The inflammatory effects of TNF-α and complement component 3 on coagulation

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Tissue necrosis factor-α (TNF-α) and complement component 3 (C3) are two well-known pro-inflammatory molecules. When TNF-α is upregulated, it contributes to changes in coagulation and causes C3 induction. They both interact with receptors on platelets and erythrocytes (RBCs). Here, we look at the individual effects of C3 and TNF-α, by adding low levels of the molecules to whole blood and platelet poor plasma. We used thromboelastography, wide-field microscopy and scanning electron microscopy to study blood clot formation, as well as structural changes to RBCs and platelets. Clot formation was significantly different from the naïve sample for both the molecules. Furthermore, TNF-α exposure to whole blood resulted in platelet clumping and activation and we noted spontaneous plasma protein dense matted deposits. C3 exposure did not cause platelet aggregation, and only slight pseudopodia formation was noted. Therefore, although C3 presence has an important function to cause TNF-α release, it does not necessarily by itself cause platelet activation or RBC damage at these low concentrations. We conclude by suggesting that our laboratory results can be translated into clinical practice by incorporating C3 and TNF-α measurements into broad spectrum analysis assays, like multiplex technology, as a step closer to a patient-orientated, precision medicine approach.
superfamily member 1A (TNFRSF1A) or CD120a, being the chief receptor. While RBCs do not express these receptors\(^ \text{23} \), TNF can induce platelet consumption, and platelets do express TNFR1 and TNFR2\(^ \text{22,23} \). TNF1 can express on other cells also causes the release of factors with agonist activity for platelets\(^ \text{24} \), and TNF-\( \alpha \) is able to activate platelets through stimulation of the arachidonic acid pathway\(^ \text{22,23} \).

Turning to complement, RBCs carry the complement receptor 1 (CR1), also known as C3b/C4b receptor or CD35, on its membrane\(^ \text{25} \). Immune complexes, which have reacted with complement and bear C3b fragments also bind to the CR1 on human RBCs, and CR1 on RBCs serves as a transport system for immune complexes in the circulation to prevent immune complex deposition outside the fixed macrophage system\(^ \text{26–29} \). Complement also interacts with the surface of activated platelets as well as with other components of the complement system including, C1q, C4, C3, and C9, which bind to activated platelets\(^ \text{41–28} \). Furthermore, thrombin-activated platelets can actually initiate the complement cascade\(^ \text{39,35} \), and C3a and its derivative C3a-des-Arg, induce platelet activation and aggregation in vitro\(^ \text{31} \). Platelets express complement receptors C3aR, CR4, as well as a receptor for iC3b and C5a, and the C1q receptors gC1qR and cC1qR on their membranes. cC1qR, in particular, was shown to mediate platelet aggregating and activating effects\(^ \text{29} \). Of importance is that platelets may also interact with the complement system via proteins that are not considered classical complement receptors, such as P-selectin\(^ \text{20} \) or GP1b\(^ \text{33} \).

In a series of papers, we have investigated the individual effects of inflammatory molecules on coagulation\(^ \text{34,35} \). During inflammation, various circulating inflammatory molecules are upregulated, and a crucial result of this combination of molecules is a pathological haematological system that ultimately translates to hypercoagulation, RBC dysfunction and platelet hyperactivation – all hallmarks of inflammation and cytokine upregulation. However, for clinical intervention, it is essential to know what the individual effects on these pro-inflammatory molecules are, to pinpoint possible biochemical interventions. Here we look at the individual effects of C3 and TNF-\( \alpha \), by adding low levels of the molecules to blood.

### Materials and methods

#### Ethical statement.

The ethics committees of the University of Pretoria and Stellenbosch University (South Africa) approved this study (ethics clearance number 298/2016). A written form of informed consent was obtained from all healthy donors (available on request). The methods were carried out in accordance with the approved guidelines. Blood was collected and methods were carried out in accordance with the relevant guidelines of the ethics committee. We adhered strictly to the Declaration of Helsinki.

#### Sample and blood collection.

Blood was collected from 14 healthy individuals who voluntarily enrolled for this study. Exclusion criteria for the healthy population were: known (chronic and acute) inflammatory conditions such as asthma, human immunodeficiency virus (HIV) or tuberculosis; risk factors associated with metabolic syndrome; smoking; and, if female, being on contraceptive or hormone replacement treatment. This population did not take any anti-inflammatory medication. Based on satisfying the exclusion criteria, the control donors were classified as ostensibly healthy. We therefore assumed that the TNF-\( \alpha \) and C3 levels in our chosen sample population were in the ranges previously reported by researchers (see Table 1). Our added final concentrations of the two products therefore slightly increased their intrinsic levels to simulate a state of low-grade chronic inflammation.

Blood was collected in citrate tubes and a plasma poor isolate was derived by centrifuging whole blood for 15 minutes at 3000g. Platelet poor plasma was stored at \(-80^\circ \text{C}\) prior to experimentation.

#### Products: TNF-\( \alpha \) and C3.

We exposed whole blood and plasma separately to either TNF-\( \alpha \) (Sigma T6674) or C3 (Sigma C2910) at levels that represent low-grade chronic inflammation. Our final TNF-\( \alpha \) exposure

| Reference ranges for circulating C3 and TNF-\( \alpha \) levels in humans |
|---------------------------------------------------------------|
| **TNF-\( \alpha \) levels in controls** |
| 5.6 ± 2.0 pg ml\(^{-1} \) |
| 13.4 ± 0.81 pg ml\(^{-1} \) in males and 13.9 ± 4.5 pg ml\(^{-1} \) in females |
| 14.80 ± 4.74 pg ml\(^{-1} \) |
| 3.9 ± 2.5 pg ml\(^{-1} \) |
| 0.7 ± 0.2 pg ml\(^{-1} \) |
| **TNF-\( \alpha \) levels in disease** |
| Type 2 diabetes: 4.28 ± 5.01 pg mL\(^{-1} \) |
| Chronic liver failure, TNF-\( \alpha \) was found to be 53.50 ± 73.49 pg mL\(^{-1} \) amongst survivors |
| Metabolic syndrome: 6.3 ± 1.9 pg ml\(^{-1} \) |
| Aortic stenosis patients: 2.1 ± 1.6 pg ml\(^{-1} \) and mitral regurgitation: 1.3 ± 0.7 pg ml\(^{-1} \) |
| **C3 levels in controls** |
| Healthy individuals 18 years and older: 88–201 mg·dl\(^{-1} \) |
| 88.5 ± 19 mg·dl\(^{-1} \) |
| 95 (94.5–95.5) mg·dl\(^{-1} \) |
| **C3 levels in disease** |
| Prediabetes: 103.2 (102.5–03.8) mg·dl\(^{-1} \) |
| Diabetes: 1.35 (1.10–1.60) g·L\(^{-1} \) |

Table 1. Circulating concentrations of TNF-\( \alpha \) and C3 in health and disease.
concentration in blood and plasma was 1 pg·mL\(^{-1}\) and our final C3 exposure concentration was 0.0025 mg·mL\(^{-1}\). We exposed blood to higher concentrations of TNF-\(\alpha\) (15 and 30 pg·mL\(^{-1}\) final exposure concentration) and also to a higher final exposure concentration of 0.2 mg·mL\(^{-1}\) C3. These higher concentrations did not allow a clot to be formed on the TEG, as we could not obtain a clot R-time, suggesting that the added high concentrations were causing the clot to form too fast. However, we do believe that we simulated low-grade chronic inflammation with our low TNF-\(\alpha\) and C3 final exposure concentration; but we do recognise that the physiological ranges during disease can be much higher.

**Thromboelastography of whole blood and platelet poor plasma.** Viscoelastic assessment of clot kinetics was performed using thromboelastography (TEG). Whole blood (WB) and platelet poor plasma (PPP) from healthy donors was incubated with TNF-\(\alpha\) or C3 for 10 minutes prior to assessment. WB was left at room temperature for 15 minutes following collection before being incubated. PPP was first thawed to room temperature from storage at \(-80^\circ\text{C}\) before incubation. 340 \(\mu\)L of naïve (untreated) or product-exposed WB or PPP was mixed with 20 \(\mu\)L of 0.2M CaCl\(_2\) in a disposable TEG cup. Recalcification of blood is necessary to reverse the effect of the sodium citrate collecting tube anticoagulation method and consequently initiate coagulation. The samples were then placed in the computer-controlled Thromboelastograph\(^{R}\) 5000 Hemostasis Analyzer System (Haemonetics Inc., Braintree, MA, USA) for analysis at 37 °C. Table 2 summarises the seven clot parameters that were studied\(^{36–38}\).

| Parameters                                      | Explanation                                                                 |
|-------------------------------------------------|-----------------------------------------------------------------------------|
| R value: reaction time; measured in minutes     | Time of latency from start of test to initial fibrin formation (amplitude of 2 mm); i.e. initiation time |
| K value: kinetics; measured in minutes          | Time taken to achieve a certain level of clot strength (amplitude of 20 mm); i.e. amplification   |
| Angle (\(\alpha\)/Alpha): slope between the traces represented by R and K; measured in degrees | The angle measures the speed at which fibrin build up and cross linking takes place, hence assesses the rate of clot formation; i.e. thrombin burst |
| Maximal Amplitude (MA): measured in mm         | Maximum strength/stiffness of clot. Reflects the ultimate strength of the fibrin clot, i.e. overall stability of the clot |
| Maximum rate of thrombus generation (MRTG): measured in Dyn.cm\(^{-2}\)·s\(^{-1}\) | The maximum velocity of clot growth observed or maximum rate of thrombus generation using G, where G is the elastic modulus strength of the thrombus in dynes per cm\(^{-2}\) |
| Time to maximum rate of thrombus generation | The time interval observed before the maximum speed of the clot growth           |
| Total thrombus generation (TTG): measured in Dyn.cm\(^{-2}\) | The clot strength: the amount of total resistance (to movement of the cup and pin) generated during clot formation. This is the total area under the velocity curve during clot growth, representing the amount of clot strength generated during clot growth |

**Table 2. TEG clot parameters for whole blood and platelet poor plasma (adapted from\(^{39}\)).**

Statistical analysis. TEG parameters were analysed by the repeat measures One-Way ANOVA with the Holm-Sidak test (and this includes corrections such as the Greenhouse-Geisser correction for sphericity/equal variability of differences). This type of analysis allows us to compare each product exposure with the control (GraphPad Prism 7), with statistical significance taken as \(p \leq 0.05\).

Data sharing. Raw data, extensive SOPs for TEG and SEM, including original images without color and micrographs can be accessed at: https://1drv.ms/f/s!AgoCOmY3bkKHuh5Av5hYQUS5UQqdG. Raw data can also be accessed at the corresponding author’s researchgate link: https://www.researchgate.net/profile/Etheresia_Pretorius.

Results

**Thromboelastography of whole blood and platelet poor plasma.** WB TEG parameters reflect clot properties due to both the cellular components (viz. platelets and RBC), as well as the plasma protein components\(^{38}\); while PPP clot results reflect only the effect of the plasma proteins, chiefly fibrinogen. Table 3 shows sample demographics and TEG results for both WB and PPP, with significant p-values in bold.
Table 3. Sample demographics and TEG results for both WB and PPP before and after exposure to TNF-α or C3. Values presented as median ± standard deviation.

| Demographic Data of Healthy Individuals | N = 14; Age 24.0 ± 12.2; M = 57%; F = 43% |
| TEG results for naïve whole blood exposed to TNF-α or C3 for 10 minutes at room temperature |
| Naïve (n = 8) | TNF-α (n = 8) | Naïve vs TNF-α P-value | Complement 3 (n = 8) | Naïve vs C3 P-value |
| R | 9.30 ± 1.47 | 8.40 ± 1.09 | 0.077 | 8.85 ± 0.93 | < 0.0001 |
| K | 3.80 ± 0.95 | 3.45 ± 1.02 | 0.16 | 3.55 ± 1.04 | 0.24 |
| Angle | 45.10 ± 5.37 | 48.50 ± 7.57 | 0.16 | 47.20 ± 7.66 | 0.16 |
| MA | 52.25 ± 5.43 | 54.35 ± 8.03 | 0.96 | 53.30 ± 5.32 | 0.76 |
| MRTG | 3.35 ± 0.79 | 3.82 ± 1.03 | 0.17 | 3.89 ± 1.06 | 0.17 |
| TMRTG | 13.84 ± 3.49 | 12.54 ± 3.66 | 0.37 | 13.17 ± 2.46 | 0.45 |
| TTG | 547.03 ± 141.48 | 596.87 ± 150.22 | 0.86 | 594.38 ± 127.38 | 0.77 |

| TEG results for naïve platelet poor plasma exposed to TNF-α or C3 for 10 minutes at room temperature |
| Naïve (n = 11) | TNF-α (n = 11) | Naïve vs TNF-α P-value | Complement 3 (n = 11) | Naïve vs C3 P-value |
| R | 13.20 ± 3.31 | 11.80 ± 1.97 | 0.03 | 10.60 ± 2.58 | 0.03 |
| K | 4.50 ± 2.31 | 4.60 ± 1.31 | 0.60 | 4.80 ± 2.17 | 0.94 |
| Angle | 43.60 ± 13.73 | 47.80 ± 8.55 | 0.11 | 52.00 ± 10.00 | 0.12 |
| MA | 23.80 ± 5.51 | 24.60 ± 4.92 | 0.99 | 22.90 ± 6.32 | 0.91 |
| MRTG | 3.11 ± 1.35 | 3.16 ± 1.62 | 0.05 | 3.41 ± 1.75 | 0.03 |
| TMRTG | 13.92 ± 3.51 | 13.33 ± 2.33 | 0.02 | 11.83 ± 3.18 | 0.13 |
| TTG | 155.64 ± 56.98 | 164.16 ± 54.24 | 0.83 | 148.62 ± 64.15 | 0.83 |

Figure 1. Wide-field microscopy using the Zeiss CellDiscoverer 7, before and 3 minutes after exposure to TNF-α or C3.
Wide-field microscopy using the Zeiss CellDiscoverer 7. Figure 1 shows the wide-field microscopy results before and after exposure to TNF-α and C3. This equipment gives us the option to, in real-time, observe changes as the inflammatory molecules are added. We could not detect any changes to RBCs after exposure to the low physiological concentrations of the products. We followed up this experiment with scanning electron microscopy, where we could look at cellular interactions between platelets and RBCs and at membrane changes in these cells, using high magnification.

Scanning electron microscopy of whole blood. Figure 2A shows a typical healthy whole blood smear, at low magnification, and Fig. 2B and C, higher magnification of RBCs and platelets. Figure 3 shows micrographs from healthy whole blood exposed to TNF-α and C3. TNF-α exposure resulted in both RBC damage and platelet hyperactivation and clumping, while C3 exposure only resulted in a slightly increased platelet pseudopodia formation.

Discussion
During inflammation, various circulating inflammatory molecules are upregulated, and a crucial result of this combination of molecules is a pathological haematological system that ultimately translates to hypercoagulation, RBC dysfunction and platelet hyperactivation – all hallmarks of inflammation and cytokine upregulation. However, for clinical intervention, it is essential to know what the individual effects on these pro-inflammatory molecules are, to pinpoint possible biochemical interventions. Here we look at the individual effects of C3 and TNF-α, by adding low levels of the molecules to blood. In a series of papers, we have investigated the individual effects of inflammatory molecules on coagulation. In our previous research we noted that the presence of all inflammatory molecules such as IL-1β, IL-6, IL-8 and IL-12 cause platelets to be hyperactivated, clumped or aggregated. In the current investigation, only TNF-α exposure, resulted in platelet clumping and activation (See Fig. 3A). TNF-α exposure also resulted in spontaneous plasma protein dense matted deposits, that surrounded and covered RBCs and showed interactions with platelet pseudopodia (Fig. 3B and C). C3, at the physiological levels that we used, did not cause platelet aggregation, and only slight pseudopodia formation was noted. This is an important observation, as C3 and TNF-α activity are
interlinked, and examples of complement-dependent TNF-α release are well-known, as discussed in the introduction. Therefore, although C3 presence has an important function to cause TNF-α release, it does not necessarily by itself cause platelet activation.

Increased levels of TNF-α are known to activate platelets\(^{40,41}\), and during inflammation, complement is activated on the surface of platelets, despite the presence of multiple regulators of complement activation\(^{11}\). A recent paper also showed that C3 plays specific roles in platelet activation\(^{42}\). The authors showed, in a tissue factor-dependent model of flow restriction-induced venous thrombosis, that complement factors play a role in

Figure 3. (A to D) Healthy whole blood smear after exposure to TNF-α and then prepared for scanning electron microscopy. (E to H) Healthy whole blood exposed to complement component 3.
platelet activation and fibrin deposition. In our paper, we did not additionally quantitatively assess platelet activation in the presence of our chosen molecules, but showed the effect visually using SEM.

We have also previously shown that RBCs may be affected by cytokines and that they can become eryptotic in the presence of cytokines like IL-8 or agglutinated in the presence of IL-12. In the current investigation, we did not observe any eryptosis, however, TNF-α did cause membrane changes and this was visible as membrane disintegration (see Fig. 3D). This was, however, not present in the majority of the cells. C3 did not cause any structural membrane changes after exposure. The results were confirmed with the wide-field microscopy results.

SEM allows the visualization of samples at a very detailed resolution. With TNF-α exposure, the RBC morphology did not appear to be the primary change. Rather, the plasma proteins appeared be changed and covered some of the RBCs. Also, with SEM we noted greater platelet reactivity. Since the wide-field visualization was based only on RBC auto-fluorescence and that it is not as sensitive as SEM, these changes were also not readily apparent with this technique.

Our TEG results showed that there were no significant differences in naïve whole blood when compared with whole blood samples with added TNF-α; although the R-time showed a trend towards a faster clotting initiation time (p = 0.077). When C3 was added to whole blood, the R-time shorted significantly. Overall these two products seem to promote a faster clot initiation. There were significant differences in the TEG results when platelet poor plasma (PPP) was analysed. It is known that the diagnosis of hypercoagulability are based on changes in variables of hemostatic monitors such as a decrease in the time to clot initiation (reaction time, R), an increase in the speed of clot propagation (angle), or an increase in clot strength (amplitude, A; or shear elastic modulus G). This indicates an enhancement of hemostasis or hypercoagulability as defined with TEG[13,41]. Here we show a significant decrease in the R-time, MRTG and the TMRTG with PPP and added TNF-α. Also a significant increase in MRTG with PPP and added C3. According to the results, the PPP samples with the added C3 and TNF-α showed that more parameters point towards hypercoagulation, thus suggesting these molecules have a more profound effect on fibrin formation.

Defining and understanding the role of cytokines and other inflammatory mediators, like TNF-α and C3 in (inflammatory) diseases, is becoming increasingly important, especially in patient-oriented and precision-based therapy initiatives[45]. We conclude by suggesting that our laboratory results can be translated into clinical practice by incorporating C3 and TNF-α measurements into broad spectrum analysis assays, like multiplex technology, that measures various cytokines (e.g. interleukins). Developments in inflammatory marker quantification technology, like multiplex assays and arrays, allow for an improved evaluation and understanding of the dynamic nature of inflammatory responses[46,47]. Furthermore, multiplexed protein array assays provide high sensitivity and specificity using low sample volumes in a high throughput analysis and, in addition, can be used during therapeutic drug monitoring. With such approaches, therapy outcomes can also be followed with more accuracy, and in the long run, reduce treatment costs, as well as expedite wellness outcomes.

Considering that our results show that these two molecules have a pro-thrombotic effect on blood, in addition to a place in diagnostics, it might be of importance to evaluate further the biochemical mechanisms by which C3 and TNF-α modulate pro-thrombotic pathways. This might reveal a therapeutic potential for lowering the pro-thrombotic state due to C3 and TNF-α, present in inflammatory diseases.

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Author Contributions
M.J.P.: Acquisition and analysis of all data, writing and editing of paper; J.B.: Acquisition and analysis of SEM data; E.P.: Study leader, SEM analysis, draft and writing and critical revision of paper.

Additional Information
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