The Prothrombotic State in Atrial Fibrillation – Observations on Fibrin Clot Structure and the Relationship to Renal Dysfunction

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I. Abstract

Atrial fibrillation (AF) is the most common arrhythmia and is closely associated with chronic kidney disease. The mainstay pharmacological agent to prevent AF-related stroke and thromboembolism is the use of oral anticoagulants, but may result in an increased risk of haemorrhage. Therefore, this MD research thesis is a comprehensive study of the changes in thrombogenesis and fibrin clot structure related to AF and CKD, as well as the potential impact of exposure to different classes of oral anticoagulant.

III Acknowledgements

"*No man ever steps in the same river twice*"

Heraclitus

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VI List of abbreviations used (in alphabetical order)

AF: atrial fibrillation

aPTT: activated partial thromboplasmin time

CAD: coronary artery disease

CKD: chronic kidney disease

CV: coefficient of variance

EMP: endothelial microparticles

ESRD: end-stage renal disease

GFR: glomerular filtration rate

INR: International Normalised Ratio

KDIGO: kidney disease international global outcomes

LFT: liver function test

LT: Lag-time

LY30 (%): percentage of clot lysed after 30 minutes

LY60 (%): percentage of clot lysed after 60 minutes

MA: Maximum amplitude

MOD: Maximum Optical Density

NOACs: non Vitamin K antagonist oral anticoagulants

NSAIDs: non-steroidal anti-inflammatory drugs

PMP: platelet microparticles

RAAS: renin-angiotensin-aldosterone system

RCD: Rate of Clot Dissolution

RCF: Rate of Clot Formation

RRT: renal replacement therapy

SEM: scanning electron microscopy

TEG: thromboelastography

tPA: tissue plasminogen activator

T50: time for 50% fibrin clot lysis

VKA: vitamin K antagonist

vWF: von Willebrand factor

Section 1: Literature Review

1.1 Atrial Fibrillation and Chronic Kidney Disease – pathophysiology and clinical implications

Introduction

Chronic Kidney Disease (CKD) is defined by the Kidney Disease Improving Global Outcomes (KDIGO) as a reduction in renal function; with a reduction in glomerular filtration rate(GFR) <60ml/min per 1.73m^2 for 3 months or longer, or with the presence of albuminuria (1, 2). CKD has potential for gradual progression to End-stage Renal Disease (ESRD) which requires dialysis to correct accompanying fluid and electrolyte imbalance. The increasing incidence and prevalence of CKD is also associated with a parallel rise in incident atrial fibrillation (AF) occurrence (3-6). The main reason for this epidemiological coupling is most likely the improving longevity achieved in the western countries, resulting in a rapidly growing older population, with a contemporary increase in the collective risk factors, shared by both conditions, such as diabetes mellitus and hypertension.

Unsurprisingly, it has been demonstrated that CKD and AF are not independent, as several studies and national registries have highlighted the increased incidence of AF among those with worsening renal function (7-14). For example, recent data show the incidence of AF development can be as high as 12.1 per 1000 patient-years in ESRD as compared to 5.0 per 1000 patient-years in controls (15). Likewise, a new diagnosis of AF not only heralds the progression of CKD, but seems also to hasten the development of ESRD (16-18). AF also leads to progression of CKD, even among those with relatively “normal renal function” with no detectable proteinuria on

dipstick at baseline (19). Thus, a bidirectional relationship exists between these two conditions.

AF per se can result in increased risk of ischaemic stroke and systemic thromboembolism by five-fold, and is implicated in 15-20% of all ischaemic strokes (20). However, the concurrent presence of both AF and CKD further exacerbates the stroke and mortality risk, with upwards of 66% increase in relative risk of death (21-24).

Hence, the intersection of both conditions results in an increase in the propensity for thromboembolism-related adverse events (including stroke, systemic thromboembolism, myocardial infarction and death) but in addition, a paradoxical increased risk of haemorrhagic sequelae.

Stroke and thromboembolic risk can be assessed using the CHA_2DS_2-VASc Score while bleeding risk can be assessed by the HAS-BLED Score, to allow for careful risk stratification of the patient requiring thromboprophylaxis (25, 26). Oral anticoagulants (whether Vitamin K antagonists (VKA) and Non-VKA oral anticoagulants (NOACs)) have been demonstrated to be effective in mild-moderate renal dysfunction, in both clinical trials and observational studies (27, 28).

Patients with severe renal impairment were excluded from the Phase 3 randomised trials of NOACs, so limited trial data are available.

This chapter will initially discuss the pathophysiological and clinical basis behind the increased risk of thromboembolism and haemorrhage amongst AF patients with

CKD. Second, we review the data on the use of oral anticoagulants for stroke prevention in AF across the spectrum of renal dysfunction.

Search Strategy

A comprehensive literature search by using electronic bibliographic databases (i.e., Pubmed, Medline, Embase, DARE, Cochrane database), scanning reference lists from included articles, and hand searching abstracts from national and international cardiovascular meetings. Search terms used include “atrial fibrillation”, “chronic kidney disease”, “renal failure”, “anti-thrombotic treatment”. Bibliographies of all selected articles and review articles were reviewed for other relevant articles. Finally, the supplements of major journals were hand searched to identify relevant abstracts that had not been published as peer-reviewed articles.

Pathophysiology and epidemiology of thromboembolism in CKD: A brief overview

Pathophysiological insights

AF confers a prothrombotic or hypercoagulable state through numerous pathophysiological pathways fulfilling ‘Virchow’s triad for thrombogenesis’, as evidenced by abnormalities in vessel wall, abnormalities in flow and abnormalities in blood constituents (29). The propensity for thrombus formation is further enhanced by CKD [**Table 1.1.1**] due to additional changes to the flow within the left atrium and left atrial appendage, damage to vessel wall and subsequent endothelial dysfunction, or upward regulation of platelet and coagulation factors [**Figure 1.1.1**].

Firstly, in relation to changes in blood flow, worsening GFR in AF is associated with reduced left atrial appendage emptying velocity and formation of dense spontaneous echocardiographic contrast, signifying significantly increased thrombogenic risk (30, 31).

Secondly, CKD-related endothelial dysfunction and damage to the vessel wall may manifest directly as reduction in endothelial dilatation or increased pulse-wave velocity (32-36), or indirectly by elevated levels of endothelin and vWF (33, 37). Endothelial dysfunction can also be reflected by the increased intima media thickening (38), which subsequently has been shown to predict upwards of 10-fold (Odds-ratio 10.20 (95% CI, 3.67 to 28.3)) increased cardiovascular mortality in ESRD (39, 40).

Thirdly, increased thrombogenesis in CKD is also related to increased platelet and coagulation abnormalities in several pathways: increased pro-coagulant and inflammatory complexes (41-45), up-regulation of tissue factor pathway and its interaction with platelets (46, 47), reduction of antithrombin III and PAI-1 levels (47, 48), reduced vWF degradation (49) and increased platelet aggregability (50).

Furthermore, CKD per se is associated with various other factors contributing to an increased thromboembolic risk: for example, activation of the renin-angiotensin-aldosterone-system (RAAS) (51) and chronic inflammation (43), aortic or vascular calcification plus dysfunction of calcium-phosphate-mineral metabolism related to renal dysfunction (52-54). Given the aforementioned pathophysiological pathways,

it is perhaps unsurprising for CKD to result in an elevated risk of ischaemic stroke and systemic thromboembolism.

Table 1.1.1: Pathophysiology of Thromboembolism in Chronic Kidney Disease

Study (Year)	Study Type	N	Population	Findings
(a) Blood stasis in left atrium and atrial appendage				
Yagishita et al (2010) (30)	Observational	321	Patients with persistent atrial fibrillation	GFR an independent predictor of reduced left atrial appendage emptying velocity and presence of left atrium spontaneous echo contrast
Providência et al (2013) (31)	Observational	372	Patients with nonvalvular atrial fibrillation	eGFR is positively associated with dense spontaneous echocardiographic contrast, and low flow velocities in the left atrial
(b) Damage to vessel wall and endothelial damage/dysfunction				
Heintz et al (1994) (37)	Comparative	40	CKD and healthy controls	CKD patients have higher endogenous levels of ET-1, plasma cAMP, and enhanced ET-1 stimulated ADP-induced platelet aggregation than healthy control
Blacher et al (1999) (32)	Observational	241	ESRD patients	Increased aortic PWV in ESRD predicts all-cause cardiovascular mortality.
Bolton et al (2000) (33)	Cross-sectional	67	23 HD patients, 16 NDD patients and 28 healthy control	Reduced flow-mediated EDD in HD and NDD patients compared to control. Increased vWF and adhesion molecules in renal dysfunction.

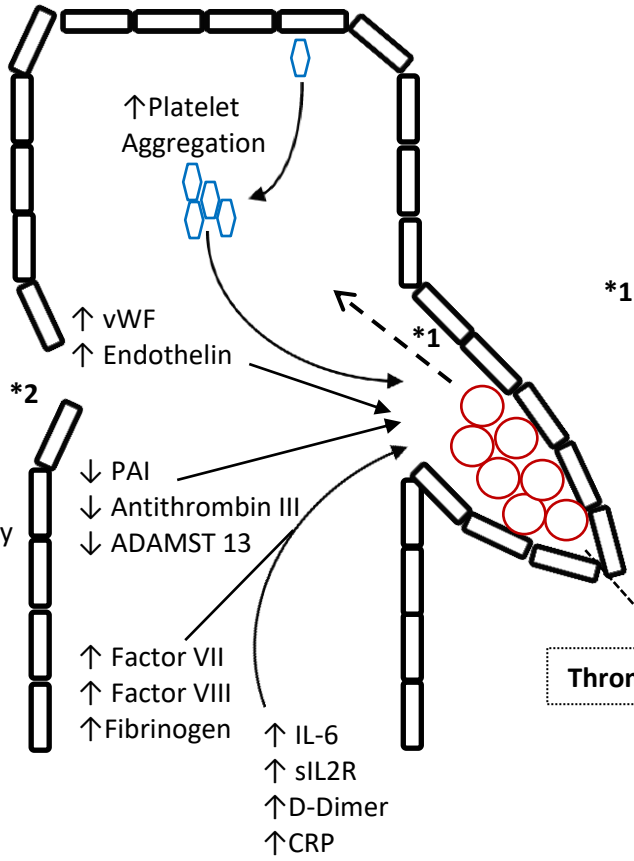
Yildiz et al (2003) (34)	Comparative	104	104 HD patients vs 49 healthy controls	Reduced flow-mediated EDD and EID in haemodialysis patients compared to control.
Hrafnkelsdóttir et al (2004) (55)	Comparative	18	Non-diabetic, non-smoking CKD patients and age-matched control	Maximal release of active tPA and capacity for active tPA release markedly impaired in CKD patients vs controls
Wang et al (2005) (35)	Observational	102	NDD CKD patients with various GFR	Decreased GFR was independently associated with an increased PWV.
Carrero et al (2012) (36)	Observational	630	NDD CKD vs ESRD	Prolactin levels increased along with reduced kidney function, related to FMD, PWD and increased risk of cardiovascular events and mortality.
Recio-Mayoral et al (2011) (38)	Comparative	141	76 CKD vs 65 age and gender matched control	CKD patients had increased CRP levels, reduced FMD and increased IMT values compared to controls
(c) Platelet and coagulation abnormalities				
Shlipak et al (2003) (41)	Cross-sectional	5888	Population-based cohort of age >65 years old	CRP, fibrinogen, IL-6, Factor VII, Factor VIII, plasmin-antiplasmin complex, and D-Dimer levels significantly higher in CKD
Pecoits-Filho et al (2003) (42)	Observational	176	176 NDD patients	Lower GFR associated with increased CRP, IL-6, hyaluronan and neopterin levels.
Keller et al (2008) (43)	Cross-sectional	6814	Population-based cohort 45-84	CRP, IL-6, TNF, TNF-αR1, intercellular adhesion molecule-1, fibrinogen, and Factor VIII levels are significantly higher in

				CKD
Landray et al (2004) (44)	Comparative	522	334 CKD patients, 92 CAD patients, 96 healthy control with no prior CV or renal disease	CKD is associated with higher fibrinogen, plasma vWF, soluble P-selectin, but not CRP
Tanaka et al (2009) (45)	Observational	190	Patients not receiving oral anticoagulant stratified to CCr	Decreased GFR predicts for elevation of TAT and D-Dimer in patients with AF
Mercier et al (2001) (46)	Cross-sectional	150	50 ESRD patients, 50 NDD CKD and 50 healthy controls	Reduced renal function associated with enhance tissue factor coagulation to platelet, monocyte and endothelial injury.
Costa et al (2008) (48)	Observational	50	50 ESRD patients vs 25 healthy controls	Higher levels of CRP, s-IL2R, IL-6 and D-dimers, and significantly lower levels of PAI-1 in ESRD patients. The tPA/PAI-1 ratio was also significantly higher in ESRD patients.
Adams et al (2008) (47)	Comparative	102	66 CKD stage 4&5 vs 36 healthy controls	Up-regulation of the tissue factor pathway, increased prothrombin fragment 1+2 and reduction in antithrombin III in CKD compared to healthy controls
Shen et al (2012) (49)	Observational	104	104 NDD vs 32 healthy controls	Increased vWF-antigen level and decreased ADAMTS13 activity in CKD.

Yagmur et al (2015) (50)	Comparative	84	30 HD patients, 34 renal transplant recipients, 20 healthy controls	Increased platelet hyperaggregability in CKD.

Abbreviations: ADAMTS13, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; ADP, adenosine diphosphate; AF, atrial fibrillation; CAD, coronary artery disease; cAMP, cyclic adenosine monophosphate; CCr, Creatinine clearance; CKD, chronic kidney disease; CV, cardiovascular; CRP, C-reactive protein; EDD, endothelium-dependent dilatation; eGFR, estimated glomerular filtration rate; EID, endothelium-independent dilatation; ESRD, End-stage renal disease; ET-1, Endothelin 1; FMD, flow-mediated dilation; GFR, glomerular filtration rate; HD, haemodialysis; IL-6, interleukin-6; intima-media thickness, IMT; MDRD, Modification of Diet in Renal Disease; NDD, non-dialysis dependent; PWV, pulse wave velocity; s-IL2R, serum interleukin-2 receptor ;TAT, thrombin-antithrombin complex; TNF-αR1, tumour necrosis factor-α soluble receptor 1; tPA, tissue plasminogen activator; vWF, von Willebrand factor.

Figure 1.1.1: Pathophysiology of Thromboembolism in Chronic Kidney Disease and Atrial Fibrillation



***1.** Reduced left atrial appendage emptying velocity induces stasi

As evidenced by increased spontaneous echocardiographi contrast. This reflects stasis of blood and spontaneous thrombus formation

***2.** Endothelial dysfunction/damageAs evidenced by increased intima media thickening, increased pulse wave velocity and reduced endothelial dilatation, resulting in endothelial dysfunction.

***3.** Increased coagulation and platelet abnormalities

Epidemiological insights

The increase in stroke risk with progressive severe CKD amongst AF patients has been reported by several large observational studies, as shown in **Table 1.1.2**.

The AnTicoagulation and Risk factors In Atrial fibrillation (ATRIA) study (56) found that the presence of proteinuria increased risk of thromboembolism in AF by 54%; and progressive worsening of GFR was also associated with increased risk of stroke, so much so that those with GFR <45 mL/min/1.73 m^2 conferred an increased risk of 39% as compared to those with GFR >60 mL/min/1.73 m^2.

In separate analyses from the Danish nationwide cohort, Olesen et al (57) and Bonde et al (58) reported that patients with concurrent AF and CKD experience significantly higher rates of stroke, thromboembolism, haemorrhage and death, as compared with those without renal disease. Those with ESRD requiring renal replacement therapy fared the worst as they are twice as likely to experience stroke and thromboembolism (58) compared to those without renal dysfunction, with an incidence rate up to 6.9 per 100 patient-years (59).

Similar relationships between increased incidences of AF with progressive renal failure, with resultant increased in adverse events were exhibited amongst the Swedish national cohort study as well as some Asian countries (15, 60-62).

Furthermore, amongst those with CKD (GFR <60ml/min per 1.73m^2), the sequential deterioration of renal function over time has been shown to be equally pertinent, as an absolute reduction in eGFR ≥25 mL/min/1.73 m^2 or a relative reduction of eGFR ≥25% effectively more than doubles the risk of ischaemic stroke when compared to

those with relatively “stable” renal function over 6 months period (18). Even among AF patients treated with effective anticoagulation, every 30ml/min per 1.73m^2 reduction in eGFR confers an increased risk of thrombotic or vascular events (HR 1.42; 95%CI 1.11 – 1.83) (63).

More recent evidence also reveals that worsening renal clearance not only is an independent, reliable predictor of stroke mortality, but is also associated with a worse adverse clinical outcome after stroke (64, 65).

Table 1.1.2: Epidemiological insight into stroke risk in patients with atrial fibrillation and chronic kidney disease

Stroke Risk in AF with CKD			
Study	Study Type	N	Findings
Go et al (2009) (56)	Retrospective	10908 AF with CKD	Comparing with GFR ≥ 60 mL/min/1.73 m^2: eGFR 45-59mL/min, RR 1.16 (95% CI, 0.95 to 1.40) eGFR < 45mL/min, RR 1.39 (95% CI, 1.13 to 1.71 (P = 0.0082 for trend).
Friberg et al (2012) (66)	Retrospective	182678 AF patients (out of which 8113 had CKD)	CKD Stage 1 and below: Multivariate HR 1.11(95% CI 0.99-1.25)
Olesen et al (2012) (57)	Retrospective	132372 AF patients (out of which 3587 NDD CKD, 901 ESRD)	Comparing with GFR ≥ 90 mL/min/1.73 m^2: NDD CKD, HR 1.49 (95% CI 1.38-1.59) ESRD, HR 1.83 (95% CI, 1.57 to 2.14)
Guo et al (2013) (18)	Prospective	617 AF patients	Risk of stroke or death: HR 2.90 (95% CI 1.88-4.48) Risk of stroke in 6 months: Absolute decrease eGFR ≥ 25 mL/min/1.73 m^2: HR 2.77 (95% CI 1.26-

			6.09) Relative decrease eGFR ≥ 25%: HR 2.57 (95% CI 1.14 – 5.80)
Roldán V et al (2013) (63)	Prospective	978 AF patients on VKA	Every decrease eGFR 30 mL/min/1.73 m^2 : HR 1.42 (95% CI 1.11 - 1.83)
Bonde at al (2014) (58)	Retrospective	154254 non-valvular AF patients (out of which 148598 NRD, 4519 NDD, 1142 on RRT)	Comparing with GFR ≥ 60 mL/min/1.73 m^2: NDD CKD, HR 1.32 (95% CI 1.23–1.42) RRT, HR 2.01 (95% CI 1.74–2.33)
Chao TF et al (2014) (59)	Retrospective	10999 AF patients with ESRD in Taiwan	11.7% of patients experienced ischaemic stroke. Absolute stroke and thromboembolism event rate: 6.9 per 100 patient year
Banerjee A et al (2014) (67)	Prospective	8962 AF patients (out of which 2982 with CKD)	Comparing with GFR ≥ 60 mL/min/1.73 m^2: eGFR 30-59 mL/min HR 1.53 (95% CI 1.10-2.12) eGFR < 30 mL/min HR 1.78 (95% CI 0.99-3.19)

Abbreviations: CI, confidence interval; CKD, chronic kidney disease; ESRD, end-stage renal disease; HR, hazard ratio; NDD, non-dialysis dependent; NRD, no renal disease; pts, patients; RR, relative risk; RRT, renal replacement therapy

The haemorrhagic tendency in CKD

Though CKD does increase the risk of thromboembolism and ischaemic stroke in AF, paradoxically it is also associated with an important increased risk of haemorrhagic sequelae as explained below.

Evidence from both the Rotterdam Study and the Japanese CIRCS Study show that the mere presence of reduced renal function (GFR <60 mL/min/1.73 m^2) resulted in an increased risk of haemorrhagic stroke with hazard ratios over 4-fold in male and 7-fold in female patients (68, 69), while patients undergoing chronic dialysis had a relative risk of intracerebral haemorrhage that can be over 10-fold higher (70). There is also mounting evidence that worsening renal function and associated vascular dysfunction may result in increased tendency for formation of MRI-defined cerebral micro-bleeds, which have the potential of contributing to subsequent intra-cerebral haemorrhage (71).

In addition, gastrointestinal (GI) bleeding is also increased, either peptic ulcer or non-peptic ulcer-related and non-variceal bleeds; overall, the recurrence, frequency and severity of such episodes are all closely linked to impairment of renal function (72-74). Similar to intracranial haemorrhage, both forms of renal replacement therapy (peritoneal dialysis and haemodialysis) is associated with an increased risk of gastrointestinal haemorrhage, conferring a hazard ratio of 3.71 (95 % CI 2.00 - 6.87) and 11.96 (95% CI 7.04 - 20.31), respectively (75).

The pathophysiological causes of the increased risk of haemorrhagic sequelae are clearly multifactorial. These can be a direct result of uremia-related platelet

dysfunction or impaired platelet adhesion and aggregation, impaired platelet glycoprotein IIb-IIIa receptor activation and subsequent binding to glycoprotein, altered von Willebrand factor and nitric oxide metabolism (76-78).

Extrinsically, the propensity to bleed can be a result of concurrent use of anti-platelets or non-steroidal anti-inflammatory drugs for other associated disaease processes. Moreover, patients with ESRD would be subjected to frequent invasive diagnostic and treatment strategies, such as central venous access and haemodialysis (plus subsequent frequent heparin exposure), that could increase their bleeding risk.

Stroke and bleeding risk stratification in AF with CKD

In the AF population, the risk of stroke is increased by 5-fold overall, according to the presence or absence of various stroke risk factors. The common risk factors have been used to formulate stroke risk stratification scheme, to help decision making on whether OAC should be recommended or not, for stroke prevention.

However, all clinical risk scores have modest predictive value for identifying 'high risk' patients who sustain events. AF guidelines have adopted the use of the CHA_2DS_2-VASc score [**Table 1.1.3**] given that it can reliably identify truly "low risk" patients (i.e. CHA_2DS_2-VASc score 0 in males, 1 in females), who do not need antithrombotic therapy (79, 80). Subsequent to this step, effective stroke prevention can be offered to those AF patients with ≥1 stroke risk factor. Effective

stroke prevention means OAC, whether as well-controlled VKA therapy or one of the NOACs.

Despite being a contributor to an increased thromboembolic risk, moderate-severe renal impairment is not included in the CHA_2DS_2-VASc score, attempts to incorporate renal impairment into stroke risk stratification scheme did not suggest an independent additive predictive value for renal impairment over the CHA_2DS_2-VASc score components, probably due to the fact that CKD is strongly associated with the single risk factor components of the CHA_2DS_2-VASc score (i.e. heart failure, hypertension, diabetes, etc).

One study advocating the addition of renal impairment for stroke risk stratification proposed the R_2CHADS_2 score (with the additional "R" for impaired renal function, and given 2 points). In its initial derivation study, the R_2CHADS_2 score also modestly improved c-index (prediction value) of the $CHADS_2$ and CHA_2DS_2-VASc scores (81-83). Nonetheless the aforementioned studies were either derived from an anticoagulated clinical trial population with exclusion of those with severe CKD (ROCKET-AF trial) (81), inclusion of a small sample size of ESRD cohort (82) or from a highly selected cohort undergoing invasive catheter ablation of AF (83). However, the positive results of R_2CHADS_2 score were not replicated in other similar studies (84, 85). Also, in other "real-world" non-anticoagulated AF cohorts, renal dysfunction did not independently improve predictive value of $CHADS_2$ and CHA_2DS_2-VASc score (86, 87).

Regarding haemorrhagic risk stratification in AF, guidelines currently recommend the use of the HAS-BLED score for assessing bleeding risk [**Table 1.1.4**]. Nevertheless, a high HAS-BLED score should not lead to withholding of effective oral anticoagulation

therapy. Instead, a high HAS-BLED score amongst those with CKD should ‘flag up’ the patients potentially at risk of bleeding for careful review or follow-up and for correction of modifiable risk factors such as uncontrolled hypertension (the H of the HAS-BLED), labile INRs, alcohol excess or concomitant use of NSAIDs and aspirin.

For those patients who did experience previous gastrointestinal haemorrhagic sequelae, the resumption of anticoagulant treatment was actually associated with a significant reduction of mortality and thromboembolic events (88, 89). Similar findings have also been seen in ESRD cohort upon restarting warfarin amongst patients with an increased risk of recurrence of gastrointestinal bleed (90).

Table 1.1.3: The CHA_2DS_2-VASc score

Congestive Heart Failure	1
Hypertension	1
Age ≥ 75 years	2
Diabetes Mellitus	1
History of **S**troke/TIA/thromboembolism	2
Vascular disease (previous myocardial infarction, peripheral vascular disease or aortic plaque)	1
Age (64-74 years)	1
Sex **c**ategory (female)	1

Maximum score 9

Table 1.1.4: The HAS-BLED Score

Hypertension	1
Abnormal renal and liver function (one point each)	1
Stroke	2
Bleeding history or propensity	1
Labile INR	2
Elderly (age > 65 or frail condition)	1
Drugs or alcohol concomitant use (one point each)	1

Maximum score 9

Oral Anticoagulation in CKD: using Vitamin K antagonists (VKA)

Though oral anticoagulant is the mainstay of treatment in the prevention of ischaemic stroke and thromboembolic-related adverse outcomes in patients with AF population, less evidence exists for those with significant renal impairment, given that such patients were excluded from randomised trials. Hence, the prescription of classical oral anticoagulants (essentially VKAs e.g. warfarin) amongst those with significant renal impairment varies from as low as 2% in Germany to as high as 37% in Canada[58]. This heterogeneity in clinical practice reflects the uncertainty about the risks and benefits of anticoagulation use within this patient group.

For AF patients with concomitant ESRD, requiring renal replacement therapy, conflicting findings exist from observational studies relating to the safety associated with use of VKA [**Table 1.1.5**].

Of the 17 studies reviewed, only Abbott et al (91) showed a clear mortality benefit, while Olesen et al (92), Bonde et al (58), Genovesi et al (93), Chan et al (94) and Findlay et al (95) demonstrated a reduction in event rate for stroke or thromboembolism. Other studies involving ESRD and VKA thromboprophylaxis have demonstrated either equivocal results (96-98) or even suggested that VKA can potentially cause harm in CKD patients with ESRD (96, 99-106).

One large observational study (100) and several multinational cohort studies (101, 104) have demonstrated that AF patients who are on haemodialysis and taking warfarin experienced more than two-fold increase in the risk of ischaemic stroke as compared to non-VKA users. Elderly patients (aged >75 years) appeared to be

particularly at risk as compared to those below 65 years (101). At the same time, those exposed to VKA whilst on haemodialysis may also face a higher risk of haemorrhagic stroke rather than thromboembolic event (103).

Possible explanations for the lack of efficacy of VKA in protection against stroke and thromboembolism, and potentially contributing to the increase in stroke risk may be due to the low time in therapeutic range for patients receiving renal replacement therapy. Indeed, the Swedish AF cohort study suggests that the improved quality of anticoagulation control, as reflected by high time in therapeutic range (TTR), is associated with lower risk of thromboembolism and haemorrhage (107).

Another possible explanation related to the lack of efficacy in reducing ischaemic stroke and haemorrhagic risk may potentially be dependent on the modality of renal replacement therapy used. A recent Hong Kong cohort suggested that patients receiving peritoneal dialysis have similar thrombotic risk as non-CKD counterparts while warfarin use in this particular patient group not only provided protection against ischaemic stroke, but does not appear to increase the risk of intracranial haemorrhage (94).

Indeed, amongst non-dialysis dependent CKD patient with AF, there appears to be more robust data favouring the use of dose-adjusted VKA in AF [**Table 1.1.6**]. In all 5 studies and 1 meta-analysis reviewed, dose-adjusted warfarin provided better protection against ischaemic stroke and systemic embolism, than no VKA- (57, 58, 105, 108-110). The efficacy of warfarin in reducing thromboembolism has to be balanced against a small but significant increase in haemorrhage tendency (19% – 36%), with even higher event rate if there is concurrent antiplatelet use (57, 105).

Therefore, though VKA can be beneficial in non-dialysis dependent patients with AF, the propensity for harm due to VKA in ESRD is yet to be fully defined, especially in a patient on haemodialysis. Thus the use of VKA for thromboprophylaxis against stroke and systemic thromboembolism in CKD patients requiring renal replacement therapy requires a careful evaluation of stroke and bleeding risks and the correction of reversible bleeding risk factors.

What are the pathophysiological mechanisms?

Murine and human studies have demonstrated that VKA contributes to vascular calcification through inactivation of matrix Gla protein (MGP) (111, 112). As MGP is a potent inhibitor of vascular calcification, the use of VKA will inhibit the final vitamin K dependent pathway, thus preventing phosphorylation and carboxylation of MGP into its active form (113, 114).

The increased vascular calcification, either as a direct result of development of ESRD or due to concurrent VKA usage may potentially increase the likelihood of development of non-cardioembolic stroke, which will not be remedied by VKA use. Moreover, VKA administration has been implicated in development of calciphylaxis, a painful and lethal complication among patients with ESRD as cutaneous arteries and arterioles undergo calcification and occlusion (115, 116). The pathophysiology resulting in calciphylaxis remains poorly understood.

Table 1.1.5: Vitamin K antagonist use and stroke rates in end-stage renal disease

VKA use in AF with ESRD			
Study	Study Type	Number (% with AF)	Findings
Wiesholzer et al (2001) (99)	Retrospective observational	430 (14.3%)	Stroke rate per 100 patient year: AF with VKA: 4.46, AF without VKA: 1.0
Abbott et al (2003) (91)	Retrospective observational	3374 (1.25%)	3-year survival rate: AF with VKA: 70% AF without VKA: 55%
Chan et al (2009) (100)	Retrospective observational	48825 (3.42%)	90-day HR – AF with VKA [a]: 1.93 (95% CI 1.29 – 2.90)
Wizemann et al (2010) (101)	Observational (DOPPS)	17513 (12.5%)	Stroke rate in >75 years old: Warfarin user: 2.17 (95% CI 1.04 – 4.53)
Phelan et al (2011) (102)	Retrospective	845 requiring dialysis (141 on warfarin)	Stroke rate per 100 patient year: VKA user: 1.7 versus Non-VKA user: 0.7 (p = 0.636)

			Major haemorrhage rate per 100 patient year: VKA user: 10.8 versus Non-VKA user: 8.0 (p = 0.593)
Winkelmayer et al (2011) (103)	Retrospective observational	2313 ESRD patients with new AF	HR for ischaemic stroke: VKA user 0.92 (95% CI 0.61 – 1.37) HR for haemorrhagic stroke: VKA user 2.38 (95% CI 1.15 – 4.96)
Olesen et al (2012) (57)	Subgroup analysis	901 patients with AF requiring dialysis	HR comparing with no antithrombotic, dialysis dependent pts: VKA: 0.44 (95% CI 0.69 – 1.01)
Knoll et al (2012) (96)	Prospective	235 patients on dialysis (19.6% on VKA)	No stroke or bleed experienced. HR for mortality in VKA user: 0.80 (95% CI 0.28 - 2.29)
Sood et al (2013) (104)	Observational (DOPPS)	41844 (9.71%)	Stroke rate per 100 patient year: VKA [b]: 3.3 No VKA or antiplatelet: 2.1
Bonde at al (2014) (58)	Retrospective	154254 non-valvular AF patients (out of which 1142 on RRT and 260	Stroke and thromboembolic risk in non-VKA users:

		receiving VKA)	HR [c]: 1.82 (95% CI 1.58 - 2.12)
Shah et al (2014) (105)	Retrospective	1626 patients with AF on RRT (out of which 756 VKA users)	HR for ischemic stroke comparing VKA vs non-VKA users: 1.14 (95% CI 0.78 – 1.67) HR for bleeding: 1.44 (95% CI 1.13 - 1.85)
Chen et al (2014) (98)	Retrospective	500 with AF and ESRD (out of which 250 receiving VKA)	Comparing with group of control (no VKA or antiplatelet): HR for ischemic stroke: 1.017 (95% CI 0.673–1.537)
Wakasugi et al (2014) (106)	Prospective	60 Japanese patients with AF requiring dialysis (out of which 28 VKA users)	Comparing VKA vs non-VKA users HR for ischemic stroke: 3.36 (95% CI 0.67–16.66)
Chan et al (2015) (94)	Retrospective	271 patients with AF on peritoneal dialysis (70 on VKA)	Comparing VKA vs aspirin user: HR for ischaemic stroke: 0.16 (95% CI 0.04 – 0.66) Comparing VKS vs non-user of antithrombotic agents: HR for ischaemic stroke: 0.19 (95% CI 0.06 – 0.65)
Findlay et al (2015) (95)	Retrospective	1382 patients with ESRD out of which 293 with AF (118 on VKA,	Stroke rate:

		while 175 without VKA)	AF with VKA: 11.4% AF without VKA: 14.4%
Genovesi et al (2015) (93)	Prospective	290 patients with AF requiring dialysis (out of which 134 on VKA at recruitment)	Comparing VKA vs non-VKA users: HR for stroke/thromboembolic events: 0.12 (95% CI 0.00-3.59)
Shen et al (2015) (97)	Retrospective	12284 pts on RRT (1383 started on VKA)	Comparing VKA vs non-VKA users: HR for ischemic stroke: 0.68 (95% CI 0.47 - 0.99) HR for mortality: 0.84 (95% CI 0.73 - 0.97)

Abbreviations: AF, atrial fibrillation; CI, confidence interval; DOPPS, Dialysis Outcomes and Practice Pattern Study; ESRD, end-stage renal disease; HR, hazard ratio; RRT, renal replacement therapy; VKA, Vitamin K antagonist

[a] AF with VKA covariate adjusted model: adjusted for $CHADS_2$ score, gender, race, Charlson comorbidity index, entry date, body mass index, facility standardised mortality ratio, cardiovascular drugs, dialysis adequacy, baseline laboratory values, heparin dosage and heparin regimes.

[b] VKA user includes patients with atrial fibrillation, thromboembolic disease or central vascular catheter.

[c] Adjusted for Aspirin treatment and all risk factors included in CHA_2DS_2VASc score.

Table 1.1.6: Vitamin K antagonist use and stroke/thromboembolic event rate in non-dialysis dependent chronic kidney disease

VKA and event rate in non-dialysis dependent CKD			
Study	Study Type	Number	Findings
Lai et al (2009) (108)	Observational	307	Stroke event rate (% per year): Dose-adjusted warfarin (INR target 2-3): 3.48 No VKA: 13.57
Hart et al (2011) (109)	Post-hoc analysis	516	Stroke/embolic event rate (% per year): Dose-adjusted warfarin: 1.45 Dose-adjusted warfarin plus aspirin: 7.05
Olesen et al (2012) (92)	Subgroup analysis	3587	HR of stroke comparing with no antithrombotic NDD CKD: Warfarin only: 0.84 (95% CI 0.69 – 1.01) Warfarin plus aspirin: 0.76 (0.56 – 1.03) Aspirin only: 1.25 (1.07 – 1.47)
Bonde et al (2014) (58)	Retrospective	154254 non-valvular AF pts (out of which 4519 on NES CKD, 1130 on VKA)	Stroke and thromboembolic risk in non-VKA user: HR [a]: 1.31 (95% CI 1.22 – 1.41)

Shah et al (2014) (105)	Retrospective	204 210 NES pts with AF (103 652 on VKA)	Stroke risk with warfarin use in non-dialysis patient: HR: 0.87 (95% CI 0.85 – 0.90) Bleeding risk in non-dialysis patient: HR: 1.19 (95% CI 1.13 – 1.85)
Providencia et al (2014) (110)	Meta-analysis	19 studies – 379506 patients with CKD and AF	Stroke and thromboembolic risk in non-NES VKA user: HR 0.39 (95% CI 0.18 – 0.86)

Abbreviations: CKD, chronic kidney disease; INR, international normalised ratio; NDD, non-dialysis dependent; NES, non end stage; VKA, vitamin K antagonist

[a] Adjusted for Aspirin treatment and all risk factors included in CHA_2DS_2VASc score.

Non-VKA Oral Anticoagulants

With the introduction of the non-VKA oral anticoagulants (NOACs), namely the direct thrombin inhibitor (dabigatran) and Factor Xa inhibitors (rivaroxaban, apixaban and edoxaban), these were considered as viable alternatives for patients with mild to moderate CKD requiring oral anticoagulant for thromboprophylaxis.

All four agents [**Table 1.1.7**] demonstrated non-inferiority or even superiority in stroke prevention, and non-inferiority (or in some cases, superiority) in bleeding profile as compared to warfarin (117-121). Even amongst those with moderately reduced renal function (as low as GFR 30mL/min), apixaban and edoxaban subgroups of ARISTOTLE and ENGAGE AF-TIMI 38 trials had demonstrated a reduced bleeding risk compared to warfarin. Additional benefits include medication delivery in fixed doses, not requiring monitoring and a lower propensity for interaction with food or other medications (122, 123).

Nonetheless, as all NOACs have a degree of renal excretion (varying from 25% in apixaban to 80% in dabigatran), in their respective trials, those with severe renal dysfunction or ESRD were excluded. Therefore, the European guidelines recommend that the NOACs are best not be used where severe renal impairment (GFR <25–30 mL/min) is present (79, 124). Among those with moderately impaired renal function, (GFR 30 – 49mL/min), dose alteration as per manufacturer's recommendation is advised.

For patients in the USA, the FDA has approved dabigatran 75mg bid, rivaroxaban 15mg od and apixaban 2.5mg bid for patients with a creatinine clearance of 15-

29mL/min. That is based on no clinical trial outcome data but on pharmacological modelling data in patients alone. Recently, based on the latest pharmacokinetic findings in patients receiving haemodialysis, the FDA has also approved the use of apixaban 5mg bid (no dose adjustment) in AF patients receiving chronic, stable dialysis treatment (125).

A recent meta-analysis has demonstrated the relative safety and efficacy of all the four NOAC agents over warfarin across various degrees of renal impairment (27). Although there is currently no head to head clinical trial comparing one NOAC with another, the same analysis also revealed that in “moderate renal dysfunction” (creatinine clearance 25 – 49ml/min), apixaban possessed better safety profile while retaining similar power of efficacy in protection against thromboembolic event. Nonetheless, in those with “mild renal dysfunction” (creatinine clearance 50 – 79 ml/min), dabigatran 110mg, apixaban, rivaroxaban and edoxaban 30mg are comparable.

Table 1.1.7: Randomised Controlled Trials for NOAC in atrial fibrillation

Study	Connolly et al (2009) (118) RE-LY	Connolly et al (2011) (117) AVERROES	Granger et al (2011) (119) ARISTOTLE	Patel et al (2011) (120) ROCKET AF	Giugliano et al (2013) (121) ENGAGE AF-TIMI 38
Number	18113	5999	18201	14264	21108
Dosage	Dabigatran 150 mg twice daily Dabigatran 110mg twice daily Dose-adjusted Warfarin	Apixaban 5mg twice daily Aspirin 81-324mg daily	Apixaban 5mg twice daily Apixaban 2.5mg twice daily (eGFR <50mL/min) Dose-adjusted Warfarin	Rivaroxaban 20mg once daily Rivaroxaban 15mg once daily(eGFR 30-49mL/min) Dose-adjusted Warfarin	Edoxaban 60mg once daily Edoxaban 30mg once daily Dose-adjusted Warfarin
F/U (months)	24	13.2	21.6	23.5	Median F/U 2.8 years
CKD stages studied	eGFR 30-50mL/min eGFR 50-79mL/min	eGFR 30-60mL/min	eGFR 25-30mL/min eGFR 31-51mL/min	eGFR 30-49mL/min eGFR ≥50-mL/min	eGFR 30 - ≤50mL/min

			eGFR 51-80mL/min		
Pharmacokinetics	80% renally excreted	25% renally excreted	25% renally excreted	33% renally excreted	35% renally excreted
Key Results (Event rate %/year)	Superior to warfarin in reducing ischaemic stroke and thromboembolism (1.11 vs 1.53 vs 1.69) Non-inferior in bleeding events (3.11 vs 2.71 vs 3.36)	Superior to warfarin in reducing ischaemic stroke and thromboembolism (1.6 vs 3.7) Non-inferior in bleeding events (1.4 vs 1.2)	Superior to warfarin in reducing ischaemic stroke and thromboembolism (1.27 vs 1.6) Lower incidence of bleeding events (2.13 vs 3.09)	Non-inferior to warfarin in reducing ischaemic stroke and thromboembolism (2.2 vs 2.4) Non-inferior in bleeding events (14.9 vs 14.5)	Non-inferior to warfarin in both doses in reducing ischaemic stroke and thromboembolism (1.49 vs 1.91 vs 1.69) Lower incidence of bleeding events (2.75 vs 1.61 vs 3.43)
Outcomes in subset with CKD	No difference in primary outcome	Lower stroke risk with no increase in bleeding risk	Non-inferior in stroke risk, but reduced bleeding risk for eGFR >30mL/min	No difference in primary outcome	Lower bleeding risk at reduced dose

Conclusion

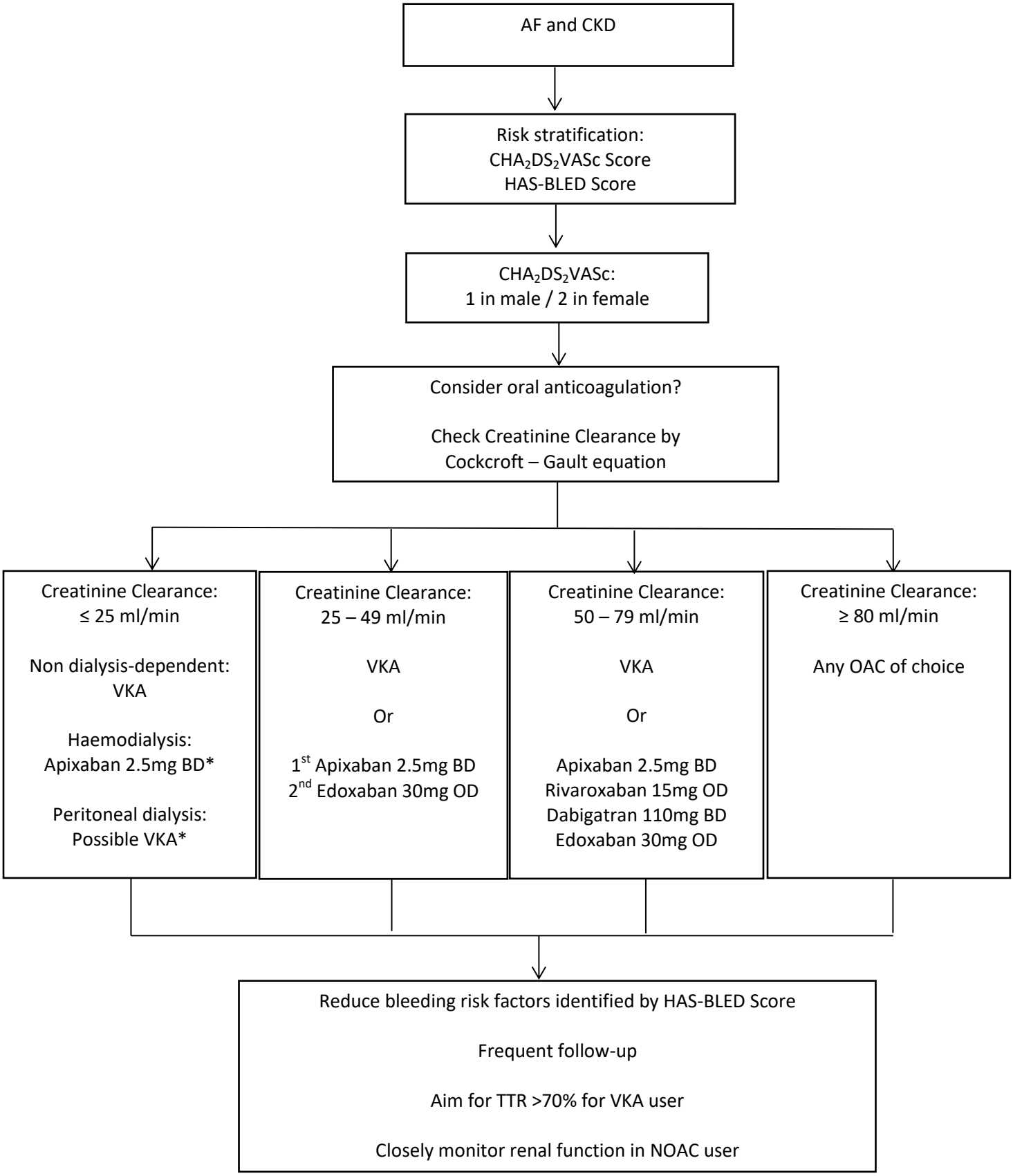
Renal dysfunction and AF commonly coexist and the concurrent existence of both conditions result in a paradoxical increase in both thromboembolic and haemorrhagic risks. Several pathophysiological factors have been demonstrated to induce a prothrombotic state while increasing bleeding sequelae. The thromboembolic and haemorrhagic risks are particularly high amongst dialysis-dependent patients with ESRD. However, at this juncture, data supporting the long-term use of VKA for thromboprophylaxis in AF in ESRD remains limited. Any potential benefit conferred by VKA appears to be outweighed by a disproportional increase in bleeding risk (and thrombotic events). Nonetheless, potential use of specific NOAC (apixaban) has recently been licensed though its use in haemodialysis individuals is based on limited pharmacokinetic studies only.

Conversely, the use of various oral anticoagulant (be it VKA or NOACs) among mild CKD patients with AF have shown a reduction in morbidity and mortality from stroke and systemic thromboembolism. Even in moderately impaired CKD, apixaban and edoxaban appear to have a good safety profile [**Figure 1.1.2**].

The key would be for careful patient selection through the use of risk stratification scores (CHA_2DS_2-VASc and HAS-BLED scores). Upon initiation of oral anticoagulation treatment, ensure substantial steps are taken to reduce bleeding risk (such as aiming for a high Time in Therapeutic Range, >70%(126)) plus regular monitoring of renal function if NOAC is chosen so as to allow for dose alteration if needed. Patients with CKD and AF are prone to experience fluctuation in renal function due to acute illness (127), thus making timely dose alteration vital to prevent adverse events (128).

As a result of an increasingly older global population, the complexity of managing AF with concurrent CKD will appear to further increase in the future. Hence a search for the best pharmacological approach to prevent stroke, systemic thromboembolism and bleeding events is clearly needed.

Figure 1.1.2: Anticoagulant choice in atrial fibrillation and chronic kidney disease



*Limited data based on observational studies or pharmacodynamics modelling only.

1.2 Fibrin Clot – formation, degradation and changes in disease

Introduction

In the healthy human body, there is a delicate balance between fibrin formation and degradation. Abnormal thrombotic or haemorrhagic phenomena will occur if one of the two processes takes precedence over the other.

The concept of coagulation and fibrinolysis is through the activation of pro-enzymes in a step-wise cascade which results in the aggregation of fibrin into fibrin clot (coagulation), or through the degradation of fibrin clot (fibrinolysis). In the classical coagulation cascade, it is subdivided into two initial pathways: the intrinsic pathway and extrinsic pathway.

The intrinsic pathway involves kallikrein and a negatively-charged surface activating the surface-bound Factor XII. Factor XII in turns activate Factor XI, which subsequently activates Factor IX. Activated Factor IX, in the presence of activated Factor VIII, will activate Factor X.

The extrinsic pathway involves release of tissue factor which will in combination with Factor VII, activate Factor X. In this common pathway of coagulation, Factor X will activate thrombin from inactive prothrombin, by cleaving off prothrombin fragment 1 and 2 from the pro-enzyme (prothrombin, Factor II). This process will end with the initiation of fibrinogen conversion to fibrin monomer by thrombin (Factor IIa) and subsequent lateral aggregation into fibrin fibre.

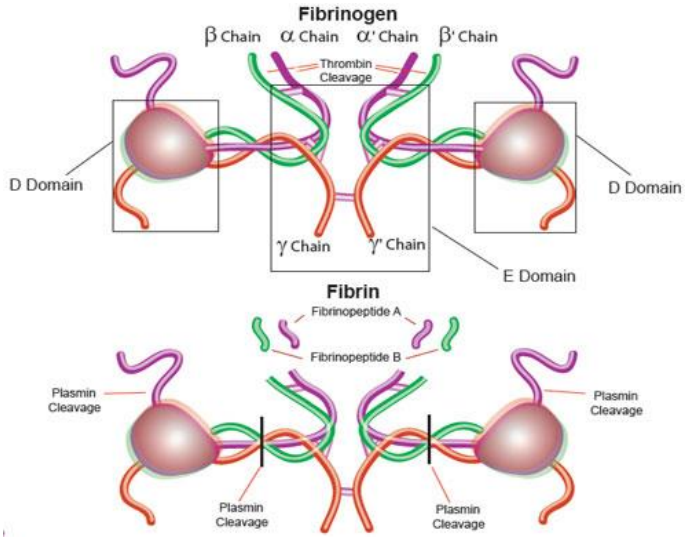
Fibrinogen and Fibrin Clot formation

Fibrinogen is a dimeric molecule consisting of identical pairs of three separate chains (Aα, Bβ and γ-chains), all of which are linked by disulfide bonds (129). This molecule exists as an

elongated 45-nm structure, circulates within the plasma at 200-450mg/dL and is generated by a functioning liver. It is also subdivided into three domains, one E-domain and two D-domains, which contain binding sites that participate in fibrinogen conversion to fibrin, fibrin assembly, crosslinking, and platelet interactions (130) (Figure 1.2.1).

The E –domain contains the N-termini of all the fibrinogen chain and is located at the centre of the dimeric molecule, whereas the D-domain located at the bilateral portion of the molecule, contains the C-termini of the Bβ and γ-chains. On the other hand, the C-termini of the Aα-chain loops back towards the E-domain. The N-termini of the Aα-chains and Bβ-chains are named Fibrinopeptide A and fibrinopeptide B (FPA and FPB respectively), which will be cleaved during activation of fibrinogen to fibrin monomers.

Figure 1.2.1: Fibrinogen and Fibrin monomer (131)



Source: Putnam FW. The Plasma Proteins. 4 ed. New York: Elsevier, 2012: 127-137

During the final stage of the coagulation cascade, the presence of thrombin will cleave off FPA and FPB from fibrinogen, resulting in formation of fibrin monomer. This allowsexposure of polymerisation sites in the E-domain (E_A and E_B) which will bind non-covalently with corresponding D_A and D_B sites located at the D-domain of neighbouring fibrin

monomer(132). These fibrin oligomers subsequently grow into a two-stranded fibrin protofibrils and after a certain length undergo lateral polymerisation and aggregation into three-dimensional fibrin fibre and fibrin bundles (133, 134).

Thrombin, besides initiating the formation of fibrin from fibrinogen, can also activate Factor XIII. Factor XIII in combination with calcium will predominantly allow for formation of cross-bridges between C-termini of γ-chains of neighbouring fibrin monomers through covalent bonds between a glutamine residue and a lysine residue of these neighbouring monomers; to a lesser extent, Factor XIII also stimulates cross-bridges between α-chains of neighbouring monomers (135, 136). This progressive cross-linkage involving both α-chains and γ-chains causes the fibrin fibres to become less susceptible to plasmin-induced fibrinolysis, forming insoluble fibrin, which further reinforced by the binding of several fibrinolysis inhibitors (all of which are induced by Factor XIII)(135).

The formation and folding of fibrin fibres will finally allow them to acquire a three-dimensional configuration, giving it the unique structural and biochemical properties. Fibrin network which are more “coarse” will result in greater opacity in light, as compared to those “fine” network which are more translucent. These “coarse” networks have been demonstrated to consist of fibres of thicker diameter and arranging in a “looser” configuration, as compared to the “fine” network which comprises of more fibres per volume of fibrin clot (137).

Fibrin Clot Degradation

Similar to the coagulation cascade, the fibrinolytic process is also governed by several proenzymes: namely tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA) and also Factor XII-related activations. All of which have the potential to cleave plasminogen to plasmin which is the key to fibrinolysis, but with tPA-related pathway being the most dominant(138).

During the fibrinolysis process, fibrin-bound plasminogen will rapidly be activated by tPA. This induces partial degradation of fibrin cross-bridges, which further expose more sites to additional plasminogen, tPA, uPA and fibrinolytic enzymes, which further accelerates the fibrinolysis process (139). The breakdown of fibrin fibre bundles into progressively smaller fragments and complexes, and thus finally to D-domains (or D-Dimers/DD fragments) and E fragments.

The rate of fibrinolysis can be influenced by several fibrin clot structure properties, such as “fine” network being more resistant to fibrinolysis than “coarse” network. Due to the laying down of layers of dense, thin fibre-matrix which possesses complex architecture that reduces permeability and thus reduces exposure of potential binding sites to tPA and plasminogen (139).

Nevertheless, the processes of formation of fibrin clot and subsequent fibrinolysis are complex. They can be altered by subtle changes in the *in vitro* environment (such as thrombin concentration, salinity of solution, pH and ionic strength), as well as through pathological conditions during human disease. Hence more research is required to provide a better understanding of fibrin clot structure and fibrinolysis.

Atrial Fibrillation and Fibrin Clot Structure in Cardiovascular Diseases

Atrial fibrillation (AF) is an atrial tachyarrhythmia characterized by rapid, chaotic and uncoordinated atrial activation with subsequent deterioration of atrial mechanical function. It is the most common cardiac arrhythmia and increasingly prevalent amongst the elderly. Up to 20% of ischaemic stroke is consequent to AF, and ischaemic stroke in association with AF is generally more severe and results in higher mortality, greater disability and longer in-patient stay than stroke occurring in the absence of this arrhythmia (20).

Though the main clinical issue in AF and its resultant sequelae is invariably thrombosis, the understanding of the effect of the three classical thrombotic factors (namely blood flow, the blood vessel wall, and the constituents of the blood) as described the Victorian pathologist Virchow are incomplete. Indeed Virchow's triad is currently being re-interpreted as having roles for the endothelium (endothelial function), platelets, and the molecules of the coagulation cascade that generate fibrin clot (140), plus their impact on thrombosis in AF have yet to be fully elucidated.

At the same time, changes in the fibrin clot structure characteristics have been demonstrated in patients with increased risk of established cardiovascular and thrombotic disease (141-145). Fibrin clots made from these "high-risk" patients are usually thicker, have earlier polymerisation and are less permeable when compared with clots made from healthy controls (141-145). However, as AF patients are at even higher thrombotic risk than patients with cardiovascular diseases, the relationship between clot structure characteristics amongst AF patients need to be investigated.

Atrial Fibrillation, Renal Dysfunction and Fibrin Clot

AF and renal dysfunction are closely interlinked. The prevalence of AF rises rapidly from 0.7%(146) in the general population age <60, to 27% (147) amongst those with end-stage renal disease. This is illustrated by the Atherosclerosis Risk in Community (ARIC) Study, showing that patients with GFR of 60 - 89, 30 - 59, and 15 - 29 mL/min/1.73 m^2 have hazard ratios of developing AF (within the 10 year follow-up period) of 1.3, 1.6, and 3.2, respectively, as compared to those with normal GFR (9).

In addition, amongst those with renal dysfunction, the existence of several pathophysiological pathways also results in a prothrombotic-hypercoagulable state. Thus, in clinical medicine, the combination of AF and renal dysfunction creates a therapeutic dilemma, as thrombotic risk amongst CKD-AF patients is not completely reduced with oral anticoagulant but the use of oral anticoagulant among them is associated with increased risk of bleeding (57). Amongst patients with ESRD, previous works by Sjoland have demonstrated altered fibrin clot structure in haemodialysis while Undas suggested fibrin clot structure in ESRD may be associated with increased mortality (148, 149). However no relationship between progressive CKD and AF has ever been explored.

Fibrin Clot Structure and Effects of Vitamin K Antagonist and Non-Vitamin K Antagonist Oral Anticoagulants

The VKA is the classic oral anticoagulant used to reduce the risk of thromboembolic complications of AF. VKA reduces thrombin generation through the inhibition of coagulation Factors II, VII, IX and X. Nonetheless, the effect of VKA on fibrin clot structure and network generation, as well as subsequent fibrinolysis, have yet to be investigated.

However, the advent of NOACs in the past five years has completely changed the landscape of antithrombotic treatment. By directly and competitively inhibiting specific pathways along the coagulation cascade, such as Factor Xa (by rivaroxaban, rpixaban and edoxaban), or inhibiting thrombin (such as dabigatran), they have been shown to be as efficacious as VKA, and result in a lower risk of haemorrhage. The effects of NOACs on fibrin clot structure, function and haemostasis will be of interest.

Conclusion

Fibrin clot formation and degradation is a result of complex proenzyme-related pathways. Changes in fibrin clot structure have been demonstrated at several patient populations who are at-risk of thrombotic events. The impact of AF on clot structure, the associated relation with progressive renal dysfunction and concurrent choice anticoagulant treatment on clot structure can be studied to assess their potential clinical significance.

Section 2: Study proposal and Hypotheses to be tested

Aim

To study the different aspects of fibrin clot structure and fibrinolysis in AF and its relationship with renal dysfunction, primarily through the assessment of the fibroelastic strength of clot, fibrin clot thickness and density, rate of fibrin clot built-up and fibrinolysis. So as to enable us to appreciate the changes in fibrin clot structure to the paradoxical increase in thrombotic and haemorrhagic phenomena associated with oral anticoagulation use in AF and CKD.

Background

Atrial Fibrillation (AF) results in an increased risk of ischaemic stroke and systemic thromboembolism. Ischaemic stroke as a result of AF is usually more debilitating and results in worse functional outcome than non-AF related ischaemic stroke (20). The improving longevity and expansion of elderly population globally is resulting in increasing incidence of AF and paralleled by the rise in CKD (150-152), as both conditions share similar risk factors (such as hypertension and diabetes (153)) which are associated with increasing age. Moreover, a bidirectional relationship exists between deterioration of renal function and AF (17), further reinforcing the close relationship of these two conditions.

AF per se confers a prothrombotic or hypercoagulable state through numerous pathophysiological pathways fulfilling 'Virchow's triad for thrombogenesis', as evidenced by abnormalities in vessel wall, abnormalities in flow and abnormalities in blood constituents (29). The propensity for thrombus formation is further enhanced by CKD due to additional changes to the flow within left atrium and left atrium appendage, damage to vessel wall and

subsequent endothelial dysfunction, or upwards regulation of platelet and coagulation factors (30, 31, 36, 43, 47, 48).

The corner stone for management of AF is through the use of chronic oral anticoagulation, classically with Vitamin K antagonist such as warfarin (79). As discussed previously in Section 1, the use of oral anticoagulant in patients with concurrent renal dysfunction is complicated by the lack of efficacy and also having to contend with a corresponding rise in haemorrhagic risk (105). Thus, this creates a therapeutic dilemma.

Hence to better understand the reason behind these paradoxical changes to thrombosis and bleeding, there is a need to investigate the potential impact of renal dysfunction (in concurrent AF) on fibrin clot formation and fibrin clot strength.

Traditionally, assessment of fibrin clot formation has been simply through determining the time taken for a clot to form from plasma such as prothrombin time and the activated partial thrombplastin time. Although of undoubted value, this approach has little regard for the roles of other endothelial/inflammatory components involved in the coagulation cascade, nor the physical strength of the fibrin clot formed.

On the other hand, with respect to fibrin clot structure, there have been a number of publications demonstrating alterations in fibrin clot structure and clot strength in relation to cardiovascular disease and severe renal failure (143-145, 148, 149, 154-159).

In accordance with previous works by Mills et al. and Collet et al., they have independently demonstrated that in healthy relatives of patients presenting with premature coronary artery disease, there is significant alteration in fibrin clot properties. The fibres formed are usually thicker in diameter and forming fibrin clots of lower permeability (though less

dense) with relative to controls (143). The fibres are also stiffer as assessed by torsion pendulum, and underwent slower rate of fibrinolysis ex vivo (159). These findings are also supplemented reports by Fatah et al. and Undas et al., confirming similar findings in patients with established coronary artery disease (144, 145). Thus, confirming the association between changes in fibrin clot structures and the increased thrombotic risk in relation to coronary disease.

Subsequently, with regards to renal disease, Sjoland et al has also reported that in patients with ESRD, the fibrin clot made from plasma has higher clot density which is less susceptible to ex-vivo fibrinolysis compared to healthy control(148). These fibrin clots from patients with ESRD also exhibited increased fibre thickness and underwent fibrin protofibril formation much quicker (149). The altered fibrin clot also associated with increased mortality during the 3 years follow-up. Thus, these findings suggested the relationship regarding ERSD with changes to fibrin clot properties and worse clinical outcome.

Therefore, from our current knowledge, we can appreciate that changes in fibrin clot structure can relate to increased CAD risk as well as ESRD. However, no previous work has been done with regards to fibrin structure and strength with regards to AF and progressive renal dysfunction. No previous has work has been done investigating changes to fibrin clot structure in relation to choice of anticoagulant or antiplatelet therapy.

Beside investigation of changes to fibrin clot, assessment of markers of platelets activation and endothelial dysfunction plus microparticles will also be done. In an adult, the endothelium is an organ composed of 1-6 x 10^{13} endothelial cells, weighing approximately 1kg and covering a surface area of approximately 4000 to 7000 m^2 (160). Its functions can be broadly classified into those pertaining to blood pressure control, and those relating to

haemostasis, and accordingly endothelial damage/dysfunction leads to hypertension and coagulopathy (161, 162). Hence, von Willebrand Factor (vWF) and endothelial-selectin (E-selectin) levels, surrogates for endothelial injury/dysfunction will be assessed to investigate its role in CKD-AF patients.

On the other hand, platelet activation is implicated in myocardial infarction and elevated levels signify increased thrombotic potential (163-166). Thus soluble platelet-selectin (p-selectin) levels will be assessed to investigate if changes to fibrin clot structure are in any way related to increase in platelet activation in relation to CKD and AF.

Subsequently, microparticles are heterogeneous vesicles, derived from cellular membrane where the parent cells had undergone apoptosis or activation (167, 168). Owing to the nature of their parent cells, different microparticles subsets possess unique composition and content, which vary in their hemostatic and thrombotic potentials (169-171). Thus, different microparticles subsets can modulate coagulation by directly facilitating formation of coagulation complexes or via modulation of tissue factor dependent pathways (172, 173).

While microparticles levels are increased in ESRD and correspond with increased cardiovascular mortality (174), a contradictory relationship exists between non-valvular AF and levels of circulating microparticles (175, 176). A potential relationship between worsening degrees of non-dialysis dependent renal dysfunction and microparticles amongst non-valvular AF patients, as well as the subsequent effect of levels of various microparticles subsets has yet to be investigated.

Summary

Haemostasis demands the correct balance between thrombogenesis and fibrinolysis as failure leads to thrombosis and haemorrhage respectively. This failure of haemostasis may relate to external factors such as type and intensity of anticoagulation treatment, renal function and vascular/endothelial function. The present study extends previous work by focussing on understanding clot structure/function and the relation to pathogenesis of thrombogenesis in AF.

Hence this research project will utilise thromboelastography (TEG), turbidimetric and fibrinolysis assay to investigate several aspects of coagulation, fibrinolysis and fibrin clot structure, assessment of vascular function through ELISA, flow cytometry for assessment of microparticles levels and finally Scanning Electron Microscopy to visualise the fibrin clot in detail.

Hypothesis to be tested

First hypothesis: Patients with AF (anticoagulation-naive) have different thrombogenesis, fibrinolysis and fibrin clot structure characteristics compared to patients with established cardiovascular disease.

Second hypothesis: Variability in certain aspects of haemostasis in AF patients treated with warfarin, with abnormal indices of thrombogenesis, fibrinolysis and fibrin clot structure, can be explained by altered renal and/or endothelial function.

Third hypothesis: Clot structure, function and haemostasis in AF patients before and after becoming therapeutically anticoagulated depends on the class of oral anticoagulant used (VKA versus NOACs).

Section 3: Methodology

3.1 Materials and study design

For my **First Hypothesis,** I recruited subjects as followed: (i) 50 AF patients were non-OAC user (aspirin) with normal-mildly impaired renal function (eGFR≥60 ml/min/1.73m^2), comparing them with (ii) 50 patients with established, stable coronary artery disease (defined as those who suffer from angina with over 50% stenosis of at least 1 coronary artery disease on coronary angiogram) who were on aspirin.

My **Second Hypothesis** was tested in a case-control cross sectional study of 200 patients with AF on classic VKA, warfarin. There were 50 patients in four distinct quartiles depending on renal function as defined by their creatinine clearance as described by Cockcroft-Gault formula.

The **Third Hypothesis** was tested in 50 patients naïve to anticoagulation, of whom 25 were started on warfarin, 25 on NOAC based on clinical criteria or physician's choice. The effects of these drugs on clot structure and function was assessed after 4 weeks treatment. Compliance among NOAC users was assessed through interviews and verbal confirmation of adherence to treatment. Warfarin user would have achieved stable INR through regular monitoring in anticoagulation clinics.

All subjects were recruited predominantly from (and not isolated to) patients attending AF and cardiology clinics at City Hospital. Data collected included full clinical and demographic details (age, gender, ethnicity, body weight, BMI, systolic and diastolic blood pressure, tobacco use, family history of CVD), concurrent disease and medication history. In all cases full routine bloods were taken for FBC, ESR, PT (INR), APTT, U&E's, total and HDL-cholesterol

and LFTs. Renal function will be defined by creatinine clearance, calculated using the Cockcroft-Gault equation.

Diagnosis of heart failure was defined as a clinical syndrome consisting of typical symptoms (dyspnea, oedema and fatigue) and signs (pulmonary oedema and pulmonary crackles), due to established structural or functional cardiac abnormality (as confirmed by Echocardiogram) resulting in reduced cardiac output.

Inclusion criteria:

- Aged 18 or over, and able to provide informed, written consent
- For AF cohort: previous or current AF established on 12-lead ECG (inclusive of paroxysmal or persistent AF)
- For coronary artery disease cohort: established coronary artery disease (previous-Myocardial infarction over 12 months ago or angina with >50% stenosis defined by coronary angiogram)

Exclusion criteria:

- Patients receiving renal replacement therapy/ undergoing dialysis (peritoneal or haemodialysis)
- Presence of potential cofounders – significant co-existing medical conditions: systemic connective tissue disease, ongoing neoplasia, recent (<3 months) surgery or acute cardiovascular event (established myocardial infarction by troponin elevation or dynamic ECG-changes to ST segments), presence of a prothrombotic/haemorrhagic phenomenon (such as lupus anticoagulant, anti-

phospholipid syndrome, coagulation Factor deficiencies), overt liver disease (deranged LFTs with/without deranged INR), deranged FBC (anaemia, polycythaemia, thrombocythaemia or thrombocytopaenia), acute sepsis, NSAIDs or oral steroids, subtherapeutic INR upon reviewAdministration of parenteral anticoagulant (such as low molecular weight heparin, bivalirudin), platelet aggregation inhibitor (abxicimab) of ADP receptor antagonists (clopidogrel, prasugrel or ticagrelor).

3.2 Laboratory Methods

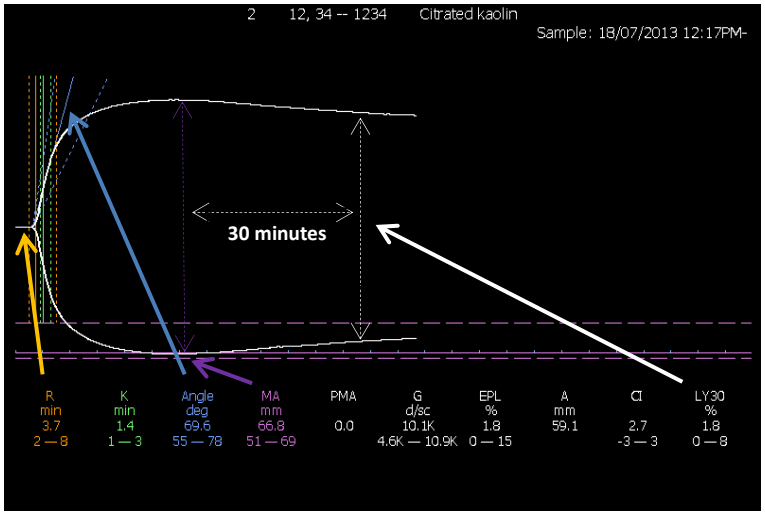
First and foremost, adequate written consent was obtained by mysef prior to twenty mL of blood taken from an antecubital vein into 0.9% citrate bottle at room temperature for assay of coagulation, fibrinolysis, vascular function, and fibrin clot structure. Platelet-poor plasma was obtained after centrifugation at 3000g for 20 minutes, and aliquots of 0.5 mL were frozen at -70°C until assay. The plasma was the source material for a comprehensive panel of markers that provided information on the following processes. Out of which five mL of fresh blood was kept aside for thromboelastography (TEG).

3.2.1 Thromboelastography (TEG)

Thromboelastography (TEG) is a whole-blood viscosity assay which has been widely used in the assessment of coagulation and fibrinolysis (177). TEG has been used previously to investigate patients with end-stage renal disease (178), and in patients with ischaemic stroke (179), but never before being used in AF subjects.

In this assay, 340 µL of citrated whole blood was added to a reaction cuvette, to which 20 µL of 0.1 M calcium chloride solution was added to reverse anticoagulation by citrate (Haemonetics, Lanarkshire, UK). The reaction proceeded immediately and monitored in real time by the analyser with results fed directly to a microcomputer. A typical TEG graphical printout is presented (**Figure 3.2.1.1**), and shows the formation of clot, the increasing physical strength of the developing clot on the vertical axis over time, and finally clot autolysis. Together with the graphical printout, 21 TEG indices are generated, of which 5 are selected due to their direct assessment of coagulation and fibrinolysis (namely R-time, K-time, Angle, MA, and LY60 indices). Further explanation of these indices provided in **Section 4**.

Figure 3.2.1.1: TEG Graphical Tracing: Amplitude against Time



Key:
A, Amplitude; CI, Coagulation Index; EPL, Estimated Potential Lysis; G, G-parameter; K, K-time;
LY30, Percentage of lysis 30 minutes post maximum amplitude is attained;
MA, Maximum Amplitude; PMA, Projected maximum amplitude; R, R-time

3.2.2 Turbidimetric Analysis and Fibrinolysis Assay

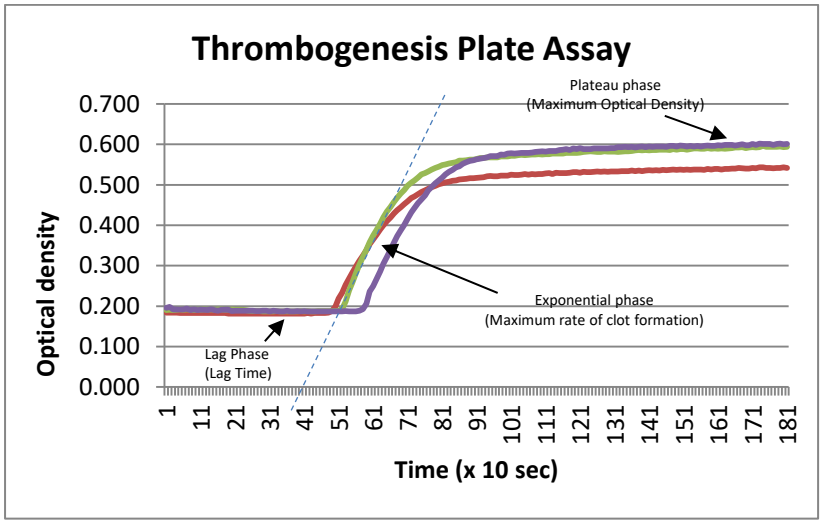
When plasma is supplemented with thrombin, polymerisation of fibrin will occur, and this process can be quantitatively measured by measuring the amount of light passing through this intervening, clotting substance.

In this turbidimetric assay, 25 µL of platelet-poor plasma was added to the well of a standard ELISA-quality 96-well microtitre plate (R&D Systems Europe Ltd, Abingdon. UK), followed by 75 µL of a TRIS-NaCl buffer (1.51 g Tris-HCl, 1.75 g NaCl, 200 mL distilled water). Coagulation was initiated by the addition of 50µL of a thrombin/calcium solution.

The plate was immediately loaded into a Tecan Sunrise (Tecan Group Ltd, Männedorf, Switzerland) plate reader at 37^{o}C programmed to measure the optical density (OD) at 340nm every six seconds (with an intermediate two-second shaking period) for 30 minutes. As fibrinogen is converted to insoluble fibrin matrix, the absorbance of light will occur and this is demonstrated by an initial lag phase, followed by a rapid exponential increase phase which result from the lateral polymerisation and aggregation of protofibrils into fibrin fibre, finally is followed by a plateau-phase (**Figure 3.2.2.1**).

The final value of the optical density at the plateau phase is directly related to the average size of the fibres (180), with high level of absorbance demonstrated to be directly proportional to larger cross-sectional area of fibrin fibres (181).

Figure 3.2.2.1: Turbidimetric Analysis (Triplicates of one sample)



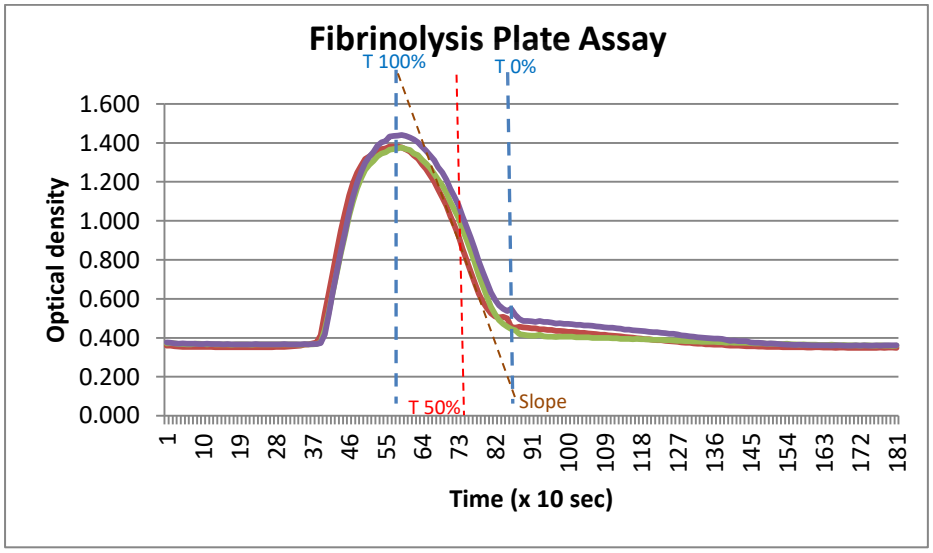
Subsequently, fibrinolysis assay calls for 75 µL of plasma to be added to the well of a microtitre plate. To this is added 75 µL of a Tris/NaCl/calcium buffer supplemented with thrombin and tPA. The plate is also immediately loaded into a Tecan Sunrise plate reader as for the turbidimetric assay, and data collected for 30 minutes.

A typical graphical print-out is presented in **Figure 3.2.2.2,** which demonstrates change in OD over time as the fibrin clot is initially formed and then lysed. The data is post-processed to plot into line charts, and from these the rate of clot dissolution (RCD), being the slope of the right hand portion of the graph, and the time for 50% clot lysis (T50) can be determined.

Structural features of fibrin clot which may contribute to fibrinolysis or suggests increase thrombogenic potential will be visualised by Scanning Electron Microscopy (see below).

Nevertheless, potential limitation of turbidimetric and fibrinolysis assay is the lack of assessment of humoral factors contributing to thrombosis and fibrinolysis, such as levels of fibrinogen, PAI, TNF-α, cytokines and interleukin.

Figure 3.2.2.2: Fibrinolysis Assay (Triplicates of one sample)



The plot shows changes in optical density as the fibrin clot forms. Triplicate plots are shown.
T100% is the time to maximum absorbance, T0% is the return of the optical denity to near-baseline.

T50% is (T100% - T0%)/2.

The slope is the sharpest fall in optical density over time under the effect of exogenous tPA.

3.2.3 Vascular/Endothelial dysfunction

For enzyme-linked immunosorbent assay (ELISA) blood samples were centrifuged within 30 min of collection at 1,500 g for 20 min at 4°C. The resultant plasma was then collected and stored at –70°C until later batch processing by ELISA to measure soluble E-selectin and soluble P-selectin (R&D Systems, Minneapolis, MN, USA) as per department's protocol (182).

Vascular function was be assessed by levels of endothelial products von Willebrand factor (vWf) and tissue plasminogen activator (tPA) (both ELISA assays) as dictated by established standard operating procedures (SOPs) developed in the department.

3.2.4 Microparticles

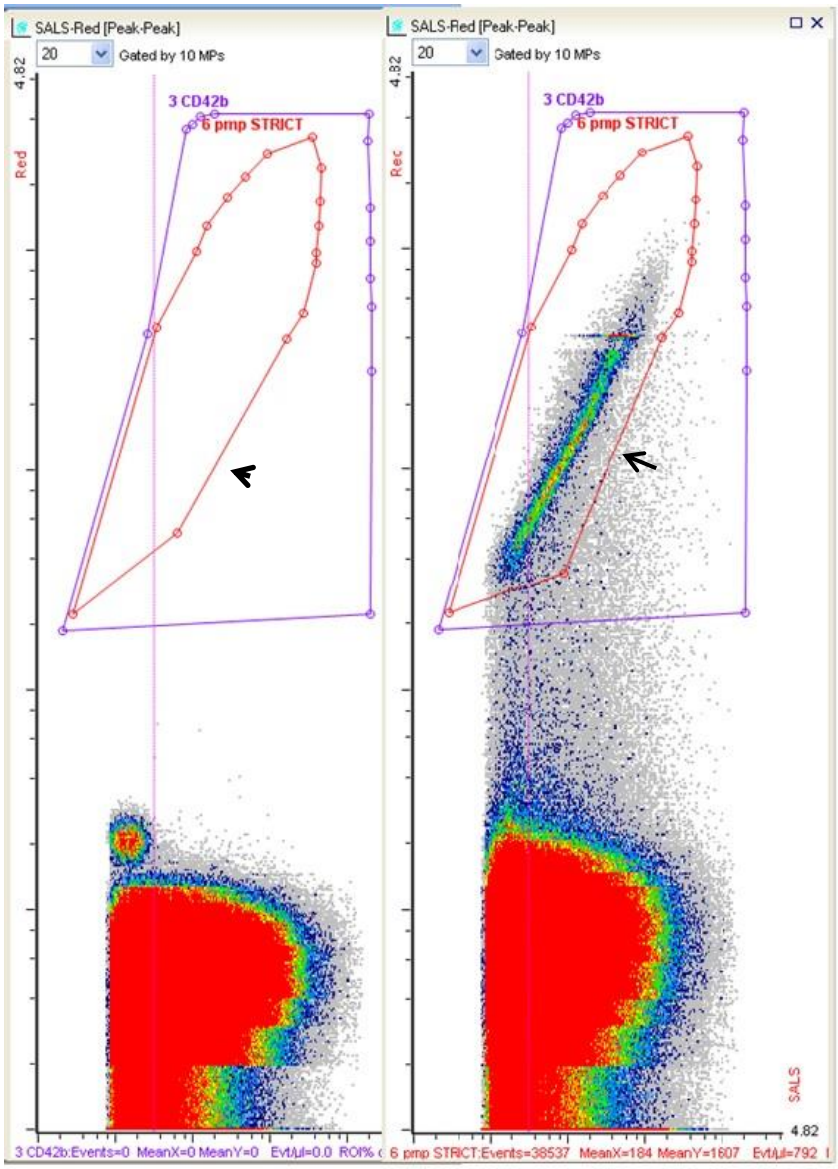
For microparticle detection, platelet-poor plasma (PPP) was obtained after 20 min centrifugation of citrated blood at 3,000 g and further centrifugation of PPP at 13,000 g for 2 minutes to remove residual cellular fragments to obtain platelet-free plasma (PFP). Aliquots of the plasmas were frozen at –70°C for subsequent batch analysis and underwent a single-freeze thaw cycle.

PFP was initially incubated separately for 30 min with 0.5 µg of biotinylated anti-human CD42b antibody (Abcam, Cambridge, UK) for platelet-derived microparticles (PMP), or 0.5 µg of biotinylated anti-human CD31 antibody (Abcam, Cambridge, UK) for endothelial-derived microparticles (EMP). This was followed by a second incubation with 0.25 µg of Streptavidin-Alexa Fluor-647nm-R-Phycoerythrin conjugate (Life Technology, Paisley, UK) for 30 min and then diluted with 990 µl filtered PBS (final dilution 1:100).

MP analysis was promptly performed using the Apogee A50 flow cytometer (Apogee Flow Systems). Polystyrene beads of 110, 200, 500 nm and 1 µm diameter (Apogee Flow Systems) were used to set up the MP-size gate and small-size MP defined as events with size between 110 and 500 nm. Triplicate of measurements (Events/uL) were recorded from region of interest (**Figure 3.2.4.1**).

Detailed instruction regarding gating selection has previously been described by department's publication (183).

Figure 3.2.4.1 Flow Cytometry – Microparticles detection



Arrowhead: Lack of excitation of MP in Region of Interest
Arrow: Excitation of PMP-fluorochrome conjugate

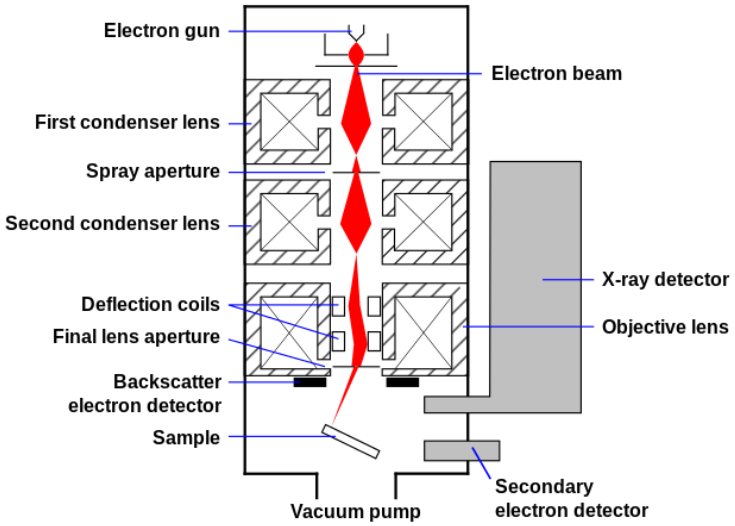
3.2.5 Scanning Electron Microscope (SEM)

To assess the fibrin clot structure using SEM in University of Leeds LIGHT Institute, fibrin clot can be prepared using the following method. First, 25 µL of plasma is inserted into the cap from aliquot tubes. Small holes were drilled through the bottom of the cap and covered in parafilm. After initial 2 hours of incubation in a moist environment, the clot will be washed

several times, before being fixed with 2% gluteraldehyde and subsequently dehydrated. Critical point drying with CO_2 in Critical Point Drying Apparatus is done prior to mounting for SEM.

The scanning electron microscope (SEM) was first developed in 1935 by focusing a narrow beam of electrons across a sample surface by magnetic deflection. A typical SEM (Figure 3.2.5.1) allows for a small stream of electrons to be emitted via the electron gun while focused on a small spot (about 5 – 10nm). The SEM is maintained under vacuum to ensure the beam electrons have minimal interaction with any intervening gas molecules on their way to the sample. Several detectors are set up to pick up secondary electrons generated though electron-sample interactions. The varied number of secondary electrons released through the interaction will determine different gray-white intensity as per assigned at the particular scan position. This will then be post-processed by adjacent computer into a SEM image.

Figure 3.2.5.1: Schematic of an Scanning Electron Microscope (184)



Diameter of fibres within the fibrin clot can then be determined by direct measurement using open-source software (ImageJ programme, Rasband, National Institute of Health, USA). Each clot type studied was photographed in at least 5 different areas at 5,000 and 20,000x magnifications using an FEI Quanta 200 FEG SEM (FEI, Hillsboro, OR).

Average fibre diameters were measured from at least 20 random fibresin each sample using ImageJ software. A region of a micrograph is selected at random and the diameter of every fibre in that area is measured. This will ensure no bias towards, for example, thicker or well-focused fibres, thus minimising any sampling effects. The operator and analyst of SEM were blinded to source of plasma and associated background demographics to reduce operator-related selection bias. Unblinding procedure took place only during final data analysis.

3.3 Statistical Analysis

Results are expressed as mean (SD, standard deviation) or median (IQR, interquartile range). Data of all subjects were analysed by t-testing, one-way ANOVA or Mann-Whitney U tests and Kruskal-Wallis tests, with post-hoc Tukey's analysis as appropriate:

Gaussian distribution was assessed by plotting of histogram, assessment of skewness and kurtosis, subsequently checked for normality by Anderson-Darling test. Continuous data between two groups assessed by t-test when normally distributed or by Mann-Whitney U test when non-normally distributed. Continuous, normally distributed data between more than two groups are assessed by ANOVA (with post-hoc Tukey's method for multiple

comparisons), while non-normally distributed data were assessed by Kruskal-Wallis test. Categorical data assessed by Chi-Square test. Correlations were performed by Spearman's correlation method and all statistical calculations were performed on a microcomputer using commercially available statistical package (Minitab, Minitab Inc, PA, USA). A value of $P < 0.05$ was considered significant in all statistical analyses unless specified.

Statistical methods utilised are cross-checked by statistician.

3.4 Ethical Considerations

The study was conducted in accordance with the declaration of Helsinki. Ethical approval was obtained from the National Research Ethics Service (NRES Committee Midlands), as well as from the Sandwell and West Birmingham Local Research Ethic Committee.

Written consent was obtained from all patients involved in this study. Appendices (in **Section X**) contains the REC ethics approval letter, patient information leaflet and consent form.

REC Reference: 13/WM/0379; IRAS ID: 134460

Section 4: Validation Results

4. Validation of laboratory methods

Coefficient of Variation (CVs) of TEG, turbidimetric and fibrinolysis analysis

As thromboelastography (TEG), turbidimetric and fibrinolysis analysis assays were all new techniques, extensive validation exercises had been done during assay and SOP development.

My results as demonstrated in **Tables 4.1** and **Table 4.2**. Coefficient of variations (CV) was calculated by the ratio of the standard deviation to the mean. A greater CV equates to greater variability/spread in the results thus reduces the reliability of assay used.

For turbidimetric and fibrinolysis analysis, frozen plasma demonstrated better median intra and inter-assay CV at 2.8 and 5.3%, while fresh plasma intra and inter-assay CV ranges at 3.6 and 9.9%. Whereas for TEG, the median intra and inter-assay CV for fresh whole blood at 16.4 and 18.4%, for fresh plasma at 19.3 and 23.7%, while frozen plasma at 20.9 and 21.7%. In TEG, 21 indices (**Table 4.3**) were evaluated but only 5 clinically significant and relevant, independent indices were selected for analysis, namely R-time, K-time, Angle, Maximum Amplitude (MA) and percentage lysed at 60 minutes (LY60).

Other validation aspects including studies of diurnal variation, exercise-related changes in coagulation, fresh vs frozen plasma, transiently frozen to short-term frozen plasma and time delay to indices of coagulation are described further below.

Coefficient of Variations of flow cytometry for microparticles detection

As microparticle detection by Apogee 50 flow cytometry was well-developed in the department, extensive validation was not required. However, mean intra and inter-assay CVs for Platelet Microparticles and Endothelial Microparticles are at 18.2 and 24.6% for PMP, while EMP at 9.3 and 12.9%, respectively.

Diurnal variation and TEG indices

To assess if diurnal variation in coagulation indices exist, fresh blood samples were collected from 10 healthy volunteers within the research department every 6 hours over a 24-hour period, and those samples were processed by TEG. All TEG indices demonstrated to show no statistical difference or diurnal variation (**Table 4.4**). Consent Form in Appendices.

Exercise and coagulation indices (assessed by TEG)

To assess if exercise will have any impact on coagulation indices, fresh blood samples were obtained from 10 healthy volunteers at baseline, at peak exercise on treadmill (defined as 85% of maximum target heart rate achieved after corrected to age and gender), 2 hours post-exercise, 4 hours post-exercise, 12 hours post-exercise and 24 hours post-exercise. All of which were processed by TEG and resultant indices obtained.

As demonstrated by **Table 4.5**, peak exercise resulted only in reduction of LY60. At 2 hours post-exercise, it resulted in the reduction of R-time, K-time and LY60, but increased Angle and MA. At 4 hours post-exercise, R-time and K-time remain reduced while MA and reduced

LY60 remain elevated. By 12 hours post exercise, all coagulation indices as assessed by TEG have returned to normal, except for reduced LY60.

Fresh vs frozen plasma samples for turbidimetric and fibrinolysis analyses

As turbidimetric and fibrinolysis assay were carried out on 96-well plate and read on an ELISA reader, steps were taken to optimise laboratory processes to allow for batch analysis. Thus 15 fresh plasma samples and respective thawed frozen samples were assessed by turbidimetric and fibrinolysis assay, which demonstrated no statistical differences between all indices measured.

Transiently frozen vs short-term frozen plasma samples

To assess if duration of freezing has any impact on coagulation and fibrinolysis indices as assessed by turbidimetric and fibrinolysis assay, 15 plasma samples were transiently flash-frozen in -70 degrees freezer for 1 hour before being thawed for analysis, and compared to respective thawed samples which were frozen for 1 week in similar freezing condition. All indices analysed by turbidimetric and fibrinolysis assay demonstrated no statistical differences between both groups.

Effect of time on indices as assessed by turbidimetric and fibrinolysis analyses or TEG

Finally, to assess if the delay between venipuncture and analysis of samples by TEG and turbidimetric and fibrinolysis assay, fresh blood and corresponding plasma samples were

collected and periodically assessed by TEG and plate assays over a 24 hour period. Results are illustrated on **Table 4.6** and **Table 4.7**

As demonstrated by **Table 4.6**, the turbidimetric and fibrinolysis analyses demonstrated that processing samples collected over time results in a progressive shortening of the lag time (LT), a slower rate of clot formation (RCF) but increased maximum clot density (MCD). Resultant clot formed over time was also demonstrated to be more resistant to lysis by tPA, shown by increased T50% and reduced rate of clot dissolution (RCD).

As compared to **Table 4.7**, parallel TEG data were broadly speaking stable to 24 hours after preparation. The only index that did show a statistical change was the Angle, which demonstrated that fibrin samples aggregate more rapidly at 3 and 24 hours after preparation (both p=0.019).

Discussion

From the result of the validation exercise, several important aspects of TEG and turbidimetric/fibrinolysis assay have been investigated.

Firstly, addressing the CV for TEG, the local TEG exhibited CV of similar characteristics as previous publication, with Anderson et al. demonstrated that depending on choice of indices studied, the inter-assay CVs for TEG can range from 4 – 22% (185). Moreover, it is known that CVs have been altered with the use of kaolin as an haemostasis accelerant in the accordance to TEG protocol (186). On the other hand, as for local developed turbidimetric and fibrinolysis assay, the demonstrated inter-assay CV is good while comparable to previous publications which can range from 4.9% (187) to 28.6% (188).

Thus based on the most reliable CV, fresh whole blood was selected for analysis via TEG, over the use of fresh plasma or frozen plasma. Whereas for turbidimetric and fibrinolysis assay, frozen plasma was selected as choice for analysis due to its excellent CV demonstrated and the ability to allow for batch analysis.

Secondly, although our study showed no diurnal differences in coagulation indices when blood samples are assessed by TEG among 10 healthy samples, this result is incongruent to findings from contemporary published works involving healthy subjects: such as variation of over 22% in level of von Willebrand factor (vWF) over 24 hours period(189), or platelet activity and aggregability, levels of coagulation system markers such as Prothrombin fragment 1+2, Factor VIIa, and Fibrinogen which are also shown to be elevated in especially in the late-morning (190). This is further supported by increased incidence of cardiovascular events (such as myocardial infarction and sudden cardiac death) and ischaemic stroke among at-risk population group in the morning (191). Otherwise, the lack of diurnal variation may reflect the combination of small sample size studied and fairly high median CV (up to 24%) for thromboelastography.

Thirdly, the reduction in R-time and K-time, together with increased in Angle and Maximum Amplitude as assessed by TEG post-exercise, suggested that post-exercise is associated with transient but significant increase in coagulation potential. This pro-coagulant state persists for up to 12 hours in our healthy subjects before returning to baseline. This result is in line with contemporary understanding regarding the relationship between acute, strenuous exercise and haemostatic response. Previous Norwegian studies involving 800 healthy young individual demonstrated that acute exercise is associated with reduced Activated Partial Thromboplasmin Time (aPTT) as well as increased in D-dimer levels, suggesting an

increased in coagulation and fibrinolytic activities post-exertion (192). That reinforced previous small scale study among 10 healthy adolescents, demonstrating that following exercise, platelet counts, aPTT, FVIII, vWF and tPA were significantly elevated in contrast to plasminogen activator inhibitor, which decreased significantly until 1h after exercise (193).

Fourthly, as fresh plasma and freshly-thawed frozen plasma demonstrate no significant difference in indices assessed by turbidimetric and fibrinolysis assay. It is possible to use frozen plasma instead of fresh plasma for batch analysis. This is further supported by subsequent validation exercise demonstrating no difference amongst those indices between transiently frozen plasma and short-term frozen plasma. At the writing of the work for this thesis, there is yet to be any published work in this specific field.

Finally, the validation results also demonstrated adverse effect of time on indices as assessed by TEG plus turbidimetric and fibrinolysis assays. This is also in line with previous reports by Zambroni et al(194) and Wasowicz et al(195), suggesting the stability of fresh and citrated blood for up to 2 hours before processing.

A summary of the chosen 10 indices for TEG, turbidimetric and fibrinolysis assay is summarized and described in **Table 4.8** .

Table 4.1: Intra- and inter- assay CV's of turbidimetric and fibrinolysis assay indices using fresh plasma or frozen plasma

Index (unit)	Fresh plasma Intra-assay	Fresh plasma Inter-assay	Frozen plasma Intra-assay	Frozen plasma Inter-assay
Turbidimetric				
Lag time (sec)	2.7	10.3	2.4	6.1
Rate of clot formation (OD unit/sec)	9.0	7.7	5.1	6.5
Maximum clot density (OD unit)	2.6	4.9	1.3	2.2
Fibrinolysis				
Rate of clot dissolution (OD unit/sec)	12.0	10.8	3.5	5.3
T50% (sec)	3.6	9.9	2.8	5.1
Median (IQR)	3.6 (2.6-10.5)	9.9 (6.2-10.5)	2.8 (1.8-4.3)	5.3 (3.6-6.3)

Data are %. CV = coefficient of variation, OD = optical density
IQR = inter-quartile range

Table 4.2: TEG intra- and inter-assay CVs

Index (unit)	Whole blood		Fresh plasma		Frozen plasma	
	Intra - Inter		Intra - Inter		Intra - Inter	
R (min)	21.8	16.4	19.3	24.5	24.6	29.5
K (min)	18.4	24.0	23.0	23.7	31.0	26.5
Angle (degrees)	10.4	14.3	9.4	10.0	12.4	11.4
MA (mm)	5.5	7.8	12.2	8.6	17.2	17.0
LY60 (%)	102.5	637.9	73.8	94.3	*	*
Median (IQR)	18.4 (8.0-62.2)	16.4 (11.1-330.1)	19.3 (10.8-48.4)	23.7 (9.3-59.4)	20.9 (13.6-29.4)	21.7 (12.8-28.7)

Data are %. *No reliable data obtained
MA = maximum amplitude, IQR = interquartile range
CV = coefficient of variation

Table 4.3: Thromboelastography (TEG) indices

Index	Function in haemostasis
SP-time	Split point time. The time from insertion of sample into TEG until initial clot formation.
R-time	The time from when the sample is put on the TEG until the first sign of clot formation (amplitude of 2 mm) is reached.
K- time	The time from the R or beginning of clot formation to a fixed level of clot firmness (amplitude of 20 mm) is reached.
Angle (α)	The rate of clot growth.
MA (Maximum Amplitude)	Maximum strength or stiffness (maximum shear modulus) of the developed clot. MA measures the strength or elasticity of the clot is measured in mm.
Time to MA	Time to MA, a global measurement of the dynamics of clot kinetics or can be described as the time needed to form a stable clot.
G parameter	Actual measure of clot strength, in terms of shear elastic modulus strength, and a derivation of A. It is measured in $Kdyn/cm^2$.
E parameter	A normalized G parameter: referred to as elasticity constant.
Thrombodynamic Potential Index (TPI)	Describes the patient's global coagulation whether the patient has normal haemostasis (TPI between 6 - 15), is hypocoagulable (TPI < 6), or is hypercoagulable (TPI >15).
Estimated Percent Lysis	The estimated percent lysis at 30 minutes after MA.
A30	Amplitude of the TEG tracing at 30 minutes.
CL30	Presents the values of A30 relative to MA. CL30 = 100 x (A30 **/** MA)
LY30	Measures percent lysis at 30 minutes after MA is reached.
A60	Amplitudes of the TEG tracing at 60 minutes.
CL60	Presents the values of A60 relative to MA. CL60 = 100 x (A60 **/** MA)
LY60	Measures percent lysis at 60 minutes after MA is reached.
Clot Lysis Time (CLT)	The elapsed time between MA and 2 mm amplitude or less post MA.
A parameter	Measures the width of the tracing at the latest time point. Amplitude (A) is a function of clot strength or elasticity and is measured in mm.
Coagulation Index (CI)	Describes the patient's overall coagulation is the coagulation index derived from the R, K, MA and Angle (α) of native or kaolin-activated blood samples.
Projected MA(PMA)	Estimator of MA, that is, whether the MA value will achieve at least the lower limit of the normal value for samples treated with kaolin.
Lysis Time Estimate (LTE)	An estimate of CLT. LTE is derived by calculating the slope of the tracing and extrapolating to an amplitude of 2 mm.

Table 4.4 Diurnal Variation by TEG

	Baseline (0600hr)	1200hr	1800hr	2400hr
R-time (min)	9.03	9.01	8.81	9.79
K-time (min)	3.09	2.87	3.04	2.78
Angle (degree)	52.3	50.7	52.3	52.7
MA (mm)	54.7	54.6	53.6	57.4
LY60 (%)	8.57	8.11	7.64	6.57

* no statistical difference in the 5 TEG indices described

Table 4.5: Exercise and coagulation indices by TEG

	Baseline	Peak Exercise	2 hours Post Exercise	4 hours Post Exercise	12 hours Post Exercise	24 hours Post Exercise
R-time (min)	8.28	8.42	6.26*	7.41*	8.56	8.31
K-time (min)	2.51	2.70	1.54*†	1.83*	2.54	2.75
Angle (degree)	55.0	53.7	67.1*	57.4	56.4	55.6
MA (mm)	54.3	56.2	67.8*	64.7*	52.6	54.1
LY60 (%)	7.62	5.55*	3.64*†	5.44*	5.22*	6.94

* $p<0.05$ compared to baseline, † $p<0.01$ compared to baseline

Table 4.6: Effect of time on indices assessed by turbidimetric and fibrinolysis assay

	Turbidimetric assay			Fibrinolysis assay	
Time point (hours)	**LT (secs)**	**RCF (OD/sec)**	**MCD (OD)**	**RCD (OD/sec)**	**T50 (secs)**
T = 0	540 (92)	11.4 (3.1)	0.59 (0.1)	2.7 (0.9)	132 (19)
T + 3	535 (93)	10.3 (2.4)	0.59 (0.1)	2.6 (0.8)	143 (24)
T + 6	535 (104)	9.5 (2.0)	0.61 (0.1)	2.6 (0.9)	143 (21)
T + 12	449 (83)	7.5 (1.0)	0.62 (0.12)	2.4 (0.8)	173 (17)
T +24	378 (26)*	6.3 (1.2)*	0.67 (0.12)	1.5 (0.6)	207 (22)*
P for linear trend	0.0021	0.0002	0.703	0.0332	0.0048

Data are mean (standard deviation). LT = lag time, MCD = maximum clot density, RCD = rate of clot dissolution, RCF = rate of clot formation, T50 = time for 50% of the clot to be lysed, OD = optical density.

*$p<0.001$ compared to baseline.

Table 4.7: Effect of time on indices assessed by TEG

	Thromboelastography (TEG)				
Time point (hours)	**R (minutes)**	**K (minutes)**	**Angle (degree)**	**MA (mm)**	**LY 30 (%)**
T = 0	12.9 (4.6)	3.4 (1.5)	45.3 (9.9)	59.7 (7.3)	2.32 (1.9)
T + 3	12.0 (3.3)	2.7 (0.8)	55.8 (9.0)*	59.7 (7.2)	2.32 (2.5)
T + 6	10.4 (2.4)	3.2 (1.0)	51.3 (8.8)	54.2 (11.5)	3.00 (1.1)
T + 12	10.0 (2.00)	3.2 (1.2)	48.6 (17.7)	56.8 (10.2)	2.98 (1.7)
T +24	9.5 (2.2)*	2.7 (1.1)	55.4 (10.9)*	59.1 (6.1)	1.52 (0.5)
P for linear trend	0.0525	0.624	0.442	0.742	0.816

Data are mean (standard deviation). R = Reaction time, K = K time, LY 60 = percent lysis at 60 minutes after MA is reached, MA = Maximum amplitude.

*$p < 0.05$ compared to baseline.

Table 4.8: Summary of chosen indices

	Process assessing
TEG indices	
R - time (minutes)	The time from when the sample is put on the TEG until the first sign of clot formation (amplitude of 2 mm) is reached.
K - time (minutes)	The time from the R time or beginning of clot formation to a fixed level of clot firmness (amplitude of 20 mm) is reached.
Angle (degrees)	Angle formed by the slope of a tangent line traced from the R time to the K time: reflects the rate at which the clot forms.
MA (mm)	Maximum amplitude of the clot dynamics, reflecting fibroelastic clot strength
LY60 (%)	Percentage of the clot that has lysed 60 minutes after the maximum amplitude achieved.
Turbidimetric and Fibrinolysis indices	
L time (minutes)	Lag time from the initiation of the test to the start of clot formation
RCF (OD/second)	Rate of clot formation: change in optical density over time from the beginning of clot formation to maximum optical density
MOD (units)	Maximum optical density, reflecting clot thickness
RCD (OD/second)	Rate of clot dissolution: reduction in optical density from maximum to the plateau phase.
T50 (minutes)	Time for 50% of the clot to lyse.

Section 5: Clinical Studies

5.1 Clot structure: Atrial fibrillation versus coronary artery disease - aspirin user (anticoagulation naïve)

Abstract

Atrial fibrillation and coronary artery disease are prothrombotic states, but they possess different fibrin clot profiles and lead to different clinical risks. 50 patients with atrial fibrillation and 50 patients with coronary artery disease were recruited, all of whom were taking aspirin. To assess the different fibrin clot profile and clot strength, whole blood and plasma samples were obtained and analysed by thromboelastography, turbidimetric and fibrinolysis analysis. Using thromboelastography no statistical significant difference were detected. However, using turbidimetric and fibrinolysis assay, atrial fibrillation confers greater rate of fibrin clot formation, fibrin clot formed of greater optical density, more resistant to fibrinolysis and requires longer time for 50% clot lysis (all $p < 0.005$). Despite exposure to aspirin, atrial fibrillation confers formation of fibrin clot of greater thrombotic potential which are also more resistant to fibrinolysis, compared to those from coronary artery disease.

Introduction

Patients with atrial fibrillation (AF) and coronary artery disease (CAD) are known to have increased thrombotic risk. Both conditions share similar risk factors, such as hypertension, increasing age and diabetes; both conditions also exhibited alterations in clot profile which increased the associated risk of thrombotic phenomena (be it stroke or myocardial dysfunction) (144, 145, 196-198).

In CAD, positive changes to fibrin clot structure (increased fibrin clot permeability and clot lysis) have been associated with the use of prognostically beneficial medications (such as ACE-inhibitor, fibrates and statins)(199, 200), while adverse fibrin clot structure with increased fibrin network density and reduced fibrinolysis have been observed among those patients with failure of antiplatelet treatment(201). While for AF, regardless of the types of atrial fibrillation (permanent, persistent or paroxysmal AF), fibrin clots are denser and more resistant to fibrinolysis as compared to control (202, 203), with subsequent modulation of fibrin clot structure shown as early as day 3 after exposure to anticoagulation treatment (202). However, despite both conditions sharing similar comorbidities and being closely related to each other (204-207), they exhibit different thrombotic and thromboembolic profiles. Hence, to better understand thrombotic potential of AF and CAD, there is a need to investigate the fibrin clot structure, in the absence of oral anticoagulant.

I hypothesized that the differences in fibrin clot structure in AF and CAD can explain the variation in thrombotic risk, with patients with AF exhibiting fibrin clot which has higher clot density and lower tendency to undergo fibrinolysis. These changes can be detected by TEG, turbidimetric and fibrinolysis assay.

Subjects and Methods

Over the course of 18 months (January 2014 – July 2015), 50 AF patients taking only aspirin as antithrombotic treatment (oral anticoagulant naïve), and 50 CAD patients (taking aspirin only) were recruited from the cardiology clinics at the City Hospital, Birmingham.

Definition of AF and CAD has been described previously in chapter 3.

Exclusion criteria were age <18 years, diagnosis of valvular AF (severe rheumatic stenosis, metallic prosthetic valve, mitral/tricuspid ring repair), active or recent (<12 months) malignancy, active immunological disease, chronic liver disease, recent or chronic infections, chronic inflammatory disease, connective tissue disease, recent stroke/acute coronary syndrome (within two months), active bleeding, recent arterial/venous thrombosis or recent surgery, known haemophilia or thrombophilia (such as Factor V Leiden, Protein C/S/anti-thrombin deficiency, anti-phospholipid syndrome), use of anticoagulant (including vitamin K antagonist, non-vitamin K antagonist oral anticoagulant, low molecular weight heparin or heparin)

Laboratory Methods

Citrated venous blood was collected by venepuncture and analysed for indices of thrombogenesis and fibrinolysis within 2 hours of collection. The TEG was used according to the manufacturer's instructions. Thrombus formation and autolysis were monitored for up to 60 minutes after the addition of calcium to whole blood supplemented with kaolin. The TEG delivers numerous indices of haemostasis. Five that pertain to the initiation of

thrombosis were selected, the rate of thrombus formation, the physical strength of the clot once formed, and the rate of autolysis (**Table 4.8**).

For the fibrinolysis and turbidity assay, citrated plasma was obtained from venous blood by centrifugation at 2500 rpm for 15 minutes. The turbidimetric and fibrinolysis assay, which was conducted at 37°C throughout, consists of two parts (208). Firstly, in the turbidimetric assay, 25µl plasma, 75µl TRIS-NaCl buffer, and 50µl thrombin were added to the wells of 96-well micro-titre plate in triplicate. Fibrin clot formation was followed for 30 minutes in a micro-titre plate reader by changes in optical density. Secondly, the fibrinolysis assay required 75µL of plasma and 75µL of a Tris/NaCl/calcium buffer supplemented with thrombin and tissue plasminogen activator (tPA). The plates were immediately loaded into a plate reader and data collected for 30 minutes. The data from turbidimetric and fibrinolysis assays were post-processed by plotting into line charts, and from these, five indices were obtained, three pertaining to the clot formation and density, whilst two refer to fibrinolysis (**Table 4.8**). Further details of the relationship between the TEG, turbidimetric and fibrinolysis assays, have previously been published(208).

Power calculations and statistical analyses

For a power of 80% (1 – β) and a 5% level of significance (α), based on the current data available, n = 50 provides a power for difference of 0.5 of a standard deviation between the two groups.

Following a test for statistical normality, data were expressed as a mean (± standard deviation, SD) or median (inter quartile range, IQR), as appropriate. Student t test was used for continuous data when normally distributed or Mann-Whitney U test in the case of non-

normal distribution (such as Creatinine, K-time and LY60). Chi-square test was used to assess intergroup differences in categorical variables. A P- value <0.05 was considered statistically significant.

Results

Baseline characteristics

AF patients did not differ from CAD patients with regards to age, gender or other basic demographics. When comparing concurrent comorbidity, it is unsurprising to note that CAD group has a higher proportion of formal diagnosis of ischaemic heart disease (100% vs 34%, p <0.001) and Type 2 diabetes mellitus (42% vs 16%, p < 0.001) as compared to the AF group. Other comorbidities including hypertension, heart failure, chronic obstructive pulmonary disease and cigarette use were no different between the two groups (**Table 5.1.1**). There was no difference between statin, ACE-i/ARB or beta-clocker use between two groups.

Laboratory indices

However, as shown in **Table 5.1.2**, when assessing the haemostasis and fibrin clot characteristics with TEG, turbidimetric and fibrinolysis analysis, several differences between AF and CAD subjects can be seen. Although no difference in TEG indices(R-time through to LY 60%) and no difference in lag-time have been demonstrated between both groups, AF patients have faster rate of clot formation (39.7 OD/sec vs 30.5 OD/sec, p = 0.004), formation of fibrin clot with greater maximum OD (0.49 OD vs 0.38 OD, p <0.001), indicating faster protofibril formation and thicker fibrin fibre formation, respectively. Slower rate of

clot dissolution (37.8 OD/sec vs 44.5 OD/sec, p = 0.002) and longer time for 50% lysis of fibrin clot (266.4 sec vs 215.6 sec, p <0.001) was also observed in AF group as compared to CAD group, suggestive increased resistance to fibrinolysis.

Discussion

The current study demonstrated that in patients with AF naïve to oral anticoagulant, fibrin clot structures are markedly different compared to those patients with CAD. Relating to AF and fibrin clot structure, it is demonstrated that AF patients who are anticoagulant-naïve (treated with aspirin only), possesses similar R-time and Lag Time as those CAD control. That is due to the lack of oral anticoagulation treatment to impede coagulation pathways. More importantly, greater RCF and increased Maximum OD, are also accompanied with the reduced RCD and prolongation of T50%, all these indices suggest that AF patient without oral anticoagulant are at greater prothrombotic potential, and result in greater rate of protofibril polymerization and subsequent formation of a “coarser” fibrin clot, which is also more resistant to subsequent fibrinolysis by exogenous tPA.

Though there was no prior published work investigating fibrin clot structure in AF as compared to CAD group, there are several published studies involving patients with thrombotic (such as those with antiphospholipid syndrome, residual vein thrombosis and premature peripheral vascular diseases (141, 209, 210)) or prothrombotic phenomena (such as Type 2 Diabetes and arterial hypertension (211, 212)) demonstrating increasing prothrombotic fibrin clot phenotype and delay in clot lysis. Thus it can be deduced that from the results obtained, a prothrombotic and hypofibrinolytic state exist among anticoagulant-naïve AF patients, as compared to CAD control. This greater propensity for thrombosis in AF

persists despite having fewer concurrent diagnosed diabetes (16% vs 42%) as compared to those with CAD.

The inability for whole blood TEG to detect any significant differences in thrombotic properties may be because the resultant whole blood clots formed from both ailments are invariably different, with clot formed from AF patients are more fibrin rich ("red clot") and CAD being more platelet rich ("white clot") (29). Thus any increased in thrombotic propensity in plasma from AF may have been counterbalanced with an increased in platelet aggregation and activation related to CAD. Moreover, it has been demonstrated that even though there is increased in platelet activation in AF, the increase may not be significantly more that than seen with the associated vascular disease or risk factors (213). Moreover, the relatively high median CV associated with TEG may make it unable to detect any small changes in prothrombotic state.

This study presents several limitations. Firstly, the size of the study groups is small, however, the number of subjects was sufficient to detect differences between AF and CAD groups given the results of power calculation.

Secondly, all laboratory assessment of fibrin clot structure is done at a single time point, and thus there is a likelihood that fibrin clot features may change with time.

Thirdly, this study did not differentiate types of AF (paroxysmal, persistent or permanent) as the duration of sustained atrial fibrillation or potential likelihood of transient sinus rhythm might have an impact of the fibrin clot structure. However, even though there are increased haemostatic factors conferring prothrombotic state in AF as compared to control , there is no convincing data to suggest increased prothrombotic/pro-inflammatory factor level (such

as von Willebrand Factor, vWF) in persistent or permanent AF compared to paroxysmal AF (182, 214-216). Crucially, Drabit L et al. have recently demonstrated despite paroxysmal or persistent AF subjects remaining in sinus rhythm, their fibrin clot still revealed increased fibrin clot density and reduced clot lysis time (203). Hence these data suggests that despite not differentiating the types of AF, the findings from current study comparing AF and CAD remains novel and credible.

Fourthly, there is no measurement of associated endothelial function (such as vWF) or thrombin levels, as vWF is known to key component for haemostasis in vivo by affecting Factor VIII plasma level (217, 218) while increased thrombin level has been associated with increased clot lysis time in venous thrombosis(219). Moreover, elevation of vWF has been detected in peripheral blood and within left atrial cavity of persistent AF and paroxysmal AF individuals (220, 221). Thus further studies, longitudinal in design, involving assessment of plasma vWF and thrombin levels will be helpful in firmly establishing the causal relationship between AF and altered fibrin clot structure.

In conclusion, the current study demonstrates that individuals with AF have greater tendency to form fibrin clots which are thicker and more resistant to lysis as compared to those with CAD, thus potentially explain the increased ischaemic stroke and thromboembolic risk associated with AF.

Table 5.1.1: Demographic data for patients with AF (aspirin) and CAD patients

	AF (aspirin)	CAD	*p* - value
N	50	50	
Age, years	74 (13.2)	70 (10.5)	0.126
Gender	74% male	58% male	0.121
Weight, kg	74.5 (17.4)	80.3 (18.6)	0.115
Creatinine, umol/L	88 (75.5 – 111)	87 (72 – 99)	0.300
Creatinine Clearance (Cockroft-Gault), mL/min	68.1 (37.4)	79.1 (29.9)	0.106
Coronary artery disease	34%	100%	**<0.001**
Type 2 Diabetes	16%	42%	**<0.001**
Hypertension	83.7%	90.6%	0.318
Heart Failure	20.4%	14.0%	0.415
Chronic obstructive pulmonary disease	10.2%	14.0%	0.580
Current Smoker	6.12%	13.9%	0.207
Medication			
ACE-i/ARB	64%	72%	0.391
Statin	82%	90%	0.246
Beta-blocker	64%	60%	0.680

Key:
Normal distribution – mean (SD), t-test
Non-normal distribution – median (IQR), Mann-Whitney
Categorical data – Chi Square test

Angiotensin converting enzyme inhibitor, ACE-I; Angiotensin receptor blocker, ARB

Table 5.1.2: AF (aspirin) vs CAD – indices by TEG, turbidity and fibrinolysis

	AF (aspirin)	CAD	*p* - value
R-time (min)	4.96 (1.51)	5.26 (1.30)	0.32
K-time (min)	1.35 (1.0 – 1.8)	1.5 (1.2-1.8)	0.25
Angle (degrees)	69.1 (7.2)	67.1 (6.3)	0.18
MA (mm)	67.9 (6.3)	67.9 (5.2)	1.00
LY 60 (%)	3.6 (2.3 – 5.2)	3.5 (1.4 – 4.6)	0.25
Lag Time (sec)	332 (55.8)	313 (65.5)	0.17
RCF (OD/s)	39.7 (12.1)	30.5 (15.0)	**0.004**
Max OD	0.49 (0.11)	0.38 (0.08)	**<0.001**
RCD (OD/s)	37.8 (10.9)	44.5 (14.6)	**0.0023**
Time 50% Lysis (sec)	266.4 (44.5)	215.6 (36.7)	**<0.001**

Key:
Normal distribution – mean (SD), t-test
Non-normal distribution – median (IQR), Mann-Whitney
LY60, percentage of clot lyse 60 minutes after maximum amplitude attained; MA, maximum amplitude; RCD, rate of clot dissolution; RCF, rate of clot formation.

5.2 Clot structure in Patients with Atrial fibrillation and renal dysfunction

Abstract

Atrial fibrillation confers increased thromboembolic risk, which is further increased with concurrent diagnosis of chronic kidney disease. However, the use of warfarin against thromboembolism in this patient group does not provide complete protection against ischaemic stroke and thromboembolism while resulting in increased risk of haemorrhagic sequelae. Thus potential changes to fibrin clot structure and clot strength may be related to worsening renal function.

200 patients with concurrent atrial fibrillation (anticoagulated with warfarin) and renal dysfunction were recruited. Fibrin clot structure and strength were assessed by thromboelastography, turbidimetric and fibrinolysis assay, while samples of fibrin clots were analysed by scanning electron microscopy.

Increased fibrin clot thrombotic potential and clot strength, with paradoxical increased fibrinolysis, relating to worsening renal function were demonstrated. Creatinine clearance (by Cockcroft-Gault equation) was strongly correlated with laboratory indices obtained, with subsequent regression analysis confirming creatinine clearance as a modest but independent predictor of changes in these laboratory indices. Representative fibrin clot visualized by scanning electron microscopy confirmed increased density and increased fibre thickness among those with worst renal function as compared to those with mildest renal dysfunction.

Clinically, 2-years follow-up demonstrated increased mortality among those with worst renal dysfunction, with creatinine clearance and changes to fibrin clot structure (K-time) as independent predictors of mortality.

Introduction

With the improving longevity and a subsequent increasingly elderly population, the frequency of atrial fibrillation (AF) and chronic kidney disease (CKD) are increasing, and are becoming recognized as important causes of mortality and morbidity. Furthermore, AF and CKD are inter-related, in that deteriorating renal function is associated with a greater than three-fold increased risk of developing AF, and the diagnosis of new AF in patients with CKD heralds the rapid deterioration of renal function (8, 18). AF *per se* confers a prothrombotic state and results in ischaemic stroke and thromboembolic events, and this increased thromboembolic risk is further exacerbated by worsening renal function and also amongst those who are dialysis dependent, and can also result in adverse clinical outcome(55-58).

In health, a delicate balance (homeostasis) exists involving optimal activation of coagulation factors, subsequent formation of fibrin, and timely fibrinolysis. Several pathophysiological mechanisms that influence haemostasis (such as endothelial dysfunction and elevated coagulation factors) are altered in those with CKD, resulting in a pro-thrombotic state (43, 44, 55). However, the relationship between CKD and the final end-product of coagulation, involving the formation and degradation of fibrin, and intrinsic fibrin clot properties remain poorly understood. In end-stage renal disease (ESRD), fibrin clots are of higher density, are less permeable, and are less susceptible to fibrinolysis as compared to healthy controls (148). Similarly, unfavorable altered fibrin clot properties have been linked to increased cardiovascular mortality in patients with ESRD (149). Whole blood clotting, as defined by thromboelastography (TEG) in patients with CKD also demonstrated increased clot stiffness and accelerated rate of clot formation (222). These data focus on ESRD or patients requiring renal replacement therapy, while the relationship between a broad spectrum of renal

function (ranging from normal to severely dysfunctional) and fibrin and whole blood clot formation, integrity and thrombolysis has not been studied.

The risk of ischemic stroke and systemic thromboembolism in AF can be reduced by the use of dose-adjusted vitamin K antagonists (VKA) such as warfarin (223, 224). However, any benefit derived from VKA use amongst AF patients with CKD must be balanced by an increased rate of bleeding, especially haemorrhagic stroke amongst those with ESRD (103, 225). Moreover, the ability to identity those whose haemostasis is out of balance will be a valuable clinical tool. Indeed, a paradox is evident by the increased propensity to thrombosis, yet bleeding risk is increased in CKD (28).

Thus, I hypothesized that deteriorating renal function has an adverse effect on clot structure, fibrin clot formation and fibrinolysis in AF patients anticoagulated with warfarin.

Patients and Methods

Subjects

Two hundred subjects with AF were recruited from routine out-patient clinics, and all had been taking the VKA warfarin for at least 12 weeks. Dose-adjustment for warfarin was managed with the aim of achieving a stable international normalised ratio (INR) between 2 and 3. INR was determined on the day of recruitment.

Exclusion criteria were age <18 years, diagnosis of valvular AF (severe rheumatic stenosis, metallic prosthetic valve, mitral/tricuspid ring repair), active or recent (<12 months) malignancy, active immunological disease, chronic liver disease, recent or chronic infections, chronic inflammatory disease, connective tissue disease, recent stroke/acute coronary syndrome (within two months), active bleeding, recent arterial/venous thrombosis or recent surgery, known haemophilia or thrombophilia (such as Factor V Leiden, Protein C/S/anti-

thrombin deficiency, anti-phospholipid syndrome), significant abnormal haemoglobin or platelet count, use of non-vitamin K antagonist oral anticoagulant. Standard clinical and demographic data were obtained.

A routine blood sample was taken for assess renal function, with subsequent calculation of creatinine clearance by Cockcroft-Gault equation (226, 227) and of estimated glomerular filtration rate (eGFR) according to the modification of diet in renal disease equation (1).

The renal function and relation to clot structure was analysed as a continuous variable and subsequently, assessed as 4 separate quartiles (CKD Group 1 to Group 4) depending on the calculated Creatinine Clearance, CKD Group 1 with the best renal clearance with CKD Group 4 with the worst.

The project was approved by the Local Research Ethics Committee and informed written consent was obtained from each subject.

Laboratory method

Citrated venous blood was collected by venepuncture and analysed for indices of thrombogenesis and fibrinolysis within 2 hours of collection. The TEG was used according to the manufacturer's instructions. Thrombus formation and autolysis were monitored for up to 60 minutes after the addition of calcium to whole blood supplemented with kaolin. The TEG delivers numerous indices of haemostasis. Five of which pertaining to the initiation of thrombosis were selected **(Table 4.8)**.

For the fibrinolysis and turbidity assay, citrated plasma was obtained from venous blood by centrifugation at 2500 rpm for 15 minutes. The turbidimetric and fibrinolysis assay, which was conducted at 37°C throughout, consists of two parts (208). Firstly, in the turbidimetric assay, 25µl plasma, 75µl TRIS-NaCl buffer, and 50µl thrombin are added to the wells of 96-

well micro-titre plate in triplicate. Fibrin clot formation is followed for 30 minutes in a micro-titre plate reader by changes in optical density. Secondly, the fibrinolysis assay calls for 75µL of plasma and 75µL of a Tris/NaCl/calcium buffer supplemented with thrombin and tissue plasminogen activator (tPA). The plates are immediately loaded into a plate reader and data collected for 30 minutes. The data from turbidimetric and fibrinolysis assays are post-processed by plotting into line charts, and from these, five indices were obtained, three pertaining to the clot formation and density, whilst two refer to fibrinolysis **(Table 4.8**).

To assess the fibrin clot structure in SEM, fibrin clot can be prepared using the following method. First, 25 µL of plasma is inserted into the cap from aliquot tubes. Small holes would have been drilled through the bottom of the cap and covered in parafilm. Samples were washed for 1 hour with 0.9% saline (w/v) and then fixed with 2% (v/v) glutaraldehyde in sodium cacodylate (10.7 g L-1, pH 7.40) for 2 hours. Before dehydration, samples were then washed further with sodium cacodylate for 1 hour. Dehydration was performed by immersing samples in successive dilutions of acetone for 15 minutes (30%, 50%, 70%, 80%, 90%, 95% and 100%). Samples were critical-point dried with CO2, and sputter coated with platinum using a Cressington 208HR high resolution sputter coater (Cressington Scientific Instruments, Watford, UK). Samples were then imaged at 5000 and 20000x magnifications using a FEI Quanta 200 FEG Scanning electron microscope (FEI, Hillsboro, USA). Note that though this technique was acquired, fibrin clot formation from plasma and SEM was completed by experienced collaborators in University of Leeds so as to reduce operator-dependent factors (such as level of technical expertise) and to ensure reliable results.

Diameter of fibres within the fibrin clot can then be determined by direct measurement using open-source software (ImageJ programme, Rasband, National Institute of Health, USA).

Average fibre diameters were measured from at least 20 random fibres in each sample using ImageJ software. A region of a micrograph is selected at random and the diameter of every fibre in that area was measured. That will ensure no bias towards, for example, thicker or well-focused fibres thus minimising any sampling effects.

The operator and analyst of SEM were blinded to source of plasma and associated background demographics to reduce operator-related selection bias. Unblinding procedure took place only during final data analysis.

Statistical Analysis

For a 1 – β power of 85% and a 5% level of significance (α), based on the known data, n = 96 provides adequate power to detect a statistically significant correlation coefficient of > 0.3 between any of three renal indices and any of the 10 haemostasis indices. In view of multiple analyses and the likelihood of multiple interactions between the laboratory methods (208), and so to ensure extra confidence, the sample size has been doubled to 200.

Statistical analysis techniques have previously been described in **Section 3**. Continuously, normally distributed, variable data are expressed as mean and standard deviation (SD), or as median and interquartile range (IQR) for non-normal distribution. Categorical data assessed by Chi-Square test. As the four quartiles of CKD derived from the same population as defined by creatinine clearance, assessment of changes in the variables across all four CKD quartiles was done by manual calculation of linear trend (L-statistics and t-value initially),

with subsequent tabulation of corresponding p-value for assessment of significance of linear trend. As the focus is assessing the changes in variable against the worsening CKD quartiles, not between each individual CKD quartiles, thus ANOVA and post-hoc Tukey's test were not utilised.

Correlations were sought using Spearman's method. Stepwise multivariate regression analysis to determine which of three renal function indices (serum creatinine, creatinine clearance, eGFR) were associated most strongly and independently with the haemostasis indices. Analyses were performed on Minitab version 17.

Results

Clinical and demographic details of the 200 AF patients are shown in **Table 5.2.1**, indices of haemostasis (as assessed on the TEG and the turbidimetric and fibrinolysis assay) in **Table 5.2.2**.

Most data from demographics and indices of haemostasis are normally distributed, besides age, INR and LY30.

Regarding TEG indices, due to the similar degree of oral anticoagulation as reflected by similar median INR, there is no difference between all 4 CKD groups for R-time. Nonetheless, with worsening renal function across the 4 quartiles, TEG revealed shortening of K-time ($p = 0.025$), steeper α Angle ($p = 0.009$), and steeper Maximum amplitude ($p = 0.024$). No statistical difference in LY30 was noted.

Regarding the indices obtained the turbidimetric analysis, it demonstrated similar degree of anticoagulation also brought about similar degree of Lag Time across all 4 CKD groups.

However, worsening renal dysfunction across 4 quartiles was also associated with increased Rate of Clot Formation ($p = 0.014$) and greater Maximum Optical Density ($p = 0.015$). Fibrinolysis assay however revealed increasing Rate of Clot Dissolution ($p = 0.016$) and reduction in Time to 50% clot lysed ($p = 0.030$) with worsening renal dysfunction.

Correlation analysis

Relationships between the three indices of renal function (serum creatinine, eGFR and creatinine clearance) the haemostasis indices are shown in **Table 5.2.3**. Three indices obtained via TEG (namely K-time, angle, maximum amplitude (MA)) and four indices obtained via turbidimetric and fibrinolysis assay (namely rate of clot formation (RCF), maximum optical density (MOD), rate of clot dissolution (RCD) and time for 50% clot lysis T50)) were moderately (mean $r>0.25$, $p<0.001$) correlated to one or more of the renal function indices.

Unsurprisingly, the eGFR correlated strongly with serum creatinine ($r= -0.821$) and creatinine clearance ($r= 0.839$), whilst creatinine clearance correlated strongly ($r= -0.685$) with serum creatinine (all $p<0.001$). INR was not related to any renal function indices.

Multiple regression analysis

Subsequently, stepwise multiple regression analysis was carried out to assess for other independent predictors for haemostasis indices (**Table 5.2.4**). Systolic blood pressure measurement, presence of diabetes mellitus, hypertension, cigarette smoking were included in the model due to previous publications relating to their potential of resulting in more adverse clot structure (201, 212, 228). Creatinine Clearance (Cockcroft Gault) was selected over eGFR and Creatinine based on above correlation analysis (**Table 5.2.3**). Age

and body weight were excluded from the model to prevent multiple collinearity (with creatinine clearance). Various haemostatic indices (namely R-time, LY30 and Lag time) which do not correlate to dependent variables were removed from multiple regression analysis.

Creatinine clearance was subsequently demonstrated to independently predict subsequent alteration of fibrin clot structure, albeit the effect is fairly modest (R^2 = 4.91% - 14.47%, p <0.001). Other selected demographic factors and comorbidities did not possess any significant impact on indices of fibrin clot structure (**Table 5.2.4**).

Scanning Electron Microscopy

SEM was utilized to assess fibrin clot structure in detail. **Figure 5.2.5** shows representative SEM images from patient plasma with variable CKD group (Top left for CKD Group 1, Bottom Right for CKD Group 4) at 5,000x magnification. Qualitatively, the fibrin clot from those with the worst renal function appears to possess higher fibrin clot density, made of thicker fibres.

Subsequent magnification to 20,000x magnification (**Figure 5.2.6**) confirms that the fibrin clot density is 6.8 (4.2 – 7.9) fibres per μm^2 in CKD group 1 compared to 11.8 (10.4 – 14.3) fibres per μm^2 in CKD group 4 (p <0.001). The fibre diameter in the images were also thicker for those with the worst renal function at 135.4 (±21.5) nm versus 99.3 (±10.5) nm in those with preserved renal function (p <0.001).

Follow-up and Clinical Outcomes

Subjects recruited were followed-up for up to 24 months, survival from recruitment was recorded (**Figure 5.2.7).** The Kaplan-Meier survival curve demonstrated early separation of CKD Group 4 (as early as 1st month of follow-up period) from the rest, indicating increased all-cause mortality risk amongst Group 4. This trend persisted throughout the follow-up

period. On the other hand, **Table 5.2.8** shows the stepwise binary regression analysis for all-cause mortality. Significant independent predictors of all-cause mortality were creatinine clearance by Cockroft-Gault equation (OR = 0.9) and K-time (OR = 1.6). Increasing age was not an independent predictor of all-cause mortality.

Discussion

The current study shows that in AF patients on warfarin, whole blood and fibrin clot properties and structure are markedly altered with progressive renal dysfunction. Fresh whole blood samples from patients with worsening renal function, displayed significantly accelerated rate of clot formation (greater K-time and angle) and increased clot stiffness (greater maximum amplitude) as assessed by TEG. Similarly, in plasma, turbidimetric and fibrinolysis indices of the rate of clot formation, the maximum density the rate of clot dissolution and the time for 50% of the clot to lyse are all influenced by renal function. The reduction of renal function and adverse clot structure is associated with increased all-cause mortality over time.

The current data corroborate well with previous findings involving thromboelastography (222, 229) and other techniques (148, 149), demonstrating the increased final clot strength in those with worst renal function. However, comparing and contrasting with Holloway (222) and Chapman (229), we are able to demonstrate the progressive alteration in properties of whole blood thrombogenesis, such that more rapid rate of whole blood clot formation (k-time and α Angle, $p = 0.025$ and 0.009, respectively) associating with worsening renal function (from CKD stage 1 to 4).

Regarding fibrin clot properties, the current data is novel and will extends findings of previous work in ESRD by Sjoland and Undas (148, 149), by demonstrating progressive deterioration of fibrin clot properties by stepwise regression of renal function. Representative SEMs of fibrin clot structure provide further documentary evidence relating to the structural changes as initially described by turbidimetric analysis in the presence of progressive renal failure.

However, some discrepancies exist and require consideration. Firstly, fibrinogen level has been demonstrated to be significantly higher in CKD (41, 43), which has been shown to lead to significantly faster fibrin monomer assembly rate and thus reduction in lag time (149). The current study, nonetheless, demonstrate no significant variation in lag time, potentially highlighting the overwhelming anticoagulation effect of VKA use in subjects with AF.

Secondly, ESRD has been demonstrated to result in formation of fibrin clot comprising of thicker fibres which are more resilient to fibrinolysis (148, 149). Our novel data confirms the greater rate of fibrin monomer lateral polymerisation (Rate of Clot Formation, $p = 0.014$) progressively thicker fibre (Maximum Optical Density, $p = 0.015$) with worsening renal function, but these are more susceptible to fibrinolysis. Notably, this analysis failed to find any effect of renal function on the INR, although this effect may be method-specific as other reported, using a different method, an association between the INR and fibrin clot permeability and fibrinolysis (202).

In relation to clinical outcomes, it is unsurprising that the group with the worst renal clearance have the highest rate of all-cause mortality over time. This has previously been demonstrated in several cohort studies (18, 58, 230, 231) . However, the results are the first to suggest the increased in rate of protofibril build-up (K-time as assessed by TEG) as an independent predictor of mortality in AF patients with CKD.

Limitation

Several limitations were noted to this study, the principal one being that the fibrin clot, by definition, is not physiological, a criticism levelled at all fibrin clot data. Although fibrin clots formed *in vitro* for permeation analysis are done in static conditions, the resultant clot structure will be profoundly different from those *in vivo* due to the presence of shear force by blood flow (232). Although the TEG assay is formed in rotating whole blood, and the micro-titre plate of the turbidimetric and fibrinolysis assay is shaken/vibrated at intervals to simulate a form of dynamics, neither can be said to reflect flowing blood.

With each quartile of worsening renal function, the median age of the subjects is progressively older, which may be associated with increased markers of inflammation (such as interleukin 6 and CRP) and co-morbidities, and these may influence the clot structure and properties (41). However, our findings reflect the real clinical world, with progressive loss of renal function in elderly patient groups.

Although the fibrinolysis assay uses exogenous tPA to promote fibrinolysis, its concentration, although standard throughout the project, does not necessarily reflect tPA levels *in vivo* and so may not be directly physiological.

Despite these limitations, the current study demonstrated deterioration in clot properties and structures over a wide range of renal function amongst anticoagulated AF patients. However, despite the broad result that worsening renal function has a deleterious effect on haemostasis, although statistically significant, these renal function indices at best account for at best 14.5% of the variability of the particular index of clot structure and function (**table 5.2.4**).

Nevertheless, our data provides insights into the phenomena of persistently increased risk of ischaemic stroke and thromboembolism amongst CKD or ESRD patients despite successful

anticoagulation, and may also help to explain the increases spontaneous haemorrhagic episodes experienced by these patients as a result of anticoagulation. It also reinforces the view that renal function is an important factor in cardiovascular disease (18, 58, 233-235). The inability of INR to relate to renal function or clot indices indicates that there may be a place for the TEG and/or the turbidimetric and fibrinolysis assay to provide an individualised risk of thrombosis and/or haemorrhage.

In conclusion, in AF patients while on warfarin, worsening renal function results in altered fibrin clot structure and clot properties. Accelerated clot formation and faster fibrin clot polymerisation despite adequate anticoagulation may explain the increased ischaemic stroke and thromboembolic risk in AF patients with CKD. The thicker fibrin clot formed is actually more sensitive to fibrinolysis in these patients also illustrate the increased risk of haemorrhage experienced amongst anticoagulated patients with renal dysfunction. Thus changes in clot structure may explain the increased mortality observed.

Table 5.2.1 Demographics and clinical details

	Overall	CKD Group 1	CKD Group 2	CKD Group 3	CKD Group 4	p – value for trend
N	200	50	50	50	50	
Features						
Age, years	73 (11.4)	65 (52 - 71)	72 (66 – 79)	78 (74 – 80)	82 (78 - 86)	***<0.001***
Gender	55% male	60% male	64% male	54% male	44% male	0.0652
Weight, kg	83.6 (22.1)	100 (27.8)	84 (11.7)	78.7 (15.8)	69.9 (17.8)	***<0.001***
INR	2.5 (0.7)	2.4 (2.1 – 3.2)	2.5 (2.1 – 2.8)	2.3 (2.0 – 2.9)	2.4 (1.9 – 2.9)	0.550
Creatinine, umol/L	107 (47)	76.5 (16.5)	87.4 (14.9)	106.9 (29.4)	159.2 (59.8)	***<0.001***
Cr Cl (CG)	71 (38)	121.3 (35.2)	76.8 (5.1)	54.7 (6.9)	30.6 (8.5)	***<0.001***
Comorbidities						
Ischaemic Heart Disease	43%	30%	42%	48%	48%	0.0546
Type 2 Diabetes Mellitus	39%	38%	32%	38%	48%	0.5822
Hypertension	83%	70%	86%	84%	86%	0.0614
Heart Failure	38%	30%	32%	42%	48%	0.0371
Chronic Obstructive Pulmonary Disease	16%	18%	8%	18%	18%	0.6622
Current Smoker	3.5%	4%	0%	2%	8%	0.2284
Medication						
Concurrent Antiplatelet	6%	8%	4%	6%	6%	0.79
Beta-blocker	76.5%	74%	80%	78%	74%	0.9406
ACE-i/ARB	72.5%	70%	74%	72%	74%	0.7234

Statin	64%	64%	64%	62%	64%	0.9476
CCB	13%	10%	14%	14%	14%	0.8508
Diuretics	34.5%	30%	34%	32%	42%	0.2581
Digoxin	22.5%	24%	22%	24%	20%	0.7050

ACE-i, Angiotensin converting enzyme inhibitor; ARB, Angiotensin receptor blocker; CCB, Calcium channel blocker; CKD, Chronic kidney disease; INR, International normalised ratio.

Table 5.2.2: AF and renal dysfunction – Indices by TEG, turbidimetric and fibrinolysis assay

	Overall Mean	CKD Group 1	CKD Group 2	CKD Group 3	CKD Group 4	p – value for trend
TEG Indices						
R-time (min)	7.244 (3.8)	7.38 (3.56)	7.78 (4.57)	6.55 (2.35)	7.27 (4.29)	0.562
K-time (min)	1.90 (0.95)	2.19 (0.54)	2.15 (1.49)	1.77 (0.74)	1.50 (0.55)	**0.025**
α Angle (angle)	63.2 (8.7)	59.0 (4.7)	60.9 (11.0)	64.7 (8.7)	68.0 (6.4)	**0.009**
Maximum Amplitude	64.5 (10.6)	60.0 (4.5)	63.4 (12.8)	66.4 (10.3)	68.3 (11.4)	**0.024**
LY 30 (%)	0.20 (0 – 0.6)	0.2 (0 – 0.7)	0.1 (0 – 0.6)	0.1 (0 – 0.5)	0.2 (0- 0.7)	1.0
Turbidimetric & Fibrinolysis Indices						
Lag Time (sec)	523 (195)	521 (220)	496 (136)	518 (180)	557 (230)	0.371
Rate of Clot Formation (OD/sec)	17.3 (9.6)	13.0 (5.6)	16.2 (10.1)	18.0 (10.0)	22.0 (9.9)	**0.014**
Max Optical Density	0.413 (0.113)	0.372 (0.093)	0.393 (0.091)	0.404 (0.121)	0.482 (0.116)	**0.015**
Rate of Clot Dissolution (OD/sec)	46.4 (18.0)	39.2 (12.7)	43.4 (18.3)	47.2 (12.9)	55.7 (12.1)	**0.016**
Time for 50% Clot Lysis (sec)	201.0 (41.5)	218.4 (46.4)	201.8 (26.5)	196.9 (47.8)	186.9 (36.4)	**0.030**

Table 5.2.3: Correlations between haemostasis indices and renal indices

Feature	Creatinine	eGFR	Creatinine clearance
TEG indices			
R-time	-0.033, 0.640	0.039, 0.582	0.095, 0.180
K-time	-0.271, <0.001	0.38, <0.001	0.452, <0.001
α Angle	0.251, <0.001	-0.414, <0.001	-0.487, <0.001
Maximum Amplitude	0.273, <0.001	-0.425, <0.001	-0.495, <0.001
LY 30	0.001, 0.99	-0.039, 0.58	-0.002, 0.975
Turbidimetric & Fibrinolysis Indices			
Lag Time	0.062, 0.385	-0.063, 0.372	-0.031, 0.662
Rate of Clot Formation	0.229, <0.001	-0.332, p<0.001	-0.338, p<0.001
Max Optical Density	0.304, <0.001	-0.332, <0.001	-0.320, <0.001
Rate of Clot Dissolution	0.361, <0.001	-0.365, <0.001	-0.342, <0.001
Time for 50% Clot Lysis	-0.258, <0.001	0.233, 0.001	0.264 <0.001

Table 5.2.4: Stepwise Multiple Regression Model

Index		Step 1 p value	Step 2 p value	Step 3 p value	R^2 (adjusted)
TEG indices					
K time	Cr Cl	<0.001	<0.001	<0.001	9.59%
	Systolic BP	0.52	0.58		
	T2DM	0.82			
Angle	Cr Cl	<0.001	<0.001		14.47%
	Systolic BP	0.141			
Maximum Amplitude	Cr Cl	<0.001			7.49%
Turbidimetric & Fibrinolysis Indices					
Rate of Clot Formation	Cr Cl	<0.001	<0.001		8.14%
	Smoking	0.091			
Max Optical Density	Cr Cl	<0.001	<0.001		9.01%
	Smoking	0.051			
Rate of Clot Dissolution	Cr Cl	<0.001	<0.001	0.001	7.75%
	Smoking	0.055	0.5		
	T2DM	0.08			
Time for 50% Clot Lysis	Cr Cl	0.001			4.91%

Blood pressure, BP; Creatinine Clearance, Cr Cl; Type 2 Diabetes Mellitus, T2DM,

Figure 5.2.5: Representative SEM of Fibrin Clots (5,000x magnification)

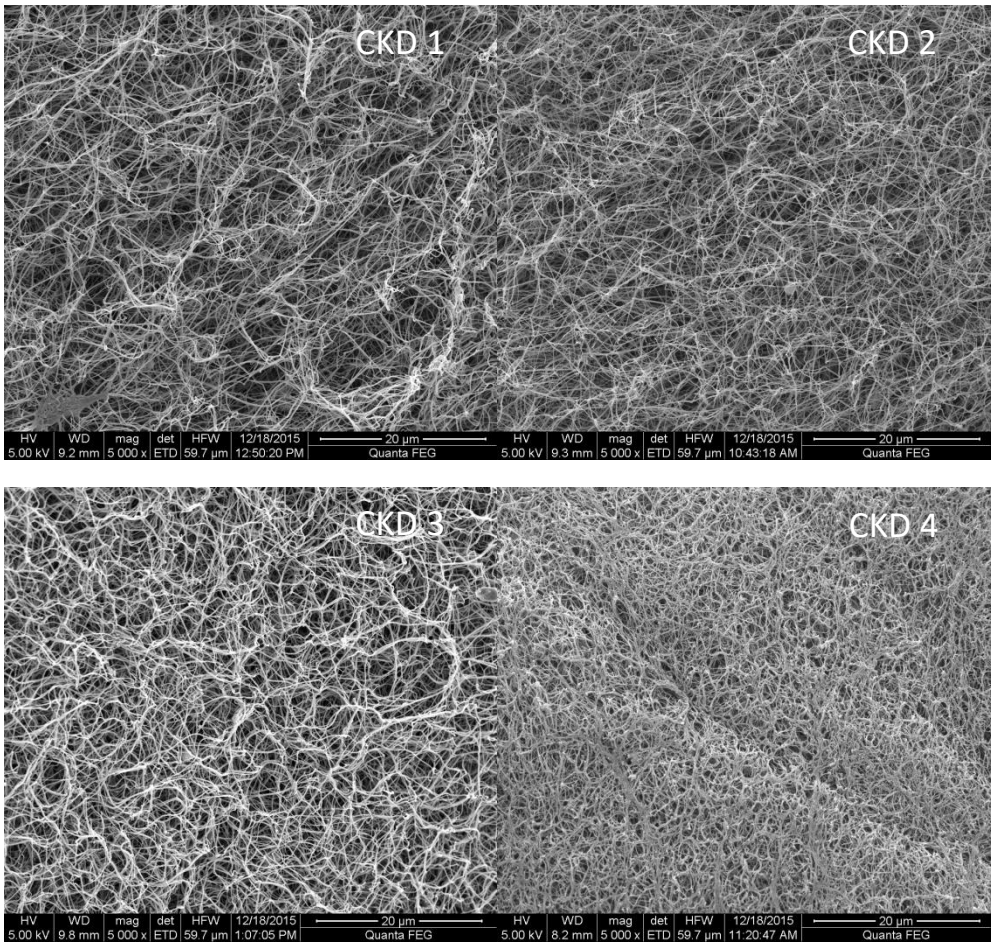


Figure 5.2.6: Representative SEM of Fibrin Clot (20,000x magnification)

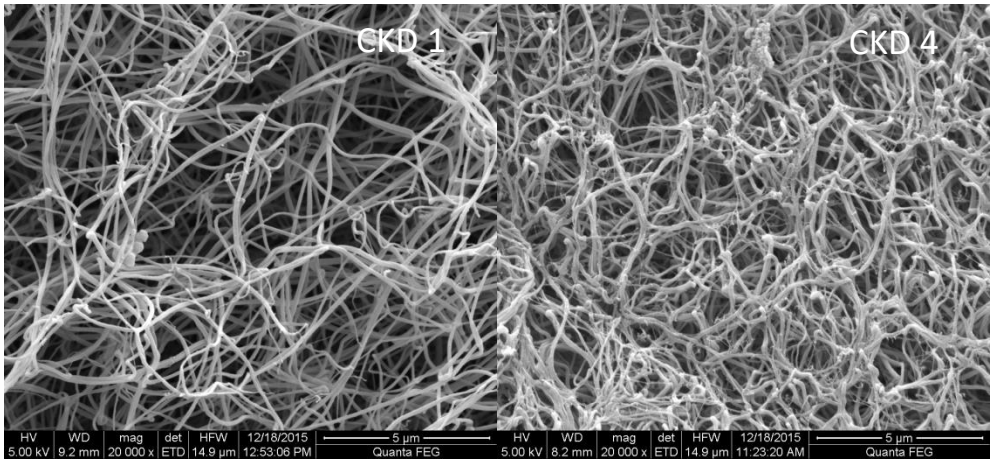


Figure 5.2.7: Kaplan-Meier Survival Curve for CKD Groups over time

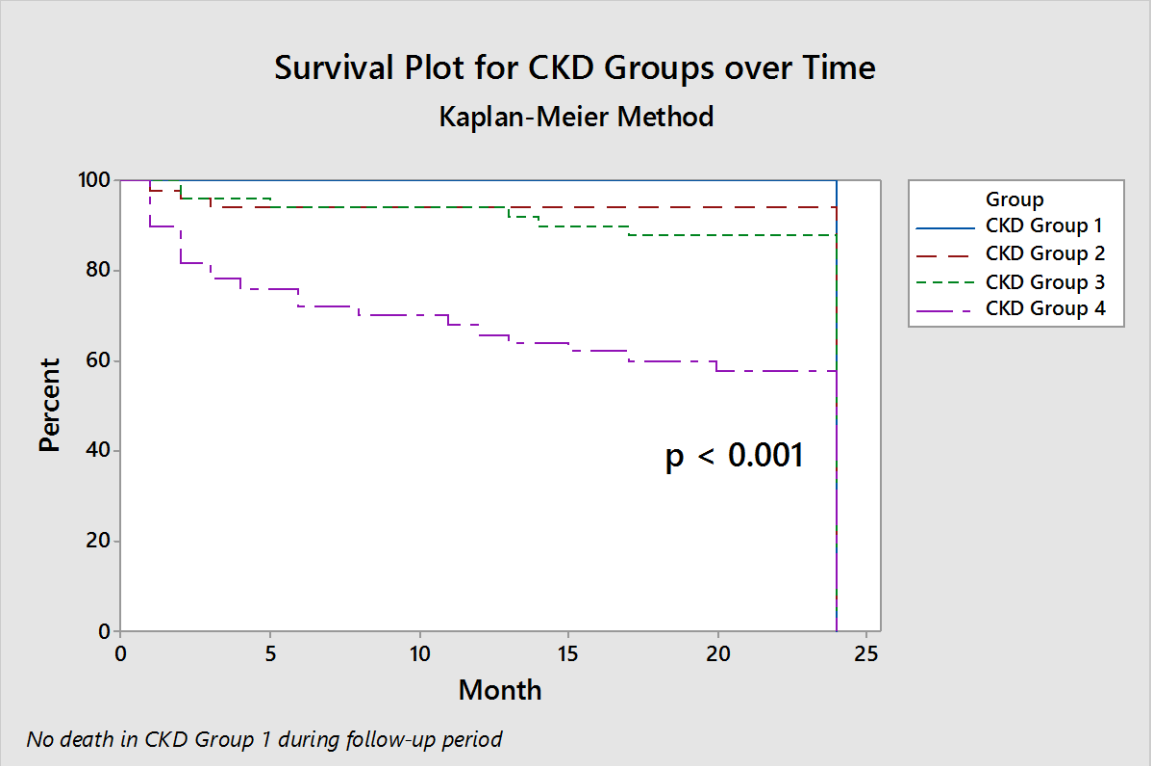


Table 5.2.8: Stepwise binary regression analysis for all-cause mortality

Parameter	Coefficient	Standard Error	p - value	Odds ratio	95% Confidence interval
Creatinine Clearance	-0.0692	0.0138	<0.001	0.9332	0.9083, 0.9587
K - time	0.4510	0.2030	0.034	1.5659	1.0533, 2.3385

5.3 AF and CKD: Microparticles, soluble P-selectin, E-selectin and von Willebrand Factor

Abstract

Atrial fibrillation and chronic kidney disease are closely related, and any associated risk of stroke and thromboembolism due to atrial fibrillation is enhanced by concurrent renal dysfunction. The relationship between levels of circulating endothelial and platelet microparticles, and soluble P selectin (reflecting platelet activation) and E-selectin (reflecting endothelial activation) with progressive renal dysfunction has yet to be investigated.

160 AF subjects with variable degrees of renal function were recruited. Blood samples were obtained, platelet and endothelial-derived microparticles were detected by flow cytometry, soluble P-selectin, E-selectin levels and von Willibrand factor were obtained by enzyme-linked immunosorbent assay.

Endothelial microparticle levels were significantly higher and demonstrated a linear trend of increase among those with progressively worse renal function (creatinine clearance) ($p = 0.03$). Endothelial microparticles were only modestly correlated with renal function (creatinine clearance) (r_s -0.28, $p < 0.001$). Platelet microparticles, P-selectin and E-selectin levels were not significantly different across various groups of renal dysfunction, and no significant correlations with renal function were evident ($p = 0.186$, $p = 0.561$, $p = 0.746$ respectively). Despite modest correlation, stepwise multivariable regression analysis demonstrated that worsening creatinine clearance was an independent predictor of endothelial microparticles levels (R^2 8.26%, $p < 0.001$).

In well-anticoagulated atrial fibrillation patients, there is relationship between endothelial function (as judged by endothelial microparticle levels) and renal function. Other markers of prothombotic state or cellular activation, such as platelet micriparticles, P-selectin, E-selectin and von Willebrand factor levels were not significantly different across the various degree of renal dysfunction.

Introduction

Non-valvular atrial fibrillation (AF) is associated with an elevated risk of ischaemic stroke and systemic thromboembolism (236, 237). This risk is further increased by concurrent diagnosis of chronic kidney disease (CKD) or end-stage renal failure (ESRD) (56, 57) and results in a worse clinical outcome (58).

The mechanism(s) underlying the worse outcomes in AF patients with ESRD requires further investigation. Dialysis-dependent ESRD can result in increased levels of circulating microparticles (238). These microparticles are heterogeneous vesicles, derived from cellular membrane where the parent cells had undergone apoptosis or activation (167, 168). Owing to the nature of their parent cells, different microparticles subsets possess unique composition and content, which vary in their hemostatic and thrombotic potentials (169-171). Thus, different microparticles subsets can modulate coagulation by directly facilitating formation of coagulation complexes or via modulation of tissue factor dependent pathways (172, 173).

Even though microparticles levels are increased in ESRD and correspond with increased cardiovascular mortality (174), different publications have reported inconsistent results involving non-valvular AF and levels of circulating microparticles (175, 176). A potential

relationship between worsening degrees of non-dialysis dependent renal dysfunction and microparticles amongst non-valvular AF patients, as well as the subsequent effect on levels of various microparticles subsets has yet to be investigated.

The selectins (P, E and L-selectins) mediate adhesion of haematopoietic cells to vascular surfaces and to each other (239, 240). P-selectin derives from α granules of platelets, as well as endothelial cells (241, 242) while E-selectin derives from endothelial cells activated by cytokines, heathy micro-vessels or as an essential component of angiogenesis (243-245). Hence, variations in P and E-selectin level may be present in relation to worsening renal function, complicated by non-valvular AF.

In this study, the hypotheses are as followed: (i) among non-valvular AF worsening class of renal dysfunction is associated with a step-wise increase in microparticles, (ii) differences in levels of platelet-derived microparticles and endothelial-derived microparticles observed can be related to worsening renal function, independent of other comorbidities; and (iii) indices of platelet and endothelial activation plus endothelial dysfunction, as measured soluble P-selectin, E-selectin levels and von Willebrand Factor may be related to worsening renal function.

Patients and Methods

Subjects

All 160 subjects with non-valvular AF were recruited from routine out-patient clinics, and all had been taking VKA (warfarin) for at least 12 weeks. Dose-adjustment for warfarin was done in specialised nurse-led anticoagulation clinic, achieving a stable international

normalised ratio (INR) between 2 and 3. INR was again determined on the day of testing to assess effective anticoagulation.

Exclusion criteria were age <18 years, diagnosis of valvular AF (severe rheumatic stenosis, metallic prosthetic valve, mitral/tricuspid ring repair), active or recent (<12 months) malignancy, active immunological disease, pregnancy, chronic liver disease, recent or chronic infections, chronic inflammatory disease, connective tissue disease, recent stroke/acute coronary syndrome (within two months), active bleeding, recent arterial/venous thrombosis or recent surgery, known haemophilia or thrombophilia (such as Factor V Leiden, Protein C/S/anti-thrombin deficiency, anti-phospholipid syndrome), use of an anti-platelet agent other than aspirin, use of NOAC, and dual anti-thrombotic therapy.

Standard clinical and demographic data were obtained.

A routine blood test was taken to assess renal function, with subsequent calculation of Creatinine Clearance by Cockcroft-Gault equation (226, 227). Subsequently, all 160 subjects were separated into 4 quartiles (CKD Group 1 to Group 4) depending on the calculated Creatinine Clearance, CKD Group 1 with the best renal clearance with CKD Group 4 with the worst. The project was approved by the Local Research Ethics Committee and informed consent was obtained from each subject.

Laboratory methods

Blood samples were collected from a large antecubital vein using a 21-gauge needle directly into Vacutainer® tubes (Becton Dickinson, UK) containing 0.5ml 3.2% sodium citrate. For microparticle detection, platelet-poor plasma (PPP) was obtained after 15 min centrifugation of citrated blood at 2,800 g and further centrifugation of PPP at 13,000 g for 2 minutes to remove residual cellular fragments to obtain platelet-free plasma (PFP). Aliquots

of the plasmas were frozen at –70°C for subsequent batch analysis and undergone a single-freeze thaw cycle.

PFP was initially incubated separately for 30 min with 0.5 µg of biotinylated anti-human CD42b antibody (Abcam, Cambridge, UK) for platelet-derived microparticles (PMP), or 0.5 µg of biotinylated anti-human CD31 antibody (Abcam, Cambridge, UK) for endothelial-derived microparticles (EMP). This was followed by a second incubation with 0.25 µg of Streptavidin-Alexa Fluor-647nm-R-Phycoerythrin conjugate (Life Technology, Paisley, UK) for 30 min and then diluted with 990 µl filtered PBS (final dilution 1:100). MP analysis was promptly performed using the Apogee A50 flow cytometer (Apogee Flow Systems). Polystyrene beads of 110, 200, 500 nm and 1 µm diameter (Apogee Flow Systems) were used to set up the MP-size gate and small-size MP defined as events with size between 110 and 500 nm. Detailed instruction regarding gating selection has previously been described (183).

For enzyme-linked immunosorbent assay (ELISA) blood samples were centrifuged within 30 min from collection at 1,500 g for 20 min at 4°C. The resultant plasma was then collected and stored at –70°C until later batch processing by ELISA to measure soluble E-selectin, soluble P-selectin and von Willebrand Factor(vWF) (R&D Systems, Minneapolis, MN, USA). (182).

Statistical Analysis

Continuously variable data are expressed as mean and standard deviation (SD) or median and interquartile range (IQR) dependent on normal or non-normal distribution.. Similar to previous **chapter 5.2**, assessment of across all four CKD quartiles was done by manual calculation of linear trend (L-statistics and t-value initially), with subsequent tabulation of

corresponding p-value for assessment of significance of linear trend. Categorical indices were analysed by the Chi-squared test. Correlations were sought using Spearman's method. Stepwise regression analyses were performed on Minitab 17, and, in view of the multiple analyses, $p \leq 0.01$ was considered as significant.

Results

Clinical and demographic details of the 160 AF patients with concurrent CKD treated with VKA are shown in **Table 5.3.1**. There were no significant differences in INR, gender, comorbidities, nicotine use or concurrent antiplatelet between all 4 quartiles of renal dysfunction. As expected, there was worsening renal function with lower creatinine clearance and higher creatinine level across the 4 CKD groups, and those with worse renal function are also associated with lower body weight and increased age. Of all the clinical demographics data, only INR is non-normally distributed, while laboratory indices are all non-normally distributed.

Overall, increasing EMP levels were evident across the 4 groups of worsening renal clearance ($p = 0.03$ for linear trend) (**Figure 5.3.1** and **5.3.2**). PMP levels, vWF, soluble P-selectin and E-selectin levels demonstrated no significant difference across the 4 groups (all p = not significant for linear trend) (**Table 5.3.2**).

There was a significant, though modest, negative correlation between creatinine clearance and EMP levels (Spearman, $r_s = 0.278$, $p < 0.001$) (**Table 5.3.3**). No significant correlations between changes in renal clearance with PMP, soluble P-selectin or E-selectin levels were evident (all p = not significant). However, there appears to be a trend towards negative correlation between creatinine clearance and vWF (Spearman, $r_s = 0.151$, $p = 0.058$).

Using stepwise regression analysis, independent predictors for EMP levels was the presence of worsening creatinine clearance ($R^2 = 8.26\%$, $p < 0.001$). Other demographic factors and comorbidities did not any significant impact on EMP levels.

Discussion

Among AF patients receiving oral anticoagulation, progressive worsening of renal function (as defined by renal clearance using the Cockcroft-Gault equation) was demonstrated to be associated with a linear trend for increasing levels of EMP. On stepwise regression analyses, renal clearance emerged as the only independent determinant of EMP levels. PMP, soluble P-selectin and E-selectin levels were not significantly associated with worsening renal function.

AF and CKD are closely linked, and progressive renal failure is implicated in an increased bleeding diathesis and thromboembolic risk despite anticoagulation. Thus, there is a strong need to identify potential markers that enables us to assess the decline of renal function and alteration(s) of thrombotic potential.

This current finding confirms some previous studies demonstrating elevated microparticle levels in those with renal dysfunction/renal failure (238, 246) and contrasts with the lack of positive findings in other studies (247, 248) which may be due to heterogeneity among study subjects or resulting from smaller population size. As EMPs are produced by endothelial cells in response to damage, the presence of greater vascular and endothelial injury associated with (or as a result of) progressive renal dysfunction will cause alteration in EMP levels. Hence, this study also extends previous work by demonstrating a progressive, step-wise, increase in EMP levels among those with worsening degrees of renal failure. Nevertheless, elevated EMP levels may be a surrogate of cellular injury due to cardiovascular diseases (249), but this can be accounted for in the study by the similar comorbidities between the 4 studied groups.

Regarding PMP, the current study demonstrates the lack of significant change in PMP levels in relation with worsening renal function among AF patients. Previous studies (175, 250) have shown that PMP levels are affected less by arrhythmia and more due to underlying cardiovascular diseases. Subsequently, PMP levels between AF-CKD subjects were compared with demographic-matched (inclusive of age and renal function), ischaemic heart disease-CKD (IHD-CKD) control who were recruited as part of the study), and this reveals significantly lower PMP levels in the AF-CKD cohort as compared to IHD-CKD control [281 (134 – 1348) events/uL vs 7177 (1292 – 15684) events/uL, p = 0.006], further reinforcing the association between ischaemic heart disease and associated risk factors and PMP levels.

The lack of alterations in soluble P-selectin and E-selectin levels across subjects with worsening renal function suggests that these biomarkers of platelet and endothelial cells activation may be less affected by other renal (dys)function. At the same juncture, the lack of significant increase in vWF (marker for endothelial dysfunction) with worsening renal function appears to run contrary to published reports (251, 252). Nonetheless, it may be due to cofounding factors such as chronic low level inflammation across all stages of CKD(49), or an over-riding elevation of vWF associated with chronic AF (215, 253, 254) may have outweigh the effect of progressive renal failure.

Limitations

The main limitation of this study is the lack of information regarding the potential roles of other microparticles besides PMP and EMP, such as those related to lymphocytes, leukocytes or monocyte/macrophage-derived subsets. Nonetheless, these subsets have not been previously related to a prothrombotic state in AF, which is the focus of this chapter.

Further studies should be performed to investigate their potential involvement in the relationship of progressive renal dysfunction and AF.

Secondly, as this study is a single time-point sampling, changes in levels of microparticles, selectin levels and vWF secondary to intervention cannot be ruled out.

In conclusion, among well-anticoagulated AF patients, there is relationship between endothelial function (as judged by EMPs) and renal function. Other markers of prothombotic state or cellular activation, such as PMP, P-selectin, E-selectin levels and von Willebrand factor were not significantly different across the various degree of renal dysfunction.

Table 5.3.1 Clinical and demographic details

	CKD Group 1st Quartile	CKD Group 2nd Quartile	CKD Group 3rd Quartile	CKD Group 4th Quartile	p – value for linear trend
N	40	40	40	40	
Age, years	65 ± 11	73 ± 8	77 ± 5	81 ± 6	***<0.001***
Gender	55% male	60% male	55% male	50% male	0.0652
Weight, kg	101 (25.2)	86.3 (10.4)	81.0 (15.6)	70.4 (17.2)	***<0.001***
INR	2.4 (2.2 – 3.2)	2.5 (2.1 – 2.8)	2.4 (2.0 – 3.0)	2.4 (2.0 – 2.9)	0.550
Creatinine	73.5 (16.5)	85.3 (13.8)	110.0 (30.6)	161.9 (62.8)	***<0.001***
CrCl (CG)	121.3 (34.7)	76.5 (4.9)	55.1 (6.7)	30.8 (8.3)	***<0.001***
Comorbidities					
Ischaemic Heart Disease	37.5%	35%	47.5%	47.5%	0.243
Type 2 Diabetes Mellitus	40%	27.5%	42.5%	55%	0.389
Hypertension	82.5%	90%	80%	85%	0.961
Heart Failure	35%	30%	40%	45%	0.770
Chronic Obstructive Pulmonary Disease	20%	5%	22.5%	20%	0.741
Concurrent Antiplatelet	10%	7.5%	5%	10%	0.948
Current Smoker	7.5%	2.5%	0%	5%	0.710

Key: CKD, chronic kidney disease; CrCl, Creatinine Clearence; INR, International Normalised Ratio

Table 5.3.2 Microparticles, P-selectin and E-selectin levels in patients with various degree of renal dysfunction

	CKD Group 1	CKD Group 2	CKD Group 3	CKD Group 4	p – value for linear trend
Platelet Microparticles (Event/uL)	281 (134 – 1348)	2123 (324 – 4079)	1476 (82 – 5041)	209 (29 – 2043)	0.897
Endothelial Microparticles (Event/uL)	5181 (737 – 12352)	6520 (4222 – 11667)	6095 (415 – 24446)	21048 (6429 – 29712)	0.034
Soluble P-selectin (ng/mL)	9.09 (7.31 – 11)	8.97 (7.58 – 10.4)	9.12 (7.19 – 11.4)	8.28 (6.18 – 10.8)	0.368
Soluble E-selectin (ng/mL)	39 (29 – 105)	39 (27 – 74)	43 (29 – 63)	39 (26 – 45)	0.930
Von Willebrand Factor (IU/dL)	117.3 (84.4 – 145.4)	126.9 (86.1 – 146.5)	121.6 (81.7 – 151.4)	135.4 (102.3– 171.7)	0.288

CKD, chronic kidney disease

Table 5.3.3 Spearman correlations between Creatinine Clearance and microparticles, P-selectin, E-selectin levels and von Willebrand Factor

	r_s	*p*
Platelet Microparticles	0.105	0.186
Endothelial Microparticles	-0.278	**<0.001**
Soluble P-selectin	0.046	0.561
Soluble E-selectin	0.026	0.746
Von Willebrand Factor	-0.151	0.057

Figure 5.3.1 Endothelial Microparticles vs CKD Group

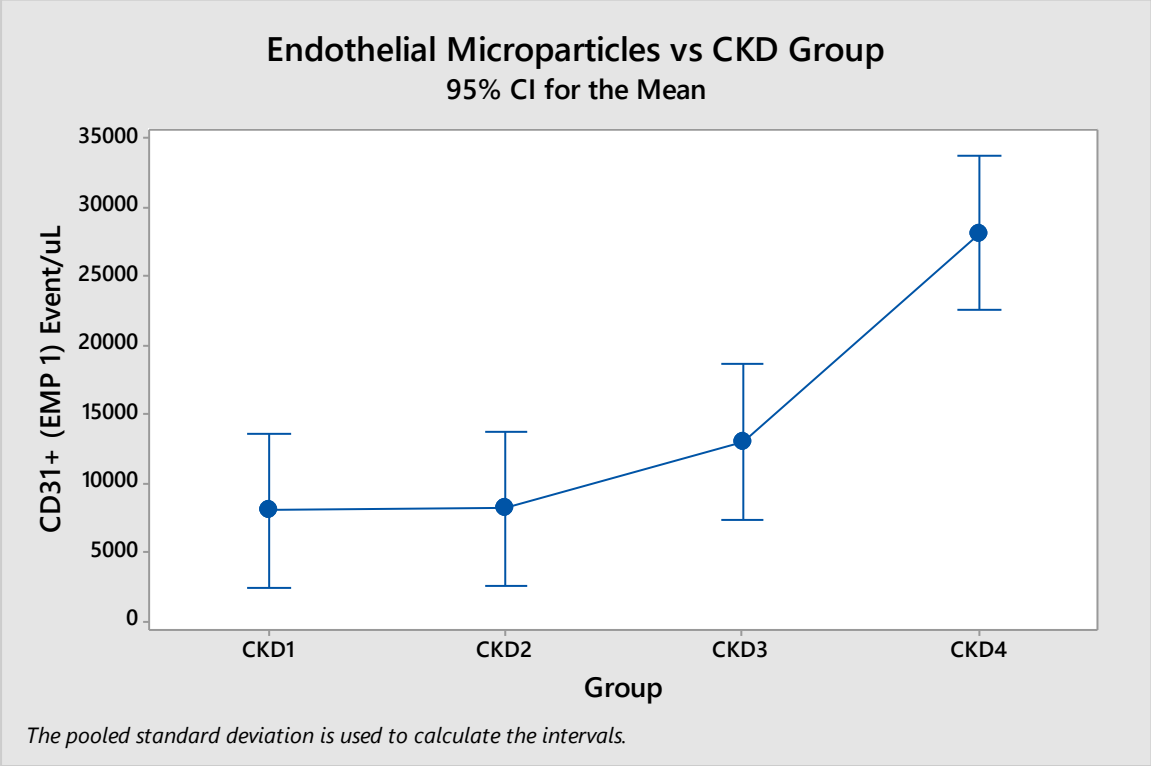
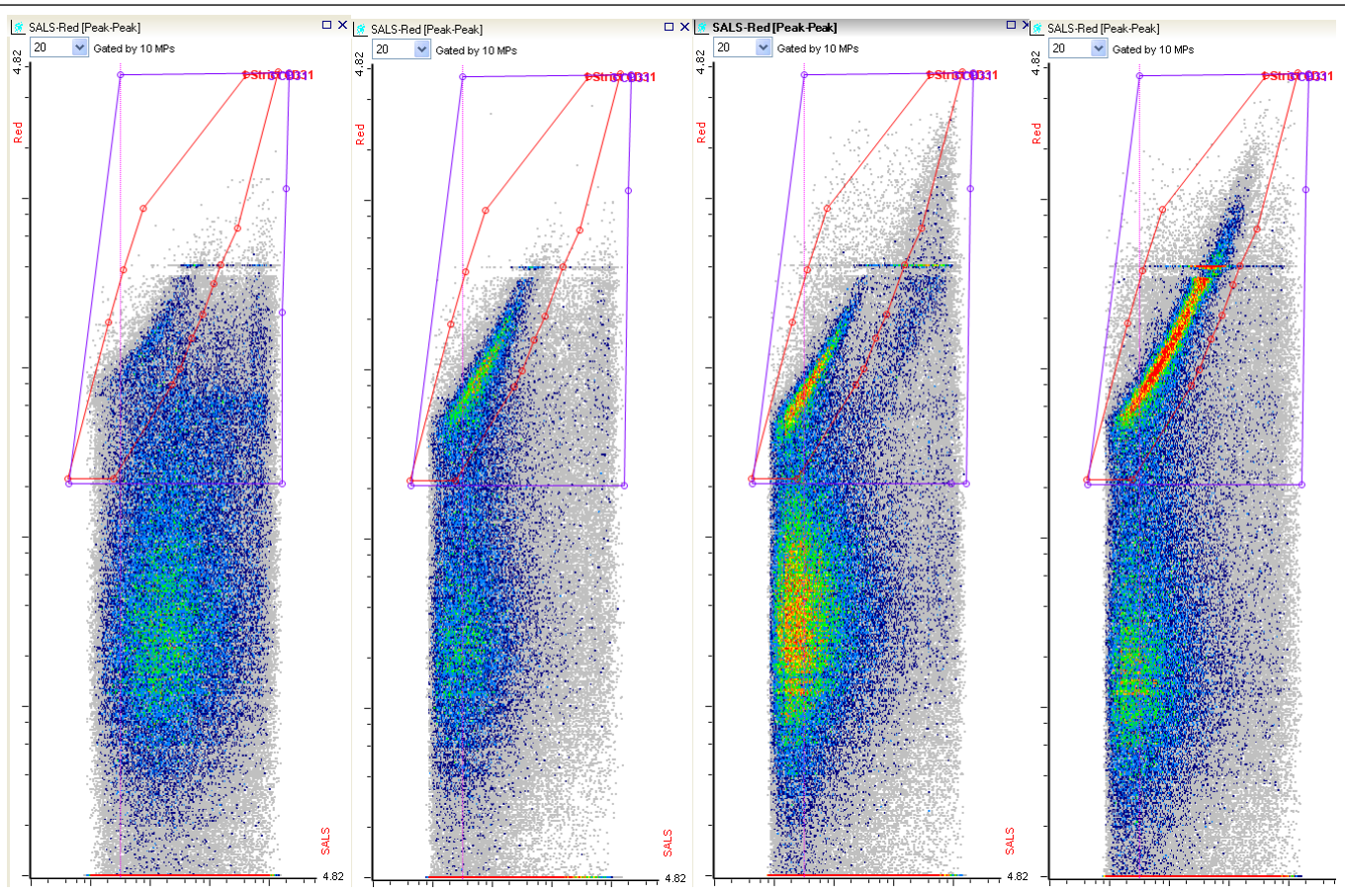


Figure 5.3.2 Excerpts of EMP Flow Cytometry Printout



CKD 1st Quartile CKD 2nd Quartile CKD 3rd Quartile CKD 4th Quartile

5.4 Clot Structure in AF: Effects of Warfarin and NOACs

Abstract

Non-Vitamin K antagonist oral anticoagulants (NOACs) have several advantages over warfarin as an oral anticoagulant for thromboprophylaxis in atrial fibrillation. However, little is known about their potential effects on fibrin clot structure and clot strength. Furthermore, effect of clot structure pre- and post-exposure to oral anticoagulant in "real patient cohort" have not been assessed.

82 patients on NOACs, 50 on warfarin and 41 on aspirin only were recruited. Their whole blood and plasma samples were analysed by thromboelastography, turbidimetric and fibrinolysis assay.

Of the 41 patients who were naïve to oral anticoagulant, 10 of whom were started on warfarin and 10 others on apixaban. The blood and plasmas samples were analysed at 4th and 12^{th} week post-exposure to oral anticoagulant.

Anticoagulation with NOACs confers slower formation of fibrin clot which are more sensitive fibrinolysis, and formation of whole blood clot which underwent greater autolysis. Whereas aspirin are generally result in greater thrombotic and lower fibrinolytic potential as compared to warfarin or NOACs. Of those receiving NOACs, differences in several laboratory indices exist between each agent used (i.e. apixaban, rivaroxaban and dabigatran).

Post-exposure to oral anticoagulant also result in formation of blood clot and fibrin clot which are more "favorable", by demonstrating features of less thrombotic potential and more sensitive to fibrinolysis.

Introduction

The introduction of non-vitamin K antagonist oral anticoagulants (NOACs) has fundamentally changed clinical practice in the use of oral anticoagulation therapy in the prevention of ischaemic stroke and thromboembolism in atrial fibrillation (AF). Unlike vitamin K antagonists (VKAs, such as warfarin), which non-specifically suppress hepatic production of functional coagulation Factors II, VII, IX and X by inhibiting vitamin K metabolism (255), NOACs target specific molecules of the coagulation cascade: dabigatran is a direct thrombin inhibitor whilst apixaban, rivaroxaban and edoxaban target Factor Xa (118-121).

NOACs have several advantages over VKAs: they possess a predictable pharmacological profile thus can be given at a fixed dose, have lower propensity for interactions with food or medication and their predictable pharmacokinetics allows for elimination of routine anticoagulation monitoring (124, 256, 257). More importantly, clinically trials of NOACs compared to warfarin, have demonstrated lower risk of intracranial haemorrhage and may result in lowering overall mortality.

Nonetheless, little is known about the potential effect of NOACs on the fibrin clot structure or fibroelastic strength of whole blood clot. There are no previous study investigating potential changes to the clot structure in AF patients before and after exposure to NOAC, or comparing it between NOACs.

Moreover, in the event of bleeding diathesis secondary to NOACs or suspected overdose, classical laboratory tests (such as the prothrombin time and the activated partial thromboplastin time) lack sensitivity and specificity to detect any effect of anticoagulation (258, 259). Other more specific assays (such as ecarin clotting time and anti-Factor Xa activity) are not routinely available, can be costly and lack standardization between

laboratories (260-263). Thus, TEG and turbidimetric-fibrinolysis assays may potentially provide a novel perspective on the actions of these agents.

Therefore, we hypothesized that the TEG, turbidimetric and fibrinolysis assays will demonstrate difference in indices of clot formation, integrity and lysis between blood and plasma from AF patients taking aspirin, warfarin and the three commonly used NOACs (namely Dabigatran, Apixaban and Rivaroxaban).

Secondly, among those who are naïve to oral anticoagulant, there will be difference in haemostasis indices post exposure to oral anticoagulant.

Patients and Methods

Subjects

Patients with AF taking oral anticoagulants were recruited from routine out-patient cardiology clinics, and all had been taking their anti-thrombotic for at least 4 weeks. Doses were according to UK guidelines, e.g. apixaban 5 mg BD, dabigatran 150 mg BD, rivaroxaban 20 mg OD while warfarin titrated to achieve an international normalised ratio (INR) between 2 and 3. For those on warfarin, INR was determined on the day of testing to assess effective anticoagulation. For those receiving NOACs, venepuncture took place 4 – 6 hours after the daily dose of the drug.

For patients with AF who are naïve to oral anticoagulants, those taking aspirin (as only antiplatelet agent) were recruited. They were prescribed oral anticoagulant based on their personal preference or clinical requirement. Venepuncture will be done on day of recruitment, and subsequently be followed up in 4 and 12 weeks post exposure to oral anticoagulant.

Exclusion criteria had previously been shown in **Chapter 3**.

Standard clinical and demographic data were obtained, and a routine blood test was taken for renal function. The project has been approved by the Local Research Ethics Committee and informed consent was obtained from each participant.

Laboratory methods

Laboratory methods involving TEG, turbidimetric and fibrinolysis assays have previously been described in **Chapter 3**.

Statistical analysis

Continuously variable data are expressed as mean and standard deviation (SD) or median and interquartile range (IQR) dependent on distribution. Differences between various NOAC agents and three main antithrombotic treatments were analysed by analysis of variance. Differences between warfarin and NOAC group were analysed by Student t test for continuous data when normally distributed or Mann-Whitney U test in the case of non-normal distribution. Chi-square test was used to assess intergroup differences in categorical variables. A P - value <0.05 was considered statistically significant. Analysis was performed on Minitab 17.

Results

VKA vs NOAC

Basic clinical demographics of warfarin and NOAC users are illustrated in **Table 5.4.1**. Overall, there was no significant demographic difference between warfarin and various NOAC users regarding age through renal function. Relating to comorbidities, only ischaemic

heart diseases demonstrate a significant difference between groups, with apixaban user having the lowest established history of coronary disease.

Nonetheless, when comparing warfarin and NOACs in accordance to investigative methods, NOACs in general bring about greater percentage of autolysis of whole-blood clot as assessed by TEG, and longer Lag-time plus shorter time for 50% lysis of fibrin clot when assessed by turbidimetric and fibrinolysis assay (**Table 5.4.2**).

When comparing whole blood and fibrin clots made from three antithrombotic treatments, aspirin treatment resulted in changes in thrombosis as assessed by TEG: shorter R-time, K-time, and steeper α-angle; and several changes to fibrin clot structure as assessed by turbidity and fibrinolysis asses: shorter Lag-time, faster rate of clot formation, greater Maximum Optical Density, and longer time for 50% lysis of fibrin clot.

Contrasting between individual NOACs (**Table 5.4.3**), only 1 of the TEG indices differed between the NOACs, with shorter R-time in those on apixaban than in those of dabigatran. However, when using turbidimetric and fibrinolysis assays, Lag-time was shorter with greater Maximum Optical Density in those on apixaban or rivaroxaban, than in those subjects partaking dabigatran. The Rate of Clot Formation was slower in those on rivaroxaban than in the two other NOACs. The Rate of Clot Dissolution was slower in those on rivaroxaban than in those on apixaban.

Post oral anticoagulant exposure

For patients who were naïve to oral anticoagulation, their risk of stroke was stratified according to theCHA_2DS_2VASc score and they werestarted on appropriate oral anticoagulant according to physician or patient choice and clinical needs.

Out of which, 20 patients completed 2 subsequent follow-ups at 4th and 12th weeks. Differences in fibrin clot structure as assessed by TEG, turbidimetric and fibrinolysis analysis pre-exposure, 4 weeks and 12 weeks post exposure to oral anticoagulants are shown in **Table 5.4.4.** With regards to TEG, post-exposure to oral anticoagulation led to a prolongation of R-time, K-time and less steep α-angle. Whereas turbidimetric and fibrinolysis assay revealed a longer lag-time, lower rate of clot formation, greater rate of clot dissolution and shorter time for 50% lysis of fibrin clot.

Of the 20 patients who have completed 12th weeks follow-up, 10 were taking warfarin and the rest apixaban. There is no statistical difference between these 2 groups regarding basic demographics and comorbidities. However, differences in the fibrin clot structure post-exposure to warfarin (classic VKA) and apixaban (NOAC) are shown in **Table 5.4.5**, with warfarin user demonstrating lower rate of clot formation and higher tendency to form clots of greater maximum optical density.

Discussion

In this study, the whole blood clot and fibrin clot structure formed from VKA and NOAC users possessed several structural differences. Using TEG, turbidity and fibrinolysis assays, some structural differences (and similarities) can be demonstrated from whole blood and fibrin clots formed from NOAC users as compared to VKA users.

Firstly, NOAC resulted in similar degree of delay in the initiation of coagulation as well-controlled VKA user, as shown by similar R-time. However, the effect on the fibrin clot per se (devoid of activated platelets in platelet poor plasma) is potentially more profound, and thus led to a prolongation of Lag-time. Moreover, the resultant whole blood and fibrin clot structure (as influenced by NOAC) is also less resistant to autolysis and ex vivo fibrinolysis by tPA, as demonstrated by changes in LY60(%) and T50. This increased susceptibility to fibrinolysis is consistent with previous findings involving dabigatran and rivaroxaban (264, 265).

Unsurprisingly, for AF patients who are not anticoagulated, and only receiving aspirin as antithrombotic agent, they exhibited increased tendency for thrombogenesis as demonstrated by shorter R-time and Lag-time (time to initiation of coagulation and fibrin clot formation), steeper angle, K-time and greater RCF (relating to fibrin clot polymerisation), thicker fibre (as reflected by greater optical density) and more resistant to fibrinolysis (longer time for 50% clot lysis).

Within NOAC groups, numerous structural changes have been detected: dabigatran confers the greatest delay to initiation of fibrin clot formation (Lag-time) and thinnest fibre (lowest maximum optical density), rivaroxaban confers greatest impact in reducing the rate of

thrombogenesis (lowest rate of clot formation), while apixaban allows for formation of fibrin clot most susceptible to fibrinolysis (highest rate of clot dissolution). These may provide some explanation for the perceived superiority of dabigatran at reducing thromboembolic events. Nonetheless, these results do not explain the reduced haemorrhagic risk associated with NOAC uses, as increased susceptibility to fibrinolysis may not only allow for revascularisation (and establishment of circulation) but also increased haemorrhagic risk.

When comparing pre and post-exposure blood and plasma results, the effect of oral anticoagulation on the whole blood and fibrin clot structure is unequivocal, as the transformation of "adverse clot structures" to one that possess less thrombotic potential, demonstrated by changes in rate of fibrin/clot built-up and increased sensitivity to fibrinolysis. These would explain the modulation of ischaemic stroke and thromboembolic risk shortly after successful anticoagulation therapy. Nonetheless, due to the small number of follow-up in both warfarin and apixaban cohorts, the lack of differences in clot structure between both groups may be a false negative error.

Limitation

Although the strength of this study on the fairly large number of patients recruited (n >130) with suitable oral anticoagulant, the lack of demonstrable differences between each NOAC agent as detected by TEG could be due to smaller cohort in apixaban and dabigatran groups. The aforementioned study (266) also utilised blood samples from healthy volunteers spiked with anticoagulant, as compared to "real-life" patients who may not be fully compliant to treatment regime, thus resulting in the lack of difference detected using TEG in the population studied.

Therefore, head-to-head comparison between NOAC agents will not be possible, and further work is warranted.

In conclusion, this study is able to demonstrate structural and mechanistic differences in whole blood and fibrin clot structure involving NOAC and OAC, as well as between each commonly prescribed NOAC agents. Changes in fibrin clot and whole blood clot structure pre and post exposure to oral anticoagulant have also been shown.

Table 5.4.1 Clinical demographics – Warfarin and NOACs

	Warfarin (n=50)	Apixaban (n=17)	Dabigatran (n=19)	Rivaroxaban (n=46)	p - value
Age (years)	73.9 (9.0)	76.9 (10.6)	73.9 (9.3)	71.6 (8.6)	0.226
Sex (male/female)	32/18	8/9	15/4	26/20	0.208
SBP (mm Hg)	130 (19)	135 (18)	126 (22)	132 (18)	0.513
DBP (mm Hg)	73 (12)	76 (15)	73 (15)	73 (11)	0.839
BMI (kg/m^2)	29.6 (4.3)	27.5 (5.4)	26.7 (4.4)	30.1 (7.3)	0.097
Creatinine (µmol/L)	87 (15)	86 (30)	90 (22)	89 (22)	0.944
Creatinine Clearance - Cockcroft Gault (ml/min/1.73)	77 (10)	74 (19)	72 (14)	82 (15)	0.580
IHD (yes/no)	21/29	3/14	6/13	8/38	**0.041**
Smoking (yes/no)	1/49	0/17	1/18	3/43	*
Diabetes (yes/no)	16/34	6/11	3/16	12/34	0.506
Hypertension (yes/no)	43/7	15/2	14/5	33/13	0.241
Heart failure (yes/no)	15/35	1/16	1/18	10/36	0.051
Valve disease (yes/no)	3/47	0/17	0/19	3/43	*
Pulmonary disease (yes/no)	4/46	3/14	1/18	5/41	0.602

Data presented as mean (standard deviation) or number of patients. P values by analysis of variance or the chi-squared test.

* = analysis unreliable.

Body Mass Index, BMI; Diastolic blood pressure, DBP; Ischaemic Heart Disease, IHD; Systolic Blood Pressure, systolic blood pressure.

Table 5.4.2 : Analysis according to anticoagulant classes (VKA vs NOAC) and antithrombotic agents (VKA vs NOAC vs Aspirin)

	NOAC (n=82)	Warfarin (n=50)	p – value (NOAC vs VKA)	Aspirin (n = 41)	P – value (NOAC vs VKA vs Aspirin)
Clinical and demographic					
Age (years)	73.5 (10.3)	71.6 (8.6)	0.234	73.2 (13.2)	0.571
Sex (male/female)	49/33	32/18	0.627	30/11	0.343
Creatinine Clearance (Cockcroft-Gault)	78 (36)	77 (5)	0.801	73 (37)	0.753
Creatinine	88 (24)	87 (15)	0.815	94 (45)	0.491
TEG Indices					
R (min)	8.6 (4.0)	7.8 (4.6)	0.291	4.9 (1.5)	**<0.001***
K (min)	1.8 (1.47-2.2)	1.8 (1.5-2.3)	0.465	1.3 (1.15 – 1.7)	**0.003***
Angle (degrees)	63.1 (8.2)	60.9 (11.0)	0.228	69.2 (6.0)	**<0.001***
MA (mm)	65.3 (7.3)	63.4 (12.8)	0.339	67.2 (5.2)	0.122
LY60 (%)	3.3 (1.8-5.2)	2.0 (0.9-3.5)	**0.005**	3.7 (2.55 – 5.4)	**0.004†**
Turbidimetric & Fibrinolysis Indices					
Lag time (min)	9.6 (7.8-13.0)	8.3 (6.8-9.5)	**<0.001**	5.3 (4.7-5.8)	**<0.001***
RCF (units/sec)	15.5 (9.6 - 26.2)	14.3 (9.5 - 21.6)	0.241	39.7 (36.3 - 44.4)	**<0.001***
MOD (units)	0.39 (0.13)	0.39 (0.09)	0.726	0.49 (0.10)	**<0.001***
RCD (units/sec)	41.4 (16.5)	43.4 (18.3)	0.194	37.7 (10.2)	0.269
T50 (min)	3.0 (0.7)	3.4 (0.4)	**<0.001**	4.4 (0.8)	**<0.001***

Data presented as mean (standard deviation) or median (interquartile range). P values analysed by the t-test, the Kruskall-Wallis test or analysis of variance.

Between group analysis by Tukey's post-hoc test:

***p<0.05 between Aspirin and two other groups**

†p<0.05 between Warfarin and two other groups

Table 5.4.3 : Analysis according to NOAC classes

	Apixaban (n=17)	Dabigatran (n=19)	Rivaroxaban (n=46)	p - value
Clinical and demographic				
Age (years)	76.9 (10.6)	73.9 (9.2)	72.1 (10.6)	0.262
Sex (male/female)	8/9	15/4	26/20	0.119
Creatinine Clearance (Cockcroft-Gault)	49.0 (41.0 – 99.5)	72.0 (53.3 – 83.4)	75.7 (54.0 – 97.1)	0.550
Creatinine (µmol/L)	86 (30)	90 (22)	88 (21)	0.879
TEG Indices				
R (min)	6.7 (1.6)	10.7 (5.9)	8.5 (3.2)	**0.009**[a]
K (min)	1.7 (0.6)	2.2 (0,9)	2.0 (0.8)	0.175
Angle (degrees)	64.4 (8.4)	60.9 (7.9)	63.5 (8.2)	0.371
MA (mm)	66.2 (6.5)	64.7 (5.8)	65.1 (8.1)	0.828
LY60 (%)	3.0 (1.4-4.2)	3.7 (2.0-6.1)	3.25 (1.8-4.9)	0.772
Turbidimetric & Fibrinolysis Indices				
Lag time (min)	8.0 (7.6-9.2)	23.0 (9.8-30.3)	9.8 (7.8-12.1)	**<0.001**[b]
RCF (units/sec)	22.0 (20.0-29.0)	28.5 (16.2-30.7)	12.4 (7.0-15.3)	**<0.001**[c]
MOD (units)	0.37 (0.09)	0.27 (0.09)	0.44 (0.13)	**<0.001**[b]
RCD (units/sec)	51.8 (13.9)	42.6 (12.1)	36.8 (17.3)	**0.005**[d]
T50 (min)	2.9 (0.45)	3.3 (0.37)	2.9 (0.75)	0.161

Data presented as mean (standard deviation) or median (interquartile range). P values by analysis of variance or the Kruskall-Wallis test.

Between group analysis by Tukey's post-hoc test:

[a] **p<0.05 between apixaban and dabigatran.**

[b] **p<0.05 between dabigatran and the two other groups.**

[c] **p<0.05 between rivaroxaban and the two other groups.**

[d] **p<0.05 between apixaban and rivaroxaban.**

Table 5.4.4: Pre and Post exposure to oral anticoagulation

	Baseline	Week 4	Week 12	p – value for trend
TEG Indices				
R (min)	5.01 (1.38)	8.96 (1.45)	7.95 (1.79)	**0.008**
K (min)	1.41 (0.36)	1.96 (0.86)	2.56 (1.03)	**0.023**
Angle (degrees)	67.9 (6.9)	64.9 (10.7)	59.0 (11.8)	**0.024**
MA (mm)	67.0 (5.5)	66.3 (8.7)	60.9 (13.8)	0.128
LY60 (%)	4.0 (3.2 – 6.5)	2.7 (1.3 – 4.0)	1.2 (0.3 – 4.2)	0.649
Turbidimetric & Fibrinolysis Indices				
Lag time (min)	6.3 (1.6)	8.5 (1.2)	8.2 (0.9)	**<0.001**
RCF (units/sec)	29.7 (8.5)	17.4 (7.5)	17.1 (6.4)	**<0.001**
MOD (units)	0.34 (0.08)	0.39 (0.10)	0.35 (0.06)	0.173
RCD (units/sec)	36.2 (9.4)	50.3 (12.2)	50.2 (16.0)	**0.001**
T50 (min)	4.1 (0.8)	3.0 (0.5)	3.0 (0.5)	**<0.001**

Data presented as mean (standard deviation) or median (interquartile range). P values as assessed for trend.

Table 5.4.5: Fibrin clot at week 12 (Warfarin vs Apixaban)

	Warfarin (n = 10)	Apixaban (n = 10)	p - value
Age (years)	68.6 (17.1)	76.6 (11.5)	0.238
Sex (male/female)	7/3	6/4	0.639
SBP (mm Hg)	123.3 (16.4)	129.6 (17.8)	0.422
BMI (kg/m^2)	27.2 (8.0)	24.8 (4.0)	0.418
Creatinine (µmol/L)	86.7 (15.7)	86.1 (29.3)	0.955
Creatinine Clearance - Cockcroft Gault (ml/min/1.73)	70.3 (51.3 – 85.7)	65.2 (41.6 – 90.7)	0.403
TEG Indices			
R (min)	8.66 (2.22)	7.16 (1.40)	0.094
K (min)	2.05 (1.48 – 3.18)	1.80 (1.60 – 2.70)	0.652
Angle (degrees)	56.0 (14.4)	62.3 (7.5)	0.250
MA (mm)	57.9 (17.7)	60.6 (5.2)	0.659
LY60 (%)	0.90 (0.23 – 3.5)	1.9 (0.55 – 5.1)	0.413
Turbidimetric & Fibrinolysis Indices			
Lag time (min)	8.5 (1.0)	8.0 (0.7)	0.215
RCF (units/sec)	14.2 (7.0)	20.2 (4.0)	**0.036**
MOD (units)	0.376 (0.068)	0.326 (0.045)	0.072
RCD (units/sec)	50.4 (10.8)	50.2 (14.3)	0.969
T50 (min)	3.1 (0.4)	2.9 (0.5)	0.494

Data presented as mean (standard deviation) or median (interquartile range). P values analysed by t-test or the Kruskall-Wallis test.

*No statistical difference between both groups regarding comorbidities.

Section 6: Summary of findings and suggestions for future study

6.1 Summary of Findings

The results of the fibrin clot structure in atrial fibrillation – effects of renal dysfunction can be summarised as follows:

- An oral anticoagulant naïve individuals, AF is associated with formation of whole blood clot and fibrin clot which are of greater thrombotic potential and more resistant to lysis as compared to coronary artery disease (Section 5.1)
- The adverse clot structure in AF can be shown to be modulated by exposure to warfarin or NOAC, with formation of fibrin clot and whole blood clot which are slower to form and more sensitive to fibrinolysis (Section 5.4).
- Different oral anticoagulation agents result in formation of whole blood and fibrin clot with various structure changes. NOACs generally form clots which are more sensitive to fibrinolysis as compared to warfarin, however significant differences exist between individual NOAC used (Section 5.4).
- In concurrent chronic kidney disease and AF, despite adequate oral anticoagulation with warfarin, changes to fibrin clot structure are present. Clot structure associated with renal dysfunction, in the presence of warfarin, possess greater thrombotic potential whilst more sensitive to fibrinolysis (Section 5.2).
- Clot structure as demonstrated by SEM was of greater fibrin density and thicker fibres in those with worst renal dysfunction as compared to those with mildest CKD (Section 5.2).
- Besides worsening creatinine clearance, structural changes to fibrin clots are shown to be independently associated with increased all-cause mortality over 2-year follow-up period (Section 5.2).

- Relating to microparticles, worsening renal function was also shown to be associated with increased endothelial dysfunction as demonstrated by increased endothelial microparticle levels. However, other markers of prothrombotic state and cellular activations were demonstrated not to be significantly different across various degree of renal dysfunction (Section 5.3).
- The current study is novel for two main reasons:
    - One: This study is first to demonstrate changes in fibrin clot structure as an independent predictor of all-cause mortality in patients with concurrent AF and CKD. Thus, with longer follow-up, adverse features in fibrin clot structure can potentially be a new prognostic marker.
    - Second: This study is also the first study to demonstrate favourable changes in fibrin clot for patients using NOACs as compared to VKA – thus future interventional study, fibrin clot structure has the potential to guide choice of anticoagulant use.

6.2 Suggestions for future study

Based on the above findings, several future studies can be suggested:

Firstly, to establish differences in whole blood clot structure and strength (assessed by TEG) as a result of each NOAC agent used, larger number of patients studied receiving dabigatran and apixaban can be recruited. The difference in fibrin clot structure as revealed by turbidimetric and fibrinolysis assay can also be visually confirmed by SEM of representative fibrin clots formed from NOAC users.

Secondly, the difference between NOAC and VKA pre- and post-exposure is at most modest. Hence, to clarify and establish the changes to fibrin clot structure and strength relating to NOAC or VKA, larger longitudinal study involving more patients in both groups will be needed.

Thirdly, due to the shorter half-life of NOAC (as short as 12 hours) as compared to VKA, alteration to changes to fibrin clot structure may be apparent within 36 – 72 hours of exposure. Thus to test this hypothesis, a short-term study, with frequent blood and plasma sampling every 12-24 hours will be able to demonstrate changes to haemostatic indices over time.

Fourthly, with the increasing number of patients receiving NOAC, potential changes to clot structure in worsening degree of CKD amongst NOAC users will need to be assessed.

Fifth, if NOAC proves to provide more favourable in fibrin clot structure as compared to VKA amongst patients with worsened CKD class, an interventional study can be done, randomising oral anticoagulant naïve individuals to NOAC or VKA. This allow for hard end-point such as ischaemic stroke and thromboembolism, mortality, haemorrhagic complications to be documented.

Sixth in relation to CKD patients with AF undergoing haemodialysis, the use of apixaban has been approved in the United States by the FDA. Thus a comparison of fibrin clot structure in haemodialysis

patients receiving VKA or NOAC will be useful to better understand potential the effect of choice of renal replacement therapy and oral anticoagulant on haemostatic indices.

Finally, for the 200 patients(anticoagulated with warfarin) who have been recruited with concurrent AF and CKD, long-term follow-up can be done to further investigate the impact of fibrin clot structure to risk of long-term mortality, strokes and major adverse cardiac events.

6.3 Conclusion

Patients with AF and CKD experience an excessive risk of ischaemic stroke and thromboembolism. This thrombotic risk is not mitigated by the use of oral anticoagulation, but conferred in increased haemorrhagic sequelae. Meanwhile, patients with coronary disease and end-stage renal disease have been shown to possess changes to fibrin clot structure and characteristics.

Therefore in the present MD thesis, fibrin clot structure and characteristics were studied in a variety of AF patients, by means of thromboelastography, turbidimetric and fibrinolysis assay, together with Scanning Electron Microscopy. In conjunction detection of markers for endothelial and platelet turnovers, and microparticles were also made.

Therefore, based on the results above, it can be concluded that AF resulted in adverse clot structure which can increased thrombotic risk. And the clinical use of oral anticoagulant may aid risk mitigation due to favourable changes in resultant clot structure. However, for patients with the worst renal function and AF, the coexistence of both conditions and use of oral anticoagulation resulted in clot structures which are pro-thrombotic, but also more sensitive to fibrinolysis. Thus, this might potentially explain the paradox of increased thromboembolic and haemorrhagic risk in this cohort.

To improve our understanding on the impact of structural changes to fibrin and whole blood clot and resultant morbidity and mortality benefit relating to renal failure and NOACs, further studies will be warranted.

VII Publications from these studies

Original Research:

1. Ranjit P, Lau Y, Lip GY, Blann AD. Development and validation of a new assay for assessing clot integrity. Vascul Pharmacol. 2015; 71: 102-7
2. Lau Y; Xiong Q; Ranjit P; Lip GY; Blann AD. Laboratory assessment of anti-thrombotic therapy in heart failure, atrial fibrillation and coronary artery disease: Insights using thromboelastography and a micro-titre plate assay of thrombogenesis and fibrinolysis. The Journal of Thrombosis and Thrombolysis. 2016; 42: 233-44
3. Lau Y; Xiong Q; Shantsila E; Lip GY; Blann AD. Effects of Non-Vitamin K Antagonist Oral Anticoagulants (NOACs) on fibrin clot and whole blood clot formation, integrity and thrombolysis in patients with atrial fibrillation. J Thromb Thrombolysis. 2016 Nov;42(4):535-44
4. Lau Y; Xiong Q; Lip GY; Blann AD. Effect of renal function on whole blood and fibrin clot formation in atrial fibrillation patients on warfarin. Annals of Medicine. 2016; 48: 275-81
5. Lau Y; Xiong Q; Blann AD; Lip GY. Relationship between renal function and circulating microparticles, soluble P-selectin and E-selectin levels in patients with atrial fibrillation. J Thromb Thrombolysis. 2016 Sep 26. (Epub ahead of print)

Related review articles:

1. Lau YC, Lip GY. Management of Atrial Fibrillation in Patients with Kidney Disease. Journal A. Fib. May 2014

2. Lau YC, Lip GY. New advances in the treatment of atrial fibrillation: focus on stroke prevention. Expert Opin Pharmacother. 2014; 15: 2193-204
3. Lau YC, Lip GY. Which drug should we use for stroke prevention in atrial fibrillation? Curr Opin Cardiol. 2014; 29: 293-300
4. Xiong Q, Lau YC, Lip GY. Pharmacodynamic profile and drug interactions with non-vitamin K antagonist oral anticoagulants: implications for patients with atrial fibrillation. Expert Opin Drug Metab Toxicol. 2015; 11: 937-48
5. Lau YC, Proietti M, Guiducci E, Blann AD, Lip GY. Atrial Fibrillation and Thromboembolism in Patients With Chronic Kidney Disease. J Am Coll Cardiol. 2016; 68: 1452-64.

Book Chapter:

Lau YC, Lip GY. Atrial Fibrillation. Medicine 2014 October 42(10): 598-603. ISSN 1357-3039

VIII Presentation and abstracts from these studies

Oral presentation:

Heart Rhythm Society 2014, Birmingham (UK), 5th Oct 2014

Title: Effects of Warfarin and Rivaroxaban on Clot Structure in AF: Assessment by TEG

Abstracts:

22nd International Congress on Fibrinolysis & Proteolysis – Marseille (France), 6-9th July 2014.

Title: Rivaroxaban on fibrin clot structure*

Title: Free-thaw action on fibrin clot structure

Canadian Cardiovascular Congress 2014 - Vancouver (Canada), 25-28th October 2014

Title: Abnormal fibrin clot structure in atrial fibrillation*

EHRA Europace 2015 – Milan (Italy), 21-24th June 2015

Title: Effects of worsening renal function on fibrin clot structure in atrial fibrillation patients receiving warfarin

European Society of Cardiology Congress 2015 – London (UK), 29th Aug – 2nd September 2015

Title: Effect of NOAC and warfarin on fibrin clot structure

60th Annual Meeting of the Society of Thrombosis and Haemostasis Research – Muenster (Germany), 17-20th February 2016

Title: Levels of circulating endothelial and platelet microparticles in chronic kidney disease*

*Highlighted poster for discussion

IX Reference:

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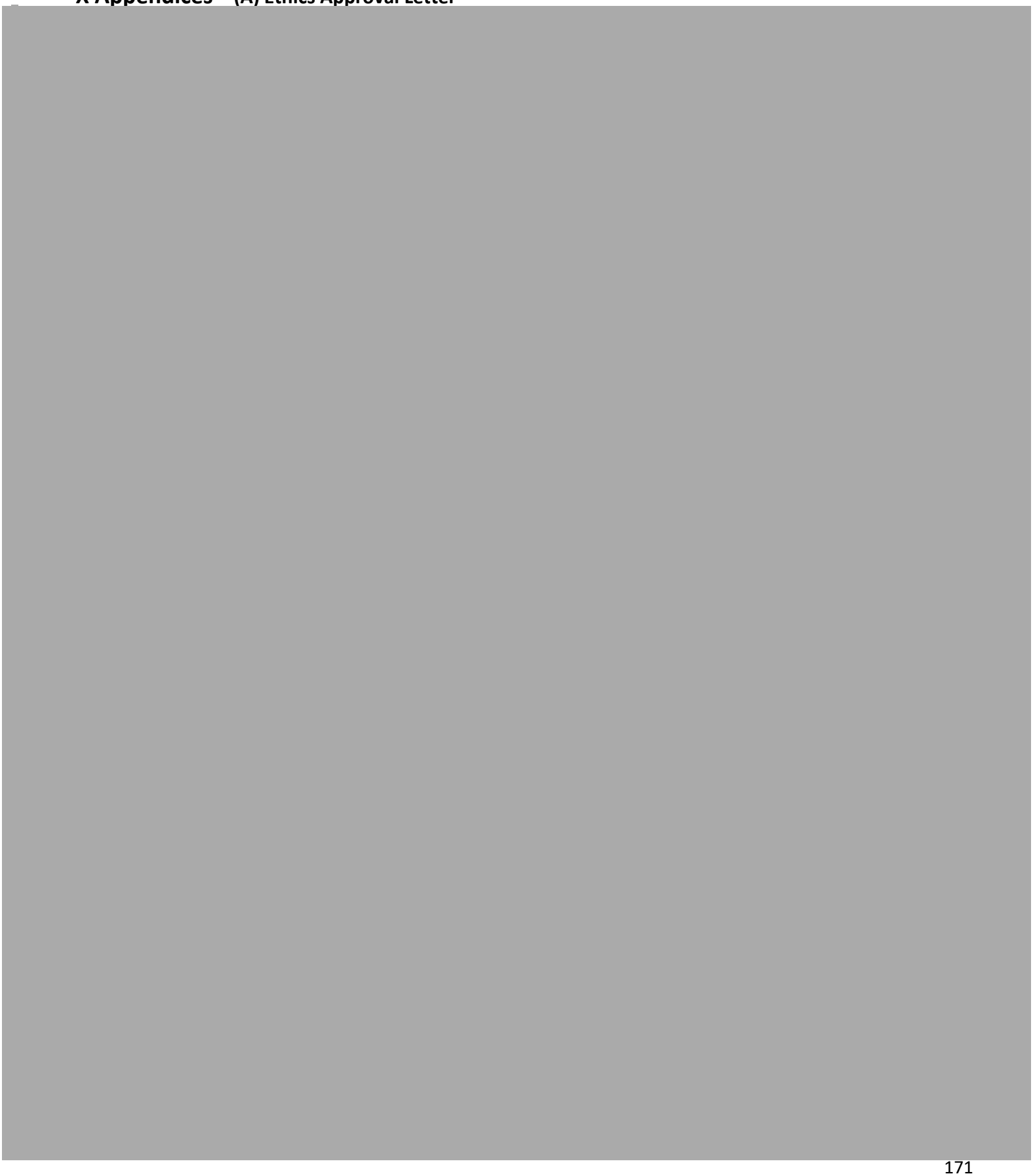
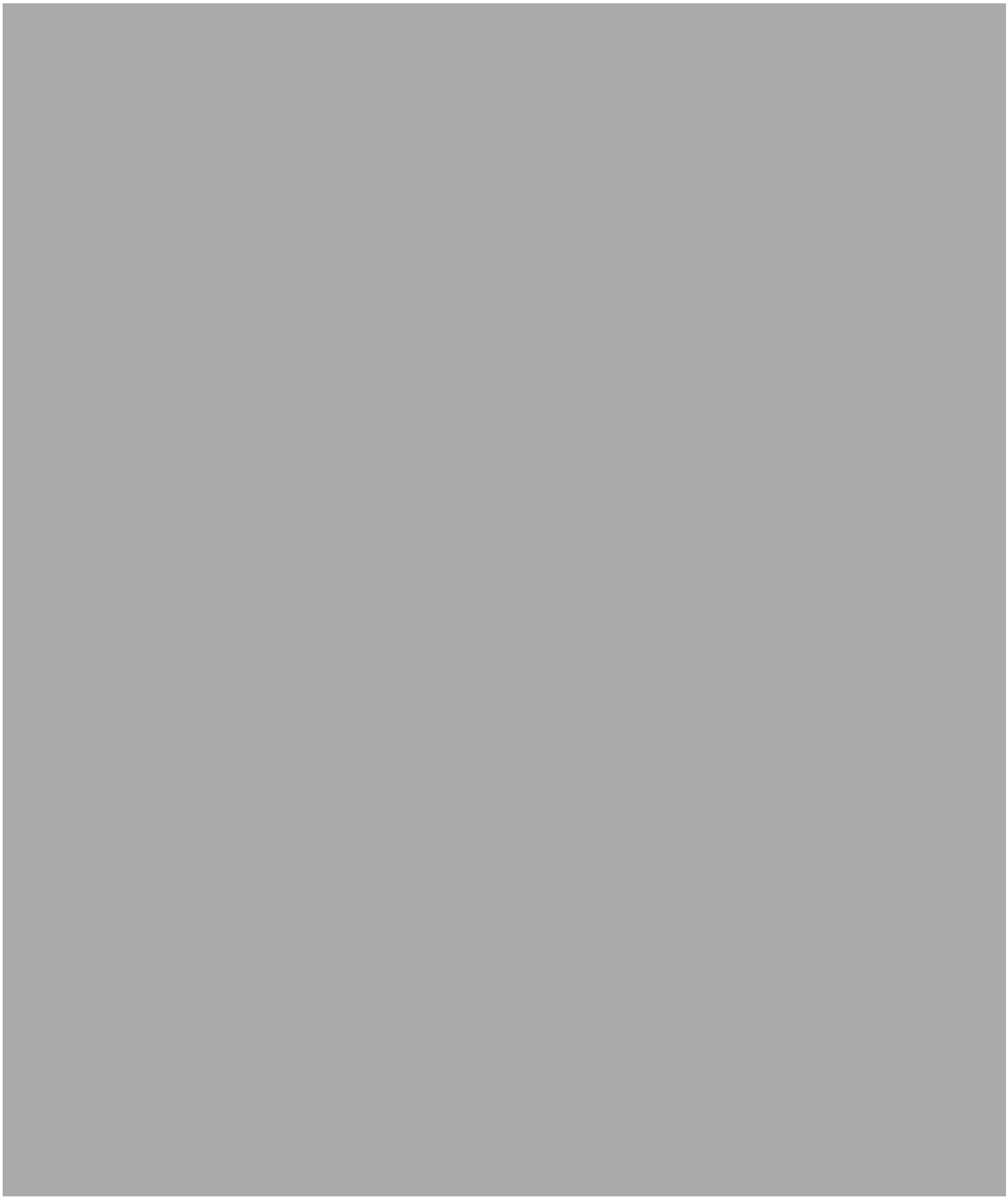
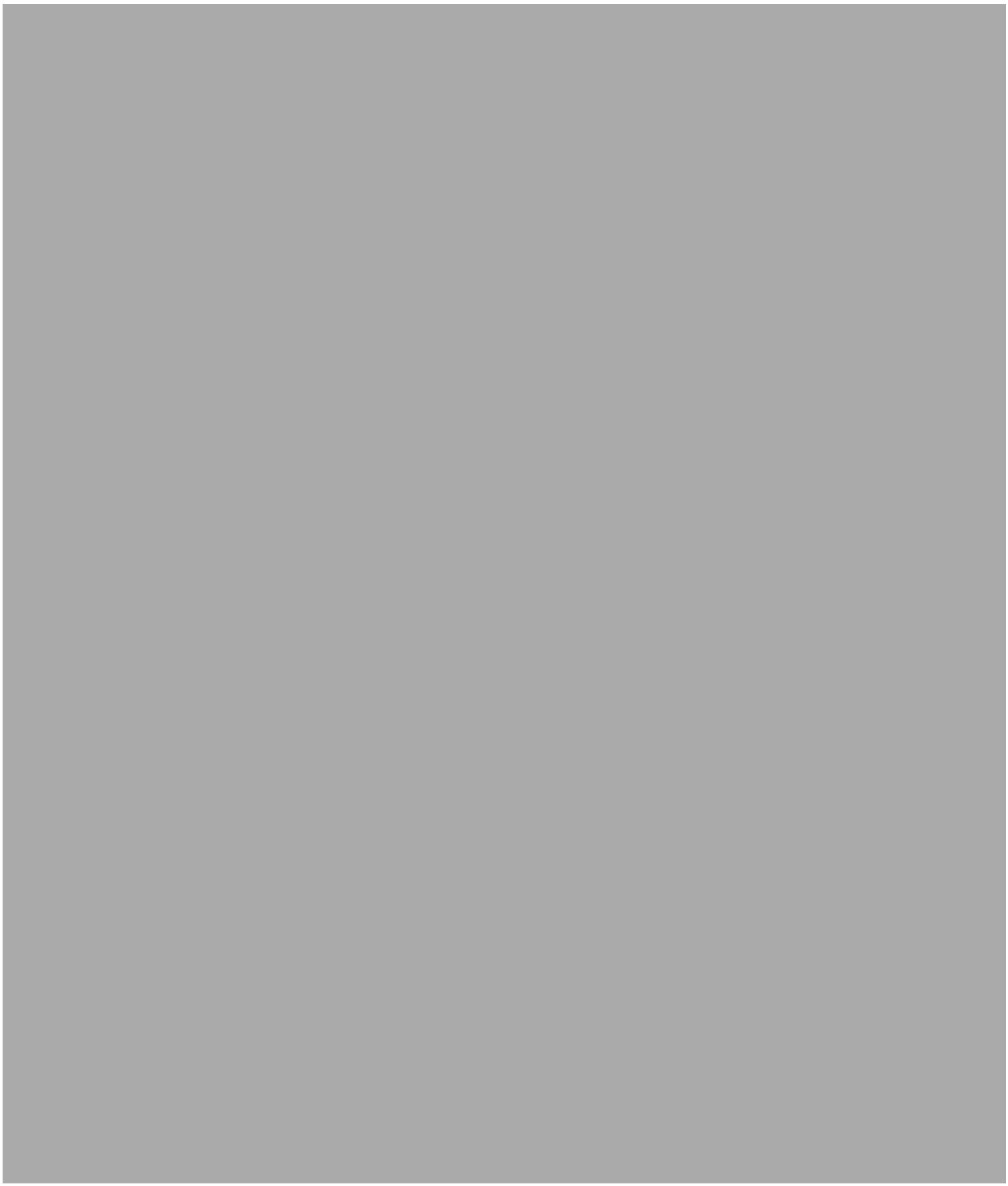
X Appendices – (A) Ethics Approval Letter







Sandwell & West Birmingham Hospitals NHS
NHS Trust

PARTICIPANT INFORMATION SHEET

1. **Study title:** CLOT STRUCTURE IN ATRIAL FIBRILLATION

2. **Invitation paragraph**

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

3. **What is the purpose of the study?**

To find out the extent to which there is variation in clot formation in people with atrial fibrillation (AF) compared to people with cardiovascular disease (such as having had a heart attack or stroke, or have problems with the arteries of their legs)

4. **Why have I been chosen?**

You have been chosen because you have AF and/or cardiovascular disease

5. **Do I have to take part?**

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect your professional relationship with the researcher or the Trust.

6. **What will happen to me if I take part?**

After having signed the consent form, all we ask in a sample of your blood. In some people (and this might be you) we will ask for a second sample in about a month's time, and a third in about three month's time. This is summarised in a diagram on the last page.

You will also be given a copy of this information sheet and the signed informed consent form. Carefully retain these documents.

7. **What do I have to do?**

There is no restriction to your work pattern or any suggestion to change this. Continue to take all your medications and attend hospital and your GP exactly as you have been doing.

8. **What is the procedure that is being assessed?**

We want to find out if the ability of your blood to form a good firm clot depends on factors such as how well your kidneys work, and according to what drugs you are taking.

9. **What are the effects of participating in the study?**

None really: all we ask is a blood sample, but in some we will ask for three samples.

10. **What are the possible disadvantages and risks of taking part?**

Only that of a bruise in your arm as a result of the blood sample being taken.

11. **What are the possible benefits of taking part?**

None. However, it is possible we may find out you have some problems you are unaware of (such as diabetes), and if so we will inform you and your GP.

12. **What if new information becomes available?**

Sometimes during the course of a research project, new information may become available about blood clotting. If this happens, the researcher will tell you about it and discuss with you whether you want to continue in the study.

13. **What happens when the research study stops?**

You will continue life just like before the study began, taking the same tablets and having the same hospital and GP appointments. You will continue to be cared for by your Doctors as if nothing had happened.

14. **What if something goes wrong?**

If you are harmed due to someone's negligent comment, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you. However there are no special compensation arrangements if you are harmed by simply participating in this research project.

15. **Will my taking part in this study be kept confidential?**

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital/surgery will have your name and address removed so that you cannot be recognised from it.

16. **What will happen to the results of the research study?**

The result of the study will be presented to our hospital colleagues and may be presented at scientific conferences, and published in peer reviewed scientific journals. However, no individual participant will be identified in any of the reports.

17. **Who is organising and funding the research?**

The project has been organised by a group of Doctors at Sandwell and West Birmingham NHS Trust. It is being funded by Departmental funds. There is no drug company involvement.

18. **Who has reviewed the study?**

This study has been reviewed by a committee of people independent from your doctor (the Local Research Ethic Committee), whose primary concerns are the safety, rights and welfare of patients in this study. This committee has reviewed and approved all written material about this study including this information sheet and consent form.

19. **Contact for Further Information**

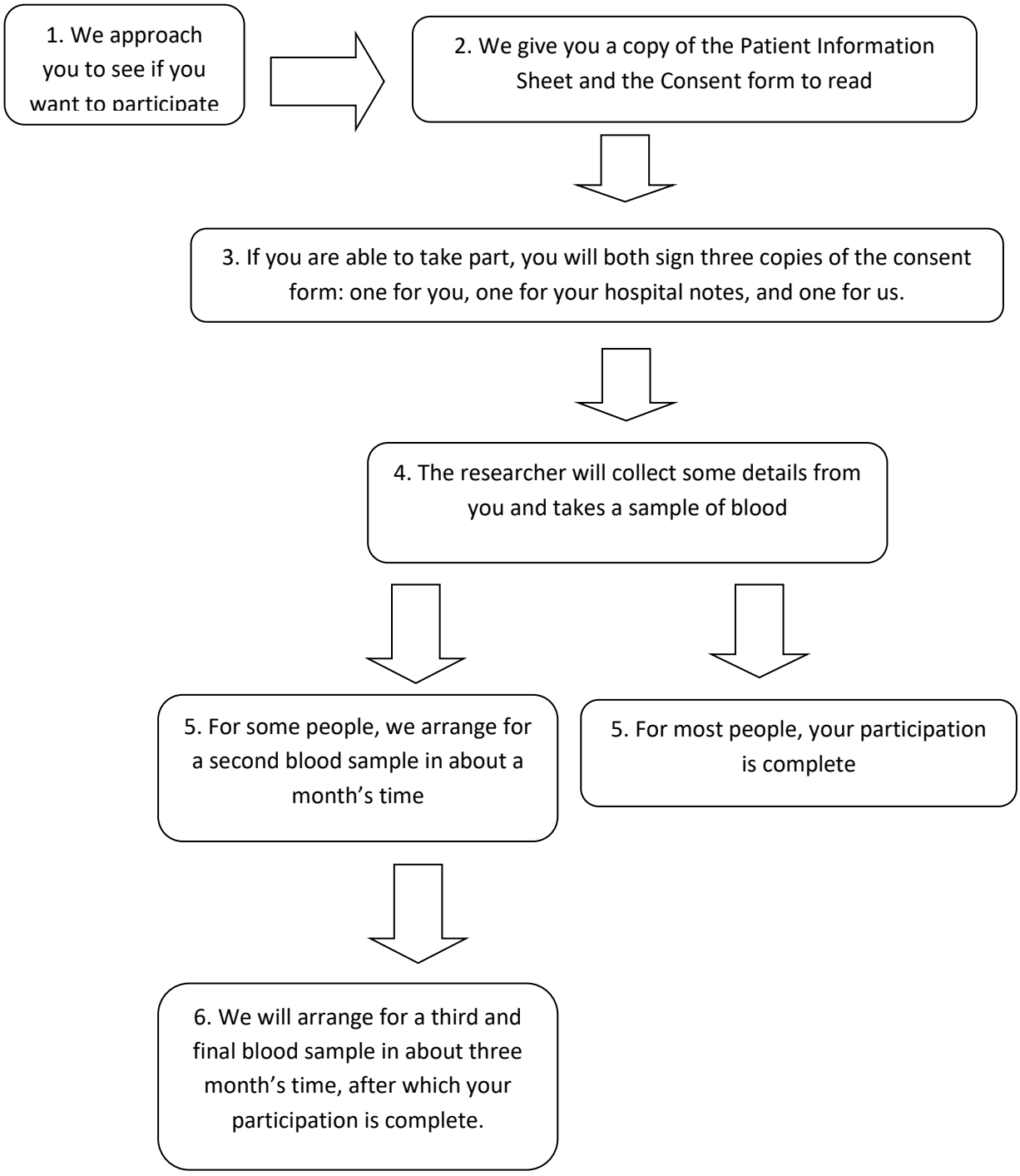
If you would like more information about the research you could contact:

- Dr Jocelyn Bell (The Head of our Department of Research and Development) on [redacted]
- Dr Andrew Blann (The principle investigator) on [redacted]. His E-mail address is [redacted]
- Professor GYH Lip (Our Head of Department) on [redacted]
- The Hospital Patient Advice and Liaison Service on 0121 507 5836. Their E-mail address is pals@swbh.nhs.uk. You also have the right to complain – and if you wish to then this is the unit your should contact

Thank you for taking part in our study…..

Participant Flow Chart

This chart shows what will happen to you if you decide to take part



X Appendices – (C) Patient Consent Form

Sandwell and West Birmingham Hospitals NHS
NHS Trust

Gregory YH Lip	MD FRCP FACC	*Professor of Cardiovascular Medicine*	University of Birmingham Centre
Paulus Kirchhof	MD FRCP	*Professor of Cardiovascular Medicine*	for Cardiovascular Sciences
Andrew D Blann	PhD FRCPath	*Senior Lecturer in Medicine*	City Hospital, Birmingham
Russell C Davis	MD MRCP	*Senior Clinical Lecturer in Medicine*	B18 7QH, United Kingdom
Deirdre Lane	MSc PhD	*Lecturer in Medicine*	Departmental Secretary Ms S Cartwright
Ronnie Haynes	RGN MICR CMS	*Departmental Manager/Trials Co-ordinator*	Tel [redacted] Fax +[redacted]
			Direct Line to Dr Blann
			Tel/fax [redacted]

Patient ID for this study:

CONSENT FORM

Title of Project: Clot Structure in Atrial Fibrillation

Name of Researcher: Dr Yee Cheng Lau & Dr Andrew Blann

Please initial all boxes

1. I confirm that I have read and understand the information sheet dated 29th August 2013 (version 1 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I agree to my GP being informed of my participation in the study. ☐

4. I agree to take part in the above study. ☐

5. I understand that to enable the study to be properly monitored and regulated, sections of my medical notes relevant to my taking part in this research and data collected during the study may be looked at by members of the research team, the NHS Trust where I will take part in the study, and regulatory agencies.
I give permission for these individuals to have access to my records. ☐

Name of Participant	Date	Signature
Name of Person	Date	Signature taking consent.

X Appendices – Consent Form (Healthy Control)

THE UNIVERSITY OF BIRMINGHAM

Sandwell and West Birmingham Hospitals NHS
NHS Trust

Gregory YH Lip	MD FRCP FESC FACC	*Professor of Cardiovascular Medicine*
Paulus Kirchhof	MD FESC FHRS	*Professor of Cardiovascular Medicine*
Andrew D Blann	PhD FRCPath	*Senior Lecturer in Medicine*
Russell C Davis	MD MRCP	*Senior Clinical Lecturer in Medicine*
Deirdre Lane	BSc PhD	*Lecturer in Cardiovascular Health*
Ronnie Haynes	PGN MICR CMS	*Department Manager / Trials Coordinator*

University of Birmingham
Centre for Cardiovascular Sciences
City Hospital
Birmingham B18 7QH
England United Kingdom
Tel [redacted]
Fax [redacted]
www.swbh.nhs.uk

CONSENT FOR A BLOOD SAMPLE TO BE OBTAINED AND USED IN CLINICAL RESEARCH

SUBJECT'S NAME:_____

INVESTIGATOR'S NAME:_____

Please initial the box

I confirm that I have read and understand the information sheet (potentially on the rear of this page). I confirm that the need for a sample has been explained to my satisfaction and I have had the opportunity to ask any questions. I know who to contact if any questions occur to me later. ☐

I understand that my participation is voluntary and that I am free to withdraw at any time without giving reason, without my medical care or legal rights being affected. ☐

I understand that the Investigator will inform me of any untoward routine results that may come about as part of my participation in a research project. ☐

I understand that any sample may be used by more than one researcher or procedure, that it will be kept in a secure laboratory, and that it will be destroyed after 5 years. ☐

I understand that my personal details will be held by the Department on secure computers that are Subject to the Data Protection Act, and that such details are confidential to the members of the research team. ☐

By signing hereunder I accept the contents of this consent form and agree to provide a blood sample.

_____ _____
Signature of the Subject - Printed Date: day/month/year

_____ _____
Signature of the Consenter - Printed Date: day/month/year

Three copies: one copy for the researcher, one for the subject, one for Ms Haynes

A University of Birmingham Teaching Hospital

Version 1: 9th May 2013 by ADB/RH