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Acute HIV infection in non-subtype B populations:

Viral dynamics, laboratory staging and early detection

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Abstract:

Background: Acute HIV infection is a field of intense study due to the high probability for transmission as well as possible beneficial health outcomes with early treatment initiation. While detection of acute HIV infection remains a challenge, newer methodologies are available that reduce the detection window.

Methods: Individuals at high risk of HIV infection from Tanzania, Uganda, Kenya and Thailand were screened twice-weekly by a highly sensitive HIV-1 qualitative RNA assay. Following a reactive RNA test, serial samples were collected twice-weekly for the first month, then less frequently. Serial samples were assayed for viral load, immune phenotype and by a panel of diagnostics tests.

Results: Evaluation of viral dynamics showed that viral load upslope and post-peak down slope were not correlated with set point viral load or disease outcome. Peak viral load was weakly associated with set point, which was established within the first 42 days of infection. CD4 absolute counts declined and CD8 counts rose coincident with peak viral load; neither subset returned to initial levels. Regional differences in viral load and lymphocyte parameters were observed. Higher viral load set point and lower CD4 counts at one year were associated with disease progression. A 4th generation EIA reduced the detection window by 8 days from a 3rd generation EIA. The Determine Combo Rapid test performed poorly in detecting acute infection. The new U.S. CDC HIV algorithm performed well in acute infection detection, although a window of detection remains. A new acute infection staging system was proposed to replace the outdated Fiebig staging system and incorporate newly available diagnostic tests.

Conclusions: Events within the first month of HIV infection play a role in disease outcome. Detection of acute infection has improved, however additional advances are required to further reduce the infection window of detection.

Key words: Acute HIV infection, HIV diagnosis, Viral load dynamics, Lymphocyte absolute counts

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ABBREVIATIONS

4th gen	4th generation EIA
3rd gen	3rd generation EIA
Ab	Antibody
Abs Cnt	Absolute Count
ACASI	Audio Computer Assisted Self Interview
ACD	Acid citrate dextrose
AFSSAP	L'Agence française de sécurité sanitaire des produits de santé
AHI	Acute HIV infection
AIDS	Acquired Immunodeficiency Syndrome
Ag	Antigen
ARS	Acute Retroviral Syndrome
ARV	Antiretroviral
CD	Cluster of Differentiation
CDC	Center for Disease Control
CO2	Carbon Dioxide
Combo RT	Determine Combo Rapid Test
CRF	Circulating Recombinant Form
DNA	Deoxyribonucleic acid
EA	East Africa
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EIA	Enzyme Immunoassay
Env	Envelope
FDA	Food and Drug Administration
gp160	Glycoprotein 160
gp41	Glycoprotein 41
HDRL	HIV Diagnostics and Reference Laboratory
HAART	Highly Active Anti-retroviral Treatment
HBsAg	Hepatitis B surface Antigen
HSV	Herpes Simplex Virus 2
HIV	Human Immunodeficiency Virus
HTLV	Human T-lymphotropic Virus Type I
HLA	Human Leukocyte Antigen
IA	Immunoassay
IATA	International Air Transport Association
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IQR	Interquartile Range
IRB	Institutional Review Board
kD	Kilodalton
LAV	Lymphadenopathy-associated virus
LBV	Large Blood Volume
LTNP	Long term non-progressors
MARPS	Most At Risk Populations
MHRP	United States Military HIV Research Program

mL	mililiter
MSM	Men who have sex with men
NAAT	Nucleic acid amplification test
NK	Natural Killer
nm	Nanometers
PBS	Phoshate Buffered Saline
pg	Picograms
pol	polymerase
RDT	Rapid Device Test
RNA	Ribonucleic acid
SBV	Small Blood Volume
s/co	Signal to Cutoff
SIV	Simian Immunodeficiency Virus
STI	Sexually Transmitted Infection
TG	Transgender
TH	Thailand
UNAIDS	Joint United Nations Programme on HIV/AIDS
U.S.	United States
VL	Viral Load
WRAIR	Walter Reed Army Institute of Research
WB	Western Blot
WHO	World Health Organization

1. Introduction

1.1. Global History of HIV

Acquired Immune Deficiency Syndrome (AIDS) was first described in a group of homosexual men and intravenous drug users who acquired Pneumocystis carinii Pneumonia [1, 2], an infection which was typically seen in individuals with extreme immnosuppression. In 1981, a virus was isolated from a lymph node of an individual who was in the early stages of AIDS [3]. The virus belonged to a group of T-lymphotropic retorviruses known as human T-cell leukemia virus (HTLV), however the virus core proteins were found to be immunologically distinct form HTLV and thus were named, lymphadenopathy-associated virus (LAV). In 1983-4 Robert Gallo's group provided evidence suggesting HTLV-like viruses could be isolated from peripheral blood lymphocytes of patients exhibiting AIDS like symptoms[4, 5]; thus naming the virus, HTLV-III. Both of these viruses, HTLV-III and LAV, were renamed Human Immunodeficiency Virus (HIV). In 1986, HIV-2, found in West Africa, was isolated and also found to cause AIDS [6-8].

In the 2013 Joint United National Programme on HIV/AIDS (UNAIDS) report, it was reported that approximately 35 million people are living with HIV/AIDS across the globe. New HIV infections throughout the world peaked in 1996, while deaths from HIV/AIDS peaked in 2005 [9]. Although there was a decline in new infections in 2012 compared to previous years, there were still 2.3 million new infections throughout the world [10]. Death rates related to AIDS have also declined over the years, however 1.6 million deaths were still reported in 2012 [10]. Sub-Saharan Africa remains the most affected region in the world, with 70% of the world's new infections reported in 2012 [10]. South Africa remains disproportionately impacted by HIV/AIDS with a prevalence of 12.2% in 2012 and 1.2 million new infections reported between 2008 and 2012 [11].

1.2. Origin of HIV

HIV belongs to the genus Lentivirus (meaning slow growing virus) and family Retroviridae. There are two species of HIV, HIV-1 and HIV-2, with a 60% genetic homology in the conserved regions between the two species. HIV-1 demonstrates higher virulence and infectivity compared to HIV-2 and is responsible for most of the HIV infections throughout the world. HIV originated from zoonotic transmission from contact between primates and humans. Through studies of phylogentics, it was found

that HIV-1 originated from Simian Immunodeficiency Virus (SIV) from chimpanzees (Pan troglodytes troglodytes) [12, 13] while HIV-2 originated from the sooty mangabey (Cercocebus atys) [14, 15].

HIV-1 is currently defined by four different groups; M (Major), N (not M and not O), O (Outlier), and the recently discovered Group P [16]. The vast majority of HIV-1 infections throughout the world are due to group M. Within group M, there are at least 9 different clades or subtypes (A, B, C, D, F, G, H, J, and K), sub-subtypes (A1, A2, F1 and F2), as well as numerous circulating recombinant forms (CRF), of which at least 20 have been identified, and contribute to extreme viral diversity of HIV-1 [17]. These subtypes and recombinant forms are generally clustered in geographic areas although all subtypes can be found in Africa [18]. The most prevalent is subtype C, found in Southern Africa, East Africa and India and accounts for ~50% of worldwide HIV-1 infections[17]. Subtype B is the most common subtype in Western Europe and North America. Subtype A is geographically distributed within East and West Africa as well as the former Soviet Union. Subtype D is localized in East and West Africa while the two most common circulating recombinant forms, CRF01_AE and CRF02_AG, are found in Southeast Asia and West Africa respectively [19]. HIV-2 is mainly localized to West Africa and is a relatively minor contributor to the AIDS epidemic due to its lower pathogenicity and less severe disease [20, 21].

1.3. HIV structure

The HIV virion has a spherical shape and a diameter of 80-110 nm surrounded by two layers of lipid molecules [22]. These lipid molecules are derived from the host cell and throughout this lipid bilayer are viral proteins called the envelope. Each virion contains two copies of a positive sense Ribonucleic Acid (RNA) that are contained within a conical core called the capsid. The approximately 9 kilobase RNA genome encodes structural, regulatory and accessory proteins. These structural proteins are the targets of diagnostic assays, vaccines and antiviral drugs and are described in more detail below. The regulatory and accessory proteins (vif, vpu, vpr, tat, rev and nef) contribute to the genetic complexity of HIV-1 and their functions are critical for HIV replication [22].

1.3.1. Envelope

The HIV envelope (*env*) gene codes for the glycoprotein 160 (gp160) protein. The protein is synthesized in the endoplasmic reticulum and is glycosylated in the

Golgi complex. This glycosylation is necessary for infection of host cells. The gp160 protein is later cleaved by host cell proteases to form two smaller proteins, glycoprotein 120 (gp120) and glycoprotein 41 (gp41). Envelope proteins protrude through the lipid bilayer so that gp120 remains on the outside of the virion while gp41 remains embedded in the membrane and serves as an anchor for the envelope complex. The envelope complex exists as trimers on the surface of the virion and allows the virus to fuse with target cells [22].

1.3.2. Gag

The *gag* gene encodes for p55, a 55 kilodalton (kD) protein. The p55 protein is involved in the budding of the virion from the cell and the recruitment of the two copies of viral RNA. After the budding process, viral proteases cleave p55 into several smaller proteins called the matrix protein (p17), the capsid protein (p24), the nucleocapsid (p7/p9) as well as others. p24 forms the capsid around the strands of RNA. There are approximately 2000 molecules of p24 in each virion. The p17 matrix protein is located between the capsid and the viral envelope and is involved in maintaining the integrity of the virus particle. The nucleocapsid proteins p7/p9 are involved in binding the viral RNA [22].

1.3.3. Polymerase

The polymerase (*pol*) gene encodes reverse transcriptase, RNase H, integrase and protease. The reverse transcriptase is the enzyme required to transcribe RNA into Deoxyribonucleic Acid (DNA). RNase H is required to cleave a DNA/RNA hybrid that is formed during DNA synthesis. The integrase protein facilitates integration of HIV genetic material to into the DNA of the infected host cell. Lastly, protease cleaves polyproteins produced in the HIV life cycle such as the gag and polymerase polyproteins [22]. Due to its essential role in HIV replication, the protease was targeted early as an avenue for treatment and many protease inhibitors are currently used in the treatment of HIV.

1.4. HIV life cycle

Cluster of differentiation 4 (CD4) is the primary receptor for HIV-1[23]. CD4 is found on the surface of helper T cells, macrophages and dendritic cells. The trimeric gp120 on the virion binds to CD4 on the target cells. Binding of the envelope to CD4 triggers a conformational change in the envelope that allows for interaction and binding to chemokine receptors (CXCR4 or CCR5) on the target cell. gp41 undergoes a

conformational change which ultimately results in the fusion of the target cell membrane with the membrane of the virion [22]. The HIV RNA genome and several HIV enzymes (reverse transcriptase, integrase, ribonuclease, and protease), enter the cytoplasm of the host cell where the viral RNA is transcribed using the reverse transcriptase enzyme into a complementary strand of DNA, then double stranded DNA. The double stranded DNA enters the nucleus of the cell and integrates into the host DNA were it may remain dormant in latently infected cells [22]. This HIV DNA is referred to as provirus and replicates when the cell divides. New RNA is produced in the nucleus of the host cells and is transported out of the nucleus. Viral RNA transcription is most efficient and rapid in activated CD4+ T cells [24]. Some of this RNA is translated and processed using host mechanisms to produce proteins that are necessary for assembly of new virus particles. The viral RNA and proteins gather at the surface of the cell and new HIV particles bud out of the cell, using the cell membrane to form the lipid bilayer of the virion. After release from the cell, the virion matures by using virus proteases to release individual HIV proteins [22].

HIV replicates rapidly, with over a billion virus particles made per day in the height of HIV infection [25]. The reverse transcriptase that copies the RNA into DNA before integration has a very high error rate of ~0.2 errors per genome per replication cycle [26] and RNA polymerase II, a host enzyme needed for replication, also introduces mutations to a lesser extent [27]. The error rate in conjunction with a high replication rate results in an accumulation of different, but closely related viruses, referred to as viral quasispecies; thus, contributing to explosive viral diversity. Another factor that contributes to viral diversity is recombination, due to reverse transcriptase switching between alternative RNA genomes during viral replication when multiple viruses infect a cell [28]. The viral diversity and constant, dynamic evolution of the HIV virus contributes to its ability to evade the immune systems and also presents a significant challenge for vaccine development.

1.5. Transmission

The main route of transmission of HIV is through sexual intercourse via the genital or rectal mucosa, accounting for the vast majority of infections throughout the world. Other routes of infection include mother-child transmission in-utero or through breast milk, intravenous drug use, and blood transfusions. Detailed sequencing studies have shown that 80% of mucosal transmissions are caused by infection from a single virus [29]. Infection at the genital or rectal mucosa targets CCR5 positive CD4+ T cells and

dendritic cells [30], which transport the virus from the mucosa to lymph nodes where additional target cells can be found. This period between infection and dissemination in the plasma is called the eclipse phase of infection, where infection has occurred, but virus is not yet detectable within the plasma. This phase is thought to last for approximately 10 days [31]. Virus continues to replicate at a furious pace, before the innate and adaptive immune responses are able to control viremia. Virus is transported to the lymph nodes, where it encounters additional CCR5 CD4+ T cells and further viral replication takes place [32, 33]. Peak viremia is thought to occur approximately 21-28 days after infection, after which plasma viral load decreases to a set point level[32]. During the time of peak viral replication, virus is disseminated throughout the body and specifically targets the CD4+ T cell rich gut associated lymphoid tissue. The gut associated lymphoid tissue is one of the main targets for HIV, resulting in massive destruction of CD4+ T cells in the gut in the initial stages of infection [34]. During peak viral replication, CD4+ T cells are rapidly depleted in the periphery, but also in the immune effector sites of the body including the gut, lung and genital tract [35]. SIV models have shown that HIV depletes 50% of memory T cells directly by viral infection during the acute phase of infection [36], however killing also occurs via indirect mechanisms, such as apoptosis [37]. The timing of the dramatic depletion of CD4 T cells with the peak of viral load suggest that the availability of target cells, in addition to cellular immune responses, may play a role in decrease viral load after peak and determination of viral load set point [38]. CD4+ T cells in the periphery rebound somewhat after the depletion in acute infection, however the CD4+ T cells in the gut do not rebound [34]. During the time of early infection, the viral reservoir in the lymphoid tissues and CD4+ T cells is established [39].

1.6. Progression to AIDS

In many cases of HIV infection, the progression to AIDS in treatment naïve individuals may not occur until 8-10 years after infection. After a time of clinical quiescence that varies based on a range of factors, viral load begins to increase and CD4+T cells in the periphery begin to decline. CD4+ T cells are critical components of the immune system that direct other cells of the immune system to provide the appropriate immune response against invading pathogens. CD4+ T cells are killed directly by virus either by lysis of the cell due to large amounts of virus budding out of the cells causing disruption of the membrane, or by interference with normal cellular activity due to hijacking of the host cell machinery. Other causes of CD4+ T cell death include apoptosis of uninfected bystander cells and killing of infected CD4+ T cells by

cytotoxic CD8+ T cells [40]. CD4+ T cell depletion will eventually lead to severe immunodeficiency, leaving infected individuals susceptible to opportunistic infections that are a hallmark of AIDS.

In some cases, progression may be delayed or accelerated based on the genetics of the HIV infected individual. For example, individuals with a heterozygous deletion of 32 base pairs their CCR5 gene, CCR5- Δ32, demonstrate a delay in progression to AIDS of up to 2 years [41], while individuals with certain Human Leukocyte Antigen (HLA) types, such as HLA-B*57 and HLA-B*27, also show delayed progression to AIDS [42]. Conversely, individual with HLA-B*35 tend to have accelerated progression to AIDS [42]. Infecting virus may also play a role in the length of time to progression to AIDS, although the mechanisms are not fully understood. In Uganda, it has been shown that subtype D and multiple or recombinant infections progressed more rapidly than subtype A infections [43]. Similarly, CRF01_AE infections have been shown to progress more rapidly than non-CRF01_AE infections [44]. Co-infections may also play a role in disease progression, especially in the absence of anti-retroviral treatment [45].

Based on these differential disease progression rates, several terms have been applied to the different classes of individuals with differential rates of progression. Rapid progressors are generally defined as individuals who progress to AIDS within 3 years of infection while long term non-progressors (LTNP) are individuals who remain AIDS free for greater than ten years [46]. Within the LTNP classification, which makes up 5-15% of all infections [47], elite controllers are individuals who maintain an undetectable viral load without antiretroviral therapy for at least ten years. Viremic controllers are individuals with detectable viral load, but who maintain viral loads of less than 2,000 copies/ml [46]. Understanding the immunology, host genetics, and virology of early events that may lead to differential outcomes is essential for development of prevention and treatment intervention strategies.

1.7. Definition of AIDS

Both the Center for Disease Control (CDC) and World Health Organization (WHO) have developed clinical staging systems in order to define the progression to AIDS and to assess eligibility for treatment. The WHO system [48] relies on clinical symptoms and manifestation of opportunistic infections in order to allow assessments in developing countries where laboratory technologies, such as CD4 testing, are not available [48].

WHO stage 1 is defined as asymptomatic or persistent generalized lymphadenopathy and Stage 2 is defined as having mild symptoms that may include recurrent upper respiratory infections and mucocutaneous manifestations. Stage 3 presents with more advanced symptoms including persistent weight loss, diarrhea, bacterial infections such as tuberculousis and CD4 count <350 cells/ul if available. AIDS, or Stage 4, involves severe symptoms that can include toxoplasmosis, candidiasis of the esophagus, Kaposi's sarcoma and a CD4 count 200, if available. The CDC staging system relies on CD4 counts and breaks the system into three categories[49]; Stage 1 for CD4 >500 and no AIDS defining illness, Stage 2 for CD4 count between 200-500 and no AIDS defining illness and Stage 3 is CD4 <200 OR AIDS defining illness. There are over 20 different conditions/illnesses that are considered AIDS defining including the examples listed above.

1.8. Treatment

Major progress has been made in the treatment of HIV infection since it's discovery 30 years ago. The implementation of Highly Active Anti-retroviral Treatment (HAART) has shown to dramatically reduce morbidity and mortality in HIV infected patients with low CD4 counts [50]. There are several classes of drugs included in HAART; nucleoside/nucleotide reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, entry/fusion inhibitors, and integrase inhibitors. Multiple drug classes are used in concert in order to interfere with different steps within the HIV life cycle and to prevent development of drug resistance. Guidelines for initiation of treatment differ by country/region. In the United States (U.S.), treatment is recommended in all HIV-infected patients regardless of CD4 count in order to reduce the risk of disease progression as well as for the prevention of transmission [51]. Most developing countries rely on the guidance from the World Health Organization. In June 2013, WHO released updated guidelines supporting initiation of treatment in patients with a confirmed HIV diagnosis and a CD4 count of less than 500 cells/microliter rather than at 350 cells/microliter per previous guidance issued in 2010 [52]. Treatment is also recommended in HIV infected patients with active tuberculosis, Hepatitis B co-infection with severe liver disease, pregnant and breastfeeding women, children less than five years of age, as well as individuals with HIV in a sero-discordant relationship [52]. Recent studies indicate that infected individuals with proper care and treatment can have a similar life-span as an uninfected individual [53]. In developing countries, the cost and access to antiretroviral treatments can be prohibitive however the U.S. government's Presidents

Emergency Plan for AIDS Relief initiated in 2003 has provided 15 billion dollars over the last decade to developing countries for prevention, care and treatment of HIV. Additional complications in the success of treatment include drug side effects, development of drug resistance, and lack of drug adherence leading to emergence of viral resistance.

1.9. Prevention

Numerous prevention strategies, both social and pharmaceutical, have reduced the number of new HIV infections. Social prevention strategies include messages in sexual education, abstinence, use of condoms, and use of needle exchange programs. In Uganda, the "ABC" strategy promotion, Abstinence, Be faithful, and use Condom, may have resulted in an overall decrease in the country's HIV prevalence rates, although the true effectiveness of this strategy remains controversial [54]. In addition to social strategies, medical intervention strategies have been shown to be effective in reducing HIV acquisition. For example, several studies demonstrate that HIV uninfected individuals at risk of HIV infection can greatly reduce their chances of becoming infected by taking daily anti-retrovirals [55, 56]. Pre-exposure prophylaxis is approved in the U.S. as a prevention method, but roll out within the developing world has proved to be challenging. An additional prevention strategy termed "Treatment as Prevention" involves treatment of HIV infected individuals regardless of CD4 count or WHO/CDC stage as suppression of viral loads has been shown to reduce HIV transmission rates. In 2011, Cohen et al. showed that early treatment of HIV infected partners in sero-discordant couples reduced transmission by 96% [57]. Similarly, a recent community level study in South Africa showed that HIV acquisition risk was significantly decreased in a community with high levels of treatment coverage [58]. Microbicides have been widely studied as a prevention method targeted for resource poor settings. Although study results to date have been inconclusive, there are 23 microbicide products in various phases of clinical trials [59]. Medical male circumcision has been a successful prevention strategy [60-62] leading to an overall risk reduction of 58% [63] and implementation is currently occurring throughout the developing world. In 2009, the first evidence of a successful vaccine to prevent HIV-1 was also described although the efficacy was modest at 30% [64]. Although efficacy in the overall study was minimal, a post-hoc analysis indicated an efficacy of 60.5% for the first year [65]. Studies with this candidate vaccine are ongoing. While these prevention strategies are promising, none provided complete protection and with the

exception of male circumcision may be difficult and expensive to roll out and scale up, especially in the developing world.

1.10. Acute infection

There are several definitions of acute HIV infection, with the most common being the time period between infection and the first detectable HIV specific antibody response [31]. During the time of high viral replication in early HIV infection, approximately 65% of individuals display clinical symptoms resembling mononucleosis. Common signs and symptoms of HIV-1 primary infection include fever, myalgia, lymphadenopathy, headache, nausea, diarrhea, vomiting, and rash [66]. In most cases these clinical symptoms decline after viral load decreases towards set-point. A few studies suggest that the level of viremia in acute infection is associated with clinical symptoms and also viral load set point [67]. Acute HIV infection (AHI) is the focus of intense study for many reasons. First, HIV transmission is more likely during acute infection due to the high level of HIV viremia and higher transmissibility of virus [68-71]. One study reported that half of the observed transmissions in a study in North America were the result early infections [68] while other studies have shown that individuals in AHI are 10-30 times more likely than chronically infected individuals to transmit HIV infection [69, 71]. Interventions during this highly infectious period could have a major impact on the spread of HIV. Secondly, new studies have shown that treatment in acute infection may reduce the viral reservoir and potentially impact the clinical course of disease [72-74]. Lastly, studying individuals in acute infection provides an opportunity to understand transmission events such as characterization of the infecting virus and innate immune responses [32]. Understanding early events in acute infection and their role in disease progression may lead to improved targets for prevention, intervention, and treatment.

1.11. Viral dynamics during HIV infection

Several studies have attempted to describe the relationship of HIV-1 viral load with long-term health outcome. In several studies conducted during the 1990's, RNA was found to be a stronger predictor of time to AIDS and disease progression [75, 76]. Most studies have focused on relationships of viral load peak, nadir and set-point to disease outcome. Viral load peak is defined as the highest measured viral load while viral load nadir is the lowest measured post-peak viral load in early infection. Set point viral load is generally defined as the steady state viral load obtained after early infection, within 4 months to one year [77-80]. In a study where viral load modeling

was implemented, peak and nadir viral loads were found to be associated with viral load at 6 and 12 months[81]. Rapid post-peak viral clearance has also been associated with lower viral load set point and delayed progression to AIDS [82], while higher viral load set points have been shown to be associated with faster disease progression [75, 83]. In addition, several studies have shown that the first measured RNA levels are predictive of disease outcome [83, 84]. The limitation of most studies investigating early events after HIV infection has been inaccurate estimations for the date of sero-conversion and as well as varying definitions of viral load parameters due to large gaps in sampling intervals. In addition, most studies have been conducted in subtype B populations. Due to the difficulty in studying early events during HIV infection in humans, simian immunodeficiency virus/primate models have been used extensively to dissect early events of AIDS virus infection due to its resemblance to HIV. Several SIV studies have demonstrated viral dynamics and relationship to health outcome with varying results. Studies have shown associations of outcome with levels of early viral load [85], post-peak viral load decline [86], and steady state post-peak viral load [87].

1.12. Lymphocytes subsets in HIV infection

The immune response to HIV involves both the humoral and cellular arms of the immune response and is mediated by several lymphocyte classes including adaptive components (CD4, CD8 and B cells) as well as innate components such as Natural Killer (NK) cells. Monitoring of total immune cells has been shown to be clinically useful in monitoring the general immune status of individuals as well as for monitoring anti-retroviral treatment and immunosuppressive therapy [88]. CD4+ T cells are also known as T helper cells due to their role in signaling other immune cells by secretion of cytokines and soluble factors that stimulate CD8+ T cells, B cells and other immune cells. The loss of CD4+ T cells in AIDS in the absence of treatment leads to incompetence in immune cell subsets that eventually leads to immunodeficiency with subsequent acquisition of opportunistic infections. It has long been known that levels of CD4+ T cells, as the targets of the HIV virus, are predictive of the development of AIDS [89, 90]. CD8+ T cells are also known as cytolytic cells that kill infected or damaged cells. HIV-specific CD8+ T cell responses are detected in acute infection around the time of peak viremia and have been associated with control of virus [91, 92]. B cells are responsible for production of antibodies against foreign antigens and also play a role in presenting antigens to T cells. Dysregulation of B cells can result in autoimmune disease, however their role in controlling HIV viremia remains unclear.

Early studies have shown B cell hyperactivation and dysfunction in patients with AIDS [93]. In addition, recent studies have shown that HIV infection causes an expansion and accumulation of T follicular helper cells in the lymph node, which leads to, altered B cell differentiation and activation [94, 95]. NK cells are cytotoxic lymphocytes involved in the innate arm of the immune system that provide the first responses to virally infected cells through both cytolytic activity and secretion of cytokines involved in the inflammatory response. NK cells deficiencies, although rare, result in a fatal infection during childhood, indicating their importance in the immune system [96]. While it is clear that NK cell dysregulation occurs during HIV infection, as with B cells, the role of NK cells in controlling viremia is not known [97].

1.13. Diagnosis

A significant proportion of HIV infections throughout the world are undiagnosed. Even in the United States, up to 20% of infected individuals are not aware of their HIV infection status [98] while 60% of infected individuals in resource limited settings are undiagnosed [99]. Individuals unaware of their infection status are 3 to 7 more times likely to transmit HIV infection than those who know their status [100] and in the U.S., an estimated 44-66% of new infections are transmitted by undiagnosed individuals [100, 101]. There are multiple reasons for the large number of undiagnosed infections. In resource-limited settings, the most common issues are access to counseling and testing facilities as well as fear of a diagnosed HIV infection and the associated stigma following a positive test. Adding to the issue are difficulties in diagnosing acute HIV infection, as the most commonly used HIV tests are unable to detect the earliest stages of infection.

The diagnosis of HIV infection is achieved through detection of several common markers in whole blood, serum, or plasma of HIV infected individuals including viral RNA and/or DNA, p24 antigen, and antibodies specific to HIV proteins. Not all markers are detectable throughout the entire course of infection. The time to detection of each of the markers can be variable due to host genetics, infecting virus, as well as the test method employed. In addition, the sensitivity of various assays can differ among vendors and technologies.

1.13.1. NAAT

The first marker of HIV infection detected in the plasma is viral RNA and this is detected by nucleic acid amplification testing (NAAT). While NAAT testing is

extremely informative, the test methods are highly complex, labor-intensive, and expensive. NAAT testing is not considered a realistic testing option in many areas in the developing world, although in some countries, a few specialized, central laboratories support NAAT testing.

1.13.1.1. HIV-DNA

In most cases of HIV infection, regardless of treatment status, proviral HIV DNA is detectable in whole blood or peripheral blood mononuclear cells. There is no highly defined time line for detection of this marker in AHI. HIV DNA tests detect both cellular and integrated DNA and are both qualitative and quantitative. The assay can be a useful in determining infection status, as it will detect proviral HIV DNA, even in those with undetectable viral RNA or serological markers. HIV DNA is also routinely used in HIV diagnosis of infants and is employed in complicated diagnostic cases. HIV DNA tests, however, are not commonly used in standard HIV diagnosis in the U.S. as there are no Federal Drug Administration (FDA) approved HIV DNA tests however national guidelines in the U.S. recommend the use of HIV DNA or RNA testing in infant diagnosis.

1.13.1.2. HIV RNA

The first marker of HIV infection that can be detected in the plasma is viral RNA. Both quantitative (referred to as viral load) and qualitative RNA tests are used in monitoring HIV infection. Only the Gen-Probe transcription mediated assay, a qualitative test, is approved by the FDA for use in the diagnosis of acute and early acute HIV infection (AHI) in the absence of serology. There are a number of other FDA approved HIV RNA platforms/assays, some for use in monitoring of viral loads and others for use in blood screening [102]. The principle use of RNA testing is for monitoring HIV-1 viral loads in previously diagnosed HIV infection. Viral load assays have differing lower limits of detection, therefore selection of the appropriate test is critical. HIV RNA is generally detectable in plasma within 7-14 days of infection. In intravenous SIV infection in primates, plasma viremia is detected in 4-7 days from infection [86, 103] while detection of RNA in plasma is more variable with infection via mucosal routes [104-106]. The amount of HIV RNA in plasma is related to the amount of virus actively replicating and is useful surrogate marker for determining the stage of infection and the effectiveness of HIV antiretroviral treatment. RNA tests are also use for blood donor screening purposes, described further in section 1.13.7.

1.13.2. p24 Antigen (Ag) assays

The next marker detected is the soluble HIV-1 *gag* protein, p24, which is detectable approximately 5-7 days detection of RNA. p24 rises in early infection with increasing viral load. Detection in the plasma is transient and generally not measurable after 4-5 weeks as it forms immune complexes with p24 antibodies; thus, is undetectable as RNA levels decline [107]. In some cases, p24 antigen is detected in late stages of infection and progression to AIDS as p24 antibodies decrease allowing free p24 antigen to circulate [108]. In order to overcome the false negative results due to formation of p24 antigen/antibody immune complexes, a dissociation step has been added to most p24 testing assay to increase assay sensitivity [109, 110], however little improvement has been made. Due to the short detection window, p24 assays are not often used in standard HIV testing, but may be useful in diagnosis of acute HIV infection. In addition, when nucleic acid testing is not available, detection of p24 antigen may be useful in infant diagnosis of HIV infection [111, 112].

1.13.3. HIV Antibody ELISA/IA

Enzyme Linked ImmunoSorbent Assays (ELISA)s and immunoassays (IAs) are commonly used in the clinical laboratory to detect antibodies or antigens to many pathogens. ELISA/IA antibody tests have evolved significantly since the first commercially available HIV ELISA developed in the 1980s. These tests are used widely in the developed world and require high-tech equipment such as spectrophotometers, automated plate washers, and autoanalyzers. ELISAs generally take 3-4 hours to complete and are usually performed in batch on 96 well microplates. In addition to the technical disadvantages, patients do not receive their HIV resting results on the same day of the blood draw. The first HIV specific antibody to develop is Immuglobulin M (IgM). IgM is the first antibody produced by B cells in response to antigens and is therefore useful in detecting early infection. The next detectable marker is anti-HIV-specific Immunglobulin G (IgG), which becomes detectable approximately 3-5 weeks after infection, and in most cases remains detectable throughout the course of infection.

1.13.3.1. First Generation ELISA

First generation HIV ELISAs detect IgG HIV-1 antibodies by immobilizing HIV-1 viral lysate antigens on a microwell plates or strips added to microwell plates.

Addition of serum or plasma from an individual with HIV, containing anti-HIV specific IgG antibodies, results in the binding an anti-HIV antibody to the immobilized antigen. After washing away unbound anti-HIV antibody, an anti-IgG antibody conjugate with an enzymatic marker is added that binds to the Fc region of the bound anti-HIV specific antibody. After incubation, the unbound conjugate is washed away and a substrate is added that will produce a colormetric change upon reaction with the enzyme tagged conjugate. The colormetric output is read on a spectrophotometer. The absorbance, optical density, is directly related to the amount of HIV specific antibody present in the sample.

1.13.3.2. Second generation ELISA

Second generation tests are identical to first generation tests except that both HIV-1 and HIV-2 can be detected by immobilizing HIV-1 and HIV-2 synthetic peptides or recombinant protein antigens on the microwell plate/strips. The other steps of the process are identical to first generation tests. Second generation ELISAs reduced the detection window to 25-35 days compared to the first generation ELISA where the window was 35-45 days [113].

1.13.3.3. Third generation ELISA/IAs

Third generation HIV ELISA/IA tests were modified to achieve higher sensitivity through detection of IgM antibodies in addition to IgG antibodies. IgM antibodies arise in HIV infection several days before IgG antibodies therefore allowing HIV specific antibodies to be detected sooner. In this test, HIV-1 and HIV-2 are immobilized on the microwell plates/strips, paramagnetic particles, and/or microparticles for chemiluminescent immunoassays. Patient serum or plasma containing anti-HIV specific IgM and IgG antibodies is added. Instead of a secondary antibody conjugate, as is used in the first and second generation ELISAs, a conjugated HIV-1 and HIV-2 antigen conjugated to an enzyme is added which is then detected by adding a substrate. The improvements between the second and third generation ELISA/IA reduced the detection window from 25-35 days to 20-30 days [113].

1.13.3.4. Fourth generation ELISA/IAs.

The most recent development in HIV detection are fourth generation ELISA/IAs where both HIV-1 and HIV-2 IgG and IgM antibodies as well as p24 antigen can be detected. The detection of HIV-1 and HIV-2 IgG and IgM is identical to that in the

3rd generation ELISA/IA. In addition to the HIV-1 and HIV-2 antigen, antibody to p24 is also mobilized on microwell plates or microparticles. Serum or plasma is added to the plate and HIV-1 specific IgG and/or IgM will bind to the HIV-1/HIV-2 antigen and p24 antigen will bind to the p24 antibody. A conjugate containing the HIV-1/HIV-2 antigen, as described in the 3rd generation ELISA/IA, as well as an antibody to the p24 antigen is added and then detected by adding a substrate. This HIV detection window was further reduced to 15-20 days by addition of the p24 antigen to the 4th generation ELISA/IA [113].

1.13.4. Western Blot

Western Blots (WBs) have been used for diagnosis of HIV since very early in the epidemic [114, 115]. WBs are commonly used as a supplemental test for confirmation after a reactive ELISA/IA. WBs are less sensitive than 3rd and 4th generation ELISAs/IAs as only IgG is detected. One advantage to WBs, however, is the ability to visualize antibody reactivity to individual HIV proteins. The proteins on a WB are generally gp160 (envelope), gp120 (envelope), p65 (Reverse transcriptase), p55 (gag precursor), gp41 (env transmembrane), p40 (core), p31 (endonuclease), p24 (gag), and p18 (core matrix). Most manufacturers require the presence of at least two bands from gp160/gp120, gp41, or p24 in order to classify a sample as positive. Some research has suggested that antigen reactivity on HIV-1 WB is useful in determining the duration since HIV infection [116, 117].

1.13.5. Rapid tests

Another common method of detection of HIV antibodies is through point of care rapid device tests (RDTs). Due to their ease of use and low cost, RDTs are the most widely used method of HIV testing, especially in the developing world. Nurses and non-laboratory trained individuals often perform RDTs, as no detailed training or equipment is required. The sample used for testing is often whole blood from a fingerstick. Testing can be performed in the presence of the patient with results within 5-20 minutes. The use of RDTs has been essential to the roll out of counseling and testing programs in developing countries. The performance of rapid tests have been demonstrated in many studies and exhibit varied sensitivity and specificity [118-123], however seven are approved by the FDA. A composite list of RDTs which met WHO prequalification requirements are listed on the WHO website [124].

Despite the success of RDTs in facilitating roll out of HIV testing programs and dramatic increases in access to counseling and treatment, the tests have limitations. One major disadvantage of most tests is the inability to detect acute infection. Most standard RDTs do not become reactive until several weeks post infection; thus, are less sensitive than 3rd generation ELISAs/IAs. To reduce the window of detection with the possibility of detecting acute infection, RDTs have recently been developed which detect both HIV-1/HIV-2 antibody and p24 antigen [125]. There have been several studies reporting on the performance of these new rapid tests, however none of the published studies have reported results from serial sampling at short intervals during acute infection [126-132].

Another limitation of rapid tests, as well as ELISA/IAs tests, is the ability to distinguish HIV-1 and HIV-2 infection. The Mulispot assay is a rapid test that can differentiate HIV-1 and HIV-2 antibodies, although it is not considered a point of care test as separation of blood into serum or plasma is required. This test was approved by the FDA in 2004 and has recently gained popularity in testing algorithms, as it is one of the few HIV-1/HIV-2 discriminatory assays on the market. It is generally used as a confirmatory test after a reactive screening assay, but is not able to detect acute infection [133-135].

1.13.6. Diagnostic Algorithms in the U.S.

In the US and Europe, the recommended HIV testing algorithm for the last 20 years has been HIV antibody immunoassay followed confirmation with Western Blot. Although the HIV antibody immunassays have improved over the years, the failure to detect acute infection when antibody remains undetectable, and the inability to discriminate between HIV-1 and HIV-2 infection has prompted the US CDC to propose a new HIV testing algorithm [118, 136, 137] (See Figure 4.20). The proposed algorithm reduces the detection window by including a 4th generation immunoassay as a screening step, thus permitting detection of HIV infection up to one week earlier than previous 3rd generation immunoassays [113]. In addition, the Multispot Rapid test has been incorporated post a 4th generation reactive test in order to discriminate between HIV-1 from HIV-2. The HIV-1 Western blot will no longer be required. In the case where the 4th generation ELISA is reactive and the Multispot is non-reactive, a nucleic acid test will be performed to resolve HIV infection status.

1.13.7. Detection of acute HIV infection

Detection of AHI remains a major challenge in the field, yet is critical in certain circumstances. Since the discovery that HIV could be transmitted via blood transfusion, the safety of the blood supply has become a concern. While all blood units could be easily tested for HIV antibodies using sensitive and inexpensive methods, blood units collected during acute infection would be non-reactive by these tests. Thus, in the late 1990s blood banks implemented testing methods that included nucleic acid testing of blood units that were negative for HIV antibodies to rule out acute HIV infection. The nucleic acid tests employed by the blood banks are usually multiplexed assays to detect HIV and Hepatitis. Due to the high cost of NAAT testing, a pooling strategy is usually employed where up to 96 samples are pooled and tested by NAAT with reactive pools then parsed in subsequent assays to determine the reactive sample [31]. Recently, testing centers have moved to using pools of less than 96 samples in order to improve sensitivity of NAAT. This method of testing pooled samples for detection in acute infection has been applied in settings outside of blood donation with success [138, 139]. Detection of acute infection in the absence of NAAT testing, however, remains a challenge. New rapid tests are being developed that incorporate detection of p24 antigen, however the sensitivity of these tests are not yet sufficient to reduce the detection window [128]. Several point of care viral load devices are under development, however none are available on the commercial market. These will be useful in cases of suspected AHI as well as in monitoring effectiveness of antiretroviral treatment. A new blood enhancement test has been developed, SMARTube (SMART Technology), which uses a proprietary cocktail of reagents to stimulate production of antibody from in vivo primed B lymphocytes in fresh whole blood under culture during the window period and before antibody is typically detected by commercially available serological assays [140, 141].

1.14. Fiebig staging

In order to classify the different stages of acute HIV infection, Fiebig et al. developed a staging systems based on the sequential appearance of HIV specific markers in the plasma of donors who were later found to be HIV infected [142]. This staging system is widely used by clinicians and researchers in order to classify the stage of acute infection. The assays used in Fiebig's staging system were HIV-1 RNA viral load, p24 antigen, 3rd generation EIA, and HIV-1 Western Blot. Stage 1 is the time period when RNA is detectable in the plasma, but all other markers remain negative. Stage 2 begins

when p24 antigen becomes detectable but antibody remains negative. Stage 3 begins when the 3rd generation ELISA becomes reactive and Western Blot remains negative. In stage 4, Western Blot is interpreted as Indeterminate. Finally, in stages 5 Western Blot is interpreted as positive, and in stage 6, the p31 (endonuclease) band on the Western Blot becomes reactive [142]. Despite the value of this system for delineating the stages of acute infection, there are limitations in the system including the use of assays that are now outdated, and the use of HIV plasma from only subtype B infected plasma donors. While staging by Fiebig employed the most sensitive/specific FDA approved screening methods available at the time, state-of-art screening/diagnostic platforms are now available which further narrow the detection window for RNA and HIV antigen/antibody with higher sensitivity and specificity for diverse subtypes. It is important to provide updates to this staging system based on newly available diagnostic assays/platforms and to determine relevance in non-subtype B infections.

2. Objectives

2.1. Describe viral dynamics in non-subtype B acute HIV-1 infection and determine relationships between early virological events, immune system integrity, and long-term clinical course.

2.2. Describe/re-define current laboratory staging definitions for acute non-subtype B HIV-1 infection based on newly available, more sensitive test assays and technologies including serological Point of Care devices.

2.3. Examine the role of novel diagnostics in detecting/diagnosing HIV infection in the eclipse phase or very early acute/primary infection using SMARTube technology.

2.4. Enhance understanding of acute infection by delineating HIV-1 exposure events through detection of RNA in self-administered vaginal/rectal swabs immediately after exposure events and examining the relationship of swab viral load to viral dynamics after infection.

3. Methods

3.1. Site and protocol description

The United States Military HIV Research Program (MHRP) has a primary mission of developing a vaccine to prevent HIV infection worldwide. Towards this goal, several international sites have been developed to study the epidemic in each country and also to develop cohorts for future vaccine efficacy studies. Current plans for HIV vaccine efficacy studies are focusing on populations with a high annual incidence of HIV infection (>3%) and minimal loss to follow-up. Therefore, MHRP attempted to

develop cohorts with study volunteers at high risk of infection, also called "most at risk populations" (MARPS) in four established clinical research sites in East Africa (EA), including Uganda, Kenya and Tanzania and Thailand (TH).

The samples and data used in this research were collected under the protocol entitled: "RV217- HIV-1 Prevalence, Incidence, Cohort Retention, and Host Genetics and Viral Diversity in Cohorts in East Africa and Thailand". The study was a multi-center, non-randomized, clinical observational study with a primary purpose of characterizing recruitment, retention, HIV prevalence, HIV incidence, and biological characteristics of acute HIV infection in high-risk volunteers. The study population consisted of men and women, aged 18-50 years old, who were members of the following high risk groups: female and male sex workers , barworkers , transgenders (TG), and men who have sex with men (MSM). Risk behavior information was collected and evaluated through the use of a questionnaire administered using Audio-Computer Assisted Self-Interview (ACASI). To be considered as high risk and enrolled in the study, participants had to meet one of the following criteria; 1) exchange of sex for goods, services or money, 2) unprotected sex with a known HIV infected partner, 3) sex with 3 or more partners in the prior 3 months or 4) diagnosis or symptoms of a sexually transmitted infection (STI) in the last 3 months.

The main study activity, or phase I, was the observational cohort or surveillance activity. In contrast to standard cohort studies that are designed with regular visits at monthly to six monthly intervals, this study collected very small blood samples via finger stick collections. These "small blood volume" (SBV) visits occurred twice weekly and afforded the opportunity to diagnose HIV infection prior to the advent of detectable antibody by testing SBVs plasma with a nucleic acid assay, the Aptima HIV-1 Qualitative RNA test (Gen-Probe, San Diego, CA). Participants with incident HIV infections observed during phase I of the study were asked to enter an intensive follow with twice weekly large blood volume collections called phase Ib to ensure diagnosis; then long-term follow-up in phase II of the study. Phase II of the study observed HIV infected individuals for an extended period to evaluate early events, chronic viral burden, and early disease progression.

3.2. Role in research

My role in the research described in this thesis included:

- Contribution to development of RV217 protocol and blood collection schedules
- Development of laboratory manual of operations that was implemented in the four international research laboratories where samples were collected and some assays were performed (See Table 3.1)
- Oversight of conduct of RV217 in the international laboratories
- Managed inventories and requested samples for shipment from the international sites to the repository in the US
- Selected samples and developed testing plan for the testing in the US laboratories (See Table 3.1)
- Collated and organized data
- Performed all data analysis including statistics as well as generation of all tables and figures

3.3. Ethical approvals

Ethical approval for RV217 was obtained by the Walter Reed Army Institute of Research (WRAIR) Institutional Review Board (IRB) in the United States, as well as within each country where the study was performed. This included:

- Uganda: National HIV/AIDS Research Committee and Uganda National Council for Science and Technology
- Kenya: Kenya Medical Research Institute
- Tanzania: Mbeya Medical Research and Ethics Committee and National Institute of Medical Research
- Thailand: The Institutional Review Board, Royal Thai Army Medical Department

Individual consents were signed by volunteers for entry into the study and for entry into phase II for long term follow up. In addition, a separate consent was signed for participation in the swab collection component of the study. The study opened in Thailand in June 2009, Kenya and Tanzania in August and September of 2009, and Uganda in December of 2009. In 2013, a protocol amendment was approved by WRAIR and local IRBs that expanded the protocol to allow for swab and SMARTube collection.

3.4. Identification acute infection/sampling

After two initial visits to determine study eligibility, HIV negative MARPS study volunteers (and a small subset of HIV positive volunteers for masking purposes) were

enrolled in twice-weekly blood collections by fingerstick for one year; then, monthly during the second year of surveillance. The SBV samples were tested for HIV viral RNA within 24-48 hours of collection at the international sites using a high-throughput qualitative nucleic acid test, the Aptima platform from Gen-Probe (San Diego, CA) with a 50% detection sensitivity for HIV-1 RNA of 5 copies/milliliter (mL) (Dr. Sheila Peel, personal communication). Detection sensitivity for protocol 1:5 dilution of participant plasma:Phosphate Buffered Saline is an expected 20 RNA copies/ml (Dr. Sheila Peel, personal communication). If the Aptima test was reactive, the volunteer was immediately entered into an intensive period of blood collection every 3-4 days for one month called phase Ib or the stutter phase. This phase was called the stutter phase due to Fiebig's observation that HIV-1 viral load in early acute infection can "stutter" between detected and not detected [143]. If HIV infection was confirmed after 1 month using the standard HIV diagnosis algorithm of EIA and Western Blot, participants moved into the next phase where samples were collected at gradually more prolonged intervals during chronic infection and followed for 5 years. If HIV infection was not confirmed, participants were entered back into surveillance phase (See Figure 3.1).

Figure 3.1: RV217 schematic and blood draw schedule

Figure 3.1: Schematic of RV217 events. SBV=Small blood volume and d=days from first RNA reactive. Timepoints in green (d17, d42 and d84) were larger blood volume collections compared to other visits.

In addition to the SBV samples, visits with larger blood volumes were collected every three months during surveillance. At each blood collection, samples were sent to the international research laboratories at each site where serum, plasma and peripheral blood mononuclear cells were harvested and cryopreserved. HIV diagnostics, including HIV EIA and Western Blot, for the purposes of informing the volunteers of HIV status were performed at the international sites' diagnostic laboratories. All laboratories were compliant with Good Clinical Laboratory Practices, accredited by the College of American Pathologists, and enrolled in numerous external quality assurance programs. Initial Fiebig stages were assigned to each sample based on the HIV-1 viral load, 3rd generation EIA, and Western Blot performed at the sites. p24 antigen was performed only on a subset of cryopreserved samples once shipped back to the US, therefore in the overall study, no differentiation was made between Fiebig I/II samples. A central laboratory manual of operations was developed to ensure that all international sites were following the same procedures for processing, aliquoting, labeling, and cryopreservation of samples. All blood samples for objective one and two were collected with an EDTA anti-coagulant. Plasma was separated by centrifugation of the whole blood at 1200 times gravity for 10-15 minutes. All plasma samples were separated within 8 hours of collection.

3.5. Shipment samples from sites

After ensuring all proper ethical approvals and material transfer agreements were in place, samples were shipped from the international sites to the MHRP repository in Rockville, MD, U.S. All shipments of plasma occurred on dry ice and according to International Air Transport Association (IATA) regulations. Samples remained in cryogenic storage at the repository until sample requests were made at which point specific aliquots were pulled and sent to the testing laboratories.

3.6. Samples included in analyses:

Between 2009 and December 2013, 95 incident cases were detected. Cases were categorized into 4 subgroups;

- Cases consisting of volunteers with at least 2 Fiebig I/II large blood volume (LBV) draws, a known date of last HIV negative RNA, and viral loads performed out to at least 6 months;
- Cases meeting criteria above, but only 1 Fiebig I/II blood draw;
- Cases with at least one Fiebig I/II sample, but no HIV RNA negative collection,
- Cases with no Fiebig I/II samples or lost to follow up.

For the purposes of the studies in Objective 1 and Objective 2 listed below, only cases from the first subgroup were included, as these allowed for a precise definition of viral load upslope and peak viral load as well as provided sufficient follow up to allow for evaluation of early disease outcome. All subgroups were included in Objectives 3 and 4, however these collections only began in 2013 with the amendment to the protocol and therefore sample numbers were limited. In addition, after the amendment in Thailand, participants were offered co-enrollment in another study that provided antiretroviral treatment in acute infection and therefore these samples were not available for evaluation in Objective #3 and 4.

3.6.1. *Objective 1*

For Viral Load and Flow cytometry analysis, longitudinal samples from 42 acute cases were included. Timepoints included in the analysis varied for each assay with the earliest being the first blood collection after a reactive Aptima out to 4 years.

3.6.2. *Objective 2*

For the diagnostics and staging assays, longitudinal samples from 29 acute cases were included. These 29 cases were also included in Objective 1. At the time of sample analysis, only 29 cases were available for this objective. Timepoints included in this analysis are from the first blood collection in acute infection out to 6 months. A pre-infection sample was also included in WB analysis.

3.6.3. *Objective 3*

The SMART tube sampling was performed for new incident cases after ethical approval was obtained.

3.6.4. *Objective 4*

Self collected swabs were collected consenting participants after ethical approval was obtained. The number of swabs collected and tested are described in Table 4.9.

3.7. Lab testing overview

Laboratories involved in testing are indicated in Table 3.1. Both the international laboratories and the Department of Laboratory Diagnostic and Monitoring Laboratory are College of American Pathologist accredited.

Table 3.1: Laboratories involved in analysis

Laboratory	Tests performed
Site International Laboratories	
Makerere University Walter Reed Project Laboratory (Uganda) Kericho CRC Laboratory (Kenya) Mbeya Medical Research Center Laboratory (Tanzania) AFRIMS laboratory (Thailand)	Plasma Aptima assays to identify acute infection Biorad 1/2 + O 3rd generation EIA/Western Blot Immune Phenotyping SMART tube collection Swab processing and Aptima testing
US based reference Laboratories	
Department of Laboratory Diagnostic and Monitoring, US Military HIV Research Program	HIV-1 Viral Load Biorad 4th generation EIA p24 antigen ELISA HIV Western Blot HIV 1/2 Multispot Rapid test Determine Combo rapid test
Department of Molecular Virology, US Military HIV Research Program	Single genome amplification sequencing to determine subtype*

*Sequencing performed as previously described [144].

With the exception of the p24 quantitative kit, the Aptima performed on the swab samples, and the SMARTube, all test kits used were FDA approved. Procedures from the manufacturer's product instructions were employed without alteration, with the except of the Aptima testing on SBV plasma samples where a 1:5 dilution was made before testing due to the low volume of plasma available from the fingerstick blood collection. Based on the available data from the international sites (3rd generation EIA and Western Blot) and projected reactivity in each assay, a testing matrix was developed for each sample in order to preserve sample volume and save on testing costs. Final HIV diagnosis for the purposes of reporting results to the volunteer was performed using the international sites' standard methodology. All other diagnostic assays were performed for research purposes only.

3.8. HIV diagnosis and co-infections at international sites

HIV testing for the purposes of reporting HIV status to the volunteers was performed at the international sites using a 3rd generation EIA with supplemental Western Blot assay for confirmation. Screening was performed using the Genetic Systems HIV-1/HIV-2 Plus O EIA (Bio-Rad Laboratories, Hercules, CA), on screening and surveillance visits as well as at selected visits during stutter phase. This EIA kit uses recombinant proteins and peptides to detect both IgG and IgM antibodies employing a direct antibody sandwich amplification technique. EIA reactive samples were repeated in duplicate using the same kit and confirmed with the Genetic Systems HIV-1 Western Blot (Bio-Rad Laboratories). The Western Blot data generated at the international sites was used for HIV diagnosis, but was not used for the analysis in this project. See section 3.13.

Hepatitis B surface antigen (HBsAg) was evaluated using the Genetic Systems BioRad Hepatitis B surface Antigen EIA 3.0 kit and confirmed using the Genetic Systems BioRad Hepatitis B surface Antigen Confirmatory Assay 3.0 (Bio-Rad Laboratories, Hercules, CA). Antibodies to Herpes Simplex Virus 2 (HSV-2) screening was evaluated using an EIA assay from Focus Diagnostics (Cypress, California, U.S.), however a higher-cut-off of 3.4 was used to assess reactivity based on the paper by Layendecker [145] that confers a higher level of specificity than the cut-off suggested by the manufacturer.

3.9. Immune Phenotyping at international sites

Clinical immunophenotyping for assessment of CD4, CD8, NK and B cell absolute counts and percentages was performed on fresh EDTA anti-coagulated whole blood using Becton Dickinson Multitest and TruCount tubes (San Jose, CA). Briefly, in two separate tubes containing TruCount beads, CD3/CD8/CD45/CD4 or CD3/CD16+CD56/CD45/CD19 monoclonal antibodies were added to whole blood. CD45 is a marker for lymphocytes and CD3 antibody stains T lymphoctyes that are further differentiated between helper T cells (CD4) and suppressor T cells (CD8). CD19 is a marker for B lymphocytes and CD3-CD16+ and/or CD56+ is the phenotype for NK cells. After a 15 minute incubation with the monoclonal antibodies, a buffer was added to lyse red blood cells for an additional 15 minutes; then, the samples were run on a 4-color FACSCalibur (Becton Dickinson) flow cytometer and data acquired using Multi-set software. Analysis was performed at the first visit in phase 1b, and then subsequently at 2 weeks, 4 weeks, 6 weeks, 12 weeks, 36 weeks and then every 3 months.

3.10. HIV-1 Viral Load

Viral Load was performed on cryopreserved longitudinal EDTA anti-coagulated plasma samples at the Department of Laboratory Diagnostics and Monitoring, Walter Reed Army Institute of Research, Silver Spring, MD using the Abbott Real-Time HIV-1 Assay on the automated m2000 platform (Abbott Laboratories, Abbott Park, Illinois) with a lower detection limit of 40 copies/ml and an upper assay limit of 10,000,000 copies/ml. This assay is a reverse transcriptase polymerase chain reaction (RT-PCR) with real-time fluorescent detection that allows for detection of a diverse group of M subtypes as well as group O. This test has been shown to reliably detect group M subtypes A, B, C, D, CRF01-AE, F, CRF02-AG, and G [146]. The target of the Abbot real

time assay is the integrase region of polymerase, a highly conserved region of HIV-1. Plasma samples were prepared using the m2000sp automated instrument that uses magnetic techniques to isolate and purify nucleic acids. An internal control is included with each sample to ensure that amplification has occurred and that inhibition is not present. The m2000sp automated system also prepares the master mix and dispenses it into the plate containing the extracted RNA which is then transferred manually to the real time PCR machine which carries out the amplification and detection via fluorescent probes. A calibration curve is implemented with each assay that includes two assay calibrators that are run in triplicate to generate the calibration curve and to calculate the RNA concentration in each sample. Any samples that were above the assay limit were diluted in normal human plasma and repeated for more accurate quantitative assessment. The viral load was multiplied by the dilution factor to obtain the final viral load in these samples. All samples from one subject were run on the same run from first timepoint through 6 months in order to reduce inter-assay variability within a subject during the acute phase of HIV infection.

3.11. 4th generation EIA

Crypreserved EDTA-coagulated plasma were run on the Genetic Systems HIV-1/2 Combo Antigen/Antibody EIA (Biorad Laboratories, Redmond, Washington) according to the manufacturer's instructions. This 4th generation EIA simultaneously detects HIV-1 p24 antigen as well as IgG and IgM antibodies to HIV-1 and HIV-2 antigens using the sandwich technique but is not able to differentiate antigen from antibody detection, nor does it distinguish HIV-1 from HIV-2 infection. The assay is able to detect antibodies/antigens from 15 different HIV-1subtypes/circulating recombinants (subtypes A, A1, B, C, D, F1, F2, G, CRF01, CRF02, CRF05, CRF06, CRF09, CRF11, and CRF13) as well as HIV-2 and Group O samples [147]. The assay cut-off for each assay is determined by the averaging the triplicate run of the cutoff calibrator that is run in each assay. Samples with absorbance values above the cutoff calibrator are considered reactive for HIV-1 and/or HIV-2. Sequential samples from each acute infection case were run from first available sample out to one visit past timepoint that 3rd generation EIA from step 3.7 became reactive. Although the package insert calls for repeat testing in duplicate when using this kit for HIV diagnosis, repeats were not performed on these samples as this testing was for research only and sample volume was limited.

3.12. p24 quantitative assay

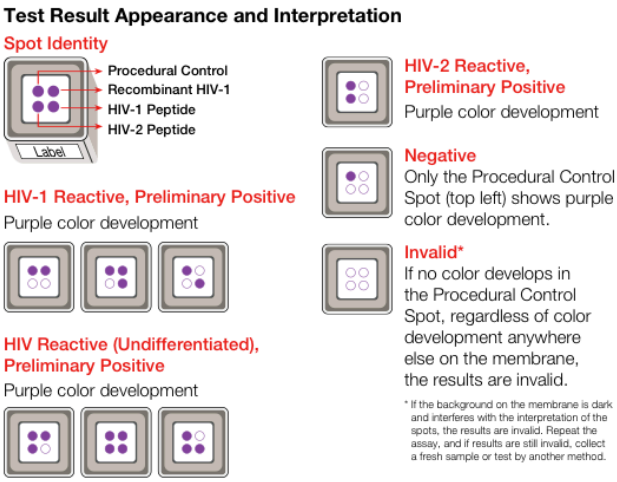
In order to evaluate p24 concentrations over time, the Genscreen HIV-1 Antigen (BioRad Laboratories, Marnes, France) EIA quantitative assay was run on serial samples from each subject. In the Genscreen assay, anti-p24 antibody is coated on the surface of the EIA plates. Serum/plasma is added to the microwell plates and any p24 antigen present in the samples will bind to the microwell. After washing, a biotinylated anti-p24 antibody is added and followed by a second conjugate containing a peroxidase tagged avidin, allowing the detection of any antibody-antigen complexes that are bound to the well with a substrate detection step. An HIV-1 antigen standard was also run in each assay in order to prepare a calibration curve for quantification of p24 antigen concentrations. Only sample concentrations between 10 and 100 picoggrams (pg)/ml, the dynamic range of the assay, were interpreted. Samples with more than 100pg/ml of p24 were diluted and re-tested in order to have concentrations that fall within the reportable range of the assay and then multiplied by the dilution factor to obtain the final concentration. All samples from one individual were run in the same assay to reduce chances of inter-assay variability. This assay is not approved by the U.S. FDA, but is the French national standard approved by AFSSAP (L'Agence française de sécurité sanitaire des produits de santé).

3.13. Multispot HIV-1/HIV-2 Rapid Test

In order to also evaluate the presence of HIV-2 in addition to HIV-1, the Multispot HIV-1/HIV-2 Rapid test (Biorad Laboratories, Redmond, Washington) was performed on serial cryopreserved samples according to manufacturer's instructions. The Multispot assay is a detection and discriminatory assay for HIV-1 and HIV-2 antibodies in serum or plasma [148]. The rapid test is based on the principle of ImmunoConcentration. Microparticles coated with either Recombinant HIV-1, HIV-1 peptide, HIV-2 peptide or a procedural internal control (anti-IgG), are immobilized in four different sections of a reaction membrane on the Multipspot Cartridge (See Figure 3.2). Samples are diluted and then added to a pre-filter in the cartridge. If antibodies to HIV-1/HIV-2 are present, they will bind to one of the antigens on the cartridge membrane. Next, a conjugate containing an alkaline phosphatase tagged anti-IgG is added which will bind to the antigen-antibody complex immobilized on the spots on the membrane. After a wash step, a substrate is added to the cartridge that will produce a color change in the spots if HIV-1/HIV-2 antibodies are present. A stopping solution is then added to halt the development of color. In order for a test to be valid, the procedural control spot

must show purple color development (See Figure 3.2). If no other spots show color, this is a negative result. If one or both of the HIV-1 spots show a purple color development, this samples is considered to be positive for HIV-1 antibodies. If the HIV-2 peptide spot shows color development, the test is considered to be positive for HIV-2 antibodies. If both the HIV-1 and HIV-2 spots show color, the sample is considered to be HIV Reactive, Undifferentiated. Differentiation can be attempted by performing dilution of the sample 1:10 and retesting; if it remains undifferentiated, then sample can be diluted 1:100 and retested. If it remains so again, then the result is HIV undifferentiated. As this test is known to be of similar or slightly less sensitive than the 3rd generation EIA, the first sample chosen to run in the sample set was the first 3rd generation EIA reactive sample and the subsequent 3-4 serial time points until the sample was positive. If the sample was positive by Multispot at the same timepoint as the 3rd generation EIA, earlier collected samples were run until a negative test was obtained.

Figure 3.2: Multispot format and interpretation



http://www.bio-rad.com/webroot/web/pdf/cdg/literature/P-120_Multispot_HIV-1_HIV-2_Rapid_Test.pdf (Permission for reproduction granted from BioRad)

3.14. Western Blot

The Genetic Systems HIV-1 Western Blot Kit (BioRad Laboratories, Redmond, Washington) was run on cryopreserved samples in acute HIV infection cases. The Western Blot detects HIV-1 antibodies to gp160, gp120, p65, p55, p51, gp41, p40, p31, p24, and p18. HIV-1 lysate was electrophoresed on protein gels, transferred to nitrocellulose membranes, then cut into individual strips. Plasma/serum samples are

diluted 1:100, incubated with individual nitrocellulose strips, washed, then incubated with anti-IgG conjugated to peroxidase. After an additional wash step, antibody-conjugate complexes bound to the strips are detected using a substrate that will produce a colorimetric change. The position and intensity of each antigen "band" is then compared to the two positive control and negative control strips. Each individual band is scored as a 0 (band absent), 0.5 (band less than the intensity of the low positive control band), 1 (band at least as intense as the gp120 band on the low positive control), 2 (band greater than or equal to the gp120 band on the high positive control). Each strip is then evaluated based on the presence and intensity of certain bands. Samples are considered negative if no bands are present on the strip. Samples are considered positive if at least two following bands (gp160 or gp120, gp41 or p24) are present in an intensity greater than or equal to "1". The reference reactivity for a score of "1" is the gp120 antigen band on the low positive control strip; thus, the reference is internal to each WB run. The band for gp41 must be broad and diffuse. The sample is considered indeterminate if any bands are present but do not meet criteria for positive. Although Western Blot is traditionally only run in HIV diagnostics on EIA reactive samples, for this study, Western Blot was run at all time points, including a pre-infection sample, out to 6 months to analyze the evolution of the banding patterns.

3.15. Determine HIV-1/2 Antigen (Ag)/Antibody (Ab) Combo Rapid Test

The Determine HIV-1/2 Ag/Ab Combo (Alere, Inc, Waltham, MA, U.S.) test is a lateral flow immunochromatographic rapid test for the detection of p24 antigen and/or antibodies to HIV-1 and HIV-2. This test does not discriminate between HIV-1 and HIV-2 infection. Performance characteristics of this kit demonstrate sensitivity with over 12 HIV-1 subtypes in addition to HIV-1 Group O and HIV-2 [125]. Samples are added to the sample pad, and then migrate by capillary action through the conjugate pad to the nitrocellulose membrane (See Figure 3.3). If antibodies to HIV-1 and/or HIV-2 are present in the sample, they will bind to the immobilized recombinant HIV antigen and peptides-colloidial selenium conjugates from the conjugate pad. This complex then migrates through the nitrocellulose until it is captured by immobilized HIV-1 and HIV-2 synthetic antigens and forms a red line in the HIV antibody window. If p24 is present in the sample, p24 will bind to the biotinylated anti-p24 in the sample pad and then the selenium colloid anti-p24 antibody in the conjugate pad. The complex migrates until is captured by the immobilized streptavidin to form a red line at the HIV Antigen window. A procedural control is also included with each rapid test

and must be positive to consider a test to be valid. Cryopreserved plasma samples from the 29 acute cases were run on samples from the earliest timepoint available with adequate volume of sample. The Determine Combo was run at all available time points up until 2 samples past 3rd generation EIA reactivity. Antigen and Antibody reactivity were evaluated separately. These samples were run in August of 2012, before the test was FDA approved.

Figure 3.3: Determine Combo Rapid Test Format and Interpretation

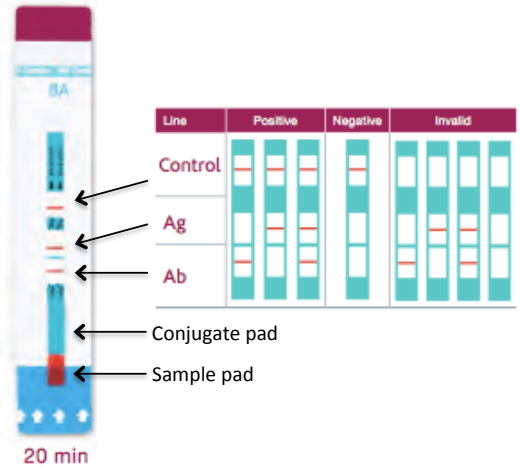


Figure 3.3: Ag=Antigen, Ab=Antibody, figure modified with permission from Alere. http://alerehiv.s3.amazonaws.com/wp-content/uploads/12000140-v03-Alere-Determine-HIV-12-AgAb-Combo-Broc.pdf

3.16. SMART tubes collection and analysis

The SMARTube is intended for use as a pre-treatment, blood enhancement device for blood that will undergo anti-HIV testing. The proprietary contents of the SMART tube activate lymphocytes in vitro leading to proliferation of the B cells and production of antibodies earlier than would normally occur in vivo. 1ml of whole blood is added to the SMARTube that is then incubated at 37 degrees for five days. After incubation, the SMARTube was centrifuged at 1,100 x g and the supernatant fluid (called SMART-plasma) was removed and stored at -80°C until testing. The SMART plasma was run on the 3rd and 4th generation EIA tests mentioned above. When a volunteer entered the stutter phase, 1ml of EDTA at visits 1 and 3 (approximately days 2 and 7) whole blood was transferred aseptically to a SMART tube.

3.17. Swab collection and analysis

Volunteers were asked to participate in a sub-study where self-administered vaginal/rectal swabs would be collected twice-weekly for the first four months of surveillance in the study. The volunteers were given the swab kits and instructions for self administration. They were requested to perform swabbing after sexual encounters, taking the sample from the most recent route of exposure. The swabs used for this purpose were the APTIMA Vaginal Swab Specimen Collection kit and included a swab and a transport tube containing 2.9mls of transport medium. The transport medium most likely contains nucleic acid stabilizing agents, although the actual contents of the medium were not disclosed by the manufacturer. The use of these swabs was for research purposes only. The swabs in transport medium were then returned to the clinic at the next small blood volume visit and were stable at room temperature for up to 60 days. Swabs were transferred to the laboratory and placed on a testing program. Due to the number of swabs collected and expense of the test, not all swab samples could be tested. All swabs in transport medium were saved for 60 days at room temperature and in case of a reactive Aptima plasma test, the transport medium from each swab available from that individual was tested to see if RNA could be detected in the swab samples before the plasma sample was reactive. Swab samples were also collected after the first reactive plasma Aptima during stutter phase. The transport medium from these swabs were aliquoted and stored at -80°C for future testing.

In addition, swab samples were chosen at random so that eight swabs for every volunteer participating in swab collection was tested by Aptima on site. One swab every other week from each volunteer was chosen and tested by Aptima at the international sites. If any of the swabs were reactive, transport medium from reactive swabs from that individual were aliquoted and frozen at -80 for follow-up testing.

3.18. Statistical definitions

Data analysis definitions/calculations

- Day: Day zero is defined as the first reactive RNA result. In most cases, this is the Aptima test. As this is a qualitative test, for graphical purposes, a value of $1.6\log_{10}$ copies/ml was assigned. All days are with a reference of day 0 as the first reactive RNA test.
- Peak Viral Load: The maximum viral load
- Nadir Viral Load: The minimum post-peak viral load before day 60.

- Viral Load Upslope: The rate of change in viral load from the last known negative sample to peak viral load. Five (5) of the 42 cases where the interval of days between last negative and first positive were greater than 10 days were excluded from viral load upslope analysis.
- Viral Load Downslope: The rate of change in viral load from the peak viral load to the nadir viral load.
- Set point: The average viral load between day 80 and day 365 and prior to HAART initiation. Set points were only determined when more than one data point was available for the calculation.
- CD4, CD8, NK or B cell at 1 year: This is the absolute count measured closest to 1 year. Subjects that have begun HAART or have not reached 1 year of follow up are not included in this analysis.
- Time to CD4<350 or start of HAART: This is the number of days from the first reactive Aptima to two consecutive CD4 absolute count of less than 350 cells/ul, or the day of start of HAART for low CD4 counts, or WHO criteria. Several women were started on HAART due to pregnancy and these women are excluded from this analysis.
- CD4 nadir: The lowest CD4 absolute count prior to visit 10 (median 41 days from first reactive Aptima).
- CD8 peak: The peak CD8 absolute count prior to visit 10 (median 41 days from first reactive Aptima).
- NK cell peak: The peak NK absolute count prior to visit 10 (median 41 days from first reactive Aptima).
- B cell nadir: The lowest B cell absolute count prior to visit 10 (median 41 days from first reactive Aptima).

3.19. Statistical methods

All graphs and statistics were performed in GraphPad Prism, Version 6.0a, GraphPad Software, La Jolla, CA. Medians, Interquartile ranges (IQR) and ranges were reported for summary statistics. Spearman correlations were used throughout the data analysis as not all data parameters were normally distributed. All Spearman correlations were performed using a two-tailed method with an alpha of 0.05. All t tests also used an alpha of 0.05. As this project is a hypothesis generating project, no corrections for multiple comparisons were performed. Data after start of antiretroviral treatment was censored. The statistical tests used in analysis are described in the text and/or the

figure/table legends. Due to the nature of the data, several of the analyses are descriptive in nature.

4. Results

4.1. Study sample overview

In the RV217 study conducted in Kenya, Tanzania, Uganda and Thailand, 2065 high-risk participants were enrolled for HIV acute infection surveillance. Small blood volumes were collected twice weekly and tested by Aptima. Between June 2009 and December 2013, 95 incident cases were observed. In order to ensure precise and accurate definition of viral load upslope, peak and down slope, only samples with at least two Fiebig 1/2 samples were included in the analysis. In addition, only samples with viral loads performed out to 6 months were included to allow for calculation of set point viral load. This resulted in forty-two (42) cases included in analysis of objective #1. A subset of these, 29 subjects, were included in objective #2 as described in section 3.5. At the time of running the assays for objective #2, only these 29 cases had samples collected through 6 months and were available for testing in the U.S. Table 4.1 shows the summary demographic information and co-infection status information for the 42 acute cases.

Table 4.1: Demographic and co-infection summary

	East Africa	Thailand	Total
Acute cases in analysis #	25 (Uganda-6) (Kenya-13) (Tanzania-6)	17	42
Male #	0	16 (8 MSM, 8 TG)**	16
Female #	25	1	26
Age, median years (range)	24 (18-34)	23 (18-48)	24 (18-48)
Pregnancy (% in region)*	13 (52%)	0	n/a
Started HAART treatment (% in region)	7 (28%)	7 (41%)	14 (33%)
Hepatitis BsAg+ # (% in region)	1 (4%)	0	1 (2.4%)
HSV-2 Ab + # (% in region)	20 (80%)	6 (35.2%)	26 (62%)
Syphilis + # (% in region)	5 (20%)	6 (35%)	11 (26%)
HIV Subtypes* (N=41)**	9-A 2-C 2-AC 8-AD 4-ACD	14-CRF01_AE 1-CRF01_AE/B 1-B	
Median days between last negative and first reactive RNA (range)	4 (3-32)	4 (2-9)	4 (2-32)

*13/25 women in East Africa were pregnant during time of follow up.
**MSM=Men Who Have Sex With Men, TG=Transgender
***Sequences determined by full length sequencing/single genome amplification as previously described [144, 149]

Of the 42 acute infections, 25 were from East Africa (13 from Kenya, 6 from Uganda, and 6 from Tanzania). The median days between the last negative and first reactive RNA test was 4 days (range 2-32). For some analyses, comparisons were made

between the regions of East Africa and Thailand although there were many confounding differences between the two regions. All of the cases in East Africa were women while 16 out of 17 cases in Thailand were male. The exposure in the women in East Africa was heterosexual while the Thailand cases were all men who have sex with men (MSM) and Transgender (TG). The median age of the 42 cases was 24 (range of 18-48). Thirteen (13) pregnancies were observed in East Africa, with pregnancy arising at different stages during infection. Fourteen (14) individuals were initiated on HAART treatment. Five (5) initiated HAART due to pregnancy while the remaining started due to CD4 counts less than 350 cells/ul based on 2010 WHO recommendations and/or per country guidelines. One case in Thailand initiated HAART due to personal preference, as this is allowed by Thailand national guidelines. All data after HAART initiation was censored. Hepatitis B prevalence was low (2.3%) while syphilis prevalence was 26%. HSV-2 prevalence was high (62%), especially in East Africa where 20/25 (80%) of cases were positive for HSV-2, even when using the higher cut-off to improve specificity [145]. HIV subtyping was performed on 41/42 cases using full length sequencing and single genome amplification techniques [144, 149]; 14/16 of the cases in Thailand were subtype CRF01_AE while the subtype distribution in East Africa was diverse, with 9 pure subtype A infections and 14 cases as recombinants containing subtype A. One pure subtype C infection was identified in Tanzania and another in Kenya. The division of analysis by regions, while informative, was confounded due to differences in sex, infecting subtype, host genetics, and possible environmental differences.

4.2. HIV-1 Viral Load Dynamics

Longitudinal viral loads were performed on 42 acute cases from the first timepoint after reactive Aptima out to at least 6 months. Viral loads for each subject were plotted verses the number of days since the first reactive Aptima. Figure 4.1 shows example plots for 9 different participants out to 400 days and shows the heterogeneity in viral load peaks and resulting set points.

Figure 4.1: Individual viral load dynamics

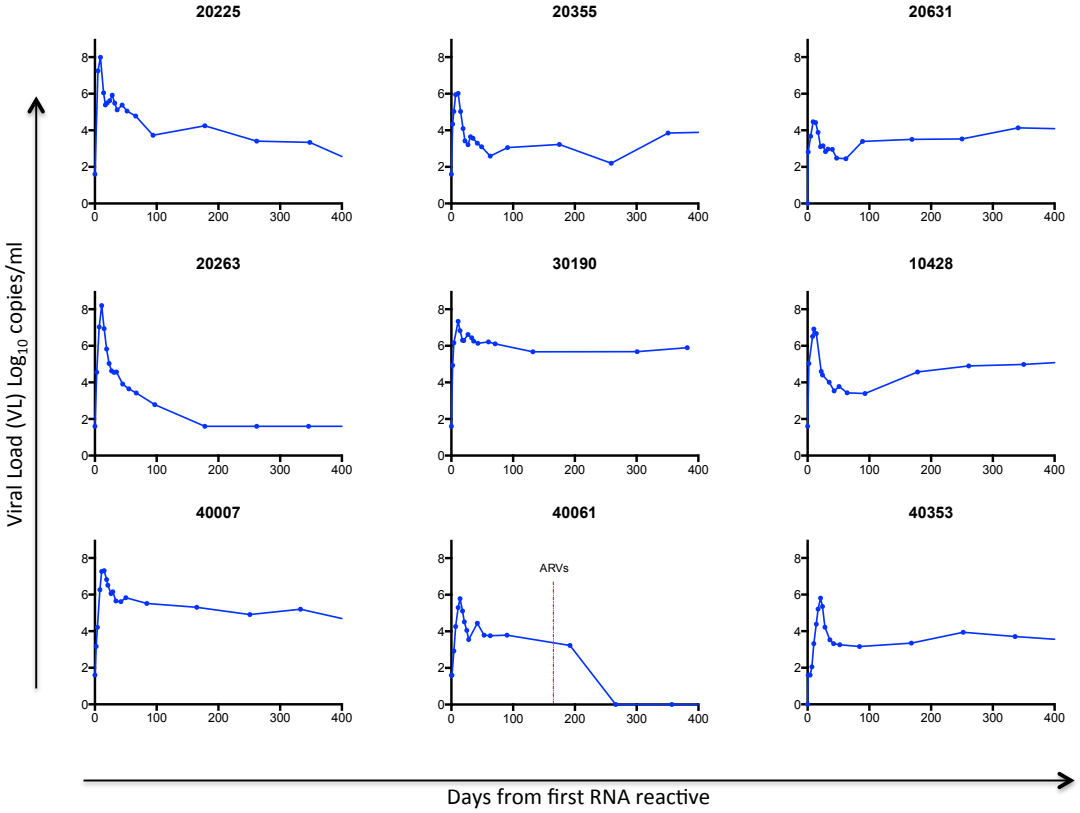


Figure 4.1: Days from first reactive Aptima are plotted on the x-axis and the log_{10} VL copies/ml are plotted on the y axis. Viral load trajectories are shown for 9 individuals out to 400 days from the first reactive. The red line in subject 40061 indicates the time that ARV treatment was started. Limit of detect for viral load was 40 copies/ml (1.6 log_{10} copies/ml).

Subject 20225 had a peak viral load of 100 million copies/ml and achieved a set point of approximately 10,000 copies/ml by day 100, then showed a gradual continuous decrease in viral load to almost 100 copies/ml by 400 days. Subject 20355 had a peak viral load of only 1,000,000 copies/ml, however the set point viral load was similar to that achieved with 20225 with nearly a two log higher peak viral load. Subject 20631 had the lowest peak viral load in 42 cases studied (29,500 copies/ml), however the set point viral load was similar to that of subjects with higher peak viral loads. Subjects 30190 and 40007 had a peak viral load near 10,000,000 copies/ml, but the set point viral load remained at 1,000,000 copies/ml. Subject 40353 had a peak viral load of less than 1,000,000 copies/ml, however the set point was similar to 20225. Subject 20263 had a very unique viral load profile as she had one of the highest peak viral loads (158,000,000 copies/ml) however by day 180, viral load had been controlled to less

than 40 copies/ml without antiretroviral treatment. This participant is seen at the Kericho, Kenya research center, which is the only source of HAART in this area of Kenya; medical staff were confident that she was not on antiretroviral treatment. In addition, several frozen plasma samples were sent to a diagnostic laboratory in the U.S. to confirm absence of antiretroviral drugs in the plasma (data not shown). Subject 40061 had a peak load of only 1,000,000 copies/ml, however by 6 months was put on antiretroviral treatment due to two consecutive CD4 counts below 350 cells/ul. The aggregate viral load trajectories for the first 100 days are shown in Figure 4.2.

Figure 4.2: Aggregate Viral Load Trajectories

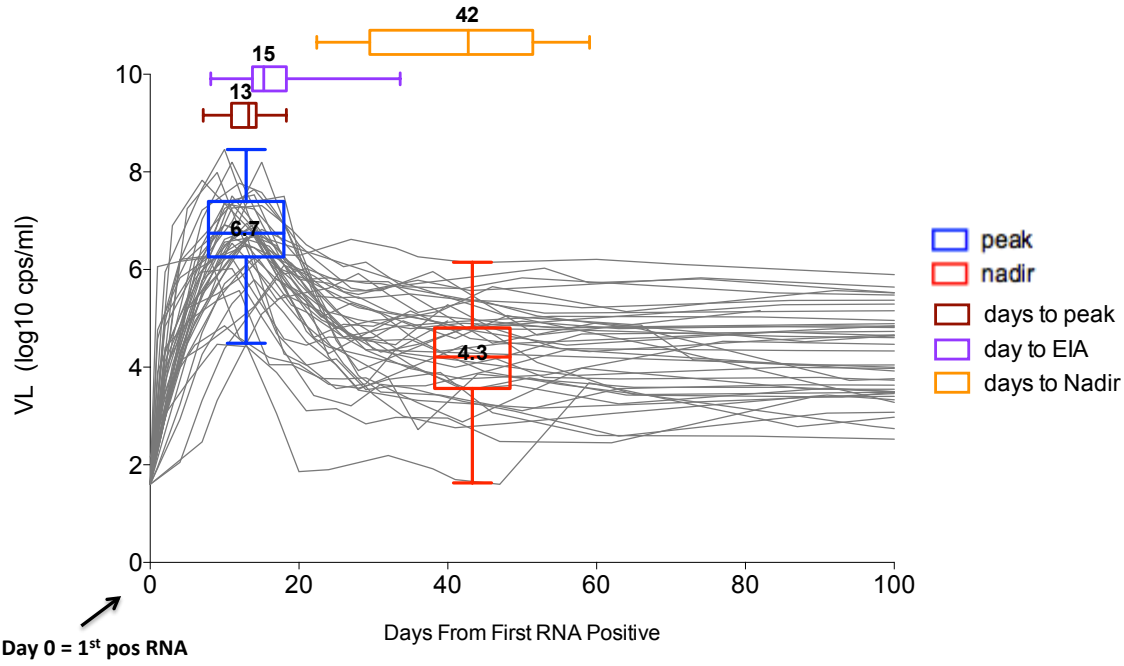


Figure 4.2: Longitudinal viral load values (log_{10}copies/ml) are plotted verses the number of days from first reactive RNA for n=42 cases. In 41/42 cases, the first positive RNA was detected by Aptima and a value of 1.6 log_{10} copies/ml was assigned. The line in the middle of the box plots show the median, the ouside of the boxes shows the interquartile range and the whiskers show the min/max for each parameter. The vertical boxes show peak (blue) and nadir (red) viral loads while the horizontal boxes plots show the ranges of days to peak (brown), EIA reactivity (purple), and Nadir Viral Load (Yellow).

First measured viral loads were median 3.2 log_{10} copies/ml (range 1.6-6.21) and occurred a median 2 days (range 1-6 days) after the first reactive Aptima test. The longitudinal viral load analysis showed a remarkable homogeneity in timing of the viral load dynamics with samples reaching median peak viral load of 6.7 log_{10} copies/ml (range 4.47-8.46 log_{10} copies/ml) by median day 13 (range 7-18)(Table 4.2). In all but two cases, EIA reactivity coincided with or followed viral load peak and

became reactive on median day 15 (range 8-33). Following peak, the viral load then fell to a nadir, defined as the lowest post-peak viral load within 60 days, of 4.3 log_{10} copies/ml (range 1.6-6.14 log_{10} copies/ml) by a median 42 days (range 22-58). Viral load set-point was defined as the mean value of all available viral load measurements after day 80 through 1 year post-seroconversion, prior to initiation of HAART, and with at least 2 measurement required for set point assignment, resulting in a median set point of 4.38 log_{10}copies/ml (range 1.67-5.68 log_{10} copies/ml). Figure 4.3 shows the aggregate viral load plots out to day 720 as well as the window for determining the set-point.

Figure 4.3: Set point viral load and long-term viral load trajectories

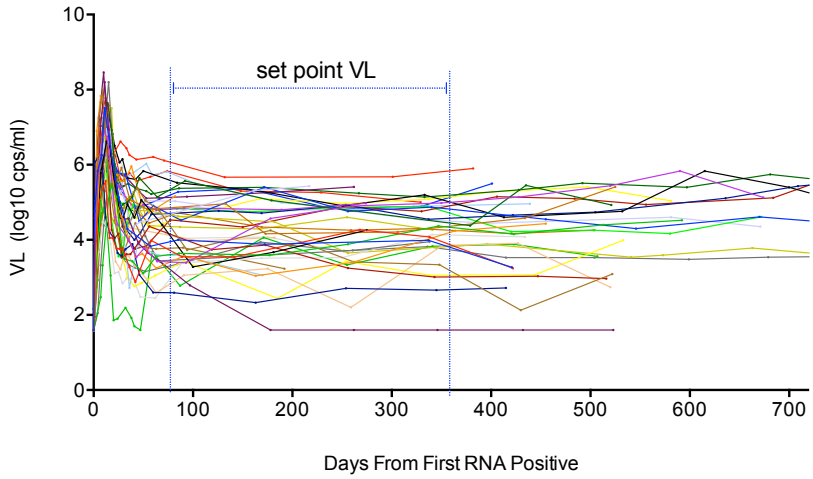


Figure 4.3: Viral load (VL) trajectories for n=42 subjects out to 700 days are shown. Longitudinal viral load values (log_{10}copies/ml) are plotted verses the number of days from first reactive RNA for n=42 cases. Set point viral load was defined as the average viral load between day 80 and 365, prior to HAART.

The viral loads between day 80 and day 365 in each subject were remarkably stable in most cases. Regional differences between Thailand and East Africa were not observed for peak viral load, however there was a regional difference in set point viral load, with Thailand having a significantly higher set point viral load (4.81 log_{10} copies/ml) than East Africa (3.84 log_{10} copies/ml, p=0.0040 Mann-Whitney test) (Figure 4.4).

Figure 4.4: Regional differences between peak and set point viral load

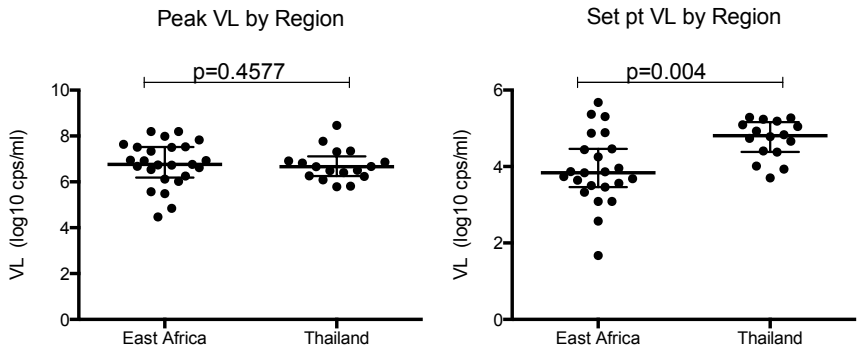


Figure 4.4: Regional differences between Peak Viral Load and Set Point Viral Load are compared using a Mann-Whitney Test. The lines show the median and interquartile range.

Viral load upslope was calculated as the change in viral load from the last negative to the peak viral load, excluding values where the interval between first positive and last negative were more than 10 days. Viral load downslope was calculated as the rate of change from viral load peak to nadir viral load. No regional differences were seen between viral load upslope or downslope (p=0.2996 and p=0.2216, respectively in Mann-Whitney test). See Table 4.2 for a summary of all viral load values separated by region.

Table 4.2: Summary of Viral Load values by region

	All	**East Africa (EA)**	**Thailand (TH)**	
	Median (range)	**Median (range)**	**Median (range)**	**p* value**
Peak VL (log_{10} copies/ml) (n=42)	6.74 (4.47-8.46)	6.76 (4.47-8.20)	6.66 (5.79-8.46)	0.4577
Peak Day (n=42)	13 (7-18)	13 (7-18)	14 (7-18)	0.3132
Nadir VL (log_{10} copies/ml) (n=42)	4.31 (1.60-6.14)	3.65 (1.6-6.14)	4.62 (3.16-5.61)	***0.0055***
Nadir Day (n=42)	42 (22-58)	43 (22-57)	41 (24-58)	0.8143
Set-Point VL (log_{10} copies/ml) (n=39)**	4.38 (1.67-5.68)	3.84 (1.67-5.68)	4.81 (3.71-5.29)	***0.0040***
VL upslope (n=37)***	0.39 (0.25-0.61)	0.30 (0.25-0.61)	0.38 (0.28-0.57)	0.2996
VL downslope (n=42)	-0.10 (-0.03- -0.36)	-0.10 (-0.04 - -0.36)	-0.09 (-0.03 - -0.03)	0.2216

VL=viral load, mL=milliliters,
*Mann-Whitney T test
**3 subjects excluded due to start of HAART before set point
***5 subjects excluded as interval between last negative and first reactive RNA was too long to evaluate viral load upslope.

4.3. Association of VL dynamics to VL set-point

In order to determine the relationships of early viral load time points to viral load set point, several Spearman correlations were performed and are shown in Figure 4.5. There was no association with age and viral load set point in this cohort (rho= -0.07, p=0.66). Peak viral load was correlated to set point viral load (rho -0.3565, p=0.0259), however this relationship was much stronger in Thailand cases compared to East African cases (rho=0.6676, p=0.0059 vs rho=0.3124, p=0.1467). Viral load upslope was not associated with set point (rho=0.1490, p=0.4078) however a trend was observed in the Thailand cases (rho = 0.4897, p =0.056). Viral load downslope was not associated with set point (rho-0.1263, p=0.4078) overall or when broken out by region (data not shown). The most striking association was between nadir viral load and set point viral load (rho=0.7576, p<0.0001) and associations remained strong in both regions. In fact, nadir viral load and set point viral load were found to be not different (p=0.2136, Wilcoxon matched pairs signed rank test) suggesting that set point viral load was established by day 42 of infection.

Figure 4.5: Association of early viral load events with set point viral load

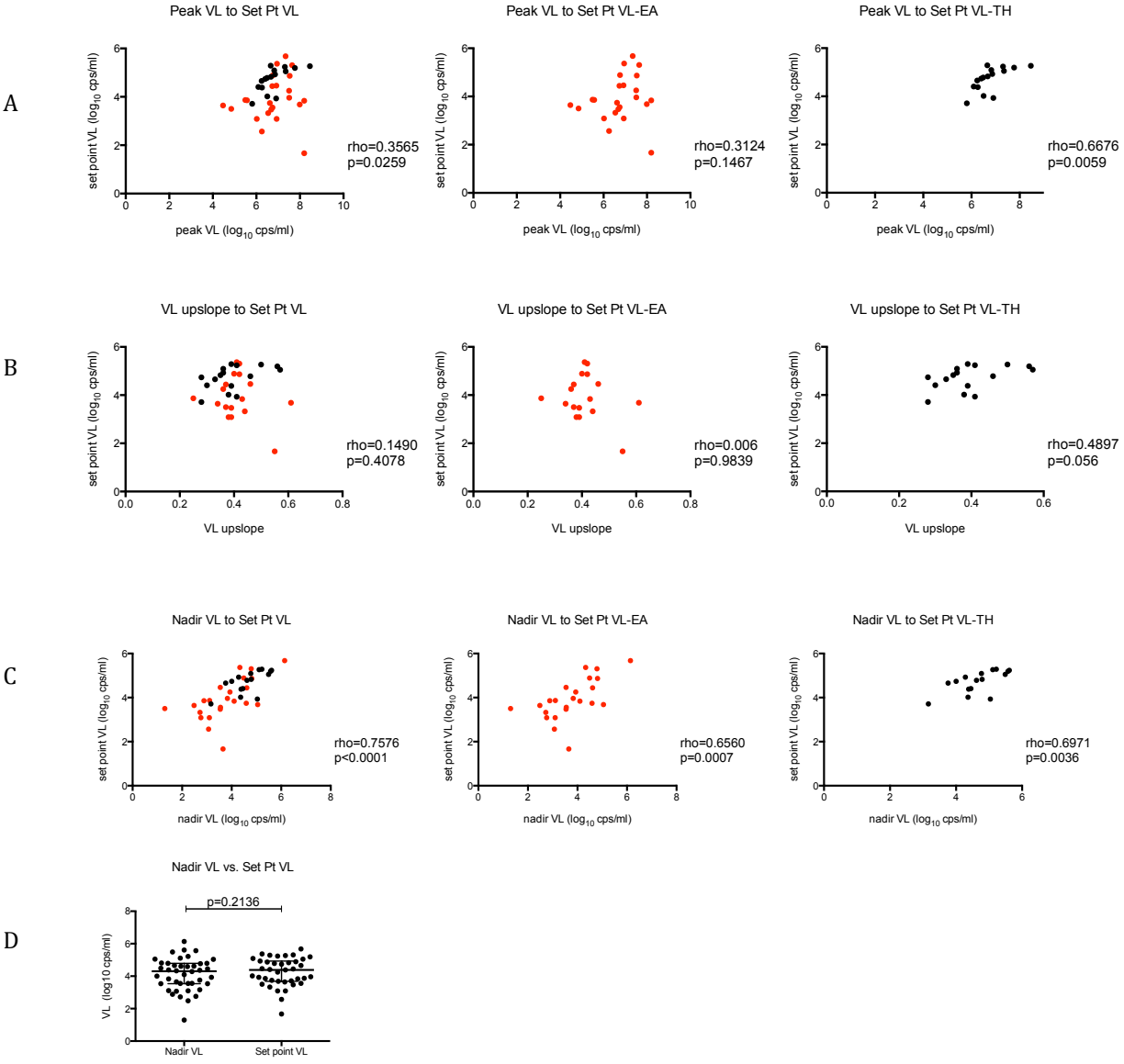


Figure 4.5: Panels A-C are configured with the n=42 cases in the left panel, the n=25 from East Africa (EA) in the middle panel and the n=17 from Thailand (TH) in the right panel. Spearman correlations were used for panels A-C. A shows correlation of peak viral load to set point viral load, B shows the correlation of viral load upslope to set point viral load, C shows the correlation of nadir viral load to set point viral load. Panel D is a Wilcoxon matched pairs signed rank test to show that the nadir viral load and set point viral load are not different. The lines show the median and interquartile range.

4.4. Immunophenotyping

Clinical immunophenotyping (Absolute and percentage CD4, CD8, NK and B cells) was performed at multiple visits during acute infection, including the first visit after a positive Aptima. No pre-infection data was collected, however the first visit was median 2 days (range: 1-6 days) from the first reactive Aptima result. Most (37/42) cases had CD4 values at this visit within expected normal range [150, 151]. Figure 4.6 shows eight representative plots of absolute lymphocyte counts and percentages relative to viral load in the first 300 days. All four participants showed a drop in CD4 absolute counts and percentages coincident with viral load peak while CD8 absolute counts and percentages showed an increase arising slightly after peak viral load. The levels to which CD4s decreased and CD8s increased was variable between subjects however the CD4 drop and CD8 increase were not associated (rho= -0.004, p=0.98). The dynamics of NK cells and B cells were more variable with transient increases and/or decreases coincident with viral load peak.

Figure 4.6: Example Lymphocyte immunophenotyping-4 subjects

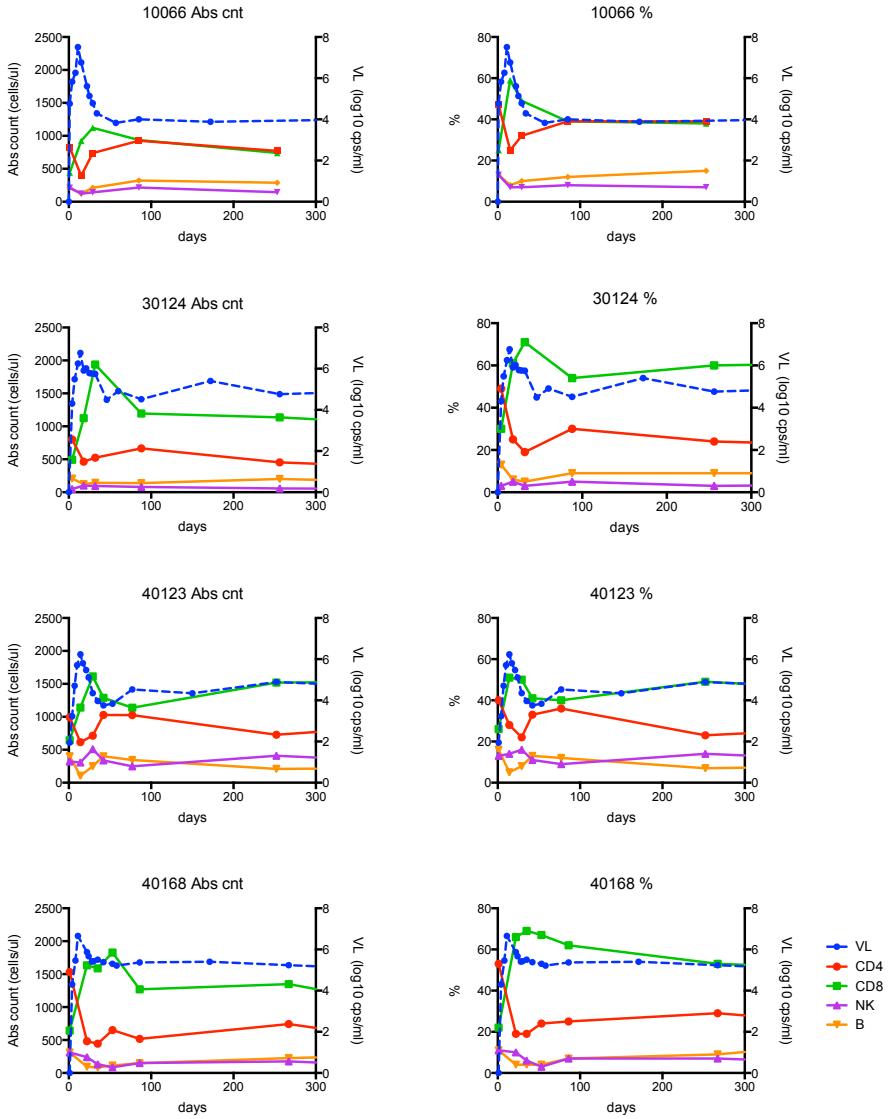


Figure 4.6: Abs count=Absolute count. Clinical Lymphocyte immunophenotyping results for 4 individuals are shown in comparison to viral load. Days are on x-axis and show days from first Aptima reactive The plots on the left are absolute counts and the plots on the right are percentages. The viral load (blue dotted line) is plotted on the right y-axis. CD4s are shown in red, CD8s in green, NK cells in purple and B cells in orange.

Evaluation of lymphocyte immunophenotyping data in aggregate using samples with matching timepoints throughout the time course (N=32) is shown in Figure 4.7. The CD4 absolute counts showed a significant overall drop from the initial visit to the visit coincident with peak viral load. 31 out of 42 subjects exhibited this decrease in CD4 count from the initial visit with a delta of median 339 cells/ul (range:12-1050 cells/ul). Individuals that did not show a decrease in CD4 counts from the initial visit had viral loads close to 1 million copies/ml at the initial visit, indicating that these individuals were further along in infection. CD4 absolute counts transiently rose by

day 97 to initial levels, however counts were significantly decreased again by day 258. CD4 counts appeared to remain depressed at day 518, however the statistics were not evaluated due to the lower number of sample pairings. The CD8 Absolute counts, in contrast, displayed a significant increase coincident with peak viral load on day 17. 34/39 participants showed an increase in CD8 counts between day 2 and day 17; 39/39 participants showed an overall increase in CD8 counts by day 32. The absolute counts began to fall incrementally with each visit, however by day 258, the CD8 counts had not reached initial levels. NK absolute counts showed a non-significant overall increase at day 17, however the dynamics of each individual were variable. All NK counts returned to original levels by day 258. B cells, on the other hand, displayed a significant drop in absolute counts coincident with viral load peak with a median overall drop of 99 cells/ul, but counts returned to normal levels by day 97.

Figure 4.7: Aggregate lymphocyte immunophenotyping data

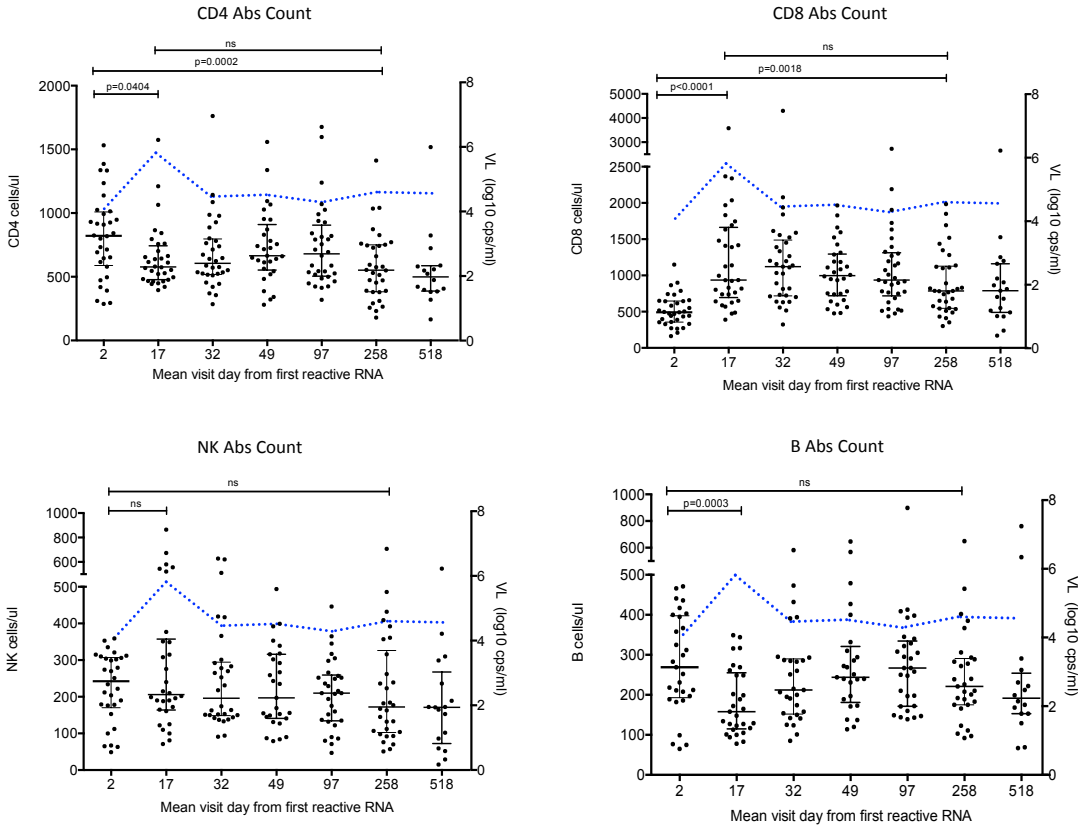


Figure 4.7: The absolute counts for CD4, CD8, NK and CB cells in 32 subjects were plotted by the median visit day on the x-axis. The median VL at each visit is plotted in blue on the right Y-axis. A non-parametric one-way anova test was performed for multiple paired comparisons. The p-values were adjusted to account for multiple comparisons. No statistics were performed for day 518, as not enough paired data was available. The median and interquartile range for each visit day is shown. ns=not significant

Regional differences between lymphocytes subsets were also examined and are shown in Table 4.3.

Table 4.3: Summary of lymphocyte immunophentoyping by region

	All Median* (range)	East Africa (EA) Median (range)	Thailand (TH) Median (range)	p value**
CD4 initial (n=41)	909 (287-1532)	842 (287-1386)	926 (419-1532)	0.6450
CD4 nadir (n=41)	483.5 (142-1211)	501 (285-1211)	511 (312-1093)	0.9531
CD4 12 months (n=37)	525 (180-1412)	606 (186-1412)	433 (180-971)	0.0741
CD8 initial (n=41)	497 (162-1150)	458 (162-742)	612 (271-1150)	***0.0283***
CD8 peak (n=41)	1390 (486-4299)	1235 (486-4299)	1409 (635-2367)	0.9843
CD8 12 months (n=37)	814 (301-2572)	618 (301-1983)	1065 (694-2572)	***0.0002***
NK initial (n=40)	261 (49-516)	197 (49-359)	*309 (81-516)*	***0.0050***
NK peak (N=41)	316 (91-864)	300 (91-864)	377 (154-673)	***0.0255***
NK 12 months (n=35)	183 (51-707)	133 (51-486)	402 (97-707)	***0.0039***
B cell initial (n=40)	306 (65-636)	261.5 (65-636)	354 (182-592)	0.069
B cell nadir (n=38)	138.5 (15-344)	139.5 (65-344)	148 (78-331)	0.9115
B cell 12 months (n=35)	92 (92-649)	201 (92-649)	311 (194-523)	**0.0075**

*All values are absolute counts (cells/ul), initial=first measured value after Aptima reactive, 12 months=samples closest to 12 months
**Mann-Whitney test

At the initial visit immediately after infection, the absolute counts of CD8 and NKs were significantly higher in Thailand than in East Africa, however no differences were observed in CD4 or B cells at this early visit. CD4 counts between regions did not differ at nadir or 12 months although there was a trend. CD8 and NK absolute counts remained higher in Thailand compared with East Africa at the first visit and 1 year timepoint. B cell counts were also higher in Thailand verses East Africa at 12 months. Associations between lymphocyte subsets and viral load data showed that neither initial CD4 absolute counts nor CD4 nadir correlated with set point viral load (rho= -0.06, p=0.7029 and rho= 0.07, p=0.6837, respectively) however overall CD4 count at 1 year correlated inversely with set point viral load (rho= -0.6559, p<0.0001) as shown in Figure 4.8. CD8 peak, NK peak and B cell nadir were not associated with setpoint viral load nor were 1 year timepoints for CD8, NK or B cells (data not shown).

Figure 4.8: Correlations of lymphocyte parameters to set point viral load

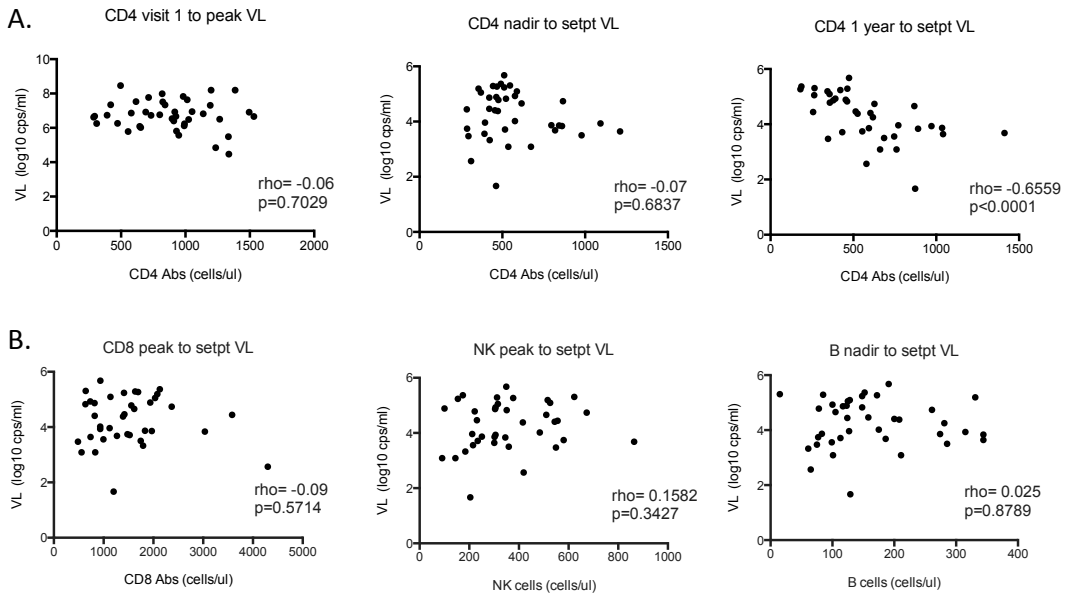


Figure 4.8: Absolute counts (cells/ul) (x-axis) were plotted against viral load (log_{10} copies/ml) (Y-axis). Spearman correlations were performed. Panel A shows relationship of CD4 initial visit after Aptima reactive (Visit 1), CD4 nadir and CD4 at 1 year to viral load set point. Panel B shows correlation between peak CD8 count, NK cell peak and B cell nadir viral load set point.

4.5. Associations of viral load and immunphenotyping with clinical outcome

A surrogate clinical endpoint/outcome was defined as the time to two consecutive timepoints with CD4<350, excluding volunteers that were pregnant or started HAART for reasons other than CD4 less than 350 cells/ul. 13 (9 from Thailand and 4 from East Africa) out of 36 participants reached the CD4<350 endpoint in a median time of 462 days (range 53-933 days). There was no difference in time of follow up of those that reached endpoint and those that did not (p=0.5131). Peak viral load did not differ in those that reached the endpoint, however set point VL was significantly higher (median 4.99 log_{10} copies/ml vs 3.9 log_{10} copies/ml, p=0.0004) in those that reached the endpoint. The CD4 absolute counts at one year were lower (median 357 cells/ul vs 618cells/ul, p=0.0003) and CD8 peaks were higher (median 1693 cells/ul vs 1202 cells/ul, p =0.0254) in those that reached endpoint. NK and B cells at peak/nadir and at one year were not different in those that reached the endpoint. See Table 4.4.

Table 4.4: Summary relationship of viral load and lymphocyte immunophenotyping parameters to surrogate clinical endpoint

	Endpoint*	No endpoint	p value**
# cases* **	13 (9 Th, 4 EA)	23 (7 TH, 16 EA)	n/a
Total days follow up	952 (439-1226)	747 (29-1371)	0.5131
VL peak	6.82 (5.79-8.46)	6.68 (4.47-8.2)	0.6667
VL set point	4.99 (4.4-5.37)	3.9 (1.67-5.68)	***0.0004***
CD4 nadir	464 (285-866)	516 (142-1211)	0.2108
CD4 1 year	357 (180-627)	618 (265-1412)	***0.0003***
CD8 peak	1693 (733-3581)	1202 (486-4299)	***0.0254***
CD8 1 year	1005 (301-2572)	745 (353-2503)	0.2471
NK peak	312 (100-673)	354 (91-864)	0.3937
NK 1 year	183 (57-431)	147 (51-707)	0.6644
B nadir	128 (78-331)	175 (15-344)	0.6432
B cell 1 year	251 (123-523)	225 (92-649)	0.6043

*Endpoint is defined as reaching 2 consecutive CD4 counts less than 350 cells/ul
**Mann-Whitney test
***Cases started on ARVs due to pregnancy or preference excluded

Taking individuals that had been followed with CD4 counts for at least 2 years (n=20 total), 4 cases could be identified as rapid progressors as their CD4 counts declined to less than 350 within 2 years. An additional 4 cases progressed between two and three years. 12 cases did not progress within the follow up period. Set point viral load was higher, although not statistically significant, and CD4 counts were lower at 1 year in those individuals that progressed to CD4< 350 in 2 years. No differences were seen in set point viral load or CD4 counts at 12 months in those that progressed in 2 years verses 3 years.

Figure 4.9: Viral load set point in rapid progressors

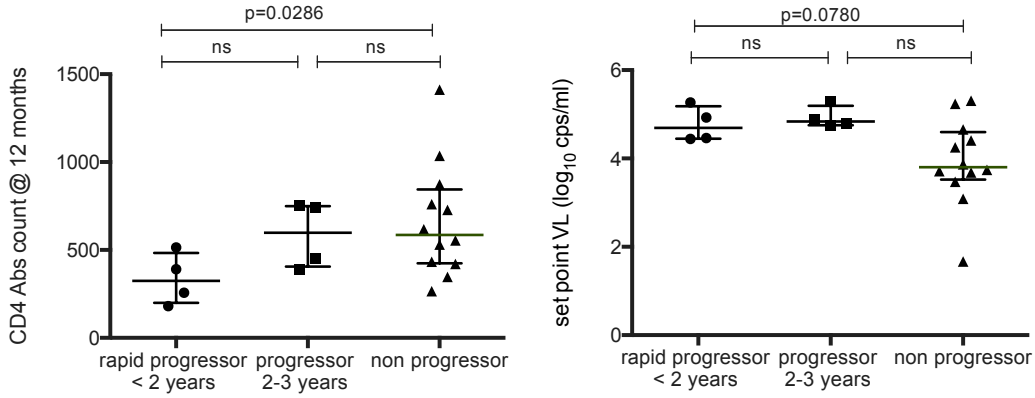
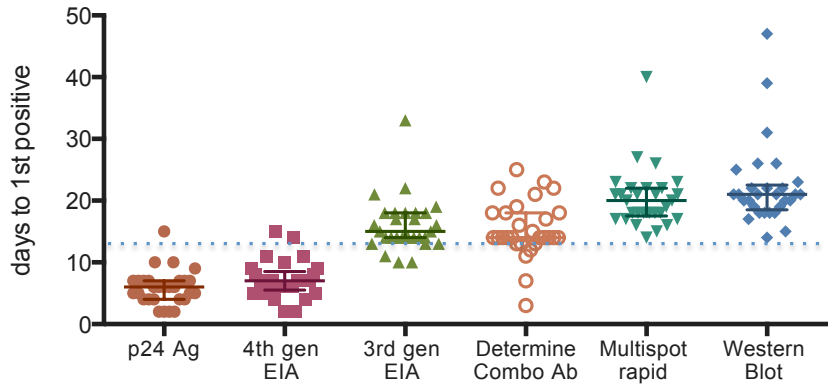


Figure 4.9: Subjects included with at least 2 years of follow up (n=20). Rapid progressors had CD4 counts <350 within 2 years (range 53-590 days). Progressors in 2-3 years had CD4 counts <350 between 2-3 years (range 758 -933 days). The non-progressors did not reach CD4 counts <350 in the time followed (747-1372 days). A Mann-Whitney test was used to test the different in set point viral load and CD4 counts at 12 months in rapid progressors verses non-progressors. The lines show the median and interquartile range in each category.

4.6. Performance of diagnostic testing

In order to evaluate the performance of diagnostic tests in acute infection, the following assays were run on 29 acute case longitudinal samples; p24 antigen quantitative, BioRad 1/2 PLUS O 3rd generation EIA, BioRad Genetic Systems 4th generation EIA, Multispot Rapid, HIV-1 Western Blot and the Determine Combo assay. The time to first reactive result was measured from the time of the first reactive Aptima test. Figure 4.10 shows the days until p24 antigen, 4th generation EIA, 3rd generation EIA Multispot rapid test, Western Blot and the Determine Combo Rapid (antibody only) test. p24 was the first reactive test on median day 6 (range 2-15) from the first reactive Aptima. 4th generation EIA became reactive on and median of 7 days (range 2-15 days), slightly after p24 antigen. 3rd generation EIA did not become reactive until a median 8 days later on day 15 (range 10-33 days), followed by Multi-spot on day 20 (range 14-40). Western Blot became reactive on median day 21 (range 14-47), shortly after Multispot reactivity. No HIV-2 infections were observed by Multispot in these 29 cases. In 28/29 cases, Western Blot was reactive at the same timepoint or after Multispot reactivity.

Figure 4.10: Summary of days to first reactive diagnostic test



	p24 Ag	4th gen EIA	3rd gen EIA	Determine Combo Ab	Multispot rapid	Western Blot
Minimum	2.000	2.000	10.00	3.000	14.00	14.00
25% Percentile	4.000	5.500	14.00	13.50	17.50	18.50
Median	6.000	7.000	15.00	14.00	20.00	21.00
75% Percentile	7.000	8.500	18.00	18.00	22.00	22.50
Maximum	15.00	15.00	33.00	25.00	40.00	47.00

Figure 4.10: Days to first positive/reactive test for p24 Ag test, 4th generation EIA, 3rd generation EIA, Determine Combo, Multispot Rapid and Western Blot. Day 0 is the first reactive Aptima test. The horizontal blue dotted line shows the median day of peak viral load. The lines show the median and interquartile range of days to reactivity for each test.

In evaluation of performance of the 4th generation EIA in 29 Acute HIV infection cases, only 7 (24%) were Reactive by 4th gen EIA at the earliest timepoint (median 2 days past reactive Aptima) with a detectable viral load (Table 4.5).

Table 4.5: Summary of 4th gen EIA reactivity

	# cases (%) 4th gen +	**# cases (%) 4th gen -**
Earliest Viral Load positive timepoint (median 2 days past R Aptima) (n=29)	7 (24%)	22 (76%)
1st timepoint with VL over 100,000 copies/ml (n=29)	27 (93%)	2 (7%)
timepoint with VL between 10,000-100,000 copies/ml (n=15)	1 (7%)	14 (93%)
timepoint with VL between 1,000-10,000 copies/ml (n=9)	1 (11%)	8 (89%)
p24 concentration between 1-50 pg/ml (n=17)	5 (29%)	12 (71%)
p24 concentration between 50-100 pg/ml (n=10)	10 (100%)	0 (0%)

HIV-1 viral loads in these very early non-reactive 4th generation EIA (4th gen) samples ranged from 2.16 to 5.95 $\log_{10}$ copies/ml and were on the early viral upslope before peak viremia. In the 22 4th gen non-reactive cases, p24 concentrations were lower than the package insert sensitivity of 50pg/ml. Five of seven (5/7) cases, however, demonstrated reactive 4th gen results with p24 levels less than 50pg/ml (range 25-49

pg/ml). Samples with viral loads at the first timepoint greater than 100,000 copies/ml were reactive in 93% of cases (n=29). In contrast, with viral loads between 10,000-100,000 copies/ml, 4th gen was reactive in only 7% of cases (n=15). Samples with p24 concentrations between 50-100pg/ml were reactive with 4th gen in 100% cases (n=10).

Figure 4.11 shows the assay reactivity for each of the diagnostic tests for two individuals. In Panel A, this volunteer had increasing HIV RNA viral loads from the time of Aptima reactivity to a peak viral load of 617,000 copies/ml at day 14. The viral load subsequently decreased to a nadir of 3,500 copies/ml on day 28. p24 antigen was detected on day 11 with a concentration of 83 pg/ml which increased to peak on day 14, coincident with peak viral load, and then began to decrease. 4th generation ELISA also became reactive on day 11 and 3rd generation EIA became reactive by day 18. Multispot became reactive on day 21 and Western Blot by day 25. Most of the other acute infection cases studied follow profiles similar to this individual, with the exception of subject 20263, who had a very interesting and unique diagnostic profile (See Figure 4.11-B). Subject 20263 was a pregnant woman who was diagnosed with acute HIV infection in her second trimester. The 4th generation EIA became reactive at day 14, similar to other cases, however by day 18, the 4th generation was non-reactive for a period of 12 days during which the 3rd generation EIA was non-reactive. The 4th generation EIA was reactive again, likely due to the emergence of an antibody response, on day 29 with the 3rd generation demonstrating reactivity on day 33. The Multispot and Western blot reactivity were also delayed and did not demonstrate reactivity until days 40 and 47, respectively. Thus, this individual had a second diagnostic window during days 17-29 where all diagnostic tests were non-reactive, but HIV-1 RNA was still detected.

Figure 4.11: Reactivity of all diagnostic markers in two individuals

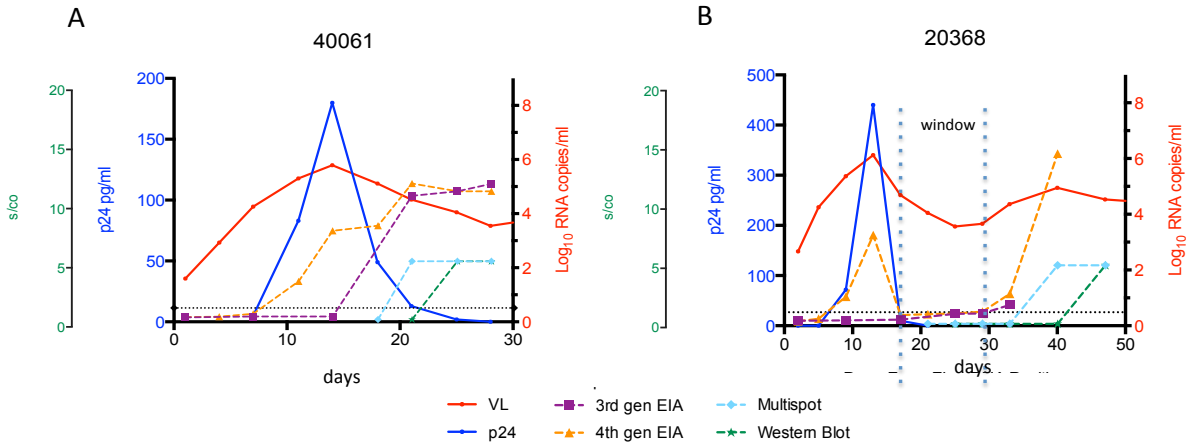


Figure 4.11: Days from first reactive Aptima are shown on the x-axis. The red line shows the viral load (VL) on the right Y axis. p24 concentration (pg/ml) is shown as the blue line on the left x-axis. The dashed lines show the signal to cut off ratios and are plotted on the far left axis and the black dotted horizontal line is the threshold for a positive signal to cut-off in the EIA tests (a s/co of 1 is considered reactive). Panel A represents a typical evolution of diagnostic markers and Panel B shows a unique diagnostic case with a second detection window. The vertical blue dotted lines in Panel B show the window period, where all diagnostic tests, other than RNA, are negative.

4.7. p24 Antigenemia

p24 antigen was measured in 29 acutely infected individuals from the initial timepoint after reactive RNA to 180 days and became reactive on median day 6 (range 2-15). Figure 4.12-A shows aggregate p24 concentration over time. The p24 antigen peaks near the time of peak viral load with p24 peak coincident with viral load peak in 21/29 cases. The peak p24 concentration was highly variable (median 1200 pg/ml, range: 89-24,000 pg/ml) and was strongly correlated to peak viral load (see Figure 4.12-B). Peak p24 concentration, however, did not correlate with set point viral load (data not shown). The p24 antigen duration was transient, with 24/29 subjects reaching undetectable p24 antigen levels by day 35.

Figure 4.12: Aggregate p24 concentrations over time and relationship of p24 to viral load

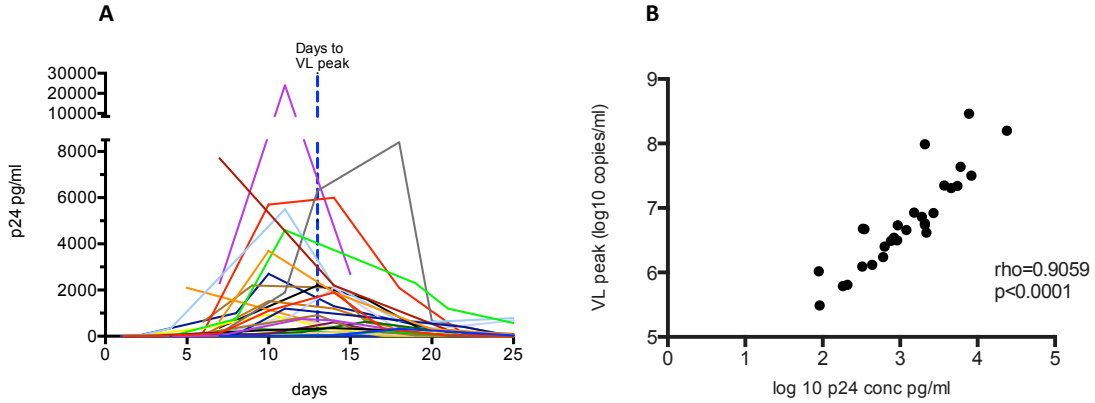


Figure 4:12: Panel A: Days from first reactive Aptima are shown on the x-axis. P24 concentration (pg/ml) is plotted for 29 acute cases. Days to peak viral load is shown as blue dashed line. Panel B: Viral Load peak (log_{10} copies/ml) was plotted against log p24 concentration (pg/ml). A spearman correlation was performed.

Individual p24 curves were plotted against corresponding viral loads for six subjects in Figure 4.13.

Figure 4.13: Example p24 concentrations

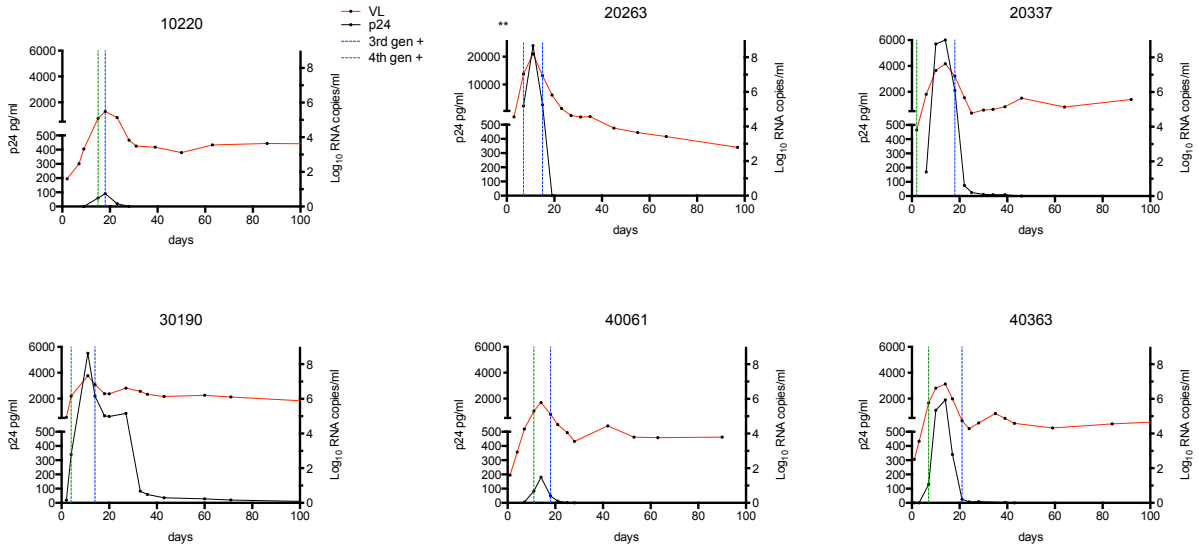


Figure 4.13: Note the different p24 scale in 20263. Longitudinal p24 antigen concentration (black line) is plotted with viral load (red line) with days from first reactive on the x-axis. p24 and VL were run in parallel on the same timepoints. The blue dotted line is the first day of reactivity of the 3rd generation EIA and the green dotted line is the first day of reactivity of the 4th generation EIA.

The initial reactivity of the 4th generation EIA and 3rd generation EIA are also shown in these plots. The 4th generation became reactive after p24 increased and then p24 decreased after 3rd generation EIA becomes reactive, as antibody concentrations increase and antibody-antigen complexes were formed that remove free p24 antigen from circulation. Interestingly, the highest p24 peak concentration (24,000 pg/ml) was subject 20263, the subject that has spontaneously controlled viral load to undetectable levels by day 180 in the absence of HAART. 10220 and 40061 had relatively low peak viral loads (5.49 and 5.79 $\log_{10}$ copies/ml, respectively) and also had very low p24 peak concentrations (91 and 180 pg/ml, respectively). The length of p24 antigenemia was longest in subject 30190, with a low level positive result out to 70 days. In contrast, the other 5 examples had a very transient p24 antigenemia, which disappeared from circulation shortly after day 20. The length of p24 antigenemia was correlated with both peak viral load (rho=0.6170, p=0.0006) and set point viral load (rho=0.5243, p=0.0035), with higher viral loads likely driving antigen persistence.

4.8. Western blot analysis

Western Blots were run on serial samples from the first timepoint after RNA positivity to approximately 180 days in order to determine the patterns of reactivity in acute

infection. Example western blots on longitudinal samples from two subjects are shown in Figure 4.14.

Figure 4.14: Example Western Blot from two subjects

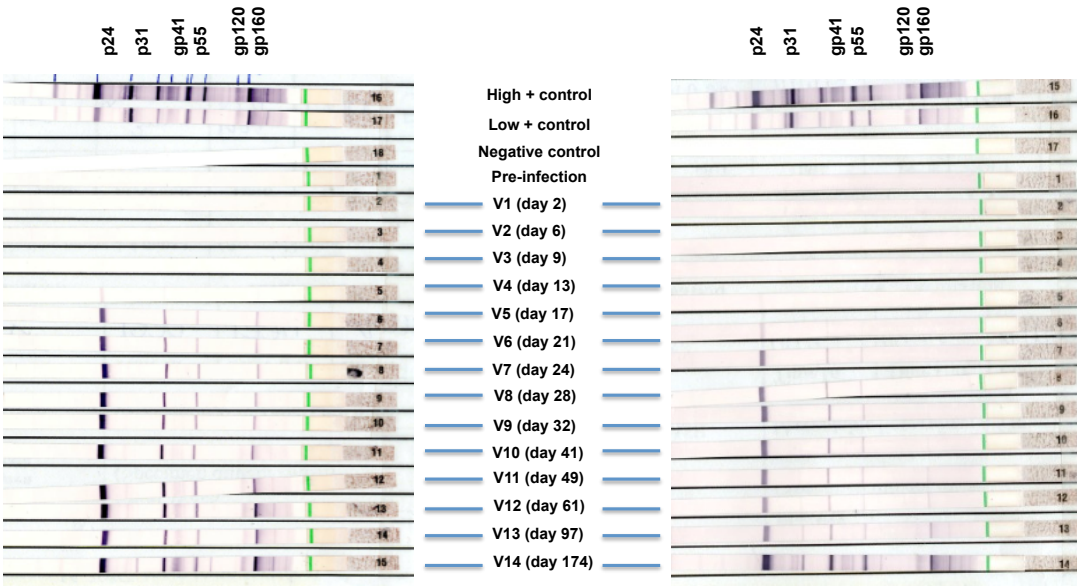
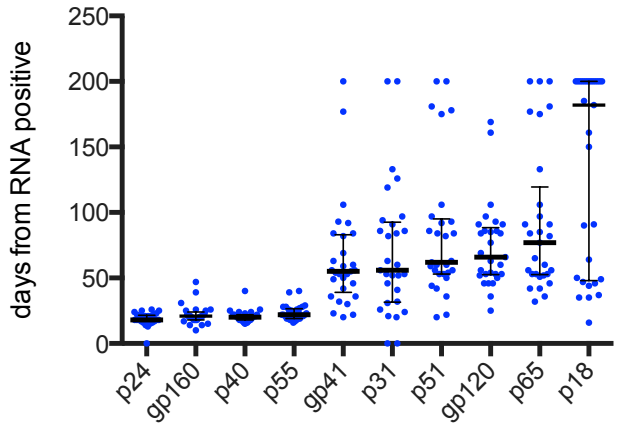


Figure 4.14: Western Blots from longitudinal samples over the course of 6 months are shown. V=visit and median days of this visit in n=29 participants is shown in parenthesis.

As shown previously in figure 4.10, the median time to a positive western blot was median 21 days (range 14-47 days). p24 and gp160 were the first detectable antibody responses, with the first bands appearing as early as 10 days after the first RNA positive timepoint. There was no difference in viral load setpoint in subjects that develop p24 before gp160 antibodies (data not shown). Figure 4.16 shows an example of the evolution of western blot bands in two acute infection cases. In the blot on the left, the p24 band developed first at visit 4, resulting in a final interpretation of Indeterminate. By Visit 5, both gp160 and p24 bands had developed, resulting in a positive interpretation. gp120 and gp41 appeared by visit 12, which wa 69 days from the first reactive Aptima test. In the blot on the right, the p24 band developed by visit 4 (day 15) however the gp160 band did not develop until visit 10 (day 39), gp120 developed on day 86 and gp41 by day 177. In 27/29 cases, the gp120 band developed at least 45 days after the first positive RNA test. The consistent appearance of the gp120 band several weeks after infection may be useful in determining the time from actual HIV infection during early infection. 17/29 samples at baseline had no reactivity to any bands, while 9/29 had a 0.5 score for at least one band. The

remaining 3 cases had a 1+ score or greater at baseline in at least one band, two had a single band with a score of 1 in p31 and one had a p24 band with a score of 1. A summary of the time for each band to become reactive is indicated in Figure 4.15. If a band did not become reactive within 180 days, a value of 200 days was assigned. p24, gp160, p40 and p55 bands became reactive within the first 22 days from first RNA positive and were followed by the remaining bands. The timing of the appearance of these remaining bands was quite variable, with some not detected by day 180. The p18 was the slowest band to develop and was only detected in 16/29 individuals by day 180.

Figure 4.15: Time to western blot band reactivity



	p24	gp160	p40	p55	gp41	p31	p51	gp120	p65	p18
Minimum	0.0	10.00	15.00	16.00	20.00	0.0	20.00	25.00	32.00	16.00
25% Percentile	17.00	18.00	18.00	19.00	39.00	31.50	53.00	52.50	52.50	48.00
Median	18.00	21.00	20.00	22.00	55.00	56.00	62.00	66.00	77.00	182.0
75% Percentile	21.50	24.00	22.00	26.50	83.00	92.50	95.00	88.50	119.5	200.0
Maximum	26.00	47.00	40.00	40.00	200.0	200.0	200.0	169.0	200.0	200.0

Figure 4.15: The time for each individual (n=29) to reach a positive result (>0.5 score) western blot band is shown in this figure. The lines show the median and interquartile range. Since western blots were performed out to day 180, if an individual did not have reactivity to that band by day 180, a value of 200 days was assigned. Individuals who had background reactivity to a band were assigned a day 0 for that band. Only three cases had a detectable (>0.5) band before infection, two with reactivity to p31 and one with reactivity to p24.

As a crude measure of determining recentness from infection the sum of the scoring for all bands from one timepoint in one individual was calculated and several cut-off points were determined in order to approximate the number of days from the first positive RNA test (See Figure 4.16). With a total Western Blot band score of less than or equal to 2, all 29 cases (100% sensitivity) were within 29 days of infection and with a total Western Blot band score of greater than 2 and less than 10, 28/29 (97% sensitivity) cases were less than 55 days from infection. This information may be

useful in early infection to determine the recency of infection, especially after 3rd generation EIA becomes reactive at day 16. This method for determining recency would need to be validated on a larger sample set with samples from chronic infection, as Western Blot scores may decrease over time and with the onset of AIDS [152, 153].

Figure 4.16: Use of Western Blot score to predict recentness from infection in early HIV infection

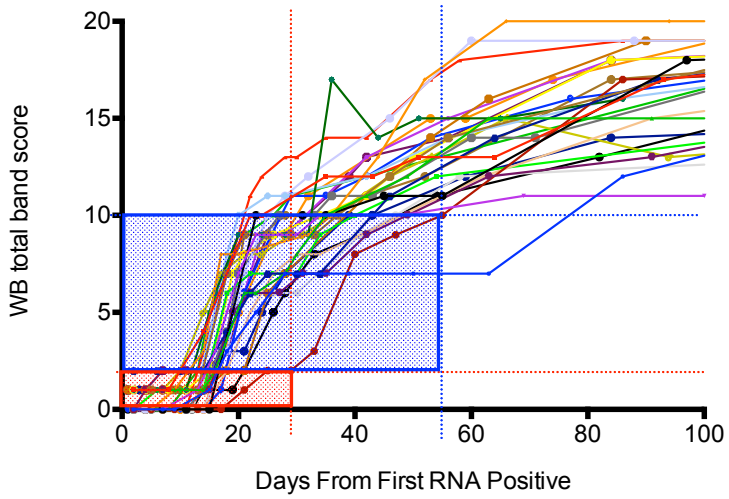


Figure 4.16: The longitudinal Western Blot band score is plotted for the n=29 acute cases verses the days from first reactive Aptima. The Western Blot band score is the sum of the scoring for each Western Blot band for one timepoint. For example, a blot with a 1+ band for gp160 and a 1+ band for p24 would have a Western Blot score of 2. Two cutoffs were determined by visual inspection of the longitudinal data. All timepoints in the red box have a Western Blot band score of 2 or less and are within 29 days of the first reactive Aptima. All but one timepoint in the blue box have a Western Blot band score between 2 and 10 and are within 55 days of infection.

4.9. Performance of the Determine Combo Rapid Test

In order to evaluate the performance of the Determine Combo Rapid test (Combo RT), cryopreserved longitudinal plasma samples were also run and results were compared to other diagnostic tests. The Antigen and Antibody components of the Determine test were evaluated separately. Representative diagnostic profiles from four acute infection cases are shown in Tables 4.6 a-d.

Table 4.6: Longitudinal diagnostic assay profiles for 4 exemplary acute infection cases

Table 4.6.a. Example Diagnostic Profile #1

Days from 1st RNA +	Viral Load (Cps/ml)	p24 conc (pg/ml)	Combo RT Ag	Combo RT Ab	3rd gen EIA	Multi-Spot
2	5.04	28	NR	NR	NR	NR
8	6.51	1000	NR	NR	NR	NR
10	6.92	2700	R	NR	R	NR
14	6.67	1300	NR	R	R	NR
22	4.6	NR	NR	R	R	R
24	4.41	NR	NR	R	R	n/d
35	4.01	NR	NR	R	R	n/d

Table 4.6.b. Example Diagnostic Profile #2

Days from 1st RNA +	Viral Load (Cps/ml)	p24 conc (pg/ml)	Combo RT Ag	Combo RT Ab	3rd gen EIA	Multi-Spot
2	4.93	19	NR	NR	NR	NR
4	6.16	340	NR	NR	NR	NR
11	7.34	5500	R	NR	NR	NR
14	6.83	2200	R	R	R	NR
18	6.3	670	NR	R	R	R
20	6.29	620	NR	R	R	R
27	6.62	850	NR	R	R	n/d

Table 4.6.c. Example Diagnostic Profile #3

Days from 1st RNA +	Viral Load (Cps/ml)	p24 conc (pg/ml)	Combo RT Ag	Combo RT Ab	3rd gen EIA	Multi-Spot
2	4.4	NR	NR	NR	NR	n/d
6	5.09	40	NR	NR	NR	n/d
10	6.5	1520	NR	NR	NR	n/d
14	6.93	1200	NR	NR	R	NR
18	5.79	290	NR	R	R	NR
22	4.93	17	NR	R	R	R
26	4.07	NR	NR	R	R	R

Table 4.6.d. Example Diagnostic Profile #4

Days from 1st RNA +	Viral Load (Cps/ml)	p24 conc (pg/ml)	Combo RT Ag	Combo RT Ab	3rd gen EIA	Multi-Spot
2	3.82	n/d	NR	NR	NR	NR
6	5.87	170	NR	NR	NR	NR
10	7.25	5700	NR	NR	NR	NR
14	7.64	6000	NR	R	R	NR
18	6.92	2100	NR	R	R	R
22	5.67	75	NR	R	R	R
25	4.79	26	NR	R	R	n/d

Table 4.6.a shows a profile where the Combo RT HIV-1 p24 antigen component was reactive at only one measured time point coincident with peak of viral load (6.92 log_{10}copies/ml) and a measured p24 antigenima of 2700pg/ml. Despite a high p24 concentration of 1300 pg/ml at the subsequent visit, the Combo RT Antigen component was non-reactive. This demonstrates the transient, narrow detection window for Antigen in the Combo RT. Table 4.6.b shows a profile where the Combo RT Antigen was reactive over two visits. On the second visit, the Combo Antibody was also positive. Table 4.6.c and 4.6.d show profiles with similar peak viral load and p24 antigen levels to those in Tables 4.6.a and 4.6.b, however Combo RT Antigen was not detected. There was no obvious association with HIV subtype and Combo RT Antigen reactivity (data not shown).

The Combo RT identified acute infection in only 4/29 (13.7%) subjects by Antigen target alone and 1/29 (0.3%) subjects with a positive Antigen and Antibody test (Ag range: 210-5,500 pg/ml) despite device claims of a limit of detection of 12.5-25 pg/ml. The Antibody component of the Combo RT was reactive on median day 14 while Multispot was not reactive until median day 20. Of the 5 subjects with a positive Combo RT Antigen test, only 3 persisted over multiple visits, indicating a very narrow window of detection for p24 antigen by this device. In all five cases, the Combo Ag positive test was detected at peak viral load and near peak p24 antigenimia, however viral load were not different (p=0.10, Mann-Whitney test) in Combo RT that were

Antigen positive versus Antigen negative. Similarly, p24 levels were not different (p=0.16, Mann-Whitney test).

The Antibody component of the Combo RT was positive on median day 14 (range: 3-25), see Figure 4.10. This window to reactivity was significantly lower (p<0.0001) than Multispot (day 20, range: 14-40), but similar to 3rd generation EIA (p=0.76). The number of days to 3rd generation ELISA reactivity was significantly lower than Multispot (p<0.0001). Thus, the Antibody component of the Combo RT performs as well as the 3rd generation EIA test. Both these tests detect Antibody sooner than the Multispot rapid test (see figure 4.17) and also before most other rapid tests on the market [154].

Figure 4.17: Comparison of days to reactivity for Determine Combo rapid test verses 3rd generation EIA and Multispot

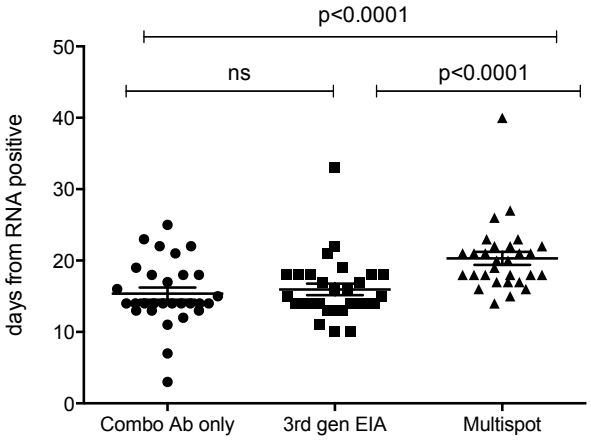


Figure 4.17: The days to reactivity for Determine Combo Antibody, 3rd generation EIA and Multispot are shown. Mann-Whitney tests are performed to detect differences in days to reactivity. The median and interquartile ranges are shown.

In Table 4.5, a summary of test performance at different diagnostic stages is shown. At peak HIV-1 viral load, both Antigen and Antibody Combo RT were negative in 37% of acute infections while 44% were positive for Antibody only. Only 13.7% samples were antigen positive at peak viral load. At the first p24 reactive test, 96.6% of the Combo tests were negative for both Antibody and Antigen. In 12 cases where p24 levels were greater than 2000 pg/ml, only 4 samples were detected by the Combo RT Antigen test, despite the manufacturer's claim of a limit of detection of 25 pg/ml. 85.2% of the Combo RT Ab tests were positive at the first reactive 3rd generation EIA test and 92.8% of Ab tests were positive at the first reactive Multispot test.

Table 4.7: Summary of Combo RT Antigen and Antibody performance

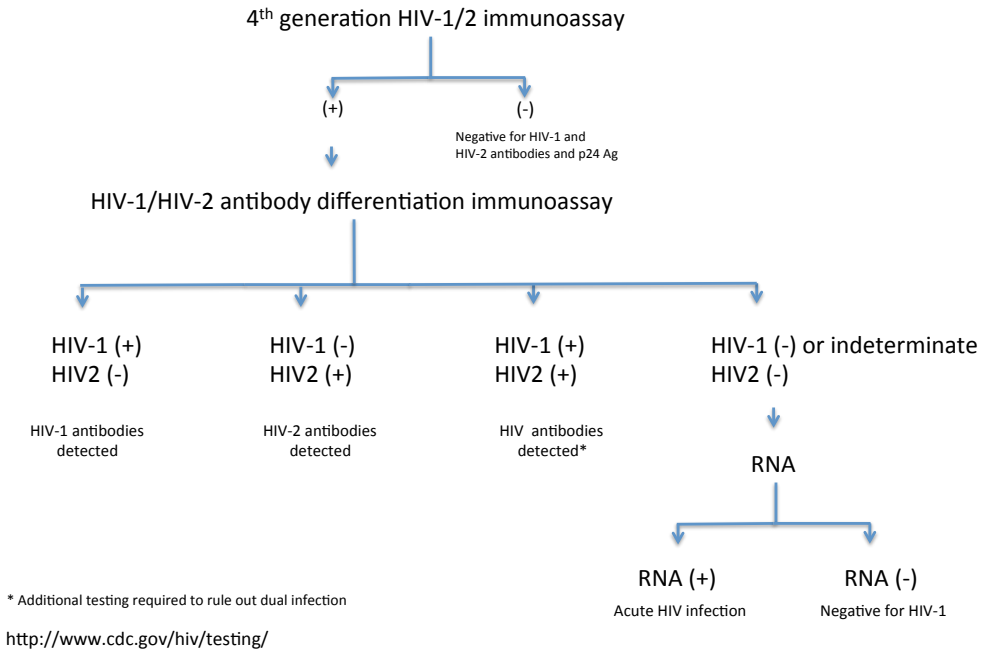
	Combo RT Result			
	Ag+/Ab-	Ag+/Ab+	Ag-/Ab+	Ag-/Ab-
First quantitative p24 +* (n=29)	0 (0%)	0 (0%)	1 (3.4%)	28 (96.6%)
Peak VL (n=27)*	4 (13.7%)	1 (0.3%)	12 (44.4%)	10 (37.0%)
First p24 > 2000 pg/ml (n=12)	4 (33.3%)	0 (0%)	2 (16.7%)	6 (50%)
First 3rd gen + (n=27)**	1 (3.7%)	3 (11.1%)	20 (74.1%)	3 (11.1%)

*Quantitative p24 Genscreen BioRad assay
**2 subjects did not have a Combo RT test at peak VL
***2 subjects did not have a Combo RT at the first 3rd gen EIA +

4.10. Evaluation of newly proposed CDC algorithm

A new HIV diagnostic algorithm has been proposed by the U.S. CDC [136] to improve acute HIV infection (AHI) detection and type differentiation of anti-HIV-1 from HIV-2 antibodies. The algorithm employs a 4th generation HIV-1/2 IA (4th gen) followed by a HIV-1/2 type differentiation immunoassay, and nucleic acid testing, if indicated. See Figure 4.18 for a schematic of the proposed testing algorithm.

Figure 4.18: Schematic of CDC's proposed HIV diagnostic algorithm



In the proposed CDC algorithm, an individual is considered HIV uninfected if the 4th gen immunoassay is non-reactive, and positive for HIV infection if a repeat reactive 4th

gen IA is confirmed by positive Multispot result. An individual is considered to be an AHI case, if the repeat reactive 4th gen IA is reactive, Multispot is negative, and HIV nucleic acid is detected. The simulated algorithm was evaluated by evaluating data previously obtained from the Viral Load, 4th generation EIA, and the Multispot, however no repeat testing was performed for the 4th generation EIA as recommended in the algorithm due to sample volume limitations.

Using the simulated proposed algorithm, maximum AHI detection (reactive 4th gen and negative Mulispot) occurred 5-15 days from the first reactive RNA result. (See Figure 4.19) The algorithm detected 29/29 acute infections in at least one timepoint within the first 15 days post detection of RNA in specimens exhibiting p24 levels above 50pg/ml. After 20 days, most samples were Multispot positive resulting in a "HIV antibodies detected" classification. No HIV-2 infections were observed. One sample was non-reactive by 4th gen EIA between days 16-25, exhibiting a second detection window due to a delayed antibody response.

Figure 4.19: Performance of proposed CDC algorithm in acute HIV cases

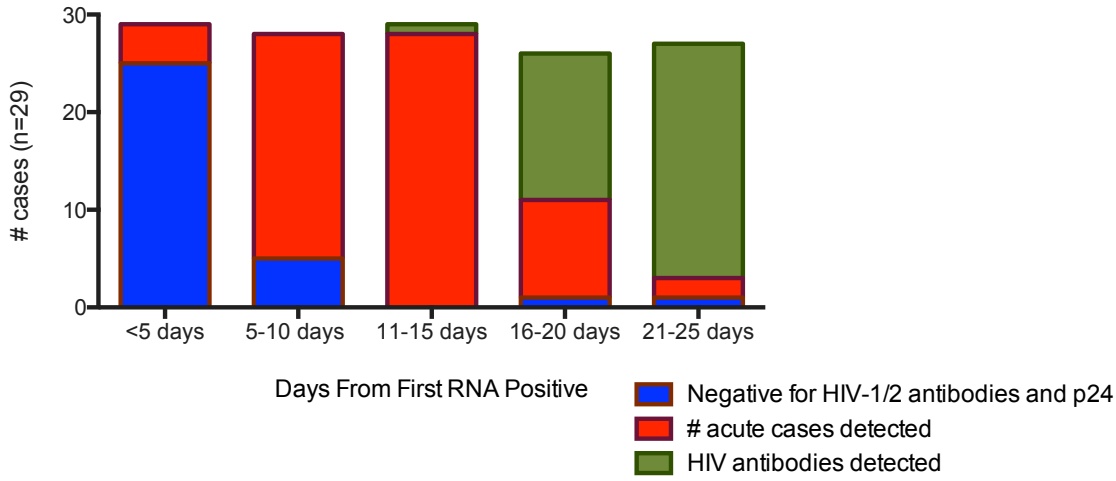


Figure 4.19: Classification using CDC algorithm segregating samples in 5 different categories of time from first reactive Aptima test. Samples from the same individual are represented in different categories. N=29 for most categories, however fewer were available in the 5-10 (n=28), 16-20 (n=26) and 21-25 (n=27) day categories.

4.11. Evaluation of Fiebig staging with RV217 samples

Using the RNA data, p24 antigen, 3rd generation, and Western Blot data from the 28 subjects (one subject, 20368, excluded due to second diagnostic window described previously), samples were grouped according to the previously published Fiebig stage [142] and timing of each stage was evaluated (See Table 4.7). The length of each stage

was compared to the data published in the Fiebig stages and some differences were noted. The most notable difference was in the length of stages 2 and 3, which were longer in the RV217 data compared with the data presented in the Fiebig paper. Stage 3 was concluded by day 19 in the RV217 data whereas it was completed by day 13.5 in the Fiebig data. This difference is likely due to the different kits that were used and the multiple subtypes present in the RV217 data. By stage 4 the timing is similar, ending by day 23 compared with day 19.1 in the Fiebig data.

Table 4.8: Evaluation of Fiebig staging using RV217 samples*

	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6
HIV RNA	+	+	+	+	+	+
P24	-	+	+/-	+/-	+/-	+/-
3rd gen EIA	-	-	+	+	+	+
Western Blot	-	-	-	IND	R (no p31)	R (w/ p31)
Median start day from 1st RNA reactive (range)	0	7 (2-15)	14 (11-15)	15 (10-19)	21 (14-39)	55 (20-133)
Median length stage (range)	7 (2-15)	8 (3-16)	4 (3-8)	4 (2-21)	33 (2-107)	
Cumulative length in days	7	15	19	23	56	
Fiebig cumulative length in days	5	10.3	13.5	19.1	88.6	

*n=28. Subject 20368 excluded due to second diagnostic window from figure 4.11.

4.12. Proposal of new staging using newly available diagnostic kits

The longitudinal evaluation of multiple diagnostic kits in acute infection allowed for generation of a new diagnostic staging system as an update to the previously published Fiebig staging system [142]. Due to the similar reactivity timing for some of the tests, multiple tests were grouped together to allow flexibility in testing algorithms; 4th generation EIA and p24 antigen, 3rd generation EIA and Determine Combo Rapid test antibody component, Multispot and Western Blot. The following are the proposed stages based on the sequential appearance of diagnostic markers from the 28 acute cases described in section 4.11 (Table 4.8).

Table 4.9: Proposed new diagnostic staging of AHI

Assay	Stage 1	Stage 2	Stage 3	Stage 4
HIV RNA	+	+	+	+
4th gen EIA	-	+	+	+
P24	-	+	+/-	+/-
3rd gen EIA	-	-	+	+
Determine Combo Antibody	-	-	+	+
Multispot	-	-	-	+
Western Blot	-	-	-	+
Median start day from 1st RNA reactive (range)*	0	7 (2-15)	14 (7-19)	20 (14-27)
Median length stage (range)	7 (4-15)	8 (3-16)	4 (2-20)	
Median VL (range)	3.56 (1.6-5.950)	5.9 (3.66-8.2)	6.035 (3.42-8.46)	5.43** (3.21-7.5)

** Analysis performed on n=28 cases. **First timepoint in stage 4 only*

The following are descriptions of each stage: Stage 1: RNA positive only, Stage 2: RNA and 4th generation IA or p24 antigen positive (>50pg/ml), antibody IA/rapid negative Stage 3: RNA, 4th generation and/or p24 positive, 3rd generation EIA and/or Determine Antibody Combo positive, Stage 4: as stage 3 except Multispot or Western Blot become reactive. Stage 1 starts with the first reactive RNA test on day 0 as lasts for a median 7 days (range 4-15); Stage 2 begins on median day 7 (range 2-15) and lasts for a median 9 days (range 3-24 days); Stage 3 begins on median day 14 (range 7-33) and lasts for a median 4 days (range 2-20); Stage 4 begins on day 20 (range 14-40) and no length was defined for this stage. As shown in Figure 4.18, analysis of the Western Blot bands may differentiate early from late infection once stage 4 is reached. The viral loads of each stage are shown in Figure 4.20, with peak viral load in stage 2 or 3 in 26/29 cases and viral loads show decline by stage 4. The Absolute counts for CD4 absolute counts in each stage are shown in Figure 4.20, with a decrease seen in stage 2 and slight recovery by stage 4.

Figure 4.20: Viral load and CD4 counts in new diagnostic staging system

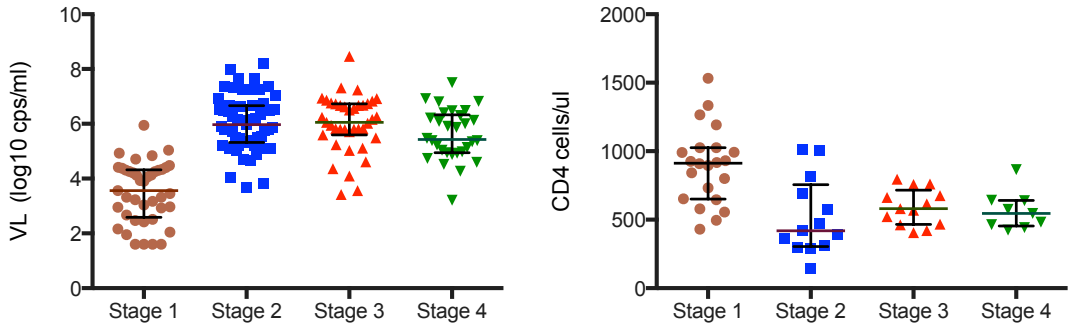


Figure 20: Panel A shows the Viral Load (VL) in each of the newly defined stages. Panel B shows the CD4 absolute count in each of the newly defined stages. The median is and Interquartile ranges are shown.

4.13. SMART plasma

Since the approval of the amendment to the RV217 protocol that allowed SMART tube analysis between 2013 and the end of January 2014, 14 new acute cases were acquired, however most of these cases were acquired after the stage of Fiebig 1/2 , after antibody became reactive by the standard 3rd generation EIA (3rd gen), preventing evaluation of SMART tubes. In addition, incident cases in Thailand were placed on antiretroviral therapy in a separate protocol where SMART tubes were not collected. Only one sample from Uganda could be evaluated for detection of antibody with SMART tube plasma vs normal plasma and no difference in reactivity was seen using the 3rd generation EIA. 4th generation EIA was reactive on the SMART tube plasma, however this sample would also likely be reactive on normal plasma, as the viral load was > 5 $\log_{10}$ copies/ml (Table 4.5). Further evaluate of this method to enhance detection of HIV antibodies is needed.

4.14. Self administered mucosal swabs

Self-administered vaginal or rectal swabs were collected on a total of 237 individuals, 5 of which were individuals who became acutely infected (See Table 4.9). Only one of five cases with swabs preceding the first plasma reactive Aptima timepoint had a swab reactive by Aptima and RNA was detected in the swabs at the timepoint coincident with the plasma reactive RNA test in 2/5 cases. In addition to swabs tested prior to acute infection, swabs of HIV uninfected individuals and prevalent cases were tested during surveillance to determine if exposure events could be detected. Over 1200 swabs were tested during surveillance phase from all four countries. In Kenya, one

subject had an Aptima reactive swab during surveillance but the plasma was non-reactive and all subsequent swab samples were non-reactive. Besides this single reactive swab in an HIV negative subject, the only positive swabs during surveillance were in volunteers with prevalent HIV infection that were enrolled in the study for masking purposes.

Table 4.10: Summary of Swab testing

	Kenya	Uganda	Tanzania	Thailand	Total
# swabs collected	738	1318	100	2500	4656
# swabs tested	283	588	66	283	1220
# subjects tested	110	52	7	68	237
# incident cases with swabs collected	1	2	0	2	5
# incident cases with swab reactive prior to plasma reactive	1	0	n/a	0	1
# incident cases with swab reactive at same time as plasma reactive	0	2	n/a	0	2
# swabs reactive in prevalent/masking case	11	0	0	0	11
# of participants in surveillance (HIV negative) with reactive swab	0	0	0	0	0
# of participants in surveillance (HIV negative) with non-reactive swab	109	50	7	66	232

5. Discussion

5.1. Comparison of cohorts to study acute infection

Acute HIV infection has become the focus of intense research in recent years. The higher transmissibility of HIV in acutely infected individuals [68, 70] makes detection of acute infection a high priority. Intervention in acute infection with antiretroviral therapy may improve clinical outcome by reducing or preventing the establishment of the viral reservoir [72-74, 155]. Study of acute infection also affords the opportunity to understand the early viral and host events in infection that may determine disease progression, leading to insights in possible prevention or treatment interventions. The study of AHI has remained a challenge in the field due to the difficulty in identifying individuals in early infection. Several strategies have been attempted to study early infection including identifying patients presenting at clinics with symptoms common to Acute Retroviral Syndrome (ARS), cross sectional studies sampling individuals accessing routine clinic or community voluntary counseling and testing venues, as well as prospective cohort studies recruiting high risk seronegative individuals who are then followed longitudinally and screened for AHI [31].

Use of clinical symptoms to detect AHI

ARS occurs in a subset of individuals with AHI [66] and may be useful tool for identification of individuals early in infection. In a recent study in Kenya, a risk score reflecting clinical symptoms common to AHI was assigned to patients seeking care at

health care facilities and those meeting criteria were screened for HIV using rapid testing [156]. Seronegative and discordant rapid tests were subjected to p24 antigen and HIV-1 RNA analysis to identify individuals with AHI. This technique identified 5 acute HIV infections from over 3600 individuals screened [156]. Similarly, individuals in Uganda referred for malaria blood smears based on a febrile illness were enrolled in a cross sectional study; 30 AHI cases were identified out of 1000 cases screened [157]. While this strategy may identify individuals in AHI, the presentations of clinical symptoms occurs around the time of peak viremia [67] and may be too late for successful treatment interventions as well as for studying the earliest events in HIV infection.

Cross sectional surveillance to detect AHI

Cross sectional studies have been designed to capture AHI, but most have been inefficient in capturing early samples. In recent years, NAAT pooling techniques, routinely used in blood bank settings to screen for AHI, have been used to identify individuals in AHI for studies outside of blood donors [138, 139]. A significant disadvantage to pooling strategies is the additional time required to de-convolute RNA positive pools for re-test of each pool member independently resulting in a delay in diagnosis. In a study performed in the Southeastern United States, acutely infected individuals were identified by pooled NAAT or by clinical presentation of AHI and EIA seronegative or EIA seroreactive and an indeterminate western blot. In this study, 155 AHI cases were identified, however the time from estimated infection to presentation was 18 days with a range of 4-68 days [158]. In a Nigerian AHI study, clinic and community sites were screened using pooling techniques on serology negative and rapid test discordant individuals. A total of approximately 30,000 individuals were screened and 13 acute infection cases were detected, however viral load upslope and peak viral load could not be evaluated due to the delay in acquisition of appropriate samples [159].

Prospective Cohorts to detect AHI

Additional methods for detection of AHI are prospective cohort studies that follow individuals at high risk for HIV infection with frequent follow up visits and HIV NAAT testing. In Cohen's review of the detection of acute HIV infection, major challenges in developing these cohorts were described including the reduction in acquisition due to repeated HIV counseling, the cost of following uninfected participants prospectively, as well as identifying subjects close to the window of infection[31]. Several cohorts

have attempted to capture seroconverters by following subjects longitudinally with hopes of obtaining individuals early in HIV infection. In a study performed in Italy in 1999, seroconverters were followed longitudinally to study the impacts of early infection, however only one viral load was performed within 2 years of seroconversion [160]. Similarly, in the Multicenter AIDS Cohort Study, homosexual male seroconverters had a window between last negative and first positive 7 months or less [160] and therefore early acute infection was not captured. One of the few acute infection prospective cohorts in the developing world is the Centre for the AIDS Programme of Research in South Africa cohort. In this study, women at high risk were followed monthly, however the definition of acute infection was quite broad with either detection of antibodies within 5 months of the last negative HIV test or HIV RNA positive/antibody negative [161]. In this study, 57 cases of acute infection were identified, however the median time to enrollment from the time from infection was 41 days [162]. None of these studies, however, captured sufficient early AHI samples with frequent blood collections to measure the earliest events in HIV infection.

RV217

The RV217 study bridged the aforementioned gap in obtaining the very earliest samples in AHI using a novel prospective cohort method of twice weekly fingersticks and NAAT testing on high-risk individuals, leading to acquisition of samples at frequent intervals during early infection. The study reliably obtained samples in AHI where the median day between the last RNA negative visit and the first reactive RNA was 4 days in the 42 cases studied. The median number of samples obtained on the upslope of viral load before peak was 3 (range 1-5), permitting a clear definition of upslope and peak viral load. Similarly, the highly precise and accurate definition of peak viral load and frequent sampling permitted calculation of viral load downslope and nadir without reliance upon complicated mathematical modeling that has been performed in previous studies [81, 82, 163]. The collection of these early samples allowed, for the first time in human cohorts with diverse subtypes, a comprehensive evaluation of the impact of early events in AHI as well as methods for detection of acute infection.

5.2. Viral Load Dynamics

Most information to date regarding viral dynamics of acute infection has been obtained by study of primate models of Simian Immunodeficiency Virus due to the ability to infect animals at a defined time and collect blood and tissues at frequent

intervals. It is difficult, however, to compare the absolute values of viral dynamic landmarks such as upslope, peak viral load, downslope and set point values to those in HIV infection, as the infecting SIV strains and type of primate infected have different viral properties from those of human infection. The basic dynamics, however, of viral load increasing to a peak viral load then subsequently decreaseing to a steady state level of varying levels is similar between SIV and HIV infection [85, 87].

The association of set point viral load to disease progression has been well established [75, 77] however the impact of early events and determinants of set point viral load in acute infection have not been well studied due to the lack of collection of early samples. The most informative studies to date regarding the relationship of early viral load dynamics to set point viral load and disease progression have occurred in the SIV model. Several early SIV studies in the 1990s focused on the relationship of early viral dynamics with long-term progression, however results have not been not consistent. Lifson, et al. showed that viral load on day seven post-infection in SIV infection was associated with post-acute viral load [82], suggesting that events occurring within the first week of infection were influencing long term course of disease. Viral dynamics post acute infection were also shown to be crucial in SIV infection in two separate studies. In the first, peak viral load was not correlated with long term survival, however viral load at 6 weeks was predictive of survival [87]. Strapans, et al. demonstrated in SIV infected monkeys that the total post-peak decline of virus was a predictor of disease progression while viral upslope was not associated with viral downslope or long-term outcome [86]. Differences in viral dynamics and disease outcomes in primates, when infections are performed with the same virus, reemphasize the role that host mechanisms play in the pathogenesis of SIV/HIV [103].

Viral Load Upslope, Peak and Downslope

As noted, few previous human studies have been able to calculate upslope of viral load due to the inability to capture individuals early enough in infection, however RV217, due to frequent early sampling, was able to define viral load upslope. Using plasma donor panels, Fiebig calculated the upslope of viral load at an average of 0.35 $\log_{10}$ copies/ml per day [142]. This is very similar to the calculated upslope in RV217 of 0.39 $\log_{10}$ copies/ml per day. In RV217, the viral load upslope did not demonstrate an association with viral load set point in the overall sample set, however there was a trend towards significance in the Thailand region (see Figure 4.5). This suggests that events occurring weeks after infection, such as adaptive immune responses, are also

involved in determining the course of HIV infection, as strong associations of upslope and set point would have downplayed the importance of these later events.

Several studies refer to peak viral load as being the highest viral load measured within the first months of AHI [84, 164, 165], however in most cases, true peak viral load has not been not captured. Interestingly, the Fiebig study did not define a peak viral load, although viral load in the different stages were described with the highest median viral load occurring in stages 2 and 3 in the range of 5.4-5.6 $\log_{10}$ copies/ml [142]. Kaufmann, et al. were able obtain samples prior to peak viral load and in this group of 41 patients, the peak viral load was determined to be 6.35 $\log_{10}$ copies/ml by use of mathematical modeling [163]. A follow up study by the same investigator showed similar viral load results [77]. In these two Kaufmann studies, the timing to peak was 12.2 and 10 days respectively, however day 0 was not clearly defined; thus, it was difficult to compare this data to that obtained from RV217 which demonstrated peak viral load occurring on median day 13 (range 7-18) of 6.7 $\log_{10}$ copies/ml. Several studies have attempted to associate peak viral load with outcome, however study results are conflicting. Linback et al. showed that peak viral load, obtained through modeling, was correlated to set point viral load [166]. Similarly, in a 2007 study, the earliest viral load collected during symptomatic phase of AHI and therefore near peak viremia, predicted set point [67]. In contrast, peak viral load levels in the first 120 days were not predictive of disease course in a study by Schacker et al., however true peak was not determined in this study as no samples were available on the upslope to accurately define peak [79]. In one study by Kaufman [163], no association was seen with peak viral load or downslope with set point viral load, however in a follow up study by the same study group, peak viral load was weakly associated with viral load at 12 months [81].

In RV217, peak viral load was associated with viral load set point (Figure 4.5), although the relationship was much stronger in Thailand than in East Africa. In studying individual viral load profiles, especially in East Africa, it was clear that peak and set point viral load were not always associated. For example, in subject 20263, this peak viral load was one of the highest observed in the 42 cases (8.2 $\log_{10}$ copies/ml), however this individual was able to control viral replication to undetectable levels by day 180. This individual does not have an HLA type previously shown to be associated with viral control (data not shown). Additionally, plasma samples tested for presence of HAART were negative in this subject. In contrast,

subject 30190 had a peak load of almost 8 log_{10} copies/ml, but maintained a viral load set point of almost 6 log_{10} copies/ml. The correlation of peak viral load to set point viral load suggests that a combination of early factors (viral, host, adaptive immune responses) may be important in determining viral load set point, as adaptive immune responses are just beginning the time of peak viral load and are known to be essential in control of viremia [32, 91, 92].

Similar to the analysis of upslope and peak viral load, the determination of post viral load peak downslope requires an accurate peak viral load, which has not been precisely determined with early and frequent RNA measurement in most previous studies. The Kaufman studies were able to predict peak viral load and therefore able to calculate a downslope of approximately -0.07 log_{10} copies/ml per day [81, 163]. The day of nadir in these studies was farther out from the time of infection (day 76 vs day 42 in RV217) due to differing definitions of nadir viral load and as a result, the RV217 downslope is steeper at -0.010 log_{10} copies/ml per day. If CD8+T cells play a role in the control of viremia, one might postulate that the ability to more quickly suppress viral replication might lead to lower viral set point and better disease outcome. Blattner et al. showed that the rate of viral clearance was associated with a longer post-nadir steady state viral load and also with the progression to AIDS [82], however in contrast, downslope was not correlated with viral load at 6 months or 12 months in the study by Kaufmann [81]. The data from RV217 showed that the downslope of viral load from peak to nadir had no association with set point viral load. Further studies should be performed to determine if viral load upslope, peak or downslope are related to establishment of the viral reservoir.

Viral Load Nadir and Set point

Viral load nadir has been well described, as many studies were able to identify individuals on the post peak viral load downslope, allowing for accurate definition of nadir, however definitions are disparate. The Kaufmann studies used modeling to determine nadir viral load and derived values of 4.32 and 4.5 log_{10} copies/ml, respectively [81, 163] using the definition of the lowest post-peak viral load in the first year of infection. In a Nigerian population, nadir viral load was 3.5 log_{10} copies/ml on day 76 [159] while in subtype C infection, the nadir at day 60 was calculated to be 5.1 log_{10} copies/ml[167]. In RV217, viral load nadir in RV217 was calculated as the lowest post-peak viral load within the first 60 days and was 4.31 log_{10}copies/ml on median day 42. Few studies have evaluated the relationship between nadir viral load and set

point, however Kaufmann et al. demonstrated that nadir viral load was strongly associated with VL at 6 and 12 months [81]. One of the most striking associations in the viral load dynamics in RV217 was the association of viral load nadir and set point. While perhaps it is not surprising that these values were highly correlated, the values are, in-fact, not different (Figure 4.5), suggesting that viral load set point and therefore likely disease outcome [75] was established within the first 42 days of infection.

In addition to calculations of very early events in AHI, the nature of sampling in RV217 allowed for a detailed assessment of VL set point. The overall setpoint in RV217 was a median of 4.38 $\log_{10}$ copies/ml (range 1.6-5.68, Table 4.2), which is very similar to the set points described in other studies. Richardson et al. reported a set point viral load of 4.6 $\log_{10}$ copies/ml in a study of Kenyan adults [164] while a set point of 4.76 $\log_{10}$ copies/ml was observed in a study in HIV infected sex-workers in Kenya [77]. In a small study of 8 AHI cases in subtype C infection, the viral load set point was 4 $\log_{10}$copies/ml [165] and in a Nigeria study, set point viral load was 4.5 $\log_{10}$ copies/ml [159].

RV217 is the first known human study to precisely determine the viral load upslope, peak viral load, and downslope in early HIV infection. While previous studies have examined some of these parameters, few, if any, have had the frequency of sampling to determine these parameters with accuracy. The peak, downslope, and nadir viral load calculated in RV217 are similar to the values modeled in the Kaufmann studies in the late 1990s. In RV217, the time to peak viral load appears to be fairly uniform, however the actual values of peak and set point viral load are extremely diverse showing a range of almost 4 logs between the minimum and maximum values (See figure 4.4). When separated by region, the range of peak and set point viral load spans approximately 4 logs in East Africa, while the range is only 2 logs in Thailand. There are many factors that could contribute to diverse viral replication including viral genetics, host genetics, HIV specific immune responses, and immune activation status. East Africa is comprised of more diverse subtypes including single and recombinant subtypes containing A, C and D, whereas the subtypes in Thailand are almost exclusively CRF01_AE. There are conflicting reports as to whether infections with different subtypes of HIV lead to different viral load set points [168-173]. In a study conducted in Uganda and Zimbabwe, viral load set point (overall mean 4.2 $\log_{10}$ copies/ml) was found to be different with subtype D infections having almost a half log higher viral load set point than subtype A infections [171]. Kiwanuuka, on the

other hand, found no difference in viral load set points between subtype A and subtype D infection [173]. A study in southern Africa found no difference between viral load set point in subtype C verses non-subtype C infection [172]. Several studies have also suggested different disease outcomes [43, 174-176] based upon viral subtype. In addition to viral differences, host genetics are likely to contribute to viral load variability as HLA types [177-184] and Killer Immunoglobulin Receptor haplotypes [182, 185, 186] have also been linked to different viral load outcomes. For example, in a study in 2003, HLA-B57 expression resulted in viremic control of less than 5000 copies/ml in the absence of HAART in six out of nine individuals [179]. HIV specific immune responses are known to influence viremia slightly before peak viral load and HIV specific T cell responses are associated with viral load set point [91, 92, 187]. The type of HIV specific immune response generated also appears to have influence over the impact on viremia. In a study by Kipeiela, breadth of gag specific CD8+T cells responses were associated with viral load control while increased breadth of env specific responses were associated with increased viremia[188]. Immune activation has been shown to be an important factor in the pathogenesis of HIV [189, 190] and may be contributing to the broad dynamic range within RV217 viral load data. The role of cytokines is unclear, but research shows that certain cytokines may play a role in determining viral load set point and therefore disease outcome[191-193]. Cytokines may also be important in determining viral loads as their production can activate and recruit CD4 T cells; therefore, increasing the pool of target cells available for HIV infection. In contrast, other cytokine display anti-viral activity and reduced set point viral load in SIV models [194]. A comprehensive analysis of all of the innate and adaptive immune responses, viral genetics, and host genetics are being performed on the RV217 samples in order to understand all of the different factors that may be contributing to viral load set point and disease outcome.

Regional differences between Thailand and East Africa were present for set point viral load, with higher viral loads identified in Thai participants. It is difficult, however, to draw conclusions from these findings based upon regional differences in infecting subtypes, sex of the subjects, host genetic differences, as well as routes of exposure. While it has previously been reported that women have lower viral loads than men [80, 195, 196], we cannot yet distinguish what is contributing to the regional differences seen in this study.

Fiebig, et al. described a low level intermittent viremia that was present before the sharp increase in viral load towards peak viral load [143]. The blood collection schedule in RV217 was specifically designed to detect this "stutter" in RNA detection, however in AHI 95 cases identified by RV217, this phenomemon was not observed. The limit of detection for the Aptima assay, 20 copies per ml plasma, employed in RV217 was not as sensitive as that of the VL assay used by Fiebig. While unlikely, the "stutter" observed by Fiebig could have been missed in our assays. No published reports have confirmed Fiebig's observation of intermittent viremia.

5.3. Immunophenotyping

In addition to careful study of the viral dynamics in acute HIV infection, RV217 afforded the opportunity to study the dynamics of lymphocytes early in infection and their relationship to viral load and disease outcome. Although baseline samples were not measured for lymphocyte counts, the median time of sampling was 2 days from the first Aptima reactive and most participants had lymphocyte values within the normal reference range. In contrast to viral dynamics, sampling was not frequent enough to determine upslope and downslope in the lymphocyte subsets as measurements were taken every two weeks. It is likely, therefore, that lymphocyte true peak/nadir were not determined in this data set. Lymphocyte absolute count data was the focus of the analysis, especially for CD4 counts, as this measure is the most clinically relevant parameter in HIV infection.

CD4+T cells are well known to be the target for HIV infection. Levels of CD4+T cells are known to decline in early infection in the circulation as well as in the gastrointestinal tract [33, 34, 197]. The decline of CD4+T cells is though to arise from several mechanisms including the direct killing of virally infected cells, either by immune cell killing of the virally infected cell or by virus induced cell death [198]. In addition to virally infected cells, uninfected CD4+T cells may be depleted by mechanisms including: apoptosis by interaction of the gp120 from infected cells with the CD4 receptor on uninfected cells; or, via bystander killing due to toxicity of viral proteins and locally produced cytokines as a result of immune activation [197]. Destroyed cells may not be readily replaced as production of new CD4+T cells is impaired by destruction of progenitor cells as well as due to damage in the lymphoid organs that produce new CD4+T cells [197]. During HIV infection some CD4+T cells will traffic to the lymph nodes, leading to a depletion in CD4+T cells in circulation [199]. The eventual immunodeficiency in untreated individuals is though to be the

result of "dysregulation and ultimately failure of host homeostatic mechanisms and cellular immune networks" caused by the inability of the central memory cells to regenerate the effector population [200].

During acute infection, while the virus is replicating to peak levels, there is a profound reduction of CD4 cells in circulation. In addition, during this phase of infection, the virus preferentially targets CD4+T cells in the gastrointestinal tract, were 20 percent of CD4 cells are directly killed by virus and another 60% are killed via indirect mechanisms [32]. It is thought that this enormous depletion of CD4+T cells in the mucosa and in circulation is a major detriment to the integrity of the immune system and that course of disease may already be set at this early stage [35]. During acute infection, the viral reservoir is also established where resting CD4+T cells and gastrointestinal associated lymphoid tissue become latently infected with HIV [39].

Given the importance of CD4+ T cells in HIV disease pathogenesis and progression, many studies have attempted to use CD4 counts early in infection to predict disease outcome. In a study by Kaufmann et al., CD4 absolute counts reached a nadir of 418 cells/ul 17 days after clinical symptoms of AHI and by 12 months, the median CD4 count was 470 cells/ul [81]. In this study, CD4 counts in the first 30 days and nadir CD4 count were associated with CD4 counts at 6 and 12 moths [81]. Similarly, in RV217, the CD4 counts dropped significantly between the first sampling timepoint shortly after infection and the next timepoint, approximately 2 weeks later, coincident with the peak of viral load. The CD4 cells slowly began to recover several weeks later, however CD4 counts never returned to the initial levels (Figure 4.7). The CD4 nadir was highly associated with CD4 count at 12 months (rho=0.5864, p=0.0002), however the drop from the initial timepoint shortly after infection to nadir was not associated with CD4 counts at 12 months. Subtype differences in CD4 counts during infection have previously been described. In one study, CD4 counts were higher for subtype CRF01_AE compared to subtype B while subtype C had lower CD4 counts at seroconversion [176]. Kiwanuka, et al. also showed differences in CD4 counts between subype A, subtype D and recombinant infections as well as differences in rates of CD4 decline [173]. In RV217, no differences were observed between the East Africa regions and Thailand in initial CD4 count, CD4 nadir and CD4 count at 12 months, although a trend was seen for higher CD4 counts in East Africa at 12 months. A comparison of subtypes was not possible due to the diverse subtypes present in East Africa. Given the higher set-point viral loads in Thailand and the overall relationship between CD4

counts at 12 months and set point viral load, it is surprising that there was not a statistical difference between CD4 counts at 12 months in East African and Thailand. These differences may appear at a later timepoint.

The decrease in viremia during acute HIV infection has been linked to the emergence of HIV-specific CD8 positive T cell responses [91, 92]. The specificity of this response has been associated with viral load set point and disease progression [187]. The importance of CD8+T cells in control of viremia was also demonstrated in a primate study where anti-CD8 monoclonal antibody was administered during SIV infection [201]. The reduction in CD8+T cells caused elevations in viral load in peripheral blood as well as in lymphoid tissues compared to control animals that did not receive the antibody [201]. The total CD8+T cell response has not been well described during AHI, as much focus has been placed on the specific subsets of CD8+T cells as well as HIV specific CD8+T cell responses. In one case study, an acutely infected individual with severe clinical symptoms had a CD8 count of 14,000 cells/ul (normal range is approximately 200-1000 [150, 151]), which then declined drastically as peak viremia declined [202]. In a study by Kaufman et al., CD8+T cell peak was reported as 1700 cells/ul, which occurred approximately 2 weeks after peak viral load, then declined to 1023 cells/ul at 12 months [81]. In this study, CD8 absolute counts were not associated with CD4 counts or RVA levels at 6 or 12 months [81]. In RV217, CD8 absolute counts rose to peak levels, median 1390 cells/ul, coincident with peak viremia however a wide range of absolute counts of CD8 peak was observed (range 486-4299). This wide range in peak values may be due to the infrequent sampling intervals for lymphocyte immunophenotyping and inability to capture true peak counts in every individual. CD8 absolute counts continued to decrease with each 2 week measurement, but did not reach initial levels by day 258. Regional differences in the initial and 12 month CD8 absolute counts occurred, with higher counts evident in Thai participants in both cases. Causation for this difference is difficult to define given the many confounding factors between the two populations. The higher CD8 count at 12 months in Thai participants may be related to the higher viral load set point with CD8 T cell increases being driven by viral antigen. Peak CD8 count and CD8 count at 12 months were not associated with the CD4 count at 12 months in the RV217 data set.

NK cells are innate cells that are the first responders in viral infection. NK cells can eliminate HIV infected cells through direct killing, production of antiviral cytokines

and through antibody dependent cytotoxicity [203]. Few studies have been performed to characterize the role of NK cells in acute infection. Alter, et al. showed an increase in NK cell percentages in acute HIV infection compared to controls including treated and untreated chronic as well as HIV uninfected individuals [204]. In a follow up study, the same group showed that NK absolute percentages and absolute counts were increased in individuals with acute HIV infection and this rise was found to precede the rise in CD8+T cells [205]. The limitation in these two studies was that longitudinal samples were not evaluated and therefore dynamics of NK cell changes could not be evaluated. In an SIV study of early infection, no changes were observed in NK absolute counts before and after infection [206]. In RV217, a non-significant transient rise in NK cells occurred that quickly returned to initial levels by day 32. It is likely that any substantial rise in NK cells might have been missed due to the lack of measurements between day 2 and day 17. There was a significant difference in the levels of NK cells at the initial, peak and 12 month timepoint between Thailand and East Africa, with Thai participants demonstrating higher NK cell counts. Higher levels of NK cells in HIV infected and uninfected Thais compared to North Americans has been previously reported [207], however no studies have shown differences in these population between Africans and Thais.

Several studies have shown that B cell dysfunction and dysregulation are evident in chronic HIV infection [93], however few studies have examined the role of B cells in early infection. As early as the 1990s, it was shown that B cells numbers were below normal reference levels in primary infection [208]. A follow up study in 2010 showed decreased B cells in early and chronic HIV infected patients compared to uninfected controls and B cell populations increased after initiation of antiretroviral therapy [209]. Several studies in acute SIV infection have shown a decrease in B cells during the first weeks of infection [206, 210, 211] however there have been conflicting results on recovery of B cells after acute infection. In RV217, a significant decline in absolute B cell counts was observed between the initial visit and day 17, coincident with peak viral load. The B cells quickly recovered, however, returning to initial levels by day 32, and remained at consistent levels through day 258. There were regional differences observed in the B cell counts at 12 months, with higher counts in Thailand. Again, given the multiple differences between regions, it is difficult to know the cause of this difference. In contrast to effector cells such as NK cells and CD8 cells, it has not been reported that B cells counts would be higher in presence of greater viral antigen.

5.4. Relationship of viral load and lymphocyte subsets

In RV217 participants, there were no associations between CD8, NK or B cell values at initial levels, peak/nadir, or 12 month visit with set point viral load (Figure 4.8), or CD4 counts at 12 months (data not shown). Surprisingly, there was also no association of viral load peak with CD8 peak or CD4 nadir. Given the role of CD4 cell populations as targets for viral replication, an association between the viral load peak and CD4 nadir might have been expected. There are limitations in these analyses, as described earlier, in that frequent measurements of lymphocyte counts were not made; thus, it is possible that true peaks and nadirs were not evaluated in these lymphocyte subsets. Particular subsets within these cell populations may well be associated with viral load peak, set point and/or CD4 count at 12 months, however only total absolute counts were evaluated in this study. Only one study to date by Kaufmann evaluated CD8 counts in acute infection in relation to CD4 counts at 1 year and set point viral load and found no relationship [81]. No available literature describes the association of these total subset populations to viral load set point and disease outcome; thus, it is not possible to compare these results with other studies. Currently, several studies are ongoing within RV217 that examine lymphocyte subsets in greater detail including the role of HIV specific cells in controlling viremia and predicting outcome.

Not surprisingly, associations were evident between CD4 counts and viral load. Previous studies have shown associations between VL and CD4 counts at various times during infection [81, 83, 163, 165]. Initial and nadir CD4 counts, however, were not associated with peak or set point viral load. An association of the initial CD4 count and peak or set point viral load might have suggested that the number of targets available could influence viral replication. It is possible that the frequency of particular subsets of CD4 cells, such as the activated CCR5+ memory subset that is the main target for infection, may be predictive of viral load set point, but that was not studied here. Neither viral load upslope, peak, or downslope were associated with the CD4 count at one year, however viral load at nadir and set point were inversely correlated with CD4 count at 1 year (Figure 4.8). As described earlier, the CD4 nadir was also associated with CD4 at 12 months. In summary, then, the only events analyzed in AHI in the RV217 study that were associated with viral load set point were the viral load peak, viral load nadir, and CD4 count at 12 months. Likewise, the only early viral load events associated with CD4 counts at 12 months were viral load set point and CD4 nadir.

5.5. Disease progression

There has been debate in the field as to the best predictor of disease progression in HIV infection. Historically, the most widely accepted prognostic marker used clinically is the CD4 count [48, 52]. CD4 count, until recently with the introduction of test and treat strategies [212], has been used to guide initiation of antiretroviral treatment as well as used in monitoring effectiveness of treatment. In the 1990s, several papers showed that both plasma viral load and CD4 counts were prognostic markers for development of AIDS [75, 83]. Mellors et al. showed that plasma viral load was the best predictor of disease progression and a strong relationship was also observed between viral load and the decline of CD4 counts [83], however the samples used in these predictions were acquired at least one year after infection. In a study conducted in France, set point viral load was highly predictive of progression to death [78]. Several studies have suggested that RNA levels after seroconversion, rather than earlier RNA measures, are better indicators of progression than early viral loads [213, 214]. In a 2005 study, high set point viral load and early low CD4 count predicted death in a group of African women [77].

Although RV217 is early in follow up, a preliminary analysis was performed to assess the relationship of events in AHI early infection with disease progression. A surrogate endpoint was defined as the number of days until reaching two consecutive CD4 counts less than 350, excluding individuals who had initiated HAART due to pregnancy or other reasons. Thirteen individuals out of 36 reached the surrogate endpoint. Of all parameters analyzed, higher viral load set point and lower CD4 absolute count at 1 year were associated with reaching the surrogate endpoint. CD8 absolute counts were also slightly higher in individuals reaching endpoint. These data confirm previously published reports emphasizing the importance of viral load set point and CD4 count in determining clinical progression. Lack of early events predicting outcome suggests that it is a later homeostatic viral replication and immune function that determines outcome and not one single parameter in early infection.

Clinical progression of HIV infection can be divided into several groups, including progressors, long-term non-progressors and rapid progressors [46, 215]. Rapid progression is defined as CD4 count decline to less than 350cells/ul within 3 years of infection [46]. In RV217 participants followed for at least two years (n=20), four

individuals progressed to CD4s<350 cells/ul within 2 years and another four individuals progressed between 2-3 years. Twelve participants did not progress during the follow up period of at least three years. Consistent with other published studies [46, 215], RV217 participants with rapid progression had elevated viral loads and decreased CD4 counts, however no difference was seen in viral loads or CD4 counts in those that progressed in two years verses three years.

5.6. Performance of diagnostic testing

There are many benefits associated with efficient diagnosis of HIV infection, however an estimated 60% of worldwide HIV infections are undiagnosed [216]. Knowledge of HIV status has proven beneficial in reducing transmission. Several studies have shown that individuals who are aware of their HIV status are less likely to engage in risky behaviors [101, 217]. On the individual level, knowledge of HIV status and linkage to care and treatment are essential in improving clinical outcomes of HIV infection [218, 219].

HIV testing has evolved since the first tests were approved in the 1980s. Technologies in HIV testing have improved over time, with each generation of ELISA/EIA further reducing the window of detection. The first generation ELISA detected infection after 35-45 days and is no longer on the market, while the second generation EIA technology reduced this window to 25-35 days [113]. Second generation ELISA technology is generally no longer used however, most rapid diagnostics have a technology similar to second generation ELISAs, as only IgG is detected. The 3rd generation EIA, which became available in the 2000s, reduced the detection window to 15-20 days from the time of infection by including detection of IgM antibodies that appear sooner in infection than IgG antibodies [113]. Although the 3rd generation tests were an improvement, they were not useful in detection of acute infection. In addition, antibody testing alone has been shown to miss a substantial number of HIV infections [220].

While detection of acute infection remains a challenge, it remains a priority due to the public health impact of increased transmissibility during acute infection [68-71]. Some have proposed used of clinical score algorithms combined with rapid testing to identify acute infection [221-223], however clinical symptoms are not present in all AHI cases [66] and algorithms may not be effective in all settings. In RV217, for example, the number of ARS symptoms reported by participants were much fewer

than have been reported in other studies (personal communication, Dr. Merlin Robb, US Military HIV Research Program); thus, a detection system based on clinical symptoms in this setting would have been ineffective. HIV markers that can be detected earlier in infection than IgM antibodies are p24 antigen and viral RNA. The p24 antigen assays is rarely used for screening/diagnosis due to the transient nature of p24 antigen in serum/plasma, however it can be extremely useful marker when attempting to diagnose acute infection. In order to further reduce the detection window, 4th generation IAs were developed that add detection of p24 antigen to the detection of IgG and IgM antibodies. The 4th generation tests allow for simultaneous detection of p24 antigen and HIV-1/HIV-2 antibodies, but do not permit discrimination between antigen and antibody detection. These tests have been used widely in Europe since the late 1990s.

Two studies in 1998 described the performance of 4th generation tests from Europe and reported a reduction in window period of approximately 4-5 days from that of 3rd generation tests [224, 225]. To date, several 4th generation tests have been marketed in the U.S. and their performance has been well established, showing an overall reduction in the detection window[226]. The first 4th generation EIAs to be approved by the U.S. FDA were the Architect HIV Ag/Ab Combo Assay (Abbott Diagnostics, Abbott Park, IL, U.S.)[227] in 2010 and the BioRad Genetic Systems Ag/Ab Combo EIA (BioRad, Redmond, WA)[228] in 2011. The extremely high sensitivity and specificity of these two tests in diagnosing established infection (antibody reactive) has been documented in several studies [229-231], although most studies have occurred in the U.S. with subtype B samples. The few studies conducted in non-subtype B settings also demonstrated excellent performance in established infection [231-233].

The detection of AHI has improved with the use of 4th generation tests. In a study by Eshleman, 13/21 acute samples were detected using the Architect assay and undetected samples had a viral load lower than the detected samples [234]. Similarly, a separate study found that 80% of samples with positive viral loads and negative antibody were detected with the Architect assay. These remaining 20% of samples that were non-reactive in the Architect had lower viral loads when compared with the reactive tests [235]. Moreover, Pilcher et al. demonstrated that 87% of acute cases could be detected by the Architect EIA [121], while another study indicated that the same assay could detect 97% of acute cases with viral loads greater than 30,700 copies/ml [236]. In a comprehensive study on the performance of the BioRad GS

Combo EIA, the test demonstrated varying sensitivity in detecting AHI in different populations, but showed increased detection of AHI over 3rd generation tests, and reduction of the detection window [231]. The Architect assay and the BioRad GS combo have been directly compared and although both assays perform very well, the GS Combo has been shown to be slightly more sensitive and specific in low prevalence populations [237].

In RV217, serially collected samples in AHI were run on several different diagnostic platforms (See Figure 4.10). The BioRad 4th gen GS Combo EIA demonstrated improved detection of AHI over the 3rd generation EIA and HIV-1 Western blot. The 4th generation EIA became reactive on median day 7 (range 2-15) after the first reactive Aptima result, one day after median p24 reactivity and 8 days before 3rd generation reactivity. The 3rd generation EIA was reactive on median day 15 (range 10-33), coincident with peak viral load or slightly later and 6 days before the Western Blot was reactive.

As shown in previous studies [231, 234], the detection of p24 Ag by the 4th gen EIA in RV217 was dependent on viral load and/or p24 concentration (Table 4.5). Samples with viral loads between 10,000-100,000 copies/ml and p24 concentrations below 50pg/ml were only reactive in the 4th gen EIA in 7% and 29% of cases, respectively. The reported p24 limit of detection in the 4th gen IA is 50 pg/ml, but 5/17 cases below this level were reactive regardless. In contrast, with a viral load of over 100,000 copies/ml and a p24 concentration between 50-100 pg/ml, sensitivity was 93% and 100% respectively in detecting AHI, before the rise of antibody. The 4th gen EIA, therefore, was highly effective in reducing the window of detection in a diverse viral subtype sample set. The evolution of diagnostic markers is shown in two cases in figure 4.11 and 4.12. Figure 4.11 shows a typical sequence of reactivity for the tests used in these analyses. As predicted by previous studies [121, 142, 231, 235], viral RNA is the first maker to be detected soon after infection, followed by a transient p24 antigenemmia. The 4th gen EIA becomes reactive when adequate p24 levels are reached, followed sequentially by the 3rd gen EIA, Multispot and Western Blot.

Figure 4.12 shows an individual with a second diagnostic window, where 4th gen EIA becomes non-reactive after p24 antigenemia declines and before antibody tests become reactive. Therefore, for a roughly 14 day window, the participant was non-reactive or negative by all tests studied, except for RNA. The individual is a Kenyan

woman that was diagnosed with HIV infection in her second trimester of pregnancy. Pregnancy is known to disrupt the immune system [238-240] and could be contributing to the delayed antibody response. The second diagnostic window has been reported in several other cases [225, 241, 242] with 4th generation tests. The benefits of 4th gen testing are apparent, however a small percentage of cases might be misclassified as HIV uninfected due to this second window. Therefore, it is important for clinicians to consider risk, patient status, and clinical symptoms when deciding if repeat testing or additional testing, such as p24 antigen or RNA, are required.

p24 antigen levels

p24 antigen levels in plasma are known to be associated with the level of HIV replication, especially in acute infection [107]. The role of p24 antigenemia testing in diagnosis of AHI has been limited due to the lack of availability of approved kits in the U.S. as well as the transient detection window for p24 antigen. p24 antigen tests have been used to assist in diagnosis of potentially HIV infected infants [111, 112, 243] and may have utility in diagnosis of AHI if 4th gen tests and/or RNA testing are not available. In RV217, p24 concentration was measured from the first timepoint after a reactive Aptima to approximately 180 days. As shown in Figure 4.13 and 4.15, the p24 antigenemia is transient and rises and falls coincident with viral load. As shown in other studies [107, 142], peak viral load was highly correlated with peak viremia (Figure 4.12), but interestingly, peak p24 antigen did not correlate with set point viral load despite the previously shown association with peak viral load and set point viral load. This is in contrast to other studies that have shown a relationship between levels of antigenemia and disease progression, although not in acute infection [244, 245]. The duration of p24 antigenemia in RV217, however, was related to peak viral load and set point viral load, which is likely due to persistence of antigen in the presence of viral replication.

Western blot analysis

The HIV-1/2 western blot has traditionally been used since the 1980s for supplemental confirmation of HIV screening antibody test reactivity [114, 115]. In RV217, the appearance of antibodies to viral antigens in early acute infection was carefully evaluated. Figure 4.17 shows the timing of the evolution of specific Western Blot bands. Our data confirms, in non-subtype B AHI, previous findings that p24, gp160 and p55 bands [116, 117, 142, 246] are usually the first Western Blot antigen to be detected. p18 was the slowest band to develop and was only detectable in 16/29

(55.2%) individuals by day 180. p31 antigen reactivity has been proposed by Fiebig as the viral antigen to differentiate late stages of acute/early infection [142], however our data show that p31 is detected over a wide range of days and therefore may not be a reliable indicator of later stage infection. Fiebig's analysis was conducted on subtype B plasma donors and could account for the differences in findings. In our non-B data-set, there were not sufficient numbers to analyze by subtype, however no obvious associations were observed with time to reactivity and geographical region. Previous studies have shown that the reactivity or lack of certain antigen reactivity may be indicative of clinical progression or stage of infection [152, 153, 247] while others have proposed the use of Western Blot to predict time from infection in early infection [116, 117, 142]. In addition to evaluating Western Blot responses separately in RV217, a total score for each individual Western Blot timepoint was assigned by summing the score for each individual antigen (Figure 4.18). Analysis shows that a total score of 2 or less is indicative of a sample within the first 29 days of infection, while a score between 2-10 was indicative of samples within 55 days of infection. When Hecht et al. used a slightly different methodology, where the number of antigen bands (not taking into account intensity of the bands) were analyzed compared to days from infection, detection of 3 antigen bands or less was 43% sensitive and 98% specific for infection within 30 days, while 6 antigen bands or less were 79% sensitive and 86% specific for infection within 30 days[117]. Our current data set does not permit calculations of specificity. Sensitivity when using a score of 2 to detect infection within 29 days is 100%. Using a score of >2-10 to detect infection within 55 days yields a sensitivity of 97%. The disadvantage of using this determination of recency is that once Western Blot antigen reactivity begins to evolve, it is difficult to determine how recently an individual has been infected. This method would not be useful once antigen reactivity is florid. Intensive research is now focused on development or modification of recency assays for samples which are 6 months or farther from infection [31].

5.7 Performance of the Determine Combo Rapid Test

Several strategies are available for detection of acute infection, including NAAT, 4th generation IAs, and p24 assays. The burden of HIV infections, however, is highest in developing countries [10] where access to these technologies is not widely available. The use of rapid tests has been invaluable in the role out of HIV care and treatment programs in developing countries. The performance of rapid tests has been well established, however their failure to detect acute infection has been reported [220,

248]. Recently, the Alere Determine HIV-1/2 Ag/Ab Combo rapid test (Combo RT) was approved by the Food and Drug Administration for detection of antibodies to HIV-1/2 as well as p24 antigen [249]. In contrast to the 4th generation IA tests, the Combo RT allows for distinct interpretation of antigen and antibody results, theoretically allowing diagnosis of acute infection if the p24 antigen is reactive and the antibody component of the test is non-reactive. There has been considerable excitement regarding the Combo RT and therefore several studies have attempted to verify the manufacturer's claims of 92.2% sensitivity to detected antigen positive, antibody negative samples [125]. While one of the early studies showed a sensitivity of 86.6% to detect antigen positive, antibody negative samples [130], subsequent studies have reported much lower sensitivities for the antigen component of the test. Rosenberg et al. reported that in a field study in Malawi, 8/8 cases of AHI were missed when using the Combo RT antigen only, resulting in a sensitivity of 0% for detection of acute infection despite 5/8 having detectable levels of p24 antigen in a separate p24 antigen assay. In addition, the antigen only component had a specificity of 98.3 %, misclassifying 14 uninfected individuals as acutely infected. Ironically, the antibody portion alone demonstrated better sensitivity and specificity for detecting acute infection and also performed very well in detecting established infection (99.4% sensitive and 99.2% specific) [128]. Similar poor sensitivities were detected in the United Kingdom [126] and in a study conducted on p24 culture supernatants of differing subtypes [126, 127]. A more recent study evaluated the performed of the Combo RT on subtype B longitudinal samples and found an improved detection of 72.2% AHI cases with the p24 component of the test [250].

RV217 afforded the opportunity to evaluate the performance of the Combo RT on longitudinal samples of multiple subtypes collected very early in infection and to compare performance with multiple diagnostics tests. Our results confirm the poor sensitivity of the antigen component of the test shown by other investigators, as only 4/29 (13.7%) of subjects were detected as antigen +/ antibody – and 1/29 as antigen +/antibody + despite p24 concentrations well above the device claims for limit of detection in all 29 samples. The 5 cases with positive antigen detection on the Combo RT were reactive at peak p24 and near peak viremia, however the viral load and p24 levels were not different in samples that were antigen positive verses antigen negative on the Combo RT. The Antibody component of the Combo RT performed equally well as the 3rd generation EIA test in detection of antibody, becoming reactive 6 days before the Multispot Rapid Test (Figure 4.10). Specificity could not be evaluated in this

sample set due to lack of HIV negative sampling. Another limitation of this analysis was performance of testing cryopreserved plasma samples rather than whole blood. Our findings, as well as others, indicate the Combo RT would greatly benefit from use of a dissociation step to increase sensitivity of the Ag component of the test. [109, 110]. In addition, the vendor has personally communicated that a later version of the test, released in 2013, may be more sensitive in detection of antigen and therefore further evaluation of this test may be warranted.

5.8 Evaluation of new proposed CDC algorithm

Until recently, the recommended HIV testing algorithm in U.S. for diagnosis of HIV infection included repeatedly reactive immunoassays with a supplemental confirmatory test such as the Western Blot [115], however these supplementary tests can be labor intensive and expensive. Given the importance of detection of acute infection, an algorithm that will detect AHI is desirable as is the ability to type differentiate HIV-1 from HIV-2. Given these requirements, a new screening algorithm has been proposed that employs a 4th generation IA, a HIV-1/HIV-2 type differentiation assay, and NAAT detection assays (see Figure 4.19) [137]. Several studies in the U.S. have evaluated this algorithm and have demonstrated excellent sensitivity and specificity as well as increased ability to detect AHI [118, 251, 252]. No published studies, however, have evaluated performance of the algorithm in non-subtype B samples. Using a simulated algorithm on data generated by 4th generation IA, Mulitispot, and NAAT , the proposed algorithm detected acute infection during at least one visit in 29/29 individuals when p24 concentration was greater than 50pg/ml. Maximum AHI detection occurred between 5-15 days from the first positive RNA, after which the result was predominately HIV antibodies detected. If improvements are made to the Determine Combo RT Antigen component, this rapid test could serve as an alternative test in the algorithm replacing the 4th gen or p24 assay, especially in less developed settings.

5.9 Diagnostic staging of acute HIV Infection

Evaluation of Fiebig staging system and proposal of new staging system

Fiebig et al., in 2003, performed a comprehensive evaluation of HIV diagnostics in acute infection, however since this time, new HIV diagnostic technologies have become available [142]. A common criticism of the Fiebig staging system has been the development based on subtype B plasma donors and the applicability to non-subtype B populations is unknown [31, 142]. RV217 allowed evaluation of the Fiebig staging

system on non-subtype B samples. In our study, the same basic stages were observed; however the length of each stage was different than that observed by Fiebig. In particular, the lengths of stage 2 and 3 were of a longer duration than those reported by Fiebig. In addition, as described previously, in RV217 it was observed that p31 reactivity on the Western Blot is not a marker that only appears later in infection (Stage 6) as it is detected as early as day 20. There are several possible explanations for the differences observed between the RV217 study and the Fiebig study including the use of different testing platforms as well as the multiple subtypes evaluated in RV217. The Fiebig staging system has become outdated with implementation of 4th generation EIA screening becoming more widespread. In addition, in the proposed CDC testing algorithm, the Western Blot will be used less in the clinical settings as it is not required Following in the footsteps of another publication describing a new 4th generation staging system[253], a staging system was developed based on RV217 data that has 4 distinct stages, but allows for flexibility in usage of assays due to the similar reactivity profiles of certain tests. Stage 1 is RNA positive only, similar to stage 1 in Fiebig's system. Stage 2 is RNA positive and either 4th gen IA or p24 reactive, while remaining antibody negative by 3rd gen EIA or Determine Combo and stage 3 adds 3rd this Antibody reactivity. The 4th and final stage occurs when Multispot and/or Western Blot becomes reactive. This proposed system allows the use of tests that are being recommended in the new CDC algorithm, and also allows flexibility in use of test.

5.10 Swab testing

The measurement HIV virus in semen has been previously described [254-257], however the actual infecting inoculum after a sexual exposure and linkage to transmitted virus has never been studied in humans. Frequent measurement of virus in infecting inoculum may give valuable information about the viral quasispecies before infection as well allow the study of frequency of exposure events before a productive infection. Self-administered vaginal or rectal swabs were collected in a subset of volunteers that were willing to consent to these procedures. Self-administered swabbing has been used successfully in detection of other diseases [258-261], but no published literature describes testing for HIV RNA with this method. Uptake of swabbing procedures was highest in Kenya and lowest in Tanzania, where only 7 participants consented. The overall results were disappointing, as only one positive test was observed during routine surveillance as a possible exposure event and only 1/5 HIV incident cases yielded a swab reactive Aptima result 7 days before the plasma reactive Aptima test. Due to the low number of positive swab results

obtained, the analysis of exposure events was not possible. There are several possible explanations and limitations of these procedures. First, only a subset of the swabs (25%) of total swabs acquired during surveillance were tested by Aptima due to the high cost and amount of labor involved, so it is possible that reactive specimens may not have been identified. Second, swabbing was self-administered outside of the research center. It was not possible to monitor technique, nor verify the timing of swab collection. In the one case where a reactive swab was obtained before the plasma reactive sample, viral sequencing to determine the quasispecies of virus present compared to the virus that was actually transmitted will provide interesting insights.

6. Conclusions

- RV217 is an extremely unique cohort that identified individuals in very early acute infection and followed them longitudinally at close intervals, which allowed precise definition of the viral load upslope, peak, downslope, nadir and set point viral load.
- Analyses of viral dynamics revealed that the only viral events measured in this study in early infection related to set point were peak and nadir viral load.
- Nadir and set point viral load were equivalent, suggesting that the long-term course of infection was determined within the first 6 weeks of infection.
- Early events in clinical lymphocyte immunophenotying (Total CD4 cells, CD8 cells, NK cells and B cells) were not related to viral load set point. Specific subsets of each cells type were not measured in this study.
- CD4 count at one year and viral load set point were inversely correlated. CD4 at one year and set point viral load, but not early events such as viral load upslope, viral load peak, or viral load set point, predicted volunteers that would reach CD4 <350. This suggests that it is not only the virus replicative capacity and initial innate defenses that impact outcome, but likely a complex interaction and balance between adaptive immunity, viral properties, and innate immune defenses that determine progression.
- Individuals that progressed to CD4 <350 in 2 years had a significantly lower CD4 absolute count at one year than those that did not progress.
- The 4th generation EIA reduced the diagnostic window from 3rd generation EIA by 8 days and reactivity of 4th gen EIA was dependent on p24 concentrations and viral load.
- The possibility of a second diagnostic window must be taken into consideration when performing HIV testing algorithms in certain settings.

- Western blot analysis shows a sequential appearance of antigen reactivity. A scoring system based on number and intensity of antigen reactivity may be useful in predicting recency of early infection.
- The current formulation of the Determine Combo Rapid Test, labeled as 4th gen test, does not adequately detect p24 antigen, however the antibody portion of the test is equally as sensitive as a 3rd generation EIA. An improved rapid test is required for enhanced detection of AHI, perhaps with the inclusion of a disassociation step of immune complexes of p24 antigen/anti-p24 antibody.
- A simulated evaluation of the new CDC HIV testing algorithm detected 29/29 acute cases with most efficient detection between 5-15 days from first positive RNA test. This was the first known evaluation of the algorithm with non-subtype B samples.
- Evaluation of the Fiebig staging system with non-subtype B samples revealed the same distinction in stages, however different lengths of each stage were detected using newer test kits and non-subtype B samples. p31 detection occurred as early 20 days, suggesting that the differentiation between Fiebig's stage 5 and 6 employing p31 may not be suitable in non-subtype B samples.
- As new testing kits are now available, a new HIV diagnostic staging system is required. A new system is proposed consisting of 4 stages which allows a choice of currently available diagnostic tests including those suggested within the CDC testing algorithm.
- Additional evaluation of the SMART plasma is necessary as the samples acquired in this study were not adequate for evaluating the product's ability to reduce the detection window based on anti-HIV antibody detection.
- Self-administered vaginal swabs were able to detect RNA by Aptima, however all but one reactive result was coincident with the plasma reactive sample and may reflect genital shedding. Detection of exposure events was not possible due to the few reactive swabs identified.

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