Variability of Nitric Oxide Signalling in Atrial Fibrillation: Potential Modulation

Nathan Procter

A thesis submitted in fulfilment of the requirements of the degree, Doctor of Philosophy, through The University of Adelaide.

Contents

Thesis 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.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Nathan Procter

Acknowledgments

"Space, the final frontier. These are the voyages of the starship Enterprise. Its continuing mission: to explore strange new worlds, to seek out new life and new civilizations, to boldly go where no one has gone before!" – Captain Jean-Luc Picard, USS Enterprise.

Thematically, this is much like how my PhD has been, starting out riding a wave of optimism and hope, fuelled with a desire to explore the realm of cardiovascular research and making discoveries that would revolutionise not only cardiovascular medicine, but the world as we know it, even reshaping the very foundations from which we understand the universe. Alas, my achievements are somewhat more humble than those of Jean-Luc Picard: no new civilizations were discovered, wars prevented or catastrophes averted as a result of this research.

Needless to say, there are a number of people I wish to thank because of their support over the course of this journey. My deepest gratitude goes to Professor John Horowitz who first gambled on the prospect that I have a mind at work and Dr Yuliy Chirkov for his invaluable advice throughout this project.

My family and friends were limitless supplies of love and support when I first announced that I was to undertake a PhD, one of whom proudly announced "nothing irritates me more than people who fail to strive to reach their full potential", before wrapping me up in a bear hug. Their ongoing encouragement has helped me through this 'period of awakening'.

Lastly, I would like to thank the staff and colleagues of the Cardiology Department and Laboratory from the Basil Hetzel Institute and The Queen Elizabeth Hospital for their help and support.

Nathan Edward Kevin Procter.

P.S. Cheers to all the ‘crew’ who were always down for a bit of a chin-wag about all things non-sciencey, when we probably should have been discussing things sciencey. Ooroo.

P.P.S. *“For God hath not given us the spirit of fear; but of power, and of love, and of a sound mind.” 2 Timothy 1:7.*

List of Abbreviations

Abbreviation	Term
ACE	Angiotensin-converting Enzyme
ACS	Acute Coronary Syndromes
ADMA	Asymmetric Dimethylarginine
ADP	Adenosine Diphosphate
AF	Atrial Fibrillation
Agxt2	Amino-glyoxylate Aminotransferase 2
ANCOVA	Analysis of Covariance
Ang II	Angiotensin II
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BH_4	Tetrahydrobiopterin
BNP	B-type Natriuretic Peptide
Ca^{2+}	Calcium
cGMP	3'5'-Cyclic Guanosine Monophosphate
$CHADS_2$	Congestive Heart Failure, Hypertension, Aged ≥ 75 years, Diabetes Mellitus, Prior Stroke/Transient Ischemic Attack
CHA_2DS_2VASc	Congestive Heart Failure, Hypertension, Aged ≥ 75 years, Diabetes Mellitus, Prior Stroke/Transient Ischemic Attack, Vascular Disease, Sex Category: Female
CRP	C-reactive Protein
DDAH	Dimethylarginine Dimethylaminohydrolase
ELISA	Enzyme-linked Immunosorbent Assay
FAD	Flavin Adenine Dinucleotide
FMN	Flavin Mononucleotide

GTP	Guanosine Triphosphate
IQR	Interquartile Range
L-NMMA	N-monomethyl-L-arginine
MPO	Myeloperoxidase
mRNA	Messenger Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NLRP3	Nod-like Receptor Protein 3
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
O_2^-	Superoxide
PBS	Phosphate Buffered Saline
PKG	3'5'-Cyclic Guanosine Monophosphate-dependent Protein Kinase
PMN	Polymorphonuclear Neutrophil
PRMT	Protein Arginine Methyltransferases
RAAS	Renin-Angiotensin-Aldosterone System
ROS	Reactive Oxygen Species
SAFETY	Standard vs. Atrial Fibrillation spEcific managemenT studY
SDMA	Symmetric Dimethylarginine
SDS-PAGE	Sodium Dodceylsulfate-Polyacrylamide Gel Electrophoresis
S.E.M.	Standard Error of the Mean
sGC	Soluble Guanylate Cyclase
SNP	Sodium Nitroprusside
TIA	Transient Ischemic Attack

TNFα	Tumour Necrosis Factor-α
TSP-1	Thrombospondin-1
Txnip	Thioredoxin-interacting Protein

List of Figures

List of Tables

Abstract

Understanding of the biochemical bases of thromboembolic risk in atrial fibrillation (AF) is incomplete. In a cohort of AF patients admitted to hospital, integrity of platelet nitric oxide (NO) signalling, and its modulation by dimethylarginines, myeloperoxidase, thrombospondin-1and thioredoxin-interacting protein, was investigated. This study identified that, (1) new onset AF is associated with impaired platelet NO response; (2) gender-specific platelet dysfunction exists in AF where females display increased platelet aggregability and impaired NO response compared to males; (3) plasma symmetric dimethylarginine correlated inversely with platelet aggregability in chronic AF patients. Abnormalities of NO signalling and its various determinants occur frequently in AF patients at risk of thromboembolism.

Keywords

Atrial fibrillation; platelet; aggregation; nitric oxide; asymmetric dimethylarginine; myeloperoxidase; thrombospondin-1; thioredoxin-interacting protein.

1: Literature Review

1.1 Atrial Fibrillation

1.1.1 History

Understanding the pathophysiology of atrial fibrillation (AF), as well as the development of theoretical concepts to explain its development, has progressed exponentially and been the subject of numerous reviews (Lip *et al.*, 1995; Nattel, 2002; Nattel *et al.*, 2000; Prystowsky, 2008). The first recognised reference to the condition arguably dates back to the period of 1699-2598BC, where it was observed in '*The Yellow Emperor's Classic of Internal Medicine*' that: "when the pulse is irregular and tremulous and the beats occur at intervals, then the impulse of life fades". In more modern times however, William Harvey (1628) observed "fibrillation of the auricles" in animals. This was followed much later with work done by Alfred Vulpian (1874), who observed induction of atrial and ventricular fibrillation through the use of electric shocks. The invention of the electrocardiograph (ECG) allowed for the first time direct observation of the electrical properties of AF, with Willem Einthoven publishing the first ECG recording of a patient in AF (1906). Further analysis by Thomas Lewis resulted in the suggestion that the diastolic oscillations apparent on the recording might be due to fibrillation of the atria.

It was not until the turn of the 20th century that theories regarding the specific mechanisms underlying the pathogenesis of AF first began to be proposed. The different conceptual frameworks provided by these theories allowed for the specific investigation of pathological mechanisms underlying the development of AF. Investigations by Thomas Lewis identified a 'rapid single circuit re-entry mechanism' as being the potential basis for AF (Lewis *et al.*, 1920). Contrasted with this were studies conducted by Walter Garrey and George Mines that resulted in the proposition of a 'multiple re-entrant circuit mechanism' for AF. Alternatively, studies by Walter Garrey and later by David Scherf identified the existence of focal ectopic nuclei capable of initiating the arrhythmia. By 1959 Moe and Abildskov had induced AF

experimentally in a canine model using multiple circuit re-entry and/or rapid pacing methods, resulting in the proposal of the 'multiple wavelet hypothesis'. Current theories on the mechanisms of AF are known as the 'automatic focus theory' (acknowledging the existence of ectopic nuclei) and the 'multiple wavelet hypothesis' (referring to the extensive structural, electrical and biochemical remodelling that allows for heterogeneous and fragmentary conduction of electrical impulses through the atria)(Iwasaki *et al.*, 2011; Jalife, 2011).

1.1.2 Definition

AF has been defined as a supraventricular tachyarrhythmia displaying uncoordinated atrial activation and deterioration of atrial mechanical function. On an ECG it can be characterised through the loss of consistent 'p' waves by rapid oscillations (known as fibrillatory waves) that can vary in amplitude, shape and timing. An ECG of someone in AF may display irregular R-R intervals as well as a variable and short (<200ms) interval between atrial activations. Initial presentation of the arrhythmia is quite often associated with a rapid and irregular ventricular rate (European Heart Rhythm *et al.*, 2010; January *et al.*, 2014).

1.1.3 Epidemiology

The global prevalence of AF is estimated to be from 0.4-2.0% of the general population, though due to the asymptomatic nature of the illness (at least initially), it's possible that the actual prevalence of AF is underestimated (Chugh *et al.*, 2014; Fitzmaurice, 2009; Lip *et al.*, 2012). Although these estimates of global AF prevalence were derived primarily from European and North American based studies, similar prevalence has been observed elsewhere, ranging from 0.6% in Japan through to 4% in Australia (Lip *et al.*, 2012). The reported prevalence of AF in India is relatively low at a modest 0.1%, while Chinese and Thai populations have reported prevalence rates ranging from 0.8-2.8%, or 0.4-2.2%, respectively (Lip *et al.*, 2012). Given the strong association between incidence of AF and ageing, the

median age and life expectancy of differing populations may affect the observed prevalence of AF. Global prevalence rates for AF are also increasing (predominantly in 'developed' countries), and are consistently reported as higher in males than females (Chugh *et al.*, 2014). The period from 1990 through to 2010 saw global prevalence of AF increase from 569.5 [95%CI 532.8-612.7] (per 100000 population) to 596.2 [95%CI 558.4-636.7] in males, and from 359.9 [95%CI 334.7-392.6] to 373.1 [95%CI 347.9-402.2] in females. Although prevalence of AF is lower in females than in males, thromboembolic risk is higher. A recent meta-analysis has reported that females experience a 1.31 fold increased stroke risk, compared to males (Wagstaff *et al.*, 2014). Similarly, another study has reported a 1.28 fold increased stroke risk in warfarinised females (compared to males), though interestingly this difference in stroke risk disappeared when new oral anticoagulants were considered (Pancholy *et al.*, 2014). Part of the increased stroke risk experienced by females with AF may explained by age at time of diagnosis: a recent study observed that 24% of males were ≥ 80 years at time of AF diagnosis, compared with 41% of females, although reported comorbidities were generally higher in males than females (Renoux *et al.*, 2014). Lower rates of anticoagulation in females may contribute to thromboembolic risk: the older age at which females tend to be diagnosed with AF has also been identified as a bleeding risk (DiMarco *et al.*, 2005). However, rates and patterns of warfarin use have been reported as broadly similar with respect to gender (Avgil Tsadok *et al.*, 2012; Inoue *et al.*, 2014).

Increasing prevalence of AF with age has also been observed globally with 0.7% of individuals under the age of 50 being diagnosed with AF, rising to as many as 15% over the age of 80 (Chugh *et al.*, 2014; Fitzmaurice, 2009; Lip *et al.*, 2012). The Australian experience testifies to this: hospitalisation because of AF increased by 8% p.a. from 1993-2007 (Wong *et al.*, 2012).

Improved screening in developing countries for rheumatic heart disease has resulted in reports of much higher prevalence with respect to developed countries (Colquhoun *et al.*, 2014;

Ferratini *et al.*, 2013). Given the implication that limited resources in these settings has resulted in significant underestimation of the true prevalence of rheumatic heart disease, it is reasonable to postulate that similar underestimation of the prevalence of atrial fibrillation (rheumatic or non-rheumatic) may also be occurring in developing countries. Indeed, a recent Australian investigation has identified that prevalence of AF is much higher among indigenous than non-indigenous populations (Wong *et al.*, 2014): rheumatic heart disease is also much more prevalent among indigenous than non-indigenous Australians (Davies *et al.*, 2014).

AF has been identified as an independent risk factor for thromboembolism, conferring a 2-7 fold increased risk relative to non-AF populations and an annual stroke rate of 3-8% (Fitzmaurice, 2009; Naccarelli *et al.*, 2009; Wolf *et al.*, 1991). Numerous epidemiological studies have been performed investigating thromboembolic risk in AF populations, specifically seeking to identify those clinical characteristics that are associated with stroke incidence. Work by the Atrial Fibrillation Investigators (Laupacis *et al.*, 1994) evaluated data from early anticoagulation clinical trials and upon multivariate analysis, identified heart disease, previous myocardial infarction, congestive heart failure, diabetes mellitus, increasing age, previous/current hypertension and prior stroke/transient ischemic attack (TIA) as being associated with stroke incidence in AF populations (Laupacis *et al.*, 1994). Similarly in a pooled analysis of the AF populations recruited in the Stroke Prevention in Atrial Fibrillation trials (R. G. Hart *et al.*, 1999), increasing age, female sex, previous/current hypertension and prior stroke were all multivariate correlates of stroke incidence (R. G. Hart *et al.*, 1999). In contrast to these studies, an investigation specifically into warfarin and aspirin naïve AF patients identified age, female gender, diabetes mellitus and prior stroke/TIA as being associated with stroke incidence (T. J. Wang *et al.*, 2003a). These investigations resulted in the empirically derived $CHADS_2$ (congestive heart failure, hypertension, age $\geq$ 75 years, diabetes mellitus and prior stroke/TIA) and CHA_2DS_2VASc (congestive heart failure,

hypertension, age ≥ 75 years, diabetes mellitus, prior stroke/TIA, vascular disease, aged 65-74 years, sex category: female) stroke risk algorithms (Gage *et al.*, 2001; Lip *et al.*, 2010). The global trend of increasing AF incidence, combined with increased hospitalisation due to AF, pose a significant burden on healthcare systems globally, especially when management of AF-related stroke is considered (Ball *et al.*, 2013; Cadilhac, 2012; Cadilhac *et al.*, 2009; Wolowacz *et al.*, 2011; Wong *et al.*, 2012).

1.1.4 Classification

AF may be classified according to the predominant pattern of arrhythmia or according to the presence or absence of underlying structural heart disease (European Heart Rhythm *et al.*, 2010; January *et al.*, 2014). Initially, a determination is made on whether the current episode of AF constitutes *de novo* detection (i.e. 'new onset') and whether it is asymptomatic. Frequency and duration of the arrhythmia impact significantly upon clinical classification, with two or more AF episodes being considered recurrent AF, and self-terminating episodes (usually within 48 hours) being considered paroxysmal. AF episodes that last more than a week in duration are considered persistent and require intervention in order to terminate the arrhythmia. AF episodes that have lasted for a year or more may be considered long standing persistent, or even (somewhat arbitrarily) permanent, depending upon the views of the patient and treating physician (European Heart Rhythm *et al.*, 2010; January *et al.*, 2014). "Lone AF", while unusual, is the presence of the arrhythmia in the absence of any other identifiable cardiovascular disease. These classifications are distinct from secondary AF, which can be directly attributed to a specific cardiac trauma, where treatment of the primary cardiac illness can result in alleviation of the arrhythmia (January *et al.*, 2014).

The majority of cases of AF reflect inflammatory/distensive changes within the atria associated with ageing and hypertensive heart disease. However, it is also important to

recognise other significant associations of AF (see Table 1), such as hyperthyroidism, rheumatic mitral valve stenosis and the acute effects of intake of large quantities of alcohol.

Table 1: Disease states frequently complicated by, or predisposing to, development of atrial fibrillation

Heart failure
Diabetes mellitus
Stroke (ischaemic/haemorrhagic)
Valvular heart disease
Acute coronary syndromes
Hyperthyroidism
Wolff-Parkinson-White syndrome
Hypertrophic cardiomyopathy
Dilated cardiomyopathy
Pulmonary disease
Coronary artery disease
Hypertension
Congenital heart disease

Furthermore, it has progressively emerged that AF may occur in the absence of other structural heart disease ("lone AF"), and that many such cases carry a genetic basis (Ellinor *et al.*, 2010; Potpara *et al.*, 2014). AF is traditionally classified not only on the basis of its being intermittent or sustained, but also relative to the presence structural heart disease (the latter potentially inducing atrial distension and remodelling). On this basis, patients with no evidence of structural abnormalities may be considered to have "lone" AF (Potpara *et al.*, 2014). The major implication of this diagnosis may be a lower risk of thromboembolic events in "lone" AF (Jahangir *et al.*, 2007). The recent finding that a substantial minority of cases of AF represent an underlying genetic predisposition complicates the issue of "lone" AF: traditionally this has been purely a diagnosis of exclusion (European Heart Rhythm *et al.*, 2010; January *et al.*, 2014), but genotyping implies a "positive" basis for predicting AF in such individuals.

1.1.5 Pathogenesis of Atrial Fibrillation

The development of AF occurs as a result of the emergence of an 'atrial substrate' that is conducive to the initiation and maintenance of the arrhythmia. Such a substrate develops through an extensive process referred to as remodelling, and encompasses electrical/biochemical and structural changes within the atria. The net result of these changes is an environment vulnerable to and supportive of the emergence of arrhythmia. These changes will be summarised briefly in the context of AF (Table 2 summarises key research papers).

1.1.5.1 Electrical Remodelling

Currently there are two main, non-mutually exclusive, theories regarding the mechanisms underlying the genesis and perpetuation of AF, these being the 'automatic focus theory' and the 'multiple wavelet hypothesis' (Jalife, 2011). The automatic focus theory suggests that focal ectopic nuclei are responsible for induction of the arrhythmia by initiating aberrant depolarisations, e.g. pulmonary vein nuclei, with isolation of these nuclei via pulmonary vein ablation displaying reasonable success in restoring sinus rhythm (Haissaguerre *et al.*, 1998). The multiple wavelet hypothesis describes AF as being the result of the propagation of a 'mother wave' of electrical activity through the atria that fractionates upon encountering tissue refractory to electrical conductance (such as fibrosis, scar tissue, etc.), resulting in the formation of self-sustaining 'daughter wavelets' (Issac *et al.*, 2007; Iwasaki *et al.*, 2011; Jalife, 2011; Jalife *et al.*, 2002; Nattel, 2002; Nattel *et al.*, 2000). A common feature to each of these theories is the requirement for heterogeneity of conduction through atrial tissue. Heterogeneous conduction of electrical impulses is a key feature of AF and allows for the formation of re-entrant circuits (the circular conduction of electrical impulses through atrial tissue), thus providing a substrate for the emergence of the arrhythmia (Burstein *et al.*, 2008; Nattel, 2002; Nattel *et al.*, 2000; Veenhuyzen *et al.*, 2004).

The emergence of re-entrant circuits occurs due to changes in ion channel density and function, affecting cardiomyocyte membrane potential and susceptibility to re-activation. The expression and function of these ion channels can be affected by external and/or internal stimuli, resulting in short term preservation of cardiomyocyte function but facilitating the long term emergence of re-entrant circuits. Repeated cardiomyocyte activation as a result of atrial tachycardia can result in accumulation of intracellular cytosolic calcium (Ca^{2+}), necessitating adaptation in order to minimise the threat this poses, e.g. induction of apoptosis (Greiser *et al.*, 2013; Iwasaki *et al.*, 2011). This adaptation takes the form of increased sequestration of Ca^{2+} in intracellular stores (e.g. sarcoplasmic reticulum) and down-regulation of L-type Ca^{2+} channels (Casaclang-Verzosa *et al.*, 2008; Greiser *et al.*, 2013; Iwasaki *et al.*, 2011; Nattel *et al.*, 2000). These adaptations render the cardiomyocyte vulnerable to reactivation however, as the down-regulation of L-type Ca^{2+} channels contributes to a shortening of action potential duration and effective refractory period. The increased intracellular Ca^{2+} load also results in increased frequency of Ca^{2+} 'sparks' or 'leaks' from sarcoplasmic reticulum, raising the possibility of aberrant cardiomyocyte activation (Greiser *et al.*, 2013; Iwasaki *et al.*, 2011). These maladaptive changes in Ca^{2+} handling by cardiomyocytes result in an environment vulnerable to the emergence of re-entrant circuits. The existence of these re-entrant circuits thus facilitates the development of AF. Cardiac remodelling, encompassing electrophysiological and structural changes, is an energy intensive process that places significant demands upon cardiomyocytes (Azevedo *et al.*, 2013; Casaclang-Verzosa *et al.*, 2008). Additionally, the regulation of ionic currents by cardiomyocytes is increasingly being associated with cardiac metabolism (Barth *et al.*, 2009). The net deficit of adenosine triphosphate (ATP) within cardiomyocytes under these conditions can result in impaired Ca^{2+} handling (contributing to impaired atrial contractility and reduced atrial effective refractory period), as well as uncoupling of mitochondrial function from ATP generation (promoting reactive oxygen species [ROS] generation and

Table 2: Major contributors to pathogenesis of atrial fibrillation

Factor	Key Studies	Seminal Findings
Electrical Remodelling		
	(Wijffels *et al.*, 1995)	Electrical induction of atrial fibrillation in goats becomes self-sustained.
	(Kostin *et al.*, 2002)	Atrial fibrillation patients display altered expression of mechanical- and gap-junction proteins, contributing to abnormal electrical conduction.
	(Sun *et al.*, 2001)	Atrial tachycardia produces alterations in myocyte calcium handling, resulting in abnormal contractile function.
Structural Remodelling		
	(Ausma *et al.*, 1997)	Sustained atrial fibrillation in goats resulted in altered atrial myocyte cellular structure, including loss of myofibrils, accumulation of glycogen and altered mitochondrial density.
	(Logan *et al.*, 1965)	Left atrial contractility exhibits short-term diminution following cardioversion of atrial fibrillation patients.
	(Schotten *et al.*, 2004)	Atrial dilation occurs due to increased atrial compliance as a result of diminished atrial contractility during atrial fibrillation.
Inflammation		
	(V. Rudolph *et al.*, 2010)	Myeloperoxidase contributes to pathogenesis of atrial fibrillation by mediating development of inflammation and fibrosis.
	(Yamashita *et al.*, 2010)	Inflammatory cells infiltrate the fibrillating human atria.
	(Ozaydin *et al.*, 2008)	N-acetylcysteine (a hypochlorous acid scavenger) infusion reduces the incidence of post-operative atrial fibrillation in coronary artery bypass graft patients.

subsequent inflammation) (Barth *et al.*, 2009; Casaclang-Verzosa *et al.*, 2008; Korantzopoulos *et al.*, 2007). Neurohumoral stimuli, such as autonomic nervous system activity, are among the external factors that can influence atrial activation rates. Changes in cardiac innervation are well documented in AF, with increased sympathetic innervation having been observed, as well as an altered balance between sympathetic and parasympathetic activity, resulting in increased conduction heterogeneity and decreased atrial effective refractory periods (Nattel *et al.*, 2000; Schotten *et al.*, 2011; Tan *et al.*, 2013). As well as affecting atrial activation rates, increased sympathetic tone (in the form of elevated epinephrine and norepinephrine levels) may predispose the cardiac environment to conditions of elevated redox stress, scavenging available nitric oxide (NO) and contributing to cardiac remodelling (Cai *et al.*, 2002; J. L. Mehta *et al.*, 2001; Ridnour *et al.*, 2007).

1.1.5.2 Structural Remodelling

Structural remodelling refers to anatomical changes in the myocardium that facilitate the emergence of re-entrant circuits and the establishment of AF. These structural alterations range from macroscopic changes, such as increases in the dimensions of the left atrium, through to microscopic changes, such as the development of fibrosis. The commonality of these structural changes is that they promote heterogeneous conduction of electrical impulses through the myocardium.

In the presence of conditions associated with 'hemodynamic overload', chronic increases in atrial pressure and/or volume drive structural change in the myocardium, allowing for the establishment of AF (De Jong *et al.*, 2011). Left atrial dilation and cardiomyocyte hypertrophy are classic examples of the overt structural changes that may occur. In the presence of tachycardia diminished cardiomyocyte contractility develops, contributing to left atrial compliance and facilitating further left atrial dilation (Burstein *et al.*, 2008; Casaclang-Verzosa *et al.*, 2008; De Jong *et al.*, 2011; Greiser *et al.*, 2013; Schotten *et al.*, 2011).

Additionally, atrial tachycardia can induce de-differentiation of cardiomyocytes into a 'foetal-like' phenotype, characterised by diminished contractility, altered mitochondrial number and function, as well as increased sensitivity to Ca^{2+}-induced activation. Increased rates of cardiomyocyte apoptosis and necrosis are also observed in AF, contributing not only to the development of fibrosis, but also to inflammation, which can enable further structural remodelling of the atria (Burstein *et al.*, 2008; Casaclang-Verzosa *et al.*, 2008; De Jong *et al.*, 2011; Schotten *et al.*, 2011).

Chronic inflammation is an established feature of AF, with various cytokines being elevated in the presence of AF, as well as correlating with thromboembolic risk (Issac *et al.*, 2007; Korantzopoulos *et al.*, 2007; Li *et al.*, 2010; Maehama *et al.*, 2010; Neuman *et al.*, 2007; V. Rudolph *et al.*, 2010; Yamashita *et al.*, 2010). Inflammation has also been suggested to play a direct role in the pathogenesis of AF: increased leukocyte infiltration of the myocardium, as well as increased expression of pro-inflammatory, pro-fibrotic mediators such as angiotensin II (Ang II) and transforming growth factor β1, induce structural changes conducive to the establishment of AF (Casaclang-Verzosa *et al.*, 2008; Ferrario *et al.*, 2006; Friedrichs *et al.*, 2012; Issac *et al.*, 2007; V. Rudolph *et al.*, 2010). Additionally, oxidative stress associated with inflammation (e.g. via activation of the enzymes nicotinamide adenine dinucleotide phosphate [NADPH] oxidase and myeloperoxidase [MPO], which is released primarily from leukocytes) can adversely affect atrial structure, via the development of fibrosis and the activation of enzymes responsible for the degradation of the extracellular matrix (matrix metalloproteinases), contributing to biochemical/structural remodelling (Ferrario *et al.*, 2006; Korantzopoulos *et al.*, 2007; V. Rudolph *et al.*, 2010; Veenhuyzen *et al.*, 2004).

1.1.6 Pathogenesis of Thromboembolic Complications in Atrial Fibrillation

It has been recognised for some time now that cardiovascular disease carries with it an increased risk for thromboembolism and with the discovery of warfarin, the need and

capability for anticoagulant therapy (Askey *et al.*, 1950; Daley *et al.*, 1951). With regards to AF specifically, the benefit of reversion to sinus rhythm as well as anticoagulant therapy has also been established for some time (Freeman *et al.*, 1967). However, these early studies were primarily considering the issue through the spectrum of rheumatic heart disease and it wasn't until much later that increased thromboembolic risk in AF (in the absence of rheumatic heart disease) was recognised (Wolf *et al.*, 1991; Wolf *et al.*, 1978). With identification of AF as an independent determinant of thromboembolic risk, the need for chronic anticoagulant therapy became apparent with numerous studies evaluating warfarin, aspirin, or both for safety and efficacy as potential anticoagulants in AF (Connolly *et al.*, 1991; Ezekowitz *et al.*, 1992; Mcbride, 1991; Petersen *et al.*, 1989; Singer *et al.*, 1992). With the need and capability for chronic anticoagulation in AF, it became necessary for clinicians to stratify thromboembolic risk in these patients. Several studies evaluated the incidence of thromboembolism in large populations of AF patients and sought to identify those clinical parameters which were associated with incidence of stroke/TIA. Identification of those clinical factors associated with thromboembolic risk would thus enable clinicians to adapt anticoagulant therapy accordingly (R. G. Hart *et al.*, 2003; Laupacis *et al.*, 1994). These early studies were refined into what is known as the $CHADS_2$ score, combining those clinical factors associated with thromboembolic risk into a factorial algorithm, that was then prospectively validated in a new patient cohort (Gage *et al.*, 2001). However, this particular stroke risk scheme arguably lacks discriminatory power for patients considered to be at low thromboembolic risk: patients with $CHADS_2$=0 have still experienced cerebrovascular accidents. This lack of discriminatory power is addressed to some extent by the CHA_2DS_2VASc score, a refinement of the Birmingham/NICE stroke risk stratification scheme (Lip *et al.*, 2010). Notably, the CHA_2DS_2VASc stroke risk stratification scheme improved upon the $CHADS_2$ scheme in being able to more accurately discriminate AF patients at low risk of thromboembolic complications.

However, while considerable progress has been made in terms of identifying clinical features that predispose AF patients to thromboembolism, these studies failed to directly address the physiological mechanisms underlying increased thromboembolic risk in AF. Virchow's triad refers to the physiological conditions required for the pathological formation of thrombus, being the presence of blood stasis, endothelial damage/dysfunction and hypercoagulability (the latter proposed initially on the basis of reported elevated markers of haemostasis present in AF, and that these markers decreased with reversion to sinus rhythm (Lip, 1995))(Lowe, 2003). These conditions are all present in AF and have been summarised with respect to thromboembolic risk, while also considering the potential contribution of inflammation to thrombogenesis (Watson *et al.*, 2009). In essence, a nexus is present in AF that results in an environment that favours thrombus formation.

1.1.7 Virchow's Triad in Atrial Fibrillation

1.1.7.1 Blood Stasis in Atrial Fibrillation

Turbulent blood flow and/or blood stasis is notably present in the left atrium during AF, favouring thrombus formation which can be observed through the presence of spontaneous echo contrast (Black *et al.*, 1991), correlating directly with clinical measures of thromboembolic risk (Maehama *et al.*, 2010; Ohara *et al.*, 2009; Zabalgoitia *et al.*, 1998). Increased left atrial size and loss of mechanical function contribute to decreased blood velocity and blood stasis, resulting in increased propensity for thrombus formation and occurrence of stroke or embolism (Hirose *et al.*, 2012; Ohara *et al.*, 2009; Providencia *et al.*, 2013; Shih *et al.*, 2011; Shively *et al.*, 1996; Wysokinski *et al.*, 2010).

1.1.7.2 Endothelial Dysfunction in Atrial Fibrillation

The disturbed blood flow patterns that occur as a result of these changes in atrial structure contribute significantly to the development of endothelial dysfunction in AF. Increased levels

of plasma von Willebrand Factor (a biochemical marker of endothelial damage/dysfunction) and soluble E-selectin (a biochemical marker of leukocyte adhesion to the endothelium) have been observed in AF (Freestone *et al.*, 2008), as well as disturbances in vascular function being apparent when measured *in vivo* (Takahashi *et al.*, 2001; Yoshino *et al.*, 2013). Moreover, restoration of sinus rhythm in AF patients has been reported to be accompanied by partial improvement in endothelial function (Takahashi *et al.*, 2001; Yoshino *et al.*, 2013). At least part of the physiological mechanisms underlying this are the result of disturbed vs. steady blood flow in AF. Laminar blood flow is associated with increased endothelial NOS (NOS), cyclooxygenase-2 and superoxide dismutase expression (Gimbrone *et al.*, 2013), as well as being necessary for stimulation of NO production (Guazzi *et al.*, 2009). Disturbed blood flow however, is associated with increased ROS generation, loss of NO production associated with increased expression of thioredoxin-interacting protein (Txnip) and increased expression of adhesion molecules on the surface membrane of endothelial cells (Guazzi *et al.*, 2009; Heo *et al.*, 2011; X. Q. Wang *et al.*, 2012; Yamawaki *et al.*, 2005).

1.1.7.3 Hypercoagulability in Atrial Fibrillation

Additionally, blood stasis facilitates the formation of platelet aggregates within stationary blood and the adhesion of platelets and leukocytes to the endothelium, precipitating thrombus formation (Bovill *et al.*, 2011; Esmon *et al.*, 2011; Phillipson *et al.*, 2011). The loss of the endogenous anticoagulants (i.e. inhibitors of platelet adhesion and activation) produced by the endothelium in response to laminar blood flow contributes significantly to the hypercoagulability and platelet hyper-reactivity, characteristic of AF. Plasma markers of platelet activation such as platelet factor-4, soluble P-selectin and β-thromboglobulin are all elevated in AF compared to controls (Kamath, Blann, *et al.*, 2002; Kamath, Chin, *et al.*, 2002; Marin *et al.*, 2004; Ohara *et al.*, 2008). Plasma D-dimer levels, an indicator of fibrinolysis, have also consistently been observed to be increased in AF and have also correlated with

clinical measures of stroke risk (Kamath, Blann, *et al.*, 2002; Kamath, Chin, *et al.*, 2002; Ohara *et al.*, 2008). Furthermore, the hypercoagulability and platelet hyper-reactivity present in AF are to some extent independent of the existence of the arrhythmia. Plasma indicators of platelet activation, as well as clot formation and breakdown, remain elevated in AF patients 30 days post successful cardioversion (Marin *et al.*, 2004).

1.1.7.4 Inflammation in Atrial Fibrillation

As part of their role in the maintenance of haemostasis, platelets are intimately involved in mediating inflammation through the release of chemokines and cytokines upon activation (Angiolillo *et al.*, 2010; Davi *et al.*, 2007; Gawaz *et al.*, 2005). Moreover, activation of the renin-angiotensin-aldosterone system (RAAS) has been associated with the development of atrial fibrosis, is involved in the recruitment of inflammatory cells to sites of vascular injury, as well as endothelial expression of adhesion molecules (Ferrario *et al.*, 2006; V. Rudolph *et al.*, 2010; Watson *et al.*, 2009). Additionally, the leukocyte-derived inflammatory enzyme MPO has a pivotal role in the pathogenesis of AF (V. Rudolph *et al.*, 2010), as well as being linked to endothelial dysfunction and reduced bioavailability of NO (Eiserich *et al.*, 2002; Eiserich *et al.*, 1998; T. K. Rudolph *et al.*, 2010). Indeed, NO is able to modulate the activity of matrix metalloproteinases (Ridnour *et al.*, 2007), and deficiency in NO availability has been implicated in the pathogenesis of AF (Cai *et al.*, 2002).

Essentially, the development of AF, comprised of atrial distension and mechanical dysfunction, gives rise to a pro-thrombotic milieu and the development of chronic inflammation. This nexus of biochemical factors further drives the remodelling process, establishing the arrhythmia and the pro-thrombotic environment that accompanies it.

1.2 Atrial Fibrillation as a Biochemical Disorder

1.2.1 The Nitric Oxide Signalling Pathway

Deficiencies in NO signalling (Table 3) are a key aspect of numerous cardiovascular diseases: endothelial dysfunction, characterised by a loss of NO production and concomitant increased ROS generation, partially due to uncoupling of endothelial NOS (Heo *et al.*, 2011), is present in AF (Takahashi *et al.*, 2001; Yoshino *et al.*, 2013), hypertension (Taddei *et al.*, 1995), diabetes mellitus (Bellin *et al.*, 2006), and polycystic ovarian syndrome (Rajendran *et al.*, 2009). Additionally, impaired platelet response to NO has been observed in heart failure (Chirkov *et al.*, 2004), stable angina pectoris (Chirkov *et al.*, 1997; Chirkov *et al.*, 1999), acute coronary syndromes (ACS) (Chirkov *et al.*, 2001; Willoughby *et al.*, 2005), and has come to be referred to as "NO resistance" (Chirkov *et al.*, 2007; Rajendran *et al.*, 2008). The impairment of NO signalling has also been implicated in the pathogenesis of AF through down-regulation of expression and activity of endothelial NOS (Cai *et al.*, 2002). This is in accordance with observations that endogenously produced NO has an inhibitory effect on matrix metalloproteinase-9 (an enzyme involved in maintenance of myocardial extracellular matrix and implicated in cardiac remodelling (Sundstrom *et al.*, 2004)) expression and activity (Eberhardt *et al.*, 2000; Ridnour *et al.*, 2007). Furthermore, uncoupling of endothelial NOS has been found to contribute to activation of matrixmetalloproteinases-2 and -9 (Takimoto *et al.*, 2005). Given the significant role of dysfunctional NO signalling in the pathogenesis of AF, a brief overview including potential mechanisms of impairment is appropriate.

1.2.1.1 Generation of Nitric Oxide: Nitric Oxide Synthase

NOSs are the major physiological source of NO. There are three isoforms of NOS (neuronal, endothelial and inducible), though it is the expression and function of endothelial NOS that

Table 3: Bases for disordered nitric oxide production and/or signalling

Factor	Key Studies	Seminal Findings
Generation of Nitric Oxide		
	(Landmesser *et al.*, 2003)	Oxidation of tetrahydrobiopterin (BH_4) results in uncoupling of nitric oxide synthase activity from production of nitric oxide, leading to production of superoxide.
	(Vallance *et al.*, 1992)	Asymmetric dimethylarginine is a competitive antagonist of nitric oxide synthase.
	(Buga *et al.*, 1996)	Arginase (a cataboliser of L-arginine) is constitutively expressed in endothelial cells, and its activity is increased in response to inflammatory stimuli.
	(Du *et al.*, 2001)	Hyperglycaemia-induced posttranslational modification of endothelial nitric oxide synthase results in loss of nitric oxide production and increased superoxide production.
Nitric Oxide Signalling		
	(Chirkov *et al.*, 1999)	Addition of the superoxide scavenger, superoxide dismutase, to blood samples of stable angina pectoris patients restored anti-aggregatory response to the nitric oxide donor, sodium nitroprusside.
	(Stone *et al.*, 1994)	Oxidation of the heme moiety in soluble guanylate cyclase prevents binding (thus inhibiting activation) of nitric oxide.
	(Ignarro *et al.*, 1982)	Activation of soluble guanylate cyclase by nitric oxide requires the heme moiety to be present.

has been most closely studied in association with cardiovascular disease (Balligand *et al.*, 2009; Forstermann, 2010).

Structurally (Figure 1), endothelial NOS consists of N-terminal oxygenase and C-terminal reductase domains (Balligand *et al.*, 2009; Forstermann, 2010). The reductase domain binds the cofactors NADPH, flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). The oxygenase domain contains a heme moiety which binds molecular oxygen, as well as the cofactor tetrahydrobiopterin (BH_4) and the substrate L-arginine (Balligand *et al.*, 2009; Forstermann, 2010). NO is generated by endothelial NOS through the transfer of electrons from NADPH to L-arginine, facilitated by the calcium-dependent binding of calmodulin, resulting in the formation of L-citrulline and liberation of NO (Balligand *et al.*, 2009; Forstermann, 2010).

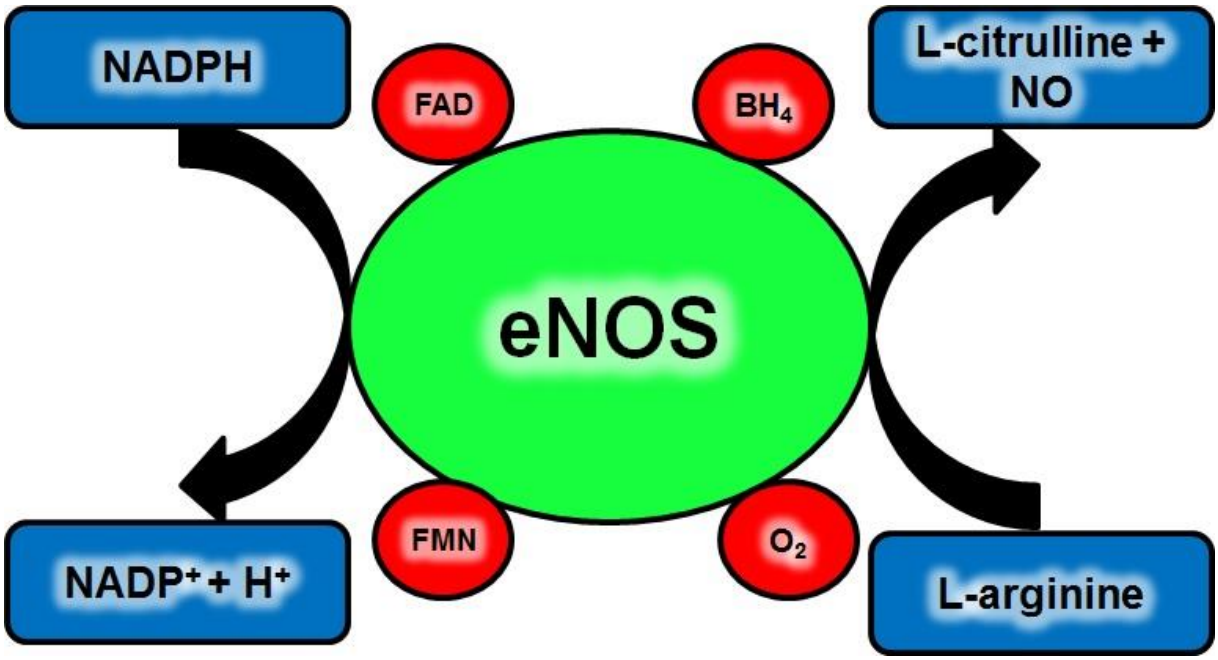

Figure 1: Structural representation of the endothelial nitric oxide synthase enzyme.

As well as being stimulated by laminar flow, endothelial NOS activity and expression is also subject to post-translational modifications which regulate its activity. Nitrosylation and glycosylation both result in inhibition of endothelial NOS activity (Federici, 2002; Ravi *et al.*,

2004). Phosphorylation of serine and/or threonine residues also profoundly affect the activation state of endothelial NOS, with phosphorylation of serine residue 1177 in particular resulting in significantly increased activity (Z.-P. Chen *et al.*, 1999; Dimmeler *et al.*, 1999). In a process referred to as 'uncoupling', the transfer of electrons across endothelial NOS can be removed from L-arginine as the substrate, instead resulting in the reduction of molecular oxygen to superoxide (O_2^-) (Balligand *et al.*, 2009; Forstermann, 2010). A number of different stimuli have been identified as being capable of uncoupling of endothelial NOS: turbulent blood flow (Heo *et al.*, 2011), depletion of cofactors such as BH_4 (Landmesser *et al.*, 2003), reduced L-arginine levels (e.g. increased arginase activity (Kim *et al.*, 2009)) and increased asymmetric dimethylarginine (ADMA, an endogenous inhibitor of endothelial NOS (Vallance *et al.*, 1992)) levels can all induce uncoupling of endothelial NOS, resulting in loss of NO production and O_2^- generation. Additionally, the product of MPO activity, hypochlorous acid, can also induce uncoupling of endothelial NOS (Xu *et al.*, 2006), as can the reactive molecule peroxynitrite (Leopold *et al.*, 2009). The resultant alteration in endothelial NOS function, where O_2^- is produced primarily instead of NO, impacts significantly upon net vascular function and also on platelet aggregability, contributing significantly to the development cardiovascular disease.

1.2.1.2 Nitric Oxide as an Effector Molecule: Activation of Soluble Guanylate Cyclase

Once generated, NO is able to influence cellular function through 3',5'-cyclic guanosine monophosphate (cGMP)-dependent and -independent mechanisms. cGMP-independent mechanisms include post-translational modifications such as S-nitrosylation, whereby the formation of S-nitrosothiols at protein cysteine residues directly alter protein activity (Godecke *et al.*, 2008; Hill *et al.*, 2010). cGMP-dependent mechanisms involve the activation of soluble guanylate cyclase (sGC, Figure 2), accumulation of cGMP and subsequent cellular effects through the increased activity of cGMP-dependent protein kinases (PKG).

Structurally, sGC is a heterodimer comprised of α and β subunits, with a prosthetic heme group attached to the β subunit (Garthwaite, 2010; Potter, 2011). Binding of NO to sGC is redox dependent, requiring heme to be in a reduced state (i.e. Fe^{2+}) in order to enable NO binding (Schrammel *et al.*, 1996). Once bound, NO induces a structural shift in sGC that allows the binding of guanosine triphosphate (GTP), which subsequently is converted to cGMP with the release of inorganic pyrophosphate (Garthwaite, 2010; Potter, 2011).

The capacity of sGC to generate cGMP from GTP is subject to oxidative impairment, which can take a variety of forms. Oxidation and/or depletion of heme from sGC will render the enzyme unable to bind NO, thus preventing its activation (Ignarro *et al.*, 1982; Mellion *et al.*, 1983; Schrammel *et al.*, 1996; Weber *et al.*, 2001). Furthermore, post-translational

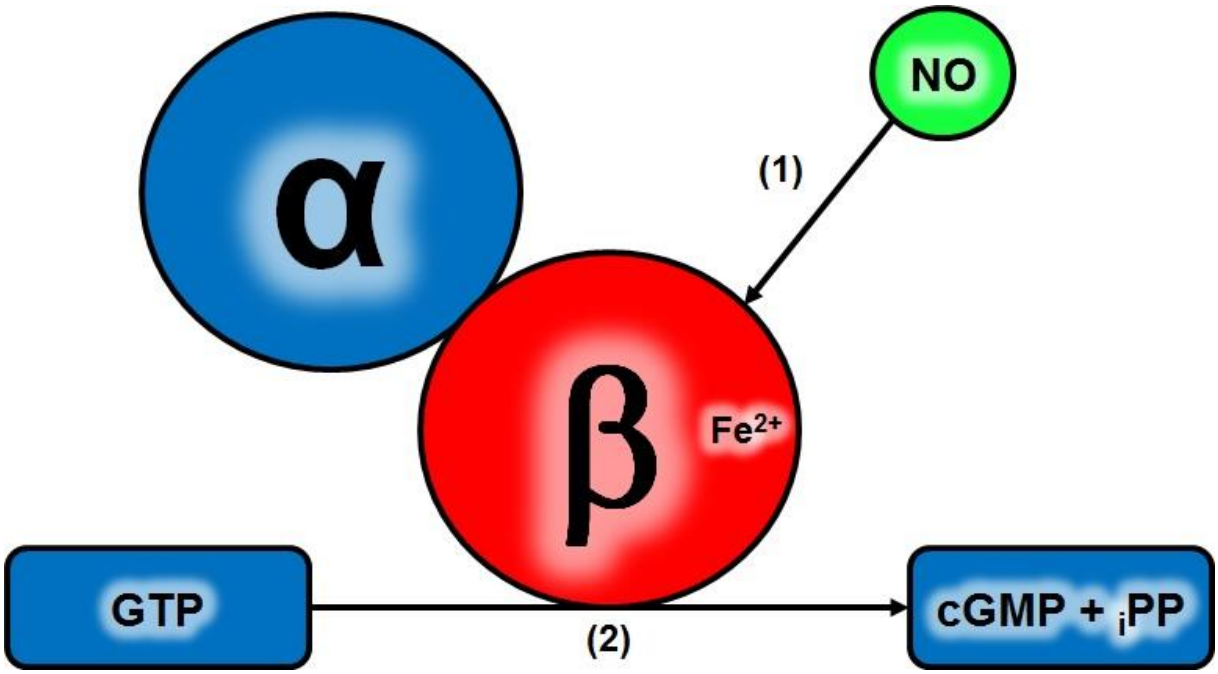

Figure 2: Structural representation of the soluble guanylate cyclase enzyme. Binding of nitric oxide to the heme containing β subunit (1) enables the catalytic conversion of GTP to cGMP (2).

modification of cysteine residues on the α and β subunits of sGC has also been shown to have inhibitory effects upon the activity of sGC: oxidation and/or S-nitrosylation of thiols significantly impairs the ability of sGC to generate cGMP (Brandwein *et al.*, 1981; Maron *et al.*, 2009; Sayed *et al.*, 2007; Sayed *et al.*, 2008).

1.2.1.3 Other Sources of Impairment of the Nitric Oxide Signalling Pathway

Bioavailability of NO can be diminished by ROS. Specifically, the free radical O_2^- may "scavenge" NO, thus contributing to the impairment of this signalling pathway (Figure 3). Furthermore, peroxynitrite, formed from the reaction of O_2^- with NO, inhibits sGC activity through oxidation of heme (Weber *et al.*, 2001). Potential sources of ROS include the mitochondrial electron transport chain, NADPH oxidase and xanthine oxidase, as well as uncoupled endothelial NOS. Angiotensin II is known to stimulate NADPH oxidase activity (Dikalov *et al.*, 2014; Lassegue *et al.*, 2001), aldosterone stimulates increased mitochondrial

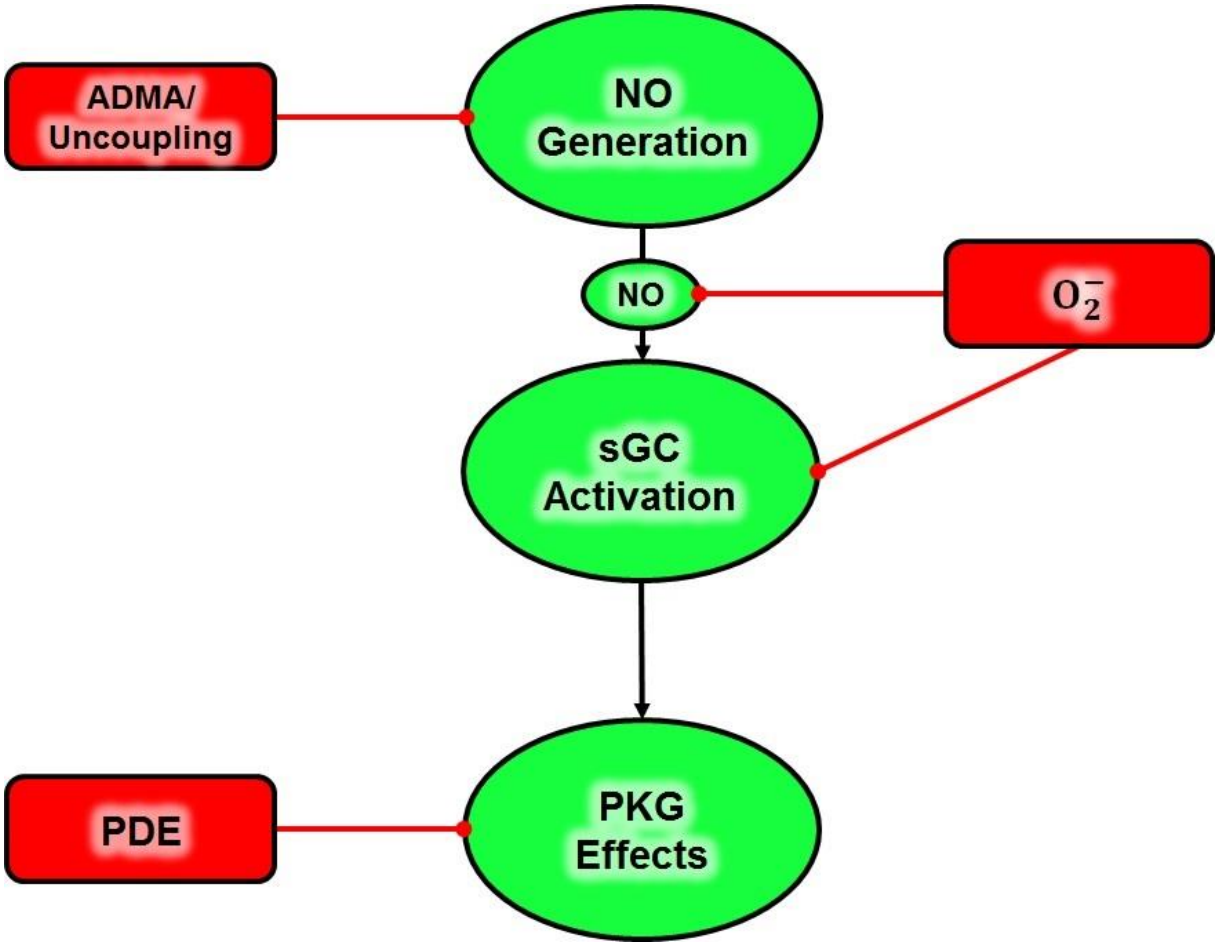

Figure 3: Other potential sources of impairment of the nitric oxide signalling pathway. Inhibition and/or uncoupling of endothelial nitric oxide synthase, scavenging of nitric oxide and/or oxidation of soluble guanylate cyclase, and aberrant phosphodiesterase activity may all contribute to loss of nitric oxide effect.

ROS generation (Nolly *et al.*, 2014), while presence of the arrhythmia promotes xanthine oxidase activity (Dudley *et al.*, 2005). Due to the strong association of AF with increased

sympathetic and/or parasympathetic activity (Jayachandran *et al.*, 2000; Sharifov *et al.*, 2004; Workman, 2010), catecholamine-induced ROS generation is also a possibility that may contribute to the scavenging of available NO. Catecholamine stimulation has been observed to increase NADPH oxidase activity and induce endothelial NOS uncoupling in vascular smooth muscle (Bleeke *et al.*, 2004) and endothelial cells (J. L. Mehta *et al.*, 2001), as well as in cardiomyocytes (Lu *et al.*, 2009; Xiao *et al.*, 2002).

Clearance of cGMP occurs via a group of enzymes known as phosphodiesterases. Altered activity and/or expression of phosphodiesterases have been observed in the pathogenesis of cardiovascular disease, most notably heart failure. For example, up-regulation of phosphodiesterase 2 (cAMP/cGMP phosphodiesterase), associated with β-adrenergic receptor signalling, has been implicated in the pathogenesis of heart failure (Lehnart *et al.*, 2005; Mehel *et al.*, 2013; Molina *et al.*, 2012), while up-regulation of phosphodiesterase 5, a cGMP specific phosphodiesterase, has been observed in right ventricular hypertrophy (Nagendran *et al.*, 2007). However, no direct impact upon the NO signalling pathway has been suggested. Phosphorylation of phosphodiesterase 5 (promoting increased clearance of cGMP) in platelets is associated with rapid desensitization of the NO signalling pathway (Mullershausen *et al.*, 2001), however this phenomenon has not yet been observed in the pathological settings of cardiovascular disease. To date, no link has been established between potentially aberrant phosphodiesterase activity and development of impaired NO signalling, in the context of cardiovascular disease.

1.2.1.4 Therapeutic Amelioration of Impaired Nitric Oxide Signalling

Therapeutic strategies that result in the restoration of impaired NO signalling provide clinical benefit: improvements in vascular tone and platelet homeostasis are apparent (Chirkov *et al.*, 2007). The use of NO donors such as sodium nitroprusside (SNP) or organic nitrates may restore NO availability. However, these agents do not address the major impairment of the

NO signalling cascade, NO resistance, in the target tissue. Additionally, organic nitrates are subject to the development of nitrate tolerance (Chirkov *et al.*, 1997; Chirkov *et al.*, 1999). In the context of AF, a number of different strategies may be employed with the net effect of improving NO signalling:

a) Successful reversion of AF to sinus rhythm has been shown to improve endothelial function (Takahashi *et al.*, 2001; Yoshino *et al.*, 2013) and reduce platelet reactivity. This effect is likely due to the restoration of laminar flow and subsequent up-regulation/re-coupling of endothelial NOS (Cai *et al.*, 2002; Goette *et al.*, 2012; Heo *et al.*, 2011), resulting in improved vascular tone and platelet reactivity.

b) Angiotensin-converting enzyme (ACE) inhibitors improve endothelial function and ameliorate impaired platelet NO signalling (Anderson *et al.*, 2000; Chirkov *et al.*, 2004; Hornig *et al.*, 2001; Sverdlov *et al.*, 2013a; Willoughby *et al.*, 2012). Likely mechanisms include a reduction in activation of the RAAS which, as reflected through plasma Ang II levels, is a potent stimulator of ROS production (Dikalov *et al.*, 2014; Luo *et al.*, 2010; Veresh *et al.*, 2008). Alternatively, blockade of the RAAS may result in increased expression of the O_2^- scavenger, superoxide dismutase (Hornig *et al.*, 2001).

c) Statin therapy has also been shown to restore NO signalling under conditions where it is impaired: use of statins ameliorates endothelial dysfunction (Ceriello *et al.*, 2005; Holowatz *et al.*, 2011), and has improved platelet response to NO in patients with ACS (Stepien *et al.*, 2003). These effects are likely distinct from the lipid lowering benefits of statin therapy (Landmesser *et al.*, 2005), and instead could be due to a reduction in ADMA levels (Ocuz *et al.*, 2008; W. Xia *et al.*, 2009), down-regulation of angiotensin type 1 receptors resulting in decreased activation of NADPH oxidase (Wassmann *et al.*, 2001), or by stimulation of endothelial NOS expression (Meda *et al.*, 2010; Murata *et al.*, 2005).

1.2.2 Endogenous Modulation of Nitric Oxide Signalling: Pathological Implications

1.2.2.1 Asymmetric and Symmetric Dimethylarginine

Post-translational methylation of arginine occurs while arginine residues are still bound up in proteins, as a result of the activity of protein arginine methyltransferases (PRMTs). PRMTs catalyse the methylation of arginine residues and can be grouped into two major types: type 1, primarily responsible for formation of ADMA and type 2, primarily responsible for formation of symmetric dimethylarginine (SDMA) (Bedford *et al.*, 2005; Vallance *et al.*, 2004). Both types of PRMT are capable of generating monomethylarginine. The activity of these enzymes is stimulated in the presence of various cardiovascular risk factors and/or diseases, such as diabetes mellitus (Y. Chen *et al.*, 2009), elevated plasma lipid levels (Boger *et al.*, 2000), hypertension (Osanai *et al.*, 2003) and conditions of oxidative stress (Jia *et al.*, 2006), all of which can result in higher levels of methylarginines being generated. Clearance of ADMA occurs via renal excretion, or metabolically through the actions of dimethylarginine dimethylaminohydrolases (DDAH) which convert ADMA to L-citrulline and dimethylamine (Bedford *et al.*, 2005; Blackwell, 2010; Teerlink *et al.*, 2009; Vallance *et al.*, 2004). These enzymes themselves are vulnerable to oxidative impairment (Luo *et al.*, 2010), contributing significantly to accumulation of ADMA.

Plasma ADMA levels have an established relationship with cardiovascular disease and outcomes. Elevated plasma ADMA levels have been observed in hypertension (Achan *et al.*, 2003; Kielstein, Donnerstag, *et al.*, 2006), diabetes mellitus (Lin *et al.*, 2002), stroke (Yoo *et al.*, 2001) and AF (Goette *et al.*, 2012; Lim *et al.*, 2013), as well as being predictive of adverse cardiovascular events (Horowitz *et al.*, 2013; Siegerink *et al.*, 2013). The link between ADMA and pathogenesis of cardiovascular disease is thought to be due to its ability to competitively inhibit NOS activity (Vallance *et al.*, 1992) and contribute to endothelial dysfunction (Antoniades *et al.*, 2009; Boger *et al.*, 2009). ADMA may also induce uncoupling of endothelial NOS, resulting in decreased NO generation, increased ROS generation

(Antoniades *et al.*, 2009; Druhan *et al.*, 2008), and may also induce activation of NADPH oxidase (Veresh *et al.*, 2008). Intriguingly, expression of the inflammatory enzyme MPO (implicated in the pathogenesis of AF (V. Rudolph *et al.*, 2010)), correlates with ADMA concentrations in clinical populations (van der Zwan *et al.*, 2011), an effect that may be due to oxidative impairment of DDAH by MPO (von Leitner *et al.*, 2011).

SDMA is the structural isomer of ADMA and similarly, is formed through post-translational modification of arginine residues on proteins (Bedford *et al.*, 2005; Vallance *et al.*, 2004). ADMA has long been known to be an endogenous inhibitor of NOS (Vallance *et al.*, 1992). In contrast, SDMA was thought to be physiologically inert and, due to the belief that plasma SDMA was eliminated almost entirely via the kidneys, was thought to be a reasonable measure of renal function (Kielstein, Salpeter, *et al.*, 2006). However, not only has metabolic elimination of SDMA via the enzyme alanine-glyoxylate aminotransferase 2 (Agxt2) been identified (Kittel *et al.*, 2013), but evidence is accumulating supporting a physiological role for SDMA. SDMA has been shown to stimulate production of tumour necrosis factor-α (TNFα), an effect inhibited by treatment with the hypochlorous acid scavenger, *N*-acetylcysteine (Schepers *et al.*, 2009). SDMA has also been shown to stimulate ROS generation in monocytes (Schepers *et al.*, 2011). The physiological role of SDMA would appear to be intimately involved with generation of, and signalling by, ROS – and by extension, may impact upon NO effect via the "scavenging" of available NO by ROS (Chirkov *et al.*, 2007; Rajendran *et al.*, 2008).

1.2.2.2 Myeloperoxidase

MPO is an enzyme released from neutrophils upon activation as a component of the neutrophil "oxidative burst", which contributes to oxidative stress associated with neutrophil infiltration of tissues (Suzuki *et al.*, 2003; Yamashita *et al.*, 2010). Increased plasma MPO levels have been associated with incidence of cardiovascular disease in population studies:

people deficient for expression of MPO experienced much lower incidence of cardiovascular disease, at the expense of being more susceptible to infection (Kutter *et al.*, 2000). MPO is able to catalyse the oxidation of high- and low-density lipoproteins (van der Veen *et al.*, 2009), contributing significantly to the development of atherosclerotic plaques (Leopold *et al.*, 2009). MPO activity has also been implicated in the development of endothelial dysfunction. Treatment of hospital inpatients with enoxaparin improved endothelial function with a concomitant increase in plasma MPO, implying that enoxaparin had 'liberated' MPO from the endothelium, thus restoring endothelial function (T. K. Rudolph *et al.*, 2010). The possible mechanisms underlying this phenomenon include restored Ca^{2+} homeostasis (Cook *et al.*, 2012), or recoupling of endothelial NOS (Xu *et al.*, 2006). MPO has also been implicated in the pathogenesis of AF: infusion of Ang II in mice induced AF in an MPO-dependent mechanism, an effect that paralleled clinical studies observing increased atrial MPO content in individuals with AF (V. Rudolph *et al.*, 2010).

MPO is able to influence NO signalling. Generation of hypochlorous acid by MPO can induce the uncoupling of endothelial NOS (Xu *et al.*, 2006), resulting in loss of NO production and ROS generation. Additionally, MPO is also capable of catabolizing NO (Eiserich *et al.*, 2002) via the formation of reactive nitrogen species (Eiserich *et al.*, 1998).

1.2.2.3 Thrombospondin-1

Thrombospondin-1 (TSP-1) is a matricellular protein found primarily in platelets, and to a lesser extent in leukocytes (Jaffe *et al.*, 1985; Suchard *et al.*, 1991) and endothelial cells (Kirsch *et al.*, 2010). Due to the presence of numerous binding sites, TSP-1 is considered a somewhat "promiscuous" protein, capable of influencing various cellular processes through binding to different receptors. TSP-1 has been implicated in a number of settings as having a modulatory effect on the vascular and/or myocardial remodelling that results in the

development of cardiovascular disease. TSP-1, through binding to the receptor CD47, contributes to the development of pulmonary arterial hypertension in animal models through uncoupling of endothelial NOS and/or activation of NADPH oxidase (P. M. Bauer *et al.*, 2012; Csanyi *et al.*, 2012). These findings correlated with higher levels of TSP-1 and CD47 being found in lung tissue of patients with pulmonary arterial hypertension (P. M. Bauer *et al.*, 2012). In contrast, TSP-1 appears to have a limiting effect on the development of cardiac hypertrophy in diabetic cardiomyopathy (Gonzalez-Quesada *et al.*, 2013) or in conditions resulting in pressure overloaded myocardium (Y. Xia *et al.*, 2011). TSP-1 also has a pivotal role in mediating the recruitment (Kirsch *et al.*, 2010; Mansfield *et al.*, 1990), adhesion (Narizhneva *et al.*, 2005) and tissue infiltration (Liu *et al.*, 2001) of inflammatory cells.

A large body of work supports the role of TSP-1 in the regulation of vascular tone through modulation of the NO signalling pathway. Upon binding to the receptor CD47, TSP-1 has been shown to inhibit the phosphorylation of endothelial NOS, thus limiting its activity and contributing to increased blood pressure (E. M. Bauer *et al.*, 2010; Isenberg *et al.*, 2009). TSP-1 can also modulate the activation of sGC (Miller *et al.*, 2010; Ramanathan *et al.*, 2011). TSP-1 has been shown to attenuate NO signalling in leukocytes (Ridnour *et al.*, 2007), platelets (Isenberg, Romeo, *et al.*, 2008), endothelium (Isenberg *et al.*, 2005) and vascular smooth muscle (Isenberg *et al.*, 2006) cells.

1.2.2.4 Thioredoxin-interacting Protein

Txnip is an α-arrestin, pro-inflammatory protein that is intimately associated with regulating the activity of the oxidoreductase thioredoxin (Nishiyama *et al.*, 1999; Patwari *et al.*, 2006). As such, Txnip has an integral role in maintaining cellular redox status.

Expression of Txnip is strongly up-regulated in conditions of high glucose, an effect associated with increased ROS generation, caspase activation and initiation of apoptosis in

pancreatic β cells (Cha-Molstad *et al.*, 2009; J. Chen *et al.*, 2008; Minn *et al.*, 2005; P. C. Schulze *et al.*, 2004). The mechanism underlying this effect is likely the ability of Txnip to displace thioredoxin from binding to apoptosis signal-regulating kinase 1, thus allowing the subsequent initiation of apoptosis (Liu *et al.*, 2002; Saxena *et al.*, 2010). In the pathogenesis of cardiovascular disease, Txnip has diverse roles. Vascular Txnip expression is up-regulated by non-laminar flow and stimulates the expression of cell adhesion molecules and subsequent recruitment of inflammatory cells (X. Q. Wang *et al.*, 2012; Yamawaki *et al.*, 2005). Txnip expression is also up-regulated in a rabbit model of aortic valve stenosis (Ngo *et al.*, 2008), an effect attenuated by administration of the ACE inhibitor ramipril (Ngo *et al.*, 2011). Conversely, Txnip expression is down-regulated in cardiomyocytes in response to myocardial ischemia (Xiang *et al.*, 2005) or biomechanical strain (Yoshioka *et al.*, 2004), allowing for promotion of thioredoxin-dependent growth and survival. Txnip has also been identified as a bridge between oxidative stress and inflammation, as it is an integral component to activation of the nod-like receptor protein 3 (NLRP3) inflammasome and production of interleukin-1β (Zhou *et al.*, 2010).

Txnip has an interesting relationship with NO. Expression of Txnip is suppressed at the level of mRNA transcription by NO under conditions of normoglycaemia (P. C. Schulze *et al.*, 2006), an effect that is potentiated by insulin signalling (in pancreatic β cells) (Shaked *et al.*, 2009). Reciprocally, through regulation of thioredoxin activity, Txnip has already demonstrated its capacity for modulating cGMP-independent mechanisms of NO signalling such as S-nitrosation (Forrester *et al.*, 2009). Similar mechanisms may also (theoretically) apply to the regulation of sGC activity. The oxidation of thiols on the α and β subunits of sGC have been shown to impair its ability to generate cGMP (Brandwein *et al.*, 1981; Maron *et al.*, 2009): Txnip, via its regulation of the activity of thioredoxin (Nishiyama *et al.*, 1999), can influence the oxidative state of these thiols. Txnip has been implicated in the induction of apoptosis in pancreatic β cells (J. Chen *et al.*, 2008; Minn *et al.*, 2005); this effect is

dependent, in part, on the subcellular localization of Txnip (Saxena *et al.*, 2010). Recent work has identified that nitrosative or oxidative stress, such as might occur in cardiovascular diseases, has a significant effect on the subcellular localization of thioredoxin; this effect is dependent on Txnip (Ogata *et al.*, 2013).

1.3 Scope of the Present Study

The presence of a hypercoagulable state in AF has been supported by numerous clinical studies. Kamath *et al* observed that soluble glycoprotein V and β-thromboglobulin (both markers of platelet activation) and fibrin D-dimer (a marker of fibrinolysis) were all elevated in the presence of AF, compared to sinus rhythm patients (Kamath, Blann, *et al.*, 2002; Kamath, Chin, *et al.*, 2002). Indices of inflammation are also elevated in AF: platelet expression of CD40, the vascular adhesion molecules (intercellular adhesion molecule-1 and vascular cell adhesion molecule-1), as well as C-reactive protein (CRP) are all increased in the presence of AF (Hammwohner *et al.*, 2007). Similarly, interleukins-6, -8 and -10, monocyte chemotactic protein-1, vascular endothelial growth factor, B-type natriuretic peptide (BNP) and TNFα were all increased in AF (Li *et al.*, 2010), as were indicators of oxidative stress, oxidized glutathione and cysteine (Neuman *et al.*, 2007). *However, when it comes to correlating indicators of inflammation and thrombogenicity with measures of thromboembolic risk in AF, available data are much more limited.*

Plasma high sensitivity CRP levels have been observed to correlate with $CHADS_2$ scores (Maehama *et al.*, 2010), as has D-dimer (Ohara *et al.*, 2008) and von Willebrand Factor (Roldan *et al.*, 2005). Additionally, the presence of left atrial distension, decreased left atrial flow velocity and presence of left atrial thrombus has been found to directly correlate with $CHADS_2$ scores (Maehama *et al.*, 2010; Ohara *et al.*, 2009). *To date, studies have not*

established a link between absolute measures of platelet reactivity and incremental clinical indicators of thromboembolic risk.

Notably, the importance of the integrity of inhibitory signalling pathways, such as the NO signalling pathway, remains a relatively unexplored area when investigating thrombogenicity in AF. Given the association of impaired NO signalling with development of cardiovascular disease, and that potential mechanisms contributing to the development of "NO resistance" are all present in AF (see section 1.2.1), this seems to be an obvious priority. Also of note is a recently identified familial mutation in the 'α' subunit of sGC, impairing NO signalling and predisposing its carriers to myocardial infarction due to increased thrombus formation (Erdmann *et al.*, 2013).

It was the intention of the current project, therefore, to evaluate the integrity of platelet NO signalling in AF, specifically seeking to identify correlates of clinical indicators of incremental thromboembolic risk. Furthermore, endogenous modulators of NO signalling were investigated for their potential pathological effects in the setting of AF (see section 1.2.2).

Specifically, the current project sought to evaluate the following hypotheses (expanded upon in section 2.8.1):

1) Platelet NO signalling in AF patients will be modulated, in part, any or all of ADMA, MPO, TSP-1 and Txnip.
2) Platelet NO signalling in AF patients will correlate inversely with clinical indices of thromboembolic risk.

These questions were addressed *a priori* in a cohort of AF patients admitted to hospital, thus enabling detailed clinical characterization, evaluation of platelet function (i.e. aggregability and response to the inhibitory effect of NO donor), and determination of plasma levels of ADMA, MPO, TSP-1 and platelet Txnip content.

In addressing this gap in the understanding of the pathology accompanying AF, it was hoped that biochemical measures of incremental thromboembolic risk, as well as novel therapeutic strategies, might be identified and subsequently developed for evaluation in clinical contexts.

2: Methods

2.1 Patient Recruitment

The investigation was conducted as a single centre mechanistic sub-study of SAFETY (Standard vs. Atrial Fibrillation spEcific managemenT studY), an investigation of non-pharmacological management strategies in patients hospitalised with AF (Carrington *et al.*, 2013; Stewart *et al.*, 2014). Patients were considered for inclusion if they were admitted to hospital for reasons relating to AF. Exclusion criteria for SAFETY were: age < 45 years, primary diagnosis of valvular heart disease, scheduled catheter ablation, pre-existing NYHA class III – IV heart failure with a documented left ventricular ejection fraction (LVEF) < 45%, alcohol-induced AF and terminal illness requiring palliative care. Additionally, patients receiving P_2Y_{12} receptor antagonists were excluded from the investigation due to the impact of such agents on platelet function. As the primary objective of the current project was to evaluate platelet NO signalling in atrial fibrillation patients, there was no perceived need to exclude patients on aspirin therapy from the current analysis. Indeed, previous research has established that aspirin therapy does not interfere with the methods employed in this study to determine platelet NO response (Chirkov *et al.*, 1999). Furthermore, no significant interaction between aspirin therapy and platelet aggregability was observed in the present study (see section 3).

The study was approved by the institutional Ethics of Human Research committee and written informed consent was obtained in all cases.

2.2 Patient Venesection

Venesection was performed upon patients following enrolment into SAFETY at a median of 38 [7, 94] days post index enrolment as well as at 11.8 [11.4, 12.1] months follow-up. Patients were required to be resting, in a recumbent position with venous blood accessed via an antecubital vein using a slow draw technique in order to minimize platelet activation.

Collected blood was dispensed into EDTA, heparin or acid citrate anticoagulant (4mM citric acid, 6mM trisodium citrate, final concentrations) and mixed by inversion. Further manipulation of blood samples is detailed according to each relevant section.

2.3 Whole Blood Impedance Aggregometry

Whole blood impedance aggregometry was performed in this investigation as described previously (Cardinal *et al.*, 1980). Whole blood impedance aggregometry is a technique that measures platelet aggregation as the change in electrical resistance across a closed system. Electrical probes containing exposed portions of conducting wires are inserted into test samples and a constant voltage (millivolts) applied. A platelet monolayer forms across the exposed wires, resulting in a stable baseline impedance, or electrical resistance, value being obtained. The addition of agonists capable of inducing platelet aggregation results in the accumulation of platelets onto this monolayer, which is reflected by increasing resistance within the system (proportional to the extent of platelet aggregation). This technique allows for the *ex vivo* assessment of platelet function. Specific procedures will be detailed below.

2.3.1 Platelet Response to Adenosine Diphosphate

Venous blood was collected into 10ml tubes containing 1ml acid citrate anticoagulant (4mM citric acid, 6mM trisodium citrate, final concentrations). After collection, blood was allowed to sit at room temperature for 20 minutes prior to aggregation studies. Aggregation was assessed using dual-channel or quad-channel impedance aggregometers (Chrono-log Corporation, series 500 or series 700). Prior to aggregation, 450µl whole blood was added to 500µl 0.9% (w/v) NaCl in 1ml polystyrene cuvettes (Chrono-log Corporation, US) and warmed to 37^{o}C. Electrodes were inserted into the samples and stirred using siliconized stir bars (Chrono-log Corporation, US) at a speed of 900rpm. After an equilibration period of approximately 2 minutes, the aggregometer was calibrated for each sample relative to a

resistance of 20Ω. Aggregation was induced using adenosine diphosphate (ADP) at a final concentration of 2.5μM. Extent of aggregation was assessed as the net change in resistance, measured in ohms (Ω). All tests were run in duplicate.

2.3.2 Platelet Response to Sodium Nitroprusside

In order to assess the function of the sGC signalling pathway in platelets, the nitric oxide donor SNP was used. Blood samples were prepared as outlined previously and incubated with 10μM SNP for 1 minute prior to addition of ADP. The inhibitory effect of SNP on platelet aggregation was calculated as a percentage, comparing maximal aggregation in the presence and absence of SNP.

2.4 Determination of Plasma Asymmetric Dimethylarginine, Symmetric Dimethylarginine and L-arginine

Plasma ADMA, SDMA and L-arginine concentrations were determined by high performance liquid chromatography as previously described (Heresztyn *et al.*, 2004). Blood was collected into heparinised anticoagulant tubes and placed on ice. Samples were centrifuged at 1800g for 15 minutes at 4^{o}C. The plasma was collected and stored at -70^{o}C until assayed. The reported limits of detection for this assay were 100nM for ADMA and SDMA, with inter-assay CV of 6% for ADMA, 4.5% for SDMA.

2.4.1 Sample Extraction and Derivatization

Plasma samples were diluted by adding 150μl of plasma to 1.4ml of distilled H_2O, as well as 60μl of 5μg/ml N-monomethyl-L-arginine (L-NMMA) as an internal control. Plasma proteins were precipitated from solution by addition of 300μl 10% (w/v) 5-sulfosalicyclic acid and incubated on ice for 10 minutes. Samples were centrifuged at 9000g for 2 minutes at room temperature and the supernatant retained. Samples underwent solid phase extraction using a

Gilson GX-274 ASPEC Liquid Handler (run using Trilution LH version 2.0 software, Gilson) and Bond Elut SCX cartridges (Agilent Technologies, US). The solid phase extraction cartridges were washed in 0.1M phosphate buffer, pH 6.0 and methanol prior to eluting the analytes with 2% (w/v) triethylamine/65% (v/v) methanol in distilled H_2O. The eluent was evaporated under nitrogen gas at 55^oC and reconstituted in distilled H_2O. Samples were centrifuged at 9000g for 2 minutes at room temperature and 50µl supernatant transferred into fresh vials to be derivatized using the AccQ-Fluor Reagent Kit (Waters, UK).

2.4.2 Chromatographic Separation and Fluorescent Detection

Samples were loaded onto an 1100 series HPLC system (Agilent Technologies, US) with a 1200 series fluorescence detector (Agilent Technologies, US) using a 717plus Autosampler (Waters, UK) maintained at 12^oC and the analytes separated on a Luna 5µm C18(2) column (Phenomenex, US) using a gradient of 4% (v/v) acetonitrile in 0.1M sodium acetate, pH 6.0 (Mobile Phase A) and 30% (v/v) acetonitrile in 0.1M sodium acetate, pH 6.0 (Mobile Phase B) at a flow rate of 1.0ml/min. Column temperature was maintained using a TCM-004055 incubator (Waters, UK) and set at 40^oC for ADMA/SDMA (20µl injection volume) determination and 30^oC for L-arginine (2.5µl injection volume) determination, with separate runs being performed for ADMA/SDMA and L-arginine determinations. Fluorescent detection of derivatized sample was achieved using excitation at λ=250nm and emission at λ=395nm. The system was managed using ChemStation for LC 3D Systems software, version Rev B.03.02[341].

2.4.3 Sample Analysis

Data analysis was also performed using ChemStation for LC 3D Systems software, version Rev B.03.02[341]. Generation of standard curves involved measuring the area under the curve for known concentrations of ADMA, SDMA and L-arginine and calculating ratios relative to

the area under the curve for the L-NMMA internal standard. Sample ADMA, SDMA and L-arginine concentrations were calculated from sample: internal standard ratios using the standard curve to determine ADMA, SDMA and L-arginine concentrations.

2.5 Determination of Plasma Myeloperoxidase

Plasma myeloperoxidase levels were determined by ELISA according to manufacturer's instruction (Mercodia, Sweden). Briefly, blood was collected into heparinised anticoagulant and placed on ice. Samples were centrifuged at 1800g for 15 minutes at 4^{o}C. The supernatant was retained and centrifuged again at 10000g for 10 minutes at 4^{o}C. The supernatant was collected and stored at -70^{o}C until assayed. Samples were assayed in triplicate and coefficients of variation determined from 5 replicate samples over 5 consecutive assays. The reported limit of detection for this assay was 3.66ng/ml, intra-assay CV was 7.6% and inter-assay CV was 8.6%.

2.6 Determination of Plasma Thrombospondin-1

Plasma thrombospondin-1 levels were determined by ELISA according to manufacturer's instructions (Quantikine®, R&D Systems, and US). Briefly, blood was collected into heparinised anticoagulant and placed on ice. Samples were centrifuged at 1800g for 15 minutes at 4^{o}C. The supernatant was retained and centrifuged again at 10000g for 10 minutes at 4^{o}C. The supernatant was collected and stored at -70^{o}C until assayed. Samples were assayed in duplicate with coefficients of variation determined from 4 replicate samples over 3 consecutive runs. The reported limit of detection was 0.949ng/ml, intra-assay CV was 2.8% and inter-assay CV was 3.2%.

2.7 Determination of Platelet Thioredoxin-interacting Protein Content

Platelet Txnip content was determined semi-quantitatively using immunohistochemistry (see Figure 4) as previously described (Sverdlov *et al.*, 2013f) and validated using immunoblotting (see Figure 5).

2.7.1 Immunohistochemistry

2.7.1.1 Slide Preparation

Blood was collected into EDTA anticoagulant and platelet rich plasma generated by centrifuging at 130g for 7 minutes at 22^{o}C. 10µl of platelet rich plasma was smeared onto untreated slides (in triplicate) and allowed to air dry at room temperature before being fixed using 4% (w/v) paraformaldehyde in PBS for 30 minutes at room temperature. The slides were air dried at room temperature then stored at -70^{o}C until assayed.

2.7.1.2 Thioredoxin-interacting Protein Determination

Slides were thawed at room temperature and washed 3 times in PBS, then blocked using 100µl 20% (v/v) goat serum in PBS for 30 minutes at room temperature. The blocking solution was discarded, 100µl 5µg/ml polyclonal rabbit anti-human Vitamin D3 Up-regulated Protein (Invitrogen™, Life Technologies, US), 1% (w/v) BSA in PBS was added and the slides incubated overnight at 2-4^{o}C. The slides were then washed 3 times in PBS and 100µl 1.5mg/ml RPE-conjugated monoclonal mouse anti-human CD41 (Dako, US) in PBS and 100µl 0.81mg/ml FITC-conjugated polyclonal swine anti-rabbit IgG (Dako, US) added per slide. Slides were incubated for 60 minutes in the dark before being washed 3 times in PBS.

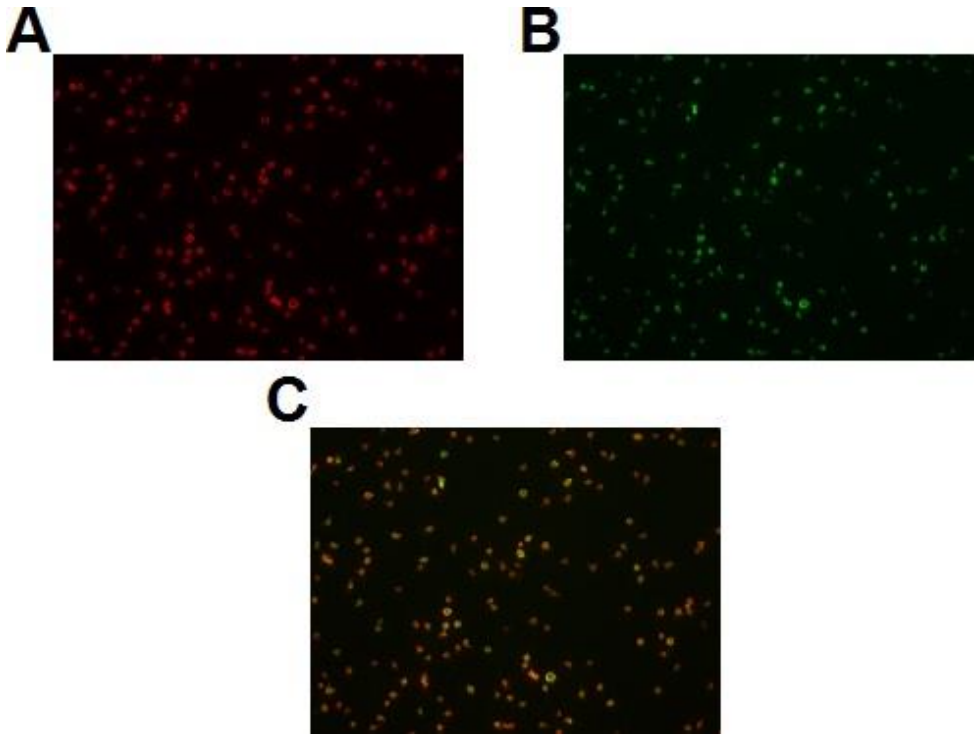

Figure 4: Determination of platelet Txnip via immunohistochemistry. **A**, CD41, **B**, Txnip, **C**, CD41 and Txnip (merged images).

Fluorescence was developed by adding 'fluorescent mounting medium' (Dako, US) to each slide and the slides incubated for a further 10 minutes in the dark. Images were acquired at 400x magnification using an Axio Scope.A1 microscope with apotome and AxioVision 4.8 software (Zeiss, Germany). Images were analysed for densitometric fluorescence using AxioVision LE software. The intra-assay CV was 8.49% and the inter-assay CV was 18.62%.

2.7.2 Immunoblotting

Blood was collected in EDTA anticoagulant and platelet rich plasma was generated by centrifuging at 130g for 7 minutes at 22^{o}C. The plasma was collected and centrifuged at 800g for 15 minutes at 22^{o}C. The pellet was retained and washed twice in 36mM Citric Acid, 5mM Glucose, 5mM KCl, 90mM NaCl, 1μM Prostaglandin E_1, pH 6.5 at 800g for 15 minutes at 22^{o}C. The pellet was lysed in 20mM Tris-HCL, 150mM NaCl, 1mM EDTA, 1mM EGTA, 2.5mM $Na_4P_2O_7$, 0.93mM β-glycerophosphate, 1% (v/v) Triton X-100, 1% (v/v) Phosphatase Inhibitor Cocktail 1 (Sigma, US), 1% (v/v) Phosphatase Inhibitor Cocktail 2 (Sigma, US), 1%

(v/v) Protease Inhibitor Cocktail (Sigma, US), pH 7.4 and stored at -70^{o}C. Protein determination was done according to the Bicinchronic Acid Protein Determination Assay (Sigma, US). Samples were solubilised using Laemmli buffer containing β-mercaptoethanol (Bio-Rad Laboratories, US) by heating at 95^{o}C for 5 minutes and loaded at a concentration of 1mg/ml (20μg protein in total per lane). Proteins were resolved using discontinuous SDS-PAGE with a 4% stacking gel (4.5% (w/v) bis-acrylamide, 97mM Tris-HCl, 1% (w/v) SDS, pH 6.8) and a 12.5% resolving gel (12.5% (w/v), 38mM Tris-base, 1% (w/v) SDS, pH 8.8) by applying a constant current of 40mA (Bio-Rad Laboratories, US) in cold running buffer (0.3% (w/v) Tris-base, 1.4% (w/v) Glycine, 0.1% (w/v) SDS). Proteins were transferred from the acrylamide gel onto nitrocellulose membrane with 0.2μm pore size (Advantec MFS Inc., US) in cold transfer buffer (25mM Tris-Base, 192mM Glycine, 20% (v/v) methanol) using a constant current of 380mA for 2 hours. The nitrocellulose membrane was then blocked overnight in 5% (w/v) milk powder, 100mM Tris-base, 150mM NaCl, 0.1% (v/v) TWEEN, pH 7.4 at 2-4^{o}C. The nitrocellulose membrane was washed 3 times in 100mM Tris-base, 150mM NaCl, 0.1% (v/v) TWEEN, pH 7.4 prior to being incubated with 125ng/ml polyclonal rabbit anti-human Vitamin D3 Upregulated Protein (Invitrogen™, Life Technologies, US), 2% (w/v) skim milk in 100mM Tris-base, 150mM NaCl, 0.1% (v/v) TWEEN, pH 7.4 for 60 minutes at room temperature. The membrane was washed 3 times in 100mM Tris-base, 150mM NaCl, 0.1% (v/v) TWEEN, pH 7.4 before being incubated with 40ng/ml horseradish peroxidase-conjugated monoclonal goat anti-rabbit IgG (Santa Cruz Biotechnology, US) 2% (w/v) skim milk in 100mM Tris-base, 150mM NaCl, 0.1% (v/v) TWEEN, pH 7.4 for 60 minutes. The nitrocellulose membrane was washed 3 times in 100mM Tris-base, 150mM NaCl, 0.1% (v/v) TWEEN, pH 7.4. Membrane chemiluminescence was developed using Amersham™ ECL Advance™ Western Blotting Detection Kit (GE Healthcare, UK) and imaged using an LAS-4000 luminescent imager (GE Healthcare, UK) and Image Reader

version 2.0 software (FujiFilm, Japan). Images were analysed using Multi Gauge version 3.0 software (FujiFilm, Japan).

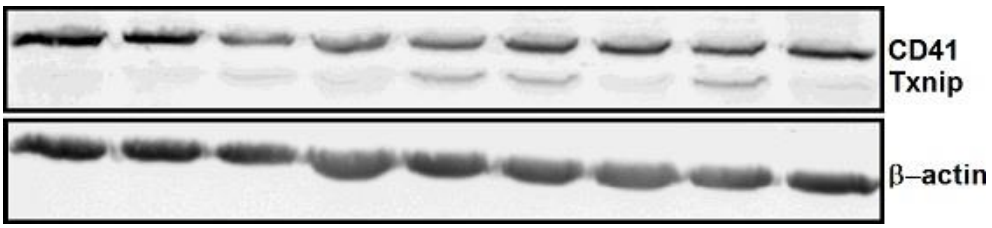

Figure 5: Example blots showing CD41, Txnip and β-actin protein bands.

2.7.3 Validation of Immunohistochemistry using Immunoblotting for Determination of Platelet Thioredoxin-interacting Protein Content

Semi-quantitative determination of platelet Txnip levels by immunohistochemistry was validated by use of western blotting. Platelet Txnip was determined from patient samples using immunohistochemistry and western blotting techniques. The values generated by each technique were normalised such that the minimum and maximum values for each technique were equivalent to 0% and 100%, respectively. These values were used to evaluate the agreement between techniques via Pearson correlation (Figure 6) and bias between techniques using Bland-Altman analysis (Figure 7).

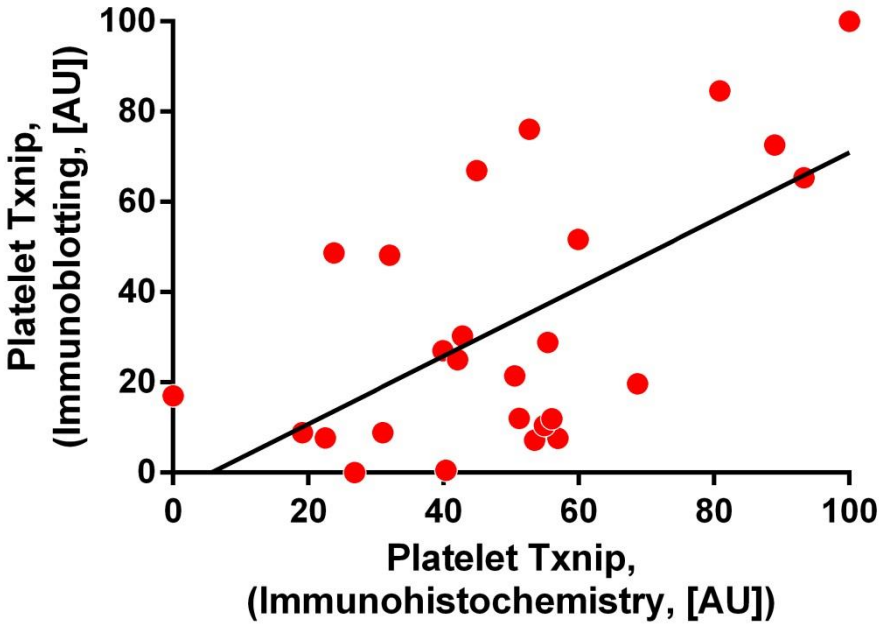

Figure 6: Agreement between immunohistochemical and immunoblotting techniques for determination of platelet Txnip content. A significant positive correlation (r=0.612, p<0.001, n=26) was observed between immunohistochemical and western blotting techniques for determination of platelet Txnip levels.

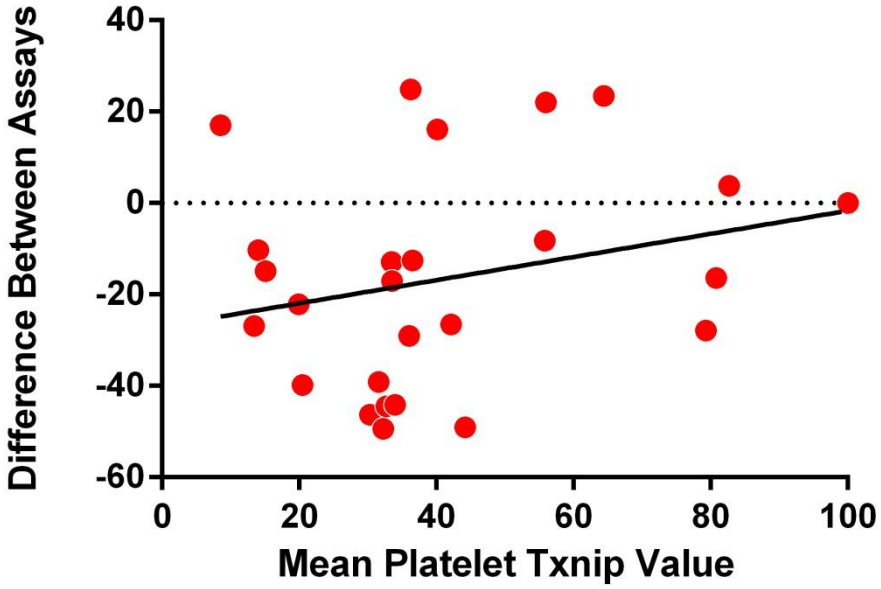

Figure 7: Bias between immunohistochemical and immunoblotting techniques for determination of platelet Txnip content. Bland-Altman analysis indicated there was some bias (r=0.253, p=0.212) towards over-estimation of platelet Txnip at lower levels by immunohistochemical techniques, compared to immunoblotting techniques.

2.8 Statistical Methods

2.8.1 Statistical Analysis

This study tested the following mechanistic (null) hypotheses regarding the population assessed:

(1) Platelet response to NO is independent of concentrations of:

   a) ADMA

   b) MPO

   c) TSP-1

   d) Txnip

This hypothesis sought to evaluate the biochemical interactions modulating the anti-aggregatory effects of NO in this population.

(2) Platelet response to NO is independent of "stroke risk" scores:

   a) $CHADS_2$

   b) CHA_2DS_2VASc

(3) While the primary endpoint for SAFETY was stroke/TIA/systemic embolism, it was uncertain whether sufficient of these events would occur in the current sub-study to permit evaluation of relationship with NO response.

(4) It was also intended that the study identify significant determinants of platelet NO response in this population. Apart from the biochemical parameters in (1), specific focus was directed to patient age and sex, and to presumptive acuity of AF. *Given that the evaluation of potential determinants of NO response was not equivalent to prospective hypothesis testing, the study design included the capacity to subsequently and specifically test any association that emerged, using a separate "validation" cohort of patients, and employing a prospective, hypothesis-testing design. An example of this is given on page 75, section 3.2.5.*

Plasma levels of ADMA, MPO, TSP-1 and Txnip were determined at baseline and follow-up and evaluated with respect to adverse outcomes in patients with AF and for impact upon platelet NO signalling.

Data are reported as mean ± S.E.M or median (interquartile range, [IQR]) where appropriate. Normality of distribution for continuous variables was assessed using the Shapiro-Wilk test. Comparisons between groups were performed using non-paired/paired t-test or Mann-Whitney U/Wilcoxon Signed Rank test, as appropriate. ANOVA, mixed models ANOVA, ANCOVA or independent samples Kruskal-Wallis test were used for categorical variables with more than 2 levels. Differences in frequencies between groups were assessed using the χ^2 test. Univariate correlates between continuous variables were assessed using Pearson or spearman correlation, or linear regression. Where possible, transformed data (using Log, Ln, Lnγ or $\surd$ functions) were used for non-Gaussian distributions. Multivariate analysis was performed using backward stepwise multiple logistic regression or multiple logistic regression. Data were analysed using IBM SPSS Statistics 19 and GraphPad Prism 5 software packages.

2.8.2 Estimated Power of the Analysis

The primary measure within this study was platelet response to nitric oxide. From a cohort of 106 patients, mean platelet response to NO was 22.9±28.4 [SD]% inhibition. In order to detect a difference of 1 standard deviation associated with differing demographics or treatment with 80% confidence at α=5%, a population of n=34 would be required. For a hypothetical population of n=40 individuals per group, the probability of detecting a significant difference can be observed in figure 8.

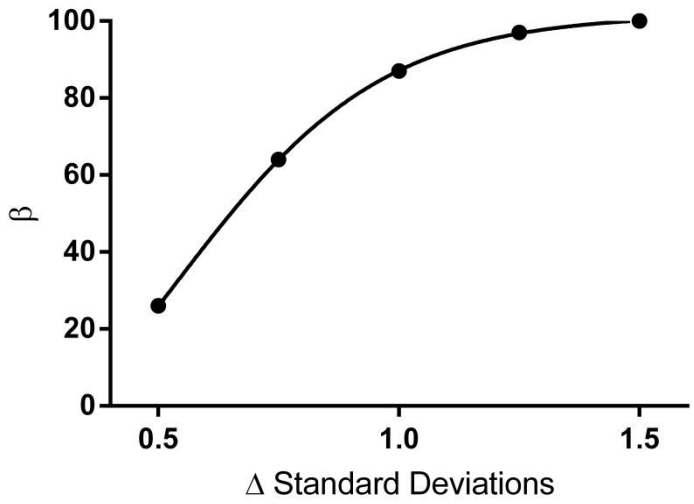

Figure 8: Sensitivity curve for detecting significant (α=5%) differences between two groups comprised of n=40 individuals each.

Therefore, based upon these assumptions, this study had a 26% chance of detecting a 14.2% (0.5SD) difference between groups, 64% chance of detecting a 21.3% (0.75SD) difference between groups and an 87% chance of detecting a 28.4% (1.0SD) difference between groups.

3: Results

3.1 Functional Integrity of Platelet Nitric Oxide Signalling in Atrial Fibrillation

The primary study sought to evaluate platelet function in the context of AF by evaluating the integrity of platelet NO signalling in these patients. The overall intention of this study was to identify biochemical factors in a cohort of AF patients which might potentially contribute to heterogeneity of associated thromboembolic risk, focussing on potential modulators of NO signalling. Since the cohort studied here was only 106 patients followed for a median of 11.8 months, it was recognised prospectively that the actual thromboembolic rate within the study would not permit direct clinical validation of these biochemical variables.

3.1.1 Patient Demographics and Clinical Events: Primary Study Cohort

3.1.1.1 Patient Characteristics

The characteristics of the study population are summarised in Table 4. Both genders were equally represented in this cohort, while marked heterogeneity was observed regarding age, admission heart rate, plasma CRP and plasma creatinine concentrations. Overall, the cohort was likely to be at relatively low thromboembolic risk (as reflected by their $CHADS_2$/CHA_2DS_2VASc scores) with hypertension followed by advancing age being the most common clinical risk factors for stroke. Of the cohort, 21.7% presented with 'new onset AF': that is, the *de novo* detection and diagnosis of AF.

Table 4: Clinical characteristics of the population included in this study

Socio-demographic Profile	**Total Cohort (n=106)**
Gender, n (% male)	54 (50.9)
Age (years)	72 [65, 81]
Aged ≥ 75 years, n (%)	46 (43.4)
Comorbidities	
Congestive Heart Failure, n (%)	9 (8.5)
Hypertension, n (%)	74 (69.8)
Diabetes Mellitus, n (%)	27 (25.5)
Prior Stroke/TIA, n (%)	14 (13.2)
Clinical Presentation	
Admission Heart Rate (bpm)	89 [71, 130]
LVEF (%)	59 [52, 65]$_{(n=62)}$
Plasma Creatinine (μM)	82 [67, 111]
Plasma CRP (mg/l)	3.4 [1.5, 9.1]
$CHADS_2$ Score	2 [1, 2]
$CHADS_2$ = 0, n (%)	15 (14.2)
$CHADS_2$ = 1-2, n (%)	68 (64.2)
$CHADS_2$ ≥ 3, n (%)	23 (21.7)
CHA_2DS_2VASc Score	3 [2, 4]
CHA_2DS_2VASc = 0, n (%)	5 (4.7)
CHA_2DS_2VASc = 1, n (%)	15 (14.2)
CHA_2DS_2VASc ≥ 2, n (%)	86 (81.1)
New onset AF, n (%)	23 (21.7)

Note: n = number of participants, (%) = proportion of total cohort, median [IQR].

Table 5 summarises the major forms of pharmacotherapy for this group of patients (at time of hospital discharge). At the time of initiation of the study, warfarin was the only oral anticoagulant generally available. As regards agents which might potentially modify platelet response to NO (Stepien *et al.*, 2003; Willoughby *et al.*, 2012), approximately half the cohort were receiving statin therapy and one third ACE inhibitor therapy. No patients were on non-steroidal anti-inflammatory therapy for the duration of the study.

Table 5: Medication use in the study population

Pharmacotherapy: Summary	
Antithrombotic therapy	
Aspirin, n (%)	34 (32.1)
Warfarin, n (%)	63 (59.4)
Rate and/or Rhythm Control therapy	
Class 3 Anti-arrhythmics, n (%)	23 (21.7)
Class 1c Anti-arrhythmics, n (%)	6 (5.7)
β Receptor Antagonists, n (%)	64 (60.4)
Digoxin, n (%)	37 (34.9)
Calcium Channel Antagonists, n (%)	28 (26.4)
RAAS Inhibitors	
ACE Inhibitors, n (%)	35 (33.0)
Angiotensin Receptor Antagonists, n (%)	28 (26.4)
Other Medications	
Statins, n (%)	55 (51.9)
Diuretics, n (%)	36 (34.0)
Proton Pump Inhibitors, n (%)	28 (26.4)
Metformin, n (%)	16 (15.1)
Nitrates, n (%)	12 (11.3)
Paracetamol, n (%)	12 (11.3)
Opioid Receptor Agonists, n (%)	10 (9.4)

Note: n = number of participants, (%) = proportion of total cohort.

Low rates of antiplatelet and oral anticoagulant therapy were observed in the cohort, perhaps reflective of the relatively mild stroke risk observed in the population (Table 4). Rate control strategies were more common than rhythm control strategies and RAAS inhibition was employed in approximately 66% of the population in the form of ACE inhibitor or angiotensin-receptor antagonist therapies.

3.1.1.2 Clinical Events

It is important to recognise at the outset that this study was not designed to correlate patient demographics, biochemistry/physiology, and outcome events in AF. Although such an evaluation would be desirable, it would require a far larger data set. However, it was felt appropriate to record clinical events, with biochemical correlations despite the limited value of such analysis.

Within this cohort six deaths occurred from cardiovascular (n=3) and non-cardiovascular (n=3) causes during a median follow-up of 11.8 months. **No stroke/TIA was observed during this period.** The characteristics of surviving/non-surviving patients are compared in Table 6. Notably, patients who died appeared to be more elderly, with elevated plasma creatinine and CRP concentrations compared to the rest of the cohort. Due to the limited sample size, this portion of the analysis was not considered further.

Among the overall cohort, 67.0% experienced hospital readmission on at least one occasion with median length of stay being 2.2 [1.6, 4.5] days. As summarised in Table 7, no clinical difference between readmission and non-readmission groups was apparent. Additionally, no significant difference was observed for platelet aggregation, platelet response to NO, plasma levels of ADMA, MPO, TSP-1, or platelet Txnip content (Table 8).

From the overall cohort, 58.5% underwent emergency hospital readmission, with a median length of stay of 2.0 [1.3, 5.0] days. 42.1% of these readmissions were cardiac related, with 40.9% of cardiac-related admissions being as a result of complications due to AF. Clinical characteristics were similar between groups (Table 9). Similarly, biochemical characteristics did not differ significantly between patients who experienced emergency hospital readmission with those who did not (Table 10). However, in this case, elevated platelet Txnip content was of borderline ($p=0.052$) significance as a correlate of emergency readmission.

Evaluation of potential determinants of emergency hospital readmission via multiple logistic regression, including platelet aggregation, platelet response to NO, plasma MPO concentrations and platelet Txnip content indicated that none of these factors were significant correlates, although a trend was observed in the case of elevated Txnip (OR 1.105, 95% CI: 0.985-1.240, $p=0.088$).

Using linear regression, age ($r=0.299$, $p<0.05$), $CHADS_2$ score ($r=0.341$, $p<0.01$), plasma SDMA ($r=0.331$, $p<0.01$) and platelet response to NO ($r=0.321$, $p<0.05$) were all found to correlate directly with increasing mean length of stay during emergency readmission, whereas

plasma ADMA: SDMA ratio was inversely correlated ($r=-0.273$, $p<0.05$). However, none of these univariate correlates were found to be determinants of emergency hospital readmission mean length of stay upon backward stepwise multiple logistic regression.

Table 6: Clinical characteristics of patients who died during follow-up compared with those who did not

Socio-demographic Profile	**Non-Deceased Cohort (n=100)**	**Deceased Cohort (n=6)**
Gender, n (% male)	50 (50.0)	4 (66.7)
Age (years)	72 [63, 81]	79 [72, 83]
Aged ≥ 75 years, n (%)	42 (42.0)	4 (66.7)
Comorbidities		
Congestive Heart Failure, n (%)	6 (6.0)	3 (50.0)
Hypertension, n (%)	71 (71.0)	3 (50.0)
Diabetes Mellitus, n (%)	25 (25.0)	2 (33.3)
Prior Stroke/TIA, n (%)	14 (14.0)	0 (0.0)
Clinical Presentation		
Admission Heart Rate (bpm)	91 [72, 131]	77 [61, 111]
LVEF (%)	60 [55, 65]$_{(n=59)}$	45 [42, 45]$_{(n=3)}$
Plasma Creatinine (μM)	80 [67, 105]	138 [101, 220]
Plasma CRP (mg/l)	3.3 [1.5, 8.5]	15.5 [5.3, 30.2]
$CHADS_2$ Score	2 [1, 2]	2
CHA_2DS_2VASc Score	3 [2, 4]	3 [3, 4]
New onset AF, n (%)	23 (23.0)	0 (0.0)

Note: n = number of study participants, (%) = proportion of deceased/non-deceased cohorts.

Table 7: Clinical characteristics among patients who underwent hospital readmission compared with those who did not

Socio-demographic Profile	**Readmission (n=71)**	**No Readmission (n=35)**	**p***
Gender, n (% male)	32 (45.1)	22 (62.9)	0.085
Age (years)	72 [65, 81]	72 [62, 82]	0.801
Aged ≥ 75 years, n (%)	31 (43.7)	15 (42.9)	0.937
Comorbidities			
Congestive Heart Failure, n (%)	7 (9.9)	2 (5.7)	0.472
Hypertension, n (%)	48 (67.6)	26 (74.3)	0.481
Diabetes Mellitus, n (%)	16 (22.5)	11 (31.4)	0.323
Prior Stroke/TIA, n (%)	9 (12.7)	5 (14.3)	0.818
Clinical Presentation			
Admission Heart Rate (bpm)	95 [70, 133]	84 [73, 116]	0.521
LVEF	60 [51, 64]$_{(n=38)}$	60 [52, 68]$_{(n=24)}$	0.607
Plasma Creatinine (μM)	81 [66, 112]	87 [71, 106]	0.745
Plasma CRP (mg/l)	3.9 [1.7, 12.0]	2.7 [1.2, 6.4]	0.069
$CHADS_2$ Score	2 [1, 2]	2 [1, 2]	0.562
CHA_2DS_2VASc Score	3 [2, 4]	3 [2, 4]	0.802
New onset AF, n (%)	16 (22.5)	7 (20.0)	0.766

Note: n = number of study participants, (%) = proportion of readmission/non-readmission patients, * = χ^2 test.

Table 8: Biochemical characteristics among patients who underwent hospital readmission and those who did not

Biochemical Parameters	Readmission (n=71)	No Readmission (n=35)	p
Platelet Aggregation (Ω, males)	7.3 [5.0, 9.6]	8.2 [5.8, 11.4]	0.418
Platelet Aggregation (Ω, females)	9.8 [7.2, 12.2]	9.6 [6.5, 11.3]	0.416
Platelet Response to NO (% inhibition)	19.7±3.7	29.2±4.7	0.127
Plasma ADMA (nM)	625±13	612±23	0.611
Plasma SDMA (nM)	584 [504, 906]	572 [448, 788]	0.508
Plasma ADMA: SDMA Ratio	1.02±0.03	1.01±0.05	0.984
Plasma MPO (ng/ml)	54 [44, 65]	59 [47, 71]	0.206
Plasma TSP-1 (ng/ml)	150 [106, 222]	120.12 [74, 224]	0.159
Platelet Txnip (AU)	337 [251, 420]	267 [170, 382]	0.085

Note: n = number of study participants, (%) = proportion of readmission/non-readmission patients, independent samples t-test for normal variables, Mann-Whitney U test for non-normal variables.

Table 9: Clinical characteristics among patients who experienced emergency hospital readmission compared with those who did not

Socio-demographic Profile	**Emergency Readmission (n=62)**	**No Emergency Readmission (n=44)**	**p**
Gender, n (% male)	29 (46.8)	25 (56.8)	0.308
Age (years)	72 [65, 81]	73 [63, 81]	0.695
Aged ≥ 75 years, n (%)	26 (41.9)	20 (45.5)	0.719
Comorbidities			
Congestive Heart Failure, n (%)	6 (9.7)	3 (6.8)	0.603
Hypertension, n (%)	43 (69.4)	31 (70.5)	0.903
Diabetes Mellitus, n (%)	15 (24.2)	12 (27.3)	0.720
Prior Stroke/TIA, n (%)	8 (12.9)	6 (13.6)	0.913
Clinical Presentation			
Admission Heart Rate (bpm)	96 [71, 137]	84 [72, 117]	0.371
LVEF (%)	59 [49, 65]$_{(n=35)}$	60 [53, 68]$_{(n=27)}$	0.326
Plasma Creatinine (μM)	82 [67, 114]	84 (68, 104)	0.700
Plasma CRP (mg/l)	3.9 [1.9, 11.5]	3.1 [1.2, 7.5]	0.114
$CHADS_2$ Score	2 [1, 2]	2 [1, 2]	0.829
CHA_2DS_2VASc Score	4 [2, 4]$_{(n=58)}$	3 [2, 4]	0.663
New onset AF, n (%)	13 (21.0)	10 (22.7)	0.829

Note: n = number of study participants, (%) = proportion of readmission/non-readmission patients, χ^2 test for categorical variables, Mann-Whitney U test for continuous variables.

Table 10: Biochemical characteristics among patients who experienced emergency hospital readmission and those who did not

Biochemical Parameters	Emergency Readmission (n=62)	No Emergency Readmission (n=44)	p*
Platelet Aggregation (Ω, males)	6.4 [4.6, 9.3]	8.2 [5.8, 11.4]	0.112
Platelet Aggregation (Ω, females)	10.0 [8.3, 12.7]	9.0 [4.8, 11.2]	0.085
Platelet Response to NO (% inhibition)	19.1±4.0	28.2±4.1	0.127
Plasma ADMA (nM)	623±14	617±20	0.807
Plasma SDMA (nM)	601 [511, 813]	573 [456, 747]	0.323
Plasma ADMA: SDMA	1.00±0.03	1.03±0.05	0.618
Plasma MPO (ng/ml)	54 [42, 66]	58 [48, 72]	0.120
Plasma TSP-1 (ng/ml)	149 [105, 223]	130 [91, 223]	0.584
Platelet Txnip (AU)	337 [264, 423]	276 [173, 393]	0.052

Note: n = number of study participants, (%) = proportion of readmission/non-readmission patients, independent samples t-test for normal variables, Mann-Whitney U test for non-normal variables.

.

3.1.2 Platelet Aggregation and its Determinants in Atrial Fibrillation

This and platelet NO response represent the central areas of investigation in the current thesis.

3.1.2.1 Platelet Aggregation

Platelet aggregability in AF was assessed through the use of 2.5μM ADP. Median platelet aggregation was 8.9 [5.6, 11.3]Ω for the overall cohort. Consistent with previous reports (Becker *et al.*, 2006; Otahbachi *et al.*, 2010), females displayed increased aggregation compared to males (9.7 [7.5, 11.6]Ω vs. 7.8 [5.4, 10.1]Ω respectively, $p<0.05$, Mann-Whitney U test) (Figure 9). Controlling for gender, neither the antiplatelet agent aspirin ($p=0.674$, two-way ANOVA), nor warfarin ($p=0.155$, two-way ANOVA) significantly interacted with platelet aggregation.

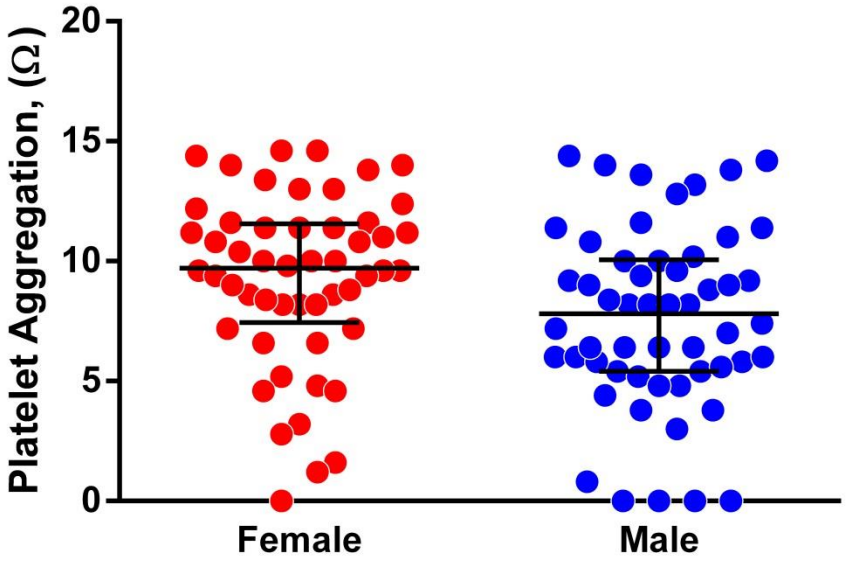

Figure 9: Median platelet aggregation in male and female AF patients. ADP-induced aggregation was 9.7 [7.5, 11.6]Ω in females and 7.8 [5.4, 10.1]Ω in males.

Given that platelet aggregation in response to ADP appeared to be gender-specific in this cohort, clinical and biochemical differences between males and females that may account for this were sought (Table 11). Females displayed increased LVEF and lower plasma creatinine concentrations than males. As expected, CHA_2DS_2VASc score were higher in females than

males. No differences were observed between males and females for biochemical parameters measured (Table 12).

Table 11: Clinical characteristics of male and female atrial fibrillation patients

Socio-demographic Profile	**Males (n=54)**	**Females (n=52)**	**p**
Age (years)	72 [62, 81]	72 [65, 81]	0.485
Aged ≥ 75 years, n (%)	23 (42.6)	23 (44.2)	0.865
Comorbidities			
Congestive Heart Failure, n (%)	3 (5.6)	6 (11.5)	0.269
Hypertension, n (%)	34 (63.0)	40 (76.9)	0.118
Diabetes Mellitus, n (%)	16 (29.6)	11 (21.2)	0.317
Prior Stroke/TIA, n (%)	8 (14.8)	6 (11.5)	0.618
Clinical Presentation			
LVEF (%)	59 [49, 60]	60 [55, 68]	<0.05
Plasma Creatinine (mM)	94 [74, 117]	75 [60, 96]	<0.001
Plasma CRP (mg/l)	2.7 [1.2, 6.5]	4.8 [1.8, 12]	0.058
$CHADS_2$ Score	2 [1, 2]	2 [1, 2]	0.836
CHA_2DS_2VASc Score	3 [1, 4]	4 [3, 5]	<0.01
New onset AF, n (%)	11 (20.4)	12 (23.1)	0.735

Note: Mann-Whitney U test for continuous variables, χ^2 test for categorical variables.

Table 12: Biochemical profiles of male and female atrial fibrillation patients

Biochemical Parameters	Males (n=54)	Females (n=52)	p
Platelet Response to NO (% inhibition)	28.0±4.1	18.1±4.1	0.092
Plasma ADMA (nM)	618±15	623±18	0.803
Plasma SDMA (nM)	603 [492, 743]	582 [484, 826]	0.970
Plasma ADMA: SDMA	1.01±0.04	1.02±0.04	0.942
Plasma MPO (ng/ml)	56 [44, 66]	55 [44, 71]	0.691
Plasma TSP-1 (ng/ml)	134 [95, 223]	155 [104, 221]	0.467
Platelet Txnip (AU)	300 [212, 415]	335 [259, 400]	0.334

Note: independent samples t-test for normal data, Mann-Whitney U test for non-normal data.

Platelet aggregation relative to CHA_2DS_2VASc score is shown in Figure 10. Platelet aggregability did not alter with increasing CHA_2DS_2VASc score in males (H[2] =5.366, p=0.068, Kruskal-Wallis test) or females (Mann-Whitney U test, p=0.208).

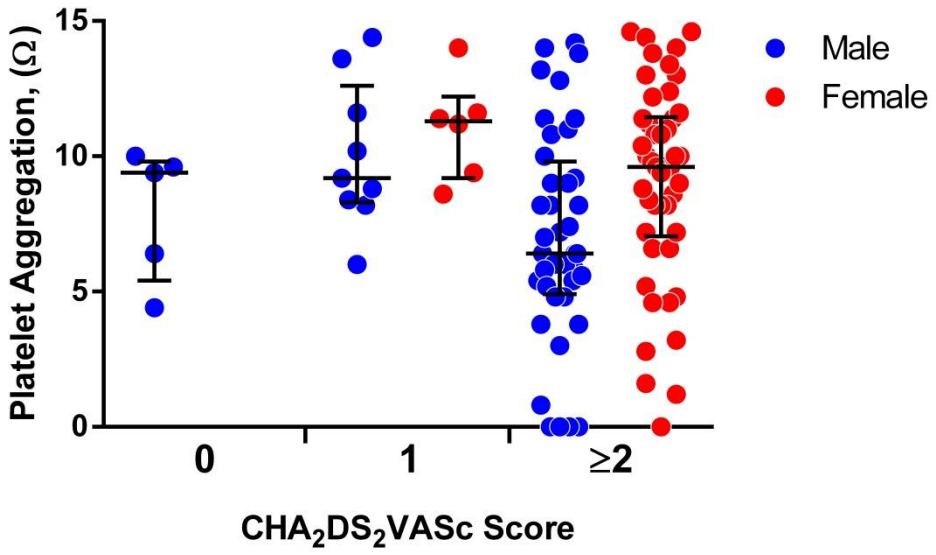

Figure 10: Platelet aggregation in males and females relative to CHA_2DS_2VASc score. Median platelet aggregation for males with CHA_2DS_2VASc score = 0 was 9.4 [5.4, 9.8]Ω, CHA_2DS_2VASc score = 1 was 9.2 [8.3, 12.6]Ω and CHA_2DS_2VASc score ≥ 2 was 6.4 [4.9, 9.8]Ω. Median platelet aggregation for females with CHA_2DS_2VASc score = 1 was 11.3 [9.2, 12.2]Ω and CHA_2DS_2VASc score ≥ 2 was 9.6 [7.1, 11.5]Ω. Note: female atrial fibrillation patients cannot be given CHA_2DS_2VASc = 0.

3.1.2.2 Determinants of Platelet Aggregation in Atrial Fibrillation

In order to determine those mechanisms affecting platelet aggregability in AF, correlates of ADP-induced aggregation were sought within this cohort. Platelet aggregation was positively correlated with admission heart rate and plasma TSP-1 concentrations and inversely correlated with plasma creatinine, ADMA and SDMA concentrations, and platelet response to NO (Figure 11).

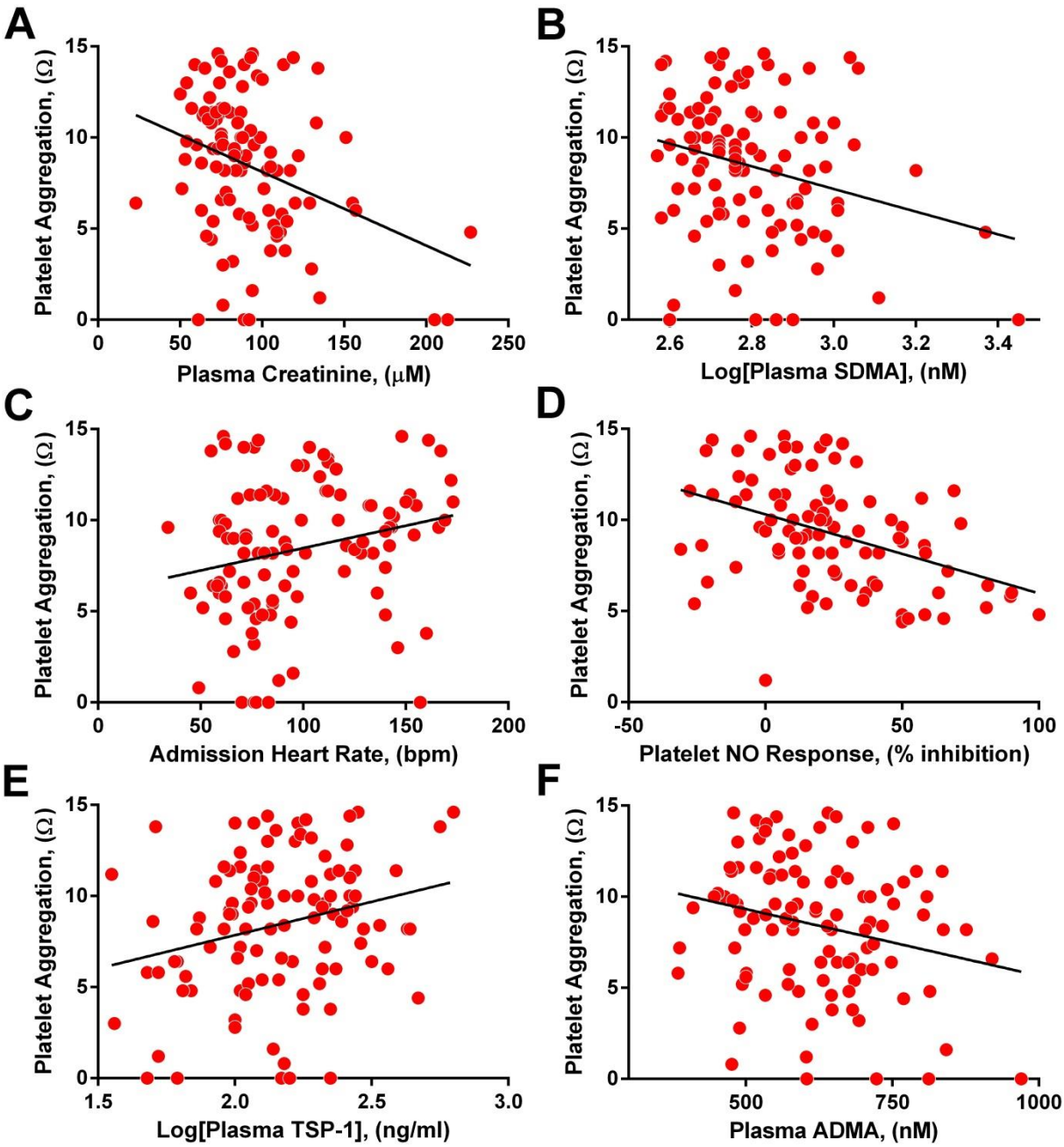

Figure 11: Correlates of ADP-induced aggregation in AF. Platelet aggregation was significantly associated with: **A**, plasma creatinine (r=-0.301, p<0.01, Pearson); **B**, plasma SDMA (r=-0.249, p<0.05, Pearson); **C**, admission heart rate (r=0.247, p<0.05, Pearson); **D**, platelet NO response (r=-0.422, p<0.001, Pearson); **E**, plasma TSP-1 (r=0.254, p<0.01, Pearson); **F**, Plasma ADMA (r=-0.225, p<0.05, Pearson).

Platelet aggregability was also observed to decline with increasing age (Figure 12).

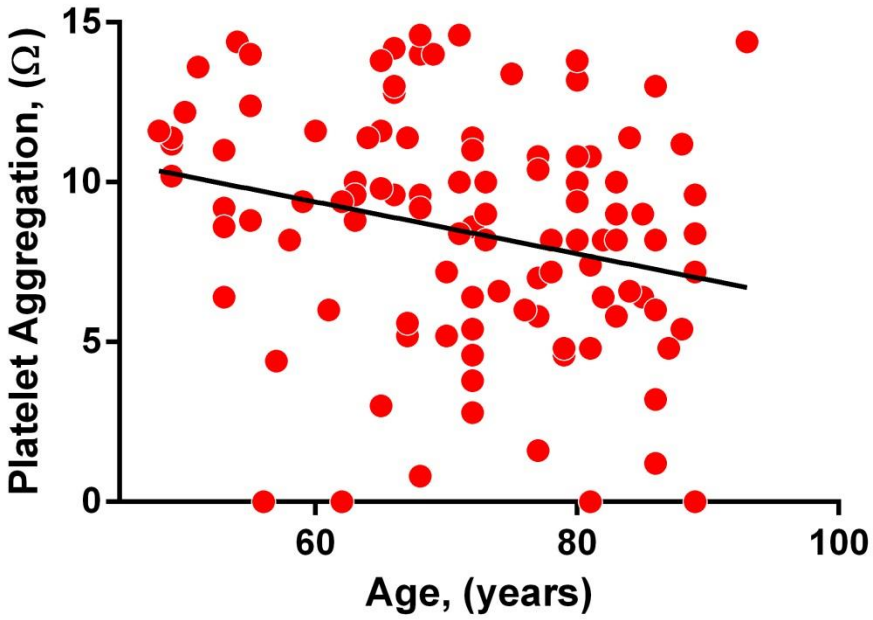

Figure 12: Age and platelet aggregability in AF. ADP-induced aggregation decreased with age in AF patients (r=-0.245, p<0.05, Pearson).

3.1.3 Inhibition of Aggregation by Nitric Oxide and its Determinants

3.1.3.1 Platelet Response to Nitric Oxide

The principal endpoint for this study was platelet response to NO, measured using the NO donor SNP and relative to 2.5μM ADP-induced aggregation. Mean platelet response to NO was 22.9±2.9% inhibition for the overall cohort. New onset AF was associated with significantly diminished platelet response to NO compared to chronic AF (7.7±5.4% inhibition vs. 27.3±3.3% inhibition respectively, p<0.01, independent samples t-test). Although previously documented to potentiate NO signalling (Chirkov *et al.*, 2004; Stepien *et al.*, 2003), neither ACE inhibitor nor statin therapy were observed to have significantly altered platelet response to NO within this cohort. Similarly, neither aspirin (p=0.255, two-way ANOVA) nor warfarin (p=0.789, two-way ANOVA) therapies interacted with platelet NO response.

Platelet response to NO did not change significantly with increasing CHA_2DS_2VASc score (F[2, 91] =1.206, p=0.304, ANOVA) (Figure 13); there was no suggestion of any diminution of NO response with increasing CHA_2DS_2VASc score.

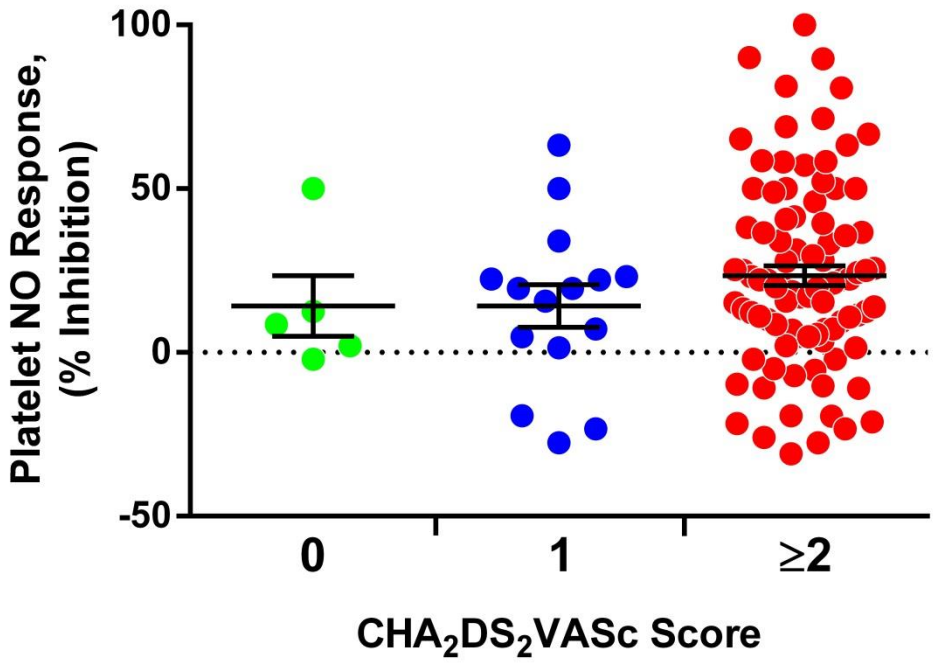

Figure 13: Platelet response to NO stratified according to CHA_2DS_2VASc score. Mean platelet response to NO among patients with CHA_2DS_2VASc score = 0 was 14.2±9.3% inhibition, CHA_2DS_2VASc score = 1 was 14.2±6.5% inhibition and CHA_2DS_2VASc score ≥ 2 was 25.3±3.4% inhibition.

3.1.3.2 Determinants of Nitric Oxide Response: Plasma Levels of Dimethylarginines

In view of the well documented ability of ADMA to impair NO generation via competitive inhibition of the NOS enzyme (see review, (Teerlink *et al.*, 2009)), plasma ADMA levels were determined in this cohort in order to evaluate the potential effect of ADMA on overall platelet function. Plasma SDMA was also determined, as evidence increasingly underlines its relevance as a marker of cardiac events, although data on SDMA in AF are limited, and the physiological role of ADMA remains unclear (Horowitz *et al.*, 2013).

Mean plasma ADMA concentrations were 620±11nM, median plasma SDMA concentrations were 584 [491, 792]nM and the mean ADMA: SDMA ratio was 1.01±0.03 for this cohort. Plasma ADMA (F[2, 103] =4.291, p<0.05, ANOVA) and SDMA (H[2] =10.991 p<0.01,

Kruskal-Wallis test) concentrations were correlated with tertiles of CHA_2DS_2VASC score, while the plasma ADMA: SDMA ratio (F[2, 103] =2.744, p=0.069, ANOVA) was not (Figure 14).

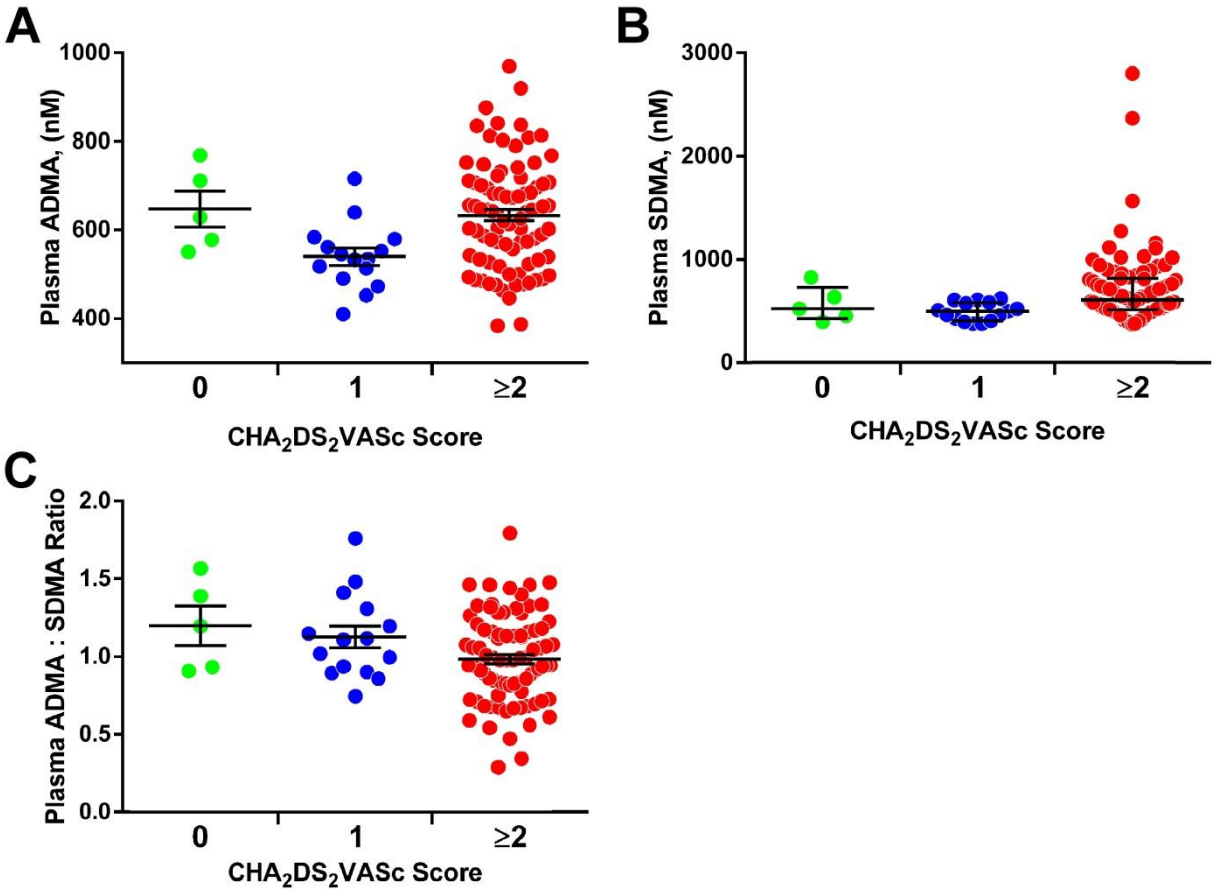

Figure 14: Plasma dimethylated arginine concentrations and their ratio, relative to CHA_2DS_2VASc score. **A**, Plasma ADMA concentrations, **B**, plasma SDMA concentrations, **C**, plasma ADMA: SDMA ratio. Patients with CHA_2DS_2VASc score = 0 had plasma ADMA of 647±41nM, plasma SDMA of 525 [425, 731]nM and plasma ADMA: SDMA ratio of 1.20±0.13. Patients with CHA_2DS_2VASc score = 1 had plasma ADMA of 540±19nM, plasma SDMA of 498 [406, 583]nM and plasma ADMA: SDMA ratio of 1.13±0.07. Patients with CHA_2DS_2VASc score ≥ 2 had plasma ADMA of 633±13nM, plasma SDMA of 609 [513, 820]nM and plasma ADMA: SDMA ratio of 0.98±0.03.

Neither plasma ADMA, nor the plasma ADMA: SDMA ratio (a postulated marker of oxidative stress) correlated with platelet response to NO. However, plasma SDMA was a direct correlate (Figure 15).

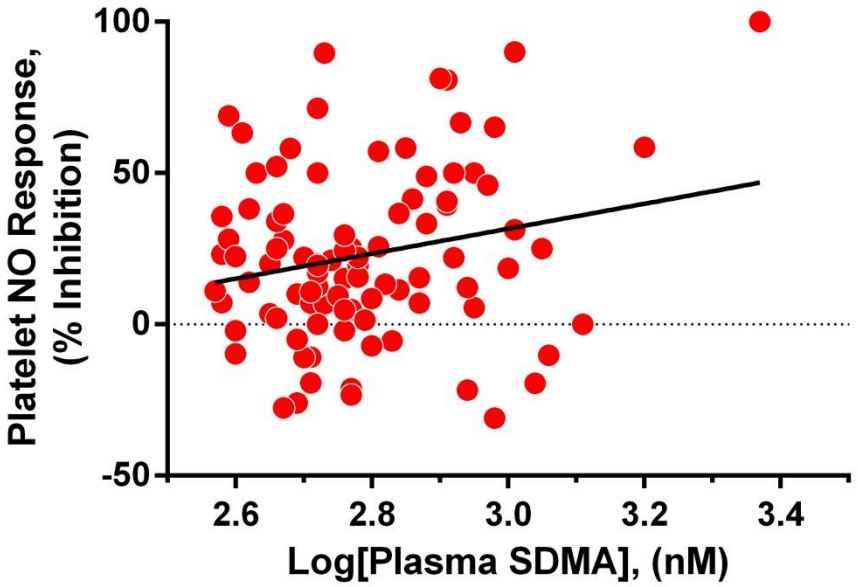

Figure 15: Plasma SDMA and platelet response to NO. Platelet response to NO correlated positively with plasma SDMA (r=0.216, p<0.05, Pearson).

3.1.3.3 Determinants of Nitric Oxide Response: Plasma Levels of Myeloperoxidase

Previous research has identified that the inflammatory enzyme, MPO, is capable of catabolising NO (Eiserich *et al.*, 2002; Eiserich *et al.*, 1998) as well as having a pivotal role in the pathogenesis of AF (V. Rudolph *et al.*, 2010). Because of this, plasma MPO was evaluated in this cohort for potential impact upon platelet NO signalling. Median plasma MPO concentrations in the cohort were 56 [44, 67]ng/ml.

Plasma MPO concentrations did not change with increasing CHA_2DS_2VASc score (H[2] =5.479, p=0.065, Kruskal-Wallis test) (Figure 16).

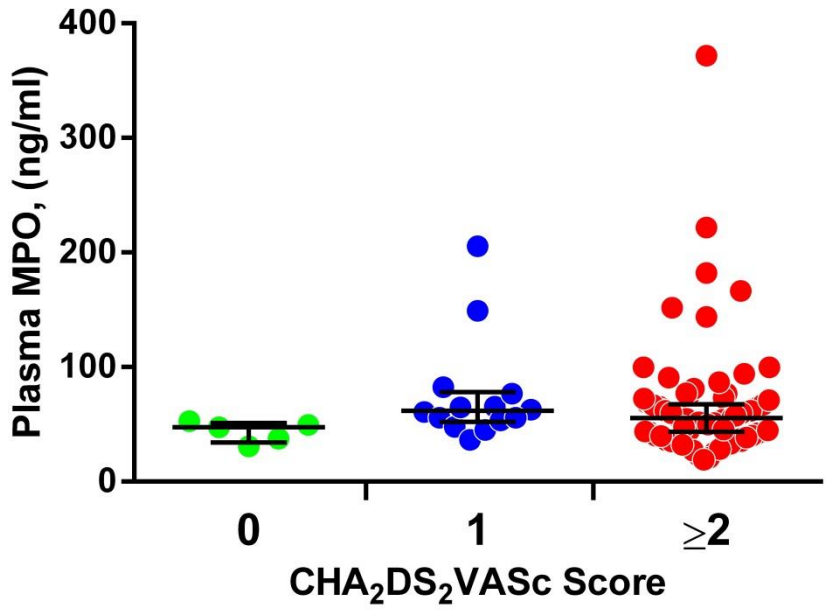

Figure 16: Plasma MPO concentrations, relative to tertiles of CHA_2DS_2VASc score. Plasma MPO concentrations for patients with CHA_2DS_2VASc score = 0 were 48 [34, 51]ng/ml, CHA_2DS_2VASc score = 1 were 62 [52, 78]ng/ml and CHA_2DS_2VASc score ≥ 2 were 55 [43, 68]ng/ml.

Plasma MPO concentrations did not correlate with platelet NO response, though correlated positively with plasma CRP concentrations (r=0.222, p<0.05, Pearson).

3.1.3.4 Determinants of Nitric Oxide Response: Platelet Content of Thioredoxin-interacting Protein

As previously summarised, previous research has identified that Txnip, an endogenous inhibitor of the oxidoreductase thioredoxin, interacts with NO (Forrester *et al.*, 2009; P. C. Schulze *et al.*, 2006). Intracellular platelet Txnip content was therefore determined within this cohort. Median platelet Txnip levels for the cohort were 315 [240, 407]AU. Platelet Txnip content did not vary with increasing CHA_2DS_2VASc score (H[2] =4.367, p=0.113, Kruskal-Wallis test) (Figure 17).

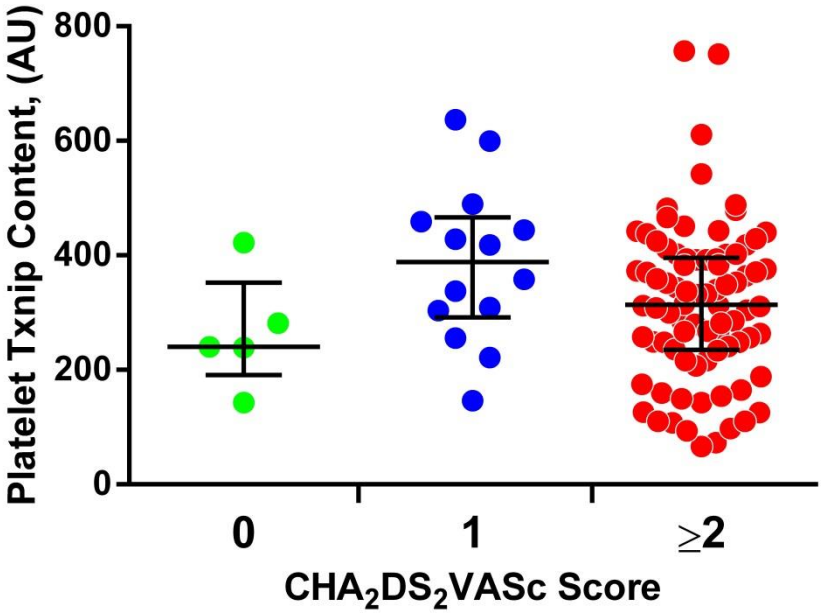

Figure 17: Median platelet Txnip levels in AF according to tertiles of CHA_2DS_2VASc score. Platelet Txnip content for patients with CHA_2DS_2VASc score = 0 was 240 [191, 352]AU, CHA_2DS_2VASc score = 1 was 388 [292, 467]AU and CHA_2DS_2VASc score ≥ 2 was 314 [235, 396]AU.

Evidence exists that pharmacological therapy sometimes used in AF patients may alter Txnip expression (Chai *et al.*, 2012; Sverdlov *et al.*, 2013f). In view of this, both platelet NO response and platelet Txnip content correlations were sought between Txnip levels and therapy with either metformin or ramipril. No significant correlations were found. Similarly, hyperglycaemia, which occurs more frequently in diabetics, stimulates increased Txnip expression. In the current study however, there was no significant variability in Txnip levels according to diabetic status or concentrations of glycosylated haemoglobin.

Consistent with previous observations (Sverdlov *et al.*, 2013f) platelet Txnip content correlated inversely with platelet response to NO (Figure 18).

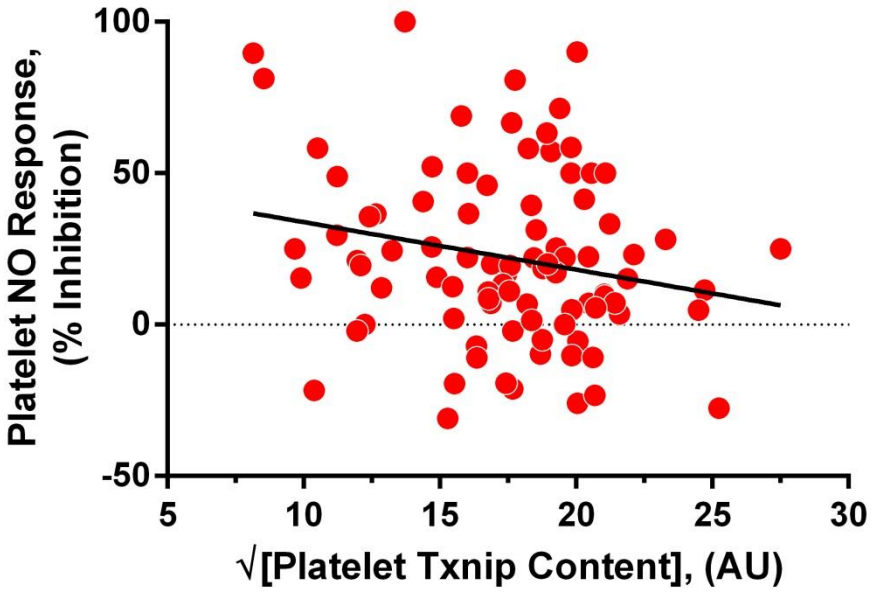

Figure 18: Platelet Txnip content and platelet response to NO. Platelet Txnip content correlated negatively with platelet response to NO (r=-0.211, p<0.05, Pearson).

3.1.3.5 Determinants of Nitric Oxide Response: Plasma Levels of Thrombospondin-1

TSP-1 has been observed to modulate several steps of the NO signalling pathway, including NOS (E. M. Bauer *et al.*, 2010) and sGC (Miller *et al.*, 2010) activity as well as having a role in potentiating platelet aggregability (Isenberg, Romeo, *et al.*, 2008; Roberts *et al.*, 2010). Median plasma TSP-1 concentrations for the cohort were 143 [100, 222]ng/ml. Plasma TSP-1 concentrations did not change in response to increasing $CHADS_2$ score (H[2] =1.663, p=0.435, Kruskal-Wallis test) (Figure 19). Due to the association between platelet activation and TSP-1 release, potential interactions with plasma TSP-1 concentrations were sought for concomitant aspirin or ACE inhibitor therapy: neither was significantly correlated.

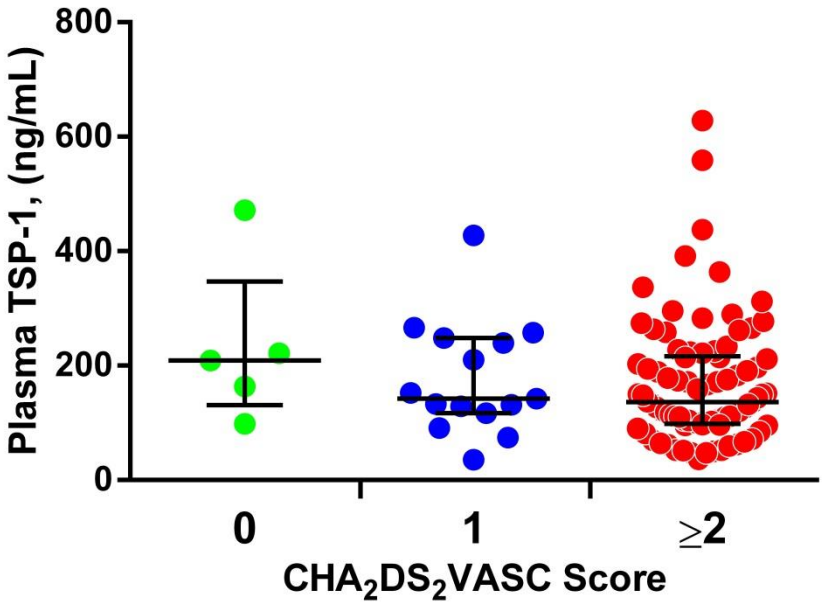

Figure 19: Plasma TSP-1 concentrations relative to tertiles of CHA_2DS_2VASc score. Median plasma TSP-1 concentrations for patients with CHA_2DS_2VASc score = 0 were 209 [131, 347]ng/ml, CHA_2DS_2VASc score = 1 were 143 [117, 248]ng/ml and for CHA_2DS_2VASc score ≥ 2 were 137 [99, 216]ng/ml.

3.1.4 Multivariate Analyses: Determinants of Platelet Function in Atrial Fibrillation

3.1.4.1 Multivariate Determinants of Platelet Nitric Oxide Response

In order to identify determinants of platelet NO response, backward stepwise multiple logistic regression was performed. ADMA, TSP-1, MPO and Txnip were included in the analysis as they formed part of the initial hypothesis as potential modulators of platelet NO signalling (Table 13A). Platelet response to ADP as well as plasma creatinine and plasma SDMA were also included in the analysis due to their univariate correlation with platelet NO response. New onset AF and admission heart rate were excluded from the model due to varying duration between initial AF diagnosis, index study enrolment and patient sample collection that might result in artefact. From this model extent of ADP-induced platelet aggregation (β=-0.344, $p<0.01$) was identified as a multivariate determinant of platelet NO response, with plasma creatinine trending towards significance as a multivariate determinant (β=0.203, $p=0.051$).

3.1.4.2 Multivariate Determinants of Platelet Aggregation

Determinants of platelet aggregation in AF were also sought via backward stepwise multiple logistic regression (Table 13B). Due to their univariate correlations with platelet aggregation, the following parameters were included in the model: sex, age, plasma TSP-1, plasma creatinine, and plasma ADMA and SDMA concentrations. Admission heart rate was excluded from the model in order to avoid artefact for reasons previously stated. Based upon this model, plasma SDMA (β=-0.292, $p<0.05$) was an inverse determinant of platelet aggregation, while gender (β=0.199, $p<0.05$) and plasma TSP-1 (β=0.276, $p<0.05$) were direct determinants.

Table 13: Multivariate correlates of platelet function in AF patients

Multivariate Correlates of Platelet Function		
A) Platelet NO Signalling		
Correlate	**β**	**P**
Platelet Aggregation	0.344	<0.01
Plasma Creatinine	0.203	0.051
B) Platelet Aggregation		
Plasma SDMA	-0.292	<0.05
Plasma TSP-1	0.276	<0.05
Female Gender	0.199	<0.05

3.1.5 Other Notable Associations

Age (Table 14A) within the cohort was observed to correlate with plasma creatinine concentrations ($r=0.454$, $p<0.001$, Pearson) and arginine metabolism as reflected by an association between age and plasma ADMA ($r=0.371$, $p<0.001$, Pearson), plasma SDMA ($r=0.438$, $p<0.001$, Pearson) and plasma ADMA: SDMA ratio ($r=-0.386$, $p<0.001$, Pearson). Plasma creatinine concentrations (Table 14B) were associated with plasma ADMA ($r=0.315$, $p<0.01$, Pearson), SDMA ($r=0.698$, $p<0.001$, Pearson) and plasma ADMA: SDMA ratio ($r=-0.648$, $p<0.001$, Pearson), as well as platelet Txnip content ($r=-0.205$, $p<0.05$, Pearson).

Table 14: Notable univariate correlates within the study population

Other Notable Correlates		
A) Correlates with Age	**r**	**P**
Plasma Creatinine	0.454	<0.001
Plasma ADMA	0.371	<0.001
Plasma SDMA	0.438	<0.001
Plasma ADMA: SDMA Ratio	-0.386	<0.001
B) Correlates with Creatinine		
Plasma ADMA	0.315	<0.01
Plasma SDMA	0.698	<0.001
Plasma ADMA: SDMA Ratio	-0.648	<0.001
Platelet Txnip	-0.205	<0.05
C) Correlates with MPO		
Plasma ADMA	0.219	<0.05
Plasma TSP-1	0.221	<0.05

Plasma MPO concentrations (Table 12C) were correlated significantly and directly with plasma ADMA concentrations ($r=0.219$, $p<0.05$, Pearson) and plasma TSP-1 concentrations ($r=0.221$, $p<0.05$, Pearson) (Figure 20).

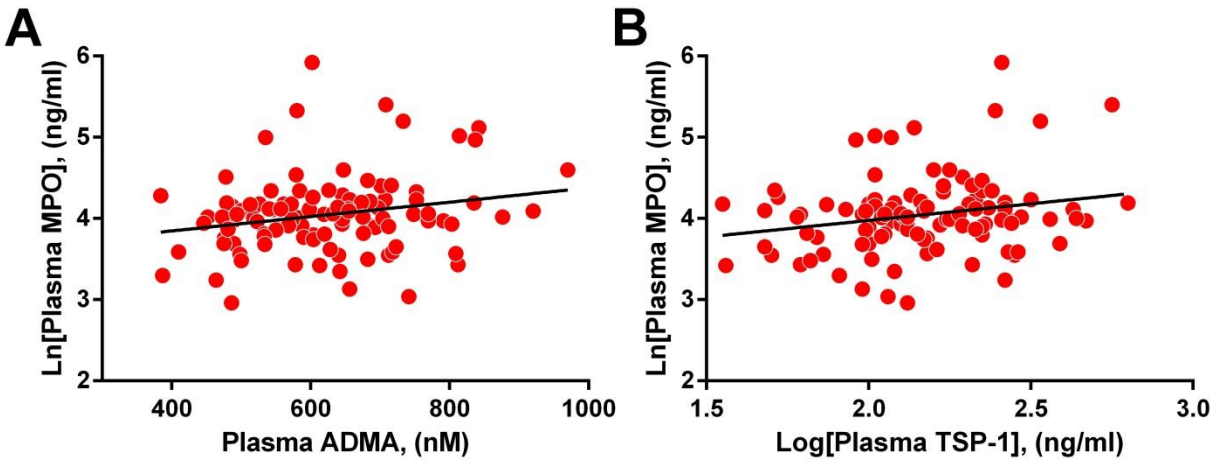

Figure 20: Correlates of plasma MPO concentrations. Plasma MPO concentrations were found to correlate with **A**, plasma ADMA concentrations and **B**, plasma TSP-1 concentrations.

3.2 Impairment of Platelet Nitric Oxide Response in New Onset Atrial Fibrillation

In section 3.1.3.1, a potential interaction between newly diagnosed AF patients and diminished platelet response to NO was observed. Interpretation of this data was potentially limited by the fact that there was no primary prospective plan to evaluate the role of new onset AF and that there was heterogeneity of the interval between presumptive onset of AF and initial blood sampling. From a clinical point of view there is evidence, summarized by Garcia *et al.* (2010), to support the concept that new onset AF carries incremental thromboembolic risk. Recent work exploring the potential for elevated thromboembolic risk in newly diagnosed AF patients identified a period of four months following index diagnosis where mortality rates were markedly increased (Miyasaka *et al.*, 2007). With this knowledge in mind, a further cohort was generated from the primary study cohort, limiting evaluation to samples obtained within four months of index enrolment into SAFETY, enabling more reliable exploration of the association between new onset AF patients and diminished platelet NO response.

3.2.1 Patient Demographics

Restricting the cohort to those patients whose samples were obtained within four months of index enrolment into SAFETY did not affect the overall clinical (Table 15) or pharmacological (Table 16) characteristics of the cohort. Compared to chronic AF patients, patients presenting to hospital with new onset AF were younger, with elevated heart rates and lower plasma creatinine levels (Table 17). There were no differences in pharmacological therapy between patients with new onset compared with chronic AF (Table 18).

Table 15: Comparison of the entire study cohort ('primary cohort') with individuals whose index blood sample was taken within four months of study entry ('secondary cohort')

Socio-demographic Profile	**Primary Cohort (n=106)**	**Secondary Cohort (n=87)**	**p**
Gender, n (% male)	54 (50.9)	45 (51.7)	0.914
Age (years)	72 [65, 81]	72 [64, 81]	0.802
Aged ≥ 75 years, n (%)	46 (43.4)	39 (44.8)	0.842
Comorbidities			
Congestive Heart Failure, n (%)	9 (8.5)	7 (8.0)	0.911
Hypertension, n (%)	74 (69.8)	59 (67.8)	0.766
Diabetes Mellitus, n (%)	27 (25.5)	21 (24.1)	0.831
Prior Stroke/TIA, n (%)	14 (13.2)	9 (10.3)	0.541
Clinical Presentation			
Admission Heart Rate (bpm)	89 [71, 130]	88 [71, 134]	0.839
LVEF (%)	59 [52, 65]$_{(n=62)}$	60 [53, 65]$_{(n=53)}$	0.957
Plasma Creatinine (μM)	82 [67, 111]	79 [67, 111]	0.701
Plasma CRP (mg/l)	3.4 [1.5, 9.1]	3.7 [1.3, 11.0]	0.804
$CHADS_2$ Score	2 [1, 2]	2 [1, 2]	0.628
CHA_2DS_2VASc Score	3 [2, 4]	3 [2, 4]	0.600
New onset AF, n (%)	23 (21.7)	22 (25.3)	0.557

Note: n = number of study participants, (%) = proportion of primary/secondary cohorts, Mann-Whitney U test for continuous variables, χ^2 test for categorical variables.

.

Table 16: Pharmacotherapy in primary and secondary cohorts

Pharmacotherapy: Summary			
Antithrombotic therapy	**Primary Cohort (n=106)**	**Secondary Cohort (n=87)**	**p**
Aspirin, n (%)	34 (32.1)	29 (33.3)	0.853
Warfarin, n (%)	63 (59.4)	50 (57.5)	0.783
Rate and/or Rhythm Control therapy			
Class 3 Anti-arrhythmics, n (%)	23 (21.7)	20 (23.0)	0.830
Class 1c Anti-arrhythmics, n (%)	6 (5.7)	5 (5.7)	0.979
β Receptor Antagonists, n (%)	64 (60.4)	51 (58.6)	0.805
Digoxin, n (%)	37 (34.9)	29 (33.3)	0.819
Calcium Channel Antagonists, n (%)	28 (26.4)	22 (25.3)	0.859
RAAS Inhibitors			
ACE Inhibitors, n (%)	35 (33.0)	31 (35.6)	0.703
Angiotensin Receptor Antagonists, n (%)	28 (26.4)	22 (25.3)	0.859
Other Medications			
Statins, n (%)	55 (51.9)	47 (54.0)	0.767
Diuretics, n (%)	36 (34.0)	30 (34.5)	0.940
Proton Pump Inhibitors, n (%)	28 (26.4)	22 (25.3)	0.859
Metformin, n (%)	16 (15.1)	11 (12.6)	0.625
Nitrates, n (%)	12 (11.3)	9 (10.3)	0.828
Paracetamol, n (%)	12 (11.3)	11 (12.6)	0.778
Opioid Receptor Agonists, n (%)	10 (9.4)	9 (10.3)	0.833

Note: n = number of study participants, (%) = proportion of primary/secondary cohorts, Mann-Whitney U test for continuous variables, χ^2 test for categorical variables.

.

Table 17: Clinical characteristics of new onset vs. chronic AF patients

Socio-demographic Profile	New Onset AF (n = 22)	Chronic AF (n = 65)	p
Gender, n (% male)	11 (50.0)	34 (52.3)	0.851
Age (years)	65 [57, 78]	74 [68, 83]	<0.01
Aged ≥ 75 years, n (%)	7 (31.8)	32 (49.2)	0.156
Comorbidities			
Congestive Heart Failure, n (%)	3 (13.6)	4 (6.2)	0.265
Hypertension, n (%)	15 (68.2)	44 (67.7)	0.966
Diabetes Mellitus, n (%)	5 (22.7)	16 (24.6)	0.858
Prior Stroke/TIA, n (%)	1 (4.5)	8 (12.3)	0.301
Clinical Presentation			
Admission Heart Rate (bpm)	132 [97, 156]	80 [65, 112]	<0.001
LVEF (%)	60 [55, 65]$_{(n=16)}$	59 [52, 64]$_{(n=37)}$	0.346
Plasma Creatinine (μM)	69 [63, 79]	90 [72, 116]	<0.01
Plasma CRP (mg/l)	4.1 [1.2, 11.5]	3.4 [1.5, 11.0]	0.934
$CHADS_2$ Score	2 [1, 2]	2 [1, 2]	0.381
CHA_2DS_2VASc Score	2 [1, 4]	3 [2, 4]	0.052

Note: n = number of study participants, (%) = proportion of new onset/chronic AF cohorts, Mann-Whitney U test for continuous variables, χ^2 test for categorical variables.

.

Table 18: Pharmacotherapy in new onset vs. chronic AF patients

Pharmacotherapy: Summary			
Antithrombotic therapy	**New Onset AF (n = 22)**	**Chronic AF (n = 65)**	**p**
Aspirin, n (%)	7 (31.8)	22 (33.8)	0.862
Warfarin, n (%)	13 (59.1)	37 (56.9)	0.859
Rate and/or Rhythm Control therapy			
Class 3 Anti-arrhythmics, n (%)	4 (18.2)	16 (24.6)	0.535
Class 1c Anti-arrhythmics, n (%)	2 (9.1)	3 (4.6)	0.436
β Receptor Antagonists, n (%)	15 (68.2)	36 (55.4)	0.292
Digoxin, n (%)	8 (36.4)	21 (32.3)	0.727
Calcium Channel Antagonists, n (%)	4 (18.2)	18 (27.7)	0.375
RAAS Inhibitors			
ACE Inhibitors, n (%)	8 (36.4)	23 (35.4)	0.934
Angiotensin Receptor Antagonists, n (%)	5 (22.7)	17 (26.2)	0.749
Other Medications			
Statins, n (%)	11 (50.0)	36 (55.4)	0.661
Diuretics, n (%)	6 (27.3)	24 (36.9)	0.410
Proton Pump Inhibitors, n (%)	7 (31.8)	15 (23.1)	0.415
Metformin, n (%)	3 (13.6)	8 (12.3)	0.871
Nitrates, n (%)	1 (4.5)	8 (12.3)	0.301
Paracetamol, n (%)	1 (4.5)	10 (15.4)	0.186
Opioid Receptor Agonists, n (%)	3 (13.6)	6 (9.2)	0.558

Note: n = number of study participants, (%) = proportion of new onset/chronic AF cohorts, χ^2 test for categorical variables.

3.2.2 Platelet Aggregability in New Onset Atrial Fibrillation

In patients with new onset AF, platelet aggregability in whole blood was assessed in response to 2.5μM ADP. Controlling for gender, no significant difference in platelet aggregation was observed in new onset AF compared to chronic AF (p=0.778, two-way ANOVA). Platelet aggregability was greater in females compared to males (9.7 [8.0, 11.5]Ω vs. 7.4 [5.3, 10.1]Ω, p<0.05, Mann-Whitney U test) within this cohort (Figure 21).

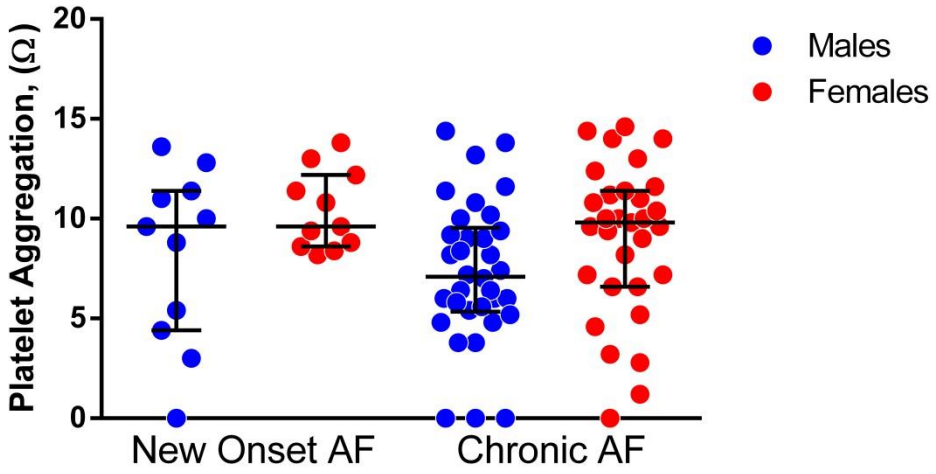

Figure 21: Median platelet aggregation (with IQR) in new onset and chronic AF patients.

3.2.3 Correlates of Platelet Aggregation

Extent of platelet aggregation correlated significantly with age, admission heart rate, plasma creatinine, TSP-1, ADMA and SDMA concentrations (Figure 22). These correlations were direct only in the cases of heart rate and TSP-1.

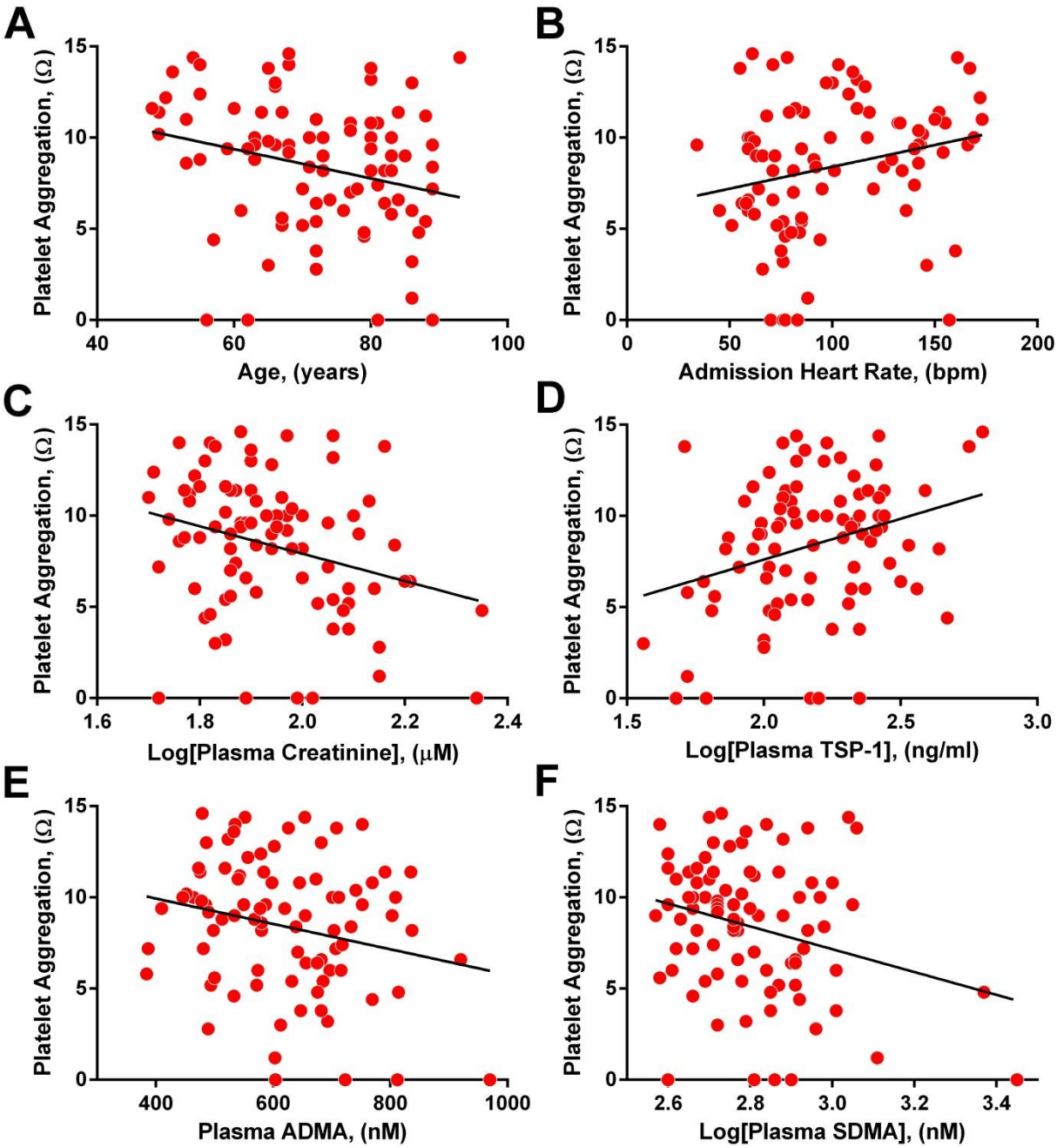

Figure 22: Correlates of platelet aggregation in AF patients. **A**, age (r=-0.250, p<0.05, Pearson); **B**, admission heart rate (0.235, p<0.001, Pearson); **C**, plasma creatinine (r=-0.290, p<0.01, Pearson); **D**, plasma TSP-1 (r=0.305, p<0.01, Pearson); **E**, plasma ADMA (r=-0.226, p<0.05, Pearson); **F**, plasma SDMA (r=-0.268, p<0.05, Pearson).

3.2.4 Platelet Response to Nitric Oxide: Inhibition of Aggregation

Platelet response to the NO donor, SNP, was markedly lower in new onset compared to chronic AF (5.7±5.2% inhibition vs. 25.2±3.8% inhibition, p<0.01, independent samples t-test), as well as in females compared to males (13.3±4.3% inhibition 27.1±4.6% inhibition, p<0.05, independent samples t-test) (Figure 23). Subsequent analysis by two-way ANOVA to

determine if there was an interaction between acuity of AF and gender revealed no significant association (p=0.986).

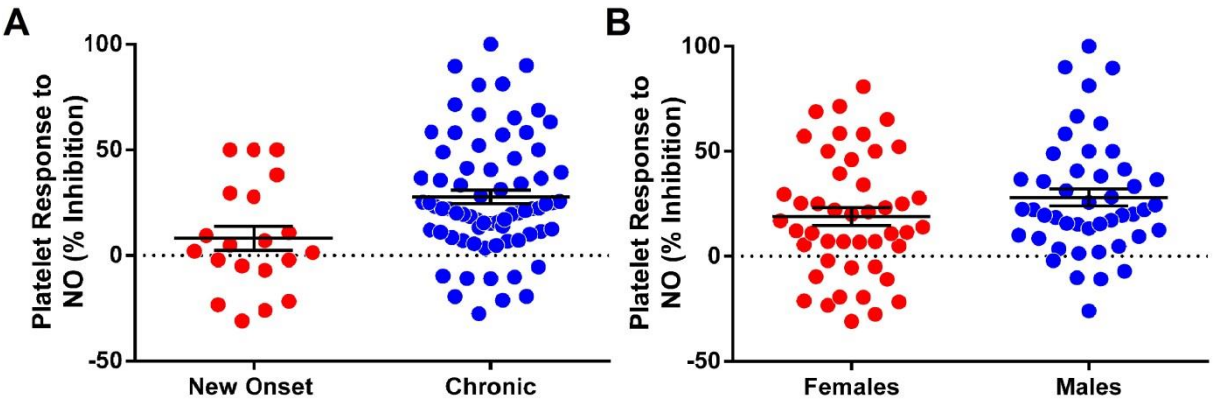

Figure 23: Platelet response to NO in, **A**, new onset and chronic AF patients, **B**, males and females.

3.2.5 Validation of Impaired Platelet Nitric Oxide Response in New Onset Atrial Fibrillation

The possibility that acuity of AF may significantly impact upon platelet NO response was not initially sought prospectively in this study. In order to determine the validity of this finding, additional patient cohorts were considered comprised of ACS (Amsterdam *et al.*, 2014; Hamm *et al.*, 2011), acute heart failure (Yancy *et al.*, 2013), and consecutively recruited new onset AF (section 3.1.1.1) patients. Patients with ACS and acute heart failure were chosen, as previous research has observed that patients with these conditions also display impaired NO response (Chirkov *et al.*, 2001; Tamargo *et al.*, 2010). The characteristics of this "validation cohort" are summarised in Table 19.

Table 19: Patient characteristics of the “validation cohort”

Validation Cohort				
Socio-demographic profile	**Chronic AF (n = 65)**	**ACS (n = 31)**	**Acute HF (n = 25)**	**New Onset AF (n=15)**
Gender (% male)	34 (52.3)	20 (64.5)	16 (64.0)	10 (66.7)
Age (years; median, IQR)	74 (68, 83)	64 (55, 77)*	71 (57, 80)	71 (65, 75)
Aged ≥ 75 years (%)	32 (49.2)	9 (29.0)	11 (44.0)	4 (26.7)
Co-morbidities				
Heart Failure (%)	4 (6.2)	0 (0.0)	25 (100.0)	2 (13.3)
Hypertension (%)	44 (67.7)	23 (74.2)	12 (48.0)	8 (53.3)
Diabetes Mellitus (%)	16 (24.6)	6 (19.4)	10 (40.0)	5 (33.3)
Prior Stroke/Transient Ischemic Attack (%)	8 (12.3)	0 (0.0)	0 (0.0)	1 (6.7)
Clinical presentation				
Admission Heart Rate (bpm; median, IQR)	80 (65, 112)	75 (66, 85)	76 (74, 97)	91 (62, 146)*
LVEF (median, IQR)	59 (52, 64)	54 (45, 65)	29 (20, 38)*	63 (44, 71)
Plasma Creatinine (μM; median, IQR)	90 (72, 116)	78 (71, 95)	123 (85, 193)*	83 (72, 119)
CHADS2 Score (median, IQR)	2 (1, 2)	1 (1, 2)	2 (2, 3)	2 (1, 2)
CHA_2DS_2VASc Score (median, IQR)	3 (2, 4)	3 (2, 5)	3 (2, 5)	2 (1, 4)
Pharmacological therapy				
Aspirin (%)	22 (33.8)	25 (80.6)	9 (36.0)	4 (26.7)
Oral Anticoagulants* (%)	38 (58.5)	0 (0.0)	3 (12.0)	12 (80.0)
Digoxin (%)	21 (32.3)	0 (0.0)	4 (16.0)	6 (40.0)
Calcium Channel Antagonists (%)	18 (27.7)	14 (45.2)	0 (0.0)	3 (20.0)
β Receptor Antagonists (%)	16 (24.6)	12 (38.7)	13 (52.0)	7 (46.7)
Anti-arrhythmics (%)	19 (29.3)	0 (0.0)	0 (0.0)	5 (33.3)
Statins (%)	36 (55.4)	25 (80.6)	10 (40.0)	4 (26.7)
ACE inhibitors (%)	23 (35.4)	11 (35.5)	10 (40.0)	5 (33.3)

Note: ‘*’ indicates $p<0.05$ compared with chronic AF, controlling for multiple comparisons (Dunnett’s test).

Patients with new onset AF from the validation cohort displayed significantly impaired platelet NO response compared with Chronic AF (5.0±6.9% inhibition vs. 25.2±3.8% inhibition respectively, $p<0.05$; Dunnett's test for multiple comparisons). Patients with ACS or acute heart failure did not display significant platelet NO signalling impairment when compared with chronic AF (Figure 24).

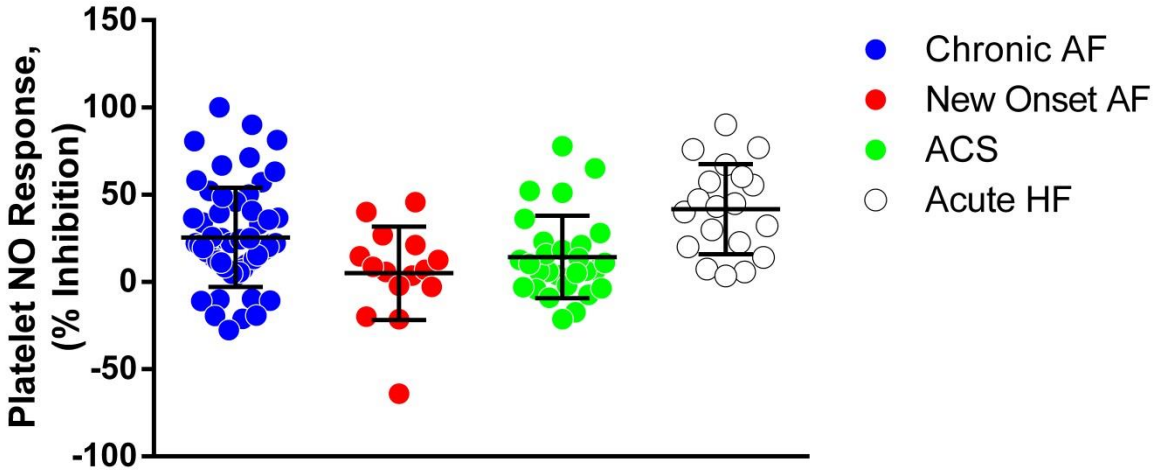

Figure 24: Platelet NO response in chronic (25.2±3.8% inhibition) and new onset (5.0±6.9% inhibition) AF, ACS (13.0±4.1% inhibition) and acute heart failure (39.7±6.0% inhibition).

3.2.6 Correlates of Platelet Nitric Oxide Response

Platelet NO response correlated significantly with admission heart rate, plasma creatinine and SDMA concentrations, and platelet aggregation (Figure 25); direct correlations applied for creatinine and SDMA concentrations.

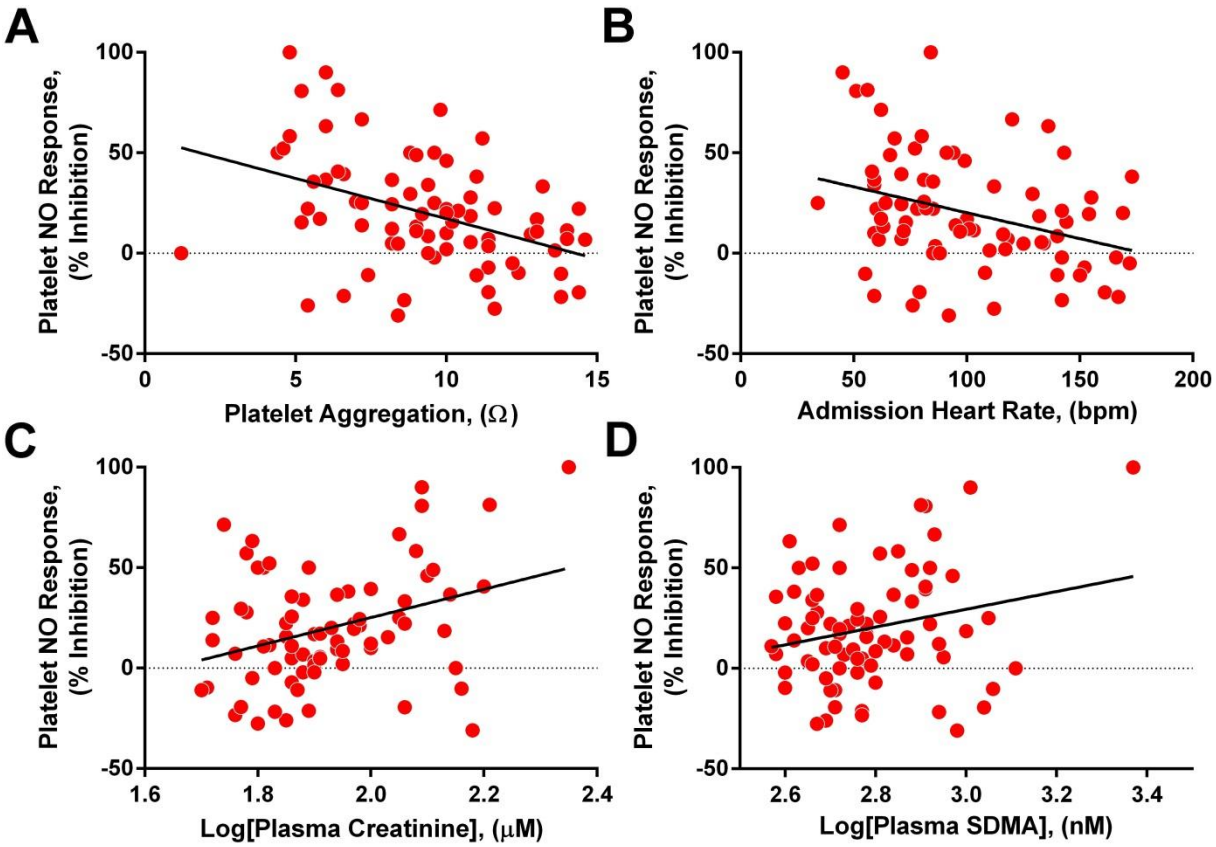

Figure 25: Correlates of platelet NO response. Platelet NO response in AF patients was associated with **A**, platelet aggregation (r=-0.411, p<0.001, Pearson); **B**, admission heart rate (r=-0.331, p<0.01, Pearson); **C**, plasma creatinine (r=0.338, p<0.01, Pearson); **D**, plasma SDMA (r=0.228, p<0.05, Pearson).

3.2.7 The Effect of Heart Rate upon Platelet Function

Due to the apparent differences between acuity of AF, gender, heart rate and platelet function within this cohort, exploratory analyses were performed to identify any associations. Table 20 and Table 21 below show that women were more likely to have higher LVEF and lower plasma creatinine concentrations compared to men, as well higher CHA_2DS_2VASc scores. Pharmacotherapy profiles for male and female AF patients were broadly similar, with diuretic use being more common in women than men. Patient demographics according to new onset vs. chronic AF have been previously outlined in Table 17 and Table 18.

Table 20: Clinical profiles of male and female AF patients

Socio-demographic Profile	Male (n=45)	Female (n=42)	p
Age (years)	72 [63, 81]	73 [65, 82]	0.683
Aged ≥ 75 years, n (%)	20 (44.4)	19 (45.2)	0.941
Comorbidities			
Congestive Heart Failure, n (%)	3 (6.7)	4 (9.5)	0.624
Hypertension, n (%)	27 (60.0)	32 (76.2)	0.106
Diabetes Mellitus, n (%)	13 (28.9)	8 (19.0)	0.284
Prior Stroke/TIA, n (%)	5 (11.1)	4 (9.5)	0.808
Clinical Presentation			
Admission Heart Rate (bpm)	85 [72, 134]	96 [70, 136]	0.683
LVEF (%)	58 [49, 60]	62 [58, 68]	0.003
Plasma Creatinine (μM)	91 [74, 119]	73 [60, 94]	0.001
Plasma CRP (mg/l)	2.6 [1.2, 6.9]	5.0 [1.5, 12.3]	0.152
$CHADS_2$ Score	2 [1, 2]	2 [1, 2]	0.929
CHA_2DS_2VASc Score	3 [1, 4]	4 [2, 5]	0.008
New onset AF, n (%)	11 (24.4)	11 (26.2)	0.851

Note: n = number of study participants, (%) = proportion of male/female cohorts, Mann-Whitney U test for continuous variables, χ^2 test for categorical variables.

.

Table 21: Pharmacological profiles of male and female AF patients

Pharmacotherapy: Summary			
Antithrombotic therapy	**Male (n=45)**	**Female (n=42)**	**p**
Aspirin, n (%)	13 (28.9)	16 (38.1)	0.363
Warfarin, n (%)	24 (53.3)	26 (61.9)	0.419
Rate and/or Rhythm Control therapy			
Class 3 Anti-arrhythmics, n (%)	9 (20.0)	11 (26.2)	0.493
Class 1c Anti-arrhythmics, n (%)	2 (4.4)	3 (7.1)	0.589
β Receptor Antagonists, n (%)	27 (60.0)	24 (57.1)	0.787
Digoxin, n (%)	14 (31.1)	15 (35.7)	0.649
Calcium Channel Antagonists, n (%)	9 (20.0)	13 (31.0)	0.240
RAAS Inhibitors			
ACE Inhibitors, n (%)	18 (40.0)	13 (31.0)	0.379
Angiotensin Receptor Antagonists, n (%)	8 (17.8)	14 (33.3)	0.095
Other Medications			
Statins, n (%)	20 (44.4)	27 (64.3)	0.064
Diuretics, n (%)	9 (20.0)	21 (50.0)	0.003
Proton Pump Inhibitors, n (%)	8 (17.8)	14 (33.3)	0.095
Metformin, n (%)	6 (13.3)	5 (11.9)	0.841
Nitrates, n (%)	5 (11.1)	4 (9.50	0.808
Paracetamol, n (%)	8 (17.8)	3 (7.1)	0.136
Opioid Receptor Agonists, n (%)	5 (11.1)	4 (9.5)	0.808

Note: n = number of study participants, (%) = proportion of male/female cohorts, Mann-Whitney U test for continuous variables, χ^2 test for categorical variables.

Gender and tachycardia, as well as acuity of AF and (potentially associated) tachycardia, were evaluated by ANCOVA for their effects on platelet function. Using ANCOVA, females displayed platelet hyperaggregability and diminished platelet response to NO compared to males for all levels of heart rate (Figure 26).

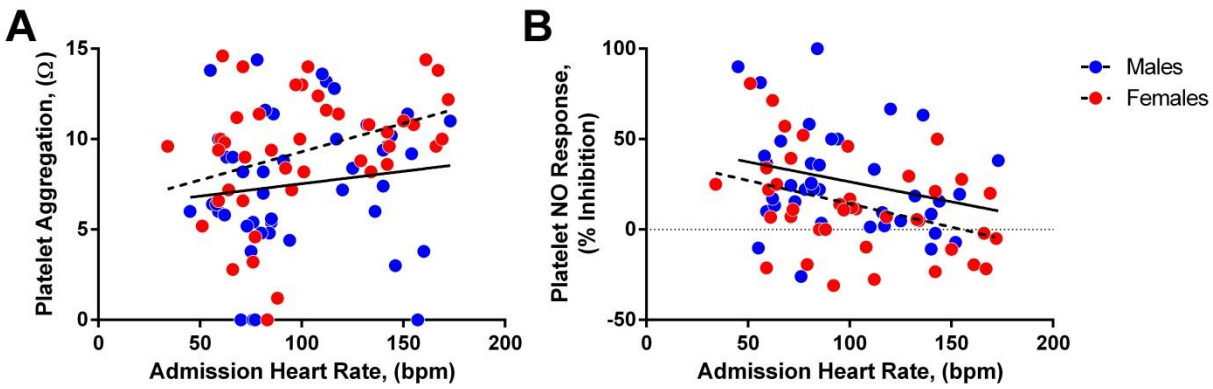

Figure 26: Exploration of potential heart rate: gender interactions in AF. **A**, platelet aggregation (gender: $F[1, 84]=5.449$, $p<0.05$; heart rate: $F[1, 84]=4.808$, $p<0.05$; interaction: $F[1, 83]=0.702$, $p=0.405$), **B**, platelet response to NO (gender: $F[1, 74]=4.031$, $p<0.05$; heart rate: $F[1, 74]=8.385$, $p<0.05$; interaction: $F[1, 73]=0.049$, $p=0.825$).

These data therefore show that both increasing heart rate and female gender were associated with increased ADP response, but that the heart rate: gender interaction did not vary with tachycardia. Analogously, both tachycardia and female gender were associated with impaired NO response, without significant tachycardia-related variability of this interaction.

Acuity of AF was also considered in relation to heart rate. This evaluation was important, given the frequent association of new onset AF with tachycardia. Acuity of AF was associated strongly ($p<0.001$) with hyperaggregability, and that there is also a strong association ($p<0.01$) with impaired NO response (Figure 27). While tachycardia tends to be associated with hyperaggregability, this association is weakened by consideration of acute vs. chronic AF status, while the significant association between tachycardia and impaired NO response persists.

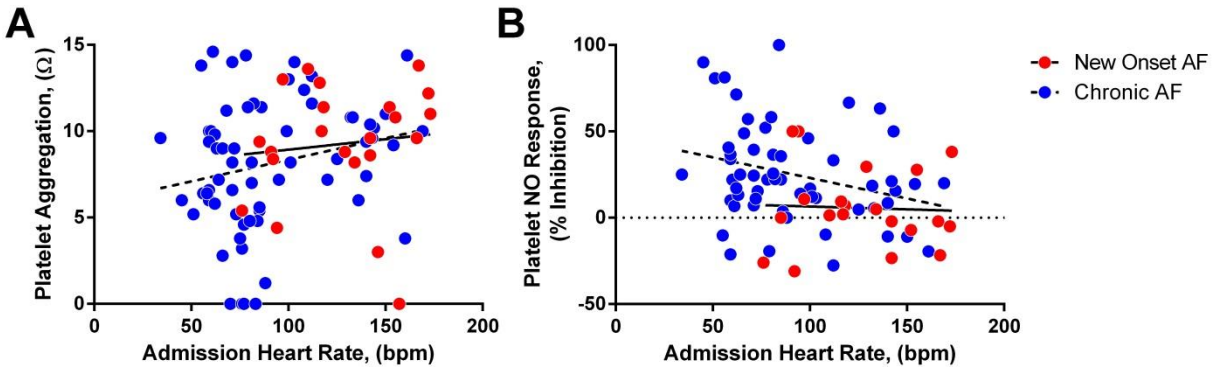

Figure 27: Exploration of potential heart rate: acuity of AF interactions. **A**, platelet aggregation (acuity of AF: F[1, 84]=20.019, p<0.001; heart rate: F[1, 84]=3.353, p=0.071; interaction: F[1, 83]=0.102, p=0.750), **B**, platelet response to NO (acuity of AF: F[1, 74]=11.624, p<0.01; heart rate: F[1, 74]=4.306, p<0.05; interaction: F[1, 73]=2.792, p=0.099).

3.2.8 Multivariate Determinants of Platelet Aggregation

Clinical and biochemical factors that achieved statistical significance on univariate analysis were included in the model: these were gender, age, admission heart rate, plasma creatinine, plasma TSP-1, and plasma ADMA and plasma SDMA concentrations. Based upon this model, plasma SDMA concentration was an inverse determinant of platelet aggregation (β=-0.240, p<0.05), whereas plasma TSP-1 (β=0.317, p<0.01), admission heart rate (β=0.204, p<0.05) and female gender (β=0.196, p<0.05) were identified as direct determinants of platelet aggregation using backwards stepwise multiple logistic regression (Table 22).

Table 22: Clinical and biochemical correlates of platelet aggregability in AF

Multivariate Correlates of Platelet Aggregation		
Correlate	**β**	**P**
Plasma TSP-1	0.317	<0.01
Plasma SDMA	-0.240	<0.05
Admission Heart Rate	0.204	<0.05
Female Gender	0.196	<0.05

3.2.9 Multivariate Determinants of Platelet Nitric Oxide Response

Univariate correlates that achieved statistical significance were evaluated via backwards stepwise multiple logistic regression, with the following parameters included in the model: gender, new onset AF, admission heart rate, platelet aggregation, plasma creatinine and plasma SDMA concentrations. New onset AF (β=-0.245, $p<0.05$) and ADP-induced aggregation (β=-0.372, $p<0.01$) were identified as determinants of platelet NO response (Table 23).

Table 23: Clinical and biochemical correlates of platelet NO response in AF

Multivariate Correlates of Platelet Nitric Oxide Response		
Correlate	**β**	**p**
Platelet Aggregation	-0.372	<0.01
New Onset AF	-0.245	<0.05

3.3 Determinants of Platelet Nitric Oxide Response in Chronic Atrial Fibrillation

The data described in section 3.2 therefore establish that new onset AF is an independent correlate of impaired platelet NO response (but not of platelet hyperaggregability). Additional determinants of platelet hyperaggregability and NO response were also sought among the 83 patients with chronic AF: this was a post hoc analysis, and therefore should be regarded as hypothesis generating.

3.3.1 Patient Demographics

As shown in table 24 and table 25, excluding patients with new onset AF did not substantially affect the clinical characteristics or types of pharmacotherapy used when the chronic AF cohort was compared with the primary study cohort.

Table 24: Clinical profile of the primary cohort compared with the chronic AF population

Socio-demographic Profile	**Primary Cohort (n=106)**	**Chronic AF Cohort (n=83)**	**p**
Gender, n (% male)	54 (50.9)	43 (51.8)	0.906
Age (years)	72 [65, 81]	73 [67, 81]	0.440
Aged ≥ 75 years, n (%)	46 (43.4)	38 (45.8)	0.743
Comorbidities			
Congestive Heart Failure, n (%)	9 (8.5)	6 (7.2)	0.750
Hypertension, n (%)	74 (69.8)	58 (69.9)	0.992
Diabetes Mellitus, n (%)	27 (25.5)	22 (26.5)	0.872
Prior Stroke/TIA, n (%)	14 (13.2)	13 (15.7)	0.632
Clinical Presentation			
Admission Heart Rate (bpm)	89 [71, 130]	81 [66, 112]	0.105
LVEF (%)	59 [52, 65]$_{(n=62)}$	59 [52, 65]$_{(n=46)}$	0.732
Plasma Creatinine (μM)	82 [67, 111]	94 [72, 114]	0.281
Plasma CRP (mg/l)	3.4 [1.5, 9.1]	8.9 [3.4, 34.6]	0.989
$CHADS_2$ Score	2 [1, 2]	2 [1, 2]	0.703
CHA_2DS_2VASc Score	3 [2, 4]	3 [2, 4]$_{(n=80)}$	0.481

Note: Mann-Whitney U test for continuous variables, χ^2 test for categorical variables.

Table 25: Pharmacotherapy of the primary cohort compared with the chronic AF population

Pharmacotherapy: Summary			
Antithrombotic therapy	**Primary Cohort (n=106)**	**Chronic AF Cohort (n=83)**	**p**
Aspirin, n (%)	34 (32.1)	27 (32.5)	0.947
Warfarin, n (%)	63 (59.4)	49 (59.0)	0.956
Rate and/or Rhythm Control therapy			
Class 3 Anti-arrhythmics, n (%)	23 (21.7)	18 (21.7)	0.998
Class 1c Anti-arrhythmics, n (%)	6 (5.7)	4 (4.8)	0.798
β Receptor Antagonists, n (%)	64 (60.4)	48 (57.8)	0.724
Digoxin, n (%)	37 (34.9)	29 (34.9)	0.996
Calcium Channel Antagonists, n (%)	28 (26.4)	24 (28.9)	0.702
RAAS Inhibitors			
ACE Inhibitors, n (%)	35 (33.0)	26 (31.3)	0.805
Angiotensin Receptor Antagonists, n (%)	28 (26.4)	23 (27.7)	0.842
Other Medications			
Statins, n (%)	55 (51.9)	44 (53.0)	0.878
Diuretics, n (%)	36 (34.0)	30 (36.1)	0.755
Proton Pump Inhibitors, n (%)	28 (26.4)	20 (24.1)	0.716
Metformin, n (%)	16 (15.1)	13 (15.7)	0.914
Nitrates, n (%)	12 (11.3)	11 (13.3)	0.687
Paracetamol, n (%)	12 (11.3)	10 (12.0)	0.877
Opioid Receptor Agonists, n (%)	10 (9.4)	7 (8.4)	0.811

Note: n = number of study participants, (%) = proportion of primary/chronic cohorts, χ^2 test for categorical variables.

3.3.2 Platelet Aggregation in Chronic Atrial Fibrillation

Consistent with previous results (Becker *et al.*, 2006; Otahbachi *et al.*, 2010; Yee *et al.*, 2006; Yee *et al.*, 2005), females were hyperaggregable compared to males (Figure 28). Controlling for gender, aspirin therapy did not significantly affect platelet aggregation (p=0.875, two-way ANOVA).

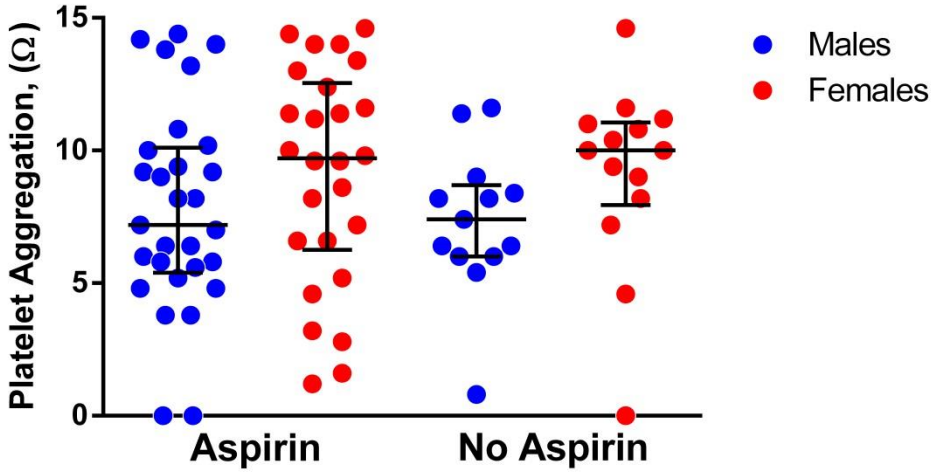

Figure 28: Median (with IQR) platelet aggregation in male and female chronic AF patients. No effect of aspirin on platelet aggregability was observed in this cohort. Platelet aggregation was 9.9 [6.8, 11.6]Ω in females and 7.2 [5.6, 9.4]Ω in males (p<0.05).

Platelet aggregability correlated inversely with plasma creatinine, plasma ADMA and SDMA concentrations, and directly with platelet Txnip content, as well as plasma TSP-1 concentrations (Figure 29).

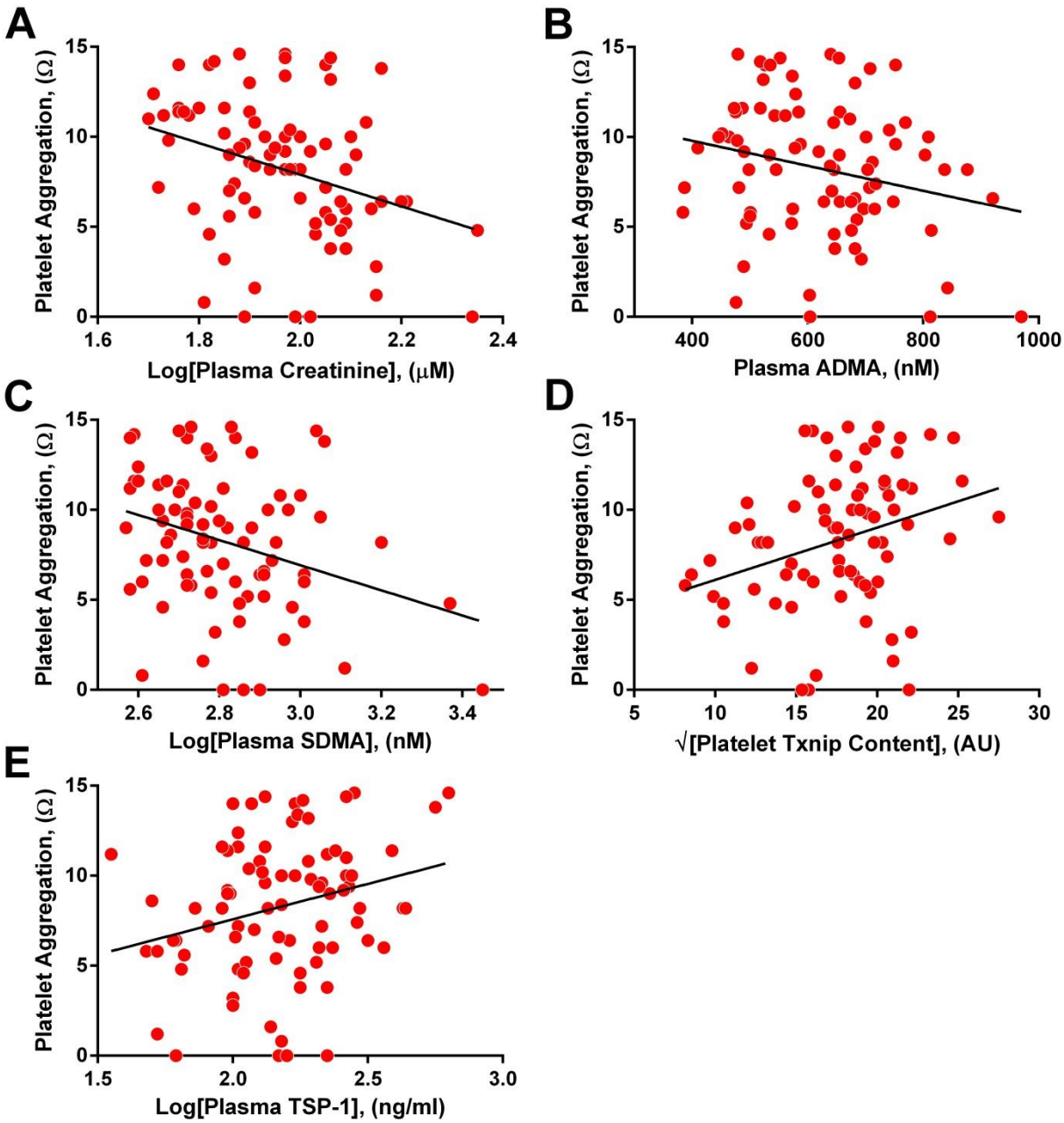

Figure 29: Correlates of platelet aggregation in chronic AF patients. **A**, plasma creatinine (r=-0.308, p<0.01, Pearson); **B**, plasma ADMA (r=-0.226, p<0.05, Pearson); **C**, plasma SDMA (r=-0.308, p<0.01, Pearson); **D**, platelet Txnip content (r=0.298, p<0.01, Pearson); **E**, plasma TSP-1 (r=0.262, p<0.01, Pearson).

Subjecting these factors to backward stepwise multiple logistic regression indicated that platelet Txnip content (β=0.277, p<0.01), plasma TSP-1 concentrations (β=0.217, p<0.05) and plasma SDMA concentrations (β=-0.306, p<0.01) remained significant determinants of platelet aggregation in chronic AF patients.

3.3.3 Platelet Response to Nitric Oxide in Chronic Atrial Fibrillation

Median platelet response to nitric oxide in this cohort was 22.2 [10.6, 43.7] % inhibition. Platelet Txnip content was the only univariate (inverse) correlate of platelet NO response (r=-0.301, p<0.05, Pearson). As a result, multivariate correlates of platelet NO responses were not sought among this cohort.

3.3.4 Other Notable Associations

Renal function correlated significantly with dimethylarginines in chronic AF patients, with plasma creatinine concentrations being associated with plasma ADMA and SDMA concentrations. Plasma ADMA concentrations also correlated with plasma MPO concentrations (Figure 30).

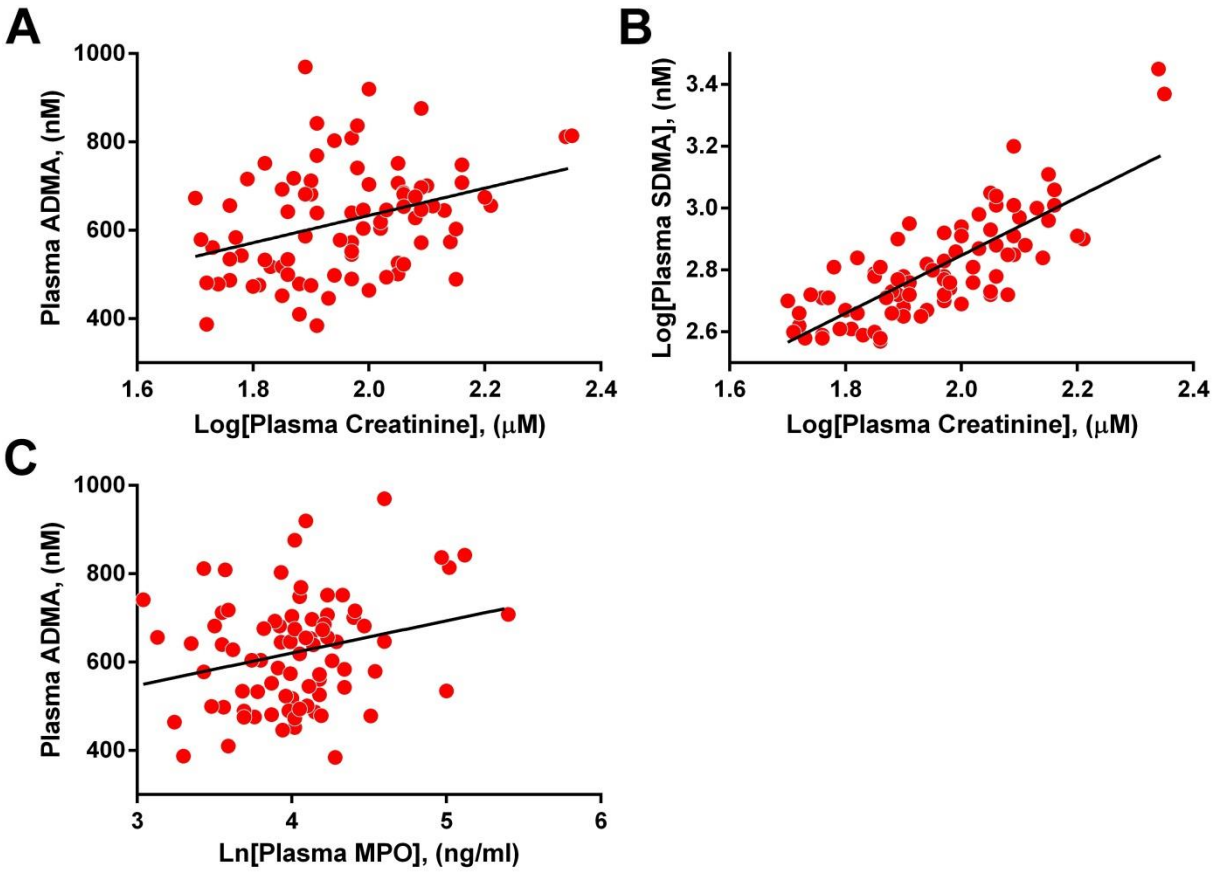

Figure 30: Correlates of plasma dimethylarginines in chronic AF patients. **A**, plasma ADMA and plasma creatinine (r=0.351, p<0.01, Pearson); **B**, plasma SDMA and plasma creatinine (r=0.717, p<0.001, Pearson); **C**, plasma ADMA and plasma MPO (r=0.254, p<0.05, Pearson).

Plasma SDMA concentrations correlated with $CHADS_2$ and CHA_2DS_2VASc scores (Figure 27).

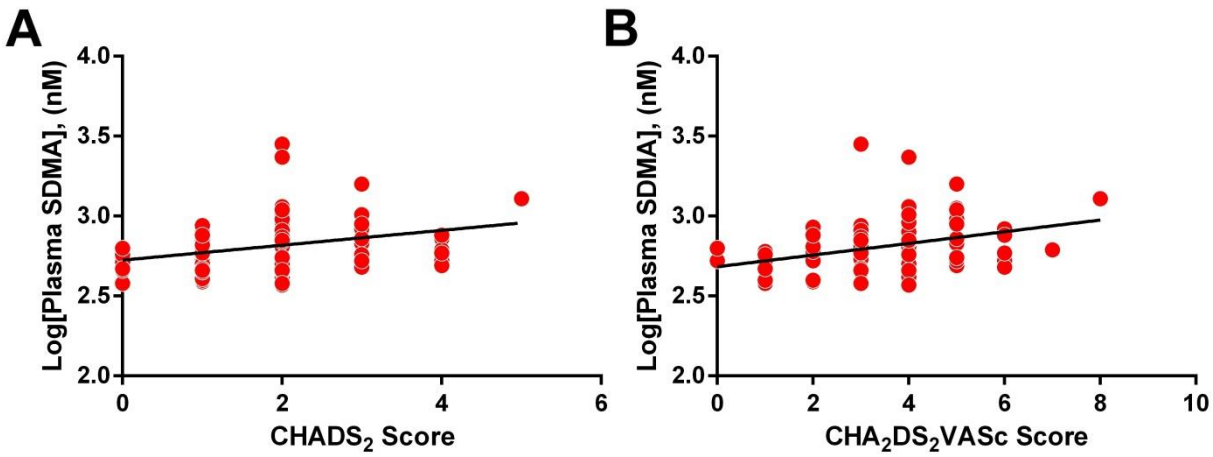

Figure 31: Plasma SDMA and clinical measures of thromboembolic risk. Plasma SDMA concentrations correlated positively with **A**, $CHADS_2$ scores ($r=0.320$, $p<0.01$, Pearson) and **B**, CHA_2DS_2VASc scores ($r=0.353$, $p<0.01$, Pearson).

3.3.5 Hospital Readmission in Chronic Atrial Fibrillation

Among chronic AF patients, 66.3% experienced hospital readmission on at least one occasion. Hospital readmission among this cohort was associated with significantly elevated platelet Txnip content (339 [259, 428]AU vs. 280 [161, 371]AU, $p<0.05$, Mann-Whitney U test). However, no other difference was observed among clinical, pharmacological or biochemical factors. Emergency hospital readmission was observed in 59.0% of patients. However, this was not associated with any clinical or pharmacological differences; length of hospital stay after emergency readmission was directly associated with $CHADS_2$ and CHA_2DS_2VASc scores, and plasma SDMA concentrations. Surprisingly, platelet NO response correlated directly with length of readmission (Figure 32).

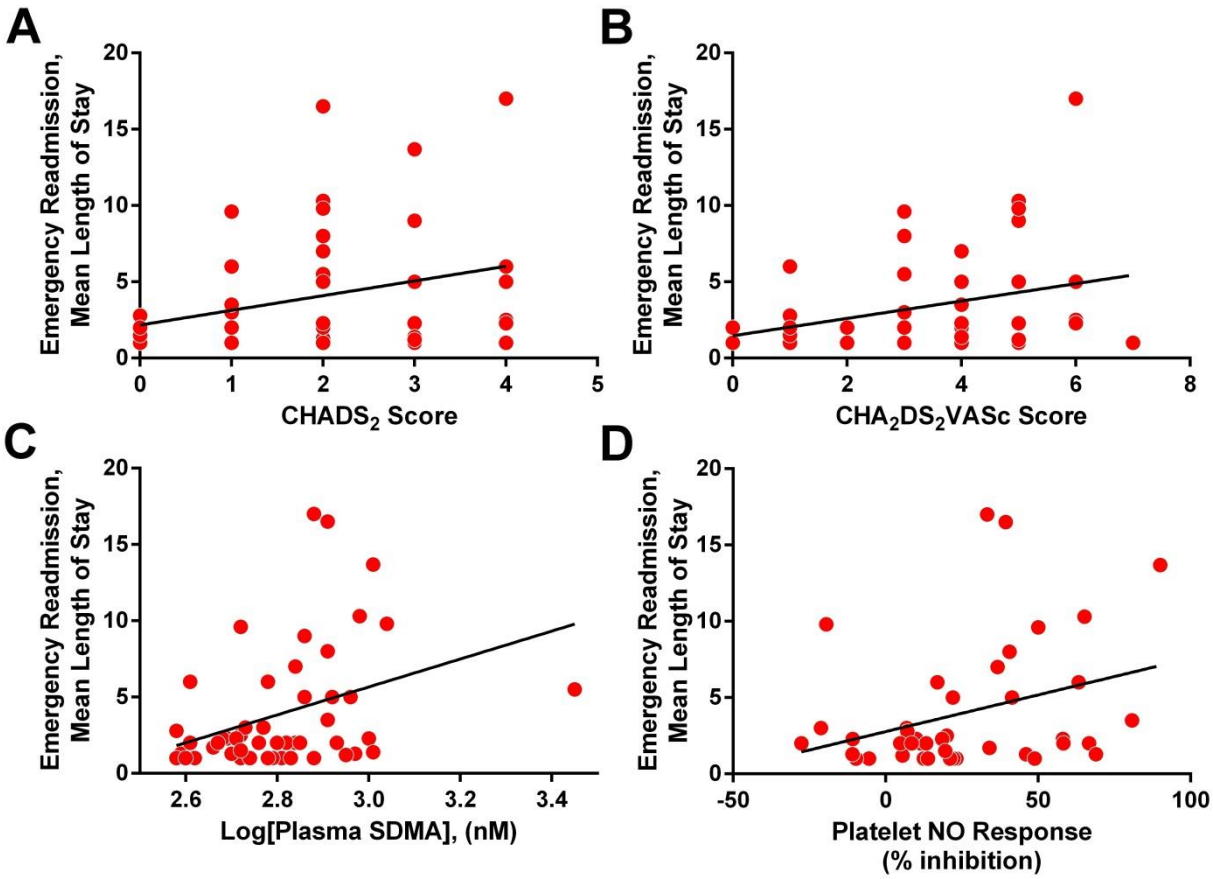

Figure 32: Correlates of emergency hospital readmission, mean length of stay. **A**, $CHADS_2$ score (r=0.299, p<0.05, Pearson); **B**, CHA_2DS_2VASc score (r=0.299, p<0.05, Pearson); **C**, plasma SDMA (r=0.360, p<0.05, Pearson); **D**, platelet response to NO (r=0.328, p<0.05, Pearson).

Upon backward stepwise multiple logistic regression, plasma SDMA concentrations were the only significant determinant of emergency hospital readmission mean length of stay (β=0.439, p<0.01).

3.4 Follow-up Study: Biochemical Correlates of Platelet Nitric Oxide Response in Atrial Fibrillation

Sixty two of the initially investigated cohort of patients, including 21% with new onset AF at initial assessment, were available for follow up in order to determine stability or otherwise of the potential biomarkers of NO signalling and inflammation during long term treatment. Follow-up occurred at median 11.8 [11.4, 12.1] months following initial assessment. Platelet aggregometry, as well as determination of ADMA, MPO, TSP-1 and Txnip, were repeated. Clinical events during the interim period were also summarised in each case, together with changes in medication.

3.4.1 Patient Demographics

Table 26 summarizes baseline demographics for patients available at follow-up. Changes in cardioactive medication in the follow-up cohort (over the intervening period) are summarised in Table 27. Importantly, the proportion of patients receiving warfarin, relatively low at discharge, did not increase markedly at follow-up (median 11.8 months).

Table 26: Baseline clinical profiles of the follow-up cohort

Socio-demographic Profile	**Follow-up Cohort (n=62)**
Gender, n (% male)	33 (53.2)
Age (years)	70 [63, 77]
Aged ≥ 75 years, n (%)	19 (30.6)
Comorbidities	
Congestive Heart Failure, n (%)	5 (8.1)
Hypertension, n (%)	42 (67.7)
Diabetes Mellitus, n (%)	13 (21.0)
Prior Stroke/TIA, n (%)	10 (16.1)
Clinical Presentation	
Admission Heart Rate (bpm)	95 [74, 137]
LVEF (%)	60 [50, 63]
Plasma Creatinine (μM)	79 [66, 102]
Plasma CRP (mg/l)	3.2 [1.5, 8.2]
$CHADS_2$ Score	1 [1, 3]
CHA_2DS_2VASc Score	3 [2, 4]
New onset AF, n (%)	13 (21.0)

Note: n = number of study participants, (%) = proportion of cohort.

Table 27: Medication use in AF patients over time

	Baseline Pharmacotherapy	Pharmacotherapy During Follow-up		
		Unchanged	Ceased	Initiated
Antithrombotic therapy				
Aspirin, n (%)	24 (38.7)	50 (72.5)	16 (23.2)	3 (4.3)
Warfarin, n (%)	34 (54.8)	52 (75.4)	8 (11.6)	9 (13.7)
Rate and/or Rhythm Control therapy				
Class 3 Anti-arrhythmics, n (%)	13 (21.0)	58 (84.1)	5 (7.2)	6 (8.7)
Class 1c Anti-arrhythmics, n (%)	4 (6.5)	59 (85.5)	3 (4.3)	7 (10.1)
β Receptor Antagonists, n (%)	36 (58.1)	51 (73.9)	7 (10.1)	11 (15.9)
Digoxin, n (%)	21 (33.9)	58 (84.1)	8 (11.6)	3 (4.3)
Calcium Channel Antagonists, n (%)	18 (29.0)	56 (81.2)	6 (8.7)	7 (10.1)
RAAS Inhibitors				
ACE Inhibitors, n (%)	20 (32.3)	57 (82.6)	6 (8.7)	6 (8.7)
Angiotensin Receptor Antagonists, n (%)	18 (29.0)	57 (82.6)	3 (4.3)	9 (13.0)
Other Medications				
Statins, n (%)	31 (50.0)	59 (85.5)	6 (8.7)	4 (5.8)
Diuretics, n (%)	17 (27.4)	47 (68.1)	5 (7.2)	17 (24.6)
Proton Pump Inhibitors, n (%)	15 (24.2)	60 (87.0)	4 (5.8)	5 (7.2)
Metformin, n (%)	9 (14.5)	67 (97.1)	0 (0.0)	2 (2.9)
Nitrates, n (%)	5 (8.1)	67 (97.1)	0 (0.0)	2 (2.9)
Paracetamol, n (%)	8 (12.9)	53 (76.8)	6 (8.7)	10 (14.5)
Opioid Receptor Agonists, n (%)	4 (6.5)	61 (88.4)	2 (2.9)	6 (8.7)

Note: data from patients (n=62) in whom follow-up data was complete

As regards unscheduled hospital admissions and thromboembolic events, no patients had suffered stroke or systemic embolus, while 67.7% had unscheduled hospital admission in the interim period.

3.4.2 Platelet Function in Atrial Fibrillation: Baseline and Follow-up

Median platelet aggregability declined significantly between baseline and follow-up in males ($p<0.05$, Wilcoxon Signed Rank test), and females ($p<0.01$, Wilcoxon Signed Rank test) (Figure 33).

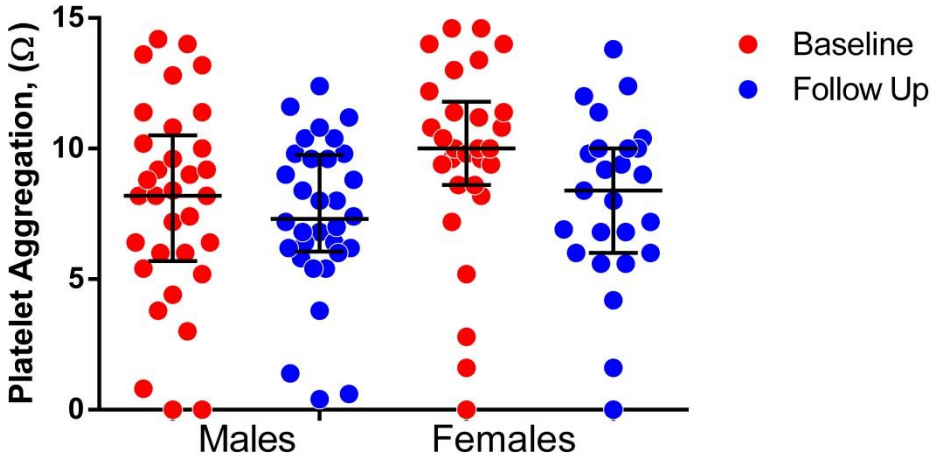

Figure 33: Median platelet aggregation (with IQR) at baseline and follow-up for males and females. Median platelet aggregation for males was 8.2 [5.5, 10.2]Ω at baseline and 6.9 [5.9, 9.6]Ω at follow-up. Median platelet aggregation for females was 10.0 [8.6, 11.5]Ω at baseline and 8.0 [6.0, 10.0]Ω at follow-up.

No significant difference was observed between baseline and follow-up platelet NO response (Figure 34).

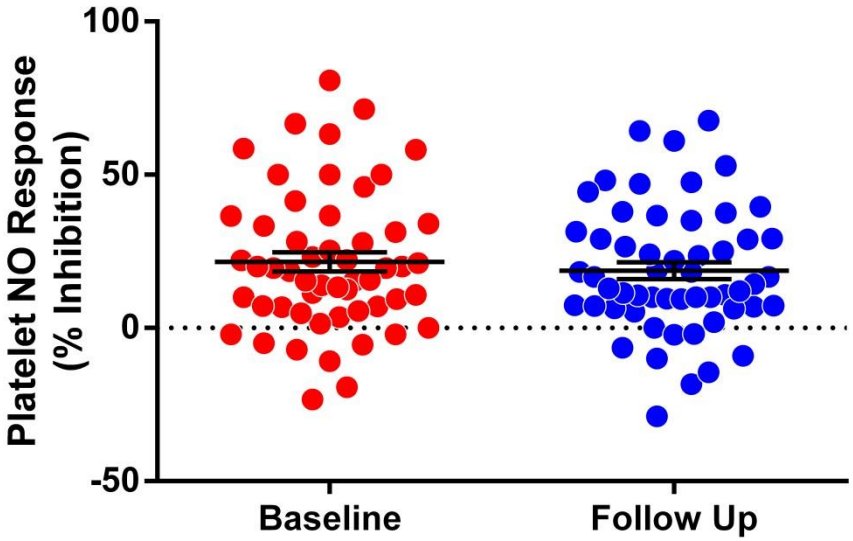

Figure 34: Platelet NO response at baseline and follow-up. Platelet response to NO did not differ significantly between baseline (21.8±3.0% inhibition) and follow-up (18.5±2.6% inhibition) in AF patients (p=0.107, paired samples t-test).

Controlling for acuity of AF (i.e. new onset vs. chronic AF) via two way (mixed models) ANOVA indicated that platelet NO response remained unchanged between baseline and follow-up time points ($F[1, 56] = 2.056$, $p=0.157$). These data therefore suggest that the differences between new onset and chronic AF patients are only partially attenuated after a median follow-up period of 11.8 [11.4, 12.1] months (Figure 35).

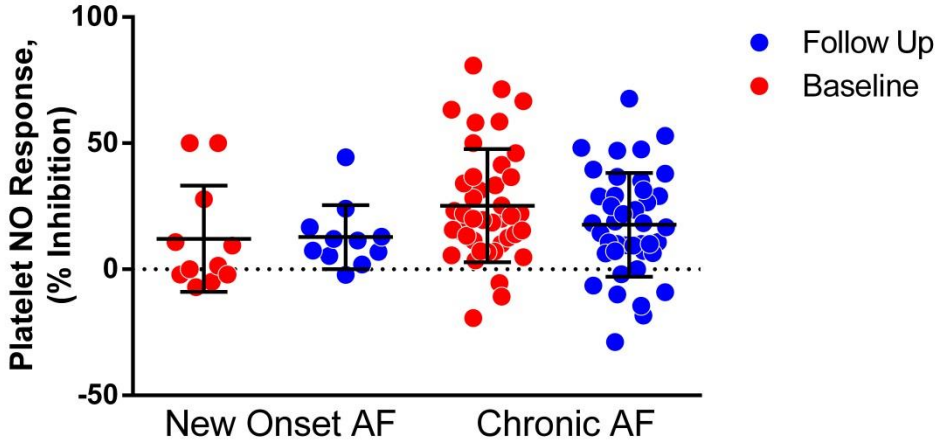

Figure 35: Platelet NO response and acuity of AF over time. Mean platelet NO response in chronic AF patients appeared to decline between baseline (24.7±3.5% inhibition) and follow-up (17.5±2.8% inhibition) however, no difference was observed for new onset AF patients between baseline (10.7±6.4% inhibition) and follow-up (13.7±5.1% inhibition).

No effect was observed for either ACE inhibitor (p=0.319, two-way mixed models ANOVA) or statin (p=0.141, two-way mixed models ANOVA) therapies in the current cohort between baseline and follow-up.

The relationship between change in platelet aggregability and NO response from initial to follow-up assessment was also explored (Figure 36). Follow-up revealed no net sensitization of platelets to NO, either overall or among the new onset AF subset.

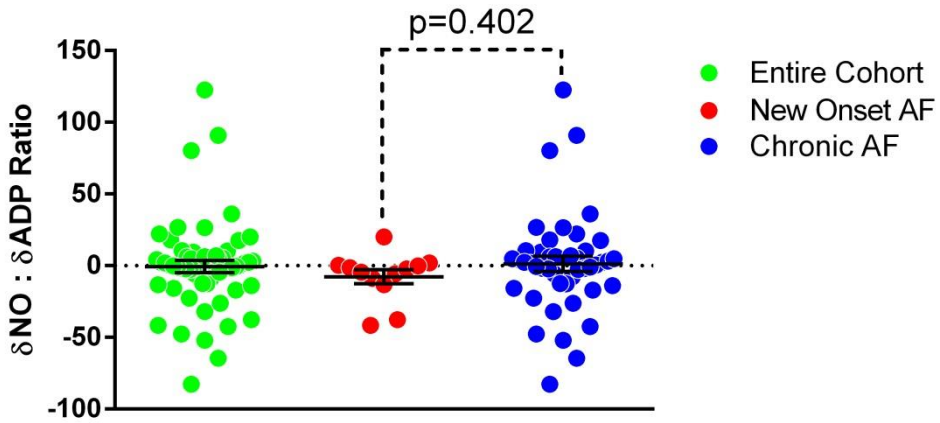

Figure 36: Relationship between changes in NO and ADP responses over patient follow-up. There was no significant difference from zero (one sample t-test, p=0.881). Post hoc analysis showed no difference between changes for new onset vs. chronic AF patients (paired samples t-test).

3.4.3 Dimethylarginines in Atrial Fibrillation: Baseline and Follow-up

A significant increase in both plasma ADMA and plasma SDMA concentrations was observed over follow-up (Figure 37).

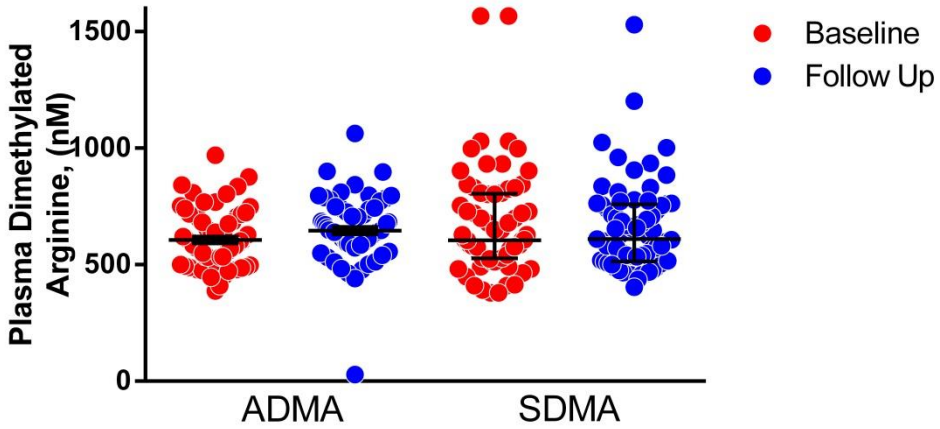

Figure 37: Plasma dimethylarginines in AF patients over time. Increases in plasma concentrations between baseline and follow-up were observed for plasma ADMA (609±18nM vs. 647±18nM respectively, $p<0.01$, paired samples t-test) and SDMA (574 [491, 723]nM vs. 617 [515, 762]nM respectively, $p<0.05$, Wilcoxon Signed Rank test).

3.4.4 Inflammation in Atrial Fibrillation: Baseline and Follow-up

No difference was observed between baseline and follow-up in plasma MPO concentrations (Figure 38). There was, however, a significant decrease in plasma CRP concentrations between baseline and follow-up (3.2 [1.5, 8.2]mg/l vs. 2.6 [1.1, 5.2]mg/l, $p<0.05$, Wilcoxon Signed Rank test).

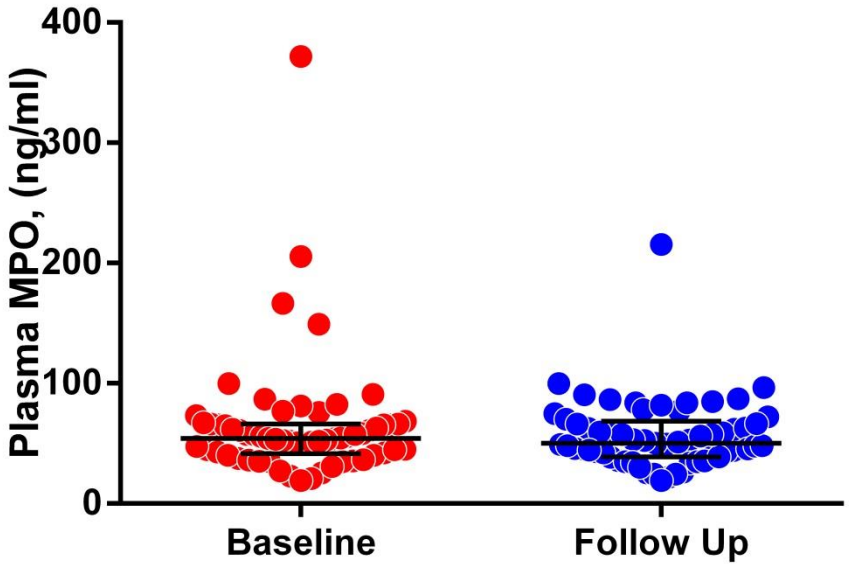

Figure 38: Plasma MPO concentrations at baseline and follow-up in AF patients. Plasma MPO concentrations did not differ between baseline and follow-up (54 [42, 66]ng/ml vs. 50 [39, 68]ng/ml respectively, p=0.669, Wilcoxon Signed Rank test).

3.4.5 Plasma Thrombospondin-1 Levels: Baseline and Follow-up

Plasma TSP-1 was observed to decrease between baseline and follow-up (Figure 39).

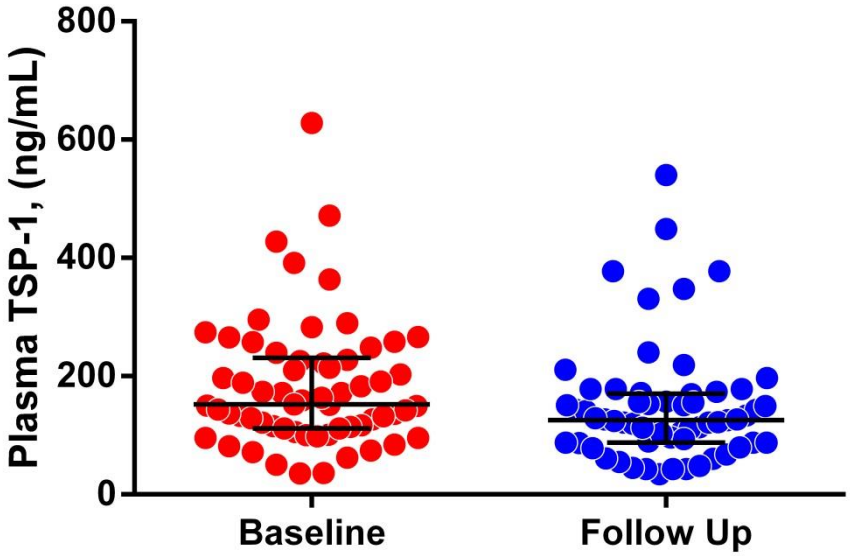

Figure 39: Plasma TSP-1 concentrations at baseline and follow-up in AF patients. Plasma TSP-1 concentrations decreased between baseline (152 [112, 231]ng/ml) and follow-up (126 [88, 171]ng/ml, p<0.05, Wilcoxon Signed Rank test).

3.4.6 Platelet Thioredoxin-interacting Protein Content: Baseline and Follow-up

No difference was observed in platelet Txnip content between baseline and follow-up (Figure 40).

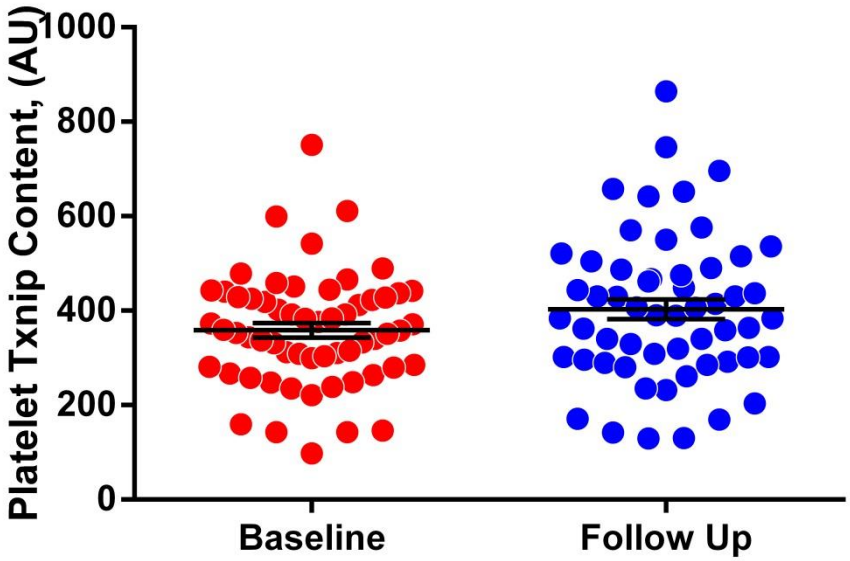

Figure 40: Platelet Txnip content at baseline and follow-up. Platelet Txnip content did not differ significantly between baseline and follow-up (357±16 [AU] vs. 404±22 [AU], p=0.076, paired samples t-test).

Additionally, platelet Txnip content did not correlate significantly with platelet NO response at follow-up (p=0.342, Pearson).

3.4.7 Correlates of Platelet Function in Atrial Fibrillation: Baseline and Follow-up

Platelet NO response was observed to correlate inversely with platelet aggregation and directly with plasma SDMA concentrations (Figure 41). ADP-induced aggregation did not correlate with any clinical or biochemical factors at follow-up.

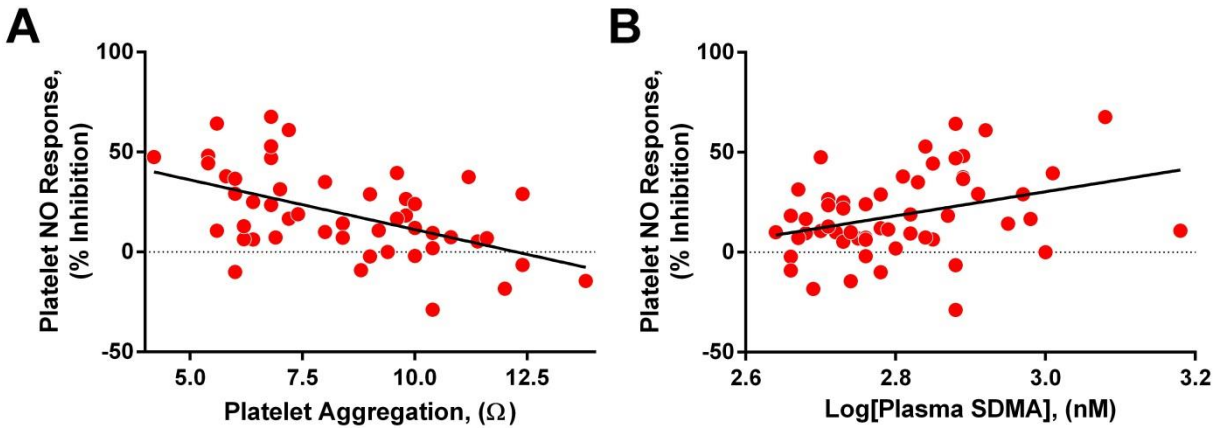

Figure 41: Correlates of platelet NO response at follow-up. Platelet NO response was observed to correlate with **A**, platelet aggregability (r=-0.478, p<0.001, Pearson) and **B**, plasma SDMA concentrations (r=0.334, p<0.05, Pearson).

3.4.8 Adverse Events in Atrial Fibrillation Patients: Baseline and Follow-up

Consistent with baseline measures, patients who experienced hospital readmission did not display any significant differences in plasma ADMA and SDMA concentrations or the plasma ADMA: SDMA ratio. Nor were any differences apparent for plasma MPO or TSP-1 concentrations, or for platelet Txnip content. The same was true when screening specifically for emergency hospital readmission. Additionally, upon hospital readmission, no relationship was observed between these biochemical parameters (i.e. ADMA, SDMA, MPO, TSP-1 and Txnip) and mean length of stay. Due to the lack of significant univariate correlates, no further analysis was conducted regarding adverse events and follow-up data.

4: Discussion

The experiments described in the preceding sections have yielded a number of important findings which are summarised below.

4.1 Summary of Findings

4.1.1 Hospital Readmission in Chronic Atrial Fibrillation

During a median follow-up period of 11.8 months, 66.3% of chronic AF patients experienced hospital readmission; of these, 89.1% were emergency readmissions. Intriguingly, platelet Txnip content was elevated in AF patients who underwent hospital readmission during follow-up, compared with those who didn't, suggesting an inflammatory basis for readmission risk. Univariate analysis also identified that increasing $CHADS_2$ and CHA_2DS_2VASc scores, as well as increasing plasma SDMA concentrations, correlated with length of hospital stay upon emergency hospital readmission. Somewhat surprisingly, platelet NO response was also directly correlated with duration of hospital stay upon emergency hospital readmission. Multivariate analysis of these factors indicated that plasma SDMA was the strongest determinant of duration of hospital stay following emergency hospital readmission. Plasma SDMA concentrations also correlated directly with both the $CHADS_2$ and CHA_2DS_2VASc stroke risk scores.

4.1.2 Platelet Nitric Oxide Response in Atrial Fibrillation

Loss of integrity of NO signalling appears to be critical to the pathogenesis of AF (Cai *et al.*, 2002) and may play a part in its association with thromboembolic risk (Horowitz *et al.*, 2013). This study therefore set out initially to explore potential mechanisms affecting platelet NO signalling in AF patients, specifically seeking to evaluate, (1), correlations with clinical algorithms for thromboembolic risk and, (2), the potential impact of plasma ADMA, MPO and TSP-1 concentrations, as well as platelet Txnip content. There was no relationship

between platelet response to the NO donor SNP and either $CHADS_2$ or CHA_2DS_2VASc scores. As regards potential biochemical correlates, the only univariate correlate was Txnip (see figure 14), but none of these biochemical factors survived multivariate analysis. Plasma creatinine concentrations and platelet aggregability in response to ADP were significant inverse correlates of platelet NO response on multivariate analysis (see section 3.1.4).

4.1.3 Platelet Hyperaggregability in Atrial Fibrillation

Given that platelet aggregability was a multivariate correlate of platelet NO response, identification of those factors which may contribute to platelet hyperaggregability in AF was undertaken. Plasma SDMA and plasma TSP-1 concentrations, as well as female sex, were found to be multivariate correlates of platelet aggregability in patients with AF (see section 3.1.4); of these, plasma SDMA concentrations were inversely correlated with ADP response.

4.1.4 Inflammation and Endothelial Dysfunction

The study also provided data linking several of the parameters assessed. Significant correlations between concentrations of the matricellular protein TSP-1 and those of the pro-oxidant enzyme MPO, as well as between MPO and the endogenous NOS inhibitor ADMA, were observed in this cohort (see Figure 20).

4.1.5 Platelet Nitric Oxide Response in New Onset Atrial Fibrillation

While it has been established that the sudden onset of newly diagnosed AF is associated with incremental thromboembolic risk (R. H. Mehta *et al.*, 2003; Miyasaka *et al.*, 2007; Mrdovic *et al.*, 2012; T. J. Wang *et al.*, 2003b), potential physiological mechanisms that account for this risk have been lacking. The elevated thromboembolic risk present in new onset AF has proven to be independent of gender (Avgil Tsadok *et al.*, 2012; Humphries *et al.*, 2001) or anticoagulant status (T. J. Wang *et al.*, 2003b) and is associated with poorer outcomes in ACS

(Lehto *et al.*, 2005; R. H. Mehta *et al.*, 2003; Mrdovic *et al.*, 2012). Thus far, new onset AF has been shown to be associated with impaired left atrial function, including decreased left atrial strain (Hirose *et al.*, 2012), cardiac distress (e.g. elevated plasma BNP) (Shibazaki *et al.*, 2012) and a pro-thrombotic state consistent with what has been observed in chronic AF (Marin *et al.*, 2004).

In the current study, when compared with chronic AF, new onset AF displayed severely impaired platelet NO response. This impairment existed despite there being no observable platelet hyperaggregability in association with acuity of AF, an observation consistent with previous research establishing no association between platelet reactivity and incremental thromboembolic risk in AF (Kamath, Chin, *et al.*, 2002; Ohara *et al.*, 2008). The impairment of platelet NO response in new onset AF was apparent despite relatively well-preserved kidney function (as reflected by plasma creatinine concentrations). Given that new onset AF was also strongly associated with tachycardia, the issue arose that the primary basis for NO resistance might be tachycardia as a result of associated inflammation/oxidative stress and/or increased plasma catecholamine levels. However, impairment of NO response to the extent seen in new onset AF did not depend on the extent of tachycardia.

Due to the retrospective nature of this initial finding, prospective validation was performed through the consecutive recruitment of additional new onset AF patients, confirming that impaired platelet NO signalling is a feature distinctive of new onset AF, even when compared with other acute cardiac illnesses such as ACS or acute decompensated heart failure.

4.1.6 Gender-specific Platelet Dysfunction in Atrial Fibrillation

Female gender is an established risk factor for thromboembolism in atrial fibrillation (Lip *et al.*, 2010; Wolf *et al.*, 1978), yet understanding of the physiological basis for this risk remains elusive. Platelet hyperaggregability has been observed in AF (Kamath, Blann, *et al.*, 2002; Ohara *et al.*, 2008) and in females compared with males (Becker *et al.*, 2006; Otahbachi *et al.*,

2010; Yee *et al.*, 2006; Yee *et al.*, 2005), yet the prospect of gender-specific platelet dysfunction in AF has not been investigated previously. This research identified that females with AF displayed significant platelet hyperaggregability and impaired platelet NO response when compared with males. Additionally, this gender-specific difference in platelet function was independent of heart rate or acuity of AF.

4.1.7 Multivariate Determinants of Platelet Function in Atrial Fibrillation

When considering clinical and biochemical correlates of platelet function in AF in the primary study (see section 3.1), certain clinical characteristics were not considered (such as new onset AF status and admission heart rate), as there was significant variation within the cohort between initial diagnosis of AF and hospital admission, index enrolment into SAFETY, and patient blood sample collection. In this refined cohort controlling for these factors, platelet function has been evaluated with the additional consideration of the effects of heart rate and acuity of AF. Plasma SDMA was retained as a significant inverse correlate of platelet aggregability, while admission heart rate, female gender and plasma TSP-1 were all direct correlates. Platelet NO response was diminished in the presence of both new onset AF and platelet hyperaggregability.

4.1.8 Platelet Function in Chronic Atrial Fibrillation

New onset AF is associated with severely impaired platelet NO response when compared with chronic AF (section 4.2.1), and in a cohort comprised of new onset and chronic AF patients, platelet response to NO was correlated on multivariate analysis with extent of platelet aggregation and presence of new onset AF. This finding highlights the possibility that determinants of platelet NO response in a cohort of AF patients may be modified in the presence of new onset AF. Therefore, potential determinants of platelet NO signalling were re-evaluated in a *post hoc* analysis of chronic AF patients, in whom platelet Txnip content

was observed to be a univariate correlate of platelet NO response. Although no platelet hyperaggregability was apparent in new onset AF compared with chronic AF, determinants of platelet aggregability were also sought. Plasma TSP-1 and platelet Txnip content correlated directly with ADP-induced aggregation, whereas plasma SDMA was an inverse correlate.

4.1.9 Changes in Platelet Function over Time in Atrial Fibrillation

Platelet aggregability declined between baseline and follow-up time points. This decline in platelet aggregability was accompanied by a decrease in plasma TSP-1 concentrations. Platelet NO response did not change between baseline and follow-up even when controlling for new onset AF status. Furthermore, the relationship between platelet aggregability, and inhibition of platelet aggregation by NO (i.e. physiological antagonism), did not alter with time (Figure 32). Platelet NO response at follow-up correlated inversely with extent of platelet aggregation and plasma SDMA levels.

4.1.10 Changes in Plasma Asymmetric and Symmetric Dimethylarginines over Time in Atrial Fibrillation

Between baseline and follow-up time points, there was an increase in plasma ADMA and SDMA concentrations (well documented markers of cardiovascular morbidity, mortality and thromboembolic risk in AF (Horowitz *et al.*, 2013)). This increase occurred despite the use of ACE inhibitor therapy in the study population (previously shown to decrease plasma ADMA concentrations) (Willoughby *et al.*, 2012), and was independent of markers of inflammation and oxidative stress such as MPO or CRP.

4.2 Implications

4.2.1 Hospital Readmission in Chronic Atrial Fibrillation

Hospital readmission rates due to AF have been increasing, in Australia and world-wide (Lip *et al.*, 2012; Wong *et al.*, 2012), posing a significant cost burden on health systems, particularly when incidence of stroke in AF is considered (Cadilhac, 2012; Cadilhac *et al.*, 2009). The likelihood of hospital readmission among AF patients has largely been attributed to increased cardiovascular comorbidity burden (Johnson *et al.*, 2013; Naccarelli *et al.*, 2012). Differing strategies have been explored in order to address this phenomenon, with varying levels of success. Pharmacotherapy for the primary prevention of AF has explored the potential benefits of statin therapy, as well as RAAS inhibition through application of angiotensin-receptor antagonists or ACE inhibitors. While both statin use and angiotensin-receptor antagonists have failed to reduce the incidence of AF (Bang *et al.*, 2011; Khatib *et al.*, 2013), some benefit seemed to be associated with ACE inhibitor therapy (Khatib *et al.*, 2013). Similarly, with regards to secondary prevention of AF (i.e. maintenance of sinus rhythm) no significant effect has been observed with angiotensin-receptor antagonist therapy (Disertori *et al.*, 2012), while ACE inhibitor and statin therapies seem to show some benefit (Disertori *et al.*, 2012; Fauchier *et al.*, 2013). 53.0% of the present cohort received statin therapy while 31.3% of the cohort received ACE inhibitor therapy. Clearly, there is a need to identify patients who may benefit more from aggressive and/or novel therapeutic strategies.

The finding that platelet Txnip content was elevated in patients that subsequently required hospital readmission may have clinical utility. The most defined function of Txnip is its inhibition of thioredoxin activity (Nishiyama *et al.*, 1999; P. C. Schulze *et al.*, 2004), through which it can affect numerous cellular processes (Spindel *et al.*, 2012). In the context of cardiovascular function, Txnip is upregulated in response to hyperglycaemia (Cha-Molstad *et al.*, 2009; J. Chen *et al.*, 2009; Minn *et al.*, 2005), has roles in regulating vascular smooth muscle proliferation (P. C. Schulze, 2002), cardiomyocyte hypertrophy (Yoshioka *et al.*, 2004) and apoptosis (Y. Wang *et al.*, 2002; Xiang *et al.*, 2005), as well as correlating with aortic valve calcification (Ngo *et al.*, 2008). Notably, Txnip is also involved in the expression

of adhesive molecules by endothelial cells in response to non-laminar blood flow (X. Q. Wang *et al.*, 2012; Yamawaki *et al.*, 2005), as well as promoting the secretion of interleukin-1β via the NLRP3 inflammasome (Zhou *et al.*, 2010). The association of Txnip with oxidative stress is particularly interesting, given that expression of Txnip is negatively regulated by NO (Forrester *et al.*, 2009; P. C. Schulze *et al.*, 2006), lending the assumption that conditions displaying oxidative stress as well as being associated with impaired NO signalling will also be associated with elevated Txnip levels. Indeed, this association has been observed before, where ACE inhibitor therapy in conditions of high cardiovascular risk has improved NO response, with a concomitant decrease in Txnip expression (Sverdlov *et al.*, 2013a; Willoughby *et al.*, 2012). These findings are consistent with the present observation that increased platelet Txnip content is associated with increased likelihood of hospital readmission, considering previous observations that hospital readmission in AF correlates with increased comorbidity burden (Johnson *et al.*, 2013; Naccarelli *et al.*, 2012).

The length of hospital stay upon readmission is also a significant factor when evaluating the cost burden that AF poses on hospital systems (Cadilhac, 2012). Consistent with what has been reported previously (Johnson *et al.*, 2013), indices of thromboembolic risk (i.e. $CHADS_2$ and CHA_2DS_2VASc) correlated directly with length of hospital readmission within the current study. Upon multivariate analysis though, plasma SDMA concentrations were found to be the strongest predictive factor for length of hospital readmission, an intriguing result given that plasma SDMA concentrations also correlated directly with both $CHADS_2$ and CHA_2DS_2VASc scores. Considering these findings, it is possible that plasma SDMA could be a sensitive biochemical risk marker of events such as ischaemic stroke in AF patients, given the correlations with scores of thromboembolic risk and length of hospital readmission. One explanation for this association that should be considered is that of renal impairment: SDMA is known to be eliminated primarily, though not exclusively (Kittel *et al.*, 2013; Rodionov *et al.*, 2010), via renal excretion and for this reason has been postulated as a potential marker of

renal function (Kielstein, Salpeter, *et al.*, 2006). However, while plasma SDMA correlated with both $CHADS_2$ and CHA_2DS_2VASc scores, plasma creatinine (another marker of renal function) did not, seemingly weakening the argument that the direct correlations between SDMA, $CHADS_2$ and CHA_2DS_2VASc scores, and length of hospital readmission are indicative of increasing disease burden, reflected by progressive renal impairment.

Alternatively, the oxidative stress that typically accompanies AF (Korantzopoulos *et al.*, 2007; Neuman *et al.*, 2007) may also stimulate the up-regulation of PRMTs, resulting not only in impairment of DDAHs but also increased methylation of arginine residues (Luo *et al.*, 2010), contributing to accumulation of ADMA and SDMA. Arguably, progressive increases in oxidative stress associated with increasing thromboembolic risk in AF may therefore be reflected in increasing plasma SDMA levels. While the direct association between SDMA levels and O_2^- generation has been reported (Schepers *et al.*, 2011; Schepers *et al.*, 2009; von Leitner *et al.*, 2011), no other detailed correlative studies with oxidative stress are currently available.

4.2.2 Platelet Nitric Oxide Response in Atrial Fibrillation

Net platelet aggregability conceptually can be considered as the net result of a 'tug of war' between opposing forces stimulating platelet aggregation or its inhibition (Rajendran *et al.*, 2008). This 'tug of war' occurs through functionally distinct pathways, that do not interact with each other directly until they intersect with their differing effects upon Ca^{2+} flux in the platelet: pro-aggregants such as ADP promoting Ca^{2+}-induced Ca^{2+} release, resulting in platelet activation, whereas inhibitors of aggregation such as NO stimulate the suppression of this Ca^{2+}flux (Chirkov *et al.*, 2007). This 'tug of war' effect may therefore be referred to as 'physiological antagonism', that is opposing effects produced by two different agonists (Henry *et al.*, 1985).

It is important to emphasize that diminished platelet NO response in the presence of hyperaggregability does not in any way imply impairment of NO related mechanisms, but rather increases in 'opposing forces'. For example, the impact of constrictor/dilator interactions in isolated smooth muscle preparations have been modelled in order to derive the expected reductions in dilator effects, with given increases in constrictor tone (Henry *et al.*, 1985). However, the problem here is that in many circumstances platelet hyperaggregability coexists with impaired NO response, that is, for reasons independent of physiological antagonism. One such example is polycystic ovarian syndrome (Chan *et al.*, 2013; Rajendran *et al.*, 2009). It is therefore important to state clearly that in the current study, no biochemical association of impaired NO response was found, while elevated creatinine levels represented an association with increased NO response.

4.2.3 Platelet Hyperaggregability in Atrial Fibrillation

Platelet hyperaggregability is an established pathophysiological feature of AF (Hammwohner *et al.*, 2007; Kamath, Blann, *et al.*, 2002; Ohara *et al.*, 2008). However, extent of platelet reactivity has not been observed to associate with incremental thromboembolic risk: currently, no correlation has been observed between measures of platelet reactivity and $CHADS_2$ score (Ohara *et al.*, 2008), or clinical categories of AF (i.e. paroxysmal, persistent, permanent) (Kamath, Chin, *et al.*, 2002). Thus far, efforts to associate platelet hyperaggregability with clinical measures of thromboembolic risk have largely been unsuccessful.

Gender has long been identified as a factor influencing stroke risk in AF, with females experiencing elevated risk compared to males, a feature that has been incorporated into the CHA_2DS_2VASc stroke risk algorithm (Lip *et al.*, 2010). Awareness of this gender-specific stroke risk has long been present, with initial studies evaluating epidemiological factors associated with stroke incidence in AF identifying female gender, along with coronary heart disease and age, as being significantly associated with thromboembolic risk (Wolf *et al.*,

1991). Female gender has continued to be identified as a risk factor for thromboembolism even when controlling for rates of anticoagulation (Avgil Tsadok *et al.*, 2012), or potential selection bias by evaluating populations naïve to anticoagulation (T. J. Wang *et al.*, 2003b), and even appears to be predictive of the likelihood of post-cardioversion complications (Airaksinen *et al.*, 2013). In this study, it has been observed that gender is a determinant of platelet reactivity, with females aggregating more strongly than males. This result is consistent with previous reports (Becker *et al.*, 2006; Otahbachi *et al.*, 2010). However, this is the first time that gender has been identified as a determinant of platelet reactivity in AF. It remains possible that platelet hyperaggregability may contribute to the increased propensity for thrombogenesis in females with AF.

Thrombospondin-1 is a 'promiscuous' matricellular protein that is stored primarily in the dense granules in platelets, and is released upon platelet activation, when dense granules fuse with the platelet extracellular membrane, expelling their contents (H. Chen *et al.*, 2000; Isenberg, Frazier, *et al.*, 2008).

In the current study, the expectation might have been of an inverse relationship between plasma TSP-1 and platelet NO response, reflecting previous work exploring the role of TSP-1 in modulating NO signalling. In vascular tissue, TSP-1 has been shown to inhibit endothelial NOS activation and attenuate sGC activity, as well as stimulate ROS generation via NADPH oxidase 1 activity (E. M. Bauer *et al.*, 2010; Csanyi *et al.*, 2012; Isenberg *et al.*, 2006; Miller *et al.*, 2010; Ramanathan *et al.*, 2011). Similarly, TSP-1 has been observed to suppress cAMP- and cGMP-dependent inhibitory signalling pathways in platelets (Isenberg, Romeo, *et al.*, 2008; Roberts *et al.*, 2010). However, no association between TSP-1 and platelet NO signalling was observed in this study. Rather, the main finding with TSP-1 was a strong association with extent of platelet aggregation. This result may be reflective of the hyperaggregable state present in AF: TSP-1 is stored primarily in platelets and released into plasma as a result of degranulation upon platelet activation (Davi *et al.*, 2007; Isenberg,

Frazier, *et al.*, 2008). Supporting this view is research identifying that circulating plasma levels of 'microparticles' (membrane vesicles with pro-coagulant, pro-inflammatory properties that are shed by cells in response to stress (see (Montoro-Garcia *et al.*, 2011)), are elevated in AF (Ederhy *et al.*, 2007; Hayashi *et al.*, 2011), and that the release of these microparticles is potentiated by TSP-1 (Camus *et al.*, 2012). Thus, TSP-1 may have a role in modulating platelet hyper-reactivity in AF. As regards potential mechanisms linking TSP-1 and platelet hyperaggregability, the lack of association between TSP-1 and NO response suggests that any such mechanism is not cGMP-dependent. cAMP-dependent mechanisms of platelet inhibition were not investigated in this study; as such the potentiation of platelet aggregability via suppression of cAMP-dependent signalling within this cohort remains a possibility.

In the current study, SDMA concentrations were inversely and independently correlated with extent of platelet aggregation (that is, after correcting for creatinine levels). Plasma SDMA was long thought to be a physiologically inert arginine residue, accumulating within organisms as a result of the breakdown of proteins containing methylated arginines. Recent studies supported this view (Veldink *et al.*, 2013), maintaining that the primary clinical value in determining plasma SDMA is as a measure of renal function (Kielstein, Salpeter, *et al.*, 2006). This underscores the possibility that the association between plasma SDMA and platelet aggregability is merely reflective of diminished platelet function in the presence of renal impairment. However, it has emerged that SDMA is partially cleared by metabolism, limiting its capacity to be regarded as an endogenous measure of renal function (Kittel *et al.*, 2013). Evidence is also accumulating that suggests that SDMA may not be physiologically inert, as was previously understood. Clinically, plasma SDMA has been associated with risk of major bleeding events and cardiac death in patients with AF (Horowitz *et al.*, 2013), as a marker of adverse outcomes in the acute phase of ischemic stroke in renal failure patients (Luneburg *et al.*, 2012), and is inversely predictive of long-term event free survival in

coronary heart disease patients (Siegerink *et al.*, 2013). Plasma SDMA has also been observed to stimulate ROS production in endothelial cells (Bode-Boger *et al.*, 2006), and monocytes (Schepers *et al.*, 2009) as well as promoting inflammatory activation of monocytes via NF-κB (Schepers *et al.*, 2011; Schepers *et al.*, 2009). This evidence of a physiological role for SDMA also allows for the possibility of a direct effect of SDMA on platelet aggregability.

Furthermore, it has long been established that platelet aggregation is impaired in patients undergoing haemodialysis (Lindsay *et al.*, 1975), and consistent with this, risk of haemorrhage is increased in AF patients with chronic kidney disease (Olesen *et al.*, 2012), a condition associated with SDMA accumulation (Duranton *et al.*, 2014; Eiselt *et al.*, 2010; Kielstein, Boger, *et al.*, 2004). However, the current data in no way explain precisely how SDMA might impair platelet aggregation. This issue would best be addressed by direct investigation of the potential SDMA/ADP platelet interaction, which is now a high priority in this field.

4.2.4 Endothelial Dysfunction and Inflammation in Atrial Fibrillation

Endothelial dysfunction and inflammation are both associated with AF; however the evidence for their direct interaction is limited. Biochemical surrogates of endothelial dysfunction (such as the endogenous NOS inhibitor, ADMA) and inflammation (the inflammatory enzyme, MPO) have been observed to interact with each other in an *in vitro* setting (von Leitner *et al.*, 2011). In spite of the modulatory roles platelets have regarding inflammation (Gawaz *et al.*, 2005; Lievens *et al.*, 2010; Mansfield *et al.*, 1990) and endothelial function (E. M. Bauer *et al.*, 2010; Csanyi *et al.*, 2012; Isenberg *et al.*, 2009; Isenberg *et al.*, 2005), their influence regarding the interaction between inflammation and endothelial dysfunction remains relatively unexplored in the context of AF.

ADMA is associated with impaired endothelial function due to its ability to competitively inhibit NOS, and can also contribute to the uncoupling of endothelial NOS, resulting in

increased O_2^- production (Antoniades *et al.*, 2009; Kielstein, Donnerstag, *et al.*, 2006; Veresh *et al.*, 2008). Clinically, ADMA has been shown to be predictive of cardiovascular morbidity and mortality (Horowitz *et al.*, 2013; Siegerink *et al.*, 2013; Yoo *et al.*, 2001). Metabolic clearance of ADMA may be influenced by the presence of oxidative stress, such as in diabetes mellitus (Lin *et al.*, 2002) and/or the activity of ROS generating enzymes NADPH oxidase or MPO (both associated with inflammation), resulting in impairment of DDAH activity and accumulation of ADMA (Lin *et al.*, 2002; Luo *et al.*, 2010; von Leitner *et al.*, 2011).

As for platelets, neutrophil activation is subject to modulation by NO, inhibiting neutrophil activation and suppressing ROS generation by NADPH oxidase (Clancy *et al.*, 1992; Moilanen *et al.*, 1993). TSP-1 and MPO (stored primarily in platelets and neutrophils, respectively) also have well documented effects on NO signalling. TSP-1 has been shown to inhibit the activity of several enzymes involved in the NO signalling pathway (E. M. Bauer *et al.*, 2010; Miller *et al.*, 2010), as well as to attenuate the inhibitory effect of NO on leukocyte activation (Ridnour *et al.*, 2007). Similarly, MPO has been implicated in endothelial NOS uncoupling (Xu *et al.*, 2006), shown to be a source of reactive nitrogen species (Eiserich *et al.*, 1998) as well as catabolising bioavailable NO (Eiserich *et al.*, 2002). Furthermore, TSP-1 and MPO have specific roles in modulating inflammation. TSP-1, through the receptor CD47, displays chemokine qualities in the recruitment and transmigration of leukocytes (Cooper *et al.*, 1995; Kirsch *et al.*, 2010; Liu *et al.*, 2001; Mansfield *et al.*, 1990). MPO is able to contribute to maintenance of neutrophil activation through catabolism of bioavailable NO as well as the binding of MPO to CD11b/CD18 receptors on neutrophils (Lau *et al.*, 2005).

In this patient cohort, mean plasma ADMA concentrations were 620±11nM, compared with 503±3nM in normal populations (Horowitz *et al.*, 2007). In effect, what may have been observed within this cohort is the endogenous inhibition of endothelial NOS due to these elevated ADMA levels, resulting in loss of tonic inhibition of neutrophil activation and ROS release, as well as platelet activation (Figure 42). Transient activation of these cell

populations would therefore increase, reflected through increased plasma concentrations of MPO and TSP-1. The increased plasma levels of MPO and TSP-1 would then be able to exert further influence on this dynamic in a feedback mechanism, further impairing the integrity of the NO signalling pathway.

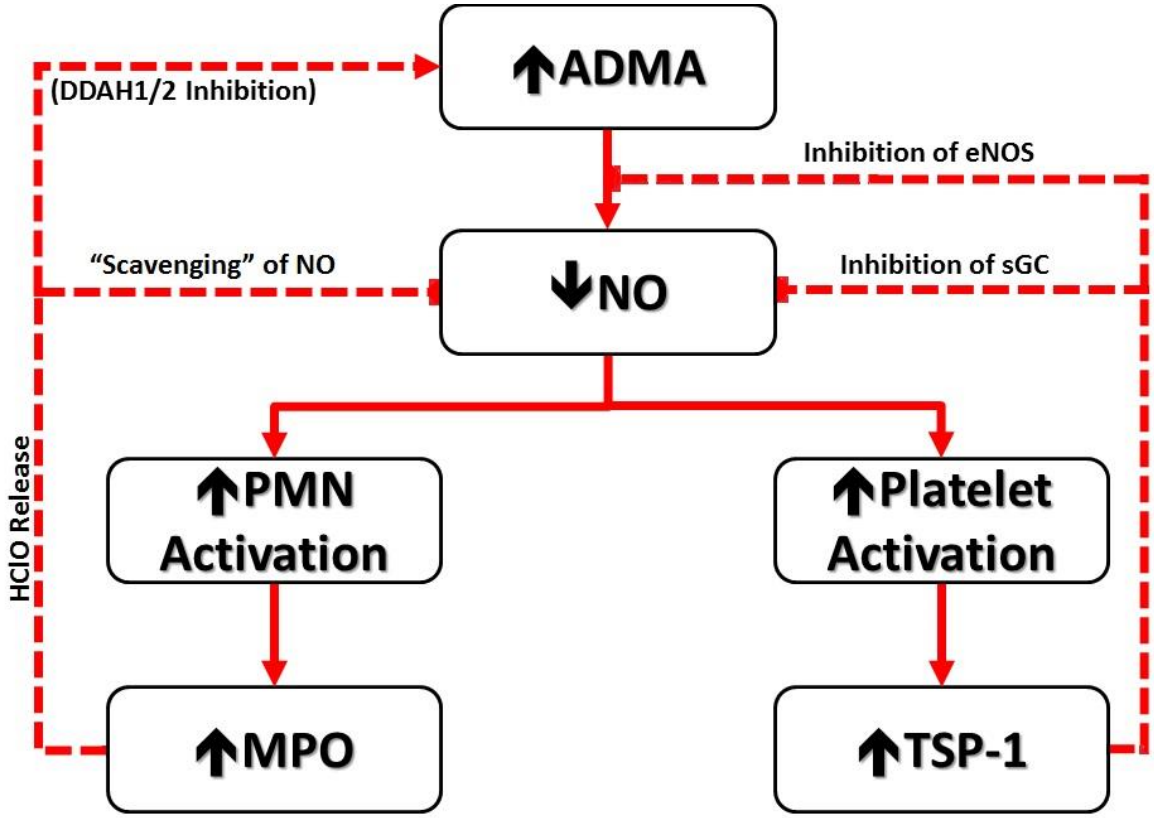

Figure 42: A proposed mechanism accounting for the nexus between TSP-1, MPO and ADMA. Transient activation of platelets and neutrophils results in the release of TSP-1 and MPO respectively, further impairing the integrity NO signalling through feedback mechanisms (Potentially, this nexus may have an important role in the pathophysiology of AF: endothelial dysfunction and MPO release have been implicated in the pathogenesis of AF (Cai *et al.*, 2002; V. Rudolph *et al.*, 2010) and platelet hyperaggregability is a known feature of the arrhythmia (Hammwohner *et al.*, 2007; Kamath, Blann*, et al.*, 2002; Kamath, Chin*, et al.*, 2002). However, any mechanistic link between endothelial dysfunction/inflammation (identified in these patients) and the pathophysiology of AF remains speculative, given the impracticality of specific mechanistic manipulation in this clinical setting).

4.2.5 Platelet Nitric Oxide Response in New Onset Atrial Fibrillation

The association of new onset AF with severe impairment of platelet NO signalling was not a prospectively sought interaction. However, this result was subsequently validated through the

prospective recruitment of additional new onset AF patients. Additionally, comparison with other acute cardiovascular diseases (e.g. ACS, acute heart failure) in which impairment of NO-mediated signalling has also been documented (Chirkov *et al.*, 2001; Tamargo *et al.*, 2010) has identified that this impairment in platelet NO response appears to be a distinct feature of new onset AF and, in spite of strong univariate correlations, to be independent of heart rate. Factors which might contribute to this phenomenon are either a surge in plasma catecholamine levels, acute inflammation and oxidative stress, or a combination of both, accompanying the new onset of AF.

It has been shown that the generation of atrial tachyarrhythmia and induction of AF is accompanied by increased sympathetic and parasympathetic activity (Jayachandran *et al.*, 2000; Sharifov *et al.*, 2004; Tan *et al.*, 2013; Workman, 2010). It is therefore plausible that new onset AF is accompanied by increased sympathetic and parasympathetic activity, associated with tachycardia, and contributing to platelet dysfunction. One possible contributory factor is catecholamine-induced platelet hyperaggregability. Platelet hyper-reactivity has been observed in aggregation experiments in response to epinephrine and norepinephrine (Béres *et al.*, 2008; Ikarugi *et al.*, 1999; Larsson *et al.*, 1994; Willoughby *et al.*, 1996; Yee *et al.*, 2006). Moreover, adrenergic stimulation of platelets (via α2A receptors) is known to result in the inhibition of adenylyl cyclase, the enzyme responsible for accumulation of intracellular cAMP, which induces inhibition of platelet activation (Keularts *et al.*, 2000). A limitation of this theory though, is that catecholamines are rapidly cleared from plasma: in the context of *ex vivo* studies it is unlikely that residual effects of catecholamines will be detectable by the time aggregation is evaluated.

An alternative mechanism involves catecholamine-induced alterations in vascular tissue function, with resultant effects on platelet function. Catecholamines have been observed to stimulate ROS generation in endothelial (J. L. Mehta *et al.*, 2001), vascular smooth muscle (Bleeke *et al.*, 2004) and myocardial cells (Lu *et al.*, 2009), as well as leukocytes (Deo *et al.*,

2013). Adrenergic and noradrenergic stimulation of endothelial cells has been linked to uncoupling of endothelial NOS and decreased production of NO (Lu *et al.*, 2009; Nickel *et al.*, 2009). Additionally, AF and atrial pacing have been associated with increased peroxynitrite formation (Carnes *et al.*, 2001; Mihm *et al.*, 2001), characteristic of uncoupled NOS activity. The effects of catecholamines upon vascular tissue, that is the stimulation of increased ROS generation, may result in scavenging of available NO and oxidative impairment of sGC (Chirkov *et al.*, 2007; Weber *et al.*, 2001). Thus, acute catecholamine surges accompanying new onset AF may contribute to long-lasting impairment of the NO signalling pathway in platelets.

There is also the possibility that acute inflammation and associated oxidative stress that accompanies the new onset of AF could result in depletion of available NO and impairment of NO signalling in platelets through scavenging by ROS or catabolism by active MPO (Eiserich *et al.*, 2002; Eiserich *et al.*, 1998), as well as inducing the uncoupling of endothelial NOS (Boulden *et al.*, 2006; Xu *et al.*, 2006), resulting in impairment of NO signalling. However, within the current cohort, no significant differences between new onset and chronic AF were observed in plasma MPO or CRP levels.

Research has consistently indicated that with the *de novo* detection and diagnosis of AF, there is an immediate period of elevated thromboembolic risk (R. H. Mehta *et al.*, 2003; Miyasaka *et al.*, 2007; Mrdovic *et al.*, 2012). While this phenomenon could be partially attributed to a delay between initiation of anti-thrombotic therapy and beneficial effect, the current research has identified specific physiological phenomena present in the acute onset of AF that may contribute to increased thromboembolic risk. Such understanding may provide the basis for new strategies in the pharmacological management of AF, such as the acute application of agents known to potentiate NO signalling (Chirkov *et al.*, 2004; Stepien *et al.*, 2003; Willoughby *et al.*, 2012), and/or the earlier application of oral anticoagulation therapies.

4.2.6 Gender-specific Platelet Dysfunction in Atrial Fibrillation

The association of female gender with thromboembolic risk in AF has been known for some time (Wolf *et al.*, 1991). This gender-specific stroke risk has been consistently identified whether strokes are ischaemic or haemorrhagic (R. G. Hart *et al.*, 1999; Humphries *et al.*, 2001), controlling for rates of anticoagulant therapy (Avgil Tsadok *et al.*, 2012), or in patients naive to previous anticoagulation (T. J. Wang *et al.*, 2003b), and persists after controlling for other clinical stroke risk factors (Fang *et al.*, 2005). Recently, sex category has been incorporated into the CHA_2DS_2VASc stroke risk score with the intention of enabling the more accurate stratification of AF patients at low risk of stroke/TIA (Lip *et al.*, 2010). The physiological bases for this risk are as yet unclear, however.

Female patients typically display increased platelet aggregability when compared with male patients (Becker *et al.*, 2006; Otahbachi *et al.*, 2010; Yee *et al.*, 2006), and as mentioned previously, stimulation of platelets with catecholamines can result in the production of a hyper-reactive phenotype (Béres *et al.*, 2008; Ikarugi *et al.*, 1999; Yee *et al.*, 2005). Arguably, based upon this, females would display much more pronounced hyperaggregability in response to a 'catecholamine' surge than males – however, the same rationale applies in this situation that applied when considering this possibility as a potential mechanism for new onset AF: catecholamine reuptake from plasma is quite rapid, with any effect between sample collection and platelet aggregometry likely to have dissipated. Similarly, the potential for catecholamines to stimulate ROS generation in endothelial and vascular smooth muscle cells (Bleeke *et al.*, 2004; J. L. Mehta *et al.*, 2001), resulting in impaired NO signalling, is also a possibility. Pre-menopausal females display decreased sensitivity to α-adrenergic and increased sensitivity to β-adrenergic stimuli relative to males (Freedman *et al.*, 1987; Kneale *et al.*, 2000). α-adrenergic stimulation of vascular tissue has been associated with increased ROS production (Bleeke *et al.*, 2004; Lu *et al.*, 2009), while β-adrenergic stimulation in pre-menopausal females has previously been associated with increased NO generation (Calderone

et al., 2002; Freedman *et al.*, 1987). However, these gender-specific differences in adrenergic signalling may largely be lost in post-menopausal females (Calderone *et al.*, 2002; McKee *et al.*, 2003). β-adrenergic signalling becomes impaired in hypertensive individuals (Bohm *et al.*, 1994; Bohm *et al.*, 1996), and in post-menopausal females (E. C. Hart *et al.*, 2011). Thus, the decline in function of β-adrenergic signalling (in association with cardiovascular diseases such as hypertension), combined with increased sensitivity of α-adrenergic, and decreased sensitivity of β-adrenergic, signalling pathways post-menopause (Calderone *et al.*, 2002; McKee *et al.*, 2003) may render females particularly vulnerable to impairment of the NO signalling pathway, as a result of catecholamine-stimulated ROS generation by vascular tissue (Bleeke *et al.*, 2004; Lu *et al.*, 2009; J. L. Mehta *et al.*, 2001). This potential mechanism may explain, in part, the impairment of platelet NO signalling in females compared to males in the current cohort. The possibility of gender-specific variability in catecholamine sensitivity is also distinctly plausible and may be contributing to the increased thromboembolic risk experienced by female AF patients.

4.2.7 Multivariate Determinants of Platelet Function in Atrial Fibrillation

Consistent with what was observed in the primary cohort, physiological antagonism between pro- and anti-aggregatory stimuli is a significant feature affecting platelet function in AF. Previous research has identified platelet hyperaggregability as a pathophysiological feature of AF (Kamath, Blann, *et al.*, 2002; Ohara *et al.*, 2008), though noticeably no association with incidence of thromboembolism has been established. While the study was not powered to evaluate potential associations between platelet NO response and clinical outcomes in AF, work has been done in other cardiovascular diseases that may be applicable. Impaired platelet NO response in ACS has been independently associated with all-cause mortality (Willoughby *et al.*, 2005). Furthermore, increasing bioavailability of NO through the use of NO donors (Zhang *et al.*, 1994) and/or agents that improve NO response (Aktas *et al.*, 2003) have been

observed to improve outcomes in stroke, and may also partially underlie the therapeutic benefit observed with statin and ACE inhibitor therapies (Chirkov *et al.*, 2004; Stepien *et al.*, 2003; Willoughby *et al.*, 2012). Thus, impaired platelet NO signalling in AF represents a potential therapeutic target.

Similar to what was observed in the primary cohort, plasma TSP-1 (see section 4.1.2) and female gender (see section 4.2.2) were both directly correlated with platelet aggregability, while plasma SDMA was inversely correlated.

The association between admission heart rate and platelet function within this cohort strongly intersected with the incidence of new onset AF: admission heart rate was noticeably elevated in new onset AF patients when compared with chronic AF patients. Significantly, though heart rate proved to be a multivariate correlate of platelet aggregability, when evaluating determinants of platelet NO response it failed to survive the analysis. Part of this may be attributable to the inclusion of acuity of AF as a variable in the analysis: new onset AF is associated with severely impaired platelet response to NO, and this relationship has proven to be independent of heart rate (see figure 23). The implications of this are unclear. Due to the endothelial dysfunction present in AF, it is possible that elevations in heart rate are accompanied by increased platelet aggregability as a result of the inability of the endothelium to adapt synchronously to increased sheer stress with increased NO and/or prostacyclin production. Previously, it has been observed that in spite of elevations in plasma epinephrine and norepinephrine in healthy adults in response to exercise, net platelet aggregability remained unchanged (Siess *et al.*, 1982). By contrast, platelet aggregability has been shown to increase in response to exercise in patients with coronary artery disease (Kumpuris *et al.*, 1980). A similar effect may be occurring in AF, where increases in heart rate result in increased platelet aggregability. While a similar sheer-dependent mechanism may account for the inverse relationship between heart rate and platelet NO response, this potential mechanism would be distinct from what is observed in new onset AF. As has been stated, the impairment

of platelet NO signalling in new onset AF is independent of heart rate. Taken together, these findings support the possibility of a relationship between the presence of tachycardia and platelet dysfunction in AF.

4.2.8 Platelet Function in Chronic Atrial Fibrillation

Ever since the need for chronic anticoagulation was established in AF (Askey *et al.*, 1950), there has also been an associated bleeding risk. For example, the risk of bleeding and/or haemorrhage in connection with warfarin use has previously been identified as higher in females (Humphries *et al.*, 2001), and the elderly (Fang *et al.*, 2004). In order to address the increased bleeding risk that accompanies anticoagulation in AF, numerous bleeding risk scores have been developed. One such score is known as HEMORR$_2$HAGES (Hepatic or Renal Disease, Ethanol Abuse, Malignancy, Older Age, Reduced Platelet Count or Function, Re-Bleeding, Hypertension, Anaemia, Genetic Factors, Excessive Fall Risk and Stroke), developed through the amalgamation of previous generic bleeding risk schemes and prospectively validated in a cohort of AF patients (Gage *et al.*, 2006). Although displaying modest predictive power for the occurrence of bleeding events, it has been criticised as being overly complicated and impractical in clinical practice, resulting in the derivation of the HAS-BLED (Hypertension, Abnormal Renal/Liver Function, Stroke, Bleeding History or Predisposition, Labile International Normalized Ratio, Elderly, Drugs/Alcohol) risk score for occurrence of haemorrhage (Lip *et al.*, 2011). Parallel to the development of HAS-BLED, an alternative bleeding risk score was also derived from the ATRIA (Anticoagulation and Risk Factors in Atrial Fibrillation) cohort (Fang *et al.*, 2011).

However, thromboembolic and haemorrhagic risk scores have displayed significant, direct, correlations with each other (Marcucci *et al.*, 2013), suggesting that their combined utility in determining appropriate anti-thrombotic therapy is limited. Recently, evidence has been accumulating supporting a potential role for SDMA in evaluating cardiovascular risk. Plasma

SDMA has been directly associated with poorer prognosis following acute stroke (Luneburg *et al.*, 2012; F. Schulze *et al.*, 2010) and inversely associated with event free survival in coronary heart disease patients (Siegerink *et al.*, 2013). Recently, plasma SDMA levels have also been recognised as predictive of bleeding risk in patients with AF (Horowitz *et al.*, 2013). Controversy exists on whether SDMA has a physiological role: arguments have been made supporting plasma SDMA as a measure of renal function (Kielstein, Salpeter, *et al.*, 2006) and indeed, plasma SDMA has been associated with renal impairment in coronary artery disease (Bode-Boger *et al.*, 2006). Infusions of SDMA have also been observed to have limited effects on blood pressure regulation, cardiac or renal function (Veldink *et al.*, 2013). However, SDMA has been shown to stimulate ROS generation in monocytes (Schepers *et al.*, 2009; von Leitner *et al.*, 2011) and inflammatory cytokine production in leukocytes (an effect inhibited by the hypochlorous acid scavenger N-acetylcysteine, interestingly), as well as to correlate with plasma levels of interleukin-6 and TNFα in patients with chronic kidney disease (Schepers *et al.*, 2011). The possibility of SDMA having a physiological role therefore remains viable.

As well as being directly associated with plasma TSP-1 concentrations and platelet Txnip content, platelet aggregability in chronic AF was inversely associated with plasma SDMA levels. Platelet Txnip content also correlated with platelet NO response, thus the association between Txnip and platelet aggregability may be reflective of the inhibitory role Txnip has on NO signalling (Forrester *et al.*, 2009; P. C. Schulze *et al.*, 2006; Sverdlov *et al.*, 2013a). However, physiological mechanisms accounting for the interaction of SDMA and platelet aggregability were not clear: two distinct possibilities are apparent. Firstly, declining platelet aggregability and increasing plasma SDMA concentrations may be occurring as a result of renal impairment. As stated previously, plasma SDMA has been proposed as a potential marker of renal impairment: the risk of both ischaemic and haemorrhagic stroke are greatly increased in AF patients with chronic kidney disease (Olesen *et al.*, 2012), a state associated

with elevated plasma SDMA levels (Eiselt *et al.*, 2010; Kielstein, Boger, *et al.*, 2004; Kielstein, Salpeter, *et al.*, 2006; Schepers *et al.*, 2011). Additionally, platelet aggregability is known to decline in patients with severe renal impairment (Lindsay *et al.*, 1975). Thus the occurrence of increasing plasma SDMA levels in association with declining platelet aggregability may both be reflective of renal impairment. Alternatively, SDMA may be exerting physiological effects upon platelet aggregability: SDMA has been shown to stimulate ROS production in leukocytes, which could potentially scavenge available NO, promoting platelet hyperaggregability. SDMA may also limit the cellular uptake of L-arginine via competitive antagonism of the cationic amino acid transporter, depriving endothelial NOS of substrate (Kielstein, Salpeter, *et al.*, 2006), thereby contributing to uncoupling of NOS. This may limit NO production and promote ROS generation, thus resulting in increased platelet aggregability.

These possibilities remain speculative at this stage and require further research to determine the physiological role of SDMA. The inverse association of platelet aggregability with plasma SDMA, in light of previous findings establishing plasma SDMA as predictive of bleeding events, represents a potential physiological mechanism that could in part account for haemorrhagic risk in AF.

4.2.9 Changes in Platelet Function over Time in Atrial Fibrillation

As has been mentioned previously (see section 4.1.2.1), the strong correlation documented between the extent of platelet aggregation and platelet response to NO most likely reflects physiological antagonism (Henry *et al.*, 1985), i.e. opposing effects resulting from the stimulation of functionally distinct signalling pathways. The consistency of this relationship over time can be considered parallel to what has previously been reported regarding platelet reactivity in AF: studies so far have not established a relationship between clinical indications of thromboembolic risk and levels of platelet reactivity (Kamath, Blann, *et al.*, 2002; Kamath,

Chin, *et al.*, 2002; Lip *et al.*, 2007; Ohara *et al.*, 2008). This finding also implies the independence of platelet NO response from ADP-induced aggregation, particularly with regards to new onset AF, as no difference in 'levels' of physiological antagonism was observed between new onset and chronic AF (see figure 32).

Interestingly, the marked impairment in baseline platelet NO signalling associated with new onset AF was only partially attenuated at follow-up, with a trend towards improvement that did not reach statistical significance. This suggests that impairment of platelet NO response may persist for up to one year post onset of AF. Due to the novel finding that platelet NO response is impaired in new onset compared with chronic AF patients, it is unclear to what extent the continued impairment of platelet NO signalling in new onset AF patients represents the pathophysiology of new onset AF, or to what extent (potentially) inadequate pharmacotherapy. ACE inhibitor and statin therapies were applied in approximately 1/3 and 1/2 of the follow-up AF population, respectively, and are known to potentiate platelet NO response, particularly where that response is severely impaired (Chirkov *et al.*, 2004; Stepien *et al.*, 2003; Willoughby *et al.*, 2012). These therapies had little apparent effect on observed platelet physiology in the current cohort, however (see section 3.4.2).

Recurrence rates for AF following successful cardioversion have been documented at approximately 40-50% (Loffredo *et al.*, 2012; Masson *et al.*, 2010), with incidence of AF previously associated with increased sympathetic and parasympathetic activity (Jayachandran *et al.*, 2000; Sharifov *et al.*, 2004). Reversion to AF, accompanied by surges in plasma catecholamine levels, could therefore in theory impair the recovery of platelet NO response. The limited recovery in platelet NO signalling exhibited by new onset AF patients at follow-up underscores the need to re-evaluate treatment strategies in these patients, and potentially to ensure that anticoagulation is initiated as soon as possible in new cases of AF.

4.2.10 Changes in Plasma Asymmetric and Symmetric Dimethylarginines over Time in Atrial Fibrillation

Endothelial dysfunction is a known feature of AF and is in part due to the uncoupling of the endothelial NOS enzyme, resulting in O_2^- generation as opposed to NO production (Freestone *et al.*, 2008; Matsue *et al.*, 2011; Takahashi *et al.*, 2001). The presence of endothelial dysfunction in AF to some extent depends upon the existence of the arrhythmia itself, as reversion to sinus rhythm has resulted in improved vascular function (Yoshino *et al.*, 2013). ADMA, an endogenous inhibitor of NOS, has been linked to endothelial dysfunction and the promotion of ROS generation (Antoniades *et al.*, 2009). ADMA, and its structural isomer SDMA, have both been associated with increased morbidity and mortality in the context of cardiovascular disease. ADMA is elevated in AF (Goette *et al.*, 2012), diabetes mellitus (Lin *et al.*, 2002), and in stroke (Yoo *et al.*, 2001), and has been associated with incidence of thromboembolism, major bleeding events and cardiac related death in AF (Horowitz *et al.*, 2013). Similarly, SDMA has been correlated with renal dysfunction and extent of coronary artery disease (Bode-Boger *et al.*, 2006), poor prognosis following acute ischaemic stroke (Luneburg *et al.*, 2012; F. Schulze *et al.*, 2010), and has also been associated with incidence of major bleeding events and cardiac related death in AF patients (Horowitz *et al.*, 2013). Decline in endothelial function with age has been observed in normotensive and hypertensive patients (Taddei *et al.*, 1995). However it is uncertain why plasma ADMA and SDMA concentrations (as biochemical surrogates of endothelial function) increased during follow-up in the current study. Renal dysfunction could contribute to accumulating plasma ADMA and SDMA levels, through reduced renal clearance or impairment of metabolic clearance enzymes via oxidative stress (Blackwell, 2010). However, in the current cohort, no consistent increase in plasma creatinine levels was observed between baseline and follow-up time points, making deteriorating renal function an unlikely possibility.

Progressive endothelial dysfunction, reflected by increasing plasma ADMA and SDMA concentrations, may constitute one biochemical aspect of incremental thromboembolic risk associated with age.

4.2.11 Secondary Hypotheses: Conclusions

The present study also sought to investigate the impact of known modulators of NO signalling on the integrity of the NO signalling pathway:

4.2.11.1 Asymmetric Dimethylarginine

ADMA is potentially able to influence the integrity of NO signalling via competitive inhibition of NOS, resulting in uncoupling and the production of superoxide (Antoniades *et al.*, 2009; Veresh *et al.*, 2008). It has been correlated clinically with endothelial dysfunction (Achan *et al.*, 2003; Kielstein, Donnerstag, *et al.*, 2006) and plasma levels of ADMA correlate directly with incidence of adverse cardiovascular events (Achan *et al.*, 2003; Boger *et al.*, 2009; Kielstein, Impraim, *et al.*, 2004; Siegerink *et al.*, 2013; Yoo *et al.*, 2001). Because of the impact ADMA may have on bioavailability of NO, a discernible effect of ADMA on platelet function was plausible, either through platelet hyperaggregability (due to depletion of local NO) or impaired platelet NO response (as a result of oxidative scavenging of NO due to uncoupled endothelial NOS). Previously, ACE inhibitor therapy (J. W. Chen *et al.*, 2002; Willoughby *et al.*, 2012) and diuretic use (Khan *et al.*, 2012) have been observed to reduce plasma ADMA levels. However the use of ACE inhibitors did not correspond with lower plasma ADMA levels in this cohort. This may simply reflect selective use of ACE inhibitors

in high risk patients. Additionally, no correlation was observed between plasma ADMA levels and platelet NO response.

4.2.11.2 Myeloperoxidase

MPO has been identified as a significant factor in the pathogenesis of AF in animal models, and to some extent in humans (Ozaydin *et al.*, 2008; V. Rudolph *et al.*, 2010), is capable of catabolising NO (Eiserich *et al.*, 2002) and can contribute to the uncoupling of endothelial NOS (Xu *et al.*, 2006), which has also been implicated in the pathogenesis of AF (Cai *et al.*, 2002). Although a correlation was observed between plasma MPO and ADMA levels, reinforcing previous research regarding the interaction of MPO and ADMA (von Leitner *et al.*, 2011), no association was observed between MPO and platelet NO response. However, the only probable basis for such an association would be NO "scavenging" by hypochlorous acid produced by MPO: the kinetics of this process and its interface with release of NO from SNP are unknown at present.

4.2.11.3 Thrombospondin-1

Previous work in *in vitro* settings has outlined the effect TSP-1 has on platelet aggregability, involving the exogenous addition of TSP-1 and determining that TSP-1 potentiates platelet activation through inhibition of both cAMP and cGMP signalling pathways (Isenberg, Romeo, *et al.*, 2008; Roberts *et al.*, 2010). However, limited evidence to date has been obtained for potentially pathological roles of TSP-1: recent work has identified the involvement of TSP-1 in cardiac remodelling (Y. Xia *et al.*, 2011) and pulmonary arterial hypertension (P. M. Bauer *et al.*, 2012). Within the current study, although a correlation was observed between TSP-1 and platelet aggregability, no such association was observed for platelet NO response. Part of this result may be attributed to alternative sources of

endogenous TSP-1 apart from platelets (Jaffe *et al.*, 1985; Kirsch *et al.*, 2010). Additionally, agents that can affect platelet reactivity, such as aspirin, had no effect on plasma TSP-1 levels.

4.2.11.4 Thioredoxin-interacting Protein

A relationship between Txnip and NO signalling has been documented in experimental (Forrester *et al.*, 2009; P. C. Schulze *et al.*, 2006) and clinical (Sverdlov *et al.*, 2013a) settings. Notably, Txnip has been identified in clinical contexts as a correlate of platelet NO response. However in the current cohort, while Txnip was identified as an inverse univariate correlate of platelet NO response, it failed to survive multivariate analysis. A number of factors may have contributed to produce this result, such as diabetic status (P. C. Schulze *et al.*, 2004), medical therapies including ACE inhibition (Sverdlov *et al.*, 2013a), metformin (Chai *et al.*, 2012) and Ca^{2+} channel antagonists (J. Chen *et al.*, 2009), all of which suppress Txnip expression. Potentially, even the duration patients spend in AF as opposed to sinus rhythm may have an effect upon Txnip expression: Turbulent blood flow has been shown to stimulate Txnip expression (X. Q. Wang *et al.*, 2012; Yamawaki *et al.*, 2005). In summary, NO is probably not the main modulator of Txnip expression in platelets, with many other interacting factors implicated in the study.

4.2.11.5 Clinical Stroke Risk Scores and Integrity of Platelet Nitric Oxide Signalling

As a surrogate measure in the absence of thromboembolic events, platelet NO response was compared against the $CHADS_2$ and CHA_2DS_2VASc stroke risk scores. No significant correlation was observed between platelet NO response and perceived stroke risk, as seen from these clinical indices: therefore this hypothesis was not supported. This negative finding is important, as it raises the issue of the biochemical bases of clinical risk scores.

4.2.11.6 New Onset Atrial Fibrillation, Tachycardia and Platelet Function

Although acuity of AF and heart rate were observed to correlate with platelet NO response within this cohort, the potential for artefact (due to the timing between onset of AF, hospital admission and study enrolment) precluded these factors from being explored by this study (these factors were explored further after controlling for duration of time between onset of AF and index enrolment into the study, see section 3.2). No conclusions can be made on clinical associations with platelet NO response at this stage.

Plasma levels of ADMA, MPO, TSP-1 and platelet Txnip content were not significant multivariate correlates of platelet NO signalling within this cohort. Platelet aggregability and plasma creatinine concentrations were, however, suggesting that the primary factors affecting platelet NO signalling in AF patients are the hyperaggregability known to be present in AF, and overall renal function.

4.3 Possible Study Limitations

4.3.1 Correlation vs. Causation?

This was an observational study originally designed to evaluate the potential impact of known modulators of the NO signalling pathway (i.e. ADMA, MPO, TSP-1 and Txnip), on the integrity of platelet NO signalling in the clinical context of AF. As such, it is difficult to establish causal mechanisms based upon this data. Furthermore, specific experiments are required to explore this aspect of these results in order to satisfy the 'Koch's Postulates' of impaired NO signalling in AF.

4.3.2 Lack of Association between Platelet Function and Clinical Factors

No association between platelet NO signalling and clinical factors (i.e. $CHADS_2$ score, hospital readmission, stroke/TIA incidence and mortality) was established in this study. Similarly, platelet aggregability also did not correlate with clinical features of the patient cohort. The finding that platelet aggregability did not correlate with clinical features of AF

nor with measures of stroke/TIA risk is consistent with previous research (Kamath, Blann, *et al.*, 2002; Kamath, Chin, *et al.*, 2002; Ohara *et al.*, 2008). However the integrity of platelet NO signalling in the context of AF has not previously been investigated. In this context, it is worth noting that the study had a limited follow-up period as well as limited sample size. The narrow scope of this study in terms of clinical outcomes weakens any attempt to reach definitive conclusions about the role of impaired platelet NO signalling with regards to thromboembolic risk and clinical outcomes in AF. Nevertheless, the present study has identified significant determinants of platelet function (i.e. platelet aggregability and its inhibition by NO) in the context of AF.

4.3.3 Methodological Limitations: Aggregometry

Whole blood impedance aggregometry allows for evaluation of platelet function in the context of interactions with other blood components, allowing for a more "physiologically relevant" exploration of platelet NO signalling in AF. However, the use of whole blood impedance aggregometry (Cardinal *et al.*, 1980), as opposed to optical aggregometry (Born, 1962), limits the ability of the study to identify platelet-specific effects due to the presence of the rest of the blood milieu.

Additionally, the use of ADP as the primary agonist of platelet aggregation and SNP as the NO donor may also be causes for concern. The decision to use ADP as the agonist for platelet aggregation reflected its being the most abundant primary agonist of aggregation (via release from vasculature, etc.) as well as a secondary agonist (being released by platelets as a result of activation) facilitating positive feedback. The use of alternative agonists (e.g. thrombin-receptor activating peptide, or the thromboxane A2 analogue U46619) in addition to ADP was not considered for this project, as the investigation was primarily focused on exploring the functional integrity of NO signalling in platelets.

Use of SNP as the NO donor was determined based upon the requirement for intracellular metabolism of SNP in order to liberate NO (Kowaluk *et al.*, 1992). Alternative NO donors

such as diethylamine diazeniumdiolate or S-nitrosoglutathione, are capable of inhibiting platelet aggregation through cGMP-independent mechanisms (Crane *et al.*, 2005; Sogo *et al.*, 2000), rendering them unsuitable for the current study. Additionally, the use of SNP as the NO donor has not been associated with the problem of tolerance induction seen with organic nitrates (Chirkov *et al.*, 1997). Thus the use of SNP as the NO donor allows for the specific assessment of the functional integrity of sGC and cGMP-dependent signalling pathways in the context of AF.

This project constituted a sub-study of SAFETY (Carrington *et al.*, 2013), with the aim of evaluating, in a subset of the trial population, potential determinants of platelet NO response upon their admission to hospital. Alcohol intake was zero for at least 24 hours prior to initial blood sampling. However, variability on the basis of diurnal changes could not be excluded, given that the time of blood sampling was not standardised.

4.3.4 Methodological Limitations: Functional Status of Soluble Guanylate Cyclase

Notably, this study did not assess the functional status of sGC (e.g. through determination of cGMP levels). However previous research has shown that impairment of the NO-sGC signalling pathway to be the net result of oxidative stress and/or functional impairment of sGC (see review, Chirkov *et al.* (2007)). In this regard, stimulation of sGC through the recently characterised redox-sensitive site via agents other than NO (such as hydrogen peroxide and S-nitrosocysteine) remains a possibility, given the association of AF with redox stress (Burke *et al.*, 1987; Mellion *et al.*, 1983).

4.3.5 New Onset Atrial Fibrillation

It should be noted that the original observation that new onset AF is associated with significantly impaired platelet NO response (when compared with chronic AF) was not a prospectively defined consideration at the initiation of the current investigation. As such, the

finding is potentially subject to selection bias. *However, the generation of a 'validation cohort' comprised of prospectively and consecutively recruited new onset AF patients, as well as patients with ACS and acute heart failure, has confirmed these initial findings.*

Furthermore, as shown in Tables 9 and 10, limiting the primary cohort to AF patients whose blood samples were obtained within four months of index arrhythmia did not fundamentally alter the characteristics of the study cohort.

At the exact time of sample collection it was not established whether the patient was in AF or sinus rhythm. Theoretically this may have an effect on platelet function, as previous research has identified that markers of inflammation are elevated in patients in AF compared with sinus rhythm (Li *et al.*, 2010). Controversy exists regarding the effects of AF (vs. sinus rhythm) on platelet reactivity in patients with AF, with conflicting reports on whether the presence of the arrhythmia has an immediate effect on platelet reactivity (Hammwohner *et al.*, 2007; Kamath, Chin, *et al.*, 2002). Recently it has been reported that restoration of sinus rhythm in AF patients results in an improvement in endothelial function (Yoshino *et al.*, 2013), so by extension, the presence or absence of arrhythmia at time of sample collection could certainly have an effect on platelet response to NO.

Plasma catecholamine levels were not determined in this study, so it is not possible to establish within the current cohort that new onset AF is accompanied by surges in catecholamine levels. However, as outlined previously (section 4.2.2.1), it has been reported that induction of AF is accompanied by increased sympathetic and parasympathetic stimulation, and also that epinephrine and norepinephrine can induce ROS generation, as well as potentiate platelet aggregation.

4.3.6 Gender-specific Platelet Dysfunction

This investigation identified that female patients with AF display platelet hyperaggregability combined with impaired platelet NO response when compared with male patients. The

mechanisms underlying this are not clear: it has been established previously that females are hyperaggregable when compared with males (Becker *et al.*, 2006; Otahbachi *et al.*, 2010; Yee *et al.*, 2006; Yee *et al.*, 2005), however gender-specific differences in platelet NO signalling have not been apparent. Although theoretical associations can be made between these findings and increased risk of thromboembolism in female AF patients, the current study has not evaluated this potential relationship. Limitations in sample size and duration of follow-up of this cohort conspired to prevent the further investigation of gender-specific forms of thromboembolic risk in AF.

4.3.7 Chronic Atrial Fibrillation

The present investigation was undertaken due to the finding that new onset AF was associated with severely impaired platelet NO response, providing the basis for a *post hoc* re-evaluation of platelet function in a cohort of chronic AF patients. As such, the current study is vulnerable to type 1 error (i.e. false positives) from the lack of pre-specified hypotheses. However, restricting the study cohort to patients with chronic AF did not significantly alter the clinical characteristics nor the rates and types of pharmacotherapy in comparison with the primary cohort. This lessens the likelihood of selection bias, decreasing the possibility of type 1 error. This study identified an inverse correlation between plasma SDMA concentrations and platelet aggregability and highlighted the potential clinical utility of this finding, given recent reports that plasma SDMA is predictive of bleeding risk in AF patients (Horowitz *et al.*, 2013). It should be noted that there were no haemorrhagic events recorded during the follow-up for the present study, nor were there any clinically relevant major bleeding events recorded. As such, the present study is not powered to establish definitively a relationship between plasma SDMA, platelet aggregability and clinically relevant bleeding events. Nevertheless, this remains an important finding that warrants further investigation.

4.3.8 Follow-Up Cohort

The present investigation was performed upon a subset of the primary AF cohort for whom follow-up data were available. The smaller sample size of the follow-up cohort weakens any definitive conclusions regarding clinical endpoints being drawn from the current investigation. However, the follow-up cohort was similar in clinical profile and rates of pharmacotherapy to the primary study cohort (at baseline). Additionally, changes in biochemical parameters (i.e. ADMA, MPO, TSP-1 and Txnip, as well as changes in platelet function) were evaluated in AF patients over time using paired analysis: as such, the findings listed here are considered robust.

Adherence to pharmacotherapy was not assessed during follow-up, with several medications potentially interacting with the biological parameters that were assessed. ACE inhibitor and statin therapy can potentiate NO response (Chirkov *et al.*, 2004; Stepien *et al.*, 2003; Willoughby *et al.*, 2012), Ca^{2+} channel antagonists can suppress Txnip expression (J. Chen *et al.*, 2009), and proton pump inhibitors can elevate plasma ADMA levels (Ghebremariam *et al.*, 2013). Additionally, anti-platelet and anti-inflammatory agents could potentially affect plasma TSP-1 and MPO levels (by suppressing platelet and neutrophil activation, respectively).

Although plasma ADMA concentrations have previously been shown to correlate with vascular endothelial function (Boger *et al.*, 1998), this association was not evaluated in the current study: hence all conclusions regarding endothelial function rest on the biochemical surrogate, i.e. ADMA concentrations. The association of AF with endothelial dysfunction is not controversial.

4.4 Future Directions

4.4.1 Potential Dysfunction of Platelet cAMP-dependent Signalling in Atrial Fibrillation

This study evaluated the integrity of cGMP-dependent signalling via activation of the NO-sGC signalling pathway in the context of AF. However, also important is the role of cAMP-dependent signalling in determining platelet aggregation. TSP-1 has been observed to potentiate platelet aggregation via inhibition of cAMP accumulation (Roberts *et al.*, 2010), as has adrenergic stimulation of platelets via α2-adrenergic receptors (Keularts *et al.*, 2000). Furthermore, interaction between cAMP- and cGMP-dependent signalling pathways has been observed at numerous levels (Cuzzocrea *et al.*, 2007; Salvemini *et al.*, 2013; Zaccolo *et al.*, 2007). Thus, the role of cAMP-dependent signalling and its effect on overall platelet function in the context of AF warrants further investigation.

4.4.2 Plasma Symmetric Dimethylarginine and Platelet Aggregability

The association between plasma SDMA levels and platelet aggregability deserves further examination in order to determine the precise relationship between these two variables. One possibility is that plasma SDMA levels are merely reflective of the extent of renal impairment: previous work has identified that platelet aggregability is impaired in patients with chronic renal dysfunction (Lindsay *et al.*, 1975), and that bleeding risk is increased in AF patients with chronic kidney disease (Olesen *et al.*, 2012). These clinical observations do not presuppose any biological activity of SDMA. However, this would be ignoring recent work identifying potential roles for SDMA in inflammation (Schepers *et al.*, 2011; Schepers *et al.*, 2009). Exploratory work to determine the potential impact of SDMA upon platelet aggregability should therefore be considered.

4.4.3 Inflammation and Endothelial Dysfunction as Mediators of Platelet Hyperaggregability

Identifying clinical evidence of the link between endothelial dysfunction and inflammation, a link that may be modulated by platelet activation via TSP-1, is an intriguing result that merits further analysis. No mechanism was established by this investigation between endothelial

dysfunction/inflammation and either the pathogenesis of AF or the potential thromboembolic complications of AF. Considering that impairment of NO signalling has been associated with pathogenesis of AF (Cai *et al.*, 2002), and that TSP-1, MPO and ADMA have potentially negative impacts upon NO signalling, further investigation should be able to identify any causal associations between the pathogenesis of AF and its complications, and the nexus of endothelial dysfunction and inflammation identified in this study.

The new oral anticoagulants such as the direct thrombin inhibitor Dabigatran (Connolly, Ezekowitz, *et al.*, 2009), or the Factor Xa inhibitor Apixaban (Granger *et al.*, 2011) have not been evaluated in this investigation for their impact upon platelet function. For example, Dabigatran (via inhibition of thrombin) may have a potent effect upon platelet activation (Hankey *et al.*, 2011), potentially affecting immune function and vascular reactivity via suppression of platelet activation. The effects these new oral anticoagulants may have on apparent NO signalling has not been evaluated as of yet.

4.4.4 Mechanisms Contributing to Impaired Nitric Oxide Response in New Onset Atrial Fibrillation

Previous research has identified that new onset AF is itself a risk factor for thromboembolism, one that is independent of gender or anticoagulant status (Garcia *et al.*, 2010). The possibility that impaired platelet NO signalling in patients with new onset AF may contribute to thromboembolic risk is worth prospectively exploring, and may allow for evaluation of new treatment paradigms in these patients. The use of medications that have been shown to potentiate NO response (Chirkov *et al.*, 2004; Stepien *et al.*, 2003; Willoughby *et al.*, 2012) could be evaluated in this context, as well as the effects newer anticoagulants (Connolly, Ezekowitz, *et al.*, 2009; Granger *et al.*, 2011) may have on mortality rates in patients with new onset AF.

Further exploration of the physiological mechanisms underlying increased thromboembolic risk in new onset AF patients should also be undertaken. The possibilities suggested here (a combination of acute inflammation, oxidative stress and surges in plasma catecholamine levels), are certainly plausible but lack evidentiary basis from this study. Further investigation of these possibilities will deepen understanding of the mechanisms of thrombogenesis in AF, particularly new onset AF, and may allow for the identification of novel avenues of therapy in these types of patients. Additionally, although there is a clearly defined gender aspect to thromboembolic risk in patients with AF, understanding of the physiological bases for this are also lacking. While this study has focussed on one potential factor (impaired platelet function) that may contribute to increased thromboembolic risk, the current study has not established a link with incidence of thromboembolism. A holistic approach, incorporating platelet, vascular and neurohumoral studies may be warranted.

4.4.5 Gender-specific Therapeutic Strategies in Atrial Fibrillation

The present study has clear clinical implications due to platelet dysfunction, in the form of impaired NO signalling, impacting upon both female AF patients as well as patients with new onset AF, where both categories of patients have documented incremental thromboembolic risk (Fang *et al.*, 2005; Garcia *et al.*, 2010; Pancholy *et al.*, 2014). These findings provide rationale for the re-evaluation of thromboprophylactic strategies in AF, as well as novel interpretation of existing therapies.

4.4.6 Thioredoxin-interacting Protein as a Biomarker for Disease Prognosis?

The present study has identified biochemical markers that may be associated with likelihood of hospital readmission (Txnip) and length of hospital stay upon readmission (SDMA). Given the cost burden that AF poses upon hospital systems, these markers should be evaluated prospectively for their predictive power regarding hospital readmission and duration of

hospital stay. Such an investigation would provide valuable information on the potential clinical utility of biochemical markers in determining appropriate therapy for AF patients.

4.4.7 Specific Therapeutic Strategies for Patients with New Onset Atrial Fibrillation

The most important issue raised by this work is whether the impairment of NO response associated with new onset AF can be ameliorated either *de novo* or via pharmacotherapy. Many patients become acutely ill with onset of AF, and perhaps the autonomic disturbance of such illness may contribute to oxidative stress and impairment of NO signalling. In this regard, it would have been desirable to investigate changes in Txnip expression serially following onset of AF, as turbulent flow may stimulate Txnip expression (X. Q. Wang *et al.*, 2012) and this might in theory be non-synchronous with the duration of suppression of NO effect. Interestingly, dronedarone, whatever its disadvantages (Hohnloser *et al.*, 2009), appears to reduce symptoms associated with recurrence of AF (Connolly, Crijns, *et al.*, 2009). As regards strategies to improve NO response, ACE inhibitors have not achieved universal application in AF patients to date: the current data provide a basis for their more widespread use.

4.5 Concluding Remarks

Pathogenesis of AF was initially attributed to atrial distension resulting in mechanical dysfunction: structural remodelling of the atria can indeed occur in response to 'atrial stretch' (De Jong *et al.*, 2011), and left atrial size has been identified as a predictor of the both the emergence of new onset AF, as well as the likelihood of AF recurrence following successful reversion to sinus rhythm (Hirose *et al.*, 2012; Marchese *et al.*, 2011). Thromboembolic risk in AF was also regarded as a result of mechanical dysfunction, whereby atrial blood stasis could promote thrombus formation: the occurrence of spontaneous echo contrast, particularly

in the left atrial appendage supports this view (Black *et al.*, 1991; Wysokinski *et al.*, 2010), as does the association between left atrial strain and stroke incidence (Shih *et al.*, 2011). However, a significant inflammatory component has emerged in association with the pathogenesis of AF. A number of inflammatory biomarkers have been positively correlated with the incidence of AF (Schnabel *et al.*, 2010), as well as being elevated in patients with AF compared against sinus rhythm (Li *et al.*, 2010). Infiltration of the atrial myocardium by leukocytes has been associated with the extent of atrial fibrosis (Yamashita *et al.*, 2010), while MPO has been identified as having a pivotal role in the pathogenesis of AF by inducing atrial remodelling (V. Rudolph *et al.*, 2010). The inflammatory component of AF contributes to thromboembolic risk as well: CRP has been correlated with incidence of left atrial thrombus (Maehama *et al.*, 2010), as well as with clinical measures of thromboembolic risk in AF patients (Crandall *et al.*, 2009; Lip *et al.*, 2007). Currently, assessment of thromboembolic risk in AF patients is largely based upon clinical factors that were derived from population based studies seeking to identify correlates of stroke incidence (Gage *et al.*, 2001; Lip *et al.*, 2010). In spite of the success observed in stratifying stroke risk using these clinical scores, a complete understanding of the physiological basis of thromboembolic mechanisms in AF remains elusive.

This project sought to evaluate the integrity of platelet NO signalling in AF and to identify clinical and biochemical factors that may contribute to its impairment. Deficient NO signalling has been identified as a feature of AF: both in terms of endothelial dysfunction (Freestone *et al.*, 2008; Guazzi *et al.*, 2009), as well as being implicated in the pathogenesis of AF (Cai *et al.*, 2002; Ridnour *et al.*, 2007). Interestingly, considering the pivotal role of MPO in the pathogenesis of AF (V. Rudolph *et al.*, 2010), as well as the catabolic activity MPO has for NO (Eiserich *et al.*, 2002; Eiserich *et al.*, 1998), a direct interaction between MPO and NO signalling could have been hypothesised in the context of AF. However, the present study does not support any such interaction.

Of additional interest was investigating the possibility that impairment of platelet NO signalling may contribute to thromboembolic risk, thereby providing physiological bases for measures of thromboembolic risk such as $CHADS_2$ and CHA_2DS_2VASc. Previous research has sought to expand upon these stroke risk schema, evaluating the potential contributions biochemical markers of endothelial damage/dysfunction (i.e. von Willebrand Factor, soluble E-selectin), or inflammation may have upon the predictive accuracy of stroke risk scores (Crandall *et al.*, 2009; Lip *et al.*, 2007; Roldan *et al.*, 2005). Measures of platelet reactivity have been assessed previously with regards to scores of thromboembolic risk in AF (Kamath, Blann, *et al.*, 2002; Lip *et al.*, 2007; Ohara *et al.*, 2008), with no interaction having been established. The integrity of platelet NO signalling has not been investigated previously for its potential association with $CHADS_2$ and CHA_2DS_2VASc scores. No association was observed between integrity of platelet NO signalling and measures of thromboembolic risk such as $CHADS_2$ and CHA_2DS_2VASc. As such, it can be concluded that impaired platelet NO signalling does not constitute a physiological basis for the $CHADS_2$ and CHA_2DS_2VASc stroke risk scores.

The current project was able to support a number of key findings that have clinical relevance and contribute significantly to the understanding of AF pathogenesis:

1) There is now prevalent recognition of a period <u>following new onset AF</u> during which patients experience incremental thromboembolic risk with respect to chronic AF patients (Conen *et al.*, 2011; Elesber *et al.*, 2006; Lehto *et al.*, 2005; Miyasaka *et al.*, 2007; Mrdovic *et al.*, 2012; T. J. Wang *et al.*, 2003b). The current project has identified that new onset AF is accompanied by severe impairment in platelet NO signalling in comparison with chronic AF. Furthermore, the diminution in platelet NO response experienced by new onset AF patients was only partially attenuated at a median 11.8 months follow-up. Of additional concern in this respect, there are no specific recommendations for the management of new onset AF patients with

regards to their increased thromboembolic risk, distinct from chronic AF patients. Indeed, pharmacotherapy between new onset and chronic AF patients were broadly similar in the current project. The current findings constitute a plausible physiological basis for the increased thromboembolic risk present in new onset AF and underscore the need to re-evaluate therapeutic strategies in these patients.

2) A gender-dependent interaction with platelet function was identified by this project: female AF patients were both more aggregable and less responsive to NO than male AF patients. This finding is clinically relevant due to the incremental thromboembolic risk female AF patients experience when compared to male patients, a fact reflected by the inclusion of gender into the CHA_2DS_2VASc stroke risk score. This particular finding cannot be accounted for by different rates of pharmacotherapy, nor new onset AF status, as these factors were equivalent across genders. Variations in adrenergic signalling between male and female AF patients could account for the differences in platelet aggregability and response to NO observed between genders, at least in part, and may form the physiological basis for elevated stroke risk in female AF patients.
3) Bleeding risk in association with chronic anticoagulation of AF patients is an ongoing clinical concern. Recently, plasma SDMA concentrations were found to positively correlate with the incidence of clinically relevant major bleeding events in AF patients (Horowitz *et al.*, 2013). The current project has identified an interaction between plasma SDMA concentrations and platelet aggregability that may contribute to the increased bleeding risk experienced by anticoagulated AF patients. Previous indications for bleeding risk were based upon clinical presentation and experienced significant overlap with thromboembolic risk scores (Marcucci *et al.*, 2013). Plasma SDMA concentrations represent a novel biochemical marker of

bleeding risk for AF patients that may in fact have a physiological basis, via diminished platelet aggregability.

Evaluation of the role of platelets in the pathophysiology of AF thus far has been limited primarily to measures of platelet aggregability/reactivity (Kamath, Blann, *et al.*, 2002; Kamath, Chin, *et al.*, 2002; Lip *et al.*, 2007; Ohara *et al.*, 2008), and whether these measures correlate with categories of thromboembolic risk. Similarly, while the current project has investigated platelet aggregability, the primary focus has been on integrity of NO signalling. In the context of platelet function, this represents a previously unexplored area of AF: in addressing this issue, the current project has identified key findings (already outlined) which contribute critically to the clinical and pathophysiological understanding of AF. Furthermore, key questions arise with respect to these findings that should be addressed by further research, specifically re-evaluation of disease management strategies in subsets of AF patients (i.e. new onset AF and female AF patients), as well as the (potential) physiological role of SDMA in modulating platelet aggregability. Addressing these issues will contribute to improved management of AF patients.

5: References

Achan, V., Broadhead, M., Malaki, M., Whitley, G., Leiper, J., MacAllister, R., & Vallance, P. (2003). Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively metabolized by dimethylarginine dimethylaminohydrolase. *Arterioscler Thromb Vasc Biol,* **23**(8): 1455-1459.

Airaksinen, K. E., Gronberg, T., Nuotio, I., Nikkinen, M., Ylitalo, A., Biancari, F., & Hartikainen, J. E. (2013). Thromboembolic complications after cardioversion of acute atrial fibrillation: the FinCV (Finnish CardioVersion) study. *Journal of the American College of Cardiology,* **62**(13): 1187-1192.

Aktas, B., Utz, A., Hoenig-Liedl, P., Walter, U., & Geiger, J. (2003). Dipyridamole enhances NO/cGMP-mediated vasodilator-stimulated phosphoprotein phosphorylation and signaling in human platelets: in vitro and in vivo/ex vivo studies. *Stroke,* **34**(3): 764-769.

Amsterdam, E. A., Wenger, N. K., Brindis, R. G., Casey, D. E., Jr., Ganiats, T. G., Holmes, D. R., Jr., Jaffe, A. S., Jneid, H., Kelly, R. F., Kontos, M. C., Levine, G. N., Liebson, P. R., Mukherjee, D., Peterson, E. D., Sabatine, M. S., Smalling, R. W., Zieman, S. J., American College of, C., American Heart Association Task Force on Practice, G., Society for Cardiovascular, A., Interventions, Society of Thoracic, S., & American Association for Clinical, C. (2014). 2014 AHA/ACC Guideline for the Management of Patients with Non-ST-Elevation Acute Coronary Syndromes: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Journal of the American College of Cardiology,* **64**(24): e139-228.

Anderson, T. J., Elstein, E., Haber, H., & Charbonneau, F. (2000). Comparative study of ACE-inhibition, angiotensin II antagonism, and calcium channel blockade on flow-mediated vasodilation in patients with coronary disease (BANFF study). *Journal of the American College of Cardiology,* **35**(1): 60-66.

Angiolillo, D. J., Ueno, M., & Goto, S. (2010). Basic principles of platelet biology and clinical implications. *Circulation Journal,* **74**(4): 597-607.

Antoniades, C., Shirodaria, C., Leeson, P., Antonopoulos, A., Warrick, N., Van-Assche, T., Cunnington, C., Tousoulis, D., Pillai, R., Ratnatunga, C., Stefanadis, C., & Channon, K. M. (2009). Association of plasma asymmetrical dimethylarginine (ADMA) with elevated vascular superoxide production and endothelial nitric oxide synthase uncoupling: implications for endothelial function in human atherosclerosis. *European Heart Journal,* **30**(9): 1142-1150.

Askey, J. M., & Cherry, C. B. (1950). Thromboembolism associated with auricular fibrillation; continuous anticoagulant therapy. *J Am Med Assoc,* **144**(2): 97-100.

Ausma, J., Wijffels, M., Thone, F., Wouters, L., Allessie, M., & Borgers, M. (1997). Structural changes of atrial myocardium due to sustained atrial fibrillation in the goat. *Circulation,* **96**(9): 3157-3163.

Avgil Tsadok, M., Jackevicius, C. A., Rahme, E., Humphries, K. H., Behlouli, H., & Pilote, L. (2012). Sex differences in stroke risk among older patients with recently diagnosed atrial fibrillation. *JAMA,* **307**(18): 1952-1958.

Azevedo, P. S., Minicucci, M. F., Santos, P. P., Paiva, S. A., & Zornoff, L. A. (2013). Energy metabolism in cardiac remodeling and heart failure. *Cardiology in Review,* **21**(3): 135-140.

Ball, J., Carrington, M. J., McMurray, J. J., & Stewart, S. (2013). Atrial fibrillation: profile and burden of an evolving epidemic in the 21st century. *Int J Cardiol,* **167**(5): 1807-1824.

Balligand, J. L., Feron, O., & Dessy, C. (2009). eNOS activation by physical forces: from short-term regulation of contraction to chronic remodeling of cardiovascular tissues. *Physiol Rev,* **89**(2): 481-534.

Bang, C. N., Abdulla, J., Greve, A., Kober, L., Gislason, G., & Wachtell, K. (2011). The preventive effect of statin therapy on new-onset and recurrent atrial fibrillation in patients not undergoing invasive procedures - a systematic review and meta-analysis. *European Heart Journal,* **32**: 622-623.

Barth, A. S., & Tomaselli, G. F. (2009). Cardiac metabolism and arrhythmias. *Circ Arrhythm Electrophysiol,* **2**(3): 327-335.

Bauer, E. M., Qin, Y., Miller, T. W., Bandle, R. W., Csanyi, G., Pagano, P. J., Bauer, P. M., Schnermann, J., Roberts, D. D., & Isenberg, J. S. (2010). Thrombospondin-1 supports blood pressure by limiting eNOS activation and endothelial-dependent vasorelaxation. *Cardiovasc Res,* **88**(3): 471-481.

Bauer, P. M., Bauer, E. M., Rogers, N. M., Yao, M., Feijoo-Cuaresma, M., Pilewski, J. M., Champion, H. C., Zuckerbraun, B. S., Calzada, M. J., & Isenberg, J. S. (2012). Activated CD47 promotes pulmonary arterial hypertension through targeting caveolin-1. *Cardiovasc Res,* **93**(4): 682-693.

Becker, D. M., Segal, J., Vaidya, D., Yanek, L. R., Herrera-Galeano, J. E., Bray, P. F., Moy, T. F., Becker, L. C., & Faraday, N. (2006). Sex differences in platelet reactivity and response to low-dose aspirin therapy. *JAMA,* **295**(12): 1420-1427.

Bedford, M. T., & Richard, S. (2005). Arginine methylation an emerging regulator of protein function. *Molecular Cell,* **18**(3): 263-272.

Bellin, C., de Wiza, D. H., Wiernsperger, N. F., & Rosen, P. (2006). Generation of reactive oxygen species by endothelial and smooth muscle cells: influence of hyperglycemia and metformin. *Horm Metab Res,* **38**(11): 732-739.

Béres, B. J., Tóth-Zsámboki, E., Vargová, K., László, Á., Masszi, T., Kerecsen, G., Préda, I., & Kiss, R. G. (2008). Analysis of platelet alpha-2 adrenergic receptor activity in stable coronary artery disease patients on dual antiplatelet therapy. *Thrombosis and Haemostasis,* **100**(5): 829-836.

Black, I. W., Hopkins, A. P., Lee, L. C., & Walsh, W. F. (1991). Left atrial spontaneous echo contrast: a clinical and echocardiographic analysis. *Journal of the American College of Cardiology,* **18**(2): 398-404.

Blackwell, S. (2010). The biochemistry, measurement and current clinical significance of asymmetric dimethylarginine. *Annals of Clinical Biochemistry,* **47**(Pt 1): 17-28.

Bleeke, T., Zhang, H., Madamanchi, N., Patterson, C., & Faber, J. E. (2004). Catecholamine-induced vascular wall growth is dependent on generation of reactive oxygen species. *Circulation Research,* **94**(1): 37-45.

Bode-Boger, S. M., Scalera, F., Kielstein, J. T., Martens-Lobenhoffer, J., Breithardt, G., Fobker, M., & Reinecke, H. (2006). Symmetrical dimethylarginine: a new combined parameter for renal function and extent of coronary artery disease. *Journal of the American Society of Nephrology,* **17**(4): 1128-1134.

Boger, R. H., Bode-Boger, S. M., Szuba, A., Tsao, P. S., Chan, J. R., Tangphao, O., Blaschke, T. F., & Cooke, J. P. (1998). Asymmetric dimethylarginine (ADMA): a novel risk factor for endothelial dysfunction: its role in hypercholesterolemia. *Circulation,* **98**(18): 1842-1847.

Boger, R. H., Sullivan, L. M., Schwedhelm, E., Wang, T. J., Maas, R., Benjamin, E. J., Schulze, F., Xanthakis, V., Benndorf, R. A., & Vasan, R. S. (2009). Plasma asymmetric dimethylarginine and incidence of cardiovascular disease and death in the community. *Circulation,* **119**(12): 1592-1600.

Boger, R. H., Sydow, K., Borlak, J., Thum, T., Lenzen, H., Schubert, B., Tsikas, D., & Bode-Boger, S. M. (2000). LDL cholesterol upregulates synthesis of asymmetrical dimethylarginine in human endothelial cells: involvement of S-adenosylmethionine-dependent methyltransferases. *Circulation Research,* **87**(2): 99-105.

Bohm, M., Castellano, M., Paul, M., & Erdmann, E. (1994). Cardiac norepinephrine, beta-adrenoceptors, and Gi alpha-proteins in prehypertensive and hypertensive spontaneously hypertensive rats. *J Cardiovasc Pharmacol,* **23**(6): 980-987.

Bohm, M., Flesch, M., & Schnabel, P. (1996). Role of G-proteins in altered beta-adrenergic responsiveness in the failing and hypertrophied myocardium. *Basic Research in Cardiology,* **91 Suppl 2**: 47-51.

Born, G. V. (1962). Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature,* **194**: 927-929.

Boulden, B. M., Widder, J. D., Allen, J. C., Smith, D. A., Al-Baldawi, R. N., Harrison, D. G., Dikalov, S. I., Jo, H., & Dudley, S. C., Jr. (2006). Early determinants of H2O2-induced endothelial dysfunction. *Free Radic Biol Med,* **41**(5): 810-817.

Bovill, E. G., & van der Vliet, A. (2011). Venous valvular stasis-associated hypoxia and thrombosis: what is the link? *Annual Review of Physiology,* **73**: 527-545.

Brandwein, H. J., Lewicki, J. A., & Murad, F. (1981). Reversible inactivation of guanylate cyclase by mixed disulfide formation. *Journal of Biological Chemistry,* **256**(6): 2958-2962.

Buga, G. M., Singh, R., Pervin, S., Rogers, N. E., Schmitz, D. A., Jenkinson, C. P., Cederbaum, S. D., & Ignarro, L. J. (1996). Arginase activity in endothelial cells: inhibition by NG-hydroxy-L-arginine during high-output NO production. *American Journal of Physiology,* **271**(5 Pt 2): H1988-1998.

Burke, T. M., & Wolin, M. S. (1987). Hydrogen peroxide elicits pulmonary arterial relaxation and guanylate cyclase activation. *American Journal of Physiology,* **252**(4 Pt 2): H721-732.

Burstein, B., & Nattel, S. (2008). Atrial fibrosis: mechanisms and clinical relevance in atrial fibrillation. *Journal of the American College of Cardiology,* **51**(8): 802-809.

Cadilhac, D. A. (2012). The economics of atrial fibrillation: a time for review and prioritization. *International Journal of Stroke,* **7**(6): 477-479.

Cadilhac, D. A., Carter, R., Thrift, A. G., & Dewey, H. M. (2009). Estimating the long-term costs of ischemic and hemorrhagic stroke for Australia: new evidence derived from the North East Melbourne Stroke Incidence Study (NEMESIS). *Stroke,* **40**(3): 915-921.

Cai, H., Li, Z., Goette, A., Mera, F., Honeycutt, C., Feterik, K., Wilcox, J. N., Dudley, S. C., Jr., Harrison, D. G., & Langberg, J. J. (2002). Downregulation of endocardial nitric oxide synthase expression and nitric oxide production in atrial fibrillation: potential mechanisms for atrial thrombosis and stroke. *Circulation,* **106**(22): 2854-2858.

Calderone, V., Baragatti, B., Breschi, M. C., Nieri, P., & Martinotti, E. (2002). Hormonal influence on the release of endothelial nitric oxide: gender-related dimorphic sensitivity of rat aorta for noradrenaline. *J Pharm Pharmacol,* **54**(4): 523-528.

Camus, S. M., Gausseres, B., Bonnin, P., Loufrani, L., Grimaud, L., Charue, D., De Moraes, J. A., Renard, J. M., Tedgui, A., Boulanger, C. M., Tharaux, P. L., & Blanc-Brude, O. P. (2012). Erythrocyte microparticles can induce kidney vaso-occlusions in a murine model of sickle cell disease. *Blood,* **120**(25): 5050-5058.

Cardinal, D. C., & Flower, R. J. (1980). The electronic aggregometer: a novel device for assessing platelet behavior in blood. *J Pharmacol Methods,* **3**(2): 135-158.

Carnes, C. A., Chung, M. K., Nakayama, T., Nakayama, H., Baliga, R. S., Piao, S., Kanderian, A., Pavia, S., Hamlin, R. L., McCarthy, P. M., Bauer, J. A., & Van Wagoner, D. R. (2001). Ascorbate attenuates atrial pacing-induced peroxynitrite formation and electrical remodeling and decreases the incidence of postoperative atrial fibrillation. *Circulation Research,* **89**(6): E32-38.

Carrington, M. J., Ball, J., Horowitz, J. D., Marwick, T. H., Mahadevan, G., Wong, C., Abhayaratna, W. P., Haluska, B., Thompson, D. R., Scuffham, P. A., & Stewart, S. (2013). Navigating the fine line between benefit and risk in chronic atrial fibrillation: rationale and design of the Standard versus Atrial Fibrillation spEcific managemenT studY (SAFETY). *Int J Cardiol,* **166**(2): 359-365.

Casaclang-Verzosa, G., Gersh, B. J., & Tsang, T. S. (2008). Structural and functional remodeling of the left atrium: clinical and therapeutic implications for atrial fibrillation. *Journal of the American College of Cardiology,* **51**(1): 1-11.

Ceriello, A., Assaloni, R., Da Ros, R., Maier, A., Piconi, L., Quagliaro, L., Esposito, K., & Giugliano, D. (2005). Effect of atorvastatin and irbesartan, alone and in combination, on postprandial endothelial dysfunction, oxidative stress, and inflammation in type 2 diabetic patients. *Circulation,* **111**(19): 2518-2524.

Cha-Molstad, H., Saxena, G., Chen, J., & Shalev, A. (2009). Glucose-stimulated expression of Txnip is mediated by carbohydrate response element-binding protein, p300, and histone H4 acetylation in pancreatic beta cells. *Journal of Biological Chemistry,* **284**(25): 16898-16905.

Chai, T. F., Hong, S. Y., He, H., Zheng, L., Hagen, T., Luo, Y., & Yu, F. X. (2012). A potential mechanism of metformin-mediated regulation of glucose homeostasis: inhibition of Thioredoxin-interacting protein (Txnip) gene expression. *Cellular Signalling,* **24**(8): 1700-1705.

Chan, W. P., Ngo, D. T., Sverdlov, A. L., Rajendran, S., Stafford, I., Heresztyn, T., Chirkov, Y. Y., & Horowitz, J. D. (2013). Premature aging of cardiovascular/platelet function in polycystic ovarian syndrome. *American Journal of Medicine,* **126**(7): 640 e641-647.

Chen, H., Herndon, M. E., & Lawler, J. (2000). The cell biology of thrombospondin-1. *Matrix Biology,* **19**(7): 597-614.

Chen, J., Cha-Molstad, H., Szabo, A., & Shalev, A. (2009). Diabetes induces and calcium channel blockers prevent cardiac expression of proapoptotic thioredoxin-interacting protein. *Am J Physiol Endocrinol Metab,* **296**(5): E1133-1139.

Chen, J., Saxena, G., Mungrue, I. N., Lusis, A. J., & Shalev, A. (2008). Thioredoxin-interacting protein: a critical link between glucose toxicity and beta-cell apoptosis. *Diabetes,* **57**(4): 938-944.

Chen, J. W., Hsu, N. W., Wu, T. C., Lin, S. J., & Chang, M. S. (2002). Long-term angiotensin-converting enzyme inhibition reduces plasma asymmetric dimethylarginine and improves endothelial nitric oxide bioavailability and coronary microvascular function in patients with syndrome X. *American Journal of Cardiology,* **90**(9): 974-982.

Chen, Y., Xu, X., Sheng, M., Zhang, X., Gu, Q., & Zheng, Z. (2009). PRMT-1 and DDAHs-induced ADMA upregulation is involved in ROS- and RAS-mediated diabetic retinopathy. *Exp Eye Res*, **89**(6): 1028-1034.

Chen, Z.-P., Mitchelhill, K. I., Michell, B. J., Stapleton, D., Rodriguez-Crespo, I., Witters, L. A., Power, D. A., Ortiz de Montellano, P. R., & Kemp, B. E. (1999). AMP-activated protein kinase phosphorylation of endothelial NO synthase. *Febs Letters,* **443**(3): 285-289.

Chirkov, Y. Y., Chirkova, L. P., & Horowitz, J. D. (1997). Nitroglycerin tolerance at the platelet level in patients with angina pectoris. *American Journal of Cardiology*, **80**(2): 128-131.

Chirkov, Y. Y., Holmes, A. S., Chirkova, L. P., & Horowitz, J. D. (1999). Nitrate resistance in platelets from patients with stable angina pectoris. *Circulation,* **100**(2): 129-134.

Chirkov, Y. Y., Holmes, A. S., Martelli, J. D., & Horowitz, J. D. (2004). Effect of perindopril on platelet nitric oxide resistance in patients with chronic heart failure secondary to ischemic left ventricular dysfunction. *American Journal of Cardiology*, **93**(11): 1438-1440, A1410.

Chirkov, Y. Y., Holmes, A. S., Willoughby, S. R., Stewart, S., Wuttke, R. D., Sage, P. R., & Horowitz, J. D. (2001). Stable angina and acute coronary syndromes are associated with nitric oxide resistance in platelets. *Journal of the American College of Cardiology,* **37**(7): 1851-1857.

Chirkov, Y. Y., & Horowitz, J. D. (2007). Impaired tissue responsiveness to organic nitrates and nitric oxide: a new therapeutic frontier? *Pharmacol Ther,* **116**(2): 287-305.

Chugh, S. S., Havmoeller, R., Narayanan, K., Singh, D., Rienstra, M., Benjamin, E. J., Gillum, R. F., Kim, Y. H., McAnulty, J. H., Jr., Zheng, Z. J., Forouzanfar, M. H., Naghavi, M., Mensah, G. A., Ezzati, M., & Murray, C. J. (2014). Worldwide epidemiology of atrial fibrillation: a Global Burden of Disease 2010 Study. *Circulation*, **129**(8): 837-847.

Clancy, R. M., Leszczynska-Piziak, J., & Abramson, S. B. (1992). Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. *Journal of Clinical Investigation*, **90**(3): 1116-1121.

Colquhoun, S. M., Kado, J. H., Remenyi, B., Wilson, N. J., Carapetis, J. R., & Steer, A. C. (2014). Echocardiographic screening in a resource poor setting: borderline rheumatic heart disease could be a normal variant. *Int J Cardiol*, **173**(2): 284-289.

Conen, D., Chae, C. U., Glynn, R. J., Tedrow, U. B., Everett, B. M., Buring, J. E., & Albert, C. M. (2011). Risk of death and cardiovascular events in initially healthy women with new-onset atrial fibrillation. *JAMA*, **305**(20): 2080-2087.

Connolly, S. J., Crijns, H. J., Torp-Pedersen, C., van Eickels, M., Gaudin, C., Page, R. L., Hohnloser, S. H., & Investigators, A. (2009). Analysis of stroke in ATHENA: a placebo-controlled, double-blind, parallel-arm trial to assess the efficacy of dronedarone 400 mg BID for the prevention of cardiovascular hospitalization or death from any cause in patients with atrial fibrillation/atrial flutter. *Circulation*, **120**(13): 1174-1180.

Connolly, S. J., Ezekowitz, M. D., Yusuf, S., Eikelboom, J., Oldgren, J., Parekh, A., Pogue, J., Reilly, P. A., Themeles, E., Varrone, J., Wang, S., Alings, M., Xavier, D., Zhu, J., Diaz, R., Lewis, B. S., Darius, H., Diener, H. C., Joyner, C. D., Wallentin, L., Committee, R.-L. S., & Investigators. (2009). Dabigatran versus warfarin in patients with atrial fibrillation. *N Engl J Med*, **361**(12): 1139-1151.

Connolly, S. J., Laupacis, A., Gent, M., Roberts, R. S., Cairns, J. A., & Joyner, C. (1991). Canadian Atrial Fibrillation Anticoagulation (CAFA) Study. *Journal of the American College of Cardiology*, **18**(2): 349-355.

Cook, N. L., Viola, H. M., Sharov, V. S., Hool, L. C., Schoneich, C., & Davies, M. J. (2012). Myeloperoxidase-derived oxidants inhibit sarco/endoplasmic reticulum Ca2+-ATPase activity and perturb Ca2+ homeostasis in human coronary artery endothelial cells. *Free Radic Biol Med*, **52**(5): 951-961.

Cooper, D., Lindberg, F. P., Gamble, J. R., Brown, E. J., & Vadas, M. A. (1995). Transendothelial migration of neutrophils involves integrin-associated protein (CD47). *Proc Natl Acad Sci U S A*, **92**(9): 3978-3982.

Crandall, M. A., Horne, B. D., Day, J. D., Anderson, J. L., Muhlestein, J. B., Crandall, B. G., Weiss, J. P., Lappe, D. L., & Bunch, T. J. (2009). Atrial fibrillation and CHADS2 risk factors are associated with highly sensitive C-reactive protein incrementally and independently. *Pacing Clin Electrophysiol*, **32**(5): 648-652.

Crane, M. S., Rossi, A. G., & Megson, I. L. (2005). A potential role for extracellular nitric oxide generation in cGMP-independent inhibition of human platelet aggregation: biochemical and pharmacological considerations. *Br J Pharmacol*, **144**(6): 849-859.

Csanyi, G., Yao, M., Rodriguez, A. I., Al Ghouleh, I., Sharifi-Sanjani, M., Frazziano, G., Huang, X., Kelley, E. E., Isenberg, J. S., & Pagano, P. J. (2012). Thrombospondin-1 regulates blood flow via CD47 receptor-mediated activation of NADPH oxidase 1. *Arterioscler Thromb Vasc Biol,* **32**(12): 2966-2973.

Cuzzocrea, S., & Salvemini, D. (2007). Molecular mechanisms involved in the reciprocal regulation of cyclooxygenase and nitric oxide synthase enzymes. *Kidney International,* **71**(4): 290-297.

Daley, R., Mattingly, T. W., Holt, C. L., Bland, E. F., & White, P. D. (1951). Systemic arterial embolism in rheumatic heart disease. *American Heart Journal,* **42**(4): 566-581.

Davi, G., & Patrono, C. (2007). Platelet activation and atherothrombosis. *N Engl J Med,* **357**(24): 2482-2494.

Davies, S. B., Hofer, A., & Reeve, C. (2014). Mortality attributable to rheumatic heart disease in the Kimberley: a data linkage approach. *Intern Med J,* **44**(11): 1074-1080.

De Jong, A. M., Maass, A. H., Oberdorf-Maass, S. U., Van Veldhuisen, D. J., Van Gilst, W. H., & Van Gelder, I. C. (2011). Mechanisms of atrial structural changes caused by stretch occurring before and during early atrial fibrillation. *Cardiovasc Res,* **89**(4): 754-765.

Deo, S. H., Jenkins, N. T., Padilla, J., Parrish, A. R., & Fadel, P. J. (2013). Norepinephrine increases NADPH oxidase-derived superoxide in human peripheral blood mononuclear cells via alpha-adrenergic receptors. *Am J Physiol Regul Integr Comp Physiol,* **305**(10): R1124-1132.

Dikalov, S. I., Nazarewicz, R. R., Bikineyeva, A., Hilenski, L., Lassegue, B., Griendling, K. K., Harrison, D. G., & Dikalova, A. E. (2014). Nox2-induced production of mitochondrial superoxide in angiotensin II-mediated endothelial oxidative stress and hypertension. *Antioxid Redox Signal,* **20**(2): 281-294.

DiMarco, J. P., Flaker, G., Waldo, A. L., Corley, S. D., Greene, H. L., Safford, R. E., Rosenfeld, L. E., Mitrani, G., Nemeth, M., & Investigators, A. (2005). Factors affecting bleeding risk during anticoagulant therapy in patients with atrial fibrillation: observations from the Atrial Fibrillation Follow-up Investigation of Rhythm Management (AFFIRM) study. *American Heart Journal,* **149**(4): 650-656.

Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., & Zeiher, A. M. (1999). Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature,* **399**(6736): 601-605.

Disertori, M., Barlera, S., Staszewsky, L., Latini, R., Quintarelli, S., & Franzosi, M. G. (2012). Systematic review and meta-analysis: renin-Angiotensin system inhibitors in the

prevention of atrial fibrillation recurrences: an unfulfilled hope. *Cardiovasc Drugs Ther,* **26**(1): 47-54.

Druhan, L. J., Forbes, S. P., Pope, A. J., Chen, C. A., Zweier, J. L., & Cardounel, A. J. (2008). Regulation of eNOS-derived superoxide by endogenous methylarginines. *Biochemistry,* **47**(27): 7256-7263.

Du, X. L., Edelstein, D., Dimmeler, S., Ju, Q., Sui, C., & Brownlee, M. (2001). Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site. *Journal of Clinical Investigation,* **108**(9): 1341-1348.

Dudley, S. C., Jr., Hoch, N. E., McCann, L. A., Honeycutt, C., Diamandopoulos, L., Fukai, T., Harrison, D. G., Dikalov, S. I., & Langberg, J. (2005). Atrial fibrillation increases production of superoxide by the left atrium and left atrial appendage: role of the NADPH and xanthine oxidases. *Circulation,* **112**(9): 1266-1273.

Duranton, F., Lundin, U., Gayrard, N., Mischak, H., Aparicio, M., Mourad, G., Daures, J. P., Weinberger, K. M., & Argiles, A. (2014). Plasma and urinary amino acid metabolomic profiling in patients with different levels of kidney function. *Clin J Am Soc Nephrol,* **9**(1): 37-45.

Eberhardt, W., Beeg, T., Beck, K. F., Walpen, S., Gauer, S., Bohles, H., & Pfeilschifter, J. (2000). Nitric oxide modulates expression of matrix metalloproteinase-9 in rat mesangial cells. *Kidney International,* **57**(1): 59-69.

Ederhy, S., Di Angelantonio, E., Mallat, Z., Hugel, B., Janower, S., Meuleman, C., Boccara, F., Freyssinet, J. M., Tedgui, A., & Cohen, A. (2007). Levels of circulating procoagulant microparticles in nonvalvular atrial fibrillation. *American Journal of Cardiology,* **100**(6): 989-994.

Eiselt, J., Rajdl, D., Racek, J., Siroka, R., Trefil, L., & Opatrna, S. (2010). Asymmetric dimethylarginine in hemodialysis, hemodiafiltration, and peritoneal dialysis. *Artificial Organs,* **34**(5): 420-425.

Eiserich, J. P., Baldus, S., Brennan, M. L., Ma, W., Zhang, C., Tousson, A., Castro, L., Lusis, A. J., Nauseef, W. M., White, C. R., & Freeman, B. A. (2002). Myeloperoxidase, a leukocyte-derived vascular NO oxidase. *Science,* **296**(5577): 2391-2394.

Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., & van der Vliet, A. (1998). Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature,* **391**(6665): 393-397.

Elesber, A. A., Rosales, A. G., Herges, R. M., Shen, W. K., Moon, B. S., Malouf, J. F., Ammash, N. M., Somers, V., Hodge, D. O., Gersh, B. J., Hammill, S. C., & Friedman, P. A.

(2006). Relapse and mortality following cardioversion of new-onset vs. recurrent atrial fibrillation and atrial flutter in the elderly. *European Heart Journal,* **27**(7): 854-860.

Ellinor, P. T., Lunetta, K. L., Glazer, N. L., Pfeufer, A., Alonso, A., Chung, M. K., Sinner, M. F., de Bakker, P. I., Mueller, M., Lubitz, S. A., Fox, E., Darbar, D., Smith, N. L., Smith, J. D., Schnabel, R. B., Soliman, E. Z., Rice, K. M., Van Wagoner, D. R., Beckmann, B. M., van Noord, C., Wang, K., Ehret, G. B., Rotter, J. I., Hazen, S. L., Steinbeck, G., Smith, A. V., Launer, L. J., Harris, T. B., Makino, S., Nelis, M., Milan, D. J., Perz, S., Esko, T., Kottgen, A., Moebus, S., Newton-Cheh, C., Li, M., Mohlenkamp, S., Wang, T. J., Kao, W. H., Vasan, R. S., Nothen, M. M., MacRae, C. A., Stricker, B. H., Hofman, A., Uitterlinden, A. G., Levy, D., Boerwinkle, E., Metspalu, A., Topol, E. J., Chakravarti, A., Gudnason, V., Psaty, B. M., Roden, D. M., Meitinger, T., Wichmann, H. E., Witteman, J. C., Barnard, J., Arking, D. E., Benjamin, E. J., Heckbert, S. R., & Kaab, S. (2010). Common variants in KCNN3 are associated with lone atrial fibrillation. *Nature Genetics,* **42**(3): 240-244.

Erdmann, J., Stark, K., Esslinger, U. B., Rumpf, P. M., Koesling, D., de Wit, C., Kaiser, F. J., Braunholz, D., Medack, A., Fischer, M., Zimmermann, M. E., Tennstedt, S., Graf, E., Eck, S., Aherrahrou, Z., Nahrstaedt, J., Willenborg, C., Bruse, P., Braenne, I., Nothen, M. M., Hofmann, P., Braund, P. S., Mergia, E., Reinhard, W., Burgdorf, C., Schreiber, S., Balmforth, A. J., Hall, A. S., Bertram, L., Steinhagen-Thiessen, E., Li, S. C., Marz, W., Reilly, M., Kathiresan, S., McPherson, R., Walter, U., CardioGram, Ott, J., Samani, N. J., Strom, T. M., Meitinger, T., Hengstenberg, C., & Schunkert, H. (2013). Dysfunctional nitric oxide signalling increases risk of myocardial infarction. *Nature,* **504**(7480): 432-436.

Esmon, C. T., & Esmon, N. L. (2011). The link between vascular features and thrombosis. *Annual Review of Physiology*, **73**: 503-514.

European Heart Rhythm, A., European Association for Cardio-Thoracic, S., Camm, A. J., Kirchhof, P., Lip, G. Y., Schotten, U., Savelieva, I., Ernst, S., Van Gelder, I. C., Al-Attar, N., Hindricks, G., Prendergast, B., Heidbuchel, H., Alfieri, O., Angelini, A., Atar, D., Colonna, P., De Caterina, R., De Sutter, J., Goette, A., Gorenek, B., Heldal, M., Hohloser, S. H., Kolh, P., Le Heuzey, J. Y., Ponikowski, P., & Rutten, F. H. (2010). Guidelines for the management of atrial fibrillation: the Task Force for the Management of Atrial Fibrillation of the European Society of Cardiology (ESC). *European Heart Journal,* **31**(19): 2369-2429.

Ezekowitz, M. D., Bridgers, S. L., James, K. E., Carliner, N. H., Colling, C. L., Gornick, C. C., Krause-Steinrauf, H., Kurtzke, J. F., Nazarian, S. M., Radford, M. J., & et al. (1992). Warfarin in the prevention of stroke associated with nonrheumatic atrial fibrillation. Veterans Affairs Stroke Prevention in Nonrheumatic Atrial Fibrillation Investigators. *N Engl J Med,* **327**(20): 1406-1412.

Fang, M. C., Chang, Y. C., Hylek, E. M., Rosand, J., Greenberg, S. M., Go, A. S., & Singer, D. E. (2004). Advanced age, anticoagulation intensity, and risk for intracranial hemorrhage among patients taking warfarin for atrial fibrillation. *Annals of Internal Medicine,* **141**(10): 745-752.

Fang, M. C., Go, A. S., Chang, Y., Borowsky, L. H., Pomernacki, N. K., Udaltsova, N., & Singer, D. E. (2011). A new risk scheme to predict warfarin-associated hemorrhage: The ATRIA (Anticoagulation and Risk Factors in Atrial Fibrillation) Study. *Journal of the American College of Cardiology,* **58**(4): 395-401.

Fang, M. C., Singer, D. E., Chang, Y., Hylek, E. M., Henault, L. E., Jensvold, N. G., & Go, A. S. (2005). Gender differences in the risk of ischemic stroke and peripheral embolism in atrial fibrillation: the AnTicoagulation and Risk factors In Atrial fibrillation (ATRIA) study. *Circulation,* **112**(12): 1687-1691.

Fauchier, L., Clementy, N., & Babuty, D. (2013). Statin therapy and atrial fibrillation: systematic review and updated meta-analysis of published randomized controlled trials. *Current Opinion in Cardiology,* **28**(1): 7-18.

Federici, M. (2002). Insulin-Dependent Activation of Endothelial Nitric Oxide Synthase Is Impaired by O-Linked Glycosylation Modification of Signaling Proteins in Human Coronary Endothelial Cells. *Circulation,* **106**(4): 466-472.

Ferrario, C. M., & Strawn, W. B. (2006). Role of the renin-angiotensin-aldosterone system and proinflammatory mediators in cardiovascular disease. *American Journal of Cardiology,* **98**(1): 121-128.

Ferratini, M., Marianeschi, S., Santoro, F., Vitali, E., Ripamonti, V., De Maria, R., Torri, A., Pezzano, A., Moraschi, A., Tavano, D., Pesaresi, M., & Martinelli, L. (2013). Valvulopathies in sub-Saharan African children: patterns, humanitarian interventions and cardiac surgical problems. *Int J Cardiol,* **165**(2): 237-241.

Fitzmaurice, D. (2009). Atrial fibrillation and coagulation: who and when? *Blood Reviews,* **23**(6): 241-244.

Forrester, M. T., Seth, D., Hausladen, A., Eyler, C. E., Foster, M. W., Matsumoto, A., Benhar, M., Marshall, H. E., & Stamler, J. S. (2009). Thioredoxin-interacting protein (Txnip) is a feedback regulator of S-nitrosylation. *Journal of Biological Chemistry,* **284**(52): 36160-36166.

Forstermann, U. (2010). Nitric oxide and oxidative stress in vascular disease. *Pflugers Arch,* **459**(6): 923-939.

Freedman, R. R., Sabharwal, S. C., & Desai, N. (1987). Sex differences in peripheral vascular adrenergic receptors. *Circulation Research,* **61**(4): 581-585.

Freeman, I., & Wexler, J. (1967). Atrial fibrillation: anticoagulation and quinidinization. *South Med J,* **60**(1): 13-17.

Freestone, B., Chong, A. Y., Nuttall, S., & Lip, G. Y. (2008). Impaired flow mediated dilatation as evidence of endothelial dysfunction in chronic atrial fibrillation: relationship to plasma von Willebrand factor and soluble E-selectin levels. *Thrombosis Research,* **122**(1): 85-90.

Friedrichs, K., Baldus, S., & Klinke, A. (2012). Fibrosis in Atrial Fibrillation - Role of Reactive Species and MPO. *Front Physiol,* **3**: 214.

Gage, B. F., Waterman, A. D., Shannon, W., Boechler, M., Rich, M. W., & Radford, M. J. (2001). Validation of clinical classification schemes for predicting stroke: results from the National Registry of Atrial Fibrillation. *JAMA,* **285**(22): 2864-2870.

Gage, B. F., Yan, Y., Milligan, P. E., Waterman, A. D., Culverhouse, R., Rich, M. W., & Radford, M. J. (2006). Clinical classification schemes for predicting hemorrhage: results from the National Registry of Atrial Fibrillation (NRAF). *American Heart Journal,* **151**(3): 713-719.

Garcia, D. A., Lopes, R. D., & Hylek, E. M. (2010). New-onset atrial fibrillation and warfarin initiation: high risk periods and implications for new antithrombotic drugs. *Thromb Haemost,* **104**(6): 1099-1105.

Garthwaite, J. (2010). New insight into the functioning of nitric oxide-receptive guanylyl cyclase: physiological and pharmacological implications. *Molecular and Cellular Biochemistry,* **334**(1-2): 221-232.

Gawaz, M., Langer, H., & May, A. E. (2005). Platelets in inflammation and atherogenesis. *Journal of Clinical Investigation,* **115**(12): 3378-3384.

Ghebremariam, Y. T., LePendu, P., Lee, J. C., Erlanson, D. A., Slaviero, A., Shah, N. H., Leiper, J., & Cooke, J. P. (2013). Unexpected effect of proton pump inhibitors: elevation of the cardiovascular risk factor asymmetric dimethylarginine. *Circulation,* **128**(8): 845-853.

Gimbrone, M. A., Jr., & Garcia-Cardena, G. (2013). Vascular endothelium, hemodynamics, and the pathobiology of atherosclerosis. *Cardiovascular Pathology,* **22**(1): 9-15.

Godecke, A., Schrader, J., & Reinartz, M. (2008). Nitric oxide-mediated protein modification in cardiovascular physiology and pathology. *Proteomics Clin Appl,* **2**(6): 811-822.

Goette, A., Hammwohner, M., Bukowska, A., Scalera, F., Martens-Lobenhoffer, J., Dobrev, D., Ravens, U., Weinert, S., Medunjanin, S., Lendeckel, U., & Bode-Boger, S. M. (2012). The impact of rapid atrial pacing on ADMA and endothelial NOS. *Int J Cardiol,* **154**(2): 141-146.

Gonzalez-Quesada, C., Cavalera, M., Biernacka, A., Kong, P., Lee, D. W., Saxena, A., Frunza, O., Dobaczewski, M., Shinde, A., & Frangogiannis, N. G. (2013). Thrombospondin-1

induction in the diabetic myocardium stabilizes the cardiac matrix in addition to promoting vascular rarefaction through angiopoietin-2 upregulation. *Circulation Research,* **113**(12): 1331-1344.

Granger, C. B., Alexander, J. H., McMurray, J. J., Lopes, R. D., Hylek, E. M., Hanna, M., Al-Khalidi, H. R., Ansell, J., Atar, D., Avezum, A., Bahit, M. C., Diaz, R., Easton, J. D., Ezekowitz, J. A., Flaker, G., Garcia, D., Geraldes, M., Gersh, B. J., Golitsyn, S., Goto, S., Hermosillo, A. G., Hohnloser, S. H., Horowitz, J., Mohan, P., Jansky, P., Lewis, B. S., Lopez-Sendon, J. L., Pais, P., Parkhomenko, A., Verheugt, F. W., Zhu, J., Wallentin, L., Committees, A., & Investigators. (2011). Apixaban versus warfarin in patients with atrial fibrillation. *N Engl J Med,* **365**(11): 981-992.

Greiser, M., & Schotten, U. (2013). Dynamic remodeling of intracellular Ca(2)(+) signaling during atrial fibrillation. *Journal of Molecular and Cellular Cardiology,* **58**: 134-142.

Guazzi, M., & Arena, R. (2009). Endothelial dysfunction and pathophysiological correlates in atrial fibrillation. *Heart,* **95**(2): 102-106.

Haissaguerre, M., Jais, P., Shah, D. C., Takahashi, A., Hocini, M., Quiniou, G., Garrigue, S., Le Mouroux, A., Le Metayer, P., & Clementy, J. (1998). Spontaneous initiation of atrial fibrillation by ectopic beats originating in the pulmonary veins. *N Engl J Med,* **339**(10): 659-666.

Hamm, C. W., Bassand, J. P., Agewall, S., Bax, J., Boersma, E., Bueno, H., Caso, P., Dudek, D., Gielen, S., Huber, K., Ohman, M., Petrie, M. C., Sonntag, F., Uva, M. S., Storey, R. F., Wijns, W., Zahger, D., & Guidelines, E. S. C. C. f. P. (2011). ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: The Task Force for the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC). *European Heart Journal,* **32**(23): 2999-3054.

Hammwohner, M., Ittenson, A., Dierkes, J., Bukowska, A., Klein, H. U., Lendeckel, U., & Goette, A. (2007). Platelet expression of CD40/CD40 ligand and its relation to inflammatory markers and adhesion molecules in patients with atrial fibrillation. *Exp Biol Med (Maywood),* **232**(4): 581-589.

Hankey, G. J., & Eikelboom, J. W. (2011). Dabigatran etexilate: a new oral thrombin inhibitor. *Circulation,* **123**(13): 1436-1450.

Hart, E. C., Charkoudian, N., Wallin, B. G., Curry, T. B., Eisenach, J., & Joyner, M. J. (2011). Sex and ageing differences in resting arterial pressure regulation: the role of the beta-adrenergic receptors. *J Physiol,* **589**(Pt 21): 5285-5297.

Hart, R. G., Halperin, J. L., Pearce, L. A., Anderson, D. C., Kronmal, R. A., McBride, R., Nasco, E., Sherman, D. G., Talbert, R. L., Marler, J. R., & Stroke Prevention in Atrial

Fibrillation, I. (2003). Lessons from the Stroke Prevention in Atrial Fibrillation trials. *Annals of Internal Medicine,* **138**(10): 831-838.

Hart, R. G., Pearce, L. A., McBride, R., Rothbart, R. M., & Asinger, R. W. (1999). Factors associated with ischemic stroke during aspirin therapy in atrial fibrillation: analysis of 2012 participants in the SPAF I-III clinical trials. The Stroke Prevention in Atrial Fibrillation (SPAF) Investigators. *Stroke,* **30**(6): 1223-1229.

Hayashi, M., Takeshita, K., Inden, Y., Ishii, H., Cheng, X. W., Yamamoto, K., & Murohara, T. (2011). Platelet activation and induction of tissue factor in acute and chronic atrial fibrillation: involvement of mononuclear cell-platelet interaction. *Thrombosis Research,* **128**(6): e113-118.

Henry, P. J., Lulich, K. M., & Paterson, J. W. (1985). Experimental testing of Mackay's model for functional antagonism in the isolated costo-uterus of the rat. *Br J Pharmacol,* **86**(1): 131-139.

Heo, K. S., Fujiwara, K., & Abe, J. (2011). Disturbed-flow-mediated vascular reactive oxygen species induce endothelial dysfunction. *Circulation Journal,* **75**(12): 2722-2730.

Heresztyn, T., Worthley, M. I., & Horowitz, J. D. (2004). Determination of l-arginine and NG, NG - and NG, NG' -dimethyl-L-arginine in plasma by liquid chromatography as AccQ-Fluor fluorescent derivatives. *J Chromatogr B Analyt Technol Biomed Life Sci,* **805**(2): 325-329.

Hill, B. G., Dranka, B. P., Bailey, S. M., Lancaster, J. R., Jr., & Darley-Usmar, V. M. (2010). What part of NO don't you understand? Some answers to the cardinal questions in nitric oxide biology. *Journal of Biological Chemistry,* **285**(26): 19699-19704.

Hirose, T., Kawasaki, M., Tanaka, R., Ono, K., Watanabe, T., Iwama, M., Noda, T., Watanabe, S., Takemura, G., & Minatoguchi, S. (2012). Left atrial function assessed by speckle tracking echocardiography as a predictor of new-onset non-valvular atrial fibrillation: results from a prospective study in 580 adults. *Eur Heart J Cardiovasc Imaging,* **13**(3): 243-250.

Hohnloser, S. H., Crijns, H. J., van Eickels, M., Gaudin, C., Page, R. L., Torp-Pedersen, C., Connolly, S. J., & Investigators, A. (2009). Effect of dronedarone on cardiovascular events in atrial fibrillation. *N Engl J Med,* **360**(7): 668-678.

Holowatz, L. A., Santhanam, L., Webb, A., Berkowitz, D. E., & Kenney, W. L. (2011). Oral atorvastatin therapy restores cutaneous microvascular function by decreasing arginase activity in hypercholesterolaemic humans. *J Physiol,* **589**(Pt 8): 2093-2103.

Hornig, B., Landmesser, U., Kohler, C., Ahlersmann, D., Spiekermann, S., Christoph, A., Tatge, H., & Drexler, H. (2001). Comparative effect of ace inhibition and angiotensin II type

1 receptor antagonism on bioavailability of nitric oxide in patients with coronary artery disease: role of superoxide dismutase. *Circulation,* **103**(6): 799-805.

Horowitz, J. D., De Caterina, R., Heresztyn, T., Andersson, U., Lopes, R., Hylek, E., Mohan, P., Hanna, M., Granger, C. B., Wallentin, L., & Investigators, A. (2013). ADMA and SDMA predict outcomes in patients with chronic atrial fibrillation: an ARISTOTLE substudy. *European Heart Journal,* **34**: 1040-1041.

Horowitz, J. D., & Heresztyn, T. (2007). An overview of plasma concentrations of asymmetric dimethylarginine (ADMA) in health and disease and in clinical studies: methodological considerations. *J Chromatogr B Analyt Technol Biomed Life Sci,* **851**(1-2): 42-50.

Humphries, K. H., Kerr, C. R., Connolly, S. J., Klein, G., Boone, J. A., Green, M., Sheldon, R., Talajic, M., Dorian, P., & Newman, D. (2001). New-onset atrial fibrillation: sex differences in presentation, treatment, and outcome. *Circulation,* **103**(19): 2365-2370.

Ignarro, L. J., Degnan, J. N., Baricos, W. H., Kadowitz, P. J., & Wolin, M. S. (1982). Activation of purified guanylate cyclase by nitric oxide requires heme. Comparison of heme-deficient, heme-reconstituted and heme-containing forms of soluble enzyme from bovine lung. *Biochimica Et Biophysica Acta,* **718**(1): 49-59.

Ikarugi, H., Taka, T., Nakajima, S., Noguchi, T., Watanabe, S., Sasaki, Y., Haga, S., Ueda, T., Seki, J., & Yamamoto, J. (1999). Norepinephrine, but not epinephrine, enhances platelet reactivity and coagulation after exercise in humans. *Journal of Applied Physiology,* **86**(1): 133-138.

Inoue, H., Atarashi, H., Okumura, K., Yamashita, T., Origasa, H., Kumagai, N., Sakurai, M., Kawamura, Y., Kubota, I., Matsumoto, K., Kaneko, Y., Ogawa, S., Aizawa, Y., Chinushi, M., Kodama, I., Watanabe, E., Koretsune, Y., Okuyama, Y., Shimizu, A., Igawa, O., Bando, S., Fukatani, M., Saikawa, T., Chishaki, A., & Investigators, J. R. R. (2014). Impact of gender on the prognosis of patients with nonvalvular atrial fibrillation. *American Journal of Cardiology,* **113**(6): 957-962.

Isenberg, J. S., Frazier, W. A., & Roberts, D. D. (2008). Thrombospondin-1: a physiological regulator of nitric oxide signaling. *Cellular and Molecular Life Sciences,* **65**(5): 728-742.

Isenberg, J. S., Qin, Y., Maxhimer, J. B., Sipes, J. M., Despres, D., Schnermann, J., Frazier, W. A., & Roberts, D. D. (2009). Thrombospondin-1 and CD47 regulate blood pressure and cardiac responses to vasoactive stress. *Matrix Biology,* **28**(2): 110-119.

Isenberg, J. S., Ridnour, L. A., Perruccio, E. M., Espey, M. G., Wink, D. A., & Roberts, D. D. (2005). Thrombospondin-1 inhibits endothelial cell responses to nitric oxide in a cGMP-dependent manner. *Proc Natl Acad Sci U S A,* **102**(37): 13141-13146.

Isenberg, J. S., Romeo, M. J., Yu, C., Yu, C. K., Nghiem, K., Monsale, J., Rick, M. E., Wink, D. A., Frazier, W. A., & Roberts, D. D. (2008). Thrombospondin-1 stimulates platelet aggregation by blocking the antithrombotic activity of nitric oxide/cGMP signaling. *Blood,* **111**(2): 613-623.

Isenberg, J. S., Wink, D. A., & Roberts, D. D. (2006). Thrombospondin-1 antagonizes nitric oxide-stimulated vascular smooth muscle cell responses. *Cardiovasc Res,* **71**(4): 785-793.

Issac, T. T., Dokainish, H., & Lakkis, N. M. (2007). Role of inflammation in initiation and perpetuation of atrial fibrillation: a systematic review of the published data. *Journal of the American College of Cardiology,* **50**(21): 2021-2028.

Iwasaki, Y. K., Nishida, K., Kato, T., & Nattel, S. (2011). Atrial fibrillation pathophysiology: implications for management. *Circulation,* **124**(20): 2264-2274.

Jaffe, E. A., Ruggiero, J. T., & Falcone, D. J. (1985). Monocytes and macrophages synthesize and secrete thrombospondin. *Blood,* **65**(1): 79-84.

Jahangir, A., Lee, V., Friedman, P. A., Trusty, J. M., Hodge, D. O., Kopecky, S. L., Packer, D. L., Hammill, S. C., Shen, W. K., & Gersh, B. J. (2007). Long-term progression and outcomes with aging in patients with lone atrial fibrillation: a 30-year follow-up study. *Circulation,* **115**(24): 3050-3056.

Jalife, J. (2011). Deja vu in the theories of atrial fibrillation dynamics. *Cardiovasc Res,* **89**(4): 766-775.

Jalife, J., Berenfeld, O., & Mansour, M. (2002). Mother rotors and fibrillatory conduction: a mechanism of atrial fibrillation. *Cardiovasc Res,* **54**(2): 204-216.

January, C. T., Wann, L. S., Alpert, J. S., Calkins, H., Cigarroa, J. E., Cleveland, J. C., Jr., Conti, J. B., Ellinor, P. T., Ezekowitz, M. D., Field, M. E., Murray, K. T., Sacco, R. L., Stevenson, W. G., Tchou, P. J., Tracy, C. M., Yancy, C. W., & American College of Cardiology/American Heart Association Task Force on Practice, G. (2014). 2014 AHA/ACC/HRS guideline for the management of patients with atrial fibrillation: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and the Heart Rhythm Society. *Journal of the American College of Cardiology,* **64**(21): e1-76.

Jayachandran, J. V., Sih, H. J., Winkle, W., Zipes, D. P., Hutchins, G. D., & Olgin, J. E. (2000). Atrial fibrillation produced by prolonged rapid atrial pacing is associated with heterogeneous changes in atrial sympathetic innervation. *Circulation,* **101**(10): 1185-1191.

Jia, S. J., Jiang, D. J., Hu, C. P., Zhang, X. H., Deng, H. W., & Li, Y. J. (2006). Lysophosphatidylcholine-induced elevation of asymmetric dimethylarginine level by the NADPH oxidase pathway in endothelial cells. *Vascul Pharmacol,* **44**(3): 143-148.

Johnson, B. H., Smoyer-Tomic, K. E., Siu, K., Walker, D. R., Sander, S., Huse, D., Smith, D. M., Song, X., & Amin, A. (2013). Readmission among hospitalized patients with nonvalvular atrial fibrillation. *Am J Health Syst Pharm,* **70**(5): 414-422.

Kamath, S., Blann, A. D., Chin, B. S., Lanza, F., Aleil, B., Cazenave, J. P., & Lip, G. Y. (2002). A study of platelet activation in atrial fibrillation and the effects of antithrombotic therapy. *European Heart Journal,* **23**(22): 1788-1795.

Kamath, S., Chin, B. S., Blann, A. D., & Lip, G. Y. (2002). A study of platelet activation in paroxysmal, persistent and permanent atrial fibrillation. *Blood Coagul Fibrinolysis,* **13**(7): 627-636.

Keularts, I. M., van Gorp, R. M., Feijge, M. A., Vuist, W. M., & Heemskerk, J. W. (2000). alpha(2A)-adrenergic receptor stimulation potentiates calcium release in platelets by modulating cAMP levels. *Journal of Biological Chemistry,* **275**(3): 1763-1772.

Khan, B. V., Rahman, S. T., Haque, T., Merchant, N., Bhaheetharan, S., Harris, J., 3rd, Umar, K., Wahi, J., & Ferdinand, K. C. (2012). Vascular effects of nebivolol added to hydrochlorothiazide in African Americans with hypertension and echocardiographic evidence of diastolic dysfunction: the NASAA study. *J Cardiovasc Pharmacol Ther,* **17**(3): 291-297.

Khatib, R., Joseph, P., Briel, M., Yusuf, S., & Healey, J. (2013). Blockade of the renin-angiotensin-aldosterone system (RAAS) for primary prevention of non-valvular atrial fibrillation: a systematic review and meta analysis of randomized controlled trials. *Int J Cardiol,* **165**(1): 17-24.

Kielstein, J. T., Boger, R. H., Bode-Boger, S. M., Martens-Lobenhoffer, J., Lonnemann, G., Frolich, J. C., Haller, H., & Fliser, D. (2004). Low dialysance of asymmetric dimethylarginine (ADMA)--in vivo and in vitro evidence of significant protein binding. *Clin Nephrol,* **62**(4): 295-300.

Kielstein, J. T., Donnerstag, F., Gasper, S., Menne, J., Kielstein, A., Martens-Lobenhoffer, J., Scalera, F., Cooke, J. P., Fliser, D., & Bode-Boger, S. M. (2006). ADMA increases arterial stiffness and decreases cerebral blood flow in humans. *Stroke,* **37**(8): 2024-2029.

Kielstein, J. T., Impraim, B., Simmel, S., Bode-Boger, S. M., Tsikas, D., Frolich, J. C., Hoeper, M. M., Haller, H., & Fliser, D. (2004). Cardiovascular effects of systemic nitric oxide synthase inhibition with asymmetrical dimethylarginine in humans. *Circulation,* **109**(2): 172-177.

Kielstein, J. T., Salpeter, S. R., Bode-Boeger, S. M., Cooke, J. P., & Fliser, D. (2006). Symmetric dimethylarginine (SDMA) as endogenous marker of renal function--a meta-analysis. *Nephrol Dial Transplant,* **21**(9): 2446-2451.

Kim, J. H., Bugaj, L. J., Oh, Y. J., Bivalacqua, T. J., Ryoo, S., Soucy, K. G., Santhanam, L., Webb, A., Camara, A., Sikka, G., Nyhan, D., Shoukas, A. A., Ilies, M., Christianson, D. W., Champion, H. C., & Berkowitz, D. E. (2009). Arginase inhibition restores NOS coupling and reverses endothelial dysfunction and vascular stiffness in old rats. *J Appl Physiol (1985)*, **107**(4): 1249-1257.

Kirsch, T., Woywodt, A., Klose, J., Wyss, K., Beese, M., Erdbruegger, U., Grossheim, M., Haller, H., & Haubitz, M. (2010). Endothelial-derived thrombospondin-1 promotes macrophage recruitment and apoptotic cell clearance. *Journal of Cellular and Molecular Medicine*, **14**(7): 1922-1934.

Kittel, A., Maas, R., Konig, J., Mieth, M., Weiss, N., Jarzebska, N., Hohenstein, B., Martens-Lobenhoffer, J., Bode-Boger, S. M., & Rodionov, R. N. (2013). In vivo evidence that Agxt2 can regulate plasma levels of dimethylarginines in mice. *Biochem Biophys Res Commun*, **430**(1): 84-89.

Kneale, B. J., Chowienczyk, P. J., Brett, S. E., Coltart, D. J., & Ritter, J. M. (2000). Gender differences in sensitivity to adrenergic agonists of forearm resistance vasculature. *Journal of the American College of Cardiology*, **36**(4): 1233-1238.

Korantzopoulos, P., Kolettis, T. M., Galaris, D., & Goudevenos, J. A. (2007). The role of oxidative stress in the pathogenesis and perpetuation of atrial fibrillation. *Int J Cardiol*, **115**(2): 135-143.

Kostin, S., Klein, G., Szalay, Z., Hein, S., Bauer, E. P., & Schaper, J. (2002). Structural correlate of atrial fibrillation in human patients. *Cardiovasc Res*, **54**(2): 361-379.

Kowaluk, E. A., Seth, P., & Fung, H. L. (1992). Metabolic activation of sodium nitroprusside to nitric oxide in vascular smooth muscle. *Journal of Pharmacology and Experimental Therapeutics*, **262**(3): 916-922.

Kumpuris, A. G., Luchi, R. J., Waddell, C. C., & Miller, R. R. (1980). Production of circulating platelet aggregates by exercise in coronary patients. *Circulation*, **61**(1): 62-65.

Kutter, D., Devaquet, P., Vanderstocken, G., Paulus, J. M., Marchal, V., & Gothot, A. (2000). Consequences of total and subtotal myeloperoxidase deficiency: risk or benefit ? *Acta Haematol*, **104**(1): 10-15.

Landmesser, U., Bahlmann, F., Mueller, M., Spiekermann, S., Kirchhoff, N., Schulz, S., Manes, C., Fischer, D., de Groot, K., Fliser, D., Fauler, G., Marz, W., & Drexler, H. (2005). Simvastatin versus ezetimibe: pleiotropic and lipid-lowering effects on endothelial function in humans. *Circulation*, **111**(18): 2356-2363.

Landmesser, U., Dikalov, S., Price, S. R., McCann, L., Fukai, T., Holland, S. M., Mitch, W. E., & Harrison, D. G. (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of

endothelial cell nitric oxide synthase in hypertension. *Journal of Clinical Investigation,* **111**(8): 1201-1209.

Larsson, P. T., Wallen, N. H., & Hjemdahl, P. (1994). Norepinephrine-Induced Human Platelet Activation in-Vivo Is Only Partly Counteracted by Aspirin. *Circulation,* **89**(5): 1951-1957.

Lassegue, B., Sorescu, D., Szocs, K., Yin, Q., Akers, M., Zhang, Y., Grant, S. L., Lambeth, J. D., & Griendling, K. K. (2001). Novel gp91(phox) homologues in vascular smooth muscle cells : nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circulation Research,* **88**(9): 888-894.

Lau, D., Mollnau, H., Eiserich, J. P., Freeman, B. A., Daiber, A., Gehling, U. M., Brummer, J., Rudolph, V., Munzel, T., Heitzer, T., Meinertz, T., & Baldus, S. (2005). Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. *Proc Natl Acad Sci U S A,* **102**(2): 431-436.

Laupacis, A., Boysen, G., Connolly, S., Ezekowitz, M., Hart, R., James, K., Kistler, P., Kronmal, R., Petersen, P., Singer, D., Godtfredsen, J., Andersen, E., Andersen, B., Hughes, R., Gress, D., Sheehan, M., Oertel, L., Maraventano, S., Blewett, D., Rosner, B., Gent, M., Roberts, R., Cairns, J., Joyner, C., Bridgers, S., Colling, C., & Krausesteinrauf, H. (1994). Risk-Factors for Stroke and Efficacy of Antithrombotic Therapy in Atrial-Fibrillation - Analysis of Pooled Data from 5 Randomized Controlled Trials. *Archives of Internal Medicine,* **154**(13): 1449-1457.

Lehnart, S. E., Wehrens, X. H., Reiken, S., Warrier, S., Belevych, A. E., Harvey, R. D., Richter, W., Jin, S. L., Conti, M., & Marks, A. R. (2005). Phosphodiesterase 4D deficiency in the ryanodine-receptor complex promotes heart failure and arrhythmias. *Cell,* **123**(1): 25-35.

Lehto, M., Snapinn, S., Dickstein, K., Swedberg, K., Nieminen, M. S., & investigators, O. (2005). Prognostic risk of atrial fibrillation in acute myocardial infarction complicated by left ventricular dysfunction: the OPTIMAAL experience. *European Heart Journal,* **26**(4): 350-356.

Leopold, J. A., & Loscalzo, J. (2009). Oxidative risk for atherothrombotic cardiovascular disease. *Free Radic Biol Med,* **47**(12): 1673-1706.

Lewis, T., Feil, H. S., & Stroud, W. D. (1920). Observations upon flutter and fibrillation. Part II. The nature of auricular flutter. *Heart-a Journal for the Study of the Circulation,* **7**(4): 191-345.

Li, J., Solus, J., Chen, Q., Rho, Y. H., Milne, G., Stein, C. M., & Darbar, D. (2010). Role of inflammation and oxidative stress in atrial fibrillation. *Heart Rhythm,* **7**(4): 438-444.

Lievens, D., Zernecke, A., Seijkens, T., Soehnlein, O., Beckers, L., Munnix, I. C., Wijnands, E., Goossens, P., van Kruchten, R., Thevissen, L., Boon, L., Flavell, R. A., Noelle, R. J., Gerdes, N., Biessen, E. A., Daemen, M. J., Heemskerk, J. W., Weber, C., & Lutgens, E. (2010). Platelet CD40L mediates thrombotic and inflammatory processes in atherosclerosis. *Blood,* **116**(20): 4317-4327.

Lim, H. S., Willoughby, S. R., Schultz, C., Gan, C., Alasady, M., Lau, D. H., Leong, D. P., Brooks, A. G., Young, G. D., Kistler, P. M., Kalman, J. M., Worthley, M. I., & Sanders, P. (2013). Effect of atrial fibrillation on atrial thrombogenesis in humans: impact of rate and rhythm. *Journal of the American College of Cardiology,* **61**(8): 852-860.

Lin, K. Y., Ito, A., Asagami, T., Tsao, P. S., Adimoolam, S., Kimoto, M., Tsuji, H., Reaven, G. M., & Cooke, J. P. (2002). Impaired nitric oxide synthase pathway in diabetes mellitus: role of asymmetric dimethylarginine and dimethylarginine dimethylaminohydrolase. *Circulation,* **106**(8): 987-992.

Lindsay, R. M., Moorthy, A. V., Koens, F., & Linton, A. L. (1975). Platelet function in dialyzed and non-dialyzed patients with chronic renal failure. *Clin Nephrol,* **4**(2): 52-57.

Lip, G. Y. (1995). Does atrial fibrillation confer a hypercoagulable state? *Lancet,* **346**(8986): 1313-1314.

Lip, G. Y., & Beevers, D. G. (1995). ABC of atrial fibrillation. History, epidemiology, and importance of atrial fibrillation. *BMJ,* **311**(7016): 1361-1363.

Lip, G. Y., Brechin, C. M., & Lane, D. A. (2012). The global burden of atrial fibrillation and stroke: a systematic review of the epidemiology of atrial fibrillation in regions outside North America and Europe. *Chest,* **142**(6): 1489-1498.

Lip, G. Y., Frison, L., Halperin, J. L., & Lane, D. A. (2011). Comparative validation of a novel risk score for predicting bleeding risk in anticoagulated patients with atrial fibrillation: the HAS-BLED (Hypertension, Abnormal Renal/Liver Function, Stroke, Bleeding History or Predisposition, Labile INR, Elderly, Drugs/Alcohol Concomitantly) score. *Journal of the American College of Cardiology,* **57**(2): 173-180.

Lip, G. Y., Nieuwlaat, R., Pisters, R., Lane, D. A., & Crijns, H. J. (2010). Refining clinical risk stratification for predicting stroke and thromboembolism in atrial fibrillation using a novel risk factor-based approach: the euro heart survey on atrial fibrillation. *Chest,* **137**(2): 263-272.

Lip, G. Y., Patel, J. V., Hughes, E., & Hart, R. G. (2007). High-sensitivity C-reactive protein and soluble CD40 ligand as indices of inflammation and platelet activation in 880 patients with nonvalvular atrial fibrillation: relationship to stroke risk factors, stroke risk stratification schema, and prognosis. *Stroke,* **38**(4): 1229-1237.

Liu, Y., Merlin, D., Burst, S. L., Pochet, M., Madara, J. L., & Parkos, C. A. (2001). The role of CD47 in neutrophil transmigration. Increased rate of migration correlates with increased cell surface expression of CD47. *Journal of Biological Chemistry*, **276**(43): 40156-40166.

Liu, Y., & Min, W. (2002). Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. *Circulation Research*, **90**(12): 1259-1266.

Loffredo, L., Angelico, F., Perri, L., & Violi, F. (2012). Upstream therapy with statin and recurrence of atrial fibrillation after electrical cardioversion. Review of the literature and meta-analysis. *BMC Cardiovasc Disord*, **12**: 107.

Logan, W. F., Rowlands, D. J., Howitt, G., & Holmes, A. M. (1965). Left Atrial Activity Following Cardioversion. *Lancet*, **2**(7410): 471-473.

Lowe, G. D. (2003). Virchow's triad revisited: abnormal flow. *Pathophysiol Haemost Thromb*, **33**(5-6): 455-457.

Lu, Y. M., Han, F., Shioda, N., Moriguchi, S., Shirasaki, Y., Qin, Z. H., & Fukunaga, K. (2009). Phenylephrine-induced cardiomyocyte injury is triggered by superoxide generation through uncoupled endothelial nitric-oxide synthase and ameliorated by 3-[2-[4-(3-chloro-2-methylphenyl)-1-piperazinyl]ethyl]-5,6-dimethoxyindazole (DY-9836), a novel calmodulin antagonist. *Molecular Pharmacology*, **75**(1): 101-112.

Luneburg, N., von Holten, R. A., Topper, R. F., Schwedhelm, E., Maas, R., & Boger, R. H. (2012). Symmetric dimethylarginine is a marker of detrimental outcome in the acute phase after ischaemic stroke: role of renal function. *Clin Sci (Lond)*, **122**(3): 105-111.

Luo, Z., Teerlink, T., Griendling, K., Aslam, S., Welch, W. J., & Wilcox, C. S. (2010). Angiotensin II and NADPH oxidase increase ADMA in vascular smooth muscle cells. *Hypertension*, **56**(3): 498-504.

Maehama, T., Okura, H., Imai, K., Yamada, R., Obase, K., Saito, K., Hayashida, A., Neishi, Y., Kawamoto, T., & Yoshida, K. (2010). Usefulness of CHADS2 score to predict C-reactive protein, left atrial blood stasis, and prognosis in patients with nonrheumatic atrial fibrillation. *American Journal of Cardiology*, **106**(4): 535-538.

Mansfield, P. J., Boxer, L. A., & Suchard, S. J. (1990). Thrombospondin stimulates motility of human neutrophils. *Journal of Cell Biology*, **111**(6 Pt 2): 3077-3086.

Marchese, P., Bursi, F., Delle Donne, G., Malavasi, V., Casali, E., Barbieri, A., Melandri, F., & Modena, M. G. (2011). Indexed left atrial volume predicts the recurrence of non-valvular atrial fibrillation after successful cardioversion. *Eur J Echocardiogr*, **12**(3): 214-221.

Marcucci, M., Nobili, A., Tettamanti, M., Iorio, A., Pasina, L., Djade, C. D., Franchi, C., Marengoni, A., Salerno, F., Corrao, S., Violi, F., Mannucci, P. M., & Investigators, R. (2013). Joint use of cardio-embolic and bleeding risk scores in elderly patients with atrial fibrillation. *European Journal of Internal Medicine,* **24**(8): 800-806.

Marin, F., Roldan, V., Climent, V. E., Ibanez, A., Garcia, A., Marco, P., Sogorb, F., & Lip, G. Y. (2004). Plasma von Willebrand factor, soluble thrombomodulin, and fibrin D-dimer concentrations in acute onset non-rheumatic atrial fibrillation. *Heart,* **90**(10): 1162-1166.

Maron, B. A., Zhang, Y. Y., Handy, D. E., Beuve, A., Tang, S. S., Loscalzo, J., & Leopold, J. A. (2009). Aldosterone increases oxidant stress to impair guanylyl cyclase activity by cysteinyl thiol oxidation in vascular smooth muscle cells. *Journal of Biological Chemistry,* **284**(12): 7665-7672.

Masson, S., Aleksova, A., Favero, C., Staszewsky, L., Bernardinangeli, M., Belvito, C., Cioffi, G., Sinagra, G., Mazzone, C., Bertocchi, F., Vago, T., Peri, G., Cuccovillo, I., Masuda, N., Barlera, S., Mantovani, A., Maggioni, A. P., Franzosi, M. G., Disertori, M., Latini, R., & investigators, G.-A. (2010). Predicting atrial fibrillation recurrence with circulating inflammatory markers in patients in sinus rhythm at high risk for atrial fibrillation: data from the GISSI atrial fibrillation trial. *Heart,* **96**(23): 1909-1914.

Matsue, Y., Suzuki, M., Abe, M., Ono, M., Seya, M., Nakamura, T., Iwatsuka, R., Mizukami, A., Toyama, K., Kumasaka, L., Handa, K., Nagahori, W., Ohno, M., Matsumura, A., & Hashimoto, Y. (2011). Endothelial Dysfunction in Paroxysmal Atrial Fibrillation as a Prothrombotic State-Comparison with Permanent/Persistent Atrial Fibrillation. *Journal of Atherosclerosis and Thrombosis,* **18**(4): 298-304.

Mcbride, R. (1991). Stroke Prevention in Atrial-Fibrillation Study - Final Results. *Circulation,* **84**(2): 527-539.

McKee, A. P., Van Riper, D. A., Davison, C. A., & Singer, H. A. (2003). Gender-dependent modulation of alpha 1-adrenergic responses in rat mesenteric arteries. *Am J Physiol Heart Circ Physiol,* **284**(5): H1737-1743.

Meda, C., Plank, C., Mykhaylyk, O., Schmidt, K., & Mayer, B. (2010). Effects of statins on nitric oxide/cGMP signaling in human umbilical vein endothelial cells. *Pharmacol Rep,* **62**(1): 100-112.

Mehel, H., Emons, J., Vettel, C., Wittkopper, K., Seppelt, D., Dewenter, M., Lutz, S., Sossalla, S., Maier, L. S., Lechene, P., Leroy, J., Lefebvre, F., Varin, A., Eschenhagen, T., Nattel, S., Dobrev, D., Zimmermann, W. H., Nikolaev, V. O., Vandecasteele, G., Fischmeister, R., & El-Armouche, A. (2013). Phosphodiesterase-2 is up-regulated in human failing hearts and blunts beta-adrenergic responses in cardiomyocytes. *Journal of the American College of Cardiology*, **62**(17): 1596-1606.

Mehta, J. L., & Li, D. (2001). Epinephrine upregulates superoxide dismutase in human coronary artery endothelial cells. *Free Radic Biol Med*, **30**(2): 148-153.

Mehta, R. H., Dabbous, O. H., Granger, C. B., Kuznetsova, P., Kline-Rogers, E. M., Anderson, F. A., Fox, K. A. A., Gore, J. M., Goldberg, R. J., & Eagle, K. A. (2003). Comparison of outcomes of patients with acute coronary syndromes with and without atrial fibrillation. *The American Journal of Cardiology*, **92**(9): 1031-1036.

Mellion, B. T., Ignarro, L. J., Myers, C. B., Ohlstein, E. H., Ballot, B. A., Hyman, A. L., & Kadowitz, P. J. (1983). Inhibition of human platelet aggregation by S-nitrosothiols. Heme-dependent activation of soluble guanylate cyclase and stimulation of cyclic GMP accumulation. *Molecular Pharmacology*, **23**(3): 653-664.

Mihm, M. J., Yu, F., Carnes, C. A., Reiser, P. J., McCarthy, P. M., Van Wagoner, D. R., & Bauer, J. A. (2001). Impaired myofibrillar energetics and oxidative injury during human atrial fibrillation. *Circulation*, **104**(2): 174-180.

Miller, T. W., Isenberg, J. S., & Roberts, D. D. (2010). Thrombospondin-1 is an inhibitor of pharmacological activation of soluble guanylate cyclase. *Br J Pharmacol*, **159**(7): 1542-1547.

Minn, A. H., Hafele, C., & Shalev, A. (2005). Thioredoxin-interacting protein is stimulated by glucose through a carbohydrate response element and induces beta-cell apoptosis. *Endocrinology*, **146**(5): 2397-2405.

Miyasaka, Y., Barnes, M. E., Bailey, K. R., Cha, S. S., Gersh, B. J., Seward, J. B., & Tsang, T. S. (2007). Mortality trends in patients diagnosed with first atrial fibrillation: a 21-year community-based study. *Journal of the American College of Cardiology*, **49**(9): 986-992.

Moilanen, E., Vuorinen, P., Kankaanranta, H., Metsaketela, T., & Vapaatalo, H. (1993). Inhibition by Nitric-Oxide Donors of Human Polymorphonuclear Leukocyte Functions. *British Journal of Pharmacology*, **109**(3): 852-858.

Molina, C. E., Leroy, J., Richter, W., Xie, M., Scheitrum, C., Lee, I. O., Maack, C., Rucker-Martin, C., Donzeau-Gouge, P., Verde, I., Llach, A., Hove-Madsen, L., Conti, M., Vandecasteele, G., & Fischmeister, R. (2012). Cyclic adenosine monophosphate phosphodiesterase type 4 protects against atrial arrhythmias. *Journal of the American College of Cardiology*, **59**(24): 2182-2190.

Montoro-Garcia, S., Shantsila, E., Marin, F., Blann, A., & Lip, G. Y. (2011). Circulating microparticles: new insights into the biochemical basis of microparticle release and activity. *Basic Research in Cardiology*, **106**(6): 911-923.

Mrdovic, I., Savic, L., Krljanac, G., Perunicic, J., Asanin, M., Lasica, R., Antonijevic, N., Kocev, N., Marinkovic, J., Vasiljevic, Z., & Ostojic, M. (2012). Incidence, predictors, and 30-

day outcomes of new-onset atrial fibrillation after primary percutaneous coronary intervention: insight into the RISK-PCI trial. *Coron Artery Dis,* **23**(1): 1-8.

Mullershausen, F., Russwurm, M., Thompson, W. J., Liu, L., Koesling, D., & Friebe, A. (2001). Rapid nitric oxide-induced desensitization of the cGMP response is caused by increased activity of phosphodiesterase type 5 paralleled by phosphorylation of the enzyme. *Journal of Cell Biology,* **155**(2): 271-278.

Murata, T., Kinoshita, K., Hori, M., Kuwahara, M., Tsubone, H., Karaki, H., & Ozaki, H. (2005). Statin protects endothelial nitric oxide synthase activity in hypoxia-induced pulmonary hypertension. *Arterioscler Thromb Vasc Biol,* **25**(11): 2335-2342.

Naccarelli, G. V., Johnston, S. S., Dalal, M., Lin, J., & Patel, P. P. (2012). Rates and implications for hospitalization of patients >/=65 years of age with atrial fibrillation/flutter. *American Journal of Cardiology,* **109**(4): 543-549.

Naccarelli, G. V., Varker, H., Lin, J., & Schulman, K. L. (2009). Increasing prevalence of atrial fibrillation and flutter in the United States. *American Journal of Cardiology,* **104**(11): 1534-1539.

Nagendran, J., Archer, S. L., Soliman, D., Gurtu, V., Moudgil, R., Haromy, A., St Aubin, C., Webster, L., Rebeyka, I. M., Ross, D. B., Light, P. E., Dyck, J. R., & Michelakis, E. D. (2007). Phosphodiesterase type 5 is highly expressed in the hypertrophied human right ventricle, and acute inhibition of phosphodiesterase type 5 improves contractility. *Circulation,* **116**(3): 238-248.

Narizhneva, N. V., Razorenova, O. V., Podrez, E. A., Chen, J., Chandrasekharan, U. M., DiCorleto, P. E., Plow, E. F., Topol, E. J., & Byzova, T. V. (2005). Thrombospondin-1 up-regulates expression of cell adhesion molecules and promotes monocyte binding to endothelium. *Federation Proceedings,* **19**(9): 1158-1160.

Nattel, S. (2002). Spotlight on atrial fibrillation—the 'complete arrhythmia'. *Cardiovascular Research,* **54**(2): 197-203.

Nattel, S., Li, D., & Yue, L. (2000). Basic mechanisms of atrial fibrillation--very new insights into very old ideas. *Annual Review of Physiology,* **62**: 51-77.

Neuman, R. B., Bloom, H. L., Shukrullah, I., Darrow, L. A., Kleinbaum, D., Jones, D. P., & Dudley, S. C., Jr. (2007). Oxidative stress markers are associated with persistent atrial fibrillation. *Clinical Chemistry,* **53**(9): 1652-1657.

Ngo, D. T., Stafford, I., Kelly, D. J., Sverdlov, A. L., Wuttke, R. D., Weedon, H., Nightingale, A. K., Rosenkranz, A. C., Smith, M. D., Chirkov, Y. Y., Kennedy, J. A., & Horowitz, J. D. (2008). Vitamin D(2) supplementation induces the development of aortic

stenosis in rabbits: interactions with endothelial function and thioredoxin-interacting protein. *Eur J Pharmacol,* **590**(1-3): 290-296.

Ngo, D. T., Stafford, I., Sverdlov, A. L., Qi, W., Wuttke, R. D., Zhang, Y., Kelly, D. J., Weedon, H., Smith, M. D., Kennedy, J. A., & Horowitz, J. D. (2011). Ramipril retards development of aortic valve stenosis in a rabbit model: mechanistic considerations. *Br J Pharmacol,* **162**(3): 722-732.

Nickel, T., Deutschmann, A., Hanssen, H., Summo, C., & Wilbert-Lampen, U. (2009). Modification of endothelial biology by acute and chronic stress hormones. *Microvascular Research,* **78**(3): 364-369.

Nishiyama, A., Matsui, M., Iwata, S., Hirota, K., Masutani, H., Nakamura, H., Takagi, Y., Sono, H., Gon, Y., & Yodoi, J. (1999). Identification of Thioredoxin-binding Protein-2/Vitamin D3 Up-regulated Protein 1 as a Negative Regulator of Thioredoxin Function and Expression. *Journal of Biological Chemistry,* **274**(31): 21645-21650.

Nolly, M. B., Caldiz, C. I., Yeves, A. M., Villa-Abrille, M. C., Morgan, P. E., Amado Mondaca, N., Portiansky, E. L., Chiappe de Cingolani, G. E., Cingolani, H. E., & Ennis, I. L. (2014). The signaling pathway for aldosterone-induced mitochondrial production of superoxide anion in the myocardium. *Journal of Molecular and Cellular Cardiology,* **67**: 60-68.

Ocuz, A., & Uzunlulu, M. (2008). Short term fluvastatin treatment lowers serum asymmetric dimethylarginine levels in patients with metabolic syndrome. *International Heart Journal,* **49**(3): 303-311.

Ogata, F. T., Batista, W. L., Sartori, A., Gesteira, T. F., Masutani, H., Arai, R. J., Yodoi, J., Stern, A., & Monteiro, H. P. (2013). Nitrosative/oxidative stress conditions regulate thioredoxin-interacting protein (TXNIP) expression and thioredoxin-1 (TRX-1) nuclear localization. *PLoS One,* **8**(12): e84588.

Ohara, K., Hirai, T., Fukuda, N., Sakurai, K., Nakagawa, K., Nozawa, T., & Inoue, H. (2009). Relation of left atrial blood stasis to clinical risk factors in atrial fibrillation. *Int J Cardiol,* **132**(2): 210-215.

Ohara, K., Inoue, H., Nozawa, T., Hirai, T., Iwasa, A., Okumura, K., Lee, J. D., Shimizu, A., Hayano, M., & Yano, K. (2008). Accumulation of risk factors enhances the prothrombotic state in atrial fibrillation. *Int J Cardiol,* **126**(3): 316-321.

Olesen, J. B., Lip, G. Y., Kamper, A. L., Hommel, K., Kober, L., Lane, D. A., Lindhardsen, J., Gislason, G. H., & Torp-Pedersen, C. (2012). Stroke and bleeding in atrial fibrillation with chronic kidney disease. *N Engl J Med,* **367**(7): 625-635.

Osanai, T., Saitoh, M., Sasaki, S., Tomita, H., Matsunaga, T., & Okumura, K. (2003). Effect of shear stress on asymmetric dimethylarginine release from vascular endothelial cells. *Hypertension,* **42**(5): 985-990.

Otahbachi, M., Simoni, J., Simoni, G., Moeller, J. F., Cevik, C., Meyerrose, G. E., & Roongsritong, C. (2010). Gender differences in platelet aggregation in healthy individuals. *J Thromb Thrombolysis,* **30**(2): 184-191.

Ozaydin, M., Peker, O., Erdogan, D., Kapan, S., Turker, Y., Varol, E., Ozguner, F., Dogan, A., & Ibrisim, E. (2008). N-acetylcysteine for the prevention of postoperative atrial fibrillation: a prospective, randomized, placebo-controlled pilot study. *European Heart Journal,* **29**(5): 625-631.

Pancholy, S. B., Sharma, P. S., Pancholy, D. S., Patel, T. M., Callans, D. J., & Marchlinski, F. E. (2014). Meta-analysis of gender differences in residual stroke risk and major bleeding in patients with nonvalvular atrial fibrillation treated with oral anticoagulants. *American Journal of Cardiology,* **113**(3): 485-490.

Patwari, P., Higgins, L. J., Chutkow, W. A., Yoshioka, J., & Lee, R. T. (2006). The interaction of thioredoxin with Txnip. Evidence for formation of a mixed disulfide by disulfide exchange. *Journal of Biological Chemistry,* **281**(31): 21884-21891.

Petersen, P., Boysen, G., Godtfredsen, J., Andersen, E. D., & Andersen, B. (1989). Placebo-Controlled, Randomized Trial of Warfarin and Aspirin for Prevention of Thromboembolic Complications in Chronic Atrial-Fibrillation. *Lancet,* **1**(8631): 175-179.

Phillipson, M., & Kubes, P. (2011). The neutrophil in vascular inflammation. *Nature Medicine,* **17**(11): 1381-1390.

Potpara, T. S., & Lip, G. Y. (2014). Lone atrial fibrillation - an overview. *Int J Clin Pract,* **68**(4): 418-433.

Potter, L. R. (2011). Guanylyl cyclase structure, function and regulation. *Cellular Signalling,* **23**(12): 1921-1926.

Providencia, R., Faustino, A., Ferreira, M. J., Goncalves, L., Trigo, J., Botelho, A., Barra, S., & Boveda, S. (2013). Evaluation of left atrial deformation to predict left atrial stasis in patients with non-valvular atrial fibrillation - a pilot-study. *Cardiovasc Ultrasound,* **11**: 44.

Prystowsky, E. N. (2008). The history of atrial fibrillation: the last 100 years. *J Cardiovasc Electrophysiol,* **19**(6): 575-582.

Rajendran, S., & Chirkov, Y. Y. (2008). Platelet hyperaggregability: impaired responsiveness to nitric oxide ("platelet NO resistance") as a therapeutic target. *Cardiovasc Drugs Ther,* **22**(3): 193-203.

Rajendran, S., Willoughby, S. R., Chan, W. P., Liberts, E. A., Heresztyn, T., Saha, M., Marber, M. S., Norman, R. J., & Horowitz, J. D. (2009). Polycystic ovary syndrome is associated with severe platelet and endothelial dysfunction in both obese and lean subjects. *Atherosclerosis,* **204**(2): 509-514.

Ramanathan, S., Mazzalupo, S., Boitano, S., & Montfort, W. R. (2011). Thrombospondin-1 and angiotensin II inhibit soluble guanylyl cyclase through an increase in intracellular calcium concentration. *Biochemistry,* **50**(36): 7787-7799.

Ravi, K., Brennan, L. A., Levic, S., Ross, P. A., & Black, S. M. (2004). S-nitrosylation of endothelial nitric oxide synthase is associated with monomerization and decreased enzyme activity. *Proceedings of the National Academy of Sciences,* **101**(8): 2619-2624.

Renoux, C., Patenaude, V., & Suissa, S. (2014). Incidence, mortality, and sex differences of non-valvular atrial fibrillation: a population-based study. *J Am Heart Assoc,* **3**(6): e001402.

Ridnour, L. A., Windhausen, A. N., Isenberg, J. S., Yeung, N., Thomas, D. D., Vitek, M. P., Roberts, D. D., & Wink, D. A. (2007). Nitric oxide regulates matrix metalloproteinase-9 activity by guanylyl-cyclase-dependent and -independent pathways. *Proc Natl Acad Sci U S A,* **104**(43): 16898-16903.

Roberts, W., Magwenzi, S., Aburima, A., & Naseem, K. M. (2010). Thrombospondin-1 induces platelet activation through CD36-dependent inhibition of the cAMP/protein kinase A signaling cascade. *Blood,* **116**(20): 4297-4306.

Rodionov, R. N., Murry, D. J., Vaulman, S. F., Stevens, J. W., & Lentz, S. R. (2010). Human alanine-glyoxylate aminotransferase 2 lowers asymmetric dimethylarginine and protects from inhibition of nitric oxide production. *Journal of Biological Chemistry,* **285**(8): 5385-5391.

Roldan, V., Marin, F., Garcia-Herola, A., & Lip, G. Y. (2005). Correlation of plasma von Willebrand factor levels, an index of endothelial damage/dysfunction, with two point-based stroke risk stratification scores in atrial fibrillation. *Thrombosis Research,* **116**(4): 321-325.

Rudolph, T. K., Rudolph, V., Witte, A., Klinke, A., Szoecs, K., Lau, D., Heitzer, T., Meinertz, T., & Baldus, S. (2010). Liberation of vessel adherent myeloperoxidase by enoxaparin improves endothelial function. *Int J Cardiol,* **140**(1): 42-47.

Rudolph, V., Andrie, R. P., Rudolph, T. K., Friedrichs, K., Klinke, A., Hirsch-Hoffmann, B., Schwoerer, A. P., Lau, D., Fu, X., Klingel, K., Sydow, K., Didie, M., Seniuk, A., von Leitner, E. C., Szoecs, K., Schrickel, J. W., Treede, H., Wenzel, U., Lewalter, T., Nickenig, G., Zimmermann, W. H., Meinertz, T., Boger, R. H., Reichenspurner, H., Freeman, B. A.,

Eschenhagen, T., Ehmke, H., Hazen, S. L., Willems, S., & Baldus, S. (2010). Myeloperoxidase acts as a profibrotic mediator of atrial fibrillation. *Nature Medicine,* **16**(4): 470-474.

Salvemini, D., Kim, S. F., & Mollace, V. (2013). Reciprocal regulation of the nitric oxide and cyclooxygenase pathway in pathophysiology: relevance and clinical implications. *Am J Physiol Regul Integr Comp Physiol,* **304**(7): R473-487.

Saxena, G., Chen, J., & Shalev, A. (2010). Intracellular shuttling and mitochondrial function of thioredoxin-interacting protein. *Journal of Biological Chemistry,* **285**(6): 3997-4005.

Sayed, N., Baskaran, P., Ma, X., van den Akker, F., & Beuve, A. (2007). Desensitization of soluble guanylyl cyclase, the NO receptor, by S-nitrosylation. *Proc Natl Acad Sci U S A,* **104**(30): 12312-12317.

Sayed, N., Kim, D. D., Fioramonti, X., Iwahashi, T., Duran, W. N., & Beuve, A. (2008). Nitroglycerin-induced S-nitrosylation and desensitization of soluble guanylyl cyclase contribute to nitrate tolerance. *Circulation Research,* **103**(6): 606-614.

Schepers, E., Barreto, D. V., Liabeuf, S., Glorieux, G., Eloot, S., Barreto, F. C., Massy, Z., Vanholder, R., & European Uremic Toxin Work, G. (2011). Symmetric dimethylarginine as a proinflammatory agent in chronic kidney disease. *Clin J Am Soc Nephrol,* **6**(10): 2374-2383.

Schepers, E., Glorieux, G., Dhondt, A., Leybaert, L., & Vanholder, R. (2009). Role of symmetric dimethylarginine in vascular damage by increasing ROS via store-operated calcium influx in monocytes. *Nephrol Dial Transplant,* **24**(5): 1429-1435.

Schnabel, R. B., Larson, M. G., Yamamoto, J. F., Sullivan, L. M., Pencina, M. J., Meigs, J. B., Tofler, G. H., Selhub, J., Jacques, P. F., Wolf, P. A., Magnani, J. W., Ellinor, P. T., Wang, T. J., Levy, D., Vasan, R. S., & Benjamin, E. J. (2010). Relations of biomarkers of distinct pathophysiological pathways and atrial fibrillation incidence in the community. *Circulation,* **121**(2): 200-207.

Schotten, U., de Haan, S., Neuberger, H. R., Eijsbouts, S., Blaauw, Y., Tieleman, R., & Allessie, M. (2004). Loss of atrial contractility is primary cause of atrial dilatation during first days of atrial fibrillation. *Am J Physiol Heart Circ Physiol,* **287**(5): H2324-2331.

Schotten, U., Verheule, S., Kirchhof, P., & Goette, A. (2011). Pathophysiological mechanisms of atrial fibrillation: a translational appraisal. *Physiol Rev,* **91**(1): 265-325.

Schrammel, A., Behrends, S., Schmidt, K., Koesling, D., & Mayer, B. (1996). Characterization of 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one as a heme-site inhibitor of nitric oxide-sensitive guanylyl cyclase. *Molecular Pharmacology,* **50**(1): 1-5.

Schulze, F., Carter, A. M., Schwedhelm, E., Ajjan, R., Maas, R., von Holten, R. A., Atzler, D., Grant, P. J., & Boger, R. H. (2010). Symmetric dimethylarginine predicts all-cause mortality following ischemic stroke. *Atherosclerosis*, **208**(2): 518-523.

Schulze, P. C. (2002). Vitamin D3-Upregulated Protein-1 (VDUP-1) Regulates Redox-Dependent Vascular Smooth Muscle Cell Proliferation Through Interaction With Thioredoxin. *Circulation Research*, **91**(8): 689-695.

Schulze, P. C., Liu, H., Choe, E., Yoshioka, J., Shalev, A., Bloch, K. D., & Lee, R. T. (2006). Nitric oxide-dependent suppression of thioredoxin-interacting protein expression enhances thioredoxin activity. *Arterioscler Thromb Vasc Biol*, **26**(12): 2666-2672.

Schulze, P. C., Yoshioka, J., Takahashi, T., He, Z., King, G. L., & Lee, R. T. (2004). Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. *Journal of Biological Chemistry*, **279**(29): 30369-30374.

Shaked, M., Ketzinel-Gilad, M., Ariav, Y., Cerasi, E., Kaiser, N., & Leibowitz, G. (2009). Insulin counteracts glucotoxic effects by suppressing thioredoxin-interacting protein production in INS-1E beta cells and in Psammomys obesus pancreatic islets. *Diabetologia*, **52**(4): 636-644.

Sharifov, O. F., Fedorov, V. V., Beloshapko, G. G., Glukhov, A. V., Yushmanova, A. V., & Rosenshtraukh, L. V. (2004). Roles of adrenergic and cholinergic stimulation in spontaneous atrial fibrillation in dogs. *Journal of the American College of Cardiology*, **43**(3): 483-490.

Shibazaki, K., Kimura, K., Fujii, S., Sakai, K., & Iguchi, Y. (2012). Brain natriuretic peptide levels as a predictor for new atrial fibrillation during hospitalization in patients with acute ischemic stroke. *American Journal of Cardiology*, **109**(9): 1303-1307.

Shih, J. Y., Tsai, W. C., Huang, Y. Y., Liu, Y. W., Lin, C. C., Huang, Y. S., Tsai, L. M., & Lin, L. J. (2011). Association of decreased left atrial strain and strain rate with stroke in chronic atrial fibrillation. *J Am Soc Echocardiogr*, **24**(5): 513-519.

Shively, B. K., Gelgand, E. A., & Crawford, M. H. (1996). Regional left atrial stasis during atrial fibrillation and flutter: determinants and relation to stroke. *Journal of the American College of Cardiology*, **27**(7): 1722-1729.

Siegerink, B., Maas, R., Vossen, C. Y., Schwedhelm, E., Koenig, W., Boger, R., Rothenbacher, D., Brenner, H., & Breitling, L. P. (2013). Asymmetric and symmetric dimethylarginine and risk of secondary cardiovascular disease events and mortality in patients with stable coronary heart disease: the KAROLA follow-up study. *Clinical Research in Cardiology*, **102**(3): 193-202.

Siess, W., Lorenz, R., Roth, P., & Weber, P. C. (1982). Plasma catecholamines, platelet aggregation and associated thromboxane formation after physical exercise, smoking or norepinephrine infusion. *Circulation,* **66**(1): 44-48.

Singer, D. E., Hughes, R. A., Gress, D. R., Sheehan, M. A., Oertel, L. B., Maraventano, S. W., Blewett, D. R., Rosner, B., & Kistler, J. P. (1992). The effect of aspirin on the risk of stroke in patients with nonrheumatic atrial fibrillation: The BAATAF Study. *American Heart Journal,* **124**(6): 1567-1573.

Sogo, N., Magid, K. S., Shaw, C. A., Webb, D. J., & Megson, I. L. (2000). Inhibition of human platelet aggregation by nitric oxide donor drugs: relative contribution of cGMP-independent mechanisms. *Biochem Biophys Res Commun,* **279**(2): 412-419.

Spindel, O. N., World, C., & Berk, B. C. (2012). Thioredoxin interacting protein: redox dependent and independent regulatory mechanisms. *Antioxid Redox Signal,* **16**(6): 587-596.

Stepien, J. M., Prideaux, R. M., Willoughby, S. R., Chirkov, Y. Y., & Horowitz, J. D. (2003). Pilot study examining the effect of cholesterol lowering on platelet nitric oxide responsiveness and arterial stiffness in subjects with isolated mild hypercholesterolaemia. *Clin Exp Pharmacol Physiol,* **30**(7): 507-512.

Stewart, S., Ball, J., Horowitz, J. D., Marwick, T. H., Mahadevan, G., Wong, C., Abhayaratna, W. P., Chan, Y. K., Esterman, A., Thompson, D. R., Scuffham, P. A., & Carrington, M. J. (2014). Standard versus atrial fibrillation-specific management strategy (SAFETY) to reduce recurrent admission and prolong survival: pragmatic, multicentre, randomised controlled trial. *Lancet,* **In Press**.

Stone, J. R., & Marletta, M. A. (1994). Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. *Biochemistry,* **33**(18): 5636-5640.

Suchard, S. J., Boxer, L. A., & Dixit, V. M. (1991). Activation of human neutrophils increases thrombospondin receptor expression. *Journal of Immunology,* **147**(2): 651-659.

Sun, H., Chartier, D., Leblanc, N., & Nattel, S. (2001). Intracellular calcium changes and tachycardia-induced contractile dysfunction in canine atrial myocytes. *Cardiovasc Res,* **49**(4): 751-761.

Sundstrom, J., Evans, J. C., Benjamin, E. J., Levy, D., Larson, M. G., Sawyer, D. B., Siwik, D. A., Colucci, W. S., Sutherland, P., Wilson, P. W., & Vasan, R. S. (2004). Relations of plasma matrix metalloproteinase-9 to clinical cardiovascular risk factors and echocardiographic left ventricular measures: the Framingham Heart Study. *Circulation,* **109**(23): 2850-2856.

Suzuki, Y., Ruiz-Ortega, M., Lorenzo, O., Ruperez, M., Esteban, V., & Egido, J. (2003). Inflammation and angiotensin II. *Int J Biochem Cell Biol,* **35**(6): 881-900.

Sverdlov, A. L., Chan, W. P., Procter, N. E., Chirkov, Y. Y., Ngo, D. T., & Horowitz, J. D. (2013a). Reciprocal regulation of NO signaling and TXNIP expression in humans: impact of aging and ramipril therapy. *Int J Cardiol,* **168**(5): 4624-4630.

Sverdlov, A. L., Chan, W. P., Procter, N. E. K., Chirkov, Y. Y., Ngo, D. T. M., & Horowitz, J. D. (2013f). Reciprocal regulation of NO signaling and TXNIP expression in humans: impact of aging and ramipril therapy. *International Journal of Cardiology*, **In Press**.

Taddei, S., Virdis, A., Mattei, P., Ghiadoni, L., Gennari, A., Fasolo, C. B., Sudano, I., & Salvetti, A. (1995). Aging and endothelial function in normotensive subjects and patients with essential hypertension. *Circulation,* **91**(7): 1981-1987.

Takahashi, N., Ishibashi, Y., Shimada, T., Sakane, T., Ohata, S., Sugamori, T., Ohta, Y., Inoue, S., Nakamura, K., Shimizu, H., Katoh, H., Sano, K., Murakami, Y., & Hashimoto, M. (2001). Atrial fibrillation impairs endothelial function of forearm vessels in humans. *Journal of Cardiac Failure,* **7**(1): 45-54.

Takimoto, E., Champion, H. C., Li, M., Ren, S., Rodriguez, E. R., Tavazzi, B., Lazzarino, G., Paolocci, N., Gabrielson, K. L., Wang, Y., & Kass, D. A. (2005). Oxidant stress from nitric oxide synthase-3 uncoupling stimulates cardiac pathologic remodeling from chronic pressure load. *Journal of Clinical Investigation,* **115**(5): 1221-1231.

Tamargo, J., Duarte, J., Caballero, R., & Delpon, E. (2010). Cinaciguat, a soluble guanylate cyclase activator for the potential treatment of acute heart failure. *Curr Opin Investig Drugs,* **11**(9): 1039-1047.

Tan, A. Y., & Verrier, R. L. (2013). The role of the autonomic nervous system in cardiac arrhythmias. *Handb Clin Neurol,* **117**: 135-145.

Teerlink, T., Luo, Z., Palm, F., & Wilcox, C. S. (2009). Cellular ADMA: regulation and action. *Pharmacological Research,* **60**(6): 448-460.

Vallance, P., & Leiper, J. (2004). Cardiovascular biology of the asymmetric dimethylarginine:dimethylarginine dimethylaminohydrolase pathway. *Arterioscler Thromb Vasc Biol,* **24**(6): 1023-1030.

Vallance, P., Leone, A., Calver, A., Collier, J., & Moncada, S. (1992). Endogenous Dimethylarginine as an Inhibitor of Nitric-Oxide Synthesis. *Journal of Cardiovascular Pharmacology,* **20**: S60-S62.

van der Veen, B. S., de Winther, M. P., & Heeringa, P. (2009). Myeloperoxidase: molecular mechanisms of action and their relevance to human health and disease. *Antioxid Redox Signal,* **11**(11): 2899-2937.

van der Zwan, L. P., Scheffer, P. G., Dekker, J. M., Stehouwer, C. D., Heine, R. J., & Teerlink, T. (2011). Systemic inflammation is linked to low arginine and high ADMA plasma levels resulting in an unfavourable NOS substrate-to-inhibitor ratio: the Hoorn Study. *Clin Sci (Lond),* **121**(2): 71-78.

Veenhuyzen, G. D., Simpson, C. S., & Abdollah, H. (2004). Atrial fibrillation. *CMAJ,* **171**(7): 755-760.

Veldink, H., Faulhaber-Walter, R., Park, J. K., Martens-Lobenhoffer, J., Bode-Boger, S., Schuett, H., Haghikia, A., Hilfiker-Kleiner, D., & Kielstein, J. T. (2013). Effects of chronic SDMA infusion on glomerular filtration rate, blood pressure, myocardial function and renal histology in C57BL6/J mice. *Nephrol Dial Transplant,* **28**(6): 1434-1439.

Veresh, Z., Racz, A., Lotz, G., & Koller, A. (2008). ADMA impairs nitric oxide-mediated arteriolar function due to increased superoxide production by angiotensin II-NAD(P)H oxidase pathway. *Hypertension,* **52**(5): 960-966.

von Leitner, E. C., Klinke, A., Atzler, D., Slocum, J. L., Lund, N., Kielstein, J. T., Maas, R., Schmidt-Haupt, R., Pekarova, M., Hellwinkel, O., Tsikas, D., D'Alecy, L. G., Lau, D., Willems, S., Kubala, L., Ehmke, H., Meinertz, T., Blankenberg, S., Schwedhelm, E., Gadegbeku, C. A., Boger, R. H., Baldus, S., & Sydow, K. (2011). Pathogenic cycle between the endogenous nitric oxide synthase inhibitor asymmetrical dimethylarginine and the leukocyte-derived hemoprotein myeloperoxidase. *Circulation,* **124**(24): 2735-2745.

Wagstaff, A. J., Overvad, T. F., Lip, G. Y., & Lane, D. A. (2014). Is female sex a risk factor for stroke and thromboembolism in patients with atrial fibrillation? A systematic review and meta-analysis. *QJM,* **107**(12): 955-967.

Wang, T. J., Massaro, J. M., Levy, D., Vasan, R. S., Wolf, P. A., D'Agostino, R. B., Larson, M. G., Kannel, W. B., & Benjamin, E. J. (2003a). A risk score for predicting stroke or death in individuals with new-onset atrial fibrillation in the community - The Framingham Heart Study. *Jama-Journal of the American Medical Association,* **290**(8): 1049-1056.

Wang, T. J., Massaro, J. M., Levy, D., Vasan, R. S., Wolf, P. A., D'Agostino, R. B., Larson, M. G., Kannel, W. B., & Benjamin, E. J. (2003b). A risk score for predicting stroke or death in individuals with new-onset atrial fibrillation in the community: the Framingham Heart Study. *JAMA,* **290**(8): 1049-1056.

Wang, X. Q., Nigro, P., World, C., Fujiwara, K., Yan, C., & Berk, B. C. (2012). Thioredoxin interacting protein promotes endothelial cell inflammation in response to disturbed flow by increasing leukocyte adhesion and repressing Kruppel-like factor 2. *Circulation Research,* **110**(4): 560-568.

Wang, Y., De Keulenaer, G. W., & Lee, R. T. (2002). Vitamin D(3)-up-regulated protein-1 is a stress-responsive gene that regulates cardiomyocyte viability through interaction with thioredoxin. *Journal of Biological Chemistry,* **277**(29): 26496-26500.

Wassmann, S., Laufs, U., Baumer, A. T., Muller, K., Ahlbory, K., Linz, W., Itter, G., Rosen, R., Bohm, M., & Nickenig, G. (2001). HMG-CoA reductase inhibitors improve endothelial dysfunction in normocholesterolemic hypertension via reduced production of reactive oxygen species. *Hypertension,* **37**(6): 1450-1457.

Watson, T., Shantsila, E., & Lip, G. Y. (2009). Mechanisms of thrombogenesis in atrial fibrillation: Virchow's triad revisited. *Lancet,* **373**(9658): 155-166.

Weber, M., Lauer, N., Mulsch, A., & Kojda, G. (2001). The effect of peroxynitrite on the catalytic activity of soluble guanylyl cyclase. *Free Radic Biol Med,* **31**(11): 1360-1367.

Wijffels, M. C., Kirchhof, C. J., Dorland, R., & Allessie, M. A. (1995). Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. *Circulation,* **92**(7): 1954-1968.

Willoughby, S. R., Chirkova, L. P., Horowitz, J. D., & Chirkov, Y. Y. (1996). Multiple agonist induction of aggregation: an approach to examine anti-aggregating effects in vitro. *Platelets,* **7**(5-6): 329-333.

Willoughby, S. R., Rajendran, S., Chan, W. P., Procter, N., Leslie, S., Liberts, E. A., Heresztyn, T., Chirkov, Y. Y., & Horowitz, J. D. (2012). Ramipril sensitizes platelets to nitric oxide: implications for therapy in high-risk patients. *Journal of the American College of Cardiology,* **60**(10): 887-894.

Willoughby, S. R., Stewart, S., Holmes, A. S., Chirkov, Y. Y., & Horowitz, J. D. (2005). Platelet nitric oxide responsiveness: a novel prognostic marker in acute coronary syndromes. *Arterioscler Thromb Vasc Biol,* **25**(12): 2661-2666.

Wolf, P. A., Abbott, R. D., & Kannel, W. B. (1991). Atrial-Fibrillation as an Independent Risk Factor for Stroke - the Framingham-Study. *Stroke,* **22**(8): 983-988.

Wolf, P. A., Dawber, T. R., Thomas, H. E., Jr., & Kannel, W. B. (1978). Epidemiologic assessment of chronic atrial fibrillation and risk of stroke: the Framingham study. *Neurology,* **28**(10): 973-977.

Wolowacz, S. E., Samuel, M., Brennan, V. K., Jasso-Mosqueda, J. G., & Van Gelder, I. C. (2011). The cost of illness of atrial fibrillation: a systematic review of the recent literature. *Europace,* **13**(10): 1375-1385.

Wong, C. X., Brooks, A. G., Cheng, Y. H., Lau, D. H., Rangnekar, G., Roberts-Thomson, K. C., Kalman, J. M., Brown, A., & Sanders, P. (2014). Atrial fibrillation in Indigenous and non-Indigenous Australians: a cross-sectional study. *BMJ Open*, **4**(10): e006242.

Wong, C. X., Brooks, A. G., Leong, D. P., Roberts-Thomson, K. C., & Sanders, P. (2012). The Increasing Burden of Atrial Fibrillation Compared With Heart Failure and Myocardial Infarction: A 15-Year Study of All Hospitalizations in Australia. *Archives of Internal Medicine,* **172**(9): 739-740.

Workman, A. J. (2010). Cardiac adrenergic control and atrial fibrillation. *Naunyn Schmiedebergs Arch Pharmacol,* **381**(3): 235-249.

Wysokinski, W. E., Ammash, N., Sobande, F., Kalsi, H., Hodge, D., & McBane, R. D. (2010). Predicting left atrial thrombi in atrial fibrillation. *American Heart Journal,* **159**(4): 665-671.

Xia, W., Yin, Z., Li, J., Song, Y., & Qu, X. (2009). Effects of rosuvastatin on asymmetric dimethylarginine levels and early atrial fibrillation recurrence after electrical cardioversion. *Pacing Clin Electrophysiol,* **32**(12): 1562-1566.

Xia, Y., Dobaczewski, M., Gonzalez-Quesada, C., Chen, W., Biernacka, A., Li, N., Lee, D. W., & Frangogiannis, N. G. (2011). Endogenous thrombospondin 1 protects the pressure-overloaded myocardium by modulating fibroblast phenotype and matrix metabolism. *Hypertension,* **58**(5): 902-911.

Xiang, G., Seki, T., Schuster, M. D., Witkowski, P., Boyle, A. J., See, F., Martens, T. P., Kocher, A., Sondermeijer, H., Krum, H., & Itescu, S. (2005). Catalytic degradation of vitamin D up-regulated protein 1 mRNA enhances cardiomyocyte survival and prevents left ventricular remodeling after myocardial ischemia. *Journal of Biological Chemistry,* **280**(47): 39394-39402.

Xiao, L., Pimentel, D. R., Wang, J., Singh, K., Colucci, W. S., & Sawyer, D. B. (2002). Role of reactive oxygen species and NAD(P)H oxidase in alpha(1)-adrenoceptor signaling in adult rat cardiac myocytes. *Am J Physiol Cell Physiol,* **282**(4): C926-934.

Xu, J., Xie, Z., Reece, R., Pimental, D., & Zou, M. H. (2006). Uncoupling of endothelial nitric oxidase synthase by hypochlorous acid: role of NAD(P)H oxidase-derived superoxide and peroxynitrite. *Arterioscler Thromb Vasc Biol,* **26**(12): 2688-2695.

Yamashita, T., Sekiguchi, A., Iwasaki, Y. K., Date, T., Sagara, K., Tanabe, H., Suma, H., Sawada, H., & Aizawa, T. (2010). Recruitment of immune cells across atrial endocardium in human atrial fibrillation. *Circulation Journal,* **74**(2): 262-270.

Yamawaki, H., Pan, S., Lee, R. T., & Berk, B. C. (2005). Fluid shear stress inhibits vascular inflammation by decreasing thioredoxin-interacting protein in endothelial cells. *Journal of Clinical Investigation,* **115**(3): 733-738.

Yancy, C. W., Jessup, M., Bozkurt, B., Butler, J., Casey, D. E., Jr., Drazner, M. H., Fonarow, G. C., Geraci, S. A., Horwich, T., Januzzi, J. L., Johnson, M. R., Kasper, E. K., Levy, W. C., Masoudi, F. A., McBride, P. E., McMurray, J. J., Mitchell, J. E., Peterson, P. N., Riegel, B., Sam, F., Stevenson, L. W., Tang, W. H., Tsai, E. J., Wilkoff, B. L., American College of Cardiology, F., & American Heart Association Task Force on Practice, G. (2013). 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *Journal of the American College of Cardiology,* **62**(16): e147-239.

Yee, D. L., Bergeron, A. L., Sun, C. W., Dong, J. F., & Bray, P. F. (2006). Platelet hyperreactivity generalizes to multiple forms of stimulation. *Journal of Thrombosis and Haemostasis,* **4**(9): 2043-2050.

Yee, D. L., Sun, C. W., Bergeron, A. L., Dong, J. F., & Bray, P. F. (2005). Aggregometry detects platelet hyperreactivity in healthy individuals. *Blood,* **106**(8): 2723-2729.

Yoo, J. H., & Lee, S. C. (2001). Elevated levels of plasma homocyst(e)ine and asymmetric dimethylarginine in elderly patients with stroke. *Atherosclerosis,* **158**(2): 425-430.

Yoshino, S., Yoshikawa, A., Hamasaki, S., Ishida, S., Oketani, N., Saihara, K., Okui, H., Kuwahata, S., Fujita, S., Ichiki, H., Ueya, N., Iriki, Y., Maenosono, R., Miyata, M., & Tei, C. (2013). Atrial fibrillation-induced endothelial dysfunction improves after restoration of sinus rhythm. *Int J Cardiol,* **168**(2): 1280-1285.

Yoshioka, J., Schulze, P. C., Cupesi, M., Sylvan, J. D., MacGillivray, C., Gannon, J., Huang, H., & Lee, R. T. (2004). Thioredoxin-interacting protein controls cardiac hypertrophy through regulation of thioredoxin activity. *Circulation,* **109**(21): 2581-2586.

Zabalgoitia, M., Halperin, J. L., Pearce, L. A., Blackshear, J. L., Asinger, R. W., & Hart, R. G. (1998). Transesophageal echocardiographic correlates of clinical risk of thromboembolism in nonvalvular atrial fibrillation. Stroke Prevention in Atrial Fibrillation III Investigators. *Journal of the American College of Cardiology,* **31**(7): 1622-1626.

Zaccolo, M., & Movsesian, M. A. (2007). cAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology. *Circulation Research,* **100**(11): 1569-1578.

Zhang, F. Y., White, J. G., & Iadecola, C. (1994). Nitric-Oxide Donors Increase Blood-Flow and Reduce Brain-Damage in Focal Ischemia - Evidence That Nitric-Oxide Is Beneficial in the Early Stages of Cerebral-Ischemia. *Journal of Cerebral Blood Flow and Metabolism,* **14**(2): 217-226.

Zhou, R., Tardivel, A., Thorens, B., Choi, I., & Tschopp, J. (2010). Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nature Immunology*, **11**(2): 136-140.