FcγRIIa and FcγRIIIa genetic polymorphisms and HIV-1 disease progression in Kenyan female sex workers

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Chapter 1

Introduction and Background

Specific Aims

This study explores a potential correlation between genetic polymorphisms of the Fc gamma receptors IIa and IIIa and the rate of disease progression in HIV-infected female sex workers (FSW) from Kenya. Longitudinal data is used from a prospective cohort following HIV-1 seroconverters, with the end-points of progression to AIDS or mortality. Using several regression techniques, we examine the relationship between genotype and set point plasma viral load, plasma viral load increase over time, and CD4+ T cell decline over time. We also determine if FcγRIIa or FcγRIIIa genotypes are predictive of the overall rate of HIV-1 disease progression in this cohort using independent outcomes of decline to CD4<200 cells/mm^3, death, and a combined outcome of time to CD4<200 cells/mm^2, death, or initiation of antiretroviral therapy (ART).

Background: HIV-1 Disease Progression

Chronic infection with Human Immunodeficiency Virus type 1 (HIV-1) is clinically characterized by a gradual increase in plasma viral load and a corresponding decline in CD4+ T cells, leading to acquired immune deficiency syndrome (AIDS). HIV-1 affects approximately 34 million people worldwide, and more than 25 million lives have been lost since the beginning of the epidemic. The center of the epidemic currently resides in Sub-Saharan Africa, where nearly 1 in 20 individuals are living with an HIV-1 infection.[1]

HIV-1 disease progression can be divided into three time periods. Acute HIV-1 infection occurs within 2-4 weeks after a transmission event, and is characterized by rapid replication of virus and a steep drop in CD4 cells.[2-4] Individuals can present clinically with influenza-like symptoms as

the body initially responds to the virus. The higher the viral load during acute infection, the worse the symptoms can be. [5] Eventually viral replication is controlled by the human immune system, and remains steady at a level called viral set point. Set point is the beginning of a period of clinical latency, and in this study will be defined beginning at 4 months post initial infection. At set point, CD4 cells will have rebounded following acute infection, but do not achieve full recovery. [6,7] High viral loads at set point can predict faster rates of HIV-1 disease progression. [6,8]

The second period of an HIV-1 infection is an extended time of clinical latency, where virus replicates in low levels and CD4 cells decline slowly. This is the beginning of chronic infection, where the immune system constantly works to control the virus, but with a gradually increasing toll on CD4 cells.[1] A complex interplay between host and virus occurs during this time, affecting the length of clinical latency.[9] This becomes an arms race between the human immune system and the virus. High viral load throughout chronic infection is indicative of poor immune control and is associated with faster disease progression. [10] The inverse is also true; the number of circulating CD4 cells in the blood at any given time is a reflection of viral pathogenesis. Lower than average CD4 cell count throughout an HIV-1 infection is associated with faster than average disease progression. [11-14] Due to these associations, viral load and CD4 cell count are important markers of disease progression and are used as such in this study.

The last stage of HIV-1 disease progression begins as CD4 cells dip below 200 cells per cubic millimeter of blood (200 cells/mm^3) as the immune system loses control and viral replication accelerates.[15] At this time infected individuals become symptomatic and are susceptible to opportunistic infections such as Kaposi's Sarcoma or Pneumocystic jirovecii pneumonia. This is the clinical definition of AIDS. [16] Figure 1 shows the stages of an HIV infection and disease progression as described.

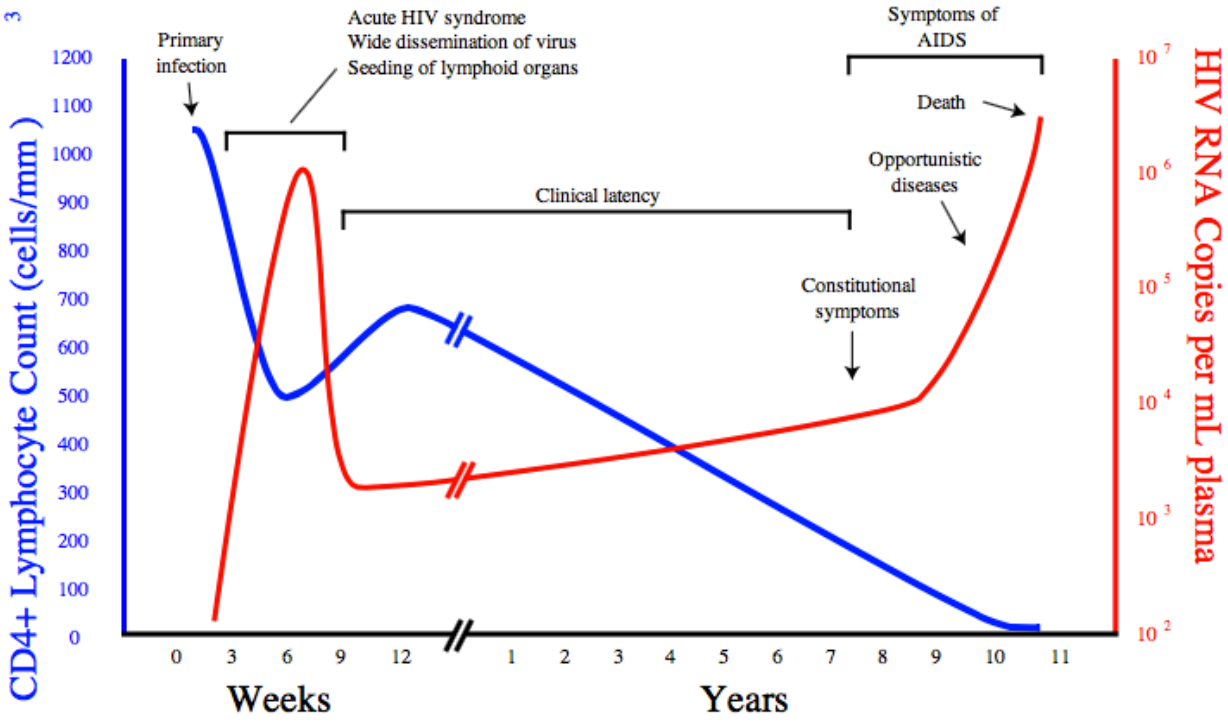


Figure 1. HIV disease progression over time, without treatment with antiretrovirals. Primary infection is directly followed by acute HIV syndrome, where viral replication is very quick and CD4 cells sharply drop. This is followed by a long period of clinical latency, which ranges from 7-12 years on average. Eventually, viral replication increases and CD4 cells decline dramatically, allowing for opportunistic infections to occur. This is clinical AIDS. Figure from Wikipedia Commons.

Human Genetics and Progression

The rate of CD4 cell decline can vary drastically between infected individuals. [17,18] Without antiretroviral therapy controlling viral replication, CD4 cell decline takes on average between 7-12 years post initial infection.[19] However, the range can be from a few years to decades. The latter group encompasses a small percentage of infected individuals who are able to naturally control the virus and maintain high levels of CD4 cells without treatment. [20] Individuals like this progress to AIDS much more slowly than the average infected person. These long-term non-progressors, or even "elite controllers" have sparked interest in researching host immune factors that may contribute to the observed variation in viral control and disease progression. Identifying specific

human genes with polymorphisms that offer protection against HIV-1 disease progression may in turn present opportunities to develop novel therapies and perhaps even new targets for vaccines. [21-23]

A handful of such genes have been discovered. The most well known and biologically important to date is a 32 base-pair deletion in the coding region for the CCR5 gene. CCR5 is the viral coreceptor and is needed for entry into CD4+ T cells. The delta32 deletion renders CCR5 unusable by the virus, and shuts down viral entry into otherwise susceptible CD4 cells.[24] Approximately 1% of Caucasians carry two copies of the modified gene, with no apparent negative health affects. The discovery of the delta32 deletion came from studies of highly exposed seronegative (HESN) individuals who had resisted infection despite high levels of exposure. A common feature of many of these HESN individuals was double CCR5-delta32, giving them virtual immunity to HIV-1 infection. [25,26] This is in part due to transmitted viruses preferentially using CCR5 as the coreceptor, as opposed to the alternate coreceptor CXCR4 that the virus can evolve to use later in infection.[27]

Expressing even one copy of CCR5-delta32 has been shown to confer some protection against infection, and is associated with a slower disease progression to AIDS. [28] The discovery of CCR5-delta32 led to the development of an entire class of treatments called CCR5 antagonists. While at least one CCR5-delta32 allele is present in up to 20% of Caucasians, it is all but absent in Africans and Asians.[29] However, all populations have benefited from treatments targeting the coreceptor, which points to the value of conducting genetic studies in many different populations.

In recent years, many new candidate genes have been discovered with polymorphisms that demonstrate some level of comparative protection against HIV-1 disease progression. [21-23,30] Genes with known important immunological consequences have been reexamined in this way. These include HLA genes, Toll-like receptors, KIRs, and CCR5 ligands. Reviews of such studies have also

suggested an additive effect of many genes functioning separately or in epistasis with each other to confer some protection against the virus.[31]

Fcγ Receptor Mediated Processes

Of particular interest in this study are the genes encoding surface cell receptors on immune cells that interact with HIV-1 through cross-linking antibodies. These include macrophages, monocytes and dendritic cells, all of which can engage in phagocytosis of virions that have been targeted by human antibodies (IgG). Phagocytosis of virus-IgG complexes leads to the degradation of the virus, which by itself is a central immune function. Additionally, phagocytosis stimulates further immune response through directing antigenic peptides to be presented on the surface of the immune cell.[32] Increased ability to phagocytose may lead to better viral control. [33]

Another cell type that interacts with HIV-1 is the natural killer cell (NK). These cells interact with IgG as well, but not to phagocytose virions. Instead, the NK cell cross-links antibodies bound to infected cells presenting HIV-1 antigens. This stimulates the NK cell to release cytotoxic chemicals to kill the infected cell, and cytokines to activate the immune system.[34] This mechanism is called antibody-dependent cellular cytotoxicity (ADCC) and is also thought to be an important contributor to viral control and disease progression.[35-37]

Both immune complex phagocytosis and ADCC are mediated through the Fc gamma receptors (FcγR), cell surface glycoproteins that selectively bind the constant fragment (Fc) of activated IgG.[33,38] Contact between IgG and an Fcγ receptor is only made after the antigen-binding (Fab) portion of IgG binds the target. In the case of phagocytosis, that target is the virus itself. In ADCC, the targets are HIV-1 genetic material and protein fragments presented on the plasma membrane of an infected CD4 cell. Figure 2 illustrates the physical locations of the Fc and Fab portions of IgG.

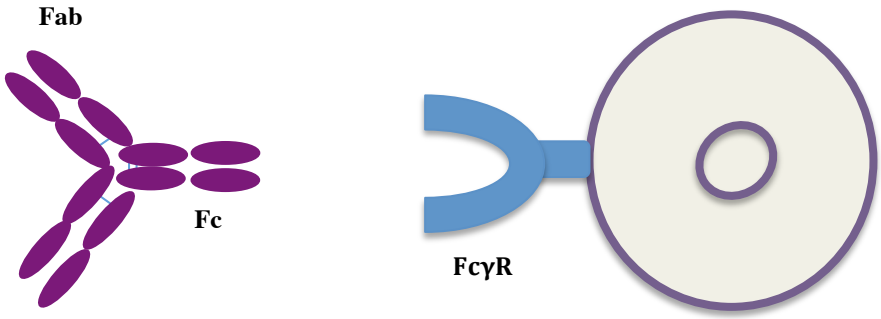


Figure 2. Cartoon of an antibody (IgG) showing both the antigen binding fragment (Fab) and the constant region (Fc). The cell surface Fcγ receptors expressed on several types of immune cells bind the Fc portion after the Fab portion has bound HIV-1 virus or an infected CD4 cell (not drawn to scale).

There are three classes of Fcγ receptors: I, II, and III. Classes II and III can each be further divided into subclasses A and B. FcγRI, IIa, and IIIa all activate phagocytosis, while their "b" forms act as phagocytosis inhibitors.[38] FcγRIIIa is the only activating FcγR expressed on NK cells, thus the only one that is involved in ADCC. Since these receptors play distinct roles in the immune response to HIV-1, we hypothesize that they play a role in the complex host-virus dynamic during chronic infection.

FcγRIIa and IIIa genetic polymorphisms

Previous studies have shown that polymorphisms in the Fcγ receptor genes are associated with severity or susceptibility to a number of infectious and autoimmune diseases.[39] FcγRIIa and FcγRIIIa both contain coding-region single nucleotide polymorphisms (SNPs) that affect their affinity to IgG. [40,41] FcγRIIa has a SNP that encodes either a Histidine (H) or an Arginine (R) at AA position 131.[42] A receptor with Histidine at AA site 131 has a higher affinity for all IgG subclasses than that with Arginine.[43] In fact, at least one allele with the H in position 131 is required for IgG2 binding, and even one H allele allows IgG3 binding with higher affinity than an RR homozygote.[44]

Worldwide prevalence shows RR and HH each in ~25% of the population, and RH ~50%, including Kenyan populations. [45-48]

The difference in affinity for IgG has been linked to efficiency in phagocytosis and subsequent immune activation, which can have clinical consequences[49,50]. FcγRIIa 131 RR low-affinity receptor is a reported susceptibility factor for Systemic Lupus Erythematosus (SLE) in Caucasians, African-Americans, and Koreans. The RR phenotype is also a potential risk factor for development of Multiple sclerosis (MS) and rheumatoid arthritis, and the severity of Guillian-Barré syndrome (GBS). [51] In addition, several cancer studies have shown that high-affinity HH homozygotes may have a general lower risk for cancer invasiveness and a better response to chemotherapy.[41] To date, the meta-analyses conducted on these topics have produced conflicting results and findings have not yet become clinically actionable. [45]

As the HIV research field examines host factors that may represent correlates of immunity against the virus, FcγRIIa has been investigated because of the above-stated immunological consequences of the SNP at amino acid 131. A handful of studies using data from longitudinal cohorts of HIV-1 infected individuals have measured rates of HIV-1 acquisition or disease progression comparing HH, HR, and RR individuals. Forthal *et al* demonstrated that FcγRIIa is predictive of progression towards CD4<200 cells/mm^3 in a US-based cohort of men who have sex with men (MSM), with the low-affinity RR homozygotes progressing more quickly than HR or HH[52]. Additionally, they demonstrated that RR homozygotes had less efficient phagocytosis of HIV-1/IgG complexes. However, Poonia *et al* found no association between the distribution of FcγRIIa genotypes in a group of "normal HIV-1 progressors" and a cohort of Natural Virus Suppressors (NVS)[53]. NVS can suppress the virus to very low levels without taking ART, delaying disease progression like the before-mentioned elite controllers. While the methods of these two studies are

not entirely comparable, these findings suggest that the role of the FcγRIIa genotype in HIV-1 disease progression deserves closer attention.

Similarly to FcγRIIa, FcγRIIIa also has a SNP in the IgG binding region that affects binding affinity. A substitution of a Guanine (G) for a Thymine (T) in the FcγRIIIa gene encodes either a Valine (V) or phenylalanine (F) at AA position 158. IgG1 and IgG3 bind with higher affinity to V-containing receptors than F-containing receptors[43]. VV is present in ~8-12% of the population, with African- Americans reported at 8-10% VV, and Caucasians 10-12% VV.[53-55] Kenyan populations are not well studied for this SNP. FV and FF are each present in ~45% of reported global populations.[45,51,53,56] While cross-linking FcγRIIIa can participate in phagocytosis, the expression on NK cells is the differentiating factor between FcγRIIa and FcγRIIIa. Polymorphisms in FcγRIIIa have also been associated with increased risk of disease. Individuals homozygous for the high affinity VV receptors may be at increased risk of rheumatoid arthritis, but there are discrepancies in the literature.[51,56,57]

Higher affinity V receptors are associated with higher levels of ADCC.[58] In HIV-1 infection, increased levels of ADCC is indicative of HIV-1 viral control. [36] Impaired ADCC has also been associated with HIV-1 disease progression.[59] Therefore, HIV-1 infected individuals homozygous for the V receptor may show increased levels of ADCC, leading to better viral control and slower progression to AIDS than those with FV or FF.

However, the Forthal *et al* study in MSM found no association between FcγRIIIa genotype and disease progression, and in fact reported an association between VV and incident Kaposi's Sarcoma, the most frequent opportunistic condition in that cohort. Poonia *et al* found increased risk of infection and of disease progression in VV homozygotes, again by comparing genotype distributions in a cohort of Natural Virus Suppressors with a group of "normal progressors". This suggests that while increased levels of ADCC is effective at killing target cells, it may also lead to

immune activation resulting in recruitment of susceptible CD4 cells, thus propagating infection. The complex relationship between FcγRIIa, FcγRIIIa and HIV-1 chronic infection makes these genes ideal candidates for studies in long-term cohorts.

Objective and Hypothesis

By investigating the role of Fc gamma receptor polymorphisms in a population of HIV-1 infected Kenyan female sex workers (FSW), we hope to learn more about the impact of the FcγRIIa and FcγRIIIa genotypes on long-term health and viral control. Based upon previous studies, we predict that the FcγRIIa and FcγRIIIa genotype will be associated with disease progression among this cohort. Noting discrepancies in the literature, we predict that individuals with the FcγRIIa RR (low affinity) and FcγRIIIa FF (low affinity) genotypes will progress, on average, to a CD4 cell count of $<200/mm^3$ or death faster than those with one or more higher affinity alleles. We predict that set point viral load and viral load over time will be higher on average in women with the RR and FF genotypes (respectively) than women with one or more higher affinity alleles.

Chapter 2

Methods

Study Population

This study population is from a 20+ year ongoing prospective cohort of initially HIV-1 seronegative female commercial sex workers in Mombasa, Kenya.[60] This cohort began enrolling women in February 1993, and continues to enroll seronegative women and monitor those who have seroconverted while participating in the cohort. Women are followed quarterly and data is collected regarding sexual risk behaviors, physical exam findings, genital infections, and HIV status. They are provided prevention services, including condoms and counseling on risk reduction.

When a woman does seroconvert, she receives post-infection counseling and continues follow-up with additional data collection on viral load, CD4 count, and other health-related factors. Blood is collected at monthly clinical visits, and CD4 counts are measured quarterly in the Mombasa laboratory. The clinic provides antiretroviral treatment as women become eligible based upon their CD4 cell levels. Plasma samples are stored at -80°C or in liquid nitrogen until shipment to Seattle and testing using the Gen-Probe HIV-1 RNA assay.

This particular study utilizes the data of women who seroconverted in the Mombasa cohort. All women who had previously extracted DNA available in the laboratory and additional women who met study criteria (>=2 viral loads *and* >=2 CD4 counts) and had samples available in Seattle were genotyped for FcγR polymorphisms. Inclusion criteria balanced the need to include as many women as possible with the need to meet the timeline of this project. Of the 322 seroconverters that have been followed, 253 women met the above criteria. When previously extracted DNA was not available, DNA was obtained through an extraction of patient plasma samples.

This cohort has been approved by the human subjects research committees of the Kenyatta National Hospital in Kenya, as well as the University of Washington and Fred Hutchinson Cancer Research Center in Seattle. All participating women provided written, informed consent for research including genetic testing.

SNP locations and characteristics

The FcγRIIa single nucleotide polymorphism rs1801274 codes for the low affinity IIa receptor (CD32) of the Fc fragment of IgG, in chromosomal region 1q23. The SNP represents a substitution of an Adenine to a Guanine, coding for Histidine or an Arginine at amino acid position 131, presented as H131R. The FcγRIIIa SNP rs396991 codes for the low affinity IIIa receptor (CD16) of the Fc fragment of IgG, at chromosomal location 1q23. The SNP is a substitution of Thymine for Guanine, coding for a change to Phenylalanine from Valine at amino acid position 158. Table 1 gives a more detailed look at the SNPs in these two genes.

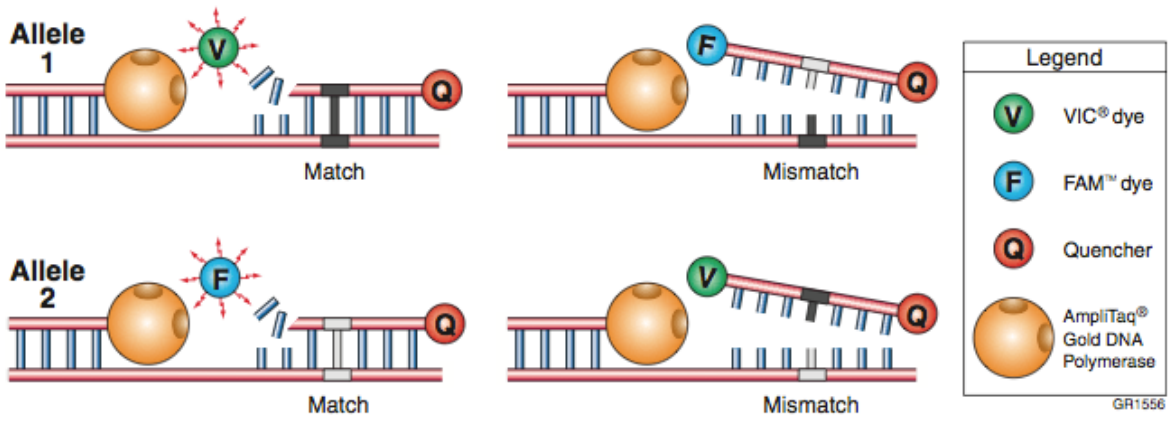
Table 1. FcγRIIa and FcγRIIIa expression and polymorphism details

	FcγRIIa	**FcγRIIIa**
Cellular expression	Macrophage, Monocyte, Dendritic Cells	Natural Killer, Macrophage, Monocyte, Dendritic Cells
Receptor	CD32	CD16
Alleles	Amino acid 131: H (high affinity) or R	Amino Acid 158: V (high affinity) or F
SNP#	rs1801274	rs396991
Entrez ID	2212	2214
Chromosomal location	1q23, negative strand Position: 161479745	1q23, negative strand Position: 161514542
Mutation	A→G	G→T
AA position	H131R	V158F
Genotype Frequencies	HH/HR/RR 25/50/25	VV/VF/FF 12/48/40
Flanking sequence	TCCCAGAAATTCTCCC*[A/G]*TTTGGATC CCACCTTCTCCATCCC	TTCTGCAGGGGGCTT*[G/T]*TTGGG AGTAAAAATGTGTC

Laboratory Methods

A TaqMan allelic discrimination assay was used, purchased through Life Technologies (Formerly Applied Biosystems, 5791 Van Allen Way, Carlsbad, CA). The assay utilized a Life Technologies Real Time PCR machine at the Fred Hutchinson Cancer Research Center using SDS 2.4 software. The software and Real Time machine runs an allelic discrimination assay, utilizing a common forward primer and allele-specific reverse primer probes with fluorescent reporters bound to quenchers.

As the PCR proceeds (as seen in Figure 3), if the allele-specific probe matches the SNP, polymerase will cleave the reporter from the quencher, and a fluorescent signal is observed. If there is a mismatch polymerase will not cleave the reporter and a signal will not be detected. The reporters VIC and FAM are used, and are both present in every reaction. A pre-read is taken to record background levels of fluorescence, followed by a period of amplification. After a post-read, a visual plot is constructed, displaying fluorescence in two dimensions on the x and y-axis. This visual plot allows the SDS 2.4 software or the user to make "calls" and to check for quality control of the assay.



The table below shows the correlation between fluorescence signals and sequences in the sample.

A substantial increase in...	Indicates...
VIC® dye fluorescence only	Homozygosity for allele 1
FAM™ dye fluorescence only	Homozygosity for allele 2
Both fluorescence signals	Heterozygosity allele 1-allele 2

Figure 3. The above Taq-man figure displays the fluorescent consequences of the probe matching or mis-matching the SNP. When there is a match, the allele-specific dye is cleaved from the quencher, releasing detectable fluorescence. The fluorescence is plotted on a two-dimensional axis, allowing for visual allelic descrimination. Figure from Life Technologies.

The two-dimensional plot displayed in Figure 4 is an example of the allele distribution visualization using SDS 2.4. Homozygotes for the Allele Y, representing the nucleotide Thymine in the FcγRIIIa assay, are displayed as blue dots, clustering together high on the Y-axis and below zero on the X-axis. The red dots, clustering high on the X-axis and low on the Y-axis, represent homozygotes of Allele X; in the FcγRIIIa assay this is Guanine. Heterozygotes show fluorescence from allele Y and from allele X, and appear clustered in the middle of the plot. Thymine-Guanine heterozygotes in the FcγRIIIa assay are shown as green clusters in Figure 4.

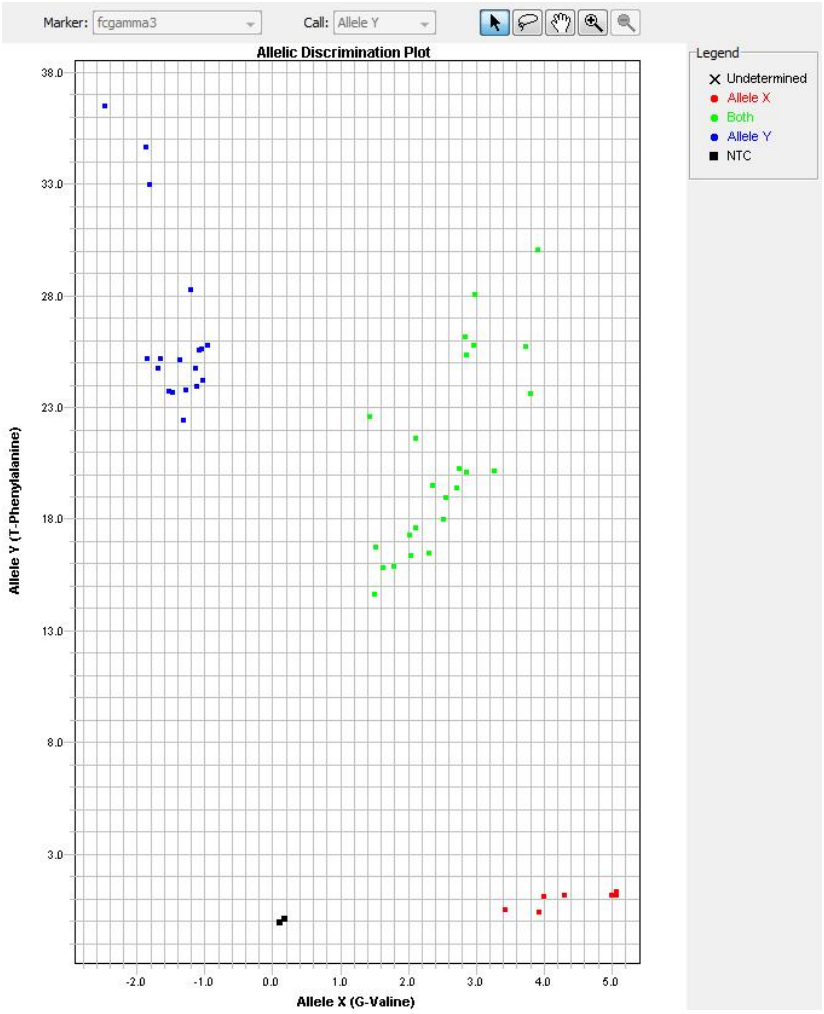


Figure 4. Example of genotyping data collected using the Taq-Man assay. This two dimensional plot allows for visualization of fluorescence of dye cleaved from quenchers as the PCR amplification proceeds. Fluorescence indicates a match on the SNP, and detectable difference in fluorescence is evidenced by clusters of samples showing homozygosity for allele Y (blue), allele X (red), or heterozygosity Y/X (green).

Controls

Controls were obtained from Puget Sound Blood Center donor peripheral blood mononuclear cells (PBMCs). DNA was isolated from the PBMCs using a Qiagen DNeasy Blood and Tissue Kit. All controls were amplified and sequenced prior to use in the assay using an Applied Biosystems 2720 thermalcycler. Primers used for amplification and cycling conditions were as follows: FcγRIIa was amplified using forward primer TTGGGATCTATCCTTACAAC, reverse primer CCTACTTGTTGGTCAATACT amplifying a region 470bp long, at 94°C for 60 seconds,

followed by 30 cycles at 94°C (60 s), 47°C (60 s), and 72°C (60 s), then held at 72°C for 8 minutes. FcγRIIIa was amplified using forward primer: TTGGGATCTATCCTTACAAC, reverse primer: CCTACTTGTTGGTCAATACT, amplifying a region 412bp long, at 94°C for 60 seconds, followed by 30 cycles at 94°C (60 s), 57°C (60 s), and 72°C (60 s), then held at 72°C for 8 minutes. After amplification, the regions of interest were purified of primers and enzymes using a Qiagen PCR Purification Kit, and sequenced using the shared Genomic Resource of the Fred Hutchinson Cancer Research Center using a Big Dye kit and protocol. Sequences were analyzed and compared with Sequencher (Gene Codes Corporation).

Genotyping Real-Time PCR

Every genotyping run contained 9 controls: 3 of each genotype (GG, AG, AA for FcγRIIa and TT, GT, and GG for FcγRIIIa) at 1ng, 10ng, and 50ng, which represents the range recommended by the manufacturer. The first 57 patient samples had been previously genotyped by Dr. Donald Forthal's laboratory at UC Irvine. Blindly genotyping these 57 patients first allowed for an additional assay quality control. DNA concentrations ranged from 12ng/μl to 200ng/μl. A standard 2μl of each was run in the assay and ambiguous calls were followed with a re-run and if still ambiguous were confirmed by sequencing using the previously mentioned parameters. Data was obtained from 189 women using DNA from lysed PBMCs.

Plasma DNA:

After genotyping all available DNA samples, the remaining list of seroconverters (133 women) were assessed for follow-up data and sample availability. 67 of those 133 women had

follow-up data fitting the criteria of >=2 viral loads obtained, >=2 CD4 counts obtained, and available plasma samples in Seattle.

All plasma DNA was extracted from patient samples in a Bio Safety Level 3 Laboratory using sterile techniques. 25µl plasma was used in the extraction, yielding approximately 5ng genomic DNA (average 0.1ng/µl in 50µl). 0.2ng DNA from plasma was used in each reaction well. In addition to the controls run in the PBMC DNA genotyping experiments, two 0.1ng controls were run for each genotype.

Initially, 12 samples were run with DNA extracted from plasma alongside DNA extracted from PBMCs to confirm that plasma extraction produced measurable, quality DNA for accurate genotyping. Figure 3 shows a plot of DNA from plasma run in the TaqMan assay. While the separation between genotypes was less clear than with higher concentration DNA, most results were easily classified. Ambiguous samples were rerun using up to 1ng DNA, and those with persistent ambiguity were amplified and sequenced as previously described in the methods for control verification. Data was obtained from 67 women using DNA extracted from plasma.

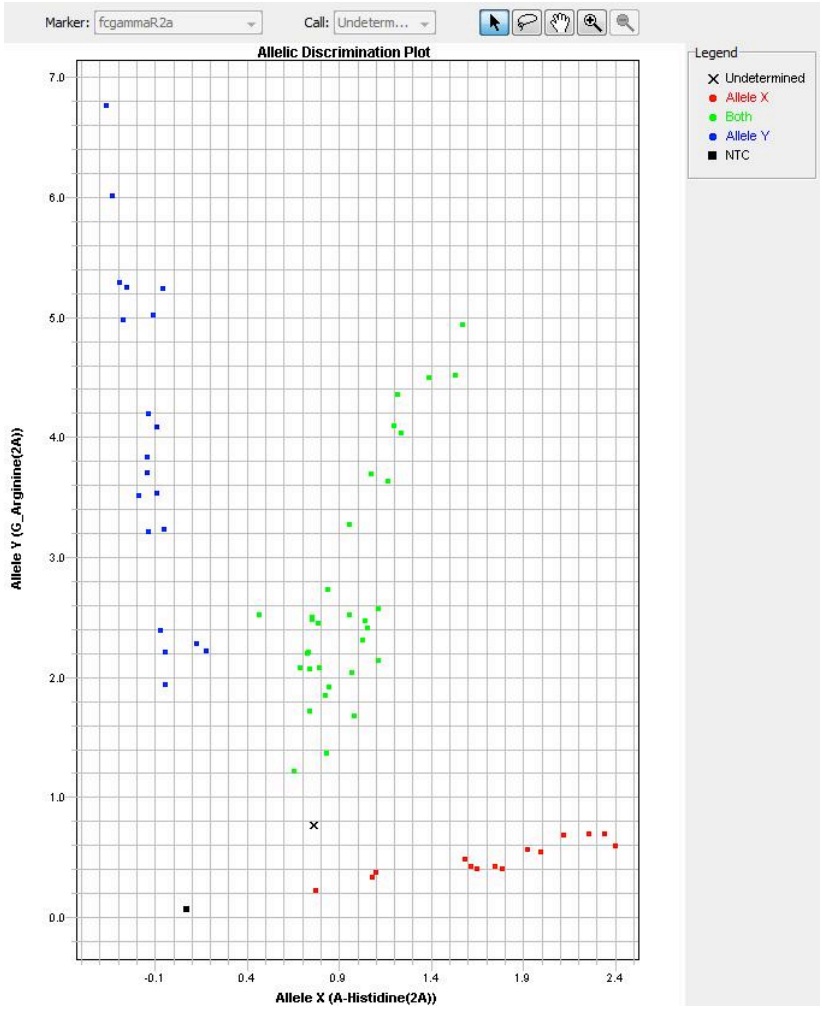


Figure 5. The above plot is an example of the Taq-Man assay using low concentration DNA samples extracted from patient plasma. Ambiguous samples were rerun, and if they remained ambiguous were sequenced for verification of genotype. This study demonstrates that enough quality DNA can be extracted from patient plasma to genotype using the TaqMan assay.

Analysis Methods

This study examines the relationship between genotype and set point plasma viral load, plasma viral load change over time, CD4+ T cell decline over time, and aims to determine if FcγRIIa or FcγRIIIa genotype is predictive of the overall rate of HIV-1 disease progression. All analysis was done using STATA software (StataCorp LP, 4905 Lakeway Drive, College Station, TX).

Primary predictors were FcγRIIa genotype (RR, HH, or RH) and FcγRIIIa genotype (VV, FV, or FF). Table 2 details the questions asked, types of tests used, and specifications and restrictions.

Table 2. Statistical analysis: questions, tests and specification.

Analysis	Question	Type of analysis	Specifications
1	Set point viral load	Linear regression/ ANOVA	First plasma viral load measured between 4-24m PI
2	Viral load incline over time	Linear Mixed Effects	Restrict to women with >=2 VL, censor at ART
3	CD4+ T-cell decline over time	Linear Mixed Effects	Restrict to women with >=2 CD4, censor at ART
4	Disease progression analysis	Cox Proportional Hazards	Outcomes: time to death, time to CD4<200, time to ART

Preliminary quality control tests were performed prior to the tests shown in Table 2. These included testing the sample population to confirm the SNPs were in Hardy-Weinberg equilibrium (HWE), and a check for evidence of linkage disequilibrium (LD) between FcγRIIa and FcγRIIIa. To test for Hardy-Weinberg a Pearson X^2 was performed as well as a Likelihood ratio test and a test for exact significance. This addresses the possibility that the sample population is currently under selective pressure for a particular trait, which could invalidate findings of an association between genotype and viral control or disease progression. Utilizing these tests is consistent with the literature.

A check for linkage disequilibrium was also done. A Pearson X^2 test for independence is used, which assesses the probability that the SNPs do not segregate independently, and are in fact inherited together due to LD. A finding of LD would not invalidate findings in this study, but it could pose difficulty in predicting which gene is really responsible for the effect seen, depending on how strong the LD is. FcγRIIa and FcγRIIIa have been previously reported to be in weak LD.

Analysis 1. Set point viral load between genotype groups

The set point was determined as the first viral load obtained clinically between 4-24 months post infection. The viral loads were log10 transformed to yield an approximately normal distribution. Set point plasma viral load was compared between FcγR genotype groups because it is an important predictor of disease progression, and directly indicative of viral control early in infection.[6,8,10] A linear regression was used to compare genotype groups for both FcγRIIa and FcγRIIIa, using indicator variables. The FcγRIIa reference group was HH and FcγRIIIa reference group was FF, shown in the literature to be the least risky genotypes.

The linear regression was performed first without adjustment and then after adjustment for covariates established *a priori* to be associated with viral load in this cohort, and thus potential confounders of the effect of Fcγ receptor genotype on viral load set point. These *a priori* confounders were: age at infection, use of hormonal contraception (depo provera, oral contraception, norplant) within a 70-day window of infection, and presence of genital ulcer disease at the time of infection. [61]

A separate analysis was conducted with restriction of the dataset to a subset of genotyped seroconverters who had also been evaluated for viral subtype and superinfection. Because subtype D has been associated with faster HIV-1 disease progression in this cohort, we dichotomized subtype into two categories: D and non-D. Several superinfection events have occurred in this cohort. Superinfection status is included in this study as a potential confounder because infection from a second source partner may increase rate of disease progression (Ronen, in preparation). Table 3 summarizes all covariates used in the analyses.

Table 3. Covariates used in analysis selected *a priori* based upon previously published work with this cohort.

Predictors	Variable type
Age at infection in years	Continuous
Genital ulcer diseases at infection	Categorical: (0,1) for N, Y
Depo-Provera exposure at infection	Categorical: (0,1) for N, Y
Oral contraceptive exposure at infection	Categorical: (0,1) for N, Y
Norplant hormonal contraceptive exposure at infection	Categorical: (0,1) for N, Y
Viral subtype	Categorical: (0,1) for non-D or D
Super-infection status	Categorical: (0,1) for N, Y

Analysis 2. Viral load over time: Linear Mixed Effects Model

A linear mixed effects model (LME) was used to determine the relationship between FcγRIIa and FcγRIIIa polymorphisms and plasma viral load as it changes throughout a chronic HIV-1 infection in the study population. LME is uniquely advantageous in that it can allow random effects as well as fixed effects for both the intercept and the slope of the regression. Fixed effects refer to sample population averages, while random effects are subject-specific. LME is particularly useful in longitudinal studies where there are multiple entries per subject. In this study, each woman's viral load trajectory is taken into account as well as group averages. An interaction term between genotype and days since infection is used to measure differences in viral load increase over time, which is the slope of the regression. The genotype main effect in this model allows for a comparison at the intercept, which is another way to model the set point viral load.

Because acute HIV-1 infection leads to a very high viral load, which later drops to a set point and slowly increases over time if untreated, viral load does not follow a linear trajectory when examined in full. Therefore, in order to utilize a linear approximation, all viral load observations prior to set point (<4 months post infection) were excluded. Since ART acts to control viral replication, women were censored at ART initiation.

Covariates in the adjusted model are those from the linear regression: age at infection, hormonal contraception exposure at infection, and presence of ulcerative genital diseases at infection. A separate sub-analysis was performed restricted to the subset of women for whom data on subtype and superinfection status were available.

Analysis 3. CD4+ T cells over time: Linear Mixed Effects Model

A linear mixed effects model was also used to determine the relationship between FcγRIIa and FcγRIIIa polymorphisms and CD4+ T cell decline over time throughout a chronic HIV-1 infection. Again, the LME allows for random effects for both the intercept and the slope of the regression. An interaction between genotype and time is also used to measure differences in CD4+ T cell decline over time, a clinical predictor of HIV-1 disease progression.

Similarly to the viral loads analysis, CD4 data from acute infection were excluded to allow for a linear approximation of cell decline. Women are censored at ART initiation, and covariates used in the adjusted model are consistent with the previous models: age at infection, hormonal contraception exposure at infection, and presence of ulcerative genital diseases at infection. A separate sub-analysis was performed with subtype data and superinfection status.

Analysis 4. HIV-1 Disease progression: Cox Proportional Hazards Model

To compare survival time between genotypes, a Cox Proportional Hazards model was used. This method uses time-to-event data to describe differences in the relative hazard of an event occurring between groups. In this study, the baseline hazard of time to an event was modeled and compared between FcγRIIa and FcγRIIIa genotype groups. Failures, person-years contributed, and incidence rates were measured for each group as well as hazard ratios. This allows for a direct comparison of overall disease progression between genotype groups. Table 4 illustrates the three

outcomes used in this analysis: time to CD4< 200 cells/mm^3, time to death, and a combined outcome of time to CD4<200 cells/mm^3, death or initiation of antiretroviral treatment (ART). In the combined outcome, whichever event occurred first for a woman became her failure event. In addition to the Cox Proportional Hazards analysis, Kaplan-Meier survival estimate curves were generated for a visual representation of the data.

Table 4. Three different outcomes were used in the Cox Proportional Hazards model.

Analysis	Cox Proportional Hazards Outcome
A	Time to CD4 <200
B	Time to death
C	Combined outcome of time to death, CD4<200, or ART initiation if earlier than CD4<200

Chapter 3

Results

Demographics and Initial SNP Tests

Clinical data from over 20 years was used in this study. Table 5 presents characteristics of the cohort as well as predictors of viral load and/or disease progression that have been previously reported in this cohort.

Table 5. Characteristics and exposures at time of HIV-1 acquisition in 253 Kenyan women, a subset of 322 women that have been followed since seroconversion.

Study population characteristics	**Median**	**IQR**
Age at infection, years	30	27-34
Time in cohort before infection, months	14.9	5.0-32.1
Post-infection follow-up time, months	69	28.1-123.8
Set point plasma viral load (log_{10} HIV-1 copies/ml)	4.6	4.1-5.3
Plasma viral load (log_{10} HIV-1 copies/ml)	4.8	4.2-5.4
CD4+ T cell count (cells/mm^3)	387	262-552
Square root of CD4+ T cell count (cells/mm^3)	19.7	16.2-23.5
	N	**%**
Genital ulcer disease exposure at infection	19	7.5%
Depo-provera exposure at infection	75	32%
Oral Contraceptive exposure at infection	37	16%
Norplant contraceptive exposure at infection	4	1.7%
Subtype D	24/184 screened	13%
Superinfection	21/142 screened	14.8%

a. Numbers and % are provided for categorical variables and medians and interquartile ranges are provided for continuous variables.

The sample population was compared to available data from the International Hap-Map Project and frequencies reported in previous studies (http://hapmap.ncbi.nlm.nih.gov). The expected SNP frequencies based on these data in Caucasians, African Americans, and Kenyans are: FcγRIIa genotype: ~25% HH, ~50% HR, ~25% RR. African populations are not well represented by the FcγRIIIa data reported in Hap-Map, but based upon data from other populations in Hap-Map and from previous studies of both African-Americans and Caucasians we would expect to see frequencies in the range of ~40% FF, ~48% FV, ~8-12% VV.[53,54,62,63] Table 6 presents the number of women and percentage of participating women that had each genotype, with a comparison to Hap-Map data. Figure 4 displays the same information graphically. 253 women were successfully genotyped for FcγRIIa and 249 for FcγRIIIa. We were unable to genotype 4 women for FcγRIIIa were due to difficulties in sequencing their plasma samples.

Table 6. Experimental SNP frequencies compared to Hap-Map data.

N=256	**HH (#/%)**	**HR (#/%)**	**RR (#/%)**	**Total**
FcγRIIa	51 / 20%	131 / 52%	71 / 28%	253
Hap-Map	25%	50%	25%	
N=253	**VV (#/%)**	**FV (#/%)**	**FF (#/%)**	
FcγRIIIa	34 / 13%	123 / 49%	92 / 37%	249
Hap-Map	8-12%	48%	40%	

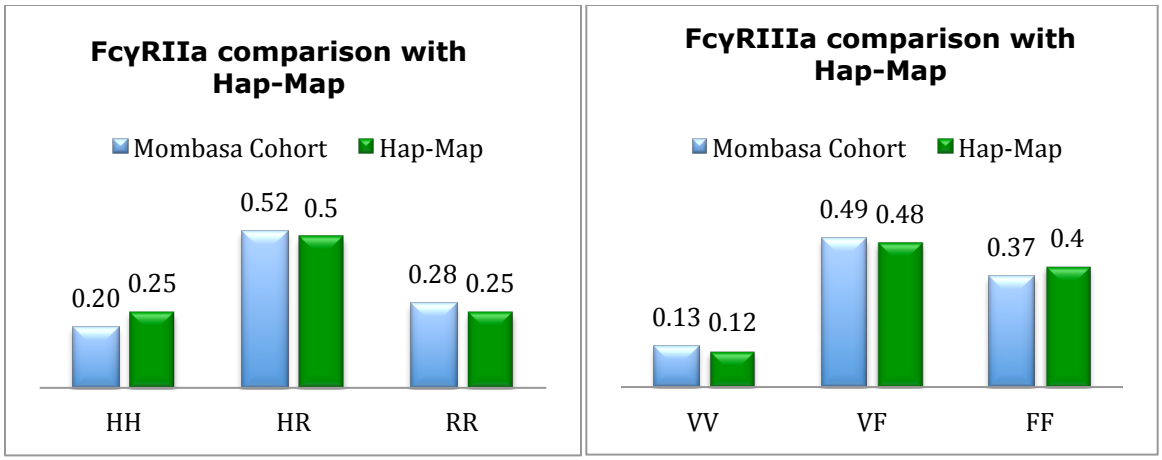


Figure 6. Comparison of FcγRIIa and FcγRIIIa SNP frequencies in this study population versus frequencies reported in the Hap-Map database (http://hapmap.ncbi.nlm.nih.gov)

Test for Hardy-Weinberg Equilibrium: A Pearson X^2 was performed, as well as a Likelihood ratio test and exact significance. These tests were performed assuming Hardy-Weinberg Equilibrium exists and that allele frequencies of these SNPs do not differ significantly from expected. The results provided sufficient evidence that the SNPs are in Hardy-Weinberg Equilibrium.

Table 7. Tests performed to evaluate for Hardy-Weinberg Equilibrium

Polymorphism	Pearson chi2	LR chi2	Exact
FcγRIIa (n=253)	X^2=0.49, p= 0.50	X^2=0.45, p=0.50	0.53
FcγRIIIa (n=249)	X^2=0.50, p=0.48	X^2=0.50, p=0.48	0.59

Next, a test for linkage disequilibrium (LD) was performed. A Pearson X^2 test for independence was run on the two genes, with a total sample size of 253. Table 8 illustrates the breakdown between different haplotypes. Using a chi2 test with 4 degrees of freedom (df), chi2= 10.23, p= 0.04. This illustrates the potential for linked inheritance, which is consistent with the literature reporting weak linkage disequilibrium between the genes. The data show a higher frequency of HH/VV compared to RR/VV than would be expected based upon the HH and RR frequencies. However, the sample size in this study is too small for a formal haplotype analysis.

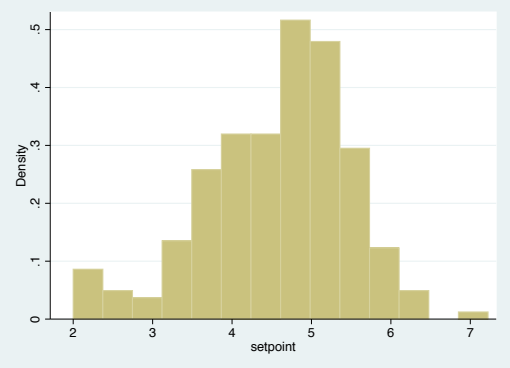
Table 8: Pearson's Chi2 test for Independence. FcγRIIa is presented as its HH, HR, and RR alleles in the first column, and FcγRIIIa is presented as FF, VF, and VV on the first row. The test results show some evidence of LD.

	FF	VF	VV	Total
HH	16	23	11	50
HR	41	71	17	129
RR	35	29	6	70
Total	92	123	34	249

X^2 (4df)= 10.23, p= 0.04

Analysis 1. Set point viral load between genotype groups

220 of the 253 women genotyped were found to have viral load data between 4-24 months post-infection. The remaining 39 had no clinic visits during that time or were lost to follow up. Figure 7 displays the distribution of $\log_{10}$ set point plasma viral load and descriptive statistics for the 220 women included.



Set point	Mean $\log_{10}$ HIV/mL	Std. Deviation	Range
220/253 women	4.53	0.92	<2-7.22

Figure 7. Log_{10} distribution of set point plasma viral load and summary statistics

The box plots in Figure 6 depict the 75th percentile (upper hinge), median (solid line), and 25th percentile (lower hinge) of $\log_{10}$ viral load at set point amongst women in each genotype group. The whiskers represent the upper and lower datum still within the 1.5 inter-quartile range. Outlier values are presented as dots outside the whiskers. There was a large amount of variation in set point,

as evidenced by the span of the whiskers. Table 9 displays unadjusted and adjusted regression results. There was no significant effect of genotype on set point plasma viral load.

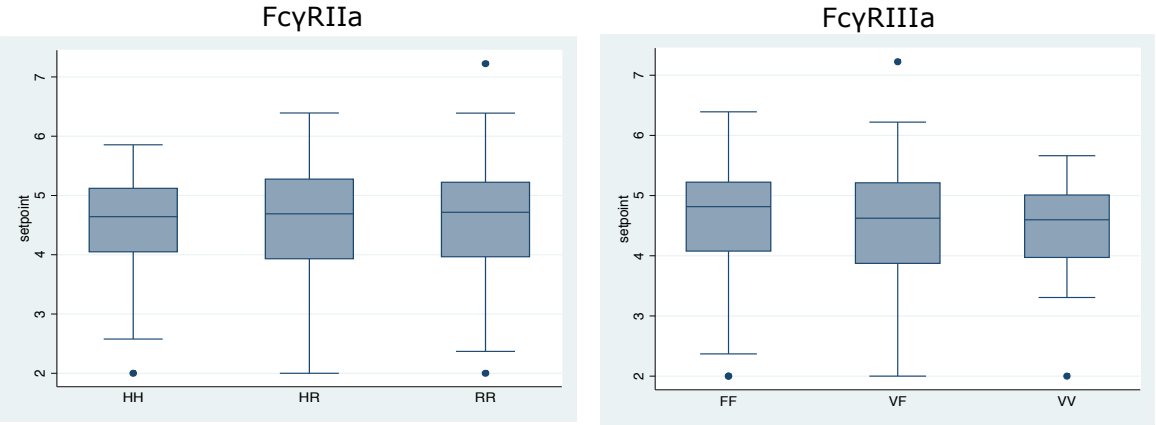


Figure 8. Boxplots of set point viral load by Fcγ genotype. No significant effect of genotype on set point plasma viral load was observed (Table 6)

Table 9. Set point plasma viral load linear regression results are shown below, including mean log_{10} set point plasma viral load for each group by genotype. Point estimates, 95% confidence intervals and p-values are reported.

FcγR genotype	Patients with Set Point	Mean set point (log10 HIV/ml)	Unadjusted regression	Unadjusted, together[a]	Adjusted for covariates, separate[b]	Adjusted for covariates, together[c]	Subtype and SI, separate[d]	Subtype and SI, together[e]
FcγRIIa HH	43	4.50	Reference	Reference	Reference	Reference	Reference	Reference
FcγRIIa HR	114	4.56	+0.03 (-0.27-0.32) p=0.87	+0.06 (-0.25-0.36) p=0.72	-0.02 (-0.32-0.29) p=0.92	+0.01 (-0.32- 0.32) p=0.99	+0.09 (-0.35- 0.53) p= 0.68	+0.05 (-0.42- 0.51) p= 0.84
FcγRIIa RR	63	4.58	+0.06 (-0.28-0.40) p=0.70	+0.07 (-0.27-0.40), p=0.70	+0.04 (-0.31- 0.38), p= 0.84	+0.03 (-0.31- 0.38) p= 0.85	+0.09 (-0.36- 0.54) p= 0.69	+0.01 (-0.47- 0.48) p= 0.98
FcγRIIIa FF	78	4.67	Reference	Reference	Reference	Reference	Reference	Reference
FcγRIIIa FV	109	4.50	-0.17 (-0.44-0.098) p=0.21	-0.17 (-0.45-0.11) p=0.22	-0.14 (-0.43- 0.15) p= 0.35	-0.13 (-0.43- 0.16) p= 0.38	-0.10 (-0.47- 0.27) p= 0.60	-0.10 (-0.50- 0.29), p= 0.60
FcγRIIIa VV	30	4.45	-0.22 (-0.57-0.13) p=0.22	-0.21 (-0.56- 0.14) p=0.23	-0.28 (-0.67- 0.11) p= 0.16	-0.27 (-0.67- 0.12) p= 0.18	-0.41 (-0.93- 0.10) p= 0.11	-0.41 (-0.96- 0.13) p= 0.14

a. Regression was performed with FcγRIIa and FcγRIIIa adjusted for each other, described as "together".
b. Regression was performed for each FcγR separately, with adjustment for *a priori* covariates: age at infection, use of hormonal contraception (DMPA, oral contraception, Norplant) within a 70-day window of infection, and genital ulcer disease at the time of infection.
c. Regression was performed with FcγRIIa and FcγRIIIa adjusted for each other, with adjustment for covariates as in (b).
d. Regression was performed for each FcγR separately in an analysis restricted to women for whom data on subtype and SI status were available (N=125).
e. Regression was performed with FcγRIIa and FcγRIIIa adjusted for each other in the separate subtype and SI analysis (N=125).

Analysis 2. Viral load over time: Linear Mixed Effects Model

Results from the linear mixed effects model are displayed in Table 10. Each FcγR gene was run in the model alone, adjusted for the other FcγR, adjusted for covariates but run alone, and adjusted for covariates including the other FcγR. The covariates in the final adjusted model were days since infection, age at infection, exposure to genital ulcer diseases at infection, and exposure to hormonal contraceptives at infection. The hormonal contraceptives were: DMPA, oral contraceptives, and Norplant. Finally, a subanalysis restricted to women with data on viral subtype and superinfection status was performed, both separately for each FcγR and together. There was no significant difference in viral load intercept or increase over time when comparing the genotype groups. Figure 9 displays viral load changes over time per woman, separated by genotype.

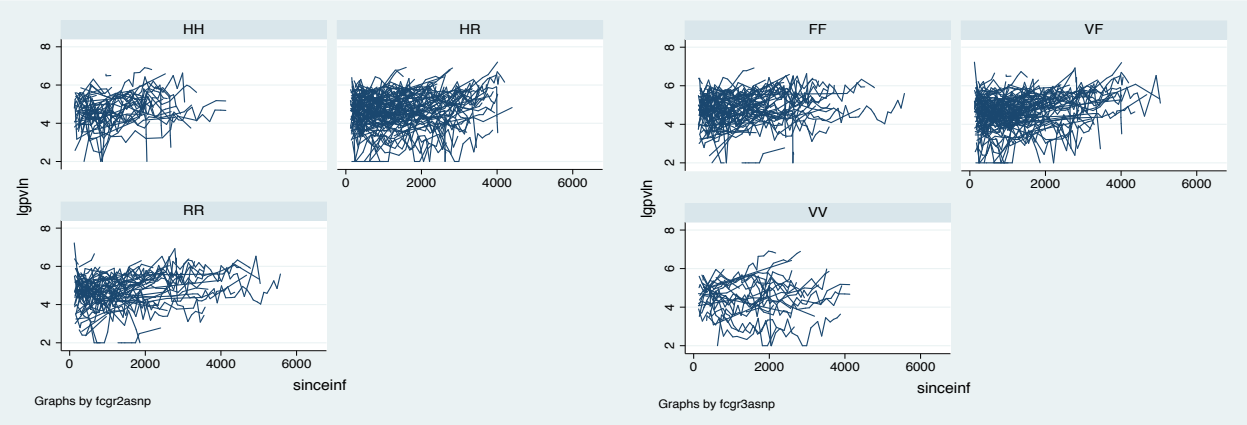


Figure 9. Viral load trajectories by FcγR genotype. The left panel is split by FcγRIIa, the right panel by FcγRIIIa. Acute infection has been removed (any viral loads prior to 4 months post-infection) and women have been censored at treatment initiation.

Table 10. Linear Mixed Effects results for viral load over time, with FcγRIIa genotype HH as reference group and FcγRIIIa genotype FF as reference group. Fcγ receptor genotypes were run as described in Table 6. Point estimates, 95% confidence intervals and p-values are reported.

FcγRIIa (N= 223), FcγRIIa (N= 221)	**Unadjusted, run separately**	**Unadjusted, run together (N=221)**	**Adjusted for *a priori* covariates, separate (N=203; 201)**	**Adjusted for *a priori* covariates, together (N=201)**	**Subtype and SI, separate (n=132)**	**Subtype and SI, together (n=132)**
FcγRIIa HH	Reference	Reference	Reference	Reference	Reference	Reference
FcγRIIa HR	-0.21 (-0.30-0.26) p=0.88	0.01 (-0.28-0.30) p=0.95	-0.06 (-0.36-0.28) p=0.72	-0.04 (-0.35-0.27) p=0.80	-0.04 (-0.35-0.27) p=0.80	-0.04 (-0.35-0.27) p=0.80
FcγRIIa RR	-0.02 (-0.33-0.29) p=0.91	-0.01 (-0.33-0.30) p=0.93	-0.06 (-0.40-0.27) p=0.71	-0.07 (-0.41-0.27) p=0.69	-0.07 (-0.41-0.27) p=0.69	-0.07 (-0.41-0.27) p=0.69
FcγRIIa HR*Days since infection	-0.000072 (-0.00020-0.000081) p=0.35	-0.00094 (-0.0023-0.00050) p=0.22	-0.000094 (-0.00023-0.00050) p=0.22	-0.00010 (-0.00030-0.00051) p=0.16	-0.00010 (-0.00030-0.00051) p=0.16	-0.00010 (-0.00030-0.00051 p=0.16
FcγRIIa RR*Days since infection	-0.000091 (-0.00024- 0.00081) p=0.26	-0.00010 (-0.00031-0.00062) p=0.21	-0.000073 (-0.00022-0.000091) p=0.38	-0.000080 (-0.00032-0.0011) p=0.38	-0.000080 (-0.00032-0.0011) p=0.38	-0.000080 (-0.00032-0.0011) p=0.38
FcγRIIIa FF	Reference	Reference	Reference	Reference	Reference	Reference
FcγRIIIa FV	-0.17 (0.45-0.06) p=0.15	-0.17 (-0.45-0.06) p=0.15	-0.12 (-0.37-0.13) p=0.34	-0.13 (-0.38-0.13) p=0.34	-0.13 (-0.38-0.13) p=0.34	-0.13 (-0.38-0.13) p=0.34
FcγRIIIa VV	-0.10 (-0.44-0.24) p=0.57	-0.10 (-0.44-0.24) p=0.56	-0.13 (-0.50-0.24) p=0.49	-0.14 (-0.52-0.23) p=0.45	-0.14 (-0.52-0.23) p=0.45	-0.14 (-0.52-0.23) p=0.45
FcγRIIIa FV*Days since infection	0.000093 (-0.00031-0.00023) p=0.17	0.00081 (-0.00042-0.00021) p=0.18	0.00054 (-0.00082-0.00023) p=0.41	0.00062 (-0.00073-0.0011) p=0.37	0.00062 (-0.00073-0.0011) p=0.37	0.00062 (-0.00073-0.0011) p=0.37
FcγRIIIa VV*Days since infection	-0.0000084 (0.00022– 0.00024), p=0.92	-0.000032 (-0.00021-0.00011), p=0.70	-0.0000031 (-0.00020-0.00011), p=0.97	-0.000012 (-0.00021-0.00023) p=0.85	-0.000011 (-0.00021-0.00023) p=0.85	-0.00001 (-0.0002-0.0002) p=0.85

Analysis 3. CD4+ T cells over time: Linear Mixed Effects Model

Results from the linear mixed effects model for CD4+ T cells are displayed in Table 11. There was not a significant difference in square root CD4 cell trajectories when compared between genotype groups. Because the CD4 cell data were skewed, the square root of CD4+ T-cells/mm^3 was used to better approximate a normal distribution. Figure 10 displays CD4 cell decline over time per woman, separated by genotype as in the viral load analysis. Table 11 gives summary statistics for each analysis performed.

Each FcγR gene was run in the model alone, adjusted for the other FcγR, adjusted for covariates but run alone, and adjusted for covariates including the other FcγR. The covariates in the final adjusted model were days since infection, age at infection, exposure to genital ulcer diseases at infection, and exposure to hormonal contraceptives at infection. The hormonal contraceptives were: depo provera, oral contraceptives, and norplant. Finally, a subanalysis restricted to women for whom data on viral subtype and superinfection status were available was performed, both separately for each FcγR and together. There was a modestly higher slope for the FcγRIIa HR group that was significant only in the unadjusted analysis. In addition, the intercept in the FcγRIIa VV group was higher compared to the reference group (FF). This finding was significant in the adjusted analysis, both with and without inclusion of subtype and SI status.

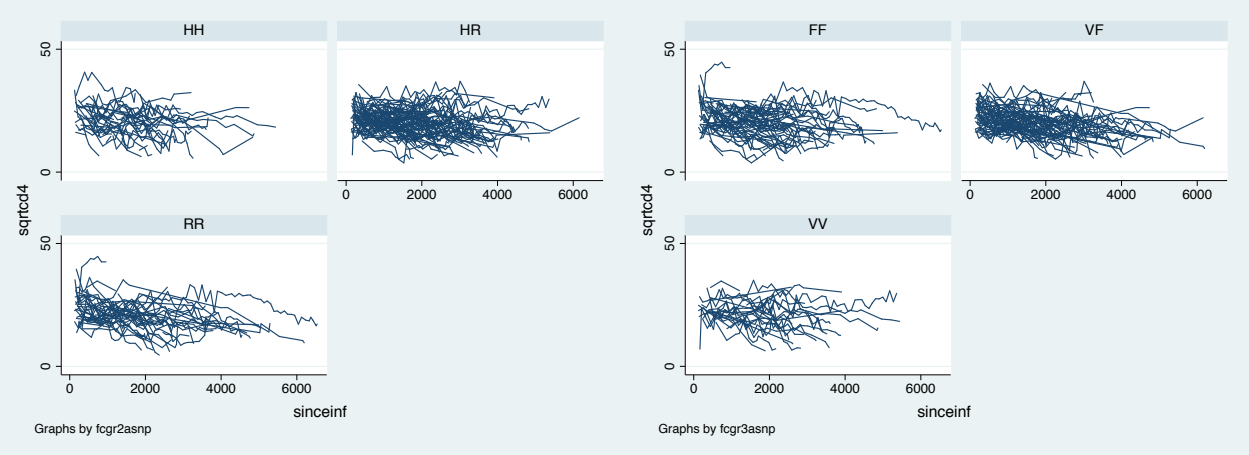


Figure 10. CD4 cell trajectories by FcγR genotype. The left panel is split by FcγRIIa, the right panel by FcγRIIIa. Acute infection has been removed and women have been censored at treatment initiation.

Table 11. Summary Statistics of LME CD4+ T cells over time, using FcγRIIa genotype HH as reference group and FcγRIIIa genotype FF as reference group. Fcγ receptor genotypes were run as described in Table 6. Point estimates and 95% confidence intervals are reported.

FcγRIIa (N= 194), FcγRIIa (N= 191)	**Unadjusted, run separately**	**Unadjusted, run together (N=191)**	**Adjusted for *a priori* covariates, separate (N=176)**	**Adjusted for *a priori* covariates, together (N=176)**	**Subtype and SI analysis, separate (n=118)**	**Subtype and SI analysis, together (n=118)**
FcγRIIa HH	Reference	Reference	Reference	Reference	Reference	Reference
FcγRIIa HR	-1.34 (-3.24-0.57) p=0.17	-0.71 (-2.63-1.21) p=0.47	-1.74 (-3.76-0.28) p=0.091	-0.93 (-2.97-1.12) p=0.38	-1.87 (-4.31-0.57) p=0.13	-1.44 (-3.89-1.02) p=0.25
FcγRIIa RR	+0.57 (-1.55-2.68) p=0.60	+1.47 (-0.70-3.64) p=0.18	+0.68 (-1.52-2.90) p=0.54	+1.88 (-0.39-4.16) p=0.10	+1.16 (-1.4-3.7) p=0.37	+2.12 (-0.54-4.78) p=0.12
FcγRIIa HR*Days since infection	+0.0012 (0.00044-0.0025) p=0.042	+0.0012 (-0.00007-0.0025) p=0.063	+0.0012 (-0.000026-0.0025) p=0.055	+0.0012 (-0.00012-0.0025) p=0.075	+0.0014 (-0.000072-0.0029) p=0.062	+0.0013 (-0.00014-0.0028) p=0.075
FcγRIIa RR*Days since infection	+0.0008 (-0.00055- 0.0022) p=0.24	+0.00066 (-0.00082-0.0021) p=0.38	+0.00055 (-0.00082-0.0024) p=0.54	+0.00043 (-0.0011-0.0023) p=0.58	+0.00046 (-0.0011-0.002) p=0.57	+0.00027 (-0.0014-0.002) p=0.74
FcγRIIIa FF	Reference	Reference	Reference	Reference	Reference	Reference
FcγRIIIa FV	+0.13 (-1.5-1.75) p=0.88	+0.50 (-1.12-2.12) p=0.55	+0.37 (-1.3-2.04) p=0.67	+0.85 (-0.81-2.51) p=0.32	+1.01 (-1.09-3.11) p=0.35	+1.45 (-0.59-3.48) p=0.16
FcγRIIIa VV	+1.70 (-0.53-3.93) p=0.14	+2.21 (-0.062-4.48) p=0.057	+2.00 (-0.33-4.33) p=0.092	+2.62 (0.26-4.97) p=0.029	+2.51 (-0.36-5.38) p=0.086	+3.47 (0.54-6.39) p=0.020
FcγRIIIa FV*Days since infection	-0.000063 (-0.0013-0.0010) p=0.99	-0.000082 (-0.011-0.0010) p=0.87	-0.000070 (-0.014-0.0011) p=0.90	-0.000070 (-0.014-0.0012) p=0.91	-0.00017 (-0.0014-0.001) p=0.79	-0.00027 (-0.0015-0.0012) p=0.66
FcγRIIIa VV*Days since infection	-0.00033 (-0.0018– 0.001) p=0.64	-0.00032 (-0.0017-0.0012) p=0.72	-0.00051 (-0.0015-0.0014) p=0.72	-0.00072 (-0.0015-0.0014) p=0.93	-0.00030 (-0.0014-0.0013) p=0.72	-0.00046 (-0.0021-0.0012) p=0.59

Analysis 4. HIV-1 Disease progression: Cox Proportional Hazards Model

Three different analyses were used to give an approximation of overall disease progression. Those analyses are summarized in Table 4 in the methods section, displayed again below. Each analysis is presented with Kaplan-Meier survival estimate curves and a table of Cox Proportional Hazards statistics. Each analysis included events and total person-years per genotype group, incidence rates, crude hazard ratios, and hazard ratios adjusted for covariates.

Table 4. Three different outcomes were used in the Cox Proportional Hazards model.

Analysis	Cox Proportional Hazards Outcome
A	Time to CD4 <200
B	Time to death
C	Combined outcome of time to death, CD4<200, or ART initiation if earlier than CD4<200

A. Time to CD4 < 200 cells/mm^3

Of the 253 seroconverters genotyped, 209 met the criteria of >=1 CD4+ T-cell count from the clinic and were included in this analysis. 81/209 women had a CD4+ T-cell count of <200 cells/mm^3 during follow-up. The Kaplan-Meier survival curve shown compares survival estimates for time to CD4<200 for each group (Figure 11). No difference in survival time between genotype groups was observed. The Cox proportional hazards model was run both without adjustment and with adjustment for age at infection and viral set point, both known predictors of disease progression. Additionally, failures per person year and incidence per 100 person-years were also calculated. Table 12 displays Cox model statistics. There was not a significant difference between genotype groups.

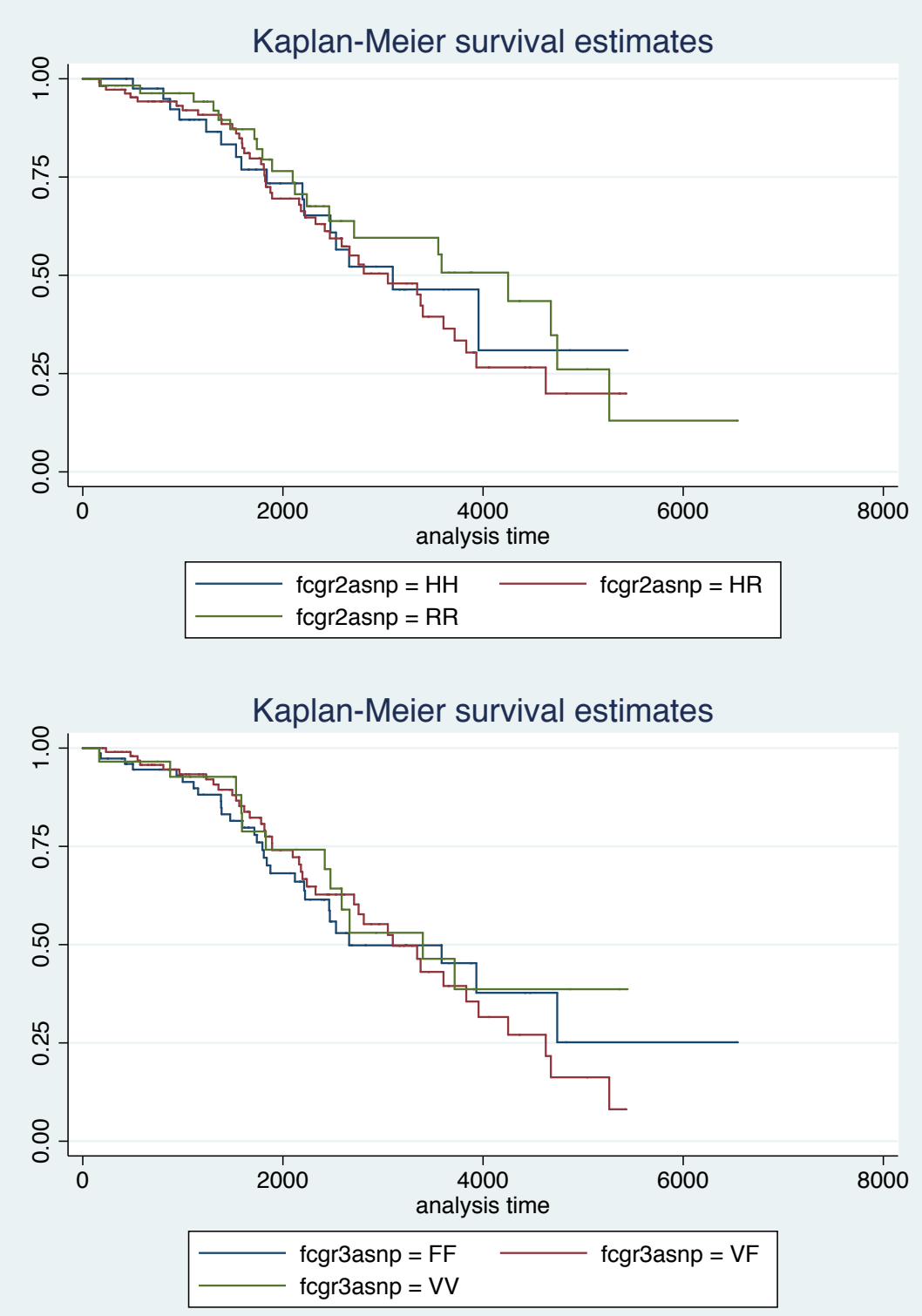


Figure 11. Kaplan-Meier CD4 cell decline survival estimates by FcγRIIa and FcγRIIIa genotypes. Failure is defined as the first time a woman's CD4 count goes below 200 cells/mm^3. There is no significant difference in hazard ratio between genotypes (Table 12)

Table 12. Cox proportional hazards of the relationship between FcγRIIa and FcγRIIIa genotypes and time to CD4<200 cells/mm^3

FcγR	Women who progressed to CD4<200/PY	Incidence/100 person years (95% CI)	Crude Hazard Ratio (95% CI)	Adjusted Hazard Ratio* (95% CI)
FcγRIIa HH	16/237.2	6.7 (4.1-11.0)	Reference	Reference
FcγRIIa HR	44/590.1	7.5 (5.5-10.0)	1.13 (0.63-2.01) p=0.69	1.36 (0.71- 2.60) p=0.35
FcγRIIa RR	21/343.4	6.1 (4.0-9.4)	0.86 (0.44-1.66) p=0.66	0.98 (0.48-2.02) p=0.96
FcγRIIIa FF	29/416.1	7.0 (4.8-10.0)	Reference	Reference
FcγRIIIa FV	39/548.5	7.1 (5.4-9.7)	1.01 (0.62-1.63) p=0.97	1.01 (0.65-1.83) p=0.72
FcγRIIIa VV	12/189.6	6.3 (3.6-11.1)	0.80 (0.41-1.57) p=0.51	0.87 (0.40-1.87) p=0.71

***Adjusted for age at infection and viral set point**

B. Time to death

54 of the 253 genotyped women died during follow-up. The Kaplan-Meier survival curve depicts the overall survival estimate, with an indication of failure at the date of death, measured in days since infection. No difference in survival estimates between genotype groups was observed (Figure 12). The Cox proportional hazards model was run both without adjustment and with adjustment for age at infection and viral set point. Additionally, failures per person year and incidence per 100 person-years were also calculated. Table 13 displays Cox model statistics. Although FcγRIIa RR was associated with a lower incidence rate (2.6 deaths/100 person-years vs. 4.2 deaths/100 person-years for HH), the adjusted hazard ratio of 0.50 was not statistically significant.

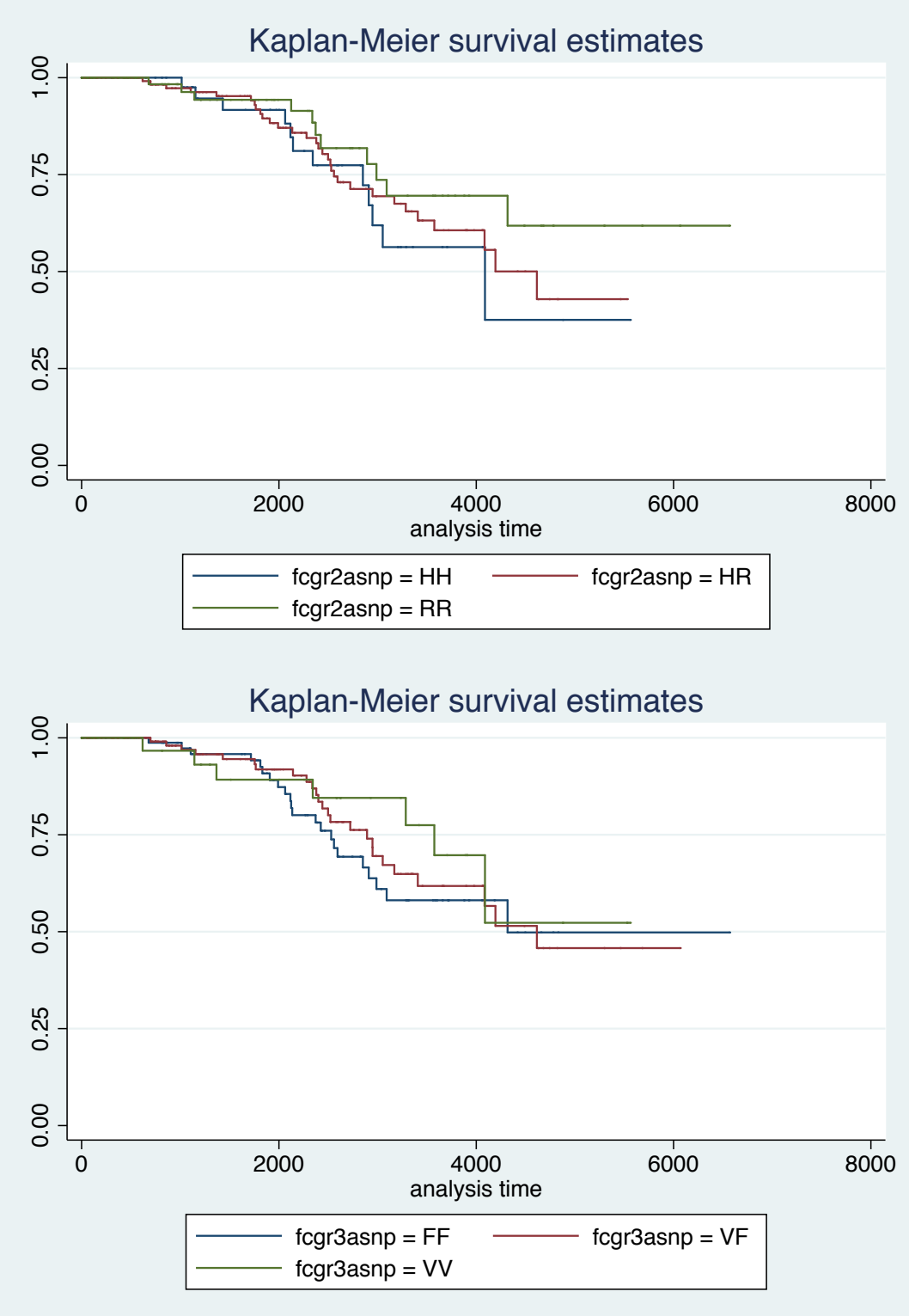


Figure 12. Kaplan-Meier survival estimates by FcγRIIa and FcγRIIIa genotypes. Death dates were reported to the clinic and are as accurate as possible. There is no significant difference in hazard ratio between genotypes (Table 13).

Table 13. Cox proportional hazards of the relationship between FcγRIIa and FcγRIIIa genotypes and time to death

FcγR	Deaths/PY	Deaths/100 person years (95% CI)	Crude Hazard Ratio (95% CI)	Adjusted Hazard Ratio* (95% CI)
FcγRIIa HH	12/288.3	4.2 (2.4-7.3)	Reference	Reference
FcγRIIa HR	31/795.9	3.9 (2.7-5.5)	0.88 (0.45-1.70) p=0.70	0.72 (0.35-1.50) p=0.37
FcγRIIa RR	11/422.3	2.6 (1.4-4.7)	0.60 (0.26-1.36) p=0.23	0.50 (0.21-1.20) p=0.12
FcγRIIIa FF	22/542.7	4.1 (2.7-6.2)	Reference	Reference
FcγRIIIa FV	25/714.8	3.5 (2.4-5.2)	0.86 (0.48-1.50) p=0.60	0.74 (0.40-1.40) p=0.34
FcγRIIIa VV	7/231.8	3.0 (1.4-6.3)	0.67 (0.29-1.60) p=0.36	0.45 (0.17-1.20) p=0.12

***Adjusted for age at infection and viral set point**

C. Combination outcome: time to CD4 < 200 cells/mm^3, ART initiation, or death

During the course of follow-up, 136 of the 253 (53.8%) genotyped women reached at least one of the three failure criteria for the combination outcome (CD4<200, death or initiation of antiretroviral treatment). In this model, whichever event occurred first for a woman became her failure event. Figure 13 displays Kaplan-Meier survival estimates and Table 14 gives summary statistics. There was no significant affect of genotype on disease progression in this model.

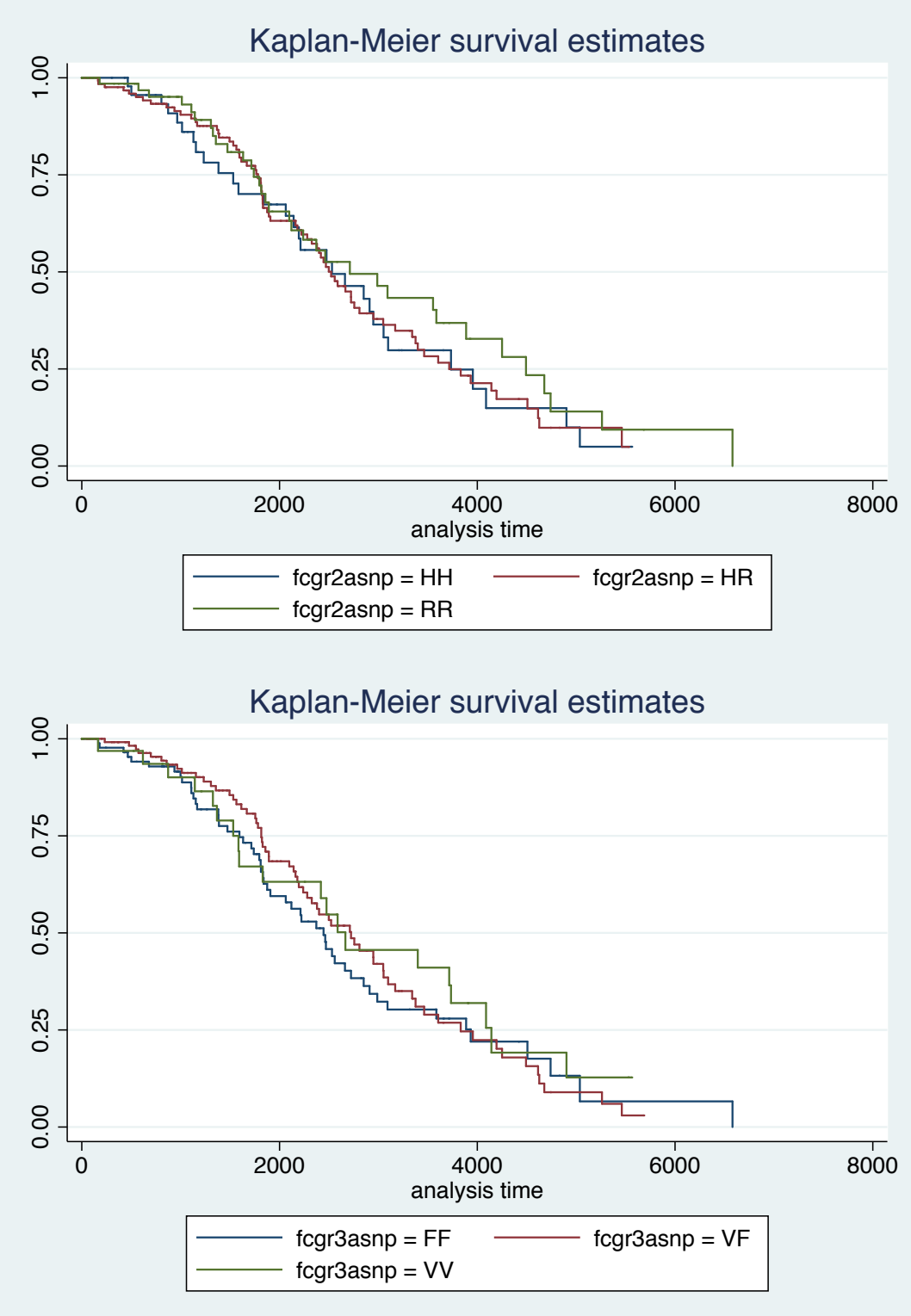


Figure 13. Kaplan-Meier combination outcome survival estimates by FcγRIIa and FcγRIIIa genotypes. There is not a significant difference in hazard ratio between genotypes (Table 14).

Table 14. Cox proportional hazards of the relationship between FcγRIIa and FcγRIIIa genotypes and a combination outcome of time to death, CD4<200, or time to treatment, whichever outcome came first.

FcγR	Events/PY	Incidence/100 person years (95% CI)	Crude Hazard Ratio (95% CI)	Adjusted Hazard Ratio* (95% CI)
FcγRIIa HH	30/247	11.0 (7.7-15.7)	Reference	Reference
FcγRIIa HR	73/707.5	10.3 (8.2-13.0)	0.94 (0.61- 1.52) p= 0.79	1.03 (0.64- 1.70) p= 0.89
FcγRIIa RR	34/378	9.0 (6.4-12.6)	0.72 (0.43- 1.21) p=0.22	0.79 (0.46- 1.37) p=0.40
FcγRIIIa FF	52/484.3	10.7 (8.2-14.1)	Reference	Reference
FcγRIIIa FV	64/651.2	9.8 (7.7-12.6)	0.87 (0.60 - 1.27) p= 0.47	0.97 (0.65 - 1.46) p= 0.89
FcγRIIIa VV	20/207.9	9.6 (6.2-14.9)	0.71 (0.41 -1.22) p= 0.22	0.71 (0.39 -1.31) p= 0.28

***Adjusted for age at infection and viral set point**

Chapter 4

Discussion

Summary of findings

In summary, we have found there to be no association between FcγR genotype and set point viral load, viral load change over time, or overall disease progression. We detected an association between FcγRIIIa VV genotype and a higher CD4 cell count after acute infection (intercept) in the linear mixed effects model (Table 11). This association was of borderline significance when FcγRIIIa genotype was adjusted for FcγRIIa genotype (p=0.057), but became statistically significant after adjustment for covariates (p=0.029) and after further adjustment for viral subtype and superinfection status (p=0.020). However, no association was observed between the FcγRIIIa VV genotype and change in CD4 cell count over time. In addition, no association was seen between the FcγRIIIa VV genotype and set point plasma viral load, viral load over time, or survival, although the hazard estimate for the VV genotype was consistently lower than for the FF or FV genotypes. There were also no associations between FcγRIIa and any of the measures of disease progression, despite a small protective effect among HR heterozygotes for CD4 decrease over time in the unadjusted analysis (p=0.045, Table 11).

The association seen between FcγRIIIa VV and CD4 cell count at the beginning of chronic infection (intercept of linear mixed effect model) does not have a clear biological explanation. Women with the VV genotype could have had higher CD4 cell counts prior to infection, so that an equivalent CD4 cell drop during acute infection appeared as though VV was protective. This would explain why a difference was detected at the beginning of chronic infection but not in the slope, since the mechanism would presumably be independent of HIV-specific ADCC. However, this is not supported by evidence from other studies in FcγR genotypes and we do not have pre-infection

CD4 data on these women for comparison. Alternately, women with the VV genotype could be experiencing less CD4 cell loss during acute infection than women with VF or FF genotypes. It is known that impaired ADCC is associated with disease progression, so ADCC may play a greater role in viral control early in infection.[59] If this is true, the greatest difference in CD4 cell count due high affinity or low affinity FcγRIIIa receptors may be during acute infection or early in chronic infection, which is what we see here.

Strengths, limitations and other studies

This study utilizes the tremendous resource of a 20-year ongoing cohort of HIV-1 seroconverters from a high-risk population. Many women in this study have been followed for more than a decade, with several that have been followed longer than 6000 days post-infection, over 16 years. However, female sex workers are a socially marginalized population, and women have frequently become lost to follow up, or failed to attend clinic for long periods of time. 30 women in this study were only able to contribute 2 visits of viral load and CD4 data to the linear mixed effects models once we had restricted the analysis to >4months post infection. Additionally, CD4 cell counts only became a routine part of clinical follow-up in 1998. This subjects the data to calendar year bias since the CD4 linear mixed model and CD4 survival analyses examine the cohort later in the epidemic than the viral load analyses do. It is possible that we have missed seeing an association because of these limitations.

Our findings suggest that the FcγRIIa and FcγRIIIa genotypes do not play an important role in predicting HIV-1 disease progression Kenyan women. While Forthal *et al* observed that FcγRIIa RR was associated with faster progression to CD4<200 cells/mm^3 than RH or HH, the study population was the Multicenter AIDS Cohort Study (MACS), which is male-only and predominantly Caucasian, with infection almost exclusively due to Subtype B[52]. That study included 558 men

genotyped for FcγRIIa and 557 genotyped for FcγRIIIa who were enrolled in MACS between 1984 and 2006. Our cohort in Mombasa is female only and of Kenyan descent, with infections that were predominantly Subtype A. We were able to genotype 253 women for FcγRIIa and 249 for FcγRIIIa. Enrollment began in 1993, nearly a decade later than MACS. Differences between sex, ethnic make-up, or infection subtype in these cohorts may explain the some of the differences between our findings.

A sample size comparison of the two studies highlights that we may not have had the power to detect the difference that Forthal *et al* described in their CD4 progression analysis (using PS version 3.0.43). Forthal *et al* included 550 men with both CD4 and genotype data, while we only had CD4 and genotype data on 209 women. Forthal *et al* found a hazard ratio of FcγRIIa RR=1.6 ($p=0.001$) using HH as the reference, of whose median time to CD4<200 cells/mm^3 was approximately 8 years. Assuming the true hazard ratio between RR and HH is 1.6, for 90% power we would have needed 220 women with each homozygous genotype (880 total women with a genotype distribution of 25% HH, 25% RR and 50% HR) to reach 95% significance ($\alpha=0.05$). Of the 209 women contributing time to this analysis, 59 were RR and 50 were HH, giving 40% power to detect this difference if the true hazard ratio is 1.6. However, since the effect estimate in our study for FcγRIIa RR is 0.86 compared to HH, it would be surprising to see the estimate change so much in the opposite direction. Accurate baseline incidence and follow-up time from the Forthal study were not readily available, so these comparisons are approximate. Based upon the two studies' comparative data, a larger study is warranted.

Our findings also do not support those of Poonia *et al*, who found a significant association between the FcγRIIIa VV genotype and HIV-1 disease progression. [53] Poonia *et al* examined genotype distributions between groups termed "normal progressors" and "Natural Virus Suppressors", using a different method (cross-sectional) than our prospective cohort study or the

prospective cohort study by Forthal *et al*. The population in the study by Poonia *et al* included both males and females in approximately equal distributions, was almost entirely African-American and comprised of 43 Natural Virus Suppressors (NVS) and 59 Progressors. No information on viral subtype was given. Poonia *et al* did not find any VV homozygotes in the pool of NVS, but found 6 in the Progressor group and 2 in the uninfected control groups (0/43, 6/59, and 2/70, respectively). The SNP distribution for healthy African-Americans has been reported as 8-10% VV. [45,54] While Poonia *et al* reported a statistically significant difference in VV distribution between the infected and uninfected groups ($p=0.025$), their study population was small. Additionally, NVS sample size was only 43, which may not have been large enough to include sufficient VV individuals. With these small sample sizes, it could be chance that this group does not have any VV homozygotes. We believe that our larger study shows in a more robust fashion that FcγRIIIa VV is not associated with increased risk of HIV-1 disease progression.

It has also been proposed that FcγRs may be acting in epistasis with other genes to confer some protection against HIV-1 disease progression. For example, Deepe *et al* examined the distribution FcγRIIa and FcγRIIIa genotypes along with SNPs in the Fc region of the antibody itself and found evidence for the GM (IgG γ chain) allotypes influencing the association between FcγR genotype and the probability of being a viral controller. [64] We have noticed in our study that when both FcγRs are included in the CD4 mixed effects model, the effect estimate for FcγRIIIa VV increases and becomes more significant. This may suggest that the receptors are acting collaboratively, in opposition to one another, or each act with a third party. A future study with this data could be to explore the relationship between GM allotypes, FcγR genotypes, and HIV-1 disease progression.

Implications and future directions

This study adds to the body of knowledge regarding the roles of FcγRIIa and FcγRIIIa genetic polymorphisms and HIV-1 disease progression, particularly in African women. The data we show suggest that polymorphisms in the FcγRs may be less important to viral control or health status than has been seen in other populations, and it implies that there may be other host or viral factors more important in these women's ability to control virus and keep a healthy CD4 count. These factors might even include FcγR relationships with other polymorphic genes, such as those in the HLA region, KIRs, CCR5 ligands, or Toll-like receptors. Although these receptors and ligands dot not act directly with the FcγRs in the way that the GM allotypes do, examining them together may be revealing of additive effects.

As a follow-up study, we would recommend repeating these analyses using a similar cohort with larger numbers. For example, the ongoing CAPRISA 002 cohort of women in South Africa could be a good comparison using a similar population. The Partners PrEP study could also be a candidate for this analysis if the research team continues to follow seroconverters longitudinally.

Finally, we plan to continue this study by genotyping a subset of uninfected women from the Mombasa cohort and analyzing the relationship between FcγRIIa and FcγRIIIa genotypes and risk of HIV-1 acquisition. We plan to use a case cohort study design, which allows for relative risk estimation by comparing cases to a random sample of the study population including both cases and non-cases. Relative risk is a more informative estimate than an odds ratio and more appropriate when the outcome is not rare. Since women in the Mombasa cohort are at high risk for HIV-1 acquisition and since both seronegative and seropositive women have been followed longitudinally, a case-cohort study will allow us to accurately measure the impact of FcγRIIa and FcγRIIIa genotypes on the risk of acquiring HIV-1 in this population.

Ethical issues and clinical implications

As the HIV research field continues to examine host factors, it is likely that more polymorphic genes predictive of HIV-1 disease progression will be thoroughly researched in many different populations. The question remains in what to do with the genetic information these studies are generating. If it is known that promoter polymorphisms in CCR5 can delay progression to AIDS, is it the role of the study to return results to the study participants? Or might it only be appropriate to take action on a gene that increases rate of progression, such as the HLA-B variant *57? If polymorphic genes are predictive of HIV-1 disease progression, guidelines must be created regarding what to do with the genetic information, particularly if it may be clinically actionable or of value to the participants. While debates regarding return of results are not new to medical research involving cancer or cognitive decline, they have recently emerged in infectious disease research on human subjects in developing nations. [65-67]

Using a virtue ethics framework from the viewpoint of the researcher, the decision to return results should be based upon how the researcher interprets and applies established guidelines. In 2006, the National Heart, Lung, and Blood Institute (NHLBI) gave recommendations to help the researcher decide when to return results.[68] The NHLBI recommendations focused on proven health interventions and significance of disease risk associated with the genetic variant, but suggested each case be examined individually. The professional guidelines provide a rule-based parameter for action, but it remains up to the individual researcher to make a judgment call regarding what he or she believes is the best thing to do given the evidence and impact on participants.

Another example of an approach to guidelines is the Burke, Pinsky, and Press (BPP) model. The BPP model provides a categorical framework to assist return of results decision-making. BPP gave guidelines for return of genetic results based upon two criteria: clinical validity and clinical utility.[69] Clinical validity has two parts: the quality of the literature associating the genetic

polymorphism with disease risk, and the sensitivity and specificity of the field-based genetic test or laboratory genotyping. Clinical utility refers to the ability to act on the knowledge. If there is no known method to reduce disease risk upon knowledge of genetic predisposition, the clinical utility is low. If the knowledge allows for preventive measures or clinical action to be taken that can change the course of the disease, clinical utility is high. These criteria were first developed in 2002 to inform emerging guidelines for clinical genetics, but can also be applied to data from clinic-based research cohorts.

The BPP model can be utilized to examine the ethical question of return of results in genetic studies like ours. Breaking down the BPP to this specific situation, we can assess the clinical validity and utility of returning results of genotype predicting rate of HIV-1 disease progression.

In this case, clinical validity is informed by robust science that biologically validates a gene associated with disease progression. Criteria for robust science could include a highly significant finding that is supported by several independent studies conducted in the same human population. To illustrate, if we were to genotype the Mombasa cohort for HLA-B alleles and find that those women with *57 are progressing to CD4<200 cells/mm^3 or to death at a rate much more quickly than those who do not have *57, the BPP may guide us to open up a conversation around the return of these results. This would be based upon the level of significance and the quality of supporting literature, of which there are several other studies. [20,70-72] However, if we had found in our study of Fcγ receptors that genotype was highly predictive of progression, based upon these criteria we would still need more studies in Africans and in women to validate our findings before any discussion of return of results should begin.

If there is sufficient evidence that women with a particular genetic profile are at risk of progressing to AIDS more quickly than others, there may be a clinically valid reason to return results to allow for a change in care. This leads us to the question of clinical utility; whether clinical

intervention would change health outcomes for women with a high-risk genotype. That change in care might be simple: a recommendation to initiate antiretroviral therapy (ART) earlier than women with a low-risk genotype. Currently the Mombasa clinic provides ART for women once they have declined to a CD4 cell count <350 cells/mm^3. In order to give a clinically valid recommendation to initiate ART earlier we would also need research supporting when those high-risk women should initiate ART. Additionally, it would be important to validate that treating earlier will have a significant health impact for women with the high-risk genotype. Finally, the recommendation to begin ART early is only applicable if there are available resources to allocate ART to some women earlier than others. This brings up many other ethical issues around allocation of resources, opportunity costs, and treating people unequally based upon genotype.

For the virtue-based researchers, if genetic studies yield information that meets the BPP clinical guidelines for returning results (both high validity and high utility), it would be unethical to not provide information to the participants because of the potential health benefit. In our study, if we had found that the Fcγ receptors were highly predictive of disease progression and we did not inform women possessing high-risk alleles, we risk being negligent in addressing something that could benefit the health of our study participants. On the other hand, if the BPP guidelines are not met, returning results could actually be harmful. If we rushed to inform participants of results that were not verified properly or were not clinically actionable, we risk adding anxiety that might be unfounded.

The BPP model does leave out some important considerations. In the absence of clinical utility, there might be other reasons why participants would want their genetic results. For example, individuals may gain personal utility from their genetic information if it is clinically valid. [73,74] In the case of high clinical validity and low clinical utility, study participants might still find it important to

know their risk of quickened HIV-1 disease progression. Likewise, there may be reasons why people do not want the information, despite meeting the BPP guidelines for returning results.

Our recommendations are that guidelines established to address these concerns use the BPP model but also take into account participant preferences. In order to do that, we would need to conduct a research study of the Mombasa Cohort participants to assess whether they would want the information. In this way, we can develop practices that are both evidence-based and participant-driven.

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