**Effects of antithrombotic drugs on the prothrombotic state in patients with atrial fibrillation: the west Birmingham Atrial Fibrillation Project**

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**Abstract**

Background: Direct oral anticoagulants (DOACs) are known to prevent thrombosis but there is limited information about their activity on the clot formation and lysis cascade.

Objectives: This study assesses the role of apixaban, one of the four licenced DOACs, on clot dynamics in patients with atrial fibrillation (AF).

Methods: We compared haemostatic and clot lysis characteristics between a group of patients with AF (n = 47) and a “disease control” group with ischaemic heart disease but in sinus rhythm (n = 39). Subsequently, we conducted clot structure studies in 3 groups of patients with AF on different antithrombotic drugs: warfarin (n = 60), apixaban (n = 60) or antiplatelets (n = 62) and in patients with AF naïve to oral anticoagulants before and after 3-months treatment with apixaban (n = 32). Haemostasis was investigated by a viscoelastic, whole blood technique (Thromboelastography/TEG), a “microplate-reader based”, citrated plasma technique (microplate assay), immunoassays to determine plasma concentrations of plasminogen activator inhibitor-1 (PAI-1), tissue- Plasminogen Activator (t-PA), D-dimer and finally platelet derived and apoptotic microparticles.

Results: Patients with AF have more potent thrombogenesis based on microplate assay indices [Rate of clot formation (p = 0.03, ƞ2 = 0.06), Maximum optical density (p < 0.001, ƞ2 = 0.05)] and delayed fibrinolysis [Rate of clot dissolution (p = 0.005, ƞ2 = 0.17)] with increased levels of apoptotic microparticles (p = 0.02, ƞ2 = 0.06) compared with the ‘disease control’ group. Apixaban was more effective in attenuating prothrombotic characteristics assessed by TEG {R (ε2 = 0.21), K (ε2 = 0.16) and angle [mean difference (MD), 95% Confidence Intervals (CI), vs warfarin 5, 0.96–8.6 and 8, 3.8–11.4 vs antiplatelets], (p < 0.001 for all indices)} compared with the other treatment groups. Patients on apixaban had lower D-dimer (p < 0.001, ε2 = 0.17) and tPA (p = 0.03, MD 90, 95%CI 6–150 vs warfarin and MD 90, 95% CI 4–150 vs antiplatelets) levels. From the microplate assay analysis, warfarin and apixaban demonstrated comparable activity based on multiple indices, both superior to antiplatelets. However, warfarin was associated with reduced fibrin network robustness (Max. optical density p < 0.001, ε2 = 0.1). Apixaban inhibited thrombosis, amplified fibrinolysis and decreased D-dimer (p = 0.001, r = 0.4) levels in the follow up study.

Conclusions: Patients with AF have impaired haemostasis and elevated levels of apoptotic microparticles. Apixaban appears to affect plasma prothrombotic characteristics in a distinctive manner compared with warfarin and to reduce biomarkers associated with adverse cardiovascular events.

**Key words:** atrial fibrillation, apixaban, warfarin, clot structure, haemostasis

**Introduction**

In atrial fibrillation (AF), endothelial inflammation and imbalance of pro- and anticoagulant constituents contribute to impaired thrombo- genesis [1]. Prevention of ischaemic stroke and haemorrhage requires efficient haemostasis with timely fibrin clot formation but also prompt lysis of the clot, once its role in maintaining the haemodynamic integrity is completed [2]. To reduce the risk of stroke, patients with AF are treated with oral anticoagulation (OAC) [3]. Apixaban, one of the licenced direct oral anticoagulants (DOACs), has showed consistent efficacy and safety in non-valvular AF (NVAF) stroke prevention phase III clinical trials and real world data [4,5].

Accumulating evidence indicates that abnormalities in fibrin clot structure may reflect a potential risk for thromboembolic disease [6]. DOACs are known to reduce thromboembolism but their influence on fibrin clot structure is still not completely described, especially in comparison with other oral antithrombotics [7,8]. One of the reason is that the traditionally used laboratory coagulation tests, such as inter- national normalised ratio (INR) are of limited value for the assessment of the antithrombotic properties of DOACs [9]. Other techniques, for example thromboelastography (TEG) [10] and microplate assay [11,12] may give more insight on how antithrombotic agents affect haemostasis and thrombolysis. TEG utilises citrated whole blood and microplate assay plasma, in order to characterise 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. Thromboelastography assesses viscoelastic properties of the whole blood which includes platelet activity. We used caolin as activator of haemostasis in TEG which mainly initiate the intrinsic coagulation pathway but also has antiplatelet activation properties [13]. Microplate assay describes haemostasis 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 tissue plasminogen activator (t-PA) [10,11]. Microplate assay mainly describes alteration in fibrin clot dynamics which occur during the common pathway of coagulation and the lysis phase [14]. The two methods assess haemostasis from different perspective and they appear complementary in order to approach an understanding of thrombogenesis and fibrinolysis [15]. 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 [16].

In addition to dynamic assays, an alternative assessment of fibrinolysis focusses on levels of individual molecules such as t-PA and plasminogen activator inhibitor-1 (PAI-1). Indeed, this imbalance may contribute to the clinical manifestations of cardiovascular diseases [17,18]. Furthermore, increased levels of D-dimers are found in many pro-thrombotic conditions, being predictive of cardiovascular events and may also interfere with the t-PA and PAI-1 dynamics [19].

Another mechanism that affects haemostasis is related to the presence of blood microparticles [20]. 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 [21].

In this study, we used the above dynamic assays, biomarkers and microparticles to characterise the haemostatic profile and coagulation potential of patients with AF and control subjects. First, we test the hypothesis that the effect of apixaban in the thrombosis-fibrinolysis pathway is different compared to warfarin and aspirin. Second, we compare haemostatic and thrombosis dynamics between AF patients and a ‘disease control’ group in sinus rhythm with ischaemic heart disease (IHD). Finally, we evaluate the changes on the coagulation and lysis kinetics before and after initiation of apixaban.

**Materials and methods**

*2.1. Study populations*

The state of haemostasis and fibrinolysis was tested in two cross- sectional studies and a follow up study. In the first cross-sectional study we compared patients with NVAF receiving one of three antithrombotic treatments: warfarin (n = 60), apixaban (n = 60) or anti- platelets only (n = 62). In the second cross-sectional study we compared patients in AF (n = 47) with age and sex matched controls in sinus rhythm, without history of AF (but with IHD defined by history of angina or previous myocardial infraction with angiographically confirmed coronary artery stenosis). Both groups received antiplatelets (aspirin or clopidogrel). Patients with AF on antiplatelets had either low thromboembolic risk (CHA2DS2VASc score of ≤1) or were recruited during their first visit to cardiology clinic prior to switching to OAC. AF patients managed by warfarin group were only recruited if the INR was within the therapeutic range (2.1–2.9) at the time of recruitment. The follow up study included 32 patients with AF assessed before and 3 months after initiation of DOAC (apixaban 2.5 or 5 mg bid as clinically indicated).

All participants were recruited from cardiology clinics in City Hospital in Birmingham, from January 2016 to April 2017. Analyses involved patients with permanent or paroxysmal AF. All permanent AF patients had satisfactory heart rate control (60–100 bpm). Subjects managed by warfarin had INR measured by the hospital haematology laboratory on the day of recruitment.

In addition and to exclude other causes of coagulopathy we checked full blood count, activated partial thromboplastin time, renal function and electrolytes, fibrinogen, C-Reactive protein and liver function tests in each participant. Exclusion criteria were end-stage chronic kidney disease, connective tissue disease, neoplasms, 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), current use of antibiotics, non-steroid anti-inflammatory drugs or steroids. The study was performed in accordance with standards of the 1964 Helsinki declaration and its later amendments. Ethical approval for the study was granted by the NRES Committee West Midlands – Coventry & Warwickshire (REC reference 13/WM/0379). All participants provided written informed consent.

*2.2. Thromboelastography*

Thromboelastography was performed using a commercial thromboelastograph (TEG® 5000 Thrombelastograph® Haemostasis Analyzer System), the manufacturer reagents and following the manufacturer instructions. Citrate-anticoagulated venous blood was collected by venepuncture and processed within 2 h. 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) (Table 1 and Supplementary appendix).

*2.3. Micro-plate assay*

Microplate assay was conducted with citrate-anticoagulated plasma and included two parts: thrombogenesis and fibrinolysis assays. For the thrombogenesis assay that reflects fibrin clot formation, changes in the sample optical density were recorded for 30 min after addition of thrombin using a micro-titre plate reader. For the fibrinolysis assay, tissue plasminogen activator was added to the sample after thrombin- induced thrombus formation with fibrinolysis recorded by the plate reader as changes in the optical density.

The following indices were assessed for thrombogenesis: lag time (sec), rate of clot formation (units/s) and maximum optical density (units). Fibrinolysis indices included the rate of clot dissolution (units/s) and the Time for 50% of the clot to lyse from maximum to the plateau phase (Table 1 and Supplementary appendix).

*2.4. Enzyme-linked immunosorbent assay (ELISA)*

Commercial ELISA kits were used to measure concentrations of tissue type plasminogen activator (tPA, Human t-Plasminogen Activator/tPA Quantikine ELISA Kit, R&D systems, Abingdon, UK), plasminogen activator inhibitor 1 (PAI1, Human Serpin E1/PAI-1 Quantikine ELISA Kit, R&D systems, Abingdon, UK), and D-Dimer (D-Dimer 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).

*2.5 Microparticles*

Microparticles were quantified from platelet-poor plasma using Apogee A50 flow cytometer (Apogee Flow Systems, High Wycombe, UK). Plasma aliquots were frozen at − 70 ◦C for subsequent batch analysis. 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-R Phycoerythrin 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. (Supplementary appendix).

*2.6. Statistical analysis*

The study power focus on the extent to which the degree of anti- coagulation influences the methods outlined above. A sample size of 60 brings α < 0.02 and 1-β = 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. In the third (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.

Shapiro-Wilk test was used to assess data distribution. Normal data are presented as means and standard deviations, non-normal data are presented as medians and inter-quartile ranges. For comparisons of three 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. Analysis was conducted with IBM SPSS Statistics version 25 software and p value less than 0.05 was considered significant. Effect size measurement was re- ported along with p value where key differences were noted [Mean difference and 95% confidence intervals for ANOVA, epsilon2 (ε2) for Kruskall–Wallis test, eta2 (ƞ2) for Mann-Whitney U test, effect size r for Wilcoxon Signed Rank test and Cohen’s d for t-test].

**3. Results**

*3.1. Cross-sectional study of antithrombotic agents in AF*

There were no significant differences between the three study groups regarding demographic and clinical characteristics (Table 2). Thrombosis assessment of whole blood by TEG showed delayed thrombo- genesis in patients receiving apixaban compared with patients on warfarin or antiplatelets, based on K (p < 0.001, ε2 = 0.16) and angle [p < 0.001, mean difference (MD), 95% Confidence Intervals (CI) 5, 0.96–8.6 vs warfarin and 8, 3.8–11.4 vs antiplatelets] indices (Table 3). Patients on apixaban and warfarin had higher R index compared with patients on antiplatelets (p < 0.001, ε2 = 0.21). From microplate assay, the warfarin treatment group observed to have longer Lag time (p < 0.001, ε2 = 0.31), less dense thrombus [expressed as maximum optical density] (p < 0.001, ε2 = 0.1) and faster clot dissolution (p < 0.001, ε2 = 0.11) compared with patients on the other antithrombotics. Based on different indices, patients receiving apixaban had slower fibrin clot production (Rate of clot formation p = 0.005, ε2 = 0.41), with faster lysis time (Time 50% lysed index), compared patients on warfarin and anti- platelets (p < 0.001, ε2 = 0. MD 70, 95%CI 47.5–92 vs warfarin; MD 92, 95%CI 70–114). Apixaban group had lower levels of D-dimer compared with warfarin and antiplatelet group (p < 0.001, ε2 = 0.17) and t-PA (p = 0.03, MD 90, 95%CI 6–150 vs warfarin and MD 90, 95% CI 4–150 vs antiplatelets). There was no difference between the groups for the microparticle levels (p > 0.05 for all).

*3.2. Comparison of AF patients with patients in sinus rhythm*

Data of the participants from the second cross sectional study (patients in AF on antiplatelets vs patients with sinus rhythm and IHD on antiplatelets) are shown in Table 2. The 2 groups were well matched in respect of demographic and clinical characteristics. There were no sig- nificant differences in thromboelastography parameters between the two groups (Table 4). On the microplate assay, patients in AF had higher rate of clot formation (p = 0.027, ƞ2 = 0.06) and maximum optical density (p < 0.001, ƞ2 = 0.05) and slower rate of clot dissolution (p = 0.005, ƞ2 = 0.06). PAI-1 was lower in the group with AF (p = 0.01, ƞ2 = 0.08). Patients with AF had higher counts of apoptotic MPs compared with those in sinus rhythm (p = 0.023, ƞ2 = 0.06); the PMPs counts were similar in the two groups (p = 0.28).

*3.3. Effect of apixaban (follow up study)*

Clinical and baseline laboratory characteristics of participants of the follow up study are shown in Table 5. Most patients (78%) received 5 mg apixaban BID. Treatment with apixaban did not affect CRP levels and white cell count (p > 0.05). A small drop in haemoglobin (p = 0.01) and creatinine clearance (p = 0.001) noticed (Table 5). There was a prolongation of the R (p<0.001,r=0.5) and K (p=0.047,r=0.24) indices measured by TEG, suggesting attenuation of fibrin polymerisation followed initiation of Apixaban (Table 6). More pronounced changes were observed with microplate assay, where there was 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, r = 0.42 and 0.5 respectively) and rate of clot dissolution (p = 0.035, r = 0.26) and Time 50% lysed (p = 0.012, Cohen’s-d = 0.75) for fibrinolysis. Additionally, there was a significant reduction of D-dimer (p = 0.001, r = 0.4). There were no substantial changes in the levels of the microparticles.

**Discussion**

The principal findings of the study are: (i) patients with NVAF ap- pears to have elevated prothrombotic potential with higher levels of apoptotic microparticles, (ii) apixaban affects haemostasis in a different manner compare with warfarin and antiplatelets and (iii) reduces biomarker levels that are related with adverse cardiovascular events.

We found several similarities and differences between antithrombotics regarding their influence on the clot structure. Considering the methods we used, TEG evaluates the viscoelastic properties of unfractionated blood during clot formation and microplate assay is turbidimetry analysis of clot formation and lysis performed with plasma.

The values of thrombosis and lysis indices from both techniques suggest that oral anticoagulants are more potent in delaying thrombosis and amplifying clot lysis compared to antiplatelets. In microplate assay this is expected as the analysis is conducted in platelet poor plasma. From TEG, clot’s maximum amplitude appears similar among antithrombotics, reflecting the limitations of TEG for the assessment of the anticoagulant effect of VKAs [22].

The biochemical environment in vivo can affect the initial phase of the fibrin polymerisation [23]. In microplate assay under the absence of activated platelets, warfarin prolongs the lag time as inhibits multiple coagulation factors, including thrombin. This activity is not evident in TEG where R time (equivalent to lag time) appears to have similar duration for the anticoagulants (warfarin and apixaban). This finding may be explained by an antiplatelet effect of apixaban which has been described in other studies [24] and contributes to augmentation of apixaban’s antithrombotic activity in TEG.

The maximum absorbance of clot in microplate assay was higher in apixaban compare to warfarin, a difference which has not been seen in TEG (expressed as maximum amplitude), suggestive of less robust fibrin polymer architecture in warfarin anticoagulated plasma. A major difference between the 2 assays is that turbidimetry assesses coagulation potential, as the fibrin polymerisation process is initiated by exogenous thrombin and bypasses endogenous factors of the coagulation cascade. Maximum absorbance is mainly influenced by fibrinogen concentration, however there are other factors that may affect the maximum clot density as fiber thickness and clot stiffness [25]. Nevertheless, turbidimetry and clot density appears important for assessment of various pro-coagulant conditions [26].

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 [27–29]. Warfarin, overall, demonstrated higher anticoagulant activity compared with apixaban, as was more effective in reduction of the maximum thrombus density, despite that the two anticoagulants share similar characteristics in the clot augmentation and degradation phase. However, the desired role of anticoagulants is to prevent thrombosis but also to maintain an effective clot dynamic in order to avoid haemorrhage [30]. The main mortality benefit of apixaban against warfarin from the large clinical trials in patients with NVAF arose from the reduction of haemorrhagic events [4,31]. Stable thrombus is associated with less haemorrhagic episodes [32]. Slower clot formation and faster clot dissolution with a combination of minor effect on the overall thrombus stability may be important characteristics of an anticoagulant in order to treat prothrombotic conditions without disrupting the fine balance between thrombosis and haemorrhage.

Apixaban appears to affect all stages of thrombosis and fibrinolysis but does not affect the density of the fibrin clot based on the findings from the follow up and the cross sectional study. Research demonstrated changes in the clot robustness related with various diseases which car- rying increase risk of thromboembolism [27]. Several parameters have been assessed in research setting such as permeability, compaction and fiber thickness in order to express the clot robustness [33–35]. However, more robust clot is not always associated with acceleration of fibrin polymerisation or reduced fibrin polymer lysis [36]. The latter components of haemostasis appears to be equally important as regard the thrombotic tendency and outcomes that are related with multiple cardiovascular diseases [37,38].

In the apixaban treatment group was observed significantly lower levels of tPA and D-Dimer compared with the other antithrombotics. Elevated levels of both biomarkers are associated with worse outcomes in patients with cardiovascular diseases [39–41], suggesting high rates of fibrin degradation due to increased clot formation. In line with the findings of the cross sectional study are the results of the follow up study. The reduction of D-Dimer has been reported with Dabigatran as well and it is likely characteristic of DOACs [39].

The stability of the vasculature is dependent on the integrity of haemostasis which may affected by different pathophysiological mechanisms [42]. We used TEG and microplate assay to detect differences between the haemostatic status of NVAF and sinus rhythm. Comparison of coagulation and lysis indices showed that there is heterogeneity in the clot structure between the two conditions. Although analysis with TEG did not reach level of significance for any of thrombosis indices, data from microplate assay showed remarkable dissimilarities in haemostatic parameters between the two conditions. Microplate assay, in contrast with TEG, measures the overall haemostatic potential in a platelet poor plasma after initiation of the cascade by exogenous thrombin and tPA [26]. Our turbidity results showed that fibrin polymerises faster, pro- duces a more robust clot which dissolves slower in the plasma of patients with NVAF compared with those in sinus rhythm. Microplate assay have been used to detect differences on clot architecture between healthy individuals and patients with coronary artery disease [12]. The overall haemostasis potential measured by plasma turbidity methods may be a sensitive marker in hypofibrinolytic conditions in order to guide OAC [26].

Imbalance of plasma procoagulant molecules is well described in AF [1]. We demonstrated that Annexin-V + microparticle levels appears to be higher in AF compared with sinus rhythm. AF is associated with higher levels of thrombin and inflammatory cytokines [43,44]. Thrombin and interleukin-6 are known stimuli for generation of microparticles [45], fact that may explain the higher levels of phosphatidylserine positive microparticles in AF. The circulating negatively charged phosphatidylserine, electrostatically attracts the positive segment of clotting proteins and promotes thrombosis [21]. The high levels of plasminogen activator inhibitor-1 in IHD have been described [46].

In this analysis, we demonstrated that patients with NVAF have higher thrombogenic activity and elevated levels of apoptotic microparticles compared to patients with sinus rhythm. Apixaban ap- pears to influence the prothrombotic characteristics by different manner compare to warfarin and antiplatelets, including the reduction of D- Dimer. Assessment of haemostatic indices and clot structure characteristics in conjunction with efficacy and safety outcomes, such as mortality, ischaemic stroke and intracranial haemorrhage may support the utilisation of dynamic assays and clot structure assessment techniques as clinical tools. This may provide the potential to identify high risk patients for adverse cardiovascular events and tailoring OAC therapy.

*Limitations*

As an observational study this analysis carries a risk for confounding and 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 5 mg twice a day. We acknowledge potential effects of other medications and of other pathology (such as hyperlipidaemia). The groups with NVAF consisted of patients with permanent or paroxysmal AF and the dose of apixaban was 5 mg or 2.5 mg which may result in differences in haemostatic parameters. IHD patients in sinus rhythm, our “disease” control group, were receiving regular antiplatelet therapy which may influence thrombosis and lysis indices compared to healthy subjects. Assessment of the clot structure changes induced by antithrombotic drugs was performed in vitro. In vivo, clot structure may have different response to anticoagulants as other physiological or non- physiological factors may intervene.

**Conflict of interest:** GYHL reports acting as a Consultant for Bayer/Janssen, BMS/Pfizer, Medtronic, Boehringer Ingelheim, Novartis, Verseon and Daiichi-Sankyo; Speaker for Bayer, BMS/Pfizer, Medtronic, Boehringer Ingelheim, and Daiichi-Sankyo. No fees are directly received personally. ES and CV declare no competing interests.

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**References**

[1] A.A. Khan, G.Y.H. Lip, The prothrombotic state in atrial fibrillation: pathophysiological and management implications, Cardiovasc. Res. 115 (1) (2019 Jan 1) 31–45.

[2] K.G. Mann, Thrombin generation in hemorrhage control and vascular occlusion, Circulation. 124 (2) (2011 Jul 12) 225–235.

[3] R.G. Hart, L.A. Pearce, M.I. Aguilar, Meta-analysis: antithrombotic therapy to prevent stroke in patients who have nonvalvular atrial fibrillation, Ann. Intern. Med. 146 (12) (2007 Jun 19) 857–867.

[4] C.B.Granger,J.H.Alexander,J.J.V.McMurray,R.D.Lopes,E.M.Hylek,M.Hanna, et al., Apixaban versus warfarin in patients with atrial fibrillation, N. Engl. J. Med. 365 (11) (2011 Sep) 981–992.

[5] P.G.Tepper,J.Mardekian,C.Masseria,H.Phatak,S.Kamble,Y.Abdulsattar,etal., Real-world comparison of bleeding risks among non-valvular atrial fibrillation patients prescribed apixaban, dabigatran, or rivaroxaban, PLoS One 13 (11) (2018), e0205989.

[6] S. Kattula, J.R. Byrnes, A.S. Wolberg, Fibrinogen and fibrin in hemostasis and thrombosis, Arterioscler. Thromb. Vasc. Biol. 37 (3) (2017) e13–e21.

[7] M. Blomb ̈ack, S. He, N. Bark, H.N. Wallen, M. Elg, Effects on fibrin network porosity of anticoagulants with different modes of action and reversal by activated coagulation factor concentrate\*, Br. J. Haematol. 152 (6) (2011 Mar 1) 758–765.

[8] S. Williams, K. Fatah, P. Hjemdahl, M. Blomba ̈ck, Better increase in fibrin gel porosity by low dose than intermediate dose acetylsalicylic acid, Eur. Heart J. 19 (11) (1998 Nov) 1666–1672.

[9] S. Kitchen, E. Gray, I. Mackie, T. Baglin, M. Makris, Measurement of non-Coumarin anticoagulants and their effects on tests of Haemostasis: guidance from the British Committee for Standards in Haematology, Br. J. Haematol. 166 (6) (2014 Sep 1)

830–841.

[10] R.J. Luddington, Thrombelastography/thromboelastometry, Clin. Lab. Haematol.

27 (2) (2005 Apr) 81–90.

[11] P. Ranjit, Y. Lau, G.Y.H.Y.H. Lip, A.D.D. Blann, Development and validation of a

new assay for assessing clot integrity, Vasc. Pharmacol. 71 (2015 Aug) 102–107.

[12] M. Pieters, H. Philippou, A. Undas, Z. de Lange, D.C. Rijken, N.J. Mutch, et al., An

international study on the feasibility of a standardized combined plasma clot turbidity and lysis assay: communication from the SSC of the ISTH, J. Thromb. Haemost. 16 (5) (2018 May 15) 1007–1012.

[13] R.M. Hardisty, R.A. Hutton, The kaolin clotting time of platelet-rich plasma: a test of platelet Factor-3 availability, Br. J. Haematol. 11 (3) (1965 May 1) 258–268.

[14] A. Undas, K. Szułdrzynski, E. Stepien, J. Zalewski, J. Godlewski, W. Tracz, et al., Reduced clot permeability and susceptibility to lysis in patients with acute coronary syndrome: effects of inflammation and oxidative stress, Atherosclerosis. 196 (2) (2008 Feb) 551–557.

[15] Y.C. Lau, Q. Xiong, P. Ranjit, G.Y.H. Lip, A.D. Blann, Laboratory assessment of anti- thrombotic therapy in heart failure, atrial fibrillation and coronary artery disease: insights using thrombelastography and a micro-titre plate assay of thrombogenesis and fibrinolysis, J. Thromb. Thrombolysis 42 (2) (2016 Aug 4) 233–244.

[16] R.A.S. Ari ̈ens, Fibrin(ogen) and thrombotic disease, J. Thromb. Haemost. 11 (2013 Jun) 294–305.

[17] A. Boland, Y. Dundar, A. Bagust, A. Haycox, R. Hill, R. Mujica Mota, et al., Early thrombolysis for the treatment of acute myocardial infarction: a systematic review and economic evaluation, Health Technol. Assess. 7 (15) (2003 Jan) 1–136.

[18] D.E. Vaughan, PAI-1 and atherothrombosis, J. Thromb. Haemost. 3 (8) (2005 Aug) 1879–1883.

[19] J.I. Weitz, B. Leslie, J. Ginsberg, Soluble fibrin degradation products potentiate tissue plasminogen activator-induced fibrinogen proteolysis 87 (3) (1991 Mar 1).

[20] H.H. Versteeg, J.W.M. Heemskerk, M. Levi, P.H. Reitsma, New fundamentals in hemostasis, Physiol. Rev. 93 (1) (2013 Jan) 327–358.

[21] Voukalis C, Shantsila E, Lip GYH. Microparticles and cardiovascular diseases. Ann Med. 2019;51(3–4).

[22] B. Korpallova ́, M. Samoˇs, T. Bolek, I. Sˇkornˇova ́, F. Kov ́aˇr, P. Kubisz, et al., Role of Thromboelastography and rotational thromboelastometry in the management of cardiovascular diseases, Clin. Appl. Thromb. Hemost. 24 (8) (2018 Nov) 1199–1207.

[23] A.S. Wolberg, R.A. Campbell, Thrombin generation, fibrin clot formation and hemostasis, Transfus. Apher. Sci. 38 (1) (2008 Feb) 15–23.

[24] L. Pujadas-Mestres, I. Lopez-Vilchez, E. Arellano-Rodrigo, J.C. Reverter, A. Lopez- Farre, M. Diaz-Ricart, et al., Differential inhibitory action of apixaban on platelet and fibrin components of forming thrombi: studies with circulating blood and in a platelet-based model of thrombin generation, PLoS One 12 (2) (2017), e0171486.

[25] M. Pieters, M. Guthold, C.M. Nunes, Z. de Lange, Interpretation and validation of maximum absorbance data obtained from turbidimetry analysis of plasma clots, Thromb. Haemost. 120 (1) (2020) 44.

[26] A. Ilich, I. Bokarev, N.S. Key, Global assays of fibrinolysis, Int. J. Lab. Hematol. 39 (5) (2017 Oct 1) 441–447.

[27] A. Undas, R.A.S. Ari ̈ens, Fibrin clot structure and function: a role in the pathophysiology of arterial and venous thromboembolic diseases, Arterioscler. Thromb. Vasc. Biol. 31 (12) (2011 Dec) e88–e99.

[28] S.T. Lord, Molecular mechanisms affecting fibrin structure and stability, Arterioscler. Thromb. Vasc. Biol. 31 (3) (2011 Mar) 494–499.

[29] A. Undas, M. Kolarz, G. Kope ́c, W. Tracz, Altered fibrin clot properties in patients on long-term haemodialysis: relation to cardiovascular mortality, Nephrol. Dial. Transplant. 23 (6) (2008 Jun) 2010–2015.

[30] G.Y.H. Lip, D.A. Lane, Assessing bleeding risk in atrial fibrillation with the HAS- BLED and ORBIT scores: clinical application requires focus on the reversible bleeding risk factors, Eur. Heart J. 36 (46) (2015) 3258–3264.

[31] R.D. Lopes, G. Heizer, R. Aronson, A.N. Vora, T. Massaro, R. Mehran, et al., Antithrombotic therapy after acute coronary syndrome or PCI in atrial fibrillation, N. Engl. J. Med. 380 (16) (2019 Apr 18) 1509–1524.

[32] D.A. Gorog, Potentiation of thrombus instability: a contributory mechanism to the effectiveness of antithrombotic medications, J. Thromb. Thrombolysis 45 (4) (2018 May) 593–602.

[33] A. Undas, T. Nowakowski, M. Cie ́sla-Dul, J. Sadowski, Abnormal plasma fibrin clot characteristics are associated with worse clinical outcome in patients with peripheral arterial disease and thromboangiitis obliterans, Atherosclerosis. 215 (2) (2011 Apr 1) 481–486.

[34] K. Fatah, A. Hamsten, B. Blomb ̈ack, M. Blomba ̈ck, Fibrin gel network characteristics and coronary heart disease: relations to plasma fibrinogen concentration, acute phase protein, serum lipoproteins and coronary atherosclerosis, Thromb. Haemost. 68 (2) (1992 Aug 3) 130–135.

[35] K. Fatah, A. Silveira, P. Tornvall, F. Karpe, M. Blomb ̈ack, A. Hamsten, Proneness to formation of tight and rigid fibrin gel structures in men with myocardial infarction at a young age, Thromb. Haemost. 76 (4) (1996 Oct) 535–540.

[36] K. Schuett, A. Savvaidis, S. Maxeiner, K. Lysaja, V. Jankowski, S.H. Schirmer, et al., Clot structure: a potent mortality risk factor in patients on hemodialysis, J. Am. Soc. Nephrol. 28 (5) (2017 May) 1622–1630.

[37] A.H.C. Guimar ̃aes, E.L.E. de Bruijne, T. Lisman, D.W.J. Dippel, J.W. Deckers,

D. Poldermans, et al., Hypofibrinolysis is a risk factor for arterial thrombosis at young age, Br. J. Haematol. 145 (1) (2009 Apr) 115–120.

[38] M.E. Meltzer, T. Lisman, P.G. de Groot, J.C.M. Meijers, S. le Cessie, C.J.M. Doggen, et al., Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1, Blood. 116 (1) (2010 Jul 8) 113–121.

[39] A. Siegbahn, J. Oldgren, U. Andersson, M.D. Ezekowitz, P.A. Reilly, S.J. Connolly, et al., D-dimer and factor VIIa in atrial fibrillation – prognostic values for cardiovascular events and effects of anticoagulation therapy, Thromb. Haemost. 115 (05) (2016 Oct 26) 921–930.

[40] A.M. Carter, A.J. Catto, P.J. Grant, Determinants of tPA antigen and associations with coronary artery disease and acute cerebrovascular disease, Thromb. Haemost. 80 (4) (1998 Oct) 632–636.

[41] R. Halaby, C.J. Popma, A. Cohen, G. Chi, M.R. Zacarkim, G. Romero, et al., D-dimer elevation and adverse outcomes, J. Thromb. Thrombolysis 39 (1) (2015 Jan) 55–59.

[42] E. Previtali, P. Bucciarelli, S.M. Passamonti, I. Martinelli, Risk factors for venous and arterial thrombosis, Blood Transfus. 9 (2) (2011 Apr) 120–138.

[43] J.G. Akar, W. Jeske, D.J. Wilber, Acute onset human atrial fibrillation is associated with local cardiac platelet activation and endothelial dysfunction, J. Am. Coll. Cardiol. 51 (18) (2008 May 6) 1790–1793.

[44] D. Kaireviciute, A. D. Blann, B. Balakrishnan, D.A. Lane, J.V. Patel, G. Uzdavinys, et al., Characterisation and validity of inflammatory biomarkers in the prediction of post-operative atrial fibrillation in coronary artery disease patients, Thromb. Haemost. 104 (07) (2010 Nov 23) 122–127.

[45] P.-E. Rautou, A.-C. Vion, N. Amabile, G. Chironi, A. Simon, A. Tedgui, et al., Microparticles, vascular function, and atherothrombosis, Circ. Res. 109 (5) (2011 Aug 19) 593–606.

[46] R.G. Jung, P. Motazedian, F.D. Ramirez, T. Simard, P. Di Santo, S. Visintini, et al., Association between plasminogen activator inhibitor-1 and cardiovascular events: a systematic review and meta-analysis, Thromb. J. 16 (1) (2018 Dec 5) 12.

**Table 1. Indices of thromboelastography and microplate assay**

|  |  |  |
| --- | --- | --- |
| *Thromboelastography* | | |
| 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 (units) | Maximum amplitude of the clot dynamics, reflecting clot strength |
| *Microplate assay* | | |
| 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 |

**Table 2. Baseline clinical characteristics of patients in cross-sectional studies**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | Cross-sectional study of antithrombotic agents in AF | | | | Cross-sectional study of AF vs SR | | |
|  | Warfarin  (n=60) | Apixaban  (n=60) | Antiplatelets  (n=62) | p value | AF  (n=47) | SR  (n=39) | p value |
| Age (years) | 75 (9) | 77 (8) | 75 (12) | 0.39 | 73 (13) | 70 (10.5) | 0.06 |
| Female gender, n (%) | 50 (83%) | 43 (72%) | 31 (50%) | 0.09 | 32 (68%) | 49 (%) | 0.11 |
| Ethnicity, n (%)  *White*  *Asian*  *Afro-Caribbean* | 54 (%)  4 (%)  2 (%) | 57 (%)  2 (%)  1 (%) | 51 (%)  8 (%)  3 (%) | 0.26 | 38 (%)  8 (%)  1 (%) | 24 (%)  11 (%)  4 (%) | 0.09 |
| Ischaemic heart disease, n (%) | 12 (20%) | 8 (13%) | 12 (20%) | 0.22 | 0 | 39 (100%) | - |
| Diabetes, n (%) | 20 (33%) | 20 (33%) | 15 (24%) | 0.31 | 12 (26%) | 41 (16%) | 0.13 |
| Hypertension, n (%) | 51 (85%) | 53 (88%) | 54 (87%) | 0.73 | 43 (91%) | 36 (92%) | 0.89 |
| Heart failure, n (%) | 14 (23%) | 5 (8%) | 8 (14%) | 0.08 | 7 (15%) | 6 (15%) | 0.95 |
| Current smokers, n (%) | 1 (2%) | 5 (8%) | 3 (5%) | 0.23 | 2 (4%) | 3 (8%) | 0.50 |
| Systolic blood pressure (mmHg) | 136 (19) | 133 (17) | 131 (17) | 0.30 | 129 (17) | 137 (20) | 0.06 |
| Diastolic blood pressure (mmHg) | 74 (12) | 78 (12) | 76 (12) | 0.19 | 77 (13) | 74 (13) | 0.26 |
| Body mass index (kg/m2) | 29 (6) | 29 (5) | 27 (6) | 0.25 | 27 (6) | 28.2 (6) | 0.37 |
| Creatinine clearance (ml/min) | 71 (47-84) | 72 (45-88) | 68 (45-88) | 0.89 | 72 (46-93) | 79 (58-100) | 0.28 |

Values are shown as mean (SD) or median (interquartile intervals) unless stated otherwise

AF, atrial fibrillation; SR, sinus rhythm

**Table 3. Haemostatic parameters in patients with atrial fibrillation on different antithrombotic treatments**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Group | Warfarin  (VKA) | Apixaban (NOAC) | Antiplatelets  (APL) | p value | 95% Confidence Intervals of difference  or epsilon-squared |
| Index / Biomarker |
| *Thromboelastography* | | | | | |
| R(min) | 6.2 (5.3-7.7)b | 6.8 (5.7-7.8)b | 4.9 (4.1-6.0) | <0.001 | 0.21 |
| K(min) | 1.8 (1.3-2.1)a | 2.2 (1.8-2.9)b | 1.6 (1.2-1.9) | <0.001 | 0.16 |
| Angle (deg) | 64 (9)a | 59 (9)b | 67 (9) | <0.001 | VKA vs NOAC 0.96 - 8.6  APL vs NOAC 3.8 -11.4 |
| MA (mm) | 66 (10) | 66 (5) | 67 (6) | 0.5 | 0.007 |
| *Microplate assay* | | | | | |
| Lag time (sec) | 445 (396-580)a,b | 357 (279-459)b | 320 (260-354) | <0.001 | 0.31 |
| Rate of clot formation (units/sec) | 17 (12-23)a,b | 8 (7-20)b | 37 (20-42) | <0.001 | 0.41 |
| Max. optical density (units) | 0.42 (0.34-0.51)a,b | 0.55 (0.44-0.64) | 0.48 (0.42-0.58) | <0.001 | 0.1 |
| Rate of clot dissolution (units/sec) | 46 (37-59)a,b | 35 (28-45) | 35 (27-42) | <0.001 | 0.11 |
| Time 50% lysed (sec) | 207 (36)a,b | 137 (41)b | 229 (67) | <0.001 | VKA vs NOAC 47.5-92  APL-VKA 0.5 – 44.5  APL vs NOAC 70-114 |
| *Enzyme-linked immunosorbent assay* | | | | | |
| D-Dimer (ng/ml) | 180(102-343)a | 78(60-112)b | 156(78-284) | <0.001 | 0.17 |
| Tissue plasminogen activator (pg/ml) | 540 (180)a | 460 (130) | 540 (210) | 0.03 | VKA vs NOAC 6-150  APL vs NOAC 0.004-0.15 |
| Plasminogen activator inhibitor 1 (pg/ml) | 17.5 (9.9-26.6) | 16.3 (11.2-22) | 16.1(12.2-23.4) | 0.73 | 0.004 |
| *Microparticles* | | | | | |
| Platelet derived microparticles (103/ml) | 15 (4-63) | 19 (2-53) | 16 (4-69) | 0.98 | <0.001 |
| Apoptotic microparticles (103/ml) | 509 (235-1067) | 327 (158-1456) | 469 (293-1171) | 0.35 | 0.013 |

Values are shown as mean (standard deviation) or median (interquartile intervals); ap <0.05 vs. apixaban; bp <0.05 vs. antiplatelets

**Table 4.** **Haemostatic parameters in patients with atrial fibrillation (AF) and sinus rhythm**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Indices/Biomarkers | AF | Sinus Rhythm | p value | Effect size (ƞ2) |
| *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 | 0.06 |
| Max optical density (units) | 0.48 (0.42-0.56) | 0.38 (0.33-0.45) | <0.001 | 0.05 |
| Rate of clot dissolution  (units/sec) | 33 (22-42) | 42 (33-47) | 0.005 | 0.17 |
| Time 50% lysed (sec) | 224 (81) | 212 (42) | 0.42 |  |
| *Enzyme-linked immunosorbent assay* | | | |  |
| D-Dimer (ng/ml) | 156(78-285) | 155(91-280) | 0.87 |  |
| Tissue plasminogen activator (pg/ml) | 480 (420-620) | 540 (460-620) | 0.34 |  |
| Plasminogen activator inhibitor 1 (pg/ml) | 16.1 (11.9-23.3) | 20.3(14.4-38.9) | 0.01 | 0.08 |
| *Microparticles* | | | |  |
| Platelet derived microparticles (103/ml) | 20 (5-71) | 7 (3-47) | 0.28 |  |
| Apoptotic microparticles (103/ml) | 480 (290-1191) | 144 (54-933) | 0.02 | 0.06 |

Values are shown as mean (standard deviation) or median (interquartile intervals)

**Table 5. Demographic and clinic characteristics of follow up study population**

|  |  |
| --- | --- |
| Characteristics | Values |
| Age (years) | 78 (6.6) |
| Female gender, n (%) | 13 (31%) |
| Ethnicity, n (%) *White*  *Black*  *Asian* | 29 (90%)  2 (6%)  1 (3%) |
| 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/m2) | 29 (5) |
| Apixaban dose, n (%) 2.*5mg*  *5 mg* | 7 (22%)  25 (78%) |
| Atrial fibrillation type, n (%) *Permanent*  *Paroxysmal* | 17 (54%)  15 (46%) |
| C-reactive protein (mg/L) *Baseline*  *Follow up* | 3 (1-8)  4 (1-7)a |
| White cell count (x109/L) *Baseline*  *Follow up* | 7.2 (6.2-8.6)  7.6 (6.2-8.8) a |
| Creatinine clearance (ml/min) *Baseline*  *Follow up* | 76 (48-91)  71 (46-89)b |
| Haemoglobin (g/L) *Baseline*  *Follow up* | 133 (126-149)  131 (122-140)c |

Values are shown as mean (standard deviation) or median (interquartile intervals) unless stated otherwise

ap=0.07, bp=0.001, cp=0.01

**Table 6. Haemostatic parameters in patients with atrial fibrillation at baseline and after 3-month treatment with apixaban**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Indices/Biomarkers | Baseline | Follow up | p value | Effect size (r) |
| *Thromboelastography* | | | |  |
| R (min) | 5.3 (4.2-6) | 6.8 (5.7-7.8) | <0.001 | -0.5 |
| K (min) | 1.9 (1.5-3) | 2.4 (2-3.6) | 0.047 | -0.24 |
| 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 | -0.42 |
| Rate of clot formation (units/sec) | 16 (13-23) | 7 (6-9) | <0.001 | -0.5 |
| 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 | -0.26 |
| Time 50% lysed (sec) | 139 (33) | 115 (31) | 0.01 | -0.31 |
| *Enzyme-linked immunosorbent assay* | | | |  |
| D-Dimer (ng/ml) | 132(88.4-206) | 82 (56-125) | 0.001 | -0.4 |
| Tissue plasminogen activator (ng/ml) | 0.42 (0.07) | 0.45 (0.08) | 0.12 |  |
| Plasminogen activator inhibitor 1 (pg/ml) | 16.8 (13.5-20.2) | 14.1 (9.5-17.6) | 0.1 |  |
| *Microparticles* | | | |  |
| Platelet derived microparticles (103/ml) | 20.4 (4.2-50.5) | 27.6 (2.8-63.0) | 0.30 |  |
| Apoptotic microparticles (103/ml) | 359 (161-663) | 275 (163-629) | 0.23 |  |

Values are shown as mean (standard deviation) or median (interquartile intervals)

Supplementary Appendix

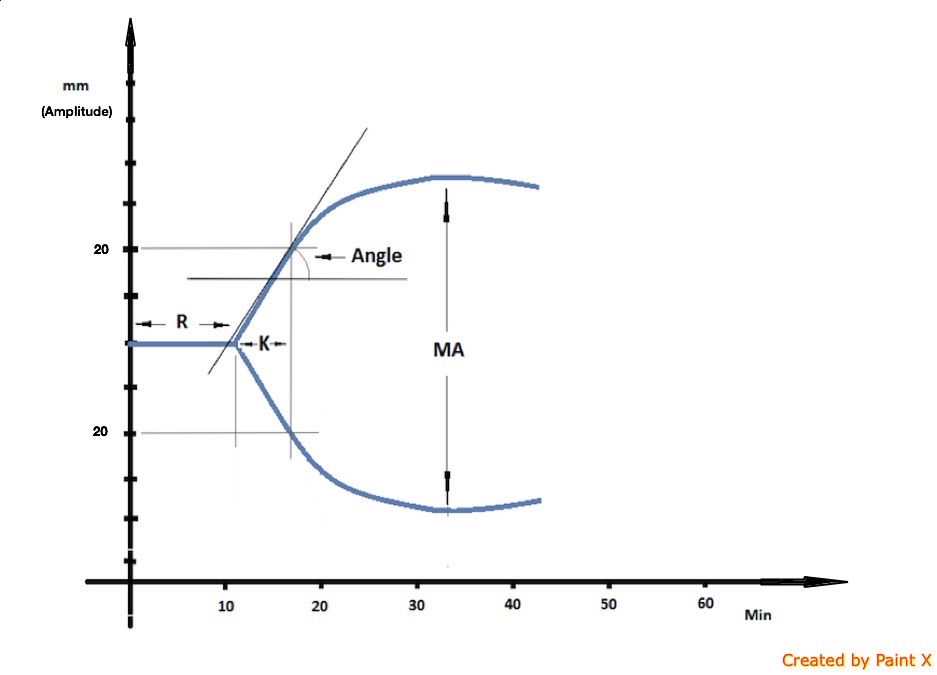
*Thromboelastography*

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 1). In the current study, citrated venous blood was analysed by TEG 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.

The following parameters were recorded (Figure 1):

* 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 1. Graphic representation of thrombus viscosity with time, based on TEG report



|  |  |
| --- | --- |
| R time (min):  K time (min)  Angle (degrees)  Maximum Amplitude [MA] (units) | Time from the initiation of the test until the point where the clot begins to form  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 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 of the clot dynamics, reflecting clot strength |

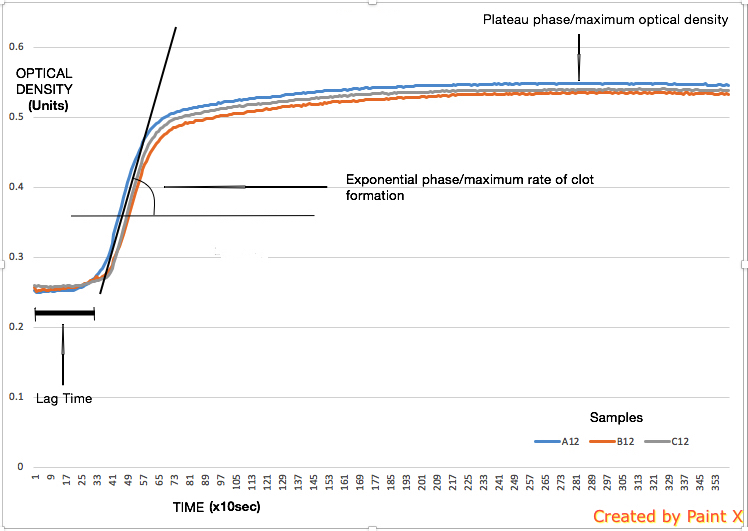
*Micro-plate assay*

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 0C and divided into two individual techniques.

The first technique is the thrombogenesis assay (Figure 2) 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 2) which reflects the fibrin clot formation. The different turbimetry variables were determined manually (after plotting the curves in MS Excel software). The determination was done in triplicates and for every index the mean value was estimated.

Figure 2. Microplate assay analysis of fibrin polymerisation (Triplicates of one sample)



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

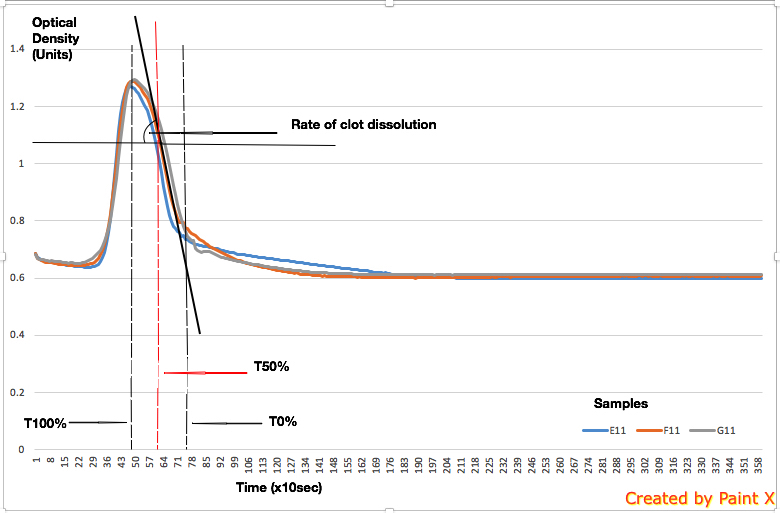
The second technique is the fibrinolysis assay (Figure 10). Each well in the micro-titre plate contains 75 μl of plasma and 75 μl of a Tris/NaCl/calcium buffer. After the addition of thrombin and tissue plasminogen activator the plate is immediately loaded into the plate reader. Optical density data are recorded for 30 min and similarly to the thrombogenesis, fibrinolysis assay can be plotted into a line chart (Figure 2 and 3).

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.

The variables above were determined manually (after plotting the curves in MS Excel software). The determination was done in triplicates and for every index the mean value was estimated.

Figure 3. Microplate assay fibrinolysis analysis



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

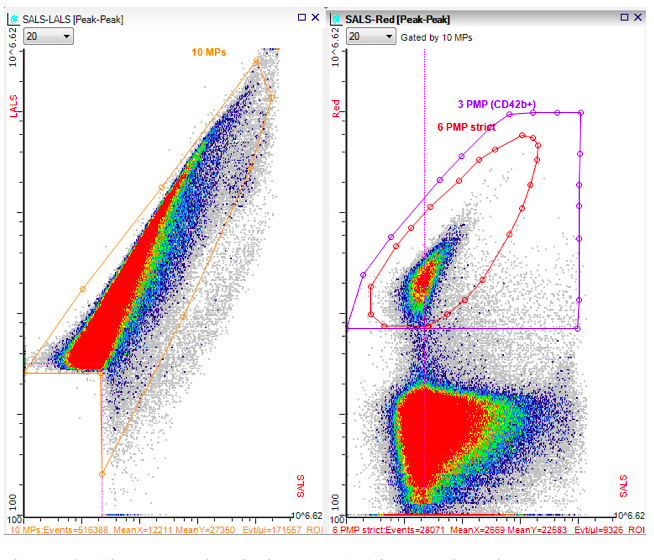
*Microparticles*

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 4.

Figure 4. 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.

*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.

Mean intra assay performed by processing the same sample for four times on the same day (Table 1). 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).

|  |  |  |  |
| --- | --- | --- | --- |
| Table 1. 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 |