Title: The effect of the acute inflammatory response of burns and its treatment on clot characteristics and quality: a prospective case controlled study

Authors: NJ Marsden[ab], M Lawrence[ac] N Davies[ac], G Davies[a], K Morris[c], PR Williams[d], IS Whitaker[b], PA Evans[ac+]

Afilliations:

a) Haemostasis Biomedical Research Unit, Welsh Centre for Emergency Medicine Research, Morriston Hospital, Swansea, UK.

b) Welsh Centre for Burns and Plastic Surgery, Morriston Hospital, Swansea, UK.

c) Swansea University Medical School, Swansea, UK.

c) Cardiff School of Health Sciences, Cardiff Metropolitan University, Cardiff, UK

d) School of Engineering, Swansea University, Swansea, UK

[+] Corresponding Author:

Professor Phillip A Evans[+] MBBS MD FRCS FCEM[1]

Director Haemostasis Biomedical Research Unit, Welsh Centre for Emergency Medicine Research, Morriston Hospital, ABMU Health Board, Swansea, UK.

Tel/Fax: +441792702222 Email: Phillip.Evans2@wales.nhs.uk

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ABSTRACT

Introduction:

Burns are known to have an effect on coagulation in the early post burn period. Current coagulation tests have been criticised in acute burns due to their inherent limitations. This study aims to investigate the potential for a new quantitative functional biomarker of clot quality, fractal dimension, to identify changes in clot microstructure as a result of the burn inflammatory response and its treatment.

Methods:

A total of fifty-eight burn patients were included in this prospective case-controlled study. The control group (29 patients mean TBSA 1%), and case group (29 patients mean TBSA 30%) were compared at baseline and the case group investigated further over four time points (baseline, 12 hours, 24 hours and 5-7 days). Fractal analysis was performed, as well as current markers of coagulation, inflammatory markers and point-of-care tests, Thromboelastography and Multiplate analysis.

Results:

Fractal dimension did not differ between groups at admission (1.73 ± 0.06 and 1.72 ± 0.1), and fell within the healthy index normal range (1.74 ± 0.7), suggesting a normal clot microstructure in the early post burn period. Fractal dimension significantly reduced from baseline over the first 24 hours post burn (1.59 ± 0.03 $p < 0.005$), indicating a significant reduction in mechanical clot strength and functionality consistent with a hypocoagulable state, not identified with other markers.

Conclusions:

This is the first study to quantify the changes in clot microstructure following burn injury. This study confirms clot microstructure is significantly altered during the first 24 hours post burn, with the production of a weaker, more porous fibrin clot, consistent with a hypocoagulable state.

Keywords: Burns, thermal injury, coagulation, clot microstructure, biomarker

1. INTRODUCTION

Burn injury involves a complex interplay between early activation of uncontrolled coagulation and fibrinolysis, coupled with increases in inflammatory mediators [1-3]. Alterations in the coagulation system contribute to the progression of organ failure and increased morbidity and mortality in burns patients [4-7]. A combination of reducing blood loss and tailoring blood product administration could improve patient outcome and reduce mortality. Accurate and early identification of coagulation abnormalities is essential in reducing morbidity and mortality. Clinical markers (PT and APTT) are commonly used for monitoring anticoagulation, however they do not represent a global picture of coagulation and are insensitive to acute alterations in coagulation defects [8,9]. Point-of-care tests of coagulation, such as rotational thromboelastometry (ROTEM), which provides bedside information on clot development and fibrinolysis, are increasingly used in the clinical setting.

Recent rheological studies have highlighted the ability to scientifically and accurately quantify the quality and arrangement of clot microstructure that occur during clot development by measuring its fractal dimension (D_f) [10]. D_f has demonstrated a significant effect of changes in temperature and fluid dilution on clot microstructure [11-13] and more recently in clinical studies, to detect abnormal clot microstructure in response to the inflammatory changes seen in cancer, stroke and sepsis patients, even when standard laboratory tests have not [14-16]. The effect of burn injury on clot microstructure and clot quality has not previously been quantified. This study aims to quantify for the first time, the changes in clot microstructure as a result of burn injury, and to assess changes following pathophysiological progression and therapeutic intervention in the early post-burn period.

2. METHODOLOGY

2.1 Study design & ethical approval

The single centre, prospective, case-controlled study was approved by the South West Wales Research Ethics Committee (07/WMW02/34). This study was performed in the emergency department and regional burns centre of a large teaching hospital. Informed two-stage written consent was sought before enrolment to the study,

unless capacity was lacking (e.g. acute transfer sedated/intubated) in which case assent was sought from legal, personal or professional representatives.

2.2 Recruitment of burns patients

Inclusion criteria included patients aged over 18 years, presenting within 24 hours of a cutaneous burn injury. Exclusion criteria included patients admitted without cutaneous burns e.g. pure inhalational injury; patients presenting after 24 hours; patients with known co-morbidities affecting coagulation e.g. liver disease, malignancy; and any patient on anticoagulant medication. Patients with total body surface area (TBSA) 3% and below were classified into the control group, patients with 6% TBSA burns and over made up the case group. All patients had baseline blood samples (time point 0), and the case group had a maximum of three further samples, at time points 1 (6-12 hours post first sample), 2 (at 24 hours) and 3 (5 – 7 days) to assess the effect of pathophysiological progression and continuing therapeutic intervention. Patient demographics and details of injury were collected at enrolment. Case group patient had details of interventions and therapy recorded until their final sample point at 5-7 days post burn.

2.3 Blood Sampling

A 20ml blood sample was obtained atraumatically from either venepuncture using an 18-gauge needle and syringe, or via an arterial/central venous line when one was already in place. The first 5mL of blood was discarded and the following 9mL was immediately transferred into the rheometer for rheological analysis. Further blood was 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) for standard laboratory markers of coagulation, and further samples were collected for ROTEM and Multiplate analysis.

2.4 Gel Point and Fractal Dimension measurements

Gel point analysis was performed using a TA Instruments AR-G2 controlled-stress rheometer (TA Instruments, New Castle, DE, USA). This technique detects the gel point, from which the time to gel point (T_{GP}), G'_{GP} (a measure of the

strength/elasticity of the incipient clot, and fractal dimension (D_f) of the fibrin clot are derived. This methodology used has been described in greater detail previously [10,11,17].

2.5 Standard laboratory tests

Haematological profile was analysed on a Sysmex XE 2100 (TOA Medical Electronics) automated haematology analyzer and routine clotting tests were undertaken using a Sysmex CA1500 analyzer (Siemens), within 2 hours of collection. D-dimers were measured using the TriniLIA Auto-Dimer® turbidimetric assay with a Sysmex CA1500, and is reported as either a positive or negative result. Factor VIII was determined using an aPTT-based one-stage assay using appropriate factor deficient plasma and Actin FS APTT reagent (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany).

2.6 Inflammatory markers

The inflammatory markers IL-6, TNF-α and Procalcitonin (PCT) were chosen to measure inflammation based on previous literature on the effect of burns[18-20]. Concentrations of each marker were measured in platelet poor plasma using enzyme-linked immunosorbent assay (ELISA) kits for IL-6, TNF-α and PCT. All materials were equilibrated to room temperature (18 – 25^{o}C) prior to use, and the standard procedure supplied was followed.

2.7 Rotational Thromboelastometry

Thromboelastometry was performed using a ROTEM delta whole blood haemostasis system and blood was activated by both intrinsic (INTEM) and extrinsic (EXTEM) activation. Tests were carried out in accordance with the manufacturer's recommendations. The Clotting Time (CT), Maximum Clot Firmness (MCF) and Maximum Lysis (ML) were recorded to assess the kinetic, structural and fibrinolytic aspects of clot development.

2.8 Whole blood platelet aggregometry (Multiplate)

Hirudinised blood was transferred 20 minutes after sampling to the Multiplate test cell (Roche Diagnostics GmbH, Mannheim, Germany, REF: 06675590), followed by 500µL of normal saline and then left to incubate for three minutes in individual test cells. The appropriate agonist (Adeonsine Diphosphate (ADP), Arachadonic acid (ASPI) and collagen (Coll)) was then added to the test cells using an automated pipette system. The area under the curve (AUC) was calculated, and reported as arbitrary aggregation units (U).

2.9 Blood clot imaging: scanning electron microscopy (SEM)

SEM samples were prepared from 21 µL of whole blood using the methodology described previously [15]. The resultant dehydrated blood samples were coated with gold palladium and then imaged using a Hitachi ultra- high resolution FE-SEM S-4800.

2.10 Sample size

The primary outcome of this study was to investigate the hypothesis that D_f will be increased in subjects with a greater than 6% burn area as compared with those less than 3% and that therapeutic intervention will result in a further modulation in D_f in the subjects with greater than 6% burns. Data from previous studies suggests we would expect a difference in D_f of at least 0.03 and an SD of 0.04 in the case group as compared with the smaller control burns with essentially normal coagulation. Using these figures as the basis of calculation, a two-sample t-test to detect between group differences at baseline $\alpha = 0.8$ and significance level of 0.05 we calculated two samples of 29 required to undertake this study.

2.11 Statistical analysis

Statistical analysis was performed using Minitab® version 16 software (Havertown, PA). Between group differences was determined at baseline using the two-sample t-test for normally distributed and the Mann-Whitney test for non-normally distributed data. Within group analysis was undertaken on the case group to determine the effect of therapeutic intervention on the various markers using one-way ANOVA on normally distributed data and Kruskal-Wallis for non-normally

distributed data. Correlations were investigated using Pearson's method for normally distributed and Spearman's method for non-normally distributed data. Statistical significance was defined as $p<0.05$ throughout.

3. RESULTS

3.1 Baseline characteristics of groups

The baseline characteristics including demographics and burn injury details are shown in **Table 1**. Fifty-eight patients were included in the study, comprising 29 in each group. The patient demographics were well matched between groups, and the baseline burn characteristics reflected the more severe burns in the case group, with a higher total body surface area burn (% TBSA), full thickness burns (% FTB), more inhalation injuries, higher volume of fluid resuscitation and mortality rate.

3.2 Changes in standard laboratory markers between groups

The results for the standard laboratory tests, inflammatory markers, ROTEM, Multiplate and rheological data for both groups at baseline are presented in **Table 2**. The white cell count was significantly higher ($p<0.005$) in the case group, compared to the controls. Haemaglobin and haemaocrit did not differ significantly. The standard kinetic markers of coagulation, PT ($p<0.005$) and APTT ($p = 0.015$) were significantly prolonged in the case group, however both fell within the normal limits. Fibrinogen levels were within the normal range for both groups and did not differ significantly. Factor VIII (FVIII) activity was significantly increased ($p<0.01$) in the case group, and above the normal range. There were a significantly higher proportion of patients in the case group (86%) compared to the control group (63%) with a positive D-dimer result, indicating both increased clot activity and breakdown in the case group.

All of the inflammatory markers were significantly elevated in the case group, indicating a more marked inflammatory response in the larger burns.

Significant changes were seen in all ROTEM parameters between the two groups, except for CT and MCF activated with INTEM. These results suggest the case group

produced a weaker clot, with less kinetic activity resulting in a prolonged clotting time than the control group. However, all results for both groups fell within the normal ROTEM parameters, so although there was a significant difference between groups, the ROTEM did not identify a coagulopathy in the case group.

All of the platelet aggregometry results were significantly reduced in the case group, and were below the normal range in the ADP and ASPI tests, whereas the control groups fell within the normal range.

D_f did not significantly differ, and both groups fell within the normal range at admission, which has been determined from previous studies [10]. The T_{GP} was shorter in the case group, although not significant, and the G'_{GP} was significantly lower ($p = 0.002$) in the case group. This indicates that the case group produced structurally weaker clots, with less elasticity than the controls.

3.3 Changes in markers over time in the case group

The changes in the case groups routine coagulation markers and D_f over the four time points, are demonstrated in **Figure 1.** APTT and fibrinogen levels significantly increased over time, with fibrinogen levels peaking at 5-7days. Although APTT levels changed significantly over time, they reached their highest at 24hours and appeared to be decreasing again by 5-7 days. PT and the proportion of positive D-dimer levels did not alter significantly over time. Factor VIII significantly decreased over the first 24hours but then was increased well above baseline at 5-7days. CRP was the only inflammatory marker that showed significant changes over the study period, with a gradual increase from admission to one week (21 ± 5 to 219.8 ± 17.9, $p<0.005$). There was little change observed in the other inflammatory markers over the four time periods.

Of the ROTEM parameters, only MCF and CT showed a significant change over time. There was a significant increase in the kinetic properties of clot formation, demonstrated by increasing CT. Significant reductions in the structural properties of the clot were demonstrated over the first 24 hours by a reduction in MCF, however

even at its lowest, it was still within the normal range. MCF results had returned to above baseline by 5-7 days. All ROTEM parameters were within normal limits for the duration of the study.

The only Multiplate parameter that showed a significant change was ADP-AUC. There was a significant reduction in platelet activity using the ADP to activate, seen in the first 24 hours, which then had increased above baseline by 5 – 7 days. The same pattern was mirrored in all other parameters, however they did not reach significance. These results demonstrate platelet activity reduces over the first 24 hours following burn injury, but then by a week post burn, activity has returned to levels above baseline and to a level within or close to the normal range.

There was a significant change in D_f over time. D_f reduced significantly over the first 24hours, to a mean of 1.59, which is well below the normal range for a healthy clot, but appeared to be increasing back towards baseline by 5-7 days.

There was a significant change from baseline in the volume of fluid given as time progressed, with the highest volume being given prior to the first sample. **Figure 2** demonstrates the changes in D_f over the four time periods in response to total fluid volume and volume of colloid given over the four time points.

3.4 Clot imaging: Scanning Electron Microscopy

Previous studies have demonstrated the relationship between D_f and fibrin fibre width as measured with SEM, particularly in inflammatory conditions whereby fibre width decreased with decreasing D_f in more severe inflammatory states, such as septic shock [12,14,16]. Scanning Electron microscopy (SEM) was used to image the mature clots of a single patient from the case group over three time points for illustrational purposes, to demonstrate the fibrin structure observed and the corresponding D_f (**Figure 3**). The images illustrate the change in clot microstructure, with a denser clot with more branch points corresponding to a D_f of 1.69, to a porous clot with fewer fibrin fibres and branch points, corresponding to a D_f of 1.39.

4. DISCUSSION

Previous studies have demonstrated D_f to be an accurate method of quantifying the developing and final clot architecture in a number of conditions. Increased D_f is associated with hypercoagulable states with increased clot strength and more densely formed clots, seen in inflammatory and vascular conditions, such as sepsis, lung cancer and stroke [14-16]. Reduced D_f is associated with hypocoagulable states, with weakened clot properties, such as in septic shock and with the use of anticoagulants [10,16]. This study demonstrates for the first time that the mechanical properties of the clot in both the hyper- and hypo-coagulable phases after burn injury can be quantified using D_f as a functional biomarker of clot microstructure.

This study demonstrated no difference in clot microstructure between smaller and larger burns at admission, with D_f falling within normal limits in both groups and a normal standard coagulation profile in both at baseline. This correlated with previous literature, which suggests that burn patients do not exhibit a coagulopathy at presentation based on routine coagulation markers [4,21,22]. Thereafter, in the larger burn group, D_f significantly reduced over the next 24 hours, corresponding to a hypocoagulable state, with formation of a loose and structurally weaker clot that would potentially be more prone to fibrinolysis. One previous study of patients with >20% TBSA found patients to be hypercoagulable on day 1 post burn based on specific coagulation factor assays, such as tissue plasminogen activator and thrombin-antithrombin III complex. However they only analysed results on day 1 and 7 and did not present baseline results, and did not compare with routine coagulation markers [5]. After 5-7 days, D_f appeared to be increasing and was found to be in the lower range of normal limits. This was comparable to the routine markers, which were normal at day 5-7, and also previous literature, which demonstrated a normalized coagulation profile 1 week post burn [1,21]. Comparison of D_f against routine laboratory coagulation markers demonstrated that during the hypocoagulable phase, PT and platelet count were within normal limits, with APTT just above the normal range, despite a significant reduction in D_f. There were a significantly higher proportion of patients in the case group (86%) compared to the

control group (63%) with a positive D-dimer result, indicating both increased clot activity and breakdown in the case group. D-dimer has previously been shown to be increased in the early post burn period, with levels normalizing by one-week post burn, which was also seen in this study [23]. Apart from APTT at 24hrs post burn, all other markers were within the normal range for the duration of the study period. This suggests a lack of sensitivity of these standard markers of coagulation to hypocoagulable changes seen in burn patients. The MCF from the ROTEM data, mirrored the changes in D_f over the four time periods, however, although it demonstrated reduced clot firmness after 24-hours, this was still within normal limits, compared to D_f at 24-hours which was significantly below the normal range for a healthy clot. This suggests that ROTEM may not be sensitive enough to as identify the production of a poor clot structure in the early post burn period. Although there was a significant reduction in platelet count after 24 hours in the case group, the findings in this chapter also showed a significant decrease in platelet activity over the same time period. The platelet count dropped to the lower end of normal at 24 hours, whereas the platelet aggregation was initially lower than the normal range at presentation, decreased further over 24 hours and then had returned to within normal levels by 5 – 7 days, which was associated with a normal platelet count that had reached above baseline levels after one week. This data supports the findings of previous literature that platelet count is typically within normal range at presentation but then decreases within 3 days and has returned to near normal within one week [24,25].

Fibrinogen levels were within normal limits at admission, and remained at a similar level during the hypocoagulable phase, when D_f was significantly reduced. This indicates that despite normal concentrations of fibrinogen in burns patients, there is reduced organisation of the fibrin microstructure, leading to weak and porous clots, demonstrated by a low Df. This study confirms previous findings that measuring the physical properties of fibrin polymerisation and how the clot is organised, gives a more useful and functional indication of the quality of the clot than a simple measure of the fibrinogen concentration available [13,14,16].

The inflammatory response to burns is widely reported and well known, and has been inherently linked to coagulation changes [1,3,20]. The extent of the inflammatory response has been associated with the size and severity of the burn [18]. In this study, despite increased inflammatory response seen, there was a significant reduction in D_f at 24 hours post burn. This could be partly down to the fact burn patients require large volumes of fluid resuscitation in the first 24-36 hours. Previous in vitro studies have shown that fluid dilution has a significant effect on reducing clot microstructure, with colloids having more of an effect than the same dilution of crystalloids [13]. This demonstrated the effect wasn't purely dilutional, but due to the intrinsic properties of colloids that have an inhibitory effect on fibrin polymerization [12,13,26]. In this study, the lowest recorded values for D_f (1.39) were comparable to the in vitro results achieved following 60% dilution of whole blood with albumin. The protocol for fluid resuscitation in the study centre is Parkland formula for the first eight hours post burn, then switching to the Albumin based Muir & Barclay resuscitation formula. This switch to Albumin resuscitation during the first 24 hours may play a part in the reduction in D_f seen over this period, as demonstrated in **Figure 2**. This could potentially lead to increased risk of blood loss during early excisional surgery, performed during the resuscitation phase, when using Albumin. However, firm conclusions cannot be made based on the size of this study.

It appears that the changes in D_f are small, ranging from 1.39 to 1.88 in this study, however it is important to remember that D_f has a non-integer value with a non-linear relationship with the amount of fibrin mass incorporated within the incipient clot (**Figure 4**). This means that substantial increases in mass are required to generate small changes in D_f [27,28]. This modelling indicated that after 24 hours (D_f = 1.59), the incipient clot had a fibrin mass of less than 20% of that incorporated into that formed at baseline. For the highest values of D_f observed in this study (D_f = 1.88), computer modelling indicated a corresponding 450% increase in fibrin mass incorporated into the clot, whereas for the lowest values (D_f = 1.39), the incipient clot had a fibrin mass of less than 5% of that incorporated into a healthy clot.

Two further factors, which could potentially influence changes in Df, were investigated; comorbidities including cardiac, respiratory, or chronic inflammatory conditions such as inflammatory bowel disease and rheumatoid arthritis and the regular use of either Aspirin or Clopidogrel medication. Patients with significant co-morbidities had a lower mean Df on admission compared to those without (1.69 versus 1.73, p = 0.11) and patients who were already taking either Aspirin or Clopidogrel had a lower mean Df than those without (1.69 versus 1.73, p = 0.27), although neither reached significance. The numbers in the co-morbidities group (n = 13) and the Aspirin/Clopidogrel groups (n = 8) were small and so little can be gained from this data.

This study has several limitations. Firstly this was a single-centre case-controlled proof of concept study, and was not powered to generate any clinical outcome data. Furthermore, it was outside the scope of this study to seek mechanistic conclusions to the studies findings. Like other similar studies before, the inherent problem of this study is the heterogeneity seen in burns, its treatment, concomitant medications and co-morbidities. To assess these effects fully and to build on the findings of this study, a much larger, multi-centre prospective study would be required.

5. CONCLUSION

This is the first study to quantify the changes in clot microstructure following burn injury. On presentation, D_f was not significantly associated with TBSA burnt. This study confirms that clot quality is significantly altered during the first 24 hours post burn, with the production of a weaker, more porous fibrin clot, consistent with a hypocoagulable state, which wasn't identified with other current markers of coagulation. Larger studies are required to investigate the factors that determine these changes in clot microstructure and also to investigate the affect of treatment on clot quality and clinical outcome.

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Legends for figures

Figure 1: Differences in standard coagulation markers and Df in the case group, over the four time points (0 = Baseline, 1 = 6-12hours, 2 = 24hours, 3 = 5-7 days). Data represented as means ± standard deviation error bars (*denotes significance change from baseline $p<0.05$ using one-way ANOVA). Normal ranges are demonstrated between dotted lines.

Figure 2: Comparison of the changes in D_f alongside the total volume of fluid (ml) given (above) and colloid (below) in the case group over the four time periods. D_f reported as mean ± SD and fluid volume represented as median. Dashed line represents the normal range for D_f.

Figure 3: Representative SEM images of fully formed clots taken from the same patient, at different time points. The micrograph scale bar in the left hand image applies to all images and is 10μm long.

Figure 4: Computational analysis of a fibrin mass curve, illustrating the non-linear relationship between the fibrin mass incorporated into the developing clot and D_f. Mass, represented on the y-axis is normalised to the baseline D_f of the case group (1.72). The following results of D_f were put into the model: $D_f = 1.72$ (baseline for the case group), $D_f = 1.59$ (case group after 24 hours), $D_f = 1.69$ (case group at 5 – 7 days).