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

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Manuscripts

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3 **Platelet reactivity influences clot structure as assessed by fractal analysis of**  
4 **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 ( $-35\pm 16\%$  change,  $p=0.04$ ) and for aspirin plus ticagrelor ( $-45\pm 14\%$  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.

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## 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<sub>2</sub> (TXA<sub>2</sub>) production [9] whilst P2Y<sub>12</sub> 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

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3 fibres are associated with thrombotic events [18, 19] and clot structure appears  
4 unfavourably altered in coronary artery disease and other conditions associated with  
5 thrombosis [20-23] and is also associated with adverse events following  
6 percutaneous coronary intervention. It has therefore, been suggested that there is a  
7 definitive diagnostic potential in characterising clot structure and modulation of clot  
8 architecture as a possible treatment for thrombosis [24]. However, many techniques  
9 that assess the mechanics and quality of clot architecture rely on processed samples  
10 using altered blood in remote laboratories, limiting their clinical use.  
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23 Viscoelastic properties have been shown to be the most sensitive measures of fibrin  
24 polymerisation and blood clot structure [25] and recent advances in our  
25 understanding of the viscoelastic changes taking place in coagulation have resulted  
26 in the development of new methods for the analysis of clot characteristics. These  
27 properties can be quantified using small amplitude oscillatory shear rheometry to  
28 measure the gel point (GP), a multi-faceted biomarker that defines the transition  
29 between viscoelastic fluid and viscoelastic solid states during gelation. The technique  
30 uses unadulterated whole blood and is measured immediately at the bedside during  
31 real-time clot formation, providing a rapid assessment of coagulation that can be  
32 more readily performed than current methods. In coagulating blood the GP marks  
33 the establishment of the incipient clot and provides three important characteristics of  
34 the clot, all calculated from one measurement: time taken to reach GP or gel time  
35 ( $T_{GP}$ ); clot elasticity/strength ( $G'_{GP}$ ) and the fractal dimension ( $d_f$ ) of the incipient clot  
36 [26]. Fractal dimension is a common technique used in biology to characterize  
37 nonlinear growth in branching network structures [27]. Importantly the measurement  
38 of  $d_f$  at the GP provides a quantitative assessment of clot structure where in studies  
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3 using viscoelastic and imaging data it has been shown to act as the template for  
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5 mature clot development [26][27]. Reduced  $d_f$  is associated with more permeable,  
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7 less branched and mechanically weaker clots and a raised  $d_f$  corresponds to a tightly  
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9 packed, highly branched clot that is less permeable and stronger. Several studies  
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11 have highlighted the potential of  $d_f$  as a biomarker in determining the effects of  
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13 disease and therapeutic intervention on clot quality [26][28-31]. However, we are yet  
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15 to establish the effects of individual anti-platelet therapies on clot structure and  
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17 whether these are quantifiable by our novel GP technique.  
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22 Here, we therefore apply the GP technique together with standard measures of  
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24 platelet reactivity in blood samples taken from healthy volunteers treated with  
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26 standard anti-platelet therapies, to investigate the complex interplay of platelets in  
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28 the formation of clot structure and further validate the role of GP as a possible  
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30 clinical biomarker. Such an approach may well provide a globally representative  
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32 marker of haemostasis and thrombosis that could permit a more personalised  
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34 approach to antiplatelet therapy.  
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## 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 through 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*

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3 In all cases blood was obtained from an antecubital vein via a 19-gauge butterfly  
4 needle and all experiments were completed within 2 hours of blood collection.  
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#### 8 9 *Laboratory markers*

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

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29 Blood for platelet aggregation was collected into tri-sodium citrate (0.32 % final;  
30 Sigma, Poole, Dorset, UK). Platelet-rich plasma (PRP) was obtained by  
31 centrifugation at 175 x g for 15 min at 25°C. Platelet-poor plasma (PPP) was  
32 obtained by centrifugation of PRP at 15 000 x g for 2 min.  
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#### 40 *Light Transmission Aggregometry (LTA)*

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

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10 ADP+ATP secretion of PRP was evaluated by luminescence in the presence of  
11 Chrono-Lume reagent (0.2  $\mu$ M luciferin/luciferase; Chronolog) after stimulation with  
12 collagen (4  $\mu$ g/mL) in an optical lumi-aggregometer (560 CA; Chronolog, Havertown,  
13 PA, USA) before commencing and on completion of therapy.  
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21 *P-Selectin Expression and GPIIb/IIIa Activation*

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23 PRP was activated with TRAP-6 (25  $\mu$ M) under slow stirring conditions (300 rpm, 37  
24 °C). After 2 minutes, the reaction was stopped by dilution in ice cold 0.9% saline.  
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26 Platelets were immediately stained with anti-CD61-allophycocyanin (CD61-APC;  
27 eBioscience, Hatfield, UK), PAC-1-FITC (BD Bioscience, Oxford, UK), and anti-p-  
28 selectin-PE (eBioscience) for 15 min at 4°C and then fixed in 2% (vol/vol) formalin  
29 (Sigma). PAC-1-FITC and anti-p-selectin-PE immunoreactivity was measured by  
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flow cytometry using a FACSCalibur instrument (Becton Dickenson, Oxford, UK).

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63 *Rheometry*

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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( $\pm$ 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

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3 were determined by detecting the Gel Point (GP) of samples of whole unadulterated  
4 blood using small amplitude oscillatory shear rheometry [32]. From which the values  
5 of  $T_{GP}$  and  $d_f$  were measured. The GP technique used in this study has been  
6 previously validated for use with blood elsewhere in several other studies [26,29-32].  
7 The value of  $d_f$  measured at the GP can be equated to the mass of the clot. In  
8 previous studies we have shown there is an exponential relationship between  $d_f$  and  
9 clot mass [33]. In the present study we recreated this relationship and used it to  
10 calculate the relative mass (RM) for each of the  $d_f$  values. In this approach the mass  
11 of a random fractal aggregate (RFA) varies as a function of fractal dimension, via the  
12 following scaling law  $M(\varepsilon) \sim \varepsilon^{d_f}$ , where  $\varepsilon$  are the characteristic length scales of the  
13 RFA (we assume the fibrin structure to be fractal over the range (100nm - 60 $\mu$ m)  
14 commensurate with light scattering experiments conducted by Ferri et al. [34].  
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### 32 *Scanning Electron Microscopy*

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34 For scanning electron microscopy (SEM) samples at each time point were allowed to  
35 clot at 37°C for a minimum of 15 mins. Samples were then washed three times with  
36 2 cocadylate buffer pH 7.2 for the removal of excess salt and fixed for a minimum of  
37 4 h in 2% glutaraldehyde solution. The clots were then rinsed with cocadylate buffer  
38 and dehydrated in a series of ethanol concentrations from 30 to 100%. The clots  
39 were then critical point dried with hexamethyldisilazane for 45 min and placed in a  
40 fume hood for 24 hrs. Finally, the clots were mounted to 0.5" SEM stubs (Agar  
41 Scientific, UK) and sputter coated with gold palladium. All samples were investigated  
42 with a Hitachi S4800 scanning electron microscope (Hitachi, High-Technologies  
43 Corporation, Tokoyo, Japan) [35]. All images were acquired with a constant  
44 resolution of 5.0kV (accelerating voltage) and a semi-in-lens achieving ultra-high  
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3 resolution. A combination of upper and lower signal detectors were used to collect  
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5 the electron signals.  
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### 9 10 *Statistics and Data Analysis*

11 Clot fractal dimension ( $d_f$ ), relative mass (RM) and haematological and haemostatic  
12 data were analysed by Wilcoxon signed rank tests. Pearson correlation coefficients  
13 were calculated for  $d_f$  and  $T_{GP}$  versus haematological and haemostatic parameters.  
14 Flow cytometry data was analysed using FlowJo v8.7 (Treestar, Ashland, USA)  
15 where the “single platelet” population was gated based on forward scatter and CD61-  
16 APC immunoreactivity (FL-4 mean fluorescence intensity). Unless otherwise stated,  
17 platelet response data in LTA, lumi-aggregometry and flow cytometry was analysed  
18 by paired t-test, using Prism v6.0e (Graphpad software, USA). Statistical significance  
19 was determined as described above and data sets considered different if  $p < 0.05$ .  
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## 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  $d_f$  ( $1.71 \pm 0.01$  to  $1.69 \pm 0.01$ ,  $p=0.41$ ) (*Supplementary, Table 7*) or the relative mass ( $-8 \pm 19\%$  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  $d_f$  ( $1.72 \pm 0.02$  to  $1.67 \pm 0.01$ ,  $p = 0.03$ ) (*Supplementary, Table 7*) and

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3 mean relative mass values ( $-40\pm 11\%$  change,  $p=0.03$ ) for incipient clots formed  
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5 following prasugrel therapy.  
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10 *Characterisation of dual anti-platelet therapy with aspirin (75mg) plus prasugrel (10*  
11 *mg) on platelet reactivity and fractal analysis of incipient clots*

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13  
14 Platelet aggregation induced by AA (1mM), collagen (0.4  $\mu\text{g/ml}$ ) and ADP (5  $\mu\text{M}$  and  
15  
16 20  $\mu\text{M}$ ) in LTA were abolished in individuals taking prasugrel plus aspirin whilst  
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18 responses to collagen (4  $\mu\text{g/ml}$ ), U46619 (10  $\mu\text{M}$ ) and TRAP-6 (25  $\mu\text{M}$ ) were also all  
19  
20 significantly inhibited (*Figure 3A*). TRAP-6 (25  $\mu\text{M}$ ) induced p-selectin expression  
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22 was also decreased (*Figure 3B*). There was a significant reduction in the  $d_f$  value  
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24 ( $1.73\pm 0.02$  to  $1.68\pm 0.02$ ,  $p=0.03$ ) (*Supplementary, Table 7*) for incipient clots and a  
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26 resulting decrease in mean relative mass ( $-35\pm 16\%$  change,  $p=0.04$ ).  
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28 Haematological parameters, and selected haemostatic parameters and gel time  
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30 were not altered (*Supplementary, Table 4 & 6*).  
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36 *Characterisation of dual anti-platelet therapy with aspirin (75mg) plus ticagrelor (90*  
37 *mg) on platelet reactivity and fractal analysis of incipient clots*

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40 Following dual anti-platelet therapy with aspirin plus ticagrelor platelet responses  
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42 followed a similar pattern to those in individuals taking aspirin plus prasugrel;  
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44 aggregations induced by AA (1mM), collagen (0.4  $\mu\text{g/ml}$ ) and ADP (5  $\mu\text{M}$  and 20  $\mu\text{M}$ )  
45  
46 were powerfully inhibited and those to collagen (4  $\mu\text{g/ml}$ ), U46619 (10  $\mu\text{M}$ ) and  
47  
48 **TRAP-6** (25  $\mu\text{M}$ ) were significantly reduced (*Figure 4A*). These reductions were  
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50 associated with a reduction in p-selectin expression ( $p<0.05$ ; *Figure 4B*) induced by  
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52 TRAP-6 (25  $\mu\text{M}$ ). Similarly, there were significant decreases in  $d_f$  for incipient clots  
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54 formed ( $1.72\pm 0.03$  to  $1.62\pm 0.02$ ,  $p=0.04$ ) (*Supplementary, Table 7*) and marked  
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3 reductions in mean mass values ( $-45\pm 14\%$  change,  $p=0.04$ ). Haematological  
4 parameters, and selected haemostatic parameters and gel time were not altered  
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7 (*Supplementary, Table 5 & 6*)  
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#### 10 11 *Clot structure analysis by scanning electron microscopy and fractal analysis*

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13 Scanning electron microscopy demonstrated that dual anti-platelet therapy resulted  
14 in formation of clots with reduced fibrin density and increased fibrin strand diameter  
15  
16 (*Figure 5*). This reduction in clot density was consistent with the  $d_f$  measurements  
17  
18 and projections of random fractal aggregates for the aspirin plus prasugrel and  
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20 aspirin plus ticagrelor groups showing a reduction in the density of connectedness  
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22 compared to that projected from blood collected under control conditions (*Figure 6*).  
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#### 28 29 *Correlation analysis between fractal analysis and other parameters*

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31 The relationships between pre and post treatment values of  $d_f$  and the haemostatic,  
32  
33 haematological and platelet parameters are shown in Table 1. The only haemostatic  
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35 or haematological parameter that showed a clear correlation with  $d_f$  was fibrinogen  
36  
37 ( $0.376$ ,  $p=0.04$ ). Conversely, regarding the relationships between  $d_f$  and the various  
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39 platelet activity tests significant correlations were found with AA (1mM) ( $0.332$ ,  
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41  $p=0.006$ ), ADP (5 $\mu$ M) ( $0.366$ ,  $p=0.002$ ), ADP (20 $\mu$ M) ( $0.369$ ,  $0.002$ ), collagen  
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43 ( $0.4\mu$ g/ml) ( $0.343$ ,  $p=0.004$ ) and TRAP-6 (25 $\mu$ M) ( $0.309$ ,  $p=0.01$ ).  
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## 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<sup>40</sup>; 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  $d_f = 1.72 (\pm 0.05)$ . 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

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3 concentrations of ADP and collagen. ATP release, activation of GP IIb/IIIa receptors  
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5 and p-selectin expression were not significantly affected. In accordance with these  
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7 limited effects on platelet function, aspirin produced no significant change in clot  
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9 structure as detected by  $d_f$  ( $1.71 \pm 0.01$  to  $1.69 \pm 0.01$ ,  $p=0.41$ ). The change in mean  
10  
11 relative mass was also non-significant ( $-8 \pm 19\%$  change,  $p=0.47$ ). Prasugrel  
12  
13 monotherapy substantially blunted platelet aggregation responses to all agonists  
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15 tested apart from TRAP-6 and caused significant reductions in ATP release, p-  
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17 selectin expression and GP IIb/IIIa receptor activation. Platelet inhibition was  
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19 associated with both a significant reduction in  $d_f$  ( $1.72 \pm 0.02$  to  $1.67 \pm 0.01$ ,  $p=0.03$ )  
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21 and a considerable change in the mean relative mass of incipient clots ( $-40 \pm 11\%$   
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23 change,  $p=0.03$ ) indicative of a substantial change in structure.  
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30 Following our monotherapy studies, which indicated a strong association between  
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32 platelet activity and clot architecture we then sought to characterise the influences of  
33  
34 dual anti-platelet therapy. Administration of aspirin plus prasugrel or aspirin plus  
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36 ticagrelor resulted in significant levels of platelet inhibition to all agonists, abolishing  
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38 responses to weaker agonists tested. P-selectin expression was also significantly  
39  
40 reduced in both groups. Again, significant platelet inhibition was coupled with a  
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42 reduction in  $d_f$ : aspirin plus prasugrel ( $1.73 \pm 0.02$  to  $1.68 \pm 0.02$ ,  $p=0.03$ ) and aspirin  
43  
44 plus ticagrelor ( $1.72 \pm 0.03$  to  $1.62 \pm 0.02$ ,  $p=0.04$ ). There were also significant  
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46 reductions in mean relative mass both for aspirin plus prasugrel ( $-35 \pm 16\%$  change,  
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48  $p=0.04$ ) and for aspirin plus ticagrelor ( $-45 \pm 14\%$  change,  $p=0.04$ ).  
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54 Overall the anti-platelet therapies had little effect on haematological and haemostatic  
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56 parameters (*Supplemental Tables 2,3,4,5 & 7*) but the changes in  $D_f$  did correlate  
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3 with changes in the platelet reactivity tests (see Table 1). Whilst as noted above,  $d_f$   
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5 correlates with fibrinogen concentration it is important to note that fibrinogen  
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7 concentrations did not change between the pre- and post- measurements for any of  
8  
9 the anti-platelet drugs tested (aspirin, prasugrel, aspirin plus prasugrel, and aspirin  
10  
11 plus ticagrelor). This indicates that the magnitude of  $d_f$  is not solely dependent on the  
12  
13 amount of fibrinogen present, but also how the processes through which fibrinogen  
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15 forms a fibrin network dependent upon platelet reactivity.  
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21 Overall our results clearly demonstrate that stronger inhibition of platelet function is  
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23 associated with the formation of less dense clots comprised of a more open/porous  
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25 network of fibrin strands. Where the inhibition of platelet function could affect other  
26  
27 activities such as coagulation and thrombin activation which would the regulate fibrin  
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29 strand formation. It is important to recognise that  $d_f$  has a very narrow range and to  
30  
31 appreciate the non-linear relationship between  $d_f$  and fibrin mass. Figure 6 helps in  
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33 this regard by providing a visual projection of how relatively small changes in fractal  
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35 dimension would underlie large changes in the mass of random fractal aggregates  
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37 (RFA); notably the lowest fractal dimension has a lower frequency of high-density  
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39 regions (i.e. the number of nearest neighbours is relatively low) consequently the  
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41 projected form is relatively sparse and open which would equate to a mechanically  
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43 weaker and more highly friable clot. On the contrary, the RFA with the highest  $d_f$  is  
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45 relatively more compact, due to the larger number of high density regions within the  
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47 structure. The density regions constrain the possible positional configurations of  
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49 adjacent constituent particles, i.e. reducing on average the spacing between  
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51 adjacent particles resulting in a relatively denser structure which would correspond to  
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53 a mechanically stronger clot less susceptible to breakdown. This predicted link  
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3 between  $d_f$  and thrombus structure is confirmed by scanning electron micrographs  
4 (SEM), which demonstrate a more open clot structure following treatment with anti-  
5 platelet therapies (*Figure 5*). These SEM images, in keeping with our previous  
6 studies highlight that incipient clot structure quantified by  $d_f$  acts as a template to  
7 predict the structure of the mature clot. Notably, the fibrin strands are much thicker in  
8 the post- as opposed to the pre-treatment images ( $d_f$  1.67 versus  $d_f$  1.63) and the  
9 number of small round particles, which represent platelets, is much reduced. This is  
10 in keeping with the association of thick fibrin strands with more porous clots and  
11 reduced platelet-fibrin interactions which we could expect following DAPT with  
12 aspirin plus ticagrelor in our healthy volunteer subject.  
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27 Results from our studies using standard single and dual anti-platelet therapy  
28 demonstrate that it is activation of P2Y<sub>12</sub> receptors that is the key driver of platelet  
29 interactions in clot formation, in keeping with the contribution of ADP but not TXA<sub>2</sub>  
30 receptors in the initiation of intravascular coagulation [38]. We did not observe a  
31 significant microstructural change in volunteers taking aspirin 75mg, in keeping with  
32 a minimal reduction in platelet reactivity. This contrasts with a recent study, where  
33 treatment with 300mg aspirin in patients with ischaemic stroke produced a significant  
34 reduction in  $d_f$ . [28] Aspirin primarily acts to inhibit platelet cyclo-oxygenase-1  
35 however, at higher doses it exerts other effects such as acetylation of fibrinogen,  
36 prothrombin and other coagulation factors [39] [40]. Therefore, we could anticipate to  
37 detect this direct role in clot modulation with higher doses of aspirin (300mg) but  
38 perhaps not at lower doses (75mg) and this difference was readily detectable by our  
39 global haemorheological marker of clot structure. It is also notable that addition of  
40 aspirin to prasugrel produced no further reduction in markers of clot density despite  
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3 additional inhibition of platelet thromboxane  $A_2$  pathways. As we have suggested  
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5 previously this may indicate that in the presence of strong  $P2Y_{12}$  receptor blockade  
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7 aspirin produces little or no additional therapeutic benefit [41, 42].  
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11 Our results are readily understandable as platelets are central to several key  
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13 coagulation processes. Activated platelets expose phosphatidylserine to support the  
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15 function of the prothrombinase complex greatly enhancing coagulation [43], interact  
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17 with many coagulation factors [44, 45] and associate with tissue factor [46]. Platelets  
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19 are instrumental in providing a surface for large scale thrombin production [47] and  
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21 are involved in fibrin formation and organisation with activated GPIIb/IIIa receptors  
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23 regulating clot retraction [48]. Previous studies have shown that platelets are  
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25 important in fibrin fibre growth where the fibres originate from the platelet aggregates  
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27 in clots formed in platelet-rich plasma [49]. This is an important consideration in  
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29 understanding  $d_f$  as it is derived from a viscoelastic measurement of the incipient clot  
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31 formation, where the fibrin network provides the dominant viscoelastic response [26].  
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33 Dense regions of fibres would inevitably influence the viscoelastic properties and  
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35 consequently the value of  $d_f$ . Therefore, platelets as the promoters of fibrin fibre  
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37 growth would contribute to an increase in elasticity and thus a corresponding  
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39 increase in the  $d_f$ . Consequently, in the presence of platelet inhibition and hence a  
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41 reduction in the number, or absence, of platelet aggregates a lower value of  $d_f$  would  
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43 be expected. In the present study comparison of responses in the absence and  
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45 presence of different levels of platelet inhibition identifies a significant relationship  
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47 between platelet function and the complex, highly disordered structure of incipient  
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49 clots in whole unadulterated blood from healthy volunteers, echoing a previous  
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51 report that altered clot structures are associated with aspirin treatment failure [50].  
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3 As well as providing further mechanistic insight into the links between platelet  
4 reactivity and clot formation, our data indicates that the therapeutic benefits of anti-  
5 platelet therapies are dependent upon their abilities to reduce thrombus density.  
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7 Analysis of our SEM micrographs further confirms that these anti-thrombotic effects  
8 are partly attributable to the formation of thicker fibrin fibres, increased volume of  
9 pore spaces and an increased permeability of the clot's fibrinolytic components. This  
10 deeper understanding of the role of platelets in thrombus formation could be useful in  
11 individual patients to determine the functional relationship between platelet reactivity,  
12 eventual clot quality and clinical outcome.  
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25 Given the numerous mechanisms by which platelets and the coagulation system  
26 interact, a more comprehensive marker of coagulation, thrombosis and the response  
27 to therapeutic manipulation, could prove important in the prediction of  
28 thromboembolic complications. Our observations support that indices of fractal  
29 analysis are intricately linked and reflect clot structure and strength and are  
30 representative of the dynamic and interwoven properties of the fibrin clot and platelet  
31 reactivity. Importantly, this study has shown that  $d_f$  can quantify the effects of  
32 individual therapeutic interventions. The development of  $d_f$  as a point of care test to  
33 assess clot structure may have potential as a future tool in quantifying thrombotic  
34 risk, through identifying those patients who form abnormal clot structures that are  
35 associated with impaired clot lysis and increased potential to embolise. Exploited in  
36 this way,  $d_f$  could prove a rapid means of influencing clinical decisions in the  
37 management of bleeding and thrombotic risk, providing more insight into the  
38 combined effect of clotting factors and cellular components in terms of functional  
39 activity of the pathological clot and its properties. Rather than simply measuring  
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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.

For Peer Review Only

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TDW has received consultancy fees from AstraZeneca relating to clinical development of P2Y<sub>12</sub> inhibitors in the previous 3 years. PAE received a grant from National Institute of Social Care and Health Research.

## 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.



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3 **Tables**  
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8 **Table 1. Pearson correlation coefficients for  $d_f$  versus haemostatic,**  
9 **haematological and platelet parameters**  
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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 $\mu$ M)	0.366*
ADP (20 $\mu$ M)	0.369*
Collagen (0.4 $\mu$ g/ml)	0.343*
Collagen (4 $\mu$ g/ml)	0.237
U46619 (10 $\mu$ M)	0.231
TRAP-6 (25 $\mu$ M)	0.309*
P Selectin Exp.	0.079
PAC-1 Binding	0.335
ATP Release	0.286

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47 \* = Significant result ( $p < 0.05$ )  
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49 PT indicates prothrombin time; APPT activated partial thromboplastin time;  $d_f$  fractal  
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## Figure Legends

**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  $\mu$ M), collagen (0.4 and 4  $\mu$ g/mL), U46619 (10  $\mu$ M) and TRAP-6 amide (25  $\mu$ M); B) P-selectin expression; C) PAC-1 binding and D) ATP release after collagen (4  $\mu$ g/ml) stimulation. Data are presented as final aggregation (%), ATP release (nmol, mean $\pm$ SEM) or geometric mean fluorescence index (MFI) (units, mean $\pm$ SEM). Significance is shown as \*  $p < 0.05$  vs non-treated throughout.

**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  $\mu$ M), collagen (0.4 and 4  $\mu$ g/mL), U46619 (10  $\mu$ M) and TRAP-6 amide (25  $\mu$ M); B) P-selectin expression; C) PAC-1 binding and D) ATP release after collagen (4  $\mu$ g/ml) stimulation. Data are presented as final aggregation (%), ATP release (nmol, mean $\pm$ SEM) or geometric mean fluorescence index (MFI) (units, mean $\pm$ SEM). Significance is shown as \*  $p < 0.05$  vs non-treated throughout.

**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  $\mu$ M), collagen (0.4, and 4  $\mu$ g/mL), TRAP-6 amide (25  $\mu$ M) and U46619 (10  $\mu$ M); and B) TRAP-6 (25  $\mu$ M) induced P-selectin expression. Data are presented as final aggregation (%), ATP release (nmol, mean $\pm$ SEM) or geometric mean fluorescence

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3 index (MFI) (units, mean±SEM). Significance is shown as \*  $p < 0.05$  vs non-treated  
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11 **Figure 4. Effect of dual antiplatelet therapy with aspirin (75mg) plus ticagrelor**  
12 **(90 mg) on platelet reactivity.** Bar graphs depicting platelet responses prior to and  
13 following treatment as measured by: A) standard light transmission aggregometry to  
14 AA (1 mM), ADP (5 and 20  $\mu$ M), collagen (0.4, and 4  $\mu$ g/mL), TRAP-6 amide (25  $\mu$ M)  
15 and U46619 (10  $\mu$ M); and B) TRAP-6 (25  $\mu$ M) induced P-selectin expression. Data  
16 are presented as final aggregation (% , mean±SEM) or geometric mean fluorescence  
17 index (MFI) (units, mean±SEM). Significance is shown as \*  $p < 0.05$  vs non-treated  
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33 **Figure 5. Effect of dual antiplatelet therapy with aspirin (75 mg) plus ticagrelor**  
34 **(90 mg) on clot structure.** Corresponding SEM micrographs of clots formed in  
35 blood taken from one participant, before (panels (a) and (c);  $d_f$  1.67) and after  
36 (panels (b) and (d);  $d_f$  1.63) treatment with aspirin (75mg) plus ticagrelor (90 mg).  
37 Micrographs were captured at two different magnifications for the pre- and post-dual  
38 antiplatelet therapy with aspirin and ticagrelor. The magnifications chosen were 5K  
39 (panels (a) and (b)) and 10K (panels (c) and (d)). Sections of the micrograph  
40 showing only the fibrin network were chosen to highlight the differences in the pre-  
41 and post- condition on clot microstructural development.  
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3 **Figure 6: Visualisation of the relationship between fractal dimension and**  
4 **mass.** Figure (a) shows the variation of the mass of a random fractal aggregate  
5 (RFA) as a function of fractal dimension. The magnitude of the RFA is normalized to  
6 the mean healthy value,  $d_f = 1.72$  (green circle). Additionally two further points are  
7 highlighted on Figure (a) showing the mass values of two RFA's with  $d_f$  values of  
8 1.68 (red square) and 1.63 (black triangle). The red square being equivalent to the  
9  $d_f$  found for the aspirin + prasugrel and the black triangle being equivalent to the  
10 aspirin + ticagrelor group. Figures (b), (c) and (d) are numerical realisations of RFAs  
11 corresponding to fractal dimensions of (b)1.63 (aspirin + ticagrelor), (c) 1.68 (aspirin  
12 + prasugrel) and (d) 1.72 (normal). Each numerical realisation of RFA is comprised  
13 of several constituent particles of unit diameter all of which are connected to at least  
14 one other particle. The colour index in the RFA realisations represents the local  
15 densities of constituent connected particles (green and red shades represent low  
16 and high density regions respectively). A RFA realisation containing many red areas  
17 denotes a more compact, densely connected structure than a RFA with  
18 predominately green areas.  
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Figure 1

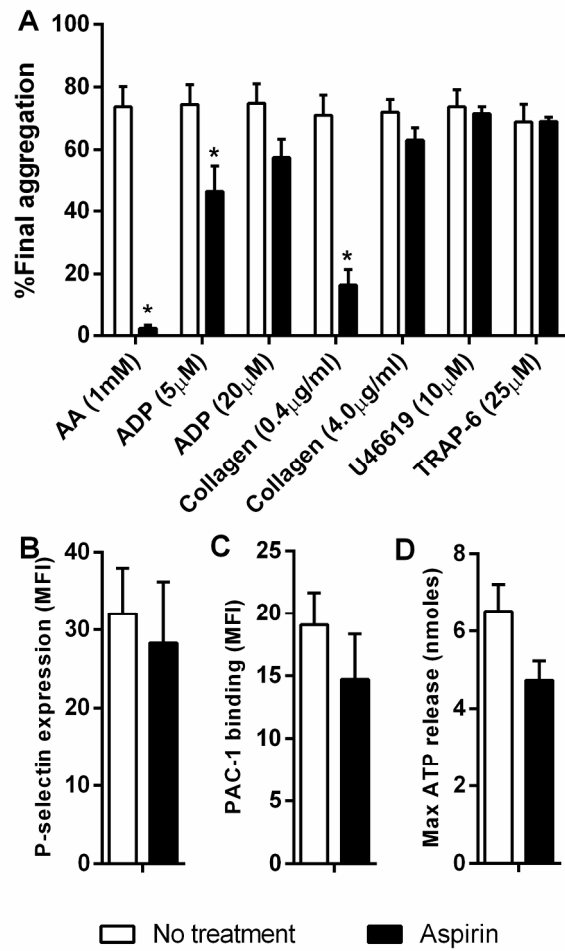


Figure 1. Effects of aspirin (75 mg) therapy on platelet reactivity.

162x300mm (300 x 300 DPI)

Figure 2

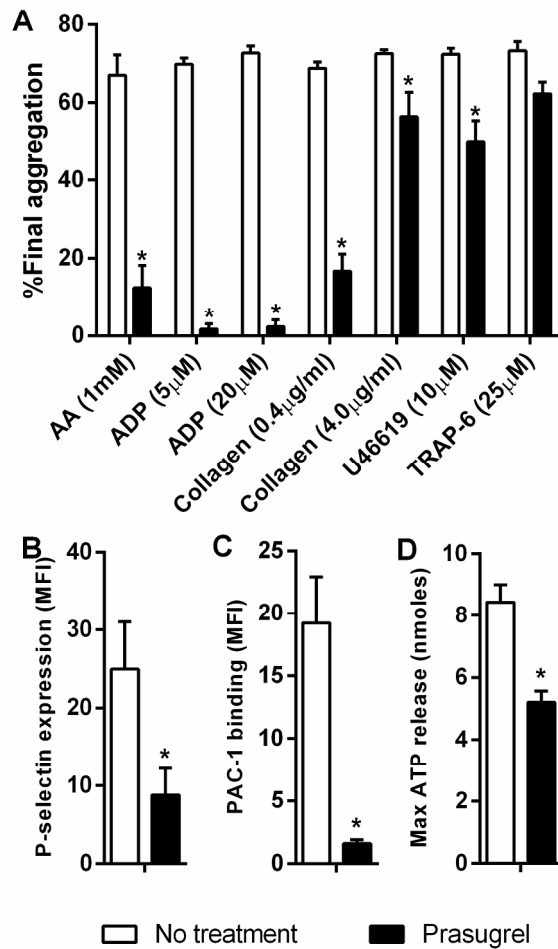


Figure 2. Effects of prasugrel (10 mg) therapy on platelet reactivity.

162x304mm (300 x 300 DPI)

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Figure 3

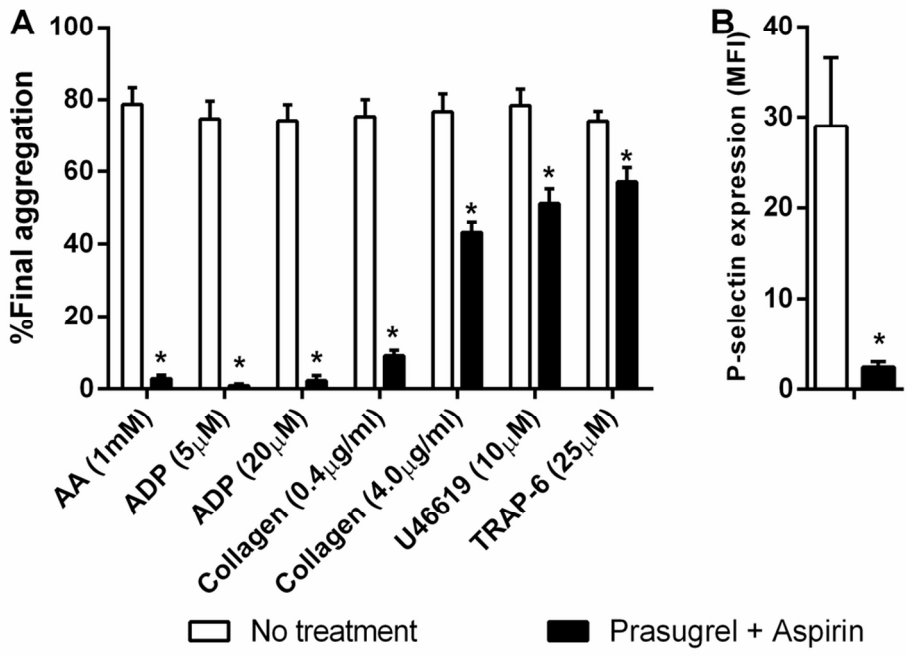


Figure 3. Effect of dual antiplatelet therapy with aspirin (75mg) plus prasugrel (10 mg) on platelet reactivity.

100x87mm (300 x 300 DPI)

Figure 4

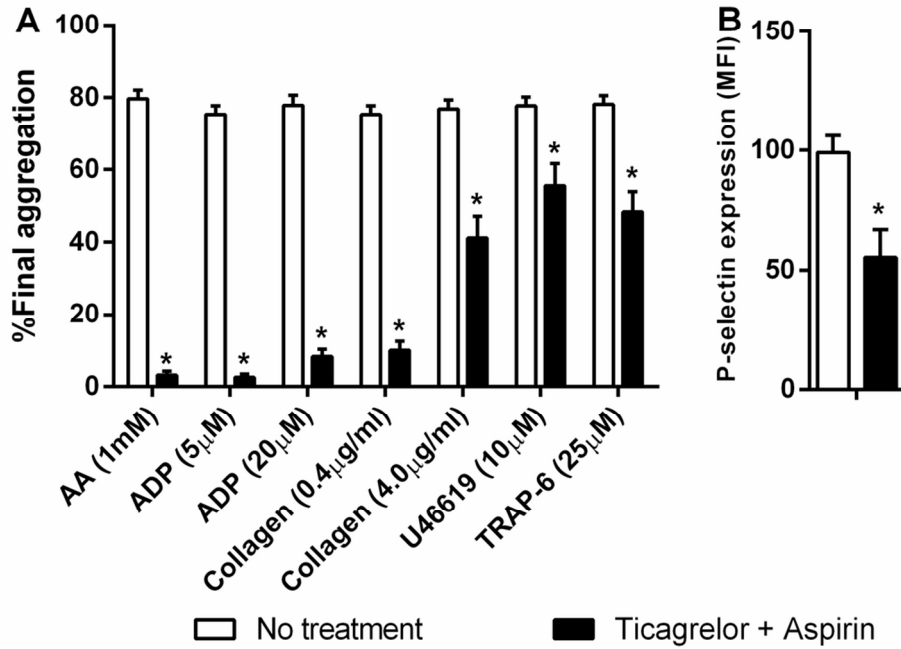


Figure 4. Effect of dual antiplatelet therapy with aspirin (75mg) plus ticagrelor (90 mg) on platelet reactivity.

100x87mm (300 x 300 DPI)

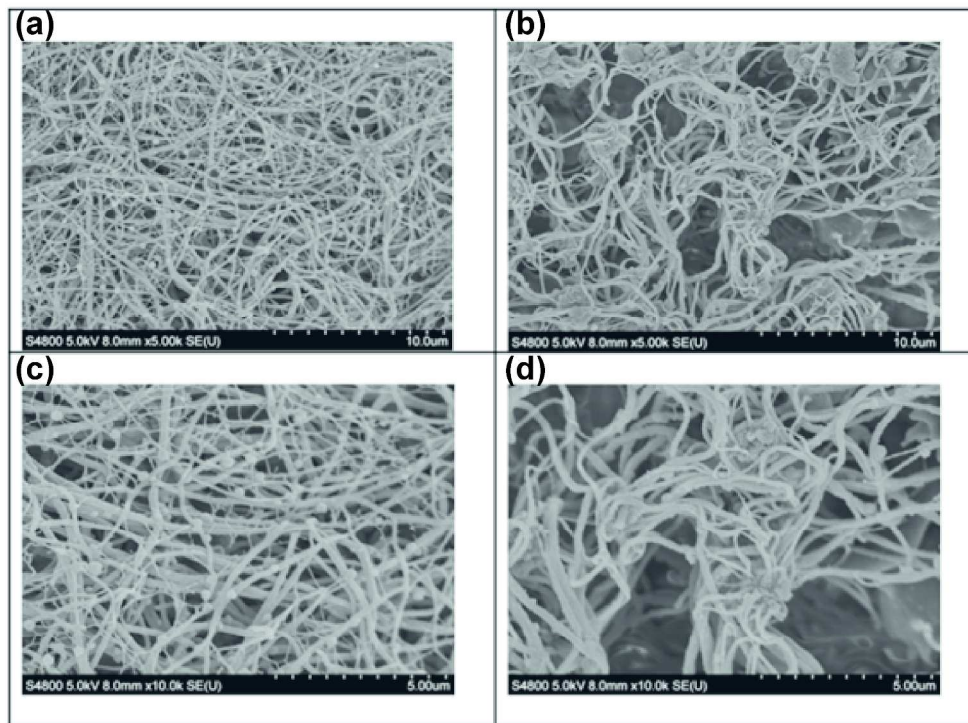


Figure 5. Effect of dual antiplatelet therapy with aspirin (75 mg) plus ticagrelor (90 mg) on clot structure. Corresponding SEM micrographs of clots formed in blood taken from one participant, before (panels (a) and (c); df 1.67) and after (panels (b) and (d); df 1.63) treatment with aspirin (75mg) plus 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.

646x481mm (150 x 150 DPI)

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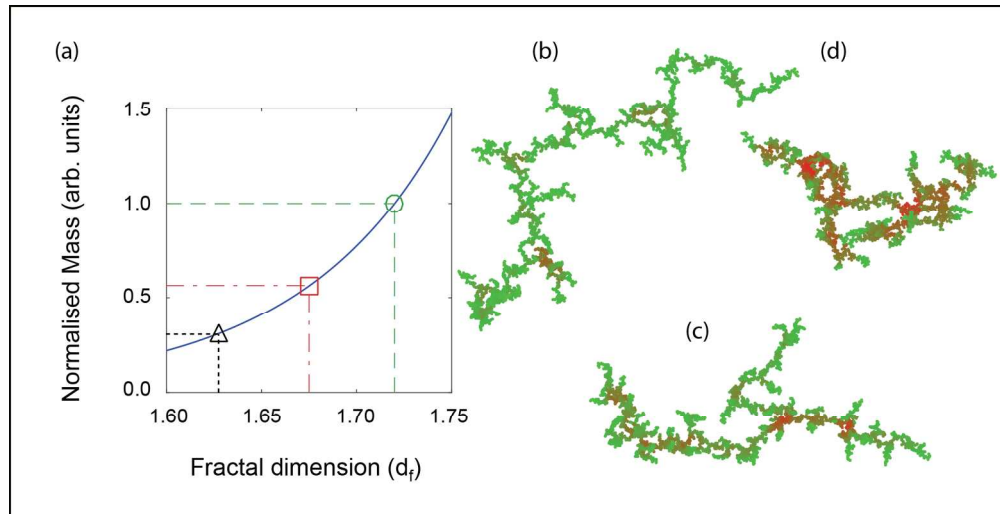


Figure 6: Visualisation of the relationship between fractal dimension and mass. Figure (a) shows the variation of the mass of a random fractal aggregate (RFA) as a function of fractal dimension. The magnitude of the RFA is normalized to the mean healthy value,  $df = 1.72$  (green circle). Additionally two further points are highlighted on Figure (a) showing the mass values of two RFA's with  $df$  values of 1.68 (red square) and 1.63 (black triangle). The red square being equivalent to the  $df$  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.

175x90mm (300 x 300 DPI)

## Supplemental data

Table 2. Haematological and haemostatic parameters pre and post aspirin therapy.

	No treatment	Aspirin
Hb	15.1 ± 0.36	14.9 ± 0.31
HCT	0.44 ± 0.01	0.43 ± 0.01
Plt	210 ± 16.1	217 ± 13.5
PT	10.6 ± 0.2	11 ± 0.22
APPT	27.88 ± 0.92	28.63 ± 0.89
APPT <sub>r</sub>	1.09 ± 0.03	1.1 ± 0.03
INR	0.99 ± 0.02	1.03 ± 0.03
Fibrinogen	1.98 ± 0.16	1.88 ± 0.15

Table 3. Haematological and haemostatic parameters pre and post prasugrel therapy.

	No treatment	Prasugrel
Hb	14.9 ± 0.28	14.4 ± 0.25
HCT	0.44 ± 0.01	0.42 ± 0.01
Plt	237 ± 9.56	237 ± 11.6
PT	10.6 ± 0.08	11 ± 0.15
APPT	27.25 ± 0.75	27.50 ± 0.73
APPT <sub>r</sub>	1.05 ± 0.03	1.05 ± 0.03
INR	1.00 ± 0.00	1.01 ± 0.01
Fibrinogen	2.04 ± 0.14	1.98 ± 0.13

Table 4. Haematological and haemostatic parameters pre and post DAPT with aspirin and prasugrel therapy.

	No treatment	Aspirin + Prasugrel
Hb	15.6 ± 0.34	15.5 ± 0.36
HCT	0.47 ± 0.01	0.48 ± 0.01
Plt	173 ± 25	175 ± 25.5
PT	11.2 ± 0.23	11 ± 0.28
APPT	29 ± 1.00	28 ± 0.9
APPT <sub>r</sub>	1.11 ± 0.04	1.06 ± 0.04
INR	1.09 ± 0.03	1.06 ± 0.03
Fibrinogen	2.03 ± 0.07	2.16 ± 0.17

**Table 5. Haematological and haemostatic parameters pre and post DAPT with aspirin and ticagrelor therapy.**

	No treatment	Aspirin + Ticagrelor
Hb	14.8 ± 0.41	14.7 ± 0.37
HCT	0.42 ± 0.01	0.41 ± 0.01
Plt	208 ± 7.2	218 ± 7.5
PT	10.7 ± 0.14	10.7 ± 0.17
APPT	28.0 ± 0.42	26.8 ± 0.55
APPT <sub>r</sub>	1.09 ± 0.01	1.04 ± 0.02
INR	1.07 ± 0.02	1.03 ± 0.02
Fibrinogen	2.05 ± 0.23	2.10 ± 0.14

**Table 6. Gel time (T<sub>GP</sub>) rheological parameter pre and post aspirin, DAPT with aspirin and prasugrel therapy (A+P) & DAPT with aspirin and ticagrelor therapy (A+T).**

	Pre (sec)	Post (sec)
Aspirin	162 ± 41	174 ± 43
Prasugrel	152 ± 64	166 ± 28
A+P	193 ± 78	183 ± 81
A+T	219 ± 100	201 ± 79

**Table 7. Fractal dimension (d<sub>f</sub>) rheological parameter pre and post aspirin, DAPT with aspirin and prasugrel therapy (A+P) & DAPT with aspirin and ticagrelor therapy (A+T).**

	Pre (sec)	Post (sec)
Aspirin	1.71 ± 0.01	1.69 ± 0.01
Prasugrel	1.72 ± 0.02	1.67 ± 0.01* (p=0.03)
A+P	1.73 ± 0.02	1.68 ± 0.02* (p=0.03)
A+T	1.72 ± 0.03	1.61 ± 0.02* (p=0.04)