Original Thesis for the Degree of Doctor of Medicine

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CIRCULATING PROGENITOR CELLS IN ATRIAL FIBRILLATION:

Relationship to endothelial dysfunction, thrombogenesis and inflammation.

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Abstract

Atrial fibrillation (AF) is the most common arrhythmia encountered in clinical practice with rapidly rising prevalence and incidence predominantly due to advancing age in Western populations. Of particular concern however is the strong relationship between AF and stroke. This relates to a number of factors, but there is an emerging body of evidence to suggest that AF confers a hypercoagulable state. Disruption of endothelial homeostasis (damage *vs*. repair) is thought to be central to this process. The endothelium appears to be damaged both by AF and various other vascular diseases (e.g. hypertension) that frequently co-exist with the arrhythmia, with similar disruption to endothelial repair (normally effected by endothelial progenitor cells). Endothelial damage seems to be an essential prerequisite to thrombogenesis in AF. Significantly, the endothelium also links a number of processes including inflammation, growth factors, the renin-angiotensin-aldosterone system among others, which may directly or indirectly lead to activation of the coagulation cascade.

This thesis investigates the relationship between the temporal pattern of AF (paroxysmal, persistent, permanent) and established markers of endothelial dysfunction (vonWillebrand factor, vWf; soluble E-selectin, sEsel), angiogenesis (vascular Endothelial Growth Factor, VEGF), apoptosis (soluble Fas/Fas ligand, sFas/sFasL) and inflammation (C-reactive protein, CRP; Interleukin-6, IL-6) in AF with particular reference to circulating progenitor cells (CPCs) as a novel marker of endothelial health/angiogenesis. Additionally the impact of restoration of sinus rhythm using

electrical cardioversion on these indices and the relevance of the AF arrhythmia burden in influencing these markers is investigated.

In conclusion, the endothelium seems to be a central link through which all three components of Virchow's triad interact in AF. This thesis finds a possible link for CPCs to interact with various other reported aberrancies of the hypercoagulable state in this process. Also reported is a modest alteration in CPC counts following restoration of sinus rhythm, however, only limited numbers of patients were assessed and this requires examination with a more in depth study. Finally, the thesis has also examined the role of paroxysmal AF in influencing surrogate markers of the hypercoagulable state, but failed to find any significant differences on the basis of the arrhythmia burden. These findings must however been considered in light of numerous study limitations, the most notable of which is limited statistical power.

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Table of Contents

Section II: MD Hypotheses, Study Design and Methods

Section III: Laboratory Studies

Section IV: Clinical Studies

Section V: Conclusion

Glossary

a) List of Abbreviations

ACTIVE	Atrial Fibrillation Clopidogrel Trial With Irbesartan for Prevention of Vascular Events
ADMA	Asymmetric DiMethylArginine
AF	Atrial Fibrillation
AFB	Atrial Fibrillation Arrhythmia Burden
AFFIRM	Atrial Fibrillation Follow-up Investigation of Rhythm Management clinical trial
ATRIA	AnTicoagulation and Risk Factors In Atrial Fibrillation clinical trial
AV	AtrioVentricular
BAFTA	Birmingham Atrial Fibrillation Treatment of the Aged clinical trial
CABG	Coronary Artery Bypass Grafting
CAD	Coronary Artery Disease
$CHADS_2$	Congestive Cardiac Failure, Hypertension, Age (≥75), Diabetes Mellitus, $Stroke_2$. A stroke risk Score
CI	Confidence Interval
CPC	Circulating Progenitor Cell
CRP	C-reactive Protein
CVA	CerebroVascular Accident
CXR	Chest X-Ray

DiLDL	Di-actylated Low Density Lipoprotein
EC-CFU	Endothelial Cell Colony Forming Unit
ECG	ElectroCardioGram
EPC	Endothelial Progenitor Cell
FITC	Fluoroscein IsoThioCyanate
HDL	High Density Lipoprotein
HMG-CoA	3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A
HRP	Horseradish Peroxidase
hsCRP	high sensitivity C-reactive Protein
IL	InterLeukin
INR	International Normalized Ratio
KDR	Kinase-insert Domain Receptor
LA	Left Atrium
LAA	Left Atrial Appendage
LDL	Low Density Lipoprotein
LIFE	Losartan Intervention For Endpoint clinical trial
LMWH	Low Molecular Weight Heparin
MMP	Matrix MetalloProteinase
NO	Nitric Oxide
OPD	o-Phenylenediamine Dihydrochloride
PAI	Plasminogen Activator Inhibitor
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline - Tween
PCI	Percutaneous Coronary Intervention
MNC	MonoNuclear Cell

mRNA	messenger RiboNucleic Acid
NICE	National Institute for health and Clinical Excellence
RAA	Right Atrial Appendage
RELY	Randomized Evaluation of Long-Term Anticoagulation Therapy clinical trial
PE	PhycoErythrin
PECy5	PhycoErythrin Cy5
RAA	Right Atrial Appendage
RAAS	Renin-Angiotensin-Aldosterone System
SD	Standard Deviation
SEC	Spontaneous Echo Contrast
sE-selectin	soluble E selectin
SPAF	Stroke Prevention in Atrial Fibrillation clinical trial
sP-selectin	soluble P selectin
TF	Tissue Factor
TIMP	Tissue Inhibitor of MetalloProteinase
TNF	Tumor Necrosis Factor
TOE	TransOesophageal Echocardiogram
TRF-2	Telomeric Repeat Binding Factor-2
t-PA	tissue Plasminogen Activator
VE	Vascular Endothelial
VEGF	Vascular Endothelial Growth Factor
vWf	von Willebrand factor

b) List of Figures

c) List of Tables

d) Publications related to this thesis

Watson T, Shanstila E, Lip GY. Modern management of atrial fibrillation. *Clin Med*. 2007;7:28-34.

Watson T, Lip GY. Management of atrial fibrillation. *Herz*. 2006;31:849-56.

Watson T, Shantsila E, Lip GY. Mechanisms of thrombogenesis in atrial fibrillation: Virchow's triad revisited. *Lancet*. 2009;373:155-66.

Shantsila E, Watson T, Lip GY. Endothelial progenitor cells in cardiovascular disorders. *J Am Coll Cardiol*. 2007;49:741-52

Watson T, Goon PK, Lip GY. Endothelial progenitor cells, endothelial dysfunction, inflammation, and oxidative stress in hypertension. *Antioxid Redox Signal*. 2008;10:1079-88.

Shantsila E, Watson T, Tse HF, Lip GY. Endothelial colony forming units: are they a reliable marker of endothelial progenitor cell numbers?. *Ann Med*. 2007;39:474-9.

Watson T, Shantsila E, Karthikeyan VJ, Jessani S, Goon PK, Lip GYH. The effects of exercise stress testing, diurnal variation and temporal decline on Circulating Progenitor Cells. *Thromb Haem*. 2010;103:419-25

Watson T, Shantsila E, Blann AD, Lip GY. Circulating progenitor cells in patients with atrial fibrillation and their relation with serum markers of inflammation and angiogenesis. Thromb Haemost. 2010;104:327-34

Watson T, Arya A, Sulke N, Lip GY. Relationship of indices of inflammation and thrombogenesis to arrhythmia burden in paroxysmal atrial fibrillation. *Chest.* 2010;137:869-76

Watson T, Karthikeyan VJ, Lip GY, Beevers DG. Atrial fibrillation in primary aldosteronism. *J Renin Angiotensin Aldosterone Syst*. 2009;10:190-4.

Section I: Literature Appraisal

1.1 Atrial Fibrillation: An Overview

1.1.1 Introduction

Atrial Fibrillation (AF) is the commonest arrhythmia encountered in clinical practice and is increasingly considered as an emerging health epidemic. Despite rapidly evolving treatment strategies, AF presents a complex management challenge to the physician and is now attracting substantial clinical and academic interest because of a strong association with substantial mortality and morbidity. Imortantly, AF And direct complications (primarily stroke) leads to considerable expenditure, at present amounting to approximately 1% of the United Kingdom National Health Service (NHS) expenditure[1].

Cross-sectional studies have shown that the prevalence and incidence of AF continues to surge, correlating in particular with the advancing age of Western populations. In those over 65, approximately 5% have AF, while in those aged over 80 years, this figure rises to around 10%[2]. For those over 40 years of age, approximately 1 in 4 will develop AF at some point during the remainder of their lives[3, 4].

This chapter provides an overview of the management of AF, with reference to published literature and to evidence-based clinical guidelines[5, 6].

1.1.2 Risk factors for AF

Many risk factors for AF have been identified. As well as advancing age, there is a strong link with hypertension, ischaemic heart disease, structural/functional heart

disease, valvular heart disease and hyperadrenergic states (such as thyrotoxicosis or illicit drug use), to name but a few. In some patients, alcohol excess may provide an adequate trigger for acute AF. In fact, the array of associations with AF is enormous (table 1) and in part explains why a detailed knowledge of this condition is essential for both the generalist and specialist alike; not only will cardiologists frequently encounter AF, but so will colleagues in other specialties.

Table 1. Common risk factors for developing Atrial Fibrillation

- Age
- Male sex
- Body mass index
- Excess alcohol
- Hyperthyroidism
- Respiratory disease (e.g. chronic lung disease, carcinomatosis, pulmonary embolus)
- Diabetes
- Cardiovascular (e.g. ischaemic heart disease, hypertensive heart disease, valvular/structural heart disease, heart failure and cor pulmonale, intracardiac masses/tumours, pericardial disease, cerebrovascular disease, peripheral vascular disease)
- Recent surgery

1.1.3 Symptoms and presentation

The incidental finding of asymptomatic AF remains relatively common, often detected during medical attendance for unrelated matters, e.g. during preoperative assessment or simply following pulse palpation. Increasingly though, AF is diagnosed during investigation for symptoms such as palpitations, dyspnoea, dizziness or syncope. This list is by no means exhaustive and it is frequently argued that AF may contribute to or exacerbate numerous complaints, including lethargy, fatigue and anxiety. Additionally, it is important to remember that other conductive or atrioventricular nodal diseases may coexist with AF and in those who complain of syncopal episodes this should be considered as a co-diagnosis. Table 2 illustrates the most common symptoms reported in association with AF.

Table 2. Common symptoms reported in Atrial Fibrillation.

- Palpitations
- Dyspnoea
- Poor exercise capacity
- Dizziness/syncope
- Fatigue
- Anxiety

1.1.4 Classification

The classification of AF has been through a period of flux. Most are now agreed on a clinical classification system, based on the temporal patterns of AF, summarized as follows[7]: The treatment implications for each AF subtype is outlined in table 3.

•**Recent / Acute onset AF** (i.e. within the preceding 48 hours)

•**Paroxysmal AF** (bouts of AF less than 7 days duration with interceding sinus rhythm)

•**Persistent AF** (lasting seven days or more, but potentially cardiovertable to sinus rhythm)

•**Permanent AF** (Duration greater than 1 year or refractory to cardioversion attempts).

Regardless of classification, the management of patients with AF should be guided by many considerations, including symptoms, the presence or absence of haemodynamic compromise, and associated co-morbidities. The (sometimes artificial) classification system purely gives an idea of the time course of the AF but not the ultimate clinical outcome, and re-emphasizes the fact that the management of AF should be guided by assessment of the individual patient.

Table 3. Treatment Implications by Temporal Pattern of Atrial Fibrillation.

Terminology	Clinical Features	Arrhythmia pattern	Therapeutic implications
Initial event (first detected episode)	Symptomatic Asymptomatic (first detected) Onset unknown (first detected)	May or may not recur	Antiarrhythmic therapy for prevention is not needed except if severe symptoms.
Paroxysmal	Spontaneous termination (<7 days) and most often <48 hours	Recurrent	Prevention of recurrences. Rate control and anticoagulation if needed
Persistent	Not self terminating Lasting >7 days or prior cardioversion	Recurrent	Rate control and anticoagulation if needed and/or cardioversion and prophylactic antiarrhythmic therapy
Permanent	Not terminated Terminated but relapsed No cardioversion attempt	Established	Rate control and anticoagulation as needed

Adapted from Levy S et al.[7]

The clinical subtypes of AF can help define the objectives of management and therapeutic strategies[8]. For example, the management objective in paroxysmal AF is the reduction of paroxysms and the long-term maintenance of sinus rhythm, and hence antiarrhythmic drugs (or increasingly non-pharmacological approaches such as radiofrequency ablation) are used. In persistent AF, the management objective is usually sinus rhythm restoration, and hence cardioversion is generally attempted at an appropriate point (i.e. after adequate anticoagulation). In permanent AF, the objective is heart rate control. This is generally achieved with antiarrhythmic drugs, however, non-pharmacological approaches may be required in some patients – usually where the support of a permanent pacemaker is required to protect from bradycardia whilst allowing pharmacotherapy at adequate dose to limit tachycardia. In all patients with AF, appropriate antithrombotic therapy use is mandatory, and is normally based on assessment of risk factors for stroke and thromboembolism.

1.1.5 Investigation of the patient with atrial fibrillation

As discussed, the potential causes for AF are many and the symptom profile is potentially complex. Thus, assessment of the patient with AF needs to be methodical. A thorough history taking and clinical examination are essential, as they will not only help alert the physician to any potential trigger for AF, but it will also help guide any further investigation and the treatment strategy to be pursued.

A 12-lead electrocardiogram (ECG), blood investigations (including full blood count, electrolytes and thyroid function tests), chest X-ray and echocardiogram are essential. Other tests such as 24-hour (or sometimes, seven-day) Holter monitoring or exercise testing may be required in certain patients – often for those where a diagnosis of

paroxysmal AF is suspected but not initially apparent or to assess the AF burden as part of risk assessment for anticoagulation. Table 4 summarizes relevant investigations for AF.

Table 4. Investigating the patient with atrial fibrillation.

History

- Symptoms and severity
- Risk factors for AF (including symptoms of thyroid disease)
- Drugs (and alcohol)

Examination

- Pulse (rate is better assessed apically in AF)
- Abnormal cardiac or respiratory findings

12-lead electrocardiogram

- Confirmation of rhythm
- Rate (at rest)
- Detection of pre-excitation arrhythmias
- Evidence of left ventricular hypertrophy (usually secondary to hypertension or aortic stenosis)
- Signs of ischaemic heart disease

Chest X-ray

- Screen for carcinomatosis and chronic lung disease

Blood investigations

- Full blood count
- Electrolyte profile
- Thyroid function tests

Echocardiogram (not always necessary)

- Structural/hypertensive/valvular heart disease
- Left atrial size
- Left ventricular size and function
- Pericardium

The following may be useful in some patients:

Holter monitor

- Useful to assess rate control
- Assessment of AF burden in paroxysmal AF

Exercise test

- Exercise/ischaemia induced AF

Assessment of rate control during exercise

1.1.6 Rate control or rhythm control

Much of the current dilemma for the physician is deciding whether to pursue a 'rate control' or 'rhythm control' strategy. Theoretically, restoration of sinus rhythm in all patients seems ideal, as this should allow improved haemodynamics and provide symptom relief while potentially reducing the thromboembolic tendency and stroke risk. However, the trend in many patients with AF is towards recurrence (often asymptomatic), even if a rhythm control strategy initially succeeds. Despite the use of potent antiarrhythmics and an aggressive serial cardioversion strategy for early relapse, for example, only approximately 50% of patients remain in sinus rhythm at one year[9]. At five years, the figures are dire, with only 25% remaining free of AF[9], bringing into question whether it is ever appropriate to stop thromboembolic prophylaxis even if rhythm control seemingly succeeds.

This knowledge presents a dilemma. Should all patients be offered rhythm control, or would it be more prudent to accept that many patients will eventually succumb to permanent AF, and adopt a rate control strategy for all? This subject has been addressed by several randomized controlled trials. The largest of these, the Atrial Fibrillation Follow-up Investigation of Rhythm Management (AFFIRM) trial, investigated 4,060 participants with recurrent AF and one or more risk factors for stroke[10]; this trial showed no difference in the primary endpoint of overall mortality at five years in those randomized to rate or rhythm control strategies. Furthermore, there was no significant difference in the composite secondary outcome measure of death, disabling stroke, disabling anoxic encephalopathy, major bleeding and cardiac arrest between the two study arms. Notably, a rhythm control strategy was associated with a higher rate of hospitalisation and more frequent adverse drug reactions associated

with antiarrhythmic drug use. In a, post hoc, analysis, both anticoagulation use and sinus rhythm emerged as significant predictors of survival. However, a higher mortality was associated with the use of antiarrhythmic drugs which offset the survival advantage of being in sinus rhythm[10]. Nonetheless, some caution should be exercised before extrapolating the data from the rate control versus rhythm control trials to the entire AF population. This is particularly important giventhat many participants in the AFFIRM study were elderly and thus important degenerative changes within the structure of the atrial tissue may have occurred (e.g. atrial dilatation) limitng the potential for sinus rhythm maintenance.

In this and other trials, a large proportion of patients in the rhythm control arms did not maintain sinus rhythm, but were continued in that arm of the trial for the 'intention to treat' analysis. Most participants were older patients with risk factors for stroke and more than likely other comorbidities as well, or who had previous failed attempts at rhythm control. Crossing over from rhythm control to rate control treatment was also common.

The United Kingdom National Institute for Health and Clinical Excellence (NICE) has systematically reviewed the evidence and provided guidance for which treatment strategy (rhythm control or rate control) is most appropriate (Table 5)[5]. These indications are not mutually exclusive, and in appropriate circumstances, the possibility of restoring sinus rhythm (e.g. using catheter ablation) can be considered, especially in symptomatic patients. Recommendations remain as guidance for appropriate treatment strategies for the majority of patients with AF. Some patients will have different requirements and will require a tailored treatment strategy.

Table 5. Rate or Rhythm Control for Atrial Fibrillation?

A *rate control* strategy should be the preferred initial option in the following patients with persistent AF:

- over 65
- with coronary artery disease
- with contraindications to antiarrhythmic drugs
- unsuitable for cardioversion
- without congestive heart failure.

A *rhythm control* strategy should be the preferred initial option in the following patients with persistent AF:

- symptomatic
- younger
- presenting for the first time with lone AF
- AF secondary to a treated/corrected precipitant
- congestive heart failure.

Adapted from UK NICE guideline on AF management[5].

1.1.7 Pharmacological Management of Atrial Fibrillation

Acute atrial fibrillation

The strategy for management of acute AF owes much to an accurate assessment of the patient's haemodynamic status and cardiac stability, as well as any associated complications such as angina or heart failure. In patients with life-threatening features, emergency electrical cardioversion should always be considered, irrespective of duration of AF. Some patients with AF and an uncontrolled ventricular response develop acute decompensation with peripheral or pulmonary oedema. Although the rapid ventricular response clearly plays a major role, these patients often have underlying cardiac disease and rapid clinical improvement can be achieved by reducing ventricular rate. These patients may include those with recent onset AF, those with paroxysmal AF who present with a fast paroxysm, and those with previously stable persistent/permanent AF who have become tachycardic. In the latter group this may be due to a hyperadrenergic state (e.g. sepsis) or hypovolaemia and so complete assessment of the patient is mandatory.

Until relatively recently, the mainstay of drug therapy for AF was the cardiac glycoside, digoxin (Vaughan Williams class V); however, this drug is of limited efficacy in the context of thyrotoxicosis, fever, perioperatively and most importantly during exercise[11]. As such, it is only in the elderly sedentary patient that digoxin monotherapy is likely to offer adequate rate control. A rate-limiting calcium antagonist (Vaughan Williams class IV) or beta-blocker (Vaughan Williams class II) should be tried as first line in stable patients, but where these are inappropriate (e.g. pulmonary oedema and hypotension), intravenous amiodarone (Vaughan Williams

class III) should be considered. This drug is a potent irritant and should ideally be administered through central venous access. In other patients, particularly those who are younger without any structural heart disease, it is important to consider Wolff-Parkinson-White syndrome, as fast AF may be the initial presenting rhythm in this condition. Atrioventricular node blocking agents (such as diltiazem, verapamil, or digoxin) should not be used in this circumstanceas these may exacerbate the ventricular rate by accelerating conduction through the accessory pathway and could be dangerous. Here it would be appropriate to consider intravenous flecainide (Vaughan Williams class 1c) for attempting pharmacological cardioversion. This drug is also helpful in restoring sinus rhythm in younger patients in the absence of structural or ischaemic heart disease.

Rate control in atrial fibrillation

Clearly optimal cardiovascular status is best achieved by restoration of sinus rhythm; however, this is not always feasible or successful and in these circumstances the ventricular rate should be controlled to that where cardiac output is optimal and the patient's symptoms are adequately controlled. It is generally accepted that a resting heart rate below 90 bpm is optimal, while on exertion the heart rate should not exceed 110 bpm in the sedentary patient or '200 minus age' in the ambulatory patient[12]. Beta-blockers are effective at controlling ventricular rate and are increasingly being used as first-line agents. Beta-blockers also offer some protection against recurrence following successful cardioversion (be it spontaneous, pharmacological or electrical) and are often used as first-line prophylactic agents in paroxysmal AF. They are also useful in the perioperative setting, to reduce the likelihood of developing AF in those deemed at risk. As AF commonly coexists with ischaemic heart disease, hypertension

or heart failure due to systolic dysfunction, beta-blockers may also be part of the therapeutic management in such patients. The rate-limiting, non-dihydropyridine calcium channel blockers (diltiazem, verapamil) are also frequently used to optimise rate control in those unable to take or tolerate a betablocker. Although digoxin is no longer considered first line in most patients, it remains a useful adjunct in those who remain tachycardic at rest and can be used in combination with either a beta-blocker or calcium channel blockers (the so-called 'hybrid' approach). Despite such strategies, a small minority of patients continue to have poor control of ventricular rate, or in the case of paroxysmal AF, may suffer frequent distressing relapses. This sub-group of patients tend to be very symptomatic and other agents such as amiodarone are sometimes useful. Side effects with this drug are, however, common, particularly with prolonged exposure, when hepatic, ophthalmic, pulmonary, thyroid and dermatological complications may occur. Thus, it is important to keep these patients under long-term follow-up with regular assessment usually under the care of a cardiologist. Alternative non-pharmacological strategies may also be employed in this setting (see section 1.1.8).

Rhythm control in atrial fibrillation

Rhythm control aims to restore, and achieve long-term maintenance of sinus rhythm. Potential candidates for a rhythm control strategy are: younger patients; those with lone AF, or AF secondary to a corrected precipitant (e.g. alcohol); patients with symptoms despite optimal rate control; patients with heart failure. In persistent AF, cardioversion can be performed electrically or pharmacologically. Electrical cardioversion may be successful in 75–93% of patients, but success rate depends on duration of AF, left atrial size and coexisting structural heart disease[13]. The outcome

is significantly lower in patients with AF duration of more than one year. The rate of recurrence of AF after electrical cardioversion is high and maintenance of sinus rhythm may be improved by concomitant administration of antiarrhythmic drugs, such as amiodarone or sometimes beta-blockers. Pharmacological cardioversion can be achieved using a number of drugs including: disopyramide (Vaughan Williams class IA), flecainide and propafenone (Vaughan Williams class IC), dofetilide, ibutilide, sotalol and amiodarone (Vaughan Williams class III). Those most commonly prescribed in the UK are flecainide, sotalol and amiodarone. As with electrical cardioversion, earlier administration improves the chance of success, with (generally) little to choose between oral and intravenous administration, except speed of conversion. In fact, successful cardioversion is reported in up to 80% with oral antiarrhythmic drugs, rising only to approximately 90% with intravenous administration[14].

As relapse following cardioversion is common, antiarrhythmic drugs are used in both persistent and paroxysmal AF to enhance long-term maintenance of sinus rhythm. The class IC and III drugs are preferred to class IA drugs, in view of their better safety profile (table 6)[15]. A number of studies have demonstrated that flecainide and propafenone are effective for preventing recurrence of AF[16-18]. The effectiveness of flecainide is comparable to quinidine, but with fewer side effects. Propafenone is more effective at maintaining sinus rhythm than quinidine, and is as effective as sotalol. Given the relatively high risk of proarrhythmia, class IC drugs should not be used in patients with ischaemic heart disease or left ventricular dysfunction. All current class IA, IC, and III antiarrhythmic drugs have potential for significant side effects. Major complications include proarrhythmia and non-cardiovascular effects.

Most antiarrhythmic medicines prevent or terminate AF by alteration of function of potassium or sodium channels of atrial cells. Blocking of potassium channels prolongs repolarisation and the refractory period that may cause QT and QRS prolongation. QT prolongation with potassium-channel blockers (e.g. sotalol) may result in the life-threatening proarrhythmia (such as torsades de pointes) in up to 5% of patients[19]. Factors known to enhance risk for development of torsades de pointes are hypokalaemia or hypomagnesaemia, congenitally prolonged QT intervals, bradycardia, congestive heart failure, female gender, and pauses associated with the conversion of AF to sinus rhythm. The risk of QT prolongation and torsades de pointes may be further increased by concomitant use of wide range of medications, especially ones, which interfere with the hepatic metabolism of antiarrhythmic drugs. It is therefore advisable that these drugs are not prescribed to patients taking other substances known to promote QT prolongation (such as erythromycin) and that a 12-lead ECG be requested after starting treatment. In some, particularly in the presence of renal impairment, class IC drugs can turn AF into atrial flutter with 1:1 atrioventricular nodal conduction and haemodynamic instability. For this reason, the concomitant use of atrioventricular nodal blocking agents, such as beta-blockers and rate-reducing calcium antagonists, is recommended.

Table 6. Pharmacological Therapy in Atrial Fibrillation by Vaughan-Williams Classification

Vaughan-Willams	Examples	Rate Control	Rhythm Control	Side Effects
Ia	Quinidine Disopyramide	-	+++	Potential for multiple side-effects across drug class. Most importantly is risk of pro-arrhythmia or atrial flutter with 1:1 conduction. Procainamide can induce lupus like syndrome. Side effects least with class IC
Ib		-	++	
Ic	Flecainide Propafenone	-	+++	
II	Atenolol Bisoprolol Sotalol	+++	+	Bradycardia; lethargy; exacerbation of asthma; impotence
III	Amiodarone Sotalol*	++	+++	Potential for multiple side-effects with amiodarone (thyroid / liver / pulmonary / ophthalmic / neurological / dermatological.
IV	Verapamil Diltiazem	+++	-	Bradycardia; gum hyperplasia; pedal oedema
V [Miscellaneous]	Digoxin	+++	-	Gastrointestinal upset - nausea, vomiting

* Low-dose sotalol primarily has class II actions, at higher dosages, class III effects predominate.

1.1.8 Non-pharmacological approaches

For those who continue to be heavily symptomatic or where antiarrhythmic drugs are ineffective or not tolerated, non-pharmacological options can be considered. It has long been recognized that the pulmonary veins appear to have a crucial role in the aetiology of AF[20]. This has led to the emergence of non-pharmacological approaches to AF management.

Catheter approaches

Ablation of the atrioventricular (AV) node with permanent pacemaker implantation is an established and effective option that ensures good rate control, alleviates symptoms and improves quality of life[21]. This technique is particularly useful in those with poor rate control despite multi-drug therapy. In some, AV node modification rather than ablation may be attempted. This technique involves preferential ablation of the electrical inputs to the AV node with the shortest refractory period, thereby implying an upper limit to ventricular rate response[22]. Of course, there is a significant incidence of complete heart block requiring subsequent pacing and also risk of AF recurrence, limiting the feasibility of this approach.

Pulmonary vein isolation has been used as first-line management for recurrent AF, with impressive results. Häissaguerre *et al*. reported that 94% of ectopic triggers for AF were located in the myocardial tissue around the pulmonary veins[23]. Ablation of these foci prevented AF recurrence in 62% of patients. Unfortunately, the procedures remain long and arduous, and often two or more attempts are required to achieve long-term success. Furthermore, complications (e.g. pulmonary vein stenosis) remain relatively common and can occur in up to 6% of patients[24]. In addition, some go on to

have recurrent episodes of AF, which may be asymptomatic and difficult to detect clinically, thus making an absolute assessment of stroke risk difficult[25, 26]. More recently, Pappone *et al* demonstrated that robotic magnetic navigation was feasible for AF ablation[27]. This allowed circumferential pulmonary vein ablation from a remote operator and illustrates the potential for expansion of catheter techniques.

Surgery.

Prior to the expansion of ablation techniques, surgical procedures allowed the potential to offer a 'cure' for recurrent AF. The 'corridor' procedure, introduced in 1985, involves surgical isolation of the atrial-free walls from the septum through a series of sutures[28]. In the remaining corridor, a conduction pathway between the sinus and AV nodes is maintained. Unfortunately, later development of other atrial arrhythmias was common and affected up to 27% after five years[29]. Subsequent work by Cox *et al.* led to the development of the 'maze' procedure which involves a series of incisions within the atria with subsequent fibrosis, thereby creating lines of conduction block[30]. This later evolved into the Maze-III operation, which involves excision of the left and right atrial appendages, isolation of the pulmonary veins and several additional incisions to prevent atrial re-entry. This is now the surgical procedure of choice for medically refractory AF. However, because of the requirement for an invasive approach, these techniques are probably best reserved for those undergoing thoracotomy for other reasons, for example, those requiring coronary artery bypass grafting or valve surgery.

1.1.9 Antithrombotic therapy

The presence of AF increases the risk of stroke by up to five-fold across all age groups; indeed, AF accounts for up to 10–15% of all ischaemic strokes[31]. This association continues to strengthen with age and in those over 80, AF accounts for nearly 35% of strokes, especially in the presence of comorbidities[31]. Of greater concern is that those with AF who have a stroke have a significantly worse outcome in terms of both morbidity and mortality.

Warfarin vs. placebo

6 randomized trials (n=2,900 participants) have assessed the efficacy of warfarin *vs*. placebo or no treatment. Meta-analysis of this data demonstrated that adjusted-dose warfarin offered a significant reduction in risk of ischaemic stroke or systemic embolism (67%, 95% CI 54 to 77%)[32]. As expected, the absolute risk reduction for all stroke was far greater for secondary prevention (8.4% per year, number needed to treat (NNT) for one year to prevent one stroke 12) when compared with primary prevention (2.7% per year, NNT=37)[32]. A target INR (international normalized ratio) of 2.0–2.5 should be maintained in most patients, as the risk of stroke increases two-fold in those with INR 1.5–2.0, and is even higher with INRs <1.5[33].

Antiplatelet therapy vs. placebo

Eight trials (n=4,876 participants) have investigated the efficacy of antiplatelet therapy compared to placebo of which seven (n=3,990 participants) specifically investigated aspirin (at various dosages). Meta-analysis of this data reveals that aspirin confers only a modest reduction in stroke compared to placebo of around 19% (95% CI -1 to 35%)[32]. This figure is similar to that seen for stroke reduction with

antiplatelet therapy for patients with vascular disease in sinus rhythm, and thus the aspirin effect may simply reflect this. In fact, much of the evidence supporting the use of aspirin for stroke prevention in AF stems from the Stroke Prevention in Atrial Fibrillation (SPAF I clinical trial, where inconsistencies in the randomization process are evident[34]. Notably patients eligible to receive anticoagulants (arm I) were randomized to warfarin, aspirin or placebo whereas those deemed ineligible for anticoagulants (based on safety grounds or patient preference) received either aspirin or placebo (arm II). By pooling data from both groups, the trialists were able to report a headline 42% stroke risk reduction with aspirin *vs.* placebo, although analysis of data from arm II alone revealed only a modest 8% reduction.

Warfarin vs. Antiplatelet agents

12 trials (n=11,748 participants) have compared adjusted-dose warfarin to antiplatelet therapy (predominantly aspirin and at varying dosages). Meta-analysis of pooled data from these trials demonstrates that adjusted-dose warfarin is associated with a 37% (95% CI 23 to 48%) reduction in stroke[32]. Combination of antiplatelet agents (e.g. aspirin plus clopidogrel), although of some benefit compared to aspirin alone, is also inferior to warfarin for stroke prevention[35].

The benefits of anticoagulation are further underlined in a recent Cochrane review of antithrombotic therapy in AF, which concluded that aspirin only provided a modest reduction in stroke risk, at best approaching 25% compared to no treatment, whereas adjusted-dose warfarin reduced risk of stroke by about one third when compared to antiplatelet therapy[36]. Thus, antiplatelet agents should therefore be considered an inferior substitute in terms of stroke prevention among high-risk subjects with AF.

Despite the evidence, there is a tendency for physicians to shy away from prescribing anticoagulation – the main concern being that of significant haemorrhage. In particular, anticoagulation is less likely to be offered to the elderly and those with a history of falls, recent history of bleeding, or poor compliance. Each of these patient factors is more common in the elderly, but it is this very patient group that is also at the highest risk from thromboembolic events. The recent Birmingham Atrial Fibrillation Treatment of the Aged (BAFTA) study has been able in part to address this very issue[37]. In agreement with other AF anticoagulation trials, this study again demonstrated the superior efficacy of warfarin over aspirin for stroke prevention. The focus in this trial however, was the over 75s (mean age 81·5 years, SD 4·2) and it is notable the observed stroke risk reduction was not at the expense of excess haemorrhagic stroke or major bleeding events. Thereby providing at least some reassurance that age alone should not be a bar to anticoagulation prescription. However, one important caveat is that entry to this trial was General Practionar assessment that warfarin would not be unsafe and thus caution should be exercised in extrapolating this data to the broader population.

Some further reassurance is provided by new data from the AnTicoagulation and Risk Factors In Atrial Fibrillation (ATRIA) study where the net clinical benefit of warfarin anticoagulation (i.e. the rate of ischaemic stroke / systemic embolus prevention whilst also accounting for harm due warfarin in relation to excess haemorrhagic stroke) was assessed[38]. The investigators report an overall net benefit of warfarin for the overall cohort of 0.68% per annum (95% CI, 0.34% to 0.87%). The net benefit was greater still when assessing subgroups of advancing age and higher untreated stroke risk. The

criteria for calculation of net benefit were not simple and based upon calculation of benefit/harm using a complex algorithm which again is unlikely to be applicable outside the setting of a clinical trial.

The requirement for regular therapeutic monitoring, lifestyle restrictions (especially alcohol and various foodstuffs) and compliance all play a role in the decision whether or not to anticoagulate. Of note, up to 40% of patients would prefer not to receive warfarin[39] and thus may pressurize the doctor advocating the (often inappropriate) use of aspirin, however many patients see a moderate–severe stroke as a fate worse than death[40]. Indeed, the importance of compliance should be stressed to each patient, as an erratic INR leads to an excessive risk of bleeding events. Furthermore, a 10% increase in time out of the therapeutic INR range can be associated with an increased risk of mortality, ischaemic stroke and hospitalisation among those patients anticoagulated for AF[41].

Other important considerations relate to the onset of AF. In fact, there appears to be a clustering of embolic events around the time of AF onset in some patients and therefore a decision regarding anticoagulation should not be excessively delayed. In addition, the greater application of percutaneous coronary intervention (PCI) for ischaemic heart disease has led to debate regarding the optimal choice for anticoagulation in this setting and indeed, many patients with AF have co-existing coronary artery disease.

For PCI, generally aspirin and clopidogrel are recommended, to reduce the risk of stent thrombosis, however, the efficacy of these drugs for stroke prevention in AF is

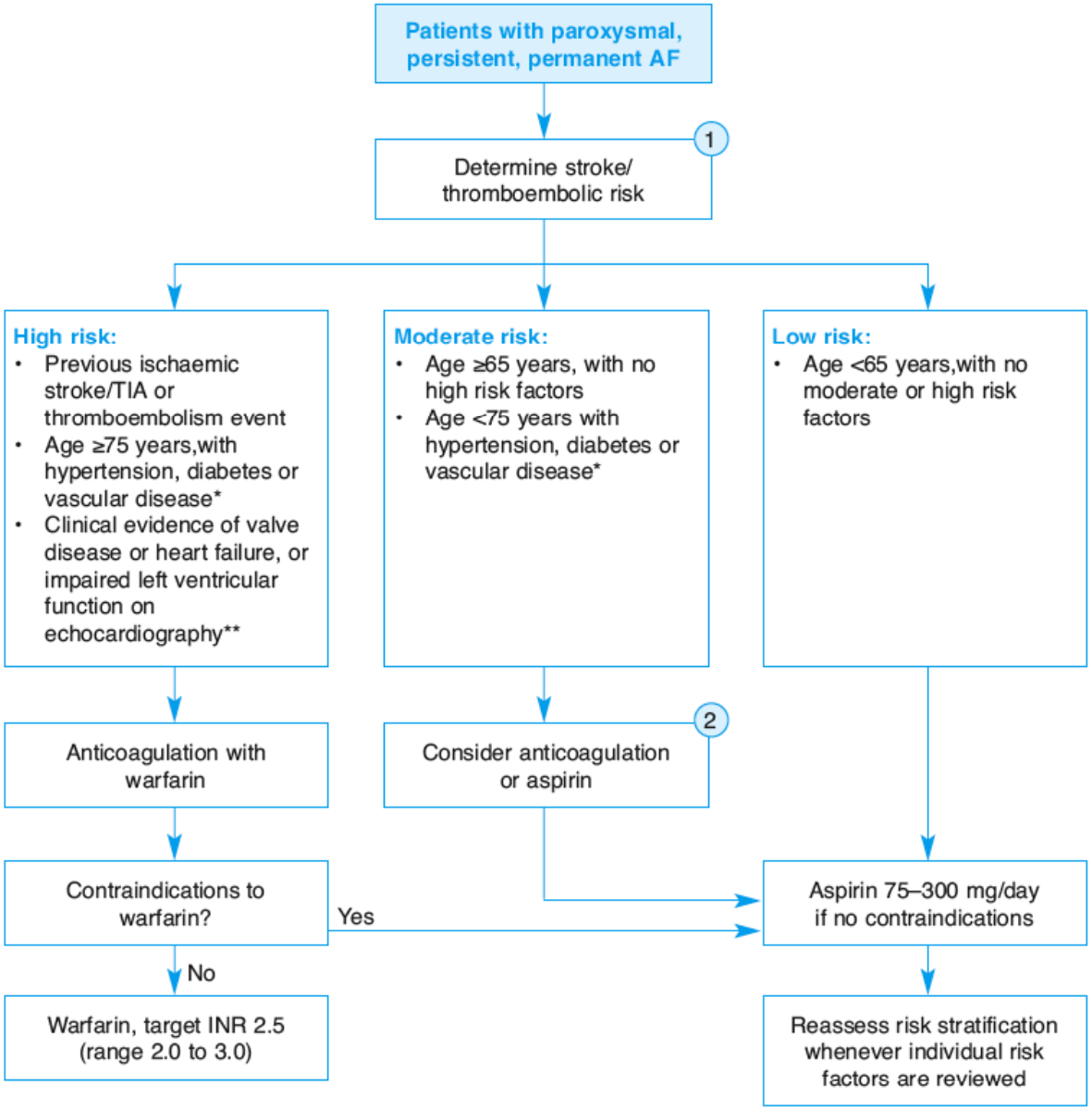
questionable, while triple therapy with aspirin, clopidogrel, and warfarin is associated with more bleeding events[42]. Various suggestions regarding treatment have been made, however these are based upon assumptions regarding risk of stroke and subsequent cardiac events. It is clear that for this common clinical scenario, guidelines formulated from evidence based on clinical trials are urgently required.

A number of novel anticoagulants are under development or being assessed in clinical trials. Much interest has been focused on the potential for factor Xa and direct thrombin inhibition. The recently reported Randomized Evaluation of Long-Term Anticoagulation Therapy (RELY) trial investigated the efficacy of dabigatran – a direct thrombin inhibitor – in comparison to open-label warfarin[43]. The trial reports that dabigatran 110mg b.d. was associated with rates of stroke and systemic embolism similar to those seen with warfarin but with a lower rate of major haemorrhage. At a higher dose of 150mg b.d., dabigatran was associated with a lower stroke and systemic embolism rate than warfarin, with similar rate of major haemorrhage. This trial was a non-inferiority study and although therefore it cannot be claimed that dabigatran is more efficacious than warfarin, the results are very encouraging. It is hoped that with the evolution of safe efficacious drugs that do not require regular therapeutic monitoring, the stranglehold of warfarin on the anticoagulation market in AF may soon be at an end. In theory, this may encourage more appropriate prescribing.

Numerous risk stratification schema have been proposed in an effort to identify high-risk patients with AF who should be targeted for anticoagulation – some derived directly from event rate analyses, others based on expert consensus. None of the

published schemes are ideal, with marked variability in terms of both complexity and patient categorisation as low-, medium-, or high-risk leading to inconsistencies in recommendations for thromboprohylaxis. Almost all schema include previous stroke or TIA, age, hypertension and diabetes mellitus as consistent independent clinical predictors for stroke[44]. It is recognized that AF commonly associates with other risk factors for stroke and investigators have proposed combining these to form a scoring system. For example, in the $CHADS_2$ scoring system, one point is assigned for the presence of congestive heart failure, hypertension, age >75 years and diabetes mellitus, while two points are assigned for a history of stroke or transient ischaemic attack. The stroke risk per 100 patient years in this schema increased by a factor of 1.5 for each one-point increase in the score, highlighting the importance of accurate risk stratification[45]. Whilst no direct comparison of the predictive accuracy of all available schema is available, it is also recognized that the usefulness of a particular score relates to the composition of the patient cohort to which it is applied – i.e. the proportion of elderly patients with multiple risk factors when compared to younger patients with fewer risk factors and to the ratio of primary to secondary prevention. In the UK, the current NICE guideline favours a more practical and pragmatic algorithm-based risk stratification approach, which is shown in figure 1. The $CHADS_2$ score and NICE risk stratification schema (the latter based on the Birmingham risk stratification schema[31]) have a similar predictive value for stroke and vascular events, when tested in a prospective cohort of AF patients.

Figure 1. UK Practical guidelines for antithrombotic therapy in non-valvular AF.



(1) The risk factors are not mutually exclusive, and are additive to each other in producing a composite risk. Since the incidence of stroke and thromboembolic events in patients with thyrotoxicosis appears similar to other aetiologies of atrial fibrillation (AF), antithrombotic treatments should be chosen based on the presence of validated stroke risk factors. (2) Owing to lack of sufficient clear-cut evidence, treatment may be decided on an individual basis, and the physician must balance the risk and benefits of warfarin versus aspirin. As stroke risk factors are cumulative, warfarin may, for example, be used in the presence of two or more risk factors. Referral and echocardiography may help in cases of uncertainty. *Coronary artery disease or peripheral artery disease. **An echocardiogram is not needed for routine assessment, but refines clinical risk stratification in the case of moderate or severe left ventricular dysfunction and valve disease. INR = international normalized ratio; TIA = transient ischaemic attack. Adapted from UK NICE guideline on AF management[5]

1.1.10 Data Appraisal

Much of the data presented in this chapter regarding the epidemiology of AF and the strong assosciation with stroke has been derived from highly regarded studies. Although some relevant publications are now historical, the populations concerned remain under active follow-up and close scrutiny. Importantly more contemporary data is also available and has broadly reiterated previous findings.

Guidelines for the management of AF have been formulated by consensus expert opinion following review of the available literature. Studies considered have been of variable design and quality and thus it is not always easy to make straightforward comparisons between treatment strategies. For example, data concerning the safety of class I antiarrhythmic drugs has been extrapolated from investigation of these drugs in other settings. Similarly, inconsistent study design has also led to some controvery as to the true value of aspirin for thromboprophylaxis in AF, whereas the efficacy of warfarin is less contentious. Further evidence of disparity in the literature is evident with consideration of the various stroke rosk stratification models that are currently available.

Finally, the role of catheter ablation for AF will continue to evolve – particularly with enhanced techniques and equipment and also with improved operator availability. Thus the available contemporary data may not truly reflect current clinical practice and merits further exploration particularly as these techniques may have some impact in patient selection regarding rate *vs.* rhythm control

1.1.11 Summary

AF is a common arrhythmia, with increasing frequency of diagnosis – both in asymptomatic and symptomatic patients. The are numerous risk factors and co-morbidities which associate with AF, in particular advancing age, hypertension, ischaemic heart disease, diabetes mellitus and heart failure. Importantly each of these also enhances risk of stroke and all are more frequent in a population advancing in age. Thus the burden of AF on healthcare systems is rising – both in relation to the arrhythmia itself and due to direct complications (e.g. stroke).

The mechanisms that enhance stroke risk in AF are complex and remain poorly understood and thus merit in depth exploration in chapter 1.2.

1.2 Mechanisms of Thrombogenesis in Atrial Fibrillation

1.2.1 Introduction

The association between atrial fibrillation (AF) and the risk of stroke and thromboembolism has long been recognized and has already been described in section 1.1. However, the pathogenesis of thrombus formation (thrombogenesis) in AF is multi-factorial and is not only related to stasis in a poorly contractile left atrium (LA). Indeed, there is an increasing body of evidence to support the presence of a 'prothrombotic' or 'hypercoagulable' state in AF[46].

1.2.2 Virchow's Triad for Thrombogenesis

Over 150 years ago, Rudolf Virchow proposed a triad of events needed for thrombus formation - i.e. abnormal changes of the vessel wall, blood flow, and blood constituents[47]. In the 21st century, we now recognize Virchow's triad as: endothelial or endocardial damage or dysfunction (and related structural abnormalities); abnormal blood stasis; and abnormal haemostasis, platelets, and fibrinolysis. Extensive alterations in these variables are clearly evident in AF. Thus, AF could, in fact, drive a prothrombotic or hypercoagulable state, by virtue of its fulfillment of Virchow's triad for thrombogenesis[48].

1.2.3 Anatomical and Structural Considerations

Attached to each atria is a blind-ended passage known as an appendage. The left atrial appendage (LAA) is long with a narrow inlet, thereby predisposing to blood stasis. Thus, the LAA is the most common site of intra-atrial thrombus formation, not only in AF, but also in patients in sinus rhythm[49, 50]. Changes in the dimensions of the LA and LAA occur as a consequence of AF, with some correlation to subsequent

thromboembolism. Detailed descriptions of endothelial damage in the context of AF are well described and can be visualized by scanning electron microscopy, especially within the appendages. Goldsmith *et al*. reported more severe endocardial changes in the LAA than in the right-atrial appendage[51] especially in subjects in AF (compared with sinus rhythm) and in mitral stenosis (compared with mitral regurgitation). This data seems to be very reproducible and Masawa *et al.* later described a 'rough endocardium' with a wrinkled appearance attributable to oedema and fibrinous transformation; small areas of endothelial denudation and thrombotic aggregation have also been noted in patients with AF and cerebral embolism[52]. However, the populations analyzed in these two studies were clearly highly selected in that they were undergoing cardio-pulmonary bypass – often for valvular heart disease.

However, subsequent work has confirmed that these changes are present, even in those without valvular heart disease[53]. Other changes, including myocytic hypertrophy or necrosis and a mononuclear cell infiltrate, are also evident[54]. These structural changes (with or without electrical changes) could explain the delay in return of atrial (mechanical) systole after successful cardioversion[55, 56]. The occurrence of such cardiac stunning highlights the importance of adequate anticoagulation even after successful restoration of sinus rhythm[57].

Not all structural changes in AF are cardiac. Complex aortic plaque identified by transoesophageal echocardiography (TOE) is common and occurs in up to 57% of patients with AF, of whom about 25% have complex plaque (i.e., thicker than 4 mm and with ulceration, pedunculation, or mobile elements)[58]. Oddly, the presence of complex plaque on the descending (rather than ascending) aorta is a risk factor for

stroke[59]. Thus it is likely that aortic plaque simply aids identification of patients who are at high stroke risk by virtue of the presence of associated vascular risk factors or atherothrombotic disease, in addition to AF.

Altered extracellular matrix turnover could also be implicated in the structural changes associated with AF. The extracellular matrix provides support scaffolding for myocytes, maintaining the structural and geometrical integrity of the heart[60]. Disruption of the extracellular matrix therefore, has the potential not only to result in conduction defects (perpetuating AF), but also to induce fibrosis and infiltration of the endocardium, and thereby promote thrombogenesis by activation of the coagulation cascade. Several studies have shown that patients with AF have altered amounts of collagen degradation products and impaired matrix degradation, with abnormal plasma concentrations of various matrix metalloproteinases (MMPs), their inhibitors (tissue inhibitor of MMPs (TIMPs), and various growth factors (e.g., transforming growth factor β1) reported[61-63]. These proteins are important in the breakdown of various collagens and hence their regulation is key to ensuring healthy matrix turnover. Evidence suggests that abnormal changes in the extracellular matrix are not related to the presence of AF itself, but are probably a consequence of various coexisting co-morbidities (e.g. hypertension). Nevertheless, MMPs and TIMPs could have a link with the prothrombotic state, as exemplified by a correlation with prothrombin fragments 1 and 2, markers of thrombogenesis[62]. Further studies have identified disruption of other extracellular matrix components, although most have focused on these factors as a cause for the arrhythmia or explanation for remodeling and chamber dilatation[64-67]. One study suggested that some of the changes in MMPs were due to concomitant mitral valve disease[66], whereas another reported changes in

the ventricular myocardium, albeit to a lesser extent[67]. Similarly, in patients with ventricular dysfunction (a potent risk factor for AF), various studies have also shown striking atrial structural changes[68, 69]. This may relate to diastolic dysfunction or to back pressure through functional mitral regurgitation.

1.2.4 Abnormal Blood Stasis

In addition to stasis consequent on the failure of atrial systole, the presence of non-valvular AF seems to promote progressive left atrial (LA) dilatation[70], thus further amplifying the potential for stasis. In the presence of mitral stenosis, LA dilatation is increased and leads to further stasis and propensity to thrombosis[71]. The contribution of LA dilatation to thrombogenesis (at least, in non-valvular AF) is indicated by the finding that atrial size corrected for body surface area is an independent risk factor for stroke [72, 73]. Valvular heart disease is also an important contributory factor to thrombogenesis in AF and cannot be ignored. In mitral stenosis, up to 75% of patients with cerebral emboli on computed tomography or autopsy are identified to have AF, presumably due to alterations in LA emptying and transmitral flow[74]. However, this data must be interpreted with caution given that 'stroke' was defined on the basis of radiological, rather than clinical findings. In contrast to this, moderate to severe (non-rheumatic) mitral regurgitation seems to reduce the risk of stroke with AF[75]. Defining patients with AF and mitral valve disease who are at the greatest risk of stroke has proved complex. The risk of emboli increases with age and in individuals with a lower cardiac index (a measure of cardiac output corrected for body surface area), but seems to correlate poorly with clinical classification or mitral valve area. Studies assessing the degree of LA dilatation have also proven inconsistent. However, an initial embolic event is highly predictive for subsequent or recurrent thromboemboli[76].

Abnormal stasis in the LA and LAA can be visualized on TOE with spontaneous echo contrast (SEC) or pulsed-wave Doppler during paroxysms of AF[50, 77-79]. In sinus rhythm, a quadriphasic pattern of blood flow can be seen in the LAA, affording minimal blood stasis[80]. This pattern of blood flow is thought to be related to the intimate, yet slightly delayed, relationship between atrial and ventricular passive and active filling. In AF, SEC has been shown to independently predict increased risk of thromboembolism[59]. SEC is thought to be related to increased interaction between fibrinogen and erythrocytes and seems to relate to the relative concentrations of each, with more fibrinogen needed to induce the same effect at lower haematocrits[81-83]. Since some patients with AF can be intravascularly deplete (for example, as a result of co-prescription of diuretics), this finding could contribute to the increased stroke rate seen in this patient population[84]. Crucially, SEC is also highly dependent on flow rate and thus more likely to occur in patients predisposed to stasis and in AF, can also be seen after restoration of sinus rhythm and can still occur in up to 37% of this cohort at 3 months[85]. With additional stroke risk factors, this proportion is higher still, illustrating the need for continuing anticoagulation despite apparent maintenance of sinus rhythm. This data again must be interpreted with some caution, as SEC is highly operator dependent and as with a number of other echocardiographic phenomena, may have become more notable with improved resolution available with modern digital equipment.

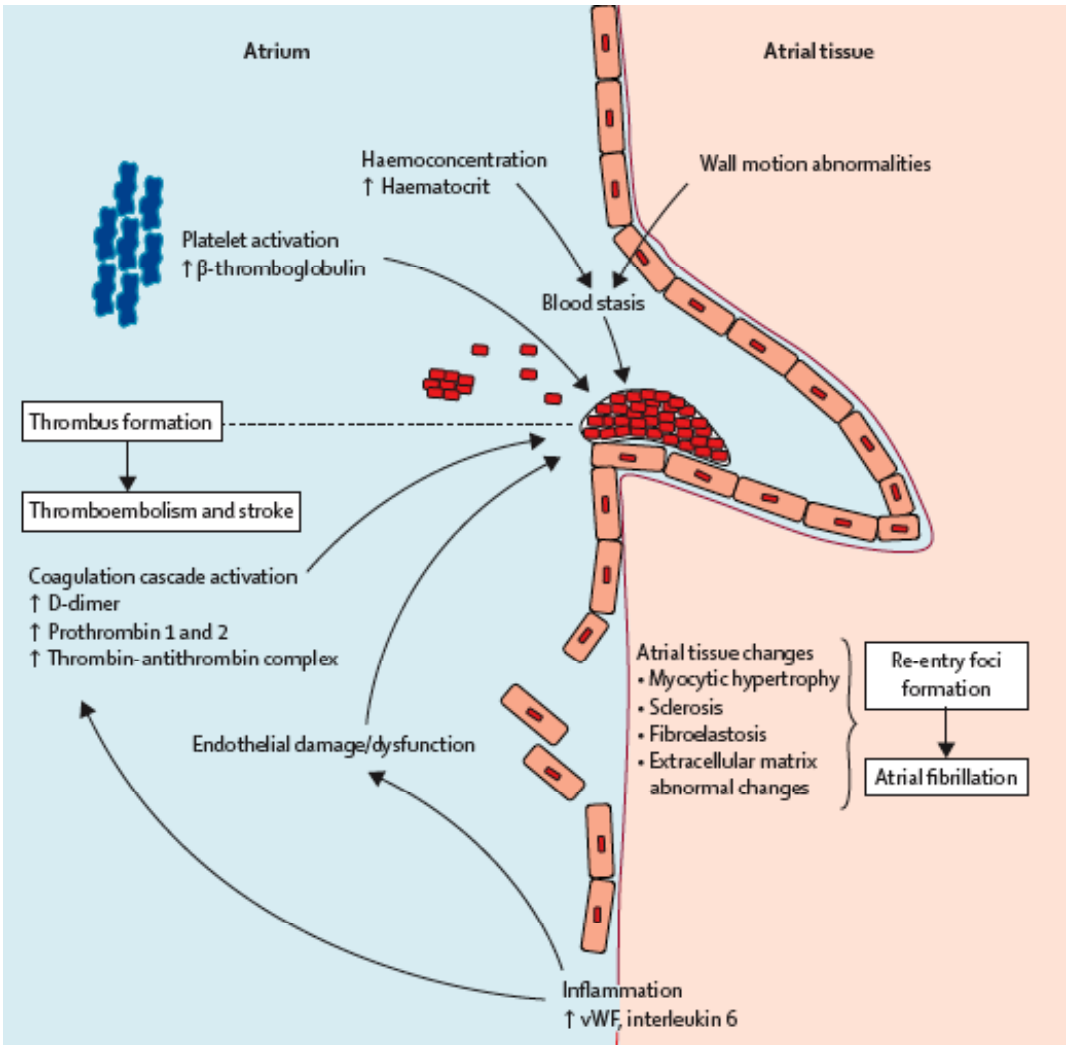
1.2.5 Abnormal Blood Constituents

The main intravascular promoters of thrombogenesis are platelets and the various proteins of the coagulation cascade. In AF, abnormal changes in all these parameters and numerous other blood constituents (e.g. inflammatory cytokines, growth factors) are evident, thereby completing Virchow's triad.

Abnormal changes in coagulation

Abnormal haemostasis and coagulation are well described in AF (figure 2 and table 7). In particular, increased fibrin turnover has been reported in patients with acute onset or chronic AF[86-92]. These changes initially seemed to be unrelated to the cause of AF or structural heart disease[91, 92]. However, abnormal concentrations of prothrombotic indices (e.g., prothrombin fragments 1 and 2 and thrombin-antithrombin complexes) are more prominent in patients with stroke who have AF than in those who have sinus rhythm[93], as well as in patients with AF and many stroke risk factors (e.g., diabetes plus heart failure) compared with either risk factor alone[94-96]. Furthermore, some prothrombotic indices are abnormal in the patients with AF only[97, 98] and in those with paroxysmal AF[99] in the absence of detectable risk factors. The data for some markers has been considered reasonable robust and they have been proposed as suitable candidates to refine various stroke risk stratification schema, many of which are reasonably able to identify patients at low risk or high risk of stroke, but poor at identifying patients at moderate risk[100].

Figure 2 Components of Virchow's triad for thrombogenesis in AF



Abnormal changes shown in the vessel wall (e.g. atrial tissue changes, endothelial damage and dysfunction), in flow (e.g. stasis in the left atrial appendage), and in blood constituents (e.g. haemoconcentration, platelets, coagulation cascade activation, inflammation); all factors contribute to propensity for thrombus formation (thrombogenesis) in atrial fibrillation. vWf=von Willebrand factor.

An association between various prothrombotic indices, stasis, and intra-cardiac thrombus has been described[101, 102]. In one study, congestive cardiac failure, a history of recent embolus, and fibrin D-dimer were both shown to independently predict the presence of LAA thrombi on TOE, leading the researchers to conclude that D-dimer could be useful in predicting the absence of LAA thrombi[102]. This is of potential importance when considering whether to offer cardioversion for recent onset AF in the absence of therapeutic anticoagulation. However, this has not been translated into a change in clinical practice – perhaps reflecting the relatively small size of this study.

The prothrombotic state also correlates with the degree of LAA dysfunction [103, 104]. Furthermore, a relation to TOE indices of stroke risk has been described. For example, SEC that is visible during TOE shows a significant correlation to prothrombin fragments 1 and 2, fibrinopeptide A, and thrombin-antithrombin III complex in non-valvular AF[105, 106]. Patients with atrial flutter and impaired LAA function (shown by pulsed-wave Doppler) have increased amounts of D-dimer and β-thromboglobulin[106]. In accordance with clinical data suggesting that mitral regurgitation protects against stroke in AF, a greater degree of mitral regurgitation is associated with reduced coagulation activity as estimated by fibrin D-dimer amounts[107], again highlighting the important contribution of stasis. Again, such echocardiographic parameters are notoriously operator dependent (see above). Additonally, echocardiographic assessment of the severity of mitral regurgitation can also depend on many factors including heart rate, blood pressure and intravascular volume etc. Thus the correlations observed here may not be as robust if applied to other populations or by other researchers.

Anticoagulant treatment has been shown to reduce concentrations of some prothrombotic markers [92, 108, 109]. This finding is true even with low-intensity anticoagulation (international normalized ratio [INR] 1·5–1·9), which has been shown to suppress prothombin fragments 1 and 2 and D-dimer[110]. Notably, some indices of hypercoagulability could be useful in investigating the efficacy of antithrombotic treatment for AF. For example, the Atrial Fibrillation, Aspirin, AntiCoagulation (AFASAK-2) substudy reported that only dose-adjusted warfarin (INR 2–3) had an effect on the amounts of prothrombin fragments 1 and 2 after 3 months of treatment. Fixed low-dose warfarin, combined low-dose warfarin and aspirin, or aspirin alone had little effect[111]. Similarly, fixed low-dose warfarin or aspirin-warfarin combination treatment did not substantially reduce other markers of thrombogenesis in AF, whereas dose-adjusted warfarin did[112]. Additionally, warfarin greatly decreased plasma concentrations of coagulation factor-related prothrombotic indices (more so than platelet-related indices), which implies that activation of the coagulation cascade, rather than platelets, is key to the excess thromboembolic risk in AF[113, 114]. These findings should come as little surprise and are consistent with reports that warfarin (as a modulator of the coagulation cascade) is more efficacious than aspirin (a platelet inhibitor) in thromboembolic prophylaxis in AF (section 1.1.9).

In chronic AF, D-dimer amounts remain in broadly the same range over time and seem to be a useful parameter for assessing the degree of hypercoagulability irrespective of patient age[87]. Combined with clinical risk factors, D-dimer has also been shown to predict subsequent thromboembolic events in patients with non-valvular AF, including those already receiving treatment with warfarin[115-117]. The application of D-dimer seemed to be especially important in patients without

conventional risk factors for stroke (e.g., age, cardiomyopathy, previous stroke), where a low D-dimer implied a low risk of stroke (0.7% per year). Conversely, in the same patient group, the stroke rate rose to 3.8% per year when D-dimer was elevated. In patients with clinical risk factors for stroke, the event rate was less than 5% per annum, irrespective of D-dimer concentration. In another study of patients with chronic AF, both D-dimer and age were important predictors of mortality. High amounts of D-dimer during treatment with oral anticoagulants was also a predictor of combined cardiovascular events[115]. Thus, D-dimer could be useful as a screening method to identify those patients with AF at low risk of intra-cardiac thrombus who can then be safely cardioverted without anticoagulation. Notably, Somlói et al. have suggested that D-dimer measurements compare favourably with the use of a TOE-guided strategy with a negative predictive value of 98%[118]. However, discrepancies between these small studies have limited application of this strategy in clinical practice.

von Willebrand factor (vWf)

Further insight into the hypercoagulable state in AF is provided by studies of vWf, which is a well-established index of endothelial damage and dysfunction. Raised vWf concentrations independently predict presence of LAA thrombus in AF[101]. Furthermore, increased LAA endocardial expression of vWf has been described[119], especially in those with an overloaded appendage, which seems to correlate with the presence of adherent platelet thrombus. Furthermore, increased expression of vWf in the endocardium has been shown to associate with enlarged LA dimensions in mitral valve disease and increased myocyte diameter[120]. Both vWf and tissue factor are over-expressed in the atrial endothelium in patients with AF who have a history of

cardiogenic thromboembolism—specifically in the endothelial sites containing inflammatory cells and denuded endocardium, which indicate features of persistent myocarditis[121].

Plasma vWf and D-dimer are also positively correlated in patients receiving either aspirin or no antithrombotic treatment, but not in those receiving warfarin[92], further indicating the ability of warfarin to modulate the thrombogenic process. Furthermore, a positive association between AF and plasma vWf was seen in the Rotterdam study[122]. This relation was most apparent in female patients, which could explain the excess risk of stroke due to AF in women compared with men. Furthermore, plasma vWf amounts were associated with the presence of four independent risk factors for stroke (heart failure, previous stroke, age, and diabetes) and stroke risk stratification schema[123, 124]. Follow-up data from this study suggests that vWf concentrations might independently predict subsequent stroke and vascular events[124, 125]. However, such applications will probably be hampered by the non-specificity of vWf, concentrations of which are also increased in various other disorders [126, 127]. Many of the studies investigating vWf have been of substantial size and the data is broadly consistent and generally considered robust. Some smaller studies studies have been contradictory and further discussion is reserved for later in this thesis (section 4.1.5).

Abnormal changes in fibrinolysis

Few studies have focused on fibrinolytic function in atrial fibrillation. Enhanced fibrinolysis, shown by increased concentrations of tissue-plaminogen activator (t-PA) antigen and t-PA inhibitor (PAI)-1 and reduced amounts of plasmin-antiplasmin complex can be attributable to a pathophysiological response to the prothrombotic

state[128, 129]. However, the available data are not consistent and conflicting results have also been reported[88]. In the Stroke Prevention in Atrial Fibrillation (SPAF) III study, increased concentrations of plasmin-antiplasmin complexes were independently associated with thromboembolic risk factors such as older age (>75 years), recent congestive heart failure, decreased fractional shortening, and recent onset of AF[130]. A significant correlation can be also shown between t-PA amounts and left-atrial diameter in AF[88]. Predictably, anticoagulation leads to some improvement in fibrinolytic markers in rheumatic AF[131]. Increased amounts of t-PA and PAI-1 can indicate the coexistence of confounders, such as hypertension, heart failure, or ischaemic heart disease, all of which can cause endothelial dysfunction, damage, and inflammation. However, studies in patients with AF only confirm that presence of the disorder does modulate these markers[88, 131]. Thus, the high amounts of t-PA and PAI-1 in AF could be a consequence of endothelial damage and dysfunction or represent systemic inflammation[132, 133]. Lower PAI-1 concentrations are also predictive of successful cardioversion[134], and are independent predictors of the development of AF after cardiopulmonary bypass[135].

It is unclear whether increased amounts of t-PA or PAI-1 in AF are due to endothelial dysfunction, inflammation, fibrinolysis, or vascular disease, or a combination as none of these studies has been large enough or broad enough to fully address the impact of confounding variables. Nevertheless, abnormal changes in the fibrinolytic system might relate not only to thrombogenesis but also to structural remodeling of the atria, in view of the strong links to extracellular matrix turnover.

Platelets

Many studies indicate a potential role for platelets in the hypercoagulable state (table 8). However, the results of the majority of these studies have been conflicting, representing the diverse aspects of platelet physiology that have been measured. Additional limitations that apply to all platelet studies relate directly to the various assays available, each of which allows for assessment of diverse aspects of platelet function and (patho) physiology and thereby demonstrate little agreement. Nonetheless, the available data support the notion that abnormal changes of platelets in AF do exist, but the relation between these measures and increased thrombotic risk remains uncertain, and many of such abnormal changes could simply indicate underlying vascular co-morbidities.

For example, Choudhury and colleagues[136] recently showed that patients with AF had far higher amounts of platelet microparticles and soluble P-selectin than healthy controls in sinus rhythm, but no difference was seen between patients with AF and disease-matched controls, implying that the abnormal changes detected were a consequence of the underlying co-morbidities rather than AF itself. Increased amounts of β-thromboglobulin, a platelet-specific protein that indicates platelet activation and is released from α-granules during platelet aggregation and subsequent thrombus formation, have been shown in patients with both valvular and non-valvular AF compared with controls in sinus rhythm[84, 104, 114, 137-140]. Substantially higher β-thromboglobulin amounts have been measured in patients with the lowest LAA flow velocities, who had greater left-atrial dimensions[104] suggesting that platelet activation could be enhanced in patients with a greater degree of intra-atrial stasis. Notably, antithrombotic treatment modulates only some of these abnormal changes, and

Kamath and co-workers did not show a beneficial effect of warfarin on plasma β-thromboglobulin concentrations[113]. In the same study, *in vitro* measures of platelet aggregation were not significantly increased in AF, once again questioning the importance of platelets in enhancing the thrombogenic tendency in this setting.

In other studies, oral anticoagulation also did not reduce platelet activation in AF, despite pronounced inhibition of other coagulation variables[141, 142]. By contrast, aspirin reduced concentrations of soluble P-selectin compared with warfarin in AF[143]. Notably, some patients given aspirin 325 mg per day still do not show complete inhibition of platelet aggregation, whereas others have hyper-aggregable platelets[144]. In view of recent interest in aspirin resistance, these findings raise the possibility of platelet-dependent mechanisms for aspirin and warfarin failure to prevent stroke in patients with AF.

Although combined antiplatelet treatment with aspirin and clopidogrel is more effective than aspirin monotherapy in inhibiting platelet function[145], this strategy does not substantially modulate the various markers of the coagulation cascade (e.g. platelet-dependent thrombin generation, antithrombin III, thrombin-antithrombin III complex, prothrombin fragments 1 and 2) in patients with AF. Such data accord with clinical trials, in which combined antiplatelet treatment with aspirin plus clopidogrel was shown to be less effective than warfarin for stroke prevention in AF[35]. These findings are also supported by data showing that patients show changes in plasma markers of platelet function but not platelet aggregation, which are unaffected by anticoagulation with warfarin[113].

The use of digoxin in patients with AF seems to be associated with platelet activation, with increased amounts of CD62P (P-selectin) expression on platelets and platelet-leucocyte conjugates, which could predispose to thrombosis and vascular events[142]. Enhancement of platelet activity and coagulability occur within 12 h of onset of AF[146], whereas after restoration of sinus rhythm, substantial reduction in platelet activity is seen compared with controls at 24 h after cardioversion[147]. During radiofrequency ablation of AF, persistent platelet activation is reported, but is not apparent during cryoablation[148]. Despite the presence of enhanced platelet activation in AF, any firm clinical evidence indicating that it directly enhances thrombotic risk is lacking. A substudy from the Stroke Prevention in Atrial Fibrillation III (SPAF-III) trial[149] recorded no association between plasma β-thromboglobulin amounts and subsequent thromboembolic events. By contrast, the population-based Rotterdam study[150] showed that plasma concentrations of soluble P-selectin were predictive of adverse clinical outcomes in elderly patients with AF. In view of the close links between platelet activation and the atherothrombotic vascular co-morbidities related to AF, the platelet activation seen in this arrhythmia could contribute to thrombogenesis indirectly. For example, increased expression of P-selectin on platelets associated with reduced concentrations of nitric oxide has also been shown to be a risk factor for silent cerebral infarction in patients with AF[151]. Moreover, raised amounts of P-selectin and CD63 have both been associated with the embolic and pre-embolic status of patients with non-rheumatic AF[152].

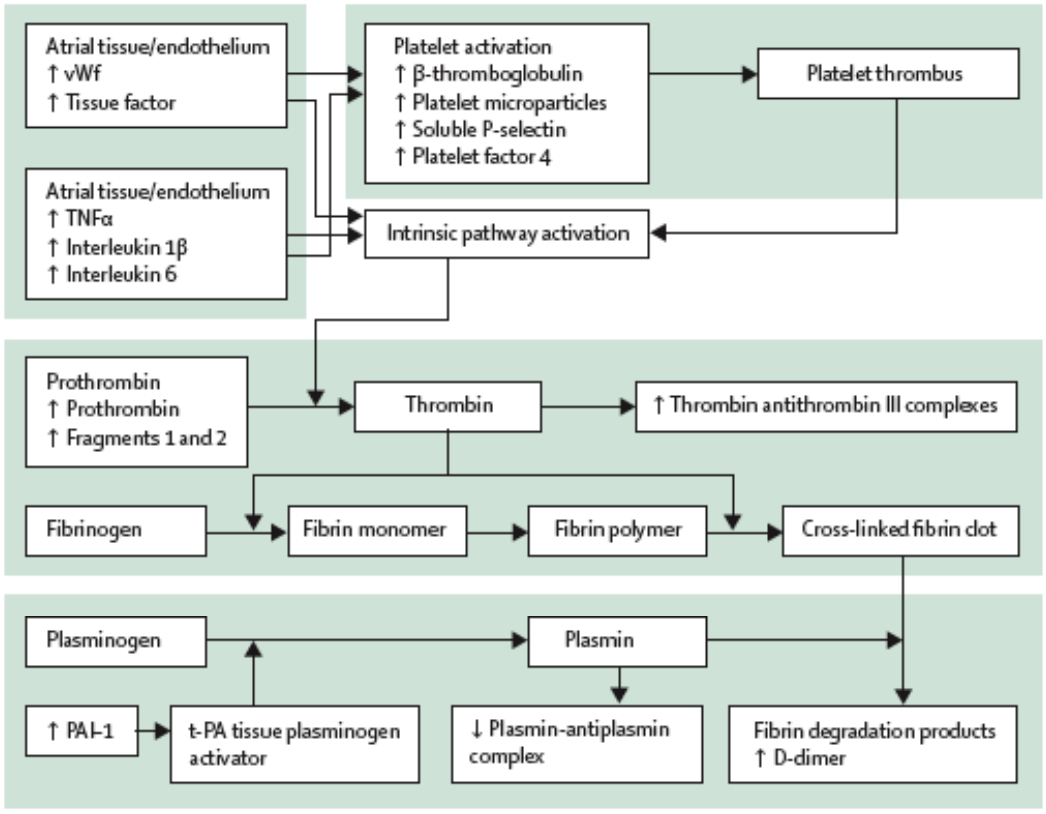
Restoration of sinus rhythm

Some evidence suggests that the coagulation system could be activated by cardioversion of AF[153]. Electrical cardioversion has been associated with more prominent activation of the coagulation system than a pharmacological strategy[154]. One study found a positive correlation between the energy delivered for cardioversion to sinus rhythm and plasma D-dimer values on day 7[154]. However, it could equally be argued that if more energy is required to restore sinus rhythm, the worse the 'state of AF'. An extended duration of AF could also lead to a more prominent hypercoagulable state (estimated by D-dimer value) after cardioversion[155], which of course may relate to delayed restoration of mechanical systole (section 1.1.6). The hypercoagulable state after cardioversion has even been seen despite optimum anticoagulation with warfarin[156]. Nevertheless, patients receiving therapeutic low-molecular-weight heparin (LMWH) before cardioversion seem to have reduced hypercoagulability[157]. In atrial flutter, limited data suggest that plasma amounts of D-dimer, platelet factor 4, β-thromboglobulin, thrombin-antithrombin III complex, and prothrombin fragments 1 and 2 remain raised, but do not seem to increase further with cardioversion of this arrhythmia[158]. Atrial flutter however is generally less thrombogenic than AF and so direct comparison needs particular caution.

1.2.6 What drives the prothrombotic state in AF?

Several mechanisms have been purported to drive the prothrombotic state in AF (figure 3), but recent evidence has focused on the potential role of inflammation and the release of various growth factors.

Figure 3 Abnormal changes in coagulation during Atrial Fibrillation



Inflammation

In AF, inflammation might not only result in endothelial damage, dysfunction, or activation, but also be linked directly to thrombogenesis. Increasing evidence has supported a link between inflammation and the initiation and perpetuation of AF[159-163]. Furthermore, abnormal changes in systemic inflammation have been related to prothrombotic indices in AF, suggesting that inflammation could drive the prothrombotic state in AF[159].

Although most cases of AF are associated with various co-morbidities, many of which could also enhance the baseline inflammatory state, there may be an underlying direct link between AF and inflammation. Interleukin-6 (IL-6) concentrations are abnormal in AF, with some prognostic implications shown in one study[164]. Many studies have also shown that amounts of high-sensitivity C-reactive protein (hs-CRP) are greater in patients with AF than in controls in sinus rhythm, with a stepwise increase in hs-CRP with the transition from patient groups with an increasing arrhythmia burden (sinus rhythm to paroxysmal then persistent AF)[132].

Raised hs-CRP amounts consistently correlate with cardiovascular risk, although not with future AF[159]. Also, reduced concentrations of both hs-CRP and E-selectin at baseline are associated with an increased probability of maintenance of sinus rhythm at 6 months after electrical cardioversion for AF, although the maintenance of sinus rhythm seems to have no effect on hs-CRP[165]. More recently, high hs-CRP amounts were shown to be predictive of mortality and vascular death in AF, but not stroke itself[166].

Both CRP and IL-6 stimulate tissue factor production from monocytes *in vitro*[167, 168]. Furthermore, IL-6 increases platelet production and sensitivity to thrombin[169], stimulates transcription of fibrinogen[170], and is linked to both endothelial activation and damage[171, 172]. However, no link seems to exist between hs-CRP and thrombin-antithrombin complexes[173]. Tissue factor and high stroke risk are also independent associates of IL-6, whereas fibrinogen and plasma viscosity are independent associates of hs-CRP amounts[174].

Growth factors

Another potential driver for thrombogenesis could be growth factors. In cancer biology, various pro-angiogenic factors, in particular vascular endothelial growth factor (VEGF), act as potent stimulants for tissue factor expression[175]. This activity could partly explain the enhanced thrombogenic risk often associated with cancer. Various pro-angiogenic factors have been identified; concentrations of some of these factors have been shown to alter in AF[176-178]. VEGF is largely produced by activated platelets[179] and results in up-regulation of tissue factor mRNA production and subsequent expression of this compound on the endothelial membrane[180].

VEGF amounts are substantially increased in both persistent and permanent AF, with a corresponding increase in tissue factor[176]. Additionally, raised serum concentrations of transforming growth factor-β1[177], and angiopoietin 2 (but not angiopoetin 1)[178] are also recorded in AF, showing the depth and complexity of modulation of growth factor amounts. Although the requirements for enhanced angiogenesis in AF are unknown, in view of the intimate association between VEGF and tissue factor, enhanced growth factors could be a crucial driving force behind the hypercoagulable

state. Notably, tissue factor acts as a cofactor to factor VIIa and is widely regarded as the physiological trigger to thrombin formation[181].

Why are factors such as the angiopoietins involved? Angiopoietin 1 and 2 are natural co-antagonists and both compete for the same binding site on Tie-2, an endothelial tyrosine kinase receptor. With an excess of angiopoietin 1, stability of the endothelium is favoured, whereas the converse is true with an excess of angiopoietin 2[178]. In these circumstances, the balance could ultimately favour endothelial destabilization and therefore the action of cytokines such as VEGF.

Extracellular matrix turnover

The extracellular matrix is a dynamic structure, which continually undergoes a process of structural remodeling[182]. Atrial remodeling has already been discussed (section1.2.3), and this process could contribute to the hypercoagulable state, by virtue of both enhanced blood stasis and an abnormal endocardium. Impaired matrix degradation in AF is well documented[61-67, 183]. These changes could, therefore, be important in atrial remodeling and therefore indirectly contribute to thrombogenesis. Perhaps more importantly, MMPs could be directly implicated in thrombogenesis by virtue of several known interactions with the coagulation cascade, most notably with plasmin[184].

The first matrix proteins studied were MMP-1 and TIMP-1 in patients with non-valvular AF, not receiving anticoagulation[62]. This study demonstrated evidence of impaired matrix degradation in patients with AF, but this finding was not independently associated with the presence of AF on multivariate analysis. However,

a significant correlation was seen between the MMP/TIMP system and echocardiographic measures of left-ventricular hypertrophy and ventricular remodeling, but with no relation to atrial dimension or function. Notably, an independent relationship was also shown between the MMP/TIMP system and the prothrombotic state, as assessed by prothrombin fragments 1 and 2. Similarly enhanced MMP-2 and MMP-9 are also associated with reduced PAI-1 activity, offering further links with thrombogenesis[185]. Other MMPs have also been investigated - e.g. up-regulation of myocardial MMP-9 and TIMP-3 shown in the left atrium of explanted hearts from patients with AF undergoing heart transplantation[67]. Additionally, MMP-14 concentrations in the right atrium were reduced. These results should be interpreted with caution since all patients had advanced heart failure; however, AF could be associated with chamber-specific alterations in myocardial collagen content and MMP and TIMP amounts, indicative of differential remodeling and altered collagen metabolism.

Nitric oxide

Nitric oxide is synthesized by nitric oxide synthase, which is present in large concentrations in the endothelium. The expression of nitric oxide synthase is regulated by flow-mediated shear stress and is consequently downregulated at sites with low flow velocity[186]. Nitric oxide shows potent antithrombotic effects in arterial endothelium, and nitric oxide released from activated platelets inhibits platelet recruitment to the growing thrombus[187], while also inhibiting expression of PAI-1[188].

In animal models of AF, the loss of atrial contraction and consequent reduction in shear stress seems to reduce LA expression of nitric oxide synthase with a

corresponding decrease in nitric oxide bioavailability and increase in PAI-1 expression[189]. In the LAA, nitric oxide concentrations were also significantly reduced compared with control animals, but this finding did not indicate decreased expression of nitric oxide synthase at this site. Since atrial thrombus is frequently formed in the LAA, this finding still has no adequate explanation.

Renin-angiotensin-aldosterone System (RAAS)

The RAAS is now appreciated as key to the pathophysiology of various cardiovascular disease states, related predominantly to the reduction in angiotensin-II amounts. Atrial tissue has the capacity to produce and use this hormone with local expression of acetylcholinesterase and angiotensin-II receptors, both of which could be up-regulated in AF[190]. RAAS could be mechanistically implicated in initiation and perpetuation of AF[190-192], as well as providing the link to other mechanisms promoting the prothrombotic state in AF. Angiotensin II has been shown to possess several pro-inflammatory properties and increases the production of pro-inflammatory cytokines (e.g., IL-6 and tumour necrosis factor α [TNFα]), adhesion molecules (e.g., vascular-cell adhesion molecule 1), monocyte chemoattractant protein 1, and selectins (e.g., P-selectin)[193-195]. Similarly, through release of various chemokines (e.g. cytokine-induced neutrophil chemoattractant), angiotensin II can initiate neutrophil recruitment[195]. Expression of angiotensin-II receptors has also been linked with increased atrial cell death and leucocyte infiltration[196]. These data potentially support a complex relation between RAAS, inflammation, and AF.

Additionally, RAAS has been implicated in the activation of various MMPs and thromboxane A_2 (a prothrombotic signaling molecule produced by activated

platelets). These processes could occur both as a direct effect of angiotensin II and also through induction of interleukin 6[197]. Furthermore, angiotensin II could accelerate degradation of nitric oxide through production of reactive oxygen species and thereby impair endothelium dependent vasodilatation[198]. Likewise, activation of RAAS increases synthesis of PAI-1, possibly indicating either enhanced endothelial damage or impaired fibrinolysis in AF[199]. Unsurprisingly, modulation of the RAAS cascade has beneficial clinical outcomes[191, 192].

A substudy of the Losartan Intervention For Endpoint reduction in hypertension (LIFE) trial assessed a cohort of patients with AF and ECG left-ventricular hypertrophy assigned to either losartan or atenolol[200]. The rate of cardiovascular morbidity, mortality, and stroke was significantly reduced in patients receiving losartan despite similar reductions in blood pressure between the two trial groups. Thus, RAAS modulatory drugs are often considered in patients with AF for both stroke reduction and rhythm suppression.

1.2.7 Data Appraisal

The literature investigating the hypercoagulable state in AF is vast. Whilst some studies are of reasonable power – e.g. where laboratory indices have been measured in patients recruited for large trials, many studies are small and have recruited only limited numbers of patients. AF is not a discrete entity and generally associates with one or more co-morbidites (section 1.1). In studies with low power, this has led to inevitable difficulties in establishing precisely whether the abnormalities detected are more likely a consequence of the arrhythmia or to co-morbidities and thus some links (e.g. between markers) remain speculative. This has been noted within the text where

this is of particuar concern.

Similar difficulties arise when the nature of the various laboratory indices are considered. Many molecules do not reflect a single process in isolation – e.g. inflammation is mediated through numerous interleukins and cytokines, some of which also interact with other pathways – potentially amplifying the effect. Thus alterations observed in, for example, IL-6 levels might not translate into similar alterations in hs-CRP. Many of the studies reported have only assessed a small handful of markers relevant to one particular process, rather than assessing the full complement. This is somewhat inevitable – assays are expensive and supply of patient's blood finite and therefore laboratory assessment is usually confined to markers deemed of direct relevance to the hypotheses being immediately tested.

Additionally, many small studies have relied upon multivariate analyses to test for association. However, with multiple variables and low power in some studies this form of statistical analysis needs to be interpreted with caution as only limited conclusions may be drawn. This process is further hampered in some studies, by suboptimal study design leading to later difficulty in data interpretation. For example, some studies have tried to compare healthy controls to a cohort of patients with AF and co-morbidities thus automatically limiting the feasibility of establishing whether a finding is due to the presence / absence of AF or due to the presence / absence of co-morbidities.

Finally, not all studies referenced in this section are of clear, logical design. For example, some have tried to compare healthy controls to patients with AF and co-

morbidities, whilst others have not included a chorort of controls at all. For reference, the most important studies, their design and findings have been listed in tables 7 and 8.

1.2.8 Future directions

There is increasingly strong evidence for the presence of a prothrombotic or hypercoagulable state in AF. The presence of various flow and structural defects has been used to refine clinical risk stratification models for stroke and thromboembolism, or to help predict the likelihood of success for cardioversion and the long-term maintenance of sinus rhythm[201]. However, the clinical role of indices of the prothrombotic or hypercoagulable state is emerging, although more data are clearly needed. For example, plasma vWf[125] and D-dimer[116, 202] have been used to refine clinical stroke risk stratification. The availability of these biomarkers would be of particular value in patients classed as moderate risk, in whom clinical guidelines state that the use of aspirin or warfarin is possible, but measurement of high vWf amounts, for example, could reclassify such patients as high risk[125]. Also, these biomarkers could serve as indices of ongoing thrombogenesis, to test antithrombotic regimens (e.g., warfarin plus an antiplatelet drug)[112] or new antithrombotic drugs (e.g., oral thrombin inhibitors, oral factor Xa inhibitors), and help decision making on dose selection[203]. Application of such surrogate markers to test antithrombotic regimens has been evident in studies[97, 145] which have suggested that oral anticoagulation would be better than aspirin-clopidogrel combination therapy in reducing thrombogenesis in AF, a finding later confirmed in clinical trials showing the effectiveness of warfarin for stroke prevention[35]. The potential of such an approach was recognized in recommendations from a consensus conference organized by the German Atrial

Fibrillation Competence NETwork and the European Heart rhythm Association on defining outcome parameters for trials in AF[204].

1.2.9 Summary

The mechanisms underlying the tendency towards thrombogenesis in AF are clearly complex and remain only partly understood. That this process is related purely to blood stasis is no longer accepted and various abnormal changes are observed in terms of blood flow, the endocardium/endothelium and blood constituents such that Virchow's triad for thrombogenesis is fulfilled. Many of these abnormalities are thought to relate directly to AF, but undoubtedly the various (often vascular) co-morbidities that frequently co-exist with this arrhythmia are also important. Together there would appear to be a synergistic interaction between these processes, further heightening the state of hypercoagulability. This process is all too clinically evident through the enhanced stroke event rate seen in the context of this arrhythmia and is only partially remediated by the use of potent antithrombotic agents such as warfarin.

Given these factors, a number of challenges need to be addressed by research both laboratory based and clinical. The first is to continue to develop our understanding of the mechanistic processes that result in the hypercoagulable state. In particular this requires further exploration of the interaction between the various components of the coagulation cascade, the endothelium, platelets and, of course, the end product thrombosis. In addition, further insight into the various pathways (e.g. inflammation, RAAS, growth factors etc) that may drive the hypercoagulable state would also prove invaluable.

Finally, the quest to identify those at greatest risk of stroke continues as does the struggle to develop a potent antithrombotic agent that reliably reduces stroke event rates, but without the cumbersome approach required with alternatives such as warfarin for therapeutic monitoring. Of course, such agents also need to have a favourable side effect profile and, in particular, a low incidence of bleeding.

Table 7. Coagulation abnormalities in atrial fibrillation

Author	Study design	DD	PF1+2	TF	s-thrombo-modulin	TATIII complex	vWf	Comment
Gustafsson C[205]	20 AF patients with stroke 20 AF patients without stroke 20 stroke patients with SR 40 normal controls	+					+	increased in nonvalvular AF patients with and with out stroke
Kumagai K[91]	73 AF patients 73 control subjects	+						
Asakura H[206]	83 AF patients vs. normal controls		+			+		
Sohara H[207]	13 paroxysmal AF patients vs. normal subjects	NS				NS		
Lip GY[92]	87 AF patients 158 control subjects	+					+	
Lip GY[114]	51 AF patients 26 healthy controls	+						

Table 7. Coagulation abnormalities in atrial fibrillation (continued)

Author	Study design	DD	PF1+2	TF	s-thrombo-modulin	TATIII complex	vWf	Comment
Kahn SR[89]	75 NV AF patients with or without prior embolic events 42 control patients with or without prior thrombotic stroke						+	in AF patients after stroke vWf was higher when compared to controls without stroke and similar to controls after stroke
Heppell RM[101]	109 AF patient with or without thrombus in left atrium	+				+	+	increased in patients with left atrial thrombus when compared to patients without thrombus
Shinohara H[104]	45 NV AF patients	+				+		increased in patients with low LAA velocity vs. patients high LAA velocity
Feinberg WM[149]	1531 AF patients		NS					PF1+2 was not associated with thromboembolism
Mondillo S[98]	45 AF patients 35 healthy controls	+			+		+	
Fukuchi M[119]	AF patients vs. patients without AF						+	increased in atrial appendage tissue
Conway D[123]	1321 AF patients						+	increased in high-risk group for stroke

Table 7. Coagulation abnormalities in atrial fibrillation (continued)

Author	Study design	DD	PF1+2	TF	s-thrombo-modulin	TATIII complex	vWf	Comment
Kamath S[113]	93 AF patients 50 normal subjects	+						
Vene N[115]	113 AF patients	+						DD were in AF patients having cardiovascular events vs. not having ones
Nakamura Y[121]	LAA tissue of 7 NV AF patients vs. patients without AF			+			+	
Conway DS[124]	994 AF patients						+	vWf levels were not a significant predictor of stroke and vascular events
Kamath S[129]	No abstract available	+						DD higher in permanent AF but not acute AF compared to controls.
Sakurai K[106]	28 patients with AFL 27 patients with SR	+	+			+		DD levels were higher in patients with impaired LAA function
Inoue H[95]	246 NV AF patients 111 control subjects	+	NS					DD levels were higher in NVAF patients having risk factors
Kumagai K[120]	AF patient						+	vWf mRNA and protein were increased in AF patients with enlarged atrium

Table 7. Coagulation abnormalities in atrial fibrillation (continued)

Author	Study design	DD	PF1+2	TF	s-thrombo-modulin	TATIII complex	vWf	Comment
Marin F[86]	24 acute onset AF patients 24 chronic AF patients vs. 24 IHD patients in sinus rhythm 24 healthy controls	+			+		+	
Nozawa T[110]	509 AF patients 111 healthy controls	+	NS					
Freestone B[178]	59 AF patients 40 healthy controls						+	
Nozawa T[116]	509 NVAF patients	+	NS					Predictive significance for thromboembolic events was evaluated

Table 8. Studies of platelet function in atrial fibrillation

Author	Study design	BTG	PF4	sP-sel	mP-sel	sGPV	Platelet aggregation	Comment
Yamauchi K[140]	26 V AF patients 73 NV AF patients 57 normal subjects	+	NS					
Furui H[128]	20 lone AF patients 15 normal controls	+						after treadmill exercise the increase of BTG was greater in AF than in normals
Gustafsson C[205]	20 AF patients with stroke 20 AF patients without stroke 20 stroke patients with SR 40 normal controls	+	+					increased in nonvalvular AF patients with and with out stroke
Sohara H[207]	13 paroxysmal AF patients vs. normal subjects	NS	NS					
Lip GY[114]	51 AF patients 26 healthy controls	+						
Heppell RM[101]	109 AF patient with or without thrombus in left atrium	+	+					increased in patients with left atrial thrombus when compared to patients without thrombus
Minamino T[151]	25 AF patients vs. healthy controls				+			

Table 8. Studies of platelet function in atrial fibrillation (continued)

Author	Study design	BTG	PF4	sP-sel	mP-sel	sGPV	Platelet aggregation	Comment
Shinohara H[104]	45 NV AF patients	+	+					increased in patients with low LAA velocity vs. patients high LAA velocity
Feinberg WM[149]	1531 AF patients	NS						BTG was not associated with thromboembolism
Minamino T[139]	28 AF patients	+			+			
Mondillo S[98]	45 AF patients 35 healthy controls	+	+					
Kamath S[113]	93 AF patients 50 normal subjects	+				+	NS	
Conway DS[123]	1321 AF patients			NS				was independent of risk for stroke
Conway DS[124]	994 AF patients			NS				sP-sel levels were not a significant predictor of stroke and vascular events

Table 8. Studies of platelet function in atrial fibrillation (continued)

Author	Study design	BTG	PF4	sP-sel	mP-sel	sGPV	Platelet aggregation	Comment
Atalar E[147]	15 paroxysmal atrial fibrillation 14 paroxysmal supraventricular tachycardia 25 chronic AF patients 22 healthy controls	+	+					BTG and PF4 were higher in chronic AF than in paroxysmal AF and after conversion to sinus rhythm
Nozawa T[110]	509 AF patients 111 healthy controls	+	+					
Sakurai K[106]	28 patients with AFL 27 patients with SR	+	+					BTG levels were higher in patients with impaired LAA function
Inoue H[95]	246 NV AF patients 111 control subjects	+	NS					
Nozawa T[116]	509 NVAF patients	+	+					Predictive significance for thromboembolic events was evaluated

1.3 Endothelial Progenitor Cells and Cardiovascular Disease

1.3.1 Introduction

The crucial role played by the endothelium in cardiovascular biology is becoming increasingly appreciated[208]. Indeed, endothelial injury has been implicated in atherosclerosis, thrombosis, and hypertension, and the balance between endothelial injury and endothelial recovery is of paramount importance for reducing cardiovascular events[209]. Moreover the importance of the endothelium in relation to the hypercoagulable state in AF has already been discussed in section 1.2.

Of note, mature endothelial cells possess limited regenerative capacity[210, 211]. There is therefore growing interest into circulating endothelial progenitor cells (EPCs), especially into their purported role in maintenance of endothelial integrity, function, and postnatal neovascularization[212]. Other studies are also providing intriguing and encouraging insight into the potential use of EPCs in the clinical setting. Indeed, there is accumulating evidence for reduced availability and impaired EPC function in the presence of both cardiovascular disease and associated co-morbid risk factors. This chapter aims to provide an overview of data relevant to the clinical role of EPCs and perspectives for treatment of cardiovascular disorders as a whole.

1.3.2 Definition of the Endothelial Progenitor Cell

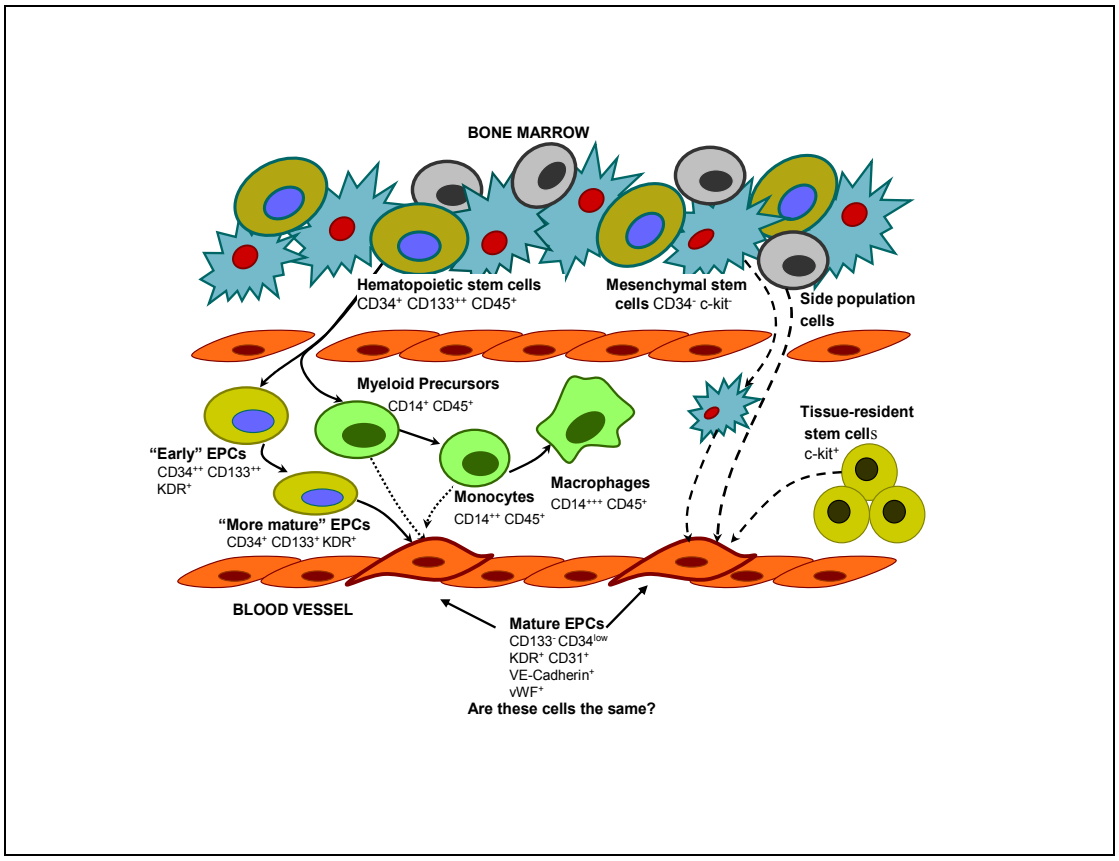
The precise definition of EPC has been through a period of flux and remains under intense debate, not least because the origin and differentiation of this cell line is complex. It is therefore important to understand some of the basic considerations that relate to this cell population.

Endothelial Markers

In 1997, Asahara et al. were the first to report isolation of endothelial precursor cells (EPCs) from peripheral blood when they found that $CD34^+$ hematopoietic progenitor cells could differentiate into cells with endothelial characteristics[213]. EPCs were defined as cells positive for both hematopoietic stem cell and endothelial cell markers, such as CD34 and vascular endothelial growth factor (VEGF) receptor-2, respectively. The latter, VEGF receptor-2, is often referred to as kinase insert domain receptor (KDR).

The putative $CD34^+$ EPC is able to proliferate and differentiate to mature endothelial cells with expression of different endothelial markers (Figure 4) such as KDR[214, 215], platelet-endothelial cell adhesion molecule (CD31)[214, 216], von Willebrand factor[214, 215, 217], VE-cadherin[214, 215], caveolin-1[216, 218], and endothelial nitric oxide synthase[216, 218]. While *in vitro*, EPCs can form vascular-like structures[215, 218], and *in vivo*, incorporate into new vessels at sites of tissue ischaemia[215, 217, 219]. Of note, CD34 antigen density is highest on early progenitors and decreases progressively as cells mature[220]; however, CD34 is expressed not only on EPCs but on mature endothelial cells, albeit at a lower density[221]. Therefore, an early hematopoietic stem cells marker, CD133, was adopted as an alternative additional marker to indicate a "true" EPC[222, 223].

Figure 4 Potential Origin and Differentiation of Endothelial Progenitor Cells



EPC - endothelial progenitor cell; KDR - kinase insert domain receptor; VE - vascular endothelial; vWf - von Willebrand factor.

The marker CD133 (also known as prominin or AC133) is a 120-kDa transmembrane polypeptide with an (as yet) unknown biological function. It is expressed on hematopoietic stem and progenitor cells from human bone marrow, fetal liver, and peripheral blood[224]. As progenitors develop to more mature endothelium-like cells, CD133 is rapidly downregulated[223]. The $CD133^+$ cells are able to form both early and late outgrowing colonies *in vitro*[223]. Thus, CD133 might provide a more reliable means of defining and tracking human angioblast-like EPCs and distinguishing these from mature endothelial or monocytic cells. The combination of CD34, CD133, and KDR is commonly used for this purpose, and this represents a rare subset of peripheral blood cells. Interestingly, the $CD34^-/133^+$ subpopulation of endothelial progenitors was recently found to be a possible precursor of classic $CD34^+/133^+$ EPCs, but to possess even more potent angioregenerative properties *in vivo* than on the latter[225].

Haematopoietic Markers

Haematopoietic cell markers such as CD34, CD133, and CD117 (c-kit) and monocytic/macrophagic cell markers such as CD11 and CD14 are expressed by early immature EPCs, and are subsequently lost in the more differentiated state[223, 224]. Paradoxically, expression of endothelial markers progressively increases with EPC maturation[215]. Subtypes of these cells that do not express VE-cadherin and von Willebrand factor seem to be an early subpopulation of EPCs localized predominantly in the bone marrow or detected immediately after their migration into the systemic circulation[226]. More mature circulating CD34-positive EPCs also present CD31, CD146, VE-cadherin, and endothelial nitric oxide synthase, as well as begin to

express von Willebrand factor[223, 224]. Thus, the surface marker profile of EPCs seems to depend predominantly on their stage of differentiation[227]. The current most detailed phenotypic description of circulating EPCs proposes the co-expression of several common hematopoietic and endothelial antigens: CD34, CD133, CD31, CD38, CD45, KDR, VE-cadherin, c-kit, and Flt-1[228].

Cultivation of peripheral blood mononuclear cells (MNCs) in medium favoring endothelial growth is another approach widely used for definition and quantitative analysis of EPCs. Adherent cells grow colonies and have been shown to possess endothelial characteristics, such as expression of von Willebrand factor and staining for Dil-acetylated low-density lipoprotein and Fluorescein isothiocyanate (FITC)-conjugated BS-lectin[229-232]. Despite the relatively low numbers of $CD34^+$ of circulating endothelial precursors in peripheral blood (100 to 500/ml), relatively large numbers of adherent cells are found during culture (approximately 100,000 from 1 mL blood). This raises some controversy with respect to the identification and the origin of isolated EPCs, as these cells seem to reflect a functional subpopulation within the blood MNCs that have the potential to differentiate into an endothelial phenotype *in vivo*[232-236].

There are at least 2 morphologically and functionally distinct endothelial cell populations can be grown from circulating MNCs[214]. The early spindle-like outgrowth cells possess a relatively low proliferative capacity and low ability to express mature endothelial proteins[215]. These cells presumably represent cells of different lineage, which include a subset of $CD14^+/CD34^-$ monocytic cells, which have the potential to

differentiate (transdifferentiate) into endothelial-like cells under certain environmental condition in the presence of special growth factors (e.g., VEGF, fibroblast growth factor, and so on)[235]. Late "outgrowth cells" show a high proliferative potential and originate predominantly from bone marrow donors and are considered as circulating angioblasts[214].

It is important to appreciate that although monocyte-derived EPCs have a lower *in vitro* proliferation potential than hematopoietic stem cells or cord-blood-derived EPCs[237], the different progenitor types seem to have a similar ability to enhance neovascularization in experimental models[215, 238, 239]. One may speculate that proliferation capacity is not the decisive factor and that the reduced proliferation of the monocyte-derived EPCs is likely to be attributable to increased release of growth factors, which may act in a paracrine manner to support angiogenesis and arteriogenesis[240].

The expression of common markers by hematopoietic and endothelial progenitor cells in embryonic development and transdifferentiation potential of monocytes into cells with endothelial characteristics would suggest a possible common origin from a bone marrow precursor, perhaps a putative hemangioblast[241]. Bone marrow also contains mesenchymal cells, which have been shown to differentiate into endothelial cells[242], improve vascularization *in vivo*[243, 244], and contribute to tissue repair[245]. Likewise, other bone-marrow-derived EPCs mesenchymal stem cells release a variety of angiogenic growth factors[246]. In addition to bone-marrow-derived cells, other cell populations, such as fat tissue[247, 248], cardiac tissue[249], neural stem cells[250], and fetal

liver cells[251], can give rise to endothelial cells, suggesting that tissue-resident stem/progenitor cells can contribute to vascular growth in the adult.

In summary, a universal single or complex EPC marker still remains to be identified, showing the heterogeneous nature of endothelial precursors. As a result, various different surface markers and cell culture properties have been used by research workers to define EPCs. This has obvious implications when interpreting data as there are marked inconsistencies in cellular definition and in assessment techniques. Thus it is not possible to completely compare and contrast studies in a direct manner and it is necessary to compile a descriptive analysis of the literature. Further discussion regarding the definition of the putative EPC and implications for the studies to be conducted within this thesis is made in section 3.1.

1.3.3 Physiological Considerations

Because of the rarity of EPCs and the difficulties in identification, limited information is available about the normal range and functional characteristics of different types of EPCs in humans.

Ageing associated decline in EPC number/function

The available data suggest that age may affect the availability and function of EPCs and this is a broadly consistent finding across the literature regardless of antigenic EPC definition[252-254]. Ageing is associated with a reduced number of circulating EPCs in patients with coronary artery disease (CAD). For example, Vasa *et al*. reported age-associated depression in circulating CD34/kinase insert domain receptor (KDR)-

positive cells in a mixed group of healthy probands and CAD patients[231]. Scheubel *et al.* have reported an age-dependent loss of circulating EPCs in stable CAD[252]. Moreover, the number of EPCs mobilized after coronary artery bypass grafting was significantly decreased in older patients in this study. The ageing-associated impairment of cardiac angiogenic capacity in older mice, estimated as neovascularisation of cardiac allografts, can be restored by implantation of bone marrow-derived EPCs from young adult animals[253]. Progression of atherosclerosis in apolipoprotein E -/- mice with persistent hypercholesterolemia seems delayed by chronic administration of bone marrow-derived progenitor cells from young mice[254]. This treatment was much less effective when donors were older animals with atherosclerosis, indicating that progressive age-dependent reduction in EPCs may accelerate the development of atherosclerosis, particularly in the presence of risk factors (e.g., hypercholesterolemia)[254].

Multiple factors are thought to be involved in the ageing associated deterioration of EPC quantity and function with a number of mechanisms proposed (table 9). Chronic exposure to risk factors continuously damages endothelial cells and requires their intensive replacement. Conversely, risk factors may possibly affect EPC mobilization, integration in injured vascular sites, and angiogenic capacity. EPC dysfunction may also be result of their accelerated senescence and apoptosis, as well as exhaustion of the pool of progenitor cells available in the bone marrow[232, 255, 256]. Reduced levels of angiogenic and mobilizing cytokines have also been related to age-dependent impairment of EPC mobilization *in vivo*. Both vascular endothelial growth factor (VEGF) and nitric oxide (NO) production have been reported to decrease with age[252, 256-260], and these factors are thought to play synergistic roles in the mobilization,

migration, proliferation, and survival of endothelial cells[256, 259]. The alteration of constitutive human telomerase reverse transcriptase activity can also affect the regenerative capacity of EPCs[261]. Thus, the impaired mobility of EPCs may be a consequence of inadequate stimuli normally involved in their physiology.

Physical Training and Exercise

It is well known that physical training improves endothelial function, exercise tolerance, and collateralization in patients with CAD, chronic heart failure[262, 263], and peripheral artery disease[264, 265]. Exercise upregulates circulating EPCs in patients with CAD[266], and increases the number of EPCs in bone marrow, peripheral blood, and the spleen (at least in mice)[267]. The upregulation of EPCs by exercise may be dependent on endothelial NO and VEGF levels or a decreased rate of EPC apoptosis[266]. In a recent clinical study, physical exertion in patients with peripheral arterial occlusive disease resulted in a 5.2-fold increase in EPCs and improvement of their function. However, subischaemic exercise training in revascularized patients did not affect EPC number, although in vitro vascular tube formation was enhanced[268]. These data imply that a positive impact of regular physical training on cardiovascular performance may be attributable at least partly to the improved behavior of EPCs[268]. Many of these studies are small in a highly selected group of patients, using a variety of methods and definitions for EPC assessment leading to inconsistencies within the data. Further exploration of the role of exercise of EPC release is discussed in section 3.2.

1.3.4 Pathophysiological Considerations

An increasing body of evidence suggests that cardiovascular risk factors affect the number and properties of EPCs. An inverse correlation is found between the number (and functional activity) of EPCs and cardiovascular risk factors among apparently healthy people and in patients with CAD[231, 232]. The number of EPCs correlates with endothelial function and is a better predictor for this than the patient's combined Framingham risk factor score[232].

Lipids

Multiple studies have consistently reported an association between lipid metabolism and the biology of human EPCs. The numbers of EPC colony forming units (EC-CFUs) are significantly reduced in relatively healthy subjects with elevated serum cholesterol levels[232]. In CAD, low-density lipoprotein (LDL) cholesterol inversely correlates with the number of circulating EPCs[231]. In addition, the functional characteristics of isolated EPCs, such as proliferation, migration, adhesion, and *in vitro* vasculogenic capacity, are also impaired in patients with hypercholesterolemia[231, 269]. Exposure of cultured EPC to oxidized LDL induces a dose-dependent impairment of their functional activity, accelerates the rate of EPC senescence, possibly by telomerase inactivation, and can be associated with up to a 70% reduction in EPC numbers[270, 271]. In addition, oxidized LDL impairs VEGF-induced EPC differentiation via the deactivation of Akt[272]. Plasma levels of high-density lipoprotein cholesterol and triglycerides positively correlate with the number of EPC-CFUs, but not with the number of $CD34^+/CD133^+$ progenitor cells[273]. Although this may of course represent fundamental differences between EPC assessment by culture and flow cytometry.

Hypertension.

Among various risk factors, hypertension is the strongest and most consistent predictor of EPC migratory impairment[231]. Angiotensin-II diminishes telomerase activity in EPCs and accelerates the onset of EPC senescence through an increase in oxidative stress. Some controversy exists about the effects of angiotensin-II on *in vitro* EPC proliferation. Although angiotensin-II inhibited EPC proliferation in one study, it enhanced VEGF-induced EPC proliferation in another[274, 275]. Angiotensin II also potentiates VEGF-induced network formation by EPCs, probably by up-regulation of KDR[275]. Both these studies originate from the same research group investigating the effect of various hormones on EPCs cultured *in vitro* in a limited number of samples. Consequently, results may not be directly applicable to clinical practice and may feasibly be influenced by the use of exogenous hormones at non-physiological levels.

Diabetes mellitus.

Diabetes mellitus, another important cardiovascular risk factor, is a disease in which impairment of ischaemia-induced neo-vascularization has been described[276, 277]. The number of EPCs is reduced in both type 1 and type 2 diabetes[278, 279]. Furthermore, marked EPC dysfunction may underlie new mechanisms involved in the pathogenesis of vascular complications in diabetic patients. Indeed, EPC proliferation, adhesion, and angiogenic properties are impaired in this setting[278-280]. EPCs can facilitate angiogenesis in a paracrine fashion by secretion of angiogenic factors to mobilize bone-marrow progenitors and to activate mature endothelial cells[281, 282]. Of note, the media from EPC culture of type 1 diabetic patients not only possesses evidence of reduced angiogenic capacity, but also contains an inhibitor for *in vitro* tube

formation[278]. Interestingly, diabetes was not associated with enhanced apoptosis in this study. Similarly, Tepper *et al.* showed impaired ability of mature endothelial cells to incorporate into tubules in type 2 diabetes[279]. In both studies, decreased number and dysfunction of EPCs was inversely related to the levels of haemoglobin A_1c, implying that the degree of glycaemic dysregulation was associated with EPC pathophysiology. These findings are important and may aid our understanding of why patients with poor glycaemic control have worse outcome.

Further evidence of the negative impact of hyperglycemia on EPCs was provided by Kränkel *et al.*, who showed that cultivation of peripheral blood mononuclear cells (MNCs) from healthy donors under hyperglycemic conditions was associated with significant reduction in EPC numbers, inhibition of NO production, and matrix metalloproteinase-9 activity, as well as an impairment of the migrational and integrative capacities of the cells[283].

Smoking

Smoking is a significant predictor of reduced circulating and cultured endothelial progenitors[231]. The number of circulating EPCs correlates inversely with the number of cigarettes consumed in one study[284]. However, naturally, this is likely to be a chance finding as it is clearly difficult to prove such an association when numerous other risk factors are unlikely to have been fully accounted for. However, EPCs from heavy smokers appear to die prematurely during the early phase of culture[284]. Similarly, smoking cessation is associated with an increase of EPC numbers, and these changes are most marked in those who smoked the least. However, if smoking is resumed, EPC numbers rapidly decrease to levels seen before smoking cessation[284].

Of note, nicotine effects on the activity and function of EPCs also seems to be dose dependent. Lower doses of nicotine have a positive influence on EPC numbers, proliferation, migration, and *in vitro* vasculogenesis with the peak effect at concentrations of nicotine 10 8 mol/l, similar to that found in the blood of smokers[285]. However, cytotoxicity was observed at higher nicotine concentrations[285]. Homocysteine, which is another common cardiovascular risk factor, was shown to decrease numbers and impair activity of EPCs from human peripheral blood[286]. Asymmetric dimethylarginine (ADMA), an endogenous NO synthase inhibitor, contributes to endothelial dysfunction and inhibition of angiogenesis and is an independent biomarker of future major adverse cardiovascular events or death[287]. Of note, circulating ADMA levels inversely correlate with the number of progenitor cells, and ADMA inhibits EPC function, at least *in vitro*[288].

1.3.5 EPCs and established cardiovascular disease

Abnormalities in quantity and function of EPCs have been shown in a number of studies of various cardiovascular disorders (table 10).

Stable Coronary Artery Disease

Despite numbers of circulating $CD34^+$ / $CD45^+$ and $CD133^+$ / $CD34^+$ progenitor cells and EPCs in patients with severe chronic CAD being similar to those in control subjects, *in vitro* functional capacity of bone marrow MNCs is significantly reduced and transplantation of bone-marrow MNCs from patients with CAD into ischaemic nude-mice high limb showed a markedly impaired ability to restore tissue perfusion[289, 290].

Unstable Coronary Artery Disease

In patients with unstable angina, an increase in numbers of EPC-CFUs, but no change in adhesive properties, has been shown; however, the number of EPCs were reduced by almost 50%, after clinical stabilization[291]. Correlations were also noted between systemic C-reactive protein (CRP) levels and circulating EPC numbers, but not with their adhesive capacity, implying that systemic inflammation may play a role in the mobilization of EPCs in patients with unstable angina[291]. On the contrary, CRP was found to inhibit EPC proliferation, survival, differentiation, and function, suggesting a possible role in the development of cardiovascular disease[292]. These apparently conflicting findings may relate to the differing contexts in which these parameters were assessed.

In myocardial infarction, the number of circulating EPCs is markedly increased from the early phase of the disease to peak levels on day 7[289, 229]. Subsequently, EPC numbers reduce and become similar to levels seen in control subjects within 60 days[229]. Of note, plasma levels of VEGF (a growth factor associated with angiogenesis) are closely related to circulating EPC numbers, and levels also peak on day 7 [229]. These data show the important role for VEGF in EPC mobilization in acute coronary syndromes. However, given that most patients with myocardial infarction are treated with EPC mobilizing drugs, such as statins or angiotensin-converting enzyme inhibitors, the primary driving factor for peripheral EPC elevation in myocardial infarction is uncertain. In a rat model, the number and function of EPCs were depressed after myocardial infarction in those given placebo, whereas treatment with either an angiotensin-converting enzyme inhibitor or a statin was associated with significant stimulation of the amount and activity of the EPCs[293]. Moreover,

mesenchymal stem cells, which also possess the potential to differentiate to endothelial cells, are decreased on day 7 after acute ST segment elevation myocardial infarction[294].

The functional role of the bone marrow cells in myocardial infarction may be attributable not only to their angiogenic properties and release of growth factors and cytokines, but also to their ability to restore the population of cardiac progenitor cells by selective homing to specific areas of myocardial injury and conversion to the phenotype of cardiac side-population cells[295]. Bone marrow-derived hematopoietic cells may generate cardiomyocytes (albeit at a very low frequency) within the infarcted myocardium in some animal models[296], although others fail to show trans-differentiation of hematopoietic stem cells into cardiac myocytes after myocardial infarction[297]. Such data has not been reproducible in human studies.

Heart Failure

The numbers of EPCs are elevated in patients with acute heart failure, which significantly correlates with levels of the cytokine tumor necrosis factor alpha (TNF-α)[298]. Differences in the quantity of EPCs can be related to the stage of heart failure, with relatively higher numbers in the early stage of heart failure (New York Heart Association, NYHA, functional class I and II), with levels progressively decreasing with NYHA functional class III and IV heart failure[298]. Higher levels of brain natriuretic peptide are associated with depression of circulating EPCs with no effect of medical therapy or etiology of heart failure[298].

Other Disease States

Reduced numbers of EPCs are found in patients with erectile dysfunction[299], those with instent restenosis[300], and in cardiac transplantation patients with vasculopathy[301]. The number of EPCs does not seem to be associated with the degree of cerebrovascular atherosclerosis *per se*[302]. However, EPC levels are significantly decreased in patients after stroke[303] and in those with atherosclerotic patients (including ones without clinical stroke) in whom areas of cerebral infarction as determined by positron emission tomography were found[302]. In the latter study, EPC numbers also correlated with regional blood flow in areas of chronic hypoperfusion of the brain[302]. Each of these studies has assessed limited numbers of patients in a somewhat unstructured manner and thus interpretation should be made with caution.

1.3.6 Effects of Drug Therapies

Drug therapies may influence EPC physiology, as summarized in table 11. These changes need to be placed in context to explain the possible therapeutic benefit(s) of these drugs, and to justify the effects on clinical outcomes, good or bad.

3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors.

Many primary and secondary prevention trials have suggested that statins possess favorable (pleiotropic) effects, which include the improvement of endothelial function and an anti-thrombotic effect, independent of their impact on cholesterol reduction[304, 305]. Along with direct effects on endothelial cells, stimulation of EPC activity may be an additional mechanism of the beneficial influence of statins on endothelial performance. Indeed, different statins have been shown to enhance the proliferative capacity of EPCs *in vitro*[255, 306, 307]. Moreover, the effect of statins seems to be

comparable with VEGF[306], which is known to augment the number of EPCs[213, 308].

Statins stimulate EPC proliferation through the cell cycle regulatory genes[255]. Additionally, statins induce EPC differentiation via the PI 3-kinase/Akt pathway[306, 307], as well as enhance adhesiveness by increased integrin expression[309], and improve migratory activity by upregulation of the telomere repeat-binding factor TRF2 in EPCs[230, 310]. As previously mentioned, the pleiotropic effects of statins on EPC activity are independent of their impact on reduction in LDL cholesterol, as shown by comparison of simvastatin and ezetimibe[311]. Finally, atorvastatin or mevastatin dose dependently inhibit the onset of EPC senescence in culture[255]. Thus, one may potentially consider the use statins to augment the functional potential of EPCs for transplantation therapy.

The renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system [RAAS] is an important pathophysiological mechanism related to many cardiovascular disorders, and may also be involved in EPC (dys)function[274, 275]. Indeed, treatment with the angiotensin II receptor antagonists, olmesartan or irbesartan, has been shown to significantly increase the number of EPCs[312]. Also, valsartan was reported to reduce angiotensin II accelerated senescence of EPCs via upregulation of telomerase activity[275]. The administration of ramipril, an angiotensin-converting enzyme inhibitor, increases the number and improves the functional capacity of EPCs in patients with CAD, independent of any impact on blood pressure[313]. Importantly, RAAS may be implicated in the thormbogenic state in AF (section 1.2.6)

Oestrogens

No direct studies of effect of oestrogen therapy on EPCs in humans are available, but increased blood oestrogen levels in women do correlate with numbers of circulating EPCs[314]. In an animal carotid injury model, oestradiol treatment showed stimulatory effects on EPC mobilization, proliferation, mitogenic activity, and migration activity, as well as inhibited EPC apoptosis[315]. These findings require further investigation with larger structured studies.

Miscellaneous drugs.

Enhancement of EPC activity has been shown with treatment with vardenafil (a phosphodiesterase inhibitor)[299], puerarin[316], and *Ginkgo biloba* extract[317]. In contrast, rapamycin inhibits proliferation and differentiation of human EPCs *in vitro*[318]. The administration of rosiglitazone, a peroxisome proliferator activated receptor gamma agonist, in patients with type 2 diabetes not only increases the number and migratory activity of cultured EPCs[280], but also can attenuate the detrimental effects of C-reactive protein on endothelial progenitors[292]. Finally a correlation between erythropoietin levels and EPC numbers, as well as functional activity, has been reported[319, 320]. The administration of erythropoietin also increases the number of functionally active EPCs in patients with renal anemia, as well as in healthy subjects[321]. Of course, each of these effects may simply be a consequence of altering underlying pathophysiology – e.g. by optimizing glycaemic control.

1.3.7 EPC Transplantation

Many pre-clinical studies have shown therapeutic efficacy of EPCs in ischaemic disorders and vascular injury in animal models[239, 322, 323]. Several small-scale phase 1 trials of bone marrow MNC transplantation in treatment of myocardial infarction, peripheral limb ischaemia, severe stable CAD, and heart failure providing preliminary evidence of feasibility and safety of EPC transplantation have been performed. However, many clinical studies have yielded disappointing results despite such early theoretical promise. A Full discussion of this topic is out of the scope of this thesis.

1.3.8 Summary

The endothelium would appear to be of central importance in various purported mechanisms leading to cardiovascular events. Endothelial injury evidenced by a wide variety of investigative techniques is documented in numerous disease states and vascular processes, including but not limited to hypertension, diabetes mellitus, atherosclerosis, thrombosis, coronary artery disease and stroke. Notably endothelial damage is clearly evident in patients with AF (section 1.2) and may provide a key link between this arrhythmia and the hypercoagulable state. Moreover, various processes which may provide a central drive to the hypercoagulable state (e.g. RAAS, inflammation) may also link directly to EPC release / activity.

Of further importance, EPCs may provide a novel (albeit thus far untapped) therapeutic avenue and thus it is vital that research be continued into this exciting cell line.

Table 9 Cardiovascular Risk Factors and Endothelial Progenitor Cells

Study	Risk factor	Patients	Effects on EPC number	Effect on function
Vasa M[231]	LDL hypertension smoking	CAD	↓$CD34^+/KDR^+$ cells, NE CFU NE ↓circulating $CD34^+/KDR^+$ cells, ↓in culture	↓migration ↓migration NE on migration
Hill JM[232]	total cholesterol, LDL	Healthy	↓CFU	ND
Chen JZ[269]	total cholesterol	CAD	↓in culture	↓proliferation, migration, adhesion, in vitro vasculogenic capacity
Pellegatta F[273]	HDL, triglycerides	Healthy	↓CFU	ND
Loomans CJM[278]	Diabetes	Type I diabetes mellitus	↓in culture	↓ in vitro vasculogenic capacity
Tepper OM[279]	Diabetes	Type II diabetes mellitus	↓in culture	↓ in vitro vasculogenic capacity
Pistrosch F[280]	Diabetes	Type II diabetes mellitus	NE	↓ adhesion
Kondo T[284]	Smoking	Healthy	↓circulating $CD45low/CD34^+/CD133^+/KDR^+$	ND

CFU – colony-forming units of EPCs; NE – no effect; ND – no data

Table 10 Cardiovascular Disorders and Endothelial Progenitor Cells

Study	Patients	Effects on EPC number	Effect on function
Heeschen C[290]	Stable CAD	↓CFU	↓migration, in vivo vasculogenic capacity
George J[291]	Unstable angina	↑CFU	NE adhesion
Massa M[289]	Myocardial infarction Stable CAD	↑ $CD34^+/KDR^+$ cells NE	ND
Shintani S[229]	Myocardial infarction	↑circulating $CD34^+$ cells, ↑CFU	ND
Valgimigli M[298]	Heart failure	CFU and $CD34^+/CD133^+/KDR^+$ cells ↑ in NYHA class I, ↓ in NYHA class III-IV	ND
Foresta C[299]	Erectile dysfunction	↓circulating EPCs	ND
George J[300]	Diffuse in-stent restenosis	↓CFU	ND
Simper D[301]	Transplant arteriopathy	↓CFU	ND
Taguchi A[302]	Cerebrovascular atherosclerosis	$CD34^+/CD133^+$ cells ↓ in cerebral infarction No correlation with the degree of atherosclerosis	ND
Ghani U[303]	Stroke	↓CFU	ND

CFU – colony-forming units of EPCs, ND – no data

Table 11 Drug Therapies and Endothelial Progenitor Cells

Study	Risk factor	Patients	Effects on EPC number	Effect on EPC function
Llevadot J[307]	ND	simvastatin	↑proliferation	↑ migration
Dimmeler S[306]	healthy	simvastatin, mevastatin, atorvastatin	↑proliferation	ND
Vasa M[231]	CAD	atorvastatin	↑EPC number	↑migration
Assmus B[255]	healthy	atorvastatin mevastatin	↑proliferation NE	↓senescence ↓senescence
Walter DH[309]	ND	simvastatin	ND	↑adhesion
Spyridopoulos I[310]	healthy	atorvastatin, mevastatin	ND	↑migration
Landmesser U[311]	heart failure	atorvastatin ezetimibe	↑in culture NE	ND
Bahlmann FH[312]	Diabetes melitus	olmesartan, irbesartan	↑in culture	ND
Imanishi T[270]	healthy	valsartan	ND	↓senescence
Min TQ[313]	CAD	ramipril	↑in culture	↑proliferation, migration, adhesion, in vitro vasculogenic capacity
Bahlmann FH[321]	renal anaemia, healthy	erythropoietin	↑circulating CD34+/CD45+ cells, ↑in culture	↑ in vitro vasculogenic capacity

Table 11 Drug Therapies and Endothelial Progenitor Cells (continued)

Study	Risk factor	Patients	Effects on EPC number	Effect on EPC function
Pistrosch F[280]	diabetes	rosiglitazone	↑in culture	↑ migration
Foresta C[299]	healthy	vardenafil	↑circulating EPCs	ND
Zhu JH[316]	ND	puerarin	↑in culture	↑migration, adhesion, in vitro vasculogenic capacity
Chen J[317]	ND	Ginkgo biloba	↑in culture	↑ migration, adhesion, in vitro vasculogenic capacity
Butzal M[318]	ND	rapamycin	↓in culture	↓differentiation, adhesion, ↑apoptosis

CFU – colony-forming units of EPCs, NE- no effect, ND – no data

Section II: Study Rationale, Hypotheses and Study Design

2.1 Study Rationale and Hypotheses

2.1.1 Introduction

The importance of AF as a clinical entity has been discussed in detail in the preceding section. It is clear that this arrhythmia, which previously was once assumed to be relatively benign, does, in fact, have an important impact on healthcare.

Although AF may be directly implicated to and may exacerbate a number of symptoms, the most important consideration is the high risk of stroke and thromboembolism. As discussed earlier, it seems increasingly clear that AF may in fact drive or co-exist with a hypercoagulable state, thereby fulfilling Virchow's triad for thrombogenesis with endothelial damage / dysfunction being a central theme or hallmark of this process. Whether this relates to the arrhythmia *per se* or to associated co-morbidities remains unclear. Markers and models of endothelial function continue to evolve and many have been tested in AF with various abnormalities reported. EPCs, however, hold potential to reflect two co-existing processes – not only may these cells reflect underlying vascular health, but in addition, but they may alter in number/function depending upon the extent of associated endothelial damage. Thus it is planned to investigate the role of EPCs, inflammation and apoptosis in AF.

2.1.2 Aim

To investigate the inter-relationships between EPCs in AF and various pathophysiological mechanisms (endothelial perturbation, inflammation, apoptosis and angiogenesis) potentially leading to thrombogenesis in this arrhythmia.

2.1.3 Original Hypotheses

1. Abnormalities of endothelial activation/dysfunction and EPCs (quantity, functionality) are present in permanent AF compared to controls (both 'disease matched controls' and 'healthy controls', in sinus rhythm)
2. EPCs correlate with plasma markers of endothelial perturbation (E-selectin, vWf), angiogenesis (VEGF), apoptosis (Fas/Fas ligand) and inflammation (IL6) in AF.
3. Similar abnormalities are present in lone AF patients, compared to matched 'healthy controls' in sinus rhythm.
4. The numbers of circulating EPCs may predict the outcome following cardioversion of AF.
5. That in paroxysmal AF, levels of surrogate markers for the hypercoagulable state (hs-CRP, E-selectin, vWf) are enhanced with a greater AF arrhythmia burden.

2.2 Study Design

2.2.1 Cross-sectional Study

50 patients with permanent AF were studied, and compared to 50 'disease controls' in sinus rhythm [matched for associated cardiovascular disease and risk factors (age/sex, prior vascular disease, hypertension, diabetes, etc)] and 50 age/sex matched healthy controls in sinus rhythm.

In addition, 30 subjects with 'lone AF' (normotensive patients with no risk factors for AF and normal chest X-ray and echocardiogram) were studied, and compared to 30 age-matched healthy controls in sinus rhythm.

Exclusion criteria were haemodynamically significant valvular disease, or other conditions which may adversely affect the endothelium, such as severe uncontrolled hypertension, carcinomatosis, recent (i.e. <3 months) stroke, myocardial infarction or surgery.

2.2.2 Effects of Cardioversion:

20 patients with newly diagnosed non-rheumatic AF lasting > 4 weeks of either sex, aged 18-85 years old planned for elective cardioversion were recruited. Blood sampling was performed at baseline, immediately after cardioversion and at 4 weeks after successful cardioversion.

Exclusion criteria were as per study (a), plus any contraindication to cardioversion (e.g. known permanent AF)

2.2.3 AF Arrhythmia Burden Study

Consecutive patients (n=100) aged 18-85 with permanent pacemakers incorporating advanced AF detection algorithms (Vitatron 'T70' or Selection 9000) were recruited from routine pacemaker follow-up clinics. These devices are able to accurately record episodes of AF and allow calculation of an exact atrial fibrillation arrhythmia burden (AFB), without the need for an extended period of ambulatory monitoring. Suitable patients were identified as those who had previous documented evidence of paroxysmal AF with at least two episodes within the last three months. Anti-arrhythmic therapy had been stable in all patients for at least five half-lives prior to study entry.

Exclusion criteria included any condition that may adversely affect the endothelium (e.g. recent (<3 months) stroke, thromboembolism, myocardial infarction, uncontrolled hypertension etc.) or activate or suppress the inflammatory cascade (e.g. concurrent infection, vasculitis, malignancy, corticosteroid usage etc.). Similarly patients with significant structural cardiac abnormalities (e.g. significant valvular disease, hypertrophic cardiomyopathy or left ventricular ejection fraction < 40%) were also excluded.

2.2.4 Validation Studies

The two commonest techniques used for assessing EPCs are flow cytometry and cell culture. Each technique has its relative merits, although the primary advantage of the latter is the ability to assess cellular function. There is no international consensus on either the best method for assessing EPCs, or their precise antigenic definition.

Various technical considerations in identifying rare cells and their heterogenous nature (with flux in their antigenic profile with maturation) are also important considerations.

The initial work for this thesis will therefore involve a comparison of laboratory assays for EPC enumeration by cell culture and flow cytometry. Concurrently, various physiological properties of EPCs will be assessed, including temporal decline once samples are held *ex vivo*, diurnal variation and response to short burst exercise.

2.2.5 Ethical Approval and Consent

The Sandwell and West Birmingham research Ethics Committee evaluated this study and offered a favourable opinion. Consent for the study was sought from the Sandwell and West Birmingham Hospitals NHS Trust Research and Development Department. Written informed consent was obtained from all patients who enrolled in the study.

2.3 Power Calculation, Data Analysis and Statistical Methods

2.3.1 Power Calculation.

In the cross-sectional study, the number of patients to be recruited was calculated from the hypothesized difference in EPCs/plasma markers between the groups (e.g. 0.25 of a standard deviation in a normally-distributed index such as vWf, or a 25% difference in a non-normally distributed index such as EPCs). Anticipated levels for EPCs was taken from a pilot feasibility study run by our sister laboratory in Hong Kong. They found the following using a similar flow cytometer protocol to ours: AF – mean 150 (90-331) cells/mL; coronary artery disease – mean 90 (60-125) cells/mL; healthy control subjects – mean 424 (185-673) cells/mL[324]. Also previous work from our laboratory investigating vWf had required a sample size of between 20-40 patients, with a lower sample size still when circulating endothelial cells (CECs – an equally rare cell) were investigated. We therefore calculated that 40 patients per group would be required – although planned to recruit 50.

Similarly in the longitudinal study, previous work within the department had shown a significant fall in both vWf and CECs at 4-weeks following cardioversion in a cohort of 20 patients[325]. Based on these parameters, we calculated that a similar number of patients would be required. Although it was acknowledged that new guidelines aimed at stratifying who underwent cardioversion may have some impact on numbers we would be able to recruit (section 1.1.6). Power criteria are a minimum of 0.05 for alpha error and 0.8 for 1-beta error.

In the AF burden study, the number of patients to be recruited was calculated from the hypothesized difference in plasma markers between groups (i.e. 0.25 of a standard deviation in a normally distributed index (vWf) and a 25% difference in a non-normally distributed index (IL-6). Data were taken from previously published work comparing sinus rhythm (with co-morbidities) *vs.* permanent AF[326]. Power criteria were a minimum of 0.05 for alpha error and 0.8 for 1-beta error. Based on these parameters we hypothesized that 100 patients (25 per group) would be required.

2.3.2 Data Analysis

General

Following a test of statistical normality (Kolmogorov-Smirnov test), data were expressed as mean with standard deviation (SD) or as median with inter-quartile range (IQR) for the normally distributed data and non-parametrically distributed data respectively. Comparisons between groups were analysed by 2-way repeated ANOVA or Kruskal–Wallis test, as appropriate. Repeated measures were tested using Friedman's method. For paired data, the Wilcoxon matched-pairs signed-ranks or paired t-test were used. A P value <0.05 was considered as statistically significant.

Correlations and Multivariable Regression

Correlations between indices were performed by Spearman's or Pearson's method as appropriate. Additionally, a step-wise multi-variate analysis will be performed to assess for potential inter-relationships. Naturally, with large numbers of statistical comparisons, the probability of chance findings becomes important. To minimize this, for these analyses a P value <0.01 will be considered statistically significant.

Inter / intra-assay coefficient of variation

The inter-assay and intra-assay co-efficients of variability are calculated for all ELISA laboratory indices are generally <5% and <10% respectively. Data are collected on a regular basis and analyzed in-house by the laboratory head to ensure each assays remains robust. For the flow cytometric analysis, the inter-assay and intra-assay co-efficients of variability were both <10%[327].

All analyses were performed using SPSS version 17.0 for Windows©. Graphpad Prism version 5.00 for Windows© was used for graphical illustrations..

2.4 Methodology

2.4.1 General

In all participants, a detailed history, physical examination, resting 12 lead electrocardiogram (ECG) and surface echocardiogram was obtained.

2.4.2 Venesection

A 20ml blood sample was drawn from each participant. Blood samples were collected from a single puncture to a large antecubital vein using a 21-gauge needle directly into Vacutainer® tubes (Becton Dickinson, UK) containing 7.2mg tri-potassium (K_3) Ethylene Diamine Triacetic Acid (EDTA), 0.5mL 3.2% sodium citrate or Z serum clot activator. The Vacutainer® system ensures that moderate standardized negative pressure was used for each sample, thereby preventing venous collapse and thus minimizing endothelial activation / damage due to trauma. In those patients where serial sampling was required, care was taken not to re-enter the previous point of venepuncture.

For enzyme-linked immunosorbent assay (ELISA) blood samples were centrifuged within 30 minutes from collection at 1,500g for 20 minutes at 4°C. The resultant supernatant (plasma/serum) was then collected and stored at -70°C until later batch processing. For endothelial cell measurements, samples were processed as below.

2.4.3 Endothelial Progenitor Cell Quantification

Flow Cytemetry

For flow cytometry, 1 ml of freshly drawn K_3 EDTA anticoagulated venous blood was used for enumerating EPCs by flow cytometry. Red blood cells were lysed with 10 mL of BD lysing solution® (Becton Dickinson, UK). The sample was gently inverted continually for 10 minutes followed centrifugation at 700g for 5 minutes. The resultant cell pellet was then washed with 10mL of buffer solution [phosphate buffered saline (PBS), 5% bovine serum albumin BSA)] and then centrifuged again at 700g for 5 minutes. This washing step was repeated twice. The resulting pellet was re-suspended and the white cells blocked with 20µL of the Fc-receptor blocking antibody Octagam® (Octapharma, Lachen, Switzerland) and 200µL 10% mouse blocking serum (Sigma-Aldrich, Gillingham, UK) for a minimum of 20 minutes at room temperature. Next, the sample was incubated with 10µL each of CD133-PE (PhycoErythrin), CD45-FITC (Fluorescein IsoThioCyanate) and CD34-PECy5 (PhycoErythrin Cy5) fluorochrome-labelled monoclonal antibodies for 20 minutes in the dark at room temperature. The sample was then washed with buffer solution and centrifuged as before. The resulting cell pellet was re-suspended and fixed in 200µL of 2% paraformaldehyde solution for 20 minutes at 4°C and then made up to a final volume of 1mL with buffer solution, ready for immediate flow cytometric analysis.

Each sample was analysed using a 3-colour FACScan™ flow cytometer (Becton Dickinson, Oxford, UK) for a minimum of 500,000 mononuclear cellular events and a pre-determined time period, corresponding to a known volume of sample. The latter was determined from previous validation studies (data not shown). Cells were plotted according to forward scatter (FSC) and side scatter (SSC) profiles (each a measure of

size and granularity respectively) and gated to include only mono- and polymorpho-nuclear events, thereby excluding cell doublets, platelets and cellular debris. EPCs were enumerated as a count of $CD34^+$, $CD133^+$, $CD45^-$ events per mL of blood.

The gating strategy used was based upon a modified version of the ISHAGE protocol for haematopoietic progenitor / stem cell enumeration which is well established and has international consensus. In the initial plot all events are plotted according to CD45-FITC vs. SSC. The primary acquisition gate (R, region 1) is centred on all leukocytes, thereby excluding debris and other cell lines. Events from R1 are then sorted as CD34-PE Cy5 vs. SSC. The secondary acquisition gate (R2) is set around the $CD34^+$ population of cells with a low SSC. The third plot is of cells gated through R1 and then R2, with R3 selecting those cells which are $CD45^-$ and $CD133^+$ (CD133-PE). The final gate (R4) displays cells from additive regions 1-3 with boundaries set by identifying the lymphocyte population. This method was designed to ensure that only cells with a FSC compatible with lymphocytes were included in the final analysis thereby providing a further point at which to exclude debris, dead cells and platelets etc. (figure 5). Fluorochrome-matched isotype controls (FITC-IgG_1, PE-IgG_1, Pe-CY5-IgG_1, BD, Abingdon, UK) and unstained samples were used to set various gate parameters and also served as negative controls. A simplified summary of this protocol is illustrated in Figure 6.

Figure 5. Flow Cytometry Gating Strategy for Endothelial Progenitor Cell Capture

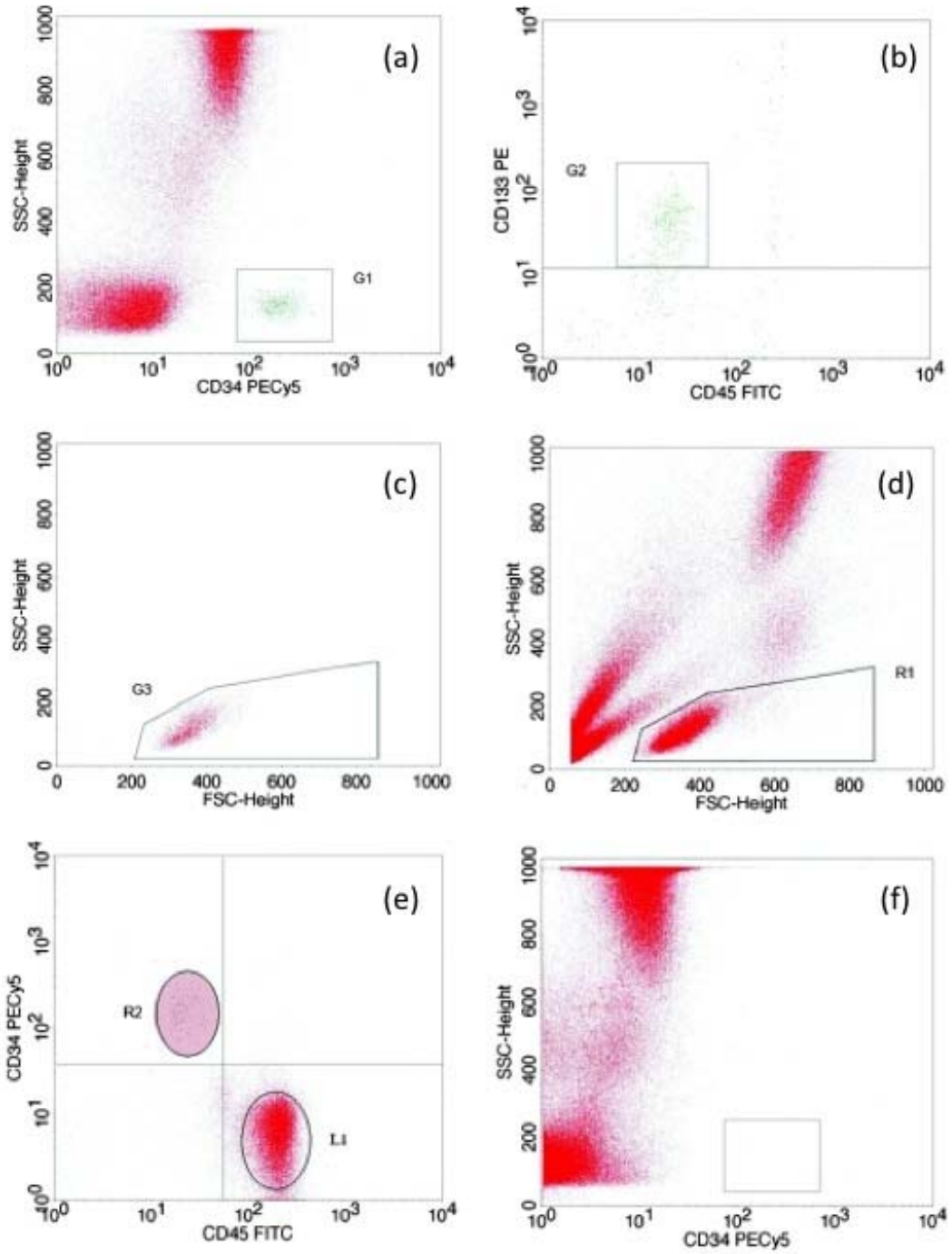
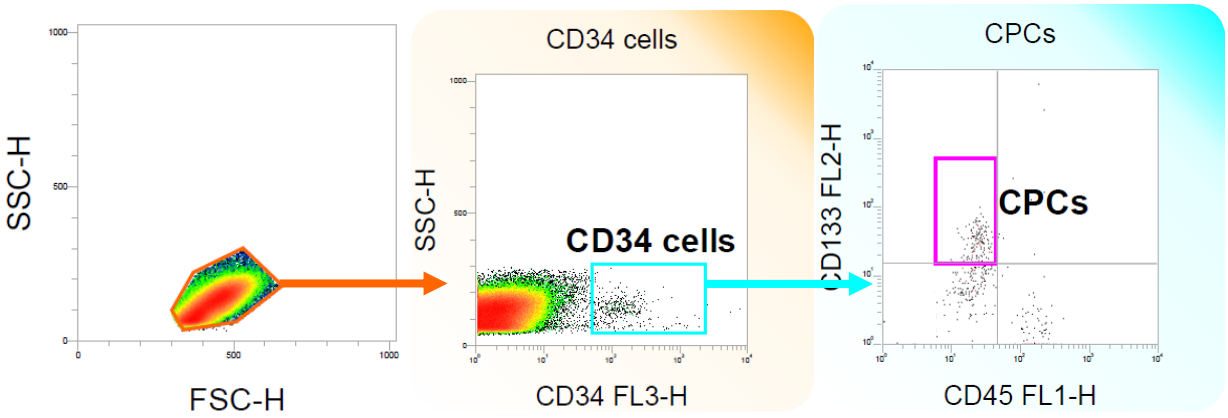


Image courtesy of Mr PKY Goon.

Figure 6. Simplified Flow Cytometry Gating Strategy for Endothelial Progenitor Cell Capture.



Cell Culture

12 ml of freshly drawn K_3 EDTA anticoagulated venous blood was transferred directly to the laboratory for processing. All work was performed in a 'clean environment' using appropriate sterile techniques and a negative pressure extraction hood designed specifically for cell culture.

In brief, Mononuclear cells (MNCs) were isolated by density gradient centrifugation. 4 mL of peripheral blood was diluted with an equal volume of Hank's balanced salt solution (Sigma-Aldrich, Gillingham, UK). This was then carefully layered on to 3 mL Ficoll® (GE-Healthcare, Little Chalfont, UK) in a 15 mL centrifuge tube, ensuring that the two solutions did not mix. The tube was centrifuged at 400 g for 40 minutes at 18°C with no brake. The resultant buffy cell layer was removed and washed twice with 6 mL Hank's balanced salt solution, followed by centrifugation at 100 g for 10 minutes at 18°C to remove excess Ficoll®, serum and platelets. The resultant cell fraction was then re-suspended in 1 mL endothelium specific culture medium and, following an initial cell count, was plated on 24-well culture plates precoated with human fibronectin (Sigma-Aldrich). This process was repeated three times for each patient.

The endothelium-specific culture medium used was Medium M199 (Gibco (Invitrogen), Paisley, UK) supplemented with 5 mg/100 mL endothelial growth supplement (Upstate, Buckinghamshire, UK), 15% foetal bovine serum (Sigma-Aldrich) and penicillin/streptomycin (Sigma-Aldrich, Gillingham, UK). At 48 hours media were changed.

After 6 days in culture, media and non-adherent cells were removed and adherent cells underwent cytochemical analysis. Cells were consequently incubated with DiLDL (Invitrogen, Paisley, UK) (2.4 mg/mL) for 2 hours and with FITC-labelled lectin Ulex europaeus (Sigma-Aldrich) (10 mg/mL) for 1 hour and then fixed with 2% paraformaldehyde for 20 minutes. Dual-staining cells positive for both lectin and DiLDL were considered as EPCs. Additionally, expression of vascular endothelial (VE) cadherin was measured. Adherent cells were detached from the culture plate using 1 mmol/L EDTA diluted in phosphate buffered saline. Following a wash, cells were then incubated first with 10 μL Octagam® (Octapharma) and 200 μL 10% mouse serum (Sigma-Aldrich), and then in the dark for 20 minutes with 10 μL phycoerythrin (PE)-conjugated VE cadherin (Becton-Dickinson, Oxford, UK). Following a cell wash to remove excess antibody, the sample was immediately run on a Becton Dickinson (BD) FACScan flow cytometer.

2.4.4 Enzyme-linked Immunosorbent Assays (ELISA)

The ELISA assays all used commercially available antibodies and each has been fully validated in-house. ELISAs to be used in this thesis include: vWf, VEGF, Soluble Fas, Soluble Fas ligand, Soluble E-selectin and IL-6.

In addition, hs-CRP was measured for the AFB study using an automated analyser from Roche Diagnostics. Brief standard operating procedures for each ELISA are included within the appendix for reference.

Section III: Laboratory Studies

3.1 Investigating the biology of Progenitor Cells

3.1.1 Introduction

EPCs make up only about 0.0001% of circulating mononuclear cells[328]. Their rarity in peripheral bloods presents a challenge to researchers, many of whom prefer a pre-enrichment phase or their reproduction through cell culture techniques prior to enumeration. In addition, the definition of EPCs relies upon a precise phenotypic definition. This antigen profile however varies widely across the literature. This in part relates to similarities between EPCs and other progenitor cell populations (e.g. haematopoietic stem cells, HSCs) which may also demonstrate similar functional characteristics or even cross-differentiate. Profiling is further hampered by a process of antigenic shift during the process of maturation (section 1.3.2). However, given the exciting potential for EPCs (particularly in view of their as yet unrealized therapeutic potential), it is vital that these cells are defined accurately and that our understanding of basic biological functions be enhanced.

3.1.2 Antibody Selection

EPCs are believed to predominantly originate from haematopoietic stem cells in bone marrow and, indeed, recent evidence suggests that the haemangioblast is a common progenitor for both haematopoietic and endothelial cells[329]. However, the definition of EPCs continues to evolve and has shifted significantly following their initial characterization by Asahara *et al*. in 1997[213]. EPCs were first identified and enumerated by their expression of both haematopoietic stem cell and endothelial cell markers, such as CD34, CD133 and vascular endothelial growth factor receptor-2

(KDR)[213]. Later expression of other endothelial markers (e.g. vascular endothelial (VE) cadherin, von Willebrand factor (vWf), CD146) in the process of maturation of endothelial progenitors was demonstrated[214]. However, given the heterogeneity of the EPC population, a robust definition still does not yet exist[225]. The situation is further complicated as expression of cell surface markers changes with EPC maturation[221-224]. There is also some evidence that cells of other lineages can cross-differentiate in to EPCs[234, 242, 247]. These markers are easily detected using flow cytometry, which essentially estimates the number of cells from peripheral blood, bone marrow, or other tissues that possess a specific cell type profile of surface markers. The expression of common markers by haematopoietic and endothelial progenitor cells in embryonic development and the trans-differentiation potential of monocytes into cells with endothelial characteristics would suggest a possible common origin from a bone marrow precursor, perhaps a putative haemangioblast[329]. Bone marrow also contains mesenchymal cells, which can differentiate into endothelial cells[242] and improve vascularization *in vivo*[243, 244], as well as release a variety of angiogenic growth factors[246] and contribute to tissue repair[245]. In addition to bone marrow-derived cells, other cell populations, such as fat tissue[247, 248], cardiac tissue[249], and neural stem cells[250] can also give rise to endothelial cells, suggesting that tissue-resident stem/progenitor cells can contribute to vascular growth in the adult. Table 12 summarizes the various phenotypic profiles currently employed for EPC enumeration.

Given the lack of universal agreement on the definition of true EPCs and the difficulty in reliably identifying a subpopulation with ongoing antigenic shift, it was decided that the term circulating progenitor cell (CPC) would best reflect these characteristics.

It was considered important that the antibody panel in flow cytometry reflect potential of CPCs for angiogenesis. Some reports indicate that most EPCs are a very early subset of progenitor cells and could therefore be defined as $CD45^{dim}/CD133^{+}/CD34^{+}$ CPCs[244, 284, 330]. Other investigators have shown that $CD45^{dim}/CD34^{+}$ progenitor cells evolve into mature endothelial cells with a mature endothelial cell phenotype ($CD45^{-}/CD31^{+}/CD105^{+}/KDR^{+}/CD34^{+}$)[331, 332]. Additionally, most EPCs are $CD34^{+}$ [333], and nearly all $CD133^{+}$ cells are also $CD34^{+}$ [224]. The CD133 antigen appears to be shed during maturation thereby reliably identifying the earliest progenitors [334].

3.1.3 Conclusion

On the basis of this data, we opted to investigate CPCs as an early precursor of the EPC using the definition: $CD133^{+}/CD34^{+}/CD45^{dim}$. Although the addition of a fourth marker (e.g. KDR/VEGF-R2) may be considered preferable, this could still not be viewed as definitive definition for a progenitor committed to endothelial differentiation as this too is non-specific. Additionally, further complexity of the antibody profiling was not possible given that the BD FACScan flow cytomter allows for only 3 colour channels.

Table 12 Flux of antigenic markers used to distinguish endothelial progenitor cells with maturation.

	HSCs	CPCs (early)	CPCs (late)
CD133	++	-	-
CD117	++	-	-
Sca-1	++	+	+
CD34	++	+	+
CXCR-4	+	+	+
VEGFR-2/KDR	+	+	+
CD31	+	+	+
VE-cadherin	-	+	+
vWf	-	+	+
E-selectin	-	+	+
ENOS	-	+	+
DiLDL	+	+	+
Lectin	+	+	+
CD105	-	+	+
CD45	-	+	-
CD14	-	+	-

3.2 Assessment of Cell Culture

Abstract

Background Flow cytometry and cell culture, the two main laboratory techniques employed for counting Circulating Progenitor Cells (CPCs), both have serious limitations. Mononuclear cells cultured in media favouring endothelial growth allow cells to replicate and differentiate/mature. CPCs under these circumstances tend to form groups of cells called endothelial colony forming units (EC-CFUs). EC-CFUs are widely accepted as a surrogate estimate of CPC number and function. However, some important limitations may restrict the assumption that EC-CFUs reflect CPC numbers accurately. Similarly, flow cytometry allows for a CPC count, using a panel predetermined markers. However, assessment of CPCs by this method alone litis complete assessment of CPCs by an inability to gauge their ability for replication and differentiation. The purpose of this study was to compare and contrast flow cytometry and cell culture for CPC enumeration and report our early findings.

Methods 60 patients (62% male, mean age 67.5 ± 6.8 years) were recruited, 42% of whom had coronary artery disease were recruited. 20mL of blood was taken through a large antecubital vein and samples processed immediately in the laboratory. In brief, the mononuclear cell layer was isolated using density centrifugation. Cells were resuspended in a medium designed to enhance growth of endothelial progenitors (Medium M199 (Gibco), enhanced with Endothlial Growth Supplement (Upstate)) and then plated on to cell culture plates precoated in human fibronectin. Non-adherent cells were removed at 24 hours and media changed every 48 hours. At Day 5, EC-CFUs were counted per plate. CPCs were confirmed by dual staining for Dil-

acetylated low-density lipoprotein and fluorescein isothiocyanate-labelled lectin from Bandeiraea simplicifolia. Adherent cells were then detached from the cell culture plate using EDTA and stainined with VE-cadherin conjugated to FITC for 20 minutes in the dark. Following a washing stage to remove excess fluorochrome, samples underwent immediate flow cytometric analysis to assess the overall cell count.

Results Across the entire cohort recruited, there was a median of 14 (12-16) EC-CFUs per plate. In contrast, flow cytometry of the same samples returned a median of 285 (201-416) $CD144^+$ detached cells per mL. Spearman's correlation method was applied and did not demonstrate any correlation between EC-CFU per plate and $CD144^+$ CPCs ($r=0.011$, $P=0.931$). Additionally, we observed that the size and number of EC-CFUs appeared to greatly fluctuate - even on the same cell culture plate - varying from only a few individual cells to large colonies. We also noted that, thin, spindle shaped endothelial-like cells are often located separately from EC-CFUs within the same plate.

Conclusions EC-CFU counts represent the cumulative assessment of CPC quantity and their functional characteristics, including rate of differentiation, proliferation and senescence, and migrational activity. However, in the present analysis we observed large discrepancies between EC-CFU counts between samples on the same patient and a clear lack of correlation between EC-CFUs and cell counts from flow cytomtery. We therefore suggest that EC-CFU counts may not be reliably used for the estimation of CPC numbers in peripheral blood and until stronger definitions of CPCs emerge, flow cytometry may be the more optimal assessment modality, although this does not allow assessment of CPC functionality.

3.2.1 Introduction

Flow cytometry and cell culture are the two main laboratory techniques employed for counting and functional assessment of CPCs (section 2.4.3). Both of these techniques, however, have serious limitations, often overlooked by the scientific community. This chapter explores the potential pitfalls encountered in counting CPCs following cell culture.

Cultivation of peripheral blood mononuclear cells (MNCs) in media favouring endothelial growth is a widely used approach for the definition and quantitative analysis of CPCs [232, 234, 281]. Adherent cells have been shown to possess endothelial characteristics, such as the expression of vWf and staining for Dil-acetylated low-density lipoprotein (DiLDL) and fluorescein isothiocyanate-labelled lectin from Bandeiraea simplicifolia (FITC-BS-lectin) [229-232]. Despite the relatively low numbers of $CD34^+$ of circulating endothelial precursors in peripheral blood (100–500 per mL), relatively large numbers of adherent cells are found during culture (<100,000 from 1 mL of blood). This raises some controversy with respect to the identification and the origin of isolated CPCs, as these cells appear to reflect a functional subpopulation within the blood MNC pool that have the potential to differentiate into an endothelial phenotype *in vivo* [222, 232, 233, 235, 236, 281]. Indeed, early endothelial-like cells are often derived from peripheral blood monocytes[281]. Nonetheless, a clear advantage then of the cell culture approach is that it permits quantification of all cells capable differentiating into endothelial cells. Differentiated endothelial cells tend to form groups of cells, which are called endothelial cell colony forming units (EC-CFUs). As the number of single endothelial cells in culture is too large to count under light or fluorescent microscopy, the number of EC-CFUs is a widely accepted surrogate

estimate for the quantity of CPCs in cell culture. However, do EC-CFUs really reflect the number of CPCs following cell culture? The purpose of this work was to compare and contrast flow cytometry and cell culture for CPC enumeration and report our early experiences.

3.2.2 Methods

We investigated whether EC-CFUs were truly reflective of the number of CPCs following cell culture in patients with different temporal patterns of AF, recruited consecutively from the AF clinic. As the main focus of the present study was to compare and contrast methods for CPC enumeration, AF represented an ideal disease group choice due to the frequent association of this arrhythmia with various cardiovascular co-morbidities.

Cell Culture and Flow Cytometric Analysis of Circulating Progenitor Cells

The technique for cell culture and flow cytometric analysis has been discussed in depth previously (section 2.4.3). For each patient, samples were plated on at least two separate culture plates and the mean EC-CFU count taken.

Flow Cytometry

Following cell culture and EC-CFU counts on day 5, adherent cells were detached from the cell culture plate as per section 2.4.3 and stainined with VE-cadherin. Following this, samples underwent immediate flow cytometric analysis to assess the overall cell count.

3.2.3 Results

60 patients (62% male, mean age 67.5 ± 6.8 years) were recruited, 42% of whom had coronary artery disease (table 13). Across the entire cohort recruited, there was a median of 14 (12-16) EC-CFUs per plate. In contrast, flow cytometry of the same samples returned a median of 286 (201-416) $CD144^{+}$ detached cells per mL (table 14).

Data for EC-CFUs and CPCs were both non-parametric in distribution (kolmogorov-smirnov). Spearman's correlation method was applied to mean EC-CFU per plate and mean $CD144^{+}$ CPCs ($r=0.011$, $P=0.931$), whilst logarithmic correlation is illustrated in figure 7.

Additionally, we observed that the size and number of EC-CFUs appeared to greatly fluctuate - even on the same cell culture plate - varying from only a few cells (Figure 8(a)) to large colonies (Figures 8(b) and 8(c)). We also noted that, thin, spindle shaped endothelial-like cells are often located separately from EC-CFUs within the same plate (Figure 8(d)).

Table 13 Baseline Demographics of cell culture patients

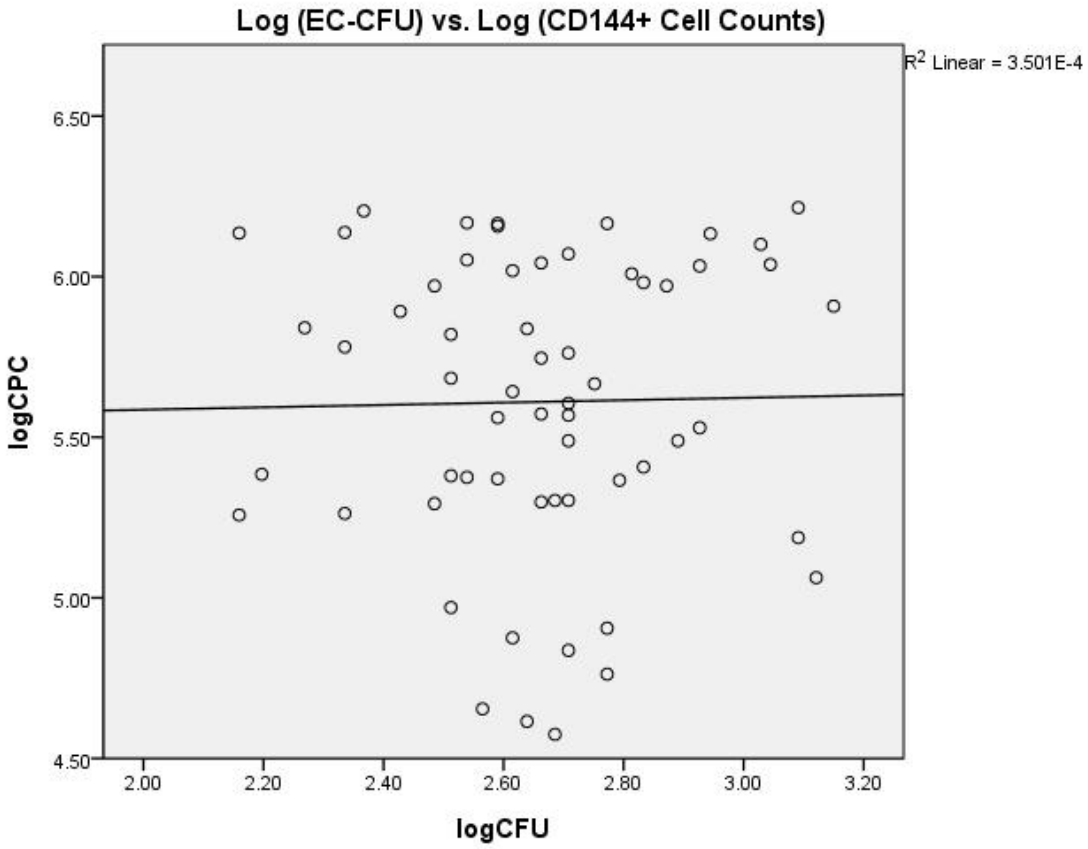
Characteristics	
n	60
Age	67.5 ± 9.8
Male	37 (62%)
Diabetes	6 (10%)
Hypertension	51 (85%)
Hyperlipidaemia	33 (55%)
Known IHD	25 (42%)
Previous stroke	3 (5%)
Smoker	34 (57%)
Aspirin	21 (38%)
Warfarin	29 (48%)

Table 14. Comparison of median cell counts during cell culture and subsequent flow cytometry.

	Number per sample		Correlation	
n	60		*r*	0.011
EC-CFUs	14 (12.4 – 16)			
CPCs	286 (201-416)		P	0.931

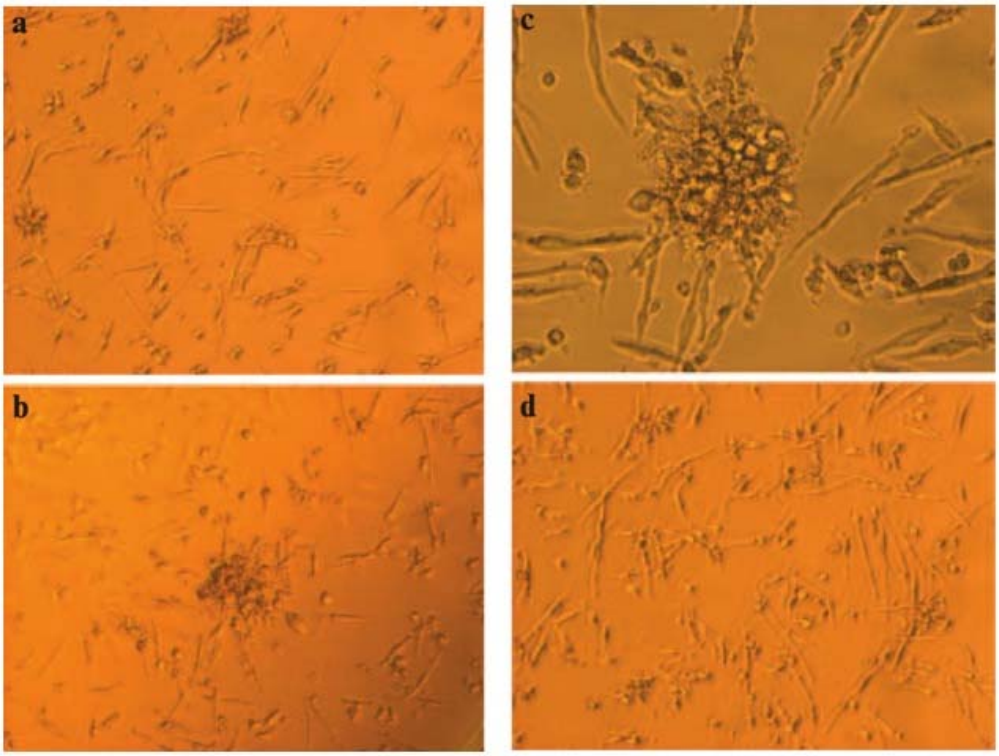
EC-CFU – Endothelial Cell Colony Forming Units; CPC – Circulating Progenitor Cells. Data refers to median (InterQuartile Range). Correlation Co-efficient by Spearman's method.

Figure 7 Correlation of cell counts by cell culture and flow cytometry.



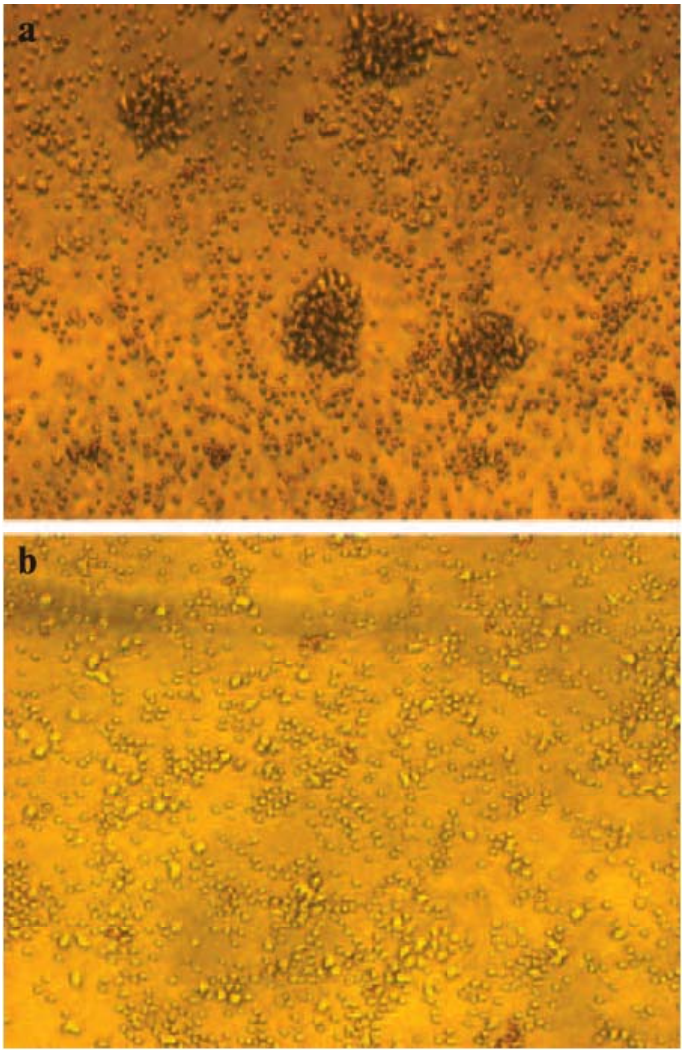
Scatterplot of mean EC-CFU per plate vs. mean $CD144^{+}$ CPCs following logarithmic transformation. Correlation by Spearman's method, r=0.011, P=0.931.

Figure 8 Endothelial Progenitor cells and endothelial colony forming units under light microscopy.



Note that the size of endothelial cell colony forming units (EC-CFUs) varies widely. (a): small CFUs consisting of a few cells (40x); (b) and (c): bigger CFUs (40x, 100x); (d): predominantly single located endothelial cells without accumulation in EC-CFUs (40x).

Figure 9 Progenitor cell migration and colony forming units (EC-CFUs).



(a): Migration of EPCs to each other before forming CFUs (40x); (b). b: This process may be impaired in some patients (40x).

3.2.4 Discussion

Large EC-CFUs are often demonstrated on research publications, and sometimes represent very large cell accumulations. Indeed, variations in the size of EC-CFUs amongst different samples are common, and the migratory capacity of CPCs can be changed in different pathological states[231, 269, 290]. The ability of CPCs to migrate to each other to form a colony may significantly affect the number of EC-CFUs formed in culture experiments, despite a similar number of CPCs. The importance of culture cell migration for colony forming can be proven visibly by the poor EC-CFU forming capacity of MNCs before their differentiation into endothelial cells (Figure 9) and the fact that EC-CFUs can include different proportions of total CPCs (Figure 8). The subjectivity regarding which clusters should be considered as EC-CFUs may make this approach less reliable. Furthermore, recent studies have demonstrated that EC-CFUs have limited differentiation capacity into endothelial cells and may retain some myeloid progenitor activity[335].

Most researchers perform CPC counts (based on numbers of EC-CFUs) relative to the day of initiation of cell culture (generally between day 4 and day 7). The rate of CPC differentiation depends on the functional condition of CPCs, culture media used, and endothelial growth supplements. Simultaneous with CPC differentiation, the process of cell proliferation (another functional characteristic of CPCs) begins. Thus, the number of EC-CFUs depends not only on CPC counts and migratory activity, but also on the rate of CPC proliferation and differentiation. Amongst adherent MNCs, there may also be some mature circulating endothelial cells (CECs), and thus some researchers prefer to perform pre-plating of MNCs to reduce contamination with CECs, which also express endothelial markers, including CD34 [225]. How effective this

approach is remains unclear, but the loss of some CPCs during the pre-plating stage may significantly affect total PC counts.

There are alternatives to EC-CFU counts. A count of the total number of cultured endothelial cells (single cells plus cells inside the EC-CFU) may be more accurate in terms of CPC quantification, but numbers of single CPCs in cell culture are too great for an accurate manual count under light microscope. As an alternative, the count of differentiated cultured CPCs by flow cytometry can be used, with specific mature endothelial markers (e.g. CD146, CD144 (VE cadherin) or vWf). This approach has been tested in our department (with VE cadherin as a marker) and permits count of both single CPCs and those CPCs grouped in EC-CFUs together. CD146 is expressed on non-differentiated endothelial progenitors, although in small amounts[336]. The rate of vWf expression is slower, and its use can thus underestimate the true CPC count. Thus, VE cadherin may be the optimal marker, from those currently available for use in this setting. Indeed, VE cadherin is an adhesion molecule that plays a fundamental role in microvascular permeability and in the morphogenic/proliferative events associated with angiogenesis, suggesting its importance for endothelial integrity. VE cadherin is exclusively expressed by endothelial cells in adults [337-339]. In addition, embryo yolk sac cells with haemoangiogenic potential have been shown to lose VE cadherin expression when differentiating to haematopoietic progenitor cells[340]. This analytical approach permits the exclusion of the effect of migratory activity on CPC counts, but cannot exclude the effect of CPC proliferation and CEC numbers.

It is impossible to fully eliminate the contamination of peripheral blood MNCs with CECs, but we can estimate the proportion of CECs by flow cytometric count of cells

with pure markers of mature endothelial cells (e.g. CD146 or VE cadherin) after the first media change (in 24–48 hours), when non-adherent MNCs are removed and CPCs have still not differentiated into mature endothelial cells. At this stage, CD146 or VE cadherin^{+} cells will reflect CEC contamination. Any increase in VE cadherin^{+} or CD146^{+} cells will mainly represent differentiated ECs, as CECs proliferation seems to be relatively low or rare. As there is no marker which can reliably distinguish mature CPCs from their generic endothelial cells as a result of CPC proliferation, it seems reasonable to standardize the day of the PC count (e.g. days 4–7) and use media/supplement to make the impact of CPC proliferation less significant.

It can be argued that one of the major characteristics of CPCs is their ability to proliferate, differentiate and integrate within the endothelium. These characteristics however, cannot be assessed by flow cytometry alone and require supplemental data – usually from cell culture experiments. The apparent lack of agreement observed within the present analysis is clearly of concern if the two techniques are to be used in parallel. Other researchers have also reported their concern regarding discrepancies observed between cell culture and cell counts returned following flow cytometry, using similar techniques to those employed here[341]. However, there is again inconsistency and other groups have suggested a strong correlation between the two techniques, albeit using alternative cell marker profiles[342]. Such discrepancies may have numerous explanations. This includes variable techniques for culture and inconsistencies within the antigenic profile used for flow cytometry. The latter is unlikely to be resolved until a universal definition for CPCs becomes established

It is therefore feasible that cells undergoing proliferation on the cell culture plate are predominantly of a different subset compared to those cells that are being enumerated

by flow cytometry.

Sample contamination with yeasts and bacteria can also inhibit cellular proliferation – thus necessitating meticulous attention to sterility and the addition of anti-fungal and bactericidal to cell culture media. Nonetheless, despite these steps the release of various substances by bacteria into the cell culture media, does offer the potential to imply variable inhibition of proliferation of CPCs.

One further methodoligcal consideration that merits discussion is the effect of cell loss by sequential handling. This is important, as each time a sample is handled, transferred to an alternative transport tube or washed, there will be loss of a number of cells of interest. This can be due to failure to transfer the entire sample – cells may adhere to the walls of the transport tube, or preparation tubes during centrifugation. Similarly during media changes and washing steps, partially adherent cells may be lost. Alternatively cells may be destroyed following detachment with EDTA and subsequent transfer from the culture plates, although this is likely to be particularly evident with flow cytometry samples as these have undergone additional processing steps after the initial cell count for EC-CFUs, thereby introducing further potential for inconsistencies.

Finally, one pragmatic solution to these methodolgical limitations would be to first count and sort CPCs using flow cytometric methods and then take the same cells and culture these in a standard way. However, it is likely that the processing involved in preparation of a sample for flow analysis may well alter the physical properties of the cells or their ability to differentiate – particularly if chemical such as

paraformaldehyde are used to 'fix' cells as in our protocol (section 2.4.3). More recently, cells have been partially pre-selected and then cultured (with some success) using magnetic sorting techniques that entail the use of an antibody conjugated to magnetic beads. The advent of further technological advances are awaited.

3.2.5 **Conclusion**

EC-CFU counts represent the cumulative assessment of CPC quantity and their functional characteristics, including rate of differentiation, proliferation and senescence, and migrational activity. However, in the present analysis we observed large discrepancies between EC-CFU counts between samples on the same patient and a clear lack of correlation between EC-CFUs and cell counts from flow cytomtery. We therefore suggest that EC-CFU counts cannot be reliably used in the setting of the present study for the estimation of CPC numbers in peripheral blood. Therefore until stronger definition(s) of bone marrow or peripheral blood population(s) of CPCs are developed, flow cytometry may be the more optimal technique for CPC quantification, despite a clear inability to assess for functional capacity of these cells. Given these findings, it was decided to base CPC enumeration for the remainder of this thesis on assessment by flow cytometry rather than use cell culture.

3.3 Physiology of Circulating Progenitor Cells

Abstract

Background Circulating progenitor cells (CPCs) are thought to help to maintain endothelial homeostasis. However, our knowledge of CPC physiology remains immature. This study aimed to define the influence of exercise treadmill testing (ETT) on CPC levels; to assess the diurnal variation of CPCs; and to investigate the rate of temporal decline in CPCs once *ex-vivo*.

Methods The dynamics of CPC count changes following an ETT were assessed on 20 patients (70% male, age 69.9±7.8 years) with suspected angina pectoris. Venous blood samples were taken pre-exercise, immediately post-exercise and at 30 minutes post-exercise. Diurnal variation in CPCs was assessed in 13 stable in-hospital patients (46% male, age 69.1±7.5 years). Blood samples were taken 5 times – once every 6 hours for a complete 24-hour cycle. To investigate the temporal decline, blood samples from 12 patients with chronic atrial fibrillation (58.3% male, age 69.9±7.9 years) were reprocessed for CPC counts at 4 and 24 hours after sample collection. Plasma levels of von Willebrand factor (vWf), soluble E-selectin (sE-selectin) were assessed by ELISA. CPCs were enumerated by flow cytometry as $CD34^{+}$, $CD133^{+}$, $CD45^{dim}$ events.

Results Exercise led to significant increases in systolic blood pressure, heart rate, vWF and sE-selectin levels, but no significant influence on CPC counts were observed. Baseline CPC numbers demonstrated a negative correlation with vWf (r=-0.551, p=0.012). Baseline CPC numbers also correlated significantly with their levels

immediately post, and 30 minutes post exercise (r=0.968 and r=0.955 respectively, both P<0.001). Similar observations applied to vWf (r=0.720, P<0.001 and r=0.471, P=0.036 respectively) and sEselectin (r=0.772 and r=0.874 respectively, both P<0.001). An *exploratory* subgroup analysis assessed patients with a negative ETT (n=9) *vs*. those unable complete the protocol (positive or inconclusive ETT, n=11). The cohort with a positive ETT had higher median CPC counts across all three timepoints, although the ΔCPC (from rest to peak exercise) were similar. None of these differences reached statistical significance. There were no major differences in sE-selectin or Vwf levels across the three timepoints, although patients with a positive test had borderline significantly lower vWf levels immediately after the test.

Median CPC counts showed significant diurnal variation (P=0.011), being significantly higher at 12am compared to 12pm (150 [90-237] *vs*. 142 [38-167]; p=0.046) and 12pm compared to 6pm (150 [90-237] *vs*. 96 [79-188]; p=0.023). Delayed sample preparation by 4 hours did not significantly affect the number of CPCs detected, compared to baseline, but there was a significant decline in CPC recovery observed when sample processing was delayed by 24 hours (P=0.019).

Conclusions Routine exercise stress testing does not appear to significantly affect CPC counts and usual physical activity prior blood sample collection is unlikely to affect their levels. Peripheral CPC levels showed a degree of diurnal variation and delays in sample preparation for CPC counts should be avoided as they may influence the accuracy of the test by resulting in a significant decline in CPC recovery. However data needs to be considered in light of low sample numbers and broad data spread, hence larger studies are required for confirmation of these findings.

3.3.1 Introduction

As with CECs, CPCs would appear to reflect vascular health with numerous studies demonstrating fewer numbers in patients with multiple cardiovascular risk factors or overt cardiovascular disease[231, 232]. Conversely, during periods of tissue ischaemia, CPC numbers generally seem to increase – presumably to effect repair to the vascular endothelium [343, 344]. Natural physiological processes also seem to have an impact on CPCs, for example, ageing has been shown to reduce both CPC numbers and function[252, 254]. Conversely during physical exercise, circulating CPC numbers rise – both in healthy patients and those with cardiovascular disease[266, 345]. The explanations for these and many other observations are varied and may relate to aberrations in CPC production, release, destruction or endothelial integration[232, 255, 344]. In addition, the role of various chemokines including endothelial nitric oxide or Vascular Endothelial Growth Factor [VEGF] may be important.

Physical training has consistently been shown to improve endothelial function, exercise tolerance and collateralization of blood supply to patients with coronary artery disease, heart failure and peripheral vascular disease[263, 265, 346]. Similarly, during exercise, circulating CPC numbers also rise – both in healthy patients or subjects with cardiovascular risk factors or overt cardiovascular disease and it has been speculated that this effect may relate to enhanced release of endothelial nitric oxide or VEGF or even due to reduced EPC apoptosis[347-349]. This effect may also be mediated through enhanced endothelial sheer stress or due to enhanced stimulation of growth factors or inflammatory mediators.

It is vital that CPCs are accurately defined in terms of origin, basic biological function and kinetics if we are to appreciate their full potential. In this study, we examined the influence of exercise treadmill testing (an established screening tool to identify patients likely to have flow-limiting coronary artery disease) on peripheral CPC numbers. In addition, we assess the diurnal variation of peripheral CPC counts in patients with risk factors for cardiovascular disease and investigate the rate of temporal decline in CPCs once *ex-vivo.* We hypothesized that exercise might provoke a rise in CPC counts. Secondly, we hypothesized significant diurnal aberrations in CPC counts throughout the 24-hour period studied. Thirdly, we hypothesized that there would be a steady temporal decline in CPC counts once the cells were held/stored *ex vivo*.

3.3.2 Methods

Effects of physical exercise on CPC count

The dynamics of CPC counts following an exercise treadmill test (ETT) were assessed on 20 consecutive patients referred to our 'rapid-access' chest pain clinic with suspected angina pectoris, who satisfied study criteria. The ETT was performed according to the Bruce protocol (an established screening tool used to identify patients likely to have flow-limiting coronary artery disease) as previously described[350, 351]. Real-time 12-lead and computer-averaged Electrocardiogram [ECG] recordings were taken during and after exercise testing. Blood pressure was measured immediately pre-procedure and at 3-minute intervals thereafter until complete recovery following the ETT. The ETT was considered positive in patients with exercise-related ECG changes diagnostic of ischaemia (>1 mm horizontal or down-sloping ST-segment depression or elevation for >60–80 ms after the end of the QRS, significant

arrhythmia, systolic blood pressure decrease of >10 mmHg or significant symptoms[352]. Venous blood samples were taken from supine patients immediately pre-exercise, immediately post-exercise and at 30 minutes post-exercise.

Exclusion criteria included recent (<3 months) stroke, thromboembolism, acute coronary syndrome, myocardial infarction, uncontrolled hypertension (BP ≥140/90 mm Hg), left or right bundle branch block on resting ECG, presence of structural cardiac abnormalities (e.g. significant valvular disease, hypertrophic cardiomyopathy or left ventricular ejection fraction <40%), and inflammation-related conditions (active infection, rheumatoid arthritis, connective tissue disorders, vasculitis, malignancy, corticosteroid usage).

Diurnal CPC variation

13 patients were studied for assessment of diurnal variation in CPCs. All patients were hospital in-patients who had been stable for at least 1 week following their initial admission and were similar in terms of age, gender and co-morbidities to participants of the exercise test arm. Blood samples were taken 5 times every 6 hours starting from at 12pm and finishing 12pm following day [5 sampling points per patient].

Effects of delay of sample preparation on CPC count

In a subset of 12 patients, the blood sample was reprocessed for CPC counts at 4 hours and 24 hours after collection in order to assess for temporal decline of CPC counts once *ex vivo*. During this time, samples were kept in a controlled environment at 4◦C.

Laboratory processing for circulating progenitor cells

The technique for blood sampling and enumeration of CPCs by flow cytometry has been previously described (section 2.4.3)

Statistical analysis

The statistical methods used have been previously described (section 2.3.2)

3.3.3 Results

Effects of exercise

20 patients were recruited into this arm of the study (age 69.9±7.8 [range 51-80], 70% male) years (table 15). The ETT was negative in 9 patients, positive in 8 cases and was considered sub-maximal or inconclusive in 3 subjects. Exercise led to significant increases in systolic blood pressure, heart rate, vWF, and sE-selectin, but no significant dynamics on CPC count were observed in relation to exercise (table 16).

Baseline CPC numbers demonstrated a negative correlation with baseline vWf (r=-0.551, p=0.012). Baseline CPC numbers also correlated significantly with their levels immediately post, and 30 minutes post exercise (r=0.968 and r=0.955 respectively, both P<0.001). Similar observations applied to vWf (r=0.720, P<0.001 and r=0.471, P=0.036 respectively) and sE-selectin (r=0.772 and r=0.874 respectively, both P<0.001) (table 17). No baseline risk factors were predictive of exercise induced alterations in research indices on multiple linear regression analysis (table 18)).

An *exploratory* subgroup analysis assessed patients with a negative ETT (n=9) versus those unable complete the protocol (positive or inconclusive ETT, n=11). Baseline

demographic data revealed no major differences between the two cohorts aside from a higher number of smokers amongst the latter group (11% *vs*. 54%, P=0.001, table 19). Those with a negative test had a peak blood pressure 17mmHg higher than those with a positive test, although this did not reach statistical significance. The cohort with a positive ETT had higher median CPC counts across all three timepoints, although the ΔCPC (from rest to peak exercise) were similar. None of these differences reached statistical significance. There were no major differences in sE-selectin or Vwf levels across the three timepoints, although patients with a positive test had borderline significantly lower vWf levels immediately after the test (table 19).

Table 15. Characteristics of the patient participating in the exercise study

Characteristics	
n	20
Age	69.9±7.88
Male	14 (70%)
Diabetes	2 (10%)
Hypertension	17 (85%)
Hyperlipidaemia	12 (60%)
Known IHD	5 (25%)
Atrial fibrillation	1 (5%)
Previous stroke	2 (10%)
Aspirin	12 (60%)
Beta-blockers	10 (50%)
Calcium antagonists	6 (30%)
Statins	8 (40%)
ACEI/ARB	12 (60%)
Current smokers	7 (35%)
Haemoglobin	14.1±1.41
Leukocytes	7.42±2.14
Platelets	235±77.6
Creatinine	93.4±19.2

IHD – Ischaemic Heart Disease; ACE-I – Angiotensin Converting Enzyme inhibitor; ARB – Angiotensin-2 receptor blocker. Values are expressed as mean ± standard deviation or No. (%)

Table 16. Effects of exercise on Haemodynamic Parameters and marker levels

Parameter	Before test	Immediately	30 min after test	P-value
HR (beats/min)	70.4 ± 16.52	145.1 ± 19.03	70.4 ± 9.96	<0.01[a,c]
BP (mmHg)	136.3 ± 18.53	170.6 ± 16.03	134.0 ± 11.3	<0.01[a,c]
CPC (mL^{-1})	117.5 (36-172)	132 (64-165)	102 (61-142)	0.279
vWf (IU/dL)	116.9±27.2	131.4±23.3	123.5±19.8	0.014[a,b]
sE-selectin (ng/mL)	52.0 (28.0-66.0)	59.0 (41.0-71.0)	55.0 (30.5-59.5)	0.004[a,c]

HR – Heart Rate; BP – Blood Pressure; CPC – Circulating Progenitor Cells; vWf – von Willebrand factor; sE-selectin – soluble E-selectin. Values are expressed as mean ± standard deviation, Median (Interquartile Range). P-value refers to the results of repeated measures ANOVA. For significant differences (Tukey post hoc P<0.05): [a]pre and immediate post; [b]pre and 30 minutes post; [c]immediate post and 30 minutes post.

Table 17. Spearman Correlation of test variables

			Baseline		Immediate post			30 minutes post		
	Variables		**vWf**	**sEsel**	**CPCs**	**vWf**	**sEsel**	**CPCs**	**vWf**	**sEsel**
Baseline	**CPCs**	*r*	-.551	-.501	.968	-.456	-.262	.955	-.202	-.328
		p	.012	.024	.000	.043	.265	.000	.394	.158
	vWf	*r*		.106	-.509	.720	.069	-.555	.471	.010
		p		.656	.022	.000	.773	.011	.036	.966
	sEsel	*r*			-.499	-.076	.772	-.454	-.063	.874
		p			.025	.749	.000	.044	.791	.000
Immediate post	**CPCs**	*r*				-.424	-.274	.963	-.109	-.332
		p				.062	.242	.000	.648	.153
	vWf	*r*					-.090	-.411	.205	-.084
		p					.705	.072	.387	.725
	sEsel	*r*						-.270	.205	.791
		p						.250	.387	.000
30-minutes	**CPCs**	*r*							-.203	-.314
		p							.390	.177
	vWf	*r*								-.214
		p								.365

CPC- Circulating Progenitor cells; vWf – vonWillebrand factor; sEsel – soluble E-selectin; Data refers to Spearman correlation of test variables at baseline. A $P<0.01$ was considered statistically significant

Table 18. Regression analysis of exercise induced alterations in research indices to risk factors.

		Research Indices		
Variables		**ΔvWf**	**ΔsEsel**	**ΔCPCs**
Age	β	.226	.419	.493
	P	.425	.193	.140
BMI	β	.603	.161	.099
	P	.041	.582	.742
BP	R	.168	.238	.230
	β	.568	.466	.492
DM	R	.246	.127	.332
	β	.417	.701	.338
↑lipidaemia	β	.376	.611	-.420
	P	.243	.099	.250
IHD	β	.222	.212	.211
	P	.455	.517	.528
Smoker	β	.191	.185	.165
	P	.574	.623	.669
R^2		.527	.648	.391

BP – Blood Pressure; BMI – Body Mass Index (Kg/m^2); DM – Diabetes Mellitus; IHD – Ischaemic Heart Disease; CPC- Circulating Progenitor Cells; vWf – vonWillebrand factor; sEsel – soluble E-selectin; Δ indicates the change in research indices from baseline to peak exercise. Data refers to standardized β co-efficients. A $P<0.01$ was considered statistically significant**.**

Table 19. Study characteristics in relation to exercise test results

Variable	Negative test	Positive test	P-value
N	9	11	
Age	72.7±6.23	67.7±8.66	0.45
Male	6 (67%)	8 (72%)	0.59
Diabetes	1 (11%)	1 (9%)	0.79
Hypertension	8 (89%)	9 (82%)	0.40
Hyperlipidaemia	6 (67%)	6 (54%)	0.34
BMI (Kg/m^2)	27.4±3.43	32.4±8.09	0.03
Sinus rhythm	9 (100%)	10 (91%)	0.06
Previous stroke	0 (0%)	1 (9%)	0.78
Known CCF	3 (33%)	5 (45%)	0.34
Smoking	1 (11%)	6 (54%)	0.001
Heart rate			
At rest	72.4±18.2	68.9±16.0	0.94
Peak exercise	146±12.9	144.1±23.5	0.07
BP systolic (mmHg)			
At rest	138.3±16.10	134.5±20.1	0.21
Peak exercise	180±14.7	162.6±12.5	0.60
CPC counts (mL^{-1})			
At rest	76 (34-156)	130 (86-180)	0.34
Immediately after ETT	64 (41-168)	148 (104-168)	0.20
30 min after ETT	78 (56-127)	124 (82-150)	0.16
ΔCPC	10 (-10-19)	6 (-14-27)	0.82
vWf (IU/dL)			
rest	108.4±34.1	123.7±18.9	0.13
Immediately after ETT	133.7±32.3	129.6±13.7	0.04
30 min after ETT	116.9±22.1	128.8±16.8	0.08
ΔvWF	25.22±12.8	5.82±19.6	0.31
sE-selectin (ng/mL)			
rest	*25.1±9.2*	24.1±9.4	0.82
Immediately after ETT	24.3±8.2	23.1±8.0	0.40
30 min after ETT	27.4±3.4	28.9±8.1	0.99
ΔsE-selectin	-0.78±5.8	-1.0±3.2	0.22

CPC – Circulating Progenitor Cells; vWf – vonWillebrand factor; sE-selectin – soluble E-selectin; BMI – Body Mass Index; CCF – Congestive Cardiac Failure; BP – Blood Pressure; ETT – Exercise Tolerance Test; Δ – Change in value from pre-exercise to peak exercise; Values are expressed as mean ± standard deviation (SD), Median (InterQuartile Range, IQR) or No. (%) as appropriate unless otherwise indicated. Analyses were performed using the χ^2 test for categorical data or unpaired t-test for continuous parametric data. The Mann-Whitney U test was used for non-parametric data. A p value < 0.05 was considered statistically significant

Diurnal Variation

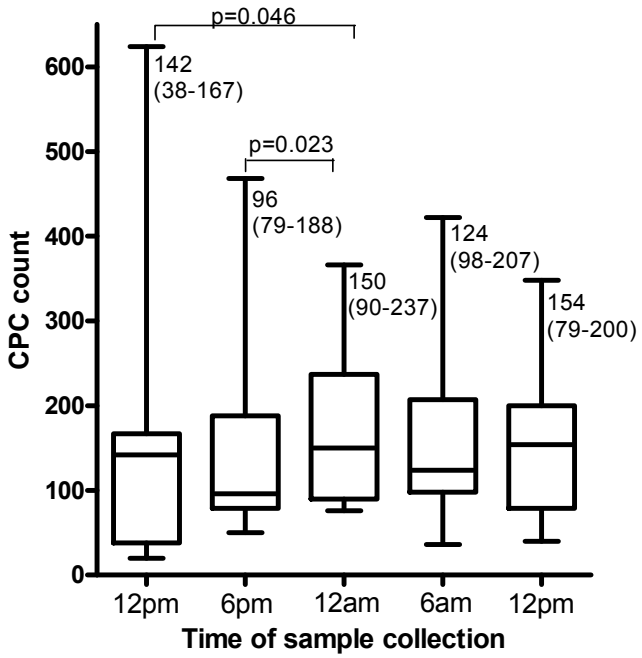
For this study, 13 patients were recruited (46% male, age 69.1±7.5 years) during the rehabilitation phase of a hospital admission with coronary artery disease. Median CPC counts showed a significant diurnal variation (table 20, figure 10, Friedman's repeated measures ANOVA, p=0.011) being significantly higher at 12am compared to 12pm (150 [90-237] *vs*. 142 [38-167]; p=0.046) and 12pm compared to 6pm (150 [90-237] *vs*. 96 [79-188]; p=0.023).

Table 20 Circulating progenitor cell counts during the diurnal variation analysis.

	Timepoint					
N=13	**12pm**	**6pm**	**12am**	**6am**	**12pm (+24 hrs)**	**P-value**
CPCs mL^{-1}	142 (38-167)	96 (79-188)	150 (90-237)	124 (98-207)	154 (79-200)	0.011

CPCs – Circulating Progenitor Cells; Values are expressed as median (InterQuartile Range, IQR). P-value refers to the results of Friedman's repeated measures ANOVA. For significant differences (Tukey post hoc P<0.05): [a]12pm-6pm; [b]6pm-12am; [c]12am-6am; [d]6am-12pm (+24hours); [e]12pm-12am; [f]12pm-6am; [g]12pm-12pm (+24 hours); [h]6pm-6am; [i]6pm-12pm (+24 hours); [j]12am-12pm (+24 hours).

Figure 10 Diurnal variation in circulating progenitor cell counts

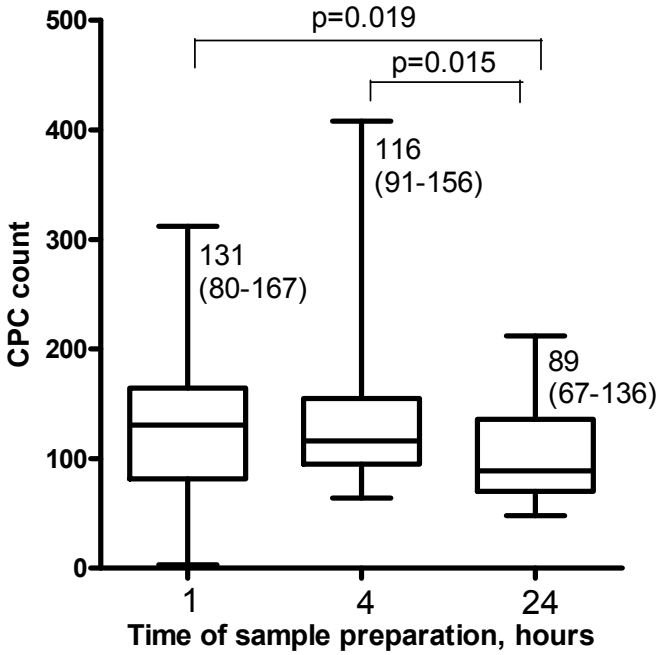


CPCs – Circulating Progenitor Cells; Values are expressed as median (InterQuartile Range)

Temporal Decline

To evaluate the effect of the delayed blood sample preparation of CPC recovery samples from a further 12 patients with chronic atrial fibrillation (58.3% male, mean age 69.9±7.9 years) were analysed sequentially at 4 hours and at 24 hours following venepuncture (figure 11). There were no significant changes in CPC numbers detected at 4 hours after sample collection, but there was a significant decline in CPC recovery observed when sample processing was delayed by 24 hours (P=0.019).

Figure 11 Temporal decline in circulating progenitor cell counts.



CPCs – Circulating Progenitor Cells; Values are expressed as median (InterQuartile Range)

3.3.4 Discussion

Our results demonstrate that median CPC numbers appear to trend towards an increase (albeit not significant) following a single 'one off' exercise stimulus (to exhaustion) using the Bruce protocol regimen. However this increase is not sustained at 30 minutes post exercise. Our data also show that CPCs demonstrate a degree of diurnal variation with respect to the circadian clock, with an apparent peak in the evening and slightly lower levels throughout the remainder of the day. Finally, we show a significant decline in CPC recovery when sample processing was delayed by 24 hours.

In the exercise study, there was a notable increase in both systolic blood pressure and heart rate in all our patients. Oddly the rise in BP appeared to be greater amongst those with a negative test comared to those with a positive test. This latter observation was not significant despite an apparent 17mmHg difference, perhaps reflecting low numbers within each subgroup. Importantly, both of these parameters may have resulted in enhanced endothelial 'shear stress' that could have accounted for the observed changes in the various research indices tested. Indeed, a rise in both mean vWf and soluble E-selectin may reflect the impact of exercise upon the endothelium and/or acted as a signal to promote CPC release and recruitment into the endothelium. This endothelial 'reaction' to exercise has previously been demonstrated in several other small studies which have assessed sEsel, vWf flow-mediated dilatation and CECs respectively[350, 353, 354].

However, there are differential effects of moderate and extreme exercise on EPC levels. In healthy subjects, intensive and moderate exercise for 30 minutes, but not for

10 minutes, acutely increases circulating levels of EPCs[355]. Generally, EPC mobilisation seems to reflect the intensity of physical activity. For example, a brief exhaustive exercise increases levels of circulating EPCs by almost 4-fold[267]. However, extreme exercise may have a different effect, as no significant changes in EPC levels were observed in marathon runners, whilst $CD34^+$ and $CD133^+$ CPCs were significantly downregulated in parallel to VEGF levels[356].

Physical activity may differentially affect levels of various EPC subsets and an increase in $CD133^+KDR^+$ but not $CD34^+KDR^+$ EPCs was associated with the intensity of habitual exercise[357]. These indicate that data obtained for one population of circulating progenitors can not automatically be extended to all subsets. For example, more intensive daily physical rehabilitation up-regulates the number of $CD34^+133^+$ CPCs[353]. The present analysis suggests that exercise in short bursts may also enhance CPC mobilization, although this response is clearly not maintained after 30 minutes.

Our data suggesting a diurnal variation in CPCs is in agreement with findings from another small study investigating KDR^+ EPCs[358]. It is plausible that the observed effects relate to relative dehydration given that consumption of fluids is generally lower in the early evening and overnight. However, this possibility is considered less likely given that the 6am sample – which follows the longest period of relative dehydration (overnight) – does not demonstrate the lowest counts.

Similar diurnal patterns are seen with other cell lines (again in small studies) and, peripheral neutrophil, monocyte and lymphocyte numbers also appear to peak during

the evening rather than in the early morning[359, 360]. The explanation for these findings is unknown. Fluctuations in counts of various cell lines may be a consequence of alterations in the very stimuli that are known to promote their release. In the case of CPCs, this includes factors such as GM-colony stimulating factor (CSF), G-CSF and corticosteroids (amongst other factors) all of which have also been shown to express a degree of diurnal variation [361, 362]. A further alternative possibility is that endothelial damage (and consequently repair) is greatest overnight, given that vascular events such as myocardial infarction, ischaemic stroke and sudden cardiac death also demonstrate marked circadian variation with a peak in the early morning hours [363, 364].

In addition, the pattern of CPC diurnal variability, if present, may be more complex than is immediately apparent. Data from the 12pm (+24hr) sample counts were broadly similar to those seen at midnight the previous evening and are also slightly greater than those observed at 12pm the previous day (table 19). This observation is interesting and may imply that the suggestion of diurnal variation from statistical analysis may be due to small data samples with a broad spread.

The significant decline in CPCs on serial sampling is also in keeping with other (small) studies from our research group assessing the temporal decline in CECs, where significant reductions were seen over a 24-hour period of cells being held *ex-vivo*[365]. Again, there are several potential explanations for these findings, including progressive cellular destruction and cell clumping leading to alterations in both forward scatter and side scatter (essentially cell size and granularity) observed using flow cytometry. In both circumstances, such cells would have been excluded from our gating analysis. Alternatively, cells may adhere to the wall of the Vacutainer® tube or

the physical properties of CPCs could alter such that the fluorochromes used bind less avidly, leading to reduced capture of CPCs. Cell clumping in the presence of EDTA is most commonly recognized with red cells or platelets and only extremely rarely occurs with lymphocytes[366]. Moreover the phenomenon of clumping is assumed to be more likely to occur during culture experiments (where EDTA and trypsin are required to dislodge adherent cells) rather than when cells are held in transit tubes.

Of note, our blood samples were held at 4°C and stored away from the influence of direct sunlight. The blood sampling tube used is employed routinely in clinical practice worldwide and has a proven track record for reliable transport of samples [367, 368]. Indeed, blood samples may sit for several hours prior to clinical laboratory processing without undue influence on full blood count assessments[369, 370]. Lymphocyte counts in particular are considered very reliable even up to 3 days after blood sampling[371]. However, the cells assessed routinely under such circumstances are frequently occurring cells and not rare sub-populations such as CPCs. Cellular destruction could, of course, occur due to alteration in the physiological properties of the suspension medium (whole blood) through the addition of K_3 EDTA or even other contaminants. K_3 EDTA does result in a small degree of shrinkage in red blood cells and slight increase in cell volume for other cells on standing [367, 368]. Additionally, some of the effects seen with EDTA occur after a number of hours and could explain the temporal decline in cell counts. K_3 EDTA as a liquid anticoagulant does partially dilute the blood sample and has been shown to return blood counts of approximately 1-2% less than with di-potassium (K_2) EDTA, again introducing (albeit consistent) potential for error[368, 372].

Finally, it is also possible that sequential insertion of large bore needles has the potential to strip cells from the vascular wall at the point of needle insertion. To overcome this many investigators routinely discard the first few mLs of blood drawn – particularly when measuring CECs. However for CPCs, this argument holds less strongly, given that the presumed origins of the majority of these cells is from direct bone-marrow release.

The implications from these data are that analysis of CPCs should be performed promptly after blood sampling. In addition, samples should where possible be stored during transit from clinic to laboratory in a consistent physical environment, to ensure regulation of temperature and exposure to other physical parameters to ensure reproducibility and consistency of results. Similarly our data suggest that sampling time may also be important, with variable CPC counts across the circadian clock.

Study Limitations

This study has a number of limitations. The first relates to study design - in particular to the small sample sizes in each of the analyses. To enlarge the sample size is certainly feasible, although it should be noted that the diurnal variation experiment required a significant amount of goodwill from participants due to the frequency and timing of venepuncture. However, greater numbers in each individual experiment would certainly add to the value of the work and potentially remove uncertainty regarding some findings – for example table 19 documents an apparent sizeable difference in some of the parameters between the two groups (e.g. peak exercise BP, resting vWf etc.) without a significant P value. This likely represents broad spread of

data in cohorts with a small sample size (i.e. inadequate power). Such concerns may equally be applied to the diurnal and temporal decline experiments also.

The application of statistical tests here and the apparent finding of significant differences may be considered chance – particularly given that the median values across the timepoints for the diurnal experiment are broadly similar. Again the broad spread of data that is inherent when measuring CPCs implies that larger sample numbers are required for further validation of these findings. In the diurnal experiment, repeating the measurements on the same series of patients over a period of several days may enhance validity, although logistics may prove limiting.

Our analysis was not confined to a population of patients with a single pathology. This was a deliberate strategy given that results from such scientific data may require extrapolation to the broader population. It should however be noted that this approach does not allow for a detailed analysis of data on specific patient profiles. Consequently, such analyses were avoided due to inadequate statistical power.

Finally, the antigenic definition of CPCs is highly variable across the literature, with no universally accepted profile as yet. Thus it is not possible to definitively extrapolate our (or others') data and apply this to all progenitor cell populations. Finally, our study has analysed CPCs using only one laboratory technique and in particular has only considered cell counts and has not taken into account cell function – i.e. the potential for CPCs to reproduce and integrate into the endothelium. Such assessment is usually based on cell culture experiments, but is an important characteristic to define in CPCs.

3.3.5 Conclusion

Routine exercise stress testing does not affect significantly CPCs counts and usual physical activity prior blood sample collection is unlikely to affect their recovery. Peripheral CPC levels showed a degree of diurnal variation, whilst evidence of temporal decline in CPC counts was apparent at 24 hours. The implications from these data are that analysis of CPCs should be performed promptly after blood sampling. In addition, samples should where possible be stored during transit from clinic to laboratory in a consistent physical environment, to ensure regulation of temperature and exposure to other physical parameters to ensure reproducibility and consistency of results. Similarly, our data suggest that sampling time may also be important, with variable CPC counts across the circadian clock. Thus, various factors may affect accuracy of CPC enumeration. However, this interpretation must be considered with the knowledge that assessment of data with a broad spread in a small population can easily induce statistical error and thus further larger studies are required.

Section IV: Clinical Studies

4.1 Cross-sectional Analysis

Abstract

Background The pathophysiological inter-relationships and underlying 'drivers' of the prothrombotic state in atrial fibrillation (AF) are poorly understood. There appears to be a complex series of alterations in a number of pathways all of which may ultimately contribute to thrombogenesis. The endothelium may be a central theme linking some of these parameters. We hypothesized that circulating progenitor cells (CPCs) numbers may be reduced amongst patients with more persistent forms of AF compared to paroxysmal AF or 'disease controls' in sinus rhythm. Secondly, we hypothesized that markers of endothelial damage/dysfunction (von Willebrand factor, soluble E-selectin), apoptosis (soluble Fas), soluble Fas ligand) and angiogenesis (vascular endothelial growth factor) would be reduced whilst serum markers of inflammation (interleukin-6) would be increased in the same groups.

Results 135 consecutive patients with AF were recruited: 60 had permanent AF (mean age 69.6±8.4 years, 39 (65%) male), 22 persistent AF (mean age 66.2±10.3 years, 16 (65%) male, 39 paroxysmal AF (mean age 70.2±8.8years, 24 (62%) male) and 14 had lone AF (mean age 46.7±11.8 years, 9 (64%) male). 33 'disease' controls (mean age 67.9±9.6 years, 21 (64%) male) and 13 healthy volunteers (mean age 50.9±9.4 years, 9 (69%) male) were also recruited.

Analysis of non-lone AF to the disease control group demonstrated a significantly lower level of sFas in the former (3.8 ng/mL (3.0-4.8) *vs*. 5.0 ng/mL (4.0-5.1) P<0.001), with the lowest levels of the marker observed in patients with persistent (3.6 ng/mL (3.0-4.6) or permanent AF (3.3 ng/mL (3.0-4.5). Patients with lone AF had similar levels of all the research indices to the healthy control arm, except for sFas ligand (275 (200-400) vs. 125 (100-250) ρg/mL) and the sFas:sFas ligand ratio (103.8 (78.9-127.3) vs. 56.2 (37.2-79.5)), both of which were significantly higher amongst the healthy controls (P=0.006 and P=0.014 respectively). Median CPC levels were significantly higher in lone AF compared to 'non-lone AF' (211/mL (120-361) vs. 85/mL (42-149), P<0.001). Lower levels of both sE-selectin (43.0 ng/mL (31.5-67.5) *vs*. 68.0 ng/mL (44.0-75.0), P=0.042); and vWf (116.3 ± 23.5 IU/dL *vs*. 135.4 ± 26.3 IU/dL, P=.013) were seen in lone compared to non-lone AF patients The presence/absence of AF was not predictive of any of the research indices tested.

Conclusion This study has a number of limitations. Many of these relate to study design and statistical power. The latter despite our pre-specified recruitment numbers being broadly met, it was apparent that many analyses had a broader spread than anticipated. Despite this, it would appear that CPC counts and one index of apoptosis (sFas) appear to be abnormal in AF compared to healthy controls although these indices – as well as abnormalities of other markers including indices of endothelial damage/dysfunction, apoptosis, angiogenesis and inflammation – are probably more related to associated co-morbidities rather than AF *per se*.

4.1.1 Introduction

Atrial fibrillation (AF) confers a 'hypercoagulable' or 'prothrombotic' state and consequent high risk of stroke and systemic thromboembolism. This is reflected by up-regulation of various blood indices including pro-thrombotic markers, platelet activation, blood stasis within the atria and ultimately by impairment of both cardiac and vascular endothelium[373]. Although the pathophysiological inter-relationships between endothelial/endocardial impairment and atrial electrical abnormalities are still elusive (i.e. cause or effect), endothelial damage/dysfunction in AF clearly represents a major potential driver of the 'prothrombotic' state, by virtue of interactions with the coagulation cascade at a number of levels[373].

Endothelial damage/dysfunction has been identified in many disease states many of which frequently associate with AF (e.g. hypertension, atherosclerosis, diabetes mellitus) and is often associated with worse cardiovascular outcomes[374]. However, altered endothelial function has also been shown to exist in AF even in the absence of co-morbidities. Indeed, high levels of plasma markers of endothelial damage/dysfunction, such as von Willebrand factor (vWf) and soluble E-selectin (sEsel) and abnormalities of flow mediated dilatation are consistently reported in patients with AF and importantly, may relate to both the pathogenesis of the arrhythmia and provide a central link for the various processes implicated in the hypercoagulable state[373, 375] (section 1.2).

The mature endothelium has limited regenerative capacity and it has been suggested that a specific subset of bone marrow-derived progenitors (circulating progenitor cells) might promote maintenance of endothelial integrity[231, 232] (section 1.3).

Intriguingly, AF has been linked to the presence of systemic inflammation[159, 166, 376] and to up-regulation of angiogenic growth factors (e.g. vascular endothelial growth factor, VEGF) both of which also seem to be important in triggering release and/or recruitment of CPCs in other disease states[344] (section 1.2.6). CPCs seem resistant to both apoptosis and oxidative stress and therefore (in adequate numbers) may potentially ameliorate the negative impact of such cardiovascular risk factors/co-morbidities on endothelial health in AF. Conversely, CPC mobilization and function may also be impaired by various cardiovascular co-morbidities and thereby reflect another mechanism through which endothelial dysfunction in AF is enhanced[344]. Thus the balance of factors promoting endothelial repair (e.g. angiogenic factors) and damage (e.g. cardiovascular co-morbidities, inflammation) in AF is difficult to predict and detailed analysis has not yet been performed.

The purpose of this study was to investigate the pattern CPCs in relation to markers endothelial damage/dysfunction (vWf, sEselectin), inflammation (IL-6), angiogenesis (VEGF) and apoptosis (sFas, sFas ligand) in patients with different temporal patterns of AF.

4.1.2 Hypotheses

These have been discussed in detail in section 2.1.3.

4.1.3 Methods

This has been described previously in section 2.2.1. Classification of temporal pattern of AF was as per internationally accepted criteria outlined in section 1.1.4 Patients below 55 years of age without established risk factors for AF were considered to have

lone AF. Reclassification was allowed if it later became apparent that the patient, for example, had paroxysmal, rather than a more persistent form of AF.

Patients were compared to a cohort of disease-matched and healthy controls. These patients were recruited from general cardiology and hypertension clinics, or as attendees to pre-assessment clinics for minor operations e.g. hernia repair. Exclusion criteria have been discussed previously (section 2.2.1)

Patients were treated according to established treatment protocols within the framework of national and international guidelines. The use of anticoagulants and anti-platelet agents was left to the discretion of the treating physician based upon assessment of individual patient's thromboembolic risk (section 1.1.9). Where patients were treated with warfarin, the target International Normalized Ratio (INR) was 2.0-3.0. If possible and unless clinical risk dictated (e.g significant coronary artery disease) anti-platelet agents were discontinued in those patients prescribed warfarin.

Laboratory

Laboratory analyses were performed as described in section 2.4 and appendix.

Statistical Methods

This has been described previously in section 2.3.

4.1.4 Results

We recruited 135 consecutive patients with AF: 60 had permanent AF (mean age 69.6±8.4 years, 39 (65%) male), 22 persistent AF (mean age 66.2±10.3 years, 16 (65%) male, 39 paroxysmal AF (mean age 70.2±8.8years, 24 (62%) male) and 14 had lone AF (mean age 46.7±11.8 years, 9 (64%) male) (tables 21, 24). 33 'disease' controls (mean age 67.9±9.6 years, 21 (64%) male) were recruited to allow for comparison against the non-lone AF patients (table 21) and 13 healthy volunteers (mean age 50.9±9.4 years, 9 (69%) male) were recruited as healthy controls to compare against the cohort of patients with lone AF (table 24).

Cross-sectional analysis

There were no significant differences in age, gender or major co-morbidities between non-lone AF patients and 'disease controls' except for a higher prevalence of coronary artery disease in the latter group (table 21). AF patients more often received warfarin, and the antiarrhythmic drugs digoxin and amiodarone, but were less often treated with anti-platelet agents.

Analysis of the entire cohort of non-lone AF compared to the disease control group demonstrated a significantly lower level of sFas in the former (3.8 ng/mL (3.0-4.8) *vs*. 5.0 ng/mL (4.0-5.1) $P<0.001$), with the lowest levels of the marker observed in patients with persistent (3.6 ng/mL (3.0-4.6) or permanent AF (3.3 ng/mL (3.0-4.5) (tables 22 & 23). No significant difference in levels of the other research indices was observed.

Table 21. Demographic Data for AF Patients and Disease-matched Control Subjects

	Variable	Disease-matched Controls	Paroxysmal AF	Persistent AF	Permanent AF	*P* value
	n	33	39	23	60	
Baseline Data	Age (SD)	67.9 ± 9.6	70.2 ± 8.8	66.2 ± 10.3	69. 6± 8.4	.320
	Age > 75 (%)	10 (30)	16 (41)	4 (17)	22 (37)	.289
	Male (%)	21 (64)	24 (62)	16 (65)	39 (65)	.989
	Body Mass Index (Kg/m^2)	30.0 ± 7.2	28.2 ± 4.1	28.7 ± 6.1	29.0 ± 6.8	.782
Risk Factors No. (%)	Smoking	13 (40)	11 (28)	9 (39)	22 (37)	.588
	IHD (Angina / MI / PCI)	15 (46)	9 (23)	11 (48)	10 (17)	.017
	Previous CVA	4 (12)	4 (10)	2 (9)	10 (17)	.395
	CCF	2 (6)	4 (10)	2 (9)	17 (18)	.106
	Hypertension	28 (85)	31 (80)	20 (87)	50 (83)	.413
	Diabetes Mellitus	4 (12)	1 (3)	3 (13)	4 (7)	.219
	Hyperlipidaemia	19 (58)	18 (46)	15 (65)	24 (40)	.645
	$CHADS_2$ risk	1 (1-2)	2 (1-2)	1 (1-2)	2 (1-3)	.181
Medication, No. (%)	Antiplatelets	18 (55)	8 (21)	13 (57)	31 (52)	.001
	Warfarin	2 (6)	20 (51)	23 (100)	25 (42)	.000
	Beta-blocker	14 (42)	22 (56)	9 (39)	26 (45)	.506
	Calcium-channel blocker	16 (48)	13 (33)	11 (48)	23 (38)	.412
	Digitalis	0 (0)	2 (5)	4 (18)	18 (30)	.000
	Flecainide	0 (0)	2 (5)	3 (14)	2 (3)	.134
	Amiodarone	1 (3)	7 (18)	1 (4)	2 (3)	.010
	ACE-i/ARB	21 (64)	24 (62)	15 (65)	35 (58)	.997
	Statin	13 (39)	20 (51)	13 (57)	21 (34)	.170

Paroxysmal AF was defined as at least two electrocardiographically documented episodes of AF with interceding sinus rhythm. *Persistent AF* was defined as AF lasting >7 days without spontaneous reversion to sinus rhythm, suitable for and requiring pharmacologic or direct-current cardioversion to restore sinus rhythm. *Permanent AF* was defined as AF lasting for >1 year in a patient in whom cardioversion was considered to be inappropriate or who had previously been unsuccessful.

Abbreviations: IHD – Ischaemic Heart Disease; MI – Myocardial Infarction; PCI – Percutaneous Coronary Intervention; ACE-I – Angiotensin Converting Enzyme inhibitor; ARB – Angiotensin-II Receptor Blocker; CCF – Congestive Cardiac Failure; CVA – Cerebrovascular Accident; $CHADS_2$: 1 point is assigned for: Congestive cardiac failure, Age≥75 years, Diabetes mellitus, with prior Stroke or transient ischaemic attack being assigned 2 points.

All Data expressed as Mean ± Standard Deviation (SD), No. (%) or Median (InterQuartile Range, IQR) unless otherwise stated. Comparisons between groups using the one-way analysis of variance or Kruskal-Wallis testing as appropriate. A p value < 0.05 was considered statistically significant.

Table 22. A comparison of Research Indices in all AF vs. Disease-matched controls

	Variable	Disease-matched Controls	All AF*	P value
	n	33	122	
Research Indices	CPCs (cells/mL)	78 (30-149)	85 (42-149)	.500
	vWf (IU/dL)	133.7 ± 23.7	135.4 ± 26.3	.737
	sEselectin (ng/mL)	68.0 (41.0-76.0)	68.0 (44.0-75.0)	.871
	VEGF (ρg/mL)	60 (5-142.5)	70 (20-250)	.211
	IL-6 (ρg/mL)	15 (10-18)	10 (2.25-20)	.083
	sFas (ng/mL)	5.0 (4.0-5.1)	3.8 (3.0-4.8)	<.001
	sFas ligand (ρg/mL)	200 (125-375)	250 (125-375)	.473
	sFas:sFas ligand	45.5 (30.6-94.4)	66.7 (34.5-125.0)	.115

*Lone AF excluded from this analysis due to absence of co-morbidities.

CPC – Circulating Progenitor Cells; vWf – von Willebrand factor; IL-6 – InterLeukin-6; VEGF – Vascular Endothelial Growth Factor. All data expressed as mean ± standard deviation (SD), or median (InterQuartile Range, IQR) unless stated otherwise; P-value refers to the results of unpaired t-test or Mann-Whitney U test for parametric and non-parametric data as appropriate. A P-value < 0.05 was considered statistically significant.

Table 23. A comparison of Research Indices by temporal pattern of AF

	Variable	Disease-matched Controls	Paroxysmal AF	Persistent AF	Permanent AF	P value
	n	33	39	23	60	
Research Indices	CPCs (cells/mL)	78 (30-149)	90 (51-164)	85 (29-153)	82 (38-134)	.754
	vWf (IU/dL)	133.7 ± 23.7	138.7 ± 22.7	136.6 ± 13.5	132.9 ± 31.5	.710
	sEselectin (ng/mL)	68.0 (41.0-76.0)	68.0 (52.0-73.0)	64.0 (39.5-76.0)	66.0 (39.5-82.5)	.939
	VEGF (ρg/mL)	60 (5-142.5)	70 (5-140)	76.3 (20-290)	80 (18.8-297.5)	.457
	IL-6 (ρg/mL)	15 (10-18)	10 (10-20)	15 (10-24)	10 (1-15)	.069
	sFas (ng/mL)	5.0 (4.0-5.1)	4.5 (3.5-5.0)	3.6 (3.0-4.6)	3.3 (3.0-4.5)	<.001
	sFas ligand (ρg/mL)	200 (125-375)	300 (125-500)	188 (119-188)	250 (125-400)	.474
	sFas:sFas ligand	45.5 (30.6-94.4)	70.0 (36.4-110.0)	48.2 (31.9-123.9)	71.4 (31.8-132.1)	.373

CPC – Circulating Progenitor Cells; vWf – von Willebrand factor; IL-6 – InterLeukin-6; VEGF – Vascular Endothelial Growth Factor. All data expressed as mean ± standard deviation (SD), or median (InterQuartile Range, IQR) unless stated otherwise; P-value refers to the results of one-way analysis of variance (ANOVA) or Kruskal-Wallis test for parametric and non-parametric data as appropriate. A P-value < 0.05 was considered statistically significant.

Lone AF vs. Healthy Controls

The healthy control group and lone AF groups were both comparable in baseline demographic parameters (table 24). Patients with lone AF had similar levels of all the research indices to the healthy control arm, except for sFas ligand (275 (200-400) vs. 125 (100-250) ρg/mL) and the sFas:sFas ligand ratio (103.8 (78.9-127.3) vs. 56.2 (37.2-79.5)), both of which were significantly higher amongst the healthy controls(P=0.006 and P=0.014 respectively), (table 24). Median VEGF was approximately 50% higher in the lone AF group (132.5 (10-187.5) ρg/mL *vs*. 85 (53-290) ρg/mL, however this did not reach statistical significance. There was no difference in levels of the other research indices between these two groups.

Table 24. Demography and Research Indices in Lone Atrial fibrillation Patients and Healthy Control Subjects.

	Variable	Healthy Controls	Lone AF	*P* value
	n	13	14	
Baseline Data	Age (SD)	50.9 ± 9.4	46.7 11.8	0.324
	Age > 75 (%)	0 (0)	0 (0)	1.000
	Male (%)	9 (69)	9 (64)	0.795
	Body Mass Index	27.6 ± 5.9	25.1 ± 4.3	0.355
Risk Factors No. (%)	Smoking	2 (15)	4 (29)	0.430
	IHD (Angina / MI / PCI)	0 (0)	0 (0)	1.000
	Previous CVA	0 (0)	0 (0)	1.000
	CCF	0 (0)	0 (0)	1.000
	Hypertension	0 (0)	0 (0)	1.000
	Diabetes Mellitus	0 (0)	0 (0)	1.000
	Hyperlipidaemia	0 (0)	0 (0)	1.000
	$CHADS_2$	0 (0)	0 (0)	1.000
Medication, No. (%)	Antiplatelets	3 (16)	4 (28)	0.382
	Warfarin	1 (5)	2 (14)	0.094
	Beta-blocker	2 (11)	7 (50)	0.013
	Calcium-channel blocker	0 (0)	0 (0)	1.000
	Digitalis	0 (0)	0 (0)	1.000
	Flecainide	0 (0)	5 (36)	0.005
	Amiodarone	0 (0)	0 (0)	1.000
	ACE-i/ARB	0 (0)	1 (7)	0.244
	Statin	0 (0)	0 (0)	1.000
Research Indices	CPCs (cells/mL)	158 (75-336)	211 (120-361)	0.353
	vWf (IU/dL)	126.0 ± 32.6	116.3 ± 23.5	0.693
	sEselectin (ng/mL)	46.0 (26.0-64.0)	43.0 (31.5-67.5)	1.000
	VEGF (ρg/mL)	85 (53-290)	132.5 (10-187.5)	0.707
	IL-6 (ρg/mL)	10 (0-15)	15 (10-20.5)	0.083
	sFas (ng/mL)	2.8 (2.0-3.0)	3.1 (2.3-3.3)	0.214
	sFas ligand (ρg/mL)	275 (200-400)	125 (100-250)	0.006
	sFas:sFas ligand	103.8 (78.9-127.3)	56.2 (37.2-79.5)	0.014

CPC – Circulating Progenitor Cells; vWf – von Willebrand factor; IL-6 – InterLeukin-6; VEGF – Vascular Endothelial Growth Factor. All data expressed as mean ± standard deviation (SD), or median (InterQuartile Range, IQR) unless stated otherwise; P-value refers to the results of one-way analysis of variance (ANOVA) or Kruskal-Wallis test for parametric and non-parametric data as appropriate. A P-value < 0.05 was considered statistically significant. All data expressed as mean ± standard deviation (SD), or median (InterQuartile Range, IQR) unless stated otherwise; P-value refers to the results of unpaired t-test or Mann-Whitney U test as appropriate. A P-value < 0.05 was considered statistically significant.

Lone vs. non-lone AF

Median CPC levels were significantly higher in lone AF compared to 'non-lone AF' (211/mL (120-361) vs. 85/mL (42-149), P<0.001). Lower levels of both sE-selectin (43.0 ng/mL (31.5-67.5) *vs*. 68.0 ng/mL (44.0-75.0), P=0.042); and vWf (116.3 ± 23.5 IU/dL *vs*. 135.4 ± 26.3 IU/dL, P=.013) were seen in lone compared to non-lone AF patients (table 25).

Table 25. Research Indices in Lone AF vs. non-lone AF.

	Variable	Non-lone AF	Lone AF	*P* value
	n	122	14	
Research Indices	CPCs (cells/mL)	85 (30-149)	211 (120-361)	<.001
	vWf (IU/dL)	135.4 ± 26.3	116.3 ± 23.5	.013
	sEselectin (ng/mL)	68.0 (44.0-75.0)	43.0 (31.5-67.5)	.042
	VEGF (ρg/mL)	70 (20-250)	132.5 (10-187.5)	.766
	IL-6 (ρg/mL)	10 (2.25-20)	15 (10-20.5)	.296
	sFas (ng/mL)	3.8 (3.0-4.8)	3.1 (2.3-3.3)	.001
	sFas ligand (ρg/mL)	250 (125-375)	125 (100-250)	0.24
	sFas:sFas ligand	66.7 (34.5-125.0)	56.2 (37.2-79.5)	.535

CPC – Circulating Progenitor Cells; vWf – von Willebrand factor; IL-6 – InterLeukin-6; VEGF – Vascular Endothelial Growth Factor. All data expressed as mean ± standard deviation (SD), or median (InterQuartile Range, IQR) unless stated otherwise; P-value refers to the results of unpaired t-test or Mann-Whitney U test for parametric and non-parametric data as appropriate. A P-value < 0.05 was considered statistically significant.

Univariate and stepwise multiple regression analyses

For the whole AF cohort (table 26), age correlated with vWf (r=0.174, p=0.045) and inversely with CPCs (r=-0.267, p=0.002). The presence of hypertension was associated with a lower CPC count (r=-0.293, p=0.001) and higher levels of sEselectin (r=0.209, p=0.022), and sFas (r=0.191, p=0.030). Of interest, the CPC count correlated with the $CHADS_2$ stroke risk score (r=-0.345, p<0.001) and presence of congestive heart failure in AF (r=-0.180, p=0.049), despite small numbers of such patients. Additionally, CPC numbers significantly negatively correlated with sFas levels (r=-0.207, p=0.020).

In the disease matched control group (table 27), hypertension correlated to VEGF (r=-0.344, p=0.050), and smoking correlated to sFas (r=-0.385, p=0.027). There was a strong correlation between sFasL and the sFas:sFasL ratio (r=0.931, p<0.001). In the healthy controls in sinus rhythm group (table 28), age correlated to IL-6 (r=0.522, p=0.022), smoking correlated to CPCs (r=0.530, p=0.024) and sFasL (r=-0.153, p=0.002). There was also a correlation between sEselectin and VEGF (r=0.649, p=0.009) and sFas to the sFas:sFasL ratio (r=0.933, p<0.001).

Following a stepwise multiple regression analysis of the entire cross-sectional cohort of patients, age (β=-0.272, P=0.014), hyperlipidaemia (β=0.369, P=0.001) and statin prescription (β=-0.260, P=0.014) emerged as significant predictors of CPCs. Age was also predictive of vWf (β=0.219, P=0.05). sE-selectin was predicted by both hypertension (β=0.354, P=0.019) and smoking (β=0.266, P=0.001). However, the presence/absence of AF was not predictive of any of the research indices tested (table 29).

Table 26. Correlations of research indices to demographic data for all atrial fibrillation.

Variables		vWf	sEsel	CPCs	sFas	sFasL	Fas:FasL	VEGF	IL-6
Age	*r*	.174	.113	-.267	.160	.026	-.041	.029	-.101
	p	.045	.212	.002	.065	.770	.644	.749	.260
IHD	*r*	.093	-.009	-.147	.047	.014	-.019	.114	-.001
	p	.298	.921	.108	.595	.674	.832	.215	.989
HTN	*r*	.104	.209	-.293	.191	.108	.021	-.089	-.166
	p	.243	.022	.001	.030	.232	.818	.333	.067
CVA	*r*	-.056	.033	-.043	-.097	-.079	-.049	-.077	-.100
	p	.527	.721	.635	.272	.380	.587	.397	.271
CCF	*r*	-.009	.086	-.180	.100	-.048	-.063	.040	-.047
	p	.924	.355	.049	.261	.593	.486	.659	.607
DM	*r*	-.067	.065	-.093	-.015	.070	.057	-.173	-.186
	p	.451	.481	.312	.865	.438	.527	.058	.040
↑ Lipids	*r*	-.026	.180	.029	.050	-.038	-.058	-.234	-.171
	p	.769	.050	.749	.575	.674	.524	.010	.060
BMI	*r*	-.074	.093	.056	-.053	-.006	.008	.167	-.092
	p	.475	.383	.596	.609	.953	.941	.064	.385
CHADS2	*r*	.071	.134	-.345	.150	-.008	-.074	-.053	-.159
	p	.426	.147	.000	.091	.933	.412	.562	.080
Warfarin	*r*	.183	.087	-.017	.100	.198	.153	.166	.110
	p	.039	.350	.855	.262	.027	.090	.069	.231
Aspirin	*r*	.030	.169	.162	-.118	.060	.059	-.031	-.205
	p	.750	.085	.099	.214	.533	.539	.757	.035
Statin	*r*	-.076	.107	-.076	.007	-.077	-.082	-.122	-.031
	p	.407	.244	.407	.940	.389	.543	.182	.731
ACE-i / ARB	*r*	-.283	.134	-.283	.118	.076	.027	-.124	.066
	p	.002	.150	.002	.185	.401	.768	.180	.473
Smoking	*r*	.039	-.258	-.080	-.073	-.036	.001	-.017	.038
	p	.663	.150	.386	.416	.695	.768	.852	.682

Table 26. Correlations of research indices to demographic data for all atrial fibrillation (continued).

Variables		sEsel	CPCs	sFas	sFasL	Fas:FasL	VEGF	IL-6
vWf	*r*	-.020	.086	-.047	.100	.095	.189	.051
	p	826	.337	.588	.257	.283	.034	.572
sEsel	*r*		-.045	.018	.079	.085	.007	.049
	p		.630	.842	.384	.352	.939	.601
CPCs	*r*			-.207	-.020	.055	-.014	-.025
	p			.020	.823	.543	.880	.786
sFas	*r*				.029	-.301	-.134	.043
	p				.746	.000	.132	.626
sFasL	*r*					.029	.132	-.062
	p					.746	.146	.493
Fas:FasL	*r*						.167	-.060
	p						.064	.505
VEGF	*r*							.186
	p							.037

IHD – Ischaemic heart disease; HTN – Hypertension; CVA – CerebroVascular Accident; CCF – Congestive Cardiac Failure; DM – Diabetes Mellitus; $CHADS_2$: Stroke risk algorithm (see text); BMI – Body Mass Index; ARB / ACE-i: Angiotensin-2 receptor blocker / Angiotensin converting enzyme inhibitor; vWf – vonWillebrand factor, sEsel – soluble E selectin; sFas – soluble Fas; sFasL – soluble Fas ligand; VEGF – Vascular Endothelial Growth Factor; IL-6 – Interleukin 6; CPC – Circulating Progenitor Cell. Data refers to Spearman correlation.

Table 27. Correlations of research indices to demographic data for disease-matched controls in sinus rhythm.

Variables		vWf	sEsel	CPCs	sFas	sFasL	Fas:FasL	VEGF	IL-6
Age	*r*	.108	-.170	-.207	-.003	-.111	-.097	.220	.186
	p	.549	.353	.273	.986	.539	.592	.218	.301
IHD	*r*	-.080	-.003	.311	.104	.170	.051	.328	-.180
	p	.658	.985	.094	.553	.347	.777	.062	.315
HTN	*r*	.147	.225	.010	.122	.027	-.018	-.344	.227
	p	.416	.216	.957	.498	.883	.922	.050	.204
CVA	*r*	-.063	-.077	-.221	-.015	-.367	-.312	.029	.030
	p	.726	.675	.241	.934	.036	.077	.871	.868
CCF	*r*	.127	.112	N/A	.238	-.114	-.133	-.047	.007
	p	.482	.540	N/A	.182	.529	.459	.795	.970
DM	*r*	.020	.062	.119	-.065	-.249	-.278	.157	-.025
	p	.914	.737	.531	.721	.162	.117	.382	.890
↑ Lipids	*r*	.071	.073	.232	.154	-.107	-.203	.227	-.122
	p	.695	.693	.218	.391	.555	.257	.204	.498
Warfarin	*r*	.013	.055	-.236	-.136	-.013	.053	.255	-.055
	p	.941	.760	.209	.450	.941	.768	.152	.762
Aspirin	*r*	.208	-.212	-.132	-.029	-.333	-.345	.071	.187
	p	.246	.243	.486	.871	.058	.049	.695	.298
Statin	*r*	-.159	-.133	-.177	.040	.139	.132	-.154	-.185
	p	.469	.547	.455	.855	.526	.547	.482	.399
ACE-i / ARB	*r*	-.096	-.067	-.184	.078	.179	.159	-.220	.014
	p	.595	.717	.331	.668	.319	.377	.219	.940
Smoking	*r*	.195	.038	.228	-.385	-.265	-.199	-.128	-.030
	p	.276	.836	.225	.027	.137	.267	.478	.868
BMI	*r*	.142	.027	.036	.021	-.325	-.295	.293	-.058
	p	.489	.898	.869	.918	.105	.144	.147	.777
vWf	*r*		-.173	.070	-.049	-.179	-.137	-.209	.160
	p		.344	.714	.786	.319	.446	.243	.373
sEsel	*r*			.041	-.170	.133	.180	.019	-.150
	p			.834	.352	.467	.323	.916	.413
CPCs	*r*				-.140	-.022	-.035	-.038	.191
	p				.462	.908	.854	.841	.312

Table 27. Correlations of research indices to demographic data for disease-matched controls in sinus rhythm (continued).

Variables		vWf	sEsel	CPCs	sFas	sFasL	Fas:FasL	VEGF	IL-6
sFas	*r*					.130	-.129	-.122	-.178
	p					.472	.474	.499	.321
sFasL	*r*						.931	.243	-.436
	p						.000	.173	.011
Fas:FasL	*r*							.276	-.315
	p							.120	.074
VEGF	*r*								-.251
	p								.158

IHD – Ischaemic heart disease; HTN – Hypertension; CVA – CerebroVascular Accident; CCF – Congestive Cardiac Failure; DM – Diabetes Mellitus; BMI – Body Mass Index; ARB / ACE-i: Angiotensin-2 receptor blocker / Angiotensin converting enzyme inhibitor; vWf – vonWillebrand factor, sEsel – soluble E selectin; sFas – soluble Fas; sFasL, soluble Fas ligand; VEGF – Vascular Endothelial Growth Factor; IL-6 – Interleukin 6; CPC – Circulating Progenitor Cell. Data refers to Spearman correlation.

Table 28. Correlations of research indices to demographic data for healthy controls in sinus rhythm

Variables		vWf	sEsel	CPCs	sFas	sFasL	Fas:FasL	VEGF	IL-6
Age	*r*	.065	-.249	-.658	.147	.159	.294	.208	.522
	p	.791	.353	.003	.648	.587	.442	.407	.022
BMI	*r*	.442	-.018	-.487	.114	.315	.800	.456	.081
	p	.150	.957	.109	.787	.447	.104	.136	.802
Aspirin	*r*	-.105	-.261	-.461	-.524	.035	N/A	.130	.467
	p	.668	.329	.054	.081	.907	N/A	.608	.044
Smoking	*r*	-.157	-.082	.530	.088	-.153	-.274	-.308	-.180
	p	.522	.762	.024	.785	.602	.476	.214	.462
vWf	*r*		.069	-.232	-.124	.272	.433	.101	-.012
	p		.799	.355	.702	.347	.244	.689	.961
sEsel	*r*			.083	.146	.057	-.679	.649	-.360
	p			.767	.687	.861	.094	.009	.170
CPCs	*r*				-.365	-.240	-.357	-.390	-.458
	p				.270	.429	.385	.122	.056
sFas	*r*					.479	.227	.498	.146
	p					.192	.557	.119	.651
sFasL	*r*						.933	.364	.498
	p						.000	.222	.119
Fas:FasL	*r*							.263	.417
	P							.528	.265
VEGF									.000
									.998

BMI – Body Mass Index; vWf – vonWillebrand factor, sEsel – soluble E selectin; sFas – soluble Fas; sFasL, soluble Fas ligand; VEGF – Vascular Endothelial Growth Factor; IL-6 – Interleukin 6CPC – Circulating Progenitor Cell. Data refers to Spearman correlation.

Table 29. Multiple Linear Regression Analysis

Variables		vWf	sEsel	sFas	sFasL	VEGF	IL6	CPCs
Age	β*	.219	-.030	.119	.202	.115	.061	-.272
	P	.050	.787	.278	.087	.301	.595	.014
Hypertension	β*	.198	.354	.263	.154	-.094	-.011	-.155
	P	.194	.019	.063	.307	.538	.943	.305
Diabetes	β*	.032	.111	-.045	.081	.006	-.036	-.074
	P	.749	.261	.636	.434	.954	.727	.441
Stroke	β*	.094	-.027	-.171	.008	-.112	-.147	-.049
	P	.566	.866	.281	.963	.494	.388	.770
IHD	β*	.066	-.030	.051	-.014	.098	-.029	.005
	P	.435	.717	.534	.877	.255	.741	.950
CCF	β*	.107	.086	.081	.063	.209	-.062	-.117
	P	.389	.493	.498	.625	.099	.630	.311
↑ lipidaemia	β*	-.064	.132	.086	-.101	-.212	-.163	.369
	P	.567	.220	.421	.381	.066	.173	.001
Statin	β*	.047	-.075	-.030	.043	.054	.054	-.260
	P	.654	.456	.767	.687	.615	.629	.014
Smoking	β*	.090	-.266	-.133	-.001	-.094	-.055	.060
	P	.273	.001	.094	.986	.259	.520	.450
AF	β*	-.007	.103	-.100	.043	.022	-.012	.043
	P	.930	.210	.214	.619	.801	.894	.601
R^2		.075	.172	.171	.047	.109	.035	.192

*Numbers are standardized β co-efficients.
IHD – Ischaemic heart disease; CCF – Congestive Cardiac Failure; BMI – Body Mass Index; $CHADS_2$: Stroke risk algorithm (see text); ARB / ACE-i: Angiotensin-2 receptor blocker / Angiotensin converting enzyme inhibitor; AF – Atrial Fibrillation.

4.1.5 Discussion

To our knowledge, this is the first study to assess both the relationship between the temporal pattern of AF and CPCs. We demonstrate similar levels of CPCs across our cohort of patients with AF and co-morbidities, but significantly suppressed levels compared to subjects with lone AF and healthy controls. Furthermore we report similar levels of IL-6 and VEGF amongst all our patients with AF and co-morbidities. Our analysis also confirms previously reported observations of enhanced endothelial damage/dysfunction (vWf), and endothelial activation (sEsel), in patients with non-lone AF in comparison to both healthy control subjects in sinus rhythm and to lone AF (although levels of both indices were lower than that observed in AF with co-morbidities).

Finally, we have also investigated the relationship between AF and markers of Fas-mediated apoptosis. We report a complex series of observations with significantly higher levels of sFas amongst disease-matched controls compared to all non-lone AF and to lone AF, but lower levels of both sFas ligand and the sFas:sFas ligand ratio in lone AF than in healthy controls.

The present study suggests that both CPC counts and one index of apoptosis (sFas) are abnormal in (non-lone) AF compared to controls although these indices – as well as indices of endothelial damage/dysfunction, apoptosis, angiogenesis and inflammation – are probably more related to associated co-morbidities rather than to AF *per se*. We also confirm previously reported observations of enhanced endothelial damage/dysfunction (vWf), and endothelial activation (sEsel), in patients with AF in comparison to healthy control subjects in sinus rhythm and to lone AF.

CPC counts were lowest amongst the subgroups of patients with AF who had various co-morbidities and amongst disease-matched controls, when compared to those patients with either lone AF or healthy controls where higher counts were seen. The reasons for the observed CPC reduction in non-lone AF patients (impaired mobilisation or increased consumption) in the presence of greater endothelial damage/dysfunction/ activation are unknown, but our findings are consistent with diminished levels of CPCs and other populations of progenitors with angiogenic potential in other cardiovascular disorders and risk factors[231, 232].

Generally, CPCs probably reflect the co-existence of co-morbid disorders rather than relating solely to the presence of AF. Importantly, these findings parallel increased levels of vWf, sEsel and IL-6 levels in both non-lone AF and in disease controls. Similarly, the higher levels of CPCs seen in healthy controls and in patients with lone-AF mirror evidence of reduced endothelial damage (vWf) and endothelial activation (sEsel) in these subjects. This is contradictory to other research studies which have observed enhanced inflammation and coagulability in similar cohorts, but may simply be reflective of their younger age compared to other groups[99, 171, 326].

In keeping with previous studies we have shown raised levels of vWf amongst all patients with non-lone AF, with no significant difference based on temporal pattern[99, 326]. We also note levels of vWf in lone AF to be similar to that seen in healthy controls – in keeping with data from the Framingham study where co-morbidities rather than AF was felt to be the primary mechanism for vWf release[377]. sEsel is an index of endothelial activation, not normally expressed by resting endothelial cells,

but detectable at low levels in normal healthy controls[378]. As with vWf, raised levels are seen amongst patients with AF, but there remains some debate as to whether this relates to the presence of co-morbidities or relates to arrhythmia itself[379]. In keeping with this data, we also report raised levels of sEsel in AF patients and controls with co-morbidities but not in healthy controls or lone AF.

Regardless of whether endothelial 'activation' or 'damage/dysfunction' predominates, both processes have the potential to mediate thrombogenesis either directly by exposure of denuded endothelium to components of the coagulation cascade or through release of other regulatory molecules such as IL-6 or VEGF, both of which may also link to thrombogenesis[373] and even CPC release[171, 344]. In the present analysis, we did not find any association between AF temporal pattern, nor the presence / absence of co-morbidities on levels of either marker. Again, this presumably relates to a complex series of interactions clouded by the presence of numerous co-morbidities.

The Fas/Fas ligand pathway is one of a number of regulatory mechanisms through which programmed cell death (apoptosis) occurs[380]. Fas and its ligand are predominantly membrane bound and the apoptotic function of these molecules is well documented. Fas and its ligand are also found in soluble forms, but the precise function of these molecules remains unclear[381]. However, the ratio between these two molecules has been cited as a potential measure to assess cellular apoptosis[382]. In the present analysis, lower levels of sFas were seen in non-lone AF compared to disease-matched controls. sFas ligand levels and the sFas:sFas ligand ratio were both lower in lone AF compared to healthy controls, but levels of sFas were generally higher

amongst AF patients in the presence of co-morbidities. The relationship between AF and Fas-mediated apoptosis is therefore decidedly complex and it would appear that neither the temporal pattern of AF, nor the presence of the arrhythmia *per se* seem to have any influence on this process. The assumption therefore is that the various observed alterations in these markers likely reflect underlying co-morbid factors.

Study limitations

The cross-sectional nature of this study allows only for associations to be made rather than identification of cause and effect. The vast majority of patients with AF have a large number of co-morbidities. This in part, relates to the age of patients studied, where inevitably, other confounders such as ischaemic heart disease, hypertension, hyperlipidaemia are more likely to become apparent or at least become a target for therapeutic intervention. Also, AF may also occur as a consequence of many of these confounding variables and thus in a small study it is clearly not possible to establish the precise relationships between all variables. To accommodate for this, we recruited a number of patients with lone AF and also healthy control subjects. Both are difficult to recruit. The former because lone AF is a relatively rare entity; the latter because healthy controls are often not seen in a hospital setting where the majority of recruitable subjects are attending for treatment or investigation of important illness.

Similarly, each of the various parameters discussed may also influence one or more of the indices researched in this study either directly (e.g. endothelial damage as a direct consequence of hypertension) or indirectly (e.g. secondary repair mechanisms triggered as a consequence of such damage) and may also link to one another (section 1.2.6). It is therefore impossible to establish clear links.

Additionally, as there is no commonly accepted definition of circulating progenitors with angiogenic potential, it is possible that our results may not be applicable to all populations of angiogenic progenitors..

It was not possible to meet our pre-specified power calculation for every subtype of AF, although in some cases (e.g. permanent AF), the target was exceeded. This relates in part to reclassification of patients. For example, it became apparent that some patients initially assumed to have paroxysmal AF had in fact permanent AF. Also a number of patients, assumed to have persistent AF of recent onset were later felt so unlikely to maintain sinus rhythm (usually based on echocardiographuc appearances) and thus were only offered rate control. This strategy by default implies permanence of AF and thus again such patients were reclassified. This has inevitable implications when interpreting data with a broad spread in a relatively small sample and may explain why fluctuations in some indices (e.g. VEGF) although relatively profound in some of the analyses did not reach statistical significance. Similarly, the power calculation was based on anticipated variation in CPC numbers, as this was the main focus of the present work and thus may have been inadequate to allow full assessment of other parameters

Further limitations may be applied to the application of multiple statistical analyses. Indeed, the concept of $P<0.05$ implying significance means a 5% (or 1 in 20) chance that the observed finding is due to chance alone. This work inevitably required numerous statistical comparisons. The greatest potential for error relates to univariate and multivariate comparisons where a large number of statistical comparisons are made. For this analysis a $P<0.01$ was considered significant. However, despite this

deliberate choice, 1 in 100 analyses may still relate to chance and thus caution needs to be applied to prevent over-interpretation.

4.1.6 Conclusion

CPC counts and indices of apoptosis (sFas) appear to be abnormal in AF compared to healthy controls although these indices – as well as abnormalities of other markers including indices of endothelial damage/dysfunction, apoptosis, angiogenesis and inflammation – are probably more related to associated co-morbidities rather than AF *per se*. This data continues to add to the complexity of the hypercoagulable state in AF. It is clear that there are pertubations of a number of physiological regulatory mechanisms and cellular pathways leading to endothelial damage, activation, inflammation, apoptosis, angiogenesis and ultimately activation of the coagulation cascade.

4.2 Effects of Rhythm Change

Abstract

Background: Atrial Fibrillation (AF) is associated with a high rate of stroke, which may relate to a hypercoagulable state. The purpose of this study was to investigate whether restoration of sinus rhythm altered indices that may be reflective of the hypercoagulable state. We hypothesized that restoration of sinus rhythm would improve plasma indices of endothelial damage (vonWillebrand factor, vWf, soluble Eselectin, sEsel), apoptosis (soluble Fas/Fas Ligand, sFas/sFasL), inflammation (Interleukin-6, IL-6) and angiogenesis (Circulating Progenitor Cells, CPCs).

Methods: 22 consecutive patients (mean age 66.2 (10.3) years, 14 (64%) male) with persistent AF deemed suitable for direct current cardioversion (DCCV) were recruited. All patients were established on warfarin (target International Normalized Ratio, INR 2.0-3.0) for a minimum of 4 weeks prior to DCCV. Blood sampling was performed pre, immediately post and at 4-weeks post DCCV. Baseline results were compared to a cohort of 23 matched controls (mean age 67.1 (9.8) years, 16 (70%) male) who were known to maintain sinus rhythm.

Results: Patients and controls were comparable in all major parameters aside from use of anti-arrhythmic drugs (P=0.049) and anticoagulants (P<0.001) for which there was significant excess amongst the DCCV cohort. Anti-platelet usage was significantly higher amongst the disease-matched control arm (P=0.001). There was no significant difference in baseline levels of vWf, CPCs, sE-selectin, IL-6, or sFas/sFas ligand ratio between either groups. Control subjects had modestly higher

levels of sFas (P=0.003), but not sFas ligand compared to the AF cohort. Patients with AF had almost a 2-fold higher median level of VEGF at baseline compared to the controls, but this difference did not reach statistical significance.

Of the 22 patients who underwent cardioversion, there was a significant increase in median levels of CPCs (P=0.004) but no significant alteration in any of the other markers tested across the three timepoints. Despite an apparent rise in median VEGF levels of approximately 50% across the three timepoints, this was not a statistically significant. At discharge from hospital (2 hours following DCCV), 20 patients (91%) were in sinus rhythm. At 4 weeks, 17 patients (77%) continued to maintain sinus rhythm. Subsequent exploratory sub-group analysis was based on success at 4 weeks, and so the subjects with early relapse into AF were assumed to have failed cardioversion. Of the 17 patients who were successfully cardioverted into sinus rhythm, the increase in CPCs from baseline was significant (p=0.003), but not for those patients who relapsed back into AF (p=0.715). There was no significant change from baseline in any of the other markers studied.

Conclusion: DCCV seems to lead to higher levels of CPCs with a rise seen immediately following the procedure. This appears to increase further at 4-weeks. This does not appear to be associated with any significant change in vWf, sE-selectin, VEGF, IL-6 or sFas/sFas ligand. The present analysis is in part agreement and in part contradictory to other available data and likely reflects the notorious difficulty in studying a diverse population where multiple co-morbidities are present. Nonetheless, the increase in CPCs with cardioversion success may reflect improved vascular health and this warrants exploration with a larger study with greater statistical power.

4.2.1 Introduction

AF tends to be progressive and recurrent with a trend from paroxysmal towards more persistent or permanent forms of the arrhythmia with time. Cardioversion is an established treatment for AF aimed at restoration of sinus rhythm. Although this may occur spontaneously, in many cases this requires the use of pharmacological agents or an R-wave synchronized direct current shock (DCCV) under sedation (section 1.1.6). With successful restoration and maintenance of sinus rhythm, the effects of the arrhythmia in driving the three components of Virchow's triad are theoretically removed, potentially leading to lower thromboembolic risk. Although in practice, the high recurrence rate and delayed restoration of mechanical atrial systole have led to various guidelines recommending an extended period of anticoagulation[5, 6] (section 1.1.9).

The purpose of this study was to investigate whether restoration of sinus rhythm altered indices that may be reflective of the hypercoagulable state or which may be implicated in the pathogenesis of AF.

4.2.2 Hypotheses

These have been detailed previously in section 2.1.3. In brief, we hypothesized that successful restoration of sinus rhythm would improve plasma indices of endothelial damage / dysfunction (vWf, sEsel), apoptosis (soluble Fas/Fas Ligand), inflammation (IL-6) and angiogenesis (CPCs).

4.2.3 Methods

This has been described previously in section 2.2.2. In brief, 20 consecutive patients with persistent AF deemed suitable for DCCV. Persistent AF was defined as presence of AF on two 12-lead electrocardiograms (ECG) at least 7 days apart in patients where restoration of sinus rhythm was deemed attainable. Exclusion criteria are as previously described (section 2.2.2).

All patients were established on warfarin (target International Normalized Ration, INR 2.0-3.0) for a minimum of 4 weeks prior to DCCV. All patients underwent synchronized DCCV under brief general anaesthetic (propofol), using a Medtronik Physio-Control LifePak 9 monopasic difibrillator (Redmond, USA). Paddles were placed in the antero-lateral position and up to a maximum of 4 sequential shocks were delivered (100J, 200J, 360J, 360J).

Baseline results were compared to a cohort of age and sex-matched controls who had established cardiovascular risk factors, but who were known to maintain sinus rhythm.

Laboratory

Venous blood was drawn at baseline, immediately following DCCV and again at 4 weeks as described in section 2.4. Analysis for CPCs and ELISAs was performed as described in the appendix.

Power Calculation

This has been described previously in section 2.3.1.

Statistical Methods

This has been described previously in section 2.3.2.

4.2.4 Results

22 consecutive patients (mean age 66.2 (10.3) years, 14 (64%) male) with persistent AF undergoing elective DCCV were recruited. A group of 23 age- and sex-matched control subjects (mean age 67.1 (9.8) years, 16 (70%) male) were used for baseline comparison as disease-matched controls (table 30). Patients and controls were comparable in all major parameters aside from use of anti-arrhythmic drugs (P=0.049) and anticoagulants (P<0.001) for which there was a significant excess amongst the DCCV cohort. Anti-platelet usage was significantly higher amongst the disease-matched control arm (P=0.001). There was no significant difference in baseline levels of vWf, CPCs, sE-selectin, IL-6, or sFas/sFas ligand ratio between either group (table 31). The controls however had modestly higher levels of sFas (P=0.003), but not sFas ligand compared to the AF cohort. Also patients with AF had almost a 2-fold higher median level of VEGF at baseline compared to the controls, but given a broad spread, this difference did not reach statistical significance.

Table 30. Demographic Data for AF patients and control subjects

	Variable	Controls	AF	*P* value
	N	23	22	
Baseline Data	Age (SD)	67.1 ± 9.8	66.2 ± 10.3	.753
	Age > 75 (%)	6 (26)	4 (18)	.391
	Male (%)	16 (70)	14 (64)	.758
	Body Mass Index	30.5 ± 7.7	28.7 ± 6.1	.441
Risk Factors No. (%)	Smoking	9 (39)	12 (55)	.408
	IHD (Angina / MI / PCI)	11 (48)	4 (18)	.057
	Previous CVA	2 (9)	1 (5)	.517
	CCF	2 (9)	6 (27)	.135
	Hypertension	20 (87)	17 (77)	.459
	Diabetes Mellitus	3 (13)	0 (0)	.233
	Hyperlipidaemia	15 (65)	10 (45)	.236
	$CHADS_2$	1 (1-2)	1 (1-2)	.615
Medication, No. (%)	Antiplatelets	13 (57)	2 (9)	.001
	Warfarin	1 (4)	22 (100)	.000
	Beta-blocker	9 (39)	13 (59)	.238
	Calcium-channel blocker	11 (48)	6 (27)	.221
	Digitalis	0 (0)	4 (18)	.049
	Flecainide	0 (0)	3 (14)	.109
	Amiodarone	1 (4)	5 (23)	.096
	ACE-i/ARB	15 (65)	15 (67)	.586
	Statin	13 (57)	14 (64)	.763

Abbreviations: IHD – Ischaemic Heart Disease; MI – Myocardial Infarction; PCI – Percutaneous Coronary Intervention; ACE-I – Angiotensin Converting Enzyme inhibitor; ARB – Angiotensin-II Receptor Blocker; CCF – Congestive Cardiac Failure; CVA – Cerebrovascular Accident; $CHADS_2$: Stroke risk algorithm (see text). All Data expressed as Mean ± Standard Deviation (SD), No. (%) or Median (InterQuartile Range, IQR) unless otherwise stated. Comparisons between groups using the χ^2 test for categorical data or unpaired t-test for continuous parametric data. The Mann-Whitney U test was used for non-parametric data. A p value < 0.05 was considered statistically significant.

Table 31. Baseline Research Indices

	Variable	Controls	AF	*P* value
	n	23	22	
Research Indices	**vWf (IU/dL)**	139.8 ± 17.5	136.6 ± 13.5	.492
	sE-selectin (ng/mL)	64.0 (40.0-76.0)	64.0 (39.5-76.0)	.674
	CPCs (cells/mL)	66 (30-187)	85 (29-153)	.880
	VEGF (ρg/mL)	31 (0-125)	76.25 (20-290)	.114
	IL-6 (ρg/mL)	15 (10-16)	15 (10-24.25)	.766
	sFas (ng/mL)	5.0 (4.0-5.25)	3.6 (3.0-4.6)	.003
	sFas Ligand (ρg/mL)	200 (125-300)	188 (119-188)	.945
	sFas/sFasL ratio	44.4 (30.0-70.0)	48.2 (31.9-123.9)	.488

CPC – Circulating Progenitor Cells; vWf – von Willebrand factor; sEsel – soluble E selectin; IL-6 – InterLeukin-6, sFas – soluble Fas, sFasL – soluble Fas Ligand, VEGF – Vascular Endothelial Growth Factor. All data expressed as mean ± standard deviation (SD), or median (InterQuartile Range, IQR) unless stated otherwise; P-value refers to the results of unpaired t-test or Mann-Whitney U test as appropriate. A P-value < 0.05 was considered statistically significant.

Table 32 illustrates the alteration in markers studied for the cohort of the 22 patients who underwent cardioversion at three time points –pre, immediately post and 4-weeks post DCCV. There was no significant alteration in levels of vWf, sE-selectin, IL-6, sFas, sFas ligand or the sFas:sFas ligand ratio. Despite an apparent rise in median VEGF levels of approximately 50% across the three timepoints, this was not a statistically significant change. There was a significant increase in median levels of CPCs (P=0.004) across all three timepoints (figure 12).

At discharge from hospital (2 hours following DCCV), 20 patients (91%) were in sinus rhythm. At 4 weeks, 17 patients (77%) continued to maintain sinus rhythm. Subsequent exploratory sub-group analysis was based on success at 4 weeks, and so the subjects with early relapse into AF were assumed to have failed cardioversion (table 33). Of the 17 patients who were successfully cardioverted into sinus rhythm, the increase in CPCs from baseline was significant (p=0.003), but not for those patients who relapsed back into AF (p=0.715). There was no significant change from baseline in any of the other markers studied.

Correlations and regression analysis was not performed as this was completed on the entire study population as part of the cross-sectional study (section 4.1).

Table 32. The Effects of Direct Current Cardioversion

	Effects of DCCV			
Variable	**Pre**	**Immediate post**	**4-weeks post**	**P-value**
Heart rate	75 ± 20	72 ± 15	76 ± 20	.508
$BP_{systolic}$ (mmHg)	134 ± 17	132 ± 17	134 ± 16	.830
CPCs (/mL)	85 (29-153)	102 (78-195)	123 (70-215)	.004[a,b]
vWf (IU/dL)	136.6 ± 13.5	137.9 ± 26.1	137.5 ± 21.3	.979
sEsel (ng/mL)	64.0 (39.5-76.0)	64.0 (55.5-76.5)	68.0 (46.0-80.0)	.706
sFas (ng/mL)	3.6 (3.0-4.6)	3.5 (3.0-4.8)	4.5 (3.5-5.0)	.156
sFasL (ρg/mL)	188 (119-188)	200 (125-400)	300 (141-500)	.635
sFas:sFasL	48.2 (31.9-123.9)	50.0 (32.8-108.0)	70.5 (33.8-127.1)	.944
IL-6 (ρg/mL)	15 (10-24.25)	10 (0-17)	15 (10-22.5)	.302
VEGF (ρg/mL)	76.25 (20-290)	95 (27.5-136.25)	125 (62-232.5)	.665

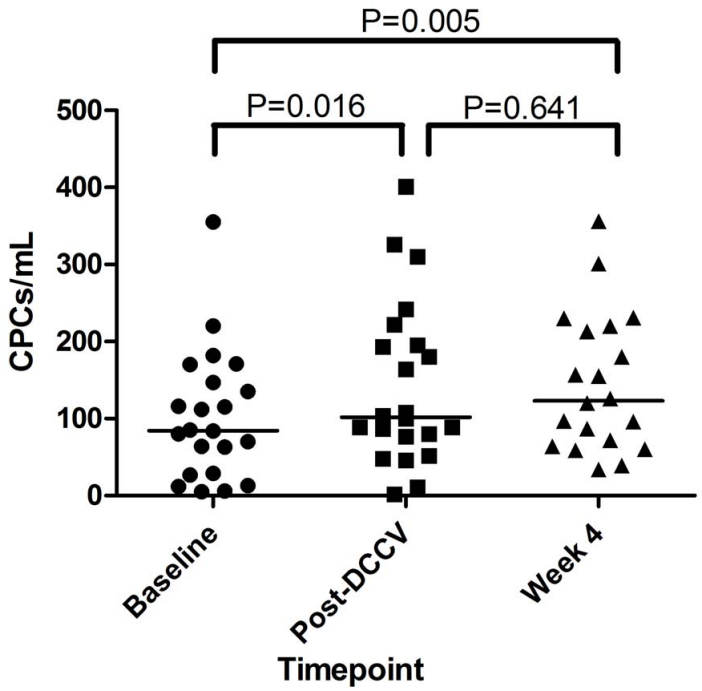
CPC – Circulating Progenitor Cells; vWf – von Willebrand factor; sEsel – soluble E selectin; IL-6 – InterLeukin-6, sFas – soluble Fas, sFasL – soluble Fas Ligand, VEGF – Vascular Endothelial Growth Factor, BP – Blood Pressure; DCCV – Direct Current Cardioversion. All data expressed as mean ± standard deviation (SD) or median (InterQuartile Range, IQR) unless otherwise stated; P-value refers to the results of Friedman's repeated measures ANOVA. A $P < 0.05$ was considered statistically significant and where appropriate Tukey's post hoc analysis applied: [a]pre and immediate post; [b]pre and 4-weeks post; [c]immediate post and 4-weeks post.

Table 33. Effect of cardioversion success on research indices

	Successful DCCV at 4-weeks			Unsuccessful DCCV at 4-weeks		
Variable	**Baseline**	**4-weeks**	**P-value**		**AF**	**P-value**
n	17			5		
CPCs (/mL)	82.0 (17-116)	140 (66-205)	.003	171 (46-269)	164 (50-284)	.715
vWf (IU/dL)	135.7 ± 15.3	133.9 ± 22.6	.462	138.2 ± 7.5	146.2 ± 16.4	.462
sEsel (ng/mL	34.0 (25.0-37.0)	35.5 (25.0-40.0)	.753	19.0 (16.0-37.5)	46.0 (26.0-82.0)	.785
sFas (ng/mL)	3.5 (3.0-4.5)	4.5 (3.3-4.8)	.117	4.5 (3.2-4.8)	4.0 (3.6-5.0)	.715
sFasL (ρg/mL)	187.5 (125.0-400.0)	325 (135-500)	.551	175 (87.5-475)	175 (148-450)	.686
sFas:sFasL	48.6 (34.6-138.3)	78.9 (36-131)	.605	40.0 (22.8-110.7)	42.9 (32-117)	.686
IL-6 (ρg/mL)	15.0 (7.5-22.75)	15 (10-20)	.894	20 (5-65)	20 (5-38)	.285
VEGF (ρg/mL)	120.0 (20.0-342.5)	132.5 (71.75-257.5)	.529	20.0 (10.0-895)	62 (35-512.5)	.686

CPC – Circulating Progenitor Cells; vWf – von Willebrand factor; sEsel – soluble E selectin; IL-6 – InterLeukin-6, sFas – soluble Fas, sFasL – soluble Fas Ligand, VEGF – Vascular Endothelial Growth Factor. All data expressed as mean ± standard deviation (SD) or median (InterQuartile Range, IQR) unless otherwise stated. Comparison from baseline values made using paired t-test or Wilcoxon test for parametric and non-parametric data respectively.

Figure 12 Impact of Cardioversion on Circulating Progenitor Cells



Scatter plot illustrating CPC counts for the entire cohort undergoing DC cardioversion. P-values from paired Wilcoxon test. P=0.004 using Friedman's repeated measures ANOVA for entire cohort.

4.2.5 Discussion

Although in theory, restoration of sinus rhythm offers the premise of reduced thromboemolic risk, the benefits of an aggressive cardioversion strategy have not translated into improvements in clinical outcome. The purported explanations for this have been discussed previously in section 1.1.6.

A number of previous studies have assessed the effect of cardioversion on various parameters including atrial endocrine function, platelet activation, and numerous coagulation and endothelial parameters[84, 154, 325, 383-387]. Alterations in some indices have been cited as reflective of improved 'vascular health', but the data is not consistent and although modest improvements in some markers are seen, this does not apply across the board and in other series there is a detrimental effect (section 1.2.5).

Our findings show a significant step-wise increase in CPCs following DCCV. Although an increase in CPCs also occurred in the DCCV failure group, this was a modest change and did not reach statistical significance. By week 4, there was a further significant rise in CPCs from baseline amongst those patients where DCCV had proven successful, but not in those where DCCV failed.

Similar work from other research groups observing $CD34^+$ cells following DCCV reported a downward trend in cell counts following DCCV success[388]. The argument from these and some other researchers is that endothelial damage is a potent stimulus for $CD34^+$ cell release and therefore in response to removal of this stimulus, circulating $CD34^+$ numbers decline[389, 390]. Of course, $CD34^+$ cells are a mixed population of various lineages and therefore only limited comparisons with our work

(where cells have been more strategically gated, albeit still not pure endothelial progenitors) is possible (section 3.1).

Although an increase in median VEGF levels across the entire cohort undergoing DCCV was observed, this did not reach statistical significance. This fluctuation was not observed in the subsequent subgroup analysis comparing those patients who maintained sinus rhythm at 4-weeks *vs*. those who reverted to AF (although the latter had VEGF levels approximately half of the former). This observation may therefore be a consequence of underpowering (although we exceded of our pre-specified power calculation) or be reflective of the rather broad spread in VEGF levels.

There was also no clear fluctuation in levels of the other research indices tested following DCCV. This may be considered surprising, particularly as some may link to the hypercoagulable state in AF[373], whilst others (i.e. VEGF) may be important in regulating CPC release[344]. Previous studies have observed alteration in levels of vWf in patients with AF and indeed alteration with DCCV[325], but again this is not a consistent finding across the literature [377] and therefore may represent publication bias of a larger negative study *vs*. a smaller later study with a chance finding. It is also plausible that failure to detect any difference was a result of low power.

Other researchers have assessed the influence of DCCV on levels of sE-selectin and found no significant alterations in plasma levels[325]. In keeping with this, we too have seen no alteration in sE-selectin levels following DCCV regardless of outcome. This together with no change in vWf levels permits speculation that the observed fluctuation in CPCs peri-DCCV may not be a consequence of alterations in peripheral

stimuli such as endothelial damage/activation. It is therefore presumed that the major contributor to endothelial damage/dysfunction (and hence vWf / other molecule release) amongst this cohort was the presence of co-morbidities rather than the arrhythmia. This would be in keeping with data from Framingham and the cross-sectional analysis previously performed (section 4.1). Although it is noteworthy that on multivariate analysis, a relationship between AF and CPCs was not defined (table 29). Thus it is possible that the alterations in CPCs observed following DCCV are not a true finding and merely a reflection of bias due to small numbers of highly selected patients.

In the present analysis, we observe similar levels of IL-6 amongst controls and patients undergoing DCCV, with little change in this serum marker of inflammation with cardioversion. Other studies have shown that markers of inflammation (e.g. hs-CRP & IL-6) can predict DCCV success[391-393]. Although once again, there is some disparity amongst available data with others showing no association[394]. The explanation for this observation may be similar to that used to explain the lack of fluctuation in other markers tested in this study. Most importantly, the heterogeneous nature of our population and in particular the number of co-morbidities associated with these patients – such that rhythm disturbance is not the predominant means for IL-6 release in this series of patients.

Significantly lower levels of sFas, but not sFas ligand, nor the ratio between the two were seen amongst the cohort of patients with persistent AF. Following DCCV, an upward trend in both median levels of sFas, sFas ligand and the ratio between the two was observed, although this did not reach significance either amongst those patients

who remained in sinus rhythm at 4 weeks or amongst those who reverted to AF. It is therefore assumed that cardioversion makes little or no impact on this mechanism for cellular apoptosis. This is perhaps not surprising given the complexity of the hypercoagulable state in AF, but given the increase in CPCs seen during the same timeframe, it could also be argued that lower Fas mediated apoptosis is unlikely to be the explanation for this observation and that the increase in CPCs likely reflects greater bone-marrow production / cellular release, for an as yet undefined stimulus.

Study limitations

The present study has a number of limitations. Once again, the first limitation relates to study design, which by virtue of the relatively small numbers included and large number of co-morbidities allows only for associations to be made rather than identification of cause and effect. Similar pitfalls also apply to the previous study and have already been discussed in detail (section 4.1.5). Such limitations are difficult to rectify, particularly as the number of patients referred for DCCV has dwindled in light of recent clinical trials, which have dramatically altered the perception of clinicians as to the effectiveness of a rhythm control strategy. Consequently, in our unit, the patients referred for DCCV are carefully selected and therefore may not be reflective of every patient deemed to have persistent AF or indeed those referred for cardioversion in other centres where application of guidelines may be less rigid. This may also explain the high cardioversion success rate seen at 4 weeks amongst the cohort of patient studied.

In addition, the highly selected nature of the cohort of patients undergoing DCCV may infer bias. This is particularly notable when the observed alteration of CPCs in

the present analysis is considered in light of the previous study (section 4.1.5, table 29) where no association between AF and CPCs was evident on multivariate analysis.

Although a series of controls were recruited for baseline comparison, these subjects were not followed longitudinally over the same time-course. Thus it is possible that observed alterations in the various markers tested simply reflects natural fluctuations in these parameters. Thus by studying small numbers of patients, we may have simply witnessed chance findings or conversely may have failed to detect a difference.

As discussed previously (section 4.1.5 and above) there remain important limitations related to power. Although our power calculation was exceeded, this was based purported alteration in CPCs from data initially analysed in a different laboratory using a different series of patients and different equipment. We also supported this calculation with previous studies from our unit that had detected a difference in vWf using a similar sample size to ours (section 2.3.1). However, many of the parameters tested had a broad spread and thus even given this initial calculation, the present study would appear to be underpowered. It is also feasible that previous studies demonstrating alterations in a number of parameters with cardioversion were also underpowered as these observations have not always been evident in larger studies (section 4.2.5).

Further limitations may be applied to the application of statistical analyses in subgroups as this will have a further detrimental effect on power and thus it is essential to acknowledge that such analyses were undertaken in an exploratory fashion.

4.2.6 Conclusion

DCCV seems to lead to higher levels of CPCs with a rise seen immediately following the procedure, but this is sustained and even increases further at 4-weeks. This does not appear to be associated with any significant change in vWf, sE-selectin, VEGF, IL-6 or sFas/sFas ligand. The present analysis is in part agreement and in part contradictory to other available data and likely reflects the notorious difficulty in studying a diverse population where multiple co-morbidities are present. Nonetheless, the increase in CPCs with cardioversion success may reflect improved vascular health and warrants exploration with a larger study with greater statistical power.

4.3 Inflammation and Arrhythmia Burden

Abstract

Background: Atrial Fibrillation (AF) is associated with a high risk of stroke and is thought to confer a hyercoagulable state fulfilling all three components of Virchow's triad (stasis, abnormalities of the endocardium and abnormalities of blood constituents). However, stroke risk is variable and underlying co-morbid factors seem to play a key role. In sustained forms of AF, the contribution of the arrhythmia to stroke appears clear and has been documented repeatedly in epidemiological studies. However, in paroxysmal AF, presently available data have yet to identify precisely what proportion of time spent in AF (i.e. arrhythmia burden, AFB) is of clinical relevance. We aimed to assess this relationship using surrogate blood markers for the hypercoagulable state associated with AF.

Methods: 121 consecutive outpatients [mean age 74.7 ± 7.8 years; 73 (60.3%) male] with pacemakers capable of arrhythmia detection were recruited. AFB was assessed over a 1-month period and classified as AFB=0% (sinus rhythm), 0.1-10% (low), 10.1-50% (medium), >50% (high AFB), to broadly reflect the patterns of AF commonly encountered in clinical practice.

Results: There was no statistically significant difference in age, gender, or co-morbidities. Stroke risk scores (calculated from $CHADS_2$) were similar in all groups. Rates of anticoagulation use were also comparable between groups except for the 'medium' AFB group where a larger number of patients receiving warfarin were observed. There were no significant differences in indices of endothelial damage (vonWillebrand factor, vWf), endothelial activation (soluble E-selectin, sEsel) or

inflammation (high sensitivity C-reactive protein, hsCRP) across the groups. Median levels of Interleukin-6 (IL-6) reduced in a stepwise fashion between groups with increasing AF burden (P=0.476). A sub-analysis of the 58 patients with 'true' paroxysmal AF (that is, excluding those with sinus rhythm or persistent AF from the analysis) demonstrated that the median number of AF episodes per day was 0.1 (Inter Quartile Range (IQR) 0-2.1) and median duration of these episodes was 0.5 (IQR 0.2-2.1) hours.

Conclusion: Data from the present analysis are potentially of importance and of direct clinical relevance when considering patients with paroxysmal and pacemaker defined AF. Although our understanding regarding the complexity of the hypercoagulable state in AF remains incomplete, we report variable aberration of prothrombotic markers in this cohort of patients with AF. However, given no appreciable relationship between these prothrombotic markers and AFB, it is it is plausible that these abnormalities do in fact relate primarily to underlying risk factors, and that such patients should be anticoagulated if risk factors dictate. Thus, AFB should probably not directly influence the decision to anticoagulate, but rather the presence of AF combined with clinical risk scoring algorithms should remain the predominant tool for stroke risk assessment. However, these findings must be interpreted with caution: the population studied was highly selected and data may not be applicable to the general population. Similarly, the data points had a broad spread and even though the pre-specified power calculation was met, it is clear that for some analyses to be interpreted, greater numbers still are required.

4.3.1 Introduction

Although the diagnosis of AF often follows the report of symptoms from the patient, frequently, this is not the case and AF is an asymptomatic incidental finding (section 1.1.3). Therefore, the true prevalence of this arrhythmia relates to how hard the clinician looks – in many cases, AF may only be detected on 24-hour (or longer) periods of ambulatory monitoring. This claim is strongly supported by the literature where up to 70% of AF episodes detected by trans-telephonic transmission are found to be asymptomatic[395].

In patients with symptomatic paroxysmal AF, many also have frequent bouts of asymptomatic arrhythmia[396, 397]. This can lull both the patient and clinician into a false sense of security and may even prompt premature discontinuation of anticoagulation for those patients believed to be maintaining sinus rhythm. This may, in part, explain the higher rate of stroke seen in the 'rhythm control' arm of recent clinical trials[398] (section 1.1.6). Thus, some clinicians routinely continue long-term anticoagulation for all AF patients deemed at significant stroke risk even if the arrhythmia is deemed 'cured', as only limited data exists to establish what frequency/duration of AF episodes (that is, AF arrhythmia burden) is of clinical significance[399].

The purpose of this study was to investigate whether a greater proportion of time spent in AF (that is, AF arrhythmia burden (AFB)) led to more extensive abnormalities of surrogate markers for enhanced coagulability.

4.3.2 Hypotheses

These have been detailed previously in section 2.1.3. In brief, we tested the hypothesis that in paroxysmal AF, the arrhythmia burden may influence the extent of systemic inflammation (IL-6 and hsCRP), endothelial damage (vonWillebrand factor (vWf)) and endothelial activation (soluble E-selectin (sE-selectin)).

4.3.3 Methods

The present study required access to a large cohort of patients with permanent pacemakers incorporating advanced AF detection algorithms (Vitatron 'T70' or Selection 9000 – see *Device Characteristics,* below). This required collaboration with another unit (Eastbourne District General Hospital) as our hospital only had access to limited numbers of suitable patients. Consecutive outpatients aged 18-85 were identified as those who had previous documented evidence of paroxysmal AF with at least two episodes within the last three months. Anti-arrhythmic therapy had been stable in all patients for at least five half-lives prior to study entry. Exclusion criteria have been discussed previously (section 2.2.3).

Pacemaker interrogation was performed at baseline to establish the 'AF burden' (AFB). The pacemaker Holter memory was then cleared. Following completion of the recruitment phase of the study, the pacemaker was interrogated and the AFB calculated once again. Patient groups were categorized on the basis of pacemaker interrogation as follows: sinus rhythm only [that is, AFB=0], 'low' AFB [0.1-10% time in AF], 'moderate' AFB [10.1-50% time in AF] and 'high' AFB [>50% time in AF]. These AFB groupings were selected to reflect commonly encountered

presentations of paroxysmal AF – i.e. patients with only occasional arrhythmia, those with regular episodes of AF and those with frequent recurrence of AF.

Device Characteristics

The Vitatron 'T70' or Selection 9000 pacemakers are dual chamber pacemakers with sophisticated algorithms for AF detection and prevention, and incorporate advanced AF detection and therapy algorithms. These devices have software tailored to detect the common mechanisms through which episodes of paroxysmal AF are initiated – by premature atrial complexes (PAC), by bradycardia or immediate reinitiation of AF[400]. AF detection is based upon atrial rate, whereby atrial tachyarrhythmias are detected when the median atrial cycle length is less than the programmed AT or AF detection interval and when the atrioventricular ratio is greater than 1:1 for a predefined number of cycles. Anti-AF pacing is then provided either in response to 'triggers' or by continuous overdrive atrial pacing. This study used the diagnostic capacity of these devices to determine AF burden, rather than their therapeutic functions. The algorithms for AF prevention therefore remained stable for the duration of the study.

Laboratory

Venous blood was drawn both at baseline and again at 4 weeks as described in section 2.4 and processed according to protocols in 2.4.4. As blood samples were obtained from patients in another unit some distance away it was not possible to analyses samples for CPCs.

Statistical Methods

This has been described previously in section 2.3.2.

Power Calculation

This has been described previously in section 2.3.1.

4.3.4 Results

121 patients [mean age 74.7 ± 7.8 years; 73 (60.3%) male] were recruited. There was no statistically significant difference in age, gender, or co-morbidities. Stroke risk scores (calculated from $CHADS_2$) were similar in all groups [table 34]. Rates of anticoagulation use were also comparable between groups except for the 'medium' AFB group where a larger number of patients receiving warfarin were observed. The use of anticoagulation in the sinus rhythm group reflects the design of the study where all patients included were documented to have AF at some point.

There were no significant differences in indices of endothelial damage (vWf), endothelial activation (sE-selectin) or inflammation (hsCRP) across the 4 patient groups [table 35]. Median levels of IL-6 reduced in a stepwise fashion between groups with increasing AF burden. This change however, did not reach statistical significance (P=0.476). A sub-analysis of the 58 patients with 'true' paroxysmal AF (that is, excluding those with sinus rhythm or persistent AF from the analysis) demonstrated that the median number of AF episodes per day was 0.1 (Inter Quartile Range (IQR) 0-2.1) and median duration of these episodes was 0.5 (IQR 0.2-2.1) hours.

Table 34. Baseline Characteristics of Subjects in relation to Atrial Fibrillation Burden

	Sinus rhythm [AFB=0]	AFB 0.1-10%	AFB 10.1-50%	AFB >50%	P-value
N	38	34	24	25	
Age	76.1 ± 8.4	73.4 ± 8.2	73.2 ± 7.4	75.7 ± 6.8	.317
Male	22 (57.9)	18 (52.9)	15 (62.5)	15 (60.0)	.897
AF Burden / %	0 ± 0	2.7 ± 3.3	32.5 ± 13.9	99.2 ± 4.20	.000
Hypertension	19 (50.0)	20 (58.8)	14 (58.3)	21 (84.0)	.055
LAd / cm	3.83 ± .67	4.1 ± .89	4.2 ± .55	4.4 ± .50	.182
LVFS / %	33.6 ± 9.4	35.6 ± 7.5	34.1 ± 7.6	30.4 ± 14.3	.575
Diabetes	5 (13.2)	3 (8.8)	0 (0)	4 (16.0)	.249
Hyperlipidaemia	14 (36.8)	17 (50)	11 (45.8)	14 (56.0)	.446
CAD	11 (28.9)	6 (17.6)	3 (12.5)	10 (40.0)	.119
Prior stroke	6 (15.8)	5 (14.7)	3 (12.5)	2 (8.0)	.820
$CHADS_2$ score	1.87 (1.45)	1.88 (1.49)	1.50 (1.22)	2.12 (1.01)	.299
Anticoagulation	16 (42.1)	16 (47.1)	21 (87.5)	14 (56.0)	.004
Smoker	6 (15.8)	1 (2.9)	1 (4.2)	1 (4.0)	.187
Statin use	22 (57.9)	16 (47.1)	17 (70.8)	15 (60.0)	.409
ARB/ACE-i	14 (36.8)	17 (50.0)	9 (37.5)	17 (68.0)	.199

AFB: atrial fibrillation burden; LVFS: Left ventricular fractional shortening from M-mode in parasternal long-axis plane; LAd: Left atrial diameter measured in parasternal long-axis plane; $CHADS_2$: Stroke risk algorithm score (see text); ARB: Angiotensin-2 Receptor Blocker; ACE-i: Angiotensin converting enzyme inhibitor.

Values are expressed as mean ± standard deviation or No. (%) unless otherwise indicated. Analyses were performed using the χ^2 test for categorical data or one-way analysis of variance for continuous data. Tukey's post hoc analysis was performed where appropriate. A p value < 0.05 was considered statistically significant.

Table 35. Endothelial, inflammatory and coagulation indices, in relation to Atrial Fibrillation Burden

	Sinus rhythm [AFB=0]	AFB 0.1-10%	AFB 10.1-50%	AFB >50%	P-value
vWf (IU/dL)	133.2 (14.3)	136.3 (18.3)	132.0 (26.9)	139.5 (19.1)	.518
Sol E-selectin (ng/mL)	56.0 (38.0-68.0)	68.0 (50.0-74.0)	64.0 (30.0-76.0)	68.0 (45.0-84.0)	.296
Il-6 (ρg/mL)	15.6 (7.0-60.0)	11.0 (5.1-33.3)	10.0 (5.0-38.0)	9.0 (4.0-30.0)	.476
hsCRP (μg/mL)	2.4 (3.3)	3.0 (3.8)	2.5 (3.8)	2.5 (3.0)	.329

All data are mean (SD) or median (IQR) as appropriate. AFB, atrial fibrillation burden; vWf, von Willebrand factor, IL-6, interleukin-6; CRP, C-reactive protein; sol, soluble. Analysis by oneway ANOVA or Kruskall-Wallis for parametric and non-parametric data respectively

No correlations were observed between levels of sE-selectin and vWF (r = -0.015, P = 0.886) or between hsCRP and IL-6 (r = -0.021, P = 0.835) [table 36]. A positive correlation was observed between levels of sE-selectin and both hsCRP (r =0.296, P = 0.003) and IL-6 (r = 0.306, P = 0.003).

Following a step-wise multiple linear regression analysis, age emerged as a significant predictor of vWf (β = 0.354, P=0.010*)*. Left ventricular ejection fraction was predictive of sE-selectin (β=0.335, P=0.020). Anticoagulation was a predictor of vWf levels (β=0.387, P=0.005) [table 37].

Table 36. Correlations between research indices

Variables		sEsel	IL-6	vWf
hsCRP	*R*	.296	-.021	.139
	P	.003	.835	.144
sEsel	*R*		.306	.007
	P		.003	.946
IL-6	*R*			-.129
	P			.186

vWf – von Willebrand factor; IL-6 – interleukin-6; CRP – high sensitivity C-reactive protein; sEsel – soluble E Selectin.Correlations relate to Spearman's method

Table 37. Multiple Linear Regression Analysis

Variables		vWf	hsCRP	sEsel	IL-6
Arrhythmia burden (%)	*β**	.039	.157	.044	.266
	P	.877	.581	.751	.376
Age	*β**	.354	.018	-.281	.028
	P	.010	.899	.105	.856
Gender	*β**	-.173	-.257	.108	.269
	P	.217	.103	.455	.107
Hypertension	*β**	-.190	-.062	-.067	.023
	P	.169	.681	.765	.890
Diabetes	*β**	.063	-.028	.000	-.087
	P	.621	.843	.998	.554
Stroke	*β**	.009	-.043	-.172	-.036
	P	.946	.762	.457	.816
$CHADS_2$	*β**	.374	-.540	.338	.058
	P	.321	.188	.361	.896
ARB / ACE-i	*β**	.140	.027	.020	-.207
	P	.293	.850	.893	.837
IHD	*β**	-.060	.122	.052	-.145
	P	.663	.412	.710	.365
LVFS	*β**	.004	.135	.335	.072
	P	.975	.332	.020	.626
↑ lipidaemia	*β**	.158	.184	-.064	.003
	P	.223	.197	.678	.982
Warfarin	*β**	.387	-.165	.018	.048
	P	.005	.258	.897	.763
R^2		.251	.105	.199	.115

Table 34. Multiple Linear Regression Analysis (continued)

Variables		vWf	hsCRP	sEsel	IL-6
Smoking	*β**	.226	-.033	.096	.125
	P	.090	.974	.495	.416
Statin	*β**	.142	.167	-.126	.115
	P	.292	.868	.416	.456
LA diameter	*β**	.128	-.140	.195	.036
	P	.322	.320	.183	.812

* Numbers are standardized β co-efficients
LA – Left atrium; LVFS – Left ventricular fractional shortening; IHD – Ischaemic heart disease; CHADS2: Stroke risk algorithm (see text); ARB / ACE-i: Angiotensin-2 receptor blocker / Angiotensin converting enzyme inhibitor.

4.3.5 Discussion

Our analysis shows no significant inter-group difference in indices of inflammation, endothelial damage or endothelial activation. These findings may support the notion that the abnormalities of the prothrombotic state seen in AF may simply be reflective of underlying co-morbidities[174], rather than being reflective of the presence or absence of AF *per se*. Indeed, it is relatively uncommon to encounter patients with AF who do not have at least one (and usually multiple) co-morbidities (section 1.1.2). Importantly, such conditions tend to be predominantly of a 'vascular' nature (e.g. hypertension) and consequently may have the potential to drive low-grade systemic inflammation, generate endothelial damage and promote platelet activation and therefore may, in their own right, lead to a state of enhanced coagulability[46, 159] (section 1.2.2). Also, this study could be of importance in defining the role of pacemaker-detected AF (and burden) in clinical practice as this concept has not been adequately explored.

The body of literature available addressing arrhythmia burden in paroxysmal AF and risk of stroke is limited. Nonetheless, there are ample available data to conclude that paroxysmal AF should be considered as an important clinical entity[398, 399]. The data investigating AFB to stroke risk is particularly sparse and it is therefore not possible from the presently available literature to conclude precisely what AFB increases the stroke risk in paroxysmal AF to levels seen with sustained forms of AF. The absence of significant inter-group differences in surrogate markers of the hypercoagulable state seen in our analysis, adds further weight to the concept that arrhythmia burden alone is *unhelpful* in establishing stroke risk and that the decision to anticoagulate should therefore be based upon assessment of clinical risk factors.

Inflammatory molecules, in particular IL-6 may be important in linking inflammation and thrombogenesis in AF by enhancing platelet production of and sensitivity to thrombin[169] and stimulating transcription of fibrinogen[170]. IL-6 is also linked to endothelial activation[171] offering a further plausible link with activation of the coagulation cascade as the process of endothelial damage may occur as a consequence of thrombosis and thereby enhance availability of molecules such as tissue factor (TF). It is interesting that IL-6 seemed to trend downwards in this study amongst patients with increasing AFB, particularly given that other studies have suggested the converse to be true – amongst patients with increasing temporal patterns of AF (i.e. paroxysmal vs. persistent vs permanent) – see section 1.2.6.

Nonetheless, another important link between inflammation and thrombogenesis is provided by study of E-selectin. This glycoprotein is expressed solely on endothelial cells and only following activation by inflammatory cytokines (e.g. IL-1β, TNF-α) or endotoxin[401, 402]. Expression of E-selectin is transitory and appears to mediate leukocyte adhesion thereby promoting or reflecting localized inflammation[403]. E-selectin may therefore reflect either endothelial damage or activation and may also link inflammation and thrombogenesis in AF[375]. Thus there would appear to be an intimate relationship between AF, inflammation, endothelial damage and activation of the coagulation cascade (section 1.2.2).

The general lack of correlation between the various markers and clinical characteristics of the patients studied is interesting but perhaps not surprising. To interpret this, one must consider that previous studies have also demonstrated diversity in the strength of association between clinical risk factors and various blood

markers tested[46, 159]. In addition it is important to note that some markers reflect more than one pathophysiological process. Thus, there may be subtle and diverse differences in the molecular mechanisms that result in release of each molecule, and a process that (for example) drives the release of vWf may only minimally alter levels of sE-selectin and vice versa. Similar arguments may also apply to hsCRP and IL-6, although despite this, modest correlations were seen between these two markers. Additionally weak correlations were also observed between IL-6 and sE-selectin, and hsCRP and sE-selectin. The implication therefore is that low-grade systemic inflammation may 'drive' or even be a consequence of endothelial damage/dysfunction – a concept that has already been suggested (section 1.2.6). Similarly, it could be concluded that inflammation (or even endothelial damage) could lead to platelet activation and such claims would be in keeping with existing data suggesting a complex and intimate relationship between the various vascular risk factors, AF and the hypercoagulable state[46, 159]. In addition, there was no correlation between vWf and frequency of AF episodes nor with median episode duration. Again this may suggest that patient baseline characteristics are an essential component of the hypercoagulable state.

On multiple linear regression analysis, age appeared to be predictive of endothelial damage (vWf). There also appeared to be a relationship between anticoagulation and vWf, which is difficult to explain. However, patients receiving anticoagulation are in fact older with a higher AF burden and more co-morbidities, each of which could potentially mediate vWf release through greater degrees of endothelial damage or dysfunction. Alternatively, these findings could relate to chance given that numerous parmeters were assessed..

Study limitations

The main limitation of this study is its cross-sectional design and that the present analysis does not include a matched cohort of 'healthy control' subjects in sinus rhythm to act as a baseline comparator. However, each of the markers assessed in this study have already been extensively investigated in patients with AF and aberrancies of each have been consistently reported compared to healthy control subjects. However, many of these aforementioned studies are of variable size and quality and therefore precise relationships between markers and AF remains open to debate. Indeed, as previously discussed in section 4.1.5 and section 4.2.5, there are notable inconsistencies amongst the published literature.

This study is also relatively small sized but we are in fulfilment of our prespecified power calculation. Nonetheless, some of the markers tested had a wide range and thus a power calculation based on vWf and IL-6 may not apply universally to all markers tested as each will have different characteristics and laboratory assays may differ.

The patient cohort considered here is highly selected – each had a permanent pacemaker and thus may not only have a different spectrum of ill health or co-morbidities to the general population with AF, they may also have been followed up in a different manner too. Some may remain under active follow-up in a cardiology clinic, whilst others may remain under follow-up with a pacing clinic where the pacemaker is interrogated periodically for function and battery life. Either option may imply key differences between this cohort and other patients with paroxysmal AF.

It would be also be difficult to generalize these observations to all paroxysmal AF patients, given the wide spectrum of this disorder and given the importance of "the state of AF" as a risk factor for thrombotic events, especially in the presence of co-morbidities[404]. Also there remains an important distinction between 'device-defined AF' and paroxysmal AF in the traditional sense as the former is based upon a series of algorithms.

Finally, the half-life of many of the markers studied is considerably shorter than the 1 month period over which AFB was assessed. Thus the influence of AF on the research indices may be lessened, particularly in cases with a very low AFB. This consideration would be difficult to overcome given the broad range of AFB observed amongst the cohort of patients studied.

4.3.6 Conclusion

Data from the present analysis are of particular importance and of direct clinical relevance when considering patients with paroxysmal and pacemaker defined AF. Although our understanding regarding the complexity of the hypercoagulable state in AF remains incomplete, we again report variable aberration of prothrombotic markers in this cohort of patients with AF. However, given no appreciable relationship between these prothrombotic markers and AFB, it is it is plausible that these abnormalities do in fact relate primarily to underlying risk factors, and that such patients should be anticoagulated if risk factors dictate. Thus, AFB should probably not directly influence the decision to anticoagulate, but rather the presence of AF combined with clinical risk scoring algorithms should remain the predominant tool for stroke risk assessment.

Section V: Conclusion

5.1 Summary of Findings

The main objective of this work was to further dissect the complex hypercoagulable state that seems to be associated with AF and establish novel mechanisms that may link and drive underlying processes such as inflammation and angiogenesis. This work has involved several comprehensive literature reviews, each of which has already been published in peer-reviewed scientific journals.

5.1.1 Literature appraisal

The first of the literature reviews (section 1.1) investigated the importance of AF as a clinical entity and outlined comprehensively the management of this arrhythmia, which at times can be particularly challenging and often requires specialist opinion and/or intervention. In particular this review emphasized the clinical sequelae of AF, the most important and evident being stroke. In section 1.1.9, the review continues to offer a literature appraisal of thromboprophylaxis by both antiplatelet agents (namely aspirin) and Vitamin K antagonists (warfarin). This review emphasises that a thromboprophylaxis decision is not always clear-cut and that individual assessment with reference to the published literature is essential to provide the most appropriate management strategy to our patients.

The second literature review (section 1.2) focussed on the hypercoagulable state in AF and in particular considered how, through very complex mechanisms and interactions, this arrhythmia is able to satisfy all three components of Virchow's triad for thrombogenesis. As previously discussed, stasis remains the most evident and

obvious component of Virchow's triad during periods of AF (section 1.2.4). However, it is clear that a broad spectrum of abnormalities do in fact exist ranging from evidence of vessel wall (endocardial / endothelial) disruption to aberrancies of various blood constituents (sections 1.2.3 & 1.2.5). The latter range in diversity from those seemingly connected directly to AF and the coagulation cascade to factors that have may provide a link between various mechanistic processes. Although not fully understood, in section 1.2.6, the review has discussed in detail various processes that may be involved in driving the thrombogenic tendency. Such a comprehensive review has not been published previously and it is hoped that this will provide a framework for other investigators to build upon.

The final literature review (section 1.3) has assessed the relevance of EPCs within cardiovascular medicine and discussed the data supporting these rare cells as important identifiers of patients at high cardiovascular risk. More importantly, this review discusses the purported role for these cells in replenishing the endothelium – a vital organ and which is implicated in the pathophysiology of numerous cardiovascular diseases, including one that may be central to thrombogenesis in AF.

EPCs have evolved in definition since first being characterized in early experiments. This has had inevitable consequences as many studies discussed in this review have used differing antigenic definitions and thus cells assessed by one research group are not necessarily the same as those assessed in other experiments. Additionally, many of the published studies have been on small highly selected groups of patients or have been conducted *in vitro*. Thus inconsistencies within some of the published literature are to be anticipated. Nonetheless, EPCs are still considered an important

physiological tool for endothelial regeneration and strong, consistent correlations with outcome continue to ignite excitement about their potential as a therapeutic tool.

5.1.2 Laboratory Studies

In section 3.1, the phenotypic definition of EPCs has been discussed, as has the concept of 'antigenic shift' whereby the surface markers of EPCs changes over time and with cellular maturation (table 12). This, of course, presents a significant problem to researchers and choosing which subpopulation of EPCs to investigate can be challenging. Particularly given the angiogenic potential of cells also alters as they mature[221-224]. Thus investigating a more mature population of cells (which by this point have become highly selected and some of which may have already become integrated into the endothelium), may not allow for assessment of the true capacity of EPCs for neoangiogenesis. Importantly, given the heterogeneity of the EPC population, a robust definition still does not yet exist[225]. The lack of universal agreement on the definition of true EPCs and the difficulty in reliably identifying a subpopulation with an evolving phenotype was recognized early in this work. In response to this observation, we opted to change nomenclature from EPC to CPC for the remainder of the study (section 3.1.2).

In section 3.2, the two main laboratory techniques for enumerating CPCs were compared and contrasted. Both processes have a steep learning curve and require meticulous attention to detail. Cell culture in addition requires a sterile working environment, utilized effectively to minimize contamination and growth of bacteria and yeasts. Despite this, reproducible results for either technique were achievable. We however report very little correlation between the two modalities and observed

frequent inconsistencies in various parameters during cell culture: (1) the size of EC-CFUs and proportion of single cells fluctuate significantly, even on the same culture plate; (2) the ability of CPCs to migrate towards one another to form EC-CFUs varies; and (3) the rate of CPC differentiation and proliferation may affect the number of EC-CFUs, despite similarities in CPC counts on separate plates. In contrast, we considered that the count of differentiated cultured CPCs by flow cytometry with specific mature endothelial markers was a potentially more objective alternative. It was on that basis, we continued the remainder of the study using only flow cytometry. Naturally, this may have limitations given that one important aspect of CPC biology is the ability of these cells to replicate and differentiate. This functional aspect of CPCs cannot be assessed by flow cytometry, which allows only assessment of cell counts at a static time point rather than allowing dynamic assessment. Also, the lack of universal agreement on EPC/CPC definition by either flow, or cell culture implies that one cannot be certain that the two subsets are comparable or complimentary when assessed simultaneously. One potential way to overcome this may have been to count CPCs and perform a cell sort using the flow cytometer at baseline. These cells could then be plated and undergo cell culture for later characterization. However, this was not possible with the available equipment within our laboratory. Also, cell culture may have been influenced by the conjugation of antibodies and additional processing steps.

Although much has been learnt about the endothelium and about CPCs, there remain some startling gaps in our knowledge. In section 3.3, we sought to define further some of the basic (patho) physiological properties of the group of CPCs that were to be studied in the remainder of the project. In particular, we investigated the influence of

exercise treadmill testing (ETT) on peripheral CPC levels, diurnal variation of CPC counts and also the rate of temporal decline in CPCs once held in blood transit tubes *ex-vivo*. The latter two components were considered of fundamental importance when assessing CPCs in the laboratory.

Exercise led to significant increases in systolic blood pressure, heart rate, vWF and sE-selectin levels, but no significant influence on CPC counts were observed (section 3.3.3). An *exploratory* subgroup analysis assessed patients with a negative ETT *vs*. those unable complete the protocol (positive or inconclusive ETT). The cohort with a positive ETT had higher median CPC counts across all three time-points, although the ΔCPC (from rest to peak exercise) were similar. None of these differences reached statistical significance – possibly due to being underpowered. There were no major differences in sE-selectin or Vwf levels across the three timepoints, although patients with a positive test had borderline significantly lower vWf levels immediately after the test.

Median CPC counts showed significant diurnal variation (P=0.011), being significantly higher at 12am compared to 12pm (P=0.046) and 12pm compared to 6pm (P=0.023). Delayed sample preparation by 4 hours did not significantly affect the number of CPCs detected, compared to baseline, but there was a significant decline in CPC recovery observed when sample processing was delayed by 24 hours (P=0.019).

These initial results were used to help standardize our assessment of CPCs during the remainder of the project. It was felt that this was of particular importance given the rarity of CPCs and thus essential to ensure that all patients, sampling, techniques and

measurement errors were consistent. In all cases, patients were asked to have been resting for at least 30 minutes prior to blood sampling, venepuncture was, where possible, completed at comparable time points and blood processing completed promptly.

5.1.3 Cross-sectional analysis

The cross-sectional study presented in section 4.1 was designed to allow analysis of patients with AF (classified by temporal pattern, section 1.1.4) and both healthy and disease-matched controls in sinus rhythm and aimed to establish association between CPCs, endothelial damage/activation and other processes that may link to the hypercoagulable state – namely inflammation, angiogenesis and apoptosis and to further explore whether previously reported aberrations in these and similar parameters may be a consequence of AF or due to associated co-morbidities (section 1.2.5).

In this study (section 4.1.5), comparison of all non-lone AF to the disease control group demonstrated a significantly lower level of sFas in the former ($P<0.001$), with the lowest levels of the marker observed in patients with persistent or permanent AF. Patients with lone AF had similar levels of all the research indices to the healthy control arm, except for sFas ligand and the sFas:sFas ligand ratio, both of which were significantly higher amongst the healthy controls ($P=0.006$ and $P=0.014$ respectively).

Median CPC levels were significantly higher in lone AF compared to 'non-lone AF' ($P<0.001$). Lower levels of both sE-selectin ($P=0.042$); and vWf ($P=.013$) were seen

in lone compared to non-lone AF patients The presence/absence of AF was not predictive of any of the research indices tested.

This study therefore suggests that CPC counts and one index of apoptosis (sFas) appear to be abnormal in AF compared to healthy controls, although these indices – as well as abnormalities of other markers indirectly assessing endothelial damage/dysfunction, apoptosis, angiogenesis and inflammation – are probably more related to associated co-morbidities than to AF or indeed the temporal pattern of the arrhythmia *per se*. However, these preliminary conclusions need to be considered in light of a number of limitations. Many of these relate to study design, the application of statistical analyses and power. The latter, despite our pre-specified recruitment numbers being broadly met, it was clear that many analyses had a broader spread than was initially anticipated.

There are numerous reports of increased vWf and sE-selectin amongst patients with AF with no relationship to the temporal pattern of the arrhythmia[99, 326, 377, 379]. The data from this analysis would be in keeping with this assessment. However, we also report that levels of vWf and sE-selectin in lone AF are similar to levels seen in healthy controls (and lower than levels seen in non-lone AF) and this raises the possibility that the predominant driver of vWf / sE-selectin release is in fact co-morbidities rather than the rhythm disturbance. This finding is in contrast with previous work from our research group where elevated levels of vWf were seen in lone AF[326], but is in agreement with data from the much larger Framingham study[377]. These two seemingly contradictory observations may relate to 'publication bias' – a

small study with a positive finding has a better chance of being published and therefore it may be prudent to accept the stronger Framingham study as more reliable.

There are presently no other significant studies assessing CPCs in AF and therefore the findings in the present analysis reporting suppressed levels of CPCs amongst the subgroups of patients with AF who had various co-morbidities and amongst disease-matched controls compared to those patients with lone AF or healthy controls where higher levels were seen are novel. The lack of difference in CPC numbers between temporal subtypes of AF observed in this study is similar to that seen with the other endothelial markers studied.

Importantly, the differential CPC numbers observed between those with and without co-morbidities raises the possibility that CPCs may somehow be implicated in the hypercoagulable state (note that the patients with lone AF did by definition have a $CHADS_2$ score of 0). This of course may simply reflect the known utility of CPCs to reflect vascular health – but such implications can be drawn from other studied markers also. It is, tempting to speculate that with enhanced endothelial damage/activation in the presence of AF and co-morbidities, reduced CPC numbers simply reflects consumption. Investigating which of these options is the most plausible is difficult, in part due to significant confounders and in part due to diversity of the progenitor cell population.

Previous investigators have also reported that levels of various inflammatory cytokines are related to the temporal pattern of AF (sections 1.2.5 and 1.2.6), although again, the data is not consistent and there are some discrepancies between studies and

between the various molecules tested[159]. In this analysis, we found no alteration in IL-6 based on temporal pattern of AF and interestingly found levels in lone AF and in healthy controls to be similar to levels seen in non-lone AF, suggesting in this cohort at least that IL-6 mediated inflammation was not an important mechanism.

There are numerous plausible explanations for these findings and the discrepancies in IL-6 / vWf / sE-selectin data. It is important to note that patients considered to have lone AF are highly selected, younger and have no co-morbidities aside from rhythm disturbance and therefore, the research indices measured are more likely to reflect the arrhythmia. One must also consider the notion that 'lone AF' is perhaps a misnomer. Indeed, it is a particularly rare entity and if these patients are kept under follow-up then it is likely many of them will develop a number of co-morbidities over time. It may therefore be argued that at least some are 'pre' diabetic or 'pre' hypertensive and that their co-morbidities have not yet become clinically detectable. Also, some of these patients had paroxysmal AF and were in sinus rhythm at the time of blood sampling – potentially masking the effects of the arrhythmia on the parameters measured.

In addition, section 1.1.2 documents some of the vast array of co-morbidities that frequently associate with AF and many of these were not assessed or corrected for in this analysis and it is possible that these parameters act as significant confounders thereby driving release of various molecules.

In summary, this data adds to the complexity of the hypercoagulable state and now permits us to consider the possibility that aberrations in CPCs may also be implicated.

However, the precise link between these various pathways is not clear and it is not possible to identify cause or effect, nor exclude the interactions from various co-morbidities.

5.1.4 Longitudinal study

The cross-sectional study presented in section 4.2 was designed to allow analysis of patients with persistent AF undergoing elective DCCV and allow comparison with a cohort of disease-matched controls in sinus rhythm. The aim of this study was to try and establish whether AF may directly influence the parameters that have already been considered as part of the longitudinal study. Analysis of a cohort of patients undergoing DCCV allows for intra-individual assessment over a period of time during which attempts were made to re-establish sinus rhythm. Such an approach potentially allows for more accurate assessment of whether the change in rhythm was of direct relevance to the various research parameters.

Our results indicate that there was no significant difference in baseline levels of vWf, CPCs, sE-selectin, IL-6, VEGF or sFas/sFas ligand ratio between the cohort with persistent AF undergoing DCCV and the disease-matched controls in sinus rhythm. The latter group however had modestly higher levels of sFas, but not sFas ligand. Also patients with persistent AF had an almost 2-fold higher level of VEGF at baseline, although this did not reach statistical significance.

For the persistent AF group, there was a significant increase in median levels of CPCs across the peri-DCCV period (P=0.022). Althoguh there was an increase in VEGF across all three timepoints this was not significant and there was no change in the

other research parameters. Of the 17 (77%) patients who successfully maintained sinus rhythm at 4 weeks, the increase in CPCs from baseline was significant (p=0.003), but not for those patients who relapsed back into AF (p=0.715). There was no significant change from baseline in any of the other markers studied.

Previous research has investigated various parameters peri-DCCV (section 4.2.5) and has reported variable response of vWf, VEGF and IL-6. In studies where improvements in these indices are seen, investigators have cited their findings as reflective of improved vascular health following restoration of sinus rhythm. However results are not consistent across the literature with a number of conflicting outcomes. Other research groups have investigated CPCs (albeit using an alternative definition) peri-DCCV and demonstrated that the number of these cells trends downwards following successful restoration of sinus rhythm. This is in contrast to our analysis where the opposite effect is seen, but this is not mirrored by improvements in the other research indices studied and therefore the interpretation of these findings remains open to debate.

It is possible that the increase in CPCs may reflect improved vascular health of patients maintaining sinus rhythm. However, section 4.1 has already considered various parameters including CPCs and analysed results according to AF temporal pattern and the presence / absence of co-morbidities. This study was highly suggestive that it was factors other than arrhythmia that led to alterations in CPCs and other markers. It therefore seems less plausible that rhythm change in this analysis was the sole cause for alteration in CPC counts.

As discussed previously (section 5.1.3), it is likely that similar limitations to those discussed in the longitudinal study also apply to this analysis including difficulty in assessing patients with a broad spectrum of co-morbidities, and various concerns that relate to CPC definition.. This includes the highly selected nature of these patients where the diagnosis of persistent AF and suitability for cardioversion was based upon history and clinician perception of cardioversion potential.

Also, the study design meant that patients undergoing DCCV only had a comparator group at baseline and therefore there was only a limited control group. Further strength may have been added by also following the control group for serial sampling over the 4-week period with repeat blood sampling.

In summary therefore, DCCV seems to lead to higher levels of CPCs with a rise seen immediately following the procedure. This appears to increase further at 4-weeks, but does not appear to be associated with any significant change in vWf, sE-selectin, VEGF, IL-6 or sFas/sFas ligand. The present analysis is in part agreement and in part contradictory to other available data and likely reflects the notorious difficulty in studying a diverse population where multiple co-morbidities are present. Nonetheless, the increase in CPCs with DCCV success *may* reflect improved vascular health and this warrants exploration with a larger study with greater statistical power.

5.1.5 Arrhythmia burden study

The AF arrhythmia burden study is presented in section 4.3. As discussed previously, it is unclear from the literature what AF episode frequency / duration is of clinical significance. Essentially this study was designed to try and establish whether indices implicated in the hypercoagulable state were altered to a similar extent when analysed in subgroups with different AFBs ranging from predominantly sinus rhythm to predominantly AF. It was unfortunately not possible to assess these patients for CPCs as they had to be recruited in collaboration with another centre some distance away. Thus we only had the ability to store and process frozen serum.

Based on an analysis comparing sinus rhythm to low (1-10%), medium (10.1-50%) and high (>50%) AF arrhythmia burden, our data show no significant differences in indices vWf, sEsel or hsCRP across the groups of patients . Median levels of IL-6 reduced in a stepwise fashion between groups with increasing AF burden, but this was not a significant finding (P=0.476).

The body of literature available addressing arrhythmia burden in paroxysmal AF and risk of stroke is particularly sparse and it is therefore not possible to conclude precisely what AFB increases the stroke risk in paroxysmal AF to levels seen with sustained forms of AF. Nonetheless, there are ample available data to conclude that paroxysmal AF should be considered as an important clinical entity (sections 1.1.9 and 4.3.6)[398, 399].

Data from the present analysis are potentially of importance and of direct clinical relevance when considering patients with paroxysmal and pacemaker defined AF.

Although our understanding regarding the complexity of the hypercoagulable state in AF remains incomplete, we report variable aberration of prothrombotic markers in this cohort of patients with AF. However, given no appreciable relationship between these prothrombotic markers and AFB, it is it is plausible that these abnormalities do in fact relate primarily to underlying risk factors, and that such patients should be anticoagulated if risk factors dictate. This point adds further weight to the concept that arrhythmia burden alone is *unhelpful* in establishing stroke risk and that the presence of AF combined with clinical risk scoring algorithms should remain the predominant tool for stroke risk assessment and hence the decision to anticoagulate. However, these findings must be interpreted with caution: the population studied was highly selected and data may not therefore be applicable to the general population or even those patients with non-pacemaker defined paroxysmal AF. Similarly, even though the pre-specified power calculation was met, it is clear that for some analyses to be interpreted with full confidence, greater numbers still are required.

5.2 Study Limitations

All studies can be improved and hindsight lends an ideal opportunity to identify methodological flaws. If this research was to be reproduced, several alterations should be considered and these are discussed in detail:

a) Definition of AF

The classification of patients according to the temporal pattern of AF is an important consideration. Although this method is somewhat arbitrary, given the trend in AF towards recurrence (and thereby more persistent forms of AF) it was felt that labelling patients based upon the chronicity of AF was the most pragmatic solution to allow for assessment of AF 'severity'. It is of course, fully acknowledged that such a strategy may have no bearing on thromboembolic risk and the reasons for this have been explored in depth previously (sections 1.1.9, 1.2.2 and 4.3.5).

In the present study, the temporal pattern of AF was based on established international guidelines and nomenclature[7]. However, this is somewhat arbitrary and depends on a number of factors – in particular the expertise and experience of the treating cardiologist and upon patient age. For example, patients who are younger are more likely to be thought to have persistent AF and thus be offered cardioversion, whereas more elderly patients are more likely to be assumed to have permanent AF and thereby be offered only a rate control strategy. Although this would seem entirely reasonable based upon the relative merits of each strategy and success of each strategy (section 1.1.6), this does allow for early introduction of recruitment bias.

This is particularly evident in this analysis where patients with persistent AF were younger with less co-morbidity and this is perhaps reflected in the obviously high immediate success rates seen for cardioversion. If further studies based upon AF temporal pattern are to be conducted, then it may be important to consider a highly aggressive cardioversion strategy from the outset, with patients only characterized as having permanent or paroxysmal AF if cardioversion fails or if there is evidence to support these diagnoses from an early stage (e.g. Holter study). However, given data from clinical trials suggesting that aggressive rhythm control strategies may be inferior for many patients, such an approach would require careful consultation and consideration of ethical matters.

An alternative approach may be to consider an animal model of AFto help further understand some of the more basic concepts of the hypercoagulable state in a controlled fashion. This has been done successfully in the past using advanced pacing techniques. Although this is somewhat artificial and detached from a 'real-world' assessment, such an approach would allow for assessment of the direct influence of the arrhythmia on various parameters being tested and potentially allow for removal of confounding factors by greater standardization. Again this would require careful ethical consultation.

Similarly the definition of lone AF may be open to debate. In most studies lone AF is considered to be AF in persons below the age of 55 years with a structurally normal heart and without any com-morbidities. AF by this definition is a rare entity and it is therefore extremely difficult to recruit such persons, many of whom tend to have paroxysmal forms of the arrhythmia. The potential sequelae of this are that some

patients with lone AF will inevitably be in sinus rhythm at the time of recruitment and indeed may predominantly be in sinus rhythm with only have sporadic bouts of AF. Also, there are some doubts as to whether lone AF is ever truly 'lone' - as many will develop co-morbidities such as hypertension or diabetes mellitus if they are followed up. Thus they may be considered pre-hypertensive. or pre-diabetic, potentially skewing any analysis of these patients on the basis that they are otherwise healthy.

b) Mode of patient recruitment

Each of the patients in this study was carefully recruited from a pool of patients attending our AF outpatient clinic. This strategy again induces recruitment bias for a number of reasons. Firstly, patients referred into this clinic represent those where AF has been detected. This may have been an incidental finding when the patient attended a doctor for other matters – but this automatically implies co-morbid illness. Alternatively, AF may have been the primary problem – i.e. the patient had reported symptoms related to AF. In either situation, the patients recruited become less representative of the general population as they have been pre-selected. Additionally, few units within the UK have a dedicated AF clinic or a particular interest/expertise. Again, this implies bias – as provision of a particular service means that patients are more likely to be referred or specialist advice sought. Additionally, due to the highly specialized nature of our AF clinic, patients may be managed in a slightly different manner compared to elsewhere in secondary care. Recruitment in a multi-centre fashion may help avoid the inevitable bias of recruiting solely from a specialized centre.

c) Study design

The studies within this thesis were designed to test the hypotheses (section 2.1.3) in as logical a manner as possible. However, the very design and even conduct of these studies may be a source of bias and limitations. For example, the cross-sectional study (section 4.1) offers a comparison of patients with differing temporal patterns of AF. This definition of AF can be somewhat arbitrary this has already been discussed as a limitation above. However, were the study to be conducted by another researcher, some patients may have been classified as having an alternative temporal form of AF as this is a particularly subjective diagnosis.

Also the cross-sectional design, did not allow for patients to be matched – i.e. patients were recruited consecutively and classified as appropriate, but this did not necessarily lead to similar proportions of patients with various co-morbidities in each group. This is difficult to correct for, aside from boosting recruitment to a higher level.

Similar concerns can be applied to the longitdunal study (section 4.2). Also, in this study, patients undergoing cardioversion only had a control group at the first timepoint. Where sequential measurements were taken, this had to be compared back to the samples taken at baseline – i.e. each patient was his/her own control. This does not allow consideration of other parameters that may have influenced biomarker levels (e.g. time or assay varation) and therefore to perform serial ampling on the control group would have improved the study.

In the arrhythmia burden study (section 4.3), the group of patients considered to have sinus rhythm had had previously documented bouts of AF (although none during the

study period) and therefore may not be considered pure controls. Also patients being recruited in another centre, prevented us from running samples on the flow cytometer for CPCs and this therefore limits the ability to fully mate findings from this study with the remainder of this thesis.

d) Power / Statistical Analyses

For each study, hypotheses were to be tested by recruitment of patients to a level pre-specified from a detailed power calculation. In each case, this was based upon data from previously published studies and/or pilot data from our own or related research groups. However, these calculations were primarily based on data for CPCs and not necessarily for other markers. This was not entirely unreasonable, as similar markers run in the department previously had not required patient numbers hugely different to those calculated here. Although the power calculation was broadly met in each study, it is clear that the data spread for the markers studied was broad. This is clearly obvious in some analyses (e.g. VEGF section 4.2) where a trend is clearly apparent and the P value remains well above 0.05. Thus if the study were to be repeated, larger patient numbers would be recommended.

In each study, established statistical analyses were applied. In all cases, the test used was appropriate for the data being handled (e.g. median, interquartile range and non-paramteric tests were applied to non-parametric data and so on). Nonetheless, in some analyses, especially correlations and multiple regression, large numbers of statistical comparisons were made, thereby increasing the probability of finding a positive association by chance alone. This was in part accounted for by reducing the P-vaue considered as significant to less than 0.01 for these particular analyses. However,

even with this change 1 in 100 tests is likely to prove 'significant' by chance. It is therefore essential to consider these analyses as illustrative only. If more detailed association is required, entering fewer variables into the equation may improve accuracy.

e) Substitute indices for hypercoagulability

The present analysis used a number of laboratory indices in assessment of endothelial function, inflammation and hypercoagulability. The interaction and importance of each of these processes in terms thrombogenesis has been discussed in detail in section 1.2 but remains particularly complex and poorly understood. Moreover, it is not clear whether each index directly relates to clinical outcome (i.e. stroke) in AF. Of course, such links are now harder to establish given that based on trial evidence various interventions (e.g. anticoagulation) are offered to reduce event rates.

Of the many indices tested, each plays a subtle and discrete role which may not fully depict the process each molecule represents. For example, IL-6 release only reflects one molecular pathway in an inflammatory response where there is release of numerous pro-inflammatory molecules and cytokines, including other interleukins and CRP. However, the relative function, activity and interaction of each molecule has not yet been fully defined. Thus it is plausible that the complexity of the various pathways is lost by over-simplification of measurement of individual assays. Moreover, each assay reflects concentration of each molecule rather than functionality and the former does not necessarily imply the latter. Such considerations also apply to markers that represent endothelial function and activation of the coagulation cascade.

The design of the present study was to explore various interactions at the molecular level and thereby help guide future research directions which are discussed in detail in the next section. To overcome limitations based upon assessment of only a handful of indices which do not necessarily reflect all elements of a given process is difficult. However as various interactions become detangled and more defined this will no doubt become a less daunting task.

To use hard clinical endpoints (e.g. stroke) in a population patients with numerous co-morbidities requires very large patient numbers and long duration of follow-up in order to observe an adequate event rate. Even then, cause and effect remains difficult to define and this is plainly obvious in the diversity of stroke-risk algorithms that are available some of which have been based on evidence from large studies (section 1.1).

f) Circulating Progenitor Cells

A number of pitfalls need to be discussed when considering CPCs. The first relates to cellular definition. Our understanding of cellular and endothelial rejuvenation remains incompletely understood. Although CPCs are thought to play a significant role, their precise origin, function and antigen expression remains a matter of debate. The complex flux in the latter that seems to occur during cellular maturation until ultimate integration within the endothelium has further hampered research efforts. Thus individual research groups have used a varied definition for CPCs and thus could be measuring cells of entirely diverse functionality.

The method used to detect CPCs is also important. In this thesis we have discussed the relative merits of flow cytometry vs. cell culture. On the basis of early laboratory

work, our experiments continued without cell culture given significant concern about the accuracy of data collected. However, the major advantage of cell culture is the ability to assess cellular functionality.

Both techniques have a number of common pitfalls. Sample processing in both techniques requires a number of wash stages, which has the potential to lose cells – this is of vital importance when measuring cells as rare as CPCs. Additionally, cells may lyse during centrifugation or pipetting again leading to aberrant results. In cell culture, it is possible to culture cells other than CPCs, although these should not show the characteristic morphology, migration and staining characteristics. In flow cytometry, sample error may be introduced by inadequate washout from the previous sample (which we were careful to avoid). Similarly delays in processing, cell fixation with paraformaldehyde etc. may lead to alteration in cellular characteristics. Again, we were careful to minimize such events to enhance reproducibility.

Naturally, other limitations are evident. Some relate to laboratory methodology regarding ELISA analysis, although each assay was rigorously tested within the department to ensure reproducibility. Other considerations must include use of statistical analyses which can only imply association, and not cause and effect.

5.3 Future Research Directions

Given the rising prevalence of AF and the strong association of this arrhythmia with stroke, research into both the mechanisms of AF and the pathophysiological interactions leading to stroke must remain high on the research agenda. However, it has become very clear through the conduct of this study that investigating AF as a clinical entity is particularly challenging. The reasons for this have already been explored in depth, but primarily relate to difficulties in examining data, which may (or may not) relate to one or more of the many co-morbidities that frequently co-exist with AF. Thus it is imperative that future research be driven by sound hypotheses and a methodical approach. Avenues that may merit exploration in future research are discussed in brief:

a) Use of biomarkers in refining stroke risk

The concept of stroke risk stratification to target populations deemed at significant risk of thromboembolic events has been discussed in detail in section 1.1. Although numerous schema are available, none is ideal with significant discrepancies in terms of patient categorization. This is most evident amongst patients categorized likely to be categorized as either low- or high-risk for stroke. This potentially can have serious implications – excessive anticoagulation in low risk patients can cause harm through increased bleeding events, whilst inadequate anticoagulation amongst high-risk patients can result in inadequate stroke prophylaxis often by the use of antiplatelet agents when anticoagulation (or novel agents) would be more appropriate.

Many stroke-risk schema follow a common theme and primarily base assessment upon clinical parameters (e.g. age, blood pressure, diabetes etc.), although some also

consider imaging modalities such as echocardiography or brain imaging. The use of biomarkers to refine stroke risk schema has also been discussed and is an area of active research[405]. A number of indices have been considered useful in this setting and include vWf and D-dimer, both of which appear to complement existing stroke risk stratification schema[116, 124, 125]. vWf, have limited evidence for . The latter however is usually modulated by warfarin and therefore may not be ideal for use in the setting of patients already receiving anticoagulation[406]. Given the spectrum of biomarker abnormalities evident in AF – particularly AF with co-morbidities (i.e. those known to be at enhanced stroke risk) – the quest to identify a biomarker to reliably indicate those patients at greatest stroke risk is an important one and should continue in earnest. Of course, any marker used must be robust with an assay that is easy to implement in healthcare laboratories. This would ideally be studied in the setting of a large trial such that the markers tested can be linked to clinical endpoints (stroke).

b) Atrial fibrillation arrhythmia burden.

One major concern for clinicians when dealing with patients who exhibit paroxysmal AF, relates to thromboembolic risk. Indeed, it is not clear from the available literature exactly what frequency / duration of AF episodes puts a given individual at significant stroke risk to merit anticoagulation. Similar considerations also apply in patients with permanent pacemakers where very brief episodes of AF are sometimes identified at routine pacemaker interrogation and there is only limited evidence in the literature assessing the clinical importance of this entity. Part of this thesis has investigated such considerations and finds no relationship between the prothrombotic indices tested and arrhythmia burden. However, it is not possible to directly extrapolate this data in to clinical management.

Certainly this topic requires further research and is presently the subject of a number of clinical trials[407, 408]. The recently reported TRENDS study assessed AF burden defined by algorithms within existing implantable devices (pacemakers, defibrillators etc.) against thromboembolic events collated in a prospective manner[408]. The investigators conclude that the risk of events was low in patients with device defined AF compared to AF diagnosed in a more traditional manner, but that greater AF burden did correlate to increased risk of embolic events. In a similar study which is still in the recruitment phase, investigators are assessing the potential for remote device monitoring in AF detection and consequent initiation of early anticoagulation[407]. It is hoped that these trials will further our understanding and help identify the importance of the arrhythmia burden in a clinical context.

c) Modulation of stroke risk with antiarrhythmic drugs

Early trials assessing rate *vs*. rhythm control in sustained forms of AF did not demonstrate any difference in stroke event rates between the two arms, although in AFFIRM, there was a trend towards higher mortality and significantly more arrhythmic events and hospitalizations in the rhythm control arm (section 1.1)[10]. Thus in many patients rate control may be considered superior. In paroxysmal AF, the management strategy is rhythm management (i.e. maintenance of sinus rhythm) by default. This is usually achieved through the prescription of various antiarrhythmic agents, or ablation, but despite these AF can (and frequently does) recur. In patients where long-term maintenance of sinus rhythm is achieved, the stasis component to Virchow's triad is removed. Similarly coagulation abnormalities due directly to AF could also be moderated. There is some clinical evidence to support this notion. A

post-hoc analysis of a recent trial investigating dronedarone (a novel antiarrhythmic) *vs*. placebo reported a significant 34% reduction in stroke risk amongst those taking the treatment[409]. Thus further research assessing the impact of successful rhythm control on stroke rates should be encouraged.

In addition, there are other avenues that merit exploration in particular the impact of patients who have undergone AF ablation and are known to be successfully maintaining sinus rhythm. Such a cohort could be readily compared pre- and post-ablation using a number of laboratory assays and thereby help clarify whether various abnormalities truly relate to AF or to co-morbidities. Additionally, these patients should be followed prospectively to assess for event rates. This is particularly important, as long-term maintenance of sinus rhythm following various ablation techniques is still unknown given the relative youth of these procedures.

5.4 Conclusion

It is clear that AF satisfies all 3 components of Virchow's triad for thrombogenesis. Although stasis is the most evident feature of this arrhythmia, it is no longer accepted that this is the only means through which thrombogenesis occurs. Research has demonstrated aberrancies of various pathways, which may be implicated in thrombogenesis either directly, or indirectly. However, the endothelium seems to be a central link through which these processes interact. The present study has implicated a possible link for CPCs in this process. It has, however, not been possible to establish cause or effect and formal documentation of the precise interaction of various processes involved in thrombogenesis remains elusive.

Finally, the present analysis has established that of the laboratory indices tested in paroxysmal AF, are unhelpful in trying to establish what AF arrhythmia burden is of enhanced thrombogenic risk and concludes that clinical risk stratification should remain the primary tool for assessing stroke risk in any given individual.

Appendix

a) vWf ELISA

Brief Method:

1. Coat microtitre plate with 100 µl of a dilution of primary antisera (35 µl in 20ml pH 9.6 coating buffer) at room temperature for >60 minutes or overnight in the fridge.
2. Wash 3x, add 100 µl 1/40 serum or plasma in pbs/tween, or neat tissue culture fluid, incubate for >60 minutes at room temperature.
3. Wash 3x, add 100 µl peroxidase-labelled conjugate (35 µl in 20ml PBS) for >45 minutes at room temperature.
4. Wash, add 100 µl substrate (OPD, hydrogen peroxide, citrate buffer). The colour develops almost immediately.
5. Stop with 100 µl acid. Read at 492 nm

Expected values:

In citrated normal plasma in the region of 100 $\pm$ 30 IU/dL stable atherosclerosis typically 130 $\pm$ 35 IU/dL, acute coronary syndromes often 150 $\pm$ 40. Data generally of normal distribution.

b) VEGF ELISA

Brief Method:

1. Coat microtitre plate with 112 µl of primary antisera l in 20 ml PBS buffer for 2 plates overnight in the fridge.
2. Wash, block with 100 µl /well of 5% Marvel (1g in 20mls PBS-T for 2 plates) for 1 hour at room temperature.
3. Wash, add 100 µl of neat plasma, or neat tissue culture fluid, and recombinant standards for 2 hours at room temperature. Standards are double diluted down the plate. Use fresh tips for each sample.
4. Wash, add 112 µl of biotinylated anti-human VEGF antibody in 20 ml PBS tween for 90 minutes at room temperature.
5. Wash, add Streptavidin (100µl/well) for 45 minutes at room temperature.
6. Wash, add 100 µl substrate (Solutions A and B). Blue colour develops well with in 20 to 30 minutes
7. Stop with 50 µl/well acid. Colour goes yellow. Read at 450 nm.

Expected values:

Data usually not normally distributed. Controls generally have median values of about 30-50 ρg/ml (but IQR may be over 200 ρg/ml) Patients' median values generally 100 to over 200 ρg/ml. Again, wide IQRs.

c) soluble E-selectin ELISA

Brief Method:

1. Coat microtitre plate with 100 µl of capture polyclonal antiserum in pH 9.6 buffer in the fridge (4^{o}C) overnight or 1.5 hours at room temperature.
2. Wash, add 100 µl serum/plasma (diluted 1/5 [20% plasma: 80% blue or pbs-tween buffer] on the plate) or standards (top 'prepared' 50 ng/mL) for 1.5 hours at room temperature. Also, prepare a sample of the 'Universal' plasma.
3. Wash, add 100 µl detection antibody (one vial in 20 mls PBS/Tween for two plates) for 1.5 hours at room temperature
4. Wash, add 100 µl Streptavidin-HRP conjugate (diluted 1/200 in PBS/Tween, i.e. 100 µl plus 20 mls) for a minimum of 20 minutes at room temperature in the dark
5. Wash, add 100µl substrate (made up from equal volumes of reagents A and B). It should go blue. Wait 3 – 5 minutes. The key definition is a clear gradation of blue colour from the top to the blank.
6. Stop with 75µl Acid. It will go yellow. Read at 450 nm.

Expected Values:

About 20-40ng/mL. The Universal is about 40 ng/mL.

d) soluble Fas Ligand ELISA

Brief Method:

1. Coat microtitre plate with 100µl of primary antisera (56µl of reconstituted primary antibody in 10ml PBS for 1 plate) room temperature (working concentration 2µg/ml).
2. Wash, block with 200µl /well of 5% Marvel (2.5g in 50mls PBS-T for 2 plates) for 1 hour at room temperature.
3. Wash, add 100µl serum and 100 µl recombinant standards for 2 hours at room temperature.
4. Wash with PBS-T and add 56µl of reconstituted secondary Ab in 10ml PBS-T then incubate for 2 hours at room temperature (working concentration 50ng/ml).
5. Wash, add 100ul/well of streptavidin-HRP (50ul strep-HRP in 10mls of PBS-T for 1 plate) and incubate for 20 minutes at room temperature (avoid direct light).
6. Wash, add 100µl substrate solution 5 mls Colour regent A + 5 mls B for 1 plate. Colour will develop in less than 5 minutes.
7. Stop with 75µl/well acid. Read at 450 nm

Expected values:

Data usually not normally distributed. Controls generally have median values of about 5mcg/ml Patients' median values generally lower.

e) soluble Fas ELISA

Brief Method:

1. Coat microtitre plate with 100µl of primary antisera (56µl of reconstituted primary Ab in 10ml PBS for 1 plate) room temperature (working concentration 1µg/ml).
2. Wash, block with 200µl /well of 5% Marvel (2.5g in 50mls PBS-T for 2 plates) for 1 hours at RT.
3. Wash, add 100µL serum (or EDTA plasma) diluted to 1 in 5 (by adding 20µL of sample to 80µL PBS-T) and recombinant standards for 2 hours at RT.
4. Wash with PBS-T, add 100µl 56µl of reconstituted secondary Ab in 10ml PBS-T for 2 hours at RT (working concentration 50ng/ml).
5. Wash, add 100ul/well of streptavidin-HRP (50ul strep-HRP in 10mls of PBS-T for 1 plate) and incubate for 20 minutes at RT (avoid direct light).
6. Wash, add 100µl substrate solution 5 mls Colour regent A + 5 mls B for 1 plate. Colour will develop in less than 5 minutes.
7. Stop with 50µl/well acid. Read at 450 nm

Expected values:

Data usually not normally distributed. Controls generally have median values of about 5mcg/ml Patients' median values generally lower.

f) Interleukin-6 ELISA

Brief method:

1. Coat microtitre plate with 100µL capture antibody (4g/mL murine monoclonal anti-human IL-6) and leave overnight at room temperature
2. Wash 3 times with PBS-tween
3. Add 100µL undiluted sample plasma to each well and leave for one hour at room temperature.
4. Wash 3 times with PBS-tween
5. Add 100µL detection antibody (50ng/mL biotinylated anti-human IL-6) to each well and incubate for 2 hours at room temperature
6. Wash 3 times with PBS-tween
7. Dilute 100µL streptavidin/HRP in just over 20mLs PBS-tween. Add 100µL to each well and incubate for 20 minutes at room temperature
8. Wash 3 times with PBS-tween
9. Mix 10.5mL reagent A with 10.5mL reagent B. Add 100µL to each well. Reaction will take about 30 minutes at room temperature
10. Stop with 50µL 1M hydrochloric acid. Read at 450nm OD

References

1. Stewart S, Murphy N, Walker A, McGuire A, McMurray JJV. Cost of an emerging epidemic: an economic analysis of atrial fibrillation in the UK. *Heart.* 2004;90(3):286-292.

2. Ostrander LD Jr, Brandt RL, Kjelsber.Mo, Epstein FH. Electrocardiographic findings among adult population of a total natural community Tecumseh Michigan.. *Circulation.* 1965;31(6):888-&.

3. Lloyd-Jones DM, Wang TJ, Leip EP, Larson MG, Levy D, Vasan RS, D'Agostino RB, Massaro JM, Beiser A, Wolf PA, Benjamin EJ. Lifetime risk for development of atrial fibrillation - The Framingham Heart Study. *Circulation.* 2004;110(9):1042-1046.

4. Heeringa J, van der Kuip DAM, Hofman A, Kors JA, van Herpen G, Stricker BHC, Stijnen T, Lip GYH, Witteman JCM. Prevalence, incidence and lifetime risk of atrial fibrillation: the Rotterdam study. *European Heart Journal.* 2006;27(8):949-953.

5. National Collaborating Centre for Chronic Conditions. Atrial fibrillation: national clinical guideline for management in primary and secondary care.: Royal College of Physicians.; 2006.

6. Fuster V, Ryden LE, Cannom DS, Crijns HJ, Curtis AB, Ellenbogen KA, Halperin JL, Le Heuzey JY, Kay GN, Lowe JE, Olsson SB, Prystowsky EN, Tamargo JL, Wann S, As ACCAH, European Soc C. ACC/AHA/ESC 2006 guidelines for the management of patients with atrial fibrillation: full text. *Europace.* 2006;8(9):651-745.

7. Levy S, Camm AJ, Saksena S, Aliot E, Breithardt G, Crijns H, Davies W, Kay N, Prystowsky E, Sutton R, Waldo A, Wyse DG. International consensus on nomenclature and classification of atrial fibrillation - A collaborative project of the Working Group on Arrhythmias and the Working Group on Cardiac Pacing of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. *Europace.* 2003;5(2):119-122.

8. Lip GYH, Tello-Montoliu A. Management of atrial fibrillation. *Heart.* 2006;92(8):1177-1182.

9. Bertaglia E, D'Este D, Zerbo F, Zoppo F, Delise P, Pascotto P. Success of serial external electrical cardioversion of persistent atrial fibrillation in maintaining sinus rhythm - A randomized study. *European Heart Journal.* 2002;23(19):1522-1528.

10. Corley SD, Epstein AE, DiMarco JP, Domanski MJ, Geller N, Greene HL, Josephson RA, Kellen JC, Klein RC, Krahn AD, Mickel M, Mitchell LB, Nelson JD, Rosenberg Y, Schron E, Shemanski L, Waldo AL, Wyse DG, Investigators A. Relationships between sinus rhythm, treatment, and survival

in the atrial fibrillation follow-up investigation of rhythm management (AFFIRM) study. *Circulation.* 2004;109(12):1509-1513.

11. Lim HS, Hamaad A, Lip GYH. Clinical review: Clinical management of atrial fibrillation - rate control versus rhythm control. *Critical Care.* 2004;8(4):271-279.

12. Farshi R, Kistner D, Sarma JSM, Longmate JA, Singh BN. Ventricular rate control in chronic atrial fibrillation during daily activity and programmed exercise: A crossover open-label study of five drug regimens. *Journal of the American College of Cardiology.* 1999;33(2):304-310.

13. Dittrich HC, Erickson JS, Schneiderman T, Blacky AR, Savides T, Nicod PH. Echocardiographic and clinical predictors for outcome of elective cardioversion of atrial fibrillation. *American Journal of Cardiology.* 1989;63(3):193-197.

14. Hersi A, Wyse DG. Management of atrial fibrillation. *Current Problems in Cardiology.* 2005;30(4):175-233.

15. Lee SH, Chen SA, Chiang CE, Tal CT, Wen ZC, Wang SP, Chang MS. Comparisons of oral propafenone and quinidine as an initial treatment option in patients with symptomatic paroxysmal atrial fibrillation: A double-blind, randomized trial. *Journal of Internal Medicine.* 1996;239(3):253-260.

16. Anderson JL, Gilbert EM, Alpert BL, Henthorn RW, Waldo AL, Bhandari AK, Hawkinson RW, Pritchett ELC. Prevention of symptomatic recurrences of paroxysmal atrial-fibrillation in patients initially tolerating antiarrhythmic therapy – A multicenter, double-blind, crossover study of flecainide and placebo with transtelephonic monitoring. *Circulation.* 1989;80(6):1557-1570.

17. Cobbe SM, Rae AP, Poloniecki JD, Chong E, Balnave K, Moriarty A, Blackwood R, Cahill N, Cobbe S, Pye M, Sedgwick M, Cooper D, Flint J, Gould B, Hutchison S, Irvine N, Lewis P, Okeefe B, Sanderson J, Rae A, Lip G, Gemmill J, Shiu G, Silas J, Watkins J, Stephens J. A randomized placebo-controlled trial of propafenone in the prophylaxis of paroxysmal supraventricular tachycardia and paroxysmal atrial-fibrillation. *Circulation.* 1995;92(9):2550-2557.

18. Pietersen AH, Hellemann H. Usefulness of flecainide for prevention of paroxysmal atrial-fibrillation and flutter. *American Journal of Cardiology.* 1991;67(8):713-717.

19. Friedman PL, Stevenson WG. Proarrhythmia. Paper presented at: Symposium on Atrial Fibrillation: Mechanism and Management; May 05, 1998; San Diego, California.

20. Haissaguerre M, Marcus FI, Fischer B, Clementy J. Radiofrequency catheter ablation in unusual mechanisms of atrial-fibrillation - report of 3 cases. *Journal of Cardiovascular Electrophysiology.* 1994;5(9):743-751.

21. Wood MA, Brown-Mahoney C, Kay GN, Ellenbogen KA. Clinical outcomes after ablation and pacing therapy for atrial fibrillation - A meta-analysis. *Circulation.* 2000;101(10):1138-1144.

22. Markowitz SM, Stein KM, Lerman BB. Mechanism of ventricular rate control after radiofrequency modification of atrioventricular conduction in patients with atrial fibrillation. *Circulation.* 1996;94(11):2856-2864.

23. Haissaguerre M, Jais P, Shah DC, Takahashi A, Hocini M, Quiniou G, Garrigue S, Le Mouroux A, Le Metayer P, Clementy J. Spontaneous initiation of atrial fibrillation by ectopic beats originating in the pulmonary veins. *New England Journal of Medicine.* 1998;339(10):659-666.

24. Cappato R, Calkins H, Chen SA, Davies W, Iesaka Y, Kalman J, Kim YH, Klein G, Packer D, Skanes A. Worldwide survey on the methods, efficacy, and safety of catheter ablation for human atrial fibrillation. *Circulation.* 2005;111(9):1100-1105.

25. Hindricks G, Piorkowski C, Tanner H, Kobza R, Gerds-Li JH, Carbucicchio C, Kottkamp H. Perception of atrial fibrillation before and after radiofrequency catheter ablation - Relevance of asymptomatic arrhythmia recurrence. *Circulation.* 2005;112(3):307-313.

26. Karch MR, Zrenner B, Deisenhofer I, Schreieck JR, Ndrepepa G, Dong J, Lamprecht K, Barthel P, Luciani E, Schomig A, Schmitt C. Freedom from atrial tachyarrhythmias after catheter ablation of atrial fibrillation - A randomized comparison between 2 current ablation strategies. *Circulation.* 2005;111(22):2875-2880.

27. Pappone C, Vicedomini G, Manguso F, Gugliotta F, Mazzone P, Gulletta S, Sora N, Sala S, Marzi A, Augello G, Livolsi L, Santagostino A, Santinelli V. Robotic magnetic navigation for atrial fibrillation ablation. *Journal of the American College of Cardiology.* 2006;47(7):1390-1400.

28. Leitch JW, Klein G, Yee R, Guiraudon G. Sinus node-atrioventricular node isolation - long-term results with the corridor operation for atrial-fibrillation. *Journal of the American College of Cardiology.* 1991;17(4):970-975.

29. vanHemel NM, Defauw J, Guiraudon GM, Kelder JC, Jessurun ER, Ernst J. Long-term follow-up of corridor operation for lone atrial fibrillation: Evidence for progression of disease? *Journal of Cardiovascular Electrophysiology.* 1997;8(9):967-973.

30. Cox JL, Ad N, Palazzo T, Fitzpatrick S, Suyderhoud JP, DeGroot KW, Pirovic EA, Lou HC, Duvall WZ, Kim YD. Current status of the Maze procedure for the treatment of atrial fibrillation. *Semin Thorac Cardiovasc Surg.* 2000;12(1):15-19.

31. Lip GYH, Boos CJ. Antithrombotic treatment in atrial fibrillation. *Heart.* 2006;92(2):155-161.

32. Hart RG, Pearce LA, Aguilar MI. Meta-analysis: Antithrombotic therapy to prevent stroke in patients who have nonvalvular atrial fibrillation. *Ann. Intern. Med.* 2007;146(12):857-867.

33. Oden A, Fahlen M, Hart RG. Optimal INR for prevention of stroke and death in atrial fibrillation: a critical appraisal. *Thrombosis Research.* 2006;117(5):493-499.

34. The SPAF Investigators. A differential effect of aspirin in prevention of stroke on atrial fibrillation. *Journal of Stroke and Cerebrovascular Disease*. 1993;3:181-188.

35. Connolly S, Pogue J, Hart R, Pfeffer M, Hohnloser S, Chrolavicius S, Yusuf S, Active Writing Grp AI. Clopidogrel plus aspirin versus oral anticoagulation for atrial fibrillation in the Atrial fibrillation Clopidogrel Trial with Irbesartan for prevention of Vascular Events (ACTIVE W): a randomised controlled trial. *Lancet.* 2006;367(9526):1903-1912.

36. Aguilar MI, Hart R, Pearce LA. Oral anticoagulants versus antiplatelet therapy for preventing stoke in patients with non-valvular artial fibrillation and no history of stroke or transient ischemic attacks. *Cochrane Database of Systematic Reviews.* 2007(3).

37. Mant J, Hobbs FDR, Fletcher K, Roalfe A, Fitzmaurice D, Lip GYH, Murray E, Bafta. Warfarin versus aspirin for stroke prevention in an elderly community population with atrial fibrillation (the Birmingham Atrial Fibrillation Treatment of the Aged Study, BAFTA): a randomised controlled trial. *Lancet.* 2007;370(9586):493-503.

38. Singer DE, Chang YC, Fang MC, Borowsky LH, Pomernacki NK, Udaltsova N, Go AS. The Net Clinical Benefit of Warfarin Anticoagulation in Atrial Fibrillation. *Ann. Intern. Med.* 2009;151(5):297-U216.

39. Protheroe J, Fahey T, Montgomery AA, Peters TJ. The impact of patients' preferences on the treatment of atrial fibrillation: observational study of patient based decision analysis. *British Medical Journal.* 2000;320(7246):1380-1384.

40. Solomon NA, Glick HA, Russo CJ, Lee J, Schulman KA. Patient preferences for stroke outcomes. *Stroke.* 1994;25(9):1721-1725.

41. Jones M, McEwan P, Morgan CL, Peters JR, Goodfellow J, Currie CJ. Evaluation of the pattern of treatment, level of anticoagulation control, and outcome of treatment with warfarin in patients with non-valvar atrial fibrillation: a record linkage study in a large British population. *Heart.* 2005;91(4):472-477.

42. Lip GYH, Karpha M. Anticoagulant and antiplatelet therapy use in patients with atrial fibrillation undergoing percutaneous coronary intervention - The need for consensus and a management guideline. *Chest.* 2006;130(6):1823-1827.

43. Connolly SJ, Ezekowitz MD, Yusuf S, Eikelboom J, Oldgren J, Parekh A, Pogue J, Reilly PA, Themeles E, Varrone J, Wang S, Alings M, Xavier D, Zhu J, Diaz R, Lewis BS, Darius H, Diener HC, Joyner CD, Wallentin L, Comm R-LS, Investigator. Dabigatran versus Warfarin in Patients with Atrial Fibrillation. *New England Journal of Medicine.* 2009;361(12):1139-1151.

44. Hart RG, Pearce LA, Halperin JL, Hylek EM, Albers GW, Anderson DC, Connolly SJ, Friday GH, Gage BF, Go AS, Goldstein LB, Gronseth G, Lip GYH, Sherman DG, Singer DE, van Walraven C, Stroke Risk Atrial F. Comparison of 12 risk stratification schemes to predict stroke in patients with nonvalvular atrial fibrillation. *Stroke.* 2008;39(6):1901-1910.

45. Gage BF, Waterman AD, Shannon W, Boechler M, Rich MW, Radford MJ. Validation of clinical classification schemes for predicting stroke - Results from the national registry of Atrial Fibrillation. *Jama-Journal of the American Medical Association.* 2001;285(22):2864-2870.

46. Lip GYH. Does atrial-fibrillation confer a hypercoagulable state? *Lancet.* 1995;346(8986):1313-1314.

47. Brotman DJ, Deitcher SR, Lip GYH, Matzdorff AC. Virchow's triad revisited. *Southern Medical Journal.* 2004;97(2):213-214.

48. Choudhury A, Lip GYH. Atrial fibrillation and the hypercoagulable state: From basic science to clinical practice. *Pathophysiology of Haemostasis and Thrombosis*. 2003-2004;33(5-6):282-9..

49. Blackshear JL, Odell JA. Appendage obliteration to reduce stroke in cardiac surgical patients with atrial fibrillation. *Annals of Thoracic Surgery.* 1996;61(2):755-759.

50. Pollick C, Taylor D. Assessment of left atrial appendage function by transesophageal echocardiography - implications for the development of thrombus. *Circulation.* 1991;84(1):223-231.

51. Goldsmith IRA, Blann AD, Patel RL, Lip GYH. Von Willebrand factor, fibrinogen, and soluble P-selectin levels after mitral valve replacement versus mitral valve repair. *American Journal of Cardiology.* 2000;85(10):1218-1222.

52. Masawa N, Yoshida Y, Yamada T, Joshita T, Ooneda G. Diagnosis of cardiac thrombosis in patients with atrial-fibrillation in the absence of macroscopically visible thrombi. *Virchows Archiv a-Pathological Anatomy and Histopathology.* 1993;422(1):67-71.

53. Boldt A, Wetzel U, Lauschke J, Weigl J, Gummert J, Hindricks G, Kottkamp H, Dhein S. Fibrosis in left atrial tissue of patients with atrial fibrillation with and without underlying mitral valve disease. *Heart.* 2004;90(4):400-405.

54. Frustaci A, Chimenti C, Bellocci F, Morgante E, Russo MA, Maseri A. Histological substrate of atrial biopsies in patients with lone atrial fibrillation. *Circulation.* 1997;96(4):1180-1184.

55. Khan IA. Atrial stunning: Determinants and cellular mechanisms. *American Heart Journal.* 2003;145(5):787-794.

56. Schotten U, Haase H, Frechen D, Greiser M, Stellbrink C, Vazguez-Jimenez JF, Morano I, Allessie MA, Hanrath P. The L-type Ca2+-channel subunits alpha(1C) and beta(2) are not downregulated in atrial myocardiurn of patients with chronic atrial fibrillation. *Journal of Molecular and Cellular Cardiology.* 2003;35(5):437-443.

57. Stellbrink C, Hanrath P. The optimal management of cardioversion of atrial fibrillation of flutter: still a 'stunning' problem. *European Heart Journal.* 2000;21(10):795-798.

58. Blackshear JL, Pearce LA, Hart RG, Zabalgoitia M, Labovitz A, Asinger RW, Halperin JL, Stroke Prevention Atrial Fibrillation I. Aortic plaque in atrial fibrillation - Prevalence, predictors, and thromboembolic implications. *Stroke.* 1999;30(4):834-840.

59. Zabalgoitia M, Leonard A, Blackshear JL, Safford R, Baker VS, Fenster P, Pennock GD, Ohm J, Huerta BJ, Strauss R, McKenzie M, Hart-McArthur P, Gramberg M, Houston H, Chan KL, Biggs J, Ives A, Rothbart R, Murray D, Loutfi H, Lynch JJ, Carlson L, Labovitz AJ, Vigil A, Asinger RW, Dick CD, Newburg SM, Fifield J, Khandheria BK, Holland AE, Tucker KR, Goldman ME, Rothlauf EB, Hess D, Young L, Mego DM, Boyd S, Wilson D, Nolte B, Edwards C, Mezei L, Riggio S, Giraud G, Marchant C, Timberg J, Dittrich HC, Keen W, Kerridge C, Bernard S, Battinelli N, Atwood JE, Quaglietti S, Alpert MA, Massey CV, Hupp C, Koehler J, Pearce LA, Halperin JL, Hart RG, Marler JR, DeMaria AN, Tajik AJ, Weyman A, Stroke Prev Atr Fibr Invest Comm E. Transesophageal echocardiographic correlates of thromboembolism in high-risk patients with nonvalvular atrial fibrillation. *Annals of Internal Medicine.* 1998;128(8):639-+.

60. Sackner-Bernstein JD. The myocardial matrix and the development and progression of ventricular remodeling. *Curr Cardiol Rep.* 2000;2(2):112-119.

61. Tziakas DN, Chalikias GK, Papanas N, Stakos DA, Chatzikyriakou SV, Maltezos E. Circulating levels of collagen type I degradation marker depend on the type of atrial fibrillation. *Europace.* 2007;9(8):589-596.

62. Marin F, Roldan V, Climent V, Garcia A, Marco P, Lip GYH. Is thrombogenesis in atrial fibrillation related to matrix metalloproteinase-1 and its inhibitor, TIMP-1? *Stroke.* 2003;34(5):1181-1186.

63. Li XP, Ma CS, Dong JZ, Liu XP, Long DY, Tian Y, Yu RH. The fibrosis and atrial fibrillation: Is the transforming growth factor-beta(1) a candidate etiology of atrial fibrillation. *Medical Hypotheses.* 2008;70(2):317-319.

64. Xu J, Cui GG, Esmailian F, Plunkett M, Marelli D, Ardehali A, Odim J, Laks H, Sen L. Atrial extracellular matrix remodeling and the maintenance of atrial fibrillation. *Circulation.* 2004;109(3):363-368.

65. Nakano Y, Niida S, Dote K, Takenaka S, Hirao H, Miura F, Ishida M, Shingu T, Sueda T, Yoshizumi M, Chayama K. Matrix metalloproteinase-9 contributes to human atrial remodeling during atrial fibrillation. *Journal of the American College of Cardiology.* 2004;43(5):818-825.

66. Anne W, Willems R, Roskams T, Sergeant P, Herijgers P, Holemans P, Ector H, Heidbuchel H. Matrix metalloproteinases and atrial remodeling in patients with mitral valve disease and atrial fibrillation. *Cardiovascular Research.* 2005;67(4):655-666.

67. Mukherjee R, Herron AR, Lowry AS, Stroud RE, Stroud MR, Wharton JM, Ikonomidis JS, Crumbley AJ, Spinale FG, Gold MR. Selective induction of matrix metalloproteinases and tissue inhibitor of metalloproteinases in atrial and ventricular myocardium in patients with atrial fibrillation. *American Journal of Cardiology.* 2006;97(4):532-537.

68. Li DS, Fareh S, Leung TK, Nattel S. Promotion of atrial fibrillation by heart failure in dogs - Atrial remodeling of a different sort. *Circulation.* 1999;100(1):87-95.

69. Sanders P, Morton JB, Davidson NC, Spence SJ, Vohra JK, Sparks PB, Kalman JM. Electrical remodeling of the atria in congestive heart failure - Electrophysiological and electroanatomic mapping in humans. *Circulation.* 2003;108(12):1461-1468.

70. Sanfilippo AJ, Abascal VM, Sheehan M, Oertel LB, Harrigan P, Hughes RA, Weyman AE. Atrial enlargement as a consequence of atrial-fibrillation - a prospective echocardiographic study. *Circulation.* 1990;82(3):792-797.

71. Keren G, Etzion T, Sherez J, Zelcer AA, Megidish R, Miller HI, Laniado S. Atrial-fibrillation and atrial enlargement in patients with mitral-stenosis. *American Heart Journal.* 1987;114(5):1146-1155.

72. Anderson DC, Asinger RW, Newburg SM, Farmer CC, Wang K, Bundlie SR, Koller RL, Jagiella WM, Kreher S, Jorgensen CR, Sharkey SW, Flaker GC, Webel R, Nolte B, Stevenson P, Byer J, Wright W, Chesebro JH, Wiebers DO, Holland AE, Miller DM, Bardsley WT, Litin SC, Meissner I, Zerbe DM, McAnulty JH, Marchant C, Coull BM, Feldman G, Hayward A, Gandara E, Macmillan K, Blank N, Leonard AD, Kanter MC, Isensee LM, Quiroga ES, Presti CH, Tegeler CH, Logan WR, Hamilton WP, Green BJ, Bacon RS, Redd RM, Cadell DJ, Gomez CR, Janosik DL, Labovitz AJ, Kelley RE, Chahine R, Cristo L, Palermo M, Perez O, Feinberg WM, Vold BK, Kern KB, Appleton C, Miller VT, Hockersmith CJ, Cohen BA, Martin GJ, Pawlow AJ, Halperin JL, Rothlauf EB, Weinberger JM, Goldman ME, Fuster V, Dittrich HC, Rothrock JF, Hagenhoff C, Helgason CM, Kondos GT, Hoff J, Kaufmann L, Rabjohns RR, McRae RP, Ghali J, Adams HP, Theilen EO, Biller J, Brown DD, Marsh EE, Sirna SJ, Mitchell VL, Rothbart RM, Bailey GH, Burkhardt C, Blackshear JL, Weaver L, Lee G, Lane G, Rubino F, Safford R, Kronmal RA, McBride R, Pearce LA, Nasco E, Hart RG, Sherman CP, Sherman DG, Talbert RL, Dacy TL, Heberling PA. Predictors of thromboembolism in atrial-

fibrillation: II. echocardiographic features of patoents at risk. *Annals of Internal Medicine.* 1992;116(1):6-12.

73. Di Tullio MR, Sacco RL, Sciacca RR, Homma S. Left atrial size and the risk of ischaemic stroke in an ethnically mixed population. *Stroke.* 1999;30(10):2019-2024.

74. Salem DN, Stein PD, Al-Ahmad A, Bussey HI, Horstkotte D, Miller N, Pauker SG. Antithrombotic therapy in vaivular heart disease - Native and prosthetic. *Chest.* 2004;126(3):457S-482S.

75. Nakagami H, Yamamoto K, Ikeda U, Mitsuhashi T, Goto T, Shimada K. Mitral regurgitation reduces the risk of stroke in patients with nonrheumatic atrial fibrillation. *American Heart Journal.* 1998;136(3):528-532.

76. Chiang CW, Lo SK, Ko YS, Cheng NJ, Lin PJ, Chang CH. Predictors of systemic embolism in patients with mitral stenosis - A prospective study. *Annals of Internal Medicine.* 1998;128(11):885-+.

77. Asinger RW, Koehler J, Pearce LA, Zabalgoitia M, Blackshear JL, Fenster PE, Strauss R, Hess D, Pennock GD, Rothbart RM, Halperin JL, Stroke Prevention Atrial F. Pathophysiologic correlates of thromboembolism in nonvalvular atrial fibrillation: II. Dense spontaneous echocardiographic contrast (The Stroke Prevention in Atrial Fibrillation SPAF-III study). *Journal of the American Society of Echocardiography.* 1999;12(12):1088-1096.

78. Handke M, Harloff A, Hetzel A, Olschewski M, Bode C, Geibel A. Left atrial appendage flow velocity as a quantitative surrogate parameter for thromboembolic risk: Determinants and relationship to spontaneous echocontrast and thrombus formation - A transesophageal echocardiographic study in 500 patients with cerebral ischemia. *Journal of the American Society of Echocardiography.* 2005;18(12):1366-1372.

79. Obarski TP, Salcedo EE, Castle LW, Stewart WJ. Spontaneous echo contrast in the left atrium during paroxysmal atrial-fibrillation. *American Heart Journal.* 1990;120(4):988-990.

80. Jue J, Winslow T, Fazio G, Redberg RF, Foster E, Schiller NB. Pulsed Doppler characterization of left atrial appendage flow. *J Am Soc Echocardiogr.* 1993;6(3 Pt 1):237-244.

81. Black IW, Chesterman CN, Hopkins AP, Lee LCL, Chong BH, Walsh WF. Hematological correlates of left atrial spontaneous echo contrast and thromboembolism in nonvalvular atrial-fibrillation. *Journal of the American College of Cardiology.* 1993;21(2):451-457.

82. Rastegar R, Harnick DJ, Weidermann P, Fuster V, Coller B, Badimon JJ, Chesebro J, Goldman ME. Spontaneous echo contrast videodensity is flow-related and is dependent on the relative concentrations of fibrinogen and red blood cells. *Journal of the American College of Cardiology.* 2003;41(4):603-610.

83. Merino A, Hauptman P, Badimon L, Badimon JJ, Cohen M, Fuster V, Goldman M. Echocardiographic smoke is produced by an interaction of erythrocytes and plasma-proteins modulated by shear forces. *Journal of the American College of Cardiology.* 1992;20(7):1661-1668.

84. Kamath S, Chin BSP, Blann AD, Lip GYH. A study of platelet activation in paroxysmal, persistent and permanent atrial fibrillation. *Blood Coagulation & Fibrinolysis.* 2002;13(7):627-636.

85. Wang YC, Lin JL, Hwang JJ, Lin MS, Tseng CD, Huang SKS, Lai LP. Left atrial dysfunction in patients with atrial fibrillation after successful rhythm control for > 3 months. *Chest.* 2005;128(4):2551-2556.

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

87. Mahe I, Drouet L, Chassany O, Mazoyer E, Simoneau G, Knellwolf AL, Caulin C, Bergmann JF. D-dimer: a characteristic of the coagulation state of each patient with chronic atrial fibrillation. *Thrombosis Research.* 2002;107(1-2):1-6.

88. Roldan V, Marin F, Marco P, Martinez JG, Calatayud R, Sogorb F. Hypofibrinolysis in atrial fibrillation. *American Heart Journal.* 1998;136(6):956-960.

89. Kahn SR, Solymoss S, Flegel KM. Nonvalvular atrial fibrillation: evidence for a prothrombotic state. *Canadian Medical Association Journal.* 1997;157(6):673-681.

90. Mitusch R, Siemens HJ, Garbe M, Wagner T, Sheikhzadeh A, Diederich KW. Detection of a hypercoagulable state in nonvalvular atrial fibrillation and the effect of anticoagulant therapy. *Thrombosis and Haemostasis.* 1996;75(2):219-223.

91. Kumagai K, Fukunami M, Ohmori M, Kitabatake A, Kamada T, Hoki N. Increased intracardiovascular clotting in patients with chronic atrial-fibrillation. *Journal of the American College of Cardiology.* 1990;16(2):377-380.

92. Lip GYH, Lowe GDO, Rumley A, Dunn FG. Increased markers of thrombogenesis in chronic atrial-fibrillation - effects of warfarin treatment. *British Heart Journal.* 1995;73(6):527-533.

93. Turgut N, Akdemir O, Turgut B, Demir M, Ekuklu G, Vural O, Ozbay G, Utku U. Hypercoagulopathy in stroke patients with nonvalvular atrial fibrillation: Hematologic and cardiologic investigations. *Clinical and Applied Thrombosis-Hemostasis.* 2006;12(1):15-20.

94. Varughese GI, Patel JV, Tomson J, Lip GYH. The prothrombotic risk of diabetes mellitus in atrial fibrillation and heart failure. *Journal of Thrombosis and Haemostasis.* 2005;3(12):2811-2813.

95. Inoue H, Nozawa T, Okumura K, Lee JD, Shimizu A, Yano K. Prothrombotic activity is increased in patients with nonvalvular atrial fibrillation and risk factors for embolism. *Chest.* 2004;126(3):687-692.

96. Ohara K, Inoue H, Nozawa T, Hirai T, Iwasa A, Okumura K, Lee JD, Shimizu A, Hayano M, Yano K. Accumulation of risk factors enhances the prothrombotic state in atrial fibrillation. *International Journal of Cardiology.* 2008;126(3):316-321.

97. Kamath S, Blann AD, Chin BSP, Lip GYH. A prospective randomized trial of aspirin-clopidogrel combination therapy and dose-adjusted warfarin on indices of thrombogenesis and platelet activation in atrial fibrillation. *Journal of the American College of Cardiology.* 2002;40(3):484-490.

98. Mondillo S, Sabatini L, Agricola E, Ammaturo T, Guerrini F, Barbati R, Pastore M, Fineschi D, Nami R. Correlation between left atrial size, prothrombotic state and markers of endothelial dysfunction in patients with lone chronic nonrheumatic atrial fibrillation. *International Journal of Cardiology.* 2000;75(2-3):227-232.

99. Li-Saw-Hee FL, Blann AD, Gurney D, Lip GYH. Plasma von Willebrand factor, fibrinogen and soluble P-selectin levels in paroxysmal, persistent and permanent atrial fibrillation - Effects of cardioversion and return of left atrial function. *European Heart Journal.* 2001;22(18):1741-1747.

100. Roldan V, Marin F, Garcia-Herola A, Lip GYH. 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.* 2005;116(4):321-325.

101. Heppell RM, Berkin KE, McLenachan JM, Davies JA. Haemostatic and haemodynamic abnormalities associated with left atrial thrombosis in non-rheumatic atrial fibrillation. *Heart.* 1997;77(5):407-411.

102. Habara S, Dote K, Kato M, Sasaki S, Goto K, Takemoto H, Hasegawa D, Matsuda O. Prediction of left atrial appendage thrombi in non-valvular atrial fibrillation. *European Heart Journal.* 2007;28(18):2217-2222.

103. Igarashi Y, Kashimura K, Makiyama Y, Sato T, Ojima K, Aizawa Y. Left atrial appendage dysfunction in chronic nonvalvular atrial fibrillation is significantly associated with an elevated level of brain natriuretic peptide and a prothrombotic state. *Japanese Circulation Journal-English Edition.* 2001;65(9):788-792.

104. Shinohara H, Fukuda N, Soeki T, Takeichi N, Yui Y, Tamura Y, Oki T. Relationship between flow dynamics in the left atrium and hemostatic

abnormalities in patients with nonvalvular atrial fibrillation. *Japanese Heart Journal.* 1998;39(6):721-730.

105. Tsai LM, Chen JH, Tsao CJ. Relation of left atrial spontaneous echo contrast prethrombotic state in atrial fibrillation associated with systemic hypertension, idiopathic dilated cardiomyopathy, or no identifiable cause (Lone). *American Journal of Cardiology.* 1998;81(10):1249-+.

106. Sakurai K, Hirai T, Nakagawa K, Kameyama T, Nozawa T, Asanoi H, Inoue H. Left atrial appendage function and abnormal hypercoagulability in patients with atrial flutter. *Chest.* 2003;124(5):1670-1674.

107. Lip GYH, Rumley A, Dunn FG, Lowe GDO. Thrombogenesis in mitral regurgitation and aortic stenosis. *Angiology.* 1996;47(12):1117-1125.

108. Roldan V, Marin F, Marco P, Climent V, Martinez JG, Monmeneu JV, Bodi V, Garri FS. Anticoagulant therapy modifies fibrinolytic dysfunction in chronic atrial fibrillation. *Haemostasis.* 2000;30(4):219-224.

109. Zeuthen EL, Lassen JF, Husted SE. Is there a hypercoagulable phase during initiation of antithrombotic therapy with oral anticoagulants in patients with atrial fibrillation? *Thrombosis Research.* 2003;109(5-6):241-246.

110. Nozawa T, Inoue H, Iwasa A, Okumura K, Lee JD, Shimizu A, Hayano M, Yano K. Effects of anticoagulation intensity on hemostatic markers in patients with non-valvular atrial fibrillation. *Circulation Journal.* 2004;68(1):29-34.

111. Koefoed BG, Feddersen C, Gullov AL, Petersen P. Effect of fixed minidose warfarin, conventional dose warfarin and aspirin on INR and prothrombin fragment 1+2 in patients with atrial fibrillation. *Thrombosis and Haemostasis.* 1997;77(5):845-848.

112. Li-Saw-Hee FL, Blann AD, Lip GYH. Effects of fixed low-dose warfarin, aspirin-warfarin combination therapy, and dose-adjusted warfarin on thrombogenesis in chronic atrial fibrillation. *Stroke.* 2000;31(4):828-833.

113. Kamath S, Blann AD, Chin BSP, Lanza F, Aleil B, Cazenave JR, Lip GYH. A study of platelet activation in atrial fibrillation and the effects of antithrombotic therapy. *European Heart Journal.* 2002;23(22):1788-1795.

114. Lip GYH, Lip PL, Zarifis J, Watson RDS, Bareford D, Lowe GDO, Beevers DG. Fibrin D-dimer and beta-thromboglobulin as markers of thrombogenesis and platelet activation in atrial fibrillation - Effects of introducing ultra-low-dose warfarin and aspirin. *Circulation.* 1996;94(3):425-431.

115. Vene N, Mavri A, Kosmelj K, Stegnar M. High D-dimer levels predict cardiovascular events in patients with chronic atrial fibrillation during oral anticoagulant therapy. *Thrombosis and Haemostasis.* 2003;90(6):1163-1172.

116. Nozawa T, Inoue H, Hirai T, Iwasa A, Okumura K, Lee JD, Shimizu A, Hayano M, Yano K. D-dimer level influences thromboembolic events in

patients with atrial fibrillation. *International Journal of Cardiology.* 2006;109(1):59-65.

117. Nozawa T, Inoue H. Reply: Fibrin D-dimer levels and thromboembolic events in patients with atrial fibrillation. *International Journal of Cardiology.* 2007;120(1):125-126.

118. Somloi M, Tomcsayi J, Nagy E, Bodo I, Bezzegh A. D-dimer determination as a screening tool to exclude atrial thrombi in atrial fibrillation. *American Journal of Cardiology.* 2003;92(1):85-87.

119. Fukuchi M, Watanabe J, Kumagai K, Katori Y, Baba S, Fukuda K, Yagi T, Iguchi A, Yokoyama H, Miura M, Kagaya Y, Sato S, Tabayashi K, Shirato K. Increased von Willebrand factor in the endocardium as a local predisposing factor for thrombogenesis in overloaded human atrial appendage. *Journal of the American College of Cardiology.* 2001;37(5):1436-1442.

120. Kumagai K, Fukuchi M, Ohta J, Baba S, Oda K, Akimoto H, Kagaya Y, Watanabe J, Tabayashi K, Shirato K. Expression of the von Willebrand factor in atrial endocardium is increased in atrial fibrillation depending on the extent of structural remodeling. *Circulation Journal.* 2004;68(4):321-327.

121. Nakamura Y, Nakamura K, Fukushima-Kusano K, Ohta K, Matsubara H, Hamuro T, Yutani C, Ohe T. Tissue factor expression in atrial endothelia associated with nonvalvular atrial fibrillation: possible involvement in intracardiac thrombogenesis. *Thrombosis Research.* 2003;111(3):137-142.

122. Conway DSG, Heeringa J, Van Der Kuip DAM, Chin BSP, Hofman A, Witteman JCM, Lip GYH. Atrial fibrillation and the prothrombotic state in the elderly - The Rotterdam Study. *Stroke.* 2003;34(2):413-417.

123. Conway DSG, Pearce LA, Chin BSP, Hart RG, Lip GYH. Plasma von Willebrand factor and soluble P-selectin as indices of endothelial damage and platelet activation in 1321 patients with nonvalvular atrial fibrillation - Relationship to stroke risk factors. *Circulation.* 2002;106(15):1962-1967.

124. Conway DSG, Pearce LA, Chin BSP, Hart RG, Lip GYH. Prognostic value of plasma von Willebrand factor and soluble P-selectin as indices of endothelial damage and platelet activation in 994 patients with nonvalvular atrial fibrillation. *Circulation.* 2003;107(25):3141-3145.

125. Lip GYH, Lane D, Van Walraven C, Hart RG. Additive role of plasma von Willebrand factor levels to clinical factors for risk stratification of patients with atrial fibrillation. *Stroke.* 2006;37(9):2294-2300.

126. Varughese GI, Patel JV, Tomson J, Lip GYH. Effects of blood pressure on the prothrombotic risk in 1235 patients with non-valvular atrial fibrillation. *Heart.* 2007;93(4):495-499.

127. Lip GYH, Pearce LA, Chin BSP, Conway DSG, Hart RG. Effects of congestive heart failure on plasma von Willebrand factor and soluble P-

selectin concentrations in patients with non-valvar atrial fibrillation. *Heart.* 2005;91(6):759-763.

128. Furui H, Taniguchi N, Yamauchi K, Sotobata I, Saito H, Inagaki H. Effects of treadmill-exercise on platelet-function, blood-coagulability and fibrinloytic activity in patients with atrial-fibrillation. *Japanese Heart Journal.* 1987;28(2):177-184.

129. Kamath S, Blann AD, Chin BSP, Lip GYH. Platelet activation, haemorheology and thrombogenesis in acute atrial fibrillation: a comparison with permanent atrial fibrillation. *Heart.* 2003;89(9):1093-1095.

130. Feinberg WM, Macy E, Cornell ES, Nightingale SD, Pearce LA, Tracy RP, Bovill EG, Stroke Prevention Atrial Fibrillation I. Plasmin-alpha(2)-antiplasmin complex in patients with atrial fibrillation. *Thrombosis and Haemostasis.* 1999;82(1):100-103.

131. Ortuno FM, Schilling VR, Vera PM, Martinez JGM, Noguera AT, Rico FGD, Sendra RC, Garri FS. Improvement of fibrinolytical function in chronic rheumatic atrial fibrillation after anticoagulation. *Revista Espanola De Cardiologia.* 1999;52(1):25-30.

132. Chung MK, Martin DO, Sprecher D, Wazni O, Kanderian A, Carnes CA, Bauer JA, Tchou PJ, Niebauer MJ, Natale A, Van Wagoner DR. C-reactive protein elevation in patients with atrial arrhythmias - Inflammatory mechanisms and persistence of atrial fibrillation. *Circulation.* 2001;104(24):2886-2891.

133. Dernellis J, Panaretou M. C-reactive protein and paroxysmal atrial fibrillation: evidence of the implication of an inflammatory process in paroxysmal atrial fibrillation. *Acta Cardiol.* 2001;56(6):375-380.

134. Tveit A, Seljeflot I, Grundvold I, Abdelnoor M, Smith P, Arnesen H. Levels of PAI-1 and outcome after electrical cardioversion for atrial fibrillation. *Thrombosis Research.* 2008;121(4):447-453.

135. Pretorius M, Donahue BS, Yu C, Greelish JP, Roden DM, Brown NJ. Plasminogen activator inhibitor-1 as a predictor of postoperative atrial fibrillation after cardiopulmonary bypass. *Circulation.* 2007;116(11):I1-I7.

136. Choudhury A, Chung I, Blann AD, Lip GYH. Elevated platelet microparticle levels in nonvalvular atrial fibrillation - Relationship to P-selectin and antithrombotic therapy. *Chest.* 2007;131(3):809-815.

137. Kaplan KL, Nossel HL, Drillings M, Lesznik G. Radioimmunoassay of platelet factor-4 and beta-thromboglobulin - development and application to studies of platelet-release in relation to fibrinopeptide-a generation. *British Journal of Haematology.* 1978;39(1):129-146.

138. Kunishima S, Hattori M, Kobayashi S, Hattori H, Iwama Y, Imai Y, Ogawa K, Naoe T, Ohno R. Activation and destruction of platelets in patients with rheumatic heart-disease. *European Heart Journal.* 1994;15(3):335-338.

139. Minamino T, Kitakaze M, Asanuma H, Ueda Y, Koretsune Y, Kuzuya T, Hori M. Plasma adenosine levels and platelet activation in patients with atrial fibrillation. *American Journal of Cardiology.* 1999;83(2):194-198.

140. Yamauchi K, Furui H, Taniguchi N, Sotobata I. Plasma beta-thromboglobulin and platelet factor-iv concentrations in patients with atrial-fibrillation. *Japanese Heart Journal.* 1986;27(4):481-487.

141. Choudhury A, Chung I, Blann A, Lip GYH. Platelet adhesion in atrial fibrillation. *Thrombosis Research.* 2007;120(4):623-629.

142. Chirinos JA, Castrellon A, Zambrano JP, Jimenez JJ, Jy W, Horstman LL, Willens HJ, Castellanos A, Myerburg RJ, Ahn YS. Digoxin use is associated with increased platelet and endothelial cell activation in patients with nonvalvular atrial fibrillation. *Heart Rhythm.* 2005;2(5):525-529.

143. Kamath S, Blann AD, Caine GJ, Gurney D, Chin BSP, Lip GYH. Platelet P-selectin levels in relation to plasma soluble P-selectin and beta-thromboglobulin levels in atrial fibrillation. *Stroke.* 2002;33(5):1237-1242.

144. Helgason CM, Hoff JA, Kondos GT, Brace LD. Platelet-aggregation in patients with atrial-fibrillation taking aspirin or warfarin. *Stroke.* 1993;24(10):1458-1461.

145. Muller I, Massberg S, Zierhut W, Binz C, Schuster A, Rudiger-von Hoch S, Braun S, Gawaz M. Effects of aspirin and clopidogrel versus oral anticoagulation on platelet function and on coagulation in patients with nonvalvular atrial fibrillation (CLAFIB). *Pathophysiology of Haemostasis and Thrombosis.* 2002;32(1):16-24.

146. Sohara H, Amitani S, Kurose M, Miyahara K. Atrial fibrillation activates platelets and coagulation in a time-dependent manner: A study in patients with paroxysmal atrial fibrillation. *Journal of the American College of Cardiology.* 1997;29(1):106-112.

147. Atalar E, Haznedaroglu IC, Acil T, Ozer N, Kilic H, Ovunc K, Aksoyek S, Nazli N, Kes S, Kabakci G, Kirazli S, Ozmen F. Patients with paroxysmal atrial fibrillation but not paroxysmal supraventricular tachycardia display evidence of platelet activation during arrhythmia. *Platelets.* 2003;14(7-8):407-411.

148. Tse HF, Kwong YL, Lau CP. Transvenous cryoablation reduces platelet activation during pulmonary vein ablation compared with radiofrequency energy in patients with atrial fibrillation. *Journal of Cardiovascular Electrophysiology.* 2005;16(10):1064-1070.

149. Feinberg WM, Pearce LA, Hart RG, Cushman M, Cornell ES, Lip GYH, Bovill EG, Stroke Prevention Atrial F. Markers of thrombin and platelet activity in patients with atrial fibrillation - Correlation with stroke among 1531 participants in the stroke prevention in atrial fibrillation III study. *Stroke.* 1999;30(12):2547-2553.

150. Heeringa J, Conway DSG, van der Kuip DAM, Hofman A, Breteler MMB, Lip GYH, Witteman JCM. A longitudinal population-based study of prothrombotic factors in elderly subjects with atrial fibrillation: the Rotterdam Study 1990-1999. *Journal of Thrombosis and Haemostasis.* 2006;4(9):1944-1949.

151. Minamino T, Kitakaze M, Sanada S, Asanuama H, Kurotobi T, Koretsune Y, Fukunami M, Kuzuya T, Hoki N, Hori M. Increased expression of P-selectin on platelets is a risk factor for silent cerebral infarction in patients with atrial fibrillation - Role of nitric oxide. *Circulation.* 1998;98(17):1721-1727.

152. Pongratz G, BrandtPohlmann M, Henneke KH, Pohle C, Zink D, Gehling G, Bachmann K. Platelet activation in embolic and preembolic status of patients with nonrheumatic atrial fibrillation. *Chest.* 1997;111(4):929-933.

153. Oltrona L, Broccolino M, Merlini PA, Spinola A, Pezzano A, Mannucci PM. Activation of the hemostatic mechanism after pharmacological cardioversion of acute nonvalvular atrial fibrillation. *Circulation.* 1997;95(8):2003-2006.

154. Giansante C, Fiotti N, Miccio M, Altamura N, Salvi R, Guarnieri G. Coagulation indicators in patients with paroxysmal atrial fibrillation: Effects of electric and pharmacologic cardioversion. *American Heart Journal.* 2000;140(3):423-429.

155. Hatzinikolaou-Kotsakou E, Kartasis Z, Tziakas D, Stakos D, Hotidis A, Chalikias G, Bourikas G, Hatseras D. Clotting state after cardioversion of atrial fibrillation: a haemostasis index could detect the relationship with the arrhythmia duration. *Thrombosis Journal*. 2005;3(1):2.

156. Jacob K, Talwar S, Copplestone A, Gilbert TJ, Haywood GA. Activation of coagulation occurs after electrical cardioversion in patients with chronic atrial fibrillation despite optimal anticoagulation with warfarin. *International Journal of Cardiology.* 2004;95(1):83-88.

157. Zeuthen EL, Lassen JF, Husted SE. Haemostatic activity in patients with atrial fibrillation treated with low-molecular-weight heparin before and after electrical cardioversion. *Journal of Thrombosis and Thrombolysis.* 2004;17(3):185-189.

158. Sakurai K, Hirai T, Nakagawa K, Kameyama T, Nozawa T, Asanoi H, Inoue H. Prolonged activation of hemostatic markers following conversion of atrial flutter to sinus rhythm. *Circulation Journal.* 2004;68(11):1041-1044.

159. Boos CJ, Anderson RA, Lip GYH. Is atrial fibrillation an inflammatory disorder? *Eur. Heart J.* 2006;27(2):136-149.

160. Engelmann MDM, Svendsen JH. Inflammation in the genesis and perpetuation of atrial fibrillation. *European Heart Journal.* 2005;26(20):2083-2092.

161. Gedikli O, Dogan A, Altuntas I, Altinbas A, Ozaydin M, Akturk O, Acar G. Inflammatory markers according to types of atrial fibrillation. *International Journal of Cardiology.* 2007;120(2):193-197.

162. Bruins P, Velthuis HT, Yazdanbakhsh AP, Jansen PGM, vanHardevelt FWJ, deBeaumont E, Wildevuur CRH, Eijsman L, Trouwborst A, Hack CE. Activation of the complement system during and after cardiopulmonary bypass surgery - Postsurgery activation involves C-reactive protein and is associated with postoperative arrhythmia. *Circulation.* 1997;96(10):3542-3548.

163. Hernandez Madrid A, Moro C. Atrial fibrillation and C-reactive protein: searching for local inflammation. *J Am Coll Cardiol.* 2007;49(15):1649-1650.

164. Conway DSG, Buggins P, Hughes E, Lip GYH. Prognostic significance of raised plasma levels of interleukin-6 and C-reactive protein in atrial fibrillation. *American Heart Journal.* 2004;148(3):462-466.

165. Tveit A, Seljeflot I, Grundvold I, Abdelnoor M, Smith P, Arnesen H. Effect of candesartan and various inflammatory markers on maintenance of sinus rhythm after electrical cardioversion for atrial fibrillation. *American Journal of Cardiology.* 2007;99(11):1544-1548.

166. Lip GYH, Patel JV, Hughes E, Hart RG. 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.* 2007;38(4):1229-1237.

167. Cermak J, Key NS, Bach RR, Balla J, Jacob HS, Vercellotti GM. C-reactive protein induces human peripheral-blood monocytes to synthesize tissue factor. *Blood.* 1993;82(2):513-520.

168. Neumann FJ, Ott I, Marx N, Luther T, Kenngott S, Gawaz M, Kotzsch M, Schomig A. Effect of human recombinant interleukin-6 and interleukin-8 on monocyte procoagulant activity. *Arteriosclerosis Thrombosis and Vascular Biology.* 1997;17(12):3399-3405.

169. Burstein SA. Cytokines, platelet production and hemostasis. *Platelets.* 1997;8(2-3):93-104.

170. Amrani DL. Regulation of fibrinogen biosynthesis: glucocorticoid and interleukin-6 control. *Blood Coagul Fibrinolysis.* 1990;1(4-5):443-446.

171. Burstein SA, Peng J, Friese P, Wolf RF, Harrison P, Downs T, Hamilton K, Comp P, Dale GL. Cytokine-induced alteration of platelet and hemostatic function. *Stem Cells.* 1996;14 Suppl 1:154-162.

172. Yudkin JS, Stehouwer CDA, Emeis JJ, Coppack SW. C-reactive protein in wealthy subjects: Associations with obesity, insulin resistance, and endothelial dysfunction - A potential role for cytokines originating from adipose tissue? *Arteriosclerosis Thrombosis and Vascular Biology.* 1999;19(4):972-978.

173. Acevedo M, Corbalan R, Braun S, Pereira J, Navarrete C, Gonzalez I. C-reactive protein and atrial fibrillation: "Evidence for the presence of

inflammation in the perpetuation of the arrhythmia". *International Journal of Cardiology.* 2006;108(3):326-331.

174. Conway DSG, Buggins P, Hughes E, Lip GYH. Relationship of interleukin-6 and C-reactive protein to the prothrombotic state in chronic atrial fibrillation. *Journal of the American College of Cardiology.* 2004;43(11):2075-2082.

175. Lip GYH, Chin BSP, Blann AD. Cancer and the prothrombotic state. *Lancet Oncology.* 2002;3(1):27-34.

176. Chung NAY, Belgore F, Hee F, Conway DSG, Blann AD, Lip GYH. Is the hypercoagulable state in atrial fibrillation mediated by vascular endothelial growth factor? *Stroke.* 2002;33(9):2187-2191.

177. Seko Y, Nishimura H, Takahashi N, Ashida T, Nagai R. Serum levels of vascular endothelial growth factor and transforming growth factor-beta 1 in patients with atrial fibrillation undergoing defibrillation therapy. *Japanese Heart Journal.* 2000;41(1):27-32.

178. Freestone B, Chong AY, Lim HS, Blann A, Lip GYH. Angiogenic factors in atrial fibrillation: A possible role in thrombogenesis? *Annals of Medicine.* 2005;37(5):365-372.

179. Webb NJA, Bottomley MJ, Watson CJ, Brenchley PEC. Vascular endothelial growth factor (VEGF) is released from platelets during blood clotting: implications for measurement of circulating VEGF levels in clinical disease. *Clinical Science.* 1998;94(4):395-404.

180. Armesilla AL, Lorenzo E, del Arco PG, Martinez-Martinez S, Alfranca A, Redondo JM. Vascular endothelial growth factor activates nuclear factor of activated T cells in human endothelial cells: a role for tissue factor gene expression. *Molecular and Cellular Biology.* 1999;19(3):2032-2043.

181. Morrissey JH. Tissue factor: An enzyme cofactor and a true receptor. Paper presented at: XVIIIth Congress of the International-Society-on-Thrombosis-and-Haemostasis; Jul 06-12, 2001; Paris, France.

182. Dollery CM, McEwan JR, Henney AM. Matrix metalloproteinases and cardiovascular-disease. *Circulation Research.* 1995;77(5):863-868.

183. Arndt M, Lendeckel U, Rocken C, Nepple K, Wolke C, Spiess A, Huth C, Ansorge S, Klein HU, Goette A. Altered expression of ADAMS (A Disintegrin And Metalloproteinase) in fibrillating human atria. *Circulation.* 2002;105(6):720-725.

184. Lijnen HR. Molecular interactions between the plasminogen/plasmin and matrix metalloproteinase systems. Paper presented at: 15th Congress of the International-Society-for-Fibrinolysis-and-Proteolysis; Jun 25-29, 2000; Hamamatsu, Japan.

185. Gramley F, Lorenzen J, Plisiene J, Rakauskas M, Benetis R, Schmid M, Autschbach R, Knackstedt C, Schimpf T, Mischke K, Gressner A, Hanrath P,

Kelm M, Schauerte P. Decreased plasminogen activator inhibitor and tissue metalloproteinase inhibitor expression may promote increased metalloproteinase activity with increasing duration of human atrial fibrillation. *Journal of Cardiovascular Electrophysiology.* 2007;18:1076-1082.

186. Davis ME, Cai H, Drummond GR, Harrison DG. Shear stress regulates endothelial nitric oxide synthase expression through c-Src by divergent signaling pathways. *Circulation Research.* 2001;89(11):1073-1080.

187. Freedman JE, Loscalzo J, Barnard MR, Alpert C, Keaney JF, Michelson AD. Nitric oxide released from activated platelets inhibits platelet recruitment. *Journal of Clinical Investigation.* 1997;100(2):350-356.

188. Swiatkowska M, Cierniewska-Cieslak A, Pawlowska Z, Cierniewski CS. Dual regulatory effects of nitric oxide on plasminogen activator inhibitor type 1 expression in endothelial cells. *European Journal of Biochemistry.* 2000;267(4):1001-1007.

189. Cai H, Li ZM, Goette A, Mera F, Honeycutt C, Feterik K, Wilcox JN, Dudley SC, Harrison DG, Langberg JJ. Downregulation of endocardial nitric oxide synthase expression and nitric oxide production in atrial fibrillation - Potential mechanisms for atrial thrombosis and stroke. Paper presented at: 74th Annual Scientific Sessions of the American-Heart-Association; Nov 11-14, 2001; Anaheim, California.

190. Goette A, Staack T, Rocken C, Arndt M, Geller JC, Huth C, Ansorge S, Klein HU, Lendeckel U. Increased expression of extracellular signal-regulated kinase and angiotensin-converting enzyme in human atria during atrial fibrillation. *Journal of the American College of Cardiology.* 2000;35(6):1669-1677.

191. Choudhury A, Varughese GI, Lip GYH. Targeting the renin-angiotensin-aldosterone-system in atrial fibrillation: a shift from electrical to structural therapy? *Expert Opinion on Pharmacotherapy.* 2005;6(13):2193-2207.

192. Healey JS, Baranchuk A, Crystal E, Morillo CA, Garfinkle M, Yusuf S, Connolly SJ. Prevention of atrial fibrillation with angiotensin-converting enzyme inhibitors and angiotensin receptor blockers - A meta-analysis. *Journal of the American College of Cardiology.* 2005;45(11):1832-1839.

193. Das UN. COX-2 inhibitors and metabolism of essential fatty acids. *Med Sci Monit.* 2005;11(7):RA233-237.

194. Tamarat R, Silvestre JS, Duriez M, Levy BI. Angiotensin II angiogenic effect in vivo involves vascular endothelial growth factor- and inflammation-related pathways. *Laboratory Investigation.* 2002;82(6):747-756.

195. Suzuki Y, Ruiz-Ortega M, Lorenzo O, Ruperez M, Esteban V, Egido J. Inflammation and angiotensin II. *International Journal of Biochemistry & Cell Biology.* 2003;35(6):881-900.

196. Cardin S, Li DS, Thorin-Trescases N, Leung TK, Thorin E, Nattel S. Evolution of the atrial fibrillation substrate in experimental congestive heart failure: angiotensin-dependent and -independent pathways. *Cardiovascular Research.* 2003;60(2):315-325.

197. Takagishi T, Murahashi N, Azagami S, Morimatsu M, Sasaguri Y. Effect of angiotensin-II and thromboxane-A2 on the production of matrix metalloproteinase by human aortic smooth-muscle cells. *Biochemistry and Molecular Biology International.* 1995;35(2):265-273.

198. Griendling KK, Alexander RW. Oxidative stress and cardiovascular disease. *Circulation.* 1997;96(10):3264-3265.

199. Dzau VJ, Bernstein K, Celermajer D, Cohen J, Dahlof B, Deanfield J, Diez J, Drexler H, Ferrari R, van Gilst W, Hansson L, Hornig B, Husain A, Johnston C, Lazar H, Lonn E, Luscher T, Mancini J, Mimran A, Pepine C, Rabelink T, Remme W, Ruilope L, Ruzicka M, Schunkert H, Swedberg K, Unger T, Vaughan D, Weber M, Working Group on Tissue Angiotensin-converting enzyme ISoCP. The relevance of tissue angiotensin-converting enzyme: manifestations in mechanistic and endpoint data. *Am J Cardiol.* 2001;88(9A):1L-20L.

200. Wachtell K, Hornestam B, Lehto M, Slotwiner DJ, Gerdts E, Olsen MH, Aurup P, Dahlof B, Ibsen H, Julius S, Kjeldsen SE, Lindholm LH, Nieminen MS, Rokkedal J, Devereux RB. Cardiovascular morbidity and mortality in hypertensive patients with a history of atrial fibrillation - The Losartan Intervention For End point reduction in hypertension (LIFE) study. *Journal of the American College of Cardiology.* 2005;45(5):705-711.

201. Dewar RI, Lip GYH, Guidelines Dev Grp Ncg. Identification, diagnosis and assessment of atrial fibrillation. *Heart.* 2007;93(1):25-28.

202. Cohen A, Ederhy S, Meuleman C, Di Angelantonio E, Dufaitre G, Boccara F. D-dimers in atrial fibrillation: a further step in risk stratification of thrombo-embolism? *European Heart Journal.* 2007;28(18):2179-2180.

203. Ezekowitz MD, Reilly PA, Nehmiz G, Simmers TA, Nagarakanti R, Parcham-Azad K, Pedersen KE, Lionetti DA, Stangier J, Wallentin L. Dabigatran with or without concomitant Aspirin compared with Warfarin alone in patients with nonvalvular atrial fibrillation (PETRO study). *American Journal of Cardiology.* 2007;100(9):1419-1426.

204. Kirchhof P, Auricchio A, Bax J, Crijns H, Camm J, Diener HC, Goette A, Hindricks G, Hohnloser S, Kappenberger L, Kuck KH, Lip GYH, Olsson B, Meinertz T, Priori S, Ravens U, Steinbeck G, Svernhage E, Tijssen J, Vincent A, Breithardt G. Outcome parameters for trials in atrial fibrillation - Recommendations from a consensus conference organized by the German atrial fibrillation competence NETwork and the European Heart Rhythm Association. *Europace.* 2007;9(11):1006-1023.

205. Gustafsson C, Blomback M, Britton M, Hamsten A, Svensson J. Coagulation-factors and the increased risk of stroke in onvalvular atrial-fibrillation. *Stroke.* 1990;21(1):47-51.

206. Asakura H, Hifumi S, Jokaji H, Saito M, Kumabashiri I, Uotani C, Morishita E, Yamazaki M, Shibata K, Mizuhashi K, Mifune J, Matsuda T. Prothrombin fragment f1+2 and thrombin antithrombin-III complex are useful markers of the hypercoagulable state in atrial-fibrillation. *Blood Coagulation & Fibrinolysis.* 1992;3(4):469-473.

207. Sohara H, Miyahara K. Effect of atrial-fibrillation on the fibrino-coagulation system - study in patients with paroxysmal atrial-fibrillation. *Japanese Circulation Journal-English Edition.* 1994;58(11):821-826.

208. Drexler H, Hornig B. Endothelial dysfunction in human disease. Paper presented at: Symposium on Endothelial Dysfunction - A Novel Therapeutic Target; May 27, 1998; Rhodes, Greece.

209. Endemann DH, Schiffrin EL. Endothelial dysfunction. *Journal of the American Society of Nephrology.* 2004;15(8):1983-1992.

210. Caplan BA, Schwartz CJ. Increased endothelial cell turnover in areas of in-vivo evans-blue uptake in pig aorta. *Atherosclerosis.* 1973;17(3):401-417.

211. Dimmeler S, Zeiher AM. Vascular repair by circulating endothelial progenitor cells: the missing link in atherosclerosis? *Journal of Molecular Medicine-Jmm.* 2004;82(10):671-677.

212. Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nature Medicine.* 2003;9(6):702-712.

213. Asahara T, Murohara T, Sullivan A, Silver M, vanderZee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science.* 1997;275(5302):964-967.

214. Lin Y, Weisdorf D, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. *Journal of Clinical Investigation.* 2000;105(1):71-77.

215. Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, Oh BH, Lee MM, Park YB. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. *Arteriosclerosis Thrombosis and Vascular Biology.* 2004;24(2):288-293.

216. Gulati R, Jevremovic D, Peterson TE, Witt TA, Kleppe LS, Mueske CS, Lerman A, Vile RG, Simari RD. Autologous culture-modified mononuclear cells confer vascular protection after arterial injury. *Circulation.* 2003;108(12):1520-1526.

217. Shi Q, Rafii S, Wu MHD, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MAS, Storb RF, Hammond WP. Evidence for

circulating bone marrow-derived endothelial cells. *Blood.* 1998;92(2):362-367.

218. Gulati R, Jevremovic D, Peterson TE, Chatterjee S, Shah V, Vile RG, Simari RD. Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. *Circulation Research.* 2003;93(11):1023-1025.

219. Zhang ZG, Zhang L, Jiang Q, Chopp M. Bone marrow-derived endothelial progenitor cells participate in cerebral neovascularization after focal cerebral ischemia in the adult mouse. *Circulation Research.* 2002;90(3):284-288.

220. Civin CI, Banquerigo ML, Strauss LC, Loken MR. Antigenic analysis of hematopoiesis. VI. Flow cytometric characterization of my-10-positive progenitor cells in normal human-bone marrow. *Experimental Hematology.* 1987;15(1):10-17.

221. Fina L, Molgaard HV, Robertson D, Bradley NJ, Monaghan P, Delia D, Sutherland DR, Baker MA, Greaves MF. Expression of the CD34 gene in vascular endothelial-cells. *Blood.* 1990;75(12):2417-2426.

222. Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, Bray RA, Waller EK, Buck DW. A novel five-transmembrane hematopoietic stem cell antigen: Isolation, characterization, and molecular cloning. *Blood.* 1997;90(12):5013-5021.

223. Peichev M, Naiyer AJ, Pereira D, Zhu ZP, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MAS, Rafii S. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood.* 2000;95(3):952-958.

224. Yin AH, Miraglia S, Zanjani ED, AlmeidaPorada G, Ogawa M, Leary AG, Olweus J, Kearney J, Buck DW. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood.* 1997;90(12):5002-5012.

225. Friedrich EB, Walenta K, Scharlau J, Nickenig G, Werner N. CD34(-)/CD133(+)/VEGFR-2(+) endothelial progenitor cell subpopulation with potent vasoregenerative capacities. *Circulation Research.* 2006;98(3):E20-E25.

226. Quirici N, Soligo D, Caneva L, Servida F, Bossolasco P, Deliliers GL. Differentiation and expansion of endothelial cells from human bone marrow CD133(+) cells. *British Journal of Haematology.* 2001;115(1):186-194.

227. Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H, Onitsuka I, Matsui K, Imaizumi T. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. *Journal of Clinical Investigation.* 2000;105(11):1527-1536.

228. Burger PE, Coetzee S, McKeehan WL, Kan M, Cook P, Fan Y, Suda T, Hebbel RP, Novitzky N, Muller WA, Wilson EL. Fibroblast growth factor receptor-1 is expressed by endothelial progenitor cells. *Blood.* 2002;100(10):3527-3535.

229. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki KI, Shimada T, Oike Y, Imaizumi T. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation.* 2001;103(23):2776-2779.

230. Vasa M, Fichtlscherer S, Adler K, Aicher A, Martin H, Zeiher AM, Dimmeler S. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation.* 2001;103(24):2885-2890.

231. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM, Dimmeler S. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circulation Research.* 2001;89(1):E1-E7.

232. Hill JM, Zalos G, Halcox JPJ, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *New England Journal of Medicine.* 2003;348(7):593-600.

233. Rookmaaker MB, Vergeer M, van Zonneveld AJ, Rabelink TJ, Verhaar MC. Endothelial progenitor cells: Mainly derived from the monocytemacrophage-containing CD34(-) mononuclear cell population and only in part from the hematopoietic stem cell-containing CD34(+) mononuclear cell population. *Circulation.* 2003;108(21):E150-E150.

234. Fernandez Pujol B, Lucibello FC, Gehling UM, Lindemann K, Weidner N, Zuzarte ML, Adamkiewicz J, Elsasser HP, Muller R, Havemann K. Endothelial-like cells derived from human CD14 positive monocytes. *Differentiation.* 2000;65(5):287-300.

235. Harraz M, Jiao CH, Hanlon HD, Hartley RS, Schatteman GC. CD34(-) blood-derived human endothelial cell progenitors. *Stem Cells.* 2001;19(4):304-312.

236. Schmeisser A, Garlichs CD, Zhang H, Eskafi S, Graffy C, Ludwig J, Strasser RH, Daniel WG. Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel (R) under angiogenic conditions. *Cardiovascular Research.* 2001;49(3):671-680.

237. Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, Pollok K, Ferkowicz MJ, Gilley D, Yoder MC. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood.* 2004;104(9):2752-2760.

238. Urbich C, Heeschen C, Aicher A, Dernbach E, Zeiher AM, Dimmeler S. Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. *Circulation.* 2003;108(20):2511-2516.

239. Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, Asahara T. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proceedings of the*

National Academy of Sciences of the United States of America. 2000;97(7):3422-3427.

240. Urbich C, Dimmeler S. Endothelial progenitor cells - Functional characterization. *Trends in Cardiovascular Medicine.* 2004;14(8):318-322.

241. Nishikawa SI. A complex linkage in the developmental pathway of endothelial and hematopoietic cells. *Current Opinion in Cell Biology.* 2001;13(6):673-678.

242. Oswald J, Boxberger S, Jorgensen B, Feldmann S, Ehninger G, Bornhauser M, Werner C. Mesenchymal stem cells can be differentiated into endothelial cells in vitro. *Stem Cells.* 2004;22(3):377-384.

243. Al-Khaldi A, Eliopoulos N, Martineau D, Lejeune L, Lachapelle K, Galipeau J. Postnatal bone marrow stromal cells elicit a potent VEGF-dependent neoangiogenic response in vivo. *Gene Therapy.* 2003;10(8):621-629.

244. Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelian progenitors in human postnatal bone marrow. *Journal of Clinical Investigation.* 2002;109(3):337-346.

245. Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P, Robey PG. Circulating skeletal stem cells. *Journal of Cell Biology.* 2001;153(5):1133-1139.

246. Kinnaird T, Stabile E, Burnett MS, Shou M, Lee CW, Barr S, Fuchs S, Epstein SE. Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation.* 2004;109(12):1543-1549.

247. Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ, Benhaim P, Lorenz HP, Hedrick MH. Multilineage cells from human adipose tissue: Implications for cell-based therapies. *Tissue Engineering.* 2001;7(2):211-228.

248. Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M, Tedgui A, Levy B, Penicaud L, Casteilla L. Plasticity of human adipose lineage cells toward endothelial cells - Physiological and therapeutic perspectives. *Circulation.* 2004;109(5):656-663.

249. Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell.* 2003;114(6):763-776.

250. Wurmser AE, Nakashima K, Summers RG, Toni N, D'Amour KA, Lie DC, Gage FH. Cell fusion-independent differentiation of neural stem cells to the endothelial lineage. *Nature.* 2004;430(6997):350-356.

251. Dan YY, Riehle KJ, Lazaro C, Teoh N, Haque J, Campbell JS, Fausto N. Isolation of multipotent progenitor cells from human fetal liver capable of

differentiating into liver and mesenchymal lineages. *Proceedings of the National Academy of Sciences of the United States of America.* 2006;103(26):9912-9917.

252. Scheubel RJ, Zorn H, Silber RE, Kuss O, Morawietz H, Holtz J, Simm A. Age-dependent depression in circulating endothelial progenitor cells in patients undergoing coronary artery bypass grafting. *Journal of the American College of Cardiology.* 2003;42(12):2073-2080.

253. Edelberg JM, Tang LL, Hattori K, Lyden D, Rafii S. Young adult bone marrow-derived endothelial precursor cells restore aging-impaired cardiac angiogenic function. *Circulation Research.* 2002;90(10):E89-E93.

254. Rauscher FM, Goldschmidt-Clermont PJ, Davis BH, Tang W, Gregg D, Ramaswami R, Pippen AM, Annex BH, Dong CM, Taylor DA. Aging, progenitor cell exhaustion, and atherosclerosis. *Circulation.* 2003;108(4):457-463.

255. Assmus B, Urbich C, Aicher A, Hofmann WK, Haendeler J, Rossig L, Spyridopoulos I, Zeiher AM, Dimmeler S. HMG-CoA reductase inhibitors reduce senescence and increase proliferation of endothelial progenitor cells via regulation of cell cycle regulatory genes. *Circulation Research.* 2003;92(9):1049-1055.

256. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM, Dimmeler S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nature Medicine.* 2003;9(11):1370-1376.

257. Hoffmann J, Haendeler J, Aicher A, Rossig L, Vasa M, Zeiher AM, Dimmeler S. Aging enhances the sensitivity of endothelial cells toward apoptotic stimuli - Important role of nitric oxide. *Circulation Research.* 2001;89(8):709-715.

258. Rivard A, Berthou-Soulie L, Principe N, Kearney M, Curry C, Branellec D, Semenza GL, Isner JM. Age-dependent defect in vascular endothelial growth factor expression is associated with reduced hypoxia-inducible factor 1 activity. *Journal of Biological Chemistry.* 2000;275(38):29643-29647.

259. Cooke JP, Losordo DW. Nitric oxide and angiogenesis. *Circulation.* 2002;105(18):2133-2135.

260. Tschudi MR, Barton M, Bersinger NA, Moreau P, Cosentino F, Noll G, Malinski T, Luscher TF. Effect of age on kinetics of nitric oxide release in rat aorta and pulmonary artery. *Journal of Clinical Investigation.* 1996;98(4):899-905.

261. Murasawa S, Llevadot J, Silver M, Isner JM, Losordo DW, Asahara T. Constitutive human telomerase reverse transcriptase expression enhances regenerative properties of endothelial progenitor cells. *Circulation.* 2002;106(9):1133-1139.

262. Hornig B, Maier V, Drexler H. Physical training improves endothelial function in patients with chronic heart failure. *Circulation.* 1996;93(2):210-214.

263. Hambrecht R, Fiehn E, Weigl C, Gielen S, Hamann C, Kaiser R, Yu JT, Adams V, Niebauer J, Schuler G. Regular physical exercise corrects endothelial dysfunction and improves exercise capacity in patients with chronic heart failure. *Circulation.* 1998;98(24):2709-2715.

264. Brendle DC, Joseph LJO, Corretti MC, Gardner AW, Katzel LI. Effects of exercise rehabilitation on endothelial reactivity in older patients with peripheral arterial disease. *American Journal of Cardiology.* 2001;87(3):324-329.

265. Stewart KJ, Hiatt WR, Regensteiner JG, Hirsch AT. Medical progress: Exercise training for claudication. *New England Journal of Medicine.* 2002;347(24):1941-1951.

266. Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K, Miche E, Bohm M, Nickenig G. Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation.* 2004;109(2):220-226.

267. Rehman J, Li JL, Parvathaneni L, Karlsson G, Panchal VR, Temm CJ, Mahenthiran J, March KL. Exercise acutely increases circulating endothelial progenitor cells and monocyte-/macrophage-derived angiogenic cells. *Journal of the American College of Cardiology.* 2004;43(12):2314-2318.

268. Sandri M, Adams V, Gielen S, Linke A, Lenk K, Krankel N, Lenz D, Erbs S, Scheinert D, Mohr FW, Schuler G, Hambrecht R. Effects of exercise and ischemia on mobilization and functional activation of blood-derived progenitor cells in patients with ischemic syndromes - Results of 3 randomized studies. *Circulation.* 2005;111(25):3391-3399.

269. Chen JZ, Zhang FR, Tao QM, Wang XX, Zhu JH. Number and activity of endothelial progenitor cells from peripheral blood in patients with hypercholesterolaemia. *Clinical Science.* 2004;107(3):273-280.

270. Imanishi T, Hano T, Sawamura T, Nishio I. Oxidized low-density lipoprotein induces endothelial progenitor cell senescence, leading to cellular dysfunction. *Clinical and Experimental Pharmacology and Physiology.* 2004;31(7):407-413.

271. Wang XX, Chen JZ, Tao QM, Zhu JG, Shang YP. Effects of Ox-LDL on number and activity of circulating endothelial progenitor cells. *Drug and Chemical Toxicology.* 2004;27(3):243-255.

272. Imanishi T, Hano T, Matsuo Y, Nishio I. Oxidized low-density lipoprotein inhibits vascular endothelial growth factor-induced endothelial progenitor cell differentiation. *Clinical and Experimental Pharmacology and Physiology.* 2003;30(9):665-670.

273. Pellegatta F, Bragheri M, Grigore L, Raselli S, Maggi FM, Brambilla C, Reduzzi A, Pirillo A, Norata GD, Catapano AL. In vitro isolation of circulating endothelial progenitor cells is related to the high density lipoprotein plasma levels. *International Journal of Molecular Medicine.* 2006;17(2):203-208.

274. Imanishi T, Hano T, Nishio I. Angiotensin II potentiates vascular endothelial growth factor-induced proliferation and network formation of endothelial progenitor cells. *Hypertension Research.* 2004;27(2):101-108.

275. Imanishi T, Hano T, Nishio I. Angiotensin II accelerates endothelial progenitor cell senescence through induction of oxidative stress. *Journal of Hypertension.* 2005;23(1):97-104.

276. Waltenberger J. Impaired collateral vessel development in diabetes: potential cellular mechanisms and therapeutic implications. *Cardiovascular Research.* 2001;49(3):554-560.

277. Abaci A, Oguzhan A, Kahraman S, Eryol NK, Unal S, Arinc H, Ergin A. Effect of diabetes mellitus on formation of coronary collateral vessels. *Circulation.* 1999;99(17):2239-2242.

278. Loomans CJM, de Koning EJP, Staal FJT, Rookmaaker MB, Verseyden C, de Boer HC, Verhaar MC, Braam B, Rabelink TJ, van Zonneveld AJ. Endothelial progenitor cell dysfunction - A novel concept in the pathogenesis of vascular complications of type 1 diabetes. *Diabetes.* 2004;53(1):195-199.

279. Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP, Gurtner GC. Human endothelial progenitor exhibit impaired proliferation, cells from type II diabetics adhesion, and incorporation into vascular structures. *Circulation.* 2002;106(22):2781-2786.

280. Pistrosch F, Herbrig K, Oelschlaegel U, Richter S, Passauer J, Fischer S, Gross P. PPAR gamma-agonist rosiglitazone increases number and migratory activity of cultured endothelial progenitor cells. *Atherosclerosis.* 2005;183(1):163-167.

281. Rehman J, Li JL, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation.* 2003;107(8):1164-1169.

282. Iba O, Matsubara H, Nozawa Y, Fujiyama S, Amano K, Mori Y, Kojima H, Iwasaka T. Angiogenesis by implantation of peripheral blood mononuclear cells and platelets into ischemic limbs. *Circulation.* 2002;106(15):2019-2025.

283. Krankel N, Adams V, Linke A, Gielen S, Erbs S, Lenk K, Schuler G, Hambrecht R. Hyperglycemia reduces survival and impairs function of circulating blood-derived progenitor cells. *Arteriosclerosis Thrombosis and Vascular Biology.* 2005;25(4):698-703.

284. Kondo T, Hayashi M, Takeshita K, Numaguchi Y, Kobayashi K, Iino S, Inden Y, Murohara T. Smoking cessation rapidly increases circulating progenitor

cells in peripheral blood in chronic smokers. *Arteriosclerosis Thrombosis and Vascular Biology.* 2004;24(8):1442-1447.

285. Wang XX, Zhu JH, Chen JZ, Shang YP. Effects of nicotine on the number and activity of circulating endothelial progenitor cells. *Journal of Clinical Pharmacology.* 2004;44(8):881-889.

286. Chen JZ, Zhu JH, Wang XX, Xie XD, Sun J, Shang YP, Guo XG, Dai HM, Hu SJ. Effects of homocysteine on number and activity of endothelial progenitor cells from peripheral blood. *Journal of Molecular and Cellular Cardiology.* 2004;36(2):233-239.

287. Schnabel R, Blankenberg S, Lubos E, Lackner KJ, Rupprecht HJ, Espinola-Klein C, Jachmann N, Post F, Peetz D, Bickel C, Cambien F, Tiret L, Munzel T. Asymmetric dimethylarginine and the risk of cardiovascular events and death in patients with coronary artery disease - Results from the AtheroGene study. *Circulation Research.* 2005;97(5):53-59.

288. Thum T, Tsikas D, Stein S, Schultheiss M, Eigenthaler M, Anker SD, Poole-Wilson PA, Ertl G, Bauersachs J. Suppression of endothelial progenitor cells in human coronary artery disease by the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine. *Journal of the American College of Cardiology.* 2005;46(9):1693-1701.

289. Massa M, Rosti V, Ferrario M, Campanelli R, Ramajoli I, Rosso R, De Ferrari GM, Ferlini M, Goffredo L, Bertoletti A, Klersy C, Pecci A, Moratti R, Tavazzi L. Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction. *Blood.* 2005;105(1):199-206.

290. Heeschen C, Lehmann R, Honold J, Assmus B, Aicher A, Walter DH, Martin H, Zeiher AM, Dimmeler S. Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease. *Circulation.* 2004;109(13):1615-1622.

291. George J, Goldstein E, Abashidze S, Deutsch V, Shmilovich H, Finkelstein A, Herz I, Miller H, Keren G. Circulating endothelial progenitor cells in patients with unstable angina: association with systemic inflammation. *European Heart Journal.* 2004;25(12):1003-1008.

292. Verma S, Kuliszewski MA, Li SH, Szmitko PE, Zucco L, Wang CH, Badiwala MV, Mickle DAG, Weisel RD, Fedak PWM, Stewart DJ, Kutryk MJB. C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function - Further evidence of a mechanistic link between C-reactive protein and cardiovascular disease. *Circulation.* 2004;109(17):2058-2067.

293. Thum T, Fraccarollo D, Galuppo P, Tsikas D, Frantz S, Ertl G, Bauersachs J. Bone marrow molecular alterations after myocardial infarction: Impact on endothelial progenitor cells. *Cardiovascular Research.* 2006;70(1):50-60.

294. Wang Y, Johnsen HE, Mortensen S, Bindslev L, Ripa RS, Haack-Sorensen M, Jorgensen E, Fang W, Kastrup J. Changes in circulating mesenchymal stem cells, stem cell homing factor, and vascular growth factors in patients with acute ST elevation myocardial infarction treated with primary percutaneous coronary intervention. *Heart.* 2006;92(6):768-774.

295. Mouquet F, Pfister O, Jain M, Oikonomopoulos A, Ngoy S, Summer R, Fine A, Liao RL. Restoration of cardiac progenitor cells after myocardial infarction by self-proliferation and selective homing of bone marrow-derived stem cells. *Circulation Research.* 2005;97(11):1090-1092.

296. Nygren JM, Jovinge S, Breitbach M, Sawen P, Roll W, Hescheler J, Taneera J, Fleischmann BK, Jacobsen SEW. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. *Nature Medicine.* 2004;10(5):494-501.

297. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KBS, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, Field LJ. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature.* 2004;428(6983):664-668.

298. Valgimigli M, Rigolin GM, Fucili A, Della Porta M, Soukhomovskaia O, Malagutti P, Bugli AM, Bragotti LZ, Francolini G, Mauro E, Castoldi G, Ferrari R. CD34(+) and endothelial progenitor cells in patients with various degrees of congestive heart failure. *Circulation.* 2004;110(10):1209-1212.

299. Foresta C, Caretta N, Lana A, Cabrelle A, Palu G, Ferlin A. Circulating endothelial progenitor cells in subjects with erectile dysfunction. *International Journal of Impotence Research.* 2005;17(3):288-290.

300. George J, Herz I, Goldstein E, Abashidze S, Deutch V, Finkelstein A, Michowitz Y, Miller H, Keren G. Number and adhesive properties of circulating endothelial progenitor cells in patients with in-stent restenosis. *Arteriosclerosis Thrombosis and Vascular Biology.* 2003;23(12):E57-E60.

301. Simper D, Wang SH, Deb A, Holmes D, McGregor C, Frantz R, Kushwaha SS, Caplice NM. Endothelial progenitor cells are decreased in blood of cardiac allograft patients with vasculopathy and endothelial cells of noncardiac origin are enriched in transplant atherosclerosis. *Circulation.* 2003;108(2):143-149.

302. Taguchi A, Matsuyama T, Moriwaki H, Hayashi T, Hayashida K, Nagatsuka K, Todo K, Mori K, Stern DM, Soma T, Naritomi H. Circulating CD34-positive cells provide an index of cerebrovascular function. *Circulation.* 2004;109(24):2972-2975.

303. Ghani U, Shuaib A, Salam A, Nasir A, Shuaib U, Jeerakathil T, Sher F, O'Rourke F, Nasser AM, Schwindt B, Todd K. Endothelial progenitor cells during cerebrovascular disease. *Stroke.* 2005;36(1):151-153.

304. Grundy SM. Statin trials and goals of cholesterol-lowering therapy. *Circulation.* 1998;97(15):1436-1439.

305. Maron DJ, Fazio S, Linton MF. Current perspectives on statins. *Circulation.* 2000;101(2):207-213.

306. Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rutten H, Fichtlscherer S, Martin H, Zeiher AM. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *Journal of Clinical Investigation.* 2001;108(3):391-397.

307. Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, Walsh K, Isner JM, Asahara T. HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. *Journal of Clinical Investigation.* 2001;108(3):399-405.

308. Kalka C, Masuda H, Takahashi T, Gordon R, Tepper O, Gravereaux E, Pieczek A, Iwaguro H, Hayashi SI, Isner JM, Asahara T. Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. *Circulation Research.* 2000;86(12):1198-1202.

309. Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, Nishimura H, Losordo DW, Asahara T, Isner JM. Statin therapy accelerates reendothelialization - A novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. *Circulation.* 2002;105(25):3017-3024.

310. Spyridopoulos I, Haendeler J, Urbich C, Brummendorf TH, Oh H, Schneider MD, Zeiher AM, Dimmeler S. Statins enhance migratory capacity by upregulation of the telomere repeat-binding factor TRF2 in endothelial progenitor cells. *Circulation.* 2004;110(19):3136-3142.

311. 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. Simvastatin versus ezetimibe - Pleiotropic and lipid-lowering effects on endothelial function in humans. *Circulation.* 2005;111(18):2356-2363.

312. Bahlmann FH, de Groot K, Mueller O, Hertel B, Haller H, Fliser D. Stimulation of endothelial progenitor cells - A new putative therapeutic effect of angiotensin II receptor antagonists. *Hypertension.* 2005;45(4):526-529.

313. Min TQ, Zhu CJ, Xiang WX, Hui ZJ, Peng SY. Improvement in endothelial progenitor cells from peripheral blood by ramipril therapy in patients with stable coronary artery disease. *Cardiovascular Drugs and Therapy.* 2004;18(3):203-209.

314. Strehlow K, Werner N, Berweiler J, Link A, Dirnagl U, Priller J, Laufs K, Ghaeni L, Milosevic M, Bohm M, Nickenig G. Estrogen increases bone marrow-derived endothelial progenitor cell production and diminishes neointima formation. *Circulation.* 2003;107(24):3059-3065.

315. Iwakura A, Luedemann C, Shastry S, Hanley A, Kearney M, Aikawa R, Isner JM, Asahara T, Losordo DW. Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury. *Circulation.* 2003;108(25):3115-3121.

316. Zhu JH, Wang XX, Chen JZ, Shang YP, Guo XG, Sun J. Effects of puerarin on number and activity of endothelial progenitor cells from peripheral blood. *Acta Pharmacologica Sinica.* 2004;25(8):1045-1051.

317. Chen JZ, Wang XX, Zhu JH, Shang YP, Guo XG, Sun J. Effects of Ginkgo biloba extract on number and activity of endothelial progenitor cells from peripheral blood. *Journal of Cardiovascular Pharmacology.* 2004;43(3):347-352.

318. Butzal M, Loges S, Schweizer M, Fischer U, Gehling UM, Hossfeld DK, Fiedler W. Rapamycin inhibits proliferation and differentiation of human endothelial progenitor cells in vitro. *Experimental Cell Research.* 2004;300(1):65-71.

319. Bahlmann FH, DeGroot K, Duckert T, Niemczyk E, Bahlmann E, Boehm SM, Haller H, Fliser D. Endothelial progenitor cell proliferation and differentiation is regulated by erythropoietin - Rapid communication. *Kidney International.* 2003;64(5):1648-1652.

320. Heeschen C, Aicher A, Lehmann R, Fichtischerer S, Vasa M, Urbich C, Mildner-Rihm C, Martin H, Zeiher AM, Dimmeler S. Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood.* 2003;102(4):1340-1346.

321. Bahlmann FH, de Groot K, Spandau JM, Landry AL, Hertel B, Duckett T, Boehm SM, Menne J, Haller H, Fliser D. Erythropoietin regulates endothelial progenitor cells. *Blood.* 2004;103(3):921-926.

322. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circulation Research.* 1999;85(3):221-228.

323. Iwaguro H, Yamaguchi J, Kalka C, Murasawa S, Masuda H, Hayashi S, Silver M, Li T, Isner JM, Asahara T. Endothelial progenitor cell vascular endothelial growth factor gene transfer for vascular regeneration. *Circulation.* 2002;105(6):732-738.

324. Siu CW, Watson T, Lai WH, Lee YK, Chan YH, Ng KM, Lau CP, Lip GYH, Tse HF. Relationship of circulating endothelial progenitor cells to the recurrence of atrial fibrillation after successful conversion and maintenance of sinus rhythm. *Europace.* 2010;12(4):517-521.

325. Freestone B, Chong AY, Blann AD, Lip GYH. The effects of direct current cardioversion for persistent atrial fibrillation on indices of endothelial damage/dysfunction. *Thromb. Res.* 2006;118(4):479-485.

326. Freestone B, Chong AY, Nuttall S, Blann AD, Lip GYH. Soluble E-selectin, von Willebrand factor, soluble thrombomodulin, and total body nitrate/nitrite product as indices of endothelial damage/dysfunction in paroxysmal, persistent, and permanent atrial fibrillation. *Chest.* 2007;132:1253-1258.

327. Goon P, Lip GYH, Stonelake PS, Blann A. Circulating Endothelial Cells and Circulating Progenitor Cells in Breast Cancer: Relationship to Endothelial Damage/Dysfunction/Apoptosis, Clinicopathologic Factors, and the Nottingham Prognostic Index. *Neoplasia.* 2009;11(8):771-779.

328. Khan SS, Smith MS, Reda D, Suffredini AF, McCoy JP. Multiplex bead array assays for detection of soluble cytokines: Comparisons of sensitivity and quantitative values among kits from multiple manufacturers. *Cytometry Part B-Clinical Cytometry.* 2004;61B(1):35-39.

329. Vogeli KM, Jin SW, Martin GR, Stainier DYR. A common progenitor for haematopoietic and endothelial lineages in the zebrafish gastrula. *Nature.* 2006;443(7109):337-339.

330. Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schafer B, Hossfeld DK, Fiedler W. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood.* 2000;95(10):3106-3112.

331. Neumuller J, Neumueller-Guber SE, Lipovac M, Mosgoeller W, Vetterlein M, Pavelka M, Huber J. Immunological and ultrastructural characterization of endothelial cell cultures differentiated from human cord blood derived endothelial progenitor cells. *Histochemistry and Cell Biology.* 2006;126(6):649-664.

332. Porat Y, Porozov S, Belkin D, Shimoni D, Fisher Y, Belleli A, Czeiger D, Silverman WF, Belkin M, Battler A, Fulga V, Savion N. Isolation of an adult blood-derived progenitor cell population capable of differentiation into angiogenic, myocardial and neural lineages. *British Journal of Haematology.* 2006;135(5):703-714.

333. Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, Silver M, Ma H, Kearney M, Isner JM, Asahara T. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation.* 2001;103(5):634-637.

334. Handgretinger R, Gordon PR, Leimig T, Chen XH, Buhring HJ, Niethammer D, Kuci S. Biology and plasticity of CD133(+) hematopoietic stem cells. *Hematopoietic Stem Cells 2002: Genetics and Function.* 2003;996:141-151.

335. Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT, Ingram DA. Redefining endothelial progenitor cells via

clonal analysis and hematopoietic stem/progenitor cell principals. *Blood.* 2007;109(5):1801-1809.

336. Delorme B, Basire A, Gentile C, Sabatier F, Monsonis F, Desouches C, Blot-Chabaud M, Uzan G, Sampol J, Dignat-George F. Presence of endothelial progenitor cells, distinct from mature endothelial cells, within human CD146(+) blood cells. *Thrombosis and Haemostasis.* 2005;94(6):1270-1279.

337. Stevens T, Garcia JGN, Shasby DM, Bhattacharya J, Malik AB. Mechanisms regulating endothelial cell barrier function. *American Journal of Physiology-Lung Cellular and Molecular Physiology.* 2000;279(3):L419-L422.

338. Dejana E, Bazzoni G, Lampugnani MG. Vascular endothelial (VE)-cadherin: Only an intercellular glue? *Experimental Cell Research.* 1999;252(1):13-19.

339. Dejana E, Spagnuolo R, Bazzoni G. Interendothelial junctions and their role in the control of angiogenesis, vascular permeability and leukocyte transmigration. Paper presented at: XVIIIth Congress of the International-Society-on-Thrombosis-and-Haemostasis; Jul 06-12, 2001; Paris, France.

340. Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1(+)VE-cadherin(+) cells at a diverging point of endothelial and hemopoietic lineages. *Development.* 1998;125(9):1747-1757.

341. Fadini GP, Baesso I, Albiero M, Sartore S, Agostini C, Avogaro A. Technical notes on endothelial progenitor cells: Ways to escape from the knowledge plateau. *Atherosclerosis.* 2008;197(2):496-503.

342. Rustemeyer P, Wittkowski W, Jurk K, Koller A. Optimized flow cytometric analysis of endothelial progenitor cells in peripheral blood. *Journal of Immunoassay & Immunochemistry.* 2006;27(1):77-88.

343. Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, Girardi L, Yurt R, Himel H, Rafii S. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. *Circulation Research.* 2001;88(2):167-174.

344. Shantsila E, Watson T, Lip GYH. Endothelial progenitor cells in cardiovascular disorders. *Journal of the American College of Cardiology.* 2007;49(7):741-752.

345. Adams V, Lenk K, Linke A, Lenz D, Erbs S, Sandri M, Tarnok A, Gielen S, Emmrich F, Schuler G, Hambrecht R. Increase of circulating endothelial progenitor cells in patients with coronary artery disease after exercise-induced ischemia. *Arteriosclerosis Thrombosis and Vascular Biology.* 2004;24(4):684-690.

346. Hambrecht R, Wolf A, Gielen S, Linke A, Hofer J, Erbs S, Schoene N, Schuler G. Effect of exercise on coronary endothelial function in patients with coronary artery disease. *New England Journal of Medicine.* 2000;342(7):454-460.

347. Hoetzer GL, Van Guilder GP, Irmiger HM, Keith RS, Stauffer BL, DeSouza CA. Aging, exercise, and endothelial progenitor cell clonogenic and migratory capacity in men. *Journal of Applied Physiology.* 2007;102(3):847-852.

348. Yang Z, Wang JM, Chen L, Luo CF, Tang AL, Tao J. Acute exercise-induced nitric oxide production contributes to upregulation of circulating endothelial progenitor cells in healthy subjects. *Journal of Human Hypertension.* 2007;21(6):452-460.

349. Steiner S, Niessner A, Ziegler S, Richter B, Seidinger D, Pleiner J, Penka M, Wolzt M, Huber K, Wojta J, Minar E, Kopp CW. Endurance training increases the number of endothelial progenitor cells in patients with cardiovascular risk and coronary artery disease. *Atherosclerosis.* 2005;181(2):305-310.

350. Boos CJ, Balakrishnan B, Lip GYH. The effects of exercise stress testing on soluble E-selectin, von Willebrand factor, and circulating endothelial cells as indices of endothelial damage/dysfunction. *Annals of Medicine.* 2008;40(1):66-73.

351. Bruce RA, Blackmon JR, Jones JW, Strait G. Exercising testing in adult normal subjects and cardiac patients. *Pediatrics.* 1963;32:SUPPL 742-756.

352. Fox K, Garcia MAA, Ardissino D, Buszman P, Katowice, Camici PG, Crea F, Daly C, De Backer G, Ghent, Hjemdahl P, Lopez-Sendon J, Marco J, Morais J, Leiria, Pepper J, Sechtem U, Simoons M, Thygesen K, European Soc C. Guidelines on the management of stable angina pectoris: executive summary. *European Heart Journal.* 2006;27(11):1341-1381.

353. Collins P, Ford I, Ball D, Macaulay E, Greaves M, Brittenden J. A preliminary study on the effects of exercising to maximum walking distance on platelet and endothelial function in patients with intermittent claudication. *European Journal of Vascular and Endovascular Surgery.* 2006;31(3):266-273.

354. Jilma B, Eichler HG, Stohlawetz P, Dirnberger E, Kapiotis S, Wagner OF, Schutz W, Krejcy K. Effects of exercise on circulating vascular adhesion molecules in healthy men. *Immunobiology.* 1997;197(5):505-512.

355. Laufs U, Urhausen A, Werner N, Scharhag J, Heitz A, Kissner G, Bohn M, Kindermann W, Nickenig G. Running exercise of different duration and intensity: effect on endothelial progenitor cells in healthy subjects. *European Journal of Cardiovascular Prevention & Rehabilitation.* 2005;12(4):407-414.

356. Adams V, Linke A, Breuckmann F, Leineweber K, Erbs S, Krankel N, Brocker-Preuss M, Woitek F, Erbel R, Heusch G, Hambrecht R, Schuler G, Mohlenkamp S. Circulating progenitor cells decrease immediately after marathon race in advanced-age marathon runners. *European Journal of Cardiovascular Prevention & Rehabilitation.* 2008;15(5):602-607.

357. Luk TH, Dai YL, Siu CW, Yiu KH, Chan HT, Fong DYT, Lee SWL, Li SW, Tam S, Lau CP, Tse HF. Habitual physical activity is associated with endothelial function and endothelial progenitor cells in patients with stable

coronary artery disease. *European Journal of Cardiovascular Prevention & Rehabilitation.* 2009;16(4):464-471.

358. Thomas HE, Redgrave R, Cunnington MS, Avery P, Keavney BD, Arthur HM. Circulating endothelial progenitor cells exhibit diurnal variation. *Arteriosclerosis Thrombosis and Vascular Biology.* 2008;28(3):E21-E22.

359. Born J, Lange T, Hansen K, Molle M, Fehm HL. Effects of sleep and circadian rhythm on human circulating immune cells. *Journal of Immunology.* 1997;158(9):4454-4464.

360. Haus E, Lakatua DJ, Swoyer J, Sackettlundeen L. Chronobiology in hematology and immunology. *American Journal of Anatomy.* 1983;168(4):467-517.

361. Dincol D, Akbulut H, Buyukcelik A, Icli F. Diurnal variations of serum GM-CSF levels. *Cytokine.* 2000;12(7):1151-1155.

362. Takeuchi M, Tanimoto Y, Tada A, Soda R, Takahashi K. Circadian rhythm of plasma levels of endogenous granulocyte colony stimulating factor in healthy volunteers. *International Journal of Hematology.* 1996;64(3-4):293-295.

363. Muller JE. Circadian variation in cardiovascular events. *American Journal of Hypertension.* 1999;12(2):35S-42S.

364. Peters RW, Zoble RG, Liebson PR, Pawitan Y, Brooks MM, Proschan M. Identification of a secondary peak in myocardial-infarction onset 11 to 12 hours after awakening - the cardiac-arrhythmia suppression trial (cast) experience. *Journal of the American College of Cardiology.* 1993;22(4):998-1003.

365. Boos CJ, Lane DA, Kang D, Goon PKY, Blann AD, Lip GYH. Temporal and venepuncture-related decline in circulating endothelial cell capture from mixed venous blood. *Journal of Thrombosis and Thrombolysis.* 2006;22(2):125-131.

366. Wenburg JJ, Go RS. EDTA-dependent lymphocyte clumping. *Haematologica.* 2003;88(8):EIM09.

367. NCCLS. NCCLS, Evacuated Tubes and Additive for Blood Specimen Collection – Fourth Edition. 1996;No. 13:H1-A4.

368. Recommendations of the International Council for Standardization in Haematology for Ethylenediaminetetraacetic Acid Anticoagulation of Blood for Blood Cell Counting and Sizing. International Council for Standardization in Haematology: Expert Panel on Cytometry. *Am J Clin Pathol.* 1993;100(4):371-372.

369. Gulati GL, Hyland LJ, Kocher W, Schwarting R. Changes in automated complete blood cell count and differential leukocyte count results induced by storage of blood at room temperature. *Archives of Pathology & Laboratory Medicine.* 2002;126(3):336-342.

370. de Baca ME, Gulati G, Kocher W, Schwarting R. Effects of storage of blood at room temperature on hematologic parameters measured on Sysmex XE-2100. *Labmedicine.* 2006;37(1):28-+.

371. Reardon DM, Warner B, Trowbridge EA. EDTA, The traditional anticoagulant of hematology - with increased automation is it time for a review? *Medical Laboratory Sciences.* 1991;48(1):72-75.

372. Sears D, Charache S, Perlstein M. Electronic blood-cell counters - faulty calibration due to type and amount of anticoagulant in collection tubes. *Archives of Pathology & Laboratory Medicine.* 1985;109(3):247-249.

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

374. Felmeden DC, Lip GYH. Endothelial function and its assessment. *Expert Opinion on Investigational Drugs.* 2005;14(11):1319-1336.

375. Freestone B, Chong AY, Nuttall S, Lip GYH. 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.* 2008;122(1):85-90.

376. Anderson JL, Maycock CAA, Lappe DL, Crandall BG, Horne BD, Bair TL, Morris SR, Li QY, Muhlestein JB, Intermountain Heart Collab GRP. Frequency of elevation of C-reactive protein in atrial fibrillation. *American Journal of Cardiology.* 2004;94(10):1255-1259.

377. Feng DL, D'Agostino RB, Silbershatz H, Lipinska I, Massaro J, Levy D, Benjamin EJ, Wolf PA, Tofler GH. Hemostatic state and atrial fibrillation (The Framingham Offspring Study). *American Journal of Cardiology.* 2001;87(2):168-171.

378. Blann AD, Yip GYH. The endothelium in atherothrombotic disease: assessment of function, mechanisms and clinical implications. *Blood Coagulation & Fibrinolysis.* 1998;9(4):297-306.

379. Roldan V, Marin F, Blann AD, Garcia A, Marco P, Sogorb F, Lip GYH. Interleukin-6, endothelial activation and thrombogenesis in chronic atrial fibrillation. *Eur. Heart J.* 2003;24(14):1373-1380.

380. Choy JC, Granville DJ, Hunt DWC, McManus BM. Endothelial cell apoptosis: Biochemical characteristics and potential implications for atherosclerosis. *Journal of Molecular and Cellular Cardiology.* 2001;33(9):1673-1690.

381. Askenasy N, Yolcu ES, Yaniv I, Shirwan H. Induction of tolerance using Fas ligand: a double-edged immunomodulator. *Blood.* 2005;105(4):1396-1404.

382. Nadal C, Maurel J, Gallego R, Castells A, Longaron R, Marmol M, Sanz S, Molina R, Martin-Richard M, Gascon P. FAS/FAS ligand ratio: A marker of

oxaliplatin-based intrinsic and acquired resistance in advanced colorectal cancer. *Clinical Cancer Research.* 2005;11(13):4770-4774.

383. Noris M, Morigi M, Donadelli R, Aiello S, Foppolo M, Todeschini M, Orisio S, Remuzzi G, Remuzzi A. Nitric-oxide synthesis by cultured endothelial-cells is modulated by flow conditions. *Circulation Research.* 1995;76(4):536-543.

384. Sampath R, Kukielka GL, Smith CW, Eskin SG, McIntire LV. Shear stress-mediated changes in the expression of leukocyte adhesion receptors on human umbilical vein endothelial-cells in-vitro. *Annals of Biomedical Engineering.* 1995;23(3):247-256.

385. Nishino M, Hoshida S, Tanouchi J, Ito T, Kato J, Iwai K, Tanahashi H, Hori M, Yamada Y, Kamada T. time to recover from atrial hormonal, mechanical, and electrical dysfunction after successful electrical cardioversion of persistent atrial fibrillation. *American Journal of Cardiology.* 2000;85(12):1451-1454.

386. Wozakowska-Kaplon B, Opolski G. Concomitant recovery of atrial mechanical and endocrine function after cardioversion in patients with persistent atrial fibrillation. *Journal of the American College of Cardiology.* 2003;41(10):1716-1720.

387. Nikitovic D, Zacharis EA, Manios EG, Malliaraki NE, Kanoupakis EM, Sfiridaki KI, Skalidis EI, Margioris AN, Vardas PE. Plasma levels of nitrites/nitrates in patients with chronic atrial fibrillation are increased after electrical restoration of sinus rhythm. *Journal of Interventional Cardiac Electrophysiology.* 2002;7(2):171-176.

388. Goette A, Jentsch-Ullrich K, Lendeckel U, Rocken C, Agbaria M, Auricchio A, Mohren M, Franke A, Klein HU. Effect of atrial fibrillation on hematopoietic progenitor cells - A novel pathophysiological role of the atrial natriuretic peptide? *Circulation.* 2003;108(20):2446-2449.

389. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nature Medicine.* 2001;7(4):430-436.

390. Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, Kajstura J, Leri A, Anversa P. Chimerism of the transplanted heart. *New England Journal of Medicine.* 2002;346(1):5-15.

391. Watanabe E, Arakawa T, Uchiyama T, Kodama I, Hishida H. High-sensitivity C-reactive protein is predictive of successful cardioversion for atrial fibrillation and maintenance of sinus rhythm after conversion. *International Journal of Cardiology.* 2006;108(3):346-353.

392. Wazni O, Martin DO, Marrouche NF, Shaaraoui M, Chung MK, Almahameed S, Schweikert RA, Saliba WI, Natale A. C reactive protein concentration and

recurrence of atrial fibrillation after electrical cardioversion. *Heart.* 2005;91(10):1303-1305.

393. Psychari SN, Apostolou TS, Sinos L, Hamodraka E, Liakos G, Kremastinos DT. Relation of elevated C-reactive protein and interleukin-6 levels to left atrial size and duration of episodes in patients with atrial fibrillation. *American Journal of Cardiology.* 2005;95(6):764-767.

394. Cosgrave J, Foley JB, Bahadur K, Bennett K, Crean P, Walsh MJ. Inflammatory markers are not associated with outcomes following elective external cardioversion. *International Journal of Cardiology.* 2006;110(3):373-377.

395. Fetsch T, Bauer P, Engberding R, Koch HP, Lukl J, Meinertzf T, Oeff M, Seipel L, Trappe HJ, Treese N, Breithardt G, Prevention Atrial F. Prevention of atrial fibrillation after cardioversion: results of the PAFAC trial. *European Heart Journal.* 2004;25(16):1385-1394.

396. Page RL, Wilkinson WE, Clair WK, McCarthy EA, Pritchett ELC. Asymptomatic arrhythmias in patients with symptomatic paroxysmal atrial-fibrillation and paroxysmal supraventricular tachycardia. *Circulation.* 1994;89(1):224-227.

397. Page RL, Tilsch TW, Connolly SJ, Schnell DJ, Marcello SR, Wilkinson WE, Pritchett ELC, Azimilide Supraventricular A. Asymptomatic or "silent" atrial fibrillation - Frequency in untreated patients and patients receiving azimilide. *Circulation.* 2003;107(8):1141-1145.

398. Wyse DG, Waldo AL, DiMarco JP, Domanski MJ, Rosenberg Y, Schron EB, Kellen JC, Greene HL, Mickel MC, Dalquist JE, Corley SD, Atrial Fibrillation Follow I. A comparison of rate control and rhythm control in patients with atrial fibrillation. *New England Journal of Medicine.* 2002;347(23):1825-1833.

399. Hohnloser SH, Pajitnev D, Pogue J, Healey JS, Pfeffer MA, Yusuf S, Connolly SJ. Incidence of stroke in paroxysmal versus sustained atrial fibrillation in patients taking oral anticoagulation or combined antiplatelet therapy. *Journal of the American College of Cardiology.* 2007;50:2156-2161.

400. Mitchell ARJ, Sulke N. How do atrial pacing algorithms prevent atrial arrhythmias? *Europace.* 2004;6(4):351-362.

401. Bevilacqua MP, Nelson RM. Selectins. *Journal of Clinical Investigation.* 1993;91(2):379-387.

402. McEver RP. Selectins. *Current Opinion in Immunology.* 1994;6(1):75-84.

403. Ruchaud-Sparagano MH, Drost EM, Donnelly SC, Bird MI, Haslett C, Dransfield I. Potential pro-inflammatory effects of soluble E-selectin upon neutrophil function. *European Journal of Immunology.* 1998;28(1):80-89.

404. Goto S, Hatt DL, Rother J, Alberts M, Hill MD, Ikeda Y, Uchiyama S, D'Agostino R, Ohman EM, Liau CS, Hirsch AT, Mas JL, Wilson PWF, Corbalan R, Aichner F, Steg PG, Invest RR. Prevalence, clinical profile, and cardiovascular outcomes of atrial fibrillation patients with atherothrombosis. *American Heart Journal.* 2008;156(5):855-863.

405. Boos CJ, Lip GYH. Biomarkers in atrial fibrillation: Further observations on biologic plausibility, cause and effect. *Journal of Thrombosis and Thrombolysis.* 2005;20(3):189-190.

406. Watson T, Shantsila E, Lip GYH. Fibrin D-dimer levels and thromboembolic events in patients with atrial fibrillation. *Int. J. Cardiol.* 2007;120(1):123-124.

407. Halperin J, Ip J. The IMPACT of BIOTRONIK Home Monitoring Guided Anticoagulation on Stroke Risk in Patients With Implanted ICD and CRT-D Devices. *ClinicalTrials.gov*. Available at: http://clinicaltrials.gov/ct2/show/study/NCT00559988?term=I. Accessed 30th October, 2009.

408. Glotzer TV, Daoud EG, Wyse DG, Singer DE, Ezekowitz MD, Hilker C, Miller C, Qi D, Ziegler PD. The relationship between daily atrial tachyarrhythmia burden from implantable device diagnostics and stroke risk: the TRENDS study. *Circ Arrhythm Electrophysiol.* 2009;2(5):474-480.

409. Connolly SJ, Crijns H, Torp-Pedersen C, van Eickels M, Gaudin C, Page RL, Hohnloser SH, Investigators A. 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.* 2009;120(13):1174-1180.