Original Article

A new structural biomarker that quantifies and predicts changes in clot strength and quality in a model of progressive haemodilution

Matthew J. Lawrence[1,2], Sendhil Kumar[3], Karl Hawkins[2], Stuart Boden[4], Harvey Rutt[4], Gavin Mills[1], Ahmed Sabra[1,2], Roger H.K. Morris[5], Simon J. Davidson[6], Nafieseh Badiei[7], Martin R. Brown[7], Phylip R. Williams[7], Phillip A. Evans[1,2,3]*

1. NISCHR Haemostasis Biomedical Research Unit, Morriston Hospital, Abertawe Bro Morgannwg University Health Board, Swansea, UK; 2. NISCHR Haemostasis Biomedical Research Unit, College of Medicine, Swansea University, Swansea, UK; 3.Emergency Department, Morriston Hospital, Abertawe Bro Morgannwg University Health Board, Swansea, UK; 4. ECS, Faculty of Physical and Applied Sciences, University of Southampton, Southampton, UK; 5. School of Applied Science, University of Wales Institute Cardiff, Cardiff, UK; 6.Department of Haematology, Royal Brompton Hospital, Royal Brompton and Harefield NHS Foundation Trust, London, UK; 7.College of Engineering, Swansea University, Swansea, UK

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Corresponding Author:
Name: Phillip Adrian Evans
Department: Director, NISCHR Haemostasis Biomedical Research Unit
Institution: Abertawe Bro Morgannwg University Health Board
Mailing Address: NISCHR Haemostasis Biomedical Research Unit, Emergency Dept., Morriston Hospital, Abertawe Bro Morgannwg University Health Board, Swansea, UK, SA6 6NL
Phone: (+44) 1792 703418
Email: phillip.evans2@wales.nhs.uk

Abstract (244)

Introduction: We investigated the effect of progressive haemodilution on the dynamics of fibrin clot formation and clot microstructure using a novel rheological method. The technique measures clotting time (T_{GP}), clot strength ($G`_{GP}$), and quantifies clot microstructure (d_f) at the incipient stages of fibrin formation. We use computational modelling to examine the relationship between structure and mass, as well as helium ion microscopy (HIM) to compare morphological changes in the fully formed clot to that of the incipient clot.

Methods: This is an *in vitro* study; 90 healthy volunteers were recruited with informed consent and a 20ml sample of whole blood obtained from each volunteer. Five clinically relevant dilutions were investigated using 0.9w.v isotonic saline (0, 10, 20, 40 and 60%, n=18 for each dilution). The rheological method of assessing structural clot changes was compared against conventional coagulation screen and fibrinogen estimation.

Results: Fractal dimension (d_f) and final clot microstructure both decreased with progressive dilution (significant at a dilution of 20%) with similar relationships observed for final clot characteristics in HIM images. Significant correlations were observed between d_f and $G`_{GP}$ (clot strength) (0.345, p=0.02), as well as clotting time (PT: -0.690, p>0.001; APTT: -0.672, p>0.001; T_{GP}: -0.385, p=0.006).

Conclusions: This study provides new insight into the effects of haemodilution by isotonic saline on clotting time (T_{GP}), clot strength (G'_{GP}) and clot microstructure (d_f). Previous studies have

attempted to link clot microstructure to clot quality/strength, however this study provides a significant step in quantifying these relationships.

Keywords: Dilution, Fluid Resuscitation, Microstructure, Fractal Dimension

Abbreviations: fractal dimension (d_f), gel point (GP), time to gel point (T_{GP}), clot strength ($G`_{GP}$)

Introduction

Recent advances in our understanding of haemorheological changes in coagulation have resulted in the development of new methods for the analysis of clot characteristics, including clot microstructure.[1-4] In the present study we exploit a novel haemorheological technique that uses oscillatory shear to detect the establishment of the *incipient* clot (the first point at which a solid clot is formed that can achieve its haemostatic function).[3-6] This technique provides three separate but related biomarkers of clot properties, the rate of coagulation (T_{GP}), clot elasticity/strength ($G`_{GP}$) and clot microstructure (d_f), which are all taken from one measurement, the Gel Point (GP). The d_f represents a quantitative measure of *clot microstructure*, a decrease in d_f corresponding to more permeable, less branched and mechanically weaker clot and a raised d_f giving a tightly packed, highly branched clot that is less permeable and stronger.[3-6] Previous studies have highlighted the importance of clot microstructure and how it affects the rate of clot lysis, where densely organised clots have been linked to thromboembolic disease.[7-10]

This study investigates the *in-vitro* effect of dilution with saline on the organization and arrangement of clot microstructure and how this affects the mechanical properties of the developing clot. The aim is to determine whether quantifying the changes in clot microstructure provides an improved clinical biomarker of haemostasis and clot quality, particularly in the setting of surgery, trauma and critical illness where the administration of resuscitation fluids and the associated effect on coagulation and clot quality still poses a significant clinical issue.[11-15] In this study the incipient clot measurements of clotting time (T_{GP}), clot strength (G'_{GP}) and clot microstructure (d_f), are used to investigate the effects of progressive haemodilution by a commonly used crystalloid fluid, saline. Furthermore computational simulation was used to illustrate the

relationship between clot microstructure (d_f) and mass incorporated into the structure during progressive dilution. We investigated the role of the incipient clot microstructure (d_f) as a template of further clot development by comparing it to images of the mature clot using helium ion microscopy (HIM).

Materials and Methods

Study Design and Population Group

The study was approved by local Research Ethics Committee. Two-stage fully informed and written consent was obtained from all subjects before enrolment. Blood was obtained from (n=90) healthy volunteers. The inclusion criteria included all volunteers to be 18 years and above, individuals with no history of an acute or chronic condition known to effect coagulation (e.g. cancer, hepatic and/or renal dysfunction) and with no personal or family history of bleeding or thromboembolic disorders, with all participants having a normal full blood count and biochemistry profile. Subjects were excluded if currently undergoing any anti-platelet or anti-coagulation treatment.

Blood Sampling

Prior to taking any sample, each subject was assigned to *one* of 5 test dilutions, these included 0% (n=18), 10% (n=18), 20% (n=18), 40% (n=18) and 60% (n=18). Each test dilution is based on a volume percentage of isotonic saline (Baxter, Viaflo, FKE1323 Sodium Chloride 0.9% w/v pH 5.5) in blood. A 20ml sample of blood was obtained atraumatically from the antecubital vein via an 18 -gauge needle for each volunteer. The sample was added to a specified volume of isotonic saline and gently inverted 3 times to aid mixing. The diluted blood was divided into two aliquots. The first (7ml) was immediately transferred to the measuring geometry of a Rheometer (see *Gel Point Measurements: Fractal Dimension and Clot formation time*). The second aliquot (≈10ml) was used to obtain a coagulation screen and full blood count (FBC) (see *Laboratory Markers*). The entire process, from taking the blood to loading the diluted whole blood within the rheometer, was in all cases <60seconds. To avoid any diurnal variations blood samples were obtained in the

same laboratory, at the same time of day. To ensure all participants had a normal haematological profile, a baseline coagulation screen (including Clauss fibrinogen) and FBC was obtained using an aliquot of *undiluted* blood taken prior to mixing with the fluid. Furthermore for 3 of the volunteers an additional aliquot of undiluted blood was used to gather images of the mature (fully formed) clot (see *Helium Ion Microscopy Imaging of Whole Blood*).

Gel Point Measurements: Fractal Dimension and Clot formation time

The haemorheological method was performed using whole physiological native blood and has been described previously.[3] Briefly a 6.6 ml aliquot of diluted blood was immediately loaded into a double-gap concentric cylinder of a TA Instruments AR-G2 controlled-stress rheometer at 37 ºC (± 0.1ºC). Immediately after sample loading, small amplitude oscillatory shear measurements were performed at test frequencies 2Hz, 0.93Hz, 0.43Hz and 0.2Hz with a peak stress amplitude of 0.03Pa. The measurements provided values of the shear elastic modulus, G' (a measure of clot strength) and the loss modulus, G'' (a measure of the viscous properties of the sample), and phase angle, δ ($\tan\delta = G''/G'$)(see Figure 1). The method detects the Gel Point (GP), by measuring the change in δ at the different frequencies, with respect to time, and recording the attainment of a frequency independent δ (see Figure 2).[2,3,16] The GP marks the first time that the fluid transitions to a solid. In coagulating blood this transition corresponds to the formation of the incipient clot, whose sample-spanning fibrin network provides the mechanical basis for attaining a haemostatic function.[3-6] A recent study involving analysis of GP and imaging data has established that the incipient clot acts as a template for ensuing clot development.[5,6]

The ability to detect the GP allows us to quantify how the fibrin clot is organised using fractal analysis. Fractal analysis is a method commonly used in biology to describe how complex structures develop and perform their specific biological role.[17,18] The incipient clot's fractal dimension, d_f, was calculated from the frequency independent value of δ at the GP using the equation $d_f = (D + 2)(2\theta - D)/2(\theta - D)$ where D is the space dimension (D = 3 herein) and the exponent, θ, is obtained from the equation $\delta = \theta\pi/2$.[17,18]

The following parameters were recorded: (i) the time taken to reach the GP (the incipient clot formation time), T_{GP}, which is a measure of the biochemical reaction of the clotting enzymes; (ii) the shear elastic modulus at the GP, G'_{GP}, which is a measure of the strength of the incipient clot;and (iii) the value of δ at the GP which is used to calculate, d_f, which is a measure of the biophysical change in clot structure. We have previously shown that the determination of d_f is highly reproducible over the measurement range.[15] All GP data was corroborated by an experienced, blinded-assessor, independent of the study.

A previously published computational simulation exploring the relationship between d_f and mass was also used.[5,19] The simulation provides computer images of clots at relevant values of d_f and defines the relationship between d_f and the amount of fibrin mass incorporated into the structure of the incipient clot.

Helium Ion Microscopy Imaging of Whole Blood

To obtain images of the mature clot microstructure at different dilutions, aliquots of whole undiluted blood (<2.5ml) from 3 of the volunteers was used. Each aliquot was divided into 5 parts

and diluted with isotonic saline to one of the 5 different dilutions (0%, 10%, 20%, 40% and 60%). 7µl of the diluted bloods were immediately transferred to plexiglass microdialysis cells perforated for solvent perfusion. The samples were left undisturbed at 37°C and allowed to clot for approximately 30minutes. The clots were then fixed using 2.5% glutaraldehyde and dried with ethanol using the same sample preparation as used in a previous study.[20] Following this process, samples were coated in 3 nm of platinum using an ion beam sputter coating system (PECS™, Gatan, Inc.). The samples were then imaged using a helium ion microscope (HIM, Orion Plus™, Carl Zeiss), with a beam energy of approximately 33 keV, a beam current of 0.4 -0.6 pA and a working distance of between 6.9 and 8.9 mm. The beam limiting aperture diameter was 10 µm, the field of view was 14 µm and image size was 1024 × 1024 pixels. An integrated Everhart Thornley in-chamber secondary electron detector was used with a grid bias of 500 V, dwell time of 0.5 µs and line averaging of 128.

HIM is a technique conceptually similar to scanning electron microscopy, however it is also capable of higher resolution imaging.[21] It was chosen for this work as its smaller beam convergence angle leads to a superior depth of field (five times that of a typical scanning electron microscope (SEM)).[21] A large depth of field is particularly useful when imaging blood clot samples because of their inherent 3D nature and high degree of surface topography. More fibrils within the image can be measured because more appear in focus, an advantage already applied to imaging other biological specimens.[22-24]

Assessment of the arrangement of the clot microstructure was achieved by qualitative assessment of the images by visual inspection focusing on the general topography of the clot, and through

quantification of the fibre widths in the clot. Fibre width were measured using the computer software program ImageJ® (Rasband WS, ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA) measuring widths of 100 different fibres at each dilution, using randomly generated x,y coordinates from within multiple fields of view. [25]

Coagulation Screen and Full Blood Count

A 4 ml aliquot of blood was taken from the undiluted bulk sample and used for FBC analysis, samples being collected into plastic, dipotassium EDTA Vacuettes (Greiner Bio-One, Stonehouse, UK Ref: 454286). FBC was analysed using a Sysmex XE 2100 (Sysmex UK, Milton Keynes, UK) automated haematology analyser.

An additional 4.5ml of blood was used for routine coagulation studies, being transferred immediately into citrated siliconized glass Vacutainers (0.109M) (Becton-Dickinson, Plymouth, UK Ref: 367691). Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT) and Clauss fibrinogen were measured using a Sysmex CA1500 analyser (Sysmex UK, Milton Keynes, UK). Fibrinogen calibration was verified against the 2nd International Fibrinogen Standard Version 4 (NIBSC code 96/612). All reagents were obtained from Siemens, (Frimley, UK). All testing was performed within 2 hours of sample collection.

Statistics and Sample Size Estimation

Statistical analysis was performed using Minitab® version 16 software (Havertown, PA). Data was normally distributed and one-way ANOVA was used to compare mean differences between blood at the various dilutions and *post-hoc* pairwise analysis used to determine when true differences

arose. Pearson correlation was undertaken to explore associations between d_f and standard markers of coagulation. Significant deviation from the baseline value of undiluted blood was determined using one-way ANOVA and Tukey's pairwise analysis. Data was assumed to be significant when $p<0.05$ unless otherwise stated. All results including fibre diameter are reported as mean and standard deviation unless otherwise stated. Correlation analysis to investigate significant relationships between the various parameters was assessed using Pearson's method. Sample size was calculated based on a mean difference in d_f of 0.1 (an expected change based on some preliminary data, not shown) between 0% and 60% diluted blood, with an estimated standard deviation of 0.05 to achieve a power of 0.9 and significance set to a value of 0.05.

Results

The results of the standard and specific laboratory markers and rheological measurements are shown in Table 1. As expected, the standard laboratory markers PT, APTT Haematocrit, Fibrinogen, and platelet count all show a progressive linear change as dilution is increased. Significant changes from baseline for the standard markers of coagulation are shown in Table 1. PT and APTT only show statistically significant changes at dilutions of >40%, whereas Fibrinogen concentration, Haematocrit and platelet count show a significant change at a dilution of >20%.

The effect of dilution on the measure of clotting time, T_{GP}, indicates no significant changes compared to baseline (0%) up to a dilution of 40%, whereas at 60% a significant increase in T_{GP} is recorded. An overall decrease in the measure of clot microstructure, d_f, is observed as dilution increases, (Figure 3), the exception being at 10% dilution where no significant change in d_f is noted. The d_f for baseline was 1.74 ± 0.033, a result commensurate with the value reported for a healthy cohort in a previous study, where d_f = 1.74 ± 0.07.[3] The strength of the incipient clot, G'_{GP}, reduces with increasing dilution, this reduction corresponding to the change in d_f and confirming that the mechanical properties or strength of the incipient clot are related to its microstructural organization.

Visual inspection of the HIM images (Figure 4) provides a qualitative description of the effect of dilution on mature clot microstructure. Figure 4a shows a progressive change as dilution increases, where an apparent looser, more open clot is formed with a large decrease in the amount of polymerized fibrin held within the clot network. Quantifying these morphological changes in clot structure is carried out by measuring the average fibre widths in these images (Figure 4b) reveals

a progressive decrease from 290nm ± 26nm at 0% to 105nm ± 19nm at 60% dilution (where 271nm ± 37nm at 10%, 228nm ± 35nm at 20%, 157nm ± 11nm at 40%). The images and fibre width calculations clearly demonstrate a gross change in clot structure with progressive dilution which shows agreement with the results of d_f.

The results of the computational model used to relate d_f to the mass incorporated into the clot structure can be seen in Figure 5. The Figures 5(a), (b) & (c) show 3 different clot structures which have d_f values of 1.74, 1.65 and 1.55 respectively. These images illustrate clear differences in clot microstructure with higher values of d_f producing denser, less porous clot structures. Furthermore, Figure 5 shows the exponential relationship between d_f and mass incorporated into the clot microstructure (where a structure with a d_f of 1.74 has 10 times more mass than a structure with a d_f of 1.55).

Correlation analysis

T_{GP} was significantly correlated with Fibrinogen concentration (correlation coefficient, ρ =0.302, p=0.004), Platelet Count (-0.278, p>0.01), Haematocrit (-0.300, p=0.005), PT (0.356, p=0.001), APTT (0.394, p>0.001) across the whole range of dilution. d_f correlated significantly with Fibrinogen concentration (0.674 p=0.001), Platelet Count (0.557, p=0.001), Haematocrit (0.662, p>0.001), PT (-0.690, p=0.001), APTT (-0.672, p>0.001), $G`_{GP}$ (0.345, p=0.02), and T_{GP} (-0.385, p=0.006).

Discussion

In this *in vitro* study, we quantify for the first time how progressive dilution of whole blood by a clinically used isotonic crystalloid solution causes a significant change in organization and arrangement of clot microstructure and quality. Analysis of the haemorheological data reveals no significant change in d_f at the lowest dilution (10%). At dilutions greater than 10% a significant reduction in d_f is recorded compared to baseline (1.74 ± 0.033 at 0% dilution to 1.69 ± 0.032 at 20% dilution), indicating a substantial change in clot microstructure. We report several significant correlations between d_f and standard time based markers of kinetic coagulation, which would be expected due to the large scale effect of haemodilution on haemostasis over the range of dilution studied. Interestingly we also show that d_f significantly correlated with G'_{GP}, both decreasing as dilution increased. This finding provides further evidence of the relationship between clot microstructure, d_f (the spatial and structural configuration of the clot's fibrin network) and its mechanical properties, $G`_{GP}$, (clot strength) which underpins the clot's haemostatic functionality.[26-27] In this study the rate based assessments of coagulation (PT, APTT and T_{GP}) show a general prolongation with progressive dilution, however the changes only become significant at much larger dilutions (≥40%) than that seen for clot microstructure (≥20%).

Previous studies investigating changes in coagulation induced by dilution of blood have indicated that the amount of available fibrinogen is critical in maintaining or retaining 'normal' clot function and/or strength.[28-30] However, in surgery and trauma the fibrinogen concentration required to provide a stable haemostatic clot is uncertain. Several studies investigating the optimal fibrinogen concentration have recommended a range values (>1.0 g/L to <2.5 g/L).[31-34] In the present study we report significant changes in the incipient clot microstructure, d_f, at a dilution of ≥20%

(see Figure 3). The d_f changes from 1.74 ± 0.033 (at 0% dilution) to 1.69 ± 0.032 (at 20% dilution) with a corresponding change in fibrinogen concentration of 2.6 ± 0.3 g/L (at 0% dilution) to 2.1 ± 0.5 g/L (at 20% dilution). This highlights that d_f is highly responsive to small changes in fibrinogen concentration. The significant reduction in d_f even at a fibrinogen concentration of 2.1± 0.5 g/L is striking, as this concentration resides in the higher range of values required to provide stable haemostasis quoted by other studies. When interpreting d_f it is important to consider that it is a measure of how fibrinogen is incorporated and organised into the incipient fibrin clot microstructure and not a simple measure of the amount of fibrinogen available.[5-6] It is noteworthy that only some 15-20% of the total available fibrinogen is estimated to be incorporated within the *incipient clot,* an important point when taking into account further clot development and the templating role of the incipient clot in mature clot development.[27]

Further analysis of the effect of dilution on clot microstructure was carried out by visual inspection of HIM images (Figure 4a) of mature (i.e. fully formed) blood clots. The images show little apparent change in clot microstructure at 10% dilution compared to 0% dilution. At dilutions of 20% or greater, changes in the clot microstructure are evident, with a progressive change to smaller fibres and less fibrin mass incorporated in the network. This study shows that the range of dilution employed in this study produces changes in clot structure as observed by the HIM images and d_f . (Figure 3 & 4b). These results confirm that changes caused by dilution at the incipient clot stage of development are reflected by the changes in the mature clot's characteristics, providing evidence of the incipient clot's microstructural templating role in predicting clot development and quality/mechanical strength.[6]

Studying the relationship between d_f and the amount of fibrin mass incorporated into the structure (Figure 5), reveals that small changes in d_f are associated with large changes in mass. A change in d_f from 1.74 at 0% dilution to 1.55 at 60% dilution corresponds to a reduction in incipient clot mass of approximately 90%(see Figure 5). From a clinical perspective, such a large reduction in mass incorporated within the incipient clot is significant, given its role as a microstructural template for ensuing clot development.[5,6] These reductions in clot mass would have a direct impact on clot properties, leading to looser, mechanically weak clots that would be readily broken down via the body's natural fibrinolytic pathways.[34,35] The change in the incipient clot microstructure and mass reported in this study (Figure 3 & 5) confer with the wholesale changes observed in the mature clot, through qualitative examination of the images and quantification by reduction in fibre width (Figure 4).

In conclusion this *in vitro* study provides new insight into the effects of haemodilution by isotonic saline on clotting time (T_{GP}), clot microstructure (d_f) and the mechanical strength of the clot ($G`_{GP}$). Previous studies have attempted to link coagulation changes to clot microstructure, however this study provides a significant step in quantifying these dynamic relationships. We show that even when the fibrinogen concentration is relatively high (2.1 g/L) the effect of small amounts of dilution can induce significant changes in clot microstructure and strength. This suggests that simply measuring fibrinogen concentration is inadequate and that understanding how the fibrin is structurally organised would be more informative in determining the effects and outcomes of dilution in the clinical setting. Furthermore, the relationship between the incipient (d_f) and mature (HIM) clot characteristics provides further supporting evidence of the template effect of incipient clot microstructure in clot development.[3] Fluid replacement and dilution is known to effect coagulation and clinical outcome, by combining measurements of clot formation time, clot microstructure and elasticity in a single measurement, this new technique has potential to provides the clinician with a rapid means of assessing the haemostatic effect of component replacement on clot properties in the management of blood loss in the acute setting. Moreover the results of this test are obtained using native untreated blood in a near patient setting. The new test therefore provides new hitherto unreported information on several significant clot parameters that may give the clinician a new basis for assessing volume and component replacement. Further work is underway to determine the different extrinsic and intrinsic effects of various replacement colloids independently alter clot microstructure and quality.

Authors' Contributions

MJL: study design and analysis, data collection (rheology), drafting of the article; SK: volunteer recruitment, blood sample collection, data collection; KH & AS: revising the article for scientific and intellectual content, interpretation of the data; SB & HR: Data collection (HIM Imaging), revising the article for scientific and intellectual content (imaging); GM: volunteer recruitment, data analysis (HIM images); DT: study design and analysis, drafting of the article; RHKM: study design and statistical analysis; SJD; revising the article for scientific and intellectual content (laboratory markers), interpretation of the data (laboratory markers); NB: sample preparation and analysis (HIM images), PRW: Study design and data analysis, revising the article for scientific and intellectual content (rheology); PAE: Idea initiation, study design and data analysis, final approval of the version to be published. All authors read and approved the final manuscript.

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Conflict of Interest

PAE and PRW have signed the International Committee of Medical Journal Editors (ICMJE) form for declaration of interest. All other authors declare no competing conflicts of interest.

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Ethics

The study was reviewed and approved by the South West Wales Research Ethics Committee. (South West Wales REC, Floor 8, National Institute for Social Care and Health Research, Research Ethics Service (NISCHR RES), 36 Orchard Street, Swansea, SA1 5AQ, Tel 01792 607416 l, Fax 01792 607533 l, WHTN 1780 7416)

Tables

	Standard Laboratory Markers					Rheology	
Dilution Vol %	Fibrinogen (clauss) g/l Mean (SD)	Haematocrit l/l Mean (SD)	Platelet Count $x10^9$ Mean (SD)	Prothrombin Time, PT sec Mean (SD)	Activated Partial Thromboplastin Time APTT sec Mean (SD)	$T_{GP,}$ Clotting Time sec Mean (SD)	$G'_{GP,}$ Clot Strength Pa Mean (SD)
0%	2.7 (0.3)	0.411 (0.033)	259 (61)	10.4 (0.4)	25.7 (1.7)	219 (73)	0.007 (0.003)
10%	2.3 (0.5)	0.370 (0.024)	226 (51)	11.3 (0.7)	26.3 (2.7)	178 (51)	0.005 (0.002)
20%	2.1 (0.5)*	0.347 (0.021)*	199 (37)*	11.8 (0.6)	29.6 (2.8)	217 (70)	0.004 (0.002)
40%	1.2 (0.3)*	0.261 (0.020)*	158 (29)*	13.9 (1.0)*	34.0 (3.0)*	238 (83)	0.003 (0.002)
60%	0.7 (0.2)*	0.197 (0.034)*	94 (16)*	19.1 (3.0)*	50.8 (8.9)*	267 (70)	0.001 (0.001)

Table 1 – Table of results for the standard laboratory markers, rheological measurements and imaging results for all dilutions. (n=18 for each dilution) *denotes a significant deviation from the baseline value using one way ANOVA Tukey's pairwise analysis where $p<0.05$).

Figure 1 –Haemorheological analysis of whole blood. A representation of a small amplitude oscillatory shear measurement being carried out on a blood sample between two testing surfaces in a rheometer. Representing the stress and strain waveforms (red and blue respectively); where the phase angle, δ, is a measure of viscoelastic response to imposed stress.

Figure 2 –GP Trace: Figure 2 provides a typical representation of a GP curve showing the change in phase angle for the different testing frequencies with respect to time. The initial response is characteristic of a viscoelastic liquid, where Figure 2 shows the measurement of the phase angle, δ, with respect to time (δ being a measure of the viscoelastic response to imposed stress). The frequency dependence of δ decreases as clotting proceeds and δ becomes frequency independent as the incipient clot is established at the GP. Thereafter the frequency dependence is characteristic of a viscoelastic solid. The structural property of the incipient clot (in terms of its fractal dimension, d_f) is derived from the frequency independent value of δ.

Figure 3 – Graph of fractal dimension (d_f) *vs.* dilution. Graphical representation of the effect of dilution on incipient clot microstructure (d_f), with a progressive decrease in d_f observed as dilution is increased. (*denotes a significant deviation from the baseline value of 0% dilution, $p<0.05$ 1-way ANOVA Tukey's pairwise analysis).

Figure 4 – (a) Representative HIM images of blood clots at 0, 10, 20, 40 and 60 vol% & (b) Graph of average fibre width (taken from HIM images) vs. dilution. Figure 4(a) presents images showing the progressive change in the arrangement of the mature clot microstructure from

0% to 60% dilution (The scale bar in the 0% picture applies to all five images and is 2 μm long). Figure 4(b) shows a progressive decrease in fibre width as dilution is increased.

Figure 5 – Computational simulation of d_f *vs.* mass. (a) Graph showing the relationship between d_f and mass incorporated into the structure. (b), (c) and (d) Illustrations of different incipient clot microstructures at particular values of d_f (1.74, 1.65 and 1.55 respectively) corresponding to the range of actual values measured in this study. (a), (b), (c) and (d) show the substantial increases in mass required to generate even small increments of d_f.

References

1. Evans PA, Hawkins K, Lawrence M, Barrow MS, Williams PR, Williams RL. Studies of whole blood coagulation by oscillatory shear, thromboelastography and free oscillation rheometry. Clin Hemorheol Microcirc 2008; 38: 267-277

2. Evans PA, Hawkins K, Williams PR, Williams RL. Rheometrical detection of incipient blood clot formation by Fourier transform mechanical spectroscopy. J Nonnewton Fluid Mech 2008; 148:122-127

3. Evans PA, Hawkins K, Morris RHK, Thirumalai N, Munro R, Wakeman L, Lawrence MJ, Williams PR. Gel point and fractal microstructure of incipient clots are significant new markers of hemostasis for healthy and anticoagulated blood. Blood 2010; 116:3341-3344

4. Weisel JW. 'Ta panta rhei'. Blood 2010;116: 3123-3124

5. Curtis DJ, Brown MR, Hawkins K, Evans PA, Lawrence MJ, Rees P, Williams PR. Rheometrical and molecular dynamics simulation studies of incipient clot formation in fibrin-thrombin gels: An activation limited aggregation approach. J Nonnewton Fluid Mech 2011; 166: 932-928

6. Curtis DJ, Williams PR, Badiei N, Campbell AI, Hawkins K, Evans PA, Brown MR. A study of microstructural templating in fibrin–thrombin gel networks by spectral and viscoelastic analysis. Soft Matter 2013; 9: 4883-4889

7. 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. Arterio Thromb Vasc Biol 2000; 20:1354-1361

8. Gabriel DA, Muga K, Boothroyd EM. The effect of fibrin structure on fibrinolysis, J Biol Chem 1992; 267:24259-24263

9. 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: 4272-4278/28.

10. Mills JD, Ariëns RAS, Mansfield MW, Gran PJ. Altered fibrin clot structure in the healthy relatives of patients with premature coronary artery disease. Circulation. 2002; 106: 1938-1942.

11. Harris T, Thomas GOR, Brohi K. Early fluid resuscitation in severe trauma. BMJ 2012; 345:e5752

12. Hess JR, Brohi K, Dutton RP, Hauser CJ, Holcomb JB, Kluger Y, Mackway-Jones K, Parr MJ, Rizoli SB, Yukioka T, Hoyt DB, Bouillion. The coagulopathy of trauma: a review of mechanisms. J Trauma 2008; 65:748 –754.

13. Kauvar DS, Lefering R,Wade CE. Impact of haemorrhage on trauma outcome: an overview of epidemiology, clinical presentations and therapeutic considerations. J Trauma 2006; 60: S3-11.

14. Santry HP, Alam HB. Fluid resuscitation: past, present, and the future. Shock 2010; 33:229 – 241.

15. Mardel SN, Saunders F, Ollerenshaw L, Edwards C, Baddeley DT. Reduced quality of clot formation with gelatin-based substitutes. Br J Anaesth 1998; 80: 204:207

16. Winter HH, Chambon F. Analysis of linear viscoelasticity of a cross-linking polymer at the Gel-Point. J Rheol 1986; 30:367-382

17. Muthukumar M, Winter HH. Fractal dimension of a crosslinking polymer at the gel point. Macromolecules 1986; 19:1284–1285

18. Scanlan JC, Winter HH. Composition dependence of the viscoelasticity of end-linked poly(dimethylsiloxane) at the gel point. Macromolecules 1991; 24:47-54

19. Brown MR, Curtis DJ, Rees P, Summers HD, Hawkins K, Evans PA, Williams PR. Fractal discrimination of random fractal aggregates and its application in biomarker analysis for blood coagulation. Chaos Solitons & Fractals 2012; 45:1025-1032.

20. Langer, B. G., J. W. Weisel, P. A. Dinauer, C. Nagaswami, and W. R. Bell. Deglycosylation of fibrinogen accelerates polymerization and increases lateral aggregation of fibrin fibers. J. Bio. Chem 1988; **263**: 15056-15063.

21. Ward BA, Notte JA, Economou NP. Helium ion microscope : A new tool for nanoscale microscopy and metrology. J Vac Sci Technol B 2006; 24: 2871–2874

22. Vanden Berg-Foels WS, Scipioni L, Huynh C, Wen X. Helium ion microscopy for high-resolution visualization of the articular cartilage collagen network. J Microsc 2012; 246: 168–76

23. Bazou D, Behan G, Reid C, Boland JJ, Zhang HZ. Imaging of human colon cancer cells using He-Ion scanning microscopy. J Microsc 2011; 242: 290–4

24. Boden SA, Asadollahbaik A, Rutt HN, Bagnall DM. Helium ion microscopy of Lepidoptera scales. Scanning 2011; 33: 1–14

25. Standeven KF, Grant PJ, Carter AM, Scheiner T, Weisel JW, Arëins RA. Functional analysis of the fibrinogen Aα Thr312Ala polymorphism effects on fibrin structure and function. Circulation 2003; 107:2326-2330

26. Weisel JW, Litvinov RI. Mechanisms of fibrin polymerization and clinical implications. Blood 2013; 121:1712-9

27. Weisel JW. The mechanical properties of fibrin for basic scientists and clinicians. Biophys Chem. 2004; 112: 267-76.

28. Fries D, Martini WZ. Role of fibrinogen in trauma-induced coagulopathy. Br J Anaesth 2010; 105:116–21

29. Bolliger D, Gorlinger K, Tanakan KA. Pathophysiology and treatment of coagulopathy in massive hemorrhage and hemodilution. Anesthesiology 2010; 113: 1205-1219

30. Mittermayr M, Streif W, Haas T, Fries D, Velik-Salchner C, Klingler A, Oswald E, Bach C, Schnapka-Koepf M, Innerhofer P. Hemostatic changes after crystalloid or colloid fluid administration during major orthopedic surgery: the role of fibrinogen administration. Anesth Analg 2007;105:905–17

31. Bolliger D, Szlam F, Molinaro RJ, Rahe-Meyer N, Levy JH, Tanaka KA.. Finding the optimal concentration range for fibrinogen replacement after severe haemodilution: an in vitro model. Br J Anaesth 2009;102:793-799

32. Neilson VG, Cohen BM, Cohen E. Effects of coagulation factor deficiency on plasma coagulation kinetics determined via thromboelastography: critical roles of fibrinogen and factors II, VII, X and XII. Acta Anaesthesiol Scand. 2005; 49:222-31

33. Yang L, Vuylsteke A, Gerrard C, Besser M, Baglin T. Postoperative fibrinogen level is associated with postoperative bleeding following cardiothoracic surgery and the effect of fibrinogen replacement therapy remains uncertain. J Thromb Haemost 2013;11:1519-1526

34. 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. Arterio Thromb Vasc Biol 2000; 20:1354-1361

35. Gabriel DA, Muga K, Boothroyd EM. The effect of fibrin structure on fibrinolysis, J Biol Chem 1992; 267:24259-24263