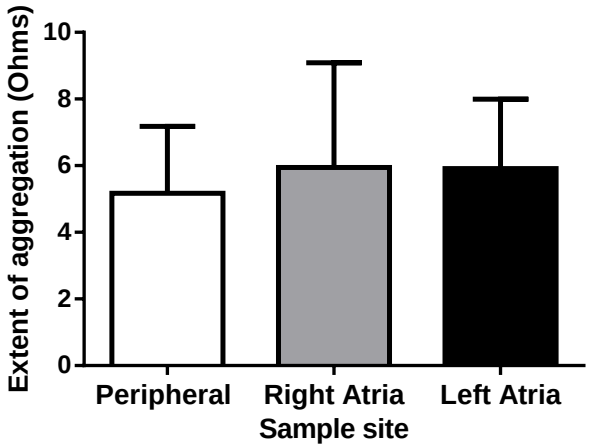
C.


sCD40 Ligand
sCD 40 Ligand concentration(pg/mL)
500
400
300
200
100
0
Peripheral
Right atria
Left atria
Sample site

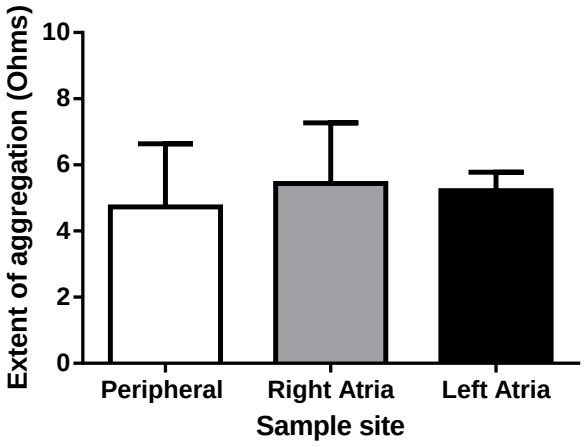
Platelet Aggregation

Figure 2: Plasma levels of platelet aggregation within the LA, RA and femoral vein (peripheral) of patients with SVT, determined by ADP induced impedance aggregometry. There was no difference in low (2.5μM, A) or high (5μM, B) dose ADP induced aggregation between any of the sample sites. Figure 2A (n=8): peripheral vs. RA; p=0.19, RA vs. LA; p=0.95, peripheral vs.LA; p=0.51. Figure 2B (n=8): peripheral vs. RA; p=0.62, RA vs. LA; p=0.78, peripheral vs. LA; p=0.56.

A.



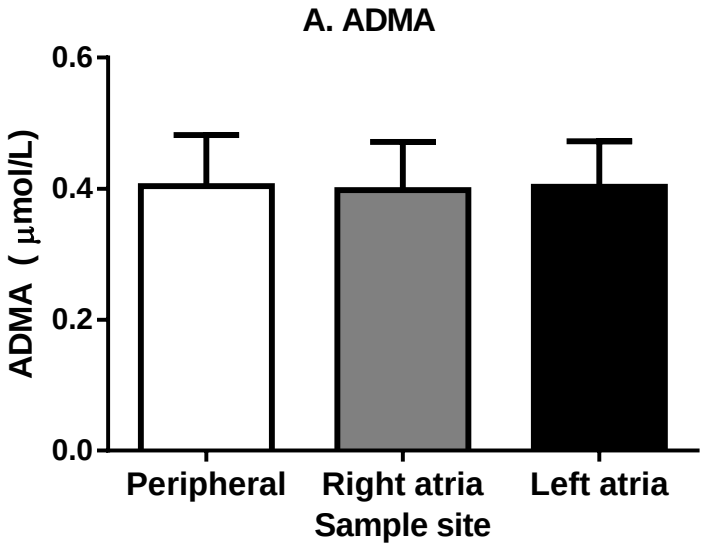
B.



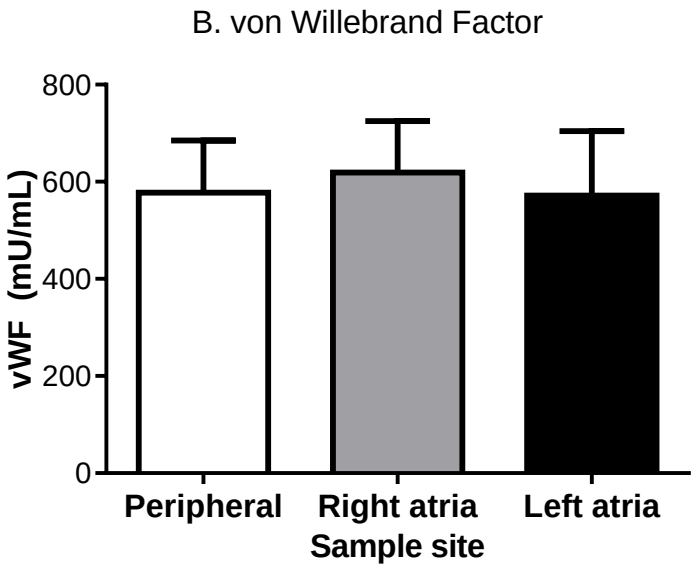
Endothelial Function

Figure 3: Plasma levels of ADMA (A), vWF (B), and nitric oxide (C). No significant difference in plasma levels of ADMA, vWF of NO were found between the LA, RA or peripheral circulations. Figure 3A: peripheral vs. RA; p= 0.84, RA vs. LA p= 0.86, peripheral vs. LA; p= 0.97. Figure 3B: peripheral vs. RA; p=0.52, peripheral vs. LA; p=0.93, RA vs. LA; p=0.51 and Figure 3C [n=8]: peripheral vs. RA; p=0.58, P vs. LA; p=0.38, RA vs. LA p=063)

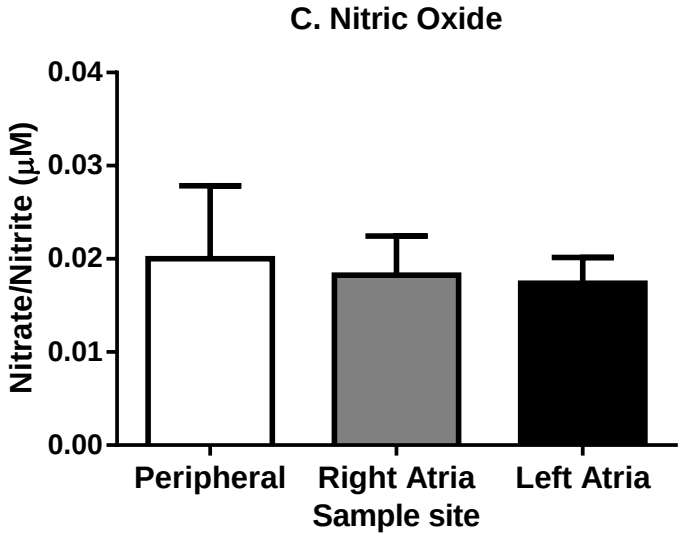
A.



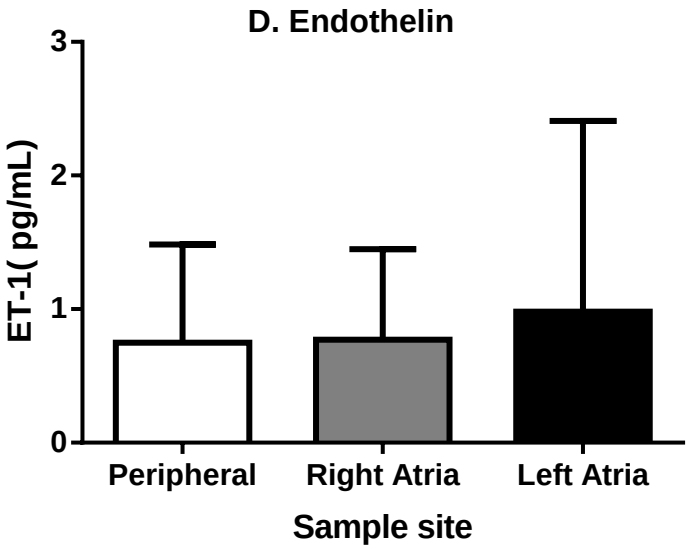
B.


B. von Willebrand Factor
800
600
400
200
0
vWF (mU/mL)
Peripheral
Right atria
Left atria
Sample site

C.


C. Nitric Oxide
0.04
0.03
0.02
0.01
0.00
Nitrate/Nitrite (μM)
Peripheral
Right Atria
Left Atria
Sample site

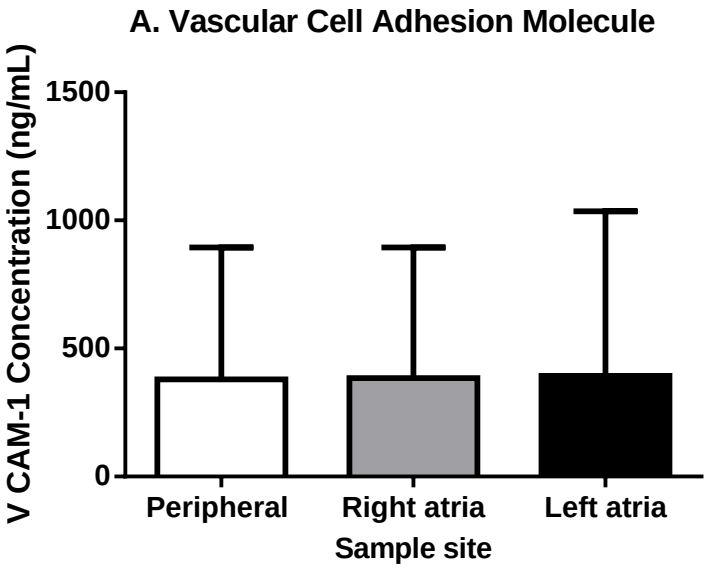
D.


D. Endothelin
ET-1(pg/mL)
3
2
1
0
Peripheral
Right Atria
Left Atria
Sample site

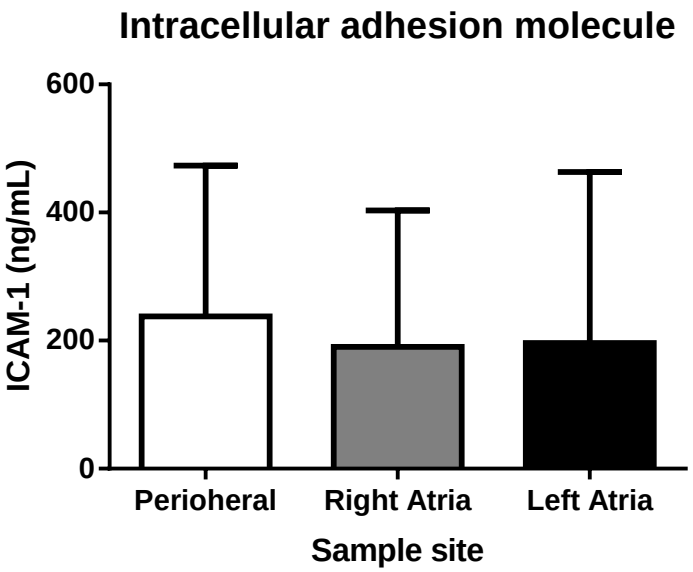
Inflammation

Figure 4: Plasma levels of inflammation in the LA, RA and peripheral circulation of SVT patients measured through VCAM-1 and ICAM-1. No difference in the levels of VCAM-1(A) (LA vs. RA; p=0.96, LA vs. peripheral; p=0.60, P vs. RA; p=0.61) or ICAM-1 (B) (LA vs. RA; p=0.79, LA vs. peripheral; p=0.90, peripheral vs. RA; p=0.69) were found between peripheral and cardiac sampling sites in patients with SVT. IL-6 no difference (LA vs. RA; p=0.79, LA vs. peripheral; p=0.90, peripheral vs. RA; p=0.69

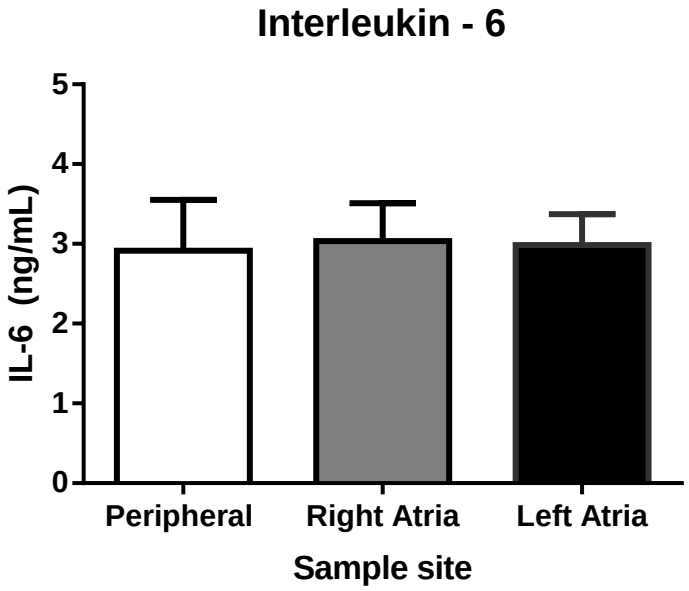
A.



B.


Intracellular adhesion molecule
600
400
200
0
ICAM-1 (ng/mL)
Perioheral
Right Atria
Left Atria
Sample site

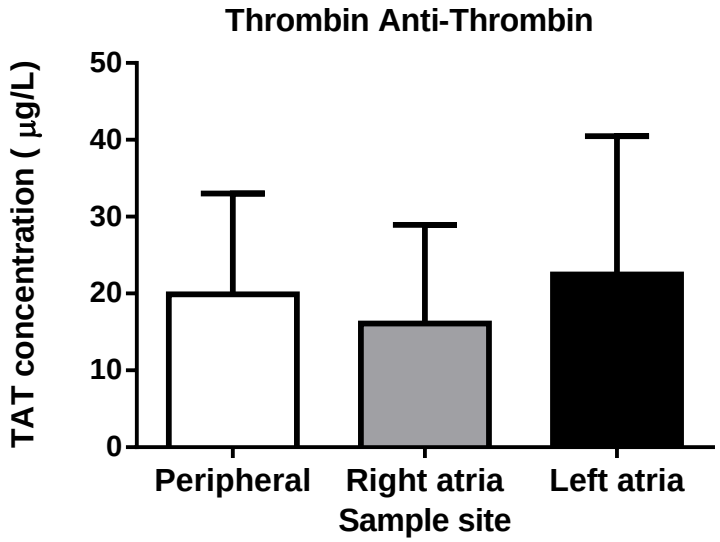
C.


Interleukin - 6
5
4
3
2
1
0
IL-6 (ng/mL)
Peripheral
Right Atria
Left Atria
Sample site

Thrombin Generation

Figure 5: TAT was used as a measured of thrombin generation. There was no difference in thrombin generation between the peripheral and atrial sites in the SVT patients (LA vs. RA; p=0.31, LA vs. peripheral; p=0.68, peripheral vs. RA; p=0.47)

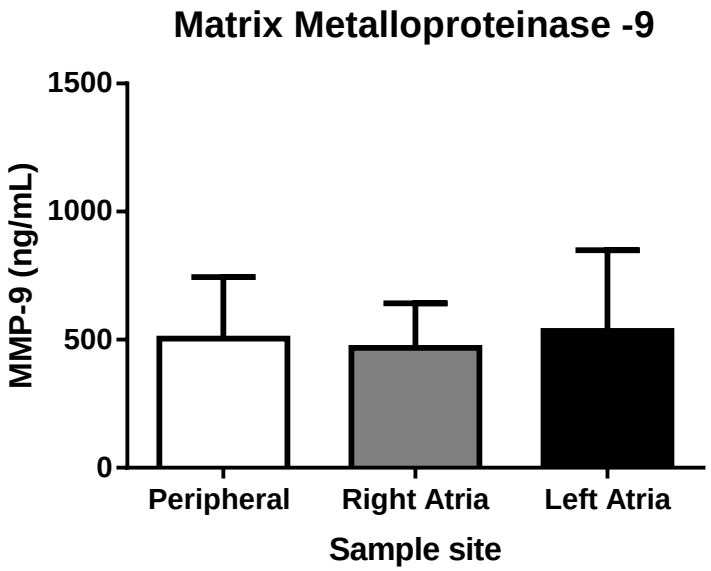
A.



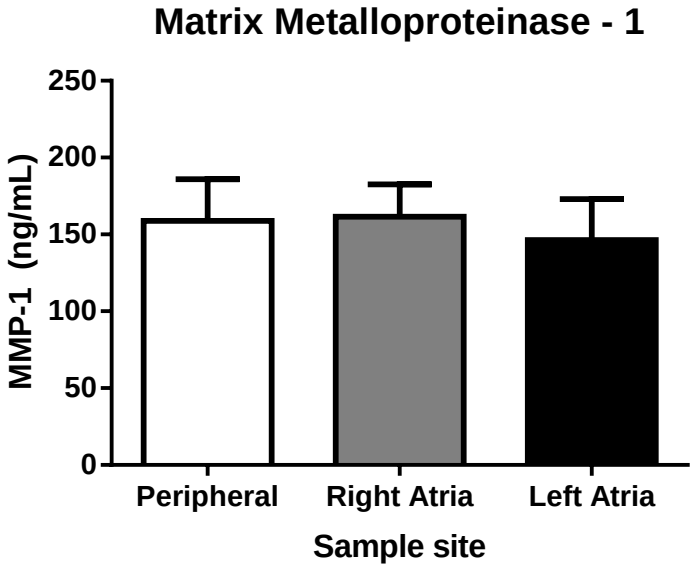
Tissue Remodelling

Figure 6: There was no change in remodelling at any of the sample sites in SVT patients for MMP-9 (p=0.79), MMP-1 (p=0.40) or TIMP-1 (p=0.12)

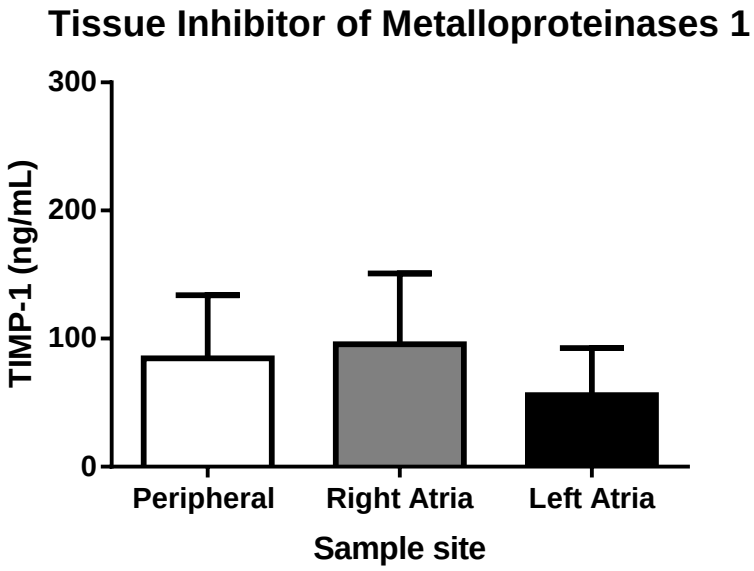
A.



B.



C.


Tissue Inhibitor of Metalloproteinases 1
TIMP-1 (ng/mL)
300
200
100
0
Peripheral
Right Atria
Left Atria
Sample site

4 CHAPTER FOUR

Influence of Ethnic Background on Inflammation, Endothelial Function and Thrombogenesis

4.1 Overview

Introduction: It has been suggested that ethnicity can make a significant difference to thrombogenesis within stroke related diseases. Ethnic differences have been shown to alter inflammatory and haemostatic factors; these may reflect differences in cardiovascular risk factors, pathophysiologic processes and stroke risk characterized by each ethnic group. However, there have been no specific studies which have determined this within a normal population of differing ethnicities. Importantly, the intra-cardiac differences in thrombogenesis remain unexplored.

Methods: We studied patients undergoing ablation of supraventricular tachycardia (SVT) in Australia (Caucasian population) and India (Indian population). Twenty-eight patients from the Royal Adelaide Hospital, South Australia, Australia and twenty four patients from the Christian Medical College, Vellore, India were studies. All patients had diagnosed with SVT due to left sided accessory pathways and were undergoing clinically indicated electrophysiological study and ablation procedure. Blood samples were taken simultaneously from the femoral vein, RA and LA, immediately following transseptal puncture and prior

to heparin bolus administration. Blood samples were analysed for the markers of endothelial function (ADMA, ET-1), inflammation (CD40L, VCAM-1, ICAM-1), and tissue remodelling (MMP-9, TIMP-1). With all markers measured by ELISA.

Results: There was no difference in tissue remodelling which was reflected in no differences in the echocardiographic measurements showed that there were no atrial structural changes in the LA and RA size and volume, as well as ejection fraction between the two ethnicities. There was no difference in endothelial function, Inflammation, between any of the sample sites between the two SVT populations.

Conclusion: We demonstrated that there is no influence of ethnicity on thrombogenic endothelial and inflammatory markers. This study found that there was no ethnic difference in peripheral and atrial makers of endothelial function, inflammation and atrial remodelling between Caucasians and Indians. In addition, there were no atrial or ventricular structural changes between the groups.

4.2 Background

The alteration in thrombogenesis due to ethnicity has been a novel and controversial topic. It has recently been showing that ethnicity can alter cardiovascular risk factors, risk of AF and stroke risk. However there is a need to increase data from minority groups in order to reduce racial and ethnic disparities in cardiovascular outcomes. Recent studies have also found that significant racial differences in baseline characteristics, treatments, and outcomes of patients hospitalized with AF. There appear to be important racial disparities in the care of minorities who are hospitalized with AF that require further investigation. Previously it has been shown that when compared with Caucasians, Afro-Caribbean's and people of African descent have an elevated cardiovascular risk factors (1.5 to 2.5 times greater), with higher rates of hypertension and diabetes mellitus, as well as related complications such as stroke, insulin resistance, and end-stage renal failure, all known risk factors for AF.[257-261] Yet paradoxically, they have a far lower incidence of coronary artery disease than both South Asians (originating in the Indian subcontinent) and white Caucasians.[261]

Previous studies have determined various ethnic differences in the incidence of AF. Showing that whites in particular have an increased risk of AF when compared to Blacks, Asians, or Hispanics.[262] With this heightened AF risk among Whites being most pronounced in the absence of further cardiovascular comorbidities. Suggesting that AF and its associated stroke risk may also alter between ethnicities. A

further study has which shown that Africans, Chinese, and Japanese had lower incidence of AF to that of Europeans. In this study it was further shown that in the case of Black Africans, this decrease incidence of AF is despite an increased prevalence of AF risk factors.[263] Further to this the prevalence of heart failure and AF in the under-researched South Asian and Black African -Caribbean minority communities in the UK appears comparable to that of the general population. HF and AF will continue to be a major cause of morbidity in all ethnic groups due to ageing of the population.[264]

Further to the risk of AF in different ethnicities, there are racial and ethnic differences in the incidence of strokes. A significant difference in myocardial infarction and symptomatic intracerebral haemorrhage recurrence among different race-ethnic groups has been shown, with the risk of recurrent ischemic and haemorrhagic stroke was greater in Asians with high blood pressure.[265] Further to this the Reasons for Geographic and Racial Differences in Stroke (REGARDS) investigators found that stroke risk is 3 times higher among blacks compared to whites for every 10-mm Hg increase in systolic blood pressure.[266] Previously it has been found that the total stroke incidence among the Hispanic and Black populations is approximately twice as high as that of the White population. The incidence of both ischaemic stroke and intracranial haemorrhage (ICH) are greater in the Hispanic population than in the White population. The same is also true for the Black population. Conversely, Asian populations are more prone to ICH.[267]

With haemorrhagic strokes comprising approximately 20% of all strokes in the White population.[268] Further to this, the vascular risk factor prevalence among different racial and ethnic groups explains disparities found in the prevalence of some stroke subtypes.[269] Race does not affect the treatment of strokes, with a study from the Neurological Disorders and Stroke and the European Co-operative Acute Stroke III trials, finding that the interaction of tissue plasminogen activator with race ethnicity was non-significant.[270] Despite this the treatment of stroke risk in AF through anticoagulation (mainly warfarin) has been reported to be less efficacious in preventing strokes in non-White patients with AF.[271] Possible reasons for this lack of effect have not been explored.

The Indian population is known to have an altered diseases risk profile to that of the Caucasian population. According to the World Health Organization, cardiovascular disease will be the number one cause of morbidity and mortality in the world by the year 2015 and it is assumed that Indians would be the most affected amongst all ethnic population. Hypertension and diabetes are highly prevalent among Asian Indian population, which may explain their high rate of stroke and heart attack in India. However there is little known about the prevalence on AF in an Indian population. A survey of a general practice Indo-Asian population of approximately 14670 patients, 12 patients with known AF were found, concluding that more information on the clinical epidemiology of AF in non-caucasian groups is still needed and urgently required.[272]

However these are no studies which have directly determined any ethnical differences in between normal populations, with particularly focus on the atria.

This study aimed to determine if there is variation in atrial and peripheral thrombogenesis through endothelial function, Inflammation and atrial remodelling between Indian and Caucasian population.

4.3 Methods

4.3.1 Patient Characteristics

This study population consisted of 52 patients with structurally normal hearts, diagnosed with supraventricular tachycardia (SVT) due to a left-sided accessory pathway-mediated undergoing elective electrophysiological study and ablation. Twenty eight patients were Caucasian recruited from the Centre for Heart Rhythm Disorders. Royal Adelaide Hospital, Adelaide, Australia, and 24 Indian origin patients were recruited from the Christian Medical College, Vellore, India. The following exclusion criteria were used: age less than 18 years; SVT not requiring LA cannulation such as atrioventricular nodal re-entry tachycardia; previous clinical evidence of AF; or structural heart disease. All patients underwent echocardiography prior to the procedure to determine left and right atrial and ventricular dimensions, to verify normal parameters of atrial size and function, any patients who had abnormal cardiac dimensions via echocardiography were also excluded. Only patients with no symptomatic arrhythmia in the 48 hours

prior to the study were included. No patients were taking antiarrhythmic or anticoagulant/platelet medication at the time of procedure.

All patients provided written informed consent to the study protocol that was approved by the following Human Research Ethics Committees: Royal Adelaide Hospital, Adelaide, Australia; University of Adelaide, Adelaide Australia; and the Christian Medical College, Vellore, India.

4.3.2 Electrophysiology Study

Electrophysiological studies were performed in a fasted conscious state. Access to the right femoral vein was achieved using conventional (Seldinger) technique. The following catheters were routinely positioned: (i) 10-pole catheter was positioned within the coronary sinus; (ii) 4-pole catheter at the right ventricular apex; and (iii) 4-pole catheter at the His location. Conventional electrophysiology mechanisms were utilized to determine the tachycardia mechanisms and identify the presence of a left sided accessory pathway.

Following confirmation of the need for LA access, a conventional transseptal puncture was performed with a SLO sheath and BRK-1 needle (St Jude Medical, ST Paul, MN). The study protocol was performed immediately following transeptal access. The SVT ablation procedure is described in detail elsewhere.[192]

4.3.3 Study Protocol

Immediately following transseptal puncture and before the administration of heparin simultaneous blood samples (20mls) were collected through the sheaths in the left atria (LA), right atria (RA) and femoral vein (peripheral). No ablation had been performed prior to the study protocol sampling. Blood was collected utilizing a slow withdrawal technique (approximately 1 ml per second) and transferred into tubes containing 3.8% sodium citrate (ratio 1: 9).

Analysis of Endothelial function/ Inflammation/ Tissue Remodelling by Enzyme-linked absorbance assay (ELISA)

The obtained blood samples were centrifuged at 2500g for 15 min at 4^{o}C and stored at -80 ^{o}C for batch analysis utilising enzyme-linked Immunosorbent assay (ELISA).Platelet activation was measured through soluble CD40 Ligand (CD40L), endothelial function through asymmetric dimethylarginine and Endothelin-1 (ADMA and ET-1), inflammation through Vascular and Intracellular adhesion molecules (VCAM-1 and ICAM-1) and tissue remodelling via matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1(MMP-9 and TIMP-1), via commercially available ELISA [for further details on specific markers see methods section 2.6].

4.3.4 Statistical Analysis

Data is shown as mean ± standard deviation. A two way ANOVA, with multiple comparisons was used to determine the difference between

the two populations. If appropriate Bonferroni's post hoc analysis was used to compare each of the two matching sample sites in each SVT group. Patient characteristics were compared using students T-test for continuous data or a fisher's exact test for categorical data. All data was tested for normality by a D'Agostino-Pearsons normality test, and log-transformed as appropriate Statistical analysis was performed using GraphPad Prism Version 5.0 (GraphPad Software). Statistical significance was defined as $p<0.05$.

4.4 Results

4.4.1 Patient Characteristics

There were no differences between the two populations in respect to demographic characteristics and cardiovascular risk factors [Table 1: Patient characteristics of control patients from Australia and India]

4.4.2 Echocardiographic Characteristics

Table 2 presents information on the structural and functional differences between the groups. There were no differences in the echocardiographic characteristics between the Caucasian and Indian populations. There was no difference in LA and RA size ($p=0.14$ and $p=0.07$), and volume ($p=0.08$), along with no difference in ejection fraction ($p=0.37$). [Table 2: Echocardiographic measurements]

4.4.3 Endothelial Function

Figure 1 demonstrates the findings with regards to endothelial function. There was no change in endothelial function between the Caucasian and Indian. This was consistent for both ADMA (p=0.38) and ET-1(p=0.12) concentrations and between the P, RA and LA circulation [Fig 1A&B].

4.4.4 Inflammation

Figure 2 demonstrates the findings with regard to inflammation. There were no differences in the levels of platelet derived inflammation (CD40L [Fig 2A], p=0.8), or vascular and intracellular inflammation (VCAM-1 or ICAM-1 [Fig 2B& C], p=0.7 and p=0.4, respectively) between the P, RA and LA between Caucasian and Indian populations.

4.4.5 Atrial Remodelling

Figure 3 demonstrates there was no change in atrial structural remodelling as assessed by levels of MMP-9 and TIMP-1 between the Caucasian and Indian populations. This was consistent for levels of MMP-9 (3A: p=0.9) and TIMP-1 (3B: p=0.6).

4.5 Discussion

4.5.1 Major findings

This study undertook detailed intra-cardiac and peripheral sampling to evaluate endothelial, structural and thrombogenic markers in matched Caucasian and Indian populations to characterise the potential sources

of ethnic variability in cardiovascular disease and stroke. It found the following between these two diverse ethnic groups;

(i) No difference in endothelial function

(ii) No difference in inflammation

(iii) No difference in tissue remodelling

The lack of changes suggests that the ethnic differences that are described in cardiovascular disease are likely to be related to the prevalence of comorbid risk rather than so specific ethnic differences.

4.5.2 Ethnic Differences in Cardiovascular Disease

Previously it has been shown that a person's ethnicity can alter cardiovascular disease prevalence and risk factors for AF.[260,273] However this has not been previously described in the setting of a control population. It has been suggested that by 2015 Indians would be the most affected amongst all ethnic population.[274] Further to this it has been shown that there is a heightened risk of the development of AF among Whites, when compared to Blacks, Asians, or Hispanics is most pronounced in the absence of cardiovascular comorbidities.[262] The patients in this study did not have cardiovascular risk factors and has shown that between two ethnicities there was no alteration to the structure and function of the heart. Where previous studies have shown cardiovascular diseases between Afro-Caribbean and European, where

by Afro-Caribbean's have poorer microvascular structure and function, unexplained by conventional risk factors. Yet paradoxically, they have a far lower incidence of coronary artery disease than both South Asians (originating in the Indian subcontinent) and white Caucasians.[261] Within these two different ethnic populations the cardiovascular disease risk factors were not different, suggesting it alterations in chance of particular diseases happening maybe dependent however between Caucasians and Indians there is not underlying disease profile risk, as seen in these control patents.

4.5.3 Ethnic Differences in Stroke

Further to the risk of AF in different ethnicities, there are racial and ethnic differences in the incidence of strokes. It has been shown that there is a significant difference in myocardial infarction and symptomatic intracerebral haemorrhage recurrence among different race-ethnic groups.[265] When compared with Caucasians, Afro-Caribbean's and people of African descent have an elevated risk (1.5 to 2.5 times greater) of hypertension and diabetes mellitus, as well as related complications such as stroke, insulin resistance, and end-stage renal failure. Hypertension and diabetes are highly prevalent among Asian Indian population, which may explain the high rate of stroke and heart attack in India.[274] Our result are in contrast to a previous study by Lip et al found that there are ethnic differences in inflammatory and haemostatic factors, between a South Asian and African Caribbean's.[275] This is mostly likely to the poor matching risk factors in

these populations. Further to this the Reasons for Geographic and Racial Differences in Stroke (REGARDS) investigators found that stroke risk that is 3 times higher among blacks compared to whites for every 10-mm Hg increase in systolic blood pressure.[266] The current study has shown that known the underling mechanisms (endothelial function, inflammation and remodelling) leading to thrombus formation in the LA are not altered in Caucasian or Indian controls, with recent clinical data supporting our findings.[276] However this is the first study to have directly determined any ethnical differences in baseline normal population, particularly within the atrial circulation within the heart.

4.6 Study Limitations

The major limitation of this study is obtaining a normal cohort; where access to blood sampling of the atria is available. Patient with SVT have echographically and structural normal hearts and sinus rhythm with a normal resting heart rate, but still have a defined electrical abnormality.

4.7 Conclusion

This is one of the first studies to analyse the differences in markers of endothelial function, inflammation and remodelling in these different two ethnicities within a group of patients who have structurally normal hearts. The thrombogenic properties did not differ between the peripheral, RA or LA circulation between the Caucasians and Indians.

These results suggest in young, risk factor matched population have no predisposing thrombotic factors that would independently account for increased risk of cardiovascular disease, AF or stroke in the future.

Table 1: Patient characteristics of control patients from Australia and India.

	Caucasian population N=28	Indian population N= 24	P Value
Age (years)	40 ± 13	36 ± 9	0.22
Sex (M: F)	14 : 14	16 : 8	0.6
Comorbidities			
Heart Failure	0	0	-
Coronary artery disease	0	0	-
Diabetes mellitus	0	0	-
Smoking	1	0	0.35
Hyperlipidaemia	0	3	0.055
Medications			
Beta blockers	9	11	0.2
Asprin	13	23	<0.0001
Warfarin	3	0	0.1
ACEI/ARB	2	0	0.2

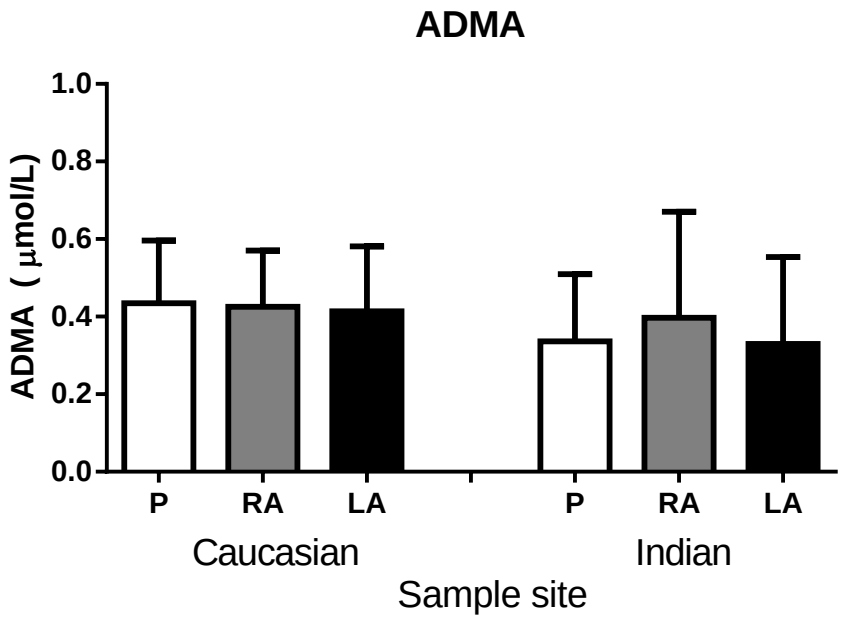
Table 2: Echocardiographic measurements

Echocardiographic Parameters	Caucasian population N=28	Indian population N= 24	P Value
LA Area (cm^2)	19.1 ± 2.4	24.1 ± 7.8	0.1
RA Area (cm^2)	17.5 ± 4.3	20.6 ± 4.5	0.07
LA diameter (mm)	31.0 ± 10.5	36.4 ± 5.1	0.08
LVEF (%)	67.3 ± 6.7	60.8 ± 9.7	0.4

Figure 1: Endothelial Function

ADMA was not altered in any of the samples sites between the two SVT groups (Fig1A, p=0.38).There was no change in ET-1 levels between Australian and Indian SVT patients, (Fig1B, p=0.12)

1A: Asymmetric dimethylarginine



1B: Endothelin-1

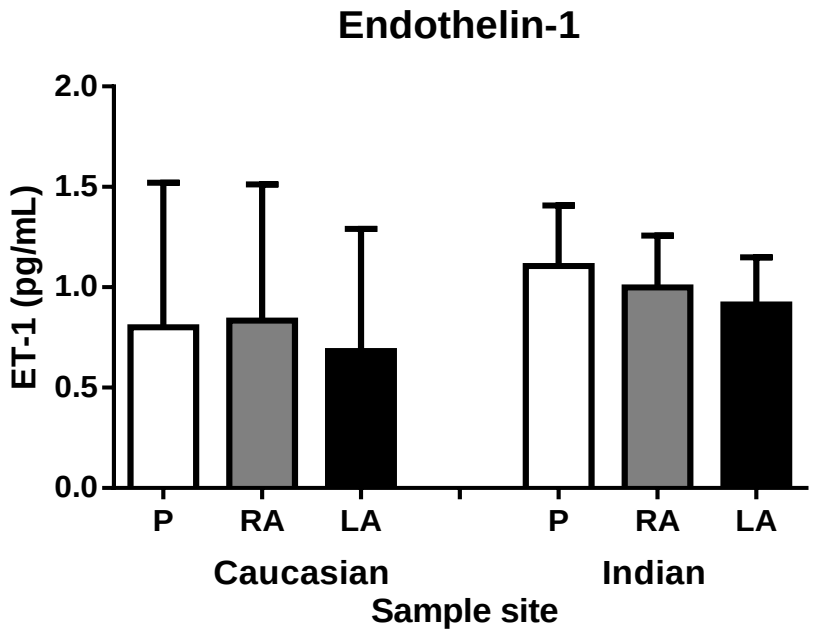
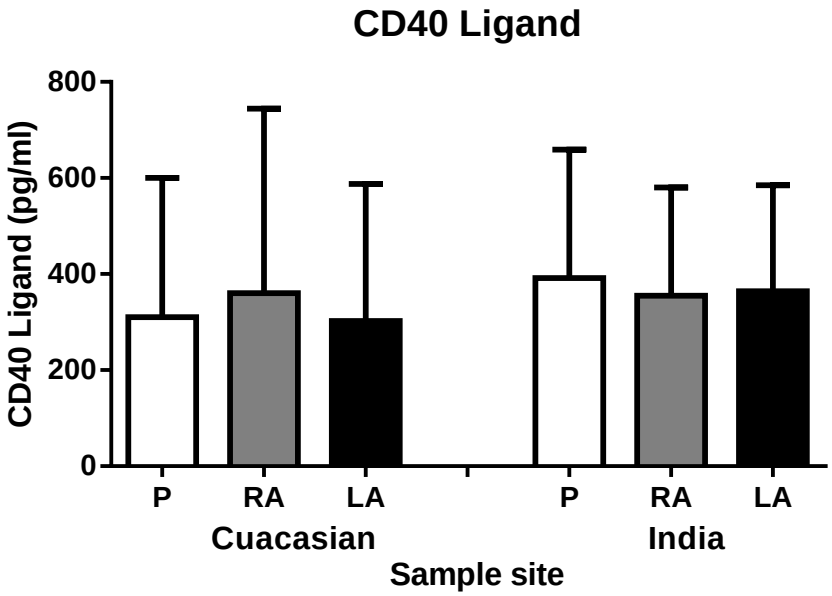


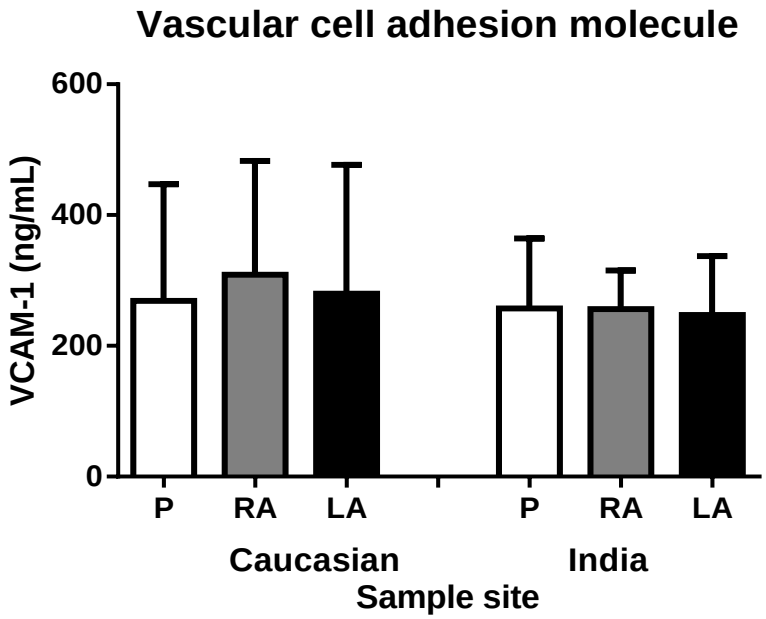
Figure 2: Inflammation

There was no change in CD 40 L levels between any of the sample sites. (Fig 2A, p=0.89), or V-CAM (Fig 2B, p=0.73) as well as for I-CAM-1 (Fig2C, p=0.64).

A: Soluble CD40 Ligand



B: Vascular cell adhesion molecule



C: Intracellular adhesion molecule

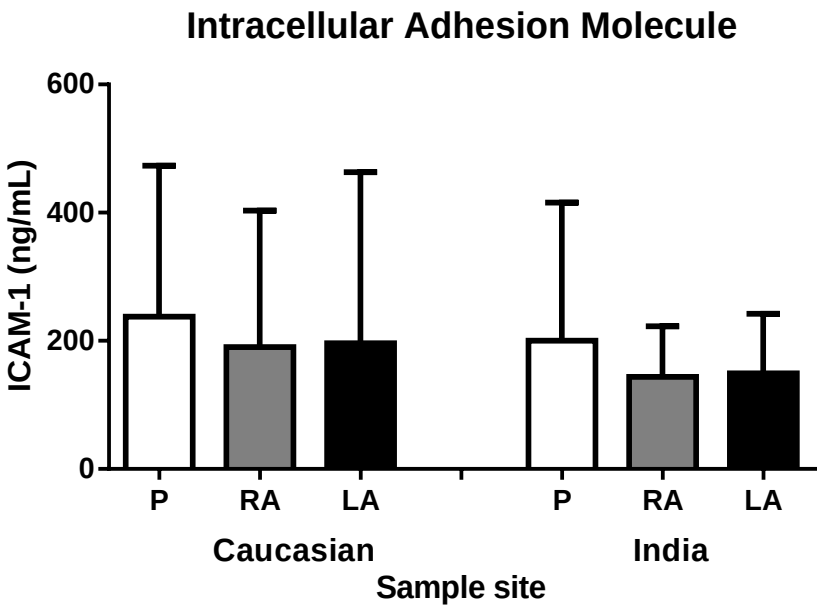
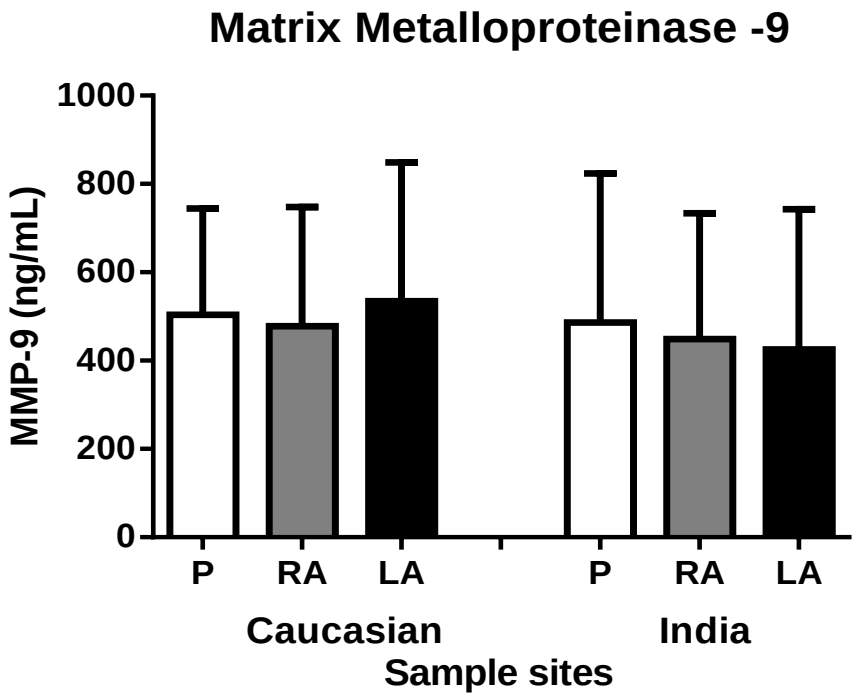


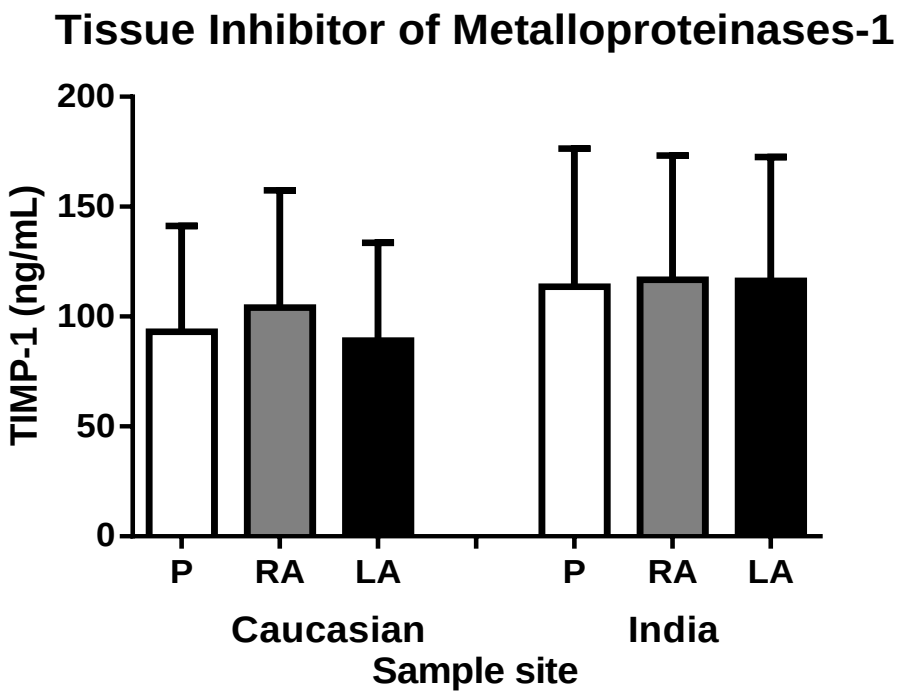
Figure 3: Tissue Remodelling

There was no difference in the levels of MMP-9 (Fig 3A, p=0.9) or TIMP-1 (Fig 3B, p=0.6) concentrations between any of the sample sites between the Australian and Indian SVT populations.

A: Matrix Metalloproteinase -9



B: Tissue Inhibitor Metalloproteinase - 1



5 CHAPTER FIVE

Risk of Thrombogenesis in Mitral Stenosis

5.1 Overview

***Introduction**:* Mitral stenosis (MS) is one of the leading causes of valvular AF in the developing world.[277] Enlargement of the LA is one of the most common structural changes that occurs in MS[29] and is known to lead to fibrosis and oxidative stress.[278,279] These alterations can also cause atrial electrical remodelling leading to the development of AF.[31] Patients with MS have been shown to have an increase in thrombogenic properties which include platelet reactivity, inflammation and endothelial dysfunction, although this is predominantly in the peripheral circulation.[40,41,235,280] In MS there exists a stroke risk in the absence of arrhythmia, the mechanisms of which remain unclear

***Methods**:* Fifty-nine patients with severe mitral stenosis undergoing clinically indicted balloon mitral valvuloplasty, and twenty four patients with supraventricular tachycardia due to a left sided accessory pathway (control) undergoing clinically indicated electrophysiological study and ablation procedure, were studied. All patients underwent echocardiography prior to the procedure. For the study protocol, 10mls of blood was collected from the peripheral (femoral vein), RA and LA, at the beginning of the procedure, post transeptal puncture and before the administration of heparin. Plasma samples were then collected and

frozen (-80) for batch analysis via ELISA. ELISA for endothelial function, inflammation and atrial remodelling were performed.

***Results**:* Compared to the control patients, MS patients had significantly larger LA size and LA volume ($p<0.0001$). MS patients also had significant endothelial dysfunction through both ADMA ($p=0.04$) and ET-1 ($p<0.0001$), at all sites. Similarly inflammation markers were atrial sites in inflammation through VCAM-1 ($p<0.0001$), IL-6 ($p<0.0001$) and MPO ($p=0.003$). This was specifically within the LA for these markers, despite this ICAM-1 ($p=0.4$) and CD40L ($p=0.2$) did not alter. Patients with MS had increased level of MMP-2 at all sites compared to controls ($p<0.0001$). There were also no differences in the markers of tissue remodelling through MMP-9 ($p=0.5$) and TIMP-1 ($p=0.7$) at any site between the control and MS patients.

***Conclusion**:* Patients with MS demonstrate the potential for a pro-arrhythmic environment. MS patients showed LA enlargement with increased inflammation within their LA. This study demonstrates that MS patients have abnormal thrombogenic properties within their LA which may predispose them to early thrombus formation. This abnormal profile may, in part explain the increased thrombogenic risk in patents with MS.

5.2 Background

Almost all cases of mitral stenosis (MS) are due to disease in the heart secondary to rheumatic fever and the consequent rheumatic heart disease or more rarely from a congenital heart disease.[29] MS is characterized by the narrowing of the orifice of the mitral valve of the heart.[281] Rheumatic heart disease was once a disease that plagued all world population; however, with improved availability of antibiotics and public health measures has now become confined to selected groups. The Australian Aboriginal population is reported to harbour the highest frequency of rheumatic heart disease in the world. It is a well-established cause of AF. Valvular AF confers the highest risk of thrombus formation and stroke. Population studies have demonstrated a 17.5 fold higher risk of stroke compared with the normal population (5 fold higher in patients with non-valvular AF).[48] AF is known to occur in 40-75% of patients who have MS. This is primarily caused by the alteration in the wall of the atria, due the chronic atrial stretch due to pressure and volume overload, caused by the diminished mitral valve function in MS. Atrial enlargement is a consistent feature which is shared in all type of AF. In addition, pathological studies have identified a direct injury to the myocardium that results from rheumatic fever.[282] Such areas of stretch and scar results in disruption of the normal conduction and therefore result in AF. MS is a disease known to have prothrombotic properties, leading to an increase risk of stroke,[38] and in the presence of AF it has been shown that patients with MS and AF together are more likely to have LASEC and LA clot formation.[57]

This study aims to determine if chronic atrial stretch due to MS is associated with elevated LA markers of thrombogenesis (endothelial dysfunction, inflammation and tissue remodelling) when compared to the RA and peripheral sampling sites.

5.3 Methods

5.3.1 Patient Characteristics

Fifty-nine patients with known severe rheumatic mitral stenosis (MS). [mitral valve area<1.2cm^2] and were undergoing clinically indicated balloon valvuloplasty (BMV) at the Christian Medical Centre in Vellore, India, were enrolled in the study.24 patients with structurally normal hearts, diagnosed with supraventricular tachycardia (SVT) due to a left-sided accessory pathway undergoing elective electrophysiological study and ablation were also recruited as a control group from the Christian Medical College. Patients had an average disease history of 10 years. Patients were selected on the basis of having severe MS with a mitral valve area of <1.5 cm^2 with significant symptoms (NYHA class ≥2) and mitral valve morphology suitable for PBMC as determined by the Wilkins criteria (score <10).

Patients were excluded if they had of the following:

- Age <18
- Patients with MS due to a non-rheumatic etiology.

- Patients with congenital heart disease, atrial fibrillation, LV systolic dysfunction, aortic stenosis or aortic regurgitation.
- Patients with a previous history of myocardial infarction.
- Individuals who are taking oral phosphodiesterase 5 inhibitors [i.e. sildenafil, vardenafil or tadalafil]
- Patients with peripheral vascular disease, hypertension, diabetes or vasculitis.
- Individuals who smoke.

All patients had no previous history of any arrhythmias documented by 12 lead ECG recordings 3 months prior to, at the time of, and during BMV procedureAll patients underwent transthoracic; M-mode, 2-dimentional (2D) and Doppler echocardiographic studies will be performed 1-2 weeks before the procedure, as per the American Society of Echocardiography criteria.[283] In MS patients the mean trans-mitral valve gradient, the mitral valve area (MVA) will be calculated from the echocardiographic Doppler study using the pressure half time method, and using the short axis 2D echocardiographic view.

All patients provided written informed consent for the study protocol that was approved by the Clinical Research Ethics Committees of the Christian Medical College, Vellore, India, and the University of Adelaide, Adelaide, Australia.

5.3.2 Balloon Mitral Valvuloplasty

A Balloon Mitral Valvuloplasty (BMV) was performed on all patients in a fasted and sedated state. The BMV was performed by the transeptal approach with the use of a Joseph mitral valvuloplasty balloon catheter. Details of the procedure have been described previously.[284] The BMV procedure was performed under local anaesthesia. Right heart catheterization precedes BMV, and was repeated, along with oximetry which was run after BMV. Heparin was administered intravenously after completion of the transeptal puncture. A modified back up wire is placed in the left ventricle through a Swan Ganz catheter. A Joseph's catheter balloon was then sent over the wire and inflated across the valve orifice. When additional balloon dilatation was required, the balloon would have been exchanged for a larger one and the same procedure would be repeated. Invasive pressure measurements were performed immediately before and after valvuloplasty.[284] Blood samples were analysed for the markers of endothelial function, inflammation and tissue remodelling, With all markers measured by Elisa [see Chapter 2.6, for further details].

5.3.3 Electrophysiology Study

The electrophysiological study was performed while patients were in a fasted and sedated state. Patients were administered local anaesthetic and were given sedation. Access to the right femoral vein was achieved using conventional (Seldinger) technique. A conventional transseptal puncture was performed to access the left atrium with a

SLO sheath and BRK-1 needle (St Jude Medical, St Paul, MN). Following transseptal puncture blood samples were collected immediately from the LA, RA and femoral vein (Peripheral, P). The SVT ablation procedure is described in detail elsewhere. [192]

5.3.4 Study Protocol

At the beginning of the procedure blood samples were taken from the sheath within the femoral vein (peripheral) and through the sheaths placed within the right (RA) and left atria (LA) for the valvuloplasty procedure. These samples were taken simultaneously, immediately following the transeptal puncture; no heparin was administered until all of the blood samples were collected. No alteration to the mitral valve had been performed prior to the study protocol sampling. Blood was collected utilizing a slow withdrawal technique (approximately 1 ml per second) and transferred into tubes containing 3.8% sodium citrate (ratio 1: 9). Blood samples were then spun to obtain plasma and this was then frozen for batch analysis for various markers of thrombogenesis via ELISA. ELISA methods are described in detail in chapter 2.

Analysis of Endothelial function/ Inflammation/ Tissue Remodelling by Enzyme-linked absorbance assay (ELISA)

The obtained blood samples were centrifuged at 2500g for 15 min at 4^{o}C and stored at -80^{o}C for batch analysis utilising enzyme-linked Immunosorbent assay (ELISA).Endothelial function through

asymmetric dimethylarginine and Endothelin-1 (ADMA and ET-1), inflammation through soluble CD40 Ligand (CD40L), Vascular and Intracellular adhesion molecules (VCAM-1 and ICAM-1), Interleukin-6 (IL-6) and Myeloperoxidase (MPO).Tissue remodelling was measured via matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1(MMP-9 and TIMP-1), via commercially available ELISA [for further details see methods section 2.6].

5.3.5 Statistical Analysis

Data is shown as mean ± standard deviation. Comparisons between the three sample sites in the MS patients and the control (SVT) patients were performed using a two-way ANOVA with multiple comparisons, with post hoc analysis via Bonferroni's multiple comparisons test where necessary. Patient characteristics were compared using students T-test for continuous data or a chi-squared test for categorical data. All data was tested for normality by a D'Agostino-Pearsons normality test, and log-transformed as appropriate. Statistical analysis was performed in GraphPad Prism Version 6.0 (GraphPad Software). Statistical significance was defined as $p<0.05$.

5.4 Results

5.4.1 Patient Characteristics

The patients included in this study consisted of 28 females and 31 males, with an average age of 32 years. Baseline characteristics of the study groups are presented in Table 1. Almost all (85%) patients had

documented pulmonary hypertension with an average NYHA score of 2.1. Approximately half the patients were on a beta blocker at the time of procedure. No patient was on antiplatelet or anti-coagulation medication. MS patients had a greater frequency of hyperlipidaemia. [Table 1]

5.4.2 Echocardiographic Characteristics

Compared with SVT patients, MS patients had severely dilated LA, and significantly increased LA volumes (Table 2). All patients had normal LV ejection fractions. The RA size of MS patients was shown to be normal to mildly dilated, with normal RA volumes.[252] [Table 2]

5.4.3 Endothelial Function

Despite the presence of the severely dilated LA there were no changes in markers of endothelial function between the peripheral, RA and LA circulation at baseline for MS patents. Endothelin-1was significantly higher in MS patients compared to the SVT ($p=<0.0001$), this was at all sites. [Fig: 1B]. With patients comparisons finding no difference between site within in ADMA $p=0.8$ or ET-1, $p=0.5$. ADMA concentration showed comparison between the sample sites between the control and MS patients has a significant difference in sample site in ADMA concentrations ($p=0.04$); however, there was no difference though post hoc analysis at any of the sample sites [Fig:1A].

5.4.4 Inflammation

Levels of inflammation were found to be higher within the atria of patients with MS compared with the peripheral circulation.Il-6 was found to have a significant difference between the sites within the MS population with the LA having significantly higher levels than the peripheral ($p=0.02$). with levels of vascular adhesion molecules (VCAM-1 [Fig, 2B] $p<0.0001$), Interleukin-6, [Fig, 2D, $p<0.0001$] and MPO [Fig 2E, $p=0.003$] common markers of an acute inflammatory response showed a stepwise increase in the sites comparison between the control and MP populations.

With further analysis, it was found that these changes were significant between the LA of the controls and MS patients in each marker, VCAM-1 $p=0.008$, IL-6 $p<0.0001$, and MPO $p=0.01$. This was also the case for the RA comparison in VCAM-1 $p=0.02$, however not or IL-6 or MPO.

5.4.5 Tissue Remodelling

There was also no difference found in MMP-9 or TIMP-1 between the control and MS patients at any sample site (MMP-9; P; $p=0.9$, RA; $p=0.8$ and LA $p=0.3$TIMP-1; P; $p=0.9$, RA; $p=0.9$ and LA $p=0.4$), however MMP-2 was significantly increased at each site compared to the MS patients (P $p<0.0001$, RA $p<0.0001$ and LA $p<0.0001$). There was no change in the level of MMP-9, MMP-2 or TIMP-1 between the three sample sites in MS patients ($p=0.76$, $p=0.80$ and $p=0.62$,

respectively). There was some evidence of a stepwise increase in remodelling in MMP-9 and TIMP-1 from the peripheral to LA circulation [Fig 3 A and C].

5.5 Discussion

5.5.1 Major Findings

This study was undertaken to determine how endothelial function, inflammation and tissue remodelling along with echocardiographic measurements are altered in MS compared to a control population, and further to detail atrial and peripheral levels. To determine how the structural changes which occur in MS may alter the risk of thrombus formation within the atria.

It found that in MS patents:

i) Compared with the SVT population, MS patients have significantly larger LA size and volumes.

ii) Endothelial function was increased at all sites compared to control patients.

iii) Increased level of MPO, IL-6 and VCAM-1 within the LA, compared with both the RA and peripheral circulation suggesting that MS affects LA inflammation.

iv) No change in tissue remodelling between the peripheral and atrial circulation in MMP-9 and TIMP-1, however at all sites in the MMP-2 levels.

Previously it has been shown that there is an increased risk of thrombus formation within the LA of patients with MS, most commonly seen via left atria spontaneous echocardiographic contrast (LASEC).[171,285]This has been shown to be related to an increased risk of stroke, though thrombus formation and embolization. In addition, a previous study demonstrated increased LA specific platelet reactivity compared with the RA and peripheral sites. Previous studies that have shown alterations in peripheral markers of coagulation, fibrinolytic system, platelet activation, and endothelial dysfunction in MS patients; however none of these studies have included atrial sampling to assess levels within the atria.[41,46]Asin AF, there is little specific research into these and other pro-thrombotic factors within the LA and how these factors contribute to thrombus formation in MS. Hence, this study aims to gain knowledge of the thrombogenic properties of the atria of patients who have MS.

Despite significant alterations in LA structure no difference was found in the markers of endothelial dysfunction between the three sampling sites in the current study. ADMA and ET-1 levels did not alter across the three sampling sites, ($p=0.8$ and $p=0.5$, respectively), This was unexpected as the obvious anatomical changes which occur as part of

MS, led us to assume that there may be significant endothelial dysfunction within the LA compared with the periphery. However when compared to the control population all sites showed significant increase in endothelial dysfunction.

In chronic disease ADMA and ET-1 are at stable levels, and it would only be expected that there would be regional differences after a local stimulus, for example after the reduction of chronic atrial stretch. The changes in LA size and function have previously been reported and where characterized by LA enlargement, loss of myocardium, and scarring associated with widespread and site-specific conduction abnormalities. However there was no change or an increase in effective refractory period. These abnormalities were associated with a heightened inducibility of AF.[31] There is limited research into specific endothelial markers in MS patients prior to treatment. Previous studies founds that von Willebrand Factor levels were significantly greater in patients with MS with sinus rhythm or AF, and where LASEC is present. Subsequently, it was found that levels of coagulation activation, platelet activation, and endothelial dysfunction in MS patients were similar in patients with all cause AF.[46] I would speculate that the lack of difference was a consequence of the duration of disease time and thus stability of structural remodelling. All patients had long term MS and it is probable that atrial remodelling caused by the atrial overload has occurred earlier in the disease.

MPO, IL-6 andVCAM-1 was found to be significantly higher within the LA of MS patients at the beginning of their BMV procedure compared to the controls. Demonstration that there is a significant inflammation occurring in MS patients, with all markers increased within the LA compared with the peripheral circulation. Despite these alterations there was no difference in the other markers of inflammation (CD40L and ICAM-1). There results could be due to the length of time that patients have had MS as higher levels of inflammation to be associated with the acute phase of the disease. These results are consistent with earlier studies that have had mixed results with thrombogenic markers in MS patients.[29,40] MS may affect different thrombogenic markers to that of AF, however these is limited studied which have investigated these markers in a comparable populations.

Tissue remodelling is known to occur in MS, with enlargement of the LA a very common consequence of the dysfunctional stenotic mitral valve, as seen in our echocardiographic characteristics. The remodelling has been shown to be able to be reversed after treatment for MS.[36] All patients had severely dilated LA leading to increase in LA volumes; however this did not affect the LV ejection fraction. Although our results show there was no change in remodelling markers MMP-9 and TIMP-1, MMP-2 was significant at all sites, this would have been expected to be seen though all markers due to the significant structural changes to the LA, which possibly occurred earlier in the disease possess. This may be due the duration of the disease, as this study

only compared sites within patients with chronic disease, and did not compare patients with varying duration of disease. In previous studies where MS patients have the collagen volume fraction of fibrosis significantly increased, these studies also show increased fibrosis within the LA. [286,287]These patients having more advanced stenosis, therefore requiring the balloon valvuloplasty procedure and it is expected that most of the remodelling as already occurred.

5.6 Limitations

All of the above mentioned factors that we measured are a part of the changes in haemodynamic occurring in relation to thrombus formation in MS patients. These patients have stable and long term stenosis of their mitral valve. All MS patients in our study were on beta blockers as a routine therapy to reduce the chance of them progressing into AF or to reduce burden of AF,[288] (none of our patients had documented AF) and this may be a factor in the inflammatory results. It is believed that due to the stability of the disease we found no changes in the markers of tissue remodelling, as it had already occurred and is now permanent damage to the endothelium. This may also explained why no change in endothelial dysfunction and inflammation was found, as inflammation had become stable over the time course of the disease state. Another area of interest, which has yet to be investigated in MS patients, is tissue sampling. Ability to derive structural damage, along with endothelial dysfunction and damage in atrial tissue may significantly increase our knowledge of how chronic atrial stretch from MS affects

atrial thrombus formation. Another factor which may be altering these makers is the age range of this study population; the mean age of our population is 32years, as rheumatic favour is known to be predominantly a childhood condition, and the duration from onset to testing may explain the lack significant of results.

Due to testing methods and time restraints we were only able to use blood samples from only 44 patients of the 59 patients were analysed for all measurements of ELISA markers.

5.7 Conclusion

Mitral stenosis is known to be associated with a significant increase in prothrombotic tendencies, with the progression to valvular AF having a significant increase in stroke risk, from LA thrombi. These results suggest that MS patients may indeed have increased risk of thrombus formation, however this may not be due to specific LA thrombogenesis, but RA and LA increased prothrombotic environment.

Table 1

Baseline Characteristics for MS Patient Cohort

	MS N = 59	SVT N= 24	P Value
Age (years)	32.6 ± 8.6	36 ± 9	0.08
Sex (M: F)	28 : 31	16 : 8	0.14
NYHA (average)	2.1 ± .4	1.9 ± .1	0.02 *
<u>Comorbidities</u>			
Heart Failure	0	0	-
Coronary artery disease	0	0	-
Diabetes mellitus	0	0	-
Smoking	0	0	-
Hyperlipidaemia	0	3	0.005*
<u>Medications</u>			
Beta blockers	27	11	0.9
Asprin	0	23	<0.0001*
Warfarin	0	0	-
ACEI/ARB	5	0	0.1

Data is shown as mean ± standard deviation or n (%).

Table 2

Echocardiographic parameters between control and MS patients.

Echocardiographic Parameters	Mitral Stenosis	Control (SVT)	P Value
LA Area (cm^2)	60.4 ± 11	24.1 ± 7.8	<0.0001
RA Area (cm^2)	46.1 ± 7	20.6 ± 4.5	<0.0001
LVEF (%)	61.2 ± 7.5	62.0± 9.6	0.6

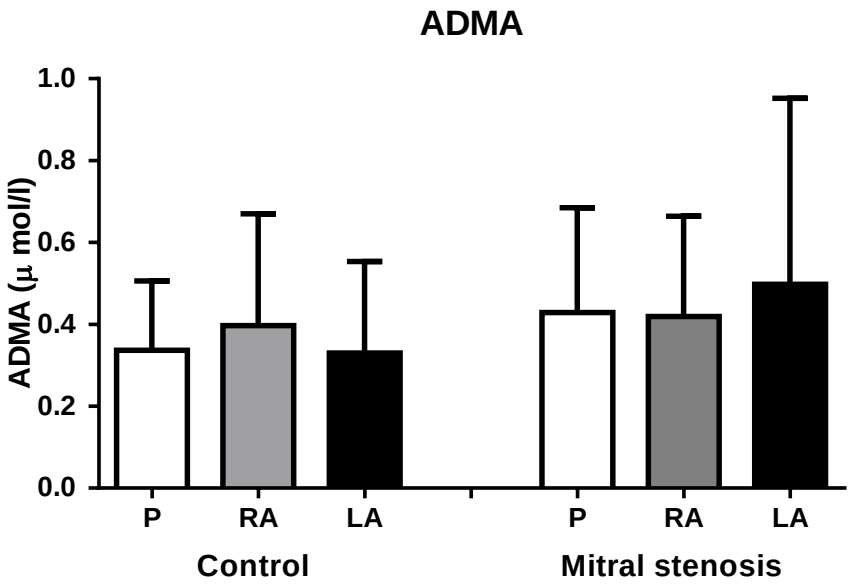
Figures

All figures are set out at peripheral, RA and LA, in white, grey and black respectively along the *x* axis, with the measurement of the marker on the *y* axis.* represents significance.

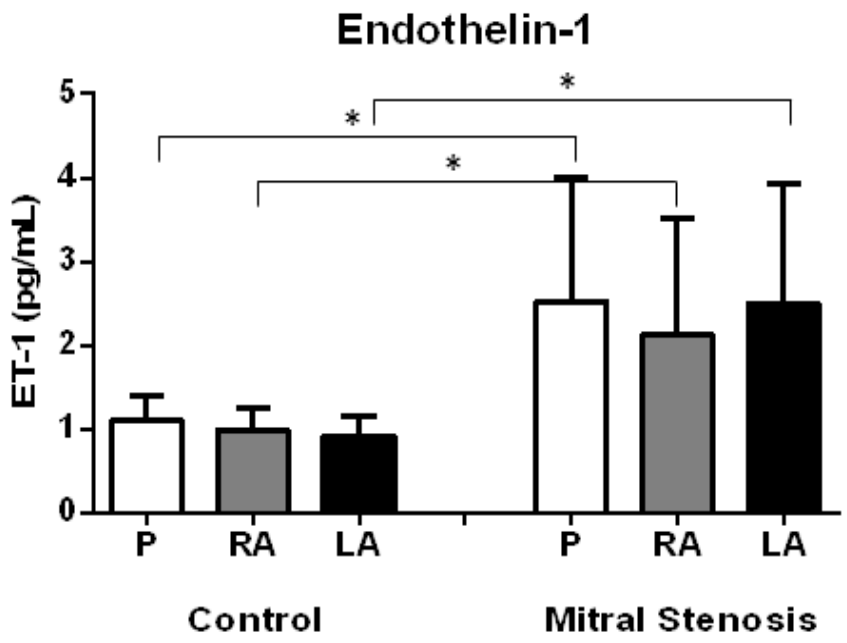
Endothelial Function

Figure 1, (A) ADMA concentrations were not significantly altered between the sites in MS patients, p=0.8, or (B) Endothelin levels p = 0.50. However there was a significant difference between the sites in both ADMA (p=0.04) and ET-1 (p<0.0001), with further analysis in ADMA this was not significant ant any of the sample sites, although was in all sites in ET-1 levels.

A.



B.

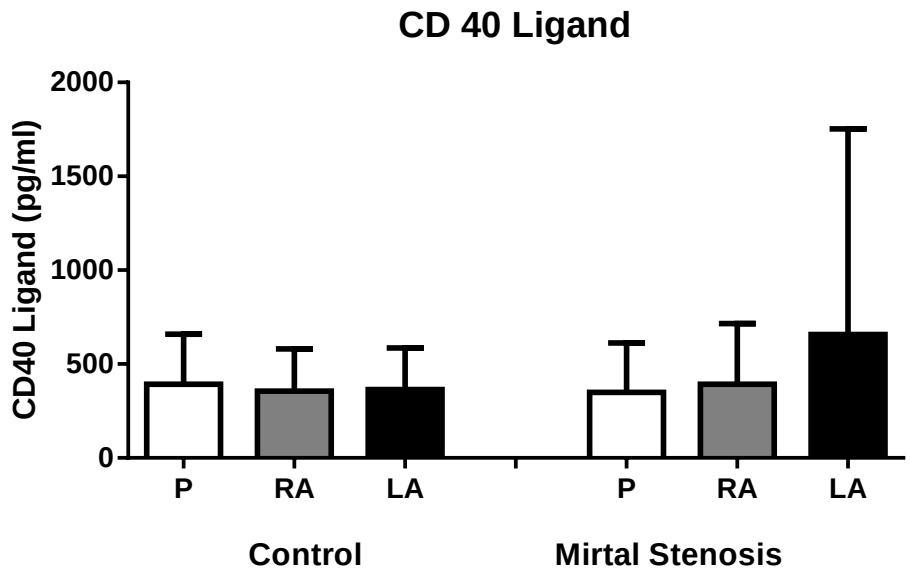


P; p<0.0001, RA; p=0.001 and LA p<0.0001

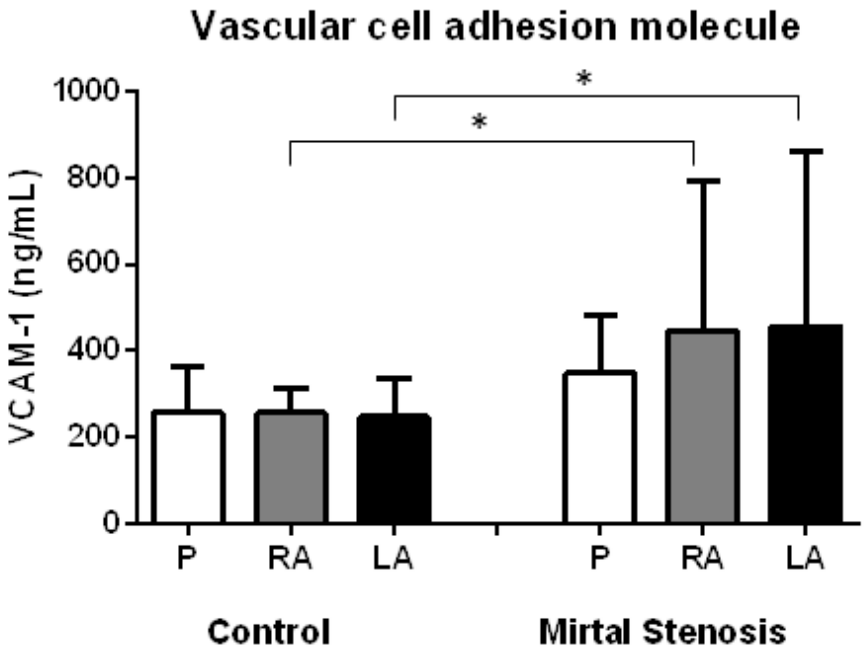
Inflammation

Figure 2: (A) Soluble CD40L levels no changed between the patients (p=0.3) or the site (p=0.2), similarly Intracellular inflammation (C: ICAM-1) was not altered between the sites at baseline in MS patients (p=0.4) or the site (p=0.4). However there was an increase in VCAM -1 (B) concentration between the sites in the MS patients (p<0.0001), however not between the sites (p=0.5), this was consistent in MPO (E) concentration between the patients p=0.0003 and site p=0.6. Interestingly there was a stepwise increase in IL-6 (D) from peripheral to LA in the MS patients (p=0.02), there was also a significant difference between the site (p<0.0001).

A.

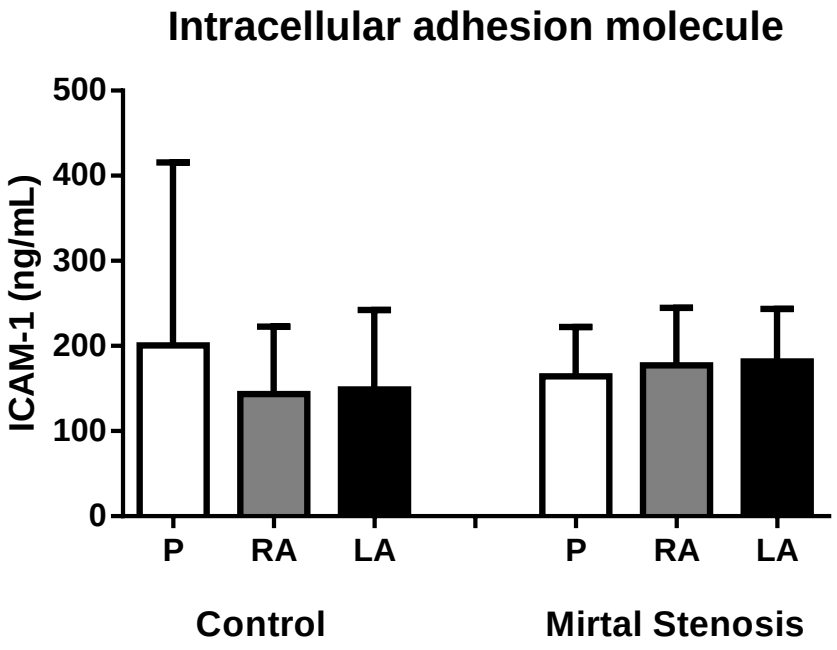


B.

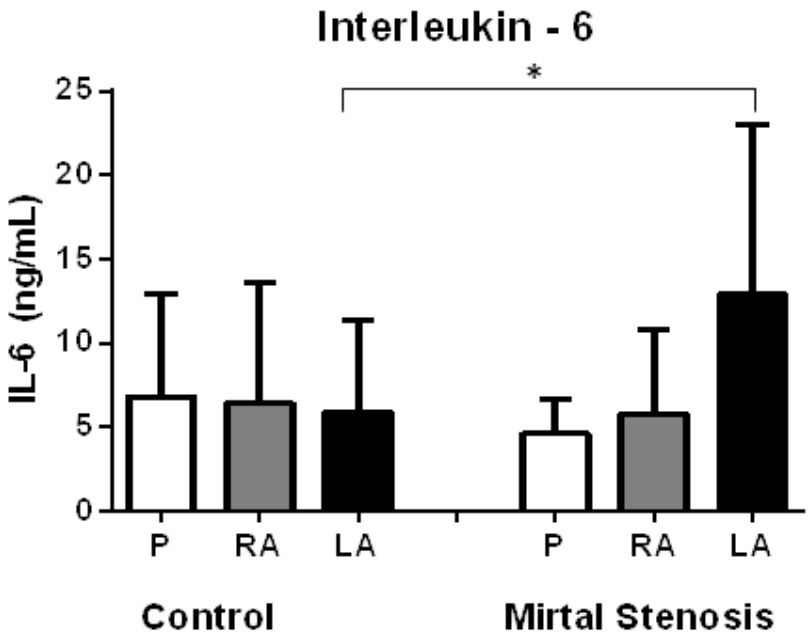


RA p=0.02, LA p=0.008

C.

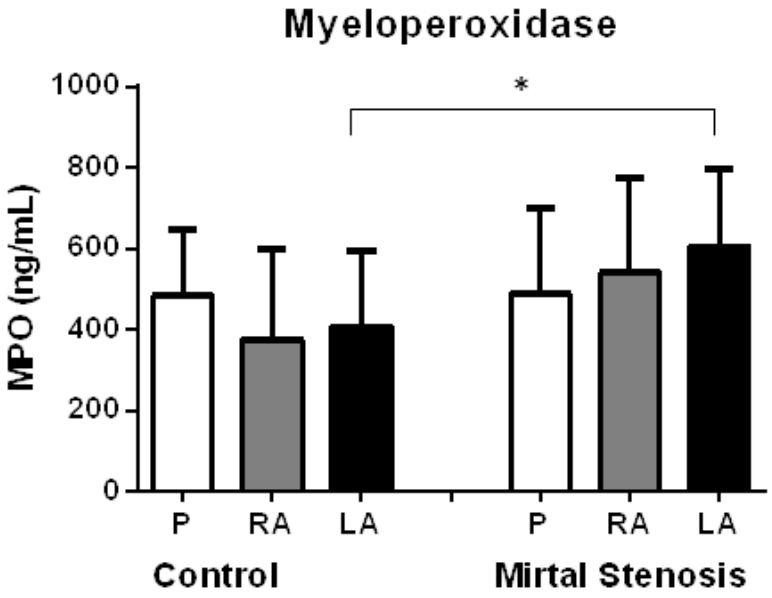


D.


Interleukin - 6
*
IL-6 (ng/mL)
25
20
15
10
5
0
P
RA
LA
P
RA
LA
Control
Mirtal Stenosis

LA p<0.0001

E.

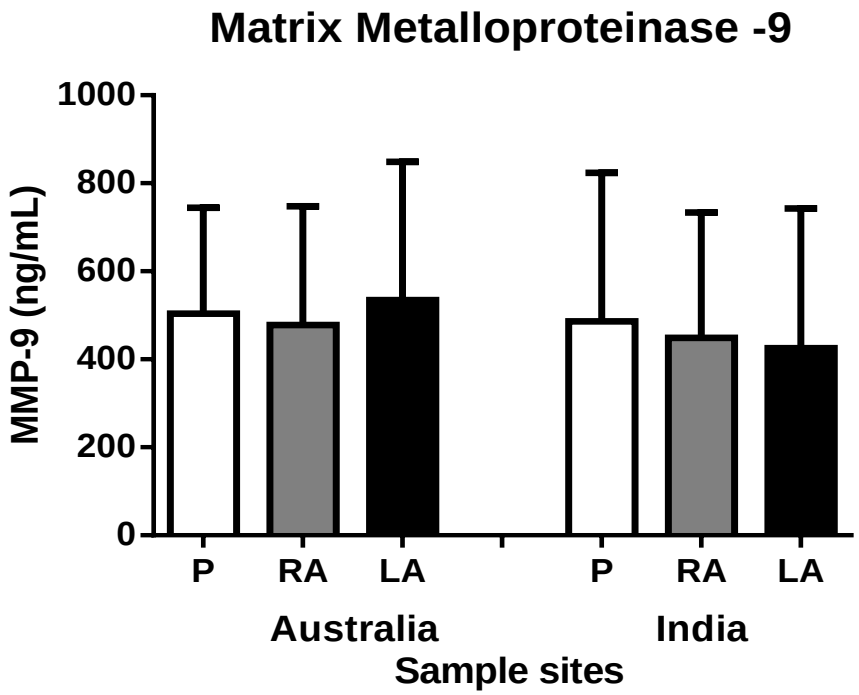

Myeloperoxidase
MPO (ng/mL)
1000
800
600
400
200
0
*
P
RA
LA
P
RA
LA
Control
Mirtal Stenosis

LA p=0.01

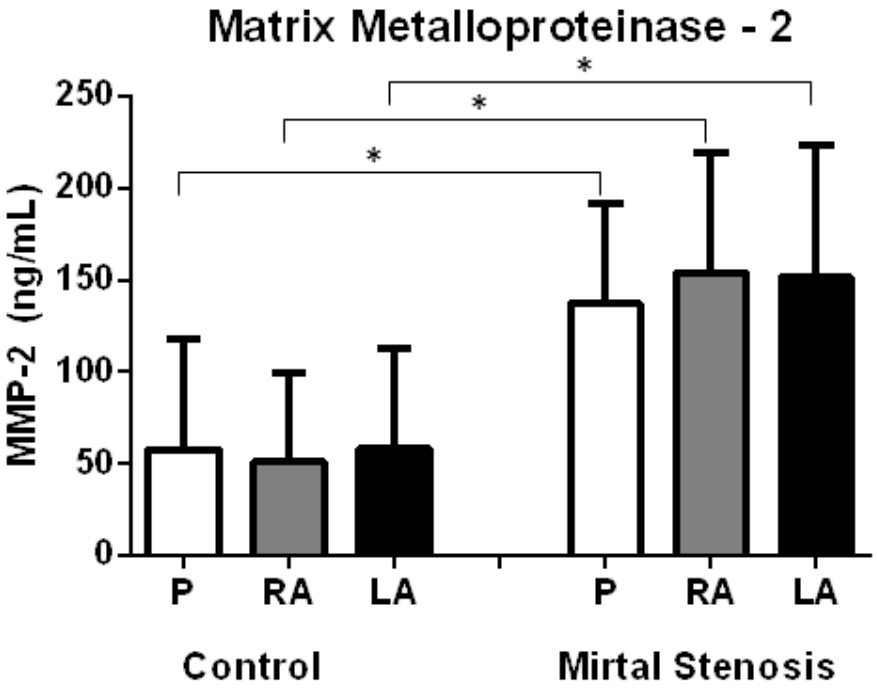
Tissue Remodelling

Figure 3: (A) MMP-9 levels did not alter between peripheral and atrial sites in between the MS and control patients, (p=0.9) and the no difference in the patients groups (p=0.5). Along with this TIMP-1 (C) concentrations were not significantly decreased at in the MS or control patients (p=0.1) or between the sites p=0.7. However there was also difference at all sites in MMP-2 (B) concentration between MS patients and SVT. (p<0.001) this change was not seen within each patients group p=0.8.

A.

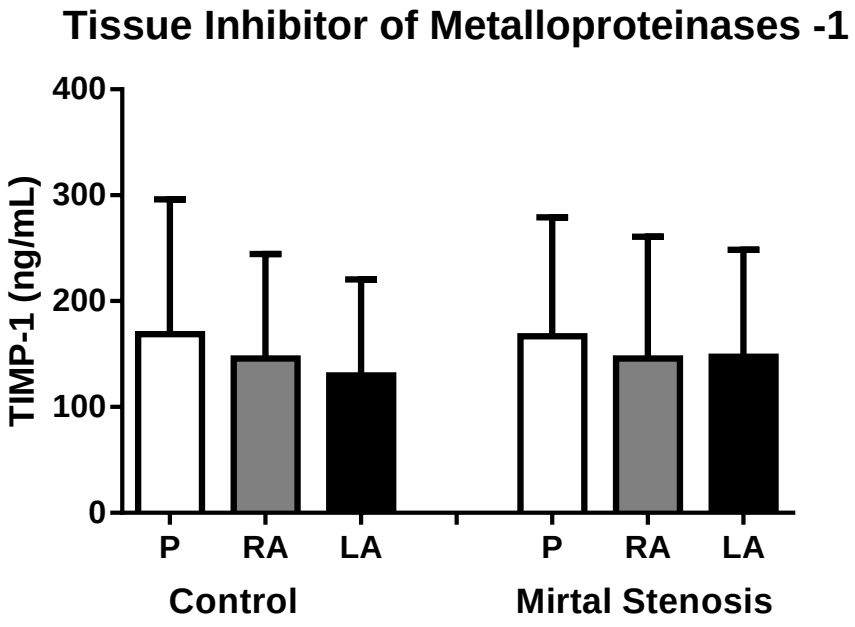


B.



P p<0.0001, RA p<0.0001, LA p<0.0001

C.



6 CHAPTER SIX

Reversal of Chronic Atrial Stretch Alters Thrombogenic Profile of Patients with Mitral Stenosis

6.1 Overview

Introduction: Chronic atrial stretch is an established substrate for atrial fibrillation (AF) and increased thrombogenic risk. In mitral stenosis (MS), recent studies have shown that the long term outcome in patients following a successful balloon mitral valvuloplasty (BMV) is associated with a reduced risk of developing AF.[235,289,290] This is coupled with a dramatic reduction in LA size and volume, showing improved atrial function and lessening of LASEC, seen as a further marker of LA thrombus formation.[37,291] Despite the knowledge of structural alteration in MS patients following BMV, there are, no studies assessing the direct impact of reversing the stretch stimulus on thrombogenesis.

This study investigates the immediate and short term (24 hour) effect of the reversal of chronic atrial stretch through BMV on the thrombogenic profile of patients with MS.

Methods: 59 Patients with mitral stenosis (MS) undergoing a balloon valvuloplasty at the Christian Medical Centre in Vellore, India, were enrolled in the study. Blood samples (10mls) were collected from the peripheral (P, femoral vein), right atrial (RA) and left atrium (LA), before and after successful balloon valvuloplasty In addition, a follow up blood

sample (peripheral only) was taken at 24 hours post procedure. All patients had an echocardiograph prior to and 24 hours following the procedure.

Results: This study found a significant rise in inflammation (MPO, $p<0.0001$) at all sites immediately following BMV, with this decreasing at 24 hours, returning to around baseline levels, however IL-6 concentration did not alter at end of procedure, but had a significant increase at 24 hours following the procedure, ($p<0.001$). There was no changes found in other markers of inflammation (CD40L $p=0.9$, VCAM-1$p=0.9$, ICAM $P=0.5$) at end of procedure. Further to this endothelial function did showed a significant decrease at 24 hours (ET-1 $p<0.001$), despite no change in ADMA ($p=0.05$). There was also a significant decrease in LA size, LA volume and LA pressure, together with a decrease in LV ejection fraction ($p<0.0001$, for all). However, there were no changes in the biomarkers of tissue remodelling from the baseline to the end of procedure at any of the sample sites, and at 24 hours following BMV.

Conclusion: Following a successful BMV procedure in MS patients is associated with an acute increase in inflammation. While there is a gradual return to baseline, endothelial dysfunction is impaired after 24 hours. Patients showed a significant decrease in LA size, volume and pressure. However, no changes in markers of tissue remodelling, at the end of procedure at any of the sample sites, and at 24 hours following

BMV were found. These findings suggest that while acutely following the procedure there may be a pro-thrombotic state this seems to rapidly resolve.

6.2 Background

Mitral stenosis (MS) is known to be a leading cause of thrombus formation potentially leading to stroke. MS itself along with the progression of disease to MA with AF have been shown to further increase the stroke risk and be pro-arrhythmic, with 40-75% of patients with MS developing valvular AF.[34,292] The progression to valvular AF is also known to additionally increase a patient's risk of stroke.[9] Chronic atrial stretch is one of the main factors in the progression from MS to AF. [36] Stretch of the LA in MS has been associated with LA enlargement and a loss of myocardium, with scarring associated with widespread and site-specific conduction abnormalities, without a change or an increase in effective refractory period; all factors known to be linked to the development of AF. The development of AF in a patient with MS is associated with a significant increased thrombotic risk, as reported in the Framingham study, where people with valvular AF had a 17. 5 fold increase risk of stroke.[9]

It has been demonstrated in patients with MS that a reduction in the size of the LA following balloon valvuloplasty is associated with reduced chance of developing AF.[293]ACC/AHA guidelines suggest that balloon mitral valvuloplasty (BMV) in a therapeutic option for patients with asymptomatic MS prior to development of or recent occurs of AF.[293] After successfully BMV procedure, it has been shown that there is improvement in LA function, as evidenced by the reduced size of LA volume,[37] improved left appendage flow velocity,[294] and decreased

intensity of spontaneous echocardiographic contrast [291] potentially reducing the chance of thrombus formation.

It is known that there is an improvement in the LA function following BMV these studies have only analysed the alteration in the structure of the LA following and not the altered risk of thrombogenesis. Following the normalisation of LA function post BMV it has been assumed that there would be a decreased risk of thrombogenesis and stroke. One study demonstrated that successful BMV was strongly associated with a decreased thromboembolic risk in patients with MS and AF, with a mean follow up of 36 months,[289] and a further study showed that MS patients are at a reduced embolic risk one month following BMV.[290] No study has previously focused on immediate and short term alterations or the alterations in atrial thrombogenesis following BMV.As valvular AF has a different pathophysiology to non-valvular AF, the mechanisms which lead to thrombus in each disease could be different.

Therefore we investigated the immediate and short term (24 hour) effect of the reversal of chronic atrial stretch following a BMV procedure on the thrombogenic profile of patients with MS.

6.3 Methods

6.3.1 Patient Characteristics

Fifty-nine patients with known severe rheumatic mitral stenosis (MS).[mitral valve area< 1.2cm^2] and were undergoing clinically

indicated balloon valvuloplasty (BMV) at the Christian Medical Centre in Vellore, India, were enrolled in the study. Patients had an average disease history of 10 years. Patients were selected on the basis of having severe MS with a mitral valve area of <1.5 cm2 with significant symptoms (NYHA class ≥2) and mitral valve morphology suitable for PBMC as determined by the Wilkins criteria (score <10).

Patients were excluded if they had of the following:

- Age<18
- Patients with MS due to a non-rheumatic etiology.
- Patients with congenital heart disease, atrial fibrillation, LV systolic dysfunction, aortic stenosis or aortic regurgitation.
- Patients with a previous history of myocardial infarction.
- Patients who developed a complication during BMV including grade II mitral regurgitation or more.
- Individuals who are taking oral phosphodiesterase 5 inhibitors [i.e. sildenafil, vardenafil or tadalafil]
- Patients with peripheral vascular disease, hypertension, diabetes or vasculitis.
- Patients who are not followed up in Christian Medical College after their procedure.
- Individuals who smoke.

All patients had no previous history of any arrhythmias documented by 12 lead ECG recordings 3 months prior to, at the time of, and during BMV procedureAll patients underwent transthoracic; M-mode, 2-

dimentional (2D) and Doppler echocardiographic studies will be performed 1-2 weeks before the procedure, as per the American Society of Echocardiography criteria.[283] In MS patients the mean trans-mitral valve gradient, the mitral valve area (MVA) will be calculated from the echocardiographic Doppler study using the pressure half time method, and using the short axis 2D echocardiographic view.

All patients provided written informed consent for the study protocol that was approved by the Clinical Research Ethics Committees of the Christian Medical College, Vellore, India.

6.3.2 Balloon Mitral Valvuloplasty

A Balloon Mitral Valvuloplasty (BMV) will be performed on all patients in a fasted and sedated state. The BMV was performed by the transeptal approach with the use of a Joseph mitral valvuloplasty balloon catheter. Details of the procedure have been described previously. The BMV procedure will be performed under local anaesthesia. Right heart catheterization precedes BMV, and was repeated, along with oximetry which was run after BMV. Heparin is administered intravenously after completion of the transeptal puncture. A modified back up wire is placed in the left ventricle through a Swan Ganz catheter. A Joseph's catheter balloon is then sent over the wire and inflated across the valve orifice. When additional balloon dilatation is required, the balloon is exchanged for a larger one and the same

procedure will be repeated. Invasive pressure measurements were performed immediately before and after valvuloplasty.[284]

6.3.2.1 **Protocol**

At the beginning of the procedure blood samples were taken from the sheath within the femoral vein (peripheral) and through the sheaths placed within the right (RA) and left atria (LA) for the valvuloplasty procedure. These samples were taken simultaneously, immediately following the transeptal puncture; no heparin was administered until all of the blood samples were collected. No alteration to the mitral valve had been performed prior to the study protocol sampling. A follow up blood sample was then taken from the three sites via the same sheaths following a successful BMV procedure. Successful BMV was usually defined as a post procedure mitral valve area of >1.5 cm2 with no more than moderate mitral regurgitation. A further follow up blood sample (peripheral only) was taken at 24 hours post procedure, at this time a further echocardiograph and ECG were taken to assess for changes in heart structure, function and LA pressure. Plasma was collected and frozen for batch analysis via ELISA.

6.3.2.2 **Blood Sampling: End of Procedure and Follow-up**

Blood samples will be obtained from the femoral vein (P), right atria (RA) and left atria (LA) after the finalisation of the BMV procedure; further to this another RA and LA blood will be obtained via sheaths inserted for the clinical procedure (Balloon Mitral Valvuloplasty).

Irrespective of sampling site the first 5mls of blood was discarded to ensure appropriate catheter flushing. Each patient was reviewed at 24 hours after their procedure. Blood samples were obtained from all patients in a fasting, non-sedated state at approximately the same time. Peripheral venous blood was drawn under minimal tourniquet pressure from an antecubital vein using a 21-gauge butterfly needle with the study patient in the supine position for at least 20min. 34ml of blood was collected after the first 2ml is discarded. The first 27mls was be transferred into tubes containing 3.8% sodium citrate (1:9, volume) with the remaining blood will be collected into lithium heparin tubes (Vacuette) for analysis. Plasma was collected and frozen for batch analysis via ELISA.

Analysis of Endothelial function/ Inflammation/ Tissue Remodelling by Enzyme-linked absorbance assay (ELISA)

The obtained blood samples were centrifuged at 2500g for 15 min at 4^{o}C and stored at -80^{o}C for batch analysis utilising enzyme-linked Immunosorbent assay (ELISA).Endothelial function through asymmetric dimethylarginine and Endothelin-1 (ADMA and ET-1), inflammation through soluble CD40 Ligand (CD40L), Vascular and Intracellular adhesion molecules (VCAM-1 and ICAM-1), Interleukin-6 (IL-6) and Myeloperoxidase (MPO).Tissue remodelling was measured via matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1(MMP-9 and TIMP-1), via commercially available ELISA [for further details see methods section 2.6].

6.3.2.1 **Statistical Analysis**

Data is shown as mean ± standard deviation, unless otherwise stated. Comparisons between the sample sites over time was performed by a two-way repeat measures ANOVA, with post hoc analysis via Bonferroni's multiple comparisons test where necessary. Patient characteristics were compared using students T-test for continuous data or a fisher's exact test for categorical data. All data was tested for normality by a D'Agostino-Pearsons normality test, and log-transformed as appropriate. All data was analysed using GraphPad Prism Version 5.0 (GraphPad Software) and was normally distributed. Statistical significance was set at $p<0.05$.

6.4 Results

6.4.1 Patient Characteristics

The patients included in this study consisted of 28 females and 31 males, with an average age of 32 years. Almost all (90%) patients had documented pulmonary hypertension with an average NYHA score of 2.1.

6.4.2 Echocardiographic Characteristics

The mitral valve area significantly increased in all patients having a valvuloplasty ($p<0.0001$). Associated with this, there was significant decrease in LA size ($p=0.02$), increase in LV ejection fraction ($p=0.03$)

and decrease in LA pressures (p<0.0001) with a successful BMV. There was no change in RA size and volume. [Table 2]

6.4.3 Endothelial Function

There was no change in ADMA or ET-1 concentration at any of the sample sites from the beginning (Base) of the procedure to the end of procedure (End) [Site: p=0.93, Time: p=0.75, Fig 1A, Site: p=0.30, Time: p=0.74, Fig: 1B]. Furthermore, there was no change in the ADMA concentration within the peripheral circulation, between baseline, end of procedure and at 24 hours following successful BMV, Fig2A: p=0.5, However there was a significant decrease in ET-1 levels at 24 hours following BMV procedure. Fig2B: p=<0.0001, with no change from baseline to end of procedure.

6.4.4 Inflammation

Levels of Inflammation were not significantly altered between the baseline (BL) and the end of procedure (End) at any of the sample sites in levels of VCAM-1, ICAM-1 and IL-6 (Site: p=0.27, Time: p=0.93, Site: p=0.61, Time: p=0.29, Site: p=0.95, Time: p=0.07, respectively). However the CD40L was found to be significantly increased within the LA Site: p=0.12, Time: p=0.03, LA: p=0.006) and MPO concentrations were found to be significantly increased at the End of procedure at all of the sample sites (Site: p=0.23, Time: p<0.0001, Fig 3E).

When observing the peripheral inflammatory response24 hour following a successful BMV procedure there again was no change in the levels of CD40L, VCAM-1 or ICAM-1 (CD40 L, p=0.9, VCAM-1, p=0.9 and ICAM-1, p=0.5). There was a significant rise in IL-6 concentration 24 hours following the procedure compared with the peripheral levels at Base and End, confirming a significant rise in inflammation following the procedure ($p<0.001$, Fig 4D). Interestingly compared with, baseline values there was a rise in MPO immediately following BMV ($p<0.0001$), this decreased again at 24 hours, returning to around baseline levels (p=0.008, Fig 4E)

6.4.5 Structural Remodelling

When comparing the levels of atrial remodelling between the three samples sites at Baseline and at the end of procedure we found that in MMP-9 had a significant difference at the three sites from baseline to the end of procedure (Fig5A, site p=0.98, time p=0.0003), and with further analysis this was only at the peripheral site (P: p=0.003, RA: p=0.21, LA: p=0.53).Despite this there was no alteration in the levels of, MMP-2 (Fig 5B, Site: p=0.91, Time: p=0.07) orTIMP-1 (Fig 5CSite: p=0.17, Time: p=0.79)

The levels of MMP-9 showed a progressive rise in levels from BL, to end of procedure and further more at 24 hours following BMV, however this did not reach significance (p=0.1) [Fig 6A], conversely levels of

MMP-2 appeared to drop at 24 hours (p=0.1) [Fig 6B], with no change TIMP-1 at any of these time points (p=0.8) [Fig 6C].

6.4.6 Correlation with LA Pressure

There was no correlation between the decrease in LA pressure and the significant change in MPO at the end of procedure (r=0.21, p=0.16), nor the decrease in ET-1 nor IL-6 24 hours following the BMV procedure (r=-0.12 p=0.15, r=-0.006 p=0.96 respectively).

6.5 Discussion

6.5.1 Major Findings

This study found that following BMV and reduction of the chronic stretch stimulus, MS patients have an immediate reduction in markers of endothelial dysfunction and rise in inflammation which alters at 24 hours. Potentially altering blood haemostasis and the mechanism for thrombus formation of MS patients following a BMV.

It found the following from the baseline, end of procedure and at 24 hours following a successful BMV procedure MS patients had:

I. A significant decrease in LA size, volume and pressure in MS patient 24 hours following BMV.

II. A rise in Inflammation which continues to increase at the 24 hours following the procedure.

III. An immediate increase endothelial dysfunction at the end of procedure which resolved to baseline levels at 24 hours.

IV. No changes in markers of tissue remodelling, at the end of procedure or 24 hours following BMV.

Reversal of the chronic stretch process in MS through a MBV procedure has an effect on both the structure of the heart and the chance of a patients developing further atrial arrhythmia as one of the most common substrates of valvular AF.[31,295] Further to this it is known that with the development of AF, a MS patient a significantly higher chance of having atrial emboli leading to stroke. However, unlike AF the specific alterations in MS patients which produce this increased thrombotic risk are still unknown, and how the reversal of the stenotic valve can alter the risk of further AF development and thrombotic risk.[296] MS itself has been shown to have increases in peripheral and LA specific platelet reactivity, as a basis for the increase in thrombosis.[35,40] However, there has been no in-depth research into other markers of thrombosis or coagulation in MS itself. This is also seen in the assessment of the reversal of MS through BMV, with long term studies finding reduction of thrombotic risk, through a reduction in the mean platelet volume and decreased thrombin anti-thrombin complex.[34,290] However this study also found that the level of platelet

factor 4 and beta-thromboglobulin were increased after the procedure but did not achieve statistical significance.[290]

This study is one of the first to evaluate the markers of endothelial function through a blood bio-marker in MS patients post BMV. We found that immediately following BMV there was no change in endothelial function (ADMA and ET-1). This was consistent for ADMA levels at 24 hours following BMV, however ET-1 dropped significantly from the end of procedure to 24 hours following ($p<0.0001$). In patients with AF it where there was a change in ADMA levels within 15 minutes following the induction of AF;[196] it would have been anticipated that a change would have been seen post procedure, if not by 24 hour following the procedure. As there was such a significant decrease in size of the atria it would be expected that there would be some alterations to the function of the endothelium. Consistent with our results for ET-1 levels, a previous study which examined von Willebrand factor 24 hour post BMV found that levels were significantly decreased at 24 hours, suggesting that there is an improvement of endothelial compliance post BMV.[296]

However, inflammation had differing results dependent on our markers; platelet derived Inflammation, vascular inflammation and Intracellular inflammation were shown not to change at any site from baseline (BL), End and 24 hours (Fig 4 A,B and C). However, a rise in MPO was seen at all sites following BMV ($p<0.001$), and this decreased again

peripherally at 24 hours, returning to around baseline levels. This study is the first to shows a stepwise rise in IL-6 concentrations from the Base and End to the levels at 24 hours following, indicating a significant rise in inflammation following the procedure, which continued through to 24 hours. This is consistent with previous AF studies finding that the induction of AF increased inflammation using sCD40L ($p<0.001$) where levels increased significantly at all sites. Oxidative stress and inflammation factors were shown to be involved in atrial remodelling in MS.[278] This may account for the different results for various markers at differing stages of the procedure. Additionally, the nature of the procedure and how each of these markers is involved in the inflammatory process may account for the variations found in each of these markers acutely following BMV. As MPO is most abundant within neutrophils as a part of the innate immune system, and is the first line of defence in inflammation, this may explain why there is a sharp increase in MPO at the end of procedure and this reduces progressively by 24 hours. IL-6 can be involved in both the acute phase of inflammation as well as the chronic stages of inflammation. However, there is no short term study this may be why IL-6 continues to increase following BMV though MPO does not. In AF, inflammatory biomarkers (C-reactive protein and interleukin-6) have been shown to be associated with the future development, recurrence and burden of AF, and the likelihood of successful cardioversion.[297] A long term study in patients with MS and chronic heart failure found that levels of IL-6 significantly decreased 6 months after BMV, but no significant

difference was found between the levels in the patient group after BMV and that of normal subjects.[42]

Previously echocardiographic studies have shown that post BMV, there is a reduction in LA size and volume, alluding to an improvement in LA function, decreasing the risk of AF generation.[36] More recently, it has been shown that the atrial electrophysiological and electroanatomic abnormalities that result from chronic stretch due to MS reverses after BMV. These observations suggest that the substrate predisposing to atrial arrhythmias might be reversed.[36] Our study has shown similar echocardiographic results, with significant reductions in LA size ($p=0.02$), volume ($p=0.006$) and pressure ($p<0.0001$) 24 hours following BMV [Table 2] This was also associated with an increase in LV ejection fraction ($p=0.03$). However despite these significant reductions in the LA structure, there was no change in tissue markers of structural remodelling [Fig 6 A, B and C]. These results may be explained by the short follow-up time points of end of procedure and 24 hours following the procedure, as there may have been insufficient time post procedure to see changes in remodelling markers despite the initial drop in LA size and volume do to the recovery of normal mitral valve function. This is despite the significant decrease in LA pressure seen at the end of the BMV, along with significant changes in MPO at the end of procedure and ET-1 and IL-6 at 24 hours post BMV. There was no correlation found between the alteration in any of these makers and the reduction in LA pressure.

6.6 Conclusions

Mitral stenosis (MS) is known to be a leading substrate in the development of AF. The significant likelihood of atrial thrombus development, which is associated with stroke, is known to be perpetuated with AF development, however if the reversal of MS can alter this thrombogenic potential is still unknown. This study found that acutely following a BVM procedure there is significant alteration in thrombogenic mechanisms which may potentially alter the risk of thrombus formation and AF development in MS patients.

Table 1

Patient characteristics

Characteristics	Mitral Stenosis Patients (n=59)
Age (years)	32.6 ± 8.6
Gender (F:M)	28 : 31
Comorbidities	
NYHA (average)	2.1 ± .4
Pulmonary Hypertension (%)	50 (85%)
Diabetes Mellitus	0
Smoking	0
Stroke	0
Medications	
B-Blockers (%)	26 (44%)
Asprin	0
Warfarin	0

Table 2

Mitral Stenosis Patients Characteristics Prior to and Following a Balloon Valvuloplasty

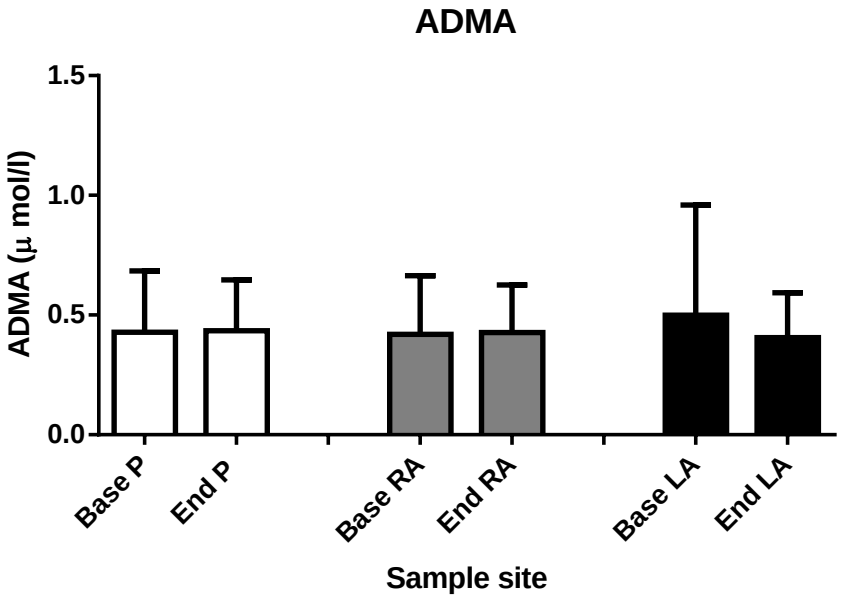
Echocardiography Characteristics	**Baseline**	**24 hours Post BMV**	**P Value**
Mitral Valve Area (cm^2)	0.82 ± 0.2	1.71 ± 0.02	<0.0001
LA Area (cm^2)	60.4 ± 11	55.8 ± 10	0.02 *
RA Area (cm^2)	46.1 ± 7	45.6 ± 8	0.7
LVEF (%)	61.2 ± 7.5	64.1 ± 6.9	0.03*
LVESD	28.4 ± 5.4	28.8 ± 4.5	0.6
LVEDD	42.0± 6.1	43.8± 4.9	0.1
LA Pressure	22.8 ± 6.9	9.3 ± 4.3	< 0.0001 *

*significant difference in between pre and post BMV

Endothelial Function

Figure1: There was no change in ADMA between the BL and End levels at any of the samples sites (site comparison p=0.93, time comparison p=0.75), this was consistent for the ET-1 levels between sites at BL and End (site comparison p=0.31, time comparison p=0.74)

A.



B.

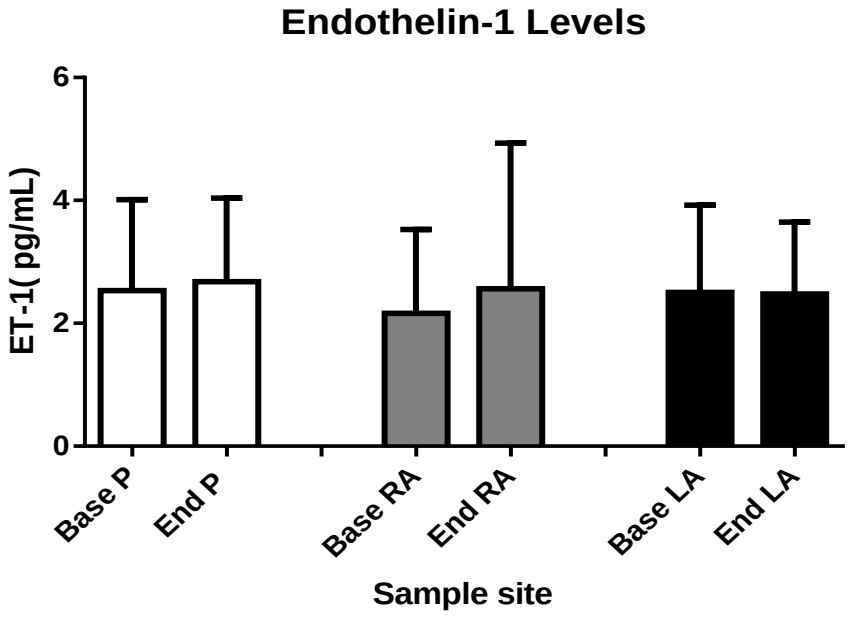
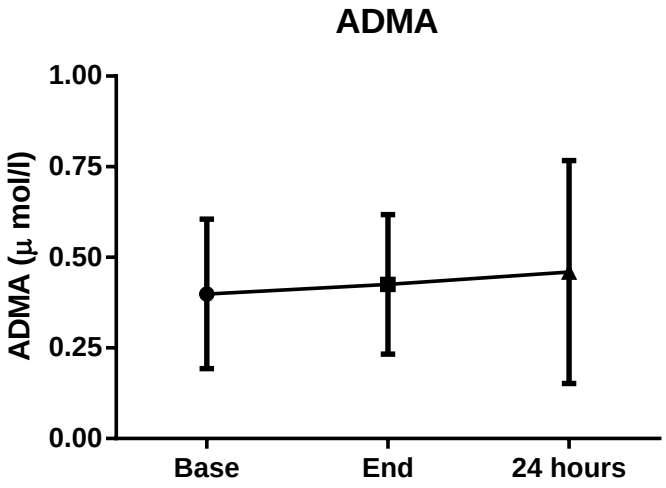


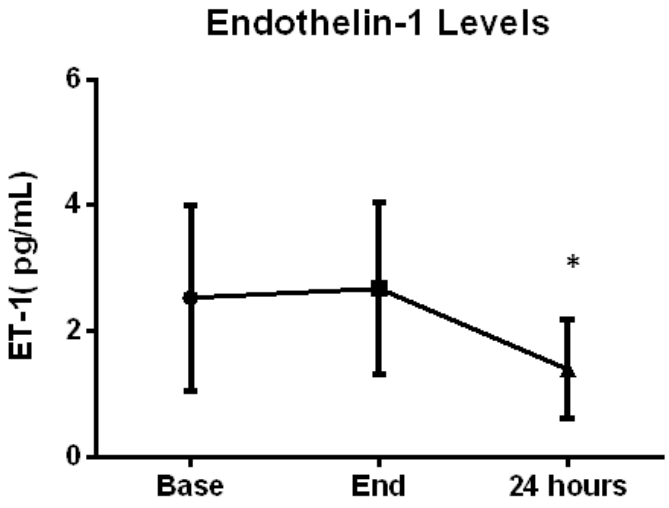
Figure 2: Endothelial function 24 hours following BMV

There was no alteration in ADMA levels from baseline, end of procedure and 24 hours following successful BMV, A: p=0.5, However there was a significant decrease in ET-1 levels at 24 hours following BMV procedure. B: p=<0.0001, with no change from baseline to end of procedure.

A.



B.

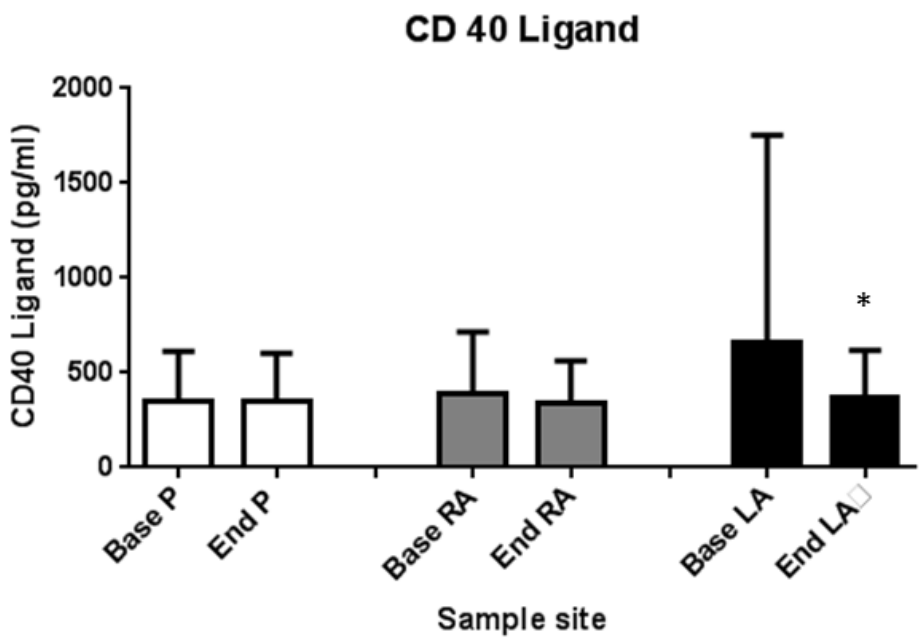


Inflammation

Figure 3: Inflammation: Baseline to End of Procedure.

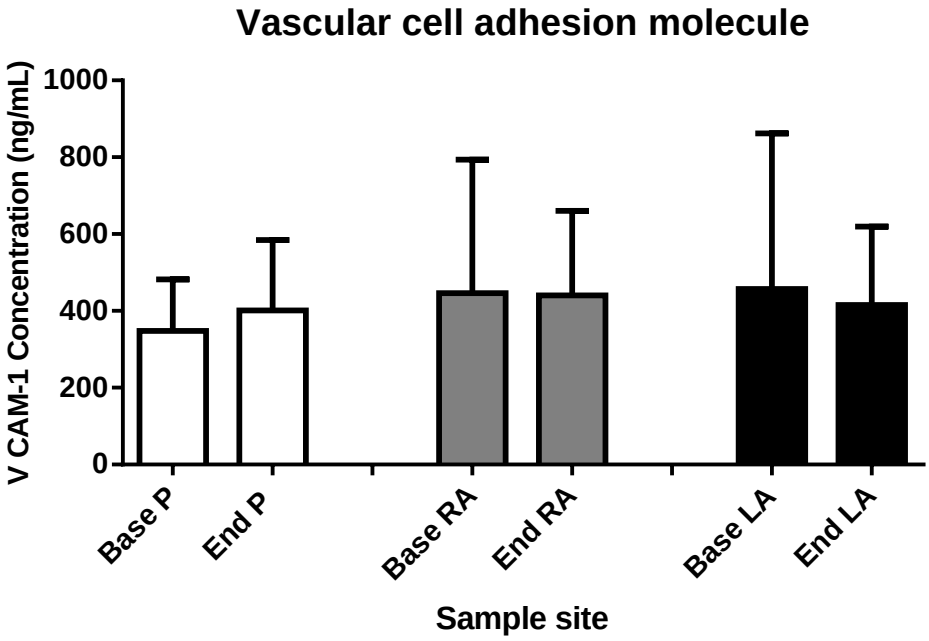
A: CD40L was not changed at the end of the procedure in MS patients between any of the samples sites, (site comparison p=0.12, time comparison p=0.03), with a significant comparison between the baseline and end of procedure LA samples (p=0.006) however not at the peripheral and RA sites (p>0.999, for both).B: There was no change in VCAM-1 concentration was seen in MS patients at any of the sample sites the End, (site comparison p=0.27, time comparison p=0.93).C: ICAM-1 concentration did not change from Base to End at any of the sample sites, (site comparison p=0.61, time comparison p=0.29). D: Again there was no change in IL-6 concentration form BL to End at any of the sample sites, (P: site comparison p=0.95, time comparison p=0.05). Unlike all other markers MPO concentration was significantly increases at the End at all three sampling sites (Interaction p=0.02, site comparison p=0.23, time comparison p<0.0001 Fig E).

A.

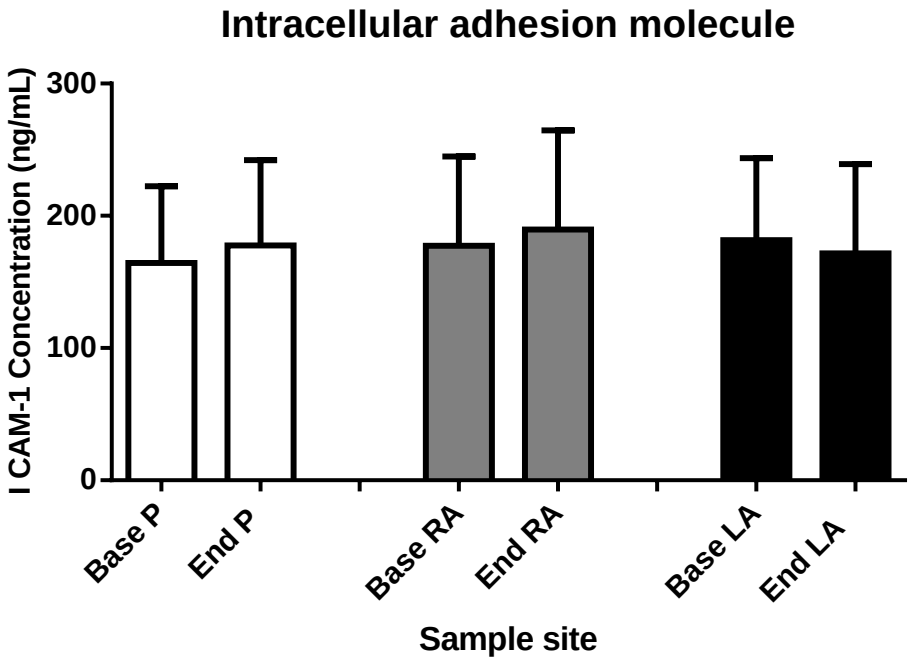

CD 40 Ligand
CD40 Ligand (pg/ml)
2000
1500
1000
500
0
*
Base P
End P
Base RA
End RA
Base LA
End LA
Sample site

LA: p=0.006

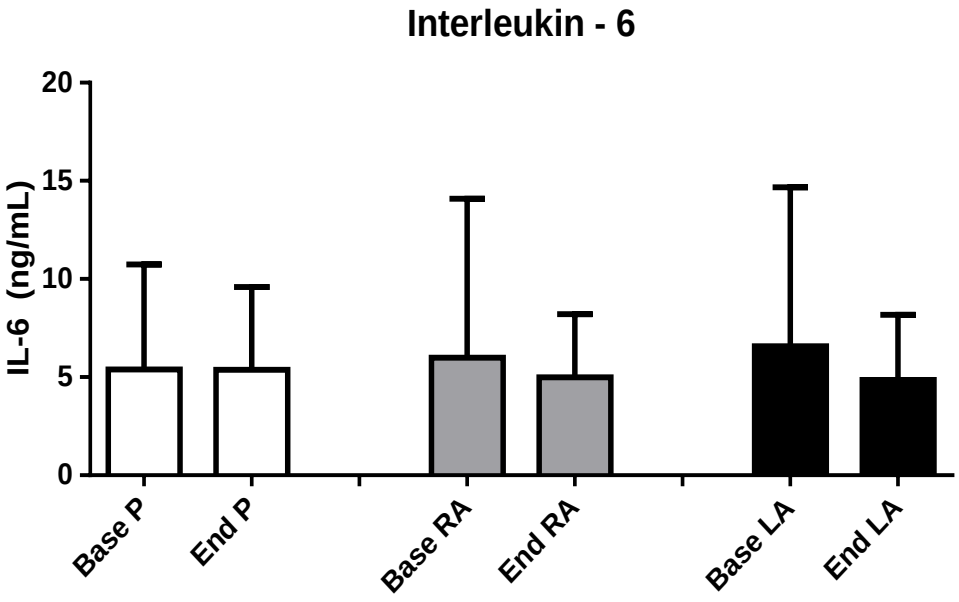
B.


Vascular cell adhesion molecule
V CAM-1 Concentration (ng/mL)
1000
800
600
400
200
0
Base P
End P
Base RA
End RA
Base LA
End LA
Sample site

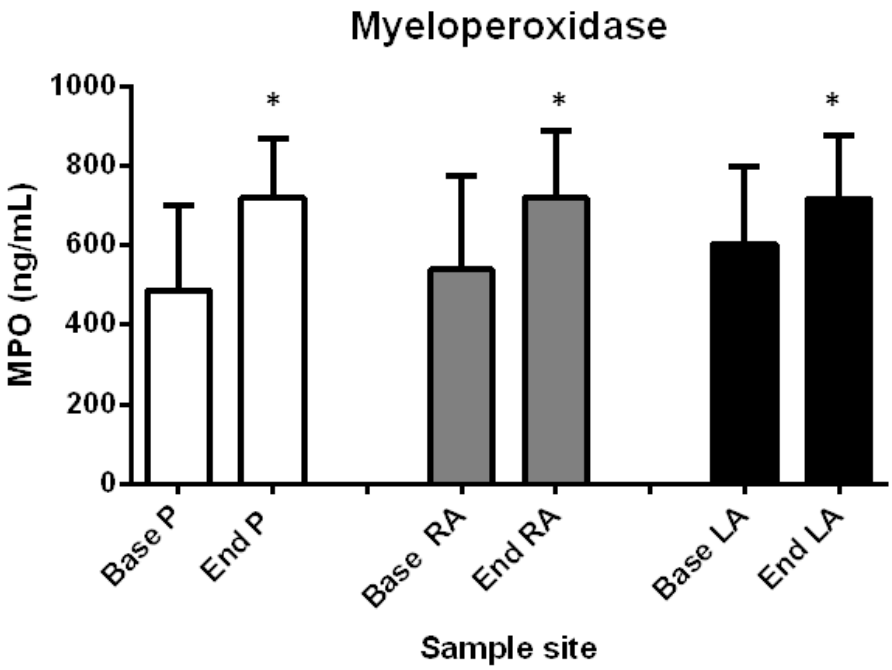
C.


Intracellular adhesion molecule
I CAM-1 Concentration (ng/mL)
300
200
100
0
Base P
End P
Base RA
End RA
Base LA
End LA
Sample site

D.


Interleukin - 6
IL-6 (ng/mL)
20
15
10
5
0
Base P
End P
Base RA
End RA
Base LA
End LA

E.

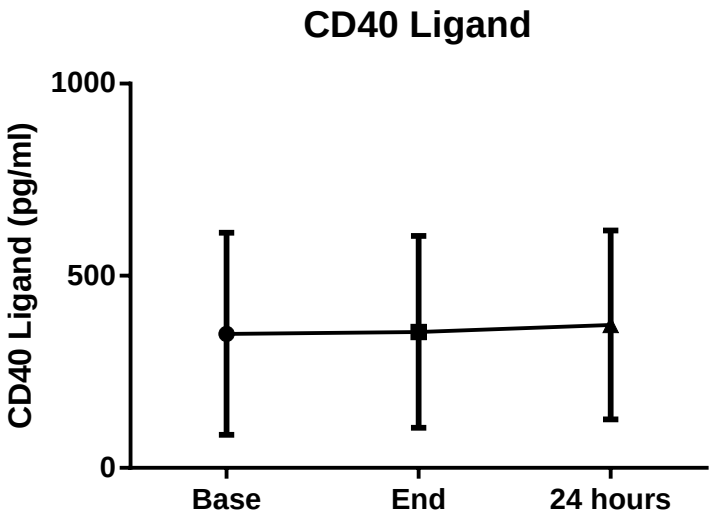


P: p<0.0001, RA: p<0.0001, LA: p=0.008

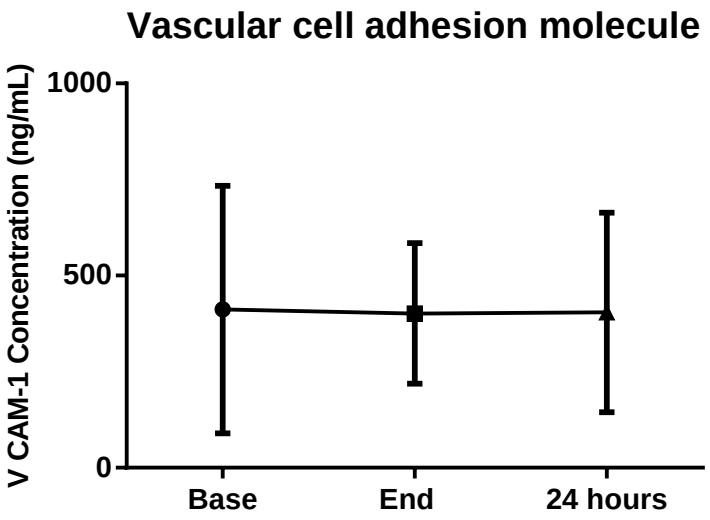
Figure 4: Inflammation 24 hours following BMV

CD40L was not changed over time in MS patients, p=0.9. B: There was no change VCAM-1 concentration was seen in MS patients (p=0.9), C: ICAM-1 concentration did not alter from baseline to End or at 24 hours following the BMV (p=0.5). D: lL-6 was significantly increased from baseline, to end of procedure and then again at 24 hours following the BMV. p< 0.0001. E: There was a significant change in the levels of MPO from baseline, where it increased at the end of procedure, and then decreased at 24 hour p<0.001.

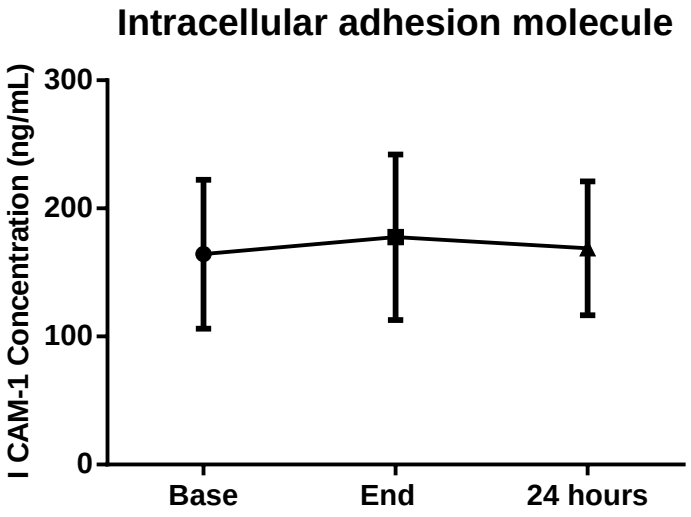
A.



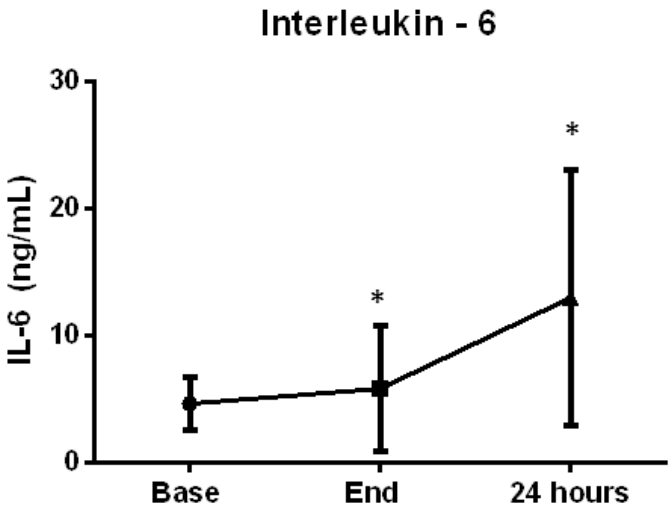
B.


Vascular cell adhesion molecule
V CAM-1 Concentration (ng/mL)
1000
500
0
Base
End
24 hours

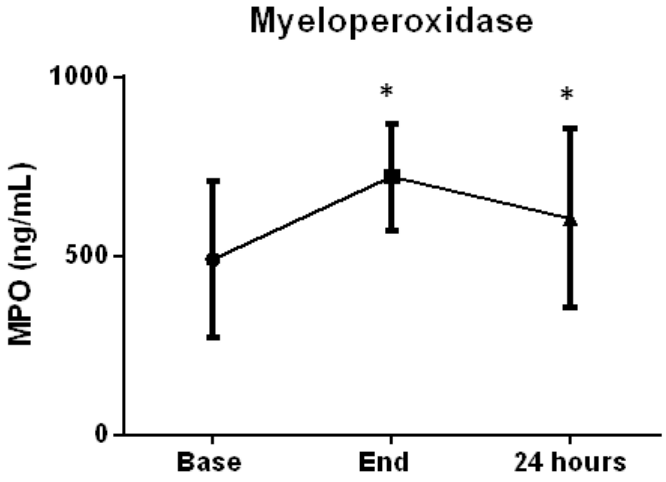
C.


Intracellular adhesion molecule
I CAM-1 Concentration (ng/mL)
300
200
100
0
Base
End
24 hours

D.


Interleukin - 6
IL-6 (ng/mL)
30
20
10
0
*
*
Base
End
24 hours

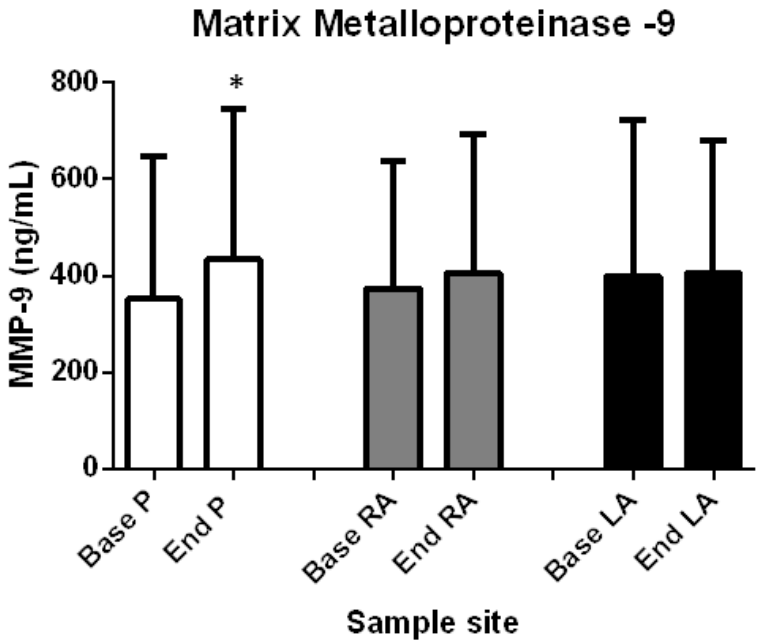
E.


Myeloperoxidase
MPO (ng/mL)
1000
500
0
*
*
Base
End
24 hours

Tissue Remodelling

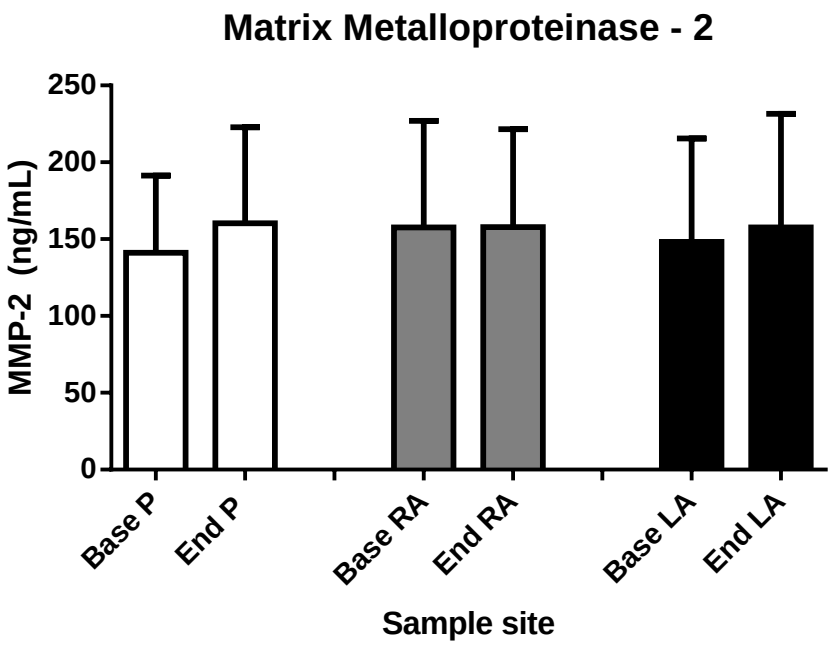
Figure 5: MMP-9 was the only in biomarkers of tissue remodelling seen to have any difference at any sample site immediately following BMV. MMP-9(A) (site comparison p=0.98, time comparison p=0.0003), and with further analysis this was only at the peripheral site. MMP-2(B) (site comparison p=0.91, time comparison p=0.07), and TIMP-1(C) (site comparison p=0.17, time comparison p=0.79).

A.



P: p=0.003

B.


Matrix Metalloproteinase - 2
MMP-2 (ng/mL)
250
200
150
100
50
0
Base P
End P
Base RA
End RA
Base LA
End LA
Sample site

C.

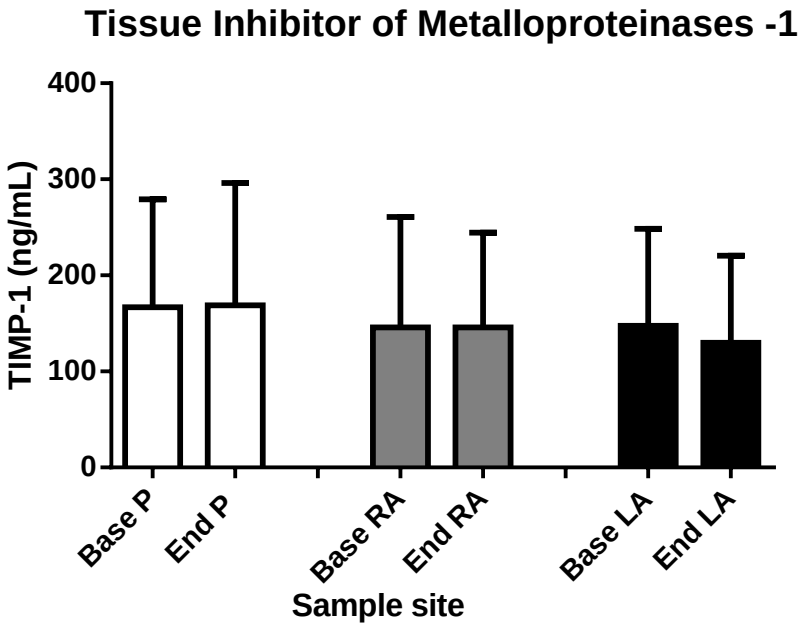
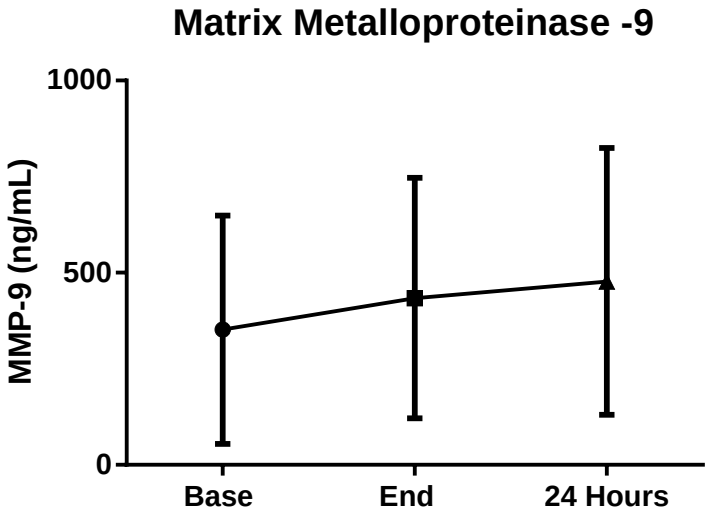
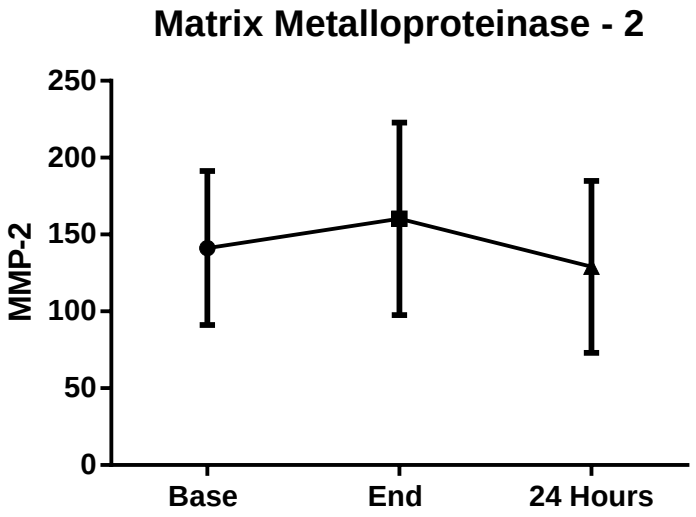

Tissue Inhibitor of Metalloproteinases -1
TIMP-1 (ng/mL)
400
300
200
100
0
Base P
End P
Base RA
End RA
Base LA
End LA
Sample site

Figure 6: Tissue remodelling: Base, End and 24 hours. Despite a rise in MMP-9 [A] concentration this was not significant (p=0.1). Along with this there was no change in MMP-2 [B] or TIMP-1 [C] concentration between the Base, End and 24 hour peripheral samples.(p=0.1 & 0.8, respectively).

A.



B.



C.

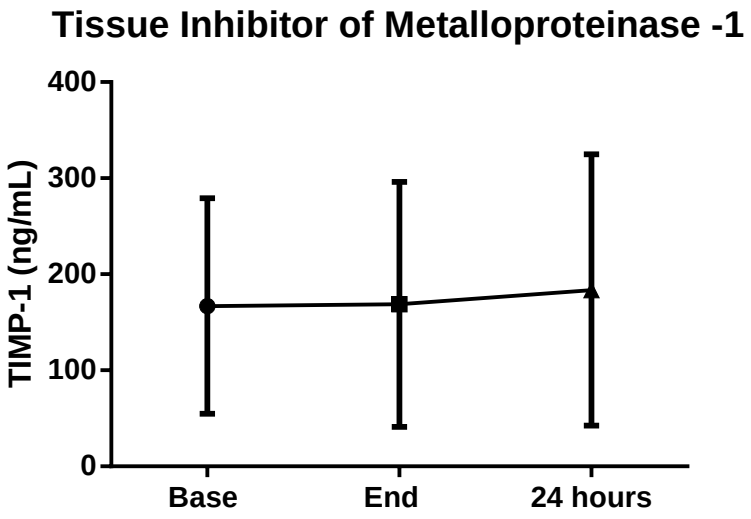
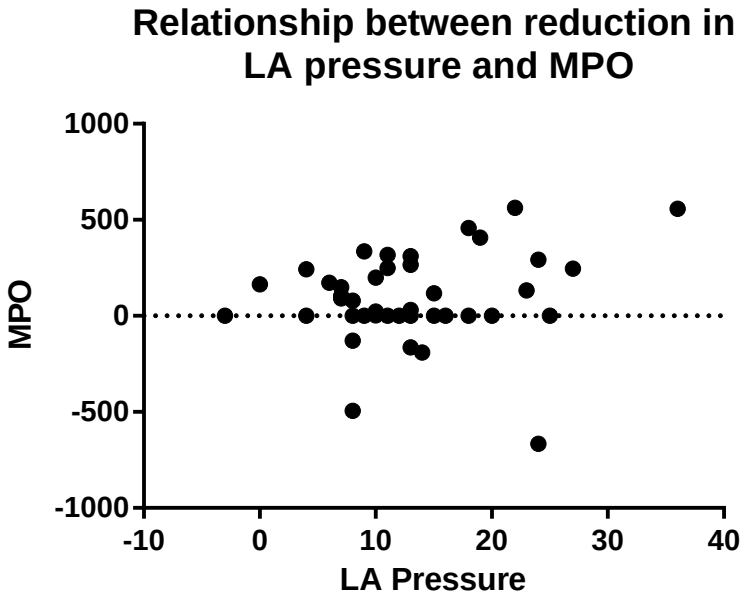

Tissue Inhibitor of Metalloproteinase -1
TIMP-1 (ng/mL)
400
300
200
100
0
Base
End
24 hours

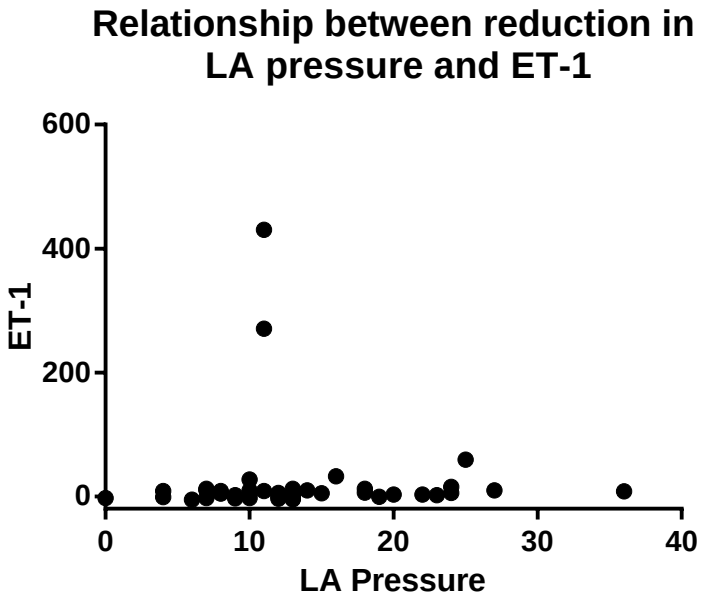
Figure 7: Correlations with LA Pressure

There was no relationship between the change in LA pleasure following a BMV and MPO at the end of procedure (A: r=0.21, p=0.16), the decrease in ET-1 (B) or IL-6 (C) at 24 hours following a BMV procedure (r=-0.12 p=0.15, r=-0.006 p=0.96 respectively).

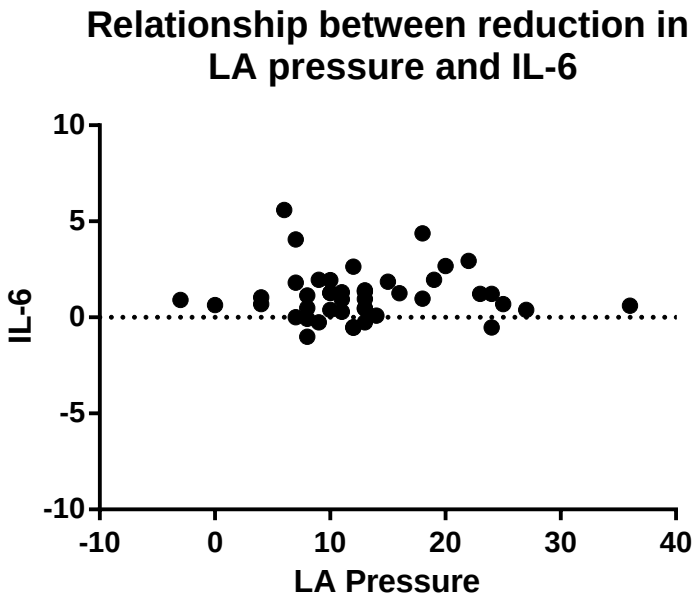
A.



B.



C.


Relationship between reduction in LA pressure and IL-6
IL-6
10
5
0
-5
-10
-10
0
10
20
30
40
LA Pressure

7 CHAPTER SEVEN

Atrial Platelet Reactivity, Endothelial Function and Inflammation in Atrial Fibrillation: Potential Thrombotic Risk

7.1 Overview

Introduction: Atrial fibrillation (AF) is characterized by chaotic electrical activation of the atria that results in rapid and irregular beating of the atria of the heart. It leads to atrial mechanical dysfunction and scarring, which further perpetuates AF. The most devastating consequence of this arrhythmia is thrombotic stroke. While stasis has been implicated in the genesis of thrombus formation within the left atrium (LA) or left atrial appendage (LAA) little is known about the thrombogenic milieu within the atria. In addition, clinically thrombus is seen within the left atria but rarely in the right atria; the reasons for this differential observation has remained elusive.

This study aimed to determine the atrial specific alteration of haemostatic mechanisms (platelet activation, endothelial dysfunction and inflammation), as well as tissue remodelling in patients with a history of paroxysmal AF.

***Methods*:**104 patients undergoing a radiofrequency ablation procedure were enrolled in this study, 55 with paroxysmal AF and 49 with SVT (controls).All patients were in sinus rhythm, had ceased any

anticoagulation therapies 7 days prior to the procedure, and had no symptoms of arrhythmia for 7 days prior to the procedure. Blood samples were collected from the peripheral circulation (femoral vein), from within the left atria (LA) at the beginning of the procedure prior to any heparin administration. Blood samples were prepared for whole blood flow cytometry (platelet reactivity, CD62P [p-selectin], and glycoprotein IIB/IIIA) and plasma was collected for batch analysis with haemostatic markers of endothelial function (ADMA, vWF and ET-1), inflammation (VCAM-1, ICAM-1, CD40L, IL-6 and MPO) and tissue remodelling (MMP-9, MMP-1 and TIMP-1). With all markers measured by commercially available ELISA.

***Results*:** This study found that patients with AF, in the absence of arrhythmia have an enlargement of the LA, increased diameter and volume, which is consistent with the result in the markers of tissue remodelling finding that AF patients significantly increased the amount of LA remodelling than that of SVT (MMP-9 $p=0.01$, MMP-1 $p=0.0004$). This was further associated with increased LA levels of platelet activation through P-selectin ($p=0.01$), CD40L ($p=0.02$) and Glycoprotein IIb/IIIA ($p<0.0001$) and endothelial function through ADMA ($p=0.0009$), ET-1 ($p=0.04$) and vWF ($p=0.006$) compared to control patients. There was also evidence of increased levels LA inflammation observed through VCAM-1($p<0.0001$) ICAM-1 ($p=.03$), and IL-6 ($p=0.04$) when compared to the periphery.

***Conclusion*:** Patients with AF have increased levels of platelet reactivity, endothelial dysfunction and inflammation with the LA compared to the peripheral circulation. The increase in LA size, volume and diameter is associated with significant changes in LA remodelling, further giving evidence for the basis for LA specific thrombus formation in AF patients. Atrial fibrillation is known to significantly increase the risk of LA thrombus formation leading to stroke. This study suggests that in addition to the risks associated with arrhythmia itself, patients with AF have an underlying mileu that is conducive to thrombogenesis within the LA.

7.2 Background

Atrial fibrillation (AF) is the most common cardiac arrhythmia.[15] AF is characterised by disordered electrical activity of the atria, desynchronised beating and elevated heart rate. AF is recognised as the most important cause of ischaemic stroke, primarily derived from thromboembolism (clot) from within the left atrium (LA).[8,298] The risk of thrombus in AF can be determined via many factors and are generally classified via the CHADS-VASc risk score, low risk patients generally have fewer comorbidities and are younger, whereas patients who are right risk tend to have more confounding diseased such as hypertension, diabetes and vascular disease and are older. However stroke risk does not seem to time with the episode of AF.[299] There have been few studies which have looked specifically at mechanisms of atrial thrombus formation in the AF population, despite the knowledge that thrombus formation is derived almost specifically within the LA. Virchow's triad explaining the many various components of thrombus formation within the LA potentially develops a pro-thrombogenic environment through increased platelet activation, Inflammation, endothelial dysfunction in AF patients even with lower risk scores. However, platelets are only one part of the clotting cascade associated with thrombus formation. There are many other components of the disease of AF that may be altering the thrombogenic properties; these include arrhythmia, disordered contraction of the heart leading to alterations in blood flow and damage to the atrial wall structure. There is a clear activation of factors as a result of arrhythmia.[196] However,

there is data to suggest that there does not seem to be a temporal relationship between arrhythmia and stroke. The TRENDS trial was designed to evaluate the relationship between comprehensive arrhythmia burden detected by cardiac implanted electronic devices and thromboembolic risk, and to determine if there is a threshold value of arrhythmia burden that increases thromboembolic risk.[300] This study showed that that thromboembolic risk is a quantitative function of arrhythmia burden. However, additional studies are needed to more precisely investigate the relationship between stroke risk and arrhythmia burden.[299]

The risk of clot formation, thromboembolism and ultimately stroke is managed in most patients, through oral anti-platelet and/or anti coagulation therapies. Despite these therapies patients are still at a risk of ischemic stroke, and whilst on these medications there is a further risk of haemorrhagic stroke.

This study aimed to determine the atrial specific alteration of haemostatic mechanisms (platelet activation, endothelial dysfunction and inflammation), as well as tissue remodelling in a population of AF patients who would be considered low risk (CHA_2DS_2 VASc ≠0-2) with a history of paroxysmal AF, currently sinus rhythm.

7.3 Methods

55 patients with a history of paroxysmal AF and 49 with SVT (controls) were enrolled. All patients were in sinus rhythm for a minimum of 48 hours prior to enrolment and at the beginning of the procedure; assessed by ECG recordings. At the beginning of the electrophysiology and ablation procedure blood samples were taken from the femoral vein (peripheral sheath) and through the catheters placed within the left atria for the electrophysiology study and ablation. These samples were taken simultaneously, immediately following the transeptal puncture; no heparin was administered until the final blood sample was collected.

Blood samples were analysed for the markers of platelet reactivity and aggregation, endothelial function (ADMA, ET-1), inflammation (CD40L, VCAM-1, ICAM-1), and tissue remodelling (MMP-9, TIMP-1). All markers were measured by ELISA.

7.3.1 Patient Characteristics

7.3.1.1 **Patients**

This study included 55 patients with documented paroxysmal AF undergoing routine elective radiofrequency ablation, 49 patients with left sided supraventricular tachycardia (SVT) were included as control patients. All patients were in sinus rhythm a minimum of 48 hours prior to and at the time of the procedure. Patients were excluded if they were younger than 18 years, hadn't ceased taking anti-platelet therapy for one week prior to procedure, had known bleeding abnormalities, and

had an acute cardiovascular or cerebrovascular event (myocardial infarction or stroke) within the last 3 months. In addition, all patients underwent trans-oesophageal echocardiography to exclude the existence of intracardiac thrombus. Paroxysmal AF is defined as recurrent AF (2 or more episodes) that terminated spontaneously within seven days.[26] All AF patients underwent anticoagulation with warfarin to maintain their international normalised ratio (INR) between 2 and 4 for ≥6 weeks prior to the procedure. All ceased warfarin therapy 7 days and enoxaparin 12 hours prior to the procedure. All antiarrhythmic were ceased 5 half-lives prior to the procedure.

All patients provided written informed consent to the study protocol that was approved by the Human Research Ethics Committees at the Royal Adelaide Hospital, Adelaide, Australia; University of Adelaide, Adelaide Australia.

7.3.1.2 **Electrophysiological Study**

The electrophysiological study was performed while patients were in a fasted and sedated state. Patients were administered local anaesthesia and general sedation. Access to the right femoral vein was achieved using a conventional Seldinger[301] technique with the following sheaths: 6F, 7F and 8F sheaths. A conventional transeptal puncture was performed to access the left atrium with a SLO sheath and BRK-1 needle (SLO, St Jude Medical). Following transeptal puncture blood samples were collected immediately from the LA and femoral vein (LA,

P). A standard ablation was performed, with the aim of pulmonary vein isolation with or without substrate modification, with ablation of AF in our laboratory as previously described.[302] The following catheters were utilised for patients with AF: (i) 10 pole catheter (Daig Electrophysiology) positioned within the coronary sinus; (ii) 10 pole circumferential catheter (Lasso; Biosense-Webster) to map the pulmonary veins; and (iii) 3.5 mm tip externally irrigated ablation catheter (Thermocool, Biosense-Webster) for ablation.

7.3.1.3 **Study Protocol**

As part of the data collecting process, patient demographics were collected. Thereafter they had an ECG done to document their baseline cardiac rhythm; transthoracic, M mode, 2D echo and Doppler echocardiographic studies were performed 1-2 weeks before the procedure.

At the beginning of the ablation procedure, blood samples were obtained from the femoral vein after puncture through the inducer sheath after the patients were in supine position for at least 20 min. LA blood samples were obtained after transseptal puncture via catheters inserted for the clinical procedure (see 7.3.1.2Electrophysiological Study) using a slow withdrawal technique (approximately 1 ml per second), before any heparin was administered. Irrespective of sampling site the first 5mls of blood was discarded to ensure appropriate catheter flushing. 10ml of blood was collected from each site and

transferred into a 10ml polystyrene tubes (Falcon; Becton Dickinson), containing 3.8% sodium citrate (1:9 volume). Plasma was then frozen for further analysis via ELISA.

Analysis of Endothelial function/ Inflammation/ Tissue Remodelling by Enzyme-linked absorbance assay (ELISA)

Blood samples were analysed for the markers of endothelial function through asymmetric dimethylarginine and Endothelin-1 (ADMA and ET-1), inflammation through soluble CD40 Ligand (CD40L), Vascular and Intracellular adhesion molecules (VCAM-1 and ICAM-1),and tissue remodelling (MMP-9, TIMP-1), with all markers measured by Elisa [see section chapter 2 further details], and tissue remodelling was measured via matrix metalloproteinase-9, matrix metalloproteinase-1 and tissue inhibitor of metalloproteinase-1(MMP-9, MMP-2 and TIMP-1), via commercially available ELISA [for further details see methods section 2.6]

7.3.1.4 **Statistical Analysis**

Data is shown as mean ± standard deviation, unless otherwise stated.

Comparisons between the sample sites in the AF patients and the control (SVT) patients were performed using a two-way ANOVA with multiple comparisons, with post hoc analysis via Bonferroni's multiple comparisons test where necessary. Patient characteristics were compared using students T-test for continuous data or a Fisher's exact test for categorical data all data was tested for normality by a

D'Agostino-Pearsons normality test, and log-transformed as appropriate. All data was analysed using GraphPad Prism Version 5.0 (GraphPad Software) and was normally distributed. Statistical significance was set at $p<0.05$.

7.4 Results

7.4.1 Patient Characteristics

The AF patients were significantly older then the SVT patients with a mean age of 59 years and 38 for SVT ($p<0.0001$). Most patients were free of any comorbidity with 15 having hypertension and one with diabetes, which was significantly more than that of SVT patients ($p<0.0001$). The mean CHA_2DS_2-VASc score for these patients was 0.6, and this is considered low risk. The AF patients had significantly higher levels of anticoagulant medications, where SVT patients were more likely to be on Asprin and taking Sotalol, ($p<0.0001$) (Table 1).

7.4.2 Echocardiographic Characterisations

Patients with AF were shown to have an enlarged LA size and volume, which is consistent with the structural changes that occur as a part of the disease, however on the LA volume was significantly increased compared the control patients ($p<0.001$). The RA area is just at the upper limit of normal, with normal being classified at 18 cm^2, and the median of our AF patients being 18.6 cm^2.[303] The left ventricular ejection fraction was considered normal for all patients (>50%). [Table 1]

7.4.3 Platelet Reactivity

Overall patients with AF had significant increased platelet reactivity compared to the control population. P-selectin levels were significantly higher between the LA of control and AF patients (p=0.001) [Fig: 1A].Glycoprotein IIb/IIIA was also increased within the between control and AF patient at both the P and LA [p<0.0001, Fig: 1B]. Further to this there was a significant difference between the P and LA within the AF population (p=0.03). Similar to this CD40L, a platelet activity and inflammation marker showed an increase between the peripheral sampling site of the SVT and AF patients (p=0.02).

7.4.4 Endothelial Function

Endothelial function markers were all found to be increased within the LA of AF patients. Levels of ADMA (p=0.0009), or vWF (p=0.006) and ET-1 (p=0.0004) have increase concentrations within the LA of AF patients when compared to SVT control patients. However there was no difference between the P and LA within the AF population.

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7.4.5 Inflammation

There was an increase in the levels of LA inflammation observed in markers of inflammation. ICAM-1 (p=0.03) and IL-6 (p=0.04) had similar results were this was LA specific. HoweverVCAM-1 (p<0.0001) levels were increased in the AF population at both the P and LA sites. Conversely MPO (p=0.04) however had higher levels within the SVT

patients than the AF. IL-6 was the only inflammation marker which further had a significant increased levels within the AF population form the P to the LA (p=0.01).

7.4.6 Tissue Remodelling

There was a significant increase in the Levels of MMP-9, and MMP-1 markers of tissue remodelling within the LA of AF patients (p=0.04 and p=0.0004, respectively). Although additionally for MMP-1 had a significant higher levels in the LA compared to the P (p=0.02).TIMP-1 concentration however did not alter at either sample site between or within the SVT or AF populations (p=0.1).

7.5 Discussion

7.5.1 Major Findings

This study was undertaken to detail left atrial and peripheral sampling to evaluate platelet reactivity, endothelial function, inflammation and structural markers in a low risk paroxysmal AF population.

It found the following between these the various sample sites patients with AF have;

(i) Significantly increased LA platelet activation compared with controls.

(ii) Significantly increased endothelial dysfunction in the LA AF patients over that of the controls.

(iii) There was an increase in the levels of LA inflammation as well as between the P and LA sites in the AF population.

(iv) Significantly increase levels of LA tissue remodelling markers compared to the peripheral circulation in all markers.

This study provides further evidence for the hypothesis that LA specific thrombus formation in AF patients is predominantly due to changes which occur in haemostatic markers specifically within the LA. Also suggest that it is AF and not comorbidities that drives thrombus formation.

Thrombogenesis in AF is known to be caused by various factors that alter the process of clot formation. There are many other components of the disease of AF that may be altering the thrombogenic properties; these include arrhythmia, disordered contraction of the heart leading to alterations in blood flow and damage to the atrial wall structure. There is a clear activation of factors as a result of arrhythmia.[196] However, there is data to suggest that there does not seem to be a temporal relationship between arrhythmia and stroke. The TRENDS trial was designed to evaluate the relationship between comprehensive arrhythmia burden detected by cardiac implanted electronic devices and thromboembolic risk, and to determine if there is a threshold value

of arrhythmia burden that increases thromboembolic risk.[300] Despite the knowledge that these changes seem to occur specifically within the LA, leading to potentially devastating strokes there are few studies have taken the further step to identify what thrombogenic changes are involved within the LA of AF patients, and how these alterations may differ in the peripheral circulation. Further to this, weather these changes also affect the likelihood to thrombus formation within the LA. The few studies which have looked into atrial differences were the first to discover that indeed platelet reactivity and activation in the LA is increased independently of the peripheral circulation in AF patients. [75,238]These studies have fostered that progression to look at various changes in haemostatic markers which are involved in thrombus formation; these include platelet reactivity, thrombin generation, endothelial function and inflammation, as well as analysing the structural changes which have occurred.

Previously it has been shown that in the AF population there is an increased risk of thrombus formation through alterations in platelet activation,[194,254] endothelial function,[129,194] and inflammation[164,200] within the peripheral circulation; which are all important contributors to thrombus formation. Our results in atrial platelet reactivity as seen with p-selectin, Glycoprotein IIB/IIIA and CD40L [Fig: 1A, B & C] show platelet reactivity was significantly increased within the LA of AF patients compared with that of controls. There has only been the limited studies which have investigated the atrial specific mechanism of the

thrombus formation,[11,75,238] and these studies have mainly focused on platelet reactivity, and these results are consistent with the findings of our study. A further study also found that the induction of short duration AF significantly increased endothelial dysfunction through increased ADMA levels;[196] ADMA levels were found to be higher in the LA in this study, further to this ET-1 and vWF concentrations were significantly increased within the LA in AF patients. Further to this Inflammation (ICAM-1 and IL-6), where these makers were significantly increased in the LA, the remaining markers (VCAM-1and MPO) increased within the P and LA in the AF patents. This supports the likelihood that it is atrial specific changes in the prothrombotic state in AF that leads to the risk of stroke. This is compounded by structural and mechanical changes[180,182] which are already known to occur in AF, further increasing a person's likelihood of thrombus formation leading to stroke.

Inflammation has become one of the newest focuses in the establishment and perpetuation of AF and AF related thrombosis and stroke,[200] with inflammatory process have long been associated with the development of thrombus and the risk of stroke. [201] Along with this, furthermore it has been shown that after the first diagnosis of AF, high CRP was associated with reoccurrence[206] of AF at 1 year follow up. The results of this study have shown that inflammation does indeed play a role in changes in haemostasis which occur in AF, and I found that there was an increase in the levels of LA inflammation observed in

both ICAM-1 and IL-6 specifically between the LA (p=0.004 and p=0.01 respectively).VCAM-1 levels this occurred at both the P and LA. With only IL-6 showing a significant difference between the P and LA within the AF population. These results show the association between LA inflammation and AF within a low risk population. Further revealing the role inflammation plays in the process of AF and the risk of thrombus formation within the LA.

The markers of structural remodelling were all significantly increased across with the LA of AF patients (MMP-9: p=0.0004, and MMP-1; p=0.04) These findings are consistent with the echocardiographic results which showed that these patients have enlarged LA with an increased volume. This is consistent with previous investigation in LA area and function in AF.[304]A previous study found that with the restoration of sinus rhythm by ablation for isolated AF patients, there was reverse morphological remodelling of the LA and improvement of LV diastolic and systolic functions.[305] Although we did not see a change in the reverse remodelling marker TIMP-1 (p=0.1) However all patients in the study were in sinus rhythm, showing that despite the rhythm patients with a history of AF were still at a heightened risk. The alterations which during AF in the structure of the LA are known to cause scarring, and alter tissue remodelling and function. Further to this, a higher amount of LA scarring is also associated with a lower ejection fraction, larger LA size, and increased inflammatory markers.[14]

This study has shown how AF patents are at a significantly increased risk of thrombus formation through a rise in platelet reactivity, endothelial dysfunction and inflammation, further showing how AF patients have increases in many factors within the Virchow's triad.

7.6 Conclusions

Atrial fibrillation is known to be a significant cause of LA thrombus which may lead to devastating and deadly strokes. This study was able to define how each of the markers of thrombogenesis is altered within the LA, of low risk AF patients. AF patients have increased levels of platelet reactivity, endothelial dysfunction and inflammation within the LA compared to the peripheral circulation. We also found that AF patients have an increased in LA area and volume, which were associated with changes in plasma markers of tissue remodelling within the LA, providing further evidence for the hypothesis that LA specific thrombus formation in AF patients is predominantly due to changes which occur in haemostatic markers specifically within the LA .

Table 1

Baseline Characteristics for AF Patients

Characteristics	AF (n=55)	SVT (n=49)	P-value
Age (Years)	59.4 ± 13	38.6 ± 11.6	<0.0001
Gender (F:M)	21 : 34	19 : 30	0.1
Comorbidities			
Hypertension (n)	15	0	<0.0001
Diabetes mellitus (n)	1	0	<0.0001
Previous stroke (n)	0	0	-
CHA_2DS_2-VASc	0.6 ± 0.05	0	<0.0001
BMI (±SD), kg/m^2	30 ± 4.2	25 ± 4.7	0.05
Medications			
Anti-Arrhythmic (n)	48	6	<0.0001
Warfarin (n)	46	0	<0.0001
Asprin (n)	7	32	<0.0001
New Oral Anticoagulants (n)	2	0	<0.0001
Flecainide (n)	14	2	<0.0001
Sotalol (n)	12	18	0.04

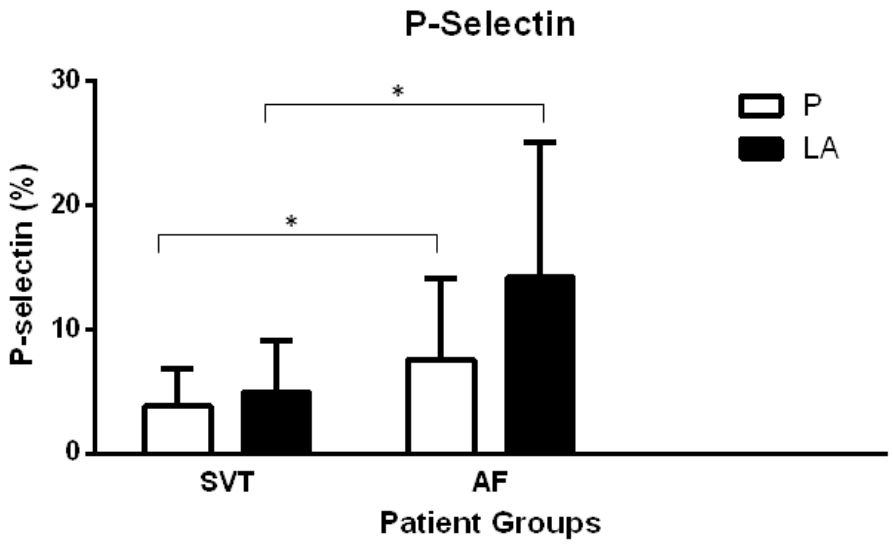
Echocardiographic Parameters			
LA Area (cm^2)	23.2 ± 9.4	25.0 ± 9.5	0.4
RA Area (cm^2)	18.6 ± 6.0	19.3 ± 4.5	0.7
LA volume	62.9 ±27.8	34.7 ± 6.9	<0.0001
LVEF (%)	60.8 ± 8.9	61.6 ± 9.1	0.7
LA Pressure (at TS puncture)	7.3 ± 4.8	Not recorded	-

Platelet Reactivity

Figure1: P selectin (A) expression between patents (p=0.001), CD40L levels (C) (p=0.02) and GP IIb/IIIA (B) (p<0.0001) significantly increased compared with the SVT patients. However not between the sites in search patients group for CD40L (A: p=0.5 and B: p=0.4) except for in GP IIb/IIIA, which was significantly increased within the LA of AF patients over that of the P (p=0.03).

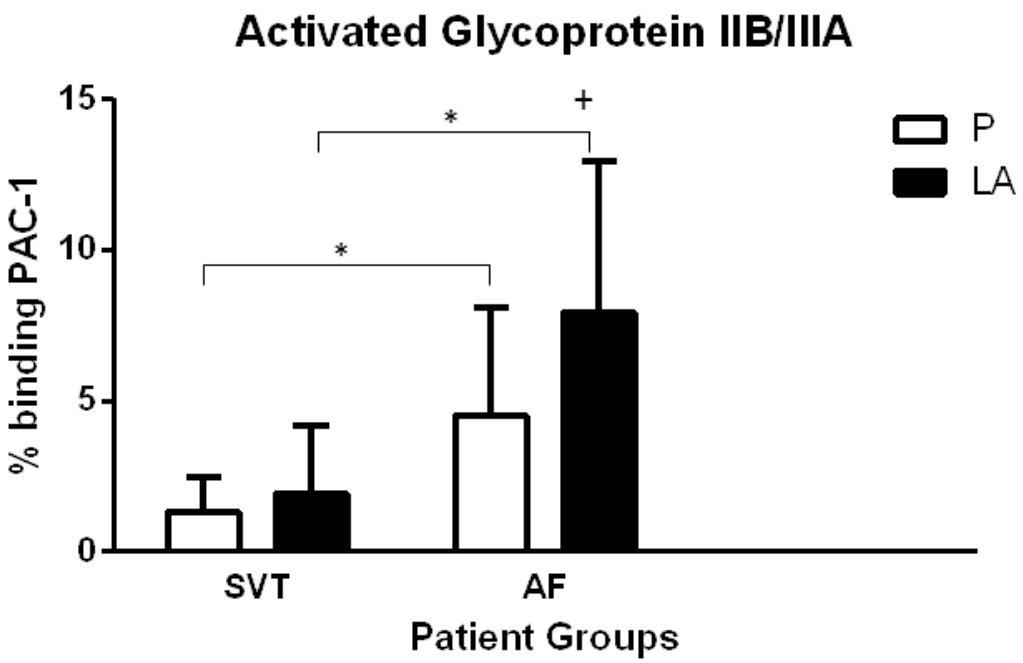
With post hoc analysis, this was significant within the P for all markers and the LA for p-secretin and GP IIb/IIIA.

A.



P p=0.02 and LA p=0.01

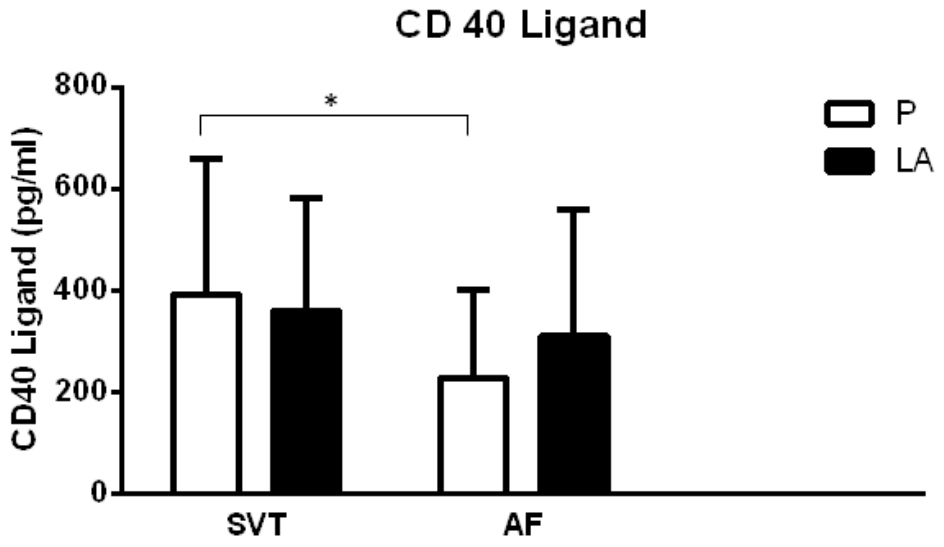
B.



P p=0.03 and LA p<0.0001

+ AF, P vs LA, p=0.03

C.



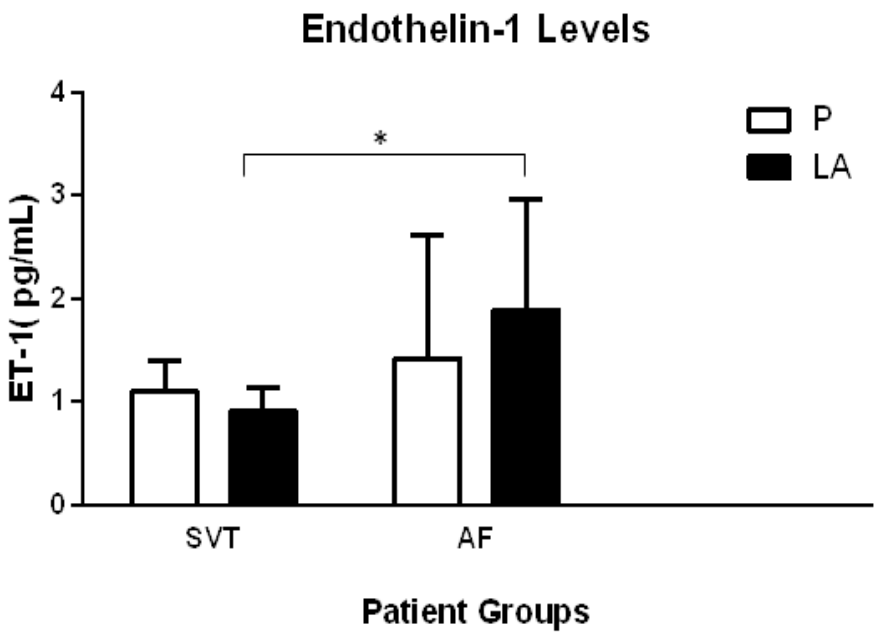
P; p=0.02.

Endothelial Function

Figure 2: Endothelial function was significant increased between the SVT and AF patients through ET-1 (A) (p=0.0004) ADMA (B) (p=0.0009) and vWF (C) (p=0.006) not changes between sites in the patient groups (p=0.4, p=0.4 and p=0.6).

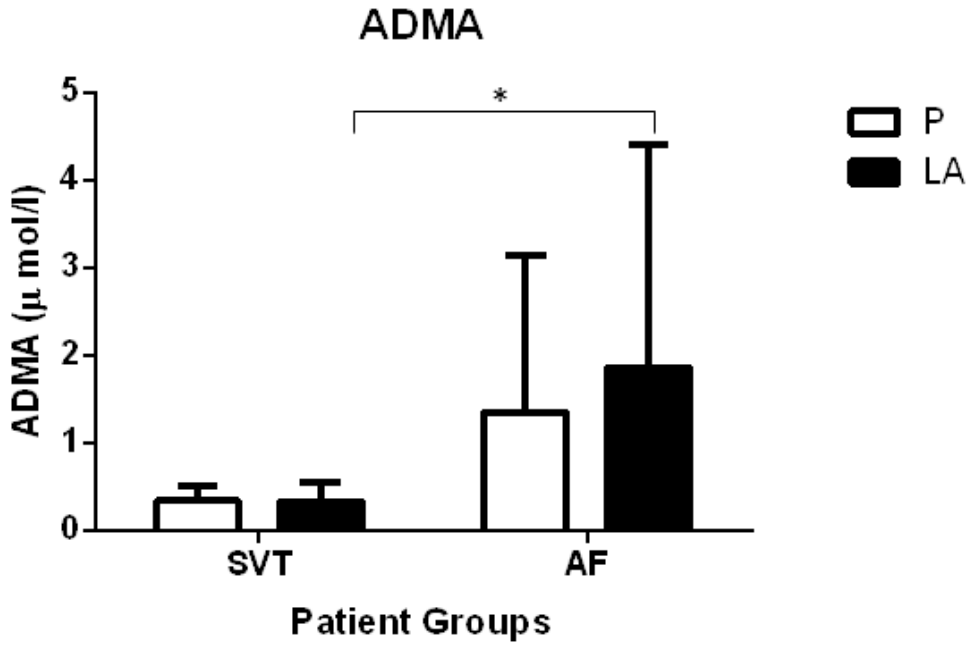
With further analysis this changes was only seen at the LA of the AF patients compared to the SVT, but not the P site.

A.



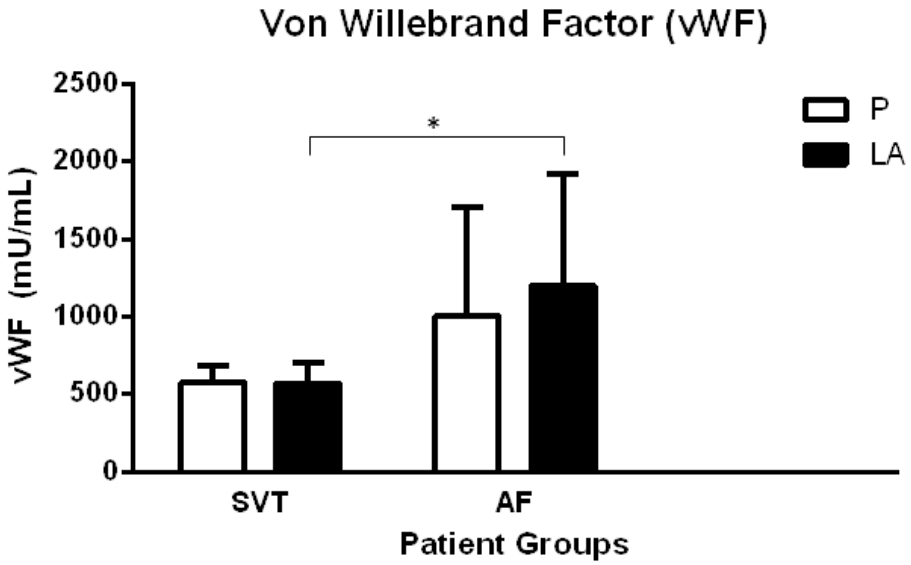
LA p=0.0004

B.



LA p=0.0004

C.



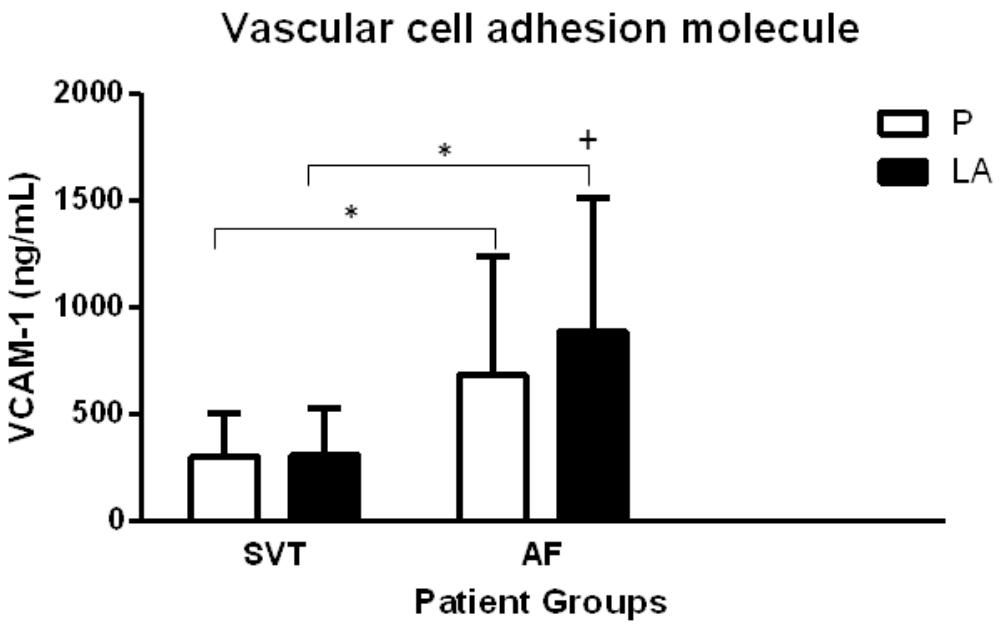
LA p=0.04

Inflammation

Figure 3: A significant increase in the levels of inflammation observed over that of the SVT patients in all markers of infmallationVCAM_1 (a) (p=<0.0001), ICAM-1 (B) (p=0.03), IL-6 (C) (p=0, 04).MPO (D) was an exception where there was higher in SVT patients than the AF patients (p=0.04). Il-6 was the only marker which had a significant difference between the P and LA of the AF patients.

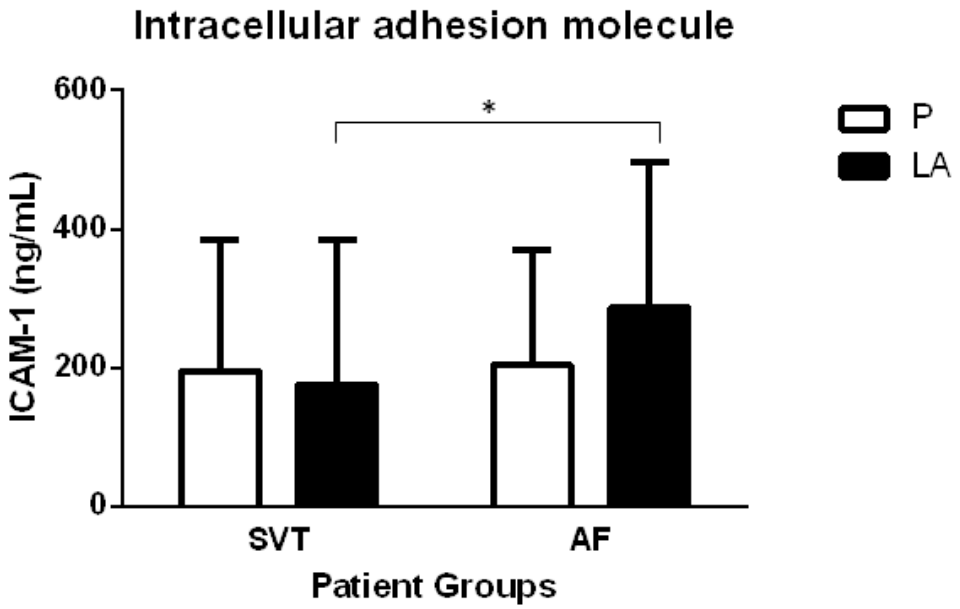
With post hoc analysis this difference was in the LA in all markers and the P in VCAM-1. With no change at either site in MPO.

A.



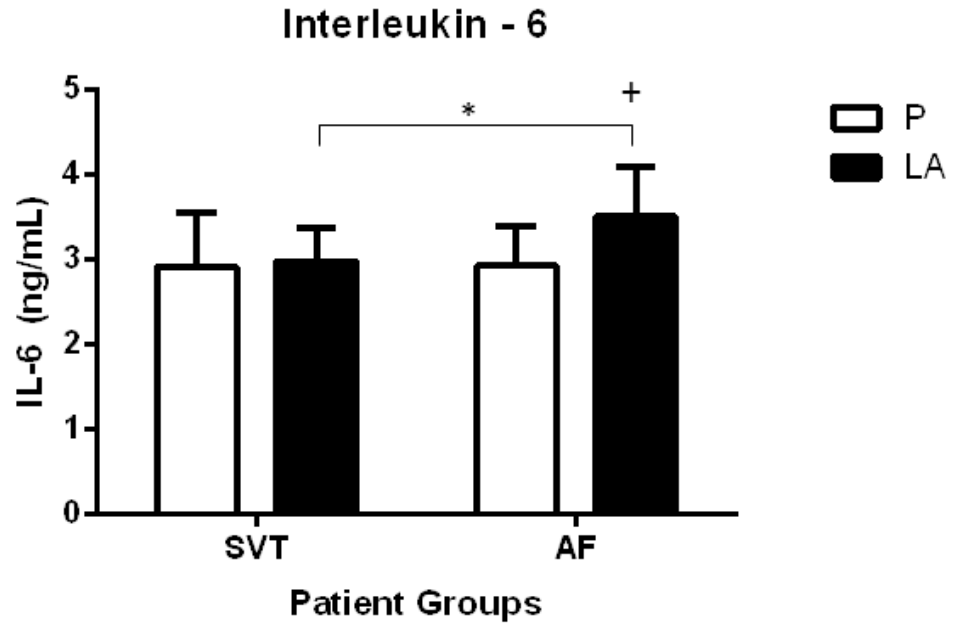
P p=0.0002, LA p<0.00001

B



LA p=0.01

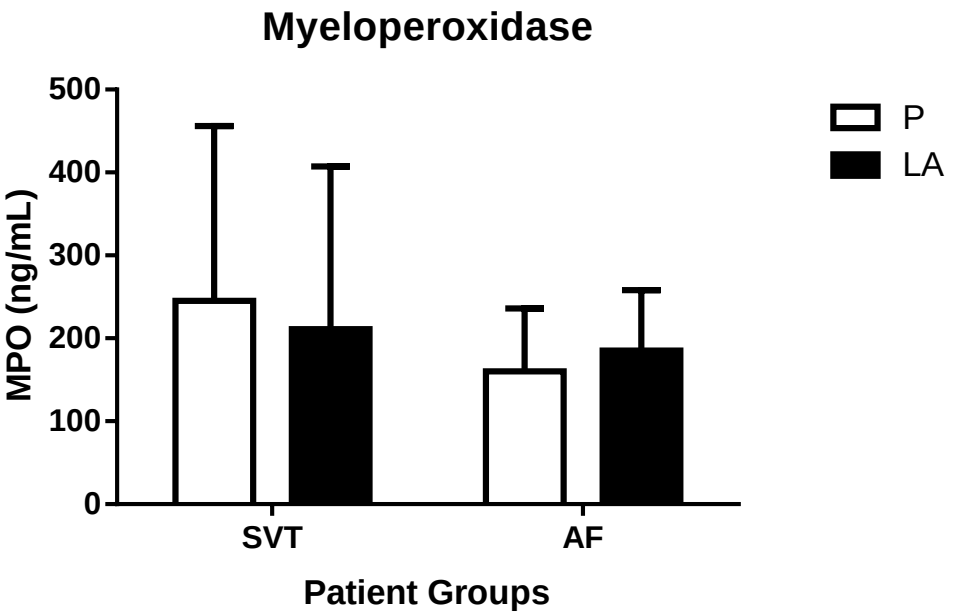
C.



LA 0.01

+ AF P vs LA p=0.1

D.


Myeloperoxidase
500
400
300
200
100
0
MPO (ng/mL)
SVT
AF
Patient Groups
P
LA

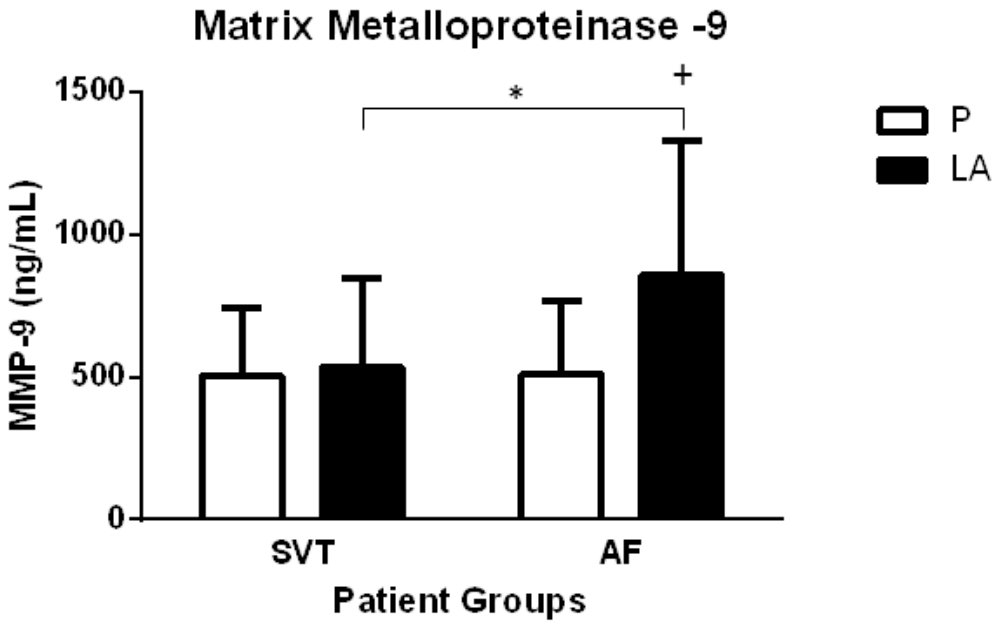
.

Tissue Remodelling

Figure 4: There was a significant increase in levels of remodelling within the LA of AF patients. This was consistent for all markers (MMP-9 (A) p=0.04, MMP-1(B) p=0.0004, although this was not for reverse remodelling in TIMP-1 (p=0.1). There was a site specific change in the MMP-9 concentrations (p=0.01) between the P and LA of the AF patients.

Post hoc analyisis showed the af patients MMP-9 and MMP-2 had significant higher LA levels than that of the SVT controls.

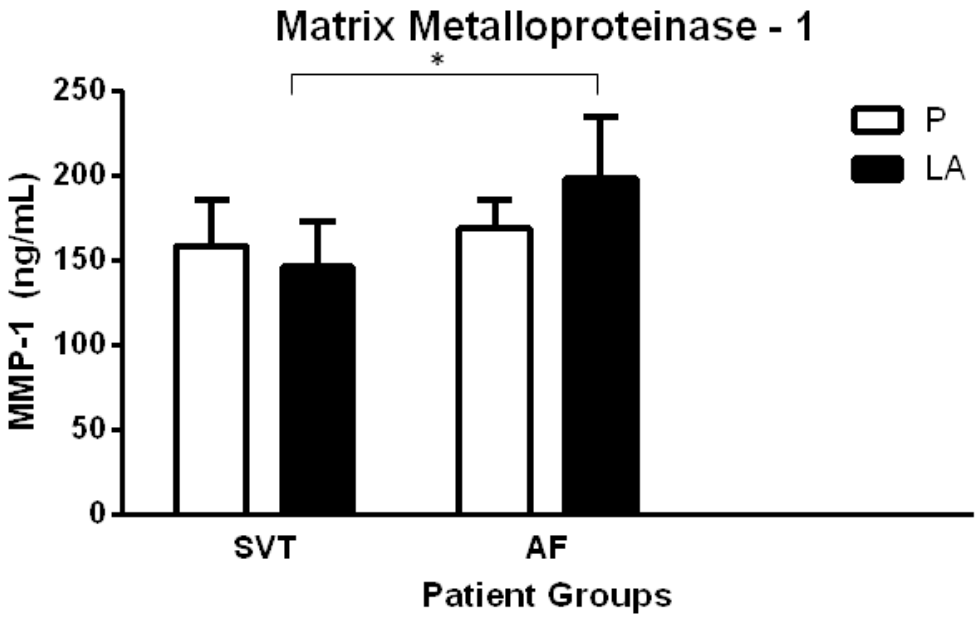
A.



LA p=0.01

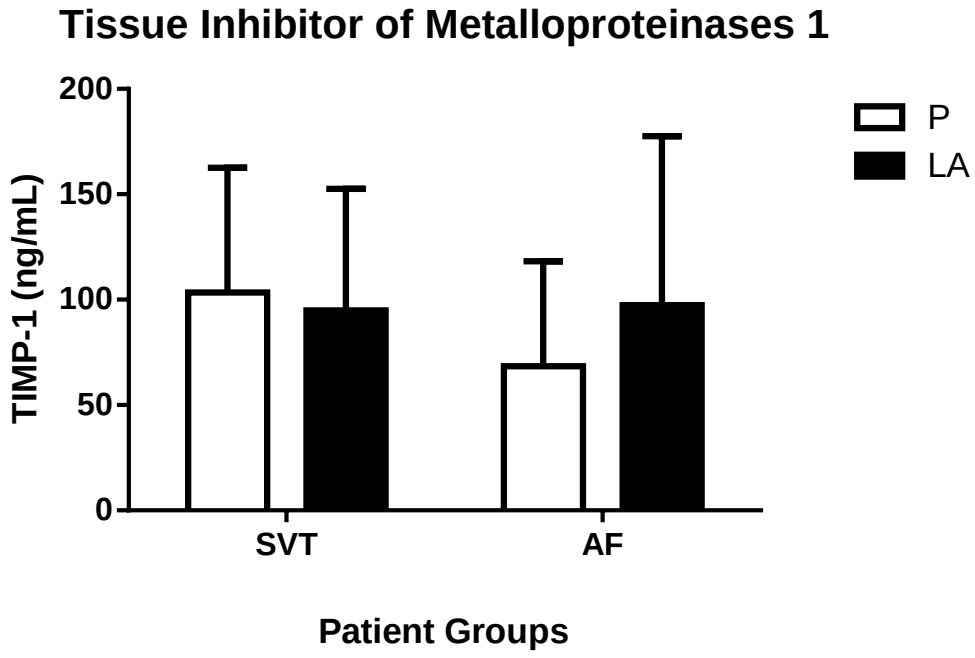
+ AF LA vs P p=0.02

B.


Matrix Metalloproteinase - 1
*
250
200
150
100
50
0
MMP-1 (ng/mL)
P
LA
SVT
AF
Patient Groups

LA p<0.0001

C.


Tissue Inhibitor of Metalloproteinases 1
200
150
100
50
0
TIMP-1 (ng/mL)
P
LA
SVT
AF
Patient Groups

8 CHAPTER EIGHT

Comparison of Atrial Endothelial Function and Inflammation between Atrial Fibrillation and its Substrate Mitral Stenosis

8.1 Overview

Introduction: Atrial fibrillation (AF) is the most common sustained arrhythmia which affects more than 2% of the general population, carrying an approximately 5-fold increased risk of stroke.[48] The most common substrate of AF in the Asian Indian population as well as 3rd world populations is mitral stenosis (MS). MS alone is known to increase a patient's risk of thrombus formation, along with an increased a risk of AF, and with concurrent diseases having a more than 17.5-fold increased risk of stroke. It is not known if the mechanisms altering haemostasis in MS are the same as in AF. Therefore, this study aimed to determine if markers of thrombogenesis (endothelial function and inflammation), are consistent between AF, and its valvular substrate MS.

Methods: A total of 166 patients were enrolled in this study, 52 with supraventricular tachycardia (SVT) due to left sided accessory pathway were included as controls; 59 had documented Mitral stenosis (MS, with no AF); and 55 had a history of paroxysmal atrial fibrillation (AF). Blood samples were collected from the peripheral circulation (femoral vein), from within the right atria (RA) and left atria (LA) at the beginning

of the cardiac procedure, relevant for each disease, and prior to any heparin administration. Blood samples were collected for batch analysis for markers of endothelial function (ADMA, ET-1), inflammation (CD40L, VCAM-1, ICAM-1, IL-6 and MPO) and tissue remodelling (MMP-9 and TIMP-1) via ELISA technique. Echocardiographic studies were used for atrial structural measurements.

Results: Patients with AF were found to have significantly higher levels of endothelial dysfunction, at all sites, compared to the SVT and MS patients ($p<0.0001$).This study had variable results in the markers of inflammation. VCAM-1 and ICAM-1 (RA $p=0.004$, LA $p=0.0038$) were significantly higher in AF compared with SVT and MS. In contrast, CD40L, MPO and IL-6 were significant higher with in the LA of MS patients compared the AF and SVT, (CD40L $p=0.03$, MPO: $p<0.0001$ and IL-6 LA $p=0.03$). MS patient also had significantly larger LA and RA area, LA volume and LA pressure compared with AF and SVT patients ($p<0.0001$), and this was consistent with tissue remodelling marker TIMP-1 ($p<0.0001$). MMP-9 levels were significantly increased within the RA and LA of patients with AF compared with that of MS and SVT patients (RA and LA $p<0.0001$).

Conclusion: This study has shown that AF and the valvular AF substrate MS have two discreetly different mechanisms that promote atrial thrombus formation. AF patients have increased endothelial

dysfunction and inflammatory adhesion molecules, whilst MS patients have significantly higher levels of markers of inflammation, and tissue structural remodelling. This study suggests that the pathophysiology of MS and AF are different. MS (as a substrate for valvular AF) impacts on atrial thrombus formation through tissue remodelling and inflammation whereas non-valvular paroxysmal AF alters endothelial function and tissue inflammation.

8.2 Background

The most dangerous complication arising from AF is that of thromboembolic stroke, which is a consequence of a prothrombotic state found within the heart of patients with AF.[306] A person with non-valvular AF has an increased risk of stroke up to 5-times greater than a person without AF; however, this risk is increased to 17.5-times in valvular AF.[9,307] Thromboembolism or thrombus formation primarily originates in the left atria (LA) of patients with AF[15] but the mechanisms of this increased risk are not fully understood. Previous studies have determined that some of the main events contributing to thrombus formation within the LA are: changes in haemostasis and platelet activation (a hypercoagulable state) and endothelial dysfunction.[75,238]

The most common valvular substrate of valvular AF, mitral stenosis (MS) has been shown to be associated with thrombogenic abnormalities.[9] Although mitral stenosis has known thrombogenic properties, little known about the stroke risk until these patients have progressed into AF. With MS is known to increase the risk of AF.[9]

This study aimed to determine if markers of thrombogenesis, through endothelial function and inflammation, are consistent between AF, and its valvular substrate MS. In addition, the regional difference in the expression profile of these markers in each group was also examined.

8.3 Methods

We enrolled 166 patients in the following groups: 55 patients who were undergoing going a radiofrequency ablation for paroxysmal AF; 59 patients with severe mitral stenosis (MS) undergoing a balloon valvuloplasty; and 52 patients with structurally normal hearts undergoing ablation of SVT due to left sided accessory pathways (controls).

All patients provided written informed consent to the study protocol that was reviewed and approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, University of Adelaide, Adelaide, Australia, and the Christian Medical College, Vellore, India.

8.3.1 Patient Characteristics

8.3.1.1 **SVT patients**

52 patients with structurally normal hearts undergoing ablation of SVT due to left sided accessory pathways (controls) were enrolled. Patients were excluded if they were younger than 18 years, had a clinical diagnosis of right sided disease, such as atrio-ventricular nodal re-entry tachycardia, if they had any previous clinical evidence of AF, or had structural heart disease. All patients underwent an echocardiography prior to the procedure to determine left and right atrial and ventricular dimensions, and to verify normal parameters of atrial size and function; any patients who had abnormal cardiac dimensions via echocardiography were also excluded. All patients were not taking

antiarrhythmic or anticoagulant/platelet medication at the time of procedure.

8.3.1.1.1 Protocol: Electrophysiology study

The electrophysiological study was performed in a fasted state. Patients were administered local anaesthesia and general sedation. Access to the right femoral vein was achieved using conventional "Seldinger" technique with the following sheaths: 6F, 7F and 8F sheaths. A conventional transseptal puncture was performed to access the left atrium with a SLO sheath and BRK-1 needle (SLO, St Jude Medical). Following transseptal puncture blood samples were collected immediately from the left atria (LA), right atria (RA) and femoral vein (P). The SVT ablation procedure is described in detail as per description chapter 3.[192]Blood samples were analysed for the markers of endothelial function, inflammation and tissue remodelling, With all markers measured by Elisa [see Chapter 2.6, for further details].

8.3.1.2 Mitral Stenosis Patients

Fifty-nine patients with known severe rheumatic mitral stenosis (MS).[mitral valve area<1.2cm^2] and were undergoing clinically indicated balloon valvuloplasty (BMV) at the Christian Medical Centre in Vellore, India, were enrolled in the study. Patients had an average disease history of 10 years. Patients were selected on the basis of having severe MS with a mitral valve area of <1.5 cm2 with significant

symptoms (NYHA class ≥2) and mitral valve morphology suitable for PBMC as determined by the Wilkins criteria (score <10).

Patients were excluded if they had of the following:

- Age<18
- Patients with MS due to a non-rheumatic etiology.
- Patients with congenital heart disease, atrial fibrillation, LV systolic dysfunction, aortic stenosis or aortic regurgitation.
- Patients with a previous history of myocardial infarction.
- Patients who developed a complication during BMV including grade II mitral regurgitation or more.
- Individuals who are taking oral phosphodiesterase 5 inhibitors [i.e. sildenafil, vardenafil or tadalafil]
- Patients with peripheral vascular disease, hypertension, diabetes or vasculitis.
- Patients who are not followed up in Christian Medical College after their procedure.
- Individuals who smoke.

All patients had no previous history of any arrhythmias documented by 12 lead ECG recordings 3 months prior to, at the time of, and during BMV procedureAll patients underwent transthoracic; M-mode, 2-dimentional (2D) and Doppler echocardiographic studies will be performed 1-2 weeks before the procedure, as per the American Society of Echocardiography criteria.[283] In MS patients the mean trans-mitral valve gradient, the mitral valve area (MVA) will be calculated

from the echocardiographic Doppler study using the pressure half time method, and using the short axis 2D echocardiographic view.

All patients provided written informed consent for the study protocol that was approved by the Clinical Research Ethics Committees of the Christian Medical College, Vellore, India.

8.3.1.2.1 Protocol: Balloon Mitral Valvuloplasty (BMV)

A Balloon Mitral Valvuloplasty (BMV) was performed on all patients in a fasted and sedated state. The BMV was performed by the transeptal approach with the use of a Joseph mitral valvuloplasty balloon catheter. Details of the procedure have been described previously.[284] The BMV procedure was performed under local anaesthesia. Right heart catheterization precedes BMV, and was repeated, along with oximetry which was run after BMV. Heparin was administered intravenously after completion of the transeptal puncture. A modified back up wire is placed in the left ventricle through a Swan Ganz catheter. A Joseph's catheter balloon was then sent over the wire and inflated across the valve orifice. When additional balloon dilatation was required, the balloon would have been exchanged for a larger one and the same procedure would be repeated. Invasive pressure measurements were performed immediately before and after valvuloplasty.[284] Blood samples were analysed for the markers of endothelial function, inflammation and tissue remodelling, With all markers measured by Elisa [see Chapter 2.6, for further details].

8.3.1.3 **Atrial Fibrillation Patients**

This study included 55 patients with documented paroxysmal AF undergoing routine elective radiofrequency ablation. All patients were in sinus rhythm a minimum of 48 hours prior to and at the time of the procedure. Patients were excluded if they were younger than 18 years, hadn't ceased taking anti-platelet therapy for one week prior to procedure, had known bleeding abnormalities, and had an acute cardiovascular or cerebrovascular event (myocardial infarction or stroke) within the last 3 months. In addition, all patients underwent trans-oesophageal echocardiography to exclude the existence of intracardiac thrombus. Paroxysmal AF is defined as recurrent AF (2 or more episodes) that terminated spontaneously within seven days.[26] All AF patients underwent anticoagulation with warfarin to maintain their international normalised ratio (INR) between 2 and 4 for ≥6 weeks prior to the procedure. All ceased warfarin therapy 7 days and enoxaparin 12 hours prior to the procedure. All antiarrhythmic were ceased 5 half-lives prior to the procedure.

All patients provided written informed consent to the study protocol that was approved by the Human Research Ethics Committees at the Royal Adelaide Hospital, Adelaide, Australia; University of Adelaide, Adelaide Australia.

8.3.1.3.1 Protocol: Electrophysiological Study

The electrophysiological study was performed while AF patients were in a fasted state. Patients were administered local anaesthesia and general sedation. Access to the right femoral vein was achieved using conventional (Seldinger[301]) technique with the following sheaths: 6F, 7F and 8F sheaths. A conventional transeptal puncture was performed to access the left atrium with a SLO sheath and BRK-1 needle (SLO, St Jude Medical). Following transeptal puncture blood samples were collected immediately from the femoral vein, LA and RA. A standard ablation was performed, with the aim of pulmonary vein isolation with or without substrate modification, with ablation of AF in our laboratory as previously described.[302] The following catheters were utilized for patients with AF: (i) 10 pole catheter (Daig Electrophysiology) positioned within the coronary sinus; (ii) 10 pole circumferential catheter (Lasso; Biosense-Webster) to map the pulmonary veins; and (iii) 3.5 mm tip externally irrigated ablation catheter (Thermocool, Biosense-Webster) for ablation. Blood samples were analysed for the markers of endothelial function, inflammation and tissue remodelling, With all markers measured by Elisa [see Chapter 2.6, for further details].

8.3.1.4 **Study Protocol**

As part of the data collecting process, patient demographics were collected. Thereafter they had an ECG performed to document their baseline cardiac rhythm. Transthoracic, M mode, 2D echo and Doppler

echocardiographic studies were performed 1-2 weeks before the procedure.

At the beginning of the procedure, blood samples were obtained from the femoral vein after puncture through the inducer sheath. RA blood was obtained via catheters inserted for the clinical procedure, using a slow withdrawal technique (approximately 1 ml per second). LA blood samples were obtained after transeptal puncture before heparin was administered. Irrespective of sampling site the first 5mls of blood was discarded to ensure appropriate catheter flushing. 10ml of blood was collected from each site and was transferred into a 10ml polystyrene tubes (Falcon; Becton Dickinson), containing 3.8% sodium citrate (1:9 volume).Blood samples were analysed for the markers of endothelial function, inflammation, and tissue remodelling by ELISA.

Analysis of Endothelial function/ Inflammation/ Tissue Remodelling by Enzyme-linked absorbance assay (ELISA)

The obtained blood samples were centrifuged at 2500g for 15 min at $4^{o}C$ and stored at $-80^{o}C$ for batch analysis utilising enzyme-linked Immunosorbent assay (ELISA).Endothelial function through asymmetric dimethylarginine and Endothelin-1 (ADMA and ET-1), inflammation through soluble CD40 Ligand (CD40L), Vascular and Intracellular adhesion molecules (VCAM-1 and ICAM-1),Interleukin-6 (IL-6) and Myeloperoxidase (MPO).With tissue remodelling via matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1(MMP-9,

MMP-1 and TIMP-1), via commercially available ELISA [for further details see methods Chapter 2.6].

8.3.1.5 **Statistical Analysis**

Data is shown as mean ± standard deviation, unless otherwise stated. Comparisons between the sample sites in the different patient groups were performed by a three-way ANOVA, with multiple comparisons, with post hoc analysis via Bonferroni's multiple comparisons test where necessary. Patient characteristics were compared using students T-test for continuous data or a Fisher's exact test for categorical data. All data was tested for normality by a D'Agostino-Pearsons normality test, and log-transformed as appropriate. All data was analysed using GraphPad Prism Version 5.0 (GraphPad Software) and was normally distributed. Statistical significance was set at $p<0.05$.

8.4 Results

8.4.1 Patients Characteristics

There was a significant difference in the age of the three patient groups, with the AF patients being significantly older to that of both the SVT and the MS patient groups ($p<0.0001$, Table 1). All patients were well matched for gender.

8.4.2 Echocardiographic Characterisations

The patients with MS were found to have structural abnormalities compared to that of the SVT and AF populations. MS patients had

significantly larger LA area as well as RA areas compared to the SVT and AF patients ($p<0.0001$ for both).The LA volume was significantly larger in the MS patients compared to the AF and SVT, with the AF and also having a significantly higher LA volume than the SVT. LA pressures in patients with MS also had significantly higher than that of the AF patients ($p<0.0001$). There were no differences in the ejection fraction between the three patient groups ($p=0.94$) Table 1.

8.4.3 Endothelial Function

ADMA and ET-1 were measured as markers of endothelial function. ADMA levels were significantly elevated at all sampling sites in the AF patient group compared with the SVT and MS patient groups ($p<0.0001$) [Fig 1A]. Post hoc analysis of ADMA showed at all sites SVT and MS had significantly lower levels than the AF. ADMA levels if not differ between the SVT and MS patients at any site (Fig 1 B, C, D). Again there was a significant difference in ET-1 levels between the patients groups ($P<0.0001$), however when assessing ET-1 the MS patients had and significantly higher levels from the SVT and AF patient group, at all sites ($p<0.001$) [Fig 2A]. With post hoc analysis showing that there was no difference in P and RA measurements between the SVT and AF patients, but a significant increase within the LA of AF patients (Fig 2 B, C, D). This was seen despite no difference between the three sites within each individual patient group.

8.4.4 Inflammation

Compared to SVT and MS patients VCAM-1 levels were significantly increased in AF patients at all sample sites [p<0.0001, Fig 3 A]. Post hoc analysis showed that at all sites SVT and MS had significantly lower levels than the AF. There was no difference between SVT and MS (Fig 3 B, C, D). Results for ICAM-1 were similar to VCAM-1. ICAM levels were significantly higher in AF patients compared to SVT and MS patients (P<0.0001, Fig 4A). However, this only seen with a significant increase in the RA and LA of AF patients compared to the SVT and MS patients (SVT vs AF; RA p=0.0009, LA p=0.03, MS vs AF RA p=0.001, LA p=0.004,) [Fig 4 C and D). There was no difference between the SVT and MS patients at any site.

Interestingly, MS patients were found to have significantly increased levels of MPO at all sites (p<0.0001, all sites MPO, Fig 6A). This was significant at all site comparisons between the SVT and AF patients against the MS. However no direct difference between any of the sample sites between the SVT and AF patents was found. IL-6 were also found to be significantly higher at the site comparison (Fig 7A p <0.0001). Post hoc analysis showed that there was significant only at the P sampling site between the MS and AF patients, and the LS between all three patients groups. Consistent with the other inflammatory markers, CD40L was significantly different in site comparison (p=0.002, Fig 5A), however this was only in the LA between the SVT and MS patients P=0.0008, and the MS and AF patients p=0.004 patient groups (Fig 5D).

8.4.5 Tissue Remodelling

Tissue remodelling was measured using MMP-9 and TIMP-1 markers. There was a significant difference between the sample site in MMP-9 levels ($p<0.0001$, Fig 8A) However, this was only significant at the RA and LA when the SVT and MS patients were compared to AF. (Fig 8C and 8D). No difference was seen within Peripheral measurements. TIMP-1 results were similar to that of MMP-9. TIMP-1 levels were significantly higher at all sites ($p<0.0001$). Post hoc analysis showed TIMP-1 levels were significantly higher in the MS patients compared to the SVT and paroxysmal AF patient populations at the peripheral and LA sites (Fig 9B, 9D), although only in the RA between the MS and AF population (Fig 9C).

8.5 Discussion

8.5.1 Major Findings

This study demonstrates that AF and the major valvular substrates for AF, MS affect mechanisms that contribute to thrombus formation this distinct patterns of expression.

(i) AF patients have increased endothelial function, acute inflammation, and tissue remodelling compared to MS and SVT.

(ii) MS patients have increased levels of Inflammation (chronic) and higher levels reverse remodelling factors compared to AF and SVT patient groups.

(iii) LA size and volume were significant larger in the MS patients compared to AF and SVT.

Endothelial dysfunction leading to tissue remodelling is known to be associated with the structural changes which occur in both AF and MS.[112,216] Tissue remodelling is known to occur in MS, with enlargement of the LA a very common consequence of the dysfunctional stenotic mitral valve, leading to fibrosis and therefore electrical remodelling.[31] Endothelial damage or dysfunction may and contribute to this increased risk of thromboembolism mediated through either a prothrombotic or hypercoagulable state.[4] The intra-atrial endothelium could be rendered dysfunctional in AF, by a localised mechanism involving blood flow stasis or turbulent blood flow in the left atrium or perhaps due to turbulent flow and reduction in shear stresses on the atrial wall, or simply by the associated cardiovascular risk factors. With damage to the endothelium creating an environment for platelet activation, inflammation and further fulfilling the Virchow's triad of mechanistic risk factors for clot formation. It has been shown that in AF VWf is associated with both an increased incidence of AF, as well as increasing the likelihood of thrombus formation.[202,220] Additionally ADMA is increased in patients with both paroxysmal and persistent

AF.[222] This is consistent with our finding that patients with AF were found to have significantly higher levels of endothelial dysfunction, at all sites, compared with the SVT and MS patients (Table 2). A further study found that four recognised risk factors for stroke in AF (advancing age, prior cerebral ischemia, recent heart failure, and diabetes) were independently associated with raised plasma vWF showing how many different factors and diseases potentially contribute to thrombus formation.[308]

AF and its major substrates are known to have a significantly high risk of clot formation within the heart which potentially leads to stroke. Valvular AF with the major substrate of MS confer a 17.5 fold higher risk of stroke than the normal population, and 12.5 fold higher than that of non-valvular AF.[9] AF is known to occur in 40-75% of patients who have MS, further reflecting how these two disease states are intertwined.[34] A previous study has suggested that the onset of LA dilatation in MS is the result of an early increase in LA pressure, and that AF, which develops irrespective of the severity of the MS, contributes to a further enlargement of the left and right atria, and thrombus formation.[33] Further to this it has been shown that tachycardia-induced structural remodelling takes place in a different time domain (weeks to months).[117]With myocardial fibrosis shown to be predictive of severe MS, lower ejection fraction, increased pulmonary artery pressure, and poor functional class.[309] All factors which are known to be associated with endothelial dysfunction and can be

predictive of the development of AF. My results clearly show that there is significant enlargement in the LA and RA in MS patients; this coincided with an increase in LA pressure and LA volume [Table 1]. This increase was significant higher than that of the SVT and AF patients.

Thrombotic abnormalities leading to colt formation are commonly seen in patients with MS; it has previously been shown that patients with MS have visualised clot formation within the atria, even when free of AF,[39] mainly through visible LASEC on echocardiography in MS patients.[285,291] This is a similar to AF in where it is known that clot formation occurs almost exclusively within the LA.[11,172,174] Studies have found that factors including platelet aggregation, thrombin-antithrombin III complex and fibrinogen are all increased in MS, shows how MS is altering the thrombotic state independently if AF.[29,40,41] Previous studies that have investigated abnormalities in haemostasis, platelets activation and aggregation, endothelial function and inflammation have all being described in the setting of AF.[55,73,163-165] Our results and other studies show the alteration in LA specific changes which occur differently in AF patients compared to MS patients without AF.[72,75] This study has shown earlier (chapter 7) that platelet reactivity was increased within the LA of AF patients. It is thought that as in AF, the change in hemodynamic factors leading to thrombus formation in MS originates from within the left atrium.[29,35,40] A study by Chen *et al* showed that in MS patients LA platelet activation

was significantly increased compared to the RA and peripheral circulation.[35]

My results show that MS patients without AF, specifically affects LA various haemodynamic factors differently from of patients with AF and SVT (Table 2). This is shown through key markers of platelet activation and inflammation where there was specific increase in CD40L concentrations. This was only increased within the LA of MS patients, but not within the periphery or RA [Fig5C]. CD40L is 95% derived from platelets, however are also is also found on monocytes, macrophages and endothelial cells, and can contribute to thrombus formation through the increased reactivity of platelets and inflammation[310] has been shown to be potential factor in the increase thrombotic risk.[311] Previous platelet specific studies have found this platelet reactivity to be increased in the LA of AF as well as in MS patients, which differ from my results.[35,72,75] This demonstration a need or a further studies aiming to determine specifically how platelet reactivity between AF and MS patients is altered , as platelet reactivity plays such a large role in both disease states, and in clot formation.

Inflammation through the recruitment of leukocytes with the release of reactive oxygen species, cytokines and growth factors, leads to adverse atrial remodelling and suggests that inflammatory pathways are a prerequisite for AF,[164] with leukocyte activation is considered an important inflammatory pathway underlying AF. Biochemical pathways

such as CD40L and P-selectin are considered important mediators of platelet-leukocyte interactions in the setting of AF.[66] The neutrophil/lymphocyte ratio was significantly higher among persons with stroke compared with individuals without stroke showing that inflammation is an emerging marker associated with thromboembolic stroke in non-valvular AF patients.[233] This was further supported by a review showing that inflammatory cytokines, such as interleukins and CRP could be driving the prothrombotic state in AF, but further work is ongoing in this important area.[220]

Inflammation is a robust process mediated by many factors; in this study we found that the disease states of MS and AF have differing effects of the inflammatory pathways. MS was shown to have increased levels of MPO and IL-6, whereas AF had increased levels of VCAM-1 and ICAM-1 [Table 2]. Inflammation is a relative newcomer to the research in AF and thrombus causing stroke and it is now recognised that inflammatory pathways could be also considered as therapeutic targets in an effort to reduce the clinical consequences of thromboembolism and improve outcomes in AF.[66] However inflammation studies of MS patients have shown inconsistent results, a study by Yamamoto *et al* showed that although some markers for coagulation factors were increased in the LA compared to the RA and peripheral circulation, this was not consistent for all markers, or across the sample sites in the patients groups.[40]

MPO is most abundantly expressed in neutrophils and is released in response to tissue injury or infection, these can also be released to the outside of the cell, where they may attack normal tissue and thus contribute to the pathogenesis of disease.[312] MPO and IL-6 were both significantly higher in MS patients at all sites compared to SVT and AF patients. These cells are more likely to be increased in MS due to the pathology of the rheumatic disease which precedes the development of MS. Previously a study defining MPO in determining strokes grouped MS and AF together with other cardiac related causes of stroke, and found that MPO was associated with the extent of brain damage and the functional outcome.[313] MPO in the setting of AF has been determined to be a part of the fibrotic process, and the perpetuation and recurrence of AF.[314-316] IL-6 increases in MS patients compared with SVT and AF patients, is known to be secreted by T cells and macrophages to stimulate the immune response. For example, after trauma or during tissue damage, leading to inflammation.[317]IL-6 is responsible for the attraction of neutrophils, and therefore is linked to the process MPO plays in inflammation in MS patients compared with inflammation in AF. Previous studies have shown that circulating levels of tumour necrosis factor-α and IL-6 were elevated in patients with isolated MS and chronic heart failure of rheumatic origin.[42] Consistent with my results, this was reversed with a percutaneous mitral valvulotomy procedure.[42] Previously IL-6 levels have been found to be higher in all AF patients compared with controls,[65,207] although these studies utilised non-rheumatic low to moderate risk AF patients. This is

the first comparative study to define these factors with a valvular substrate prior to the development of AF (MS) and an AF population.

In contrast to the inflammatory profile of MS, AF was shown to have increased inflammation through adhesion molecules VCAM-1 and ICAM-1. VCAM-1 is expressed on both large and small blood vessels only after the endothelial cells are stimulated by cytokines, whereas ICAM-1 is a cell surface glycoprotein which is typically expressed on endothelial cells and cells of the immune system. When activated, leukocytes bind to endothelial cells via ICAM-1 and then transmigrate into tissue. [318]Previously it has been shown that similar to my results VCAM-1 and ICAM-1 significantly increase during AF (persistent AF patients), reaching highest levels in patients with atrial thrombi.[311] Inflammation has previously been associated the initiation and progression of AF [319] rather than the thrombotic risk, where platelet and endothelial functions have been a main focus. MS studies have previously found that the venous plasma soluble VCAM-1 level of patients with MS was significantly higher than that of healthy volunteers or patients with lone AF.[43,44] The results of this study show a stepwise increase in VCAM-1 concentrations, with MS having significant higher levels than that of SVT and AF patients have higher levels of VCAM-1 than MS and SVT patients. This may be explained by the length of time these patients have AF and comorbidities. The previously mentioned study only included lone AF, where as our patients are considered low

risk however not all are lone AF, which could potentially increase VCAM-1 concentrations.

Structural remodelling of atria tissue is associated the propagation and perpetuation of AF. Atrial tissue remodelling also contributes to the thrombotic risk in AF. The current study used MMP-9 and TIMP-1 assesses tissue remodelling. We found that AF and MS patient have differing results for the two markers (Table 2). MMP-9 levels were significantly higher in the RA and LA of AF patients compared with the SVT and MS patient groups, with no difference in the peripheral samples. This is consistent with a previous study finding that pre-cardioversionMMP-9 was higher and TIMP-4 lower in AF patients.[229] MMP's are a part of the early remodelling phase. TIMP's are more involved in the chronic remodelling process where they work against most MMP's.[320] TIMP-1 levels were higher in the MS patients compared with SVT and AF patient groups. These results are then consistent with the disease progression of our AF and MS patients. As shown in previous studies where MS patients have the collagen volume fraction of fibrosis significantly increased in MS, with other studies showing increased fibrosis, and therefore with this chronic remodelling MS patents have higher levels of TIMP-1.[286,287] This was found to be consistent with the echocardiographic measurements where it was found that there was difference in the LA and RA area as well as LA volume and LA pressure between the three groups of patients (Table1). With the MS patients having significantly higher levels than that of the

SVT and AF patients. It is known that with MS and AF there is a significant increase in LA size.[31,182] This was also a predictors of AF in MS as is has been shown that patients with MS with a large LA diameter (P<0.0001) were more likely to develop AF.[321] The LA pressure was not available for SVT patients; however the patients with MS had a significantly higher LA pressure than that of the AF patients (p<0.0001). It is known that significant structural changes occur in MS patients particularly within the LA. This result is consistent with previous studies.[35-37] LA structural changes result in LA electrical remodelling believed to be the origin of the progression from MS to valvular AF.[31,36]As MS is a chronic condition and the changes which occur in the LA size and function occur earlier in the disease it was not perhaps surprising that these levels of remodelling were found as the changes may have already occurred and the atria become chronically remodelled and fibrotic. Conversely AF patients would be more likely to be in an acute remodelling phase of their disease, consistent with higher levels of MMP-9. This has been shown in previous studies where elevated levels of MMP-9 are independently associated with increased risk of AF.[322] Originally endothelial function was believed to be one of the largest contributors to the thrombotic process in AF, due to the structural changes which occur, as well as the abnormal or fibrillating atrial wall, as well as the alteration in blood flow. Not surprisingly when compared to SVT patients markers of endothelial dysfunction were significantly increased in AF patients at all sample sites. However quite surprisingly, the AF patients had a significant

increase in endothelial dysfunction at all site compared with MS patients. With many previous studies having linked endothelial dysfunction and AF.[4,165,194,196,211,308,323] This chronic vs. acute changes is also shown through the remodelling markers.

8.6 Limitations

The current study has several limitations. Although we demonstrated the expression profile of thrombogenic markers differs between MS and AF patients the relative contribution and distribution of these markers to atrial thrombus formation in these disease states is unknown. It was not possible to measure platelet function as part of the study. Abnormalities in platelet function are involved in the thrombogenic processing in AF,[72,75] and also MS,[35] though there is no data with a direct comparison.

8.7 Conclusion

The current study showed that AF and its valvular AF substrate mitral stenosis (MS) have two significantly different mechanisms of atrial thrombus formation. AF was shown to have increased endothelial dysfunction and Inflammation whilst MS has significantly higher levels of other inflammatory markers and remodelling if the atrial structure. Further work to explore the contribution of these markers to atrial thrombus formation in AF and MS is needed.

Table 1

Patient characteristics for all SVT, MS and AF patients

Characteristics	SVT N= 52	MS N= 59	AF N= 55	P value
Age (years)	38.6 ± 11.6	32.6 ± 8.6	59.4 ± 13.6	<0.0001
Sex (M: F)	30 : 22	27 : 31	21 : 34	0.12
Echocardiography Characteristics				
LA Area (cm^2)	25.0 ± 9.5	60.4 ± 11	23.2 ± 9.4	<0.0001
RA Area (cm^2)	19.3 ± 4.5	46.1 ± 7	18.6 ± 6.0	<0.0001
LA volume	34.7 ± 6.9	85.1 ± 35	62.9 ± 27.8	<0.0001
LVEF (%)	61.6 ± 9.1	61.2 ± 7.5	60.8 ± 8.9	0.94
LA Pressure	not recorded *	22.8 ± 6.9	7.3 ± 4.8	<0.0001

* LA pressures are not routinely recorded during the SVT electrophysiology procedure; therefore we do not have this for SVT patients.

Table 2: summary of results

Thrombogenic Markers	Supraventricular Tachycardia	Mitral Stenosis	Atrial Fibrillation
ENDOTHELIAL DYSFUNCTION			
ADMA	↔	↔	↑↑↑
ET-1	↔	↑	↑↑↑
INFLAMMATION			
CD40 Ligand	↔	↑↑↑ (LA)	↔
VCAM-1	↔	↑	↑↑↑
ICAM-1	↔	↔	↑↑↑
IL-6	↔	↑↑↑	↔
MPO	↔	↑↑↑	↔
TISSUE REMODELLING			
MMP-9	↔	↔	↑↑↑ (RA/LA)
TIMP-1	↔	↑↑↑	↔

Figures

All graphs for each marker will be shown per site, with SVT patients in White, MS in Grey and AF in Black.

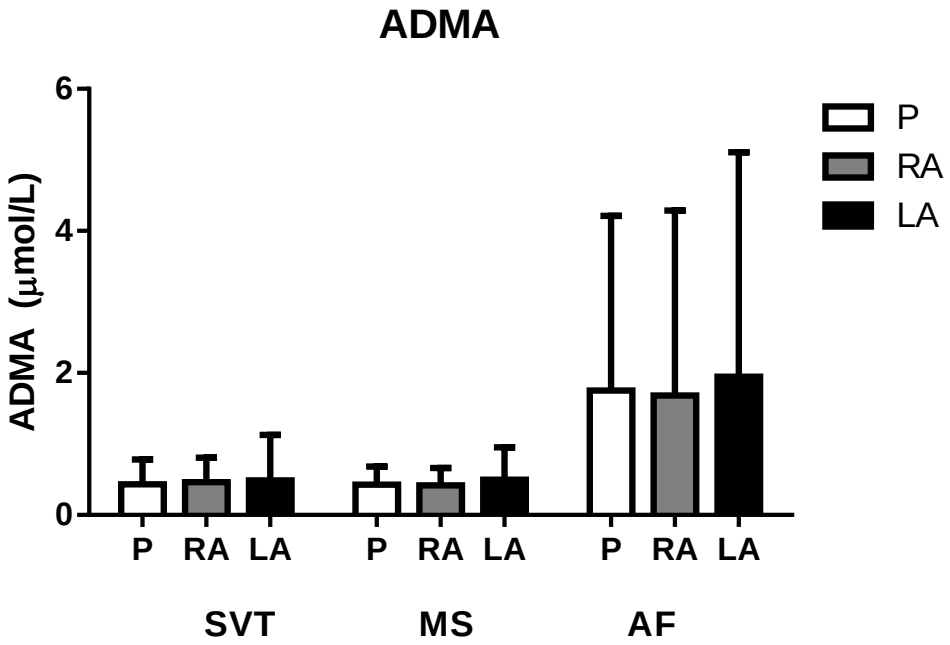
Figure 1: Endothelial function: ADMA

Patients with AF has significantly increased levels of endothelial dysfunction through ADMA at all sample sites compared to both the SVT and MS patients ($p<0.0001$, at all sites).

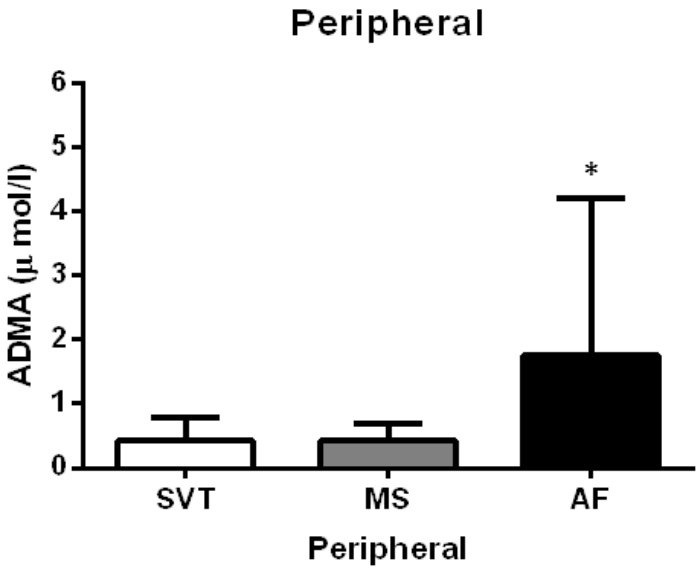
Patient and site interaction ($p=0.9$)

Post hoc analysis showed at all sites SVT and MS had significantly lower levels than the AF. However not between the SVT and MS patients at any site.

A.

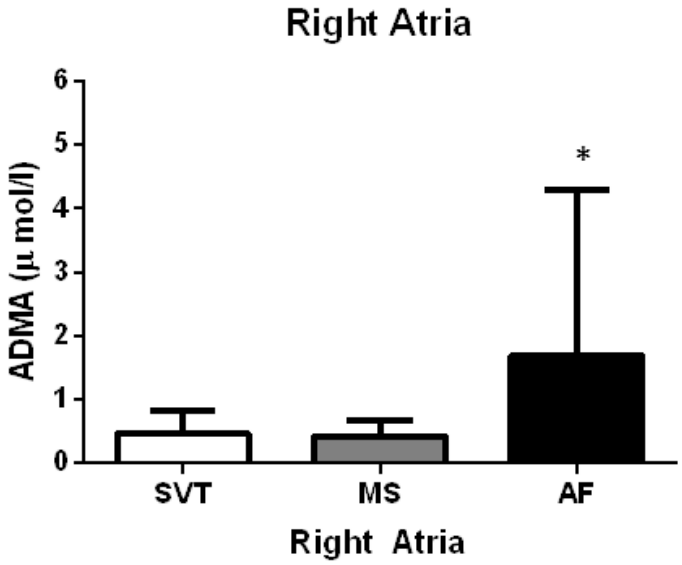


B.



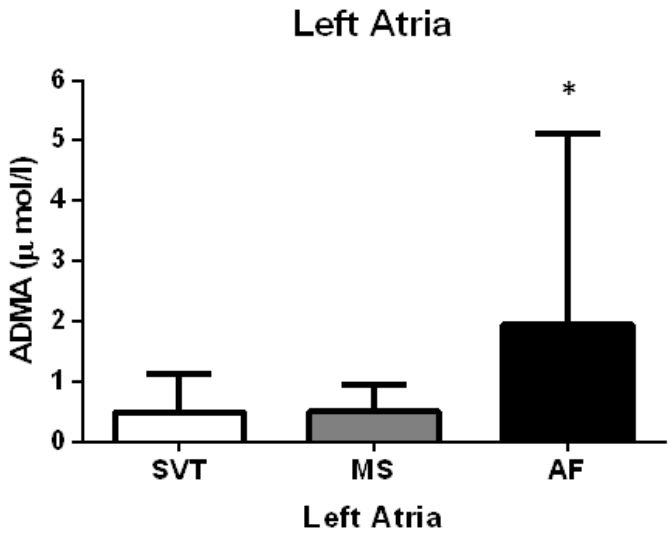
SVT vs AF: p=0.0006, MS vs AF: p=0.0005

C.



SVT vs AF: p=0.001, MS vs AF: p=0.0001

D.



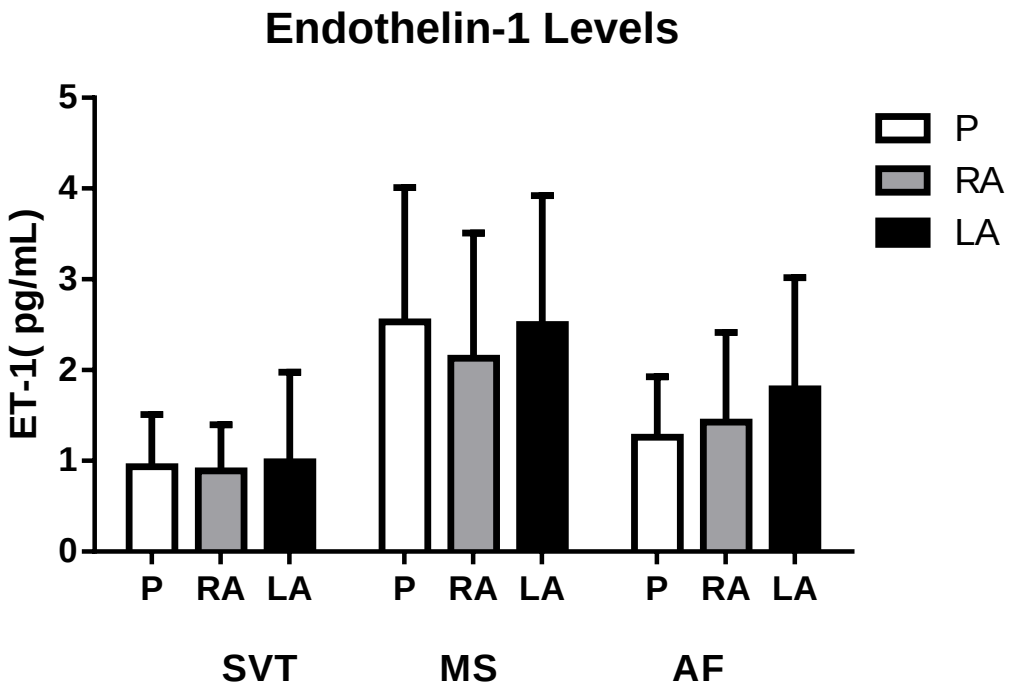
SVT vs AF: p=0.0001, MS vs AF: p=0.0001

Figure 2: Endothelial function: ET-1

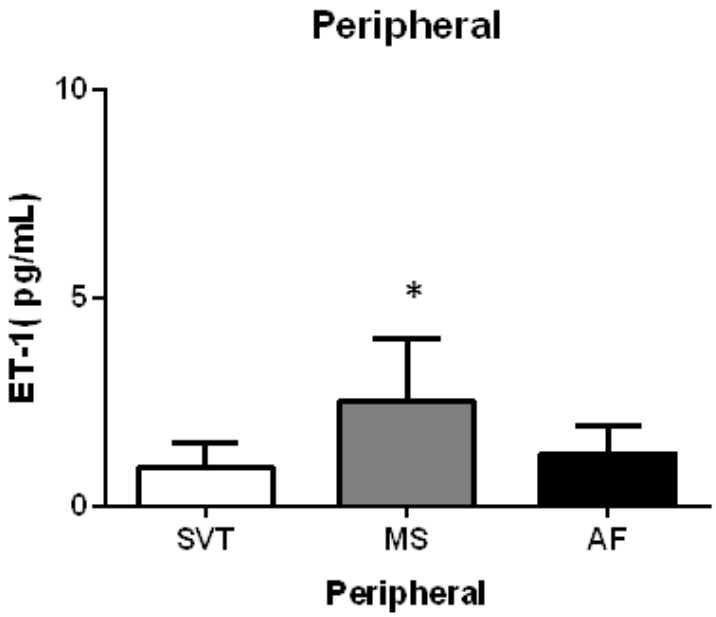
There was a significant difference between the patients groups (P<0.0001), however when assessing ET-1 the MS patients had and significantly higher levels from the SVT and AF patient group, at all sites (p<0.001) [Fig: 2A, B, C]. Patients site interaction (p=0.4)

Post hoc analysis showed that there was no difference in P and RA measurements between the SVT and AF patients, but a significant increase within the LA of AF patients.

A.

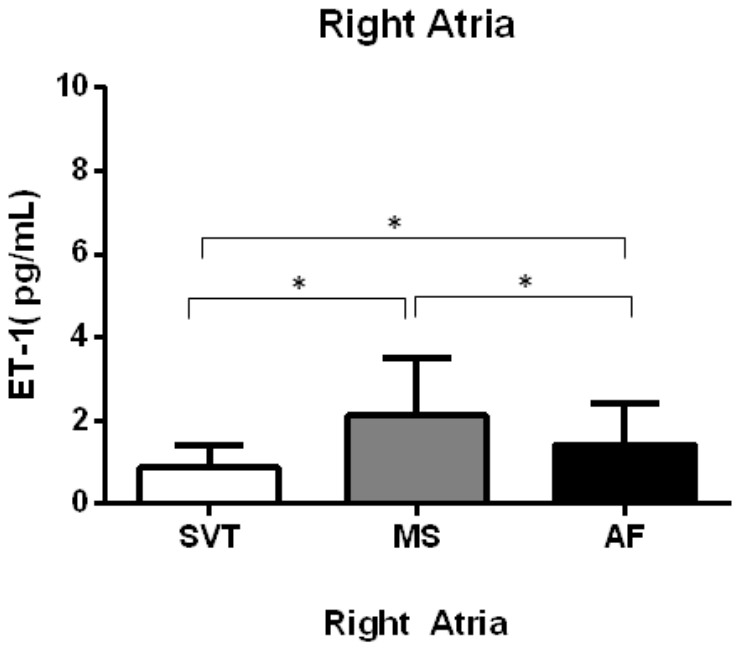


B.



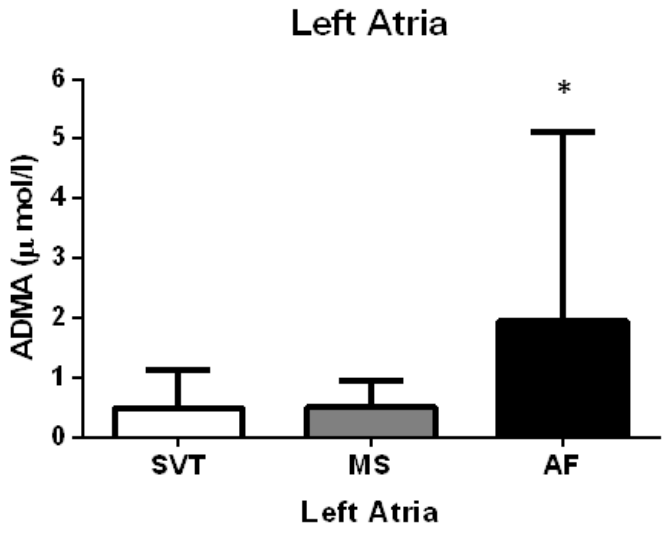
SVT vs MS: p<0.0001, MS vs AF: p<0.0001

C.



SVT vs MS: p<0.0001, SVT vs AF: p=0.1, MS vs AF: p=0.02

D.



SVT vs MS: p<0.0001, SVT vs AF: p=0.008, MS vs AF: p=0.02

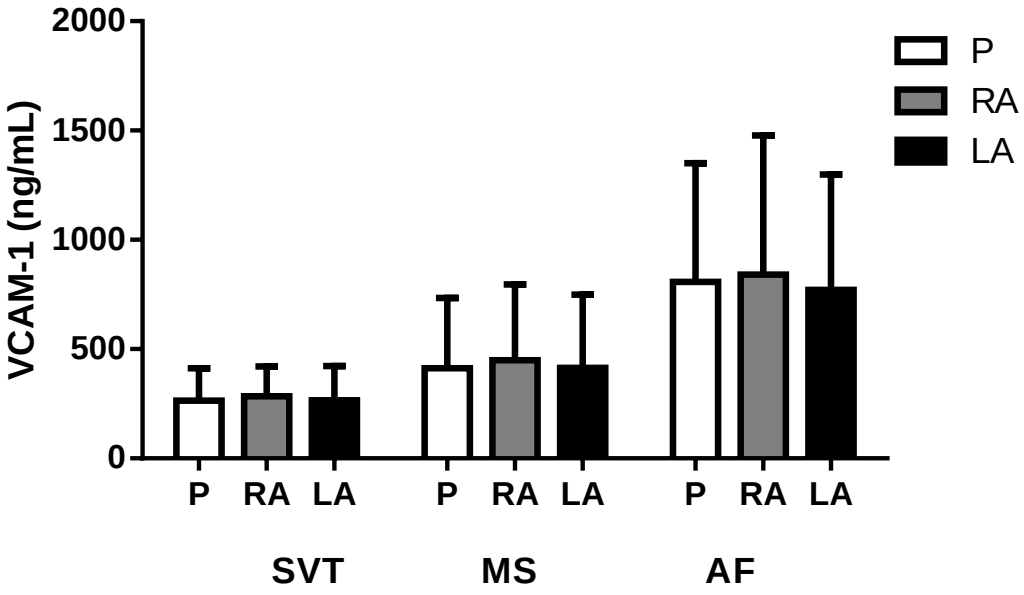
Inflammation

Figure 3: Inflammation: VCAM-1

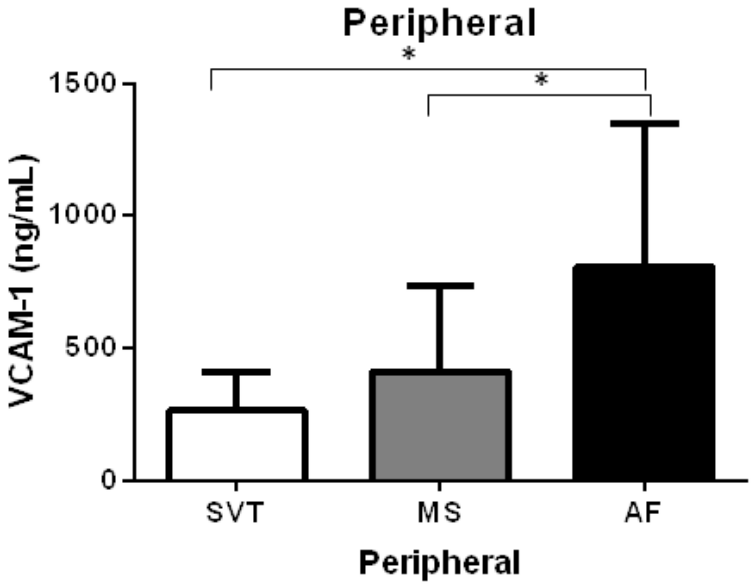
Inflammation was significantly increased in AF patients at all sample sites when measured through VCAM-1 concentration [p<0.0001, Fig3 A].patients site comparison (p=0.9).

Post hoc analysis showed at all sites SVT and MS had significantly lower levels than the AF. However no between the SVT and MS patients at any site. (3B, C,)

A.

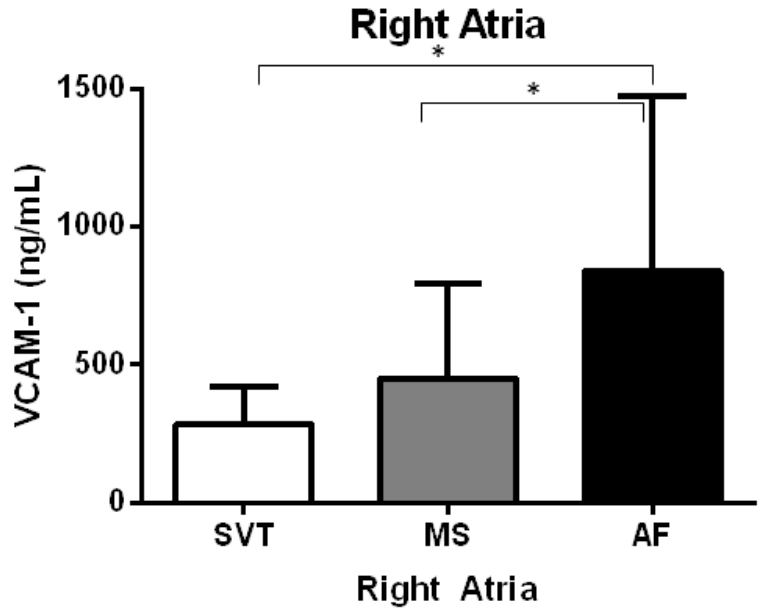


B.



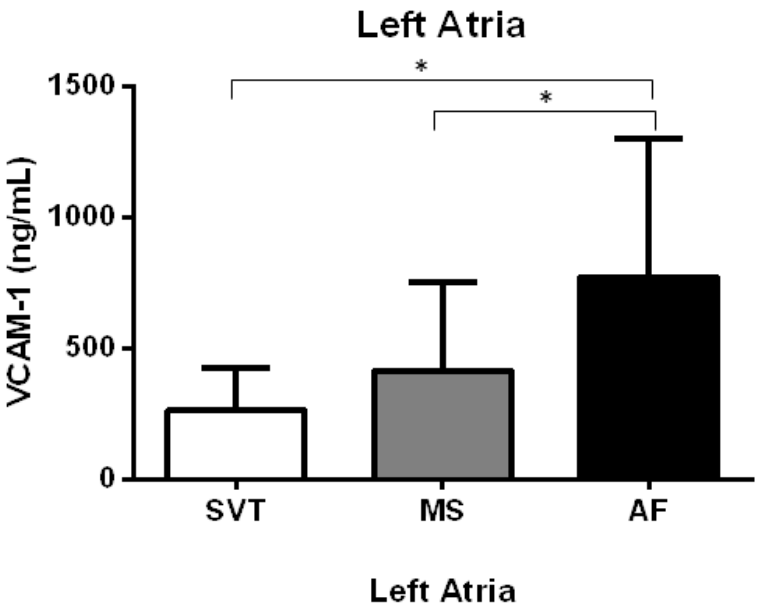
SVT vs AF: p<0.0001, MS vs AF: p<0.0001

C.



SVT vs AF: p<0.0001, MS vs AF: p<0.0001

D.



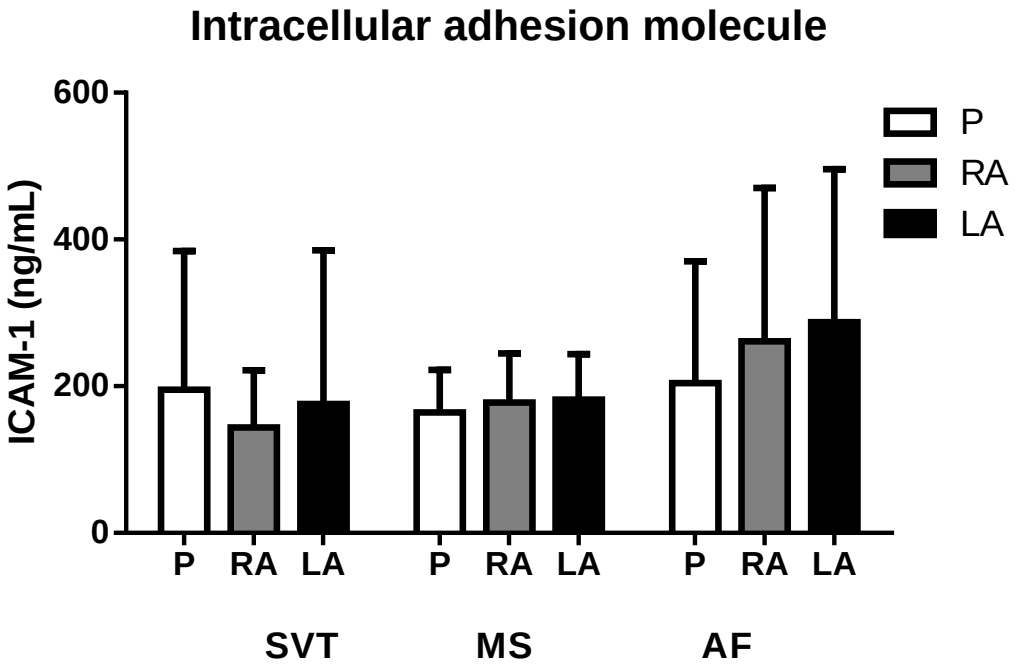
SVT vs AF: p<0.0001, MS vs AF: p<0.0001

Figure 4: Inflammation ICAM-1

ICAM-1 concentrations in the AF patients; however, this only seen with a significant increase in the RA and LA of AF patients compared to the SVT and MS patients (SVT vs AF;RA p=0.0009, LA p=0.03, MS vs AF RA p=0.001, LA p=0.004,) [Fig 4 B, C).patient site comparison (p=0.1)

With further post hoc analysis there was no between the SVT and MS patients at any site.

A.



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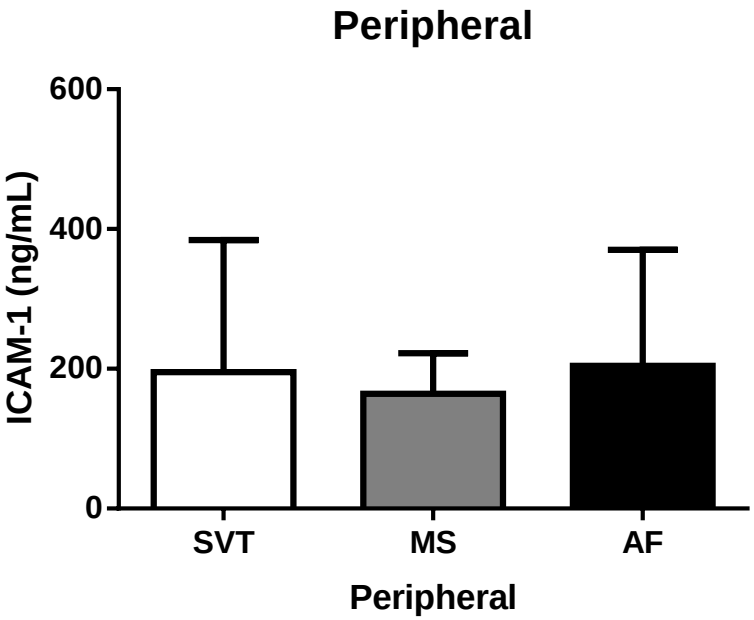
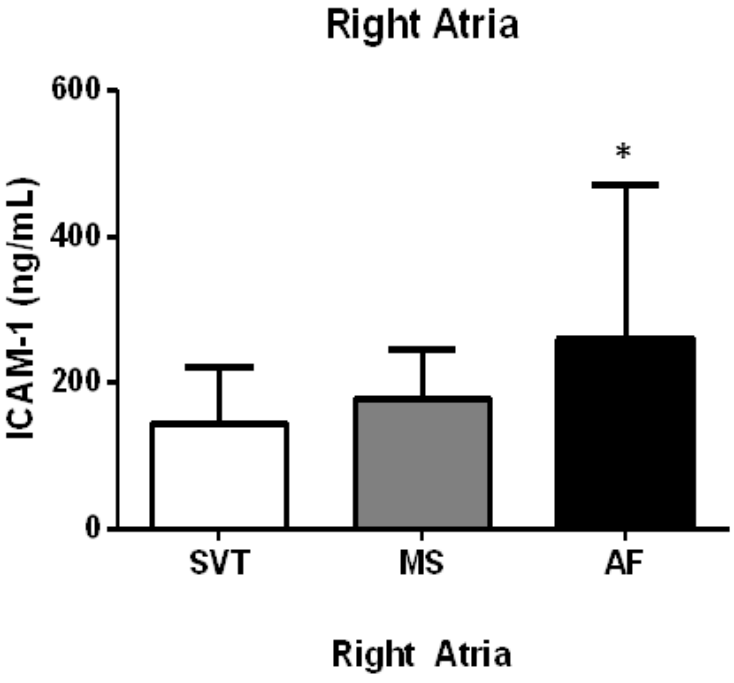
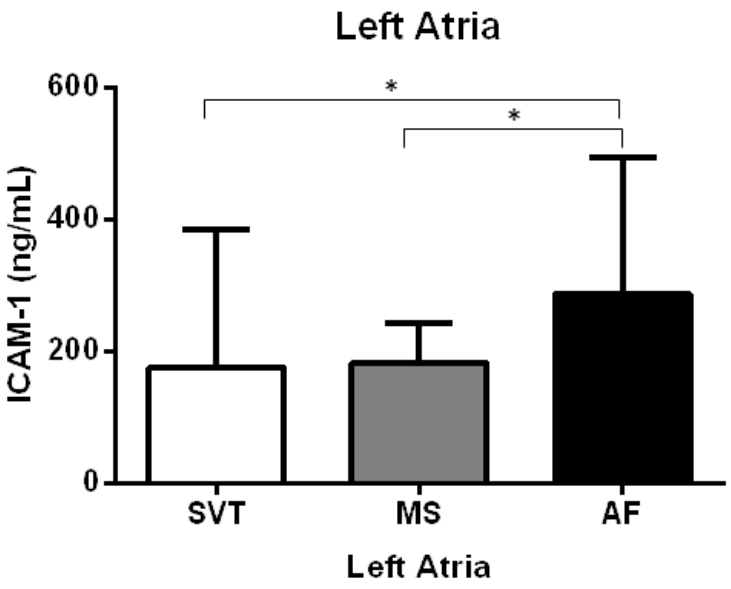


C.



SVT vs AF: p=0.0009, MS vs AF: p=0.03

D.

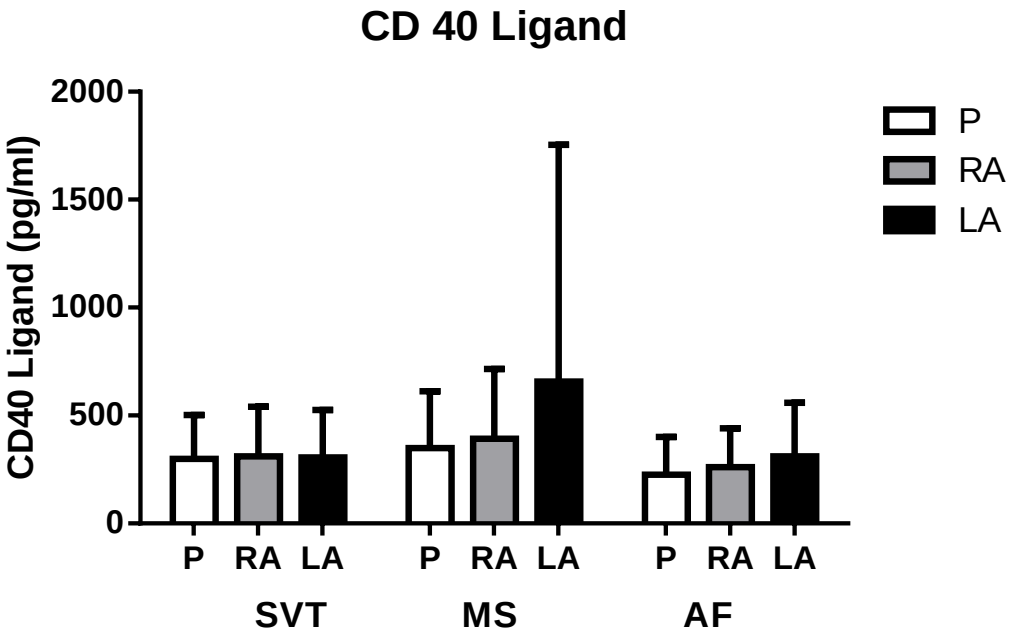


SVT vs AF: p=0.0001, MS vs AF: p=0.004

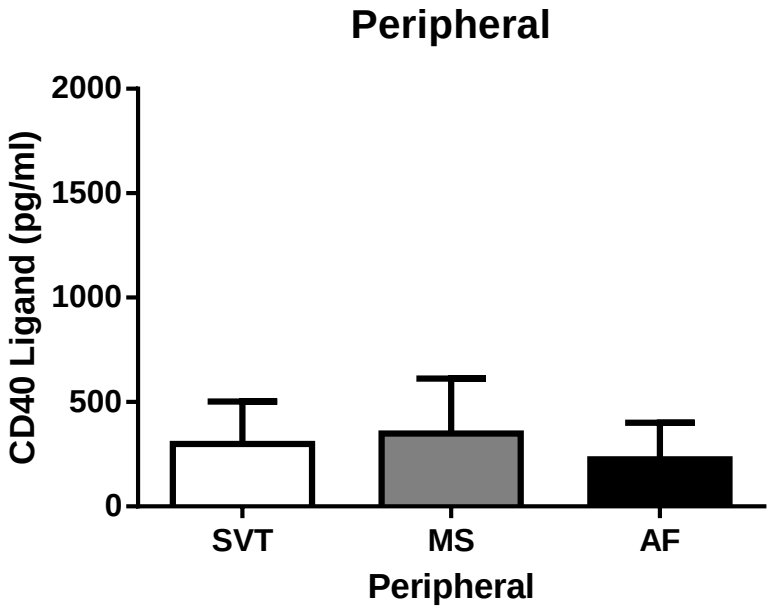
Figure 5: Inflammation CD40 Ligand

There was a significant increase in CD40L levels in the LA of MS patients compared to the SVT and AF patents. CD40L was significantly different in site comparison (p=0.002, fig 5), however this was only in the LA between the patient groups (Fig 5C p<0.001).patient site comparison (p=0.2)

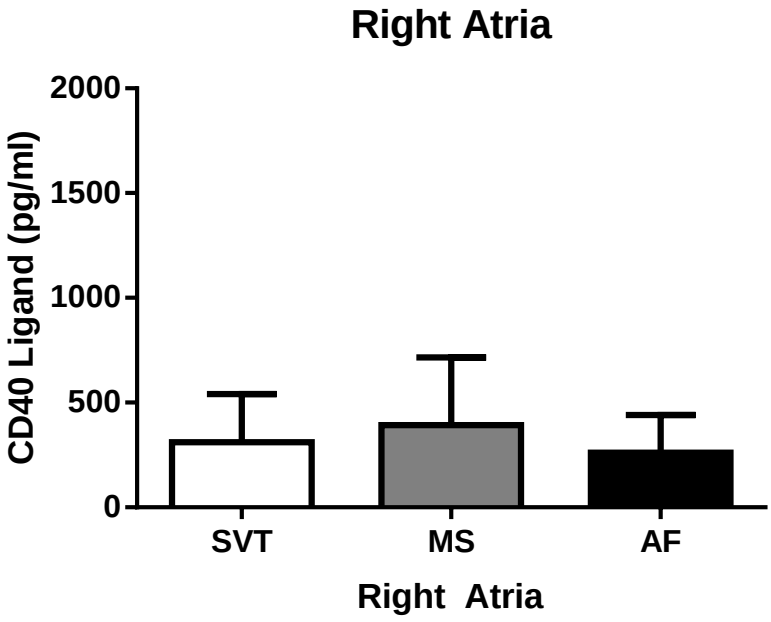
A.



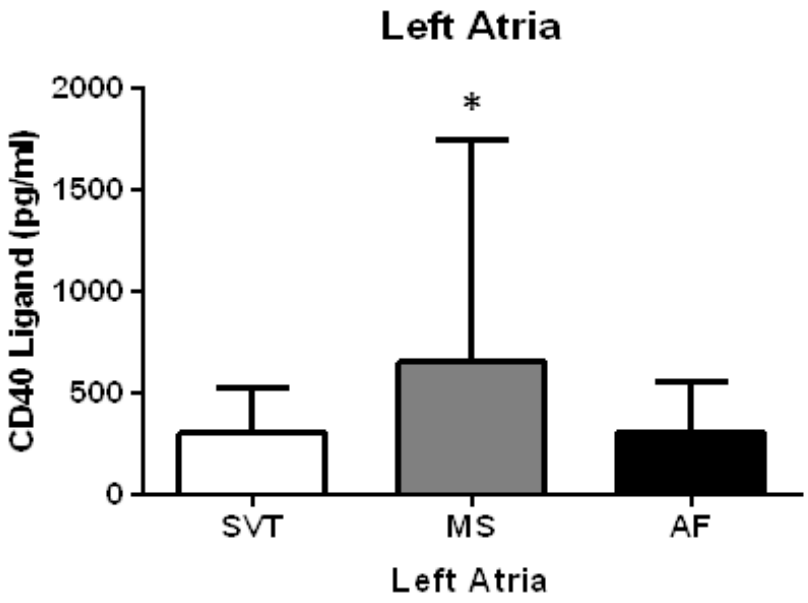
B.


Peripheral
2000
1500
1000
500
0
CD40 Ligand (pg/ml)
SVT
MS
AF
Peripheral

C.


Right Atria
2000
1500
1000
500
0
CD40 Ligand (pg/ml)
SVT
MS
AF
Right Atria

D.

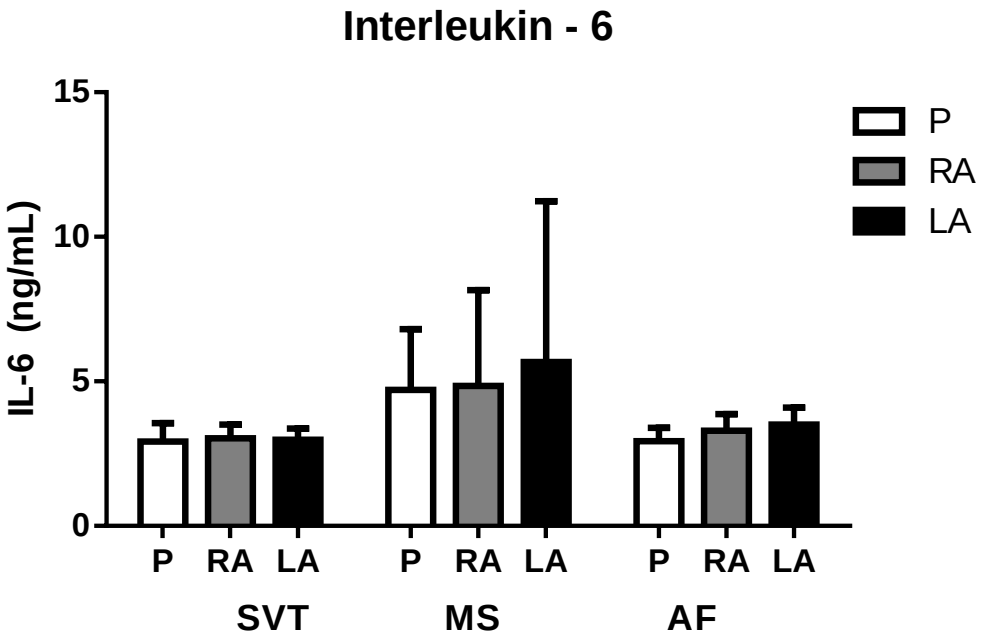


SVT vs MS: p=0.008, MS vs AF: p=0.004

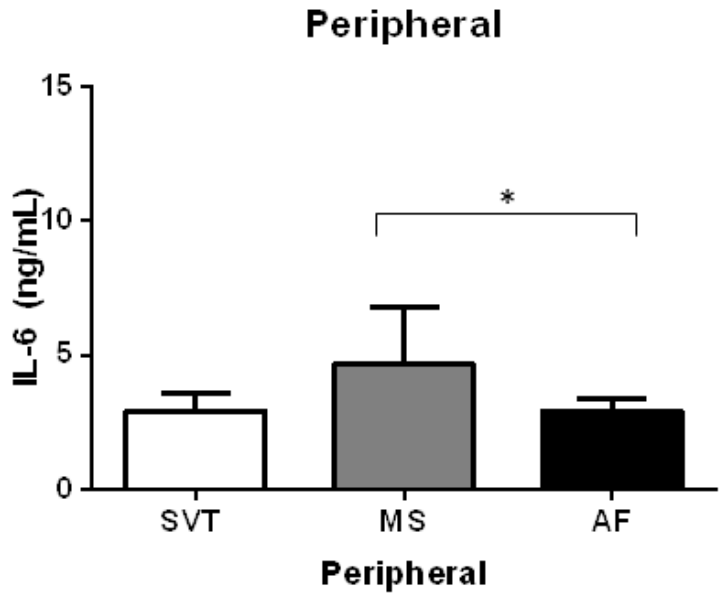
Figure 6: Inflammation Interleukin – 6

IL-6 measurements were also found to be significantly higher at the site comparison (Fig 7 p <0.0001). patient and site comparison (p=0.9) With post hoc analysis showing that whish was only significantly higher P between the MS and AF patients, and was significantly increase in the LA of MS patient groups over both AF and SVT.

A.

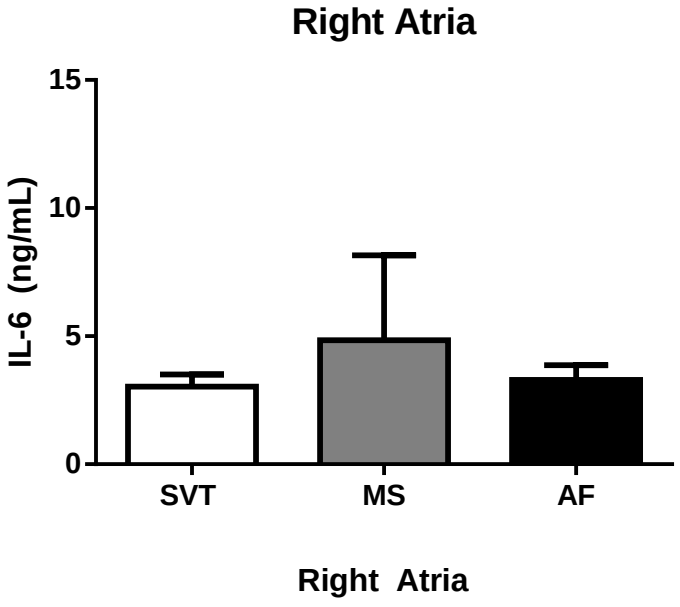


B.

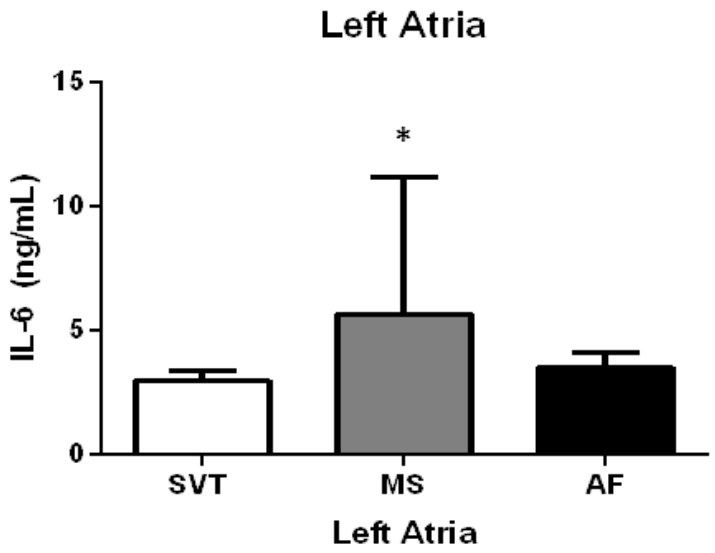


MS vs AF: p=0.04

C.



D.

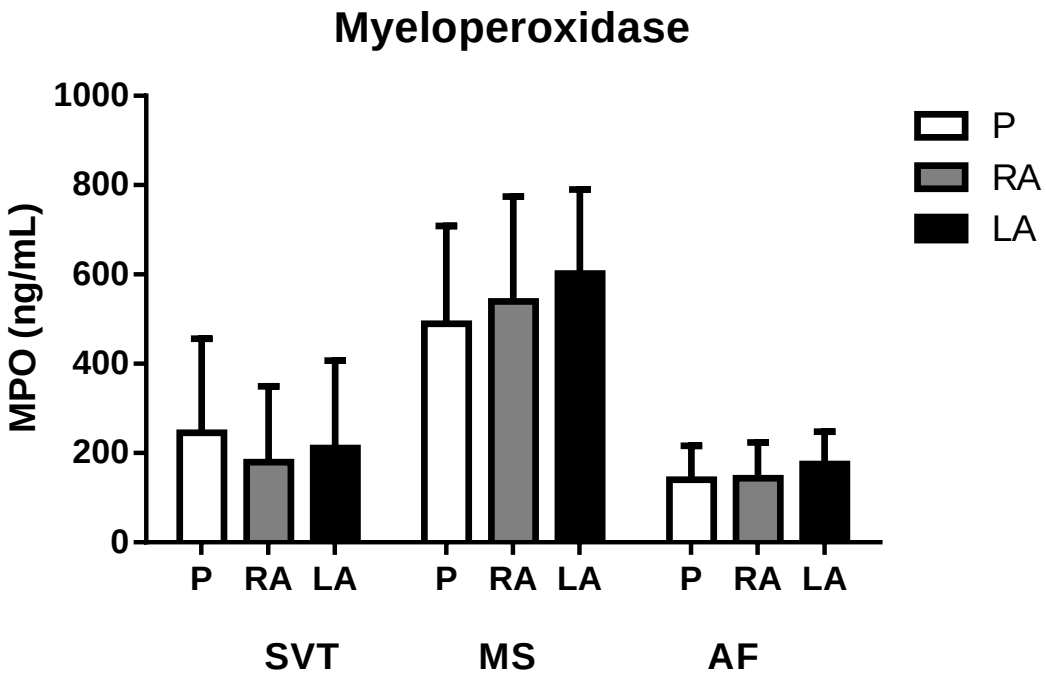


SVT vs MS: p=0.01, MS vs AF: p=0.009

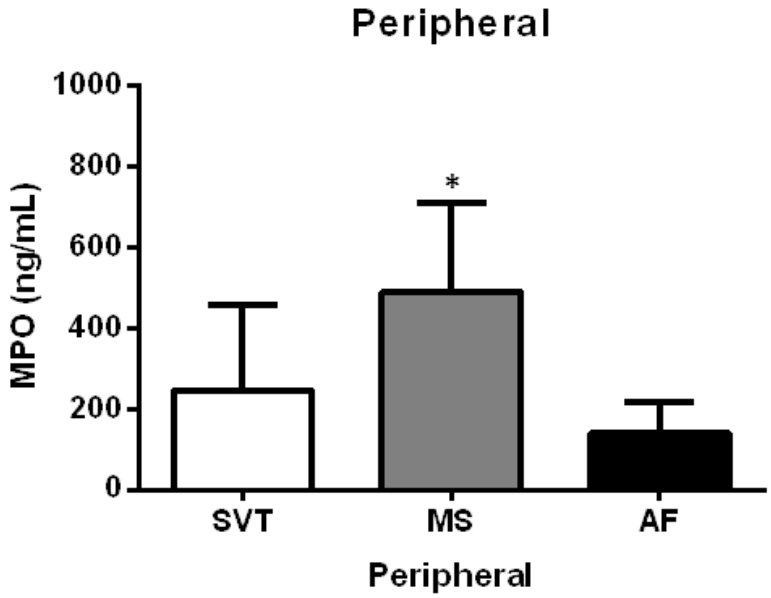
Figure 7: Myeloperoxidase (MPO)

MS patients were found to have significantly increased levels of MPO at all sites ($p<0.0001$, all sites MPO, Fig A). This was significant at all site comparisons between the SVT and AF patients against the MS. Patient and site comparison ($p=0.1$). However no direct difference between any of the sample sites between the SVT and AF patients was found.

A.

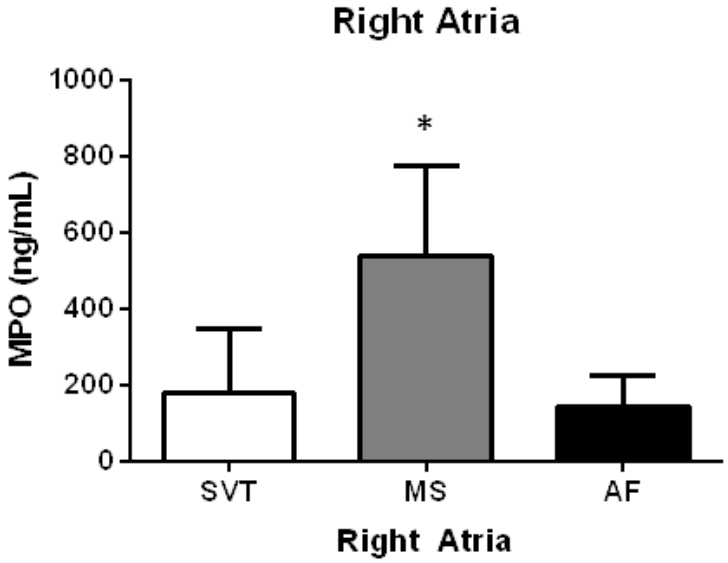


B.



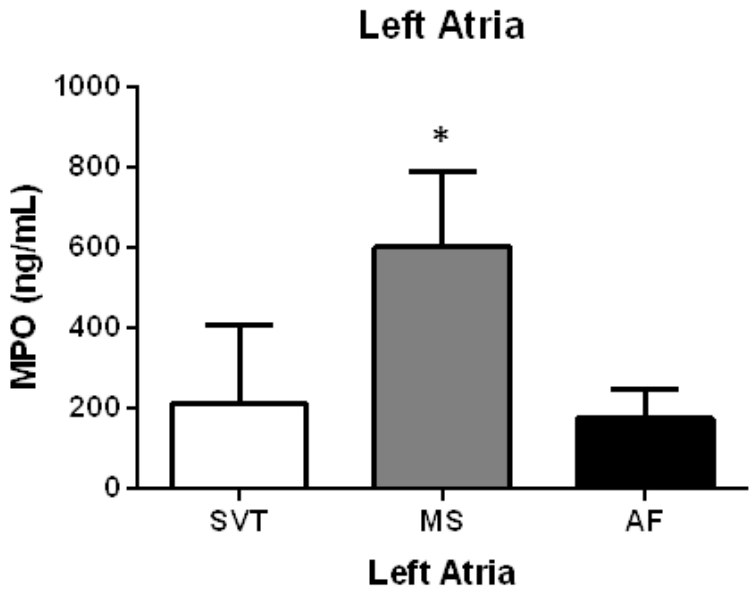
SVT vs MS: p>0.0001, MS vs AF: p>0.0001

C.



SVT vs MS: p>0.0001, MS vs AF: p>0.0001

D.

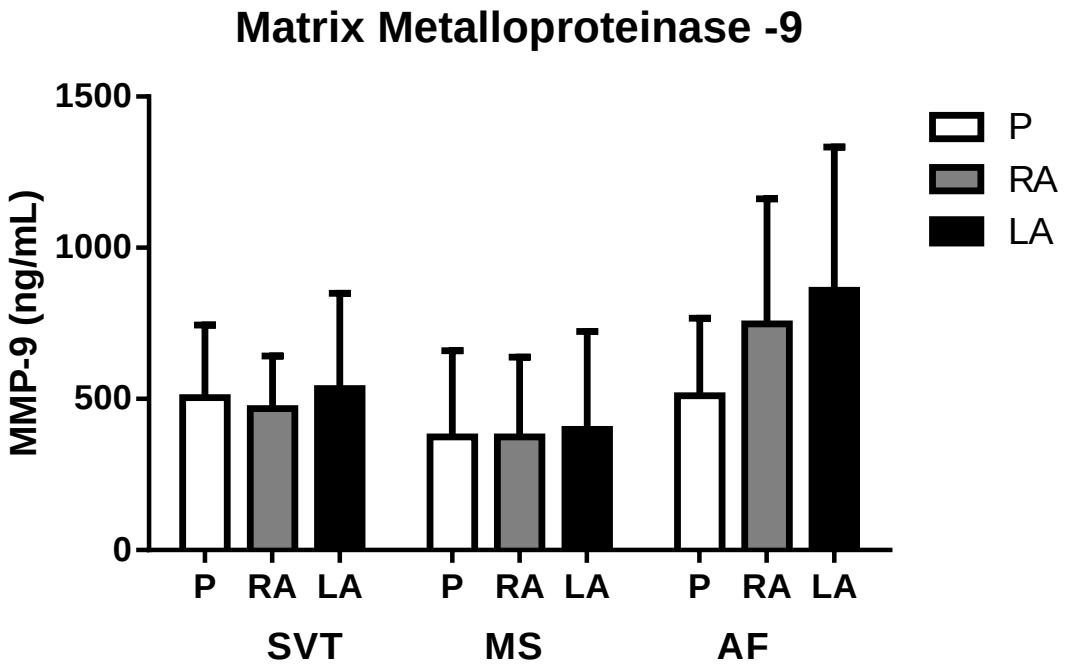


SVT vs MS: p>0.0001, MS vs AF: p>0.0001

Figure 8: Tissue remodelling MMP-9

MMP-9 levels were significantly increased within the RA and LA of patients with AF compared with that of MS and SVT patient groups (Fig 8B; RA and 8C: LA $p<0.0001$), with no difference seen within Peripheral measurements. Patient and site comparison ($p=0.08$)

A.



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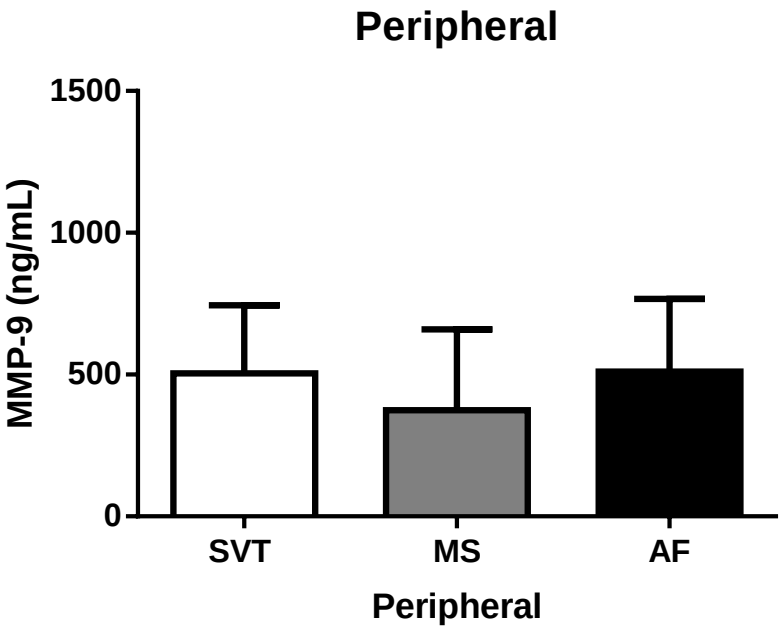

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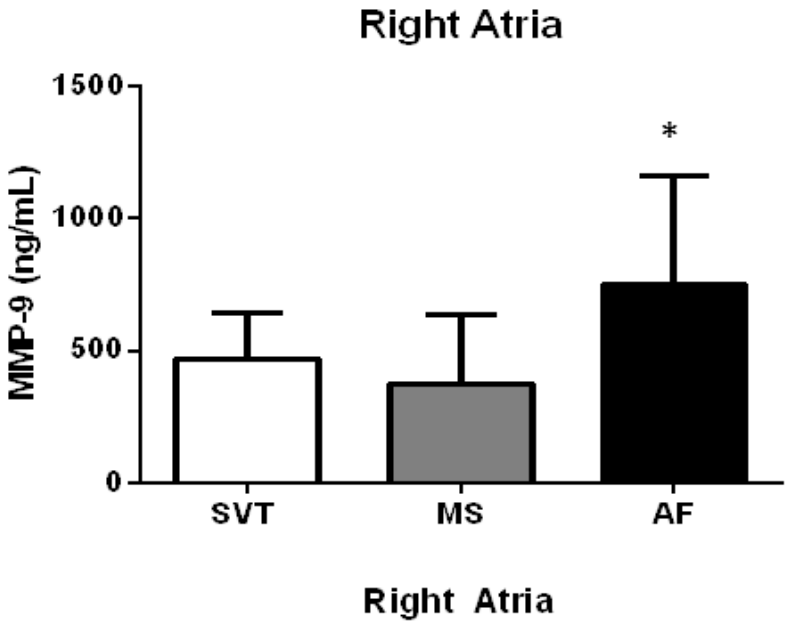
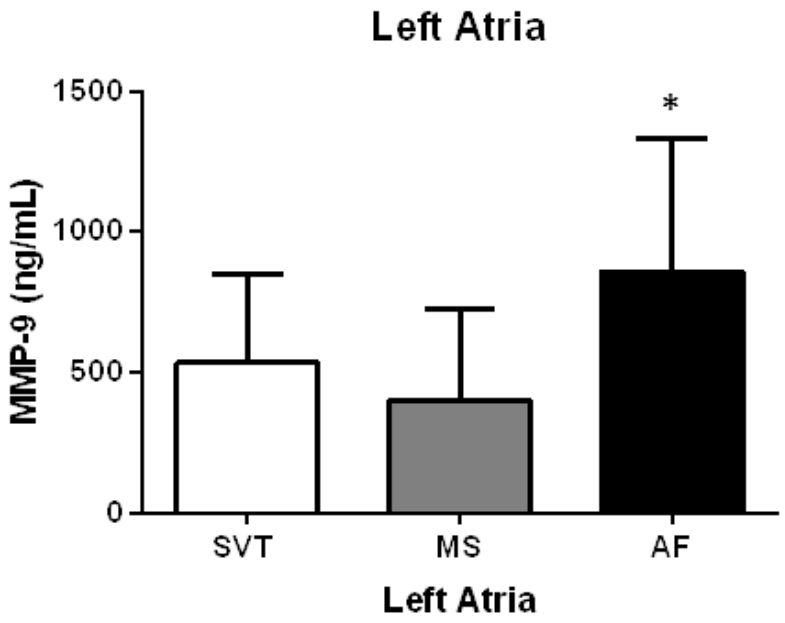


SVT vs AF: p=0.02, MS vs AF: p<0.0001

D.

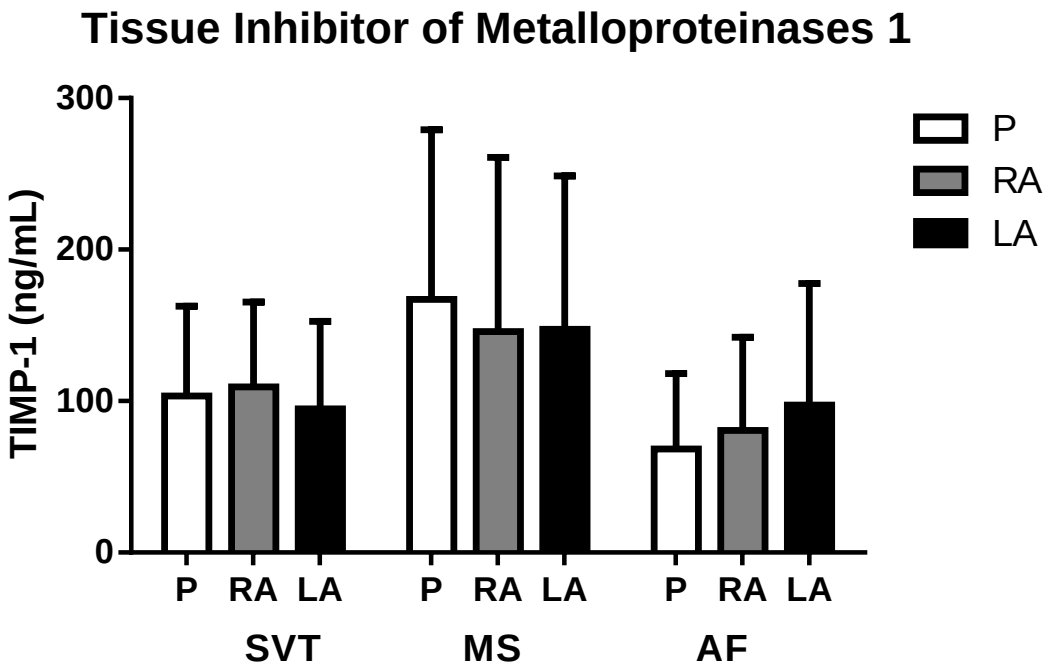


SVT vs AF: p=0.007, MS vs AF: p<0.0001

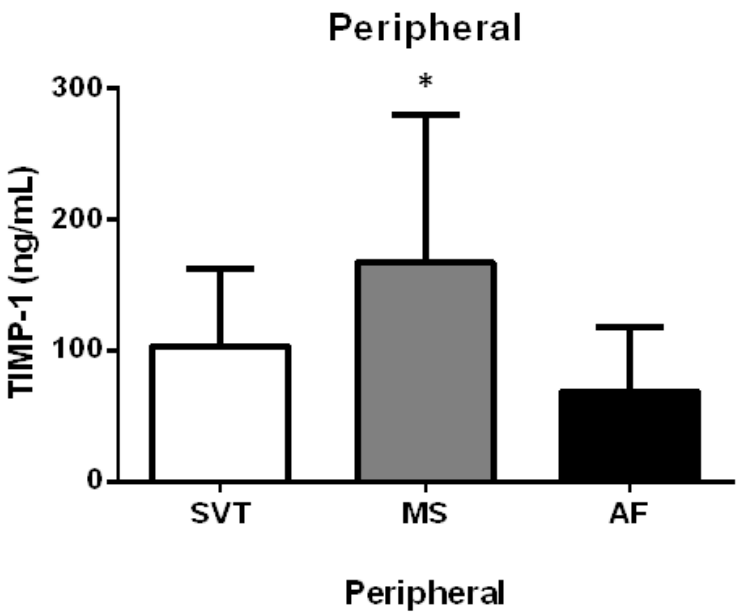
Figure 9: Tissue Remodelling TIMP-1

TIMP-1 results show that there was a significantly higher amount of remodelling at all sites ($p<0.0001$) in the MS patients compared to the SVT and paroxysmal AF patient populations at the peripheral and LA sites (Fig 9B, $p<0.0001$ and Fig 9D $p=0.003$ respectively), although only at the RA between the MS and AF population (Fig 9C). Patient and site comparison ($p=0.4$)

A.

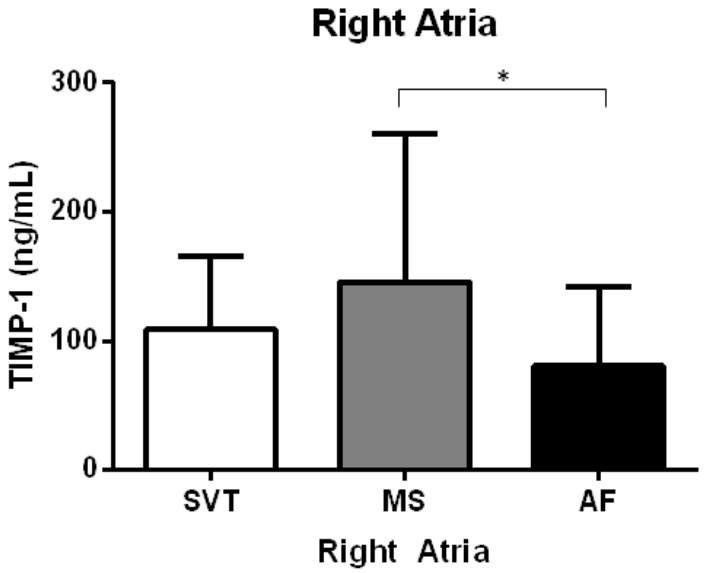


B.



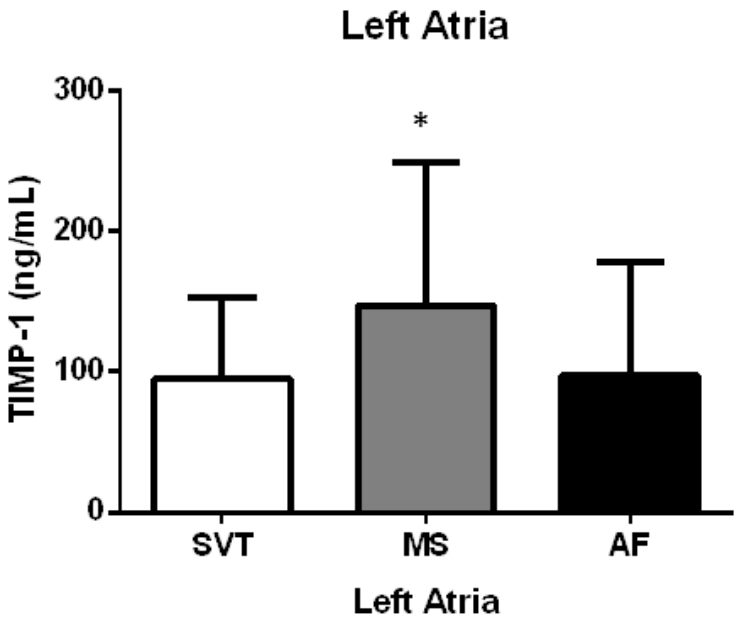
SVT vs MS: p=0.003, MS vs AF: p<0.0001

C.



MS vs AF: p=0.003

D.



SVT vs MS: p=0.02, MS vs AF: p=0.04

9 CHAPTER NINE

Discussion and Limitations

9.1 Discussion

AF is the most common atrial arrhythmia affecting the global population.[2] Currently 1 in 5 people over the age of 80 has AF and the incidence is progressively increasing with the prediction that by 2050 over 15 million people in the United Stated alone will be affected by AF.[3] Despite there being a large amount of research into AF and the risk of stroke there are still large gaps in our knowledge of the mechanisms behind thrombus formation.

This thesis investigated the mechanisms involved in atrial thrombus formation in atrial fibrillation (AF) and its valvular substrate mitral stenosis (MS). These studies have specific emphasis in highlighting the alterations in thrombogenic and haemostatic markers within the left atrium (LA), where thrombus formation is primarily seen in these patients. We also utilised a control population of patients with a structurally normal heart with a history of supraventricular tachycardia (SVT), where direct RA and LA comparison were able to be made. Throughout the course of the thesis AF and MS, as high risk thrombotic populations were compared to the control group to determine if specific haemostatic parameters were altered. In addition, the expression profile of haemostatic markers was compared between the AF and MS group.

The control population with SVT showed no regional differences in markers of thrombogenesis, (**chapter 3**) demonstrating that the normal heart has stable haemostatic function between the arterial and venous circulations and hence no predisposing thrombogenic properties. Markers for endothelial function, inflammation and atrial remodelling did not differ between the periphery, RA nor LA. This study also found there was no ethnic difference in the thrombogenic properties (Inflammation, endothelial function or tissue remodelling) between the Caucasian (Australia) and India populations with SVT (**chapter 4**). Although previous studies showing differences in the rate of cardiovascular related diseases and risk factors between the two ethnically different populations. This is one of the first studies to analyse the differences in markers of endothelial function, inflammation and remodelling in these different two ethnicities in a group of patients who have structurally normal hearts. This provides further evidence that despite the electrical changes which occur during the initiation of SVT, it does not alter the normal function of the circulation or thrombotic tendencies.

AF is classified as valvular and non-valvular. This is defined by whether the valves within the heart are affected as part of the primary disease leading to AF.[20] The term valvular AF is used to imply that AF is related to valvular disease (predominantly mitral stenosis).[21] In contrast, non-valvular AF, the most common form of AF which is caused by

progression of atrial remodelling (structural or electrical) by many other substrates.

The studies included in this thesis which examined thrombogenic properties within the atria of a valvular substrate (MS) are some of the first to fully describe mechanistic alterations in this population (Chapter 5 and 6). This study has found (**chapter 5**) that patients with MS in have increased inflammation within their LA, seen with an increase in MPO levels. Previous studies have found an increase in platelet reactivity in MS; our results for CD40L were in keeping with this however this did not quite reach significance. Consistent with previous studies, this study has also shown that MS significantly increases the LA area and volume measurements, providing a potential pro-thrombotic and pro-arrhythmic environment. This data suggests that indeed MS patients may have increased risk of thrombus formation, but at this late stage of MS disease process when these patients were sampled; I was unable to isolate any blood abnormalities in LA.

Further to this, this study also found that following balloon mitral valvuloplasty (BMV)procedure (**chapter 6**) MS patients have increases in inflammation through MPO and IL-6 at the end of procedure (End) and 24 hours following, with a decrease in endothelial dysfunction through ET-1 seen at 24 hours following BMV. These results are consistent with previous studies of changes post BMV.[296] This study also further showed interesting that there were no changes in markers

of tissue remodelling at the end of procedure at any of the sample sites, and at 24 hours following BMV, despite a decrease in LA size, volume and pressure.

In addition to valvular AF we assessed the LA thrombogenic properties in non-valvular AF (**chapter 7**). The mechanisms of the thrombogenesis in AF patients have previously been explored, with the creation of the Virchow's triad explaining the many various components of thrombus formation within the LA. This study was able to redefine how each of the markers of thrombogenesis was altered within the LA, RA and peripheral circulation of AF patients, showing that consistent with previous reports AF patients have increased levels of platelet reactivity, endothelial dysfunction and inflammation in the LA compared with the peripheral circulation. In addition, non-valvular AF patients have increased LA area, volume and diameter. These abnormalities are associated with significant changes in plasma marker of tissue remodelling within the LA. These findings provides further evidence that the basis for LA specific thrombus formation in AF patients is predominantly due to changes occurring (in haemostatic markers) specifically within the LA.

The culmination of these studies (**chapter 8**) allowed comparison of the levels of inflammation, endothelial function and structural remodelling within a select group of non-valvular AF patients together with one of its major valvular substrates (MS) against a control population. It observed

that within the AF population there is increased thrombogenic markers within the atria compared to the peripheral circulation. Atrial platelet reactivity measured by p-selectin is consistent with previous findings [Chapter 7, Fig: 1A],[75,238] where platelet reactivity was significantly increased within the LA of AF patients compared with that of peripheral and RA sites. This was also consistent for endothelial function (ADMA) and Inflammation (ICAM-1 and IL-6), where these markers were significantly increased in the LA compared with periphery. These results further supports the likelihood that it is LA specific changes AF that leads to a prothrombotic state, thus increasing the risk of stroke.

When MS and AF populations were compared to each other we showed that different thrombogenic profile in each group. Patients with AF were found to have significantly higher levels of endothelial dysfunction at all sites, compared with the MS and control (SVT) patients. This was consistent for both ADMA and ET-1. We also found in the markers of inflammation, VCAM-1 and ICAM-1 in AF patients compared with that of the MS and control (SVT) patients. Conversely, other markers of inflammation CD40L, MPO and IL-6 were significantly higher levels in MS patients compared with AF and control (SVT) patients.

Despite AF patients showing an increase in platelet reactivity and endothelial dysfunction, some markers of inflammation and the structural remodelling were more pronounced within the MS population.

This study has shown that despite having a similar predisposition to LA thrombus formation, AF and its substrates affect the thrombogenic process differently. This shows that a person with both diseases (valvular- AF) has a compounded risk of LA thrombus formation and thus stroke. This study shown there is a need for further research into other common substrates, and how these could be affecting stroke risk in the population.

9.2 Limitations

The major limitation of this study is common in most patient derived study cohorts, disease states affect individuals differently and this has a major influence on their management and treatments. Patients were necessarily also collected from two different continents where disease progress can be altered by lifestyle factors and possibly access to health care.

9.2.1 SVT

The major limitation for the control cohort was obtaining LA access for sampling, this is necessary for a true comparison between a study and control cohort. SVT patients have an echocardiographically structural normal hearts, are sinus rhythm with a normal resting heart rate. However they still have an intracardiac electrical abnormality, which can lead to sudden cardiac death which is why patients undergo a curative electrophysiology procedure which provides access to the LA milieu. In a normal person the risk of this procedure including the need

for transseptal puncture rules out the ability of collecting LA blood samples from a control group.

9.2.2 MS and AF

All markers measured are representative of the changes in haemodynamic in relation to thrombus formation in AF and MS patients. The MS patients have stable and long term (10 years +) stenosis of their mitral valve. It is believed that due to the stability of the disease we found no changes in the markers of tissue remodelling as it had already occurred and is now permanent damage to the endothelium. This may also be why no change in endothelial dysfunction and inflammation was found, as inflammation became stable over the preceding time course of the disease state. Another factor which has yet to be investigated in MS patients is atrial tissue sampling in regard to thrombogenesis and thrombus formation. The ability to quantify the amount of structural damage, together with endothelial damage on the tissue would enable us to significantly increase our knowledge of how chronic atrial stretch from MS is able to effects atrial thrombus formation. Another factor which could be altering these makers is the age of this population, they are a younger population with a mean age of 32 years, and despite the length of disease this may explain the lack of results.

The relative contribution and distribution in AF and its substrates is different, with further epidemiological work needed in this area.

Unfortunately the current section of the study (Chapter 8) did not include the platelet studies. Platelet function is known to be heavily involved in thrombogenic processing in AF,[72,75] and has been implied to be involved in MS,[35] though there is no data enabling a direct comparison.

10 CHAPTER TEN

Future Directions

10.1 Future Directions

This study into how the substrates of AF alters the thrombogenic properties within the LA presented in this thesis have provided key insights into alteration in peripheral and atrial platelet reactivity, endothelial function and inflammation, along with tissue and structural remodelling. It defines the role of these markers in assessing the risk of thrombus formation in AF and its valvular substrate MS. AF together with MS are known increase a person's stroke risk in various and deferring ways, with this thesis giving further insight as to how stroke risk if altered each disease state, when comparing thrombogenic and haemostatic markers.

AF is a disease with a wide variety of clinical presentations and subtypes, and these can all affect AF progression and thrombotic risk differently. It can be difficult to distinguish between the influences of the AF and patients other cardiovascular comorbidities. In this study I aimed to get as close as possible to lone AF (with no comorbidities), however this was not always possible and patients with no more than one risk factor were included. Ideally a lone AF population would give a more accurate idea of the alteration of markers and thrombogenic risk for AF.

A further arm of this study would be in look directly at patients who have progressed from pre-existing MS and have then developed valvular-AF. This would determine if indeed the factors seen in this study are the compounded in the valvular AF population. The limitation in this circumstance is that patients with valvular AF are very often sick and often too unwell to undergo a procedure.

This study could be furthered by including differing substrates, particularly ones that affect the majority of AF sufferers such as hypertension and heart failure, as well as lifestyle factors such as obesity and alcohol consumption. The contribution of lifestyle factors and multiple cardiovascular comorbidities, the induction of AF and disease progression, and their impact on stroke risk needs further investigation.

A more direct assessment of the alter alteration which are occurring to the atrium requites access to atrial tissue samples in AF and its substrates. This could prove very important in the understanding of specific atrial thrombogenic mechanisms. Access to tissue sampling of the atrial endothelium in the setting of AF may produce enlightening thrombogenic profile in these patients.

The greater understanding of the mechanisms promoting atrial thrombogenesis in AF and its major valvular substrate MS, which are diverse and multifaceted, will provide additional insights in the

underlying process and mechanism in both disease states, and result in improved understanding and treatment for all.

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12 Published Paper (First Author)

Characterization of Thrombogenic, Endothelial and Inflammatory Markers within the Normal Human Hearts.

Carlee D Schultz, Han S Lim; Angelique Fraudeau, Glenn Young, Kurt Roberts-Thomson, Matthew Worthley, Prashanthan Sanders, Scott R Willoughby.

Clinical and Experimental Pharmacology and Physiology, 2014(May 14, Epub ahead of print)

Schultz, C.D., Rangneker, G., Lim, H.S., Fraudeau, A., Young, G., Roberts-Thomson, K., John, B., Worthley, M., Sanders, P. & Willoughby, S.R. (2014) Characterization of thrombogenic, endothelial and inflammatory markers in supraventricular tachycardia: a study in patients with structurally normal hearts
Clinical and Experimental Pharmacology and Physiology, v. 41 (8), pp. 551-557