

Fractal Dimension (d_f) as a new structural biomarker of clot microstructure in lung cancer and its stages.

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Running Title: Fractal dimension lung cancer

Key Words: Lung cancer, venous thromboembolism, risk assessment, biomarker, fractal

Funding: This translational work was supported by the NISCHR Biomedical Research Unit (BRU) grant (BRO1).

What is known on this topic?	What this paper adds?
<ul style="list-style-type: none"> • Patients with lung cancer are at increased risk of developing venous thromboembolism (VTE), significantly affecting morbidity and mortality. 	<ul style="list-style-type: none"> • Fractal dimension of blood clots is significantly higher in patients with lung cancer compared to healthy controls, suggesting an abnormal clot structure.
<ul style="list-style-type: none"> • There are currently no reliable markers which accurately quantify pathological clot structure. 	<ul style="list-style-type: none"> • Patients with extensive (stage III & IV) lung cancer had significantly higher d_f as compared to those with limited disease (stages I & II).
<ul style="list-style-type: none"> • Fractal dimension (d_f) is a new technique previously demonstrated to quantify clot microstructure, shown to be associated with changes observed by scanning electron microscopy (SEM). 	<ul style="list-style-type: none"> • Changes in d_f were associated with similar changes in SEM images, reaffirming the link between incipient and mature clot structure.

Abstract

Background: Venous thromboembolism (VTE) is common in cancer patients, and is the second commonest cause of death associated with the disease. Patients with chronic inflammation, such as cancer, have been shown to have pathological clot structures with modulated mechanical properties. Fractal dimension (d_f) is a new technique which has been shown to act as a marker of the microstructure and mechanical properties of blood clots, and can be performed more readily than current methods such as scanning electron microscopy (SEM).

Methods: This study compares d_f in 87 patients with newly diagnosed lung cancer to 47 matched-controls, in addition to analysis based on stage of disease. Results were compared with conventional markers of coagulation, fibrinolysis and SEM images.

Results: Significantly increased d_f was observed in lung cancer patients compared with controls. Significantly increased d_f was observed in patients with extensive (stage 3/4) compared with limited disease (stage 1/2) ($p < 0.05$), whilst conventional markers failed to distinguish between these groups. The relationship between d_f of the incipient clot and mature clot microstructure was

confirmed by SEM and computational modelling: higher d_f was associated with highly dense clots formed of smaller fibrin fibres in lung cancer patients compared to controls.

Conclusion: This study demonstrates that d_f is a sensitive technique which quantifies the structure and mechanical properties of blood clots in lung cancer patients, which appears to be modulated by stage of disease. This data suggests a potential role in identifying and managing patients with a high d_f , and therefore abnormal clot structure.

Introduction

Venous thromboembolism (VTE), comprising deep vein thrombosis (DVT) and pulmonary embolus (PE) is a common phenomenon affecting 6.5 million people worldwide annually (1,2). The incidence is higher in people with cancer who account for 18% of all cases of VTE (3), with an estimated 4 to 20% of cancer patients developing VTE during the course of their disease (4). VTE contributes significantly to morbidity and mortality in patients with cancer, in whom it remains the second commonest cause of death (5). Risk factors for VTE include advancing age, metastatic burden, surgery and chemotherapy (3). Cancers are also known to vary in thrombogenicity with histological type and primary tumour site. Compared with the general population, lung cancer confers a 22-fold increased risk of VTE, second only to haematological malignancies (6).

Despite the well-documented incidence of VTE in patients with cancer, little is known on the effects of differences in global coagulation on the structure of clots formed, and if these are pathologically abnormal compared to healthy individuals. Factors that increase the thrombogenicity in malignancy result from release of tumour procoagulants such as tissue factor (TF), microparticles and factor VIII (7-10). In addition, chronic inflammation has long been associated with cancer (11-13), having an important role in modulating the tumour microenvironment and its impact on systemic processes, including proliferation and migration (12). It has also been suggested that as cancers metastasize and grow, inflammatory processes may be increased (13), which may have greater impact on systemic processes such as haemostasis. Previous studies on patients with inflammatory disorders and vascular disease have demonstrated the development of pathological clots of a dense, highly polymerised and less porous microstructure (14), which may alter mechanical properties of the clot (15-18). Further

studies have suggested that fibrinolytic properties may also be reduced in blood clots in with abnormal structure (19,20). It is therefore likely that patients with cancer also form pathological clots with similar arrangement and resulting structural properties, which may be modulated by stage of disease and accompanying chronic inflammation. There is however, currently no method of investigating or quantifying the link between chronic inflammation and its global effect on processes such as haemostasis.

Attempts have been undertaken using viscoelastic techniques to investigate clot formation in patients with cancer. Several studies using thromboelastography (TEG) and rotational thromboelastometry (ROTEM) have suggested its use in predicting thromboembolic complications in cancer patients, (21-23) whilst others have demonstrated no differences (24,25). However, conflicting results on whether these techniques can be used to accurately assess changes in global coagulation in patients with cancer and the relatively small populations of patients with mixed primary tumour sites, limit their clinical utility. A larger study of ROTEM parameters demonstrated that patients with lung cancer had decreased clotting time and increased maximum clot firmness than healthy controls (26). These parameters were further altered in patients with extensive lung cancer compared to those with limited disease and in patients who went on to develop VTE, but cannot be used to identify differences in coagulation depending on stage of disease (26). In combination, these studies suggest that not only do patients with cancer form clots faster, but that physical clot properties may also be different in these patients, leading to pathological clot formation.

Recent advances in our understanding of viscoelastic changes in coagulation have resulted in the development of multi-faceted biomarker of clot characteristics, including clot microstructure, measured by fractal dimension (d_f), clot formation time (T_{GP}) and viscoelastic clot strength (G^*_{GP})

which are all calculated from the gel point (*GP*) (27-30). In contrast to standard coagulation assays, this technique uses unadulterated whole blood and is measured immediately at the bedside during real-time formation of the clot. In addition, structure of the early clot, as quantified by d_f , has been established as providing a template for the mature clot when analysed based on viscoelastic and imaging data (31,32). We have previously shown that d_f quantified the microstructure of the incipient clot in normal and anticoagulated blood in a sensitive and highly reproducible manner (30,32), and that this is important in quantifying the underlying pathological effect on clot structure (33). Similarly, increased d_f , has been demonstrated in patients with inflammatory vascular disorders (34,35), correlating with scanning electron microscopy (SEM) images of the mature blood clot. Measurement of global coagulation and resulting clot structure in using this biomarker may therefore be a means of investigating and quantifying the effect of chronic inflammation on haemostasis. As far as we are aware, this technique has not been applied to the study of blood clot formation in patients with malignancy.

We hypothesised that blood from lung cancer patients could have modulated mechanical properties at its *GP* compared with healthy controls, given the increased inflammation in these patients. In order to test this hypothesis, the present study was designed to investigate whether d_f was significantly different in patients with lung cancer compared to appropriately matched controls, and if changes varied with stage of lung cancer. In order to investigate whether high d_f values predict pathological abnormalities in mature clot structure, SEM imaging was also performed.

Materials and Methods

Patients

We undertook a prospective, observational study of consecutive patients with histologically or cytologically confirmed primary lung cancer diagnosed by the Swansea Multi-Disciplinary Lung Cancer Clinic. Exclusion criteria were as follows:

- Previous history of cancer
- Active disease known to alter coagulation (e.g. hepatic, renal or haematological disorders)
- Personal/family history of VTE
- Anticoagulant therapy (e.g. warfarin, heparin)

Demographic and clinical information was recorded and anonymised for each patient on initial presentation (Table 1). Tumours were staged according to the TNM classification system of the American Joint Committee on Cancer (36). Of 105 patients screened, 18 were excluded (14 had a history of other malignancy, two samples were not suitable for rheometric analysis, one patient had chronic kidney disease and one had a past history of VTE). Patients were sub-divided into those with limited disease (stages I or II) and those with extensive disease (stages III or IV). Forty-seven age and sex matched healthy volunteers (20 current smokers and 27 non-smokers) with no history of cancer or VTE acted as controls. The study was approved by Wales Research Ethics Committee 6 (REC Number 07/WMW02/34), and sponsored by Abertawe Bro Morgannwg University Health Board Research and Development Office. All subjects gave written, informed consent.

Given the high proportion of patients with smoking history, we undertook a preliminary study to compare levels of d_f in smoking and non-smoking controls. In order to address the effect of co-morbidities, we compared patients with limited lung cancer (stages I & II) and those with

extensive disease (stages III & IV), as the prevalence of co-morbidities appeared similar in both groups. A number of patients were on taking therapy for hypertension, hyperlipidaemia and prophylactic doses of aspirin (75mg), representative of the population of the study locality. These patients were not excluded as it was thought that aspirin therapy would favour the null hypothesis.

Venesection

Blood was taken from the antecubital vein via a Vacutainer® Needle following diagnosis and before any specific therapy was offered. The first 3-5 mls were discarded as waste. Whole venous blood was transferred immediately for rheometric analysis. Further samples were transferred into vacuum-sealed tubes containing sodium citrate (3.2%) or K₂EDTA (Greiner Bio-one, Stonehouse, UK) for measurement of standard markers of coagulation and fibrinolysis.

Rheometric analysis

The biomarkers (T_{GP} , G'_{GP} & d_f) which are the focus of this study are obtained from the measurement of viscoelastic properties at of the gel point obtained from clotting blood (27,29,30,32). 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 $37^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$) in a near patient setting. All rheometric sampling was carried out by members of the research team who were appropriately trained and experienced in rheometric analysis. In order to eliminate bias and enhance accuracy of measurement, data was anonymised and reviewed independently by three haemorheologists blinded to the sample origin, and the mean used for further analysis.

Computational analysis

In order to investigate the relatively small change in d_f and the previously reported increase in fibrin mass of the mature clot (33), computational analysis was carried out. Previously, a numerical technique has been used to generate random fractal aggregates where the fractal dimension is fixed a priori is presented (37,38). The algorithm utilizes the box-counting measure of the fractal dimension to determine the number of hypercubes required to encompass the aggregate, on a set of length scales, over which the structure is deemed to be fractal. At each length scale the number of required hypercubes are randomly chosen and importantly linked using a simple random walk in the embedding dimension. This late step ensures connectivity of the random fractal aggregate on all considered length scales. The algorithm is highly efficient and overcomes the limitations on the achievable magnitude of the fractal dimension encountered by alternative techniques. This provides a visual illustration of clot structure, based on the d_f values measured on whole blood, and allows the corresponding fibrin mass to be calculated.

Coagulation screen

Standard time based kinetic markers of clot initiation, propagation and fibrinolysis were carried out. PT, APTT and Clauss fibrinogen were measured using a Sysmex CA1500 analyser within 2 hrs of collection. All reagents were obtained from Siemens, (Frimley, UK).

Thrombin Generation

Thrombin generation was measured using the Thrombin Generation Assay (TGA, Technoclone Diagnostics, Vienna, Austria). 40 μ L of citrated plasma was dispensed into a 96 well ELISA plate, prewarmed to 37°C (NUNC F16 maxisorp black fluorescence plates, Pathway Diagnostics, Dorking, UK). Ten μ L of TF was added to a final concentration of 5pM followed by 50 μ L of fluorogenic substrate 1mM Z-G-G-R-AMC (Technoclone Diagnostics, Vienna, Austria). Plates were loaded into the fluorogenic plate reader TECAN infinite F200 pro (Labtech International,

Uckfield, UK) and read every 60seconds for 1 hour. TGA® software was used to calculate individual thrombin generation curves.

Factor VIII Analysis

Factor VIII was performed as one-stage factor assays on the ACL TOP 500 according to manufacturer's instructions using Instrumentation laboratory calibration plasma, quality control material and factor deficient substrate plasma (Instrumentation Laboratory, Warrington, UK).

Fibrinolytic Markers

PAI-1 antigen was measured using an ELISA assay performed according to manufacturer's instructions (Hyphen Biomed, Quadragech, Epsom, UK). D-Dimer analysis was carried out using Latex immunoturbidimetric assay Hemosil HS D-dimer (Instrumentation Laboratory, Warrington, UK). The D-dimer assay was performed on an ACL TOP 500 (Instrumentation Laboratory, Warrington, UK).

Microparticle assay

ELISA bio-immunoassay for MP-TF in plasma was performed according to manufacturers instructions using calibration and positive and negative controls (Hyphen Biomed, Quadragech, Epsom, UK).

Scanning electron microscopy

SEM samples were prepared by allowing 12µl of whole blood to clot for 15 minutes at 37°C. Resulting clots were washed with cacodylate buffer and fixed with gluteraldehyde, before point-critical dehydration with ethanol (30-100%) and hexamethyldisilazane (Sigma Aldrich, UK).

Samples were coated with gold palladium, imaged using a Hitachi Ultra-high resolution FE-SEM S-4800, and fibre width calculated as previously described (39).

Statistical analysis

Power calculations were performed to achieve the primary aim of identifying differences in d_f between a healthy individuals and a pilot group of patients with lung cancer. Parametric data are reported as mean (\pm SD) unless otherwise stated and compared using two-sample t-tests. Pearson correlation was undertaken to explore associations between d_f and standard markers of coagulation where significant differences were identified between those with limited compared to extensive disease. Statistical analysis was performed using Minitab version 15 software (Havertown, PA) and deemed significant when $p < 0.05$.

Results

Rheometric Analysis (d_f)

Mean d_f was 1.73 ± 0.04 in healthy individuals and comparable with levels reported in our previous study of healthy individuals from the same locality (30). There was no significant difference in d_f between healthy non-smokers (1.73 ± 0.04) and healthy smokers (1.74 ± 0.03 , $p = 0.10$), therefore both groups were combined to make a larger group of healthy controls ($n = 47$).

Lung cancer patients had a significantly higher d_f than controls (1.76 ± 0.06 v 1.73 ± 0.04 , $p < 0.05$; Figure 1a), but there were no differences in d_f between patients with non-small cell and small cell lung cancer (1.76 ± 0.06 v 1.78 ± 0.04 , $p = 0.266$; Figure 1b). Figure 1c shows that d_f was also significantly increased in patients with extensive disease compared to those with limited disease (1.79 ± 0.05 v 1.73 ± 0.05 , $p < 0.05$) along with an observed increase in G'_{GP} (0.013 ± 0.006 v 0.008 ± 0.004 , $p < 0.05$). T_{GP} was not significantly different between controls and lung cancer patients (231 ± 78 v 216 ± 83 s, $p = 0.243$). T_{GP} was higher in patients with extensive disease

compared with limited disease (245 ± 107 s v 197 ± 63 s, Table 2) but the difference failed to reach statistical significance ($p=0.07$). Whilst the study was not powered as a clinical outcome study, it is noteworthy that d_f was significantly increased in patients who died within 12 months of diagnosis (1.79 ± 0.06 v 1.74 ± 0.06 , $p<0.05$).

Computational analysis

In patients with lung cancer, relatively small changes in d_f related to large increases in fibrin mass, as calculated by computation analysis. Fibrin mass was on average 150% greater than controls and up to 215% in those with extensive disease. In the patient with the highest d_f , this equated to over 700% fibrin mass of healthy controls. Computational models illustrated that highly dense clot structures are generated at the highest d_f values identified in this study, compared to those of controls (Figure 2). These images clearly show a significant difference in the structures of the clots formed over this range, the high density of red (nodes) in the 1.91 image indicates an area of increased cross connectivity, creating a very strong clot, in contrast to that of the 1.73 image where there are significantly fewer dense (red) areas.

Laboratory markers

All healthy controls were within normal reference ranges (Table 3). No significant differences were observed in conventional tests of coagulation between controls and patients with lung cancer (PT: 10.4 ± 0.5 s v 10.6 ± 0.6 s, $p=0.0784$ APTT: 25.8 ± 1.9 s v 25.9 ± 1.0 s, $p=0.845$). Nor were there any differences between those with limited and extensive disease (PT: 10.6 ± 0.5 s v 10.5 ± 0.6 s, $p=0.722$; APTT: 26.1 ± 2.8 s v 25.7 ± 3.2 s, $p=0.538$). Fibrinogen and platelets were significantly higher in patients with lung cancer compared with controls (Fibrinogen 4.2 ± 1.1 v 3.2 ± 0.5 ; Platelets: $319 \pm 96 \times 10^9/l$ v $244 \pm 51 \times 10^9/l$, $p<0.05$ in both cases), but were not significantly higher in extensive compared with limited disease (Fibrinogen: 4.2 ± 1.1 g/l v 4.2 ± 1.0 g/l, $p=0.956$; Platelets: 326 ± 105 v 310 ± 81 , $p=0.438$). No statistical differences were

identified between patients with limited or extensive disease with regard to markers of coagulation and fibrinolysis including thrombin generation, FVIII, MP-TF and D-dimer (Table 4). Conversely, PAI-1 was significantly raised in those with extensive compared to those with limited disease (10.2 ± 5.9 v 17.6 ± 10.3 , $p < 0.05$). No significant differences were observed between those alive at 12 months and those who had died 12 month from diagnosis (data not shown).

Correlation analysis

PAI-1 was significantly correlated with d_f in patients with extensive lung cancer ($r = 0.399$, $p = 0.02$). No significant correlation was identified between PAI-1 and d_f in those with limited disease (correlation coefficient = 0.245, $p = 0.248$).

Scanning electron microscopy of mature clot structure

Clots formed from blood from patients with extensive lung cancer were denser and had a more highly branching fibrin network with smaller pore areas than those from patients with limited disease or controls (Figure 3). Fibrin fibres were thinner in patients with extensive disease ($0.16 \pm 0.02 \mu\text{M}$) compared to those with limited disease ($0.24 \pm 0.06 \mu\text{M}$) or controls ($0.27 \pm 0.05 \mu\text{M}$).

Discussion

This is the first study to demonstrate differences in microstructural arrangement and mechanical properties of blood clots in patients with lung cancer, compared to healthy, age-matched controls, as demonstrated by a significantly increased d_f . Significantly increased d_f values were observed in patients with extensive lung cancer compared to those with limited disease whilst the two groups appeared evenly matched for confounding variables such as aspirin therapy and cardiovascular risk factors. This provides evidence that not only does advanced cancer confer an increased risk of VTE (1-5), but also that pathological clots formed are of abnormally dense structure, particularly

in those with extensive disease. These findings were also confirmed by images generated by SEM, demonstrating clots of highly dense, polymerised structures in patients with high d_f values.

In contrast to rheometric data, we observed no significant differences in standard time-based kinetic markers of coagulation between patient groups and controls. These findings were also consistent in patients with extensive compared to limited disease. Whilst patients had significantly higher levels of platelets and fibrinogen compared to controls, it is noteworthy that all results fell within local normal ranges. Further markers of thrombus initiation (FVIII, TF-MPs), propagation (TGA) or fibrinolysis (D-dimer), did also not differentiate between patients with limited compared to extensive lung cancer. However, results obtained were markedly elevated in patients with cancer, particularly those with extensive disease, despite remaining within normal ranges. Whilst there was no difference in Factor VIII levels between limited and extensive disease, a number of patients had levels near or above the normal range. This is consistent with previous publications (7-10), in which patients with malignancies found that increased Factor VIII was associated with a 14% cumulative probability of VTE (10), and may also be indicative of the chronic inflammation present in these patients. D-dimer has also been described to be increased in patients with cancer (40), but our current data suggest that it cannot be used to differentiate between local and extensive disease. In contrast to the elevated levels of both Factor VIII and D-dimer described, d_f did differentiate between stages of disease, emphasizing its specificity in this population.

PAI-1 was significantly higher in those with extensive lung cancer, suggesting increased suppression of fibrinolytic activity in this group. This is in keeping with previous studies, which have identified increased PAI-1 in various malignancies (41-43), a decrease in fibrinolytic efficiency in clots from patients with gastrointestinal cancer (45). Furthermore, PAI-1

significantly correlate with d_f in patients with extensive lung cancer, all be it a weak correlation. This may suggest that d_f takes into account suppression of the fibrinolytic system, in combination with quantifying the microstructural arrangement of the clot. In our previous study, d_f was shown to be an accurate marker of fibrinolysis and anticoagulation therapy (30,33), and as such may have potential to not only quantify thrombogenicity, but also to guide and improve anticoagulation therapy. These findings suggest that manipulation of the clot structure may be an effective target in therapeutic intervention, given the pathological differences in clot structure observed.

The present study provides additional evidence to support previous studies demonstrating that the clot structure, quantified by d_f , acts as a template predicting the structure of the mature clot demonstrated in SEM images. Blood clots from patients with lung cancer who had progressively higher levels of d_f , were found on SEM to be composed of progressively thinner fibrin fibres that displayed increased numbers of branching points creating a denser, less porous structure than those from healthy individuals. Patients with extensive disease produced clots of the highest density with small porous spaces, suggested to be representative of the increased inflammation present in those with highest tumour burden, as previously described (12,13). This is consistent with previous publications demonstrating that increased d_f translates to highly polymerised, dense clots in patients with inflammatory vascular disease (34,35). These observations are supported by the by the significant positive correlation between d_f and G'_{GP} ($p < 0.001$), the highest values of d_f corresponding to the highest values of clot strength, taking the elastic modulus, G'_{GP} , as analogous to that of elasticity or clot 'strength'.

Computational modelling of d_f values obtained in the study was carried out in order to relate the small changes in d_f to changes in fibrin mass. Increases of fibrin mass of up to 900% were

calculated in patients with the highest d_f values, which were also associated with changes in microstructural arrangement demonstrate by computational images. These representative images confirm that patients with increased d_f form clots with areas of high cross-connectivity of fibrin fibres as illustrated by the increased presence of red (nodal) areas. At d_f values of 1.73 and 1.79 there are only a handful of red areas, representing relatively few areas of fibrin cross connectivity. At 1.91 the clot is comprised of mainly red nodal areas, representing multiple areas of fibrin connectivity, which would translate to clots with highly elastic properties. Consequently these clots will mechanically stronger and less susceptible to breakdown. The rheological findings, in combination with SEM images and computation analysis, support previous studies identifying clots of abnormally dense structure in patients with inflammatory disorders (14,15,19,34,35), which may increase resistance of these clots to fibrinolysis (16-18). This supports previous reports that not only does chronic inflammation in cancer impact upon tumour progression, but also has an effect on systemic process such as haemostasis (11-13), which may have an indirect effect on efficiency of the fibrinolytic system.

Our preliminary study comparing d_f in healthy smokers and non-smokers showed that there was no significant difference between the two groups. This is in keeping with a recent epidemiological study suggesting that only heavy smoking conveyed an increased VTE risk (46). This suggests that the changes in d_f we observed between lung cancer patients and controls are due to increased thrombogenicity of malignancy, and not smoking *per se*. Furthermore, this study did not find significant differences in measures of viscoelasticity or standard measures of coagulation between patients with different lung cancer cell types, in contrast to a previous report non-small cell tumours are more thrombogenic than small cell tumours (47).

There are several advantages to using d_f in this regard: firstly, it is a rapid and convenient assay on unadulterated blood; secondly, it is a global measure of the functional clot properties rather than a measure of an individual coagulation component as in conventional coagulation tests. This takes into account all aspects of global coagulation, including fibrinolytic activity, assessing all functions of the haemostatic system; thirdly, small changes in d_f represent exponential changes in clot mass—physical properties which are likely to influence subsequent clot lysis and potential to embolisation.

Whilst our findings indicate the utility of d_f in assessing clot microstructure in lung cancer patients, there are several limitations to this study. Firstly, the numbers of subjects investigated are relatively small and our findings will need to be confirmed and validated by larger studies and in other malignancies. Secondly, we have not provided prospective information on incidence of VTE, which would be required to confirm the utility of d_f potential biomarker of thrombogenicity in clinical practice. In addition, whilst the study was not powered for patient mortality, d_f was significantly increased in patients who died within 12 months of diagnosis. Although this may be associated with the increased d_f in patients with extensive disease, and therefore a poorer prognosis, a larger outcome study would provide additional information on whether d_f is truly associated with survival.

In conclusion, this proof of concept study demonstrates that d_f has the potential to quantify differences in pathological clot microstructure in patients with cancer. Furthermore, our biomarker may be a means of investigating and quantifying the increasing effects of chronic inflammation observed in different stages of lung cancer on global coagulation. We believe it lays the foundation for a paradigm shift away from measuring individual coagulation factors towards the measurement of functional activity of pathological clot and its properties. Further studies will be

required to investigate the use of d_f as a marker of clot microstructure and potential thrombogenicity in other malignancies, to further validate its reproducibility and define its narrow reference range for use in clinical practice.

Acknowledgements

We would like to acknowledge the outstanding support provided to us by the ABMU Health Board. We are grateful to the staff of the NISCHR Haemostasis Biomedical Research Unit and the South West Wales Cancer Multi Disciplinary Team. This translational work was supported by the NISCHR Biomedical Research Unit (BRU) grant (BRO1).

Author Contributions

NAD: study design and analysis, blood sample collection, data collection (rheology), drafting of the article; NKH: volunteer recruitment, revising the article for scientific and intellectual content, interpretation of the data; SN: revising the article for scientific and intellectual content, interpretation of the data; MJL: study design and analysis, interpretation of the data (rheology), revising the article for scientific and intellectual content; LD: Data collection and analysis (SEM Imaging); RHKM: study design and statistical analysis; MRB: Computational modelling and interpretation; SJD; revising the article for scientific and intellectual content (laboratory markers), interpretation of the data (laboratory markers); KH: revising the article for scientific and intellectual content, interpretation of the data (rheology); 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.

Conflicts of interest

SN has previously received honoraria and acted as an advisor for Leo Pharma and Pfizer, and received research fund from Leo Pharma for studies not related to this one; SJD has previously received honoraria from Werfen, Bayer, and Mitsubishi Pharma and acted as an advisor for Werfen and Mitsubishi Pharma; PRW and PAE currently hold a patent on a method of analysing coagulating blood; No other authors have anything to disclose.

References

- 1 Silverstein MD, Heit JA, Mohr DN, et al. Trends in the incidence of deep vein thrombosis and pulmonary embolism: a 25-year population-based study. *Arch Intern Med* 1998; 158: 585-593.
- 2 White RH. The epidemiology of venous thromboembolism. *Circulation* 2003; 107: 14-8.
- 3 Noble S, Pasi J. Epidemiology and pathophysiology of cancer-associated thrombosis. *Br J Cancer* 2010; 102: S2-9.
- 4 Chew HK, Davies AM, Wun T, et al. The incidence of venous thromboembolism among patients with primary lung cancer. *J Thromb Haemost* 2008; 6: 601-608.
- 5 Khorana AA, Francis CW, Culakova E, et al. Thromboembolism is a leading cause of death in cancer patients receiving outpatient chemotherapy. *J Thromb Haemost* 2007; 5: 632-634.
- 6 Blom JW, Doggen CJ, Osanto S, et al. Malignancies, prothrombotic mutations, and the risk of venous thrombosis. *JAMA* 2005; 293: 715-722.
- 7 Bick RL. Alterations of hemostasis associated with malignancy: etiology, pathophysiology, diagnosis and management. *Semin Thromb Hemost* 1978; 5: 1-26.
- 8 Zwicker JI. Tissue factor-bearing microparticles and cancer. *Semin Thromb Hemost* 2008; 34: 195-8.
- 9 Vormittag R, Simanek R, Ay C, et al. High factor VIII levels independently predict venous thrombembolism in cancer patients. *Arterioscler Thromb Vasc Biol* 2009; 29: 2176-2181.
- 10 Geddings JE, Mackman, N. Tumor-derived tissue factor-positive microparticles and venous thrombosis in cancer patients. *Blood* 2013; 122: 1873-80.
- 11 Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001; 357: 539-545
- 12 Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002; 420:860-867.
- 13 Rakoff-Nouham, S. Why cancer and inflammation? *Yale J Biol Med* 2006; 79:123-130.
- 14 Mills JD, Ariëns RAS, Mansfield MW, et al. Altered fibrin clot structure in the healthy relatives of patients with premature coronary artery disease. *Circulation* 2002; 106: 1938-1942.
- 15 Undas A, Szuldrzynski K, Stepien E, et al. Reduced clot permeability and susceptibility to lysis in patients with acute coronary syndrome: effects of inflammation and oxidative stress. *Atherosclerosis* 2008; 196: 551-557.
- 16 Undas A, Zawilska K, Ciesla-Dul M, et al. Altered fibrin clot structure/function in patients with idiopathic venous thromboembolism and in their relatives. *Blood* 2009; 114: 4272-4278.
- 17 Weisel JW. The mechanical properties of fibrin for basic scientists and clinicians. *Biophys Chem* 2004; 112: 267-276.
- 18 Weisel JW, Litvinov RI. Mechanisms of fibrin polymerization and clinical implications. *Blood* 2013;

121: 1712-1719.

- 19 Collet JP, Park D, Lesty C, et al. Influence of fibrin network conformation and fibrin fiber diameter on fibrinolysis speed: dynamic and structural approaches by confocal microscopy. *Arterioscler Thromb Vasc Biol* 2000; 20: 1354-1361.
- 20 Martinez MR, Cuker A, Mills AM, et al. Enhanced lysis and accelerated establishment of viscoelastic properties of fibrin clots associated with pulmonary embolism. *Am J Physiol Lung Cell Mol Physiol* 2014; L397-404.
- 21 Ustuner Z, Akay OM, Keskin M, Kus E, Bal C, Gulbas Z. Evaluating coagulation disorders in the use of bevacizumab for metastatic colorectal cancer by thromboelastography. *Med Oncol* 2002; 29: 3125-3128.
- 22 Toukh M, Siemens DR, Black A, Robb S, Leveridge M, Graham CH, Othman M. Thromboelastography identifies hypercoagulability and predicts thromboembolic complications in patients with prostate cancer. *Thromb Res* 2014; 133: 88-95.
- 23 Thorson CM, Van Haren RM, Ryan ML, Curia E, Sleeman D, Levi JU, Livingstone AS, Proctor KG. Persistence of hypercoagulable state after resection of intra-abdominal malignancies. *J Am Coll Surgeons* 2013; 216: 580-589
- 24 Papa ML, Capasso F, Pudore L, Torre S, Mango S, Russo V, Delrio P, Palaia R, Ruffolo F, d'Eufemia MD, De Lucia D, Napolitano M, Di Micco P, Parisi V. Thromboelastographic profiles as a tool for thrombotic risk in digestive tract cancer. *Exp Oncol* 2007; 29: 111-115.
- 25 Akay OM, Ustuner Z, Canturk Z, Mutlu FS, Gulbas Z. Laboratory investigation of hypercoagulability in cancer patients using rotation thromboelastography. *Med Oncol* 2009; 26: 258-364
- 26 Davies NA, Harrison NK, Sabra A, Lawrence MJ, Noble S, Davidson SJ, Evans VJ, Morris RHK, Hawkins K, Williams PR, Evans PA. Application of ROTEM to assess hypercoagulability in patients with lung cancer. *Thromb Res* 2015; DOI: 10.1016/j.thromres.2015.02.021
- 27 Winter HH, Chambon F. Analysis of linear viscoelasticity of a cross-linking polymer at the Gel-Point. *J Rheol* 1986; 30: 367-382.
- 28 Muthukumar M, Winter HH. Fractal dimension of a crosslinking polymer at the gel point. *Macromolecules* 1986; 19: 1284-1285.
- 29 Evans PA, Hawkins K, Williams PR, et al. Rheometrical detection of incipient blood clot formation by Fourier transform mechanical spectroscopy. *J Nonnewton Fluid Mech* 2008; 148: 122-126.
- 30 Evans PA, Hawkins K, Morris RH, et al. Gel point and fractal microstructure of incipient clots are significant new markers of hemostasis for healthy and anticoagulated blood. *Blood* 2010; 116: 3341-3344.
- 31 Curtis DJ, Williams PR, Badiei N, et al. A study of microstructural templating in fibrin-thrombin gel networks by spectral and viscoelastic analysis. *Soft Matter* 2013; 9: 4883-4889.
- 32 Lawrence MJ, Kumar S, Hawkins K, et al. A new structural biomarker that quantifies and predicts changes in clot strength and quality in a model of progressive haemodilution. *Thromb Res* 2014; 134: 488-94.
- 33 Lawrence MJ, Sabra A, Mills G, Pillai SG, Abdullah W, Hawkins K, Morris RHK, Davidson SJ, D'Silva LA, Curtis DJ, Brown MR, Weisel JW, Williams PR, Evans PA. A new biomarker quantifies difference in clot microstructure in patients with venous thromboembolism. *British Journal of Haematology* 2014; 168 (4); 571-575.

- 34 Stanford SN, Sabra A, D'Silva LA, Lawrence MJ, Morris RHK, Storton S, Brown MR, Evans VJ, Hawkins K, Williams PR, Davidson SJ, Wani M, Potter JF, Evans PA. The changed in clot microstructure in patients with ischaemic stroke and the effects of therapeutic intervention: a prospective observational study. *BMC Neurology* 2015; DOI:10.1186/s12883-015-0289-1
- 35 Lawrence MJ, Sabra A, Thomas P, et al. Fractal dimension: A novel clot microstructure use in ST elevation myocardial infarction patients. *Atherosclerosis* 2015; DOI: 10.1016/j.atherosclerosis.2015.04.012
- 36 Edge S, Byrd DR, Compton CC, et al. *AJCC Cancer staging manual*. 7th edition. Springer publications 2010.
- 37 Curtis DJ, Brown MR, Hawkins K, et al. 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.
- 38 Brown MR, Curtis DJ, Rees P, et al. Fractal discrimination of random fractal aggregates and its application in biomarker analysis for blood coagulation. *Chaos Solitons Fractals* 2012; 45: 1025-1032.
- 39 Schneider, C.A., Rasband, W.S., Eliceiri, K.W. NIH Image to Image: 25 years of image analysis. *Nat Methods* 2012; 9: 671-675.
- 40 Ay, C, Dunkler D, Pirker R, Thaler J, Quehenberger P, Wagner O, Zielinski C, Pabinger I. High D Dimer levels are associated with poor prognosis in cancer patients. *Haematologica* 2012. 97(8): 1158-1164
- 41 Janicke F, Schmitt M, Pache L, et al. Urokinase (uPA) and its inhibitor PAI-1 are strong and independent prognostic factors in node-negative breast cancer. *Breast Cancer Res Treat* 1993; 24: 195-208.
- 42 Kuhn W, Pache L, Schmalfeldt B, et al. Urokinase (uPA) and PAI-1 predict survival in advanced ovarian cancer patients (FIGO III) after radical surgery and platinum-based chemotherapy. *Gynecol Oncol* 1993; 55: 401-409.
- 43 Andreasen PA, Kjoller L, Christensen L, et al. The urokinase- type plasminogen activator system in cancer metastasis: a review. *Int J Cancer* 1997; 72:1–22.
- 44 Schmitt M, Harbeck N, Thomssen C, et al. Clinical impact of the plasminogen activation system in tumour invasion and metastasis: prognostic relevance and target for therapy. *Thromb Haemost* 1997; 78: 285-96.
- 45 Gronostaj K, Richter P, Nowak W, et al. Altered plasma fibrin clot properties in patients with digestive tract cancers: links with the increased thrombin generation. *Thromb Res* 2013; 131: 262-267.
- 46 Cheng Y, Liu Z, Yao, F, et al. Current and former smoking and risk for venous thromboembolism: A systematic review and meta-analysis. *PLOS Medicine* 2013; 10: 1371.
- 47 Tesselaar ME, Osanto S. Risk of venous thromboembolism in lung cancer. *Curr opin pulm med* 2007; 13: 362-367

Figure Legends

Figure 1: Graph illustrating the changes in d_f observed in patients with lung cancer compared to healthy controls. Dot plots illustrating the change in d_f in A: patients with lung cancer (1.76 ± 0.06) compared to healthy controls (1.73 ± 0.04 , $p < 0.05$), and B: patients with extensive lung cancer (1.79 ± 0.05) compared to patients with limited disease (1.73 ± 0.05 , $p < 0.05$). The stars represent the mean value of the data for each time point. * denotes a significant deviation ($p < 0.05$) analysed using a two sample t-test.

Figure 2: Computational analyses of fibrin mass and clot structure A: Graph illustrating the non-linear relationship between the fractal properties of the incipient fibrin clot measured by d_f and the amount of mass, incorporated into the structure. The mass value on the y-axis is normalised for the control $d_f = 1.73$, with the d_f values of 1.73 (healthy controls and patients with limited disease) and 1.79 (patients with extensive disease) annotated. B: Illustrations of fibrin mass at values of $d_f = 1.73$ (healthy controls and patients with limited disease), 1.79 (patients with extensive disease) and 1.91 (highest value obtained in the present study). The colour of each node (unit sphere) within the fractal represents the local density of constituent nodes within a sphere of radii 5 units, the colour ranges from green (1 neighbouring node) to red (20 neighbouring nodes).

Figure 3: SEM micrographs of mature blood clots. Scanning electron micrographs of mature clots in A: Healthy individual, $d_f = 1.73$, B: patient with stage I lung adenocarcinoma, $d_f = 1.74$ and

C: patient with stage IV lung adenocarcinoma, $d_f = 1.78$, all at x5.00k magnification using a Hitachi Ultra-high resolution FE-SEM S-4800. Clots were allowed to form at 37°C then imaged at room temperature.

Tables

Characteristic	Limited	Extensive
<i>n</i>	35	52
Age	71.7±7.5	69.2±8.0
Male (%)	16 (46.0)	27 (48.1)
Non smoker (%)	4 (11.0)	1 (1.9)
Hypertension (%)	16 (45.7)	17 (32.7)
Hyperlipidaemia (%)	11 (31.4)	15 (28.8)
Type 2 Diabetes (%)	4 (11.4)	6 (11.5)
12 month mortality	6 (17.1)	28 (53.8)
Aspirin use	9 (25.7)	6 (11.8)
<u>Histopathological tissue type</u>		
Adenocarcinoma	18 (51.4)	22 (42.3)
Squamous cell carcinoma	12 (34.3)	15 (28.8)
Small cell carcinoma	1 (2.9)	13 (25.0)
NSCLC NOS	4 (11.4)	2 (3.8)
<u>TNM Classification</u>		
Stage 1	23 (65.7)	-
Stage 2	12 (34.3)	-
Stage 3	-	30 (57.7)
Stage 4	-	22 (42.3)

Table 1: Baseline demographics and tumour information of the lung cancer cohort. (NSCLC

NOS: Non small cell lung cancer not otherwise specified, TNM: Tumour Nodes Metastases)

	d_f	p-value	T_{GP} (secs)	p-value
Healthy controls	1.73 ± 0.04	<0.05	231 ± 78	0.243
(Range)	(1.63-1.79)		(90-369)	
Lung cancer	1.76 ± 0.06		216 ± 83	
(Range)	(1.64-1.91)		(80-366)	
Non-small cell lung cancer	1.76 ± 0.06	0.266	209 ± 84	0.552
(Range)	(1.64-1.91)		(80-344)	
Small cell lung cancer	1.78 ± 0.04		228 ± 84	
(Range)	(1.72-1.82)		(136-366)	
Limited (Stages 1&2)	1.73 ± 0.05	<0.05	245 ± 107	0.072
(Range)	(1.64-1.81)		(113-344)	
Extensive (Stages 3&4)	1.79 ± 0.05		197 ± 63	
(Range)	(1.71-1.91)		(80-366)	
Alive at 12 months	1.74 ± 0.06	<0.05		
RIP at 12 months	1.79 ± 0.06			

Table 2: Rheometric data for patients with lung cancer and healthy controls. Rheometric data (d_f : fractal dimension, T_{GP} : Gel time) as measured in the lung cancer cohort compared to healthy controls, and analysed based on tissue type and stage of disease.

	PT (sec)	p-value	APTT (sec)	p-value	Clauss fibrinogen (g/l)	p-value	Platelets (x10 ⁹)	p-value
Healthy	10.4 ± 0.5	0.784	25.8 ± 1.9	0.845	3.2 ± 0.5	<0.05	244±51	<0.05
Lung Cancer	10.6 ± 0.6		25.9 ± 1.0		4.2 ± 1.1		319 ± 96	
Limited (stages 1& 2)	10.6 ± 0.5	0.722	26.1 ± 2.8	0.538	4.2 ± 1.0	0.956	310 ± 81	0.438
Extensive (stages 3&4)	10.5 ± 0.6		25.7 ± 3.2		4.2 ± 1.1		326 ± 105	

Table 3: Conventional markers of coagulation as measured in healthy controls compared to lung cancer patients and patients with limited disease (stages 1&2) compared to extensive disease (stages 3&4). (PT: Prothrombin time; APTT: Activated partial thromboplastin time)

	Limited (Stages 1&2)	Extensive (Stages 3&4)	p-value	Normal range
Thrombin generation (nM)	400 ±133	425 ±138	0.489	140-440
FVIII (IU/L)	131.3 ± 39.7	143.3 ± 48.4	0.311	50-150
TF MP	0.417 ± 0.381	0.594 ± 0.401	0.096	<2.0
<u>Fibrinolytic markers</u>				
PAI-1	10.2 ± 5.9	17.6 ± 10.3	<0.005	<25.0
D-dimer	280 ± 162	461 ± 670	0.096	<240

Table 4: Additional measures of haemostasis in patients with limited (stages 1&2) compared to extensive (stages 3&4) lung cancer. (FVIII: Factor VIII; TF MP: Tissue Factor Microparticles; PAI-1: Plasminogen activator inhibitor-1)