HYPERCOAGULABILITY USING BIOPHYSICAL PARAMETERS IN HIV POSITIVE VERSUS HIV NEGATIVE PATIENTS WITH DEEP VEIN THROMBOSIS

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Hypercoagulability using biophysical parameters in HIV positive versus HIV negative patients with deep vein thrombosis

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Abstract

BACKROUND: Patients infected with the Human immunodeficiency virus (HIV) are more prone to developing a hypercoagulable state which results in thromboembolic disease, especially deep vein thrombosis (DVT). This hypercoagulable state may be attributable to HIV positive patients having an upregulated inflammatory system resulting in a different coagulation profile compared to HIV negative patients. The effects of antiretroviral medication (ARV) should theoretically improve this hypercoagulable state though ARV treatment may not restore full health. The study of coagulation in the presence of HIV infection is ever changing. A better understanding of hypercoagulability will result in improved patient care in HIV infected patients.

HYPOTHESIS: HIV positive patients have a different coagulation profile compared to HIV negative patients which results in hypercoagulability and DVT.

OBJECTIVES: To compare the following: inflammatory markers; haematological markers; viscoelastic properties of whole blood (WB) and platelet poor plasma (PPP); light microscopy smears; ultrastructure of platelets and red blood cells; WB with thrombin to the viscoelastic results; and PPP with thrombin to the viscoelastic results.

METHODS: A descriptive comparative prospective study recruiting symptomatic confirmed DVT adult patients with HIV status. There will be three groups: Controls, HIV-negative, and HIV-positive. The objectives, as mentioned above, were achieved as follows:

1. Inflammatory markers of serum iron, fibrinogen, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) as measured by an automated analyser.
2. Haematological results using a haematology analyser

3. Viscoelastic properties, using a thromboelastogram (TEG), of WB and PPP.
4. Light microscopy smears of WB with Giemsa stain for differential staining of the blood cells.
5. Surface morphology of platelets and red blood cells using WB smears with Scanning Electron Microscope (SEM).
6. SEM, using WB with thrombin, to the viscoelastic results to assess the interaction of fibrin strands with the red blood cells.
7. SEM, using PPP with thrombin, to the viscoelastic results to assess the fibrin strand ultrastructure and diameter.

RESULTS: Fifty DVT patients were recruited, thirty-five HIV negative and fifteen HIV positive. In the HIV positive group there were 2 patients not on ARV treatment and 13 on the primary ARV regimen (Emtricitabine, Tenofovir and Efavirenz).

DVT patients have anaemia, raised inflammatory markers and iron deficiency. HIV positive compared to HIV negative patients with DVT have raised C-reactive protein and ferritin concentrations; and a microcytic hypochromic anaemia. There was no significant change in platelet concentration in any of the groups.

DVT patients have a hypercoagulable profile on the viscoelastic test whether using WB or PPP. The viscoelastic tests did not demonstrate a significant difference between the HIV positive and HIV negative groups.

The light microscopy demonstrated red blood cell changes in the DVT groups, which were more extreme in the HIV positive group. These findings were confirmed and more pronounced by examining the ultrastructure of red blood cells. Activated platelet ultrastructure in HIV positive group also demonstrated significant changes, although not correlating with the TEG results. Hypercoagulable changes can also be seen with the increase fibrin fibre diameter, dense matted deposits and the dense compact fibrin network.

CONCLUSION: Patients with deep vein thrombosis are in a state of inflammation. Whether the HIV is the cause of the DVT or the DVT is the cause of the inflammatory changes is not

certain. However, HIV infection is linked to inflammation and inflammation is linked with a hypercoagulable state.

Patients with DVT are hypercoagulable regardless of HIV infection and there does not appear to be any significant difference between HIV positive and HIV negative patients with regard to the viscoelastic studies. The scanning electron microscopy is able to detect changes when other tests such as haematological markers and viscoelastic test (TEG) are unable to. The ultrastructural changes of the red blood cell, platelets and fibrin fibre network indicates that there are specific differences in patients infected with HIV. HIV positive patients do have a different coagulation profile compared to HIV negative patients which can result in hypercoagulability and deep vein thrombosis.

Declaration

I, Brandon Spencer Jackson, hereby declare that this research dissertation is my own work and has not been presented for any degree at another University

Signed: 

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Abbreviations

ABC	Abacavir
AIDS	Acquired immunodeficiency syndrome
ACT	Activated clotting time
aPTT	Activated partial thromboplastin time
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ARV	Antiretroviral medication
CaCl2	Calcium chloride
CD	Cluster of differentiation
G	Clot elasticity
CFT	Clot formation time
CR	Clot rate
CT	Clotting time
CRP	C-reactive protein
DVD	Deep vein thrombosis
DMD	Dense matted deposits
DNA	Deoxyribonucleic acid
ddl	Didanosine
FTC	Emtricitabine
ETP	Endogenous thrombin potential
ELIZA	Enzyme-linked immunosorbent assay
ESR	Erythrocyte sedimentation rate
EDTA	Ethylenediaminetetraacetic acid
e.g.	Example
etc.	Et cetera
Fe3+	Ferric ion
FBC	Full blood count
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
Hb	Haemoglobin
Hct	Haematocrit
HMDS	Hexamethyldisilazane

HIV	Human immunodeficiency virus
IL	Interleukin
INR	International normalized ratio
Fe	Iron
K-time	Kinetics time
3TC	Lamivudine
Ly30	Lysis at 30 minutes
MA	Maximum amplitude
MCE	Maximum clot elasticity
MCF	Maximum clot firmness
MTG/MRTGG	Maximum rate of thrombus generation
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MPV	Mean platelet volume
NHLS	National health laboratory services
ns	Not statistically significant
NRTI	Nucleoside reverse transcriptase inhibitors
NNRTI	Non-nucleoside reverse transcriptase inhibitors
N	Number
OsO4	Osmium tetroxide
PA	Peak amplitude
PBS	Phosphate buffered solution
PAF	Platelet activating factor
PDGF	Platelet-derived growth factor
PDW	Platelet distribution width
PF	Platelet function
PPP	Platelet poor plasma
Plt	Platelets
PI	Protease inhibitors
PT	Prothrombin time
PE	Pulmonary embolism
R- time	Reaction time

RBC	Red blood cells
RCDW	Red cell distribution width
RNA	Ribonucleic acid
ROTEM	Rotational Thromboelastometry
SEM	Scanning Electron Microscope
D4T	Stavudine
START	Strategic timing of antiviral therapy
TDF	Tenofovir
TG	Thrombin generation
TEG	Thromboelastography
TMG/TMRTGG	Time to maximum rate of thrombus generation(s).
TP	Time to peak
TGG	Total thrombus generation
TGF	Transforming growth factor
vWF	von Willebrand factor
WCC	White cell count
AZT	Zidovudine

Measurement abbreviations

mm3	Cubic millimeter
Dyn.cm.s	Dyne per centimeter per second
fL	Femtolitre
kV	Kilovolts
L	Liters
g/dL	Grams per deciliter
nm	Nanometer
nmol/L	Nanomoles per liter
µg/L	Micrograms per liter
µL	Microlitres
µm	Micrometer
umol/L	Micromoles
mg/L	Milligrams per liter
ml	Milliliters
mm/hr	Millimeter per hour
mm	Millimeters
mM	Millimoles
min	Minutes
M	Molar
pM	Particulate matter
%	Percentage
pg	Picograms
sec	Seconds

CHAPTER 1

1.1) INTRODUCTION

In 2016 the amount of people recorded with human immunodeficiency virus (HIV) infection had totalled approximately 36 million people, which is half of the total number recorded since the start of the epidemic. The mortality from HIV-related illnesses in 2016 was at 1 million people.[1] Sub-Saharan Africa has just under 21 million people infected with HIV.[2] New HIV infections are approximately 2.6 million per year globally.[3]

HIV is a retrovirus that targets the immune system and infects principally T lymphocyte cells, that bear the CD4 receptor (CD4+), and monocytes/macrophages destroying or impairing their function. The viral replication and the antigenic stimulation by the virus stimulates a host inflammatory response.[4,5] Proinflammatory cytokines concentrations are known to be raised in their plasma. These inflammatory cytokines include interleukin 6 (IL-6), interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNFα).[6-10] An untreated HIV infection results in an exhausted and weakened immune system.[4] The deteriorating immune system is reflected by the low CD4+ count and an increased risk of opportunistic infections.[11] The immune status of HIV positive patients can be classified by measuring the absolute number of CD4+ lymphocytes (per mm3) (**Table 1**). Acquired Immunodeficiency Syndrome, AIDS, is the term used for the severely immunocompromised patients.[2]

Table 1: World Health Organisation (WHO) immunological classification according to the absolute number of CD4+ lymphocytes (adjusted from [11])

HIV immunodeficiency classification	CD4+ cells (per mm^3)
None or non-significant	Greater than 500
Mild	Between 350 to 499
Advanced	Between 200 to 349
Severe	Lower than 200

In 2007 already, the WHO published a clinical staging system of HIV/AIDS. The clinical criteria are divided into 4 stages. Asymptomatic patients are classified as stage 1. Those presenting with mild clinical features are classified as stage 2. Stage 3 has advanced symptoms and Stage 4 has severe symptoms. Stages 3 and 4 are associated with advanced HIV infection[11] (**Table 2**).

Table 2: World Health Organization Clinical staging of HIV/AIDS[11]

Clinical stage 1
Asymptomatic Persistent generalized lymphadenopathy
Clinical stage 2
Moderate unexplained weight loss (<10% of presumed or measured body weight) Recurrent respiratory tract infections sinusitis, tonsillitis, otitis media and pharyngitis) Herpes zoster Angular cheilitis Recurrent oral ulceration Papular pruritic eruptions Seborrhoeic dermatitis Fungal nail infections
Clinical Stage 3
Unexplained severe weight loss (>10% of presumed or measured body weight) Unexplained chronic diarrhoea for longer than one month Unexplained persistent fever (above 37.6°C intermittent or constant, for longer than one month) Persistent oral candidiasis Oral hairy leukoplakia Pulmonary tuberculosis (current) Severe bacterial infections (such as pneumonia, empyema, pyomyositis, bone or joint infection, meningitis or bacteraemia) Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis Unexplained anaemia (<8 g/dl), neutropaenia (<0.5 × 10^9 per litre) or chronic thrombocytopaenia (<50 × 10^9 per litre)

Table 2: World Health Organization Clinical staging of HIV/AIDS[11] continued…

Clinical stage 4
HIV wasting syndrome
Pneumocystis pneumonia
Recurrent severe bacterial pneumonia
Chronic herpes simplex infection (orolabial, genital or anorectal
of more than one month's duration or visceral at any site)
Oesophageal candidiasis (or candidiasis of trachea, bronchi or lungs)
Extrapulmonary tuberculosis
Kaposi's sarcoma
Cytomegalovirus infection (retinitis or infection of other organs)
Central nervous system toxoplasmosis
HIV encephalopathy
Extrapulmonary cryptococcosis including meningitis
Disseminated non-tuberculous mycobacterial infection
Progressive multifocal leukoencephalopathy
Chronic cryptosporidiosis (with diarrhoea)
Chronic isosporiasis
Disseminated mycosis (coccidiomycosis or histoplasmosis)
Recurrent non-typhoidal Salmonella bacteraemia
Lymphoma (cerebral or B-cell non-Hodgkin) or other solid HIV-associated tumours
Invasive cervical carcinoma
Atypical disseminated leishmaniasis
Symptomatic HIV-associated nephropathy or symptomatic HIV-associated cardiomyopathy

The prevalence of HIV in South Africa, as well as worldwide, has escalated the already overwhelming influx of patients into hospitals. Highly effective antiretroviral (ARV) therapy was introduced around 1995-1996.[12] In South Africa, there are multiple classes of ARVs available (**Table 3**)**.** First-line ARV regimen as recommended by the Southern African HIV Clinicians Society, conforming with international guidelines , recommends the combination of 2 nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) and a non-nucleoside reverse transcriptase inhibitor (NNRTI). The following combinations are recommended:

- Lamivudine (3TC) with Tenofovir (TDF), Zidovudine (AZT) or Abacavir (ABC)
- Emtricitabine (FTC) with Tenofovir in a fixed-dose combination or with the addition of Efavirenz as a combined pill[13].

In Pretoria, the First-line ARV regimen combination most frequently used is Efavirenz (NNRTI), Emtricitabine (NRTI) and Tenofovir (NRTI).

Table 3: Classes of ARV agents available in Southern Africa[13]

Class	Mechanism of action	Specific action	ARV agents available in Southern Africa
Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs and NtRTIs)	Reverse transcriptase inhibition	Nucleic acid analogues that mimic the normal building blocks of DNA, preventing transcription of viral RNA to DNA	Zidovudine (AZT), Didanosine (ddI), Lamivudine (3TC), Stavudine (D4T), Abacavir (ABC), Tenofovir (TDF), Emtricitabine (FTC)
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Reverse transcriptase inhibition	Small compounds shaped to fit into the genomic HIV binding site of reverse transcriptase and directly inhibit its action	Nevirapine (NVP), Efavirenz (EFV), Etravirine (ETV),
Protease inhibitors (PIs)	Protease inhibition	Inhibit the final maturation stages of HIV replication, resulting in the formation of non-infective viral particles	Indinavir (IDV), Atazanavir (ATV), Lopinavir/ritonavir (LPV/r), Darunavir (DRV), Saquinavir (SQV),
Integrase inhibitors (also termed integrase strand transfer inhibitors) (InSTIs)	Inhibit viral integration	Prevent the transfer of proviral DNA strands into the host chromosomal DNA	Raltegravir (RAL)

ARV = antiretroviral; DNA = deoxyribonucleic acid; RNA = ribonucleic acid.

ARV's suppresses HIV replication, but also has multiple side effects (**Table 4**).[3] ARV's are also known to be associated with coagulation abnormalities in HIV infected patients (**Table 5**).[14-17]

Table 4: **Side effects of Antiretroviral medication**

Class	**Medication name**	**Side effects**
NRTIs	Zidovudine	fever, rash, and Nausea and vomiting[15,16]
		Fatty liver change, hepatomegaly, elevated liver function tests[15]
		Nail hyperpigmentation[15]
		Myelosuppression[15, 16]
		Diabetes Mellitus and Insulin Resistance[16]
		Dyslipidaemia[16]
		Steatosis[16]
		Lactic acidosis[15,16]
		Myopathy[15, 16]
		Stevens- Johnson Syndrome/ Toxic Epidermal Necrosis[16]
	Didanosine	Pancreatitis[15, 16]
		Deranged liver function tests[15]
		Hyperuricemia, abdominal pain and abnormalities in cardiac function[15]
		Fulminant hepatitis[15]
		Cardiovascular Disease (increased risk of MI)[16]
		Diabetes Mellitus and Insulin Resistance[16]
		Nausea and vomiting[16]
		Steatosis[16]
		Lactic acidosis[16]
		Stevens- Johnson Syndrome/ Toxic Epidermal Necrosis[16]
		Noncirrhotic portal hypertension and oesophageal varices[16]
		Peripheral neuropathy[15]
		Optic neuritis[15]
	Lamivudine	Lactic acidosis[16]
		Elevated liver function tests[16]
		Pancreatitis[16]
	Stavudine	Peripheral neuropathy[15]
		Nervous System/ Psychiatric Effects[16]
		Dyslipidaemia and hyperglycaemia[17]
		Diabetes Mellitus and Insulin Resistance[16]
		Dyslipidaemia[16]

Table 4: **Side effects of Antiretroviral medication** continued…

Class	Medication name	Side effects
NRTIs	Stavudine	Lactic acidosis[15, 16]
		Lipodystrophy[15, 16]
	Abacavir	Cardiovascular Disease (increased risk of MI) [16]
		Dyslipidaemia[16]
		Hypersensitivity Reaction[16]
	Tenofovir	loss of Bone Density[16]
		Dyslipidaemia[16]
		↑ Serum Creatinine, Proteinuria[16]
		Hypophosphatemia and urinary phosphate wasting[16]
		Glycosuria[16]
		Hypokalaemia, and non-anion gap metabolic acidosis[16]
	Emtricitabine	Hyperpigmentation
NNRTI	Nevirapine	Hypersensitivity rash. Hepatotoxicity [16, 17]
		Diarrhoea[16]
		Hypersensitivity Reaction[16]
		Stevens- Johnson Syndrome/ Toxic Epidermal Necrosis[16]
	Efavirenz	Neuropsychiatric disorders[16, 17]
		Cardiac conduction effects [16]
		Dyslipidaemia[16]
		Hepatic failure[16]
		Hypersensitivity rash[16]
	Etravirine	Hypersensitivity rash[16]
		Stevens- Johnson Syndrome/ Toxic Epidermal Necrosis[16]
PI	Indinavir	Associated with cardiovascular events[16]
		Drug-induced hepatitis[16]
		↑ Serum creatinine, pyuria, renal atrophy, or hydronephrosis[16]
		Urinary stone or crystal formation[16]
		Hypersensitivity rash[16]
	Atazanavir	Cardiac conduction effects[16]
		Diarrhoea[16]
		Drug-induced hepatitis[16]
		Hypersensitivity rash[16]
		Chronic kidney disease[16]
		Urinary stone or crystal formation[16]

Table 4: **Side effects of Antiretroviral medication** continued…

Class	**Medication name**	**Side effects**
PI	Lopinavir/ritonavir	Cardiac conduction effects[16]
		Associated with cardiovascular events[16]
		Dyslipidaemia[16]
		Diarrhoea[16]
		Drug-induced hepatitis[16]
		Hypersensitivity rash[16]
		Chronic kidney disease[16]
	Darunavir	Associated with cardiovascular events[16]
		Diarrhoea[16]
		Drug-induced hepatitis[16]
		Hypersensitivity rash[16]
	Saquinavir	Cardiac conduction effects[16]
		Drug-induced hepatitis[16]
InSTIs	Raltegravir	Hypersensitivity Reaction[16]
		Lipohypertophy[16]
		↑ CPK, rhabdomyolysis, and myopathy or myositis[16]
		Stevens- Johnson Syndrome/ Toxic Epidermal Necrosis[16]

NRTIs= Nucleoside and nucleotide reverse transcriptase inhibitors, NNRTIs=Non-nucleoside reverse transcriptase inhibitors, PIs=Protease inhibitors, InSTIs=Integrase inhibitors (integrase strand transfer inhibitors)

Table 5: Coagulation parameters and ARV regimens[18, 19, 20, 21]

Parameters	**NRTI regimen**	**NRTI + NNRTI regimen**	**NRTI + PI regimen**
Coagulation markers PT, aPTT, vWF, factor VIII, Fibrinogen, D-dimer and endogenous thrombin potential	No significant differences	No significant differences between combinations	Increased fibrinogen levels
Endothelial markers vWF, sVCAM-1 and sICAM-1		Decreased	Decreased
Platelet markers CD40 ligand and P-selectin		No significant differences	No significant differences

HIV is directly related to the increased risk of oppertunistic infections by decreasing the host immune system. There are also non-infective conditions that are associated with the increased HIV proinflammatory state. HIV-related inflammatory states increase mortality and morbidity. Raised inflammatory markers (such as interleukin- 6, d-dimers and macrophage activation markers) are linked to cardiovascular diseases, type 2 diabetes, non-AIDS related malignancy, neurocognitive dyfunction, multiple end-organ disease and venous thromboembolic disease such as deep vein thrombosis.[5]

Deep vein thrombosis (DVT) is abnormal clotting in the deep venous system, most commonly in the lower limbs. Acute DVT is a common and potentially life-threatening condition. A complication of DVT is pulmonary embolism that commonly complicates the course of patients in hospital. The pathophysiology of DVTs can be classified by “Virchow’s

triad" which consists of venous endothelial damage, hypercoagulability and decreased venous flow.[2]

Deep vein thrombosis is associated with increased concentrations of inflammatory markers whether the patient is infected with HIV or not. Raised plasma levels of C-reactive protein (CRP), D-dimer, tumour necrosis factor alpha (TNFα), IL-6, IL-8 and monocyte chemotactic protein have been found in patients with DVT, especially at the onset of DVT.[22,23,24,25] Inflammation and coagulation are closely linked. Inflammation can activate coagulation by cytokine (IL-6 and TNFα) induced tissue factor expression, downregulating protein C and inhibiting fibrinolysis. Conversly, coagulation factors and products (thrombin and fibrin) can also activate inflammation.[25,26]

The proinflammatory state in HIV infected patients, with upregulated cytokines and other inflammatory molecules present in the blood, are therefore stongly associated with DVT formation. The elevated inflammatory markers in the haematological system are therefore as a good measuring tool to assess inflammation in HIV infected patients.

Inflammation is one of the hallmarks of hypercoagulation. A hypercoagulable state can be considered as a condition where the components of coagulation are increased/activated and/or the anticoagulation pathways decreased/inactivated resulting in a haematological system that is prone to thromboembolism. Hypercoagulable conditions include protein C deficiency, antiphospholipid syndrome, increased von Willebrand factor and malignancy. Hypercoagulabilty is commonly measured by plasma-based (prothombin time, partial thromboplastin time and international normalised ratio) and whole blood (viscoelastic tests) based coagulation tests, each with their limitations[27] (**Table: 6**).

Table 6: Commonly used coagulation tests and their limitations.

Coagulation tests	Limitations
Bleeding Time (BT)	Patients using antiplatelet therapy, operator dependant, frail skin[28]
Prothrombin Time (PT)	Laboratory variations in PT results vary with the type of reagent used in combination with the instrument[28]
Activated Partial Thromboplastin Time (aPTT)	Laboratory variations in PT results vary with the type of reagent used in combination with the instrument[28]
International normalized ratio (INR)	INR differences due to incorrect prothrombin time ratios; incorrect thromboplastin reagent/test system[29]
Thromboelastography	Unable to identify platelet adhesion and Von Willebrand factor abnormalities[30]

Screening for a hypercoagulable state in HIV patients that may develop a DVT is not currently performed unless the patient presents with a symptomatic venous thromboembolic disease. However, prophylactic DVT screening and/or early detection of a DVT in HIV infected patients would improve patient care and decrease morbidity and mortality. A better understanding of hypercoagulability will result in improved patient care in HIV infected patients.

1.2) **HYPOTHESIS AND OBJECTIVES**

Hypothesis: HIV positive patients have a different coagulation profile compared to HIV negative patients which results in hypercoagulability and deep vein thrombosis.

Objectives:

1. To compare inflammatory markers:
    - Iron (Iron saturation, transferrin and serum ferritin)
    - Fibrinogen
    - C-reactive protein (CRP)
    - Erythrocyte sedimentation rate (ESR)
2. Compare haematological results between the groups.
3. Compare viscoelastic properties of whole blood and platelet poor plasma (PPP).
4. Compare light microscopy smears between groups.
5. Compare ultrastructure of platelets and red blood cells (using whole blood smears) with the scanning electron microscopy (SEM).
6. Compare whole blood with thrombin using SEM to the viscoelastic results.
7. Compare platelet poor plasma with thrombin using SEM to the viscoelastic results.

CHAPTER 2: LITERATURE REVIEW

This thesis investigates the pathology in the haematological system, with specific focus on the coagulation system in HIV infected patients with DVT. In this chapter the literature pertaining to the coagulation system and tools to measure changes will be discussed. The current DVT screening and diagnostic investigations will be described. The interaction of the inflammatory and coagulation system will be reviewed, describing the factors that generate a hypercoagulable state.

2.1) Cells and plasma proteins in the blood

Whole blood consists of 45% formed elements (blood cells) and 55% plasma. Plasma consists of 7% proteins, 91.5% water and 1.5% of other solutes. Blood cells consists of red blood cells, white blood cells and platelets[31].

Red blood cells (erythrocytes) are enucleate cells with a function of carrying haemoglobin throughout the body allowing transport of oxygen from the lungs and to the tissues, and transporting carbon dioxide from tissues to the lungs. Red blood cells are derived from proerythroblasts. The absence of a nucleus results in a biconcave disc shaped cell which can deform in order to pass through capillary beds. The lifespan of a red blood cell in the circulation is 120 days.[32]

White blood cells (leukocytes) function as the body's defence system. The white blood cells are derived from the common myeloid progenitor cell which develop into granulocytes and monocytes; and common lymphoid precursor cells which develop into lymphocytes. Granulocytes (neutrophils, eosinophils and basophils) have multiple granules in their cytoplasm and have multilobed nuclei. Monocytes and lymphocytes do not have cytoplasmic granules. Monocytes can differentiate into macrophages. Granulocytes and monocytes are the body's innate immune response through phagocytosis and killing. The lymphocytes function as the adaptive immune response providing specific cellular and humoral immunity.[32]

Platelets (thrombocytes) are involved in haemostasis and are derived from megakaryoblasts that transforms into metamegakaryocytes which splinters into fragments. Platelets are disc-

shaped with no nucleus but have multiple granules. Platelets have a half-life in the blood of 4 days (and life span of 8-12 days) are then removed by macrophages in the spleen[33].

Platelets are tightly linked to coagulation. Coagulation involves multiple plasma proteins, coagulation factors, which are activated by one another. The activation of these proteins results in a positive feedback forming a large thrombus. Many of the plasma proteins are identified by Roman numerals according to their order of discovery. Anticoagulation factors are also plasma protein that have the reverse effect on the coagulation system thus allowing the body to maintain homeostasis.[31]

The literature review will focus on the association of platelets and red blood cells, coagulation system and HIV infection with pathological clotting in the form of DVT. Platelets and red blood cells will be reviewed as well as the role they play in inflammation and the how these cells are affected by circulating inflammatory molecules. The different plasma proteins involved in coagulation and the models of the process of coagulation will also be discussed in the following paragraphs.

2. 2) **Platelets**

Platelet function and platelet number varies with sex differences. Males have been reported to have raised platelet concentrations and quicker clotting times compared to females, thereby making them more susceptible to thrombosis. The differences are possibly due to the concentration of growth hormone secretion between males (pulsatile secretion) and females (sustained secretion). The differences on growth hormone secretion may also have differences in coagulation regulator production.[34]

Platelet activation occurs in three stages.[35] In the initiation stage platelets become activated when they come into contact with vascular wall collagen fibres or damaged endothelial cells.[33, 36] Von Willebrand (vWF) factor assists the platelets to attach to the injury site.[36]

In the extension phase, activated platelets swell, develop pseudopods protruding from their surface, release multiple active factors e.g. ADP, thromboxane A2, and serotonin; and become sticky and attach to surrounding platelets. ADP and thromboxane A2 activate

nearby platelets forming platelet aggregates[35]. As platelets are recruited and accumulate, a platelet plug is formed which contributes to hemostasis[33].

The last stage is the perturbation phase. In this phase stabilization of the clot takes place.[35]

2.3) **Platelet recruitment pathways**

The platelet cytoplasm contains different granules, alpha granules and dense granules (**Table 7**). The alpha granules contain clotting factors (V and VIII) and platelet-derived growth factor (PDGF). The dense granules contain adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin and calcium ions. Also present in the cytoplasm are enzymes that produce thromboxane A2 and fibrin-stabilising factor (factor XIII).[35]

ADP binds to platelet membrane receptors $P2Y_1$ and $P2Y_{12}$. The activation of receptor $P2Y_1$ causes the shape of the platelets to change. Receptor $P2Y_1$ also amplifies platelet aggregation by increasing the release of thromboxane A2 and stimulating the coagulation cascade.[35]

Thromboxane A2 is a prostaglandin. Thromboxane A2 binds to platelet receptors TPα and TPβ, which causes platelet recruitment and platelet adherence. Thromboxane A2 also functions as a vasoconstrictor.[31, 35]

Serotonin binds to platelet receptor 5HT-2. This interaction causes platelet recruitment and platelet shape change. Serotonin also activates the coagulation system and can function as a vasoconstrictor.[31, 35]

Vascular collagen fibres indirectly activates platelets via von Willebrand factor (vWF). VWF attaches to the receptors glycoprotein Ib and GP IIb/IIIa, found on the activated platelet surface and the vascular subendothelium.[35]

Thrombin binds to platelet receptors PAR-1 activating platelets and causing platelet aggregation. Thrombin also binds to platelet receptor PAR-4 which also has a procoagulant activity and also causes release of serotonin and epinephrine[35].

Table 7: Platelet Granules and their components[35]

PLATELET GRANULES	CLASS	TYPE	FUNCTION
Alpha granules	Adhesion molecules	P-selectin	Platelet-platelet interaction
		Von Willebrand Factor Thrombospondin-1 Fibronectin Platelet factor-4	Platelet interactions with other blood cells
	Mitogenic factors	Platelet derived growth factor	Cell growth and cell division
	Coagulation factors	Fibrinogen Plasminogen Factors V,VII,XI,XIII	Coagulation
	Protease inhibitors	Plasminogen activator inhibitor-1, Thrombin activatable fibrinolysis inhibitor	Fibrinolysis inhibition
Dense granules	Nucleotides	Adenosine diphosphate and Adenosine triphosphate	Amplification of platelet aggregation
		Guanosine diphosphate (GDP) and Guanosine triphosphate (GTP)	Modulation of vascular endothelium and leukocyte function
	Amines	Serotonin Histamine	
	Bivalent cations	Calcium ions Magnesium ions	
Lysosomes	Hydrolytic enzymes	Glycosidase Proteases	Digest material in platelet aggregates through hydrolytic degradation
	Proteins	Cationic proteins	Bactericidal activity

2.4) **Thrombocytopenia**

Thrombocytopenia is a common manifestation of HIV infection. Thrombocytopenia (platelet count of less than 100 x 10^9/L) is a common manifestation of HIV infection. Severe thrombocytopenia is classified as less than 50x 10^9/L, and a high risk for spontaneous bleeding below 10x 10^9/L.[37] Thrombocytopenia in the presence of HIV can be caused by megakaryocyte infection by the HIV virus in the bone marrow, bone marrow infiltration from opportunistic infections or lymphoma, hypersplenism, myelosuppression effects of medications or by immune-mediated peripheral platelet-specific antiglycoprotein antibodies[38, 39] Platelet destruction is predominantly early in the HIV disease course, whereas decreased platelet production is the main cause later in the disease course.[40]

2.5) **Immunity Role of Platelets**

Besides haemostasis, platelets also appear to have a role in the immune system.[38] Low-grade inflammatory conditions are associated with a high mean platelet volume (MPV).[36] Platelets can also be classified as natural inflammatory cells. They have the ability to aggregate/gather around microorganisms, and to assist with the removal from the blood.[38] In the presence of infection, platelets become activated and their production is increased by certain inflammatory mediators, such as interleukin 6.[36] Platelets aggregate with leukocytes in areas of ischemia/reperfusion injury.[34] The activated platelets interact with and signal to other inflammatory cells, such as leukocytes, and release their granules in high concentrations. Approximately 0.26 to 7.6% of HIV infected patients will have venous thromboembolism and activated platelets have been suggested as the etiology.[38]

Several chemokines are stored in very significant amounts in alpha granules in the platelet cytoplasm which possess virucidal and suppressive activity against HIV[37]. Activated platelets release cytokines such as IL-1-β, IL-7, IL-8, platelet activating factor (PAF) and transforming growth factor (TGF)-β.[38] The chemokines released activates leukocytes, specifically neutrophils and monocytes. P-selectin, a cell adhesion molecule, mediated by thrombin, is expressed on activated platelets which increase the platelet's adhesion to endothelial cells

and to neutrophils. Activated platelets release a pro-inflammatory-mediator CD40 ligand which induces the production of IL-6, IL-8 and the synthesis of tissue factor.[36] Platelets therefore play a role in surveillance and amplifying the immune response during viral infections. However, platelets and cytokines, such as IL-18, can also cause dysregulation of the immune response to HIV.[38]

Platelets attach HIV via 2 receptors, fibronectin and CXCR4, a fundamental co-receptor required for the virus to enter into cells. Activated platelets can then endocytose the HIV virus. P-selectin is then expressed on the platelet membrane surface which can activate surrounding macrophages. This leads to enhanced viral clearance by the activated macrophages.[41] On the other hand, platelets can also enhance the spread of the virus. Platelets attached to HIV can infect CD4+ cells directly or endocytose and deliver the viral particles elsewhere in the body.[38]

Thrombocytopaenia can occur when the glycoproteins on the platelet membrane surface are targeted by the antibodies that are supposed to be directed toward the viral glycoproteins.[37]

A recent study done on patients with essential thrombocythaemia found evidence of platelet leucocyte interaction. The interaction resulted in aggregates different from the usual platelet aggregates that may affect haemostasis. This platelet-leucocyte interaction has not been visualised in detail previously.[42, 43]

2.6) **Antiretrovirals and Platelets**

Antiretroviral therapy usually improves HIV-related thrombocytopenia but the effect may not be the same for all HIV infected patients.[38] Before ARV the prevalence of HIV associated thrombocytopaenia was 5-30%, with ARV the prevalence is 3.2%. HIV patients may however have an increased risk of bleeding with ARV's which indicates that ARV's or the different combinations of ARV's alter the nature of the observed thrombocytopenia.[37]

Zidovudine, NRTI, results in resolution of 24-34% of HIV associated thrombocytopenia and a partial response in 33–44%. Didanosine commonly results in a rise in the platelet concentration. Protease inhibitors (PIs) are generally associated with an increased rate of thrombosis, however increased bleeding risks have also been documented.[37]

2.7) **Ultrastructure of Platelets**

The morphology of activated platelets is characterised by membrane blebbing, platelet spreading and extensive pseudopodia formation.[36] These ultrastructural changes have been documented in diseases like asthma, cancer, thrombotic disease, and bleeding disorders.[44] In addition to the morphological changes seen in activated platelets, HIV infection is also associated with platelets undergoing apoptosis (cell death) where procoagulant vesicles are released and membrane fragmentation occur. [39, 45]

A study of 4 HIV infected patients using a Scanning Electron Microscopy was reported in 2008 by the University of Pretoria which examined the ultrastructural changes of platelet aggregates. There were areas of intact membranes adjacent to membrane blebbing with areas where the membrane was torn compared to the control platelet aggregates which showed smooth membranes. Both HIV positive and HIV negative platelet aggregates had pseudopodia and openings of the open canalicular system channels. HIV infected patients therefore have platelet aggregates that appear apoptotic which could be due to direct viral damage or to patient antibody cross-reaction.[39]

Not much research has been documented on the platelet ultrastructure in HIV patients while on ARV therapy. One study looked at the morphology of platelets with the use of an immunomodulator, Canova, in HIV patients. Canova is a herbal drug which augments the activation of the patient's immune system. Once again the HIV patients platelet morphology showed membrane blebbing and ruptured platelet membranes were observed which is indicative of apoptosis. There were minor morphological changes observed in fibrin networks suggesting that HIV does not impact on the fragility of the fibrin network.[39]

Patients treated with Canova had intact platelet morphology including decreased platelet membrane blebbing and intact smooth membranes. The findings suggest that the use of Canova prevents the destructive effects of the virus.[39, 46] The effects of ARV's may show similar effects on platelet ultrastructure as Canova.

The proposed study seeks to demonstrate these morphological changes on platelets from HIV infected patients and the effect of ARV treatment thereupon.

2.8) **Red Blood Cells (Erythrocytes)**

Platelets are influenced by physical and chemical properties of red blood cells (RBCs). RBCs can release ADP and therefore has prothrombotic properties by activating platelets resulting in a prothrombotic state. RBCs may stimulate platelet granule release which will recruit additional platelets into the developing thrombus. The interaction between platelets and RBCs are initiated by platelet activation. As the platelets become activated, the coagulation pathways are stimulated resulting in fibrin network trapping more platelets and RBCs. The interaction of activated platelets with RBCs therefore has a positive feedback on the coagulation system.[47]

Fibrinogen expresses binding sites for the membrane receptors of cells involved in inflammation. Fibrinogen enhances adhesion of the RBCs by binding to the erythrocyte membrane.[47] Red blood cells aggregation thereby increases in the presence of fibrinogen. The erythrocyte sedimentation rate (ESR) is a marker of inflammation. Since inflammation can increase the concentration of fibrinogen, the ESR can assess fibrinogen levels indirectly.[27]

Red blood cell membranes appear smooth in non-pregnant females and granular in pregnant females, it is possible that the same appearance may be present in HIV patients who are also hypercoagulable.[47]

2.9) **Fibrin**

Fibrinogen is a large centrosymmetric glycoprotein. Fibrinopeptides A and B are released when thrombin cleaves the fibrinogen molecules. Fibrinopeptide A is an early indicator of fibrin formation. Cleavage of the fibrinopeptides allows fibrin monomers to attach forming protofibrils. Bundles of protofibrils form the fibrin fibres.[36] Soluble fibrinogen is therefore converted to insoluble fibrin fibres.[31, 48] The fibrin clot consists of thick fibres, which form the majority of the clot, and thin minor fibres which are arranged among the major fibres.[49] Thrombin concentration appears to influence the density of the fibrin clot by increasing the thickness of each of the fibrin fibres.[36]

In the anticoagulation pathway antithrombin III inactivates thrombin, but 85-90% the fibrin fibres absorbs thrombin while the clot is being formed thereby preventing spread of the clot. Lysis of the clot occurs with activation of plasminogen, a plasma protein, into its active form plasmin in the presence of tissue plasminogen activator. Plasmin causes proteolysis of the fibrin fibres into soluble degradation products.[33]

The nature of the clot can be influenced by changes in the concentration of fibrinogen; a higher concentration of fibrinogen will lead to rapid clot formation with decreased spaces between the fibrin strands. Clot characteristics can also be influenced by fibrinogen amino acid sequence changes and divalent cations (e.g. iron ions) changes that interact with fibrinogen and its conversion into fibrin fibres. Ionic strength can influence the clot pore size as fibrinogen at a neutral pH has a negative charge. In diabetes, the nature of fibrinogen can change due to glycation.[50] Hormonal changes, such as an increase in oestrogen, causes a granular fibrin network.[46]

Fibrin clot proteolysis in hypercoagulable conditions may be inhibited or delayed. Scanning Electron Microscopy reveals an associated altered fibrin network.[49] The delayed/inhibited breakdown of the fibrin clot may be due to inflammatory cellular elements that are trapped in the fibrin fibre network that stimulates a firm clot formation resistant to anti-thrombotic agents.[51] In stroke patients, it was noticed that an abnormal matted fibrin network was present even without the addition of thrombin, indicating the presence of other plasma factors. In disease free individuals this abnormal matted fibrin network can only be recreated when thrombin is added to citrated blood.[52]

A thick matted layered fibrin network is seen which is due to the increased volume of the thin minor fibrin fibres. The result is the appearance of the thick major fibres being covered by a net of thickened minor fibres. These fibrin changes are documented in patients with inflammatory or other conditions such as arthritis, asthma and smoking.[49]

2.10) **Coagulation models and monitoring**

The following paragraphs will discuss the coagulation models that explain how various plasma molecules are involved to form blood clots. Clot formation is a normal and healthy physiological process, however, when inflammogens are present, it acts on the structure of the plasma proteins and causes hypercoagulation. The following coagulation models will be discussed in the next paragraphs: cascade/waterfall model and cell based model. The methods used to investigate the different model functioning will also be mentioned.

2.10.1) **Coagulation models**

There are 2 theories which explain the coagulation process with the end result of a fibrin clot. The initial cascade/waterfall model describes the coagulation process in an extrinsic and an intrinsic pathway which results in activation of a common pathway. The cell-based model, includes the activated platelets and considers the coagulation pathway as a single intricate process. Tissue factor, an integral membrane protein, appears to initiate the coagulation pathway in both models. Tissue factor is present in the blood vessel wall, platelet membrane and also the matrix of platelet α-granules.[36]

2.10.1.1) **The cascade/waterfall model**

The extrinsic pathway is activated with the release of tissue factor on exposed vascular subendothelium which activates factor VII. Activated factor VII-tissue factor complex activates factor X. Activated factor X combines with activated factor V, which is activated by thrombin, and together with calcium ions forms the enzyme prothrombinase.[36]

The intrinsic pathway is initiated by damaged platelets, resulting in the release of phospholipids by the platelets, or damaged endothelial cells that expose collagen in the surrounding basal lamina which activates factor XII. Activated factor XII activates factor XI in the presence of calcium ions. Activated factor XI, with calcium ions, activates factor IX. Activated factor IX in the presence of activated factor VIII, which is activated by thrombin, together with calcium ions as a co-factor, activates factor X. Activated factor X combines with activated factor V, in the presence of calcium ions forms the enzyme prothrombinase.[31, 36]

Both extrinsic and intrinsic pathways now follow a common pathway once prothrombinase is formed. Prothrombinase, together with calcium ions, converts prothrombin (factor II) to thrombin (activated factor II). Thrombin, together with calcium ions, converts soluble fibrinogen (factor I) to insoluble loose fibrin threads (activated factor I). Factor XIII (fibrin stabilizing factor) is activated by thrombin and promotes crosslinking of fibrin threads to form a stable fibrin network.[31, 36]

2.10.1.2) **Cell-based model**

The cell-based model of coagulation occurs in 3 stages: initiation, amplification and propagation. In the initiation stage the tissue factor on a tissue-factor bearing cell combines with activated factor VII which activates factor IX. Tissue factor and activated factor VII complex also activates factor X. Activated factor X together with activated factor V converts prothrombin (factor II) to thrombin (activated factor II).[31,36]

In the amplification stage thrombin activates factor V and factor XI. Calcium ions is required in the activation of factor V. Thrombin also assists with the cleavage of factor VIII from vWF and thereby activating factor VIII.[31,36]

The propagation stage occurs on the platelet surface as thrombin formed in the initiation stage activates resting platelets. The activated platelets release platelet-derived acidic phospholipids which drives the propagation stage. Activated factor XI activates factor IX. Activated factor IX together with activated factor VIII activates factor X. Once again, activated factor X together with activated factor V converts prothrombin (factor II) to thrombin (activated factor II), driving a positive feedback process.[36]

Thrombin, in the presence of calcium ions, converts fibrinogen (factor I) into loose fibrin threads/fibres (activated factor I). Factor XIII (fibrin stabilizing factor) is activated by thrombin and promotes crosslinking of fibrin threads to form a network of stable fibrin fibres. Thrombin assists in primary plug formation by activating resting platelets and mediating the expression of P-selectin, a cell adhesion molecule, on endothelial cells and platelets. Thrombin also assists in secondary plug formation by activating fibrinogen to ultimately form the fibrin network and maintains the coagulation process by initiating the

production of endothelial cytokine Interleukin-6 (IL-6) that promotes tissue factor expression.[31, 36]

2.10.2) **Methods to monitor coagulation**

Methods to monitor coagulation and coagulopathies will require plasma or whole blood. Plasma-based coagulation tests include activated partial thromboplastin time (aPTT), prothrombin time (PT) and the international normalized ratio (INR). Whole blood based coagulation tests includes Thromboelastography (TEG), Rotational Thromboelastometry (ROTEM) and the Sonoclot instruments. These whole blood tests can determine the viscoelastic properties of the clot directly.[27]

TEG was invented around 1940. The clinical application was limited at the time until approximately 60 years later due to technical limitations.[27] The TEG is used to identify specific areas of functioning in the clotting process.[30] The method of TEG has been standardized and results are reproducible.[53] The ROTEM, developed at the Ludwig-Maximillian University in Munich. ROTEM can detect coagulation abnormalities as well as fibrinolysis changes. There a few drawbacks of the ROTEM, it is unable to identify platelet adhesion and vWF abnormalities during primary haemostasis[30]. The parameters of whole blood and platelet poor plasma in viscoelastic tests are shown in **Table 8**.[54-59]

There is a difference between the TEG and the ROTEM (**figure 1**). In the TEG the cuvette, which holds the blood, is moving around a stationary pin and in the ROTEM the pin is rotated with a stationary cuvette. The movement of the cuvette or the pin is unobstructed, but as the fibrin fibres form, the movement becomes restricted[30]. Therefore the more firm the clot is, the slower the rotation of the cuvette or pin. The TEG produces a trace recording of coagulation and lysis of the clot, the y-axis represents the viscosity of the clot and the x-axis the time. The clot time (R-reaction time) measures from the initiation of the coagulation process to 2mm amplitude on the trace recording. The clot kinetics (K-kinetics) records the time from the R time to 20mm amplitude. The alpha angle (which is shown as the angle between R time and K time) measures the intensity of the clot forming. The clot strength is defined by the maximal amplitude (mm) of the tracing (MA-maximum amplitude). Clot lysis (Ly30, Ly60) measures amplitude reduction at 30 and 60 minutes after the MA. The velocity

thrombin formation and thrombin generation concentration can also be measured (**Figure 2 and 3**). The ROTEM presents a similar trace recording but with different terminology of the variables measured (**Table 9**)[27]. In addition, the ROTEM also has 4 measuring channels which are temperature controlled that can assess whole blood or plasma. ROTEM can thereby also determine abnormal functioning of platelets, anticoagulant defects, defective fibrinogen activation and hyperfibrinolysis (**Table 10**). Tissue factor is used to activate the extrinsic pathway, whereas kaolin is used as an intrinsic pathway activator in the ROTEM.[60] The ROTEM instrument is also more durable than the classical thromboelastrography equipment as it can be transported over rough terrain and maintains accuracy.[30]

The Sonoclot instruments are different where the viscoelastic probe vibrates vertically. The tracing recorded from the Sonoclot instruments is in the form of peaks (**Figure 4**).[27]

Nielsen and colleagues developed the clot lifespan model which is able to determine increased coagulability and decreased fibrinolysis in the same sample (**Figure 5**). Tissue factor is used as a clotting stimulus and tissue plasminogen activator as a fibrinolytic stimulus to assess the changes in clot resistance directly during clot lysis. The test lasts approximately 30 minutes.[27]

Table 8: Parameters of Whole blood and Platelet poor plasma in viscoelastic tests[54-59]

	PARAMETER	**WHOLE BLOOD**	**PLATELET POOR PLASMA**
TEG	Clot time time to 2mm amplitude)	R (reaction time, min)	R (reaction time, min)
	Clot kinetics time from 2-20mm amplitude	K (kinetics) α (slope between R and K)	K (kinetics) α (slope between R and K)
	Clot strength Maximum strength Clot elasticity	MA (maximum amplitude) G	MA (maximum amplitude) G
	Clot lysis Lysis (at fixed time)	Ly30, Ly60 (amplitude reduction 3/60 min after MA)	Ly30, Ly60 (amplitude reduction 3/60 min after MA)
Thrombin generation (TG) curve		Lag time Time to peak height	Lag time Time to peak height
	Maximum amount of thrombin generated within the sample at any one time	Peak height	Peak height
	Maximum amount of thrombin that the sample can potentially generate.	ETP-Endogenous thrombin potential (area under the TG curve)	ETP-Endogenous thrombin potential (area under the TG curve)
		Time to tail of graph	
Velocity profile curve	Maximum rate of thrombus generation	MTG	
	Total thrombus generation	TTG	
	Time to maximum rate of thrombus generation(s).	TMG	

Figure 1: Thromboelastography (TEG) and thromboelastometry (ROTEM) [27]

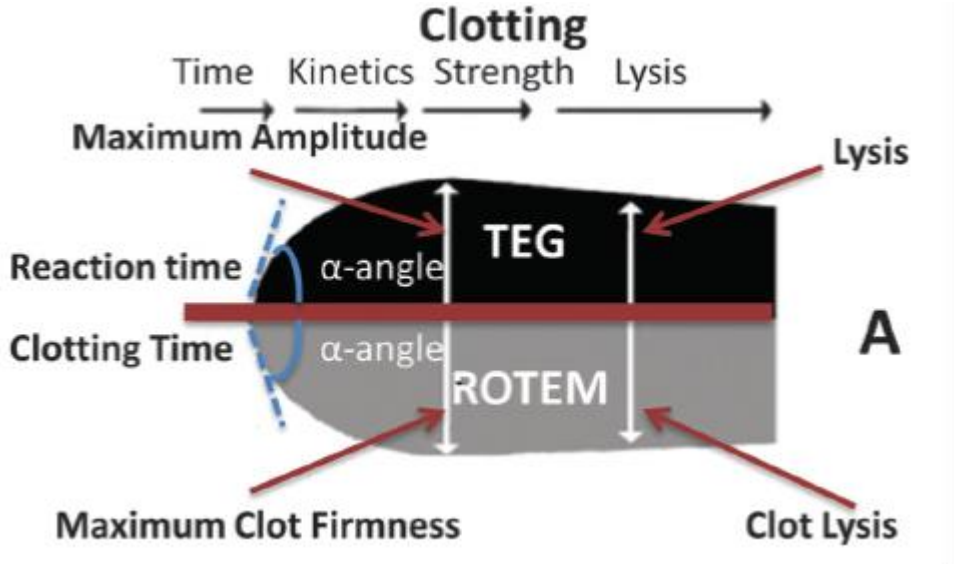


Table 9: Terminologies of the TEG, ROTEM and Sonoclot[27]

PARAMETER	**TEG**	**ROTEM**	**SONOCLOT**
Clot time time to 2mm amplitude)	R (reaction time, min)	CT (clotting time, sec)	ACT (activated clotting time, sec)
Clot kinetics time from 2-20mm amplitude	K (kinetics) α (slope between R and K)	CFT (clot formation time) α (slope of tangent at 2mm amplitude)	CR (clot rate, U min^{-1}) TP (time to peak, min)
Clot strength Maximum strength Clot elasticity	MA (maximum amplitude) G [Shear clot modulus; G=(5000xA)/(100-A)]	MCF (maximum clot firmness) MCE (maximum clot elasticity)	PA (peak amplitude)
Clot lysis Lysis (at fixed time)	Ly30, Ly60 (amplitude reduction 3/60 min after MA)	CL30,CL60 (amplitude reduction 30/60 min after MCF)	

Figure 2: A representative thrombelastograph with the grey area representing the velocity curve. (MTG: maximum rate of thrombus generation; TTG: total area under the curve, measuring total thrombus generation; TMG: time to maximum rate of thrombus generation.)[54]

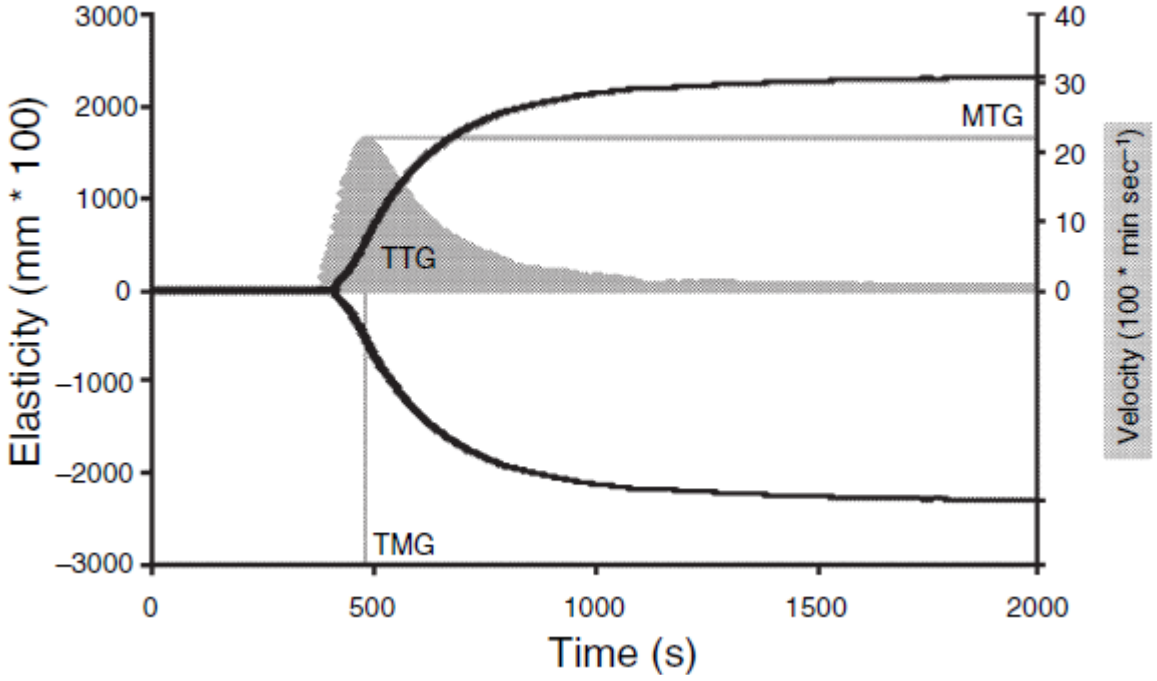


Figure 3: Thrombin generation curves[55]

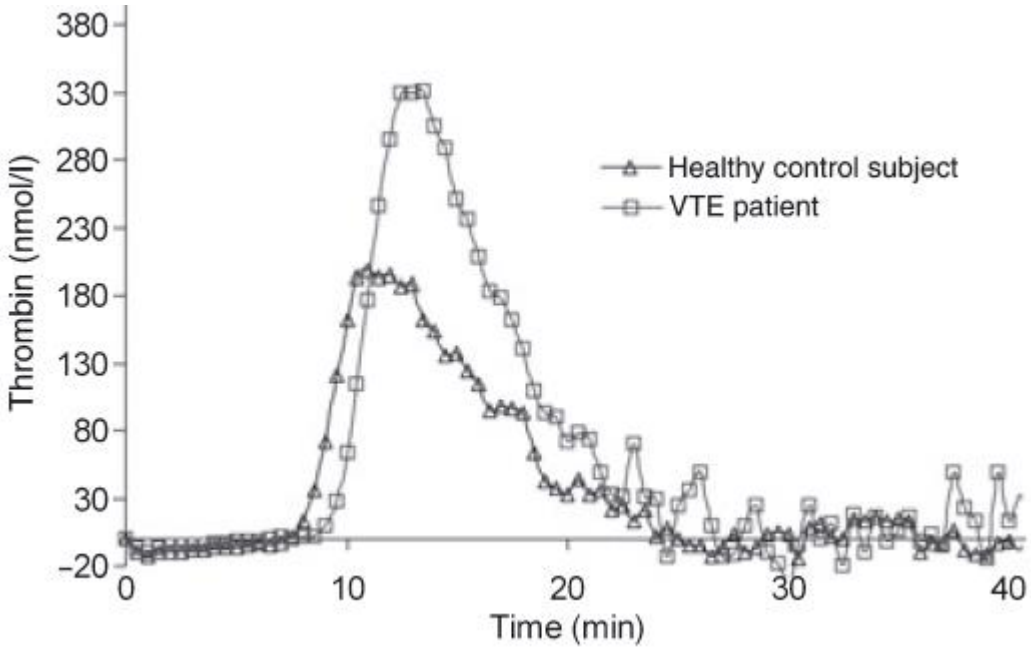


Table 10: The methods and objectives of ROTEM analysis[30].

TEST	METHOD	OBJECTIVE
Extem	Activation of clot formation with tissue factor	Assessment of factors VII, X, V, II, I, platelets and fibrinolysis
Intem	Activation of clot formation via the contact phase	Assessment of factors XII, XI, VII, X, V, II, I, platelets and fibrinolysis
Fibtem	Activation as in Extem with addition of a platelet-blocking substance	Fibrinogen function
Aptem	Activation as in Extem with addition of an antifibrinolytic (aprotinin)	Recognition of hyper-fibrinolysis
Heptem	Activation as with Intem with the addition of heparinise	Detection of heparin and heparin-related substances

Figure 4: A typical Sonoclot signature. (ACT= activated clotting time; CR= clot rate; PF= platelet function) [61]

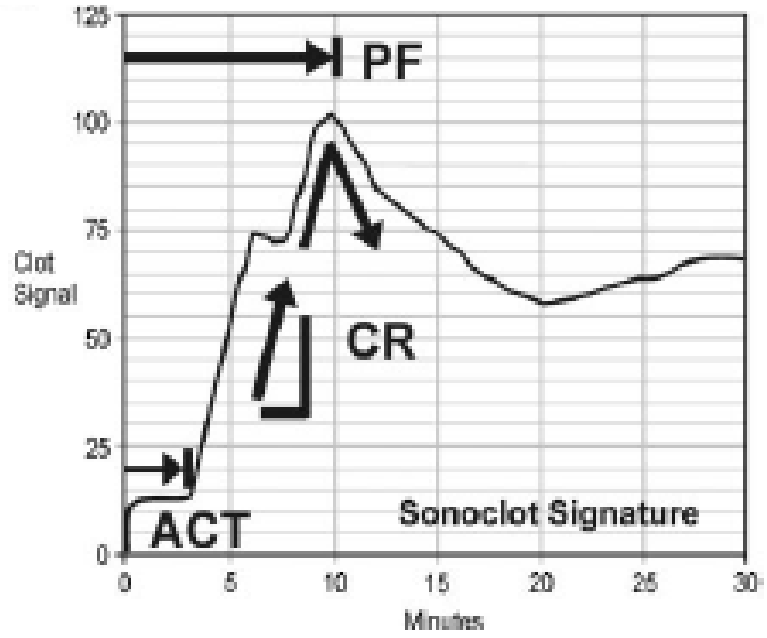
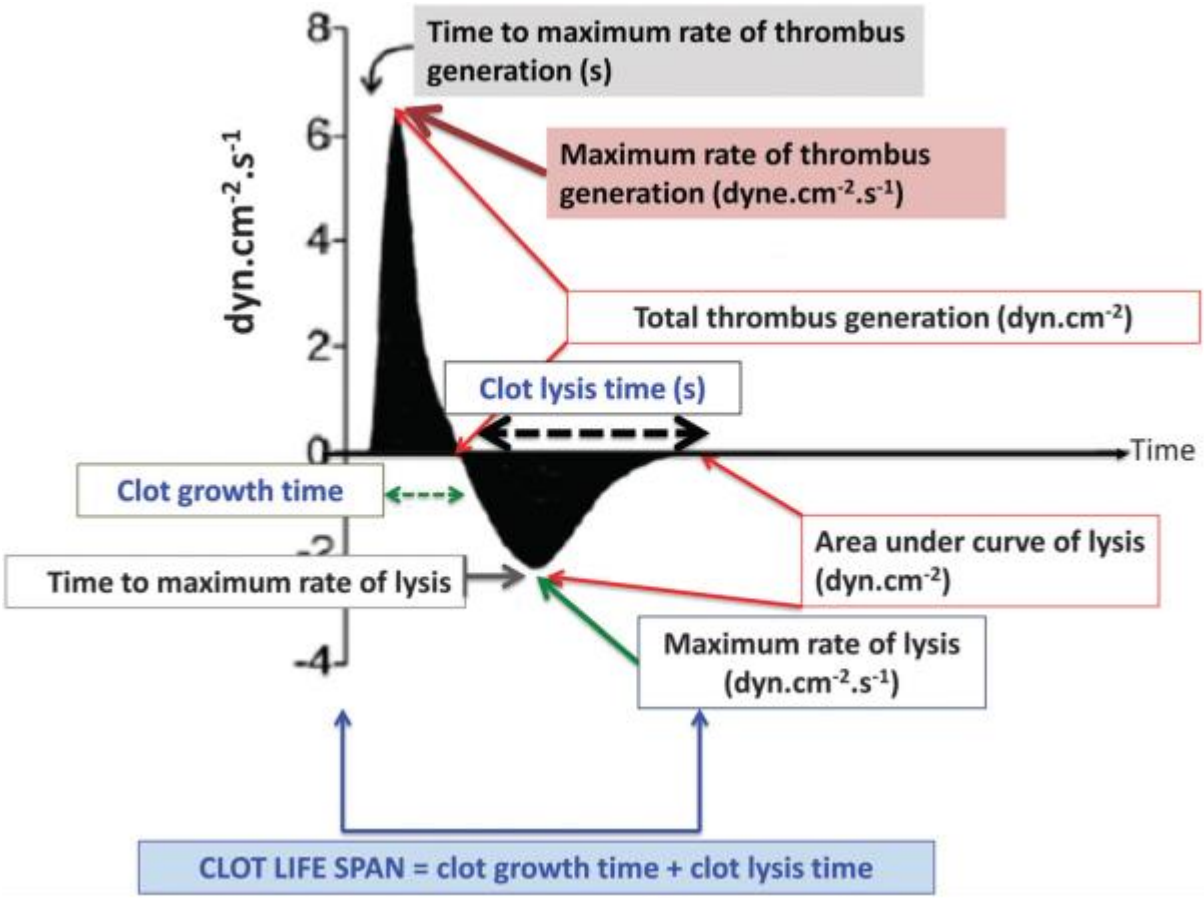


Figure 5: The clot lifespan model by Nielsen and colleagues[27]



The above paragraphs have dealt with the models and the cells affected by hypercoagulation/ inflammation. The next paragraphs will discuss the various circulating inflammatory molecules like iron, microparticles and the inflammatory cytokines.

2.11) **Inflammatory-coagulation link**

Inflammation is associated with oxidative stress which is reflected in the raised markers of inflammation. Inflammatory conditions with hypercoagulation has been documented in patients with thrombo-embolic stroke, diabetes, systemic lupus erythematosus, Parkinson's disease and Alzheimer-type dementias.[50, 51, 62, 63, 64]

Pro-inflammatory cytokines are associated with hypercoagulability esp. interleukin-6 which is involved in mediating both the coagulation and inflammation pathways[27]. Platelet production is increased by interleukin 6.[36] Interleukin-6 also stimulates the synthesis of fibrinogen by liver cells and other cells thereby influencing the concentration of this inflammatory marker (an acute-phase protein). Hypofibrinolysis is therefore closely linked with inflammation.[27]

The inflammatory system is also stimulated by the coagulation system. As mentioned previously, activated platelets are able to release pro-inflammatory cytokines too [specifically interleukin-1-beta, interleukin-7, interleukin-8, transforming growth factor (TGF)-beta and platelet activating factor (PAF)].[38] Activated platelets also release a pro-inflammatory-mediator CD40 ligand which induces the synthesis of tissue factor and stimulates inflammatory cytokine production such as IL-6 and IL-8.[36] The inflammatory system and the coagulation system therefore are involved in a positive cycle response.

2.12) **Fibrin and Iron**

Iron can cause abnormal clotting characteristics, dynamics and the kinetics in plasma clot formation. Iron molecules can alter the mechanical properties of the clot by interacting with fibrinogen resulting in changes in the fibrin fibre diameter, fibrin network pore size, fibrin network rigidity and fibrinolysis. These changes in the morphological structure of the fibrin clot may result in reduced fibrinolysis.[27]

Iron overload can cause spontaneous activation of non-enzymatic blood coagulation by precipitating plasma proteins which results in the formation of fibrin-like dense matted deposits (DMD). Inflammatory diseases typically has poorly liganded iron, which can interact with fibrin(ogen) during the formation of a clot. During the inflammatory process activation of IL-6, which is mediated by IL-1, also stimulates hepcidin biosynthesis which is linked with iron regulation.[65] Increase free iron (chelatable) levels may also be from damaged cells as a leakage product.[50]

The addition of ferric ions (Fe^{3+}) can cause these deposits which have been seen on scanning electron microscopy. These DMDs are resistant to proteolytic and chemical degradation.[66] In keeping with the effect of iron on the coagulation pathways is the result of iron-chelating agents which prevents DMDs formation in the plasma.[48]

Fibrin-like dense matted deposits are also associated with red blood cells, altering the morphology of their cell membranes to trap fibrin fibres. It was noted in whole blood smears with added iron (Fe^{3+}), as well as in diabetic patients, that more than 70% of red blood cells were tightly bound to fibrin fibres had a changed ultrastructure. Normally a minority (3%) of red blood cells are usually irreversibly trapped within the fibrin clot mesh. Such interactions seen in prothrombotic states, such as conditions of diabetes mellitus and stroke, affect clot properties, making it more resistant to fibrinolysis.[67]

2.13) **Microparticles/microvesicles**

Increased microparticles/microvesicles levels are seen in HIV infected patients. Microparticles are microscopic cellular remnants found circulating in the plasma. They appear to derive from CD4 lymphocytes, endothelial cells and platelets. Microparticles have an affinity for factor V. Microparticles derived from the endothelium are thought to contain von Willebrand factor.[68] Platelet-derived microparticles express several membrane proteins (glycoprotein (GP) Ib, platelet endothelium adhesion molecule-1, GPIIb-IIIa, P-selectin, CD63, CD41 and CD61). CD41 is a very specific marker for platelet-derived microparticles[51]. Microparticles appear to enhance thrombin generation which enhances the production of endothelial cytokine interleukin-6 that promotes tissue factor expression and enhances the systemic inflammatory response .[31, 36, 69]

2.14) **Deep Vein Thrombosis (DVT)**

The following paragraphs will review literature regarding DVT, as this occurrence is seen to be driven by a pathological coagulation process, involving an aberrant coagulation system, hyperactivated platelets and eryptotic RBCs.

Acute DVT is a common and potentially life-threatening condition. A complication of DVT is pulmonary embolism that commonly complicates the course of patients in hospital. Up to 30% of general surgery patients may develop a symptomatic or non-symptomatic DVT.[70] A systematic review of HIV positive patients in 2005 found a DVT incidence ranging from 0.19% to 18% whereas in the HIV negative population the DVT risk is reported to be approximately 0.0005% (5/10 000).[68] Embolization of DVT to the pulmonary arteries, results in pulmonary embolism, which is a severe and life-threatening complication. Up to 40% of patients with DVT can develop pulmonary embolism.[71]

As mentioned previously, the pathophysiology of DVTs can be classified by "Virchow's triad" which consists of venous endothelial damage, hypercoagulability and decreased venous flow.[2] Patients with HIV are more prone to a hypercoagulable state compared to HIV negative patients. Factors (Virchow's triad) predisposing to a procoagulable state have been documented in HIV infected patients

- antiphospholipid antibodies
- lupus anticoagulant
- von Willebrand factor
- d-dimers;

and deficiencies of

- protein C
- protein S
- heparin cofactor II

- anti-thrombin.

Elevated concentrations of activated platelets (raised levels of p-selectin, microparticles, and aminophospholipids) are present in HIV positive patients. With the addition of elevated levels of vWF these activated platelets easily aggregate and adhere to each other and the surrounding endothelium.[68]

There is a positive correlation, the more severe the viral immune suppression, the more severe the coagulation abnormalities. The risk of thrombosis can therefore also occur in uncommon sites and in those patients that do not have known risk factors for DVT.[14] This provides another challenge in an already difficult clinical situation.

2.15) **Screening tests for deep vein thrombosis**

Patients with symptomatic deep vein thrombosis present typically with a swollen oedematous, painful limb of recent onset. A typical suspicious history of immobility, previous DVT, malignancy, oral contraceptive use, etc. may be present, but some patients do not present with a typical history. A Well's clinical model of the patient having a DVT may assist in diagnosing the condition by assigning a score to predict the probability (**Table 11**).[72]

Table 11: Clinical model for predicting pre-test probability for DVT[73]

Clinical features	**Points**
Currently has cancer	1
Major surgery within 4 weeks/ bedridden for more than 3 days	1
Immobilization of the lower limbs	1
Deep venous system localized tenderness	1
Ipsilateral swollen leg	1
Ipsilateral leg swollen more than 3 cm compared to the contralateral leg	1
Non-varicose superficial veins	1
Oedema that is pitting	1
Previously diagnosed DVT	1
Alternative diagnosis as likely as DVT	-2
• 0 points or less: Low probability, ± 3% DVT risk. • 1-2 points: Moderate probability, ± 17% DVT risk. • 3 points or more: High probability, ± 75% DVT risk.	

DVT=Deep vein thrombosis

2.15.1) **D-dimer test**

D-dimers are fibrin degradation products produced by the action of plasmin on fibrin. Raised d-dimers are sensitive but not specific for DVT as they can rise in inflammatory conditions. Nonetheless, due to their high negative predictive value, it can assist the clinician to rule-out thromboembolism when used together with clinical probability and/or ultrasonography.[73]

2.15.2) **Definitive investigations for deep vein thrombosis**

a) **Invasive tests: Venography**

Contrast venography is the gold standard for diagnosing a DVT. Contrast venography is, however, an invasive procedure requiring available expertise. As contrast is used, the procedure therefore also has a risk of causing an allergic reaction. Another disadvantage is the risk of causing a DVT.[73]

b) **Non-invasive tests**

The best non-invasive cost-effective diagnostic method to diagnose deep vein thrombosis is ultrasonography. Ultrasonography compared to contrast venography has been shown to be an acceptable diagnostic tool with a 97% sensitivity and specificity.[73]

Three ultrasonography techniques are used to improve accuracy:

Compression ultrasound—The Ultrasonographer applies gentle pressure with the ultrasound probe over the vein. In the absence of a venous thrombosis, the vascular lumen is fully compressible. If the vascular lumen is not compressible, then it indicates a venous thrombus is present.[73]

Duplex ultrasonography—The same technique is used as with the conventional compression ultrasound with the addition of the pulsed Doppler signal, which produces the images to evaluate blood flow characteristics. Blood flow in normal veins with respiration has a phasic pattern, spontaneous and the flow pattern can be altered with distal compression. A venous thrombosis causes an outflow obstruction which results in the absence of the phasic pattern and the flow becomes continuous.[73]

Colour flow duplex imaging— the same method is used as with the Duplex ultrasonography with the addition of superimposing colour to the grey image. When a Doppler shift, change in the direction of the blood flow, is seen, a red and blue colour is each assigned to the 2 flow directions i.e. to or away from the ultrasound probe. The addition of colour assists to identify the veins easier.[73]

Spiral computed tomography venography or magnetic resonance imaging are also accurate at diagnosing deep vein thrombosis but due to costs are not likely to be used widely.[73, 74]

2.16) **Relevance and Motivation**

The knowledge and understanding of the role of HIV is improving and the medical field has to keep researching the effects of this disease. Hypercoagulable changes are present in the body before an actual thromboembolic event occurs. The importance of the investigations into the molecular and fibre-level origins of hypercoagulability have only recently been realized. Platelet ultrastructure in HIV positive patients have shown differences compared to HIV negative patients but previous study samples have been small. Besides the morphology and ultrastructure of platelets and fibrin fibres, the interaction of activated platelets with the fibrin fibres as well as the interactions with other blood cells, such as red blood cells and white blood cells, could have important implications of hypercoagulability.

Ultrastructural investigation should be used as a complementary investigation with the tests more commonly used for diagnosing coagulation abnormalities. This will result in a more complete method of understanding and diagnosing hypercoagulability and hypofibrinolysis. A predictive screening assessment of HIV patients could then possibly be determined for those at risk of a thromboembolic event.

Platelet ultrastructure has not been compared in HIV patients on ARV's which would improve understanding of the effect of ARV's on platelets functioning and coagulation. Studying the components of the coagulation system in HIV patients will assist in the assessment of HIV treatment strategies, i.e. antiretrovirals, on a hypercoagulable state.

Screening methods can be used for early pick-up of HIV patients developing thromboembolic disease. Treatment strategies may also be improved as well as monitoring of those strategies. A better understanding of hypercoagulability of HIV infected patients is therefore needed.

3) METHODOLOGY

3.1) Study Design

A descriptive comparative prospective study.

3.2) Research Site

Pretoria Tertiary hospitals:

- Steve Biko Academic Hospital
- Kalafong Hospital.

3.3) Subjects and Patient selection

3.3a) DVT group:

Inclusions:

- All **symptomatic** deep vein thrombosis patients that are admitted to hospital.
- Symptomatic deep vein thrombosis confirmed with Doppler Ultrasound or comparable imaging.
- 18 years or older
- Confirmed HIV status by HIV ELIZA 4th generation test.
- If HIV positive (in order to exclude opportunistic infections as a confounding factor):

  -World Health Organization Clinical stage 1; and

  - >170 CD4+ cells absolute number (per mm3)

- HIV patients on ARV: Only ARV combination of Emtricitabine (FTC), Tenofovir (TDF) and Efavirenz (EFA). (**Table 9**)

Exclusions:

- Patients less than 18 years of age (Paediatrics).
- Patients whose HIV status are not known or patients who refuse to undergo HIV testing.
- Patients currently on anticoagulation.
- HIV positive patients with moderate to severe disease that have increased risk of opportunistic infections:
  -World Health Organization Clinical stage 2 or higher ; or
  -World Health Organization Immunological staging <170 CD4+ cells absolute number (per mm3).
- HIV patients on ARVs: All ARV combinations excluding Emtricitabine (FTC), Tenofovir (TDF) and Efavirenz (EFA).
- Medical history suspicious of hereditary coagulation disorders, e.g. haemophilia, hereditary thrombophilia.

3.3b) Control group:

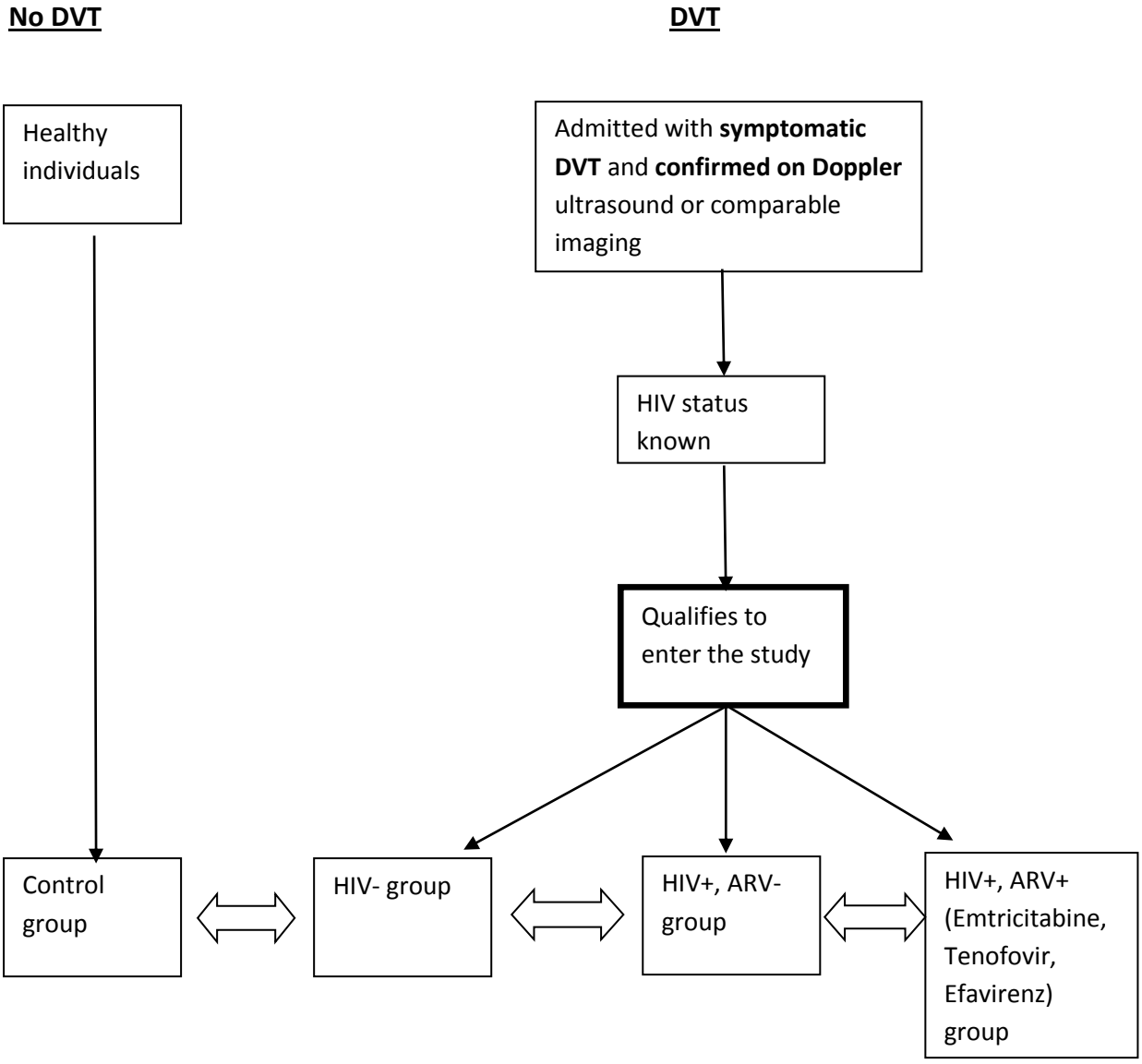
Inclusions:

- 18 years or older.
- Known HIV negative.
- No medical history of any chronic diseases.

Exclusions:

- Less than 18 years.
- Medical history of any chronic disease.
- Medical history suspicious of hereditary coagulation disorders or previously diagnosed with DVT.

Summary of Subjects and Patient selection



3.4) Materials and Methods

- Informed consent was taken from each patient (**Appendix 1**).
- Data Collection Form completed (**Appendix 2**)
- Five blood tubes of venous blood were withdrawn for full blood count (4ml EDTA tube-purple top), ESR (4ml EDTA tube-purple top), whole blood sample (4.5ml buffered tri-sodium citrate solution tube-blue top), fibrinogen levels (4.5ml buffered tri-sodium citrate solution tube-blue top), iron levels and CRP (5ml serum separator tube-yellow top).

3.5) Ethical Considerations

Ethics approval obtained from the University of Pretoria Ethics Committee. Ethics Reference Number: 547/2015.

Superintendent consent from the relevant hospitals was obtained to perform the study.

Informed consent from the patients was obtained. (**Appendix 1**)

3.6) Financing

Professor Etheresia Pretorius, Department of Physiology, sponsored all the laboratory cost (viscoelastic studies, light microscopy and SEM) from the National Research Fund.
Department of Surgery, Pretoria, sponsored all other costs not related to routine clinical management of the patients.

CHAPTER 4: HAEMATOLOGICAL MARKERS

Introduction

HIV is a retrovirus that targets the immune system and infects principally T lymphocyte cells, that bear the CD4 receptor (CD4+), and monocytes/macrophages destroying or impairing their function. An untreated HIV infection results in an exhausted and weakened immune system.[2,4] In 1984 Robert Gallo reported on the human immunodeficiency virus (HIV) and showed evidence that this virus was the cause of Acquired Immune Deficiency Syndrome (AIDS). In 1983 Luc Montagnier had isolated the virus in a patient with lymphadenopathy (enlarged pathological lymph nodes).[75] The severe stage of HIV infection is known as the AIDS.[2] AIDS has caused more than 25 million deaths globally with 2.6 million new infections per year.[3]

HIV infection has an effect directly or indirectly on the haematological system. HIV may decrease erythrocyte and platelet concentrations. These changes may be due to disrupted marrow production, increased removal or increase destruction of the cells.[76] Whether the HIV infected patient is on treatment with ARV medication or not, there is still an association with changes in the levels of circulating markers of anti-coagulation, coagulation, endothelial activation, fibrinolysis and platelet functioning.[77] Literature has shown conflicting reports on coagulation/anticoagulation status of HIV positive patients. Some studies report on a hypocoagulable state whereas others report on a hypercoagulable changes.[77, 78, 79, 80]

The risk of a venous thromboembolism in a HIV infected patient has been reported to be two to ten times compared to the general population.[81] Factors such as a low CD4 count, protein C deficiency and opportunistic infections appears to increase the risk of thromboembolism (**Table 12**). ARV therapy may improve the immune status of the HIV positive patient but has many side effects. Different ARV therapy combinations may have different effects on the coagulation system. Some studies report decreased coagulation abnormalities with ARV therapy.[21] Other studies have reported a higher risk of venous thromboembolism in HIV infected patients on ARV therapy.[79] However, in all these studies the ARV regimen was not standardised.

Table 12: HIV-related thromboembolism risk factors[81]

HIV-related hypercoagulable state	Protein C deficiency
	Protein S deficiency
	Antithrombin deficiency
	Antiphospholipid syndrome
	Tissue factor increase
	Homocysteine increase
	Microparticles increase
Viral risk factors	CD4 <200
	Detectable viral load
Opportunistic infections	Cytomegalovirus
	Tuberculosis
	Pneumocystis jiorveci
Endothelial dysfunctions	P-selectin
	Von Willebrand Factor
	Plasminogen activator inhibitor-1
HIV-related malignancies	Kaposi Sarcoma
	Non-Hodgkin Lymphoma
	Solid Tumours
Acquired traditional risk factors	Advancing age
	Obesity
	Previous thrombosis
	Cigarette smoking
	Immobilization
	Injecting drug abuse
	Pregnancy

Patients with a venous thrombosis are in a pro-inflammatory state even without HIV infection. A hypercoagulable state has been documented in other conditions of inflammation.[22, 82] Changes in serum iron concentrations has been reported to be associated with hypercoagulable states. Iron dysregulation has also been associated with multiple conditions where inflammation was the central process.[50, 51, 62, 63] Iron overload has been reported to spontaneously stimulate the coagulation system.[50]

With the addition of HIV infection in patients with a DVT, the inflammatory state of the body may be significantly raised to result in multiple haematological changes.[83-92] The NHLS reference ranges are shown in **Table 13.** Screening for a hypercoagulable state in HIV positive patients is not performed routinely until the patient presents with a venous thromboembolism.

The inflammatory changes may result in a different haematological profile between patients with a DVT and those without, as well as in patients with no HIV infection compared to patients with HIV infection. If this is possible, then using the haematological profile, or changes thereof, may lead to improved screening, diagnosis and management of DVT patients who are HIV negative or HIV positive. Thus the following objectives directs this chapter.

Table 13: National Health Laboratory Service reference ranges.[93]

Haematological Marker		**Reference ranges**
Full Blood Count (FBC)	White cell count (WCC)	(m) 3.92-10.40 x10^9/L (f) 3.9-12.6 x10^9/L
	Red cell count (RCC)	(m) 4.19-5.85 x10^{12}/L (f) 3.93-5.40 x10^{12}/L
	Haemoglobin (Hb)	(m) 13.4-17.5 g/dL (f) 11.6-16.4 g/dL
	Haematocrit (Hct)	(m) 0.39-0.51 L/L (f) 0.34-0.48 L/L
	Mean cell volume (MCV)	(m) 83.1-101.6 fL (f) 78.9-98.5 fL
	Mean corpuscular haemoglobin (MCH)	(m) 27.8-34.8 pg (f) 26.1-33.5 pg
	Mean corpuscular haemoglobin concentration (MCHC)	(m) 27.8-34.8 g/dL (f) 32.7-34.9 g/dL
	Red cell distribution width (RCDW)	(m) 12.1-16.3% (f) 12.4-17.3%
	Platelets count (Plt)	(m) 171-388 x 10^9/L (f) 186-454 x 10^9/L
	Mean platelet volume (MPV)	(m) 7.1-11.0 fL (f) 7.3-11.3 fL
Iron studies	Serum iron (Fe)	(m) (11.6-31.3 µmol/L (f) 9.0-30.4 µmol/L
	Transferrin	(m) 2.15-31.3 g/L (f) 2.50-3.80 g/L
	% Saturation	(m) 20-50% (f) 15-50%
	Ferritin	(m) 26-388 µg/L (f) 8-252 µg/L
C-reactive protein (CRP)		<10 mg/L
Erythrocyte sedimentation rate (ESR)		0-10 mm/hr
Fibrinogen		2-4 g/L

Chapter objectives

1. To compare inflammatory markers:
    - Iron (Iron saturation, transferrin and serum ferritin)
    - Fibrinogen
    - CRP
    - ESR
2. Compare haematological results between the groups.

Materials and Methods

Five blood tubes of venous blood were drawn for full blood count (4ml EDTA tube-purple top), ESR (4ml EDTA tube-purple top), whole blood sample (4.5ml buffered tri-sodium citrate solution tube-blue top), fibrinogen levels (4.5ml buffered tri-sodium citrate solution tube-blue top), iron levels and CRP (5ml serum separator tube-yellow top). The test tubes collected from each patient was sent to the National Health Laboratory Service (NHLS).

Objective 1

In order to answer the first objective, namely to compare inflammatory markers, 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 AAII-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 between the different groups.

Objective 2

The second objective is to compare haematological results between the groups. This was accomplished by using a haematology analyser to process venous blood in the EDTA tube. The haematology analyser is located in the department of physiology laboratory. Full blood count values which includes white cell count (and its differential count), red blood cell count, haemoglobin level, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), the mean corpuscular haemoglobin concentration (MCHC), platelet count and mean platelet volume (MPV).

RESULTS

Table 14: Demographics

Groups	N	Male	Female	Age	CD4 count
Controls	25	7	18	29	N/A
HIV-	35	11	24	52	N/A
HIV+	15	2	13	39	353

There were 25 healthy volunteers recruited for the control group, 35 patients recruited in the HIV negative group with a DVT and 15 patients recruited in the HIV positive group with a DVT (**Table 14**). The HIV positive patients consisted of 2 patients not on ARV's and 13 patients that were on the primary ARV regimen (Emtricitabine, Tenofovir and Efavirenz). All 3 groups consisted with a majority of females. The median age of 29 in the control group, 52 in the HIV negative group with DVT and 39 in the HIV positive group. The median CD4 count in the HIV positive patients was 353.

The haematological markers in the control, HIV negative with DVT and HIV positive with DVT groups are shown in **Tables 15 to 17**. The Tables shows the number of patients, the median, minimum, maximum values and the standard deviation of each haematological marker. For the comparison between groups, **Tables 18-20**, p-values were calculated using the Mann-Whitney test with a significance level of 0.05.

Table 15: Control group haematological markers

Haematological marker	N	Median	Minimum	Maximum	Standard deviation
WCC (x10^9/L)	24	5.98	3.17	11.01	1.963
RCC (x10^{12}/L)	24	4.89	4.17	5.68	0.4405
Hb (g/dL)	24	14.45	10.3	16.4	1.436
Hct (L/L)	24	0.444	0.356	0.543	0.04392
MCV (fL)	24	92.05	74	108.2	6.916
MCH (pg)	24	29.9	21.6	32.5	2.368
MCHC (g/dL)	24	32.2	24.5	35	2.251
RCDW (%)	24	14.1	11.5	16.9	1.493
Platelets (x 10^9/L)	24	247	154	469	63.99
MPV (fL)	24	9	6.9	10.9	1.032
Serum iron (µmol/L)	23	17.7	3	33	7.587
Transferrin (g/L)	23	2.54	2.1	4.49	0.6391
% Saturation (%)	20	25	3	53	12.34
Ferritin (µg/L)	22	82.5	3	412	99.15
CRP (mg/L)	24	1.5	1	47	9.422
ESR (mm/hr)	20	4	1	25	5.432
Fibrinogen (g/L)	23	2.6	1.7	4.3	0.6412

Table 16: HIV negative group with DVT haematological markers

Haematological marker	N	Median	Minimum	Maximum	Standard deviation
WCC ($x10^9$/L)	35	8.26	4.55	13.73	2.375
RCC ($x10^{12}$/L)	35	4.4	2.19	6.98	0.9182
Hb (g/dL)	35	12.7	4.6	18.5	3.23
Hct (L/L)	35	0.393	0.175	0.617	0.09156
MCV (fL)	35	91.7	57.7	103.8	7.854
MCH (pg)	35	29.3	15.3	36	3.705
MCHC (g/dL)	35	32.4	26.2	35.5	2.172
RCDW (%)	35	14	11.9	22.1	2.371
Platelets (x 10^9/L)	35	268	36	690	128.2
MPV (fL)	35	8.6	7.1	11.4	1.089
Serum iron (µmol/L)	30	7.2	2	29	6.279
Transferrin (g/L)	28	2.27	0.99	3.89	0.6489
% Saturation (%)	27	12	3	97	19.31
Ferritin (µg/L)	26	96	1.3	1101	239.7
CRP (mg/L)	32	47	1	193	59.13
ESR (mm/hr)	32	13	2	139	36.59
Fibrinogen (g/L)	32	3.7	1	7	1.258

Table 17: HIV positive group with DVT haematological markers

Haematological marker	N	Median	Minimum	Maximum	Standard deviation
WCC (x10^9/L)	14	8.285	3.98	33.43	7.739
RCC (x10^{12}/L)	14	3.57	2.2	4.99	0.8327
Hb (g/dL)	14	9.75	4.1	16.7	3.518
Hct (L/L)	14	0.308	0.159	0.517	0.09656
MCV (fL)	14	86.85	63.1	103.6	10.53
MCH (pg)	14	26.95	15.5	33.5	5.054
MCHC (g/dL)	14	30.95	23.3	33.9	3.075
RCDW (%)	14	16.5	12.1	25.6	3.848
Platelets (x 10^9/L)	14	316	149	796	171.8
MPV (fL)	14	8.6	7.9	10.8	0.7564
Serum iron (µmol/L)	12	5	1.8	13	3.335
Transferrin (g/L)	11	1.79	0.59	3.22	0.774
% Saturation (%)	10	11	2	88	25.18
Ferritin (µg/L)	11	239	7	487	150.1
CRP (mg/L)	14	109.5	7	245	78.09
ESR (mm/hr)	12	36	9	116	36.83
Fibrinogen (g/L)	13	3.3	1.5	5.3	1.221

Table 18 compares the haematological markers in the control group with the HIV negative group patients that have a DVT. The HIV negative group had statistically significant elevated inflammatory markers which includes WCC (p-value of 0.0026), CRP (p-value of <0.0001), ESR (p-value of 0.0006) and fibrinogen (p-value of 0.0014). The HIV negative group had statistically significant decrease in RCC, Hb and Hct with p-values of 0.0025, 0.0063 and 0.0018 respectively. The MCV, MCH, MCHC, RCDW, platelets and MPV all had non-significant changes compared to the control group. The serum iron, transferrin and percentage of iron saturation had a statistically significant decrease with p-values of <0.0001, 0.0158 and 0.0007 respectively, with a non-statistically significant rise in the serum ferritin level.

Table 18: Control group vs. HIV negative DVT group

Haematological marker	Controls	HIV-	P-value	Significant difference
WCC ($x10^9$/L)	5.98	8.26	0.0026	Yes
RCC ($x10^{12}$/L)	4.89	4.4	0.0025	Yes
Hb (g/dL)	14.45	12.7	0.0063	Yes
Hct (L/L)	0.444	0.393	0.0018	Yes
MCV (fL)	92.05	91.7	0.4682	No
MCH (pg)	29.9	29.3	0.4266	No
MCHC (g/dL)	32.2	32.4	0.2346	No
RCDW (%)	14.1	14	0.2501	No
Platelets (x 10^9/L)	247	268	0.445	No
MPV (fL)	9	8.6	0.2265	No
Serum iron (µmol/L)	17.7	7.2	< 0.0001	Yes
Transferrin (g/L)	2.54	2.27	0.0158	Yes
% Saturation (%)	25	12	0.0007	Yes
Ferritin (µg/L)	82.5	96	0.2032	No
CRP (mg/L)	1.5	47	< 0.0001	Yes
ESR (mm/hr)	4	13	0.0006	Yes
Fibrinogen (g/L)	2.6	3.7	0.0014	Yes

Table 19 compares the haematological markers in the control group with the HIV positive group patients that had a diagnosed DVT. As with the HIV negative group, the HIV positive group also had statistically significant increase in the inflammatory markers which included WCC (p-value of 0.012), CRP (p-value of < 0.0001) and ESR (p-value of < 0.0001). However the fibrinogen concentration, although raised, was not statistically significant (p-value of 0.0672). The HIV positive group had a decrease concentrations of RCC, Hb and Hct which were statistically significant, with p-values of <0.0001, <0.0001 and <0.0001 respectively. This anaemic picture correlates with the low MCV (p-value of 0.0206) and MCH (p-value of 0.0016), which is also in keeping with the low serum iron (p-value of <0.0001), transferrin (p-value of 0.001) and percentage of iron saturation (p-value of 0.0059). The MCHC however did not demonstrate a significant change. In contrast to the decreased serum iron and transferrin, the serum ferritin concentration had a statistically significant increase with a p-value of 0.0231. The RCDW was significantly raised with a p-value of 0.0035. The platelet count and the MPV did not show a significant change compared to the control group.

Table 19: Control group vs. HIV positive DVT group

Haematological marker	Controls	HIV+	P-value	Significant difference
WCC ($x10^9$/L)	5.98	8.285	0.012	Yes
RCC ($x10^{12}$/L)	4.89	3.57	< 0.0001	Yes
Hb (g/dL)	14.45	9.75	< 0.0001	Yes
Hct (L/L)	0.444	0.308	< 0.0001	Yes
MCV (fL)	92.05	86.85	0.0206	Yes
MCH (pg)	29.9	26.95	0.0016	Yes
MCHC (g/dL)	32.2	30.95	0.1878	No
RCDW (%)	14.1	16.5	0.0035	Yes
Platelets (x 10^9/L)	247	316	0.0718	No
MPV (fL)	9	8.6	0.6599	No
Serum iron (µmol/L)	17.7	5	< 0.0001	Yes
Transferrin (g/L)	2.54	1.79	0.001	Yes
% Saturation (%)	25	11	0.0059	Yes
Ferritin (µg/L)	82.5	239	0.0231	Yes
CRP (mg/L)	1.5	109.5	< 0.0001	Yes
ESR (mm/hr)	4	36	< 0.0001	Yes
Fibrinogen (g/L)	2.6	3.3	0.0672	No

Table 20 compares the two study DVT groups, HIV negative and HIV positive groups. There were no significant differences in the inflammatory markers of WCC, ESR and fibrinogen. However CRP had a statistically significant rise in the HIV positive group compared to the HIV negative group with a p-value of 0.0389. There was a statistically significantly decrease in the RCC, Hb, Hct, MCV, MCH, MCHC and RCDW with p-values of 0.0163, 0.0061, 0.0124, 0.0261, 0.0043, 0.026 and 0.0284 respectively. The platelet count and MPV had no significant difference in the HIV positive and HIV negative groups. The serum iron, percentage of iron saturation and serum ferritin concentrations showed no significant difference. However, the transferrin concentration was significantly decreased in the HIV positive group.

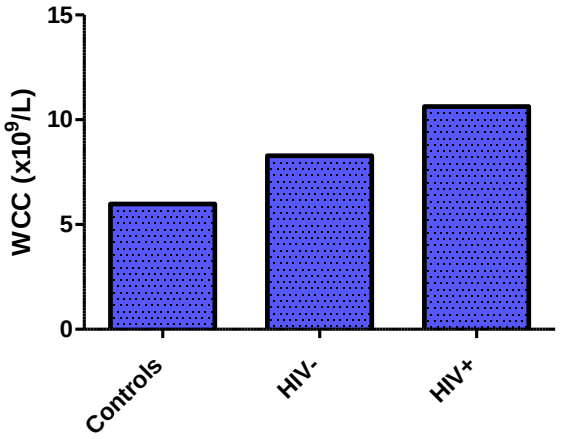
Table 20: HIV negative DVT group vs. HIV positive DVT group

Haematological marker	**HIV-**	**HIV+**	**P-value**	**Significant difference**
WCC ($x10^9$/L)	8.26	8.285	0.7651	No
RCC ($x10^{12}$/L)	4.4	3.57	0.0163	Yes
Hb (g/dL)	12.7	9.75	0.0061	Yes
Hct (L/L)	0.393	0.308	0.0124	Yes
MCV (fL)	91.7	86.85	0.0261	Yes
MCH (pg)	29.3	26.95	0.0043	Yes
MCHC (g/dL)	32.4	30.95	0.026	Yes
RCDW (%)	14	16.5	0.0284	Yes
Platelets (x 10^9/L)	268	316	0.2931	No
MPV (fL)	8.6	8.6	0.4121	No
Serum iron (µmol/L)	7.2	5	0.1253	No
Transferrin (g/L)	2.27	1.79	0.0379	Yes
% Saturation (%)	12	11	0.4822	No
Ferritin (µg/L)	96	239	0.173	No
CRP (mg/L)	47	109.5	0.0389	Yes
ESR (mm/hr)	13	36	0.1046	No
Fibrinogen (g/L)	3.7	3.3	0.841	No

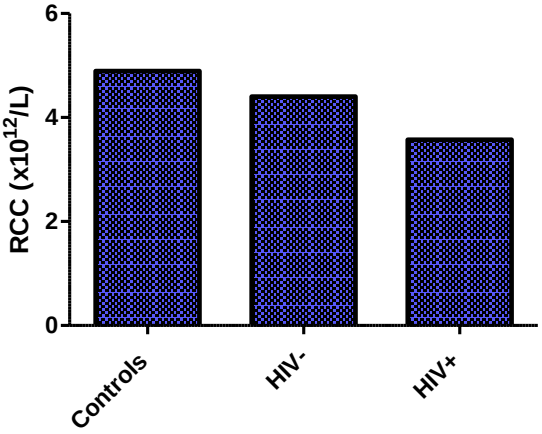
Graph 1 demonstrates the comparison of the haematological marker between the 3 groups.

Graph 1: Bar graph comparison of haematological markers

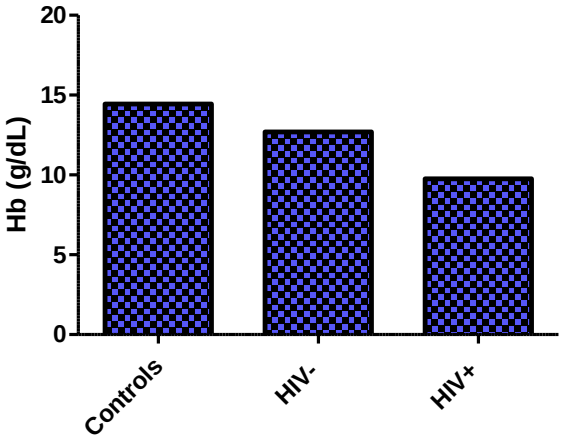
A) White cell count



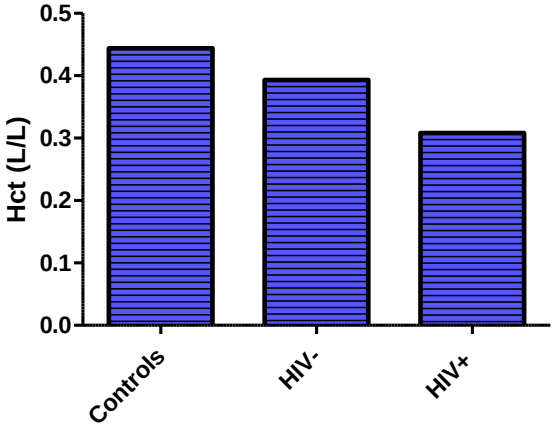
B) Red cell count



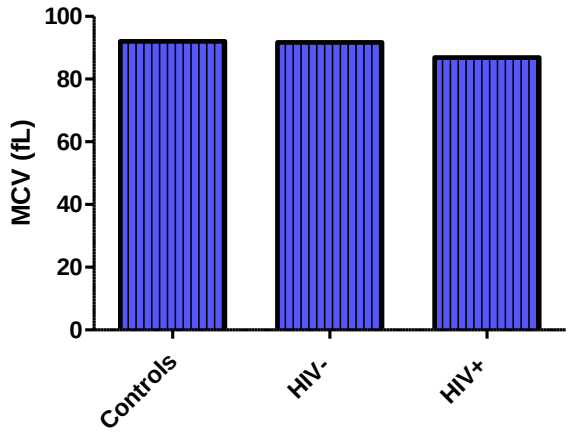
C) Haemoglobin



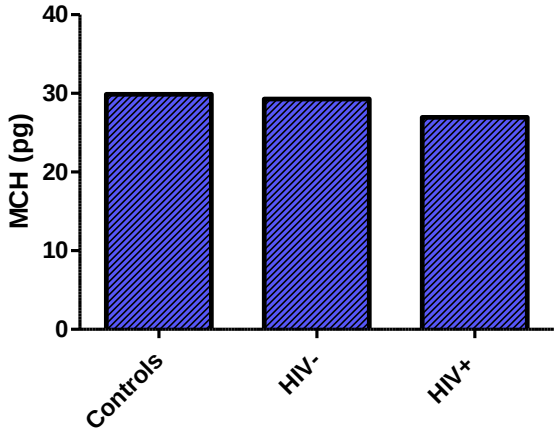
D) Haematocrit



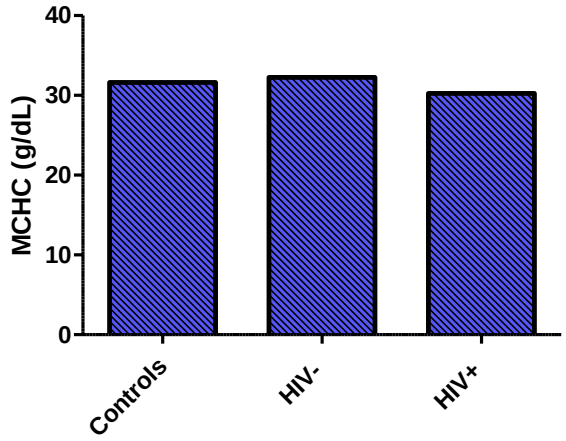
E) Mean cell volume



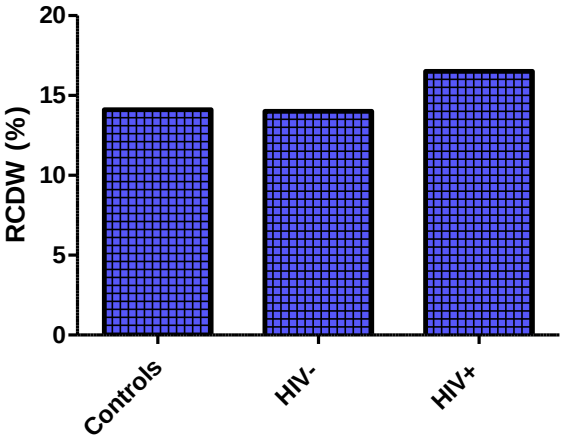
F) Mean cell haemoglobin



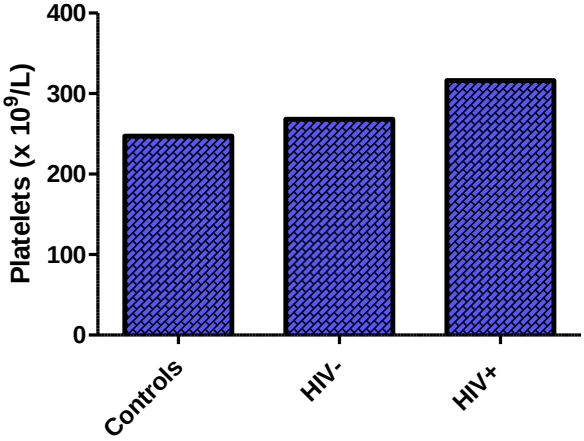
G) Mean cell haemoglobin concentration



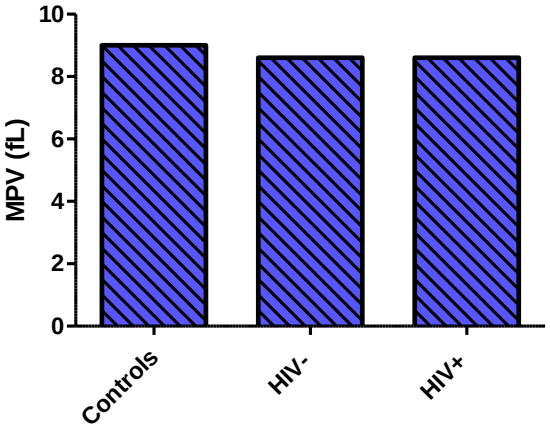
H) Red cell distribution width



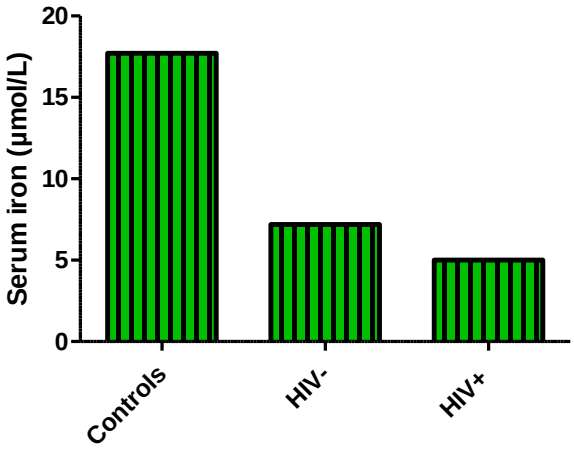
I) Platelet count



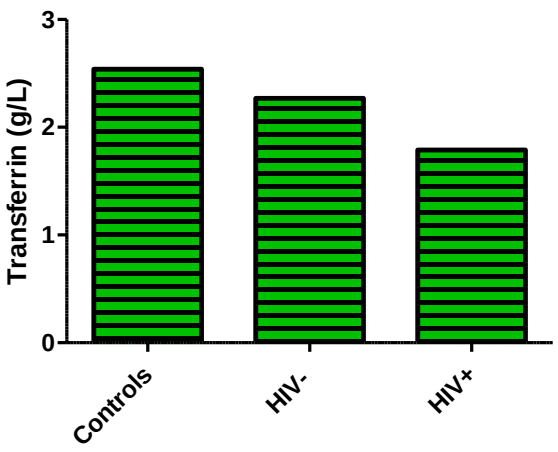
J) Mean platelet volume



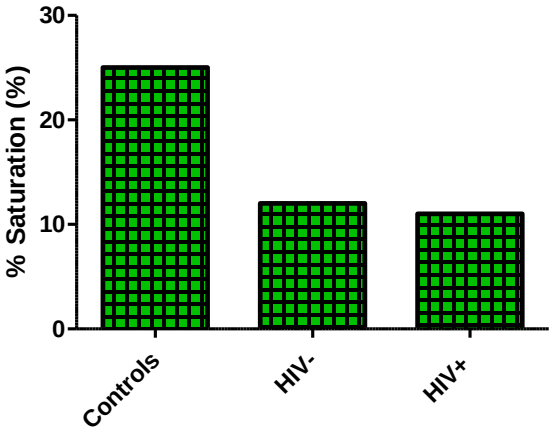
K) Serum iron concentration



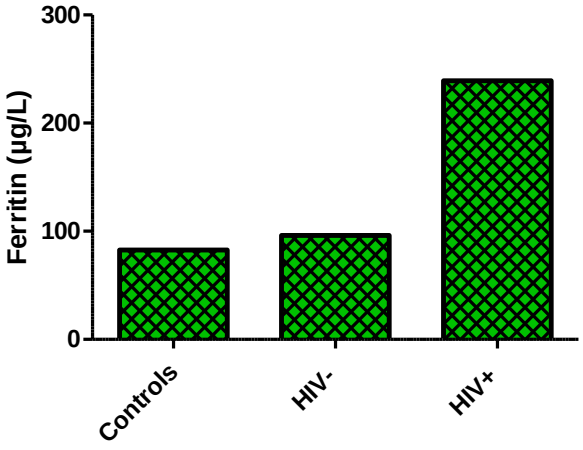
L) Transferrin concentration



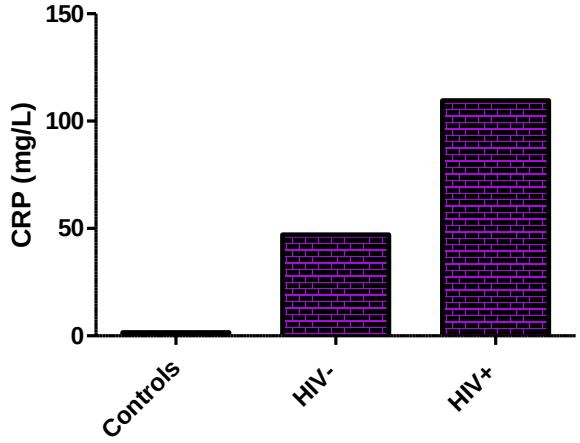
M) Percentage iron saturation



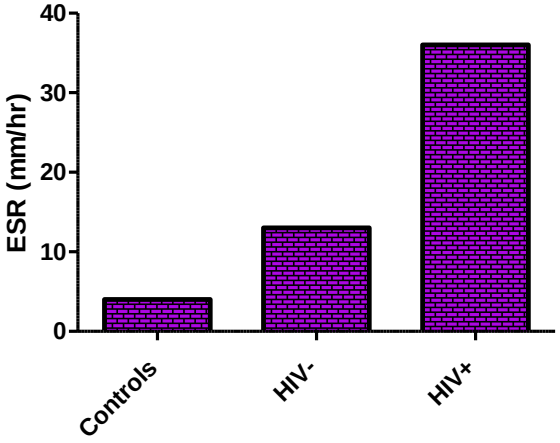
N) Serum ferritin concentration



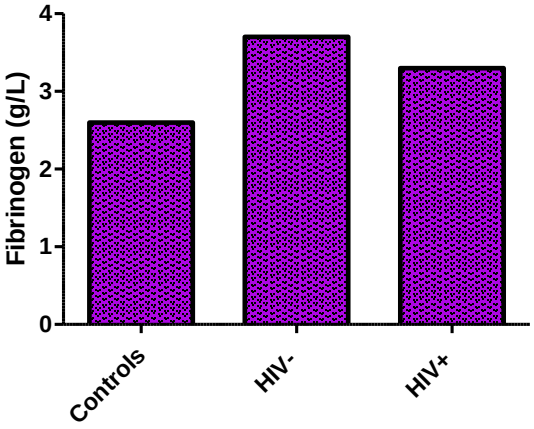
O) C-reactive protein concentration



P) Erythrocyte sedimentation rate



Q) Fibrinogen concentration



DISCUSSION

Anaemia

Both DVT groups, HIV- and HIV+ groups, have a significantly lower red cell count (RCC), haemoglobin (Hb) and haematocrit [the volume of blood that contains erythrocytes when packed by centrifugation (Hct)] levels than the control group indicating an anaemia in the HIV- and HIV+ groups (**Table 18 and 19**; and **Graph 1**). The values of these 3 parameters in the HIV- group, even though lower than the control group, are still within the reference ranges (**Table 13**) indicating a relative anaemia. The RCC, Hb and Hct in the HIV+ group is significantly lower than the HIV negative and control groups; and lower than the reference ranges indicating a true anaemia.

Anaemia is commonly found in HIV positive patients. The cause of the anaemia is not always clear.[76, 94-100] An inadequate erythropoietin feedback mechanism is suspected to be a major contributor in HIV related anaemia. The low reticulocyte count is commonly found with associated polychromasia (abnormally high number of immature red blood cells) indicating an under-producing bone marrow.[76, 101-104] Other factors that contribute to HIV associated anaemia includes intestinal malabsorption, autoimmune haemolysis, bone marrow malignancies, blood loss, opportunistic complications, etc.[76, 105, 106]

Even with the significantly decreased RCC, Hb and Hct levels in the HIV negative DVT group, the control group and the HIV- groups have no significant difference with the comparative values of Mean cell volume (MCV), Mean corpuscular haemoglobin (MCH) and Mean corpuscular haemoglobin concentration (MCHC) (**Table 18** and **Graph 1**). The changes in the HIV- group are in keeping with anaemia of inflammation, previously known as anaemia of chronic disorders.[107] This type of anaemia is common in conditions of acute inflammation, in this case, the response to the DVT**.** The mechanisms involved in the anaemia of inflammation are due to decreased availability of iron, decreased erythropoietin production, inadequate response from the bone marrow to erythropoietin and increased clearance of RBCs.[107] These mechanisms are in keeping with the decreased serum iron, transferrin and iron saturation concentrations but a normal ferritin concentrations seen in the HIV negative DVT group.

There has been documented association with venous thrombo-embolism and low serum iron levels or iron deficiency anaemia but the pathogenesis is unclear.[108] Potential explanations for the association include iron deficiency stimulating a thrombocytosis, which is probably unlikely for a venous thrombosis, as in this study, as venous clots are coagulation factor/fibrin based.[109, 110] Another explanation for the association is the iron deficiency increasing plasminogen activator inhibitor-1.[109, 111] Both explanations imply the iron deficiency caused the thromboembolism. On the other hand, if the thromboembolic phenomena caused the iron deficiency, then possibly a consumptive mechanism from the thrombosis may be involved.

Iron

Iron deficiency appears to be the main contributor to anaemia in the HIV+ group. The HIV+ group has significantly decrease in MCV, MCH and MCHC values when compared to the control group (**Table 19**) and the HIV- groups (**Table 20**). This indicates that the HIV+ group appears to have a microcytic (decrease erythrocyte size) hypochromic (decreased erythrocyte haemoglobin content) anaemia.[112] This is in keeping with the low serum iron levels in the HIV+ group. HIV is known to contribute to iron deficiency anaemia.[113, 114]

A low serum iron, transferrin and iron saturation percentage but with a raised ferritin level seen in the HIV positive group are not uncommon in patients infected with HIV.[105, 115, 116] Ferritin is an intracellular structure capable of storing iron atoms.[117] The concentration of serum ferritin is related to the reticuloendothelial iron stores.[118]

A low serum iron level with a raised ferritin level in the HIV group can be explained by an immunologically altered iron metabolism where the body has adequate or increased iron stores but are unable to utilize those stores.[115, 119, 120, 121] Hepcidin is a 25 amino acid peptide hormone involved with iron distribution at the cellular level.[122] Raised hepcidin concentrations are associated with inflammatory conditions, like HIV infective state. The elevated hepcidin results in inhibition of iron efflux from duodenal enterocytes and circulating iron is redistributed intracellularly, specifically macrophages.[123] The result is a decrease in serum iron and transferrin but an increase in ferritin levels. This functional iron deficiency can be considered a host defence mechanism by withholding iron from possible

pathogens.[124, 125] However, as iron is required for normal immune function, iron deficiency can also increase the risk of infection.[125] Chang et al. reported a study with different findings where HIV positive patients had increased serum iron and transferrin saturation levels. Their results were based on 10 HIV positive patients though. Interestingly, with the addition of different ARV combination regimens, there were no effect on the iron derangements on these 10 HIV patients that were present before ARV therapy was started.[126] ARV therapy therefore does not appear to influence iron studies. This is an important finding as our study's HIV positive group was a combination of patients with and without ARV therapy.

Increased serum iron tends to upregulate prostanoid signalling molecules, e.g. thromboxane, which increases platelet activation resulting in a hypercoagulable state.[124] Serum iron levels were actually decreased in the study group indicating that the hypercoagulable state is probably not from the influence of serum iron.

Serum iron levels are usually reflected by the serum ferritin levels. Ferritin is not synthesised in the serum but yet it is present in the serum. Kell and Pretorius hypothesised that ferritin leaks into the serum with cellular damage. Serum ferritin lacks most of the iron it contained intracellularly.[117] There is usually a correlation between disease status, in this case HIV infection, and the serum ferritin level. In the HIV+ group the serum ferritin levels are significantly raised compared to the control group but the serum iron levels are decreased (**Table 19**). Serum ferritin and iron concentrations have acute phase responses to inflammation.[118] This is in keeping with the view that serum ferritin is a better marker of inflammation than iron status.[117] During the inflammatory process, iron concentrations may decrease with ferritin remaining normal or increasing independently of the reticuloendothial stores. This may explain the changes in iron concentration and serum ferritin in the HIV- and HIV+ groups.[118]

Red cell distribution width

The red cell distribution width (RCDW) is the coefficient of variation of erythrocyte volume. The higher the value, the more anisocytosis (unequal erythrocyte sizes) present.[127] The RCDW of the HIV positive group (16.5 $x10^{12}$/L) was significantly greater that the control (14.1 $x10^{12}$/L) and HIV negative (14 $x10^{12}$/L) DVT groups, with a p value of 0.0035 and 0.0284

respectively (**Table 19** and **Table 20**). A raised RCDW is associated with a decrease in haemoglobin and MCV concentration; but with a raised white cell count, fibrinogen and C-reactive protein (CRP).[127] This correlates with the haematological markers found in the HIV positive group (**Table 17**). RCDW is strongly associated with mortality. Patel and colleagues reported the all-cause mortality risk increases by 22% for every 1% increase in RCDW. The physiological association between RCDW and mortality have been hypothesised to be related to the systemic factors involved in inflammatory conditions and oxidative stress which affects erythrocyte maturation and degradation.[127, 128, 129]

Inflammatory markers

The HIV- (8.28 x10^9/L) and HIV+ (8.285 x10^9/L) groups both had an elevated leukocyte count compared to the control group (5.98 x109/L), with p-values of 0.0026 and 0.0120 respectively (**Table 18** and **19**). Similar to patients with ischemic strokes, thromboembolism results in an inflammatory reaction with raised leukocyte count and C-reactive protein.[22, 82] HIV is usually associated with a decreased immune function**.** The elevated leukocyte count in these HIV positive patients may be due to the HIV infection itself, or the action of the ARV's or to opportunistic infections, regardless of whether the patient has a DVT or not. However, the risk of coexisting opportunistic infections/inflammatory conditions was minimised or absent by excluding patients that are classified as a WHO clinical stage 2 to 4. Also, with the ARV treatment the HIV virus is suppressed and the lymphocytes, particularly the CD4 lymphocytes, increase in turnover.[130, 131, 132]

C-reactive protein (CRP) is an acute-phase protein.[133] The concentration of CRP is increased by cytokines that are proinflammatory, such as interleukin 1 and 6.[134] The HIV negative group has an inflammatory response to the DVT which is reflected by the statistically significant raised CRP levels (47mg/L) compared to the control group, with a p-value of <0.0001 (**Table 18**). The same argument can be made for the raised CRP in the HIV positive group, however the CRP concentration (as well as fibrinogen) is commonly raised in HIV positive patients compared to the general population even without a DVT.[135-139] The raised CRP in HIV positive patients (**Table 19**) indicates a sustained acute phase response.[137] This is statistically significant in the HIV positive group (109.5mg/L) compared to the control group (1.5mg/L) as well as the HIV negative DVT groups (47mg/L), p-values of <0.0001 and 0.0389

respectively. Increasing CRP concentrations have been reported with HIV disease progression, and this increase does not appear to be affected by ARV treatment.[134] 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.[140, 141]

Fibrinogen, a high molecular weight plasma protein, levels influences the ESR. Increased fibrinogen levels are associated with thrombo-embolic events. Fibrinogen tends to adhere to the erythrocytes, which becomes "heavier" resulting in an increased sedimentation rate and blood viscosity.[94, 142-145] The fibrinogen levels, although within the recommended normal range, were greater in the HIV negative (3.7g/L) and HIV positive (3.3g/L) DVT groups compared to the control (2.6g/L) group, with p values of 0.0014 and 0.0672 respectively (**Table 18** and **19**; **Graph 1**). Fibrinogen concentration increases in the inflammatory response, which can explain the raised fibrinogen concentration in the HIV positive group which contributed to the DVT. Whether the HIV positive group was on ARV medication or not has been shown not to affect the fibrinogen concentration.[146] The HIV negative 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 increase in fibrinogen concentration correlates with the increased erythrocyte sedimentation rate (ESR) in the HIV negative and positive DVT groups.

The ESR is the extent in which erythrocytes sediment in one hour.[147] The ESR in both the HIV negative (13mm/hr) and HIV positive (36mm/hr) DVT groups were significantly raised compared to the control group (4mm/hr), p values of 0.0006 and <0.0001 respectively (**Table 18** and **19**). In inflammatory conditions the ESR rises as the erythrocytes become sticky and adhere to each other which can be seen as rouleaux formation.[148, 149, 150] The rouleaux formation in the HIV positive group can be seen under the scanning electron microscope, which will be discussed in the following chapter.

Considering all the inflammatory markers (WCC, fibrinogen, CRP and ESR), each marker was statistically significantly raised in the HIV negative DVT group compared with the control group, as well as the HIV positive group compared with the control group (**Table 18** and **19; Graph 1**). Interestingly, CRP is the only inflammatory marker that has a statistically

significant increase in the HIV positive group compared to the HIV negative group (**Table 20**). CRP may therefore be a more sensitive acute phase marker to differentiate significant an inflammatory condition between HIV negative and HIV positive patients.

Platelets

Thrombocytopenia is commonly found in HIV positive patients. This may be due to increased destruction or decrease production of platelet cells. Osime and colleagues reported a decrease in the platelet count and the platelet cell volume in patients on ARV's compared to HIV patients not on ARV's, although the decrease was not statistically significant.[146] However in this study, both the HIV negative and HIV positive DVT groups had a non-statistically significant rise in the platelet count. Platelet count is not always associated with an increased risk of DVT though.[151]

Mean platelet volume (MPV) is a common platelet activation marker.[152-157] Platelets tend to enlarge when activated**.**[151, 158] An elevated MPV is associated with low-grade inflammation as well as thrombosis.[36, 159] However both HIV negative (8.6fL) and HIV positive (8.6fL) DVT groups also had a non-statistically significant slight decrease in the mean platelet volume compared to the control group (9fL) (**Table 18** and **19**). These results indicate that the platelets in both groups were not activated to a significant degree to cause a change in the MPV. This is in keeping with a venous thrombosis where the thrombus is due to activation of the coagulation cascade instead of platelets.[110] It should also be kept in mind that platelets shape and volume do vary, even in healthy persons.[158]

Conclusion

There are haematological changes between the groups as seen in the simplified summary (**Table 21**). The DVT groups, i.e. the HIV negative and HIV positive groups, both have anaemia. The cause of the anaemia appears to be due to different causes. The HIV negative groups appears to have an anaemia of inflammation (anaemia of chronic disorders) whereas the HIV positive group is in keeping with an iron deficiency anaemia. The changes in serum iron, transferrin and ferritin reflects systemic iron status but serum ferritin appears to be a better marker of inflammation than iron status. The red cell distribution width is an indication of the inflammatory process on the erythrocytes but also increased mortality. The inflammatory process, whether from the DVT or the HIV infection, is reflected by the raised white cell count, fibrinogen concentration, C-reactive protein and the erythrocyte sedimentation rate. However considering these inflammatory markers, only CRP was statistically significant raised in the HIV positive group compared to the HIV negative group. Surprisingly, the platelet count was not decreased in the HIV positive group as expected where HIV thrombocytopaenia is usually common. Even the mean platelet volume did not reflect an increase in platelet activation.

There are definite haematological changes seen in all patients with deep vein thrombosis. With the addition of the HIV infection (and possibly the antiretroviral medication), different haematological changes are reflected.

Table 21: Simplified Summary of Haematological markers

Haematological marker	Control	HIV-	HIV+
WCC	↔	↑	↑
RCC	↔	↓	↓
Hb	↔	↓	↓
Hct	↔	↓	↓
MCV	↔	↓ (ns)	↓
MCH	↔	↓ (ns)	↓
MCHC	↔	↑ (ns)	↓ (ns)
RCDW	↔	↓ (ns)	↑
Platelets	↔	↑ (ns)	↑ (ns)
MPV	↔	↓ (ns)	↓ (ns)
Serum iron	↔	↓	↓
Transferrin	↔	↓	↓
% Saturation	↔	↓	↓
Ferritin	↔	↑ (ns)	↑
CRP	↔	↑	↑
ESR	↔	↑	↑
Fibrinogen	↔	↑	↑

ns = not statistically significant

CHAPTER 5: VISCOELASTIC, LIGHT MICROSCOPY AND ULTRASTRUCTURAL PROPERTIES

Introduction

There is a relationship between coagulation and inflammation. Inflammation can result in red blood cell changes. Red blood cell aggregation increases in the presence of fibrinogen, which is a marker that increases during inflammatory conditions. Fibrinogen enhances the adhesion of the red blood cells by binding to the red blood cell membrane.[43] Changes in the morphology of red blood cells have been shown in inflammatory conditions.[50, 51, 62, 63] Physiological changes such as pregnancy have also been shown to influence the morphology of red blood cells, changing the normal smooth membrane to a granular morphology.[43]

The red blood cell-platelet interaction and have an important role in the coagulation system. This relationship is usually initiated by platelet activation. As the platelets become activated, the coagulation pathways are stimulated resulting in fibrin network trapping more platelets and red blood cells. However, red blood cells can also release ADP which is prothrombotic by activating the platelets.[43]

Platelets undergo different morphological stages when activated:

- Stage 1: resembles a disk.
- Stage 2: has a spheroidal shape.
- Stage 3: early spheroidal shape with scanty, short pseudopods developing.
- Stage 4: late spheroidal shape with many, long pseudopods.
- Stage 5: cytoplasm moves from the center to the pseudopodia.
- Stage 6: a central small elevation with the rest of the cell becoming flattened.
- Stage 7: a final flat configuration[43]

A study of 4 HIV infected patients in 2008 revealed an altered platelet morphology.[36] There were areas of intact membranes adjacent to membrane blebbing with areas where the membrane was torn compared to the control platelet aggregates which showed smooth membranes.[39] These structural changes are in keeping with features of apoptosis, cell death.

There is limited research on the ultrastructure of erythrocytes and platelets in HIV patients on ARV treatment. One study did look at the morphology of platelets with the use of an immunomodulator, Canova, in HIV patients. The reported findings suggested that platelet morphology is kept intact with the use of Canova and it prevents the cyto-destructive effects of the HIV disease.[39, 46] The effects of ARV's may therefore show similar effects in red blood cells and platelet ultrastructure. Previous studies, specifically with patients on NRTI + NNRTI regimens, have not shown significant differences on coagulation markers (prothrombin time, activated partial thromboplastin time, von Willebrand factor, factor VIII, fibrinogen, D-dimer and endogenous thrombin potential).[18, 19, 20, 21] The proposed study seeks to demonstrate if any morphological changes occur in platelets and red blood cells from HIV infected patients and the effect of ARV treatment thereupon.

Whole blood based coagulation tests such as the thromboelastography (TEG) can determine the viscoelastic properties of the clot directly. The TEG was invented around 1940 and is used in some emergency centers as a point of care testing, i.e. performed at or near the patient.[27] The test produces results quickly and gives an extensive evaluation of the patient's coagulation (and anticoagulation) status. The clot time (R-reaction time) measures from the initiation of the coagulation process to 2mm amplitude on the trace recording. The clot kinetics (K-kinetics) records the time from the R time to 20mm amplitude. The alpha angle (slope between R and K) measures the intensity of the clot forming. The clot strength is defined by the maximal amplitude (mm) of the tracing (MA-maximum amplitude). The TEG can also give results on maximum rate of thrombus generation (MRTGG), total thrombus generation (TTG) and the time to maximum rate of thrombus generation (TMRTGG).[54] A disadvantage of the TEG is the inability to identify platelet adhesion and Von Willebrand factor abnormalities during primary haemostasis.[30]

Whole blood can be centrifuged to produce a sample of platelet poor plasma (PPP). PPP is the plasma without cells and is therefore useful to test the coagulation system without the influence of platelets or red blood cells. By testing whole blood and PPP, specific abnormalities in the coagulation system can be detected.[50, 51, 63, 160]

The light microscopy allows for the identification of specific molecules and cells but the resolution is typically submicrometer. The light microscopy also allows for the

determination of changes of the red blood cell shape by assessing the axial ratios of each red blood cell. The scanning electron microscope (SEM) allows ultrastructural analysis at a high resolution. However, the disadvantage of SEM is that the images are on grayscale and finding rare events in space and time is difficult. By combining the two methods of microscopy allows improved analysis and decreases the limitations of each method.[161]

The TEG results together with the light microscopy and the ultrastructural changes seen on the scanning electron microscope can give a combined evaluation of the patient's coagulation status. By using these research methods and applying them to the 3 groups (controls, HIV negative and HIV positive), a predictive screening assessment could then possibly be determined for those at risk of a thromboembolic event. The following objectives therefore directs this chapter.

Chapter objectives

3. Compare viscoelastic properties of whole blood and platelet poor plasma (PPP).
4. Compare light microscopy smears between groups.
5. Compare ultrastructure of platelets and red blood cells (using whole blood smears) with the scanning electron microscopy (SEM).
6. Compare whole blood with thrombin using SEM to the viscoelastic results.

Materials and methods

Objective 3

- Objective 3 is to compare viscoelastic properties of whole blood and platelet poor plasma (PPP) between the groups.
- This objective was accomplished by using the Thromboelastogram (TEG) which is located in the department of physiology laboratory.
- Whole blood as well as platelet poor plasma were used.
- Whole blood in the citrate tube was centrifuged to obtain platelet poor plasma.
- Whole blood was used to assess the full coagulation process
- Platelet-poor plasma was used to assess coagulation without the influence of platelets on the coagulation pathways.
- Whole blood/Platelet-poor plasma was inserted into the cuvette
- The cuvette containing the blood then moves with a stationary pin
- Activator (calcium chloride) was added to the blood sample
- The resistance of the pin in the blood sample was recorded on the connected computer
- The data was presented as a curve with its numerical parameters.
- Refer to Table 2: Parameters of Whole blood and Platelet poor plasma in viscoelastic tests for the data analysed from the viscoelastic parameters.

Objective 4

Objective 4 is to compare light microscopy smears between the groups. Whole blood was used to form a blood smear on a microscope glass slide which was stained with Giemsa stain for differential staining of the blood cells. The slide was examined under a light microscope to assess the shapes of the different blood cells.

Axial ratios of the red blood cells were determined from the light microscopy micrographs, by using a program written in the C# programming language. The longest axis from each red blood cell was measured and referenced as the major axis. The minor axis length was then measured by a line drawn perpendicularly to the centre of the major axis. The axial ratio for each cell was calculated by dividing the major axis length by the minor axis length. A value

of 1 represents a perfect circle. The axial ratio of 50 different red blood cells were measured per subject.

Objective 5

Objective 5 is to compare the ultrastructure of platelets and red blood cells using whole blood smears with the scanning electron microscopy (SEM). Whole blood samples (citrate tube) for SEM were prepared as follows:

- 10 µl of whole blood was placed directly on a glass cover slip.
- The whole blood on the cover slips was then incubated for 3 minutes at room temperature.
- The cover slips were then immersion in 0.075 M sodium phosphate buffer (pH 7.4) and finally placed on a shaker for 20 minutes.
- Fixation of smears were done by using 2.5% gluteraldehyde in phosphate-buffered saline (PBS) solution with a pH of 7.4 for 30 minutes, followed by the wash step where the samples were washed 3 times in phosphate buffer for 3 minutes before the final fixation step of 15 minutes in 1% osmium tetraoxide (OsO4).
- The samples were again washed 3 times with PBS for 3 minutes and the dehydration of the samples were done with 30%, 50%, 70%, 90% and 3 times with 100% ethanol (different concentrations of ethanol are used to avoid osmotic shock of samples).
- After the dehydration step the material were immersed with hexamethyldisilazane (HMDS) for 30 minutes
- The HMDS was removed and a drop of HMDS added to the samples and then left to dry.
- After the samples were dried they were mounted on metal plates and coated with carbon to provide conduction of the samples under the microscope.
- A Zeiss ULTRA plus FEG-SEM with In Lens capabilities was used to study the surface morphology of platelets, red blood cells and white cell interactions; and micrographs were taken at 1kV. (The scanning electron microscope used for this study is located in the Microscopy and Microanalysis laboratory of the University of Pretoria, Pretoria, South Africa.)

Objective 6

Objective 6 is to compare whole blood with thrombin using SEM to the viscoelastic results.

Whole blood samples (citrate tube) for SEM were prepared as follows:

- 10 µl of whole blood was placed directly on a glass cover slip.
- Whole blood was mixed immediately with 5 µl thrombin.
- The whole blood on the cover slips was then incubated for 3 minutes.
- The rest of the SEM preparation steps were the same as for objective 5.
- The interaction of fibrin strands with the red blood cells were then assessed.

Results

TEG results

The TEG measurements using **Whole blood** for the Controls, HIV negative DVT group and the HIV positive DVT group are shown in **Tables 22, 23** and **24** respectively. The tables shows the median, standard deviation, minimum and maximum values for each TEG parameter.

For the comparison between groups, refer to **Tables 25, 26** and **27**, p-values were calculated using the Mann-Whitney test with a significance level of 0.05.

Table 25 compares the TEG parameters using whole blood in the Controls to the HIV negative group with a DVT. The HIV negative group showed a hypercoagulable TEG profile with statistically significant decreases in the R-time (p-value 0.0021), K-time (p-value 0.0323), MRTGG (p-value 0.0314) and the TMRTGG (p-value 0.0087). The alpha angle, MA and TGG, although greater than the Controls, were not significantly raised.

Table 26 compares the TEG parameters using whole blood in the Controls to the HIV positive group with a DVT. The HIV positive group showed a hypercoagulable TEG profile with statistically significant decreases in the R-time (p-value 0.0022), K-time (p-value 0.0151), MRTGG (p-value 0.0123) and the TMRTGG (p-value 0.0039). The alpha angle was significantly increased with a p-value of 0.0303. The MA and TGG, however, had no significant change.

Table 27 compares the TEG parameters using whole blood in the HIV negative DVT group to the HIV positive DVT group. Although the HIV positive group has a faster R-time, K-time, MRTGG and TMRTGG indicating hypercoagulability, there was no statistically significant difference between the HIV negative and the HIV positive groups for any of the TEG parameters. **Graph 2** compares the TEG parameters using whole blood between the 3 groups using box-and-whisker graphs.

Table 22: TEG Whole Blood- Controls

TEG parameter	N	Median	Minimum	Maximum	Standard deviation
R-time	22	7.7	4.1	12.2	2.006
K-time	22	4.2	1.5	9.8	1.963
alpha angle	22	49.85	41.1	69.4	8.382
MA	22	52.2	37.5	62.7	7.274
MRTGG	21	3.27	1.33	10.67	2.075
TMRTGG	21	10.92	5.58	20.92	3.761
TGG	21	542.8	301.6	849	165

Table 23: TEG Whole Blood- HIV negative DVT group

TEG parameter	N	Median	Minimum	Maximum	Standard deviation
R-time	29	6.2	3.7	12.2	1.824
K-time	29	2.8	1.3	11.5	2.217
alpha angle	29	52.1	17.7	78.1	14.24
MA	29	54.4	29.4	79.9	12.64
MRTGG	29	4.6	1.66	14.34	3.366
TMRTGG	29	8.83	4.33	20.08	2.972
TGG	29	559.8	101.3	1830	406.7

Table 24: TEG Whole Blood- HIV positive DVT group

TEG parameter	N	Median	Minimum	Maximum	Standard deviation
R-time	15	5.2	2.2	11.8	2.689
K-time	15	2.5	0.8	6.1	1.494
alpha angle	15	60.4	32.1	78.7	13.31
MA	15	54.1	39.2	80.9	12.07
MRTGG	15	5.09	1.87	21.73	4.929
TMRTGG	15	7.25	3.17	15.67	3.558
TGG	15	594.1	322	1999	451.8

Table 25: TEG Whole blood: Controls vs. HIV negative DVT group

TEG Parameters	Controls	HIV-	P-value	Significant difference
R-time	7.7	6.2	0.0021	Yes
K-time	4.2	2.8	0.0323	Yes
alpha angle	49.85	52.1	0.408	No
MA	52.2	54.4	0.2578	No
MRTGG	3.27	4.6	0.0314	Yes
TMRTGG	10.92	8.83	0.0087	Yes
TGG	542.8	559.8	0.9217	No

Table 26: TEG Whole blood: Controls vs. HIV positive DVT group

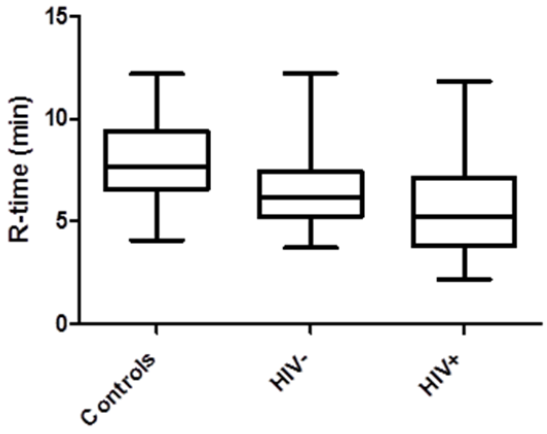
TEG Parameters	Controls	HIV+	P-value	Significant difference
R-time	7.7	5.2	0.0022	Yes
K-time	4.2	2.5	0.0151	Yes
alpha angle	49.85	60.4	0.0303	Yes
MA	52.2	54.1	0.4302	No
MRTGG	3.27	5.09	0.0123	Yes
TMRTGG	10.92	7.25	0.0039	Yes
TGG	542.8	594.1	0.4412	No

Table 27: TEG Whole blood: HIV negative DVT group vs. HIV positive DVT group

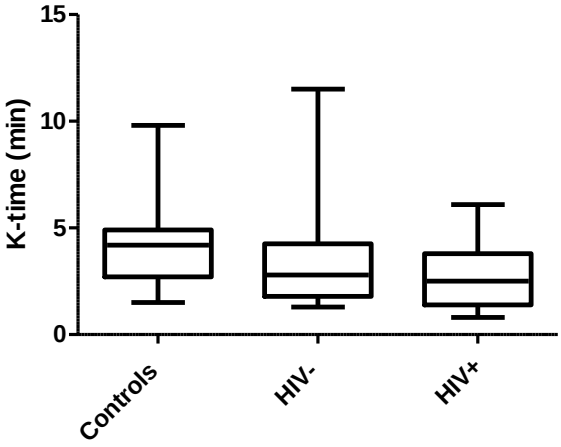
TEG Parameters	HIV-	HIV+	P-value	Significant difference
R-time	6.2	5.2	0.1157	No
K-time	2.8	2.5	0.4495	No
alpha angle	52.1	60.4	0.113	No
MA	54.4	54.1	0.9015	No
MRTGG	4.6	5.09	0.569	No
TMRTGG	8.83	7.25	0.2022	No
TGG	559.8	594.1	0.5359	No

Graph 2: Box and whiskers graphs of the comparison of TEG Whole blood: A) R-time, B) K-time, C) α angle, D) MA), E) MRTGG, F) TMRTGG, G) TGG.

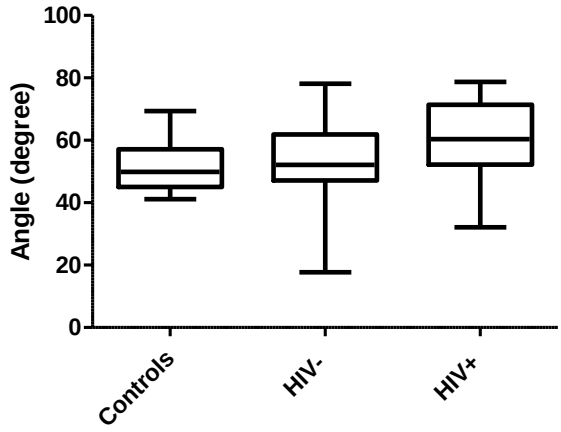
A) R-time



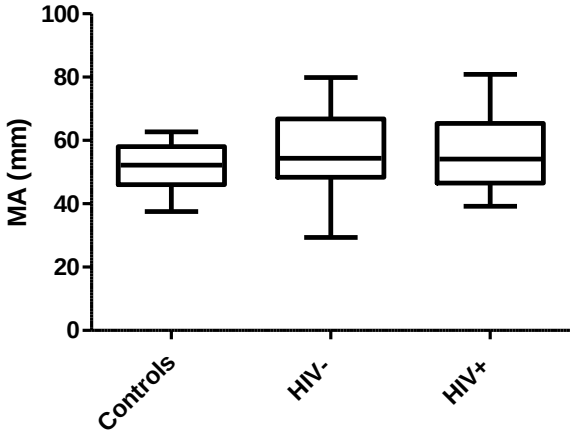
B) K-time



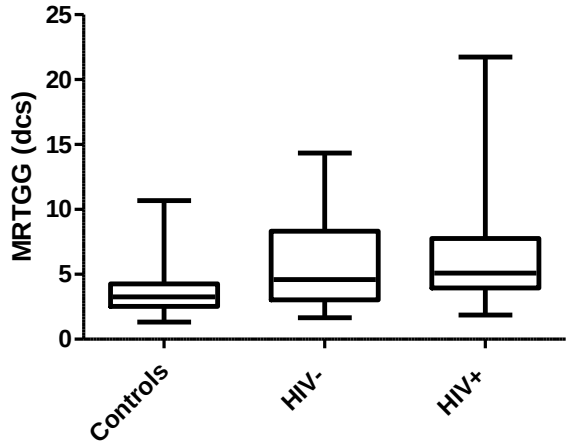
C) α angle



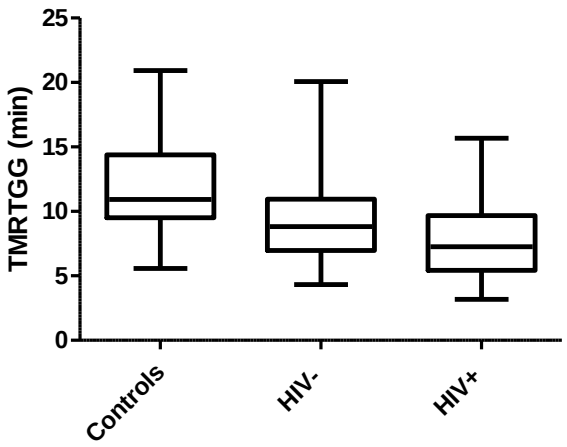
D) MA



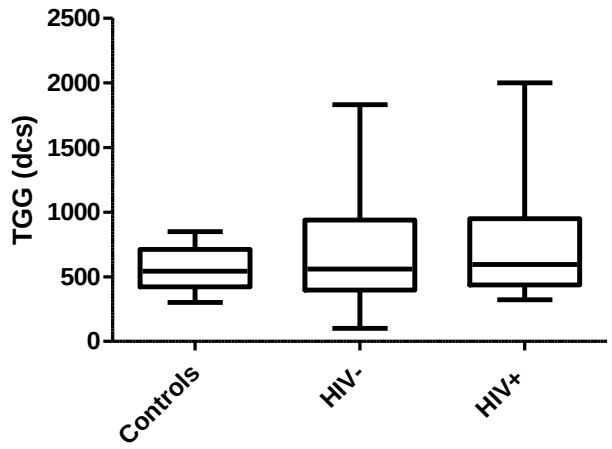
E) MRTGG



F) TMRTGG



G) TGG



The TEG measurements using **Platelet poor plasma** for the Controls, HIV negative DVT group and the HIV positive DVT group are shown in **Tables 28, 29** and **30** respectively. The tables shows the median, standard deviation, minimum and maximum values for each TEG parameter.

For the comparison between groups, refer to **Tables 31, 32** and **33**, p-values were calculated using the Mann-Whitney test with a significance level of 0.05.

Table 31 compares the TEG parameters using platelet poor plasma in the Controls to the HIV negative DVT group. The HIV negative group has a hypercoagulable profile with statistically significant changes in the R-time (p-value 0.0022), K-time (p-value 0.0002), MA (p-value of 0.0002), MRTGG (p-value 0.0001), TMRTGG (p-value 0.0041) and TGG (p-value 0.0001). The alpha angle although raised was not statistically significant.

Table 32 compares the TEG parameters using platelet poor plasma in the Controls to the HIV positive DVT group. The HIV positive group had a hypercoagulable profile with statistically significant decreases in the R-time (p-value 0.0006), K-time (p-value 0.0034), MRTGG (p-value 0.0176) and the TMRTGG (p-value 0.0016). The alpha angle, MA and TGG, although all raised, in keeping with a hypercoagulable picture, were not statistically significant.

Table 33 compares the TEG parameters using platelet poor plasma in the HIV negative DVT group to the HIV positive DVT group. There was no statistically significant difference between the HIV negative and the HIV positive groups for any of the TEG parameters. **Graph 3** compares the TEG parameters using platelet poor plasma between the 3 groups using box-and whisker graphs.

Table 28: TEG Platelet poor plasma- Controls

TEG parameter	N	Median	Minimum	Maximum	Standard deviation
R-time	21	9.8	4	14.5	2.592
K-time	21	3.5	1.4	11.4	2.978
alpha angle	21	60.1	17.7	74.7	12.75
MA	21	28.1	16.8	37	6.302
MRTGG	21	4.77	1.53	12.24	2.361
TMRTGG	21	11.58	5	19.42	3.446
TGG	21	188	101	294.3	61.15

Table 29: TEG Platelet poor plasma: HIV negative DVT group

TEG parameter	N	Median	Minimum	Maximum	Standard deviation
R-time	29	7	3.2	16.4	2.695
K-time	27	1.7	0.8	10.9	1.948
alpha angle	29	66.5	23.2	79.9	13.84
MA	29	38.9	10.1	51.8	9.468
MRTGG	29	9.37	1.29	17.24	3.753
TMRTGG	29	8.08	4.33	23	3.804
TGG	29	319.8	56.03	537.5	111.5

Table 30: TEG Platelet poor plasma: HIV positive DVT group

TEG parameter	N	Median	Minimum	Maximum	Standard deviation
R-time	14	6.1	2.2	8.6	1.909
K-time	13	1.5	0.8	6.9	1.809
alpha angle	14	69.4	31.8	82.2	15.77
MA	14	33.45	16.6	70.3	14.97
MRTGG	14	8.86	2.7	23.35	5.587
TMRTGG	14	7.625	2.83	12.67	2.511
TGG	14	250.5	100	1193	291.4

Table 31: TEG Platelet poor plasma: Controls vs. HIV negative DVT group

TEG Parameters	Controls	HIV-	P-value	Significant difference
R-time	9.8	7	0.0022	Yes
K-time	3.5	1.7	0.0002	Yes
alpha angle	60.1	66.5	0.0987	No
MA	28.1	38.9	0.0002	Yes
MRTGG	4.77	9.37	0.0001	Yes
TMRTGG	11.58	8.08	0.0041	Yes
TGG	188	319.8	0.0001	Yes

Table 32: TEG Platelet poor plasma: Controls vs. HIV positive DVT group

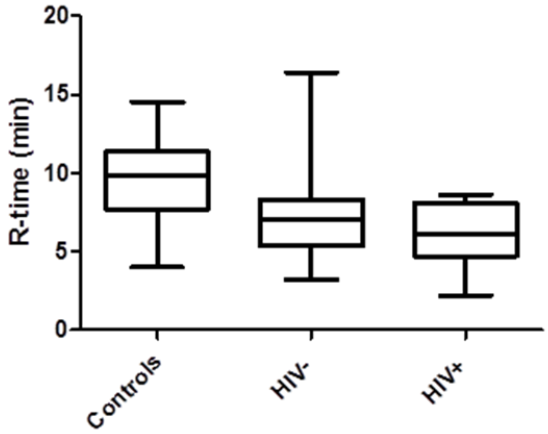
TEG Parameters	Controls	HIV+	P-value	Significant difference
R-time	9.8	6.1	0.0006	Yes
K-time	3.5	1.5	0.0034	Yes
alpha angle	60.1	69.4	0.0593	No
MA	28.1	33.45	0.0528	No
MRTGG	4.77	8.86	0.0176	Yes
TMRTGG	11.58	7.625	0.0016	Yes
TGG	188	250.5	0.0571	No

Table 33: TEG Platelet poor plasma: HIV negative DVT group vs. HIV positive DVT group

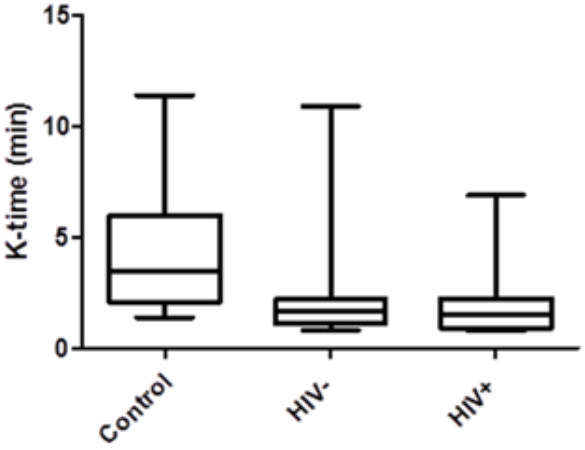
TEG Parameters	HIV-	HIV+	P-value	Significant difference
R-time	7	6.1	0.2593	No
K-time	1.7	1.5	0.7503	No
alpha angle	66.5	69.4	0.3376	No
MA	38.9	33.45	0.2134	No
MRTGG	9.37	8.86	0.6408	No
TMRTGG	8.08	7.625	0.2878	No
TGG	319.8	250.5	0.2281	No

Graph 3: Box-and-whiskers graphs of the comparison of TEG Platelet poor plasma: A) R-time, B) K-time, C) α angle, D) MA), E) MRTGG, F) TMRTGG, G) TGG.

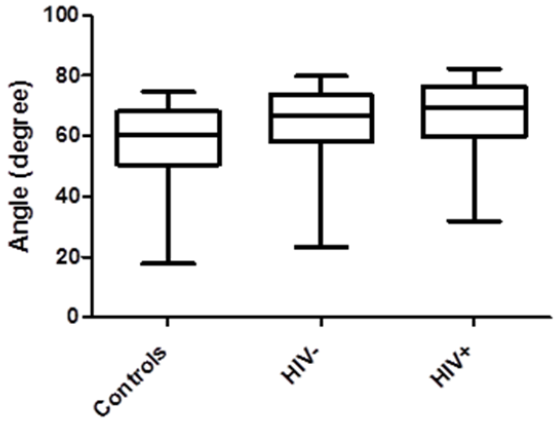
A) R-time



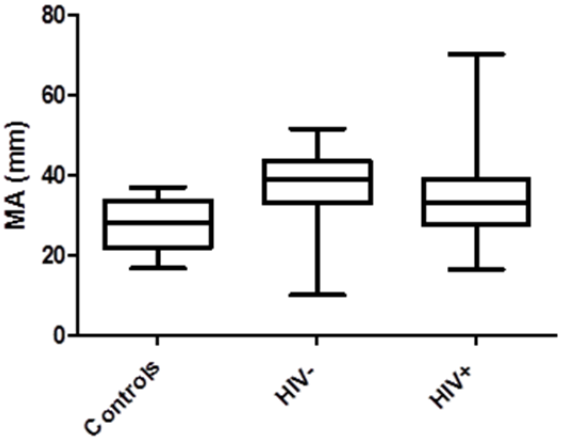
B) K-time



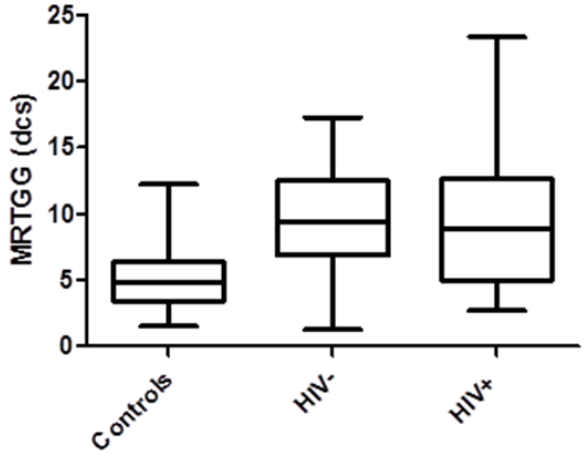
C) α angle



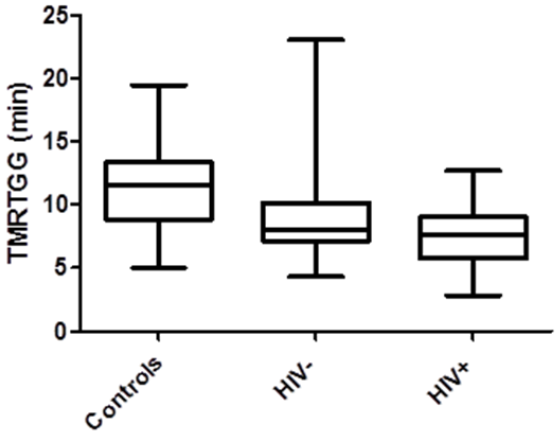
D) MA



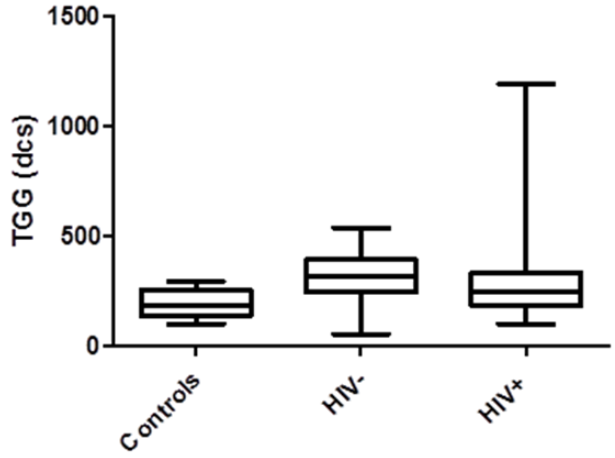
E) MRTGG



F) TMRTGG



G) TGG



Light Microscopy results

Figure 6: Light microscopy blood smear of a healthy patient. Showing normal round smooth surface red blood cells.

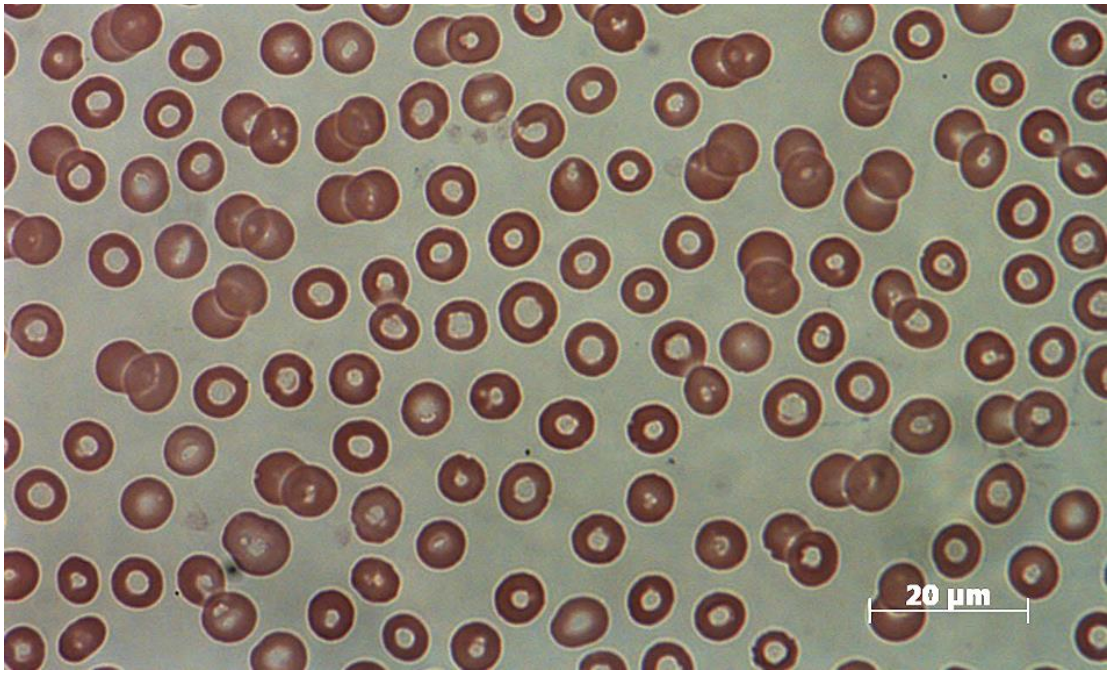


Figure 7: Light microscopy blood smear of a HIV negative DVT patient. Showing Anisocytosis and eryptosis. Label 1 showing a balloon cell with a projection from the inferior surface. Label 2 showing an eryptotic cell.

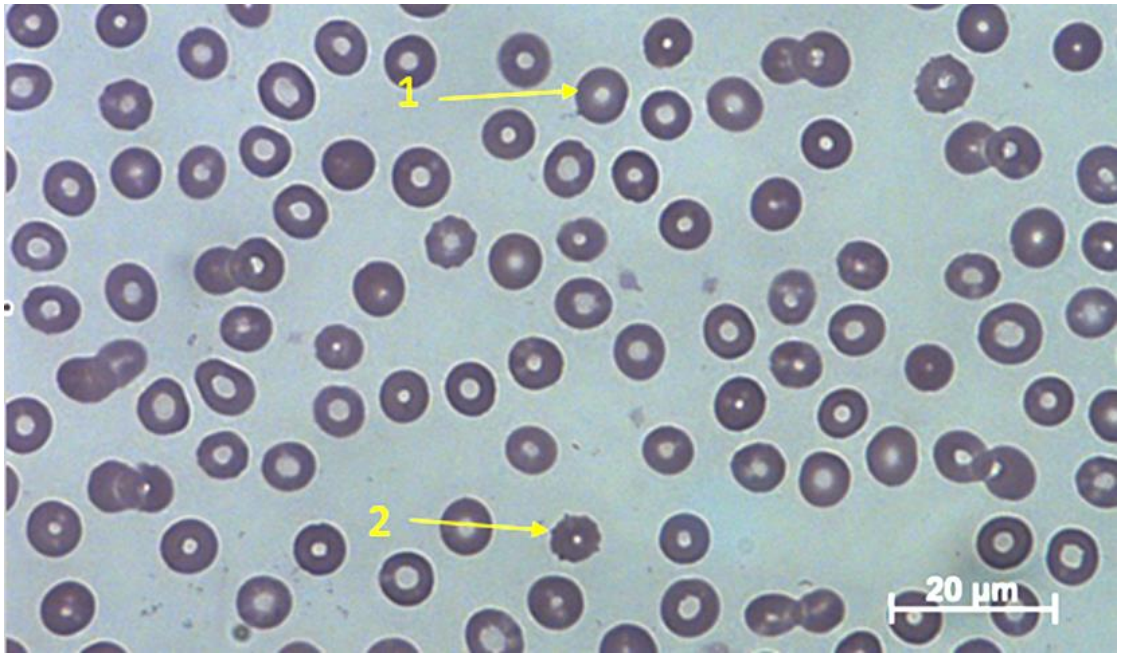


Figure 8: Light microscopy blood smear of a HIV positive DVT patient. Label 1 showing acanthocytes (irregular blunt shaped projections from the red blood cell membrane). Label 2 showing anisocytosis (unequal sized red blood cells). Label 3 showing eryptotic cell.

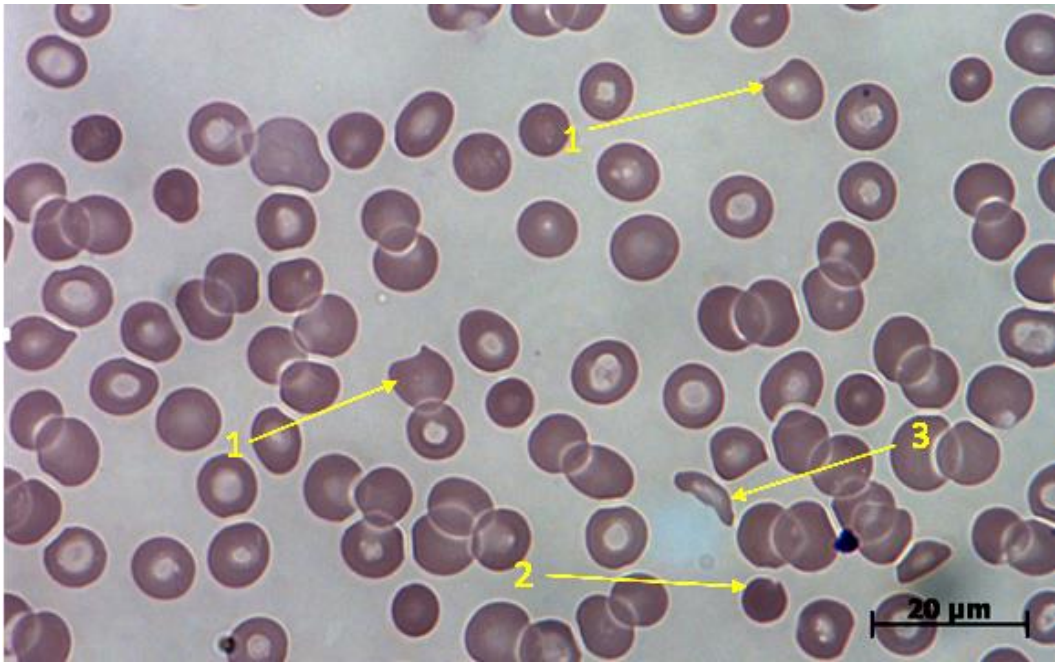


Figure 9: Light microscopy blood smear of HIV positive patient with rouleaux formation and eryptosis. Label 1 showing rouleaux (stacking of red blood cells) formation. Label 2 showing eryptotic red blood cell.

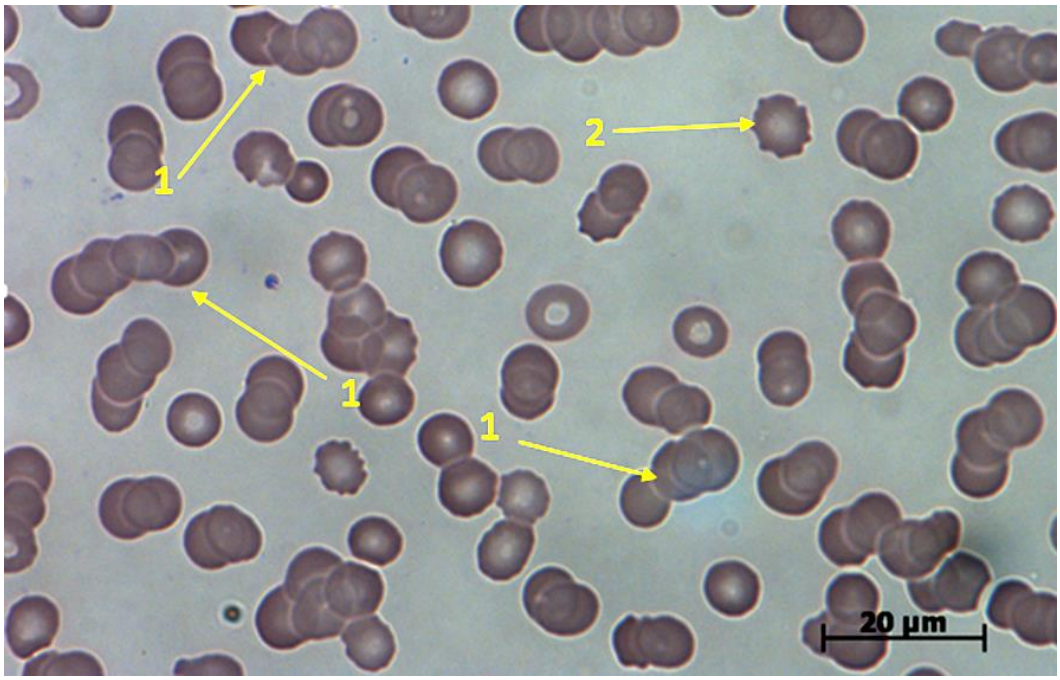


Table 34: Axial Ratios of red Blood Cells between the groups

Study groups	N	Median	Minimum	Maximum	Standard deviation
Controls	1200	1.108	1	2.093	0.132
HIV-	1600	1.081	1	2.217	0.1163
HIV+	750	1.101	1	2.377	0.1497

Table 35: Comparing the axial ratios of red blood cells

Study groups	P-value	Significant difference
Controls vs. HIV-	< 0.0001	Yes
Controls vs. HIV+	0.9082	No
HIV- vs. HIV+	< 0.0001	Yes

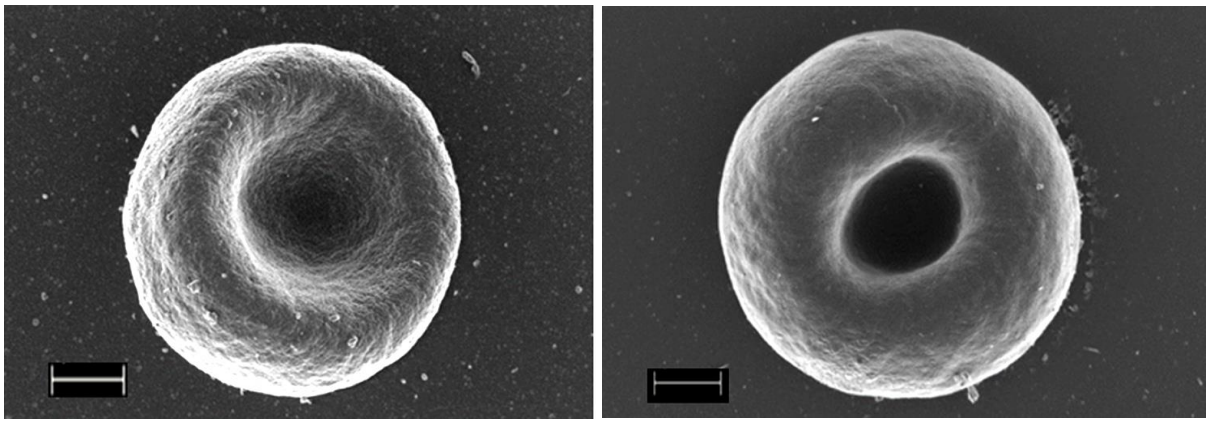
Tables 34 and **35** shows a significant difference in shape variation according to the axial ratios on light microscopy between the control group and HIV- group ($p < 0.0001$); and also the HIV- group and the HIV+ group ($p<0.0001$). There was no significant difference between the control group and the HIV+ group.

Scanning Electron Microscopy results: RED BLOOD CELLS (RBCs)

Controls

Figure 10: SEM Control RBCs. Healthy RBCs that are round with smooth membranes. All figure scale bars: 1 µm; except for C where the scale bar is 2 µm.

A) **B)**



C)

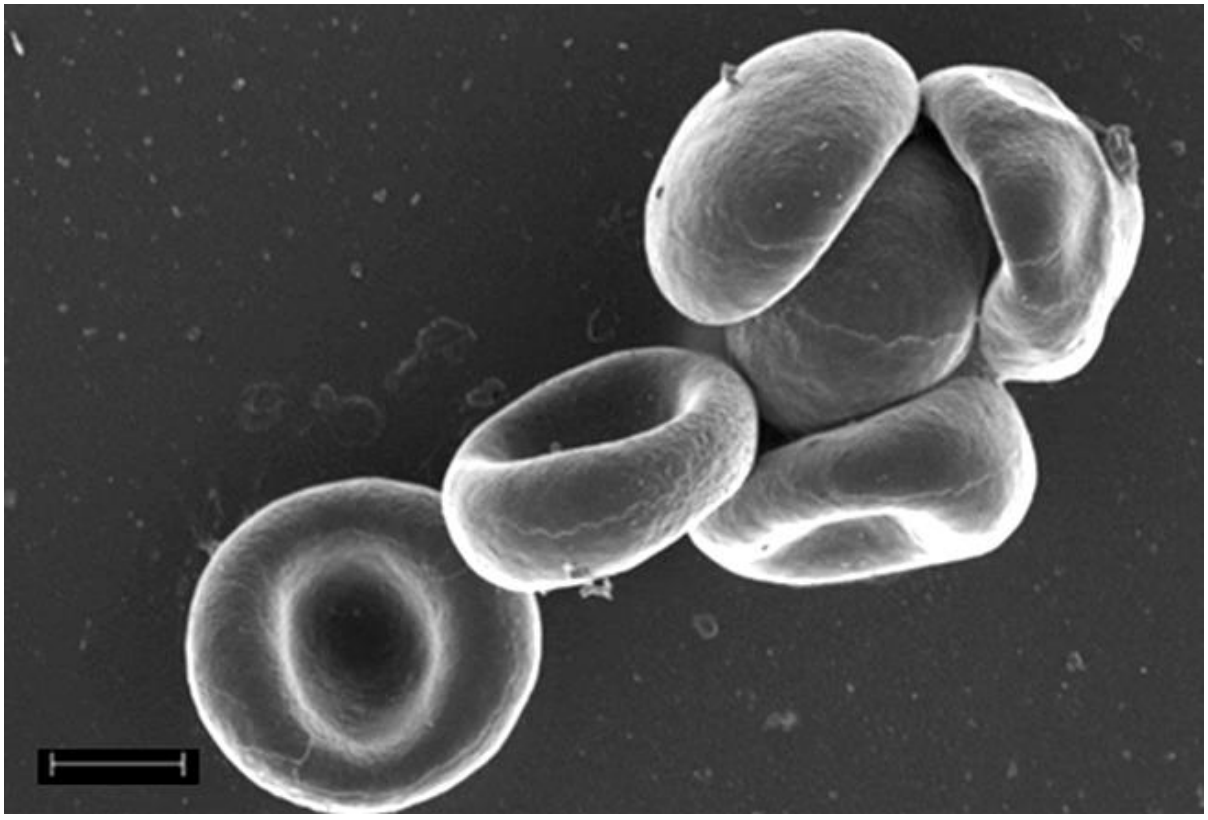
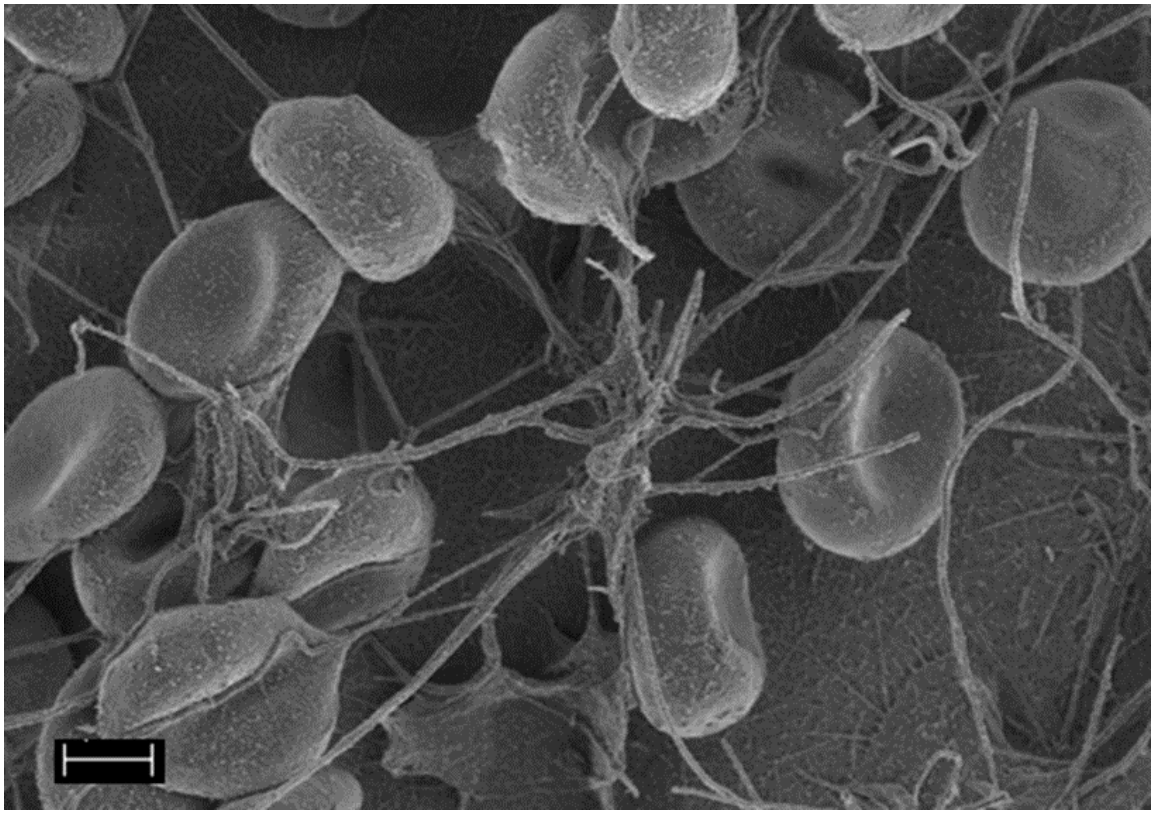


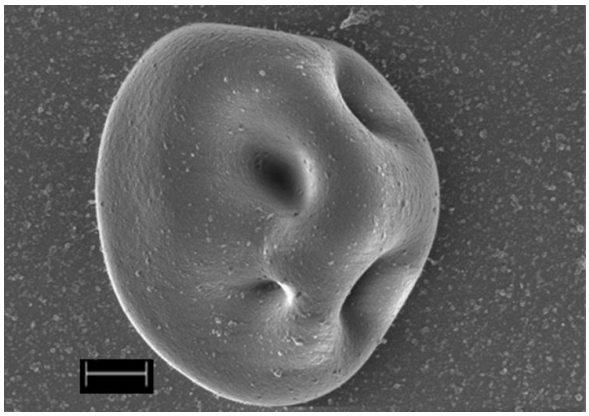
Figure 11: SEM Control- Red blood cells in a fibrin network. Figure scale bars: 2 µm.

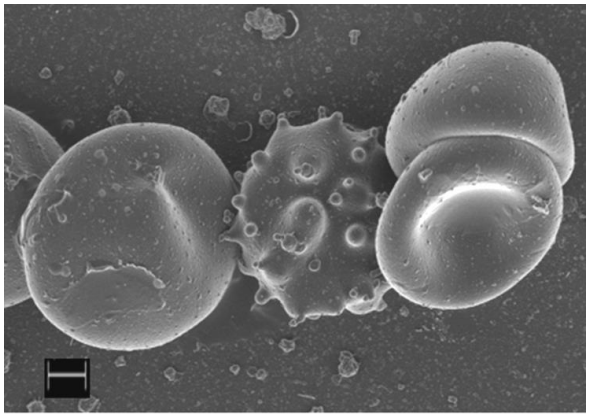


HIV negative with DVT

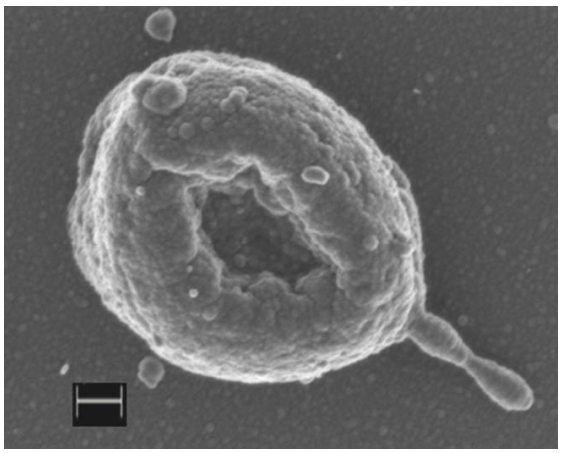
Figure 12: SEM HIV negative patients with DVT- Red blood cells. A) Irregular shape and membrane disruption appearing as holes or craters B) Eryptotic cell surrounded by normal RBCs. C) Balloon cell. D) Microparticle/microvesicle formation. E) Eryptotic RBC. F) Acanthocyte (RBC with spicules that are irregular in size, shape and distribution around the cell). G) Irregular RBC membrane with formation of microparticles/microvesicles. H) Stomacytes (abnormal RBC shapes due to loss of biconcave morphology). I) Stomacyte surrounded by fibrin strands. J) RBC interacting with fibrin. K) Polyhedrocyte surrounded by fibrin strands. All figure scale bars: 1 µm; except C and D where scale bar is 0.2 µm; and G where the scale bar is 0.1µm.

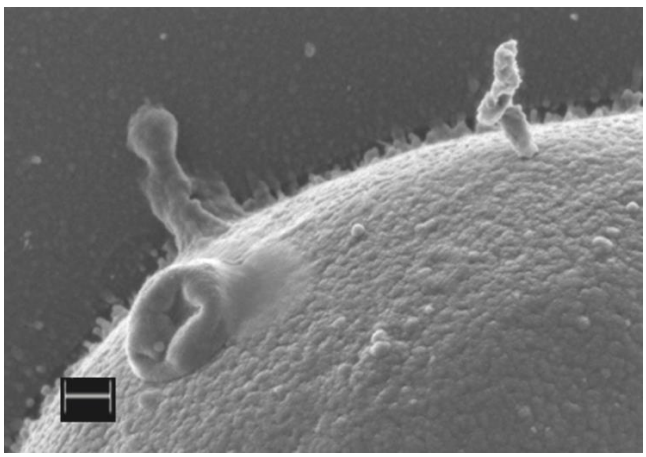
A) **B)**



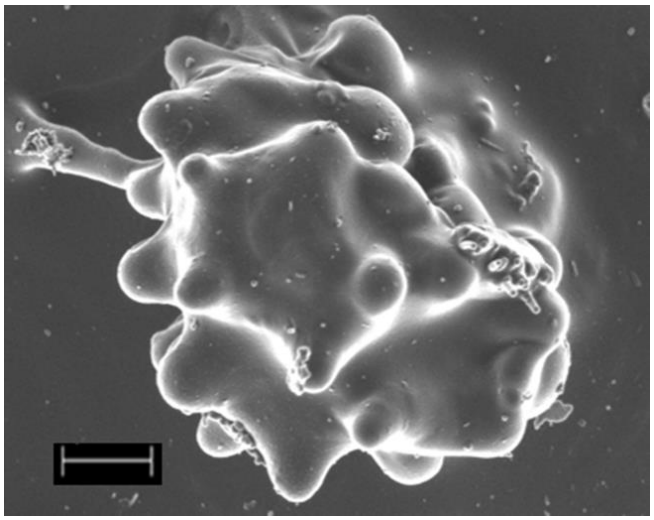


C) **D)**

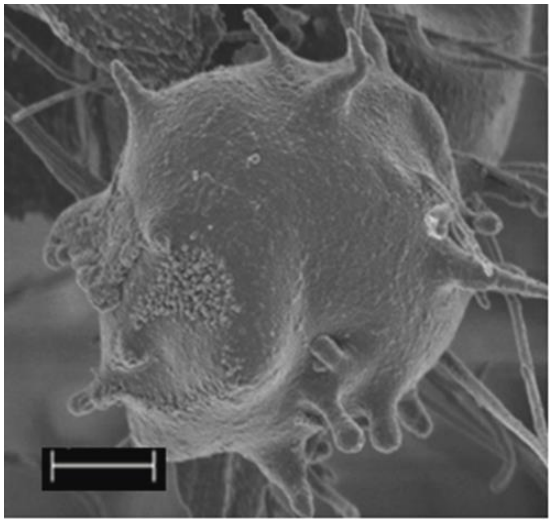




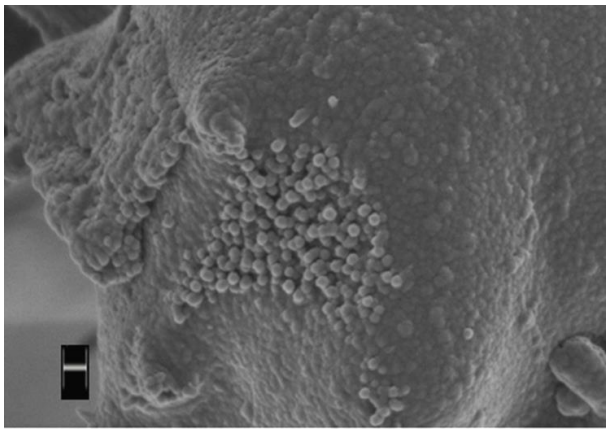
E)



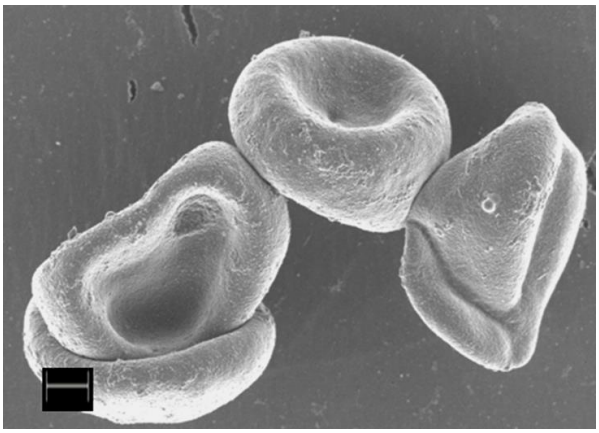
F)



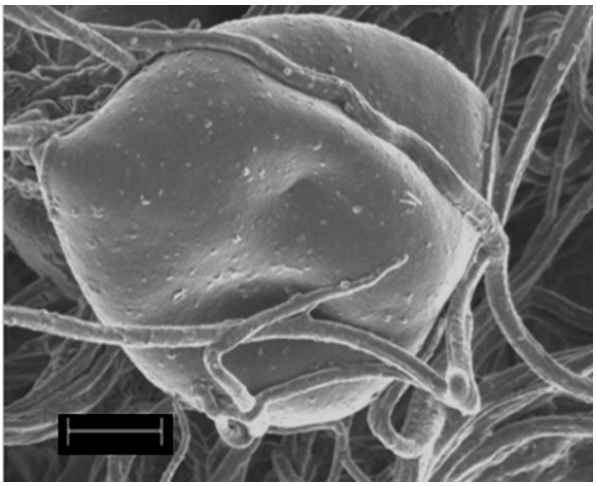
G)



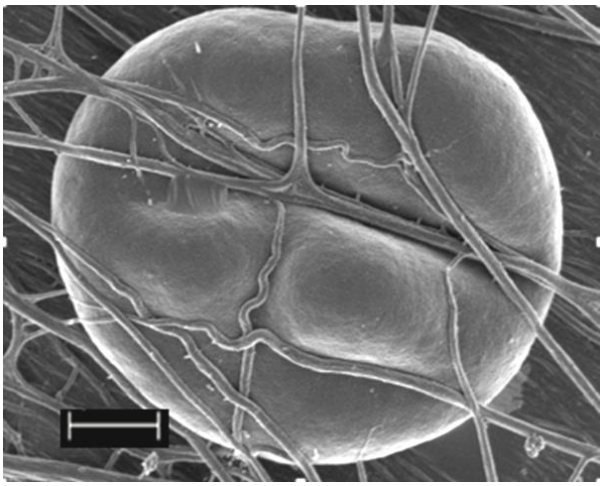
H)



I)



J)



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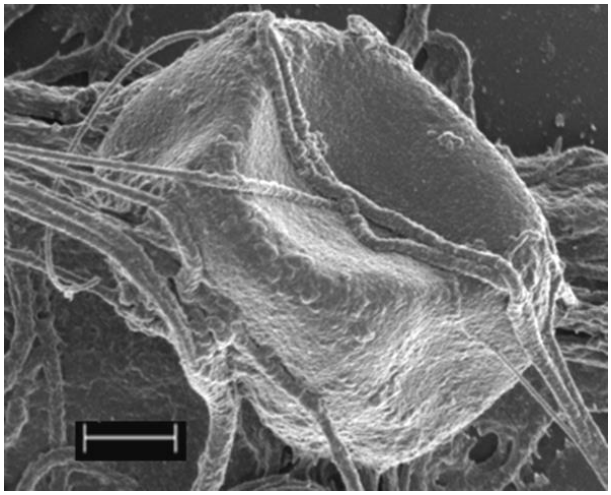


Figure 13: SEM HIV negative patients with DVT- Red blood cells in a fibrin network. Arrow showing a helmet shaped RBC. 1µm. Figure scale bars: 1 µm

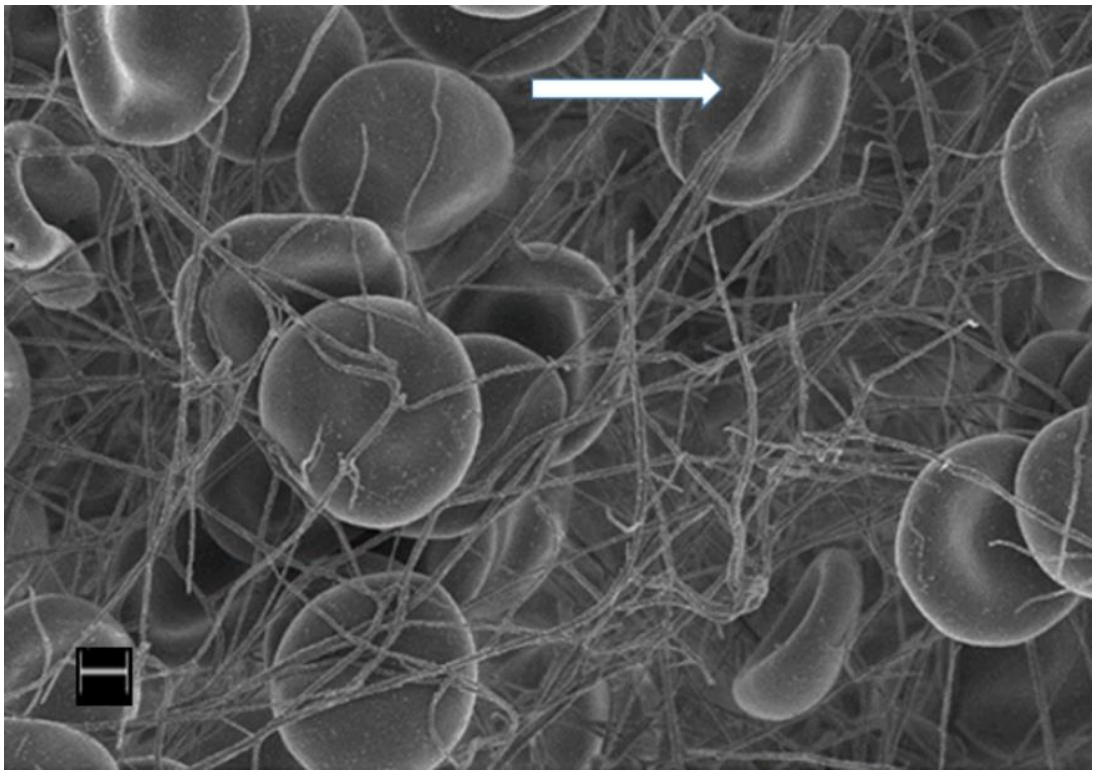
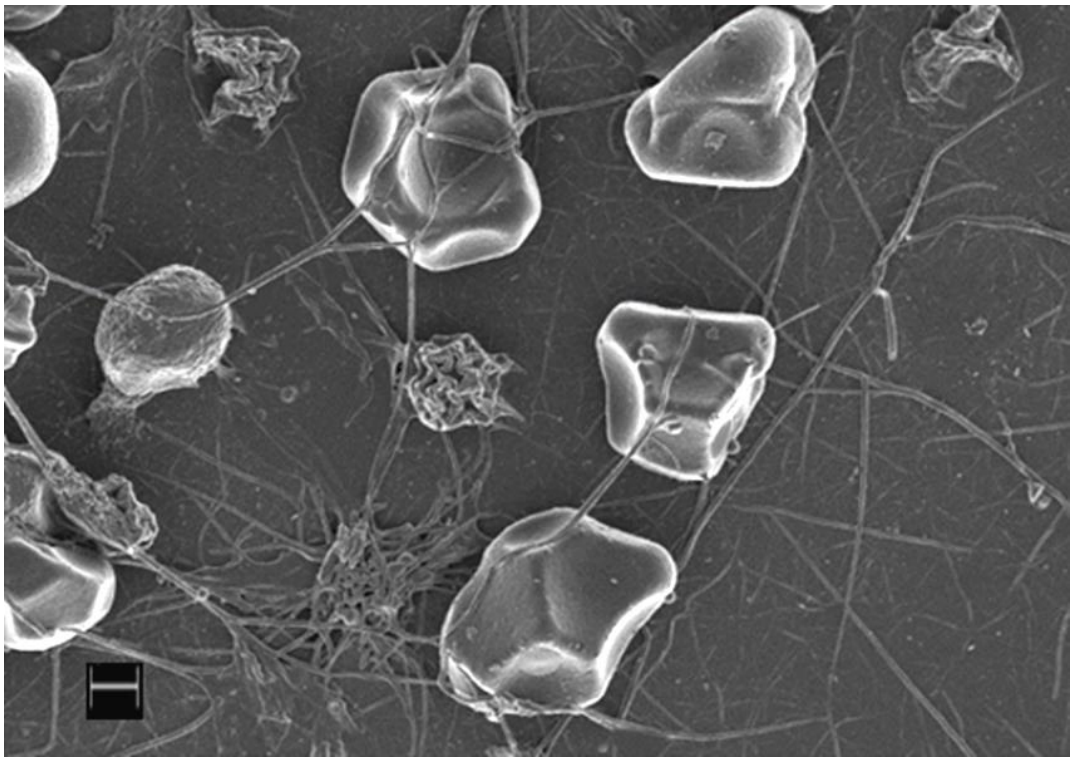


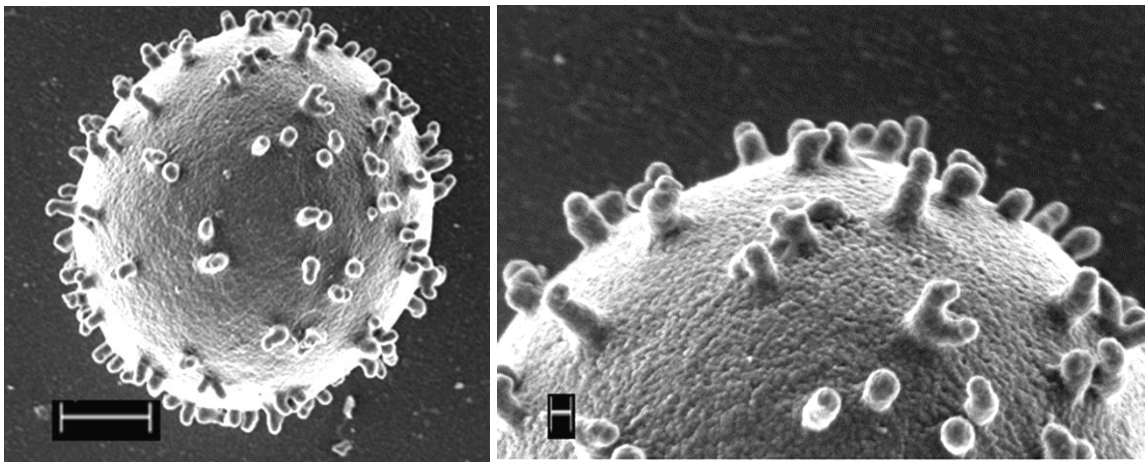
Figure 14: SEM HIV negative patients with DVT- Polyhedrocytes in a fibrin network. All figure scale bars: 1 µm



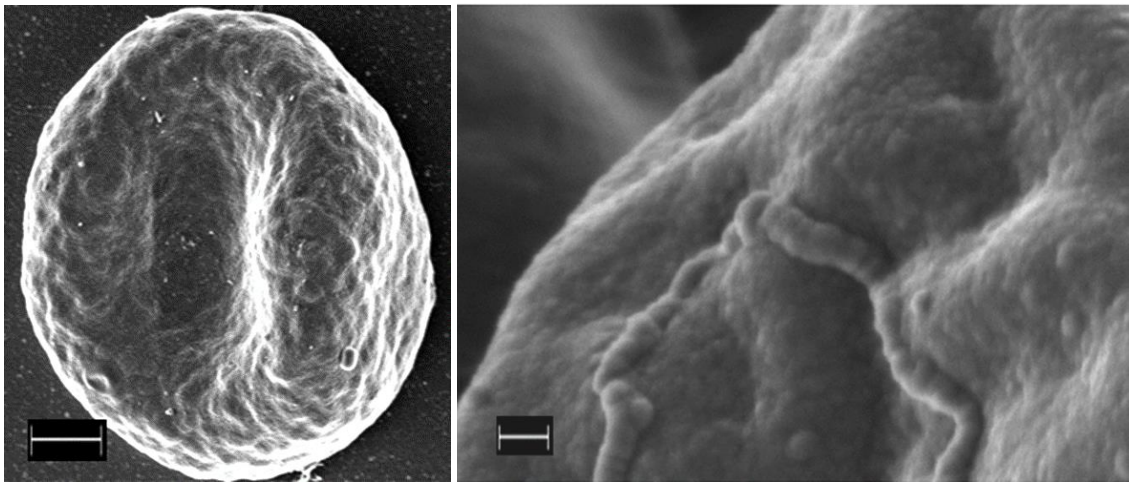
HIV positive with DVT

Figure 15: SEM HIV positive patients with DVT- Red blood cells. A) Echinocyte ("porcupine cell/hedgehog/sea urchin cell"): abnormal evenly spaced thorny membrane projections. B) High magnification of the projections in figure A. C) Early eliptocyte RBC with an irregular membrane surface. D) High magnification of the irregular RBC membrane in Figure C. E) Early eliptocyte RBC with an irregular granular surface. F) Early eliptocyte RBC with disruption of the membrane appearing as holes or craters in the membrane. G) Microparticle/microvesicle formation. H) High magnification of the Microparticle/microvesicle formation in Figure G. I) Acanthocyte (Irregular shaped blunt projection). J) Echinocyte. K) Knot cell. L) Elyptoid shape, progressing to eryptosis. All figure scale bars: 1 µm, except B, D and H where scale bar is 0.1 µm.

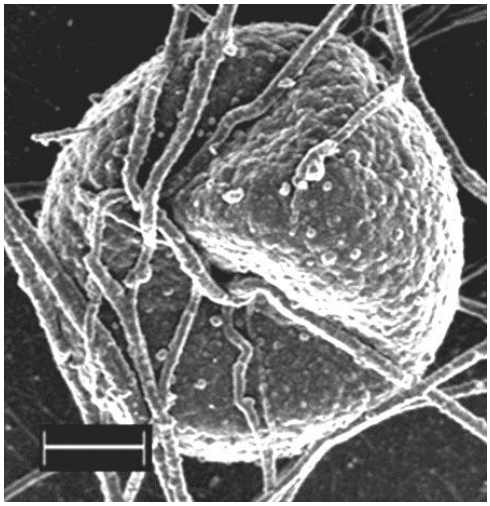
A) **B)**



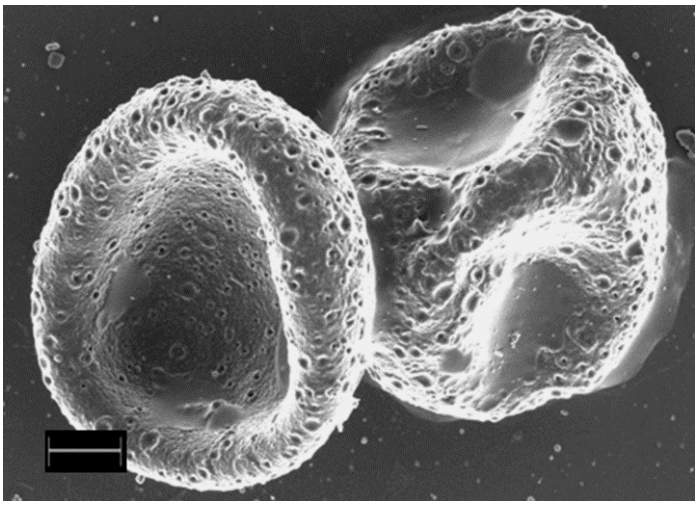
C) **D)**



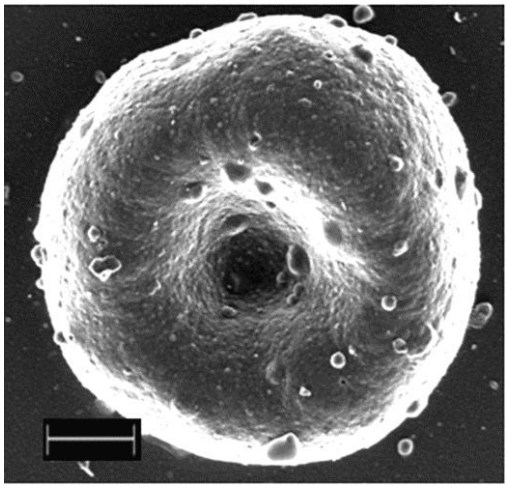
E)



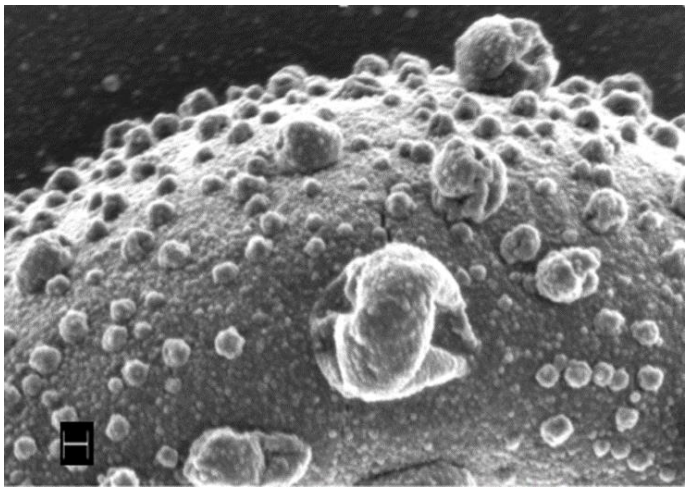
F)



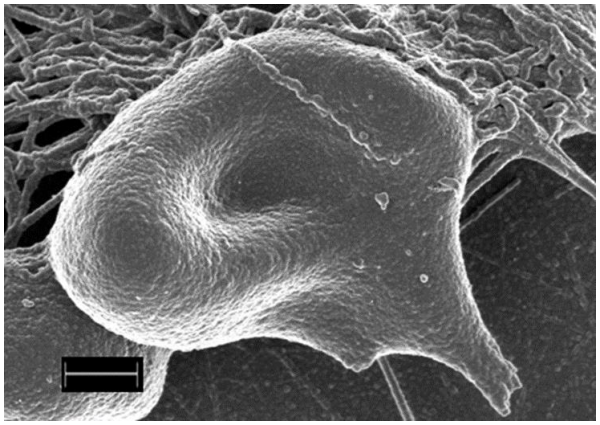
G)



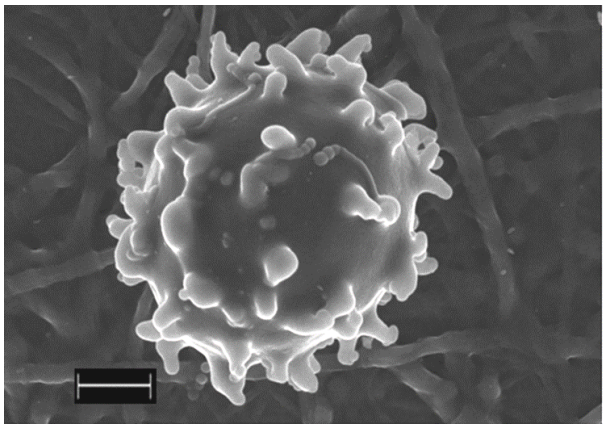
H)



I)



J)



K) **L)**

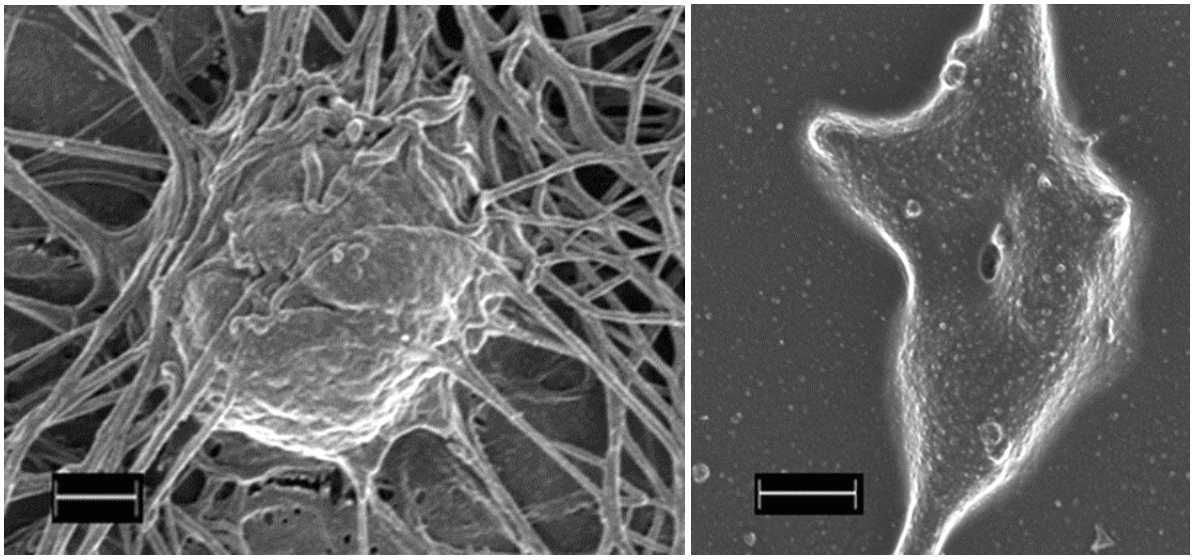


Figure 16: SEM HIV positive patients with DVT- Red blood cell rouleaux formation. Arrow indicating agglutination of the red blood cells. Arrow-head indicating the adherence of plasma proteins. Figure scale bars: 1 µm

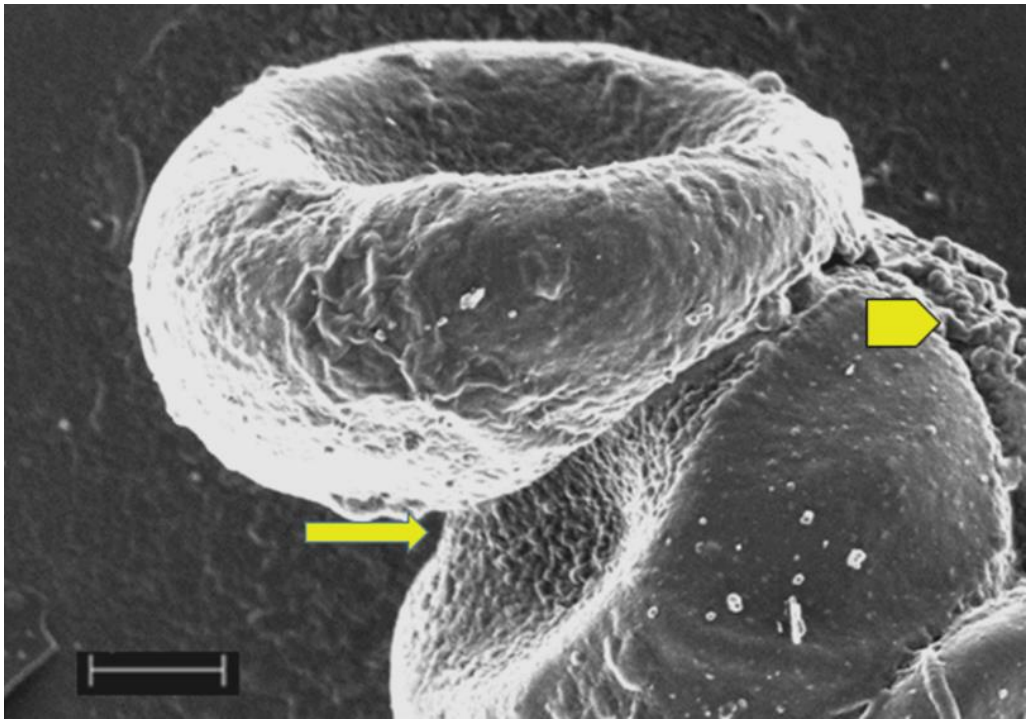


Figure 17: SEM HIV positive patients with DVT- Polyhedrocytes in a fibrin network. Figure scale bars: 2 µm.

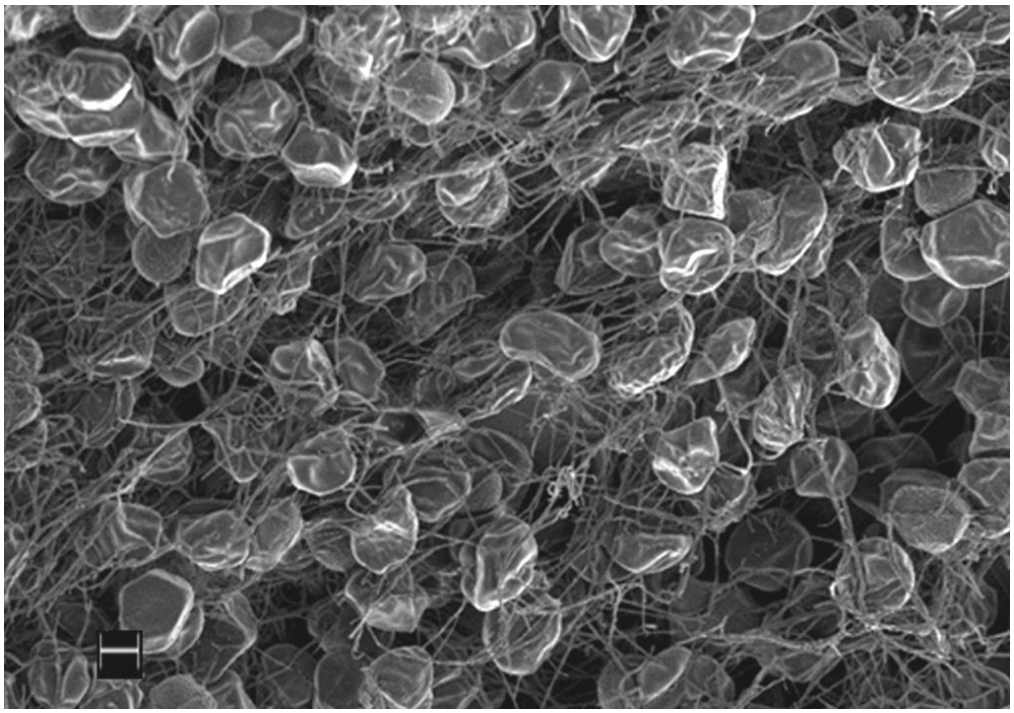
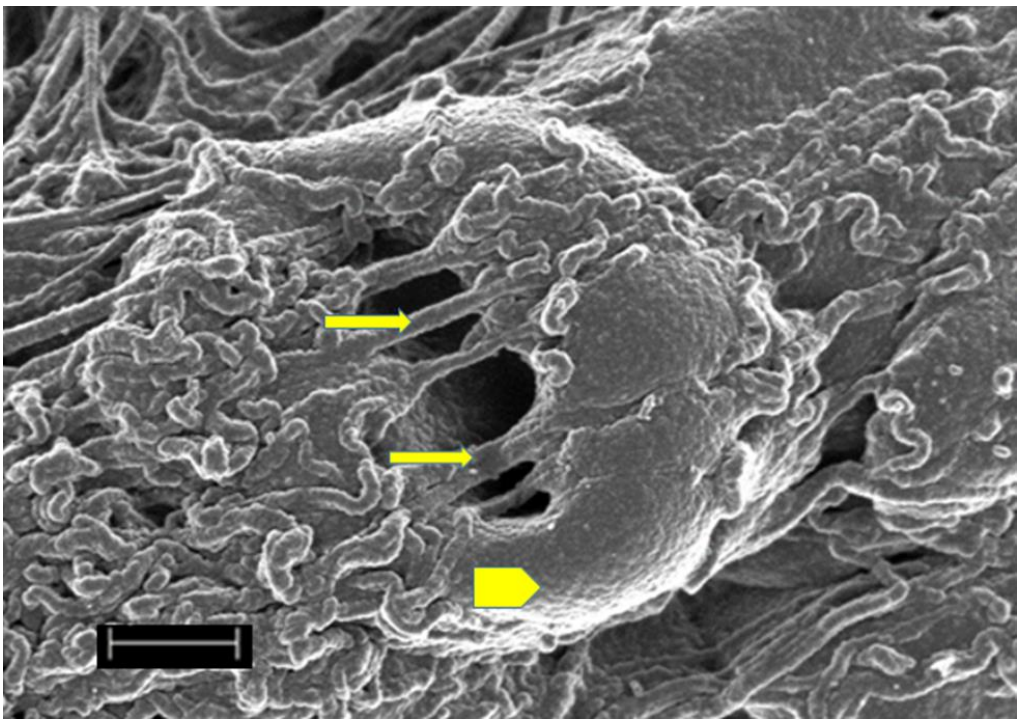


Figure 18: SEM HIV positive patients with DVT- Contraction of a red blood cell by the fibrin network. Arrow indicating the fibrin strands. Arrow head indicating the red blood cell. Figure scale bars: 1 µm.

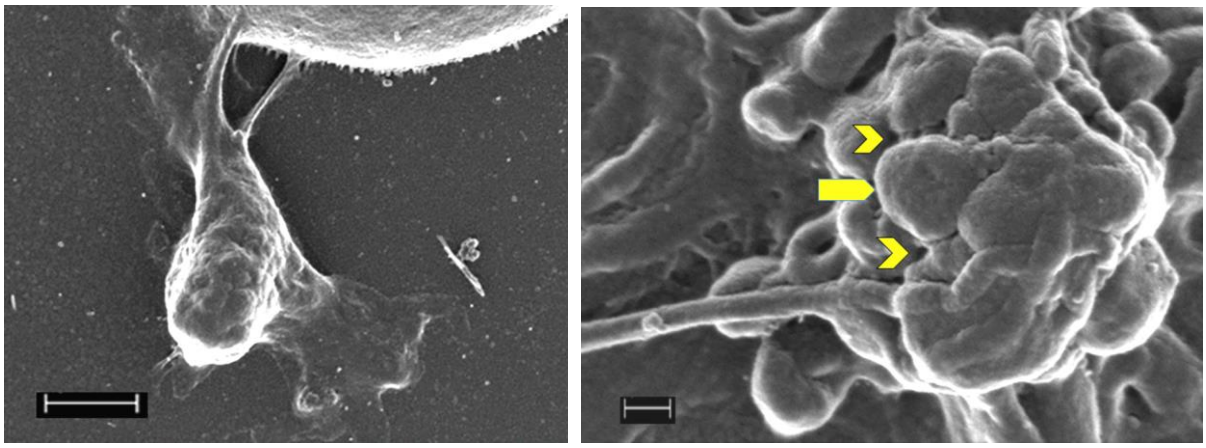


Scanning Electron Microscopy: PLATELETS

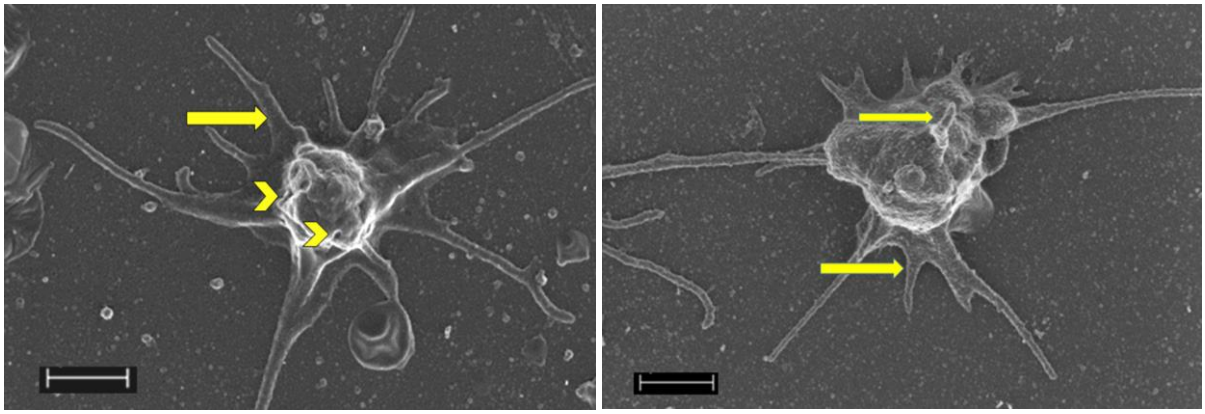
Controls

Figure 19: SEM Controls- Platelets. A) Smooth intact membrane. B) Membrane blebbing and openings of the open canalicular channels. C) Pseudopodia formation and openings of the open canalicular channels. D) Pseudopodia formation from smooth intact membrane. Arrows showing pseudopodia formation. Arrow-heads showing blebbing. Open Arrow-heads showing openings of the open canalicular channels. All figure scale bars: 1 µm, except B where scale bar is 0.2 µm.

A) **B)**



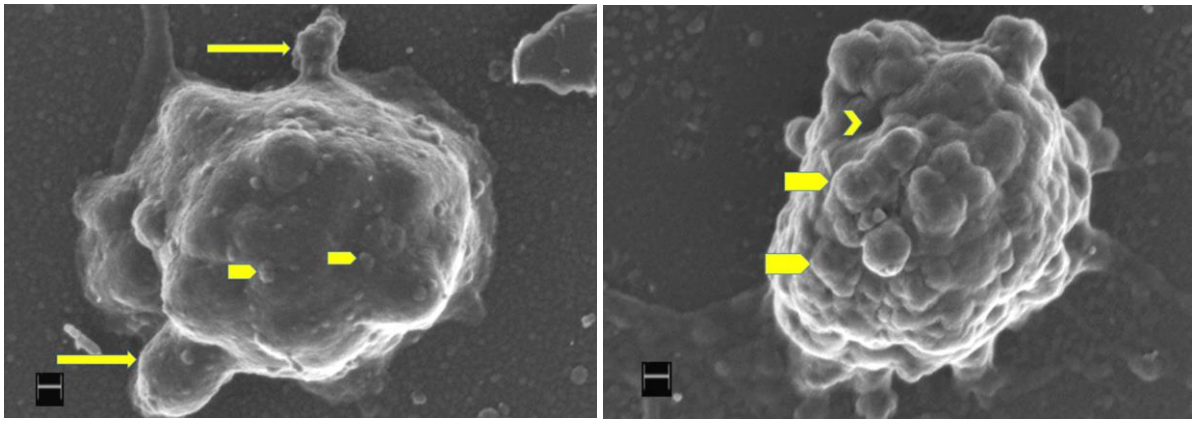
C) **D)**



HIV negative with DVT

Figure 20: SEM HIV negative patients with DVT- Platelets. A) Pseudopodia forming and membrane blebbing. B) Membrane blebbing and openings of the open canalicular channels. C) Pseudopodia and openings of the open canalicular channels in the smooth intact membrane. D) Pseudopodia with openings of the open canalicular channels in the smooth intact membrane. Arrows showing pseudopodia. Open arrow-heads showing openings of the open canalicular channels in the smooth intact membrane. Arrow-heads showing membrane blebbing. All figure scale bars: 0.1µm.

A) **B)**



C) **D)**

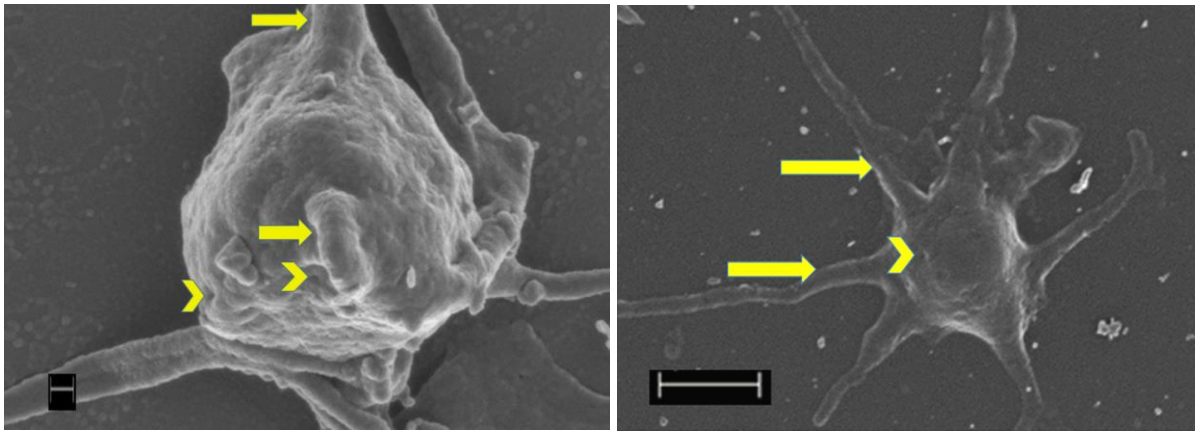


Figure 21: SEM HIV negative patients with DVT- Multiple platelets with a RBC.

Multiple platelets (arrows) with a RBC (arrow-head). All figure scale bars: 1µm.

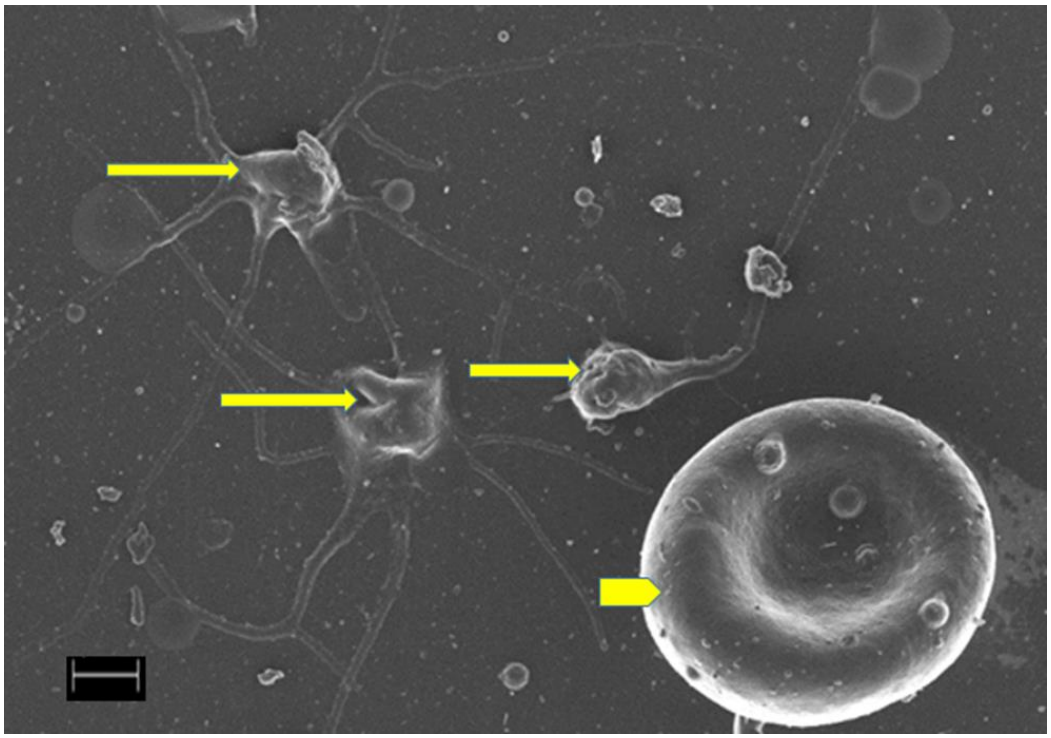
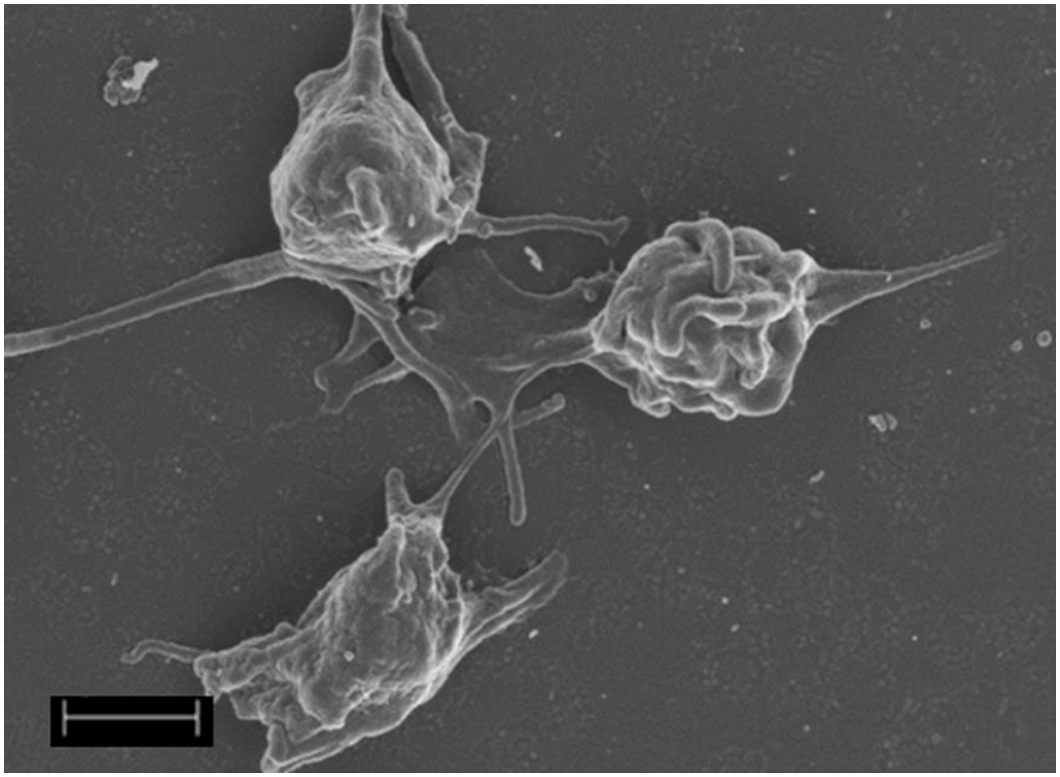


Figure 22: SEM HIV negative patients with DVT- Platelets interaction to form an aggregate.

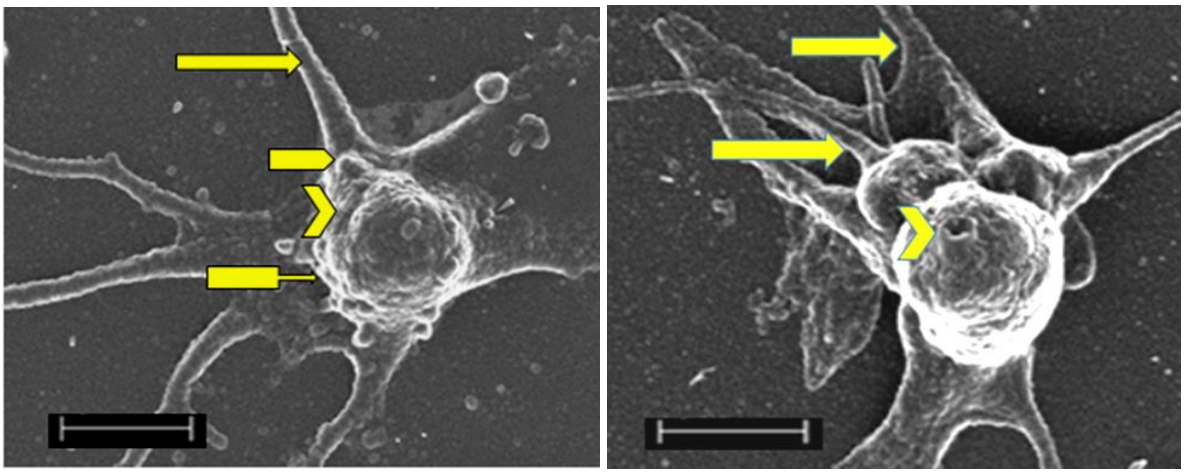
All figure scale bars: 1µm.



HIV positive with DVT

Figure 23: SEM HIV positive patients with DVT- Platelets. A) Irregular membrane with blebbing, fragmentation, openings of the open canalicular channels and pseudopodia formation. B) Openings of the open canalicular channels and membrane blebbing. C) Multiple branching pseudopodia, with irregular membrane blebbing and membrane fragmentation. D) Pseudopodia and membrane fragmentation. Arrow showing pseudopodia. Arrow-head showing membrane blebbing. Box-arrow showing membrane fragmentation. Open arrow-heads showing openings of the open canalicular channels. All figure scale bars: 1µm.

A) B)



C) D)

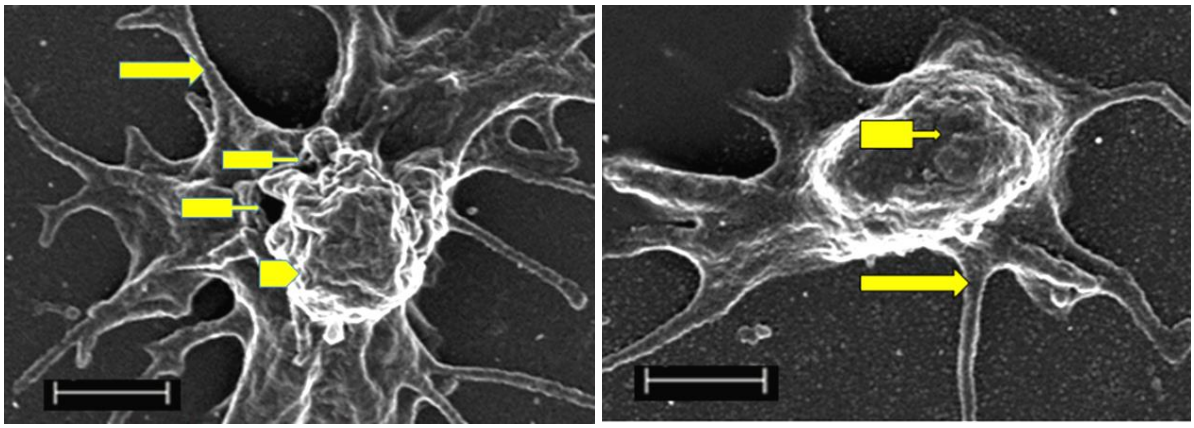
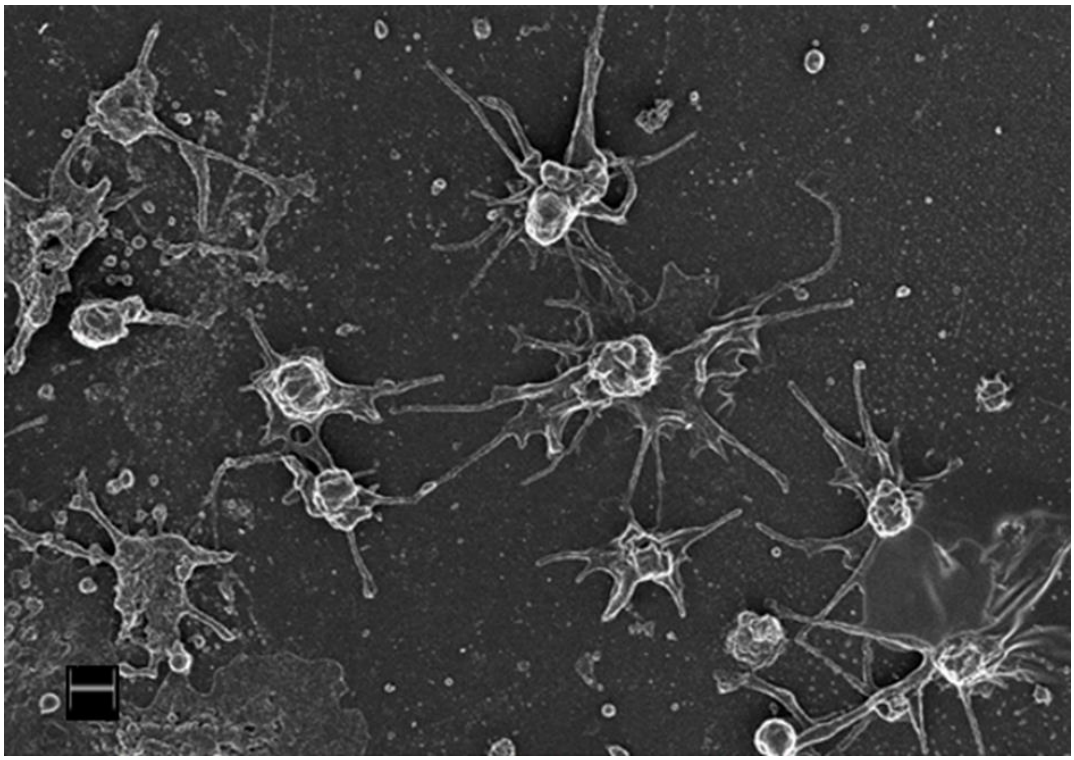


Figure 24: SEM HIV positive patients with DVT- Platelet interaction to form an aggregate.

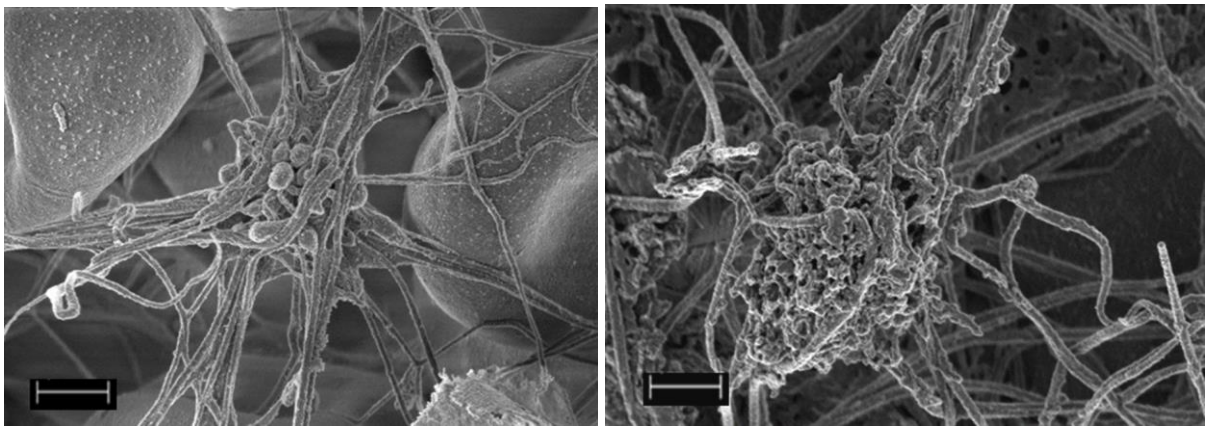
Platelets with irregular membranes, interacting with each other. All figure scale bars: 1µm.



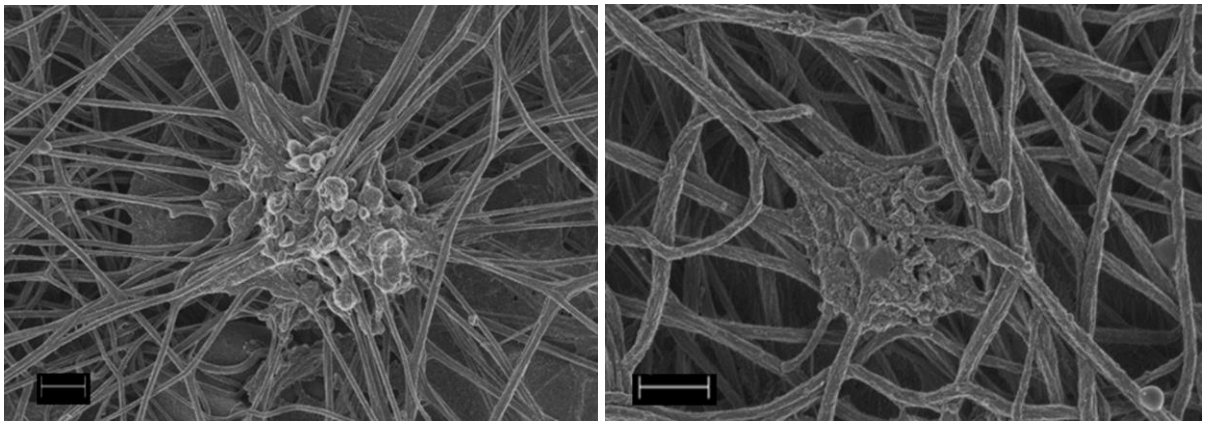
Scanning Electron Microscopy: PLATELETS WITH FIBRIN

Figure 25: SEM- Platelet aggregates merging with the fibrin network. A) Control. B) HIV positive patient with DVT. C to E) HIV negative patients with DVT. All figure scale bars: 1µm.

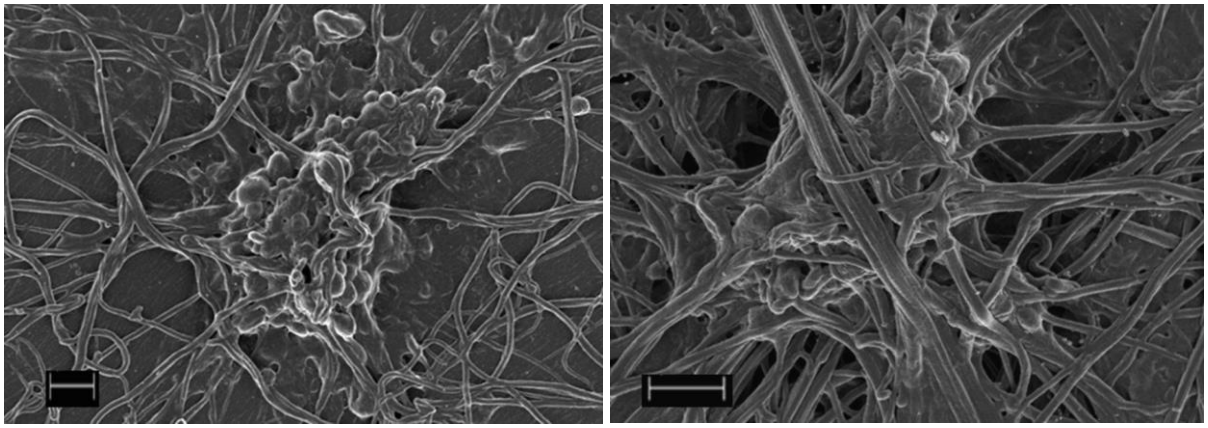
A) **B)**



C) **D)**



E) **F)**



Discussion

Red blood cells

In healthy people up to 44% of red blood cells in the plasma may be abnormal and not always discoid in shape. The red blood cells in patients with ischemic stroke patients, using SEM and light microscopy, were shown to have an atypical morphology in up to 92% of the red blood cells examined. Membrane extensions and shape changes have been seen under SEM. The changes on SEM are not always visible on the light microscopy. The SEM images allows for greater magnification.[64] Abnormal red blood cells also tend to interact with fibrin fibres that are abnormal.[162]

Elevated number of circulating white blood cells (leukocytes) can influence the morphology of the red blood cells. Activated leukocytes release oxygen metabolites and proteases which causes the red blood cells to undergo oxidative and proteolytic changes. Ischemic stroke patients have been shown to have increase number of activated leukocytes as well as abnormal red blood cell morphology.[163, 164]

The HIV- and HIV+ groups both have an elevated leukocyte count compared to the control group (p=0.0026 and p=0.0120 respectively) (**Table 18** and **19; Graph 1**). Similar to patients with ischemic strokes, the leukocyte count may be elevated due to the thromboembolism. DVT also causes an inflammatory reaction and a rise in the leukocyte count.[22] So whether the leukocytes are raised due to related HIV infection or treatment, or to the DVT itself, the effect is an increase in abnormal erythrocytes.

As a DVT forms and occludes the vein, the blood flow decreases. Blood circulating through a narrower vessel results in the surrounding cells including the red blood cells having increased contact time with the activated leukocytes products e.g. reactive oxygen species.[165] With the altered red blood cell morphology, the red blood cell has a decreased potential to deform in a narrowed occluding vein results in a progressive decrease in venous flow and a longer interaction with the activated leukocyte products.[163]

In pro-inflammatory states, reactive oxygen species such as superoxide ($O2^-$), hydrogen peroxide, hydroxyl radicals, etc. are increased which causes oxidative stress to the red blood cells.[165, 166] Systemic inflammation appears to directly translate to red blood cell (and

platelet) morphological and functional changes.[167-171] As mentioned, the effects on the red blood cells have been documented in inflammatory conditions such as thrombo-embolic stroke but also in diseases such as type II diabetes, systemic lupus erythematosus and even Parkinson's disease and Alzheimer-type dementias.[50, 51, 62, 63, 64] These diseases studied are non-communicable diseases, which were not documented in the sample group. There have not been many reports of communicable diseases, specifically HIV, and the effect on red blood cells and the coagulation system.[39, 46, 80, 172, 173, 174]

Serum iron levels can have an effect on the red blood cells. Increase total body iron levels, especially unliganded iron, have been shown to cause a hypercoagulable state as they contribute in oxygen-dependent free radical formation.[175] The results seen on red blood cells are a greater axial ratio on light microscopy and ultrastructural changes shape changes (elongated shape with pointed extentions).[176] In this study the HIV- and HIV+ groups did not have increased levels of serum iron, transferrin or percentage of iron saturation; but had lower than normal levels (**Table 18** and **19; Graph 1**). The total serum iron levels were lower in the HIV+ group compared to that of the HIV- group (the difference was significant in the transferrin levels). However, ferritin levels in the HIV+ group were higher than the HIV- group (239µg/L and 96µg/L respectively) (**Table 20**). Ferritin concentration is used as an important marker of iron overload in Hereditary Hemochromatosis and it reflects unliganded iron that has been released during or following its secretion.[177, 178, 179] This could be in keeping with the increased axial ratio on light microscopy in the HIV+ group compared to HIV- group ($p<0.0001$) (**Table 35**). However, the change in erythrocyte morphology may not be related to the serum ferritin levels as was seen in patients with Parkinson's disease.[62]

A variety of multiple abnormal shape changes and membrane abnormalities are seen in the patients with DVT (HIV- and HIV+ groups) (**Figure 10 to 18**). During inflammatory diseases, red blood cell exposed to oxidative stress and inflammatory molecules undergoes biochemical membrane changes which can result in biophysical shape changes.[84, 166] The red blood cell membrane consists of a phospholipid bilayer with an underlying spectrin-actin cytoskeletal complex.[180] The cytoskeletal complex is anchored to the inner surface of the red blood cell membrane through its association with the protein ankyrin and other trans-membrane proteins.[181-186] Certain red blood cell membrane lipids are mostly found in the

external membrane layer (sphingomyelin and phosphatidylcholine) and others in the internal membrane layer (phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine).[187] Transmembrane proteins, such as flippases, floppases and scramblases, allow for the transfer of the membrane lipids from external to internal and vice versa.[188] During inflammatory conditions there is an increase of plasma concentrations of sphingomyelinase, normally found in lysosomes of macrophages, which converts sphingomyelin into ceramide.[189, 190] Sphingomyelinase has been shown to be increased with inflammatory markers such as platelet activating factor and tumour necrosis factor alpha as well from some bacteria.[190, 192] Ceramide enhances red blood cell sensitivity to an already raised cytosolic calcium concentration.[193] This results in the activation or inhibition of the transmembrane proteins causing an abnormal expression of phosphatidylserine on the external membrane layer. The result is the formation of membrane vesicle formation and microparticle shedding, which is a procoagulant factor. Red blood cell shape changes will then occur and eventually eryptotic red blood cells can be seen.[193-197] Eryptosis is a co-ordinated red blood cell death, similar to apoptosis, that allows for the removal of infected, defective or potentially harmful cells before they undergo hemolysis.[196]

Red blood cells that display phosphatidylserine on the membrane surface contribute to the hypercoagulation state. The abnormal red blood cells provides a prothrombotic surface for the formation of thrombin.[198, 199] Besides adhering to each other, the abnormal red blood cells become “sticky” and tend to adhere to the blood vessel endothelium.[200, 201] Low flow in the vessels also contribute to the cells adhering to the endothelium as demonstrated in microcirculation.[202] The same principle would apply to the venous system, which is a low flow vessel compared to the arterial system, which contributes to the formation of a DVT.

Red blood cell-derived microvesicles or microparticles (**Figure 12D and 12G; Figure 15G and 15H**), microscopic extracellular membranous structures, is associated with the expression of phosphatidylserine.[203] Red blood cell-derived microparticles appear to enhance thrombin generation resulting in a hypercoagulable state, such as in post transfusion DVT, sickle cell disease and haemolytic anaemia.[204, 205] As the microparticles are associated with increased thrombin, the complement system can therefore also be activated and thereby enhancing the systemic inflammatory response which is also a hypercoagulable state.[69] Microparticles

are also thought to originate from CD4 lymphocytes.[68] As the HIV virus infects CD4 lymphocytes, HIV positive patients may be more prone to developing microparticles and therefore enhancing the hypercoagulable state.

The distribution of red blood cells can also reflect a coagulation status.[166] Erythrocytes agglutinating (merging) together and in a rouleaux formation are features of a hypercoagulable state as seen in **Figure 9** and **Figure 16**. The adherence of plasma proteins, such as fibrinogen, contributes to the red blood cells adhering together. The DVT groups both have a higher fibrinogen level compared to the control group, but was only significant in the HIV- group (p=0.0014) (**Table 18** and **19**). A higher fibrinogen concentration is associated with an increased incidence of thrombosis. Fibrinogen may have specific interactions with the red blood cell transmembrane protein receptors.[206-208]

Whole blood with thrombin SEM slides demonstrates the incorporation of red blood cells into the fibrin network. The red blood cells can influence the fibrin network distribution, causing the fibrin fibres to be loosely surrounding the red blood cells as seen in **Figure 11** and **Figure 13.** Fibrin fibres also tend to increase in diameter and the viscoelasticity may also be influenced by the red blood cell inclusion in the fibrin network.[198] The incorporation of red blood cells into the fibrin network stabilises and strengthens the clot by decreasing the permeability of fibrin and increasing the resistance to fibrinolysis.[209, 210] A decreased R-time is associated with trapped deformed red blood cells.[211] In the HIV- and HIV+ groups, which had decreased R-time and K-times (**Tables 25** and **26**; **Graph 2**), trapped red blood cells between the fibrin network can be seen in **Figure 12I-K** and **Figure 17.**

Under conditions of low partial pressure of oxygen, acidosis and in response to mechanical deformation, red blood cells release adenosine triphosphate (ATP) and adenosine diphosphate (ADP) activating platelets and promoting aggregation and release of platelet granules.[205] The activation of platelets, together with fibrins network, contracts the clot containing the trapped red blood cells into a tight package (**Figure 18**). The result is the formation of polyhedrocytes which is commonly found in DVT's (**Figure 14 and Figure 17**).[213]

The TEG results can be correlated with the changes seen on the SEM, although not all the TEG parameters were abnormal. 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.[211] The DVT groups (HIV- and HIV+) compared to the control group showed significant changes on the TEG using whole blood (**Table 25 and 26**; and **Graph 2**). The HIV-group compared to the control group showed significant differences with regards to R-time (p-value of 0.0021), K-time (p-value of 0.0323), MRTGG (p-value of 0.0314) and the TMRTGG (0.0087). Similarly, the HIV+ group compared to the control group showed significant differences with regards to R-time (p-value of 0.0022), K-time (p-value of 0.0151), alpha angle (p-value of 0.0303), MRTGG (p-value of 0.0123) and the TMRTGG (p-value of 0.0039). There were no significant TEG differences between the HIV- and HIV+ groups (**Table 27**). As for the SEM, where changes were seen in the DVT groups, a hypercoagulable response is observed on the TEG.

By comparing the TEG results using platelet poor plasma, to remove the effect of the blood cells from the clot formation, a hypercoagulable profile is still observed. The TEG results in the platelet poor plasma have a similar comparison profile between the 3 groups as using whole blood (**Table 31-33; Graph 3**)**.** In the HIV negative DVT group compared to the control group the changes in R-time, K-time, MRTGG and TMRTGG are statistically significant as it is using whole blood (**Table 31**). However, in the platelet poor plasma, MA and TGG also have a statistically significant increase. The MA and TGG are strongly influenced by the fibrin network. The changes in the platelet poor plasma compared to the whole blood indicates that the fibrin fibres may be abnormal. In the HIV positive group compared with the control group, both platelet poor plasma and whole blood results in changes in the same TEG parameters i.e. R-time, K-time, MRTGG and TMRTGG (**Table 32**). Whether using platelet poor plasma or whole blood there are still no statistically significant changes in any of the TEG parameters when comparing HIV negative and HIV positive DVT patients (**Table 27** and **33**).

Both DVT groups (HIV negative and HIV positive), using whole blood or platelet poor plasma, indicate a hypercoagulable profile that has a rapid initiation and amplification resulting in the rapid formation thrombin and therefore fibrin fibre network. However the concentration of thrombin that forms although increased compared to the control group is not significant except in the HIV negative DVT group using platelet poor plasma (**Table 31**).

The strength of the clot formed is also not significantly different from the control group. The exception, once again, is the HIV negative DVT group using platelet poor plasma (as mentioned above, may be due to abnormal fibrin fibres).

The TEG parameters in the HIV positive DVT group compared to the HIV negative DVT group do indicate a hypercoagulable profile but whether using whole blood or platelet poor plasma, there was no statistical significance in any of the parameters. HIV infection, according to the TEG, therefore does not appear to result in a more hypercoagulable state than already present with that of the DVT. Whether there is a difference between HIV positive patients not on ARV compared to those on ARV treatment could not be assessed as there were only 2 patients not on ARV treatment that were recruited in this study, which is a reflection of the improved South African ARV distribution. However, a study by Haugaard and colleagues did not show any functional differences (TEG), meaning the coagulopathy in HIV infected patients is not reversed with ARV treatment.[173]

Platelets

The ultrastructure of platelets in the HIV+ group has features different to that of the control group and the HIV- group (**Figure 19-24**). These changes are not due to coexisting opportunistic infections or inflammatory conditions as HIV+ patients with a WHO clinical stage 2 to 4 were excluded from the study. The control group and the HIV- groups have 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.[36] The HIV positive patients also have similar features of activated platelet aggregates 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.[39]

The 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.[39, 41, 214] 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.[41] With the latter, the ultrastructural changes may be due to the immune response of the body resulting in antibody-induced destruction of the platelets.[39]

The HIV+ group had 13 patients on the primary ARV regimen and 2 which were not on any ARV's. The platelet ultrastructure had similar features of apoptosis whether the patients were on ARV or not. The ARV treatment does not therefore appear to improve the platelet morphological changes. This finding is not in keeping with a study, by Pretorius and colleagues in 2009, on platelets and HIV+ patients that were using Canova, a herbal immune-stimulator.[46] In that study the HIV+ patients on Canova showed improved platelet morphological changes. There are differences though between the two studies. This study only included patients with a DVT, so the patients were known to be in a hypercoagulable state, also 13 HIV+ patients were on ARV's, which consists of 3 combined medications (Emtricitabine, Tenofovir and Efavirenz). Canova is also an immune-stimulator whereas the ARV's do not stimulate the immune system but rather inhibits the HIV virus.

ARV's have shown to have platelet related effects such as a decreased prevalence of HIV associated thrombocytopaenia but may have an increased bleeding risk.[37] 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.

Platelet functioning depends on their quality and the quantity.[215] The platelet count, the quantity, was within normal limits in all 3 groups (**Table 18-20**)**.** The ultrastructural changes of platelets in the DVT groups does not appear to translate to functional differences on all the TEG parameters using whole blood (**Table 25-27**). The TEG maximum amplitude (MA), indicating the strength of the clot, and the total thrombin generation (TTG) are influenced by platelets and fibrinogen.[216-219] Activated platelets release activated factor V and express phospholipids to promote thrombin generation.[220] The MA and TTG represents the platelet interaction with the fibrin network. In a whole blood clot the MA relates to the platelet-

fibrin interaction whereas the TTG relates to the overall fibrin generation.[211] The MA for the HIV- and HIV+ groups using whole blood were not significantly different from the control group (p-values of 0.2578 and 0.4302 respectively), which is similar to the SEM results where the platelets interacting with the fibrin strands appear to be similar in the different groups, although the control group fibrin strands does appear more orderly around the platelet (**Figure 25**)**.** A study by Ronsholt et al. also did not show a difference in the fibrin clot strength in HIV+ patients compared to controls.[77]

The activated platelets did not result in an overproduction of thrombin. This was represented by the TTG in the both the HIV- and HIV+ groups, which were not significantly different from the control group (p-values of 0.9217 and 0.4412 respectively). However, the rate (MRTGG) to achieve maximum thrombin generation was significantly increased. Also, the time to achieve that maximum rate of thrombin generation (TMRTGG) was significantly decreased in both the DVT (HIV- and HIV+) groups indicating a hypercoagulable state in which the activated platelets do contribute. Interestingly though, red blood cells can contribute directly to thrombin generation as they present a larger source of procoagulant cell membranes than the activated platelets.[221] Therefore it is possible that the increased MRTGG and TMRTGG may be as a result of the influence by the red blood cells, especially the abnormal red blood cells, in combination with the activated platelets.

The SEM is therefore able to detect platelet changes whereas the haematological markers of platelet activation, mean platelet volume (MPV) and the viscoelastic test (TEG) did not show significant differences. This statement is also in keeping with the finding of increased P-selectin, a marker of platelet activation, has been reported to be elevated in HIV positive patients.[222, 223] A study by Majluf-Cruz and colleagues reported no recurrence of thrombotic episodes, after previous recurrent episodes while on anticoagulants, in patients with Acquired Immunodeficiency Syndrome (AIDS) that were on antiplatelet medication. However, this observation was based on 6 patients.[172]

Conclusion

Patients with a deep venous thrombosis (DVT) have a hypercoagulable profile on the viscoelastic testing (TEG). There does not appear to be a significant difference between those with HIV infection and those without.

The light microscopy demonstrated abnormal red blood cell changes in the DVT groups, in keeping with an inflammatory state. These changes were more prominent in the HIV positive group. There were more shape variations in the HIV infected patients as demonstrated on the axial ratios. Abnormal red blood cell distribution, such as rouleaux formation, is also prominent in the HIV positive group.

The ultrastructure allowed more in-depth investigation at a high magnification. Both HIV negative and HIV positive groups demonstrated red blood cell shape and size variation, membrane abnormalities and microparticle/microvesicle formation. These changes appear to be part of a spectrum of eventual eryptosis, which is a co-ordinated suicidal death of the red blood cells, similar to apoptosis. The abnormal expression of phosphatidylserine, a membrane lipid, on the external membrane layer of red blood cells is the common finding related to this process. Both groups also demonstrated compact fibrin strand binding with the red blood cells. All these changes were more prominent in the HIV positive patients. The abnormal red blood cell findings are in keeping with changes documented in inflammatory conditions. These changes can also promote the hypercoagulable state and/or be caused by the hypercoagulable state.

The interaction between platelets and the fibrin strands between the 3 groups were similar on the SEM which was in keeping with the results of the maximum amplitude on the TEG. Both HIV negative and positive DVT groups, using whole blood, were not significantly different from the control group. However, the ultrastructure of activated platelets in the HIV infected patients demonstrated significant changes. HIV appears to cause features of apoptosis in the platelets. Release of procoagulant platelet phospholipids contributes to the hypercoagulable state. These changes may be due to altered viral infected megakaryocyte morphology or due to direct infection and damage by the HIV virus.

The SEM is able to detect changes when other tests such as haematological markers and viscoelastic test (TEG) are unable to. Assessment of HIV related hypercoagulability, and other procoagulant conditions, should include ultrastructural analysis of the red blood cells, platelets and their interaction with the fibrin network.

CHAPTER 6: VISCOELASTIC AND ULTRASTRUCTURAL PROPERTIES OF PLATELET POOR PLASMA

Introduction

HIV infection is associated with various abnormalities predisposing to a hypercoagulable state such as raised procoagulant factors (antiphospholipid antibodies, lupus anticoagulant, tissue factor, circulating microparticles, etc.) and decreased anticoagulant factors (protein C, protein S, anti-thrombin, etc.).[224] The thrombus that forms is therefore more fibrin-based, which eventually traps the red blood cells in the fibrin network. This is typical of a venous thrombosis, a red clot, compared to an arterial thrombosis, a white clot, which is more platelet-based.[225]

There is approximately 0.10% risk of DVT per year in the general population.[226-228] The incidence of DVT in patients with HIV infection increases to two to ten times that of the healthy population of comparable age.[224, 229-237]

The process of venous coagulation, whether using the cell-based model or the cascade/waterfall model with the extrinsic and intrinsic pathways, thrombin is formed which allows for the conversion of soluble fibrinogen into insoluble fibrin strands. Fibrinogen is a soluble plasma protein that is 2 to 3 nm in diameter and 45nm in length. There are 3 pairs of polypeptide chains which results in the protein with 2 distal and 1 central globular regions. The polypeptide chains are termed Aα, Bβ and Y.[238, 239] The 2 globular regions are connected to the central region by 2 coiled coils which consists of 3 α-helices each. The coiled coils are 17nm in length.[239] The whole structure is supported by 29 disulphide bonds. Fibrinopeptides, A and B, from the amine end (N-terminal) of the Aα and Bβ, respectively, are cleaved by thrombin. A fibrin monomer is then formed. The monomers attach to one another to eventually form fibrin fibres. [238, 239]

Factor XIII is bound to fibrinogen in the circulation. Activated thrombin not only activates fibrinogen but also Factor XIII.[145, 238-240] Factor XIII is a heterotetramer consisting of 2 catalytic subunits (FXIII-A) and 2 non-catalytic subunits (FXIII-B). Thrombin catalyses the release of amine (N) terminal peptides from the 2 catalytic subunits. Calcium is required for the dissociation of the non-catalytic subunits from the hydrophobic catalytic subunits. The

changes in the 4 subunits activates Factor XIII.[145, 241] Activated factor XIII stabilises the clot by covalent cross-linking fibrin α-chains and fibrin Y-chains.[31,36] Factor XIII also binds antifibrinolytic proteins; such as α2-antiplasmin, thrombin-activatable fibrinolysis inhibitor (TAFI) and α2-macroglobulin; and fibrin and thereby decreasing the lysis of the fibrin fibres by plasmin.[33, 240, 241]

Factor XIII then promotes the fibrin fibres to crosslink forming a stable fibrin fibre network.[31, 36] Plasmin, which is an activated plasma protein, lyses the fibrin fibres into soluble degradation products resulting in resolution of the clot as well as ensuring the clot does not extend beyond the initiating site,.[33]

Increase in fibrinogen concentration and delayed breakdown of the fibrin clot may result in a hypercoagulable state. Fibrinogen is an acute-phase protein which increases during inflammation. Fibrinogen synthesis is induced by the pro-inflammatory cytokine interleukin-6.[27]

Assessment of the clot structure allows for the interpretation of the overall haematological state in the patient.[36] The fibrin clot consists of fibrin fibres of different diameter. Thick fibrin fibres forms the majority of the clot and thin minor fibres are arranged among the major fibres.[49] Changes in fibrin thickness has been demonstrated in different conditions.[50, 51, 63, 160, 242] In inflammatory disease states the minor fibres can increase in volume and form a net covering the thick major fibres.[49]

The fibrin network distribution or configuration has also been shown to be present in a hypercoagulable state.[243-247] The configuration has more of an effect on the fibrinolysis rate than the thickness of the fibrin fibres.[248] A tighter configuration, compact fibrin clot, is more resistant to fibrinolysis than a loose configuration.[242, 249]

In order to assess the fibrin fibres without the influence of platelets and red blood cells, whole blood is centrifuged to isolate plasma. The platelet poor plasma therefore does not contain any cells but only the coagulation factors and thereby allowing to assess a fibrin-based thrombus. The viscoelastic properties of the platelet poor plasma should correlate with the morphological structure of the fibrin fibres and the configuration of the fibrin fibre network.

The total thrombus generation (TTG) on the TEG relates to the fibrin network configuration, the more open spaces between the branching elongated fibrin fibres should correspond with a decrease TTG. The less open spaces between the branching elongated fibrin fibres should correspond with an increased TTG.[211]

The maximum amplitude (MA) on the TEG, which represents the clot strength, is determined by platelets and the fibrin fibres together. However by using the platelet poor plasma, only the strength of the fibrin fibres are assessed. The morphological assessment of the individual fibrin fibre thickness, or diameter, correlates with the MA. An increased fibre diameter should correlate with a raised MA, and vice versa.[211]

Hypercoagulability may not cause a change in all the TEG parameters. The degree and the cause of the hypercoagulable state are related to the number of parameters and which parameter specifically is abnormal.[211] Changes not detected on the TEG may however present as significant changes on the ultrastructural examination of the fibrin strands and the fibrin network. The following objective therefore directs this chapter.

Objectives

7. Compare platelet poor plasma with thrombin using SEM to the viscoelastic results.

Materials and methods

Objective 7

Objective 7 is to compare platelet poor plasma with thrombin using SEM to the viscoelastic results. Platelet poor plasma (citrate tube) for SEM was prepared as follows:

- After the blood sample was collected it was then centrifuged to obtain platelet poor plasma (PPP).
- 10 µl of platelet poor plasma was placed directly on a glass cover slip.
- Platelet poor plasma was mixed immediately with 5 µl thrombin.
- The platelet poor plasma with thrombin smears on the cover slips were then incubated for 3 minutes.
- The cover slips were then immersion in 0.075 M sodium phosphate buffer (pH 7.4) and finally placed on a shaker for 20 minutes.
- Fixation of smears were done by using 2.5% gluteraldehyde in phosphate-buffered saline (PBS) solution with a pH of 7.4 for 30 minutes, followed by the wash step where the samples were washed 3 times in phosphate buffer for 3 minutes before the final fixation step of 15 minutes in 1% osmium tetraoxide (OsO_4).
- The samples were again washed 3 times with PBS for 3 minutes and the dehydration of the samples were done with 30%, 50%, 70%, 90% and 3 times with 100% ethanol (different concentrations of ethanol are used to avoid osmotic shock of samples).
- After the dehydration step the material were immersed with hexamethyldisilazane (HMDS) for 30 minutes
- The HMDS was taken off and a drop of HMDS was be added. The samples were then be left to dry.
- After the samples were dried they were mounted on metal plates and coated with carbon to provide conduction under the microscope.

- A Zeiss ULTRA plus FEG-SEM with In Lens capabilities was used to study the surface morphology of the fibrin strand ultrastructure and diameter; and micrographs were taken at 1kV. (The scanning electron microscope that was used for this study is located in the Microscopy and Microanalysis laboratory of the University of Pretoria, Pretoria, South Africa.)
- To quantify the density of the fibrin network, ImageJ was used. The mean and standard deviation of the intensity of the pixels in the images of the clot were calculated using the histogram function. The coefficient of variation could then be calculated (i.e. standard deviation /mean) which would represent the intensity of the clot structure.
- To measure the fibrin fibre diameter, ImageJ was used. The fibrin fibre diameters of 50 different fibres were measured per subject.

To assess the viscoelastic properties of platelet poor plasma:

- Platelet-poor plasma was inserted into the cuvette
- The cuvette containing the blood is moved with a stationary pin
- Activator (calcium chloride) was added to the blood sample
- The resistance of the pin in the blood sample was recorded on the connected computer
- The data was presented as a curve with its numerical parameters.
- Refer to Table 2: Parameters of Whole blood and Platelet poor plasma in viscoelastic tests for the data analysed from the viscoelastic parameters.

Results

For the viscoelastic results for the platelet poor plasma, refer to Chapter 5 ***Tables 28-33****; and* ***Graph 3****.*

The fibrin fibre diameters using platelet poor plasma for the Controls, HIV negative DVT group and the HIV positive DVT group are shown in **Table 36**. The fibre diameters were measured in nanometers. The table shows the median, standard deviation, minimum and maximum values for each group.

The comparison between groups are shown in **Table 37.** P-values were calculated using the Mann-Whitney test with a significance level of 0.05. The fibrin diameters in the HIV negative and HIV positive groups were both statistically significant compared to the control group, with p-values <0.0001 for both groups. There was no significant difference between the HIV negative and HIV positive DVT group though.

Table 36: Fibrin fibre diameter

Group	N	Median	Minimum	Maximum	Standard deviation
Controls	850	118.3	32.26	251	36.53
HIV-	1550	136.9	48.09	328.3	43.1
HIV+	750	135.4	48.09	365.1	43.63

nm: nanometers

Table 37: Comparison of the fibrin fibre diameters

Comparison Groups	P-value	Significant difference
Controls vs. HIV-	< 0.0001	Yes
Controls vs. HIV+	< 0.0001	Yes
HIV- vs HIV+	0.5915	No

The coefficient of variation using platelet poor plasma for the Controls, HIV negative DVT group and the HIV positive DVT group are shown in **Table 38**. The table shows the median, standard deviation, minimum and maximum values for each group.

The comparison between groups are shown in **Table 39.** P-values were calculated using the Mann-Whitney test with a significance level of 0.05. There was no significant difference between any of the groups.

Table 38: Coefficient of variation

Group	N	Median	Minimum	Maximum	Standard deviation
Controls	16	0.4833	0.4502	0.9173	0.1309
HIV-	31	0.5372	0.2837	0.9085	0.164
HIV+	15	0.6469	0.3563	1.166	0.29

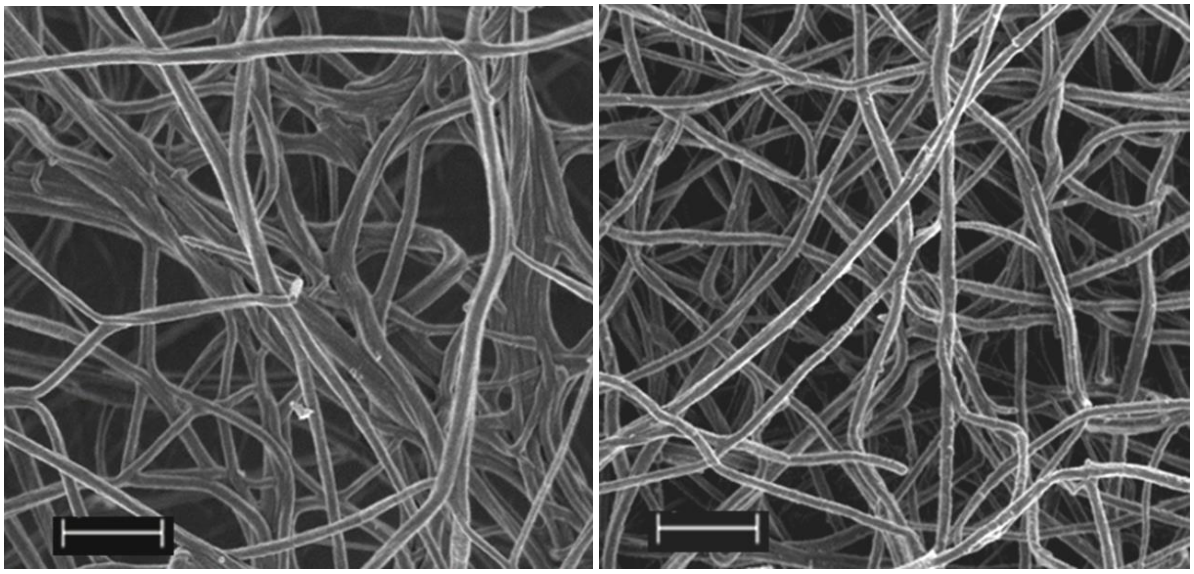
Table 39: Comparison of the coefficient of variation

Groups	P-value	Significant difference
Controls vs. HIV-	0.4656	No
Controls vs. HIV+	0.1727	No
HIV- vs HIV+	0.1399	No

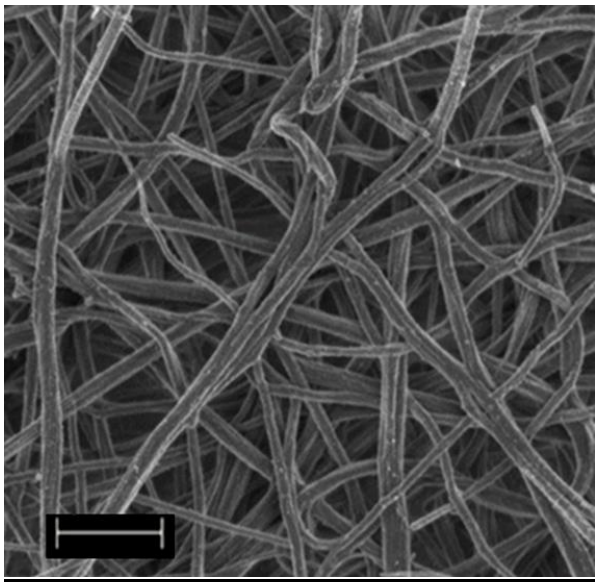
Scanning Electron Microscopy: Controls

Figure 26: SEM Controls- Fibrin fibre network. A-C) Distribution of fibrin fibre network. The fibrin fibres are smooth. All figure scale bars: 1µm.

A) **B)**



C)



Scanning Electron Microscopy: HIV negative DVT group

Figure 27: SEM HIV negative patient with DVT- Fibrin fibre diameter. Label A demonstrating the diameter measurement of a fibrin fibre. Figure scale bars: 0.1µm.

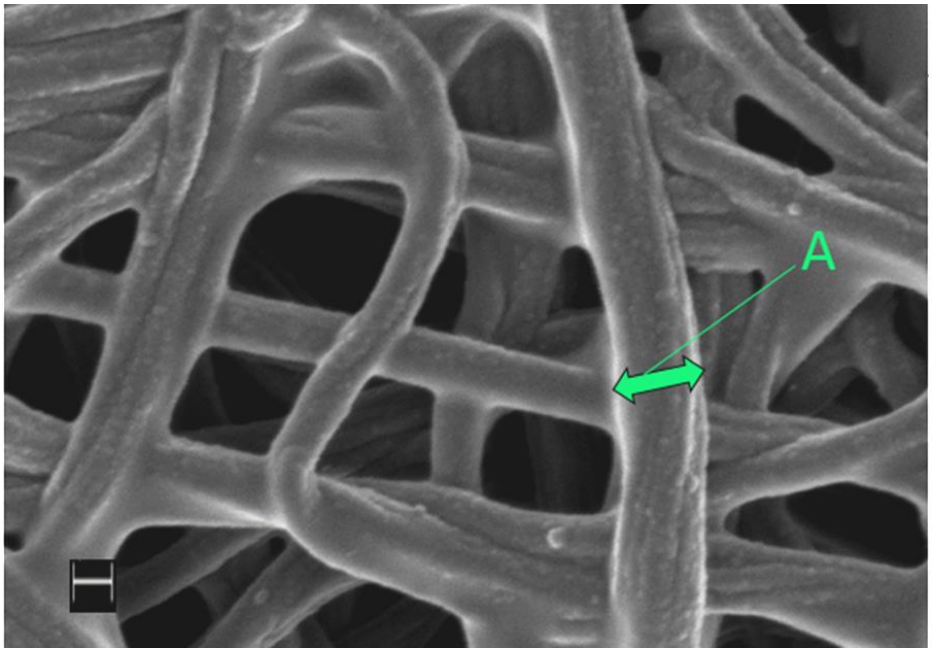


Figure 28: SEM HIV negative patient with DVT- Dense matted deposits. The open-arrow-head showing dense matted deposits. Figure scale bars: 0.1µm.

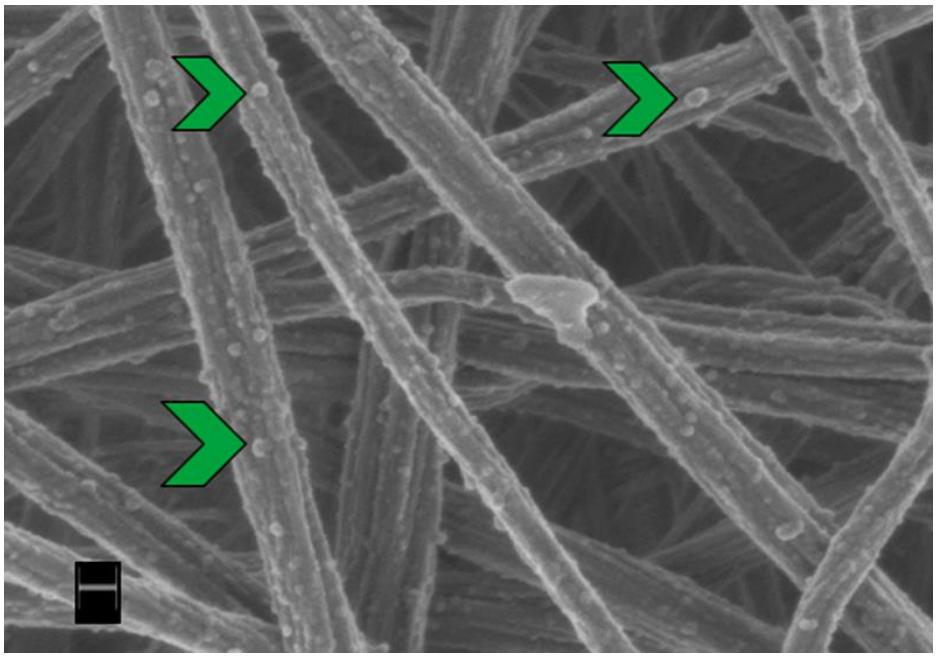
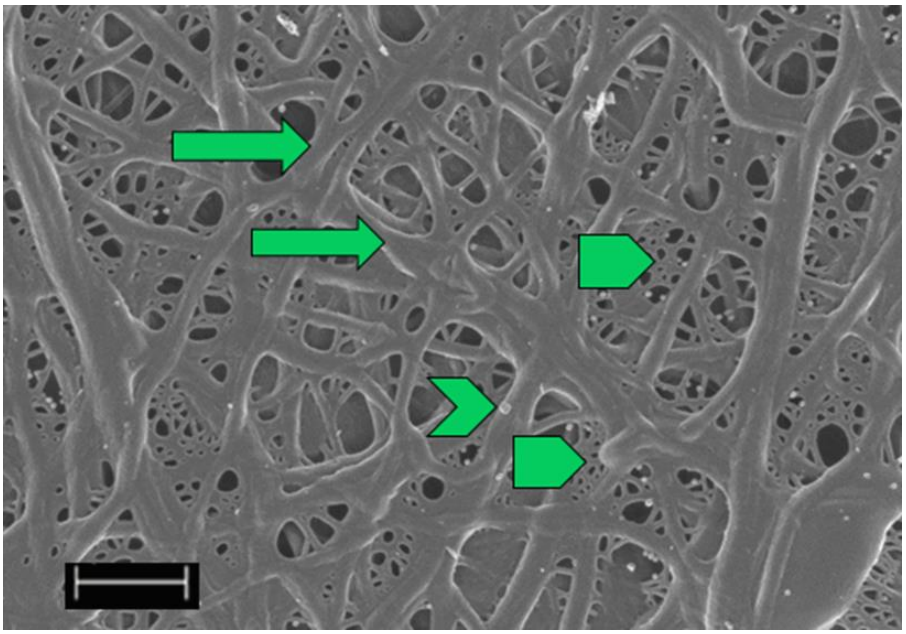


Figure 29: SEM HIV negative patient with DVT- Abnormal fibrin network. A) 34 000x magnification, B) 200 000x magnification. Dense matted deposits gives the appearance of an abnormally thick and rigid clot. The arrows showing thick fibrin fibres. The arrow-heads showing thin fibrin fibres. The open-arrow-head showing dense matted deposits. Figure A scale bar: 1µm; Figure B scale bar: 0.1µm.

A)



B)

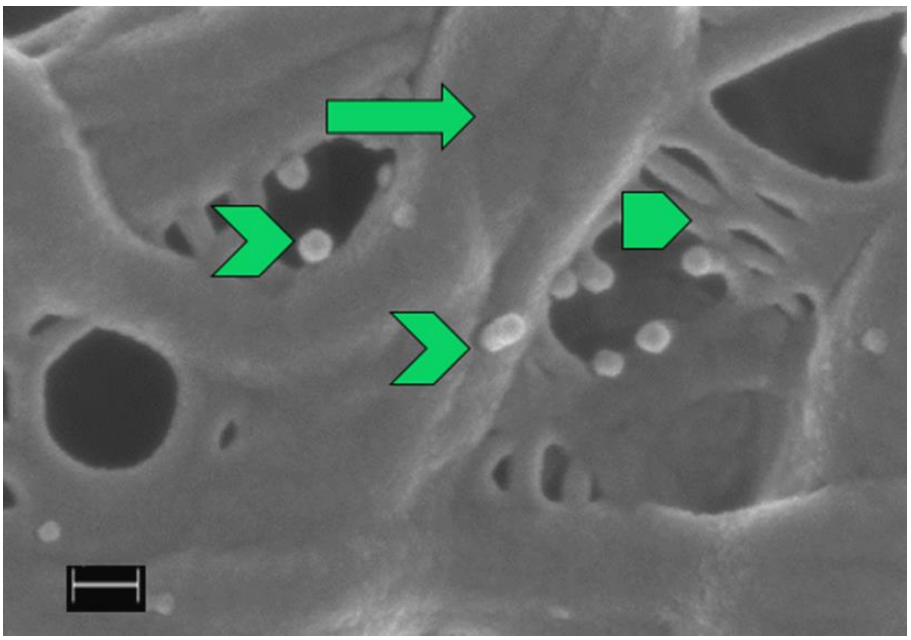
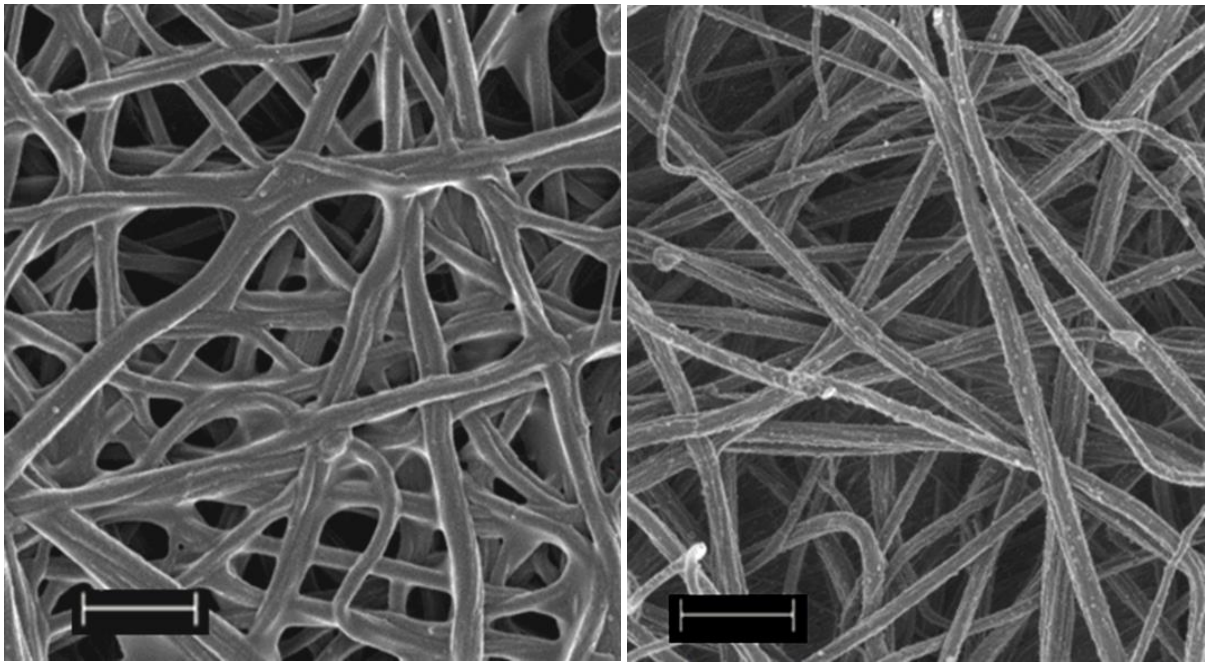
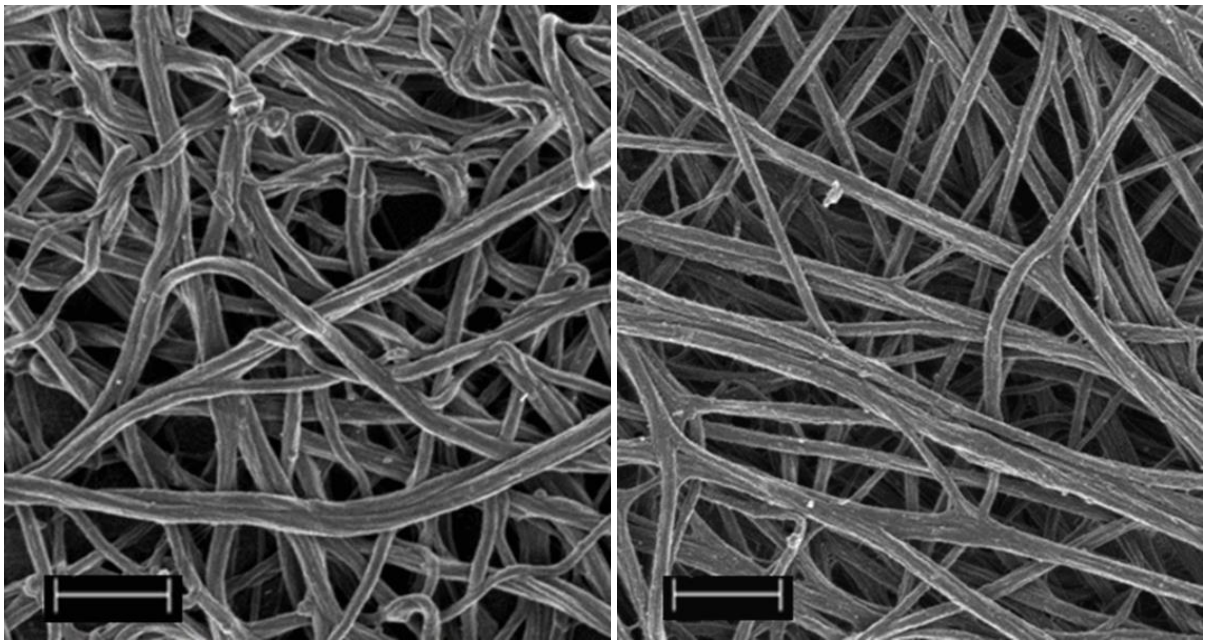


Figure 30: SEM HIV negative patients with DVT - Compact fibrin network. A-D) Dense fibrin network. The dense compact distribution of fibrin network indicating a hypercoagulable state. The fibrin network appears more compact compared to the control group. All figure scale bars: 1µm; except figure E where the scale bar is 0.1µm.

A) **B)**



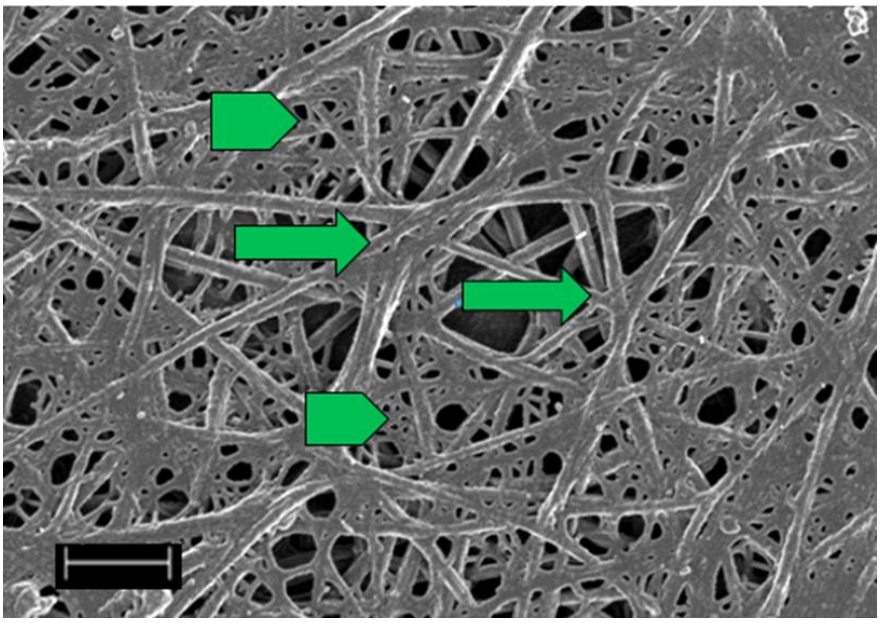
C) **D)**



Scanning Electron Microscopy: HIV positive DVT group

Figure 31: SEM HIV positive patient with DVT- Abnormal fibrin network. A) 34 000x magnification, B) 100 000x magnification. Dense matted deposits gives the appearance of an abnormally thick and rigid clot. The arrows showing thick fibrin fibres. The arrow-heads showing thin fibrin fibres. The open-arrow-heads showing dense matted deposits. Figure A scale bar: 1µm; Figure B scale bar: 0.1µm.

A)



B)

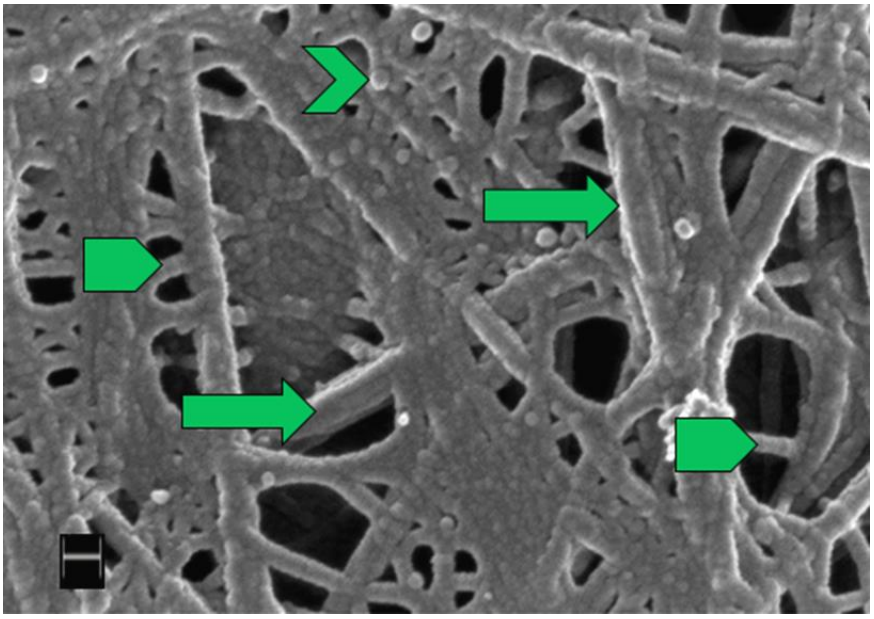


Figure 32: SEM HIV positive patient with DVT- Dense matted deposits at 100 000x magnification. Open-arrow-heads showing dense matted deposits. Scale bar: 0.1µm.

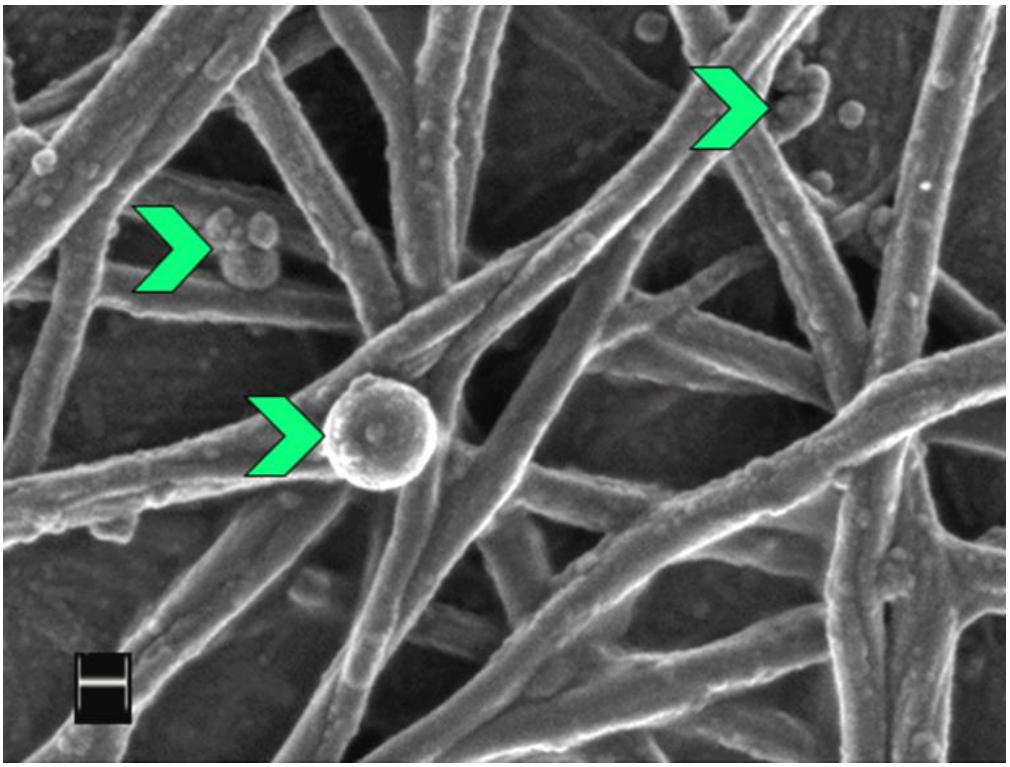
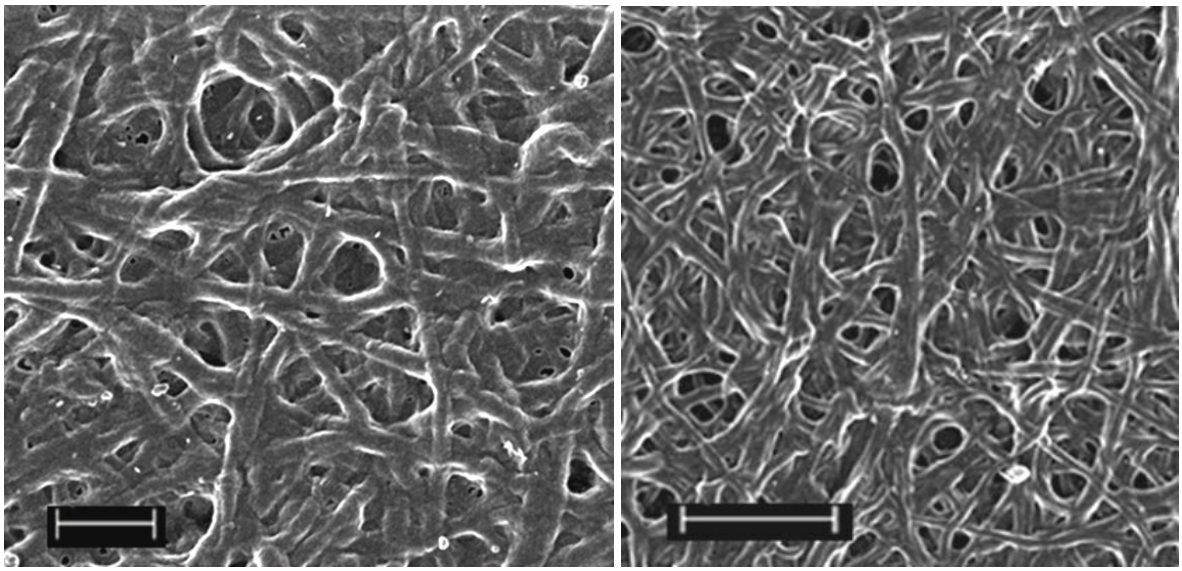
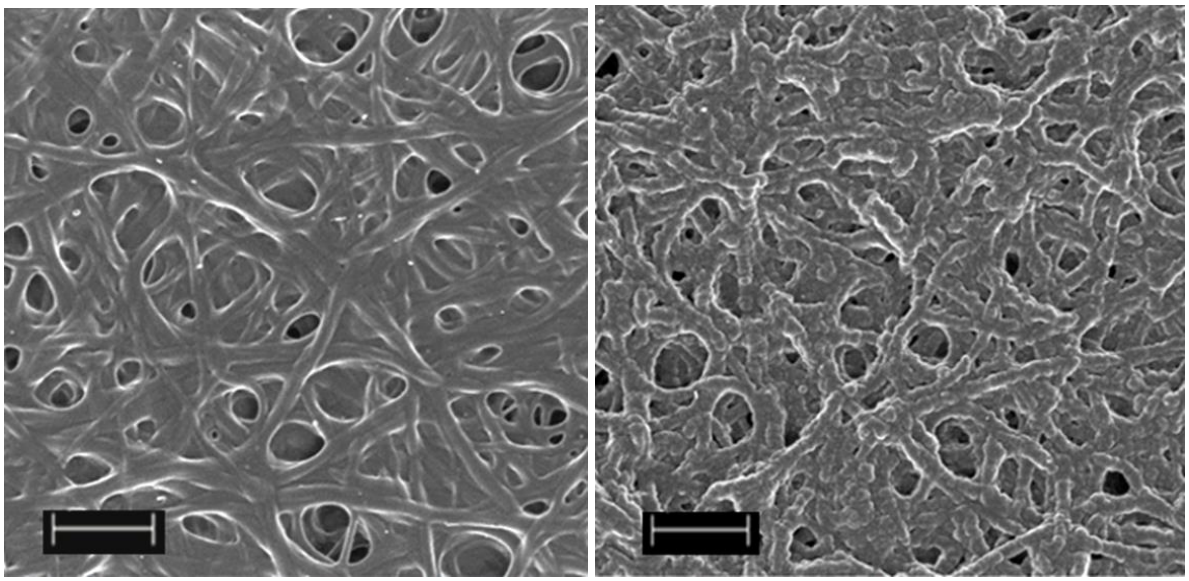


Figure 33: SEM HIV positive patients with DVT- Compact matted fibrin network. A-E) Dense matted fibrin network. The dense compact distribution of fibrin network indicating a hypercoagulable state. The abnormal fibres are thicker than normal fibres and have a different ultrastructural morphology when compared to the controls. Dense matted deposits gives the appearance of an abnormally thick and rigid clot. All figure scale bars: 1µm; except figure E where the scale bar is 0.1µm.

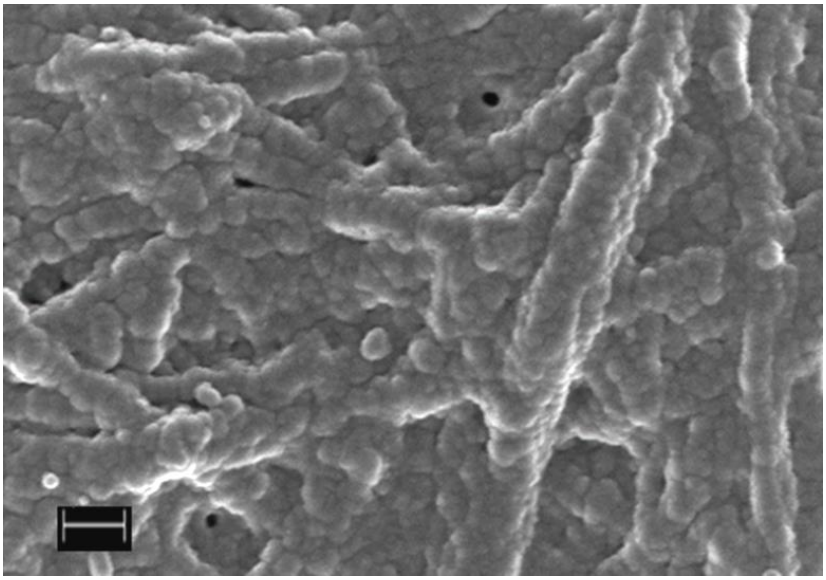
A) **B)**



C) **D)**



E)



Discussion

The viscoelastic study (TEG) does indicate a hypercoagulable state in both the HIV negative and HIV positive DVT groups. (**Table 31** and **32**). There was no statistically significant difference between the HIV negative and HIV positive groups (**Table 33**)**.** The scanning electron microscopy allowed for the ultrastructural changes to be seen. The fibrin fibre thickness is increased and the fibrin network distribution are more compact in the HIV positive and negative groups (**Table 36** and **37; Figure 26, 27 29, 30, 31 and 33**).

The normal architecture of the fibrin network contains mainly thick major fibres with thin minor fibres distributed in between.[250] Multiple factors can influence the diameter, thickness, of the fibrin fibres and also the distribution of the fibrin network.[251, 252]

Bacteria have been found to be present in the blood of patients, even though the patient may not be symptomatic of an infection and blood cultures are negative (have not grown any bacteria). These bacteria are in a dormant state can still be reactivated. Kell and colleagues has stated that dormant bacteria can periodically reactivate or awaken and proliferate before returning to a dormant state.[251]

Bacteria can release highly inflammatory cell wall components, bacterial endotoxins, known as lipopolysaccharides (LPS) which can stimulate the coagulation system.[253, 254] LPS can activate the coagulation pathways by upregulating the amount of tissue factor.[251, 255, 256] LPS can also bind directly to fibrinogen and change the protein structure to form fibrin fibres. The fibrin fibres produced however, appear to vary in diameter. A study by Pretorius et al. demonstrated the effects of adding minute amounts of LPS to fibrinogen resulted in thinner fibres.[27, 257] Fibrin fibre diameters in DVT patients are increased compared to the control group, this includes the HIV negative group ($p<0.0001$) as well as the HIV positive group ($p<0.0001$) (**Table 36** and **37**) The viscoelastic parameters in the HIV positive group compared to the control group did not always correlate significantly with the ultrastructural findings. The increased fibrin fibre diameter did correlate with the raised MA on the TEG, but was not statistically significant (p-value of 0.0528) (**Table 32**). There was no significant difference between the fibrin fibre diameters of the HIV negative DVT group and the HIV

positive DVT group (p=0.5915) (**Table 33**). The proposed mechanism for the change in fibre diameter is also suspected to be the effect of LPS on the fibrin fibres.

Bacteria in the blood, bacteraemia (usually caused by Gram-negative bacteria), are common in HIV infected patients due to the decreased immune status.[259, 260, 261] The incidence of bacteraemia in HIV infected patients can range from 0 to 27%. At least half of all HIV infected patients will be infected with bacteria during the course of their disease.[259] Bacteria, although reduced, with the treatment of ARV's are still present in many patients.[260] This can result in an increase in LPS released which can activate the coagulation system resulting in the DVT. Slotta and colleagues termed this effect as endotoxin-mediated hypercoagulation.[261] This could be the reason for the variation in fibrin fibre diameters in HIV infected patients. LPS would therefore be the reason for the change in fibre diameter which can be the cause of the DVT.

In HIV non-infected patients the presence of LPS should be minimal if any. The change in fibrin fibre diameters in this group can therefore not be attributed to the LPS. The variation in the fibres may actually be the result of one of the causes that resulted in the DVT; or to the DVT itself (remembering that the qualifying criteria for the study was that the study groups have already been diagnosed with a DVT). The anticoagulation pathways (protein C and S; plasmin; thrombomodulin; etc.) limits the spread of a DVT to a localised segment of vein. The DVT itself could, however, prime the fibrinogen systemically. The change in fibrin fibre diameter would therefore be the result of the DVT and not the cause.

Cytokine interleukin-6 and fibrinogen are both involved in the coagulation and inflammation pathways. Interleukin-6 induces fibrinogen synthesis by hepatocytes and other cells. Fibrinogen is also an inflammatory marker (an acute-phase protein) and the concentration of which is influenced by interleukin-6.[27] Fibrinogen which is produced as a result of inflammation instead of specifically for the coagulation pathway may possibly also explain the change in fibrin fibre diameters compared to the control group. Studies have shown changes in the fibrin fibre diameter as well as the architecture of the fibrin network in the presence of inflammatory conditions. Fibres have been seen to be larger (thicker) than normal in Alzheimer's type dementia.[63] Fibrin fibres can also be smaller than normal in conditions such as strokes, thermal injuries and rheumatoid arthritis.[49, 52, 250] In the HIV

infected patients a state of inflammation may be present because of the HIV infection itself or the consequence of the HIV infection. In both the HIV positive and HIV negative groups the presence of the DVT can also result in an inflammatory state.

The fibrin diameter plays a role in the efficiency of breakdown or lysis of the fibrin fibres by plasmin. The larger the diameter of the fibres, the longer it takes to breakdown. The arrangement of the fibres in the fibrin fibre network also determines the time to lyse. Fibres arranged in a compact mesh will take longer to lyse than fibres arranged in a coarse (loose) network.[27]

The fibrin network in the HIV negative DVT group (**Figure 30**) does appear more compact compared to the control group (**Figure 26**). However, the HIV positive DVT group (**Figure 33**) appears even more compact than that of the HIV negative DVT group. Although SEM analysis is typically used only as a qualitative method, the coefficient of variation can be used to quantitatively assess the density of the fibrin clot, by examining the variance between light and dark pixels. The more light pixels present, the denser is the clot. The coefficient of variation did show a trend of a denser more compact distribution of the fibrin fibres in the HIV positive group, and less so in the HIV negative group, compared to the control group (**Table 38** and **39**). These changes were not statistically significant between the 3 groups though. This non-significant result may not be truly representative as the coefficient of variation is not normally distributed in which the ImageJ programme calculates the mean and standard deviation of the intensity of the pixels in the images of the clot.

The viscoelastic parameters in the HIV positive group compared to the control group did not correlate significantly with the ultrastructural findings. The dense compact fibrin network did correlate with the raised TGG on the TEG, but was not statistically significant (p-value of 0.0528) (**Table 32**; **Figure 33**). The statistically significant rise in TGG in the HIV negative DVT group correlated with the compact fibrin network on SEM (**Table 31**; **Figure 30**).

Blood clots have a tendency to contract allowing the clot to become more stable and compact.[262] Microscopically this is seen as protein aggregation which expels water resulting in reduced volume.[239] Under tension forces of compression and extension of the fibrin clot,

protein aggregation is stimulated to accommodate strain. The central nodule is the most stable portion of the fibrin molecule and only allows minimal extension. The distal Y-nodules can undergo sequential unfolding but the main mechanism of accommodation to tension is due to changes occurring at the level of the coiled coils of the fibrin protein.[263] The α-helical structure is changed into a β-structure. The aggregation of these β-structures results in tightly packed β-sheets being formed. These β-sheet-mediated protein aggregations have been proposed to be the mechanism of the different types of fibrin deformation. β-sheets are not usually present more than 40% in fibrin under normal conditions but may do so under pathological conditions.[255] The β-sheet-mediated protein aggregation results in different fibrin fibre ultrastructure and causes fibre bundling on the SEM.[238, 255, 263] This may be result of the change in the architecture of the fibrin fibre network into a netted mat appearance.

In a study by Collet and colleagues using confocal microscopy, 75% of the tight network configuration consists of thin fibrin fibres whereas the loose (coarse) configuration consists of >50% of thick fibres. Even if the fibres are thin, lysis still requires a longer period when arranged in a tight network configuration compared to a coarse network with thick fibres. These observations were performed with platelet poor plasma and as a result the effect of blood cells (such as erythrocytes and platelets) were not studied. The presence of blood cells trapped in the fibrin fibre network could possibly influence the speed of fibrinolysis, fibre diameter and fibrin fibre configuration.[249] An abnormal clot architecture that can result in increased resistance to thrombolysis has been shown to be a cause of venous thromboembolism.[264]

Fibrin-like dense matted deposits are present in the HIV positive (**Figure 31** and **32**) and the HIV negative DVT groups (**Figure 28** and **29**)**.** There were no dense matted deposits in the control group (**Figure 26**). Fibrin-like dense matted deposits may present in inflammatory conditions specifically in conditions of iron overload. Ferric ions (Fe^{3+}) have been shown to cause these deposits on the SEM which is resistant to proteolytic degradation.[27, 66] However, both HIV negative and positive groups had a decreased iron concentration, more so in the HIV positive group. The dense matted deposits seen in both groups are therefore not due to iron overload. The only commonality between the two groups is that they both had a

venous thrombosis present. It is possible that the presence of a thrombosis would promote the formation of the fibrin-like dense matted deposits.

The change in the fibrin fibres seen in inflammatory conditions may actually be as a result of the change in the protein structure. Kell et al hypothesised that fibrinogen is a similar model to amyloid formation.[238, 265] Amyloid monomers, soluble proteins, bind together to eventually form insoluble fibrils.[238] These insoluble protein fibrils occur due to a sequence of changes in protein folding that results in amyloidosis.[266] Fibrinogen are also soluble proteins that eventually forms insoluble fibrin fibres, like amyloid, but with the exception that fibrinogen requires thrombin to be activated. Such an amyloid protein structure is characterized by the formation of multiple β-sheets, compared to α-helices. This can be observed with X-ray reflections and fluorescent amyloid markers added to pathologic fibrin clots.[267-274] It is therefore possible that in the presence of a chronic infection state, such as HIV, the protein structure is changed in fibrinogen to form an amyloid structure. This may explain the change in fibrin fibre diameter as well as the change in fibrin fibre network.[238]

In keeping with the theory of fibrin as amyloid, or amyloid-like, is the fact that fibrin behaves like amyloid by staining with Congo red.[239] Amyloid fibril proteins occur in extracellular tissue deposits and tend to stain with Congo red.[266] Also, the presence of amyloid β (Aβ) peptides which appears to resist fibrin clot lysis.[239] Aβ peptides are also present in Alzheimer's disease and from the amyloid hypothesis, Alzheimer's disease is the result of accumulation of Aβ and the imbalance of Aβ production and clearance.[275, 276] It is possible that the fibrin-like dense matted deposits may be due to amyloid formation.

Conclusion

The viscoelastic studies on platelet poor plasma indicates a hypercoagulable state in both HIV negative and HIV positive DVT groups, but no significant differences between the two groups. The scanning electron microscopy also identified hypercoagulable changes in the two groups.

The fibrin fibre diameters as seen on the scanning electron microscope are significantly larger in patients with DVT, i.e. the HIV negative and HIV positive patients. There is no significant difference between those infected with and those not infected with HIV. The increase fibre diameter correlated with a raised maximum amplitude (MA) on the viscoelastic test in the HIV negative group compared to the control group, but not in the HIV positive group.

The distribution of the fibrin network, together with the dense matted deposits, on ultrastructural assessment results in a dense compact arrangement of fibres in the DVT groups, more so in the HIV infected group. Once again the compact networks correlated with a raised total thrombin generation (TGG) in the HIV negative group compared to the control group, but not in the HIV positive group.

The distribution and the density of the fibrin fibres changes in HIV negative and HIV positive DVT patients. A dense compact clot with fibrin fibres that have an increased diameter are found in patients with DVT but the changes may be due to different causes for each group of patients. The cause of the DVT (such as HIV infection, LPS from bacteria and ARV treatment) may result in the fibrin fibre changes and the dense matted deposits. The DVT itself may also produce fibrin changes. Besides the “cause and effect” of the DVT, the amyloid theory may play a role in the fibrin behaviour in which fibrin have similarities to that of amyloid.

The viscoelastic profile may not always detect all coagulation abnormalities. The scanning electron microscope allows for examination of morphological changes at high magnification. The two modalities of assessing the platelet poor plasma allows for a more comprehensive insight into the coagulation system.

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APPENDIX 1

INFORMED CONSENT DOCUMENT

CONSENT TO PARTICIPATE IN THE RESEARCH PROJECT

1) **TITLE OF STUDY HYPERCOAGULABLITY USING BIOPHYSICAL PARAMETERS IN HIV POSITIVE VERSUS HIV NEGATIVE PATIENTS WITH DEEP VEIN THROMBOSIS** (Principal Investigator: Dr Brandon Jackson)

2) **NATURE AND PURPOSE OF THIS STUDY**

You are invited to volunteer for a research study that will influence the management of blood clots. This study will be performed in Steve Biko Academic hospital and Kalafong hospital. This information leaflet is to help you to understand and to decide if you would like to participate. The treatment of your condition will not change in any way, i.e. you will still receive anticoagulation (blood thinning medication). The aim of the study will determine if HIV positive patients have factors in their blood that causes their blood to clot more easily compared to a person that is HIV negative. Also, the study seeks to assess if Anti-retroviral treatment (ARV) has an effect on blood clotting.

The results of this study will increase the understanding of blood clots in the veins in the presence of HIV infection. This will allow the detection the condition before it begins and also to improve the management of the condition to be more efficient resulting in improved patient care.

3) **EXPLANATION OF PROCEDURES TO BE FOLLOWED**

- If you are being given this form that means your doctor has already confirmed that you have a deep vein clot and that you have already had a test for your HIV status as per standard management. You can now be included in this study.
- What is required from you? Blood (approximately 20 millilitres) is the only samples that will be required from you, which will be once-off. We will study the blood samples to examine the factors and cells involved in clotting

- Risks: Besides a needle prick, there is no risks for you to have your blood drawn. Benefits: By volunteering for this study, you will assist in determining if the management can be improved for all patients with the same condition (deep vein thrombosis).

- We will also require from you consent to access and use your results/information from your patient records. The results/information required includes:

    - Hospital number, Age and Sex
    - HIV status
    - Current CD4 count (type of blood cell which is affected by HIV)
    - Type of Anti-retrovirals treatment if any.
    - Any concurrent medication
    - Co-morbidities

4) RIGHTS AS A PARTICIPANT IN THIS TRIAL

You understand that participation in this trial is entirely voluntary and if you do not want to participate in this study, you will still receive standard treatment (Warfarin) for your illness. The investigator retains the right to withdraw you from the study if it is considered to be in your best interest. If it is detected that you did not give an accurate history or did not follow the guidelines of the trial and the regulations of the trial facility, you may be withdrawn from the trial at any time.

5) You may at any time withdraw from the study without affecting your treatment.

6) This study has been approved by the University of Pretoria ethics committee.

7) If you have any questions concerning this study, you should contact Dr B Jackson at (w) (012) 373 1046.

8) CONFIDENTIALITY

All records obtained in this study will be treated with strict confidentiality. Data or results will be published in such a fashion that patients are anonymous and remain unidentifiable.

9) CONSENT TO PARTICIPATE IN THIS STUDY

I have read or had read to me in a language that I understand the above information before signing this consent form. The content and meaning of this information have been explained to me. I have been given the opportunity to ask questions and these have been answered satisfactorily. I understand that if I do not participate it will not alter my effective treatment in any way. I hereby volunteer to take part in this study.

....

Patient signature Date

....

Person obtaining informed consent Date

....

Witness Date

APPENDIX 2

INFORMED CONSENT DOCUMENT: Control group

CONSENT TO PARTICIPATE IN THE RESEARCH PROJECT

1) TITLE OF STUDY HYPERCOAGULABLITY USING BIOPHYSICAL PARAMETERS IN HIV POSITIVE VERSUS HIV NEGATIVE PATIENTS WITH DEEP VEIN THROMBOSIS (Principal Investigator: Dr Brandon Jackson)

2) NATURE AND PURPOSE OF THIS STUDY

You are invited to volunteer for a research study that will influence the management of blood clots. This study will be performed in Steve Biko Academic hospital and Kalafong hospital. This information leaflet is to help you to understand and to decide if you would like to participate. The aim of the study will determine if HIV positive patients have factors in their blood that causes their blood to clot more easily compared to a person that is HIV negative. Also, the study seeks to assess if Anti-retroviral treatment (ARV) has an effect on blood clotting.

The results of this study will increase the understanding of blood clots in the veins in the presence of HIV infection. This will allow the detection the condition before it begins and also to improve the management of the condition to be more efficient resulting in improved patient care.

3) EXPLANATION OF PROCEDURES TO BE FOLLOWED

- If you have documented to previously been tested negative for HIV, you can be included in this study.
- What is required from you? Blood (approximately 20 millilitres) is the only samples that will be required from you, which will be once-off. We will study the blood samples to examine the factors and cells involved in clotting
- Risks: Besides a needle prick, there is no risks for you to have your blood drawn. Benefits: By volunteering for this study, you will assist in determining if the

management can be improved for all patients with the same condition (deep vein thrombosis).

4) RIGHTS AS A PARTICIPANT IN THIS TRIAL

You understand that participation in this trial is entirely voluntary. The investigator retains the right to withdraw you from the study if it is considered to be in your best interest. If it is detected that you did not give an accurate history or did not follow the guidelines of the trial and the regulations of the trial facility, you may be withdrawn from the trial at any time.

5) You may at any time withdraw from the study.

6) This study has been approved by the University of Pretoria ethics committee.

7) If you have any questions concerning this study, you should contact Dr B Jackson at (w) (012) 373 1046.

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All records obtained in this study will be treated with strict confidentiality. Data or results will be published in such a fashion that patients are anonymous and remain unidentifiable.

9) CONSENT TO PARTICIPATE IN THIS STUDY

I have read or had read to me in a language that I understand the above information before signing this consent form. The content and meaning of this information have been explained to me. I have been given the opportunity to ask questions and these have been answered satisfactorily. I hereby volunteer to take part in this study.

……. …….

Patient signature Date

……. …….

Person obtaining informed consent Date

…… …….

Witness Date

APPENDIX 3

Data Collection Form

Title of study: HYPERCOAGULABLITY USING BIOPHYSICAL PARAMETERS IN HIV POSITIVE VERSUS HIV NEGATIVE PATIENTS WITH DEEP VEIN THROMBOSIS (Principal Investigator: Dr Brandon Jackson)

Hospital number	
Telephone numbers:	**(C)** **(H)** **(W)**
Gender (M/F)	
HIV status (positive/negative)	
HIV viral load	
CD4 count	
Antiretrovirals dosage (Only ARV combination of Emtricitabine (FTC), Tenofovir (TDF) and Efavirenz (EFA)	
Current duration of ARV	
Any concurrent medication	
Co-morbidities	

APPENDIX 4: Ethics and hospital approval

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

26/11/2015

Approval Certificate
New Application

Ethics Reference No.: 547/2015

Title: HYPERCOAGULABLITY USING BIOPHYSICAL PARAMETERS IN HIV POSITIVE VERSUS HIV NEGATIVE PATIENTS WITH DEEP VEIN THROMBOSES.

Dear Brandon Jackson

The **New Application** as supported by documents specified in your cover letter dated 17/11/2015 for your research received on the 17/11/2015, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 25/11/2015.

Please note the following about your ethics approval:
- Ethics Approval is valid for 4 years.
- Please remember to use your protocol number (**547/2015**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2015 (Department of Health).

☎ 012 354 1677 🖷 0866516047 deepeka.behari@up.ac.za http://www.healthethics-up.co.za
✉ Private Bag X323, Arcadia, 0007 - 31 Bophelo Road, HW Snyman South Building, Level 2, Room 2.33, Gezina, Pretoria

082 783 7072

Permission to access Records / Files / Data base at the STEVE BIKO ACADEMIC Hospital

DR N. MASITHELA
MANAGER
MEDICAL SERVICES
S B A H

To: Chief Executive Officer/Information Officer
_____Hospital

Dr MASITHELA

From: The Investigator
SBAH / KALAFONG Hospital

Dr BRANDON JACKSON

Re: Permission to do research at STEVE BIKO ACADEMIC **Hospital**

Drs _____, _____ and I are researchers working at the _____ Unit, Department of SURGERY at SBAH/KALAFONG Hospital. I am requesting permission on behalf of all of us to conduct a study on the _____ Hospital grounds that involves access to patient records.

The request is lodged with you in terms of the requirements of the Promotion of Access to Information Act. No. 2 of 2000.

The title of the study is: HYPERCOAGULABILITY IN HIV POSITIVE VERSUS HIV NEGATIVE PATIENTS

The researchers request access to the following information:

Access to the clinical files, record book and the data base.

We intend to publish the findings of the study in a professional journal and/ or at professional meeting like symposia, congresses, or other meetings of such a nature.

We intend to protect the personal identity of the patients by assigning each patient a random code number.

We undertake not to proceed with the study until we have received approval from the Faculty of Health Sciences Research Ethics Committee, University of Pretoria.

Yours sincerely

Signature of the Principle Investigator

Permission to do the research study at this hospital and to access the information as requested, is hereby approved.

Chief Executive Officer

DR N. MASITHELA
MANAGER
MEDICAL SERVICES
S B A H

_____Hospital

Dr _____

Signature of the ~~CEO~~

GAUTENG PROVINSIALE REGERING
DEPT VAN GESONDHEID
STEVE BIKO AKADEMIESE HOSPITAAL
STEVE BIKO ACADEMIC HOSPITAL
Hospital Official Stamp
2015 -09- 18
PRIVAATSAK/PRIVATE BAG X160
PRETORIA 0001
GAUTENG PROVINCIAL GOVERNMENT
DEPT OF HEALTH

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

KALAFONG HOSPITAL
PRIVATE BAG X396
PRETORIA
0001
17 SEPTEMBER 2015

ENQUIRIES : **DR KHIN HTWE**
TEL : **012 318 6502**
FAX : **012 373 9021**
EMAIL : **Htwe.Khin@gauteng.gov.za**

TO: DR BRANDON JACKSON

RE: PERMISSION TO CONDUCT RESEARCH

TITLE: HYPERCOAGULABILITY IN HIV POSITIVE VERSUS HIV NEGATIVE PATIENTS

Permission is hereby granted for the research to be conducted at **Kalafong Provincial Tertiary Hospital**. Provided that ethics clearance is first obtained from the training institution ethics committee.

DR KHIN HTWE 17/09/2015
MEDICAL MANAGER
KALAFONG HOSPITAL

GAUTENG PROVINCIAL GOVERNMENT
KALAFONG HOSPITAL / HOSPITAAL
17 SEP 2015
PRIVATE BAG / PRIVAATSAK X396 PRETORIA 0001
SUPERINTENDENT'S OFFICE