Point-of-care Diagnosis and Monitoring of Hypofibrinolysis in the Critically Ill: Results from a Feasibility Study.

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Abstract

Background

In critical conditions such as sepsis, severe trauma, COVID-19 and non-COVID acute respiratory failure, hypofibrinolysis is associated with multi-organ dysfunction syndrome and death. The mechanisms underpinning hypofibrinolysis may include reduced tissue plasminogen activator (t-PA) and/or plasmin effect due to elevated inhibitor levels, reduced expression and/or exhaustion. This study in critically ill patients with hypofibrinolysis aimed to evaluate the ability of t-PA and plasminogen supplementation to restore fibrinolysis assessed by bedside viscoelastic testing (VET).

Methods

Prospective observational and interventional studies were undertaken in 28 critically ill patients identified as hypercoagulant and hypofibrinolytic using standard ClotPro VET. Hypercoagulation was defined as above normal values for clot amplitude on the EX-test (tissue factor (TF) activated coagulation) or FIB-test (TF activated coagulation with platelet inhibition). Hypofibrinolysis was defined as a clot lysis time > 300 seconds on the TPA-test (TF activated coagulation with t-PA accelerated fibrinolysis). In experimental VET, repeat TPA-tests were spiked with additional t-PA and/or plasminogen and the effect on lysis time determined. In a hypofibrinolytic patient, alteplase was administered intravenously over a 24-hr period with standard ClotPro VET repeated frequently throughout to monitor the effect on coagulation and fibrinolysis.

Results

In the ex-vivo studies, distinct response groups emerged with increased fibrinolysis observed following (i) additional t-PA supplementation only, or (ii) combined plasminogen and t-PA supplementation. A baseline TPA-test lysis time of > 1000 sec associated with the latter group. In the interventional study, alteplase administered as a 2-hr bolus (25 mg) followed by a 22-hr infusion (1 mg/hr) resulted in a gradual reduction in serial TPA-test lysis times.

Conclusions

ClotPro viscoelastic testing, the associated TPA-test and the novel spiked ex-vivo assays may be utilised to (i) investigate the potential mechanisms of hypofibrinolysis, (ii) guide corrective treatment, and (iii) monitor in real-time the treatment effect. Such a precision-medicine and personalised treatment approach to the management of hypofibrinolysis has the potential to increase treatment benefit, whilst minimising adverse events in hypofibrinolytic critically ill patients.

Trial Registration:
VETiPAT ARF, a clinical trial evaluating the use of ClotPro-guided tissue plasminogen activator (alteplase) administration in hypofibrinolytic patients with acute respiratory failure is ongoing (ClinicalTrials.gov NCT05540834, registered 15 September 2022, retrospectively registered).

**Introduction**

Severe infection and tissue injury induce dynamic and interconnected responses in the inflammatory, coagulation and fibrinolytic systems(1, 2). Significant perturbations in fibrinolysis may result in life-threatening haemorrhage (hyperfibrinolysis) or fatal macro or micro-thrombosis (hypofibrinolysis)(3–7), with several studies providing evidence that initial hyperfibrinolysis transitions to hypofibrinolysis due to factor consumption(7, 8). In critical conditions, such as sepsis, COVID-19 and non-COVID acute respiratory failure (ARF), and extensive trauma, hypofibrinolysis has been associated with elevated levels of plasminogen activator inhibitor-1 (PAI-1)(4–6, 9–11). In another study in COVID-19 disease, elevated levels of both PAI-1 and tissue plasminogen activator (t-PA) levels were reported, with hyperfibrinolysis associating with high t-PA levels and a poor outcome(12). A mechanism for the hypofibrinolysis seen in severe COVID-19 disease hypothesised by Medcalf et al.(13), is that of a “consumptive hypofibrinolysis”. Here, authors postulate that levels of fibrin and necrotic material generated during the infective/inflammatory process overwhelm and exhaust key fibrinolytic factors. Furthermore, that balance can be restored by enhancing plasmin formation either via administration of t-PA or its substrate, plasminogen(13). This hypothesis is supported in severe COVID-19 disease by the apparent beneficial effects of direct fibrinolytic approaches using either t-PA(14) or plasminogen(15).

To establish rapid, informative assays that evaluate hypofibrinolysis and its correction at the bedside, we undertook prospective observational and interventional studies using viscoelastic testing (VET). VET is a point-of-care technology that provides a rapid, global assessment of whole blood coagulation, thus analysis occurs in the presence of circulating blood cells and the secreted products of the endothelium and immune cells. VET is also able to detect hyper- and hypofibrinolysis. VET is sufficiently sensitive to detect COVID-19 associated coagulopathy, with several VET parameters portending severity of COVID-19 disease(16–23). We used ClotPro and its TPA-test, that measures the fibrinolytic response to added t-PA, in critically ill patients to initially select individuals with hypofibrinolysis. Further exploratory VET analyses were then undertaken with ex-vivo spiking of patient blood with additional t-PA with/without plasminogen to assess the requirements for correction of the hypofibrinolysis. In addition, in a hypofibrinolytic patient with bacterial pneumonia causing ARF, we administered alteplase (tPA) over 24 hours and used VET to monitor the effect on fibrinolysis.

We hypothesised, based on the existing literature, that hypofibrinolytic patients would display significant variation in the degree of fibrinolytic compromise, but also that the underlying defect/s could be corrected through supplementation with t-PA and/or plasminogen. Additionally, we hypothesised that the ClotPro TPA-test would offer rapid bedside capability to monitor the effect on fibrinolysis of intravenous t-PA administration in hypofibrinolytic patients thus permitting a personalised dose that maximised benefit while minimising risk.

**Methods**
ClotPro® (enicor GmbH, Munich, Germany) was used for viscoelastometry. In all individuals, venous blood was collected into 3.2% buffered sodium citrate tubes and analysed by ClotPro within 30 minutes of collection using the instruments EX-test®, FIB-test® and TPA-test® ("standard" Clotpro assays, defined in Supplementary Data) as per the manufacturer’s instructions. The manufacturer’s normal ranges were used for the EX-test and FIB-test and results recorded above or below these ranges were classified as hyper- or hypocoagulant, respectively. The platelet contribution to the clot, platelet A10, was calculated by subtracting the FIB-test A10 value from the EX-test A10 value. The TPA-test measures the fibrinolytic response to added t-PA (650 ng/mL) with the lysis time (LT), calculated by the associated software, indicating the time taken for the maximum clot formed to be reduced by 50%. The volume of the fibrin clot and endogenous blood levels of the fibrinolytic proteins, t-PA and plasminogen, and their inhibitors will influence the rate of clot lysis.

Twenty-eight patients, who were identified to be hypercoagulant and hypofibrinolytic, were included in this study. Twenty controls (healthy ICU staff members) were also studied to establish normal fibrinolysis parameters. Data from previous studies(19, 21) and from this study were used to define hypofibrinolysis as a TPA-test lysis time (LT) of > 300 sec, this value being > 4 standard deviations above the mean LT obtained in the healthy controls (mean 180.4 +/- SD 28.6 sec). (Fig. 1A).

Ex-vivo studies

Repeat viscoelastometry (‘exploratory Clotpro assays’) was performed in hypofibrinolytic patients with ex-vivo spiking of additional t-PA (650–1300 ng t-PA/mL blood) by passing blood through 1 or 2 additional TPA-test tips (enicor GmbH, Munich, Germany) with or without the addition of plasminogen (59 µg or 147 µg/mL blood - Hyphen-Biomed, France).

In-vivo study

In a hypofibrinolytic patient with ARF entered into a clinical trial of VET guided treatment using low dose alteplase, the EX-test, FIB-test and TPA-test were repeated regularly throughout the 24 hr infusion and one hour after cessation and analyses commenced within 10 minutes of blood collection.

The data were analysed using PRISM V9.0 software (GraphPad, CA). Results are reported as medians with the 95% confidence interval/interquartile range (IQR). Comparisons were made using the Mann-Whitney test for non-normally distributed data (D’Agostino and Pearson test) and reported including the median difference and its 95% confidence interval [95%CI]. Correlations were analysed by Spearman’s correlation coefficient (rho) and reported including the 95%CI. Two-sided p-values are reported for all analyses. Statistical significance was set at a two-sided p-value of < 0.05.

Results

Standard ClotPro Assays

Of the 28 hypofibrinolytic patients included in the ex-vivo study, 12 were COVID-19 positive and 16 were COVID-19 negative. No difference was found in the extent of hypofibrinolysis, as measured by the TPA-test LT,
between COVID-19 and non-COVID-19 critically ill patients (Fig. 1A), median difference 19 [95% CI -293 to 85] sec, p = 0.45.

When compared with the healthy controls (n = 20), the 28 hypofibrinolytic critically ill patients showed significantly increased (median difference 17 [95% CI 13 to 19] mm, p < 0.0001) fibrin clot amplitude (FIB-test A10), with 24 (86%) cases above the upper level of normal (ULN) (23 mm). No significant difference in FIB-test A10 was found between COVID-19 and non-COVID-19 cases (Fig. 1B) median difference 3.5 [95% CI -2 to 11] mm, p = 0.27.

In the normal healthy state, the fibrinolytic system is tightly regulated as is demonstrated by the minimal variation in LT seen in healthy controls (Fig. 1A). The FIB-test A10 of controls were all within the normal range (7–23 mm) and a correlation analysis between the FIB-test A10 and the TPA-test LT demonstrated a strong linear relationship (Fig. 1C) (rho = 0.72 [95% CI 0.39 to 0.88], p = 0.0004). In comparison, the LT in the hypofibrinolytic critically ill patients ranged from the cut-off value, 300 sec, to > 2400 sec, wherein no lysis occurred within the 40 min assay time frame (Fig. 1A). Correlation analysis between the FIB-test A10 and the TPA-test LT in hypofibrinolytic patients demonstrated no significant relationship (rho = 0.14 [95% CI -2.55 to 0.49], p = 0.47) and no patient data aligned with the extrapolated curve of best-fit derived from the healthy control data (Fig. 1C). In comparison, in critically ill patients who were not hypofibrinolytic (n = 28) (TPA-test LT < 300 sec), and hence did not undergo further study procedures, a strong correlation between the FIB-test A10 and the TPA-test LT was observed (Supplementary Fig. 1) (rho = 0.81 [95% CI 0.62 to 0.91], p > 0.0001).

The EX-test A10 was increased in hypofibrinolytic patients compared to healthy controls (median difference 11.5 [95% CI 5 to 13] mm, p < 0.0008) with no significant difference between the COVID-19 and non-COVID-19 patients (Fig. 1D) median difference 7.5 [95% CI -1 to 16] mm, p = 0.16. The platelet contribution to the clot, platelet A10, was reduced in hypofibrinolytic patients compared to healthy controls (median difference 5 [95% CI 3–8] mm, p < 0.0001). Again, no difference was found between COVID-19 and non-COVID-19 cases in the calculated platelet A10 (Fig. 1E) median difference 5 [95% CI -5 to 4] mm, p = 0.81.

**Exploratory ClotPro Assays**

TPA-tests that were spiked with additional t-PA (total 1.3 µg/mL) were undertaken in 17 patients with reductions in LT of > 30% seen in 14 (82%) cases (Table 1, Column B; Fig. 2). The 3 (18%) patients who had no response to additional t-PA supplementation responded to plasminogen supplementation of the TPA-test resulting in a > 50% reduction in LT (Fig. 3). Of the 19 cases who had plasminogen 147 µg/mL added to the TPA-test (Table 1, Column D), 6 (32%) cases demonstrated a reduction in LT of > 30%. All of these cases had a baseline TPA-test LT (Table 1, Column A) of > 1000 sec (Cases 1, 10, 25–28). Two of these cases (25 and 26) responded with a > 30% reduction in LT to additional t-PA supplementation (Table 1, Column B) as well as to plasminogen supplementation of the TPA-test (Table 1, Column D). In contrast, 10 (53%) cases demonstrated a > 30% prolongation of the lysis time in response to 147 µg/mL plasminogen supplementation (Table 1, Column D). All these patients had a baseline TPA-LT (Table 1, Column A) of < 1000 sec and all had responded to additional t-PA supplementation (Table 1, Column B) when tested. Supplementary Fig. 2 illustrates the effect of plasminogen supplementation on the TPA-test LT in patients with a baseline TPA-test LT > 1000 sec vs those with a LT < 1000 sec. No such inhibitory effect on the LT was observed in 9 cases where the TPA-test was supplemented with the lower amount of 59 µg/mL plasminogen (Table 1, Column C) with reductions in LT.
observed irrespective of the baseline TPA-test LT (Table 1, Column A), however, the reductions seen were not as substantial as those achieved with 147 µg/mL plasminogen.

The addition of plasminogen (147 µg/mL) to ClotPro assays that do not contain exogenous t-PA (the EX-test +/- FIB-test), was tested in 5 patients (Cases 1, 2, 13, 14 and 21) and resulted in changes of < 10% in the EX-test/ FIB-test A10. (Table 1, Columns E and F). A 20% reduction in EX-test A10 was seen in one individual (Case 12) who was receiving warfarin (Fig. 2C).

To summarise the ex-vivo supplementation data, two predominant groups emerged that were distinguished by the response to ex-vivo fibrinolytic protein supplementation: (i) cases that responded to supplementation with t-PA alone, the majority of which had a baseline TPA-test LT of < 1000 sec, and (ii) cases that responded to the combination of t-PA and plasminogen supplementation, the majority of which had a baseline TPA-test lysis times > 1000 sec. Plasminogen supplementation in the absence of t-PA appeared ineffective at inducing significant clot lysis.
Table 1
Effect of ex-vivo t-PA +/- plasminogen supplementation on clot lysis times and plasminogen on clot amplitude.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>COVID-19 Status (PCR)</th>
<th>Principal Diagnosis</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Positive</td>
<td>Bacterial pneumonia</td>
<td></td>
<td>&gt;2400</td>
<td>290</td>
<td>+2%</td>
<td>-6%</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Positive</td>
<td>Sub-arachnoid haemorrhage</td>
<td>383</td>
<td>175 (-44%)</td>
<td>-1%</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
<td>Bacterial pneumonia</td>
<td>453</td>
<td>713 (+57%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Positive</td>
<td>Pneumonitis</td>
<td>338</td>
<td>185 (-45%)</td>
<td>308 (-9%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5*</td>
<td>Positive</td>
<td>Pneumonitis</td>
<td></td>
<td>&gt;2400</td>
<td>&gt;2400 (0%)</td>
<td>&gt;2400 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Positive</td>
<td>Pneumonitis</td>
<td></td>
<td>&gt;2400</td>
<td>305 (-87%)</td>
<td>2400 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Positive</td>
<td>Pneumonitis</td>
<td>415</td>
<td>368 (-11%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Positive</td>
<td>Pneumonitis</td>
<td>403</td>
<td>172 (-57%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Positive</td>
<td>Diarrhoea, septic shock</td>
<td>450</td>
<td>398 (-12%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Positive</td>
<td>Hypovolaemic shock – bleed</td>
<td>2400</td>
<td>2247 (-6%)</td>
<td>668 (-72%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Positive</td>
<td>Pneumonitis</td>
<td>460</td>
<td>370 (-20%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Positive</td>
<td>Pneumonitis</td>
<td>465</td>
<td>288 (-38%)</td>
<td>298 (-36%)</td>
<td>-20% (warfarinised)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Negative</td>
<td>Bacterial pneumonia, ARF</td>
<td>450</td>
<td>655 (+46%)</td>
<td>-3%</td>
<td>0%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Negative</td>
<td>Sepsis, MODS</td>
<td>340</td>
<td>240 (-29%)</td>
<td>-3%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*warfarinised*
<table>
<thead>
<tr>
<th>Case No.</th>
<th>COVID-19 Status (PCR)</th>
<th>Principal Diagnosis</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TPA-test# (t-PA 650 ng/mL)</td>
<td>TPA-test x 2 (t-PA 1.3 µg/mL)</td>
<td>TPA-test + 59 µg Plg/mL (LT sec)</td>
<td>TPA-test + 147 µg Plg/mL (LT sec)</td>
<td>EX-test A10 + 147 µg Plg/mL (% change from baseline)</td>
<td>Fib-test A10 + 147 µg Plg/mL (% change from baseline)</td>
<td></td>
</tr>
<tr>
<td>15*</td>
<td>Negative</td>
<td>Hypoxic arrest, endocarditis</td>
<td>530</td>
<td>222 (-58%)</td>
<td>700 (+32%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>16*</td>
<td>Negative</td>
<td>Acute severe pancreatitis</td>
<td>545</td>
<td>252 (-54%)</td>
<td>553 (+1%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Negative</td>
<td>Ischaemic bowel</td>
<td>348</td>
<td>167 (-52%)</td>
<td>990 (+184%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Negative</td>
<td>Severe sepsis, MODS</td>
<td>425</td>
<td>190 (-55%)</td>
<td>580 (+37%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Negative</td>
<td>Polytrauma, sepsis</td>
<td>425</td>
<td>177 (-58%)</td>
<td>623 (+47%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Negative</td>
<td>Necrotising fasciitis</td>
<td>690</td>
<td></td>
<td>813 (+18%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Negative</td>
<td>STEMI, necrotising fasciitis</td>
<td>373</td>
<td></td>
<td>558 (+50%)</td>
<td>0%</td>
<td>-8%</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Negative</td>
<td>Trauma, multi DVTs</td>
<td>330</td>
<td>157 (-52%)</td>
<td>568 (+72%)</td>
<td></td>
<td></td>
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<tr>
<td>23</td>
<td>Negative</td>
<td>Sepsis, foot amputation</td>
<td>390</td>
<td>200 (-49%)</td>
<td>588 (+51%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>24</td>
<td>Negative</td>
<td>Polytrauma, sepsis</td>
<td>388</td>
<td>192 (-51%)</td>
<td>633 (+63%)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>25</td>
<td>Negative</td>
<td>Polytrauma</td>
<td>1125</td>
<td>425 (-62%)</td>
<td>685 (-39%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Negative</td>
<td>SIRS post cardiac bypass</td>
<td>2107</td>
<td>578 (-73%)</td>
<td>465 (-78%)</td>
<td></td>
<td></td>
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<tr>
<td>27*</td>
<td>Negative</td>
<td>Saddle PE, Influenza ARF</td>
<td>2400</td>
<td>2400 (0%)</td>
<td>710 (-70%)</td>
<td></td>
<td></td>
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<tr>
<td>28*</td>
<td>Negative</td>
<td>Necrotising cholecystitis</td>
<td>2400</td>
<td>2400 (0%)</td>
<td>995 (-59%)</td>
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</table>

*ClotPro profiles feature in Figs. 2 and 3; # baseline value against which other TPA-test results are compared with % change from # value provided in brackets; LT = lysis time; Plg = Plasminogen; A10 = Clot amplitude at

In Case 5 (bottom row) the FIB- and EX-test results were essentially normal. On the standard TPA-test (650 ng/mL), the double dose TPA-test (1.3 µg/mL) and even a triple dose TPA-test (1.95 µg/mL), a concentration-dependent reduction in the MCF was observed but did not reach 50% and, therefore, no lysis time registered. When plasminogen 59 µg/mL was added to the triple TPA-test, however, a significant increase in clot lysis occurred with a LT finally registering.

**Interventional Study of ClotPro monitoring of 24 hr alteplase infusion**

In a hypofibrinolytic patient (baseline TPA-test LT of 450 sec) with bacterial pneumonia, the lowest alteplase dosing regime (25 mg bolus of alteplase over 2 hr, followed by 1 mg/hr for a further 22 hr) in Stage I of the Phase 2 safety, dose-finding and efficacy study evaluating viscoelastic testing (VET) guided tissue plasminogen activator (t-PA) treatment in critically-ill pro-thrombotic acute respiratory failure (ARF) (VETtiPAT ARF) trial (NCT05540834) was administered. Frequent VET monitoring performed throughout the infusion and within 10 minutes of blood collection, revealed a gradual reduction in the TPA-test LT of 25% from the baseline TPA-test LT value over the 24-hr duration of the alteplase infusion with a partial relapse in LT observed 1 hr following cessation of the infusion (Fig. 4). No changes were observed in the EX-test and FIB-test parameters over the 24-hour period (data not shown) and there were no signs of bleeding.

**Discussion**

These data demonstrate that (i) hypofibrinolysis is associated with many critical conditions requiring intensive care, and occurs with equal severity in COVID-19 and non-COVID-19 patients; (ii) ex-vivo supplementation of t-PA on its own, or in combination with its substrate, plasminogen, is able to correct the majority of hypofibrinolysis cases, (iii) a TPA-test lysis time of > 300 sec appears to detect the majority of patients with hypofibrinolysis, and a TPA-test lysis time of > 1000 sec appears to predict the beneficial response to combined t-PA and fibrinogen supplementation, and (iv) ClotPro VET and the associated TPA-test can potentially be used to monitor the response and guide the dose of in-vivo t-PA supplementation that is delivered with the intention of restoring the balance of t-PA:PAI-1, thus correcting hypofibrinolysis.

Significant recent attention has focused on the hypofibrinolysis that occurs in a proportion of patients with severe COVID-19 disease, however, our study is a reminder that equally severe hypofibrinolysis occurs in critically ill patients with a range of non-COVID-19 related diagnoses that have systemic inflammatory response syndrome as a common feature. Correlation analyses between FIB-test A10 and TPA-test LT in patients with a LT < 300 sec (Supp. Figure 1) and those with a LT > 300 sec (Fig. 1C) demonstrated that in patients with a LT > 300 sec, slower t-PA-induced fibrinolysis was not associated with larger fibrin clots, as was observed in healthy controls and patients with a LT < 300 sec. Numerous factors may impact upon the rate of fibrinolysis including the depletion of fibrinolytic proteins, imbalances of fibrinolytic proteins with their inhibitors, the extent of fibrin cross-linking, and the presence of neutrophil extracellular traps, bacterial or platelet polyphosphates, von Willebrand factor (vWF) and reactive oxygen species within the blood(24–28).
As part of the acute phase inflammatory response, endothelial cells release t-PA and plasminogen activator-1 (PAI-1), the principal inhibitor of t-PA(29). Platelets also contribute significantly to increase PAI-1 levels. Thus, an imbalance of t-PA:PAI-1 levels rapidly develops resulting in hypofibrinolysis that correlates with multi-organ dysfunction syndrome and death in bacterial and viral infection(11, 30, 31).

Reductions in plasmin activity also occur in systemic inflammation and may be due to insufficient t-PA needed to convert plasminogen to plasmin, plasminogen exhaustion through consumption, and/or high levels of plasmin inhibitors. Inhibition of plasmin activity was reported in bronchoalveolar lavage samples from ARDS patients and was partially attributed to increased levels of α2-antiplasmin(32). Two studies in patients with sepsis and septic shock demonstrated decreased levels of thrombin-activatable fibrinolysis inhibitor (TAFI) implying that hypofibrinolysis in these patients is not due to increased fibrinolysis inhibition by this protein(33, 34). In a study of patients with severe sepsis and septic shock, decreased plasminogen levels and coagulation inhibitors, anti-thrombin III (ATIII) and Protein C were measured in comparison to patients with less severe sepsis, in whom fibrinolysis was strongly activated and coagulation inhibited by ATIII. The authors concluded that the exhaustion of plasminogen and coagulation inhibitors was the principal mechanism leading to hypofibrinolysis in the more severely ill patients and that the same mechanisms occurred with sepsis due to several different pathogens(8). Additional evidence for plasminogen consumption causing hypofibrinolysis was obtained in burns patients with reduced plasminogen levels associating with the extent of burn injury and development of organ dysfunction(7). Plasmin is not only involved in intravascular fibrin degradation, but also in tissue repair and several aspects of the immune response(35, 36). In fact, plasmin has been found to drive the early inflammatory response to tissue injury promoting cytokine release and inflammatory signalling(7). Also contributing to depleted plasminogen levels is the finding that the protease released from activated neutrophils, neutrophil elastase, is capable of degrading plasminogen(37).

The evidence indicates therefore, that the fibrinolytic perturbations that occur in response to systemic inflammatory response syndrome following severe infection or injury are dynamic and characterised by early stage hyperfibrinolysis which transitions to hypofibrinolysis due to factor consumption (Fig. 5). The data obtained in this study suggests that an initial phase of hypofibrinolysis occurs, that is characterised by reduced tPA activity, which may progress into a more severe phase of hypofibrinolysis that is characterised by both reduced tPA and plasmin activity.

The ClotPro TPA-test and the novel exploratory extensions of this test described herein permit identification of hypofibrinolytic patients and the corrective treatment required. The results of this study demonstrate the significant variation in the degree of hypofibrinolysis between patients, as measured by the TPA-test LT, and the amount of t-PA +/- plasminogen required to restore fibrinolysis ex-vivo. The dichotomy of the response to plasminogen supplementation may reflect endogenous plasminogen levels. A baseline LT > 1000 sec may indicate plasminogen insufficiency, thus supplementation enhanced clot lysis. In contrast, where the baseline LT < 1000 sec, supplemented plasminogen combined with sufficient endogenous levels may have resulted in the inhibition of clot lysis. We are unaware of this effect being previously described, although a previous study demonstrated inhibitory effects of high tPA levels on plasmin lysis of fibrin(38). Further ex-vivo studies are planned to correlate viscoelastometry results with laboratory-based measures of fibrinolytic proteins and to investigate the mechanism of the observed inhibitory effect.
The in-vivo data demonstrate the utility of the ClotPro TPA-test to monitor in real time the effect on fibrinolysis of in-vivo fibrinolytic protein administration, in this case alteplase. The working hypothesis for the VETtiPAT ARF trial (NCT05540834) is that the infusion of t-PA over days will correct the imbalance caused by PAI-1 inhibition resulting in the restoration of fibrinolysis but without compromising coagulation. The unchanged EX-test and FIB-test parameters measured in this patient during the alteplase infusion demonstrated that coagulation in response to tissue factor was preserved throughout. A similar method could also be used to monitor plasminogen administration or the combination of both proteins. This method mimics the use of activated partial thromboplastin clotting time (aPTT) to monitor therapeutic heparin administration, with the contemporary capacity to dose the supplemented fibrinolytic protein/s according to the TPA-test LT. This method enables the dose of the fibrinolytic protein to be tailored to the patient’s requirements, thus potentially increasing efficacy and reducing thrombosis and bleeding risk.

**Conclusions**

This study has demonstrated the potential to identify and correct hypofibrinolysis in critically ill patients. Two response groups were identified (i) those requiring t-PA supplementation alone, and (ii) those requiring both t-PA and plasminogen supplementation, with the TPA-test lysis time, a measure of the extent of hypofibrinolysis, potentially delineating these groups. The use of this methodology may enable more rapid and targeted intervention to restore fibrinolysis and preserve organ function. Additionally, this point-of-care technology enables close monitoring of the response to in-vivo fibrinolysis protein supplementation, thus permitting dose-optimisation at an individual level which will potentially translate into risk reduction and improved patient outcomes. These methods and results may have far reaching therapeutic implications for many critical conditions associated with compromised fibrinolysis.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>COVID-19</td>
<td>Coronavirus disease-19</td>
</tr>
<tr>
<td>LT</td>
<td>Lysis time</td>
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<tr>
<td>MODS</td>
<td>Multi-organ dysfunction syndrome</td>
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<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>t-PA</td>
<td>Tissue plasminogen activator</td>
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<td>VET</td>
<td>Viscoelastic testing</td>
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**Declarations**
Ethics approval and consent to participate

All research was approved by the Southwestern Sydney Local Health District Human Research Ethics Committee (2021/ETH11630) and undertaken in accordance with the National Statement on Ethical Conduct in Human Research (2007). The study was conducted in a large tertiary centre, Liverpool Hospital, Sydney, Australia, between December 2021 and August 2022. The ex-vivo VET studies were aligned with standard of care and trial eligibility screening, thus consent was not required. Written informed consent was obtained from relatives for the patient entered onto the VETtiPAT ARF study.

Consent for publication

Patient was consented to a clinical trial which included the publication of de-identified data.

Availability of data and materials

Data is provided in the manuscript. If any additional information is required, email l.coupland@unsw.edu.au

Competing interests

T. Ghent acts as a scientific consultant for Haemoview Diagnostics, Australia.

The remaining authors have no conflicts of interest to declare.

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Authors’ contributions

LAC: Project conception, design, conduct, data analysis and manuscript preparation. DJR: Intellectual input and manuscript preparation. JGS: intellectual input and manuscript preparation. PC: Intellectual input, data analysis and manuscript preparation. JJM: project feasibility and conduct. TG: Intellectual and technical support. AA: Project conception, design, conduct, finance, data analysis and manuscript preparation.

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References


2020;24(1):676.


Figures
Figure 1

Disordered coagulation and hypofibrinolysis in critically ill patients with or without COVID-19 disease.

ClotPro analysis was undertaken as per manufacturer's instructions within 30 min of blood collection from patients admitted to Intensive Care to identify patients with hypofibrinolysis (TPA-test lysis time (LT) <300 sec) (n=28) and the results compared based on the presence of COVID-19 with the results obtained from healthy ICU staff provided as a reference (n=20). A The TPA-test LT indicates the time taken in seconds for 50% clot lysis to occur in response to t-PA 650 ng/mL with coagulation activated by tissue factor. Red dotted line
represents cut-off for hypofibrinolysis inclusion. B Fib-test A10 indicates the fibrin clot amplitude 10 min after the commencement of clot formation stimulated with tissue factor. C Correlation between Fib-test A10 and TPA-test lysis time with the normal range for the Fib-test A10 shown between red dotted lines and the line of best fit for the control group shown in green dotted line. D EX-test A10 represents the fibrin and platelet clot amplitude 10 min after the commencement of clot formation stimulated with tissue factor. E Platelet A10 is calculated by subtracting the Fib-test A10 value from the Ex-test A10 value and indicates the platelet contribution to the clot. Individual data points presented, bars represent median and 95% CI. Non-parametric Mann-Whitney test performed between patient groups; Spearman’s correlation analysis (rho); ns = non-significant p value.

![Image of tables and graphs showing data for Fib-test, EX-test, TPA-tests, and comparisons with normal ranges and control group lines.](image)

**Figure 2**

**Examples of patients requiring an increased t-PA dose**

Cases 15 and 16 (Table 1) demonstrated a significant hypercoagulable state on both FIB-test and EX-test with elevations in the clot amplitude (A5, A10) and maximum clot firmness (MCF). The first of the TPA-tests was performed according to the manufacturer’s instructions and hence contained t-PA 650 ng/mL blood. In both cases the LT was significantly prolonged at 545 and 530 sec. In the second of the TPA-tests, the t-PA dose was doubled by loading the test according to the manufacturer’s instructions but passing the blood through a
second TPA-test tip twice prior to commencing the assay. In both cases the increased t-PA dose resulted in a significant shortening of the LT to that approximating the upper level seen in healthy controls. In the final TPA-tests on the right-hand side in each case, the blood was passed first through a tip containing plasminogen 147 mg/mL twice immediately prior to the TPA-test tip twice and the assay being commenced. In both cases the addition of plasminogen had a counter-productive effect on the lysis time and appeared to inhibit the effect of t-PA by increasing the MCF and the prolonging the LT further than the single t-PA dose on its own.

Figure 3

![Graph showing example of patients requiring the combination of t-PA and plasminogen](image)

**Example of patients requiring the combination of t-PA and plasminogen**
Cases 27 and 28 (top two rows) demonstrated a significant hypercoagulable state on both FIB-test and EX-test with elevations in the clot amplitude (A5, A10) and maximum clot firmness (MCF). The first of the TPA-tests was performed according to the manufacturer's instructions and hence contained t-PA 650 ng/mL blood. In both cases, little to no lysis occurred, hence a LT was not registered. In the second of the TPA-tests, the t-PA dose was doubled as described in Figure 2. In both cases the increased t-PA dose did little to induce lysis with a LT still not registering. In the final TPA-tests on the right-hand side in cases 27 and 28, the blood was passed through a tip containing plasminogen 147 mg/mL as described in Figure 2. In both cases the addition of plasminogen induced clot lysis with the LT being significantly shortened but remaining well above normal.

![Figure 4](image)

**Figure 4**

**Monitoring changes in fibrinolysis during 24 hour alteplase infusion**

A patient with bacterial pneumonia causing acute respiratory failure was identified as hypofibrinolytic using the ClotPro TPA-test with a baseline lysis time of 450 sec. Alteplase infusion was commenced with a 25 mg bolus over 2 hr, followed by a 1 mg/hr infusion over 22 hr. ClotPro analysis was repeated at the times indicated with EX-test, Fib-test and TPA-tests being performed. Analyses were undertaken within 10 minutes of blood collection. The plot depicts the results of the TPA-test lysis time presented as a percentage of the baseline value. The upper level of normal (ULN) at 50% on the Y-axis approximates the highest level measured in our healthy control population (LT range: 132-227 sec, n=20).
Figure 5

The acute phase response (APR), following isolated or severe injury or infection, and the utility of ClotPro VET to guide fibrinolytic protein supplementation to correct hypofibrinolysis.

A. After an isolated injury or infection, the APR follows a predictable and quantifiable time-course with minimal risk of complications. The biological systems activated during the APR rapidly change and can be generally divided into two biologically distinct phases: ‘survival-APR’ and ‘repair-APR’. Temporally, survival-APR precedes repair-APR, occurring during early convalescence. It involves activation of hemostasis (a) and survival
inflammatory cells (b), which work together to activate the serine protease thrombin that, in its canonical role, activates platelets and converts fibrinogen to fibrin. Together the fibrin platelet plug contains ruptured compartments, both intravascular and extravascular and prevents, or mitigates, invasion by pathogens (c). Once containment is achieved, the survival-APR transitions to repair-APR (indicated by circular arrows). In this phase, the serine protease plasmin (d) promotes repair – in its canonical role to remove fibrin (fibrinolysis). Additionally, in its non-canonical role it is used and activated by reparative inflammatory cells to degrade and remove damaged tissues, either directly or indirectly promotes angiogenesis, and tissue reconstruction by activating growth factors and other proteases (e.g. MMPs) from their precursor forms and egress of mesenchymal stem cells. B. After severe injury, e.g., Level-1 trauma, or with sepsis, the APR follows a less predictable time-course with great potential for adverse outcomes. A key biological event portending mortality in these cases are either early hyperfibrinolysis (a) or later hypofibrinolysis (b). Both of these states can be detected using bedside VET. Early hyperfibrinolysis is associated with severe bleeding and vascular leak and is a major cause of death. Later hypofibrinolysis may be caused by multiple mechanisms (see text) and is associated with macro and micro thrombosis and multi-organ dysfunction syndrome (MODS) and is also a major cause of death. C. Here we demonstrate the utility of the ClotPro test to rapidly detect hypofibrinolysis, determine ex-vivo if the addition of tPA and/or Plasminogen (PLG) are sufficient to restore fibrinolysis, and to monitor in-vivo supplementation of fibrinolysis proteins to reverse the prolonged hypofibrinolytic state, reducing the risk of multi-organ dysfunction and death.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryData.docx