

Haematological parameters and pathological clotting in deep vein thrombosis and patients with HIV

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Research

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Abstract

Background. Patients infected with the human immunodeficiency virus (HIV) are more prone to systemic inflammation and pathological clotting, and many may develop deep vein thrombosis (DVT) as a result of this dysregulated inflammatory profile. Coagulation tests are not routinely performed unless there is a specific reason.

Methods. We recruited ten healthy control subjects, 35 HIV negative patients with deep vein thrombosis (HIV negative-DVT), and 13 HIV patients with DVT (HIV positive-DVT) on the primary antiretroviral therapy (ARV) regimen- Emtricitabine, Tenofovir and Efavirenz. Serum inflammatory markers, haematological results, viscoelastic properties (using thromboelastography-TEG) and scanning electron microscopy (SEM) of whole blood (WB) were used to compare the groups.

Results. DVT patients (HIV positive and HIV negative) have anaemia with raised inflammatory markers which are more pronounced in HIV positive patients. HIV positive-DVT patients also have a microcytic hypochromic anaemia. DVT patients have a hypercoagulable profile on the TEG but no significant difference between HIV negative-DVT and HIV positive-DVT groups. The TEG analysis compared well and supported our ultrastructural results. Scanning electron microscopy of HIV positive-DVT patient's red blood cells (RBCs) and platelets demonstrates inflammatory changes including abnormal cell shapes, irregular membranes and microparticle formation. All the ultrastructural changes were more prominent in the HIV positive-DVT patients.

Conclusions. HIV positive patients have an increased hypercoagulability and DVT prevalence. Our results point to the importance of looking at the coagulation system function in HIV infected individuals with DVT. Parameters like haematological markers, coagulation tests (activated partial thromboplastin time and prothrombin time / International normalized ratio), thromboelastography and global clotting tests should be used as standard indicators of hypercoagulation and part of standard clinical practice.

Abbreviations

ARV- Antiretroviral therapy

AIDS- Acquired Immune Deficiency Syndrome

CRP- C-reactive protein

DVT- Deep vein thrombosis

ESR- Erythrocyte sedimentation rate

Haem- Haematological

Hb- Haemoglobin

HIV- Human immunodeficiency virus

IL- Interleukin

K-time- kinetic time

MA- Maximum amplitude

MCH- Mean corpuscular haemoglobin

MCHC- mean corpuscular haemoglobin concentration

MCV- Mean corpuscular volume

MPV- Mean platelet volume

MRTG- Maximum rate of thrombus generation

Plt- Platelet count

PPP- Platelet poor plasma

RBCs- Red blood cells

RCC- Red cell count

RCDW- Red cell distribution width

R-time- Reaction time

SEM- Scanning electron microscopy

TEG- Thromboelastography

TMRTG- Time to maximum rate of thrombus generation

TNF- tumor necrosis factor

TTG- total thrombus generation

UNAIDS- Joint United Nations Programme on HIV/ Acquired Immune Deficiency Syndrome

WB- Whole blood

WCC- White cell count

Clinical Implications

- HIV positive patients do have a hypercoagulable profile compared to HIV negative patients.
- Increased inflammation is present in patients with DVT
- A more detailed clinical coagulation profile should be compiled when DVT is suspected in HIV positive individuals, as this will assess the possibility of a more severe event like pulmonary embolism.

Background

Human immunodeficiency virus and Acquired Immune Deficiency Syndrome (AIDS) prevalence is a world-wide pandemic and the Joint United Nations Programme on HIV/ Acquired Immune Deficiency Syndrome (UNAIDS) estimates that 1 million AIDS-related deaths occurred during 2016 ¹ and 1.8 million [1.4 million–2.4 million] people became newly infected with HIV in 2017 (<http://www.unaids.org/en/resources/fact-sheet>). Although treatment of the infection with antiretroviral regimes (ARVs) is essential to addressing the pandemic, the condition is characterized by a large plethora of additional conditions associated, and also co-existing with the HIV infection, including the presence of systemic inflammation. Systemic inflammation is associated with an increase in circulating pro-inflammatory biomarkers, and is closely associated with an amplified propensity to form pathological blood clots (which is associated with hypercoagulability or an over-activated coagulation system) ²⁻⁸.

During HIV infection, various circulating inflammatory biomarkers, including cytokines interleukin (IL)-1 β , IL-2, IL-6, IL-8, IL-10, IL-12p70, tumor necrosis factor (TNF)- α and also other pro-inflammatory biomarkers are present ⁹. An increase in these biomarkers are also present in cardiovascular disease ^{10,11}; and it is therefore not surprising that HIV positive individuals are known to have an increased presence of cardiovascular complications ^{12,13}, including an increased risk to develop atherosclerosis and venous thromboembolic disease ¹⁴; and also DVT ¹⁵⁻¹⁷. The presence of DVT is also classified as a systemic inflammatory process ¹⁸, and associated with pathological clotting and upregulated circulating inflammatory biomarkers ¹⁹.

The prevalence of developing a DVT in HIV positive individuals is increased two to ten times compared to the general population ²⁰. HIV positive individuals also have a 43% increase in age-adjusted odds ratio for pulmonary embolism, a common complication of DVT, compared to HIV negative individuals ²¹. Multiple coagulation abnormalities have been reported in HIV positive patients such as decreased levels of protein C and S; and increased levels of von Willebrand factor ²²⁻²⁴. However, the association of these abnormalities with DVT is not always consistent ^{22,25}. Coagulation investigations are therefore not performed routinely in patients with HIV infection. Standard coagulation investigations are also not

performed routinely as part of the management in patients with DVT, with the exception of a D-dimer which is used to assist with the diagnosis²⁶⁻²⁸.

In the current paper, we therefore study the haematological profiles, including clotting and various inflammatory markers, in the presence of DVT in HIV positive and HIV negative individuals and compare the results to that of healthy individuals. We compare inflammatory markers for iron (iron saturation, transferrin and serum ferritin), fibrinogen, high-sensitive C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and haematology analyser results, together with viscoelastic properties of whole blood (WB) and platelet poor plasma (PPP). We also looked at ultrastructure of platelets and erythrocytes/red blood cells (RBCs) (using whole blood smears) with the SEM, as well as after thrombin was added to whole blood, to study clot structure.

Materials And Methods

The aim was to compare the inflammatory and haematological profile of HIV patients with DVT to HIV negative patients with DVT. An analytical and descriptive prospective case control study was used from 2 Pretoria academic hospitals, Kalafong Provincial Tertiary and Steve Biko Academic Hospital. Ten healthy control subjects, 35 HIV negative patients with DVT (HIV negative-DVT), and 13 HIV patients with DVT (HIV positive-DVT) on the primary ARV regimen- Emtricitabine, Tenofovir and Efavirenz- were recruited for the study. For each individual, five blood tubes of venous blood were drawn (this included ethylenediamine tetraacetic acid, buffered tri-sodium citrate and serum separator tubes). The Research Ethic Committee, Faculty of Health Sciences, University of Pretoria, South Africa approved the study (Ethics reference number: 547/2015). Inclusion criteria for the healthy individuals were known HIV negative status and no medical history of any chronic diseases. Patients were included to the research groups if they had confirmed HIV status and confirmed symptomatic DVT on doppler ultrasound or comparative imaging. In order to minimise opportunistic infections as a confounding factor, HIV positive patients were only included with a World Health Organization Clinical stage 1 and CD4+ cells greater than 170 absolute number (per mm³). Exclusion criteria for the healthy individuals and for DVT patients include smoking, pregnancy; or the use of any inflammatory-, anticoagulant-, antiplatelet-, hormone replacement- or oral contraceptive- medication.

Inflammatory marker analysis

Serum iron (total iron in blood) levels were measured together with iron saturation, transferrin (iron binding protein) and serum ferritin (iron storage form). Serum iron levels were measured by a modification of the automated AAI-25 colorimetric method. Fibrinogen (quantitative measurement of functional fibrinogen by automated coagulation analysers), CRP (measured by latex-enhanced nephelometry) and ESR (measured by an automated ESR analyser) levels were also assessed.

Haematology analysis

A haematology analyser (Advia 2120i, Siemens Healthcare) was used to do full blood counts, and the analysis included white cell count (and its differential count), RBC count, haemoglobin level, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), the mean corpuscular haemoglobin concentration (MCHC), as well as platelet count and mean platelet volume (MPV).

Viscoelastic properties of whole blood and platelet poor plasma using thromboelastography

Citrated WB, as well as PPP were used. Whole blood, collected in a citrate tube, was centrifuged to obtain PPP (15 minutes at 3000g). Whole blood was used to assess the full coagulation process, while PPP was used to assess coagulation without the influence of platelets and RBCs on the viscoelastic properties of the clot. Calcium chloride was added to either WB or PPP and 7 different parameters measured, which included: reaction time (R-time), kinetic time (K-time), alpha angle, maximum amplitude (MA), maximum rate of thrombus generation (MRTG), time to maximum rate of thrombus generation (TMRTG) and total thrombus generation (TTG).

Ultrastructure of platelets and red blood cells (RBCs)

The ultrastructure of platelets and RBCs were studied after preparing whole blood smears for scanning electron microscopy (SEM). 10 µl of WB was placed directly on a glass microscope slide, followed by fixing in 2.5% glutaraldehyde, dehydration (as per usual SEM preparation)²⁹ and mounting. Micrographs were taken with Zeiss Crossbeam 540 Field Emission Gun Scanning Electron Microscopy.

Statistical analysis

Graphpad 5 was used to do one-way ANOVA analysis. A post-test to compare groups was performed using Tukey's multiple comparison test.

Results

Inflammatory marker and haematological parameter analysis

Inflammatory marker analyses are shown in Table 1 and haematology analysis are shown in Table 2. Both HIV negative-DVT and HIV positive-DVT groups have anaemia. The HIV negative-DVT group appears to have anaemia due to inflammation (this type of anaemia is typically found in chronic disorders), whereas the anaemia in the HIV positive-DVT group is rather due to iron deficiency. The changes in serum iron, transferrin and ferritin in the HIV positive-DVT group reflects low systemic iron status, but the raised

serum ferritin (although not statistically significant) may be due to the inflammatory status of the individuals.

Inflammation is reflected, whether from the DVT or the HIV infection, by the raised white cell count, fibrinogen concentration, CRP and ESR. Surprisingly, the platelet count was not decreased in the HIV positive-DVT group. We expected this parameter, as well as the MPV to be markedly decreased, due to e.g. HIV thrombocytopenia, which is usually common amongst HIV patients, but in our sample this was not the case.

Table 1: Analysis of inflammatory markers using one-way ANOVA with Tukey's multiple comparison test.

Inflammatory marker	Control mean (std. dev)	HIV negative-DVT mean (std. dev)	HIV positive-DVT mean (std. dev)	P value	Significant difference <0.05		
					Control vs. HIV negative-DVT	Control vs. HIV positive-DVT	HIV negative-DVT vs. HIV positive-DVT
WCC (x10 ⁹ /L)	5.272 (0.857)	8.276 (2.375)	9.958 (7.783)	0.028	-	Yes	-
CRP (mg/L)	2.300 (2.359)	60.910 (59.130)	92.330 (76.610)	0.003	Yes	Yes	-
ESR (mm/hr)	5.000 (4.598)	32.060 (36.590)	46.360 (38.310)	0.041	-	Yes	-
Fibrinogen (g/L)	2.620 (0.722)	3.581 (1.258)	3.418 (1.216)	0.086	-	-	-
Serum iron (µmol/L)	18.010 (8.990)	8.690 (6.279)	6.110 (3.547)	0.0002	Yes	Yes	-
Transferrin (g/L)	2.962 (0.733)	2.348 (0.649)	1.896 (0.848)	0.007	-	Yes	-
Iron Saturation (%)	26.600 (15.170)	17.740 (19.310)	20.380 (28.250)	0.505	-	-	-
Ferritin (µg/L)	147.600 (131.300)	187.300 (237.600)	240.400 (159.200)	0.620	-	-	-

HIV=Human immunodeficiency virus, vs.=versus, WCC=white cell count, CRP=C-reactive protein, ESR=erythrocyte sedimentation rate, %= percentage.

Table 2: Analysis of haematological markers using one-way ANOVA with Tukey's multiple comparison test.

Haem. marker	Control mean (std. dev)	HIV negative-DVT mean (std. dev)	HIV positive-DVT mean (std. dev)	P value	Significant difference <0.05		
					Control vs. HIV negative-DVT	Control vs. HIV positive-DVT	HIV negative-DVT vs. HIV positive-DVT
RCC (x10 ¹² /L)	5.264 (0.442)	4.339 (0.918)	3.728 (0.731)	0.0003	Yes	Yes	-
Hb (g/dL)	14.940 (1.520)	12.670 (3.230)	9.800 (3.252)	0.0008	-	Yes	Yes
Hct (L/L)	0.475 (0.048)	0.391 (0.092)	0.320 (0.091)	0.0004	Yes	Yes	Yes
MCV (fL)	90.570 (9.424)	89.830 (7.854)	84.630 (11.190)	0.183	-	-	-
MCH (pg)	28.490 (2.836)	29.050 (3.705)	25.730 (5.107)	0.047	-	-	Yes
MCHC (g/dL)	31.490 (1.501)	32.230 (2.172)	30.250 (2.958)	0.038	-	-	Yes
RCDW (%)	14.360 (1.559)	14.960 (2.371)	16.820 (3.077)	0.040	-	-	-
Plt (x 10 ⁹ /L)	232.900 (35.820)	287.200 (128.200)	358.600 (182.500)	0.087	-	-	-
MPV (fL)	8.978 (1.214)	8.766 (1.089)	8.967 (0.780)	0.779	-	-	-

Haem.=Haematological, HIV=Human immunodeficiency virus, vs.=versus, RCC=red cell count, Hb=haemoglobin, Hct= haematocrit, MCV= mean corpuscular volume, MCH=mean corpuscular haemoglobin, MCHC=mean corpuscular haemoglobin concentration, RCDW=red cell distribution width, Plt=platelet count, MPV=mean platelet volume.

Thromboelastography

Table 3 shows a comparison of the WB and PPP TEG results between the various groups. The WB and PPP, in the HIV negative-DVT and HIV positive-DVT groups, is suggestive of clot hypercoagulability and it

is reflected by a rapid R-time, K-time, MRTG and TMRTG. However, with regard to the WB, only the R-time and TMRTG in the HIV-DVT group compared to the control group; and only the TMRTG in the HIV negative-DVT group compared to the control group were statistically significant. The PPP only demonstrated a statistically significant difference with the K-time in both the DVT groups (HIV positive and HIV negative) compared to the control group. Interestingly, there were no significant differences in hypercoagulability between the HIV positive-DVT and the HIV negative-DVT groups.

Table 3: TEG results comparing the 3 groups using WB and PPP using one-way ANOVA with Tukey's multiple comparison test.

Scanning electron microscopy

SEM micrographs of representative healthy RBCs and platelets are shown in Figure 1, while Figure 2 and 3 show SEM of RBCs and platelets in HIV negative-DVT and HIV positive-DVT patients.

Figure 1: Scanning electron microscopy micrographs of **(A)** representative healthy RBCs, **(B)** a representative healthy platelet and **(C and D)** after addition of thrombin to whole blood, where fibrin fibres are formed over the discoid RBCs with no cellular distortion.

Figure 2: Representative scanning electron microscopy micrographs of RBCs from HIV negative-DVT patients. **(A)** Whole blood with thrombin, showing RBC entrapped in fibrin matter, **(B)** RBCs agglutinated to each other (no thrombin), **(C)** higher magnification showing agglutinated plasma proteins attached to the RBC membrane and **(D)** a hyperactivated platelet.

Figure 3: Representative scanning electron microscopy micrographs of RBCs and platelets from HIV positive-DVT patients. **(A)** RBC with pathological membrane; **(B and C)** whole blood with thrombin, showing RBCs trapped in dense matted fibrin deposits and **(D)** a hyperactivated platelet.

Discussion

Red blood cells are able to transport oxygen around the body due to the haemoglobin it contains. Haemoglobin is a tetrameric protein consisting of four globin chains, two α and two non- α , each enclosing a haem group which binds to oxygen³⁰. Red blood cell is a measure of the number of red

blood cells in a volume of blood whereas the Hct is the ratio of the volume of RBC in the whole blood volume. The Hb, RCC and Hct assesses if the patient may be anaemic.

Both DVT groups (HIV negative and HIV positive groups), have a significantly lower RBC count, Hb and Hct levels than the control group indicating an anaemia in the HIV negative-DVT and the HIV positive-DVT groups (**Table 2**). The values of these 3 parameters in the HIV negative-DVT group, even though lower than the control group, are still within the normal reference ranges³¹. However, Hb and Hct in the HIV positive-DVT group were significantly lower than both the HIV negative-DVT and control groups; and lower than the normal reference ranges indicating a more severe anaemia. Anaemia is commonly found in HIV positive patients but the cause of the anaemia is not always clear³²⁻³⁷. An inadequate erythropoietin feedback mechanism is suspected to be a major contributor in HIV-related anaemia³². A low reticulocyte count is commonly found with associated polychromasia (abnormally high number of immature RBCs), indicating a possible under-producing bone marrow^{32,38-40}. Other factors that contribute to HIV-associated anaemia, includes intestinal malabsorption, autoimmune haemolysis, bone marrow malignancies, blood loss and opportunistic complications^{32,34,40,41}. Even with the decreased RBC count, Hb and Hct levels in the HIV negative-DVT group, there were no significant differences with MCV, MCH as well as MCHC (**Table 2**). The changes in the HIV negative-DVT group is in keeping with anaemia typically associated with inflammation, also known as anaemia of chronic disorders^{32,42}.

The RCDW is the coefficient of variation of RBC volume. The higher the value, the more anisocytosis (unequal RBC sizes) present. The RCDW of the HIV positive-DVT group was greater than the control and HIV negative-DVT groups (**Table 2**). A raised RCDW is commonly associated with a decrease in haemoglobin and MCV concentration; but with a raised CRP, fibrinogen and white cell count⁴³. This correlates with the haematological and inflammatory markers found in the HIV positive-DVT group (**Table 1 and 3**). RCDW is strongly associated with mortality. Patel and colleagues reported the all-cause mortality risk increases by 22% for every 1% increase in RCDW. Furthermore, the physiological association between RCDW and mortality has been hypothesised to be related to the systemic factors involved in inflammatory conditions and oxidative stress which affects erythrocyte maturation and degradation⁴³⁻⁴⁵.

The ESR is the extent in which erythrocytes sediment in one hour⁴⁶. The ESR in both the HIV negative-DVT and HIV positive-DVT groups were raised compared to the control group (**Table 1**). In inflammatory conditions the ESR rises as the erythrocytes become sticky and adhere to each other which can be seen as rouleaux formation⁴⁷⁻⁴⁹.

Fibrinogen, a high molecular weight plasma protein, is a crucial factor in the coagulation pathway (factor I). Increased fibrinogen levels are associated with thrombo-embolic events. Fibrinogen also has a role in inflammation as it tends to adhere to the membrane receptors of cells involved with inflammation. Fibrinogen can adhere to the RBCs, which becomes “heavier” resulting in an increased ESR and blood viscosity⁵⁰⁻⁵⁷. The fibrinogen levels were greater (but not statistically significant) in the HIV negative-DVT and HIV positive-DVT groups compared to the control group (**Table 1**) which correlates with the raised ESR levels seen in both groups. Increase fibrinogen concentration in the inflammatory response can explain the raised fibrinogen concentration in the HIV positive-DVT group which contributed to the DVT. The concurrent use of ARV medication has not been shown to affect the fibrinogen concentration⁵⁸. The HIV negative-DVT group with a raised fibrinogen concentration is either due to the DVT resulting in the inflammatory response (and the raised fibrinogen concentration) or the raised fibrinogen concentration contributing to the DVT.

The HIV negative-DVT and HIV positive-DVT groups had an elevated leukocyte count compared to the control group (**Table 1**). Similar to patients with ischemic strokes, thromboembolism results in an inflammatory reaction with raised leukocyte count and CRP^{59,60}. CRP, like the leukocyte count, is an important indicator of inflammatory conditions⁶¹. HIV is usually associated with a decreased immune function. The elevated leukocyte count in these HIV positive-DVT patients may be due to the HIV infection itself or to opportunistic infections, regardless of whether the patient has a DVT or not. Also, with the ARV treatment HIV is suppressed and the lymphocytes, particularly the CD4 lymphocytes, increase in turnover⁶²⁻⁶⁴. The concentration of CRP is increased by proinflammatory cytokines, interleukin 1 and 6⁶⁵. The HIV negative-DVT group has an inflammatory response to the DVT which is reflected by the statistically significantly raised CRP levels compared to the control group (**Table 1**). The same argument can be made for the raised CRP in the HIV positive-DVT group, however the CRP concentration (as well as fibrinogen) is commonly raised in HIV positive patients compared to the general population even without a DVT⁶⁶⁻⁷⁰. The raised CRP in HIV positive-DVT patients (**Table 1**) indicates a sustained acute phase response⁶⁸. This was statistically significant in the HIV positive-DVT group compared to the control group. The CRP in the HIV positive-DVT group was almost double compared to the HIV negative-DVT group. Increasing CRP concentrations has been reported with HIV disease progression, and this increase does not appear to be affected by ARV treatment⁶⁵. Previously it was noted that increased levels of CRP and fibrinogen are independently highly predictive of 5 year mortality risk in HIV positive patients, especially where the CD4 count is low^{71,72}.

Considering all the inflammatory markers (WCC, fibrinogen, CRP and ESR), each marker was statistically significantly raised in the HIV positive-DVT group compared to the control group, with the exception of fibrinogen (**Table 1**). In the HIV negative-DVT group compared to the control group, only CRP was

statistically significantly raised. CRP may therefore be a more sensitive acute phase marker to differentiate an inflammatory condition between DVT patients (HIV negative and HIV positive) compared to healthy subjects. Interestingly, no inflammatory marker was statistically significantly raised in the HIV positive-DVT group compared to the HIV negative-DVT group.

The transferrin, serum iron and iron saturation levels reflects the amount of iron in the body. Transferrin is a plasma protein that transports iron in the blood ⁶⁴, whereas ferritin is an intracellular structure capable of storing iron atoms. The concentration of serum ferritin is related to the reticuloendothelial iron stores ⁷³. Serum ferritin and iron concentrations are also indicators for acute phase responses to inflammation ⁷³, although serum ferritin appears to be a better marker of inflammation than iron status ⁷⁴. Changes in the various concentrations of the iron parameters were noted, in particular, the levels seen in the HIV negative-DVT group were similar to anaemia typically found during inflammation, where a decreased serum iron, transferrin and iron saturation concentration is found, but with a normal serum ferritin concentration ^{32,42,75}.

Iron deficiency appears to be the main contributor to anaemia in the HIV positive-DVT group in keeping with a microcytic (low MCV), hypochromic (low MCH) anaemia (**Table 2**) ^{76,77}. A low serum iron level, low transferrin level and a low iron saturation percentage, but with a raised ferritin level seen in the HIV positive-DVT group, can be explained by an immunologically altered iron metabolism where the body has adequate or increased iron stores but is unable to utilize those stores ^{34,39,62,63,73,78-80}. This functional iron deficiency can be considered a host defence mechanism by withholding iron from possible pathogens ^{81,82}. However, as iron is required for normal immune function, iron deficiency can also increase the risk of infection ⁸².

Although the inflammatory RBC changes have been documented in non-communicable diseases, there are only a few reports of communicable diseases, specifically HIV, and the effect on RBCs and the coagulation system ⁸³⁻⁸⁸. Multiple abnormal RBC shape changes and membrane abnormalities were noted in the patients with DVT (HIV negative and HIV positive groups) (**Figure 1 to 3**). During inflammatory diseases, RBCs exposed to oxidative stress and inflammatory molecules undergoes biochemical membrane changes which can result in biophysical shape changes and eryptotic cells ⁸⁹⁻⁹⁷. Eryptosis is a co-ordinated suicidal death of the red blood cells, similar to apoptosis, that allows for the removal of defective, infected or potentially harmful cells before they undergo haemolysis ⁹⁸⁻¹⁰². The abnormal RBCs present with an abnormal expression of phosphatidylserine, a cell membrane lipid, on the external membrane layer. RBCs that display phosphatidylserine also contribute to the hypercoagulation state and they provide a prothrombotic surface for the formation of thrombin ^{42,100,103-114}. Membrane

vesicle formation and microparticle shedding (microscopic extracellular membranous structures) were also seen in both DVT groups. RBC-derived microvesicles or microparticles, is known to be associated with the expression of phosphatidylserine¹¹⁵. RBC-derived microparticles appear to enhance thrombin generation resulting in a hypercoagulable state, such as in post transfusion DVT, sickle cell disease and haemolytic anaemia^{116,117}. As the microparticle presence might also be associated with increased thrombin presence, the complement system can therefore also be activated and thereby enhance the systemic inflammatory response which is also a hypercoagulable state¹¹⁸. Microparticles are also thought to originate from CD4 lymphocytes¹¹⁹. As the HIV virus infects CD4 lymphocytes, HIV positive patients may be more prone to developing microparticles and therefore enhancing the hypercoagulable state.

Whole blood with thrombin SEM analysis showed the incorporation of RBCs into the fibrin network. The incorporation of RBCs into the fibrin network stabilises and strengthens the clot by decreasing the permeability of fibrin and increasing the resistance to fibrinolysis¹²⁰⁻¹²². Healthy (discoid) RBCs in netted fibrin fibers are shown **Figure 1C and 1D**. However, in our HIV negative-DVT and HIV positive-DVT groups, the RBCs are trapped in a matted fibrin fiber network. During inflammation, fibrin fibres tend to increase in diameter and assume a matted rather than a netted appearance; while their viscoelasticity may also be influenced by the RBC inclusion in the fibrin network¹⁰³. Also, under conditions of low partial pressure of oxygen, acidosis and in response to mechanical deformation, RBCs release ATP and ADP activating platelets and promoting aggregation and release of platelet granules¹¹⁷. This can happen as part of the HIV and DVT pathology. The (hyper) activation of platelets, together with an abnormal matted fibrin network, contracts the clot containing the trapped pathological RBCs into a tight package (**Figure 3B and 3C**). The result is the formation of polyhedrocytes, which is commonly found in DVT¹²³.

Platelet functioning depends on the quality and the quantity of the platelets¹²⁴. Platelet count is a measure of the number of platelets in a volume of blood. Thrombocytopenia (low platelet count) is a common finding in HIV positive patients, whether it be due to increased destruction or decrease production of platelet cells⁵⁸. However, in this study both the HIV negative-DVT and HIV positive-DVT groups had a non-statistically significant increase in the platelet count (**Table 2**). It should be noted that platelet count is not always associated with an increased risk of DVT¹²⁵. The mean platelet volume measures the average size of platelets in the blood and is a common platelet activation marker¹²⁵⁻¹³². An elevated MPV is associated with low-grade inflammation as well as thrombosis¹³³. However, both HIV negative-DVT and HIV positive-DVT groups had a decrease in the mean platelet volume compared to the control group (**Table 2**). These results may be in keeping with a venous thrombosis where the thrombus is due to activation of the coagulation cascade instead of platelets¹³⁴. It should also be kept in mind that platelets shape and volume do vary, resulting in changes in MPV, even in healthy persons¹³². Together

with these results, the ultrastructure of platelets in the HIV positive-DVT group also have features different to that of the control group and the HIV negative-DVT group (**Figure 1B, 2D and 3D**). The control group and the HIV negative-DVT group have (hyper) activated platelet aggregates with smooth intact membranes, pseudopodia formation, openings of the open canalicular channels and membrane blebbing interspersed among smooth intact membranes. These are the typical morphological features of activated platelets seen in healthy individuals ¹³⁵. The HIV positive-DVT patients have activated platelet aggregates which are also seen to have the same features; but with the addition of shrivelled aggregates with irregular membranes, torn membrane surface and shedding of procoagulant vesicles. These features are suggestive of apoptosis, cell death, as was seen in the red blood cells. Similar ultrastructural changes in HIV patients were documented by Pretorius et al. in 2008 ⁸³. These ultrastructural changes may be due to altered viral infected megakaryocyte morphology or due to direct infection and damage by the HIV virus. The HIV virus may gain entry into the platelets by undergoing phagocytosis or through the openings of the open canalicular system channels ^{83,136,137}. Regardless of the way of entry, platelets containing the HIV virus are activated. It is not clear if the platelets containing the virus facilitates viral replication and spreading; or assists in clearance of the virus ¹³⁶. With the latter, the ultrastructural changes may be due to the immune response of the body resulting in antibody-induced destruction of the platelets ⁸³. Antiretroviral medication has been shown to have platelet related effects such as a decreased prevalence of HIV associated thrombocytopenia but may have an increased bleeding risk ¹³⁸. This increased bleeding risk may be explained by the ultrastructural changes seen on the SEM. It is possible that the use of different ARV combinations may result in different morphological changes observed, however to standardize the results, only patients on the primary regimen were recruited for this study.

Our TEG analysis compared well and supported our ultrastructural results (**Table 3**). The HIV negative-DVT group compared to the control group showed significant differences with regards to TMRTG, while the HIV positive-DVT group compared to the control group, showed significant differences with regards to R-time, and the TMRTG. According to Pretorius and colleagues not all the parameters need be abnormal to indicate pathological coagulability and the degree of coagulability can be related to the number of parameters that are abnormal ⁵.

Both DVT groups (HIV negative and HIV positive), using whole blood, indicate a hypercoagulable profile that has a rapid initiation and amplification, resulting in the rapid formation of thrombin. The TEG parameters in the HIV positive-DVT group compared to the HIV negative-DVT group indicate a hypercoagulable profile but there were no statistical significance in any of the parameters. In the HIV negative-DVT and HIV positive-DVT groups, both having rapid R-times and K-times (**Table 3**), trapped RBCs between a matted (hypercoagulable) fibrin network were noted. Considering that pulmonary embolism can result in up to 10% mortality ¹³⁹ and half of the patients with DVT may have non-

symptomatic (silent) pulmonary embolism¹⁴⁰, the changes found on the TEG and SEM may provide for a risk of assessment of the DVT complicating to pulmonary embolism.¹⁴¹

Conclusion

It is well-known that HIV infection is linked to inflammation and inflammation is linked with the presence of a hypercoagulable state. The presence of DVT is also associated with inflammation. Whether HIV is the cause of the DVT, as noted in our sample, is not certain. However, our results point to the importance of looking at the coagulation system function in HIV infected individuals with DVT, where parameters like haematological markers, traditional coagulation tests (activated partial thromboplastin time and Prothrombin time / International normalized ratio), and ultrastructural tests should all be used as a measure of the level of systemic inflammation and hypercoagulability, as its presence may be the preamble of more severe complications like pulmonary embolism.

Declarations

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Author contributions

BSJ and EP conceived the experiments; BSJ and JN conducted the experiments; BSJ and EP analysed the results. All authors reviewed and approved the manuscript.

Availability of data and materials

The dataset(s) supporting the conclusions of this article are available from the authors.

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

The Human Research Ethics Committee, Faculty of Health Sciences, University of Pretoria, South Africa approved the study (reference number: 547/2015). Consent was obtained from each participant. Consent was also obtained from the Chief Executive Office of each hospital.

Consent for publication

Consent obtained from each patient/person.

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Table 3

Table 3: TEG results of WB and PPP using one-way ANOVA with Tukey's multiple comparison test.

TEG Parameters	Control mean (std. dev)	HIV negative-DVT mean (std. dev)	HIV positive-DVT mean (std. dev)	P value	Significant difference <0.05		
					Control vs. HIV negative-DVT	Control vs. HIV positive-DVT	HIV negative-DVT vs. HIV positive-DVT
Whole blood analysis							
R-time (min)	8.280 (2.710)	6.331 (1.824)	5.769 (2.894)	0.032	-	Yes	-
K-time (min)	4.240 (1.078)	3.348 (2.217)	2.731 (1,573)	0.180	-	-	-
alpha angle (degrees)	53.700 (4.229)	52.920 (14.24)	58.430 (13.76)	0.437	-	-	-
MA (mm)	56.050 (6.555)	55.580 (12.640)	56.370 (12.820)	0.979	-	-	-
MRTG (dcs)	3.353 (1.053)	5.836 (3.366)	6.893 (5.249)	0.074	-	-	-
TMRTG (min)	12.550 (3.663)	9.135 (2.972)	8.244 (3.726)	0.008	Yes	Yes	-
TTG (dcs)	669.300 (168.000)	652.200 (406.700)	769.900 (478.200)	0.666	-	-	-
Platelet poor plasma analysis							
R-time (min)	7.810 (1.075)	7.234 (2.695)	6.008 (1.920)	0.166	-	-	-
K-time (min)	4.760 (4.132)	2.115 (1.948)	1.973 (1.727)	0.016	Yes	Yes	-

alpha angle (degrees)	65.870 (7.175)	63.290 (13.84)	66.48 (14.720)	0.731	-	-	-
MA (mm)	30.700 (6.503)	37.010 (9.468)	37.900 (15.960)	0.235	-	-	-
MRTG (dcs)	6.145 (4.017)	9.227 (3.753)	9.607 (5.683)	0.118	-	-	-
TMRTG (min)	9.392 (1.256)	9.008 (3.804)	7.133 (2.167)	0.164	-	-	-
TTG (dcs)	230.800 (69.280)	310.400 (111.500)	363.900 (312.200)	0.27	-	-	-

TEG=Thromboelastography, HIV=Human immunodeficiency virus, vs=versus, R=reaction, K=kinetics, MA=maximum amplitude, MRTG=Maximum rate of thrombus generation, TMRTG=Time to maximum rate of thrombus generation, TTG=Total thrombus generation, PPP=Platelet poor plasma.

Figures

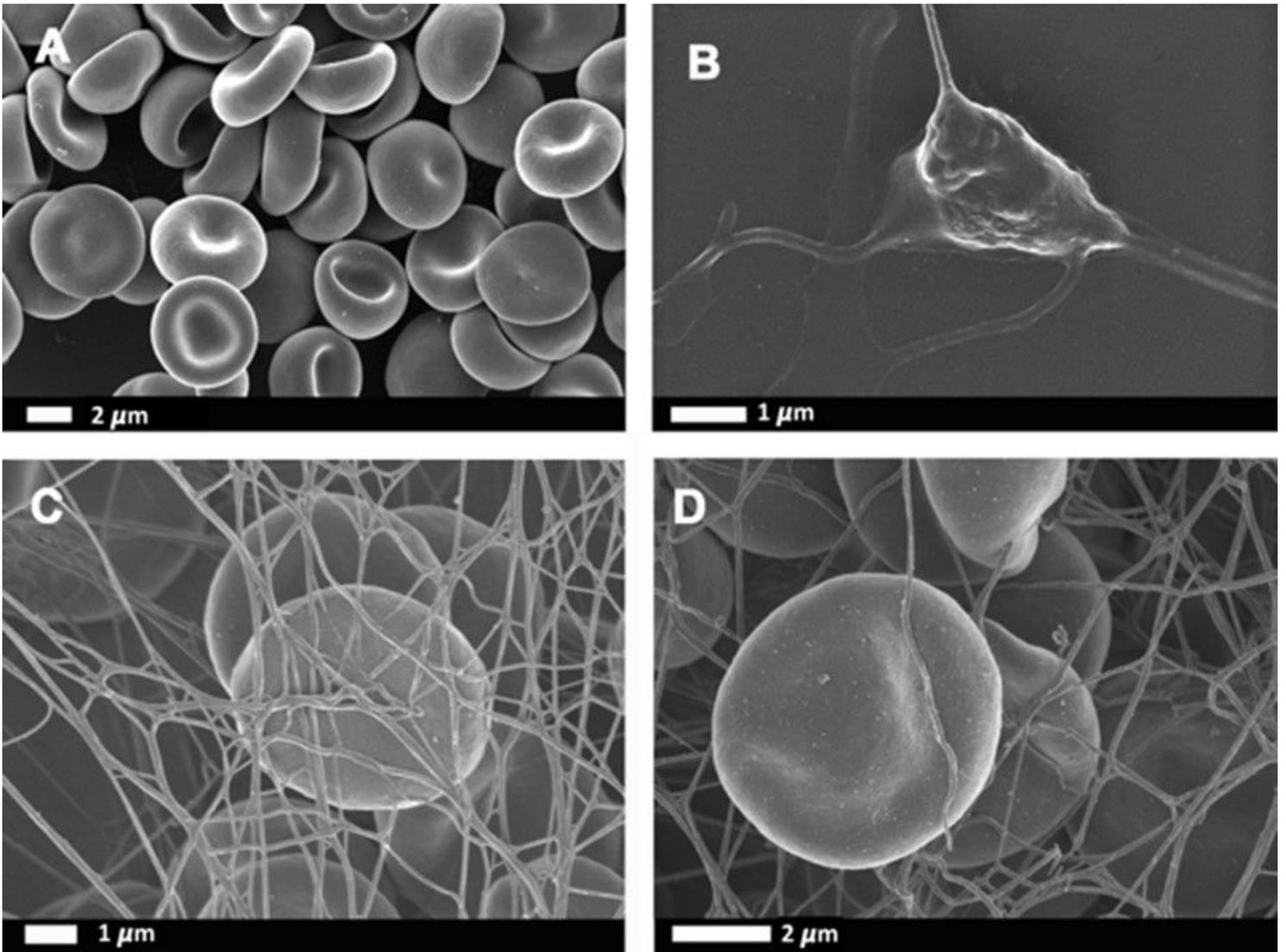


Figure 1

Scanning electron microscopy micrographs of (A) representative healthy RBCs, (B) a representative healthy platelet and (C and D) after addition of thrombin to whole blood, where fibrin fibres are formed over the discoid RBCs with no cellular distortion.

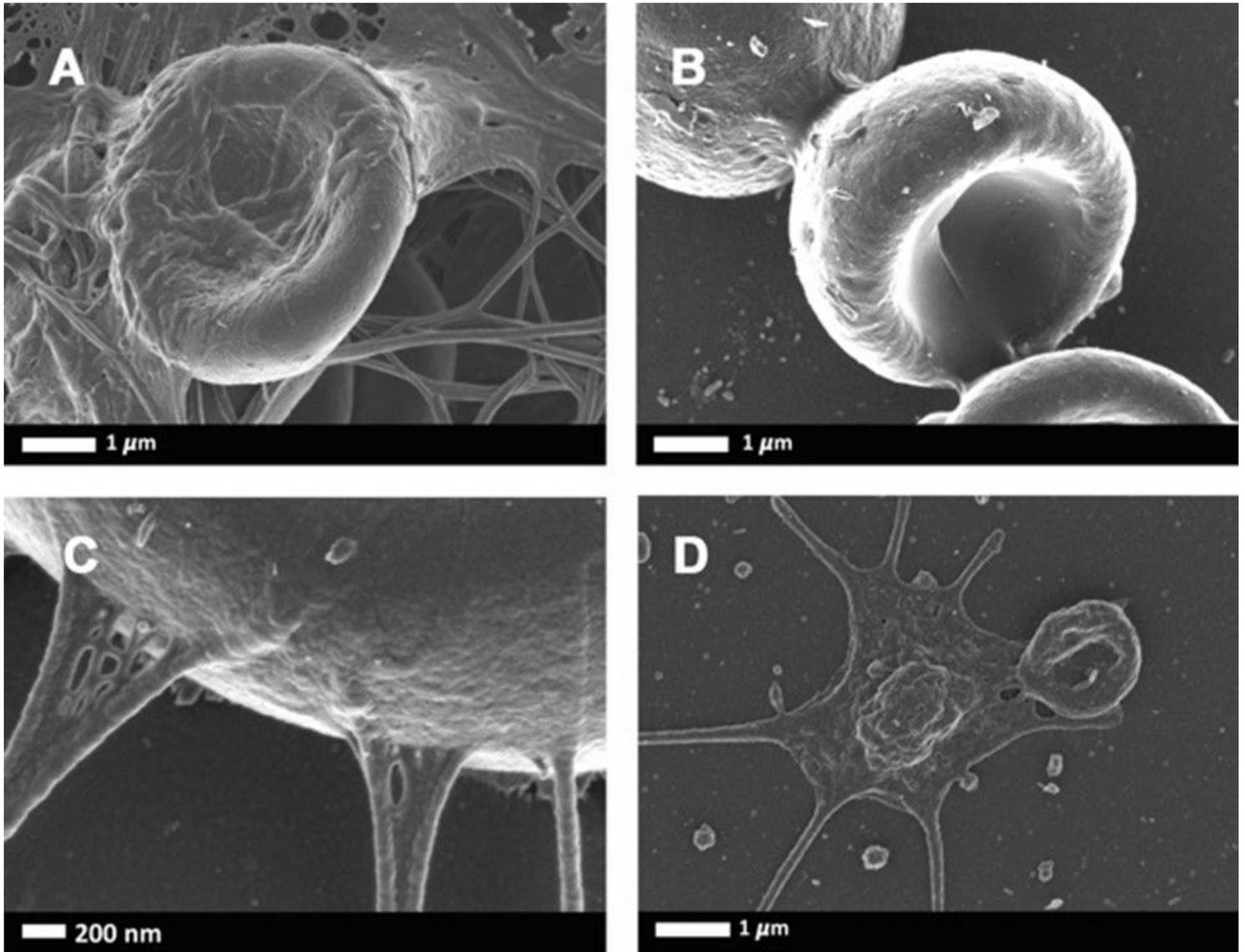


Figure 2

Representative scanning electron microscopy micrographs of RBCs from HIV negative-DVT patients. (A) Whole blood with thrombin, showing RBC entrapped in fibrin matter, (B) RBCs agglutinated to each other (no thrombin), (C) higher magnification showing agglutinated plasma proteins attached to the RBC membrane and (D) a hyperactivated platelet.

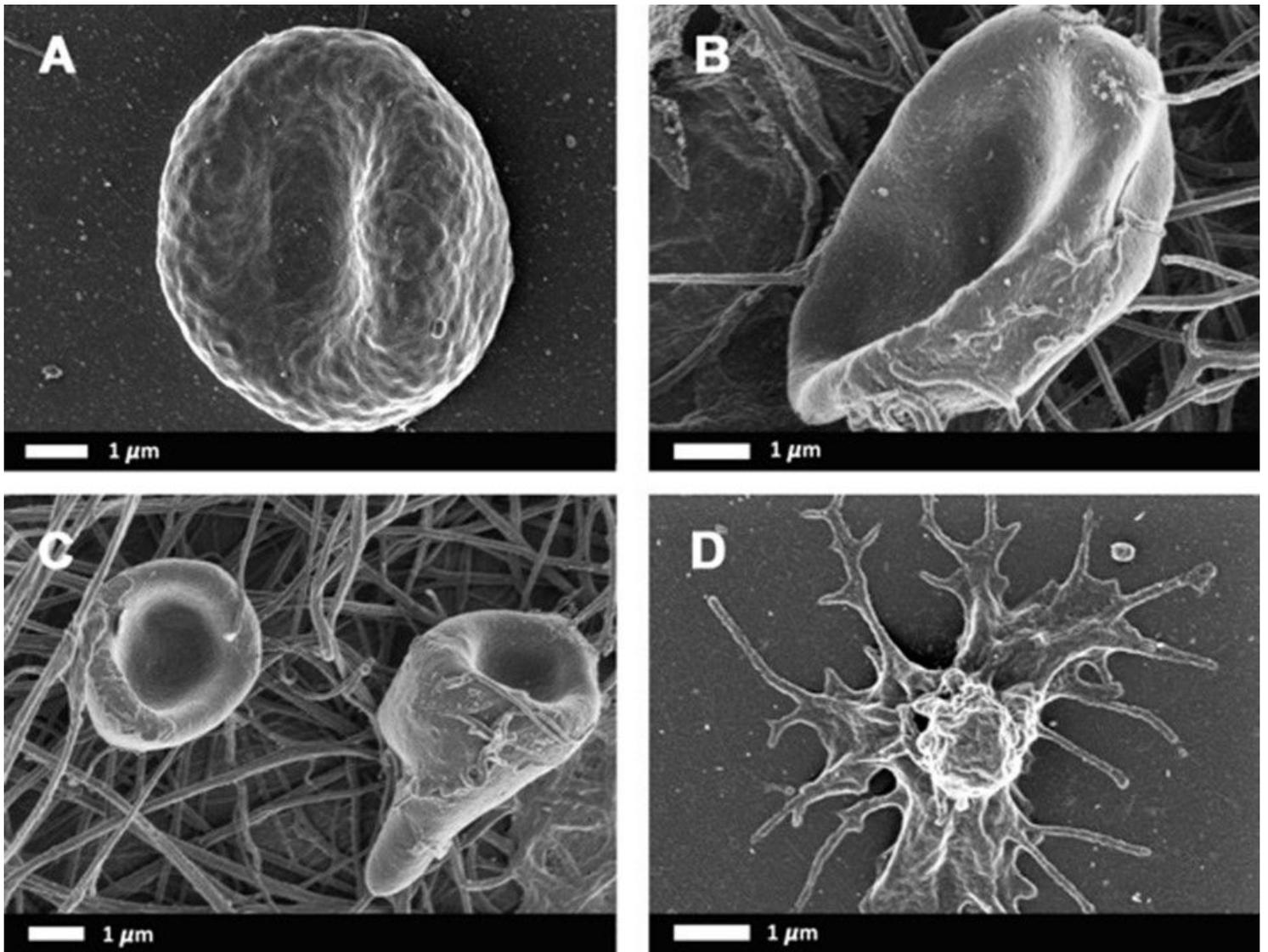


Figure 3

Representative scanning electron microscopy micrographs of RBCs and platelets from HIV positive-DVT patients. (A) RBC with pathological membrane; (B and C) whole blood with thrombin, showing RBCs trapped in dense matted fibrin deposits and (D) a hyperactivated platelet.