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FC gamma receptor genetic variability in paediatric HIV-1 infection

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FC GAMMA RECEPTOR GENETIC VARIABILITY IN PAEDIATRIC HIV-1 INFECTION

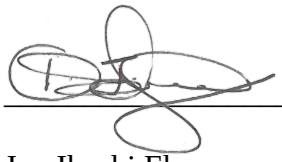
Joy Ikechi Ebonwu

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg, 2022

DECLARATION

I, Joy Ikechi Ebonwu, declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



_____ (Signature of candidate)

Joy Ikechi Ebonwu

08 August 2022

DEDICATION

My Parents: Late Sir & Lady R.A. Odimegwu

My husband: Emmanuel Ebonwu

ABSTRACT

The crystallisable fragment (Fc) region of immunoglobulin G antibodies engages with Fc gamma receptors (FcγRs) to recruit antiviral effector functions of the innate immune system. Allelic variation within the *FCGR* genes that encode FcγRs alter the potency of these effector functions by modulating the receptor surface density, binding affinity, cellular localization, glycosylation and cellular distribution. Genetic association studies of functional FcγR genetic variants can therefore provide insight into the role of Fc-mediated effector functions in affecting disease susceptibility and outcome. This study aimed to investigate FcγR variants in the context of perinatal HIV-1 acquisition, mortality, virological control and latent reservoir size during antiretroviral treatment in the early life of children living with HIV-1. A nested case-control study was conducted, combining *FCGR* genotypic data from five perinatal cohorts at two hospitals in Johannesburg, South Africa. All study participants were black South Africans and received nevirapine for prevention of mother-to-child transmission. Children with perinatally-acquired HIV-1 (cases) were compared to HIV-1-exposed uninfected children (controls). Functional variants were genotyped using a multiplex ligation-dependent probe amplification assay and Sanger sequencing. Their representation compared between groups using regression analyses. Increased odds of perinatal HIV-1 acquisition was associated with the *FCGR2C* c.134-96T-allele, *FCGR3A* gene duplication, homozygous FcγRIIb-232T allele, and FcγRIIIb-HNA1a, but not the common FcγRIIa-H166R and FcγRIIIa-F176V polymorphisms. The low-affinity FcγR variants did not associate with mortality, except for *FCGR3A* copy number variation that demonstrated a positive association. None of the FcγR variants associated with pre-treatment viral load, virologic failure, or size of the HIV-1 DNA reservoir. In contrast to the protective effect observed in the Thai RV144 trial, we found the *FCGR2C* variant c.134-96T-allele associated with increased odds of perinatal HIV-1 acquisition in South African children. These findings, taken together with a similar deleterious association found with HIV-1 disease progression in South African adults, highlight the importance of elucidating the functional relevance of this variant in different populations and vaccination/disease contexts. Our findings suggest that the FcγRIIb-232TT genotype exerts a controlling influence on infant susceptibility to HIV-1 infection. We also show a deleterious role for the less studied *FCGR3A* copy number variation and homozygous HNA1a. These findings provide additional insight into a role for FcγRs in HIV-1 infection in children.

PUBLICATIONS AND PRESENTATIONS

Publications from this thesis

- **Ebonwu J**, Lassaunière R, Paximadis M, Goosen M, Strehlau R, Gray GE, Kuhn L and Tiemessen CT (2021). An HIV vaccine protective allele in FCGR2C associates with increased odds of perinatal HIV acquisition. Front. Immunol. 12:760571. doi: 10.3389/fimmu.2021.760571
- **Ebonwu J**, Lassaunière R, Paximadis M, Strehlau R, Gray GE, Kuhn L and Tiemessen CT *FCGR3A* gene duplication, FcγRIIb-232TT and FcγRIIIb-HNA1a associate with an increased risk of vertical acquisition of HIV-1 (in press at PLOS ONE journal – manuscript number: PONE-D-22-06428)

Presentations at meetings

- *The FCGR2C allele that associates with protection against HIV-1 acquisition in the Thai RV144 HIV-1 vaccine trial is associated with increased risk of perinatal HIV-1 acquisition in South African children*. [On-demand poster presentation] 4th IAS Virtual Conference on HIV Research for Prevention HIV Research for Prevention (HIVR4P). 27-28 January and 3-4 February 2021

- *An HIV vaccine protective allele in FCGR2C associates with increased odds of perinatal HIV acquisition*. [Oral presentation] National Institute for Communicable Diseases Scientific Meeting. 24 November 2021, Johannesburg, South Africa

- *FCGR3A gene duplication, FcγRIIb-232TT and FcγRIIIb-HNA1a associate with an increased risk of vertical acquisition of HIV-1*. [poster presentation] 24th international AIDS conference. 29 July – 2 August 2022, Montreal, Canada

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LIST OF ABBREVIATIONS/NOMENCLATURE

ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
AIDS	Acquired immunodeficiency virus syndrome
AOR	Adjusted odds ratio
ART	Antiretroviral therapy
BCR	B cell antigen receptor
Bonf	Bonferroni correction
CI	Confidence interval
CNR	Copy number regions
CNV	Copy number variation
CTL	Cytotoxic T-lymphocytes
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Fab	Antigen binding fragment
Fc	Crystallisable fragment
FcγR	Fc gamma receptor
FCGR	Fc gamma receptor
FcR	Fc receptor
FcRn	Neonatal Fc receptor
GRCh38	Genome Reference Consortium Human Reference 38
Gp	Glycoproteins
GPI	Glycosylphosphatidylinositol
GWAS	genome-wide association studies
HGVS	Human Genome Variation Society
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HLA	Human leukocyte antigen
HNA	Human neutrophil antigens

HVTN	HIV Vaccine Trials Network
ICGH	International Collaboration for the Genomics of HIV
Ig	Immunoglobulin
IgG	Immunoglobulin G
IQR	Inter-quartile rang
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
LD	Linkage disequilibrium
Lopinavir-ritonavir	LPV/r
MLPA	Multiplex ligation-dependent probe amplification
MTCT	Mother-to-child transmission
NA	Neutrophil antigens
nAbs	Neutralising antibodies
NK	Natural killer cells
NVP	Nevirapine
OR	Odds ratio
ORF	Open reading frame
PCR	Polymerase chain reaction
PMTCT	Prevention of mother-to-child transmission
PRT	Paralogue ratio test
PSV	Paralogous sequence variant
RNA	Ribonucleic acid
RT	Reverse transcriptase
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
UTR	Untranslated region
VL	Viral load

AMINO ACIDS

Amino acid	**Three-letter code**	**One-letter code**
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Phenylalanine	Phe	F
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V

CHAPTER 1 - INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Human immunodeficiency virus (HIV) disease is a continuum of progressive damage to the immune system from the onset of infection to the manifestation of acquired immunodeficiency syndrome (AIDS), which is characterised by the gradual increase in plasma viral load, reduction in cluster of differentiation (CD) 4 (CD4+) T-lymphocyte cells and increased susceptibility to opportunistic infections. AIDS was first recognized in 1981 in a cluster of diseases associated with loss of cellular immunity in young, homosexual men in New York City, Los Angeles and San Francisco, who were previously healthy and had no obvious reason for presenting such immune deficiencies (1,2).

Since then, HIV has become a major cause of morbidity and mortality, and dramatically changed the global burden of disease. In 2020, there were an estimated 37.7 million people living with HIV, with 1.5 million new infections and 680,000 AIDS-related deaths. The global roll-out of antiretroviral therapy (ART) has saved many lives but efforts to prevent new HIV infections have been less successful. South Africa remains the country with the biggest HIV epidemic, with an estimated 7.8 million people (7.5 million adults and 310,000 children <15 years) living with HIV in 2020 and the largest treatment programme in the world (3).

Globally, children less than 15 years of age accounted for 1.7 million of those living with HIV in 2020, including 150,000 new infections and 99,000 AIDS-related deaths (3). Significant strides in HIV prevention have been made, with a notable 54% decline in the number of new paediatric HIV infections from 2010 to 2020. This is attributable to the substantial increase in access to ART for pregnant women living with HIV, from 45% in 2010 to 85% in 2020. Yet, some children get infected despite ART interventions and remain at a greater risk of AIDS-related mortality compared to adults. Thus, further studies are required to elucidate mechanisms of protection and identify novel targets for therapeutic interventions.

Two related but serologically and geographically distinct HIV types have been described, namely HIV type 1 (HIV-1) and HIV type 2 (HIV-2). Both HIV-1 and HIV-2 replicate in CD4+ T-lymphocyte cells and are pathogenic in infected persons, although there is higher virulence and infectivity in HIV-1-infected individuals (4). Geographically, the distribution of HIV-1 is

global whereas HIV-2 is largely restricted to West Africa (4). Further reviews and discussions in this thesis refer to HIV-1.

Multiple interacting factors determine the pathogenesis and progression of HIV-1 infection, including a complex interplay between the virulence of the infecting virus and the host immune response to the virus. Adaptive immune responses, in the form of antibodies (humoral) and virus-specific CD4+ and CD8+ T cells (cell-mediated), are key components in host defence in most infections and vaccines (5). While humoral immunity depends on antibodies, cell-mediated immunity involves the activation of antigen presenting cells (macrophages, dendritic cells), production of antigen-specific CD4+ helper and cytotoxic T-lymphocytes (CTL), and release of cytokines in response to an antigen. Different immune functions, virological factors and host genetic factors that modulate HIV-1 disease outcome have been identified (6–12). With regards to host genetics, the human leukocyte antigen (HLA) and chemokine receptors (CCR5 and CXCR4) are strong predictors of HIV-1 acquisition and disease progression (6,13,14). CD4+ and CD8+ HIV-1-specific responses are not generally adequate to control viral replication and the role of neutralising antibodies in the control of already established HIV-1 infection is unclear (5).

In spite of advances in preventing vertical transmission of HIV-1, there are still infants acquiring infection. Understanding the immune mechanisms that confer protection may provide insights for future interventions. Functional study of breastmilk has suggested that immunoglobulin G (IgG) Fc mediated effector functions may confer protection against infection through breastfeeding (15). The foetus also passively acquires HIV-1-specific IgG through trans-placental transfer from its HIV-1-infected mother, where Fc-mediated effector functions may protect against in utero (during pregnancy) and intrapartum (during delivery) HIV-1 infection. The study of protective antibody functions in mother-to-child transmission (MTCT) of HIV-1 not only provides insight into potential therapeutic targets for reducing MTCT, but offers an attractive model to study the protective role of crystallisable fragment (Fc) gamma receptors (FcγR)-mediated effector functions against HIV-1 acquisition in passively immunized individuals (16,17).

There is evidence for other antibody functions (Fc-mediated effector functions) in contributing to HIV-1 protection. Specifically, crystallisable fragment (Fc)-mediated effector functions have been shown to influence HIV-1 acquisition and post-infection control of viraemia (15,18,19).

Fc gamma receptors (FcγRs) are key immune receptors for IgG, expressed on a variety of immune cells, which regulate both humoral and innate immunity. The six classic FcγRs (FcγRI, FcγRIIa/b/c, and FcγRIIIa/b) differ by their binding affinity for IgG and signalling activities. The low affinity receptors (FcγRIIa/b/c and FcγRIIIa/b) are polymorphic and have been implicated in the genetic susceptibility of a range of chronic inflammatory and autoimmune diseases (20,21). Genetic variations of these receptors can affect their activating and inhibitory functions and the balance of the immune system (22). Fc-mediated pathways play a critical role in modulating the effector activities of an antibody, but their role in perinatal HIV-1 transmission risk is currently undefined. The functional consequences for all FcγR variants in HIV-1 infection *in vivo* is not clearly defined. Investigating the association of functional FcγR variants in HIV-1 acquisition and disease progression will contribute to a better understanding of their significance *in vivo* and inform future interventions.

This introductory chapter first provides an overview of HIV-1 biology, infection and disease progression, and MTCT of HIV-1. Next discussed are the human FcγRs and their allelic variants, genetic associations of FcγRs with HIV-1 acquisition and progression, and FcγR genotyping methods.

1.2. HUMAN IMMUNODEFICIENCY VIRUS

1.2.1. Structural biology of HIV-1

HIV-1 is a *Lentivirus*, a genus that belongs to the *Retroviridae* family. The ribonucleic acid (RNA) genome of retroviruses are transcribed into a deoxyribonucleic acid (DNA) within the cell using the viral enzyme reverse transcriptase (RT). The DNA then enters the nucleus and integrates into the genome of the host. The genetic information of the virus is contained in the genome while the capsid gives the virus its spherical shape and protects the genome. Primarily, HIV-1 infects T cells bearing the CD4 molecule and macrophages. The envelope (outer shell of the virus) is coated with glycoproteins (gp), which it uses to bind to the surface receptor CD4 and infect the host cell (23). HIV-1 is characterised by three structural genes: *gag*, comprising the viral capsid proteins; *pol*, comprising the viral enzymes (reverse transcriptase, integrase and protease); and *env*, comprising the envelope glycoproteins. Lentivirus infections typically present a chronic course of disease, characterised by long incubation periods, persistent viral replication and neurologic manifestations (24).

1.2.2. The life cycle of HIV-1

The life cycle of HIV-1 comprises of a series of steps that begin with binding of viral glycoproteins to host cell surface receptors and end with the production of infectious virions (Figure 1.1). The steps include i) binding and entry; ii) reverse transcription of the positive-sense viral RNA to DNA; iii) provirus integration into the host genome; iv) virus protein synthesis and assembly; and v) budding from the cell surface (24). HIV-1 utilizes host CD4 molecules as primary receptors and chemokine receptors on lymphoid cells - CCR5 and CXCR4 - as co-receptors. These receptor and co-receptor host proteins play important roles in immunity and inflammation. After attachment, HIV-1 RNA and several HIV-1-encoded enzymes are released into the host cell. HIV-1 reverse transcriptase transcribes viral RNA as proviral DNA, which enters the host cell's nucleus and is integrated into the host DNA. This copying mechanism is prone to a high frequency of transcription errors, which results in mutations that increase the chance of producing strains resistant to host immunity and drugs. The integrated proviral DNA is duplicated along with the host DNA with each cell division. Thereafter, the proviral HIV-1 DNA can be transcribed to HIV-1 RNA and translated to HIV-1 proteins for producing infectious virions. The final steps of HIV-1 replication involve viral assembly and the release of new progeny. The HIV-1 proteins are assembled into HIV-1 virions at the host cell inner membrane and budded from the cell surface. After budding, HIV-1 protease cleaves viral proteins, converting the immature virion into a mature, infectious virion. These mature virions later bud out of the cell and infect other cells (25).

1.2.3. HIV-1 transmission

The transmission of HIV-1 occurs through the exchange of body fluids. The three primary modes of transmission are sexual contact, exposure to blood (mainly via injection drug use and transfusion), and MTCT (26). The most common route of transmission is sexual through epithelial cells of the genitourinary and rectal mucosa (27). The risk of HIV-1 transmission depends on the biologic properties of the viral isolate, the level of the virus in the infected body fluid and the host's immune response to the virus (24).

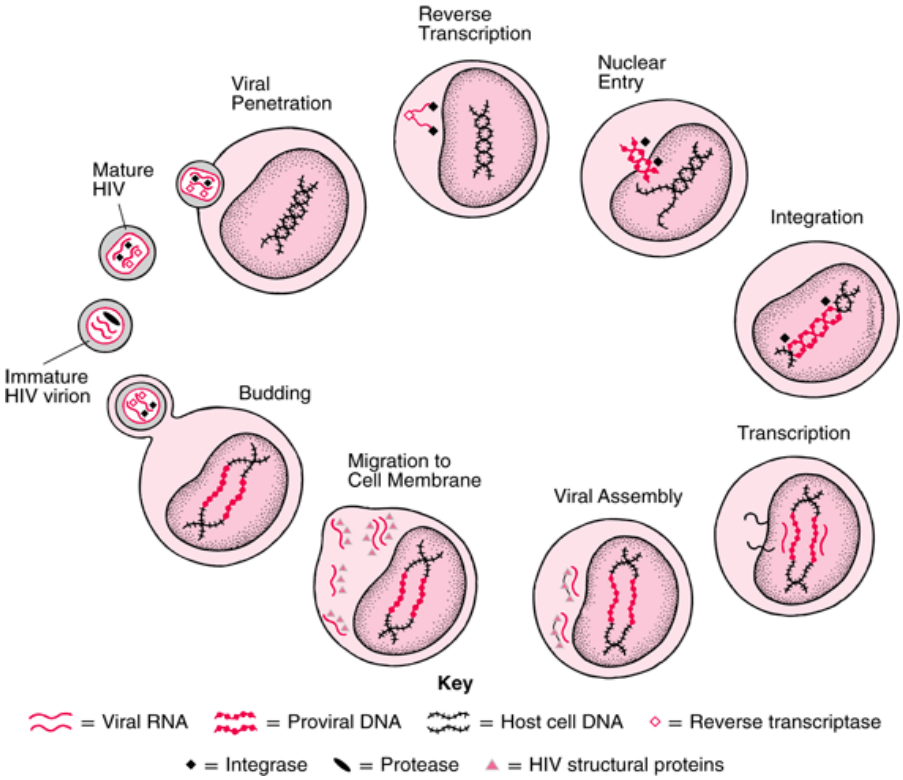

Figure 1.1 HIV-1 replication cycle.
(Reproduced from Merck Manuals professional edition) (25)

1.2.4. HIV-1 infection and disease progression in adults

The course of a HIV-1 infection involves three main clinical phases: acute, chronic and AIDS-defining illness (Figure 1.2). The acute phase of HIV-1 infection is the early period in which high virus production takes place and CD4+ T cell numbers decline. An acute self-limited flu-like or mononucleosis-like illness that lasts for a week or two usually develops in people weeks after infection with HIV-1 (28). Cellular factors associated with innate and adaptive immune responses can influence viral replication and establishment of a viral set point during the acute infection phase. This inability of the immune system to completely eliminate the virus leads to establishment of a chronic, persistent asymptomatic period of variable length, before other clinical manifestations develop (24). The frequency and severity of subsequent HIV-1-related opportunistic infections directly correlates with the degree of immune system dysfunction.

The rate of HIV-1 disease progression is highly variable among HIV-1-infected individuals and can be categorized as rapid, typical or long-term non-progression (11). This variability dictates how rapidly the virus replicates and allows it overcome host immunity and effects of ART (24,29). The majority (70-80%) of HIV-1 infected individuals experience typical disease

progression that is a period of clinical latency for 6-10 years. Rapid progressors display rapid progression to AIDS from two to three years of primary infection and account for 10-15% of HIV-1 infected individuals. About 5% are long-term non-progressors, who can maintain good control of infection for 10 years or more, in the absence of ART (11). However, children have a markedly different disease progression course compared to adults [reviewed in (30,31)].

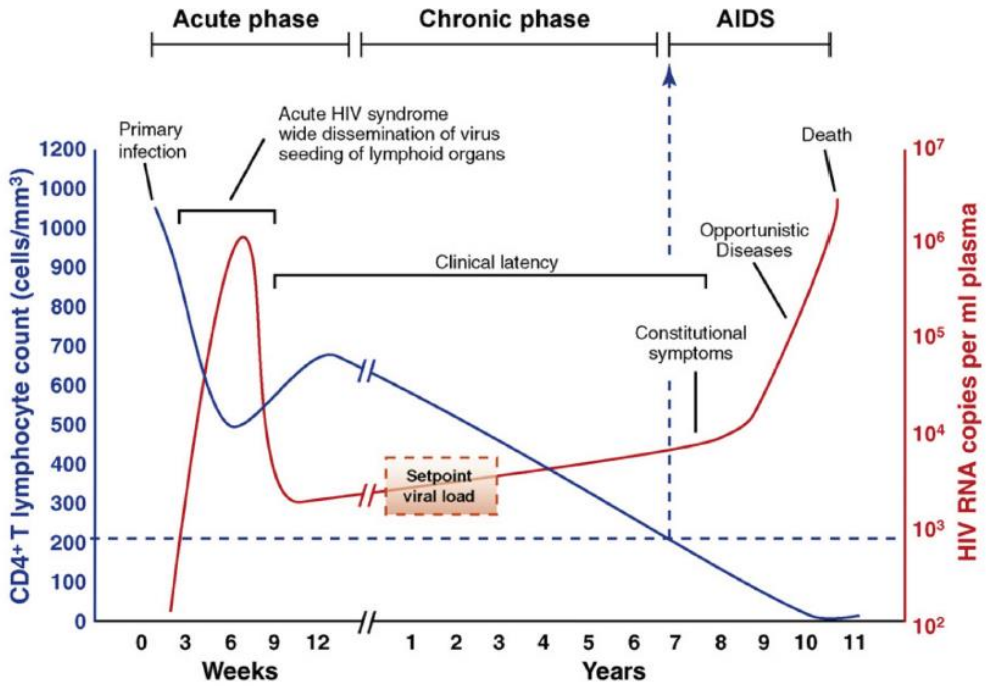

Figure 1.2 Clinical course of HIV-1 infection in adults.
Reproduced from O'Brien & Hendrickson 2013 (32)

1.3. MOTHER-TO-CHILD TRANSMISSION OF HIV-1

Paediatric HIV-1 infection remains a significant public health problem in sub-Saharan Africa, where more than 90% of all children with HIV-1 in the world live and HIV-1 sero-prevalence among pregnant women remains high (33,34). Over 90% of paediatric HIV-1 infections are acquired through MTCT (35), which can occur during pregnancy (*in utero*), labour/delivery (*intrapartum*) or breast-feeding (*postpartum*) (36). In the absence of prevention of mother-to-child transmission (PMTCT) interventions, HIV-1 infection rates range from 25-40% among breast-fed infants and 15-25% among formula-fed infants (35).

Progress has been made in scaling up PMTCT programmes in resource-poor settings, leading to a global reduction in the risk of MTCT of HIV-1 (37,38). In particular, South Africa has made considerable strides in reducing MTCT of HIV-1. By 2015, South Africa provided ART to more than 90% of pregnant women living with HIV-1 and achieved an early MTCT transmission rate of <2% (38,39). This has seen to a significant 84% reduction in new HIV-1 infections among children (less than 15 years of age) between 2009 and 2015 (39). However, complete elimination of vertical HIV-1 transmission through PMTCT interventions is hindered by the transmission of ART-resistant virus strains, inadequate maternal adherence to ART and acute maternal infection during pregnancy and breastfeeding (33,40). In the face of significant virus exposure, some infants escape infection while others do not, suggesting some maternal or foetal immune protective factors at play. Further studies are required to elucidate natural mechanisms of protection in order to identify novel targets for therapeutic interventions.

1.3.1. Paediatric HIV-1 disease progression

Infants who become infected and do not receive ART are at significantly increased risk of mortality, reaching 50% by the second year of life and peaking at 2-3 months in South Africa (41,42). Among untreated infants, mortality in the first year of life is particularly high for perinatally infected (*in utero* and *intrapartum*) infants compared to those infected through breastfeeding (48% vs. 22%) (43). Paediatric HIV-1 infections are characterized by higher HIV-1 RNA viral load and more rapid clinical disease progression and death than adult infections (30,31,44–46).

Without ART, the median time to development of AIDS is one year following paediatric HIV-1 infection compared to 10 years in adults (30,46). Unlike in adults, HIV-1 viral load is not a strong determinant of disease progression in infants because of high RNA levels and overlap in levels between children with or without rapid disease progression (47,48). In paediatrics, viral load reaches a set point after five years of infection compared to six weeks in adults (30,46) (Figure 1). Immune activation is a strong marker for HIV-1 disease progression in both adult and children but the underlying mechanisms in the two groups are different (30,49,50). In adults, HLA class 1 polymorphism plays a central role in the suppression of viral replication but the mechanism of low immune activation in the face of persistent high viral load in children is unknown (49).

It is evident that initiating ART in children soon after HIV-1 infection, at the time the immune system is most immature, reduces disease progression (51) and HIV-1-related mortality (44). Early initiation of ART in vertically HIV-1 infected children has also been shown to reduce the size of the HIV-1 reservoir (52–57) and an understanding of this benefit can be leveraged on in designing interventions aimed at achieving functional cure.

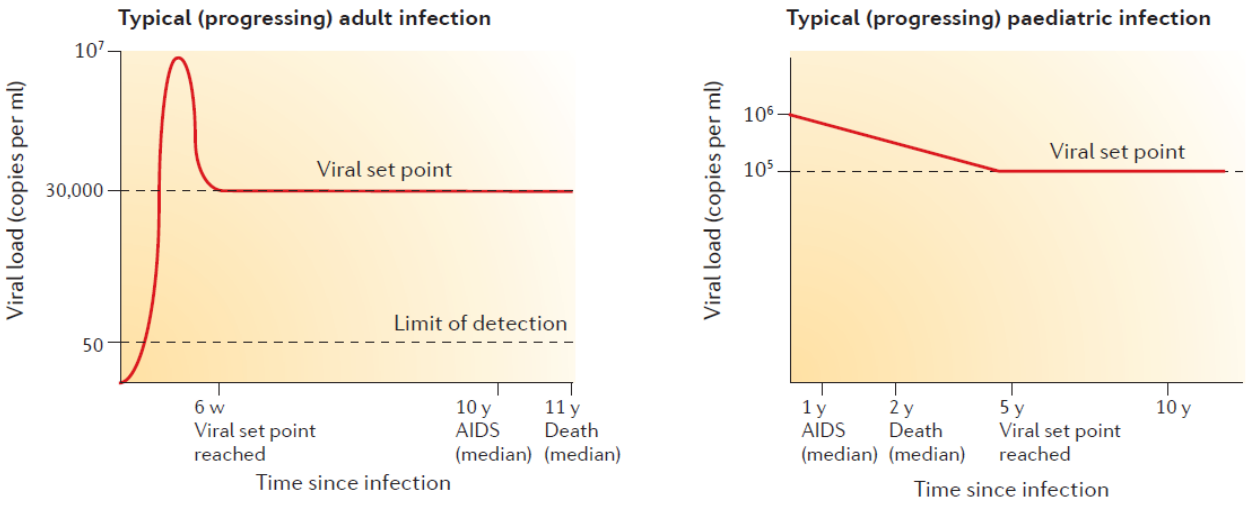

Figure 1.3 Changes in viral load following HIV-1 infection in paediatrics and adults
Reproduced from Goulder et al. 2016 (30)

1.3.2. Role of HIV antibodies in MTCT

Without interventions to prevent vertical transmission, many infants do not acquire HIV-1 infection even with persistent exposure in utero, during delivery and breastfeeding (33,45). This suggests possible role of maternal immune factors that offer protection to the infant from HIV-1 acquisition. Early in life, infants acquire protective immunity through maternal antibodies of the IgG class, transferred passively across the placenta through an active process mediated by the neonatal Fc receptor (FcRn) (58) and from IgA provided through breastmilk. HIV-specific neutralising antibodies (nAbs) bind to the HIV envelope, preventing its interaction with CD4 receptors on T-cells and entry into the cell. Previous studies have produced conflicting results on the role of nAbs in the risk of MTCT of HIV-1. While some have shown that nAbs do not predict protection from HIV-1 acquisition (59,60), others have suggested otherwise (61–63). These different outcomes may be due to differences in study design and analysis, HIV antibody measures, population and environment, virus clades,

maternal ART use, modes of transmission and maternal plasma viral load and CD4+ T cell count that impact MTCT (64,65).

Apart from neutralisation, other antibody functionalities also contribute to protection, such as antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), respiratory burst, release of pro-inflammatory mediators and enhancement of antigen presentation (18,66). These functions are induced via their antigen binding fragment (Fab) regions and through the interaction of their Fc domain with Fc receptors (FcRs) on effector cells (67). Passively acquired antibodies that mediate ADCC have been shown to correlate with protection against HIV-1 acquisition (15,68,69) and improved survival of HIV-1-infected infants (19,68,70). The role of FcγR-mediated effector functions in modulating perinatal HIV-1 transmission and acquisition, using FcγR variants as proxy for functional capacity, was investigated in South Africa (71). The study found the maternal FcγRIIIa-158V allele, which confers enhanced ADCC capacity, significantly associated with reduced odds of HIV-1 transmission to the infant. Together, these findings highlight the potential of maternal ADCC-mediating effector functions in protecting infants from acquiring HIV-1 infection and improving disease outcomes in those infected.

1.4. FC GAMMA RECEPTORS

1.4.1. Overview

IgG is the most abundant immunoglobulin class in serum. The molecule is made up of two antigen-binding fragment regions that specifically bind to antigen and an Fc region. It is the only immunoglobulin class that is tranferred across the human placenta. The Fc region of IgG interacts with activating and inhibitory FcγRs (membrane-bound glycoproteins) expressed on diverse immune cells, enabling antibodies to regulate the immune system. Four subclasses of IgG - IgG1, IgG2, IgG3 and IgG4 - are produced in response to various antigens, differing with respect to antigen binding, immune complex formation, complement activation and binding to Fc receptors (72). FcγRs are cellular receptors that bind the Fc region of IgG antibodies, linking both humoral and cellular branches of immunity. They are central regulators for modulating both pro- and anti-inflammatory responses. FcγRs are widely expressed on haematopoietic cells including monocytes, macrophages, neutrophils, natural killer (NK) cells, dendritic cells and B cells. Cross-linking of FcγRs on the cell surface initiates and

regulates immune mechanisms that include ADCC, ADCP, antibody production and B-cell activation, antigen presentation and cytokine production (67,73,74).

Generally, FcγRs are divided into three classes, each with different isoforms (produced from similar locus but encoded by different genes and have different protein coding DNA sequences): FcγRIa/b/c (CD64), FcγRIIa/b/c (CD32), and FcγRIIIa/b (CD16) (Table 1.1; (74)). The classes differ in their structural domain organisation, affinity for specific IgG subclasses, and ability to trigger activating or inhibitory signals (74,75). While FcγRI binds monomeric IgG with high affinity, both FcγRII and FcγRIII bind to IgG complexes through multivalent, low affinity, high avidity interactions. Furthermore, FcγRI, FcγRIIa/c, and FcγRIII are activating receptors, transmitting their signals through immunoreceptor tyrosine-based activation motif (ITAM), whereas FcγRIIb is an inhibitory receptor, with an immunoreceptor tyrosine-based inhibition motif (ITIM). Clearance of immune complexes is a key function of the activating FcγRs, while FcγRIIb suppresses immune complex-mediated B cell activation and regulates effector functions and pro-inflammatory cytokine release mediated by myeloid cells (76).

The low affinity FcγRs (FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa and FcγRIIIb) are encoded by five genes - *FCGR2A, FCGR2B, FCGR2C, FCGR3A* and *FCGR3B*, respectively, clustered on chromosome 1q23 (75). These low affinity FcγRs perform different roles in regulating immune responses (73). There is high sequence homology between the genes in the *FCGR2/3* loci. For instance, *FCGR2C* is a result of unequal crossover event between *FCGR2A* and *FCGR2B*, making it highly homologous to *FCGR2B* in the first six exons and *FCGR2A* in the last two exons (77). Most human FcγRs have measurable affinity for subclasses of IgG. Both IgG1 and IgG3 bind to all FcγRs; IgG2 binds to FcγRIIa and FcγRIIIa; IgG4 binds to FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa but not FcγRIIIb. The affinity for IgG1, IgG2 and IgG3 is lower for the inhibitory receptor FcγRIIb than the activating receptors (72).

1.4.2. Allelic variations in low-affinity FcγR

Functionally significant genetic variants, including copy number variations (CNVs) and single nucleotide polymorphisms (SNPs), have been described for all low affinity FcγRs. These modulate the function of FcγRs by altering receptor cell surface density, binding affinities to IgGs, glycosylation patterns, cellular distribution, or subcellular localization (67,71). Overall, the genetic variations have been associated with susceptibility to or severity of chronic

inflammatory, autoimmune and infectious diseases (20,78–80) and antibody responses to vaccinations (81–83).

Table 1.1 Genetic characteristics of the three classes of human FcγRs

	FcγRI	**FcγRII**	**FcγRIII**
Genes	*FCGR1*	*FCGR2A*	*FCGR3A*
	FCGR1B	*FCGR2B*	*FCGR3B*
	FCGR1C	*FCGR2C*	
Chromosome	1q21	1q23-24	1q23-24
Isoforms	FcγRIa	FcγRIIa	FcγRIIIa
	FcγRIb	FcγRIIb	FcγRIIIb
	FcγRIc	FcγRIIc	
Affinity for IgG	High	Low	Low

At the low affinity *FCGR* gene loci, large genomic segments are deleted or duplicated in certain individuals, contributing to inter-individual genetic variation and affecting FcγR-mediated effector functionality. CNV produces a gene-dosage effect, which leads to altered protein levels, cellular phenotype and response. To date, CNV has been demonstrated for *FCGR3A*, *FCGR3B* and *FCGR2C* but not for *FCGR2A* and *FCGR2B* (84). Phenotypically and functionally, CNV of *FCGR3A* correlates with FcγRIIIa surface expression levels on NK cells, a key mediator of ADCC (84). Similarly, CNV of *FCGR3B* directly correlates with protein expression, neutrophil adherence to and uptake of immune complexes (85). With regard to *FCGR2C,* CNV is associated with a predisposition to idiopathic thrombocytopenic purpura (80). Genes are either duplicated or deleted at the *FCGR2/3* loci within copy number variable regions (CNRs) (86,87), with CNR1 and CNR2 being the most common. CNR1 includes genes of complete *FCGR2C*, *HSPA7* and *FCGR3B.* CNR2 encompasses incomplete *FCGR2C, FCGR2A*, *HSPA6* and *FCGR3A* (87).

Duplications and deletions within CNRs lead to the creation of chimeric *FCGR* genes, with functional consequences (87,88). CNR1 deletion creates *FCGR2C/2B* chimeric genes, which leads to expression of FcγRIIb on NK cells and reduced ADCC activity (89,90). A deletion in CNR2 creates an *FCGR2A/2C* chimera that associates with decreased expression levels of

FcγRIIa and subsequent reduced activity in response to IgG, whereas a duplication creates an *FCGR2C/2A* chimeric gene (87). The *FCGR2C/2A* chimeric genes express high levels of FcγRIIc and are present in more individuals than the *FCGR2A/2C* chimeric genes. In addition to CNV, functionally significant amino acid substitutions have been reported for FcγRIIa, FcγRIIb, FcγRIIIa and FcγRIIIb and these substitutions contribute to differential binding affinity for subclasses of IgG. SNPs are either indicated by the amino acid positions in the mature (excluding the signal peptides) or full protein.

1.4.2.1. **FcγRIIa**

FcγRIIa is the most widely expressed isoform of the FcγRII class of receptors on human chromosome 1q23. It is mainly expressed on monocytes, macrophages, dendritic cells, neutrophils and platelets, where it induces a variety of cellular defense mechanisms that include phagocytosis, ADCC, cytokine production, platelet activation, and dendritic cell maturation (22,74,91,92). An arginine (R) to histidine (H) substitution at amino acid position 166 of the FcγRIIa full protein (FcγRIIa-H166R; also known as FcγRIIa-H131R on the mature protein) alters the ability of the receptor to bind human IgG2. Functionally, cells that express FcγRIIa-166H bind IgG2 more efficiently than those that express FcγRIIa-166R (93). Phagocytosis of IgG2 opsonised particles is more effective in phagocytes from homozygous FcγRIIa-166HH individuals compared to phagocytes from FcγRIIa-166RR (94).

1.4.2.2. **FcγRIIb**

FCGR2B that encodes FcγRIIb protein is located at the distal end of the *FCGR2/3* loci and, like *FCGR2A*, is not subject to CNV (84,86). FcγRIIb, the only inhibitory receptor with an ITIM in its cytoplasmic domain; it is up regulated on neutrophils and largely expressed on B cells. On B cells, FcγRIIb regulates the activating signals transmitted by B cell receptors (BCR) and ultimately antibody production. On myeloid cells, FcγRIIb regulates immune activation by inhibiting effector functions including phagocytosis and pro-inflammatory cytokine release. A SNP in *FCGR2B* substitutes an isoleucine (I) to threonine (T) at amino acid position 232 (I232T) in the transmembrane domain of the FcγRIIb protein. The FcγRIIb-232T allele affects the receptor's ability to translocate to lipid rafts (signalling microdomains on the cell surface) and consequently reduces its inhibitory capacity (95,96). The FcγRIIb-232T allele is subject to ethnic variation and has been observed more in South African blacks than Caucasians (97). The 232T allele is enriched in malaria-endemic populations, suggesting a protective role for this

allele in the context of malaria; however, the increased inflammatory response can increase susceptibility to systemic lupus erythematosus (SLE) (98).

Allelic variation in promoter regions can affect the expression levels of genes. For *FCGR2B*, two SNPs occur within its promoter at position c.−386G>C and c.−120A>T (80,99) that yield four haplotypes: -386G/-120T (*FCGR2B*.1), -386C/120T (*FCGR2B*.2), -386G/-120A (*FCGR2B*.3), and -386C/-120A (*FCGR2B*.4). The rare *FCGR2B*.4 haplotype has been shown to exhibit greater transcriptional activity than the more common *FCGR2B*.1 haplotype (99). Despite the high sequence homology shared between *FCGR2B* and *FCGR2C* in this region, they differ in their promoters such that the -120A allele is exclusively found in *FCGR2B* (80).

1.4.2.3. **FcγRIIc**

FCGR2C, described as a pseudogene, resulted from an unequal crossover between the 5' part of *FCGR2B* and 3' part of *FCGR2A* (77). It is expressed mainly on NK cells (where it is capable of inducing ADCC), neutrophils, monocytes and macrophages. A variant in exon 3 of *FCGR2C* (c.169T>C) that substitutes a premature stop codon with a glutamine at amino acid 57, results in abolished protein expression or maintenance of the open reading frame (87,100). Overall, the expression of a functional FcγRIIc molecule is dependent on a combination of three minor alleles that include the minor alleles of the c.169T>C variant in exon 3 (c.169C) and two splice variants, c.789+1A>G (c.798+1G) and c.799-1G>C (c.799-1G) (81,89). The expression of FcγRIIc protein is subject to ethnic variation as a result of significant variation in the frequencies of the minor alleles between populations (86,97). In addition to the expression variants, a SNP in intron 2 of *FCGR2C* has been identified as clinically significant i.e. c.134-96C>T, which previously was associated with vaccine efficacy in the RV144 HIV vaccine trial in Thailand (82).

1.4.2.4. **FcγRIIIa**

Similar to FcγRIIa/b/c, the FcγRIIIa is a transmembrane protein receptor. It is expressed at high levels on cytotoxic NK cells, a key mediator of ADCC, as well as on tissue macrophages and a subset of peripheral monocytes. FcγRIIIa mediates phagocytosis on mononuclear phagocytes (91). A valine (V) to phenylalanine (F) substitution at amino acid position 176 of the full protein (FcγRIII-V176F; also known as FcγRIII-V158F in the mature protein) in the membrane-proximal Ig-domain of FcγRIIIa, alters the receptors affinity for IgG1, IgG3 and IgG4. Compared to FcγRIII-176F, the FcγRIIIa-176V allele displays a greater affinity for the

aforementioned IgG subclasses and enhanced effector functions (72,75,101). Lassaunière et al., identified an intragenic haplotype in *FCGR3A* that alters surface expression levels. This intragenic haplotype is rare to absent in black South Africans, however it represents an important genetic marker for study in various disease outcomes (102).

1.4.2.5. **FcγRIIIb**

Unique among FcγRs, FcγRIIIb is a glycophosphatidylinositol (GPI)-anchored protein that lacks both transmembrane and cytoplasmic signaling domains. It is found predominantly on neutrophils at high levels. It is marked by the presence of polymorphic human neutrophil antigens (HNA or NA) within the membrane-distal Ig-like domain. A combination of five amino acid changes give rise to three allotypic variants of FcγRIIIb: HNA1a, HNA1b and HNA1c (previously designated NA1, NA2, and SH, respectively). HNA1a and HNA1b allotypes differ at five nucleotide bases (141, 147, 227, 277, and 349), predicting four amino acid substitutions (R36S, N65S, D82N and V106I) (103,104). The amino acid substitutions result in two more *N*-linked glycosylation sites in HNA1b compared to HNA1a (104). Neutrophils from HNA1a homozygous individuals display greater phagocytic capacity and higher affinity for IgG3 compared to HNA1b homozygous individuals (105,106). HNA1c is identical to HNA1b at the five nucleotide positions that distinguish HNA1a from HNA1b, but differs at amino acid position 78 with an alanine to aspartic acid (A78D) substitution of which the functional consequence is unknown. While the amino acid changes between HNA1a and HNA1b alter the primary structure of the protein, the change in HNA1c affects the tertiary structure (104,107).

1.4.3. FcγR variants and HIV-1 infection

Functional studies have suggested Fc-mediated effector functions (such as ADCC) play a protective role in HIV-1 immunity, especially those that utilized the MTCT model to assess infant outcome (15,18,19). The host factors impacting MTCT of HIV-1 are multifactorial and complex. Studies have assessed the association of polymorphic variants of FcγRs with HIV-1 acquisition (71,108,109), disease progression (78,110,111) and vaccine efficacy (82,83)cohorts, as a proxy for their functional capacity. However, the reported findings are inconsistent.

The effect of *FCGR2A* and *FCGR3A* variations on HIV-1 acquisition and disease progression remains unclear. Specifically, in the context of MTCT, infant FcγRIIa-166HH genotype

associated with HIV-1 acquisition in a Kenyan MTCT cohort (109), but this was not observed in other MTCT cohorts in Kenya (108) and South Africa (71). In addition, a large adult, sexual transmission cohort of European descent from a genome-wide association study was used to establish non-association of common *FCGR2A* and *FCGR3A* polymorphisms with HIV-1 acquisition and post-infection control (112).

Forthal *et al*. demonstrated an association between FcγRIIa genotype and a faster rate of CD4+ T cell decline and progression to AIDS in Caucasian adults. Specifically, individuals homozygous for low binding FcγRIIa-166R progressed more rapidly than those with at least one FcγRIIa-166H allele (110). This association was not observed in a study of Kenyan women (111) and adult African-American cohort (113). Poonia *et al*. reported the high-affinity FcγRIIIa-176VV genotype as a risk factor for HIV-1 infection and progression, suggesting that carriage of 176VV increases immune activation and higher susceptibility to HIV-1 transmission (113). Conversely, other studies reported that FcγRIIIa polymorphism did not predict HIV disease progression (110,111). In a nested case-control study in Rwanda and Zambia, FcγRIIa and FcγRIIIa genotypes showed no association with heterosexual HIV-1 acquisition and disease progression (114). The *FCGR2C* c.134-96C>T variant significantly associated with increased odds of HIV-1 disease progression in a black South African adult cohort (78). Overall, there is a paucity of data on Fc-mediated effector functions of FcγRIIb and FcγRIIIb on HIV-1 infection.

Accumulating evidence suggests FcγR variation in host genes impact HIV-1 acquisition differently in HIV-1 vaccine recipients (82,83,115). In a recombinant HIV-1 gp120 Vax004 vaccine trial conducted in North America among men who have sex with men, homozygosity for FcγRIIIa-176V allele in the lowest behavioural risk group associated with HIV-1 acquisition. FcγRIIa-H166R was not associated with acquisition in both vaccinees and recipients of placebo (115). Furthermore, a three-variant haplotype within *FCGR2C*, namely c.353C>T; c.391+111G>A and c.134-96C>T, associated with protection against HIV-1 acquisition in the Thai RV144 vaccine trial. The study found an estimated efficacy of 64%-91% in vaccinees bearing at least one c.134-96T minor allele compared 11%-15% among those with wild type allele (82). Conversely, in the HIV Vaccine Trials Network (HVTN) 505 vaccine trial, two variants within the haplotype (excluding c.134-96C>T) associated with increased risk of HIV-1 acquisition (83). The RV144 follow-on trial (HVTN 702 vaccine trial) in South Africa did not prevent HIV-1 infection (116). The type of vaccine regimen,

population, affinity of different IgG subclasses and the expression pattern of the FcγRs on effector cells might explain the differences in outcome (83,117). In contrast to the protective effect observed in the Thai RV144 trial, the *FCGR2C* c.134-96C>T variant significantly associated with increased odds of HIV-1 disease progression in a black South African adult cohort (78). In addition to potential different mechanisms that modulate HIV-1 acquisition risk and disease progression, genetic differences between populations may explain the two different results. Previous study has shown that the Africans, including black South Africans, are polymorphic for only one (c.134-96C>T) of the three variants within the Thai *FCGR2C* haplotype (97).

Despite ART, latently HIV-1-infected CD4+ T cells (HIV reservoirs) facilitate viral persistence that impacts negatively on achieving HIV-1 cure or remission (118). Collectively, studies have provided evidence that early diagnosis and prompt ART initiation reduces the size of the HIV-1 reservoir and predicts post-treatment virological control in adults (119–122) and children (52,53). The influence of host genetic variation on HIV-1 reservoir size and post-ART control has not been definitely studied. Descours et al identified FcγRIIa as a marker of the HIV-1 latent reservoir (123). However, subsequent independent studies were unable to replicate this finding (112,124,125).

Most association studies between polymorphic and copy number variants of FcγRs and HIV-1 infection in natural history cohorts have utilized candidate gene study designs, where specific polymorphisms of interest are investigated. Throughout this thesis, the standard technique used for genotyping the *FCGR2/3* region is the multiplex ligation-dependent probe amplification (MLPA). Gene-specific long-template polymerase chain reaction (PCR) was used where necessary.

1.5. ASSAYS FOR GENOTYPING *FCGR* GENETIC VARIANTS

The *FCGR* loci is a complex region to study due to CNV and a high degree of nucleotide sequence homology between genes. Different technologies used to genotype *FCGR* variants are summarized in Table 1.2. The technologies include Sanger sequencing, the pyrosequencing, paralogue ratio test (PRT), PCR-based hydrolysis probe genotyping (e.g. TaqMan®) and MLPA. The primary shortcomings of most assays are incapability of detecting both CNV and SNPs, analysis of only a single target at a time, and low through-put or accuracy. The MLPA

technique is robust in that it allows for the simultaneous genotyping of up to 96 samples and controls, with a 24 hour turn-around-time for results. The multiplex technique of the assay makes possible the study of several regions of the human genome in a single reaction. The principle is based on the amplification of up to 60 DNA sequences of unique lengths in a single multiplex polymerase chain reaction (PCR)-based reaction (Figure 1.4).

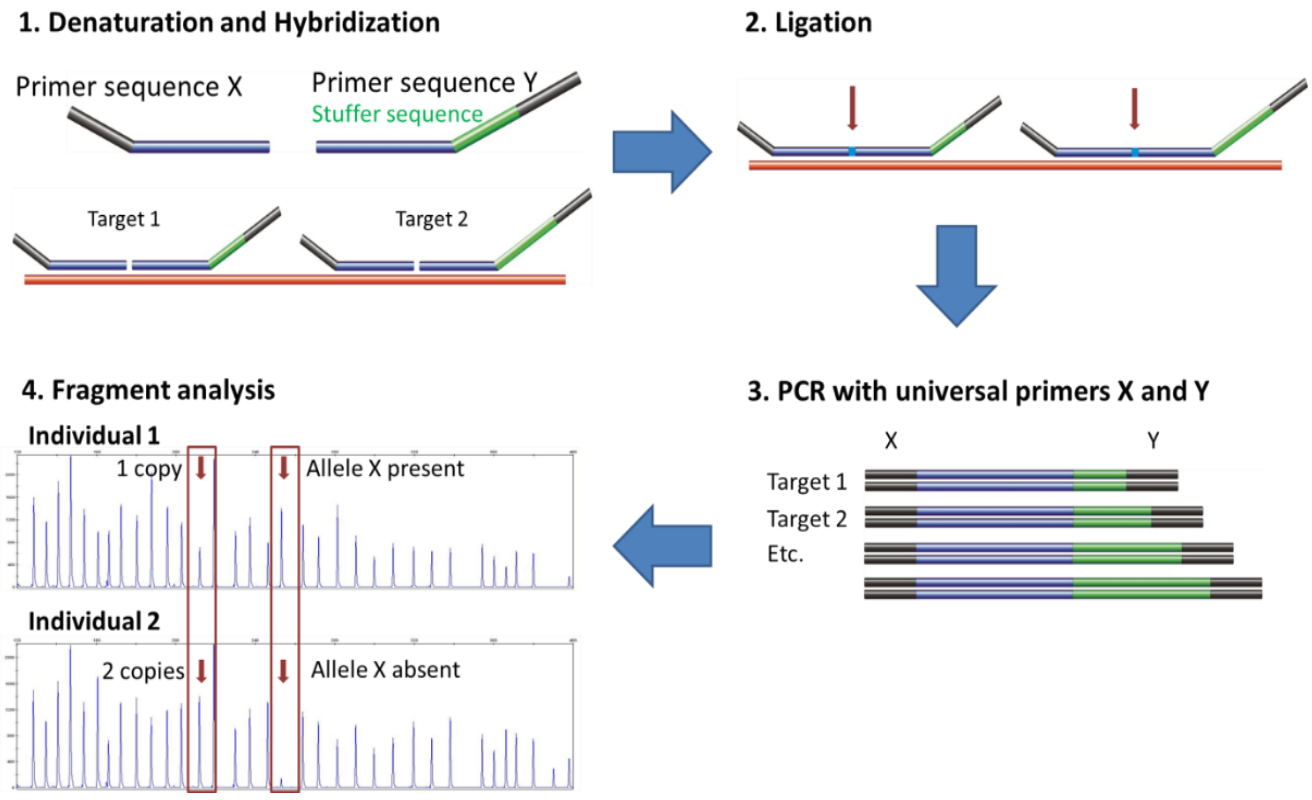

Figure 1.4 Multiplex ligation-dependent probe amplification assay principle.
(1) Sample genomic DNA is denatured and incubated with MLPA probe mix. MLPA probes are made up of two oligonucleotides that each contain a PCR primer sequence and hybridization sequence complementary to the genomic target. **(2)** The oligonucleotides hybridise to adjacent DNA target sequences and are subsequently ligated to produce a single molecule. **(3)** The ligated probes are then amplified exponentially during the PCR reaction using a fluorescently labelled PCR primer pair complementary to the flanking sequences on the probe. **(4)** The variable length fragments are separated and quantified by capillary electrophoresis. The relative peak height of each probe, compared to the mean probe peak height for all DNA samples, is used as a measure of gene copy number. Increase in relative peak heights reflects duplication while decrease indicates deletion of one or more target sequence. Adapted from Schouten *et al* (126).

Table 1.2 Comparison of current technologies for genotyping variants at the *FCGR* loci
Reproduced from Hargreaves et al. 2016 **(22)**

Method	Applications	Advantages	Disadvantages
Sanger sequencing	SNP	– Gold standard – Low template amount required	– Time-consuming – Costly – The primer design and interpretation are complicated in regions of homology
TaqMan	SNP, CNV	– High through-put – Low template amount required – Cost effective – Off-the-shelf products available	– Can only analyse one target at a time – Genotype clustering can be confounded by CNV – Short amplicons limit gene-specific designs in regions of homology
MLPA	SNP, CNV	– Detect SNPs and CNV simultaneously – Amplification of ligated probes rather than template – The products are commercially available and the analysis software is free – Enables both inter- and intra-sample normalization	– Expensive – Specific equipment required (capillary electrophoresis machine) – Challenging data analysis pipeline – SNPs in probe binding sites can affect probe binding – Requires at least 200 ng DNA
PRT	CNV	– A single primer pair amplifies target and reference loci increasing accuracy and minimizing inter-reaction variation – Primer design is aided by software	– Limited through-put – Assay design may be difficult as primers must amplify two loci
Pyrosequencing	SNP and CNV via PSV tags	– Primer pair amplifying two homologous genes determines relative amounts of PSVs and therefore each gene	– Less accurate with homopolymeric stretches of nucleotides

CNV, copy number variation; MLPA, multiplex ligation-dependent probe amplification; PRT, paralogue ratio test; PSV, paralogous sequence variant; SNP, single nucleotide polymorphism

1.6. STUDY RATIONALE AND PRELIMINARY DATA

The potential role of Fc-mediated effector functions in exerting influence on HIV-1 transmission and acquisition was first described in a South African MTCT study (71). The study found the maternal FcγRIIIa-176V allele to be significantly associated with reduced HIV-1 transmission. In both mother and infant, having an FcγRIIIb-HNA1b allotype was significantly associated with increased odds of HIV-1 transmission and susceptibility, respectively. On the other hand, infants homozygous for the FcγRIIIb-HNA1a allotype were protected against perinatal HIV-1 acquisition. Since FcγRIIIb is largely expressed in neutrophils and FcγRIIIb-HNA1b allotype has been shown to exhibit lower neutrophil-mediated effector functions (105,106), the study findings indicate possible role for neutrophils in modulating perinatal HIV-1 transmission and acquisition. However, only a small number of HIV- infected infants (n = 78) were investigated in the study. In order to further explore the role of FcR-mediated functions in paediatric HIV-1 infection, this current study addresses questions of both acquisition of HIV-1 infection as well as virologic control with ART and mortality in children with perinatally-acquired HIV-1 using a much larger cohort.

The rationale of this study is to contribute to a better understanding of the role of FcγRs in HIV-1 infection in children (protective immunity and virologic control with timing of treatment), and also gain further insights to inform future strategies for HIV-1 functional cure/remission. There are currently no data available that address the role of FcγRs in the context of HIV-1 remission, either in adults or children. This current study will therefore provide crucial and novel information to this end, especially given that therapeutic vaccination and passive immunization are strategies being considered as interventions, particularly for early-ART-treated children living with HIV-1.

1.7. STUDY AIM AND OBJECTIVES

The overall aim of this thesis was to describe the role of FcγR-mediated effector functions in modulating perinatal HIV-1 acquisition, outcomes of virologic control, mortality and latent reservoir size in the context of ART administered in early life in children living with HIV-1.

The specific objectives were:

1. To assess the association between the *FCGR2C* c.134-96C>T (rs114945036) variant and mother-to-child transmission of HIV-1. (Chapter 3)
2. To validate the previously observed associations between FcγR variants and mother-to-child transmission of HIV-1. (Chapter 4)
3. To assess the association between FcγR variants and pre-treatment viral load and CD4+ T-cell percentage, mortality, virologic control and latent reservoir size for children on ART who acquired HIV-1 vertically. (Chapter 5)

CHAPTER 2 - MATERIALS AND METHODS,

2.1. STUDY DESIGN AND POPULATION

A nested case-control study was carried out to assess the association between low affinity *FCGR* variability in HIV-exposed-infected infants (cases) and HIV-exposed-uninfected infants (controls). The data used were selected from five perinatal cohorts from past studies at two hospitals in Johannesburg, South Africa (127–130). One cohort consisted of 546 HIV-1-infected children recruited as part of clinical trials (NEVEREST 2 and 3), conducted at Rahima Moosa Mother and Child Hospital (formerly Coronation Hospital) in Johannesburg, South Africa (127–129). In the NEVEREST cohort, there were few breastfeeding infections and the data did not distinguish in utero infections from intrapartum infections. The remaining four cohorts were made up of 849 HIV-1 infected mothers and their infants (birth cohorts). They were enrolled and followed prospectively, and a total of 83 (10%) infants acquired HIV-1 (130) (Appendix A.1.). The controls were selected from the HIV-1-exposed-uninfected infants recruited as part of two perinatal HIV-1 transmission cohorts (Coro-PIPE and Bara-PIPE) at Rahima Moosa Mother and Child and Chris Hani Baragwanath hospitals in Johannesburg, South Africa. At the time of the studies, ART was not routinely administered to pregnant women living with HIV-1. All the children enrolled in the study were black and exposed to nevirapine for prevention of MTCT. The contribution of the five perinatal cohorts to the overall cohort in each study chapter is shown in Appendix A.2.

In the NEVEREST 2 study, ART-naive children less than 2 years of age were recruited, started on a started on a lopinavir-ritonavir-based regimen (LPV/r), followed up for a minimum of 12 months and observed for viral suppression. They were then randomized to either stay on the initial LPV/r-based regimen or switched to a nevirapine-based regimen if they had achieved viral suppression to less than 400 HIV-1 RNA copies/millilitre (mL) (127,128). Those recruited in the NEVEREST 3 study were nevirapine-exposed children between three and five years of age, randomised to either stay on LPV/r-based therapy or switch to efavirenz-based therapy and virally suppressed (less than 50 copies/ml) during LPV/r-based therapy. They were not followed from time of ART initiation (129).

2.2. STUDY VARIABLES

The available demographic and perinatal variables included for both cases and controls are gender, birth weight, breastfeeding status and gestation (term or pre-term). Pre-treatment viral load, CD4+ T cell percentage and treatment outcome variables were only available for the cases. Mortality analysis and virological control once on ART were done only from NEVEREST 2 study data. The deaths occurred either before or after viral suppression. Data on HIV-1 DNA levels (a marker of viral reservoir) were obtained from a previous study that quantified cell-associated HIV-1 DNA in a subset of currently older NEVEREST 3 cohort (52). Maternal blood samples, as well as clinical information (such as maternal viral load, CD4+ T cell count, parity) were collected for the four birth cohorts at enrolment but not for the NEVEREST cohort.

2.3. SAMPLE SIZE CALCULATION

The required sample size for the proposed unmatched case-control study was calculated according to the Fleiss method (131). For a two-sided confidence level of 95%, power of 80%, and control-to-case ratio of 1, the required number of cases and controls are each 160 (173 with continuity correction) when using the FcγRIIIb-HNA1a/b/c genotype frequency data from the South African cohort of 78 HIV-1 infected infants (cases) and 235 HIV-1 exposed uninfected infants (controls) (71).

2.4. ETHICS

Ethics approval for the study was obtained from the University of the Witwatersrand Human Research Ethics Committee (Reference number: M180575).

2.5. DETECTION OF CNV AND SNPS IN *FCGR*

Genomic DNA was extracted from Ethylenediaminetetraacetic acid (EDTA) anticoagulated blood samples using the QIAamp DNA Mini Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions.

2.5.1. MLPA

Gene CNV and functional SNPs within the low-affinity *FCGR* genes were genotyped using *FCGR*-specific MLPA assay (MRC Holland, Amsterdam, The Netherlands). In two reactions, the *FCGR*-specific MLPA assay detects CNV of the *FCGR2C, FCGR3A* and *FCGR3B* genes

as well as functional allelic variants: FcγRIIa-H166R, FcγRIIb-I232T, FcγRIIIa-V176F, FcγRIIIb-HNA1a/b/c and promoter variants within *FCGR2B/C* (c.-386G>C and c.-120T>A). In addition, the assay detects two *FCGR2C* gene expression variants: c.169T>C in exon 3 and c.798+1A>G, and the *FCGR2B/C* promoter variant at position c.−386G>C and c.−120A>T. In this study, *FCGR2B* and *FCGR2C* promoter sequences were not distinguished because previous findings have shown that Africans do not bear the promoter variant in *FCGR2B.* As a result, any detected c.−386G>C minor alleles would be in *FCGR2C* (97).

The MLPA assay was performed according to the manufacturer's instructions using the One-Tube protocol (80,126). In brief, DNA samples were diluted to a concentration of 20 ng/µl in Tris-EDTA buffer and 5 µl denatured at 98 °C for 5 minutes and cooled to 25 °C in a thermocycler. After addition of 1.5 µl MLPA buffer and 1.5 µl probe mix, each sample was incubated for 1 minute at 95 °C, then 18 hours at 60 °C to allow probe hybridization. Ligation of the hybridized probes was performed by adding 32 µl Ligase-65 reaction mix (25 µl dH20 + 3 µl Ligase buffer A + 3 µl Ligase buffer B + 1 µl Ligase-65 enzyme) to each sample while at 54 °C, followed by an incubation of 15 minutes at 54 °C and inactivation of the ligase enzyme for 5 minutes at 98 °C. Following ligation, samples were cooled to 20 °C and the 10 µl polymerase master mix (7.5 µl dH2O + 2 µl SALSA PCR primer mix + 0.5 µl SALSA polymerase) added. PCR amplification began immediately after the addition of the polymerase mix, with 35 cycles of 30 seconds at 95 °C, 30 seconds at 60 °C and 60 seconds at 72 °C; followed by 20 minutes at 72 °C.

After the PCR reaction, 0.8 µl of each PCR product was diluted in 9 µl HiDi Formamide and 0.2 µl GeneScan 500 LIZ size standard. The plate was sealed, incubated for 3 minutes at 86 °C and cooled for 2 min at 4 °C. Capillary electrophoresis using an ABI Genetic Analyser 3130 (Life Technologies, Applied Bio systems, Foster City, CA, USA) was used to separate the amplicons. The fragments were analysed with the Coffalyzer.NET software (MRC Holland), using peak height as a measure of gene/allele copy number.

2.5.2. Determination of CNV and SNPs from MLPA data

Two MLPA *FCGR* probe mixes (P110 and P111) were used to detect both CNVs and some frequent SNPs. For copy number determination, P110 probe length 337 (exon 1) and P111 probe length 310 (exon 5) were used for *FCGR3A.* For *FCGR3B,* P110 probe length 310 (exon 5) and 361 (exon 1) and P111 probe length 361 (exon 1) were used. P110 probe length 180

binds both *FCGR2B* and *FCGR2C* in exon 1 and since CNV has not been established in *FCGR2B,* increases or decreases in probe signal is associated with *FCGR2C* copy variation. *FCGR2C* duplication/deletion can occur in at least two different segments: One encompassing a complete *FCGR2C* copy, *HSPA7* and *FCGR3B* and the other an incomplete *FCGR2C* (excluding exon 8), distal part of *FCGR2A*, *HSPA6*, and *FCGR3A* (87). We therefore looked at the copy number of all the *FCGR2C* exons.

The P110 and P111 probe length 355 are specific for *FCGR2A*-H166R; P110 and P111 probe length 202 detects I232T in both *FCGR2B* and *FCGR2C* but the rare 232T allele is *FCGR2B* specific. In the case of *FCGR3A*-V176F, probe length 391 in P110 is specific for the 176F variant which only occurs in *FCGR3A* while the 176V variant occurs in *FCGR3B* as well and thus the 158V variant is detected in both *FCGR3A* and *FCGR3B* by the same probe in P111(80). The number of *FCGR3A*-176V copies is calculated according to *FCGR3B* copy number variation. That is by subtracting *FCGR3B* copy numbers from the total copies determined by the *FCGR3A*-176V probe.

The P110 and P111 probe length 187 and 254 are specific for the *FCGR2B/C* promoter variants c.-386G>C and c.-120T>A, respectively. The usual haplotypes are -386G-120T and -386C-120A. The MLPA assay cannot distinguish between the *FCGR2B* and *FCGR2C* promoter sequence. For the *FCGR2C*-ORF/Stop variant, P110 probe length 367 is specific for the stop variant in exon 3 (c.169T). When the ORF allele is present, this probe signal will reduce. P110 probe length 211 is specific for the splice variant c.798+1A allele, which results in incorrect slicing of *FCGR2C* transcript and no expression of the FcγRIIc protein. Since this probe is specific for the c.798+1A allele, the presence of the c.798+1G allele will result in a reduction in the probe signal, similar to what is seen for the ORF/Stop variant. Both the splice and ORF/Stop variants should be analysed according to *FCGR2C* copy number.

For the *FCGR3B* haplotypes, the P110 probe length 160 detecting the HNA1a allotype also binds to *FCGR3A* exon 3. The number of HNA1a allotype was determined by taking into account the CNV of *FCGR3A*. Whereas P111 probe length 247 only binds HNA1c, P111 probe length 166 binds both HNA1b and HNA1c allotypes. The number of HNA1b alleles were calculated by subtracting any detected number of HNA1C alleles. The total number of HNA1a, HNA1b and HNA1c allotypes should add up to the number of *FCGR3B* gene copies.

2.5.3. Sanger nucleotide sequencing

Genotyping of the *FCGR2C* c.134-96C>T variant was done via conventional PCR and Sanger nucleotide sequencing, modified from an established method (80). In brief, a 6,374 base pair fragment was amplified with the Expand Long Template PCR System (Roche, Mannheim, Germany) using the *FCGR2B/C* sense primer (5'-ATGTATGGGGTGTCTGTGTGTC-3') and *FCGR2C*-specific antisense primer (5'-CTCAAATTGGGCAGCCTTCAC-3'). The PCR reaction consisted of ~20 ng (genomic DNA as template, 3.75 U Expand Long Template enzyme mix, 5 µl 10× PCR buffer 3 (2.75 mM $MgCl_2$), 500 µM of each deoxynucleotide, 0.3 µM of each oligonucleotide primer, and molecular grade water to a final volume of 50 µl. The PCR conditions were initial denaturation at 94 °C for 2 minutes, followed by 10 cycles of 94 °C for 10 seconds (denaturation), 60 °C for 15 seconds (annealing) and 68 °C for 7 minutes (elongation). Thereafter, 25 cycles repeat process of denaturation, annealing and elongation respectively at 94 °C for 15 seconds, 60 °C for 15 seconds and 68 °C for 7 minutes plus 20 seconds cycle elongation for each successive cycle; and a final elongation cycle at 72 °C for 7 minutes. The internal antisense primer (5'-CCTCCACTGACCAGAAAGCAC-3') was used in standard BigDye Terminator v3.1 Cycle Sequencing reactions. Sequences were analysed in Sequencer version 4.5 (Gene Codes Corporation, Ann Arbor, MI) and area under the curve of the electropherogram used to determine allele count for individuals bearing more than two *FCGR2C* gene copies.

2.6. NOMENCLATURE

The description of the polymorphisms and CNVs used in this thesis is according to the Human Genome Variation Society (HGVS) guidelines and refers to the amino acid positions in the full protein (132). Copy number variation has been previously described within distinct copy number variable regions (CNRs) (86,87,97). The nucleotide numbering is based on the Genome Reference Consortium Human Reference 38 [GRCh38 (hg38)].

2.7. ANALYSIS

2.7.1. Statistical and computational analysis

For acquisition analysis, FcγR variants in children living with HIV-1 (cases) were compared to the variants in HIV-1-exposed-uninfected infants (controls). Categorical data were summarized as proportions and Chi-square and Fisher Exact tests (where appropriate) were used for comparisons. For normally distributed numerical data, the t-test was used for

comparison of means while Mann Whitney (Wilcoxon rank sum) test was used for skewed data. For all analysis, total number (n) refers to number of individuals.

For comparison of the *FCGR* genotypes, gene copies greater than two were regarded as homozygous when all the copies have the same allele or heterozygous when both alleles were present. Genotype reference groups for the di-allelic FcγRIIa-H166R, FcγRIIb-I232T, and FcγRIIIa-V176F variants were homozygosity for the major allele, while the genotype reference group for the multi-allelic FcγRIIIb-HNA1a/b/c were selected based on prevalence. Logistic regression analyses (univariate and multivariate) were carried out to ascertain factors associated with perinatal HIV-1 acquisition, pre-treatment viral load and CD4+ T-cell percentage, mortality, virological control and latent reservoir size after ART, in vertically infected infants/children. A P value < 0.05 in the multivariate analysis was regarded as statistically significant and 95% confidence intervals (CI) were used to estimate precision. Bonferroni correction was used to adjust for multiple comparisons. All analyses were performed in STATA version 15.1 (StataCorp LP, Texas, USA). Linkage disequilibrium (LD), the non-random association of alleles at different loci in a given population, between *FCGR* variants was assessed using the Haploview software package (133), expressed as D prime (D′) and square of the correlation coefficient (r^2). Hardy-Weinberg equilibrium, which states that allele and genotype frequencies in a population remain constant between generations in the absence of disturbing factors, was considered for individuals with two gene copies.

CHAPTER 3 - AN HIV VACCINE PROTECTIVE ALLELE IN *FCGR2C* ASSOCIATES WITH INCREASED ODDS OF PERINATAL HIV ACQUISITION

3.1. INTRODUCTION

The crystallisable fragment (Fc) region of immunoglobulin G (IgG) antibodies interacts with Fc gamma receptors (FcγRs) expressed on the surface of hematopoietic cells to mediate effector functions. In humans, FcγRs are divided into three classes (FcγRI, FcγRII, and FcγRIII) based on structural domain organisation, differences in affinity and specificity for IgG subclasses, and whether their binding triggers activating or inhibitory signals. The low affinity FcγRs are encoded by five genes on chromosome 1q23, namely *FCGR2A, FCGR2B, FCGR2C, FCGR3A* and *FCGR3B* (75) and play different roles in regulating immune responses (73). Functionally significant genetic variants occur for all low affinity FcγRs. These affect FcγRs by altering receptor cell surface density, binding affinities to IgGs, glycosylation patterns, cellular distribution, or subcellular localization (67,71). Apart from single nucleotide polymorphisms (SNPs), copy number variation (CNV) has been demonstrated for *FCGR2C, FCGR3A and FCGR3B* (84,89), and has been correlated with protein expression levels (134). Genes are duplicated or deleted at the *FCGR2/3* region within well-defined copy number variable regions (CNRs), namely CNR1, CNR2, CNR3 (86,87) and CNR4 (87). The most common are CNR1, which comprises genes of *FCGR2C, HSPA7* and *FCGR3B* and CNR2 that includes the distal part of *FCGR2A* (exon 8 and 3'-untranslated region [3'UTR]), *HSPA6, FCGR3A* and proximal part of *FCGR2C* (excluding exon 8 and 3'UTR) (87).

The *FCGR2C* gene, encoding FcγRIIc, is described as a pseudogene and is the product of an unequal crossover event between the 5' part of *FCGR2B* genes and 3' part of *FCGR2A* (77). Expression of the membrane-bound *FcγRIIc* protein depends on a combination of three minor alleles that include the c.169T>C variant in exon 3, which substitutes a premature stop codon with a glutamine at amino acid 57, and two splice variants in intron 7 - c.798+1A>G and c.799-1G>C (81,89). Due to significant variation of the minor allele frequencies in different populations (97), FcγRIIc protein expression is subject to ethnic variation. The splice variant c.798+1A>G minor allele rarely occurs in black Africans and East Asians, thus, few individuals in this population express FcγRIIc compared to approximately 33% of Caucasians (97). An

additional *FCGR2C* c.134-96C>T variant (also known as *FCGR2C 126C>T*) has been identified as clinically significant (82). Overall, genetic variation of *FCGR2C* has been associated with rheumatoid arthritis (135) idiopathic thrombocytopenic purpura (80), HIV-tuberculosis co-infection (79), antibody responses to vaccinations (81–83) and HIV disease progression (78).

In the RV144 vaccine trial, where the vaccine regimen was designed against HIV-1 clade B and E, a three-variant haplotype within *FCGR2C* [c.353C>T (rs138747765); c.391+111G>A (rs78603008) and c.134-96C>T (rs114945036)] reduced the risk of HIV-1 acquisition in Thai adults. The vaccine test subjects carrying at least one minor allele of the c.134-96C>T tag variant had an estimated vaccine efficacy of 91% against the CRF01_AE 169K HIV-1 strain and 64% against any HIV-1 strain, while those with wild type allele exhibited a vaccine efficacy of 15% and 11%, respectively (82). Conversely, two variants within the haplotype were associated with increased risk of HIV-1 acquisition in the HIV Vaccine Trials Network (HVTN) 505 vaccine trial (83). A follow-on trial of a similar vaccine regime to RV144 (HVTN 702 vaccine trial) tested in South Africa showed no efficacy (116). The cause underlying the different vaccine trial outcomes remains undetermined. However, differences in vaccine regimen, population, demographics and environment should be considered (83). A role for population genetics warrants consideration, since black South Africans do not possess the complete Thai *FCGR2C* haplotype and are only polymorphic for c.134-96C>T (rs114945036) (97).

The c.134-96C>T *FCGR2C* variant has been implicated in HIV-1 disease progression in a black South African cohort (78). However, unlike the protective effect observed for Thai vaccinees, the minor allele was associated with increased odds of HIV-1 disease progression in those already infected. It is unknown whether the alternate protective and deleterious roles of the *FCGR2C* c.134-96C>T variant in the Thai vaccinees and HIV-1 infected South Africans is due to different mechanisms involved before and after HIV-1 infection or whether the genetic differences associated with the haplotype alters its role in the two populations. Establishing the role of the c.134-96C>T variant in HIV-1 protective immunity in other models of persistent HIV-1 exposure, such as infants born to HIV-1 infected mothers, will be informative.

Mother-to-child transmission (MTCT) is an attractive model in which to study immune correlates of protection since both members of the transmitting dyad are known, timing of

transmission can be ascertained with reasonable precision, and it affords the opportunity to assess factors contributing to both the infectiousness of the transmitter (mother) and susceptibility of the recipient (infant) (16,17). Limitations of this model are that transmission occurs between genetically similar individuals, exposure to HIV-1 occurs at a time of early immune development, and immune circumstances during pregnancy are associated with tolerance of the foetal allograft (136). Nevertheless, it provides a unique opportunity to investigate the role of FcγR-mediated effector functions, since the individual (foetus/infant) at risk is passively immunized with HIV-1-specific antibodies through trans-placental transfer of IgG from the HIV-1 infected mother and the model is not confounded by interspecies differences as observed for non-human primate studies (137). In this study, we investigate the association between the *FCGR2C* c.134-96C>T variant and HIV-1 acquisition in black South African children born to women living with HIV.

3.2. MATERIALS AND METHODS

3.2.1. Study design and population

A nested case-control study was undertaken to investigate the association between the *FCGR2C* variants and HIV-1 perinatal acquisition in children, combining data from past studies of five perinatal cohorts at two hospitals in Johannesburg, South Africa (127–130). One of the five cohorts consists of 546 HIV-infected children who were recruited as part of two sequential randomized clinical trials (NEVEREST 2 and 3) (127–129). The remaining four cohorts comprised of 849 HIV-1 infected mothers and their infants who were recruited and followed prospectively, of whom 83 (10%) infants acquired HIV (130). In the present study, only samples that were found and with sufficient volume were genotyped. *FCGR2C* genotypic data from 99 out of 546 and 77 of 83 HIV-1-infected children (cases) from the NEVEREST and mother-infant cohorts, respectively (n = 176) were compared with 349 of the HIV-exposed uninfected children (controls).

Mode of transmission was defined according to the presence or absence of detectable HIV-1 deoxyribonucleic acid (DNA) in the infant at birth and six weeks of age. Infants that tested HIV-1 positive at six weeks of age, but who were negative at birth, were considered to be infected *intrapartum* (during labour and delivery) (n = 31), while infants that tested HIV-1 positive at birth were considered infected *in utero* (n = 19). Infants who were HIV-1 positive at six weeks, but had no birth sample, were categorized as ‘undetermined’ (n = 28). In the

'undetermined' category, 25/28 (89.2%) mothers received single-dose nevirapine or triple-drug combination therapy (two nucleoside reverse transcriptase inhibitors with either a protease inhibitor or non-nucleoside reverse transcriptase inhibitor) known to reduce *intrapartum* transmission (130,138,139). Genotyping generated a result for all the *FCGR2C* variants assessed in this study in 27 out of the 28 samples. It was thus concluded that the majority (n = 27) of infants were likely infected *in utero* and were combined with the *in utero* group to form an *in utero*-enriched group. For the NEVEREST cohort, there were no birth samples as the children were recruited from six weeks of life. They were therefore classified as mixed transmission since a few were breastfeeding infections and in utero infections could not be distinguished from intrapartum infections (n = 99). All study participants were black South Africans and received nevirapine for prevention of MTCT. Maternal antiretroviral therapy was not routinely used at the time.

3.2.2. Ethics

Ethics approval for the study was obtained from the University of the Witwatersrand Human Research Ethics Committee (Reference numbers: M180575).

3.2.3. Genotyping

FCGR2C copy number and SNPs that affect gene expression – c.169T>C (p.X57Q), c.798+1A>G, and the *FCGR2B/C* promoter variant at position c.−386G>C and c.−120A>T – were determined using the *FCGR*-specific multiplex ligation-dependent probe amplification assay (MRC Holland, Amsterdam, The Netherlands) according to manufacturer's instructions. Refer to Chapter 2, sections 2.5.1 and 2.5.2. We did not utilise gene-specific polymerase chain reactions (PCR) to distinguish *FCGR2B* and *FCGR2C* promoter sequences since earlier findings indicate that African individuals do not possess the promoter variant in *FCGR2B*, and thus any detected c.−386G>C minor alleles were in *FCGR2C* (97).

The *FCGR2C* c.134-96C>T (rs114945036) variant was genotyped through conventional PCR and Sanger nucleotide sequencing. Refer to Chapter 2, sections 2.5.3

3.2.4. Nomenclature

For the SNP nomenclature used, refer to Chapter 2, section 2.6

3.2.5. Statistical analysis

Categorical data were summarized as proportions and the Fisher's Exact test was used for comparisons between children with HIV-1-infection and children who were HIV-1-exposed uninfected. For numerical data, the t-test was used for comparison of means. Univariate and multivariate analyses were conducted to determine factors associated with perinatal HIV acquisition. Adjustment for multiple comparisons was performed using the Bonferroni correction, which considered 16 independent tests — four unrelated clinical subgroups each tested for four variants (gene copy number, c.134-96C>T, c.169T<C, and c.−386G>C). Both unadjusted and adjusted P values are reported. Analysis of an association between *FCGR2C* variants and HIV-1 acquisition was limited to variants whose allele frequencies were ≥ 5%. Due to the low frequencies of minor allele homozygotes, their effect was tested using dominant model approach, where participants were divided into two genotype groups: homozygous genotype of the major allele (CC) and the two genotypes containing at least one minor allele (CT/TT). All analyses were performed in STATA version 15.1 (StataCorp LP, Texas, USA).

Linkage disequilibrium between *FCGR2C* functional variants and CNRs was computed using the Haploview software package (133) and expressed as D prime (D′) and square of the correlation coefficient (r^2). The closer D′ is to 1 the stronger the LD between two loci. Hardy-Weinberg equilibrium was considered for individuals with two gene copies and the statistics abstracted from the Haploview analysis output. For the analysis, genotypic data with multiple gene copies were considered homozygous if all copies carried the same allele or heterozygous when both alleles were present.

3.3. RESULTS

3.3.1. Cohort

This nested case-control study investigated *FCGR2C* genotypic data from 525 children to determine the role of *FCGR2C* variants and HIV-1 acquisition in South African children born to women living with HIV-1. The cohort includes 176 HIV-1 infected (cases) and 349 HIV-exposed-uninfected (controls) children. The HIV-1 infected children comprised four transmission mode groups: *in utero* (n = 19), *in utero*-enriched (n = 46), *intrapartum* (n = 31) and mixed (n = 99). Overall, there was no significant difference in sex, gestation and breastfeeding status between the HIV-1 infected and HIV-1 uninfected cohort. However, the total HIV-1 infected and HIV-1 exposed-uninfected groups differed significantly in birth

weight at delivery. Specifically, a higher proportion of HIV-infected children had a birth weight below 2500 g (22% vs. 11%; $P = 0.001$, $P_{Bonf} = 0.016$) (Table 3.1).

3.3.2. *FCGR2C* copy number distribution and HIV-1 acquisition

The *FCGR2C* gene, highly homologous to *FCGR2B* in the first six exons and *FCGR2A* in the last two exons, is subject to CNV within previously described distinct regions (Figure 3.1). We did not observe any individual with a complete absence of the *FCGR2C* gene. Overall, *FCGR2C* CNV occurred in 166/525 (32%) children, with the frequency of duplications (n = 114) 2.2-fold higher than deletions (n = 52) (69% vs. 31%). The copy number distribution was significantly different between the HIV-1 infected and HIV-1 exposed-uninfected groups but not after Bonferroni correction ($P = 0.010$, $P_{Bonf} > 0.05$) (Table 3.2). Variation in copy number among the whole study cohort was observed more frequently in CNR1, which encompasses a complete *FCGR2C* copy, *HSPA7* and *FCGR3B* (28%; n = 147) than CNR2, with an incomplete *FCGR2C* copy, *HSPA6* and *FCGR3A* (2.7%; n = 14). In six instances (1.1%), we observed CNV for only *FCGR2C* in the absence of duplicated/deleted flanking genes, as previously described among the South African black population (97). A duplication in both CNR1 and CNR2 was observed in one individual. Given the differences between the CNRs, their copy number variability was determined separately.

Within CNR1, CNV was significantly different between the HIV-1 infected and HIV-1 exposed-uninfected groups ($P = 0.009$, $P_{Bonf} > 0.05$). This difference was primarily determined by gene deletions. There were a higher number of HIV-1 exposed-uninfected children with a single gene copy compared to HIV-1 infected children (36% vs. 14%) (Table 3.2). Using two *FCGR2C* gene copies as reference, the possession of a single gene copy was independently associated with reduced odds of HIV-1 acquisition (OR = 0.29; 95% CI 0.12-0.71; $P = 0.007$, $P_{Bonf} > 0.05$) and retained significance after controlling for birthweight and *FCGR2C* genotypes (AOR = 0.37; 95% CI 0.15-0.90; $P = 0.029$, $P_{Bonf} > 0.05$) (Table 3). The CNR2 and the novel CNR4 variability were excluded from further association analysis due to low frequencies (< 5%).

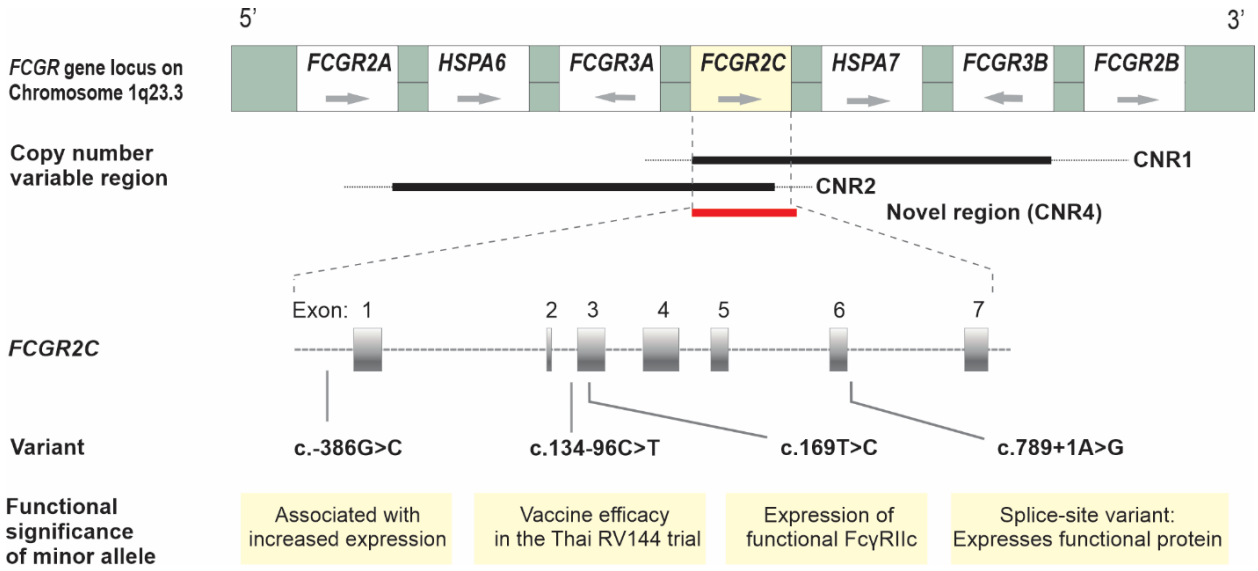

Figure 3.1 Schematic diagram of the *FCGR* gene loci on chromosome 1q23 with their orientation.
CNV of *FCGR2C* within distinct copy number variable regions – previously designated CNR1, CNR2 (86,87) and a novel region (CNR4) where only *FCGR2C* duplicated/deleted, as described among South African black population (97); Functional and clinically significant *FCGR2C* variants and their positions on the gene.

3.3.3. *FCGR2C* variants that determine the expression of surface FcγRIIc

We further genotyped functional *FCGR2C* variants that determine the expression of FcγRIIc (c.−386G>C, c.169T>C and c.798+1A>G) and the c.134-96C>T variant that associated with risk of HIV-1 acquisition in the Thai RV144 HIV-1 vaccine trial (Figure 3.1). To assess the role of the *FCGR2C* genotypes and allele distribution in perinatal HIV-1 acquisition, children with a single *FCGR2C* gene copy were considered homozygous; those with more than one *FCGR2C* gene copy were considered homozygous if all the alleles were the same or heterozygous if both alleles were present. With MLPA, we obtained genotypic data from 166 out of the 176 HIV-1 infected for c.169T>C and c.−386G>C variants.

A SNP in exon 3 (c.169T>C) that results in an open reading frame (ORF) or a stop codon determines the expression of FcγRIIc when present with the minor allele of two splice variants in intron 7 (c.798+1A>G and c.799-1G>C). While 129/515 (25%) of individuals carried at least one *FCGR2C*-ORF (c.169C allele), only 4/129 (3%) individuals possessed the c.798+1G minor allele that represents the classic *FCGR2C*-ORF and predicts FcγRIIc expression (89,140). Of the four individuals with the c.798+1G minor allele, three were HIV-1-exposed uninfected and one HIV-1 infected. Conversely, the c.169C allele co-occurred with the

c.798+1A allele in 97% (n = 125) of the participants, representing the non-classic *FCGR2C*-ORF that does not yield surface expression of FcγRIIc. The c.799-1G>C splice cite variant was not genotyped, since it has been shown that the c.169C and c.798+1A alleles are syntenic with the c.799-1G allele in South African population (97).

Table 3.1 Demographic and clinical characteristics of perinatal HIV-1 acquisition groups

Variables	**HIV-1-exposed uninfected**	**Total HIV-1 infected**		**In utero infected**		**In utero-enriched infected**		**Mixed infected**		**Intrapartum infected**	
	n = 349	n = 176	*P* value	n = 19	*P* value	n = 46	*P* value	n = 99	*P* value	n = 31	*P* value
Sex	(n = 346)	(n = 176)	1.000		0.487		0.875		0.569		0. 260
Male	160 (46)	80 (45)		7 (37)		20 (43)		42 (42)		18 (58)	
Female	189 (54)	96 (55)		12 (63)		26 (57)		57 (58)		13 (42)	
Gestation	(n = 327)	(n = 164)	0.355	(n = 18)	0.105	(n = 44)	0.788	(n = 95)	1.000	(n = 25)	**0.013**
Term	295 (90)	143 (87)		14 (78)		39 (89)		86 (91)		18 (72)	(P_{Bonf} =
Preterm (<37 weeks)	32 (10)	21 (13)		4 (22)		5 (11)		9 (9)		7 (28)	0.208)
Birth weight (g)	(n = 344)	(n = 168)	**0.001**		0.251		**0.003**	(n = 92)	**0.001**	(n = 30)	0.898
≥ 2500	307 (89)	131 (78)	**(P_{Bonf} =**	15 (79)		34 (74)	**(P_{Bonf} =**	70 (76)	**(P_{Bonf} =**	27 (90)	
< 2500	37 (11)	37 (22)	**0.016)**	4 (21)		12 (26)	**0.048)**	22 (24)	**0.016)**	3 (10)	
Breastfed	(n = 346)	(n = 170)	1.000	(n = 18)	0.384	(n = 45)	0.117	(n = 95)	0.780	(n = 30)	0.359
No	271 (78)	134 (79)		16 (89)		40 (89)		73 (77)		21 (70)	
Yes	75 (22)	36 (21)		2 (11)		5 (11)		22 (23)		9 (30)	

Data are n (%) unless otherwise specified.
The *P* values refer to comparisons between the HIV-1-exposed uninfected (control) group and each of the HIV-1 infected (case) groups.
Bold indicates statistical significance of $P < 0.05$; P_{Bonf}, Bonferroni corrected *P* value

Table 3.2 *FCGR2C* copy number distribution in HIV-1-exposed infected and uninfected infants

Variables	**Total study cohort**	**HIV-1-exposed uninfected**	**Total HIV-1 infected**		**In utero infected**		**In utero-enriched infected**		**Mixed infected**		**Intrapartum infected**	
	n = 525	n = 349	n = 176	*P* value	n = 19	*P* value	n = 46	*P* value	n = 99	*P* value	n = 31	*P* value
FcγRIIc copy number				**0.010** (P_{Bonf} =		0.672		0.142		0.095		**0.028** (P_{Bonf} =
1 copy	52 (10)	44 (12)	8 (4)	0.16**)**	1 (5.3)		3 (6)		5 (5)		0 (0)	0.448)
2 copies	359 (68)	233 (67)	126 (72)		13 (68.4)		28 (61)		71 (72)		27 (87)	
≥3 copies	114 (22)	72 (21)	42 (24)		5 (26.3)		15 (33)		23 (23)		4 (13)	
CNR1	(n = 147)	(n = 105)	(n = 42)	**0.009**	(n = 5)	0.162	(n = 15)	**0.035**	(n = 23)	0.228	(n = 4)	0.296
deletion	44 (30)	38 (36)	6 (14)	(P_{Bonf} =	0 (0)		1 (7)	(P_{Bonf} =	5 (22)		0 (0)	
duplication	103 (70)	67 (64)	36 (86)	0.144)	5 (100)		14 (93)	0.56)	18 (78)		4 (100)	
CNR2	(n = 14)	(n = 8)	(n = 6)	0.767	(n = 1)	0.444	(n = 2)	0.444	(n = 4)	0.180	(n = 0)	
deletion	5 (36)	3 (37.5)	2 (33)		1(100)		2 (100)		0		0	
duplication	9 (64)	5 (62.5)	4 (67)		0 (0)		0		4 (100)		0	
CNR4	(n = 6)	(n = 3)	(n = 3)	0.100	(n = 0)		(n = 1)	0.250	(n = 2)	0.100	(n = 0)	
deletion	3 (50)	3 (100)	0		0		0		0)		0	
duplication	3 (50)	0	3 (100)		0		1 (100)		2(100)		0	

Data are n (%) unless otherwise specified.
The *P* values refer to comparisons between the HIV-1-exposed uninfected (control) group and each of the HIV-1 infected (case) groups.
Bold indicates statistical significance of $P < 0.05$; P_{Bonf}, Bonferroni corrected *P* value

Table 3.3 Effect of *FCGR2C* CNR1 copy number distribution on perinatal HIV-1 acquisition, adjusting for birthweight and *FCGR2C* genotypes

	Univariate		Multivariate	
FcγRIIc copy number (Total group)	OR (95% CI)	P-value	Adjusted OR	P-value
1 copy	0.29 (0.12-0.71)	**0.007** (P_{Bonf} = 0.112)	0.37 (0.15-0.90)	**0.029** (P_{Bonf} = 0.464)
2 copies	Ref		Ref	
≥3 copies	0.99 (0.63-1.57)	0.978	0.74 (0.43-1.27)	0.275

OR, Odds Ratio; CI, Confidence Interval; P_{Bonf}, Bonferroni corrected *P* value
Bold indicates statistical significance of $P < 0.05$.

While the c.169T>C variant alone does not result in surface expression of FcγRIIc, it may have other unknown functional consequences and was therefore investigated for a possible association with HIV-1 perinatal transmission. The c.169T>C genotype distribution was significantly different between HIV-1 infected and HIV-1 exposed-uninfected children (P = 0.002, P_{Bonf} = 0.032) (Figure 3.2i). In a dominant model, the c.169C was overrepresented in the HIV-1 infected compared to the uninfected children (32% vs. 22%) and significantly associated with increased odds of HIV acquisition (OR = 1.68; 95% CI 1.11-2.55; P = 0.013, $P_{Bonf} > 0.05$).

The strength of association increased after adjusting for birthweight and CNR1 copy number (AOR = 2.39; 95% CI 1.45-3.95; P = 0.001, P_{Bonf} = 0.016). For *the FCGR2B/C* promoter variant at position –386G>C, which modulates gene expression levels, no significant difference in genotype frequency was observed between the HIV-1 infected and HIV-1 uninfected cohort (P = 0.288) (Figure 3.2ii). The homozygous −386 CC genotype was not observed in any individual. Due to the low frequency of the splice site variant c.798+1A>G minor allele, an association analysis was not conducted.

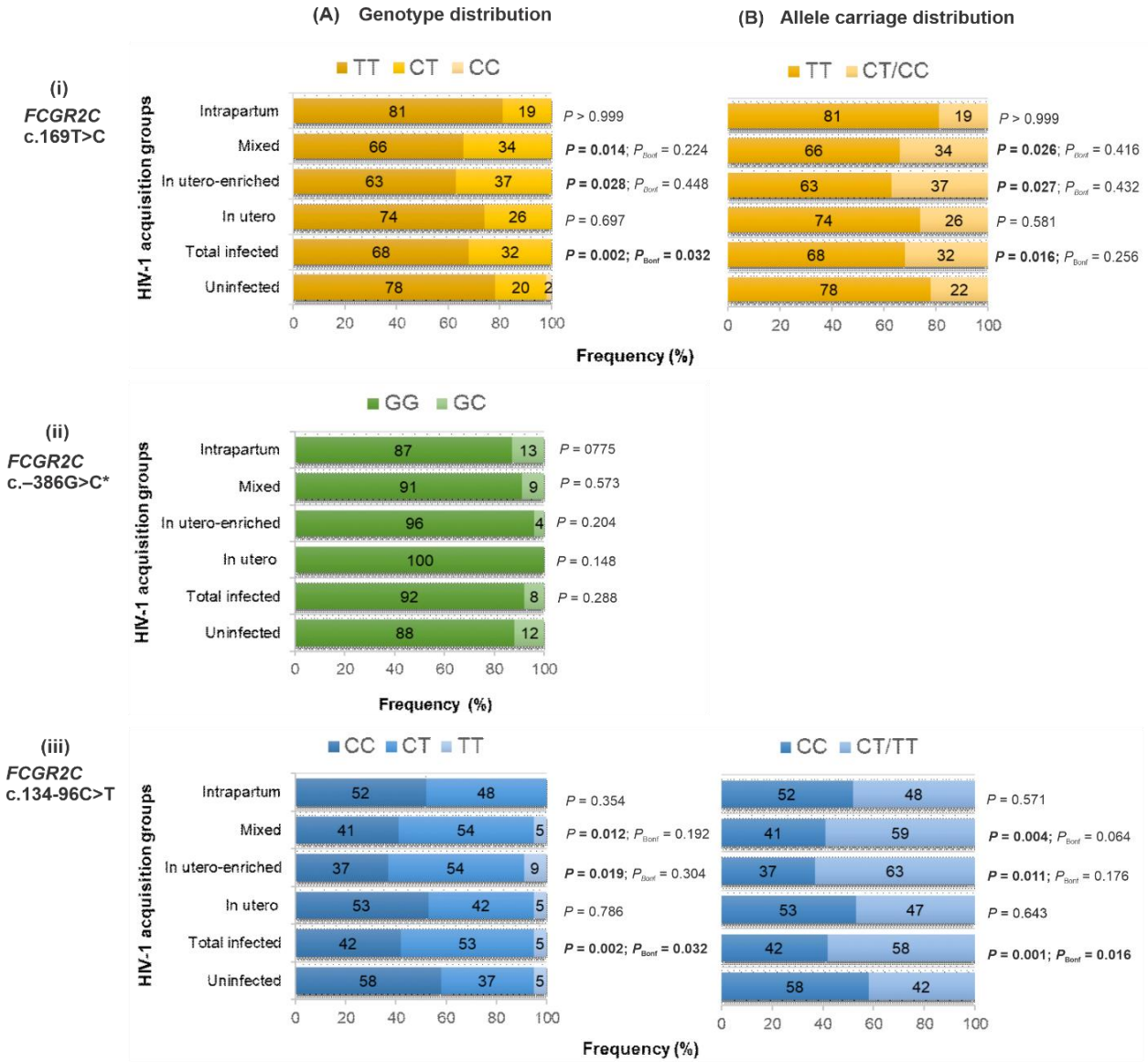

Figure 3.2 Genotype (A) and allele carriage (B) distribution of functional *FCGR2C* variants and their association with perinatal HIV-1 acquisition in black South Africans
[HIV-1-exposed uninfected (n = 349), total infected (n = 176 for c.134-96C>T; n = 166 for c.169T>C and c.−386G>C), in utero infected (n = 19), in utero-enriched infected (n = 46), mixed infected (n = 99 for c.134-96C>T; n = 89 for c.169T>C and c.−386G>C) and intrapartum infected (n = 31)]. * No individual with homozygous −386 CC genotype

3.3.4. The Thai *FCGR2C* haplotype tag variant c.134-96C>T associates with increased odds of perinatal HIV-1 transmission

The *FCGR2C* c.134-96C>T genotype distribution was significantly different between the children who acquired HIV-1 and those who did not ($P = 0.002$, $P_{Bonf} = 0.032$) (Figure 2iii). In particular, the c.134-96T-allele was overrepresented in the HIV-1-infected children compared to the exposed-uninfected children (58% vs. 42%; $P = 0.001$, $P_{Bonf} = 0.016$). The overrepresentation was primarily driven by the *in utero*-enriched (63% vs. 42%; $P = 0.011$, $P_{Bonf} > 0.05$) and mixed (59% vs. 42%; $P = 0.004$, $P_{Bonf} > 0.05$) infected groups (Figure 3.2iii). We combined the *in utero*-enriched and mixed transmission groups into a larger *in utero*-enriched group, excluding the 27 HIV-1 infected and 75 HIV-1 exposed uninfected breastfed individuals, and still observed overrepresentation of the minor allele in the HIV-1 infected children (60% vs. 43%; $P = 0.002$, $P_{Bonf} = 0.048$) (data not shown).

The association between *FCGR2C* c.134-96C>T variant and HIV acquisition was assessed in a univariate model and a multivariate model that controlled for birthweight and CNR1 copy number, which were statistically significant at univariate analysis (Table 3.4). In a dominant model, the c.134-96C>T minor allele was associated with increased odds of perinatal HIV-1 transmission at univariate (OR = 1.89; 95% CI 1.31-2.73; $P = 0.001$, $P_{Bonf} = 0.016$) and multivariate analysis (AOR = 1.89; 95% CI 1.25-2.87; $P = 0.003$, $P_{Bonf} = 0.048$). The association was specific to the *in utero*-enriched (OR = 2.34; 95% CI 1.24-4.42; $P = 0.009$, $P_{Bonf} > 0.05$) and the mixed transmission groups (OR = 1.94; 95% CI 1.24-3.06; $P = 0.004$, $P_{Bonf} > 0.05$) but not the *in utero* (OR = 1.24; 95% CI 0.49-3.12; $P = 0.653$) and *intrapartum* groups (OR = 1.29; 95% CI 0.62-2.69; $P = 0.500$). Statistical significance was retained in both *in utero*-enriched (AOR = 2.49; 95% CI 1.31-4.76; $P = 0.006$, $P_{Bonf} > 0.05$) and mixed transmission groups (AOR = 2.06; 95% CI 1.28-3.30; $P = 0.003$, $P_{Bonf} = 0.048$) after adjusting for birthweight.

Table 3.4 Univariate and multivariate analysis of the effect of *FCGR2C* c.134-96C>T on perinatal acquisition of HIV-1

	Univariate		Multivariate	
Genotype	**OR (95% CI)**	***P* value**	**Adjusted OR (95% CI)**	***P* value**
*Total infected**				
CC	Ref		Ref	
CT/TT	1.89 (1.31-2.73)	**0.001 (P_{Bonf} = 0.016)**	1.89 (1.25-2.87)	**0.003 (P_{Bonf} = 0.048)**
In utero-enriched[#]				
CC	Ref		Ref	
CT/TT	2.34 (1.24-4.42)	**0.009** (P_{Bonf} = 0.144)	2.49 (1.31-4.76)	**0.006** (P_{Bonf} = 0.064)
Mixed[#]				
CC	Ref		Ref	
CT/TT	1.94 (1.24-3.06)	**0.004** (P_{Bonf} = 0.064)	2.06 (1.28-3.30)	**0.003 (P_{Bonf} = 0.048)**
In utero				
CC	Ref			
CT/TT	1.24 (0.49-3.12)	0.653		
Intrapartum				
CC	Ref			
CT/TT	1.29 (0.62-2.69)	0.500		

OR, Odds Ratio; CI, Confidence Interval; P_{Bonf}, Bonferroni corrected *P* value
Bold indicates statistical significance of $P < 0.05$.
* adjusted for birthweight and CNR1 copy number
[#] adjusted for birthweight

3.3.5. The *FCGR2C* c.134-96C>T and c.169T>C are in high linkage disequilibrium

The observed genotype frequencies for *FCGR2C* c.–386G>C, c.134-96C>T, c.169T>C were in Hardy-Weinberg equilibrium ($P > 0.05$). We also observed that 71% (39/55) of children carrying a c.169C were heterozygous for the *FCGR2B/C* promoter variant (data not shown). We analysed linkage disequilibrium between the *FCGR2C* variants and CNRs, with and without considering the CNV. It was important to determine whether the observed variants associated with HIV-1 acquisition act independently or linkage disequilibrium plays a part. Our result demonstrated high linkage disequilibrium between c.134-96C>T and c.169T>C both without considering CNV ($D' = 0.867$; $r^2 = 0.319$) and when only those with two gene copies were included ($D' = 0.908$ and $r^2 = 0.213$) (Figure 3.3). Both c.134-96C>T and c.169T>C independently associated with increased odds of HIV-1 acquisition, but in multivariate analysis, c.134-96C>T retained significance (AOR = 1.91; 95% CI 1.23-2.96; $P = 0.004$, $P_{Bonf} > 0.05$) while c.169T>C did not (AOR = 1.14; 95% CI 0.70-1.86; $P = 0.590$).

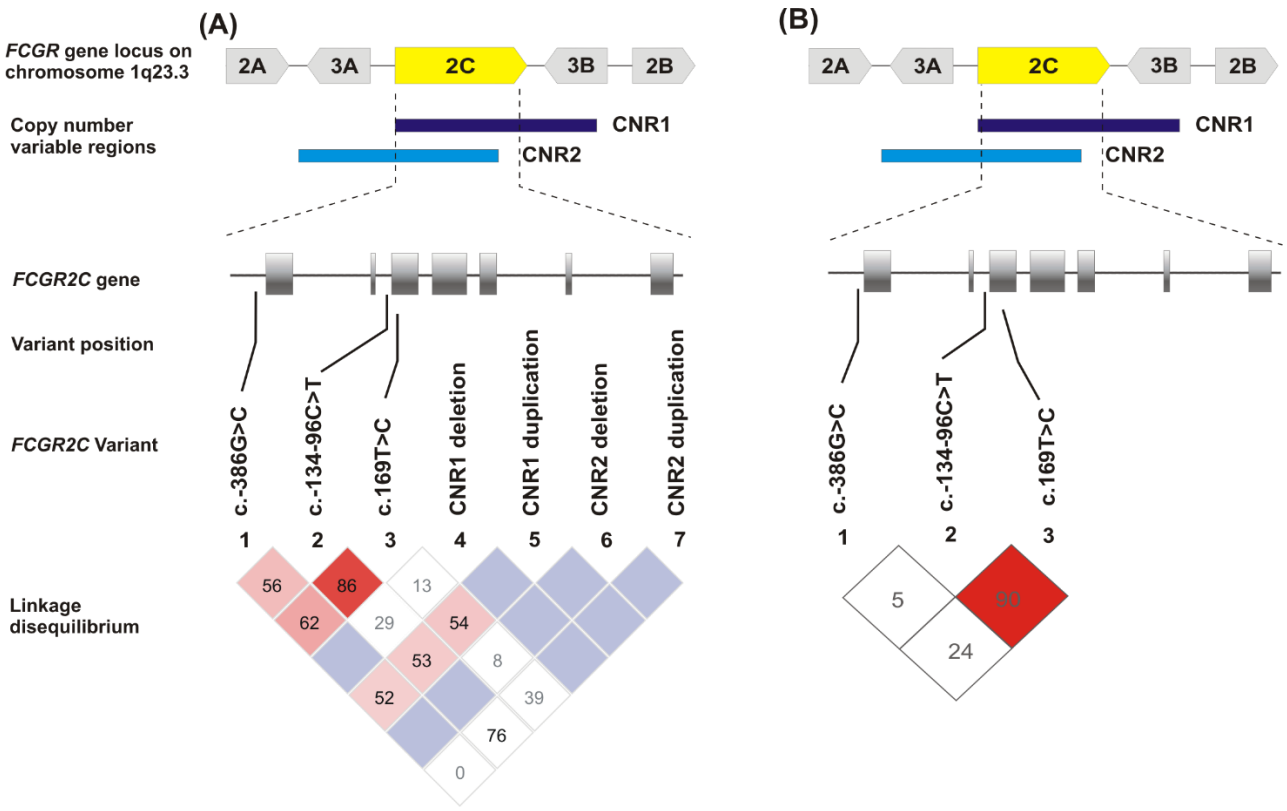

Figure 3.3 Linkage disequilibrium of the single nucleotide polymorphisms (SNPs) and copy number regions (CNRs) in *FCGR2C* gene in South African children born to women living with HIV-1.
(A) LD plots for SNPs without considering CNV and (B) when only those with two gene copies were included. Values reflect D′ measures of LD and colour in the squares given by standard D′/LOD (log of the odds of there being LD between two loci) colour scheme, with bright red colour used to display very strong LD.

3.3.6. Effect of *FCGR2C* c.134-96C>T and maternal viral load on perinatal acquisition of HIV-1

Maternal viral load is a key determinant of MTCT of HIV-1 infection. For the NEVEREST cohorts, the maternal viral load was not recorded. However, in the birth cohort with the defined modes of transmission, information on maternal HIV-1 viral load was available for 279 mothers of whom 70 transmitted HIV to their infants and 209 did not. The mean HIV-1 viral load was significantly higher in transmitting mothers than non-transmitting mothers (4.53 vs. 3.95 $\log_{10}$ copies/ml; $P < 0.0001$). The maternal *FCGR2C* c.134-96C>T variant independently associated with HIV-1 transmission (OR = 1.98; 95% CI 1.16-3.37; $P = 0.012$, $P_{Bonf} = 0.192$) and when controlled for maternal viral load and birthweight (AOR = 2.03; 95% CI 1.14-3.62; $P = 0.016$, $P_{Bonf} = 0.256$). In this small subset, the infant *FCGR2C* c.134-96C>T variant was independently associated with HIV-1 acquisition (OR = 1.92; 95% CI 1.14-3.23; $P = 0.014$,

P_{Bonf} = 0.224). The significant association remained when adjusted for maternal viral load (AOR = 2.10; 95% CI 1.13-3.87; P = 0.018, P_{Bonf} = 0.288), specifically in the in utero-enriched transmission group (AOR = 2.67; 95% CI 1.33-5.37; P = 0.006, P_{Bonf} = 0.064) (Table 3.5).

3.3.7. Mother-child *FCGR2C* c.134-96C>T genetic similarity and HIV-1 acquisition

We next evaluated mother-child *FCGR2C* c.134-96C>T genotype concordance and association with HIV-1 acquisition. Concordance was defined as mother and infant each bearing at least one T allele (mother-child: CT/TT-CT/TT) or both were homozygous for the C allele (CC). Discordance was defined as one individual within the dyad possessing a CC genotype and the other bearing a T allele (CT/TT). Possession of a T allele in both mother and infant was overrepresented in the HIV-1-infected children compared to the exposed-uninfected children (45% vs. 28%; P = 0.012, P_{Bonf} = 0.192). Independently, MTCT was more likely among the mother-child concordant CT/TT group compared to the concordant CC group (OR = 2.58; 95% CI 1.36-4.88; P = 0.004, P_{Bonf} = 0.064) and retained significance after adjusting for maternal viral load, birthweight and infant CNR1 copy number (AOR = 2.87; 95% CI 1.36-6.06; P = 0.006, P_{Bonf} = 0.096) (Table 3.6).

Table 3.5 Effect of *FCGR2C* c.134-96C>T on perinatal acquisition of HIV-1 in study cohort with information on maternal viral load

	Univariate		Multivariate	
Genotype	**OR (95% CI)**	***P* value**	**Adjusted OR (95% CI)**	***P* value**
*Total infected**				
CC	Ref		Ref	
CT/TT	1.92 (1.14-3.23)	**0.014** (P_{Bonf} = 0.224)	2.10 (1.13-3.87)	**0.018** (P_{Bonf} = 0.288)
In utero-enriched[#]				
CC	Ref		Ref	
CT/TT	2.34 (1.24-4.42)	**0.009** (P_{Bonf} = 0.144)	2.67 (1.33-5.37)	**0.006** (P_{Bonf} = 0.064)

OR, Odds Ratio; CI, Confidence Interval; P_{Bonf}, Bonferroni corrected *P* value
Bold indicates statistical significance of $P < 0.05$.
* adjusted for maternal viral load, birthweight and CNR1 copy number
[#] adjusted for maternal viral load and birthweight

Table 3.6 Mother-child *FCGR2C* c.134-96C>T genetic similarity and HIV-1 acquisition[a]

Genotype (mother-child pair)	**HIV-1-exposed uninfected n = 222**	**HIV-1 infected n = 76**	**Univariate**		**Multivariate***	
			OR (95% CI)	***P* value**	**OR (95% CI)**	***P* value**
Concordant CC	94 (42)	20 (26)	Ref		Ref	
Concordant CT/TT	62 (28)	34 (45)	2.58 (1.36-4.88)	**0.004** (P_{Bonf} = 0.064)	2.87 (1.36-6.06)	**0.006** (P_{Bonf} = 0.096)
Discordant	66 (30)	22 (29)	1.57 (0.79-3.10)	0.197	1.50 (0.68-3.29)	0.308

OR, Odds Ratio; CI, Confidence Interval; P_{Bonf}, Bonferroni corrected *P* value
Bold indicates statistical significance of $P < 0.05$.
* adjusted for maternal viral load, birthweight and CNR1 copy number
[a] "Concordant" denotes mother-child pairs with same *FCGR2C* c.134-96C>T genotype. "Discordant" denotes mother-child pairs with different genotypes.

3.4. DISCUSSION

Previous studies have reported both functional and clinical relevance of *FCGR2C* genetic variability, including single nucleotide polymorphisms and copy number variations. Expression of the membrane-bound FcγRIIc protein in individuals bearing the *FCG2C* c.169T>C minor allele (*FCGR2C*-ORF) has been associated with susceptibility to idiopathic thrombocytopenic purpura (80), Kawasaki disease (140), systemic lupus erythematosus and increased antibody responses to vaccinations (81). Furthermore, the *FCGR2C* c.134-96C>T tag variant associated with reduced risk of HIV-1 infection in the Thai phase 3 RV144 HIV-1 vaccine trial (82) and increased risk of HIV-1 disease progression in black South Africans (78). In addition, increased risk of HIV-1 acquisition in the HVTN 505 vaccine trial was associated with two variants within the Thai *FCGR2C* haplotype but not the c.134-96C>T tag variant (83).

In this study, we report further associations between *FCGR2C* variants and perinatal HIV-1 acquisition in South African children. Deletion of CNRI was significantly protective of perinatal HIV-1 acquisition compared to two gene copies, but the significance was not retained after Bonferroni correction. However, the observed association between CNR1 and perinatal HIV-1 acquisition might not be due to *FCGR2C* copy number variability because *FCGR3B* and *HSPA7* genes are also deleted with CNR1. The associations appear to be unrelated to surface expression of membrane-bound FcγRIIc. While some children carried the c.169C open reading frame allele, the co-occurrence of the splice-site variant c.798+1A allele predicted the absence of FcγRIIc expression in the majority of children. The latter allele gives rise to alternative mRNA splicing and a premature stop codon with associated loss of surface expression (89,140). Only four children carried both the c.169C open reading frame allele and the splice-site variant c.798+1G allele required for functional expression of FcγRIIc. This finding correlates with previous studies that suggested rare to absent expression of FcγRIIc in the black African population (97,140) and raises questions around the functional relevance of the c.169T>C variant.

The *FCGR2C* c.134-96T-minor allele was associated with increased odds of perinatally acquired HIV-1 acquisition in South African children. Specifically, a significant association was observed in the *in utero*-enriched and mixed transmission groups but not in the *in utero* and *intrapartum* transmission groups. The *in utero*-enriched and mixed transmission groups are very similar in terms of drug treatment, as all were exposed to nevirapine for prevention of

MTCT. Due to the nevirapine, fewer infants in the mixed group would have had *intrapartum* transmission and therefore were likely *in-utero* enriched. After adjusting for multiple comparisons using Bonferroni correction, the overall c.134-96C>T association retained significance, primarily driven by the mixed transmission group. The non-significance in the *in utero*-enriched group may potentially be due to small sample size. We postulate that the role of c.134-96C>T in HIV-1 acquisition is more pronounced during the course of pregnancy and at the maternal-foetal interface (71). In a subset of our study cohort, the observed association with *FCGR2C* c.134-96C>T variant remained when adjusted for maternal viral load, a key determinant of MTCT of HIV-1 infection. In both mother and child, the *FCGR2C* c.134-96C>T variant was associated with HIV-1 transmission and acquisition, respectively. Concordance in mother-child possession of the c.134-96T-allele further associated with a greater risk of MTCT compared to homozygosity for the C-allele in both mother and infant.

Assessing the role of the *FCGR2C* c.134-96C>T tag variant in both HIV-1 acquisition and disease progression has produced contrasting results of both protective and deleterious effects. The Thai phase 3 RV144 HIV vaccine trial provided the first clinical evidence of vaccine-induced protection, where the *FCGR2C* c.134-96C>T tag variant reduced the risk of HIV acquisition (82). Subsequently, two variants within the Thai *FCGR2C* haplotype were associated with increased risk of HIV-1 acquisition in vaccine recipients in the HVTN 505 trial but the c.134-96C>T tag variant was not. The population in this trial was predominantly Caucasian men who have sex with men (83). The *FCGR2C* c.134-96C>T tag variant was also associated with HIV-1 disease progression in South African adults (78). In this mother-child transmission model study, we establish its role in increased predisposition to HIV-1 acquisition. Whether the contrasting associations bear any functional significance is currently not clear and the modulating risk factors may not necessarily overlap (78). The differing results may be attributable to a variety of factors, including genetic differences between populations. It has been shown that two of the three variants within the Thai *FCGR2C* haplotype are absent in the African population, including black South Africans (97).

These findings may be of consequence to efforts aimed at elucidating the different outcomes of the two very similar HIV-1 vaccine efficacy trials, RV144 in Thailand and HVTN702 in South Africa. In addition to population genetic differences, the vaccine regimen evaluated in the RV144 and HVTN702 vaccine efficacy trials were also different. The RV144 vaccine candidate was specific for HIV-1 clades B and E with alum as adjuvant. This vaccine candidate

elicited robust cross-clade immune responses in South Africans (141). However, in subsequent clinical trials the RV144 vaccine regimen was modified to increase immunogenicity and improve the duration of vaccine-induced immune responses (142). The vaccine regimen was HIV-1 clade C-specific, the predominant clade in South Africa, and was adjuvanted with MF59 (142,143). The adapted ALVAC-HIV and Bivalent Subtype C gp120–MF59 vaccine regimen evaluated in HVTN 702 trial showed no efficacy among South African adults, despite previous evidence of immunogenicity (116). The differential vaccine efficacy between the RV144 and HVTN 702 trials might be due to differences in some components of the vaccine regimen and host genetics. In vaccine design, it is important to consider host genetic variation that can modulate vaccine efficacy (144). We previously established that black South Africans do not possess the complete Thai *FCGR2C* haplotype and are only polymorphic for c.134-96C>T (rs114945036) (97).

The functional mechanisms underlying the association of the variants within the Thai *FCGR2C* haplotype with HIV-1 acquisition, disease progression and vaccine efficacy is largely undefined. It raises many biological questions as to how expression of the membrane-bound FcγRIIc protein or the variants could modulate HIV-1 infection. The proposed impact of the *FCGR2C* variant haplotypes on immunity against HIV is unknown, one can only speculate. It is increasingly evident that it does not involve expression of the surface FcγRIIc receptor, since a limited number of individuals in the available genetic association studies bear the minor alleles that predict expression of the surface molecule. It is unknown whether a truncated soluble form of the FcγRIIc protein is produced in individuals with the premature stop codon or alternative splicing variants that prevent surface expression of FcγRIIc. Results from a study suggest that variants within the Thai *FCGR2C* haplotype either directly associate with the expression of *FCGR2C* in human B cells or in correlation with other causal variants that affect expression levels (144). The correlation with *FCGR2A* was observed across different populations and was specific to the c.134-96C>T variant (112,144). We have proposed that the variants modulate transcription factor binding that may alter mRNA expression (78). This may in turn affect processes regulated by mRNA from the *FCGR2C* gene.

In conclusion, the *FCGR2C* variant c.134-96T-allele, which was associated with protection in the Thai RV144 trial, was associated with increased odds of perinatal HIV-1 acquisition in black South African children. This adds to other deleterious associations found for *FCGR2C* variants in the context of HIV-1 (78,83) and warrants investigation of these variants in South

African adults actively immunized in the HVTN 702 trial (116) and individuals passively immunized with the broadly neutralizing VRC01 antibody in the Antibody Mediated Prevention (AMP) trials (145). Collectively, these intriguing results highlight the need for further mechanistic studies to establish the functional relevance of *FCGR2C* variation in different populations and more broadly in contexts of vaccination and disease.

CHAPTER 4 - *FCGR3A* GENE DUPLICATION, FcγRIIb-232TT AND FcγRIIIb-HNA1a ASSOCIATE WITH AN INCREASED RISK OF VERTICAL ACQUISITION OF HIV-1

4.1. INTRODUCTION

Antibody crystallisable fragment (Fc) gamma receptors (FcγRs) are hematopoietic cell surface glycoproteins that bind the Fc region of immunoglobulin G (IgG) antibodies, linking both humoral and cellular branches of immunity. Cross-linking of FcγRs on the cell surface initiates and regulates immune mechanisms that include antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), antibody production, B-cell activation, antigen presentation, and cytokine production (18,67,73,146). Cumulative data have highlighted the role of Fc-mediated effector functions in human immunodeficiency virus 1 (HIV-1) acquisition and post-infection control of viremia (12,15,17–19,71,109,110,147,148).

Generally, FcγRs are divided into three classes (FcγRI, FcγRII, and FcγRIII), each with different isoforms and encoded by different genes. The classes differ in structural domain organisation, affinity for specific IgG subclasses and ability to trigger activating or inhibitory signals (74,75). While FcγRI binds monomeric IgG with high affinity, both FcγRII and FcγRIII bind to IgG complexes through multivalent and low affinity interactions (72). The low affinity FcγRs located on chromosome 1q23 (FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa and FcγRIIIb) are encoded by *FCGR2A, FCGR2B, FCGR2C, FCGR3A and FCGR3B* genes, respectively (75) and their roles in the regulation of immune responses are different (73).

Functionally-relevant genetic variants, including single nucleotide polymorphisms (SNPs) and copy number variation (CNV), have been characterized in low-affinity receptors and associated with different diseases (20,22,71,75,78,80,82,83,110). Generally, CNV is considered an important factor of inter-individual differences and to date, only *FCGR2C, FCGR3A and FCGR3B* show CNV (84,89). Variation in copy number of *FCGR3A* correlates with FcγRIIIa surface expression levels on natural killer (NK) cells, a key mediator of ADCC (84). Similarly, CNV of *FCGR3B* directly correlates with protein expression and uptake of immune complexes by neutrophils (85). Functionally-significant amino acid changes have been reported for FcγRIIa, FcγRIIb, FcγRIIIa and FcγRIIIb that affect either their binding affinity for IgG or

receptor function. An arginine (R) to histidine (H) substitution at amino acid position 166 of FcγRIIa (position 131 in the mature protein), alters the receptor's affinity of IgG and its subclasses. In FcγRIIb, an isoleucine (I) to threonine (T) substitution at position 232 in the full protein reduces its inhibitory function on B cells (75). A polymorphism in FcγRIIIa results in a substitution of valine (V) to phenylalanine (F) at amino acid position 176 (position 158 in the mature protein) that alters the receptor's affinity for IgG and its subclasses (75,101). Conversely, a combination of five amino acid changes in FcγRIIIb give rise to the human neutrophil antigen 1 (HNA1) variants, HNA1a, HNA1b and HNA1c. Neutrophils from HNA1a homozygous individuals display greater phagocytic capacity compared to HNA1b homozygous individuals (105).

Accumulating evidence from mother-to-child transmission (MTCT) studies suggests that allelic variations of FcγR play a role in infant HIV-1 acquisition, but the observed results are inconsistent (71,108,109). Specifically, Brouwer et al. reported an association between the FcγRIIa H166R variant and perinatal HIV-1 acquisition in a cohort of infants in Kenya (109) that was not observed in subsequent separate studies in Kenya and South Africa (71,108).
The functional consequence for FcγR variants beyond FcγRIIa-H166R and FcγRIIIa-F176V during HIV-1 infection and acquisition *in vivo* has not been largely investigated. Further studies are therefore needed to elucidate the definitive role of *FCGR* genotypes on MTCT of HIV-1.

The potential role of Fc-mediated effector functions in exerting influence on HIV-1 transmission and acquisition was first described in a South African MTCT study (71). The study found the maternal FcγRIIIa-176V allele to be significantly associated with reduced HIV-1 transmission. In both mother and infant, having an FcγRIIIb-HNA1b allotype was significantly associated with increased odds of HIV-1 transmission and susceptibility, respectively. On the other hand, infants homozygous for the FcγRIIIb-HNA1a allotype were protected against perinatal HIV-1 acquisition. Since FcγRIIIb is largely expressed in neutrophils and FcγRIIIb-HNA1b allotype has been shown to exhibit lower neutrophil-mediated effector functions (105,106), the study findings indicate possible role for neutrophils in modulating perinatal HIV-1 transmission and acquisition. However, only a small number of HIV- infected infants (n = 78) were genotyped. This study further interrogates the role of FcγR-mediated effector functions in modulating perinatal HIV-1 acquisition, using a much larger cohort. As we strive towards the goal of elimination of vertical HIV-1 transmission, more

studies are required to elucidate natural mechanisms of protection in order to identify novel targets for preventative and therapeutic interventions.

4.2. MATERIALS AND METHODS

4.2.1. Study design and population

A nested case-control study was carried out to assess the association between low affinity *FCGR* variability and perinatal HIV-1 acquisition, combining data from past studies of three perinatal cohorts at two hospitals in Johannesburg, South Africa (127–130). The HIV-1-infected cohort (cases) consists of 546 children, enrolled in two clinical trials (NEVEREST 2 and 3) (127–129). The remaining two cohorts comprised of 566 HIV-1-exposed uninfected children (controls) (130). For this study, only available samples with sufficient material were genotyped. *FCGR* genotypic data from 395 HIV-1-infected children were compared with 312 of the HIV-1-exposed uninfected children. At the time of the studies, ART was not routinely administered to pregnant women living with HIV-1. All the children enrolled in the study were black and exposed to nevirapine for prevention of MTCT. The available demographic and perinatal variables included for both cases and controls are sex, birthweight, breastfeeding status and gestation (term or pre-term).

4.2.2. Genotyping

Functional *FCGR* variants were genotyped using the *FCGR*-specific multiplex ligation-dependent probe amplification assay (MRC Holland, Amsterdam, The Netherlands) according to manufacturer's instructions (80,126). In two reactions, the assay detects genomic copy number of *FCGR2C, FCGR3A* and *FCGR3B,* as well as functional allelic variants: FcγRIIa-H166R (alias H131R), FcγRIIb-I232T, FcγRIIIa-V176F (alias V158F) and FcγRIIIb-HNA1a/b/c. Furthermore, the assay detects *FCGR2C* expression variants – c.169T>C and c.798+1A>G, as well as the *FCGR2B/C* promoter variant at positions c.−386G>C and c.−120A>T. Refer to Chapter 2, sections 2.5.1 and 2.5.2. For the SNP nomenclature used, refer to Chapter 2, section 2.6.

4.2.3. Statistical and computational analysis

Categorical data were presented as absolute numbers and percentages. The Chi-squared and Fisher Exact tests (where appropriate) were used for comparisons between children with HIV-1-infection and children who were HIV-1-exposed uninfected. Association between functional *FCGR* variants and perinatal HIV-1 acquisition was determined by logistic regression analyses. Each *FCGR3B* genotype is defined as the combination of FcγRIIIb-HNA1a/b/c allotypes present or absent irrespective of gene copy number. Genotype reference groups for the di-allelic FcγRIIa-H166R, FcγRIIb-I232T, and FcγRIIIa-V176F variants were homozygosity for the major allele, while the genotype reference group for the multi-allelic FcγRIIIb-HNA1a/b/c were selected based on prevalence. A *P* value < 0.05 in the multivariate analysis was regarded as statistically significant and 95% confidence intervals (CI) were used to estimate precision. Bonferroni correction was used to adjust for multiple comparisons, and it considered six independent tests for the different variants - *FCGR3A* copy number, *FCGR3B* copy number, FcγRIIa-H166R, FcγRIIb-I232T, FcγRIIIa-V176F and FcγRIIIb-HNA1a/b/c. Both unadjusted and adjusted *P* values are reported. All analyses were performed in STATA version 15.1 (StataCorp LP, Texas, USA).

Linkage disequilibrium (LD), using the Haploview software package (133), was assessed LD for FcγRIIIb-HNA1a/b/c allotype using tag SNP p.N65S (amino acid change from asparagine to serine at position 65) that differentiates HNA1a (P.65N) from HNA1b|c (p.65S), and the SNP that differentiates HNA1b from HNA1c, resulting in aspartic acid replacing alanine at amino acid position 78 (p.A78D). LD is expressed as D prime (D′) and square of the correlation coefficient (r^2). For comparison of the *FCGR* genotypes, gene copies greater than two were regarded as homozygous when all the copies have the same allele or heterozygous when both alleles were present. We checked for Hardy-Weinberg equilibrium, considering only those with two gene copies.

4.3. RESULTS

4.3.1. Study population characteristics

There were no significant differences in sex and gestation between the 395 HIV-1 infected (cases) and 312 HIV-1-exposed uninfected (controls) included in this analysis. However, the proportion of HIV-infected children who had a low birth weight (<2500g) and were breastfed were higher than the controls (Table 4.1).

Table 4.1 Characteristics of perinatal HIV-1 acquisition in our study cohort

Characteristics	HIV-1-exposed uninfected n = 312	HIV-1 infected n = 395	*P* value
Sex			0. 661
Male	160 (51)	196 (49.6)	
Female	152 (49)	199 (50.4)	
Gestation	(n = 312)	(n = 389)	0.180
Term	282 (90)	339 (87)	
Preterm (<37 weeks)	30 (10)	50 (13)	
Birth weight (g)	(n = 312)	(n = 375)	**<0.001**
≥ 2500	282 (90)	295 (79)	
< 2500	30 (10)	80 (21)	
Breastfed	(n = 311)	(n = 388)	**<0.001**
No	289 (93)	297 (77)	
Yes	22 (7)	91 (23)	

Data are expressed as n (%).
Total numbers analyzed for each variable are indicated.
Bold indicates statistical significance of $P < 0.05$

4.3.2. Distribution of *FCGR* copy number variation and HIV-1 acquisition

Genes are deleted or duplicated at the *FCGR2/3* region within previously defined copy number variable regions (CNRs) (86,87,107). Figure 4.1 shows the SNPs genotyped (A) and the 4 distinct patterns of CNV in the present South African cohort: *FCGR2C/FCGR3B*, *FCGR2C/FCGR3A*, *FCGR2C/FCGR3A/FCGR3B* and *FCGR3A* only (Figure 4.1B). The most common variation was observed for the combined duplication/deletion of complete *FCGR2C* and *FCGR3B* (29.6%; 209/707), equivalent to CNR1 as described by Niederer et al. (86). Within CNR1, one or more copies were deleted in 61/209 individuals (29%) and duplicated in 148/209 individuals (71%). Thus, in the total group of 707 South African children, 8.6% carried a CNR1 deletion and 20.9% a CNR1 duplication. We observed low variation within CNR2, which encompasses the complete *FCGR3A* and exons 1 to 6 of *FCGR2C* (1.7%; 12/707; 4 deletions and 8 duplications). In seven (1%) individuals, CNV in *FCGR2C*, *FCGR3A* and *FCGR3B* was observed simultaneously, with one deletion and six duplications. Deletion or duplication of *FCGR3A* alone was noted in 16 individuals (2.3%), 11 with a gene deletion and 5 with a gene duplication.

Copy number variation in *FCGR2C* and *FCGR3B* were separately observed in 233/707 (33%) and 219/707 (31%) children, respectively, and did not associate with HIV-1 acquisition ($P > 0.05$; Table 4.2). Complete absence of *FCGR2C and FCGR3B* was observed in one HIV-1

infected child. *FCGR3A* showed low frequency in copy number variation in 33/707 (4.7%) individuals, with 16 (2.3%) carrying a single gene copy and 17 (2.4%) having three gene copies. No individual with complete absence of *FCGR3A* was observed. A significant difference in *FCGR3A* copy number distribution was observed between the HIV-1 infected and exposed-uninfected children. Using one *FCGR3A* copy as reference, gene duplication was independently associated with increased odds of HIV-1 acquisition (OR = 10.27; 95% CI 2.00-52.65; $P = 0.005$, $P_{Bonf} = 0.03$; Table 4.2).

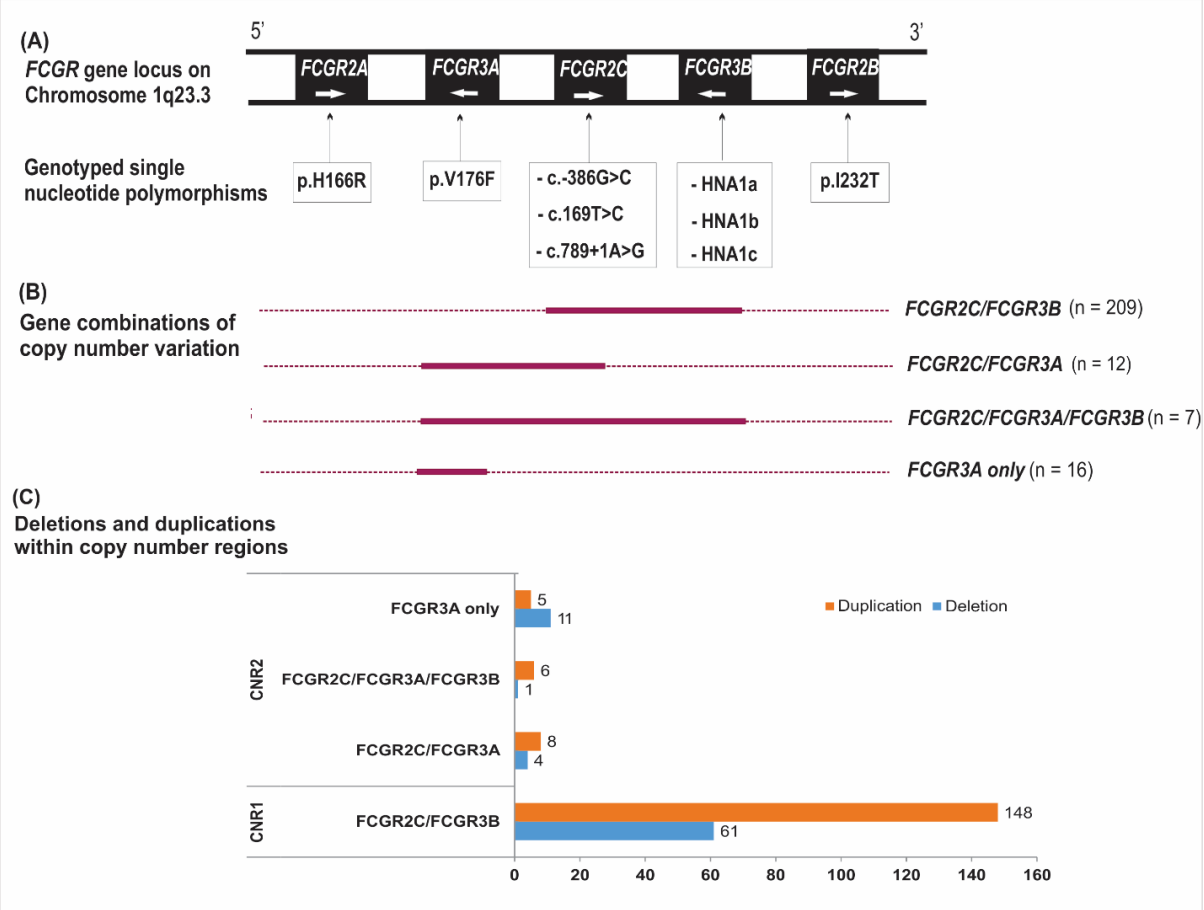

Figure 4.1 Diagrammatic representation of the FCGR2/3 loci structure and variation.
(A) The *FCGR2* and *FCGR3* on human chromosome 1q23 with their orientation and the functional SNPs genotyped in the study. Polymorphic amino acids are designated by one-letter code. **(B)** CNV has been previously described within distinct copy number variable regions (CNRs) (86,87). Four gene combinations of CNV, either duplication or deletion, were observed and are indicated as solid lines. The *FCGR2C/FCGR3B* and *FCGR2C/FCGR3A* combinations correspond to the previously designated CNR1 and CNR2, respectively. **(C)** Copy number region deletions and duplications within CNR1 and CNR2. This displays further breakdown of individuals with either a deletion or duplication within the 4 distinct gene combinations of CNV.

Table 4.2 Associations of *FCGR* variants with perinatal HIV-1 acquisition

Variants	HIV-1-exposed uninfected n = 312	HIV-1 infected n = 395	OR (95% CI)	*P* value
FCGR2C copy number				
≤ 1 copy	31 (10)	37 (9)	Ref	
2 copies	209 (67)	265 (67)	1.06 (0.64 - 1.77)	0.816
≥ 3 copies	72 (23)	93 (24)	1.08 (0.61 - 1.91)	0.785
FCGR3A copy number				
1 copy	11 (3.5)	5 (1.3)	Ref	
2 copies	298 (95.5)	378 (95.2)	2.78 (0.95 - 8.08)	0.061
3 copies	3 (1.0)	14 (3.5)	10.27 (2.00 - 52.65)	**0.005 (P_{Bonf} = 0.03)**
FCGR3B copy number				
≤ 1 copy	28 (9)	35 (9)	Ref	
2 copies	217 (70)	271 (69)	0.99 (0.59 - 1.69)	0.997
≥ 3 copies	67 (21)	89 (22)	1.06 (0.59 - 1.92)	0.840
FCGR2A genotype				
166HH	65 (20.8)	86 (21.8)	Ref	
166HR	154 (49.4)	187 (47.3)	0.92 (0.62 - 1.35)	0.663
166RR	93 (29.8)	122 (30.9)	0.99 (0.65 - 1.51)	0.968
Allele carriage				
≥ 1 166H allele	219 (70)	273 (70)	0.95 (0.69 - 1.31)	0.757
≥ 1 166R allele	247 (79)	309 (78)	0.95 (0.66 - 1.36)	0.762
FCGR2B genotype				
232II	147 (47)	165 (41.8)	Ref	
232IT	126 (40)	160 (40.5)	1.13 (0.82 - 1.56)	0.453
232TT	39 (13)	70 (17.7)	1.60 (1.02 - 2.51)	**0.041** (P_{Bonf} = 0.246**)**
Allele carriage				
≥ 1 232I allele	273 (88)	325 (82)	0.66 (0.43 - 1.01)	0.057
≥ 1 232T allele	165 (53)	230 (58)	1.24 (0.92 - 1.67)	0.156
FCGR3A genotype				
176FF	134 (43)	155 (39)	Ref	
176FV	135 (43)	176 (45)	1.13 (0.82 - 1.56)	0.467
176VV	43 (14)	64 (16)	1.29 (0.82 - 2.02)	0.273
Allele carriage				
≥ 1 176F allele	269 (86)	331 (84)	0.83 (0.54 - 1.26)	0.373
≥ 1 176V allele	178 (57)	240 (61)	1.17 (0.86 - 1.58)	0.319
FCGR3B genotype				
HNA1a+/1b+/1c-	101 (32)	116 (29.37)	Ref	
HNA1a+/1b+/1c+	13 (4)	16 (4.05)	1.07 (0.49 - 2.34)	0.862
HNA1a+/1b−/1c+	44 (14)	61 (15.44)	1.21 (0.75 - 1.93)	0.433
HNA1a+/1b-/1c-	60 (19)	98 (24.81)	1.42 (0.94 - 2.16)	0.098
HNA1a-/1b+/1c+	43 (14)	50 (12.66)	1.01 (0.62 - 1.65)	0.960
HNA1a-/1b+/1c-	28 (9)	38 (9.62)	1.18 (0.68 - 2.06)	0.556
HNA1a-/1b-c+	23 (7)	15 (3.80)	0.57 (0.28 - 1.15)	0.115
HNA1a-/1b-/1c-	0 (0)	1 (0.25)	-	-
Allele carriage				
≥1 HNA1a allotype	218 (70)	290 (73)	1.19 (0.86 - 1.66)	0.298

≥1 HNA1b allotype	185 (59)	221 (56)	0.87 (0.65 - 1.18)	0.372
≥1 HNA1c allotype	123 (39)	141 (36)	0.85 (0.63 - 1.16)	0.309

Data are expressed as n (%)
OR, Odds Ratio; CI, Confidence Interval; P_{Bonf}, Bonferroni corrected *P* value; Ref, Reference group
Bold indicates statistical significance of $P < 0.05$

4.3.3. *FCGR2A* and *FCGR3A* genotypes did not associate with perinatal HIV-1 acquisition

For the FcγRIIa-H166R genotype, 341 (48.2%) children were heterozygous (FcγRIIa-166HR), 151 (21.4%) were homozygous for the higher affinity IgG binding allele (FcγRIIa-166HH) and 215 (30.4%) homozygous for FcγRIIa-166RR. The genotype distributions of the *FCGR3A* were 311 (44%) for FcγRIIIa-176FV heterozygotes, 289 (41%) for FcγRIIIa-176FF, and 107 (15%) for FcγRIIIa-176VV. The FcγRIIa and FcγRIIIa genotype distributions observed in this study are similar to previous findings (71,108). FcγRIIa-H166R and FcγRIIIa-V176F genotype and allele carriage frequencies did not differ significantly between the HIV-1 infected and uninfected cohort (Table 4.2). Neither genotypes significantly associated with HIV-1 acquisition in the univariate or multivariate analyses ($P > 0.05$ for all comparisons).

4.3.4. Associations between *FCGR2B* and *FCGR3B* genotypes and perinatal HIV-1 acquisition

The FcγRIIb-232II was the most prevalent *FCGR2B* genotype (44.1%; n = 312), followed by 232IT (40.5%; n = 286) and 232TT (15.4%; n = 109). The FcγRIIb-232TT genotype was overrepresented in the HIV-1-infected children compared to the exposed-uninfected children (17.7% vs. 13%; Table 4.2). Compared to the FcγRIIb-232II genotype, the FcγRIIb-232TT genotype significantly associated with increased odds of HIV-1 acquisition in univariate analysis (OR = 1.60; 95% CI 1.02-2.51; $P = 0.041$, $P_{Bonf} > 0.05$). At the *FCGR3B* locus, HNA1a was the dominant allotype (72%; n = 508), followed by HNA1b (57%; n = 406) and HNA1c (37%; n = 264). We observed an overrepresentation of homozygous FcγRIIIb-HNA1a allotype in HIV-1-infected children compared to the exposed-uninfected (24.81% vs. 19%) but it did not independently associate with HIV-1 acquisition (OR = 1.42; 95% CI 0.94-2.16; $P = 0.098$, $P_{Bonf} > 0.05$; Table 4.2).

The association with homozygous FcγRIIIb-HNA1a attained significance after further assessment in a multivariate model that controlled for *FCGR3A* copy number and *FCGR2B*

genotype (AOR = 1.55; 95% CI 1.01-2.38; P = 0.044, P_{Bonf} > 0.05). Both *FCGR3A* copy number (AOR = 10.68; 95% CI 2.04-55.86; P = 0.005, P_{Bonf} = 0.03) and *FCGR2B* genotype (AOR = 1.72; 95% CI 1.07-2.76; P = 0.024, P_{Bonf} > 0.05) remained significant (Table 4.3). The strength of association for *FCGR2B* genotype increased (AOR = 2.28; 95% CI 1.11-4.69; P = 0.024, P_{Bonf} > 0.05) when adjusted for *FCGR2C* c.134-96C>T that associated with HIV-1 acquisition in our previous study (149) . We further explored the associations in a subset of the study cohort that excluded breastfed infants (91 HIV-1 infected and 22 HIV-1 exposed-uninfected; nested total n = 586) and controlled for birthweight. The FcγRIIb-232TT genotype (AOR = 1.83; 95% CI 1.13-2.97; P = 0.014, P_{Bonf} > 0.05), homozygous FcγRIIIb-HNA1a allotype (AOR = 1.66; 95% CI 1.07-2.57; P = 0.025, P_{Bonf} > 0.05) and *FCGR3A* copy number (AOR = 8.58; 95% CI 1.60-45.92; P = 0.012, P_{Bonf} > 0.05) retained significance (Table 4.4).

Table 4.3 Multivariate analysis of the effect of FCGR3A copy number, FCGR2B and FCGR3B variants on perinatal HIV-1 acquisition

Variants	Adjusted OR (95% CI)*	*P* value
FCGR3A copy number		
1 copy	Ref	
2 copies	2.81 (0.94 - 8.36)	0.064
3 copies	10.68 (2.04 – 55.86)	**0.005 (P_{Bonf} = 0.03)**
FCGR2B genotype		
232II	Ref	
232IT	1.20 (0.86 - 1.67)	0.295
232TT	1.72 (1.07 - 2.76)	**0.024** (P_{Bonf} = 0.144**)**
FCGR3B genotype		
HNA1a+/1b+/1c-	ref	
HNA1a+/1b+/1c+	1.18 (0.54 - 2.60)	0.674
HNA1a+/1b−/1c+	1.27 (0.78 - 2.05)	0.333
HNA1a+/1b-/1c-	1.55 (1.01 - 2.38)	**0.044** (P_{Bonf} = 0.264**)**
HNA1a-/1b+/1c+	1.02 (0.63 - 1.68)	0.917
HNA1a-/1b+/1c-	1.10 (0.63 - 1.95)	0.734
HNA1a-/1b-/1c+	0.64 (0.31 - 1.32)	0.228

OR, Odds Ratio; CI, Confidence Interval; P_{Bonf}, Bonferroni corrected P value
Bold indicates statistical significance of $P < 0.05$
* Multivariate model controlled for *FCGR3A* copy number, *FCGR2B* and *FCGR3B* variants

Table 4.4 Associations of *FCGR* variants with perinatal HIV-1 acquisition in non-breastfed children after adjusting for birthweight

Variants	HIV-1-exposed uninfected n = 289	HIV-1 infected n = 297	Univariate		Multivariate*	
			OR (95% CI)	***P* value**	**Adjusted OR (95% CI)**	***P* value**
FCGR3A copy number						
1 copy	**11 (3.5)**	**5 (1.3)**	**Ref**		**Ref**	
2 copies	**298 (95.5)**	**378 (95.2)**	**2.78 (0.95 - 8.08)**	**0.061**	**2.49 (0.82 - 7.54)**	**0.107**
3 copies	**3 (1.0)**	**14 (3.5)**	**10.27 (2.00 - 52.65)**	0.005 (P_{Bonf} = 0.03)	**8.58 (1.60 – 45.92)**	0.012 (**P_{Bonf} = 0.072**)
FCGR2B genotype						
232II	140 (48.4)	123 (41.4)	Ref		Ref	
232IT	113 (39.1)	122 (41.1)	1.13 (0.82 - 1.56)	0.453	1.27 (0.90 - 1.80)	0.171
232TT	36 (12.5)	52 (17.5)	1.60 (1.02 - 2.51)	0.041 (P_{Bonf} = 0.246)	1.83 (1.13 – 2.97)	0.014 (P_{Bonf} = 0.084)
FCGR3B genotype						
HNA1a+/1b+/1c-	94 (32.53)	90 (30.30)	Ref		Ref	
HNA1a+/1b+/1c+	11 (3.81)	12 (4.38)	1.07 (0.49 - 2.34)	0.862	1.27 (0.56 – 2.84)	0.568
HNA1a+/1b−/1c+	42 (14.53)	40 (13.47)	1.21 (0.75 - 1.93)	0.433	1.32 (0.80 – 2.15)	0.275
HNA1a+/1b-/1c-	57 (19.72)	76 (25.59)	1.42 (0.94 - 2.16)	0.098	1.66 (1.07 - 2.57)	0.025 (P_{Bonf} = 0.15)
HNA1a-/1b+/1c+	37 (12.8)	41 (13.8)	1.01 (0.62 - 1.65)	0.960	1.12 (0.59 - 1.63)	0.937
HNA1a-/1b+/1c-	26 (9)	26 (8.75)	1.18 (0.68 - 2.06)	0.556	0.95 (0.63 – 2.02)	0.676
HNA1a-/1b-/1c+	22 (7)	10 (3.37)	0.57 (0.28 - 1.15)	0.115	0.55 (0.30 - 1.32)	0.217
HNA1a-/1b-/1c-	0 (0)	1 (0.34)	-	-		

OR, Odds Ratio; CI, Confidence Interval; P_{Bonf}, Bonferroni corrected *P* value
Bold indicates statistical significance of $P < 0.05$
* The multivariate analysis adjusted for all 3 genetic parameters (*FCGR3A* copy number, *FCGR2B* and *FCGR3B* genotypes) simultaneously plus birthweight

4.3.5. Linkage disequilibrium of functionally relevant variants in the *FCGR2/3* region

The functionally-relevant variants in the *FCGR2/3* region have been reported to be in strong linkage disequilibrium due to physical proximity of the genes on chromosome 1q23 (97,140,150). The observed genotype frequencies for FcγRIIa-H1166R, FcγRIIIa-V176F and FcγRIIIb-HNA1a/b/c were in Hardy-Weinberg equilibrium ($P > 0.05$) but those for FcγRIIb-I232T were not ($P = 0.018$). We assessed linkage disequilibrium between *FCGR2A, FCGR2B, FCGR3A and FCGR3B* variants to determine whether the observed associations with independent *FCGR* variants are linked due to coinheritance of alleles at different loci. All participants were included irrespective of copy number; those with 3 or more copies were considered heterozygous if both alleles were present and homozygous if all copies carried the same allele. We found the FcγRIIIb-HNA1a/b/c haplotype in complete LD as expected ($D' = 1.0$; $r^2 = 0.243$). The FcγRIIb-I232T was in weak LD with FcγRIIIb-HNA1a/b ($D' = 0.254$; $r^2 = 0.032$) and FcγRIIIa-V176F ($D' = 0.486$; $r^2 = 0.077$). Similarly, weak LD was observed between FcγRIIa-H166R and FcγRIIIa-V176F ($D' = 0.280$; $r^2 = 0.052$), and the FcγRIIIb-HNA1c allotype ($D' = 0.297$; $r^2 = 0.02$). When only those with two gene copies were included, the observed LD pattern remained the same (Figure 4.2). Multivariate analysis was used to test allelic association for each variant that had some LD. The observed association remained significant for FcγRIIb-I232T (AOR = 1.69; 95% CI 1.06-2.70; $P = 0.028$, $P_{Bonf} > 0.05$) while FcγRIIIb-HNA1a did not (AOR = 1.50; 95% CI 0.98-2.30; $P = 0.060$) and remained not significant for FcγRIIa-H166R and FcγRIIIa-V176F.

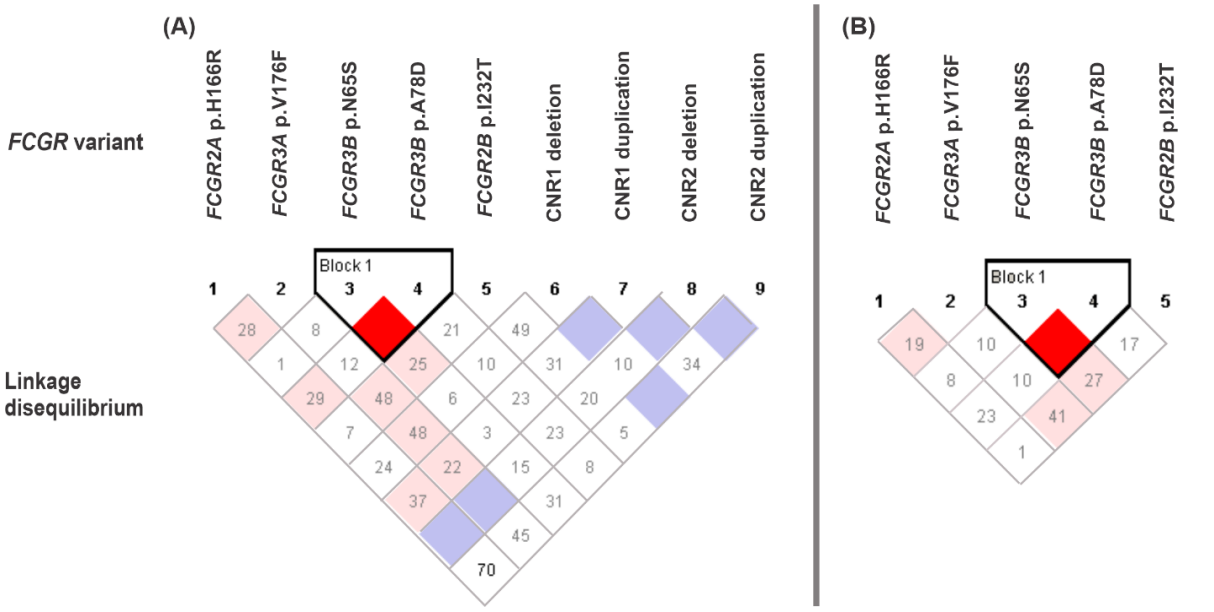

Figure 4.2 Linkage disequilibrium of functional *FCGR* variants in South African children born to women living with HIV-1.

(A) All individuals, with or without CNV (n = 707); **(B)** only individuals with two gene copies (n = 474). The black triangle illustrates a haplotype block. Values reflect D′ measures of LD and colour in the squares given by standard D′ divided by log of the odds of LD between two loci (LOD). Bright red colour indicates very strong LD.

4.4. DISCUSSION

The human *FCGR2/3* region comprise activating and inhibitory receptors that are highly polymorphic (including SNPs and CNV), with functional implications. Whilst the role of some of the genetic variants are not well understood, the functional and clinical relevance of others in the pathogenesis of autoimmune and infectious diseases has been well documented (20,80,81,85,98,140). Furthermore, functional *FCGR* variants have been investigated in the context of HIV-1 acquisition (71,108,109,149), disease progression (78,110,111,115) and response to vaccination regimens (82,83,115) with inconsistent findings. In this study, we report further associations between the functional *FCGR* variations and HIV-1 acquisition in black South African children born to women living with HIV. The analysis excluded an association with *FCGR2C* variants, which was separately assessed by the authors in another study (149). In that study, the *FCGR2C* c.134-96C>T tag variant produced a deleterious association in perinatal HIV-1 acquisition in contrast to the observed protective effect in the Thai RV144 vaccine trial (82).

The potential role of FcγR variants in modulating perinatal HIV-1 transmission and acquisition in South Africa was initially investigated by Lassaunière *et al.* (71), albeit in a small sample cohort. This present study used a larger cohort to validate the previously observed findings and determine if new FcγR variants associated with perinatal HIV-1 acquisition that may have been confounded by the earlier smaller sample size. The present study adds to a limited number of studies investigating the association between *FCGR* variants and HIV-1 acquisition in the maternal-infant HIV-1 transmission model. Whereas previous studies in Kenyan (108,109) and South African (71) cohorts used data from mother-infant pairs, only infant data was available for our study cohort.

We did not observe an association between the common *FCGR2A* and *FCGR3A* polymorphisms and HIV-1 acquisition. This is in line with findings from independent Kenyan (108) and South African (71) cohorts, as well as a large genome-wide association study of adults (112), but contrasts the increased acquisition risk associated with FcγRIIa-166HH genotype reported in another Kenyan cohort (109). Cohort differences, study design and statistical rigor employed have been suggested as possible reasons for the observed variable results (108,112).

Other FcγR variants beyond FcγRIIa-H166R and FcγRIIIa-V176F, which include FcγRIIb-I232T, FcγRIIIb-HNA1a/b/c, and gene copy number are rarely studied in the context of HIV-1, in particular MTCT. Our earlier study (71) included FcγRIIb-I232T in their study, and found that possession of at least one 232I allele was protective against in utero infection. Since this current study comprises a completely different cohort and are presumed to be predominantly in utero infected infants (since all received nevirapine at birth that reduces intrapartum transmission), our results on this larger cohort confirm those reported previously. Here we show that homozygosity for the FcγRIIb-232T minor allele associated with increased odds of perinatal acquisition of HIV-1. These findings suggest that the FcγRIIb-232TT genotype exerts a controlling influence on infant susceptibility to HIV-1 infection. FcγRIIb transmits signals via an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail. The FcγRIIb-232T polymorphism affects the receptor's ability to translocate to lipid rafts, disrupting the inhibitory function of FcγRIIb, leading to a potentially higher activation state of cells (95,96). The *FCGR2B/C* promoter variants at position c.−386G>C and c.−120A>T also influence FcγRIIb expression but such an effect would not play a role in our cohort because African individuals do not possess the promoter variant in *FCGR2B* (97).

The FcγRIIb-232T and FcγRIIIb-HNA1a alleles are subject to ethnic variation, both being more prevalent in black compared to white South Africans [FcγRIIb-232T (30.9% vs. 10.9%; FcγRIIIb-HNA1a (50.6% vs. 36.2%)] (97). The observed genotype frequencies for FcγRIIb-I232T were not in Hardy-Weinberg equilibrium possibly because of selection pressure from potent endemic infections in Africa, such as malaria (98). Significant selection pressure is a likely driver of retention of the FcγRIIb-232T allele that produced a deleterious effect on susceptibility of HIV-1 infection in South African children.

Gene CNV not only contributes to differences in expression levels but also alters the cellular distribution of FcγRs in response to activation by IgG complexes (88). Variation in copy number of *FCGR3A* has been shown to correlate with FcγRIIIa surface expression and function of NK cells (84). In the present study, duplication or deletion of *FCGR3A* occurred either alone, in combination with *FCGR2C* or simultaneously with both *FCGR2C* and *FCGR3B* and significantly associated with HIV-1 acquisition. Specifically, we observed a trend towards an association of *FCGR3A* duplications but due to the low frequency of *FCGR3A* duplication, further studies on larger sample size are needed. We also observed 8.6% of the South African children carried a CNR1 deletion, which leads to the formation of *FCGR2C/2B* chimeric genes. This results in unusual expression of inhibitory FcγRIIb on NK cells and subsequently, reduced ADCC activity (89). This genotype did not associate with HIV-1 acquisition.

The FcγRIIIb is a glycosylphosphatidylinositol (GPI)-anchored protein, expressed largely on neutrophils (146). Neutrophils from homozygous HNA1a individuals display higher affinity for IgG1 and IgG3 and greater phagocytic capacity than homozygous HNA1b individuals (105). We observed an association between FcγRIIIb-HNA1a/b/c allotype and perinatally acquired HIV-1 infection. Specifically, homozygosity for the FcγRIIIb-HNA1a allotype produced a deleterious effect on perinatal HIV-1 acquisition. This is contrary to the protective effect observed in the earlier study with a smaller South African cohort, primarily in the intrapartum infected children (71). The different observations between the two studies may be attributable to different cohort compositions. The present study cohort was exposed to nevirapine for prevention of MTCT and more likely infected in-utero, with few intrapartum infections. When the breast-fed children were excluded from the analysis, the observed significant association with FcγRIIb-232T, FcγRIIIb-HNA1a and *FCGR3A* copy number variants remained. These variants likely play a role in HIV-1 acquisition during the course of pregnancy and at the maternal-foetal interface (71).

The study has several strengths. The genotyping method utilized is robust, as the MLPA assay is able to assess functional SNPs and CNV within the *FCGR2/3* locus simultaneously, rather than investigating associations with perinatal HIV-1 infection using methodologies that use candidate gene designs. Due to high homology of *FCGR2/3* we checked for linkage disequilibrium to identify functional interaction between the independently associated polymorphisms. A limitation of the study is that maternal data were not available. In particular, we could not adjust for maternal viral load, a key determinant of MTCT of HIV-1 infection. Furthermore, we could not assess the the effect of maternal *FCGR* genotypes on transmission. Our earlier study (71) revealed a protective role for the maternal FcγRIIIa-176V allele in *in utero* HIV-1 transmission that could not be re-studied due to lack of maternal samples

The contribution of FcγRIIb to disease susceptibility has largely been studied in systemic lupus erythematosus patients (95,98) but there is paucity of data on association with HIV-1 acquisition. The findings of this study contribute to better understanding of the role of FcγRs in HIV-1 infection in children and add to the growing evidence of a potential role for Fc-mediated effector functions in modulating perinatal HIV-1 acquisition. As more FcγR variants associated with HIV-1 acquisition are reported, more studies are needed to critically evaluate their clinical relevance in the development of preventive or therapeutic interventions.

CHAPTER 5 : A ROLE FOR *FCGR3A* COPY NUMBER VARIATION IN MODULATING MORTALITY IN HIV-1 INFECTED CHILDREN ON ANTIRETROVIRAL THERAPY

5.1. INTRODUCTION

Paediatric human immunodeficiency virus type 1 (HIV-1) infection remains a significant public health problem in sub-Saharan Africa. This region has more than 90% of all children living with HIV-1 globally and the HIV-1 sero-prevalence among pregnant women remains high (33,34). Mother-to-child transmission (MTCT), either in utero, intrapartum or postpartum, accounts for most paediatric HIV-1 infections (35,36) with maternal viral load being a key predictor of transmission risk (151,152). The variability in HIV-1 infection outcome and disease progression in adults and children is influenced by multiple interacting factors, including virulence of the infecting virus and the host immune response to the virus (153). Preventing new paediatric HIV-1 infections and maintaining post-infection control of viraemia are critical for reaching the goal of eliminating MTCT (33). However, in spite of substantial progress through antiretroviral therapy (ART) interventions, some children get infected and are at great risk of mortality (154). Thus, further studies are required to elucidate mechanisms of protection and identify novel targets for therapeutic interventions.

The foetus passively acquires immunoglobulin G (IgG) through transplacental transfer from the HIV-1 infected mother that may affect viraemia or disease progression early in life, while the development of autologous antibody responses later in life may also play a role. Previous studies have produced conflicting results on the role of maternal nAbs in the risk of MTCT of HIV-1. While some have shown that maternal nAbs do not predict protection from HIV-1 acquisition (59,60), others have suggested otherwise (61–63). These different outcomes may be due to differences in study design and analysis, HIV antibody measures, population and environment, virus clades, maternal ART use, modes of transmission and maternal plasma viral load and CD4+ T cell count that impact MTCT (64,65). Beyond virus neutralisation, the crystallisable fragment (Fc) region of IgG antibodies interacts with Fc gamma receptors (FcγRs) to mediate antiviral effector functions, including antibody-dependent cellular cytotoxicity (ADCC) (18,73). Fc-mediated effector functions have been suggested to influence HIV-1 acquisition and post-infection control of viremia (12,15,17–19,109,110,147,148).

Specifically, increased passively acquired ADCC activity in HIV-1-infected infants has been shown to associate with reduced mortality (19) and reduced HIV-1 transmission risk in women with high viral loads (15). Several studies have highlighted the role of ADCC in HIV-1 post-infection control of viraemia in both adults and children[reviewed in (18)],(15,19,68,69).

A number of genetic variations exist within low affinity FcγRs, with functional implications (155). These include gene copy number variation (CNV) for *FCGR2C, FCGR3A* and *FCGR3B,* which affects expression density on effector cell surface and create chimeric *FCGRs* (84) and single nucleotide polymorphisms (SNPs) that alter binding affinity to IgG subclasses (FcγRIIa-H166R and FcγRIIIa-F176V) (93,101), the receptor's ability to translocate to lipid rafts that affect cell signalling (FcγRIIb-I232T) (95,96), *N*-linked glycosylation sites (FcγRIIIb-HNA1a/b/c) (104), and the surface expression of receptors (FcγRIIc-c.169T>C and c.798+1A>G) (89,100).

The study of FcγR genetic variants have been used as proxy for assessing Fc-mediated effector functions in modulating HIV-1 infection risk, disease progression and vaccine efficacy (71,78,82,83,109,110,149). In adults, Forthal *et al*. demonstrated an association between the low affinity binding FcγRIIa-166RR and a faster rate of CD4+ T cell decline and progression to AIDS in Caucasian men who have sex with men (110). This finding was not replicated in separate cohorts of Kenyan women (111) and adult African-Americans (113). Poonia *et al*. reported high-affinity FcγRIIIa-176VV as risk factor for HIV-1 disease progression in adult African Americans (113) that was not predicted in other studies (110,111,114). Meta-analysis of large data from the International Collaboration for the Genomics of HIV (ICGH) confirmed the non-association of allelic variants of *FCGR2A* and *FCGR3A* with control of HIV-1 infection in a large adult sexual transmission cohort of European descent [reviewed in (112)]. In contrast, the *FCGR2C* c.134-96C>T variant associated with increased odds of HIV-1 disease progression in a black South African adult cohort (78). There is currently a paucity of data on the modulation of FcγRIIb-I232T, FcγRIIIb-HNA1a/b/c and gene copy number variation in HIV-1 immunity, especially in the context of MTCT.

In addition to reducing HIV RNA viral load through killing of infected cells via ADCC, this Fc-mediated effector function, and potentially others, may contribute to limiting the HIV-1 DNA reservoir. Despite ART, latent HIV-1-infected CD4+ T cells (HIV reservoirs) facilitate viral persistence that impacts negatively on achieving HIV-1 cure or remission (118).

Collectively, studies have provided evidence that early diagnosis and prompt ART initiation reduces the size of HIV-1 reservoir and predicts post-treatment virological control in adults (119–122) and children (52,53). The influence of *FCGR* genetic variation on HIV-1 reservoir size and post-ART control has not been studied definitely. Descours et al identified FcγRIIa as a marker of HIV-1 latent reservoir (123). However, subsequent independent studies were unable to replicate this finding (112,124,125). This study was undertaken to investigate the role of FcγR variants in modulating outcomes of mortality, virologic control and latent reservoir size in the context of ART administered in early life of HIV-1 infected children.

5.2. MATERIALS AND METHODS

5.2.1. Study design and population

The HIV-1 infected children included in the study reported here were recruited as part of two sequential randomized clinical trials (NEVEREST 2 and 3) conducted in Johannesburg, South Africa (127,128,156). The children all received nevirapine for prevention of MTCT and were more likely infected in utero, with fewer intrapartum and breastfeeding infections, although the timing was not directly determined (130,138,139). Maternal ART was not routinely used at the time. In the NEVEREST 2 study, ART-naive children less than 2 years of age were recruited, started on a started on a lopinavir-ritonavir-based regimen (LPV/r), followed up for a minimum of 12 months and observed for viral suppression. They were then randomized to either stay on the initial LPV/r-based regimen or switched to a nevirapine-based regimen if they had achieved viral suppression to less than 400 HIV-1 RNA copies/millilitre (mL) (127,128). Those recruited into the NEVEREST 3 study were between three and five years of age, already on treatment and virally suppressed (less than 50 copies/mL) during LPV/r-based therapy (129). For this study, only available and sufficient stored blood samples were genotyped. A total of 395 *FCGR* genotypic data from NEVEREST 2 (n = 267) and NEVEREST 3 (n = 128) were analysed.

Available data from the initial clinical trials were used to describe the characteristics of the study population. Information on mortality and early response to ART were available only for NEVEREST 2 study data. HIV-1 DNA levels (a marker of viral reservoir) for a 104 children were available from a previous study that quantified cell-associated HIV-1 DNA in a subset of currently older NEVEREST 3 cohort (52). Maternal blood samples and clinical information were not available for the NEVEREST cohorts. Ethics approval for the study was obtained

from the University of the Witwatersrand Human Research Ethics Committee (Reference numbers: M180575).

5.2.2. Genotyping

The *FCGR*-specific multiplex ligation-dependent probe amplification (MLPA) assay (MRC Holland, Amsterdam, The Netherlands) was used to genotype the functionally relevant *FCGR* variants, according to manufacturer's instructions (80,126). Refer to Chapter 2, sections 2.5.1 and 2.5.2. Conventional PCR and Sanger nucleotide sequencing were used to genotype the *FCGR2C* c.134-96C>T (rs114945036) variant. Refer to Chapter 2, sections 2.5.3. For the SNP nomenclature used, refer to Chapter 2, section 2.6.

5.2.3. Statistical analysis

Categorical data were summarized as proportions while median and inter-quartile range (IQR) were used to describe continuous data. The outcome measures were mortality, viral suppression and size of viral reservoir. The outcomes were compared across groups using Pearson's chi-square or Fisher's exact tests for categorical variables and non-parametric Wilcoxon rank sum or Kruskal–Wallis tests for continuous variables. The non-parametric Spearman rank order correlation test was used to measure the degree of association between two continuous variables. Viral load (VL) were $\log_{10}$ transformed for all analyses to reduce the skewness of our original data. Logistic regression analyses were conducted to determine the association between functional *FCGR* variants, infant characteristics and the study outcomes. The distribution of HIV-1 DNA level was displayed using histogram and kernel density estimates. Differences in HIV-1 DNA levels by *FCGR* genotypes were illustrated using box and whiskers plot. Genotype reference groups were selected based on prevalence. A P value < 0.05 was considered statistically significant and analyses were performed in STATA version 15.1 (StataCorp LP, Texas, USA).

5.3. RESULTS

5.3.1. Study population characteristics

Of 395 HIV-1 infected, treated children included in this study, 267 (68%) were ART-naive at enrolment before two years of age and 128 (32%) were recruited three years and older, already on treatment and virally suppressed. Overall, 50.4% (199/395) were female, 13% (50/389) were born premature (less than 37 weeks of pregnancy), 21% (80/375) had low birthweight (2500 grams or less at birth) and 23% (91/388) were ever breastfed (Table 5.1). The median weight-for-age Z score, an indicator of nutritional status of children, was -2.22 (IQR: -3.43 to -1.3; a Z score of less than -2 defines undernourishment (157)). Pre-ART HIV-1 plasma VL was recorded for 340 children and pre-ART CD4+ T-cell percentage for 373 children. The median pre-treatment VL and CD4+ T-cell percentage were ≥ 750,000 RNA copies/mL, which was the upper threshold of the assay in use at the time (IQR: 270,000-≥ 750,000) and 19.8% (IQR: 13.6-27.3; moderate suppression is indicated by a CD4+ percentage of 15-24 (158)), respectively. The graphical distribution of pre-ART VL and CD4+ T cell percentage is shown in Appendix A.3. The age at ART initiation ranged from 0.79 to 31.3 months, with a median of 6.9 months (IQR: 4.2-13.1). The older the child at pre-ART VL measurement, the lower the VL, suggesting declining VL with time after birth even before ART initiation, as well as possible selection bias with healthier children only requiring treatment at older ages (rho -0.1300, $P = 0.017$).

5.3.2. Outcomes for HIV-1 infected children in our study cohort after one year of treatment

The infant characteristics of the original study cohorts that associated with mortality and viral suppression have been well described (127,128). In the study, children with pre-treatment lower weight-for-age Z scores and higher HIV-1 viral load at ART initiation were more likely to die and less likely to achieve viral suppression (virologic failure). In the cohort included in the present study, genotypic data were available for 267 children who were ART-naive at enrollment. Of these, 34 (13%) died, 18 (7%) were lost to follow-up and virologic outcome of suppression or failure was recorded for 215 (80%). Of the 215 children with recorded virologic outcome, 172 (80%) became virally suppressed (less than 400 HIV-1 RNA copies/mL) and 43 (20%) did not achieve viral suppression. All those who did not remain in follow-up were excluded from all analyses.

Table 5.1 Characteristics of the HIV-1 infected children in our study cohort at initiation of antiretroviral therapy (ART)

Characteristics	Total HIV-1 infected children (n = 395)	NEVEREST 2 Enrolled ART-naïve (n = 267)	NEVEREST 3 Enrolled virally suppressed (n = 128)	*P* value
Sex				0.161
Male	196 (49.6)	139 (52.1)	57 (44.5)	
Female	199 (50.4)	128 (47.9)	71 (55.5)	
Gestation	(n = 389)	(n = 266)	(n = 123)	0.091
Term	339 (87)	237 (89)	102 (83)	
Preterm (<37 weeks)	50 (13)	29 (11)	21 (17)	
Birth weight (g)	(n = 375)	(n = 255)	(n = 120)	**<0.001**
≥ 2500	295 (79)	214 (84)	81 (67.5)	
< 2500	80 (21)	41 (16)	39 (32.5)	
Breastfed	(n = 388)	(n = 263)	(n = 125)	**0.004**
No	297 (77)	190 (72)	107 (86)	
Yes	91 (23)	73 (28)	18 (14)	
Age at treatment start (months)				**<0.001**
< 12	275 (70)	169 (63)	106 (83)	
≥ 12	120 (30)	98 (37)	22 (17)	
Median (IQR)	6.9 (4.2-13.1)	8.8 (5.0-14.2)	4.8 (3.2-8.4)	
Weight-for-age-Z score	(n = 309)	(n = 238)	(n = 71)	0.768
≤ -4	74 (24)	59 (25)	15 (21)	
>-4 to -2	139 (45)	107 (45)	32 (45)	
> -2	96 (31)	72 (30)	24 (34)	
Median (IQR)	-2.22 (-3.43 to -1.3)	-2.25 (-3.5 to -1.33)	-1.85 (-3.1 to -1.08)	
Pre-ART HIV-1 plasma viral load (copies/ml)	(n = 340) .	(n = 230)	(n = 110)	0.130
< 100,000	34 (10)	20 (9)	14 (13)	
100,000-749,999	110 (32)	69 (30)	41 (37)	
≥ 750,000	196 (58)	141 (61)	55 (50)	
Median (IQR)	750, 000 (270,000-750,000)	750, 000 (300,000-750,000)	730, 000 (200,000-1,100,000)	
Pre-ART CD4+ T-cell percentage	(n = 373)	(n = 250)	(n = 123)	**<0.001**
< 15	109 (29)	86 (34)	23 (19)	
15 - 24	141 (38)	104 (42)	37 (30)	
≥ 25	123 (33)	60 (24)	63 (51)	
Median (IQR)	19.8 (13.6 – 27.3)	17.8 (12.8 – 24.1)	24.7 (17.1 – 30.8)	
Died	34 (13)	34 (13)	-	
Lost to follow up	18 (7)	18 (7)	-	
Virologic outcome	(n = 215)	(n = 215)	-	
Suppressed	172 (80)	172 (80)		
Failure to suppress	43 (20)	43 (20)		

Data are expressed as n (%) unless otherwise specified
Bold indicates statistical significance of $P < 0.05$
'-' Data not available

For mortality analysis, the 34 children who died were compared to the 215 children who survived. The age at ART initiation was significantly lower for children who died compared to those who survived ($P = 0.047$). Those who died were started on ART at a median age of 6.7 months (IQR: 4.9-10) compared to 9.7 months (IQR: 9.2-15) for those who survived. We hypothesize this is likely due to selection bias with healthier children surviving to older ages before becoming symptomatic and needing ART. Guidelines in place at the time this study was done only started ART on children once clinical criteria and immunosuppression criteria were reached. In addition, coverage with early nucleic acid amplification tests was low at that time and children tended to only come to clinical attention once symptomatic.

No significant difference in pre-ART CD4+ T-cell percentage was observed between the two groups ($P > 0.05$) but two groups differed significantly in pre-ART plasma viral load ($P = 0.018$). Of those who died, most had pre-ART CD4+ T-cell percentages ≥ 15% (55%; 18/33) and viral load ≥ 750,000 copies/mL (86%; 25/29). In our study cohort, age at start of ART, pre-ART viral load and weight-for-age-Z score predicted mortality (Table 5.2) whereas virologic failure was not associated with any of the assessed infant characteristics (data not shown).

Table 5.2 Infant characteristics associated with mortality in treated HIV-1 infected children

Variables	**Survived n = 215**	**Died n = 34**	**Univariate**		**Multivariate**	
			OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age at treatment start (months)						
< 12	128 (60)	28 (82)	Ref		Ref	
≥ 12	87 (40)	6 (18)	0.32 (0.13 - 0.79)	**0.014**	0.32 (0.11 - 0.93)	**0.036**
Pre-treatment viral load (log_{10} copies/mL)	(n = 186)	(n = 29)				
< 5.9	77 (41)	4 (14)	Ref		Ref	
≥ 5.9	109 (59)	25 (86)	4.42 (1.48 - 13.20)	**0.008**	3.15 (1.00 – 9.90)	**0.050**
Weight-for-age-Z score	(n = 191)	(n = 31)				
≤ -4	29 (20)	18 (58)	6.81 (2.14 - 21.64)	**0.001**	6.41 (1.91 - 21.55)	**0.003**
>-4 to -2	93 (49)	9 (29)	1.43 (0.42 - 4.85)	0.568	1.27 (0.36 - 4.51)	0.713
> -2	59 (31)	4 (13)	Ref		Ref	

Data are expressed as n (%)
OR, Odds Ratio; CI, Confidence Interval
Bold indicates statistical significance of $P < 0.05$

5.3.3. Pre-treatment viral load, CD4+ T-cell percentage and FcγR genotypes

The distribution of the functional *FCGR* genotypes and gene copy number variation for the 395 genotypic data in our study is shown in Appendix A.4. The FcγR genotypes and CNV did not significantly associate with pre-ART HIV-1 plasma viral load (Figure 5.1). Conversely, an association was observed between the FcγRIIIa-F176V genotype and pre-ART CD4+ T-cell percentage (Figure 5.2). Children who were homozygous for FcγRIIIa-176FF had the highest percentage of CD4 T-cells (median 22.6; IQR 14.8-28.4) compared to FcγRIIIa-176FV heterozygotes (median 19.6; IQR 13.6-25.8) and FcγRIIIa-176VV homozygotes (median 17; IQR 13-24.7; $P = 0.037$).

CD4 T-cell percentage prior to ART initiation was dichotomised at (< 20% vs. ≥ 20%) and logistic regression analysis carried out to determine its relationship with the FcγRIIIa genotypes (Table 5.3). The HIV-1 infected children homozygous for the higher affinity IgG binding allele (FcγRIIIa-176VV) were 2.2 times more likely to have CD4 T-cell percentage < 20 compared to those carrying FcγRIIIa-176FF genotype (OR = 2.15; 95% CI 1.17 – 3.94; $P = 0.013$).

Table 5.3 Univariate analysis of pre-ART CD4+ T-cell percentage and FcγRIIIa genotypes

Genotype	**≥ 20 CD4 T-cell percentage**	**< 20 CD4 T-cell percentage**	**Univariate**	
			OR (95% CI)	*P* value
FcγRIIIa genotype	(n =198)	(n = 175)		
176FF	87 (44)	56 (32)	Ref	
176FV	85 (43)	83 (47)	1.52 (0.97 – 2.38)	0.071
176VV	26 (13)	36 (21)	2.15 (1.17 – 3.94)	**0.013**

Data are expressed as n (%)
OR, Odds Ratio; CI, Confidence Interval
Bold indicates statistical significance of $P < 0.05$

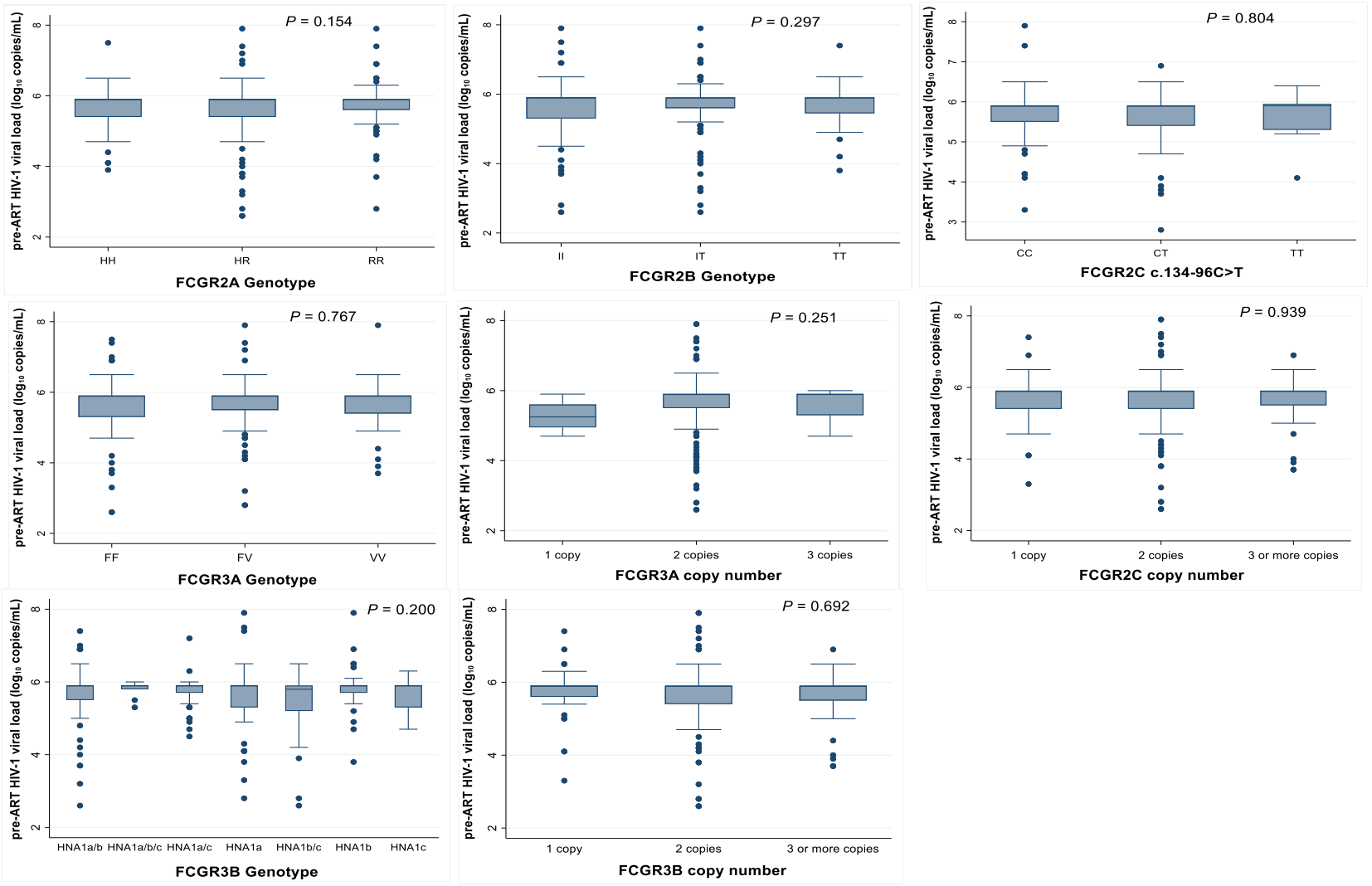

Figure 5.1 Box and whisker plot of pre-ART HIV-1 viral load (log10 copies/mL) by *FCGR* genotypes.
P values were determined using Kruskal–Wallis tests. The blue box contains the 25th to 75% percentiles (IQR) of the dataset while the horizontal line that divides the box into 2 denotes the median. The whiskers mark the minimum and maximum values and the navy blue dots indicate outliers.

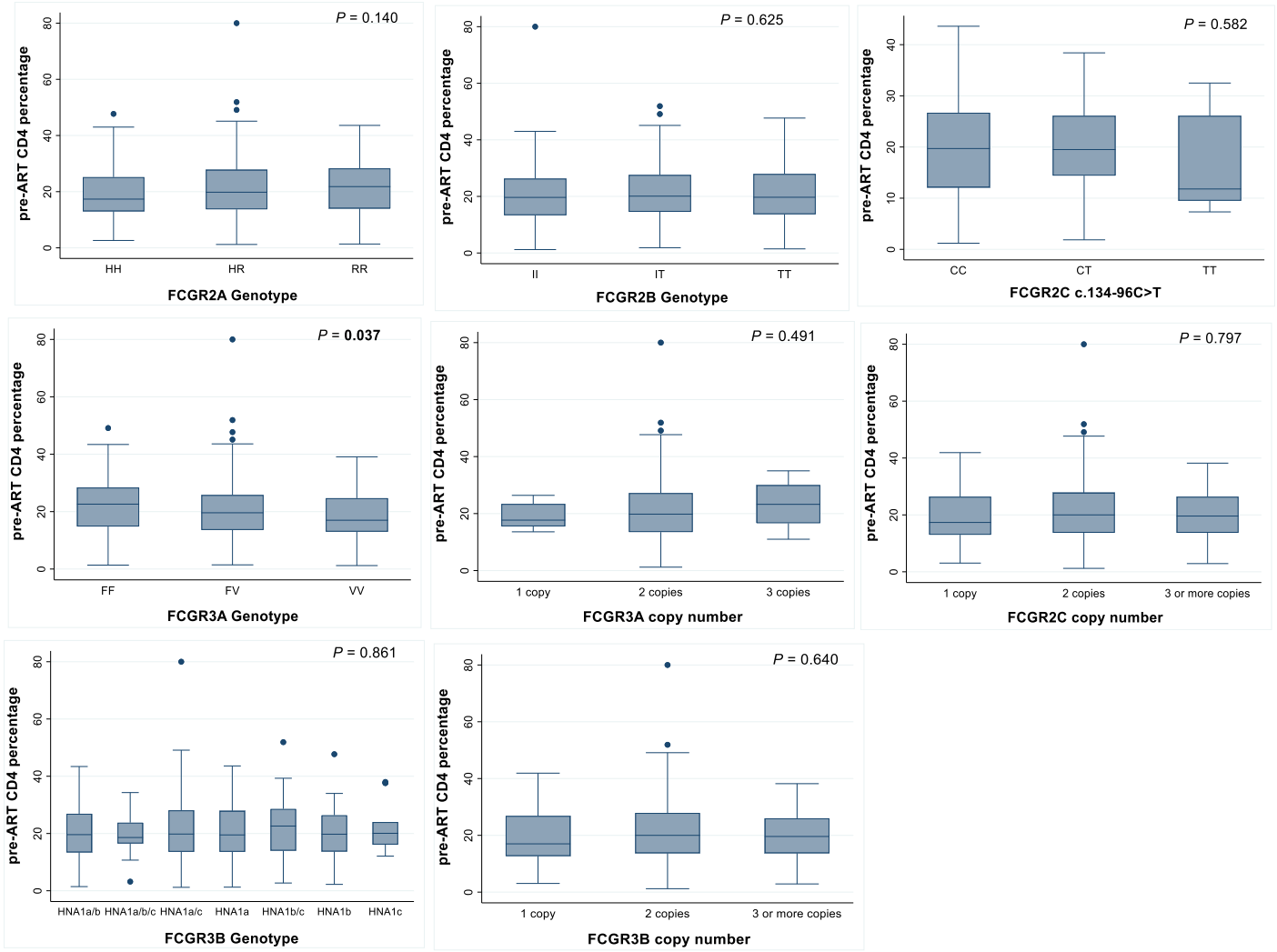

Figure 5.2 Box and whisker plot of pre-ART CD4+ T-cells percentage by *FCGR* genotypes.
P values were determined using Kruskal–Wallis tests. The blue box contains the 25th to 75% percentiles (IQR) of the dataset while the horizontal line that divides the box into 2 denotes the median. The whiskers mark the minimum and maximum values and the navy blue dots indicate outliers.

5.3.4. *FCGR3A* copy number variation associates with increased odds of mortality in HIV-1 infected children

The low-affinity FcγR genotypes and allele carriage frequencies did not differ significantly between the children who died and those who survived. Conversely, CNV (deletion or duplication) of *FCGR3A* was more frequent in cases than controls (P = 0.020; Table 5.4). In univariate analysis and using two gene copies as reference, there was a trend of association of both *FCGR3A* deletion (OR = 7.13; 95% CI 0.97 – 52.64; P = 0.054) and duplication (OR = 3.57; 95% CI 0.85 – 15.05; P = 0.083) with increased odds of mortality. Due to very low frequencies, *FCGR3A* deletion and duplication were combined as CNV and significantly associated with increased odds of mortality (OR = 4.46; 95% CI 1.37 - 14.57; P = 0.013; Figure 5.3). The association remained significant when adjusted for age at ART initiation, weight-for-age Z score and pre-ART plasma viral load that were independently associated with mortality (AOR = 15.98; 95% CI 3.72 – 68.62 P < 0.001; Table 5.2). *FCGR* variants (gene CNV, FcγR genotypes and allele carriage) did not associate with virologic failure (Table 5.4; Figure 5.3).

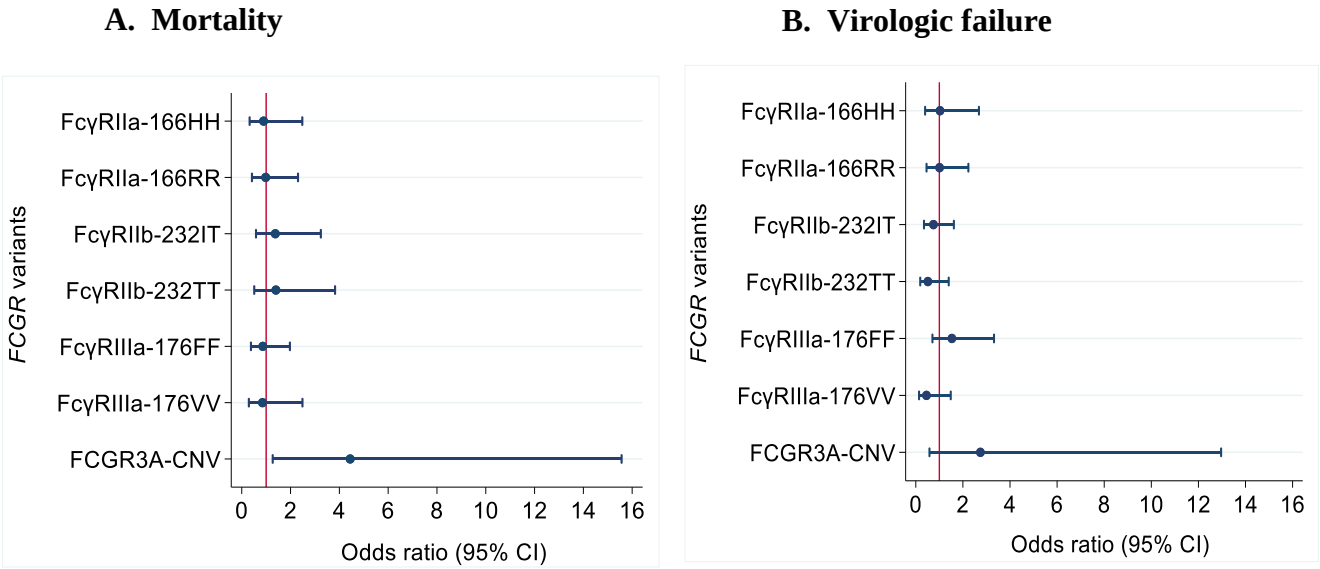

Figure 5.3 Odds ratio and confidence intervals from univariate logistic regression model for *FCGR* variants and mortality (A) and virologic failure (B) in HIV-1 infected children. The blue circle, unadjusted odds ratio; whiskers, 95% confidence interval (CI); red line, odds ratio of 1.

Table 5.4 *FCGR* genetic variants and outcomes after antiretroviral therapy initiation in HIV-1 infected children

	Mortality			Virologic control		
Variants	**Survived n = 215**	**Died n = 34**	***P* value**	**Viral suppression n = 172**	**Virologic failure n = 43**	***P* value**
FcγRIIa genotype			0.880			0.734
166HH	46 (21.4)	6 (17.6)		38 (22)	8 (19)	
166HR	104 (48.4)	17 (50)		84 (49)	20 (47)	
166RR	65 (30.2)	11 (32.4)		50 (29)	15 (35)	
Allele carriage						
≥ 1 166H allele	150 (70)	23 (68)	0.803	122 (71)	28 (65)	0.459
≥ 1 166R allele	169 (79)	28 (82)	0.617	134 (78)	35 (81)	0.618
FcγRIIb genotype			0.725			0.620
232II	85 (40)	11 (32)		66 (38)	19 (44)	
232IT	84 (39)	15 (44)		67 (39)	17 (40)	
232TT	46 (21)	8 (24)		39 (23)	7 (16)	
Allele carriage						
≥ 1 232I allele	169 (79)	26 (76)	0.779	133 (77)	36 (84)	0.363
≥ 1 232T allele	130 (60)	23 (68)	0.424	106 (62)	24 (56)	0.486
FcγRIIIa genotype			0.939			0.198
176FF	89 (41.4)	13 (38)		67 (39)	22 (51)	
176FV	89 (41.4)	15 (44)		72 (42)	17 (40)	
176VV	37 (17.2)	6 (18)		33 (19)	4 (9)	
Allele carriage						
≥ 1 176F allele	178 (83)	28 (82)	0.950	139 (82)	22 (51)	0.134
≥ 1 176V allele	126 (59)	21 (62)	0.728	105 (61)	21 (49)	0.148
FcγRIIIb genotype			0.488			0.554
HNA1a+/1b+/1c-	57 (26.51)	11 (32.35)		46 (26.74)	11 (25.58)	
HNA1a+/1b+/1c+	12 (5.58)	0 (0.00)		10 (5.81)	2 (4.65)	
HNA1a+/1b−/1c+	35 (16.28)	4 (11.76)		29 (16.86)	6 (13.95)	
HNA1a+/1b-/1c-	57 (26.51)	8 (23.53)		46 (26.74)	11 (25.58)	
HNA1a-/1b+/1c+	27 (12.56)	3 (8.82)		20 (11.63)	7 (16.28)	
HNA1a-/1b+/1c-	17 (7.91)	6 (17.65)		11 (6.40)	6 (13.95)	
HNA1a-/1b-/1c+	9 (4.19)	2 (5.88)		9 (5.23)	0 (0.00)	
HNA1a-/1b-/1c-	1 (0.47)	0 (0.00)		1 (0.58)	0 (0.00)	
Allele carriage						

≥1 HNA1a allotype	160 (74)	23 (68)	0.407	130 (76)	30 (70)	0.435
≥1 HNA1b allotype	114 (53)	20 (59)	0.529	88 (51)	26 (60)	0.276
≥1 HNA1c allotype	82 (38)	9 (26)	0.193	67 (39)	15 (35)	0.623
FcγRIIc c.134-96C>T	(n = 128)	n = 30)	0.631	(n = 101)	(n = 27)	0.598
CC	66 (51.56)	13 (43.33)		54 (53)	12 (44.4)	
CT	56 (43.75)	16 (53.33)		43 (43)	13 (48.2)	
TT	6 (4.69)	1 (3.33)		4 (4)	2 (7.4)	
FCGR2C copy number			0.700			0.771
1 copy	19 (8.8)	4 (11.8)		16 (9)	3 (7)	
2 copies	139 (64.7)	23 (67.6)		112 (65)	27 (63)	
3 or more copies	57 (26.5)	7 (20.6)		44 (26)	13 (30)	
FCGR3A copy number			**0.020**			0.140
1 copy	2 (0.9)	2 (5.9)		2 (1.16)	0 (0.00)	
2 copies	207 (96.3)	29 (85.3)		167 (97.09)	40 (93.02)	
3 copies	6 (2.8)	3 (8.8)		3 (1.74)	3 (6.98)	
FCGR3B copy number			0.515			0.906
1 copy	18 (8.4)	4 (11.7)		15 (8.7)	3 (7)	
2 copies	141 (65.5)	24 (70.6)		113 (65.7)	28 (65)	
3 or more copies	56 (26.1)	6 (17.7)		44 (25.6)	12 (28)	

Data are expressed as n (%)
OR, Odds Ratio; CI, Confidence Interval
Bold indicates statistical significance of $P < 0.05$

5.3.5. *FCGR* genetic variants and HIV-1 reservoir

Information on HIV-1 DNA level was available for 108 out of the 128 virally suppressed children in our study cohort. The HIV-1 DNA level ranged from 3 to 1077 copies/million cells, median of 54 copies/ million cells (IQR: 20-137). The distribution of the levels of HIV-1 DNA (log_{10} copies/ million cells) is shown in Appendix A.5. The children were initiated on ART at a median of 4.2 months (IQR: 2.9-5.5). HIV-1 DNA levels (log_{10} copies/ million cells) correlated with the age at start of ART (rho = 0.419, $P < 0.0001$; Spearman's rank-order correlation test). When dichotomised at median level of HIV-1 DNA, higher HIV-1 DNA levels (≥ 1.7 log_{10} copies/ million cells) occurred in those who were started on ART at ≥ 4.2 months of age (OR 3.91; 95% CI 1.74-8.79; $P = 0.001$) and had CD4+ T-cell percentage < 15 (OR 3.40; 95% CI 1.15-10.06; $P = 0.027$). In multivariate analysis, both ART start at ≥ 4.2 months of age (AOR 3.51; 95% CI 1.53-8.06; $P = 0.003$) and CD4+ T-cell percentage < 15 (AOR 3.11; 95% CI 1.01-9.57; $P = 0.048$) remained significant. As illustrated in Figure 5.4, *FCGR2A, FCGR2B, FCGR2C, FCGR3A* and *FCGR3B* genotypes, as well as CNVs for *FCGR2C, FCGR3A* and *FCGR3B* were not associated with HIV-1 DNA levels, a marker for viral reservoir.

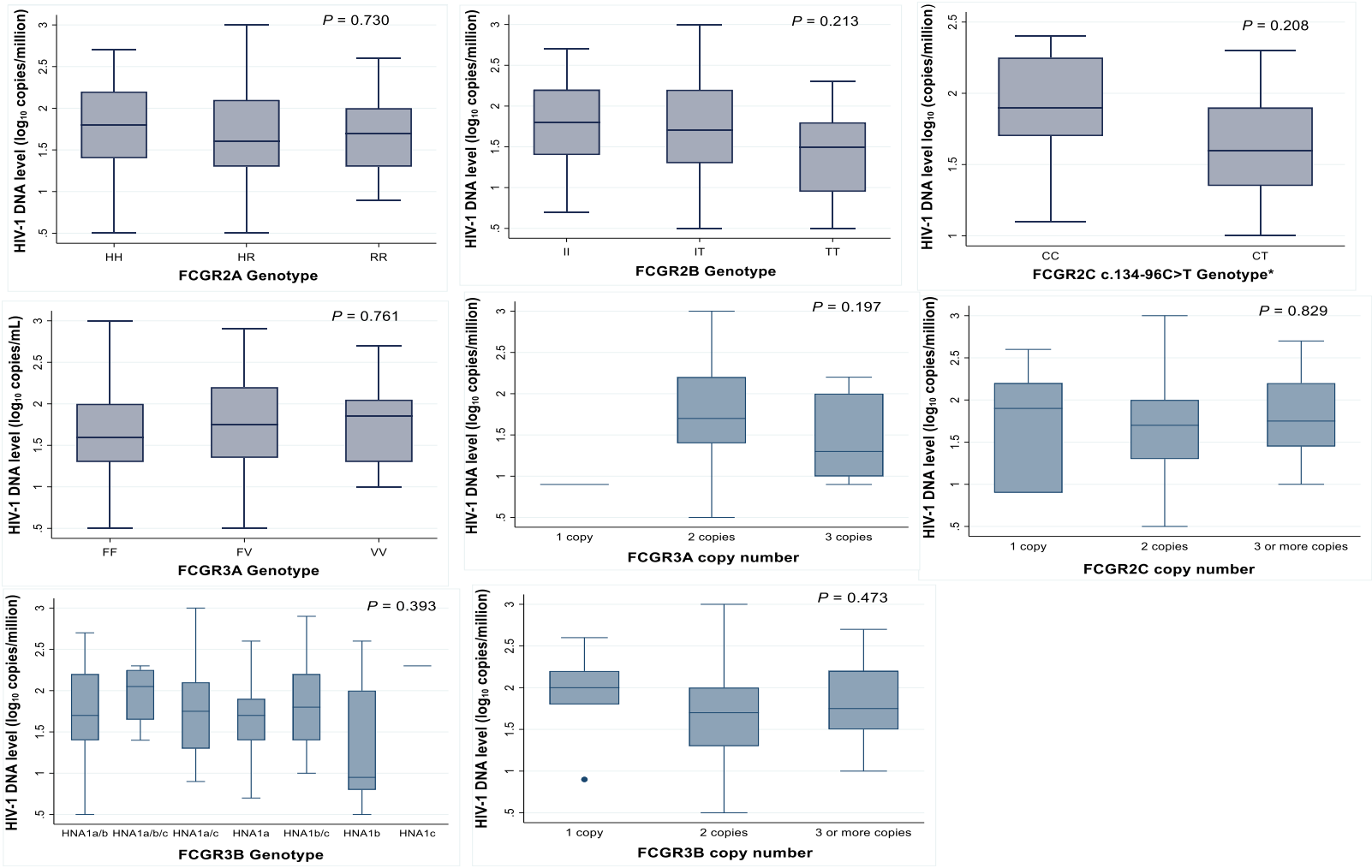

Figure 5.4 Box and whisker plot of HIV-1 DNA log_{10} copies/million cells by *FCGR* genotypes.

P values were determined using Kruskal–Wallis test except for *FCGR2C* where Wilcoxon rank sum test was used. * No *FCGR2C* c.134-96TT genotype observed. The blue box contains the 25th to 75th percentiles (IQR) of the dataset while the horizontal line that divides the box into 2 denotes the median. The whiskers mark the minimum and maximum values of the dataset and the black dot indicates outlier.

5.4. DISCUSSION

There is currently a paucity of data for the role of *FCGR* variants and HIV disease outcome in children. The few studies that investigated a potential role for FcγRIIa-R166H and FcγRIIIa-F176V genotypes and HIV-1 disease progression in children did not observe an association with either virologic control or mortality (108,109). In a large cohort of children with perinatally-acquired HIV-1 infection, we studied multiple FcγR variants (*FCGR* point mutations and gene copy number variation) relative to pre-ART HIV plasma viral load, CD4+ T cell percentage, and post-ART outcomes that include mortality, virologic control and the HIV DNA viral reservoir size. Beside *FCGR2A* and *FCGR3A* genotypes, we also assessed the role of *FCGR2B, FCGR2C, FCGR3B* and gene copy number variability that are rarely studied in the context of HIV-1. We did not observe an association between child *FCGR* point mutations and pre-ART viral load, virological failure, mortality and HIV DNA viral reservoir size. In contrast, we observed an association between *FCGR3A* CNV and mortality, as well as, FcγRIIIa-F176V genotype and pre-ART CD4+ T-cell percentage.

Generally, gene CNV can have functional implications and may contribute to inter-individual differences in gene expression (84). In our study, variation in copy number of *FCGR3A* (either gain or loss) significantly associated with increased odds of mortality. Duplication and deletion of *FCGR3A* generally occurs with the gain or loss of a distinct region of the genome, namely copy number variable region 2 (CNR2) (86). A study by Breunis et al (84) demonstrated that CNV in *FCGR3A* correlates with the expression and function of FcγRIIIa on natural killer (NK) cells. In addition to a gene dosage effect, duplication and deletion of this region results in distinct chimeric genes. A CNR2 deletion results in an *FCGR2A/2C* chimera that associates with reduced FcγRIIa surface levels and oxidative burst response (86,87). Conversely, a CNR2 duplication results in an *FCGR2C/2A* chimeric gene that increases FcγRIIc expression levels. The gain or loss of an *FCGR3A* copy may therefore perturb the immune response in several different ways, making it difficult to pinpoint the mechanism underlying the association of *FCGR3A* copy number with HIV-1 associated mortality in children. Nonetheless, our study highlights the importance of looking at *FCGR3A* copy number in relation to HIV-1 outcomes. Due to the low frequency of CNV of *FCGR3A* in our study cohort, the finding must be validated in a defined cohort with sufficiently large sample size.

The FcγRIIIa-F176V polymorphism alters the receptor's affinity for IgG. Compared to the 176F allele, the 176V allele displays increased affinity for IgG1, IgG3 and IgG4 (72,101). This increased affinity correlates with enhanced NK cell activation and ADCC activity. In this study, homozygosity for the 176V allele associated with a lower percentage of CD4+ T cells. A possible explanation may be that increased ADCC activity conferred by the 176V allele may result in increased killing of CD4+ T cells that display HIV envelope on the surface, either from infection of the cell or through bystander killing of uninfected cells presenting soluble envelope attached to CD4 receptors. Alternatively, the functional consequences of the FcγRIIIa-F176V polymorphism may be different in children and in the context of a chronic HIV-1 infection, as described for adults, and lead to altered CD4+ T cell percentages by a different mechanism (159,160). Different mechanisms at play in adults and children may also contribute to an observed association for the *FCGR2C* c.134-96T allele in disease progression in African adults, but no association of this allele with HIV-1 outcomes in children of the same ethnicity (79).

The persistence of the HIV-1 reservoir in infected individuals, in spite of ART, presents a hindrance to HIV-1 cure or remission (118). The role of FcγR-mediated effector functions in controlling the HIV-1 reservoir remains largely undefined. To date, FcγRIIa expressed on infected cells has been identified as a marker of HIV-1 latent reservoir (123), although this has not been replicated in other subsequent independent studies (124,125,161). A study conducted on ART-suppressed adult patients from the RV254 acute HIV infection cohort also did not observe an association between the FcγRIIa-R166H polymorphism and HIV-1 reservoir size (112). We do not exactly know the role of *FCGR* variants on HIV-1 disease outcomes after ART initiation, especially in the context of MTCT. Therefore, the impact of *FCGR* variation on post-ART outcomes of HIV-1 disease is an important consideration for therapeutic interventions that depend on Fc-mediated responses, which predict disease progression (15,19).

Initiating ART in children with vertically acquired HIV-1 at a younger age and high CD4+ percentages could have long-term immunologic benefit and reduce HIV-1 viral load (162). Of note, many HIV-1-infected children who initiated ART early will have a steady decline of HIV-specific antibodies and ultimately test antibody negative at older age (163) due to limited exposure to antigen necessary for antibody production. Therefore, the role of FcγR-mediated effector functions in HIV replication and other outcome measures under long-term treatment, where antibody levels are reduced because of ART, would be altered. It is very possible that

one would see an effect of various genotypes in untreated HIV-1 infected children that might not be seen in those treated.

Our study assessed the association between multiple FcγR variants and post-ART outcomes (mortality, virologic control and the HIV DNA viral reservoir size) in a cohort of South African infants born to women living with HIV-1. The study has some limitations. Data on the change from baseline CD4+ T cell percentage and plasma viral load decline after ART initiation were not available. In addition, children included in our study were initiated on ART at a median of 6.9 months (IQR: 4.2-13.1); the implication of not initiating ART earlier is greater risk of death or progression to AIDS (45). Consequently, death prior to ART initiation presents a missed opportunity to collect blood samples to produce genotypic data that would have contributed to the assessment of disease outcomes.

CHAPTER 6 : CONCLUSION

Fc receptors for IgG (FcγRs) have been identified as key role players in the mechanisms that regulate immune response. They are widely expressed on the surface of most immune cells, where they mediate diverse cellular responses such as ADCC, ADCP, triggering of cell activation, antigen presentation and regulation of B-cell activation. All human FcγRs are transmembrane proteins except FcγRIIIb, which is a GPI-anchored protein; they differ in structural domain organisation, affinity for specific IgG subclasses and ability to trigger either activating or inhibitory signals (74,75).

Allelic variants of FcγRs (SNPs and CNV) have been identified, which can alter their functionality (155). The CNV for *FCGR2C, FCGR3A* and *FCGR3B* has effect on cell surface expression (84). The presence of SNPs can alter binding affinity to IgG subclasses (FcγRIIa-H166R and FcγRIIIa-F176V) (93,101), receptor's ability to translocate to lipid rafts (FcγRIIb-I232T) (95,96) and *N*-linked glycosylation sites (FcγRIIIb-HNA1a/b/c) (104). The surface expression of receptors (FcγRIIc-c.169T>C and c.798+1A>G) can also be determined by the presence of polymorphisms (89,100). As a result, Fc-mediated antibody functions have been highlighted as relevant in many clinical conditions and specifically in HIV-1 acquisition and post-infection control of viremia (15,18,19,82). The involvement of FcγRs in a range of innate and adaptive immune responses also makes them attractive targets for developing antibody-based therapeutics [reviewed in (73)]. However, the functional and clinical relevance of *FCGR* polymorphisms in the context of HIV-1 acquisition (71,108,109,149), disease progression (78,110,111,115) and response to vaccination regimens (82,83,115) have produced inconsistent results.

The genes on the *FCGR2/3* region are in high sequence homology because of their close proximities on chromosome 1q23 and as a result, they are in linkage disequilibrium. Most association studies of the allelic variants of FcγRs with diseases have utilized candidate gene designs to assess specific allelic variants. This present study used MLPA genotyping method that can assess functional SNPs and CNV within the *FCGR2/3* region at the same time, which also provided the opportunity to check for linkage disequilibrium to identify functional interaction between the independently associated variants.

MTCT is an attractive model that can be used to study immune correlates of protection and elucidate the definitive role of *FCGR* genotypes on MTCT of HIV-1. Different MTCT studies have produced variable results on the role of allelic variations of FcγR in HIV-1 acquisition (71,108,109) and findings from the two independent Kenyan cohorts suggest the FcγR genotypes have no impact on disease progression. The present study investigated the associations between FcγR variability (*FCGR* point mutations and gene copy number variation) and perinatal HIV-1 acquisition and disease progression in a large cohort of South African infants born to women living with HIV-1. The study findings add to the growing evidence that *FCGR2A* and *FCGR3A* polymorphisms do not associate with perinatal HIV-1 acquisition or disease progression (mortality and virologic control). In addition, the observed association of FcγRIIb-232TT genotype with HIV-1 acquisition confirmed previous findings in a smaller cohort of South African children (71). Collectively, the findings are suggestive of a functional impact of the FcγRIIb-232TT genotype on perinatal HIV-1 acquisition. We also show a role for less studied variants – *FCGR3A* duplication and homozygous HNA1a. These findings provide additional insight into a deleterious role for FcγRs in HIV-1 infection in children.

Our findings suggest that the *FCGR* genotypes do not associate with HIV-1 disease outcomes of mortality, virologic failure and size of HIV-1 reservoir in children. For the first time, our study highlights the importance of looking at CNV when assessing the functional variability of FcγR in disease contexts. *FCGR3A* CNV matters in both HIV-1 acquisition and outcome. However, the findings must be validated in a much larger cohort than the small numbers available for our study.

We report further deleterious association between *FCGR2C* c.134-96T allele and perinatally-acquired HIV-1 infection, contrary to the protection from HIV-1 acquisition observed in the Thai RV144 vaccine trial (82). Similarly, a deleterious association was found with HIV-1 disease progression in South African adults (78), which was not replicated in our children cohort. The functional mechanisms modulating the impact of variants within the Thai *FCGR2C* haplotype on immunity against HIV-1 does not involve expression of the surface FcγRIIc receptor. This is because in the available genetic association studies, very few individuals carry the minor alleles that predict expression of the FcγRIIc surface molecule. Together, these findings necessitate the need to elucidate the functional significance of this variant in different populations, as well as in vaccination and disease contexts.

Other than FcRn, other receptors expressed in the placenta (FcγRs) contribute in the selective placental transfer of maternal IgG. This selective process is driven by a combination of factors that include IgG FcγR binding strength, subclass, and glycan profiles (164). Our previous study findings of perinatal acquisition of HIV-1 implicated *FCGR2C* c.134-96T-minor allele (149), homozygosity for the FcγRIIb-232T and FcγRIIIb-HNA1a alleles but these variants showed no effect on mortality and virologic control once on ART. We therefore postulate that the selective and differential transfer of maternal IgG subclasses to infants likely provide a unique setting for the variants associated with acquisition but the disease outcomes may be dominated by other factors (109).

As part of a greater study addressing markers of remission in HIV-1 infected children, the cohort under study is being evaluated for the repertoire of HIV-1 specific antibodies produced, the size of the proviral DNA reservoir, as well as other select genetic markers of interest. In addition, a large subset of children from this cohort are still in follow up, and currently 10-12 years of age, providing an ideal opportunity for more in-depth and ongoing studies that will add value, by integrating all these parameters over time.

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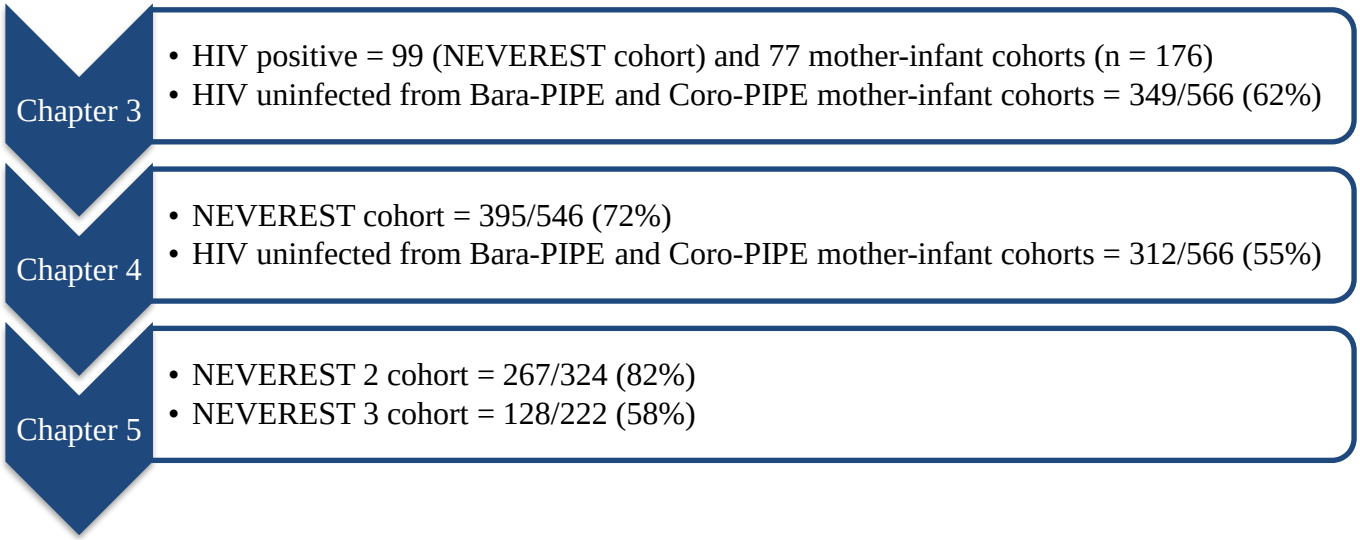
APPENDICES

Appendix A.1. Description of the five perinatal HIV-1 transmission cohorts selected for the nested-case control study

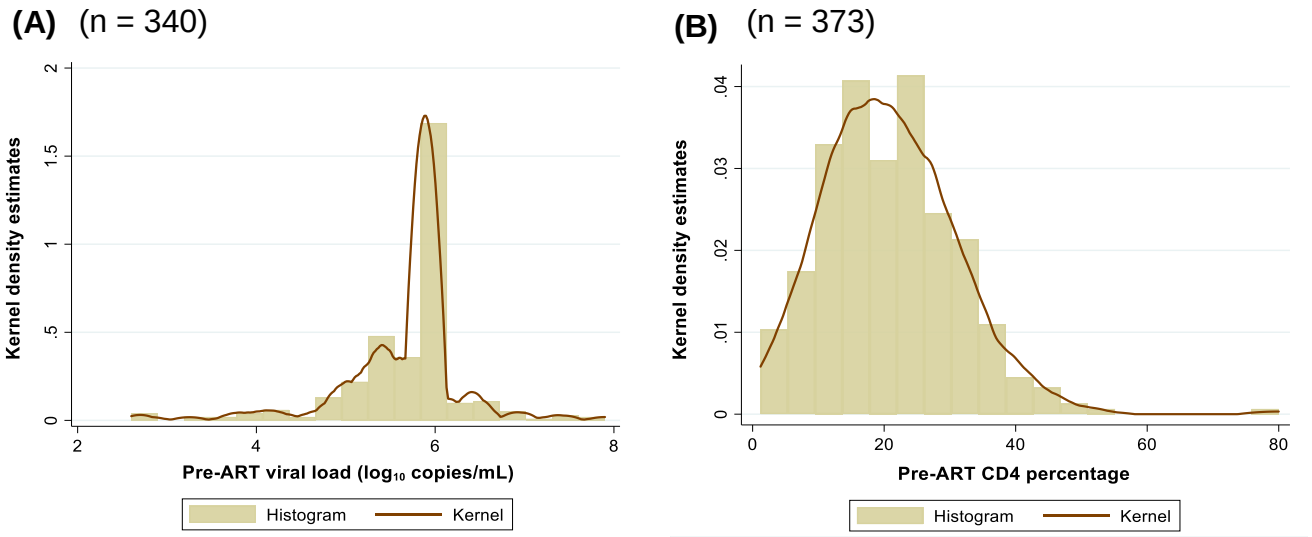
	Cohort 1	Cohort 2	Cohort 3	Cohort 4	Cohort 5*
Cohort name	PETRA (165,166)	PEP/DART (167,168)	Bara-PIPE (130)	Coro-PIPE (130)	NEVEREST (127–129)
Description	Women living with HIV were recruited at 35 weeks' gestation. They were randomised to either receive short courses of zidovudine and lamivudine or placebo.	Most mothers were recruited postpartum, before discharge from hospital. Maternal HIV status was unknown at delivery but determined after birth. The women did not receive ARVs before or during delivery. A small number of postpartum women who received NVP as part of a demonstration PMTCT project were also enrolled.	Postpartum drug naïve women living with HIV, who had not accessed antenatal PMTCT services and those that received NVP as part of routine PMTCT services were recruited.	Women living with HIV and already enrolled in PMTCT services. Recruited at 6 weeks postpartum and exposed to NVP or triple-drug combination therapy	Children with perinatally-acquired HIV-1, recruited as part of two sequential clinical trials. In NEVEREST 2 ART-naive children less than 2 years were randomized to either stay on LPV/r or switched to NVP if they achieved viral suppression after 12 months (n=324). Those in NEVEREST 3 were between 3 and 5 years, already on treatment and virally suppressed during LPV/r- therapy- (n=222)
Infant HIV testing	At birth and 6 weeks of age	At birth and 6 weeks of age	At birth and 6 weeks of age	At 6 weeks of age, no birth sample	At 6 weeks of age, no birth sample
Total cohort	31	202	284	332	546
Child infected	8	25	26	24	546
Child uninfected	23	177	258	308	N/A

Cohorts 1-4 are birth cohorts (mother-child pairs). *Maternal blood samples and clinical information were available for the four birth cohorts at enrolment but not for the NEVEREST cohort.

Appendix A.2. Contribution of the five perinatal cohorts to the overall cohort in each study chapter



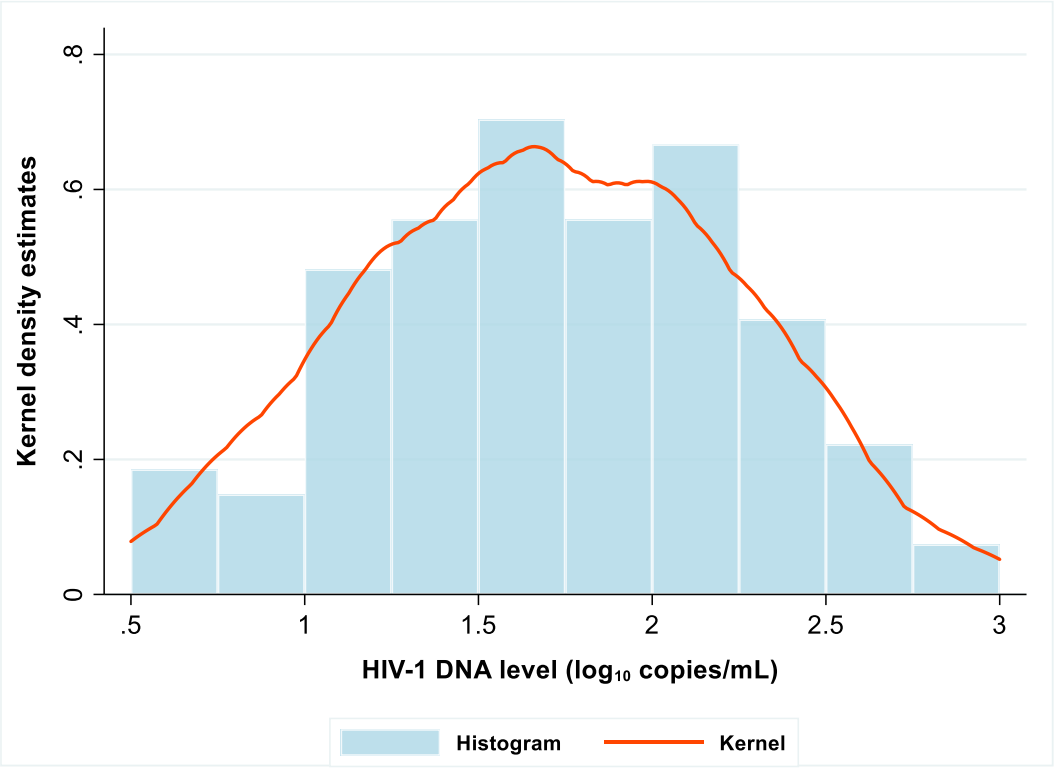
Appendix A.3. Distribution of pre-treatment viral load (log10 copies/mL) (A) and CD4+ T-cell percentage (B) in HIV-1 infected South African children



Appendix A.4. Distribution of *FCGR* variants in the 395 HIV-1 infected children

Variants	**HIV-1 infected** n (%)
FCGR2A genotype	
166HH	86 (21.8)
166HR	187 (47.3)
166RR	122 (30.9)
FCGR2B genotype	
232II	165 (41.8)
232IT	160 (40.5)
232TT	70 (17.7)
FCGR3A genotype	
176FF	155 (39)
176FV	176 (45)
176VV]	64 (16)
FCGR3B genotype	
HNA1a+/1b+/1c-	116 (29.37)
HNA1a+/1b+/1c+	16 (4.05)
HNA1a+/1b−/1c+	61 (15.44)
HNA1a+/1b-/1c-	98 (24.81)
HNA1a-/1b+/1c+	50 (12.66)
HNA1a-/1b+/1c-	38 (9.62)
HNA1a-/1b-/1c+	15 (3.80)
HNA1a-/1b-/1c-	1 (0.25)
FCGR2C c.134-96C>T	(n = 205)
CC	98 (47.8)
CT	97 (47.3)
TT	10 (4.9)
FCGR2C copy number	
≤ 1 copy	37 (9)
2 copies	265 (67)
≥ 3 copies	93 (24)
FCGR3A copy number	
1 copy	5 (1.3)
2 copies	378 (95.2)
3 copies	14 (3.5)
FCGR3B copy number	
≤ 1 copy	35 (9)
2 copies	271 (69)
≥ 3 copies	89 (22)

Appendix A.5. Histogram and estimated kernel distributions of HIV-1 DNA log10 copies/million cells



Appendix B. Ethics clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research & Post Graduate Affairs)

TO: Ms J Ebonwu and Dr R Lassaunierre
School of Pathology
Department of Virology
National Institute for Communicable Diseases
National Health Laboratory Service

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CC: Supervisor: Professor C Tiemessen <Carolinet@nicd.ac.za>
and <HREC-Medical.ResearchOffice@wits.ac.za>

FROM: Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 14/06/2018

REF: *R14/49*

PROTOCOL NO: **M180575** *(This is your ethics application study reference number. Please quote this reference number in all correspondence relating to this study)*

PROJECT TITLE: *Crystallizable fragment (FC) gamma receptor genetic variability in paediatric human immunodeficiency virus (HIV) infection*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to the Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

Appendix C. Declaration: Student's contribution to articles and agreement of co-authors

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, **Joy Ebonwu**, student number **341929**, declare that this Thesis Report is my own work and that I contributed adequately towards research findings published in the articles stated below which are included in my Thesis Report. **Signature of Student** **Date** 22/03/2022

Name of Primary Supervisor...Prof Caroline T Tiemessen

Signature of Primary Supervisor ....**Date**.....23/032022

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the articles by the student as part of her Thesis Report. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for her degree purposes.

Article 1: Title: An HIV vaccine protective allele in FCGR2C associates with increased odds of perinatal HIV acquisition

Journal name, year, volume and page numbers: Frontiers in Immunology, 2021, 12:760571

Authors	Name	Signature	Date
1st author	Ebonwu Joy		22/03/2022
2nd author	Lassaunière Ria		23/03/2022
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4th author	Goosen Mark		23/03/2022
5th author	Strehlau Renate		24/03/2022
6th author	Gray Glenda		24/03/2022
7th author	Kuhn Louise	Louise Kuhn 3/24/2022 Louise Kuhn	
8th author	Tiemessen Caroline		23/03/2022

Comments by primary supervisor:

N/A.....

.....

Article 2: Title: FCGR3A gene duplication, FcγRIIb-232TT and FcγRIIIb-HNA1a associate with an increased risk of vertical acquisition of HIV-1

Journal name, year, volume and page numbers: Under review with PLOS ONE Journal – manuscript number: PONE-D-22-06428 (Submitted March 3 2022)

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1

7th author	Tiemessen Caroline		23/03/2022

Comments by primary supervisor:

N/A.....

.....

Appendix D. Published paper

frontiers in Immunology

ORIGINAL RESEARCH
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An HIV Vaccine Protective Allele in *FCGR2C* Associates With Increased Odds of Perinatal HIV Acquisition

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In the Thai RV144 HIV-1 vaccine trial, a three-variant haplotype within the Fc gamma receptor 2C gene (*FCGR2C*) reduced the risk of HIV-1 acquisition. A follow-on trial, HVTN702, of a similar vaccine candidate found no efficacy in South Africa, where the predominant population is polymorphic for only a single variant in the haplotype, c.134-96C>T (rs114945036). To investigate a role for this variant in HIV-1 acquisition in South Africans, we used the model of maternal-infant HIV-1 transmission. A nested case-control study was conducted of infants born to mothers living with HIV-1, comparing children with perinatally-acquired HIV-1 (cases, n = 176) to HIV-1-exposed uninfected children (controls, n = 349). All had received nevirapine for prevention of mother-to-child transmission. The *FCGR2C* copy number and expression variants (c.–386G>C, c.–120A>T c.169T>C, and c.798+1A>G) were determined using a multiplex ligation-dependent probe amplification assay and the c.134-96C>T genotype with Sanger sequencing. The copy number, genotype and allele carriage were compared between groups using univariate and multivariate logistic regression. The *FCGR2C* c.134-96C>T genotype distribution and copy number differed significantly between HIV-1 cases and exposed-uninfected controls (P = 0.002, P_{Bonf} = 0.032 and P = 0.010, P_{Bonf} = > 0.05, respectively). The *FCGR2C* c.134-96T allele was overrepresented in the cases compared to the controls (58% *vs* 42%; P = 0.001, P_{Bonf} = 0.016). Adjusting for birthweight and *FCGR2C* copy number, perinatal HIV-1 acquisition was associated with the c.134-96C>T (AOR = 1.89; 95% CI 1.25-2.87; P = 0.003, P_{Bonf} = 0.048) and c.169C>T (AOR = 2.39; 95% CI 1.45-3.95; P = 0.001, P_{Bonf} = 0.016) minor alleles but not the promoter variant at position c.–386G>C. The c.134-96C>T variant was in strong linkage disequilibrium with the c.169C>T variant, but remained significantly associated with perinatal acquisition when adjusted for c.169C>T in multivariate analysis. In contrast to the protective effect

observed in the Thai RV144 trial, we found the *FCGR2C* variant c.134-96T-allele associated with increased odds of perinatal HIV-1 acquisition in South African children. These findings, taken together with a similar deleterious association found with HIV-1 disease progression in South African adults, highlight the importance of elucidating the functional relevance of this variant in different populations and vaccination/disease contexts.

Keywords: Fc gamma receptor, FCGR2C, genetic variant, polymorphism, gene copy number, perinatal HIV-1 acquisition, genetic association study, South Africa

INTRODUCTION

The crystallisable fragment (Fc) region of immunoglobulin G (IgG) antibodies interacts with Fc gamma receptors (FcγRs) expressed on the surface of hematopoietic cells to mediate effector functions. In humans, FcγRs are divided into three classes (FcγRI, FcγRII, and FcγRIII) based on structural domain organization, differences in affinity and specificity for IgG subclasses, and whether their binding triggers activating or inhibitory signals. The low affinity FcγRs are encoded by five genes on chromosome 1q23, namely *FCGR2A, FCGR2B, FCGR2C*, *FCGR3A* and *FCGR3B* (1) and play different roles in regulating immune responses (2). Functionally significant genetic variants occur for all low affinity FcγRs. These affect FcγRs by altering receptor cell surface density, binding affinities to IgGs, glycosylation patterns, cellular distribution, or subcellular localization (3, 4). Apart from single nucleotide polymorphisms (SNPs), copy number variation (CNV) has been demonstrated for *FCGR2C*, *FCGR3A and FCGR3B* (5, 6), and has been correlated with protein expression levels (7). Genes are duplicated or deleted at the *FCGR2/3* locus within well-defined copy number variable regions (CNRs), namely CNR1, CNR2, CNR3 (8, 9) and CNR4 (9). The most common are CNR1, which comprises genes of *FCGR2C*, *HSPA7* and *FCGR3B* and CNR2 that includes the distal part of *FCGR2A* (exon 8 and 3'-untranslated region [3'UTR]), *HSPA6, FCGR3A* and proximal part of *FCGR2C* (excluding exon 8 and 3'UTR) (9).

The *FCGR2C* gene, encoding FcγRIIc, is described as a pseudogene and is the product of an unequal crossover event between the 5' part of *FCGR2B* genes and 3' part of *FCGR2A* (10). Expression of the membrane-bound FcγRIIc protein depends on a combination of three minor alleles that include the c.169T>C variant in exon 3, which substitutes a premature stop codon with a glutamine at amino acid 57, and two splice variants in intron 7 - c.798+1A>G and c.799-1G>C (6, 11). Due to significant variation of the minor allele frequencies in different populations (12), FcγRIIc protein expression is subject to ethnic variation. The splice variant c.798+1A>G minor allele rarely occurs in black Africans and East Asians, thus, few individuals in this population express FcγRIIc compared to approximately 33% of Caucasians (12). An additional *FCGR2C* c.134-96C>T variant (also known as *FCGR2C* 126C>T) has been identified as clinically significant (13). Overall, genetic variation of *FCGR2C* has been associated with rheumatoid arthritis (14) idiopathic thrombocytopenic purpura (15), HIV-tuberculosis co-infection (16), antibody responses to vaccinations (11, 13, 17) and HIV disease progression (18).

In the RV144 vaccine trial, where the vaccine regimen was designed against HIV-1 clade B and E, a three-variant haplotype within *FCGR2C* [c.353C>T (rs138747765); c.391+111G>A (rs78603008) and c.134-96C>T (rs114945036)] reduced the risk of HIV-1 acquisition in Thai adults. The vaccine test subjects carrying at least one minor allele of the c.134-96C>T tag variant had an estimated vaccine efficacy of 91% against the CRF01_AE 169K HIV-1 strain and 64% against any HIV-1 strain, while those with wild type allele exhibited a vaccine efficacy of 15% and 11%, respectively (13). Conversely, two variants within the haplotype were associated with increased risk of HIV-1 acquisition in the HIV Vaccine Trials Network (HVTN) 505 vaccine trial (17). A follow-on trial of a similar vaccine regime to RV144 (HVTN 702 vaccine trial) tested in South Africa showed no efficacy (19). The cause underlying the different vaccine trial outcomes remains undetermined. However, differences in vaccine regimen, population, demographics and environment should be considered (17). A role for population genetics warrants consideration, since black South Africans do not possess the complete Thai *FCGR2C* haplotype and are only polymorphic for c.134-96C>T (rs114945036) (12).

The c.134-96C>T *FCGR2C* variant has been implicated in HIV-1 disease progression in a black South African cohort (18). However, unlike the protective effect observed for Thai vaccinees, the minor allele was associated with increased odds of HIV-1 disease progression in those already infected. It is unknown whether the alternate protective and deleterious roles of the *FCGR2C* c.134-96C>T variant in the Thai vaccinees and HIV-1 infected South Africans is due to different mechanisms involved before and after HIV-1 infection or whether the genetic differences associated with the haplotype alters its role in the two populations. Establishing the role of the c.134-96C>T variant in HIV-1 protective immunity in other models of persistent HIV-1 exposure, such as infants born to HIV-1 infected mothers, will be informative.

Mother-to-child transmission (MTCT) is an attractive model in which to study immune correlates of protection since both members of the transmitting dyad are known, timing of transmission can be ascertained with reasonable precision, and it affords the opportunity to assess factors contributing to both the infectiousness of the transmitter (mother) and susceptibility of the recipient (infant) (20, 21). Limitations of this model are that

transmission occurs between genetically similar individuals, exposure to HIV-1 occurs at a time of early immune development, and immune circumstances during pregnancy are associated with tolerance of the fetal allograft (22). Nevertheless, it provides a unique opportunity to investigate the role of FcγR-mediated effector functions, since the individual (fetus/infant) at risk is passively immunized with HIV-1-specific antibodies through trans-placental transfer of IgG from the HIV-1 infected mother and the model is not confounded by interspecies differences as observed for non-human primate studies (23). In this study, we investigate the association between the *FCGR2C* c.134-96C>T variant and HIV-1 acquisition in black South African children born to women living with HIV.

MATERIALS AND METHODS

Study Design and Population

A nested case-control study was undertaken to investigate the association between the *FCGR2C* variants and HIV-1 perinatal acquisition in children, combining data from past studies of five perinatal cohorts at two hospitals in Johannesburg, South Africa (24–27). One of the five cohorts consists of 546 HIV-infected children who were recruited as part of two sequential randomized clinical trials (NEVEREST 2 and 3) (24–26). The remaining four cohorts comprised of 849 HIV-1 infected mothers and their infants who were recruited and followed prospectively, of whom 83 (10%) infants acquired HIV (27). In the present study, only samples that were found and with sufficient volume were genotyped. *FCGR2C* genotypic data from 99 out of 546 and 77 of 83 HIV-1-infected children (cases) from the NEVEREST and mother-infant cohorts, respectively (n = 176) were compared with 349 of the HIV-exposed uninfected children (controls).

Mode of transmission was defined according to the presence or absence of detectable HIV-1 deoxyribonucleic acid (DNA) in the infant at birth and six weeks of age. Infants that tested HIV-1 positive at six weeks of age, but who were negative at birth, were considered to be infected intrapartum (during labor and delivery) (n = 31), while infants that tested HIV-1 positive at birth were considered infected *in utero* (n = 19). Infants who were HIV-1 positive at six weeks, but had no birth sample, were categorized as 'undetermined' (n = 28). In the 'undetermined' category, 25/28 (89.2%) mothers received single-dose nevirapine or triple-drug combination therapy (two nucleoside reverse transcriptase inhibitors with either a protease inhibitor or non-nucleoside reverse transcriptase inhibitor) known to reduce intrapartum transmission (27–29). Genotyping generated a result for all the *FCGR2C* variants assessed in this study in 27 out of the 28 samples. It was thus concluded that the majority (n = 27) of infants were likely infected *in utero* and were combined with the *in utero* group to form an *in utero*-enriched group. For the NEVEREST cohort, there were no birth samples as the children were recruited from six weeks of life. They were therefore classified as mixed transmission since a few were breastfeeding infections and *in utero* infections could not be distinguished from intrapartum infections (n = 99). All study participants were black South Africans and received nevirapine for prevention of MTCT. Maternal antiretroviral therapy was not routinely used at the time.

Ethics

Ethics approval for the study was obtained from the University of the Witwatersrand Human Research Ethics Committee (Reference numbers: M170585; M180575).

Genotyping

FCGR2C copy number and SNPs that affect gene expression – c.169T>C (p.X57Q), c.798+1A>G, and the *FCGR2B/C* promoter variant at position c.–386G>C and c.–120A>T – were determined using the *FCGR*-specific multiplex ligation-dependent probe amplification assay (MRC Holland, Amsterdam, The Netherlands) according to manufacturer's instructions. Amplicons were separated by capillary electrophoresis on an ABI Genetic Analyser 3130 (Life Technologies, Applied Bio systems, Foster City, CA, USA) and fragments analyzed with the Coffalyzer.NET software (MRC Holland) using peak height as a measure of gene/allele copy number. We did not utilize gene-specific polymerase chain reactions (PCR) to distinguish *FCGR2B* and *FCGR2C* promoter sequences since earlier findings indicate that African individuals do not possess the promoter variant in *FCGR2B*, and thus any detected c.–386G>C minor alleles were in *FCGR2C* (12).

The *FCGR2C* c.134-96C>T (rs114945036) variant was genotyped through conventional PCR and Sanger nucleotide sequencing. In brief, a 6,374 base pair fragment was amplified with the Expand Long Template PCR System (Roche, Mannheim, Germany) using the *FCGR2B/C* sense primer (5'-ATGTATGGGGTGTCTGTGTGTC-3') and *FCGR2C*-specific antisense primer (5'-CTCAAATTGGGCAGCCTTCAC-3') (15). The PCR reaction consisted of ~20 ng genomic DNA as template, 3.75 U Expand Long Template enzyme mix, 5 µl 10× PCR buffer 3 (2.75 mM $MgCl_2$), 500 µM of each deoxynucleotide, 0.3 µM of each oligonucleotide primer, and molecular grade water to a final volume of 50 µl. The PCR conditions were initial denaturation at 94°C for 2 minutes, followed by 10 cycles of 94°C for 10 seconds (denaturation), 60°C for 15 seconds (annealing) and 68°C for 7 minutes (elongation). Thereafter, 25 cycles repeat process of denaturation, annealing and elongation respectively at 94°C for 15 seconds, 60°C for 15 seconds and 68°C for 7 minutes plus 20 seconds cycle elongation for each successive cycle; and a final elongation cycle at 72°C for 7 minutes. The internal antisense primer (5'-CCTCCACTGACCAGAAAGCAC-3') was used in standard BigDye Terminator v3.1 Cycle Sequencing reactions. Sequences were analyzed in Sequencer version 4.5 (Gene Codes Corporation, Ann Arbor, MI) and area under the curve of the electropherogram used to determine allele count for individuals bearing more than two *FCGR2C* gene copies.

Nomenclature

The SNP nomenclature used in this manuscript refers to the amino acid positions in the full protein, in accordance with the Human Genome Variation Society (HGVS) guidelines (30).

The numbering of nucleotides is according to the Genome Reference Consortium Human Reference 38 [GRCh38 (hg38)].

Statistical Analysis

Categorical data were summarized as proportions and the Fisher's Exact test was used for comparisons. For numerical data, the t-test was used for comparison of means. Univariate and multivariate analyses were conducted to determine factors associated with perinatal HIV acquisition. Adjustment for multiple comparisons was performed using the Bonferroni correction, which considered 16 independent tests — four unrelated clinical subgroups each tested for four variants (gene copy number, c.134-96C>T, c.169T<C, and c.–386G>C). Both unadjusted and adjusted P values are reported. Analysis of an association between *FCGR2C* variants and HIV-1 acquisition was limited to variants whose allele frequencies were ≥ 5%. Due to the low frequencies of minor allele homozygotes, their effect was tested using dominant model approach, where participants were divided into two genotype groups: homozygous genotype of the major allele and the two genotypes containing at least one minor allele. All analyses were performed in STATA version 15.1 (StataCorp LP, Texas, USA).

Linkage disequilibrium between *FCGR2C* functional variants and CNRs was computed using the Haploview software package (31) and expressed as D prime (D′) and square of the correlation coefficient (r^2). The closer D′ is to 1 the stronger the LD between two loci. Hardy-Weinberg equilibrium was considered for individuals with two gene copies and the statistics abstracted from the Haploview analysis output. For the analysis, genotypic data with multiple gene copies were considered homozygous if all copies carried the same allele or heterozygous when both alleles were present.

RESULTS

Cohort

This nested case-control study investigated *FCGR2C* genotypic data from 525 children to determine the role of *FCGR2C* variants and HIV-1 acquisition in South African children born to women living with HIV-1. The cohort includes 176 HIV-1 infected (cases) and 349 HIV-exposed-uninfected (controls) children. The HIV-1 infected children comprised four transmission mode groups: *in utero* (n = 19), *in utero*-enriched (n = 46), intrapartum (n = 31) and mixed (n = 99). Overall, there was no significant difference in sex, gestation and breastfeeding status between the HIV-1 infected and HIV-1 uninfected cohort. However, the total HIV-1 infected and HIV-1 exposed-uninfected groups differed significantly in birth weight at delivery. Specifically, a higher proportion of HIV-infected children had a birth weight below 2500 g (22% *vs.* 11%; $P = 0.001$, $P_{Bonf} = 0.016$) (**Table 1**).

FCGR2C Copy Number Distribution and HIV-1 Acquisition

The *FCGR2C* gene, highly homologous to *FCGR2B* in the first six exons and *FCGR2A* in the last two exons, is subject to CNV within previously described distinct regions (**Figure 1**). We did not observe any individual with a complete absence of the *FCGR2C* gene. Overall, *FCGR2C* CNV occurred in 166/525 (32%) children, with the frequency of duplications (n = 114) 2.2-fold higher than deletions (n = 52) (69% *vs.* 31%). The copy number distribution was significantly different between the HIV-1 infected and HIV-1 exposed-uninfected groups but not after Bonferroni correction ($P = 0.010$, $P_{Bonf} > 0.05$) (**Table 2**). Variation in copy number among the whole study cohort was observed more frequently in CNR1, which encompasses a complete *FCGR2C* copy, *HSPA7* and *FCGR3B* (28%; n = 147) than CNR2, with an incomplete *FCGR2C* copy, *HSPA6* and *FCGR3A* (2.7%; n = 14). In six instances (1.1%), we observed CNV for only *FCGR2C* in the absence of duplicated/deleted flanking genes, as previously described among the South African black population (12). A duplication in both CNR1 and CNR2 was observed in one individual. Given the differences between the CNRs, their copy number variability was determined separately.

TABLE 1 | Demographic and clinical characteristics of perinatal HIV-1 acquisition groups.

Variables	HIV-1-exposed uninfected	Total HIV-1 infected		*In utero* infected		*In utero*-enriched infected		Mixed infected		Intrapartum infected	
	n = 349	n = 176	*P* value	n = 19	*P* value	n = 46	*P* value	n = 99	*P* value	n = 31	*P* value
Sex	(n = 346)	(n = 176)	1.000		0.487		0.875		0.569		0. 260
Male	160 (46)	80 (45)		7 (37)		20 (43)		42 (42)		18 (58)	
Female	189 (54)	96 (55)		12 (63)		26 (57)		57 (58)		13 (42)	
Gestation	(n = 327)	(n = 164)	0.355	(n = 18)	0.105	(n = 44)	0.788	(n = 95)	1.000	(n = 25)	**0.013**
Term	295 (90)	143 (87)		14 (78)		39 (89)		86 (91)		18 (72)	(P_{Bonf} =
Preterm (<37 weeks)	32 (10)	21 (13)		4 (22)		5 (11)		9 (9)		7 (28)	0.208)
Birth weight (g)	(n = 344)	(n = 168)	**0.001**		0.251		**0.003**	(n = 92)	**0.001**	(n = 30)	0.898
≥2500	307 (89)	131 (78)	**(P_{Bonf} =**	15 (79)		34 (74)	**(P_{Bonf} =**	70 (76)	**(P_{Bonf} =**	27 (90)	
<2500	37 (11)	37 (22)	**0.016)**	4 (21)		12 (26)	**0.048)**	22 (24)	**0.016)**	3 (10)	
Breastfed	(n = 346)	(n = 170)	1.000	(n = 18)	0.384	(n = 45)	0.117	(n = 95)	0.780	(n = 30)	0.359
No	271 (78)	134 (79)		16 (89)		40 (89)		73 (77)		21 (70)	
Yes	75 (22)	36 (21)		2 (11)		5 (11)		22 (23)		9 (30)	

Data are n (%) unless otherwise specified.
The P values refer to comparisons between the HIV-1-exposed uninfected (control) group and each of the HIV-1 infected (case) groups.
Bold indicates statistical significance of $P < 0.05$; P_{Bonf}, Bonferroni corrected P value.

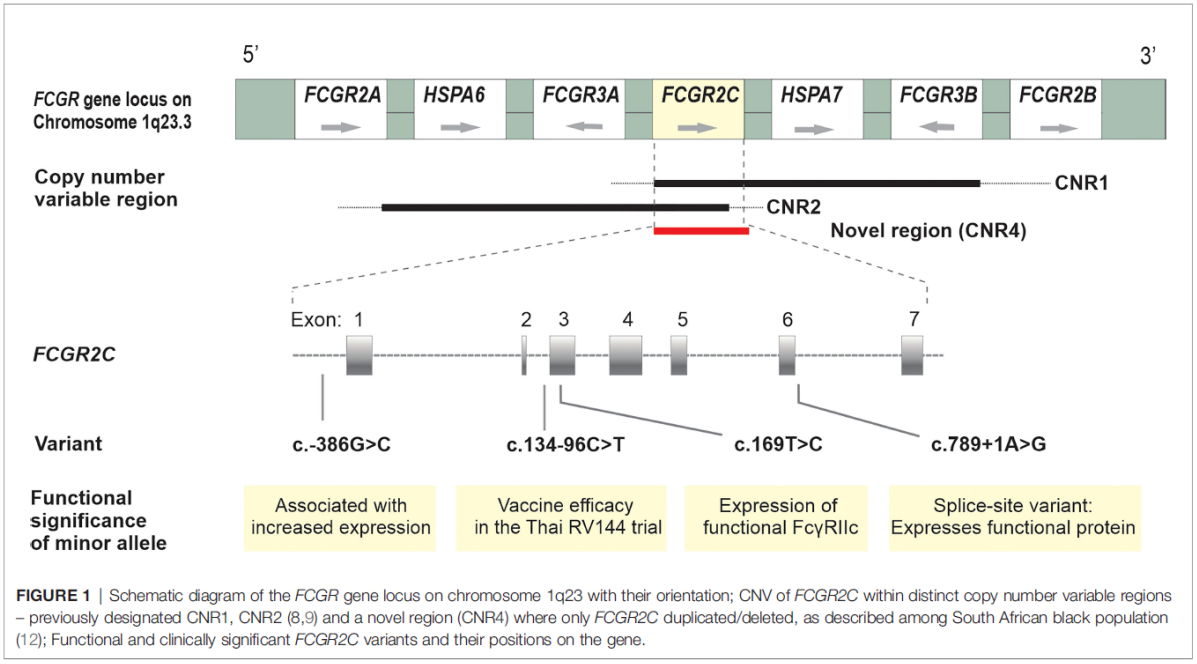

FIGURE 1 | Schematic diagram of the *FCGR* gene locus on chromosome 1q23 with their orientation; CNV of *FCGR2C* within distinct copy number variable regions – previously designated CNR1, CNR2 (8,9) and a novel region (CNR4) where only *FCGR2C* duplicated/deleted, as described among South African black population (12); Functional and clinically significant *FCGR2C* variants and their positions on the gene.

Within CNR1, CNV was significantly different between the HIV-1 infected and HIV-1 exposed-uninfected groups (P = 0.009, P_{Bonf} > 0.05). This difference was primarily determined by gene deletions. There were a higher number of HIV-1 exposed-uninfected children with a single gene copy compared to HIV-1 infected children (36% *vs.* 14%) (**Table 2**). Using two *FCGR2C* gene copies as reference, the possession of a single gene copy was independently associated with reduced odds of HIV-1 acquisition (OR = 0.29; 95% CI 0.12-0.71; P = 0.007, P_{Bonf} > 0.05) and retained significance after controlling for birthweight and *FCGR2C* genotypes (AOR = 0.37; 95% CI 0.15-0.90; P = 0.029, P_{Bonf} > 0.05) (**Table 3**). The CNR2 and the novel CNR4 variability were excluded from further association analysis due to low frequencies (< 5%).

FCGR2C Variants That Determine the Expression of Surface FcγRIIc

We further genotyped functional *FCGR2C* variants that determine the expression of FcγRIIc (c.−386G>C, c.169T>C and c.798+1A>G) and the c.134-96C>T variant that associated with risk of HIV-1 acquisition in the Thai RV144 HIV-1 vaccine trial (**Figure 1**). To assess the role of the *FCGR2C* genotypes and allele distribution in perinatal HIV-1 acquisition, children with a single *FCGR2C* gene copy were considered homozygous; those with more than one *FCGR2C* gene copy were considered homozygous if all the alleles were the same or heterozygous if both alleles were present. With MLPA, we obtained genotypic data from 166 out of the 176 HIV-1 infected for c.169T>C and c.−386G>C variants.

A SNP in exon 3 (c.169T>C) that results in an open reading frame (ORF) or a stop codon determines the expression of FcγRIIc when present with the minor allele of two splice variants in intron 7 (c.798+1A>G and c.799-1G>C). While 129/515 (25%) of individuals carried at least one *FCGR2C*-ORF (c.169C allele), only 4/129 (3%) individuals possessed the c.798 +1G minor allele that represents the classic *FCGR2C*-ORF and predicts FcγRIIc expression (6, 32). Of the four individuals with the c.798+1G minor allele, three were HIV-1-exposed uninfected and one HIV-1 infected. Conversely, the c.169C allele co-occurred with the c.798+1A allele in 97% (n = 125) of the participants, representing the non-classic *FCGR2C*-ORF that does not yield surface expression of FcγRIIc. The c.799-1G>C splice cite variant was not genotyped, since it has been shown that the c.169C and c.798+1A alleles are syntenic with the c.799-1G allele in South African population (12).

While the c.169T>C variant alone does not result in surface expression of FcγRIIc, it may have other unknown functional consequences and was therefore investigated for a possible association with HIV-1 perinatal transmission. The c.169T>C genotype distribution was significantly different between HIV-1 infected and HIV-1 exposed-uninfected children (P = 0.002, P_{Bonf} = 0.032) (**Figure 2**i). In a dominant model, the c.169C was overrepresented in the HIV-1 infected compared to the uninfected children (32% *vs.* 22%) and significantly associated with increased odds of HIV acquisition (OR = 1.68; 95% CI 1.11-2.55; P = 0.013, P_{Bonf} > 0.05). The strength of association increased after adjusting for birthweight and CNR1 copy number (AOR = 2.39; 95% CI 1.45-3.95; P = 0.001, P_{Bonf} = 0.016). For the *FCGR2B/C* promoter variant at position −386G>C, which modulates gene expression levels, no significant difference in genotype frequency was observed between the HIV-1 infected and HIV-1 uninfected cohort

TABLE 2 | *FCGR2C* copy number distribution in HIV-1-exposed infected and uninfected infants.

Variables	Total study cohort	HIV-1-exposed uninfected	Total HIV-1 infected		*In utero* infected		*In utero*-enriched infected		Mixed infected		Intrapartum infected	
	n = 525	n = 349	n = 176	*P* value	n = 19	*P* value	n = 46	*P* value	n = 99	*P* value	n = 31	*P* value
FcγRIIc copy number				**0.010** (P_{Bonf} =		0.672		0.142		0.095		**0.028** (P_{Bonf} =
1 copy	52 (10)	44 (12)	8 (4)	0.16)	1 (5.3)		3 (6)		5 (5)		0 (0)	0.448)
2 copies	359 (68)	233 (67)	126 (72)		13 (68.4)		28 (61)		71 (72)		27 (87)	
≥3 copies	114 (22)	72 (21)	42 (24)		5 (26.3)		15 (33)		23 (23)		4 (13)	
CNR1	(n = 147)	(n = 105)	(n = 42)	**0.009** (P_{Bonf} =	(n = 5)	0.162	(n = 15)	**0.035** (P_{Bonf} =	(n = 23)	0.228	(n = 4)	0.296
deletion	44 (30)	38 (36)	6 (14)	0.144)	0 (0)		1 (7)	0.56)	5 (22)		0 (0)	
duplication	103 (70)	67 (64)	36 (86)		5 (100)		14 (93)		18 (78)		4 (100)	
CNR2	(n = 14)	(n = 8)	(n = 6)	0.767	(n = 1)	0.444	(n = 2)	0.444	(n = 4)	0.180	(n = 0)	
deletion	5 (36)	3 (37.5)	2 (33)		1(100)		2 (100)		0		0	
duplication	9 (64)	5 (62.5)	4 (67)		0 (0)		0		4 (100)		0	
CNR4	(n = 6)	(n = 3)	(n = 3)	0.100	(n = 0)		(n = 1)	0.250	(n = 2)	0.100	(n = 0)	
deletion	3 (50)	3 (100)	0		0		0		0)		0	
duplication	3 (50)	0	3 (100)		0		1 (100)		2(100)		0	

Data are n (%) unless otherwise specified.
The P values refer to comparisons between the HIV-1-exposed uninfected (control) group and each of the HIV-1 infected (case) groups.
Bold indicates statistical significance of P < 0.05; P_{Bonf}, Bonferroni corrected P value.

($P = 0.288$) (**Figure 2**ii). The homozygous −386 CC genotype was not observed in any individual. Due to the low frequency of the splice site variant c.798+1A>G minor allele, an association analysis was not conducted.

The Thai *FCGR2C* Haplotype Tag Variant c.134-96C>T Associates With Increased Odds of Perinatal HIV-1 Transmission

The *FCGR2C* c.134-96C>T genotype distribution was significantly different between the children who acquired HIV-1 and those who did not ($P = 0.002$, $P_{Bonf} = 0.032$) (**Figure 2**iii). In particular, the c.134-96T-allele was overrepresented in the HIV-1-infected children compared to the exposed-uninfected children (58% *vs.* 42%; $P = 0.001$, $P_{Bonf} = 0.016$). The overrepresentation was primarily driven by the *in utero*-enriched (63% *vs.* 42%; $P = 0.011$, $P_{Bonf} > 0.05$) and mixed (59% *vs.* 42%; $P = 0.004$, $P_{Bonf} > 0.05$) infected groups (**Figure 2**iii). We combined the *in utero*-enriched and mixed transmission groups into a larger *in utero*-enriched group, excluding the 27 HIV-1 infected and 75 HIV-1 exposed uninfected breastfed individuals, and still observed overrepresentation of the minor allele in the HIV-1 infected children (60% *vs.* 43%; $P = 0.002$, $P_{Bonf} = 0.048$) (data not shown).

The association between *FCGR2C* c.134-96C>T variant and HIV acquisition was assessed in a univariate model and a multivariate model that controlled for birthweight and CNR1 copy number, which were statistically significant at univariate analysis (**Table 4**). In a dominant model, the c.134-96C>T minor allele was associated with increased odds of perinatal HIV-1 transmission at univariate (OR = 1.89; 95% CI 1.31-2.73; $P = 0.001$, $P_{Bonf} = 0.016$) and multivariate analysis (AOR = 1.89; 95% CI 1.25-2.87; $P = 0.003$, $P_{Bonf} = 0.048$). The association was specific to the *in utero*-enriched (OR = 2.34; 95% CI 1.24-4.42; $P = 0.009$, $P_{Bonf} > 0.05$) and the mixed transmission groups (OR = 1.94; 95% CI 1.24-3.06; $P = 0.004$, $P_{Bonf} > 0.05$) but not the *in utero* (OR = 1.24; 95% CI 0.49-3.12; $P = 0.653$) and intrapartum groups (OR = 1.29; 95% CI 0.62-2.69; $P = 0.500$). Statistical significance was retained in both *in utero*-enriched (AOR = 2.49; 95% CI 1.31-4.76; $P = 0.006$, $P_{Bonf} > 0.05$) and mixed transmission groups (AOR = 2.06; 95% CI 1.28-3.30; $P = 0.003$, $P_{Bonf} = 0.048$) after adjusting for birthweight.

TABLE 3 | Effect of *FCGR2C* CNR1 copy number distribution on perinatal HIV-1 acquisition, adjusting for birthweight and *FCGR2C* genotypes.

FcγRIIc copy number (Total group)	Univariate		Multivariate	
	OR (95% CI)	P-value	Adjusted OR	P-value
1 copy	0.29 (0.12-0.71)	**0.007** ($P_{Bonf} = 0.112$)	0.37 (0.15-0.90)	**0.029** ($P_{Bonf} = 0.464$)
2 copies	Ref		Ref	
≥3 copies	0.99 (0.63-1.57)	0.978	0.74 (0.43-1.27)	0.275

OR, Odds Ratio; CI, Confidence Interval; P_{Bonf}, Bonferroni corrected P value.
Bold indicates statistical significance of P < 0.05.

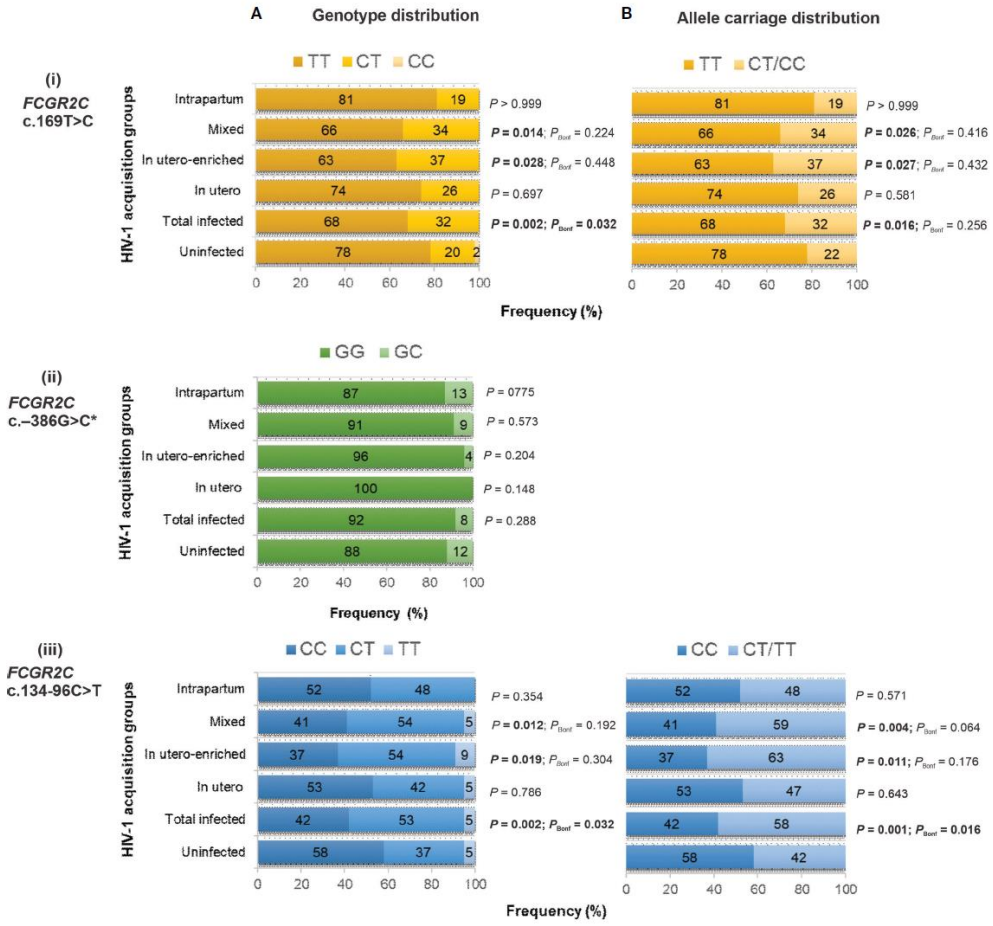

FIGURE 2 | Genotype **(A)** and allele carriage **(B)** distribution of functional *FCGR2C* variants and their association with perinatal HIV-1 acquisition in black South Africans [HIV-1-exposed uninfected (n = 349), total infected (n = 176 for c.134-96C>T; n = 166 for c.169T>C and c.–386G>C), *in utero* infected (n = 19), *in utero*-enriched infected (n = 46), mixed infected (n = 99 for c.134-96C>T; n = 89 for c.169T>C and c.–386G>C) and intrapartum infected (n = 31)]. * No individual with homozygous –386 CC genotype.

The *FCGR2C* c.134-96C>T and c.169T>C Are in High Linkage Disequilibrium

The observed genotype frequencies for *FCGR2C* c.–386G>C, c.134-96C>T, c.169T>C were in Hardy-Weinberg equilibrium ($P > 0.05$). We also observed that 71% (39/55) of children carrying a c.169C were heterozygous for the *FCGR2B/C* promoter variant (data not shown). We analyzed linkage disequilibrium between the *FCGR2C* variants and CNRs, with and without considering the CNV. It was important to determine whether the observed variants associated with HIV-1 acquisition act independently or linkage disequilibrium plays a part. Our result demonstrated high linkage disequilibrium between c.134-96C>T and c.169T>C both without considering CNV ($D' = 0.867$; $r^2 = 0.319$) and when only those with two gene copies were included ($D' = 0.908$ and $r^2 = 0.213$) (**Figure 3**). Both c.134-96C>T and c.169T>C independently associated with increased odds of HIV-1 acquisition, but in multivariate analysis, c.134-96C>T retained significance (AOR = 1.91; 95% CI 1.23-2.96; $P = 0.004$, $P_{Bonf} > 0.05$) while c.169T>C did not (AOR = 1.14; 95% CI 0.70-1.86; $P = 0.590$).

Effect of *FCGR2C* c.134-96C>T and Maternal Viral Load on Perinatal Acquisition of HIV-1

Maternal viral load is a key determinant of MTCT of HIV-1 infection. For the NEVEREST cohorts, the maternal viral load was not recorded. However, in the birth cohort with the defined modes of transmission, information on maternal HIV-1 viral

TABLE 4 | Univariate and multivariate analysis of the effect of *FCGR2C* c.134-96C>T on perinatal acquisition of HIV-1.

Genotype	Univariate		Multivariate	
	OR (95% CI)	*P* value	Adjusted OR (95% CI)	*P* value
*Total infected**				
CC	Ref		Ref	
CT/TT	1.89 (1.31-2.73)	**0.001 (P_{Bonf} = 0.016)**	1.89 (1.25-2.87)	**0.003 (P_{Bonf} = 0.048)**
In utero-enriched#				
CC	Ref		Ref	
CT/TT	2.34 (1.24-4.42)	**0.009** (P_{Bonf} = 0.144)	2.49 (1.31-4.76)	**0.006** (P_{Bonf} = 0.064)
Mixed#				
CC	Ref		Ref	
CT/TT	1.94 (1.24-3.06)	**0.004** (P_{Bonf} = 0.064)	2.06 (1.28-3.30)	**0.003 (P_{Bonf} = 0.048)**
In utero				
CC	Ref			
CT/TT	1.24 (0.49-3.12)	0.653		
Intrapartum				
CC	Ref			
CT/TT	1.29 (0.62-2.69)	0.500		

OR, Odds Ratio; CI, Confidence Interval; P_{Bonf}, Bonferroni corrected P value.
Bold indicates statistical significance of P < 0.05.
**adjusted for birthweight and CNR1 copy number.*
#adjusted for birthweight.

load was available for 279 mothers of whom 70 transmitted HIV to their infants and 209 did not. The mean HIV-1 viral load was significantly higher in transmitting mothers than non-transmitting mothers (4.53 vs. 3.95 $\log_{10}$ copies/ml; $P < 0.0001$). The maternal *FCGR2C* c.134-96C>T variant independently associated with HIV-1 transmission (OR = 1.98; 95% CI 1.16-3.37; $P = 0.012$, $P_{Bonf} = 0.192$) and when controlled for maternal viral load and birthweight (AOR = 2.03; 95% CI 1.14-3.62; $P = 0.016$, $P_{Bonf} = 0.256$). In this small subset, the infant *FCGR2C* c.134-96C>T variant was independently

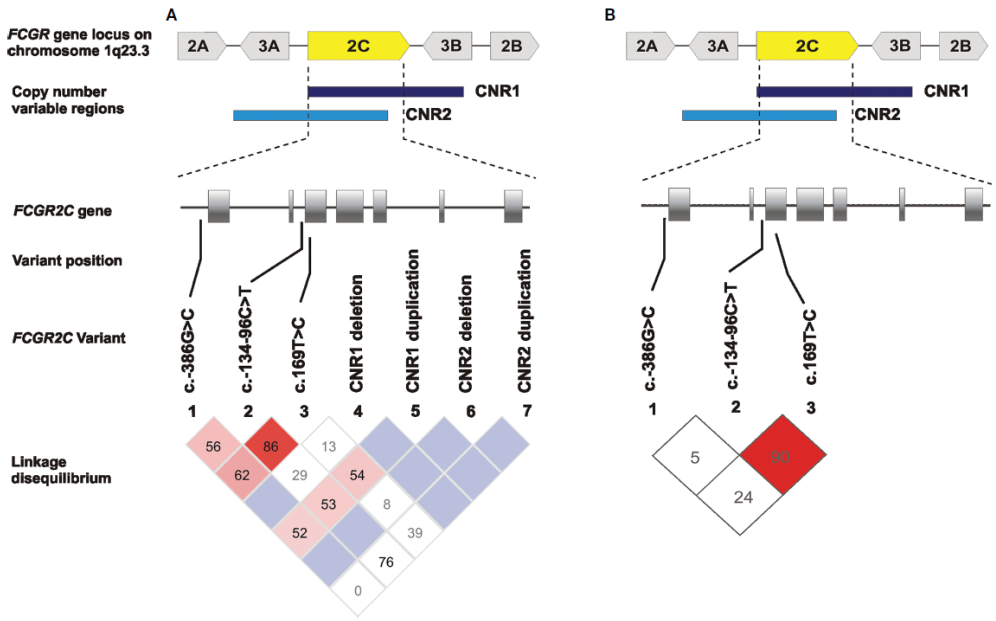

FIGURE 3 | Linkage disequilibrium of the single nucleotide polymorphisms (SNPs) and copy number regions (CNRs) in *FCGR2C* gene in South African children born to women living with HIV-1. **(A)** LD plots for SNPs without considering CNV and **(B)** when only those with two gene copies were included. Values reflect D′ measures of LD and color in the squares given by standard D′/LOD (log of the odds of there being LD between two loci) color scheme, with bright red color used to display very strong LD.

associated with HIV-1 acquisition (OR = 1.92; 95% CI 1.14-3.23; P = 0.014, P_{Bonf} = 0.224). The significant association remained when adjusted for maternal viral load (AOR = 2.10; 95% CI 1.13-3.87; P = 0.018, P_{Bonf} = 0.288), specifically in the *in utero*-enriched transmission group (AOR = 2.67; 95% CI 1.33-5.37; P = 0.006, P_{Bonf} = 0.064) (**Table 5**).

Mother-Child *FCGR2C* c.134-96C>T Genetic Similarity and HIV-1 Acquisition

We next evaluated mother-child *FCGR2C* c.134-96C>T genotype concordance and association with HIV-1 acquisition. Concordance was defined as mother and infant each bearing at least one T allele (mother-child: CT/TT-CT/TT) or both were homozygous for the C allele (CC). Discordance was defined as one individual within the dyad possessing a CC genotype and the other bearing a T allele (CT/TT). Possession of a T allele in both mother and infant was overrepresented in the HIV-1-infected children compared to the exposed-uninfected children (45% *vs.* 28%; P = 0.012, P_{Bonf} = 0.192). Independently, MTCT was more likely among the mother-child concordant CT/TT group compared to the concordant CC group (OR = 2.58; 95% CI 1.36-4.88; P = 0.004, P_{Bonf} = 0.064) and retained significance after adjusting for maternal viral load, birthweight and infant CNR1 copy number (AOR = 2.87; 95% CI 1.36-6.06; P = 0.006, P_{Bonf} = 0.096) (**Table 6**).

DISCUSSION

Previous studies have reported both functional and clinical relevance of *FCGR2C* genetic variability, including single nucleotide polymorphisms and copy number variations. Expression of the membrane-bound FcγRIIc protein in individuals bearing the *FCG2C* c.169T>C minor allele (*FCGR2C*-ORF) has been associated with susceptibility to idiopathic thrombocytopenic purpura (15), Kawasaki disease (32), systemic lupus erythematosus and increased antibody responses to vaccinations (11). Furthermore, the *FCGR2C* c.134-96C>T tag variant associated with reduced risk of HIV-1 infection in the Thai phase 3 RV144 HIV-1 vaccine trial (13) and increased risk of HIV-1 disease progression in black South Africans (18). In addition, increased risk of HIV-1 acquisition in the HVTN 505 vaccine trial was associated with two variants within the Thai *FCGR2C* haplotype but not the c.134-96C>T tag variant (17).

In this study, we report further associations between *FCGR2C* variants and perinatal HIV-1 acquisition in South African children. Deletion of CNRI was significantly protective of perinatal HIV-1 acquisition compared to two gene copies, but the significance was not retained after Bonferroni correction. However, the observed association between CNR1 and perinatal HIV-1 acquisition might not be due to *FCGR2C* copy number variability because *FCGR3B* and *HSPA7* genes are also deleted with CNR1. The associations appear to be unrelated to surface expression of membrane-bound FcγRIIc. While some children carried the c.169C open reading frame allele, the co-occurrence of the splice-site variant c.798+1A allele predicted the absence of FcγRIIc expression in the majority of children. The latter allele gives rise to alternative mRNA splicing and a premature stop codon with associated loss of surface expression (6, 32). Only four children carried both the c.169C open reading frame allele and the splice-site variant c.798+1G allele required for functional expression of FcγRIIc. This finding correlates with previous studies that suggested rare to absent expression of FcγRIIc in the black African population (12, 32) and raises questions around the functional relevance of the c.169T>C variant.

The *FCGR2C* c.134-96T-minor allele was associated with increased odds of perinatally acquired HIV-1 acquisition in South African children. Specifically, a significant association was observed in the *in utero*-enriched and mixed transmission groups but not in the *in utero* and intrapartum transmission groups. The *in utero*-enriched and mixed transmission groups are very similar in terms of drug treatment, as all were exposed to nevirapine for prevention of MTCT. Due to the nevirapine, fewer infants in the mixed group would have had intrapartum transmission and therefore were likely *in-utero* enriched. After adjusting for multiple comparisons using Bonferroni correction, the overall c.134-96C>T association retained significance, primarily driven by the mixed transmission group. The non-significance in the *in utero*-enriched group may potentially be due to small sample size. We postulate that the role of c.134-96C>T in HIV-1 acquisition is more pronounced during the course of pregnancy and at the maternal-fetal interface (3). In a subset of our study cohort, the observed association with

TABLE 5 | Effect of *FCGR2C* c.134-96C>T on perinatal acquisition of HIV-1 in study cohort with information on maternal viral load.

Genotype	Univariate		Multivariate	
	OR (95% CI)	*P* value	Adjusted OR (95% CI)	*P* value
*Total infected**				
CC	Ref		Ref	
CT/TT	1.92 (1.14-3.23)	**0.014** (P_{Bonf} = 0.224)	2.10 (1.13-3.87)	**0.018** (P_{Bonf} = 0.288)
In utero-enriched#				
CC	Ref		Ref	
CT/TT	2.34 (1.24-4.42)	**0.009** (P_{Bonf} = 0.144)	2.67 (1.33-5.37)	**0.006** (P_{Bonf} = 0.064)

OR, Odds Ratio; CI, Confidence Interval; P_{Bonf}, Bonferroni corrected P value.
Bold indicates statistical significance of P < 0.05.
**adjusted for maternal viral load, birthweight and CNR1 copy number.*
#adjusted for maternal viral load and birthweight.

TABLE 6 | Mother-child *FCGR2C* c.134-96C>T genetic similarity and HIV-1 acquisition[a].

Genotype (mother-child pair)	HIV-1-exposed uninfected n = 222	HIV-1 infected n = 76	Univariate		Multivariate*	
			OR (95% CI)	*P* value	OR (95% CI)	*P* value
Concordant CC	94 (42)	20 (26)	Ref		Ref	
Concordant CT/TT	62 (28)	34 (45)	2.58 (1.36-4.88)	**0.004** (P_{Bonf} = 0.064)	2.87 (1.36-6.06)	**0.006** (P_{Bonf} = 0.096)
Discordant	66 (30)	22 (29)	1.57 (0.79-3.10)	0.197	1.50 (0.68-3.29)	0.308

OR, Odds Ratio; CI, Confidence Interval; P_{Bonf}, Bonferroni corrected P value.
Bold indicates statistical significance of P < 0.05.
**adjusted for maternal viral load, birthweight and CNR1 copy number.*
[a]*"Concordant" denotes mother-child pairs with same FCGR2C c.134-96C>T genotype. "Discordant" denotes mother-child pairs with different genotypes.*

FCGR2C c.134-96C>T variant remained when adjusted for maternal viral load, a key determinant of MTCT of HIV-1 infection. In both mother and child, the *FCGR2C* c.134-96C>T variant was associated with HIV-1 transmission and acquisition, respectively. Concordance in mother-child possession of the c.134-96T-allele further associated with a greater risk of MTCT compared to homozygosity for the C-allele in both mother and infant.

Assessing the role of the *FCGR2C* c.134-96C>T tag variant in both HIV-1 acquisition and disease progression has produced contrasting results of both protective and deleterious effects. The Thai phase 3 RV144 HIV vaccine trial provided the first clinical evidence of vaccine-induced protection, where the *FCGR2C* c.134-96C>T tag variant reduced the risk of HIV acquisition (13). Subsequently, two variants within the Thai *FCGR2C* haplotype were associated with increased risk of HIV-1 acquisition in vaccine recipients in the HVTN 505 trial but the c.134-96C>T tag variant was not. The population in this trial was predominantly Caucasian men who have sex with men (17). The *FCGR2C* c.134-96C>T tag variant was also associated with HIV-1 disease progression in South African adults (18). In this mother-child transmission model study, we establish its role in increased predisposition to HIV-1 acquisition. Whether the contrasting associations bear any functional significance is currently not clear and the modulating risk factors may not necessarily overlap (18). The differing results may be attributable to a variety of factors, including genetic differences between populations. It has been shown that two of the three variants within the Thai *FCGR2C* haplotype are absent in the African population, including black South Africans (12).

These findings may be of consequence to efforts aimed at elucidating the different outcomes of the two very similar HIV-1 vaccine efficacy trials, RV144 in Thailand and HVTN702 in South Africa. In addition to population genetic differences, the vaccine regimen evaluated in the RV144 and HVTN702 vaccine efficacy trials were also different. The RV144 vaccine candidate was specific for HIV-1 clades B and E with alum as adjuvant. This vaccine candidate elicited robust cross-clade immune responses in South Africans (33). However, in subsequent clinical trials the RV144 vaccine regimen was modified to increase immunogenicity and improve the duration of vaccine-induced immune responses (34). The vaccine regimen was HIV-1 clade C-specific, the predominant clade in South Africa, and was adjuvanted with MF59 (34, 35). The adapted ALVAC-HIV and Bivalent Subtype C gp120–MF59 vaccine regimen evaluated in HVTN 702 trial showed no efficacy among South African adults, despite previous evidence of immunogenicity (19). The differential vaccine efficacy between the RV144 and HVTN 702 trials might be due to differences in some components of the vaccine regimen and host genetics. In vaccine design, it is important to consider host genetic variation that can modulate vaccine efficacy (36). We previously established that black South Africans do not possess the complete Thai *FCGR2C* haplotype and are only polymorphic for c.134-96C>T (rs114945036) (12).

The functional mechanisms underlying the association of the variants within the Thai *FCGR2C* haplotype with HIV-1 acquisition, disease progression and vaccine efficacy is largely undefined. It raises many biological questions as to how expression of the membrane-bound FcγRIIc protein or the variants could modulate HIV-1 infection. The proposed impact of the *FCGR2C* variant haplotypes on immunity against HIV is unknown, one can only speculate. It is increasingly evident that it does not involve expression of the surface FcγRIIc receptor, since a limited number of individuals in the available genetic association studies bear the minor alleles that predict expression of the surface molecule. It is unknown whether a truncated soluble form of the FcγRIIc protein is produced in individuals with the premature stop codon or alternative splicing variants that prevent surface expression of FcγRIIc. Results from a study suggest that variants within the Thai *FCGR2C* haplotype either directly associate with the expression of *FCGR2C* in human B cells or in correlation with other causal variants that affect expression levels (36). The correlation with *FCGR2A* was observed across different populations and was specific to the c.134-96C>T variant (36, 37). We have proposed that the variants modulate transcription factor binding that may alter mRNA expression (18). This may in turn affect processes regulated by mRNA from the *FCGR2C* gene.

In conclusion, the *FCGR2C* variant c.134-96T-allele, which was associated with protection in the Thai RV144 trial, was associated with increased odds of perinatal HIV-1 acquisition in black South African children. This adds to other deleterious associations found for *FCGR2C* variants in the context of HIV-1 (17, 18) and warrants investigation of these variants in South African adults actively immunized in the HVTN 702 trial (19) and individuals passively immunized with the broadly neutralizing VRC01 antibody in the Antibody Mediated Prevention (AMP) trials (38). Collectively, these intriguing results highlight the need for further mechanistic studies to

establish the functional relevance of *FCGR2C* variation in different populations and more broadly in contexts of vaccination and disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethics approval for the study was obtained from the University of the Witwatersrand Human Research Ethics Committee (Reference numbers: M170585; M180575). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

RS, GG, and LK recruited study participants and acquired clinical data. MG extracted DNA from blood samples. JE and MP genotyped individuals. JE managed and analyzed the data. JE and RL wrote the first draft of the manuscript. CT and RL designed the study and supervised the research. All co-authors critically revised the manuscript for intellectual content. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.760571/full#supplementary-material

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