Clot structure and plasma microparticles in atrial fibrillation

Christos Voukalis, MBChB, MRCP (UK)
ID: 1707874

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University of Birmingham Institute of Cardiovascular Sciences
City Hospital, Dudley Road,
Birmingham B18 7QH,
United Kingdom

Supervisors: Professor Gregory YH Lip, Dr Eduard Shantsila

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Dedication

I dedicate this research project to the memory of my father Dr Dimitrios Voukalis, who passed away during the writing up period of this thesis.

Acknowledgments

"As you set out for Ithaka I hope your road is a long one, full of adventure, full of discovery. Ithaka gave you the marvellous journey. Without her you would not have set out. But is not the destination, is the journey that matters"

Constantine Cavafis

Influenced by Greek mythology, I found many similarities of my research "adventure" years with Ulysses trip from Troy to Ithaka. Multiple tasks, challenges and barriers emerged, especially when try to combine research with clinical commitments; from the other hand achievements, new people and friends who accompanied me in the journey towards the completion of this MD thesis.

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I. Abstract

Chronic oral anticoagulation (OAC) is an important decision related with stroke thromboprophylaxis in non-valvular atrial fibrillation (NVAF). Almost a decade after the approval of Non vitamin K oral anticoagulants (NOACs) for prevention of thromboembolic events in NVAF, there is now confidence regarding their efficacy and safety as real word evidence complements the findings from the phase III pivotal trials. NOACs favourable safety profile against warfarin have changed the threshold of starting OAC, even with lower risk for systemic thromboembolism. Apixaban, one of the four licenced NOACs for stoke prevention in NVAF, even at the higher recommended dose (5mg BID), has significantly reduced the haemorrhagic complications but there is still a considerable risk of intracranial bleeding. This MD research thesis studies the influence of antithrombotics (aspirin, warfarin and apixaban) on the fibrin polymerisation and fibrinolysis pathway. To highlight antithrombotic activity variances, this analysis is based on dynamic assays and biomarker quantification related with clot structure features. Additionally, explores possible relationship between microparticle levels and physical status in NVAF patients. My findings suggest that NVAF is associated with impaired haemostasis and each antithrombotic class is related to different clot structure characteristics. Apixaban has distinctive anticoagulation dynamics and induces a reduction of coagulation biomarkers. My results also support that microparticles levels may be a useful marker of physical status as suggested by the relation between objective (cardiopulmonary exercise test) and subjective (quality of life questionnaire – EQ5D5L) evidence of fitness level.

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VI. List of abbreviations used (in alphabetical order)

AF	Atrial Fibrillation
HF	Heart Failure
ICH	Intracranial Haemorrhage
INR	International Normalised Ratio
LV	Left Ventricle
LVEF	Left Ventricular Ejection Fraction
MPA	Microplate Assay
NOACs	Non-Vitamin K Antagonists Oral Anticoagulants
NT-pro BNP	N-Terminal Pro B-Type Natriuretic Peptide
NVAF	Non-Valvular Atrial Fibrillation
OAC	Oral Anticoagulation or Oral Anticoagulant(S)
PAD	Peripheral Arterial Disease
PAI-1	Plasminogen Activator Inhibitor 1
PCI	Percutaneous Coronary Intervention
PFP	Platelet Free Plasma
PPP	Platelet Poor Plasma
SE	Systemic Embolism
TEG	Thromboelastography
TIA	Transient Ischaemic Attack
TTR	Time in Therapeutic Range
VKA	Vitamin K Antagonists

Section 1: Background and literature review

1.1 Introduction

The prevalence of atrial fibrillation (AF) is estimated around 2% in the Western world[1]. Considering that AF increases by 5-fold the risk of ischaemic stroke and strokes related to AF tend to be more severe, debilitating and require longer hospitalisation, this arrhythmia causes a significant burden to healthcare systems[2]. AF is sometimes the consequence or manifests itself in combination with other cardiac conditions, such as heart failure or coronary artery disease, which increases further the thromboembolic risk[3].

Undoubtedly, the last decade was revolutionary regarding the oral anticoagulation (OAC) in AF. For over 60 years, Vitamin K antagonists (VKA) were the only available agents for long-term, effective OAC. They have significantly influenced the management of AF but they also have shortcomings[4]. In order to improve the standards of care, the non-vitamin K antagonists oral anticoagulants (NOACs) were developed. Apixaban, one of the four licenced NOACs, has showed consistent efficacy and safety in non-valvular AF (NVAF) stroke prevention phase III trials and real world data[5,6]. NVAF is the most common type of AF which is not associated with mitral stenosis or prosthetic heart valve (and valve repair in North American guidelines only)[7].

Evidence supports that clot structure characteristics are important determinants for the course of cardiovascular diseases[8]. Fibrin polymerisation and lysis has fundamental role in haemostasis[9]. At present, there are no standardised techniques to assess clot structure features[10]. The traditionally used laboratory tests are of limited value in order to assess the full spectrum of the haemostatic dynamics that are related with antithrombotics [11]. For NOACs, such as apixaban, the direct thrombin inhibitor assay, ecarin clotting time and chromogenic

anti-Xa assay which currently are the recommended methods to assess their serum concentration have weaknesses and are not standardised[12]. A more mechanistic scope of how antithrombotics influence the thrombosis and thrombolysis pathway may give further insights about important parameters affecting the fine balance between AF associated hypercoagulopathy and bleeding related to OAC.

In order to assess the risk of AF associated thromboembolism, various risk factors have been identified[13] and amalgamated into a risk stratification models to guide physicians towards optimal use of oral anticoagulation (OAC)[14]. Several blood-based biomarkers have been proposed to further refine estimation of stroke risk[15–17]. Microparticles are a distinctive group of small vesicles, without nucleus, which are involved as significant modulators in several physiological and pathophysiological mechanisms, including thrombosis. The strong correlation of microparticle levels with several outcomes in cardiovascular diseases has led to their utilisation as biomarkers. The role of microparticles in the pathophysiology and clinical manifestations of AF has been described but with conflicting evidence[18,19]. Although there are robust data for relationship between microparticles and thrombosis[20], their involvement in AF thromboembolic tendency remains unclear.

The purpose of this thesis is to assess the clot dynamics under the influence of different OAC and investigate a potential significance of microparticles levels in patients with NVAF. With the view to set the full background for the current thesis, this chapter will initially discuss the role of oral anticoagulants in AF, with emphasis on NOACs, and explore the current data (literature review) related with microparticle involvement as biomarkers in cardiovascular conditions.

1.2.1 Pathophysiological aspects of hypercoagulopathy in atrial fibrillation

Several cardiac pathologies and underlying systemic conditions may promote electrical instability on atrial myocardium and induce AF[21]. Thromboembolic events is by far the most severe complication of AF[22].

German physician, Rudolph Virchow[23] described in 1856 the pathophysiology of deep venous thrombosis. His theory, which still stands among physicians a basic concept to interpret the pathophysiology of thrombosis[24], consists of three elements (triad). Blood stasis, endothelial injury and coagulopathy, related with abnormal blood constituents, complete his triad [25].

Loss of atria contraction in AF is the mechanical result of chaotic atria electrical activity. The haemodynamic consequences, as a result of blood stasis, can be assessed by echocardiography[26]. Pulse doppler interrogation of blood velocities in left atrium appendage suggest loss of the quadriphasic pattern seen in sinus rhythm[27]. In addition, the presence of spontaneous echo contrast, seen most commonly with 2D transoesophageal echocardiography, in the left atrium during AF, indicates low flow and alteration of haematological dynamics[28].

Inflammatory changes in atrial tissue have been demonstrated at microscopic and molecular levels[29]. Markers of low grade systemic inflammation such as high-sensitivity C-reactive protein (hs-CRP) and Interleukin 6 (IL-6), indicate the activity of an inflammatory process which in conjunction with impaired endothelial integrity, promote haemostatic imbalance[30].

The final part of Virchow's triad, abnormal procoagulant blood constituents, is well recognised in AF resulting to a chronic hypercoagulable state[31]. These molecules, usually part of the coagulation pathway, were found in higher levels among AF subjects. Typical examples are D-Dimer, von Willebrand factor (VWF), tissue factor and fibrinogen[32].

1.2.2 Risk factors for stroke in atrial fibrillation: A brief overview

The most common risk factors associated with stroke (eg, heart failure, hypertension, diabetes, age, prior stroke) were initially identified from treatment of naïve cohorts in randomized trials conducted 2 decades ago[33]. These trials only randomised <10% of patients screened and many common stroke risk factors were not recorded or consistently defined.

A systematic analysis from the Stroke in AF Working Group searched for independent risk factors for stroke related to AF using information from 27 studies. Of the 24 studies (although many were from trial cohorts), age was found to be an independent risk of stroke, associated with an incremental increase in risk of 1.5-fold per decade [Relative Risk (RR) 1.5 per decade; 95% Confidence Interval (CI), 1.3-1.7]. Overall stroke risk increased 2.5-fold in patients with prior stroke / transient ischaemic attack (TIA) (RR 2.5; 95%CI, 1.8-3.5). Hypertension was independently associated with stroke in 13 of 20 studies (RR, 2.0; 95% CI, 1.6-2.5)[13]. In another systematic review, history of hypertension was present in 42% to 53% (mean of 48%) of analysed subjects and was independently related to stroke in all studies included. Diabetes mellitus was present in 14% to 18% (mean of 15%) of the study cohorts and it was a significant independent risk factor for stroke (RR 1.7, 95% CI, 1.4 to 2.0)[34]. Interestingly, heart failure

(HF) and coronary artery disease did not emerge as independent predictors for stroke risk in this analysis.

Other data suggest that recent decompensated HF is indeed related to higher risk of stroke in AF, whether left ventricular (LV) ejection fraction (LVEF) was reduced or preserved[35,36]. However, moderate-to-severe systolic LV dysfunction on cardiac imaging is associated with a higher stroke risk even if asymptomatic[37]. One systematic review found that although moderate-severe LV systolic impairment on echocardiography is an independent risk factor for stroke/systemic embolism, the magnitude by which it increases the risk of stroke cannot be precisely quantified. Overall, although a clinical diagnosis of HF have not been universally predictive of stroke in AF, significant impairment of LV contractility or previous admission due to HF decompensation irrespective of LV systolic function clearly increases risk of stroke[38].

Female sex was also associated with an increased risk of stroke, especially in women aged >65 or in presence of an additional stroke risk factor. Meta-analysis of 17 studies revealed a 1.31-fold (95% CIs) elevated risk of stroke in women with AF; especially in those aged ≥75 years[39]. Overall, female sex plays limited role in prognostication in AF at age of <65 years when there no other risk factors (i.e., risk of ischaemic stroke <1%/year)[40,41,42].

A systematic review by Anandasundaram et al supports a significant association between a history of myocardial infarction (MI) and the risk of thromboembolism in AF (in 5 of 6 studies). Angina was reported as a stroke risk factor in AF by two studies[43]. Furthermore, the presence of AF in association with peripheral arterial disease (PAD) and presence of complex plaque in the descending aorta are independent predictors for stroke and thromboembolism in AF[44].

Renal failure has been assessed as a risk factor for stroke in AF patients. A correlation between decreasing glomerular filtration rate and stroke risk in conjunction with proteinuria has been reported as an independent stroke risk factor[45]. The Swedish Atrial Fibrillation cohort study confirmed such association using the composite endpoint, that included unspecified stroke, TIA and systemic embolism (SE) but not with ischaemic stroke alone[46].

Standard echocardiography contributes to refinement of stroke risk stratification. Moderate-severe LV dysfunction from 2-D echocardiography was the strongest independent predictor of later thromboembolism[47]. LV hypertrophy, defined as a LV mass >110 g/m^2 in women and >134 g/m^2 in men, was found to be a significant independent risk factor for stroke in two studies[13]. Non-unexpectedly, demonstration of left atrial thrombi, complex aortic plaque, spontaneous echo contrast or low velocities in left atrial appendage have also been associated with an increased risk of thromboembolism in AF[44].

1.2.3 Risk Stratification Models for stroke

Co-existence of several risk factors for stroke potentiates the overall risk, thus prompting development of various Risk Stratification Models. Some of these models categorize AF patients into 'high', 'moderate', and 'low' risk strata; however, stroke risk is a continuum of risk, and thus approaches based on risk quantification appear the preferred option[48].

In 2001, an amalgamation of the AF Investigators and Stroke Prevention in AF schemes from the original historical trials led to introduction of the $CHADS_2$ score[49]. The $CHADS_2$ acronym was derived from the individual stroke risk factors: Congestive heart failure, Hypertension,

Age≥ 75 years, Diabetes mellitus, and prior Stroke or TIA. Two points were given for prior stroke or TIA (hence, the subscripted “2”), and 1 point was assigned for each of the other factors[50].

The CHA_2DS_2-VASc score (Table 1) extends stroke prognostication in AF by including additional common stroke predictors to the older $CHADS_2$ score (ages 65–74, vascular disease, and female sex)[51]. The CHA_2DS_2-VASc score particularly improves the risk prediction in many patients deemed ‘low risk’ based on the $CHADS_2$ schema (Table 2)[52,53]. The CHA_2DS_2-VASc tool has been validated in multiple independent populations and is now recommended by the European[54], US[55] and UK (NICE)[56] guidelines.

Other risk stratification schemes that have been developed, such as the ATRIA[57] and the QSTROKE[58], have included further clinical and/or demographic factors, as well as different weighing strategies for primary or secondary prevention of stroke. However, perceived marginal benefits (at least statistically) of these scores are outweighed by their complexity and thus limited practicality for everyday use. All suggested risk stratification schemes have their own limitations but the major guidelines emphasize the role of CHA_2DS_2-VASc score for clinical use, in view of its better capability to identify patients with genuinely low risk who do not need any antithrombotic therapy along with ability to accurately quantify risk of stroke in subjects with multiple risk factors[33].

Table 1. CHA_2DS_2VASc score[53]

Condition or characteristic	points
Congestive heart failure	1
Hypertension	1
Age ≥75 y	2
Diabetes mellitus	1
Stroke/TIA/SE	2
Vascular disease (prior ACS, PAD, or aortic plaque)	1
Age 65-74 y	1
Sex category (ie, female sex) *	1
Maximum score	9
*** Counts only in the presence of another risk factor.**	

Abbreviations: TIA Transient Ischaemic Attack, SE Systemic Embolism, ACS Acute Coronary Syndrome, PAD Peripheral Arterial Disease.

Table 2. Event rates (95% CI) of hospital admission and death due to thromboembolism per 100 person years [Based on Olesen et al [52]]

Score/risk category	1 year's follow-up	5 years' follow-up
CHA_2DS_2-VASc:		
0	0.78 (0.58 to 1.04)	0.69 (0.59 to 0.81)
1	2.01 (1.70 to 2.36)	1.51 (1.37 to 1.67)
2	3.71 (3.36 to 4.09)	3.01 (2.83 to 3.20)
3	5.92 (5.53 to 6.34)	4.41 (4.21 to 4.61)
4	9.27 (8.71 to 9.86)	6.69 (6.41 to 6.99)
5	15.26 (14.35 to 16.24)	10.42 (9.95 to 10.91)
6	19.74 (18.21 to 21.41)	12.85 (12.07 to 13.69)
7	21.50 (18.75 to 24.64)	13.92 (12.49 to 15.51)
8	22.38 (16.29 to 30.76)	14.07 (10.80 to 18.33)
9	23.64 (10.62 to 52.61)	16.08 (8.04 to 32.15)
CHA_2DS_2-VASc:		
Low risk (0)	0.78 (0.58 to 1.04)	0.69 (0.59 to 0.81)
Intermediate risk (1)	2.01 (1.70 to 2.36)	1.51 (1.37 to 1.67)
High risk (2-9)	8.82 (8.55 to 9.09)	6.01 (5.88 to 6.14)

CHA_2DS_2-VASc : C, congestive heart failure, H, hypertension, A_2,age at least 75 years, D, diabetes, S_2, previous stroke, TIA, or systemic embolism, V, vascular disease, A, age 65 through 74 years, Sc, sex category female sex.

1.2.4 Risk of Bleeding

Despite the clinical benefit of OAC for stroke prevention in AF, major bleeding events (especially intra-cranial bleeds) may be devastating events. Identification of patients at high risk of bleeding and delineation of conditions and situations associated with bleeding risk can help to refine antithrombotic therapy in individual cases to minimize bleeding risk[59].

The first bleeding risk prediction score (ORBI score) was published in 1998 and it was developed in patients newly started on warfarin after hospitalisation for venous thromboembolism[60]. Since then, various bleeding risk scores have been described but only four $HAEMORR_2AGES$[61], HAS-BLED[62], ATRIA[63] and ORBIT[64]) were developed and verified in AF populations.

The HAS-BLED score (Table 3) demonstrated good discriminatory performance for "any clinically relevant bleeding" in anticoagulated patients with AF[65]. It also performs well in predicting bleeding events compared with older bleeding scores and the ATRIA score[66]. Other scores, such as ATRIA or ORBIT may falsely categorise some patients as ‘’low risk’ with no action needed, whilst the HAS-BLED score would flag up those subjects, particularly if labile international normalised ratios(INR) are evident[67].

Additionally, the simple HAS-BLED score was highly predictive of bleeding events in patients managed with NOACs[66], patients on triple antithrombotic therapy post percutaneous coronary intervention(PCI) (i.e., dual anti-platelets therapy and warfarin)[68] and during bridging of chronic oral anticoagulants with unfractionated or low molecular weight heparin prior to surgery[69].

A HAS-BLED score ≥3 indicates high risk of bleeding and suggest some caution with OAC in such patients with their regular review following the initiation of antithrombotic therapy[48]. A high HAS-BLED score is not a reason to withhold anticoagulation but it emphasises the need to correct potential causes of bleeding such as uncontrolled hypertension, concomitant use of other medications that can contribute to bleeding and falls, excessive alcohol consumption, etc[33].

A large number of patients with high risk of stroke/SE (i.e., CHA_2DS_2-VASc Score >3) also have a high risk of bleeding[70]. This is partly since both scores have some characteristics in common (e.g., age, hypertension and previous stroke). Nonetheless, HAS-BLED outperforms $CHADS_2$ and CHA_2DS_2-VASc for predicting bleeding[65]. Thus, a bleeding risk score (HAS-

BLED) should be used to predict bleeding, and stroke risk assessed using a specific stroke risk score (CHA_2DS_2-VASc)[70]. From a large observational study the adjusted net clinical benefit favoured OAC in patients irrespective of HAS-BLED or CHA_2DS_2-VASc score[71].

Many clinicians are reluctant to initiate appropriate antithrombotic therapy in patients with AF and risk or history of falls. In a large "real world" cohort, prior history of falls was uncommon and it was independently associated with increased risk of stroke/thromboembolism, bleeding, and mortality but not with haemorrhagic stroke in the presence of anticoagulation[72]. In another cohort, patients on oral anticoagulants at high risk of falls did not have any significant increase in risk of major bleeds, and risk of falls is usually not a valid reason to avoid OACs in AF patients[73]. In a modelling analysis, Man-Son-Hing et al. estimated that elderly patients need to fall 295 times per year for the benefit of stroke reduction to be outweighed by serious bleeding[74].

Table 3. HAS-BLED score[62]

Condition or characteristic	points
Hypertension	1
Abnormal renal and liver function (1 point each)	1 or 2
Stroke	1
Bleeding tendency /predisposition (anaemia)	1
Labile INRs (eg, TTR<60%)	1
Elderly (eg, age >65 y, frail condition)	1
Drugs* or alcohol excess (1 point each)	1 or 2
Maximum score	9
* Concomitant antiplatelet drugs, Steroids, Non-Steroidal Anti-inflammatory drugs	
Abbreviations: VKA Vitamin K Antagonist, NOAC Non-Vitamin K Oral Antagonist, TIA Transient Ischaemic Attack, SE Systemic Embolism, ACS Acute Coronary Syndrome, PAD Peripheral Arterial Disease, INR International Normalised Ratio, TTR Time in Therapeutic Range	

1.2.5 Oral anticoagulants for stroke prevention in atrial fibrillation

Vitamin K antagonists

VKAs have been the "gold standard" for prevention of stroke and SE in AF patients for more than 3 decades[4]. A meta-analysis[22] of the 6 initial (and now historical) randomised controlled trials (2900 patients), comparing warfarin with placebo or control, demonstrated that the incidence of all strokes was reduced by 64% (95% CI, 48%-72%) with a significant reduction in mortality (RR 26%, 95% CI, 3-43%). For primary prevention the number needed to treat (NNT) for 1 year patients to prevent one stroke was 37; the NNT was 12 for secondary prevention[22].

Prothrombin time was the initial assay which was used to monitor the intensity of the OAC therapy[4]. In 1983 the World Health Organisation introduced the international normalised ratio (INR) to promote standardization of assessment of anticoagulation[4]. The measured prothrombin time is converted into the INR by using the reagent-specific international sensitivity index[75]. Optimal OAC with INR between 2 and 3 is essential in order to achieve the benefits of thromboprophylaxis and to minimize the risk of bleeding[75]. Many factors affect the pharmacokinetics and pharmacodynamics of VKAs, such as genetic polymorphisms, various drugs and dietary habits, several disease states and alcohol intake[76]. The quality determinant of OAC is the estimated time spent in therapeutic range (TTR)[4]. A Swedish national registry[77] for patients on warfarin for thromboprophylaxis included 68797 patients with AF with the reported average TTR was 76,5%. With this high quality of OAC the event rates of systemic embolic events among patients with AF were 1.54% and of major bleeding 0.38%[77]. Generally, an average individual TTR above 70% is considered satisfactory[4].

Non-Vitamin K oral anticoagulants

The usage of NOACs in daily clinical practice is increasing and all the major guidelines recommend them as first line OAC therapy for stroke prevention in AF patients. Fixed dose administration, no need for routine monitoring of anticoagulation parameters and fewer drug and food interactions make these agents attractive to both physicians and patients[Table 4][78].

The first NOAC that gained approval for stroke and systemic embolism prevention in patients with NVAF was dabigatran etexilate (DE). DE is the prodrug of dabigatran, a direct thrombin inhibitor[79]. The RE-LY trial [80] was a large, multicenter, phase III trial which compared DE with warfarin in patients with AF. The RE-LY trial was followed by the ROCKET-AF trial[81] for rivaroxaban, the ARISTOTLE trial[5] for apixaban and the ENGAGE-AF trial[82] for edoxaban. Rivaroxaban, apixaban and edoxaban belong to another group of NOACs, the factor Xa inhibitors[54]. All the above phase III trials of NOACs had similar hypothesis and were designed to assess the non-inferiority of the index NOAC against warfarin. The primary efficacy endpoint was stroke or SE and the primary safety endpoint was major bleeding (plus non-major clinically relevant bleeding in the ROCKET-AF trial)[5,80–82]. The baseline characteristics and the principle results of the trials are summarized in Table 5.

In the RE-LY[80] and the ENGAGE-AF[82] studies two doses of DE (150mg and 110mg BID) and edoxaban (60mg and 30mg OD) respectively were compared with adjusted dose warfarin in a three arm trial design. The mean TTR for the warfarin group in the RE-LY study was 64% and 68.4% in the ENGAGE-AF trial. In the ROCKET-AF trial[81], patients were randomly assigned to receive either fixed-dose rivaroxaban (20 mg daily with a dose adjustment to 15

mg daily in patients with a creatinine clearance of 30 to 49 ml/min; n= 1490) or adjusted-dose warfarin. The mean TTR for the warfarin group was 55%. In the ARISTOTLE trial[5], warfarin was compared to apixaban 5-mg BID, in a two-arm trial (with a dose adjustment where 2.5mg BID was used in patients with two or more of the following criteria: age ≥80 years, a body weight <60 kg, or serum creatinine level of 133 μmol/L or higher; n= 831). The mean TTR for the warfarin group was 62.2%.

All tested NOAC doses were at least non-inferior to warfarin regarding the primary efficacy and safety endpoint and demonstrated statistically significant reduction in risk of intracranial haemorrhage (ICH)[54].

A meta-analysis of the 4 pivotal phase III trials (42411 patients on NOAC vs 29272 on warfarin), showed that NOACs significantly reduced stroke (RR 0.81, 95% CI 0.73-0.91; p<0.0001), all-cause mortality (0.90, 0.85-0.95; p=0.0003) and ICH (RR 0.48, 95% CI 0.39-0.59; p<0.0001) compared with warfarin. Low dose of NOACs demonstrated equivalent reductions in the primary endpoint compared to warfarin (1.03, 0.84-1.27; p=0.74) and a better bleeding profile (RR 0.65, 95% CI 0.43-1.00; p=0.05), but a higher incidence of ischaemic strokes (RR1.28, 95% CI 1.02-1.60; p=0.045). Thus, low dose NOACs is an option for patients with high risk of bleeding[83]. The benefits for efficacy and safety may be even greater in Asians, compared to non-Asians. In the meta-analysis by Wang et al[84], standard dose of NOACs found to reduce the incidence of stroke or SE (Odd Ratio [OR]=0.65 [0.52-0.83] versus 0.85 [0.77-0.93], P interaction= 0.045) and major bleeding (OR=0.57 [0.44-0.74] versus 0.89 [0.76-1.04], P interaction=0.004) more in the Asian compared to non-Asian population.

There are no direct head to head comparisons between different NOACs and indirect comparisons based on the phase III trials are not sufficiently reliable due to differences in the characteristics of the recruited populations, for example, demographic characteristics, comorbidities, risk factors for stroke and TTR[85]. The use of different NOACs in various subgroups of patients, participated in the above trials, was subject of several meta-analyses and post-hoc analyses. The results of these studies and knowledge of pharmacodynamics and pharmacokinetics of different NOACs can be helpful in guiding NOAC prescription in challenging clinical scenarios (Table 4)[85].

Nonetheless, randomized controlled trials recruit patient populations that may not strictly reflect patients seen routine practice[86]. Real-world observational studies and registries provide valuable confirmation of the trial results and give insight into potential inconsistencies between the data from daily clinical practice and the trials[87]. The first real world evidence cohort studies for NOACs showed lower rates of ischaemic stroke and bleeding compare to VKAs, however, they were criticized for selection bias[88]. Cross sectional studies provided further information about OAC practices, such as identifying NOAC underdosing and the OAC prescription trends[89,90].

Large registries, including the regional Dresden NOAC Registry, the national ORBIT AF I/II (Outcomes Registry for Better Informed Treatment of Atrial Fibrillation) registries and the multinational GARFIELD-AF (Global Anticoagulant Registry in the FIELD-Atrial Fibrillation), and GLORIA-AF (Global Registry on Long Term Oral Antithrombotic Treatment in Patients with Atrial Fibrillation) registries, presented useful assessments of cost effectiveness and outcomes[91–94]. Similar information with registries revealed by observational analyses from electronic health record studies[95–97].

In summary, multiple types of real world data are consistent with the results of the pivotal phase III trials of NOACS[87]. Despite limitations of the observational studies[98], these data provide an extra support to physicians for prescribing NOAC as first line OAC therapy and tailor OAC therapy according to patient risk factor profile[99].

Table 4. Pharmacokinetic properties and other profile features of Non-Vitamin K Oral Antagonists[25]

Drug	Dabigatran	Rivaroxaban	Apixaban	Edoxaban
Mechanism of action	Direct thrombin inhibitor	Direct factor Xa inhibitor	Direct factor Xa inhibitor	Direct factor Xa inhibitor
Fixed Dose	Twice daily	Once daily	Twice daily	Once daily
Half-life (h)	12–17	5–9	9–12	8–11
Time to peak effect (h)	2	2–3	1–2	1–2
Renal elimination	80%	33%	25%	35%
Drug interactions	Strong P-gp inhibitors and inducers	Strong CYP3A4 inducers, strong inhibitors of both CYP3A4 and P-gp	Strong inhibitors and inducers of CYP3A4 and P-gp	Strong P-gp inhibitors
Coagulation assay	aPTT, dTT Ecarin clotting time	PT Chromogenic anti-Xa assay	Chromogenic anti-Xa assay	Chromogenic anti-Xa assay

Abbreviations: P-gp-permeability glycoprotein, aPTT-activated Partial Thromboplastin Time, dTT-dilute Thrombin Time, PT-Prothrombin Time.

Table 5. Baseline characteristics and results of the phase III trials of the approved non-vitamin K Oral Anticoagulants against warfarin[5,80–82]

	Dabigatran			Rivaroxaban		Apixaban		Edoxaban		
Phase III trial	RE-LY			ROCKET-AF		ARISTOTLE		ENGAGE-AF		
Type of study	Randomized, open-label			Randomized, double-blind		Randomized, double-blind		Randomized, double-blind		
Dose	150mg BID	110mg BID	warfarin	20mg OD	warfarin	5 mg BID	warfarin	60mg OD	30 mg BID	warfarin
Participants	6076	6015	6022	7131	7133	9120	9081	7035	7034	7036
mean TTR	64%			55%		62.2%		68.4%		
Dose reduction				Rivaroxaban 15 mg once daily if CrCl 30-49 mL/min		Apixaban 2.5 mg twice daily if at least 2 of: age ≥80 years, body weight ≤60 kg or serum creatinine level ≥133 µmol/L		Edoxaban 30 mg reduced to 15 mg once daily, if any of the following: creatinine clearance of 30–50mL/min, body weight ≤60 kg, concomitant use of verapamil or quinidine or dronedarone		
% of elderly patients (≥75years)	40	38	39	43	43	31	31	41	40	40
% of patients with moderate CKD, 30ml/min≤CrCl≤50ml/min	19	19	19	21	21	17	17	20	19	19
Mean $CHADS_2$ score	2.2	2.1	2.1	3.5	3.5	2.1	2.1	2.8	2.8	2.8
	Event rate, %/year	Event rate, %/year	Event rate, %/year	Event rate, %/year	Event rate, %/year	Event rate, %/year	Event rate, %/year	Event rate, %/year	Event rate, %/year	Event rate, %/year
Stroke or systemic embolism	1.12 P<0.001 for non-inferiority and superiority	1.54 P<0.001 for non-inferiority	1.72	1.6 P<0.001 for non-inferiority	2.4	1.27 P <0.001 for non-inferiority, P = 0.01 for superiority	1.6	1.57 P <0.001 for non-inferiority	2.04 P = 0.005 for non-inferiority	1.8
Ischaemic stroke	0.93 P=0.03	1.34 P=0.42	1.22	1.34 P=0.58	1.42	0.97 P=0.42	1.05	1.25 P=0.97	1.77 P,0.001	1.25
Haemorrhagic stroke	0.1 P<0.001	0.12 P<0.001	0.38	0.26 P=0.024	0.44	0.24 P<0.001	0.47	0.26 P<0.001	0.16 P<0.001	0.47
Intracranial Haemorrhage	0.32 P<0.001	0.23 P<0.001	0.77	0.49 P=0.02	0.74	0.33 P<0.001	0.8	0.39 P<0.001	0.26 P<0.001	0.85
Major Bleeding	3.4 P=0.41	2.92 P=0.003	3.61	3.6 P=0.58	3.45	2.13 P<0.001	3.09	2.75 P<0.001	1.61 P<0.001	3.43
Gastrointestinal major bleeding	1.6 P<0.001	1.13 P=0.74	1.09	2.0 P<0.001	1.24	0.76 P=0.37	0.86	1.51 P=0.03	0.82 P<0.001	1.23
Myocardial infarction	0.81 P=0.12	0.82 P=0.09	0.64	0.91 P=0.12	1.12	0.53 P=0.37	0.61	0.7 P=0.6	0.89 P=0.13	0.75
All-cause mortality	3.64 P=0.051	3.75 P=0.13	4.13	1.87 P=0.07	2.21	3.52 P=0.047	3.94	3.99 P=0.08	3.8 P=0.006	4.35

Abbreviations: $CHADS_2$=stroke risk factor scoring system in which one point is given for history of congestive heart failure, hypertension, age ≥75 years, and diabetes, and two points are given for history of stroke or transient ischaemic attack, TTR=time in therapeutic range, OD= once daily, BID=twice daily, CKD= chronic kidney disease, CrCl= creatinine clearance

Antiplatelet Therapy

A meta-analysis of 11 randomised controlled trials demonstrated that adjusted-dose warfarin reduced the RR for all strokes by 37% (95% CI 23-48%) and for ischemic strokes by 52% (95% CI 41-62%) compared with aspirin[22]. From the same study, the absolute risk of ICH was only mildly increased from VKAs compared to aspirin (0.2% per year) with no overall mortality benefit from antiplatelets if they used for thromboprophylaxis in AF patients[22]. In the elderly, aspirin does not diminish the absolute risk of stroke and does not possess a lower risk for major bleeding or ICH[100]. Different doses of aspirin appear to have similar efficacy in AF thromboprophylaxis[101]. Other antiplatelets, such as clopidogrel are probably inferior to warfarin in the prevention of ischemic stroke and bear a risk of bleeding similar to that of aspirin monotherapy[102]. Aspirin combined with clopidogrel shows only modest benefit in stroke prevention compared with aspirin monotherapy in patients with AF[102].

Aspirin may be considered in combination with OAC plus clopidogrel in AF patients post myocardial infarction or percutaneous coronary intervention[103]. Addition of any antiplatelet drug to OAC increases the risk of bleeding[102]. The duration of the antiplatelet agents should be balanced between the risk of stroke and the risk of bleeding regardless the stent type[104]. In patients receiving VKA, the TTR should be maintained above 70% to avoid excess in stroke or haemorrhage[103]. The lower tested dose of the NOACs for stroke prevention is advisable (ie. Dabigatran 110mg BID, rivaroxaban 15 mg OD, apixaban 2.5mg BID, edoxaban 30mg OD), when they are used in combination with antiplatelet therapy. The combination of OACs with prasugrel or ticagrelor should be avoided[103].

1.2.6 Net clinical benefit of oral anticoagulants

A decision to advise OAC should be based upon the individual risks for stroke or other thromboembolic events and bleeding, with estimation of resultant net clinical benefit (NCB) of treatment. Whilst there are various ways to define NCB, it is most commonly estimated by balancing the risk of ischemic stroke without OAC against the risk of intracranial haemorrhage with OAC[105].

NCB is clearly in favour of OAC in patients with $CHADS_2$ score ≥1 or CHA_2DS_2-VASc score ≥2. NCB is positive or neutral for VKA therapy in patients with ≥1 stroke risk factor(s) (i.e. CHA_2DS_2-VASc score ≥1). Regardless of HAS-BLED score, there is negative NCB for OAC in patients with 'truly low risk' (i.e., CHA_2DS_2-VASc score 0)[106].

NCB analyses from recent randomised trials as well as modelling analyses applying the clinical trial data to patient cohorts all show a positive NCB for the NOACs vs warfarin, aspirin or no treatment. Indeed, one analysis estimated that the use of NOACs has lowered the threshold for starting OAC is an ischaemic stroke rate of 0.9 %/year[107]. In one modelling analysis with CHA_2DS_2-VASc=1, apixaban and both doses of dabigatran (110 mg and 150 mg bid) have a positive NCB. In patients with CHA_2DS_2-VASc≥2, all three analysed NOACs (dabigatran, rivaroxaban and apixaban) had NCB superior to warfarin, regardless of risk of bleeding as determined by HAS-BLED score[108,109].

1.2.7 Assessment of the effect of antithrombotics on the fibrin clot

Increasing evidence support that abnormalities in fibrin clot structure may reflect a potential risk for thromboembolic disease[110]. Many factors influence fibrin clot structure such as genetic, environmental, platelets, glucose concentration, smoking, inflammation and medications.[110]Atherothrombotic disease and clot structure characteristics outcomes have strongly been altered by oral antithrombotics. [111,112]

Oral antithrombotics may be divided in 2 broad groups, the antiplatelets and the anticoagulants. The main antiplatelet which has been used for decades as primary or secondary prevention of thrombosis related with atherosclerotic disease and as thromboprophylaxis for NVAF, is aspirin. Clopidogrel has also been used in conjunction with aspirin or alone, for the same reasons[113]. Despite that antiplatelets are not supported by strong evidence as mode of thromboprophylaxis in NVAF, they do affect the fibrin clot characteristics[114]. In contrast, VKAs and NOACs have robust data for significant reduction of embolic events in AF[25]. OACs affect the coagulation pathway by inhibition of different molecules in the fibrin polymerisation / fibrinolysis cascade (figure 1)[115]. Thromboelastography (TEG), a technique that measures the viscoelastic properties of fibrin clot in vitro, has been used to assess the clot structure under the influence of different conditions and antithrombotics[116]. Several useful information can be provided by TEG for the dynamics of fibrin polymerisation and lysis process[117]. However, TEG as a single method to interpret coagulation parameters has limitations and it is evident in clinical practise[118].

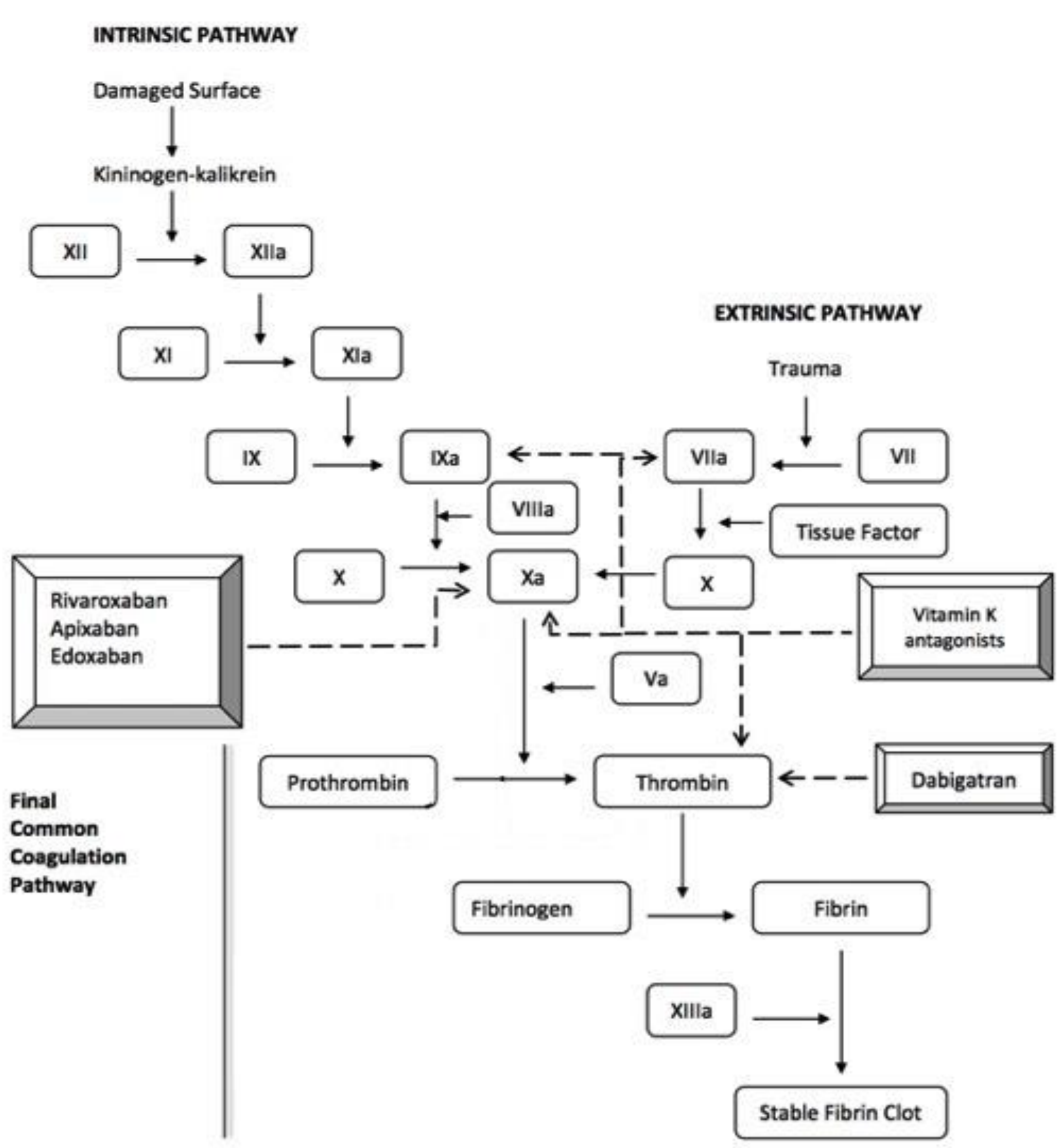
In research setting, another method to quantify fibrin polymerisation is turbidity analysis[119]. In a similar assay, when plasma of an individual is exposed to both hypercoagulable and

hyperfibrinolytic solution, it is possible to determine fibrin thrombus lysis potential. Fibrin clot structure has direct impact on fibrinolysis rate.[120] Both of these assays can be combined and performed in a 96 well plate [microplate assay (MPA)], so the amalgamation of turbidity and fibrinolysis can give a more comprehensive analysis of plasma clot structure properties[10].

MPA uses standard doses of exogenous thrombin and tPA to quantify turbidity and fibrinolysis potential of a plasma sample[10]. A complementary to MPA method for assessment of clot structure features, is by establishing the plasma concertation of tPA[121]. Increased levels of tPA are related with endothelial cell imbalance and reflect the presence of underlying endothelial injury[122]. Most of tPA in plasma circulates in complex with Plasminogen Activator Inhibitor 1 (PAI-1)[123]. The advantage of the complex is the bidirectional activity, mainly to prevent systemic haemorrhage while permitting local clot lysis. PAI-1 levels are significantly elevated during vascular injury and the presence of thromboembolic disease[124].

Different methods and clot phenotype indices have been used for the assessment of the clot structure, predominantly, in case-control studies. Turbidity, lysis assays, permeation and microscopy suggest that short lag phase of thrombosis and prolonged lysis time are consistently associated with the presence of cardiovascular diseases and worst outcomes.[110,125]

Figure 1. Oral anticoagulants and inhibitory activity on the coagulation pathway.



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1.3.1 Definition of microparticles and their nomenclature

Knowledge on extracellular vesicles have significantly expanded over the last 2 decades. However, their definition remains problematic. Even at the last meeting of International Society of Extracellular Vesicles in 2018, there was no definitive consensus about the definition of microparticles[126].

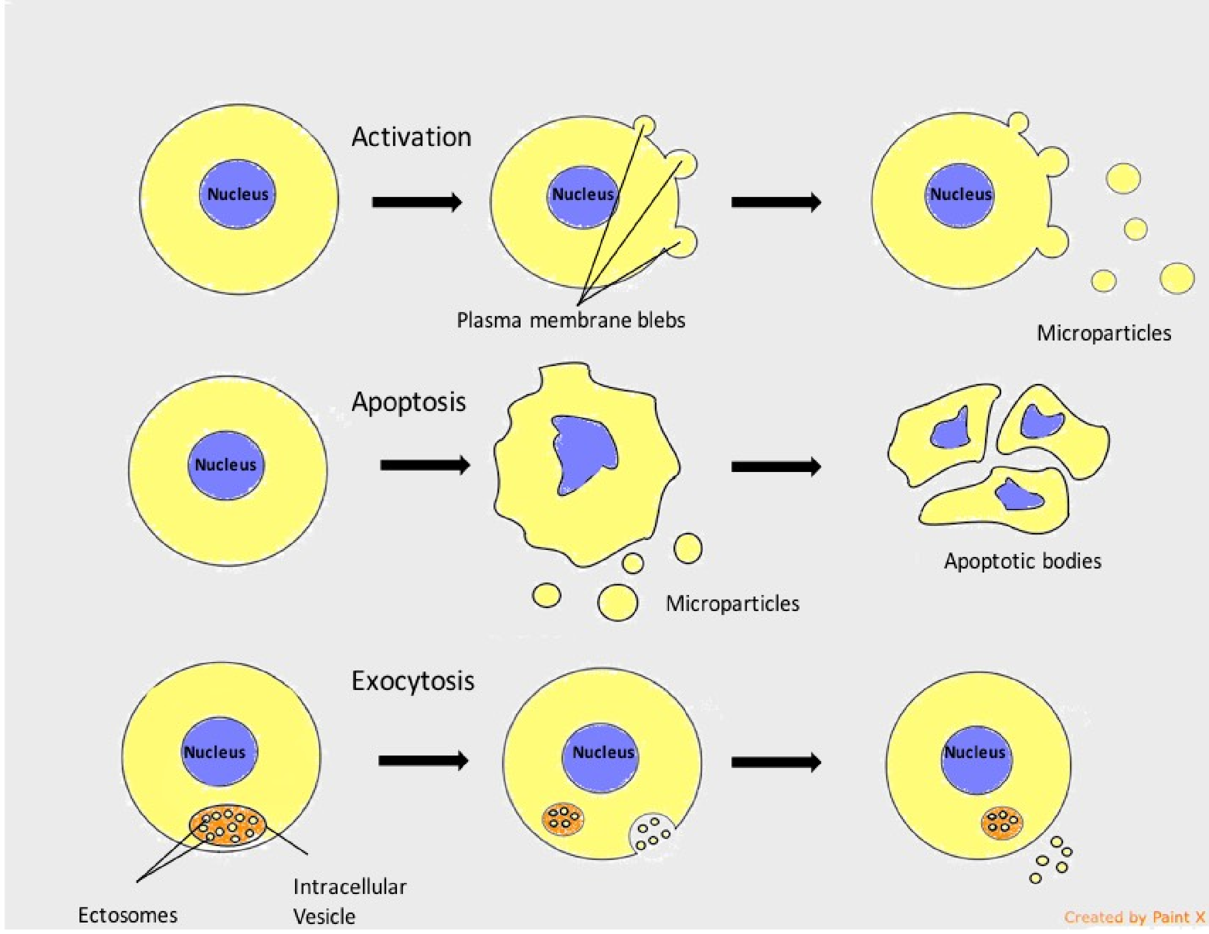
The broad term to describe particles released from cells by natural process such that their cytosol is enclosed by a lipid bilayer and lacking synthetic capacity, is “extracellular vesicles”. The term ‘microparticles’ has been used for a variety of extracellular vesicles in the past. The distinction of exosomes and ectosomes (Figure 2) is important in order to approach the nature of microparticles. Exosomes are formed after an inward bledding of the plasma membrane and stored into a bigger intracellular vesicle, the multivesicular body. Later, exosomes can be released in the extracellular environment by exocytosis. On the contrary, ectosomes are vesicles which directly released from the paternal cell by outwards rearrangement of the cellular membrane[127]. Of note, there is a significant overlap regarding size (diameter), membrane protein composition and cellular origin of the extracellular vesicles which at present makes difficult their categorisation[128]. Even their characterisation as ectosomes or exosomes is generally not advisable unless particle biogenesis is documented by a live imaging technique[126].

Other types of extracellular vesicles are the apoptotic bodies which have usually a diameter between 1-5μm. Formation of apoptotic bodies is exclusively linked to the latest stages of apoptosis where there is cellular shrinkage and nuclear fragmentation. Nuclear material, cell organelles and a permeable membrane are distinguishable characteristics of apoptotic

bodies[129]. In general, ectosomes are larger than exosomes (30-100nm) and smaller than apoptotic bodies (Figure 1). Additionally, the content may be different. For example, exosomes contain some membrane specific markers which are related with their formation process, such as lysosomal-associated membrane protein 1 and the membrane protein CD63. For microparticles, externalisation of the negatively charged phospholipid phosphatidylserine is the rule but for exosomes it is a rare structural condition. Also exosomes might contain cytosolic RNA but not like microparticles nuclear material[130]. The above "rules" have exceptions to the degree that specific identification criteria based on size and/or markers seems to be causing more confusion than consensus[131].

In this chapter, we have included research articles related with extracellular vesicles which their diameter is between 100nm-1μm and they have at least one marker to describe their membrane biochemical composition. Furthermore, the cell(s) of origin are usually known to be related either with the biomarker or with the experimental process and the analysis method. In most of the literature these vesicles are called "microparticles" and thus, we kept that term.

Figure 2. Extracellular vesicles origin



1.3.2 Biological functions of microparticles

Inducers and mechanisms of formation

Potentially, any cell of an eukaryotic organism can produce microparticles[132]. In the blood, the most common (70-90%) of the circulating microparticles are platelet derived microparticles. The rest of the blood containing microparticles are from endothelial, granulocyte, erythrocyte

and smooth-muscle cells[133]. Microparticles from epithelial, tumour cells, fibroblasts and other cellular origin have been also isolated[134,135].

Apart from the formation of the microparticles under normal circumstances, which is mainly linked with growth, differentiation and apoptosis[136], there are other non-physiological conditions that promote microparticle production. These conditions include hypoxia[137], shear stress[138], inflammation[139] and a variety of prothrombotic or proapoptotic factors[140](Table 6). Usually, the first result after exposure of the cell to these factors, is an increase of Ca^{+2} influx[141](Figure 2). High concentration of the intracellular Ca^{+2} induces molecular processes resulting in the release of the microparticles in the extracellular media[141].

The general assumption is that loss of the phospholipid asymmetrical set up of the plasma membrane, which is present during cellular relaxation, leads to the production of the microparticles[142]. Externalisation of phosphatidylserine, a negative charged phospholipid, primarily located on the inner surface of the plasma membrane of the non-activated cell, results in the membrane asymmetry[143]. Several phospholipid transporters regulate the inwards (flip) or outwards (flop) translocation of the plasma membrane lipids. An ATP-dependent "floppase" is responsible for the outwards translocation of the phosphatidylserine with simultaneous inhibition of flippase(s)[144]. Non-specific, bidirectional lipid transporters, the "scramblases" [145] along with the formation of transient membrane pores constitute another pathway of the membrane remodelling[146].

The intracellular influx of Ca^{+2} is also involved in the shaping of the plasma membrane protrusions which results in the formation of microparticles[142]. This process begins with degradation and reconfiguration of the cytoskeleton proteins[147]. The proteolysis of specific

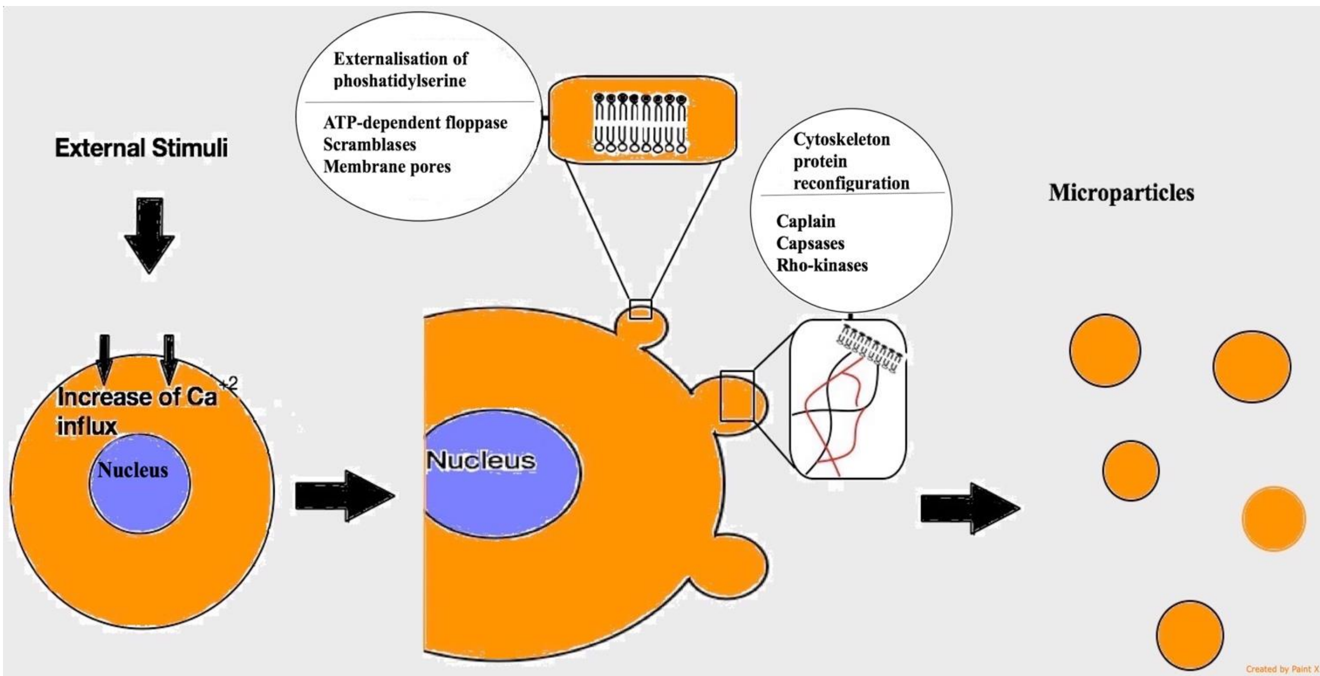
cytoskeleton network part, by calpain activation, causes separation of the membrane protrusion from the parental cell as independent vesicle into the extracellular media[148]. Another group of proteases, the capsases, is also associated with cytoskeleton ingredient lysis like talin, filamin, and gelsolin[149]. Capsases are involved in the actin-myosin cytoskeletal network reorganisation by interacting with various Rho-kinases isoforms[150,151]. Rho- kinases mediated microparticle shedding, with possible cellular nucleic acid redistribution, appears to be involved in apoptotic processes[152].

Phosphatidylserine exposure is commonly involved in microparticle formation from activated or apoptotic cells[153]. However, there are less controlled situations where stress or injury induce cellular necrosis and loss of membrane integrity with production of microparticles[154]. Also, there are populations of microparticles that are phosphatidylserine (-)ve (i.e., they do not bind Annexin-V), suggesting alternative formation processes[155]. Further evidence of other ion channel involvement in the microparticle formation cascade, apart from Ca^{+2}, support the presence of unknown mechanisms associated with plasma membrane shedding[156].

Table 6. Stimuli causing generation of microparticles in vitro or in vivo[157140]

Type of cell	**Endothelial cell**	**Platelet**	**Neutrophil**	**T-Cell**	**Monocyte and Macrophage**	**Smooth cell**
Stimuli						
	Modified LDL	Flow conditions	Pro-inflammatory cytokines	Pro-inflammatory cytokines	Cigarette extract	Modified LDL
	HDL cholesterol	Thrombin		Phytohemagglutinin	Pro-inflammatory cytokines	HDL cholesterol
	Uremic toxin	Collagen	Phorbol myristate acetate	Staurosporin	Calcimycin	
	Flow conditions	Homocysteine		Etoposide		Flow conditions
	Thrombin	Pro-inflammatory cytokines	Anti-neutrophil cytoplasmic antibodies	Actinomycin D	Lipopolysaccharides	Activated protein C
	Homocysteine	Calcimycin	Bacterial infection	Phorbol myristate acetate	Etoposide	Pro-inflammatory cytokines
	Activated protein C	collagen	N-Formyl-Methionyl-Leucyl-Phenylalanine		Fas Ligand	Fas-ligand
	Plasminogen activator inhibitor	Lipopolysaccharides				Platelet derived growth factor
	Pro-inflammatory cytokines[a]	Shiga toxin				
	Oxidative stress	sCD40 Ligand				
	High glucose	Erythropoietin				
	Uremic toxins	Noradrenaline				
	Lipopolysaccharides	acid phosphatase 5				
	Camptothecin	Reactive oxygen species				
	Angiotensin II					
	Plasminogen Activator Inhibitor 1					
	C Reactive Protein					

Figure 3. Mechanisms involved in the generation of the Microparticles



Structure and content

Microparticles contain a wide variety of biological molecules as part of their phospholipid membrane or within the cytosol that they enclose (Figure 4). These molecules are proteins (signal proteins, receptors, effector proteins), lipids and nucleic acids [158–160]. Various techniques have been tried in order to characterize the components of the microparticles [161,162]. Irrespective of the origin of microparticles, the plasma membrane is negatively charged due to translocation of phospholipids such as phosphatidylserine and phosphatidylcholine from internal to external surface[163,164]. Other phospholipids of the membrane include lysophosphatidylcholine, sphingomyelin, lysophosphatidylethanolamine, phosphatidylethanolamine, lysophosphatidylserine, and phosphatidylinositol[165]. It appears that

the bi-lipid layer of the microparticles affects the attached protein activities and the general properties of the vesicles[166].

The origin of the microparticles influences their composition. For example, platelet derived microparticles are enrich in various membrane proteins that are important in the coagulation process such us GPIb, GPII-IIIb, P-selectin, intergrins[167–169]. Similarly, microparticles from endothelial cells carry characteristic endothelial proteins (vascular endothelium cadherin, E-selectin)[170] and leucocyte derived microparticles are enriched in metalloproteinases and other proteolytic enzymes[171] involved in inflammation process. Antigenic clusters of differentiation (CD31, CD105, etc) which arise directly from the parental cell, are present in the plasma membrane of the microparticles[172] (Table 7).

The stimuli that triggers the formation of the microparticles regulates the ratio and the composition of the expressed membrane proteins. For example, monocytes have been stimulated by various substances in vitro (lipopolysaccharide, soluble P-selectin chimera, phosphate-buffered saline) and the produced microparticles expressing different membrane proteins. Similar findings were reported for microparticles derived from other cellular lines such as T cells[173], endothelial cells[172] and leucocytes[174]. However, all microparticles shared some common molecules[175].

The nucleic acids contained into the microparticles are usually result of apoptotic process[176]. Different types of RNA (ribosomal, micro and messenger) and DNA are enclosed into membrane vesicles which are protected from nuclease exposure and might be activated into the target cells. RNA packaging is influenced by the variation of the stimuli that trigger

microparticle formation[177]. This selective translocation of nucleic acids contributes to intercellular communication[178,179].

Figure 4. Microparticle content

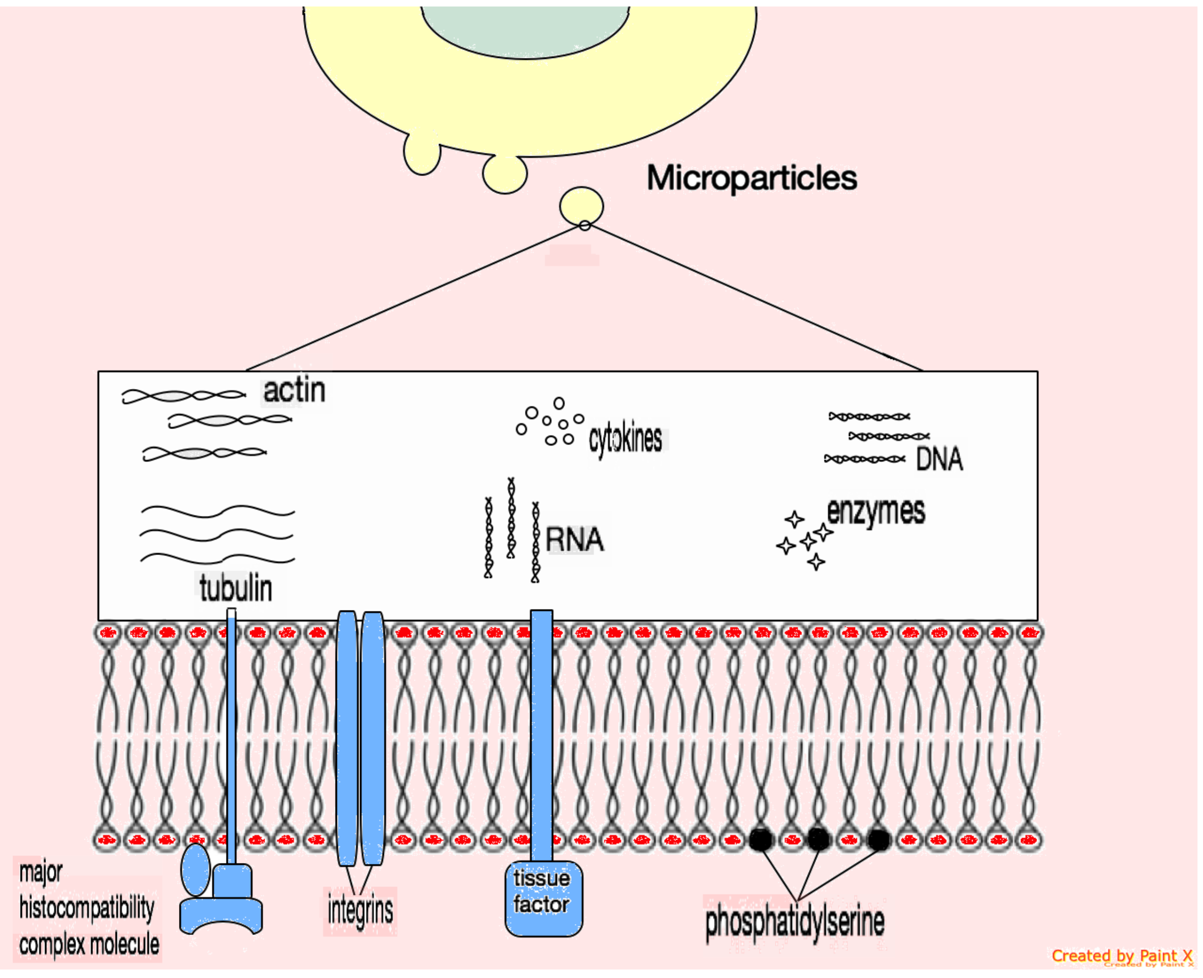


Table 7. Main antigen markers used for Microparticle cell origin determination

Cell	Cluster of differentiation (CD)
Endothelial	CD31[180]
	CD51[181]
	CD105[182]
	CD62E[182]
	CD144[183]
	CD34[184]
Platelet	CD41[185]
	CD42a[186]
	CD42b[187]
	CD61[184]
Red Cell	CD235[188]
Leukocyte	CD45[188]
Monocyte	CD14[189]
Neutrophil	CD66b[185]
T cell	CD4[190]
	CD8[191]
CD31, CD51, CD105 are not specific for endothelial cells. CD31 is also expressed on platelets, CD51 on platelets and macrophages and CD105 in activated monocytes/macrophages. For MP detection, markers are usually combined to discriminate this population from other MPs. For example, platelet MPs are CD31 positive / CD42b positive whereas endothelial MPs are CD31 positive / CD42b negative.	

1.3.3 Functions related with cardiovascular physiology

Transfer of Biological information

Several biological functions of microparticles can be summarised with the title “factors of intercellular communication and information exchange”. In principle, there are 2 ways microparticles may contribute to intercellular signalling. The first is mediated by activation of receptors on the plasma membrane of the target cell by presentation of molecules which result in alteration of the cellular function. The second way of interaction is by direct transfer to the target cell bioactive components such as proteins, lipids and nucleic acids (Table 8). The target cell can utilise these molecules which alters its biological function by activation of certain

pathways or by phenotypic modification[168,192,193]. Phenotypic modification is achieved usually by transferring membrane receptors to the recipient cell. These receptors interfere with stimuli that before the transfer did not influence cellular activity at all or not by the same way[194].

Proteins can also be carriers of biological signal. Apart from membrane proteins, microparticles might have proteins in their cytosol in various forms. After incorporation of the microparticles into the target cell by phagocytosis, the proteomic load can be in an activated form or can be cleaved and activated by proteolytic enzymes into the target cell[195,196].

Reverse transcription polymerase chain reaction and microarray analysis demonstrated that microparticles carry a specific subset of messenger RNA or microRNA from the origin cell[196]. By this manner, microparticles transfer to the target cell transcriptional information, analogous to the stimulating factor. The recipient cell will promote several processes, such as differentiation, proliferation and apoptosis by expressing different gene[197,198].

Lipids are not only components of the plasma membrane of the microparticles but actively determine the role of the ectosomes and their interaction with other cells[199]. This function is mediated by the surface provided by microparticle membrane and from bioactive lipids such as arachidonic acid, cyclooxygenase 2 and prostacyclin[200,201].

Table 8. Bioactive molecules of microparticles

Molecule	Type of cell producing Microparticles	Target cell or environment
Receptor/Membrane molecule		
chemokine receptor CCR5[194]	peripheral blood mononuclear cells	Various cells
CXCR4 receptor[202203]	Platelets	Various cells
Glycoprotein IIb/IIIa receptors[204]	Platelets	neutrophils
oncogenic receptor EGFRvIII (epidermal growth factor receptor variant III) [205]	Tumor Cells	Various cells
major histocompatibility complex (MHC) class II[193]	Immune cells	Immune cells
Tissue Factor[168]	Monocytes	Platelets
Peroxisome proliferator-activated receptor gamma[206]	Platelets	Monocytes
Cytokines		
interleukin-1beta[207196195208]	Various cells	Various cells
Chemokine (C-C motif) ligand 5[209]	Platelets	Endothelial cells
Growth factors		
Vascular endothelial growth factor[210211212]	Platelets/Tumor Cells	Endothelial cells
Basic fibroblast growth factor[210211]	Platelets/Tumor Cells	Endothelial cells
Platelet-derived growth factor[211]	Platelets	Endothelial cells
Lysis enzymes		
Matrix metalloproteinases[212]	Tumor Cells	extracellular matrix
Extracellular matrix metalloproteinase inducer[213]	Tumor Cells	extracellular matrix
Caspase 1[214]	Monocyte	Smooth muscle cell
Lipids		
Arachidonic acid[199200215]	Platelets	Various cells
Platelet activated factor[216217]	Various cells	Platelets
Ribonucleic acid (RNA)		
Messenger RNA[218158]	Stem cells	Various cells
Micro RNA[219220221]	Stem cells	Various cells

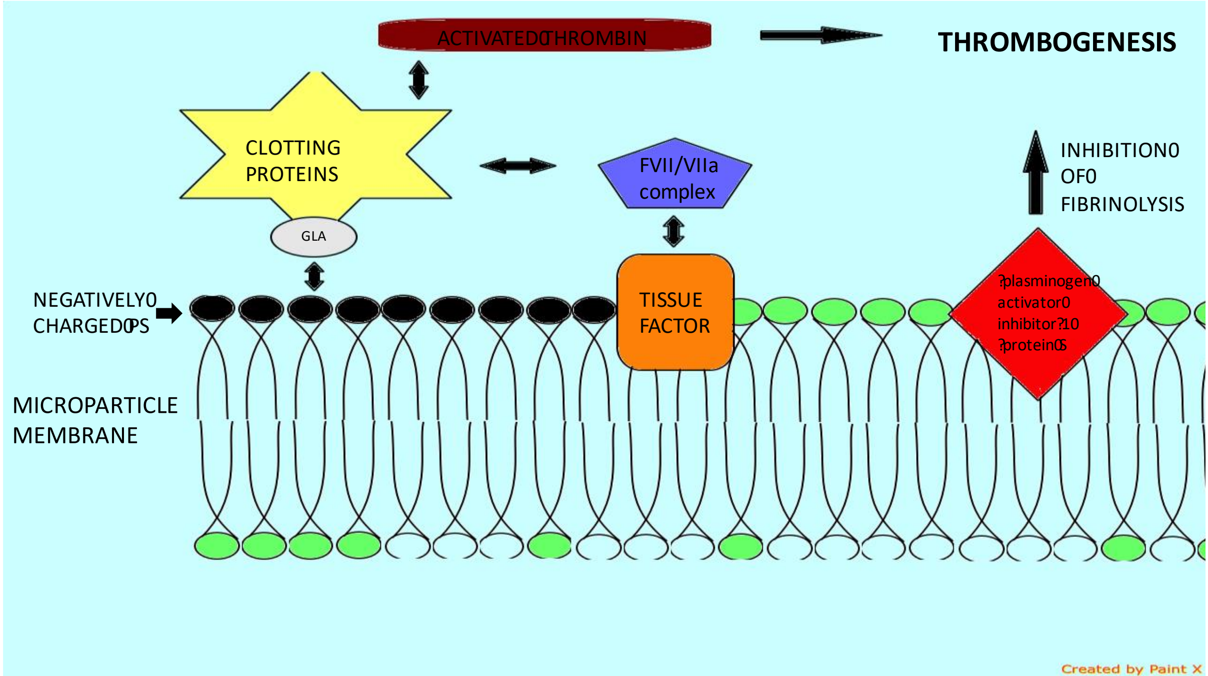
Coagulation

The involvement of microparticles in the coagulation process was noticeable from the time of their discovery[222]. The strongest procoagulant activity is mainly related to the negatively charged, external surface of their plasma membrane due to the presence of phosphatidylserine. The phosphatidylserine electrostatically attracts the positively charged segment of clotting proteins such as factors VII, IX and X, and prothrombin. The presence of γ-carboxyglutamic acid (GLA) domains creates the cationic features of these clotting factors[223](Figure 5).

Additionally, tissue factor as plasma membrane protein of the microparticles appears to play key role in the coagulation process. Tissue factor is an integral protein of the coagulation cascade and acts as a receptor of the FVII/VIIa complex, which activates both factors IX and X to initiate thrombin formation (figure 5). Tissue factor + microparticles may derive from monocytes, neutrophils, endothelial cells and platelets as response to various pathological conditions[224]. P-selectin, a cell adhesion receptor, interacts with tissue factor positive microparticles, through P-selectin glycoprotein ligand 1 (PSGL-1) on monocytes and causes further tissue factor positive microparticles generation which carry PSGL-1. These microparticles bind to activated platelets on the site of vascular injury and contribute further to thrombus expansion[225].

Finally, another mechanism of microparticles induced thrombogenic substrate is inhibition of the fibrinolytic process. Expression of proteins on the plasma membrane of microparticles like plasminogen activator inhibitor-1 and protein S, leads to amplification of thrombogenesis by suppression of fibrinolysis[226,227].

Figure 5. Mechanisms and molecules related with Microparticle induced coagulation



Inflammation and immune regulation

Microparticles possesses intercellular communication features which are related with the immune regulation. Immune and non-immune cells may produce microparticles which carry antigens. In this context, microparticles can influence immune responses to foreign[228] or self-antigens[229]. All immune cell types under certain stimuli can generate microparticles but the most effective, with regard to the regulation of immune response, are the "professional" antigen-presenting cells, such as dendritic cells, macrophages, and B cells[230]. This is achieved by binding the antigen to the cell surface or by phagocytosis[231,232].

Microparticles have pro-inflammatory effects mainly by inducing the production of cytokines and chemokines and by the activation of inflammatory cells[157](Table 9). This recruitment of

inflammatory mediators can be done without the presence of micro-organisms[233]. Administration of endothelial microparticles to rats is associated with release of pro-inflammatory cytokines IL-1β and TNF-α, acute lung injury and histological damage as evidence from the neutrophil infiltration into the perivascular space[234].

In vitro studies reported release of cytokines IL-6 and monocyte chemotactic protein from endothelial cells after stimulation by neutrophil microparticles[235]. Furthermore, pro-inflammatory cytokine secretion, such as IL-8, TNF-a and IL-1β, have been described from other cellular cultures after exposure to microparticles[163,236].

In addition to chemotactic factors, microparticles are involved in the production of specific cellular membrane proteins which promote adhesion of inflammatory cells to endothelium. Examples are the intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and E-selectin[215,237]. Bioactive lipids produced by platelet derived microparticles, like thromboxane A2 and cyclooxygenase, may act as mediators of inflammation. The target tissue is usually the endothelium[199].

Cases have been described where microparticles from polymorphonuclear leukocytes antagonise parallel pro-inflammatory stimuli by releasing cytokines like transforming growth factor beta 1. Polymorphonuclear derived microparticles also contain annexin 1, a protein with anti-inflammatory properties. Annexin-1 and transforming growth factor beta 1 inhibit macrophages. This action usually occurs during the early stages of the inflammatory process[238,239]. Microparticles from monocytes were found to induce macrophages and monocytes expression of peroxisome proliferator-activated receptor gamma (PPAR-γ) protein with anti-inflammatory action. In the same study, monocyte microparticles promoted

inflammatory [reactive oxygen species (ROS), cytokines] or anti-inflammatory (PPAR-γ) molecules in dose dependant manner[240]. Another mechanism which associated with polymorphonuclear derived microparticles mediated inflammatory process is activation of complement. Purified complement proteins like C1q from serum were found to be carried by polymorphonuclear derived microparticles[171].

Table 9. Inflammation and Microparticles (MPs)

Type of cell producing MPs	Target cell	Molecules involved in the pathogenesis of inflammation mediated by MPs
		Cytokines
Endothelial	Various inflammatory cells	IL-1β and TNF-α[234]
Leucocytes	Endothelial	IL-6 and MCP-1[235]
T cells	monocytes	IL-8, TNF-a and IL-1β[163236]
		Adhesion molecules
Monocytes Platelet	endothelial	intercellular adhesion molecule-1, vascular cell adhesion molecule-1 E-selectin[237215]
		lipids
Platelet	endothelial	thromboxane A2 and cyclooxygenase[199]
		Other
Polymorphonuclear	macrophage	transforming growth factor beta1[238]
		Annexin V[239]
Monocytes	macrophage	peroxisome proliferator-activated receptor gamma protein[240]
Polymorphonuclear	Various cells	complement proteins (C1q)[171]
Abbreviations: IL interleukin, TNF tumor necrosis factor, MCP-1 Monocyte chemoattractant protein-1,		

Angiogenesis

The platelet derived microparticles were the first group of microvesicles demonstrated angiogenetic properties in vitro. Platelets are known carriers of neovascularisation factors. Bioactive lipids from platelet derived microparticles (Table 10) are the main inducers of proliferation and tube formation in human umbilical vein endothelial cells[210,241]. Experiments in rat models reported angiogenesis in ischaemic myocardium after injection of platelet derived microparticles. The process was facilitated by vascular endothelial growth factor, basic fibroblast growth factor and inhibition of platelet factor-4[211]. Also, tissue factor + microparticles can induce endothelial cell proliferation through a beta 1-integrin and extracellular signal regulated kinase activation[242].

Another microparticle related neovascularisation mechanism is possibly associated with the morphogen Sonic Hedgehog pathway. T-cell derived microparticles, harbouring the Sonic Hedgehog antigen, promote in vitro and in vivo formation of new vascular network by regulating the nitric oxide pathway and stimulate genes coding expression of adhesion molecules and proangiogenic factors[243,244].

Endothelial derived microparticles have also angiogenic properties. Plasmin formation on the surface of the endothelial derived microparticles might activate proteolytic pathways and generation of factors which promote tube formation from endothelial cells[245]. In addition, matrix metalloproteinases carried by endothelial derived microparticles contribute to matrix-degrading proteolytic activity necessary for the angiogenic process[246]. Neovascularization programming by endothelial derived microparticles appears to be conducted in relation with direct transfer of mRNA to target endothelial cells which codifies the formation of capillary-

like structures[158]. In vivo demonstration of the angiogenic features of microparticles was reported by Leroyer et al[247]. Microparticles derived from ischaemic muscle injected into ischemic legs in a rat model resulted in enhanced neovascularization.

Adipose cell derived microparticles from rats was also found to be carriers of angiogenic molecules, such as leptin, fibroblast growth factor alpha (FGFa) and TNFa and in collaboration with tissue matrix metalloprotease (MMP)-2 and MMP-9 may promote neovascularization[248].

A combined signal transmission from microparticles that regulates the functions of angiogenesis, apoptosis, differentiation and migration might contribute to tissue regeneration and remodelling[249]. After permanent middle cerebral artery occlusion in rats, administration of platelet derived microparticles increased neurogenetic and angiogenetic activity, followed by behavioural improvement but no changes in infracted volumes[250].

The role of microparticles in angiogenesis is not always promotive but may be inhibitory. There are reports about prevention of neovascularization by microparticles. For example, vascular network proliferation from human umbilical vein endothelial cells was inhibited by the presence of endothelial derived microparticles[251]. Similarly, lymphocyte-derived microparticles were found to cause overexpression of the CD36 anti-angiogenic receptor while significantly downregulated protein levels related with angiogenesis[252]. The generation of reactive oxygen species such as hyperoxide is likely to be involved in the inhibitory process. The balance between inhibition and promotion of neovascularization, for different types of microparticles, appears to be affected by their concentration along with other potential unknown factors [246,252,253]

Table 10. Angiogenesis and Microparticles (MPs)

Type of cell producing MPs	Target cell or environment	Molecules involved in the angiogenesis mediated by MPs
Promotion of neovascularization		
Platelets	Human umbilical vein endothelial cells	bioactive lipids[210241]
Platelets	Ischaemic myocardium	vascular endothelial growth factor (VEGF), basic fibroblast growth factor (BFGF), inhibition of platelet factor-4[211]
Various cells expressing membrane Tissue Factor	Endothelial	beta1-integrin, extracellular signal regulated kinase[242]
T-cells	Endothelial	Activation of morphogen Sonic Hedgehog (Shh) pathway which promotes synthesis of adhesion molecules and proangiogenic factors[243244]
Endothelial	Endothelial	Plasmin activating factors[245]
Endothelial	Matrix	metalloproteinases[246]
Endothelial	endothelial	Messenger RNA[247]
Adipose cells	Human umbilical vein endothelial cells	Leptin, fibroblast growth factor alpha (FGFa), Tumor Necrosis Factor a, matrix metalloprotease (MMP)-2 and MMP-9 mediated activation[248]
Inhibition of neovascularization		
Lymphocyte	Endothelial	CD36 antiangiogenic receptor[252]
Abbreviations: CD cluster of differentiation, RNA Ribonucleic acid		

Regulation of vascular tone

Several studies have established a link between endothelial activity, expressed by modification of vascular tone and microparticles. Microparticles may affect the regulation of nitric oxide synthetase resulting in impaired production of nitric oxide in vivo[228,254]. Endothelial derived microparticles may also impair vasorelaxation. This was demonstrated in patients with end stage renal failure and type 2 diabetes, where different sonographic indices of arterial function have been assessed[181,255]. Furthermore, endothelial relaxation was impaired in aortic rings after exposure to endothelial derived microparticles from patients with recent myocardial infarction, in contrast with endothelial derived microparticles from non-ischaemic patients[256].

Lymphocyte derived microparticles also affect the nitric oxide synthetase activity[257,258]. The mechanism driven the synthetase downregulation is related with the phosphorylation of the extracellular signal–regulated kinase 1/2 via phosphatidylinositol-3-kinase and nuclear factor κ-light-chain-enhancer of activated B cell pathways[257]. Additionally, lymphocyte derived microparticles may induce endothelial overexpression of the integral membrane protein caveolin-1 which inhibits the nitric oxide synthetase[258].

Endothelial and platelet derived microparticles are carriers of endothelial nitric oxide synthase and in patients with cardiovascular risk factors the isolated microparticles found to have significant less levels of the synthase compared with healthy subjects[259]. Platelet derived microparticles can also influence the vascular tone as they are involved in the production of vasoactive molecules such as prostacyclin (vasodilator)[200] or thromboxane (vasoconstrictor) [260]. In experimental models, microparticles can modify cyclo-oxygenase metabolites levels through the Fas antigen and its natural ligand FasL pathway[261].

Apoptosis

Microparticles production can be the result from an apoptotic process along with the formation of apoptotic bodies. Additionally, microparticles can also induce programmed cellular death of remote cells[262,263]. Anti-apoptotic stimulation showed a reduction in the cell "blebbing" and microparticle formation in Human tonsil germinal center B cells in vitro. B cell blebs appear to have chemo-attractive properties to macrophages which carry out the apoptotic cell removal[264]. Endothelial derived microparticles can initiate apoptosis in angiogenic cells. The rich in arachidonic acid microparticles are phagocytosed by the angiogenic cells and signalise apoptosis. This action, as other characteristics of microparticles, is concentration dependant[265]. Apoptosis mediated by the lipid synthesis of the microparticle is also possible, even without involvement of phagocytosis. PtdIns(3,5)BP is a specific inhibitor of the acid sphingomyelinase which can inhibit the apoptosis pathway via upregulation of the capsase-8. Inhibition of the extracellular signal regulated kinase 1 prevents the apoptosis of macrophages in vitro. Extracellular signal regulated kinase 1 as microparticle membrane protein activate target cell membrane phospholipases and contributes to formation of arachidonic acid from phospholipids[266]. Capsase-3 protein is also involved in apoptosis[267] and has been identified as membrane protein in platelet derived microparticles and endothelial derived microparticles[268,269].

The signal for programmed cellular death to remote cells can be transmitted by microparticles through the Fas antigen and its natural ligand FasL. This mechanism was demonstrated in human tumour cells in vitro[270]. Additionally, tumour derived microparticles contain matrix

metalloproteinases responsible for matrix degradation but also adhesion molecules and receptors like the CX3CL1/fractalkine system which regulates migration and apoptosis[271].

Oxidative stress

Oxidative stress is caused when there is an imbalance between production of reactive oxygen species and the antioxidant defence mechanisms[272]. Controlled production of reactive oxygen species is important as contributes to cell growth, adhesion, differentiation, and apoptosis[273]. Brodsky et al[254] reported an active role of endothelial derived microparticles in the formation of superoxide. The p22(phox) subunit of NADPH oxidase has been detected in endothelial derived microparticles. Also, microparticles from other cellular origins, such as lymphocytes and monocytes, may lead to the production of reactive oxygen species[252,274]. Monocyte derived microparticles can induce nitrosative stress in endothelial cells in vitro. This occurs by increasing the nitration of several proteins in endothelial cells after regulating calveolin-1 expression or activation of phosphatidylinositide-3 kinase and other extracellular signal–regulated kinases[275].

1.3.4 Microparticles and cardiovascular risk factors

Essential Hypertension

Mechanisms linked with microparticles, have been described to be involved in the pathogenesis of endothelial dysfunction as a result of hypertension[276,277].

The Renin-angiotensin system in known to have a fundamental role in the regulation of arterial hypertension[278]. Angiotensin II, a potent vasoconstrictive hormone can induce the formation of microparticles from monocytes in vitro. The derived microparticles expressed tissue factor on their membrane and demonstrated procoagulant activity[279]. Procoagulant features of microparticles reported by Preston et al[280] in patients with severe hypertension. The hypertensive cohort had increased levels of platelet and endothelial derived microparticles with strong positive correlation between two types circulating microparticles (CD31+ endothelial derived microparticles and CD62P + platelet derived microparticles) and absolute levels of systolic and diastolic blood pressure. Tissue factor expression on endothelial derived microparticle and platelet factor 3 on platelet derived microparticles might explain the procoagulant features of microparticles and thrombogenicity of hypertension.

High levels of circulating endothelial derived microparticles with synchronous reduction of endothelial progenitor cells (EPC) as expressed by increased ratio of endothelial derived microparticles/EPC were detected in hypertensive patients with reduced glomerular filtration rate and microalbuminuria. The endothelial progenitor cells are considered to promote endothelial integrity and vascular repair. On the other hand, the CD31/annexin V+ apoptotic

microparticles were related with atherosclerotic disease and further deterioration of the renal function[281,282].

Diabetes mellitus

Endothelial derived microparticles(CD62E+) are higher in a pre-diabetic cohort along with elevated biomarkers of endothelial dysfunction[283](Table 11), suggesting an involvement of microvesicles in the pathogenesis of the disease. In patients with established diabetes, the absolute number of microparticles was also found to be elevated. Kurtzman et al[284] reported increased number of several microparticle phenotypes in diabetics compared with healthy controls[284]. Similar findings were demonstrated by a metanalysis of 48 studies involving 2,460 patients with Type 2 diabetes[285].

Different types of microparticles, such as endothelial, platelet, erythrocyte and monocyte derived microparticles have also been found to be elevated in diabetic populations[181,286–288]. Monocyte, endothelial and platelet derived microparticles are significantly higher in diabetic patients with related vascular complications such as nephropathy, retinopathy or neuropathy compared with diabetic patients without complications[183,287,289,290].

Patients with type 1 diabetes mellitus have not only higher but also different types of circulating microparticles compared with patients with type 2 diabetes mellitus and healthy people[185]. Platelet, endothelial and apoptotic cell (annexin V+) derived microparticles were significantly elevated in type 1 diabetes. The different microparticle phenotypes between the two conditions reflect differences in their functional and particularly their procoagulant properties. For type 2 diabetes patients, these microparticles have limited procoagulant action but for type 1 diabetes

their prothrombotic properties were positively correlated with the glycaemic control [HbA1c][185]. The relation between glycaemic control and microparticles have been demonstrated in a study of overweight subjects with type 2 diabetes. The levels of the circulating microparticles have been reduced after bariatric surgery with synchronous normalization of glycaemic control [189].

The pathophysiology of hypercoagulopathy in diabetes has been linked with the presence of microparticles[185,291,292]. In vitro studies have shown that microvesicles influence coagulation and immune pathways by abnormal production of reactive oxygen species along with protein expression on their membrane[292]. Tissue factor antigens on the membrane of circulating microparticles in patients with type 2 diabetes was reported by Cimmino et al[291] and is likely to be involved in the prothrombotic activity along with other athero-imflammatory processes observed in diabetic populations. Microparticle coagulability (expressed by the density of membrane tissue factor and membrane tissue factor pathway inhibitor ratio) was found to be high in diabetic patients with severe foot ulcers and manifestations of coronary artery disease[293]. Similar findings regarding hypercoagulability of plasma microparticles in diabetic patients reported by Tripodi et al[294] as measured and correlated by conventional coagulation tests such us antithrombin and protein C activity and levels of factors II and VIII.

Diabetic vascular complications are associated with vascular inflammation and endothelial dysfunction[295]. Endocytosis of platelet derived microparticles from endothelial cells in vitro induced expression of von Willebrand factor on the plasma membrane of the endothelial cells, which promoted adhesion of platelets and excessive production of reactive oxygen species leading to inflammation[187]. Microparticles mediated reactive oxygen species production in diabetics is involved in downregulation of NO activity affecting the vascular tone and

contribute to leukocytes chemotaxis to endothelium by expression of surface antigens[296,297]. In clinical studies, type 2 diabetes, is associated with high levels of endothelial/apoptotic cell derived microparticles and asymptomatic coronary atherosclerotic disease[298].

Microvesicles have been demonstrated to be involved in impaired neovascularization process affecting diabetic populations. Incubation of human umbilical vein endothelial cells with extracellular microvesicles from patients with diabetic foot or diabetic retinopathy induced the formation of tube networks, suggesting an important role of the vesicles in the process of angiogenesis[293]. Tissue factor positive microparticles in well controlled diabetic patients are not always involved in coagulation process and potentially have a role in signal transmission, including angiogenesis[190,299]. MiR-126, a type of RNA, which is contained in microparticles was reported to play important role in endothelial integrity and angiogenesis[300]. Reduced expression of miR-126 in endothelial derived microparticles from diabetic patients might contribute to endothelial injury, abnormal vascular remodelling and impaired angiogenesis[301,302].

Various medications were investigated regarding their effects in the levels of circulating microparticles in diabetes. Apart from the antidiabetic drugs, anti-hypertensives, statins and anti-platelets have been demonstrated to decrease the microparticles in various populations with diabetes[303] .

Table 11. Studies with Microparticles (MPs) in Diabetic populations or high glucose concentration conditions

Type of MPs	Conclusion	Reference
Endothelial derived microparticles CD62E +, CD62P +, CD142 +, CD45 + circulating MPs, their apoptotic (AnnexinV +) fractions and miRNA-126 expression.	CD62E + MPs level and miR-126-3p content in MPs are abnormal in subjects with pre-diabetes.	Giannella A et al[283]
CD3 + T- Lymphocyte MPs, CD105 + EMPs, Annexin V + MPs, CD31 + MPs, CD41a + and Annexin V/CD31/ CD41a +	Increased number of MPs in diabetic patients compare with healthy control group	Kurtzman N et al. [284]
PMPs	Association with vascular changes in T2DM/ endothelial dysfunction and activated platelets/PMPs	Nomura S et al.[286]
Monocytes derived MPs Annexin V/CD14 +, PMPs GPI +	higher in diabetic patients of with related vascular complications	Omoto S et al[287]
annexinV +, PMP CD31/CD42 +, LMP CD45+ , CD31+/CD42negative EMP, CD51+ EMP	MPs increased in patients with T2DM. EMPs levels are associated with vascular dysfunction.	Feng B et al[181]
CD36 +	CD 36 + MPs in DM patients were from erythrocyte origin compare with healthy subjects, originated from endothelial cells.	Alkhatatbeh MJ et al[288]
PMPs CD41 +, EMPs CD51 +, leukocytes derived MPs CD45 +, neutrophil derived MPs CD66b +, monocyte derived MPs CD14 + and total annexin V-+ MPs.	Different phenotypes identified between T1, T2 DM and healthy subjects. Differences in properties and particularly the procoagulant activities.	Sabatier F et al[185]
Annexin V +, MPs from non- activated platelet (CD41+), MPs from activated platelets (CD62p +), EMPs (CD144 +)	MPs properties and type (composition, content and cellular origin) are related with the type of vascular complications due to DM.	Tsimerman G et al[293]
MPs from Human umbilical vein endothelial cells	Raised glucose levels is a potent stimulus for MP formation that affects their molecular composition and may cause endothelial injury	Burger et al. [292]
PMPs CD41 +, Annexin V MPs, MPs expressing tissue factor (CD142)	DM is associated with high levels circulating MPs with procoagulant features	Tripodi A et al[294]
EMPs CD144 +, PMPs CD42b +, monocyte derived MPs CD14 +	MPs from T1DM patients promoted platelet/endothelial cell interaction with an intensity correlated with the degree of the associated vascular complications	Terrisse Ad et al[187]
Lymphocyte and plasma MPs (Surface markers: CD3, CD11a, GP1b, CD31)	Lymphocyte-derived MPs from diabetic patients or in	Martin S[258]

	vivo circulating MPs from diabetic patients reduced endothelial NO synthase expression.	
Surface markers: CD41a, CD64, CD144, CD144/CD31, Annexin V, CD144/annexin V and CD144/CD31/annexin V.	Apoptotic endothelial cell-derived were significantly increased in diabetic patients and associated with asymptomatic atherosclerosis	Berezin et al. [298]
human coronary endothelial cells derived MPs	High glucose environment increases NADPH oxidase activity in EMPs and promotes endothelial dysfunction	Jansen F et al[297]
EMPs (surface markers: CD31, CD42b, annexin V, and CD62E)	EMP levels are associated with different risk of diabetic vascular complications	Jung K-H et al[180]
PMPs CD41 (GPIIb) +, EMPs CD144 (VE-Cadherin) +	Unstable coronary artery plaques in diabetics are associated with elevated EMPs	Bernard S et al[183]
PMPs (Surface marker: antiplatelet GPIX monoclonal antibody)	Plasma PMPs are significantly higher in patient with DM compare with normal controls. Antiplatelet therapy reduces the level of PMPs.	Omoto S et al[289]
EMPs CD 144 +, Monocyte derived MPs CD 14 +, PMPs tissue factor and CD 41 +	Normalisation of glycaemic control in DM patients after bariatric surgery leads to reduction of the MPs levels	Cheng V et al[189]
PMPs, T-lymphocytes derived MPs and leukocyte derived MPs (surface markers: quadruple-stained with annexin V, CD61, anti-TF, and CD15 (ligand for P-selectin), CD66e (granulocytic marker), or CD62P (P-selectin), or with CD4 (T-lymphocytes), anti-TF, and CD11b (leukocyte marker)	TF on MPs from DM patients may be involved in processes other than coagulation such as angiogenesis	Diamant M et al[190]
TF bearing MPs from human renal mesangial cells and human dermal microvascular endothelial cells	MPs expressing TF might be a mediator to neovascularisation due to elevated glucose levels	Ettelaie C et al[299]
MPs from Human umbilical vein endothelial cells	Reduction of miR-126 from plasma vesicles might explain the impaired neo-angiogenesis in DM patients	Zampetaki A et al[301]
CD cluster of differentiation, EMP endothelial derived MP, PMP platelet derived MP, T2DM Type 2 diabetes mellitus, , GP glycoprotein, LMP leukocyte derived MP, TF Tissue factor, miR micro Ribonucleic acid, NADPH Nicotinamide adenine dinucleotide phosphate, NO Nitric monoxide		

Smoking

Smokers have significantly higher levels of plasma tissue factor concentrations[304]. Human monocytes and macrophages produce microparticles and demonstrated apoptotic activity in vitro, after exposure to tobacco smoking extract. These microparticles were found to be tissue factor positive, reflecting their procoagulant properties[305].

Smokers with normal spirometry but reduced diffusing capacity of the lung for carbon monoxide have also elevated levels of endothelial derived microparticles, likely derived from apoptotic endothelial capillary cells[306]. Another mechanism which appears to be induced by tobacco smoke inhalation is microparticle gelatinolytic and collagenolytic activities. After exposure to tobacco smoking extract in vitro, human macrophages produce microparticles with transmembrane matrix metalloproteinase 14 (MMP14). These microparticles may induce extracellular matrix destruction leading to inflammation, atherosclerotic plaque vulnerability and tissue necrosis[307]. Elevation of endothelial derived microparticles was also observed in passive smokers along with endothelial dysfunction, as assessed by flow-mediated dilation using ultrasound. In the same study, the endothelial progenitor chemotaxis toward vascular endothelial growth factor was impaired resulting in reduction of nitric oxide production and endothelial dysfunction[308].

Dyslipidaemias

Endothelial derived microparticles were found to be elevated in patients with uncomplicated type 2 diabetes mellitus after consumption of high-fat meals. This elevation was correlated with other dysmetabolic changes such as high levels of glucose, insulin, and triglycerides and

low levels of high density lipoprotein[309]. Hypercholesterolaemia along with endothelial derived microparticles has inhibitory activity in cardiac angiogenetic mechanisms through imbalance of the endothelial nitric oxide synthetase regulation[310]. Hypercholesterolaemic conditions may induced endothelial damage associated with microparticles but there is evidence that can also promote generation of prothrombotic vesicles. Aggregated low density lipoprotein was found to induce release of tissue factor positive microparticles from human vascular smooth muscle cells[311]. Similarly, monocytes enriched by cholesterol in vitro, appear to expose phosphatidylserine on their cellular membrane along with induction of apoptosis and release increased levels of tissue factor positive microparticles[312]. Additionally, phosphatidylserine expression mediated by oxidative low density lipoprotein in diabetic population was associated with elevated levels of platelet derived microparticles[313].

The relation between the endothelial protective role of high density lipoprotein and microparticles has been investigated[163,314]. High density lipoprotein was reported to inhibit the binding of the T cell derived microparticles to monocytes and sequelae monocyte activation. Furthermore, high density lipoprotein partially inhibited production of pro-inflammatory cytokines from monocytes.

In several studies which recruited patients with type 2 diabetes and hyperlipidaemias there was a reduction of the circulating microparticles after treatment with statins. For example, in patients with type 1 diabetes mellitus and hyperlipidaemia, treatment with atorvastatin reduced gpIIIa, P-selectin- and tissue factor-containing microparticles[315]. For patients with type 2 diabetes and hyperlipidaemia, treatment with pravastatin for 8 weeks did not significantly alter the blood cholesterol concentrations but reduced the gpIIb/IIIa membrane receptor in the circulating platelet derived microparticles. GpIIb/IIIa is an important receptor for fibrinogen

involved in thrombus formation. Downregulation of this receptor which is partially induced by changes to platelets and microparticles lipids membrane composition might contribute to less thrombotic risk[191]. Fluvastatin reduced microparticles in vitro from cultured human coronary artery endothelial cells by inhibition of the Rho kinase pathway which is responsible for alteration of cytoskeleton[316].

High triglycerides levels is a component of metabolic syndrome among with hyperinsulineamia, hypertension and low high-density lipoprotein cholesterol levels[317]. Elevation of endothelial[318], platelet[319] and leukocytes derived microparticles[320] in patients with metabolic syndrome contribute to vascular inflammation and hypercoagulant status [261]. In an observational study[321], patients with metabolic syndrome and dyslipidaemia have higher levels of endothelial derived microparticle (CD144+) and erythrocyte microparticle (CD235a+) compared with healthy control subjects. Obesity influenced the levels of platelet (GpIIb/IIIa+) and endothelial derived microparticles and hypertension only the endothelial derived microparticles. Levels of Annexin V+ microparticles were affected by each of the different components of metabolic syndrome.

1.3.5 Microparticles and atherosclerosis

Various mechanisms contributing to initiation, progression and clinical manifestations of atherosclerotic disease are associated with the presence and formation of microparticles (Figure 6). The disruption of the normal levels and composition of the microparticles might represent one of the initial steps of the atherosclerotic disease. The link between apoptosis and microparticle production is well-established[262]. Reduced laminar shear stress is also reported

as a signal for endothelial apoptosis[322] which potentially contributes to microparticle production and imbalance of the normal endothelial features[323].

Endothelial permeability occurs during the first phase of the atherosclerotic process[324]. Findings suggest a link between endothelial permeability and microparticles. Injection of endothelial derived microparticles in rats significantly increases the pulmonary capillary permeability and causes acute lung injury. This action is likely mediated through inhibition of nitric oxide generation and sequelae impaired vasodilation[325]. Abnormal endothelial homeostasis related with nitric oxide production have been reported with endothelial derived microparticles[256] and T lymphocyte-derived microparticles in vitro[258]. Another mechanism, induced by CD54 antigen, might play a role in atherogenesis. It was described in patients with multiple sclerosis where endothelial derived microparticle CD54+ induce inflammation and increase migration of monocytes through the endothelium[326]. Additionally, platelet derived microparticles can affect the endothelial cell barrier integrity, in a manner related with their size and protein composition[327].

Chemo-attraction of leukocytes to the inflamed endothelial segment is essential for the progression of atherosclerosis[328]. Microparticles from different cellular origins may trigger production of pro-inflammatory cytokines from the endothelium, such as IL-6 and IL-8, which attract and activate leukocytes[215,329]. Another suggested mechanism which contributes to the progression of the atheroma is linked to microparticles-induced expression of adhesion molecules on the endothelial cells. An example is platelet derived microparticles mediated upregulation of intercellular adhesion molecule-1 on the endothelial cell membrane[215,330]. Plaque microparticles isolated from endarterectomy specimens could transfer ICAM-1 to endothelium[331]. Also microparticles induce integrin expression on the surface of the

leukocytes, such as CD11a and CD11b, which interact with intercellular adhesion molecule-1[215]. Chemokines delivered from microparticles to inflamed or atherosclerotic endothelium promote further leukocyte recruitment. Mause et al described a platelet derived microparticle-associated delivery of the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) to human microvascular endothelial cells which promotes monocyte adhesion[209].

Microparticles concentrations are 200 times higher in atherosclerotic plaques than in blood[332]. Microparticles derived from leukocytes have the higher levels in plaques; 29% found to be from macrophages, 15% from lymphocytes and 8% from neutrophils. Other significant populations of microparticles concentration delivered from erythrocytes (27%), smooth muscle (13 %) and endothelial cells (8%)[332]. Microparticle origin in atherosclerotic plaques was not found to be affected by symptoms of ischaemia[332]. All plaque microparticles, regardless their origin, possess pro-coagulant activity as they express tissue factor on their external plasma membrane surface[333].

Furthermore, atherosclerotic plaque microparticles was described that contain immunoglobulins. The immunoglobulins they expressed were found to be different from the plasma circulating microparticles[334]. Co-labelling of IgG and CD14 demonstrated that the vast majority of microparticles (93±7%) containing IgG were CD14+, revealing their macrophage origin[334]. High macrophage infiltration was observed also in raptured atherosclerotic lesions with concurrent macrophage apoptosis[335]. Microparticles are involved in macrophage apoptosis but is unknown if this mechanism contributes to rapture or characterise vulnerability of the fibrous cap of the atheromatous plaques[336]. Additionally, as macrophages have been involved in the clearance of microparticles, defective phagocytosis due to increased

macrophage apoptosis may lead to accumulation of microparticles[337–339]. A point towards this hypothesis is the acceleration of atherosclerotic lesions in mice which lack lactadherin activity[340]. Lactadherin is essential protein for the removal of the microparticles[341].

Atherosclerosis is a complex immune-inflammatory disease which several type of cells are involved, including lymphocytes (T, B, Natural killer T), dendritic cells, and mast cells[342]. In vitro, endothelial derived microparticles induced dendritic cell maturation and secretion of pro-inflammatory cytokines, contributing to CD4 T cells activation and proliferation[343]. In the same study, microparticles from activated T cells or platelets failed to stimulate dendritic cell maturation[343]. Polymorphonuclear neutrophil-derived microparticles were reported to interact with human monocyte-derived dendritic cells and to promote morphologic changes which reduce monocyte phagocytic activity and increase cytokines excretion[344]. Additionally, microparticles from activated dendritic cells can interfere with resting dendritic cells and transfer antigens to them[345]. These antigens can be presented to lymphocytes leading to their activation and proliferation[345]. Microparticles from T cells may also stimulate mast cells. This action can take place by transfer of membrane biomolecules via microparticles instead of cellular contact[346].

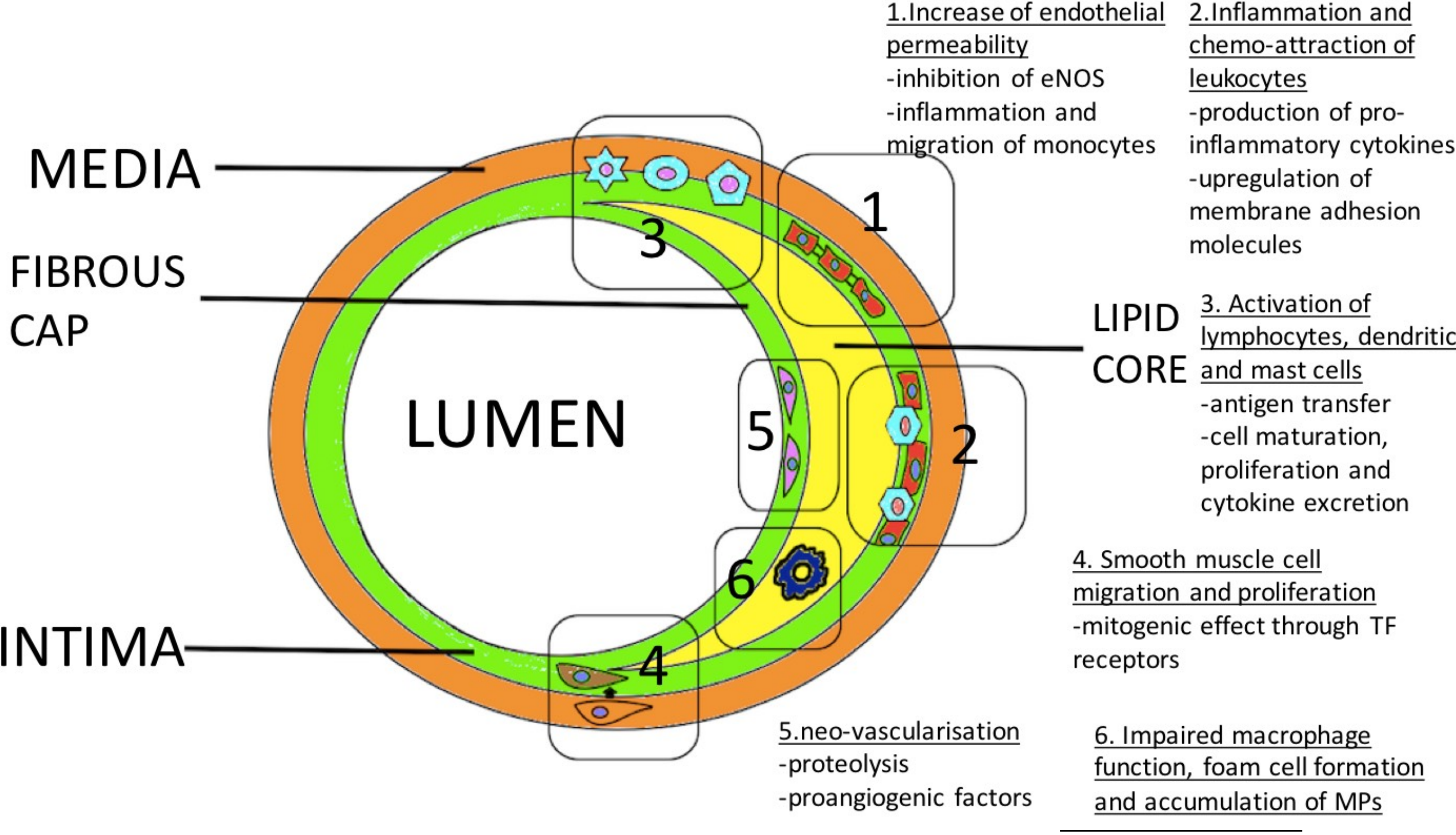
Smooth muscle cell proliferation and migration from the media to intima is essential for the formation of the atheroma[328]. Platelet derived microparticles were found to have mitogenic effect on smooth muscle cell in vitro, with no chemotactic contribution[347]. The mitogenic effect increased synergically if platelet derived microparticles were combined with serotonin or thromboxane A2[348]. The chemotaxis for smooth muscle cell migration is strongly facilitated by tissue factor receptors[349,350]. Microparticles isolated from atherosclerotic plaques express

tissue factor in their surface[333] and might act as a chemo-attractive factor for smooth muscle cell migration and proliferation.

The intraplaque neovessel formation due to hypoxia and inflammation influences the stability of the atherosclerotic lesion[351,352]. Microparticles might play a significant role in the neovascularization. They can carry proteolytic enzymes on their membrane and they have ability to induce the production of metalloproteases from other cells, so the endothelial tissue would be able to penetrate the surrounding interstitial matrix[353–355]. Furthermore, plaque microparticles mainly of macrophage origin can induce endothelial cell proliferation in vivo[356]. On the contrary, in this study circulating microparticles were unable to induce endothelial cell proliferation[356]. CD40/CD40 ligand system appears to play an important role in the microparticles endothelial cell interaction[356]. Patients with symptoms of cardiac ischaemia expressed more CD40L than asymptomatic patients and their microparticles were more potent endothelial proliferation inducers in vitro[356].

Studies of patients with early atherosclerosis and chronic coronary artery disease showed increased levels of CD144+/CD31+ endothelial derived microparticles expressing T-cadherin compared with healthy volunteers. T-cadherin was found to reflect endothelial dysfunction as measured by reactive hyperaemia following brief peripheral flow occlusion[357]. Plasma levels of CD144+/ CD42b- endothelial derived microparticles predicted the presence of coronary artery disease in asymptomatic diabetic patients [358].

Figure 6. Microparticles and atherosclerotic process



1.3.6 Atrial Fibrillation

Patients with atrial fibrillation have increased risk of ischaemic stroke and were found to have higher levels of platelet derived microparticles (CD42b+/CD61+) in contrast with healthy subjects. There was no difference in the levels of circulating platelet derived microparticles between patients with permanent or paroxysmal atrial fibrillation or between patients on treatment with aspirin or warfarin. In the same analysis there were no significant differences in platelet derived microparticles for patients in atrial fibrillation and patients with established coronary artery disease and sinus rhythm[18]. In another observational study, annexin V+ microparticle levels were higher in atrial fibrillation patients compare with healthy controls and with patients with sinus rhythm but cardiovascular risk factors (disease control). On the contrary, platelet derived microparticles (anti-glycoprotein Ib+) and endothelial derived microparticles (CD31+) were similar in patients with AF and disease control group but higher compared with healthy control subjects[19]. A recent study investigating microparticles of the same origin reported opposite results[359]. Platelet (CD41+) and endothelial (CD31+ / CD41-) derived microparticles were higher in AF patients but annexin+ microparticles were similar compared to healthy subjects. The discrepancy from the above studies is likely related with different methodology. The presence of higher levels phosphatidylserine+ microparticles, which are labelled with annexin V, has been reported in other studies with NVAF subjects. Blocking the phosphatidylserine with lactadherin resulted in inhibition of plasma procoagulant activity[360].

P-selectin microparticles were also found to be elevated in AF cohorts. P selectin has an essential role in activation of leukocytes in inflammatory process and platelets in vascular injury[361,362].

In patients with chronic AF due to mitral stenosis CD41+ platelet derived microparticles levels were higher compared to healthy controls and there was a significant direct relationship between the mitral valve area and the levels of circulating platelet derived microparticles[363], suggesting higher risk of thromboembolic events with increasing severity of mitral stenosis.

Induction of AF during electrophysiology studies increased the P-selectin+ microparticles compared with chronic AF and control patients which might reflect different procoagulant mechanism between chronic and paroxysmal AF[362].

1.3.7 Prognostic value of microparticles related with cardiovascular diseases

Several studies have demonstrated an association between microparticles and outcomes in patients with cardiovascular diseases (Table 10), with coronary artery disease is the most studied condition. From an observational study that recruited patients with cardiovascular risk factors and stable coronary artery disease, CD31+/annexin V+ microparticles (apoptotic endothelial & platelet derived microparticles) levels were correlated with higher risk of major adverse cardiovascular and cerebrovascular events and the need for revascularization[364]. In patients with ST elevation myocardial infarction post primary angioplasty, high levels of monocyte derived microparticles were strongly related with poor long term survival[365]. Additionally, post angioplasty levels of erythrocyte derived microparticles in ST elevation myocardial infarction patients were higher compared to healthy individuals and associated with higher risk of major adverse cardiovascular and cerebrovascular events[366]. Increased tissue factor+ microparticle levels were also related with poor outcomes in patients with acute coronary syndrome (unstable angina or acute MI)[367].

Montoro-Garcia et al[368] reported that in patients with non-ST elevation myocardial infarction, levels of endothelial and monocyte derived microparticles were associated with a higher risk of future admissions due to left ventricular failure and levels of platelet derived microparticles with a higher risk of major haemorrhagic events[368]. Higher counts of endothelial derived microparticles were also associated with worse outcomes in patients who presented with cardiac sounding chest pain and underwent coronary angiography for further evaluation[369].

Apart from coronary artery disease, several studies reported association between microparticles and outcomes for patients with heart failure. In acute decompensated heart failure due to coronary artery disease the levels of endothelial derived microparticles (CD31+/Annexin V+) were related with increased mortality within the 3-year follow up period[370]. Bulut et al[371] reported similar findings in patients with ischaemic cardiomyopathy. The ratio of endothelial progenitor cells to endothelial derived microparticles (CD31+/Annexin V+) was decreased in the cardiomyopathy group compared with patients with stable 3 vessel coronary artery disease and preserved left ventricular systolic function, suggesting that apart from endothelial dysfunction there is impaired vascular repair capacity. Worse outcomes were also described in patients with left ventricular assisting devices and elevated phosphatidylserine/Annexin V+ microparticles[372]. Finally, patients with pre-capillary pulmonary hypertension prior to treatment, high levels of CD62E+ endothelial derived microparticles were associated with higher risk of death and hospitalization due to right heart failure[373].

Table 12. Studies where Microparticle levels are associated with outcomes

Type of MPs	Prognostic value	Reference
Annexin V-binding MPs, MPs CD42b +, EMPs CD144 + and monocyte derived MPs CD14 +	In NSTEMI, EMP and monocyte derived MPs were independently predictive for future admissions related to heart failure	Montoro-García Set al[368]
EMPs apoptotic CD31/Annexin V +	CD31/Annexin V + MPs is an independent predictor of MACCE in stable CAD patients	Sinning J-M et al[364]
Monocyte-derived MPs (Surface markers: CD14+, CD14/CD11b and CD14/CD142)	Monocyte-derived MPs levels assessed in the acute phase of STEMI are related to the prognosis of long-term CV death	Chiva-Blanch G et al[365]
Annexin V +, CD41 + PMPs, CD235a + erythrocyte derive MPs	CD235a + erythrocyte derive MPs concentrations appear to be +ly associated with MACCE in patients with STEMI and PPCI	Giannopoulos G et al[366]
Surface markers: Annexin V, TF, CD41, CD66b	In the plasma of patients with ACS, the TF activity is a consequence of circulating MPs and is an independent predictor for MACCE	Steppich BA et al[367]
EMPs CD146, CD31	Higher EMP levels had a higher risk of MACCE in ACS patients.	Fan Y et al[369]
EMPs CD31/Annexin V +	EMPs levels were associated with increased mortality and recurrent hospitalization due to CHF	Berezin AE et al[370]
Apoptotic MPs Annexin V +	elevation of apoptotic MP levels in LVAD-supported patients are associated with increased risk for adverse events.	Nascimbene A et al[372]
EMPs CD62e +, CD144 +, CD31 + (+)/CD41 negative leukocytes-derived MPs CD45 +	Elevated levels of CD62e + EMPs in PHTN patients prior to treatment are associated with adverse clinical events	Amabile N et al[373]
Abbreviations: MACCE Major adverse cardiovascular and cerebral event , CD cluster of differentiation, EMP endothelial derived MP, NSTEMI non-ST elevation myocardial infarction, CAD coronary artery disease, STEMI ST elevation myocardial infarction, CV cardiovascular, TF tissue factor, ACS acute coronary syndrome, LVAD Left ventricular assisting device, PHTN pulmonary hypertension		

1.3.8 Isolation and Detection methods

Pre-analysis issues and isolation

Although microparticles can be found in all biological fluids, in this chapter we will focus on blood sample analysis. Blood is the major bio-fluid and studies using blood samples for microparticles analysis dominate the literature. The need of standardization of microparticles processing techniques was emphasised in the special workshop of the International Society for Extracellular Vesicles (ISEV) in New York City in October 2012. The report from the workshop attempted a standardisation starting from sample handling. Of note, for many questions consensus has not been achieved[374]. Blood cells are very sensitive to several factors and exposure to them might lead to microparticles formation which consequently can cause confounding results in analysis[161].

Tubes with anticoagulants are used after blood collection to avoid thrombosis. The antithrombotic action is based on Ca^{+2} chelation, with the most commonly used anticoagulant the sodium citrate[375].

An important dilemma is whether to determine the absolute number or a representative sample of the in vivo circulating microparticles. Estimation of the absolute number of microparticles, requires whole blood or platelet rich plasma, however these types of samples are not easy to handle in clinical trials due to instability[375]. Usually, blood cells need to be removed from plasma in order to avoid further MP formation which will not reflect true in vivo status of microparticles numbers[161]. Microparticles isolation and purification can be obtained by different methods, including ultracentrifugation, filtration, immunoaffinity isolation and

microfluidics techniques[374]. Centrifugation has been used in most of the studies as isolation technique. There are no standard protocols for the application of centrifugation[374]. Ideally, the analysis should be performed in platelet free plasma (PFP), as further microparticles might arise from platelet activation[376]. According to Clinical and Laboratory Standards Institute, PFP can be achieved by a centrifugation using 1,500 g for 20 min followed by a high speed centrifugation at 13,000 g for 2 min[377]. Platelet poor plasma (PPP) can be obtained by centrifuging blood once at 1,550 g for 20 min. PFP may contain less microparticles than PPP, probably because during the last centrifugation step, apart from platelets, a population of microparticles also being removed or destroyed[378]. Another alternative method suggest enumeration of microparticles from PPP after 15 minutes centrifugation at 4000 rpm (circa 2860g)[379]. Although freezing appears to affect microparticles levels[380], most of the time is inevitable. The current consensus support storage at -80^{0}C with minimum freeze/ thaw cycles prior to analysis[374]. Frozen samples should be compared with frozen samples and fresh with fresh samples.

Quantitative and qualitative assessment

Several optical and non-optical methods have been used for quantitative and qualitative assessment of microparticles. The most commonly used methods are flow cytometry and electron microscopy[374]. Other techniques include dynamic light scattering, nanoparticle tracking analysis, resistive pulse sensing (impedance) scanning and atomic force microscopy have been described but rarely used as lacking the ability to distinguish microparticles from

other non-vesicular structures and to identify membranous molecules specific of certain microparticle subgroups[381]. Additionally, emerging techniques like mass spectrometry-based proteomic analysis offers an opportunity to determine the protein composition of microparticles.

Flow cytometer

Flow cytometry is the most popular microparticle assessment method among researchers as (i) can distinguish if a structure is a lipid microvesicle or other cellular components, immune complex or precipitate, (ii) can determine the cellular origin and other characteristics of the microparticle, and (iii) requires reasonable effort and cost for the analysis of large number of samples[381]. The enumeration of microparticles is based on the principle of scattered light. The sample is flowing into the device and the microparticles pass a laser beam. The device receives information from the scattered light of each microparticle regarding the forward and the side direction of the scattered light. In general, forward direction mainly provides data for the size and side direction for the granularity and structural complexity[378]. Conventional flow cytometers have not been designed to measure microparticles and have a lower detection threshold around 500 nm. Newer devices can detect particles as small as 100 nm in diameter[374].

By labelling the microparticles with fluorescence antibodies is possible to determine their ancestry. More than two antibodies can be detected by the flow cytometer. The light passes through filters and detectors providing data from microparticles subpopulations[378]. Annexin V has been used as a marker for PS positive microparticles but its binding with the negatively charged lipids is highly influenced by Ca^{+2} concentrations[375]. Several studies have reported PS negative microparticles in significant numbers. Alternative labelling of microparticles with

other florescence substances like PKH67, bio-maleimide, phalloidin and calcein have been suggested with various results. Different marking antigens have been used to assess the cellular origin of the circulating microvesicles (Table 7)[378].

Combining the features of conventional flow cytometer with high-resolution imaging at the single-cell level improves the distinguish abilities of the flow cytometer and provides more morphological information compare with the standard technique[376].

2. Study aims and hypotheses

Aim

To determine the influence of different antithrombotics (apixaban, warfarin and antiplatelets) on the thrombosis and thrombolysis pathway (clot structure) and to quantify microparticle levels in patients with non-valvular AF. This study evaluates the variance of haemostatic activity related with each type of the common antithrombotics along with clot structure changes associated with AF coagulopathy. In addition, I investigate the role of microparticles as biomarkers and their potential relationship with factors reflecting level of fitness.

Background

In AF, endothelial inflammation and imbalance of pro- and anticoagulant constituents contribute to impaired haemostasis[382]. Prevention of haemorrhage and ischaemic stroke requires efficient haemostasis with timely generation of fibrin clot formation but also prompt lysis of the clot, once its role in maintaining haemodynamic integrity is complete[383]. To reduce the risk of stroke, patients with AF need OAC[22]. Apixaban, an Xa factor inhibitor, is one of the 4 NOACs that gained approval as OAC for stroke thromboprophylaxis in NVAF. Its favourable profile over warfarin on prevention of ischaemic stroke and bleeding outcomes from the ARISTOTLE trial is supported by real world evidence[384].

The traditionally used laboratory tests are of limited value in order to assess the antithrombotic properties of NOACs[11]. Other techniques such as TEG[116] and MPA[385] may provide a detailed

analysis regarding the influence of antithrombotics on haemostasis. TEG can utilise citrated whole blood and MPA plasma in order to describe the fibrin polymerisation and lysis. Both techniques have similarities regarding the indices they use to describe the coagulation cascade. However, they do have fundamental methological differences. TEG assesses viscoelastic properties of the whole blood which includes platelet function[116]. MPA describes the haemostatic process after recording changes of plasma optical density by micro-titre plate photometer reader. The optical density is positively related with fibrin polymerisation which is induced by exogenous thrombin. Similarly, fibrinolysis is activated by exogenous combination of thrombin and tPA[385]. The two methods assess haemostasis from different perspective and they appear complementary in order to approach an understanding of thrombogenesis and fibrinolysis[386].

In addition to dynamic assays, an alternative assessment of fibrinolysis focusses on levels of individual molecules such as t-PA and PAI-1. Indeed, this imbalance may contribute to the clinical manifestations of cardiovascular diseases[387,388]. Furthermore, increased levels of D-dimers are found in many pro-thrombotic conditions, predict cardiovascular events and may also interfere with the t-PA and PAI-1 dynamics[389].

Another mechanism that affect haemostasis is related with the presence of microparticles [390]. Their procoagulant properties are associated with the negatively charged external membrane surface that attracts clotting factors, the presence of Tissue Factor as membrane protein and their ability to inhibit fibrinolysis[20]. Apart from involvement in the coagulation process, microparticles have multiple other functions which are related with cardiovascular pathology. Endothelial damage, inflammation, oxidative stress are mechanisms associated with AF pathophysiology[391] and microparticle generation[20].

Different oral antithrombotics have been used as stroke thromboprophylaxis in NVAF with variable efficacy and safety but most promising results derived from NOACs trials[392]. A mechanistic analysis of the thrombosis/thrombolysis pathway by dynamic assays and thrombosis related biomarkers may identify significant differences between the effect of antithrombotics on haemostasis and clot structure. These differences reflect the functional variation of antithrombotics which influence their efficacy and safety. In addition, an approach to understand further the functional capacity of antithrombotics may assist in the quantification of their anticoagulation potential or activity which is valuable in multiple clinical circumstances.

Part of the present study is based on previous work, where was proposed as a model for the analysis of clot structure characteristics a combination of dynamic assays, TEG and MPA. We used TEG and MPA to demonstrate the effect of apixaban (NOAC), warfarin (VKA) and antiplatelets (aspirin or clopidogrel) on the coagulation process. Additionally, for the assessment of coagulation biomarkers and for enumeration of microparticles we used ELISA and flow cytometry, respectively.

The last part of my thesis involves an assessment of microparticle levels in patients with permanent AF and preserved LV systolic function. I investigate potential relations of microparticles with clinical characteristics such as exercise tolerance (assessed by cardiopulmonary exercise test and 6 minutes walking test), quality of life questionnaires and echocardiographic parameters of LV diastolic function. My experiments are completed by evaluating the effects of spironolactone on microparticle levels.

Hypotheses

First hypothesis: Patients with NVAF, not exposed to OAC, have different thrombogenesis and fibrinolysis characteristics compared to patients with established IHD in sinus rhythm.

Second hypothesis: Apixaban is a potent oral anticoagulant with different features regarding the way it affects clot dynamics, from warfarin and antiplatelets.

Third hypothesis: Microparticle levels in patients with permanent NVAF and preserved LVEF are influenced by physical status and exercise capacity. Inhibition of the effects of aldosterone by spironolactone, may affect microparticle levels.

3. Methodology

3.1 Study design and population

For first Hypothesis, a cross-sectional study was designed where I compared 47 patients with AF and 39, age and sex matched controls, with IHD but without AF. Participants in both groups were receiving antiplatelets (aspirin or clopidogrel). Patients with IHD were defined as those who suffer from angina or had previous acute myocardial infraction with over 50% stenosis of at least 1 coronary artery on coronary angiogram.

Second Hypothesis was tested in 2 studies. In a cross-sectional study, I compared three groups of patients with NVAF: 60 patients receiving warfarin, 60 apixaban and 62 antiplatelets only. In a second, follow up, study, I enrolled 32 patients with NVAF not receiving OAC. In those patients, the study tests were performed at baseline and three months after starting apixaban (2.5 or 5 mg bid as clinically indicated).

The Third Hypothesis was tested in 147 patients who were recruited as part of the double blind, randomized, controlled trial "IMPRESS-AF" (IMproved exercise tolerance in patients with PReserved Ejection fraction by Spironolactone on myocardial fibrosiS in Atrial Fibrillation). All the participants were randomised during the baseline visit to placebo or spironolactone treatment. Microparticle levels (Platelet derived microparticles, Endothelial derived microparticles, Monocyte derived microparticles and Annexin V+ microparticles) were estimated for the baseline and end of study visit (24 months after randomisation).

3.2 Clot Structure Study

The 3 studies to assess the first and the second hypothesis, conducted as part of the "clot structure" study. Inclusion criteria were aged 18 or over and able to provide informed, written consent. For the AF cohort, AF documentation on 12-lead ECG (paroxysmal or permanent AF) was essential. The definition of AF of this was determined by the European Society of Cardiology guidelines[54]. This was defined by the presence of a supraventricular tachyarrhythmia characterised by uncoordinated atrial activation. All patients had an electrocardiogram (ECG) as part of the eligibility assessment. AF was described by the replacement of consistent P waves by rapid oscillations or fibrillatory waves that vary in size, shape, and timing, associated with an irregular, ventricular response when atrioventricular (AV) conduction is intact.

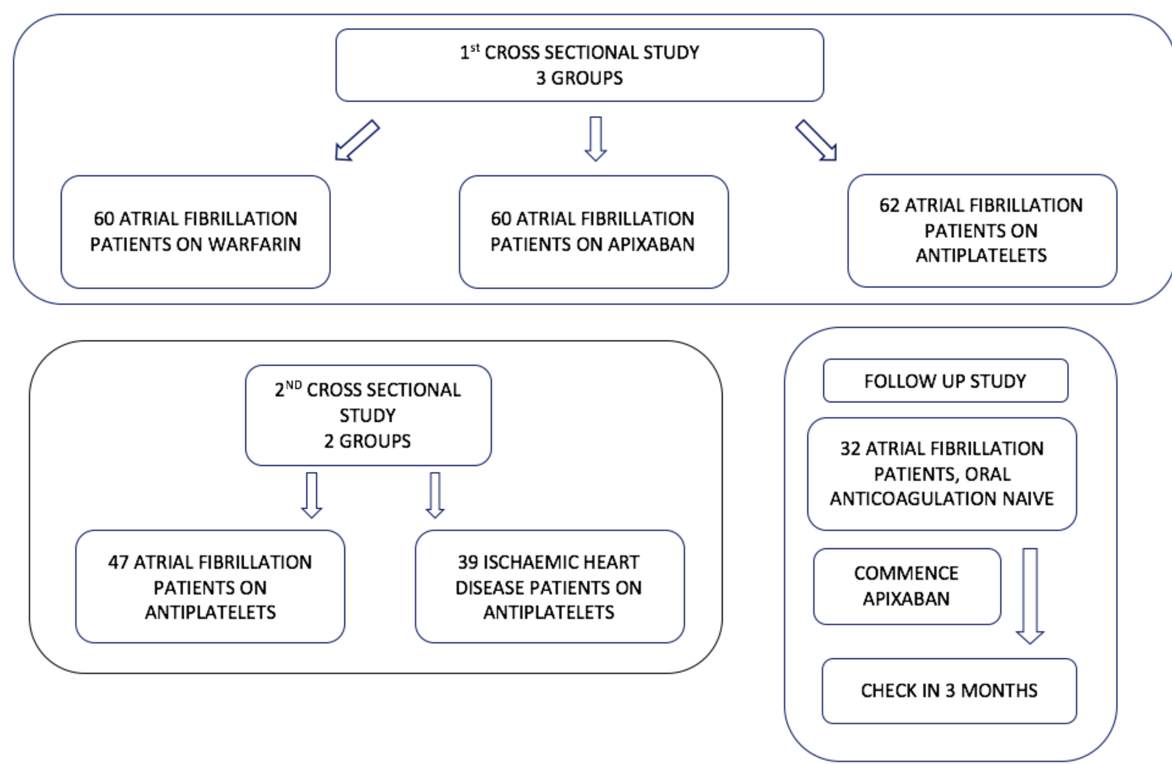
Established coronary artery disease (previous myocardial infarction due to coronary artery disease, over 12 months ago, or angina with >50% stenosis defined by coronary angiogram), in the absence of AF history were the inclusion criteria for the IHD with sinus rhythm cohort. Exclusion criteria were end-stage chronic kidney disease, connective tissue disease, neoplasia, recent (<3 months) surgery or acute cardiovascular event, presence of a prothrombotic factor (lupus anticoagulant, anti-phospholipid syndrome etc.), significant liver or haematological condition (abnormal liver function tests, cytopenia), use of antibiotics, non-steroid anti-inflammatory drugs or steroids.

All AF patients on antiplatelets had low thromboembolic risk (CHA_2DS_2–VASc score of ≤1)

or were recruited during their first visit to cardiology clinic prior to commencement on OAC. Participants were recruited predominantly from cardiology clinics. Rate control of AF patients was satisfactory (60-100 bpm). For the warfarin group, INR was determined on the day of recruitment by the haematology laboratory of the hospital. Data collected included full clinical and demographic details (age, gender, ethnicity, body weight, BMI, systolic and diastolic blood pressure, smoking and family history of coronary artery disease) and concurrent disease. In all cases citrated venous blood was collected and the following hematology and biochemistry tests were assessed: FBC, INR, APTT, U&E's, fibrinogen, CRP and LFTs. Renal function was defined by creatinine clearance, calculated using the Cockcroft-Gault equation.

Clotting and lysis parameters of samples were assessed by TEG (whole blood) and MPA (plasma). The following biomarkers tPA, PAI-1, D-Dimer, Platelet derived Microparticles (CD42b+) and Annexin V+ microparticles (apoptotic/PS+) were analysed.

Figure 7. Clot Structure study



3.3 IMPRESS-AF trial

For my thesis, I recruited patients for the IMPRESS AF trial where I was one of the 2 research fellows working on the trial . I participated as site investigator, contributing to screening, recruitment and randomisation. Additionally, I performed the initial clinical and echocardiographic assessment along with follow up visits. The single-centre IMPRESS-AF[393] trial was conducted in order to assess if treatment with spironolactone, compared to placebo, improves exercise capacity, quality of life and diastolic function in patients with permanent atrial fibrillation and preserved LV contractility. A total of 250 patients have been randomised in this double-blinded trial for 2-year treatment with spironolactone or matched placebo. Inclusion criteria were 50 years old or older, permanent atrial fibrillation and LV ejection

fraction >55%. Exclusion criteria included contraindications to aldosterone inhibitors, uncontrolled hypertension and presence of severe comorbidities with life expectancy <2 years. The primary outcome was improvement in exercise tolerance at 2 years (estimated by cardiopulmonary exercise testing and 6 minutes walking test) compare to the baseline visit. Secondary outcomes included quality of life [assessed using the EuroQol EQ-5D-5L (EQ-5D) and Minnesota Living with Heart Failure (MLWHF) questionnaires], diastolic function and all-cause hospitalisation.

Clinical assessment

At baseline, all participants had a full medical history and clinical examination. This approach allowed the collection of detailed information on demographics (age, gender, smoking status and ethnicity), comorbidities (diabetes, hypertension, hyperlipidaemia etc) and medication use. The clinical examination yielded data on peripheral pulse rate, systolic and diastolic blood pressure (BP), height, weight, body mass index (BMI), evidence of valvular disease and co-morbid lung pathology. Blood pressure readings were taken using the Omron 705IT (HEM-759P-EZ) blood pressure machine. Systolic and diastolic blood pressure were recorded and interpreted in accordance with ESC guidelines on hypertension. The device was calibrated yearly. The results were not directly indicative of the treatment (although one could argue Spironolactone reduces BP) that the patient is receiving and were therefore recorded at each visit in the proforma.

Randomisation

Patients meeting all eligibility criteria and providing written informed consent underwent a baseline visit and the process of randomisation. A secure web-based randomisation system was used to randomise patients and allocate participants to receive a unique IMP number. After the patient was randomised, the system displayed the IMP number the patient had been assigned to but did not disclose the identity of the treatment that is associated with that IMP number.

Randomisation itself was stratified based on baseline peak VO_2 performance. Participants were stratified into two groups, those with baseline peak VO_2 below or above 16 ml/min/kg. Randomisation was implemented by the statistician initially producing two lists: peak VO_2 above and below 16 ml/min/kg.

Dispensing of treatment

The Centre for cardiovascular Sciences was responsible for dispensing the trial medication to the participant on receipt of a prescription. The hospital NHS pharmacy has oversight and performed audits every 6 months.

Treatment and Dosing Schedule

Trial participants received either Spironolactone or matched placebo according to the following instructions:

- a single 25 milligram tablet
- to be taken orally once per day (typically to be taken during the morning but can be taken regularly at other time of day if preferable).
- to be taken with a drink to help swallowing of the drug
- for a 24-month period

In the case of an increase in potassium level to 5.1-6.0 mmol/L or in the presence of other non-life-threatening side effects (such as gynaecomastia) the trial drug was down-titrated to 25 mg each second day. In such cases, the investigators advised to re-up-titrate the trial medication if the reason for down-titration has resolved. Routine laboratory surveillance of serum potassium, sodium, full blood count with haematocrit, and renal function was done by protocol at each visit and within 1 week of any dose adjustment.

Drug toxicity was defined as an increase in potassium level to >6 mmol/L. In the case of toxicity or suspected toxicity, the trial medication was stopped for the duration of the trial. The patient invited to attend the remaining follow up visits at 12 months and end of study visit at 24 months.

The participants were given bottles with the trial drug sufficient for 6 months of treatment. A pill count was performed at each visit. The remaining number of pills in the pack were checked and recorded in the patient CRF for that visit. At 6, 12 and 18 months visits, the previous

bottle and any remaining pills were returned and the next supply dispensed. Bottles were returned to the nurse at the clinic visit and stored in the RC-CSS pharmacy until the end of the trial.

Adverse event reporting

Clinical aspects of the study were managed by the trials team at the University of Birmingham.

Blinding

Trial participants, trial team in contact with the patient, care providers, outcome assessors, data analysts remained blinded to the treatment after assignment to interventions. To prevent accidental unblinding, code breaks performed by the City Hospital pharmacy, who are independent to the study team.

Unblinding of the trial drug was rare, as the patients were treated symptomatically and the management not usually altered by knowing the trial compound (i.e., spironolactone or placebo). Unblinding Codebreaks were avoided unless knowledge of the trial treatment was essential for the correct clinical care of the patient e.g. in the cases of acute liver injury, acute renal failure, Stevens-Johnson syndrome or agranulocytosis, where suspected to have been caused by spironolactone. Cases that were considered serious, unexpected and possible, probably or related were unblinded.

3.4 Laboratory Methods for clot structure

After written consent was obtained, twenty mL of venous blood taken from an antecubital vein into 0.9% citrate bottles at room temperature for assay of thrombosis/fibrinolysis, coagulation biomarkers and microparticles. Platelet-poor plasma (PPP) was achieved after centrifugation at 2860g for 15 minutes, and 0.5 mL aliquots were frozen at -70°C for later batch analysis. 5 mL of fresh blood was processed, within 2 hours from venesection, by TEG.

3.4.1 Thromboelastography

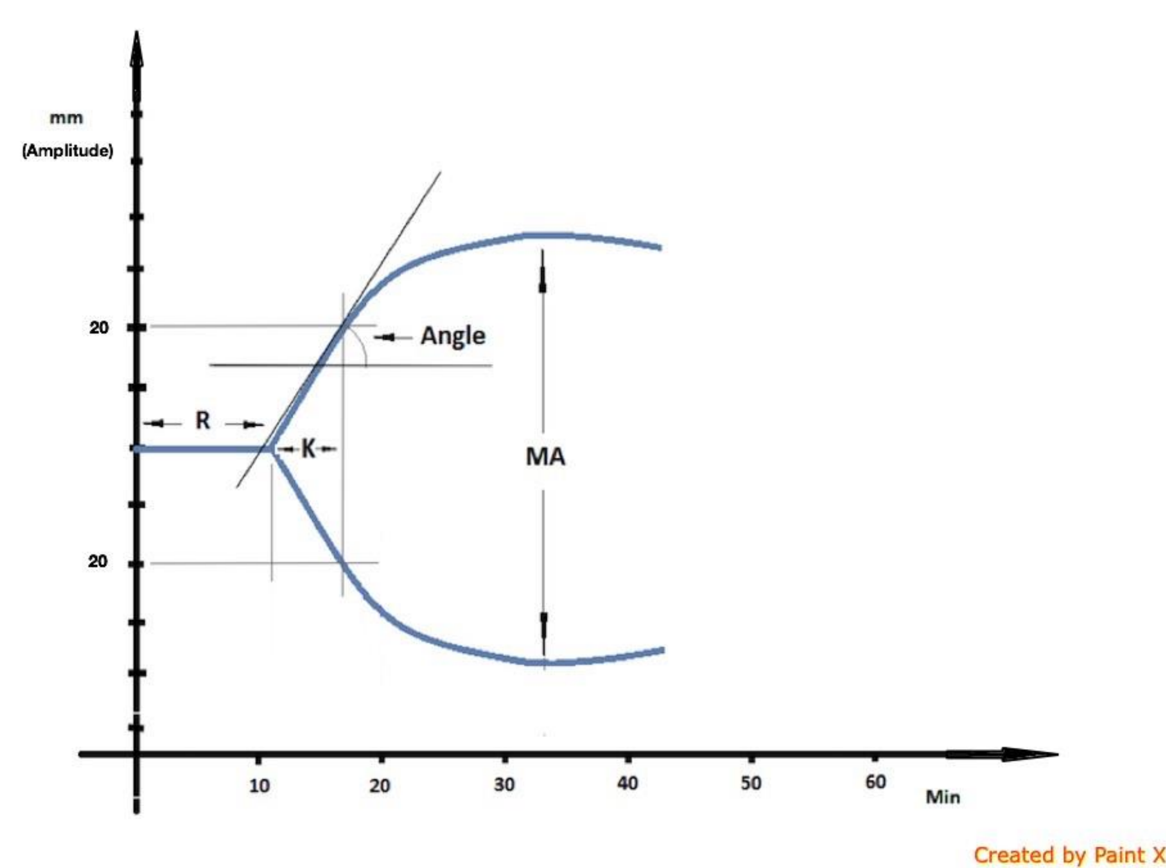
Test was performed using a specially designed device called thromboelastograph. The viscoelastic alterations that take place during the fibrin polymerization cascade correlate with thrombus formation. The values were recorded continuously and depicted by graphical representation (Figure 8). In my study, citrated venous blood was analysed by tromboelastography for its thrombogenic properties. The initiation of thrombosis was induced after the addition of calcium to whole blood supplemented with kaolin. Thrombus formation was monitored up to reach the maximum strength. The technique was performed according to the manufacturer's instructions (more details in TEG SOP, No222, appendices page 166).

The following parameters were recorded (Figure 8):

- R time (min): Time from the initiation of the test until the point where the clot begins to form
- K time (min): Interval from the split point of the test to the point where the fibrin cross-linking provides enough clot resistance to produce a 20-mm amplitude

- Angle (degrees): Angle formed by the slope of a tangent line traced from the R time to the K time and reflects the rate at which the clot forms
- MA (units): Maximum amplitude of the clot dynamics, reflecting clot strength

Figure 8. Graphic representation of thrombus viscosity with time, based on TEG report



R time (min):	Time from the initiation of the test until the point where the clot begins to form
K time (min)	Interval from the split point of the test to the point where the fibrin cross-linking provides enough clot resistance to produce a 20-mm amplitude
Angle (degrees)	Angle formed by the slope of a tangent line traced from the R time to the K time and reflects the rate at which the clot forms
Maximum Amplitude [MA] (units)	Maximum amplitude of the clot dynamics, reflecting clot strength

3.4.2 Micro-plate assay

Plasma can be processed by microplate assay, so its fibrin polymerisation and fibrinolysis potential may be determined by different indices after recording changes of the optical density, measured by micro-titre plate photometer reader.

Plasma was collected during the visit(s) and stored at −70 °C until later batch processing. Samples undergone a single-freeze thaw cycle. The procedure was performed at 37 ^{0}C and divided into two individual techniques.

The first technique is the thrombogenesis assay (Figure 9) where 25 µl plasma, 75 µl Tris–NaCl buffer and 50 µl thrombin added to the wells of 96-well micro-titre plate in triplicate. Changes in the wells optical density were recorded for 30 minutes by a micro-titre plate reader. The changes can be depicted in a graph (Figure 9) which reflects the fibrin clot formation.

Figure 9. Microplate assay analysis of fibrin polymerisation (Triplicates of one sample)

Lag Time (sec)	Time from the initiation of the test to the start of clot formation
Rate of clot formation (units/sec)	Changes in optical density over time during clot augmentation
Maximum Optical Density (units)	Maximum optical density, reflecting clot strength

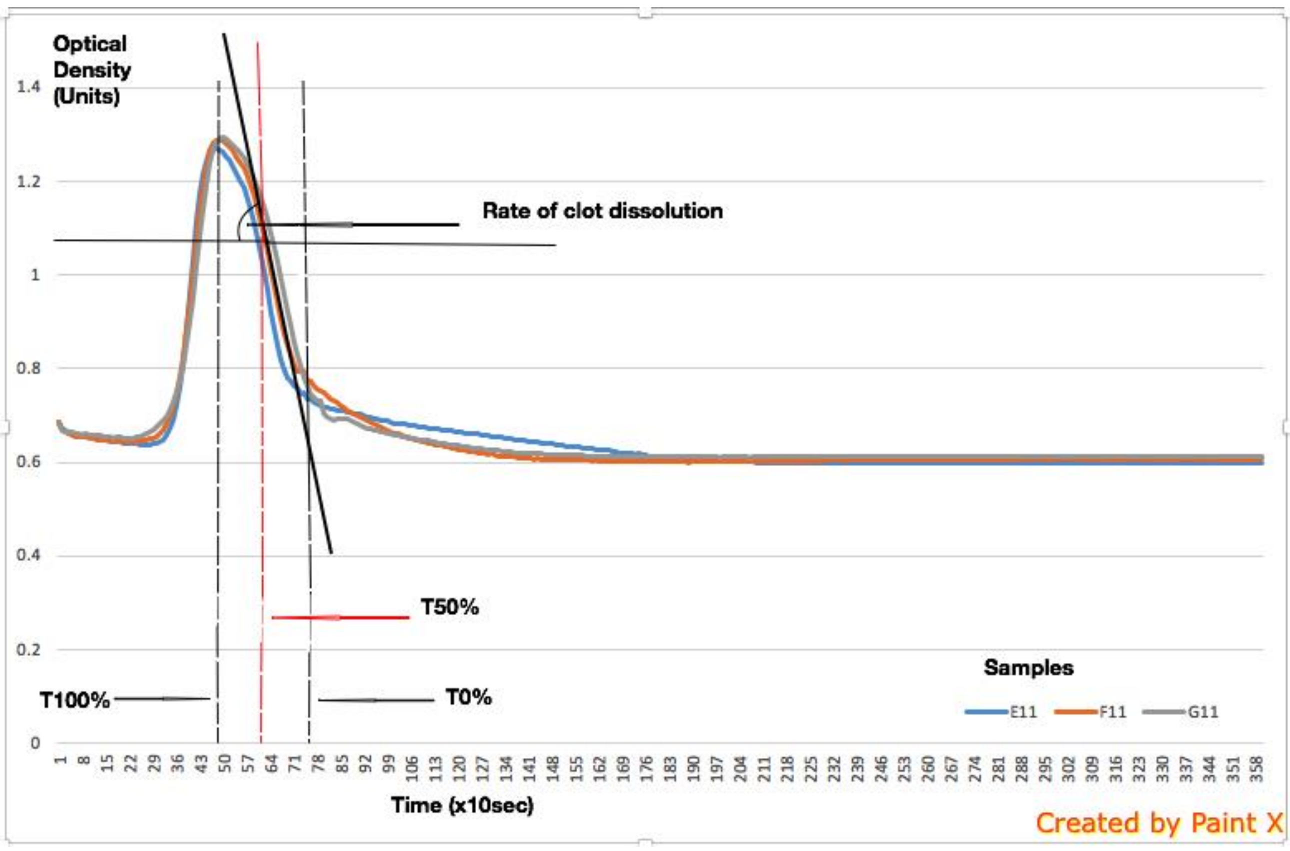
The second technique is the fibrinolysis assay (Figure 10). Each well in the micro-titre plate contains 75 µl of plasma and 75 µl of a Tris/NaCl/calcium buffer. After the addition of thrombin and tissue plasminogen activator the plate is immediately loaded into the plate reader. Optical density data are recorded for 30 min and similarly to the thrombogenesis, fibrinolysis assay can be plotted into a line chart (Figure 10).

The following indices were assessed:

- Lag Time(sec): time from the initiation of the test to the start of clot formation
- Rate of clot formation (units/sec): changes in optical density over time during clot augmentation
- Maximum Optical Density (units): Maximum optical density, reflecting clot strength
- Rate Of Clot Dissolution(units/sec): changes in optical density during the reduction of clot strength / fibrinolysis
- T50% (sec): Time for 50% of the clot to lyse from maximum to the plateau phase

(more details in Turbidity assay SOP, No223, appendices page 175 and fibrinolysis assay SOP, No 221, appendices page 184).

Figure 10. Microplate assay fibrinolysis analysis



Rate Of Clot Dissolution (units/sec)	Changes in optical density during the reduction of clot strength fibrinolysis
T50% (sec)	Time for 50% of the clot to lyse from maximum to the plateau phase. (t50%=T0%-T100%/2)

3.4.3 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. In ELISA, an antigen must be immobilized on a solid surface and then complexed with another antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate (stored serum) to produce a measurable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction[394].

ELISA was performed in 96-well polystyrene plate, which passively bind antibodies and proteins. Having the reactants of the ELISA immobilized to the microplate surface makes it easy possible to separate bound from non-bound material during the assay. This ability to wash away nonspecifically bound materials makes the ELISA a powerful tool for measuring specific analytes[395].

A detection enzyme or other tag is linked directly to the primary antibody or introduced through a secondary antibody that recognizes the primary antibody. The most used enzyme labels are horseradish peroxidase and alkaline phosphatase. Other enzymes have been used as well, but they have not gained widespread acceptance because of limited substrate options[395].

Batch analysis of plasma (stored at −70 °C) was processed by ELISA to measure t-Plasminogen Activator (R&D systems, Human t-Plasminogen Activator/tPA Quantikine ELISA Kit), Plasminogen Activator Inhibitor 1 (R&D systems, Human Serpin E1/PAI-1 Quantikine ELISA Kit) and D-Dimer (abcam – D-Dimer Human SimpleStep ELISA® Kit). Samples undergone a

single-freeze thaw cycle. The samples were analyzed according to manufacture instructions. Values of the samples from the optical photometer were processed by ELISAanalysis.com[396].

3.4.4 Microparticles

Plasma samples undergone a single-freeze thaw cycle and initially incubated separately for 30 min with 5 µL of 1/10 diluted (0.01 mg/ml), biotinylated anti-human CD42b antibody (Abcam, Cambridge, UK), for platelet-derived microparticles (PMPs). This was followed by a second incubation with 0.25 µg of Streptavidin-Alexa Fluor-647 nm-RPhycoerythrin conjugate (Life Technology, Paisley, UK) for 30 min and then diluted with 945 µl filtered PBS (final dilution 1:20).

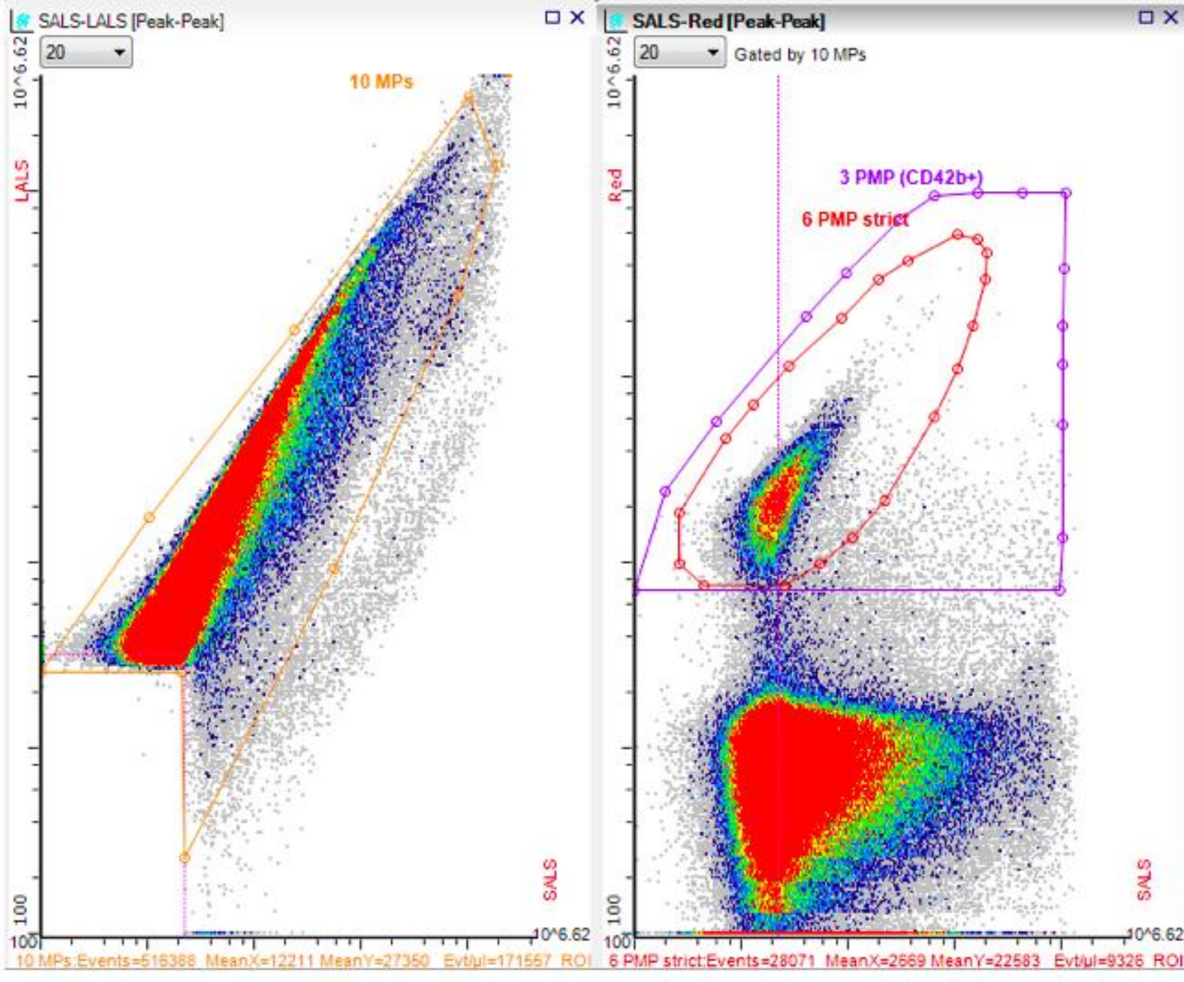
For Annexin V+ microparticles, PPP was incubated for 30 min with Annexin V binding buffer, 2x concentrated solution (Molecular Probes, Thermofisher scientific, UK) and 5 µL of Annexin V, Alexa Fluor® 488 conjugate (Molecular Probes, Thermofisher scientific, UK). Final dilution 1:20 was achieved by adding 895 µL of Annexin V dilution buffer.

Microparticle analysis was promptly performed, after achieving the required dilution, using the Apogee A50 flow cytometer (Apogee Flow Systems, High Wycombe, UK). 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. Example of Apogee A50 flow cytometer report is shown in Figure 1 (more details in Enumeration of

100

Platelet derived microparticles SOP, No209, appendices page 196 and Enumeration of Apoptotic microparticles SOP, No222, appendices page 211)

Figure 11. Analysis of CD42b positive microparticles with Apogee A50 flow cytometer



Platelets microparticles (PMPs) are enumerated by the CD42b positive events on the right window. The red gate, which defines the number of CD42b positive events has been set by the results of the previous experiments. At the right bottom of the PMPs plot, the number of positive events per µL in selected region of interest (gate) is demonstrated, i.e. 9 326 Evt/µL. Once dilution factor (x20) is applied into the calculation, the number of platelet MPs in undiluted plasma is 186 520 μL^{-1}.

3.5 Methods and laboratory processes for IMPRESS-AF trial

3.5.1 Blood Sampling

All routine haematological and biochemistry tests were undertaken by Pathology Department in City Hospital, Birmingham with regards to analysis for the trial. Routine biochemistry tests included urea, creatinine, sodium and potassium.

Prior to venepuncture, patients were rested in chair but were not required to fast. The skin was cleaned with a sterile wipe and a tourniquet applied immediately before commencement of the procedure. The blood samples were obtained by inserting a 21-gauge needle into a vein located in the anterior cubital fossa of the patient's arm. Alternative anatomical sites were used if this was not feasible. In total, 18ml of non-fasting peripheral venous blood was collected into vacutainer tubes. This consisted of 5mls collected into an ethylene-diamine tetra-acetic acid (EDTA)-containing tube, 8mls into a citrated tube and 5mls into a serum tube with silicon coated interior. Routine haematological and biochemical tests were performed on the blood samples the same day, including BNP. Remaining blood samples were separated by centrifugation and the plasma stored at –70 ^{o}C for subsequent batched analysis.

3.5.2 Brain Natriuretic Peptide

Various factors are implicated in BNP release in AF, including an increase in atrial load, tachycardia, and deterioration in systolic/diastolic function[397].

BNP was performed at screening and end of study visits. They were performed using the Roche Cobas H 232 BNP device. The BNP machines were used strictly per manufacturer instructions. The device was calibrated yearly and certified.

3.5.3 Assessment of atrial fibrillation

The definition of AF of this was guided by the European Society of Cardiology guidelines[54]. This was defined by the presence of a supraventricular tachyarrhythmia characterised by uncoordinated atrial activation. All patients had an electrocardiogram (ECG) as part of the eligibility assessment. Patients with evidence of permanent AF on ECG underwent preliminary screening to ensure preserved ejection fraction in the absence of significant valve disease prior to undergoing further baseline investigations.

3.5.4 Echocardiography

Patients in whom permanent AF was confirmed, underwent an echocardiogram and those with an LVEF of >55% with no severe valvular disease were eligible for the study (i.e. preserved ejection fraction, contractility).

M-mode, 2D, Doppler and tissue doppler imaging (TDI) transthoracic echocardiography was performed using Phillips iE33 ultrasound system (Bothel, WA, USA). Modern off-line QLAB software [Xcelera, Phillip (iE33) Ultrasound Quantification Module, USA] was used for quantification of LV systolic and diastolic function. E/E' (early mitral inflow velocity/TDI derived early septal mitral annular diastolic velocity) was used to assess diastolic function in

AF. This parameter was strongly correlated with LV diastolic filling pressure in AF ($r=0.79$, $p<0.001$) and it is relatively independent of left atrial pressure. Average values from 10 consecutive cardiac cycles were calculated for each patient.

3.5.6 Minute Walk Test

Participants undertook a 6-minute walk test as a simple measure of exercise tolerance. No specialist equipment was required for this procedure and instructions on how the test should be performed was in accordance to the trial protocol. For the trial, only the distance the patient walked needed to be recorded for analysis.

3.5.7 Cardiopulmonary Exercise Testing

Exercise capacity is a strong predictor of cardiovascular events and overall mortality[398]. Cardiopulmonary exercise testing (CPET), with the determination of peak VO_2 is the 'gold-standard' assessment of exercise capacity, providing in depth information on cardiorespiratory system during intense, yet controlled periods of metabolic stress. This makes CPET an ideal non-invasive tool to establish the functional status of the heart, which provides a wealth of clinically relevant diagnostic and prognostic information[399].

CPET testing was performed to assess peak oxygen uptake (peak VO_2). Recently, CPET has been shown to be a highly accurate and reproducible measure of exercise tolerance in participants with preserved LV contractility[399]. Exercise testing was performed with participants in the upright position on an electronically braked bicycle, with expired gas

analysis under continuous electrocardiographic monitoring. Participants were encouraged to exercise to exhaustion. Peak VO_2 values were averaged from the final 30 seconds of the exercise test. Additionally, ventilator anaerobic threshold was evaluated by standardized methods using ventilator equivalents.

The Statement of the American Thoracic Society and American College of Chest Physicians[400] recommends that an increased breathing reserve [(VE/MVV): represents the ratio between maximal ventilation during exercise (VE) and maximum voluntary ventilation (MVV) at rest] (e.g., > 85%) occurring at a relatively low work rate (e.g., 50 W) strongly suggests that ventilator factors are contributing to exercise limitation.

The CPET test was performed using the L COSMED CPET system, based on cycle ergometer procedural instructions. Modern CPX systems contain rapidly responding O_2 and CO_2 sensors that allow for the calculation of oxygen uptake and carbon dioxide output at rest, during exercise, and during recovery, as frequently as breath by breath. Although manufacturers' recommendations vary considerably regarding calibration, the CPET systems were to be calibrated immediately before each exercise test. This included calibration of airflow, volumes, and both the O_2 and CO_2 analysers.

Because ambient conditions affect the concentration of O_2 in the inspired air, temperature, barometric pressure, and humidity was considered. The CPET system automatically quantifies these conditions and make appropriate adjustments to calculate the inspired O_2 concentration.

The CPET testing for my study was performed as per the RC-CSS SOP. Interpretation of the VO_2 maximum value result was used as a measure of exercise tolerance (i.e. higher value means better exercise tolerance).

3.5.8 Microparticles

In this study, levels of 4 types of microparticles, platelet derived microparticles (CD42b+), endothelial derived microparticles (CD144+), monocyte derived microparticles (CD14+) and apoptotic or Annexin V+ microparticles were quantified. Microparticle analysis with flow cytometer is explained in 3.4.2.

For enumeration of endothelial derived microparticles, anti-CD144-biotinylated antibodies [Bio-techne, Catalog No BAM9381] and for monocyte derived microparticles, anti-CD14-biotinylated antibodies [Invitrogen, Catalog No MA1-19489] were used.

Microparticle analysis was promptly performed, after achieving the required dilution, using the Apogee A50 flow cytometer (Apogee Flow Systems, High Wycombe, UK). Polystyrene beads of 110, 200, 500 nm and 1 μm diameter (Apogee Flow Systems) were used to set up the microparticle-size gate (Detailed description of microparticle enumeration process in Appendices).

3.6 Statistical analyses

Formal training in statistical methodology was undertaken as part of the curriculum for MD students at the University of Birmingham. Statistical methodology and analyses were discussed and doubled checked by my supervisors during several steps of the project.

Shapiro-Wilk test used to assess data distribution. Normal data are presented as mean and standard deviation, non-normal data are presented as median and inter-quartile range. For comparisons of more than 2 groups, analysis of variance (ANOVA) with Tuckey's posthoc test were used for normal data and Kruskall–Wallis test with Dunn's posthoc test for non-normal data. For comparisons of two groups, t-test was used for normal data and Mann-Whitney U test for non-normal data. For follow up comparisons, t-test was used for normal data and Related-Samples Wilcoxon Signed Rank test for non-normal data. Categorical data assessed by Chi-Square test. Correlations between continuous study measures were performed using Spearman rank method. After checking for collinearity, multivariable linear or where appropriate binary regression analysis was performed to identify potential relationship between conditions, outcomes and/or variables. Statistical calculations were performed with IBM SPSS Statistics version 25 software and p value less than 0.05 was considered significant.

3.7 Ethical Considerations

Both studies were conducted in accordance with the declaration of Helsinki.

Clot Structure Study

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

IMPRESS-AF trial

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. Ethical approval was obtained from Coventry and Warwickshire Research Ethics Committee, 07/01/2015, ref: 14/WM/1211.

4. Validation Results

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.

TEG, MPA, ELISA and microparticle enumeration with flow cytometry were well-developed in the department and extensive validation was not required. Training for the used laboratory methods was provided by research fellows from Birmingham University Haemostasis-thrombosis and vascular Biology unit. Process of the patient samples for my thesis was conducted after validation studies. Mean intra assay performed by processing the same sample for four times on the same day (table 13, Figure 12 for TEG). Mean inter-assay was performed after processing four samples from the same healthy subject which were taken on different day (table 13, figure 13 for TEG).

Figure 12. TEG intra-assay

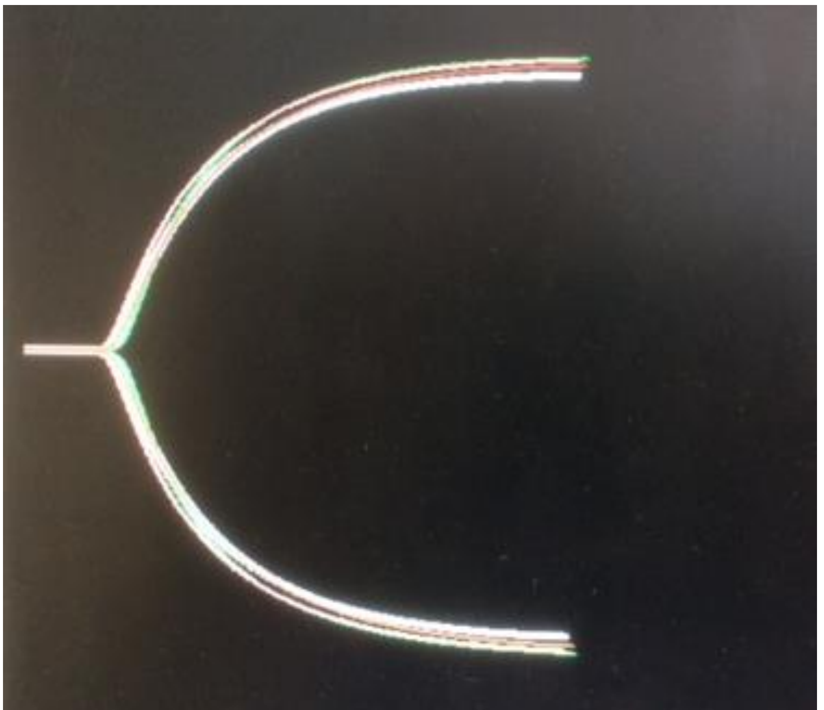


Figure 13. TEG Inter-assay

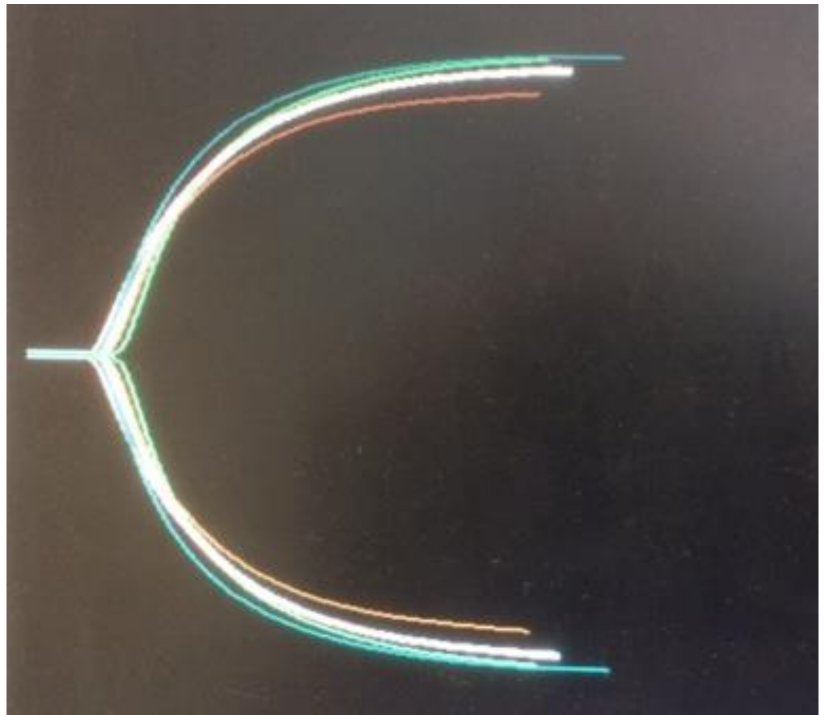


Table 13. Coefficient of variations (CV) for study methods			
Method			
	Indices/biomarker	Intra-assay coefficient of variation	Inter-assay coefficient of variation
Thromboelastography	R	6.4	12.2
	K	7.7	12.5
	Angle	2.8	4
	MA	2.3	5
Microplate assay	Lag time	9.4	14
	Rate of clot formation	11.6	16.2
	Maximum optical density	8.9	15.4
	Rate of clot dissolution	8.3	17.1
	Time 50% lysed	10.5	18.3
ELISA	Tissue Plasminogen activator inhibitor	4.8	16
	Plasminogen activator inhibitor 1	4.9	6.3
	D-Dimer	6.4	10
Microparticles	Platelet derived Microparticles (CD-42b positive)	10.1	18.2
	Apoptotic Microparticles (Annexin V positive)	9.1	14.3

5. Data chapters

5.1 Clot structure: AF patients vs 'disease control' patients in sinus rhythm with Ischaemic Heart disease taking antiplatelet drugs

Abstract

Background: Atrial fibrillation (AF) is associated with high risk of thromboembolic events. Apart from mechanical phaenomena which explain the prothrombotic nature of the condition, there is increased evidence that abnormal bloods constituents contribute to the coagulopathy. We hypothesised that patients with AF have defective haemostasis and prothrombotic microparticles are related with the pathophysiology of the disease.

Methods: 47 patients with AF and 39 age and sex matched controls with Ischaemic Heart disease (IHD) but without AF, were recruited. Participants in both groups were receiving antiplatelets (aspirin or clopidogrel). Thromboelastography (TEG), microplate assay (MPA), Enzyme Linked Immunosorbent Assay (ELISA) for tissue Plasminogen Activator (tPA), Plasminogen Activator Inhibitor-1 (PAI-1) and D-Dimer and microparticles enumeration were used to evaluate the haemostatic characteristics of the participants.

Results*:* Assessment of whole blood by TEG revealed no differences between the 2 groups. On plasma analysis by MPA, patients in AF had higher Rate Of Clot Formation ($p=0.027$) and Maximum Optical Density ($p<0.001$) and slower Rate Of Clot Dissolution ($p=0.005$). PAI-1 was lower in AF group ($p=0.008$ vs. patients in sinus rhythm). D-Dimer and tPA had similar levels among the groups (p value 0.87 and 0.34 respectively). Lastly, AF patients had higher counts of apoptotic microparticles compared with those in sinus rhythm ($p=0.023$).

Conclusions: Patients with AF appear to have impaired haemostasis and elevated levels of phosphatidylserine positive microparticles which may have a role in the pathophysiology of thromboembolism.

Introduction

The mechanisms that are involved in the hypercoagulant status of AF appear to be complex and haven't been completely understood[382]. Several prothrombotic factors have been implicated in the pathophysiology of the coagulopathy related with AF. Studies have demonstrated an association between biomarkers (related with haemostasis, inflammation and abnormal cardiac function) and adverse cardiovascular events[382]. The prognostic value of biomarkers was linked with risk stratification scores for thromboembolism in order to guide OAC management[30].

To approach a better understanding of the defective haemostasis in AF, dynamic assays may provide a mechanistic analysis of the coagulation pathway. TEG and MPA may present the different steps of thrombogenesis and fibrinolysis model in AF, in a complementary way[386]. TEG measures viscoelastic properties of whole blood which includes platelet function[116]. MPA describes haemostasis after recording changes of plasma optical density by micro-titre plate photometer reader. The optical density is positively related with fibrin polymerisation and negatively with lysis which is induced by exogenous thrombin and exogenous combination of thrombin and tPA respectively[385].

Microparticles, small anucleic formations enclosed in cellular membrane, are involved in thrombosis and inflammation[20], mechanisms that are well known to be associated with the pathophysiology of the hypercoagulopathy in AF[31]. Apoptotic and platelet derived, are types of microparticles which are involved in pathological conditions with thrombotic tendency[223]. Phosphatidylserine is negatively charged lipid which is characteristic membrane constituent of the apoptotic microparticles[401].

In this study our hypothesis is that AF compared to sinus rhythm is associated with impaired haemostasis and abnormal clot structure features along with higher levels of prothrombotic constituents, including microparticles.

Methods

- Study population

We compared 47 patients with AF with 39 age and sex matched controls with IHD but without AF. Participants in both groups were receiving antiplatelets (aspirin or clopidogrel). All AF patients on antiplatelets had low thromboembolic risk (CHA_2DS_2-VASc score of ≤1) or were recruited during their first visit to cardiology clinic prior to commencement on OAC. Patients with IHD were defined as those who suffer from angina with over 50% stenosis of at least 1 coronary artery disease on coronary angiogram or had previous type 1, acute myocardial infraction.

Inclusion and exclusion criteria are shown in chapter 3. The project has been approved by the Local Research Ethics Committee and informed consent was obtained from every subject.
Data collected, included full clinical and demographic details (age, gender, ethnicity, body weight, BMI, systolic and diastolic blood pressure, smoking and family history of coronary artery disease) and concurrent disease.

- Laboratory methods

Venepuncture was done on the day of recruitment and 20 mL of venous blood was taken from an antecubital vein. The following hematology and biochemistry tests were assessed: FBC, INR, APTT, U&E's, fibrinogen, CRP and LFTs. Patients who fulfilled the exclusion criteria not recruited. Renal function was defined by creatinine clearance, calculated using the Cockcroft-Gault equation.

PPP was achieved after centrifugation of citrated blood at 2860g for 15 minutes, and 0.5 mL aliquots were frozen at -70°C for later batch analysis. Batch analysis performed for MPA, ELISA and microparticles enumeration. 5 mL of fresh blood was processed within 2 hours from venesection by TEG. TEG was performed using a commercial thromboelastograph (TEG® 5000 Thrombelastograph® Hemostasis Analyzer System), the manufacturer reagents and following the manufacturer instructions. Thrombus formation was monitored up to reach the maximum strength after the addition of calcium to whole blood supplemented with kaolin. The following parameters were recorded: R time (min), K time (min), angle (degrees) and maximum amplitude (units).

Samples for microplate assay undergone a single-freeze thaw cycle. After addition of thrombin, changes in the sample optical density were recorded for 30 minutes by a micro-titre plate reader. For the fibrinolysis assay, thrombin and t-PA were added in the sample. The following indices were assessed for thrombogenesis: lag time (sec), rate of clot formation (units/sec) and maximum optical density (units). Fibrinolysis indices included the rate of clot dissolution (units/sec) and the Time for 50% of the clot to lyse from maximum to the plateau phase.

The following commercial ELISA kits were used to measure concentrations of tPA (TPA, Human t-Plasminogen Activator/tPA Quantikine ELISA Kit, R&D systems, Abingdon, UK), PAI 1 (PAI1, Human Serpin E1/PAI-1 Quantikine ELISA Kit, R&D systems, Abingdon, UK), and D-Dimer (D-Dimer Human SimpleStep ELISA® Kit, Abcam, Cambridge, UK).

Microparticles were quantified using Apogee A50 flow cytometer (Apogee Flow Systems, High Wycombe, UK). Samples undergone a single freeze-thaw cycle. For analysis of platelet microparticles (PMP), platelet-poor plasma was incubated with biotinylated anti-human CD42b antibody (Abcam, Cambridge, UK) for 30 min, followed by further incubation with streptavidin-Alexa Fluor-647 nm-RPhycoerythrin conjugate (Life Technology, Paisley, UK) for 30 min and dilution with filtered PBS (overall dilution 1:20). For analysis of annexin V-binding microparticles, platelet-poor plasma was incubated with annexin V binding buffer (Molecular Probes, Thermofisher scientific, UK) and annexin V-Alexa Fluor® 488 conjugate (Molecular Probes, Thermofisher scientific, UK) for 30 min. Final dilution of 1:20 was achieved by adding annexin V dilution buffer. Polystyrene beads of 110, 200, 500 nm and 1 μm diameter (Apogee Flow Systems) were used to set up the microparticle-size gate and small-size microparticles were defined as events with size between 110 and 500 nm. The microparticles count was established using the volumetric function of the flow cytometer.

- Statistical analysis

Shapiro-Wilk test used to assess data distribution. Normal data are presented as mean and standard deviation, non-normal data are presented as median and inter-quartile range. For comparisons of the two groups, t-test was used for normal data and Mann-Whitney U test for

non-normal data. Statistical analysis was performed with IBM SPSS Statistics version 25 software and p value less than 0.05 was considered significant.

Results

Data from the participants of this cross-sectional study are shown in Table 14. The 2 groups were well matched in respect of demographic and clinical characteristics. Assessment of whole blood by TEG revealed no differences as respect to initiation (R), propagation (angle and K) and maximum clot strength (MA), between the AF and sinus rhythm cohort (Table 15). On plasma analysis by MPA, patients in AF had higher Rate Of Clot Formation (p=0.027) and Maximum Optical Density (p<0.001) and slower Rate Of Clot Dissolution (p=0.005). PAI-1 was lower in AF group (p=0.008 vs. patients in sinus rhythm and IHD), in contrast with D-Dimer and tPA which have similar levels among the groups (p value 0.87 and 0.34 respectively). AF patients had higher counts of apoptotic microparticles compared with those in sinus rhythm (p=0.023); the platelets derived microparticle counts were similar in the two groups (p=0.28).

Discussion

Our comparison of the haemostatic status between AF patients and patients with IHD and sinus rhythm showed that there is heterogeneity in the clot structure characteristics between the two conditions. Although analysis with tromboelastography did not reach level of significance for any of thrombosis indices, data from microplate assay showed remarkable contrasts in

haemostatic parameters between the two conditions. Our data from MPA support that fibrin polymerises faster in AF and produces a more robust polymer which dissolves slower compare to clot from patients with sinus rhythm. This is based on the rate of clot formation, maximum density and rate of clot dissolution indices.

These findings, which derived from plasma analysis, support Virchow's triad model about hypercoagulant status in AF. Apart from the blood stasis which is the mechanical result of atrial enlargement and loss of contractility, there is an imbalance of plasma procoagulant molecules[382]. Our study showed that part of the intravascular molecular changes in AF are the higher levels of Annexin-V positive microparticles. AF is associated with higher levels of thrombin and inflammatory cytokines[402,403]. Thrombin and interleukin-6 are known stimuli for generation of microparticles, fact that may explain the higher levels of phosphatidylserine positive microparticles. The circulating negatively charged phosphatidylserine, electrostatically attracts the positive segment of clotting proteins and promotes thrombosis[20]. The high levels of Plasminogen Activator Inhibitor -1 in IHD has been described[404].

Limitations

There are certain limitations for our study. Groups are comprised of small numbers. We acknowledge potential effects of other medications and of other pathology (such as hyperlipidaemia). IHD patients, who were our control group, were receiving regular antiplatelet therapy which may influence thrombosis and lysis indices compared to healthy subjects. Part of our study was conducted with patients receiving two different antiplatelets (aspirin or clopidogrel) with potential differences in the way that affect the coagulation cascade.

Conclusion

AF appears to affect the haemostatic balance. Plasma analysis revealed that AF patients have higher thrombogenic potential with less effective fibrinolytic mechanisms. Prothrombotic factors that contribute to the hypercoagulopathy in AF are phosphatidylserine positive microparticles. These vesicles reflect apoptosis and may have a role in the pathophysiology of AF prothrombotic status.

Table 14. Baseline clinical characteristics of the 1st cross-sectional study. Patients with Atrial Fibrillation (AF) or without AF [Ischaemic heart Disease (IHD)]

Group (number of participants)	AF (47)	IHD (39)	P value AF vs IHD
Age (years)	73 (13)	70 (10.5)	0.06
Gender, male-female, n(%)	68-32	51-49	0.11
Ethnicity (White/Asian/Afro-Caribbean) n	38/8/1	24/11/4	0.09
Ischaemic Heart Disease, n(%)	0 (0)	39 (100)	
Diabetes, n(%)	12 (26)	41 (16)	0.13
Hypertension, n(%)	43 (91)	36 (92)	0.89
Heart failure, n(%)	7 (15)	6 (15)	0.95
Current Smokers, n(%)	2 (4)	3 (8)	0.5
Systolic Blood Pressure (mmHg)	129 (17)	137 (20)	0.06
Diastolic Blood Pressure (mmHg)	77 (13)	74 (13)	0.26
Body Mass Index (kg/m^2)	27.3 (6)	28.2 (5.7)	0.37
Creatinine Clearance [Cockroft-Gauld](ml/min)	72 (46-93)	79 (58-100)	0.28
Values are shown as Mean (SD) or Median (interquartile intervals) unless stated otherwise			

Table 15. Study indices of patients with Atrial Fibrillation (AF) or in sinus rhythm and ischaemic heart disease (IHD)

Method	Indices/Biomarkers	AF	Sinus rhythm and IHD	P value AF vs sinus rhythm
Thromboelastography	R (min)	4.9(4.1-5.8)	5.3(3.9-6.3)	0.53
	K (min)	1.6((1.2-2)	1.4(1.2-1.7)	0.28
	Angle (deg)	66 (8.5)	68 (5.3)	0.45
	Maximum Amplitude (mm)	67 (5.3)	68 (5.3)	0.29
Microplate assay	Lag time (sec)	320(250-351)	310(250-340)	0.84
	Rate of clot formation (units/sec)	37 (20-43)	30 (23-34)	*0.03*
	Max. Optical density (units)	0.48 (0.42-0.56)	0.38 (0.33-0.45)	*<0.001*
	Rate of Clot dissolution (units/sec)	33 (22-42)	42 (33-47)	*0.005*
	Time 50% lysed (sec)	224 (81)	212 (42)	0.42
Enzyme-linked immunosorbent assay	D-Dimer (ng/ml)	7.82 (4.19-13.61)	7.74 (4.38-11.76)	0.87
	Tissue Plasminogen Activator (ng/ml)	0.48 (0.42-0.62)	0.54 (0.46-0.62)	0.34
	Plasminogen Activator Inhibitor 1 (pg/ml)	0.16 (0.12-0.23)	0.2 (0.14-0.41)	*0.008*
Microparticles	Platelet Derived Microparticles (events/ml)	19960 (4680-71260)	7220 (2720-46640)	0.28
	Apoptotic Microparticles (events/ml)	479870 (290050-1190890)	144400 (54400-932800)	*0.02*
Values are shown as Mean (SD) or Median (interquartile intervals)				

5.2 Clot structure study: Comparison of patients with atrial fibrillation on different antithrombotic agent (warfarin, apixaban or aspirin).

Abstract

Background: Non-Vitamin K Anticoagulants (NOACs) are known to affect haemostatic mechanisms but there is no detailed description regarding their influence on the 2 main components of haemostasis, thrombosis and fibrinolysis. In this study we tested the hypothesis that the haemostatic profile of apixaban is different from warfarin and aspirin, if tested in patients with atrial fibrillation (AF).

Methods: We studied 3 groups of patients with AF on different antithrombotics: warfarin (60 patients), apixaban (60) or antiplatelet (62). Thromboelastography (TEG), microplate assay (MPA), Enzyme Linked Immunosorbent Assay (ELISA) for tissue Plasminogen Activator (tPA), Plasminogen Activator Inhibitor-1 (PAI-1) and D-Dimer were used to evaluate the haemostatic characteristics of the participants. In addition, platelet derived and apoptotic microparticle levels were quantified.

Results: Apixaban appears more effective attenuating thrombosis, in whole blood analysis by TEG, compared with warfarin and antiplatelets. Additionally, patients on apixaban had lower levels of ELISA biomarkers, D-Dimer and tPA, ($p<0.001$). In plasma analysis by MPA, warfarin and apixaban demonstrated comparable activity as anticoagulants, both superior to antiplatelets.

Conclusions: Apixaban is as effective as warfarin in deceleration of fibrin polymerisation and amplification of fibrinolysis; in addition, normalises biomarkers related with adverse cardiovascular events.

Introduction

Vitamin K antagonists (VKAs) have showed better efficacy compared to aspirin preventing non valvular atrial fibrillation (NVAF) related thromboembolism[22]. Results from phase III trials meta-analysis concluded non inferiority of Non Vitamin K Anticoagulants (NOACs) in embolic stroke prevention associated with NVAF and superiority regarding safety against warfarin[83]. Real world data support the findings from the pivotal NOACs trials[405]. Apixaban has demonstrated a favourable profile preventing stroke and haemorrhagic complications[406]. The 3 types of antithrombotics (aspirin, warfarin, apixaban) have different mechanisms of action and different influence on the thrombosis-thrombolysis pathway[407].

Apixaban activity can be measured by Anti-Xa assay. Measurements demonstrated strong linear correlation with apixaban concentration[408]. Diluted Thrombin Time (dTT) can also useful in emergency situations[409]. However, both tests have shortfalls and are not always available. In addition, the tests just provide a single number and not a detailed description of how the drugs affect the steps of the coagulation cascade[408].

A combination of dynamic assays such as TEG and MPA, and analysis of thrombosis related biomarkers (tPA, PAI-1, D-Dimer and microparticles) may be more appropriate, compare with the existing activity tests, to present the anticoagulant potential of the antithrombotics and their inpact in the coagulation process [10,386,388–390]. The above biomarkers have been linked with adverse cardiovascular events and increased mortality[410–412].

We hypothesised that each antithrombotic has unique influence on the fibrin polymerisation and lysis model which correlates with their potential as inhibitors of coagulation. In addition, each antithrombotic type is related with different levels of thrombosis related biomarkers.

Methods

-Study population

In this cross-sectional study, 3 groups of patients with AF (permanent, persistent or paroxysmal), on different antithrombotics, were recruited. The first group, comprised of 62 patients, was treated with antiplatelets (aspirin or clopidogrel) only. For these patients, naïve to oral anticoagulants (as only on antiplatelet agent), NVAF thromboprophylaxis was initiated promptly, according with the CHA_2DS_2VASC score and based on their personal preference and clinical judgment. The second group, again of 60 patients, was treated with warfarin. The INR on the day of the recruitment was within the therapeutic range (INR 2.1-2.9)(mean 2.43, SD 0.25). For the third group which was treated with apixaban for AF thromboprophylaxis, 60 patients were enrolled.

Inclusion and exclusion criteria are shown in chapter 3. The project has been approved by the Local Research Ethics Committee and informed consent was obtained from each participant. Data collected included full clinical and demographic details (age, gender, ethnicity, body weight, BMI, systolic and diastolic blood pressure, smoking and family history of coronary artery disease) and concurrent disease.

-Laboratory methods

Laboratory methods used in this study are the same with previous cross-sectional study 5.1 (AF patients vs 'disease control' patients in sinus rhythm with Ischaemic Heart disease taking antiplatelet drugs), page 113.

-Statistical analysis

Our quantified hypotheses focused on the extent to which the degree of anticoagulation influences the laboratory methods outlined above. A sample size of 60 brings $\alpha<0.02$ and $1-\beta=0.8$ for a two-sided correlation coefficient of 0.4, and $p<0.01$ for a difference of 0.5 of a standard deviation between two indices with a normal distribution.

Shapiro-Wilk test used to assess data distribution. Normal data are presented as mean and standard deviation, non-normal data are presented as median and inter-quartile range. Analysis of variance (ANOVA) with Tuckey's posthoc test were used for normal data and Kruskall–Wallis test with Dunn's posthoc test for non-normal data. To determine which of the TEG or MPA indices were most closely associated with use of OAC, a binary logistic regression analysis was performed. Data were processed with IBM SPSS Statistics version 25 software and p value less than 0.05 was considered significant.

Results

Clinical and standard laboratory characteristics of participants are shown in Table 16. The were no significant differences between the groups regarding demographics and cardiovascular related comorbidities. Thrombosis assessment of whole blood by TEG showed delayed thrombogenesis in patients receiving apixaban compared with patients on warfarin or antiplatelets, based on K (p=0.001 vs warfarin and p<0.001 vs antiplatelets) and angle (p=0.005 vs warfarin and p<0.001 vs antiplatelets) indices (Table 17). Patients on apixaban and warfarin had higher R index compared with patients on antiplatelets (p<0.001). Microplate assay showed that warfarin and apixaban outweigh each other in respect of different indices. Patients on warfarin have longer Lag time (p<0.001), less dense thrombus [expressed as maximum optical density](p<0.001, p=0.006) and faster clot dissolution (p<0.001, p=0.001) compared with patients on the other antithrombotics (antiplatelets and apixaban respectively). On the other hand, apixaban appeared to decelerate fibrin clot development (Rate of clot formation, p=0.005) and lysis time (Time 50% lysed, p<0.001), in comparison with patients on warfarin and antiplatelets. Apixaban group had lower levels of D-Dimers compared with warfarin and antiplatelets group (p<0.001 for both) and t-PA versus warfarin(p=0.03). There was no statistical difference between the groups for the microparticle levels (p>0.05 for all).

From binary logistic regression analysis, 3 MPA indices showed significance (p<0.001) as predictors of OAC (warfarin or apixaban), Lag time [95% confidence intervals (CI) 1.008-1.021], rate of clot formation (95% CI 0.719-0.849) and rate of clot dissolution (95% CI 1.065-1.206). A model to predict the presence-activity of OAC using as predictors the lag time (coefficient B=0.017, p=0.007) and rate of clot formation (B -0.17, p=0.01) indices gives a

specificity of 84% and sensitivity of 92% . Adding as extra independent variable the rate of clot dissolution, marginally increases the specificity to 87% and sensitivity to 94%.

Discussion

Analysis of unfractionated blood's thrombotic activity by tromboelastography revealed significant differences between antithrombotics. Apixaban appeared superior against warfarin and antiplatelets delaying the process of thrombosis by prolonging the first step of fibrin polymerisation and subsequently reducing the rate of clot formation. On the contrary, plasma analysis by microplate assay did not manifest similar findings suggesting that apixaban, apart from inhibition of thrombin pathway, may contribute to inhibition of platelet aggregation. It is known that inhibition of thrombin formation causes less platelet activation that subsequently blocks the fibrin polymerisation, an action which might mediated by apixaban[413].

In the AVERROES trial (Apixaban Versus Acetylsalicylic Acid to Prevent Stroke in Atrial Fibrillation for Patients Who Have Failed or Are Unsuitable for Vitamin K Antagonist Treatment)[406] Apixaban demonstrated superiority against aspirin to prevent ischaemic strokes. In the same study, the events of significant bleeding were similar among the two groups. Additionally, the number of myocardial infarctions between the treatment arms were statistically insignificant. Our results suggest that apixaban is more potent in delaying thrombosis and amplifying clot lysis compare with aspirin, without affecting the clot robustness. That may explain the findings from the AVERROES trial from "haemostatic perspective". The desired role of anticoagulants is to prevent thrombosis but also to maintain an effective clot dynamic in order to avoid haemorrhage. Differences in clot structure related

with apixaban and warfarin, demonstrated by our study, may also reflect findings from the ARISTOTLE (Apixaban for Reduction in Stroke and Other Thromboembolic Events in Atrial Fibrillation)[5] and the AUGUSTUS (Antithrombotic Therapy after Acute Coronary Syndrome or PCI in Atrial Fibrillation)[414] trials. In both trials, apixaban was compared with warfarin regarding efficacy (prevention of ischaemic/thromboembolic events) and safety (bleeding). Results confirmed that apixaban was not inferior to warfarin to prevent ischaemic events but was superior regarding safety outcomes. The important difference between apixaban and warfarin, which may contribute to the safety outcomes of the trials, is the stability of the thrombus that is produced under their influence. Stable thrombus is associated with less haemorrhagic episodes, while medication provides the substrate for slower clot formation and faster clot dissolution in order to prevent thromboembolism. Indeed, previous research in clot structure demonstrated that fast polymerisation of fibrin monomers and thrombus resistant to lysis are features of several cardiovascular diseases and associated with higher mortality[110,415,416].

Analysis of clot structure characteristics showed that values from 3 MPA indices are strongly correlated with activity of oral anticoagulants (apixaban or warfarin). A model with the indices of lag time and rate of clot formation as independent variables can predict the use of OAC with satisfactory specificity and sensitivity. These 2 indices derive from the first 2 steps of the MPA analysis, within 15 minutes from the initiation of the process. Although the assay was tested only in research setting, it may be beneficial in clinical environment as well. Decision of thrombolysis after acute ischaemic stroke with history of recent exposure to OAC and even compliance can be based on this simple model, probably as an alternative to factor Xa assay.

In addition to haemostasis assays, administration of apixaban was related with significantly lower levels of tissue Plasminogen Activator and D-Dimer compared with the other antithrombotics. Elevated levels of both biomarkers are associated with worse outcomes in patients with cardiovascular diseases[417–419]. Normalisation of the above biomarkers may explain further the efficacy and safety results of apixaban from the phase III trials and real word data[5,6].

Limitations

The size of the groups is relatively small but large enough to detect differences on the clot structure properties associated with the individual antithrombotics. Other medications or pathology (such as hyperlipidaemia) may affect the thrombosis/fibrinolysis balance. Our AF groups consistent of patients with permanent or paroxysmal AF and the dose of apixaban was 5mg or 2.5 mg which may result in heterogeneity of clot structure parameters.

Conclusion

NOACs, VKAs and antiplatelets affect the haemostasis by different manner. Warfarin and apixaban demonstrated comparable clot structure dynamics as anticoagulants, both superior to aspirin. In addition, *apixaban* significantly weakens fibrin polymerisation and amplifies fibrinolysis but also decreases levels of thrombosis related biomarkers.

Table 16. Baseline clinical characteristics of patients in 2nd cross-sectional study. Patients with AF on antithrombotics

	2nd cross-sectional study. Patients with AF on antithrombotics			
Group (number of participants)	Warfarin (n=60)	Apixaban (n=60)	Antiplatelets (n=62)	P value Between groups
Age (years)	75 (9)	77 (8)	75 (12)	0.39
Gender, male-female, n(%)	50-50	57-43	69-31	0.09
Ethnicity (White/Asian/Afro-Caribbean) n	54-4-2	57-2-1	51-8-3	0.26
Ischaemic Heart Disease, n(%)	12 (20)	8 (13)	12 (20)	0.22
Diabetes, n(%)	20 (33)	20 (33)	15 (24)	0.31
Hypertension, n(%)	51 (85)	53 (88)	54 (87)	0.73
Heart failure, n(%)	14 (23)	5 (8)	8 (14)	0.08
Current Smokers, n(%)	1 (2)	5 (8)	3 (5)	0.23
Systolic Blood Pressure (mmHg)	136 (19)	133 (17)	131 (17)	0.30
Diastolic Blood Pressure (mmHg)	74 (12)	78 (12)	76 (12)	0.19
Body Mass Index (kg/m^2)	28.8 (5.5)	29 (5.4)	27.4 (6)	0.25
Creatinine Clearance [Cockroft-Gauld](ml/min)	71.5 (47-84)	71.6 (45-88)	68 (45-88)	0.89
Values are shown as Mean (SD) or Median (interquartile intervals) unless stated otherwise				

Table 17. Haemostatic Parameters of patients with AF on antithrombotics and comparison between groups

Method	Index / Biomarker	Warfarin	Apixaban	Antiplatelets	P value Between groups[a]	P value Apixaban vs Warfarin	P value Apixaban vs Antiplatelets	P value Warfarin vs Antiplatelets
Thromboelastography	R(min)	6.2 (5.3-7.7)	6.8 (5.7-7.8)	4.9 (4.1-6)	<0.001	0.43	<0.001	<0.001
	K(min)	1.8 (1.3-2.1)	2.2 (1.8-2.9)	1.6 (1.2-1.9)	<0.001	0.001	<0.001	0.3
	Angle(deg)	64 (9)	59 (9)	67 (9)	<0.001	0.005	<0.001	0.38
	MA(mm)	65.5 (10.1)	65.5 (5.4)	66.9 (6)	0.5			
Microplate assay	Lag time(sec)	445 (396-580)	357 (279-459)	320 (260-354)	<0.001	<0.001	0.01	<0.001
	Rate of clot formation(units/sec)	16.9 (11.8-22.7)	8.4 (6.6-20)	37.3 (20-42)	<0.001	0.005	<0.001	<0.001
	Max. Optical Density (units)	0.42 (0.34-0.51)	0.55 (0.44-0.64)	0.48 (0.42-0.58)	<0.001	<0.001	0.9	0.01
	Rate of Clot Dissolution (units/sec)	45.5 (37-59)	34.5 (28-45)	35 (27-42)	<0.001	0.001	1.00	<0.001
	Time 50% lysed (sec)	207 (36)	137 (41)	229 (67)	<0.001	<0.001	<0.001	0.04
Enzyme-linked immunosorbent assay	D-Dimer (ng/ml)	9.03 (5.1-17.2)	3.94 (3-5.6)	7.82 (3.92-14.2)	<0.001	<0.001	<0.001	0.5
	Tissue Plasminogen Activator (ng/ml)	0.54 (0.18)	0.46 (0.13)	0.54 (0.21)	0.03	0.03	0.1	0.8
	Plasminogen Activator Inhibitor 1 (pg/ml)	0.17 (0.1-0.27)	0.16 (0.11-0.22)	0.16 (0.12-0.23)	0.73			
Microparticles	Platelet Derived Microparticles (events/ml)	15060 (3940-62580)	19490 (1780-53215)	15670 (3560-69200)	0.98			
	Apoptotic Microparticles (events/ml)	509240 (235260-1067120)	326920 (157720-1456225)	469360 (293220-1171140)	0.35			

Values are shown as Mean (SD) or Median (interquartile intervals)
[a]: pairwise comparison performed when p value between 3 groups is significant (<0.05)

5.3 Clot structure: Patients with AF, pre and post apixaban

Abstract

Background: Apixaban is a factor Xa inhibitor which proved to be non-inferior compared to warfarin, for non-valvular atrial fibrillation (NVAF) thromboprophylaxis. In this study we hypothesised that apixaban significantly affects the baseline characteristics of the fibrin polymerisation and fibrinolysis process (clot structure), including thrombosis related biomarkers.

Methods: 32 patients with NVAF not receiving oral anticoagulant (OAC) were enrolled. In those patients, the study tests were performed at baseline and three months after starting apixaban (2.5 or 5 mg bid as clinically indicated). Thromboelastography (TEG), microplate assay (MPA), Enzyme Linked Immunosorbent Assay (ELISA) for tissue Plasminogen Activator (tPA), Plasminogen Activator Inhibitor-1 (PAI-1) and D-Dimer and microparticles enumeration were used to measure haemostatic changes induced by apixaban.

Results: There is a significant prolongation of the R ($p<0.001$) and K ($p=0.047$) indices measured by thromboelastography after initiation of apixaban. Similarly, MPA indices such as Lag Time and Rate Of Clot Formation ($p<0.001$ for both) are suggestive of significant deceleration of thrombogenesis. Comparably, Rate Of Clot Dissolution ($p=0.035$) and Time 50% lysed ($p=0.01$), indices for fibrinolysis, support faster fibrinolysis induced by apixaban. In addition to dynamic assays, there was a significant reduction in D-dimer ($p=0.001$) and PAI-1 levels ($p=0.049$) after commencement of the anticoagulant.

Conclusions: Apixaban is an effective OAC mainly by slowing the mechanisms of thrombosis and augmenting fibrinolysis; in addition, normalises biomarkers related with adverse cardiovascular events.

Introduction

The net clinical benefit of thromboprophylaxis in NVAF has moved towards the OAC after the approval of NOACs[420]. This is supported from large observational studies[421] in addition to the phase three RCTs[83]. It appears that the benefit is mainly from reduction of major bleeding but at the same time, maintaining at least similar efficacy with VKAs preventing ischaemic stroke[422].

Based on ARISTOTLE trial[5] and real world data[384], apixaban significantly reduces intracranial haemorrhages compared to warfarin. As the most severe form of bleeding, intracranial haemorrhage is related with most deaths associated with OAC[423]. In addition to major bleeding, where the activity of the anticoagulant usually needs to be reversed, there are other clinical circumstances that the amplitude of the antithrombotics needs to be evaluated in order to proceed in further therapeutic actions. Examples of such conditions are the emergency lifesaving surgery and thrombolysis after ischaemic stroke, in the presence of OAC[12]. Evaluation of NOACs activity in such circumstances is problematic as the recommended anti Xa activity assay, which gives correlation of NOAC concentration and assumed activity, is not always available and lacks standardisation[409].

In the previous study (Comparison of patients with atrial fibrillation on different antithrombotic agent, page 120) we have demonstrated that there are substantial differences regarding the influence of antithrombotics on the haemostatic process. In order to outline further the

haemostatic profile of apixaban, I investigated the changes that apixaban induces in patients with AF naïve to OAC.

In this study, we tested our model of thrombosis/fibrinolysis dynamic assays and assessment of coagulation biomarkers in patients with AF naïve to OAC at the baseline visit and then after 3 months of continuous OAC with apixaban. We hypothesised that apixaban significantly delays the coagulation mechanisms in vitro and at the same time augments fibrinolysis.

Methods

-Study population

In this follow up study, I enrolled 32 patients with NVAF not receiving OAC. The study tests were performed at baseline and three months after starting apixaban (2.5 or 5 mg bid as clinically indicated). Participants were recruited predominantly from cardiology clinics. OAC, in the form of apixaban, was initiated after explained to the patients the risks and benefits of thromboprophylaxis in NVAF. The decision was based on the individual CHA_2DS_2VASc score, according to European guidelines. All subjects had satisfactory AF rate control (60-100 bpm). In all cases, citrated venous blood was collected and the following haematology and biochemistry tests were assessed: FBC, INR, APTT, U&E's, fibrinogen, CRP and LFTs. Renal function was defined by creatinine clearance, calculated using the Cockcroft-Gault equation.

Inclusion and exclusion criteria are shown in chapter 3. The project has been approved by the Local Research Ethics Committee and informed consent was obtained from each participant.

Data collected included full clinical and demographic details (age, gender, ethnicity, body weight, BMI, systolic and diastolic blood pressure, smoking and family history of coronary artery disease) and concurrent disease.

-Laboratory methods

Laboratory methods used in this study are the same with previous cross-sectional study 5.1 (AF patients vs 'disease control' patients in sinus rhythm with Ischaemic Heart disease taking antiplatelet drugs), page 113.

-Statistical analysis

In this longitudinal study, the sample size of 30 brings equivalent power for a change of 0.5 of a standard deviation e.g. from mean 100 units (SD 15) at baseline to 107 units or 93 units at 3 months. For follow up comparisons, t-test was used for normal data and Related-Samples Wilcoxon Signed Rank test for non-normal data. To determine which study indices were most closely associated with use of apixaban, a binary logistic regression analysis was performed. Data were processed with IBM SPSS Statistics version 25 software and p value less than 0.05 was considered significant.

Results

Table 18 shows clinical and baseline laboratory data for this follow up study. The analyses involved patients with permanent and paroxysmal AF. Most of the patients (78%) were on 5

mg of apixaban BID. Infection markers, such as CRP and white cells were similar before and after initiation of apixaban. A clinically insignificant, drop noticed in haemoglobin and creatinine clearance, 3 months after initiation of apixaban (table 18). There is a substantial prolongation of the R (p<0.001) and K (p=0.047) indices measured by thromboelastography, suggesting attenuation of fibrin polymerisation following initiation of Apixaban (table 19). More pronounced changes observed with microplate assay method. There is a marked delay of thrombogenesis and amplification of fibrinolysis after commencement on apixaban, based on Lag Time and Rate Of Clot Formation indices for thrombogenesis (p<0.001) and Rate Of Clot Dissolution (p=0.035) and Time 50% lysed (p=0.012) for fibrinolysis. As regard to thrombosis biomarkers, there was a significant reduction in D-dimer (p=0.001) and PAI-1 levels (p=0.049) after commencement of apixaban. There were no significant changes regarding the levels of the microparticles.

From binary logistic regression analysis, 2 MPA indices reached statistical significance as predictors of apixaban anticoagulation effect, Lag time [p=0.007, 95% confidence intervals (CI) 1.004-1.026] and rate of clot formation (p=0.01, 95% CI 0.645-0.897). A model to predict the presence-activity of apixaban using as predictors the lag time (B=0.015) and rate of clot formation (B=-0.27) indices gives a specificity of 84% and sensitivity of 87%.

Discussion

Our data suggest that apixaban affects all stages of thrombosis and fibrinolysis but not the density of the fibrin clot. The findings from this follow up study support our results from the previous cross sectional study, where the haemostatic profile of different antithrombotics was compared (Data chapter 5.2). Many studies have reported changes in the clot robustness related

with various disease which carrying increase risk of thromboembolism[110]. Several parameters have been assess in research setting such as permeability, compaction and fibre thickness in order to express the clot robustness[125,424,425]. However, more robust clot does not always associated with acceleration of fibrin polymerisation or reduced fibrin polymer lysis[426]. The last 2 components of haemostasis appears to be equally important as regard the thrombotic tendency and outcomes that are related with multiple cardiovascular diseases[427,428]. Apixaban, according to this study, alters clot features by attenuation of the fibrin network development and a more lysable fibrin structure.

The significant differences of various haemostatic indices, pre and post apixaban may have clinical implications regarding assessment of the anticoagulant effect of NOACs in several scenarios, such as decisions for thrombolysis, major haemorrhage and emergency surgery. At present, drug-calibrated chromogenic Anti-Xa assays are recommended as suitable methods to provide rapid quantitation of anti-Xa NOACs, like apixaban[429]. As an alternative to chromogenic assays, R index from thromboelastography has been recommended as marker to identify NOAC effect[430]. From our study, 2 microplate assay indices, lag time and rate of clot formation appear to be associated with strong, statistically significant difference ($p<0.001$ for both indices) after treatment with apixaban. In a multivariable logistic regression model, lag time and rate of clot formation predict the anticoagulant effect of apixaban. This finding supports the results from our previous study where the same indices predict the effect of warfarin and apixaban in plasma. The combination of these 2 MPA, thrombosis indices may be beneficial for quantification of the anticoagulant effect of OAC[385].

Administration of apixaban, also resulted in decreased levels of D-Dimer and PAI-1. Similar results with reduction of D-Dimer has been reported with Dabigatran and likely, is a

characteristic of NOACs[417]. The reduction of PAI-1 may be related with the enhanced fibrinolysis reported in this study, mediated by apixaban. PAI-1 in blood, forms complexes with tPA and there is strong association between levels of tPA and PAI-1 antigens[123]. The latter may explain our findings from this follow up study in combination with the previous cross-sectional study, where patients receiving apixaban appeared to have lower levels of tPA compared to other antithrombotics. Elevated levels of both biomarkers are associated with worse outcomes in a broad variety of cardiovascular diseases[419,431].

Limitations

From methodological perspective, my assessment of the clot structure changes induced by apixaban were performed in vitro. In vivo, clot structure may have different response to anticoagulants as other physiological or non-physiological factors may intervene. Additionally, MPA method utilises standard doses of thrombin and tPA. This is not physiological as variations in concentration and structure of the inducers of thrombosis and thrombolysis occur. However, the study evaluates anticoagulation potential as it compares the haemostatic process amplitude against standard doses of inducers.

Cohort size is relatively small and susceptible to selection bias. Few subjects (22%) received 2.5 mg of apixaban twice a day which has less effect on the anticoagulation potential of the samples from the full dose of 5mg twice a day. In addition, some of the patients had paroxysmal and some permanent AF.

Conclusion

Apixaban, in our model of clot structure analysis, significantly delays thrombosis and amplifies fibrinolysis without effect on maximum clot density/strength. Furthermore, can reduce thrombosis biomarkers which are related with adverse cardiovascular events. MPA indices of clot formation (Lag time and Rate of clot formation) may have value in assessment of antithrombotic activity in clinical circumstances.

Table 18. Clinical, demographic and routine laboratory data for the follow up study (Patients with Atrial fibrillation, naïve to oral anticoagulants)

Characteristic			
Age (years)	78 (6.6)		
Gender, male-female, n(%)	19-13 (59-31)		
Ethnicity (White/Black/Asian), n	29-2-1		
Diabetes, n(%)	12 (37)		
Hypertension, n(%)	30 (94)		
Ischaemic Heart Disease, n(%)	6 (19)		
Heart failure, n(%)	3 (9)		
Current Smokers, n(%)	3 (9)		
Body Mass Index (kg/m^2)	29 (5)		
Apixaban dose (2.5-5mg), n(%)	7-25 (22-78)		
Atrial Fibrillation type (permanent-paroxysmal), n(%)	17-15 (54-46)		
	Pre Apixaban	Post Apixaban	P value
C-Reactive Protein (mg/L)	3(1-8)	4(1-7)	0.7
White Cell Count ($x10^9/L$)	7.2 (6.2-8.6)	7.6 (6.2-8.8)	0.7
Creatinine Clearance [Cockroft-Gauld](ml/min)	76.2 (47.5-91)	71.2 (45.7-89.4)	0.001
Haemoglobin (g/L)	133 (126-149)	131 (122-140)	0.01
Values are shown as Mean (SD) or Median (interquartile intervals) unless stated otherwise			

Table 19. Study indices in patients with atrial fibrillation at baseline and after three months treatment with apixaban

Method	Indices/Biomarkers	Baseline	Follow up	P value baseline vs follow up
Thromboelastography	R (min)	5.3 (4.2-6)	6.8 (5.7-7.8)	<0.001
	K (min)	1.9 (1.5-3)	2.4 (2-3.6)	0.047
	Angle (deg)	59 (9)	56 (9)	0.12
	Maximum Amplitude (mm)	64.8 (6)	64.9 (9)	0.92
Microplate assay	Lag time (sec)	223(185-265)	320(270-365)	<0.001
	Rate of clot formation (units/sec)	16 (13-23)	7 (6-9)	<0.001
	Maximum Optical Density (units)	0.59 (0.54-0.68)	0.63 (0.55-0.68)	0.20
	Rate of Clot dissolution (units/sec)	28 (24-31)	31 (25-38)	0.03
	Time 50% lysed (sec)	139 (33)	115 (31)	0.01
Enzyme-linked immunosorbent assay	D-Dimer (ng/ml)	6.6 (4.2-10.3)	4.1 (2.8-6.3)	0.001
	Tissue Plasminogen Activator (pg/ml)	0.42 (0.07)	0.45 (0.08)	0.12
	Plasminogen Activator Inhibitor 1 (ng/ml)	0.16 (0.15-0.21)	0.14 (0.1-0.18)	0.049
Microparticles	Platelet Derived Microparticles (events/ml)	20380 (4240-50530)	27630 (2810-63070)	0.30
	Apoptotic Microparticles (events/ml)	359160 (160705-663410)	275160 (162525-628740)	0.23
Values are shown as Mean (SD) or Median (interquartile intervals)				

5.4 Microparticles in patients with permanent AF and preserved left ventricular systolic function. The effect of spironolactone.

Abstract

Background: Patients with permanent atrial fibrillation (AF), even with preserved left ventricular (LV) contractility, might experience heart failure symptoms, mainly due to LV diastolic dysfunction. We tested whether factors that reflecting general fitness are related with changes in microparticle levels and if administration of spironolactone due to its anti-fibrotic features can reduce the concentration of plasma extracellular vesicles.

Methods: Patients for this study were recruited as part of the double-blind, placebo-controlled IMPRESS-AF trial. The trial randomised 250 stable patients with permanent AF and preserved left ventricular ejection fraction to spironolactone 25 mg daily or placebo. The primary efficacy outcome was peak oxygen consumption (peak VO_2) on cardiopulmonary exercise testing (CPET) at two years. Secondary end points included 6-minute walk distance, diastolic left ventricular function and quality of life. We performed enumeration of four different cellular/antigenic origin (platelet, endothelial, monocyte and apoptotic) microparticles. 147 patients from the baseline visit and 103 patients from end of study visit included in the analysis.

Results: After stepwise linear regression analysis, increased resting heart rate prior to CPET is associated with lower levels of CD42b+ microparticles (p=0.007, B=-0.2). Peak VO_2 was positively correlated with CD42+ microparticle levels (p=0.04, B=0.2). Lower score in EQ5D5L questionnaire (p=0.02, B=-0.2) and lower resting heart rate (p=0.04, B=-0.2), pre CPET challenge, were associated with higher levels of endothelial derived microparticles. Peak VO_2 (p=0.03, B=-0.2) and score of EQ5D5L questionnaire (p=0.05, B=-0.2) were negatively correlated with monocyte derived and annexin V+ microparticles, respectively.

Spironolactone, compared to placebo, did not induce any significant alteration as regard the microparticle levels.

Conclusion: In patients with AF and preserved LV systolic function there is a relationship between physical status and microparticle levels. Spironolactone compared to placebo did not alter the assessed microparticle concentrations.

Introduction

The impaired haemostasis in atrial fibrillation (AF) is related with complex pathophysiological mechanisms[391]. Prothrombotic and inflammatory biomarkers have been identified in excess among AF populations[382]. In some cases, higher concentration of biological molecules is associated with worse cardiovascular outcomes[417,432].

Research studies have demonstrated strong evidence that plasma microparticles are involved in mechanisms of apoptosis, thrombosis and inflammation[20]. These small vesicles, without nucleus, carrying the antigenic characteristics of the paternal cells and can transfer biological information[162]. It appears that in stable clinical conditions microparticles have physiological functions related with homeostasis and under different stimuli their balance is disrupted[433]. The imbalance is associated with quantitative (concentration related) and qualitative (antigenic related) changes which reflect disease activity and in some cases possess a prognostic value[434,435].

Different approaches have been proposed in order to assess physical fitness in patients with cardiovascular conditions and monitoring disease activity. Cardiopulmonary exercise test

(CPET)[399] and 6 minute walking test[436] are valid methods for objective assessment of general physical status. In addition to objective, subjective assessment can be achieved by questionnaires, designed for patients with various conditions, such as the Minnesota Living With Heart Failure (MLWHF) and EQ5D5L[437,438].

The IMPRESS-AF trial[393] was single centre, double blind, randomised placebo-controlled study with aim to determine if treatment with spironolactone as compared with placebo, improves exercise tolerance, quality of life and diastolic function in patients with permanent atrial fibrillation and preserved left ventricular (LV) systolic function. From pathophysiological aspect, spironolactone can inhibit the adverse effect of aldosterone in heart failure. Aldosterone levels are elevated in patients with heart failure and higher levels are associated with LV hypertrophy, cardiac inflammation and fibrosis[439–441].

Our hypothesis is that there is a relation between microparticle levels, and the physical status as measured by objective and subjective methods. Furthermore, we tested the assumption that spironolactone reduces the microparticle levels in patients with AF and preserved LV function, as can decreases fibrosis and atrial remodelling.

In this study, we assessed the levels of 4 plasma microparticle types [platelet derived microparticles (anti CD42b+), endothelial derived microparticles (anti CD144+), monocyte derived microparticles (anti CD14+) and apoptotic microparticles (Annexin V+)] in a group of patients from the IMPRESS-AF trial.

Methods

-Study population

250 patients were recruited in total for the IMPRESS-AF trial. Inclusion criteria were 50 years old or older, permanent atrial fibrillation and LV ejection fraction >55%. Exclusion criteria included contraindications to aldosterone inhibitors, uncontrolled hypertension and presence of severe comorbidities with life expectancy <2 years. The primary outcome was improvement in exercise tolerance at 2 years (estimated by cardiopulmonary exercise testing and 6 minutes walking test) compare to the baseline visit. Secondary outcomes included quality of life [assessed using the EuroQol EQ-5D-5L (EQ-5D) and Minnesota Living with Heart Failure (MLWHF) questionnaires], diastolic function and all-cause hospitalisation. Plasma samples were processed for microparticle analysis of 147 patients from the baseline and 103 from the end of the study visit. After the baseline visit the patients were randomised blindly to spironolactone or placebo treatment. The end of the study visit was conducted 24 months after the baseline. The sample selection was random for both the baseline and the end of the study visit.

-Laboratory Methods

Routine haematological and biochemical tests (including BNP) were performed on the day of the first and last visit (baseline and 24 months). Blood collection was performed before the exercise capacity assessment. Remaining blood samples were separated by centrifugation and the plasma was stored at –70 °C for subsequent batched analysis. Patients in whom permanent

AF was confirmed underwent an echocardiogram and those with an LVEF of >55% with no severe valvular disease were eligible for the study (i.e. preserved ejection fraction, contractility). E/E' (early mitral inflow velocity/TDI derived early septal mitral annular diastolic velocity) was used to assess diastolic function in AF. Participants undertook a 6-minute walk test. As additional tool to assess the exercise tolerance, we used the VO_2 maximum value, result derived from cardiopulmonary exercise test (CPET) (using the L COSMED CPET system).

-Microparticles

In this study, I analysed the levels of 4 types of microparticles, platelet derived microparticles (CD42b+), endothelial derived microparticles (CD144+), monocyte derived microparticles (CD14+) and apoptotic or Annexin V+ microparticles. Microparticle analysis with flow cytometer is described in 3.4.2.

For enumeration of endothelial derived microparticles, anti-CD144-biotinylated antibodies [Bio-techne, Catalog No BAM9381], for monocyte derived microparticles, anti-CD14-biotinylated antibodies [Invitrogen, Catalog No MA1-19489] and for platelet-derived microparticles, biotinylated anti-human CD42b antibody (Abcam, Cambridge, UK) were used. For Annexin V-binding microparticles, platelet poor plasma was incubated for 30 min with Annexin V binding buffer, 2x concentrated solution (Molecular Probes, Thermofisher scientific, UK) and 5 µL of Annexin V, Alexa Fluor® 488 conjugate (Molecular Probes, Thermofisher scientific, UK).

Microparticle analysis was promptly performed, after achieving the required dilution, using the Apogee A50 flow cytometer (Apogee Flow Systems, High Wycombe, UK). 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 description of MP enumeration process in Appendices).

-Statistical analysis

Shapiro-Wilk test used to assess data distribution. Normal data are presented as mean and standard deviation, non-normal data are presented as median and inter-quartile range. Analysis of variance (ANOVA) with Tuckey's post-hoc test was used for parametric data and Kruskall–Wallis test with Dunn's post-hoc test for non-parametric data. Categorical indices were compared by the Chi-squared test. Correlations were sought using Spearman's method. To determine which characteristics are influence the level of microparticles, a stepwise linear regression analysis was performed. Statistical analysis was performed with IBM SPSS Statistics version 25 software and p value less than 0.05 was considered significant.

In order to assess the effect of spironolactone on the microparticle levels we divided the patients into four groups, two groups from the baseline line visit and two groups from the end of the study visit. One group, from the end of the study, was consistent of 53 patients who were treated with spironolactone for 24 months and the other group of 51 patients treated with placebo for the same period. From the baseline groups, the first was consistent from 72 patients where later were randomised to be treated with spironolactone and the second of 75 patients pre placebo administration.

Results

Demographic and clinical data along with laboratory results of the 147 patients, from the base line visit, are shown on the table 20. From univariate analysis, microparticle levels are significantly correlated with different variables-parameters that were assessed in the study (table 21). In general, there was no strong correlation (r>0.6). The level of significance remained in stepwise linear regression analysis, with small predictive value (R^2). As regard to Platelet derived microparticles (CD42b+), increased resting heart rate prior to CPET is associated with lower levels of CD42b+ microparticles (p=0.007, B=-0.2). Peak VO_2 was positively correlated with CD42+ microparticle levels (p=0.04, B=0.2). Lower score in EQ5D5L questionnaire (p=0.02, B=-0.2) and lower resting heart rate (p=0.04, B=-0.2), pre CPET challenge, were associated with higher levels of endothelial derived microparticles. Peak VO_2 (p=0.05, B=-0.2) and score of EQ5D5L questionnaire (p=0.03, B=-0.2) were negatively correlated with monocyte derived and annexin V+ microparticles, respectively.

Table 22 shows demographic, clinical and laboratory data from the groups. In general, the groups were well matched. Patients from the end of the study appear fitter compare to baseline, as regard the distance walked during the 6 min walking test, however, peak VO_2, another marker of exercise capacity, was similar between baseline and end of the study groups. Endothelial and monocyte derived microparticles are lower in both of end of the study groups; although, the statistical significance for the CD14+ microparticles difference, disappears after Bonferroni correction. There was no difference between the platelet derived and apoptotic

microparticles between the groups. Overall, administration of spironolactone does not seem to affect the assessed microparticle populations.

Discussion

The main findings of this study are that general health status as assessed subjectively with the EQ5D5L questionnaire, and objectively with the peak VO_2, is related with microparticle levels. In addition, spironolactone administration in patients with permanent AF and preserved LV contractility, resulted in no difference regarding microparticle concentrations as compared to placebo.

Enumeration of microparticles is challenging[442]. Even the definition of microparticles is under constant review. The agreed broad term to describe these formations is "extracellular vesicles" and only if the genesis of the vesicle is documented by live imaging technique then the term microparticle is accepted[126]. In most of the literature these vesicles with diameter between 100nm-1μm and at least one marker to describe their membrane biochemical composition are called "microparticles" and thus, we kept that term. The clinical utilization of microparticles as biomarkers is limited as enumeration techniques and sample handling lack standardization[20]. The interpretation of the quantitative analysis is not straight forward as the functions of the microparticles appear to be regulated by many factors, such as cell origin, stimuli and concentration. Multiple studies have suggested that microparticles, as part of the organism signalling process, are present in physiological conditions such as apoptosis and protection of deleterious external stimuli[443]. On the other hand, several non-physiological conditions as

sepsis, autoimmune disease and cardiovascular pathologies are associated with high levels of microparticles from different cellular origin[444].

In the present study, we investigated the levels of 4 microparticle subtypes in relation with general health status based on objective (six minutes walking test and CPET) and subjective (heart failure questionnaires) assessment methods. In general, patients recruited for the study were seen in outpatient setting, had stable health and were ambulatory. In particular, the end of the study population was followed up every 3 months for 2 years and suboptimal control of cardiovascular risk factors (hypertension, diabetes etc) and conditions such as anaemia and anticoagulation issues were investigated and treated. In addition, withdrawal from the trial of patients who their general health for various reasons significantly deteriorated, may explain why patients at the end of the study visit, performed better in 6 minute walking test and possibly had lower levels of CD144+ microparticles (table 22).

PeakVO_2 from regression analysis appears to influence positively the levels of CD42b+ and negatively the CD14+ microparticles (table 20). Peak VO_2 consumption correlates with disease severity and general physical fitness level[399]. It was demonstrated that CD42+ microparticles may have endothelial protective-repairing role in healthy subjects[445]. Platelet derived microparticles in vitro may transform mononuclear cell population to endothelial progenitor cells by induction of membrane proteins characteristic for endothelial cells with angiogenic properties. Similarly, CD42b+ extracellular vesicles can boost endothelial regeneration after vascular injury in vitro[446]. In contrast with the positive relation between the peak VO_2 and CD42+ microparticles, there is a negative influence of the general fitness to CD14+ microparticles. Monocyte derived microparticles is known that can promote endothelial inflammation and apoptosis which results to impaired vascular physiology[447]. Impaired

vascular physiology can inhibit normal endothelial adaptations such as angiogenesis and remodelling important for sustaining endothelial function[448].

Resting heart rate (prior to CPET) as variable is negatively related with the levels of plasma circulating endothelial and platelet derived microparticles. From the literature, resting HR, apart from marker of fitness, is a risk factor for mortality[449]. In our study, we demonstrated that higher resting AF rate is associated with lower levels of platelet and endothelial derived microparticles. In healthy subjects CD144+ microparticles may inhibit apoptosis and possess cytoprotective and anti-inflammatory activities[450].

Another marker from our study that reflects fitness is the EQ5D5L questionnaire score which offers a simple outline of a respondent's health status. Higher marking on EQ5D5L questionnaire is associated with lower levels of endothelial derived and Annexin V microparticles. Indeed, higher exercise capacity and general physical status as evidenced by chronic aerobic exercise can cause reduction of endothelial derived microparticles[451].

It appears that the relationship between health status and microparticles is complex. Due to diverse methodological approaches for enumeration of microparticles, an attempt to compare data from different studies is unlikely to produce valid results. However, there is evidence that the levels of extracellular vesicles change within the spectrum of physical status and disease activity[452]. In order to define the quantitative relation between fitness and microparticles more studies are needed. Further antigenic characterization of microparticles may also provide important information related with their role as biomarkers in disease monitoring.

In a previous study, we demonstrated higher levels of apoptotic microparticles in AF compared with a disease control group (Data chapter 5.1). Apoptosis and atrial remodelling have been described in AF[453]. In addition, shear stress and hormonal imbalance, such as angiotensin II and transforming growth factor beta 1, induce inflammation and fibrosis to myocardium. We have hypothesised that spironolactone can reduce fibrotic activity on the myocardium in patients with permanent AF and anti-fibrotic activity is associated with reduction of microparticles related with apoptosis and inflammation. Spironolactone has beneficial effects in patients with heart failure and reduced LV ejection fraction[454]. There is evidence that spironolactone may improve diastolic dysfunction in patients with heart failure and preserved LV contractility in sinus rhythm[455,456]. In the current study we demonstrated that there is no statistical difference in microparticle levels, with different antigenic background, between the placebo and the spironolactone group. This finding suggests that spironolactone did not influence microparticle levels in patients with permanent AF and preserved LV function. Significant reduction in platelet and endothelial derived microparticles have been reported after restoration of sinus rhythm in persistent AF[457]. Permanent AF is associated with more advanced atrial fibrosis which results in low efficacy of rhythm control strategies[458]. It is likely that the anti-fibrotic functions of aldosterone inhibition have no clinical benefits in permanent AF with preserved LV contractility, as assessed by changes in microparticle levels, but might be more promising in patients with paroxysmal AF[453,459].

Limitations

Exercise capacity assessment has its limitations as can be affected by various musculoskeletal problems despite every effort to perform the tests until the limits of exhaustion. In addition, specific validation of the quality of life questionnaires in the study population was not

performed. Specific microparticle levels were assessed which may affected by other clinical conditions that not considered. The enumeration of microparticles was performed from frozen samples, with one freeze/ thaw cycle.

Conclusion

In this study, we demonstrated that factors related with physical fitness such as peak VO_2 and resting heart rate from CPET along with EQ5D5L questionnaire score reflect changes in plasma circulating microparticle levels. This finding may be beneficial and have clinical relevance as can provide information about patient's health status and disease activity. Spironolactone did not affect concentration of microparticles, compared to placebo in patients with permanent AF and good LV systolic function.

Table 20. Demographic, clinical and laboratory data from baseline visit (n=147)

Age (years)	73 (7)
Ethnicity [white/other] (n%)	142/5 (97%/3%)
Gender [male/female] (n%)	112/35 (76%/24%)
Body mass index (kg/m^2)	30.6 (5.6)
Systolic Blood Pressure (mmHg)	130 (15)
Diastolic Blood Pressure (mmHg)	75 (11)
Diabetes (n%)	29 (20%)
Current smokers / Ex-smokers / Never smoked (n%)	6/78/63 (4%/53%/43%)
Alcohol use (units/week)	4 (0-12)
Haemoglobin (g/L)	142 (16)
MCV (L/cell)	91 (5.5)
Leucocytes (x10^9/L)	7.2 (6-8.7)
Neutrophils (x10^9/L)	4.8 (3.7-5.6)
Lymphocytes (x10^9/L)	1.6 (1.3-2)
Monocytes (x10^9/L)	0.6 (0.5-0.8)
Platelets (x10^9/L)	223 (178-267)
Serum Creatinine (umol/L)	90 (21)
estimated Glomerular Filtration Rate (ml/min)	69 (16)
Creatinine clearance (Cockcroft-Gault) (ml/min)	67 (15)
Urea (mmol/L)	6.3 (5.2-7.4)
Total cholesterol (mmol/L)	4.2 (1)
Low Density Lipoprotein (mmol/L)	2 (1.5-2.8)
Triglycerides (mmol/L)	1.3 (1.1-1.5)
Brain Natriuretic Peptide (pg/mL)	115 (73-210)
Distance walked in 6 minutes waking test (meters)	268 (77)
Peak VO_2 (mL/kg/min)	14.5 (4.8)
Minnesota Living with Heart Failure questionnaire score	13 (6-33)
EQ5D5L questionnaire score	0.8 (0.7-0.9)
LVEF (%)	61 (5)
E/E' ratio	10.4 (3.8)
Platelet derived (CD42+) microparticles (10^{3}xevents/ml)	5.9 (0.6-37.3)
Endothelial derived (CD144+) microparticles (10^{3}xevents/ml)	121.7 (12.9-265.5)
Monocyte derived (CD14+) microparticles (10^{3}xevents/ml)	3.3 (0.3-82.4)
Apoptotic (annexin V+) microparticles (10^{3}xevents/ml)	71 (37-116.5)
Values are shown as Mean (SD) or Median (interquartile intervals)	

Table 21. Linear regression and Univariate analysis for variables of microparticles in patients with permanent atrial fibrillation and preserved left ventricular systolic function.

Stepwise linear regression analysis						Univariate analysis	
Dependent variable	Independent Variable(s)	Standardized coefficient B	R	R^2	P value	Spearman Correlation coefficient r	P value
Platelet derived microparticles (CD42b+)	Resting heart rate	-0.2	0.2	0.03	0.007	-0.2	0.04
	Peak VO_2	0.2	0.3	0.08	0.04	0.2	0.01
Endothelial derived microparticles (CD144+)	Resting Heart Rate	-0.2	0.1	0.04	0.04	-0.1	0.01
	EQ5D5L questionnaire score	-0.2	0.2	0.07	0.02	-0.2	0.02
Monocyte derived microparticles (CD14+)	Peak VO_2	-0.2	0.2	0.03	0.048	-0.2	0.02
Annexin V+ microparticles	EQ5D5L questionnaire score	-0.2	0.2	0.05	0.01	-0.2	0.01

Table 22. Baseline and end of study (EOS) demographic, clinical and laboratory data

Group (n)	Baseline pre placebo (75)	Baseline pre spironolactone (72)	EOS placebo (51)	EOS spironolactone (53)	Difference between groups P value
Age (years)	73 (7)	73 (7)	74 (8)	75 (8)	0.3
Gender [male/female] n(%)	20/55 (27%/73%)	15/57 (21%/79%)	12/39 (24%/76%)	12/41 (23%/77%)	0.9
Ethnicity [white/other] n(%)	72/3 (96/4)	70/2 (97/3)	50/1 (98/2)	52/1 (98/2)	0.7
Diabetes (n%)	14 (19%)	15 (21%)	12 (23%)	9 (17%)	0.9
Current smokers / Ex-smokers / Never smoked n(%)	4/38/33 (7%/51%/42%)	2/40/30 (3%/56%/41%)	3/28/20 (6%/55%/39%)	1/29/23 (2%/55%/43%)	0.9
Systolic Blood Pressure (mmHg)	129 (14)	130 (16)	129 (15)	124 (19)	0.3
Diastolic Blood Pressure (mmHg)	74 (10)	76 (11)	74 (11)	73 (11)	0.5
Body mass index (kg/m^2)	31 (6)	31 (6)	31 (5)	31 (6)	0.9
Angiotensin converting enzyme Inhibitor n(%)	38 (51%)	29 (40%)	27 (53%)	21 (40%)	0.3
Angiotensin II receptor blocker n(%)	11 (15%)	9 (13%)	7 (14%)	8 (15%)	0.9
Beta blocker n(%)	37 (49%)	39 (54%)	26 (51%)	29 (55%)	0.9
Calcium channel blocker n(%)	23 (31%)	24 (33%)	17 (3150	19 (36%)	0.9
Non vitamin K oral anticoagulant n(%)	33 (44%)	35 (49%)	23 (45%)	25 (47%)	0.9
Warfarin n(%)	24 (32%)	23 (32%)	17 (33%)	17 (32%)	1
Statin n(%)	35 (47%)	40 (55%)	24 (47%)	26 (49%)	0.7
Digoxin n(%)	13 (17%)	9 (12%)	11 (21%)	7 (13%)	0.6
Diuretic n(%)	20 (27%)	19 (26%)	12 (23%)	16 (30%)	0.9
Body mass index (kg/m^2)	31 (6)	31 (6)	31 (5)	31 (6)	0.9
LVEF (%)	61 (5)	61 (6)	57 (5)	59 (5)	<0.001[a]
E/E' ratio	9.7 (7.6-13.1)	9.8 (7.9-12)	9.9 (7.5-12)	9.5 (7.2-10.7)	0.6
Distance walked in 6 minutes (meters)	270 (82)	266 (70)	326 (102)	314 (84)	<0.001[a]
Peak VO_2 (mL/kg/min)	14 (10.8-17.4)	13.8 (10.9-17.5)	14.1 (10.9-17.5)	13.2 (11.2-16.8)	1
Minnesota Living with Heart Failure questionnaire score	11.8 (4.7-28.2)	15 (7.2-34)	9.5 (1.7-26.2)	12 (2.7-29)	0.2
EQ5D5L questionnaire score	0.83 (0.15)	0.8 (0.17)	0.84 (0.16)	0.81 (0.22)	0.6
Brain Natriuretic Peptide (pg/mL)	113 (79-208)	118 (67-227)	148 (97-222)	155 (107-252)	0.1
Haemoglobin (g/L)	142 (15)	142 (17)	138 (16)	137 (14)	0.1
Leucocytes (x10^9/L)	6.9 (5.9-8.4)	7.2 (6.1-8.9)	7.1 (5.7-8.3)	7 (5.9-7.9)	0.6
Platelets (x10^9/L)	213 (66)	232 (78)	220 (56)	209 (61)	0.2
estimated Glomerular Filtration Rate (ml/min)	70 (15)	69 (17)	64 (15)	67 (13)	0.2
Creatinine clearance (Cockcroft-Gault) (ml/min)	85 (34)	82 (25)	76 (32)	77 (33)	0.3
Total cholesterol (mmol/L)	4.2 (0.9)	4.3 (1.1)	4.1 (0.8)	4 (1.1)	0.7
Low Density Lipoprotein (mmol/L)	2 (1.5-2.8)	2.1 (1.5-2.7)	2 (1.8-2.8)	2.1 (1.5-2.8)	0.8
Triglycerides (mmol/L)	1.3 (1-1.7)	1.2 (0.9-1.9)	1.2 (0.9-1.7)	1 (0.8-1.6)	0.4
Apoptotic (annexin V+) microparticles (10^{3}xevents/ml)	82.1 (38.7-138)	65.3 (36.7-94.5)	47.2 (27.5-93.5)	69.9 (31.3-122.7)	0.2
Platelet derived (CD42+) microparticles (10^{3}xevents/ml)	6.9 (0.8-39.4)	3.1 (0.5-35.8)	9.1 (1.4-40.6)	5.2 (0.6-40.1)	0.6
Endothelial derived (CD144+) microparticles (10^{3}xevents/ml)	127.3 (6.4-284)	112.5 (12.9-242.3)	7.8 (0.4-114.9)	18.6 (0.3-123.1)	<0.001[a]
Monocyte derived (CD14+) microparticles (10^{3}xevents/ml)	7.3(0.3-94.2)	2.5 (0.4-65.7)	0.5 (0.06-16.8)	1 (0.08-10.4)	0.3[b]

Values are shown as Mean (SD) or Median (interquartile intervals)
a: Statistical difference between baseline and EOS groups but not between EOS groups
b: After Bonferroni correction

Section 6: Summary of findings, conclusion and suggestions for future studies

6.1 Summary of findings

The main findings of the clot structure and microparticles in atrial fibrillation thesis can be summarized as follows:

- Patients with NVAF have higher thrombogenic potential compared to IHD patients in sinus rhythm.
- Patients with NVAF have elevated levels of apoptotic microparticles compared to patients with sinus rhythm which may contribute to higher thrombogenic risk in AF.
- In our model of assessment clot structure and OAC potential, apixaban and warfarin demonstrated more dynamic anticoagulant features compared to antiplatelets.
- Apixaban is a potent anticoagulant affecting the whole haemostatic cascade but not fibrin clot robustness.
- The effect of apixaban in the coagulation cascade and clot structure is different compared to warfarin and aspirin.
- Administration of apixaban resulted in normalisation/reduction of biomarkers, such us D-Dimer and PAI-1.
- Based on our results, microplate assay is a dynamic method with excellent reproducibility to assess the anticoagulation status in subjects. Method indices, such as lag time and rate of clot formation may be beneficial in order to monitor anticoagulation activity in several clinical situations.
- In patients with AF and preserved LV systolic function there is a relationship between physical status and microparticle levels.
- Spironolactone, compared to placebo, did not influence significantly microparticle levels in patients with permanent AF and maintained LV contractility.

6.2 Overall conclusion

The current thesis represents a detailed assessment of clot structure in AF. Haemostasis was investigated with different methods including a viscoelastic bedside technique performed in whole blood (TEG), a "microplate-reader based" technique in citrated plasma evaluating fibrin formation and lysis (MPA), immunoassays (i.e. commercial ELISAs) to determine plasma concentrations of PAI-1, tPA and D-dimer, and lastly, measurements of two different microparticle populations (CD42+ and annexin V+).

As AF is associated with increased risk of thromboembolism the initial approach was to investigate pathophysiological differences between normal sinus rhythm and loss of atrial contraction and atrioventricular desynchrony. My results suggest that patients with NVAF have more potent coagulation mechanisms, so they generate faster fibrin clot which also dissolves slower. PS+ microparticles may play a role in the hypercoagulable AF tendency. In the second part, I examined further the clot structure changes associated with different antithrombotics (antiplatelet, VKA and apixaban). Findings support that each antithrombotic affect haemostasis by different manner. Warfarin and apixaban are more efficient than antiplatelets to alter clot structure characteristics. Apixaban induces changes on the fibrin network that allow faster lysis and in addition normalises biomarkers related with adverse cardiovascular events. On the last part of my thesis, I explore a possible relationship between microparticles and the physical status in patients with permanent AF and preserved LV systolic function. The study shows that parameters reflecting fitness level such as resting heart rate and peak VO_2 from CPET and patient's self-rated health questionnaire (EQ5D5L) are related with microparticle levels.

6.3 Suggestion for future studies

Based on the above conclusions, several future studies can be suggested. It is important the standardisation of the clot structure assessment and enumeration of microparticle methods. Comparison of results from various studies with the same methodology may provide further understanding about the complex physiology of haemostasis and microparticles. Analysis of haemostatic indices in conjunction with efficacy and safety outcomes, such as mortality, ischaemic stroke and ICH can support the utilisation of dynamic assays as clinical tools. The possibility to identify high risk patients for adverse cardiovascular events due to thrombosis or haemorrhage from clot structure characteristics should be explored further. MPA demonstrated promising results as a technique to measure the potential of OAC. Further validation of the technique and data from larger studies may specify its practicality, cost effectiveness and accuracy. Direct comparison of the four different NOACs (dabigatran, rivaroxaban, apixaban and edoxaban) regarding clot structure features is also a potential important study which may assist with the selection of the OAC based on the individual haemostatic profile. The in vitro assessment of clot structure characteristics should not be limited to AF but expanded to other conditions, such as heart failure and ischaemic heart disease, that possess thrombotic and/or haemorrhagic risk. As the model we used to assess the properties of fibrin network under the influence of different types of antithrombotics showed significant differences between them it may be beneficial to explore differences between antiplatelet types (aspirin, clopidogrel, ticagrelor etc) and their combinations with anticoagulants. These combinations are quite common in the clinical setting of AF and IHD.

Furthermore, future studies in larger numbers are required to further elucidate the underlying role of plasma circulating microparticles in the pathophysiology of AF and its complications. As multiple functions of microvesicles is concentration and antigenic related, experiments involving identification of more membrane proteins may reveal further mechanisms associated with the hypercoagulant tendency in NVAF. Only after appropriate verification studies microparticles may prove to be useful biomarkers reflecting fitness level, disease state and progression.

V. Related Publications

• Microparticles and cardiovascular diseases.
Voukalis C, Shantsila E, Lip GYH.
Ann Med. 2019 May - Jun;51(3-4):193-223. doi: 10.1080/07853890.2019.1609076. Epub 2019 Jun 17. PMID: 31007084

• Clinical Stroke prevention in atrial fibrillation.
Voukalis C, Shantsila E, Lip GY.
J R Coll Physicians Edinb. 2017 Mar;47(1):13-23. doi: 10.4997/JRCPE.2017.105. Review. PMID: 28569277

• Atrial Fibrillation and End Stage Renal Failure in Patients Receiving Dialysis: Balancing the Risks and Benefits of Stroke Prevention.
Voukalis C, Lip GYH.
Can J Cardiol. 2017 Jun;33(6):705-707. doi: 10.1016/j.cjca.2017.02.010. Epub 2017 Feb 28. PMID: 28457737

• Oral anticoagulation for elderly patients with non-valvular atrial fibrillation: recent insights from randomised trials and the 'real world'.
Voukalis C, Lip GYH.
Heart. 2017 Jul;103(13):977-978. doi: 10.1136/heartjnl-2017-311162. Epub 2017 Mar 3. PMID: 28258246

• Non-vitamin K oral anticoagulants versus vitamin K antagonists in the treatment of venous thromboembolic disease.
Voukalis C, Lip GY, Shantsila E.
Expert Opin Pharmacother. 2016 Oct;17(15):2033-47. doi: 10.1080/14656566.2016.1232393. Review. PMID: 27667112

• Drug-drug interactions of non-vitamin K oral anticoagulants.
Voukalis C, Lip GY, Shantsila E.
Expert Opin Drug Metab Toxicol. 2016 Dec;12(12):1445-1461. Epub 2016 Aug 26. Review. PMID: 27535163

• Emerging Tools for Stroke Prevention in Atrial Fibrillation.
Voukalis C, Lip GY, Shantsila E.
EBioMedicine. 2016 Jan 15;4:26-39. doi: 10.1016/j.ebiom.2016.01.017. eCollection 2016 Feb. PMID:26981569

VI. Appendices

- Clot structure 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 Fax
			Direct Line to Dr Blann
			Tel/fax

Patient ID for this study:

CONSENT FORM

Title of Project: Clot Structure in Atrial Fibrillation

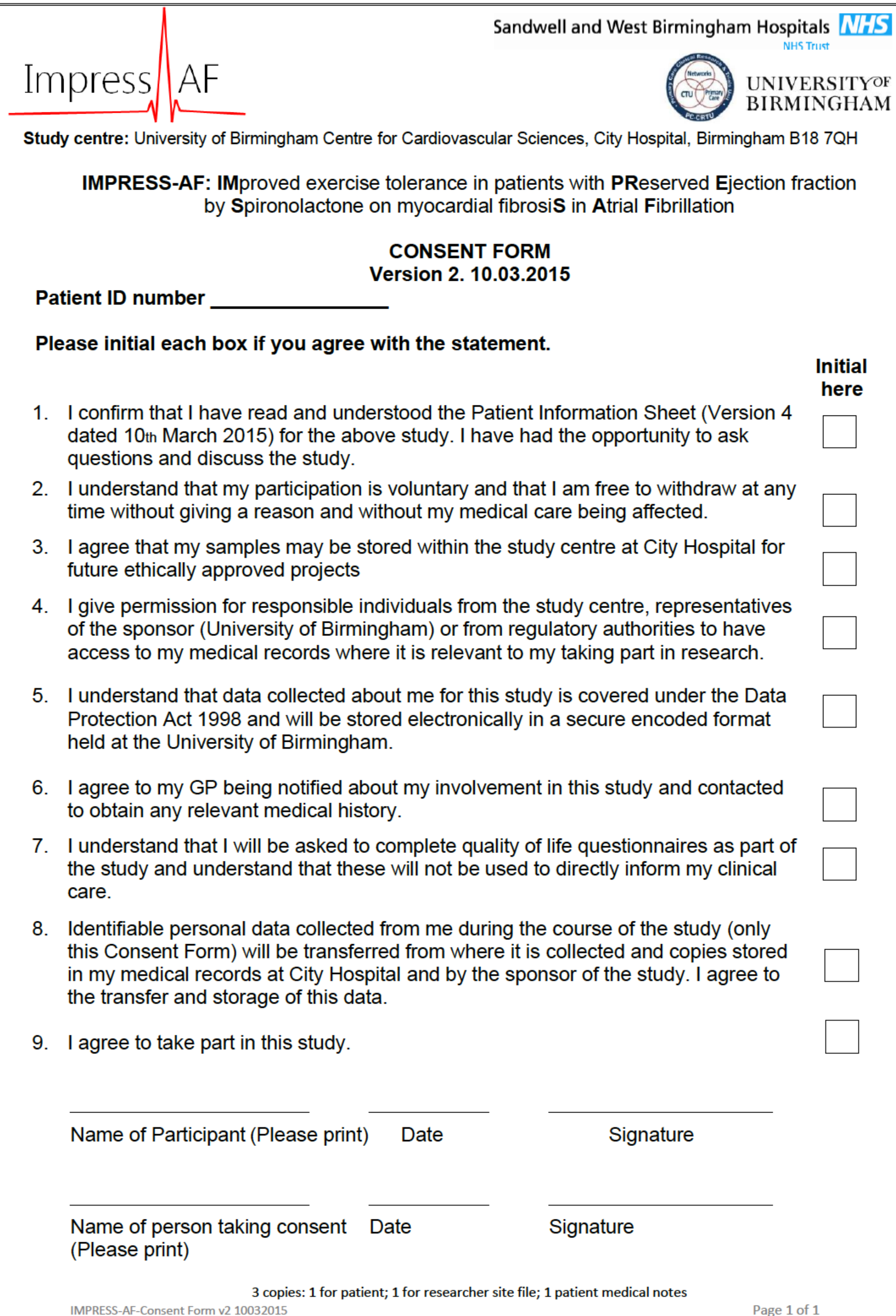
Name of Researcher:

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 mymedical care or legal rights being affected.
3. I agree to my GP being informed of my participation in thestudy.
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.

- <u>IMPRESS-AF consent form</u>



Impress AF

Sandwell and West Birmingham Hospitals NHS Trust

UNIVERSITY OF BIRMINGHAM

Study centre: University of Birmingham Centre for Cardiovascular Sciences, City Hospital, Birmingham B18 7QH

IMPRESS-AF: IMproved exercise tolerance in patients with **PR**eserved **E**jection fraction by **S**pironolactone on myocardial fibrosi**S** in **A**trial **F**ibrillation

CONSENT FORM
Version 2. 10.03.2015

Patient ID number ________________

Please initial each box if you agree with the statement.

		Initial here
1.	I confirm that I have read and understood the Patient Information Sheet (Version 4 dated 10th March 2015) for the above study. I have had the opportunity to ask questions and discuss the study.	☐
2.	I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason and without my medical care being affected.	☐
3.	I agree that my samples may be stored within the study centre at City Hospital for future ethically approved projects	☐
4.	I give permission for responsible individuals from the study centre, representatives of the sponsor (University of Birmingham) or from regulatory authorities to have access to my medical records where it is relevant to my taking part in research.	☐
5.	I understand that data collected about me for this study is covered under the Data Protection Act 1998 and will be stored electronically in a secure encoded format held at the University of Birmingham.	☐
6.	I agree to my GP being notified about my involvement in this study and contacted to obtain any relevant medical history.	☐
7.	I understand that I will be asked to complete quality of life questionnaires as part of the study and understand that these will not be used to directly inform my clinical care.	☐
8.	Identifiable personal data collected from me during the course of the study (only this Consent Form) will be transferred from where it is collected and copies stored in my medical records at City Hospital and by the sponsor of the study. I agree to the transfer and storage of this data.	☐
9.	I agree to take part in this study.	☐

________________	________	________________
Name of Participant (Please print)	Date	Signature
________________	________	________________
Name of person taking consent (Please print)	Date	Signature

3 copies: 1 for patient; 1 for researcher site file; 1 patient medical notes

IMPRESS-AF-Consent Form v2 10032015 Page 1 of 1

- **STANDARD OPERATING PROCEDURE: Thromboelastography (TEG®) (222)**

Written by Dr. Yee Cheng Lau and Dr. Praveen Ranjit: September 2013,
Updated by Dr. Christos Voukalis January 2016

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1. Introduction

Haemostasis is a dynamic and complex processes, involving several interacting factors, and balance of coagulation and fibrinolytic processes.

The Thrombelastograph® (TEG®) System, comprised of the TEG® Hemostasis Analyzer and the TEG® Analytical Software, is designed to perform a whole blood coagulation test that produces a hemostasis profile. The TEG® Hemostasis Analyzer automatically records the viscoelastic changes in a sample of whole blood, plasma or platelet-rich plasma as the sample clots, retracts and/or lyses. The resultant profile is a measure of the kinetics of clot formation and dissolution of clot quality (the ability to perform the work of coagulation). Because the TEG® system monitors shear elasticity (a physical property), it is sensitive to all the interacting cellular and plasmatic components in the blood that may affect the rate or structure of a clotting sample and its breakdown. The overall profile can be qualitatively or quantitatively interpreted in terms of the hypo, normal or hypercoagulable state of the sample, the degree of lysis, and other measurements of coagulopathy.

Brief Method

1. Confirm you are fully familiar with this SOP

2. Ensure all reagents are at room temperature and in date
3. Prepare the TEG machine and setting the heparinised Cup and Pin in position.
4. Add calcium chloride solution into the cup.
5. Then add a sample of citrated blood/plasma into the Cup, which will form a clot.
6. By activating the TEG, it will lower the Pin into the content of the Cup and start rotating at frequency of 6 revolutions per minute, simulating sluggish blood flow in vessel for thrombosis.
7. The Torsion wire above the pin will detect the tension of the fibrin formation, which then uses the mechanical-electrical transducer to monitor the signal via a computer.
8. Ensure the data is secure.

2. Materials and Supplier contact details:

- Disposable Cups and Pins for the 5000 series: Store them at room temperature (15-30°C) in the Styrofoam tray and keep covered when not in use. [Haemoscope, Catalogue No REF 6211/6212]
- Kaolin, Premeasured: The Kaolin vials are stored, until the expiration date, at 2 - 8ºC until use. [Haemoscope, Catalogue No REF 6300]
- 0.2M $CaCl_2$: Store at 2-8°C. Stable until expiration date. [Haemoscope, Catalogue No REF ####]
- TEG® Biological Controls - Level I and II: Store at 2 -8ºC. Unopened vials stable to expiration date. Reconstituted vials are stable for 2 hours at room temperature. [Haemoscope, Catalogue No REF 8001/8002]
- Clear pipette tips [Alpha Laboratories Limited, Catalogue No FR1250 1250 µL Fastrak Refill NS]
- Yellow pipette tips [Alpha Laboratories Limited Catalogue No FR1200 200 µL Fastrak Refill NS]
- Clear pipette tips [Alpha Laboratories Limited, Catalogue No FR1010 10 µL Fastrak Refill NS]

- 9 mL 9NC Coagulation sodium citrate 3.2% Vacuette [Greiner Bio-One GmbH, Ref#455322, Lot# A110509E]

3. Detailed Methods

Bring all reagents and citrated plasma samples to room temperature over 15 minutes.
Frozen samples are stored at -70°C freezer, near the door.
Samples can be transferred to 4°C fridge the night before assay for gradually thawing.

3.1. Preparation of the plasma samples

SOP on venepuncture, good clinical practice.

Centrifuge (the green centrifuge, Thermo) the “citrated” whole blood samples at 3,000g for 15 minutes, room temperature, to obtain Platelet Poor plasma (PPP).

Collect all the PPP samples from the same subjects, pool together in a larger Polypropylene tube and mixed. Subsequently transfer aliquots to clear 2ml Polypylene vial (measuring 1 mL plasma per vial), which will be stored in -70°C freezer for further assay.

A minimal of 445µL PPP sample is required to be added to 10 µL kaolin from each subject for each experiment.

Additional Note for plasma preparation

TEG® test results may be adversely affected by such factors as tissue fluids or contamination from catheter lines.
Do not obtain blood from a heparinized access line, lock, or indwelling heparin
Lock
When using Sodium Citrated samples, TEG testing must wait 15 minutes prior to analysis to allow equilibration of sample within anticoagulant.

3.2 Loading the Cups and Pins

With the lever in the load position, slide the white carrier halfway down the platform.

Pick up a disposable cup and pin from the Styrofoam tray. (Do not touch the outside of the pin or the inside of the cup.)

Place cup and pin into the cup well.

Carefully slide the white carrier all the way up, being sure that the disposable pin is standing straight up in the cup so that the skewer tip can enter smoothly.

When the top of the white carrier is in contact with the bottom of the column, push the pin firmly into place using the plastic pusher located at the bottom of the white carrier.

Counterbalance the analyzer by holding your hand on top while pushing the pin.

Make sure that the pin is correctly loaded by checking that the bottom tip of the skewer is touching the inside bottom of the disposable pin.

Slide the white carrier halfway back down and push the cup firmly into the cup well.

The cup should rest securely with the white carrier and should not pop up.

3.3 Quality Control

Ensure that the TEG® analyzer is turned on. If not, turn on the TEG® machine by pressing the green power switch. Allow the temperature for both columns to reach 37ºC.

Make sure the cup wells are clean and dry. Clean with cotton swab if needed (but DO NOT touch the pin)

Check the instrument levelling bubble and adjust if not level by turning the level adjustment legs in the front and back.

Perform a baseline test by following the prompts displayed on the computer:

3.3.1 Electronic (E) test

E test allows the machine to calibrate and align the pins of analyzer. To ensure correct alignment of the skewers, and to ensure accurate results, it needs to be done daily.

Ensure that the levelling bubble is in the middle.

Ensure that cup well is empty. DO NOT load cup and pin.

When the lever is at the ‘Load’ position, raise the white carrier to the top of the column.

Turn the level to the right into the ‘Test’ position before selecting the “Start eTest” icon to begin the test.

If eTest is satisfactory, select Done and commence with sample testing.

If eTest is unsatisfactory, ensure levelling bubble is levelled, and run eTest again. Notify TEG Customer Service if eTest results remain outside acceptable limits.

3.3.2 Liquid Control QC (Level I & Level II Control)

Two levels of liquid controls are required every fortnight of patient testing.

Liquid QC Preparation:

Remove control materials from refrigerator and allow them to reach room temperature (15 minutes).

Reconstitute lyophilized control by adding 1 vial of diluent water into each vial of lyophilized control.

Shaking the reconstitution vigorously to rehydrate the control and then letting it stand for five minutes.

Then, further shaking the reconstitution for a second time and rest for further 5 minutes before testing.

Once reconstituted, the controls are stable for only 2 hours.

Liquid QC Analysis via TEG® screen:

Enter the following fields to identify the controls for each channel to be tested.
Sample Type: Select the correct level of biological control.
Patient ID: Type in lot number of the control located at the bottom of the vial.

After the above data has been inputted, click on the first channel that will be tested so it turns blue.

Load the cup and pins as above (**Section 3.2**)

QC testing:
Pipette 20 µl of 0.2M calcium chloride into each TEG® well (with cup in-situ).

Pipette 340 µl of reconstituted control into the well of each channel.

Raise the column and move the lever to test in the first channel.
Quickly press F10 on the keyboard or select Start icon to activate the first sample.

Repeat steps 3 and 4 for other testing columns.

Terminate control after MA (15 minutes for Level I and 20 minutes for Level II) is achieved to expedite matters. Print the tracings by pressing the F6 key or copy the tracings by selecting "Capture" icon and saving a copy in Microsoft Word format.
Repeat with Level II control.

Change channels the next time controls are run. (Such as 1st run of day using Level I in channel 1/ Level II in channel 2, the next run switch channels, Level II in channel 1 and Level I in channel 2.)

Additional Note for QC analysis

Failure to obtain the expected value for 4 out of 4 parameters for Level I (3 out of 4 parameters for Level II only - R, K, alpha, and MA) may be an indication of product deterioration, TEG® instrument or procedural problems.

Check the temperature. If the temperature appears correct, re-try using fresh vial of the control and fresh calcium chloride.

If the results are remains abnormal, contact technical support.

3.4 Testing Plasma Samples (Starting the Run)

Load cups and pins **(Section 3.2)**

Add Pipette 20 µl of 0.2M calcium chloride into each TEG® well (with cup in-situ)

- For native whole blood analysis add 1 ml of native whole blood to the pre-filled kaolin vial (25 µl kaolin from Haemoscope), cap and mix by inversion five times, and then pipette 360 µl of the mixture directly into the cup. **DO NOT** add Calcium Chloride for whole blood.
- For fresh/freshly thawed citrated PPP, add 445µl into pre-filled kaolin vial (10 µl kaolin, prepared locally). Cap and mix by inversion five times, and then pipette 340 µl of the mixture directly into the cup.

Lift the white carrier carefully to the pin with the lever still in the load position.

Firmly push the white carrier up against the column.

Move the lever to the right (test position), resting your hand on top of the analyzer to counterbalance.

Press [F10] on the computer keyboard to begin the test.

If you have followed the sample loading procedure correctly, the sample information display for that channel changes from yellow to **green**. This also helps distinguish between active and inactive channels on the screen. The active channels are highlighted green for active, yellow for inactive, or white for an ended sample.

Repeat steps above for each additional column.

3.5 Terminating the test (Ending the Run)

If the test has not already been terminated by the software, or once the samples have attained pre-determined perimeters, end the test on the computer as below.

From the main screen, highlight your sample. Once selected, the channel will be highlighted **blue.**

Click on the STOP icon.

The program repeats the identifying information for the channel and asks you to confirm your selection by clicking.

The data is automatically saved

Slide the lever to the left to the "load" position and then press down on the lever to eject pin.

Slide the white carrier down to the platform. Be sure the pin has dropped into the cup.

Press the white carrier down firmly against the platform. This will release the cup from the white carrier.

When the disposable cup pops up, dispose of it into appropriate bin.

Additional Note for starting the Run:

Ensure that there are no bubbles in the cup while introducing plasma or blood by pipette.

Ensure Calcium in introduced into the middle of the well.

TEG process is highly susceptible to external vibration/shock.

- **STANDARD OPERATING PROCEDURE: Microplate Assay, Turbidity Assay (Clot Formation Time & Fibrin Clot thickness, 223)**

Written by Dr. Jackson Lau and Dr. Praveen Ranjit: January 2014
Updated by Dr. Christos Voukalis January 2016

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1. Introduction

Thrombosis and haemostasis is a result of complex interplay of both clot formation and fibrinolysis. Fibrinolysis assay (SOP 221) can quantify the ability of sample to dissolve a formed fibrin clot, while thromboelastograph (TEG) reveals the intrinsic properties of fibrin clot formation and clot strength.

However, the *actual* physical property of the fibrin clot, such as rate of lateral polymerization of protofibril, thickness of fibrin fibers and subsequent deduced pore size, which potentially have a major impact on thrombosis and fibrinolysis, can be investigated by turbidity analysis.

Turbidity is a technique to measure the level of cloudiness of a fluid, as opacification of transmitted light is due to the quantity of suspended matter. Light is passed through the solution and scattered by any intervening matter, a detector will measure the amount of transmitted light and therefore the relative amount of matter present. (Figure 1)

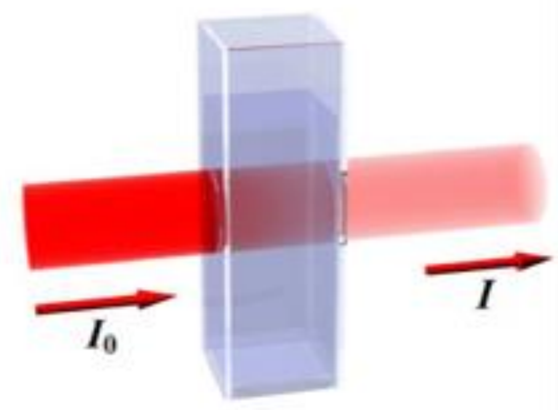


Figure 1: Schematic diagram of Turbidity analysis

Turbidity can be expressed as the absorbance A (also termed the optical density) by measuring the intensity of the incident light (I_0) and the intensity of the transmitted light (I):

$$A_\lambda = \log\left(\frac{I_0}{I}\right)$$

The objective of this standard operating procedure (SOP) is to enable the efficient quantification of the ability of a sample of plasma to form a fibrin clot, as well as the relative fiber thickness as reflected by the absorbance. Subsequent relationship between fibers size and subsequent size of pore can be confirmed by Scanning Electron Microscopy.

This experiment is based on work by Undas et al , Ryan et al , and Wiesel et al.

<u>Brief Method</u>

9. Add 25ul of plasma
10. Add 75ul of Permeation Buffer
11. Add 50ul of Activation Mix
12. Plasma will react with activation mix and start to form fibrin clot.
13. Pass the plate through a plate reader set at fixed intervals
14. Changes in light passage through the clot give an assessment of the rate of clot formation.

15. Maximal absorbance will give an assessment of fiber thickness.
16. Transfer data from XFLURO4 to Excel. Interpret the results accordingly.

2. Materials and Supplier contact details:

Tris-Hcl (Trizma® Pre-Set Crystals, pH=7.5, MW=150.6 g/mol, Sigma T7818, powder, 100g/bottle) (On the left-hand side of shelf near the sink)
NaCl (Sigma S9888, MW=58.44g/mol,powder, 500g/bottle) (On the left hand side of shelf near the sink)
$CaCl_2$ (Sigma C1016, MW=110.98 g/mol, granular, 500g/bottle) (On the left-hand side of shelf near the sink)
Bovine serum albumin (BSA, protease free) (powder, Sigma A3294, 50g/bottle) (in the 4°C fridge of culture room, middle layer)
Thrombin (THR, powder, Sigma T9549, 1506 NIH U/mg=1000 IU per vial). The vial of THR should be reconstituted with 4 mL PBS containing 0.1% BSA to produce a final stock at 250 IU/mL, 200µL aliquot it in eppendorfs and store stocks at -20˚C. (Once frozen, it is stable for maximum of 24 months, but once thawed will degrade rapidly)
96 wells clear flat bottom microtitre plates (Immunlon 2 high binding, Thermo Electron Corp #3455) (On the right-hand side of shelf in the main lab)
9 mL 9NC Coagulation sodium citrate 3.2% Vacuette (Greiner Bio-One GmbH, Ref#455322, Lot# A110509E)
Clear pipette tips [Alpha Laboratories Limited, Catalogue No FR1250 1250 µL Fastrak Refill NS]
Yellow pipette tips [Alpha Laboratories Limited Catalogue No FR1200 200 µL Fastrak Refill NS]
Clear pipette tips [Alpha Laboratories Limited, Catalogue No FR1010 10µL Fastrak Refill NS]

3. Detailed Method

Bring all reagents and citrated plasma (see below) samples to room temperature. Samples are stored at -70°C freezer No.5, near flow cytometer room, samples can be transferred to 4°C fridge the night before assay for gradually thawing

3.1. Preparation of the plasma samples

SOP on venepuncture, good clinical practice.

1. Centrifuge (the green centrifuge, Thermo) the "citrated" whole blood samples at 2,800g for 15 minutes, room temperature, to obtain Platelet Poor plasma (PPP).

1. Collect all the PPP samples from the same subjects, pool together in a larger tube and aliquots (1 mL/vial)

1. 25µL PPP sample per well from each subject for one experimental condition is required. In addition, triplicates are demanded. Hence only 75µL PPP (if doing the triplicates) from each subject is required.

1. Use fresh plasma if possible. Thawed frozen plasma (stored -70°C) may be possible. **Plasma stored in fridge or at room temperature degrades rapidly.**

3.2. Preparation of the assay buffers and reagents

1. To prepare the **Permeation buffer**; 200 mL of Tris-HCl 50mM (pH7.5) and NaCl 0.15 M buffer, add 1.51 g Tris-HCl and 1.75 g NaCl into 200 mL distilled water, mix well and wait until all powders have dissolved. This will provide the Permeation Buffer (50mM Tris & 150mM NaCl). Store this buffer in 4°C fridge.

1. To prepare the **Calcium buffer**; first add 2.77 g $CaCl_2$ into 50mL of permeation buffer (as described above), mix well and wait until all powders have dissolved. Store this buffer in 4° fridge.

1. To prepare **Activation Mix**, thaw the eppendorf tube of frozen thrombin in a 4°C fridge over 30 minutes, then add the mixture of Permeation Buffer, Calcium Buffer and thrombin, as indicated. Refer to **Appendix 2** for reference of the optimal volume of reagent required for number of wells used.

The reference chart is aimed to provide optimal condition per well for fibrin clot formation. Any variation in pH, thrombin concentration, salinity will adversely impact overall result.

Thawed thrombin degrades rapidly on heat, hence once thawed, use immediately or store it in 4°C fridge. Dispose all unused thawed thrombin at end of the day.

3.3. Turbididity assay measurement

1. Synopsis: Turbidity will be assessed as a continuous variable (not end-point time) for around 60 minutes (6 sec intervals, 360 time points) in a microtitre plate, at 340 nm (turbidity wavelength). Our plate reader will measure at 37 degrees Celsius will be altered via temperature control.

1. Detailed procedures of clot lysis assay (for example: for 4 plasma samples):

Turn on the spectrophotometer (in the right-hand side of computer, the), the switch is on the right-hand side back of the machine.
Turn on the computer, double click on the software called "XFLURO4" on the desktop.

*<u>Measurement settings:</u>
Initial shaking period: 2 sec
Measurement intervals: 6 sec

Total times of measurements: 360 times

Wavelength: stepping, kinetic, 340 nm

Temperature Control 37 ^{o}C

Add 25 μL of **plasma sample** in each well following the arranged layout (in duplication). Then add 75 μL of **permeation buffer**, lastly add 50 μL of **activation mix** to each well.

Add **Activation Mix** last, as it will react with plasma to form fibrin clot rapidly. One should take less than 10 seconds between each column, giving a total lag time from Column 1 – 12 of 110 seconds.

Double check the measurement settings of the software are correct before loading the assay buffers into the plasma samples.

Read the plate immediately (by pressing the "Start Measurement" sign on the software)

<u>Tips:</u>

Do NOT to produce bubbles in this assay is crucial, therefore please do NOT push the pipette into the end (there are 2 steps while pushing the pipette, please push only until in the end of the 1st step)

For the pipetting convenience reason, the samples layouts are suggested as below:

Once the software program has finished:

*Take the plate out from the reader, and if there are any empty wells left, keep it for next time experiment. If all wells were used, please throw it away in the yellow bin.

To save data:

Go to *File*→ select *Save* → choose the *Location (e.g. C disk or thumb drive)* and give a proper *File Name (e.g. xxx.**xls**)→Save* [This is only allowed us to save the original data following default setting layout. It will present the whole 96-wells data at different time points, i.e. it will generate 100 separate data batches (which is not convenient for later analysis and plotting)]. (Appendix A)

*To generate kinetic, excel file:

*Retrieve all the data from the computer (you can save it in your thumb drive) and turn off the spectrophotometer and the computer.

3.4. Turbidity analysis

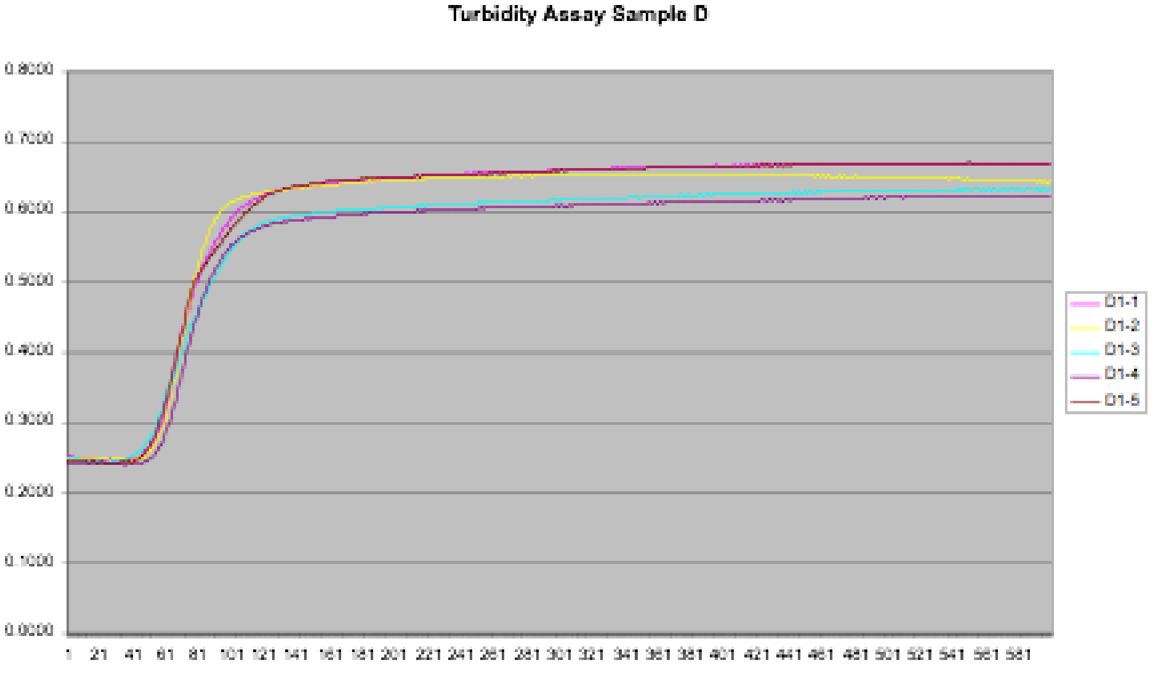
1. Assembly kinetic data can be exported as an excel file from the XFLUR4 software.

Thus, lag time and peak absorbance can thus be derived.

1. The lag time corresponds to rate of protofibrils formation, based on the lateral polymerisation of fibrin monomers, which derived from fibrinogen molecules.

1. The peak value of absorbance corresponds to the average thickness of protofibril fibers.

Figure 2: Illustrative plot of Turbidity



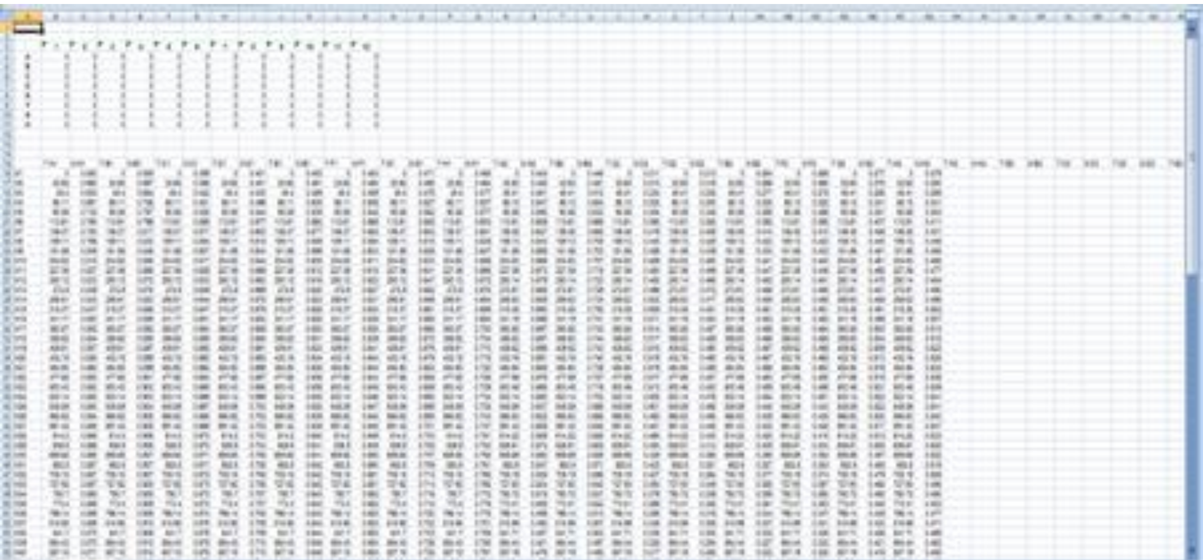
3.5. Limitations of this method

1. Factors affecting clot formation should be measured and within normal ranges. We induced clot formation *in vitro* in the presence of "optimised" thrombin (250 U/ml) and re-calcification of plasma (0.5M Ca) based on work by Wiesel et al [3]. However, there are other factors that may affect the fibrin clot structure: such as Factor XIII concentration (and perhaps genotype), fibrinogen and plasminogen intrinsic levels.

1. Several factors may also affect clot architecture, including age, fibrinogen activity and alternatively spliced γ-chain (γ') in activated fibrinogen.

Appendix 1. Kinetic assembly of turbidity for each subject

(This is how raw data appear from XFLURO4)



- **STANDARD OPERATING PROCEDURE: Fibrinolysis Assay (Clot Lysis, 221)**

Written by Dr. Yu-Wen Chen and Dr. Silvia Montero-Garcia: June 2012

Updated by Dr Jackson Lau and Dr Praveen Ranjit January 2014 and by Dr Christos Voukalis (January 2016)

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1. Introduction

Hypofibrinolysis, a common finding in patients with thrombotic tendency, may be related either to an increase plasminogen activator inhibitor (PAI) activity and/or to a deficient activity of tissue-type plasminogen activator (tPA). However, these plasma markers cannot give a direct impression of clot formation and physical structures. A fibrin clot, which is characterized mainly by the thickness of fibrin fibers and the size of pores, has a major impact on fibrinolysis.

The lysis of a plasma clot depends on two main factors: the properties of the clot and of the lytic potential of the plasma. With spectrophotometric analysis we are only able to assess the second factor (a predisposition to lysis) but not the intrinsic properties of clot formation [this will be needed to be tested with thromboelastography (TEG)]. In this sense, it is important to notice that clot structure will always represent a limitation in this spectrophotometric analysis.

The objective of this standard operating procedure (SOP) is to enable the efficient quantification of the ability of a sample of plasma to dissolve a formed fibrin clot, that is, fibrinolysis. It is based on the work of Undas et al.

Brief Method

1. Add a sample of plasma, with thrombin, to the wells of a microtitre plate. It will form a clot
2. Add a second plasma sample, as above but with added tPA. It will form a clot, but soon after it will be dissolved by the action of the tPA on plasmin
3. Pass the plate through a plate reader at set intervals
4. Changes in light passage through the clot give an assessment of the rate of lysis
5. Use these figures to drive a curve, and so the rate of lysis

2. Materials and Supplier contact details:

2.1 Tris-Hcl (Trizma® Pre-Set Crystals, pH=7.5, MW=150.6 g/mol, Sigma T7818, powder, 100g/bottle) (On the left-hand side of shelve near the sink)

2.2 NaCl (Sigma S9888, MW=58.44 g/mol, powder, 500g/bottle) (On the left-hand side of shelve near the sink)

2.3 $CaCl_2$ (Sigma C1016, MW=110.98 g/mol, granular, 500g/bottle) (On the left-hand side of shelve near the sink)

2.4 Bovine serum albumin (BSA, protease free) (powder, Sigma A3294, 50g/bottle) (in the 4°C fridge of culture room, middle layer)

2.5 Thrombin (THR, powder, Sigma T9549, 1506 NIH U/mg=1000 IU per vial). The vial of THR should be reconstituted with 1 mL PBS containing 0.1% BSA (stable for several months as described by manufacturer) to produce a final stock at 1000 U/mL, 200µL aliquot it in eppendorfs and store stocks at -20˚C.

2.6 Potassium bicarbonate ($KHCO_3$, powder, MW=100.12 g/mol, Sigma 60339, 500g/bottle) (On the left-hand side of shelve near the sink)

2.7 Tissue-type plasminogen activator (tPA) (human, recombinant) (Technoclone, TC41072, 100µg/vial) , stored in the 4° fridge of beside the computer in main lab.

Once it has been reconstituted from dried powder (with 1mL of 1M $KHCO_3$ and 1% protease-free albumin), it should be stored in -70° freezer (stable for several months as described by manufacturer).

2.8 96 wells clear flat bottom microtitre plates (Immunlon 2 high binding, Thermo Electron Corp #3455) (On the right-hand side of shelve in the main lab)

2.9 9 mL 9NC Coagulation sodium citrate 3.2% Vacuette (Greiner Bio-One GmbH, Ref#455322, Lot# A110509E)

2.10 Clear pipette tips [Alpha Laboratories Limited, Catalogue No FR1250 1250 μL Fastrak Refill NS]

2.11 Yellow pipette tips [Alpha Laboratories Limited Catalogue No FR1200 200 μL Fastrak Refill NS]

2.12 Clear pipette tips [Alpha Laboratories Limited, Catalogue No FR1010 10 μL Fastrak Refill NS]

3. Detailed Method

Bring all reagents and citrated plasma samples to room temperature.
Preferably fresh plasma is used. Frozen samples can be thawed by transferring to 4°C fridge the night before assay.

3.1. Preparation of the plasma samples

3.1.1 SOP on venepuncture, good clinical practice.

3.1.2 Centrifuge (the green centrifuge, Thermo) the “citrated” whole blood samples at 2,800g for 15 minutes, room temperature, to obtain Platelet Poor plasma (PPP).

3.1.3 Collect all the PPP samples from the same subjects, pool together in a larger tube and aliquots (1 mL/vial) can be used immediately or be stored in -70° freezer near flow cytometer room for further assay.

3.1.4 75μL PPP sample per well from each subject for one experimental condition is required. In addition, triplicates (or at least duplicates) are demanded. Hence at least 350μL PPP (if doing the duplicates) from each subject is required.

3.2. Preparation of the assay buffers and reagents

3.2.1 To prepare 200 mL of Tris-HCl 50 mM (pH7.5) and NaCl 0.15 M buffer, add 1.51 g Tris-HCl and 1.75 g NaCl into 200 mL distilled water, mix well and wait until all powders have dissolved. Store this buffer in 4° fridge (middle layer) in the culture room.

3.2.2 To prepare 50 mL of $CaCl_2$ 0.5 M in Tris-HCl (50 mM, pH7.5)+ NaCl (0.15 M) buffer, add 2.77 g $CaCl_2$ into 50 mL Tris-HCl (50 mM, pH7.5)+ NaCl (0.15 M) buffer, mix well and wait until all powders have dissolved. Store this buffer in 4° fridge (middle layer) in the culture room.

3.2.3 To prepare 10 mL 10% "protease-free" albumin stock, add 1 g albumin (protease-free) into 10 mL distilled water, wait until all powders have dissolved. Store this buffer in 4°C fridge (middle layer) in the culture room. DO NOT VORTEX, this will generate bubbles!

3.2.4 To prepare 20 mL 1M $KHCO_3$ buffer containing 1% protease-free albumin, add 2 g $KHCO_3$ into 18 mL distilled water. Then add 2 mL 10% protease-free albumin stock, mix well and wait until all powders have dissolved. Store this buffer in 4°C fridge (middle layer) in the culture room.

3.2.5 To prepare tissue-type plasminogen activator (tPA), dried tPA (100μg) in vial is reconstituted with 1mL of 1M $KHCO_3$ containing 1% protease-free albumin. Gentle mixing for 20 minutes at 25°C. Then aliquot 100 μL in eppendorfs will be stored in -70°C freezer No. 8, near lab door, upper chamber. Before using it, dilute with 300uL KHCO3+1%BSA to final concentration 25μg/mL

3.3. Turbidometric clot lysis assay measurement

3.3.1 Synopsis: Fibrinolysis will be assessed as a continuous variable (not end-point time) for 60 minutes (10 sec intervals, 360 time points) in a microtitre plate, at 340 nm, at 37℃ (physiological temperature).

3.3.2 From our previous validation assays, the optimal final working concentrations of thrombin (THR) is 1 IU/mL and tPA is 0.5µg/mL. (Appendix C)

3.3.3 For measuring the fibrinolysis, citrated plasma will be diluted in 2 conditions:

- *Without the presence of tPA* (will achieve stabilized clot, can be used as an internal turbidity control): citrated plasma will be diluted with Tris-HCl buffer (Tris-HCl 50 mM + NaCl 0.15 M) containing $CaCl_2$ 20 mM and thrombin 1 IU/mL (plasma: reagents = 1: 1 vol/vol).

- *In the presence of tPA*: citrated plasma will be diluted with Tris-HCl buffer (Tris-HCl 50 mM + NaCl 0.15 M) containing $CaCl_2$ 20 mM, thrombin 1 IU/mL and tPA 0.5µg/mL (plasma: reagents = 1: 1 vol/vol).

- 75µL mixed buffer per well for each experimental condition (with or without tPA) is requested. In addition, triplication (or at least duplication) is demanded.

- To eliminate the intra-assay errors when mixing reagents, please refer to chart (**Appendix C**).

3.3.4 Detailed procedures of clot lysis assay (for example: for 4 plasma samples):

- Turn on the spectrophotometer (in the right-hand side of computer, the), the switch is on the right-hand side back of the machine.
- Turn on the computer, double click on the software called “XFLURO4” on the desktop.

*Measurement settings:

Initial shaking period: 2 sec

Measurement intervals: 6 sec

Total times of measurements: 360 times

Wavelength: stepping, kinetic, 340 nm

Temperature Control 37 ^{o}C

- Prepare 2 troughs for the 2 assay buffers, respectively:

*<u>1 mL Assay buffer WITHOUT tPA</u>: 880µL Tris-HCl buffer (Tris-HCl 50 mM + NaCl 0.15 M) containing 80µL $CaCl_2$ 0.5 M, 2µL thrombin 1000 IU/mL and 40µL 1M KHCO3 + 1% protease-free albumin. (for 4 plasma samples, required total buffer volumes are only 600µL, however, we will use the 8-channel pipetteman to add this buffer, therefore extra volumes are needed).

*<u>1 mL Assay buffer WITH tPA</u>: 880µL Tris-HCl buffer (Tris-HCl 50 mM + NaCl 0.15 M) containing 80µL $CaCl_2$ 0.5 M, 2µL thrombin 1000 IU/mL and 40µL **tPA (25µg/mL) in 1M $KHCO_3$ + 1% protease-free albumin**. (for 4 plasma samples, required total buffer volumes are only 600µL, however, we will use the 8-channel pipetteman to add this buffer, therefore extra volumes are needed).

- Add 75µL plasma sample in each well following the arranged layout (in duplication).

- Double check the target wells and measurement parameters of the XFLURO4 software are correct before loading the assay buffers into the plasma samples.

- Use two 8-channel pipettemen to aspirate and load 75µL assay buffers (without and with tPA, respectively) for each well (which has already contained 75µL plasma).

- Read the plate immediately (by pressing the “Start Measurement” sign on the software)

Tips:

- Do NOT to produce bubbles in this assay is crucial, therefore please do NOT push the pipette into the end.
- For the pipetting convenience reason, the samples layouts are suggested as below:

*Yellow column (column 1)—WITHOUT tPA

*Pink column (column 2)—WITH tPA

*A1, B1 are duplications from the same subject

*Please use the same frame for the remaining columns (from column 3 to 12)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample_1	Sample_1										
B	Sample_1	Sample_1										
C	Sample_2	Sample_2										
D	Sample_2	Sample_2										
E	Sample_3	Sample_3										
F	Sample_3	Sample_3										
G	Sample_4	Sample_4										
H	Sample_4	Sample_4										

- Once the software program has finished:

*Take the plate out from the reader, and if there are any empty wells left, keep it for next time experiment. If all wells were used, please throw it away in the yellow bin.

*To save data:

Go to *Process Menu* → select *Sheet* → select *Save As New Sheet* → choose the *Location (e.g. C disk or thumb drive)* and give a proper *File Name (e.g. xxx.**xls**)* → *Save* [This is only allowed us to save the original data following default setting layout. It will present the whole 96-wells data at different time points, i.e. it will generate 100 separate data batches (which is not convenient for later analysis and plotting)]. (Appendix A)

*To generate kinetic, excel file:

Go to *Process Menu* → select *Organize* → select *Target Data Sheet (usually is Sheet 1)* → in *Source and Calculation* → tick *Integral by Time* → in *View* → tick *Matrix for Further Calculation* → *Apply* → *OK* → the data layout will be rearranged in a kinetic pattern by different samples → Repeat the procedures "To save data". (Appendix A)

*Retrieve all the data from the computer (you can save it in your thumb drive) and turn off the spectrophotometer and the computer.

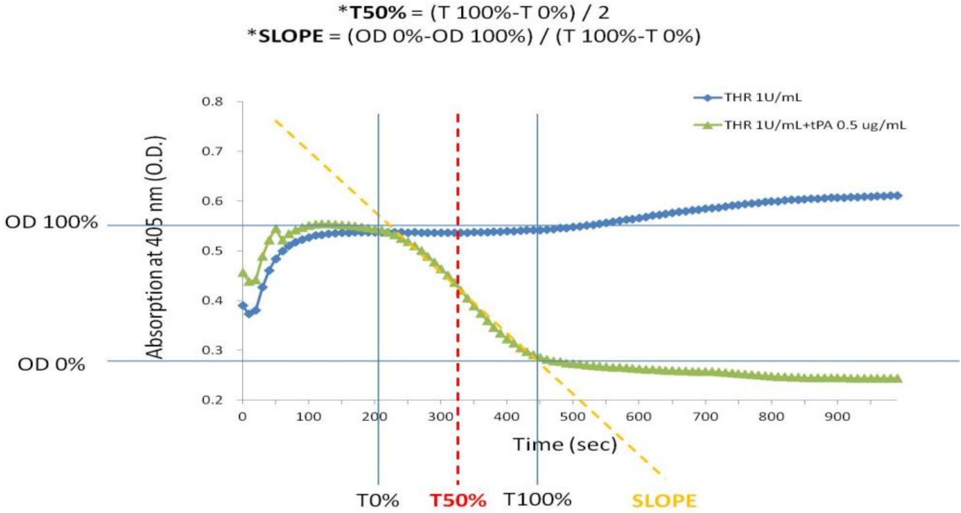
- Plasma rapidly degrades over time at room temperature or fridge.
- Thrombin degrades in room temperature, use immediately or store in ice.

3.4. Clot lysis time and slope analysis

3.4.1 Assembly kinetic data can be exported as an excel file from the Ascent software.

3.4.2 The time required for a 50% decrease in clot turbidity (T50%) from a peak value and the slope [= (the bottom value of turbidity, OD 0% − the peak value of turbidity, OD 100%) divided by (time for the bottom value, T 100% − time for the peak value, T 0%)] are derived from the data (Figure 1)

Figure 1: Illustrative plot of clot lysis



3.4.2 Please see Appendix B for the optimal thrombin and tPA concentrations validation.

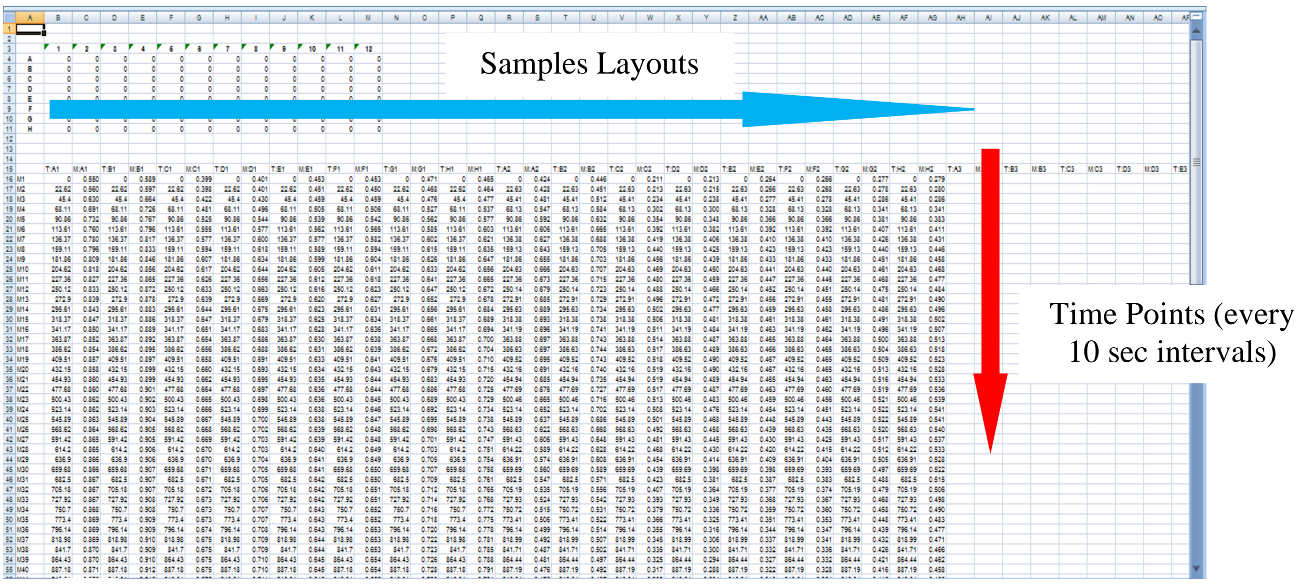
3.5. Limitations of this method

3.5.1 The analysis of this method is quite subjective, the results may be slightly different from different analyzers.

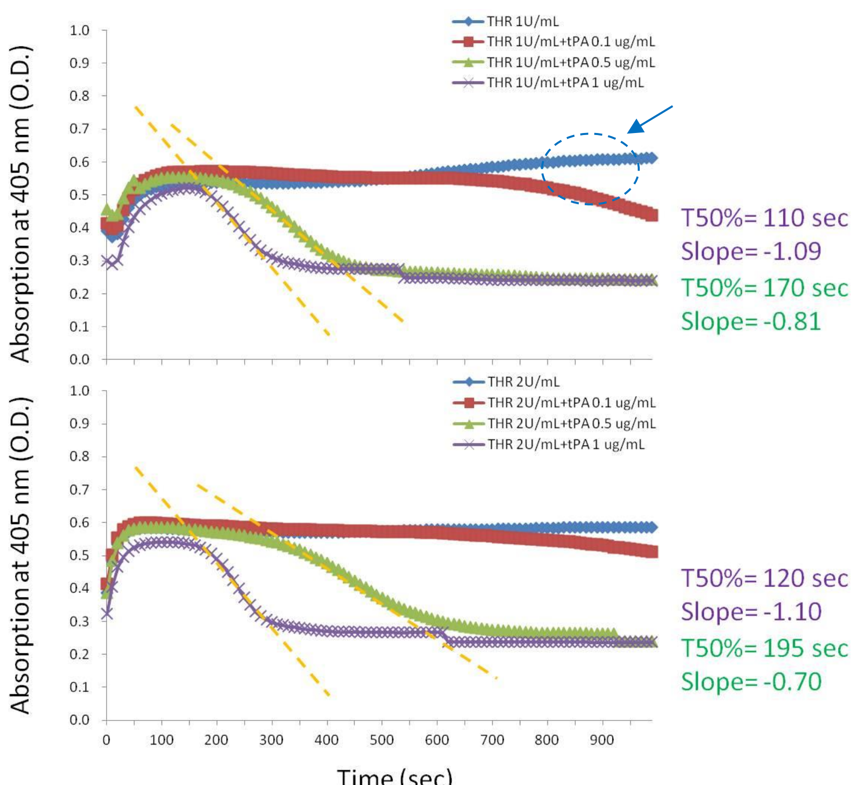
3.5.2 Factors affecting clot formation should be measured and within normal ranges. We induced abnormal clot formation *in vitro* in the presence of thrombin (unphysiological concentration) and recalcification of plasma (20 mM Ca), but the final clot factors are still affecting the structure: FXIII concentration (and perhaps genotype), fibrinogen and plasminogen intrinsic levels.

3.5.3 Several factors may also affect clot architecture and resistance to lysis, including age, fibrinogen, lipoprotein (a), C-reactive protein (CRP) and oxidative stress. Therefore, when collecting or analyzing samples, these factors should be kept in mind.

Appendix A: Raw data from photometer using XFLURO4 software



Appendix B. Illustrations of optimal thrombin and tPA concentrations validation



The optimal thrombin and tPA concentrations validation were done in 4 different samples. Here we only show one of the examples as a representative. (All four we got similar results)

1. No matter with thrombin 1 or 2 IU/mL, without tPA addition, the clot turbidity remained constant (blue lines in the chart). But with thrombin 1 IU/mL treatment, the clot formation was not stable, the secondary clot formation appeared at the middle stage. However, thrombin 2 IU/mL treatment generated firm clot from the beginning.

2. With only 0.1μg/mL tPA treatment (in both thrombin 1 or 2 IU/mL conditions), we couldn't observe obvious and complete clot lysis within the experimental period. Hence this concentration of tPA might not be an optimal concentration.

3. With 1μg/mL tPA treatment (this was the suggested concentration from the references), we couldn't discriminate the differences of clot lysis from thrombin 1 and 2 IU/mL. However, supposedly clot formation under higher thrombin treatment condition would

be thicker and stronger, which meant the clot structure wouldn't be lysed as easily as with lower thrombin concentration. Therefore, tPA 1μg/mL again is not an ideal concentration for our study.

4. With 0.5μg/mL tPA treatment, we successfully discriminate the differences of clot structure from thrombin 1 and 2 IU/mL, i.e. under thrombin 1 IU/mL condition, the clot is prone to be lysed by tPA than thrombin 2 IU/mL condition.

Therefore, in the future study, we will use thrombin 1 IU/mL and tPA 0.5μg/mL as our assay condition

Appenix C: Fibrinolysis Assay: (Active tPA) [1] Reagents Volume vs Wells Reference Chart

No. of wells	8	16	24	32	40	48	56	64	72	80	88	96
Buffer (Tris + NaCl) microliter **ul**	880 **ul**	1760 **ul** (1.76 ml)	2640 **ul** (2.64 ml)	3520 **ul** (3.52 ml)	4400 **ul** (4.40ml)	5280 **ul** (5.28ml)	6160 **ul** (6.16ml)	7040 **ul** (7.04ml)	7920 **ul** (7.92ml)	8800 **ul** (8.8ml)	9680 **ul** (9.68ml)	10560 **ul** (10.56ml)
Calcium Buffer **ul**	80 **ul**	160 **ul**	240 **ul**	320 **ul**	400 **ul**	480 **ul**	560 **ul**	640 **ul**	720 **ul**	800 **ul**	880 **ul**	960 **ul**
tPA In KHCO3 **ul**	40 **ul**	80 **ul**	120 **ul**	160 **ul**	200 **ul**	240 **ul**	280 **ul**	320 **ul**	360 **ul**	400 **ul**	440 **ul**	480 **ul**
Thrombin **ul**	2 **ul**	4 **ul**	6 **ul**	8 **ul**	10 **ul**	12 **ul**	14 **ul**	16 **ul**	18 **ul**	20 **ul**	22 **ul**	24 **ul**
Total **ul**	1002	2004	3006	4008	5010	6012	7014	8016	9018	10 020	11 022	12 024

Reagent concentration per well:
Thombin 1.0 units / mL
Calcium 3uL of 0.5M $CaCl_2$
tPA 0.5 ug/mL

Volume of plasma per well: Plasma 75 ul
Multi-channel Pipette : 75 ul

Keep Thrombin on ICE after thaw

Use tPA within 8 hours upon thaw (room temperature)

- **STANDARD OPERATING PROCEDURE: Enumeration of Platelet, Endothelial and Monocyte derived Microparticles (using Apogee A50 Flow Cytometry)(209)**

Developed by Dr Silvia Montoro Garcia and Dr Eduard Shantsila (September 2011)

Updated by Dr Jackson Lau and Dr Xiong Qinmei (May 2015);
Dr Mikhail Dzeshka (January 2017); Dr Christos Voukalis (July 2017)

Note: Use of the flow cytometry is forbidden without official training

<u>Required pre-training: </u>SOP on Venepuncture, Good Laboratory Practice, Good Analytical Practice, Good Clinical Practice, and Code of Practice for Research in the University of Birmingham

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1. Introduction

Cellular microparticles (MP) are submicron biological structures released from different types of cells (e.g., from platelets, leucocytes, endothelial cells, red blood cells) via remodelling of plasma membrane in response to numerous conditions, including activation and apoptosis. MPs are generally defined as small (0.1 to 1 μm) membrane fragments that often expose the anionic phospholipids phosphatidylserine and membrane antigens representative of their cellular origin. These characteristics discriminate MP from exosomes, which are smaller (<0.1 nm), originate from intracellular multivesicular bodies, and differ in antigenic composition.

Some MPs have prothrombotic properties irrespectively from their origin. However, presence of certain markers (e.g., tissue factor) on MP surface substantially enhances thrombogenic

properties. Flow cytometry allows identifying and enumerating different types of MP based on their size, surface antigens and, usually, utilisation of count beads.

The Apogee A50-Micro flow cytometer was shown to be superior to most conventional flow cytometer for the purpose of MP analysis due to its design specifically focused for analysis of small events [1]. Conventional flow cytometers are limited to measuring particles larger than about 500 nm by small angle light scatter (forward scatter, FS). For example, the lower limit of resolution of BD FACSCalibur is 5.0 µm. Apogee A50 discriminates 20 nm particles (latex beads), and it also benefits from a volumetric sampling system that accurately counts MP without using count beads). Additional advantage of Apogee A50 is a fluidics system that can be refilled with pure distillate water, saving reagents compare to conventional cytometers.

This SOP describes enumeration of CD42b (GPIb)$^{+}$ platelet-derived microparticles (PMP). N.B. the SOP may be amended for more detailed analysis of MP subpopulations, for example, VE-cadherin (CD144)$^{+}$ endothelial MPs or CD14^{+} monocyte-derived MPs.

2. Materials and Supplier contact details:

1. Distilled water from the still in the Tissue culture room (ensure sufficient volume of water is available to run flow cytometry continuously).
2. Eppendorf Microcentrifuge Tubes 1.5 ml (Conical Bottom, Graduated, UltraClear® copolymer polypropylene, APEX® Soft-Release Flip-Cap, Frosted writing area) *[Alpha Laboratories, Pack of 500, Catalog No LW2455]*
3. Pipette tips 0.1-10 µl (TubeGuard Top Ultrafine Tip, Extended-Length 38 mm, Non-Sterile, Fastrak® Tip Refill System) *[Alpha Laboratories, Pack size: 14 racks of 96 in a refill tower, Catalog No FR 1010]*
4. Pipette tips 1-200 µl (Universal, Bevelled, Yellow, Non-Sterile, 50 mm in length, Fastrak® Tip Refill System) *[Alpha Laboratories, Pack size: 10 racks of 96 in a refill tower, Catalog No FR 1200]*
5. Pipette tips 100-1250 µl (FlexTop Ultrafine Tip, Extended-Length 89 mm, Non-Sterile, Fastrak® Tip Refill System) *[Alpha Laboratories, Pack size: 5 racks of 96 in a refill tower, Catalog No FR 1250]*
6. Syringes 20 ml, disposable, sterile, Terumo [Appleton Woods, Pack of 50, Catalog No GS577]

7. Syringe filters 0.22 µm, 28 mm, PES (polyethersulfone), sterile, Sartorius [*Appleton Woods, Pack of 50, Catalog No FS095]*
8. Gibco® 1x (280 - 315 mOsm/kg) Phosphate Buffered Saline (PBS) solution, pH 7.4, 500 mL bottles *[Life Techonology, Catalog No 10010015]* Storage conditions: 15°C to 30°C
9. Anti-CD42b monoclonal antibodies, Biotin-conjugated, 50 µg at 0.1 mg/ml (0.5 mL vial). *[Abcam, Catalog No AB30400]*

Shipped at 4°C. Briefly spin down vial before opening. Can be separated into multiple aliquots upon receipt (aliquot volumes must be greater than 20 µl to ensure the antibody is recoverable, e.g. 50 µl each) and stored at -20°C to -80°C. Avoid freeze / thaw cycle! Once thawed, to be stored in fridge at +4°C short term (1-2 weeks).

* For enumeration of endothelial MP use anti-VE-cadherin (CD144) monoclonal antibodies, Biotin-conjugated, 100 µg. Supplied in lyophilized form. Shipped at ambient temperature. Reconstitute at 0.2 mg/mL in 0.5 mL of sterile PBS. Can be separated into multiple aliquots after reconstitution (aliquot volumes have to be greater than 20 µl to ensure the antibody is recoverable, e.g. 50 µl each) and stored at -20°C to -70°C for up to 6 months. Avoid freeze / thaw cycle! Once thawed, to be stored in fridge at +4°C short term (1-2 weeks) *[Bio-techne, Catalog No BAM9381]*

** For enumeration of monocyte MP use anti-CD14 monoclonal antibodies, Biotin-conjugated, 100 µg at 1 mg/ml (0.1 mL vial). Shipped at ambient temperature. Briefly spin down vial before opening. Upon receipt store in refrigerator at 4°C. Do NOT freeze! *[Invitrogen, Catalog No MA1-19489]*

10. Streptavidin, Alexa Fluor® 647—R-Phycoerythrin Conjugate (Alexa Fluor® 647—R-Phycoerythrin Streptavidin), 1 mg⁄ml, 100 µL vial. *[Life Technology, S20992]*

Shipped at 4°C. Briefly spin down vial before opening. Store in refrigerator (2°C to 8°C). Protect from light! Do NOT freeze!

3. Detailed method

3.1 General Preparation

1. Peripheral vein blood sample is to be collected with a 21-gauge needle without applying haemostasis into 3.2% sodium citrate BD Vacutainer™ tubes (BD Diagnostics). Discard the first vacutainer as this may have cell that are activated by

the trauma, particularly platelets (plasma samples obtained from the vacutainer collected first can be used for processing for other analytes, which are proved not to be affected significantly by the temporal vessel compression / vessel injury). Use only the second vacutainer to generate clinical data on MP levels.

2. Platelet-poor plasma (PPP) has to be prepared by centrifugation for 15 min at 2860 g (circa 4,000 rpm).
3. Aliquots should be frozen in 1.5 mL tubes (0.5 mL per tube) at -70°C until use.

3.2 Sample Preparation (to be performed in dark)

1. PPP has to be slowly thawed before sample staining at room temperature (RT) and not under hot water because of impact on MP numbers. Only one freeze / thaw cycle is allowed.

Note: platelet-free plasma (PFP) obtained via additional centrifugation of PPP at 13 000 g for 2 min in order to remove residual platelets or platelet fragments was suggested for MP enumeration in the first version of this protocol. Nonetheless, we do not recommend use of PFP now, because additional centrifugation significantly increases MP numbers due to cell damage.

2. Prepare dilutions 1/10 of the anti-CD42b biotinylated antibodies and the streptavidin on the morning of analysis, do not store the rest for other days. Calculate the required amount before analysis. In actual preparation, 10-20 µl greater volume must be prepared (to account for possible pipetting bias, surface tension, etc.). The below recipe is for the analysis of 20 samples of plasma. Increase or reduce the volumes of reagents in accordance.
    a. Mix thoroughly 12 µL of anti-CD42b-biotinylated antibodies and 108 µL of sterile and filtered PBS in a 1.5 mL Eppendorf tube, or

* Mix thoroughly 12 µL of anti-CD144-biotinylated antibodies and 108 µL of sterile and filtered PBS in a 1.5 mL Eppendorf tube, or

** Mix thoroughly 2.5 µL of anti-CD14-biotinylated antibodies 122.5 µL of sterile and filtered PBS in a 1.5 mL Eppendorf tube (1/50 dilution).

    b. Mix 6 µL of Streptavidin-Alexa Fluor® 647—R-PE conjugate and 54 µL of sterile and filtered PBS in a 1.5 mL Eppendorf tube.

3. Take 50 μL of thoroughly vortexed plasma sample with ‘wet tip’ reverse pipetting technique and add to the tube.
4. Take 5 μL of the 1/10 diluted (0.01 mg/ml) anti-CD42b biotinylated antibodies (corresponds to 0.05 μg) with ‘wet tip’ reverse pipetting technique and dispense it into plasma, or

* take 5 μL of the 1/10 diluted (0.02 mg/ml) anti-CD144 biotinylated antibodies (corresponds to 0.1 μg), or

** take 5 μL of the 1/50 diluted (0.02 mg/ml) anti-CD14 biotinylated antibodies (corresponds to 0.1 μg).

5. Gently vortex the sample. Incubate in the dark at room temperature for 30 minutes
6. Put 2.5 μL of the 1/10 diluted Streptavidin-Alexa Fluor® 647-R PE conjugate (corresponds to 0.25 μg) into the 1.5mL Eppendorf with plasma and anti-CD42b-biotinylated antibodies added at previous stages.
7. Gently vortex the sample again. Incubate in the dark at room temperature for 30 minutes.
8. Add 943 μL of filtered PBS, to achieve 1:20 dilution. Dilution factor may be higher depending on concentration of MP in the sample. Aim to keep number of events per second (reflected on the “Control” tab of the Control Panel while the sample runs) within 2 000 to 8 000 events.
9. Gently vortex the sample again. The sample is now ready to be analysed.

*   enumeration of EMPs

** enumeration of MMPs

3.3 Start-up procedure for Apogee A50 Flow Cytometry

<u>Part 1 – Restoring reagents and general preparation</u>

1. The sheath (distilled water) fluid reservoir is located at the top of the flow cytometer. Carefully lift up the top of the sheath fluid reservoir (the second deck on the top from the front of the machine) and fill it with distilled water (the level is indicated by a red plastic in the clear vertical plastic tube). Add small amount (1 plastic spatula full) of sodium azide into the sheath reservoir upon every refill of distilled water, this will retard bacterial or fungal growth in sheath and flow cell. Ensure gloves are worn and good ventilation, as pungent odour may be released upon dissolving sodium azide. Avoid direct contact with sodium azide.

The cytometer requires up to 2-3 refills over a continuous 8 hours running period (note, water is consumed when the FCM is on irrespectively of samples processing). Never allow the distilled water in the sheath reservoir to run out. If sheath fluid reservoir has got empty while FCM is on, it should be refilled with distilled water and then reloaded via the “Load new sheath” command on the FCM control window. Ensure that distilled water still is kept running. When performance of the still is getting low interruptions in continuous samples processing may occur, call for service then.

2. The waste reservoir is located on the floor under the flow cytometer. Carefully disconnect / unscrew the waste container and empty contents down sink with plenty of water. Ensure adequate ventilation as pungent fumes may be released. Never run flow cytometer with full waste container, as built up of back pressure will “aspirate” waste back into flow cell.
3. Switch on the computer by pressing black button on the front of machine. The flow cytometer will be automatically initialised after system start.

Part 2 – Apogee Histogram software preparation

4. After FCM initialisation open file with appropriate protocol (folder with protocols for enumeration of MP subtypes is available on the desktop), this will start the Apogee Histogram Software. Go to “Service” at the right-hand top corner of the screen, open it, and press “Flow Cell Clean”, this will automatically clean the system and needle before starting. When it is complete it will say so (< 5 minutes). Then press “Remove air in syringe”. Status will then move to “Idle clean”.
5. Before running any samples, ensure the following instruments checks are completed:

a. Check whether photomultiplier tubes (PMT) voltage settings are optimised to ensure most optimal detection of MP. Alteration in voltage will lead to increase excitation and emission and lead to significant alteration of results.

- Small angle light scatter (SALS) - 245.
- Large angle light scatter (LALS) - 400.
- Detector for the wavelength >650 nm (Red, suits emission spectrum of the Alexa Fluor® 647-R-PE) – 500.

Detectors for wavelength of 535±35 nm (Green) and 585±20 nm (Orng) are not used within current protocol and may have any voltage settings.

b. PMT noise level. Ensure that noise levels are <1.0 for SALS, LALS, and all detectors. Noise is due to background dust in flow cell and decreases accuracy of reading, usually increases after leaving Apogee A50 standing idle for longer time.

Reduction in noise can be achieved by multiple times of “Flush”, “Flow Cell Clean” and running sample of 10% household bleach at 10 µL/min (see 3.4 “Shut-down procedure”). Persistent elevation of noise level may require servicing to replace parts.

c. Settings in Control tab: running rate - 3 µL/min; aspirate volume - 150 µL (these settings allows for 150 µL sample to be aspirated and analysed at 3 µL/min) Increase in running rate results in lower precision.

3.4 Running Samples.

1. Open swing arm at bottom right of the flow cytometer, place the sample and put the arm to the “running position” from the “flush position” to acquire the sample from the Eppendorf tube. The system will automatically aspirate from the sample without pressing any button, once the arm is switched to the “running position”. Ensure that there is adequate volume to prevent aspiration of air.
2. The machine will say “Aspirate sample”, and then will start “Running sample”.
3. The sample will now run until the event collection is complete; the time of the acquisition will depend on the volume aspirated and running speed. If the software buffer is full (1 000 000 events) a message will appear asking whether the sample should be acquired further (with erasing the last data) or the acquisition should be stopped.
4. Press “No” and the sample will be stopped. If you want to stop the sample before reaching the limit of buffer size, press “Stop” during acquisition (at the right-hand corner of the Histogram software window). This may be required to prevent unexplained software crash that occurs occasionally when number of events per second in analysed sample is too high (e.g. greater than 16 000 evt/s).
5. After the acquisition stops, move arm to the “clean position” and discard the Eppendorf tube with the sample. The system will automatically clean the needle and tubes with pre-determined two flush cycles.
6. When autosaving is enabled the “Save window” will pop up. If not, or sample processing was discontinued intentionally by the operator, the data file should be saved by clicking in “Save data file as” under “File” menu or on the quick access toolbar. The data can be saved until automatic data clearance before the next sample has been aspirated and sample run has started. Note, data file backup on the external storage devices is needed, since free space on the computer hard disk drive is limited.

7. Absolute number of MP of interest will be expressed as events per µL at the bottom of the window with $CD42b^+$ (or $CD144^+$, or $CD14^+$) strict gate (results and gate have the same colour). This number has to be multiplied by the dilution factor (e.g. x20).
8. Obtain all the data and apply in into the appropriate spreadsheet of your project.

3.4 Shut-down procedure

1. Leave the cytometer on if to be used again later that day.
2. At the end of the day a sample of 10% household bleach (1% sodium hypochlorite) has to be run at about 10 µL/min until it finishes to clear out the protein traces from the flow cell. After moving the arm to the flush position and discarding the Eppendorf, the system will perform two predetermined flush cycles as after plasma sample.
3. Press button "Shutdown" at the right corner of the software panel and leave the system for cleaning (takes approx. 5 min). This will be done automatically. After the system has been automatically cleaned, a window will appear "Shutdown completed", close the software and the control panel.
4. Finally shut down the computer (Windows), this will automatically shut down the cytometer too.
5. Clean up the workstation.

Note, most of technical issues can be advised / resolved with the Apogee technical support. Please contact Oliver Kenyon via OJK@ApogeeFlow.com. Internet access may be required (currently not available) to fix software bugs. For more complex issues, e.g. requiring parts replacement, Apogee service (paid) can be arranged.

4. Interpretation of the results

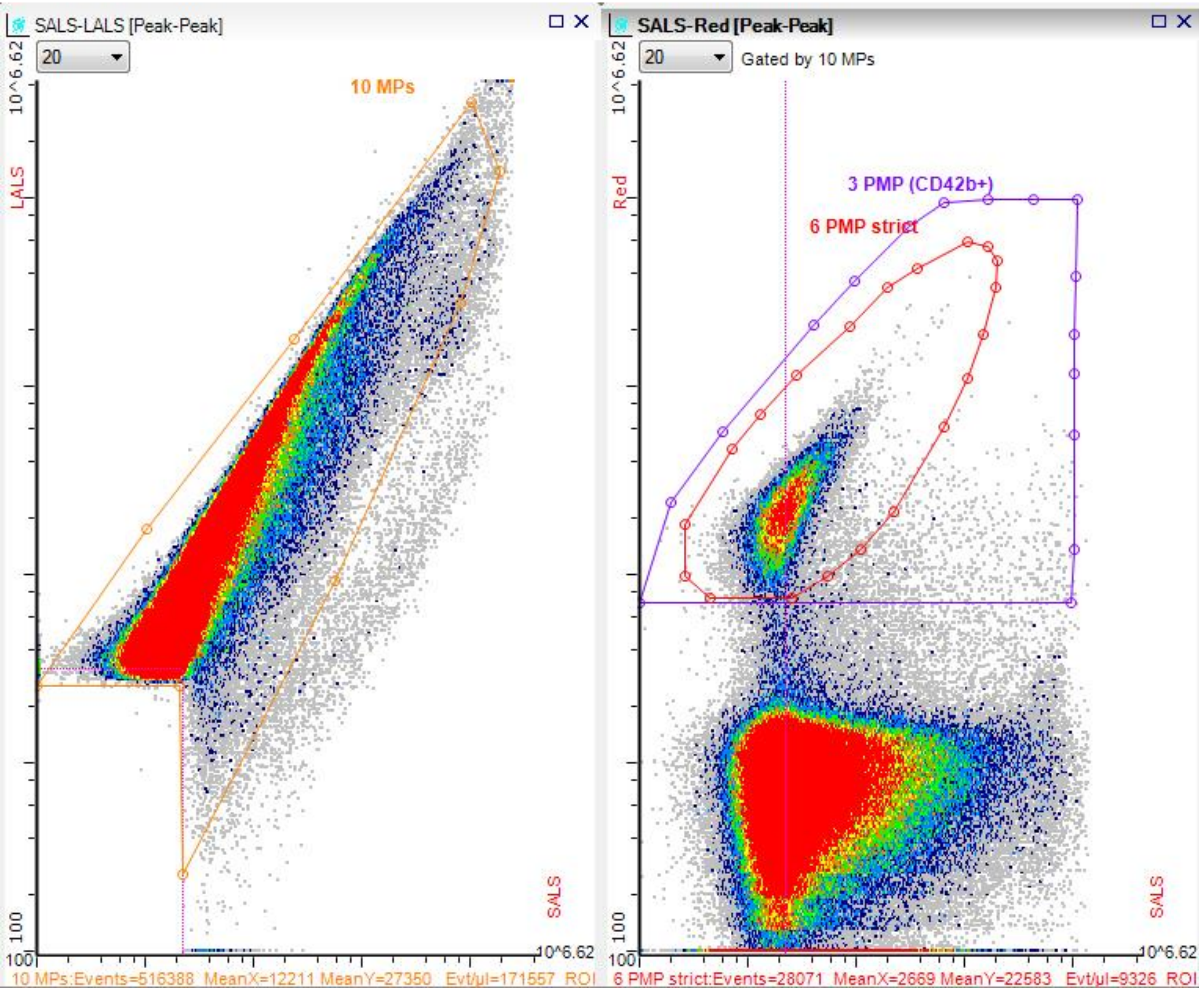

Figure 1: $CD42b^+$ event rate detection by Apogee A50 in prepared sample.

Platelets microparticles (PMPs) are determined by the CD42b positive events. The vertical (Y) axis on the right window (RED) is the staining with Streptavidin-Alexa Fluor® 647-R-PE - anti$CD42b^+$-biotin of platelet MPs. The horizontal (x) axis (SALS) is shows small angle light scatter. Note the red gate, which defines the number of CD42b positive events. This has been set by the results of the previous isotype experiments. Purple region-of-interest (ROI), signifies potential $CD42b^+$ events due to detection of excited fluorochrome. At the bottom of the PMPs plot, the number of positive events per µL in selected ROI is demonstrated, i.e. 9 326 Evt/µL. Once dilution factor (x20) is factored into the calculation, the number of platelet MPs in undiluted plasma is 186 520 $µL^{-1}$.

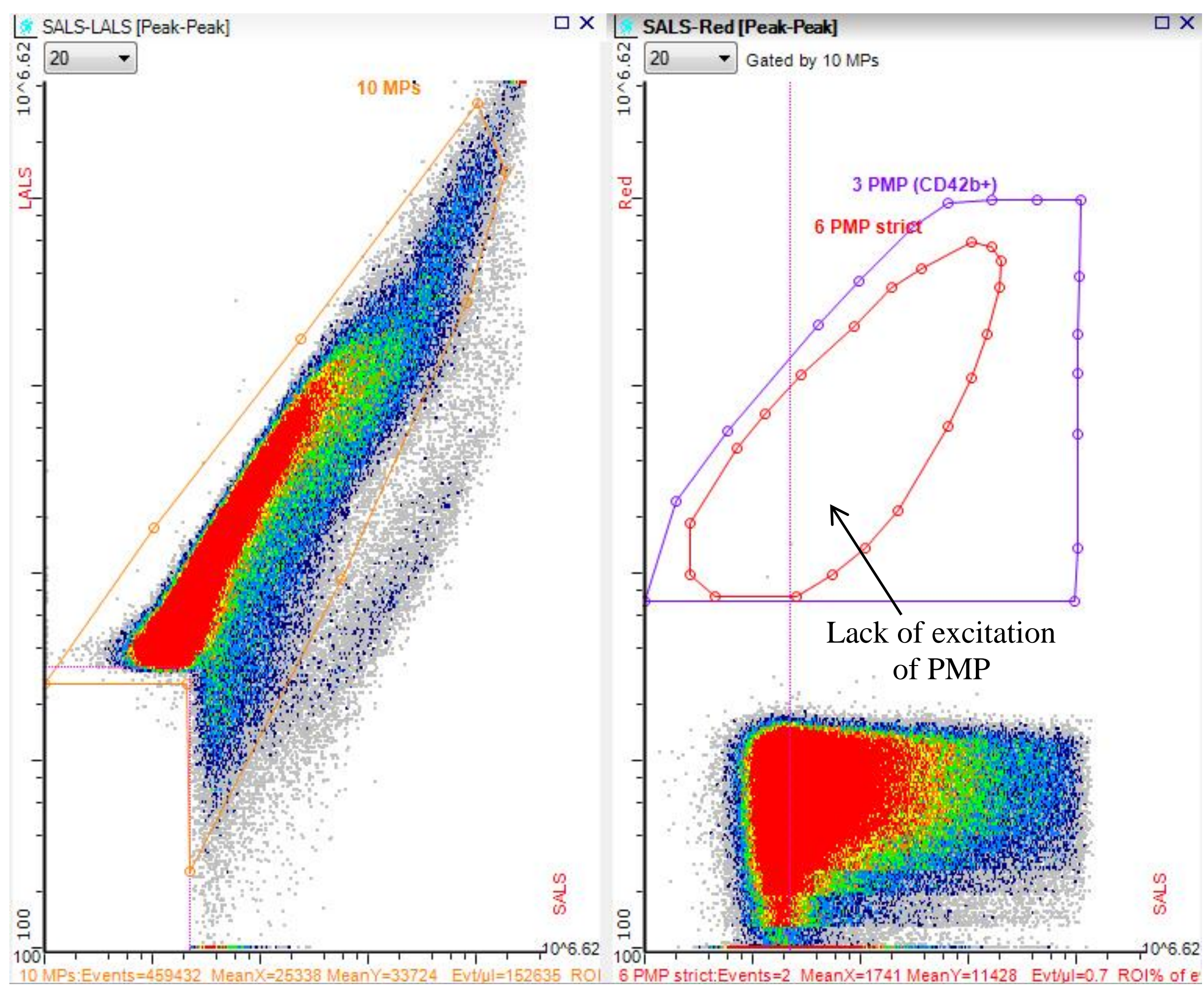

Figure 2: CD42b event rate detection by Apogee A50 in control plasma.
Note event rates in ROIs are close to zero.

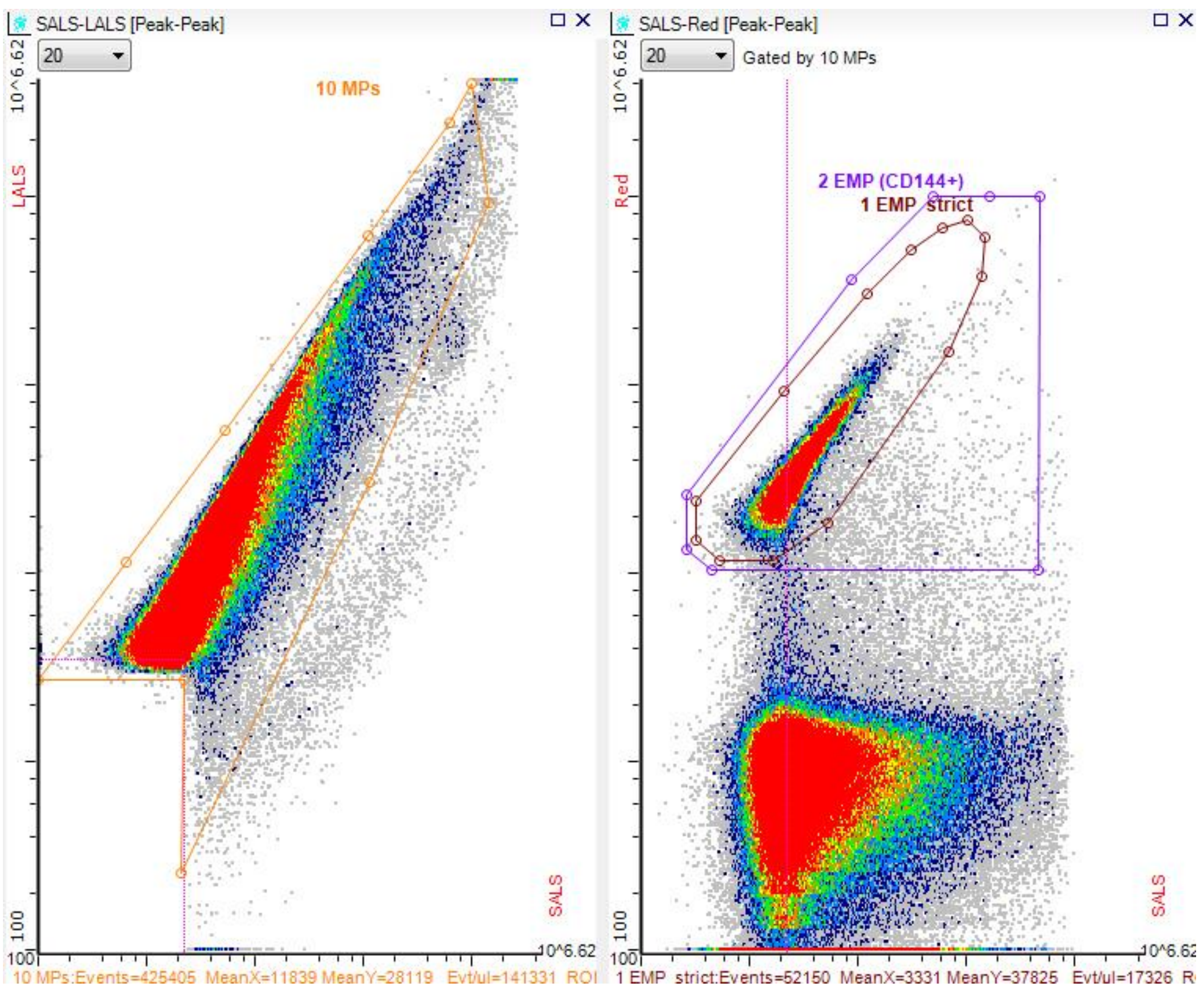

Figure 3: CD144$^+$ event rate detection by Apogee A50 in prepared sample.

The brown ROI defines the number of CD144 positive events. Purple ROI signifies potential CD144$^+$ events due to detection of excited fluorochrome. At the bottom of the EMPs plot, the number of positive events per µL in selected ROI is demonstrated, i.e. 17 326 Evt/µL. Once dilution factor (x20) is factored into the calculation, the number of endothelial MPs in undiluted plasma is 346 520 µL^{-1}.

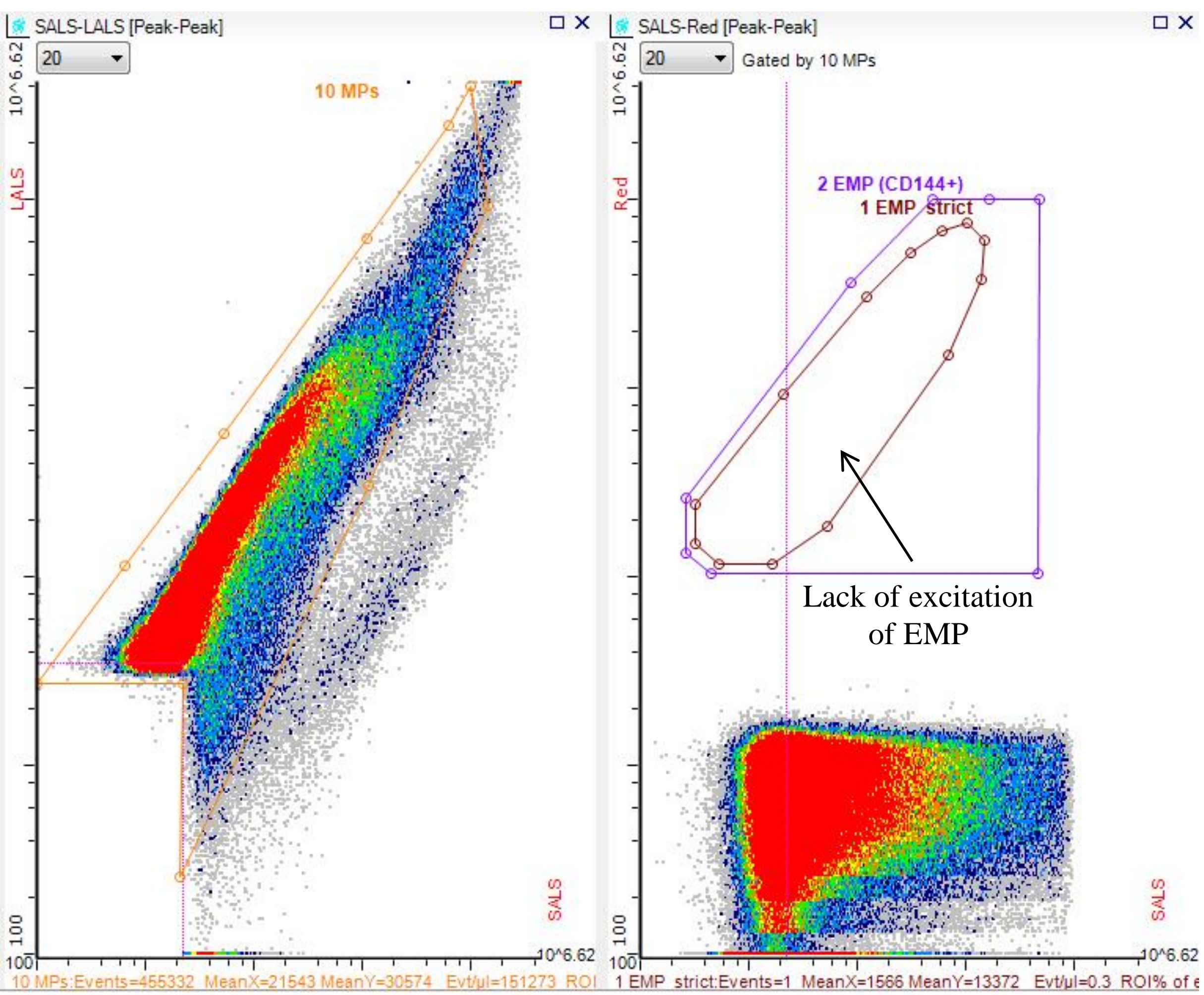

Figure 4: CD144 event rate detection by Apogee A50 in control plasma.
Note event rates in ROIs are close to zero.

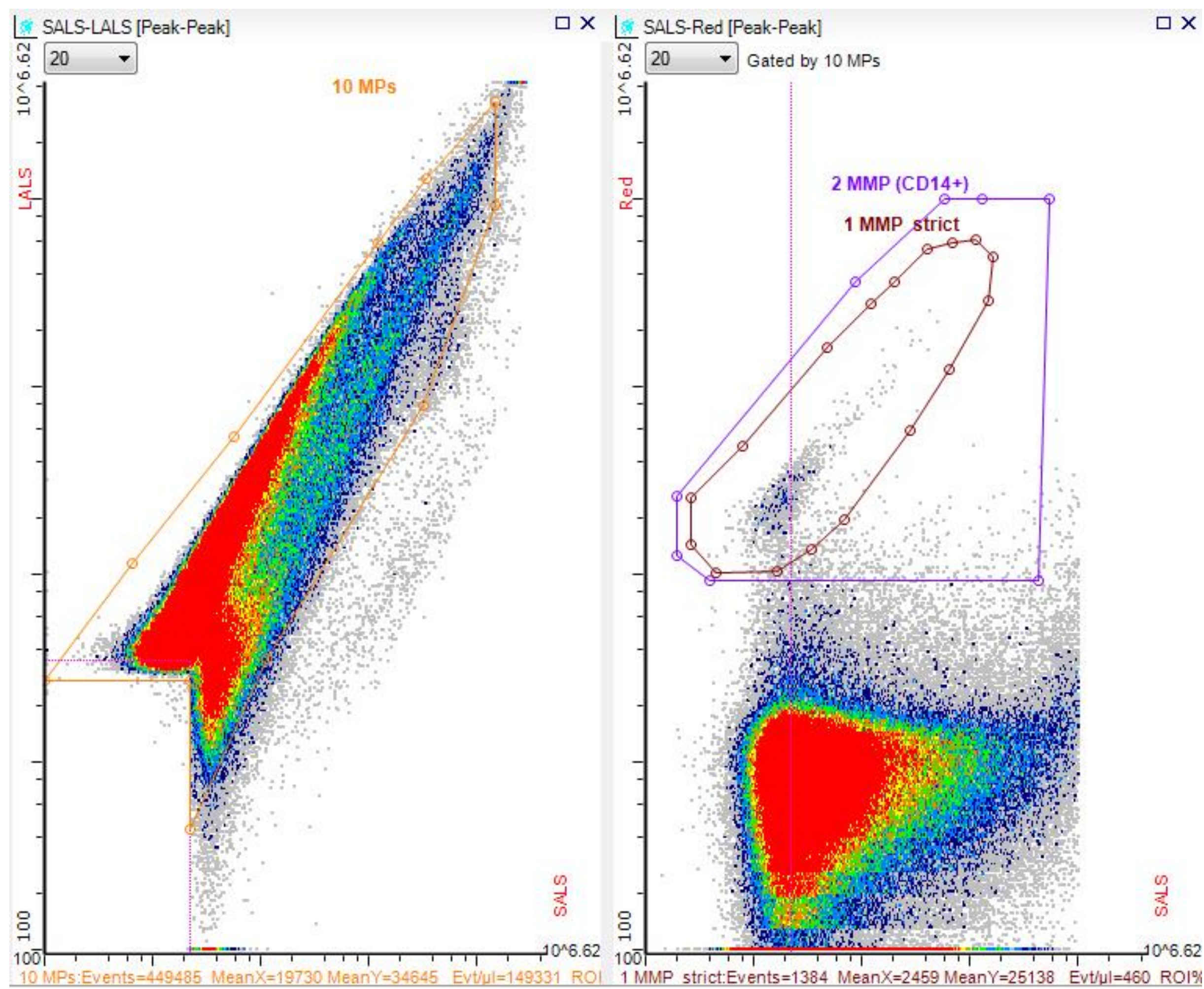

Figure 5: CD14^{+} event rate detection by Apogee A50 in prepared sample.

The brown ROI defines the number of CD14 positive events. Purple ROI signifies potential CD14^{+} events due to detection of excited fluorochrome. At the bottom of the MMPs plot, the number of positive events per µL in selected ROI is demonstrated, i.e. 460 Evt/µL. Once dilution factor (x20) is factored into the calculation, the number of monocyte-derived MPs in undiluted plasma is 9200 μL^{-1}.

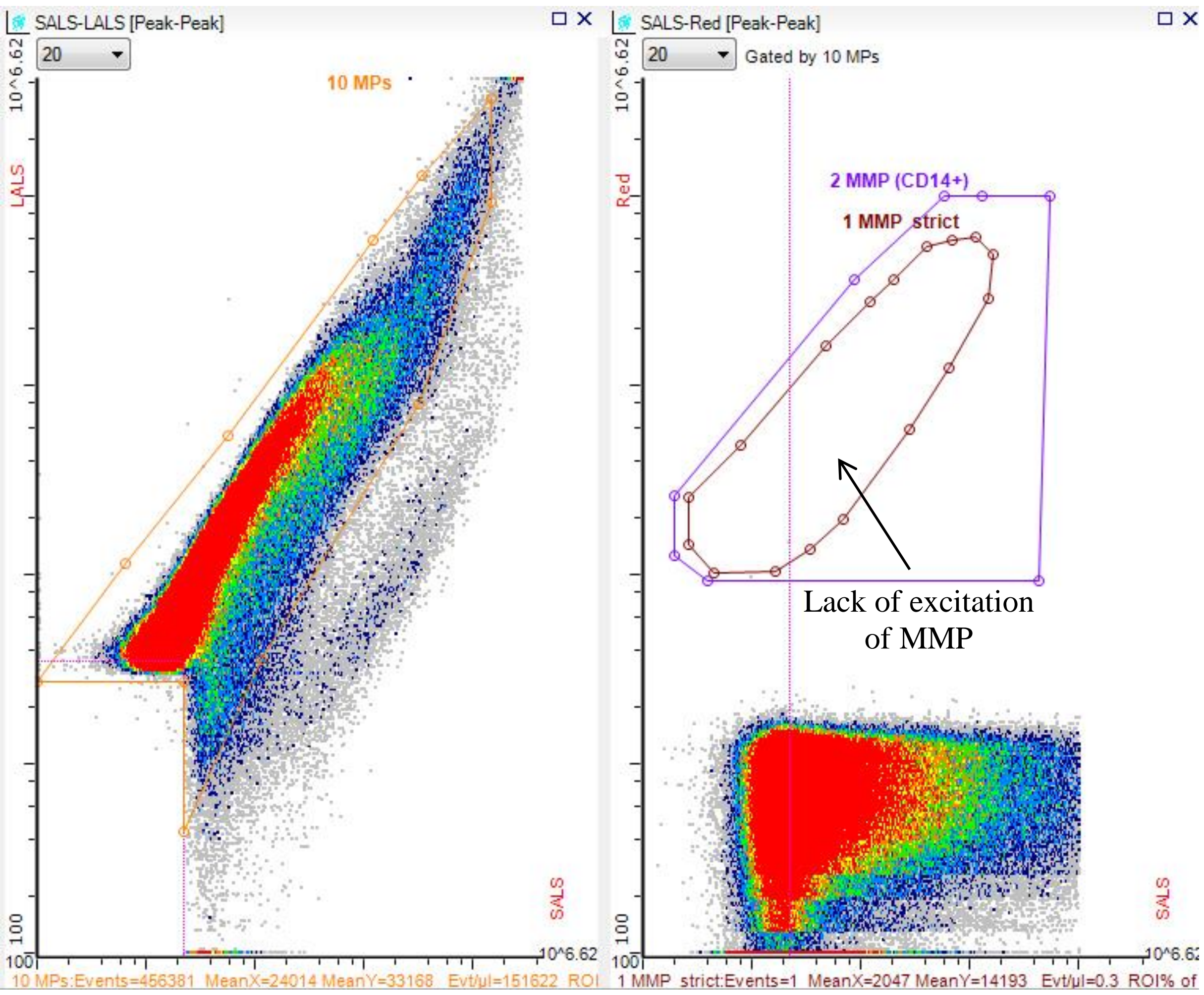

Figure 6: CD14 event rate detection by Apogee A50 in control plasma.
Note event rates in ROIs are close to zero.

- **STANDARD OPERATING PROCEDURE: Enumeration of Apoptotic Microparticles (Anexin V+) using Apogee A50 Flow Cytometry**

Developed by Dr Silvia Montoro Garcia and Dr Eduard Shantsila (September 2011)

Updated by Dr Mikhail Dzeshka (January 2017); Dr Christos Voukalis (July 2017)

Note: Use of the flow cytometry is forbidden without official training

Required pre-training: SOP on Venepuncture, Good Laboratory Practice, Good Analytical Practice, Good Clinical Practice, and Code of Practice for Research in the University of Birmingham

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1. Introduction

Cellular microparticles (MP) are submicron biological structures released from different types of cells (e.g., from platelets, leucocytes, endothelial cells, red blood cells) via remodelling of plasma membrane in response to numerous conditions, including activation and apoptosis. MPs are generally defined as small (0.1 to 1 μm) membrane fragments that often expose the anionic phospholipids phosphatidylserine and membrane antigens representative of their cellular origin. These characteristics discriminate MP from exosomes, which are smaller (<0.1 nm), originate from intracellular multivesicular bodies, and differ in antigenic composition.

Some MPs have prothrombotic properties irrespectively from their origin. However, presence of certain markers (e.g., tissue factor) on MP surface substantially enhances thrombogenic properties. Flow cytometry allows identifying and enumerating different types of MP based on their size, surface antigens and, usually, utilisation of count beads.

The A50-Micro flow cytometer was shown to be superior to most conventional flow cytometer for the purpose of MP analysis due to its design specifically focused for analysis of small events [1]. Conventional flow cytometers are limited to measuring particles larger than about 500 nm by small angle light scatter (forward scatter, FS). For example, the lower limit of resolution of BD FACSCalibur is 5.0 μm. Apogee A50 discriminates 20 nm particles (latex beads), and it also benefits from a volumetric sampling system that accurately counts MP without using count beads). Additional advantage of Apogee A50 is a fluidics system that can be refilled with pure distillate water, saving reagents compare to conventional cytometers.

This SOP describes enumeration of Annexin V-binding microparticles, usually referred as apoptotic MPs (AMP).

2. Materials and Supplier contact details:

11. Distilled water from the still in the Tissue culture room (ensure sufficient volume of water is available to run flow cytometry continuously).
12. Eppendorf Microcentrifuge Tubes 1.5 ml (Conical Bottom, Graduated, UltraClear® copolymer polypropylene, APEX® Soft-Release Flip-Cap, Frosted writing area) *[Alpha Laboratories, Pack of 500, Catalog No LW2455]*
13. Pipette tips 0.1-10 µl (TubeGuard Top Ultrafine Tip, Extended-Length 38 mm, Non-Sterile, Fastrak® Tip Refill System) *[Alpha Laboratories, Pack size: 14 racks of 96 in a refill tower, Catalog No FR 1010]*
14. Pipette tips 1-200 µl (Universal, Bevelled, Yellow, Non-Sterile, 50 mm in length, Fastrak® Tip Refill System) *[Alpha Laboratories, Pack size: 10 racks of 96 in a refill tower, Catalog No FR 1200]*
15. Pipette tips 100-1250 µl (FlexTop Ultrafine Tip, Extended-Length 89 mm, Non-Sterile, Fastrak® Tip Refill System) *[Alpha Laboratories, Pack size: 5 racks of 96 in a refill tower, Catalog No FR 1250]*
16. Syringes 20 ml, disposable, sterile, Terumo [Appleton Woods, Pack of 50, Catalog No GS577]
17. Syringe filters 0.22 µm, 28 mm, PES (polyethersulfone), sterile, Sartorius [*Appleton Woods, Pack of 50, Catalog No FS095]*
18. Gibco® 1x (280 - 315 mOsm/kg) Phosphate Buffered Saline (PBS) solution, pH 7.4, 500 mL bottles *[Life Techonology, Catalogue No 10010015]* **Storage conditions:** 15°C to 30°C
19. Annexin V Binding Buffer, 5x concentrated solution (50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4) for flow cytometry, 50 mL. Shipped at 4°C. Store at 2–8°C. *[Molecular Probes, Catalogue No V13246]*
20. Annexin V, Alexa Fluor® 488 conjugate, 500 µL. Shipped at 4°C. Store in refrigerator at 2°C to 8°C. **Protect from light! Do NOT freeze!** *[Molecular Probes, Catalog No A13201]*

3. Detailed method

3.1 General Preparation

4. Peripheral vein blood sample is to be collected with a 21-gauge needle without applying haemostasis into 3.2% sodium citrate BD Vacutainer™ tubes (BD Diagnostics). Discard the first vacutainer as this may have cell that are activated by the trauma, particularly platelets (plasma samples obtained from the vacutainer collected first can be used for processing for other analytes, which are proved not to be affected significantly by the temporal vessel compression / vessel injury). Use only the second vacutainer to generate clinical data on MP levels.
5. Platelet-poor plasma (PPP) must be prepared by centrifugation for 15 min at 2860 g (circa 4,000 rpm).
6. Aliquots should be frozen in 1.5 mL tubes (0.5 mL per tube) at -70°C until use.

3.2 Sample Preparation (to be performed in dark)

10. PPP must be slowly thawed before sample staining at room temperature (RT) and not under hot water because of impact on MP numbers. Only one freeze / thaw cycle is allowed.

Note: platelet-free plasma (PFP) obtained via additional centrifugation of PPP at 13 000 g for 2 min in order to remove residual platelets or platelet fragments was suggested for MP enumeration in the first version of this protocol. Nonetheless, we do not recommend use of PFP now, because additional centrifugation significantly increases MP numbers due to cell damage.

11. Prepare Annexin V binding buffer, 2x concentrated solution (20 mM HEPES, 280 mM NaCl, 5 mM $CaCl_2$, pH 7.4), on the morning of analysis. Excess solution can be stored at 4°C in the fridge for 1 week, storage of reconstituted solution for longer time should be discouraged.

Calculate the required amount before analysis. The below recipe is for the analysis of 20 samples of plasma. Increase or reduce the volumes of reagents in accordance. Mix thoroughly 600 µL of Annexin V binding buffer, 5x concentrated solution, and 900 µL of sterile and filtered PBS in a 1.5 mL Eppendorf tube.

12. Prepare Annexin V dilution buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4, i.e. equal to Annexin V binding buffer, 1x solution),), on the morning of analysis. Excess solution can be stored at 4°C in the fridge for 1 week, storage of reconstituted solution for longer time should be discouraged.

Calculate the required amount before analysis. The below recipe is for the analysis of 20 samples of plasma. Increase or reduce the volumes of reagents in accordance. Mix thoroughly 4 mL of Annexin V binding buffer, 5x concentrated solution, and 16 mL of sterile and filtered PBS in a 1.5 mL Eppendorf tube.

13. Take 50 µL of thoroughly vortexed plasma sample with 'wet tip' reverse pipetting technique and add to the tube.
14. Put 50 µL of the 2x concentrated Annexin V binding buffer into the 1.5mL Eppendorf with plasma.
15. Gently vortex the sample and put 5 µL of Annexin V, Alexa Fluor® 488 conjugate. Incubate in the dark at room temperature for 30 minutes.
16. Add 895 µL of Annexin V dilution buffer to achieve 1:20 dilution. Dilution factor may be higher depending on concentration of MP in the sample. Aim to keep number of events per second (reflected on the "Control" tab of the Control Panel while the sample runs) within 2 000 to 8 000 events.
17. Gently vortex the sample again. The sample is now ready to be analysed.

3.3 Start-up procedure for Apogee A50 Flow Cytometry

<u>Part 1 – Restoring reagents and general preparation</u>

6. The sheath (distilled water) fluid reservoir is located at the top of the flow cytometer. Carefully lift up the top of the sheath fluid reservoir (the second deck on the top from the front of the machine) and fill it with distilled water (the level is indicated by a red plastic in the clear vertical plastic tube). Add small amount (1 plastic spatula full) of sodium azide into the sheath reservoir upon every refill of distilled water, this will retard bacterial or fungal growth in sheath and flow cell. Ensure gloves are worn and good ventilation, as pungent odour may be released upon dissolving sodium azide. **Avoid direct contact with sodium azide.**

The cytometer requires up to 2-3 refills over a continuous 8 hours running period (note, water is consumed when the FCM is on irrespectively of samples processing). Never allow the distilled water in the sheath reservoir to run out. If sheath fluid reservoir has got empty while FCM is on, it should be refilled with distilled water and then reloaded via the "Load new sheath" command on the FCM control window. Ensure that distilled water still is kept running. When performance of the still is getting low interruptions in continuous samples processing may occur, call for service then.

7. The waste reservoir is located on the floor under the flow cytometer. Carefully disconnect / unscrew the waste container and empty contents down sink with plenty of water. Ensure adequate ventilation as pungent fumes may be released. Never run flow cytometer with full waste container, as built up of back pressure will "aspirate" waste back into flow cell.
8. Switch on the computer by pressing black button on the front of machine. The flow cytometer will be automatically initialised after system start.

Part 2 – Apogee Histogram software preparation

9. After FCM initialisation open file with appropriate protocol (folder with protocols for enumeration of MP subtypes is available on the desktop), this will start the Apogee Histogram Software. Go to "Service" at the right-hand top corner of the screen, open it, and press "Flow Cell Clean", this will automatically clean the system and needle before starting. When it is complete it will say so (< 5 minutes). Then press "Remove air in syringe". Status will then move to "Idle clean".
10. Before running any samples, ensure the following instruments checks are completed:

a. Check whether photomultiplier tubes (PMT) voltage settings are optimised to ensure most optimal detection of MP. Alteration in voltage will lead to increase excitation and emission and lead to significant alteration of results.

- Small angle light scatter (SALS) - 245.
- Large angle light scatter (LALS) - 400;
- Detector for the wavelength of 535±35 nm (Green, suits emission spectrum of the Alexa Fluor® 488) – 500.

Detectors for wavelength of 585±20 nm (Orng) and >650 nm (Green) are not used within current protocol and may have any voltage settings.

b. PMT noise level. Ensure that noise levels are <1.0 for SALS, LALS, and all detectors. Noise is due to background dust in flow cell and decreases accuracy of reading, usually increases after leaving Apogee A50 standing idle for longer time.

Reduction in noise can be achieved by multiple times of "Flush", "Flow Cell Clean" and running sample of 10% household bleach at 10 μL/min (see 3.4 "Shut-down procedure"). Persistent elevation of noise level may require servicing to replace parts.

c. Settings in Control tab: running rate - 3 μL/min; aspirate volume - 150 μL (these settings allows for 150 μL sample to be aspirated and analysed at 3 μL/min) Increase in running rate results in lower precision.

3.3 Running Samples.

9. Open swing arm at bottom right of the flow cytometer, place the sample and put the arm to the "running position" from the "flush position" to acquire the sample from the Eppendorf tube. The system will automatically aspirate from the sample without pressing any button, once the arm is switched to the "running position". **Ensure that there is adequate volume to prevent aspiration of air.**
10. The machine will say "Aspirate sample", and then will start "Running sample".

11. The sample will now run until the event collection is complete; the time of the acquisition will depend on the volume aspirated and running speed. If the software buffer is full (1 000 000 events) a message will appear asking whether the sample should be acquired further (with erasing the last data) or the acquisition should be stopped.
12. Press "No" and the sample will be stopped. If you want to stop the sample before reaching the limit of buffer size, press "Stop" during acquisition (at the right-hand corner of the Histogram software window). This may be required to prevent unexplained software crash that occurs occasionally when number of events per second in analysed sample is too high (e.g. greater than 16 000 evt/s).
13. After the acquisition stops, move arm to the "clean position" and discard the Eppendorf tube with the sample. The system will automatically clean the needle and tubes with pre-determined two flush cycles.
14. When autosaving is enabled the "Save window" will pop up. If not, or sample processing was discontinued intentionally by the operator, the data file should be saved by clicking in "Save data file as" under "File" menu or on the quick access toolbar. The data can be saved until automatic data clearance before the next sample has been aspirated and sample run has started. Note, data file backup on the external storage devices is needed, since free space on the computer hard disk drive is limited.
15. Absolute number of MP of interest will be expressed as events per µL at the bottom of the window with Annexin V strict gate (results and gate have the same colour). This number has to be multiplied by the dilution factor (e.g. x20).
16. Obtain all the data and apply in into the appropriate spreadsheet of your project.

3.4 Shut-down procedure

6. Leave the cytometer on if to be used again later that day.
7. At the end of the day a sample of 10% household bleach (1% sodium hypochlorite) has to be run at about 10 µL/min until it finishes to clear out the protein traces from the flow cell. After moving the arm to the flush position and discarding the Eppendorf, the system will perform two predetermined flush cycles as after plasma sample.
8. Press button "Shutdown" at the right corner of the software panel and leave the system for cleaning (takes approx. 5 min). This will be done automatically. After the system has been automatically cleaned, a window will appear "Shutdown completed", close the software and the control panel.
9. Finally shut down the computer (Windows), this will automatically shut down the cytometer too.
10. Clean up the workstation.

Note, most of technical issues can be advised / resolved with the Apogee technical support. Please contact Oliver Kenyon via OJK@ApogeeFlow.com. Internet access may be required (currently not available) to fix software bugs. For more complex issues, e.g. requiring parts replacement, Apogee service (paid) can be arranged.

4. Interpretation of the results

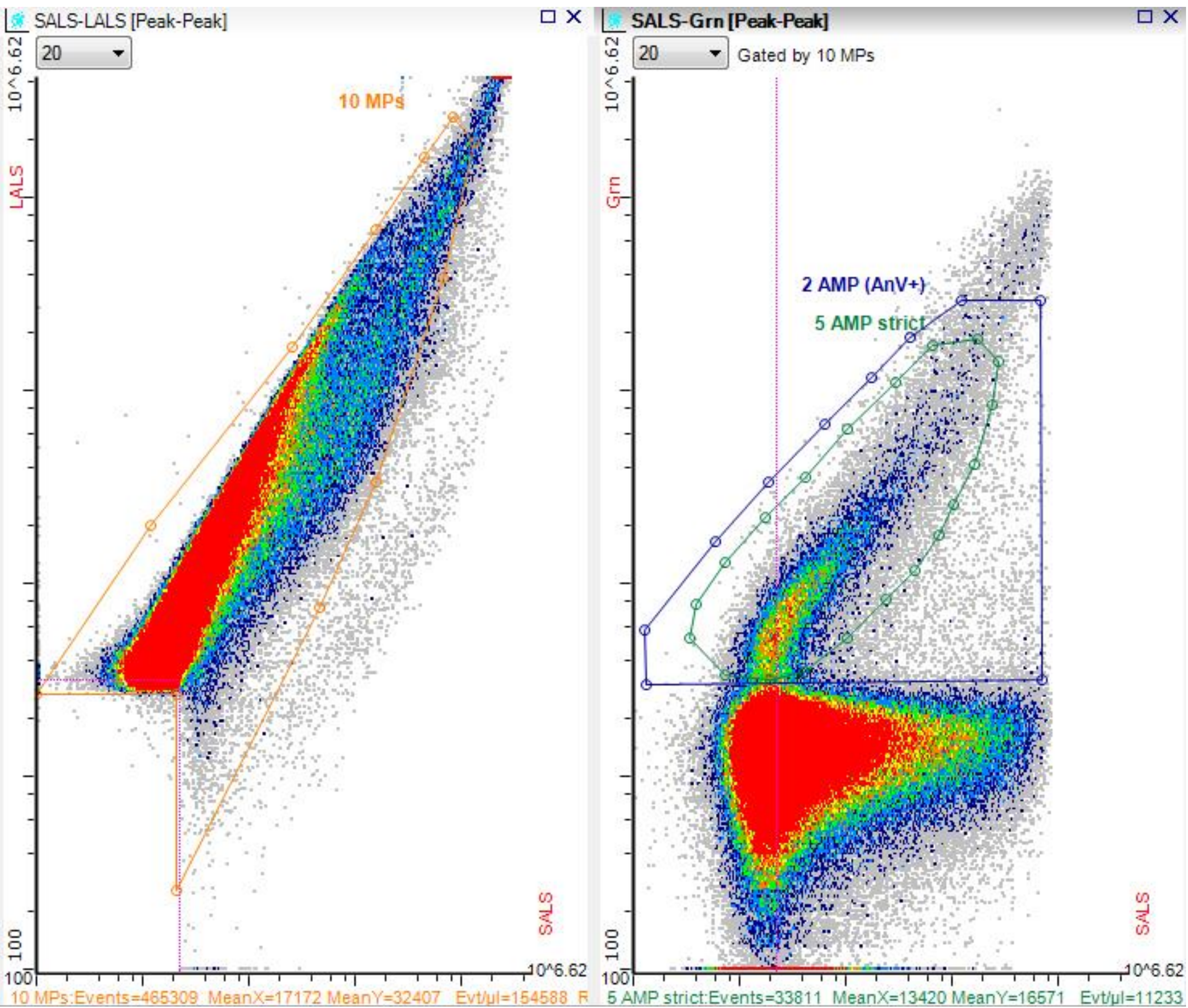

Figure 1: Annexin V event rate detection by Apogee A50 in prepared sample.

Apoptotic microparticles (AMPs) are determined by the Annexin V positive events. The vertical (Y) axis on the right window (Green) is the staining with Annexin V, Alexa Fluor® 488 conjugate of apoptotic MPs. The horizontal (x) axis (SALS) is shows small angle light scatter. Note the green ROI, which defines the number of AnV positive events. This has been set by the results of the previous isotype experiments. Purple ROI signifies potential AnV events due to detection of excited fluorochrome. At the bottom of the AMPs plot, the number of positive events per µL in selected ROI is demonstrated, i.e. 11 233 Evt/µL. Once dilution factor (x20) is factored into the calculation, the number of apoptotic MPs in undiluted plasma is 224 660 μL^{-1}.

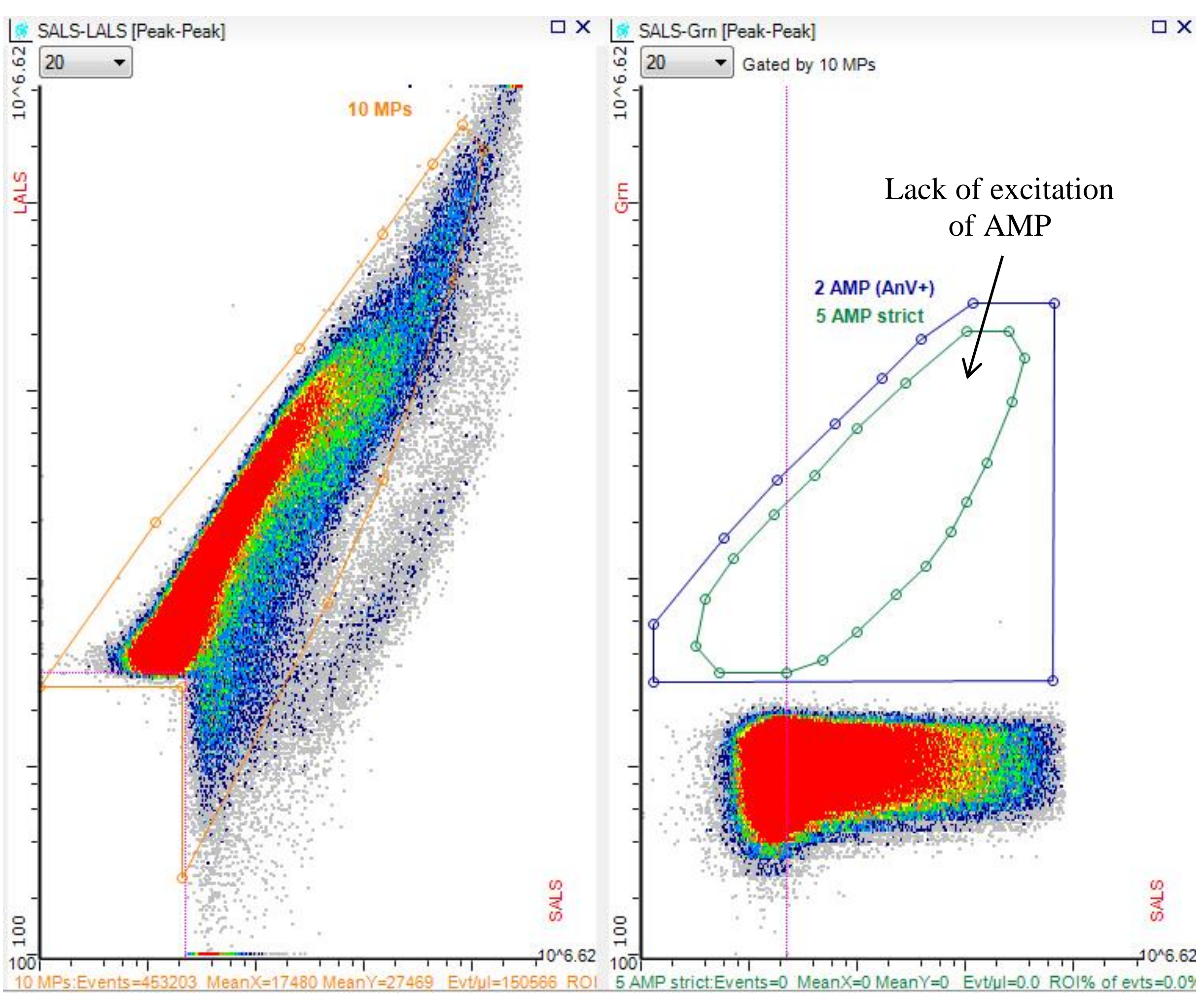

Figure 2: Annexin V event rate detection by Apogee A50 in control plasma.
Note event rates in ROIs are close to zero.

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