

# Investigating platelet function and immune activation in HIV-infection

*By*

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## DECLARATION

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## Abstract

### Introduction

In the era of antiretroviral therapy (ART), people living with Human Immunodeficiency Virus (HIV) now have prolonged life spans. An emerging trend of non-acquired immune deficiency syndrome (AIDS) related complications now prevails in the aging HIV infected population. Increased levels of inflammation and chronic immune activation are associated with HIV infection. In the era of ART people living with HIV are at an increased risk of cardiovascular disease (CVD). Platelets play a pivotal role in both inflammation and immune activation and upon activation platelets degranulate and secrete various inflammatory, coagulatory and adhesion molecules. Activated platelets express surface P-selectin (CD62P) and are a key component of the coagulation pathway and serve as a link between inflammation and thrombosis. Activated platelets have been implicated in inflammatory and cardiovascular disease and have been identified as immune cells that play a crucial role in pathogen recognition and modulation of immune cells during infections. Several antiviral and antibacterial properties of platelet alpha granule contents have been established. Platelet aggregometry remains the most widely used technique to evaluate platelet function even though this technique is limited by many pre-analytical variables. Platelet flow cytometry on the other hand offers a rapid measurement of platelet function in their physiological environment with minimal artefactual activation. Few studies have however reported on standardized methods to evaluate platelet function in the context of HIV. Platelet function remains unclear and data on HIV infected treatment naïve individuals remains scarce. The aim of this project was to examine the relationship between platelet function and immune activation in patients with HIV

### Materials and methods

This study consisted of five sub-studies, firstly platelet indices and levels of platelet activation were determined in a cohort of 330 participants (185 HIV infected ARV naïve and 145 uninfected healthy controls) using; flow cytometry and haematology analyzers. The relationship between these indices and markers of platelet activation, disease progression and immune activation were assessed. Furthermore, levels of platelet activation and aggregation were evaluated in a cohort of 82 participants (41 HIV infected (ARV naïve) individuals and 41 uninfected healthy controls), using a novel whole blood flow cytometry based functional assay. These baseline levels were then correlated with markers of immune activation and disease progression in HIV.

In a subsequent study, platelet function in a cohort consisting of 58 HIV infected (ARV naïve) and 38 uninfected controls was evaluated using flow cytometry. Platelet response was measured post stimulation with adenosine diphosphate (ADP) at concentrations known to induce reversible (0.04mM) and irreversible (0.2mM) platelet aggregation. In order to assess platelet function in HIV, platelet response was evaluated in a cohort consisting of 58 HIV infected (ARV naïve) and 38 uninfected controls. Platelets were activated using varying concentrations of ADP, arachidonic acid (AA) and collagen and platelet function was measured using flow cytometry. Levels of circulating platelet leukocyte aggregates (PLAs) were also measured using flow cytometry in a cohort consisting of 35 HIV-infected (ARV naïve) individuals and 32 uninfected healthy controls. Associations between PLAs, immune activation and disease progression in HIV infected individuals were determined. The final study evaluated platelet aggregates, platelet derived microparticles (PMPs) and microparticles (MPs) in a cohort consisting of 46 HIV infected (ARV-naïve) and 40 uninfected healthy controls. Associations between MPs, PMPs, platelet aggregates and markers of immune activation and disease progression were evaluated.

## Results

HIV infected individuals showed decreased mean platelet volume levels (HIV mean  $7.91 \pm 0.85$  vs.  $8.52 \pm 1.12$ ,  $p < 0.0001$ ) that directly correlated with CD4 counts ( $r = -0.2898$ ,  $p = 0.0075$ ) and viral load ( $r = 0.2680$ ,  $p = 0.0177$ ). Platelet distribution width (PDW) levels directly correlated ( $r = 0.3455$ ,  $p = 0.0362$ ) with active coagulation and inversely correlated ( $r = -0.3666$ ,  $p = 0.0463$ ) with platelet aggregation. HIV infected individuals showed increased levels of platelet activation (%CD62P median  $11.33[5.96-29.36]$  vs. control group  $2.48[1.56-6.04]$ ,  $p = 0.0001$ ). In HIV, platelet function is retained and platelets showed increased response to submaximal concentrations of endogenous agonists. HIV infected individuals showed increased levels of circulating platelet monocyte aggregates ( $25.26[16.16-32.28]$  vs. control group  $14.12[8.36-18.83]$ ,  $p = 0.0001$ ) that directly correlated with markers of immune activation; %CD38/8 ( $r = 0.54624$ ,  $p = 0.0155$ ), viral load ( $r = 0.633$ ,  $p < 0.009$ ). Furthermore we report on increased levels of circulating MPs (median %MPs  $1.7[0.95-2.83]$  vs. Control group  $1.12[0.63-1.57]$ ,  $p = 0.0160$ ); PMPs (median %PMPs  $26.64[11.33-36.62]$  vs. Control group  $20.02[18.08-26.08]$ ,  $p = 0.0133$ ); activated PMPs (median CD62P MFI  $3.81[3.46-4.54]$  vs. Control group  $3.41[3.16-3.6]$ ,  $p = 0.0037$ ) and platelet aggregates (Median %CD62P  $14.10[5.49-39.94]$  vs. Control group  $0.17[0.10-10.99]$ ,  $p = 0.0097$ ) in HIV infected asymptomatic individuals.

## Conclusion

This study supports the potential use of the MPV and PDW as readily available markers of platelet activation and immune activation in HIV. We also showed elevated levels of activated platelets in HIV infected individuals that were hyper responsive to endogenous agonists in a concentration dependent manner. Platelet flow cytometry is a rapid and valuable technique in the evaluation of platelet function in HIV. The measurement of platelet function using flow cytometry allows the evaluation of platelet signalling pathways that may be modified in HIV infected individuals. Lastly we describe an optimized whole blood flow cytometry based assay for the evaluation of circulating microparticles (MPs), platelet derived microparticles (PMPs) and levels of activated platelets and aggregates which mimics the *in vivo* physiological environment of MPs. To the best of our knowledge, this study is the first to report on a novel approach in evaluating platelet function in HIV using a series of optimised whole blood flow cytometry based platelet assays. In addition, minimal work has been performed previously on platelet function in the context of HIV-infection; and particularly in a cohort of asymptomatic, untreated patients as defined for this study.

## Opsomming

### Inleiding

In die era van antiretrovirale terapie (ART), het mense wat met die menslike immuuniteitsgebrekswirus (MIV) leef, 'n verlengde lewensduur. 'N opkomende neiging van nie-verworwe immuuniteitsgebreksindroom (vigs) heers nou in die verouderende MIV-besmette bevolking. Verhoogde vlakke van inflammasie en chroniese immuun aktivering word geassosieer met MIV-infeksie en in die era van ART loop mense wat met MIV leef, 'n verhoogde risiko van kardiovaskulêre siekte (KVS). Plaatjies speel 'n belangrike rol in beide inflammasie en immuun aktivering en met aktivering degranuleer en skei plaatjies verskeie inflammatoriese, coagulatory en adhesie molekule af. Geaktiveerde plaatjies druk oppervlak P-selectin (CD62P) is 'n belangrike komponent van die stollings weg en dien as 'n skakel tussen inflammasie en trombose. Geaktiveerde plaatjies is in beide inflammasie en kardiovaskulêre siekte betrokke en is geïdentifiseer as immuun selle wat 'n deurslaggewende rol speel in die patogeen erkenning en modulering van immuun selle tydens infeksies. Verskeie antivirale en antibakteriese eienskappe van plaatjie alpha korrel inhoud is vasgestel. Plaatjie aggregometry bly die mees gebruikte tegniek om plaatjie funksie te evalueer, alhoewel hierdie tegniek is beperk deur baie pre-analitiese veranderlikes. Plaatjie vloeisitometrie aan die ander kant bied 'n vinnige meting van plaatjie funksie in hul fisiologiese omgewing met 'n minimale artefaktual aktivering. Min studies het egter berig op gestandaardiseerde metodes om plaatjie funksie in die konteks van MIV te evalueer. Plaatjie funksie is steeds onduidelik en data oor MIV besmet behandeling naïef individue bly skaars. Die doel van hierdie projek was om die verhouding tussen die plaatjie funksie en immuun aktivering in pasiënte met MIV te ondersoek.

### Materiaal en metodes

Hierdie studie het bestaan uit vyf sub-studies. In die eerste plekis plaatjie indekse en vlakke van plaatjie aktivering bepaal in 'n groep van 330 deelnemers (185 MIV-besmette ARV naïef en 145 onbesmette gesonde kontrole) met behulp van vloeisitometrie en hematologie ontleders. Die verhouding tussen hierdie indekse en merkers van plaatjie aktivering, die siekte se progressie en immuun aktivering is beoordeel. Verder is die vlakke van plaatjie aktivering en samevoeging in 'n groep van 82 deelnemers (41 MIV-besmette (ARV naïef) individue en 41 onbesmette gesonde kontrole) geëvalueer, met behulp van 'n nuwe vol bloed vloeisitometrie gebaseerde funksionele toets. Hierdie basislyn vlakke is dan gekorreleer met merkers van immuun aktivering en die progressie van die siekte in MIV.

In 'n daaropvolgende studie, is plaatjie funksie in 'n groep wat bestaan uit 58 MIV besmet te (ARV naïef) en 38 onbesmette beheer geëvalueer met behulp van vloeisitometrie. Plaatjie reaksie is na stimulasie gemeet met adenosine diphosphate (ADP) by konsentrasies bekend omkeer (0.04mM) te oorreed en onomkeerbaar (0.2mM) plaatjie aggregasie. Ten einde plaatjie funksie in MIV te evalueer, is plaatjie reaksie in 'n groep wat bestaan uit 58 MIV-besmette (ARV naïef) en 38 onbesmette kontrole geëvalueer. Die plaatjies is geaktiveer deur gebruik te maak van wisselende konsentrasies van ADP, is aragidoonsuur (AA) en kollageen en plaatjie funksie gemeet met behulp van vloeisitometrie. Vlakke van sirkulerende plaatjie leukosiet gemiddeldes is ook gemeet met behulp van vloeisitometrie in 'n groep wat bestaan uit 35 MIV-positiewe (ARV naïef) individue en 32 onbesmette gesonde kontrole. Assosiasies tussen leukosiet gemiddeldes, immuun aktivering en die progressie van die siekte in MIV-besmette individue is ook bepaal. Die finale studie het plaatjie-gemiddeldes, plaatjie afgelei mikrodeeltjies en mikrodeeltjies geëvalueer in 'n groep wat bestaan uit 46 MIV besmet (ARV-naïewe) en 40 onbesmette gesonde kontrole. Assosiasies tussen mikrodeeltjies, plaatjie afgelei, plaatjie gemiddeldes en merkers van immuun aktivering en die siekte se progressie is geëvalueer.

## Resultate

MIV-besmette individue het gedaalde gemiddelde plaatjie volume vlakke getoon (HIV gemiddelde  $7,91 \pm 0,85$  teen  $8,52 \pm 1,12$  teen,  $p < 0,0001$ ) wat direk gekorreleer het met CD4-tellings ( $r = -0,2898$ ,  $p = 0,0075$ ) en virale ( $r = 0,2680$ ,  $p = 0,0177$ ). Plaatjie verspreiding breedte vlakke het direk gekorreleer met ( $r = 0,3455$ ,  $p = 0,0362$ ) met 'n aktiewe koagulasie en omgekeerd gekorreleer ( $r = -0,3666$ ,  $p = 0,0463$ ) met plaatjie aggregasie. MIV-besmette individue het verhoogde vlakke van plaatjie aktivering getoon (% CD62P mediaan  $11,33$  [5,96-29,36] teen kontrole groep  $2,48$  [1,56-6,04],  $p = 0,0001$ ). In MIV, was plaatjie funksie behou en plaatjies het 'n verhoogde reaksie op submaksimale konsentrasies van endogene agoniste getoon. MIV-besmette individue het verhoogde vlakke van sirkuleer plaatjie monosiet-gemiddeldes gedemonstreer ( $25,26$  [16,16-32,28] teen kontrole groep  $14,12$  [8,36-18,83],  $p = 0,0001$ ) wat direk gekorreleer het met merkers van immuun aktivering; % CD38 / 8 ( $r = 0,54624$ ,  $p = 0,0155$ ), virale lading ( $r = 0,633$ ,  $p < 0,009$ ). Verder rapporteer ons op verhoogde vlakke van sirkulerende mikrodeeltjies (mediaan% LP  $1,7$  [0,95-2,83] teen kontrole groep  $1,12$  [0,63-1,57],  $p = 0,0160$ ); PMPs (mediaan% PMPs  $26,64$  [11,33-36,62] teen kontrole groep  $20,02$  [18,08-26,08],  $p = 0,0133$ ); geaktiveer PMPs (mediaan CD62P MFI  $3,81$  [3,46-4,54] teen kontrole groep  $3,41$  [3,16-3,6],  $p = 0,0037$ ) en plaatjie gemiddeldes (Mediaan% CD62P  $14,10$  [5,49-39,94] teen  $0,17$  [0,10-10,99],  $p = 0,0097$ ) in MIV besmet asimptomatiese individue.

## **Gevolgtrekking**

Hierdie studie ondersteun die potensiele gebruik van die MPV en PDW as waardevolle gereedlik waardevolle merkers van plaatjie aktivering en immuun aktivering in MIV. Ons het ook getoon verhoogde vlakke van geaktiveer de plaatjies in MIV-besmette individue getoon wat hyper reageer op endogene agoniste was in 'n konsentrasie-afhanklike wyse. Plaatjie vloesitometrie is 'n vinnige en waardevolle tegniek in die evaluering van plaatjie funksie in MIV. Die meting van plaatjie funksie gebruik vloei cytometry maak die evaluering van plaatjie sein paaie wat in MIV-geïnfekteerde individue verander moontlik. Laastens het ons beskryf 'n hele bloed vloesitometrie gebaseer de toets vir die evaluering van sirkulerende mikrodeeltjies, plaatjie afgelei mikrodeeltjies en vlakke van geaktiveer plaatjies en gemiddeldes wat lyk soos die *in vivo* fisiologiese omgewing van MP's. Na die beste van ons kennis, is hierdie studie die eerste om te rapporteer oor 'n nuwe benadering in die evaluering van plaatjie funksie in MIV met behulp van 'n reeks van new hele bloed vloesitometrie gebaseer de plaatjie toetse. Daarbenewens is minimale werk voorheen uitgevoer op die plaatjie funksie in die konteks van MIV-infeksie; en veral in 'n groep van asimptomatiese, onbehandelde pasiënte soos vir hierdie studie. Hierdie projek het bewyse bygevoeg tot die teorie dat plaatjies, in MIV, kan 'n skakel wees tussen die aktiewe inflammatoriese reaksie en die toename in die aantal trombotiese en kardiovaskulêre siekte waargeneem in pasiënte wat met hierdie siekte saamleef.

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## **Dedication**

In loving memory of my late grandmother

**Hlala Smangele Hettie Nkambule**

(25 December 1920 – 1 July 2013)

## Preface

This thesis is presented in the format of five articles. One of the articles has been published in a peer-reviewed journal (Nkambule *et al.*, 2014). Another article has been considered for publication pending revision. This article has been presented as chapter 3 in this thesis and appears in the format recommended by the International Journal of laboratory Haematology. The remainder of the articles will also be submitted for publication in an international peer-reviewed journal soon.

Chapter one is a brief overview of the research topic and a statement of the research problem including the aims and approach of thesis. Chapter two provides a comprehensive review on current and previously published work relating the chosen research topic. Research gaps are also identified in this section. Chapter three is presented as an original research article evaluating platelet indices in asymptomatic HIV infected treatment naïve individuals. Chapter four is presented as an article evaluating platelet function using a novel flow cytometry based platelet assay. Chapter five is presented as an original research article evaluating platelet leukocyte aggregates using a novel platelet based flow cytometry assay. Chapter six is presented as an article evaluating platelet derived microparticles, microparticles and platelet aggregates in HIV infected asymptomatic treatment naïve individuals. Chapter seven provides a conclusion and future directions of the work presented in this thesis.

### Publications and presentations

Nkambule, Bongani B., Glenda Davison, and Hayley Ipp. "The value of flow cytometry in the measurement of platelet activation and aggregation in human immunodeficiency virus infection." *Platelets* 0 (2014): 1-8.

Nkambule BB., Davison GM and Hayley Ipp. The evaluation of platelet indices and markers of inflammation, coagulation and disease progression in treatment-naïve, asymptomatic HIV-infected individuals. *International Journal of laboratory hematology*. (2014). *In press*.

## Conference presentations

### 1. Poster presentation

#### **17th European Hematology Congress 14-17 June 2012, Amsterdam, The Netherlands**

**Platelet Flow Cytometry and markers of immune activation in asymptomatic, treatment-naïve HIV infection.** Bongani B. Nkambule<sup>1</sup>, Richard Glashoff<sup>1</sup> and Hayley Ipp<sup>1,2</sup>. Divisions of Haematology <sup>1</sup> and Virology <sup>2</sup>, Department of Pathology, Stellenbosch University and NHLS, Tygerberg, South Africa

### 2. Oral presentations

#### **Pathpoint 2012 congress, SEPT 28-30. Crystal towers hotel in Cape Town, South africa**

**Platelet Flow Cytometry and markers of immune activation in asymptomatic, treatment-naïve HIV infection.** Bongani Nkambule, RH Glashoff, H Ipp. Divisions of Haematology <sup>1</sup> and Virology <sup>2</sup>, Department of Pathology, Stellenbosch University and NHLS, Tygerberg, South Africa

#### **Laboratory medicine congress 2013, JULY 28-31. Cape Town international convention centre in Cape Town, South Africa**

**The value of Flow Cytometry in the measurement of platelet activation and aggregation in human immune-deficiency virus HIV infection.** Bongani B. Nkambule, Glenda Davidson, Hayley Ipp. Divisions of Haematology, Department of Pathology, Stellenbosch University, Tygerberg <sup>1</sup>. Department of Biomedical sciences, Faculty of Health and wellness sciences, Cape Peninsula University of Technology, Bellville, South Africa <sup>2</sup>.

## Published abstracts

### EHA 17 Amsterdam, 2012 Education Book

**Platelet flow cytometry and markers of immune activation in asymptomatic , treatment naïve HIV infection.** Bongani B Nkambule<sup>1</sup>, Richard H Glashoff<sup>2</sup> and hayley Ipp<sup>1,2</sup>

Divisions of Haematology and Virology, Department of Pathology, Stellenbosch University and NHLS, tygerberg, South Africa

**Background:** In the era of antiretroviral therapy (ART), the risk of acquired immune deficiency syndrome (AIDS)-related deaths has decreased and people living with Human Immunodeficiency Virus (HIV) have prolonged life spans. HIV-infected patients are now at increased risk of developing cardiovascular disease (CVD) and other inflammatory-associated complications. Activated platelets play a key role during infection and the inflammatory process by mediating interactions between cells of innate immunity and the endothelium. Soluble markers of platelet activation are increased during HIV-infection. P-selectin (CD62P) is expressed on the platelet surface only upon platelet activation. Activated platelets play a role in HIV-induced atherosclerosis through the expression and release of mediators that induce endothelial activation and support leukocyte adhesion to the inflamed vessel wall.

**Aim:** To determine the levels of platelet activation and function by Flow Cytometry in HIV-infected individuals as compared with uninfected controls; and further, to correlate these levels with full blood count, coagulation and other inflammatory parameters.

**Methods:** In this study, a total of 57 adult South Africans were recruited from a clinic in the Western Cape. These included 32 HIV positive, ART- naïve patients and 25 HIV negative controls. Platelet activation and platelet function were investigated using a novel whole blood Flow Cytometry assay: Platelet-specific markers CD41a and CD42b were used to ensure platelet-specific gating. CD62P expression was used to evaluate platelet activation. Platelet function was evaluated by investigating the response of platelets to endogenous agonists; adenosine diphosphate (ADP) and arachidonic acid (AA) at varying concentrations. A full blood count and differential as well as fibrinogen levels were determined using routine hematology laboratory analyzers.

**Results:** Baseline levels of CD62P expression were significantly higher in treatment-naïve HIV positive patients compared with uninfected controls (mean %CD62P  $71.74 \pm 2.18$  vs control  $54.52 \pm 2.42$ ;  $p < 0.0001$ ). CD62P expression correlated directly with platelet counts ( $r = 0.374$ ,  $p = 0.042$ ) and platelet counts showed an inverse correlation with viral loads ( $r = -0.65$ ,  $p = 0.0061$ ). Fibrinogen levels correlated with absolute WCCs ( $r = 0.659$ ,  $p = 0.0021$ ); absolute neutrophil count

( $r=0.619$ ,  $p=0.0105$ ); absolute monocyte count ( $r=0.562$ ,  $p=0.0235$ ) and hsCRP ( $r=0.688$ ,  $p=0.0011$ ). Importantly, fibrinogen showed a negative correlation with CD4 counts ( $r=-0.594$ ,  $p=0.0014$ ) and may be a valuable marker of prognosis in treatment-naïve, HIV positive patients. HsCRP levels correlated with absolute neutrophil counts ( $r=0.392$ ,  $p=0.0005$ ). The HIV Group showed an overall hyper-response to ADP at a concentration  $0.025 \mu\text{M}$  as compared to uninfected controls ( $62.34 \pm 9.7$  vs control  $36.90 \pm 5.7$ ,  $p=0.0433$ ).

**Conclusions:** We describe a novel Flow Cytometry technique that may be used to evaluate the levels of platelet activation and function in HIV-infected patients. In addition we report a cost-effective panel in the form of fibrinogen, WCC and platelets that may be valuable in predicting the progression to AIDS or other inflammatory-associated complications in treatment-naïve HIV-infected patients. This may impact on patient management prior to initiation of ART and provide a tool for monitoring responses to treatment. Longitudinal studies will be required to assess risk of thrombosis based on these assays and the potential for application in other chronic inflammatory conditions.

**Laboratory medicine congress 2013, JULY 28-31. Cape Town international convention centre in Cape Town, South Africa**

**The value of flow cytometry in the measurement of platelet activation and aggregation in human immunodeficiency virus (HIV) infection.** Bongani B. Nkambule, Glenda Davidson, Hayley Ipp. Divisions of Haematology, Department of Pathology, Stellenbosch University, Tygerberg <sup>1</sup>. Department of Biomedical sciences, Faculty of Health and wellness sciences, Cape Peninsula University of Technology, Bellville, South Africa <sup>2</sup>.

**Background:** The activation status of platelets in HIV infection could be important as HIV is associated with a higher risk for thrombosis and cardiovascular disease. Platelet flow cytometry offers the assessment of platelets in their physiological state with minimal in vitro activation. Platelet P-selectin (CD62P) and platelet glycoprotein IV (CD36) have been described as markers of platelet activation and aggregation respectively.

**Objective:** To measure platelet function in HIV using a novel whole blood platelet flow cytometry assay

**Method:** 36 (ARV naïve) HIV+ve patients and 25 HIV-ve controls were recruited from a Clinic in the Western Cape. The response of platelets to adenosine diphosphate (ADP) was assessed at two concentrations (0.04mM, 0.2mM ADP). The expression of CD62P and CD36 was used to evaluate platelet function.

**Results:** %CD62P was higher in HIV+ve patients compared with uninfected controls (mean %CD62P 47.62 + 4.214 vs control 33.58+ 3.15; p=0.00335). In addition baseline levels of CD36 expression were significantly higher in HIV+ve patients compared with controls (mean %CD36 41.73 + 2.9 vs control 34.13 + 4.04; p=0.0488). Furthermore, the HIV group showed a significantly higher overall response at 0.04mM ADP (HIV mean %CD62P 69.64 + 3.9 vs control 61.17; p=0.0476) and at 0.2mM ADP (HIV group mean %CD36 80.11 + 3.98 vs control 70.39 + 1.865; p=0.0407).

**Conclusions:** We describe a platelet flow cytometry assay that assesses and discriminates between resting and activated platelets in HIV patients. We also report that, although platelets are significantly activated in HIV, they retain their functional capacity

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**List of abbreviations**

AA	Arachodonic acid
ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
APC	Allophycocyanin
ART	Antiretroviral therapy
ARV	Antiretroviral
Ca <sup>2+</sup>	Calcium
CD	Cluster differentiation
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
FBC	Full blood count
FITC	Flourescein isothiocynate
FMO	Fluorescence minus one
FSC	Forward side scatter
GIT	Gastrointestinal tract
GP	Glycoprotein
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
hs-CRP	Highly soluble C reactive protein
IL	Interleukin
LDL	Low density lipoprotein
LPS	Lipopolyssacharide
MCP-1	Monocyte chemotactic protein-1
MFI	Mean fluorescence intensity
MFO	Mean fluorescence minus one

MgCL <sub>2</sub>	Magnesium chloride
MK	Megakaryocytes
MP	Microparticle
MPV	Mean platelet volume
MVB	Multivesicular bodies (MVBs)
NETs	Neutrophil extracellular traps
NRL	Nucleotide-binding oligomerization-domain protein like receptors
PBS	Phosphate buffered saline
PCT	Plateletcrit
PDW	Platelet distribution width
PE	Phycoerythrin
Per-CP	Peridinin chlorophyll protein
PLA	Platelet leukocyte aggregate
PLA <sub>2</sub>	Phospholipase A <sub>2</sub>
PLT	Platelet
PLWH	People living with HIV
PMA	Platelet monocyte aggregates
PMP	Platelet derived microparticles
PNA	Platelet neutrophil aggregates
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PRR	Pattern recognition receptors
PS	phosphatidyleserine
RBC	Red blood cells
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SMART	Strategies for Management of anti-Retroviral Therapy

SSC	Side scatter
TNF	Tumor necrosis factor
TLR	Toll like receptor
TPO	Thrombopoietin
TXA <sub>2</sub>	Thromboxane A2
VCAM-1	Vascular cell adhesion molecule-1
vWF	von Willebrand factor

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## CHAPTER 1

### 1.1 Introduction

South Africa has the highest number of People Living with HIV (PLWH), which is estimated at 5.3 million. This represents a quarter of the burden of HIV in sub-Saharan Africa (Appay *et al.*, 2008). The improved access to antiretroviral therapies (ART) has resulted in an increase in the life span of HIV infected patients currently on uninterrupted ART (El-Sadr *et al.*, 2006). However, analysis of data from the large SMART study has shown that markers of inflammation correlate with adverse outcomes in patients on anti-retroviral regimens (El-Sadr *et al.*, 2006). Therefore, despite adequate control of viral loads in first-world countries, an era of non-AIDS related deaths, such as myocardial infarctions and cerebrovascular accidents (CVA's) now prevails (Matta *et al.*, 2008). The incidence of coronary heart disease in developed countries is three-times more in HIV-infected patients as compared to the general population (Vitetecoq *et al.*, 2003). The on-going activation of the immune system has been identified as a key driving force in the pathogenesis of these non-AIDS related deaths (Kuller *et al.*, 2008). An emerging paradigm is the close association of inflammation with thrombosis, and platelets appear to be key role players (Jahoor *et al.*, 1998). Inflammation plays an important role in platelet activation and enhances thrombin formation which further activates platelets. This therefore provides a positive feed-back system (Jahoor *et al.*, 1998). Activated platelets are found at the sites of inflammation and release a variety of pro-inflammatory mediators which amplify the inflammatory response (Woollard *et al.*, 2008). During the inflammatory response to invading pathogens, it is important for the body to regulate the balance between pro- and anti-inflammatory mediators (Woollard *et al.*, 2008). Inflammation induces an imbalance between endothelial pro-coagulant and anti-coagulant properties which is facilitated by the interaction between leukocytes, endothelial cells and platelets (Lindemann *et al.*, 2008)). Studies have suggested that unregulated on-going inflammation is a major predisposing factor to thrombotic complications in HIV-infection (Wagner *et al.*, 2003; Corrales-Medina *et al.*, 2010).

Activated platelets play a role in HIV-induced atherosclerosis through the expression and release of mediators that induce endothelial cell activation and microparticle formation which support the adhesion of leukocytes to the inflamed vessel wall (Woollard *et al.*, 2008). Platelets are the first cellular elements to accumulate at the site of vascular damage and are key elements involved in the processes of haemostasis, inflammation, atherosclerosis and tissue repair (Vandendries *et al.*, 2004). Atherosclerotic plaques contain pro-coagulant proteins and platelet

activators that are exposed upon plaque rupture (Joven *et al.*, 2006). These findings triggered interest in platelets as potential key players in forming a link between inflammation, coagulation and thrombus formation following plaque rupture. In particular, studies which have focussed on the interactions of various cells have led to the establishment of an evolving role of platelets in the inflammatory and coagulation process (Webber *et al.*, 2008). However the relevance of these interactions is not fully understood in the context of HIV. Activated platelets form complexes with neutrophils and monocytes, with subsequent neutrophil and monocyte activation (Thornton *et al.*, 2010). In addition, they induce neutrophil activation and degranulation via the expression of certain molecules such as P-selectin (CD62P) and PSGL-1 interaction (Inwald *et al.*, 2003).

## 1.2 Statement of the problem

Human immunodeficiency virus (HIV) infection is well established as an inflammatory condition. Recent studies have highlighted an increase in non-AIDS related complications such as cardiovascular disease (CVDs) and strokes, amongst people living with HIV (PLWH). Research has also shown that platelet activation and platelet induced complexes such as platelet-monocyte aggregates (PMAs) and Platelet-Neutrophil aggregates (PNAs) induce coagulation with subsequent thrombus formation in animal models. However, these complexes have not been evaluated in the context of chronic HIV infection. Thus, investigation of the levels of circulating activated platelets, PMAs and PNAs may aid in evaluating the thrombotic risk profile of PLWH. This may add value to monitoring and stratifying HIV infected patients who are at an increased risk of thrombotic events.

Platelet aggregometry has been considered as the gold standard for the assessment of platelet aggregation and function. However, standardization of this technique has proved to be complicated. Recent advances in platelet flow cytometry has provided a technique which has the potential to standardize rapid platelet function assays and which may be utilized for the evaluation of platelet function in various inflammatory conditions and malignancies.

Patients with chronic HIV-infection are at increased risk of thrombotic complications such as myocardial infarctions and strokes. Recent work has highlighted the important association between markers of inflammation and coagulation and increased risk of these adverse events. This work may have an impact on the patient management strategies in resource-limited settings. In addition, minimal work has been performed on platelet function in the context of HIV-infection; and particularly in a cohort of asymptomatic, untreated patients as defined for this study. Therefore, the aim of this project was to evaluate the relationship between platelet

function and immune activation and disease progression in PLWH using novel flow cytometric techniques.

### **1.3 Objectives of the study**

This was achieved by the following objectives:

- I. To optimize a flow cytometry based assay to measure platelet activation and aggregation, platelet derived microparticles, PMAs and PNAs.
- II. To measure baseline levels of platelet activation by flow cytometry in HIV infected and uninfected healthy controls. To correlate these baseline levels with Fibrinogen and D-dimer levels
- III. To measure platelet kinetics in HIV, by determining differences in platelet indices between HIV infected and uninfected controls. To correlate these baseline indices with markers of immune activation and disease progression.
- IV. To determine platelet function in chronic HIV infection by stimulation with the endogenous agonists; arachidonic acid (AA); Collagen and adenosine diphosphate (ADP)
- V. To measure the baseline levels of PMAs and PNAs. To measure the levels of monocyte activation and tissue factor (TF) expression and to compare these levels with PMAs. To further evaluate PMAs and PNAs post stimulation with lipopolysaccharide (LPS).
- VI. To evaluate and measure the levels of platelet derived microparticles and to correlate these levels with CD4 counts, activated CD8 cells and viral load tests

### **1.4 Study approach**

The overall objective of the study was to determine associations between platelet function and markers of immune activation and disease progression in HIV. This was achieved by evaluating parameters of platelet activation and function such as, platelet indices; cell surface expression of markers of platelet activation (CD62P) and aggregation (CD36); platelet response to endogenous agonists; platelet leukocyte aggregates and microparticles and platelet derived microparticles. The study was divided into five cross-sectional cohorts, in which each study evaluated a specific aspect of platelet activation and function. These measurements were then correlated with well-established markers of immune activation and disease progression in HIV.

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## CHAPTER 2 Literature review

### 2. Introduction

An estimated 9.7 million people living with human immunodeficiency virus (PLWH) are currently on antiretroviral treatment (ART) in developing countries (UNAIDS, 2012). South Africa has the highest number of PLWH in Sub Saharan Africa (Losina *et al.*, 2009) and the accessibility and availability of antiretroviral drugs has led to an increase in the life expectancy of PLWH (Rehle *et al.*, 2010). The aging population of PLWH are now faced with an increasing trend of inflammatory and thrombotic complications (Savès *et al.*, 2003; Matta *et al.*, 2008). Unregulated inflammation appears to drive immune activation and may be a key predisposing factor of non-AIDS related deaths (Mallon, 2013). Growing evidence supports the involvement of platelets in both immune regulation and the propagation of inflammation (Semple *et al.*, 2011).

This review focuses on the key aspects of platelet function in inflammation, coagulation and cellular interactions. Cellular components which are involved in the maintenance of haemostasis and that directly interact with platelets, such as endothelial cells, neutrophils and monocytes, will be discussed as they may regulate immune function and the inflammatory process. Although the pathogenesis of HIV is not the primary focus of this review, aspects of the immune response are briefly dealt with. PubMed and google scholar data bases were searched using the terms; "Platelet function" HIV pathogenesis", "Immune activation", "HIV ", "Platelet activation", "platelet CD62P", "Platelet function", "aggregation", "platelet CD36", "platelet signalling", "platelet agonists" "thrombosis", "Platelets in HIV", "aggregometry". Articles citing the selected sources were also accessed to identify new knowledge and advances in the reported work. The reference list of the selected sources was used to identify key sources of the current topic.

#### 2.1. Inflammation and immune activation

In order to understand the connection between platelets and the immune response a brief introduction to inflammation is required. The inflammatory response is categorised into two phases which are identified as the acute and chronic phase (Kumar *et al.*, 2007). During inflammation, it is crucial that a fine balance between pro- and anti-inflammatory mediators is maintained (Jahoor *et al.*, 1999). Inflammation plays an essential role in resolving infection or tissue damage and maintaining a state of haemostasis (Barton *et al.*, 2008). Preferably, the inflammatory response should be rapid, microbe-specific and self-limiting to avoid consequential

damage to the host as observed in chronic infections (Nathan, 2002). The physiological function and pathological consequences of inflammation are

dependent on the type of stimulus involved (Medzhitov *et al.*, 2008). Notably, an immune response is only initiated as a result of an infection or antigen and is facilitated by receptors of the innate immune system which include toll-like receptors (TLRs), pattern recognition receptors (PRR) and nucleotide-binding oligomerization-domain protein like receptors (NLRs) (Medzhitov *et al.*, 2008, Barton *et al.*, 2008). Signal transduction, facilitated by TLRs and PRRs, are crucial in the recognition of pathogens and initiation of the inflammatory response. (Medzhitov, 2007). The acute inflammatory response coordinates the delivery and recruitment of plasma components and leukocytes to the sites of infection (Kumar *et al.*, 2007) and is coordinated by a complex regulatory network of mediators of inflammation (Keating *et al.*, 2012). These mediators are categorized based on their biochemical properties (table 2.1) (Medzhitov, 2008).

If pathogen induced stimulation persists as a result of inadequate clearance, the inflammatory process acquires new characteristics which are associated with chronic inflammation (Kumar *et al.*, 2007). The chronic inflammatory process is characterised by the infiltration of macrophages, T cells and subsequent granuloma formation (Drayton *et al.*, 2006). The inflammatory pathway mainly consists of four components which involve; inducers which are triggers (Infection or tissue injury), sensors (cell receptors such TLRs), mediators (cytokines and chemokines) and effectors (tissues and cells affected by mediators of inflammation) (Medzhitov *et al.*, 2008). However, the signalling mechanisms of these pathways and mediators are beyond the scope of this review and will not be discussed in detail.

**Table 2.1. Inflammatory mediators** (compiled by author based on work by Medzhitov, 2008)

<b>Mediator</b>	<b>Mediator</b>	<b>Effects</b>
<b>Vasoactive amines</b>	Histamine, serotonin	vascular permeability, vasodilation, vasoconstriction
<b>Vasoactive peptides</b>	Substance P, kinins	Vasodilation, Vasoconstriction
<b>Complement fragments</b>	Anaphylatoxins	Monocyte & granulocyte recruitment, mast cell degranulation
<b>Lipid mediators</b>	Eicosanoids & platelet activating factors	Vasodilation, vasoconstriction, increased vascular permeability & platelet activation
<b>Cytokines</b>	TNF $\alpha$ , IL-1, IL-6	Endothelium, leukocyte activation, induction of acute response
<b>Chemokines</b>	CXCL12, CCL9, CCL20, CCL21	Leukocyte extravasation and chemotaxis
<b>Proteolytic</b>	Elastin, cathepsins, matrix metalloproteinases	Degradation of ECM and basement-membrane proteins

### 2.1.1. Characteristics of the inflammatory response

Inflammation has been well described in microbial infections, where macrophages and mast cells which reside in tissue, recognise foreign structures and secrete inflammatory molecules (Kumar *et al.*, 2007; Medzhitov *et al.*, 2008). During injury, immune cells secrete proinflammatory cytokines; chemokines; prostaglandins and bradykinins that constitute an inflammatory milieu that induce immune activation (Chen *et al.*, 2013). These include interleukin 1 $\beta$ ; tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and monocyte chemoattractant protein-1 (MCP-1) (Liao, 2013). Cytokine induced endothelial activation results in the extravasation of neutrophils while retaining red blood cells in the vasculature (Pober *et al.*, 2007). During activation, endothelial cells express surface adhesion molecules such as VCAM-1, ICAM-1 and E selectin (Liao, 2013).

Although several haemostatic functions of inflammation remain unclear, extensive work has been done to unravel the physiological role of the host immune response in pathogen-induced inflammation (Medzhitov *et al.*, 2008). The inflammatory process is also characterised by

multiple interactions between leukocytes and platelets (Semple & Freedman, 2010) which are facilitated by P-selectin expressed on the surface of activated platelets and endothelial cells (Romo *et al.*, 1999). Platelets initiate and propagate inflammation including immune mediated inflammation (table 2.1) (Andrews *et al.*, 2006). The inflammatory process is mediated by various cytokines and chemokines which are secreted upon immune activation (Keating *et al.*, 2012). Platelets have been identified as key role players in the regulation of vascular inflammation (Semple & Freedman, 2010) and acute inflammation has been linked with activation and impairment of the coagulation system (Levi *et al.*, 2008). Mechanisms of inflammation induced coagulation, involve the extrinsic coagulation pathway in which tissue factor directly initiates the activation of the clotting cascade (Levi *et al.*, 2010). In addition, active coagulation is regulated by three main anticoagulation pathways all of which are impaired during the inflammation process (Levi & van der Poll, 2005).

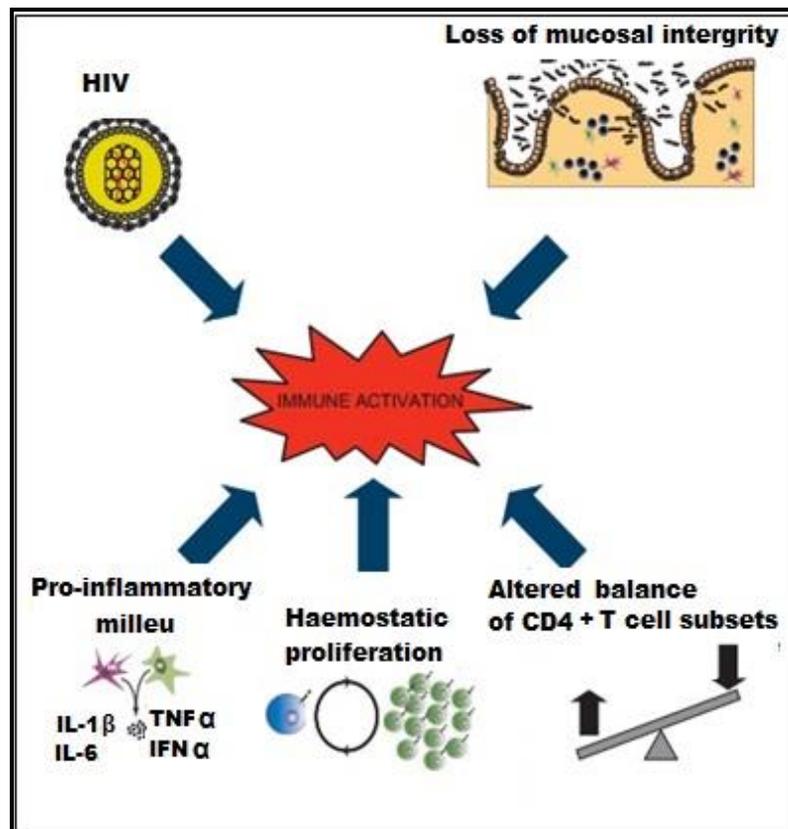
### **2.1.2. Immune activation and HIV**

HIV targets CCR5<sup>+</sup> CD4<sup>+</sup> T Cells and to a less extent macrophages and dendritic cells (Siliciano *et al.*, 2003; Gresele *et al.*, 2012). CCR5<sup>+</sup> CD4<sup>+</sup> T cells which are found in the gastrointestinal tract (GIT) are predominantly activated memory CCR5<sup>+</sup> CD4<sup>+</sup> T cells (Appy & Sauce, 2008). These memory T cells are susceptible to viral infection due to their inherent activation status (Brenchley *et al.*, 2004). The consequences of immune activation and inflammation in HIV have been comprehensively reviewed in the literature (Appy & Sauce, 2008; Ipp & Zemlin, 2013). The knowledge gained from studies conducted on simian immunodeficiency virus (simian equivalent of HIV) hosts, suggest that chronic immune activation is a key contributor to pathogenic infection (Chahroudi *et al.*, 2012). Furthermore, research has also shown that levels of T cell activation remain high in HIV infected individuals despite successful antiretroviral therapy (ART) (Sauce *et al.*, 2011).

Elevated markers of lymphocyte and monocyte activation have been described in PLWH (Grossman *et al.*, 2002; Deeks *et al.*, 2004; Funderburg *et al.*, 2010; Karim *et al.*, 2013) which has led to the theory that immune activation is a key role player in HIV infection (Rotger *et al.*, 2011; Sauce *et al.*, 2011). Previous observations highlight the vicious cycle of inflammation, immune activation and HIV replication (Decrion *et al.*, 2005). In addition, a positive feedback mechanism which involves HIV replication and immune activation resulting in CD4<sup>+</sup> T cell depletion has been described (Appy & Sauce, 2008). The gradual depletion of CD4<sup>+</sup> T cells is a characteristic feature of untreated HIV infection (Deeks *et al.*, 2004) and several direct and

indirect mechanisms may account for this phenomenon (Gasper-Smith *et al.*, 2008; Richard *et al.*, 2010).

Immune activation is a major cause of CD4<sup>+</sup> T cell depletion (Grossman *et al.*, 2002). The loss of mucosal integrity and translocation of bacterial products into the circulation have been described as a contributor to the overall immune activation in HIV (Cassol *et al.*, 2010) (figure 2.1). HIV infection is characterised by increased T cell activation which may result in the apoptosis of T cells (Duoek *et al.*, 2002). Furthermore, T cell activation and apoptosis could initiate the formation apoptotic microparticles (MPs) which have been implicated in immune suppression (Frleta *et al.*, 2012). Furthermore, many reports have indicated the survival benefits of early ART initiation on immune reconstitution. This has been demonstrated by the measurement of circulating CD4<sup>+</sup> T cells (Hunt *et al.*, 2003; Goicoechea *et al.*, 2006).



**Figure 2.1.** Proposed contributors to HIV-associated chronic immune activation (adapted from Paiardini & Müller-Trutwin, 2013)

Persistent T-cell activation induces increased CD4<sup>+</sup> T cell turn over which may result in the exhaustion of the immune system and alterations in the regenerative capacity of immune cells (McCune *et al.*, 2001). ART restores the concentration of circulating T-cells via mechanisms that

include; decreasing cell turnover; redistribution of T cells and increased thymic output (Duoek *et al.*, 2000). However, complete reconstitution of CD4<sup>+</sup> T cells still remains a major challenge (Garcia *et al.*, 2004; Moore *et al.*, 2007). Alterations in the homing of CD4<sup>+</sup> cell to the gut mucosal membrane, contributes to the mechanisms which inhibit the complete reconstitution of CD4<sup>+</sup> cells in the GIT (Mavigner *et al.*, 2012). Chronic immune activation persists in HIV infected individuals which leads to several inflammation associated complications in both ARV naïve and individuals on ART (Kaplan *et al.*, 2011). Elevated levels of pro-inflammatory cytokines have been described in HIV (table 2.1) (Paiardini & Müller-Trutwin, 2013) and this pro-inflammatory cytokine milieu further enhances the hypercoagulable state (Paiardini & Müller-Trutwin, 2013).

Elevated levels of pro-inflammatory cytokines such as; interleukin 1 $\beta$  (IL-1 $\beta$ ); interleukin 6 (IL6) and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ); have been described in HIV (Appy & Sauce, 2008). IL-1 $\beta$ , a major activator of endothelial cells is associated with CD4 T cell loss during the acute phase of HIV, whereas, IL-6 and TNF $\alpha$  are associated with disease progression in the chronic phase of the infection (Paiardini & Müller-Trutwin, 2013). Macrophages, activated by tumor Necrosis Factor  $\alpha$  (TNF $\alpha$ ), induce the expression of the surface adhesion molecules P-selectin and E-selectin on endothelial cells (Wagner *et al.*, 2003). These mediate the tethering of leukocytes to the endothelial cells and facilitate rolling along this surface (Joven *et al.*, 2006). Endothelial dysfunction and increased expression of adhesion molecules and inflammatory cytokines such as, vascular cell adhesion molecule-1 (VCAM-1), P-selectin, monocyte chemo-attractant protein-1 (MCP-1) and von Willebrand factor (VWF), have been described during chronic HIV infection (Joven *et al.*, 2006). Activated platelets secrete IL-1 $\beta$  amongst several chemokines, cytokines and coagulation factors involved in immune activation and coagulation (discussed in section 2.6.1) (Zarbock *et al.*, 2007).

### **2.1.3. Markers of immune activation in HIV**

Several markers of immune activation and disease progression have been described in HIV (Hazenberg *et al.*, 2003). Notably, markers of T cell activation and exhaustion have been shown to be markedly increased. The expression of CD38 on CD8<sup>+</sup> T cells a marker of early T cell activation has been established as a strong predictor of disease progression in HIV (Liu *et al.*, 1998; Tuailleon *et al.*, 2009). In addition, studies have shown associations between HIV, viral RNA levels and CD38 expression on CD8 + T cells (Tuailleon *et al.*, 2009). Interestingly, these included markers of inflammation and active coagulation. In the large SMART study elevated D-

dimer levels were associated with increased mortality and risks of cardiovascular disease (El-Sadr *et al.*, 2006).

Recent studies have established the pivotal role that activated platelets play in immune activation, inflammation and coagulation (Carter *et al.*, 2012; Morrell *et al.*, 2014). This has led to the recognition of platelets as immune cells that link inflammation with thrombosis (von Hundelshausen and weber, 2007; Ware *et al.*, 2013). Platelets express a repertoire of surface membrane receptors that enable them to directly interact with various cells and pathogens (Rivera *et al.*, 2009; Flaujac *et al.*, 2010) and the engulfment of HIV particles by activated platelets has been described. (Chaipan *et al.*, 2006). However, the physiological consequences of these interactions still remain unclear (Flaujac *et al.*, 2010).

#### **2.1.4. Markers of platelet activation and coagulation**

Several biomarkers of inflammation have been described in HIV (Armah *et al.*, 2012). Fibrinogen is an acute phase reactant (Jahoor *et al.*, 1999) and a precursor of the blood coagulatory protein fibrin, while the degradation of fibrin to D-dimer is a measure of the formation and subsequent dissolution of blood clots (Thorton *et al.*, 2010). Platelets interact with fibrinogen via their CD 41a (GPIIb/IIIa) receptor and this may result in platelet activation and secretion of P-selectin (CD62P). The expression of P-selectin (CD62P) on platelets is a well-recognized marker of platelet activation (Vandendries *et al.*, 2004). This adhesion molecule is of interest because of its role in modulating interactions between blood cells and the endothelium, and the possible use of the soluble form as a plasma predictor of adverse cardiovascular events (Ridker *et al.*, 2001). Although present on the external cell surface of both activated endothelium and activated platelets, it now seems clear that most, if not all, of the measured plasma P-selectin is of platelet origin (Ridker *et al.*, 2001). Increased levels of soluble P-selectin, measured in citrated plasma, have been shown to predict which patients are at risk of an adverse cardiovascular event (Ridker *et al.*, 2001; Blann *et al.*, 2003). Of interest, elevated levels of CD62P have been reported in HIV infected individuals (Mayne *et al.*, 2012; Nkambule *et al.*, 2014)

Platelet Glycoprotein (GP) IV (CD36) is a class B scavenger receptor that is involved in angiogenesis; atherosclerosis; inflammation and lipid metabolism (Podrez *et al.*, 2007). Increased CD36 expression upon platelet aggregation has been described (Berger *et al.*, 1993; Ghosh *et al.*, 2008; Nkambule *et al.*, 2014). Berger *et al.*, using thrombin-stimulated platelets demonstrated an increase CD36 expression on fully degranulated platelets (Berger *et al.*, 1993). The authors further showed that the intracellular pool of CD36 is associated with the open

canicular system and the inner surface of the  $\alpha$ -granule membrane (Berger *et al.*, 1996). A study conducted by Aiken *et al.*, showed increased platelet aggregation induced by anti-CD36 antibody clone OKM5 at a concentration of 20nmol/l in the presence of calcium and magnesium. Binding of the antibodies induced platelet aggregation and alpha granule and dense granule secretion (Aiken *et al.*, 1990). CD36 acts as a thrombospondin (TSP) receptor and is a component of the multiprotein complex (TSP/CD36/GPIIb-IIIa/Fg) found on activated platelets. This complex formed by the colocalization of TSP with GPIIb-IIIa-fibrinogen (Fg) (Leung *et al.*, 1984) and it may play a role in the consolidation of platelet aggregates (Leung *et al.*, 1982). Notably CD36 mediated signalling has been recently described in signalling events that occur post aggregation (Silverstein, 2014). Elevated levels of fibrinogen have also been correlated with higher risk of vascular inflammatory conditions such as hypertension and atherosclerosis (Menezes *et al.*, 2009) and importantly, in various inflammatory conditions increased levels of D-dimers have been shown to correlate with coagulation activity and increased risks of thrombotic events (Menezes *et al.*, 2009). Previous studies have demonstrated that oxidized lipids may induce activation of platelets via the CD36 receptor (Klinger *et al.*, 2002). The authors proposed that this could be a possible explanation for the association of high levels of low-density lipoprotein cholesterol and thromboembolic risks, in a population with high baseline levels of LDL cholesterol. Notably, data on the expression of CD36 in HIV cohorts is scarce. Increased levels of platelet CD36 have however been described in both diabetes and HIV (Alkhatatbeh *et al.*, 2013; Nkambule *et al.*, 2014).

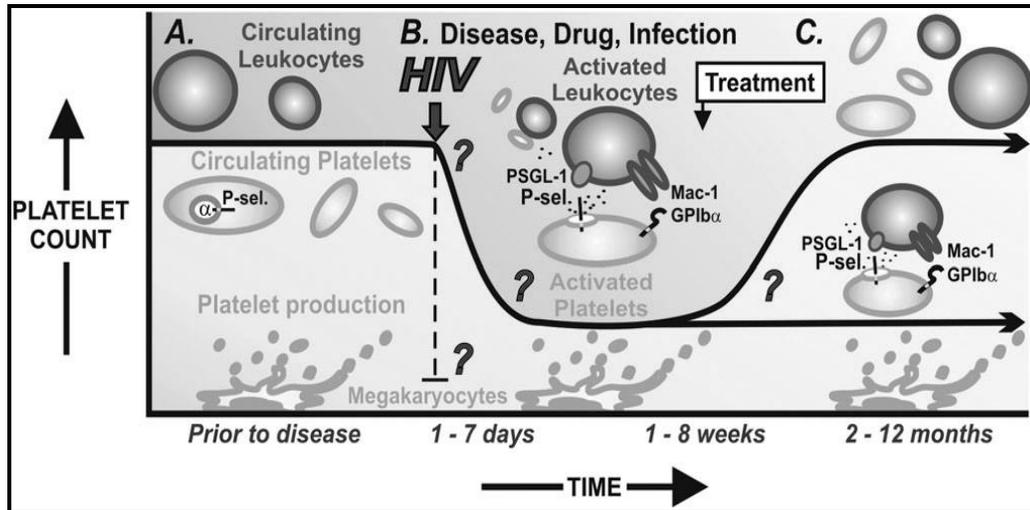
## 2.2. Megakaryopoiesis and thrombopoiesis

Megakaryocytes (MKs) are derived from hematopoietic stem cells (Geddis *et al.*, 2010). Upon differentiation into megakaryoblasts, these cells lose the capacity to proliferate and undergo maturation via endomitosis, a process involving nuclear division without cytoplasmic divisions (Bluteau *et al.*, 2009). This results in the formation of polyploid MKs in the bone marrow (Hoffmann *et al.*, 2014). The degree of MK ploidy is associated with both the rate of platelet production and peripheral platelet concentrations (Kuter, 1996; Raslova *et al.*, 2003; Hoffmann *et al.*, 2014). Megakaryoblasts proliferate and differentiate in response to thrombopoietin (TPO) stimulation (Geddis *et al.*, 2010; Morita *et al.*, 2010). TPO is a major regulator of megakaryopoiesis that binds c-Mpl, expressed on the surface of MKs (Schulze *et al.*, 2005; Machlus *et al.*, 2014). MKs are the largest hematopoietic cells present in the bone marrow milieu (Geddis *et al.*, 2010).

In normal haemostasis, MKs regulate the rate of platelet formation and maintain a constant plateletcrit (Machlus *et al.*, 2014). The exact mechanisms underlying platelet formation remain unclear, however two models of platelet formation have been proposed. Firstly, the fragmentation model in which platelets are formed as a result of MK fragmentation in the lungs and secondly, the proplatelet model which involves the formation of pseudopodial extensions of MKs that extend into marrow sinusoids (Italiano *et al.*, 1999; Richardson *et al.*, 2005; Junt *et al.*, 2007). Interestingly Schwertz *et al.*, reported on the capability of platelets to undergo cell division and generate functional progeny (Schwertz *et al.*, 2010) however the underlying biochemical mechanisms of these models are beyond the scope of this review and will not be discussed in detail.

### **2.2.1. HIV and the megakaryocyte lineage**

HIV associated thrombocytopenia (HAT) has been reported as a common clinical manifestation of HIV. In a previous meta-analysis, a HAT prevalence of 5-30% in HIV infected individuals was reported (Liebman *et al.*, 2007). However in the era of cART the prevalence of HAT has significantly declined, with a reported prevalence of 3.2% (Marks *et al.*, 2009). Thrombocytopenia is recognized as an independent predictor of mortality in untreated HIV infection (Johannessen *et al.*, 2008). Interestingly, in haemophiliacs thrombocytosis is associated with disease progression and mortality (Rieg *et al.*, 2007). Thrombocytosis has been implicated in immune reconstitution inflammatory syndrome (IRIS) and in HIV-associated CVDs (Espinosa *et al.*, 2010). HAT is dependent on the phase of HIV infection, an initial transient drop in platelet counts occurs in the acute phase of infection (Figure 2.2) with a the subsequent persistent decline during chronic infection (Wachtman *et al.*, 2006).



**Figure 2.2 Human immunodeficiency virus (HIV) related thrombocytopenia (Gardiner *et al.*, 2013).**

The figure illustrates effects of HIV on platelet kinetics. In normal healthy individuals (A) Platelets circulate in an inactivated state and retain P-selectin in the  $\alpha$ -granules. Platelet counts are maintained by the balance between clearance and megakaryocyte mediated thrombopoiesis. Thrombocytopenia as result of HIV or other causes may be associated with enhanced platelet-leukocyte aggregates (B) and megakaryopoiesis is inhibited by HIV GP120 interactions (Zhang *et al.*, 2010). HIV induces platelet activation via direct and indirect mechanisms this results in platelet degradation and exocytosis of P-selectin to the surface of activated platelets. (C) After treatment it is unpredictable whether normal platelet count recovers. In HIV, or other infection, autoimmune or drug-induced thrombocytopenia, systemic effects on platelet clearance and /or megakaryocytes/platelet production may complicate both the clinical diagnosis and monitoring of treatment.

The aetiology of HAT in the era of highly active antiretroviral therapy HAART, points to HIV RNA replication itself rather than low CD4 counts or AIDS related disease (Marks *et al.*, 2009). The underlying mechanisms are complex and are multi faceted. HIV directly interacts with megakaryocytes (MacEneaney *et al.*, 2011) and mature platelets (Flaujac *et al.*, 2010). Thrombopoietin (TPO) is a major cytokine involved in regulating thrombopoiesis and platelet counts (Michele *et al.*, 2007). MacEneaney *et al.*, demonstrated the effects of HIV GP120 protein on the differentiation of TPO induced megakaryocytes. The authors suggested that GP120 induces increased transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) and APRIL down-regulation which results in the apoptosis of GPIIb/IIIa (CD41) and CD61 subsets (MacEneaney *et al.*, 2011). Zhang *et al.*, further demonstrated that in the context of HIV, thrombocytopenia may be the result of the inhibition of megakaryopoiesis through c-Mpl facilitated inhibition (Zhang *et al.*, 2010). The authors assessed the role of the V3 loop of the GP120 envelope protein in the lineage specific inhibition of megakaryopoiesis. They constructed HIV-1 V3 loop clones from isolates obtained from thrombocytopenic HIV-1 infected patients and using the SCID-hu Thy model, the authors reported significant loss in the expression of the Tpo receptor (c-Mpl) from the SCID-hu implants. They concluded that the Tpo/ c-Mpl signalling pathway plays a pivotal role

in megakaryopoiesis and that decreased expression of c-Mpl may inhibit the differentiation of CD34+ progenitor cells into megakaryocytes (Zhang *et al.*, 2010).

### **2.3. Platelet morphology and granules**

Platelets circulate as anuclear discs with a small diameter of 2-3 $\mu$ m (Linden & Jackson *et al.*, 2010). Three distinct zones have been described in platelets which include; (1) a peripheral zone that consists of coagulation factors and endogenous platelet agonists (Rendu *et al.*, 2001); (2) sol-gel zone that contains the open canalicular and dense tubular system; (3) organelle zone that contains platelet granules and several chemokines and mitogenic factors (Cimmino & Golino, 2013). The platelet granules contain several chemokines, coagulation proteins and immunological molecules (Blair & Flaumenhaft, 2009).

#### **2.3.1. Platelet granules**

Platelets contain three distinct types of granules: alpha ( $\alpha$ ) granules, dense granules and lysosomes (figure 2.3) (Blair & Flaumenhaft, 2009; Fitch-Tewfik *et al.*, 2013). Alpha granules are the largest (200-400nm) and most abundant type of granules found in platelets (Harper *et al.*, 2010; Blair & Flaumenhaft, 2009). The dense granules are the smallest (150nm) and contain high levels of phosphate and calcium ( $\text{Ca}^{2+}$ ) amongst their granular contents (Rendu *et al.*, 2001).

Lysosomes are intermediate in size (200-250nm) and contain hydrolytic enzymes that are active towards constituents of the extracellular matrix (ECM). Lysosomes may be released during platelet activation, however a more potent stimulus is required compared to that which facilitates  $\alpha$  granule secretion (Ren *et al.*, 2007). The functional role of lysosomes remain unclear, however they have been associated with the regulation of thrombus formation and remodelling of the ECM (Ciferri *et al.*, 2000). Interestingly, Thon *et al.*, described the presence of a novel T granule that contains toll like receptor 9 (TLR9) transcripts and may play a crucial role in platelet mediated immune regulation (Thon *et al.*, 2012).

## 2.4. Platelet function

The essential role that platelets play in wound healing and inhibition of haemorrhage has been well established. Platelet receptors and endothelial cell molecules play a crucial role in inducing platelet activation and aggregation which is necessary to maintain haemostasis. Upon vascular injury, molecules present on the extracellular matrix (ECM) are exposed. Amongst these, vWF and collagen are the most potent platelet agonists which induce platelet activation and irreversible platelet aggregation (Ruggeri *et al.*, 2006; Kuijpers *et al.*, 2009). Several ECM components react with platelets such as fibronectin, laminin and thrombospondin (Jurk & Kehrel, 2005) however this review will only focus on major platelet receptors and signaling pathways involved in overall platelet function.

Platelet activation results in the secretion of platelet derived chemokines and molecules that are involved in inflammation, proliferation vasoconstriction and coagulation (Projahn & Rory, 2012). In normal haemostasis platelets monitor and maintain vascular integrity (Li *et al.*, 2010). Although platelets lack a cellular nucleus they contain mRNA and translational machinery which are necessary for protein synthesis (Semple *et al.*, 2011). Changes in normal platelet function have been described in coagulopathies (Berger *et al.*, 2010; Klovaite *et al.*, 2011). Shear stress plays a crucial role in the modulation of platelet function (Hathcock *et al.*, 2006; Reininger *et al.*, 2009). The force generating blood flow produces shear stress, which is associated with flow rate, blood viscosity and vessel diameter (Cimmino & Golino, 2013). Platelet activation may occur in pathologically high shear pressure (Brass, 2010). In general, platelet activation is facilitated by platelet agonists and surface receptor interactions (Brass, 2010).

The shear rate increases with elevated shear force and decreases with increasing blood viscosity (Cimmino & Golino, 2013). Platelets, a major contributor to arterial thrombosis in diseases such as atherosclerosis, are associated with increased shear stress (Jennings, 2009). Platelet adhesion under high shear rates is facilitated by vWF molecules which uncoil from their globular shape and stretch. This action exposes the vWF A1 domain (Schneider *et al.*, 2007) which allows the platelet GPIb $\alpha$  receptor to bind to the exposed A1 domain. This process may result in platelet activation (Nesbitt *et al.*, 2002). vWF is the only subendothelial component that is capable of binding circulating platelets to the endothelium at high shear conditions (Ruggeri *et al.*, 2006). Interestingly, under conditions of high shear rates platelet aggregation may occur independently of platelet activation (Ruggeri *et al.*, 2006). Circulating non activated platelets directly interact with soluble vWF via the GPIb $\alpha$  receptor, resulting in the formation of platelet aggregates and promoting platelet recruitment without inducing platelet activation (Reininger *et al.*, 2009). Platelet function can be classified into four distinct steps which involve several

biochemical and cellular processes. These include adhesion; activation; secretion and aggregation (Cimmino & Golino, 2013).

#### 2.4.1. Adhesion

Circulating platelets are non-adherent and are activated upon exposure to soluble platelet agonists and adhesive proteins immobilised at sites of vascular injury (Li *et al.*, 2010). Platelet adhesion and aggregation are characterised by the transition of platelets from freely circulating in blood to binding and interacting with ligands expressed on the endothelial surface (Ruggerrri *et al.*, 2007). Platelet adhesion and aggregation are two distinct biochemically complex processes which are essential for normal platelet function (Cimmino & Golino, 2013). Platelet adhesion involves the tethering of platelets on the vessel wall or the extracellular matrix upon vascular injury (Vargo-Szabo *et al.*, 2008). This process is dependent on the collective function of platelet cell surface receptors which facilitate the adhesion of platelets to the endothelial surface resulting in the activation and aggregation of platelets (Ruggerrri *et al.*, 2007). Platelet adhesion is facilitated by a receptor complex glycoprotein (GP) Ib/V/IX (Cimmino & Golino, 2013).

The initial tethering of platelets is primarily dependent on GPVI and subendothelial collagen interactions (Wu *et al.*, 2002; Vargo-Szabo *et al.*, 2008). These interactions result in the adherence of platelets onto the endothelial surface (Brass, 2010). In addition, P-selectin (CD62P) plays a crucial role in the adhesion of platelets to activated endothelial cells via PSGL-1 and GPIb receptor interactions (van Gils *et al.*, 2009). P selectin is largely responsible for platelet leukocyte interactions which lead to the rolling and subsequent activation of leukocytes bound to the activate endothelium (Maugeri *et al.*, 2009). This process is facilitated by chemokines such as monocyte chemoattractant protein I (MCP-I) and RANTES (von Hundelshausen *et al.*, 2001). Mechanisms facilitating platelet leukocyte interactions are discussed in later sections of this review.

In response to vascular injury platelets are activated by thrombin and collagen (Brass, 2010). Several collagen receptors are expressed on the surface of platelets that are involved in platelet tethering and adhesion such as; GPVI and GPIa/IIa ( $\alpha_2\beta_1$  integrin) (Vargo-Szabo *et al.*, 2008; Angiolillo *et al.*, 2010). GPIa mediates the adhesion of platelets to collagen whereas GPIV plays a crucial role in the signalling transduction which results in collagen induced platelet activation (Watson & Gibbins *et al.*, 1998).

Adhesion receptors play a crucial role in initiating platelet activation at sites of vascular injury (Li *et al.*, 2010). GPIb or platelets glycoprotein (GP) Ib $\alpha$  is a receptor densely expressed on the surface of platelets and binds to the exposed vWF A1 domain. Platelet signalling pathways

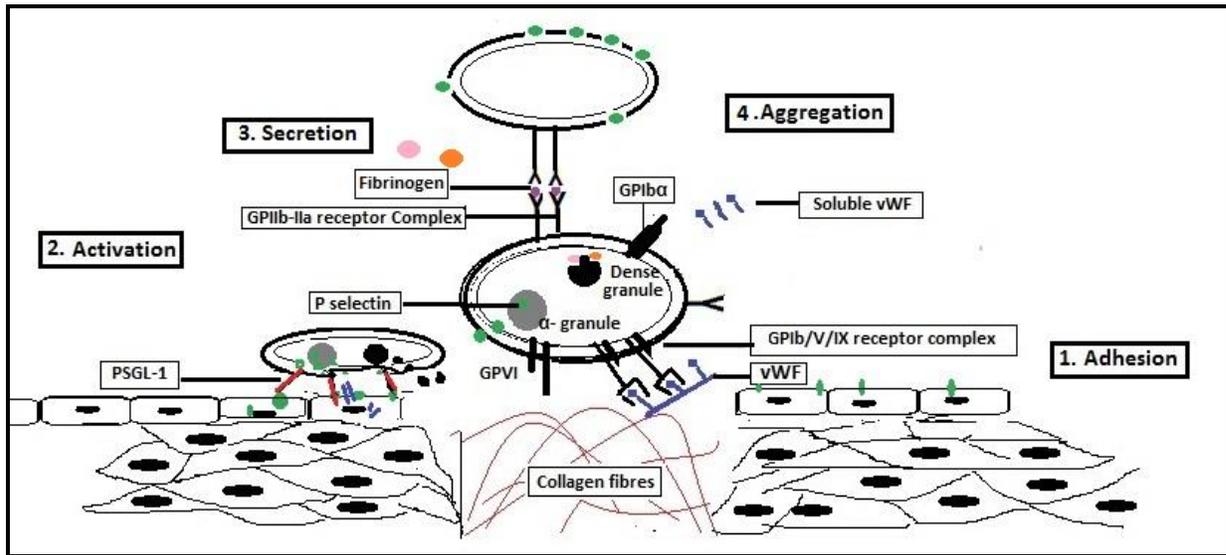
converge into similar signal transduction pathways involving Src family kinases, phosphoinositide 3-kinases and immunoreceptor tyrosine-based activation motif (ITAM) (Li *et al.*, 2010). Using an animal model, Massberg *et al.*, described the crucial role played by platelet adhesion receptors GPIIb $\alpha$  and GPIIb $\alpha$   $\beta_3$  (Massberg *et al.*, 2002). These authors further suggested that activated platelets promote atherosclerosis and that inhibition of platelet adhesion receptors including GPIIb and P-selectin (CD62P) may result in the reduction of atherosclerotic lesions (Massberg *et al.*, 2005).

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is also an adhesion receptor, expressed on endothelial cells, platelets and leukocytes (Delisser *et al.*, 1994; Watt *et al.*, 1995). PECAM-1 plays a key role in the adhesion and extravasation of leukocytes during inflammation and is a mediator of cell activation (Osawa *et al.*, 1997). The role of PECAM-1 (CD31) in cell-cell adhesion has been described and this involves hemophilic or heterophilic interactions (Albelda *et al.*, 1992; Sun *et al.*, 1996; Newton *et al.*, 1997). Using CD31-deficient mice Duncan *et al.*, demonstrated that CD31 is not crucial in the formation of platelet aggregates and vasculogenesis (Duncan *et al.*, 1999). While Mahooti *et al.*, demonstrated that in CD31-deficient mice platelet-endothelial interactions were disrupted and this resulted in an increased bleeding. Recent work by De Cuyper *et al.*, also suggested the use of CD31 as a platelet marker in the evaluation of platelet function as using monoclonals targeting this receptor do not interfere with the platelet aggregation (De Cuyper *et al.*, 2013)

#### **2.4.2. Activation**

Platelet activation is initiated by several agonists in a process facilitated by membrane receptors and complex signal transduction pathways (Varga-Szabo *et al.*, 2009). During platelet activation, platelet morphology changes from discoid to spherical with filopodia extrusions (Dunois-Larde *et al.*, 2009; Jennings *et al.*, 2009). Filopodia are enriched with GPIIb/IIIa and molecules involved interactions with platelets in the immediate environment (Brisson *et al.*, 1997). This process involves several intracellular signal transduction pathways involving; increased intracellular  $Ca^{2+}$ ; phosphoinositide metabolism; phosphorylation and nuclear proteins. Platelet shape change leads to actin polymerization and the formation of pseudopodia (Jurk & Kehrel, 2005). During platelet activation, degranulation and secretion of granular contents occurs (Nieswandt *et al.*, 2003). P-selectin stored in the platelet  $\alpha$  granules translocates to the membrane surface and mediates interactions between endothelial cells, peripheral circulating platelets and leukocytes (Linden *et al.*, 2007; Maugeri *et al.*, 2009). The interactions between vWF and collagen result in

increased affinity of collagen (Cruz *et al.*, 2005). Collagen is regarded as a potent platelet agonist (Wagner *et al.*, 1996).

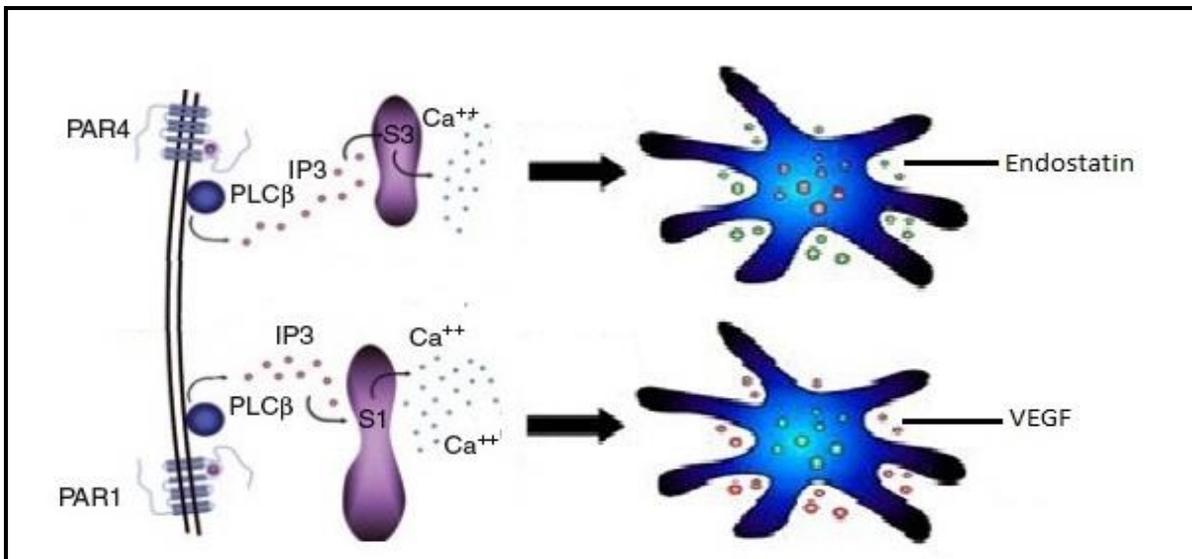


**Figure 2.5 Aspects of platelet function** (compiled by author, based on work Rugggeri *et al.*, 2007; Vargo-Szabo *et al.*, 2008). The schematic diagram illustrates the steps involved in platelet function. (1) Platelet adhesion is facilitated by adhesion receptors GPIb/V/IX and vWF interactions. (2) Activation involves the platelet degranulation and surface P-selectin expression. (3) Secretion of platelet granular contents facilitate the recruitment of circulating platelets and activation of leukocytes. (4) Aggregation occurs as a result of interactions between activated platelets involving the fibrinogen receptor GPIIb-IIIa complex. Platelet aggregation may also occur as a result of soluble vWF and GPIb $\alpha$  interactions.

### 2.4.3. Secretion

Platelet activation induces platelet degranulation and secretion of chemokines; cytokines; growth factors and coagulation factors stored in the platelet granules (Linden *et al.*, 2007). The majority of platelet chemokines are released from the  $\alpha$ -granules upon activation (Brandt *et al.*, 2000). Activated platelets secrete several endogenous agonists such as ADP, TXA<sub>2</sub> and serotonin (Dale *et al.*, 2002). The secretion of platelet granules is a highly orchestrated process and may be dependant on specific transduction signals rather than the general release of platelet granular constituents (Italiano *et al.*, 2008). PAR-4 signalling results in the release of anti-angiogenic granular contents such as endostatin while suppressing the secretion of angiogenic molecules such as vascular endothelial growth factor (VEGF) (Ma *et al.*, 2005). In contrast, PAR-1 facilitates the release of VEGF while inhibiting the secretion of endostatin (shown in Figure 2.6) (Ma *et al.*, 2005). PAR1 and PAR2 are major platelet receptors and their role in

signalling pathways are mentioned in later sections of this review. The process of platelet secretion is orchestrated and stimuli-dependant (White & Rompietti, 2007) and is critical for normal haemostasis (Cimmino & Golino, 2013).



**Figure 2.6 Signalling-dependant granule secretions** (Adapted from Italiano *et al.*, 2008; White & Rompietti, 2009). The figure illustrates differential secretion of platelet granules that is dependent on the type of signalling receptors involved. PAR1 signalling results in VEGF secretion whereas PAR 4 facilitated signalling results in endostatin secretion.

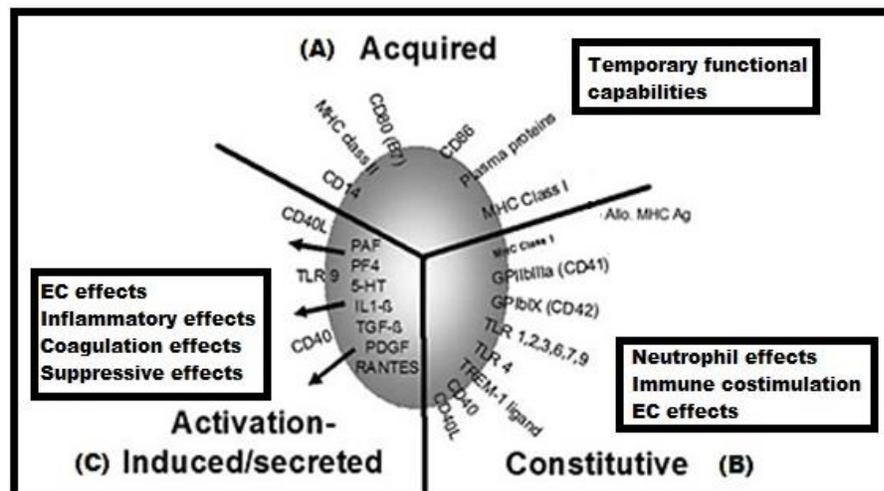
## 2.5. The immunological role of platelets in infectious disease

The function of platelets as solely role players within haemostasis has changed and they are now considered as also having a role within the immune system (Semple *et al.*, 2011). Platelets are involved in immune surveillance and the relay of pathogen associated information to innate immune cells (Beaulieu *et al.*, 2010). They express several immunoregulatory molecules on their surface which include; CD40 and CD40L. These two surface proteins are involved in both T and B cell co-stimulation (Semple *et al.*, 2011). In addition, platelets express a variety of surface toll-like receptors which contribute to the enhanced platelet activity and thrombosis (Panigrahi *et al.*, 2013). The interaction between platelets with various bacteria and viruses is facilitated by a repertoire of platelet surface receptors. These interactions subsequently result in platelet activation (Cox *et al.*, 2011) or in the enhancement of receptor function (Tilley *et al.*, 2013). The mechanisms underlying platelet-pathogen interactions involve both direct and indirect mechanisms. In direct mechanisms the platelet receptor ligands act as bridging proteins which bind to pathogens via their relevant platelet receptors (Flaujac *et al.*, 2010). The presence of the

Fc receptor FcγRIIa, enables platelets to interact with pathogens by binding to IgG in immune complexes (Tilley *et al.*, 2013). Thus platelets can interact with a broad range of bacteria and viruses (shown in figure 2.8) and although platelets are acaryote cells, they contain mRNA and are capable of synthesizing protein (Semple *et al.*, 2011; Speth *et al.*, 2013).

### 2.5.1. Platelet toll-like receptor (TLR) expression

TLRs, a family of receptors capable of recognizing pathogen associated molecular patterns are expressed on neutrophils, monocytes, dendritic cells and platelets (Medzhitov & Janeway, 2003) and are able to modulate immune activation in response to pathogens (Elzey *et al.*, 2003). Platelets express toll-like receptors (TLRs), (O' Neil *et al.*, 2006) and studies suggest that TLR expression on platelets serves as a link between platelets and the inflammatory response to pathogens (Aslam *et al.*, 2006; Shiraki *et al.*, 2004). Several studies have demonstrated TLRs expression on platelets however these findings have been contradicted by some researchers (Shiraki *et al.*, 2004; Cognasse *et al.*, 2005; Ward *et al.*, 2005). Montrucchio *et al.*, further demonstrated that platelets lack TLR4 and do not bind to LPS (Montrucchio *et al.*, 2003). Interestingly, a novel platelet T granule has been reported which contains TLR9 (Thon *et al.*, 2012).



**Figure 2.7** Linking platelets to the immune system by phenotype (adapted from Semple & Freedman, 2010). The figure illustrates several immunoregulatory molecules expressed/secreted by platelets. (A)

Molecules may be acquired by platelets via direct interactions with leukocytes or as a result of plasma adsorption. (B) Illustrates molecules expressed constitutively on platelets, while (C) represents molecules that are secreted by platelets upon activation.

### 2.5.2. The role of TLRs in platelet functions

The functionality of TLRs in platelets has been studied with a focus on TLR4 and TLR2 (Ståhl *et al.*, 2006; Clark *et al.*, 2007; Semple *et al.*, 2007). In an animal model, LPS stimulated mice platelets, which were TLR4 deficient, were unable to adhere to fibrinogen. The authors demonstrated that through TLR4, LPS can regulate platelet function (Andonegui *et al.*, 2005; Beaulieu *et al.*, 2010). TLR4 enables platelets to act as sensory cells which surround and recognize pathogens while regulating pro-inflammatory cytokine release (Beaulieu *et al.*, 2010). Interestingly, *in vitro* studies have shown that enterohemorrhagic *E. Coli* -LPS (EHEC-LPS) can bind to platelets via TLR4 and CD62P resulting in increased  $\alpha_{IIb}\beta_3$  integrin and fibrinogen binding. The same authors also demonstrate that *in vitro* LPS stimulation is able to induce ATP and CD62P secretion from platelet granules (Zhang *et al.*, 2009).

The binding of LPS to TLR4 results in platelet activation and increased platelet neutrophil cross-talk (Aslam *et al.*, 2006). This results in neutrophil activation and bacterial trapping with subsequent killing (Aslam *et al.*, 2006). Contradictory findings have been reported however, and some investigators have suggested that LPS alone does not induce platelet activation but rather enhances agonist-induced platelet aggregation (Zhang *et al.*, 2009). Cognasse *et al.*, demonstrated increased CD63 and not CD62P expression post *in vitro* LPS stimulation (Cognasse *et al.*, 2005). Therefore the effects of LPS on platelet function still remain inconclusive.

TLR2 directly activates platelets inducing platelet adhesion and aggregation via the PI3K/Akt signaling pathway (Blair *et al.*, 2009). The activation of the PI3K/Akt pathway results in increased CD62P expression, ROS production and  $\alpha_{IIb}\beta_3$  activation (Blair *et al.*, 2009).

TLR9 has been recently described as a functional receptor in platelets which plays a crucial role in modulating platelet function and thrombosis (Panigrahi *et al.*, 2013).

**Table 2.2.** Toll-Like receptors expressed on platelets

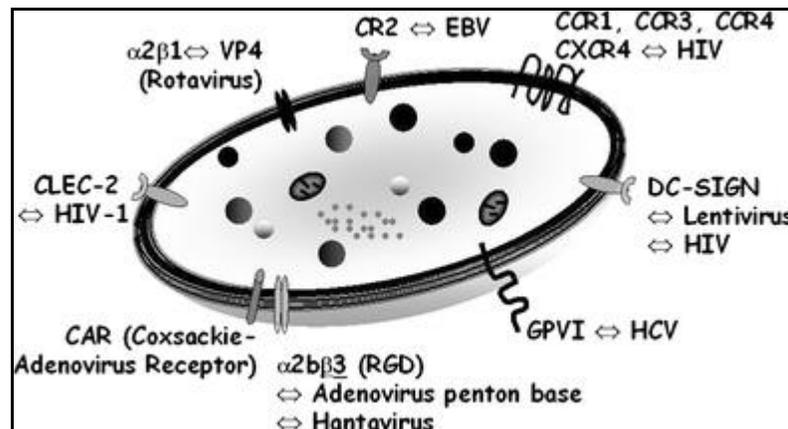
<b>Effects on platelet function</b>	<b>TLR</b>	<b>Signaling pathway</b>
Aggregation; Adhesion; $\alpha$ granule release, ROS production, heterotypic aggregation	TLR1 ,TLR2, TLR6	PI3K/Akt
Cytoskeletal rearrangement, $\alpha$ and dense granule release; heterotypic aggregation	TLR4	PKG
Modulation of platelet function and thrombosis	TLR9	MyD88

### 2.5.3. Platelet receptors interactions with pathogens and HIV

The platelet specific receptor GPIIb/IIIa binds to bacteria and viruses via indirect mechanisms that involve its ligands fibrinogen and fibronectin (Cox *et al.*, 2011). These ligands act as bridging proteins, linking the ligand bound pathogen with the platelet specific receptor (Cox *et al.*, 2011). The platelet receptor GPIIb/IIIa is also capable of directly binding to pathogens (Flaujac *et al.*, 2010) while GPIb $\alpha$  can directly bind to bacteria or vWF bound pathogens. Notably, these interactions may not induce platelet activation (Ruggeri *et al.*, 2007). Previous studies have, in addition, identified platelet integrins that enable the binding of platelets to viral particles (Flaujac *et al.*, 2009). Although numerous platelet receptors which directly interact with viruses have been described (figure 2.5) (Flaujac *et al.*, 2009), these are beyond the scope of this review.

HIV directly binds to platelets via gp 120 which interacts with CXCR4 and the fibronectin receptor expressed on platelets (Pugliese *et al.*, 1996). Previous studies have described the binding and engulfment of HIV by platelets (Boukour *et al.*, 2006; Chaipan *et al.*, 2006; Flaujac *et al.*, 2010) however the consequences of these interactions on the fate of the virus are not fully understood, as both intact and degraded engulfed HIV particles have been described (Boukour *et al.*, 2006). The C-type-like receptor 2 (CLEC-2) expressed on the surface of platelets plays a role in the engulfment of HIV particles. Interestingly, Chaipan *et al.*, showed that dendritic cell-specific intercellular adhesion molecule 3-grabing nonintegrin (DC-SIGN) (Chaipan *et al.*, 2006) (figure 2.8), is a C-type lectin receptor expressed on the surface of platelets, monocytes and dendritic cells (Boukour *et al.*, 2006). DC-SIGN plays a significant role in the binding of HIV to platelets (Chaipan *et al.*, 2006). The mechanisms of DC-SIGN mediated engulfment of HIV has been described in dendritic cells, and involve the endocytosis of HIV particles into endocytic

vesicles. This is followed by the degradation of viral particles in an acidic compartment of the vesicle (Geijzenbeek *et al.*, 2000). However the fate of this endocytosed viral particles in the platelets is not fully understood. These interactions may however result in platelet activation and alterations in platelet morphology (Ruggeri *et al.*, 2007) as increased levels of circulating activated platelets have been reported in HIV infected individuals (Holme *et al.*, 1998; Nkambule *et al.*, 2014). Activated platelets contribute to the innate immune system by secreting antiviral chemokines, such as platelet factor 4 (CXCL4), which has been reported as a broad spectrum HIV-1 inhibitor (Auerbach *et al.*, 2012).



**Figure 2.8** Platelet receptors involved in platelet-virus interactions

## 2.6. Role of platelets in inflammation

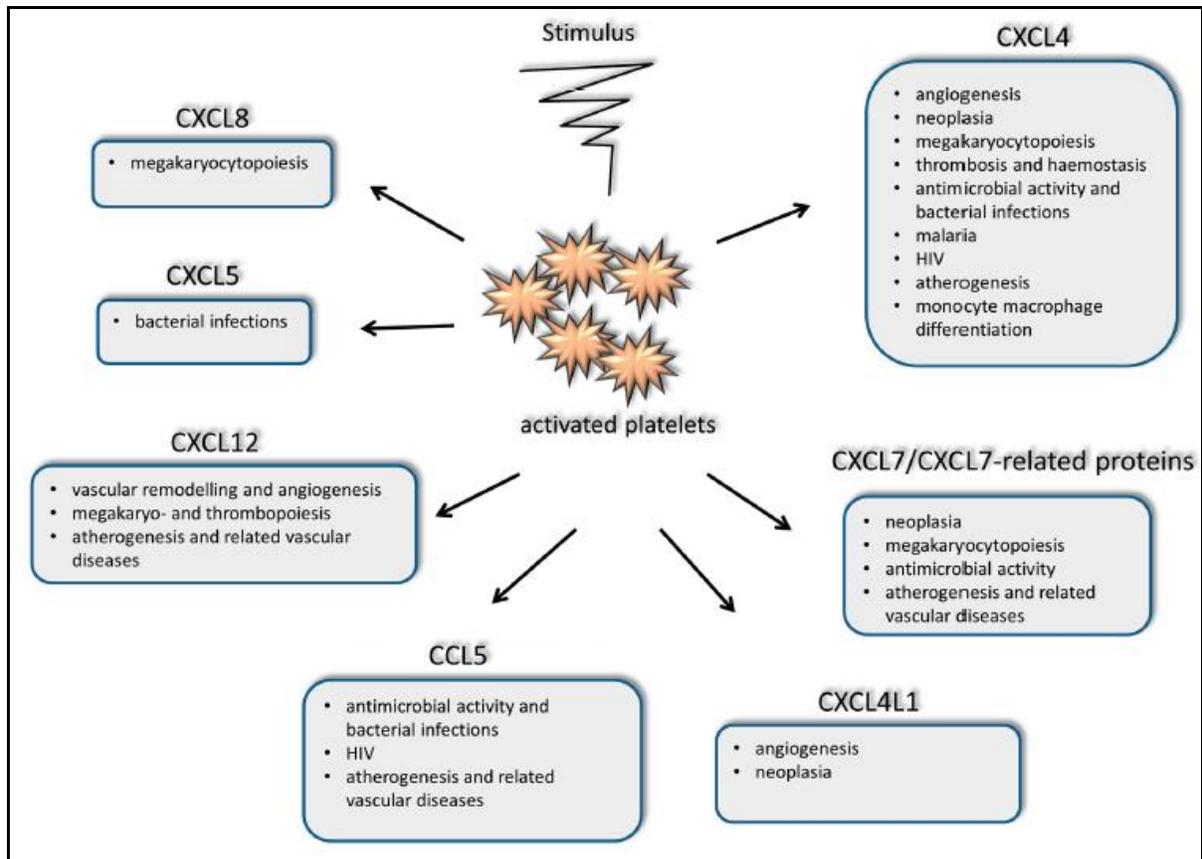
Previous studies support the involvement of platelets in the initiation and propagation of inflammation (Shi *et al.*, 2011; Rondina *et al.*, 2013). The alpha granule plays a crucial role in linking platelet activation and inflammation as it serves as a major source of adhesion receptors facilitating platelet leukocyte interactions (Blair *et al.*, 2009). These interactions play a key role in the propagation of inflammation and further promote the inflammatory phenotype of cells (Kasper *et al.*, 2007). The adhesion or interaction of immune cells with platelets results in the activation and release of chemokines (Blair *et al.*, 2009; Cerletti *et al.*, 2012). The alpha granule is a source of several chemokines such as CXCL7 ( $\beta$ -TG), CXCL4 (PF-4) and its non-allelic variant (PF4alt) that regulate the function of immune cells (Linden & Jackson, 2010; von Hundelshausen *et al.*, 2007). PF-4 induces neutrophil activation and promotes the adhesion of neutrophils to sites of inflammation (Kasper *et al.*, 2004) and is also involved in the differentiation of monocytes into foam cells (Kasper *et al.*, 2007). von Hundelshausen *et al.*, described the

combined effects of PF-4 and CCL5 (RANTES) which together induce the adhesion of monocytes to endothelial cells (von Hundelshausen *et al.*, 2007). CXCL7 is involved in the chemotaxis and adhesion of neutrophils to activated endothelial cells and their subsequent diapedesis (Schenk *et al.*, 2002).

### **2.6.1. The physiological role of platelet $\alpha$ granule contents**

Platelets play a crucial role in the formation of atherosclerotic plaques as the contents of the alpha granule drive the adherence of platelets and leukocytes to activated endothelial cells. In fact, in mouse models of atherosclerosis, deficiency in platelet P-selectin (which translocates from the alpha granule to the membrane upon platelet activation) results in alterations of fatty streak formation and reduces plaque size (Huo *et al.*, 2003). The deposition of RANTES onto inflamed endothelium is facilitated by P-selectin (Schober *et al.*, 2002) and both P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) are crucial for the interactions between neutrophils and endothelial cells (these interactions are discussed in section 2.7) (Luo *et al.*, 2012).

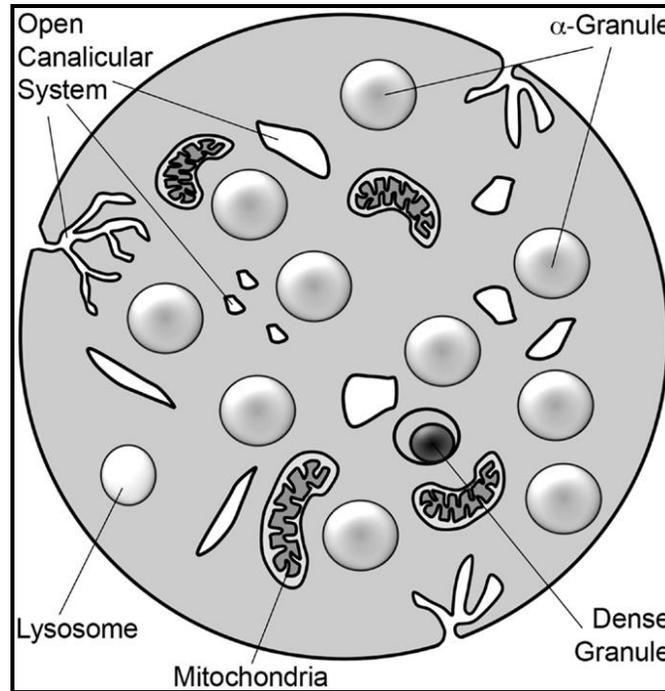
The alpha granule contents also consist of molecules that are involved in vascular repair and angiogenesis (CXCL12); neoplasia (PF-4, CXCL7); regulation of megakaryopoiesis (CXCL4, CXCL12); thrombopoiesis (PF-4) and importantly antimicrobial (PF-4; CXCL5; RANTES; CXCL7) and antiviral (PF-4; RANTES) properties (Karshovska *et al.*, 2013). Platelet derived chemokines that possess antimicrobial properties are known as thrombocidins (Yeaman *et al.*, 2007). For the purpose of this review, only the antiviral properties of chemokines in the context of HIV will be discussed.



**Figure 2.9.** Overview of platelet derived chemokines. The figure illustrates several chemokines secreted by platelets upon activation and degranulation and their physiological function. Karshovska *et al.*, 2013)

### 2.6.1.1 The alpha granule

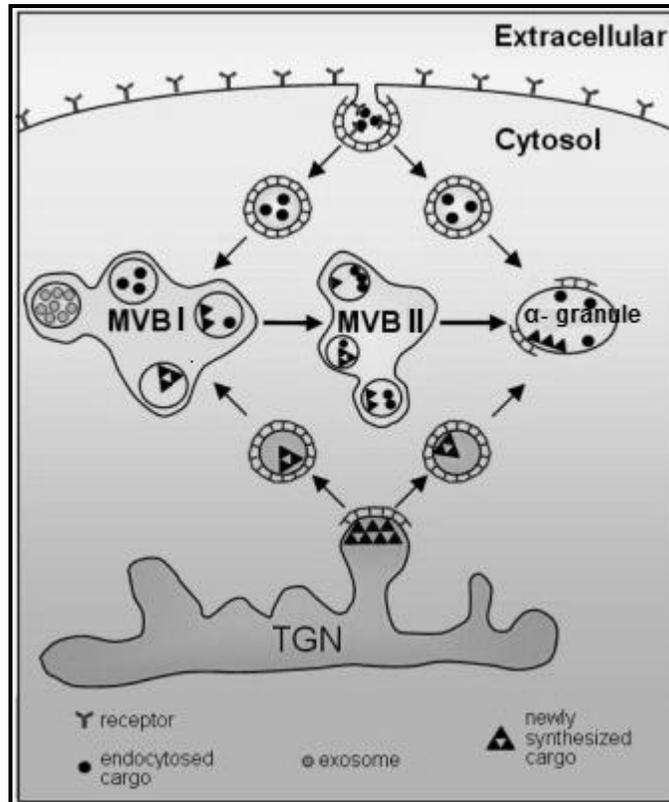
The formation of alpha granules occurs in megakaryocytes, as a result of vesicle budding from the trans-Golgi network (TGN) (Blair *et al.*, 2009). These vesicles then fuse into multivesicular bodies (MVBs) which contain endocytosed and synthesised molecules that ultimately form the alpha granule (Blair *et al.*, 2009). MVBs facilitate the sorting of both the dense and  $\alpha$  granule (Youssefian *et al.*, 2000). Maturation of  $\alpha$  granules in circulating platelets, is facilitated by the endocytosis of plasma membranes (Behnke *et al.*, 1992). Endocytosed proteins are processed from the endosome to immature MVBs (MVB1) and proceed to mature MVBs (MVB2) (Figure 2.4) and finally forming the  $\alpha$  granule (Blair & Flaumenhaft, 2009). Mechanisms modulating platelet endocytosis remain elusive (Panigada *et al.*, 2002). The  $\alpha$  granule contains coagulants and anticoagulants; proinflammatory and anti-inflammatory molecules (Fitch-Tewfik *et al.*, 2013).



**Figure 2.3 Platelet structure.** The schematic diagram illustrates (Fitch-Tewfik *et al.*, 2013)

The functional relevance of the co-localisation of molecules that are functionally antagonistic in  $\alpha$  granules, remains unclear. However, the presence of  $\alpha$  granule subpopulations has been described which suggests the presence of biologically distinct  $\alpha$  granule subtypes (Italiano *et al.*, 2008).

In addition, previous studies suggest that platelet  $\alpha$  granules contents may be sorted and stored in distinct and separate granular compartments that are differentially released in response to different agonists (Sehgal & Storrie, 2007; Italiano *et al.*, 2008; Blair & Flaumenhaft, 2009). The granular contents play a crucial role in haemostasis and platelet function (Blair & Flaumenhaft, 2009) and defects in  $\alpha$  granule formation may result in mild to moderate bleeding, as reported in patients with congenital gray platelet syndrome (Burkhart *et al.*, 2012). The  $\alpha$  granule contains coagulation factors (V, XI, XIII) and molecules that facilitate platelet based coagulation such as fibrinogen and von Willebrand factor (discussed in section 2.8) (Maynard *et al.*, 2007).



**Figure 2.4** Working model of  $\alpha$  granule formation in MKs (Blair & Flaumenhaft, 2009). The schematic diagram illustrates the formation of alpha granules in MKs. MVB maturation facilitates the processing of alpha granule contents and alpha granule formation.

### 2.6.2. Platelet derived chemokines involved in HIV

The direct interaction between platelets and HIV is facilitated by platelet surface receptors (discussed in section 2.5.3). The most predominant chemokines involved in HIV are PF-4 and RANTES (Flaujac *et al.*, 2010) and elevated levels of activated platelets and RANTES have been reported in HIV infected individuals (Holme *et al.*, 1998). The proposed mechanisms which permit the antiviral properties of these chemokines involve their inherent antiretroviral activity; the attraction of immune cells or the occupation of the receptor CCR5 by its ligand RANTES (CCL5) which inhibits the attachment of HIV envelope protein (Env) and prevents cell entry (Karshovska *et al.*, 2013). Auerbach *et al.*, described the antiviral properties of PF-4 which prevented the infection of CD4<sup>+</sup> T cells and macrophages by HIV (Auerbach *et al.*, 2012).

This may also explain a recent finding by Tsegaye *et al.*, who showed that the release of PF-4 by activated platelets inhibited the entry of HIV-1 into T cells (Tsegaye *et al.*, 2013). However, in infected macrophages PF-4 has been shown to facilitate HIV RNA replication and promotes viral spread. Interestingly, in a recent study Berre *et al.*, demonstrated that soluble antibodies directed

against CD36 inhibit the release of HIV from infected macrophages and further inhibit the transmission of viral particles to T- cells (Berre *et al.*, 2013).

## 2.7. Pathways of platelet activation

Three distinct platelet receptors mediate transduction pathways essential for platelet function and which also play a role in thrombosis (Blann *et al.*, 2003). These include two G protein coupled receptors (GPCRs) P2Y<sub>1</sub> (Gq coupled) and P2Y<sub>12</sub> (Gi coupled) and one ATP receptor P2X<sub>1</sub> (Karim *et al.*, 2009). These receptors mediate the effects of nucleotides on platelets. The P2Y<sub>1</sub> receptor facilitates partial aggregation in response to ADP, while the P2Y<sub>12</sub> receptor is responsible for complete aggregation (Varga-Szabo *et al.*, 2009). ADP plays an essential role in haemostasis and in thrombus development, extension and stabilization (Quinn *et al.*, 2005; Varga-Szabo *et al.*, 2009). The P2Y<sub>1</sub> receptor initiates platelet activation via the Gq mediated signaling pathway, which involves the activation of phospholipase C $\gamma$  (PLC $\gamma$ ). This results in further activation of downstream targets that eventually lead to thromboxane production and enhanced granule secretion (Varga-Szabo *et al.*, 2009). The major platelet receptors involved in platelet activation and aggregation are tabulated below (table 2.3).

### 2.7.1. Platelet agonists and receptors

The signalling pathways of platelets are similar to those of neurons (Ridker *et al.*, 2001, Giorgi *et al.*, 2005). Recent studies have highlighted multiple complex platelet activation pathways that regulate platelet activation (Ridker *et al.*, 2001). Soluble platelet agonists play a crucial role in platelet activation and thrombus formation. Upon platelet activation and degranulation (discussed in section 2.2.2) platelets secrete soluble agonists such as thromboxane (TXA<sub>2</sub>); adenosine diphosphate (ADP) and serotonin (Offermanns *et al.*, 2006). These soluble agonists amplify platelet activation and promote platelet recruitment (table 2.3) (Li *et al.*, 2010). Furthermore, agonists produced by both the inflammatory and coagulatory process such as thrombin and platelet activation factor also play an essential role in platelet activation and aggregation (Offermanns *et al.*, 2006). Several novel platelet agonists have been described (table 2.4) (Morrell *et al.*, 2011), however for the purpose of this review the biochemistry of these agonists will not be discussed further.

**Table 2.3.** Major platelet membrane receptors and their physiological agonists

Receptor	Agonists	Receptor Type	Signalling pathway	Role
<b>Glycoprotein (GPVI)</b>	<b>VI</b> Collagen	Ig	Tyrosine Kinase	Activation, Adhesion
$\alpha_2\beta_1$	Laminin	Integrin	Tyrosine kinase	
<b>Glycoprotein (GPIb-XI-V)</b>	<b>Ib-V-IX</b> Collagen	Leucine-rich	Tyrosine kinase	Adhesion
$\alpha_{IIb}\beta_3$	vWF	Integrin	Tyrosine kinase	Aggregation
<b>P2Y<sub>1</sub></b>	Fibrinogen, vWF	GPCR	$G\alpha_q$	Aggregation
<b>P2Y<sub>12</sub></b>	ADP	CPCR	$G\alpha_i$	aggregation
<b>P2X<sub>1</sub></b>	ATP	$Ca^{2+}$ channel		
<b>Thromboxane Receptor (TP)</b>	$TXA_2$	GPCR	$G\alpha_q, G\alpha_{12/13}$	aggregation
<b>PAR1</b>	Thrombin	GPCR	$G\alpha_q, G\alpha_{12/13}$	Aggregation
<b>PAR4</b>	Thrombin	GPCR	$G\alpha_q, G\alpha_{12/13}$	Aggregation

Thrombin and thromboxane ( $TXA_2$ ) induce platelet activation via a G-protein-coupled receptor ( $G_q$ ) and protease-activated receptor (PAR1 and PAR2). The  $G_q$  stimulates phospholipase  $\beta$  which then acts as a catalyst and releases inositol triphosphate (IP3) and diacylglycerol (DAG) (Crittenden *et al.*, 2004). This results in the activation of protein kinase and the calcium signaling pathway which leads to platelet activation and aggregation. Although the platelet agonist receptors may differ, their signaling pathways share a common intracellular signaling pathway that involves PLC (Li *et al.*, 2010).

**Table 2.4.** Recently recognized platelet agonists

<b>Agonist Class</b>	<b>Pathway</b>	<b>Defined receptor</b>
Stimulatory molecule	Glutamate, Ox-LDL	AMPA & KAR, CD36
Receptor	CLEC-2	PDPN & HIV-1
Inflammatory mediator	SDF-1, TARC, BSDL	CXCR4
Pathogen	Plasmodium, HIV-1	CD36, CXCR4 & CLEC-2

### 2.7.2. G-protein-Coupled receptors

G proteins consist of three subunits which are the  $\alpha$ ,  $\beta$ ; and  $\gamma$  (Li *et al.*, 2010). Upon activation the  $\alpha$  subunit undergoes conversion from its GDP form to its activated GTP bound form (Offermanns *et al.*, 2006). The activated  $G\alpha$  then dissociates from the receptor complex and transmits downstream GPCR signals (Stephens *et al.*, 1997). G proteins can be categorized into subfamilies (Gq, G12/G13, Gi/Gz and Gs) which are coupled with selective receptors (Offermanns *et al.*, 2006). The Gq, G12/G13, Gi/Gz subunits are coupled to agonist receptors whereas the Gs subunit is coupled to inhibitory receptors which bind to platelet inhibitors such as prostacyclin (Li *et al.*, 2010). Protease activated receptors play a crucial role in thrombin induced platelet activation and aggregation (Coughin *et al.*, 2005). In fact, platelet activation via PAR receptors induces selective secretion of platelet granule contents (described in section 2.2.3) (White & Rompietti, 2009). The prostaglandin H2 receptor (TP) that is coupled to Gq and G13 binds to  $TXA_2$  and induces platelet degranulation and activation.

### 2.7.3. Gq signaling mediated signaling

The  $P2Y_1$  receptor is a Gq coupled receptor, expressed on a wide variety of tissues including the heart, neural tissue and blood cells (Crittenden *et al.*, 2004). It is required for ADP-induced platelet aggregation (Lisa *et al.*, 2009) and induces the mobilization of  $Ca^{2+}$  stored in platelets. This process results in changes to the platelet shape and transient aggregation in response to ADP (Ohlmann *et al.*, 2010). In addition Mangin *et al.* in 2004 demonstrated that when thromboxane  $A_2$  ( $TXA_2$ ) formation is prevented, the  $P2Y_1$  receptor participates in collagen-induced platelet aggregation and shape change (Mangin *et al.*, 2004). Overall, the  $P2Y_1$  receptor is crucial for ADP-induced platelet activation, as pharmacological inhibition of this receptor results in the absence of platelet aggregation and shape change in response to ADP (Magnin *et al.*, 2004). Phospholipase C ( $PLC\beta$ ) is essential for Gq mediated signaling which

induces platelet secretion, activation of integrins and platelet aggregation (Offermanns *et al.*, 1997). However, Gq mediated signaling does not induce complete platelet aggregation in response to ADP, TXA<sub>2</sub> or sub maximal concentrations of thrombin (Li *et al.*, 2010). Gq signaling induces platelet shape change via signaling pathways involving Ca<sup>2+</sup> and p160 Rho kinase dependant pathways (Jin *et al.*, 1998; Vogt *et al.*, 2003).

#### **2.7.4. Gi mediated Signaling**

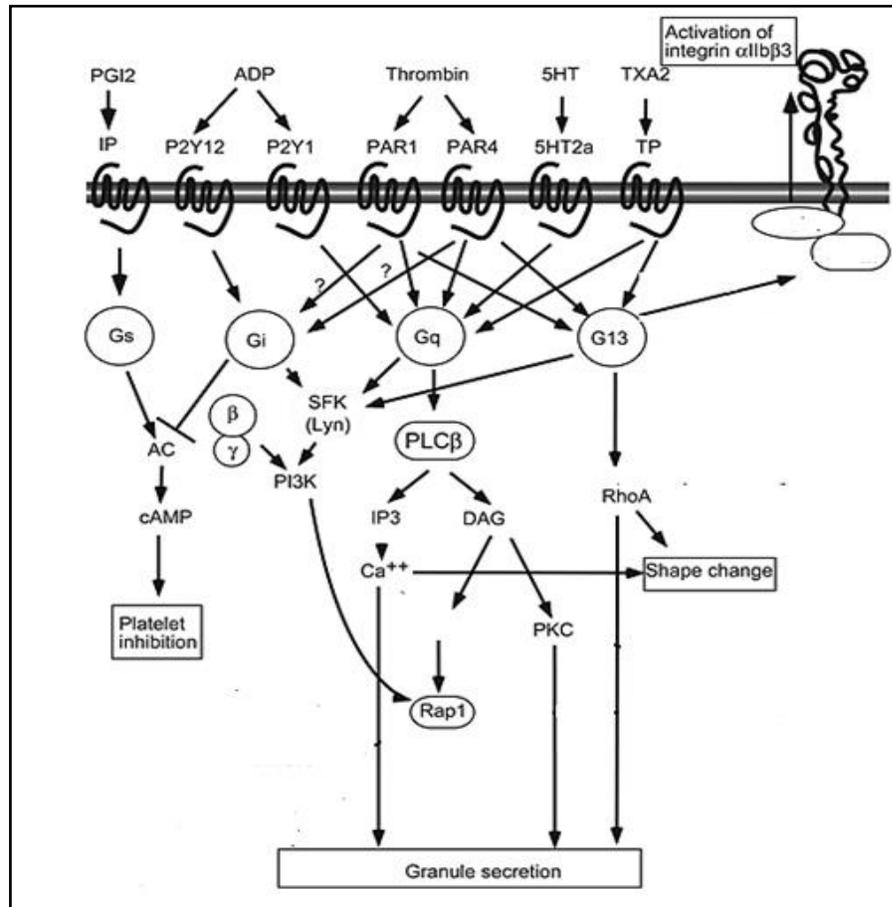
The P2Y<sub>12</sub> receptor is a Gi coupled receptor involved in platelet aggregation in response to ADP, TXA<sub>2</sub> and thrombin (Conley *et al.*, 2003; Li *et al.*, 2010). Gi mediated platelet activation involves the activation of the phosphoinositide 3-kinase (PI3K) and small GTPase Rap 1b which facilitates integrin activation (Conley *et al.*, 2003; Li *et al.*, 2010). Interestingly, Gi signalling positively regulates Gq signaling whereas Gq seems to inhibit Gi signalling (hardy *et al.*, 2004).

#### **2.7.5. G13 mediated signaling**

Gα13 mediated signalling plays a role in platelet shape change, aggregation and most importantly in platelet adhesion (Li *et al.*, 2010). Gα13 interacts with integrinβ<sub>3</sub> and mediates activity of integrins involved in inside out signaling (Gong *et al.*, 2010). G13 has been described as a regulator of RhoGTPases which have been implicated in thrombosis and malignancies (Aittaleb *et al.*, 2010; Cadamuro *et al.*, 2013).

#### **2.7.6. Common intracellular pathway involved in platelet activation**

Platelet signaling mechanisms converge to a common intracellular pathway (Varga-Szabo *et al.*, 2009). Although GCPRs signaling transduction pathways may vary the final signaling event mediating platelet activation are similar (figure 2.6) (Li *et al.*, 2010). PLC is the most common pathway activated by various agonists such as TXA<sub>2</sub>; thrombin and ADP. PLC facilitates the release of inositol triphosphate (IP3) diacylglycerol (DAG) which is involved in platelet shape change, degranulation and protein kinase C activation (Li *et al.*, 2010).



**Figure 2.10** G protein coupled receptor signaling (adapted from Li *et al.*, 2010). The schematic diagram illustrates the complex signaling pathways involved in platelet activation. Platelet receptors play a crucial role in the recognition of platelet agonists and inducing platelet activation in response to agonists. Several pathways converge to a common activation pathway that involves PLCβ and results in activation of PKC and subsequent platelet degranulation.

## 2.8. Platelets and coagulation.

The platelet *alpha* granule contains molecules involved in coagulation such as (Factors V,XI, XIII, fibrinogen and von Willebrand factor). Platelets interact with fibrinogen via the  $\alpha_{IIb} \beta_3$  integrin however the fate of integrin-bound fibrinogen remains unclear. The expression of phosphatidylserine (PS) on activated platelets promotes and enhances coagulation (Heemskerk *et al.*, 2013). PS expressing platelets provide a negatively charged phospholipid platform for the

assembly of coagulation factors which include FVIIa; FIXa; FX; FVa;FXa and prothrombin (Monroe *et al.*, 2002). Notably FX and thrombin bind specifically to platelets expressing surface PS which is involved in the cleavage of FX and prothrombin into active FX (FXa) and thrombin (Heemskerk *et al.*, 2013). Thrombin is a potent vitamin K dependant platelet agonist that induces platelet activation by PAR 1 ,PAR 4 (illustrated in figure 2.9) and GPIb mediated interactions (table 2.3) (Ravanat *et al.*, 2000).

### **2.8.1. Platelet TF expression**

The expression of TF by megakaryocytes and platelets remains highly debated, with contradictory findings being reported (Camera *et al.*, 2010, Kretz *et al.*, 2010; Østerud *et al.*, 2013). Pre-analytical variables that may explain these findings have been described and include sample preparation methods and the choice of monoclonal antibody clone used for the detection of platelet TF which may induce artefactual platelet activation. To date, three mechanisms for the presence of platelet TF have been described. These involve; (1) microparticle facilitated transfer of TF; (2) the release of TF stored within the  $\alpha$  granule and (3) the *de novo* protein synthesis of TF mRNA (Rauch *et al.*, 2000; Muller *et al.*, 2003; Panes *et al.*, 2007). In the context of HIV, where increased levels of plasma TF have been reported (Funderburg *et al.*, 2010), activated platelets may be implicated as an additional source of TF. This may further support the role of platelet activation in coagulation.

### **2.9 Platelet leukocyte aggregates**

Platelets are capable of directly or indirectly interacting with several hematopoietic cells. This may result in the formation of heterotypic aggregates which are a hallmark of inflammatory and acute coronary syndromes as upon adhering to platelets the function of leukocytes is modulated (Metcalf *et al.*, 2013). Furthermore, the pro-inflammatory activity of platelets is mediated by the direct or indirect interactions of platelets and leukocytes (Freedman & Loscalzo, 2002). Increased levels of platelet leukocyte aggregates have been described in thrombosis (Singh *et al.*, 2012) and have been proposed as a marker of cardiovascular disease (Falanga *et al.*, 2005). Platelets preferably form complexes with monocytes which is primarily due to the increased PSGL-1 density on monocytes (Kappelmayer *et al.*, 2001). Receptors involved in innate immunity such as TLR4, along with CD40L and P selectin, have been described in PLAs (Maugeri *et al.*, 2011).

### 2.9.1 Platelet neutrophil aggregates

Activated platelets release chemoattractants such as CXCL7, which recruit neutrophils to sites of inflammation and vascular injury. Neutrophils then interact with platelets via P-selectin and PSGL-1. These interactions result in neutrophil activation, degranulation and tissue factor expression (Maugeri *et al.*, 2008; Manfredi *et al.*, 2009). Neutrophils are the most abundant leucocytes in circulation (Manfredi *et al.*, 2010) and adhere to the activated endothelium via P-selectin mediated interactions (Maugeri *et al.*, 2009). The process of neutrophil rolling, adhesion and final extravasation involves a well-regulated multistep process. Briefly, P-selectin and PSGL-1 interactions facilitate the adhesion of neutrophils to the vessel wall while integrins such as  $\alpha_{IIb}\beta_3$  (GPIIb-IIIa complex) are essential for firm tethering and diapedesis (Maugeri *et al.*, 2009). P-selectin stored in the Weibel-Palade bodies of endothelial cells translocates to the endothelial cell surface upon activation (Maugeri *et al.*, 2009) and interactions between neutrophils and endothelial cells results in the activation of intracellular pathways that promote neutrophil diapedesis (Li *et al.*, 2008). As a result of the density of surface platelet P-selectin, neutrophils preferably and rapidly interact with platelets compared to activated endothelial cells (Linden *et al.*, 2007). Interestingly, activated platelets in the circulation are rapidly phagocytosed by neutrophils (Maugeri *et al.*, 2009). This phenomenon has been previously described as a possible P-selectin facilitated clearance system which seems to be dysfunctional in CVDs (Maugeri *et al.*, 2009).

### 2.9.2 Platelet monocyte aggregates

Platelet monocyte interactions may also result in the maturation of classical monocytes to an inflammatory phenotype (Singh *et al.*, 2012). Circulating platelet monocyte aggregates have been described as a more sensitive marker of platelet activation (Michelson *et al.*, 2001). Previous work by Laura *et al.*, described a rapid flow cytometry based assay for the detection of platelet monocyte aggregates (Laura *et al.*, 2009). The adherence of neutrophils and monocytes to activated platelets during PLA formation is facilitated by P-selectin and its counter receptor PSGL1 (Bournazos *et al.*, 2008; Singh *et al.*, 2012). Monocytes are also capable of adhering to activated platelets via P-selectin and CD40-CD40L ligand interactions (Li *et al.*, 2008). Interactions facilitated by P-selectin results in monocyte activation and increased tissue factor expression (Christersson *et al.*, 2008). It is postulated that P-selectin mediated PMAs may be a link between platelet activation and coagulation. Interestingly, Stephen *et al.*, demonstrated that although the inhibition of P-selectin reduces platelet-monocyte interactions, platelet induced monocyte activation is not inhibited (Stephen *et al.*, 2013). P-selectin mediated interactions

between platelets and monocytes results in the expression of cyclooxygenase (COX)-2 on monocytes (Dixon *et al.*, 2006).

Increased levels of circulating platelet monocyte aggregates (PMAs) have been associated with CVDs and inflammatory conditions (Furman *et al.*, 2001; McCabe *et al.*, 2004; Elalamy *et al.*, 2008) and in HIV (Singh *et al.*, 2012). PMAs are regarded as a more sensitive marker of platelet activation than PNAs and the expression of P-selectin (Michelson *et al.*, 2001; Lippi *et al.*, 2007). Increased levels of PMAs have been reported in PLWH despite successful ART (Singh *et al.*, 2012). Notably, data from cohorts consisting of ARV naïve HIV infected individuals is scarce and hence, associations between HIV replication and PMA formation remain elusive. Singh *et al.*, demonstrated increased PMAs levels that directly correlated with levels of activated platelets in HIV (Singh *et al.*, 2012).

## **2.10 Platelet derived Microparticles**

Microparticles (MPs) are released upon cellular activation and apoptosis and can also form as a result of membrane remodelling and fragmentation caused by necrosis. MPs are heterogenous in size and range between 0.1-1 $\mu$ m. These submicron fragments contain surface receptors of their cell of origin. Although several mechanisms involved in the formation of MPs have been proposed which include platelet activation and apoptosis (Leytin *et al.*, 2012), the physiological origin of microparticles remains unknown (Flaumenhaft *et al.*, 2006). Platelet derived microparticles (PMPs) are defined as submicron particles that express a platelet specific marker and may also express surface phosphatidyleserine (PS). PMPs that do not express surface PS have been described and are associated with inflammatory conditions (Nielsen *et al.*, 2011).

The contribution of activated platelets in PMP formation remains unclear as inhibitors of platelet activation do not completely halt the formation of PMPs in stored platelets (Bode *et al.*, 1991). The life span and kinetics involved in the clearance of PMPs also remains unknown (Flaumenhaft *et al.*, 2006). The role of PMPs in innate and adaptive immunity, have however been described (Semple *et al.*, 2010) and deficiencies in PMP formation have been demonstrated in bleeding disorders (Castaman *et al.*, 1997). Increased levels of PMP have been reported in HIV (Holme *et al.*, 1998; Pretorius *et al.*, 2008; Corrales-Medina *et al.*, 2010) with numbers of of activated PMPs being associated with levels of HIV RNA (Mayne *et al.*, 2012).

## 2.11. Research gaps

Platelet signalling and activation involves a complex network of signalling pathways. Novel platelet agonists have been described which are capable of inducing the activation and secretion of platelet derived chemokines which may regulate the function of immune cells. This may be important in the context of HIV, as platelet function could either enhance viral RNA replication or inhibit the infection by circulating T cells. Patients with chronic HIV-infection are at increased risk of thrombotic complications such as myocardial infarctions and strokes (Friis-Moller *et al.*, 2003; Mondy *et al.*, 2007; Benjamin *et al.*, 2012). The mechanisms underlying these thrombotic conditions remain unclear but could be associated with altered platelet signalling and function. Recent work has highlighted the important association between markers of inflammation and coagulation and the increased risk of these adverse events.

Therefore, by defining a panel of tests for the evaluation of platelet function and thrombotic risk, a direct impact on patient management strategies especially in resource-limited settings could be made. In addition, minimal work has been performed on platelet function in the context of HIV-infection; particularly in a cohort of asymptomatic, untreated patients. The relevance of platelet leukocyte interactions in this setting also remains unclear. In the context of HIV, the evaluation of the association between platelet leukocyte aggregates and traditional markers of immune activation and disease progression may give insight into the physiological role these interactions play in this disease. In addition, a thrombotic risk panel of tests may be of value in the assessment of specific anti-platelet therapies which could be used as preventative strategies in the management of patients at risk of inflammatory-associated complications.

## 2.12. Conclusion

The role of platelets as immune cells has led to the description of various mechanisms and receptors that explain the immunoregulatory function of platelets. Notably the establishment of activated platelets as mediators of inflammation and coagulation forms the basis of associating platelet function with inflammatory and coagulatory conditions. Extensive work has been done to identify and describe the function of various platelet receptors, chemokines and cytokines. Recent work has also described the antiviral properties of these chemokines and the potential role they play in the suppression of HIV pathogenesis. Platelet functional assays may therefore offer insight into understanding platelet function in the context of this disease. Flow cytometry allows for the rapid evaluation of platelet receptors and their biological function.

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## **CHAPTER 3: The evaluation of platelet indices and markers of inflammation, coagulation and disease progression in treatment-naïve, asymptomatic HIV infected individuals**

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## Abstract

**Introduction:** Cardiovascular disease and thrombotic events have emerged as major causes of mortality in people living with HIV. Activated platelets play a key role in both inflammation and thrombosis. Haematology analysers measure a variety of platelet indices which could be surrogate markers of platelet activation. Flow cytometry offers the discrimination of platelet subpopulations and evaluation of the activation status of platelets. This study aimed to measure platelet indices in untreated HIV infection and to evaluate their relationship with markers of immune activation and disease progression.

**Materials and methods:** One hundred and eighty five (185) antiretroviral therapy (ART)-naïve HIV infected and 145 HIV negative individuals were recruited. Platelet indices measured using the ADVIA 2120 platform consisted of, platelet count (PLT), mean platelet volume (MPV fl), platelet distribution width (PDW%) and plateletcrit (PCT%). These were correlated with CD4 count, %CD38 on CD8+(CD38/8) T-cells, viral load, fibrinogen, D-dimers and platelet CD62P and CD36, determined using flow Cytometry.

**Results:** The HIV group had decreased MPV levels (median 7.7[7.1 to 8.3] vs. control group 8.4[7.8 to 9.2],  $p < 0.0001$ ) which correlated with PCT% ( $r = 0.3038$ ,  $p = 0.0013$ ); viral load ( $r = 0.2680$ ,  $p = 0.0177$ ) and PDW% ( $r = 0.2479$ ,  $p = 0.0257$ ). Additionally, the MPV correlated with CD4 count  $r = -0.2898$ ,  $p = 0.0075$ . The HIV group had decreased PDW%, 49.35[46.40 to 52.65] vs. control group, 53.90 [50 to 56.80],  $p = 0.0170$ . In addition the PDW% showed correlations with D-dimers ( $r = 0.443$ ,  $p = 0.03$ ) and %CD36( $r = -0.3666$ ,  $p = 0.0463$ ).

**Conclusion:** Platelet indices may offer a rapid and affordable method for monitoring platelet activation and disease progression in HIV patients.

## 1.1 Introduction

Sub-Saharan Africa has an estimated 23.4 million people living with HIV (PLWH) (UNAIDS, 2012). Although uninterrupted antiretroviral therapy (ART) suppresses HIV RNA replication and successfully lowers plasma viral load (Quinn *et al.*, 2000); studies have shown that long term use of ART increases the risk of cardiovascular events in PLWH (Friss-Moller *et al.*, 2003; Triant *et al.*, 2007). In addition, PLWH are now faced with an increasing trend of non-AIDS related complications (Deeks *et al.*, 2009). The strategies for management of antiretroviral therapy (SMART) study reported increased cardiovascular events associated with higher levels of the coagulation marker D-dimer and inflammatory markers interleukin-6 and hs-CRP; highlighting the important concept of ongoing inflammation as a risk for CVD (El-Sadr *et al.*, 2006). Therefore, HIV infection should be viewed as a chronic inflammatory condition, associated with increased risks of thrombotic events (Cole *et al.*, 2004; Torriani *et al.*, 2008; Grunfeld *et al.*, 2011). Activated platelets play a key role in both inflammation and thrombosis (Van der Bom *et al.*, 2009; Rondina *et al.*, 2013) and this contributes to vascular diseases (Grunfeld *et al.*, 2011).

In normal haemostasis circulating platelets are heterogeneous in size and reactivity, with larger platelets being more active metabolically compared to smaller platelets (Leslie *et al.*, 2010; Rondina *et al.*, 2013). Large platelets are pro-thrombotic and contain high levels of thromboxane A<sub>2</sub> and B<sub>2</sub> per unit volume (Cay *et al.*, 2012). Activated platelets play a key role in the pathogenesis of thrombotic complications (Leslie *et al.*, 2010); cardiovascular disease (Cay *et al.*, 2012) and hypertension (Coban *et al.*, 2005). During platelet activation the influx of calcium into the platelet cytoplasm, triggers morphologic changes which can be measured using haematology analysers and flow cytometry (Santimone *et al.*, 2011).

Platelet indices are readily available and can be utilised to assess morphological changes during the inflammatory process (Fateh-Moghadam *et al.*, 2005). Amongst a variety of platelet indices, the mean platelet volume (MPV) and platelet distribution width (PDW) have been studied previously (Santimone *et al.*, 2011). The MPV is an established marker of platelet activation in various inflammatory and thrombotic conditions (Berger *et al.*, 2010). In active inflammatory conditions the MPV is decreased due to mechanisms that involve the migration and breakdown of the larger platelets at sites of inflammation. In contrast, in conditions where the inflammatory process is inactive, a decline in the breakdown of large, young platelets results in an elevated MPV (Gasparyan *et al.*, 2011). In addition, studies have shown that the MPV is a reliable measure of platelet function in stored platelet concentrates (Vagdatli *et al.*, 2010). The platelet distribution width has been reported as a potential surrogate marker for chronic inflammation (De Gonzalo-Calvo *et al.*, 2013) and platelet aggregation (Vagdatli *et al.*, 2010).

Haematology analysers rapidly measure a variety of platelet indices including the platelet count, PDW, plateletcrit (PCT) and MPV (Kim *et al.*, 2008). Reference intervals for these indices have been reported in previous studies; with PCT% 0.19-0.40; MPV 8.80-12.50 (Adibi *et al.*, 2007; Farias *et al.*, 2010; Naina *et al.*, 2010; Botma *et al.*, 2012). The PDW% reported in previous studies varies markedly, with reference intervals ranging from 10-56.6% (Wiwanitkit *et al.*, 2004; Farias *et al.*, 2010; Chandrashekar *et al.*, 2013). Notably, studies investigating the relevance of platelet indices in the context of HIV are scarce (Mena *et al.*, 2011). Hence, the potential prognostic value of these indices in the context of HIV remains inconclusive (Miguez *et al.*, 2005). The aim of the present study was to evaluate platelet indices in HIV and to investigate the relationship of these indices with traditional markers of disease progression, inflammation and markers of platelet activation.

## **2. Materials and methods**

### **2.1. Blood collection**

All full blood count (FBC) analysis was done on the ADVIA 2120 (Siemens Healthcare diagnostics Inc, tarrytown NY, USA) platform at the Division of Haematology, Tygerberg Hospital, which is a South African National Accreditation System (SANAS) accredited laboratory. 5ml of blood was collected into standardized tubes containing potassium ethylenediametetraacetate (EDTA) as an anticoagulant. The platelet parameters measured by the ADVIA 2120 platform consisted of the Platelet count, MPV, PDW and PCT.

### **2.2 Participant sampling**

Participants were recruited from the Emavundleni Voluntary Counselling and Testing (VCT) Clinic in Crossroads, Cape Town. The total number and demographic characteristics of the study participants are reported in Table 1.

None of the participants were on ART, aspirin, anti-inflammatory drugs or TB treatment. The study protocol was registered and underwent ethics review at both Stellenbosch University (approval number N07/09/197) and UCT (approval number 417/2006) according to the declaration of Helsinki. Participant informed consent was taken according to the University of Stellenbosch HREC regulations.

### **2.3 Study design**

In this cross-sectional study we compared the platelet indices; platelet counts (PLT), mean platelet volume (MPV), Platelet distribution width (PDW), plateletcrit (PCT) of the control group with that of the HIV group. This was followed by flow cytometric analysis of platelet aggregation and activation markers CD36 and CD62P respectively. The relationship between these markers and the indices was then assessed.

## 2.4 Antibodies and reagents

The anti-human CD31-FITC (clone 5.6E), CD36-APC (clone FA6.152), CD62P-APC (clone AK-4) antibodies were all purchased from Beckman Coulter, Miami, USA. Phosphate buffered saline (PBS) without  $\text{Ca}^{2+}$  or  $\text{MgCl}_2$  was obtained from SIGMA-ALDRICH Co, MO, USA.

## 2.5 Viral load and CD4 counts

Viral loads were determined using the NucliSensEasyQ® HIV-1 v1.2 Viral Load Test (BioMerieux Inc., Boxtel, Netherlands) at the Division of Medical Virology, Faculty of Health Sciences, and Stellenbosch University which is SANAS-accredited. The BD MultiTEST CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC reagent (BD Biosciences, San Jose, USA) and BD TruCOUNT tubes (BD Biosciences, San Jose, USA) were used for CD4 counts. CD4 count measurements were performed according to the manufacturer's instruction in the Division of Medical Virology, Faculty of Health Sciences, Stellenbosch University.

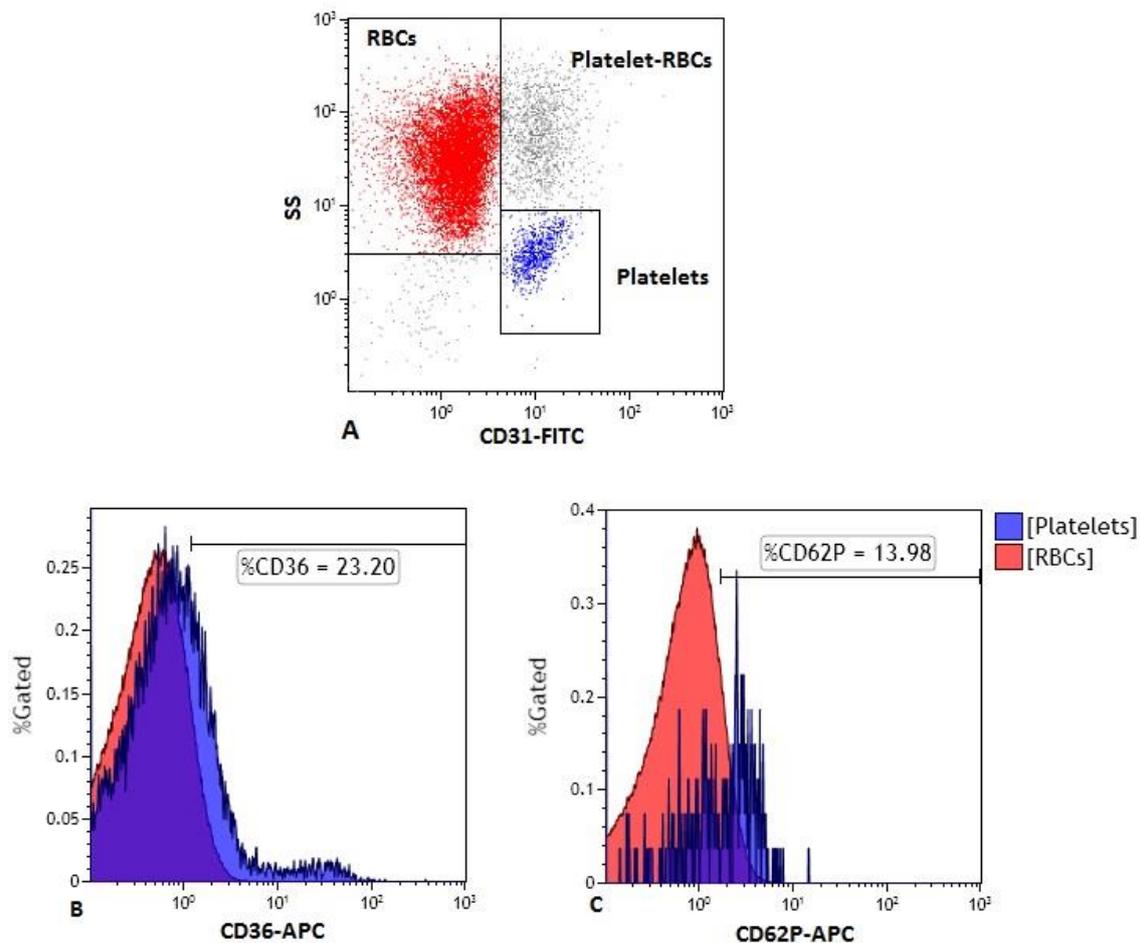
## 2.6 D-dimer and fibrinogen measurements

Quantitative D-dimer measurements were performed using the Hemosil™ Dimertest @kit (Instrumentation lab, Bedford, USA). Fibrinogen measurements were performed on the ACL TOP (Beckman Coulter, Miami, USA). All measurements were done on citrated whole blood according to the manufacturer's instruction at the National Health Laboratory Services (NHLS), Haematology coagulation laboratory, Tygerberg Hospital.

## 2.7 Flow cytometry

*Sample collection:* A total of 2-3ml of venous blood was collected by venipuncture into 4.5ml tubes containing 3.2% sodium citrate (BD Vacutainer, San Jose, CA). Sodium citrate was the anticoagulant of choice as blood collected in EDTA may show artificially elevated levels of the platelet activation marker CD62P-APC (Maurer-spurej *et al.*, 2001). Samples were kept at room temperature during transit from clinic to the laboratory. The time between sample collection and analysis was limited to 1-3hrs. *Instrument set-up and detector settings:* Flow cytometry data acquisition was performed using a Beckman Coulter FC500 flow cytometer (Beckman Coulter, Miami, USA). Flow check pro fluorescent beads of known size (Beckman Coulter, Miami, USA) were used to verify and standardise the optics and fluidics of the cytometer. Forward scatter (FSC) and side scatter (SSC) parameters were set at a log-scale, to allow the detection of platelets and red blood cells. An unstained fresh blood sample was used to set the voltages for the FSC/SSC; this allowed the separation of red blood cells and platelets based on cell size and granularity. Stained activated platelets were used to determine percentage of spectral overlap and to perform compensation. Antibody titration assays were performed to establish optimal antibody concentrations.

**Staining protocol and gating strategy:** The platelet marker CD31-FITC was used to characterize platelets as previously described and CD36-APC and CD62P-APC were used to monitor platelet aggregation and activation (De Cuyper *et al.*, 2013; Nkambule *et al.*, 2014). Briefly 50µl of citrated whole blood was stained and incubated in the dark for 20 minutes at room temperature. 500µl of PBS staining buffer (containing 1,25µl of fetal bovine serum (FBS) and 500ml of PBS which does not contain Ca<sup>2+</sup> or MgCl<sub>2</sub>) was added and samples were analysed immediately. The use of CD31 allowed the detection and separation of free platelets from red blood cells (RBCs) and platelet-bound RBCs (Platelet-RBCs) (Figure 1A).



**Figure 1. Gating strategy.** The figure illustrates the gating strategy used to discriminate platelet subpopulations based on size and internal complexity. The color dot plot A illustrates the discrimination between RBCs and Platelets using CD31 FITC and side scatter (SS) properties. Events that were positive for CD31 were gated as platelets and negative events were gated as red blood cells (RBCs), illustrated in figure A. Histogram plots C and D illustrate the use of RBC as internal controls to distinguish between positive and negative (CD36 and CD62P) cell surface expression.

## 2.8 CD38/8 measurement:

The expression of CD38 on CD8 T cells, is a well-established marker of immune activation and independent predictor of disease progression in HIV (Savarino *et al.*, 2000). The percentage of CD8+ T-cells expressing CD38 was analysed using flow cytometry. Briefly, whole blood samples were stained with a titrated monoclonal antibody cocktail containing; CD8 Per-CP; CD38 APC; CD3 FITC (BD Biosciences, San Jose, CA). Data acquisition was performed using a BD FACSCalibur instrument and analysis was done using the BD Cell Quest Pro (Version 2) software (BD Biosciences, San Jose CA, USA).

## 2.9 Statistical analysis

Statistical analysis was performed using GraphPad prism 5.00 for windows (GraphPad Software, San Diego CA, USA). The Mann-Whitney *U* test was used to compare non-parametric data and these values were reported as median and interquartile range. For parametric data an unpaired student *t* test was performed and data was reported as mean and standard deviation. Correlations were performed using a spearman rank correlation. A *p* value of <0.05 represented statistical significance.

## 3. Results

A total of 330 participants were enrolled in this study, 185 adult untreated (ARV naïve) HIV infected patients and 145 HIV negative controls. The demographics and characteristics of the control and HIV groups are illustrated in Table 1. The groups had similar age and sex distributions and were recruited from the same clinic in the Western Cape and therefore had similar socio-economic and ethnic backgrounds. The HIV group had a slightly higher PLT count mean  $299.1 \pm 78.31$  compared to the control group mean  $288.6 \pm 59.64$ , however this was not statistically significant ( $p=0.1542$ ). All the other platelet indices and markers of immune and platelet activation were statistically significant, as shown in Table 1.

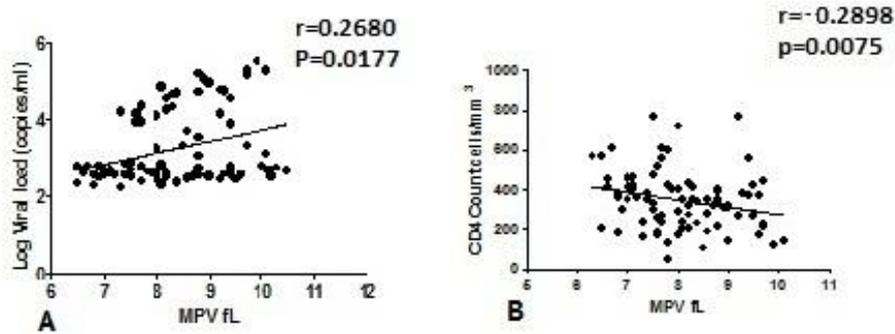
**Table 1.** Cohort characteristics; platelet parameters and disease markers

	<b>Control Group</b> <b>(n=145 )</b>	<b>HIV Group</b> <b>(n=185 )</b>	<b>Reference</b> <b>Interval</b>	<b>P-value</b>
<b>Age, years (interquartile range)</b>	28[24-35]	30[26-37]		0.0518
<b>Male: female</b>	1:1.51	1:4.5		
<b>Platelet (PLT) count x10<sup>9</sup>/L</b>	288.6 ± 59.64	299.1 ± 78.31	178 - 429	0.2082
<b>Mean platelet volume (MPV), fL</b>	8.52 ± 1.12	7.91 ± 0.85	8.80 - 12.50	<b>&lt;0.0001</b>
<b>Plateletcrit (PCT),%</b>	0.23 ± 0.03	0.26 ± 0.04	0.22 - 0.40	<b>&lt;0.0001</b>
<b>Platelet distribution width (PDW) ,%</b>	51.70[45.65-55.95]	49.40[46.40-52.65]	-	<b>0.0266</b>
<b>%CD62P</b>	0.68[0.08-3.3]	11.95[1.68-24.51]	-	<b>&lt;0.0001</b>
<b>%CD36</b>	11.22[5.59-18.25]	38.65[25.85-54.55]	-	<b>&lt;0.0001</b>
<b>CD4 count cells/mm<sup>3</sup></b>	852.9 ± 244.6	373.8 ± 168.2	500-2010	<b>&lt;0.0001</b>
<b>D-dimers</b>	0.2 [0.2-0.2]	0.23[0.2-0.33]	0.00-0.25	<b>&lt;0.0001</b>
<b>Fibrinogen</b>	2.91 ± 0.05	2.8± 0.07	1.56-4.0	0.2651
<b>%CD38/8</b>	31.13 ± 18.65	50.30 ± 22.62		<b>&lt;0.0001</b>
<b>Viral load (log)</b>	-	4.080[3.4-4.8]		-

Significant values shown in boldface

### 3.1 Decreased MPV in HIV positive individuals compared to uninfected controls

The MPV which is a measure of mean platelet volume was significantly decreased in the HIV group (n=80) compared to the control group (n=82), (HIV mean 7.91 ± 0.85 vs. 8.52 ± 1.12, p<0.0001). In the HIV group, the MPV showed a positive correlation with log viral load r=0.2680, p=0.0177 (figure 2a); PDW% r=0.2479, p=0.0257. Furthermore, the MPV showed an inverse correlation with CD4 counts r=-0.2898, p=0.0075 (figure 2b). No correlations were detected with; CD38/8 (r=0.307, p=0.53); markers of platelet activation %CD62P (r= 0.328, p=0.082) and aggregation %CD36 (r=0.248, p= 0.282); and D-dimer levels (r=0.117, p=0.445).



**Figure 2.** HIV group correlations between markers of disease progression and platelet indices. A. MPV showed a positive correlation with viral load and B a negative correlation with CD4+ T cell count.

### 3.2 Increased PCT% in HIV positive individuals compared to uninfected controls

PCT% is an indicator of the total platelet composition in blood. Although the PCT% was higher in the HIV group compared to the control group, (0.26[0.23 to 0.29] vs. 0.23 [0.21 to 0.25],  $p<0.0001$ ) the values were still within the normal reference interval and therefore the clinical significance of this finding remains to be determined.

### 3.3 Decreased PDW% levels in HIV positive individuals compared to uninfected controls

The PDW%, a measure of variation in platelet size was decreased in the HIV group compared to the control group, (HIV group median 49.35[46.40 to 52.65] vs. 53.90 [50 to 56.80],  $p=0.0170$ ). In order to establish the value of PDW as a marker of coagulation and platelet aggregation, this index was correlated with D-dimers and platelet %CD36 expression. PDW% showed a positive correlation with D-dimers and inverse relationship with %CD36 (values shown in Table 2).

**Table 2.** PDW% correlations

Parameter	Spearman r	P-value
Platelet count	-0.3474	<b>0.0002</b>
Plateletcrit (PCT) %	-0.3138	<b>0.0008</b>
%CD62P	0.1326	0.4929
%CD36	-0.3666	<b>0.0463</b>
D-Dimers	0.3455	<b>0.0362</b>
Fibrinogen	0.2318	0.1347
CD4 count	-0.1023	0.2786
CD38/8	0.307	0.530
Viral load	0.0346	0.7283

Significant values shown in boldface

#### 4. Discussion

Several investigators have assessed the value of platelet indices in platelet activation (Park *et al.*, 2002; Chapman *et al.*, 2003). However the potential value of these indices in the context of HIV-infection remains inconclusive (Miguez-Burbano *et al.*, 2005). The present study aimed at evaluating the relationship between platelet indices and traditional markers of disease progression, inflammation and platelet activation in the context of untreated HIV infection. Platelet indices are affordable and readily available as part of routine full blood counts performed using hematology analyzers. These indices offer a more rapid analysis of platelet activation using an automated system that limits observer bias (Beyan *et al.*, 2006). The MPV reflects platelet size and the rate of platelet production (Bancraft *et al.*, 2000). MPV values increase during platelet activation and have been established as a marker of platelet activation (Park *et al.*, 2002).

Interestingly, the HIV group MPV levels were significantly decreased compared to the control group (Table I), which was in contrast to the finding of significantly increased levels of activation (CD62P) and aggregation (CD36). It is possible that in the untreated HIV group; levels of inflammation were such that an increased consumption of the larger platelets occurred; effectively removing them from the circulation. In low grade inflammatory conditions, inflammatory cytokines induce thrombopoiesis resulting in increased circulating

large young platelets. However, in high grade inflammatory diseases the rapid breakdown of these larger platelets results in a lower MPV (Gasparyan *et al.*, 2011). Furthermore, persistent platelet activation in HIV may be induced by direct engulfment of HIV virions by platelets (Chaipan *et al.*, 2006). In addition, platelet activation may be a consequence of enhanced thrombin production from increased tissue factor expression on activated monocytes and endothelial cells in HIV infection (Baker *et al.*, 2013). Moreover, the up regulation of platelet receptor ligands on leukocytes in response to the increased inflammatory cytokine milieu, may further enhance the migration and adhesion of platelets to inflammatory sites (von Hundelshausen *et al.*, 2007). The increased consumption of large platelets have been previously (Baker *et al.*, 2013) as a result of the migration of large hyper-reactive platelets from the circulation into extravascular sites of inflammation (Öztürk *et al.*, 2013). This is likely to be the mechanism underlying the decrease in MPV levels measured in our HIV group. Decreased MPV levels have also been reported in several chronic inflammatory diseases (von Hundelshausen *et al.*, 2007; Lichtenstein *et al.*, 2010; Baker *et al.*, 2013; Öztürk *et al.*, 2013). In addition, our current findings are consistent with recent findings reported by Qadri *et al.*, in a cohort of HIV infected women (Qadri *et al.*, 2013). The authors reported a decreased MPV in HIV infected patients (8.66 vs. 9.05) compared to uninfected counterparts. Although these patients were all women, demographic differences in the MPV reference intervals between the cohorts exist, the MPV levels in our study were similarly decreased. Gender related differences in MPV reference intervals have been reported, with higher MPV levels in females (9.0-12.50fl) compared to [8.80-11.30] (Botma *et al.*, 2012).

In the present study, MPV levels correlated inversely with CD4 counts and directly with viral loads. This association contradicts findings reported in recent studies (von Hundelshausen *et al.*, 2007; Mena *et al.*, 2011). Mena *et al.* reported no association between MPV and CD4 counts in a cohort of ART-naïve HIV infected patients with mean baseline CD4 counts of 591 cells/mm<sup>3</sup>. The differences between baseline CD4 counts (mean 373.8 cells/mm<sup>3</sup>) in our study and that of Mena *et al.* may account for these discrepancies. In contrast to our findings, Qadri *et al.* reported no associations between MPV, CD4 counts and viral load (Qadri *et al.*, 2013). Notably, only 37 (16%) of these participants were treatment-naïve (Qadri *et al.*, 2013) which may explain the contrary findings observed in our study. The majority of the participants in the study by Qadri *et al.*, were on treatment and this may have impacted the association of MPV with CD4 counts and viral load as HIV RNA replication was controlled in the majority of these participants. The direct correlation of the MPV with viral load in our study was supported by the findings of Mena *et al.* interestingly, as a host defence mechanism; platelets are capable of directly interacting and engulfing HIV virions which may

result in platelet degranulation and a decrease in both MPV levels (Schoorl *et al.*, 2011) and HIV RNA in blood (Boukour *et al.*, 2006).

PDW has been reported as a potential measure of platelet activation and aggregation (Vagdatli *et al.*, 2010). Vagdatli *et al.*, reported PDW as a specific marker of coagulation and a decrease in PDW may indicate the presence of aggregated platelets (Vagdatli *et al.*, 2010). In the current study, the HIV group had significantly decreased levels of PDW compared to uninfected controls and this showed a correlation with the marker of aggregation (CD36). In addition, we observed a positive correlation between PDW and D-dimers, a marker of fibrinolysis and associated with increased risk of mortality in HIV infected individuals (Kuller *et al.*, 2008). This supports the relevance of PDW% as a potential marker of coagulation which may be a valuable marker in the context of HIV infection.

In the present study the PDW% index which is associated with active coagulation inversely correlated with %CD36 a marker of platelet aggregation. This suggests the value of PDW% as a rapid indicator of platelet activation. Thus, we have shown that the MPV and PDW indices may offer a more affordable, standardised and rapid platelet activation monitoring technique.

CD38 is a transmembrane glycoprotein that is upregulated during early T-cell activation (De Cuyper *et al.*, 2013). In the context of HIV, increased levels of CD38 on CD8 T cells have been described as a marker of generalised immune activation and a strong predictor disease progression (De Cuyper *et al.*, 2013; Karim *et al.*, 2013). In our study, the HIV group showed increased levels of immune activation as shown by increased percentages of CD8 T cells expressing CD38. However, the levels of immune activation were not associated with the chosen platelet indices. This may further suggest that in HIV, alterations in platelet kinetics may involve complex mechanisms that are not solely dependent on immune activation.

To the best of our knowledge, the present study forms the largest study investigating platelet indices and in particular, the MPV, in HIV treatment-naïve patients and uninfected controls. However, this study is subject to the limitations of a cross-sectional study, which include the lack of evidence of causality between the studied parameters and platelet indices. Future longitudinal follow-up studies should be done to determine the prognostic value of these indices.

In conclusion, our findings suggest that the MPV and PDW may be valuable markers of platelet and immune activation in HIV infected patients. Moreover, platelet indices may offer a rapid and affordable method of monitoring platelet activation in HIV patients.

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## Competing interests

The authors declare that there are no financial, personal or professional competing interests that may interfere with this work.

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## **CHAPTER 4: The evaluation of platelet function in HIV infected, asymptomatic treatment-naïve individuals using flow cytometry**

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## Abstract

### Introduction

Human immunodeficiency virus (HIV) induces inflammation and platelet activation. People living with HIV (PLWH) are at increased risk of thrombotic events and activated platelets link inflammation with thrombosis. Platelet function in HIV remains unclear. P-selectin (CD62P), a marker of platelet activation, and platelet glycoprotein GPIV (CD36) a marker of platelet aggregation, can be measured using flow cytometry. We aimed to evaluate platelet function in HIV using a whole blood platelet flow cytometry based assay.

### Materials and methods

Fifty-eight (58) antiretroviral therapy (ART) naïve HIV infected and 38 HIV negative individuals were recruited in a clinic in Cape Town. Platelet surface CD36 and P-selectin CD62P were measured using flow cytometry. These measurements were then correlated with CD4 count, viral load and % of CD8+ T-cells cells expressing CD38. Platelet function was evaluated using adenosine diphosphate (ADP), arachidonic acid (AA) and collagen at varying concentrations. The CD62P mean fluorescence intensity (MFI) was used as a measure of agonist induced platelet response.

### Results

The HIV group showed increased levels of circulating activated platelets, (median %CD62P 5.51[3.03– 10.11] vs. Control group 2.14[0.19 – 3.59],  $p < 0.0001$  and %CD36 21.93[11.03-44.92] vs. Control 16.15[2.24-25.37],  $p = 0.0087$ ). In the HIV group, levels of platelet activation correlated with Viral load ( $r = 0.336$ ,  $P = 0.008$ ) and showed no significant correlation with CD4 count ( $r = 0.028$ ,  $p = 0.891$ ). The HIV group also had increased levels of cells expressing CD36, a marker of platelet aggregation which directly correlated with viral load ( $r = 0.398$ ,  $p = 0.024$ ) and showed no correlation with CD4 count ( $r = 0.205$ ,  $p = 0.730$ ). There were no significant differences in baseline CD36 and CD62P MFI. The HIV group showed a hyper response to AA and collagen at various concentrations. Notably, the HIV group only showed a hyper response to ADP at a maximal concentration of 20 $\mu$ M (median CD62P MFI, 1.91[1.64-4.95] vs. Control 1.75[1.45-2.44]  $p = 0.0279$ ).

### Conclusion

We show elevated levels of activated platelets in HIV infected individuals that have enhanced reactivity to endogenous agonists in a concentration dependent manner. Platelet flow cytometry is a rapid and valuable technique in the evaluation of platelet function in HIV. The measurement of platelet function using flow cytometry allows the evaluation of platelet signalling pathways that may be modified in HIV infected individuals.

## 1. Introduction

An increasing trend of non-AIDS related deaths has been reported in the ageing population of people living with HIV (PLWH) (Satchell *et al.*, 2011; May *et al.*, 2013) and cardiovascular diseases (CVDs) are reported as the most common cause of mortality (Lohse *et al.*, 2007). Uninterrupted antiretroviral therapy (ART) successfully suppresses viral replication and improves the clinical outcome of human immunodeficiency virus (HIV) infected individuals (Fauci *et al.*, 2012). However, a decreased life expectancy has been reported in people living with HIV (PLWH), compared to uninfected counterparts (Nakagwa *et al.*, 2011). In HIV infected individuals, chronic immune activation is associated with an increased thrombotic risk that persists regardless of ART and involves factors that are both dependant and independent of viral replication (Appay *et al.*, 2008; Sauce *et al.*, 2011). Aspects of both the innate and adaptive immune response contribute to chronic immune activation and inflammation in PLWH (Sauce *et al.*, 2013).

Elevated levels of immune activation and coagulation are linked to disease progression in HIV (Deeks, 2011). Previous studies have highlighted the complications that arise as a result of chronic immune activation and long term use of ART (Hogg *et al.*, 2008). CXCR4 receptor expression on the megakaryocyte lineage fosters interactions between platelets and HIV (Gear *et al.*, 2003). These Interactions which involve various platelet receptors have resulted in the engulfment of HIV particles and subsequent platelet activation (Torre & Pugliese *et al.*, 2008; Flaujac *et al.*, 2010) which may play a crucial role in thrombosis (Satchell *et al.*, 2011).

Functional assays measure platelet responses to various agonists such as collagen, adenosine diphosphate (ADP), thromboxane A<sub>2</sub> (TXA<sub>2</sub>) and thrombin (Mangalpally *et al.*, 2010; Satchell *et al.*, 2011). The response to these agonists gives insight into the functionality of signal transduction pathways involved in platelet activation (Varga-Szabo *et al.*, 2009). The platelet response is measured and interpreted as a degree of activation and aggregation which defines the functional capacity of platelets (Satchell *et al.*, 2011). ADP and TXA<sub>2</sub> induce platelet aggregation via G-protein-coupled receptors (Crittenden *et al.*, 2004; Varga-Szabo *et al.*, 2009). The metabolism of phospholipids by phospholipase A<sub>2</sub> (PLA<sub>2</sub>) is crucial in platelet activation (Li *et al.*, 2008). Arachidonic Acid (AA) a precursor of TXA<sub>2</sub>, is released by platelets upon the PLA<sub>2</sub> facilitated cleavage of membrane phospholipids (Li *et al.*, 2008).

Platelets express surface receptors for exogenous agonists and are either activated or aggregate in response to them (Ghosh *et al.*, 2008). Platelet aggregometry remains a widely used technique to assess platelet function (Satchell *et al.*, 2010) however it is a cumbersome technique which is dependent on sample preparation and requires large volumes of blood (Brass *et al.*, 2010). Although efforts have been made to standardise the technique, poor

reproducibility of results and the time-consuming nature of this method remains a major concern (Breddin *et al.*, 2005). Platelet flow cytometry offers a rapid alternative to evaluate platelet function (De Cuyper *et al.*, 2013). P-selectin (CD62P) is an established marker of platelet activation (Maugeri *et al.*, 2009) and during activation, is released from the  $\alpha$ -granules and expressed on the surface of the platelet (Linden *et al.*, 2007). Platelet glycoprotein IV (CD36) is a scavenger receptor ubiquitously expressed on the surface of platelets (van Velzen *et al.*, 2012) and has been identified as a receptor for collagen and thrombospondin-1 which plays a crucial role in platelet adhesion and aggregation (van Velzen *et al.*, 2012). The underlying mechanism of platelet activation in the context of HIV remains complex and unclear (Corrales-Medina *et al.*, 2010; Satchell *et al.*, 2010). Therefore, using a whole blood platelet flow cytometry based assay, we sought to evaluate levels of platelet activation and aggregation in HIV and to further evaluate platelet responses to various endogenous agonists. In order to determine the role of platelets in HIV, we further assessed the relationship between activated platelets and conventional markers of immune activation and disease progression.

## **2. Methodology**

Ninety-six (96) participants were recruited. Fifty eight of these were HIV positive ARV-Naïve and thirty eight were HIV negative individuals. Patients were recruited from the Emavundleni Voluntary Counselling and Testing (VCT) Clinic in Crossroads, Cape Town which is associated with the Institute of Infectious Diseases and Molecular Medicine (IIDMM) and the Desmond Tutu HIV Centre, University of Cape Town (UCT). None of the participants were taking aspirin or anti-inflammatory drugs on the day of testing. Participant demographics and baseline characteristics are reported in table 1. The study protocol was reviewed by the health research ethics committee (HREC) at Stellenbosch University and the research ethics committees (REC) at University of Cape Town. Ethical review was performed according to the declaration of Helsinki. The study was approved by both committees and registered under the ethical approval number N07/09/197 and 417/2006, respectively. Written patient informed consent was taken according to the University of Stellenbosch HREC regulations

### **2.1 Design of the study**

Baseline platelet %CD36 and %CD62P measurements were performed on both groups in order to determine baseline levels of activation. In addition, CD4 count, viral load and % CD8+/CD38+ T cells were measured. The platelet response to agonists was then evaluated using flow cytometry. CD62P mean fluorescent intensity (MFI) levels were compared pre and post stimulation with ADP, AA and collagen at varying concentrations. These measurements were used as a measure of platelet function.

## **2.2 Sample collection**

A total of 2-3ml venous blood was collected by venipuncture into 4.5ml tubes containing 3.2% sodium citrate (BD Vacutainer, San Jose, CA). Samples were kept at room temperature during transit from clinic to the laboratory in order to limit any effects of temperature fluctuations on platelet activation. The time frame between sample collection and analysis was limited to 1-3hrs.

## **2.3 Markers of immune activation and disease progression**

**2.3.1** CD4 T-cell counts measurements were performed using the BD MultiTEST CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC reagent (BD Biosciences, San Jose, CA) and BD TruCOUNT tubes (BD Biosciences, San Jose, CA). CD4 T cell counts were performed at the Division of Medical Virology, Faculty of Health Sciences, Stellenbosch University, which is accredited by the South African National Accreditation System (SANAS).

**2.3.2** Viral load measurements: were performed according to the manufacturer instructions, using the NucliSensEasyQ® HIV-1 v1.2 Viral Load Test (BioMerieux Inc., Boxtel, Netherlands), at the Division of Medical Virology, Faculty of Health Sciences, and Stellenbosch University which is a SANAS-accredited laboratory.

**2.3.3** *CD38 on CD8<sup>+</sup> T cell measurements:* To measure the percentage of CD8<sup>+</sup> cells expressing CD38, 50µl of heparinized whole blood was stained with a titrated monoclonal antibody cocktail containing; CD8 Per-CP; CD38 APC; CD3 FITC (BD Biosciences, San Jose, CA). Data analysis was done using the BD Cell Quest Pro Version 2 software (BD Biosciences, San Jose, CA).

**2.3.4** *D-dimer measurements:* were performed using the HemosIL TM Dimertest® Kit (Instrumentation Lab) and the ACL TOP platform at the NHLS Haematology coagulation laboratory at Tygerberg Hospital which is South African National Accreditation System (SANAS) accredited.. Measurements were performed using 4.5ml of citrated blood and sample collection was done using tubes containing 3.2% sodium citrate.

## **2.4 Platelet flow cytometry**

**2.4.1 Instrument settings:** The Beckman Coulter FC500 flow cytometer (Beckman Coulter, Miami Florida) was used for data acquisition. Flow check pro (Beckman Coulter, Miami, USA) fluorescent labelled beads of known size and fluorescence intensity were used to verify optical path and laminar flow of the cytometer. Flow set beads (Beckman Coulter, Miami, USA) and the Beckman Coulter quickcomp kit (Beckman Coulter, Miami, USA) were used for voltage settings and compensation. This ensured the reporting of standardized and consistent results that were not influenced by long-term instrumental drifts.

**2.4.2 Sample preparation and Staining:** CD31-FITC (Beckman Coulter, Miami, USA) was used to characterize platelets (Fig 1A) as this monoclonal antibody does not bind to receptors that directly affect platelet aggregation (De Cuyper *et al.*, 2013). CD62P-APC (BD Biosciences, San Jose, CA) and CD36-APC (Beckman Coulter, Miami, USA) were used to monitor platelet activation and aggregation respectively. Briefly, 50µl of citrated whole blood was stained using 5µl of the antibody cocktail and incubated in the dark for 20 minutes at room temperature. 500µl of PBS (which did not contain Ca<sup>2+</sup> or MgCl<sub>2</sub>) was added and samples were analysed immediately.

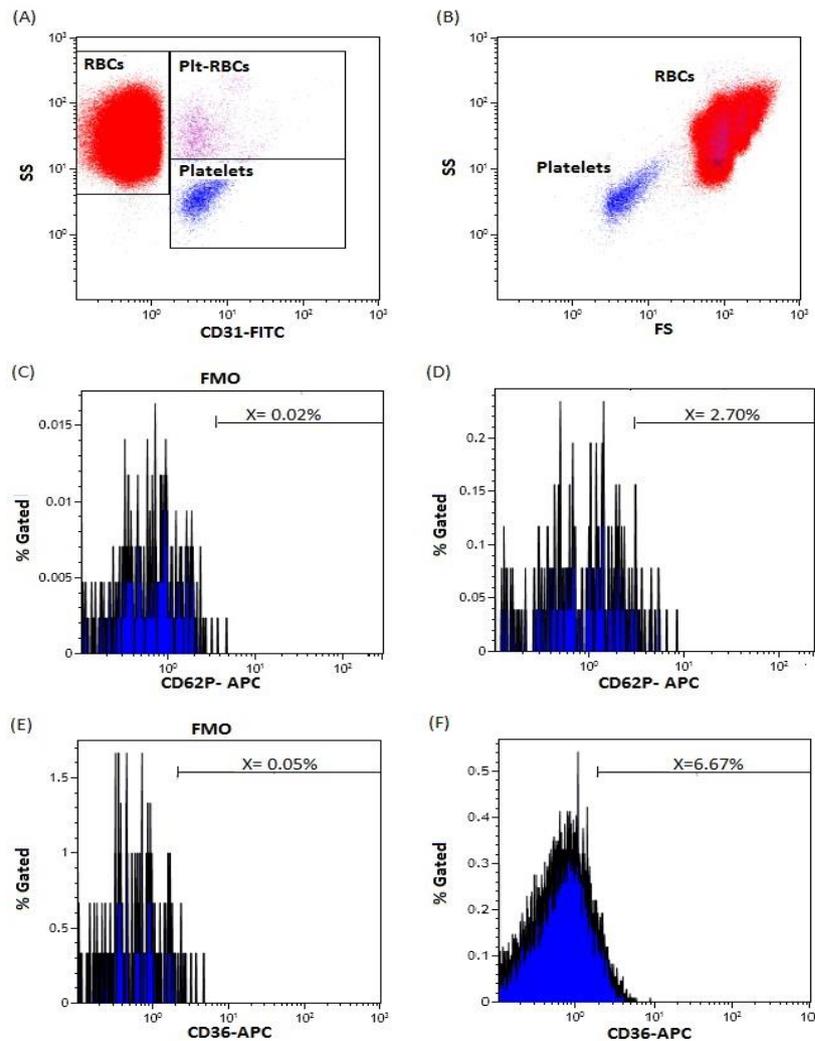
## **2.5 Platelet stimulation with endogenous agonists:**

**2.5.1 Preparation of platelet agonist concentrations:** Agonists were reconstituted according to the manufacturer instructions (all obtained from BIO/Data Corporation, Holland, The Netherlands). Briefly, serial dilutions were performed on the reconstituted agonists to obtain concentrations similar to those previously used in aggregometry based assays (Satchell *et al.*, 2010, Gaglia *et al.*, 2011; O'Brien *et al.*, 2013). The final concentrations were; ADP (5µM, 10µM, 20µM); AA (5mg/ml; 2.5mg/ml; 1.25mg/ml) and Collagen (47.5µg/ml; 95µg/ml; 190µg/ml).

**2.5.2 Platelet stimulation:** To stimulate platelets, 50µl of citrated whole blood was incubated with 20µl of ADP, AA and collagen at the varying concentrations. Samples were incubated in the dark, at room temperature for 10 minutes and were then stained and analysed immediately.

### **2.5.3 Gating Strategy**

Freely circulating platelets were gated based on side scatter, set at logarithmic scale and the expression of CD31 (PECAM-1) which is not present on the surface membrane of red blood cells (RBCs). This allowed the exclusion of platelet bound RBCs (Figure 1A). Fluorescence minus one (FMO) controls were used to determine CD62P and CD36 positive events cells (figure 1 C-E).



**Figure 1. Gating strategy.** The figure illustrates the gating strategy, applied on an individual control sample. Platelets were discriminated based on side scatter and CD31 expression (shown in figure A). The purity of the gated platelet population was ensured by gating on circulating free platelets and excluding platelet bound-RBCs from the analysis (shown in figure A). FMOs were used to distinguish between positive and negative events (figures C and E). Figures D and F illustrate the measurement of %CD62P and %CD36 expression on gated platelets.

## 2.6 Statistical analysis

The D'Agostino-Pearson Normality test was used to determine the distribution of the data. For parametric data the student unpaired t-test was used to compare the means of groups and expressed as mean  $\pm$  SD. While the Mann-Whitney test was used for non-parametric data and expressed as median and Interquartile range [IQR]. In addition, paired data tests were performed to assess the effects of platelet agonists on CD62P MFI. A paired *t* test was performed for parametric data and Wilcoxon matched data test was performed for non-parametric data. All statistical analysis was two-tailed and a *p* value  $\leq$  0.05 was considered as statistically significant. Graph pad prism 5 for windows, version 5.0 was used for statistical analysis.

### 3. Results

The mean age and platelet counts between the two groups were similar (Table 1). The HIV group had decreased levels of circulating CD 4+ T cells, mean  $357.8 \pm 192.6$  vs. Control  $768.9 \pm 214$ ,  $p < 0.0001$ . Furthermore the HIV group showed increased levels of CD8+ T-cells expressing CD38 median  $21.35[16-42.53]$  vs. Control group  $9.23[6.8-12.96]$ ,  $p < 0.0001$ . To determine the association between increased levels of immune activation with platelet activation and disease progression in the HIV group, levels of %CD38 on CD8<sup>+</sup> T cells were correlated with, %CD62P ( $r = -0.109$ ,  $p = 0.589$ ); %CD36 ( $r = 0.111$ ,  $p = 0.496$ ); CD4 count ( $r = 0.046$ ,  $p = 0.731$ ) and Viral load ( $r = 0.379$ ,  $P = 0.008$ ). Although the HIV group showed increased D-dimer levels  $0.23[0.2-0.32]$  vs. Control group  $0.2[0.2-0.2]$ ,  $p = 0.0015$ , these values were within the normal reference interval (shown in table 1).

#### 3.1 Increased levels of platelet %CD36 directly correlates with HIV RNA levels

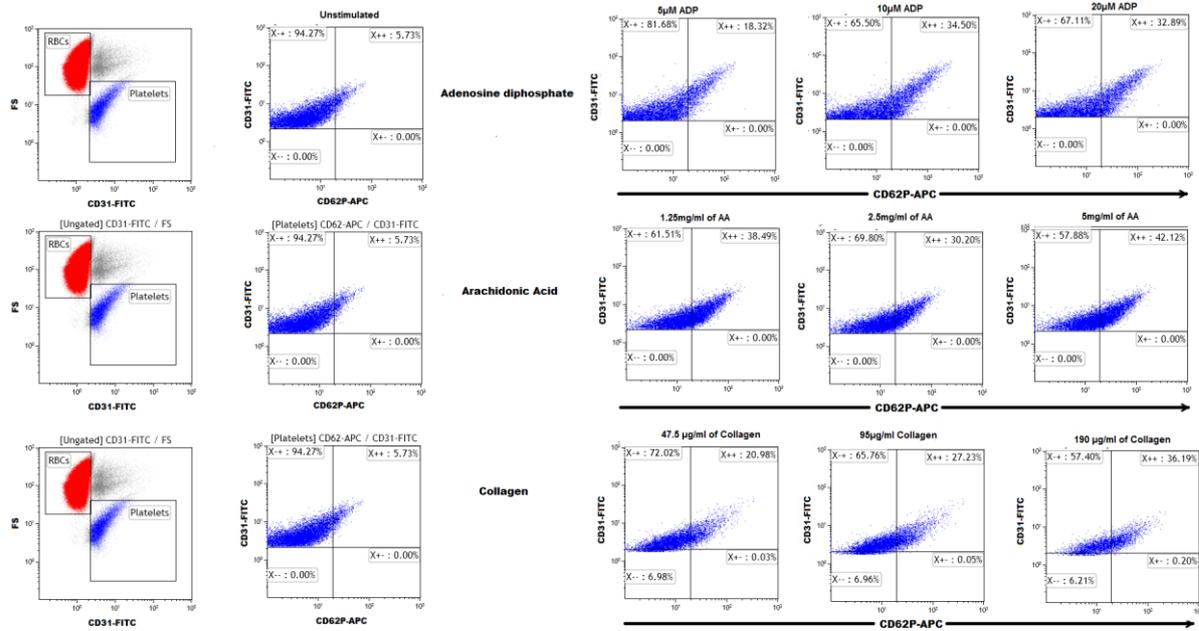
In order to determine baseline levels of platelet activation and aggregation, platelet surface %CD62P and %CD36 expression was assessed. The HIV group showed increased levels of circulating activated platelets, median %CD62P  $5.51[3.03- 10.11]$  vs. Control group  $2.14[0.19 - 3.59]$ ,  $p < 0.0001$ . To further evaluate the association between increased levels of circulating activated platelets and markers of immune activation and disease progression in the HIV group, levels of %CD62P were correlated with, CD4 count ( $r = 0.028$ ,  $p = 0.891$ ) and viral load ( $r = 0.336$ ,  $P = 0.008$ ). In addition the HIV group showed increased levels of %CD36  $21.93[11.03-44.92]$  vs. Control  $16.15[2.24-25.37]$ ,  $p = 0.0087$ . In the HIV group %CD36, a marker of platelet aggregation, showed a direct correlation with viral load ( $r = 0.398$ ,  $p = 0.024$ ) and no correlation with CD4 count ( $r = 0.205$ ,  $p = 0.730$ ).

To evaluate quantitative differences in the levels of platelet activation and aggregation, the mean fluorescence intensity of CD62P and CD36 were measured. There were no significant differences in CD62P MFI levels between the groups, HIV group CD62P MFI  $1.35[1.06-4.67]$  vs. Control group  $1.77[1.47-3.125]$ ,  $p = 0.1191$ . In addition the baseline CD36MFI levels of both groups were similar, HIV group CD36 MFI,  $1.815[1.45-4.76]$  vs.  $2.42[2.24-2.88]$ ,  $p = 0.1374$ .

Table I. Participant demographics and baseline characteristics

Parameter	Control	HIV	P- value	Reference Interval
No. of participants	38	58		
Age	30.30 ± 8.27	32.40 ± 7.34		
Gender (% males)	50	22.5	-	
Ethnicity				
Black (%)	100	100	-	-
CD4 count (cells/ $\mu$ l)	768.9 ± 214	357.8 ± 192.6	<b>&lt;0.0001</b>	500-2010
Platelet count ( $\times 10^9$ /l)	274[236-313.3]	306[226-346.5]	0.2772	178-400
%CD62P	2.14[0.19-3.59]	5.51[3.03-10.11]	<b>&lt;0.0001</b>	-
CD62P MFI	1.77[1.47-3.125]	1.35[1.06-4.67]	0.1191	
%CD36	16.15[2.24-25.37]	21.93[11.03-44.92]	<b>0.0087</b>	-
CD36 MFI	2.42[2.24-2.88]	1.815[1.45-4.76]	0.1374	
D-dimer (mg/l)	0.2[0.2-0.2]	0.23[0.2-0.32]	<b>0.0015</b>	0.00-0.25
% CD8/38	9.23[6.8-12.96]	21.35[16-42.53]	<b>&lt;0.0001</b>	
Log10 mean Viral Load(copies/ml)	-	4.08 ± 0.75	-	

Significant values ( $p < 0.05$ ), are shown in boldface

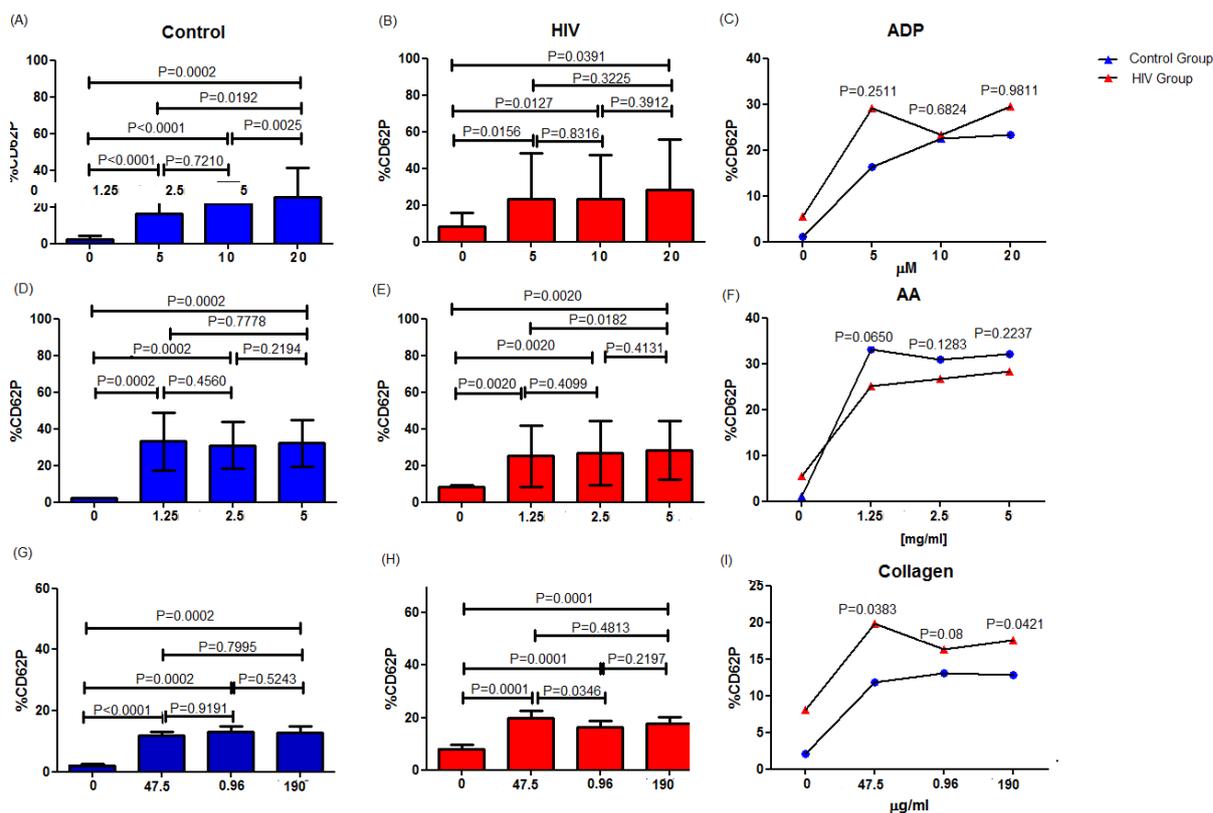


**Figure 2. Platelet response to agonists.** The color dot plots illustrate; agonist induced %CD62P surface expression on an individual control sample using the gating strategy described above (figure 1).

### 3.2 Increased platelet agonist induced activation in HIV infected individuals at a submaximal concentration of collagen

In the control group, maximal platelet activation was obtained at 20 μM of ADP (mean 25.15); 1.25 mg/ml of AA (mean 33.08); 0.48 μg/ml of collagen (median 6.23) (shown in figure 3A). In the HIV group maximal platelet activation was achieved at; 20 μM of ADP (mean 29.53); 5 mg/ml of AA (median 28.26) and at 95 μg/ml of collagen (median 9.87).

In order to evaluate the platelet response to agonists, %CD62P measurements in the control group were performed post stimulation with agonists and compared to the HIV group. The HIV group showed no significant differences in the expression of %CD62P post stimulation with varying concentrations of ADP and AA (figure 3). The HIV group showed increased levels of %CD62P expression post stimulation with collagen at; 47.5 μg/ml (the HIV group median 18.20 [8.39-29.35] vs. 9.43 [7.10-14.61], p=0.0383), and at 190 μg/ml demonstrated significantly decreased levels of %CD62P expression, (HIV median 17.60 [8.24-28.59] vs. 9.12 [5.52-16.88], p=0.0421).

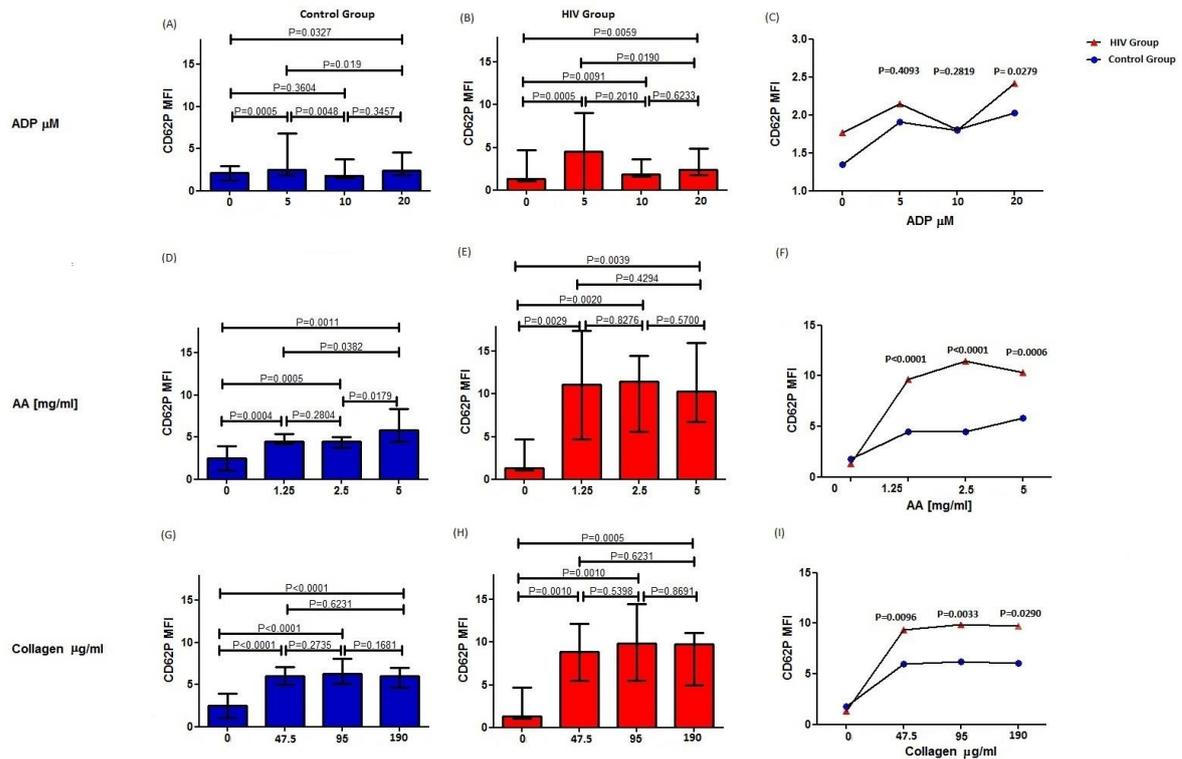


**Figure 3.** %CD62P expression post agonist stimulation. The figure illustrates platelet response measured as the changes in the levels of %CD62P expression in response to agonists at varying concentrations in both the HIV and control group. Figure A-C illustrates platelet response to ADP. Figure D-F illustrates platelet response to AA and figures G-I illustrate platelet response to collagen.

### 3.3 Increased platelet response to endogenous agonists in HIV infected individuals compared to uninfected controls

In order to assess differences in the platelet response to agonists, CD62P MFI levels post stimulation with agonists was measured in the control group and compared to the HIV group. In the control group, maximal platelet activation was obtained at 5 $\mu$ M of ADP (median 2.15); 5mg/ml of AA (median 5.86); 95mg/ml of collagen (median 6.23) (shown in figure 3A). In the HIV group maximal platelet activation was achieved at; 20 $\mu$ M of ADP (median 2.42); 2.5 mg/ml of AA (median 11.46) and at 95 $\mu$ g/ml of collagen (median 9.87).

The HIV group showed increased response to ADP at a concentration of 20 $\mu$ M (median CD62P MFI, 1.91[1.64-4.95] vs. Control 1.75[1.45-2.44] p=0.0279. However at lower concentrations of ADP there were no significant differences in platelet response, at 5 $\mu$ M of ADP (HIV group median CD62P MFI 2.42[1.72-4.84] vs. Control group 2.03[1.57-3.41], p=0.04093); and at 10 $\mu$ M of ADP (HIV group median CD62P MFI 2.24[1.59-5.16] vs. Control group 1.81[1.52-3.78], p=0.2819).



**Figure 4. Platelet response to agonists.** The figure illustrates platelet response measured as the changes in the levels of CD62P MFI in response to agonists at varying concentrations in both the HIV and control group. Figure A-C illustrates platelet response to ADP. Figure D-F illustrates platelet response to AA and figures G-I illustrate platelet response to collagen.

The HIV group showed an overall hyper-response to arachidonic acid (figure 4), with a higher CD62P MFI, at 1.25mg/ml of AA (CD62P MFI  $12.54 \pm 7.91$  vs.  $5.21 \pm 1.66$ ,  $p < 0.0001$ ); at 2.5mg/ml of AA HIV group  $12.09[5.55-14.53]$  vs. Control group  $4.60[4.02-5.48]$ ,  $p < 0.0001$  and at 5mg/ml of AA  $9.64[5.56-16.06]$  vs. Control group  $4.66[3.89-6.903]$ ,  $p = 0.0006$ . A similar trend was observed post stimulation with collagen (shown in figure 4), with increased overall CD62P MFI levels shown in the HIV group at different concentrations of collagen. At 47.5  $\mu\text{g/ml}$  the HIV group showed a hyper-response with median CD62P MFI  $9.51[5.79-11.46]$  vs. Control group  $6.02[5-7.71]$ ,  $p = 0.0096$ . At 95  $\mu\text{g/ml}$ , HIV group CD62P MFI  $9.79 \pm 4.53$  vs. Control group  $6.69 \pm 2.29$  and at 190  $\mu\text{g/ml}$ , CD62P MFI  $9.61 \pm 4.82$  vs.  $6.81 \pm 3.78$ ,  $p = 0.0290$ .

#### 4. Discussion

This study aimed at evaluating markers of platelet activation and aggregation in asymptomatic HIV infection using a whole blood platelet flow cytometry assay. Furthermore we aimed at assessing the relationship between these platelet markers and conventional markers of immune activation and disease progression. We further evaluated differences in the response of platelets to endogenous agonists in HIV, compared to uninfected controls. Flow cytometry allows the quantification and rapid analysis of platelet antigenic characteristics in whole blood (Linden *et al.*, 2004). In addition the use of whole blood minimises any artefactual *in vitro* activation that may be as a result of platelet enrichment methods that involve centrifugation of blood samples. In this study we made use of a whole blood platelet flow cytometry assay that allowed the assessment of platelet function with minimal artefactual platelet activation.

In this study, the HIV group showed decreased levels of CD4<sup>+</sup> T-cells compared to uninfected controls. In addition, the HIV group showed elevated levels of CD38<sup>+</sup> CD8<sup>+</sup> T cells, a marker of general immune activation. Increased levels of Immune activation in HIV are associated with increased all-cause mortality (Kuller *et al.*, 2008). The levels of immune activation directly correlated with viral load and showed no correlation with CD4 counts and markers of platelet activation and aggregation. We also report on elevated D-dimer levels in HIV, although these fell within the normal reference interval of 0.00-0.25 (shown in table 1). The HIV group demonstrated increased levels of circulating activated platelets compared to uninfected controls. This is consistent with previous studies where the authors reported on elevated levels of platelet activation in HIV infected patients on ART (Holme *et al.*, 1998; Mayne *et al.*, 2012). In our study both %CD62P and %CD36 correlated with viral load and showed no correlation with CD4 count. This may suggest the association between viral RNA levels and platelet activation. This is consistent with findings reported by Holme *et al.*, where the authors reported an association between increased levels of activated platelets and HIV RNA levels in HIV patients on ART (Holme *et al.*, 1998). Notably, there were no significant differences in the baseline CD36 and CD62P MFI. This may suggest the presence of quantitative rather than qualitative differences in platelet activation in HIV infected individuals, which is similar to findings reported by Corrales-Medina *et al.*, who reported no qualitative differences in the levels of platelet activation in HIV compared to uninfected controls (Carrales-Medina *et al.*, 2010).

The lack of quantitative differences between baseline platelet activation in the HIV group and control group demonstrated by CD62P MFI levels, allowed the use of CD62P MFI as a parameter to evaluate platelet response to agonists. We report on hyper activation of platelets in HIV infected individuals compared to uninfected controls which is consistent with

results reported by O'Brien *et al.*, where the authors described hyper-reactive platelets in HIV infected individuals on ART (O'Brien *et al.*, 2013). In contrast, Satchell *et al.*, demonstrated hypo-responsive platelets in HIV infected patients compared to uninfected individuals, which was dependant on the type of agonist used (Satchell *et al.*, 2010). The authors reported a decreased response to ADP and collagen in HIV infected individuals and notably, the majority of the participants were on ART and had undetectable HIV RNA levels which was defined as <50 copies/ml (Satchell *et al.*, 2010). Differences between the cohort characteristics may explain these contradictory findings. In addition, differences in the type of assay used to evaluate platelet response exist. In this study we report on platelet degranulation and surface receptor changes as a qualitative measure of platelet response, as demonstrated by the levels of platelet %CD62P expression and CD62P MFI. In contrast, Satchell *et al.*, used a light transmission based aggregometry assay that reports on light absorbance and platelet aggregation as a measure of platelet response to agonists (Satchell *et al.*, 2010).

Based on qualitative differences between the two groups, our study showed hyper-reactive platelets in HIV when compared to uninfected controls. Notably, differences between the two groups, in platelet responses to ADP were only observed when evaluating CD62P MFI at a concentration 20 $\mu$ M and no differences in the %CD62P expression were observed at this concentration of ADP ( $p=0.9811$ )(figure 2). ADP at lower concentrations of 3-5 $\mu$ M induces platelet responses which are PLA<sub>2</sub> dependent whereas at higher concentrations of 20 $\mu$ M, they are PLC dependent (Quinn *et al.*, 2005). This may suggest that in the context of HIV ADP induced platelet hyper-reactivity is dependent on PLC mediated signalling pathways. Interestingly in the HIV group maximum platelet activation was achieved at submaximal concentrations of 2.5mg/ml AA. This was unlike the control group where maximal platelet activation was achieved at a concentration of 5mg/ml. Activated PLA<sub>2</sub> facilitates the release of cell membrane AA and makes it available as a substrate that is converted into prostaglandin G<sub>2</sub> (PGG<sub>2</sub>) in the presence of cyclooxygenase (COX). PGG<sub>2</sub> is then peroxidised into prostaglandin G<sub>2</sub> (PGH<sub>2</sub>) (Cho *et al.*, 2003). Taken together this may suggest an enhanced response to submaximal AA concentrations in HIV infected individuals. Thromboxane A<sub>2</sub> synthetase converts PGH<sub>2</sub> into TXA<sub>2</sub> which is then released by activated platelets which may result in autocrine and paracrine platelet activation (Quinn *et al.*, 2005). TXA<sub>2</sub> amplifies the initial activation stimulus and promotes the recruitment of additional platelets which further promotes thrombus growth and stability (Son *et al.*, 2004). Taken together, our findings may suggest that in HIV increased platelet activation, in response to submaximal AA concentrations, may promote a pro-coagulatory phenotype and agents inhibiting TXA<sub>2</sub> activity may be useful in the management of thrombotic disorders in this

disease. In support of this CVDs have been reported in patients with increased levels of AA induced platelet activation despite being on aspirin therapy (Gaglia *et al.*, 2011).

Collagen directly activates platelets through the  $\alpha_2 \beta_1$  integrin and GPIV receptor (Podrez *et al.*, 2007). Maximal platelet activation was achieved at a similar concentration of 95 $\mu$ g/ml collagen, in both the HIV and control group (shown in figure 3). Platelets in the HIV infected group showed an overall hyper response to collagen as measured by both %CD62P expression and CD62P MFI (figures 2 and 3). CD36 signalling is PLC dependent whereas  $\alpha_2 \beta_1$  integrin mediated signalling is dependent on PLA<sub>2</sub>. Furthermore, collagen induced platelet activation exacerbates inflammation (Boilard *et al.*, 2010).. Interestingly the HIV group had increased baseline levels of %CD36 and platelet activation via this receptor may result in increased levels of inflammation. Signals generated via the CD36 (GPIV) receptor are more potent than that of the  $\alpha_2 \beta_1$  integrin (Podrez *et al.*, 2007). Moreover, platelets expressing CD36 have been reported as hyper reactive to platelet agonists (Podrez *et al.*, 2007).

The *in vitro* cross-sectional nature of the study limits the description of a causative relationship between platelet function and thrombotic events in the current cohort. Future longitudinal follow up studies will be essential in evaluating the possible thrombotic risk profiling and predictive value of this assay. In conclusion, we describe elevated levels of both activated and aggregated circulating platelets in HIV. We further show that the levels of platelet aggregation and activation directly correlate with viral load and not CD4 count. However the potential value of evaluating platelet function using the described flow cytometry assay in the monitoring of platelet activation and function in HIV will require further studies.

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### **Competing interests**

The authors declare that there are no financial, personal or professional competing interests that may interfere with this work.

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**CHAPTER 5: Platelet leukocyte aggregates and markers of and platelet aggregation immune activation and disease progression in HIV infected treatment naive asymptomatic individuals.**

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## **Abstract**

### **Background**

Platelet monocyte aggregates (PMAs) are a more sensitive marker of platelet activation compared to platelet neutrophil aggregates (PNAs) and platelet surface P-Selectin (CD62P) expression. Platelet aggregates play a crucial role in the immune defence mechanism against bacteria and viruses and increased levels of lipopolysaccharide (LPS) have been reported in human immunodeficiency virus (HIV) infected individuals. Platelets are capable of interacting with bacterial LPS and subsequently forming platelet leukocyte aggregates (PLAs). This study aimed at determining the levels of circulating PLAs in treatment naïve HIV infected individuals and to determine the relationship of PLAs in HIV infected individuals, with markers of immune activation, disease progression and platelet aggregation. A further aim was to assess the effects of LPS stimulation on the formation of PLAs

### **Methodology**

Thirty-two (32) HIV negative and 35 HIV positive individuals were recruited from a clinic in the Western Cape. All participants had normal leukocyte and platelet counts. Platelet monocyte and platelet neutrophil aggregates were measured using flow cytometry at baseline and were correlated with markers of platelet activation (CD62P) and aggregation (CD36); monocyte and neutrophil activation (CD69); monocyte tissue factor expression (CD142); immune activation (CD38 on T+ cells); CD4 count and viral load and a marker of active coagulation (D-dimers). Platelet monocyte aggregates were also measured post stimulation with lipopolysaccharide.

### **Results**

PMA levels were higher in HIV 25.26[16.16-32.28] vs. 14.12[8.36-18.83],  $p=0.0001$  when compared to negative individuals. PMAs correlated with %CD38/8 expression ( $r=0.54624$ ,  $p=0.0155$ ); CD4 count ( $r=-0.6964$ ,  $p=0.0039$ ) viral load ( $r=0.633$ ,  $P<0.009$ ) and levels of monocyte %CD69 expression ( $r=0.757$ ,  $p=0.030$ ). In addition the %PMAs correlated with platelet %CD36 expression ( $r=0.606$ ,  $p=0.017$ ). The HIV group showed increased levels of %CD62P 5.44[2.72-11.87] vs. Control 1.15 [0.19-3.59],  $p<0.0001$ ; %CD36 22.53[10.59-55.15] vs. 11.01[3.69-26.98],  $p=0.0312$  and tissue factor expression (CD142) (CD142 MFI 4.84[4.01-8.17] vs. 1.74[1.07-9.3],  $p=0.0240$ ). Stimulation with LPS induced a significant increase in PMAs in both groups; however there were no significant differences in PMAs between the two groups.

## Conclusion

We describe increased levels of circulating PMAs which directly correlates with markers of immune activation, disease progression and platelet aggregation in HIV treatment naïve individuals.

## 1. Introduction

Inflammation and chronic immune activation are associated with increased mortality and disease progression in human immunodeficiency virus (HIV) infected individuals (Ross *et al.*, 2009). Activated monocytes and platelets have been described as facilitators of both inflammation and cardiovascular disease (CVD) (Cerletti *et al.*, 2010; Funderburg *et al.*, 2014) however the association between inflammation and CVDs in HIV remains inconclusive (O'Brien *et al.*, 2013). Platelet aggregates play a crucial role in the immune defence mechanism against bacterial and viral pathogens (Beck *et al.*, 2013; David, 2013). The broad repertoire of platelet surface receptors enables the formation of interactions between platelets and invading organisms (Speth *et al.*, 2013). Scavenger and toll-like receptors (TLRs) expressed on the surface of platelets allow them to function as sensory cells for pathogens and pathogen induced inflammatory ligands (Metcalf *et al.*, 2013; Clark *et al.*, 2007; Arazna *et al.*, 2013). Consequently, platelets are able to initiate interactions with cells involved in both the innate and adaptive immune response (de Gaetano *et al.*, 2007; Bournazos *et al.*, 2008; Metcalf *et al.*, 2013). These interactions induce platelet activation and degranulation which subsequently increases the trapping of adjacent circulating leukocytes resulting in the formation of neutrophil extracellular traps (NETS) which are able to trap pathogens within the circulation (Clark *et al.*, 2007). Platelets have been described as initiators and mediators of inflammation at the vessel wall (Shi & Morrell, 2011) and alterations in normal platelet kinetics have been reported in human immunodeficiency virus (HIV) infected individuals (O'Brien *et al.*, 2013; Satchell *et al.*, 2010). Furthermore, decreases in platelet counts have been associated with elevated platelet monocyte aggregates (Metcalf *et al.*, 2013) plasma HIV RNA levels and disease progression (Reig *et al.*, 2007).

Although activated platelets preferentially form complexes with monocytes, they have the capacity to directly interact with neutrophils via P-selectin and P-Selectin glycoprotein ligand-1 (PSGL-1) receptor engagements (Singh *et al.*, 2012). Elevated platelet leukocyte aggregates (PLAs) play a crucial role in the pathogenesis of thrombotic conditions. However, platelet monocyte aggregates (PMAs) have been reported as a more sensitive marker of platelet activation (Harding *et al.*, 2007) than platelet neutrophil aggregates (PNAs) and platelet surface P-selectin(CD62P) expression (Michelson *et al.*, 2001). Furthermore,

elevated levels of circulating PMAs have been demonstrated in HIV individuals on antiretroviral therapy (ART) (Singh *et al.*, 2012).

Interactions between platelets and monocytes can often result in the activation of monocytes and the modulation of monocyte function (Metcalf *et al.*, 2013). The result of increased monocyte activation was recently linked with cardiovascular disease in a study which described an association between monocytes expressing increased levels of toll like receptor 4 (TLR4) and cardiovascular disease (Tapp *et al.*, 2013).

On the other hand, platelet-neutrophil interactions may induce neutrophil activation with the subsequent release of pro-inflammatory molecules (Nathan, 2006). Platelets are capable of interacting with bacterial LPS and forming complexes with neutrophils (PNA's) (Clark *et al.*, 2007). These PNAs, which are the result of LPS bound platelets, release reactive oxygen species (ROS) and are associated with neutrophil mediated endothelial cell damage (Clark *et al.*, 2007).

Although antiretroviral therapy (ART) suppresses HIV viral replication, chronic immune activation and bacterial translocation in HIV infected individuals persists (Cassol *et al.*, 2010). Bacterial translocation in HIV has been associated with chronic immune activation and increased levels of LPS have been reported in HIV infected individuals (Brenchley *et al.*, 2006). However, data regarding LPS induced PLAs in the context of HIV remains scarce.

Flow cytometry offers a sensitive and rapid technique for the evaluation of PLAs (Harding *et al.*, 2007; de Gaetano, 2007). However due to the sensitivity of the technique, several methodological considerations have to be considered in order to ensure that ex vivo artefacts are kept minimal (Harding *et al.*, 2007). In this study PMAs and PNAs were measured using a rapid flow cytometry technique. Efforts to minimise artefactual platelet-leukocyte aggregate formation included avoiding centrifugation and washing of samples. This study aimed at measuring baseline PNAs and PMAs in treatment naïve HIV infected individuals and to correlate these with markers of platelet activation (CD62P) and aggregation (CD36), monocyte and neutrophil activation (CD69); immune activation (CD38 on T+ cells), CD4 count, viral load and marker of active coagulation (D-dimers). We further determined the response of platelet monocyte aggregates/complexes to stimulation with LPS.

## **2. Methodology**

### **2.1 Participants and sample collection**

Participants were recruited from the Emavundleni Voluntary Counselling and testing (VCT) clinic in crossroads, Cape Town. The participant demographics are described in table 1. Participants were excluded if they were on aspirin, anti-inflammatory or anti-TB drugs. Ethical

review was performed according to the declaration of Helsinki and the study was approved by the Stellenbosch University human research ethics committee (HREC) (no. N07/09/197). The study was also registered with the University of Cape Town research ethics committee (REC) (no. 417/2006). Informed consent was obtained from each participant.

## **2.2 Study Design**

Baseline platelet-monocyte and platelet-neutrophil complexes were measured using flow cytometry. CD4 T cell count; viral load; full blood counts, D-dimers and fibrinogen were also measured. Associations between each baseline study parameter were assessed. This was followed by LPS induced monocyte activation. The levels of both PMAs and PNAs were then measured and a comparison between the post LPS stimulation sample and the baseline sample was made.

## **2.3 Sample collection**

A volume of 2-3ml of venous blood was collected by venipuncture into 4.5ml tubes containing 3.2% sodium citrate (BD Vacutainer, San Jose). Samples were kept at room temperature during transit from clinic to the laboratory. The time frame between sample collection and sample analysis was limited to 1-3hrs.

## **2.4 Antibodies and reagents**

The anti-human CD14-PC5, CD69-PC7, CD42b monoclonal antibodies were purchased from Beckman Coulter, USA. The anti-human CD16-PE, 7AAD and CD142(HTF-1) were purchased from BD Biosciences, San Jose, CA. Antibody titration assays were performed to detect the optimal antibody concentrations.

## **2.5 Markers of immune activation**

**2.5.1 CD4 T Cell count:** The BD MultiTEST CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC reagent (BD Biosciences) and BD TruCOUNT tubes (BD Biosciences, San Jose, CA) were utilised for the measurement of CD4 T cell count. CD4 T cell flow cytometry was performed at the Division of Medical Virology, Faculty of Health Sciences, Stellenbosch University, which is accredited by the South African National Accreditation System (SANAS).

**2.5.2 Viral load measurements:** Blood samples were collected into 5ml EDTA tubes, which were centrifuged at 20°C at 300g for 12 minutes. 1 ml of plasma was transferred into a Greiner Bio-one cryotube (Greiner Bio-One GmbH, Frickenhausen, Germany) and sent for viral load testing. The viral load assay performed was a NucliSensEasyQ® HIV-1 v1.2 Viral Load Test (BioMerieux Inc., Boxtel, Netherlands) which has a detection range of 1.60 to 6.7 log<sub>10</sub> copies/ml. Viral load testing was performed at the SANAS-accredited, Division of Medical Virology, Faculty of Health Sciences, Stellenbosch University.

**2.5.3 Measurements of the percentage of CD8+ T-cells expressing CD38:** This measurement was performed using flow cytometry. Briefly, 50µl of whole blood was stained with a titrated monoclonal antibody cocktail containing; CD8 Per-CP; CD38 APC; CD3 FITC (BD Biosciences, San Jose, CA). Data acquisition was performed using a BD FACSCalibur instrument and analysis was done using the BD Cell Quest Pro (Version 2) software.

**2.5.4 TF measurements:** Monocyte tissue factor expression measurements were performed using flow cytometry. Briefly 50µl of citrated whole blood was stained with 5µl of an antibody cocktail containing; CD14 PC5 (Beckman Coulter, USA) and CD142 PE (BD Biosciences, San Jose, CA). Data acquisition was performed using a Beckman Coulter FC500 cytometer and analysis was done Kaluza V 1.2 software (Beckman Coulter, USA).

**2.5.5 D-dimer measurements:** The measurement of D-dimer levels was performed on citrated blood. Blood samples were collected using 4.5ml tubes containing 3.2% sodium citrate. Measurements were performed using the HemosIL™ Dimertest® Kit (Instrumentation Lab) and the ACL TOP platform at the SANAS-accredited NHLS Haematology coagulation laboratory at Tygerberg Hospital.

## **2.6 Measurements of PMAs and PNAs**

### **2.6.1 Prior to *in vitro* monocyte and neutrophil activation**

The measurement of PMAs and PNAs was performed on whole blood. Briefly, 50µl of blood was stained with 5µl of titrated antibody cocktail. The cocktail consisted of titrated volume of monoclonal antibodies; CD16-PE, CD14-PC5, CD69-PC7 and CD42b FITC. Red blood cell lysis was performed using 500 µl of FACS lyse. 5µl of Transfix was added at the time of red blood cell lysis to limit the artefactual formation of platelet leukocyte aggregates which could be the result of red blood cell lysis. As a final step before acquisition, samples were stained with 7AAD (a viability marker) and incubated for 15 minutes. Samples were then analysed immediately. Fluorescence minus one (FMO) was used to distinguish positive from negative events and unstained whole blood was used as a control for 7AAD staining. PMAs were defined as CD14<sup>+</sup> CD42b<sup>+</sup> dual positive events while PNAs were identified as CD16<sup>+</sup> CD42b<sup>+</sup> events (shown in figure 1). The CD42b mean fluorescence intensity (MFI) and percentage (%) CD42b expression was used for qualitative and quantitative analysis of PMAs.

### **2.6.2 Gating strategy**

Viable CD14<sup>+</sup> monocytes were gated as shown in figure 1A and 1C. The expression of CD42b on monocytes was assessed (figure 1H). Monocytes expressing CD42b were regarded as platelet-monocyte complexes. CD69 expression was also evaluated to determine monocyte activation. Fluorescence minus one (FMO) control was used to distinguish between positive and negative CD69 and CD42b events. For the assessment of

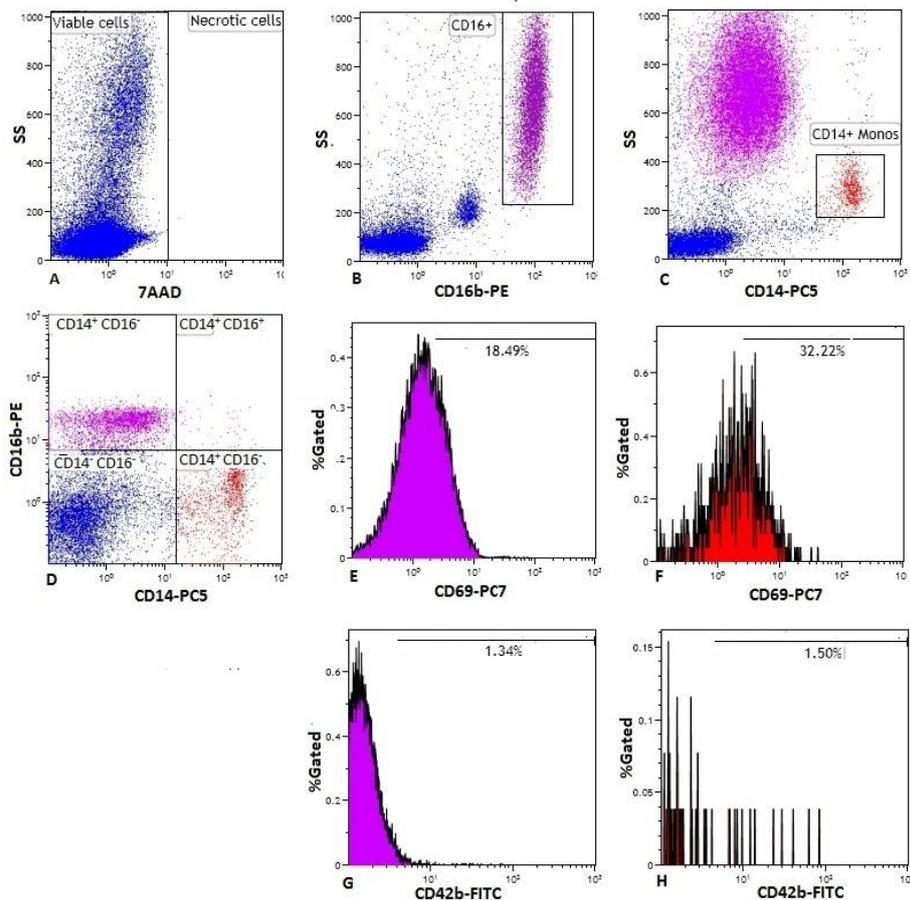
platelet-neutrophil complexes CD16<sup>+</sup> neutrophils were gated (figure 1B) and the expression of CD69 and CD42b on these neutrophils was assessed (figure 1E and G). Neutrophils expressing CD42b were regarded as platelet-neutrophil complexes.

### **2.6.3 Post *in vitro* monocyte activation**

A volume of 20 µl of LPS (2µg/ml) was used for monocyte and neutrophil stimulation. Briefly, 50 µl of blood was treated with 20 µl of LPS. This was followed by 16 hours overnight incubation at 37 °C. The blood samples were then stained as mentioned above with the addition of 7AAD for the assessment of cell viability (shown in figure 2A). The expression of CD42b on viable; monocytes (7AAD<sup>-</sup> CD14<sup>+</sup>) was measured post stimulation with LPS (figure 2C). FMO controls were used to distinguish between positive (CD14<sup>+</sup> CD42b<sup>+</sup>) and negative (CD14<sup>+</sup> CD42b<sup>-</sup>) events (shown in figure 2E). This ensured the analysis and reporting of physiologically viable and functional PMAs, induced by *in vitro* LPS stimulation.

### **2.7 Statistical analysis**

Statistical analysis was performed using Graph pad prism 5 for windows, version 5.00 software. The Mann-Whitney *U* test was used to compare non-parametric data and these values were reported as median and interquartile range. For parametric data an unpaired student *t* test was performed and data was reported as mean and standard deviation. Paired data tests were also performed. For parametric data a paired t-test analysis performed and for non-parametric data Wilcoxon matched data set analysis was performed.



**Figure 1. Gating strategy.** The figure illustrates the baseline measurement of monocyte, neutrophil activation and platelet complexes using a control sample. Figure A represents the determination of viability of cells using 7AAD. The gating strategy applied to discriminate CD16b<sup>+</sup> neutrophils and CD14<sup>+</sup> monocytes is shown in figures B-D. The histograms E and F demonstrate the measurements of monocyte activation and Neutrophil activation using CD69. Histograms G and H, demonstrate the measurement of baseline platelet neutrophil (CD16b<sup>+</sup>CD42b<sup>+</sup>) and platelet monocyte (CD14<sup>+</sup>CD42b<sup>+</sup>) complexes.

### 3. Results

The cross-sectional study consisted of a total of sixty-seven (67) participants, 32 HIV negative controls and 35 HIV positive ARV-naïve individuals. The characteristics of the study participants are tabulated in table I. The study groups demonstrated a similar mean age of 30 years and no participants were thrombocytopenic. Differences in absolute monocyte and neutrophil count were observed amongst the two groups; however these were within the normal reference intervals (shown in table 1). The HIV group showed increased levels of circulating activated platelets as demonstrated by the increased median baseline levels of platelet %CD62P expression 5.44[2.72-11.87] vs. Control group 1.150[0.19-3.59], p<0.0001. The HIV group also showed increased median levels of %CD36 a marker of platelet

aggregation, 22.53[10.59-55.15] vs. control group 11.01[3.69-26.98],  $p=0.0312$  (shown in table 1).

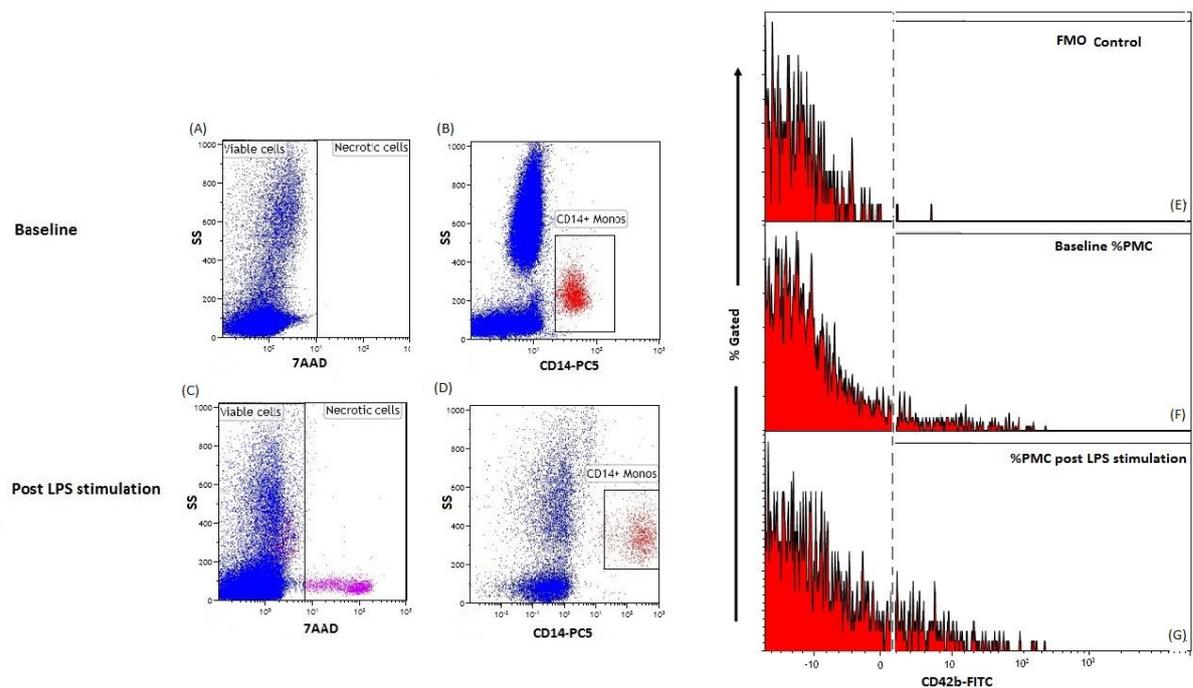
Table 1. Participant demographics and baseline characteristics

Parameter	Control	HIV	P-value	Reference Interval
<b>No. of participants</b>	32	35		
<b>Age</b>	30.00 ± 1.52	30.43 ± 1.23	0.8276	
<b>Gender (% males)</b>	52.4	33.3	-	
<b>Ethnicity</b>				
<b>Black (%)</b>	100	100	-	-
<b>CD4 count (cells/μl)</b>	743.6 ± 39.92	357.1 ± 38.77	<b>&lt;0.0001</b>	500-2010
<b>Monocytes (x10<sup>9</sup>/l)</b>	0.34[0.25-0.41]	0.28[0.19-0.33]	<b>0.0377</b>	0.18-0.80
<b>Neutrophils (x10<sup>9</sup>/l)</b>	3.13[2.38-4.56]	2.51[1.73-3.42]	<b>0.0107</b>	2-7.50
<b>Platelet count (x10<sup>9</sup>/l)</b>	281.5 ± 12.44	296.8 ± 17.0	0.4629	178-400
<b>%CD62P</b>	1.15[0.19-3.59]	5.44[2.72-11.87]	<b>&lt;0.0001</b>	-
<b>%CD36</b>	11.01[3.69-26.98]	22.53[10.59-55.15]	<b>0.0312</b>	-
<b>Monocyte CD69 MFI</b>	7.49[5.463-14.74]	16.79[15.39-25.96]	<b>0.0150</b>	-
<b>Monocyte TF expression (MFI)</b>	1.74[1.07-9.3]	4.84[4.01-8.17]	<b>0.0240</b>	
<b>Neutrophil CD69 MFI</b>	11.51 ± 8.73	34.63 ± 18.15	<b>0.0015</b>	-
<b>%Platelet monocyte aggregates</b>	14.12[8.36-18.83]	25.26[16.16-32.28]	<b>&lt;0.0001</b>	
<b>MFI Platelet-monocyte aggregates</b>	11.34[3.67-15.99]	22.29[19.26-27.39]	<b>&lt;0.0231</b>	-
<b>%Platelet neutrophil aggregates</b>	2.49[0.78-4.390]	1.78[0.52-4.390]	0.3604	
<b>MFI Platelet neutrophil aggregates</b>	23.38[17.25-32.70]	30.15[19.79-48.14]	0.6254	-
<b>D-dimer (mg/l)</b>	0.2[0.2-0.2]	2.6[2.5-3.0]	<b>&lt;0.0001</b>	0.00-0.25
<b>% CD8/38</b>	9.63[5.554-13.35]	19.45[14.02-43.32]	<b>0.0001</b>	
<b>Log10 mean Viral Load(copies/ml)</b>	-	4.08 ± 0.75	-	

Significant values (p<0.05), are shown in boldface

### 3.1 Increased levels of circulating activated platelets, monocytes and PMAs in HIV infected individuals compared to uninfected individuals

In order to compare the levels of platelet monocyte aggregates between the groups, the percentage expression of CD42b on monocytes was measured. The HIV group showed elevated levels of %PMAs 25.26[16.16-32.28] vs. Control group 14.12[8.36-18.83],  $p < 0.0001$ . To further assess the relative number of platelets bound on monocytes, differences in the mean fluorescent intensity (MFI) were analysed. The HIV group showed increased levels of PMA MFI 23.07[19.26-27.39] vs. control group 11.22[3.42-16.38],  $< 0.0001$ . This observation indicated an increased number of platelets bound to each monocyte. There were no significant differences in the levels of circulating PNAs between the two groups (shown in table 1).



**Figure 2. Platelet monocyte gating strategy.** The figure demonstrates platelet monocyte measurements prior and post LPS stimulation in a control sample. Figures A and C illustrate the use of 7AAD as a viability dye to ensure analysis of viable monocytes. Figures B and D are gated on the viable cells which are 7AAD negative. Figure E demonstrates the use of an FMO control to distinguish between positive and negative cells. While figures F-G illustrate %PMA changes in response to LPS stimulation.

### **3.2 Platelet monocyte aggregates inversely correlate with CD4 count and directly correlate with markers of immune activation, disease progression and platelet activation and aggregation**

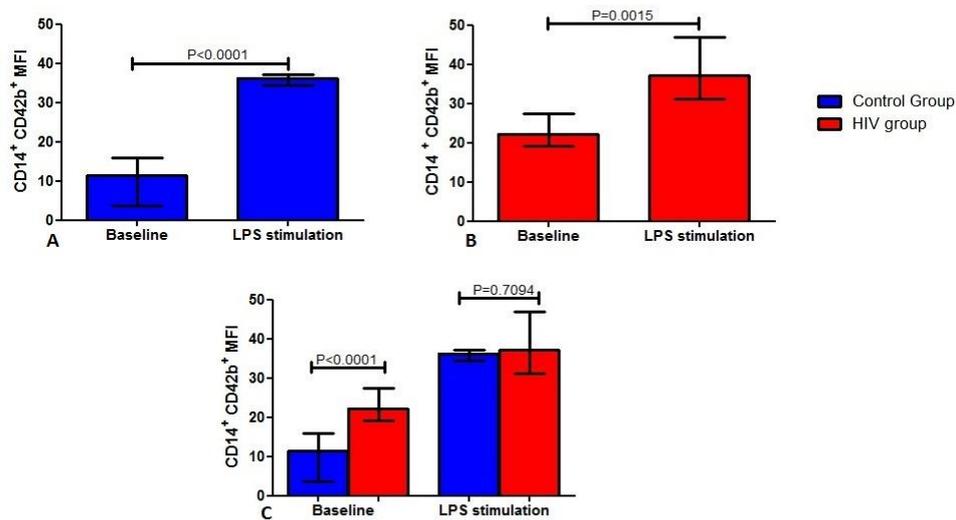
To determine the associations between PMAs and markers of immune activation and coagulation, PMAs were correlated with %CD38/8 expression, ( $r=0.54624$ ,  $p=0.0155$ ); CD4 count ( $r=-0.6964$ ,  $p=0.0039$ ) and viral load ( $r=0.633$ ,  $P<0.009$ ). The levels of monocyte activation (%CD69 expression) showed a direct correlation with PMAs ( $r=0.757$ ,  $p=0.030$ ). In order to determine the association of %PMA levels with platelet aggregation and activation, the %PMA was correlated with platelet %CD36 expression ( $r=0.606$ ,  $p=0.017$ ) and free circulating activated platelets %CD62P expression ( $r=0.236$ ,  $p=0.379$ ). The %PMA showed no significant correlation with D-dimers ( $r=-0.119$ ,  $p=0.638$ ), a marker of active coagulation.

### **3.3 Increased levels of monocyte TF expression in HIV does not correlate with the percentage of PMAs**

To determine the levels of pro-coagulant monocytes, tissue factor (CD142) expression on each cell was compared between the two groups. The HIV group showed increased TF expression as demonstrated by increased TF MFI  $4.84[4.01-8.17]$  vs.  $1.74[1.07-9.3]$ ,  $p=0.0240$ . To assess the association between monocyte TF expression and platelet monocyte interactions, PMA levels were correlated with monocyte TF expression. This analysis demonstrated that there was no relationship between PMA and TF expression on the monocytes (%PMAs ( $r=0.322$ ,  $p=0.368$ ), PMA MFI( $r=-0.479$ ,  $p=0.166$ )). In addition, there was no correlation between TF expression and viral load ( $r=-0.1364$ ,  $p=0.6937$ ); CD4 count ( $r=-0.2252$ ,  $p=0.4018$ ) or; CD38/8 ( $r=0.09890$ ,  $p=0.7479$ ).

### **3.4 LPS induces increased PMA levels in HIV infected individuals compared to uninfected controls**

To determine the effects of TLR4 ligand signalling on platelet-monocyte cross talk, the levels of platelet-monocyte complexes post-stimulation with LPS were measured. In the control group the PMA MFI was significantly increased post stimulation with LPS, median  $34.37[32.32-35.50]$  vs. baseline  $11.22[3.42-16.38]$ ,  $p=0.0010$ . Similarly in the HIV group the %PMA levels were increased post LPS stimulation, median  $37.24[31.14-46.90]$  vs.  $23.07[19.26-27.39]$ ,  $p=0.0157$ . However there were no significant differences in the %PMAs between the two groups post LPS stimulation,  $34.37[32.32-35.50]$  vs. baseline  $24.68[21.19-33.86]$ ,  $p=0.7094$ .



**Figure 3. Platelet monocyte complexes post LPS stimulation.** The graphs demonstrate the % of PMAs in the control group (figure A) and HIV group (figure B), post stimulation with LPS). Graph C illustrates the PMA MFI differences between the two groups.

In the control group the PMA MFI was significantly increased post stimulation with LPS and a similar response was observed in the HIV group (Figure 3A and B). However, post LPS stimulation there were no differences between the two groups with regard to the PMA MFI. Notably, the control group showed a greater response to LPS (figure 3A) compared to the HIV group (figure 3B). This may be as a result of increased baseline levels of circulating PMAs in the HIV group compared to the control group (table 1). Platelets and monocytes may be primed *in vivo* and therefore demonstrate minimal *in vitro* stimulation and interactions compared to that of the control group.

#### 4. Discussion

The measurement of platelet leukocyte aggregates using flow cytometry, offers a highly sensitive and rapid method for the qualitative (mean fluorescence intensity) and quantitative (%positive events) measurement of platelet leukocyte aggregates. Efforts have been made to standardize this technique (Harding *et al.*, 2007; de Gaetano, 2007; Pearson *et al.*, 2009; Majumder *et al.*, 2012; Singh *et al.*, 2012; Nagasawa *et al.*, 2013) and measurement of platelet monocyte aggregates have been demonstrated to be a more sensitive and robust measure of platelet activation than platelet neutrophil aggregates and surface P-selectin (Michelson *et al.*, 2001). The use of flow cytometry in the measurement of platelet aggregates however requires numerous pre-analytical and analytical methodological considerations in order to minimize *in vivo* artefacts (Singh *et al.*, 2012, Harding *et al.*, 2007). These include the type of anticoagulant used and the time between sample collection and immunostaining (Harding *et al.*, 2007). In the present study the anticoagulant sodium citrate was used, as a previous study had reported reduced, *in vivo* time dependant changes in

platelet monocyte aggregates when blood was collected into heparinised and EDTA tubes (Harding *et al.*, 2007). To further minimise *in vivo* induced changes in platelet aggregates, centrifugation and washing of samples was omitted. Although red blood cell lysis was performed, the addition of fixative at the time of red blood cell lysis kept the consequential platelet activation to a minimum, limiting platelet activation induced by ADP which is released upon the lysis of red blood cells. Furthermore, care was taken to ensure the viability of cells and in order to ensure that necrotic cells were excluded from the analysis 7AAD was utilised in the gating strategy (shown in figure 1a). The primary aim of this study was to determine the levels of platelet monocyte and platelet neutrophil interactions in HIV treatment naïve individuals. This was achieved by measuring the levels of platelet monocyte and platelet neutrophil aggregates and assessing the association between platelet aggregates; markers of immune activation and disease progression.

In this study we report on increased levels of activated platelets and monocytes in HIV infected individuals compared to uninfected individuals. Recent studies have described the role platelets play in the defence against invading pathogens. In these situations platelet activation and aggregation is crucial for the engulfment and killing of pathogens by activated platelets (Flaujac *et al.*, 2010; Metcalf *et al.*, 2013). In our study, HIV infected individuals had increased levels of monocyte tissue factor expression compared to their uninfected counterparts. These findings are consistent with those reported by Funderburg *et al.*, where the authors observed increased monocyte TF expression in HIV infected individuals on highly active antiretroviral therapy HAART (Funderburg *et al.*, 2010). Activated monocytes are involved in the initiation of inflammation, clearance of apoptotic cells and the facilitation of both the innate and adaptive immune response. Based on their expression of CD16, monocytes are classified into three distinct subsets; classical, intermediate and nonclassical. Specific functions and responses to bacterial and viral products between the different subsets have been well described (Ziegler-Heitbrock, 2007; Cros *et al.*, 2010) and classical monocytes are considered a source of infiltrating macrophages involved in atherosclerosis (Ingersoll *et al.*, 2011). Activated platelets express a repertoire of receptors that enable and facilitate platelet leukocyte crosstalk and as a result platelet leukocyte aggregates are formed. Therefore, platelet leukocyte aggregates may play a key role in inflammation and thrombosis (Cerletti *et al.*, 2012).

In this study, platelet monocyte aggregates were significantly increased in HIV compared to the control group. These findings are consistent with recent work published by Singh *et al.*, in which the authors reported that there were increased PMAs in HIV infected individuals on ART. The authors further observed a positive correlation between platelet CD62P expression and PMAs (Singh *et al.*, 2012). However this is contrary to our findings as in our study there was no correlation between PMAs and CD62P. Notably, Singh *et al.*, reported on a cohort of

only 8 HIV infected individuals and the differences in the size of the cohorts may explain the contradictory findings.

Our observations demonstrated that PMAs in HIV infected individuals directly correlate with viral load, %CD38/8 and inversely correlate with CD4 count. In addition, PMAs also showed a positive correlation with the % of platelets expressing CD36. The role of CD36 as a mediator of inflammation and thrombosis has been previously described (Podrez *et al.*, 2007). CD36 is a scavenger receptor expressed by monocytes, macrophages capillary endothelial cells and platelets (Febbraio *et al.*, 2001). Its expression on monocytes has been associated with the formation of foam cells which are abundantly found at sites of atherosclerosis (Rahaman *et al.*, 2006). Platelet CD36 plays a crucial role in platelet aggregation and inflammation (Febbraio *et al.*, 2001). Taken together with our current findings this may suggest that platelet CD36 expression could be a useful marker for the prediction of PMA formation. Interestingly, the HIV group showed increased baseline percentages of platelets expressing CD36. However the lack of a correlation between CD36 expression and viral load suggests that the mechanism responsible for its up-regulation is independent of viral load. However, platelet CD36 may be a valuable marker in the thrombotic risk profiling of HIV infected individuals.

The *in vivo* expression of monocyte TF expression in response to TLR4 ligands has been well described (Funderburg *et al.*, 2010). In our study, stimulation with LPS induced increased PMAs in both the HIV and control group. However, no significant differences in the levels of PMAs between the two groups, post stimulation, were observed. This may suggest that although baseline levels of PMAs are increased in HIV, LPS and TLR4 ligand interactions may induce PMA formation in a similar manner to uninfected individuals. In HIV, the persistent microbial translocation and consequential immune activation (Cassol *et al.*, 2010) would lead to the activation of both platelets and monocytes. This, taken together with our findings may suggest that the increased thrombotic risk observed in HIV infected individuals may be as a result of increased platelet- monocyte cross-talk and the subsequent increased levels of PMAs.

In support of this theory a study, of a group of HIV infected individuals, described the association of bacterial translocation across the intestinal mucosa with increased levels of LPS (Brenchley *et al.*, 2006). Further research by Ward *et al.*, demonstrated that LPS induced platelet activation had no effect on platelet aggregation (Ward *et al.*, 2005), however, contrary to this Aslam *et al.*, reported that after *in vivo* TLR4 receptor LPS-induced activation, decreased platelet counts were observed (Aslam *et al.*, 2006). Our findings suggest that a procoagulant platelet phenotype, and monocytes which have retained their functional capacity, exists in HIV infected individuals. It has been suggested that platelet activation via TLR4 receptor signalling facilitates the clearance of activated platelets and the

generation of inflammatory cytokines (Smith *et al.*, 2011). This proposal may further explain our current finding of increased PMAs which may be an indication of monocyte mediated clearance of activated platelets in HIV.

However, due to the cross-sectional design of the study, we cannot ascertain the clinical value of increased PMAs in the thrombotic risk profiling of HIV infected individuals. In this study, age and ethnicity could be excluded as confounders, since there were no significant differences in the age and ethnicity of the study participants (table 1). We also suggest that increased PMAs are dependent on immune activation and HIV RNA replication and that PMAs are associated with platelet aggregation. Our findings further imply the potential prognostic value of PMAs in HIV infected individuals, as shown by an inverse association between CD4 count and PMAs in HIV infected treatment naïve individuals. To the best of our knowledge the present study is the first to describe a direct association between increased PMAs and markers of; immune activation, platelet aggregation and disease progression in treatment naïve HIV infected individuals. The monitoring of PMAs in HIV infected individuals may be crucial and a useful marker of immune activation and thrombotic risk in HIV infected individuals not yet prioritized for treatment.

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**CHAPTER 6: Platelet derived microparticles, aggregates, markers of immune activation and disease progression in HIV infected treatment naive asymptomatic individuals.**

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## **Abstract**

### **Background**

Chronic immune activation in human immunodeficiency virus (HIV) infection induces activation of leucocytes and platelets, resulting in the formation of microparticles (MPs). Platelet microparticles (PMPs) are prothrombotic and have been described in inflammatory disease. Increased levels of platelet aggregation have been reported in HIV but evidence suggests PMP levels may be a more sensitive marker of platelet activation, compared to surface CD62P expression. We aimed to measure PMPs and platelet aggregates in HIV infected individuals, using a flow cytometry based whole blood assay. In addition, we aimed to investigate associations between MPs, PMPs, platelet aggregates and markers of immune activation with disease progression in asymptomatic treatment-naïve HIV infected individuals.

### **Materials and methods**

Forty-Eight (48) antiretroviral therapy (ART) naïve HIV infected and 40 uninfected individuals were recruited at a clinic in Cape Town. Platelet microparticles (PMPs), platelet aggregates and platelet P-selectin CD62P were measured using flow cytometry and correlated with CD4 count, viral load and %CD38 on CD8+ T cells.

### **Results**

HIV infected individuals showed increased levels of median platelet %CD62P 2.93[1.23-12.88] vs. control 1.15[0.19-3.59]; circulating MPs (HIV group median 1.7[0.95-2.83] vs. 1.12[0.63-1.57] control group,  $p=0.0160$ ); and levels of PMPs (median %PMPs 26.64[11.33-36.62] vs. control 20.02[18.08-26.08],  $p=0.0133$ ). In the HIV group, levels of activated platelets correlated with; platelet aggregates ( $r=0.5530$ ,  $p=0.011$ ) and activated PMPs ( $r=0.594$ ,  $p=0.007$ ). The baseline platelet aggregate % was similar between the two groups, however the platelet aggregates in the HIV group showed increased levels of activation (platelet aggregates median %CD62P 14.10[5.49-39.94] vs. 0.17[0-10.99] control group,  $p=0.0097$ ). The HIV group of patients demonstrated increased levels of immune activation, (median %CD38 on CD8+ cells 24.03[15.76-43.92] vs. control 9.23[6.8-12.96],  $p<0.0001$ ). In the HIV group levels of %CD38+ CD8+ T cells correlated with; %PMPs ( $r=0.4730$ ,  $p=0.0196$ ), %MPs ( $r=0.4386$ ,  $p=0.025$ ) and %PLT aggregates ( $r=0.321$ ,  $p=0.135$ ). In addition the %PMPs demonstrated no correlation with viral load ( $r=0.152$ ,  $p=0.511$ ) or CD4+ T cell count ( $r=0.0361$ ,  $p=0.07$ ).

## Conclusions

We describe an optimized whole blood flow cytometry based assay for the evaluation of circulating MPs, PMPs and levels of activated platelets and aggregates which is similar to the in vivo physiological environment of MPs. Furthermore we report on increased levels of circulating MPs and PMPs in HIV infected asymptomatic individuals together with increased levels of activated PMPs and platelet aggregates in HIV.

## 1. Introduction

Human immunodeficiency virus (HIV) infection is characterized by chronic immune activation and inflammation (López *et al.*, 2012). People living with HIV (PLWH) are at an increased risk of developing cardiovascular disease (CVD) (Hsue *et al.*, 2009) and are associated with a prothrombotic and pro-inflammatory state (Carr *et al.*, 2008). Activated platelets play a key role in both inflammation and thrombosis (Nurden *et al.*, 2011) as they release pro-inflammatory mediators that amplify the inflammatory response (Menzes *et al.*, 2009). Microparticles (MPs) are small vascular membrane-derived fragments that form upon cell activation or apoptosis (Freyssinet *et al.*, 2010). They express surface antigens of the cell of origin which allows them to be characterized (Mostefia *et al.*, 2008). Upon activation the remodeling of the platelet membrane results in the formation of sub-micrometer fragments described as platelet derived micro-particles (PMPs) (Zwaal *et al.*, 1992; Holme *et al.*, 2004). Circulating platelets are 2.0-4.0 by 5µm in size (George *et al.*, 2000) and PMPs are defined as small vesicles with a diameter of 0.1-1µm (Robert *et al.*, 2008).

Increased levels of MPs in HIV are associated with uncontrolled viral replication (López *et al.*, 2012) and PMPs represent the majority of microparticles detected in human plasma (Burnier *et al.*, 2009). Elevated levels of PMPs have been described in prothrombotic and inflammatory disease (Shantsila *et al.*, 2010). Data in the context of HIV remains inconclusive and contradictory findings in PLWH compared to uninfected individuals have been reported (Corrales-Medina *et al.*, 2010; da Silva *et al.*, 2011). PMPs have more procoagulant compared to activated platelets (Morel *et al.*, 2006; Rautou *et al.*, 2011). This is because of the abundant phosphotyrosine (PT) expressed on the surface of PMPs which acts as a potent surface for the stimulation of coagulation factors and the promotion of thrombin generation (Rautou *et al.*, 2011). PT expression has been used to define MPs, however MPs lacking PT expression have also been described (Connor *et al.*, 2010). MP's are involved in haemostasis and thrombosis whereas PMPs enhance thrombosis by inducing platelet activation and aggregation, decreasing clotting and increasing thrombus size

(Suades *et al.*, 2012). In addition, PMPs modulate both innate and adaptive immunity (Semple *et al.*, 2010).

Flow cytometry allows for the rapid measurement of microparticles and numerous studies have reported on attempts to standardize these methods (Shah *et al.*, 2008; Trummer *et al.*, 2008; Robert *et al.*, 2009; Dey-Hazra *et al.*, 2010). The majority of MP measurements are based on the preparation of platelet rich plasma (PRP) (Perez-Pujol *et al.*, 2007) or platelet poor plasma (PPP) (Iversen *et al.*, 2013) which often involves centrifugation and freezing steps (Yuana *et al.*, 2011). These processes may lead to platelet activation, destruction of MPs and fusion of MPs (Christersson *et al.*, 2013). The detection of MPs using flow cytometry is a highly sensitive technique that requires the optimization and consideration of all pre-analytical variables (Shah *et al.*, 2008). Accurate and consistent instrument calibration is required to enable the discrimination of MPs from cellular debris (Christersson *et al.*, 2013). Thus, the use of calibration beads to determine the particle size detection threshold and the use of fluorescence as a detection parameter for MPs are essential (Robert *et al.*, 2009).

Flow cytometry allows for the discrimination of aggregated cells from freely circulating cells (Wersto *et al.*, 2001). The use of a doublet strategy designed to overcome the analysis of doublets in DNA studies has been applied in the measurements of platelet monocyte aggregates (Majumder *et al.*, 2012). Ultrastructural changes in platelet aggregates have been described in HIV infected individuals (Pretorius *et al.*, 2008) and therefore application of the doublet discrimination strategy in the enumeration of platelet aggregates may be a useful tool. The present study aimed at measuring PMPs and platelet aggregates in HIV infected individuals, using a flow cytometry based whole blood assay. The assay was optimised to ensure artefactual platelet activation is minimised by omitting any centrifugation and platelet isolation steps. Moreover, we aimed at evaluating associations between PMPs, platelet aggregates and markers of immune activation and disease progression in asymptomatic treatment naïve HIV infected individuals.

## **2. Methodology**

### **2.1 Methodology**

Forty-six (46) HIV infected ARV-Naïve and 40 uninfected consenting individuals were recruited from the Emavundleni Voluntary Counselling and Testing (VCT) Clinic in Crossroads, Cape Town which is associated with the Institute of Infectious Diseases and Molecular Medicine (IIDMM) and the Desmond Tutu HIV Centre, University of Cape Town

(UCT). None of the participants were on aspirin on the day of testing and baseline participant characteristics are reported in table 1. The study protocol was reviewed by the health research ethics committee (HREC) at Stellenbosch University and the research ethics committees (REC) at University of Cape Town. Ethical review was performed according to the declaration of Helsinki and the study was approved by both committees and registered under the ethical approval number N07/09/197 and 417/2006, respectively.

## **2.2 Design of the study**

Baseline levels of platelet derived micro-particles, activated platelets and platelet aggregates were measured among all participants. In order to assess associations between these measured platelet parameters and immune activation and disease progression in HIV, PMPs and platelet aggregates were correlated with CD4 T-cell count, viral load and % of CD8+ T-cells expressing CD38.

## **2.3 Sample collection**

Whole blood was collected by venipuncture into 4.5ml tubes containing 3.2% sodium citrate (BD Vacutainer, San Jose, CA). Samples were kept at room temperature during the transportation from clinic to the laboratory. The time frame between sample collection and analysis was limited to 1-3hrs.

## **2.4 Markers of immune activation and disease progression**

**2.4.1 CD4 T-cell counts measurements:** The BD MultiTEST CD3-FITC/CD8-PE/CD45-PerCP/CD4-APC reagent (BD Biosciences) and BD TruCOUNT tubes (BD Biosciences, San Jose, CA) was used for CD4T cell count measurements. Measurements were performed at the Division of Medical Virology, Faculty of Health Sciences, Stellenbosch University, which is South African national Accreditation System (SANAS) accredited.

**2.4.2 Viral load measurements:** The NucliSensEasyQ® HIV-1 v1.2 Viral Load Test (BioMerieux Inc., Boxtel, Netherlands) was used for viral load measurements. All measurements were performed according to the manufacturer instructions at the Division of Medical Virology, Faculty of Health Sciences, and Stellenbosch University which is SANAS-accredited.

**2.4.3 CD38 on CD8+ T cell measurements:** BD FACSCalibur flow cytometry instrument was used to measure the percentage of CD8+ T-cells expressing CD38. 50µl of heparinized whole blood was stained using a titrated monoclonal antibody cocktail containing; CD8

PerCP; CD38 APC; CD3 FITC (BD Biosciences, San Jose, CA). Data analysis was done using the BD Cell Quest Pro (Version 2) software.

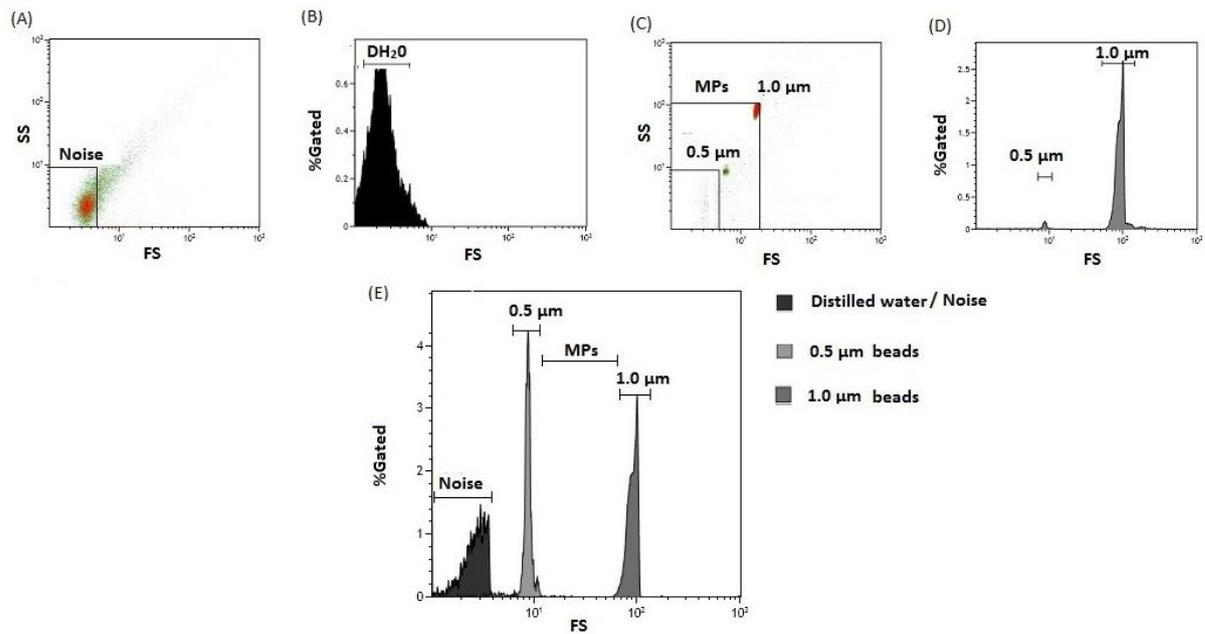
**2.6 D-dimer measurements:** The HemosIL™ Dimertest® Kit (Instrumentation Lab) and the ACL TOP platform were used for the measurement of D-dimers. All measurements were performed at the NHLS Haematology coagulation laboratory at Tygerberg Hospital which is South African National Accreditation System (SANAS) accredited laboratory.

## 2.7 Platelet flow cytometry

**2.7.1 Instrument settings:** The Beckman Coulter FC500 flow cytometer (Beckman Coulter, Miami Florida) was used for data acquisition. Flow check (Beckman Coulter, Miami, USA) fluorescent labelled beads of known size and fluorescence intensity were used to verify optical path and laminar flow of the cytometer. Flow set beads (Beckman Coulter, Miami, USA) were used to set instrument voltages and to ensure consistent instrument detector settings. The Beckman Coulter Quickcomp kit (Beckman Coulter, Miami, USA) was used to compensate for spectral overlap. This ensured the reporting of standardized results that were not influenced by long-term instrumental drifts.

**2.7.1 Sample preparation and Staining:** CD42b-FITC a platelet specific antibody was used to characterize the platelets (Fig 2B) and CD62P-APC expression was used to monitor platelet and aggregate activation levels. Briefly, 50µl of citrated whole blood was stained using 5µl of the antibody cocktail and incubated in the dark for 20 minutes at room temperature. 500µl of PBS (which did not contain Ca<sup>2+</sup> or MgCl<sub>2</sub>) was added and samples were analysed immediately.

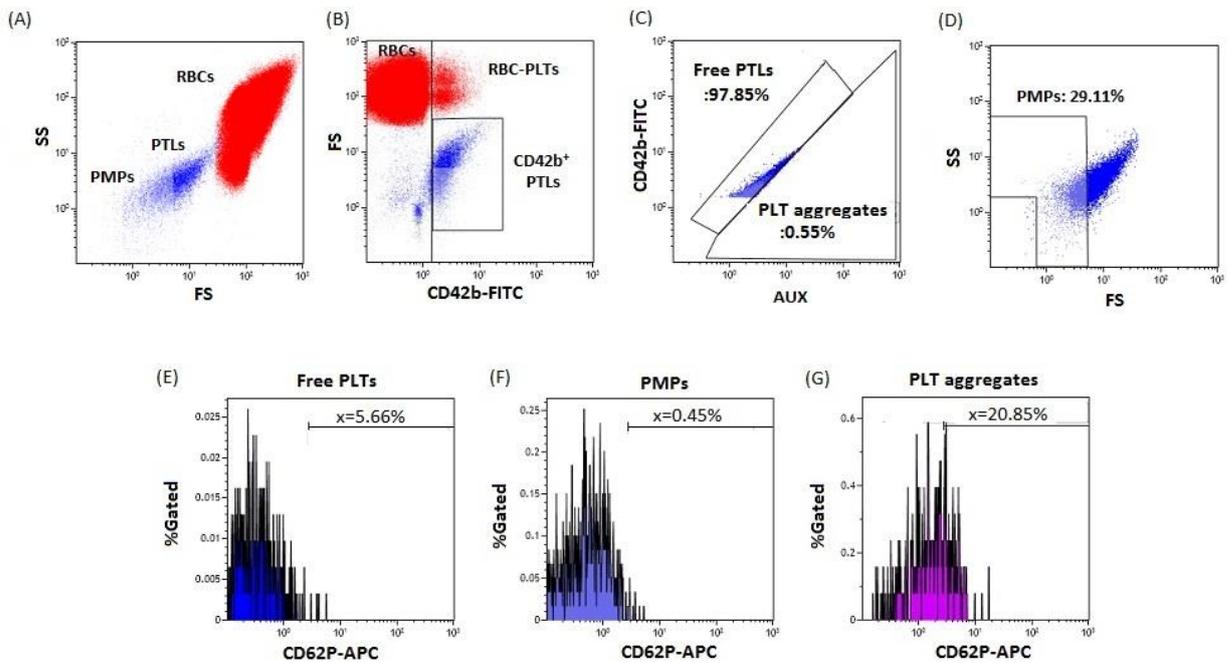
**2.7.2 Micro-particle Gating strategy:** Size calibrated beads with a diameter of 0.5µm and 1µm (Beckman Coulter, Miami, USA) were used to establish a size restricted gate in order to identify the microparticles, shown in figures 1A-E. The purity of the gated platelet population was ensured by gating on circulating free platelets (shown in figure 2A and B). In addition doublet discrimination strategy was applied to distinguish between free and aggregated platelets (shown in figure 2C). The light scatter properties of glycoprotein Iba (CD42b) positive events were used to determine the percentage of platelet derived microparticles (PMPs) (shown in figure 2C).



**Figure 1. Micro-particle discrimination and enumeration.** The figure illustrates the use of nominal beads for flow cytometric discrimination of micro-particles (MPs) based on size. Figures A and B illustrate the exclusion of buffer and background noise from analysis. Figures C, D and E illustrate the use of nominal beads to determine a size reference ladder and a micro-particle gate with a particle size limit of  $>0.5$  and  $<1.0$   $\mu\text{m}$ .

### 2.7.3 Doublet discrimination and PMP Gating strategy.

Doublet discrimination strategy was applied to distinguish platelet aggregates from freely circulating platelets (shown in figure 2B). The MP gate created using nominal latex beads was applied to determine the percentage of PMPs in freely circulating PLTs. Platelet micro-particles were defined by forward scatter properties that fell within the micro-particle gate and by the surface expression of platelet specific glycoprotein Ib (CD42b) which is platelet specific (Shown in figure 2C).



**Figure 2. Discrimination between aggregated platelets and PMPs.** The color dot plot A illustrates the forward scatter (FS) and side scatter (SS) properties distinguishing between platelet derived micro-particles (PMPs), platelets (PLTs) and red blood cells (RBCs). The dot plot B illustrates the use of platelet specific GPIb $\alpha$  (CD42b) and FS to discriminate between free platelets and RBC-bound platelets (RBC-PLTs). Dot plot C illustrates the use of the doublet discrimination strategy to distinguish between free CD42b<sup>+</sup> PTLs and aggregated platelets. Nominal beads of known size (10 $\mu$ M) that fluoresce in multiple spectra were used as controls to set the limits of the free PTLs and PLT aggregate gate. Plot D represents the use of the MP gate created using nominal beads; to determine the percentage of micro-particles in freely circulating PTLs. Dot plots E-G illustrate the measurement of CD62P on PLTs, PMPs and PLT aggregates respectively.

## 2.8 Statistical analysis

Statistical analysis was performed using Graph pad prism 5 for windows, version 5.00 software (San Diego, CA) .The Mann-Whitney *U* test was used to compare non-parametric data and these values were reported as median and interquartile range. For parametric data an unpaired student *t* test was performed and data was reported as mean and standard deviation. Paired data tests were also performed, for parametric data a paired t-test analysis was performed and for non-parametric data Wilcoxon matched data set analysis was performed.

### 3. Results

The study consisted of eighty six (86) participants, 40 HIV negative controls and 46 HIV positive ARV-naïve individuals. Participant demographics and baseline characteristics are tabulated in table 1. The study groups demonstrated a similar mean age distribution, (control group mean age 30.3 vs. 31.55 HIV group). None of the study participants were thrombocytopenic and platelet counts were similar between the groups (table 1). Differences in D-dimer levels were observed (shown in table 1); however these were within the normal reference intervals of 0.00-0.25 mg/ml. As expected the HIV group had lower levels of CD4<sup>+</sup> T- cells (768.9 ± 21 vs. 391.5 ± 195.3 control group, p=<0.0001). The HIV group demonstrated increased levels of CD8<sup>+</sup> T-cells expressing CD38 (median %CD38 on CD8<sup>+</sup> T cells 24.03[15.76-43.92] vs. control 9.23[6.8-12.96], p<0.0001). In the HIV group decreased levels of CD4<sup>+</sup> T cells correlated with increased levels of immune activation which was measured as the levels of CD38 expression on CD8<sup>+</sup> T cells (r= -0.384, p=0.015). In addition, HIV-1 viral RNA levels showed an inverse correlation with CD4<sup>+</sup> T cells (r= -0.348, p= 0.038) and a direct correlation with %CD38 on CD8<sup>+</sup> T cells (r=0.471, p=0.008). The HIV group, also displayed increased median levels of %CD36 a marker of platelet aggregation, (22.53[10.59-55.15] vs. control group 11.01[3.69-26.98], p=0.0312 (shown in table 1).

#### 3.1 Increased levels of activated platelets directly correlate with activated PMPs in HIV.

To determine the levels of platelet activation, CD62P expression on the surface of platelets was measured. The HIV group showed increased baseline levels of circulating activated platelets as demonstrated by increased median %CD62P expression (2.93[1.23-12.88] vs. control group 1.15 [0.19-3.59], p<0.0001). In the HIV group, increased percentages of platelets expressing CD62P directly correlated with PLT aggregates (r=0.5530, p=0.011) and the percentages of PMP's expressing CD62P (r=0.594, p=0.007). In addition the CD62P MFI directly correlated with PMPs CD62P MFI (r=0.694, p=0.002) but demonstrated no correlation with %PMPs (r=-0.198, p=0.389).

**Table 1.** Participant demographics and baseline characteristics

Parameter	Control	HIV	P- value	Reference Interval
No. of participants	40	46		
Age	30.3 ± 8.3	31.55 ± 6.7	-	
Gender (% males)	62.2	22.4		
Ethnicity				
Black (%)	100	100		-
CD4 count (cells/ $\mu$ l)	768.9 ± 214	391.2 ± 195.3	<b>&lt;0.0001</b>	500-2010
Platelet count ( $\times 10^9$ /l)	274[236-320.8]	313.5[232.5-365.5]	0.2620	178-400
%CD62P	1.15[0.19-3.59]	2.93[1.23-12.88]	<b>0.0058</b>	-
%PMPs	20.02[18.08-26.08]	26.64[11.33-36.62]	<b>0.0133</b>	
% Platelet aggregates	0.78[0.14-1.82]	0.7[0.33-2.31]	0.1986	
D-dimer (mg/l)	0.2[0.2-0.2]	0.24[0.2-0.33]	<b>0.0020</b>	0.00-0.25
% CD8/38	9.23[6.8-12.96]	24.03[15.76-43.92]	<b>&lt;0.0001</b>	
Log10 mean Viral Load(copies/ml)	-	4.18 ± 0.85	-	

Significant values ( $p < 0.05$ ), are shown in boldface

### 3.2 Increased percentages of platelet aggregates expressing CD62P and elevated CD62MFI on PMPs in HIV patients.

In order to determine the levels of activated platelet aggregates and PMPs, the %CD62P and CD62P MFI levels on PMPs and PLT aggregates were measured. The HIV group revealed increased levels of activated PLT aggregates 14.10[5.49-39.94] vs. 0.17[0-10.99],  $p=0.0097$  and there were no significant differences in the %CD62P expression on PMPs as shown in table 2. However the MFI of CD62 expression on PMP was increased in HIV, (3.81[3.46-4.54]) when compared to the control group (3.41[3.16-3.6],  $p=0.0037$ ). No significant differences in the MFI of CD62P expression on PLT aggregates were observed, shown in table 2.

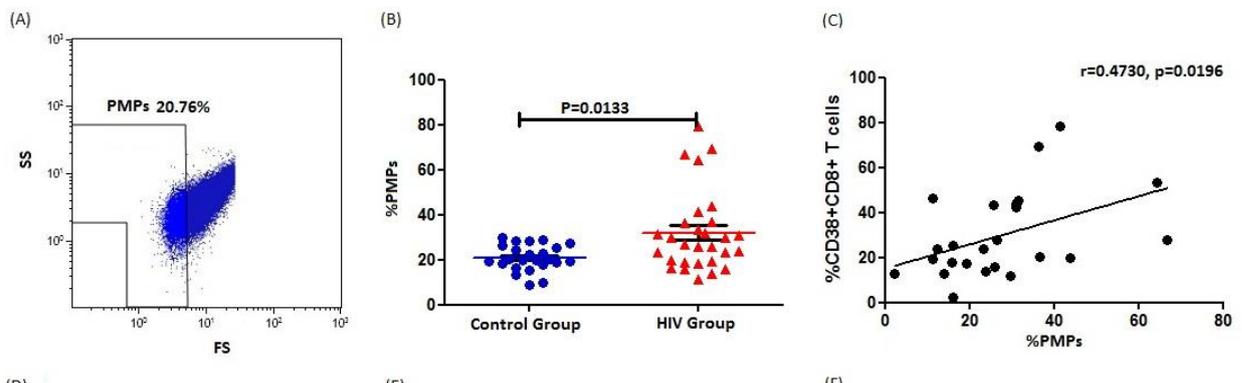
**Table 2.** Levels of CD62P expression on PMPs and PLT aggregates

	Control Group	HIV Group	P-value
<b>%CD62P</b>			
<b>%PMP</b>	0.45[0.1-0.88]	0.19[0.06-0.94]	0.5728
<b>%PLT aggregates</b>	0.17[0-10.99]	14.10[5.49-39.94]	<b>0.0097</b>
<b>CD62P MFI</b>			
<b>%PMP</b>	3.41[3.16-3.6]	3.81[3.46-4.54]	<b>0.0037</b>
<b>%PLT aggregates</b>	6.34[5.42-7.28]	7.86[4.69-12.30]	0.5967

Significant values ( $p < 0.05$ ), are shown in boldface

### 3.3 Increased levels of immune activation in HIV correlates with %PMPs

In order to determine the association between immune activation and %PMPs levels in the HIV group, baseline levels of %CD38<sup>+</sup> CD8<sup>+</sup> T cells were correlated with; %PMPs ( $r=0.4730$ ,  $p=0.0196$ ), and %PLT aggregates ( $r=0.321$ ,  $p=0.135$ ). In the HIV group, percentages of %CD38<sup>+</sup> CD8<sup>+</sup> T cells inversely correlated with CD4<sup>+</sup> T cell count ( $r=-0.348$ ,  $p=0.015$ ) and directly correlated with viral load ( $r=0.471$ ,  $p=0.008$ ).



**Figure3.** %CD38<sup>+</sup>CD8<sup>+</sup> T cells correlate with, %PMPs in HIV. Figure A and B illustrates the measurement of PMPs and figure C illustrates the direct correlation between %CD38<sup>+</sup>CD8<sup>+</sup> T cells and %PMPs ( $r=0.4730$ ,  $p=0.0196$ ), in the HIV group.

#### 4. Discussion

Several approaches to the enumeration of MPs have been described, with most of these involving the use of platelet rich (PRP) or platelet poor plasma (PPP), platelet isolation strategies and thawing of samples. The freezing and thawing of samples affects the phenotypic analysis of MPs (Gasper-Smith *et al.*, 2008). A recent study by Christersson *et al.*, demonstrated the feasibility of using a whole blood assay that mimics the *in vivo* state by omitting centrifugation and thawing of samples (Christersson *et al.*, 2013). The present study aimed at measuring PMPs and platelet aggregates in HIV infected individuals, using a flow cytometry based whole blood assay which has been optimised to ensure artefactual platelet activation is kept to a minimum. Moreover we aimed at evaluating associations between PMPs, platelet aggregates and markers of immune activation and disease progression in asymptomatic treatment naïve HIV infected individuals

We have previously reported on increased levels of activated platelets in HIV compared to uninfected controls (Nkambule *et al.*, 2014) which is consistent with previously findings (Holme *et al.*, 1998; Corrales-Medina *et al.*, 2010; Mayne *et al.*, 2012). In the HIV group, levels of platelet activation directly correlated with the levels of activated platelet aggregates which have been previously associated with thromboembolic events in HIV infected individuals (Shen *et al.*, 2004). Our study found no differences in the % of platelet aggregates between the HIV and control group. The levels of PMPs were however significantly increased in the HIV infected individuals compared to their uninfected counterparts. In addition, PMP levels in HIV infected individuals had increased qualitative CD62P expression as measured by the CD62P MFI. This may suggest an increased number of adhesion molecules (CD62P) on the surface of PMPs in HIV and is consistent with findings reported by Mayne *et al.*, who showed that PMPs in HIV infected individuals are activated and express CD62P (Mayne *et al.*, 2012). These findings may further support the described prothrombotic characteristic of PMPs. P-selectin has been described as an adhesion molecule that can bind to leukocytes, resulting in increased platelet leukocyte aggregate (PLA) formation (Totani *et al.*, 2010). Moreover our findings may suggest that PMPs expressing increased levels of P-selectin promote the adhesion and trapping of circulating platelets and leukocytes on the endothelial surface, which would consequently increase the thrombus size. Consistent with our finding of increased PMP levels in HIV, Corrales-medina *et al.*, described a cohort of HIV infected participants on ART, which had increased PMP levels compared to uninfected controls. In addition, the authors showed no significant differences in PMPs when intragroup comparisons were performed amongst HIV individuals on different highly active antiretroviral therapy (HAART) regimes (Corrales-medina *et al.*, 2010).

In our study PMPs showed a direct correlation with generalized immune activation, as measured by the expression of CD38 on CD8<sup>+</sup> T cells. As expected increased levels of

immune activation inversely correlated with the levels of CD4<sup>+</sup> T cells and directly correlated with viral RNA levels. Haematopoietic cells release MPs upon cellular activation by lipopolysaccharides (LPS) and inflammatory cytokines. This is significant in the context of HIV which is characterized by increased levels of LPS and chronic immune activation (Vassalo *et al.*, 2012). The association between levels of immune activation and PMPs further supports the link between immune activation, platelet activation and increased thrombotic risk in HIV infected individuals. The lack of an association between PMPs and viral RNA levels suggests that in HIV infected individuals MP formation may be facilitated by mechanisms involving immune activation rather than the direct effects of HIV. PMP levels demonstrated a trend towards a negative correlation with CD4 counts ( $r=-0.361$ ,  $p=0.070$ ). This finding is similar to that reported by Corrales-Medina *et al.*, who reported on increased PMPs in HIV infected individuals. Interestingly, the authors also showed that in HIV, elevated PMP levels persisted, although levels of CD62P expression declined after ART initiation (Corrales-medina *et al.*, 2010). Although differences in the mean CD4<sup>+</sup> T cell count exists between our present study (391 cells/ $\mu$ l) compared to the mean of 550 cells/ $\mu$ l CD4<sup>+</sup> T cell count reported by Corrales-Medina *et al.*, our findings may further support the fact that PMPs are a more sensitive marker of residual increased platelet activity (Corrales-Medina *et al.*, 2010) which may result in increased thrombotic risks in HIV infected individuals. PMP formation may be stimulated by sub clinical viral replication that promotes apoptosis of circulating platelets or impairs the clearance of apoptotic cells (Corrales-Medina *et al.*, 2010). Notably platelets are capable of engulfing and internalizing HIV, a process which is facilitated by their surface receptors (Flaujac *et al.*, 2010). These interactions may result in platelet activation and a subsequent increase in PMP formation. In contrast however, da Silva *et al.*, reported no differences in PMP levels between HIV infected individuals and controls (da Silva *et al.*, 2011).

The early stages of HIV infection are characterized by elevated production of proinflammatory cytokines with a delayed or compromised adaptive immune response (Stacey *et al.*, 2009; Turnbull *et al.*, 2009; McMicheal *et al.*, 2010).

In this study, platelet aggregates in HIV infected individuals showed elevated quantitative levels of activation when compared to uninfected controls. In comparison, PMPs in HIV infected individuals had increased qualitative levels of activation compared to uninfected controls (shown in table 2). Ultrastructural changes in platelet aggregates of HIV infected individuals have been described. Platelet aggregates in HIV infected individuals have been shown to have increased areas of morphological membrane blebbing, torn membranes and pseudopodia when using scanning electron microscopy (Pretorius *et al.*, 2008). These findings may suggest that circulating platelet aggregates in HIV infected individuals are activated and may contribute to the hypercoagulable state found in HIV infected patients. The cross sectional design of the study limits conclusive associations and thrombotic risk analysis

of this cohort and therefore future longitudinal studies will be required to assess and stratify individuals with increased risk of thrombotic events.

In conclusion we report on increased levels of circulating PMPs in HIV infected asymptomatic individuals. We also describe increased levels of activated PMPs and platelet aggregates in asymptomatic HIV infected individuals.

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### **Declaration of interest**

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## 7.1 Conclusions

The aim of this project was to examine the function and characteristics of platelets, in the context of HIV infection, in order to establish a link between platelet activity, the inflammatory response and the increased thrombotic events and cardiovascular disease observed in patients who have this disease.

In the work presented, the levels of platelet activation were persistently elevated in HIV infected treatment naïve individuals. This finding is consistent with that previously reported in the literature (Holme *et al.*, 1998; Mayne *et al.*, 2012). Notably the significance of the decreased MPV (a measure of platelet size and rate of production) in HIV, may suggest mechanisms involving the migration of large platelets from the circulation to extracellular sites of inflammation. Furthermore decreased MPV levels correlated inversely with CD4 counts and directly with viral load. This may further support the immunological role played by platelets during HIV infection in which, direct interactions between platelets and HIV may result in the engulfment and clearance of activated platelets by leukocytes (Torre & Pugliese *et al.*, 2008; Flaujac *et al.*, 2010).

Platelet indices have been evaluated in various inflammatory conditions however only a few studies have determined the clinical significance of these indices in HIV infected treatment naïve individuals. Our study also describes decreased levels of PDW (a measure of platelet activation and aggregation), which directly correlated with levels of platelet activation. To date this project forms the largest study evaluating platelet indices in HIV infected treatment naïve individuals. We further report on MPV and PDW as potential cost effective markers of platelet activation and immune activation.

Platelet function in HIV is not fully understood and contradictory findings have been reported. The lack of reproducibility of reported findings is mainly influenced by methodological variations between reported data. In our study we optimized a flow cytometry based assay that ensured that minimal artefactual platelet activation took place and care was taken to retain the physiological environment of the platelets. We report on a novel platelet functional assay that evaluates signaling pathways involved in platelet activation using whole blood. In addition we report on hyper responsive platelets in HIV to low doses of strong endogenous agonists (AA and collagen) and high doses of the platelet agonist (ADP). We further describe elevated levels of activated platelets in HIV which retain their functional capacity. These findings are significant and may be essential in the monitoring and thrombotic risk stratification of HIV infected individuals.

Our research revealed increased numbers of circulating activated platelets in HIV infected individuals and is the first to describe elevated levels of activated platelets in asymptomatic HIV infected treatment naïve individuals. Furthermore our findings demonstrated high levels

of D-dimer which showed a positive correlation with platelet CD36. Platelets in HIV infected individuals appear to retain their functional capacity and are able to degranulate and aggregate upon stimulation with ADP. Interestingly, the platelets in HIV demonstrated a single phase response to ADP. This may suggest that in this disease, platelets are activated *in vivo* and may respond irreversibly with submaximal concentrations of ADP.

Baseline PMAs were significantly increased in HIV infected treatment naïve individuals. In addition PMA levels directly correlated with viral load and immune activation. This may suggest that in HIV, generalized immune activation promotes monocyte and platelet crosstalk. In addition, levels of platelet CD36 directly correlated with elevated PMA levels. Notably, CD36, a scavenger receptor, is also expressed on the surface of monocytes where it is involved in the formation of foam cells in atherosclerosis (Rahaman *et al.*, 2006). We therefore suggest that platelet CD36 could be utilised as a valuable marker in the monitoring of platelet and monocyte interactions and may therefore be of use in the thrombotic risk profiling of HIV infected individuals. Furthermore, in support of this theory whole blood *in vitro* stimulation with LPS resulted in increased PMA levels in both HIV infected and control groups.

Further longitudinal studies are however required in order to ascertain the clinical value of increased PMA levels in the HIV group, Even though associations between PMA levels and markers of immune activation and disease progression were determined, PMAs showed a direct correlation with viral load and immune activation. In addition the inverse relationship between PMAs and CD4 count supports the potential value of PMAs as a marker of disease progression in HIV infected asymptomatic treatment naïve individuals. We have also demonstrated elevated levels of activated PMPs and platelet aggregates in this group of individuals which directly correlated with generalized immune activation. This may further support the link between immune activation, platelet activation and increased thrombotic risk in HIV infected individuals.

This project therefore supports previous findings of increased levels of activated platelets in HIV infected individuals (Holme *et al.*, 1998; mayne *et al.*, 2012). In addition, this work further presents a novel approach, in the form of whole blood platelet flow cytometry based assays, to study platelets and the underlying mechanisms of thrombotic complications in HIV infected individuals. Minimal work has been undertaken in this area of HIV research particularly in a cohort of asymptomatic untreated patients which have been targeted in this study. This approach may facilitate the earlier access to appropriate therapies and form the baseline for monitoring response to treatments. We propose that the above assays could be utilised as a 'thrombotic risk' panel of tests which may be of value in the assessment of specific anti-

platelet therapies used as preventative strategies in the management of patients at risk of inflammatory-associated complications.

Finally, the results of our investigations have added knowledge and evidence to the theory that platelets form an important link between the immune response and the increased levels of thrombosis in patients living with HIV. These observations could be relevant in other inflammatory diseases such as diabetes, multiple sclerosis and autoimmune diseases and future studies should focus on the role platelets play in chronic inflammatory conditions. Our findings provide new knowledge in understanding platelet function in HIV. The panel of flow cytometry based platelet assays presented in this work, provide findings that may address the research gap that exists regarding platelet activation in HIV. In addition rapidly available platelet parameters in the form of platelet indices may be a cost effective tool in the monitoring of immune suppression and disease progression in HIV. Our study focused on a cohort consisting of asymptomatic treatment naïve HIV infected patients. This allowed the exclusion of treatment as a confounding factor in our evaluation of platelet function in HIV. Our findings strongly suggest direct associations between the levels of platelet activation and markers of immune activation and disease progression in HIV. The measurement of platelet function using the described panel of flow cytometry based assays may be of value in monitoring disease progression and thrombotic risk profiling of HIV infected treatment naïve individuals.

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## ADDENDUM 1:

### RESEARCH OUTPUTS

#### CONFERENCE PRESENTATIONS

##### 3. Poster presentation

###### **17th European Hematology Congress 14-17 June 2012, Amsterdam, The Netherlands**

**Platelet Flow Cytometry and markers of immune activation in asymptomatic, treatment-naïve HIV infection.** Bongani B. Nkambule<sup>1</sup>, Richard Glashoff<sup>1</sup> and Hayley Ipp<sup>1,2</sup>. Divisions of Haematology<sup>1</sup> and Virology<sup>2</sup>, Department of Pathology, Stellenbosch University and NHLS, Tygerberg, South Africa

##### 4. Oral presentation

###### **Pathpoint 2012 congress, SEPT 28-30. Crystal towers hotel in Cape Town, South Africa**

**Platelet Flow Cytometry and markers of immune activation in asymptomatic, treatment-naïve HIV infection.** Bongani Nkambule, RH Glashoff, H Ipp. Divisions of Haematology<sup>1</sup> and Virology<sup>2</sup>, Department of Pathology, Stellenbosch University and NHLS, Tygerberg, South Africa

###### **Laboratory medicine congress 2013, JULY 28-31. Cape Town international convention centre in Cape Town, South Africa**

**The value of Flow Cytometry in the measurement of platelet activation and aggregation in human immune-deficiency virus HIV infection.** Bongani B. Nkambule, Glenda Davidson, Hayley Ipp. Divisions of Haematology, Department of Pathology, Stellenbosch University, Tygerberg<sup>1</sup>. Department of Biomedical sciences, Faculty of Health and wellness sciences, Cape Peninsula University of Technology, Bellville, South Africa<sup>2</sup>.

## ADDENDUM 2:

### Publications

Nkambule, Bongani B., Glenda Mary Davison, and Hayley Ipp. "The evaluation of platelet function in HIV infected, asymptomatic treatment-naïve individuals using flow cytometry." *Thrombosis Research* (2015).

Nkambule BB., Davison GM and Hayley Ipp. The evaluation of platelet indices and markers of inflammation, coagulation and disease progression in treatment-naïve, asymptomatic HIV-infected individuals. *International Journal of laboratory hematology*. (2014). *In press*.

Nkambule, Bongani B., Glenda Davison, and Hayley Ipp. "The value of flow cytometry in the measurement of platelet activation and aggregation in human immunodeficiency virus infection." *Platelets* 0 (2014): 1-8.

Ipp, Hayley, Bongani B. Nkambule, Timothy D. Reid, Dalene de Swardt, Linda-Gail Bekker, and Richard H. Glashoff. "CD4+ T cells in HIV infection show increased levels of expression of a receptor for vasoactive intestinal peptide, VPAC2." *Immunologic research* (2014): 1-5.

**ADDENDUM 3:**



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**Ethics Letter**

04-Mar-2013

**Ethics Reference #:** N07/09/197

**Clinical Trial Reference #:**

**Title:** "Identifying surrogate markers of the activation status of the immune system in chronic HIV-infection as correlates of risk of disease progression."

Dear Doctor Richard Glashoff,

Your letter dated 1 March 2013 refers.

The Chairperson of the Health Research Ethics Committee approved the amended documentation in accordance with the authority given to him by the Committee on 4 March 2013.

The following amendment was approved:

PhD proposal of Mr Bongani Nkambule submitted as an amendment to the study (N07/09/197).

Title: "Investigating platelet function and immune activation in HIV-infection and other chronic inflammatory conditions."

If you have any queries or need further assistance, please contact the HREC Office 0219389657.

Sincerely,

REC Coordinator  
Franklin Weber

**ADDENDUM 4:**

**Participant information leaflet and consent form**

**Title of the research project:** Modulation of activation and apoptosis of immune cells from HIV-infected individuals with the neurotransmitter Vasoactive Intestinal Peptide (VIP) and other biomediators

**Title of sub-study:** Investigating platelet function and immune activation in HIV infection and other chronic inflammatory conditions.

**Reference number:** N07/09/197

**Principal investigator:** Dr Hayley Ipp/ Dr Richard Glashoff

**Address:** Medical virology; 8<sup>th</sup> Floor Clinical Building; Tygerberg Hospital Campus

**CONTACT NUMBER:** 021 938 9356

You are being invited to take part in a research project. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This study has been approved by the **Health Research Ethics Committee (HREC) at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is this research study all about?

- **Selection of Study Volunteers** This study will enroll about 70 volunteers who have been attending the clinics attached to the Desmond Tutu HIV Centre, have CD4 counts greater than 200, are 21 years or older, and who have not yet received antiretroviral drugs.
- About 40 volunteers will also be recruited from trials that are being set up for HIV- negative people.
- *The research on the blood will take place at Tygerberg Hospital, Department of Pathology*
- **Reason for the study:** The drugs (antiretrovirals) that are now available for the treatment of HIV have saved many people's lives and continue to do so on a daily basis. However, we still need to look for different ways to help people who are HIV positive, live for longer, before they need to take the antiretroviral drugs. If a person's CD4+ T cell count is less than 200, it is very important to get the antiretroviral treatment, however, for those people whose count is still greater than 200, we will be looking for new ways in the laboratory to keep the count at this higher level, and hopefully this will help prevent the onset of full-blown AIDS.

### **Study procedures**

If you decide to take part in the study, after you read, discuss and sign or mark this form, the following procedures will take place:

- You will undergo a medical examination and short medical history.
- You will be asked a number of questions about how you may have been exposed to HIV and when you think you may have become HIV-infected. If you have completed this questionnaire on a previous study with the same investigators, you will not need to complete it again.
- You will also be asked questions about any medicines that you are taking.
- If you are a woman, your urine will be tested to see if you are pregnant. If you are or become pregnant, you will be referred to prenatal care and to programs for Prevention of Mother to Child Transmission (PMTCT) where you will be able to get medicines to prevent your baby from getting HIV.

**What will happen to your blood?** Up to 10 teaspoons of blood i.e. 50ml (one or 5 small tubes) will be drawn from your arm. The blood will then be sent to the laboratory at Tygerberg Hospital where the red blood cells will be separated from the white blood cells. The cells from the immune system will then be tested in the laboratory, by mixing them with different medications to see which medicine is the best at keeping the cells alive. Some chemistry tests will be performed to determine levels of inflammation and small clot formation. The results of these tests are for research purposes only and cannot be made available to you.

- No genetic testing will be performed.

Will you benefit from taking part in this research?

**Benefits:** You may benefit by taking part in the study from receiving counseling and a medical examination. You or others may benefit in the future from information learned in this study. You may get some personal satisfaction from being part of research on HIV. If you are a woman, and you become pregnant, you will be referred for prenatal care and for PMTCT.

**Risks and/or Discomforts:** There may be some discomfort involved in drawing blood for the tests which sometimes can cause pain and bruising where the needle goes into your arm. When blood is being drawn, you may feel dizzy or faint, but this is not common.

### **Confidentiality**

Your participation in the study, all information collected about you as well as all results of laboratory tests will be private and not available to others outside of the people listed below. You will have your own special identity number known only to you and the clinic staff. They are required to respect your confidentiality. Your identity will not be disclosed in any publication or presentation of this study. Your blood specimens will only be identified by your study number. Any documents containing your name will be locked away in a secure place.

### **Will you be paid to take part in this study and are there any costs involved?**

No you will not be paid to take part in the study but your transport and meal costs will be covered for each study visit. There will be no costs involved for you, if you do take part.

### **Is there any thing else that you should know or do?**

- You can contact Dr Hayley Ipp..... At tel ...021 9389356..... if you have any further queries or encounter any problems.
- You can contact the **Health Research Ethics Committee** at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your study doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I ..... agree to take part in a research study entitled *(insert title of study)*.

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at *(place)*.....Crossroads..... on *(date)* ..... 2010

.....  
**Signature of participant**

.....  
**Signature of witness**

Declaration by investigator

I *(name)* ..... declare that:

- I explained the information in this document to .....
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use an interpreter. *(If an interpreter is used then the interpreter must sign the declaration below.*

Signed at *(place)* .....Cross roads..... on *(date)* ..... 2010.

.....  
**Signature of investigator**

.....  
**Signature of witness**

Declaration by interpreter

I (*name*) ..... declare that:

- I assisted the investigator (*name*) ..... to explain the information in this document to (*name of participant*) .....  
Using the language medium of Afrikaans/Xhosa.
- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) ..... On (*date*) .....

.....  
**Signature of interpreter**

.....  
**Signature of witness**

## Appendix A

### Technical note: Doublet discrimination strategy

#### Introduction

The doublet discrimination technique is applied to overcome coincident analysis of doublets in DNA ploidy experiments (Wersto *et al.*, 2001). In studies where DNA staining has been performed, when a doublet passes through the flow cell during interrogation by the laser beam, it may be interpreted as a single cell with increased amount of DNA. This will result in incorrect histograms and percentage determinations for each cell cycle phase. It is essential that doublets are gated out from DNA histograms. In DNA studies doublet discrimination is performed using linear scale, however light scatter detected using linear scaling does not allow the discrimination of granulated and stimulant induced large cells (Rothe, 2009).

The principles of this technique have been used to measure platelet monocyte complexes (Majumder *et al.*, 2012). However this concept has not been used in the measurement of platelet aggregates. Flow cytometers can distinguish between doublets and singlets based on pulse processed data (Nunez, 2001). Doublets can be discriminated based on three kind of signals (figure 1), (1) Integral, total fluorescence (A); (2) Peak (H); (3) Time of flight (w) (Lopez-Sanchez *et al.*, 2013).

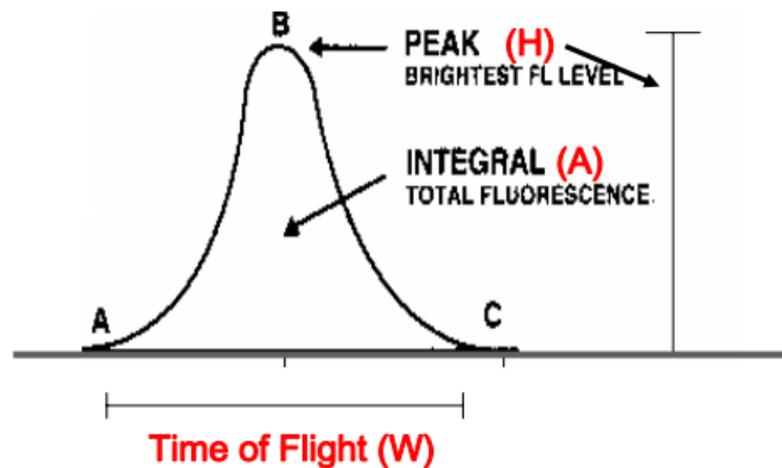
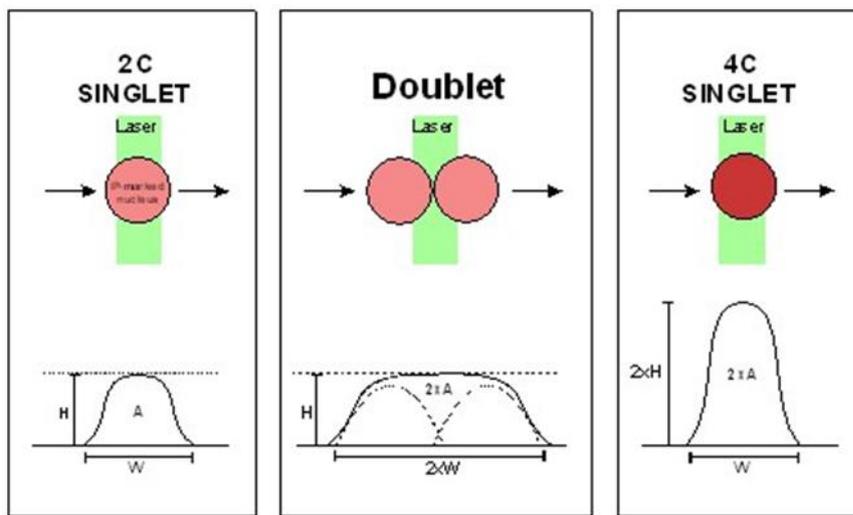


Fig 1. Three kinds of pulse signals that can be measured.

[http://www.ym.edu.tw/ymirc/flow/FC500\\_Cell\\_Cycle.pdf](http://www.ym.edu.tw/ymirc/flow/FC500_Cell_Cycle.pdf)

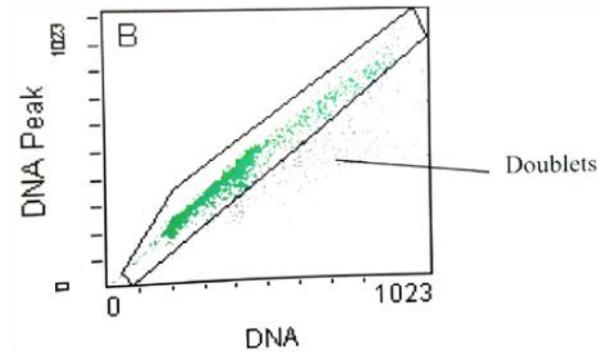
Doublets demonstrate a lower peak pulse height compared to the integral. This allows for the discrimination of doublets from large single cells. The FC500 cytometer uses integral measurements as a major measure to discriminate doublets from single cells. An auxiliary parameter can be set on the FC500 which collects peak signals of a selected parameter. In order to apply this gating technique in the analysis of platelet aggregates, the auxiliary parameter was set on FL1 using log scale. A Color dot plot was used to created to discriminate platelet doublets (aggregates) from single freely circulating platelets as described in the Beckman coulter FC500 training module. (Fig2).



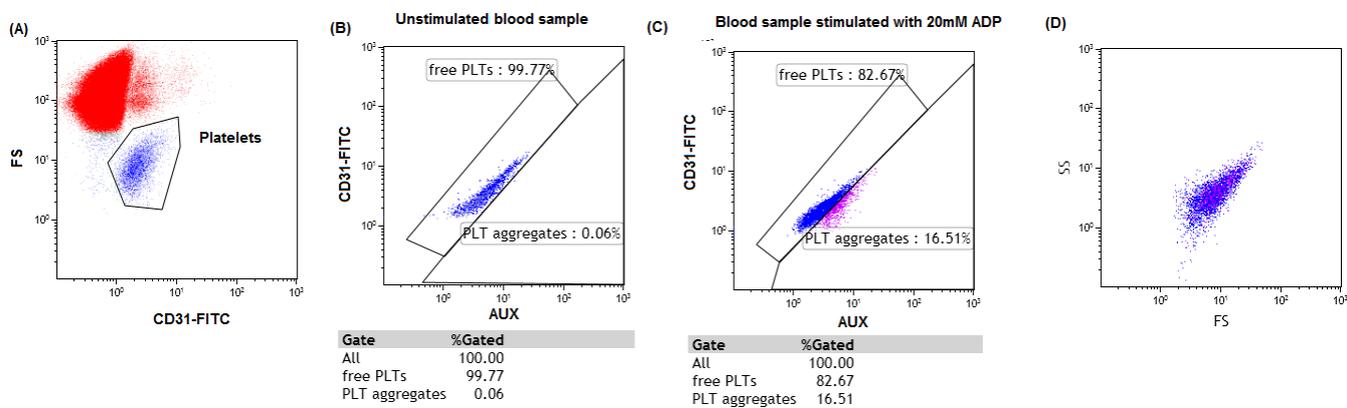
**Figure 2 .** Discrimination of singlets and doublets using flow cytometry. Doublets and large single cells have distinct peak and time of flight characteristics (Lopez-Sanchez *et al*, 2013).

## Methods

Fresh blood was stained with CD31-FITC and used to set a gate for freely circulating platelets (Figure 1). For a positive control fresh blood was stimulated with 20mM of ADP and incubated for 20 min at room temperature. The sample was then stained with CD31-FITC and analyzed immediately using the Beckman coulter FC500. The instrument flow rate was kept at low to minimize coincidental events that may be interpreted as aggregates. A doublet discrimination protocol was set as described in the Beckman coulter training protocol and modified for analysis of platelet aggregates (shown figure 5). CD31 positive platelets were gated and the doublet discrimination strategy was then applied as outlined in figure 5.



**Figure 3. Doublet discrimination peak vs. Integral (Classic method).** (taken from the Beckman coulter FC500 training module, DN-9))



**Figure 4.** illustrates the doublet discrimination strategy applied on a fresh blood sample. Figure B demonstrates the discrimination of freely circulating platelets from aggregated platelets based on CD 31 integral and Peak. Figure C demonstrates the use of ADP stimulated platelets as a positive control. Figure (D) illustrates a back gate used to evaluate the side scatter and forward scatter properties of the gated platelet aggregates.

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