



Swansea University
Prifysgol Abertawe



Cronfa - Swansea University Open Access Repository

This is an author produced version of a paper published in :
Intensive Care Medicine

Cronfa URL for this paper:
<http://cronfa.swan.ac.uk/Record/cronfa30088>

Paper:

Davies, G., Pillai, S., Lawrence, M., Mills, G., Aubrey, R., D'Silva, L., Battle, C., Williams, R., Brown, R., Thomas, D., Morris, K. & Evans, P. (2016). The effect of sepsis and its inflammatory response on mechanical clot characteristics: a prospective observational study. *Intensive Care Medicine*
<http://dx.doi.org/10.1007/s00134-016-4496-z>

This article is brought to you by Swansea University. Any person downloading material is agreeing to abide by the terms of the repository licence. Authors are personally responsible for adhering to publisher restrictions or conditions. When uploading content they are required to comply with their publisher agreement and the SHERPA RoMEO database to judge whether or not it is copyright safe to add this version of the paper to this repository.

<http://www.swansea.ac.uk/iss/researchsupport/cronfa-support/>

The Effect of Sepsis and its Inflammatory Response on Mechanical Clot Characteristics: A Prospective Observational Study

Authors:

Gareth R Davies PhD^{1,2}

Suresh Pillai³

Matthew Lawrence PhD^{1,2}

Gavin M Mills³

Robert Aubrey³

Lindsay D'Silva PhD^{1,2}

Ceri Battle PhD¹

Professor Rhodri Williams PhD⁵

Rowan Brown PhD⁵

Dafydd Thomas⁶

Keith Morris PhD⁴

Professor Phillip Adrian Evans MD FRCS FFAEM^{1,2,3*}

Affiliated Institutions

1. Haemostasis Biomedical Research Unit (HBRU), Morriston Hospital, Swansea, UK.
2. College of Medicine, Swansea University, Swansea, UK.
3. Emergency Department, ABM University Health Board, Swansea, UK.
4. School of Applied Sciences, Cardiff Metropolitan University, Cardiff, UK.
5. College of Engineering, Swansea University, Swansea, UK.
6. Cardiac Intensive Care Unit, ABM University Health Board, Swansea, UK.

*Corresponding author

e-mail: phillip.evans2@wales.nhs.uk

telephone: +441792 532419

Conflict of Interest: PAE and PRW have signed the International Committee of Medical Journal Editors (ICMJE) form for declaration of interest and have declared all conflict of interests. All other authors declare no competing conflicts of interest.

Abstract

Purpose: Sepsis and its progression are known to have a major influence on the coagulation system. Current coagulation tests are of limited use when assessing coagulation in sepsis patients. This study aims to assess the potential for a new functional biomarker of clot microstructure, fractal dimension, to identify changes in the mechanical properties of clot microstructure across the sepsis spectrum (sepsis, severe sepsis and septic shock). *Methods:* A total of 100 patients that presented acutely to a large teaching hospital were included in this prospective observational study (50 sepsis, 20 severe sepsis and 30 septic shock) against a matched control of 44 healthy volunteers. Fractal analysis was performed, as well as standard markers of coagulation, and six plasma markers of inflammation. *Results:* Fractal dimension was significantly higher in the sepsis and severe sepsis groups than the healthy control (1.78 ± 0.07 and 1.80 ± 0.05 respectively vs 1.74 ± 0.03) ($p < 0.001$), indicating a significant increase in mechanical clot strength and elasticity consistent with a hypercoagulable state. Conversely, fractal dimension was significantly lower in septic shock (1.66 ± 0.10 , $p < 0.001$), indicating a significant reduction in mechanical clot strength and functionality consistent with a hypocoagulable state. This corresponded with a significant increase in the inflammatory response. *Conclusions:* This study confirms that clot microstructure is significantly altered through the various stages of sepsis. Of particular importance was the marked change in clot development between severe sepsis and septic shock, which has not been previously reported.

Key Words

Sepsis

Biomarkers

Coagulation

Clot Microstructure

Introduction

Sepsis and its associated underlying inflammatory response are known to exert complex changes on the haemostatic system [1]. Defects in the coagulation system have been implied to contribute to the development of organ dysfunction and poor outcomes in sepsis [2], however, clinical trials aiming to correct these defects have so far failed to provide effective new treatments [3–5]. New insights into haemostasis may help to explain the lack of progress in the treatment of sepsis. Although currently used clinical markers (aPTT and PT) are useful for monitoring anticoagulation, they do not reflect the global picture of coagulation and are insensitive to clinically significant coagulation defects [6–8]. Rheometry can be used to scientifically quantify the developing mechanical properties of blood as it clots, and has recently been identified as a means for quantifying the quality and arrangement of clot microstructure through its fractal dimension (d_f) [9]. d_f is measured using whole blood, and has been shown to detect hypercoagulable effects even when conventional tests of coagulation do not [10]. Abnormal clot microstructure has recently been detected in patients with both stroke and cancer using d_f [10, 11]. However, d_f has not been used previously to quantify clot microstructure across the sepsis spectrum. This study hypothesises that the mechanical properties of clot microstructure are significantly altered by the underlying inflammatory response across the sepsis spectrum, and that d_f is able to detect and identify the changes from the earlier hypercoagulable phase and the hypocoagulable phase of septic shock in terms of clot quality.

The primary aim of this study was to quantify the changes in mechanical clot quality and strength across the stages of sepsis, by using the new marker of clot microstructure, d_f .

Some of the data related to the research in this manuscript was presented and published as an abstract in the International Symposium of Intensive Care and Emergency Medicine 2016 prior to the completion of this study [12].

Methods

Study Design

The present study was a single centre prospective observational study carried out in the Emergency Department and Intensive Care Unit of a large teaching hospital. This study had full ethical approval from the South West Wales Research Ethics Committee. Informed 2-stage written consent was given by patients with capacity to do so. Assent was obtained from personal or legal representation in cases where capacity to give informed consent was lacking.

Recruitment of Subjects with Sepsis

Subjects that met sepsis criteria as outlined by the American College of Chest Physicians/Society of Critical Care Medicine in 1991 [13] were recruited to this study within 24 hours of hospital admission. Patients (age ≥ 18 years) were included from October 2011 to March 2014. Patients on warfarin or those who had received any treatment likely to affect coagulation (anticoagulation including thromboprophylaxis, component replacement) were excluded. Patients with any pre-

existing disease likely to affect the coagulation profile e.g. chronic renal failure, chronic liver disease, malignancy or thrombotic/clotting disorder were also excluded. Furthermore, patients that were deemed insensitive to include i.e. impending death were also excluded from the study.

Patients were categorised as sepsis, severe sepsis or septic shock at the time of inclusion to the study. Categorisation was blinded, and carried out by an intensive care specialist independent of the study using the criteria established in 1991 [13]. Sepsis was defined as two or more of the SIRS criteria, with evidence of infection. Severe sepsis was defined as sepsis with at least one acute organ dysfunction [14] whereas septic shock was defined as sepsis with refractory hypotension including perfusion abnormality (lactate >2 mmol/L) in the absence of cardiogenic shock/bleeding.

A group of healthy volunteers matched for age, gender and from a demographically similar population group was recruited as a healthy control.

Blood Sampling

Blood was sampled immediately on inclusion to the study. The first 5mls of blood was discarded, with the following 6.6mls drawn and immediately placed into the rheometer to determine d_f . Further blood samples were drawn into an EDTA vacutainer (Becton, Dickinson and Company, UK, Ref: 367839) and a 3.2% sodium citrate vacutainer (Greiner Bio-One GmbH, Austria, Ref: 454327) to determine coagulation and inflammatory markers.

Laboratory Markers

Standard coagulation markers (aPTT, PT and fibrinogen) were determined using a Sysmex CA1500 automated analyser within 2 hours of collection. Platelet count was determined using a Sysmex XE 2100 automated haematology analyser within 2 hours of collection. Factor VIII activity was determined by an aPTT based one-stage assay using appropriate factor deficient plasma and Actin FS aPTT reagent (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). D-dimer concentration was determined using TriniLIA Auto-D-dimer[®] turbidimetric assay.

Inflammatory markers were determined to quantify the inflammatory response at each stage of the sepsis spectrum and were selected based on previous studies examining the inflammatory effects of sepsis. Concentration of each of the inflammatory markers was measured in platelet poor plasma using enzyme-linked immunosorbent assay (ELISA) kits for PCT, TNF- α , IL-6 and sE-Selectin. The standard methodology was followed as supplied by manufacturer.

Rheometrical analysis

Gel point analysis was performed using rheometry, to detect the gel point of the coagulating blood and quantify the fractal microstructure of the fibrin clot at this point (d_f). The methodology carried out in this study has been described in several previous publications [9], [15–17].

Computational Analysis

To assess the relationship between d_f and fibrin mass at the gel point (incipient clot mass), computer modelling was carried out. A numerical technique which has previously been used to generate random fractal aggregates was used [18, 19]. The algorithm incorporated in this technique uses the

box-counting measure of fractal dimension and allows a visual illustration of the incipient clot microstructure to be produced, based on the d_f measurements, and also allows for the corresponding fibrin mass to be calculated.

Mature Clot Imaging – Scanning Electron Microscopy

SEM samples were prepared from 12 μ L of whole blood using the methodology described previously [10]. The resultant dehydrated blood samples were coated with gold palladium, and imaged using a Hitachi ultra-high resolution FE-SEM S-4800. Fibrin fibre width was determined from the randomly selected images using the same methodology as has been described previously [20].

Statistical Analysis

This study aimed to investigate significant differences in df between subjects with sepsis, severe sepsis and septic shock. However, sample size calculation was based on our pilot data and involved detecting the smaller difference in subjects with sepsis, and severe sepsis of 0.07(\pm SD 0.06). Hence using this minimum values to determine our sample size (expected difference of 0.07, power=0.9 and SD \pm 0.06) and one-way ANOVA at three levels, a minimum sample of at least 15 subjects in each group was required for this study. The data are reported as mean and standard deviation or median and interquartile range where appropriate. Differences in frequency distribution used chi-squared analysis of nominal data across groups. Students T-test assessed differences between normally distributed groups, and Mann-Whitney U test was used for non-normally distributed groups. One-way ANOVA with Bonferroni post-hoc correction was used to analyse for multiple group differences or alternatively a Kruskal-Wallis test for non-normally distributed data. Data normality was assessed using the Shapiro-Wilk test with α value of 0.05. A binomial logistic regression using mortality as the binary response variable and PT and d_f as Continuous Predictor variables. The Goodness of Fit was determined by the Hosmer-Lemeshow and Pearson Methods and the Odds ratios together with their 95% Confidence Intervals for the continuous variables calculated. All statistical analysis was carried out using SPSS for Windows, version 22.0 (Armonk, NY: IBM Corp.).

Results

Baseline Characteristics of Patient Groups

Baseline characteristics of the included patients are shown in table 1. 100 sepsis patients in total were included in the study. This included 50 patients with sepsis, 20 with severe sepsis and 30 with septic shock. 44 healthy volunteers matched for gender and age were also recruited as a healthy control group. Baseline characteristics reflected the severity of each group, with a significantly increased SOFA score, hospital length of stay, mortality and requirement for component replacement observed from sepsis through septic shock.

Changes in Standard Markers of Coagulation across the Sepsis Spectrum

Changes in coagulation markers across the different groups are shown in figure 1. Standard kinetic markers of coagulation PT and aPTT were significantly prolonged in septic shock ($p < 0.001$) when compared to other groups. PT was also significantly prolonged in severe sepsis compared to the healthy group ($p < 0.005$). An increased fibrinogen concentration was observed in sepsis and severe sepsis ($p < 0.001$) consistent with the underlying inflammatory response of the disease process. In the septic shock group, although fibrinogen concentration was significantly lower than in sepsis ($p < 0.01$), it remained significantly higher than in the healthy group ($p < 0.05$). A significantly reduced platelet count was observed in patients with septic shock compared to all other groups ($p < 0.05$) (Figure 1). Factor VIII activity was significantly lower in septic shock than both sepsis ($p < 0.05$), and severe sepsis ($p = 0.001$). Furthermore, D-Dimer was also significantly increased in septic shock compared to sepsis ($p < 0.001$), indicating increased fibrinolytic activity.

Changes in Inflammatory Markers across the Sepsis Spectrum

Inflammatory markers were measured on a subgroup of the total patients in order to quantify an increasing or decreasing inflammatory response with severity of group (40 sepsis, 13 severe sepsis, 12 septic shock). Baseline measurements for inflammatory markers are shown in Table 2. A progressive increase in the inflammatory response from sepsis through to septic shock was observed. The inflammatory markers PCT and IL-8 were both significantly elevated in the septic shock group.

Changes in Clot Microstructure (d_f) across the Sepsis Spectrum

Changes in d_f in the sepsis groups compared to healthy volunteers are shown in Figure 1. A d_f of 1.74 ± 0.03 was observed in healthy volunteers, which is consistent with the healthy range that has been determined in previous studies [9–11]. d_f was significantly higher in subjects with sepsis and severe sepsis (1.78 ± 0.07 and 1.80 ± 0.05 respectively ($p < 0.05$)) and significantly lower d_f was observed in subjects with septic shock (1.66 ± 0.10 ($p < 0.001$)). This suggests clots formed in subjects with sepsis and severe sepsis are structurally dense, with increased fibrin branching and elasticity, whereas in septic shock clots formed are structurally weaker, with a tenuous fibrin structure with reduced branching and elasticity.

Computer Modelling of Mass Change at the Incipient Clot in Relation to d_f

It is possible to quantify both the mechanical arrangement of the incipient clot and its mass through computer modelling techniques. Using these techniques previous studies have shown that a relatively small change in d_f equates to large changes in the fibrin mass incorporated into the clot [10, 19]. Therefore, to understand and define the change in fibrin mass and connectivity of the incipient clot, computer modelling was carried out (Figure 2). Using this model for incipient clot formation indicates the patients with the highest d_f form an incipient clot with 350% of the fibrin mass of the control group, whereas the patients with the lowest d_f form an incipient clot with 4% of the normal fibrin mass.

Final Clot Imaging – Scanning Electron Microscopy

Scanning electron microscopy was used to image the mature clot as a qualitative comparison for subjects across the sepsis spectrum. Clots formed from subjects with sepsis were denser and had thinner fibres than healthy individuals. Clots formed from subjects with septic shock appeared looser, with less fibrin thinner fibres than subjects with sepsis (Figure 3).

Comparison between Survivors and non-Survivors at 28 Days

All patients were followed up for all cause 28-day mortality. Characteristics of survivors and non-survivors at 28 days are shown in Supplementary Table 1. A significant trend towards hypocoagulability was apparent in non-survivors compared to survivors. Poor outcome was associated with a prolonged PT and reduced fibrinogen concentration. A weaker clot microstructure was also significantly associated with poor outcome, as indicated by significantly lower values of d_f in patients that did not survive 28 days. Furthermore, IL-8 and PCT were significantly associated with poor outcomes.

Binary Logistic Regression was undertaken on the two most significant coagulation variables (PT and d_f) as a predictor of mortality. PT and d_f were found to be significant predictors of mortality ($p < 0.0005$) with odds ratios of 1.54 (CI 1.12 – 2.12) and 0.10 (CI 0.00 – 0.21) respectively. The odds ratio of less than 1 for d_f confirmed the significant effect of a lowered d_f on survival.

Discussion

This study demonstrates for the first time that a new functional biomarker of clot microstructure, d_f , can act as a significant biomarker for characterising changes in the mechanical properties of the clot in both the hyper and hypocoagulable phases of sepsis.

Previous studies have shown how d_f is an accurate determinant for defining both the developing and the final clot architecture in acute vascular inflammatory conditions [11]. Increases in d_f have previously been shown to be characteristically associated with hypercoagulable states of vascular inflammatory disease and increasing clot strength [10, 11], whereas reduced values of d_f are associated with hypocoagulable states and a weakening of the mechanical clot properties [11, 20], [21].

In the present study, we describe for the first time the changes that occur in clot microstructure across the clinically recognised stages of the sepsis spectrum. A significant increase in d_f was observed in patients with sepsis and severe sepsis, corresponding to formation of a tight, highly elastic incipient clot, that is potentially more resistant to fibrinolysis. In septic shock, there was a significantly lower d_f , corresponding to formation of a loose, structurally weak incipient clot, that is potentially more susceptible to fibrinolysis.

As in previous studies the present study indicated a significant increase in the inflammatory response in septic shock [22]. Despite this increased inflammatory response, there was a profound reduction in d_f and mechanical strength of the developing clot in septic shock. There are multiple factors that could contribute to this hypocoagulable effect. It has been shown that activity of clotting factors is reduced in septic shock, and this culminates in delayed and reduced thrombin generation [23]. This impairment of thrombin generation could lead to a lack of available fibrin to be incorporated into the incipient clot. Furthermore, in the present study patients with septic shock were significantly more acidotic and hypothermic, both of which have been shown to induce hypocoagulable effects and effect fibrin polymerisation and clot strength [24, 15].

In the hypercoagulable phases of sepsis and severe sepsis there was a significant increase in fibrinogen concentration, which is a known finding associated with the acute inflammatory response in sepsis [25]. In septic shock, fibrinogen concentration still remained significantly elevated when compared the healthy control, whereas there was a corresponding significant and marked reduction in d_f . This indicates that although there is an adequate concentration of fibrinogen in septic shock, the organisation of the fibrin clot microstructure formed was mechanically weak and porous compared to the matched healthy control group. This was confirmed in the scanning electron microscopy, where it was noted that there was an open clot, with visibly reduced incorporation of fibrin and branching which was also reflected by fibre width.

The present study, utilising d_f scientifically determines and quantifies how mechanical clot strength in terms of its cross linking and connectivity changes as sepsis progresses. Donze et al. showed that with increasing severity of sepsis there is an increase in prothrombotic tendency and occurrence of thromboembolic events [26]. In this study d_f indicated a significant procoagulant change in clot microstructure in sepsis and severe sepsis, which could contribute to the recognised increased incidence of thromboembolic events [26]. Previous studies have shown how patients in septic shock undergo haemostatic changes due to consumption and coagulopathic effects, which could result in bleeding tendency [27, 28]. It is difficult to assess the relative risk of bleeding in septic shock, however, with previous studies reporting the incidence of major bleeding events at between 1 and 20% [3, 29, 30], but no definitive study quantifying this bleeding risk. In this study, it was highlighted that despite sufficient platelet numbers and fibrinogen concentration in septic shock, there was still the inability to form a mechanically stable clot, as highlighted by d_f .

Comparison of d_f against standard clinical markers of coagulation showed that in the hypercoagulable phase of sepsis, the standard markers were normal, despite an increased d_f . This confirms the lack of sensitivity of the standard markers of coagulation to hypercoagulable changes in sepsis. In septic shock there was a significant prolongation of aPTT and PT, which corresponded to a significant reduction in d_f .

It is well recognised that both thrombin generation and fibrinolytic function can be impaired in septic shock [27, 31]. Overall, these complex haemostatic changes can lead to a dichotomy, where a patient may have thromboembolic risk, but still be at risk of a bleeding diathesis, both of which may be clinically difficult to evaluate in the intensive care setting. Although in this study we did not

look at clinical outcomes or underlying mechanisms, d_f clearly quantified how the changes and the balance between the pro and anti-coagulant effects of coagulation could be assessed in terms of clot characteristics and strength.

In order to investigate the structural characteristics of the mature clot across the sepsis spectrum and its relationship to d_f , we used SEM imaging. It has been shown previously that in hypercoagulable states, there is increased fibrin binding with an associated increase in fibrin mass and density of branching [32]. In this study it was confirmed that there was an alteration in clot microstructure in the different stages of sepsis, which has not been shown before. In the early hypercoagulable stages, SEM images showed increased branching, reduced pore space and thinner fibrin fibres. Conversely in septic shock, it was noted that although thinner fibrin fibres were observed, similar to in hypercoagulable states, the cross linking was reduced and pore spaces appeared larger, indicating a loosening of the clot architecture and implying a looser, weaker final clot structure due to less fibrinogen being incorporated into the polymerised clot mass.

Altered clot structure and its mechanical properties were further investigated using computer modelling, where it was indicated that higher value of d_f were associated with a much greater polymerised fibrin mass of high connectivity consistent with a prothrombotic or hypercoagulable state. For the highest values of d_f that were observed, computer modelling indicated a corresponding 350% increase in fibrin mass incorporated into the clot, whereas for the lowest values of d_f the incipient clot had a fibrin mass of less than 10% of that incorporated into a healthy clot.

In previous studies it has been shown that d_f can characterise the mechanisms of clot microstructure in inflammatory diseased states at their various stages [10]. This study again confirms and gives further evidence that characterisation of clot architecture across the sepsis spectrum may give further understanding of the effects of sepsis and its inflammatory response on the coagulation system. However, further mechanistic studies are required to explore and determine the factors that underpin these changes in clot microstructure in patients who move from severe sepsis to septic shock. Further studies are underway to explore these mechanistic possibilities and also to investigate how these changes affect clinical outcome.

Limitations

This study has a number of limitations. Firstly this was a single centre observational study, and although some outcome data were presented, they were not powered for clinical outcome, as this was a proof of concept study. Furthermore, it was outside the scope of this study to seek mechanistic conclusions. The inherent problem of this study and other similar studies is the heterogeneity of the disease, its treatment and comorbidities. It is also difficult to take in to account the possible differences in concomitant medications and treatment between the groups. To assess these effects fully a much larger study would be required. Further larger prospective studies are required to build on the findings of this study.

Acknowledgements

This study was funded by the National Institute for Social Care and Health Research (NISCHR) and was also part-funded by the European Social Fund (ESF) through the European Union's Convergence

programme administered by the Welsh Government. Our thanks go to the staff in the Emergency Department, Intensive Therapy Unit and Haemostasis Biomedical Research Unit of Morriston Hospital for their invaluable support.

References

1. Schouten M, Wiersinga WJ, Levi M, Van Der Poll T (2006) Inflammation, endothelium, and coagulation in sepsis. *J Leukoc Biol* 79:536–545
2. Dhainaut J-F, Shorr AF, Macias WL, Kollef MJ, Levi M, Reinhart K, Nelson DR (2005) Dynamic evolution of coagulopathy in the first day of severe sepsis: relationship with mortality and organ failure. *Crit Care Med* 33:341–348
3. Ranieri VM, Thompson BT, Barie PS, Dhainaut J-F, Douglas IS, Finfer S, Gårdlund B, Marshall JC, Rhodes A, Artigas A, Payen D, Tenhunen J, Al-Khalidi HR, Thompson V, Janes J, Macias WL, Vangerow B, Williams MD (2012) Drotrecogin alfa (activated) in adults with septic shock. *N Engl J Med* 366:2055–2064.
4. Rimmer E, Kumar A, Doucette S, Marshall J, Dial S, Gurka D, Dellinger RP, Sharma S, Penner C, Kramer A, Wood K, Ronald J, Kumar A, Turgeon AF, Houston DS, Zarychanski R (2012) Activated protein C and septic shock: a propensity-matched cohort study*. *Crit Care Med* 40:2974–81
5. Abraham E, Opal S, Demeyer I, Lo A, Svoboda P, Laterre F, Simon S, Light B, Peckelsen C, De Deyne C, Percell SR, Shu V, Poole L, Creasey AA (2003) Efficacy and safety of tifacogin (recombinant tissue factor pathway inhibitor) in severe sepsis: a randomized controlled trial. *JAMA* 290:238–247
6. Lipets EN, Ataullakhanov FI (2015) Global assays of hemostasis in the diagnostics of hypercoagulation and evaluation of thrombosis risk. *Thromb J* 13:4
7. Meybohm P, Zacharowski K, Weber CF (2013) Point-of-care coagulation management in intensive care medicine. *Crit Care* 17:218
8. Favaloro EJ, (Adcock) Funk DM, Lippi G (2012) Pre-analytical Variables in Coagulation Testing Associated With Diagnostic Errors in Hemostasis. *Lab Med* 43:1–10
9. Evans PA, Hawkins K, Morris RHK, Thirumalai N, Munro R, Wakeman L, Lawrence MJ, Williams PR (2010) Gel point and fractal microstructure of incipient blood clots are significant new markers of hemostasis for healthy and anticoagulated blood. *Blood* 116:3341–3346
10. Davies NA, Harrison NK, Morris RHK, Noble S, Lawrence MJ, DSilva LA, Broome L, Brown MR, Hawkins KM, Williams PR, Davidson S, Evans PA (2015) Fractal dimension (df) as a new structural biomarker of clot microstructure in different stages of lung cancer. *Thromb Haemost* 114:1251–1259
11. Stanford SN, Sabra A, DSilva LA, Lawrence M, Morris R, Storton S, Brown M, Evans V, Hawkins K, Williams P, Davidson SJ, Wani M, Potter JF, Evans PA (2015) The changes in clot microstructure in patients with ischaemic stroke and the effects of therapeutic intervention: a prospective observational study. *BMC Neurol* 15:35
12. Davies G, Pillai S, Mills G, Aubrey R, Morris K, Williams P, Evans P (2016) Fractal dimension: a new biomarker for quantifying clot microstructure in patients across the sepsis spectrum. *Crit Care* 20(Suppl 2):P031

13. Bone R, Balk R, Cerra F, Dellinger R, Fein A, Knaus W, Schein R, Sibbald W (1992) Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 20:864–874
14. Bernard GR, Vincent J-L, Laterre P-F, Larosa SP, Dhainaut J-F, Lopez-Rodriguez A, Steingrub JS, Garber GE, Helterbrand JD, Ely EW, Fisher CJ Jr (2001) Efficacy and safety of recombinant human activated protein c for severe sepsis. *N Engl J Med* 344:699–709
15. Lawrence MJ, Marsden N, Mothukuri R, Morris RHK, Davies G, Hawkins K, Curtis DJ, Brown MR, Williams PR, Evans PA (2016) The Effects of Temperature on Clot Microstructure and Strength in Healthy Volunteers. *Anesthesia and Analgesia* 122:21–26
16. Evans PA, Hawkins K, Lawrence M, Williams RL, Barrow MS, Thirumalai N, Williams PR (2008) Rheometry and associated techniques for blood coagulation studies. *Med Eng Phys* 30:671–679
17. Evans PA, Hawkins K, Lawrence M, Barrow MS, Williams PR, Williams RL (2008) Studies of whole blood coagulation by oscillatory shear, thromboelastography and free oscillation rheometry. *Clin Hemorheol Microcirc* 38:267–277
18. Curtis DJ, Brown MR, Hawkins K, Evans PA, Lawrence MJ, Rees P, Williams PR (2011) Rheometrical and molecular dynamics simulation studies of incipient clot formation in fibrin-thrombin gels: An activation limited aggregation approach. *J Nonnewton Fluid Mech* 166:932–938
19. Brown MR, Curtis DJ, Rees P, Summers HD, Hawkins K, Evans PA, Williams PR (2012) Fractal discrimination of random fractal aggregates and its application in biomarker analysis for blood coagulation. *Chaos, Solitons and Fractals* 45:1025–1032
20. Lawrence MJ, Kumar S, Hawkins K, Boden S, Rutt H, Mills G, Sabra A, Morris RHK, Davidson SJ, Badiei N, Brown MR, Williams PR, Evans PA (2014) A new structural biomarker that quantifies and predicts changes in clot strength and quality in a model of progressive haemodilution. *Thromb Res* 134:488–494
21. Lawrence MJ, Sabra A, Thomas P, Obaid DR, DSilva LA, Morris RHK, Hawkins K, Brown MR, Williams PR, Davidson SJ, Chase AJ, Smith D, Evans PA (2015) Fractal dimension: A novel clot microstructure biomarker use in ST elevation myocardial infarction patients. *Atherosclerosis* 240:402–407
22. Harbarth S, Holeckova K, Froidevaux C, Pittet D, Ricou B, Grau GE, Vadas L, Pugin J (2001) Diagnostic value of procalcitonin, interleukin-6, and interleukin-8 in critically ill patients admitted with suspected sepsis. *Am J Respir Crit Care Med* 164:396–402
23. Andersen MG, Hvas CL, Tonnesen E, Hvas AM (2014) Thromboelastometry as a supplementary tool for evaluation of hemostasis in severe sepsis and septic shock. *Acta Anaesthesiol Scand* 58:525–33
24. Dirkmann D, Hanke AA, Gorlinger K, Peters J (2008) Hypothermia and acidosis synergistically impair coagulation in whole human blood. *Anesth Analg* 106:1627–32
25. Mavrommatis AC, Theodoridis T, Orfanidou A, Roussos C, Christopoulou-Kokkinou V, Zakyntinos S (2000) Coagulation system and platelets are fully activated in uncomplicated sepsis. *Crit Care Med* 28:451–7
26. Donze JD, Ridker PM, Finlayson SRG, Bates DW (2012) Impact of sepsis on risk of postoperative arterial and venous thromboses: large prospective cohort study. *BMJ* 349:g5334

27. Massion PB, Peters P, Ledoux D, Zimmermann V, Canivet J-L, Massion PP, Damas P, Gothot A (2012) Persistent hypocoagulability in patients with septic shock predicts greater hospital mortality: impact of impaired thrombin generation. *Intensive Care Med* 38:1326–35
28. Davies GR, Mills GM, Lawrence M, Battle C, Morris K, Hawkins K, Williams PR, Davidson S, Thomas D, Evans PA (2014) The role of whole blood impedance aggregometry and its utilisation in the diagnosis and prognosis of patients with systemic inflammatory response syndrome and sepsis in acute critical illness. *PLoS One* 9:e108589
29. Annane D, Timsit JF, Mégarbane B, Martin C, Misset B, Mourvillier B, Siami S, Chagnon J-L, Constantin J-M, Petitpas F, Souweine B, Amathieu R, Forceville X, Charpentier C, Tesnière A, Chastre J, Bohe J, Colin G, Cariou A, Renault A, Brun-Buisson C, Bellissant E (2013) Recombinant human activated protein C for adults with septic shock: a randomized controlled trial. *Am J Respir Crit Care Med* 187:1091–1097
30. Hvidt LN, Perner A (2012) High dosage of dextran 70 is associated with severe bleeding in patients admitted to the intensive care unit for septic shock. *Dan Med J* 59:1–5
31. Fourrier F, Chopin C, Goudemand J, Hendrycx S, Caron C, Rime A, Marey A, Lestavel P (1992) Septic shock, multiple organ failure, and disseminated intravascular coagulation. Compared patterns of antithrombin III, protein C, and protein S deficiencies. *Chest* 101:816–23
32. Collet JP, Allali Y, Lesty C, Tanguy ML, Silvain J, Ankri A, Blanchet B, Dumaine R, Gianetti J, Payot L, Weisel JW, Montalescot G (2006) Altered fibrin architecture is associated with hypofibrinolysis and premature coronary atherothrombosis. *Arterioscler Thromb Vasc Biol* 26:2567–73

Tables

Table 1: Baseline characteristics of healthy volunteers and sepsis patients: Demographic information and baseline characteristics are shown for each of the sepsis groups and the healthy control group. Significance values assessed by One-way ANOVA, Kruskal-Wallis test and Pearson Chi-Square test where appropriate. SOFA, sepsis-related organ failure assessment.

	Healthy	Sepsis	Severe Sepsis	Septic Shock	Significance Value
N	44	50	20	30	
Age (years)	60.8 ± 18.6	59.6 ± 19.8	66.3 ± 17.4	66.6 ± 14.9	0.268
Male Gender (n [%])	22 [50]	24 [48]	9 [45]	18 [60]	0.693
Source of Infection					0.398
Respiratory	-	29 [58]	12 [60]	11 [37]	
Urinary Tract	-	8 [16]	4 [20]	7 [23]	
Abdominal	-	9 [18]	1 [5]	9 [30]	
Soft Tissue	-	2 [4]	1 [5]	0 [0]	
Other/Unknown	-	2 [4]	2 [10]	3 [10]	
SOFA Score	-	3 (1, 3.5)	5 (4, 6)	9.5 (6.75, 12)	<0.001
Hospital Length of Stay (days)	-	6.0 (2, 9)	9.0 (5, 18)	15.0 (3.75, 37.5)	0.006
28 Day Mortality (n [%])	-	1 [2]	3 [15]	13 [43]	<0.001
Required Component Replacement During Stay (n [%])	-	0	1 [5]	6 [20]	0.003

Table 2: Inflammatory markers in sepsis patients: Concentrations of the different inflammatory markers are shown for the different sepsis groups. Data is presented as median (IQR). Significance values assessed using Kruskal-Wallis test. *Significantly different from sepsis group ($p = 0.001$), **Significantly different from both sepsis ($p < 0.001$) and severe sepsis ($p < 0.05$) (Kruskal-Wallis test, post-hoc pairwise comparisons, Bonferroni correction).

	Sepsis	Severe Sepsis	Septic Shock	Significance Value
CRP (mg/dL)	79 (39.5, 221.5)	193 (124, 422)	127.5 (21.0, 127.5)	0.078
PCT (pg/mL)	307.6 (108.3, 1037.5)	702.4 (384.5, 1637.1)	1257.6 (230.3, 17295.7)*	0.001
TNF-α (pg/mL)	4.0 (2.9, 30.5)	6.2 (4.8, 34.5)	40.6 (10.4, 249.3)	0.110
IL-6 (pg/mL)	320.7 (67.6, 651.7)	202.8 (137.5, 532.6)	743.9 (103.5, 822.3)	0.062
IL-8 (pg/mL)	61.4 (20.6, 176.2)	71.1 (40.9, 103.7)	525.7 (136.1, 7997.9)**	<0.001
e-Selectin (ng/mL)	70 (37, 127)	54 (20.5, 118.5)	37 (33.8, 74.8)	0.584

Figure 1: Changes in markers of coagulation across the sepsis spectrum: Markers of coagulation across the sepsis spectrum and compared to the healthy control group are shown. Normally distributed data are presented as means plots with the error bars representing the standard deviation of the data. Non-normally distributed data are presented as box plots (median and IQR) with whiskers representing the range. Significant differences are shown (Bonferroni corrected post-hoc analysis; Multiple comparisons made using one-way ANOVA for normalised data and Kruskal-Wallis for non-normalised data).

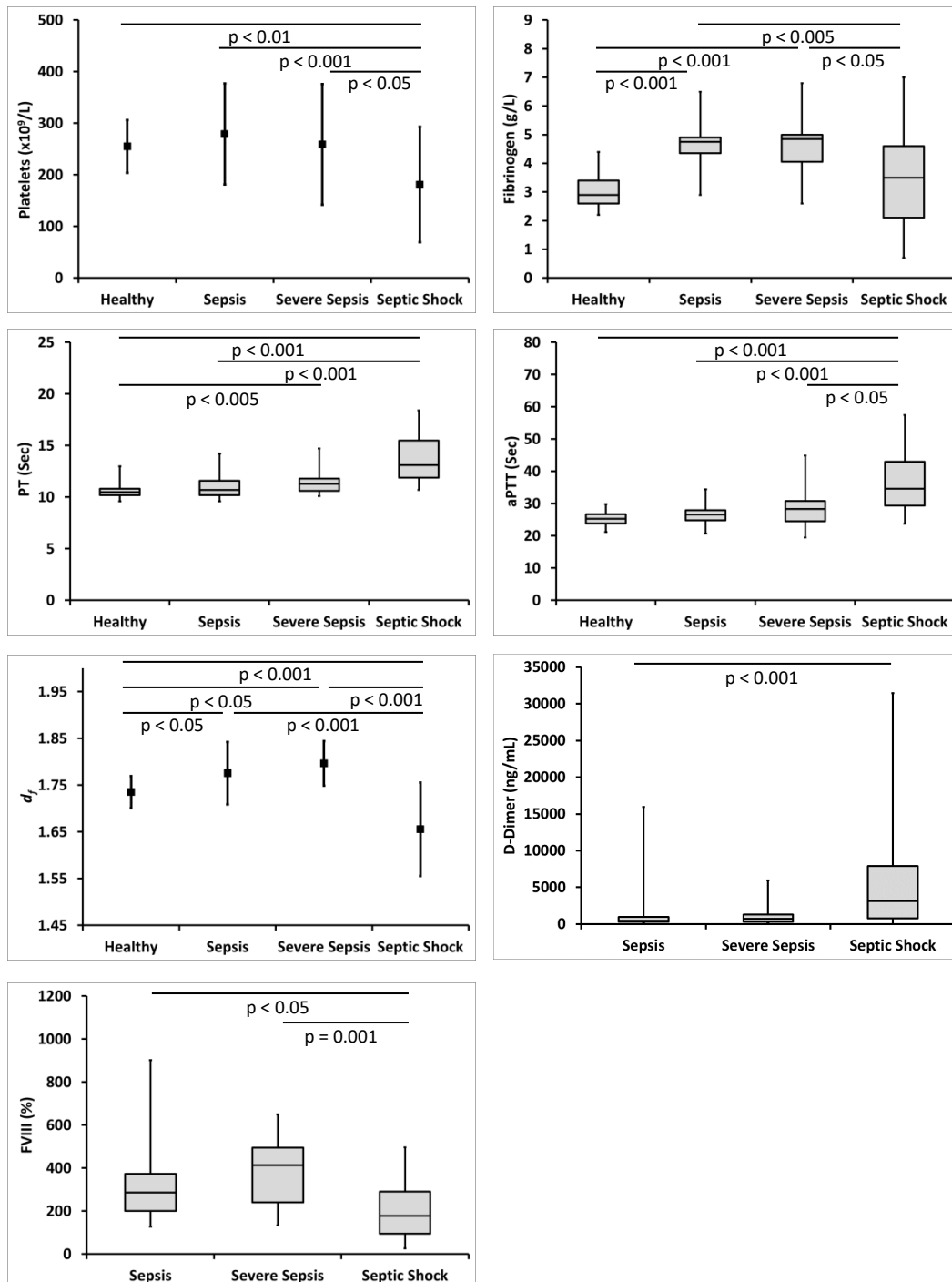


Figure 2: Computational analysis of fibrin mass curve: A) Graph illustrating the non-linear relationship between fibrin mass incorporated into the incipient clot and d_f . The mass on the y-axis is normalised for the control of $d_f = 1.74$. The following values of d_f were put into the model: $d_f = 1.74$ (healthy), $d_f = 1.45$ (septic shock) and $d_f = 1.85$ (sepsis/severe sepsis). The respective mass of each d_f is indicated by O, X and \square respectively. Accompanying diagrams of fractal structures indicate the fibrin mass incorporated into fractal structures of $d_f = 1.74$, 1.45 and 1.85. The colour of each node represents the local density of nodes within a sphere of radii 5 units, colour ranging from green (1 node) to red (20 nodes). In basic terms the red nodes represent an increased degree of connectivity in terms of elastic clot strength.

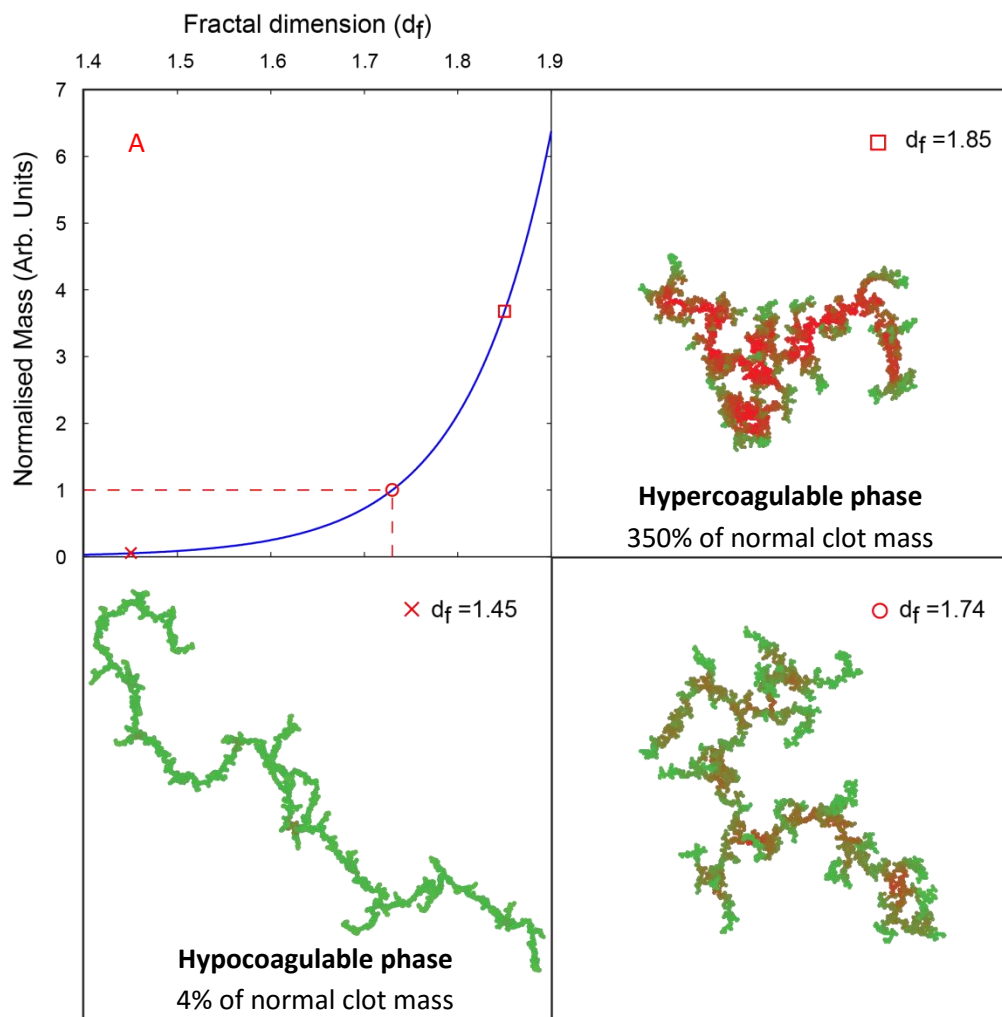
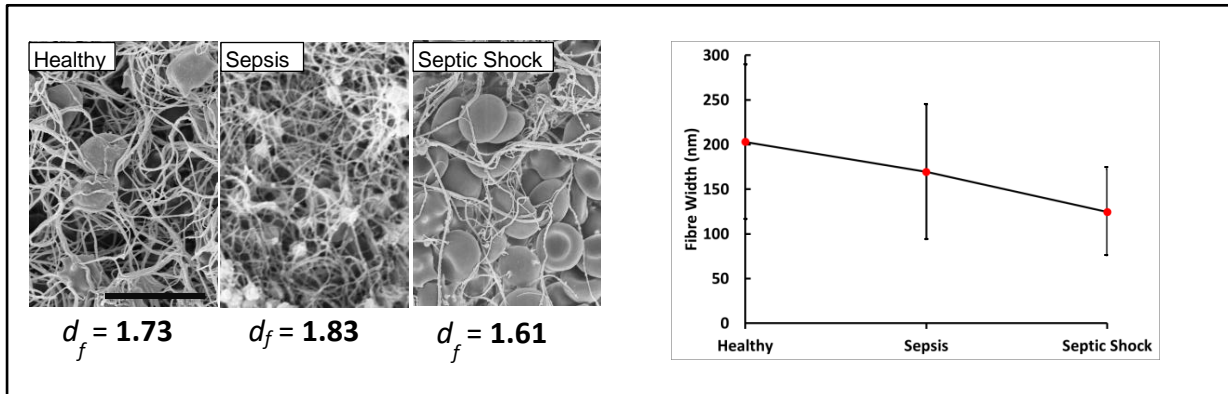


Figure 3: SEM images and corresponding fibrin fibre widths for different sepsis groups:

Representative images of mature whole blood clots are shown for a healthy individual ($d_f = 1.73$), sepsis patient ($d_f = 1.83$) and septic shock patient ($d_f = 1.61$). A means plot illustrating the changes in fibre width across the different groups is also shown ($n = 3$). A progressive decrease in fibre width was observed with increasing severity of sepsis. In septic shock, low d_f appeared to correspond with less fibrinogen incorporated into the polymerised clot mass, despite there being a sufficient fibrinogen concentration. The micrograph scale bar applies to all images and is $10\mu\text{m}$ long.



Supplementary Table 1: Relationship between coagulation parameters and outcome: Comparison of the different coagulation parameters between survivors and non-survivors at baseline. Data presented as mean \pm SD or median (IQR). Significance values assessed using Students t-test for normally distributed data, and Mann-Whitney U test for non-normally distributed data.

	Survivors (n=83)	Non-survivors (n=17)	Significance Value
aPTT (sec)	27.25 (25.5, 30.6)	30.3 (25.5, 42.4)	0.087
PT (sec)	11.2 (10.5, 12.0)	12.9 (11.6, 15.6)	0.001
Fibrinogen (g/L)	4.75 (4.025, 4.75)	3.5 (2.3, 4.775)	0.012
Platelets ($\times 10^9$/L)	249 \pm 108	227 \pm 138	0.465
d_f	1.76 \pm 0.08	1.66 \pm 0.12	<0.001