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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 (100)

This study compares patients with venous thromboembolism (VTE) to non-VTE patients using a biomarker of clot microstructure (d_f) and clot formation time (T_{GP}). d_f was the only marker that identified a significant difference ($p<0.001$) between the VTE (n=60) and non-VTE cohorts (n=69). The ‘abnormal’ clot microstructures observed in the VTE patients suggests either inadequate response to anticoagulant therapy, or the presence of a procoagulant state not detected by other markers of coagulation (i.e. INR). 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 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). The management of VTE includes the administration of oral anticoagulants such as warfarin. Warfarin dosage is monitored using the International Normalised Ratio (INR) however recurrent embolic events in patients who are fully anticoagulated according to their INR remain problematic (Kearon *et al*, 2008; Thachil, 2012).

Clots with abnormal microstructures and viscoelastic properties have been reported in patients with VTE (Undas *et al*, 2009; Martinez *et al*, 2014). A biomarker that can quantify abnormal clot microstructure could identify subjects at risk of thrombotic events and improve current management strategies by providing a more individualized therapeutic approach.

This paper reports an observational cohort study that differentiates VTE from non-VTE patients by using a new biomarker based on viscoelastic measurements of clotting blood at the Gel Point (*GP*). The *GP* measurement has been validated in healthy subjects (Evans *et al*, 2010) and quantifies both clot microstructure in terms of its fractal dimension, d_f, and clot formation time (T_{GP}). In contrast to standard coagulation assays this technique uses unadulterated whole blood in a near patient setting.

Methods

Patients

This is an observational cohort study approved by the local Research Ethics Committee (South West Wales REC 6). Patients routinely attending the anticoagulation clinic at a large UK teaching hospital were recruited following informed written consent. Patients were excluded if they were on anti-platelet therapy or acutely unwell. A 20 ml venous blood sample was obtained for *GP measurements*, *Standard Coagulation Markers, Thrombin Generation, t-PA-PAI 1, D-dimer* and *Thromboelastography*. All assays were calibrated and quality control performed according to manufacturer's instructions.

GP measurements were obtained by reproducing the methodology reported in a previous study (Lawrence *et al*, 2014). A 6.6 ml aliquot of whole blood was loaded into a double-gap concentric cylinder measuring geometry of an AR-G2 (TA Instruments, USA) controlled-stress rheometer (at 37°C ± 0.1ºC) and measurements were started immediately.

A 4.5 ml aliquot of blood was used to obtain *standard coagulation markers* including; Prothrombin Time (PT) and INR, activated partial thromboplastin time (APTT) and Clauss fibrinogen, all measured using a Sysmex CA1500 analyzer within 2 hrs of collection. All reagents were obtained from Siemens, (Frimley, UK). *Thrombin generation* was performed using the TGA assay and associated software (Technoclone Diagnostics, Austria). In summary 40µL of citrated plasma was dispensed into an ELISA plate at 37ºC (NUNC F16 maxisorp black fluorescence plates, Pathway Diagnostics, UK).10 µL tissue factor (Technoclone Diagnostics, Austria) was added (final concentration 5pM), followed by 50µL of fluorogenic substrate 1mM Z-G-G-R-AMC

(Technoclone Diagnostics, Austria).The fibrinolytic marker *t-PA-PAI* 1 complex was assessed using ELISA assay (Hyphen Biomed, Quadratech, UK). *D-Dimers* were measured using the TriniLIA Auto-Dimer® turbidimetric assay with a Sysmex CA1500 analyzer (Siemens, UK). Another 360µl aliquot of whole blood was immediately analyzed using *thromboelastography* (TEG®- Hemoscope 3000). R-time, MA (maximum amplitude), and TMA (time to maximum amplitude) were recorded.

Results are reported as mean (±SD) unless otherwise stated. Pearson correlation coefficients, two-sample t-test and Mann-Whitney U test were used for analysis. Data was deemed significant when $p<0.05$. Statistical analysis was performed using Minitab version 16 software (Havertown, USA).

Results and Discussion

Patient recruitment and clinical details

Patients were divided into a VTE cohort (n=60), those receiving warfarin for lower limb deep venous thrombosis (DVT) or pulmonary embolism (PE), and a non-VTE cohort (n=68) for patients receiving warfarin for other reasons (atrial fibrillation and heart valve disease). The VTE cohort contained 16 single and 44 recurrent VTE, 27 of which were due to DVT and 33 to PE with or without DVT. The non-VTE cohort contained 48 atrial fibrillation and 20 valve replacements. We found that all markers with the exception of d_f show no significant differences between the VTE and non-VTE cohorts (Table 1). The time in therapeutic range (TTR) (Rosendaal *et al*, 1993), for the VTE was 60.5% (IQR 51.7-72.7) and for non-VTE was 65.6% (IQR 55.2-74.6) was not significantly different (p=0.248).All analysis was first performed for all patients in a cohort then repeated for only those within therapeutic INR range, a similar trend was observed in both cases.

A significant difference in d_f is found between the VTE group and the non-VTE group.

Previously the d_f for non-anticoagulated healthy blood was found to be 1.74±0.07, where progressive in-vitro anticoagulation lowered the value ($1.55 < d_f < 1.74$) (Evans *et al*, 2010). It was expected that warfarin would cause a reduction in d_f, which it did in the non-VTEs (d_f=1.69±0.046).In contrast the d_f in the VTE cohort is essentially indistinguishable from the healthy cohort despite full anticoagulation (1.73±0.055) a value significantly different from the non-VTEs (p=0.002). Interestingly INR values of VTEs and non-VTEs are similar (INR= 2.7±1.09 and 2.7±0.73 respectively). This suggests that the VTE patients' anticoagulation may be sub-therapeutic, at least in the context that clot microstructure is not being sufficiently altered. Whilst warfarin may prolong coagulation, clinically this effect can be sub-optimal with some patients still developing thrombosis (Kearon *et al*, 2008; Thachil, 2012). It is possible that increased values of d_f are the result of an increase in thrombotic potential, one that is not adequately regulated by warfarin.

Time based markers of coagulation revealed no significant difference between the VTE and non-VTE cohorts.

No significant correlation between d_f and the standard time based markers of coagulation (INR - r=0.006, p=0.9) was found. In contrast the *GP* derived time based marker, T_{GP}, was negatively correlated with d_f (r=-0.352, p< 0.001) as found in a previous study (Evans *et al*, 2010). However, all time based markers measured herein, show no significant difference between VTE and non-VTE (Table 1). Current anticoagulant monitoring is widely performed using time based assessments of coagulation, from a clinical perspective the findings of this study suggests measuring clot microstructure provides additional information which could improve therapeutic management of VTE patients.

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

The VTE group contained 16 single VTEs (s-VTE) and 44 recurrent VTEs (r-VTE), where the value of d_f for the r-VTE (d_f =1.74±0.049) was significantly higher than the s-VTE (d_f=1.71±0.060) (p=0.001). Furthermore, 3 of the 16 s-VTE patients at the time of the GP 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) in comparison to the mean of the s-VTE patients. Increases in d_f would be expected to have significant consequences in terms of clot functionality, such as a denser structure with reduced porosity and increased clot elasticity or strength. Features such as these could lead to ineffective fibrinolysis and increased risks of embolization (Mills *et al*, 2002; Weisel *et al*, 2013). These findings suggest that d_f may be a potential indicator of increased risk of VTE recurrence.

Small changes in d_f correspond to substantial changes in clot mass

It is important to recognize what these differences in d_f represent in terms of the fibrin mass incorporated within the clot. Large amounts of mass M are required to produce small changes in d_f (Curtis *et al*, 2011), the non-linear relationship between M and d_f shown in Fig 1 revealing that a clot with d_f =1.69 (non-VTE group) has approximately half the mass of a clot for which d_f = 1.73 (VTE group). Strikingly, for d_f = 1.86 (the highest single value recorded for the VTE cohort), more than 40-times more fibrin mass is incorporated within the clot than at d_f = 1.59 (the lowest recorded, for a member of the non-VTE group). From a clinical perspective, the incorporation of substantial additional mass within the incipient clot is significant, given its role as a microstructural template for clot development (Curtis *et al*, 2013).

In conclusion while the VTE and non-VTE groups are not age and sex matched and do not directly investigate patients' risk of thromboembolic disease, the study reveals 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 VTE patients to anticoagulant therapy, or the presence of a more procoagulant state 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, which will 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 predict risk of recurrence. A larger prospective study will explore these potential clinical implications.

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Author Contributions

MJL, SP, WA, GM recruited patients. MJL, KH, SJD performed experiments. MRB, DJC performed computational analysis. AS, GM, WA, LAD collected patient data. MJL, AS, PAE, JW, PRW,RHKM analyzed and interpreted the data. PAE designed the research. All authors reviewed and approved the article.

Competing Interests

The authors have no competing interests.

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Fig 1 – 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.

	ALL Patients	**VTE**	**non-VTE**	***p value (VTE vs non-VT)***
Patient demographics†				
Mean (±SD)				
Age (years)	63.3±12.5	57.1±12.6	67.9±10.5	0.02
Male (%)	86/128 (67.2%)	35/60 (59.6%)	51/68 (75.7%)	0.37
Gel Point†				
d_f	1.72±0.053	1.73±0.055	1.69±0.046	0.002
T_{GP}	373±150	366±135	384±164	0.49
General Markers†				
INR	2.7 (±0.89)	2.7 (±1.09)	2.7 (±0.73)	0.52
APTT (sec)	37.1 (±5.15)	37.6 (±7.14)	36.2 (±4.25)	0.17
Fibrinogen(Clauss) (g/l)	3.5 (±0.68)	3.4 (±0.67)	3.6 (±0.70)	0.22
Thromboelastography†				
R-time (min)	19.2±9.5	19.0±9.1	19.4±10.7	0.84
MA (mm)	45.9±119	45.6±11.2	46.0±13.2	0.88
TMA (min)	52.3±21.8	53.1±19.0	51.9±23.6	0.88
Thrombin Generation ‡				
Median (range)				
TGA (nM)	62 (41-85)	61 (50-84)	67 (49-95.2)	0.44
Fibrinolytic Markers‡				
t-PA-PAI (mg/mL)	2.0 (0.86-3.03)	1.5 (1.56-2.00)	2.2 (1.43-3.17)	0.21
D-Dimer	20 (0-51)	23 (0-26)	25 (0-59)	0.61

Table 1: Patient Demographics, Baseline Characteristics and Gel Point Measurements for all 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.