THE CHANGING FACE OF THROMBOTIC THROMBOCYTOPENIC PURPURA: THE PATHOPHYSIOLOGICAL ROLE OF ENDOTHELIITIS AND COMPLEMENT ACTIVATION IN THE DEVELOPMENT OF HUMAN IMMUNODEFICIENCY VIRUS ASSOCIATED THROMBOTIC THROMBOCYTOPENIC PURPURA

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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

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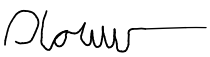
DECLARATION BY STUDENT

I, Susan Louw, 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, South Africa. It has not been submitted for any degree or examination at any other University.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12th January 2023

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Johannesburg

DEDICATION

This thesis is dedicated to my husband, Louis Wildenboer, my children Werner and Betessa and my always supportive mother, Du Borette and to my late dad, Herman Louw, who would have been so proud. Your support and steadfast love saw me through times when my usual 'can do' outlook threatened to waiver. And to the one in the wings. Thank you.
"*Family is not an important thing. It's everything*." –Michael J. Fox

And yet, the need to continue learning and explore remains.
"Live as if you were to die tomorrow. Learn as if you were to live forever." –Mahatma Gandhi

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PUBLICATIONS ARISING FROM THE RESEARCH

Thrombotic thrombocytopenic purpura (TTP)-like syndrome in the HIV era. - **Louw S**, Gounden R, Mayne ES. ***Thromb J.*** 2018;16:35. doi: 10.1186/s12959-018-0189-x
Good Fences Make Good Neighbors: Human Immunodeficiency Virus and Vascular Disease. - Mayne ES, **Louw S. Open Forum Infect Dis.** 2019;6(11):ofz303. doi: 10.1093/ofid/ofz303.
The Hemostatic Conundrum of Thrombotic Thrombocytopenic Purpura: A Case Report. - Pillay E, **Louw S**. **Clin Lab.** 2019;65(6)10.7754/Clin.Lab.2018.181222. doi: 10.7754/Clin.Lab.2018.181222
HIV-associated thrombotic thrombocytopenic purpura (HIV-TTP): A practical guide and review of the literature. - **Louw S,** Jacobson BF, Wiggill TM, Chapanduka Z, Mayne ES. ***HIV Med.*** 2022 Apr 3. doi: 10.1111/hiv.13305.
Thrombotic thrombocytopenic purpura (TTP) in Human immunodeficiency virus (HIV) infected patients: New twists on an old disease. - **Louw S,** Gededzha MP, Mayne AL, Mayne ES. **Aids.** 2022;36(10):1345-1354.1097/qad. doi:0000000000003257
Distinguishing and overlapping laboratory results of thrombotic microangiopathies in HIV infection: Can scoring systems assist? - **Louw S**, Jacobson BF, Mayne, ES. **J Clin Apher**. 2022; 1- 8. doi:10.1002/jca.22003

PRESENTATIONS ARISING FROM THE RESEARCH

Research components of the work have been presented as follows:

Conference (Authors)	Location	Date	Title (Abstract number/oral presentation)
IXth National conference of the South African Immunology Society (SAIS) **(Louw S)**	Johannesburg, SA	October, 2022	Is thrombotic thrombocytopenia (TTP) in patients infected with human immunodeficiency virus (HIV) a complementopathy? (Poster presentation)
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Southern African Society of Thrombosis and Haemostasis (SASTH) Controversies in Thrombosis and Haemostasis, National conference. **(Louw S)**	Virtual conference	November, 2020	TTP and HIV - The problem continues. (Invited oral presentation)
International Society of Thrombosis and Haemostasis (ISTH) International conference. (**Louw S**, Mayne ES, Meiring M, Mmakgabu A, Gounden R)	Virtual conference	June, 2020	ADAMTS-13 in thrombotic thrombocytopenic purpura (TTP) in South Africa in the Human Immunodeficiency Virus (HIV) era. (Abstract number: PB1886)
STAGO Scientific meeting. **(Louw S)**	Istanbul, Turkey	September, 2019	Thrombotic Thrombocytopenia (TTP) in the HIV era. (Invited oral presentation)
Southern African Society of Thrombosis and Haemostasis (SASTH) National conference. **(Louw S)**	Johannesburg, SA	October, 2018	The haemostatic conundrum of TTP in an HIV infected patient. (Abstract number: SASTH 2018_03)
56th International Congress of the Federation of South African Societies of Pathology (FSASP). **(Louw S)**	Stellenbosch, SA	August, 2018	Microangiopathic haemolytic anaemia in HIV: separating the sheep from the goats. (Invited oral presentation)
Southern African Society of Thrombosis and Haemostasis (SASTH) National conference. (Gounden R, **Louw S**)	Johannesburg, SA	November, 2015	Thrombotic thrombocytopenic purpura (TTP) in the HIV era. (Abstract number: SASTH 2015_02)

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LIST OF ACRONYMS AND ABBREVIATIONS

ADAMTS-13 – a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13

ART – antiretroviral treatment

CFH – complement Factor H

DIC – disseminated intravascular coagulation

FFP – fresh frozen plasma

HIV – human immunodeficiency virus

HIV-DIC – human immunodeficiency associated disseminated intravascular coagulation

HIV-TTP – human immunodeficiency virus associated thrombotic thrombocytopenic purpura

IL-6 – interleukin 6

MAHA – microangiopathic haemolytic anaemia

PLASMIC – reduced platelet count, red blood cell haemolysis, absence of cancer, no history of transplantation, reduced red blood cell volume, preservation of the international normalised ratio and creatinine

PLWH – people living with HIV

sICAM – soluble intracellular adhesion molecule

sVCAM – soluble vascular cell adhesion molecule

TMA – thrombotic microangiopathy

TNF-α – tumour necrosis factor alpha

TPE – therapeutic plasma exchange

TTP – thrombotic thrombocytopenic purpura

ULVWF – ultra-large von Willebrand Factor

VWF – von Willebrand Factor

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ABSTRACT

Introduction

Human Immunodeficiency virus (HIV) is a described risk factor for secondary thrombotic thrombocytopenic purpura (TTP) (HIV-TTP). The pathogenesis of this thrombotic microangiopathy (TMA) is however still unclear. The micro-thrombotic process in TTP is related to excess ultra-large von Willebrand Factor (ULVWF) multimers produced by the endothelial cells. Autoantibodies to the VWF cleaving protease, a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13 (ADAMTS-13), are postulated to be pivotal in initiating HIV-TTP. Inflammation and complement activation with resultant endothelial dysfunction and excessive release of ULVWF multimers have been implicated in other forms of TMA. These pathophysiological processes were investigated to assess the contribution to the development of HIV-TTP.

Methods

Data were collected from patients presenting with HIV-TTP in an observational cohort study to delineate the routine presenting laboratory parameters and treatment outcomes. The published literature was reviewed to ascertain the documented prevalence, postulated pathogenesis and treatment outcomes of patients with HIV-TTP. An investigational study was performed in patients (n=35) presenting with HIV-TTP. In this study, patient samples were analysed for levels of endothelial activation markers (soluble intracellular adhesion molecule [sICAM] and soluble vascular adhesion molecule [sVCAM]), inflammatory cytokines (tumour necrosis factor alpha [TNF-α] and interleukin-6 [IL-6]), and complement components C3 and 4 and complement Factor H (CFH), an inhibitor of the complement pathway. Published studies were also reviewed to define the baseline biomarker levels of endothelial dysfunction and coagulation activation in people living with HIV (PLWH) without TTP to serve as points of reference. Data were collected from 2 patient cohorts with HIV infection with either disseminated intravascular coagulation (DIC) or with HIV-TTP to assess the utility of scoring systems.

Results

In contrast to published literature which suggests that the prevalence of HIV-TTP is declining, this TMA is prevalent in South African PLWH in Johannesburg with heterogeneous clinical and laboratory presentation. Conventional scoring systems specifically the PLASMIC (reduced platelet count, red blood cell haemolysis, absence of cancer, no history of transplantation, reduced red blood cell volume, preservation of the international normalised ratio and creatinine) score, developed for detection of other acquired forms of TTP, performed inconsistently in the 35 patients assessed with considerable overlap with other TMAs, notably disseminated intravascular coagulation (DIC). ADAMTS-13 levels were undetectable in all patients and all patients had anti-ADAMTS-13 antibodies. The clinical presentation was, however, atypical. To delineate intermediate pathophysiological markers, the complement system, proinflammatory system and coagulation system, as well as markers of endothelial activation were analysed before and after therapeutic plasma exchange (TPE). Complement components C3 and -4 were consistently at the lower limit of the normal reference range in HIV-TTP patients at presentation. The complement regulatory protein, complement Factor H, was increased. Patients with HIV-TTP had significantly increased levels of proinflammatory cytokines when compared with published results in PLWH without comorbidities. Endothelial activation markers, sICAM and sVCAM, were also significantly increased in the HIV-TTP cohort. Importantly, D-dimer levels were also raised in this cohort of patients.

Conclusions

HIV-TTP remains a cause of HIV-related morbidity and mortality in South African patients. This study reports the findings on 35 PLWH who presented to hospitals in Johannesburg with suspected TTP. The clinical presentation was inconsistent with other secondary forms of TTP i.e., minimal evidence of systemic organ dysfunction. All patients presented with schistocytosis, evidence of haemolysis and thrombocytopaenia and all had low ADAMTS-13 activity levels at presentation with detectable anti-ADAMTS-13 antibodies. Biomarkers of endothelial dysfunction, proinflammatory cytokine levels and markers of coagulation were all significantly increased suggesting that the pathophysiology involves complementary proinflammatory pathways which directly impact secretion of VWF from a compromised endothelium. Activation of the coagulation system, as reflected by increased D-dimer levels, specifically suggests that there may be overlap in the pathophysiology of HIV-TTP and HIV-associated DIC. A potential strategy for differentiation of these disorders with modification of scoring systems is suggested. This study provides compelling evidence of the role of the endothelium in HIV-TTP. Future directions will include validation of the biomarkers described here in more extensive cohorts as well as investigation of these biomarkers in management of these patients.

CHAPTER 1 – INTRODUCTION

Normal endothelial cell function

Endothelial cells line the vasculature as a confluent monolayer of cells which can rapidly switch between a quiescent state, in which they function primarily as a mechanical and anticoagulant barrier, and a metabolically active state.[1] Endothelial cells also have multiple biological functions including maintenance of vascular homeostasis, production of both vasodilatory and vasoconstrictive mediators and regulation of platelet activation. In addition, endothelial cells are responsible for controlling inflammatory egress of leukocytes to underlying tissues.[2]

Role of endothelial cells in haemostasis

The endothelium produces and releases Von Willebrand Factor (VWF) thereby initiating primary haemostasis which involves the binding and subsequent activation of platelets. This coagulation factor further facilitates secondary haemostasis since it binds coagulation Factor VIII, ensuring its availability for the formation of thrombus.[3] Two endothelial transmembrane proteins, endothelium protein C receptor (EPCR) and thrombomodulin, have important anticoagulant function. Finally, endothelial cells promote clot resolution since these cells control the activity of plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (TPA), important regulators of fibrinolysis (clot breakdown).[2-4] The endothelium is therefore important in primary, platelet-based, and coagulation-factor based, secondary clot initiation and termination.

Von Willebrand Factor (VWF)

VWF is a multi-domain adhesive protein which is produced by the endothelium as ultra-large VWF (ULVWF) multimers. VWF is secreted in a basal and on-demand fashion. This protein is pivotal in normal haemostasis with binding sites for sub-endothelial connective tissue (collagen), platelets, coagulation Factor VIII and for a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13 (ADAMTS-13) which is the VWF cleaving protease.[5, 6] In the quiescent state, VWF binds sub-endothelial collagen at sites where vascular integrity is compromised. VWF then unravels under shear stress in the microvasculature with exposure of platelet-binding sites. The interaction between VWF and platelets with subsequent formation of platelet plugs constitutes primary haemostasis.[7] Unravelling of VWF exposes ADAMTS-13-binding sites followed by ADAMTS-13 cleavage of VWF to control the rate and extent of clot propagation. This is pivotal in preventing microvascular occlusion and tissue ischemia, which are important pathological processes in thrombotic thrombocytopenic purpura (TTP). Small quantities of VWF multimers do spontaneously unravel in the absence of vascular injury in the high shear-force microvasculature followed by ADAMTS-13 cleavage.[5, 8] This protease is produced and released by the liver, megakaryocytes, platelets and endothelial cells and exists in 2 conformational states namely a “closed” configuration which can be changed to an “open”, more active form when bound to its substrate, VWF.[5, 9, 10]

Endothelial cell dysfunction (ECD) related to Human Immunodeficiency Virus (HIV) infection and anti-retroviral treatment (ART)

Vascular disease related in part to endothelial cell dysfunction (ECD) is an important non-infectious complication of HIV infection manifesting in all compartments of the vascular tree i.e. the venous, arterial and micro-circulation.[11] Dysfunction of the endothelium with consequent activation of coagulation and

inflammatory pathways has been extensively documented in people living with HIV (PLWH) both prior to initiation of and on virologically suppressive anti-retroviral treatment (ART).[11-14] The putative pathophysiological mechanisms involved in HIV-related vascular disease [15] include chronic inflammation linked to HIV infection[12], presence of concomitant and opportunistic infections such as hepatitis C[16] and *Mycobacterium tuberculosis*[17] which can directly impact the endothelium and metabolic dysregulation with formation of atherosclerotic plaques, related to ART.[13, 15] Pro-inflammatory cytokines including tumour necrosis factor-alpha (TNF-α) and interleukins, like interleukin-6 (IL 6), amongst others, are increased in PLWH resulting in upregulation of endothelial adhesion molecules including, soluble intracellular adhesion molecule (sICAM) and soluble vascular adhesion molecules (sVCAM) which engage with leukocytes which, in turn, bind to the endothelial cells and also extravasate into tissues.[18, 19] HIV proteins including trans-activator of transcription (tat), negative factor (nef) and membrane glycoproteins as well as ART can damage endothelial cells by various mechanisms resulting in dysfunction and apoptosis.[20] Many biomarkers, which serve as surrogate markers of inappropriate endothelial cell activation and endothelial cell dysfunction, have been identified in association with HIV infection including soluble vascular adhesion markers, cytokines, chemokines and their receptors as well as coagulation factors and products of thrombus formation and breakdown.[15, 21-23]

The complement cascade

As a humoral effector of the innate immune system, the complement cascade plays an important role in the adaptive immune system and has the ability to inactivate and facilitate the elimination of pathogenic micro-organisms, including viruses, as well as infected and malignant cells. The complement cascade consists of more than 50 plasma and membrane-bound proteins. Activation pathways of complement include the classical, mannose-binding lectin and alternative pathways.[24, 25] The complement system is pivotal in the control of infections mediating direct pathogen lysis and pathogen opsonisation but these powerful proinflammatory mediators can damage host tissue if the system is inappropriately or chronically activated as may occur with chronic infections like HIV.[25, 26] Various control mechanisms are therefore in place. Complement Factor H (CFH) is one of the main inhibitors of complement activity.[27]

HIV infection and the complement system

Infection with HIV activates complement. The HIV envelope glycoprotein gp41, interacts with and stimulates complement component C1q.[25, 28] HIV infection further preferentially stimulates production of immunoglobulin (Ig) G subclass 1 and 3 antibodies, both potent activators of complement, as opposed to IgG subclasses 2 and 4.[29] The HIV surface glycoprotein (gp)120, binds mannose-binding lectin proteins activating the lectin complement pathway.[28] Complement-mediated virolysis occurs during the acute, as well as the chronic phase of infection and HIV viral load is inversely proportional to complement activity in the acute phase.[30] Anti-HIV antibodies also results in ongoing potent complement activation in chronic, established HIV infection.[31] Although complement assists in clearing HIV virions, this group of proteins can also mediate host-cell viral entry supporting infection. Deposition of complement components on the envelope of HIV virions facilitates adhesion and infection of cells including monocytes and dendritic cells. Viral particles opsonised with complement component C3b, increase dendritic cell mediated activation of HIV-specific cluster of differentiation 8 positive ($CD8^+$) cytotoxic T-lymphocytes upon HIV antigen presentation.[25, 32]

The final complement components, the membrane attack complex (MAC), can initiate endothelial injury culminating in the death of these cells. Healthy endothelial cells are therefore rich in surface receptors that regulate and protect against damage by complement including membrane co-factor protein (MCP)(CD46), decay accelerating factor (DAF)(CD55), and complement receptor-1 (CR1)(CD35).[26, 33, 34] Excessive complement activation, with resultant endothelial damage, has been implicated in the thrombotic microangiopathic anaemia (TMA) resembling TTP which occurs in patients with systemic lupus erythematosus (SLE)[35], as well as in congenital forms of TTP (Upshaw-Shulman disease). Patients suffering from this inherited form of TTP who manifest with renal dysfunction early in the disease course have increased complement activation.[36] Complement activation and dysregulation are also implicated in pregnancy related TMAs and TMA-like syndromes including pre-eclampsia and pregnancy related haemolytic uraemic syndrome (HUS).[37]

Thrombotic thrombocytopenic purpura (TTP) and TTP-like syndrome

Thrombotic thrombocytopenic purpura (TTP), and the related TTP-like syndrome, are thrombotic microangiopathies (TMAs) resulting in critical organ ischaemia and associated morbidity and mortality. The micro-thrombotic process in these disorders is initiated by an excess of ULVWF multimers which are produced and secreted into the circulation by endothelial cells.[38] Abundance of ULVWF multimers in TTP relates to an inherited or secondary acquired absolute or relative deficiency of the VWF-cleaving protease, ADAMTS-13. This enzyme controls the size of circulating VWF multimers thereby maintaining the haemostatic balance since VWF activity is proportional to the size of the individual molecules. The ensuing microvascular thromboses in TTP results in microvascular occlusion with a haemolytic anaemia characterised by red blood cell fragmentation and marked thrombocytopenia in the absence of another TMA, such as disseminated intravascular coagulation (DIC).[39-41] The endothelial dysfunction in TTP-like syndrome is postulated to be secondary to complement activation and inflammation with excess ULVWF multimers released from damaged cells which exhaust the proteolytic activity of ADAMTS-13. The concentration of this protease is initially normal in TTP-like syndrome but eventually decreases as it is consumed. Therefore, although the pathological abnormality in TTP and TTP-like syndrome is similar in consisting of platelet-rich ULVWF microthrombi in the circulation, the disorders have different underlying pathophysiological pathways which might warrant different diagnostic and therapeutic strategies.[42-44]

The established management guidelines for inherited or acquired TTP and TTP-like syndrome describe five clinical features - fever, haemolysis, skin purpura/additional sites of bleeding, central nervous system abnormalities and kidney failure.[41, 45] At present the combination of a reduced platelet count with Coombs negative haemolysis manifesting with increased lactate dehydrogenase (LDH) levels with features of haemolysis (anaemia with reduced haptoglobin and abundant red blood cell fragments) is sufficient to consider the presence of TTP.[46, 47] Additional TMAs, most notably DIC, should also be considered in patients presenting with these features.[41] Establishing the correct diagnosis can be difficult due to the significant overlap in symptoms and signs as well as the laboratory parameters between the syndromes are concerned. The diagnosis should however be correct in order to ensure appropriate treatment and the best outcomes for patients.[39, 41]

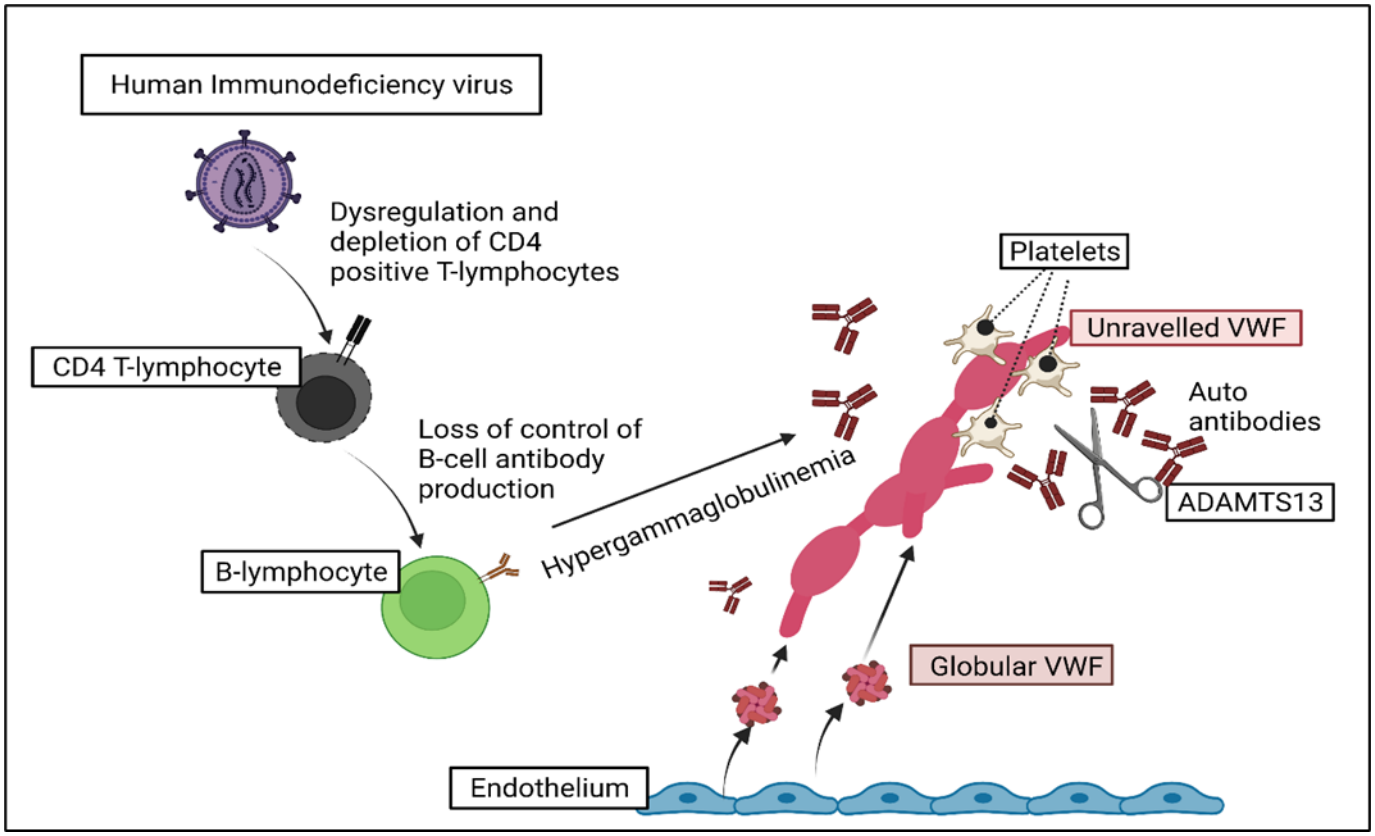
The current gold standard therapy for TTP is daily plasma therapy either as infusion or exchange. This therapy is postulated to remove or dilute ULVWF multimers and auto-antibodies as well as inflammatory

cytokines while supplementing ADAMTS-13. Immunosuppressive therapy in the form of corticosteroids is recommended for first acute episodes and relapses of acquired, immune-mediated TTP.[48] Daily plasma therapy is administered until the platelet count has recovered to above 150 x 10^9/L for 2 days with decreasing LDH levels and schistocytes.[40] Novel therapies for TTP are being tested in patients with congenital, as well as those with relapsing and or refractory secondary TTP.[49]

Thrombotic thrombocytopenic purpura (TTP) associated with HIV infection

Although TTP occurs more commonly in PLWH[40, 50, 51], the underlying pathophysiology in this setting has not been completely elucidated.[52-54] HIV-associated TTP (HIV-TTP) remains life-threatening even after the introduction of therapeutic plasma exchange (TPE).[38, 53] This therapy is expensive, not widely available and not without side-effects.[55] ADAMTS-13 activity in patients with HIV-TTP has not been adequately documented although it has been postulated that the action of auto-antibodies to this protease is pivotal in the pathogenesis of this TMA in PLWH.[53, 54] ADAMTS-13 antibodies in HIV-TTP probably relates to the immune dysregulation in PLWH who develop a hypergammaglobulinaemia but these antibodies are inconsistently detected with poorly defined pathophysiological activity.[56-58] (Figure 1)

The majority of published case series assessing the pathological variables in secondary TTP did not focus on [59, 60] or excluded[39, 61-63] patients with HIV infection. International studies and reviews have, however, concluded that the prevalence of HIV-TTP has decreased considerable after the introduction of ART which is compatible with the hypothesis that viral replication and activity are pivotal in the development of this TMA.[61, 64, 65] Local studies and anecdotal reports have documented that the prevalence of HIV-TTP has not significantly decreased in African patients and develops at variable viral loads in ART-treated and naïve PLWH.[52, 66]



HIV, Human immunodeficiency virus; ADAMTS-13, a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13; VWF, von Willebrand Factor; TTP, thrombotic thrombocytopenic purpura.

Figure 1: Currently postulated pathogenesis of Human immunodeficiency virus (HIV)-associated thrombotic thrombocytopenic purpura (TTP)

Depletion of $CD4^+$ regulatory T-lymphocytes occurs after HIV infection with resultant disinhibition of B-lymphocyte immunoglobulin production and ensuing polygammaglobulinemia. These inappropriately produced antibodies contain anti-ADAMTS-13 inhibitory antibodies resulting in reduced VWF proteolysis and an increased concentration of ultra large VWF multimers in the circulation. VWF obstructs the microvasculature manifesting as the thrombotic microangiopathy, TTP. *Created with Biorender.com*

The antigen and activity levels of ADAMTS-13 in local South African HIV-TTP cohorts vary across studies[53, 54] with inconsistent presence of auto-antibodies suggesting a different pathophysiology in HIV-TTP. Gunther et al. [53] postulated that secretion of VWF in the circulation following viral or cytokine-mediated endothelial cell injury is a pivotal initiating event in the development of HIV-TTP. The authors documented increases in D-dimer levels in patients presenting with HIV-TTP which they contributed to endothelial dysfunction and formation of pathological thrombi and further suggested that further investigations of the pivotal initiating event of this microangiopathic haemolytic anaemia (MAHA) are indicated. Only one South African observational study by Louw et al.[52] refers to the MAHA encountered in PLWH as the TTP-like syndrome described by Chang[42] in which excessive release of VWF is an important pathophysiological initiator.

Patients with HIV-TTP improve with plasma therapy administered either as infusion or as TPE together with optimisation of ART to achieve viral suppression.[38] Prolonged TPE is however often required to achieve remission.[67] The role of immunosuppression with agents such as corticosteroids has not been evaluated in controlled trials in HIV-TTP patients resulting in treatment inconsistencies despite the postulated

pathogenic role of ADAMTS-13 auto-antibodies.[68, 69] Corticosteroids are used to treat auto-immune diseases and diseases due to excess inflammation. These agents act in the nucleus where they bind to corticosteroid response elements which in turn either stimulate or suppress gene transcription ultimately suppressing production of inflammatory mediators such as various interleukins and tumour necrosis factor (TNF).[70] Diagnostic algorithms and scoring systems in patients with HIV-TTP are also incompletely validated. Clinical as well as laboratory result endpoints should be investigated. Cytopenias, including thrombocytopenia, are for example prevalent in patients infected with HIV without comorbidities such as TTP or DIC.[71, 72]

Aim

This research project aimed to investigate the pathophysiological role of inflammation and complement activation with resultant endothelial damage and excessive release of von Willebrand Factor (VWF) in patients presenting with HIV-associated thrombotic thrombocytopenic purpura (HIV-TTP). This was achieved with the following objectives:

1. To document the presenting symptoms and signs together with accompanying laboratory results and treatment outcomes in a cohort of HIV-TTP patients (Chapter 2, Publication 1).
2. To review publications in the medical literature to determine the underlying pathogenesis as well as the prevalence in both the local and international context. (Chapter 2, Publication 2).
3. To assess the levels of inflammatory mediators, adhesion molecules of the endothelium and complement components prior to and on completion of treatment for HIV-TTP and to document other potential pathogenic factors including the presence of comorbidities.
   (Chapter 2, Publication 3).
4. To perform a review of the published levels of D-dimers, inflammatory cytokines and endothelial adhesion markers in people living with HIV (PLWH) infection but without additional infections or comorbidities such as TTP in order to establish a baseline against which to interpret the levels of these biomarkers in patients with HIV-TTP. (Chapter 2, Publication 4)
5. To assess any overlap in the results of routine laboratory investigations in patients with HIV-TTP and HIV associated disseminated intravascular coagulation (DIC) in order to investigate the accuracy of diagnostic scores in HIV infected patients presenting with a microangiopathic haemolytic anaemia (MAHA). (Chapter 2, Publication 5).

These studies were conducted in 3 separate cohorts:

1. Retrospective, descriptive cohort study on 21 patients admitted to CMJAH with thrombotic thrombocytopenic purpura (TTP) over 24 months between 2016-2018. (Wits Ethics M180674)
2. Investigational cohort study on 35 patients presenting with HIV-TTP between 2019-2020 (Wits Ethics M160134).
3. Retrospective record review of the results of the investigations of patients with a diagnosis of HIV-TTP (n 71) or PLWH with disseminated intravascular coagulation (HIV-DIC) (n 88) admitted to academic hospitals in Johannesburg between 2015-2021. (Wits Ethics M180674, M160134, M160389)

CHAPTER 2: TITLES AND SYN0PSES OF PUBLICATIONS

Publication 1: Thrombotic Thrombocytopenic Purpura (TTP)-like syndrome in the HIV era

To assess the presenting clinical and laboratory features of Human immunodeficiency virus (HIV)-associated thrombotic thrombocytopenic purpura (TTP), a retrospective study detailing the clinical and laboratory findings in 21 patients presenting sequentially with TTP to the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) was performed. Sixteen patients were infected with HIV (76%). The majority of patients presented with detectable viral loads and variable CD4+ T-cell counts. Seven (43%) of the 16 HIV infected patients were antiretroviral therapy (ART) naïve at presentation with TTP. Of the 4 HIV-uninfected patients, 2 had a positive Coombs test suggesting a possible alternative diagnosis of Evans syndrome. All patients showed marked evidence of haemolysis with a median lactate dehydrogenase (LDH) level of 1,688 U/L (Interquartile range [IQR]: 1,069-2,299 U/L) (reference index [RI] 100-190 U/L), a haptoglobin of <0.1 g/L (RI 0.4-2.4 g/L) in all patients and a median red cell distribution width of 24.5 % (IQR: 20.2-27.73 %)(RI 12.4 - 17.3 %). 96.5% of the cohort of patients responded to plasma therapy with a duration of 11-17 days for remission. Neither the laboratory abnormalities nor the degree of immunosuppression was prognostic, markers being unrelated to the duration of therapy needed for remission. Concomitant steroid therapy was utilised inconsistently. In ART-naïve patients, ART was started when the clinical situation allowed for ART initiation. One patient died after a day of plasma exchange therapy (TPE). Secondary organ involvement was uncommon in this cohort of patients with only 1 patient presenting with renal failure and none with neurological symptoms. It was concluded that HIV-TTP was not uncommon in South African people living with HIV (PLWH) but was characterised by heterogeneity in viral disease parameters and presentation. In these patients, predictive scoring systems may perform inconsistently with no widespread access to confirmatory testing of the von Willebrand Factor (VWF) cleaving protease, a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13 (ADAMTS-13) and associated auto-antibody levels. Furthermore, the impact of other pathophysiological processes requires elucidation including the presence of significant inflammation since this may indicate the need for additional therapeutic measures like consistent immunosuppressive therapy with for example steroids.

Contribution of the candidate:

The candidate was responsible for conceptualisation of the study, patient recruitment, diagnosis and patient management. Co-authors assisted by collecting additional patient information and routine blood test results (RG), writing of the manuscript and critical manuscript review (EM). All 3 authors were significantly involved in the publication and no conflict of interest was declared.

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RESEARCH Open Access

Thrombotic thrombocytopenic purpura (TTP)-like syndrome in the HIV era

Susan Louw, Reenelle Gounden and Elizabeth Sarah Mayne*

Abstract

Background: The thrombotic microangiopathies (TMAs) is a heterogeneous group of relatively uncommon but serious disorders presenting with thrombocytopenia and microangiopathic haemolysis. Thrombotic thrombocytopenic purpura (TTP) is one of these microangiopathic processes. HIV infection is an acquired cause of TTP but the pathogenesis is poorly understood. HIV-associated TTP was previously described to be associated with advanced immunosuppression. The incidence of HIV-related TTP was expected to decline with access to anti-retroviral therapy (ART).

Methods: We undertook an observational study of patients with a diagnosis of TTP admitted to our hospital (CMJAH). The patient demographics, laboratory test results and treatment outcomes were recorded.

Results: Twenty-one patients were admitted with a diagnosis of TTP during the study period. All patients had schistocytes and severe thrombocytopaenia. The presenting symptoms were non-specific and renal dysfunction and neurological compromise were uncommon. 77% of the patients were HIV-infected and, in 7 patients, TTP was the index presentation. The remainder of the HIV infected patients were on ART and the majority were virologically suppressed. A significant female preponderance was present. Only 4 of the 21 patients tested HIV negative with a positive Coombs test in 2. All patients in this cohort received treatment with plasma exchange therapy for a median period of 12 days with a 96.5% survival rate. Neither the baseline laboratory features nor the degree of immunosuppression was predictive of the duration of therapy needed for remission.

Conclusion: HIV-related TTP is still a cause of morbidity and the clinical presentation is heterogeneous which may present a diagnostic challenge in the absence of sensitive biomarkers. Early treatment with plasma exchange is effective but expensive and invasive.

Introduction

The thrombotic microangiopathies (TMAs) consist of a heterogeneous group of relatively uncommon but serious disorders presenting with thrombocytopenia and microangiopathic haemolysis with resultant characteristic red cell fragments on peripheral smear morphology. The pathophysiological disorders manifesting as TMAs include thrombotic thrombocytopenic purura (TTP), haemolytic uraemic syndrome (HUS), disseminated intravascular coagulopathy (DIC) and malignant hypertension [1–3].

Thrombotic thrombocytopenic purpura (TTP) is a microangiopathic thrombotic process which can result in multi-organ failure [4] characterised by widespread microvascular thrombi consisting of platelets and von Willebrand Factor (VWF). Thrombosis in TTP is initiated when haemostatically-active ultralarge VWF multimers accumulate in the circulation because of a relative or absolute deficiency of the cleaving protease, ADAMTS-13 (a disintegrin and metalloprotease with thrombospondin type 1 repeats, member 13) [5]. The sole function of ADAMTS-13 is to cleave VWF [4, 5]. Unravelling of VWF multimers in the high shear stress microvasculature results in spontaneous formation of platelet aggregates in organs such as the kidneys, heart and brain [4, 6]. TTP is reported as rare with an annual incidence of 1 per 1,000,000 of the population [7] but it carries a high mortality rate (10–20%) [1, 8]. Approximately 5% of cases are caused by a congenital deficiency of ADAMTS-13 [4]. The remainder (over 90%) are ascribed to auto-antibody formation against ADAMTS-13 arising spontaneously or secondary to a number of states including collagen vascular disorders like systemic lupus

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erythematosus (SLE), pregnancy, post-transplantation or after drug exposure [4, 5]. Female patients of African ancestry reportedly have the highest prevalence of acquired TTP often in the context of active SLE [4].

The diagnosis of TTP is based on clinical suspicion with supporting laboratory test results [9]. The diagnostic pentad consists of anaemia, neurological symptoms, fever, thrombocytopaenia and renal dysfunction although all 5 features are seen in fewer than 10% of patients [10]. Critical organ ischaemia can also present as gastrointestinal symptoms (in 35% of patients) and cardiac symptoms (25%) [10]. The laboratory tests reveal a severe bicytopaenia (anaemia and thrombocytopaenia) which is present in almost all cases with schistocytes (red cell fragments) on the peripheral smear and an elevated red cell distribution width (RDW). Laboratory features of haemolysis are present, notably a marked increase in lactate dehydrogenase (LDH) levels [4, 5, 11].

Early recognition and treatment is critical to prevent morbidity and mortality. Standard therapy is plasma exchange to supplement ADAMTS-13 and to remove ultra-large VWF multimers. Where plasma exchange is not readily available, plasma infusions can be performed [5, 12, 13].

HIV confers an increased risk for acquired TTP with a 15–40 fold higher incidence in this patient population compared with the HIV-uninfected population [14] but the pathogenesis is poorly understood [15]. TTP is reported to be more common in HIV-infected patients with advanced disease, low $CD4^+$ T cell counts and with comorbidities (including Kaposi sarcoma and cryptococcal meningitis) [15–17]. Published case reports have documented that almost 100% of patients with acquired TTP have severe (< 10%) underlying ADAMTS-13 deficiency but levels of this protease in HIV-infected patients with TTP are variable and may be relatively preserved [18]. The incidence of HIV-related TTP was expected to decline with widespread access to antiretroviral therapy (ART) [10, 12–14] but evidence suggests that HIV is still an important cause of secondary TTP [4, 16].

The haematology ward at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), South Africa, has not experienced the predicted decline in HIV-related TTP or TTP-like syndrome [19] despite increased access to antiretroviral therapy. We decided to undertake a study to document the clinical presentation and treatment outcomes in these patients.

Methodology

This was a retrospective observational study of all patients with a diagnosis of TTP or TTP-like syndrome admitted to the haematology ward at CMJAH, a 1000 bed, tertiary care academic hospital in Johannesburg, South Africa, over a period of 24 months. Patient demographic and clinical parameters, treatment and outcome were collated using a database. As this study was retrospective, informed consent could not be obtained. Ethics approval for the study was granted by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (Clearance Certificate No. M160134).

Descriptive statistics were derived for all parameters. For continuous variables, medians and interquartile ranges were derived. Where appropriate, a Mann-Whitney U test was performed and a p value of < 0.05 was considered statistically significant.

Results

Twenty-one patients were admitted to CMJAH with a diagnosis of TTP during the study period. This hospital serves as a referral centre for healthcare facilities in Gauteng, the smallest but most densely populated province of South Africa. The hospital operates various specialist units, including haematology and oncology.

The patient demographics, laboratory test results and treatment are detailed in Table 1.

All patients presented with laboratory features of a microangiopathic thrombotic process with high numbers of schistocytes on the peripheral smear and a severe evolving thrombocytopaenia (median platelet count at diagnosis of 12×10^9/L). The majority of patients were transferred to our tertiary care facility from other clinical sites. The presenting symptoms were non-specific commonly including headache, lethargy and gastrointestinal symptoms (diffuse abdominal pain with mild diarrhoea). Only 1 patient had laboratory evidence of renal dysfunction (urea of 27.3 mmol/L (normal reference range 2.1–7.1 mmol/L) and a creatinine of 262 μmol/L (normal reference range 49–90 μmol/L)) together with confusion. This patient demised shortly after admission and could not be further investigated. Only 1 patient presented with active bleeding (ecchymoses).

Only 4 of the 21 patients tested HIV negative. All of these patients were female and were investigated extensively for underlying autoimmune disease. A positive Coombs test for IgG antibodies was seen in 2 of these patients. One patient could not be tested for HIV infection. The remaining 17 patients were HIV-infected and in 7 (47%), TTP was the initial presentation of the underlying HIV infection. These patients were untreated and had a median viral load of 228,912 (interquartile range: 131952.5–467,817.5 copies per ml) and a median $CD4^+$ T cell count of 134 cells/uL (interquartile range: 90–305 cells/uL). The HIV infected ART naïve patients were all commenced on ART with as a single, fixed-dose combination (FDC) tablet containing tenofovir (TDF), emtricitabine (FTC) and efavirenz (EFV) following appropriate pre- and post-test counselling. The remainder of the infected patients were on ART and the majority were virologically suppressed with only 2 patients having detectable viral loads. These patients did, however display a wide range of $CD4^+$ T cell counts with a median value of 184.5 cells/mm^3 and an interquartile range of 170–232 cells/uL.

Table 1 TTP-like patient demographics, laboratory test results and treatment

	Age	Sex	Hb	WCC	Plts	RDW	Fragments	INR	PTT	D-dimers	DATT	LDH	Haptoglobin	Urea	Creatinine	HIV-infected	HIV VL	CD4+ T cell count	ARV therapy	Days of PE
Units	Yrs		g/dL	$\times 10^9$/L	$\times 10^9$/L	%			s	mg/L			g/L	mmol/L	ug/L		copies/mL	Cells/ul		
Reference range			11,6 16,4	3,9 12,60	186–454	12,4 17,3	Absent		31–48	< 0.25	Negative	100–190	0,4-2,4	2.1–7.1	49–90			332–1642		
1	48	M	8.8 (11.5)	10.4	33 (276)	33.8	3+	1.02	ND	1.48	ND	2365 (300)	< 0.1	9.9	126	Yes	LTDL	141	Yes	20
2	37	F	10.5 (12.4)	12.85	18 (351)	19.6	2+	1.81	32.6	ND	Neg	1073 (320)	< 0.1	12.4	105	Yes	LTDL	346	Yes	20
3	39	M	7.1 (12.9)	5.42	12 (268)	24.5	2+	1.09	32.6	ND	Neg	698 (468)	< 0.1	4	88	Yes	321,158	194	Yes	10
4	47	F	7.2 (12.8)	7.84	8 (219)	23.3	3+	1.14	30.8	3.20	ND	2023 (311)	< 0.1	9	128	Yes	191,000	134	No	25
5	32	F	4.6 (11.5)	8.56	8 (272)	30.5	3+	1.12	32.9	1.16	ND	941 (191)	< 0.1	1.8	57	Yes	373,000	80	No	11
6	27	F	8 (14.1)	4.92	35 (244)	32.6	3+	1.01	ND	11.90	Negative	2102 (180)	< 0.1	4.7	52	Yes	8642	182	Yes	10
7	43	F	10.2 (12.8)	8.4	16 (86)	24.6	3+	1.01	30.0	0.75	IgG +	2005 (160)	< 0.1	9.1	79	No	N/A	N/A	N/A	12
8	30	F	7.3 (9.7)	6.56	15 (175)	33.9	3+	1.15	ND	11.90	Negative	1836 (178)	ND	4.8	60	Yes	42	113	Yes	10
9	35	F	5.5 (11.1)	9.17	5 (280)	26.6	3+	1.2	31.0	6.49	Negative	1768 (182)	< 0.1	3.8	71	Yes	ND	585	Yes	18
10	45	F	6.7 (10.4)	4.16	18 (105)	22.7	3+	1.06	44.3	ND	IgG +	950 (227)	< 0.1	4.1	67	Yes	228,912	54	No	20
11	43	F	7.8 (9.5)	10.4	18 (23)	23.9	1+	1.62	43.1	0.56	IgG +	1441	ND	17	211	No	N/A	N/A	N/A	12[b]
12	44	F	6.3 (14.3)	13.91	3 (290)	27.9	3+	1.21	43.1	10.00	ND	2708 (478)	< 0.1	7.8	113	Yes	2150	187	Yes	18
13	43	F	6.1 (10.4)	10.5	10 (274)	25	3+	1.17	36.9	7.13	ND	1357 [32]	< 0.1	5.5	94	Yes	72,905	264	No	12
14	40	M	7.4 (10.8)	4.15	21 (254)	20.6	2+	1.11	41.1	1.0	IgG +	1068 (273)	< 0.1	7.7	101	Yes	26,065	215	No	14
15	34	F	6.5 (10.7)	8.96	6 (270)	19	2+	1.35	33.8	17.00	Negative	1683 (257)	< 0.1	6.4	96	No	N/A	N/A	N/A	12
16	56	F	5.8 (8.5)	14.7	5 (328)	34.2	3+	1.3	33.5	ND	Negative	3339 (305)	< 0.1	6.6	80	Yes	LTDL	180	Yes	11
17	34	F	6.7 (12.5)	13.67	5 (248)	27.2	3+	1.28	31.7	0.00	IgG +	2404 (206)	< 0.1	6.5	110	Yes	562,635	421	No	11
18	44	M	7.5	19.35	25	20.2	3+	1.14	34.0	ND	ND	5472	ND	27.3	262	Unknown	ND	ND	Unknown	1[a]

Table 1 TTP-like patient demographics, laboratory test results and treatment *(Continued)*

	Age	Sex	Hb	WCC	Plts	RDW	Fragments	INR	PTT	D-dimers	DATT	LDH	Haptoglobin	Urea	Creatinine	HIV-infected	HIV VL	CD4+ T cell count	ARV therapy	Days of PE
Units	Yrs		g/dL	$\times10^9$/L	$\times10^9$/L	%			s	mg/L			g/L	mmol/L	ug/L		copies/mL	Cells/ul		
Reference range			11,6 16,4	3,9 12,60	186–454	12,4 17,3	Absent		31–48	< 0.25	Negative	100–190	0,4-2,4	2.1–7.1	49–90			332–1642		
19	33	F	6.5 (10.2)	8.9	6 (203)	19	2+	1.35	33.8	17.00	Negative	1683 (195)	< 0.1	6.4	96	No	N/A	NA	N/A	12
20	38	F	7.9 (9.5)	5.6	3 (253)	15.2	2+	1.05	43.6	1.93	IgG +	827 (244)	< 0.1	5.3	67	Yes	2,030,000	100	No	15
21	35	M	2.7 (7)	13.2	29 (134)	13.5	1+	2.38	92.3	0.97	IgG+	978 (476)	< 0.1	5.6	136	Yes	1,010,000	34	No	15
Median	39		7.1 (10.95)	9.065	12 (253.5)	24.5		1.14	33.6	2.565		1683 (244)		6.6	96		209,956	181		12
IQR (25–75%)	34–44		6,35-7725	6,56-13,2		20,2 27,725	6–20,3	1.08–1.2	32–43	0.99–10.5		1069-2299		1.2–3	73–122.7		21,709-420,409	110–227		11–18

[a]Patient demised on day 1. [b] Patient defaulted after 12 days of plasma exchange. Values in brackets denote discharge haemoglobin, platelet and LDH levels

Abbreviations: *Yrs* years, *Hb* haemoglobin, *WCC* White cell count, *RDW* red cell distribution width, *Plts* platelets, *LDH* lactate dehydrogenase, *ARV* antiretroviral therapy, *VL* viral load, *LTDL* viral load lower than detectable limit

PE:plasma exchange, *N/A* not applicable

There was a significant female preponderance in the HIV-infected patients with only 4 of these patients (23.5%) being male. Male patients presented with significantly higher platelet counts than females (median of 16×10^9/l vs 6×10^9/l, $p < 0.004$). No other significant differences between male and female patients was noted with respect to degree of anaemia, levels of LDH or duration of plasma exchange.

Haptoglobin was analysed in the 19 of the 21 patients and was consistently reduced to < 0.01 g/L (normal reference range 0.4–2.4 g/L) despite preserved liver synthetic function as indicated by normal albumin concentration.

All patients in this cohort received treatment with plasma exchange therapy for a median period of 12 days (range 1–20 days) with a 96.5% survival rate (one patient demised 24 h post-admission). One patient refused hospital care after 12 days of treatment. The remaining patients were discharged post-exchange therapy to out-patient follow-up care after their platelet counts had normalised and had remained stable for 2 days and their LDH levels had declined to normal [5, 11, 12]. Neither the baseline platelet counts, LDH level nor the degree of immunosuppression was predictive of the duration of plasma exchange needed for remission.

Discussion

Although TTP incidence was reported to be declining in HIV-infected patients with increased access to ART [4], this has not been the experience at our centre. Over a 24-month period, we admitted 21 patients with a diagnosis of TTP-like syndrome. The majority (75%) of patients admitted to CMJAH with a diagnosis of TTP were HIV-infected in keeping with previous studies at our own and other centres in South Africa [13, 15, 16, 18].

The clinical presentation in our cohort was heterogeneous with none of the patients displaying the classical diagnostic pentad [15]. Of note, only 1 patient had objective laboratory features of renal dysfunction and no patient had clear evidence of neurological dysfunction. Consistent features in our presenting cohort were severe evolving thrombocytopaenia and haemolytic anaemia with numerous schistocytes on the peripheral smear.

Applying the PLASMIC score [2, 20] consisting of the presence of thrombocytopenia and haemolysis with reduced mean red cell volume (MCV), preserved renal function and absence of underlying malignancy with no history of receiving a tissue transplant, to the patients in our cohort (Table 1) would have changed the diagnosis of TTP to another TMA for patients 1, 11 and 21. The PLASMIC score was however developed to identify patients with TMA and severe ADAMTS-13 deficiency manifesting as TTP. Previous studies at our centre have demonstrated that a significant proportion of patients with HIV-associated TTP-like syndrome do not have anti-ADAMTS-13 inhibiting antibodies [18]. Other studies have shown heterogeneity in levels of ADAMTS-13 in these patients ranging from very severe deficiency (< 5%) in up to 44% of patients to normal levels in up to 30% of patients and factors other than ADAMTS-13 deficiency are therefore postulated as pathogenic mechanisms including endothelial injury by HIV itself [21–25], damage by other opportunistic infections or endothelial activation caused by HIV-associated chronic inflammation [15, 18, 26]. This endothelial injury is postulated to results in release of stored VWF which overwhelms the capacity of ADAMTS-13 culminating in a consumptive deficiency. A similar transient deficiency in ADAMTS-13 activity can be seen in healthy volunteers after DDAVP administration which results in release of VWF from endothelial cells [27]. The application of scoring systems such as the PLASMIC score in our environment is currently inappropriate given the high mortality if definitive treatment is not promptly instituted.

Endothelial damage and local activation of coagulation probably also results in an isolated, elevated D-dimer level in these patients [18, 28]. The patients in this study had consistently raised D-dimer levels with a median level of 2.565 mg/L although other coagulation parameters were not deranged. The inconsistency between the decrease in ADAMTS-13 levels and presence of inhibiting antibodies may explain the therapeutic effect of plasma infusion without exchange therapy in HIV-related TTP [13]. The elevated D-dimer levels in the 4 HIV-uninfected patients in the current cohort may relate to sub-clinical bleeding in view of the significant thrombocytopaenia. A differential diagnosis of Evan's syndrome (autoimmune haemolytic anaemia with thrombocytopaenia) was considered in 2 of the HIV-uninfected patients although the laboratory features were non-diagnostic (specifically the Coombs testing was equivocal). The therapy for TTP is, however, effective for patients with Evan's syndrome and both patients responded well.

HIV-associated TTP or TTP-like syndrome is prevalent in our centre despite increased access to antiretroviral therapy (ART). The patients in this study did not show consistently low $CD4^+$ T cell counts or high HIV viral loads. Other co-morbid diseases like Kaposi Sarcoma and cryptococcal meningitis were not overtly evident in the HIV-infected patients in our cohort. Previous studies have shown that TTP is seen in patients with profound immunosuppression and often with AIDS-defining conditions like Kaposi Sarcoma [5, 9, 14, 18, 28, 29]. 8 of the 15 HIV-infected patients in the current study were on ART at the time of admission with TTP. The HIV viral loads ranged from below detectable limit (in the majority of these patients) to 2,030,000 copies/ml. The viral load and CD4+ T cell count did not predict the duration of plasma exchange needed to achieve remission although the HIV-uninfected patients did achieve remission after on average fewer days of

plasma exchange therapy. For 7 patients, TTP was the index presentation of their HIV infection.

The differential diagnosis for TTP includes other thrombotic microangiopathies (TMAs) importantly disseminated intravascular coagulopathy (DIC) and haemolytic uraemic syndrome (HUS) [30]. The diagnosis of TTP and TTP-like syndrome in our unit is made on the basis of severe thrombocytopaenia, elevated LDH levels and schistocytes on the peripheral smear in the correct clinical setting (including the presence of HIV infection). DIC is generally excluded when the coagulation parameters (with the exception of the D-dimers) are normal [28]. HUS would only be considered in patients with current or historical diarrhoea and severe renal dysfunction. In our cohort, only 1 patient presented with any significant renal dysfunction. The absence of a single highly sensitive and specific marker for TTP in HIV infection is a significant impediment to early diagnosis and care, since other TMAs cannot always be excluded with certainty. Of note, the Coombs test may be non-specifically positive in HIV-infected patients and the clinical and diagnostic significance of this is uncertain [31].

All 21 patients in this cohort received daily plasma exchange therapy with Fresh Frozen Plasma (FFP). In patients who responded slowly either the volume of exchange was increased to 1.5 plasma volumes and/ or cryo-poor plasma at the treating clinician's discretion [32]. Steroids (prednisolone at 1 mg/kg) were prescribed to all HIV-uninfected patients in the cohort but were used inconsistently in the HIV-infected individuals [4, 5]. Only 1 patient demised during this study after 1 day of treatment. The cause of death in this patient was not clear. This patient presented with the highest LDH levels in the cohort (5472 U/L) suggesting severe haemolysis and tissue damage [4]. All other patients responded to therapy and were discharged with normal platelet counts and LDH levels. All HIV-infected patients who were not on ART were placed on first-line therapy consisting of single, fixed-dose combination (FDC) tablet containing tenofovir (TDF), emtricitabine (FTC) and efavirenz (EFV [32]. The 4 HIV-uninfected patients responded quicker to plasma exchange. A possible differential diagnosis of Evans syndrome (autoimmune haemolytic anaemia with thrombocytopaenia) was considered in these patients.

Conclusion

TTP or TTP-like syndrome is a significant cause of morbidity in patients infected with HIV. There is no clear link to opportunistic infections in all cases or to severe levels of immunosuppression. In some cases, TTP represents the presenting complaint for patients with undiagnosed HIV infection. Early treatment with plasma exchange is highly effective but expensive and invasive (requiring insertion of large bore catheters). The absence of highly sensitive and specific biomarkers to diagnose TTP in this subset of patients is a challenge.

Limitation of the study

Only 8 (less than 50%) patients in this cohort were on ART limiting the statistical ability to draw a conclusion with regard to the effectiveness of ART in preventing the development of TTP. In addition, ADAMTS-13 antibodies and levels are not routinely measured at our centre which makes the application of scoring systems like the PLASMIC score difficult. Previous studies [18] suggest, however, that this score may need modification in our patient population due to the heterogeneity in ADAMTS-13 levels.

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Availability of data and materials

All data and material which was available under the consent guidelines was included in this publication.

Authors' contributions

SL and RG devised the study and collected the data. EM and SL analysed the data and wrote the paper. All authors read and approved the final manuscript

Ethics approval and consent to participate

This was a retrospective analysis and patients were not consented. The research was approved by the University of Witwatersrand Ethics Committee (Clearance Certificate No. M160134).

Consent for publication

All authors have reviewed and approve submission of this work.

Competing interests

The authors declare that they have no competing interests.

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Erratum:

The following additional references support the article content:

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Only 4 of the 21 patients in the cohort tested HIV negative. One patient was not tested for HIV infection. The remaining 16 patients were HIV-infected and in 7 (43%) of the patients, TTP was the initial presentation of the underlying HIV infection.

Publication 2: HIV-associated thrombotic thrombocytopenic purpura (HIV-TTP): A practical guide and review of the literature

Following the description of the laboratory and clinical presentation in the observational cohort (Publication 1), the published literature was reviewed to determine the described clinical signs and symptoms, laboratory diagnostic parameters and treatment outcomes of patients with HIV-TTP.
Ten publications were reviewed which were published between 2004 and 2019. Three were multicentre studies and the remainder were single-centre observational studies. In up to 78% of patients, TTP was the presenting manifestation of underlying HIV infection. The majority of patients presented with detectable viral loads and corresponding lower CD4+ T-cell counts but the relationship between virological control and immunological recovery with development of and prognosis in HIV-TTP was inconsistent. The CD4+ T-cell counts in most cases were below 200 cells/mm^3 but patients with higher CD4+ T-cell counts of up to 818 cells/mm^3 (Gunther et al. 2017) were also described. Diagnostic criteria in the PLASMIC (reduced platelet count, red blood cell haemolysis, absence of cancer, no history of transplantation, reduced red blood cell volume, preservation of the international normalised ratio and creatinine) score were inconsistently demonstrated in these patients and few showed classical clinical presentations like renal failure or neurological symptoms. Variable levels of ADAMTS-13 antigen and activity levels (moderately reduced to undetectable) as well as presence or absence of anti-ADAMTS13 antibodies were reported in the reviewed studies. Importantly, the majority of patients responded to plasma therapy although additional therapies and immunosuppressive steroids were not used consistently. Although the reduced prevalence in high-income countries has been ascribed to wider access to ART, the imperfect correlation between the degree of immune reconstitution and the development of HIV-TTP suggests that additional pathophysiological mechanisms may drive the disease in South Africa and these should be considered when diagnosing and managing HIV-TTP.

Contribution of candidate:

In this systematic review, the candidate was responsible for definition of the Medical Subject Heading (MESH) terms, data collection and drafting and review of the final manuscript. The co-authors (TMW, BFJ, ZC and ESM) assisted by reviewing and signing off the final draft of the manuscript.

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REVIEW ARTICLE

HIV-associated thrombotic thrombocytopenic purpura (HIV-TTP): A practical guide and review of the literature

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Abstract

Background: Thrombotic thrombocytopenic purpura (TTP), a serious thrombotic microangiopathy (TMA), is prevalent in the South African HIV-infected population. The exact pathogenesis of HIV-associated TTP (HIV-TTP) is however still unclear with diagnostic and therapeutic inconsistancies.

Methods: A systematic review of the published literature regarding HIV-TTP was performed.

Results: HIV-TTP is still associated with significant morbidity and mortality in Africa despite the availability of anti-retroviral therpy (ART). Diagnosis of HIV-TTP requires the presence of a micro-angiopathic haemolytic anaemia with significant red blood cell schistocytes and thrombocytopenia in the absence of another TMA but background activation of the coagulation system and inflammation in HIV infected people can result in diagnostic anbiguity. Plasma therapy in the form of infusion or exchange is successful but expensive, associated with side-effects and not widely available. Adjuvant immunosuppression therapy may of benefit in patients with HIV-TTP and ART must always be optimised. Endothelial dysfunction caused by chronic inflammation and complement activation most likely contributes to the development of HIV-TTP.

Conclusion: The role of adjuvant immunomodulating therpy, the therapeutic targets and pathogenic contribution from endothelial dysfunction in HIV-TTP requires further investigation.

KEYWORDS

endothelial dysfunction, HIV, inflammation, management, thrombotic thrombocytopenic purpura

BACKGROUND

Thrombotic thrombocytopenic purpura (TTP) is a thrombotic microangiopathy (TMA) associated with organ ischaemia and dysfunction. There is significant overlap between the different thrombotic microangiopathies, which also includes disseminated intravascular coagulation (DIC), in both pathogenesis and clinical presentation [1]. Secondary TTP can occur in relation to autoimmune diseases, such as systemic lupus erythematosus (SLE), chronic viral infections, notably HIV but also hepatitis and cytomegalovirus [1], and acute infections like coronavirus disease (COVID-19) [2]. Endothelial dysfunction and activation of the immune system are postulated to play pivotal roles in many of these varied conditions by promoting a hypercoagulable state [3–5]. Despite a significant amount

of research on the role of endothelium in health and disease, a significant chasm still exits from 'bench to bedside' as far as endothelial biology is concerned, as is highlighted in the potential role of endothelial dysfunction in patients with HIV-associated TTP [6].

The microthromboses in TTP are initiated by the accumulation of von Willebrand factor (VWF), a coagulation factor produced and secreted by the endothelium. Ultralarge VWF (ULVWF) multimers accumulate in TTP because of an absolute or relative deficiency of the VWF cleaving protease, a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13 (ADAMTS-13). Obstruction of the microvasculature by platelet-VWF microthrombi manifests as a haemolytic anaemia with red blood cell fragmentation and a significant thrombocytopenia. A diagnosis of TTP is made after exclusion of other forms of TMA [1].

Deficiency of ADAMTS-13 is either congenital, Upshaw–Schulman syndrome, or acquired in the background of conditions including malignancies, autoimmune diseases and infection, including HIV infection [1]. A relative deficiency of ADAMTS-13 may also occur with endothelial activation, damage and excessive release of ULVWF multimers manifesting as TTP-like syndrome, which occurs in the background of various disease processes [5]. This syndrome, in contrast to classical TTP, is mediated by complement activation with accumulation of proinflammatory C5b-9 membrane attack complexes (MACs), which form pores in endothelial cell membranes [5]. The resultant endotheliitis causes cellular apoptosis as well as activation of inflammatory pathways, which results in platelet activation and release of ULVWF multimers from endothelial stores. The platelet-rich VWF thrombi occlude the microcirculation in a process similar to TTP. There is some controversy as to whether similar pathogenic factors mediate HIV-associated TTP [7].

Vascular disease, including TMAs, related to endotheliitis is an established complication of HIV infection [8,9]. Endotheliitis, a state of endothelial cell dysfunction (ECD), is linked to a proinflammatory and procoagulant phenotype and occurs in patients with HIV infection, including virologically suppressed patients on antiretroviral therapy (ART) [8–10]. A full description of the mechanisms of endothelial damage related to HIV infection [11] is outside the scope of this review but these include chronic inflammation linked to ongoing HIV replication [12], opportunistic infections (including hepatitis C and *Mycobacterium tuberculosis*) with endothelial damage and metabolic dysregulation associated with ART. HIV proteins including trans-activator of transcription (tat), negative factor (nef) and membrane glycoproteins can damage endothelial cells, causing dysfunction and apoptosis [11]. Biomarkers of inappropriate endothelial cell activation and dysfunction are upregulated in HIV infection, including vascular adhesion markers, proinflammatory cytokines, chemokines and their receptors, coagulation factors and products of coagulation factor activation and breakdown [9,11].

PREVALENCE OF HIV-ASSOCIATED TTP

Thrombotic thrombocytopenic purpura was first described in HIV-infected cohorts in the 1980s [13,14]. The majority of these early cases occurred in men who presented with variable features of the diagnostic TTP pentad of fever, neurological and renal dysfunction, thrombocytopenia and microangiopathic haemolysis [13]. These patients showed significant heterogeneity in comorbid conditions and degree of immunosuppression although the patients treated with plasma therapy generally had good outcomes [14]. Limited investigation of the pathophysiology was, however, undertaken [14]. The relationship between HIV and TTP has been confirmed in multiple subsequent observational studies (Table 1).

Thrombotic thrombocytopenic purpura has declined in prevalence in HIV-infected patients in high-income countries [25]. This may reflect increased access to early suppressive ART. Conversely, TTP in low- and middle-income countries remains a significant cause of morbidity and mortality [21,23,24,26]. HIV-associated TTP was the commonest condition requiring therapeutic plasma exchange (TPE) provided by the South African National Blood Services (SANBS) with a calculated crude incidence of between 17.6 and 63.8 cases per million every year since 2011 [26]. This represents a considerable burden on healthcare resources, with many patients requiring admission and multiple cycles of TPE [21].

PATHOPHYSIOLOGY OF HIV-ASSOCIATED TTP

Classical TTP is caused by significantly reduced ADAMTS-13 activity either from birth or subsequent to the development of autoantibodies which are associated with a number of triggers, including infectious and autoimmune diseases as well as pregnancy [1]. The pathophysiological mechanism of TTP in HIV is not entirely clear, with variable levels of both ADAMTS-13 and associated autoantibodies creating diagnostic uncertainty [7,27,28]. It is still unclear whether a one- or two-hit model is operational in the pathophysiology of HIV-associated TTP but work by Feys et al. [29] in a primate animal model demonstrated that functional,

TABLE 1 Observational studies of patients with HIV-associated TTP

Authors (year)	Study design	No. of HIV-infected patients with TTP	Study population and major findings
Becker et al. (2004) [15]	Multicentre cohort study over 6 years	17[a]	• TMA associated with lower mean $CD4^+$ T-cell count, higher mean viral load • Comorbidities reported – *Mycobacterium avium* and hepatitis C virus
Miller et al. (2005) [16]	Single centre cohort study over 6 years	8	• 6 patients with decreased ADAMTS-13 and 1 with autoantibodies • Good response to TPE and ART
Novitsky et al. (2005) [17]	Single centre cohort study over 7 years	21 (23 HIV uninfected controls)	• HIV infected patients with TTP respond to FFP infusion quicker than HIV uninfected patients • None of the patients with HIV-associated TTP required TPE • HIV-associated microangiopathy is highly responsive to plasma infusions
Gunther et al. (2007) [18]	Single centre cohort study over 3 years	22 (3 HIV uninfected controls)	• HIV-infected patients with TTP – increased median D-dimer but normal coagulation factor assays • Lower platelet counts and haemoglobin levels compared with HIV-uninfected patients with TTP
Malak et al. (2008) [19]	Multicentre prospective cohort study	29	• 17 (58%) significantly decreased ADAMTS-13 levels; 12 (42%) with detectable ADAMTS-13 activity • Mortality in HIV-associated TMA correlated with higher ADAMTS-13 and VWF levels
Hart et al. (2011) [20]	UK TTP registry over 10 years	24	• TTP was HIV index presentation in 30% • Poor adherence to ART associated with relapse in 4 patients • Responds to TPE and ART ± steroids; Immunomodulator (e.g. rituximab) needed in 10% • Duration of TPE correlated with viral load • ADAMTS-13 activity <5% in all patients and anti-ADAMTS-13 antibodies in 80%
Masoet et al. (2017) [21]	Single centre experience over 5 years	40 (12 HIV uninfected controls)	• Overall mortality rate: 44.2% • ART associated with better outcomes • Clinical: fever commoner in HIV infected patients; neurological pathology was common in both groups • 90.2% of HIV-infected patients with TTP only received plasma infusion with good clinical response
Bade et al. (2018) [22]	Single centre experience over 12 years	28	• Median viral load: 89 500 copies/mL • Median $CD4^+$ T-cell count: 58 cells/μL with good response to TPE
Louw et al. (2018) [23]	Single centre experience over 2 years	16	• TTP was HIV index presentation in 2 (13%); 14 (87%) patients had variable virological control on ART • Female preponderance (female:male ratio: 4:1) • TPE for median of 12 days with 96.5% survival
Swart et al. (2019) [24]	Single centre experience over 5 years	41	• TTP was index presentation of HIV in 78% of patients • Median of 10 days TPE • Relapse rate of 9.8%; mortality rate of 29.3%

Abbreviations: ADAMTS-13, a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13; ART, anti-retroviral therapy; CD4, cluster of differentiation 4; HIV, human immunodeficiency virus; TMA, thrombotic microangiopathy; TPE, therapeutic plasma exchange; TTP, thrombotic thrombocytopenic purpura; UK, United Kingdom.

[a]TTP and haemolytic uraemic syndrome (HUS).

in vivo inhibition of ADAMTS-13 was sufficient to induce development and phenotypic expression of TTP in the absence of a second trigger.

A number of secondary triggers for HIV-associated TTP have been proposed. Multiple opportunistic infections including hepatitis C, cytomegalovirus, Kaposi sarcoma herpesvirus and *Mycobacterium tuberculosis* may directly activate and damage the endothelium, resulting in release of ULVWF multimers [19,22,30]. Underlying endotheliitis, associated with inflammation, is present in both ART virally suppressed and ART-naïve HIV-infected patients [3,10,31,32]. Endothelial dysfunction in HIV-associated TTP has been proposed as the major driver of microvascular disease, with studies showing a direct impact of HIV proteins on endothelial cells [33,34]. Recently, the contribution of other innate effectors, including complement, has also been highlighted, particularly in the related TMA, TTP-like syndrome [5]. Complement, a zymogenic cascade which assembles on cell membranes, can mediate loss of endothelial cell integrity and endothelial cell apoptosis [35]. Complement activation, commonly associated with HIV infection [36], also causes platelet activation and recruitment of leukocytes to the site of inflammation, resulting in the development of immunothrombosis which may present as a TMA [37].

CLINICAL AND LABORATORY PRESENTATION OF HIV-ASSOCIATED TTP

Initial guidelines for the diagnosis of congenital or acquired TTP defined a diagnostic pentad of fever, haemolytic anaemia, cutaneous (purpura) or other bleeding related to thrombocytopenia, neurological abnormalities and renal dysfunction [30]. Currently, the combination of thrombocytopenia with a microangiopathic anaemia with features of haemolysis [elevated lactate dehydrogenase (LDH), reduced haemoglobin and haptoglobin, with increased red blood cell (RBC) fragments] is sufficient for a presumptive diagnosis of TTP [38]. The differential diagnosis of TTP and TTP-like syndrome includes other TMAs, most importantly DIC. The distinction can be challenging because of the significant overlap of clinical and laboratory test results between the syndromes [30]. Correct diagnosis is essential, however, in order to ensure appropriate treatment [30]. As TTP is a diagnosis of exclusion, it is important to investigate the patient for other contributory conditions, including autoimmune disease, malignancy and infection [1]. Given the need to treat TTP urgently, these investigations are frequently conducted after initiation of therapy (Table 2).

The clinical and laboratory presentation of patients with HIV-associated TTP is heterogeneous [7]. Observational studies report varying incidences of neurological dysfunction, fever and renal failure, with neurological dysfunction and fever being prominent in certain cohorts described in South Africa [17,21,23,24]. Cardiac ischaemia has been described in up to 25% of patients with TTP and the measurement of troponin levels may be of value in patients presenting with HIV-associated TTP [1]. Although thrombocytopenia and evidence of microangiopathic haemolysis (particularly elevated LDH levels) are invariably present during the course of disease [23,24], other laboratory findings may be atypical. D-dimer levels are often raised and there may be variable activation of the coagulation pathways [18,23]. Patients with HIV-associated TTP respond to TPE but may not achieve full normalization of parameters because of the presence of dyshaematopoiesis, with persistence of thrombocytopenia and anaemia [39,40]. Viral load and CD4 T-cell count show an inconsistent relationship with diagnosis and prognosis. Although TTP is more common in patients with poor virological control, it may present in patients with viral suppression [22-24,28]. ART non-compliance is nevertheless associated with TTP relapse [7,20,21,41]. A full list of recommended investigations at our centre is included in Table 2.

Clinical predictive scores, such as the PLASMIC [platelet count, haemolysis, active cancer, mean RBC volume (MCV), international normalized ratio (INR) and creatinine] score [42], have been developed to assist in the diagnosis of TTP (Table 3). The PLASMIC score is based on clinical and routine laboratory parameters and predicts the likelihood of severe ADAMTS-13 deficiencies in patients with a TMA. The utility of this score requires validation in patients with TTP in whom ADAMTS-13 deficiency does not drive pathogenesis such as patients with TTP-like syndrome [5,7,28,42].

TREATMENT OF TTP AND TTP-LIKE SYNDROME

HIV-TTP is treated either with daily therapeutic plasma exchange (TPE) or with plasma infusion [1,17–19,35,39]. TPE removes and dilutes ULVWF multimers, autoantibodies and inflammatory cytokines while supplementing ADAMTS-13 [1,41]. TPE is, however, invasive and not available in all centres [26]. Infusion of fresh frozen plasma (FFP) alone (at a dose of 30 mL/kg per day) has been shown to be efficacious in the treatment of HIV-TTP and dilutes ULVWF multimers while supplementing ADAMTS-13 [17]. Plasma infusion can, however, result in

TABLE 2 Recommended baseline laboratory investigations

Test parameter	Expected findings
Full blood count (FBC) with manual smear review	Reduced Hb (concentration frequently < 7 g/dL) with > 10 RBC fragments per high-power field and marked thrombocytopenia (PLT count < 20×10^9/L)
Lactate dehydrogenase (LDH)	Significantly elevated (frequently > three times upper limit of normal)
End-organ damage • Kidney dysfunction (U&E) • Cardiac injury (troxponin T)	U&E generally unremarkable Underlying cardiac muscle injury has been described
Liver function test (LFT)	Unconjugated bilirubinaemia Elevated aspartate transaminase (AST)
Disseminated intravascular coagulation (DIC) screen (PT, fibrinogen, D-dimers and platelet count)	PT: preserved or mildly prolonged Fibrinogen: variable (may be elevated as acute-phase response) D-dimers: often significantly elevated Platelet count: frequently < 20×10^9/L
HIV serology/viral load/$CD4^+$ count	HIV serology: usually positive Viral load: often high CD4 T-cell count: variable
Direct antiglobulin test (DAT)	IgG and C3d variable
Haptoglobin	Usually undetectable
Possible additional triggers to consider in appropriate patient groups	
Infections	*Mycobacterium tuberculosis* – sputum (Gene Xpert® and microscopy), chest X-ray, urine and blood culture Gram-negative organisms including *Shigella* spp. and *E. coli* spp. (to exclude HUS) – bacterial culture Viral infections with endothelial cell activation/tropism including Gamma herpesviridae, hepatitis B and C – serology and viral PCR testing
Malignancy	B-cell lymphoma (lymph node biopsy, bone marrow biopsy)
Pregnancy	Beta-HCG
Autoimmune disease	Rheumatoid factor Anti-nuclear factor Anti-double-stranded DNA
Therapeutic monitoring to guide plasma therapy	
FBC and differential count	Therapeutic targets: Platelet count should be sustained above 150×10^9/L for 2 days (may not fully normalize); Scanty red cell fragments; Recovery of Hb to > 6 g/dL.
LDH	Should show a persistent downward trend and ideally be < 450 U/L (may not fully normalize)
Calcium, magnesium and phosphate	To exclude metabolic abnormalities associated with plasma therapy and citrate anticoagulation

Abbreviations: DNA, deoxyribonucleic acid; FBC, full blood count; Hb, haemoglobin; HCG, human chorionic gonadotropin; HUS, haemolytic uraemic syndrome; IgG, immunoglobulin G; PCR, polymerase chain reaction; PLT, platelet; PT, prothrombin time; RBC, red blood cells; U&E, urea and electrolytes.

the administration of insufficient amounts of plasma due to ensuing fluid overload and unavailability of FFP, resulting in poor therapeutic responses and a need to convert to TPE [17]. In HIV-infected patients, it may be difficult to achieve a normal platelet count [39], and LDH levels or schistocyte numbers may be a more appropriate target. Methods for the quantification of schistocytes are poorly standardized, however, and usually rely on subjective peripheral blood smear examination [43]. In the experience of the authors, LDH is an objective, reliable surrogate marker of haemolysis. TPE is expensive, requires large volumes of FFP, has the risk of citrate-related reactions and is not widely available in many low-income countries [26]. Furthermore, administration of FFP may have significant

TABLE 3 PLASMIC score for the prediction of thrombotic microangiopathy associated with severe a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13 (ADAMTS-13) deficiency with high probability of thrombotic thrombocytopenic purpura (TTP) [42]

Parameter	Points
Platelet count $< 30 \times 10^9$/L	1
Haemolysis: reticulocyte count > 2.5% or haptoglobin undetectable or elevated indirect bilirubin	1
No active cancer	1
No history of solid organ or stem cell transplant	1
Mean red blood cell volume (MCV) < 90 fL	1
International normalized ratio (INR) < 1.5	1
Creatinine < 176.8 μg/dL	1
• Score of 0–4: low risk of severe ADAMTS-13 deficiency • Score of 5: intermediate risk of severe ADAMTS-13 deficiency • Score of 6–7: high risk of severe ADAMTS-13 deficiency	

immunological and non-immunological adverse effects, including transfusion-associated circulatory overload, transfusion-associated lung injury and allergic responses [44]. The insertion of a large-bore catheter can result in vessel injury and predispose to thrombosis and bleeding. Drip site sepsis is another complication of indwelling intravenous catheters [45]. For these reasons, TPE should be discontinued as soon as clinically appropriate.

Although ART has an inconsistent effect on the development of HIV-associated TTP [23,24], it most likely prevents TTP relapse [7,20,21]. For this reason, ART should be initiated as soon as possible. A theoretical concern is the development of TTP following initiation of ART as an immune reconstitution inflammatory syndrome (IRIS), although haematological manifestations in IRIS are uncommon and there are no reports of TTP as IRIS in the literature [46].

The role of adjunctive treatments in HIV-associated TTP is unclear. Immunosuppression, including oral steroid therapy, is used sporadically to reduce inflammation-associated endotheliitis [7,41]. Corticosteroids are frequently employed to treat inflammation and autoimmune diseases based on their action on numerous steps in the inflammatory pathway [47,48]. Other therapies that have been employed include the anti-CD20 monoclonal antibody, rituximab, which may assist in reducing levels of autoantibodies against ADAMTS-13 [20,25,30]. However, a local study in South Africa to investigate the pathophysiological significance of ADAMTS-13 autoantibodies in HIV-TTP patients demonstrated that these antibodies were present in HIV-infected people without TTP and the exact clinical relevance remains unclear [28]. Novel therapies are in development but clinical trials focus on patients with congenital, relapsing and refractory classic TTP [30]. The monoclonal antibody caplacizumab, for example, is a humanized anti-VWF antibody which prevents platelet aggregation [49]. Although approved for TTP therapy in patients with acquired and congenital forms of the disease, no wide-scale studies have investigated its role in HIV-associated TTP [49]. Another potential avenue of exploration, is the role of complement inhibition in HIV-associated TTP syndrome, which may share a common pathogenesis with TTP-like syndrome [5]. Eculizumab, a monoclonal antibody which inhibits formation of the membrane attack complex, assists in prevention of thrombosis in paroxysmal nocturnal haemoglobinuria and may have a similar effect in TTP-like syndrome [5].

CONCLUSIONS

HIV-related TTP was relatively common prior to widespread access to ART, implying that viral replication drives pathogenesis [1,25,41,50]. Local African case series and investigational studies, however, suggest that HIV-associated TTP is still prevalent and occurs at a range of viral loads in ART-treated and -untreated patients [18,21,23,24,26,32]. ADAMTS-13 levels in these local and some international cohorts have also been variable, with an absence of inhibitory antibodies in many patients suggesting a different pathophysiology in HIV-associated, acquired TTP compared with other forms of secondary TTP [7,20,27,28]. Endothelial dysfunction with excessive release of VWF which overwhelms ADAMTS-13 proteolytic capacity has been postulated as a pivotal initiating event in HIV-associated TTP [19,25]. Infection with HIV is associated with endothelial dysfunction, which has been described extensively in the literature, and the causes include direct viral effects, the effects of opportunistic infections and inflammation including complement activation [3,9,32].

Investigation of HIV-associated TTP should include a haemolytic work-up, measurement of ADAMTS-13 levels and autoantibodies directed against ADAMTS-13 [19]. Coagulation parameters may show abnormalities [18], and other triggers (infectious and non-infectious) should be actively excluded [1]. Red cell fragments, LDH levels and platelet count are useful in monitoring therapeutic response [1,23].

Standard-of-care remains daily TPE or plasma infusion [1,17]. The applicability of international treatment regimens and the duration and target end-point of TPE require further investigation, especially in the context of thrombocytopenia being a common finding in HIV-infected patients without TTP [39]. Adjunctive therapy with immunosuppressants and novel agents shows some

benefit in small case series [1,20]. Initiation and optimization of ART remains central to preventing TTP relapse in these patients [20,21,41].

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CONFLICT OF INTEREST

SL, BFJ, TMW, ZC and ESM declare there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

SL and ESM – devising concept, literature selection and review, analysis and drafting manuscript; BJ, TW and ZC – drafting of manuscript and critical reading of manuscript.

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Erratum: This article was based on a review of the literature and not a systematic review

Publication 3: Thrombotic thrombocytopenic purpura (TTP) in Human immunodeficiency virus (HIV) infected patients: New twists on an old disease

Observational studies in patients at our centre and the literature, highlighted the need to elucidate additional pathophysiological pathways in HIV-TTP. The contribution of a number of complementary pathways was investigated – proinflammatory pathways (comprising both proinflammatory cytokines and complement activation and regulation), the coagulation pathway and the endothelial activation pathway. The concentrations of 2 inflammatory cytokines, tumour necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), 2 complement components, C3 and C4, and one complement regulatory protein, Factor H, and 2 biomarkers of endothelial activation and damage, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), along with the levels of fibrin degradation products were determined at presentation and again at the time of resolution of the TMA in 35 consecutive patients presenting with HIV-TTP between January 2019 and December 2020. Research blood samples were collected at presentation and again on completion of therapy for HIV-TTP. Analytes were assessed using Luminex Map™ technology on a Luminex Biorad 200 flow cytometer (Biorad, Hercules, California) platform using the Human Cardiovascular Disease bead array 2, the cytokine/chemokine magnetic bead panel-2 and a human complement panel (all MILLIPLEX Merck® KGaA, Darmstadt, Germany). Activation of the coagulation cascade was assessed by measuring fibrin degradation products (D-dimer) and von Willebrand Factor (VWF) on a STAGO® STA-R Max (Stago Diagnostica®, France) automated coagulation analyser. Complement proteins C3 and -4 were quantified on an BN-II immunonephelometry platform (Siemens®, Erlangen, Germany). The results are presented in the Table below.

D-dimer, von Willebrand Factor (VWF), endothelial activation markers, inflammatory cytokine and complement pre- and post-plasma therapy in HIV-TTP cohort (n 35):

	D-dimer	VWF		TNF-α		IL-6		sVCAM		sICAM		C3/C4	
RI	< 0.25 mg/L	50-160%		<27 pg/mL		<7 pg/mL		328-792 ng/mL		325-447 ng/mL		C3: 0.90-1.80 g/L C4: 0.10-0.40 g/L	
TTP cohort Pre-TPE vs. Post TPE	Pre	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Median (IQR)	8.75 (6.52-14.55)	353 (275-420)	182 (118-230)	116 (79.9-152)	29.9 (17.2-42.3)	43 (41-85)	8.4 (5-14)	1,643 (1,022-1,977)	897 (810-1,194)	1,417 (986-2,141)	453 (287-605)	C3: 1.12 (0.91-1.27) C4: 0.187 (0.143-0.269)	C3: 1.38 (1.22-1.43) C4: 0.272 (0.22-0.35)
p-value (Pre vs. Post TPE)		<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	

RI, reference index; IQR, interquartile range; TPE, therapeutic plasma exchange; VWF, von Willebrand Factor; TNF-α, tumour necrosis factor-α; IL-6, interleukin-6; sVCAM, soluble vascular adhesion molecule; sICAM, soluble intracellular adhesion molecule; C3/C4, complement fractions 3 & 4.

Biomarkers of endothelial dysfunction, inflammation and coagulation were significantly elevated when compared with the reference interval described in the package inserts. There was also significant elevation of D-dimer levels at presentation. The anti-inflammatory, complement inhibitor, Factor H (CFH), was elevated at presentation with HIV-TTP (median of 0.626 mg/ml (IQR 0.505-0.849) (RI: 0.237-0.426 mg/ml) and complement C3 and C4 levels tended to the lower limit of the reference interval. All biomarkers demonstrated a trend towards normalisation with clinical resolution of the thrombotic microangiopathy (TMA). It was concluded that inflammatory pathways and endothelial activation were contributory to the pathogenesis of HIV-TTP and that the utility of these biomarkers should be explored in a diagnostic and prognostic setting in a more extensive cohort of patients – particularly including the therapeutic value of immunosuppression. Although complement activation has been associated with TTP-like syndrome, the evidence for complement activation in this cohort was less compelling although the significant elevation of complement regulator, CFH, at presentation warrants additional investigation. The cause of the low $CD4^{+ve}$ T-cell count in the patient who suffered from systemic lupus erythematosus (SLE) could possibly have been due to steroid therapy and unfortunately testing for ART drug resistance was not performed.

Contribution of candidate:

The candidate was personally responsible for the study design and conceptualisation, selection of biomarker analytes, enrolment and consent of the participating patients as well as the collection and storage of research specimens. The candidate then, under the guidance of a co-author and co-supervisor, MPG, processed the samples for the research parameters on the Luminex platform. The candidate further assisted with the statistical data analysis together with a co-author ALM. The candidate then compiled the first draft of the manuscript with subsequent revisions by the co-author ESM prior to publication.

Thrombotic thrombocytopenic purpura in HIV-infected patients: new twists on an old disease

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Objective: Investigate the presence of inflammation, endothelial dysfunction and complement activation in patients with HIV-associated thrombotic thrombocytopenic purpura (HIV-TTP) to support the hypothesis that these processes probably contribute to the development of this thrombotic microangiopathy.

Design: A prospective, investigational cohort study of 35 consecutive patients diagnosed with HIV-associated TTP presenting to three academic, tertiary care hospitals in Johannesburg, South Africa over 2 years.

Methods: The patients with HIV-TTP received therapeutic plasma therapy and supportive treatment. Demographic data, the results of routine investigations and patient outcomes were recorded. Peripheral blood samples were collected prior to and on completion of plasma therapy and the following additional parameters were assessed at both time points: activity of the von Willebrand factor (VWF) cleaving protease, a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13 (ADAMTS-13) and the presence of ADAMTS-13 autoantibodies, levels of pro-inflammatory cytokines, interleukin-6 and tumour necrosis factor-alpha, and two endothelial cell adhesion molecules. Complement activation was assessed by sequential measurement of C3 and C4 as well as levels of the complement inhibitor, factor H.

Results: The inflammatory and endothelial activation markers were significantly ($P < 0.001$) elevated in the cohort of patients prior to plasma therapy compared with levels on discharge. Complement was activated and normalized with therapy. The ADAMTS-13 levels were reduced with significant auto-antibodies to this protease at presentation.

Conclusion: Inflammation in HIV mediates endothelial damage and complement activation. This study proposes that these processes are probably contributory to the development of HIV-TTP, which can therefore be characterized in part as a complementopathy, resembling TTP-like syndrome.

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Keywords: complement activation, endothelial dysfunction, HIV-associated thrombotic thrombocytopenic purpura, inflammation

Introduction

HIV infection is prevalent in Africa with over 26 million people with HIV (PWH) infection [1]. Ongoing chronic inflammation from low-grade viral replication, opportunistic infections and microbial translocation manifest with noncommunicable diseases including malignancies and thromboses [2–5]. Thromboses in PWH [2,3,6] occur in

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all compartments of the vascular tree presenting as arterial [3,7], venous [8,9] and microvascular events [10,11].

Thrombotic thrombocytopenic purpura (TTP) is a thrombotic microangiopathy (TMA) with critical organ ischaemia, infarction and dysfunction. This microthrombotic process is initiated by excess ultra-large von Willebrand Factor (ULVWF) multimers secreted by the endothelium [12]. VWF is a key coagulation factor in normal haemostasis [13]. The excess ULVWF in TTP relates to an absolute congenital or acquired deficiency of the VWF-cleaving protease, a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13 (ADAMTS-13). Subsequent microvascular occlusion manifests as a haemolytic anaemia with red blood cell (RBC) fragments (schistocytes) and significant thrombocytopenia (low platelet count) in the absence of another cause of TMA. TTP had a mortality rate close to 95% prior to the introduction of therapeutic plasma exchange (TPE) in the 1980s [12,14]. Infection with HIV is a well established cause of acquired TTP [12,15–19] but the exact pathogenesis has not been elucidated [10,20]. Although HIV-associated TTP (HIV-TTP) responds to plasma therapy in the form of infusion or TPE [21,22], the targets and duration of therapy and the role of adjunctive immunosuppression require further assessment [10,16] as this TMA still has a mortality rate of up to 40% despite best available treatment [18–20].

TTP-like syndrome, in contrast to classical TTP, is considered a disorder of complement dysregulation [23–25]. Complement, a zymogenic cascade, closely associates with the coagulation cascade with reciprocal activation [26,27]. Disorders of complement dysregulation include atypical haemolytic uraemic syndrome (aHUS), a TMA because of activation of the complement system with autoantibodies against an inhibitor of complement, complement factor H (CFH) in some instances [25]. CFH regulates the alternative complement pathway and protects the integrity of host cell membranes from damage by the terminal complement components C5b-9, which assemble as the membrane attack complex (MAC) [27,28]. Complement may activate the endothelium by disrupting endothelial cell integrity resulting in apoptosis and exposure of procoagulants factors, upregulation of adhesion molecules like soluble intercellular and vascular cell adhesion molecule 1 (sVCAM-1 and sICAM-1) and promotes release of ULVWF multimers [24,25,27]. Complement further stimulates release of procoagulant, proinflammatory cytokines (including interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α)) and these together activate platelets [27]. Platelet rich-VWF thrombi in TTP-like syndrome occlude the microcirculation resulting in a clinical and laboratory presentation, which resembles TTP but with initially normal ADAMTS-13 levels [23,24]. TTP-like syndrome may complicate illnesses associated with complement activation including trauma, severe infection, autoimmune diseases and sepsis [29,30] and may be difficult to distinguish from other TMAs including disseminated intravascular coagulation (DIC) [31]. Possible adjuncts to the diagnosis of HIV-TTP may include measurement of complement components and regulators, proinflammatory cytokines and secreted markers of endothelial cell activation although only limited studies have evaluated these parameters in a clinical cohort [15,23,27,32–34].

Endothelial dysfunction in PWH has been extensively investigated and is linked to multiple disease processes, which can persist despite antiretroviral therapy (ART) [3,35–37]. The pathophysiological activation of complement is also well recognized in PWH [38,39]. The contribution of endothelial and complement activation and resultant cellular damage to the development of HIV-TTP is, however, unclear. Published work highlights the lack of knowledge of the pathophysiology and diagnostic ambiguity, which exist in HIV-TTP TMAs [10,15,16,20,40]. It is in this background that we detail the results of a cohort study conducted in Johannesburg, South Africa to support the hypothesis that inflammation and endothelial dysfunction contribute to the development of HIV-TTP.

Methods

Ethics approval for this investigational cohort study was obtained from the Human Research Ethics Committee of the University of the Witwatersrand (Wits) (Protocol M160134). Consecutive eligible patients ($n = 35$) presenting to the three academic hospitals affiliated to Wits namely Charlotte Maxeke Johannesburg, Chris Hani Baragwanath and Helen Joseph hospitals between January 2019 and December 2020 were invited to participate in the study and informed signed consent was obtained. The patients were diagnosed with HIV-TTP based on the combination of thrombocytopenia with a microangiopathic anaemia, that is, haemolysis [elevated lactate dehydrogenase (LDH), reduced haemoglobin and haptoglobin, with increased RBC fragments] in the absence of features of another TMA in the background of HIV infection. The results of routine investigations, demographic data, presenting signs and symptoms and the outcome of treatment were recoded. Daily therapeutic plasma exchange (TPE) (at a volume of 1 : 1 exchange) or infusion of fresh frozen plasma (FFP) (at a dose of 30 ml/kg per day) were continued until the platelet count was above 150×10^9/l for 2 days with a normalizing LDH level and decreasing RBC fragments. The antiretroviral therapy (ART) of the patient cohort was also optimized, that is, either initiated, re-introduced or altered as soon as clinically appropriate in conjunction with plasma therapy. None of the cohort of patients received additional therapy, such as rituximab or caplacizumab. In order to exclude alternative diagnoses including other TMAs, all

patients were extensively investigated for additional infectious disease processes, end organ dysfunction and comorbidities and were followed up telephonically post-discharge.

Research blood samples were collected in Becton Dickinson (BD, Plymouth, UK) vacutainers containing EDTA and trisodium citrate anticoagulant as well as in plain tubes for the generation of plasma and serum, respectively, prior to and on completion of plasma therapy. The research samples were centrifuged for 15 min at 3500*g* in the National Health Laboratory Service (NHLS) laboratory at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) to generate platelet-poor plasma and serum, which was separated into Eppendorf polypropylene tubes and stored at −80 °C. The samples were analysed within 6 months of collection for ADAMTS-13 activity and autoantibody levels, inflammatory cytokines (IL-6 and TNF-α), endothelial activation markers (sICAM and sVCAM) and the complement inhibitor, factor H (CFH), and complement components C3 and C4.

The cytokine and endothelial activation markers and CFH were analysed with Merck Milliplex reagents on a Luminex 200 (Biorad, Hercules, California, USA) ELISA platform. The cytokine markers were evaluated using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel 2 – Assay (Merck KGaA, Darmstadt, Germany). The endothelial adhesion molecule expression was evaluated using the MILLIPLEX MAP Human Cardiovascular Disease (CVD) Magnetic Bead Panel 2 – Assay (Merck KGaA). CFH was analysed with the Human Complement Magnetic Bead Panel 2 - Multiplex Assay (Merck KGaA). Briefly, plasma was thawed according to the manufacturer instructions at either room temperature or in a waterbath at 37 °C and diluted at a ratio of 1 : 100 for the endothelial adhesion molecule and 1 : 40 000 for the CFH analysis in assay buffer. The samples for cytokine analysis were processed neat without dilution as per the instructions of the manufacturer. The standard was prepared according to the instructions of the manufacturer. Twenty-five microlitres of sample, standard or control was added to a 96 well plate with 25 μl of assay buffer and 25 μl of beads. The plate was incubated at room temperature on a rotating platform for 2 h. Fifty microlitres of detection antibodies were then added and the plate was incubated at room temperature for a further hour prior to addition of streptavidin–phycoerythrin. Following a further 30 min incubation, the plate was washed three times using a handheld magnet and the sample was acquired using a Luminex 200 (Biorad) analyser.

The complement components C3 and C4 were analysed at the Immunology laboratory of the Department of Molecular Medicine and Haematology at Wits with Siemens, Healthcare GmbH, Erlangen, Germany reagent on an immunonephelometry platform. The ADAMTS-13 activity and autoantibody levels were analysed at the research laboratory in Bloemfontein, South Africa on an ELISA platform with Technozyme ADAMTS-13 activity/antigen fluorogenic kit (Technoclone, Austria) and Technozyme anti-ADAMTS-13 autoantibody kit (Technoclone, USA), respectively.

Statistical analysis

The data analysis was performed on Stata 14.2 software (StatCorp., College Station Texas, USA). A *P* value less than 0.05 was considered significant. A Wilcoxon signed-rank test was applied to demonstrate the difference between biomarker results pre-TPE and post-TPE therapy. The pre-TPE and post-TPE therapy median values were also compared with normal biomarker reference intervals in HIV-uninfected controls provided by the manufacturer.

Results

Clinical presentation and response to treatment

The median age of the patients in the TTP cohort ($n = 35$) was 35 years [interquartile range (IQR) 31–39.5] with a 2 : 1 female : male ratio. The presenting symptoms included symptomatic anaemia, bleeding, neurologic and gastrointestinal complaints with two patients complaining of atypical chest pain and TTP was an incidental finding in one patient (Table 1).

All patients in the TTP cohort were infected with HIV with a median $CD4^+$ T-cell count of 137 cells/μl (IQR 87–173) [reference interval (RI): 332–1,642 cells/μl] and a median HIV viral load of 276 500 copies/ml (IQR 52 925–894 384). TTP was the HIV index event in 21 (60%) patients with 8 patients having defaulted ART and 6 on first line ART at the time of presenting with TTP. The median $CD4^+$ T-cell count and viral load for the six patients on ART were 92 cells/μl (IQR 78–264) and 38 550 copies/ml (IQR 20 300–52 900), respectively. Only one of the patients on ART was virologically suppressed with a lower than detectable (LDL) viral load on PCR analysis but without immune reconstitution ($CD4^+$ T-cell count of 78 cells/μl). This patient had a background history of systemic lupus erythematosus (SLE) controlled with hydroxychloroquine and steroid treatment. The remaining five patients on ART did not have viral control and had variable levels of immune reconstitution with a range of $CD4^+$ T-cell counts [median $CD4^+$ T-cell count 175 cells/μl (IQR 88–261)] and a median viral load of 24 200 copies/ml (IQR 20 300–52 900). (Fig. 1).

The diagnostic presentation of HIV-TTP were reduced haemoglobin (Hb), increased red cell distribution width (RDW) with significant RBC fragments (>10 per high

Table 1. Presenting symptoms in HIV-associated thrombotic thrombocytopenic purpura cohort ($n = 35$).

Symptom		Number of patients (% of cohort)
Symptomatic anaemia (fatigue/decreased effort tolerance)		25 (71%)
Bleeding	Bruising	4 (11%)
	Epistaxis	6 (17%)
	Oral (gum bleeding)	5 (14%)
	GIT (haematemesis)	2 (5%)
	Menorrhagia	6 (17%)
	Urological	1 (3%)
	Scalp (subgaleal haematoma)	1 (3%)
Total		25 (71%)
Neurologic	Confusion	10 (28%)
	Seizures	6 (16%)
	Headache	4 (11%)
	TIA	1 (3%)
Total		21 (60%)
GIT complaints other than bleeding	Nausea/vomiting	8 (22%)
	Diarrhoea	1 (3%)
	Epigastric pain	1 (3%)
Total		10 (28%)
Atypical chest pain		2 (6%)
Incidental finding		1 (3%)

GIT, gastrointestinal track; TIA, transient ischaemic attack.

power field at presentation in all but two patients in whom fragments developed within 24 h), thrombocytopenia, and elevated lactate dehydrogenase (LDH) with significant improvement ($P < 0.001$) with plasma therapy (Table 2). The reticulocyte count was available in 30 patients in the cohort with median result of 11.76% (IQR 7.655–16.23) ((RI: 0.50–2.00%).

The VWF antigen levels were increased at presentation and decreased with therapy ($P < 0.001$) (Table 2). The PLASMIC [Platelet count, Haemolysis, Active cancer, MCV (mean RBC volume), International normalised ratio (INR) and Creatinine] scores for the prediction of the likelihood of severely reduced ADAMTS-13 activity were at least 6 (high risk) and 5 (intermediate risk) in 32 (91%) and three (9%) of the patients, respectively (Table 2). The ADAMTS-13 levels, which were subsequently available were less than 15%, that is, severe deficiency with associated significant ADAMTS-13 autoantibody levels in all patients ($n = 35$) with improvement post treatment ($P < 0.001$) (Table 2).

The aspartate transaminase (AST) and unconjugated bilirubin results were increased in all 35 patients [median AST 108 U/l (IQR 81–161) (RI: 13–35 U/l) and median unconjugated bilirubin 32 μmol/l (IQR 21–48)

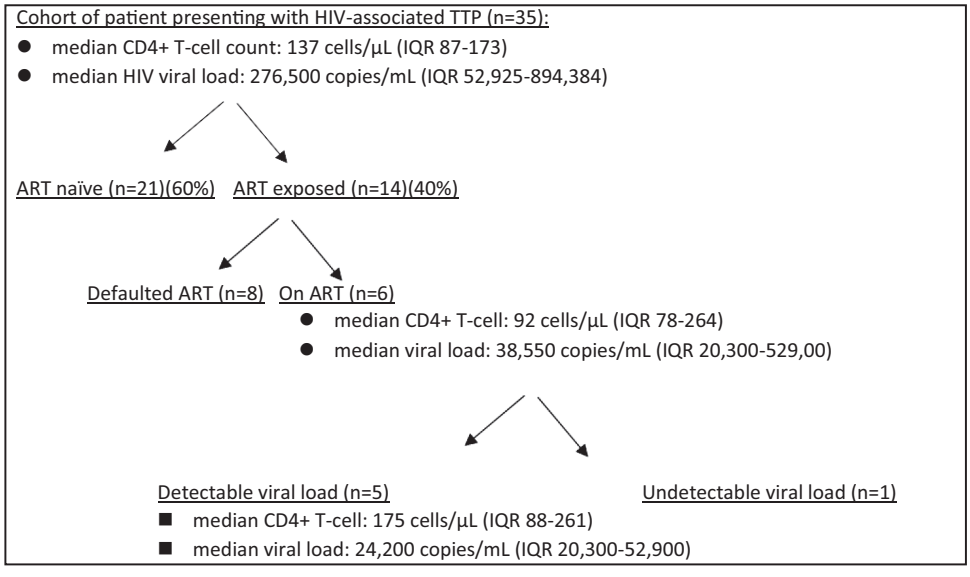

Fig. 1. Antiretroviral treatment history, $CD4^+$ T-lymphocyte count and HIV viral loads in HIV-associated thrombotic thrombocytopenic purpura cohort ($n = 35$).

Table 2. Cumulative results of the routine and ADAMTS-13 activity and autoantibody results in the HIV-associated thrombotic thrombocytopenic purpura cohort of patients ($n = 35$).

Parameter (RI)	Median result before plasma therapy (IQR)	Median result post plasma therapy (IQR)	*P* value
Haemoglobin (Hb) (11.6–16.4 g/dl)	6.0 (5–7.7)	10.2 (9.1–10.6)	<0.001
RDW (12.4–17.3%)	29.3 (24.6–32.5)	19.4 (18.1–21.9)	<0.001
Platelets count (186–454 $\times 10^9$/l)	7 (5–12)	225 (182–283)	<0.001
LDH (100–150 U/l)	1 638 (1282.5–2 217)	283 (253.5–312)	<0.001
VWF Ag (50–160%)	353 (275–420)	182 (118.5–230.5)	<0.001
ADAMTS13 activity (>50%)	0 (0–1)	41 (29–65.5)	<0.001
ADAMTS13 autoantibodies (<15 IU/ml)	58 (34.5–75)	12 (7.5–17.5)	<0.001

ADAMTS-13, a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13; IQR, 25–75 interquartile range; LDH, lactate dehydrogenase; RDW, red cell distribution width; RI, normal reference interval; VWF Ag, von Willebrand Factor antigen.

RI: 12–19 μmol/l]. Additional markers of haemolysis included an undetectable haptoglobin level in 31 (88% of the cohort) and a negative Coombs test in 26 (74% of the cohort) patients. The D-dimer level was elevated in all patients with a median result of 8.75 mg/l (IQR 6.52–14.55) (RI: <0.25 mg/dl) and the renal function was preserved or mildly deranged with a median creatinine concentration of 94 μmol/l (IQR 87.5–110) (RI: 49–90 μmol/l).

TPE was commenced in 32 (91%) of the patients with 3 (9%) only receiving plasma infusion. Plasma therapy was required for a median of 10 days (IQR 7–13). Six patients did not receive steroid therapy and had a poor response to daily TPE or suffered early TTP relapse with improved outcomes after initiation of concomitant steroid therapy. One patient, however, responded to TPE and ART without concomitant steroid therapy and remained in remission. The ART of the TTP cohort of patients was optimized with blood product transfusions and additional best supportive care including antibiotics as required.

All 35 patients in the cohort achieved remission and was discharged to outpatient specialist care and also followed up telephonically. Three patients, including a patient with concomitant coronavirus disease 2019 (COVID-19), demised within 4 months of discharge after defaulting ART with symptoms compatible with relapsed TTP. Three patients were lost to follow-up and the remaining 30 (85%) patients were well on ART at 6–24 months of out-patient follow-up.

Associated underlying conditions

The median C-reactive protein (CRP) level in the cohort was 14 mg/l (IQR 10–35) (RI: <10 mg/l). Blood cultures were performed on admission in 31 (88%) with negative results in all cases. Cerebrospinal fluid was examined in one patient with negative bacterial and fungal culture. Examination for malaria was negative in all five patients in whom this test was performed and COVID-19 PCR tests were performed in eight patients with one positive result. The patient with COVID-19 had the highest CRP of 169 mg/l and four of the remaining patients with elevated CRP results were diagnosed with underlying infection with *Mycobacterium tuberculosis*.

Routine serology testing revealed previous hepatitis B infection in two patients and chronic hepatitis C in one patient but the viral load was undetectable. Eighteen patients had IgG antibodies against Epstein–Barr virus and cytomegalovirus, although viral loads were not performed and it was not possible to exclude acute infection although the IgM results for these viral pathogens were negative. Markers for autoimmune diseases were performed in 20 patients with one patient meeting the diagnostic criteria for systemic lupus erythematosus (SLE).

Complement levels

The plasma level of CFH was elevated at presentation [median result 0.626 mg/ml (IQR 0.505–0.849) (RI: 0.237–0.426 mg/ml) and decreased to a median result of 0.444 mg/ml (IQR 0.306–0.512) on completion of plasma therapy. The complement components C3 and C4 results were at the lower limit of the RI [C3, median 1.12 g/l (IQR 0.915–1.27) (RI: 0.90–1.80 g/l) and C4, median 0.1875 g/l (IQR 0.143–0.269) (RI: 0.10–0.40 g/l)] at presentation. These components increased to a median of 1.38 g/l (IQR 1.22–1.43) and 0.2725 g/l (IQR 0.223–0.357) for C3 and C4, respectively.

Markers of endothelial cell activation and proinflammatory cytokines

The inflammatory cytokines, IL-6 and TNF-α, and the endothelial activation markers, sICAM and sVCAM, were significantly ($P < 0.001$) elevated above the normal reference interval of the assay in the cohort of patients at presentation with a trend towards normal after plasma therapy and the change was also significant ($P < 0.001$) (Table 3 and Fig. 2).

Discussion

This study aimed to investigate inflammation and associated endothelial dysfunction and complement

Table 3. Inflammatory cytokine and endothelial activation markers pre-plasma and post-plasma therapy in the HIV-associated thrombotic thrombocytopenic purpura cohort ($n = 35$).

	Interleukin-6 (IL-6)		Tumour necrosis factor-α (TNF-α)		Soluble intracellular adhesion molecule (sICAM)		Soluble vascular adhesion molecule (sVCAM)	
RI	<7 pg/ml		<27 pg/ml		325–447 ng/ml		328–792 ng/ml	
	Pre-TPE	Post-TPE	Pre-TPE	Post-TPE	Pre-TPE	Post-TPE	Pre-TPE	Post-TPE
TTP cohort	43	8.4	116	29.9	1417	453	1643	897
Median (IQR)	(41–85)	(5–14)	(79.9–152)	(17.2–42.3)	(986–2,141)	(287–605)	(1,022–1,977)	(810–1,194)
P value (pre-TPE vs. post-TPE)	<0.001 Pre-TPE > post-TPE		<0.001 Pre-TPE > post-TPE		<0.001 Pre-TPE > post-TPE		<0.001 Pre TPE > post-TPE	

IQR, 25–75 interquartile range; RI, normal reference interval; TPE, therapeutic plasma exchange; TTP, thrombotic thrombocytopenic purpura.

activation in South African patients with HIV-TTP to support the hypothesis that these processes contribute to the development of this TMA. Although TTP does occur in HIV-uninfected patients, HIV infection is the most common cause of acquired TTP in South Africa [17–19,22]. HIV-TTP remains a significant cause of morbidity and mortality in South Africa [16–19,21,22,41], although the incidence has dramatically decreased in other international centres following the availability of ART [12,42–44]. This difference may reflect reduced access to ART [1] but HIV-TTP is reported in both ART-naive and treated patients in South Africa and even in patients with virological suppression as also described in one patient in the current study [17–19].

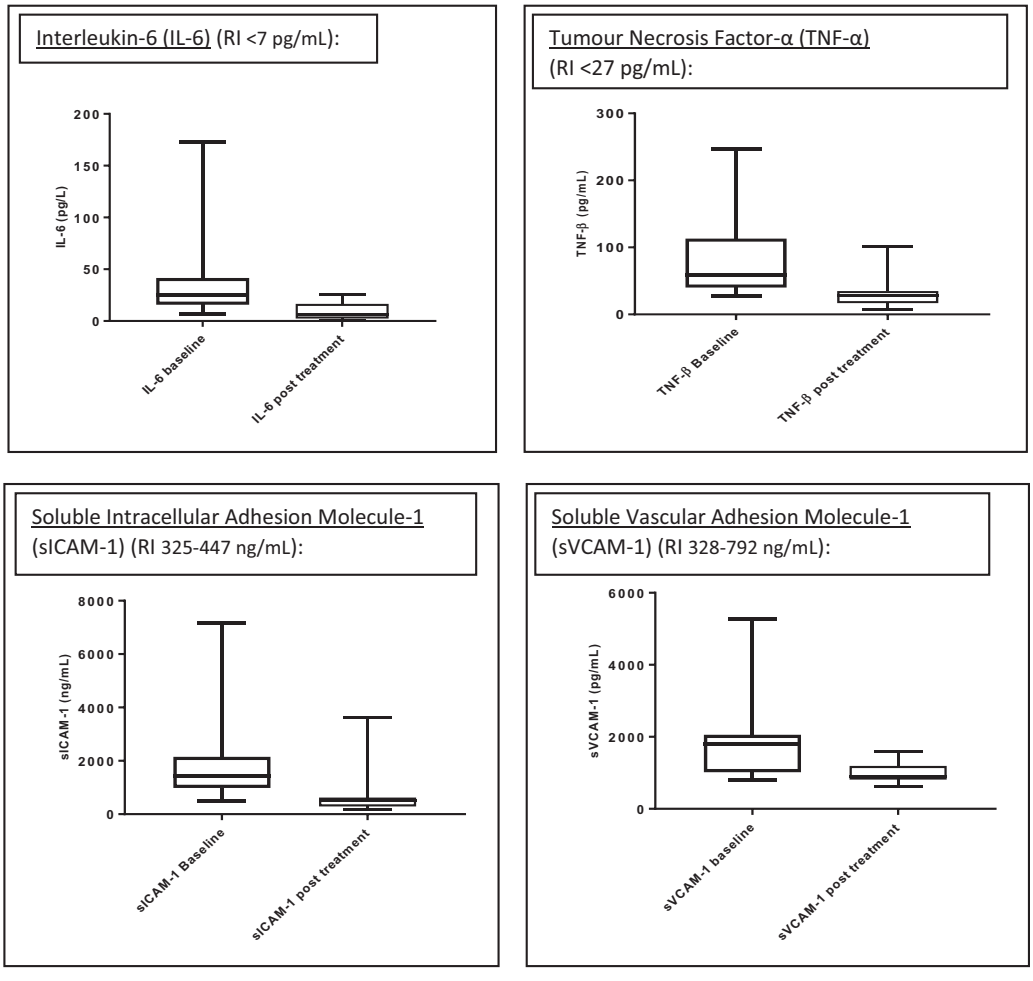

Fig. 2. Boxplots of the study parameters in the HIV-associated thrombotic thrombocytopenic purpura cohort ($n = 35$) at baseline and on completion of plasma therapy representing medians, interquartile ranges and minimum/maximum values. RI, reference interval.

Relapse appears more common with loss of virologic control though [10,19,22,45,46]. On follow-up, family members reported the death of three patients in this cohort who defaulted ART with symptoms suggestive of relapsed TTP. The reason for the lack of viral control and incomplete immune reconstitution in five of the patients on ART in this cohort was not clear as no drug resistance viral sequencing was performed. Poor compliance on drug regimens and recent ART initiation may have been contributory. The poor viral control despite ART probably played a role in the development of HIV-TTP. The patient on ART who achieved virological control without immune reconstitution was on immunosuppressive therapy for underlying SLE, which could possibly explain this finding.

HIV-TTP syndrome was characterized by large numbers of schistocytes, high levels of the enzyme LDH and a marked thrombocytopaenia. The levels of ADAMTS-13 were consistently markedly reduced and patients had ADAMTS-13 autoantibodies. Variably reduced levels of ADAMTS-13 activity has been documented in the literature [16,41,47,48], and it is therefore, not clear whether a deficiency in this VWF cleaving protease is the pivotal initiating event in the development of HIV-TTP or whether the severe reduction in the current study reflects acute consumption. ADAMTS-13 autoantibodies are not detectable in all patients described with HIV-TTP [10,16,41,46,47]. Although antibodies were present in all patients in the current cohort, it was not possible to determine their clinical relevance. It has been postulated that the antibodies in HIV may be transient reflecting background immunological activation and it is postulated that a second insult may, therefore, be required for phenotypic expression of HIV-TTP [10,16,44]. Complement activation and associated endothelial dysfunction may be pivotal in this process [10,43,49].

We hypothesized that HIV-TTP is a disorder of complement regulation constituting a TTP-like syndrome. We report an unexpectedly significantly elevated level of the complement regulator, CFH. This may reflect activation of compensatory anti-inflammatory biological pathways. C3 and C4 complement levels were low and increased following therapy. Ongoing investigation of the direct effect of complement on endothelial cells in patients with HIV-TTP is required. Complement is associated with the development of TMA [24,25], is activated in HIV [38,39,50], and is therefore, an attractive potential pathophysiological trigger of HIV-TTP.

Patients in this cohort showed markedly elevated levels of pro-inflammatory cytokines compared with reference intervals in HIV-uninfected populations. The levels of these biomarkers were also elevated when compared with the published levels in PWH without TTP [51–61]. Elevated IL-6 levels occurs in uncontrolled HIV infection and correlate with mortality [62,63]. This pleiotropic cytokine has multiple effects on the coagulation system including platelet activation, regulation of lipid mediators of inflammation, upregulation of adhesion molecules and leukocyte recruitment [64]. TNF-α, a cytokine with similar proinflammatory functions [65], was also increased in the study cohort patients. Importantly, levels of these cytokines declined with plasma therapy. It was unclear whether these factors were prognostic in the patients and this will require further longitudinal data. The therapeutic efficacy of immunosuppression with steroids was demonstrated by the poor response to TPE observed in seven patients who did not receive concomitant steroids. The significantly increased levels of inflammatory cytokines detected in the HIV-TTP cohort at presentation also supports steroid therapy in patients with HIV-TTP.

Endothelial dysfunction and damage related to direct effects of the HIV, persistent inflammation, complement activation opportunistic infections and drug side-effects have been postulated to contribute to HIV-TTP [43,47,49,66]. Endothelial activation markers, sICAM and sVCAM, were markedly increased in the current cohort of patents. These cellular adhesion molecules promote leukocyte adhesion to the endothelium [67]. Levels have been measured in PWH with and without cardiovascular disease and have been examined as a potential biomarker for arterial disease [35,68]. This is the first study, which looks at these markers in patients with HIV-TTP. The elevated D-dimers were also documented in previous cohorts of patients [17,66] with HIV-TTP and probably signals local activation of the coagulation cascade secondary to endothelial damage and inflammation.

Increased levels of cytokines [33], and evidence of endothelial activation and remodelling [34,69] have been previously documented in HIV-uninfected patients with idiopathic TTP and in TTP animal models. Shariatmadar *et al.*[33] demonstrated that cytokines, notably interleukin-8 (IL-8) and TNF-α, were elevated in patients with idiopathic TTP and postulated a pathogenic role of cytokines, involving tissue damage, in the development of TTP. Endothelial dysfunction and remodelling were prospectively studied by Widermann *et al.* [34] in patients with idiopathic TTP and concluded that the profile of circulating endothelial markers were compatible with significant endothelial activation and repair. The pathogenic mechanisms of cytokine activation and endothelial damage postulated in the current study are, therefore, not unique to patients with HIV-TTP but the extent of derangement and repair capacity in HIV-infected patients might be significantly different requiring further investigation.

This study has some limitations. A major limitation is the lack of HIV-infected controls without TTP although the study patients served as their own controls pre-plasma and post-plasma therapy. Given the neurological impact of

TTP, the patients were not necessarily accurate historians. The history of ART exposure was determined from family members and hospital records, although it was not possible to always assess whether patients received and were compliant on ART. Longitudinal repeat out-patient measurements were not available. Follow-up was telephonic and it was not possible to confirm that the three deaths was linked to TTP relapse. Some patients were lost to follow-up. Finally, not all patients were extensively investigated for opportunistic viral and mycobacterial infections. We, however, propose that the pathophysiology of HIV-TTP is multifactorial with activation of the complement system, inflammation and endotheliitis and is more similar to the TTP-like syndrome [23]. Although the levels of the complement components C3 and C4 in this study were within the normal RI, they were at the lower end of the range and increased with plasma therapy. The level of CFH was elevated at presentation and decreased after therapy and the significance of this finding requires further investigation. Future work will examine the direct effect of these different factors on endothelial cells in patients with HIV-TTP and the endothelial repair capacity in HIV-infected patients.

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Author contributions: S.L. – study design, sample collection and analysis, data collection and analysis, manuscript writing and approval of submission. M.P.G. – sample analysis, manuscript writing and critical review and approval of submission. A.L.M. – statistical analysis, manuscript writing and critical review and approval of submission. E.S.M. – study design, data collection and analysis, manuscript writing and critical review and approval of submission.

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Conflicts of interest

There are no conflicts of interest.

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Erratum:

A typographical error occurred as only 2 patients were lost to follow-up. The last paragraph on page 1349 of the 'Results section: Clinical presentation and response to treatment' of the article should therefore read as follows:

Three patients, including a patient with concomitant coronavirus disease 2019 (COVID-19), demised within 4 months of discharge after defaulting ART with symptoms compatible with relapsed TTP. Two patients were lost to follow-up and the remaining 30 (85%) patients were well on ART at 6-24 months of out-patient follow-up.

Manuscript 4: Selected inflammatory and coagulation biomarkers pre-viral suppression in people with Human immunodeficiency virus (HIV) infection (article in submissible format)

Levels of the selected laboratory markers of inflammation (tumour necrosis factor-α [TNF-α] and interleukin-6 [IL 6]), endothelial (intra-cellular and vascular cell adhesion molecules [sICAM-1 and sVCAM-1] as well as the coagulation system (D-dimers) were elevated in patients with HIV-TTP (Publication 3). To understand these findings, a systematic review of levels of these biomarkers in ART-naïve people living with HIV (PLWH) without comorbid diseases was undertaken. Forty-six studies were included for review over the study period (1994-2020). Significant heterogeneity was present with respect to method of analysis, number of participants and laboratory testing assays utilised. Forest plots were constructed to reflect the inter-assay heterogeneity and medians were weighted by study participant numbers. Of the markers measured, only D-dimer and more recently IL 6, are available as routine diagnostic assays in clinical laboratories with clearly defined normal reference indices (RIs). For all other assays, the reference interval was derived from that reported in the assay package insert. The median values in PLWH were above the upper limit of the assay normal cut-off for D-dimers in 5/15 studies reviewed, for IL-6 in 8/16 studies, for TNF-α in 5/5 studies, for sVCAM-1 in 3/6 studies and for sICAM-1 in 4/5 studies. Of interest, the elevation of these parameters in the HIV-TTP patients investigated and described in Publication 3 was statistically higher than published ranges detected in the reviewed studies in PLWH. This provides evidence that these biomarkers may show prognostic or diagnostic significance in HIV-TTP and may in future be utilised to guide therapeutic decisions.

Contribution of candidate:

The candidate defined the Medical Subject Heading (MESH) terms and selected the studies for both inclusion and exclusion. The candidate further reviewed the included studies, extracted the required data and performed the statistical analysis under the guidance of a co-author, ALM. The candidate was responsible for compiling the manuscript with assistance from co-authors BJ, ALM and ESM.

Selected inflammatory and coagulation biomarkers pre-viral suppression in people with Human immunodeficiency virus (HIV) infection

Abstract

Background

Morbidity and mortality related to infection with human immunodeficiency virus (HIV) is relatively common in Africa despite antiretroviral therapy (ART) with a prominence of non-communicable diseases such as cardiovascular diseases (CVD) with thromboses throughout the vascular system. Ongoing inflammation and endothelial dysfunction in people living with HIV (PLWH) probably contribute significantly to HIV-related CVD.

Objectives

A systematic review was conducted to inform interpretation of 5 biomarkers commonly measured in PLWH namely interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α), D-dimer, and intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) to attempt to define a range for these values in ART naïve PLWH without overt cardiovascular disease or additional comorbid disease.

Methods

A systematic search was conducted for all studies documenting the levels of the above biomarkers in ART naïve PLWH published on the PubMed database from 1994 to 2020.

Results

The number of publications that reported medians above the assay values were: 4/15 for D-dimer, 0/5 for TNF-α, 8/16 for IL-6, 3/6 for VCAM-1 and 4/5 ICAM.

Conclusion

The clinical utility of biomarkers is reduced by the lack of standardisation of the measurement of these parameters, absence of normal reference indices and the lack of uniformity of study protocols in different research centres. This review supports the ongoing use of D-dimers to predict thrombotic and bleeding events in PLWH since the weighted averages across assays suggest that the median levels do not exceed the reference range. The role of inflammatory

cytokine monitoring and measurement of endothelial adhesion markers is less clear although more recently, we have demonstrated significantly increased levels in HIV-associated TTP which is a documented thrombotic microangiopathic complication of HIV disease.

Selected inflammatory and coagulation biomarkers pre-viral suppression in people with Human immunodeficiency virus (HIV) infection

Introduction:

HIV infection remains a significant cause of morbidity and mortality in Africa, despite extensive antiretroviral therapy (ART) implementation with an estimated 300 000 people dying from AIDS-related illnesses in 2019 in the most affected regions of the continent.[1] Opportunistic infections still occur but the focus has shifted towards managing non-communicable complications of HIV infection.[2] The SMART (Strategic Management of Antiretroviral Therapy) study revealed that, in people living with HIV (PLWH) where treatment was interrupted, all-cause mortality increased and that cardiovascular disease (CVD) was the commonest cause of death in these patients. Two parameters, interleukin-6 (IL-6) and D-dimers, were most predictive of the development of CVD.[3] Subsequently, multiple studies have identified biomarkers which could indicate CVD risk in people living with HIV.[4-7] Although the pathogenesis of HIV-related CVD is incompletely understood, it is hypothesised that chronic immune system activation with resultant inflammation[8, 9] and endothelial dysfunction[10, 11] are likely to be contributory.

The cause of the inflammation and immune activation in both ART-naïve and treated PLWH infection, relates directly to low-grade ongoing HIV replication, increased levels of modified lipids such as oxidized low-density lipoprotein (oxLDL) effecting monocyte activation, concomitant infections with, for example, Hepatitis B-, C- and cytomegalovirus resulting in activation of cytotoxic $CD8^+$ T-lymphocytes and translocation of microbial products across a compromised gastrointestinal mucosal barrier.[8, 12] Pro-inflammatory cytokines, notably IL-6 and tumour necrosis factor-α (TNF-α), produced by activated lymphocytes induce the upregulation of endothelial transmembrane adhesion molecules including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). These endothelial adhesion molecules facilitate the transmigration of leukocytes, including neutrophils and monocytes, across the endothelial lining into underlying tissues.[8] Various treatment strategies have been explored to treat the chronic inflammation and resultant endothelial dysfunction in PLWH with the aim of improving patient outcomes.[7] Clinically, endothelial dysfunction and activation of the coagulation system in PLWH manifest as arterial disease (ischaemic heart disease, peripheral vascular disease and stroke), venous thromboembolic disease (VTED) and microcirculatory disorders like HIV-related thrombotic thrombocytopaenic purpura (HIV-TTP), a frequently encountered thrombotic microangiopathy (TMA) in the local African population.[7, 11, 13-15]

Inflammatory cytokines

IL-6 and TNF-α are two of the most extensively studied proinflammatory cytokines in PLWH.[16, 17] IL-6 is produced by leukocytes but also by non-immune tissues including endo- and epithelial cells, hepatocytes and osteoclasts.[18] Acute transient expression of IL-6 is a key component of an acute-phase response. Dysregulated, chronic production of IL-6 is pathophysiologic in various illnesses including autoimmune conditions like rheumatoid arthritis.[18] This inflammatory cytokine facilitates recruitment of monocytes and lymphocytes, activates endothelial cells and inhibits

T-cell apoptosis and T-regulatory cell differentiation.[18] TNF-α is produced by macrophages, lymphocytes and endothelial cells amongst others and controls immune functions and tissue homeostasis. The 2 receptors for TNF (TNFR) have distinct patterns of cellular expression with TNFR-1 being ubiquitous while TNFR-2 expression is restricted to specific cell types including endothelial and immune cells. TNFR-1 primarily promotes inflammation and tissue degeneration, whereas TNFR-2 mediates homeostasis, cell survival and tissue regeneration. The action of TNF effects both cell survival, homeostasis and regeneration but also inflammation and cell death. Although all cytokines can have pleiotropic functions, TNF and IL-6 have predominantly pro-inflammatory effects contributing to the chronic inflammatory state in PLWH.[19]

Fibrin degradation products (D-dimers)

D-dimers are the products of degradation of cross-linked fibrin by plasmin during blood clot breakdown (fibrinolysis). D-dimer levels rise with both bleeding and subsequent clotting as well as pathological thromboses and have a high negative predictive value for venous thromboembolic events (VTE).[20] PLWH are at an increased risk of thrombotic events in all compartments of the vascular tree (arteries, veins and the microcirculation) and D-dimers are prognostic of mortality and often remain elevated despite ART.[13]

Adhesion molecules

ICAM-1 and VCAM-1 are inducible cell surface glycoproteins expressed by activated endothelial cells in response to inflammation. They regulate leukocyte recruitment from the circulation.[21] Soluble forms of these markers (sICAM-1 and sVCAM-1) may be increased in PLWH reflecting underlying endothelial dysfunction.[10, 11]

These different biomarkers have been measured in heterogeneous cohorts of PLWH, both ART treated and untreated, utilising different reagents or kits, different reportable ranges and different analytical techniques.[11, 22] This can make evaluation and comparison between different studies difficult. Many of these markers are measured as research applications and reference ranges are not available even in HIV-uninfected controls.

Attempts have been made to develop predictive CVD risk scores by correlating the various markers with both clinical presentation and with other manifestations of arterial disease including carotid intimal thickness and flow-mediated dilation in different clinical cohorts of PLWH.[7, 23-26] The use of control populations and reference indices in these studies are, however, poorly standardised with inclusion of healthy controls and ART naïve as well as ART-treated participants, with differing levels of virological control and immunological reconstitution. Many of the biomarkers are not routine laboratory tests and validation of appropriate reference indices and standardization of test methodology and protocols have not been consistently performed.[8, 10] This makes comparison between studies detailing biomarker levels difficult and may limit the development of clinical guidelines.

We conducted a systematic review to inform interpretation of 5 biomarkers commonly measured in PLWH namely IL-6, TNF-α, D-dimers, and sVCAM-1 and sICAM-1 to attempt to define a range for these values in ART-naïve PLWH.

Methodology:

A systematic search was conducted for all studies documenting the levels of the above biomarkers in ART naïve PLWH published on the PubMed database from 1994 to 2020. The following MESH terms were utilised:

1. HIV and Interleukin 6 (IL-6)
2. HIV and Tumour necrosis factor alpha (TNF-α)
3. HIV and D-dimers
4. HIV and soluble vascular adhesion molecules (sVCAM and sICAM respectively)

Study selection

Over the period (1994-2020), 229 papers were reviewed and 183 studies were excluded (Figure 1). In short, all clinical studies published in English and which reported the results of the selected biomarkers in cohorts of ART-naïve PLWH were included in the review. Meta-analyses, reviews, case studies on a single subject and cohorts consisting of fewer than 30 individuals, due the statistically insignificant contribution to the median results, and pre-clinical or animal studies were excluded. Abstracts from conference proceedings as well as grey literature were also excluded. The results of subgroups within the studies were selected based on the absence of comorbidities or experimental treatment interventions. The selected studies were also scanned for potential duplicate data publications.

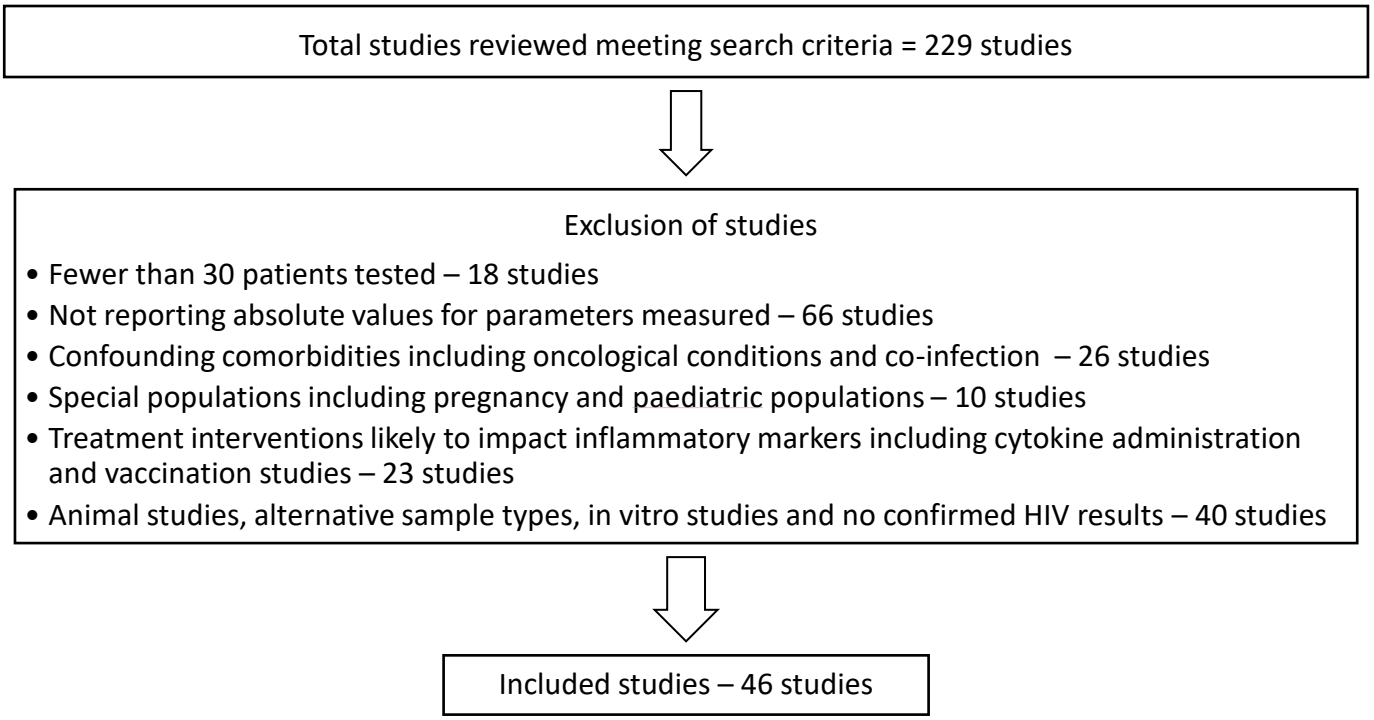


Figure 1: Review methodology of published studies reporting inflammatory biomarkers and markers of endothelial and coagulation system activation in PLWH.

Statistical analysis

Data values at 25th, 50th and 75th percentiles were recorded in Microsoft Excel® 2016 (Table 1) and formally analysed with STATA® 15 (StataCorp, College Station, Texas software). Forest plots depicting the recorded values for medians and error bars at 25th and 75th percentiles were constructed for the five biomarkers. Values in the forest plots were weighted by participant numbers and assay used. P-values for chi-square tests were based on the difference between 75th percentiles and expected values, and corresponding inverse values were disclosed for Cochrane i^2 values.[27] A p-value <0.05 and i^2>90% indicated heterogeneity in a forest plot.

Results:

Biomarkers in the reviewed studies were measured at baseline as indicators of inflammatory status in patients immediately prior to ART initiation. In none of the participants were any of these parameters utilised in diagnosis of established, overt cardiovascular disease although, in a few, an attempt was made to correlate the biomarkers with subclinical arterial disease. Forest plots for D-dimers and IL-6, with values of p=0.9272 and I^2<20% were homogeneous (Figure 2 and 3), and TNF-α, sVCAM-1 and sICAM-1 (Figures 3 and 4) with values of p=0.0001 and I^2>90% were heterogeneous. Because all forest plots are univariate and the majority are heterogeneous, a fixed effects model was used. Analysis revealed that for D-dimers, 5/15 papers showed medians above assay values; for TNF-α, 5/5 papers showed medians above assay values; for IL-6, 8/16 papers showed medians above assay values; for sVCAM-1, 3/6 papers showed medians above assay values and for sICAM-1, 4/5 papers showed values above assay values.

Table 1: Published biomarker results in anti-retroviral treatment (ART) naïve people living with human immunodeficiency virus (PLWH).

		D-dimers	IL-6	TNF-α	s-VCAM	s-ICAM
Number of studies		15	16	5	6	5
Total number of study participants		4 318	4 769	731	2 412	2 377
Reagent used (number of participants)						
	Quantikine HS; R&D Systems®		12 (3 873)	3 (400)	4 (332)	3 (297)
	Meso Scale, Discovery®		4 (896)	2 (331)	2 (2 080)	2 (2 080)
	*Additional D-dimer assays	9 (3 483)				
	Liatest D-dimers, STAGO Diagnostica®	6 (835)				
	Reagent kit not specified	2	3	0	1	0
Reported normal cut off in healthy controls		≤0.5 mg/ml *See below	Quantikine® ≤1.77 pg/mL Meso Scale® ≤1.8 pg/mL	Quantikine® ≤ 1.66 pg/mL Meso Scale® ≤4.64 pg/mL	R&D Systems® ≤ 1012 ng/mL Meso Scale® ≤ 562 ng/mL	R&D Systems® ≤ 205 ng/mL Meso Scale® ≤ 355 ng/mL
Weighted median		0.43	1.92	12.58	744	516
Weighted 25th centile		0.27	1.21	9.38	578	402
Weighted 75th centile		0.71	3.20	16.47	963	659

*biomerieux VIDAS® / Dade Behring Innovance® / Roche COBAS Integra 400 plus® / Siemens Innovance® / HemosIL TM Dimertest®.

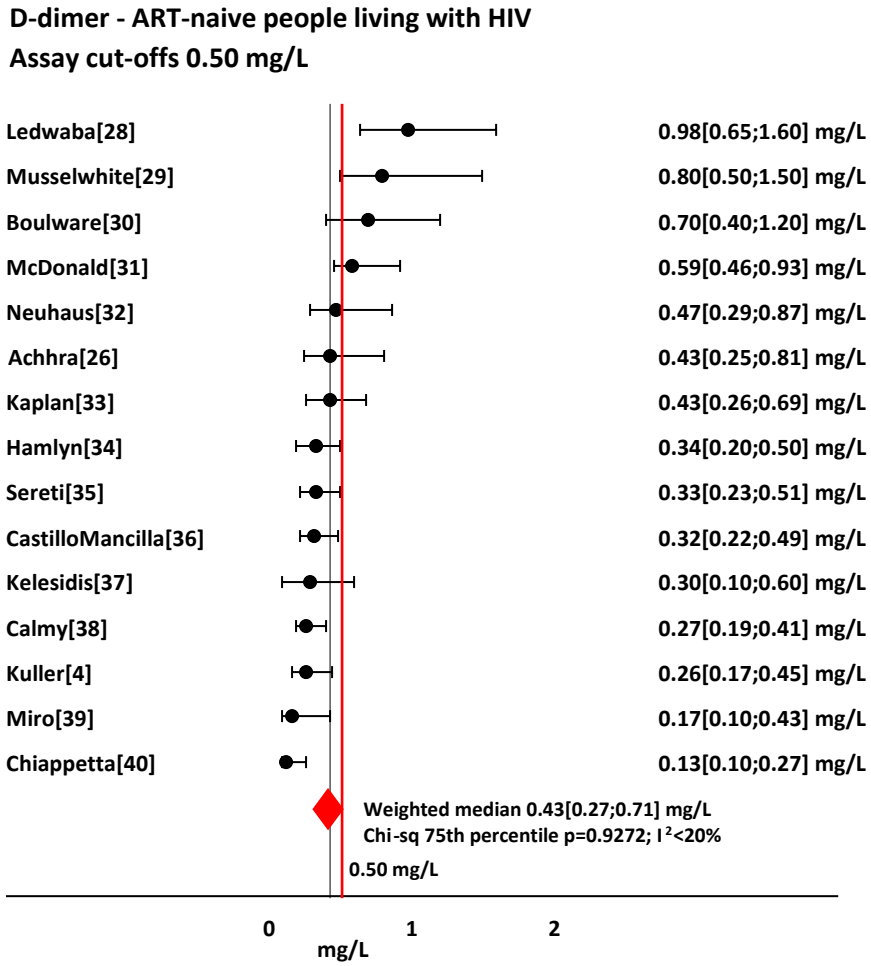
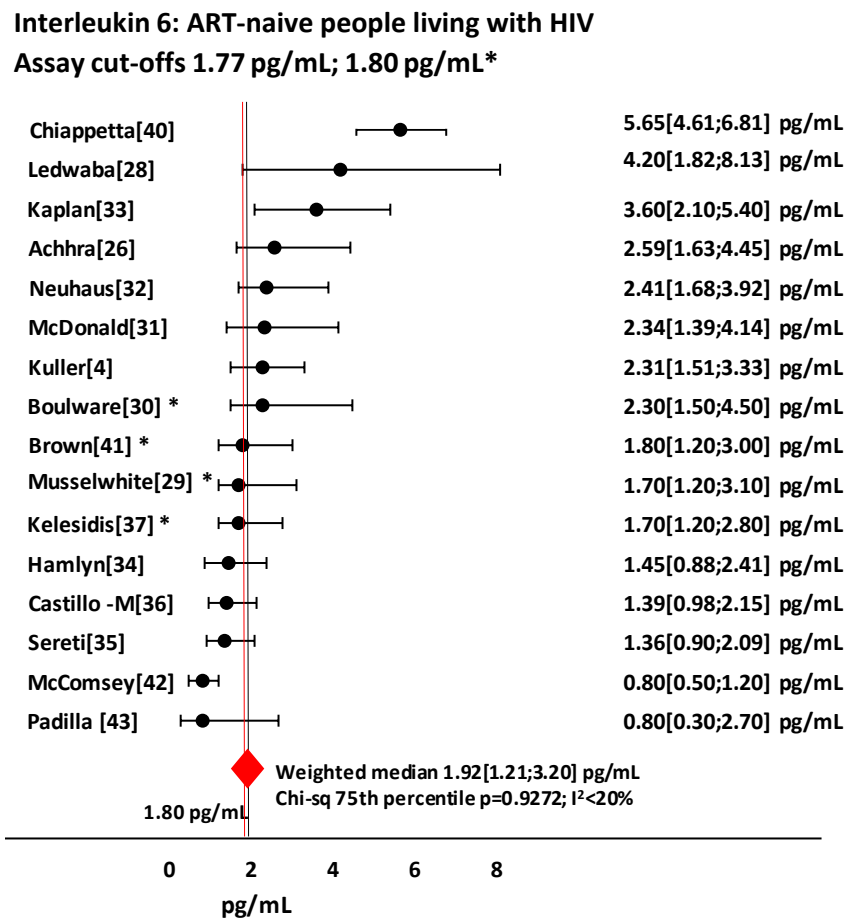


Figure 2: Fibrinolytic marker (D-dimers) in ART naïve PLWH.



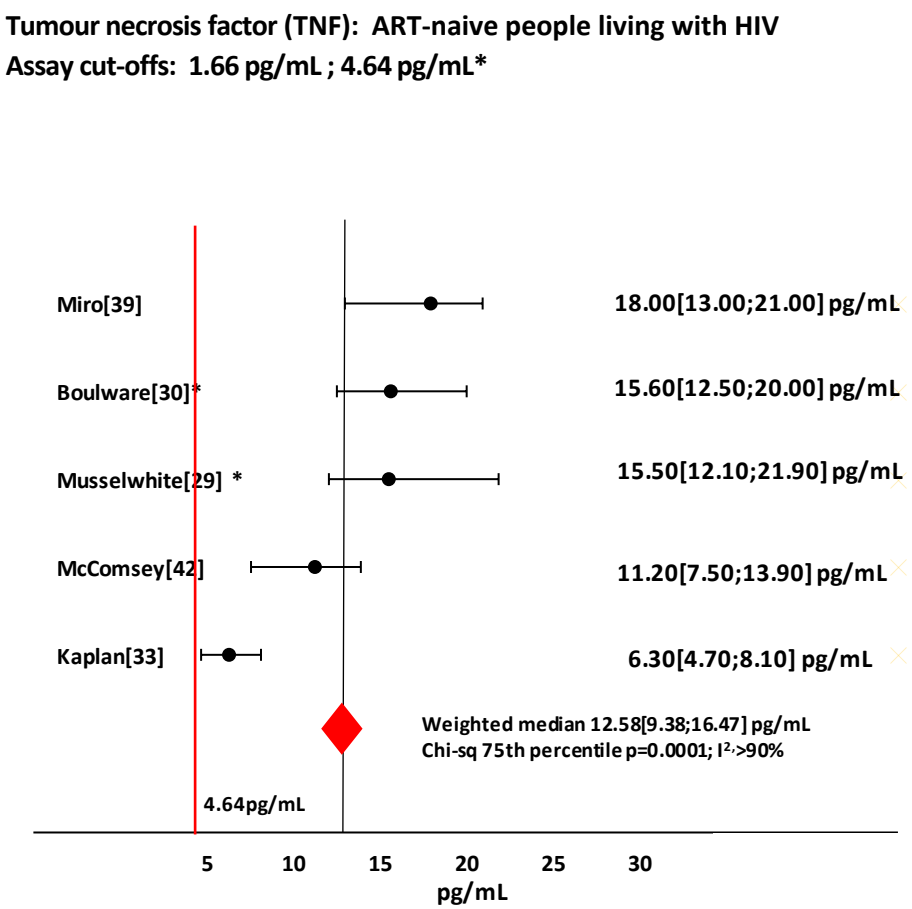
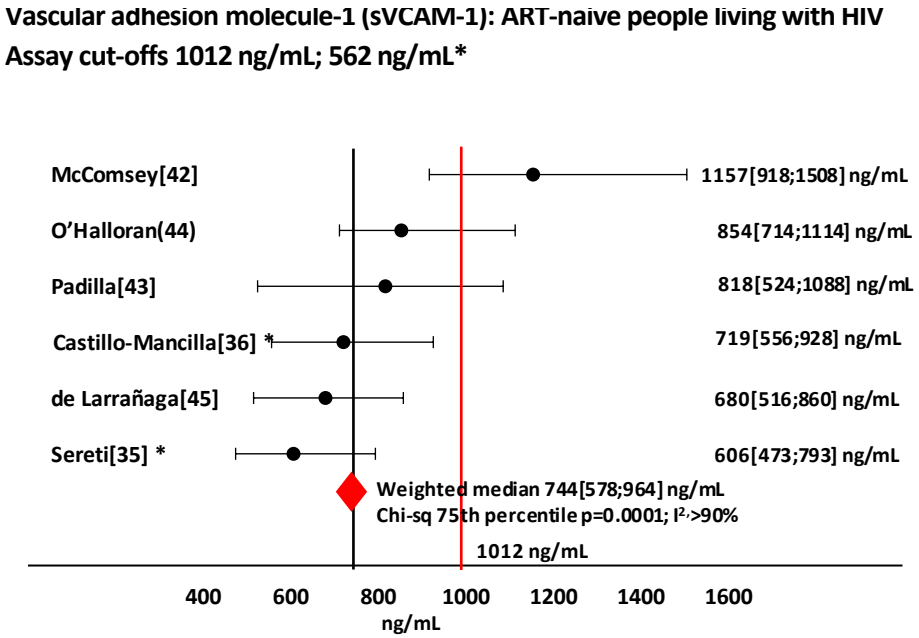


Figure 3: Inflammatory cytokines (IL-6 and TNF-α) in ART-naïve PLWH.



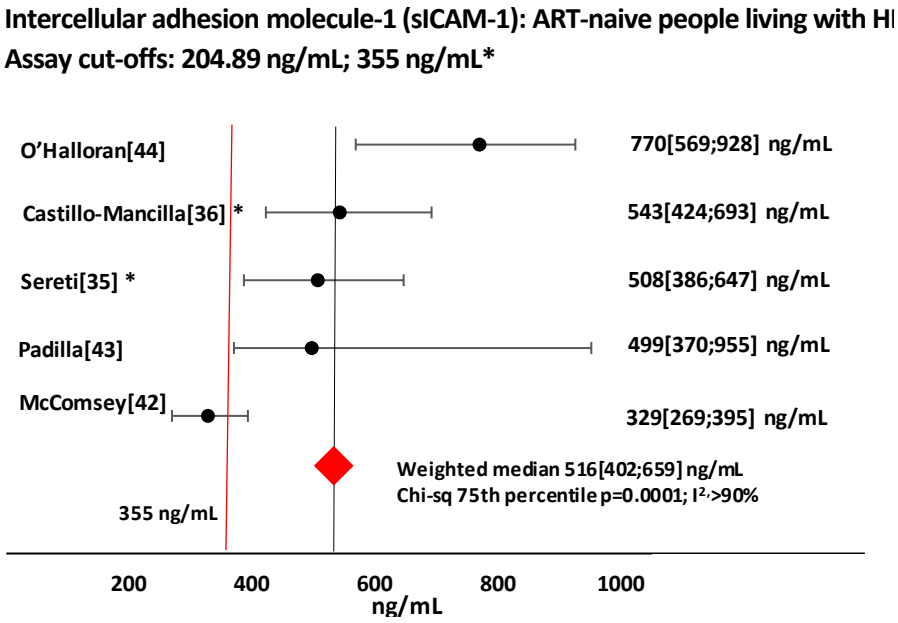


Figure 4: Adhesion molecules (sVCAM and sICAM) in ART naïve PLWH.

Discussion

Inflammation and associated endothelial dysfunction are hallmarks of infection with HIV and do not fully resolve even with early and sustained ART.[8, 10, 11] Biomarkers assist to identify subclinical diseases in at-risk populations to allow appropriate prophylaxis and therapy and may partially clarify underlying pathogenesis of comorbidities such as HIV-related CVD.[7, 22, 46] The clinical utility of biomarkers is, however, reduced by the lack of standardisation of the measurement of these parameters, absence of normal reference indices and the lack of uniformity of study protocols in different research centres.[22, 47] Despite these limitations, elevation of biomarkers denoting inflammation, endothelial as well as coagulation system activation in PLWH, have been extensively documented in the medical literature and predict all-cause mortality.[4, 7, 8, 10] The D-dimer assay is a routine laboratory test with well-established automated methodologies and reference indices. In addition, D-dimer levels are widely used for their negative predictive value to rule out venous thromboembolism.[20] Other biomarker assays, including IL-6, TNF-α and the vascular adhesion molecules, are either not offered by laboratories or are only performed in research laboratories. IL-6 assays are currently offered more widely since the advent of the COVID-19 pandemic since it has utility for prognostication in patients suffering from this viral infection.[48]

The link between various secondary diseases and biomarker derangement has been documented in multiple HIV infected cohorts.[7, 22] Prediction and detection of cardiovascular disease (CVD) i.e. cerebrovascular accidents, cardiac ischaemia and peripheral vascular disease in PLWH is currently an important focus of researchers in the aging, HIV infected population following the decrease in communicable diseases in the ART era.[2, 7, 8]

In this article, the published levels of the products of fibrinolysis, D-dimers, the inflammatory cytokines, IL-6 and TNF-α, and adhesion molecules, sVCAM and sICAM, in ART-naive PLWH without overt CVD or concomitant infections, are reviewed. This review supports the ongoing use of D-dimers to predict thrombotic and bleeding events in PLWH since the weighted averages across assays suggest that the median levels do not exceed the reference range. The role of inflammatory cytokine monitoring and measurement of endothelial adhesion markers is less clear although more recently, we have demonstrated significantly increased levels in HIV-associated TTP, a thrombotic microangiopathy which is a documented, although rare, complication of HIV infection.[49] Future prospective validation of these biomarkers in longitudinal cohort studies will be of value.

This study has some limitations. These include potential selection bias and gaps in literature search strategies that might have led to the omission of relevant research. In most cases, assay cut-off values and/or reportable ranges of the analytes were not stated although the researchers have attempted to define these (primarily from the package insert) wherever possible. In most cases, the researchers did not include an assay cut-off value and a reportable range although these were subsequently retrieved from the package inserts for the assays described. These limitations notwithstanding, this paper is the first to address the measurement of these biomarkers systematically.

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Publication 5: Distinguishing and overlapping laboratory results of thrombotic microangiopathies in HIV infection: Can scoring systems assist?

Following demonstration of high levels of D-dimers in patients presenting with HIV-TTP, it was important to identify the diagnostic implications of the overlap between HIV-TTP and other thrombotic microangiopathies (TMAs). HIV-TTP is characterised by high levels of inflammation and endothelial activation prior to plasma therapy and also in comparison with levels in ART-naïve cohorts of people living with HIV (PLWH). TTP is a diagnosis of exclusion and, in particular the TMA disseminated intravascular coagulation (DIC), associated with HIV infection shares pathophysiological pathways. Previously, we have demonstrated that DIC is common in PLWH even without additional pathogenic factors (notably concomitant infections). Two recognised scoring systems were applied retrospectively to 2 cohorts of patients – the first diagnosed with HIV-TTP and the second in patients with HIV-associated DIC. TTP and DIC in PLWH showed significant overlap with respect to activation of the coagulation system and other diagnostic criteria. Of the 152 patients included for review, 51 fulfilled the diagnostic scoring criteria based on the common DIC and PLASMIC (reduced platelet count, red blood cell haemolysis, absence of cancer, no history of transplantation, reduced red blood cell volume, preservation of the international normalised ratio and creatinine) scoring systems for both disorders. D-dimer levels, previously documented as being normal in patients with TTP, were elevated above the levels detected in patients with DIC in the background of HIV infection. Although patients with HIV-DIC were more likely to have comorbid disease including opportunistic infections and cancer, this was not consistent. Key differentiating factors utilised clinically to make a diagnosis of HIV-TTP were the presence of significant numbers of red cell fragments (schistocytes) and an elevated lactate dehydrogenase (LDH) level. This suggests that recognised international scoring systems should be reviewed and potentially modified for local relevance. Importantly, the role of measuring levels of ADAMTS-13, needs to be considered and this test potentially should be made more widely available for the diagnosis of TTP in South Africa.

Contribution of candidate:

The candidate identified, collated and analysed all the laboratory parameters of the patients included in the study and compiled the initial draft of the manuscript which was then considered and optimised by the co-authors (ESM and BFJ).

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RESEARCH ARTICLE

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Distinguishing and overlapping laboratory results of thrombotic microangiopathies in HIV infection: Can scoring systems assist?

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Abstract

Background: Patients with Human Immunodeficiency Virus (HIV) infection are at risk of thrombotic microangiopathies (TMAs) notably thrombotic thrombocytopenic purpura (TTP) and disseminated intravascular coagulation (DIC). Overlap between laboratory results exists resulting in diagnostic ambiguity.

Methods: Routine laboratory results of 71 patients with HIV-associated TTP (HIV-TTP) and 81 with DIC with concomitant HIV infection (HIV-DIC) admitted between 2015 and 2021 to academic hospitals in Johannesburg, South Africa were retrospectively reviewed. Both the PLASMIC and the International Society of Thrombosis and Haemostasis (ISTH) DIC scores were calculated.

Results: Patients with HIV-TTP had significantly ($P < .001$) increased schistocytes and features of hemolysis including elevated lactate dehydrogenase (LDH)/upper-limit-of-normal ratio (median of 9 (interquartile range [IQR] 5-12) vs 3 (IQR 2-5)) but unexpectedly lower fibrinogen (median 2.8 (IQR 2.2-3.4) vs 4 g/L (IQR 2.5-9.2)) and higher D-dimer (median 4.8 (IQR 2.4-8.1) vs 3.6 g/L (IQR 1.7-6.2)) levels vs the HIV-DIC cohort. Patients with HIV-DIC were more immunocompromised with frequent secondary infections, higher platelet and hemoglobin levels, more deranged coagulation parameters and less hemolysis. Overlap in scoring systems was however observed.

Conclusion: The laboratory parameter overlap between HIV-DIC and HIV-TTP might reflect a shared pathogenesis including endothelial dysfunction and inflammation and further research is required. Fibrinogen in DIC may be elevated as an acute phase reactant and D-dimers may reflect the extensive hemostatic activation in HIV-TTP. Inclusion of additional parameters in TMA scoring systems such the LDH/upper-limit-of-normal ratio, schistocytes count

and wider access to ADAMTS-13 testing may enhance diagnostic accuracy and ensure appropriate utilization of plasma.

KEYWORDS

diagnostic scoring systems, disseminated intravascular coagulation (DIC), thrombotic thrombocytopenic purpura (TTP), treatment decisions

1 | INTRODUCTION

Thrombotic microangiopathy (TMA) is a clinical syndrome characterized by hemolytic anemia, thrombocytopenia and microvascular thrombosis resulting in life-threatening multi-organ failure.[1,2] TMAs are heterogeneous and include congenital and acquired thrombotic thrombocytopenic purpura (TTP) and TTP-like syndromes, hemolytic uremic syndrome (HUS) and the atypical form of this disease, aHUS.[1-3] Disseminated intravascular coagulation (DIC) can also be classified as a TMA.[4] TMAs can be the manifestation of common disease processes such as hypertension and malignancy as well as develop in relation to drug exposure.[1,4] Although the distinction between different TMA syndromes is often difficult, authors have advised against grouping of these disorders under a single pathological entity underlining the need for further studies in order to improve patient outcomes.[3]

There are more than 7.7 million people in South Africa infected with human immunodeficiency virus (HIV).[5] Antiretroviral therapy (ART) is often initiated late in these patients who consequently present with advanced HIV infection and high rates of non-communicable disease, like malignancy and cardiovascular disease, and opportunistic infections as well as associated complications such as TMAs.[5-8] HIV-infected patients with laboratory features of a TMA pose a diagnostic dilemma since infection with HIV predisposes to a number of these disease processes particularly secondary TTP (HIV-TTP) and DIC with background HIV infection (HIV-DIC).[9-14] Distinguishing these conditions is important since treatment differs. DIC is managed by treatment of the underlying pathogenic cause and HIV-TTP with therapeutic plasma exchange (TPE) or plasma infusion.[1,12,13,15,16] Treatment of patients with HIV-TTP is the most frequent request for TPE in South Africa.[17] Plasma infusion alone is also of therapeutic value in patients with HIV-TTP[16] but administration of insufficient amounts of plasma due to the risk of fluid overload and limited availability of plasma frequently results in poor responses and a need to convert to TPE.[16] Adverse events related to apheresis therapy and exposure to plasma still occur despite technological and procedural developments which have made operational systems safer.[18] For these reasons and to ensure best patient outcomes, correct distinction between secondary TTP and DIC is paramount.

The microvascular thrombosis in TTP and in DIC differs in both pathogenesis and in composition of the thrombi.[1,19] In acquired TTP, the cleavage of von Willebrand Factor (VWF) multimers released by the endothelium may be impaired by a reduction in activity of the VWF proteolytic enzyme, a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13 (ADAMTS-13), mediated by auto-antibodies.[1] Excessive release of high molecular weight VWF multimers from damaged endothelium resulting in a relative deficiency of ADAMTS-13 is another postulated pathogenic factor in secondary TTP termed TTP-like syndrome.[13,20] The resultant thrombi in TTP are therefore rich in VWF and platelets with abundant red blood cell (RBC) fragments (schistocytes) and severe thrombocytopenia.[1,9,12] The microvascular thromboses in DIC in contrast consist mainly of fibrin-platelet clots following the exposure of coagulation factors to tissue factor secondary to an initiating process such as sepsis or trauma.[10,21] Excessive bleeding occurs frequently in DIC secondary to the consumption of coagulation factors as well as platelets. Intravascular clot formation is further accelerated in DIC by the loss of natural anticoagulant and fibrinolytic activity.[15] Schistocytes are present in DIC but usually constitute <10% of the RBCs.[15] In both of these disease processes, endothelial damage and dysregulation of the coagulation cascade also contribute to disease pathogenesis.[22] In addition, HIV-infected patients often have background hematological abnormalities including cytopenias, underlying bone marrow dyshematopoiesis and baseline activation of the hemostatic system contributing to diagnostic uncertainty.[6,22-24]

The PLASMIC (platelet count, hemolysis, active cancer, MCV (mean red blood cell (RBC) volume), international normalized ratio (INR) and creatinine) score (Table 1) is based on clinical and routine laboratory parameters and predicts the likelihood of severe ADAMTS-13 deficiency in patients with a TMA since testing for this parameter is not widely available.[25] This score was designed to enable the distinction between TTP and other TMAs.[25] The International Society of Thrombosis and Haemostasis (ISTH) DIC score (Table 1), is a diagnostic tool to assist in the diagnosis of DIC in an

TABLE 1 The ISTH DIC score and the PLASMIC score for prediction of thrombotic microangiopathy associated with severe ADAMTS-13 deficiency[25,26]

ISTH diagnostic score for DIC		PLASMIC score	
Parameter	**Points**	**Parameter**	**Points**
Platelet count		Platelet count $<30 \times 10^9$/L	1 point
• $>100 \times 10^9$/L	• 0 points		
• $<100 \times 10^9$/L	• 1 point	Hemolysis[b]	1 point
• $<50 \times 10^9$/L	• 2 points		
Elevated fibrin markers (D-dimer)[a]		No active cancer	1 point
• No increase	• 0 points		
• Moderate increase	• 2 points	No solid-organ or stem-cell transplant	1 point
• Strong (marked) increase	• 3 points		
Prolonged prothrombin time (PT) vs control result		MCV <90 fL	1 point
• <3 seconds	• 0 points		
• >3 but <6 seconds	• 1 point	INR <1.5	1 point
• >6 seconds	• 2 points		
Fibrinogen level			
• >1 g/L	• 0 points	Creatinine <176.8 μmol/L	1 point
• <1 g/L	• 1 point		
Score: ≥5: compatible with overt DIC: repeat score daily <5: suggestive for non-overt DIC: repeat next 1 to 2 days		Likelihood score for severe ADAMTS-13 deficiency: • 0-4: low likelihood • 5: intermediate likelihood • 6 or 7: high likelihood	

Abbreviations: ADAMTS-13, a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13; INR, international normalized ratio; MCV, mean corpuscular volume.

[a]Moderate D-dimer increase: = 0.25-1 D-dimer units (mg/L)/ Strong (marked) D-dimer increase: ≥ 1 D-Dimer units (mg/L).[26]

[b]Reticulocyte count >2.5%, or haptoglobin undetectable, or indirect bilirubin > 12.0 μmol/L.

appropriate clinical setting.[26] The utility of these scoring systems in HIV-infected patients with TMAs has not been comprehensively assessed and bedside treatment decisions are often inconsistent. It is further possible that the background hemostatic changes in HIV infected patients may alter TMA scoring system performance.[4,13,14,27] The objective of the current study was to identify distinguishing clinical and laboratory parameters to assist with the accurate diagnosis of HIV infected patients who present with a TMA suspected to be either HIV-TTP or HIV-DIC.

2 | METHODS

Approval for this study was obtained from the Human Research Ethics Committee of the University of the Witwatersrand (Wits) (Certificate numbers: M160134 and M160839). Informed individual patient consent was waived for this retrospective record review in which all patient identifiers were removed. The authors independently and retrospectively applied both the PLASMIC and the ISTH DIC scores to the available results of consecutive HIV-infected patients who were diagnosed with either HIV-associated TTP (HIV-TTP) (n = 71) or overt, uncompensated DIC with background HIV infection (HIV-DIC) (n = 81) between 2015 and 2021 at the 3 academic hospitals affiliated to Wits. The diagnoses were made by treating physicians based on clinical and routine laboratory parameters. A diagnosis of HIV-TTP was made based on laboratory features of severe thrombocytopenia (Platelets $<30 \times 10^9$/L) and abundant schistocytes (constituting >10% of the RBCs on the peripheral film) in the absence of features suggestive of another TMA in most cases. ADAMTS-13 activity and autoantibody levels were not included in the initial diagnosis. Where possible, stored plasma was sent for batch ADAMTS-13 activity and autoantibody levels performed at the University of the Free State, Research Coagulation Laboratory. Diagnosis of DIC was made in patients in the

TABLE 2 Baseline median (IQR) results of 71 patients diagnosed with HIV-TTP (including 43 (61%) with confirmed reduced ADAMTS-13 levels) and 81 with HIV-DIC

Parameter (RI)	HIV-TTP cohort with confirmed low ADAMTS-13 level (n = 43)	Total HIV-TTP cohort (n = 71)	HIV-DIC cohort (n = 81)	*P* values[a] (HIV-TTP vs HIV-DIC)
Age in years	35 (29-40)	36 (33-44)	36 (31-43)	N/S
CD_4^+ T-cells (500-2010 cells/μL)	156 (92-220)	144 (94-191) (n = 64)	68 (22-184)	$P < .001$
HIV viral load (RNA copies/mL)	276 500 (52 925-894 384)	199 000 (21 275-597 626) (n = 62)	35 397 (524-512 200) (n = 70)	$P < .001$
Hemoglobin (12.1-17.5 g/dL)	6 (5.2-7.3)	6 (5.3-7.2)	7.5 (6.4-8.5)	$P < .001$
Red blood cell distribution width (RDW) (12.4-17.3%)	29 (24.3-31.9)	28 (23.2-33.1)	18 (16.3-21.2)	$P < .001$
Platelets (186-454 × 10^9/L)	7 (6-13)	9 (6-15)	40 (18-68)	$P < .001$
Lactate dehydrogenase (LDH) (100-190 U/L)	1645 (1185-2217)	1681 (1004-2340)	513 (350-1057) (n = 51)	$P < .001$
LDH/upper-limit-of-normal ratio	9 (6-11)	9 (5-12)	3 (2-5) (n = 51)	$P < .001$
C-reactive protein (CRP) (<10 mg/L)	22 (10-42)	19 (12-41) (n = 68)	149 (62-211)	$P < .001$
Prothrombin time (14 seconds)	15.1 (14.0-16.4)	15.1 (14.1-17.1) (n = 70)	20.5 (17.5-27.0)	$P < .001$
Fibrinogen (2-4 g/L)	3.0 (2.3-3.3)	2.8 (2.2-3.4) (n = 62)	4.0 (2.5-9.2)	$P < .001$
D-dimer levels (<0.25 mg/L)	5.0 (3.0-7.8)	4.8 (2.4-8.1) (n = 70)	3.6 (1.7-6.2)	$P < .044$
PLASMIC score	6 (6-6)	6 (6-6)	4 (3-5)	$P < .001$
ISTH DIC score	4 (4-5)	4 (4-5)	6 (5-6)	$P < .001$

Abbreviations: ADAMTS-13, a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13; HIV-TTP, HIV-associated thrombotic thrombocytopenic purpura; HIV-DIC, disseminated intravascular coagulation (DIC) with background HIV infection; IQR, interquartile range (25-75%); n, number of available results if not available in all patients; N/S, not significant; RI, normal reference interval.

[a]$P \leq .05$ was deemed significant.

correct clinical context by applying the ISTH-DIC score. The available results for both cohorts, including full blood count (FBC) (performed on Sysmex XN analysers, Sysmex, Japan), peripheral smear findings, hemolytic and inflammatory markers (performed on Roche Cobas analysers, Roche, Switzerland) and coagulation assays (performed on a STAGO STA-R MAX analysers, Diagnostica Stago, France) from the accredited National Health Laboratory Service (NHLS) laboratory as part of routine patient management were collected. Summary statistics were computed for all parameters including a median and interquartile range (IQR). Results were compared using Graphpad Prism version 9 (Graphpad software, San Diego).

3 | RESULTS

The results of the 71 patients diagnosed with HIV-associated TTP (HIV-TTP) and 81 patients diagnosed with overt DIC with background of HIV infection (HIV-DIC) are included in Table 2. The patients with laboratory-confirmed DIC were less likely to have virological control and had significantly more pronounced immunodeficiency. The hemoglobin and platelet counts were also significantly higher and the prolongation of the PT was more pronounced in the DIC cohort. Although patients diagnosed with HIV-TTP showed less pronounced derangement of the coagulation system, that is,

less prolongation of the prothrombin time (PT), they presented with significantly higher D-dimer and significantly lower fibrinogen levels compared to the cohort with HIV-DIC. Underlying infection was identified in 68 (84%) of the DIC cohort. Identified pathogens included bacterial septicemia and *Mycobacterium tuberculosis*. In contrast, no secondary infection could be identified in 62 (88%) of the patients with HIV-TTP despite extensive investigations.

The diagnosis of HIV-TTP was made clinically in conjunction with routine results. In 43 of these patients (61%), ADAMTS-13 activity levels were measured retrospectively. A sub-analysis was performed comparing the results of routine parameters in patients with suspected TTP with and without confirmed ADAMTS-13 deficiency and those diagnosed with HIV-DIC. This sub-analysis confirmed that the differences persisted between HIV-DIC and HIV-TTP even when patients without confirmed ADAMTS-13 levels were excluded (P-value <.001). There was therefore no significant difference in the results of routine tests between the HIV-TTP groups with and without ADAMTS-13 results ($P > .9$). Clinically significant levels of autoantibodies to ADAMTS-13 were present in the 43 (61%) of the patients with HIV-TTP in whom ADAMTS-13 levels were measured. No ADAMTS-13 levels were measured in the patients who were diagnosed with HIV-DIC.

Although the PLASMIC score was high in 99% of the patients diagnosed with HIV-TTP (n = 71), 18 (31%) of these patients also had an ISTH DIC score of 5 or greater which is compatible with an underlying overt DIC. The PLASMIC score was also applied to the cohort of HIV infected patients diagnosed with an overt DIC as per the ISTH DIC score (n = 81) and 14 (17%) of these patients had a PLASMIC score of 5 (intermediate likelihood of severe ADAMTS-13 deficiency) and 19 (23%) had a PLASMIC score of 6 or higher (high likelihood of severe ADAMTS-13 deficiency). ADAMTS-13 levels were retrospectively available in 43 (61%) of the patients with HIV-TTP. All of these patients had levels below 15%, that is, severe ADAMTS-13 deficiency. Unfortunately, ADAMTS-13 levels were not available in the remaining 28 patients. Importantly, 69 (97%) of patients diagnosed with HIV-TTP responded to plasma therapy. Notable, exclusion of the patients without documented ADAMTS-13 levels from the final data analysis did not alter the statistical difference in parameter results between the HIV-DIC and the HIV-TTP cohorts.

The most prominent laboratory features in the cohort of patients with HIV-TTP were marked peripheral schistocytosis (>10% of RBCs) which was present on admission in 65 of 71 patients (91.5%) and developed within 24-h in five additional patients. The LDH/upper-limit-of-normal ratio was also significantly elevated in the patients with HIV-TTP compared to the patients with a HIV-DIC. LDH levels were however only performed in 51 (71%) patients in the DIC cohort.

67 (94%) of the patients with HIV-TTP were treated with fresh frozen plasma (FFP) with 64 (90%) receiving TPE and 3 (4%) plasma infusion only for a median of 10 days (IQR 7-13). 69 (97%) of the patients who received plasma therapy responded and 2 (3%) deteriorated and demised in hospital despite plasma therapy, ART and additional supportive care

4 | DISCUSSION

The differentiation between TTP and DIC represents an important diagnostic decision since TTP is managed primarily with TPE in our treatment center and delays in initiation of therapy may adversely impact patient outcomes.[1,28] Although plasma infusions may be used in DIC to correct severe hemostatic abnormalities, primary management is treatment of the underlying pathogenic cause.[15]

HIV represents a significant risk factor for both secondary TTP and DIC.[10,12,22,29] The HIV viral load results were significantly higher in the HIV-TTP group compared with the HIV-DIC cohort but despite better HIV viral control in the HIV-DIC cohort, the CD_4 positive T-cell counts were lower ($P < .001$). This finding probably reflects acute concomitant infections in the HIV-DIC cohort.

Normal D-dimer levels were previously considered a feature of TTP and, that together with preserved time-to-clot formation assays, for example, PT as well as antithrombin (AT), were suggested to be useful in distinguishing between these conditions in HIV-uninfected patients.[30] In the current study, patients with HIV-TTP however presented with significantly elevated D-dimer levels suggesting widespread microthrombosis although mucocutaneous bleeding was probably also contributory.

In this study, we demonstrate that there is significant overlap between the laboratory parameters included in diagnostic scores in patients with HIV-TTP and those with HIV-DIC with 51 of the 152 patients having scores which were diagnostic for both conditions. Important differentiators in these patients included the abundance of schistocytes and the elevated LDH/upper-limit-of-normal ratios which appeared to show a higher specificity for TTP. The prothrombin time in patients with DIC was significantly more prolonged vs the HIV-TTP cohort. Importantly, D-dimers were a poor discriminator between the two populations with TTP patients showing higher D-dimer levels than patients with DIC. Elevated D-dimer

levels in patients with HIV-associated TTP have also been observed in other studies.[13,31] Median fibrinogen levels were within the normal reference range in both cohorts but were significantly higher in patients with HIV-DIC mirroring the CRP levels most likely reflecting increased production of fibrinogen as an acute phase reactant. CRP was a distinguishing parameter between the two cohorts with elevated levels in the HIV-DIC cohort probably related to underlying concomitant infections and this routine parameter therefore could have clinical utility in distinguishing between HIV-DIC and HIV-TTP. D-dimers and fibrinogen form important components of the ISTH DIC score and should be interpreted with caution in HIV infected patients with a TMA.[11,32] The authors caution against favoring a diagnosis of HIV-DIC instead of HIV-TTP based on elevated D-dimer levels when additional features are compatible with HIV-TTP.

The overlap in laboratory parameters between acquired TTP and DIC in HIV infected patients may reflect a shared pathogenesis. Contributory factors include chronic inflammation with baseline activation of the hemostatic and complement systems as a result of ongoing viral replication, microbial translocation across a disrupted gastrointestinal mucosal barrier and opportunistic infections.[23,33,34] Inflammation and complement activation causes endothelial damage which predispose to coagulopathies including TMAs.[6] The background derangements of the coagulation and hematopoietic systems in patients with underlying HIV infection should also be considered when making diagnostic and treatment decisions in patients with HIV-TMAs.[24,35] Scoring systems standardize diagnoses to ensure appropriate therapy and improve patient outcomes.[25,27] The PLASMIC score is based on clinical parameters and the results of routine tests to predict the likelihood of significant ADAMTS-13 deficiency which is indicative of the presence of TTP in a patient with laboratory features of a TMA.[25] Although the PLASMIC score predicted a high probability of severe ADAMTS-13 deficiency in 99% of the cohort diagnosed with HIV-TTP, it also predicted a similar risk in 23% of HIV-infected patients with an overt DIC based on the ISTH DIC score. The PLASMIC score may therefore not have sufficient specificity to delineate between HIV-TTP and HIV-DIC in all cases and inclusion of the LDH/upper-limit-of-normal ratio is likely to improve the specificity and accuracy. Zhao et al[36] also demonstrated that inclusion of the LDH/upper-limit of-normal ratio improved the accuracy of the PLASMIC score in identifying patients who suffered from TTP. Increased schistocyte count was also a distinguishing feature between the TTP and DIC cohort but this parameter is poorly standardized with considerable inter-observer variability since it often relies on the subjective methodology of light microscopy and manual counting of cells.[37,38] Wider access to ADAMTS-13 testing, possibly even on a Point-of-Care-Testing (POCT) platform, could also improve the accuracy of the diagnosis of the pathophysiological cause in patients presenting with a TMA.[39] Although all 43(61%) patients in the HIV-TTP cohort who were tested for ADAMTS-13 autoantibodies had clinically significant levels, the diagnostic utility of this parameter is uncertain as it probably forms part of the HIV polygammaglobulinemia in HIV infected individuals and is present even in the absence of TTP.[12]

The limitations of this study include the retrospective nature which resulted in some results being unavailable. No ADAMTS-13 levels were performed in the DIC-cohort of patients. The details of the treatment administered and the patient outcomes for the HIV-DIC cohort were also not available and based on the PLASMIC scores, some of these patients may have benefited from plasma treatment. Unfortunately, the details of the ART regimens and duration of treatment in the HIV-DIC cohort were not available. ART status could therefore not be evaluated as a distinguishing feature between the two TMAs. Further studies in this regard are required. The study data, however, reflect the diagnostic and treatment decisions made on admission in the patient cohorts. All requests for DIC screen analysis were available to the authors but patients with a diagnosis of HIV-TTP may have been treated by attending physicians without the knowledge of the authors and were therefore not included in the study. Irrespective of these limitations, we are of the opinion that the study results reflect the overlapping findings of these serious conditions in our population with HIV infection.

5 | CONCLUSION

HIV infection is prevalent in the African context[5] with secondary HIV-associated TTP and DIC in the background of HIV infection constituting the most prevalent TMAs in this group of patients.[2,22] The diagnostic distinction between these conditions can be ambiguous resulting in inappropriate treatment due to the background activation of the coagulation system and inflammation in HIV infected patients.[9,11,40] The addition of the LDH/upper-limit-of-normal ratio and objective, automated quantification of schistocytes will probably improve the accuracy of the PLASMIC score.[28,41] The LDH/upper-limit-of-normal ratio standardizes across different reagents and reference indices. The value of longitudinal, repeated application of scoring systems in patients with a TMA in our setting must also be evaluated. The cause and significance of the elevation of D-dimers in patients with HIV-associated TTP also requires further investigation.[13,31] Based on the results of the study, the authors

support the addition of the LDH/upper-limit-of-normal ratio to the PLASMIC score for improved diagnostic accuracy and to guide urgent, but appropriate, institution of therapeutic plasma exchange (TPE) as was proposed by Zhao et al.[36]

AUTHOR CONTRIBUTIONS

Susan Louw: study design, data collection and analysis, manuscript writing and critical review, and approval of submission. Barry Frank Jacobson: study design, critical review, and approval of submission. Elizabeth Sarah Mayne: study design, data collection and analysis, manuscript writing and critical review, and approval of submission.

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CONFLICT OF INTEREST

The authors declare no conflict of interest pertaining to the study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon request.

ETHICS STATEMENT

Approval was obtained from the Human Research Ethics Committee of the University of the Witwatersrand (Wits) (Certificate numbers: M160134 and M160839). Individual patient consent was waived for this retrospective record review.

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Erratum for Results:

67 (94%) of the patients with HIV-TTP were treated with fresh frozen plasma (FFP) with 64 (90%) receiving TPE and 3 (4%) plasma infusion only for a median of 10 days (IQR 7-13). 65 (97%) of the patients who received plasma therapy responded and 2 (3%) deteriorated and demised in hospital despite plasma therapy, ART and additional supportive care.

Note: Mann-Whitney U tests were utilised for comparisons between the results of the cohorts.

CHAPTER 3: SUMMARY AND FUTURE DIRECTIONS

Secondary or acquired TTP is a serious condition associated with microangiopathic haemolysis, thrombocytopaenia and variable degrees of critical organ dysfunction. Without plasma therapy, morbidity and mortality are high. [40, 73] The pathogenesis of this condition is classically thought to involve the production of antibodies against the protease, a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13 (ADAMTS-13), which is involved in cleaving newly secreted ultra-large von Willebrand Factor (ULVWF) multimers.[74] TTP, although a rare complication of HIV infection, has been documented in multiple South African cohorts of people living with HIV (PLWH).[50, 52, 75]

This project hypothesised that the principal pathophysiologic cause of HIV-associated TTP (HIV-TTP) differs from other forms of acquired TTP. Although PLWH with a thrombotic microangiopathy (TMA) had haemolysis with numerous schistocytes with marked thrombocytopaenia, and although plasma therapy remains the mainstay of therapy in these patients, the uneven performance of scoring systems like the PLASMIC (reduced platelet count, red blood cell haemolysis, absence of cancer, no history of transplantation, reduced red blood cell volume, preservation of the international normalised ratio and creatinine) system and the presence of marked inflammation suggest that the disease differs fundamentally from non-HIV associated secondary TTP. We hypothesise that endothelial activation, linked to chronic inflammation, is the primary cause with the profuse release of ULVWF multimers causing a relative deficiency of ADAMTS-13 secondary to consumption rather than a primary deficiency which initiates the microangiopathic process.

All patients within this cohort showed elevated levels of proinflammatory markers including the cytokines interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α). These cytokines are released in response to multiple inflammatory stimuli and, in PLWH, may reflect poor virologic control, the presence of additional opportunistic infections and the inappropriate activation of the immune system caused by the translocation of microbial substances across a compromised mucosal barrier.[76, 77] Both IL-6 and TNF-α activate the endothelium with upregulation of adhesion molecules facilitating translocation of inflammatory cells and platelet recruitment.[78, 79] Intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) were significantly raised in the study patients even when compared to cohorts of ART-naïve PLWH. Activation of the coagulation system in patients with HIV-TTP is probably secondary to endothelial damage with the exposure of procoagulant sub-endothelial connective tissue elements and is reflected by elevated levels of fibrin degradation products (D-dimers).

A summary model is proposed of the complex interplay of factors which contribute to the pathogenesis of HIV-TTP in Figure 2. HIV-associated chronic inflammation with resultant T-cell depletion results in direct endothelial activation, leukocyte and platelet recruitment and complement activation.[80] The endothelial activation with endothelial disruption, manifests in upregulation of adhesion molecules like ICAM-1 and VCAM-1 and exposure of sub-endothelial tissue. In addition, there is secretion of ULVWF multimers which swamp the cleaving protease, ADAMTS-13 resulting in a relative deficiency of the enzyme. The inconsistent levels of both activity and antibodies demonstrated across previous studies[53, 67, 81] suggest that the formation of ADAMTS-13 autoantibodies is more likely to be a contributory factor rather than an initiator of the process but this will require confirmation in further studies.

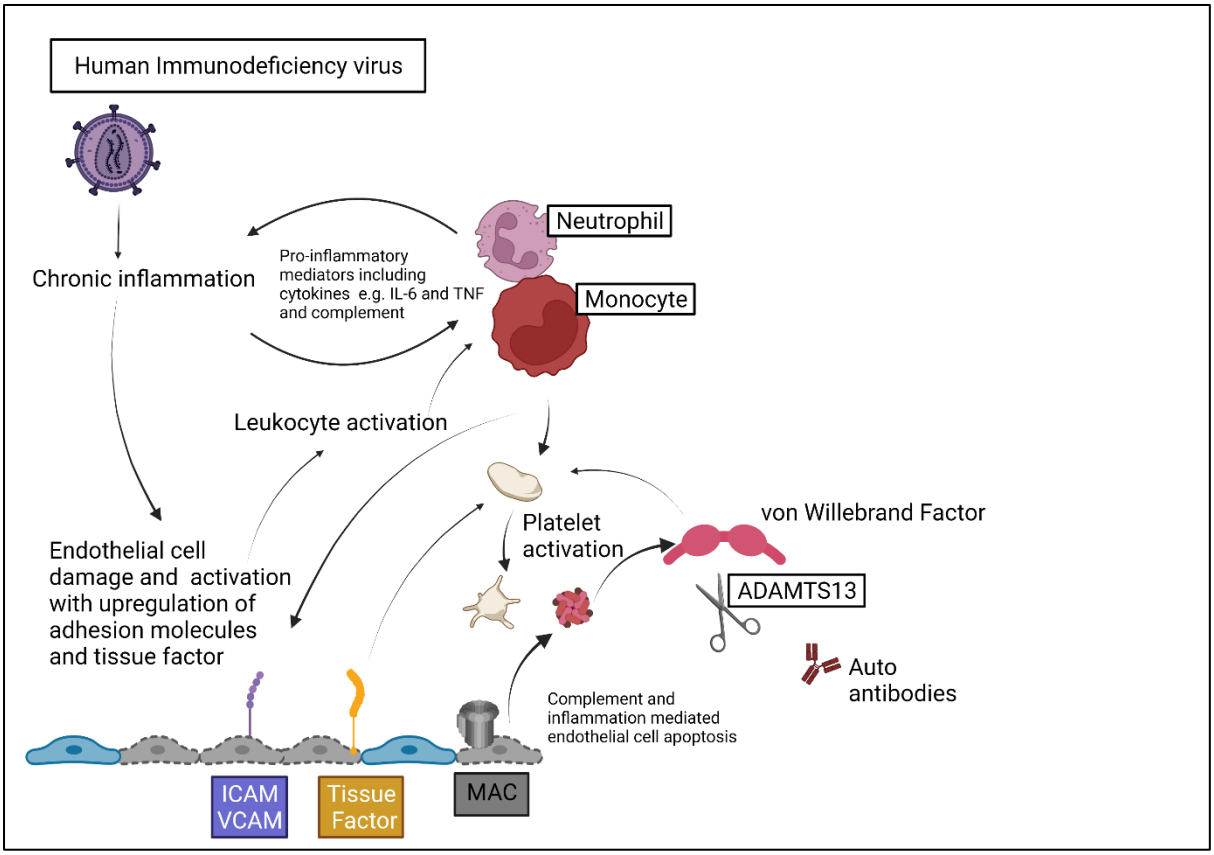

Figure 2: A revised model of the pathophysiological factors culminating in HIV-TTP

Infection with Human immunodeficiency virus (HIV) stimulates pro-inflammatory pathways including the complement system culminating in the formation of membrane attack complexes (MAC) on cell surfaces including on endothelial cells. $CD4^+$ T-lymphocytes are depleted with resultant opportunistic infections and bacterial translocation across a compromised gut membrane which support ongoing inflammation. Complement activation with release of proinflammatory cytokines, directly activates the endothelium to upregulate procoagulant and cell adhesion molecules. The compromised endothelium releases excessive amounts of ultra-large von Willebrand Factor (ULVWF) multimers which overwhelm the proteolytic capacity of the VWF cleaving protease, a-disintegrin-and-metalloproteinase-with-thrombospondin-motifs 13 (ADAMTS-13). Unravelled VWF multimers in the microcirculation bind and activate platelets to form platelet-rich micro-thrombi. Tissue factor, expressed on damaged endothelial cells and platelets, together with exposure of sub-endothelial connective tissue, activates the coagulation system. Clot formation and fibrinolysis are demonstrated by increased levels of fibrin degradation products (D-dimers). Both platelets and endothelial cells also interact with leukocytes in the circulation resulting in leukocyte infiltration of the tissue and reciprocal activation. Dysregulated B-cell responses result in the production of ADAMTS-13 autoantibodies although these antibodies are likely to be contributory rather than initiating events in TTP. Created with Biorender.com

Secondary TTP is generally a diagnosis of exclusion and should be differentiated from other primary thrombotic microangiopathies. Overt disseminated intravascular coagulation (DIC) is not uncommon in PLWH even in the absence of other causes like bacterial infection or malignancy.[72, 82] The overlap that was demonstrated between the scoring systems of TTP and DIC is probably primarily as a result of activation of the coagulation system in both conditions and presents a diagnostic dilemma for clinicians managing these patients.[82] A modification of the PLASMIC system is proposed which should include additional markers of haemolysis, primarily the lactate dehydrogenase (LDH) level, and possibly inflammatory parameters such

as the routinely performed C-reactive protein (CRP) to guide treatment decisions in PLWH presenting with a thrombotic microangiopathy (TMA).[83] Additional markers of inflammation such as IL-6 may also be of value. Recent guidelines from the Scientific Standardisation Committee of the International Society of Thrombosis and Haemostasis (ISTH) recommend utilising ADAMTS-13 activity levels to guide management in secondary TTP. Although previously, both ADAMTS-13 activity and autoantibody levels have varied in patients with HIV-TTP, ADAMTS-13 levels were consistently low in the patients in this cohort and all patients showed anti-ADAMTS-13 antibodies although these varied in titre.[83, 84] The exact epitope target and pathophysiologic effect of the detected ADAMTS-13 autoantibodies warrants further exploration as these antibodies may be part of the non-specific hypergammaglobulinaemia secondary to CD_4^+ T-lymphocyte depletion.[54, 85, 86] The ADAMTS-13 activity levels are not widely available in South Africa and it is unlikely that the test will be available in the emergency setting in which HIV-TTP presents. Future evaluation of the value of including ADAMTS-13 levels in HIV-TTP diagnosis should be evaluated in larger studies.

Although the inflammatory and endothelial biomarkers were uniformly and significantly raised in patients with HIV-TTP, there was no direct relationship with any parameter of severity of TTP including the number of days of required plasma therapy. Interestingly, the 3 patients who demised had the highest levels of IL-6 and TNF-α suggesting that more comprehensive and wide scale evaluation of these biomarkers in HIV-TTP may indicate a role in either diagnosis or prognosis.

HIV is associated with immune dysregulation including dysregulation of the complement system. Disorders of complement activation, sometimes referred to as complementopathies, are linked to endothelial activation. There is also overlap in presentation of complementopathies and thrombotic microangiopathies most importantly, atypical haemolytic uraemic syndrome (aHUS) which is related to an inherited deficiency of the regulatory complement protein, Factor H.[33, 87, 88] The patients in this study had C3 and C4 levels at the lower limits of the normal reference indices (RIs) but this is not an unexpected finding in patients with immune activation. Although complement may be contributory to HIV-TTP, we did not find indisputable evidence that it was a primary initiating event. Further assessment of the role of complement in HIV-TTP is warranted.

A number of questions remains to be answered regarding the pathogenesis of TTP in HIV. Recent work in our research unit (unpublished data) has suggested that HIV proteins, including negative factor 1 (NEF-1), may directly cause endothelial activation with certain polymorphisms of this viral protein resulting in enhanced endothelial tropism. Direct infection of endothelial cells by HIV is controversial but has not been fully excluded. Although there was no consistent evidence of a single underlying opportunistic infection in the patients with HIV-TTP like *Mycobacterium tuberculosis* or endothelial-tropic viruses, such as *gamma-herpesviridae* specifically *Kaposi-sarcoma herpesvirus,* the presence and pathogenic contribution of these concomitant infections should be considered. Another important emerging pathogen, the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) virus, is also associated with secondary TTP.[89] Since this virus is also associated with elevated proinflammatory cytokines including IL-6[90], it is tempting to hypothesise that the underlying pathogenesis in Coronavirus disease (COVID-19)-associated TTP and HIV-TTP is similar.

The treatment of HIV-TTP relies on donated plasma which is a scarce and expensive resource in the local African environment. Treatment of HIV-TTP is the commonest request for therapeutic plasma exchange (TPE) in the South African population and usually relies on up to 14 units of donated fresh frozen plasma (FFP) units per day for an average of 10 days per patient treated.[55] The current study demonstrated the therapeutic effect of immunosuppression with steroids which probably ameliorates the endothelial dysfunction and activation related to ongoing inflammation in patients with HIV-TTP. Elucidating additional pivotal underlying pathophysiological factors in HIV-TTP could potentially result in the establishment of alternative therapies as well as preventative strategies.

A final feature which must be discussed is that HIV-TTP is uncommon in South African PLWH who are ART-treated and who achieve full virologic control. This indicates the importance of commencement of ART as a keystone of management of these patients but may also provide hope that, with more widespread ART access and adherence, this condition will become rare in the African context.

Future directions will include a more comprehensive evaluation of the direct viral effects of HIV on endothelial cells in primary culture including an assessment of the presence of HIV NEF protein mutations in patients with HIV-TTP. A more widespread evaluation of the utility of diagnostic and prognostic biomarkers in HIV-TTP is indicated to ensure appropriate utilisation of resources and achieve best outcomes for patients. Assessment of patient specific factors including the activity of the natural complement inhibitors, VWF multimer sizes, as well as well as the enzymatic efficiency of ADAMTS-13, could potentially identify individuals at risk of developing HIV-TTP. The potential adverse effects of TPE on the on the immune and additional systems of patients receiving TPE must be assessed. The utility of novel therapies for acquired TTP, such as Caplacizumab, must be assessed in patients with HIV-TTP. This humanized, bivalent, immunoglobulin fragment targets the A1 domain of VWF thereby preventing interaction with the platelet glycoprotein Ib-IX-V receptor thereby preventing the ensuing microvascular thrombosis and resultant multi-organ dysfunction which occur in TTP. Evaluation of another drug, eculizumab, a monoclonal antibody and terminal complement inhibitor, is also indicated in patients with HIV-TTP. This drug binds with high affinity to the human C5 complement protein and blocks the generation of proinflammatory C5a and C5b-9. A significant limitation of the current research is the absence of control groups consisting of PLWH without TTP as well as HIV uninfected people although the patients in the investigational study detailed in publication 3 served as their own controls pre- and post TPE. This limitation should be addressed in future research.

This study does, however, provide compelling evidence that HIV-TTP is an inflammatory disorder and an important non-communicable complication of HIV infection in South Africa. It suggests that international guidelines and scoring systems may not be fully applicable in this cohort of patients but that a more comprehensive understanding of the pathogenesis may translate into improved diagnostic and management decisions. These will require more widespread investigation and an appropriate implementation plan which considers the resource-limitations in South Africa.[42]

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APPENDIX 1 – PERMISSION TO REUSE ARTICLES IN THE THESIS

Thrombotic thrombocytopenic purpura in HIV-infected patients: new twists on an old disease

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APPENDIX 2 – ETHICS APPROVAL CERTIFICATES (retrospective cohort study) M160134

R14/49 Dr Susan Louw et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160134

NAME: (Principal Investigator)	Dr Susan Louw et al
DEPARTMENT:	Molecular Medicine and Haematology Charlotte Maxeke Johannesburg Academic Hospital National Health Laboratory Service, South Africa
PROJECT TITLE:	The Changing Face of Thrombotic Thrombocytopenic Purpura (TTP)
DATE CONSIDERED:	29/01/2016
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	12/05/2016

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 the conditions under which I am/we are authorised 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 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 review in January and will therefore be due in the month of January each year.

Principal Investigator Signature

Date 26 / 09 / 2018

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr Susan Louw et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M180674

NAME: (Principal Investigator)	Dr Susan Louw et al
DEPARTMENT:	Molecular Medicine and Haematology National Health Laboratory Services Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Laboratory features and patient outcomes of thrombotic thrombocytopenia purpura (TTP)
DATE CONSIDERED:	29/06/2018
DECISION:	Approved Unconditionally
CONDITIONS:	
SUPERVISOR:	
APPROVED BY:	Professor CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	24/08/2018

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 on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, 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 **June** and will therefore be due in the month of **June** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

30/06/2018

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Ethics approval for record review study of patients with DIC (M160389)

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 10 / 05 / 2016

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES