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A new biomarker quantifies differences in clot microstructure in patients with venous thromboembolism

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<td>BLOOD COAGULATION, WARFARIN, VENOUS THROMBOSIS, HAEMOSTASIS, ANTICOAGULATION</td>
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A new biomarker quantifies differences in clot microstructure in patients with venous thromboembolism

Are current coagulation tests sufficient?

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Summary (199)

This study compares patients with venous thromboembolism (VTE) to non-VTE patients using a novel biomarker of clot microstructure ($d_f$) and incipient clot formation time ($T_{GP}$). 142 warfarinised patients were recruited where a significant difference ($p<0.001$) in $d_f$ between the VTE and non-VTE cohorts was observed ($d_f = 1.73 \pm 0.055$ and $1.69 \pm 0.046$ respectively). International Normalized Ratio (INR) and all other established tests employed did not distinguish between VTE and non-VTE patients. Time based assessments of coagulation including $T_{GP}$ did not distinguish between the two groups (367 ± 134sec and 382 ± 163sec). We identify that VTE patients who are anticoagulated with warfarin, produce ‘abnormal’ clot microstructures when compared to other patient groups with similar INR values. This suggests either an inadequate response of these VTE patients to anticoagulant therapy, or the presence of a procoagulant state which is not detected by clotting time based assessments of coagulation (i.e. INR), or both. Measurement of $d_f$ may allow clinicians to develop and assess new anticoagulation regimes in the treatment of thromboembolic conditions. Furthermore, elevated values of $d_f$ in first time VTE patients who later develop a secondary event indicates that $d_f$ may identify those at risk of VTE recurrence.

Keywords: BLOOD COAGULATION, WARFARIN, VENOUS THROMBOSIS, HAEMOSTASIS, ANTICOAGULATION
Introduction

Venous thromboembolism (VTE) is a major health problem worldwide with an annual incidence of around 1 per 1000 person-years (Silverstein et al, 1998; Heit et al, 2006). Identifying the risk of VTE and its recurrence is complex and imprecise, requiring knowledge and consideration of both patient demographics and response to multiple clinical and laboratory parameters (Zhu et al, 2009; Eichinger et al, 2010; Kyrle et al, 2010). The management and treatment of VTE includes the administration of oral anticoagulants such as warfarin. Warfarin dosage is monitored using the International Normalised Ratio (INR) (Ansell et al, 2008) but recurrent embolic events in patients who are fully anticoagulated according to their INR are still well recognised and remain problematic (Kearon et al, 2003; Kearon et al, 2008; Thachil, 2012). Furthermore, the effects of co-morbid conditions and concomitant treatment make assessment by current markers difficult.

Previous studies have identified that abnormal clot microstructures are an identifying feature of patients and individuals with a familial history of VTE (Undas et al, 2009; Undas et al, 2011; Martinez et al, 2014). However, no studies have explored the changes in incipient clot microstructure in patients who are undergoing therapeutic treatment following VTE. Currently, there is no global clotting biomarker that accurately quantifies the pro- or anti-thrombotic status due to the disease state and pharmacological intervention of a patient at a given point in time. A biomarker that improves current management strategies by providing a more individualized therapeutic approach and identifying subjects who are at risk of thrombotic events is urgently needed.
Advances in our understanding of the viscoelastic changes in coagulation have led to the development of a technique for the quantification of clot microstructure as described elsewhere (Evans et al, 2008; Evans et al, 2010; Lawrence et al, 2014). In contrast to standard coagulation assays this technique uses unadulterated whole blood in a near patient setting. In addition to quantifying clot microstructure in terms of its fractal dimension, $d_f$, the biomarker also provides measurements of incipient clot formation time ($T_{GP}$), both being calculated from a single measurement of the viscoelastic properties at the Gel Point ($GP$) (Winter et al, 1986; Evans et al, 2008; Evans et al, 2010; Brown et al, 2012; Lawrence et al, 2014). The $GP$ marks the establishment of the incipient clot, a vital stage in clot development and provides a basis for assessing perturbations in haemostasis due to disease and/or therapeutic intervention (Curtis et al, 2012; Curtis et al, 2013).

This paper reports an observational cohort study that explores the potential ability of $d_f$ to identify differences in clot microstructure between warfarinised VTE and non-VTE patients.
Methods

Patients

This is an observational cohort study, patients routinely attending the anticoagulation clinic at a large teaching hospital in the UK were identified and invited to participate in the study. Those who volunteered to participate were referred to the NISCHR Haemostasis Biomedical Research Unit (HBRU). Informed consent was obtained from each patient in accordance with the declaration of Helsinki. Patients who are on any other form of anticoagulation, anti-platelet therapy or acutely unwell were excluded. The study was approved by local Research Ethics Committee (REC Number 07/WMW02/34). Participants were divided into two cohorts; VTE and non-VTE. All the laboratory analysis was performed blinded to the indication for anticoagulation.

Sample Handling

Blood samples were obtained from the antecubital vein via an 18-gauge needle, the first 2mls of blood were discarded, following which 20ml was collected in a syringe. Immediately following obtaining the sample it was divided into 2 aliquots. The first whole blood aliquot was transferred immediately to the AR-G2 (TA instruments) rheometer for testing (see Gel Point Measurements). The second sample was transferred to Vacutainers (0.109M) (Becton-Dickinson, Plymouth, UK) and used to analyze the initiation, propagation and fibrinolytic pathways of coagulation using standard and specific markers (see Standard Coagulation Screen, Thrombin Generation, Fibrinolysis and Thromboelastography).

Gel Point Measurements
$T_{GP}$ and $d_j$ are obtained from measurements of viscoelastic properties at the $GP$ (Winter et al., 1986; Evans et al., 2010). In the present study a 6.6 ml aliquot of whole unadulterated venous blood was loaded into a double-gap concentric cylinder measuring geometry of a TA Instruments AR-G2 (TA Instruments, New Castle, DE, USA) controlled-stress rheometer (at 37°C ± 0.1°C) in a near patient setting. The methodology obtaining the $GP$ parameters followed the exact procedure reported in a previous study (Lawrence et al., 2014). The rheometric measurements were verified independently by a second operator.

**Standard Coagulation Screen**

A 4.5 ml aliquot of blood was transferred immediately into siliconised glass citrated Vacutainers (0.109M) (Becton-Dickinson, Plymouth, UK). Prothrombin Time (PT) is reported in seconds and as INR (White, 2003), activated partial thromboplastin time (APTT) and Clauss fibrinogen were measured using a Sysmex CA1500 analyzer within 2 hrs of collection. All reagents were obtained from Siemens, (Frimley, UK) and the analyzer was calibrated according to manufacturer’s instructions.

**Thrombin generation**

Thrombin generation was performed using the Thrombin Generation Assay TGA (Technoclone Diagnostics, Vienna, Austria). 40µL of citrated plasma was dispensed into a 96 well ELISA plate that had been pre warmed to 37°C (NUNC F16 maxisorp black fluorescence plates, Pathway Diagnostics, Dorking, UK). Added to this was 10 µL tissue factor at a final concentration of 5pM (Technoclone Diagnostics, Vienna, Austria) followed by 50µL of fluorogenic substrate 1mM Z-G-G-R-AMC (Technoclone Diagnostics, Vienna, Austria). The plate was loaded into the
fluorogenic plate reader TECAN infinite F200 pro (Labtech International, Uckfield, UK) and
measurement made every 60 seconds for a total of 1 hour. TGA® software was used to calculate
individual thrombin generation curves. The assay was calibrated and quality control performed
according to manufacturer’s instructions.

Fibrinolysis

The fibrinolytic marker t-PA-PAI 1 complex was an ELISA assay and performed according to
manufacturer’s instructions (Hyphen Biomed, Quadrattech, Epsom, UK). A second fibrinolytic
marker D-Dimer was carried out using the TriniLIA Auto-Dimer® turbidimetric assay with a
Sysmex CA1500 analyzer (Siemens, Frimley, UK).

Thromboelastography

A 360 µl aliquot of whole blood was immediately analyzed using thromboelastography (TEG®-
Hemoscope 3000 Clot Analyzer). The TEG® parameters recorded were: (i) the ‘R-time’ which is
the time elapsed between the start of data collection to a recorded chart deflection greater than
2 mm; (ii) MA (maximum amplitude); and (iii) TMA (the time elapsed between the start of data
collection and MA).

Statistics

Descriptive analysis was undertaken to establish baseline characteristics of both groups. Results
are reported as mean (±SD) unless otherwise stated. Pearson correlation coefficients and two-
sample t-test were calculated on data that was assumed to be normally distributed. For data not
assumed to be normally distributed, differences were compared using the Mann-Whitney U test.
(Actual probability values are quoted, for results where) data was deemed significant when 
p<0.05. Statistical analysis was performed using Minitab version 16 software (Havertown, PA) 
and GRAPHPAD PRISM® version 6.0 (GraphPad software Inc., La Jolla, CA, USA).
Results

Patient recruitment and clinical details

A total of 142 warfarinised patients were recruited into the study between October 2009 and May 2011. The patients were divided into two cohorts: one VTE cohort with patients receiving warfarin for lower limb deep venous thrombosis (DVT) or pulmonary embolism (PE), and the other a non-VTE group for patients receiving warfarin for other reasons; such as atrial fibrillation and heart valve disease. Six patients were excluded due to concurrent anti-platelet therapy. Another eight patients undergoing warfarin therapy for reasons that did not fit either of the two groups were excluded from the cohort analysis (arterial thrombosis, ischaemic limb, pulmonary hypertension and portal vein thrombosis). The VTE and non-VTE groups were comprised of 60 and 68 patients respectively. The VTE group contained 16 first time/single VTE and 44 recurrent VTE in which 27 were due to only deep vein thrombosis (DVT) and 33 had pulmonary embolism (PE) with or without DVT. The non-VTE group contained 48 atrial fibrillation and 20 valve replacement patients. Demographics and baseline characteristics, including the standard markers of coagulation for the whole group as well as both the VTE and non-VTE are presented in Table 1. We found that the time in therapeutic range (TTR), calculated using the Rosendaal method (Rosendaal et al, 1993), for the VTE (60.5% IQR 51.7-72.7) and non-VTE (65.6% IQR 55.2-74.6) was not significantly different (p=0.248). Furthermore, we found no significant difference between the VTE and non-VTE groups with regards to the laboratory markers of coagulation and thromboelastography in this study (Table 1). All analysis in this article was first carried out using all patients in a particular group, then repeated for only those within their therapeutic INR range, similar trends were observed in both cases.
No significant correlation between $d_f$ and INR was found. However a significant correlation was found between $d_f$ and $T_{GP}$

Previous studies report that $d_f$ is significantly correlated with rate based assessments of coagulation (APTT) in an in vitro model of anticoagulation (Evans et al, 2010). In the present study the value of $d_f$ for patients anticoagulated with warfarin was $d_f = 1.72\pm0.053$, not significantly different from that of a previously reported healthy index (1.74±0.07). Notably, linear Pearson correlation tests revealed no significant correlation between $d_f$ and laboratory rate based markers of coagulation (INR -r=0.006, p=0.9). In contrast the $GP$ derived measure of coagulation time, $T_{GP}$, was negatively correlated with $d_f$ (r=-0.352, p< 0.001).

A significant difference in $d_f$ is found between the VTE group and the non-VTE group whereas no difference in INR is found

The INR for the two patient sub-cohorts, VTE and non-VTE groups was the same (INR= 2.7±1.09 and 2.7±0.73 respectively) (see Table 1). Conversely, statistical analysis revealed that the value of $d_f$ for the VTE patients was significantly higher than for the non-VTE patients (p=0.002), where the mean values were 1.73±0.055 and 1.69±0.046, respectively (Fig 1).

$d_f$ is a potential indicator of increased risk of VTE recurrence in patients with a first time VTE

The VTE group contained 16 first time/ single VTEs (s-VTE) and 44 recurrent VTE (r-VTE), studying the subpopulations of the VTE cohort shows a higher value of $d_f = 1.74\pm0.049$ for the r-VTE patients compared to the s-VTE ($d_f = 1.71\pm0.060$). The $d_f$ of the r-VTE group is significantly different from the non-VTE cohort (p<0.001). Furthermore, 3 of the 16 patients who were s-VTE at the time of their test later developed into r-VTE (within a 2 year follow up),
each having a relatively high value of $d_f$ (1.74, 1.75 & 1.78) when compared to the mean of the group.
Discussion

In this study we report a measure of clot microstructure, $d_f$, that identifies significant differences in clots formed in VTE and non-VTE patients despite both groups appearing to be fully anticoagulated in terms of their INR values. We previously reported a mean value of $d_f$ for non-anticoagulated healthy blood ($d_f = 1.74\pm0.07$) where progressive in-vitro anticoagulation (using heparin) produces lower values ($1.55 < d_f < 1.74$) (Evans et al, 2010). As such anticoagulation using warfarin could be expected to cause a reduction in the value of $d_f$. Patients receiving warfarin for non-VTE related disease did have a reduced value of $d_f$ (1.69±0.046). However, the VTE cohort has a $d_f$ value of 1.73±0.055, essentially indistinguishable from the healthy cohort despite full anticoagulation (Fig 1). This finding suggests that the anticoagulation may be subtherapeutic, at least in the context that the incipient clot microstructure is not being sufficiently altered. While warfarin is effective insofar as it prolongs coagulation, as measured by INR, clinically this therapeutic effect can often be found to be suboptimal with some patients still developing thrombosis (Kearon et al, 2003; Kearon et al, 2008; Thachil, 2012). It is possible that the significant increased mean difference in $d_f$ ($p=0.0001$) we report for the VTE patients is associated with a large increase in thrombotic potential, one that is not adequately regulated by warfarin. From a clinical perspective this is an important finding as it suggests that additional tools may be needed to underpin therapeutic management in certain patient groups.

It is important to understand what these differences in $d_f$ represent in terms of the amount of fibrin mass which is incorporated within that clot. The key point is that large amounts of mass are required to produce small changes in $d_f$. To illustrate this we use the results of a previously published computational analysis (Curtis et al, 2011). Previous studies using light scattering and
microscopy have established that incipient fibrin clots have fractal properties, where the mass, M, is related to \(d_f\) by the following power law equation: \(M \sim \varepsilon^{d_f}\), where \(\varepsilon\) represents length scale values in the range 100nm to 10\(\mu\)m (Scanlan et al, 1991; Curtis et al, 2011). This non-linear relationship is presented in Fig 2 and shows that a clot with a \(d_f\) of 1.69 (a value corresponding to the non-VTE group) has approximately half the amount of fibrin incorporated into its structure compared to a \(d_f\) of 1.73 (representing the non-VTE group). Interestingly, a clot with \(d_f = 1.86\) (this being the highest single value recorded for a member of the VTE cohort) has over 40 times more fibrin mass incorporated within it than a clot for which \(d_f = 1.59\) (the lowest value recorded and a member of the non-VTE group). To further illustrate these differences representative images of fractal networks are shown in Fig 2 for three values of \(d_f\). From a clinical perspective, the incorporation of such large amounts of additional mass within the incipient clot is significant, given its established role as a microstructural template for ensuing clot development (Curtis et al, 2013). Increases in \(d_f\) and hence mass would be expected to have significant consequences in terms of clot functionality, such as reduced porosity and increased elasticity or strength, features which could lead to ineffective fibrinolysis and increased risks of embolization (Gabriel et al, 1992; Jorneskog et al, 1996; Collet et al, 2000; Mills et al, 2002; Weisel et al, 2013). In support of this statement the results of this study show that of the sixteen first time VTE patients (s-VTE), three developed a recurrent VTE event within two years. Interestingly these three patients had high \(d_f\) values (1.74, 1.75 & 1.78) in comparison to the mean of the s-VTE patients (1.71±0.060). These findings suggest that increased values of \(d_f\) are a potential indicator of increased risk of VTE recurrence.

In addition to \(d_f\), each GP measurement provides the corresponding value of the incipient clot
formation time ($T_{GP}$) (Fig 1), both of which are obtained in a near patient setting using fresh whole blood within a few minutes (Evans et al, 2010; Lawrence et al, 2014). $T_{GP}$ is significantly correlated with $d_f$, which supports previous findings (Evans et al, 2010). However, whilst $d_f$ shows a significant difference between the non-VTE and VTE patients, $T_{GP}$ does not (along with all the other clotting time parameters reported herein). The significance of this finding is that current anticoagulant monitoring is widely performed using time based assessments of coagulation. The results of the present study shows that for VTE patients assessments of clot microstructure could provide additional information for the management of patients receiving anticoagulation therapy.

While the VTE and non-VTE groups are not well matched in terms of patients’ demographics (age and sex) and the study does not directly investigate patients’ risk of thromboembolic disease, it does reveal that VTE patients anticoagulated with warfarin, produce ‘abnormal’ clot microstructures, when compared to another patient group with similar INR. This suggests either a less effective response of these VTE patients to anticoagulant therapy, or the presence of a more procoagulant state that is not detected by current tests (i.e. INR), or both. It follows that the ability to detect abnormalities in clot microstructure using whole blood as determined by $d_f$ may complement (or even replace) the available routine clotting tests based on simpler ‘kinetic’ or rate-based assessments using plasma. Measurement of $d_f$ may allow the clinician to develop and assess new anticoagulation regimes in the treatment of thromboembolic conditions. Furthermore, this study highlights that increased values of $d_f$ in patients who have suffered a first time VTE may be a potential indicator of an increased risk of VTE reoccurrence. A larger prospective study will explore these potential clinical implications.
Acknowledgements

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Author Contributions

PAE proposed the idea and designed the research. MJL, SP, WA, GM recruited the patients. MJL, KH performed the rheology tests, SJD performed the laboratory tests. MRB, DJC created the mass / $d_f$ graph and illustrations. MJL, PRW, DJC and KH provided rheological advice. AS, GM, WA, LAD collected the patient data. MJL, AS, PAE, JW, MRB, PRW and RHKM analyzed and interpreted the data. All authors reviewed and approved the article.

Disclosures

All other authors declare no competing conflicts of interest.
References


Fig 1: Fractal Dimension, $d_f$ and Gel time, $T_{GP}$: box and whisker plot illustrating the change in $d_f$ and $T_{GP}$ between the VTE and non-VTE cohorts. The star symbol represents the mean values and * represents a significant difference ($p>0.001$) is observed between the two cohorts using a two sample t-test.

Fig 2 – Graph illustrating the non-linear relationship between the fractal dimension, $d_f$ and the amount of mass, incorporated within the fractal structure. The mass value on the y-axis is normalised with respect to the healthy index value of $d_f=1.74$ (circle). Illustrations of different incipient clot microstructures at particular values of $d_f$ are provided, corresponding to the range of $d_f$ values obtained in this study. When compared with the healthy index value, a clot for which $d_f=1.60$ (cross) would be characterised by reduced mechanical strength (elasticity) and a more open, porous network structure – features typically associated with hypocoagulable states. Conversely, a clot for which $d_f=1.80$ (square) would be mechanically far stronger, with a more compact microstructure corresponding to a hypercoagulable state.
Table 1: Patient Baseline Characteristics and Demographics for all warfarin patients and the two cohorts: VTE and non-VTE groups. Showing p values for †two sample t-test and ‡Mann-Whitney U test between the VTE and non-VTE groups. {add age and sex}

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<th>non-VTE</th>
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<td>35/60 (59.6%)</td>
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<td>INR</td>
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(a) Fractal Dimension ($d_f$)

- VTE
- non-VTE

$p = 0.001$

(b) Gel Time ($T_{GP}$, sec)

- VTE
- non-VTE