Platelet reactivity influences clot structure as assessed by fractal analysis of viscoelastic properties

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Abstract

Despite the interwoven nature of platelet activation and the coagulation system in thrombosis few studies relate both analysis of protein and cellular parts of coagulation in the same population. In the present study, we use matched *ex vivo* samples to determine the influences of standard anti-platelet therapies on platelet function and use advanced rheological analyses to assess clot formation. Healthy volunteers were recruited following fully informed consent then treated for 7 days with single anti-platelet therapy of aspirin (75 mg) or prasugrel (10 mg) or with dual anti-platelet therapy (DAPT) using aspirin (75 mg) plus prasugrel (10 mg) or aspirin (75mg) plus ticagrelor (90mg). Blood samples were taken at day 0 before treatment and at day 7 following treatment. We found that aspirin plus prasugrel or aspirin plus ticagrelor inhibited platelet responses to multiple agonists and reduced P-selectin expression. Significant platelet inhibition was coupled with a reduction in fractal dimension corresponding to reductions in mean relative mass both for aspirin plus prasugrel (-35±16% change, p=0.04) and for aspirin plus ticagrelor (-45±14% change, p=0.04). Aspirin alone had no effect upon measures of clot structure whereas prasugrel reduced fractal dimension and mean relative mass. These data demonstrate that platelets are important determinants of clot structure as assessed by fractal dimension (d_f) and that effective platelet inhibition is associated with a weaker, more permeable fibrin network. This indicates a strong association between the therapeutic benefits of anti-platelet therapies and their abilities to reduce thrombus density that may be useful in individual patients to determine the functional relationship between platelet reactivity, eventual clot quality and clinical outcome. d_f could represent a novel risk stratification biomarker useful in individualising anti-platelet therapies.

Introduction

Development of the platelet-rich fibrin clot at sites of atherosclerotic plaque rupture is central to atherothrombotic disease [1]. Platelets are critical to the processes of atherothrombosis, driving thrombus formation through a cascade of complex intracellular signalling pathways and powerful positive feedback loops [2, 3]. Platelets also have a central role in cell-based regulation of the coagulation system [4-6]. Because of the fundamental involvement of platelets in atherothrombosis, antiplatelet drugs that target amplifying secondary platelet agonists are the standard of care for the prevention of thrombotic events in patients with cardiovascular disease [7, 8]. Aspirin irreversibly blocks the cyclooxygenase enzyme (COX) to inhibit thromboxane A_2 (TXA_2) production [9] whilst $P2Y_{12}$ receptor antagonists such as clopidogrel and prasugrel block the effects of pro-aggregatory ADP [10]. Together these agents constitute dual antiplatelet therapy (DAPT). Despite this approach adverse thrombotic events still occur and high on-treatment platelet reactivity to ADP [11] and aspirin resistance [12] are associated with adverse clinical outcome. This has led to questioning of the current 'one size fits all approach' to DAPT and the development of the concept of tailored antithrombotic therapies. However, to date attempts to use platelet testing to personalise therapy have repeatedly failed to provide any improvement in clinical outcome in randomised clinical trials [13-15].

Coagulation pathway changes also play central roles in clot propagation, a process that is affected by many factors both genetic and environmental [16]. Configuration of the fibrin network is a vital determinant of clot stability and susceptibility to fibrinolysis [15, 17] with clot permeability being the rate limiting factor for the activity of plasmin, the fibrin network degradation enzyme. Clots composed of compact thin fibres are

associated with thrombotic events [18, 19] and clot structure appears unfavourably altered in coronary artery disease and other conditions associated with thrombosis [20-23] and is also associated with adverse events following percutaneous coronary intervention. It has therefore, been suggested that there is a definitive diagnostic potential in characterising clot structure and modulation of clot architecture as a possible treatment for thrombosis [24]. However, many techniques that assess the mechanics and quality of clot architecture rely on processed samples using altered blood in remote laboratories, limiting their clinical use.

Viscoelastic properties have been shown to be the most sensitive measures of fibrin polymerisation and blood clot structure [25] and recent advances in our understanding of the viscoelastic changes taking place in coagulation have resulted in the development of new methods for the analysis of clot characteristics. These properties can be quantified using small amplitude oscillatory shear rheometry to measure the gel point (GP), a multi-faceted biomarker that defines the transition between viscoelastic fluid and viscoelastic solid states during gelation. The technique uses unadulterated whole blood and is measured immediately at the bedside during real-time clot formation, providing a rapid assessment of coagulation that can be more readily performed than current methods. In coagulating blood the GP marks the establishment of the incipient clot and provides three important characteristics of the clot, all calculated from one measurement: time taken to reach GP or gel time (T_{GP}); clot elasticity/strength (G'_{GP}) and the fractal dimension (d_f) of the incipient clot [26]. Fractal dimension is a common technique used in biology to characterize nonlinear growth in branching network structures [27]. Importantly the measurement of d_f at the GP provides a quantitative assessment of clot structure where in studies using

viscoelastic and imaging data it has been shown to act as the template for mature clot development [26][27]. Reduced d_f is associated with more permeable, less branched and mechanically weaker clots and a raised d_f corresponds to a tightly packed, highly branched clot that is less permeable and stronger. Several studies have highlighted the potential of d_f as a biomarker in determining the effects of disease and therapeutic intervention on clot quality [26][28-31]. However, we are yet to establish the effects of individual anti-platelet therapies on clot structure and whether these are quantifiable by our novel GP technique.

Here, we therefore apply the GP technique together with standard measures of platelet reactivity in blood samples taken from healthy volunteers treated with standard anti-platelet therapies, to investigate the complex interplay of platelets in the formation of clot structure and further validate the role of GP as a possible clinical biomarker. Such an approach may well provide a globally representative marker of haemostasis and thrombosis that could permit a more personalised approach to antiplatelet therapy.

Materials and Methods

Study participants

16 healthy, male volunteers (aged 18-40 years) participated in an initial single anti-platelet therapy study. Health status was determined though medical history and physical examination, including blood pressure, pulse rate, blood chemistry and urinalysis. Volunteers with normal clinical profiles were included in the study. Subsequently, a further 20 healthy volunteers were recruited in an identical manner for a follow-up dual anti-platelet therapy study. Both studies were approved by St Thomas's Hospital Research Ethics Committee (Ref. 07/Q0702/24) and all volunteers gave written consent before entering the study.

Study protocol

All healthy volunteers abstained from aspirin, non-steroidal anti-inflammatory drugs (NSAIDs) and any other anti-platelet therapy for 14 days before commencing the studies. Two groups of 8 volunteers received monotherapy aspirin (75 mg; Nu-Seals Cardio 75, Alliance Pharmaceuticals Ltd, Chippenham, UK) or prasugrel (10 mg; Effient®, Eli Lilly, RA Houten, The Netherlands) for 7 days. Subsequently, two groups of 10 volunteers received dual anti-platelet therapy (DAPT) with either aspirin (75 mg) plus prasugrel (10 mg) or aspirin (75mg) plus ticagrelor (90mg; Brilique®, AstraZeneca, Södertälje, Sweden), for 7 days. Compliance was assessed by interview. Blood samples were collected prior to and following drug treatment in both studies.

Blood collection

In all cases blood was obtained from an antecubital vein via a 19-gauge butterfly needle and all experiments were completed within 2 hours of blood collection.

Laboratory markers

Blood was taken for full blood count (FBC) analysis, including platelet count and haemoglobin into 4ml EDTA vacutainers and into 4.5ml citrate vacutainers (BD Biosciences, UK) for routine coagulation studies, including prothrombin time (PT), activated partial thromboplastin time (APTT), APTTr, INR and fibrinogen. Measurements were made by use of automated analysers, Sysmex XN-2000 and Sysmex CS-2100i (Lincolnshire, USA).

Platelet function

Blood for platelet aggregation was collected into tri-sodium citrate (0.32 % final; Sigma, Poole, Dorset, UK). Platelet-rich plasma (PRP) was obtained by centrifugation at 175 x *g* for 15 min at 25˚C. Platelet-poor plasma (PPP) was obtained by centrifugation of PRP at 15 000 x *g* for 2 min.

Light Transmission Aggregometry (LTA)

Baseline aggregation of PRP to arachidonic acid (AA, final concentration, 1 mM; Sigma), adenosine diphosphate (ADP, 5 and 20 µM; Labmedics, Salford, Manchester, UK), Horm collagen (0.4, 4 and 10 µg/mL; Nycomed, Linz, Austria), U46619 (10 µM; Cayman Chemical Company, Ann Arbor, MI, USA) and TRAP-6 amide specific for PAR1 (TRAP-6, 25 µM; SFLLRN, Bachem, Bubendorf, Switzerland) was measured in a Bio/Data PAP-8E turbidimetric aggregometer (5 minutes, 1200 rpm, 37°C; Alpha Laboratories, Eastleigh, UK) before and following treatment.

ADP+ATP Secretion

ADP+ATP secretion of PRP was evaluated by luminescence in the presence of Chrono-Lume reagent (0.2 µM luciferin/luciferase; Chronolog) after stimulation with collagen (4 µg/mL) in an optical lumi-aggregometer (560 CA; Chronolog, Havertown, PA, USA) before commencing and on completion of therapy.

P-Selectin Expression and GPIIb/IIIa Activation

PRP was activated with TRAP-6 (25 µM) under slow stirring conditions (300 rpm, 37 °C). After 2 minutes, the reaction was stopped by dilution in ice cold 0.9% saline. Platelets were immediately stained with anti–CD61-allophycocyanin (CD61-APC; eBioscience, Hatfield, UK), PAC-1-FITC (BD Bioscience, Oxford, UK), and anti–p-selectin-PE (eBioscience) for 15 min at 4°C and then fixed in 2% (vol/vol) formalin (Sigma). PAC-1-FITC and anti–p-selectin-PE immunoreactivity was measured by flow cytometry using a FACSCalibur instrument (Becton Dickenson, Oxford, UK).

Rheometry

9ml aliquots of blood collected into Vacuette vacutainers (Greiner Bio-One, Austria) without anti-coagulant were transferred directly and immediately (< 60 seconds) after sampling to a double-gap concentric cylinder geometry of a TA instruments Discovery HR-2 controlled stress rheometer (New Castle, DE, USA). All work was conducted at 37(±0.1) °C and all measurements were made on aliquots of the same sample by use of similar measuring geometries with identical measuring surfaces and surface preparation procedures. The viscoelastic properties of incipient clots were determined by detecting the Gel Point (GP) of samples of whole unadulterated blood using small

amplitude oscillatory shear rheometry [32]. From which the values of T_{GP} and d_f were measured. The GP technique used in this study has been previously validated for use with blood elsewhere in several other studies [26,29-32]. The value of d_f measured at the GP can be equated to the mass of the clot. In previous studies we have shown there is an exponential relationship between d_f and clot mass [33]. In the present study we recreated this relationship and used it to calculate the relative mass (RM) for each of the d_f values. In this approach the mass of a random fractal aggregate (RFA) varies as a function of fractal dimension, via the following scaling law $M(\varepsilon) \sim \varepsilon^{d_f}$, where ε are the characteristic length scales of the RFA (we assume the fibrin structure to be fractal over the range (100nm - 60μm) commensurate with light scattering experiments conducted by Ferri et al. [34].

Scanning Electron Microscopy

For scanning electron microscopy (SEM) samples at each time point were allowed to clot at 37°C for a minimum of 15 mins. Samples were then washed three times with 2 cocadylate buffer pH 7.2 for the removal of excess salt and fixed for a minimum of 4 h in 2% glutaraldehyde solution. The clots were then rinsed with cocadylate buffer and dehydrated in a series of ethanol concentrations from 30 to 100%. The clots were then critical point dried with hexamethyldisilazane for 45 min and placed in a fume hood for 24 hrs. Finally, the clots were mounted to 0.5" SEM stubs (Agar Scientific, UK) and sputter coated with gold palladium. All samples were investigated with a Hitachi S4800 scanning electron microscope (Hitachi, High-Technologies Corporation, Tokoyo, Japan) [35]. All images were acquired with a constant resolution of 5.0kV (accelerating voltage) and a semi-in-lens achieving ultra-high resolution. A

combination of upper and lower signal detectors were used to collect the electron signals.

Statistics and Data Analysis

Clot fractal dimension (d_f), relative mass (RM) and haematological and haemostatic data were analysed by Wilcoxon signed rank tests. Pearson correlation coefficients were calculated for d_f and T_{GP} versus haematological and haemostatic parameters. Flow cytometry data was analysed using FlowJo v8.7 (Treestar, Ashland, USA) where the “single platelet” population was gated based on forward scatter and CD61-APC immunoreactivity (FL-4 mean fluorescence intensity). Unless otherwise stated, platelet response data in LTA, lumi-aggregometry and flow cytometry was analysed by paired t-test, using Prism v6.0e (Graphpad software, USA). Statistical significance was determined as described above and data sets considered different if $p<0.05$.

Results

Effects of aspirin (75 mg) therapy on platelet reactivity and fractal analysis of incipient clots

In individuals taking aspirin, standard LTA responses to AA (1 mM) and collagen (0.4 µg/ml) were strongly inhibited. Responses to ADP (5 µM) were also significantly reduced whilst those to ADP (20 µM), collagen (4 µg/ml), U46619 (10 µM) and TRAP-6 (25 µM) were unaffected *(Figure 1A)*. Similarly, aspirin therapy did not affect dense granule secretion induced by collagen (4 µg/ml) as measured by ATP release (*Figure 1B),* or TRAP-6 (25 µM) induced PAC-1 binding (*Figure 1C*) and p-selectin expression (*Figure 1D*). Haematological parameters, selected haemostatic parameters and gel time were not altered (*Supplementary*, *Table 2 & 6*). Aspirin treatment also produced no significant changes in the value of d_f (1.71±0.01 to 1.69±0.01, p=0.41) *(Supplementary, Table 7)* or the relative mass (-8±19% change, p=0.47) of incipient clots.

Effects of prasugrel (10 mg) monotherapy on platelet reactivity and fractal analysis of incipient clots

Following prasugrel therapy aggregatory responses to AA (1 mM), collagen (0.4 and 4 µg/ml), ADP (5 and 20 µM), as well as U46619 (10 µM) were all significantly reduced. TRAP-6 (25 µM)-induced aggregation however, was unaffected (*Figure 2A*). ATP release induced by collagen (4 µg/ml) was significantly decreased (*Figure 2B*), as was TRAP-6 (25 µM) induced PAC-1 (*Figure 2C*) and p-selectin expression (*Figure 2D*). Haematological parameters, and selected haemostatic parameters and gel time were not altered (*Supplementary, Table 3 & 6*). There were notable reductions in d_f (1.72±0.02 to 1.67±0.01, p =0.03) *(Supplementary, Table 7)* and mean relative mass

values (-40±11% change, p=0.03) for incipient clots formed following prasugrel therapy.

Characterisation of dual anti-platelet therapy with aspirin (75mg) plus prasugrel (10 mg) on platelet reactivity and fractal analysis of incipient clots

Platelet aggregation induced by AA (1mM), collagen (0.4 µg/ml) and ADP (5 µM and 20 µM) in LTA were abolished in individuals taking prasugrel plus aspirin whilst responses to collagen (4 µg/ml), U46619 (10 µM) and TRAP-6 (25 µM) were also all significantly inhibited (*Figure 3A*). TRAP-6 (25 µM) induced p-selectin expression was also decreased (*Figure 3B*). There was a significant reduction in the d_f value (1.73±0.02 to 1.68±0.02, p=0.03) *(Supplementary, Table 7)* for incipient clots and a resulting decrease in mean relative mass (-35±16% change, p=0.04). Haematological parameters, and selected haemostatic parameters and gel time were not altered (*Supplementary, Table 4 & 6*).

Characterisation of dual anti-platelet therapy with aspirin (75mg) plus ticagrelor (90 mg) on platelet reactivity and fractal analysis of incipient clots

Following dual anti-platelet therapy with aspirin plus ticagrelor platelet responses followed a similar pattern to those in individuals taking aspirin plus prasugrel; aggregations induced by AA (1mM), collagen (0.4 µg/ml) and ADP (5 µM and 20 µM) were powerfully inhibited and those to collagen (4 µg/ml), U46619 (10 µM) and TRAP-6 (25 µM) were significantly reduced (*Figure 4A*). These reductions were associated with a reduction in p-selectin expression ($p<0.05$; *Figure 4B*) induced by TRAP-6 (25 µM). Similarly, there were significant decreases in d_f for incipient clots formed (1.72±0.03 to 1.62±0.02, p=0.04) *(Supplementary, Table 7)* and marked reductions in

mean mass values (-45±14% change, p=0.04). Haematological parameters, and selected haemostatic parameters and gel time were not altered (*Supplementary, Table 5 & 6*)

Clot structure analysis by scanning electron microscopy and fractal analysis

Scanning electron microscopy demonstrated that dual anti-platelet therapy resulted in formation of clots with reduced fibrin density and increased fibrin strand diameter (*Figure 5*). This reduction in clot density was consistent with the d_f measurements and projections of random fractal aggregates for the aspirin plus prasugrel and aspirin plus ticagrelor groups showing a reduction in the density of connectedness compared to that projected from blood collected under control conditions (*Figure 6*).

Correlation analysis between fractal analysis and other parameters

The relationships between pre and post treatment values of d_f and the haemostatic, haematological and platelet parameters are shown in Table 1. The only haemostatic or haematological parameter that showed a clear correlation with d_f was fibrinogen (0.376, p=0.04). Conversely, regarding the relationships between d_f and the various platelet activity tests significant correlations were found with AA (1mM) (0.332, p=0.006), ADP (5μM) (0.366, p=0.002), ADP (20μM) (0.369. 0.002), collagen (0.4μg/ml) (0.343, p=0.004) and TRAP-6 (25μM) (0.309, p=0.01).

Discussion

Here using an advanced viscoelastic technique we have identified that platelets are potential regulators of clot structure under clinically relevant conditions. This and previous studies show that our technique could be applied to investigate the functional relationship between coagulation pathways, platelets, eventual clot quality and outcome. This is relevant as increased platelet reactivity represents an important and potentially modifiable risk factor for thrombosis [40]; if reliably identified then it could be modified by individualised anti-platelet therapy regimes. A possible limitation of standard kinetic markers is their focus on individual components of platelet reactivity, coagulation or fibrinolysis alone which fails to distinguish abnormal clot architecture.

Our study is the first to apply the measurement of d_f to quantify differences in structural organisation and mechanical properties of blood clots secondary to individual and combined anti-platelet therapy regimes. Analysis of pre-treatment blood indicated a clearly defined d_f value within a narrow range, representing a normal index of haemostasis where d_f = 1.72 (± 0.05). Further analysis of pre and post treatment d_f values demonstrated a clear correlation with fibrinogen (0.376, p=0.04) (*Table 1*).

In control conditions we observed platelet reactivity responses within the normal ranges reported for healthy volunteers [11, 36, 37]. Following treatment with aspirin or prasugrel we noted powerful inhibition of platelet responses to AA or ADP respectively, in keeping with consensus statement indicators of effective therapy [11, 37]. Aspirin monotherapy also inhibited platelet aggregations induced by low concentrations of ADP and collagen. ATP release, activation of GP IIb/IIIa receptors and p-selectin expression were not significantly affected. In accordance with these limited effects on

platelet function, aspirin produced no significant change in clot structure as detected by d_f (1.71±0.01 to 1.69±0.01, p=0.41). The change in mean relative mass was also non-significant (-8±19% change, p=0.47). Prasugrel monotherapy substantially blunted platelet aggregation responses to all agonists tested apart from TRAP-6 and caused significant reductions in ATP release, p-selectin expression and GP IIb/IIIa receptor activation. Platelet inhibition was associated with both a significant reduction in d_f (1.72±0.02 to 1.67±0.01, p =0.03) and a considerable change in the mean relative mass of incipient clots (-40±11% change, p=0.03) indicative of a substantial change in structure.

Following our monotherapy studies, which indicated a strong association between platelet activity and clot architecture we then sought to characterise the influences of dual anti-platelet therapy. Administration of aspirin plus prasugrel or aspirin plus ticagrelor resulted in significant levels of platelet inhibition to all agonists, abolishing responses to weaker agonists tested. P-selectin expression was also significantly reduced in both groups. Again, significant platelet inhibition was coupled with a reduction in d_f: aspirin plus prasugrel (1.73±0.02 to 1.68±0.02, p=0.03) and aspirin plus ticagrelor (1.72±0.03 to 1.62±0.02, p=0.04). There were also significant reductions in mean relative mass both for aspirin plus prasugrel (-35±16% change, p=0.04) and for aspirin plus ticagrelor (-45±14% change, p=0.04).

Overall the anti-platelet therapies had little effect on haematological and haemostatic parameters (*Supplemental Tables 2,3,4,5 & 7*) but the changes in Df did correlate with changes in the platelet reactivity tests (see Table 1). Whilst as noted above, d_f correlates with fibrinogen concentration it is important to note that fibrinogen

concentrations did not change between the pre- and post- measurements for any of the anti-platelet drugs tested (aspirin, prasugrel, aspirin plus prasugrel, and aspirin plus ticagrelor). This indicates that the magnitude of d_f is not solely dependent on the amount of fibrinogen present, but also how the processes through which fibrinogen forms a fibrin network dependent upon platelet reactivity.

Overall our results clearly demonstrate that stronger inhibition of platelet function is associated with the formation of less dense clots comprised of a more open/porous network of fibrin strands. Where the inhibition of platelet function could affect other activities such as coagulation and thrombin activation which would the regulate fibrin strand formation. It is important to recognise that d_f has a very narrow range and to appreciate the non-linear relationship between d_f and fibrin mass. Figure 6 helps in this regard by providing a visual projection of how relatively small changes in fractal dimension would underlie large changes in the mass of random fractal aggregates (RFA); notably the lowest fractal dimension has a lower frequency of high-density regions (i.e. the number of nearest neighbours is relatively low) consequently the projected form is relatively sparse and open which would equate to a mechanically weaker and more highly friable clot. On the contrary, the RFA with the highest d_f is relatively more compact, due to the larger number of high density regions within the structure. The density regions constrain the possible positional configurations of adjacent constituent particles, i.e. reducing on average the spacing between adjacent particles resulting in a relatively denser structure which would correspond to a mechanically stronger clot less susceptible to breakdown. This predicted link between d_f and thrombus structure is confirmed by scanning electron micrographs (SEM), which demonstrate a more open clot structure following treatment with anti-platelet

therapies (*Figure 5*). These SEM images, in keeping with our previous studies highlight that incipient clot structure quantified by d_f acts as a template to predict the structure of the mature clot. Notably, the fibrin strands are much thicker in the post- as opposed to the pre-treatment images (d_f 1.67 versus d_f 1.63) and the number of small round particles, which represent platelets, is much reduced. This is in keeping with the association of thick fibrin strands with more porous clots and reduced platelet-fibrin interactions which we could expect following DAPT with aspirin plus ticagrelor in our healthy volunteer subject.

Results from our studies using standard single and dual anti-platelet therapy demonstrate that it is activation of $P2Y_{12}$ receptors that is the key driver of platelet interactions in clot formation, in keeping with the contribution of ADP but not TXA_2 receptors in the initiation of intravascular coagulation [38]. We did not observe a significant microstructural change in volunteers taking aspirin 75mg, in keeping with a minimal reduction in platelet reactivity. This contrasts with a recent study, where treatment with 300mg aspirin in patients with ischaemic stroke produced a significant reduction in d_f.[28] Aspirin primarily acts to inhibit platelet cyclo-oxygenase-1 however, at higher doses it exerts other effects such as acetylation of fibrinogen, prothrombin and other coagulation factors [39] [40]. Therefore, we could anticipate to detect this direct role in clot modulation with higher doses of aspirin (300mg) but perhaps not at lower doses (75mg) and this difference was readily detectable by our global haemorheological marker of clot structure. It is also notable that addition of aspirin to prasugrel produced no further reduction in markers of clot density despite additional inhibition of platelet thromboxane A_2 pathways. As we have suggested previously this

may indicate that in the presence of strong $P2Y_{12}$ receptor blockade aspirin produces little or no additional therapeutic benefit [41, 42].

Our results are readily understandable as platelets are central to several key coagulation processes. Activated platelets expose phosphatidylserine to support the function of the prothrombinase complex greatly enhancing coagulation [43], interact with many coagulation factors [44, 45] and associate with tissue factor [46]. Platelets are instrumental in providing a surface for large scale thrombin production [47] and are involved in fibrin formation and organisation with activated GPIIb/IIIa receptors regulating clot retraction [48]. Previous studies have shown that platelets are important in fibrin fibre growth where the fibres originate from the platelet aggregates in clots formed in platelet-rich plasma [49]. This is an important consideration in understanding df as it is derived from a viscoelastic measurement of the incipient clot formation, where the fibrin network provides the dominant viscoelastic response [26]. Dense regions of fibres would inevitably influence the viscoelastic properties and consequently the value of d_f. Therefore, platelets as the promotors of fibrin fibre growth would contribute to an increase in elasticity and thus a corresponding increase in the d_f. Consequently, in the presence of platelet inhibition and hence a reduction in the number, or absence, of platelet aggregates a lower value of d_f would be expected. In the present study comparison of responses in the absence and presence of different levels of platelet inhibition identifies a significant relationship between platelet function and the complex, highly disordered structure of incipient clots in whole unadulterated blood from healthy volunteers, echoing a previous report that altered clot structures are associated with aspirin treatment failure [50]. As well as providing further mechanistic insight into the links between platelet reactivity and clot formation, our

data indicates that the therapeutic benefits of anti-platelet therapies are dependent upon their abilities to reduce thrombus density. Analysis of our SEM micrographs further confirms that these anti-thrombotic effects are partly attributable to the formation of thicker fibrin fibres, increased volume of pore spaces and an increased permeability of the clot's fibrinolytic components. This deeper understanding of the role of platelets in thrombus formation could be useful in individual patients to determine the functional relationship between platelet reactivity, eventual clot quality and clinical outcome.

Given the numerous mechanisms by which platelets and the coagulation system interact, a more comprehensive marker of coagulation, thrombosis and the response to therapeutic manipulation, could prove important in the prediction of thromboembolic complications. Our observations support that indices of fractal analysis are intricately linked and reflect clot structure and strength and are representative of the dynamic and interwoven properties of the fibrin clot and platelet reactivity. Importantly, this study has shown that d_f can quantify the effects of individual therapeutic interventions. The development of d_f as a point of care test to assess clot structure may have potential as a future tool in quantifying thrombotic risk, through identifying those patients who form abnormal clot structures that are associated with impaired clot lysis and increased potential to embolise. Exploited in this way, d_f could prove a rapid means of influencing clinical decisions in the management of bleeding and thrombotic risk, providing more insight into the combined effect of clotting factors and cellular components in terms of functional activity of the pathological clot and its properties. Rather than simply measuring platelet reactivity a more thorough understanding of how the fibrin network is structurally organised could be more informative in

determining the effects and outcomes of anti-platelet therapies in the clinical setting with the potential of individualising therapies.

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Authorship

Contribution: R. B. Knowles, L. A. D'Silva, M. J. Lawrence, contributed to concept and design, analysed data and critical writing; P. M. Ferreira, M. A. Hayman, S. N. Stanford, A. Sabra, analysed data; K. M. Hawkins , P. R. Williams, A. T. Tucker, contributed to concept and design and interpreted data. T. D. Warner, P. A. Evans, contributed to concept and design, revised intellectual content and final approval of the version to be published.

References

1 Dahlback B. Blood coagulation. *Lancet*. 2000; **355**: 1627-32.
2 Davi G, Patrono C. Platelet activation and atherothrombosis. *The New England journal of medicine* 2007; **357**: 2482-2494.
3 Offermanns S. Activation of platelet function through G protein-coupled receptors. *Circulation research* 2006; **99**: 1293-1304.
4 Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. *Thrombosis and haemostasis* 2002; **88**: 186-193.
5 Monroe DM, Hoffman M, Roberts HR. Platelets and thrombin generation. *Arteriosclerosis, thrombosis, and vascular biology* 2002; **22**: 1381-1389.
6 Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood* 2004; **104**: 1606-1615.
7 Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK, Clopidogrel in Unstable Angina to Prevent Recurrent Events Trial I. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. *The New England journal of medicine* 2001; **345**: 494-502.
8 Steinhubl SR, Berger PB, Mann JT, 3rd, Fry ET, DeLago A, Wilmer C, Topol EJ, Observation CICftRoED. Early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. *Jama* 2002; **288**: 2411-2420.
9 Vane JR, Botting RM. The mechanism of action of aspirin. *Thrombosis research* 2003; **110**: 255-258.
10 Hechler B, Cattaneo M, Gachet C. The P2 receptors in platelet function. *Seminars in thrombosis and hemostasis* 2005; **31**: 150-161.
11 Tantry US, Bonello L, Aradi D, Price MJ, Jeong YH, Angiolillo DJ, Stone GW, Curzen N, Geisler T, Ten Berg J, Kirtane A, Siller-Matula J, Mahla E, Becker RC, Bhatt DL, Waksman R, Rao SV, Alexopoulos D, Marcucci R, Reny JL, Trenk D, Sibbing D, Gurbel PA. Consensus and Update on the Definition of On-Treatment Platelet Reactivity to ADP Associated with Ischemia and Bleeding. *Journal of the American College of Cardiology* 2013.
12 Michelson AD, Cattaneo M, Eikelboom JW, Gurbel P, Kottke-Marchant K, Kunicki TJ, Pulcinelli FM, Cerletti C, Rao AK, Platelet Physiology Subcommittee of the S, Standardization Committee of the International Society on T, Haemostasis, Working Group on Aspirin R. Aspirin resistance: position paper of the Working Group on Aspirin Resistance. *Journal of thrombosis and haemostasis* 2005; **3**: 1309-1311.
13 Price MJ, Angiolillo DJ, Teirstein PS, Lillie E, Manoukian SV, Berger PB, Tanguay JF, Cannon CP, Topol EJ. Platelet reactivity and cardiovascular outcomes after percutaneous coronary intervention: a time-dependent analysis of the Gauging Responsiveness with a VerifyNow P2Y12 assay: Impact on Thrombosis and Safety (GRAVITAS) trial. *Circulation* 2011; **124**: 1132-1137.
14 Collet JP, Cuisset T, Range G, Cayla G, Elhadad S, Pouillot C, Henry P, Motreff P, Carrie D, Boueri Z, Belle L, Van Belle E, Rousseau H, Aubry P, Monsegu J, Sabouret P, O'Connor SA, Abtan J, Kerneis M, Saint-Etienne C, Barthelemy O, Beygui F, Silvain J, Vicaut E, Montalescot G, Investigators A. Bedside monitoring to adjust antiplatelet therapy for coronary stenting. *The New England journal of medicine* 2012; **367**: 2100-2109.
15 Collet JP, Park D, Lesty C, Soria J, Soria C, Montalescot G, Weisel JW. Influence of fibrin network conformation and fibrin fiber diameter on fibrinolysis

speed: dynamic and structural approaches by confocal microscopy. *Arteriosclerosis, thrombosis, and vascular biology* 2000; **20**: 1354-1361.
16 Scott EM, Ariens RA, Grant PJ. Genetic and environmental determinants of fibrin structure and function: relevance to clinical disease. *Arteriosclerosis, thrombosis, and vascular biology* 2004; **24**: 1558-1566.
17 Gabriel DA, Muga K, Boothroyd EM. The effect of fibrin structure on fibrinolysis. *The Journal of biological chemistry* 1992; **267**: 24259-24263.
18 Collet JP, Lesty C, Montalescot G, Weisel JW. Dynamic changes of fibrin architecture during fibrin formation and intrinsic fibrinolysis of fibrin-rich clots. *The Journal of biological chemistry* 2003; **278**: 21331-21335.
19 Colle JP, Mishal Z, Lesty C, Mirshahi M, Peyne J, Baumelou A, Bensman A, Soria J, Soria C. Abnormal fibrin clot architecture in nephrotic patients is related to hypofibrinolysis: influence of plasma biochemical modifications: a possible mechanism for the high thrombotic tendency? *Thrombosis and haemostasis* 1999; **82**: 1482-1489.
20 Fatah K, Silveira A, Tornvall P, Karpe F, Blomback M, Hamsten A. Proneness to formation of tight and rigid fibrin gel structures in men with myocardial infarction at a young age. *Thrombosis and haemostasis* 1996; **76**: 535-540.
21 Greilich PE, Carr ME, Zekert SL, Dent RM. Quantitative assessment of platelet function and clot structure in patients with severe coronary artery disease. *The American journal of the medical sciences* 1994; **307**: 15-20.
22 Nair CH, Azhar A, Wilson JD, Dhall DP. Studies on fibrin network structure in human plasma. Part II--Clinical application: diabetes and antidiabetic drugs. *Thrombosis research* 1991; **64**: 477-485.
23 Undas A, Zawilska K, Ciesla-Dul M, Lehmann-Kopydlowska A, Skubiszak A, Ciepluch K, Tracz W. Altered fibrin clot structure/function in patients with idiopathic venous thromboembolism and in their relatives. *Blood* 2009; **114**: 4272-4278.
24 Ariens RA. Denser matters. *Blood*. 2009; **114**: 3978-3979.
25 Weisel JW. The mechanical properties of fibrin for basic scientists and clinicians. *Biophysical chemistry* 2004; **112**: 267-276.
26 Evans PA, Hawkins K, Morris RH, Thirumalai N, Munro R, Wakeman L, Lawrence MJ, Williams PR. Gel point and fractal structure of incipient blood clots are significant new markers of hemostasis for healthy and anticoagulated blood. Blood 2010; 116: 3341-3346.
27 Losa GA. The fractal geometry of life. *Riv Biol* 2009; **102**: 29-59.
28 Stanford SN, Sabra A, D'Silva L, Lawrence MJ, Morris RHK, Storton S, Brown MR, Evans V, Hawkins K, Williams PR, Davidson SJ, Wani M, Potter JF, Evans PA. The changes in clot microstructure in patients with ischaemic stroke and the effects of therapeutic intervention: a prospective observational study. BMC Neurology. (2014) 15:35
29 Lawrence MJ, Sabra A, Thomas P, Obaid DR, D'Silva LA, Morris RH, Hawkins K, Brown MR, Williams PR, Davidson SJ, Chase AJ, Smith D, Evans PA. Fractal dimension: a novel clot structure biomarker use in ST elevation myocardial infarction patients. *Atherosclerosis* 2015; **240**: 402-407.
30 Lawrence MJ, Sabra A, Mills G, Pillai SG, Abdullah W, Hawkins K, Morris RH, Davidson SJ, D'Silva LA, Curtis DJ, Brown MR, Weisel JW, Williams PR, Evans PA. A new biomarker quantifies differences in clot structure in patients with venous thromboembolism. *British journal of haematology* 2015; **168**: 571-575.
31 Lawrence MJ, Kumar S, Hawkins K, Boden S, Rutt H, Mills G, Sabra A, Morris RH, Davidson SJ, Badiei N, Brown MR, Williams PR, Evans PA. A new structural

biomarker that quantifies and predicts changes in clot strength and quality in a model of progressive haemodilution. *Thrombosis research* 2014; **134**: 488-494.
32 Davies NA, Harrison NK, Morris RH, Noble S, Lawrence MJ, D'Silva LA, Broome L, Brown MR, Hawkins KM, Williams PR, Davidson S, Evans PA. Fractal dimension (df) as a new structural biomarker of clot structure in different stages of lung cancer. *Thrombosis and haemostasis* 2015; **114**:1251-1259
33 Badiei N, Sowedan AM, Curtis DJ, Brown MR, Lawrence MJ, Campbell AI, Sabra A, Evans PA, Weisel JW, Chernysh IN, Nagaswami C, Williams PR, Hawkins K. Effects of unidirectional flow shear stresses on the formation, fractal structure and rigidity of incipient whole blood clots and fibrin gels. *Clin Hemorheol Microcirc* 2015; **60**: 451-464.
34 Ferri F, Greco M, Arcovito G, De Spirito M, Rocco M. Structure of fibrin gels studied by elastic light scattering techniques: dependence of fractal dimension, gel crossover length, fiber diameter, and fiber density on monomer concentration. *Phys Rev E Stat Nonlin Soft Matter Phys* 2002; **66**: 011913.
35 Langer BG, Weisel JW, Dinauer PA, Nagaswami C, Bell WR. Deglycosylation of fibrinogen accelerates polymerization and increases lateral aggregation of fibrin fibers. *J Biol Chem* 1988; **263**: 15056-15063.
36 Lordkipanidze M, Lowe GC, Kirkby NS, Chan MV, Lundberg MH, Morgan NV, Bem D, Nisar SP, Leo VC, Jones ML, Mundell SJ, Daly ME, Mumford AD, Warner TD, Watson SP, Genotyping UK, Phenotyping of Platelets Study G. Characterization of multiple platelet activation pathways in patients with bleeding as a high-throughput screening option: use of 96-well Optimul assay. *Blood* 2014; **123**: e11-22.
37 Cattaneo M, Cerletti C, Harrison P, Hayward CP, Kenny D, Nugent D, Nurden P, Rao AK, Schmaier AH, Watson SP, Lussana F, Pugliano MT, Michelson AD. Recommendations for the Standardization of Light Transmission Aggregometry: A Consensus of the Working Party from the Platelet Physiology Subcommittee of SSC/ISTH. *Journal of thrombosis and haemostasis* 2013.
38 Leon C, Alex M, Klocke A, Morgenstern E, Moosbauer C, Eckly A, Spannagl M, Gachet C, Engelmann B. Platelet ADP receptors contribute to the initiation of intravascular coagulation. *Blood* 2004; **103**: 594-600.
39 Bjornsson TD, Schneider DE, Berger H, Jr. Aspirin acetylates fibrinogen and enhances fibrinolysis. Fibrinolytic effect is independent of changes in plasminogen activator levels. *The Journal of pharmacology and experimental therapeutics* 1989; **250**: 154-161.
40 He S, Blomback M, Yoo G, Sinha R, Henschen-Edman AH. Modified clotting properties of fibrinogen in the presence of acetylsalicylic acid in a purified system. *Annals of the New York Academy of Sciences* 2001; **936**: 531-535.
41 Bjorkman JA, Zachrisson H, Forsberg GB, von Bahr H, Hansson GI, Warner TD, Nylander S. High-dose aspirin in dogs increases vascular resistance with limited additional anti-platelet effect when combined with potent P2Y12 inhibition. *Thrombosis research* 2013; **131**: 313-319.
42 Warner TD, Armstrong PC, Curzen NP, Mitchell JA. Dual antiplatelet therapy in cardiovascular disease: does aspirin increase clinical risk in the presence of potent P2Y12 receptor antagonists?. *Heart* 2010; **96**: 1693-1694.
43 Zwaal RF, Comfurius P, Bevers EM. Lipid-protein interactions in blood coagulation. *Biochimica et biophysica acta* 1998; **1376**: 433-453.
44 Gailani D, Ho D, Sun MF, Cheng Q, Walsh PN. Model for a factor IX activation complex on blood platelets: dimeric conformation of factor XIa is essential. *Blood* 2001; **97**: 3117-3122.

45 Monkovic DD, Tracy PB. Functional characterization of human platelet-released factor V and its activation by factor Xa and thrombin. *The Journal of biological chemistry* 1990; **265**: 17132-17140.
46 Muller I, Klocke A, Alex M, Kotzsch M, Luther T, Morgenstern E, Zieseniss S, Zahler S, Preissner K, Engelmann B. Intravascular tissue factor initiates coagulation via circulating microvesicles and platelets. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2003; **17**: 476-478.
47 Roberts HR, Monroe DM, Oliver JA, Chang JY, Hoffman M. Newer concepts of blood coagulation. *Haemophilia : the official journal of the World Federation of Hemophilia* 1998; **4**: 331-334.
48 Braaten JV, Jerome WG, Hantgan RR. Uncoupling fibrin from integrin receptors hastens fibrinolysis at the platelet-fibrin interface. *Blood* 1994; **83**: 982-993.
49 Collet J, Montalescot G, Lesty C, Weisel JW. A Structural and Dynamic Investigation of the Facilitating Effect of Glycoprotein IIb/IIIa Inhibitors in Dissolving Platelet-Rich Clots. *Circ Research* 2002; **90**: 428-434

50 Neergaard-Petersen S, Ajjan R, Hvas AM, Hess K, Larsen SB, Kristensen SD, Grove EL. Fibrin clot structure and platelet aggregation in patients with aspirin treatment failure. *PLoS One* 2013; **8**: e71150.

Tables

Table 1. Pearson correlation coefficients for d_f versus haemostatic, haematological and platelet parameters

Parameter	Correlation coefficient
Fibrinogen	0.376 *
PT	-0.301
APTT	0.154
Platelets	0.171
Haemoglobin	0.238
Haematocrit	0.252
AA (1 mM)	0.332*
ADP (5 µM)	0.366*
ADP (20 µM)	0.369*
Collagen (0.4 µg/ml)	0.343*
Collagen (4 µg/ml)	0.237
U46619 (10 µM)	0.231
TRAP-6 (25 µM)	0.309*
P Selectin Exp.	0.079
PAC-1 Binding	0.335
ATP Release	0.286

* = Significant result ($p<0.05$)

PT indicates prothrombin time; APPT activated partial thromboplastin time; d_f fractal dimension

Figure Legends

Figure 1

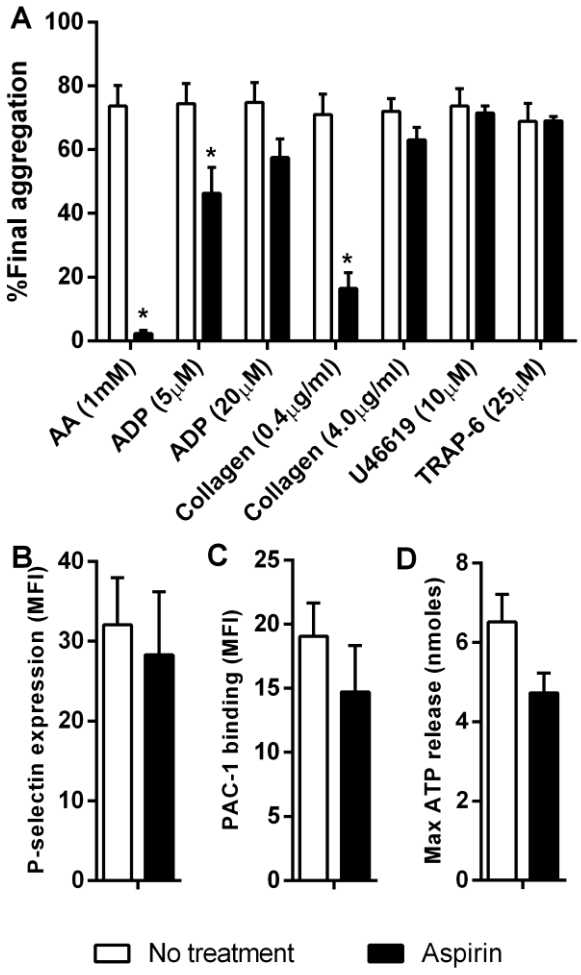

Figure 1. Effects of aspirin (75 mg) therapy on platelet reactivity. Bar graphs representing platelet responses before and after treatment measured by: A) standard light transmission aggregometry to AA (1 mM), ADP (5 and 20 µM), collagen (0.4 and 4 µg/mL), U46619 (10 µM) and TRAP-6 amide (25 µM); B) P-selectin expression; C) PAC-1 binding and D) ATP release after collagen (4 µg/ml) stimulation. Data are presented as final aggregation (%, mean±SEM), ATP release

(nmol, mean±SEM) or geometric mean fluorescence index (MFI) (units, mean±SEM). Significance is shown as * $p<0.05$ vs non-treated throughout.

Figure 2

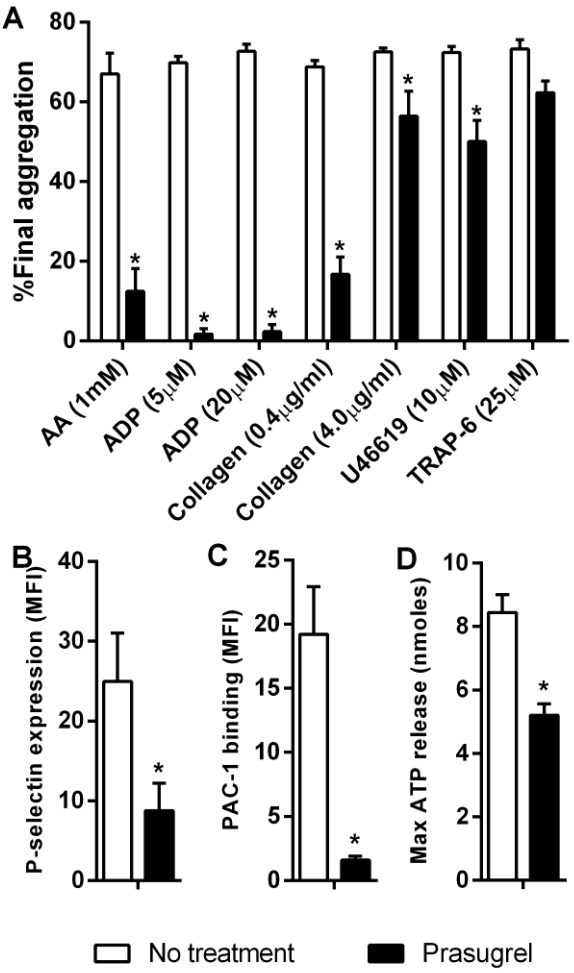

Figure 2. Effects of prasugrel (10 mg) therapy on platelet reactivity. Bar graphs representing platelet responses before and after treatment measured by: A) standard light transmission aggregometry to AA (1 mM), ADP (5 and 20 µM), collagen (0.4 and 4 µg/mL), U46619 (10 µM) and TRAP-6 amide (25 µM); B) P-selectin expression; C) PAC-1 binding and D) ATP release after collagen (4 µg/ml) stimulation. Data are presented as final aggregation (%, mean±SEM), ATP release (nmol, mean±SEM) or geometric mean fluorescence index (MFI) (units, mean±SEM). Significance is shown as * $p<0.05$ vs non-treated throughout.

Figure 3

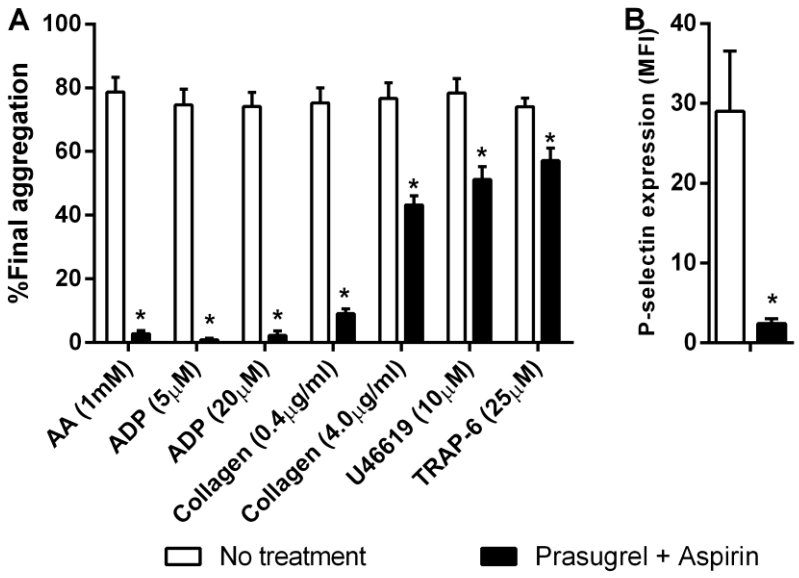

Figure 3. Effect of dual antiplatelet therapy with aspirin (75mg) plus prasugrel (10 mg) on platelet reactivity. Bar graphs demonstrating platelet responses before and after treatment measured by: A) standard light transmission aggregometry to AA (1 mM), ADP (5 and 20 µM), collagen (0.4, and 4 µg/mL), TRAP-6 amide (25 µM) and U46619 (10 µM); and B) TRAP-6 (25 µM) induced P-selectin expression. Data are presented as final aggregation (%, mean±SEM) or geometric mean fluorescence index (MFI) (units, mean±SEM). Significance is shown as * $p<0.05$ vs non-treated throughout.

Figure 4

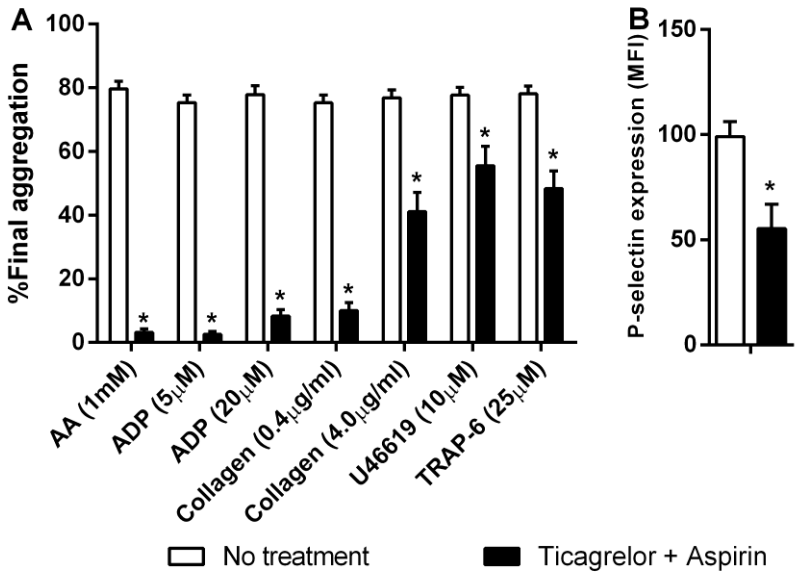

Figure 4. Effect of dual antiplatelet therapy with aspirin (75mg) plus ticagrelor (90 mg) on platelet reactivity. Bar graphs depicting platelet responses prior to and following treatment as measured by: A) standard light transmission aggregometry to AA (1 mM), ADP (5 and 20 µM), collagen (0.4, and 4 µg/mL), TRAP-6 amide (25 µM) and U46619 (10 µM); and B) TRAP-6 (25 µM) induced P-selectin expression. Data are presented as final aggregation (%, mean±SEM) or geometric mean fluorescence index (MFI) (units, mean±SEM). Significance is shown as * $p<0.05$ vs non-treated throughout.

Figure 5

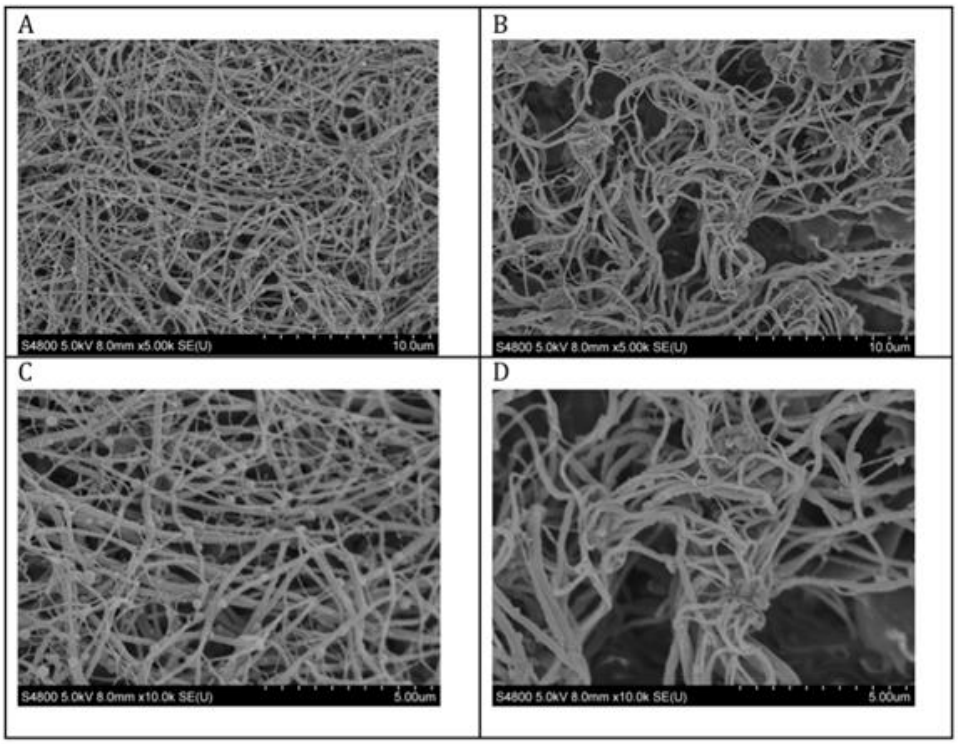

Figure 5. Effect of dual antiplatelet therapy with aspirin (75 mg) plus ticagrelor (90 mg) on clot structure. Corresponding SEM micrographs of clots formed in blood taken from one participant, before (panels (a) and (c); d_f 1.67) and after (panels (b)

and (d); d_f 1.63) treatment with aspirin (75mg) plus ticagrelor (90 mg). Micrographs were captured at two different magnifications for the pre- and post-dual antiplatelet therapy with aspirin and ticagrelor. The magnifications chosen were 5K (panels (a) and (b)) and 10K (panels (c) and (d)). Sections of the micrograph showing only the fibrin network were chosen to highlight the differences in the pre- and post- condition on clot microstructural development.

Figure 6

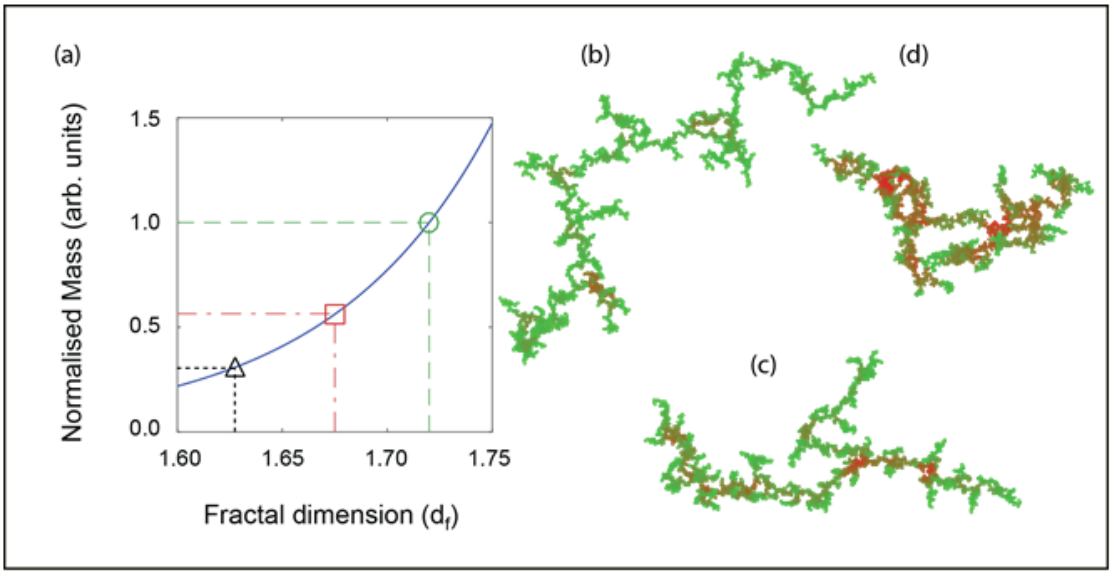

Figure 6: Visualisation of the relationship between fractal dimension and mass. Figure (a) shows the variation of the mass of a random fractal aggregate (RFA) as a function of fractal dimension. The magnitude of the RFA is normalized to

the mean healthy value, d_f = 1.72 (green circle). Additionally two further points are highlighted on Figure (a) showing the mass values of two RFA's with d_f values of 1.68 (red square) and 1.63 (black triangle). The red square being equivalent to the d_f found for the aspirin + prasugrel and the black triangle being equivalent to the aspirin + ticagrelor group. Figures (b), (c) and (d) are numerical realisations of RFAs corresponding to fractal dimensions of (b)1.63 (aspirin + ticagrelor), (c) 1.68 (aspirin + prasugrel) and (d) 1.72 (normal). Each numerical realisation of RFA is comprised of several constituent particles of unit diameter all of which are connected to at least one other particle. The colour index in the RFA realisations represents the local densities of constituent connected particles (green and red shades represent low and high density regions respectively). A RFA realisation containing many red areas denotes a more compact, densely connected structure than a RFA with predominately green areas.