PLATELET FUNCTION IN ADULT HUMAN IMMUNO-DEFICIENCY VIRUS-1 INFECTED HAART NAÏVE PATIENTS AT KENYATTA NATIONAL HOSPITAL, KENYA

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DECLARATION

This thesis is my original work and has not been presented for award of a degree or any other award in any University.

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DEDICATION

This work is dedicated to my parents Risper Aoko and Onjula Ogola.

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I thank God for his perpetual protection, guidance and strength in pursuing my goals.

To my parents, Mr. Onjula Ogola and Mrs. Risper Ogola thank you for your prayers and unconditional love. I can never find words to describe the depth and value of the sacrifices you have both made to ensure my dreams are fulfilled. Thank you for your encouragement and persistent belief in my potential and untapped dreams. May God continue to bless our family.

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LIST OF ACRONYMS

PLWH:	People living with HIV
HIV-1:	Human Immuno-Deficiency Virus-1
CVE:	Cardio-vascular event
CVD:	Cardiovascular disease
HAART:	Highly Active Anti-retroviral Therapy
TCP:	Thrombocytopenia
MI:	Myocardial infarction
KNH:	Kenyatta National Hospital
GP:	Glycoprotein.
LTA:	Light Transmission Aggregation
VWF:	Von Willebrand Factor

ABSTRACT

Occurrence of cardiovascular diseases (CVDs) in adult human immuno-deficiency virus-1 (HIV-1) infected patients is associated with 18% of HIV-1 deaths. Availability of highly active antiretroviral therapy (HAART) has prolonged and improved quality of life in HIV patients. However it has led to the appearance of pre-mature cardiovascular events (CVEs) of athero-thrombotic origin with its continued use. It is still unclear whether thrombotic risks associated with increased platelet function are as a consequence of HIV-1 infection alone or of the long-term use of these antiretroviral drugs. This study aimed at determining platelet function in adult HIV-1infected patients not on HAART. In a case-control study design, using convenient and consecutive sampling method a total of 28 participants were recruited. Fourteen adult HIV-1positive HAART naive patients and fourteen HIV negative participants attending Kenyatta National Hospital, comprehensive care clinic (CCC) were enrolled to the study after consenting. Blood samples were collected and platelet concentrates prepared from each sample. Platelet aggregation was determined by measuring time dependent light transmission in response to known agonists (adenosine tri-phosphate (ADP), collagen and arachidonic acid (A.A) on chrono-log 100 aggregometer. Platelet activation was done by measuring P-selectin expression using flow cytometry. In both groups, participants were of similar age and sex. The results show that median Platelet % P-Selectin expression levels in HIV positive group was 1.5 times that of the control group (34.5 (IQ) 10.3-63.3 vs 21.1 (IQ) 2.7-46.2),and had a positive correlation with viral load (r=0.634, P= 0.019) but not with CD4 count (r= -0.532, P= 0.7229). Among the three agonists platelet aggregation showed a significant higher response to collagen compared to arachidonic acid and ADP (P=0.00087, P=0.00056 and P=0.019) respectively. Importantly, less percentage maximum platelet aggregation was observed in HIV positive group compared to HIV negative group for all the agonists (Collagen:75±4.8% cases vs 80.4±6.7% controls at 10µg/ml; AA 64±8.1% cases vs 88.4±6.2% controls at 10µg/ml; ADP 60.7±6.7% cases, 71.1±8.1% controls at 5µmol/l). Furthermore, % maximum platelet aggregation correlated inversely with viral load (ADP r=-0.286, P=0.0424; Collagen r=-0.4663, P=0.0177 and A.A r=-0.3, P=0.259) and directly with CD4 counts ADP r= 0.614, P=0.0528; collagen r=0.384, P=0.0273 and A.A r=0.850, P=0.002).This study was able to demonstrate that platelet function in HIV-infected patients is altered. This offered insights into the complex mechanisms underlying occurrences of thrombotic events in HIV positive patients.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

There is increasing evidence that patients infected with HIV/AIDS are at a higher risk of coagulation dysfunctions. This is the case even when there are no known predisposing factors such as surgery, trauma, pregnancy or pre-existing coagulation factor deficiencies (Satchell *et al*., 2010). It's manifested by higher incidences of cardiovascular events (CVEs) among HIV infected/AIDS patients, depicted by early onset of stroke and heart attacks mainly of arterial thrombotic origin (Bozzette *et al*., 2003). Studies indicate that increased platelet reactivity in HIV sero-positive patients correlates with increased thrombotic risk. Increased platelet activation has also been associated with elevated inflammatory markers such as hyper sensitive C-reactive protein (hsCRP), D-dimers and hyper-lipidemia that consequently activate platelets in circulation creating a constant pro-coagulatory state (Hsue *et al*., 2012)

The introduction of HAART as a standard therapy for HIV infection has elicited interest in the relationship between HIV and platelet reactivity. HIV infected patients both in the pre and post-HAART eras are recognized to have increased thrombotic risk. HAART by reducing viral load and thus inflammation might be expected to down-regulate platelet hyper-reactivity (Satchell *et al*., 2011). However, these drugs also induce metabolic side effects such as alteration of the lipid profile, hyper-lipidemia and fat redistribution which paradoxically promote atherosclerosis and platelet hyper-reactivity

(Donati *et al*., 2004). Recent data reveals that patients undergoing treatment suffer high rates of deep venous thrombosis (DVT) and pulmonary embolism (PE) which further contributes to treatment failure. Whether HAART reduces platelet hyper-reactivity associated with HIV infection or contributes to further platelet activation is still a matter of controversy. It is not clear whether thrombotic risks in HIV infected patients is due to HIV infection alone, treatment with HAART or a synergistic interaction between these factors.

The study investigated and documented platelet function in adult HIV-1infected patients with a view to unraveling the disordered coagulation states seen, using a combination of platelet function assays.

1.2 Statement of the Problem

Thrombocytopenia is a common finding in patients infected with HIV/AIDS which would suggest hemorrhagic complications. However, these patients suffer from thrombo-embolic complications with a prevalence of 18% (Becker *et al*., 2011). This strongly suggests platelet hyper-reactivity in association with HIV-1 despite low count. With the introduction of HAART platelet counts improve though thrombotic incidences remain unchanged or increased. A better insight was needed into the patho-physiologic links between HIV-1 infection and platelet reactivity/function in the development of thrombotic sequelae. This helped to identify HIV-1 infected patients at greatest risk of thrombotic events for whom anti-thrombotic prophylaxis may be justified.

1.3 Justification

The introduction of HAART has significantly modified the course of HIV-1/AIDS disease, with longer survival and improved quality of life. Despite its benefits it has simultaneously lead to the appearance of previously unrecognized complications, such as ischemic cardiovascular events. It is unclear whether the altered platelet function is because of HIV or due to long term use of these antiretroviral drugs. Previous studies on platelet function in HIV have been done in Caucasian populations (Gresele *et al*., 2011; Satchell *et al*., 2011). It is not clear whether the findings were due to genetic predisposition (population differences) or natural cause of the platelet response to HIV. No studies have been done in Kenya on platelet function in HIV infected patients. Local data for Kenyan populations are warranted to enhance the clinical benefit of HAART. Proper insight would also permit anti-platelet therapy for management of thrombotic events as observed in these patients. The study further promotes related studies involving coagulation in adult HIV-1infected patients.

1.4 Research Questions

i. What is the level in platelet P-selectin expression in adult HIV-1infected HAART naïve patients compared to matched healthy controls?
ii. What is the level of optical platelet aggregation in adult HIV-1infected patients HAART naïve patients compared to matched healthy controls?
iii. How does platelet function correlate with viral load and CD4 counts in adult HIV-1infected patients HAART naïve patients?

1.5 Null Hypothesis

i. There is no difference in platelet P-selectin expression levels in adult HIV-1infected HAART naïve patients compared to matched healthy controls
ii. There is no difference in optical platelet aggregation in adult HIV-1 infected HAART naïve patients compared to matched healthy controls
iii. There is no correlation of platelet function with viral load and CD4 counts in adult HIV-1infected HAART naïve patients.

1.6 Objectives

1.6.1 General Objective

To determine platelet function in adult HIV-1infected HAART naïve patients at Kenyatta National Hospital.

1.6.2 Specific Objectives

i. To establish platelet P-selectin expression in adult HIV-1infected HAART naïve patients compared to matched healthy controls
ii. To determine optical platelet aggregation in adult HIV-1 infected HAART naïve patients compared to matched healthy controls
iii. To correlate platelet function with viral load and CD4 counts in adult HIV-1infected HAART naïve patients.

1.7 Study Output

The study was intended to contribute to a better understanding on the link between HIV-1 infection, platelet function that is leading to disordered coagulation, thrombotic events and cardiovascular risks among HIV-1 infected patients. This was expected to help in highlighting the need for targeted therapeutic management of thrombotic conditions in HIV infected/AIDS patients and their prevention.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Human immuno-deficiency virus epidemiology

Human immuno-deficiency virus (HIV) is a lentivirus, member of human retrovirus sub-type. In the recent past, there has been a massive spread of HIV infection in ripple waves all over the globe since it was first identified in 1981(Fanales-Belasio *et al*., 2010). By end of 2013, 35 million people were reported to have been infected with HIV globally. The number of people living with HIV (PLWH) is increasing due to antiretroviral therapy with reduced mortality and morbidity rate. It is estimated that about 0.8% adults of age 15–49 years are living with HIV worldwide (*The gap report*, 2013).

Of the 35 million PLWH, 24.7 million are from sub-Saharan Africa therefore being the region most affected by the epidemic. In this region, approximately one in every 20 adults is infected with the HIV. By the end of 2013, Asia and Pacific region had nearly 4.8 million PLWH. The Caribbean had 1.1% adults infected with HIV in the same year (*The gap report*, 2013).

Human immuno-deficiency virus prevalence in Sub-Saharan Africa accounts for about 71% of the total global percentage. Ten countries: South Africa, Kenya, Malawi, Nigeria, Mozambique, Tanzania, Zambia, Uganda, Zimbabwe and Ethiopia contribute approximately 81% of all PLWH in the region. Half of the total comes from Nigeria and South Africa which are the hardest hit countries with the epidemic. Statistics also indicate that in the sub-Saharan Africa more women are living with HIV (58%)

compared to men (49%). In 2015, Kenya alone had a HIV prevalence of 6% with about 1.6million PLWH (*The gap report*, 2015).

It is recorded that AIDS related mortality in sub-Saharan Africa dropped by 49% between 2005 and 2014. Kenya recorded a significant decline of 60% in 2015 (*The gap report*, 2015). This success has been attributed to successive rise in the number of PLWH on antiretroviral treatment. Averagely about 12.9 million PLWH were on antiretroviral treatment globally by the end of 2013. Globally, the percentage of PLWH not on antiretroviral therapy dropped from 90% in 2006 to 59% in 2015. In the past three years sub-Saharan region has witnessed a significant expansion in the coverage of HIV therapy (Aung *et al*., 2017). The year 2015 recorded 1.9 million additional PLWH on antiretroviral therapy. In Kenya about 42% of PLWH are on ARVs and the government has committed to registering more numbers in the coming years (*Clinical Management of HIV/AIDS*, 2015).

Significant investments have been made in Kenya regarding HIV treatment programmes. A strong health care infrastructure has been developed to provide care for PLWH (*Global AIDS response progress reporting 2015*, 2015). One of the centers put up for monitoring of these HIV patients is Comprehensive Care Clinic (CCC) at the Kenyatta National Hospital which is the largest referral hospital in the country. The CCC has approximately 200 staff which includes doctors and nurses who test, counsel and offer general care to HIV infected/AIDS patients. The clinic is open every day of the week. Currently, 6642 patients are actively being followed up at the CCC. Every week approximately 5500 patients are receiving ARV therapy and 950 patients on palliative care (*Global AIDS response progress reporting 2015*, 2015).

2.2 Human Immuno-deficiency virus biology

Human immuno-deficiency virus belongs to the group of Retroviruses (Klatt, 2016). These viruses use ribonucleic acid (RNA) to store their genetic information and not deoxyribonucleic acid (DNA) which is used by most viruses (Batki & Selwyn, 2008).

2.2.1 Structure of HIV

Human immuno-deficiency virus has an outer envelope composed of a lipid bi-layer with spikes of glycoproteins (GP) mainly, GP 41 and GP 120 protruding from the surface membrane (Lucas, 2002). The nucleo-capsid (p 17) is found inside the envelope surrounding p24, which is a central core protein (Sturdevant *et al*., 2015). Inside the core lies the virus genome in form of two copies of single-stranded RNA. Two major proteins proteins p7 and p9 are attached to the RNA strands and are thought to regulate expression of genes. The core also contains enzyme reverse transcriptase (RT) which is majorly involved in conversion of viral RNA into pro-viral DNA (Gray, 2005).

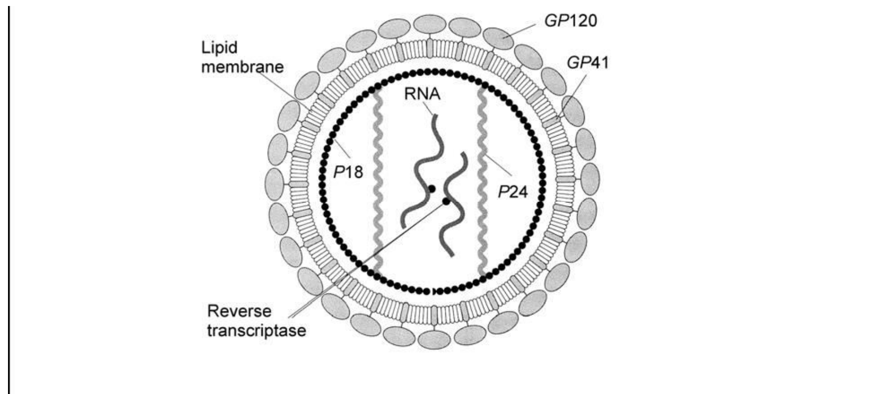

Figure1.1Structure of HIV virus (Klatt, 2016)

2.2.2 Mode of transmission

Human immuno-deficiency virus can only survive within a human body. Transmission of HIV occurs from one person to another majorly via body fluids such as semen, blood, breast milk or vaginal secretions (Naburi *et al*., 2017). The most common effective means of HIV transmission is during sexual contact with an infected person. This is because tissues found at the vagina, rectum and anus are easily injured allowing easy entry of the virus into the body (Hess *et al*., 2017). Other modes of transmission include contact with infected blood during child birth, blood transfusion/organ transplants or use of injection equipments already used by an infected person (Kozlowski *et al*., 2016).

2.2.3 Human immuno-deficiency virus infection and life cycle

Infection of HIV is particular to specific types of cells in the body. These are cells that express CD4 receptors on their surface membrane, referred to as CD4 cells or T4 cells. In the immune system, T4-lymphocytes or T-helper cells have these receptors (Fanales-Belasio *et al*., 2010). Other cells carrying the CD4 receptors include other leukocytes (monocytes and macrophages), chromaffin cells in the intestines, glial cells in the brain and Langerhans' cells in the skin. All of these cell types have been shown to be infected with HIV. Once HIV interacts with these cells, GP120 has spikes that ‘slot into’ the CD4 receptor. At this point the virus is able to bind to the host cell (Fanales-Belasio *et al*., 2010).

Once inside the cell, the virus loses its outer envelope releasing its contents (viral RNA and reverse transcriptase) into the cytoplasm of the host cell. Viral RNA acts as a template from which the enzyme reverse transcriptase produces a complementary DNA strand using the host cell's nucleotides (Maldonado-Rodriguez *et al.*, 2015). This single stranded DNA acts as a template to replicate a second DNA strand. A double stranded proviral DNA is formed which is incorporated into the host cell by enzyme intergrase. Translation of viral RNA into viral proteins occurs along with host cell genome. Catalyzed by a viral enzyme protease, new viral particles are assembled and released from the infected cells. New viral particles released into the system infect other body cells (Mohammed & Nasidi, 2007).

2.3 Clinical course of HIV

Host pathogenesis to HIV infection is influenced by factors affecting immune responses of the host to the virus and the viral properties. Different outcomes of the viral infection are determined by the effective balance between these two components thus influencing progression into AIDS and subsequent long survival (Fanales-Belasio *et al.*, 2010). Through receptor mediated mechanisms, dendritic cells and CD4+ lymphocytes are infected. This allows the virus to spread to the nearby lymph nodes and finally into the blood stream (Sturdevant *et al.*, 2015).

In the early stages of the infection, there is extensive replication of the viral particles in the infected lymph nodes and within the lymphatic tissue of infected tissue (Sturdevant *et al.*, 2015). At this stage, the viral particles can be found in the main infection targets

such as activated CD4+ T-cells and within follicular dendritic cells. Resting CD4+ T-cells become permanent reservoirs for the virus allowing establishment of a latent state. Other infected cells will undergo lysis further leading to spread of the virus (Mohammed & Nasidi, 2007).

In about 10-13 days after infection, viral RNA is traceable in blood. This plays a critical point during the HIV infection because it indicates that the infected individual can potentially transmit the virus (Mohammed & Nasidi, 2007; Simon *et al.*, 2010). The onset of viremia also suggests a chance for the infection being detected in plasma. At the phase of sero-conversion there is rapid viral replication and the level of viremia peaks to over 100 million copies per cubic centimeter (Klatt, 2016).

The high viral levels are normally short-lived as the host mounts immune response against the viral infection. Viral replication is therefore partially controlled and viremia drops to lower undetectable levels referred to as viral set point (Simon *et al.*, 2010). During the infection, several humoral and cellular antiviral immunity influence replication of the virus and its successive progression into the viral set point phase (often referred as "acute infection"). Before formation of neutralizing antibodies, the role of specific CD8+ T-cell cytotoxic activity is very pivotal in initially controlling replication of the virus (Mohammed & Nasidi, 2007).

Sero-conversion has been shown to occur ranging from 3 to 5 weeks using commercial tests. It's observed that the onset of flu-like symptoms in infected patients begins after a few weeks of exposure to HIV (Lucas, 2002; Simon *et al.*, 2010). The symptoms include; fever, weight loss, oral ulcers, lymphadenopathy, arthralgia, malaise, maculopapular rash, pharyngitis and myalgia. These clinical symptoms heterogeneously

vary among infected individuals, with those displaying severe symptoms in the acute phase infection quickly progress into AIDS (Klatt, 2016).

During acute HIV infection, the symptomatic phase lasts between 7 and 10 days but not longer than 14 days. In this phase before antiviral immune response is mounted, the CD4+ T-cell counts dramatically declines. This is associated with high viremia levels (Prebensen *et al*., 2017). The viral levels drop once an effective immune response is elicited causing the CD4 counts to increase although to levels lower than prior to the infection. Lower CD4 counts observed suggest pathogenic effects associated by the viral infection. In addition, a general dysfunction in the immune system is detected during HIV disease and other antigens indicating that the infection impairs cells of the immune system (Prebensen *et al*., 2017).

Infected individuals progress into the asymptomatic period shortly after onset of acute infection. This phase is characterized by absence of symptoms and decline in HIV viremia levels. This is attributed to the antiviral action impacted by both innate and adaptive immunity (Lecher *et al*., 2016). Virus specific neutralizing antibodies bind to the HIV antigens or elimination of infected cell by T-lymphocytes and natural killer cells via Antibody- Dependent Cellular Cytotoxicity (ADCC) (Bangham*et al*., 2009). In addition, HIV-specific T-lymphocytes recognize virus antigens on the surface of infected cells and promote their elimination by antigen-specific cytotoxic mechanisms. During the asymptomatic phase, HIV continues to replicate in various body compartments as it counteracts antiviral immunity, inducing a chronic inflammatory state (Weston & Marett, 2009).

Several reasons affect complete eradication of the infection by antiviral immunity. Majorly so, is the integration of the virus in the lymphoid compartments which act as reservoirs (Klatt, 2016). These areas have very low expression of viral antigens and the virus have very high frequency of mutation leading to escape of the virus from the immune system (Simon *et al*., 2010). Therefore, replication of the virus continues in the lymphoid compartments and so viremia is detectable in plasma even in absence of the symptoms associated with the infection. In other infected individuals there is effective control of the infection such that viremia is not detectable in plasma for many years (Mohammed & Nasidi, 2007). Individuals exhibiting such condition are referred to as 'elite controllers'. To further elucidate the mechanisms involved in control of the viral infection, these individuals are still intensely studied (Gray, 2005).

During the symptomatic phase, there is a progressive loss in CD4+ lymphocytes and a dysfunction of the immune system due to pathogenic viral effects (Prebensen *et al*., 2017). Progression of the infection is marked by destruction of the lymphoid tissue architecture, caused by viral replication and persistent activation of the immune cells. Viral diffusion is increased to surrounding CD4+ T-cells and enhancing its spread within the local and whole lymphoid area. At this stage there is intensive replication of HIV particularly in the draining lymph nodes, the gut lamina propria and submucosa (Klatt, 2016; Weston & Marett, 2009).

Capacity of the host immunity to contain viral replication by reconstituting T-cells within lymph nodes or the mucosa associated lymphoid tissue memory determines further progression of the infection (Lucas, 2002). Without containment of the virus, lymphoid system continues to be destroyed and CD4+ T-cell counts drop to levels

below <250 cells/μl. This posses a major risk for onset of opportunistic infections by pathogenic infectious agents. Overtime, the immune system also continues to get impaired and the infected individuals become susceptible to tumors (Klatt, 2016). It would be interesting to observe whether there is difference in platelet function or coagulation risk with CD4 and viral load counts.

2.4 Opportunistic infections

Acquired immune deficiency syndrome (AIDS) stage is characterized by common opportunistic infections caused by Cytomegalovirus, *Microcystiscarinii*, *Herpes zoster* or enteropathic parasites (*Isospora belli*, *Criptosporidiu* and *Giardia* species) which cause life threatening diseases. This phase is usually defined by severe loss in body weight, swelling of the lymph nodes, fever, gastro-intestinal and respiratory symptoms (Maldonado-Rodriguez *et al*., 2015). The clinical course of the infection is further worsened by neoplastic diseases, such as lymphomas and Kaposis Sarcoma due to immune deficiency state. Progression of the AIDS phase greatly varies among individuals but generally marked lymphopenia, continued drop in CD4+ T-cell count and anaemia is also observed (Hirashima *et al*., 2016).

2.5 Management of HIV

A complete history and physical examination are helpful in determining the stage of the disease. CD4+ T lymphocyte count and percentage is commonly used as a surrogate marker to assess disease progression (de Bruin *et al*., 2017). Guidelines recommend that there should be a baseline CD4+ count for all HIV infected individuals done at the

initial follow up. A positive ELISA test is used for infants born of HIV infected mothers (Batki & Selwyn, 2008).

A patient is clinically managed is dependent on the stage of the disease. To enable detection of early development of symptomatic phase, it is important to carry out early detection, continuous follow-up and monitoring of the patient. This early intervention helps to delay disease progression (Aung *et al*., 2017). For a patient who is symptomatic, recommended preliminary investigations are carried out. Immunological surrogate markers such as CD4+/CD8+ ratio, CD4+ T cell counts and percentage are performed. Polymerase chain reaction (PCR) tests are used for early detection of the virus. ELISA assays are utilized as a confirmatory test whenever indicated (Gray, 2005).

Careful consideration is done before recruiting an infected patient into antiretroviral therapy. This includes discussion with the patient or a close relative in the case of a child infected with HIV or a patient with AIDS associated dementia (Mudany *et al*., 2016). This is imperative not only due to toxicity of the antiviral drugs but also close follow up and monitoring of the patient by the physician is required. Guidelines indicate that before starting therapy, the attending clinician has to do preliminary clinical assessment. Some of the assessments done include: current patients' weight, immunological status (as evidence by the level of CD4+ T lymphocyte counts or CD4+/CD8+ ratio) and evidence of opportunistic infections (Lecher *et al*., 2016).

2.6 Human immuno-deficiency virus and cardiovascular diseases

Recent clinical studies have associated HIV positive patients with high rates of cardiovascular disease and athero-thrombosis of up to 18% (Becker *et al*., 2011).

These are patients who show no known predisposing factors such as surgery, trauma, pregnancy or a deficiency in any of the coagulation factors. The comparative incidence of coronary heart diseases (CHDs) of deep venous thrombo-embolism among HIV positive patients was recorded to be significantly four folds higher than in HIV negative controls (Aurigemma *et al*., 2007). The pathogenic mechanism leading to increased incidences of thrombotic events in HIV-infection still remains controversial (Gresele *et al*., 2011; Hsue *et al*., 2012). However it is suggested that a chronic inflammatory state leading to a hyper-coagulative state, HIV-associated endothelial injury and consequently alterations of platelet function seem to be involved (Schneida *et al*., 2009).

2.7 Human immuno-deficiency virus and risk of cardiovascular diseases

It is thought that for atherosclerosis to take place at any stage of the disease then the process of inflammation is very pivotal. Various factors can trigger Inflammation consequently leading to atherosclerosis; for example, pro-atherogenic lipid particles infiltrate the arterial intima thus activating cells of the endothelial wall (Arvind *et al*., 2013). This leads to increased expression of inflammatory molecules and cell adhesion molecules which attracts inflammatory cells to the site of inflammation. Microphages take up the lipids to form foam cells which after accumulating further contribute to formation of atheromas (Hsue *et al*., 2012). Later various T-cell subsets are recruited to

these sites leading to formation of a chronically inflamed unstable atherosclerotic plaque. A progressive chronic inflammation proceeds in the arterial wall leading to deposition of collagen, fibrosis and thickening of the wall (Gresele *et al*., 2011).

Previous cohort studies having large HIV positive patient populations demonstrated that elevated levels of inflammatory markers, more so high-sensitivity C-reactive protein (hsCRP) strongly predicts cardiovascular events and mortality (Arvind *et al*., 2013). A consistent observation also noticed that patients who suffer myocardial infarctions and other vascular complications also suffer from chronic inflammatory diseases such as rheumatoid athritis. This suggests a causal association between inflammation and cardiovascular disease (Hsue *et al*., 2012).

Elevated hsCRP has also been associated with risk of cardiovascular disease in HIV positive adults. However it is not clear whether the prognostic role of hsCRP in HIV positive adults is different from uninfected adults (Hsue *et al*., 2012). Clinical Studies compared risk of developing cardiovascular disease between individuals recruited to receive continuous Highly Active Antiretroviral Therapy (HAART) and those who receiving intermittent therapy with the aim of maintaining a CD4 T-cell counts. Contradictory reports were recorded clearly elucidating that the role of HAART in the development of cardiovascular risk during HIV infection is still unclear (Hsue *et al*., 2012).

Evidence suggests that during HIV infection, there is a higher risk of cardiovascular diseases which is associated with elevated levels of D-dimers and pro-inflamatory cytokine interleukin 6 (IL-6). These biomarkers could be potentially used in prediction

of HIV-related cardiovascular events. This observation proposes the link between inflammation and CVD in HIV diagnosis (Arvind *et al*., 2013).

2.7.1 Role of inflammation and immune activation

Several parallel pathways during HIV disease may increase potential risk to cardiovascular events. Human immuno-deficiency virus disease is associated with chronic persistent inflammatory condition (Green *et al*., 2015). Multiple pathways mediate this effect, which include immune activation caused by direct effect of viral replication and other co-pathogens. This consequently leads to deficiency in immunity, loss of mucosal integrity and chronic translocation of microbial products into the gut (Green *et al*., 2015). Emerging data shows the pivotal role of inflammation as a cause of cardiovascular diseases among the HIV negative population. Implying that chronic inflammatory condition associated with HIV is a plausible cause for increased cardiovascular events observed in adult HIV infected patients (Arvind *et al*., 2013).

2.8 Human immuno-deficiency virus and haematological manifestation.

The primary effect of HIV is on the hematological system. Characteristically, there is a decrease in T-lymphocytes especially CD4 and CD8 in patients not on treatment. This may be accompanied by a decrease in formed elements: anemia (in 70% of patients), granulocytopenia (in 50-70% of patients), thrombocytopenia (in 10-40% of patients), and lymphopenia (Parinitha & Kulkarni, 2012). These changes are caused by increased destruction of peripheral cells or a reduced production of these cells resulting from bone marrow being directly suppressed by HIV, opportunistic Pathogens, infiltration of

tumors or therapeutic treatments. Some of the therapies include antiretroviral, antimicrobial and anti-tumor agents (Zeijlstra, 2012).

Human immune-deficiency virus itself suppresses the bone marrow evidenced by an increase of cellular deficiencies with advancing disease. However, isolated thrombocytopenia is often the presenting manifestation of HIV infection and is thus not considered a criterion for advancing disease and progression to AIDS (Gresele *et al.*, 2011). Infectious agents that infiltrate bone marrow and suppress cell production or cause reticulo-endothelial dysfunction are: *Mycobacterium tuberculosis*, *Cryptococcus neoformans*, Cytomegalovirus, and *Histoplasma capsulatum* (Green *et al.*, 2015).

The metastatic diseases, which infiltrate the bone marrow further contributing to haematological manifestations linked HIV disease, include Kaposi sarcoma and lymphoma (Chabert *et al.*, 2015).

Thrombocytopenia is a common clinical manifestation during HIV disease with an etiology that is complex and multi-factorial. Initial mechanisms leading to thrombocytopenia usually takes place earlier in the disease process (Vaughan, 2010). One major pathway in destruction of platelets involves immune mediated destruction of platelets by platelet bound IgG, complement, antibody complexes or circulating immunoglobulins. Evidence suggests that molecular mimicry exists between platelet GP IIIa and HIV GP 120. Therefore, HIV auto antibodies through cross reaction are able to bind to platelet antigens (Assinger, 2014; Dominguez *et al.*, 2004).

Other possible etiologies for thrombocytopenia include decreased and defective production in the bone marrow (which also shortens platelet life-span), a side effect of

drugs taken, syndromes mimicking haemolytic uraemic syndrome, thrombotic thrombocytopenic purpura, hyper-splenism, disseminated intravascular coagulation, or an increase in the peripheral destruction and fragmentation of platelets (platelets are directly attacked by HIV through CD4 and CXCR4 receptors)(Vaughan, 2010). There might be a decrease in thrombopoietin production in hepatocytes in those HIV infected patients with liver disease (Assinger, 2014).

2.9 Platelets

Platelets are the smallest of the human blood cells (3.6-0.7 mm). They are key cellular mediators in the processes of haemostasis and thrombosis. They are released into the circulation after being formed from megakaryocytes in the bone marrow (Yeh *et al*., 2016). When the endothelial wall is damaged, recruitment of platelets to the site of damaged sub-endothelial matrix takes place. A haemostatic plug is formed at the site of vessel injury closing up the leak. Platelets are also involved in thrombus formation at sites of ruptured atherosclerotic plaques which trigger strokes and heart attacks (Conde *et al*., 2005).

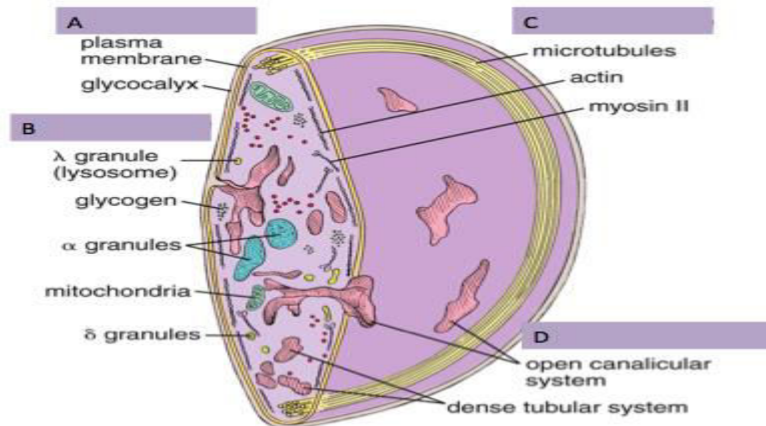

Figure1.2 Structure of a platelet (Theoret *et al*., 2006)

2.9.1 Platelet function in heamostasis

Platelets play an important role as specialized blood cells in the physiological and pathological processes of haemostasis (Wecht, 2008). In addition, they are also studied as cells that play a role in innate immunity, wound healing and central modulators during the process of inflammation. Following injury of a vessel wall the sub-endothelial collagen matrix is exposed. Platelets in the circulation are recruited, characterized by four major reactions: adhesion, activation, secretion and aggregation (Berger *et al*., 2000).

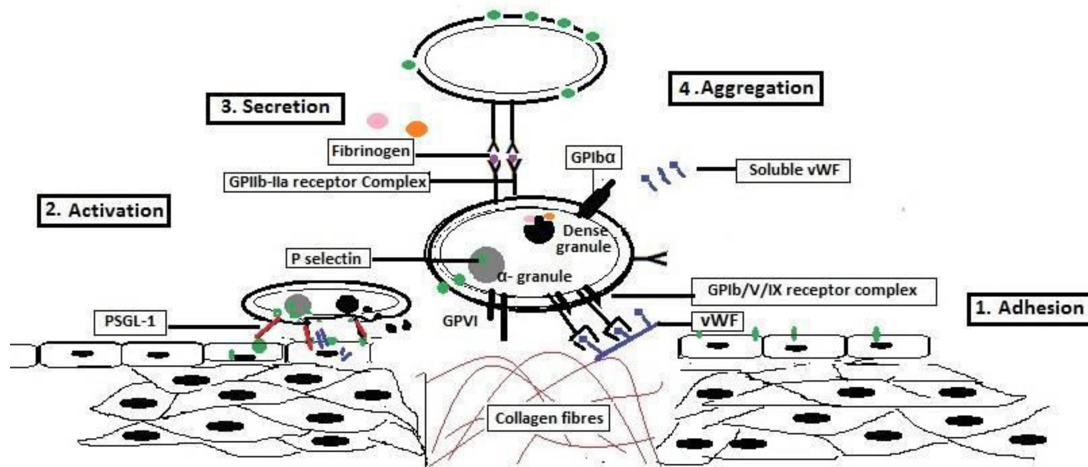

Figure1.3The schematic diagram illustrates the steps involved in platelet reaction (Nkambule, 2015)

2.9.1.1 Platelet adhesion

Platelets circulate in an inactivated quiescent state under physiological conditions. Inhibitory mediators from intact endothelial cells are released preventing untimely activation of the platelets, which then assume a discoid shape (Qiu *et al*., 2014; Ruggeri & Mendolicchio, 2007). Changes in released anti-platelet factors and endothelial dysfunction leads to expression of endothelial cell adhesion molecules and collagen associated Von willebrand factor. Platelets interact with the endothelium undergoing shape change and formation of a ‘pseudopod like appearance (Smyth *et al*., 2009).

The initial step during primary haemostasis involves adhesion of platelets to the sub-endothelial matrix. Initial adhesive receptor on the platelet is the glycoprotein GP Ib/IX/V complex which binds to collagen-associated von Willebrand Factor (vWF) found at the endothelial lesion (Jurk & Kehrel, 2005). Platelets can also bind to collagen via GP Ia/IIa under low shear conditions. Several other adhesive receptors are:

platelet surface integrins notably immobilized fibrinogen, fibronectin, vitronectin and laminin (Smyth *et al*., 2009).

2.9.1.2 Platelet activation

Following adhesion, platelets get activated by a number of soluble mediators/agonists engaging with specific receptors. The initial response is further amplified by additional platelets being recruited to the site of vessel injury (Jurk & Kehrel, 2005). One of the most powerful platelet agonists is thrombin. It is a produced from the coagulation cascade and mediated via the protease- activated receptors (PAR) 1 and 4, and also GPI b/ IX/V. Once activated, platelet's receptors for serotonin, epinephrine, vasopressin and platelet activating factor (PAF) are expressed. Each of these contributes to and enhances further activation of the platelets (Wecht, 2008).

2.9.1.3 Platelet Secretion

Platelet activation is followed by secretion and release of dense and alpha granule constituents. A large number of proteins are contained in the alpha granules such as cytokines, protease inhibitors, coagulation factors and chemokines (Berger *et al*., 2000). They all have various functions. Granular membrane fuses with the plasma membrane during the process of exocytosis (Polgar *et al*., 2005). The outcome is expression of increased numbers of proteins on the platelet surface such as CD63, CD40 ligand (CD40L) and CD62P (P-selectin). P-selectin mediates platelet binding to leukocytes, attracting them to the site of inflammation (Conde *et al*., 2005). Expression of P-selectin

on the surface of the membrane only happens upon platelet activation and thus majorly used to indicate activation.

2.9.1.4 Platelet aggregation

Platelet aggregation is mainly characterized by platelets accumulating to form a hemostatic plug (French & Seligsohn, 2000). Mechanisms mediated by ADP or thromboxane further adds to recruitment of platelets to the site of vessel injury. The platelets combine with the fragile layer of previously adherent platelets forming an aggregate or unstable haemostatic plug (Perumal *et al*., 2014). GPIIb/IIIa is the major glycoprotein involved in platelet aggregation. During aggregation, GPIIb/IIIa becomes activated and undergoes a conformational change causing expression of its adhesive domain on which protein binds. This dormain allows binding of fibrinogen, a bivalent molecule. Fibrinogen binds to GPIIb/IIIa on adjacent platelets allowing platelet-platelet interaction via fibrinogen bridges (Theoret *et al*., 2006). Additional platelets are activated by thrombin produced from the coagulation cascade which also cleaves fibrinogen that is bound to fibrin. Fibrin establishes a firm cross-link stabilizing the platelet plug formed (Smyth *et al*., 2009).

2.9.2 Platelet cell adhesion molecules

Early inflammatory events are marked by leucocytes and platelets adhering to and trans-migrating across the endothelial wall. Cell adhesion molecules (CAMs) coupled with other counter-adhesive molecules present on platelets, leucocytes and endothelial wall are key mediators of these process (Leytin *et al*., 2000).

Cell adhesion molecules can be categorized into four families of proteins: the the integrin family, selectin family, immunoglobulin (Ig) superfamily and the mucin-like family (Furie, 2005)

The selectin family has three molecules, designated as P, E, and L. vascular endothelial cells express E and P-selectin while L-selectin is expressed on leukocytes that are circulating. Platelets and endothelial cells upon activation express P-selectin (CD62p). Inactivated platelets store P-selectin in alpha granules and weibel-palade bodies. On activation of the endothelial cells by platelet activating molecules or surgical trauma, P-selectin is expressed on the surface membrane. It allows rolling and tethering of platelets and leucocytes on endothelial cells that is activated (Polgar et al., 2005). P-selectin has ligands in all leucocytes referred to as P-selectin glycoprotein ligand-1 (PSGL-1) which allows platelet-leukocyte interaction to the site of inflammation. Current clinical studies indicate plasma P-selectin could partly be used as a marker of platelet activation (Yeh *et al*., 2016). Eleveted levels of soluble P-selectin have also been shown to predict patients at greatest risk of adverse cardiovascular events (Schneida *et al*., 2009). Elevated levels of platelet P-selectin has been observed in HIV positive individuals (Mayne *et al*., 2012; Nkambule *et al*., 2015).

2.10 Platelet and thrombosis

Thrombosis or athero-thrombosis refers to the acute process caused by leakage or rapture of an atheromatous plaque causing adherence and aggregation of platelets forming a thrombus. This causes a mechanical blockage of the whole or most of the coronary artery lumen leading to fatal clinical complications such as myocardial

infarction and ischemia (Caron *et al*., 2002; Gresele *et al*., 2011). Clinical evidence records that in vivo platelet activation, increased platelet count, and hyper-reactivity of platelets further contribute to CVEs (Qiu *et al*., 2014). A plaque is formed when the mechanisms designed to prevent unwarranted extension of platelet activation are impaired leading to formation of a pathological thrombus (Abdel *et al*., 2016). Platelet aggregation could be triggered by a variety of molecules either released from platelets or were present in mileu prior to platelets activation (Furie, 2005).

During thrombosis platelets, leukocytes and coagulation proteases stimulate inflammatory responses (Abdel *et al*., 2016). Leukocytes interact with platelets to release micro-particles and other soluble mediators such as P-selectin, CD40L and cytokines among other substances (Marc van der Zee *et al*., 2006). The net effect of these mechanisms is a creation of a pro-inflammatory state and perturbation of the vascular endothelium and processes such as platelet and leukocyte adhesion and translocation are up regulated (Furie, 2005). This leads to plaque formation. Plaque formation or rapture exposes platelet activating proteins such as tissue factor and collagen into circulation which in ordered sequence is sufficient to trigger thrombosis. A stabilized arterial thrombus resistant to clot lysis is formed leading to myocardial infarction (Feng *et al*., 2001). Several studies have recorded elevated plasma levels of some biomarkers of fibrinolysis and platelet activation such as D-dimers, P-selectin, sCD40L platelet-derived micro-particles in patients with CVDs (Berger *et al*., 2000; Schneida *et al*., 2009).

2.11 Effect of HIV/AIDS on platelet reactivity

In-vivo platelet activation during HIV-1 infection is predominantly triggered by increased inflammatory response and viral effect on plasma lipids. Persistent viral Infection leads to an up regulated release of pro-inflammatory cytokines; IL-6, IL-8, IL-1 and TNF-α from T Helper 2 cells. These levels have been shown to correlate with HIV-1 viral load (Rönsholt *et al*., 2013). The action of HIV-1 protein trans-activator of transcription (Tat) activates inflammatory pathways causing endothelial dysfunction and increased monocyte expression of surface tissue factor. Local inflammation that ensues leads to atherosclerotic plaques and occlusive thrombotic events. These events are mediated by recruitment of leukocytes, platelet adhesion and aggregation, blood clotting activation, and fibrinolysis derangement (Marc van der Zee *et al*., 2006).

Platelets once activated also produce and secrete pro-inflammatory cytokines in particular, IL-1 beta and IL-18 in high amounts which contributes to further activation (Gresele *et al*., 2011). Elevated plasma levels of platelet activation markers such as surface platelet P-selectin (sPsel) platelet-derived micro-particles and levels of D-dimers and have been recorded in HIV-1 positive patients (Polgar *et al*., 2005). It reflects in-vivo fibrinolysis and clot formation (Rönsholt *et al*., 2013).

These findings triggered interest in platelets as potential key players in forming a link between inflammation, coagulation and thrombus formation following plaque rupture (Gresele *et al*., 2011). In particular, studies which have focused on the interactions of various cells have led to the establishment of an evolving role of platelets in the inflammatory and coagulation process (O'Brien *et al*., 2012). However the relevance of these interactions is not clear in the context of HIV infection. Activated platelets have

been demonstrated to form complexes with leukocytes via P-selectin (CD62P) interaction subsequently activating neutrophil and monocyte (Polgar *et al*., 2005).

2.12 Effects of highly active antiretroviral therapy (HAART) on platelet functions.

Introduction of HAART has improved the prognosis of HIV-1 positive patients with a significant decline in both mortality and morbidity rates. Generally, there is an improvement in patients' quality of life. However use of this anti-retroviral therapy has been linked with accelerated atherosclerosis, increasing the risk of CHD among adult HIV-1infected patients (Satchell *et al*., 2011). Clinical studies indicate that HAART is independently associated with a 24% relative increase in the rate of cardiovascular diseases with continued exposure, during the first 3–6 years of therapy. By reducing HIV viral load and thus inflammation, HAART also expected to down-regulate inflammatory endothelial components causing atherosclerotic plaques which trigger platelet hyper reactivity (Donati *et al*., 2004).

Treatment induced metabolic abnormalities in particular; hyper-lipidemia, hyper-glycemia and fat redistribution have been advocated as central phenomena in HAART-induced cardiovascular risk (Donati *et al*., 2004). This leads to endothelial dysfunction that triggers in-vivo activation of platelets due to absence of platelet-inhibiting mediators. Platelet pro-aggregatory and pro-thrombotic state is promoted. However previous studies have not been able to conclusively elucidate the role of HAART in relation to platelet reactivity (Gresele *et al*., 2011).

2.13 Platelet function tests

When a patient presents with clinical symptoms associated with a defect in primary haemostasis such as epistaxis, easy bruising or menorrhagia abnormality in platelets is considered and investigated (Johns, 2004). It is critical to accurately measure platelet function not only to identify patients with dysfunctional or hyper-reactive platelets but also in identifying patients at risk of thrombotic incidences. This allows the patients to benefit from anti-platelet therapy and also general monitoring (Harrison, 2000).

There are two categories of platelet disorders; qualitative and quantitative platelet disorders. Abnormalities in platelet number are classified as quantitative platelet defects whereas abnormalities in platelet function are qualitative defects (Johns, 2004). Most common cause of platelet-related bleeding is considered to be thrombocytopenia. The first step involved in determination of a defective primary haemostasis is measurement of platelet counts and evaluation of a peripheral blood smear (Peter *et al*., 2000). After confirmation of adequate number of platelets, then can one consider performing platelet function assays. Surrogate platelet function marker tests, measure and/or monitor the platelet's reactions: shape change, secretion and receptor expression (Harrison *et al*., 2013).

2.13.1 Principles of Born platelet aggregometry

Platelet aggregometry (Born, 1962) was invented in the 1960s and it revolutionized ability to diagnose defects in platelet function (Yee *et al*., 2016). It later became accepted as a standard laboratory assay for testing platelet function (Sbrana *et al*.,

2007).

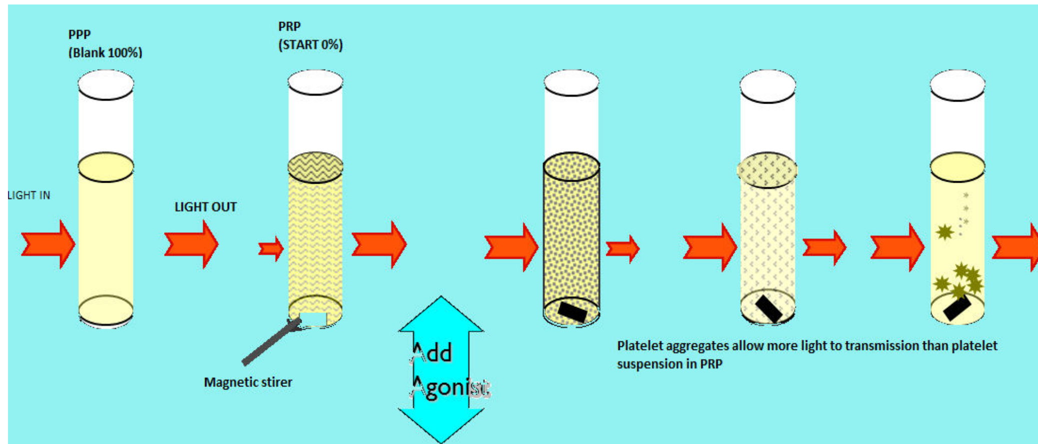

Figure1.4 Steps involved in platelet aggregometry method (Sbrana *et al*., 2007)

The basic principle of platelet aggregometry is based on measuring change in optical density (or light transmittance) in platelet rich plasma (PRP) at 37°C after adding desired agonists. Calibration is done using autologous platelet poor plasma (PPP) as baseline set at 100% light transmission while PRP is set at 0% light transmission (Perumal *et al*., 2014). During the assay, agonist (collagen, ADP and adrenaline) is added to PRP and platelets aggregation is triggered allowing light transmission through the cuvetts. Light transmission is detected and recorded over time on a chat as maximum percentage platelet aggregation. Other classical platelet responses such as maximum percentage secondary aggregation lag time and shape change are also recorded (Harrison *et al*., 2012).

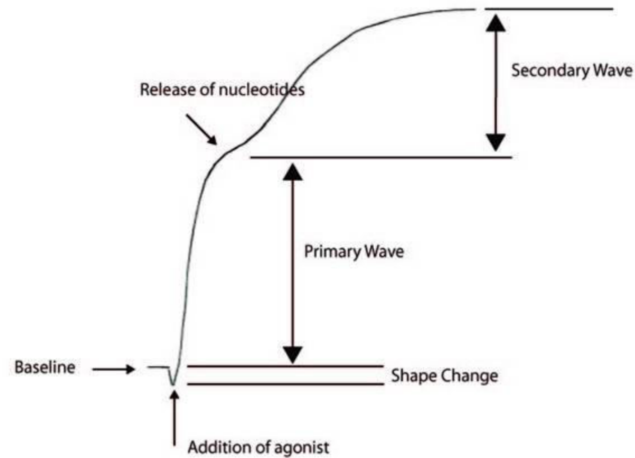

Figure1.5Typical Aggregation curve showing the events in classic biphasicaggregation (chrono-log data on file)

Various factors affect recorded responses which include: normal functioning of the platelet, presence of inhibitors and the concentration of agonist used. Based upon the patterns of the aggregation trace, one is able to detect any classical platelet disorders (Harrison *et al*., 2013). Although an enormous amount of information can be obtained with aggregometry, a major limitation is that the test is labour intensive, requires sample preparation, quality control and a fair degree of technical expertise both to perform the test and interpret the results (Javela, 2006).

2.13.2 Flow cytometry

This is a relatively new but powerful laboratory technique used for analysis of platelet function either in whole blood or platelet concentrates (Gremmel *et al*., 2015). The assay utilizes fluorescent labeled monoclonal antibodies specific to particular receptors (Harrison, 2000).

During sample analysis light beams pass across the blood sample and light scatter is recorded. The technique is useful for analysis of platelet activation markers such as P-selectin (CD62p), CD63, LAMP-1 and CD40L), glycoproteins, platelet-leukocyte aggregates, platelet turnover and platelet micro-particles (Leytin *et al*., 2000)

Flow cytometry assay in whole blood allows platelets to be analyzed in presence of other cells such as leukocytes and erythrocytes avoiding premature platelet activation during platelet concentrate preparations. It also possible to perform platelet studies on patients with thrombocytopenia due to sensitivity of the test (Peter *et al*., 2000). However a major setback is that the technique requires a fair degree of expertise and expensive equipment/reagents (Wecht, 2008). Further flow cytometry only shows presence and quantity of platelet receptors but gives no information whether they will end up or participate in aggregation which is the ultimate function.

2.13.3 Bleeding Time

The bleeding time is the time taken for a standardized skin wound to stop bleeding (Johns, 2004). The bleeding time assays are used to determine the ability of platelets to arrest bleeding. In this test both platelet function and number are analyzed. The assay is able to detect and assess acquired and congenital in-vivo platelet function disorders (Wecht, 2008).

Over time, the size and depth of incisions made during bleeding time have been standardized. However several factors still influence results obtained from this test including condition of the skin, age of the patient and environmental factors. The test lacks precision and accuracy due to too many confounding factors. Sometimes, a

questionable correlation with the patient's clinical condition is often observed (Harrison *et al*., 2013). In view of these shortcomings, it is no longer recommended.

2.13.4 Platelet Function Analyzer

The PFA 100™ system utilizes test cartridges with membranes coated with agonists (collagen, epinephrine, collagen or ADP) which stimulate aggregation of platelets. Platelet adhesion and aggregation on the membrane is monitored as whole blood is aspirated under controlled flow conditions through a microscopic aperture cut into the membrane. Time taken for the platelet plug to completely occlude the aperture is recorded (Zwaginga *et al*., 2006). This system is considered cost effective and reliable for detection of bleeding disorders such as von Willebrand disease, evaluation of other physiologic functions of activated platelets and general information pertaining to haemostatic status of a patient (Harrison, 2000). This is a screening test but gives limited information.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The study was done at Kenyatta National Hospital, Nairobi County. Kenyatta National Hospital is the largest referral and teaching hospital in Kenya with a capacity of more than 2000 beds and over 5000 members of staff. The hospital has a comprehensive care center (CCC) that handles HIV infected/AIDS patients. The center provides HIV care to address the diagnosis, treatment of opportunistic infections and other HIV/AIDS co-morbidities, antiretroviral therapy management, antiretroviral adherence counseling, laboratory management of patients and supportive counseling.

3.2 Study Population

Study population included adult HIV-1infected patients above 18 and below 65 years of age (both male and female) attending KNH comprehensive care center clinic. The control group was healthy HIV-1 negative adult blood donors above 18 and below 65 year of age from the KNH blood transfusion center. Adults above 65 years were found unsuitable for the study and excluded because of cellular aging. Due to age their cells are senesced and potentially dysfunctional.

3.3 Study Design

A case-control single-centered design was adopted in this study. There were two study groups; platelet activation and platelet aggregation done independently.

3.4 Sample Size Determination

The sample size was determined by using the formulae of Satchell *et al*. (Satchell *et al*., 2010).

$$N = \frac{(Z\ 1/22 \times p \times (1-p)}{D}$$

Where:

N= sample size required

Z= normal standard deviation

P= postulated prevalence (18%)

D= 0.05 the inverse of 95% confidence limit

$$N = \frac{[(1.96 \times 1/22 \times 0.18 \times (1-0.18) \times 10]}{0.05} = 14.46$$

A minimum sample size of 14 in each group was used (HIV positive patients and healthy controls).

3.5 Sampling Technique

Convenient sampling technique was used. Study subjects were enrolled consecutively as they came into the clinic until the sample size of 28 was reached. That is 14 from each group; adult HIV-1 HAART naïve patients (case) and HIV-1 negative control group.

3.5.1 Inclusion criteria for study participants

The following inclusion criteria were used for the study:

i. HIV positive adult patients above 18 years and below 65 years not on ARVs.

ii. Adult HIV negative blood donors above 18 years and below 65 years.

iii. Consent to participate

3.5.2 Exclusion criteria for study participants

The following exclusion criteria were used for the study:

i. HIV-1 positive/negative participants below 18 years and above 65 years of age.
ii. Refusal to give consent.
iii. HIV-1 positive patients known to have any coagulation abnormalities as defined by attending clinician.
iv. HIV-1infected patients on anti-thrombotic treatment.
v. Participants on lipid lowering agents.
vi. Trauma or surgery on any participant in the recent 4 weeks.
vii. Pregnant participants
viii. Participant on hormonal contraception.

3.6 Ethical Consideration

The study was approved by Kenyatta National Hospital/University of Nairobi ethics and research committee. Study subjects were provided with consent information document and consent form prior to enrollment.

3.7 Enrolment of Study Participants

Human immune-deficiency virus positive study participants were reviewed and screened by assigned comprehensive care clinic physician before enrollment into the study. Potential HIV-1 positive participants were enrolled into the study after a signed informed consent. During the screening, patient’s demographic data was collected including detailed medical history, age, weight, blood pressure, current use of non-HIV medications including complementary therapies. This was recorded in the participant’s medical data information form.

HIV negative blood samples to be used as comparison group were taken from blood donation blood bank at KNH Hematology and blood transfusion unit. Ten mls of blood from each participant was transferred into 3.2% citrate anti-coagulated plastic tubes and assigned a code number. This was then transported to KNH hematology laboratory for further analysis.

3.8 Blood sample collection

Blood samples from HIV positive study participants were drawn by assigned clinic Medical Officer/phlebotomist. Ten milliliters (10 ml) of Peripheral blood was drawn from study subjects using standard veni-puncture method. Blood samples were collected in 3.2% citrate anti-coagulated plastic tubes for plasma. All samples were collected from the populations available in the morning, over a two month time span. Collection, transportation and centrifugation were performed at room temperature. Hemolyzed and/or lipemic specimens were discarded as they might interfere with light transmission on the platelet aggregometer.

3.9 Blood sample analysis

3.9.1 Platelet preparation

Platelet sample preparation was done according to method described by Satchel *et al.* (Satchell *et al*., 2010). Briefly Platelet rich plasma (PRP) was prepared by spinning of anti-coagulated blood at 180g for 10 minutes at room temperature. The cloudy yellow supernatant containing the platelets was removed carefully with a disposable plastic pipette and placed into a clean polypropylene tube and capped. Care was taken not to disturb the white blood cells (WBC) and red blood cells (RBCs) cell layers when removing the PRP. Platelet-poor plasma (PPP) was prepared by centrifuging the remaining sample at 1,200g for 20 minutes at room temperature. The PRP was adjusted to a platelet count of 200 to 350 $\times 10^3/\mu l$ (200-350 $\times 10^9/L$) after addition of PPP.

3.10 Platelet function assays

Platelet function testing was completed within 3 hours after blood sample collection.

3. 10. 1 Platelet Aggregation Assays

Platelet reactivity was determined by measuring agonist-induced platelet aggregation using Chrono-log model 700 (Havertown, PA USA) according to method described by Satchell *et al*. (Satchell et al., 2010). Briefly, the four channels of the aggregometer were adjusted to 100% light transmission with platelet poor plasma. After that, 450 µl of PRP was pipetted into a cuvette containing a magnetic stirrer (stirring rate 12000 rpm/min), and pre-warmed up to 37°C for five minutes. Platelet aggregation was induced by adding to PRP 50 µL of agonists: adenosine diphosphate at concentration of 5 µg/ml, arachidonic acid at concentration of 10 µmol/l and collagen at concentration of 10 µg/ml. The aggregation reaction was followed for eight minutes. Platelet aggregation was expressed as a percentage of the maximal aggregation and the maximal aggregation velocity per minute measured. Decrease of optical density before base line was measured to determine platelet shape change. Decrease of optical density was expressed as a percentage and the duration of shape change in seconds.

3.10.2 P-Selectin Expression Analysis Using Flow Cytometry

The expression of P-selectin on platelets was determined by using 4 color flow cytometer (FACS Calibur^{TM}) according to Nkambule *et al*. (Nkambule *et al*., 2015), using conjugated monoclonal antibodies specific to the P-Selectin receptor. Briefly, Fluorescein isothiocyanate (FITC)-conjugated human anti-CD62p IgG (BD

Biosciences) was used as an independent marker for the activation of platelets. Fifty (µl) of whole blood was added to 5 µl of Anti–CD62P-FITC antibodies. The sample was then incubated in the dark room for 20 minutes. After incubation the sample was diluted by adding 500 µl of PBS Dulbescos (no Mg^{2+} and Ca^{2+}) and sample analyzed immediately. The fluorescence intensity was analyzed on the flow cytometer. The level of platelets expressing P-selectin was defined as a fraction of the 10,000 platelets sorted exhibiting specific binding (that is, CD62p) minus that exhibiting nonspecific binding (that is, the percentage defined with Imuno-globulin G(IgG)-FITC conjugate). Each experiment was repeated at least 3 times. The expression of P-selectin was recorded in percentages. The mean level of P-selectin expression in HIV positive samples was compared with HIV negative control samples.

3.11 Quality Assurance

All blood samples were collected in 3.2% citrate anticoagulant plastic tubes and correctly labeled using study code numbers. Collection, transportation and storage of blood samples were done at room temperature. Samples were analyzed within 3 hours after blood sample collection for aggregation tests and 6 hours for flow cytometry analysis. Both platelet aggregator and flow cytometer machines were setup for use as recommended in the operator's reference manual. Controls were run every morning to ensure the machines are working correctly and efficiently. All laboratory procedures for analysis of blood samples were done according to manufacturer's instructions in the kit paired with the standard operating procedures at KNH hematology laboratory. This was aimed at ensuring reliability and consistency of the results. Known normal blood

specimens (HIV negative control samples) were used to establish typical aggregation and gating patterns in platelet aggregation and flow cytometry respectively. This was to determine normal range for the test results.

Test results were in print outs and attached to the participant medical information form (Appendix V). For platelet aggregation the results were in curves clearly illustrating platelet shape change, primary aggregation (%), secondary aggregation (%), maximum velocity (%/min) and closing time (sec). Flow cytometry results were in histograms illustrating the gating patterns in P-selectin expression. During interpretation of results, normal reference values from the controls were compared with the test results. Test results showing marked variation from the normal reference range indicated platelet dysfunction. Care was taken to ensure data is entered and analyzed correctly.

3.12 Data Analysis

Percentage aggregation was calculated from absorbance values, using the light absorbance of platelet-poor plasma as a reference for 100% aggregation and absorbance at time 0 in the platelet rich plasma control wells representing 0% aggregation. Dose response curves were plotted using the mean platelet aggregation of the final 3 time points for each concentration of agonist, with the F test used to assess whether the equation of best curve fit for one group was applicable to the respective comparator group (Prism, version 5; GraphPad). Probit analysis was used to determine log10 concentration of agonist required to induce 25%, 50%, and 75% of maximal platelet aggregation (logEC25, logEC50, and logEC75, respectively).

Where there was a significant between-group difference in the F test for a given agonist, differences in platelet reactivity were assessed further. When graphs exhibited between-group differences in platelet aggregation at midrange concentrations, logEC25, logEC50, and logEC75 were determined. When the graphs demonstrated between-group differences in platelet aggregation at maximal and sub-maximal concentrations, differences in platelet aggregation between groups at those specific concentrations were determined. Where the graphs demonstrate between group differences at midrange and maximal concentrations, both sets of analyses were employed. Nonparametric Mann-Whitney Utest was applied for comparison between groups with a P value of 0.05 was regarded as significant (SPSS, Windows version 10.0). Results of flow cytometric parameters were reported as median (interquartile range). Time courses were tested with Friedman test followed by Wilcoxon rank-sum test. To corroborate these analyzes, ANOVA was performed for repeated measures, which always confirm the results of the non-parametric tests. Spearman correlation test was used to correlate platelet percentage P-selectin expression and percentage maximum platelet aggregation values with viral loads and CD4.

CHAPTER FOUR

4.0 RESULTS

4.1Platelet activation

A total of 28 participants were enrolled in this study; 14 adults HIV positive HAART naïve patients and 14 HIV negative controls from blood donors. The groups had similar age and sex distributions. The HIV group had a slightly lower platelet (PLT) count (mean 285.1±64.91) compared to the HIV negative group mean (310.0±75.07), however this was not statistically significant (P = 0.1428). On analysis, the maximum percentage P-selectin expression was 1.5 times higher in HIV positive than in HIV negative which was significant (P=0.001) (Table 4.1).

Table 4.1 Biographical and haematological data of participants in platelet aggregation group

PARAMETER	HIV NEGATIVE GROUP (n=14)	HIV POSITIVE GROUP (n=14)	P-VALUE
Median AGE (years) (IQ)	28 (24–35)	29 (26–44)	0.0581
Gender M:F	6:8	7:7	
PLT COUNT (mean ±SD)	310.0±75.07	285.1±64.91	0.1428
%CD62P (median, IQ)	21.1 (2.7-46.2)	34.5(10.3-78.36)	**0.001**
CD4 COUNTS cells/mm3 (median, IQ)	876[605.5-864.5]	386 [328-516]	**<0.0001**
mean viral load Log $_{10}$(copies/ml)	-	3.01±0.82	**-**

4.2 Platelet Aggregation.

A total of 20 participants were enrolled in this group. Ten adults HIV positive patients not on treatment and ten HIV negative participants from blood donors. The groups had similar age and sex distributions. The HIV group had a slightly lower platelet (PLT) count (mean 389±106) compared to the comparison group mean (759.6±214), however this was not statistically significant (P = 0.4629) (Table 4.2).

Table 4.2 Biographical, haematological and P-selectin profile of participants in platelet activation group

Parameter	**HIV Negative**	**HIV positive**	**p-value**
No' in group	10	10	
Median Age (IQ)	33(29-44)	37(32-51)	0.0618
Gender (M:F)	5:5	5:5	
Platelet count (mean+/-SD)	310±21.4	226.8 ± 17.0	0.4629
CD4 counts (Mean+/-SD)	759.6±214	389±106	***0.0012**
$\log_{10}$ viral load	-	9±0.4	-

On analysis, platelet aggregation showed a significant higher response to collagen compared to arachidonic acid and ADP (P=0.00087, P=0.00056 and P=0.019) respectively. Importantly, the HIV positive group showed a lower maximum percentage platelet aggregation compared to HIV negative group (Collagen:75±4.8% cases vs 80.4±6.7% controls at 10µg/ml; AA 64±8.1% cases vs 88.4±6.2% controls at 10µg/ml; ADP 60.7±6.7% cases, 71.1±8.1%controls at 5µmol/l) (Table 4.3).

Table 4.3 Platelet aggregation parameters

Agonist	**measured platelet aggregation parameter**	**HIV negative** n=10	**HIV positive** n=10	**p-value**
Adenosine diphosphate(ADP) (µM)	% Max aggregation	71.1±8.1	60.7±6.7	*0.019
	lag time	0.19 (0.03-0.39)	0.51(0.11-0.60)	**0.0001
	Slope	73.51±9.26	59.50±28.65	**0.00212
Arachidonic acid(AA) (mg/ml)	% max aggregation	88.4±6.2	64±8.1	**0.00056
	lag time	0.29(0.09-0.03)	0.22 (0.09-1.03)	*0.024
	Slope	85.22±26.20	113±34.98	*0.0030
Collagen (mg/ml)	% max aggregation	80.4±6.7	75±4.8	**0.00087
	lag time (sec)	0.35(0.03-1.10)	0.12(0.07-0.16)	*0.00328
	slope (% aggregation/sec)	76±5.39	72±26.41	*0.0182

Significant values *p≤0.05

****P≤0.001**

4.3 Correlation of platelet function with viral load and CD4 counts in adult HIV-1infected patients.

4.3.1 Correlation of maximum %CD62P expression with viral load and CD4 count in HIV 1infected patients.

P-selectin showed a positive correlation with viral load (r=0.634, P= 0.019), though the strength of the correlation was weak. Interestingly, in HIV positive participants, P-selectin expression neither correlated with CD4 T-cell count nor was it significant (r=-0.532, P= 0.7229).

(a) **(b)**

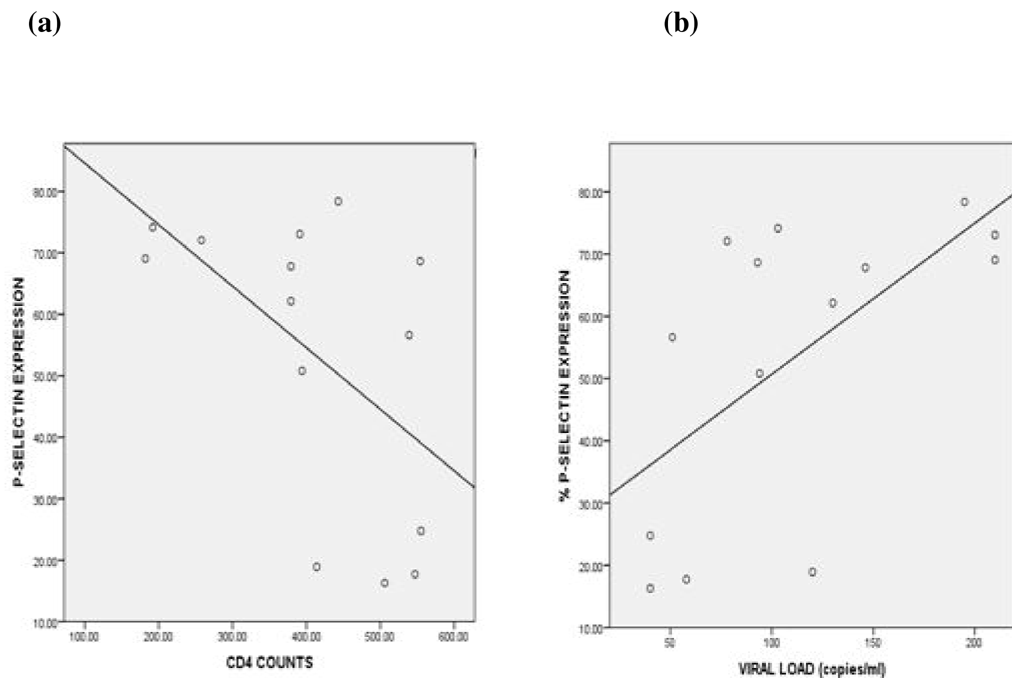

Figure 4.1 HIV group Correlation between % CD62 expression and markers of disease progression CD4 and viral load. P-selectin showed a negative correlation with CD4 counts (a) and a positive correlation with viral load (b).

4.3.2Correlation of maximum percentage platelet aggregation with viral load in respect to different agonists for the HIV positive group

All the agonists showed an inverse relationship with viral load: Collagen (r=-0.4663, P=0.0177) (a), ADP (r=-0.286, P=0.0424) (b) and Arachidonic acid(r=-0.3, P=0.0259) (c)

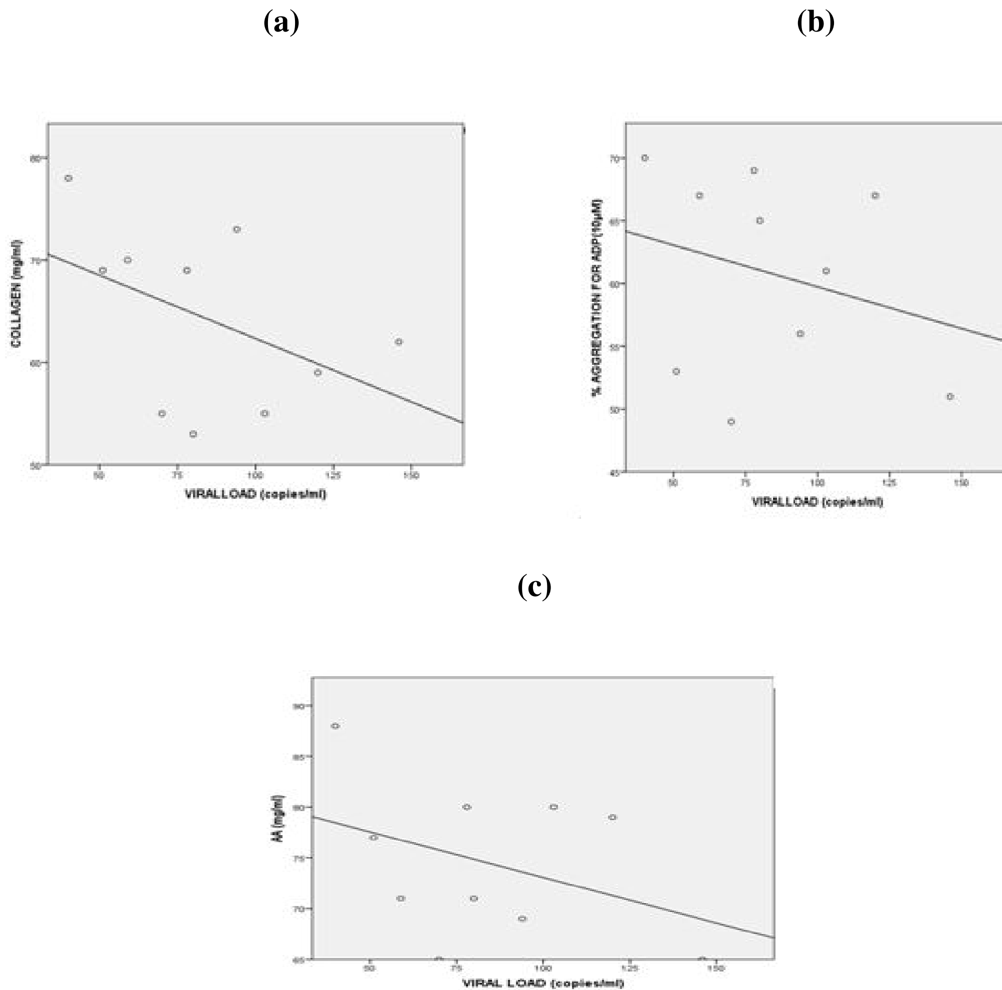

Figure 4.2 HIV group correlations between maximum percentage platelet aggregation in respect to different agonists with viral load. Maximum percentage platelet aggregation negatively correlated with CD4 counts for all agonists Collagen (a), ADP (b), Arachidonic acid (c).

4.3.3 Correlation of percentage maximum platelet aggregation with CD4 counts in HIV-1infected patients

All the agonist showed a positive correlation with CD_4 counts: ADP (r= 0.614, P=0.0528) (a), collagen (r=0.384, P=0.0273) (b) and Arachidonic acid (r=0.850, P=0.002) (c).

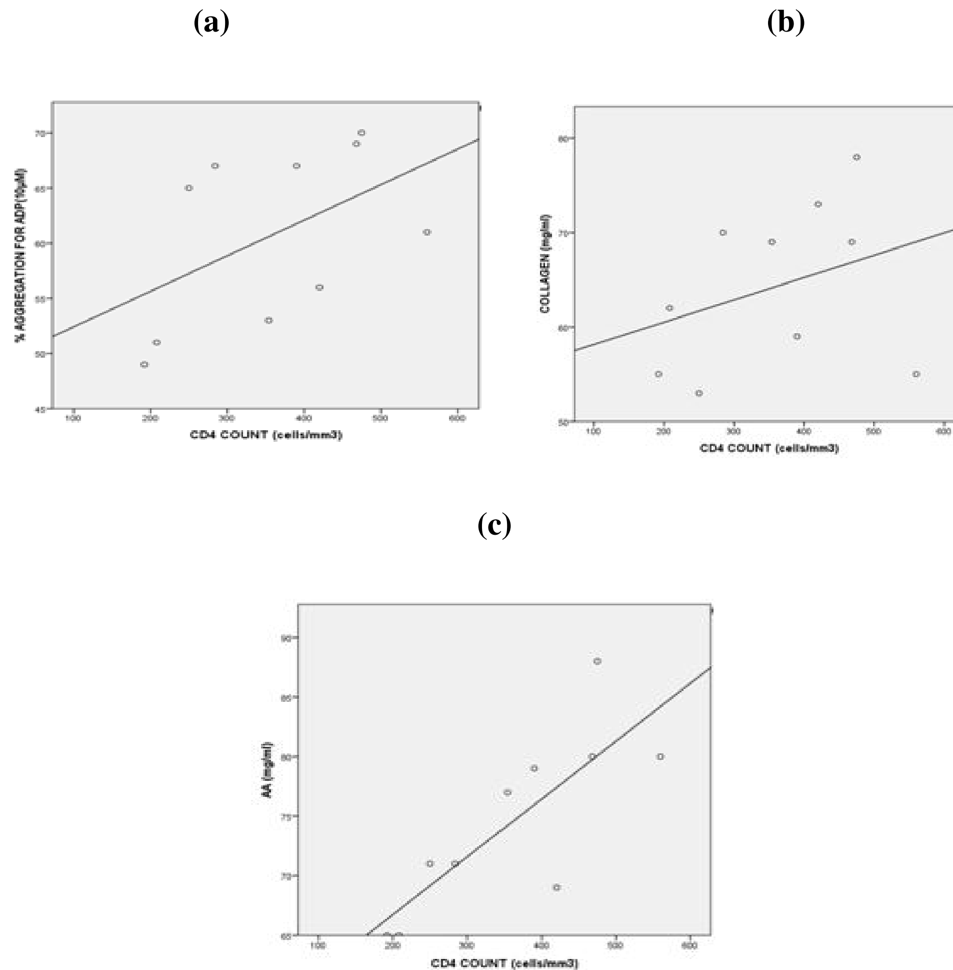

Figure 4.3 HIV group correlations between maximum percentage platelet aggregation in respect to different agonists with CD4 counts. Maximum percentage platelet aggregation positively correlated with CD4 counts for all agonists ADP (a), Collagen (b), Arachidonic acid (c).

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1.1 Discussion

The present study aimed at evaluating platelet function (activation and aggregation) and correlating it with classical markers of disease progression in the context of untreated HIV infection. This is very important with the continued rise in the prevalence of thrombotic cardiovascular events among HIV infected patients in a developing country like Kenya. A total of 28 adult participants were recruited into the study; fourteen HIV positive HAART naïve patients and fourteen HIV negative controls from blood donors. Participants in both groups were of similar age and sex.

In this study, HIV group had a slightly lower mean platelet count compared to the control group mean however, this was not statistically significant. This study compares to a report by Parinitha and Kulkarni which found that thrombocytopenia occurred in 45/250 (18%) of the HIV positive cases (Parinitha & Kulkarni, 2012). The mechanism of thrombocytopenia in HIV infection appears to involve increased platelet destruction and ineffective platelet production in the course of the viral infection. Most studies indicate that there is significant platelet sequestration and destruction in the spleen in HIV-associated thrombocytopenia. Platelet destruction is predominant early in the course of disease, whereas decreased platelet production is the predominant factor later in the course of the disease (Vaughan, 2010). Megakaryocytes have been shown to express a functional CD_4 and CXCR4 molecules thus susceptible to infection by HIV. Studies involving megakaryocytes from HIV-infected patients have shown viral RNA

and proteins suggesting that these cells are infected in vivo. This causes a marked defect in platelet production among HIV patients (Parinitha & Kulkarni, 2012; Vaughan, 2010).

In addition immunologically mediated platelet destruction has been well documented in HIV patients. Immune complexes containing anti-idiotype-like antibodies against anti-HIV-1 GP120 could bind to platelets of HIV infected individuals and increase their bridging with cells of the monocyte/macrophage system therefore facilitating their immune destruction (Chabert *et al*., 2015). Secondly Evidence suggests molecular mimicry between HIV and platelet proteins might be a possible mechanism in HIV associated thrombocytopenia. In HIV related classic immune thrombocytopenia, the occurrence of cross reactive antibodies against platelet immuno-dorminant epitopes GP IIb/IIIa complex and HIV-1 GP120/160 has been described in HIV-1 infected patients (Assinger, 2014). Other causes include marrow infiltration by opportunistic infection or lymphoma and myelosuppressive effects of drug therapy (Li *et al*., 2012).

5.1.2 P-selectin expression levels

Importantly, the study observed that percentage platelet P-selectin expression (CD62P) levels were significantly elevated in HIV positive patients compared to HIV negative controls (table 4.1). This is similar to previous findings reported by Mayne *et al.* (Mayne *et al*., 2012) where the authors reported up-regulation of platelet P-selectin expression during HIV infection. A similar observation was also reported by Nkambule *et al.* (Nkambule *et al* 2015), in which the study demonstrated that elevated levels of

activated platelets in HIV positive ARV naïve individuals correlates with indices of immune activation T cell activation (CD8+) and viral load.

The current study suggests a relation between HIV viral RNA levels, platelet counts and platelet activation in untreated HIV infection. It is possible that in untreated HIV infection there is high viral load causing intense immune activation. This leads to high thrombo-inflammatory conditions that consequently activate the platelets. Furthermore, persistent platelet activation during HIV infection may be induced by direct binding and engulfment of HIV virions by platelets (Nkambule *et al*., 2015). HIV directly binds to platelets via GP 120 which interacts with CXCR4 and the fibronectin receptor expressed on platelets (Assinger, 2014; Mayne *et al*., 2012). Previous studies have described mechanism of engulfment involving the endocytosis of HIV particles into endocytic vesicles. This is followed by the degradation of viral particles in an acidic compartment of the vesicle. However the fate of these endocytosed viral particles in the platelets is not fully understood. These interactions may result in platelet activation and alterations in platelet morphology (Nkambule, 2015).

From the present study, increased levels of circulating activated platelets in the case of untreated HIV infection has been clearly demonstrated. Given the role of platelets in thrombus formation preceding myocardial infarction, the clinical observation of increased platelet activation in treatment naïve patients provides a plausible mechanism for increased rates of thrombotic events.

5.1.3 Optical Platelet aggregation

The study also assayed platelet responses using three agonists (ADP, arachidonic acid and collagen) to induce platelet aggregation. Notably in both groups, an increased platelet aggregation was observed in response to collagen and arachidonic acid whereas platelet aggregation was reduced in response to ADP. This is consistent with a recent study by satchel *et al.* (Satchell *et al*., 2010)which demonstrated that collagen and arachidonic acid as potent inducers of platelet aggregation while ADP is a weak agonist. Induction of platelet aggregation by collagen and arachidonic acid is mediated through GPVI receptor activation, causing a conformational change in the GP IIbIIIa receptor on the platelet surface. This converts the GP IIbIIIa receptor from a low-affinity state to a high-affinity state through inside-out signalling. The consequence is alpha granule release to produce ADP and thromboxane sustaining GPIIb/IIIa activation thus increasing platelet aggregation. On the contrary ADP-induced platelet aggregation is mediated through the P2Y12 receptor which is a weak agonist hence, alpha granule release is not mediated resulting in non-amplification aggregation.

Interestingly, a markedly reduced percentage maximum platelet aggregation in HIV positive group compared to HIV negative control group was recorded. These findings are consistent with Satchel *et al*. (Satchel *et al*., 2010) where the authors reported hypo-responsive platelets in HIV positive patients on treatment in response to certain agonists at different doses compared to the HIV negative controls. However this observation

contradicts reports by Nkambule *et al.* (Nkambule *et al*., 2015) in which the author observed hyper-responsive platelets to agonists at different concentrations.

The likely mechanism underlying reduced platelet aggregation observed in HIV positive group could point to molecular mimicry between HIV-1 and a human membrane protein found in platelets. A structural homology has been demonstrated to exist between HIV GP160/120 and platelet surface glycoproteins GPIIb/IIIa involved in platelet aggregation (Assinger, 2014). During HIV infection by way of cross reactivity, patients IgG could bind specifically, through the $F(ab')_2$ portions, to a common epitope of HIV-GPI6O/ 120 and platelet GPllb/Illa (Assinger, 2014; Dominguez, Victoria *et al*., 2004). Finding that antibodies to Virus platelet-like sequences cross react with platelet Virus-like sequences implies their potential obstructive role in the proper function of platelet's GPIIb/IIIa during platelet aggregation.

Previous studies have also indicated that C-type lectin-like receptor 2 expressed on the surface of platelets has been recently identified as an HIV-1 attachment factor (Li *et al*., 2012). It promotes virus capture by platelets, mediates platelet aggregation and has the capacity to promote viral dissemination (Assinger, 2014). The current study elucidates that platelet aggregation is markedly reduced during HIV infection. The extent to which this HIV-associated platelet aggregation subverts the haemostatic machinery involved in platelet reactivity is not known, albeit our results would support a role for HIV

infection disrupting normal platelet aggregation. This is possibly through an altered mechanism involved in receptor or post-receptor signaling during platelet aggregation.

5.1.3 Correlation of platelet function with CD4 counts and viral load

5.1.3.1 Platelet P-selectin correlation with viral load and CD4 counts

Level of percentage maximum P-selectin expression was observed to correlate positively with viral loads and negatively with CD4 counts. The findings suggest that in the case of untreated HIV infection at high viral load, CD4 counts are low consequently increasing platelet activation. The lack of statistical significance of P-selectin expression with CD4 counts at high viral load, points to CD4 counts being a less predictive marker of platelet reactivity. Further studies need to be carried out to clearly observe how the level of CD4 counts and viral load correlate with expression of P-selectin.

5.1.3.2 Platelet aggregation correlation with viral load and CD4 counts

HIV positive group percentage maximum platelet aggregation correlated directly with CD4 and inversely with viral load. This is the first study to demonstrate the correlation between platelet aggregation and markers of disease progression in untreated HIV infection. The data implies that that at very high viral loads there is a massive influx of anti-HIV-1 antibodies that bind to homologous antigenic epitopes of GPllb/Illa (French & Seligsohn, 2000; Satchell *et al*., 2010). This results points to an altered mechanism

during platelet aggregation either at the receptor or post-receptor signaling during HIV infection. The association of increased viral loads with reduced agonist induced platelet aggregation suggests that either or both of these aforementioned functions being potentially compromised in HIV-infected patients.

Strikingly, the current study observed a discrepant platelet function in HIV, whereby platelet activation was observed to be increased but platelet aggregation was decreased. This is consistent with a previous study by Haugaard et al., where the author documented of a discrepant platelet function in untreated HIV infection (Haugaard *et al*., 2013). In a typical physiological reaction, platelet activation precedes platelet aggregation. During platelet aggregation GPIIb/IIa gets activated, undergoes a conformational change and dimerization causing expression of its adhesive domain on which soluble fibrinogen binds. Fibrinogen binding allows formation of platelet-platelet interaction via the fibrinogen bridges (French & Seligsohn, 2000). This current study suggests that due to molecular mimicry anti-HIV antibodies bind to GPIIb/IIa, hindering dimerization of the glycoprotein and consequently affecting platelet aggregation. Therefore though the platelets are activated it does not necessarily mean that the reaction will end in platelet aggregation.

The study was done contextually at Kenyatta National hospital, and being a single centre, the context may not allow for generalization of the study results since the populations may be different. Also the case control design of the study limits the ability to determine a definitive causative relationship between platelet function and HIV-1

infection. In addition, the small sample size prevents us from exploring in-depth associations in multivariate analyses on the observed differences in platelet function.

This was an in-vitro study and not equivalent to in-vivo because of the absence of endothelium. Other aspects of platelet interaction were not investigated such as Von willebrand factor adhesion to endothelium as well as platelet-leukocyte aggregates. Therefore the study cannot be extrapolated to clinical situations.

Use of whole blood during platelet P-selectin (CD62p) flow cytometric analysis minimized any artifactual in-vitro platelet activation through such processes as centrifugation of blood samples thus mimicking platelet in-vivo conditions. However use of whole blood limits differentiation between membrane bound P-seletin and soluble P-selectin molecules in plasma.

The study generally focused on platelets to the exclusion of other components of the haemostatic system. This may affect its translational application in etiology of in-vivo thrombosis. Nevertheless, the findings from the study provide a baseline to direct further research on platelet function in HIV.

5.2 Conclusions

i. The study confirmed that platelet P-selectin expression is increased in HIV positive patients.

ii. A decreased platelet aggregation in HIV positive patients was clearly demonstrated contrary to increased activation.

iii. P-selectin was found to correlate positively with viral load but no correlation with CD4 counts.

iv. Platelet aggregation correlated inversely with viral load but positively with CD4 counts.

5.3 Recommendations

5.3.1 Policy

i. The Ministry of health should consider including P-selectin as a marker of platelet dysfunction in HIV diagnosis.

5.3.2 Further research work

i. Longitudinal prospective clinical and in-vitro studies of platelet function in HIV patients both pre and post initiation of HAART is required to further elucidate underlying mechanisms.

ii. To correlate laboratory findings to clinical presentation

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APPENDICES

Appendix 1: KNH/UON ERC Approval

Appendix 2: Graduate school proposal approval