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Endothelial dysfunction in human immunodeficiency virus infection

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ENDOTHELIAL DYSFUNCTION IN HUMAN IMMUNODEFICIENCY VIRUS INFECTION

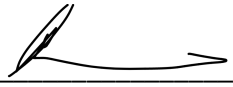
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Original published work submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg 2022

DECLARATION BY STUDENT

I, Elizabeth Sarah Mayne, declare that this Thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



_____ (Signature of candidate) _____23rd_____day of____June_____20__22_____in Johannesburg____

DEDICATION

This thesis is dedicated to my husband, Paul Marsden, my daughter, Rebecca, and my parents, Anthony and Susan Mayne with love and gratitude for their assistance and patience and to the memory of my sister, Alexandra, who left me far too soon.

"*Who is the third who walks always beside you?*
When I count, there are only you and I together
But when I look ahead up the white road
There is always another one walking beside you"

T.S. Elliott, The Waste Land

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PUBLICATIONS ARISING FROM RESEARCH

Diagnosis of human immunodeficiency virus associated disseminated intravascular coagulation. PLoS One. 2022 Jan 21;17(1):e0262306. doi: 10.1371/journal.pone.0262306. eCollection 2022.PMID: 35061794 **Mayne ES**, Mayne A, Louw S
Good Fences make Good neighbours: human immunodeficiency virus and vascular disease. Open Forum Infect Dis. 2019 Jun 25;6(11):ofz303. doi: 10.1093/ofid/ofz303. eCollection 2019 Nov. Review **Mayne ES** and Louw S
The Utility of the Lipoprotein-Associated Phospholipase A_2 (Lp-PLA_2) Assay in Detecting Abnormalities in Lipid Metabolism and Cardiovascular Risk in an HIV-Infected South African Cohort. **Mayne** ES, Moabi H, Grobbee DE, Barth RE, Klipstein-Grobusch K, Stevens WS, Vos AG, **Louw** S. Clin Appl Thromb Hemost. 2019 Jan-Dec;25:1076029619883944. doi: 10.1177/1076029619883944.
Pathogenic factors associated with development of disseminated intravascular coagulopathy (DIC) in a tertiary academic hospital in South Africa. PLoS One. 2018 Apr 12;13(4):e0195793. doi: 10.1371/journal.pone.0195793 **Mayne, E.S.**, Mayne, A, Louw, S.
Mortal allies: human immunodeficiency virus and noncommunicable diseases. Current Opinion in HIV and AIDS. 2017 Mar;12(2):148-156 **Mayne, ES** and George, JA
Altered Monocyte and Endothelial Cell Adhesion Molecule Expression is linked to Vascular Inflammation in HIV-infection Open Forum Infectious Disease. 2016 Oct 15;3(4) M Kulkarni, E Bowman, J Gabriel, T Amburgy, **E Mayne**, D.A. Zidar, C. Maierhofer, A. Turner, J. A. Bazan, S.L. Koletar, M.M. Lederman, S. F. Sieg, N.T. Funderburg
Increased Platelet and Microparticle Activation in HIV infection: Upregulation of P-selectin and Tissue Factor Expression. J.AIDS 2012 Apr 1; 59(4):340-6 **E Mayne**, N Funderburg, S Sieg, R Asaad, M Kalinowska, B Rodriguez, A Schmaier, W Stevens and M Lederman.
Increased tissue factor expression on circulating monocytes in chronic HIV infection: relationship to in vivo coagulation and immune activation. Blood. 2010 Jan 14;115(2):161-7 NT Funderburg, **E Mayne**, SF Sieg, R Asaad, W Jiang, M Kalinowska, AA Luciano, WS Stevens, B Rodriguez, JM Brenchley, DC Douek, MM Lederman.
Altered Monocyte and Endothelial Cell Adhesion Molecule Expression is linked to Vascular Inflammation in HIV-infection Open Forum Infectious Disease. 2016 Oct 15;3(4) M Kulkarni, E Bowman, J Gabriel, T Amburgy, **E Mayne**, D.A. Zidar, C. Maierhofer, A. Turner, J. A. Bazan, S.L. Koletar, M.M. Lederman, S. F. Sieg, N.T. Funderburg

PRESENTATIONS ARISING FROM THIS RESEARCH WORK

Research components of this work have been presented as follows:

Conference	Location	Month and Date	Title of Abstract
International Society of Thrombosis and Haemostasis	Online	July 2021	**Diagnosis of Disseminated Intravascular Coagulation in the context of Human Immunodeficiency Viral (HIV) infections in a South African tertiary care setting (Abstract Number 326114)**
International Society of Thrombosis and Haemostasis	Online	July, 2020	**Development of a Clinical Cohort of HIV-infected Patients with Peripheral Vascular Disease (Abstract Number: 295271)**
International Society of Thrombosis and Haemostasis	Berlin	July, 2017	**A comparison of Disseminated Intravascular Coagulation (DIC) Screening Parameters with and without Human Immunodeficiency Virus Infection in an African Academic Hospital Setting (Abstract Number: 186990)**
International Society of Thrombosis and Haemostasis	Berlin	July, 2017	**Epidemiological Evaluation of Patients Presenting with Disseminated Intravascular Coagulation (DIC) at an Academic Hospital in an African Middle-income Country (Abstract Number : 186991)**

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ABSTRACT

Introduction

Infection with Human Immunodeficiency virus (HIV) is a risk factor for endothelial disease which can manifest as venous thromboembolic disease (VTE), arterial thrombosis and atherosclerosis and microvascular disease specifically thrombotic microangiopathies (TMAs) including disseminated intravascular coagulation (DIC) and Thrombotic Thrombocytopaenic Purpura (TTP). The procoagulant state in these patients appears to be multifactorial in pathogenesis and linked to the chronic inflammatory state in these patients. The primary hypothesis is that these disparate clinical presentations reflect the baseline haemostatic activation in these patients.

Methods

Data and samples were collected from 5 different sources – HIV-infected patients without documented arterial disease, HIV-infected patients with documented arterial disease, HIV-infected patients and HIV-uninfected patients with documented thrombotic microangiopathic disease and healthy HIV-uninfected controls. Two extensive literature reviews were undertaken to identify both cellular and biochemical markers of increased coagulation The activation status of both monocytes and platelets and production of procoagulant microparticles were analysed by flow cytometry and compared with markers of HIV disease and chronic inflammation. Levels of the lipid mediators and cardiovascular biomarkers were assessed in a cohort of HIV-infected patients *ex vivo* and compared with markers of virological suppression and immunological recovery as well as markers of cardiovascular disease. The contribution of HIV as a risk factor for disseminated intravascular coagulation was assessed and the laboratory presentation was compared between HIV-infected and uninfected individuals.

Results

Thrombosis in HIV is linked to chronic inflammation and endothelial dysfunction. Markers of endothelial cell activation are increased in HIV-infected patients without overt cardiovascular disease and correlate with viral load. Patients with HIV are more likely to have platelet and monocyte activation and produce microparticles which are rich in tissue factor. Clinically, these patients present with increased risk of thrombotic microangiopathies.

Conclusions

HIV associated vascular disease is multifactorial in origin with cellular factors (specifically monocytes, neutrophils and platelets) and humoral factors (including factors like LP-PLA_2). Clinical presentation is pleomorphic but includes increased risk and more severe presentation of arterial and microvascular disease

CHAPTER 1 - INTRODUCTION

The structure and function of the endothelium

The endothelium is a confluent monolayer of cells which lines the vasculature including the venous, arterial and microvascular systems. It constitutes one of the largest organs in the human body with a surface area of over 4000 m^2.[1] Endothelial cells derive in the embryo from the mesodermal layer but undergo epigenetic imprinting linked to the physical location and physiological factors like shear stress, circulating volumes and pressure.[1, 2] These factors can activate genes including Krüppel-like factors which affect endothelial function. The endothelial cells are covered by a glycocalyx which also protects the endothelial cells.[2]

The endothelium acts a gatekeeper for the interface between the vasculature and the tissue. In addition to facilitating the diffusion of key nutrients and gases to the tissue, the endothelium also regulates both coagulation and inflammation systems.[2] Inflammatory and immunological responses are closely linked with coagulation which acts both as a sequela of infection and as a key innate immune response mechanism. As a key modulator of these systems, the endothelium interacts with cells including leukocytes and platelets both through direct cell-cell contact and through the release of a number of regulatory factors.[1, 3]

The endothelium and vascular tone

The endothelium regulates vascular tone throughout the system. Nitric oxide (NO), produced by nitric oxide synthase (NOS), results in vasorelaxation. NO both prevents vasoconstriction and inhibits coagulation associated with constriction through an inhibitory effect on platelets. It also inhibits the production of cellular adhesion molecules which can facilitate leukocyte and platelet adhesion and trafficking. Nitric oxide is released under the influence of activated platelets which secrete substances like serotonin and is a compensatory response to increased flow, temperature reduction and shear stress. It is also affected through a number of hormones including sex hormones, insulin, adiponectin and erythropoietin and the bioavailability of its substrate amino acid, arginine (reviewed in [3]). Response to NO is blunted in older individuals.

Vasoconstriction can be effected by a number of inflammatory and endogenous factors. A key vasoconstrictor is endothelin-1 which acts on 2 receptors to cause both constriction as well as changes in the associated smooth muscle including hypertrophy and smooth muscle proliferation (reviewed in [3]). Lipid mediators of inflammation including prostaglandins and leukotrienes also directly effect changes in vascular tone.[4, 5]

The endothelium and coagulation

Clotting is initiated *in vivo* through a cell surface – provided either by the endothelium or a platelet. Under certain conditions, leukocytes (specifically monocytes) may also provide prothrombotic surfaces. Clot propagation involves the activation of a zymogenic cascade which is initiated by the exposure of tissue factor with activates factor VII and proceeds through positive feedback loops.[6] A clot is comprised predominantly of fibrin which is activated by thrombin and cross-linked by factor

XIII. Arterial clots are often platelet-rich while venous clots, formed under lower pressure, are often predominantly fibrin-based.[7]

The endothelium is a key regulator of coagulation. Under physiological conditions, the endothelium is inert and predominantly functions to limit clotting throughout the vascular tree. Anticoagulants can be secreted or expressed on the endothelial cell membrane. Of these, heparan sulphates and tissue factor (TF) pathway inhibitor act primarily on factor activation with downstream inhibition of factors II and X, and TF, respectively. The endothelium produces thrombomodulin which binds to thrombin and activates the anticoagulant factor protein C which, with a cofactor Protein S, binds to and inhibits factors V and VIII. The endothelium also inhibits platelet activation and aggregation by absorbing and inactivating platelet activating agents like ADP through surface receptors like CD39 and by secretion of platelet inhibitors of which eNOS is the most potent.[2]

Following local damage and inflammation, the endothelium becomes procoagulant. Von Willebrand factor (vWF) stored in the subendothelium in Weibel-Palade bodies and subendothelial TF are exposed, triggering coagulation. Platelets are activated through exposure to vWF, TF and endothelial collagen and the endothelium upregulates expression of cell adhesion molecules which bind to platelets as well as mediating leukocyte migration into the tissues and leukocyte activation.[1]

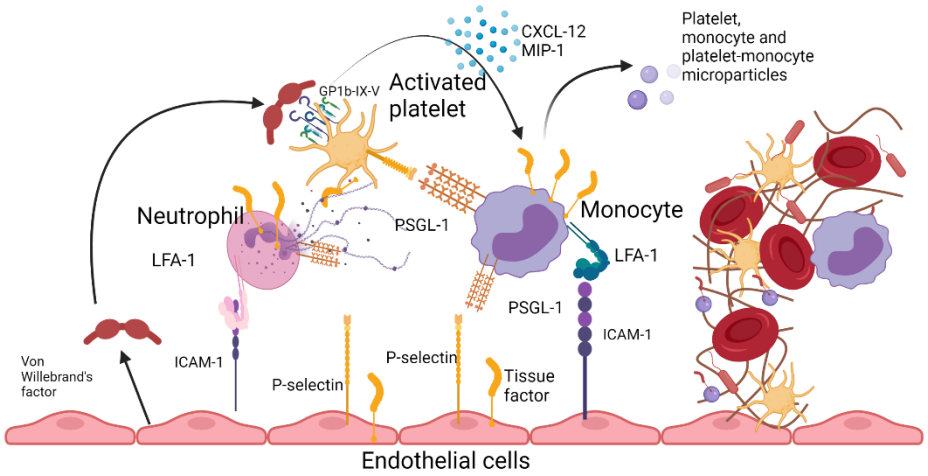
Endothelium, inflammation and immunothrombosis

Clot formation is a response to tissue injury. Clotting should be limited and processes are present to inhibit inappropriate extension with fibrinolysis and clot organisation. A procoagulant state induced by infection and acute inflammation contributes to the host defence process by preventing dissemination of pathogens, preventing transmigration of pathogens into the tissue and by localising the response.[8]

In infections where local control is not possible, a procoagulant state can result in inappropriate coagulation – a state sometimes referred to as immunothrombosis.[5, 9] This can occur throughout the vasculature manifesting as venous thromboembolic disease (VTE), arterial thrombosis and microangiopathic thrombotic disease. The clinical presentation is determined by multiple factors including the nature of the infection, the chronicity of the infection and the presence of co-morbid conditions which may accelerate or retard coagulation.

Following exposure to inflammatory stimuli, there is production of proinflammatory cytokines including tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), which are produced by a number of cells but primarily by activated macrophages and interleukin-1b (IL-1b), which is produced by both the inflamed endothelium and activated macrophages.[10] In addition, there is intravascular activation of the complement system which produces activated chemotactic agents like complement components C3a and C5a. Finally, there is activation of both the arachidonic acid and bradykinin pathways. These inflammatory mediators have pleotropic effects on the endothelium, the associated leukocytes and platelets. The endothelium secretes the Weibel-Palade bodies containing WF, exposes TF on the subendothelium and upregulates the expression of cellular

adhesion markers including Platelet (P)-selectin, Endothelial (E)-selectin, vascular intercellular adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1.[11] This results in platelet activation with release of platelet granules and expression of activated TF by platelets and associated macrophages.[6] This triggers the coagulation cascade. Leukocytes adhere to the endothelium and transcytose or become activated. In the case of neutrophils, this may result in the extrusion of the cellular genetic material as neutrophil extracellular traps (figure 1).[12]



Immunothrombosis is an interaction between the coagulation and immunological systems. Inflammation results in activation of the endothelium to produce both adhesion markers (ICAM-1 and V-CAM-1 and selectins) and to release Weibel-Palade bodies which carry the procoagulant factor vWF and tissue factor. Exposed collagen, inflammatory mediators and vWF activate platelets which upregulate expression of selectins and tissue factor. Platelets are also activated by interaction with monocytes through receptors like PSGL-1 and during netosis by neutrophils. The adhesion molecules from the endothelium and chemokines like CXCL-12 and MIP-1 from both the endothelium and platelets recruit additional leukocytes. Monocytes and neutrophils also upregulate tissue factor production and monocytes produce cellular fractions called microparticles. These, with leukocytes and platelets form cellular surfaces to initiate clotting.

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Figure 1: Immunothrombosis is a pathogenic interaction between the activated endothelium, platelets and leukocytes (*E.Mayne*)

Immunothrombosis contributes to mortality in a number of infective processes. Disseminated intravascular coagulopathy (DIC) associates strongly with sepsis, which is a dysregulated response to infection. Sepsis with DIC is often associated with bacterial infection but may also follow viral infections including chronic viral infections like HIV, hepatitis B and herpes virus infections. Immunothrombosis also predisposes patients who are admitted with infections to VTE and also to accelerated arterial thrombosis. Surrogate markers of coagulation activation including the presence of fibrin degradation products (D-dimers) are associated with poor prognosis in patients with infection.

Human Immunodeficiency Virus infection

Human immunodeficiency virus (HIV) is a human retrovirus which predominantly causes a sexually transmitted disease. The virus infects cells which express the receptor CD4 as well as the co-

receptors CCR5 or CXCR4 – primarily CD4+ T cells, macrophages and dendritic cells. Currently, there is no efficacious vaccination strategy but patients on antiretroviral therapy (ART) show good response and have a normal or near normal life expectancy. South Africa has a high HIV burden with an estimated prevalence of 8.2 million infected patients in 2020.[13] Of these, over 90% know their status and 70% are on ART with an estimated 88% showing virological suppression.

The HIV treatment strategy focuses on a test-and-treat approach and patients are placed on ART as soon as a diagnosis is made.[14] Previously, patients were treated in a $CD4^+$ T-cell count driven strategy, initially treating patients with $CD4^+$ T cell counts below 250 cells/mm^3 and then patients with $CD4^+$ T cell counts below 350 cells/mm^3.[14] Although patients in South Africa continue to present later with more advanced disease, the treatment programme is associated with a corresponding decline in some HIV-associated opportunistic infections and a corresponding increase in estimated life expectancy.[15, 16]

As HIV becomes an increasingly chronic disease seen in older patients, focus has shifted to the interaction between the viral infection and non-communicable diseases (NCDs).[17] HIV itself has been associated with the development of diseases including malignancy and cardiovascular disease.

The SMART study (Strategies for the Management of Anti-Retroviral Therapy), was a community based and National Institutes of Health (NIH) funded study looking at different treatment strategies for HIV. The two arms included a drug conservation (DC) wing and a viral suppression (VS) wing. Treatment for participants in the DC wing was interrupted when the CD4+ T cell count rose above 250 cells/mm^3 and only restarted when the CD4+ T cell count fell below this level. Participants assigned randomly to the DC wing showed increased all-cause mortality but specifically increased risk of non-AIDS events including most commonly cardiovascular disease.[18-21] This stimulated research into risk factors for cardiovascular disease in people living with HIV (PWH).

HIV and endothelial dysfunction

PWH are at increased risk of thrombosis across the vascular tree. This risk is partially mitigated at lower CD4+ T cell counts by ART but even in patients with full virological suppression, the risk of thrombosis is elevated. [22-27]

Endothelial dysfunction is typically defined as an arterial disease in which the endothelial ability to regulate vascular tone, inflammation and coagulation is variably impaired.[2, 3] A number of endothelial markers produced in this condition have been measured in individuals with atherosclerotic disease. These include markers of coagulation activation like D-dimers [28, 29], procoagulant factors released by the endothelium (vWF)[30], proinflammatory cytokines and chemokines (IL-6)[18, 19, 31, 32] and adhesion molecules (like vascular and intercellular adhesion molecules).[33, 34] Although considered primarily an arterial disease, endothelial dysfunction can be extrapolated to both the venous and the microvascular circulation. In addition to an increased risk of myocardial infarction, cerebrovascular, aneurysmal and peripheral vascular disease, increased incidences of VTE and microangiopathic thrombotic disease are also seen.[28, 35]

Endothelial dysfunction in PWH appears to be linked to ongoing chronic inflammatory processes resulting in immunothrombosis. Contributory causes for ongoing inflammation include the presence of low-grade viral replication in treated patients [36], the presence of opportunistic infections including other viral infections like Kaposi sarcoma herpes virus, cytomegalovirus and Epstein-Barr Virus,[37] and translocation of microbial products across a compromised gastrointestinal mucosal barrier. These often combine with traditional cardiovascular risk factors which are prevalent in Southern Africa including high rates of essential hypertension, diabetes mellitus type 2, hypercholesterolaemia and use of tobacco. HIV viral proteins appear to impact endothelial function directly. Negative factor protein (Nef) transcytoses between endothelial cells and causes endothelial apoptosis and endothelial activation.[38] HIV glycoprotein 120 (gp120) and transactivator of transcription (Tat) induce proinflammatory cytokine secretion, leukocyte and specifically monocyte recruitment and upregulation of endothelial adhesion molecules.[38] Although ART has been implicated in increased cardiovascular risk, particularly use of protease inhibitors, this risk appears relatively minor when compared with uncontrolled viraemia. [39, 40]

Aim

The overall aim of this research was to investigate the effects of HIV-1 infection on the human endothelium to determine whether HIV-associated cardiovascular disease is mediated by pathogenic interactions between endothelial cells, leukocytes and platelets in the context of a chronic inflammatory state. This was achieved by the following objectives:

1. To perform a literature review on the status of primary HIV-1 infection and NCDs in Africa. (Publication 1).
2. To investigate the activation status of platelet and platelet-monocyte microparticles in HIV-infected individuals (Publication 2).
3. To conduct an extensive literature review to ascertain the performance of evaluated biomarkers in HIV-infected patients with cardiovascular disease (Publication 3) to investigate which markers would be most useful to measure.
4. To measure biomarkers in a cross-sectional cohort of HIV infected patients and compare with clinical and radiographical markers of cardiovascular disease (Publication 4).
5. To study the epidemiological characteristics of the thrombotic microangiopathy (disseminated intravascular coagulation) in patients with and without HIV infection (Publication 5 and Publication 6).
6. To collect arterial and venous blood samples from a cohort of HIV-infected patients with known arterial disease for examination of the arterial wall and clinical characterisation (Future directions)

These studies were conducted on 4 separate cohorts:

1. HIV-infected and uninfected patients with peripheral vascular disease presenting for arterial bypass grafting at Charlotte Maxeke Johannesburg Academic Hospital. (M130372) (Preliminary data chapter 9)
2. HIV-uninfected controls (n=20) - donors attending the transplant donor clinic (area 561) at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). Briefly, these participants have

undergone extensive evaluation including virological, cardiovascular and biochemical testing as part of the pre-transplant work-up (M130372) (Preliminary data chapter 9)

3. Retrospective record review of patients presenting for coagulation and DIC analysis at CMJAH (M160389) (Publications 5 and 6)
4. HIV-infected patients with no known cardiovascular disease in South Africa nested within a cohort recruited in Johannesburg. Briefly this comprised 27 ART-naïve individuals and 22 participants on ART. (M160131) - Publication 4
5. HIV-infected patients with no known cardiovascular disease attending the Case Western Reserve University Special Immunology Unit (n=60) (IRB approval UH-01-98-55) – Publication 2

In conclusion, these studies evaluate the pathogenesis and clinical features of endothelial dysfunction and cardiovascular disease. The papers together present a model of immunothrombosis as a cause for coagulopathy in HIV-infected patients. We show that leukocytes and platelets in HIV-infected patients express high levels of the procoagulant TF in chronic inflammation. These cells form substrates for clot propagation. The endothelium upregulates expression of adhesion molecules like ICAM-1. Patients with HIV develop arterial disease which in our clinical cohort is more extensive and presents at an earlier age. HIV is also linked to increased biomarkers of cardiovascular disease in the absence of overt clinical disease suggesting that there may be subclinical tissue damage. They are also at significantly increased risk of other vascular diseases including microangiopathic thrombotic processes.

CHAPTER 2: SYNOPSIS OF PUBLICATIONS

Publication 1: Mortal Allies: Human Immunodeficiency virus and non-communicable disease

This publication provides an extensive literature review that was performed to investigate the impact of primary HIV infection on non-communicable diseases in Africa including cardiovascular disease. Briefly, this paper outlined some of the risk factors which contribute to increased atherosclerosis and cardiovascular disease including chronic inflammation with increased monocyte adhesion and lipid uptake, secretion of proinflammatory and proatherogenic cytokines. This literature review highlighted important pathogenic pathways in HIV-associated cardiovascular disease.

Contribution of candidate:

Responsible for literature identification and equal responsibility for drafting and reviewing of final manuscript

Mortal allies: human immunodeficiency virus and noncommunicable diseases

Elizabeth S. Mayne[a] *and Jaya A. George*[b]

Purpose of review
HIV-infected individuals have improved access to antiretroviral therapy. This has resulted in a shift in causes of mortality from infectious diseases to noncommunicable diseases including cardiovascular disease, chronic kidney disease (CKD) and malignancies. This review will look at the epidemiological shift, risk factors for the development of these diseases and examine some of the supporting laboratory diagnostic testing, which may be required.

Recent findings
Risk factors for the development of these diseases in HIV-infected patients include underlying genetic predisposition, lifestyle risk factors, chronic inflammation as a consequence of HIV infection, the presence and persistence of opportunistic infections and in some cases, highly active antiretroviral therapy, itself. Morbidity and mortality from HIV-associated conditions are increasing in low-income and middle-income countries (LMICs) with increased prevalence of HIV-associated cancers, cardiovascular disease and CKD.

Summary
Management of these conditions in LMICs requires an integrated pathology solution that will enable early screening, diagnosis and monitoring.

Keywords
cardiovascular, HIV, malignancy, nephropathy, disease

INTRODUCTION

Approximately 36.9 million people are infected with HIV. By the middle of 2015, approximately 15.8 million of these individuals had accessed antiretroviral therapy (ART). Access to ART in lower and middle-income countries (LMICs) in Africa approaches 40% of the infected population [1]. This has had a significant impact on the epidemic with a change in the causes of mortality from infectious agents to noncommunicable diseases even in LMICs [2,3]. HIV is becoming a chronic disease in older patients who have a number of coexisting comorbidities [2,4,5]. Management of these conditions requires an understanding of how HIV and these conditions interact [6]. The key is a diagnostic paradigm that integrates communicable and noncommunicable disease screening and monitoring for consolidated patient management [7]. This review will focus on three noncommunicable diseases common in HIV-infected populations in underresourced settings: cancer, cardiovascular disease and chronic kidney disease (CKD).

HIV AND INFLAMMATION

Chronic infections, which cannot be cleared by the immune system, are associated with low-grade inflammation and accelerated immunosenescence (also called inflammaging) [8–10]. HIV infection is associated with elevated levels of inflammatory markers, which persist despite virologic control and are linked to increased mortality risk [11–13]. These markers which include, among others, proinflammatory cytokines such as interleukin (IL)-6 [14] and cytokine receptors (IL-2R-α), chemokines (like CXCL12 and 13) [13,15,16] and acute phase reactants like the highly sensitive C-reactive protein [11–13,15,17]. Interestingly, increased mortality has also been linked to markers of increased coagulation activity like D-dimers and increased activation of innate effector cells like soluble CD14 released from

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KEY POINTS

- Chronic inflammation and opportunistic infections associated with HIV infection can result in the development of noncommunicable conditions such as malignancy, cardiovascular disease and CKD in genetically predisposed individuals.
- Diagnostic management of people living with HIV and AIDS should include screening and diagnostic protocols to minimize complications that can predispose towards comorbid noncommunicable diseases.

monocytes [13,18]. Figure 1 shows the potential causes of inflammation in HIV-infected individuals.

This activation has been linked to factors including an immune response to low-level viral replication (even in patients on treatment) [19,20], the presence of subclinical opportunistic infections like cytomegalovirus [21] and translocation of microbial products across compromised mucosal barriers (especially the gastrointestinal tract) [22]. Persistent immune activation, even in patients with controlled viremia, is associated with inappropriate immune cell trafficking and activation, tissue damage and dysplasia [15,23]. Eventually, extensive tissue damage leads to organ dysfunction.

The severity of the consequent disease can be impacted by known risk factors prior to HIV infection. This includes tobacco and alcohol use [24▪▪,25,26], exposure to harmful environmental and occupational stimuli (such as asbestosis, silicosis and agricultural toxins) and a genetic predisposition to certain chronic diseases. Tobacco use in HIV-infected individuals is significantly higher than in the general population [24▪▪]. In certain African population groups, higher prevalences of metabolic diseases such as type 2 diabetes mellitus and essential hypertension [4,5] predispose to chronic kidney and cardiovascular disease.

The impact of ART, itself, requires further consideration. Although the safety profile of these drugs has improved, biochemical parameters are still deranged in response to these drugs [27].

HIV AND MALIGNANCY

HIV is associated with an increased risk of malignancy [28]. This risk has been ascribed to decreased tumor immunosurveillance, coinfection and persistence of oncogenic viruses [29,30] and low levels

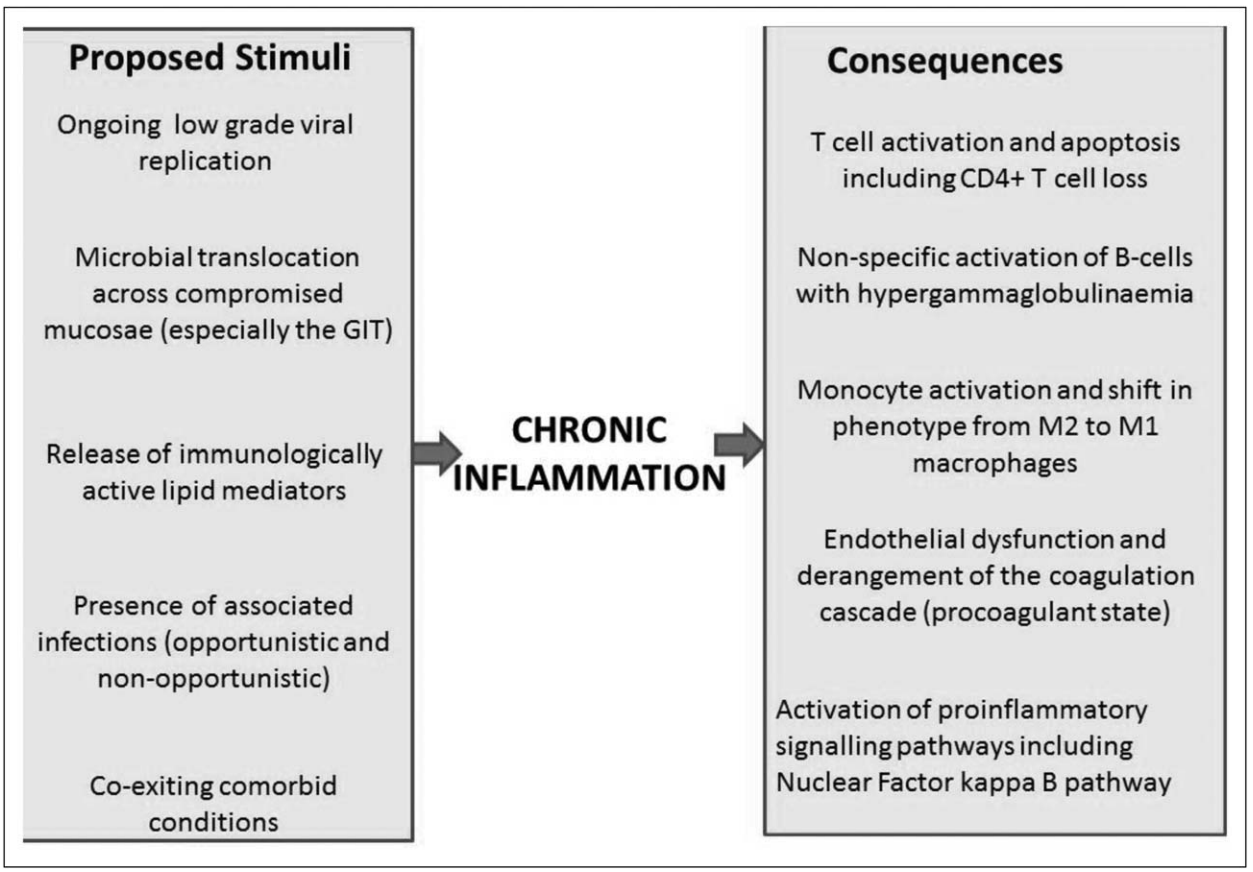


FIGURE 1. Potential causes of inflammation in HIV-infected individuals.

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of chronic inflammation. In pre-ART era, people infected with HIV present with the AIDS-defining malignancies (high-grade B-cell non-Hodgkin lymphoma, cervical carcinoma and Kaposi sarcoma). These malignancies are associated with significant $CD4^+$ T-cell lymphopenia. These tumors continue to be common in countries with a high incidence of HIV infection and with limited access to ART [31]. Studies in HIV-infected individuals with controlled viremia suggest that the prevalence of a number of non-AIDS-defining malignancies is increased in these populations [29] including anal carcinoma, non-small cell lung carcinoma, Hodgkin lymphoma and hepatocellular carcinoma [29,32].

Diagnosis of HIV-associated malignancies is generally histological, immunophenotypic and molecular. In low-resourced countries, without access to equipped anatomical disease facilities, this can present a diagnostic challenge. To tailor therapy, a tissue diagnosis is required. A surgical biopsy is generally formalin-fixed and stained with hematoxylin and eosin. The cell of origin is defined by expression of specific antigens. For hematological malignancies, these often include the cluster of differentiation antigens. Solid tumors are often characterized using a cytokeratin profile. Tables 1 and 2 [24▪▪,25,28,30,33–37,38▪▪,39–41,42,43,44▪▪,45–47] show some of the risk factors and diagnostic criteria of AIDS-defining and HIV-associated malignancies.

HIV AND CARDIOVASCULAR DISEASE

Cardiovascular disease (CVD) mortality is higher among HIV-infected people compared with the general population [48,49]. The cause includes classical risk factors such as smoking [24▪▪], diabetes, dyslipidemia as well as the effects of combination antiretroviral therapy and the effects of the virus via immune activation [23]. The risk factors for development of atherogenesis in HIV-infected individuals are presented in Fig. 2 [13,50–53].

Antiretroviral therapy

HIV-infected patients receiving antiretroviral therapy (ART) exhibit a number of metabolic complications, including lipid abnormalities, dysregulation of glucose metabolism and body-fat redistribution and renal dysfunction [50,54–56]. Putative mechanisms are summarized in Table 3 [27,50,54,56–62].

HIV AND KIDNEY DISEASE

CKD occurs as a complication of HIV infection, other comorbid disease and infections and as a consequence of treatment of HIV infection and its complications. The estimated prevalence of CKD in HIV ranges from 5% to about 18% [63,64], and is shown to be higher when proteinuria or albuminuria is used to determine CKD as opposed to estimated glomerular filtration rate (eGFR). Risk factors for CKD include black race, older age, CD4 cell count less than 200 cells/μl, high viral load, proteinuria/albuminuria, hepatitis C coinfection, Apo L1, diabetes mellitus and hypertension, eGFR less than 90 ml/min per 1.73 m^2 and drugs such as tenofovir [65,66▪▪]. This is discussed further in the article by Venter *et al.* in this issue. Diseases specific to HIV include HIV-associated nephropathy (HIVAN), which presents with a nephrotic range proteinuria and is thought to result from direct infection by HIV-1 and expression of viral genes in genetically susceptible hosts [67,68]. Other diseases specific to HIV are HIV immune complex disease, which may manifest as acute kidney injury or as proteinuria and thrombotic microangiopathy [69].

LABORATORY INVESTIGATIONS AND CHALLENGES

Cardiovascular risk assessment allows for primary and secondary health interventions and should be carried out prior to initiation of ART. Traditional tools such as the Framingham score were developed for the general population [70], and underestimate risk in HIV patients. Risk equations developed specifically for HIV-infected persons are more accurate at predicting risk than conventional risk prediction models [71].

Diagnosis of diabetes

Screening and management of diabetes and its complications are essential in HIV-positive populations. The standard for the diagnosis of diabetes is based on raised fasting blood glucose (FPG) at least 7mmol/l or a 2-h blood glucose post oral glucose tolerance test (OGTT) at least 11.1 mmol/l [72]. Although diagnosis based on FPG is affordable, widely available and easy to perform, clinicians should be aware of the pitfalls which include important facts such as: glucose shows diurnal variation, higher in the mornings, which may lead to underdiagnoses in patients seen later in the day, loss of glucose from sample containers as ongoing glycolysis is a serious and underappreciated problem, approximating 5–7%/h and blood should be collected after an overnight fast of at least 8 h. In addition, there are insufficient data to support the use of point-of-care glucose meters for the diagnosis of diabetes. OGTT measures the ability of the body to metabolize glucose, and a raised postprandial

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Table 1. Infectious agents, lifestyle risk factors and histological and immunohistochemical features of the AIDS-defining malignancies and possible screening modalities

Malignancy	Coexisting infections	Lifestyle risk factors	Histologic description and subtypes occurring in HIV	Immunophenotype	Screening modality and biomarkers
Kaposi sarcoma	KSHV [33,34]	No agents conclusively identified [35]	Sarcoma of vascular endothelial cells characterized by vascular proliferation [33]	KSHV positive (latency nuclear antigen 1) [34]	Clinical suspicion
				Endothelial cell markers – CD31 (PECAM), D2–40, FL-1	KSHV viral load evaluation [34]
				Primitive hemopoietic cell marker – CD34 [33,36]	
High-grade B-cell NHL	Epstein–Barr virus (EBV) – DLBCL, PL and BL	No agents conclusively identified [28,30]	Tumors of malignant B cells which are generally intermediate to large in size and may show plasmablastic or plasmacytic differentiation	B-cell markers: CD20	Fine needle aspiration [30]
Burkitt leukemia/ lymphoma (BL)	Kaposi sarcoma Herpes virus (KSHV) – MCD and PEL		Subtypes: BL, DLBCL and PL	Germinal center markers: CD10 and BCL-6	
Diffuse large B-cell lymphoma (DLBCL)	Malaria – BL [30]		MCC and PEL [35]	Markers of plasma cell differentiation: CD38, CD138 and BLIMP1	
Primary effusion lymphoma (PEL)				Cellular turnover – Ki67	
Plasmablastic lymphoma (PL)				c-Myc activation	
Large cell lymphoma in the context of multicentric Castleman's disease (MCD)				Viral antigens – EBV (BL, DLBCL and PL), KSHV (PEL and MCD) [30,35]	
Cervical carcinoma	HPV 16,18	Use of contraceptives	Squamous cell carcinoma [37]	Often positive for CK7 and 20	Papanicolau smear
	Other sexually transmitted infections [33]	Smoking [33]		Oncogenic HPV subtypes [37,38▪▪]	Colposcopy with visualization using acetic acid
					HPV screening (high oncogenic genotypes) [37,38▪▪]

HPV, human papillomavirus; MCD, multicentric Castleman's disease.

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Table 2. Histological and immunohistochemical features of HIV-associated malignancies and possible screening modalities

Malignancy	Coexisting infections	Lifestyle risk factors	Histologic description and subtypes seen in HIV infection	Immunophenotype	Screening modality and biomarkers
NSLC	No agents conclusively identified [39]	Smoking	Predominant subtype is adenocarcinoma with primitive duct formation. Other subtypes less commonly seen are large cell carcinoma and squamous cell carcinoma [39,41]	CD7 positive and often CD20 negative	May be role of chest CT for early diagnosis in smokers [24▪▪,40]
		Occupational exposure to carcinogens (e.g. asbestos and soot) [24▪▪,25]		Epithelial antigen expression (e.g. CEA)	
				Expression of TTF-1 [41]	
HL	EBV [30]	No agents conclusively identified [35]	Reed Steenberg cell is the pathognomic tumor cell	CD15, CD30 and IRF4	Fine needle aspiration [30]
			Subtypes – mixed cellularity, nodular sclerosis then not otherwise specified [28,35]	Viral proteins – EBL [35]	
HCC	Hepatitis B and C [37–40]	Alcohol use and smoking	Tumor cells resemble liver hepatocytes with formation of blood vessels [42]	Hepatocyte antigen	Monitoring for hepatitis B antigen and antibody
		Use of anabolic steroids		CD34	Monitoring for hepatitis C
		Aflatoxins [43]		Factor 8-related antigen	Liver enzyme levels
				Complex CK profile but generally positive for CK8 and18 and negative for 7, 19 and 20 [42]	Magnetic resonance imaging [43,44▪▪,45]
					HBV and HCV viral load
ACC	HPV [37,42]	Smoking (in women) [37]	Squamous cell carcinoma – large degree of histological heterogeneity [42]	Complex CK profile but generally CK 7 and 20 negative and 5 and 18 positive. CK13 may be of value	Pap smear
				HIV-associated ACC generally positive by FISH for HPV16/18 [42]	HPV screening (high oncogenic genotypes) [30,46,47]

ACC, anal cell carcinoma; CEA, carcinoembryonic antigen; CK, cytokeratin; CT, computed tomography; EBV, Epstein–Barr virus; FISH, fluorescence in-situ hybridization; hepatitis B virus; HCC, hepatocellj.ar carcinoma; HCV, hepatitis C virus; HL, Hodgkin lymphoma; HPV, human papillomavirus; NSLC, non-small cell lung carcinoma; TTF-1, thyroid transcription factor 1.

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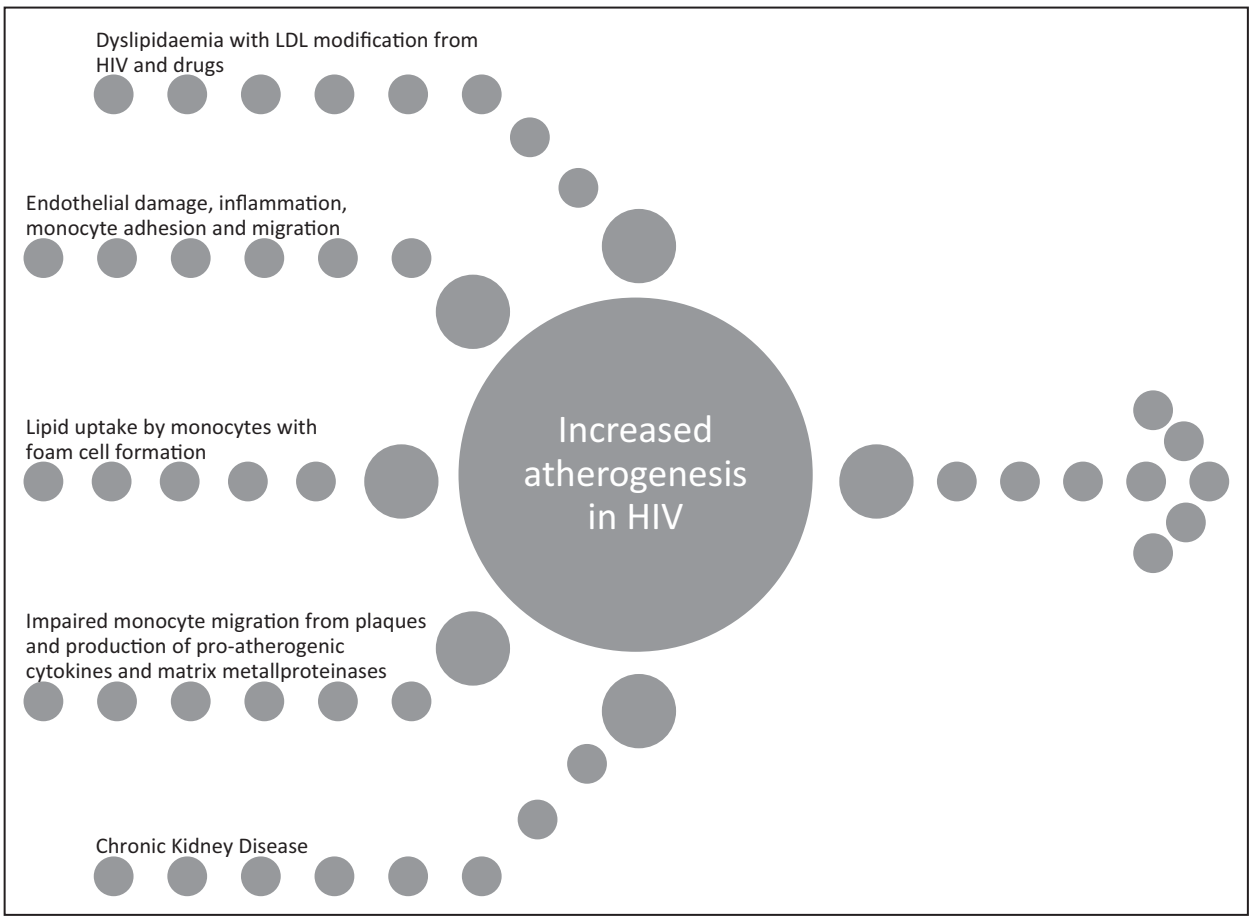


FIGURE 2. Risks factors for increased atherogenesis are increased LDL and/or reduced HDL [50], virus and cytokine-mediated arterial wall inflammation [13,51–52], platelet dysfunction, foam cell formation [53] and chronic kidney disease. HDL, high density lipoprotein; LDL, low density lipoprotein cholesterol.

Table 3. Putative mechanisms for metabolic abnormalities seen in HIV infection

Metabolic abnormality	Mechanism and reference
Body fat redistribution	Nucleoside reverse transcriptase inhibitors such as didanosine and stavudine [56]
	Protease inhibitors [54]
	Growth hormone deficiency [57]
	Mitochondrial toxicity [58]
Glucose abnormalities	Protease inhibitors [54,59]
	Cytokine-mediated insulin resistance [60]
	Hepatitis C coinfection [61]
High triglyceride and cholesterol	Protease inhibitors [27,50,54]
	Body fat redistribution [54,57]
Renal dysfunction	Tenofovir [55,62]

glucose is a sensitive indictor for the development of diabetes and a better predictor of all-cause mortality and cardiovascular mortality than FPG [73]. However, because of its poor reproducibility, inconvenience and cost, FPG is the preferred first-line test. Hemoglobin A1c (HbA1c), which measures the percentage of hemoglobin A that is glycated, is the primary index of glycemic control in patients with DM and has been recommended for the diagnosis of DM [72]. The advantage is that HbA1c is not affected by acute fluctuations in blood glucose as is seen with recent food intake or infections. However, concentrations increase with age, and may be affected by race. Recently, it has been shown that HbA1c underestimates glycemia in HIV infection [72].

Diagnosis and monitoring of renal dysfunction

The combination of eGFR and albuminuria or proteinuria allows the identification of patients at greatest risk of progression to advanced CKD.

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Table 4. Basic laboratory investigations for metabolic disease in HIV

Screening for metabolic complications	
Risk factor assessment at entry into care	Entry into care fasting blood glucose or HbA1c, fasting lipid profile
	Initiation of treatment or treatment modification: repeat blood glucose or HbA1c, and fasting lipids
	If tests are abnormal, repeat 3–6 monthly and if normal then yearly
	Carry out risk assessment for CVD
Other markers	
Laboratory monitoring for kidney disease	
	1. Serum creatinine and estimated GFR (eGFR), and urinalysis
	2. eGFR <60 ml/min/1.73m^2 or urine protein >1+ requires further evaluation:
	Urine protein or albumin/creatinine ratio
	3. Renal ultrasound, consider referral to nephrologist and renal biopsy
	4. Additional tests to determine cause if CKD is not due to HIV such as serology for hepatitis B, C, cryoglobulins, serum protein electrophoresis
Other markers	1. Urine phosphate, glucose and uric acid
1. Markers of renal tubular dysfunction	2. Tubular proteins including neutrophil gelatinase-associated lipocalin, cystatin C, α 1 microglobulin, β2 microglobulin and kidney injury molecule-1 [76]

CKD, chronic kidney disease; CVD, cardiovascular disease; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c.

Plasma creatinine is used to estimate eGFR using any one of a number of equations such as the modification of diet in renal disease equation or CKD epidemiology collaboration (CKD-EPI) equations. GFR estimating equations have not been validated in HIV-infected populations. Most published studies show that the combined cystatin C – CKD-EPI is somewhat more accurate than equations based on a single biomarker [74,75].

Urinalysis provides a semiquantitative measure of protein concentration in the urine, and is an acceptable screen for albuminuria/proteinuria. Increased fractional excretions of phosphate, uric acid and glycosuria are established markers of proximal tubule dysfunction and are easy to screen for, but they are less sensitive than tubular proteins. Urinary markers of tubular injury could potentially be useful in the early identification of kidney disease before the development of proteinuria or a decreased GFR [76].

The presence of albuminuria/proteinuria assists in staging of CKD and prognosis independent of GFR. Therapy with angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers has clinical benefits in individuals with albuminuria/proteinuria, particularly in patients with diabetes and/or hypertension, which provides a rationale for periodic urine monitoring in high-risk populations.

Ultrasound can identify evidence of obstruction, chronic infection or reflux, and polycystic kidney disease, as well as providing information on kidney size and echogenicity.

Although HIVAN classically presents with heavy proteinuria in the setting of advanced HIV disease, data from retrospective biopsy series suggest that noninvasive diagnostic testing lacks sufficient sensitivity and specificity to distinguish HIVAN from non-HIVAN diagnose, and biopsy confirmation of HIVAN is helpful to guide decisions about therapy. Table 4 [76] refers to basic metabolic investigations useful in the setting of HIV care and management.

CONCLUSION

HIV is associated with increased risk of noncommunicable diseases in the post-HAART era [2,3]. There are a number of challenges associated with effective diagnosis and monitoring of noncommunicable diseases in these settings. A significant disparity exists between the disease burden carried by LMICs and the research into those conditions in these regions of the world [77]. Although some programmes are in place to research some noncommunicable diseases in LMIC (e.g. the Health and Human Heredity in Africa project [78,79]), most studies are small and isolated to single regions. A concerted approach should build capacity to evaluate the burden of disease and the underlying etiological drivers and to produce well-curated data sets. Screening and diagnostic guidelines for noncommunicable diseases are produced for well-resourced settings and are impractical for LMIC, which may lack the infrastructure to support them adequately. Several lessons have been learnt in the diagnostic management of communicable diseases in LMIC, which can and should be translated into diagnostics for other disease processes [80,81]. Public health diagnostic strategies should focus on strengthening and integrating disease services to enable effective screening with early management of these complications [82,83].

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Conflicts of interest

The authors have no conflicts of interest.

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Publication 2: Increased platelet and microparticle activation in HIV infection: upregulation of P-selectin and tissue factor expression

Previous work, conducted by the candidate explored the upregulation of tissue factor by monocytes. Following this, the candidate focused on a key cellular effector of coagulation, platelets.

Platelets are subcellular fragments which mediate coagulation especially in arterial thrombosis. Platelets can interact with leukocytes including monocytes with reciprocal activation and may release smaller fragments (or microparticles). In this publication, platelet activation status was measured in 46 HIV-infected patients and 18 HIV-uninfected controls derived from the same cohort as described in publication 2. Platelets and platelet-monocyte microparticles were identified by scatter characteristics on flow cytometry calibrated against platelets[41] and the size of the particles were typically >0.5µm. Isotype controls were also included. It was not fully possible to distinguish monocytes from platelet-monocyte microparticles although the expression of platelet markers were generally considered as indicative of platelet monocyte microparticles. Microparticles were <3µm in size.

A greater percentage of platelets and microparticles derived from HIV-infected patients expressed TF (4.7%) and the platelet adhesion marker, P-selectin (17.3%) than HIV-uninfected controls (1.2 and 1.1% respectively) suggesting a significantly enhanced platelet activation status in HIV-infected individuals. The percentage of platelets expressing P-selectin was significantly higher in viraemic patients although the percentage of platelets expressing TF remained constant. Expression of both platelet activation markers correlated with markers of microbial translocation (soluble CD14 expression) and markers of immune activation (CD8+ T cell expression of HLA-DR and CD38). In summary, platelet and platelet-monocyte microparticles in HIV-infected individuals show activation with increased procoagulant ability and increased expression of the primary platelet adhesion marker, P-selectin.

Contribution of candidate

Candidate was personally responsible for devising the experiments, for performing the flow cytometry analysis and analysis and for drafting and reviewing the manuscript. My co-authors provided input into the assay design (NF, MK, SS, AS), assisted with collection of samples from patients (RA, BR), and were responsible for reviewing and approving the final manuscript (NF, WS, ML)

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Increased platelet and microparticle activation in HIV infection: upregulation of Pselectin and tissue factor expression

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Abstract

OBJECTIVE—HIV-1 infected patients have an increased risk for atherothrombosis and cardiovascular disease, but the mechanism behind these risks is poorly understood. We have previously reported that expression of tissue factor (TF) on circulating monocytes is increased in persons with HIV infection and that TF expression is related to immune activation, to levels of HIV in plasma, and to indices of microbial translocation. In this study, we explore the activation state of platelets in HIV disease.

METHODS—Here, using flow cytometry-based assays, we measured platelet and platelet microparticle (PMP) activation in samples from HIV-1 infected donors and controls.

RESULTS—Platelets and PMPs from HIV-1 infected patients are activated (as reflected by expression of CD62 P-selectin) and also more frequently expressed the procoagulant tissue factor (TF) than did platelets and PMPs obtained from controls. Expression of these proteins was directly related to expression of TF on monocytes, to markers of T cell activation (CD38 and HLA-DR) and to plasma levels of soluble CD14, the coreceptor for bacterial lipopolysaccharide. Platelet and microparticle expression of TF was not related to plasma levels of HIV but expression of P-selectin was; neither TF nor P-selectin expression was related to CD4 T cell count.

CONCLUSIONS—Platelets and microparticles are activated in HIV infection and this activated phenotype may contribute to the increased risk for cardiovascular and thrombotic events in this population although a role for other confounding cardiovascular risks cannot be completely excluded.

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Keywords

tissue factor; platelets; HIV-1; immune activation

Introduction

Platelets, anuclear cell fragments, participate in the localization of coagulation reactionsin the intravascular compartment. Platelet reactivity, especially within ruptured atheromatous plaques, has been implicated in the pathogenesis of atherothrombosis and cardiovascular disease [1, 2]. Many agents aimed at preventing cardiovascular disease target platelet activation [2, 3]. Upon activation, platelets increase expression of the adhesion molecule, P-selectin, thereby promoting adhesion to cell surfaces and clot formation [2, 3]. Platelets also contain pre-mRNA for the procoagulant tissue factor. Tissue factor on platelets [4], and on other surfaces, forms a complex with factor VII to serve as a cofactor for factor VIIa to activate factor X and drive clot formation [5].

P-selectin, a glycoprotein found in the α-granules of platelets, binds to P-selectin glycoprotein ligand-1 (PSGL-1) which is expressed by the endothelium and leukocytes. This interaction is integral to the recruitment of pro-inflammatory leukocytes to sites of injury and thrombosis, such as occurs in the settings of endotoxin-induced sepsis and atherosclerosis [3, 6–9]. P-selectin expressed by platelets captures microparticles, monocyte and platelet fragments, in both pathologic and non-pathologic thrombi to activate leukocytes and cause up-regulation of tissue factor expression by endothelial cells and monocytes [3, 10].

Patients with human immunodeficiency virus (HIV) infection have an increased risk of thrombotic cardiovascular disease [11, 12]. The SMART study, a clinical trial of continued versus interrupted antiretroviral treatment, found that the risk of death, including deaths due to cardiovascular events, was linked to higher plasma levels of the inflammatory cytokine interleukin-6 (IL-6), C-reactive protein, and D-dimers (markers of clot formation and lysis) [13]. We have earlier shown that HIV infection is associated with systemic translocation of microbial products such as bacterial lipopolysaccharide and bacterial DNAs from the intestinal lumen. These microbial products can bind to innate immune pattern recognition molecules such as Toll-like receptors (TLRs), resulting in activation of both innate [14] and adaptive immune cells [15]. Plasma levels of these products are correlated with indices of immune activation [16, 17].

We have also shown that freshly isolated monocytes from HIV-infected patients more frequently express cell surface tissue factor than do monocytes from uninfected controls. Monocyte TF expression correlates with markers of immune activation, with plasma levels of HIV RNA, with plasma levels of the LPS co-receptor CD14, and with levels of D-dimers [18].

Platelets, which have an important role in arterial thrombosis, interact with leukocytes [1, 2] and express functional Toll-like receptor 4 [19]. We hypothesized that platelets of HIV-infected patients might also be activated as a result of sustained exposure to microbial elements in circulation. In the present report we show that platelets and microparticles in HIV infection are activated to express both P-selectin and tissue factor and that expression of these markers correlate with indices of T cell activation and microbial translocation/ monocyte activation that are recognized predictors of morbidity in chronic HIV infection [20, 21].

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Methods

All procedures were conducted in accordance with policies of the Case Western Reserve University/University Hospitals of Cleveland Institutional Review Board.

Peripheral blood was drawn, with consent, from HIV infected patients (n=46) and from healthy controls (n=18). All samples were conveniently collected from the populations available, in the morning, over a two month time span. Blood was collected in glass tubes for preparation of serum, and in EDTA-coated or citrate-anticoagulated plastic tubes for plasma. Anticoagulated (citrate) blood was centrifuged at 180x g for 10 minutes to separate platelet-rich plasma from cells. Peripheral blood mononuclear cells were isolated on a ficoll-hypaque cushion.

Flow cytometry

Platelets, from platelet-enriched citrated plasma, were identified in plasma by small size and low complexity on forward versus side scatter plots. These elements were positively identified as platelets, or platelet microparticles, by expression of the surface marker glycoprotein 1b (CD42b) using a phycoerythrin (PE) conjugated monoclonal antibody (BD Pharmingen, San Diego, CA). In selected experiments we confirmed that these labelled elements were neither monocyte derived-MPs nor monocyte PMP aggregates by the absence of CD14 expression (not shown). Surface expression of P-selectin (CD62P) and tissue factor was measured using conjugated monoclonal antibodies to CD62P (Allophycocyanin (APC)-conjugated - BD Pharmingen) and to tissue factor (fluorescein isocyanate (FITC)-conjugated (American Diagnostica, Stamford, CT).

Red blood cells within whole blood preparations were lysed by incubation in FACS Lyse buffer (BD Biosciences, San Diego, CA) for 15 minutes on ice. Monocytes were identified by light scatter characteristics and by reactivity with anti-CD14 Peridinin-chlorophyll-protein Complex (PerCP), and with anti-HLA-DR APC (BD Pharmingen). CD4+ and CD8+ T cells were identified by binding of anti-CD3 APC and anti-CD4 Pacific Blue, or anti-CD8 APC-Cy7 and levels of T cell activation were measured using anti-CD38 PE and anti-HLA-DR FITC (BD Pharmingen). Fluorochrome-conjugated monoclonal antibodies of appropriate isotype were used to establish gating. Cell preparations were stained for 30 minutes, then washed in phosphate buffered saline containing 1% fetal calf serum and 0.1% sodium azide, and resuspended in 1% paraformaldehyde. Platelet preparations were stained for 30 minutes on ice, then washed and resuspended in 1% paraformaldehyde before analysis. Cells, platelets and PMPs were acquired and analyzed using an LSR II flow cytometer (Becton-Dickinson, San Jose, CA) and FACSDiva software version 6.1.1 (BD Biosciences, San Diego, CA).

Measurement of D-dimers and Soluble CD14

Whole blood was collected into EDTA containing tubes and plasma was prepared by centrifugation for 10 minutes at 1610×g and was then frozen at −80°C until assay. Levels of D-dimers were measured using the Asserachrom D-DI immunoassay (Diagnostic Stago Asnieres, France) and soluble CD14 was measured by ELISA using the Quantikine soluble CD14 kit (R&D Systems Minneapolis, MN, USA) following the manufacturer's instructions.

Statistical Methods

Differences between variables among patients and controls were tested with a Mann-Whitney's U test. We tested associations between pairs of continuous variables with Spearman's rank correlation. We processed the analyses using Graphpad Prism v. 5.02

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(GraphPad Software, Inc., La Jolla, CA). All tests are two-sided, and a p-value of 0.05 was considered nominally significant.

Results

Platelets in HIV infected subjects have high surface expression of P-selectin and tissue factor

Previous studies have shown that HIV infected patients have an increased risk for thrombotic and cardiovascular events [11, 12, 22–24] and since platelets and microparticles play a major role in coagulation, we prepared these anuclear fragments from the plasma of 46 HIV infected donors and 18 healthy controls and measured surface expression of P-selectin and tissue factor by flow cytometry. All of our samples were collected from patients and controls in the University Hospital/ Case Western Reserve University population. Among the patients and controls for whom we have information, 67% of each group was male. The median ages for the patient and control populations were 42 years (range 26–65) and 33 years (range 20–61) respectively (p=0.015). Forty one percent of patients had plasma RNA levels >400copies/ml. The median plasma HIV RNA level for the entire patient population was 50 copies/ml (range = 50–590,000copies/ml) and the median CD4+ T cell count was 400 cells/ul (range = 6–1,063 cells/ul). Seventy three percent of the patients were being treated with antiretroviral therapy. A greater proportion of the patient population reported that they were current smokers (29%), compared to 17% of the controls While the HIV-infected group had a greater number of patients who were defined as obese (Body Mass Index, BMI> 30, N=7 for patients, N=0 for controls), the median BMI of each group was not statistically different (patients =25.8, controls 24.5, p=0.11). Among the HIV+ group, 6 patients reported daily treatment with aspirin, 5 patients reported using statins, 4 patients had a positive screen for Hepatitis B and 6 patients were positive for Hepatitis C. Among the healthy controls, 1 control reported taking aspirin daily, 2 were receiving statins, and none was diabetic or known to be infected with Hepatitis B or C. A greater proportion of the HIV infected population was African American (51%) compared to 6% among uninfected controls. Demographic information is summarized in Table 1.

Platelets and microparticles were identified by low forward and side scatter characteristics, and by expression of CD42b (Figure 1A). Among healthy controls, these elements rarely expressed P-selectin (median 1.1% positive) or tissue factor (median 1.2% positive). The proportions of platelets and microparticles expressing P-selectin or tissue factor (17.3% and 4.7% respectively) in HIV-infected patient samples were 10 significantly greater ($p < 0.001$ for both) than among controls. Platelets and microparticles obtained from patients with detectable viremia (n =18) more frequently had detectible surface P-selectin than did platelets and PMPs obtained from patients with virologic control (HIV RNA<400 copies/ml) (n = 23, 23.3 versus 12.6%, p =0.022, Figure 1B). The proportions of TF+ platelets and PMPs were similar among patients with uncontrolled viremia and patients with virologic suppression (3.4% vs 5.0%, respectively, p=0.7, Figure 1C). The proportions of TF+ and CD62P+ platelets and microparticles were directly related when samples from all donors were analyzed (r=0.53 p<0.001, data not shown).

We previously described an increase in surface expression of TF on monocytes from HIV infected patients [18]. In this study, the proportions of monocytes and platelets/microparticles expressing tissue factor, from all donors, are directly related (r=0.4842, p=0.0078, Figure 1D), suggesting that the drivers of platelet/microparticle and monocyte expression of tissue factor may be related.

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Platelet/microparticle activation is differentially related to plasma levels of the LPS coreceptor CD14 and to plasma HIV levels, but not to CD4 T cell counts

To obtain preliminary insight into the determinants of platelet activation in HIV-1 infection, we explored potential clinical and laboratory correlates of platelet activation. Unlike monocyte TF expression, there was no relationship between platelet/PMP expression of TF and the magnitude of plasma viremia or CD4+ T cell count (r=0.1066 p=0.51 and r= −0.12, p=0.46 respectively, not shown). There was a correlation between the proportion of P-selectin positive platelets/PMPs and levels of plasma viremia (r=0.45, p=0.003) but, there was no relationship between P-selectin expression and CD4+ T cell counts(r=−0.20, p=0.21, not shown). Thus, HIV replication or its consequences may drive platelet expression of P-selectin, but not of tissue factor. We speculate that monocytes may recognize and be activated by HIV-1 through surface receptors used for viral entry or by innate immune receptors expressed by monocytes that are likely absent on platelets and PMPs.

We have previously reported that the increased plasma levels of the LPS co-receptor (sCD14) in HIV infected patients correlated with the proportion of monocytes expressing TF [18]. In the present study, we find higher plasma levels of sCD14 in HIV infection than in healthy controls (2603ng/ml versus 1364ng/ml, p=0.004). In all donors, plasma levels of sCD14 were directly related to platelet/microparticle expression of P-selectin (r=0.71, p<0.001, Figure 2A) and tissue factor (R=0.39, p=0.026, Figure 2B). The relationship between platelet/microparticle expression of P-selectin and sCD14 remained significant in the HIV infected patients population (r=0.456, p=0.029, not shown).

Plasma levels of D-dimers, markers of coagulation and fibrinolysis, were predictive of all-cause mortality in the SMART study [13, 16]. Since we had earlier linked monocyte TF expression to D-dimer levels [18], we asked if this marker also was related to platelet/PMP activation. While plasma levels of D-dimers were increased in patients compared to levels in controls (436 FEU versus 256 FEU, respectively, p=0.029) there were only modest correlations between D-dimer levels and the proportion of platelets/microparticles expressing P-selectin (r=.40, p=0.041) or tissue factor (r=0.30, p=0.115) in samples from all donors.

Platelet/microparticle activation is directly related to CD8+ T cell activation

T cell activation indices have been linked to disease course in HIV infection [25, 26]. We next asked if T cell and platelet/PMP activation could also be related. The expression of CD38 and HLA-DR on CD8+ T cells was strongly correlated with platelet/microparticle surface expression of both P-selectin (r=0.63, p=0.0006) and tissue factor (r=0.45, p=0.0175) (Figure 3A and B) in all donors, and the relationship between CD38 and HLA-DR on CD8+ T cells and the proportion of platelets/PMPs that express TF remained when analyzing the patient population alone (r=0.530, p=0.02).

Discussion

Both in the pre-HAART and in the post HAART eras, HIV infected patients have experienced an increased risk of thrombosis and cardiovascular disease, [12, 27, 28] yet, the determinants of these risks are incompletely understood. In a recent study where continuous antiretroviral therapy was compared to intermittent therapy, all cause mortality, as well as morbidity due to cardiovascular disease, were linked to increased levels of D-dimers, interleukin-6, and C-reactive protein [13], implicating both inflammation and coagulation as potential mediators of these risks.

In plasma samples from persons with chronic HIV infection, we and others have found increased levels of microbial products; evidence of microbial translocation from the

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damaged gut [16, 17, 29]. These levels were linked both to indices of immune activation and also to T cell homeostasis as reflected by a strong relationship to the magnitude of CD4+ T cell restoration after application of combination antiretroviral therapies [16, 17]. Moreover, we have demonstrated that a broad variety of microbial TLR ligands can induce an activation phenotype on both CD4+ and CD8+ T cells in vitro that is similar to the activation phenotype seen *in vivo* in chronic HIV infection [15]. More recently, we have shown that selected TLR ligands can induce tissue factor expression on blood monocytes and that monocytes from HIV infected donors commonly express high levels of cell surface TF [18]. Soluble TF levels were also increased in HIV infection and levels of monocyte TF were related to plasma levels of the LPS coreceptor sCD14 and to plasma levels of D-dimers, potentially linking this procoagulant to microbial translocation/monocyte activation and to ongoing clot formation and fibrinolysis. Recently, Hashimoto et al have also found that *in vitro* exposure to LPS could induce platelets to express P-selectin [30]. Thus, it is reasonable to propose that monocyte and platelet activation and increased procoagulant expression in chronic HIV infection may be linked at least in part to in vivo exposure to microbial products.

We identified platelets/microparticles by their size and light scatter characteristics and confirmed that these small anuclear fragments are of platelet origin by the concordant expression of CD42b, a glycoprotein that is uniquely expressed by platelets [31]. The absence of CD14 expression assured that these elements were not microparticles derived from macrophages/monocytes, nor had they bound their membrane fragments. Based on our gating strategy, we could not discriminate between platelets and PMPs. These subcellular platelet fragments are mainly produced by activated platelets and express the same cell surface antigens as do activated platelets, including CD42b, tissue factor, and P-selectin [32–34]. While we cannot distinguish activated platelets from platelet derived microparticles, these two populations are functionally and phenotypically nearly identical and the main finding of this study is that the proportion of small, CD42b+ anuclear cell fragments that express TF or P-selectin are increased in the circulation of HIV-1 infected patients, compared to these proportions in the circulation of healthy uninfected controls. High levels of P-selectin have been previously reported in plasma [35] and on the surfaces of platelets in HIV infected patients [36], in consumptive coagulopathies, [37] and in settings of direct endothelial injury and platelet activation [38]. Unlike the finding reported by Holme et al [36], we did not see a relationship between platelet/microparticle expression of P-selectin and CD4+ T cell counts among our HIV infected donors [36].

Platelet/microparticle expression of TF was not directly related to clinical indices of HIV disease (plasma viremia and CD4+ T cell count), but both TF and P-selectin expression on platelets/microparticles were related to T cell activation. This work suggests that the drivers of chronic immune activation and inflammation that are independent of the magnitude of viral replication may be important determinants of morbidity in both treated and untreated HIV infection.

While our findingsrelate the chronic immune activation of HIV-1 infection to platelet activation and coagulation in HIV disease, the results of this cross sectional study may be limited by the small sample size and by significant differences in our patient and control populations. Patients tended to be older (mean age 42 years) than our controls (mean age 33 years, p=0.015) but, age has not been associated with differences in P-selectin expression in other studies [39–42] nor did we show a significant relationship between age and platelet expression of Pselectin (r=0.159, p=0.334) or TF (r=0.035, p=0.832) in our patient population. Although there were proportionally more smokers among patients (29%) than among controls (17%), in our patient population, smokers did not have higher platelet tissue factor expression (mean= 5.7 %TF+) or platelet activation (16.29% CD62P+) than did non-

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smokers (5.5 %TF+, 20.77%CD62P+, p=0.39 and p=0.34, respectively), suggesting smoking does not influence the increased levels of platelet activation in these patients. While we also had more obese patients among the HIV infected population (N=7) than among the controls (N=0), expression of CD62P or TF on platelets from obese patients and non-obese patients within the HIV+ population was not significantly different (p= 0.43 and p=0.42, respectively). While the proportion of African Americans was much greater in our patient population (51%) than in controls (6%), we did not see a significant difference in markers of platelet activation between African Americans and Caucasians within the HIV-infected population. The proportions of CD62P+ or TF+ platelets within the African American population were 19.4+/−3% and 6.15+/−2%, and among the Caucasian population, the proportions of CD62P+ and TF+ platelets were 18.7+/−3% and 5.1+/−3%, (p=0.75 and p=0.87, respectively.) These results suggest that race/ethnicity was not a major determinant of the increased platelet activation seen here in persons with HIV infection. Several of these indices (smoking, BMI, ethnicity) contribute to an increased risk of cardiovascular disease and platelet activation. Due to the small overall sample size in this study and the differences between our HIV+ and HIV− subjects as noted above, we cannot entirely rule out some contribution of these confounders on the differences in platelet activation reported herein. Future studies, where a larger and or more comparable groups of participants are examined, will be needed to confirm our findings that platelets and microparticles are activated in HIV infection. Conceivably this activation contributes to the apparent increased cardiovascular risk experienced by persons with HIV infection that is linked both to HIV-1 replication and to other indices of immune activation.

The proposed linkage between platelet activation and microbial translocation is also limited by the fact that we did not measure LPS directly. While sCD14 levels have been correlated directly with plasma levels of LPS in some studies[16, 43] other studies were unable to find such a relationship[21, 44]. Activation of monocytes by microbial products, such as LPS, or inflammatory cytokines, such as IL-6 [44], can induce shedding of CD14. Thus, increased levels of plasma sCD14 may also reflect monocyte activation through a variety of stimuli. We have previously demonstrated a relationship between monocyte expression of TF and sCD14 [18] and in this current work, we report a relationship between platelet expression of TF and Pselectin to plasma levels of sCD14, suggesting that there may be a common “driver,” such as microbial translocation or inflammatory cytokines, that may be helping to drive monocyte and platelet activation in HIV disease.

In summary, we show here that platelets/microparticles of HIV infected persons are activated *in vivo* to express P selectin and tissue factor, and that expression of these markers of adhesion and coagulation are related to soluble levels of the LPS receptor CD14. The proportions of TF+ monocytes and TF+ platelets/microparticles appear to be directly related, and our data suggest that they may be driven, at least in part, by sustained exposure to microbial elements or represent another consequence of monocyte activation. Both platelet/microparticle and monocyte TF expression were strongly related to levels of the LPS coreceptor sCD14 while monocyte TF, [18] but not platelet/microparticle TF, was strongly correlated to plasma levels of HIV. We have recently found that monocytes can be directly activated by HIV in vitro to express tissue factor (unpublished); this is not likely the case for platelets. Additional study is needed to clarify the mechanisms of TF upregulation and platelet activation in HIV infection and to ascertain the relationship among these indices and in vivo thrombosis and cardiovascular morbidity. We suspect that the recognized increases in cardiovascular morbidities that are complicating the course of HIV infection may be at least in part, a result of the sustained exposure of these patients to the proinflammatory environment that persists even among HIV infected persons in whom viremia is well controlled by antiretroviral therapies [17].

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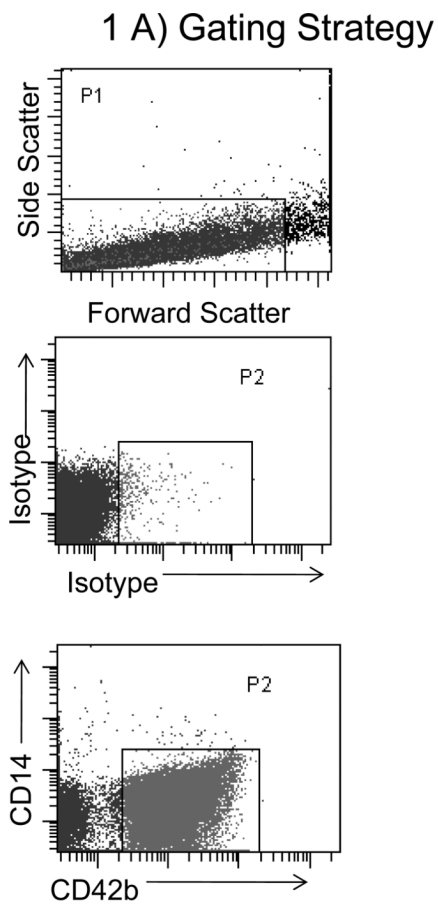
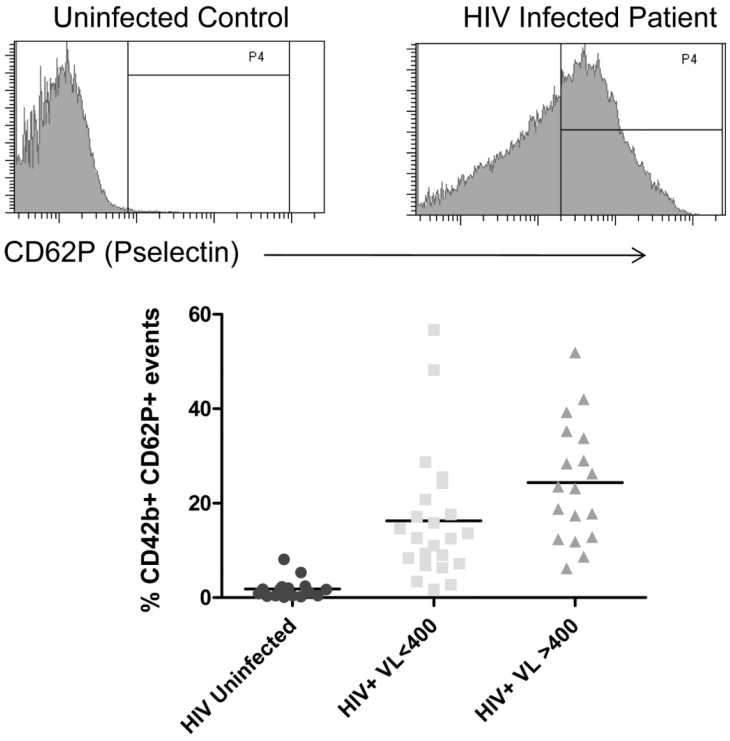


Figure 1 B)



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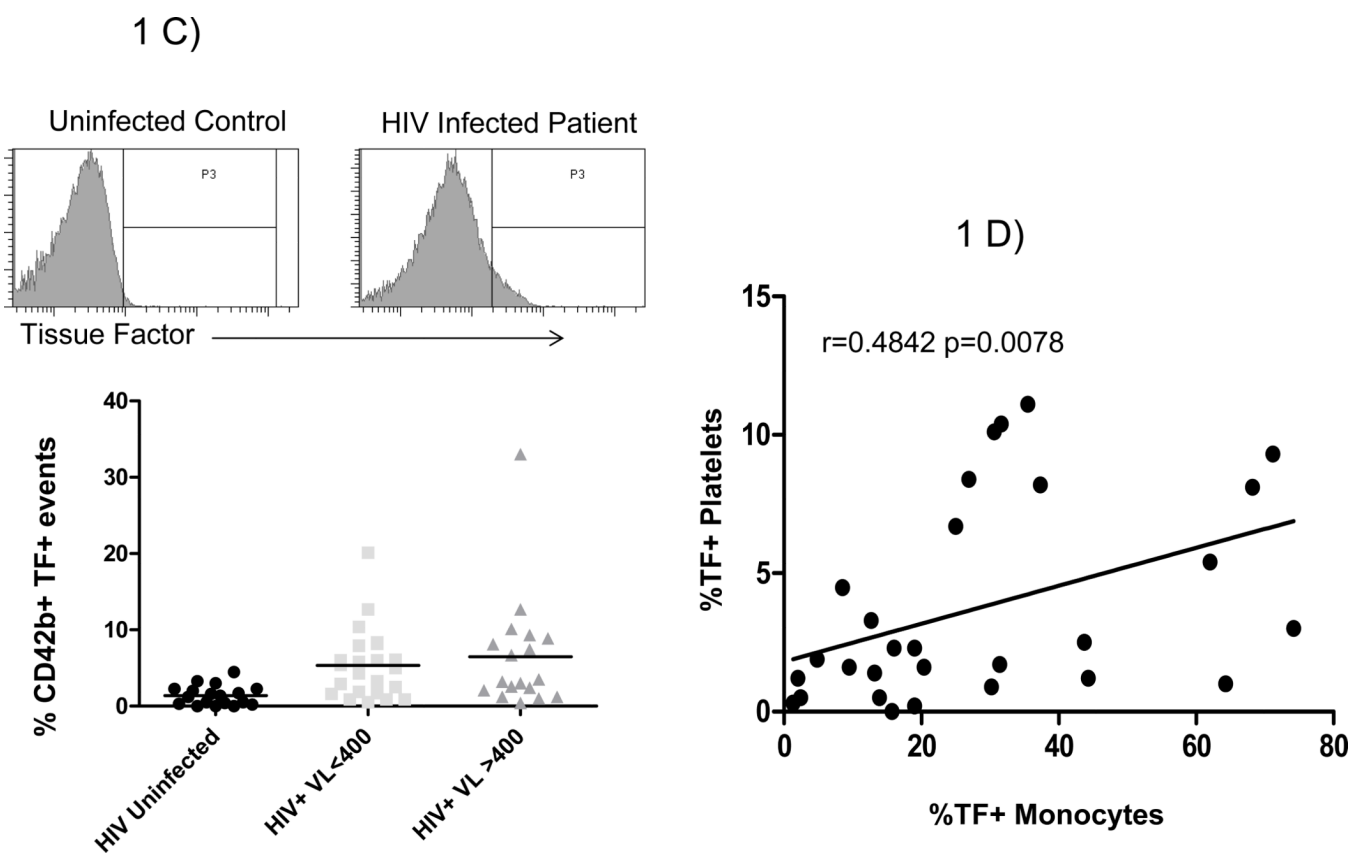


Figure 1. Platelets/microparticles from HIV infected patients have an activated phenotype

A) Platelets/microparticles are defined by their low complexity and small size in plasma and by expression of CD42b. Representative histograms and summary data among platelets/microparticles from healthy controls (N=18), patients with controlled viremia (HIV RNA<400 copies/mL, N=23), and uncontrolled viremia (HIV RNA>400 copies/mL, N=18) for surface expression of B) CD62P and C) tissue factor. The proportions of platelet/PMP that expressed CD62P in each of our patient groups were significantly different from the proportion in controls ($p<0.001$ for both). Likewise, the proportions of TF+ platelets/PMPs in each of our patient groups were significantly different from the proportion of TF+ platelets/PMPs in controls ($p>0.001$ for both) D) The proportions of TF+ platelets/microparticles and TF+ monocytes are directly related ($r=0.4842$ $p=0.0078$).

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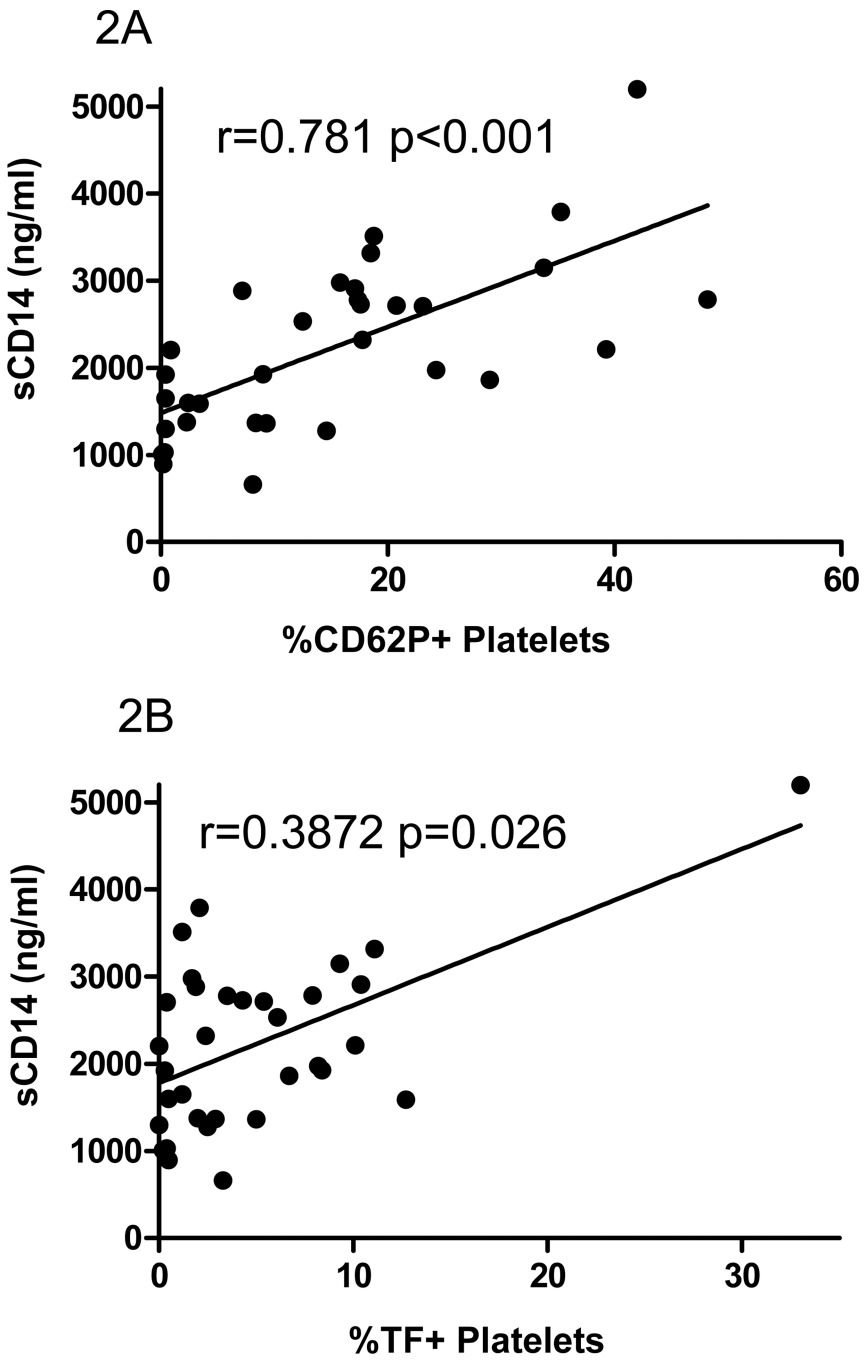


Figure 2. The proportions of platelets/microparticles expressing P-selectin or tissue factor are related to plasma levels of sCD14

Plasma samples were thawed in batch and levels of sCD14 were measured. In samples from all donors A) the proportion of P-selectin positive platelets/microparticles is directly related to plasma levels of sCD14, r=0.781 p<0.001; and B) the proportion of tissue factor positive platelets/microparticles is directly related to plasma levels of sCD14, r=0.3872 p=0.026.

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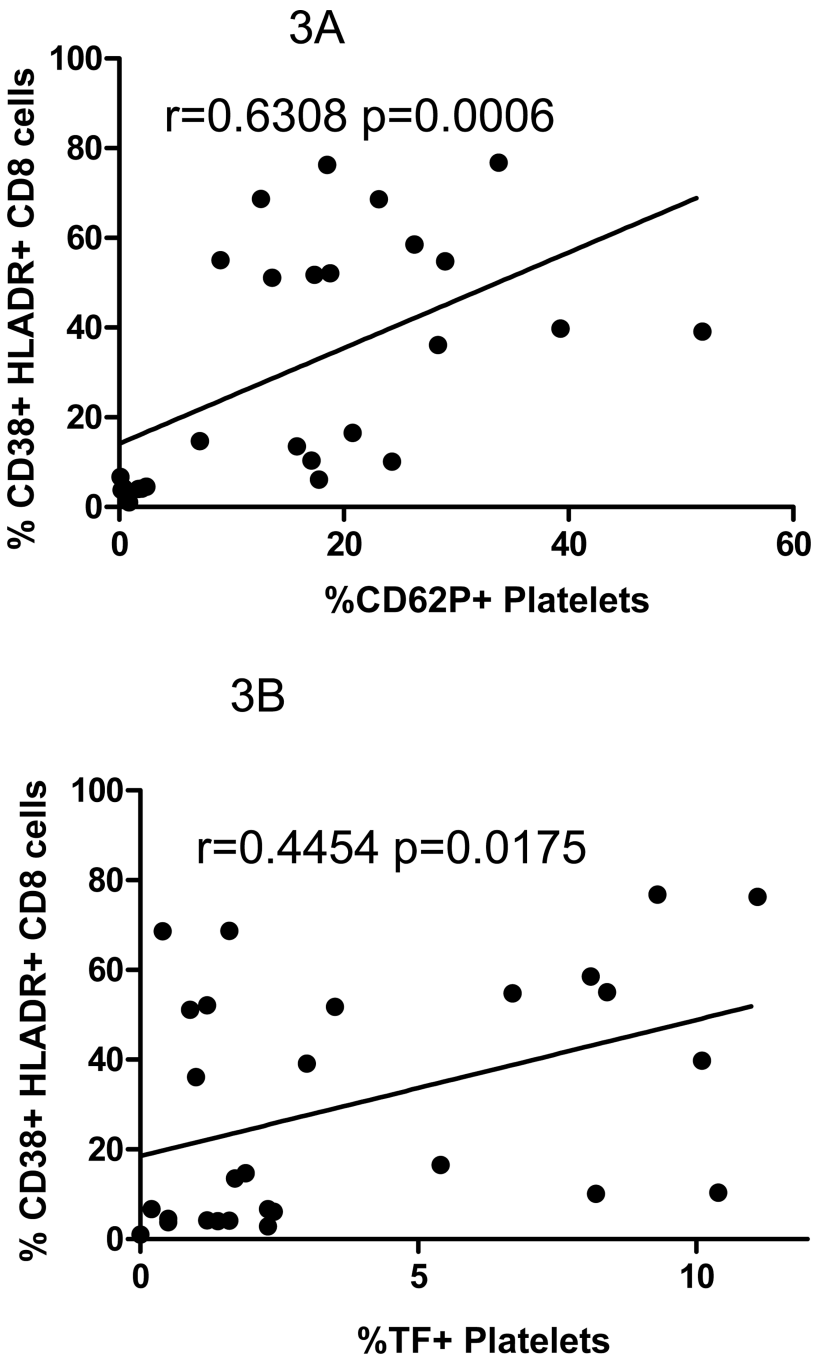


Figure 3. The proportions of platelets/microparticles expressing P-selectin or tissue factor are related to markers of T cell activation

CD8+ T lymphocytes were identified by size, complexity, and by expression of CD3 and CD8, and markers of activation (CD38 and HLA-DR) were measured on phenotypically gated cells. In samples from all donors A) the proportion of P-selectin positive platelets/microparticles is directly related to CD8+ T cell activation, r=0.6308 p=0.0006; and B) the proportion of TF positive platelets/microparticles is directly related to CD8+ T cell activation, r=0.4454 p=0.0175.

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Table 1

Demographic information of the study cohort

	Gender	Age (Median yrs)	Tobacco use	Antiretroviral usage	Ethnicity (# individuals, self reported information)	BMI (median)
Controls	7 females 11 males	33 range [20–61]	Non-smokers 15 Smokers 3	Not applicable	1- African American 12- Caucasian 2 -Hispanic/Latin 2-Asian 1 –African	24.5 range [16.7–28.3]
Patients	14 females 28 males	42 range [26–65]	Non-smokers 33 Smokers 13	55 on HAART	19 -African American 18- Caucasian	25.8 range [18.9–42.1]

Publication 3: Good Fences Make Good Neighbors: Human Immunodeficiency Virus and Vascular Disease

The clinical evaluation of patients with cardiovascular disease requires measurement of both cellular and humoral biomarkers of disease. Following the evaluation of activation status of platelets and monocytes in patients with HIV, we then undertook a comprehensive review of all clinical, humoral and cellular biomarkers of HIV-associated cardiovascular disease. This provided an important summary of these disparate markers across HIV-cohorts (including in Africa and agnostic of treatment status). Following this review, one of the critical biomarkers which linked inflammation, endothelial disease and cardiovascular risk was identified as Lipoprotein Associated Phospholipase (Lp-PLA2).

Contribution of student:

In this systematic literature review, the candidate was responsible for definition of the Medical Subject Heading (MESH) terms, data collation and for drafting and review of the final manuscript. The co-author (SL) assisted with data collation and drafting and final review of the manuscript

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REVIEW ARTICLE

Good Fences Make Good Neighbors: Human Immunodeficiency Virus and Vascular Disease

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Cardiovascular disease, venous thrombosis, and microvascular disease in people with HIV (PWH) is predicted to increase in an aging HIV-infected population. Endothelial damage and dysfunction is a risk factor for cardiovascular events in PWH and is characterized by impaired vascular relaxation and decreased nitric oxide availability. Vascular disease has been attributed to direct viral effects, opportunistic infections, chronic inflammation, effects of antiretroviral therapy, and underlying comorbid conditions, like hypertension and use of tobacco. Although biomarkers have been examined to predict and prognosticate thrombotic and cardiovascular disease in this population, more comprehensive validation of risk factors is necessary to ensure patients are managed appropriately. This review examines the pathogenesis of vascular disease in PWH and summarizes the biomarkers used to predict vascular disease in this population.

Key words: biomarkers; cardiovascular disease; endothelium; HIV; thrombosis.

INTRODUCTION

Patients with human immunodeficiency viral (HIV) infection have an improved prognosis on antiretroviral therapy (ART) [1]. This is associated with a concomitant drop in the prevalence of opportunistic infections and a corresponding increase in life expectancy [1–3]. Noncommunicable diseases have become a major cause of morbidity and mortality in these patients, including large vessel disease (occlusive vasculitides and aneurysms), cardiovascular disease (CVD), and venous thromboembolism [4–13]. Thrombotic disease is pathological clotting in the vascular system. In arteries, abnormal clotting can result in peripheral vascular disease, myocardial infarction, and cerebrovascular accidents [8]. Venous clots can dislodge and travel to the pulmonary vasculature, a phenomenon known as pulmonary thromboembolism [11]. In the microvasculature system, small disseminated clots can be seen with microangiopathic thrombotic processes, including thrombotic thrombocytopaenic purpura (TTP), TTP-like syndrome, and disseminated intravascular coagulopathy (DIC) [14]. Abnormal clotting in the entire vascular tree occurs in people with HIV (PWH). Thrombotic risk in PWH has been attributed to a number of factors, including the presence of opportunistic infections, prolonged immobility, antiretroviral drugs and other treatments, comorbid conditions (including hypertension and diabetes), and the impact of HIV, itself, on the endothelium [15–17]. This review will look at the interaction between HIV, the vascular wall, and pathogenic thrombosis.

THE ENDOTHELIUM

The endothelium is a monolayer of cells that lines the blood vessels [18, 19]. It is a highly specialized organ that is responsible for control of both inflammation and coagulation. The endothelium-lining arteries, veins, and capillaries show differential response to stressors that are physiological adaptations to the anatomical location [19]. These stressors include differential shear stress (high in the arterial system and lower in the venous system) that can result in location-specific gene transcription [19].

Under normal conditions, the endothelium is a selectively permeable, anticoagulant surface. It produces a number of molecules that act to limit clotting by inhibiting both platelet activation and coagulation factors (Table 1).

In response to pro-inflammatory stimuli or trauma, the endothelium upregulates cellular adhesion molecules and procoagulant factors and becomes more permeable (Table 2) [19]. This allows leukocytes to translocate across the endothelial surface and into the tissue. There is a local shift from

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Table 1. Anticoagulant Factors and Vasodilators Produced by the Endothelium

Anticoagulant Factor	Function
Heparan sulphate	Combines with antithrombin and inactivates coagulation factors IIa and Xa [19]
Prostacyclin	Platelet inhibition [19]
Cluster of differentiation (CD) [39]	Scavenges adenosine diphosphate released by activated platelets to inhibit platelet aggregation [20]
Endothelial protein C receptor	Binds activated protein C to potentiate its activity; complex also acts to protect endothelial cells through the activity on protease-activated receptor-1 and -2 [21]
Thrombomodulin	Forms a complex with thrombin to activate protein C, which binds and inactivates factors V and VIII (with protein S as a cofactor) [22]
Tissue factor pathway inhibitor	Inhibits the tissue factor-VIIa complex and factor Xa [23]
Primary vasodilator	
Nitric oxide (endothelium-derived)	Mediates vasodilation; inhibits platelet activation [24, 25]
Endothelium derived hyperpolarizing factor	Mediates smooth muscle relaxation and causes vasodilation [26]

an anticoagulant to a procoagulant surface [27]. Tissue factor may be exposed by trauma, secreted into the peri-endothelial space in endothelial vesicles, or upregulated on leukocytes (especially monocytes) [28, 29] and platelets, resulting in activation of the coagulation cascade and clot initiation. Platelets are activated by exposure to subendothelial tissue (primarily collagen) and provide a secondary surface for coagulation, resulting in clot propagation [30].

Following endothelial injury, a number of processes are initiated to repair the damaged endothelium and allow for clot resorption [40]. Bone-marrow–derived endothelial progenitor cells home to sites of vascular injury and stimulate angiogenesis [40–42]. Platelets and endothelial cells secrete a number of growth factors (including vascular endothelial growth factor), cytokines (including TGF-beta), and chemokines (including CXCL-12 or stromal derived factor-1) that restore barrier function and stimulate endothelial cell proliferation [43–45]. Clot resolution occurs with activation of proteases, like plasmin (through the function of tissue plasminogen activator or tPA present on endothelial cells) [46], which break down fibrin clots with resulting production of fibrin degradation products (measured as D-dimers). This process reduces local hypoxia with stabilization of hypoxia-inducible factor-1, which also can contribute to repair [19].

ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction is a state of aberrant endothelial cell activation that is associated with thrombosis [47]. Endothelial dysfunction classically is associated with arterial atherosclerosis, but it can be more broadly defined to include a prothrombotic state throughout the vasculature [48]. Multiple pathophysiological mechanisms contribute to a phenotype that is characterized by reduced dilatation, decreased arterial compliance, and local inflammation with reduced vascular repair and angiogenesis [19]. Bioavailability of nitric oxide, a key vasodilator and platelet inhibitor, is significantly reduced [25]. The pro-inflammatory mediators that are produced by the damaged endothelial tissue also act directly to upregulate tissue factor expression by leukocytes and to activate platelets [28, 49, 50]. Cyclo-oxygenase enzymatic activity is upregulated in response to various inflammatory stimuli and results in increased production of prostaglandin E2 and thromboxane A2, which stimulate platelet activation and aggregation [38, 51].

PATHOPHYSIOLOGICAL MECHANISMS ASSOCIATED WITH DEVELOPMENT OF ENDOTHELIAL DYSFUNCTION IN PWH

Chronic Inflammation Caused by HIV Infection

HIV infection is a cause of chronic inflammation [49]. A detailed description of the causes of inflammation in PWH is outside the scope of this article. Briefly, rapid depletion of $CD4^+$ T cells from the gut-associated lymphoid tissue and, especially, Th17 subsets disrupts the gastrointestinal barrier function with translocation of microbial products across the interface [49, 52]. This directly stimulates innate pattern recognition molecules, like toll-like receptors (TLRs), with activation of pro-inflammatory signaling pathways like NFκB. TLRs 7 and 9 also are activated directly by HIV, because low-grade viral replication persists even on therapy [49]. Innate immune effector cells activated through pattern recognition receptors produce pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β [53]. The presence of HIV DNA in the cytoplasm of target cells also activates caspase-1, resulting in increased apoptosis [54]. Chronic inflammation has been associated with functional and quantitative abnormalities in multiple leukocytes subsets. This includes monocyte and neutrophil activation, CD4+ T cell dysfunction and apoptosis, CD8+ T cell activation, and B cell activation [49].

Chronic inflammation results in a state of abnormal endothelial cell activation that has been compared to changes seen in aging [55]. This is characterized by reduced number and function of endothelial progenitor cells with reduced capacity to repair endothelial damage [40, 47, 56]. Damage to endothelium can result from the release of oxygen-free radicals by activated immune cells, including activated monocytes and lymphocytes. Pro-inflammatory cytokines like TNF-α bind

specific endothelial receptors triggering endothelial apoptosis and activation [50]. Neutrophil activation may result in the production of cytotoxic neutrophils extravasation traps (NETs) that interact directly with coagulation activators and may promote leukocyte adherence [57]. In animal models, neutrophil activation and NETosis is associated with increased risk of thromboembolism [58].

Direct Effects of HIV on Endothelial Cells

The ability of HIV to infect endothelial cells directly is controversial. Small preliminary studies (in vitro) suggested that some endothelial cells could harbor infectious viruses, although larger scale studies have disputed this [59, 60]. It is clear, however, that HIV viral proteins can have a detrimental effect on the endothelium with subsequent dysfunction. Tat activates endothelial signaling pathways with downstream reduction in transcription of nitric oxide synthetase and upregulation of monocyte chemoattractant protein-1 (MCP-1) and cell adhesion molecules [61, 62]. This promotes both leukocyte activation and leukocyte adhesion to the endothelium. Nef activates pro-inflammatory pathways, including NF-κB and NFAT-1, in both endothelial cells and macrophages, promoting alterations in the monocyte phenotype towards pro-inflammatory cells and release of free radicals that can directly damage the endothelium [63]. In addition, Nef can affect cholesterol transport that predisposes to the formation of foam cells [63]. The HIV envelope proteins, gp-120/41, activate the p38 map kinase pathway that has been linked to increased endothelial permeability, endothelial cell apoptosis, and vasoconstriction [64].

Large Vessel Arteritis and Thrombosis in HIV

Aneurysmal disease and occlusive large vessel disease has been well-described in PWH. Histologically, this disease is pleomorphic with some studies reporting an appearance similar to panarteritis nodosa and others reporting transmural inflammation [65]. The pathogenesis of large cell arteritis is delineated incompletely. Upregulation of chemokine secretion can result in transmural cellular infiltrates with compromise of the *vasa vasorum*. A suggestion that HIV proteins may mimic arterial proteins with a subsequent cross-reactivity. Aside from direct effects on endothelial cells, studies suggest that arterial smooth muscle cells and fibroblasts may be susceptible to direct HIV infection [65-69]. This may result in weakening of the vascular wall and subsequent dilation [67, 70]. This area does, however, require further elucidation.

HIV-Associated Communicable Diseases

HIV-associated immunodeficiency increases the predisposition to and persistence of a number of infections. Chronic infections are associated with a number of changes, including upregulation of procoagulant factors and platelet activation, and mediate a direct effect on endothelial cells. *Mycobacterium tuberculosis* has been identified in endothelial cells in extrapulmonary infection [71]. Mycobacterial infection is an independent risk factor for thromboembolism and microvascular abnormalities [71, 72]. Parasitic infections like *Toxoplasma gondii* upregulate endothelial adhesion molecules to assist with invasion [73]. In addition, dysbiosis and microbiome perturbations in the gastrointestinal, respiratory, and urogenital tracts occurring in PWH are an independent risk factor for cardiovascular disease [74].

Endothelial cells are targets for *Herpesviridae*, including cytomegalovirus, Epstein-Barr virus, Kaposi-sarcoma herpes virus (KSHV), and varicella-zoster virus. Persistent infection with these viruses have been associated, especially in aging or otherwise immunodeficient patients, with an increased risk of vasculitis [75] and endothelial dysfunction [76], mediated through inflammatory signaling pathways [73] and through endothelial cell apoptosis [78]. KSHV, specifically, secretes cytokine homologs like viral IL-6 that have been implicated in both mediating a pro-inflammatory milieu and in atypical angiogenesis [79]. Herpes simplex viruses may infect the endothelium with subsequent apoptosis [76]. Finally, other concomitant viral infections (including persistent hepatitis C viral infection) have been linked to increased risk of cardiovascular disease [81, 82].

Traditional Risk Factors for Cardiovascular Disease in the ART HIV Era

Traditional risk factors for endothelial dysfunction include the presence of diabetes mellitus, hypertension, and hyperlipidaemia. These conditions contribute significantly to CVD risk in PWH [83, 84]. Chronic inflammation (with circulating pro-inflammatory cytokines) can predispose to insulin resistance through the phosphorylation of insulin receptor substrate-1 [85, 86]. Antiretroviral therapy is associated with lipid- abnormalities and dysregulation of glucose-processing pathways, which are independently associated with an increased risk of type 2 diabetes mellitus [85]. Hypertension in PWH is common and a number of potential pathogenic mechanisms have been identified, including lipodystrophy, a pro-inflammatory state associated with the secretion of cytokines and adipokines, and renal disease [87]. HIV protease is molecularly homologous to renin, and renin levels are often inappropriately high [88, 89]. Hypertension is exacerbated by worsening endothelial dysfunction [87]. Dyslipidaemia is a common complication of both treated and untreated HIV infection [90, 91]. In ART-naïve patients, this may be mediated by direct effects of the virus and the inflammatory milieu. Lipid processing and transportation is altered in PWH. Modified lipids may directly activate pattern recognition receptors [90]. Finally, substance use, especially tobacco use, is increased in PWH [88]. Risk of arterial disease is significantly higher in PWH who smoke and there is a concomitant increased mortality [6, 93].

Antiretroviral Drugs

Antiretroviral therapy has reduced the morbidity and mortality of HIV infection, affording improved life expectancy,

but long-term therapy can result in endothelial toxicity and vascular dysfunction. This has been linked with a number of metabolic abnormalities, including lipid abnormalities and predominantly an increase in circulating low-density lipoprotein and cholesterol levels [94]. The classes of drugs most commonly implicated are protease inhibitors. The majority of inflammatory and cardiovascular disease biomarkers show a decline with effective therapy, however, confirming a benefit of ART even for cardiovascular disease outcomes [90, 95–97].

CLINICAL BIOMARKERS OF ENDOTHELIAL DYSFUNCTION IN PWH

Attempts have been made to identify appropriate biological markers to predict and monitor cardiovascular disease risk in PWH with varying results. Early findings from the SMART (strategic timing of antiretroviral treatment) trial suggested that elevated highly sensitive C-reactive protein (CRP) and D-dimer results correlated with cardiovascular mortality in HIV-infected patients [98]. Summary data on the findings of selected trials investigating biomarkers related to thrombosis and accelerated atherogenesis are presented in Table 3. The most commonly measured biomarkers included IL-6 [29, 53, 56, 97–108], highly sensitive CRP [7, 28, 98, 99, 101–104, 106, 108, 109] and D-dimer levels [7, 28, 98, 99, 101–104, 106, 108, 109]. In addition, a number of studies measured markers of endothelial adhesion or activation, or both, including soluble intercellular adhesion molecule-1 and soluble vascular cell adhesion molecule-1 [7, 15, 17, 50, 97, 106–108, 110–112], monocyte activation (sCD163, sCD14, or changes in monocyte phenotype) [15, 28, 29, 50, 56, 96, 97, 100, 107–114], and platelet activation (expression of s- and p-selectin) [30, 33, 115]. Limitations exist in many studies examining biomarkers for CVD outcomes. Confounding variables include the age of the patients at analysis, the treatment status of the patients, the ART drug regimen used, and the presence of concomitant diseases. Not all studies include an HIV-uninfected control group. There is significant variation in the selection of biomarkers, measurement modality, and timing of measurement. Prediction of CVD outcomes often is correlative looking at surrogate markers of arterial disease, like flow-mediated dilation and carotid intimal medial thickness. Importantly, in studies looking at PWH after ART initiation, however, biomarkers did not always fully normalize, which suggests ongoing inflammation [17, 49, 100]. Ongoing study protocols include combinations of these markers [116, 117].

Few biomarkers have been studied in the ART era in the context of venous thrombosis or microvascular disease, and none have been conclusively linked to diagnosis or prognostication of occlusive vasculitis or aneurysmal disease [65]. This may represent a future study focus.

CLINICAL SCORING SYSTEMS

A number of clinical scoring systems exist to assess arterial, venous, and microvascular thrombosis risk (Table 4). Although thrombosis throughout the vascular tree is described in PWH, relatively few of these scoring systems have undergone validation in this patient cohort. No published performance evaluations of venous thromboembolic scoring systems or microvasculature scoring systems exist in PWH, although small case series have

Table 2. Procoagulant Substances Produced by the Endothelium [36]

Factor	Site of Storage or Expression	Function
Activation of the coagulation cascade		
Tissue factor	Subendothelial tissue including fibroblasts. Induced on endothelial cells in vivo. Expressed by leukocytes during inflammation (specifically monocytes) [31]	Activates the extrinsic coagulation pathway resulting in thrombin generation [31]
Factor VIII	Endothelial cells [32]	Stabilizes factor IX [32]
Platelet activation		
Collagen and subendothelial matrix	Subendothelial tissue [33]	Promotes platelet adhesion, activation, and aggregation [33]
Cellular adhesion molecules, including p-selectin and e-selectin, ICAM-1, and CXC12 [34]	Endothelial cells [33]	Promotes platelet adhesion, activation, and aggregation [33]
Von Willebrand Factor [35]	Weibel-Palade bodies [35]	Enables platelet adherence to exposed collagen through its interaction with platelet receptor Ib-V-IX (protects factor VIII from degradation [36, 37])
Eicosanoids, including prostaglandins and thromboxane A2 [38]	Endothelial cells [38]	Promotes platelet aggregation [38]
Vasoconstrictive agents		
Endothelins (predominantly endothelin-1) [39]	Endothelial cells, vascular smooth muscles cells, and reproductive system [39]	Activates endothelin receptors, increases production of reactive oxygen species, and reduces bioavailability of nitric oxide [39]

Table 3. Selected Studies of Biomarkers for Cardiovascular Disease in PWH

Author and Year	Number of HIV-infected Participants and Treatment Status	Number of Uninfected Controls	Biomarkers and Measurement Modality	Major Findings
2008 Von Hentig [33]	18 HIV-infected patients pre- and post-ART initiation	—	*Platelet activation*: platelet expression of CD62P, CD40L, and CD41 (flow cytometry)	Platelet function unaltered on PI-containing ART regimen; CD40L and CD41 both increased on PI regimen
2008 Kuller [98]	250 HIV-infected patients on continuous ART and 249 patients on drug interruption protocol	—	*Cytokines*: IL-6 *Inflammatory markers*: hsCRP, amyloid-A, amyloid-P *Coagulation markers:* D-dimers, PT fragment 1,2	IL-6, CRP, and D-dimers independently predicted all-cause mortality in HIV-infected patients
2009 Francisci [15]	56 HIV-infected patients pre- and post-ART treatment on PI or NNRTI and 10 patients not on ART	28	*Cytokines:* sCD40L, MCP-1 *Endothelial markers:* P-selectin, sVCAM-1 *Coagulation markers:* vWF, tPA	CD40L and tPA within normal limits in HIV-infected patients; p-selectin was elevated at baseline and remained elevated on treatment; vCAM-1, vWF, and MCP-1 decreased significantly on treatment irrespective of regimen
2010 Jong [118]	86 HIV-infected patients pre- and post-ART initiation	71	*Coagulation markers*: vWF PT fragment 1 and 2, TAT complex, endogenous thrombin potential, APC, protein-S and -C	Significantly increased vWF and D-dimers, APC ratio, and decreased free and bound protein-C and -S in HIV-infected patients; all markers except APC ratio improved with ART initiation
2010 Funderburg [28]	60 HIV-infected patients, majority on ART	19	*Microbial products*: LPS *Monocyte activation*: sCD14, TF expression by monocytes *Coagulation markers*: D-dimers	Monocyte expression of TF correlated with sCD14 and markers of immune activation in HIV-infected patients
2012 Funderburg [29]	57 HIV-infected patients on ART	23	*Microbial products:* LPS *Monocyte activation*: sCD14, monocyte CD62P and TF expression *Cytokines*: IL-6 *Inflammatory markers*: hsCRP	HIV-infected patients showed increased frequency of non-classical and intermediate monocytes that resembled profiles in associated with acute coronary syndrome; these monocytes express CD62P and TF and are related to T-cell activation, IL-6 and viral load
2012 Mayne [115]	46 HIV-infected patients, 73% on ART	18	*Platelet activation*: patient P-selectin and TF expression	HIV-infected patients showed higher levels of platelet activation
2012 Olmo [97]	54 HIV-infected patients—34 on continuous treatment and 20 with treatment interruption	—	*Cytokines*: IL-6, IL8, sCD40L, MCP-1 *Endothelial adhesion markers*: sP-selectin, 1 sVCAM-1 sICAM-1 *Coagulation*: tPA	MCP-1 and sVCAM-1 increased relative to baseline in with treatment-interruption; sCD40L, tPA, and sP-selectin increased in both treatment arms relative to baseline
2013 Ronsholt [17]l	70 HIV-infected patients on ART with viral suppression	16	*Cytokines*: IL-8, β2-MI TNF-α *Endothelial markers*: sVCAM-1 sICAM-1, sE-selectin, sP-selectin	HIV-infected patients on long-term therapy showed increased levels of β2-MI, IL-8, and sICAM-1
2013 Baker [113]	163 HIV-infected patients—54 ART-naïve and 109 ART-treated	—	*Monocyte activation*: Monocyte microparticles with TF expression *Cytokines*: IL-6 *Coagulation markers*: D-dimers, vWF	Monocyte-microparticle TF expression correlated with inflammatory and coagulation biomarkers in HIV-infected patients
2013 Baker [119]	717 HIV-infected patients—500 on continuing ART and 271 with treatment interruption	—	*Coagulation markers:* FVIII, AT, protein C	Patients in the interrupted treatment wing had transient increases in procoagulant factors and decreases in anticoagulant factors, increasing thrombin generation potential
2015 Van den Dries [114]	Retrospective review of Dutch HIV-infected cohort	—	*Markers of monocyte activation*: sCD14, LPB *Coagulation markers*: vWF	vWF increased in all HIV-infected patients but significantly higher in patients with first and recurrent venous thrombosis; higher risk of venous thrombosis in HIV-infected patients
2015 O' Halloran [110]	25 HIV-infected patients pre and post-ART initiation	15	*Monocyte activation markers:* sCD14, sCD163 *Cytokines*: sCD40L *Endothelial adhesion markers*: sP-selectin, 1 sVCAM-1 sICAM-1 *Coagulation factors*: vWF	All biomarkers were significantly higher pre-ART initiation compared with controls and reduced after therapy in HIV-infected patients; only GPVI reduced to levels comparable to controls
2015 Nkambule [30]	58 HIV-infected patients pre-ART initiation	38	*Platelet activation*: platelet aggregation and CD62P and CD36 expression on platelets	Platelet expression of CD62P increased in HIV-infected patients; CD62P and CD36 expression correlated with viral load; response in keeping with hypersensitivity on platelet aggregation

Table 3. Continued

Author and Year	Number of HIV-infected Participants and Treatment Status	Number of Uninfected Controls	Biomarkers and Measurement Modality	Major Findings
2016 Siedner [120]	105 HIV-infected patients on ART	100	*Cytokine*: IL-6 *Monocyte activation:* Kyrenunine: tryptophan ratio, sCD14 *Sonographic*: Ankle-brachial index	Increased arterial stiffness in HIV-infected patients; declines in inflammatory markers (IL-6, KTR and sCD14s) predicted a lower CIMT and hence atherosclerotic burden
2016 Haissman [109]	50 untreated and 155 ART treated HIV-infectedpatients	105	*Monocyte activation:* sCD14 *Coagulation markers*: D-dimers *Radiological*: Myocardial perfusion defect, CIMT *Other* Asymmetric dimethylargininine	Concentrations of ADMA in infected patients and higher levels in untreated individuals; ADMA associated with viral load, sCD14, D-dimers and low CD4+ T cell count but not with CIMT or subclinical atherosclerosis
2016 Grund [101]	3766 HIV-infected on ART	—	*Cytokines*: IL-6 *Coagulation*: D-dimers *Inflammatory*: hsCRP	260 patients had significant non-AIDS events or death and this was independently associated with increased IL-6, D-dimers, and hsCRP levels
2016 Freiburg [102]	249 patients measured prior to seroconversion, prior to ART initiation and post ART initiation	—	*Cytokines*: IL-6 *Coagulation markers*: D-dimers	Increased IL-6 and D-dimer levels post-seroconversion; D-dimer levels remained elevated and were associated with non-AIDS related adverse events
2016 Borges [103]	4304 HIV-infected patients	—	*Cytokines*: IL-6 *Coagulation markers*: D-dimers	IL-6 better predictor with all-cause mortality and cardiovascular disease than D-dimers or hsCRP
2016 Kulkarni [50]	19 HIV infected patients on ART	49	*Monocyte activation/adhesion markers*: VLA-4, LFA-1, fractalkine, CD11c, sCD14, sCD163 *Endothelial adhesion markers* sICAM-1 sVCAM-1 *Other*: Lp-PLA2	Endothelial activation markers increased in HIV infected individuals; decreased levels of fractalkine expression and increased levels of LFA-1 expression on circulating monocytes
2017 Dysangco [111]	28 HIV-infected patients on ART and 44 HIV-infected patients ART-naive	39	*Arterial dilatation endothelial markers*: sVCAM-1 *Monocyte activation*: CD163 *Inflammatory markers*: β2-MI, IP10, TNFR2	HIV-infected ART naïve patients had higher levels of inflammatory and endothelial adhesion markers (including sCD163, TNFR2, TIM and VCAM-1), but there was no difference in FMD amongst the groups
2017 Baker [104]	4299 HIV-infected patients on immediate or deferred ART	—	*Cytokines*: IL-6 *Coagulation markers*: D-dimers	Increased IL-6 and D-dimer levels consistently associated with AIDS– and non–AIDS related deaths
2017 Grome [112]	70 HIV-infected patients on ART	—	*T-cell activation, senescence, and exhaustion. Macrophage activation:* sCD163, sCD14 *Chemokines*: MIP-1α *Endothelial markers*: sICAM-1 sVCAM-1 *Radiological*: Flow-mediate dilation	Decreased flow mediated dilation was associated with CD8+ T cell activation sICAM-1 and sVCAM-1 were associated with soluble markers of monocyte activation
2017 Maggi [7]	119 ART-naïve HIV-infected patients stratified to receive efavirenz, atazanavir or darunavir based-regimens	—	*Endothelial adhesion*: sVCAM-1 sICAM-1 Radiological: CIMT *Coagulation markers:* D-dimers	Patients on Darunavir at higher risk of pathological intimal thickening; endothelial markers remained static, but D-dimer levels fell consistently
2018 Viskovic [105]	181 virally suppressed HIV-infected patients on ART	—	*Cytokines:* CD40L,MCP-1,IL-8,IL-6 *Inflammatory marker:* hsCRP *Endothelial markers:* P-selectin, tPA	Markers used to construct an inflammatory burden score (IBS), which correlated positively with the presence of dyslipidaemia (total cholesterol:HDL ratio)
2018 Seang [56]	57 HIV-infected patients on ART	—	Endothelial progenitor cells *Cytokine:* IL-6 *Monocyte activation*: sCD163	Undetectable EPC levels associated with higher CVD risk, decreased IL-6 levels, and increased sCD163 (monocyte activation) in HIV-infected patients
2018 Rezer [53]	10 HIV-infected patients on long-term ART	10	*Cytokines*: IL-6, IFN-γ, IL-17, TNF-α, IL-2, IL-4, IL-10 *Inflammatory markers*: hsCRP *Cardiac markers*: Troponin, CK-MB, LDH	Increased levels of IL-6 and IFN-γ in HIV-infected patients; no increases in levels of enzymatic cardiac markers in HIV-infected patients
2018 Peterson [106]	326 ART-naïve HIV-infected patients with CD4+ T cell count >500	—	*Cytokines*: IL-6, IL-27 *Endothelial adhesion markers*: sVCAM-1, sICAM-1 *Inflammatory markers*: hsCRP, serum amyloid A *Coagulation*: D-dimers Sonographic: Radial artery waveform	Increased levels of IL-6 and hsCRP inversely related to small arterial elasticity in HIV-infected patients
2018 Mosepele [107]	112 HIV-infected patients with viral suppression on long-term ART	84	*Cytokines*: IL-6 *Monocyte activation:* sCD163 *Endothelial adhesion*: sVCAM-1 sICAM-1, sE-selectin *Radiological*: CIMT	HIV infection increased levels of sICAM-1 and sVCAM-1 but not E-selectin; IL-6 showed no relationship with biomarkers of endothelial dysfunction

Table 3. Continued

Author and Year	Number of HIV-infected Participants and Treatment Status	Number of Uninfected Controls	Biomarkers and Measurement Modality	Major Findings
2019 Subramanya [108]	452 HIV-infected patients on ART	276	*Cytokines*: IL-6, TNF-α *Endothelial markers*: sICAM-1 *Monocyte activation*: sCD163 *Inflammatory markers*: CCL2, hsCRP, TNFR1, TNFR2 *Coagulation*: Fibrinogen, D-dimers	Elevated CCL2, IL-6, sCD163, CRP increased risk of carotid plaque independent of cardiovascular risk factors sTNFR2, ICAM-1, and fibrinogen predicted CIMT in HIV uninfected men; 8 biomarkers increased significantly in HIV-infected patients

Abbreviations: ADMA, asymmetric dimethylargininine; APC, activated protein C; ART, antiretroviral therapy; AT, antithrombin; â2MI, â2-microglobulin; CD, cluster of differentiation; CIMT, coronary artery intimal medial thickness; CK-MB, creatine kinase; hsCRP, highly sensitive C-reactive protein; LDH, lactate dehydrogenase; LFA-1, leukocyte functional adhesion molecule-1; IL, interleukin; IP10, interferon-ā induced protein 10; LPB, lipopolysaccharide binding protein; Lp-PLA2, lipoprotein-associated phospholipase A2; MCP, monocyte-chemoattractant protein-1; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; PT, prothrombin; sICAM-1, soluble intercellular adhesion molecule-1; sVCAM-1, soluble vascular cell adhesion molecule-1; TAT, thrombin-antithrombin complexes; TF, tissue factor; TNFR, tumour-necrosis Factor á receptor; tPA, tissue plasminogen activator; VLA-4, very late antigen-4; vWF, von Willebrand factor.

Table 4.

Scoring System	Developer	Parameters	Studies in PLWH	Utility
Cardiovascular disease				
Framingham [123]	National Heart Institute/ Boston University	Age, tobacco use, systolic blood pressure, total cholesterol, HDL cholesterol	Modified Framingham scores generally outperformed other scoring systems in large cohorts [128] although systems often either overpredicted [129] or underpredicted [125] cardiovascular risk [123].SCORE generally performed least well [124]. SCORE and D:A:D consistently underestimated cardiovascular risk [117, 128]	10-year risk of coronary artery disease only
D:A:D* [123]	D:A:D Study Group	Modified Framingham incorporating previous tobacco use, family history, and previous or current idinavir and lopinavir treatment		5-year risk of coronary artery disease only
SCORE** [117]	European Society of Cardiology	Gender, age, systolic blood pressure, smoking status, and total cholesterol/HDL cholesterol ratio		10-year risk of coronary artery disease only
ASCVD*** [117]	American Heart Association	Age, gender, race, total cholesterol, HDL, blood pressure, and smoking		10-year risk of coronary artery disease or stroke
PROCAM**** [130]	Institute of Atherosclerosis Research at the University of Munster, Germany	Gender, age, serum HDL and LDL cholesterol and triglyceride levels, smoking status, diabetes, family history of coronary heart disease, and systolic blood pressure		10-year risk of coronary artery disease or stroke
Venous thromboembolic disease				
Caprini Score [131]	American College of Chest Physicians	Age, planned surgery and type, immobility, inherited thrombophilic state, recent stroke, presence of a cast, serious comorbidity (including malignancy), chronic obstructive pulmonary disease, inflammatory bowel disease, central venous access, use of oral contraceptives, pregnancy or recent miscarriage, swollen legs, varicose veins, or morbid obesity	Not assessed in PWH	Thromboembolic disease, especially deep-vein thrombosis
Rogers Score (Patient Safety in Surgery Score) [132]		Biochemical—albumin, bilirubin, sodium Haematological—recent tranfusion and haematocrit Patient factors—American Society Anesthesia risk, ventilation, respiratory distress Surgical factors—type, infection, complexity and emergency		Thromboembolic disease, especially deep-vein thrombosis
Microvascular circulatory disease				
DIC ISTH [14, 133]	International Society of Thrombosis and Hemostasis	Platelet count, D-dimers, and prothrombin time in correct clinical context	Utilized as a diagnostic score in PWH [14, 121, 122], but there were no validation studies	Disseminated intravascular coagulation
DIC—JSTH [134]	Japanese Society of Thrombosis and Hemostasis	Clinical features, platelet count, D-dimers, prothrombin time, and antithrombin		Disseminated intravascular coagulation
DIC JAAM [134]	Japanese Association for Acute Medicine	Septic score, platelet count, D-dimers, amd prothrombin time		Disseminated intravascular coagulation
PLASMIC [135]	Harvard TMA Research Collaborative	Clinical—no active cancer, no history of transplant Laboratory— platelet count, haemolysis, Mean Cell Volume, International normalized ratio, Creatinine		Thrombotic thrombocytopaenic purpura

*Data Collection on Adverse Events of Anti-HIV Drugs, **Sytematic COronary Risk Evaluation ***Atherosclerotic cardiovascular disease risk equation ****Prospective Cardiovascular Munster

referenced these scores [14, 121–122]. Arterial scoring systems have been more extensively studied. The Framingham risk score, based on the ongoing Framingham heart study, was initially designed to look at heart disease in nondiabetic, Caucasian participants between the ages of 30 and 69 [123]. A number of modifications have been introduced, including an ART regimen specifically for PWH—the Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D score) [123]. Other scoring systems that have been evaluated include the systematic coronary risk evaluation (SCORE), atherosclerotic cardiovascular disease risk score (ASCVD), and prospective cardiovascular munster (PROCAM) scores. In PWH, these scoring systems have shown variable performance across validation studies. The Framingham risk score has generally shown the best predictive value for CVD in PWH with D:A:D, with the ASCVD and SCORE systems showing more consistent under-prediction in large European and American cohorts [117, 124–125]. Only smaller cross-sectional evaluations have been undertaken in low and middle-income countries [123, 126–127]. Despite the relatively poor predictive power and data fit shown in many analyses, these scoring systems continue to form the basis of clinical trials in this cohort of patients.

Modeling studies suggest a significant health economic burden of cardiovascular disease in PWH that is predicted to increase as the HIV-infected population ages [136–137]. Understanding the underlying pathogenesis, assessing risk, and identification and validation of appropriate biomarkers will be important. This includes the development of risk scores for microvascular and venous thrombosis. Cardiovascular disease risk is increased in patients who are untreated or who fail to achieve or maintain viral suppression, and early initiation of ART is the mainstay of therapy. In addition, traditional cardiovascular risk factors, including tobacco use, dyslipidaemia, hypertension, and diabetes should be aggressively managed in this population along with chronic infections that can cause chronic inflammation and predispose to vascular disease [138].

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Publication 4: The Utility of the Lipoprotein-Associated Phospholipase A2 (Lp-PLA2) Assay in Detecting Abnormalities in Lipid Metabolism and Cardiovascular Risk in an HIV-Infected South African Cohort

The American College of Cardiology (ACC)/American Heart Association (AHA) have defined HIV as a risk factor for development of coronary atherosclerosis. Lipoprotein-associated phospholipase A2, a surrogate marker of endothelial dysfunction, is an enzyme linked to production of lipid mediators of inflammation and is secreted by monocytes. It has been included in the American Association of Endocrinologists Guidelines for management of dyslipidaemia and cardiovascular disease prevention. This marker is secreted by leukocytes (including monocytes) which links the inflammatory and the cardiovascular systems. Publication 4 measured the performance of this marker as assessed in a cohort of 98 South African HIV-infected patients on and off therapy and compared with HIV-uninfected controls. LP-PLA_2 did not show a correlation with carotid intimal thickness or increases in the Framingham score in HIV-infected individuals. As expected, LP-PLA2 did show a significant correlation with Low-density lipoprotein (LDL), total cholesterol and age in PWH on a protease inhibitor but when corrected for LDL, this correlation did not persist. Of more interest, LP-PLA_2 showed a significant positive correlation with HIV viral load. This suggests that this enzyme may be overproduced in patients with HIV infection and provides another link between chronic inflammation, endothelial dysfunction and cardiovascular disease risk in HIV-infected individuals.

Contribution of student:

For this paper, the candidate formulated the research question and acquired the test described. The candidate analysed the data and was responsible for drafting and review of the final manuscript. The co-authors assisted with sample acquisition (AV), laboratory analysis of the samples (HM), review of the manuscript (DG, RB, KK, WS, SL).

Erratum:

A typographical error was identified in the table 1 where the range for Lp-PLA_2 should read Mean = 127 and range =17-162?

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The Utility of the Lipoprotein-Associated Phospholipase A_2 (Lp-PLA_2) Assay in Detecting Abnormalities in Lipid Metabolism and Cardiovascular Risk in an HIV-Infected South African Cohort

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Abstract

People with HIV (PWH) have an increased prevalence of cardiovascular disease (CVD) compared to uninfected patients. Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) catalyzes the synthesis of pro-inflammatory lipids that recruit monocytes. Current guidelines for assessing cardiovascular risk in HIV-infected patients suggest that Lp-PLA_2 may be a useful surrogate marker for CVD health in this patient population. Lipoprotein-associated phospholipase A_2, lipids, glucose, physical parameters, and carotid intimal–medial thickness (CIMT) were measured in 98 participants (49 HIV-uninfected, 27 antiretroviral therapy [ART]-naive PWH, and 22 ART-treated PWH). HIV viral load (VL) and CD4+ T-cell count were measured in HIV-infected participants. Lipoprotein-associated phospholipase A_2 was increased in participants on protease inhibitor (PI) ART (median 50.5 vs 127.0 nmol/mL, $P = .05$) and correlated with age, body mass index, and cholesterol. Lipoprotein-associated phospholipase A_2 was not related to Framingham risk score or CIMT but correlated directly with VL ($r = .323$, $P = .025$) and inversely with CD4+ T-cell count ($r = -.727$, $P < .001$). Lipoprotein-associated phospholipase A_2 was increased in HIV-infected participants on PIs and correlated strongly with VL and CD4+ T-cell count suggesting that HIV-associated inflammation is linked to increased Lp-PLA_2, providing a mechanistic link between HIV and CVD.

Keywords

Lp-PLA_2, cardiovascular disease, biomarker, HIV, viral load

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Introduction

Access to antiretroviral therapy (ART) has increased the life expectancy of people with HIV (PWH).[1] Although there has been a decrease in the prevalence of opportunistic infections, PWH are more likely to develop noncommunicable diseases including cardiovascular disease (CVD). The risk of CVD is estimated to be 60% higher in patients with HIV infection, and the relative risk is increased 2-fold even in patients on ART.[2] Cardiovascular disease was the cause of mortality in patients undergoing planned interruption of ART, focusing research interest on validation of biomarkers to assess CVD risk in these patients.[3] The pathogenesis of CVD in PWH is multifactorial. Immune activation and chronic inflammation, associated with low-grade viral replication, microbial translocation, and opportunistic diseases, directly impacts the endothelial surface and activates leukocytes, specifically monocytes, which are implicated in plaque formation.[4] People with HIV may have underlying traditional risk factors and are more likely to use tobacco.[5] Although suppression of viral replication generally protects against CVD, certain ART drugs including the protease inhibitors (PIs) cause disorders in lipid metabolism.

Cardiovascular disease risk assessment tools, validated in the general population, such as the Framingham risk score (FRS), convert clinical risk factors into a summary estimate of the likelihood of a CVD event over a specified period. The scores can underestimate the individual patient risk[6] and may have limited utility in PWH, as HIV-specific risk factors including the use of PI and viral burden are not always included.[7]

Various biomarkers have been assessed in CVD risk evaluation. In PWH, inflammatory changes (cytokines and leukocyte activation), changes in coagulation proteins, presence of oxidized lipids, and adhesion markers have been measured to identify a robust predictive model.[8] Lipoprotein-associated phospholipase A_2 (Lp-PLA_2), an enzyme secreted by leukocytes and liver cells, circulates primarily complexed to low-density lipoprotein (LDL).[9] Lipoprotein-associated phospholipase A_2 hydrolyzes phospholipids to produce metabolically active lipid mediators including pro-inflammatory free fatty acids. These products recruit T-cells and monocytes, key effectors of atherosclerotic plaque formation. Lipoprotein-associated phospholipase A_2 is overexpressed in macrophages in the fibrous cap of unstable atherosclerotic coronary lesions.[10] The relationship between Lp-PLA_2 and HIV has been extensively studied with early reports in both simian and human leukocyte models suggesting that Lp-PLA_2 synthesis, promoted by CD4–HIV envelope interactions, could facilitate viral integration into host cells by destabilizing the cell membrane. This permissive effect could be further enhanced by the T-cell proliferation and activation in response to pro-inflammatory lipid mediators.[11]

Lipoprotein-associated phospholipase A_2 has been extensively evaluated in clinical cohorts. It correlates with plaque formation and CVD risk and identifies patients at risk of recurrent cardiovascular events.[10] Lipoprotein-associated phospholipase A_2 was incorporated into the American Association of Clinical Endocrinologists/American College of Endocrinology Guidelines for management of dyslipidemia and prevention of CVD as a nontraditional risk factor. Cardiovascular disease risk is elevated in patients with raised Lp-PLA_2 (generally considered as >30 nmol/mL)[12] and C-reactive protein levels, even in the presence of a moderately increased LDL.[13] Studies suggest PWH have higher Lp-PLA_2 levels than uninfected patients, independent of triglyceride and LDL levels.[14] The elevated levels persist with viral suppression. Levels of Lp-PLA_2 may also guide both ART regimen choice (tenofovir may reduce Lp-PLA_2 levels) and use of ancillary risk modifiers such as statins. Most studies measured Lp-PLA_2 levels in patients with controlled HIV viraemia.[15]

The aim of this study was to investigate Lp-PLA_2 levels of HIV-infected patients (ART-naive and treated) enrolled in large cross-sectional study in South Africa and to investigate relationships between this marker, other measures of CVD risk, and markers of viral control.

Methodology

Patient Recruitment

The study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (Protocol number M160131). Ninety-eight patients, enrolled in a larger study to evaluate CVD risk in PWH in Johannesburg, South Africa, from 2016 to 2017, were included. Twenty-seven HIV-infected, ART-naive participants and 22 HIV-infected participants on stable PI-containing ART regimen were compared with 49 age- and sex-matched uninfected controls. Participants were considered ART naive if they had received no ART or ART for less than 6 weeks. The ART-treated participants, treated with a PI-containing regimen for at least 48 weeks, showed viral suppression. Demographic and cardiovascular risk factors were collected using a modified version of the World Health Organization STEPwise approach to Surveillance (WHO STEPS) instrument.[16] Blood was collected for measurement of fasting glucose and lipid levels and Lp-PLA_2. Physical examination included measurement of height, weight, waist and hip circumference, and blood pressure. The FRS was computed to estimate a cumulative 10-year CVD risk. Left and right carotid intima–media thickness (CIMT) was measured sonographically at 3 standardized angles using the Meijer Arc. Measurements were semiautomated but manually corrected if required. Plasma Lp-PLA_2 levels were measured on the Roche Cobas analyzer using an enzymatic assay (Diazyme Laboratories, Poway, California).

Statistical Analysis

Because of the small number of participants per group, continuous variables were expressed using median with minimum and maximum values or count with percentage. Baseline variables across the 3 groups were compared using a Kruskal-Wallis test for continuous outcomes and a χ^2 test for

Table 1. Characteristics of the Patient Population at the Time of Analysis (All Values Expressed as a Median and Range Unless Otherwise Specified).

Participant Characteristics	HIV Negative, n = 49	HIV-Positive, ART Naive, n = 27	HIV-Positive, PI ART, n = 22
Female[a]	24 (49.0)	14 (51.9)	12 (54.5)
Age, years	38.9 (25.1-64.3)	38.9 (24.2-56.5)	40.5 (26.5-57.7)
Current smoking[a]	12 (25.0)	8 (29.6)	2 (9.1)
Systolic BP, mm Hg	123.5 (99.5-190.0)	119.5 (87.5-164)	120.5 (91.5-161.0)
BMI, kg/m^2	25.7 (17.0-47.2)	23.7 (17.5-35.0)	25.0 (18.7-42.1)
Waist to hip ratio	0.88 (0.69-1.00)	0.88 (0.76-0.98)	0.88 (0.62-0.97)
CD4, cells/mm^3	-	276 (15-532)	480 (121-997)
Log_VL, cp/mL, median (IQR)	-	9.53 (3.66-14.15)	3.66 (3.66-8.72)
Total cholesterol, mmol/L	4.32 (3.60-6.40)	3.66 (2.50-7.40)	4.43 (3.60-6.40)
HDL cholesterol, mmol/L	1.32 (0.90-3.20)	1.18 (0.60-3.90)	1.29 (0.80-1.80)
LDL cholesterol, mmol/L	2.23 (0.60-4.40)	2.16 (1.10-3.40)	2.74 (1.80-4.70)
Triglycerides, mmol/L	1.04 (0.50-3.70)	0.97 (0.60-1.90)	1.58 (0.80-4.90)
Random glucose, mmol/L	4.85 (2.60-9.60)	4.40 (3.40-7.80)	4.50 (4.30-9.4)
Framingham risk score (%)	3.17 (0.59-40.99)	2.50 (0.51-13.52)	2.73 (0.51-22.37)
CIMT, mm	0.514 (0.447-1.024)	0.512 (0.446-0.758)	0.547 (0.477-0.738)
Lp-PLA$_2$, nmol/mL	127.0 (52.0-196.0)	127.0 (17.0-62.0)	150.5 (47.0-219)

Abbreviations: ART, antiretroviral therapy; BMI, body mass index; BP, blood pressure; CMIT, carotid intimal–medial thickness; HDL, high-density lipoprotein; IQR, interquartile range; LDL, low-density lipoprotein; Lp-PLA$_2$, Lipoprotein-associated phospholipase A$_2$; PI, protease inhibitor; VL, viral load.
[a]Expressed as an absolute number and percentage.

categorical outcomes. The LP-PLA$_2$ was correlated with age, body mass index (BMI), waist-to-hip circumference, lipid subcomponents, and glucose using a Pearson correlation coefficient.

In a multivariable regression, the LP-PLA$_2$ levels in the 3 groups were adjusted for age, gender, LDL levels, BMI, and smoking. The relationship between Lp-PLA$_2$ levels and FRS or CIMT was examined in a multivariable regression considering HIV and ART status. Finally, the relationship between LP-PLA$_2$ levels of all viremic participants and log-viral load and CD4-cell count were analyzed using a Pearsons correlation coefficient. A P value of $\leq$.05 was considered significant. Statistical analyses were performed using IBM SPSS Statistics version 25 (SPSS, Chicago, Illinois).

Results

The baseline characteristics for the 3 participant groups are included in Table 1. With the exception of viral load and CD4+ T-cell count, no significant differences were noted between the populations.

We correlated Lp-PLA$_2$ with traditional cardiovascular risk factors, with carotid artery intimal thickness and with the FRS in all 3 population groups. The Lp-PLA$_2$ did not show a significant relationship with either CIMT or FRS in HIV-uninfected (median = 0.514 nmol/mL, P = .44 and median = 3.17 nmol/mL, P = .92, respectively), ART-naive HIV-infected participants (median = 0.512 nmol/mL, P = .52 and median = 2.50 nmol/mL, P = .92, respectively) or HIV-infected participants on second-line ART (median = 0.547 nmol/mL, P = .48 and median = 2.73 nmol/mL, P = .94).

As expected, patients on PI treatment had significantly higher LDL (median = 2.74 mmol/L, P < .001), triglycerides (median = 1.58 mmol/L, P < .006), and total cholesterol levels (median = 4.43 mmol/L, P = .02) than either uninfected patients or patients who were ART naive. Levels of Lp-PLA$_2$ were increased in patients on PI-containing ART regimens (median = 150.5 nmol/mL vs 127.0 nmol/mL, P = .05) and correlated with age (r = .25, P < .001), BMI (r = .73, P = .04), total cholesterol (r = .21, P = .04), and LDL-cholesterol (r = .40, P < .001) in this population. The significant higher levels of Lp-PLA$_2$ for participants on ART did not persist when LDL levels were considered.

Of interest, Lp-PLA$_2$ correlated with viral load (r = .323, P = .025). When patients with undetectable viremia were excluded, the correlation became more pronounced (r = .653, P < .001). There was an inverse relationship with CD4+ T-cell count, but this reached only significance in the virally unsuppressed ART-naive participants (r = .727, P < .001).

Conclusion

Lipoprotein-associated phospholipase A$_2$ is a phospholipase that complexes with LDL and catalyzes the release of pro-inflammatory lipid mediators. It has been linked to CVD risk (plaque formation and instability) specifically in patients with underlying inflammatory disease. Studies, generally performed on patients with controlled viremia, have demonstrated elevated levels of Lp-PLA$_2$.[14] In this study, we assessed Lp-PLA$_2$ utility when compared to traditional CVD risk factors and also in ART-naive, HIV-infected participants and participants on a PI-containing ART regimens.

Lipoprotein-associated phospholipase A$_2$ levels were only significantly increased in HIV-infected participants in this cross-sectional study on a PI-containing ART regimen. This increase reflected LDL levels and may indicate the general dyslipidemia associated with PI-containing drug regimens.[17]

Studies suggest that the benefits of ART initiation outweigh the risks of initiating a PI for CVD outcomes.[18] Lipoprotein-associated phospholipase A_2 correlated with LDL and total cholesterol that are risk factors for atherosclerosis, although there was no convincing relationship with the FRS or the CIMT. The FRS has been criticized for both under- and overestimating cardiovascular risk in the HIV-infected population.[19]

More importantly, Lp-PLA_2 correlated strongly with viral load and CD4+ cell count, specifically in those patients with detectable viremia. Persistent chronic inflammation secondary to viral factors including low-grade viral replication, opportunistic infections, and bacterial translocation is associated with endothelial dysfunction and coagulation abnormalities.[20-22] Lipoprotein-associated phospholipase A_2 is a lipid regulator of inflammatory pathways and is a biomarker of inflammation-induced endothelial dysfunction. The significant relationship with virological status in this population provides a link between active viremia and the mechanistic development of CVD in these patients.

This study has a number of limitations. We did not access patients who were not on a PI-containing regimen which may explain the discrepancies of our findings and other studies looking at Lp-PLA_2 in HIV-infected patients. In addition, this study was cross-sectional, and it would be useful to examine the patients with increased Lp-PLA_2 levels longitudinally to assess the contribution to CVD risk. Finally, we did not include measurement of Highly sensitive C-reactive protein (hs-CRP) which would have been an important indicator of inflammation. Despite these limitations, we are of the opinion that this enzyme may drive atherosclerosis in HIV-infected patients, and its utility in clinical care should be investigated in prospective studies.

Authors' Note

Elizabeth S. Mayne and Susan Louw contributed equally to this article.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Publication 5: Pathogenic factors associated with development of disseminated intravascular coagulopathy (DIC) in a tertiary academic hospital in South Africa

Inflammation and the hypercoagulable state has been associated with venous and microvascular disease. HIV is associated strongly with the development of acquired thrombotic thrombocytopaenic purpura (TTP) but has not been strongly associated with other forms of thrombotic microangiopathies. Disseminated intravascular coagulation (DIC) is a disorder associated with microthrombosis and consumption of platelets and coagulation factors resulting in a paradoxical bleeding diathesis. Publication 5 evaluated the prevalence of HIV infection in DIC; a retrospective audit was undertaken of all DIC screens submitted over a one-year period. Briefly, the International Society of Thrombosis and Haemostasis scoring system was used. A pathogen was isolated in 84% of patients who were confirmed to have a DIC. 56.6% of patients were infected with HIV. In 47 of the HIV-infected patients (42%), no additional pathogen could be isolated despite repeated investigations. The relative risk for development of DIC was highest in patients infected with HIV (2.7387). In summary, these findings highlight the importance of HIV in the pathogenesis of microvascular diseases like DIC.

Contribution of candidate:

For this paper, the candidate formulated the research question, collected the data and analysed the data and was responsible for drafting and review of the final manuscript. The co-authors assisted with data collection and manuscript review (SL) and with statistical support (AM).

PLOS ONE

RESEARCH ARTICLE

Pathogenic factors associated with development of disseminated intravascular coagulopathy (DIC) in a tertiary academic hospital in South Africa

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Abstract

Introduction

Disseminated intravascular coagulopathy (DIC) is a thrombotic microangiopathy arising from consumption of both coagulation factors and platelets. DIC is triggered by a number of clinical conditions including severe infection, trauma and obstetric complications. Early diagnosis and treatment of the underlying condition is paramount. A high clinical index of suspicion is needed to ensure that patients at risk of developing DIC are appropriately investigated.

Methods

In order to establish the clinical conditions most frequently associated with DIC, we reviewed all DIC screens received at a tertiary hospital in Johannesburg, South Africa over a 1 year period.

Results

The commonest clinical condition associated with DIC in our population was infection with 84% of patients infected with an identified pathogen. The most frequently diagnosed pathogen was HIV followed by *Mycobacterium tuberculosis* and other bacterial infections. In the majority of cases, bacteria were isolated from blood cultures. In 47 patients, HIV was the only pathogen which could be isolated. A relative risk ratio of 2.73 and an odds ratio of 29.97 was attributed to HIV for development of a DIC. A malignancy was present in 51 of the patients of which approximately 60% had co-existing infection. No cause could be attributed in 30 patients.

Conclusion

Infection was identified in the majority of the patients diagnosed with DIC in this study. HIV showed the highest relative risk ratio of all pathogens although previous studies have not

suggested that HIV was strongly associated with DIC. In almost half of the HIV infected patients, there was no other pathogen isolated despite extensive investigation. This suggests that HIV has a strong association with the development of DIC, warranting further research into the relationship between HIV and disseminated microvascular thrombosis.

Introduction

Disseminated intravascular coagulopathy (DIC) is a syndrome of dysregulation of the haemostatic pathways associated with formation of small vessel microthrombi and consumption of platelets and coagulation factors. [1,2] DIC has been associated with a number of clinical conditions including severe infectious disease and sepsis, [3] malignancy,[4] trauma [5] and obstetric complications [6]. The treatment of DIC consists predominantly of managing the underlying disease process and providing supportive treatment for the coagulopathy.[2, 7, 8] Diagnosis of DIC is made using both clinical and laboratory criteria and a number of scoring systems is available. Parameters measured assess the consumption of coagulation factors (prothrombin time, activated partial thromboplastin time, thombin time and fibrinogen levels), anticoagulant activity (antithrombin) and platelet numbers.

Fibrin degradation products (D-dimers) are also included which demonstrate ongoing fibrinolysis (in the context of widespread clot formation and breakdown). [8] Critically, a diagnosis of DIC should be made in the context of an appropriate clinical trigger. [2,8]

The mainstay of treatment of DIC is resolution of the clinical trigger. In patients who are bleeding or who require invasive procedures (like surgery), replacement of depleted coagulation factors and platelets may be indicated. This replacement therapy is monitored using the PTT and aPTT and the platelet count. [2]

The mechanism underlying the development of DIC is not fully understood. Inflammatory stimuli result in endothelial activation with a change from an anticoagulant to a procoagulant surface. [3, 9] This potentiates the formation of small thrombi which eventually consume coagulation factors and platelets resulting in a bleeding diathesis. Early compensatory mechanisms may delay recognition of this process and it is important for clinicians to have a high index of suspicion in the correct clinical context.[4, 9]

Currently, there are little data regarding the aetiology of DIC in South Africa. This is of concern in a setting with a high prevalence of infectious diseases including human immunodeficiency virus (HIV). Early and accurate identification of potential high risk conditions would assist in risk stratification and may assist in creating algorithmic approaches for these patients. [10] In particular, the impact of HIV and *Mycobacterial tuberculosis* infection on the risk of developing DIC is poorly understood.

In order to assess the risk factors for development of these conditions, we conducted a retrospective survey of all DIC screens submitted for analysis to the coagulation laboratory at Charlotte Maxeke Johannesburg Academic Hospital, an academic hospital in Johannesburg South Africa over a 1 year period.

Methodology

This study received ethics clearance from the Human Resource Ethics Committee of the University of the Witwatersrand (Clearance number: M160839). Participants were assigned a study number and all data were anonymised. All sequential DIC screens received at the laboratory over the period between May 2015 and April 2016 were included in the analysis (n = 401).

All coagulation testing was conducted on a STAGO EVO-R analyser (Cedex, France) on citrate plasma and the platelet count was assessed using a Siemens Advia 120 Haematology analyser (Erlangen, Germany). Where time-to-clot assays were prolonged, a manual correction using normal factor pool was performed. The following data parameters are assessed routinely on DIC panels: Activated Partial Thromboplastic Time (aPTT) in seconds, Prothrombin Time (PT) in seconds, D-dimer levels in g/dl, Antithrombin levels as a percent, Thrombin Time (TT) in seconds and platelet count. Although not allocated points in the ISTH scoring system, antithrombin levels have been utilised by other scoring systems (like the JSTH scoring system) [11] to indicate consumption of anticoagulant factors. Thrombin time has been used historically in our centre to exclude the presence of heparin contamination. D-dimer cut-off levels for moderate and strong increases were set at 0.25-1mg/L and greater than 1mg/L respectively in accordance with previously calculated interquartile ranges. [2]

Information on all screens was accessed from the laboratory information system. All screens were assessed for completeness of data and were assigned a score based on the ISTH DIC scoring system (Table 1). A score of 5 or greater was considered positive for the presence of a DIC. Screens lacking results for D-dimer levels, fibrinogen, PT or platelet count, were excluded from the analysis. Additional clinical data were retrieved from the laboratory information system including the results of bacteriological cultures, histological analysis, viral testing and biochemical testing.

Statistical analysis was conducted using the STATA statistical package (version 14.2 Statacorp, Texas, USA). Relative risk and odds ratios were calculated for all possible DIC triggers utilising the following formulae: Relative risk $\frac{(DIC\ positive\ given\ disease)/(DIC\ positive\ given\ disease\ positive\ and\ negative}{(DIC\ negative\ given\ disease)/(DIC\ negative\ given\ disease\ positive\ and\ negative)}$ and Odds Ratio: $\frac{(DIC\ positive\ given\ disease)/(DIC\ positive\ given\ no\ disease)}{(DIC\ negative\ given\ disease)/(DIC\ negative\ given\ no\ disease)}$.

Results

Over the study period, 198 of the 401 patients fulfilled the ISTH diagnostic criteria for a diagnosis of DIC. The median age of the patients at the time of presentation with a DIC was 37 years (range 1 month -76 years) with a female to male ratio of 1.2:1. In the majority of patients (84.3%), infection was present at the diagnosis of DIC. The most prevalent pathogen was HIV

Table 1. ISTH DIC scoring system [10].

Laboratory Parameter	Point allocation
Platelet Count	
> 100 x10^9/l	0
50-100x10^9/l	1
<50x10^9/l	2
Increase in Fibrin Markers	
No change	0
Moderate rise (> 0.25-1mg/L)	2
Strong rise (> 1mg/L)	3
Prothrombin Time Prolongation	
3 seconds or less	0
> 3 seconds but < 6 seconds	1
Greater than 6 seconds	2
Fibrinogen Level	
Greater than 1 g/l	0
Less than 1 g/l	1

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which was present in 56.6% of these patients. Bacterial infection (excluding mycobacterial infections) was diagnosed in 49 patients and 20 of these had underlying HIV infection. Mycobacterial infection was present in 28 patients, all of whom were HIV co-infected. Malignancy was present in 25.8% of the patients of whom 60% (30) also had co-existing infection.

The most prevalent bacterial infection in the patients was Mycobacterium tuberculosis which was diagnosed in 28 patients followed by *Klebsiella pneumoniae* which was cultured in 12 patients. (Table 2).

Fungal infections were demonstrated in 12 patients and a further 4 patients had evidence of a suspected fungal infection (an elevated beta-D glucan level) although blood and urine cultures failed to demonstrate a fungal pathogen (Table 3). Only 4 patients had evidence of fungal septicaemia.

Malignancies were present in 51 patients at the time of diagnosis. Haematological malignancies were the most commonly identified (n = 37) with Acute myeloid leukaemia predominating. The commonest solid organ malignancy identified was squamous carcinoma of the cervix which was present in 5 patients at diagnosis (Table 4).

HIV infection, itself, had the highest relative risk ratio for all possible triggers of 2.738 (95% confidence limits 2.32–2.32) followed by bacterial infection. The Odds ratio for development of DIC was highest for mycobacterial infection (Odds ratio of 35.005, 95% confidence limits of4.7–259.66) followed by HIV and then non-mycobacterial bacterial infections (Table 5).

Discussion

Over the study period, 198 patients fulfilled the ISTH DIC criteria for diagnosis of a DIC. As anticipated, the commonest potential trigger for the DIC identified in these patients was infection with a positive assay for viral, mycobacterial, fungal or bacterial infection in approximately 84% of the patients.

The commonest pathogen identified was HIV. HIV has been reported as a potential cause of DIC although this has generally been in association with an opportunistic or co-existing infection or malignancy. [12, 13] HIV infection has been reported more commonly as a trigger for other microangiopathic haemopathies specifically thrombotic thrombocytopaenic purpura (TTP). [14, 15] Early studies suggest that DIC is an uncommon complication in HIV. HIV is, however, associated with an increased risk of endothelial dysfunction and activation. [16] The increased risk of dysfunctional clotting in HIV-infected individuals has been attributed to a number of risk factors including low levels of the anticoagulant factor protein S [17], activating antibodies, reduced processing of procoagulant factors like von Willebrand factor and low level chronic inflammation. [18–20] Although a large number of HIV-infected patients (43.8%) in this study were co-infected with an additional pathogen and 16 had an underlying malignancy (14%), in 47 patients (42% of all HIV infected patients), no additional pathology could be found despite extensive investigation and repeated cultures.

The ISTH DIC diagnostic score allocates points for thrombocytopaenia, elevated fibrin degradation products (D-dimers), prolonged PT and fibrinogen. Elevated D-dimer levels have been reported in HIV patients, particularly in the context of uncontrolled viraemia. [21] The platelet count is often decreased in these patients for a number of reasons including autoimmune platelet destruction, marrow infiltration and HIV-associated dyshaemopoiesis.[22] Chronic infection with liver synthetic dysfunction in these patients could also result in perturbation of the coagulation cascade and decreased fibrinogen. [23] These factors may impact the laboratory analyses and the diagnosis of DIC in these patients may not reflect a pure consumptive coagulopathy. The clinical presentation is, however, indistinguishable with high morbidity and mortality and the treatment for these patients includes treatment of the underlying cause.

Table 2. Bacteria and mycobacteria identified at time of DIC diagnosis with site of culture and HIV status.

Organism identified	Number of patients and site of positive test	HIV status of patient at time of culture			Total
		Negative	Positive	Untested	
Mycobacterium tuberculosis	Blood (n = 8), Sputum GeneXPert (n = 18) Sputum culture (n = 1), Trephine (n = 1)	0	28	0	28
Klebsiella pneumoniae	Blood (n = 7), Urine (n = 5)	6	3	3	12
Enterococcus faecalis	Blood (n = 5), Cerebrospinal fluid (n = 1)	3	2	1	6
Acinetobacter baumanni	Blood (n = 4), sputum (n = 1)	4	1	0	5
Coagulase negative staphylococcus	Blood (n = 4), sputum (n = 1)	0	4	0	4
Escherichia coli	Blood (n = 1), urine (n = 4)	1	0	0	1
Staphylococcus aureus	Blood (n = 4)	0	4	1	5
Pseudomonas aeruginosa	Blood (n = 1), Cerebrospinal fluid (n = 1) Tracheal aspirate (n = 1), pus (n = 1),	3	1	1	5
Streptococcus pyogenes	Blood (n = 3)	0	2	1	3
Salmonella spp.	Blood (n = 4)	0	3	1	4
Streptococcus pneumoniae	Blood (n = 1), Cerebrospinal fluid (n = 1)	0	2	0	3
Neisseria meningitis	Blood (n = 2)	0	2	0	2
Providentia spp	Blood (n = 2)	0	0	2	2
Haemophilus parainfluenza	Sputum (n = 1)	0	1	0	1
Streptococcus anginosus	Blood (n = 1)	0	1	0	1
Proteus mirabilis	Urine (n = 1)	0	1	0	1
Mixed Growth	Urine (n = 3), Blood (n = 1)	1	2	1	4

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This would include treatment of all infections with initiation of chemotherapy for tuberculosis and antiretroviral therapy where appropriate.

In 30 patients, no clear trigger for the underlying DIC could be ascertained. It is suspected that, in some of these patients, investigation was limited owing to early mortality. HIV testing can only be performed after counselling and consent and it was beyond the scope of the study to test the patients retrospectively. The clinical features were, in some cases, suggestive of underlying immunosuppression.

This study has a number of limitations. Results were assessed retrospectively and no additional testing was performed. Subjects could only be identified if a sample was submitted for DIC screening and it is possible that not all patients in the hospital were appropriately investigated for DIC. This could result in bias as there is an overrepresentation of DIC screens from intensive care units and internal medicine wards where the clinical suspicion for DIC is high. There was an underrepresentation of screens from the surgical, trauma and obstetric

Table 3. Fungal pathogens identified at the time of DIC diagnosis.

Fungal pathogen	Site of identification and number of patients	HIV status			Total
		Number			
		Negative	Positive	Untested	
Candida spp	Urine (3), Sputum (1), Cervical (2) Blood (1)	1	5	1	7
Candida albicans	Blood (1), Urine (1)	-	1	1	2
Candida parapsilosis	Blood (1)	1	-	-	1
Cryptococcus neoformans	Blood (1), Cerebrospinal fluid (1)	-	2	-	2
High B-D glucan		-	4	-	4

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Table 4. Underlying malignancies present in patients with diagnostic features of a DIC.

Primary diagnosis	Staging (Solid organ malignancies)	World Health organisation subtyping (Haematological malignancies)	HIV status			Total
			Number of patients			
			Negative	Positive	Unknown	
Haematological Malignancies						
Myelodysplastic syndrome		Myelodysplastic syndrome with excess blasts	2			2
Acute myeloid leukaemia (AML)		AML with recurrent translocation t(15;17) n = 5 AML with recurrent translocation t(8;21) n = 3 AML NOS, Acute monoblastic n = 1 AML not otherwise specified n = 6	4	7	4	15
Chronic Myeloid leukaemia		CML with BCR-ABL1	-	-	2	2
B-cell Acute Lymphoblastic leukaemia (ALL)		B-cell ALL, not otherwise specified n = 2	1	-	1	2
Acute leukaemia		Not subtyped	2	-	1	3
Diffuse Large		Gastric (n = 1), Plasmablastic (n = 1), NOS (n = 1)	-	2	1	3
B-cell lymphoma						
B-cell Lymphoproliferative disorder		Not subtyped	-	1	-	1
Hairy cell leukaemia		Hairy cell leukaemia	1	-	1	2
Castleman's Disease		Castleman's Disease	1	-	-	1
Monoclonal gammopathy of uncertain significance			1	-	-	1
Hodgkin Lymphoma		Classical (n = 1), Nodular sclerosing HL (n = 1) HL NOS (n = 1)	-	3	-	3
Plasma cell myeloma			1	1	-	2
Solid Organ Malignancies						
Adenocarcinoma						
Breast Ductal	Stage 4		-	-	1	1
Ovarian	Stage 4		-	1	1	2
Prostatic	Stage 4		1	-	1	2
Squamous carcinoma						
Cervical	Stage 1 (1) Stage 2b (1) Stage 4 (3)		1	3	1	5
Oesophagus	Stage 4		-	1	-	1
No identifiable primary	Stage 4		-	-	2	2
Sarcoma						
Kaposi Sarcoma	Stage 4		-	1	-	1

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Table 5. Relative risk ratios for suspected triggers for DIC.

	Bacterial (non-mycobacterial)	*Mycobacterium tuberculosis*	Malignancy	HIV
Odds ratio	27.2199	35.0059	22.7478	29.9776
95% lower confidence limit	8.3579	4.7193	6.9607	12.7020
95% upper confidence limit	88.6490	259.6629	74.3400	70.7492
Relative risk ratio	2.3110	2.1335	2.2310	2.7387
95% lower confidence limit	2.0104	1.8738	1.9414	2.3257
95% upper confidence limit	2.6565	2.4292	2.5638	3.3257

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departments where DIC may be consequently under-diagnosed. It was not possible to determine whether patients fulfilled the criteria for diagnosis of sepsis as commonly used scoring systems require clinical data. [24] The study contained a small number of participants. Finally, it was not possible to follow patients up and the final clinical outcome of the DIC could not be evaluated. The study does, however, highlight the common triggers for DIC in our setting and may alert clinicians to ensure appropriate investigation and treatment.

Author Contributions

Conceptualization: Elizabeth S. Mayne, Susan J. Louw.

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Investigation: Elizabeth S. Mayne, Susan J. Louw.

Methodology: Elizabeth S. Mayne, Susan J. Louw.

Writing – original draft: Elizabeth S. Mayne, Susan J. Louw.

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Publication 6 – Disseminated intravascular coagulation in HIV infection

The candidate demonstrated that HIV constitutes an independent risk factor for the development of disseminated intravascular coagulation. To evaluate the laboratory presentation, 246 consecutive DIC screens were collected over a 1 year period. All patients with no confirmatory HIV testing were excluded. Patients with HIV infection presented at a significantly earlier age (41 year vs 46 years, $p<0.02$). The prothrombin time was significantly more prolonged (30.1s vs 27.3s, $p<0.05$), the d-dimer levels were substantially higher (5.89mg/L vs 4.52mg/L, $p<0.04$) and the fibrinogen (3.92g/L vs 1.73g/L, $p<0.01$) and platelet levels (64.8 vs $114.8x10^9$/l, $p<0.01$) were significantly lower in PWH. These findings suggest that even in a cohort of patients with an inflammatory thrombotic microangiopathy, PWH presented with more significant coagulation derangements. This may, in part reflect the baseline activation of the haemostatic system although liver synthetic dysfunction was also likely to be contributory.

Contribution of candidate

The candidate was responsible for devising the study, collating the data, analysing the data, drafting the manuscript and review of the manuscript. The co-authors assisted with data collection and manuscript review (SL) and with statistical support (AM).

PLOS ONE

RESEARCH ARTICLE

Diagnosis of human immunodeficiency virus associated disseminated intravascular coagulation

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Abstract

Introduction

Disseminated intravascular Coagulation (DIC) is a thrombotic microangiopathy which may complicate a number of severe disease processes including sepsis. Development of microvascular thromboses results in consumption of coagulation factors and platelets and ultimate bleeding. Patients with HIV infection (PWH) often present with baseline dysregulation of the coagulation system which may increase severity and derangement of DIC presentation. Previously, we have shown that HIV is a significant risk factor for development of DIC.

Methodology

We conducted a retrospective record review of all DIC screens submitted to our tertiary coagulation laboratory in Johannesburg, South Africa, over a one year period and compared the laboratory presentation of DIC in PWH with presentation of DIC in patients without HIV infection.

Results

Over the year, 246 patients fulfilled the International Society of Thrombosis and Haemostasis (ISTH) diagnostic criteria for DIC– 108 were confirmed HIV-infected and 77 were confirmed uninfected. PWH and DIC presented at a significantly earlier age (41 vs 46 years respectively, p<0.02). The prothrombin time was significantly more prolonged (30.1s vs 26. s), the d-dimer levels were substantially higher (5.89mg/L vs 4.52mg/L) and the fibrinogen (3.92g/L vs 1.73g/L) and platelet levels (64.8 vs 114.8x10^9/l) were significantly lower in PWH. PWH also showed significant synthetic liver dysfunction and higher background inflammation.

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Competing interests: The authors have declared that no competing interests exist.

Conclusion

PWH who fulfil the diagnostic criteria for DIC show significantly more dysregulation of the haemostatic system. This may reflect baseline abnormalities including endothelial dysfunction in the context of inflammation and liver dysfunction.

Introduction

South Africa has a high prevalence of Human immunodeficiency virus (HIV) infections with an estimated 13% or 7.8 million people with HIV (PWH) and approximately 110 000 HIV-related deaths annually [1]. The prevalence of HIV infection is even higher in the South African in-hospital population particularly in medical wards and intensive care units where the true prevalence may be over 60% [2, 3]. HIV infection status should be considered in all patients particularly since HIV can result in unusual presentations and exacerbations of pre-existing conditions [4].

HIV infection is strongly associated with pro-thrombotic states including pulmonary thromboembolic disease and deep vein thrombosis [5], arterial thrombosis manifesting as cardiovascular disease [6] and microvascular disorders including, most prominently, thrombotic thrombocytopaenic purpura (TTP) [7]. This increased propensity to pathological clotting has been attributed to a number of different features including endothelial dysfunction resulting from chronic inflammation (reviewed in [8]), associated infections (opportunistic and non-opportunistic), an imbalance between pro- and anti-coagulant factor levels [9–15] as well as platelet dysfunction [16]. These derangements, documented in individuals on antiretroviral therapy (ART) as well as in patients with uncontrolled viraemia (ART-naïve), include decreased Protein S levels, elevated levels of coagulation factors including factor VIII and von Willebrand factor [9, 14] and quantitative and qualitative platelet disorders [13, 16, 17]. The imbalance between pro- and anticoagulant factors manifests in the laboratory as elevated D-dimers levels in HIV infected individuals [18, 19].

Disseminated intravascular coagulation (DIC) is a thrombotic microangiopathic state characterised by widespread microvascular thrombosis, consumption of coagulation factors and platelets and ultimately a bleeding diathesis [20]. It is important to make the diagnosis of DIC in an appropriate clinical setting and a number of triggers have been associated with DIC development including severe infection and sepsis, malignancy, trauma and obstetric complications [21]. No single laboratory test is sufficiently robust, specific or sensitive enough to diagnose DIC. The diagnosis may be made using a number of scoring systems which assign numeric values to abnormalities in a panel of tests including the International Society of Thrombosis and Haemostasis (ISTH) Diagnostic Scoring system which assigns points for reduction in platelet count, elevation of fibrin degradation products, prolongation of the prothrombin time and the fibrinogen level (Table 1) [20].

Although HIV is associated with the development of DIC, this has been considered an indirect relationship with the majority of HIV-associated DIC cases attributed to HIV- related infections or malignancies rather than with HIV as a primary pathophysiological trigger for DIC development. We have reported the high risk of development of DIC in HIV-infected patients even in the absence of other comorbidities [21]. It is important to define the trigger accurately as the primary therapy of DIC is the treatment of the underlying cause although supportive replacement of coagulation factors and platelets may also be considered especially

Table 1. The ISTH scoring system for overt DIC.

Parameter and value	Points allocation
Platelet Count	
• >100 x 10^9/L	• 0 points
• <100 x 10^9/L	• 1 point
• <50 x 10^9/L	• 2 points
Elevation of Fibrin Degradation products (D-dimer levels)	
• No increase	• 0 points
• Moderate increase	• 2 points
• Strong (marked) increase	• 3 points
Prolongation of the Prothrombin Time (PT)	
• <3 seconds	• 0 points
• >3 but <6 seconds	• 1 point
• >6 seconds	• 2 points
Fibrinogen level	
• >1 g/L	• 0 points
• <1 g/L	• 1 point
Score:	
≥ 5: compatible with overt DIC: repeat score daily	
<5: suggestive for non-overt DIC: repeat score next 1–2 days	

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if a patient is actively bleeding [22]. PWH often show baseline activation of the coagulation and haemostatic systems which may impact the laboratory presentation of these patients.

In order to define the laboratory presentation of DIC in patients with and without HIV infection, we undertook a retrospective analysis of all DIC screens submitted to our tertiary care academic facility in Johannesburg, South Africa.

Methodology

Permission for this retrospective study was granted by the University of the Witwatersrand Human Research Ethics Committee (Certificate number: M160389). All data were retrieved from the laboratory information system for screens submitted to the coagulation laboratory at the National Health Laboratory Service (NHLS) Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) for a one year period from 2015 to 2016. This laboratory is a specialist referral facility for the academic hospital and a number of surrounding hospitals in the greater Gauteng area.

Since the study was a retrospective record review, no patient clinical data were obtained and no further testing was possible. All data were anonymized. The minimum dataset used included D-dimer levels, prothrombin time (PT), activated partial thromboplastin time (aPTT), platelet count and fibrinogen levels. The coagulation analysis was performed on a STAGO STA-R Max™ (Diagnostica Stago, Asnières-sur-Seine, France) and platelet counts were performed on the Sysmex XN analyser (Sysmex, Kobe, Japan). For each participant, the ISTH DIC scoring system was used to assess the presence or absence of DIC with patients with ISTH scores of 5 and greater considered to have overt DIC. Other data collected from the database included albumin levels (a surrogate of liver dysfunction), C-reactive protein levels, HIV status (where this had been performed) and CD4+ T cell counts and viral load levels in patients who were HIV infected. Screens where no testing for HIV had been conducted were excluded from the final analysis.

Table 2. Demographic and laboratory parameters in patients presenting with a DIC.

	HIV-infected patients (n = 108)	HIV-uninfected patients (n = 77)	P-value*	Adjusted p-value
Age at diagnosis (mean± SD)	41± 11	47± 18	<0.02	<0.001
Sex (female; male)	59; 49	44;33	<0.15	0.42
ISTH score (mean± SD)	5.97± 0.89	5.74± 0.85	>0.50	1.53
CD4+ T cells per mm^3 (mean± SD)**	159± 285	-	-	-
Viral Load, log copies/mL (mean±SD)**	685,375± $1.4x10^6$	-	-	-
ISTH DIC score parameters				
PT (s, mean± SD)	30.12± 34.26	27.30±27.45	<0.05	0.28
D-dimer (mg/L, mean± SD)	5.89± 10.89	4.52± 5.77	<0.04	0.22
Fibrinogen (g/L, mean± SD)	3.92± 1.73	4.70± 2.64	<0.01	<0.001
Platelets ($x10^9$/L, mean±; SD)	64.87±91.15	114.84± 218.36	<0.01	0.01
Additional laboratory parameters				
C-reactive protein (mg/L, mean± SD)	116.98± 83.05	115.38± 87.87	<0.12	0.69
Albumin (g/L, mean± SD)	26.75± 7.89	28.00± 10.39	<0.01	0.01

N–number, SD–standard deviation, ISTH–International Society of Thrombosis and Haemostasis, PT–prothrombin time

* p-value of <0.05 considered significant

** CD4+ T cell count and viral load available on 96 patients and 67 patients respectively

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Statistical analysis was performed using StataSE® 14.2 StataCorp. Summary statistics of all analytes including mean values and standard deviations were computed. Mean values were compared using a student's t-test after applying ranking by an upward sort to analytes by positive or negative HIV status. A p-value <0.05 was considered significant.

Results

For the period, 246 patients met the ISTH diagnostic criteria for a DIC. Of these, 61 had no recorded HIV test and were excluded from further analysis. 108 patients were HIV-infected and 77 patients were confirmed HIV-uninfected. Of the 108 HIV-infected patients, 67 viral loads and 96 CD4+ T cell counts were available. It was not possible to assess treatment status or length of infection in these patients.

The summary data are presented in Table 2. Patients with HIV-associated DIC presented at a significantly earlier age. The coagulation parameters were significantly more deranged although this did not impact the mean ISTH score. Both HIV-infected and uninfected patients presented with a high C-reactive protein, reflecting the high level of inflammation in both cohorts. Importantly, HIV-infected patients had significantly lower mean albumin levels.

Discussion

HIV-infected patients in South Africa often present for treatment with advanced disease at lower CD4+ T cell counts and with higher viral loads [23, 24]. This is associated both with an increased incidence of opportunistic and non-communicable complications of HIV infection and with longer and more severe chronic inflammation. This results in an underlying derangement of the coagulation pathways. Previously we have shown that HIV-infection can be a significant trigger for the development of the thrombotic microangiopathy (TMA), DIC [21]. In this study, we show that HIV-infected patients with DIC present at a younger age and with a more significant coagulation disorder. Although this did not significantly impact their DIC score, it is possible that the diagnosis of overt DIC may be made erroneously in these patients with significant baseline activation of the haemostatic systems. The implications of the severity

of the coagulopathy on outcomes and management of these patients should be urgently investigated.

Importantly, HIV-infected patients showed significantly lower mean albumin levels. Albumin is a negative acute phase reactant and is often reduced in severe infection. It may also, however, reflect liver synthetic dysfunction [25]. The majority of coagulation factors are synthesised in the liver and the prothrombin time, in particular, is a sensitive indicator of liver disease. Unfortunately, limited clinical data were available but liver involvement in HIV could be associated with opportunistic infections especially disseminated mycobacterial disease or malignancies like, for example, B-cell lymphoma [26–28]. As these conditions may also predispose patients to the development of DIC, this may contribute to diagnostic uncertainty in patients with HIV-associated DIC.

This study has a number of limitations. There were limited clinical data and the outcomes of the patients included is unknown. In the majority of cases, serial measurements were not available and HIV viral loads and CD4+ T cell counts were also not available on all patients. This study does, however, indicate that DIC in PWH presents with more significant derangement of coagulation parameters and this should be considered when making the diagnosis of DIC in this population.

Supporting information

S1 File. Raw data for all quantitative DIC parameters.
(XLSX)

Author Contributions

Conceptualization: Elizabeth S. Mayne, Susan Louw.

Data curation: Elizabeth S. Mayne.

Formal analysis: Elizabeth S. Mayne, Anthony Mayne, Susan Louw.

Funding acquisition: Elizabeth S. Mayne.

Methodology: Elizabeth S. Mayne, Susan Louw.

Writing – original draft: Elizabeth S. Mayne, Anthony Mayne, Susan Louw.

Writing – review & editing: Elizabeth S. Mayne, Anthony Mayne, Susan Louw.

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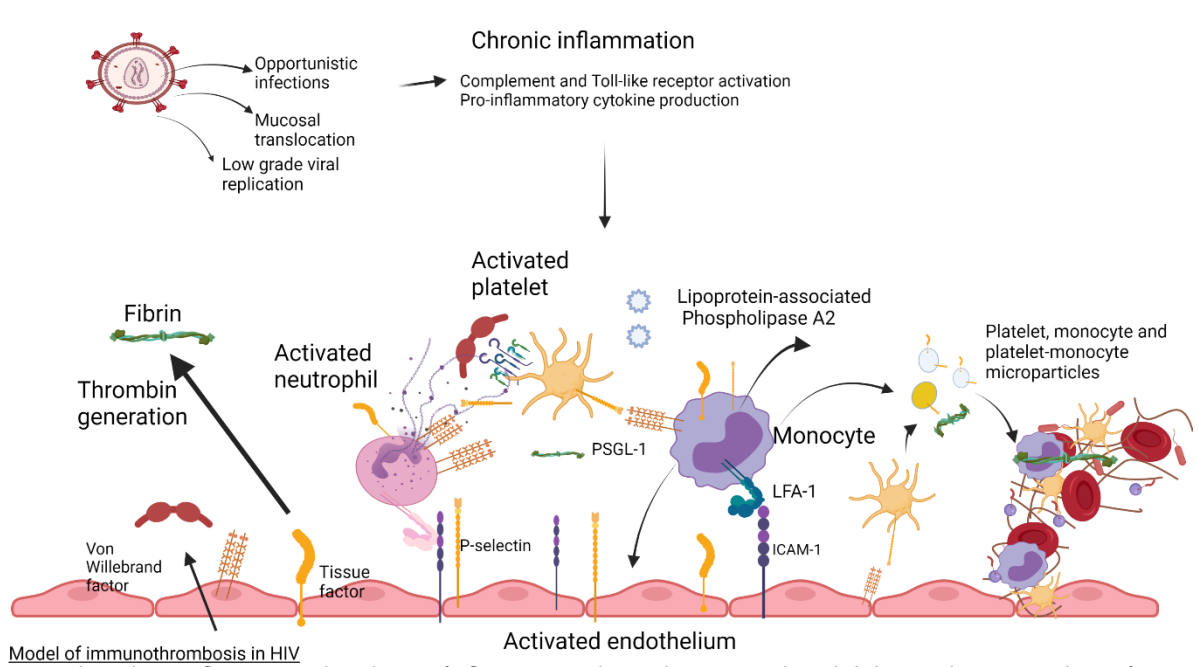
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CHAPTER 3: SUMMARY AND FUTURE DIRECTIONS

HIV-infected patients are at risk of abnormal thrombosis throughout the vascular system. This includes an increased risk of venous thromboembolic disease, microvascular thrombosis and accelerated atherosclerosis and arterial thrombosis.[22, 42, 43] This abnormal thrombosis represents in part an innate response to the chronic inflammation which is present in these patients.[26, 43] Patients enrolled in the Strategic Management of Antiretroviral Therapy study in the ART interruption wing were at increased risk of all-cause mortality but were at high risk of cardiovascular disease as a cause of morbidity and mortality. In these patients, specific poor prognostic factors included a marker of chronic inflammation (interleukin-6) and of accelerated fibrinolysis (elevated d-dimer levels). [19] Although epidemiological studies show temporal and causal relationships between HIV infection (and specifically uncontrolled HIV infection) and thrombosis, mechanisms remain to be elucidated. This is relevant to the South African population. In 2021, over 8.2 million individuals were infected with HIV and patients continue to present late and with consequently advanced disease.[13, 44] Although antiretroviral therapy does mitigate the risk of both communicable and non-communicable diseases, in our patients, the risk is not abrogated particularly in patients with associated comorbidities like essential hypertension and diabetes mellitus type 2 which are both prevalent in the South African population. [45-47]

A summary figure of the pathways described is presented in figure 2. Key questions remain with respect to cardiovascular disease risk in HIV infection. A number of biomarkers of disease have been measured internationally although there are differences within the populations sampled including the access to ART, presence of comorbidities and the measurement of cardiovascular disease.[48] Many studies use surrogates of cardiovascular disease risk like carotid intimal thickness and attempt to link the performance of biomarkers to these surrogate markers.[48] Certain vascular diseases are also excluded from extensive investigation. We have demonstrated that the levels of Lp-PLA_2, a phospholipase which is linked to chronic inflammation and which has been advocated as a marker of cardiovascular disease in HIV-infected populations by the American College of Cardiologists, links to viral load in HIV-infected patients.[49, 50] This suggests that uncontrolled viraemia remains a key risk factor for cardiovascular disease in these patients. What is unclear is whether these biomarkers translate to an increased risk of both microvascular and venous disease. Preliminary data from patients with HIV with arterial disease collected as a prospective cohort suggests that additional markers of endothelial activation and dysfunction are raised. In a cohort of 22 HIV-infected patients, sampled immediately *ex vivo*, we demonstrated that both levels of intercellular adhesion molecule-1 (sICAM-1) and growth/differentiation factor-15 (GDF-15) were significantly increased when compared with 11 uninfected, healthy controls (95.56 vs 55.33 ng/mL, p=0.0032 and1.144 vs 0.381ng/mL, p<0.001, respectively). ICAM-1 is a cellular adhesion marker which is expressed by the activated endothelium and is responsible for tight adherence of leukocytes including monocytes and neutrophils to the endothelium. GDF-15, also known as monocyte inhibitory cytokine-1, is a cytokine from the transforming growth factor-beta (TGF-β) family and has cardioprotective effects in inflammatory conditions. The raised levels in HIV suggest that this may have a compensatory protective function in this cohort of patients. Future directions will include the measurement of these and other markers of endothelial dysfunction and inflammation in HIV-infected patients with diagnosed cardiovascular disease.



Model of immunothrombosis in HIV
HIV results in chronic inflammation with production of inflammatory mediators These activate the endothelium resulting in upregulation of adhesion molecules like P-selectin, PSGL-1 and ICAM-1 which bind receptors on leukocytes (especially neutrophils and monocytes) and platelets. Activated leukocytes secrete proinflammatory mediators like Lp-PLA2 to activating platelets and other leukocytes. Platelets are also activated by von Willebrand Factor secreted by the endothelium, collagen in the subendothelium and lipid mediators of inflammation like arachidonic acid. Platelets bind leukocytes and the endothelium through P-selectin-PSGL-1 interaction. Tissue factor is released following endothelial cell apoptosis and by activated platelets responding both to activated leukocytes and the activated endothelium. Activated platelets and monocytes form microparticles rich in selectin and tissue factor . Tissue factor through its action on Factor VIIa results in thrombin generation and thrombin cleaves fibrinogen to fibrin. Consequent thrombi contain fibrin strands, platelets and leukocytes. Created with Biorender.com

Figure 2 – The Model of Immunothrombosis in HIV (E. Mayne)

Equally important to the risk of coagulopathy, are cellular effectors of coagulation. Previous work done in our group, identified the expression of tissue factor by monocytes.[51] Tissue factor, a key effector molecule in the coagulation cascade, binds to coagulation factor VII to initiated a zymogenic cascade which results in thrombin generation.[6, 52] Tissue factor expression by monocytes has been linked to thrombotic complications of infection including bacterial septicaemia and, more recently, SARS-CoV2.[8, 53] The exact extent of the contribution of monocyte-derived tissue factor to the propagation of the coagulation cascade is unclear. Other potent sources of tissue factor are the subendothelium and activated platelets. Publication 2 reported here discusses monocyte-platelet interactions[51, 54] and suggest that there is reciprocal activation between these cells. This includes the production of haemostatically active platelet and platelet-monocyte microparticles which express adhesion molecules like P-selectin and additional tissue factor.[54] These microparticles form a surface for coagulation factor activation and also activate the endothelium. In addition to the expression of tissue factor by monocytes and platelets, we have recently described significantly increased expression of tissue factor by neutrophils in HIV-infected patients. Neutrophils have only been recently appreciated as a key effector cell of thrombosis. In atherosclerosis, in particular, neutrophils have a number of different functions including the deposition of cathepsin and cathelicidin on the surface of the atherosclerotic plaque which stimulates tight monocyte adherence.[55] Neutrophils extrude cellular DNA as neutrophil extravasation traps (NETs) in a process called NETosis.[56] NETosis results in the production of many proinflammatory compounds included in neutrophil proteins which act to activate the endothelium, platelets and monocytes. Finally, neutrophils secrete the enzyme myeloperoxidase which contributes to the oxidation of low density

lipoprotein which is a key component of the atherosclerotic plaque.[57-60] Future studies are planned to examine the effect of neutrophils and neutrophil products on endothelial cell cultures.

The clinical presentation of thrombosis in HIV requires further examination. HIV is a significant risk factor for microvascular disease including disseminated intravascular coagulation (DIC). Although previously considered to be an indirect trigger for this disease, in a number of patients with HIV infection and DIC, there is no additional demonstrable cause present.[61] The predisposition is linked with background haemostatic activation and may result in HIV-infected patients with DIC presenting with more extensive defects including higher d-dimer levels, more extensive prolongation of time-to-clot assays and more significant thrombocytopaenia. Thrombocytopaenia is prevalent in the HIV-infected population.[62, 63] In DIC, the primary cause of reduced platelet count is consumption by the widespread fibrin-platelet clots, but additional HIV-related causes could be contributory including HIV bone marrow suppression, direct infection by HIV of megakaryocytes and immune-mediated destruction.[62]

To address the direct impact of HIV on the arterial endothelium, a cohort of patients with HIV-associated peripheral vascular disease (PVD) was collected from patients presenting to Charlotte Maxeke Johannesburg Academic Hospital. PVD is an atherosclerotic disorder which results in occlusion of peripheral arteries with consequent tissue hypoxia and critical ischaemia.[64] Without therapy, patients with peripheral vascular disease are at high risk of tissue loss including lower limb amputation. Samples were collected with informed consent (ethics approval certificate: M130372) and included a small segment of arterial tissue which was removed during peripheral arterial bypass grafting. The median age of the HIV-infected cohorts was 46 years and that of the uninfected controls was 64.5 years ($p<0.001$) and 90% of the HIV-infected patients were male. Patients with HIV-infection were less likely to suffer from either diabetes mellitus or hypertension (20% and 30% respectively) than HIV-uninfected patients (42 and 58.3% respectively, $p<0.001$) There was no significant difference in the use of tobacco between the groups (60% and 66% respectively). In order to evaluate the mechanism behind the PVD in these patients, arterial segments and matched plasma will be evaluated by immunohistochemistry and full RNA sequencing. We hypothesise that the arterial wall in patients with HIV will show significant inflammatory infiltrates including activated monocytes and neutrophils. To assess monocyte subsets, we will stain for markers of monocyte activation in the plaque. To assess the presence of NETosis, we plan to stain for histone-4, a key component of the NET[57] and we hope to measure cell expression markers which are upregulated in response to inflammation in this unique cohort of patients. Finally, it is important to consider the context of vascular disease in the South African population. This includes the presence of comorbid conditions specifically *Mycobacterium tuberculosis* and viral infections including Epstein-Barr Virus, cytomegalovirus and, more recently, severe acute respiratory coronavirus-2.

In summary, HIV-associated vascular disease is an extensive inflammatory condition which can affect the entire vascular system. Coagulation in these patients represents an innate response to ongoing chronic inflammation and is mediated by leukocytes including monocytes and neutrophils, platelets and the endothelium itself. Patients with HIV-associated coagulation abnormalities including arterial and microvascular disease are likely to present with more severe disease and abnormal laboratory parameters reflecting background haemostatic activation.

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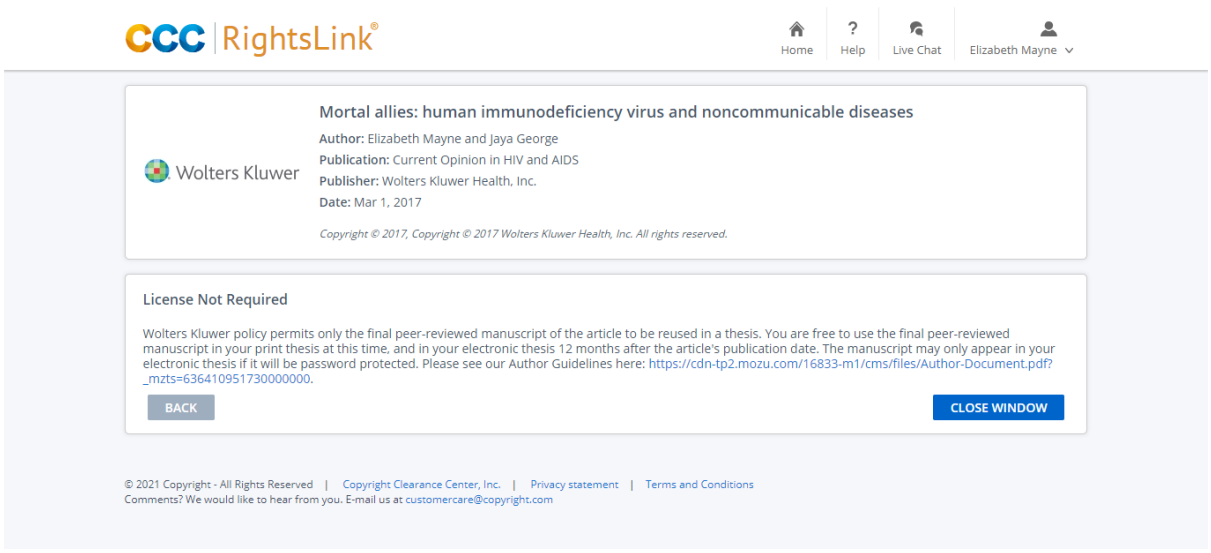
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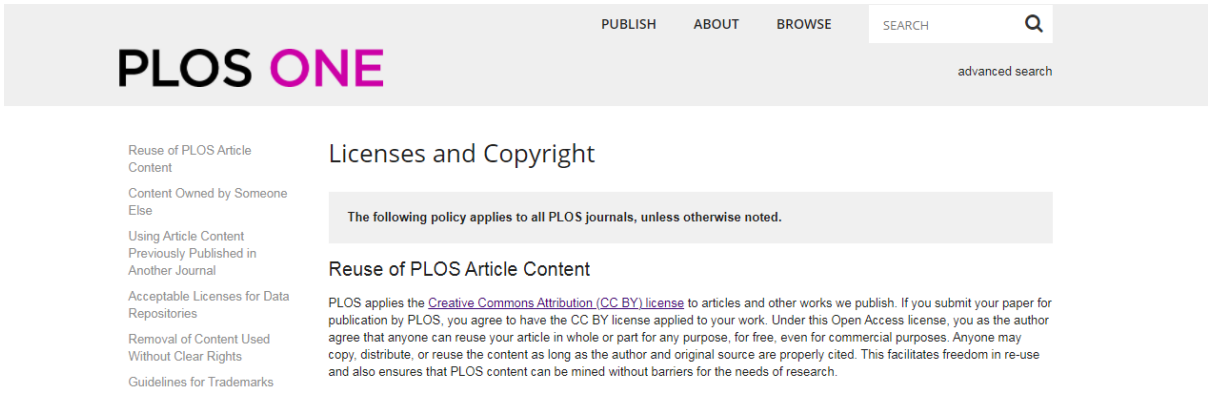
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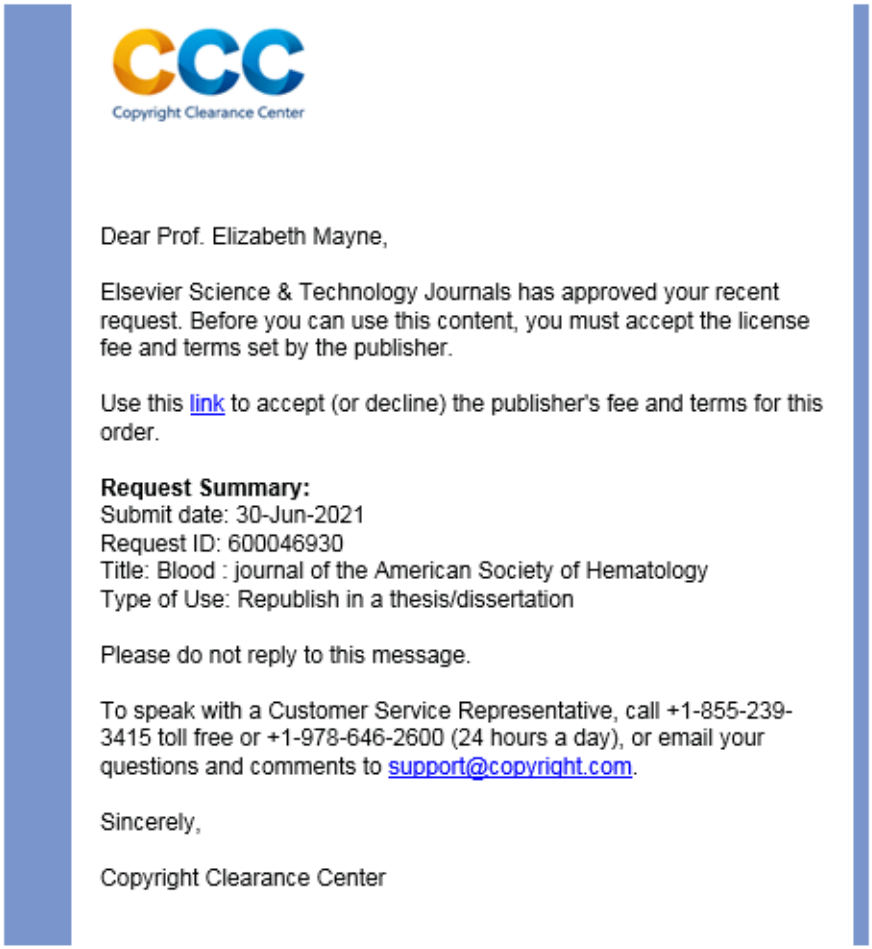
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Research Office Secretariat: Senate House Room SH 10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Phillip Tobias Building, 2nd Floor. Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

05 July 2016

Dr Elizabeth Mayne
Haematologist
Charlotte Maxeke Johannesburg Academic Hospital
National Health Laboratory Service
Parktown
Jubilee Road

Sent by email to: elizabeth.mayne@nhls.ac.za

Dear Dr Mayne

Re: **Protocol Ref no:** M130372
Protocol Title: Assessment of the Immunological Effects of Human Immunodeficiency Virus Clade on a Stable Culture of Macrovascular Arterial Endothelial Cells in Vitro
Principal Investigator: Dr Elizabeth Mayne
Protocol Amendments : Request to expand cadaveric donors and collection of arterial tissue from patients undergoing repair with peripheral vascular disease.

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has approved the following amendments on the abovementioned study, as detailed in your document dated 15 June 2016.

The following documents were received:

- Study Protocol
- Appendix 1 – Data Collection Sheet
- Appendix 2 – Information Sheet

Thank you for keeping us informed and updated,

Yours Sincerely,

L. Moeng

Mr Lebohang Moeng
Administrative Assistant
Human Research Ethics Committee (Medical)

UNIVERSITY OF THE WITWATERSRAND
OFFICE OF THE DEPUTY VICE CHANCELLOR RESEARCH
05 JUL 2016
RESEARCH OFFICE HREC (Medical)
JOHANNESBURG

Ethics Certificate: Retrospective DIC data collection

R14/49 Dr Susan Louw et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160389

NAME: (Principal Investigator)	Dr Susan Louw et al
DEPARTMENT:	Molecular Medicine and Haematology Charlotte Maxeke Johannesburg Academic Hospital National Health Laboratory Service
PROJECT TITLE:	Disseminated Intravascular Coagulopathy (DIC): A Retrospective Review of the Laboratory Parameters for DIC Diagnosis in HIV Positive vs. HIV Negative Patients
DATE CONSIDERED:	01/04/2016
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	15/04/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004, 10th floor, Senate House/2nd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in March and will therefore be due in the month of March each year.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Ethics Approval for collection of Samples at Case Western Reserve University



IRB Administration Offices
UH IRB Phone: 216.844.1529
CWRU IRB Phone: 216.368.6925

APPROVAL

Principal Investigator:	Jeffrey Jacobson
Title:	AIDS 125: SUBSTUDY A - Immunopathogenesis and Virologic Studies in HIV Infection - CFAR-sponsored Network of Integrated Clinical Systems (CNICS)
IRB Number:	01-98-55.
Submission Number:	CR00004688
IRB Office:	UH IRB
Approval Date:	6/22/2021
Effective Date:	6/22/2021
Approval End Date:	6/21/2022
Type of Submission:	Continuing Review
Type of Review:	Full Board
Documents Reviewed:	• ICF Database_01292021, Category: Consent Form; • C-NICS INFO SHEET_01292021, Category: Consent Form; • ICF Blood Draw_01292021, Category: Consent Form;

Ethics approval – biomarker analysis

R14/49 Dr Michelle Moorhouse et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160130

NAME: (Principal Investigator)	Dr Michelle Moorhouse et al
DEPARTMENT:	Clinical Medicine Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	The Influence of Human Immunodeficiency Virus Infection and Antiretroviral Treatment on Cardiovascular Profile and Pulmonary Condition in HIV-Infected Individuals in an Urban Setting in Sub-Saharan Africa. CaPuT Prevalence - and Cappuchino Study
DATE CONSIDERED:	29/01/2016
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	

UNIVERSITY OF THE WITWATERSRAND OFFICE OF THE DEPUTY VICE CHANCELLOR RESEARCH 22 JUN 2016 RESEARCH OFFICE HREC (Medical) RECEIVED JOHANNESBURG

APPROVED BY: Professor P. Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 01/06/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

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Principal Investigator Signature

Date 20 JUNE 2016

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES