

The effects of apixaban on clot characteristics in atrial fibrillation: A novel pharmacodynamic biomarker

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Abstract

Atrial fibrillation (AF) is a major risk factor for stroke. We aim to characterize AF patients and the effects of apixaban therapy in terms of clot microstructure using gel point analysis, a novel biomarker. Seventy-eight patients were included in the study, 50 Stroke with AF (AF-S), and 28 AF without stroke (AF). Pre- and post-anticoagulation samples were collected: gel point (GP) analysis was performed to obtain (i) TGP (the time taken to reach the GP or the clot formation time) and (ii) d_f , the fractal dimension of the clot, a quantification of clot fibrin microstructure at the GP. At baseline, the AF-S group had a $d_f = 1.70 (\pm 0.05)$ and TGP = 306 (± 73 s). The AF group had a $d_f = 1.70 \pm 0.05$ and TGP = 346 ± 78 s, showing a significantly shortened TGP in the stroke group ($p = .008$). For both groups, apixaban significantly prolonged TGP, $p = .005$, but resulted in no change in d_f . Apixaban prolonged clotting time while having no significant impact on the blood's ability to form stable clots (no change in d_f). This indicates that apixaban provides protection from the formation of thrombi by reducing clotting kinetics.

KEYWORDS

anticoagulation, apixaban, cerebrovascular disease, clot microstructure, gel point and fractal analysis

1 | INTRODUCTION

Stroke affects over 150 000 people in the UK each year and is the second leading cause of death worldwide.¹ Atrial fibrillation is a major stroke risk factor and current guidelines advocate the use of direct oral anticoagulants (DOACs), such as apixaban, to reduce this risk.²⁻⁴

Apixaban (1-(4-methoxyphenyl)-7-oxo-6(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]

pyridine-3-carboxamide) is a specific, direct, inhibitor of the trypsin-like serine protease, Factor Xa (FXa). Under physiological conditions, apixaban inhibits free and clot-bound FXa-mediated conversion of prothrombin into thrombin at nanomolar concentrations, directly reducing thrombus growth, through decreased thrombin formation; which also indirectly inhibits platelet aggregation.^{5,6} ARISTOTLE, a phase three randomized controlled trial, demonstrated reduced strokes, bleeding events, and overall mortality in patients with atrial

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fibrillation (AF) and one additional risk factor for stroke treated with apixaban 5 mg twice daily compared with warfarin, dosed to a target internationalized ratio (INR) of 2 to 3.⁷ Studies such as these have resulted in a significant increase in DOAC prescribing in the UK, from 9% in 2014 to 74% in 2019.⁸ Despite the advantages, bleeding risks, hemorrhage, and thromboembolic recurrence remain significant clinical issues in the long-term management of AF patients.⁹⁻¹¹ As a result, there is a need for new techniques to better address oral anticoagulation management of AF.

Previous studies have shown that the fibrin microstructure of a clot is of singular importance in the pathophysiology and outcome of bleeding and thromboembolic disease.^{12,13} The importance of clot microstructure stems from the organization of the fibrin network, which is critical in the development and characteristics of the clot.^{14,15} Recent studies have highlighted the potential of advanced viscoelastic techniques that can be used to measure the viscoelastic characteristics of the fibrin microstructure in clotting blood.¹⁶ This technique uses gel point, GP, analysis which provides a point of care, rapid assessment of viscoelastic and microstructural properties as blood clots, where the GP defines the transition from a viscoelastic liquid to a viscoelastic solid. GP analysis provides two measurable quantities of clotting characteristics (i) the time taken to reach the GP, TGP; and (ii) the fractal dimension of the clot, d_p , which is a quantification of the clot's fibrin microstructure. GP analysis has been investigated and validated in a number of acute vascular inflammatory disease states such as sepsis, myocardial infarction, and ischemic stroke.¹⁷⁻²¹

This study is designed to characterize the clotting characteristics of subjects with AF and their response to oral apixaban using GP analysis. The aims include: first to characterize the effect of apixaban on clotting characteristics and secondly to characterize clot characteristics in AF patients presenting with and without acute ischemic stroke.

2 | MATERIALS AND METHODS

2.1 | Study population

This was an observational study of ischemic stroke patients with untreated AF and newly diagnosed AF patients attending the anticoagulation and DOAC clinics, at a large teaching hospital in South Wales, UK. All strokes were diagnosed clinically, with and without radiological evidence of infarction, and diagnosed with AF using 12 lead ECG, ward-based cardiac monitoring, or previously documented history of AF. AF patients attending the anticoagulation and DOAC clinics were diagnosed using 12 lead ECG or determined by clinical general practice-based cardiac monitoring. All types of AF were included. The eligibility criteria included: (1) New patients diagnosed with AF and naive of anticoagulation treatment; (2) full informed consent; (3) over 18 years. The exclusion criteria included: (1) Taking anticoagulation drugs; (2) moderate or severe mitral stenosis, rheumatic mitral valve disease, or mechanical heart valve; (3) previous acute conditions: myocardial infarction, acute coronary events, stroke, or

having undergone a percutaneous coronary intervention within the last 30 days; (4) pre-planned invasive procedure; (5) pregnancy; (6) dual antiplatelet therapy; (7) active cancer; (8) participants currently undertaking a clinical trial of an investigational medicinal product (CTIMP) or who have completed one recently (8 weeks).

2.2 | Ethics

The study received ethical approval from Wales REC 5 (NHS) and Research Ethics Committee (REC Ref 17/WA/0236). The protocol was approved by Bristol-Myers Squibb and Pfizer (funder) and Swansea University (sponsor).

2.3 | Study design

Samples and data were collected at two time points. In the Stroke with AF (AF-S) group, a baseline sample was taken 24 h after hospital admission and before oral anticoagulation was commenced. We have shown previously in our ischemic stroke study that 24 h after admission the effects of thrombolysis or other initial treatment will have worn off and the GP parameters will have returned to baseline levels.¹⁶ In addition, we have also shown in our previous study on anti-platelets that monotherapy with a prophylactic dose of aspirin (75mg aspirin O.D.) does not have a significant effect on GP parameters.¹⁸ The second samples were taken 3-7 days after apixaban oral anticoagulation was initiated, the second sample was taken between 2 and 5 h after the patient had received their apixaban dose. In the AF without stroke (AF) group samples were taken before apixaban oral anticoagulation was commenced and a second sample was taken at 7-14 days after the patient had started their apixaban regime, the second sample was taken between 2 and 5 h after the patient had received their apixaban dose.

2.4 | Research variables

Each patient's demographics and past medical history were recorded, including: age, sex, and previous thrombotic events. Additionally, important routine clinical procedures and assessments as well as the CHA₂DS₂-VASc, HASBLED (for both groups), and NIH Stroke Scale/Score (NIHSS) were recorded.

2.5 | Study specific blood sampling

At each sampling point, a 22 ml sample of blood was obtained from the antecubital vein via an 18-gauge needle, with the first 2 mls being discarded. The 20 ml of blood was aliquoted into: 8 ml of whole blood used immediately for GP analysis; 12 ml was collected into vacuum-sealed tubes and used for the measurement of the fibrinolytic markers and laboratory markers (standard and specific).

2.6 | Standard laboratory markers

For each sample 4 ml aliquot of blood was used for full blood count analysis, samples were collected into plastic, full-draw dipotassium EDTA Vacuettes (Becton Dickinson, Plymouth, UK Ref: 367839). FBC was analyzed using a Sysmex XE 2100 (Sysmex UK, Milton Keynes, UK) automated hematology analyzer. 2.7ml of the blood was transferred into citrated siliconized glass Vacutainers (0.109 M) (Becton-Dickinson, Plymouth, UK Ref: 367691) and used for routine coagulation studies such as Prothrombin Time (PT), activated partial thromboplastin time (APTT), and Clauss fibrinogen, all measured using a Sysmex CA1500. All reagents were obtained from Siemens, (Frimley, UK). For DOAC patients, Anti-FXa chromogenic assays were carried out using Heparin LRT Kit (Biophen, UK) on a Sysmex CS5100 analyzer. D-dimer analysis was carried out using latex immune turbidimetric assay Hemosil HS d-dimer (Instrumentation Laboratory, Warrington, UK). The d-dimer assay was performed on an ACL TOP 500 (Instrumentation Laboratory, Warrington, UK).

2.7 | GP analysis

6.6ml aliquot of whole unadulterated venous blood was loaded into a double-gap concentric cylinder measuring geometry of a TA Instruments AR-G2 (TA Instruments, New Castle, DE, USA) controlled-stress rheometer (at $37\text{ }^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$) in a near-patient setting and tested immediately for GP analysis.¹⁶ Figure 1 shows the quantification of the GP. GP analysis is used to obtain (i) the time taken to reach the GP (the incipient clot formation time), TGP; (ii) the fractal dimension of the clot, d_f , which is a quantification of the clot's fibrin microstructure. df quantifies the clot microstructure of coagulating blood and shows the propensity of the person's blood to form a denser or looser clot. This is illustrated by the relationship between df and mass of the structure as shown in a previous publication using computational simulations of df and mass.^{17,21} Figure 2 shows a computational model of the relationship between df of a particular structure and the amount of mass contained within that structure. The model illustrates that small changes in df can result in large changes in clot mass and density.

2.8 | Statistical analysis

Statistical analysis was performed using GRAPHPAD PRISM® version 6.0 (GraphPad software Inc., La Jolla, CA, USA). Categorical variables were summarized using percentages and compared using chi-square tests between each of the groups. The normality of data distributions was assessed by normal probability plots and Shapiro-Wilk test of normality. For continuous variables, differences between groups either at baseline or following anticoagulation were assessed using a two-sample t-test. Any difference was assumed to

be significant at $p < .05$. Spearman's correlation analysis was performed to explore any associations between df , $\text{CHA}_2\text{DS}_2\text{VASc}$, prothrombin time (PT), activated partial thrombin time (APTT), fibrinogen concentration, and lipid profile. A correlation was assumed to be significant at $p < .05$.

3 | RESULTS

3.1 | Patient recruitment and demographics

A total of 120 patients in total were recruited into the study, this included 60 AF-S and 60 AF without stroke. A full flow diagram detailing recruitment can be found in Figure 3. In the AF-S group, 7 patients were excluded: 6 due to technical failure and 1 due to already being on anticoagulation therapy when the baseline sample was collected. Of the 53 AF-S patients 50 were prescribed apixaban. Three patients prescribed edoxaban were excluded from the analysis as they received a different anticoagulant and the numbers were too small to analyze them separately.

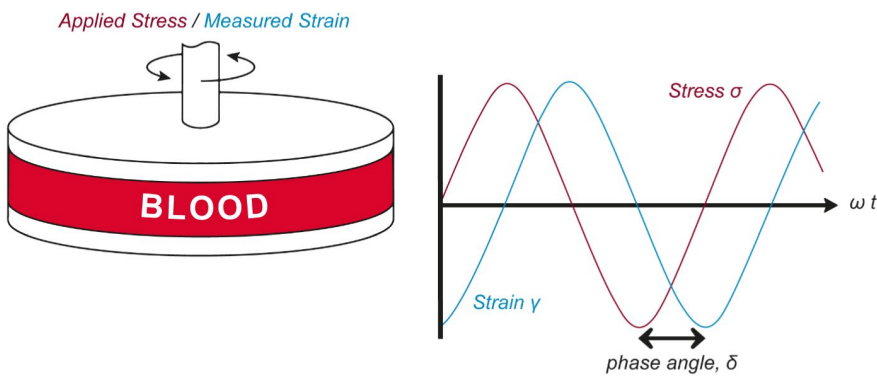
In the AF without stroke group 4 patients were excluded: 2 due to technical failure, 2 excluded as found to have taken anticoagulant before the first sample is taken. Of the 56 AF patients identified, 28 were prescribed apixaban. Twenty-seven were prescribed warfarin and one was given rivaroxaban, the rivaroxaban patient was excluded from the analysis due to the small sample number. Variation in anticoagulant agent prescribing was a result of individual clinician preference. A total of 78 patients were included in the analysis, 50 Stroke with AF (AF-S) and 28 AF without stroke (AF). All doses of apixaban received by patients in both groups were in accordance with the manufacturer's recommendations, a twice-daily dose of 5mg.

Demographics and patient data are presented in Table 1. The stroke with AF group were significantly older than the AF without stroke, $p = .001$, but there was no statistical difference in the ratio between males and females in the two groups. Further analysis showed that the GP parameters were not different when comparing those receiving antiplatelet monotherapy to those who were not. In the AF-S and AF groups, the change in df was 1.72 ± 0.05 to 1.72 ± 0.05 , $p = 0.4$, and 1.70 ± 0.04 to 1.70 ± 0.05 , $p = 0.9$, respectively. The change in TGP in the AF-S group was 340 ± 76 s to 324 ± 39 s, $p = .5$, and in the AF group, it was 339 ± 77 s to 371 ± 80 s, $p = 0.2$.

3.2 | Standard laboratory markers

The results of the standard laboratory markers can be seen in Table 2. The results show an increase in D-dimer in the AF-S group compared to the AF: AF-S group median =1214 units (IQR 624–1786); AF group median =477 units (IQR 329–995), $p = .01$. No other differences in the standard laboratory markers were observed between the baseline results for both groups. Analysis of the AF-S group for those

(A)



(B)

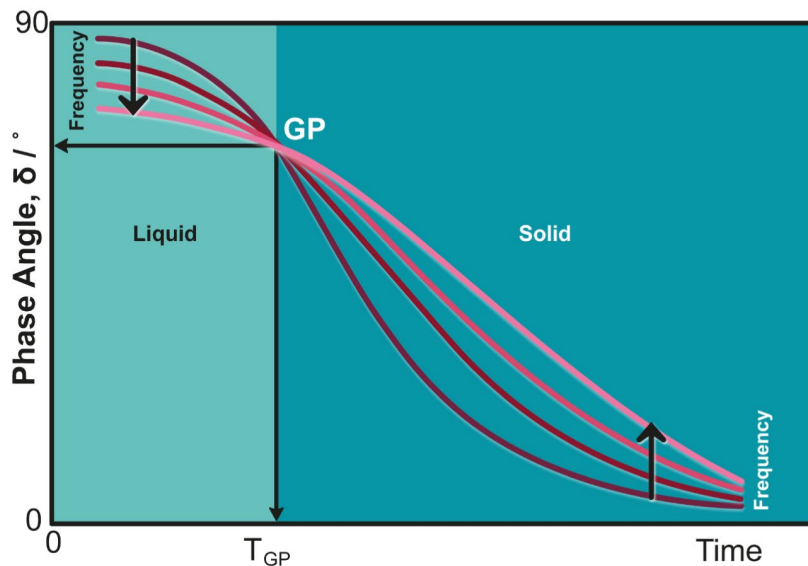


FIGURE 1 Gel point (GP) analysis: Figure 1A represents blood contained between two testing surfaces where small amplitude oscillatory stress measurements are performed and the resultant strain waveform is recorded. The differences in phase between the stress and strain waveforms are recorded as the phase angle. Figure 1B represents a typical gel point experiment result showing the change in phase angle for 4 different testing frequencies with respect to time. The GP is located where the 4 frequencies crossover. The GP defines the transition of the blood from a liquid to a solid and is the first point where a sample spanning a hematological stable clot is formed. The TGP (Time to GP) assesses the kinetic pathways during the liquid phase of clot formation and is a measure of clotting time. The phase angle at the GP is synonymous with clot structure and can be converted to fractal dimension, df

who received thrombolysis compared to those who did not show any significant differences (APTT thrombolysed vs. non thrombolysed: 23.1 ± 23 s vs. 23.3 ± 2.0 , $p = .8$ and Fibrinogen concentration thrombolysed vs. non thrombolysed: 3.8 g/L ± 1.0 vs. 3.2 ± 1.3 , $p = 0.06$). Post anticoagulation in the AF-S group only showed a significant change in APTT (from 23.3 ± 2.0 s to 26.2 ± 4.9 s, $p = .001$) post-anticoagulation. In the AF group, APTT was significantly prolonged in both the warfarin (from 23.3 ± 2.0 s to 26.2 ± 4.9 s, $p = .001$) and apixaban from (24.6 ± 2.2 s to 26.3 ± 1.9 s, $p = .02$) subgroups. In addition, in the warfarin group, significant reductions were observed in the platelet count ($245 \pm 46 \times 10^9$ vs. $190 \pm 86 \times 10^9$, $p = .02$) and hematocrit (0.42 ± 0.04 l/L vs. 0.39 ± 0.03 l/L, $p = .04$) post-anticoagulation. Conversely, no other significant changes were observed in the AF apixaban group post anticoagulation in terms of standard laboratory measures.

3.3 | Gel point analysis of atrial fibrillation

The results of the gel point analysis can be seen in Tables 2 and 3. Comparison of the baseline results shows no difference in terms of df , $p = .4$, between the AF-S and AF groups. A significantly shortened TGP is observed in the AF-S group, $p = .008$, compared to the AF group at baseline.

3.4 | Gel point analysis of oral anticoagulation

3.4.1 | AF-S group

The GP results for the pre- and post-anticoagulation with apixaban in the AF-S group show no change in df from 1.70 ± 0.05 to

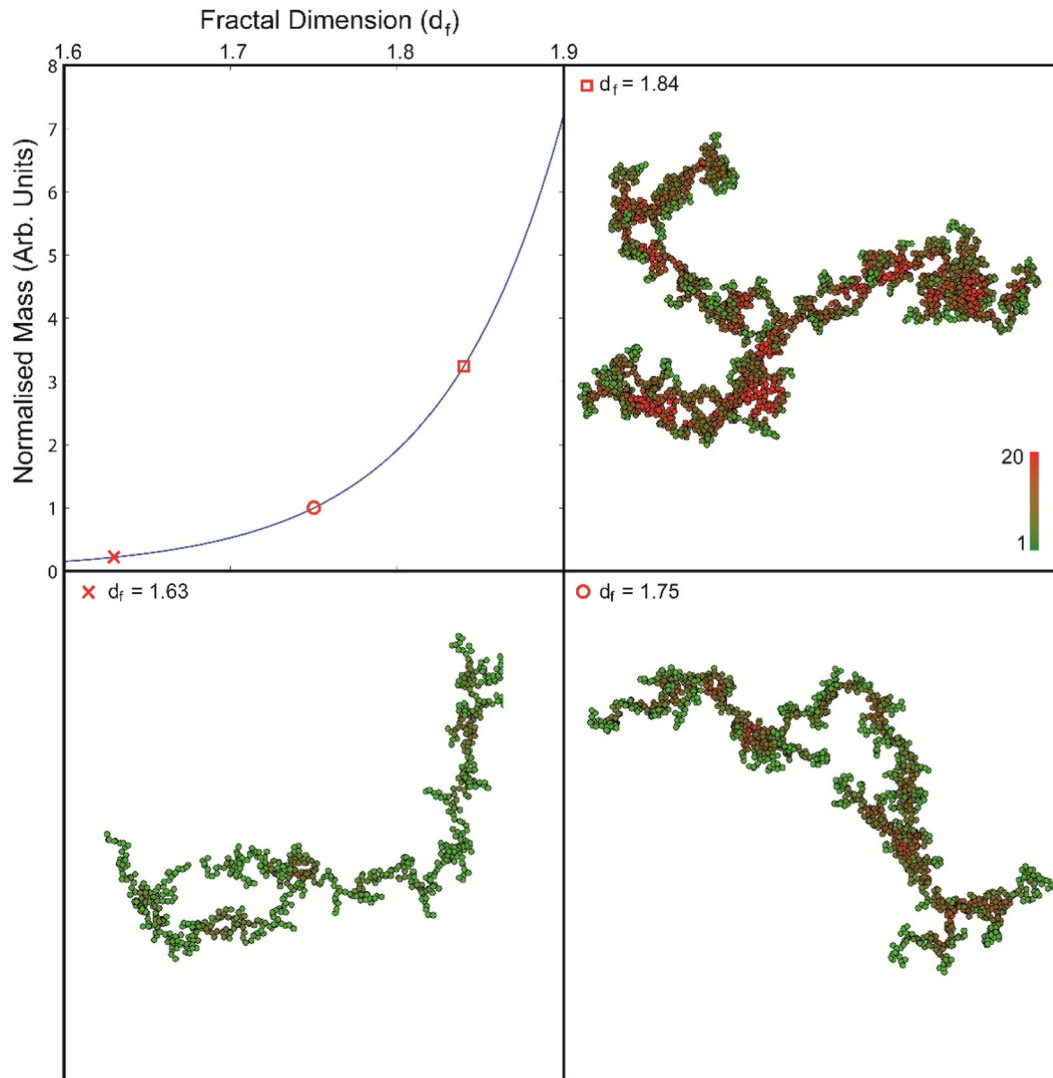


FIGURE 2 Graph of d_f vs. mass taken from MJ Lawrence et al, *Atherosclerosis*. 240 (2015) 402–407. Illustration of the non-linear relationship between d_f and the amount of mass, incorporated into the structure. Substantial increases in mass are required to generate small increments of d_f . The mass value on the y-axis is normalized for the healthy index value of d_f (≈ 1.74). Illustrations of different incipient clot microstructures at particular values of d_f are provided (cross ≈ 1.63 , circle ≈ 1.75 , and square ≈ 1.84 , respectively). The color of each node (unit sphere) within the fractal represents the local density of constituent nodes within a sphere of radii 5 units, the color ranges from green (1 neighboring node) to red (20 neighboring nodes). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

1.68 ± 0.03 , $p = .6$, and a significant prolongation of TGP from 306 ± 73 s to 386 ± 111 s, $p = .004$.

warfarin prolonged TGP, from 331 ± 69 s to 503 ± 113 s, $p < .001$, and reduced d_f from 1.70 ± 0.04 to 1.65 ± 0.04 , $p > .001$.

3.4.2 | AF group

The GP results for the pre- and post-anticoagulation in the AF group show a significant decrease in d_f from 1.70 ± 0.05 to 1.67 ± 0.05 , $p = .04$, and a significant prolongation of TGP from 346 ± 78 s to 458 ± 88 s, $p = .001$. However, in this group two types of anticoagulant were prescribed for the patient's long-term anticoagulant treatment apixaban ($n=28$) and warfarin ($n=27$). Apixaban significantly prolonged TGP from 360 ± 84 s to 432 ± 67 s, $p = .004$, but did not reduce d_f from 1.70 ± 0.05 to 1.67 ± 0.05 , $p = .3$. In comparison,

3.5 | Gel point analysis and correlation analysis

In the AF-S group, a negative correlation between TGP and D-Dimer, $r = -.39$; $p < .05$ was observed. In addition, significant positive correlations were observed between d_f and non HDL Cholesterol values, $r = .375$; $p < .05$, and d_f and LDL cholesterol, $r = .477$; $p < .005$.

In the AF without stroke, a significant negative correlation was observed between TGP and d_f , $r = -.39$; $p < .01$. In addition, d_f was positively correlated with: hemoglobin concentration, $r = .35$; $p < .05$, red blood count, $r = .41$; $p < .05$, and hematocrit, $r = .35$;

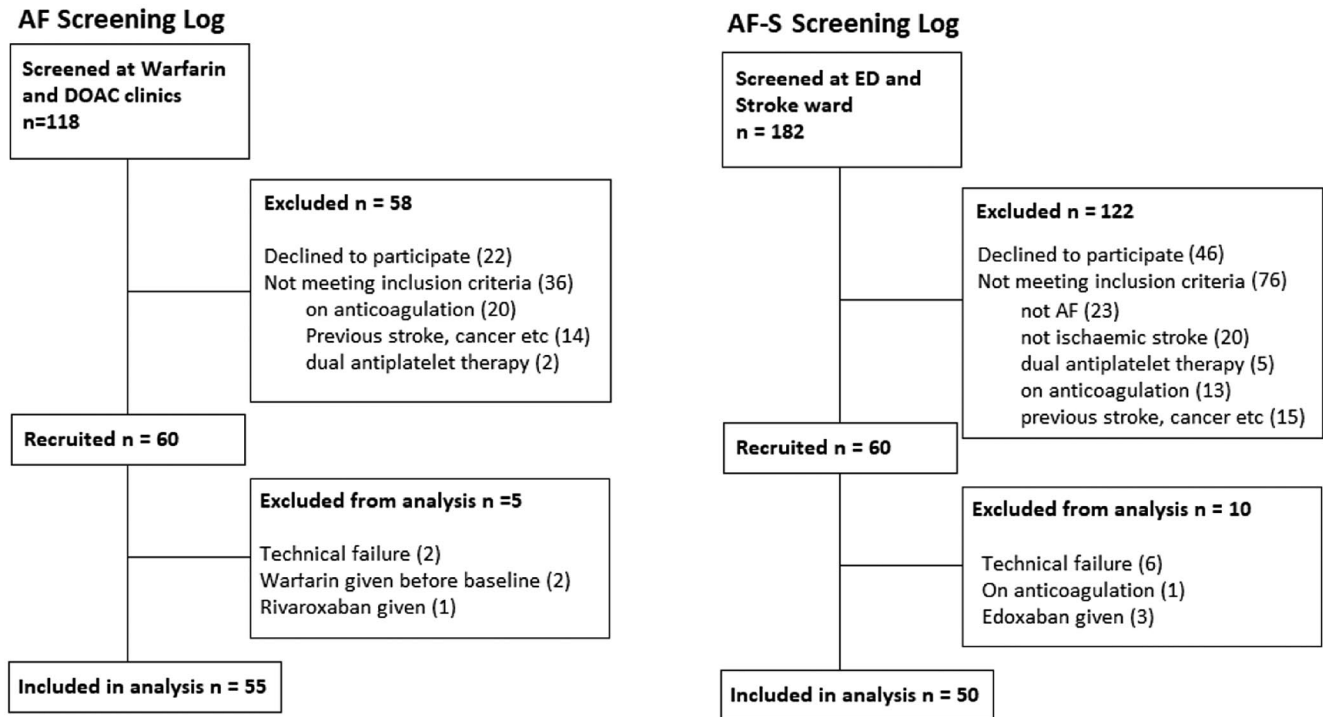


FIGURE 3 Screening log for the AF-S and AF without stroke patient groups

TABLE 1 Table of demographics and co-morbidities

	AF-S	AF without stroke	Sig. value
N	50	55	
Sex M/F	28:22	32:23	0.8
Age (years \pm SD)	78.9 \pm 10.1	73.4 \pm 8.8	0.004
BMI (\pm SD)	29.3 \pm 5.5	31.3 \pm 7.0	0.3
Comorbidities			
Hypertension	24/50	28/55	0.7
Diabetes (T2DM)	9/50	8/55	0.6
Congestive Heart Failure	2/50	4/55	0.4
Previous Stroke or TIA	5/50	5/55	0.9
Drug history			
Anti platelet medication	7/50 8 Aspirin	11/55 8Aspirin 3 Clopidogrel	0.4
Scores			
CHA2DS2-VASc	2.9 \pm 1.2	2.6 \pm 1.1	0.18
HASBLED	1.8 \pm 0.9	1.5 \pm 0.8	0.16
NIHSS on Arrival	10.7 \pm 8.9	N/A	N/A
Stroke type and treatment			
Cardioembolic	48/50*	N/A	N/A
Thromboylzed	19/50 [†]	N/A	N/A

Note: *2of uncertain diagnosis of stroke type.

[†]Those not thrombolized were treated as per NICE guidelines⁸

$p < .05$. Fibrinogen concentration was found to correlate with TGP in this group, $r = .315$; $p < .05$. No correlations between the anti-FXa test, a marker of apixaban concentration, were observed for TGP, $r = .047$; $p = 0.7$, or df , $r = -.113$; $p = .4$. Analysis of INR and drug dosage did not reveal any significant differences. We analyzed the TGP and df results to observe any correlation between them and CHA_2DS_2-VASc , TGP: $r = -.113$; $p = .4$ and df : $r = -1.51$; $p = .3$, and HASBLED, TGP: $r = -.158$; $p = 0.2$ and df : $r = -.031$; $p = .8$), however, we found no correlation in either of the study groups.

4 | DISCUSSION

4.1 | GP profile in AF and stroke

In the present study, patients with AF, both with and without stroke, produced mean GP, and df profiles were similar to those previously observed, where in previous studies, the ranges in healthy participants were 253 to 351 s and 1.68 to 1.78 for TGP and df , respectively.¹⁶ While the AF-S group was found to clot significantly quicker (reduced TGP) than the AF without stroke group; the TGP results were still within the range observed previously in healthy controls.¹⁶ Therefore, in this study, we identify that patients with AF, with or without the complication of ischemic stroke, do not appear to have a systemic pro-coagulant condition, in terms of their clotting kinetics or clot microstructural properties.

AF-related thrombus formation is stated to be caused by several mechanisms including: blood stasis, endothelial damage, and coagulation properties, all three of the components of Virchow's Triad.²² Blood stasis and altered flow in the left atrium, particularly in the blind-pouch left atrial appendage, are recognized as the major driving force behind stroke caused by AF.²³ In addition, atrial endothelial dysfunction in AF caused by several mechanisms such as nitric oxide production, upregulated prothrombotic plasminogen activator inhibitor-1, and downregulation of thrombomodulin and tissue factor pathway inhibitor is also indicated as an important contributor to AF induced stroke.²⁴ Abnormal coagulation processes, causing a potential prothrombotic state, have also been suggested to be involved²⁵ where raised levels of von Willebrand, D-dimer, and fibrinogen concentration have been observed.^{25,26} Only D-Dimer was found to be significantly different in this study, however, this did not result in elevated values of either TGP or *df* measured by the GP analysis. It was previously shown that changes in the concentration of factors such as fibrinogen are not

always related to how they are functionally incorporated into the fibrin microstructure.¹⁶

It has previously been shown that the physiological makeup of clots formed through arteriosclerotic plaques are functionally different from those formed through venous flow-related activation.^{27,28} Arteriosclerotic clots are formed rapidly and primarily consist of a dense fibrin microstructure rich in platelets, often termed "white" clots.^{27,28} The process of these clots is primarily caused by damage at the vessel wall and the release of tissue factors causing clot formation at the site of injury. Clots formed through activation brought about by restricted venous flow, blood pooling, or stasis, such as in AF and DVT, result in "red" clots which are largely populated by red blood cells within the fibrin network.^{27,28} However a recent study involving localized measurements of clot microstructure formation from the left atrium appendage showed clots with high fibrin density compared to those formed from peripheral blood.²⁹ This was in contrast to the results found in the present study for *df*, for AF patients, which was within the normal range. This can be explained by the fact that the *df* measures the systemic effect in the blood rather than the localized effect in the left atrium appendage. In a recent study, investigating the role of anticoagulants in deep vein thrombosis using GP analysis, similar results were observed in terms of TGP and *df* at baseline to those seen in the present study,³⁰ the role of stasis and altered flow requiring prolongation of clotting time being an important therapeutic goal in the management of low flow venous thrombi and AF.

TABLE 2 Hematological and coagulation biomarkers for the AF-S and AF without stroke at baseline. Values reported as mean \pm Standard Deviation. *indicates significance

Coagulation markers	AF-S	AF	Significance value
<i>df</i>	1.70 \pm 0.05	1.70 \pm 0.05	0.4
TGP (sec)	306 \pm 73	346 \pm 78	0.008
Hemoglobin (g/L)	136 \pm 20	137 \pm 19	0.6
Platelets ($\times 10^9$ /L)	251 \pm 66	243 \pm 52	0.5
Hematocrit (l/l)	0.41 \pm 0.05	0.42 \pm 0.05	0.7
PT (s)	11.7 \pm 1.0	11.8 \pm 1.6	0.8
APTT (s)	23.3 \pm 2.1	24.6 \pm 2.5	0.001
Fibrinogen (g/l)	3.6 \pm 1.2	3.4 \pm 0.8	0.4
D-Dimer (units) [§]	1214(IQR 624-1786)	477(IQR 329-995)	0.01
HDL (units)	1.6 \pm 0.6	N/A	N/A
LDL (units)	2.3 \pm 0.8	N/A	N/A

Note: [§]data presented in the median and interquartile range

4.2 | GP profile and anticoagulation

GP analysis of the AF with stroke group demonstrates that apixaban increased TGP from 235 \pm 66s to 410 \pm 105 s, $p < .05$, but did not significantly change *df*. In addition, the change in the GP profile in the AF without stroke group showed the TGP also increased from 300 \pm 92 to 395 \pm 100 s, $p = 0.009$, with no significant reduction in *df*.

The effect of apixaban on gel point analysis was consistent in both groups and the *df* results are within the previously documented healthy range.^{16,18} The study shows that apixaban slows clotting activation (increased TGP), however, not at the expense of significantly modifying clot microstructural properties measured by *df*. This supports the findings in a recently published study investigating

TABLE 3 Viscoelastic, hematological, and coagulation biomarkers for AF-S and AF without stroke pre- and post-anticoagulation with apixaban. Values reported as mean \pm Standard Deviation. Significance differences determined using independent t-tests. *indicates significance

	AF-S			AF		
	Baseline	Post apixaban	<i>p</i> -value	Baseline	Post apixaban	<i>p</i> -value
<i>df</i>	1.70 \pm 0.05	1.68 \pm 0.03	0.06	1.70 \pm 0.05	1.67 \pm 0.05	0.3
TGP (sec)	306 \pm 73	386 \pm 111	0.004*	360 \pm 84	432 \pm 67	0.004*
Anti FXa (units)	N/A	183 \pm 88		N/A	161 \pm 59	

the use of another DOAC, rivaroxaban, in the management of DVT patients.³⁰ In the DVT study, GP analysis showed that rivaroxaban prolonged TGP but did not significantly alter *df*. We have previously described that high values of *df* (outside the normal healthy range $df < 1.78$) are associated with thromboembolic disease and relate to dense tightly packed fibrin networks within the clot.^{17,18} In contrast, we have also shown that low values of *df*, outside the normal healthy range ($df > 1.68$), are associated with poor hemostatic function with the fibrin network in the clot being loose and sparsely populated and potentially linked to bleeding risk.^{16,20}

A previous study investigating the effect of unfractionated heparin, UFH, in healthy volunteer blood demonstrated a UFH concentration-dependent effect on GP analysis¹⁶ where the higher the drug concentration of UFH the larger the prolongation of TGP and reduction in *df* observed.¹⁶ In this study, a significant correlation was observed between drug concentration and TGP and *df*.

It is possible that a relationship may exist between apixaban plasma concentration and GP analysis. We were, however, unable to detect a relationship between GP analysis and anti-FXa, which correlates linearly with plasma apixaban concentrations over 0 to 400 ng/ml, potentially due to the degree of variation between apixaban concentrations at steady-state and the variation in anti-FXa activity. All patients in our study received a fixed dose of apixaban, 5 mg BD. This dose typically produces a steady-state trough concentration of 103 ng/ml (41–230 ng/ml), with a peak concentration of 171 ng/ml (91–321 ng/ml), in AF patients after three days; Anti-FXa varies between 1.5 IU/ml (0.61–3.4 IU/ml) and 2.6 IU/ml (1.4–4.8 IU/ml) over these apixaban concentrations *in vivo*.³¹

The finding that anti-Xa did not correlate with GP biomarkers suggests that the use of this biomarker as a surrogate for drug concentration measurement is not only difficult to interpret and may not adequately reflect its effects. It is possible that a relationship may exist between apixaban plasma concentration and GP analysis. We were, however, unable to detect a relationship between GP analysis and anti-FXa, which correlates linearly with plasma apixaban concentrations over 0 to 400 ng/ml, potentially due to the degree of variation between apixaban concentrations at steady-state and the variation in anti-FXa activity. All patients in our study received a fixed dose of apixaban, 5 mg BD. This dose typically produces a steady-state trough concentration of 103 ng/ml (41–230 ng/ml), with a peak concentration of 171 ng/ml (91–321 ng/ml), in AF patients after three days; Anti-FXa varies between 1.5 IU/ml (0.61–3.4 IU/ml) and 2.6 IU/ml (1.4–4.8 IU/ml) over these apixaban concentrations *in vivo*,³⁰ therefore, estimating the plasma apixaban concentration from anti-FXa concentration is likely to be influenced by the time-interval between apixaban dosing and blood draw.

Whereas GP analysis provides a functional measurement of the physiological and therapeutic effect in the body. The action of increasing TGP, or the time it takes for a clot to form, could potentially be the most important parameter in reducing the risk of thromboembolic events. As previously described, the physiology of thrombosis in AF, while multifactorial and complicated, can be explained

by the disruption of blood flow in the upper chambers of the heart. Therefore, the action of reducing clotting kinetics and prolonging clot formation time would logically be an important therapeutic goal in AF management.

Despite the reduction in the use of warfarin to treat AF in the UK, it is still used sparingly. In this observational study, we recruited a sub-group of patients who were given warfarin as part of their standard care in the AF without stroke group. We found that warfarin increased TGP and, unlike apixaban, also decreased *df*. In both oral anticoagulant agents, the clotting time is prolonged significantly, however, *df* remained stable and within the normal healthy range (1.67–1.78) for patients receiving apixaban, indicating the patients retain the ability to form viable clots.

In contrast, warfarin patients show a significant reduction in *df* that falls below the lower limit of the normal healthy range. Previous studies investigating changes in clot microstructure have shown that a low value of *df*, indicates that the ability of the blood to form a stable clot is reduced,^{16,20} where the clot microstructures being formed are mechanically weak, less dense, and more dispersed resulting in a higher chance of bleeding as the clot is not stable. The effect of warfarin reduces the *df* well below the normal healthy range for *df* placing it in a range that would indicate poor hemostatic viability, suggesting a potential for increased risk of bleeding. The result of this study comparing warfarin with apixaban supports the observation in clinical trials that, while both anticoagulants will protect against thrombotic events by increasing gel time, warfarin produced a clotting profile that is more at risk of causing major bleeding. The rationale for this stems from the fact that in both groups, AF and stroke and AF, a normal systemic coagulation profile was observed, with the TGP and *df* in the normal age-controlled range.

4.3 | Demographics factors

Analysis of risk factors and demographic data of the two patient groups show the two groups have similar proportions of major risk factors not including age, where the AF-S group is significantly older (see Table 1). CHA2DS2-VASc is the most popular scoring system used to assess the risk of stroke in AF patients and identifies age, sex, past medical history of congestive heart failure, stroke, transient ischemic attack, thromboembolism, hypertension, or vascular disease as major risk factors for stroke in AF patients.³² We found no correlation between the GP and *df* markers and CHA2DS2-VASc score, not surprising as GP and *df* did not identify a procoagulant response in the blood. However, *df* for females, a risk factor in the CHA2DS2-VASc score, was higher than in males. In most previous studies, it was generally found that *df* is higher in males.¹⁸⁻²⁰

4.4 | Standard laboratory markers

In the present study, the standard laboratory markers did identify significant increases in the values of fibrinogen and d-dimer in both

groups when compared to the healthy index. The role of local inflammatory or Systemic inflammatory response (SIRS) factors cannot be ruled out in the AF and stroke group, as white blood count was also significantly raised (white blood count AF-S = 9.4 ± 3.0 cells $\times 10^9$ /L vs. AF = 7.0 ± 1.6 cells $\times 10^9$ /L, $p = .001$). This is likely linked to neuropathic cell death caused by the insult of the thrombus in the brain. However, as previously stated this did not translate to a significantly systemic pro-coagulant GP and *df* profile, or a systemic pro-coagulant response in the other standard coagulation markers (such as PT or APTT) results (see Table 2). This study would support the rationale that dysfunction of proper flow and endothelial properties are the driving force of thrombus formation in AF, although it cannot rule out localized pro-coagulant effects within the left atrium as a potential cause of thrombus formation. The variability of cardiometabolic factors has been suggested as the risk factors for cardiovascular disease and mortality. Compared to healthy controls, patients with AF had lower blood lipid levels, especially LDL-c and HDL-c levels. In addition, we found significant positive correlations between *df* and non-HDL Cholesterol values and a very strong positive correlation between *df* and LDL cholesterol. Previous studies have indicated that hypolipoproteinaemia may increase a patient's susceptibility to develop AF.³³ This seems to be mirrored in the association with an increase in *df* observed in this study.

4.5 | Limitations and future direction

Differences were observed in key demographic and physiological categories between the AF and stroke with AF groups. This limits any conclusions which are drawn from direct comparisons between the different patient groups. This does not include comparisons between baseline and post anticoagulation results in both groups, however, limitations did arise in that not all post anticoagulation samples were able to be completed and there were no warfarin patients in the AF-S group. An important factor is that nearly 25% of patients suffering a recurrent stroke are already on anticoagulant or antiplatelet therapy. These patients suffer either a thromboembolic or hemorrhagic event indicative of therapeutic failure. This may indicate that the patients are insufficiently anticoagulated for thromboembolism or the effects of anticoagulation have contributed to a cerebral hemorrhage. Furthermore, at their time of presentation, there is no reliable test, which can be carried out to determine whether they are over- or under-coagulated. The findings of this observational study for the first time show that there may be the possibility that a test will be able to assess therapeutic efficacy in such patients. Further follow-up data from larger cohorts are required to determine the stability of measurements over time and their relationship to clinical outcome data.

5 | CONCLUSION

In conclusion, the present study has shown that in terms of GP analysis, both the stroke with AF and stroke without AF groups did not display

systemic hypercoagulable clotting characteristics. Furthermore, the study indicated that apixaban prolonged clotting time (increased TGP) while having no significant impact on the blood's ability to form stable clots (no change in *df*). This indicates that apixaban provides protection from the formation of thrombi in the left atrium by reducing clotting kinetics and thus reducing the risks caused by abnormal flow and stasis during AF. We would argue that the blood in AF is not systemically hypercoagulable as measured by GP. There may be localized hypercoagulability in the left atrium due to stasis and contact activation, but we hypothesize this is a product of the abnormal physiology of the heart and not an underlying hypercoagulable condition of the blood. Future studies are required to explore comparisons of different DOACs and also whether these changes correspond to clinical outcome measures for recurrent stroke, thromboembolic events, bleeding, or hemorrhagic events and death.

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DISCLOSURE

None declared.

PRINCIPAL INVESTIGATOR STATEMENT

The authors confirm that the Principal Investigator for this paper is Professor Phillip Adrian Evans and that he had direct clinical responsibility for patients.

ETHICAL APPROVAL

The study received ethical approval from Wales REC 5 (NHS) and Research Ethics Committee (REC Ref 17/WA/0236). The protocol was approved by Bristol-Myers Squibb and Pfizer (funder) and Swansea University (sponsor).

AUTHOR CONTRIBUTIONS

MJL: Study design and data analysis, patient recruitment, data collection, drafting of the article; VE and JW: Patient recruitment, data collection and analysis; SP, PRW, JC, MK, PS, KP: Revising the article for scientific and intellectual content; RHKM: Statistical analysis and interpretation of the data. PAE: Idea initiation, study design and data analysis, final approval of the version to be published. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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