A Longitudinal Perspective on the Impact of Immune Status on the HIV-1 Latent Reservoir and Neurocognitive Outcomes in Virologically Suppressed Children

by

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third-party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

April 2022

Abstract

Background: Children remain the most vulnerable population affected by the Human Immunodeficiency Virus-1 (HIV-1) pandemic whose reliance on lifelong therapy is accompanied by several immune abnormalities. In the absence of an effective vaccine, there is currently a strong emphasis on HIV-1 "cure" and although cART leads to viral suppression the virus is still able to establish latent reservoirs within host cells and this is considered the major barrier to achieving cure. Immune factors are considered critically important for the establishment and maintenance of HIV-1 latent reservoirs, but these factors are not well characterised, particularly in children. Delineating and understanding the immunological mechanisms that drive HIV-1 persistence and other chronic diseases such as metabolic, cardiovascular and neurodegenerative diseases is important in children approaching adolescence. Longitudinal studies evaluating the relationship between immune status, the HIV-1 latent reservoir and neurocognitive outcomes are limited, and inadequately studied, specifically within the South African subtype C context. The aim of this study was to longitudinally investigate and characterise host immune status before and after early initiated, delayed and interrupted cART in relation to HIV-1 latent reservoir size and neurocognitive outcomes in perinatally HIV-1 infected children.

Methods: Study participants originated from the well-characterised Children with HIV Early AntiRetroviral Therapy (CHER) randomised controlled trial. This was a descriptive study that employed both a longitudinal (birth to 8 years of age) and cross-sectional study design and analysed samples collected retrospectively and prospectively. For the extensive longitudinal evaluation of immunological biomarker (cytokine, chemokine and receptor antagonist) profiling, we utilised Luminex® Multiplex Assays as well as Enzyme Linked ImmunoSorbent Assays (ELISAs). Multiparameter flow cytometry was utilised for the analysis of monocyte subset distribution. The quantitative viral outgrowth assay (QVOA) was implemented on a subset of participants for measuring of the HIV-1 replication-competent viral reservoir in conjunction with a novel highly sensitive RT-qPCR single copy assay targeted at HIV-1 integrase (iSCA). In addition, HIV-1 cell-associated DNA, specific for integrase (iCAD), were measured longitudinally as a molecular biomarker of HIV-1 persistence and correlated to immune and neurocognitive parameters. Longitudinal neurocognitive assessments were completed independently and correlated to immune, virological and clinical parameters. Age and demographically matched HIV exposed uninfected (HEU) and HIV unexposed uninfected (HUU) were included at the last time point (8 years of age).

Key Findings: HIV-1 infected (HIV+) children showed significantly higher levels of biomarkers associated with generalised chronic inflammation, particularly those associated with myeloid cell activation compared to HEU and HUU controls at 8 years of age. These included hsCRP ($p=0.01$), MIP-1β ($p=0.03$), IL-1α ($p<0.001$), INF-α ($p<0.001$), CD40L ($p<0.001$), sCD14, sCD163, IL-18, IL-17F ($p<0.001$) and PDGF-BB ($p<0.00001$). We also observed a significant elevation of soluble calcium binding alarmin protein involved in the regulation of the inflammatory process and immune response, s100A8/A9, in the HIV+ group compared to the two study control groups ($p<0.001$). Within the HIV+ group, significant elevation of biomarkers associated with gut epithelial damage was observed at 8 years of age. IL-18 was the only immune biomarker that significantly correlated with HIV-1 CAD at baseline ($r = +0.35$; $p=0.04$) and at the 8-year follow-up ($r = +0.38$; $p=0.02$)

and associated to the innate IL-1 family of immune biomarkers including IL-1RA (r = +0.33; p<0.01), IL-1α (r = +0.22; p<0.01), IL-1β (r = +0.26; p<0.01), and IL-1RA (r = +0.32; p<0.01). IL-18 also significantly correlated with biomarkers of monocyte/macrophage activation and gut damage, including: sCD14 (r = +0.50; p<0.01), sCD163 (r = +0.40; p<0.01), LBP (r = +0.26; p<0.01) and MIP-1β (r = +0.19; p=0.02). IL-18 showed significant negative associations (p<0.01) with CD4 % at baseline, longitudinal CD4 count and CD4:CD8 ratio at 8 years (r = +0.35; p=0.04). For longitudinal cytokine analysis, principal component analysis indicated that about 36% of the soluble biomarkers measured including: IL-15, TNFβ, GCSF, IL-1RA, IL-5, IL-4, IL-8, IL-10 and RANTES contributed to the largest parameter change over time across all treatment groups. The key early clinical predictors of plasma biomarker expression over time include time to therapy initiation, time to viral suppression, longitudinal CD4% and absolute count and CD4 and CD8% and absolute counts at birth. Neurocognitive outcomes can be predicted by early immunological, virological and clinical parameters.

Conclusion: By investigating and profiling participants from the CHER randomised control trial cohort, we were able to provide important insights into immunological mechanisms that contribute to driving HIV-1 persistence during long-term suppressive therapy.

The findings reported in this thesis have highlighted some key features of immune abnormalities that persists after early initiated long-term ART in paediatric populations. The evaluation of the persistence of any of the key associations through and beyond adolescence will aid in building a lifelong profile of immune changes in the MTCT-infected children. This research also provides important knowledge to further exploring PHIV children as potential “cure” and remission candidates.

Opsomming

Agtergrond: Kinders bly die mees kwesbare groep wat deur die Menslike Immuniteitsgebrekvirus-1 (MIV-1) pandemie geraak word en wie se afhanklikheid van lewenslange behandeling met verskeie immuunafwykings gepaard gaan. In die afwesigheid van 'n doeltreffende entstof, is daar tans 'n sterk klem op MIV-1 "genesing" en alhoewel kombinasie antiretrovirale behandeling (cART) tot virale onderdrukking lei, is die virus steeds in staat om latente reservoirs binne gasheerselle te vestig en dit word beskou as die grootste struikelblok vir die bereiking van genesing. Immuunfaktore word as krities belangrik geag vir die vestiging en instandhouding van MIV-1 latente reservoirs en om te bepaal of 'n reservoir gevestig en in stand gehou word, maar hierdie faktore is nie goed gekarakteriseer nie, veral nie by kinders nie. Die afbakening en begrip van die immunologiese meganismes wat MIV-1-volharding en ander chroniese siektes dryf, is belangrik by kinders wat adolessensie nader. Longitudinale studies wat die verband tussen immuunstatus, die MIV-1 latente reservoir en neurokognitiewe uitkomste evalueer is beperk en onvoldoende bestudeer, spesifiek binne die Suid-Afrikaanse subtipe C konteks.

Metodes: Studiedeelnemers is uit die goedgekarakteriseerde Kinders met Vroeë AntiRetrovirale (CHER) gerandomiseerde kontroleproef geneem in ingesluit. Dit was 'n beskrywende studie wat beide 'n longitudinale (geboorte tot 8 jaar oud) en deursnee studie-ontwerp gebruik het en monsters wat retrospektief en prospektief versamel is, ontleed het. Vir die uitgebreide longitudinale evaluering van immunologiese biomerker (sitokien-, chemokien- en reseptorantagonis) profiele, het ons Luminex® multipleks en ensiem-gekoppelde immuunsorbent toetse (ELISAs) gebruik. Multiparameter vloeisitometrie is gebruik vir die ontleding van monosiet subset verspreiding. Die kwantitatiewe virale uitgroei-toets (QVOA) is op 'n subgroep deelnemers geïmplementeer vir die meting van die MIV-1-replikasie-kompetente virale reservoir in samewerking met 'n nuwe hoogs sensitiewe RT-qPKR enkelkopie-toets wat op MIV-1-integrase (iSCA) gerig is. Daarbenewens is MIV-1 sel-geassosieerde DNS, spesifiek vir integrase (iCAD), longitudinaal gemeet as 'n molekulêre biomerker van MIV-1 volharding en gekorreleer met immuun en neurokognitiewe parameters. Longitudinale neurokognitiewe assesserings is onafhanklik voltooi en gekorreleer met immuun-, virologiese en kliniese parameters. Ouderdom en demografies ooreenstemmende MIV-blootgestelde ongeïnfekteerde (HEU) en MIV-onblootgestelde ongeïnfekteerde (HUU) is op die laaste tydstip (8 jaar oud) ingesluit.

Sleutelbevindinge: MIV-1-geïnfekteerde (MIV+) kinders het aansienlik hoër vlakke van biomerkers getoon wat met algemene chroniese inflammasie geassosieer word, veral dié wat met myeloïedselaktivering geassosieer word in vergelyking met HEU- en HUU-kontroles op 8-jarige ouderdom. Dit het hsCRP ($p=0.01$), MIP-1β ($p=0.03$), IL-1α ($p<0.001$), INF-α ($p<0.001$), CD40L ($p<0.001$), sCD14, sCD163, IL-18 ingesluit, IL-17F ($p<0,001$) en PDGF-BB ($p<0,00001$). Ons het ook 'n beduidende toename van oplosbare kalsiumbindende alarmienproteïen waargeneem wat betrokke is by die regulering van die inflammatoriese proses en immuunrespons, s100A8/A9, in die MIV+-groep in vergelyking met die twee kontrolegroepe ($p<0.001$). Binne die MIV+-groep is beduidende verhoging van biomerkers geassosieer met derm-epiteelskade op 8-jarige ouderdom waargeneem. IL-18 was die enigste immuun biomerker wat beduidend gekorreleer het met MIV-1 CAD by basislyn ($r = +0.35$; $p=0.04$) en by die 8-jaar opvolg ($r = +0.38$; $p=0.02$) en geassosieer met die

aangebore IL-1 familie van immuun biomerkers insluitend IL-1RA ($r = +0.33$; $p<0.01$), IL-1α ($r = +0.22$; $p<0.01$), IL-1β ($r = +0.26$; $p<0.01$), en IL-1RA ($r = +0.32$; $p<0.01$). IL-18 het ook beduidend gekorreleer met biomerkers van monosiet/makrofageaktivering en dermskade, insluitend: sCD14 ($r = +0.50$; $p<0.01$), sCD163 ($r = +0.40$; $p<0.01$), LBP ($r = +0.26$; $p<0.01$) en MIP-1β ($r = +0.19$; $p=0.02$). IL-18 het betekenisvolle negatiewe assosiasies ($p<0.01$) getoon met CD4 % by basislyn, longitudinale CD4 telling en CD4:CD8 verhouding op 8 jaar ($r = +0.35$; $p=0.04$). Vir longitudinale sitokien-analise het hoofkomponent-analise aangedui dat ongeveer 36% van die oplosbare biomerkers gemeet insluitend: IL-15, TNFβ, GCSF, IL-1RA, IL-5, IL-4, IL-8, IL-10 en RANTES bygedra het tot die grootste parameterverandering oor tyd oor alle behandelingsgroepe. Die sleutel vroeë kliniese voorspellers van plasma-biomerkeruitdrukking oor tyd sluit in tyd tot terapie-inisiasie, tyd tot virale onderdrukking, longitudinale CD4% en absolute telling en CD4 en CD8% en absolute tellings by geboorte. Neurokognitiewe uitkomste kan deur vroeë immunologiese, virologiese en kliniese parameters voorspel word. Gevolgtrekking: Deur deelnemers van die CHER-gerandomiseerde kontroleproefkohort te ondersoek en te profileer, kon ons belangrike insigte verskaf oor immunologiese meganismes wat bydra tot die aandryf van MIV-1-volharding tydens langtermyn-onderdrukkende terapie. Die bevindinge wat in hierdie tesis gerapporteer word, het 'n paar sleutelkenmerke van immuunafwykings uitgelig wat voortduur

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2018	International AIDS Society (IAS) for conference travel scholarships
2018	South African Immunology Society (SAIS) for conference travel scholarships
2017	Conference on Retroviruses and Opportunistic Infections (CROI) for conference travel

Dedication

In loving memory of my two angel mothers, Damini Naidoo and Patsy Govender.

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List of Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ANI	Asymptomatic Neurocognitive Impairment
ANOVA	Analysis of Variance
APOBEC 3G	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G
BBB	Blood Brain Barrier
BCM	B-cell media
Beery-VMI	Beery-Buktenica Development Test for Visual-Motor Integration
bNAbs	Broadly Neutralizing Antibodies
BST-2	Bone Marrow Stromal Cell Antigen 2
CAD	Cell-associated DNA
CAMs	CNS associated macrophages
cART	Combination Antiretroviral Therapy
CCL2	C–C motif chemokine ligand 2
CCR5	C-C chemokine receptor type 5
CD4	Cluster of Differentiation 4
cDNA	Complementary DNA
CHER	Children with Early Antiretroviral Therapy
CMV	Cytomegalovirus
CNS	Central Nervous System
COVID-19	Coronavirus Disease 2019
CPM	Complete PBMC Media
CSF	Cerebrospinal Fluid
CXCL12	C-X-C Motif Chemokine Ligand 12
CXCR4	C-X-C chemokine receptor type 4
DAMPs	Damage-associated molecular patterns
DC	Dendric Cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DoH	Department of Health
EBV	Epstein-Barr virus
ELISA	Enzyme-Linked ImmunoSorbent Assay
EndoCAb	Endogenous endotoxin-core antibody
FBS	Foetal Bovine Serum
FGF	Fibroblast Growth Factor
GALT	gut-associated lymphoid tissue

GIT	Gastrointestinal Tract
GMCSF	Granulocyte-macrophage colony-stimulating factor
GMDS	G*riffiths Mental Development Scale*
HAART	Highly Active Antiretroviral Therapy
HAND	HIV-Associated Neurocognitive Disorder
HEU	HIV-1 Exposed Uninfected
HIV-1	Human Immunodeficiency Virus
HIVE	HIV encephalitis
HLA	human leukocyte antigen
HLA-DR	Human Leukocyte Antigen – DR isotype
HRP	Horseradish Peroxidase
hsCRP	Highly Sensitive C-Reactive Protein
HUU	HIV-1 Unexposed Uninfected
iCAD	integrase Cell-Associated DNA
IFABP	Intestinal Fatty-Acid Binding Protein
IFN	Interferon
IL	Interleukin
IPDA	Intact Proviral DNA Assay
IQR	Interquartile Ranges
iSCA	integrase Single Copy Assay
IUPM	Infectious Units per Million cells.
IVR	Infectious Virus Recovery
LN2	Liquid Nitrogen
LPS	Lipopolysaccharide
LSD	Least Significant Differences (Means)
LTR	Long-terminal Repeat
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
MAIT	Mucosal associated invariant T cells
MALT	Mucosa Associated Lymphoid Tissue
MCP	Monocyte chemoattractant protein-1
MDS	Multi-dimensional Scaling
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MND	mild neurocognitive disorders
MRI	Magnetic resonance imaging
MTCT	Mother-to-child Transmission

NAA	*N-acetyl-aspartate*
NK	Natural Killer
NLRP	Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRT	no reverse transcriptase control
NVP	Nevirapine
OD	Optical Density
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PD-1	Programmed Death 1
pDCs	Plasmacytoid dendritic cells
PDGF	Platelet-derived growth factor
PHA	Phytohemagglutinin
PHIV	Perinatal HIV-1 infected
PLWH	People Living with HIV-1
PMA	Phorbol 12-myristate 13-acetate
PMTCT	Prevention of Mother-to-Child Transmission
qPCR	quantitative polymerase chain reaction
QVOA	Quantitative Viral Outgrowth Assay
RANTES	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
RCAS	Replication-competent avian retrovirus
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SAMHDI	SAM domain and HD domain-containing protein 1
SANAS	South African National Accreditation System
SD	Standard Deviation
SIV	Simian Immunodeficiency Virus
START	Strategic Timing of Anti-Retroviral Treatment
STCM	Super T cell Media
Tcm	T central memory
Tem	T effector memory
TFh	T follicular helper
TGFβ	Transforming growth factor beta
Th17	T helper 17
TILDA	Tat/rev Induced Limiting Dilution Assay

TNF	Tumour Necrosis Factor
TRIM-5α	Tripartite motif-containing protein 5 alpha
Tscm	T stem cell memory
Ttm	T transitional memory
TVR	Total Virus Recovery
VEGF	Vascular endothelial growth factor
VISCONTI	Virological and immunological studies in controllers after treatment interruption
WHO	World Health Organization
WLHIV	Women Living with HIV

Research Outputs

Below is a list of research outputs completed and contributed towards during the time of this doctoral research:

Manuscripts in Preparation

1. Naidoo S., Cotton M. F., and Glashoff R. H. ***Review: Immunological dysfunction during long-term virological suppression in children with perinatal HIV-1 infection***

2. Naidoo S., Oosthuizen D., Veldsman. K.A., Maponga, T.., Laughton. B., Cotton M. F.., and Glashoff R.H. ***Evidence of persisting gut permeability associated chronic inflammation despite early, suppressive therapy in perinatally HIV-1 infected children at 8 years of age***

3. Naidoo S., Veldsman K., Van Zyl G. U., Laughton. B., Cotton M. F., and Glashoff R.H. ***Elevated IL-18 is associated with HIV-1 cell-associated DNA in pre-adolescent children: relationship with other key innate biomarkers***

4. Naidoo S., Laughton B., Cotton M. F., and Glashoff R. H. ***Longitudinal assessment of plasma inflammatory biomarker association with measures of neurocognitive impairment in virologically suppressed children with perinatal HIV-1 infection***

List of Publications (Doctoral Research Period)

1. Ruhanya V, Jacobs GB, Naidoo S, Paul RH, Joska JA, Seedat S, Nyandoro G, Engelbrecht S and Glashoff RH. *Impact of Plasma IP-10/CXCL10 and RANTES/CCL5 Levels on Neurocognitive Function in HIV Treatment-Naive Patients*. **AIDS Res Hum Retroviruses**. **2021 Feb 17**. doi: 10.1089/AID.2020.0203. Epub ahead of print. PMID: 33472520.

2. Laughton B, Naidoo S, Dobbels E, Boivin MJ, Janse van Rensburg A, Glashoff R, van Zyl GU, Kruger M and Cotton MF. *Neurodevelopment at 11 months after starting antiretroviral therapy within 3 weeks of life*. **South Afr J HIV Med. 2019 Oct 30;20(1):1008**. doi: 10.4102/sajhivmed.v20i1.1008. PMID: 31745434; PMCID: PMC6852492.

3. Veldsman KA, Janse van Rensburg A, Isaacs S, Naidoo S, Laughton B, Lombard C, Cotton MF, Mellors JW and van Zyl GU. *HIV-1 DNA decay is faster in children who initiate ART shortly after birth than later*. **J Int AIDS Soc. 2019 Aug;22(8):e25368**. doi: 10.1002/jia2.25368. PMID: 31441231; PMCID: PMC6868516.

List of Research Honours and Awards

2019 International AIDS Society (IAS) Scholarship Award, Mexico City, Mexico

2019 Polio Research Foundation (PRF) Bursary Award

2018 **L'Oréal-UNESCO for Women in Science Fellowship Award, Nairobi, Kenya**

2018 **Dominique Dormont Prize (Awarded by Françoise Barré-Sinoussi)**

2018 International AIDS Conference Scholarship Award, Amsterdam, Netherlands

2018 Polio Research Foundation (PRF) Bursary Award

2017 **Young Investigator's Scholarship Award, CROI, Seattle, Washington**

2017 International IAS Conference Scholarship Award, Paris France

2017 South African Immunology Society Scholarship Award, Cape Town South Africa

2017 Polio Research Foundation (PRF) Bursary Award

List of Conference Abstracts Accepted and Presented (Local and International)

1. Naidoo, S.; Maponga, T.; Veldsman, K.; Laughton, B.; Cotton, M.F.; Glashoff, R.H. *Decreased ex vivo T-cell proliferation and increased immune exhaustion in early treated and long-term suppressed HIV infected pre-adolescents from the CHER trial: implications for cure strategies.* **International AIDS Society (IAS) Conference on HIV Science, July 2019, Mexico City, Mexico** (Oral)

2. Laughton, B.; Naidoo, S.; Dobbels, E.F.M.; Boivin, M.J.; Janse van Rensburg, A.; Glashoff, R.H.; van Zyl, G.U.; Kruger, M.; Cotton, M.F. *Neurodevelopmental outcome at 11 months in perinatally HIV-infected infants: Does starting very early antiretroviral therapy help?* **International Workshop on HIV Pediatrics, July 2019, Mexico City, Mexico** (Poster)

3. Naidoo, S.; Maponga, T.; Veldsman, K.; Laughton, B.; Cotton, M.F.; and Glashoff, R.H. *An Assessment of unresolved immune dysregulation despite early therapy, normalised CD4 counts and low HIV-1 cell infectivity in HIV-infected children approaching adolescence in South Africa*. **VIIth Conference of the South African Immunology Society, June 2019, Durban, South Africa (Oral)**

4. *Veldsman, K.A.; Janse van Rensburg, A.; Isaacs, S.; Naidoo, S.; Laughton, B.; Lombard, C.; Cotton, M.; Mellors, J.W.; van Zyl, G.U. Early ART start in children is associated with more rapid decay of HIV-1 DNA.* **Conference on Retroviruses and Opportunistic Infections (CROI), March 2019, Seattle, Washington** (Poster)

5. Naidoo, S.; Veldsman, K, Cotton, M.F.; Glashoff, R.H. *Persistence of myeloid cell-associated inflammation in HIV-infected children after 8 years on early initiated therapy - The key role players in HIV persistence?* **International AIDS Society (IAS), July 2018, Amsterdam, Netherlands (Poster and Oral)**

6. Naidoo, S.; Katusiime, M.G.; Cotton, M.F.; Van Zyl, G.U.; Glashoff, R.H. *Upregulation of Chronic Inflammatory Cytokines Despite Early Treatment and Virological Suppression in Children: A Role in HIV Persistence?* **Faculty of Medicine and Health Science Annual Academic Year Day. September 2017, Cape Town South Africa** (Poster)

7. Naidoo, S., Sobolewski, M.D.; Simmons, T.; Cyktor, J.C.; Tosiano, M.A.; Katusiime, M.; Van Zyl, G.U.; Cotton, M.F.; Glashoff, R.H.; Mellors, J.W. *Detection of Inducible HIV in Children on ART Despite Low HIV-1 DNA.* Presented at: **Faculty of Medicine and Health Science Annual Academic Year Day. September 2017, Cape Town South Africa** (Oral)

8. Naidoo, S.; Sobolewski, M.D.; Simmons, T.; Cyktor, J.C.; Tosiano, M.A.; Katusiime, MG.; Cotton, M.F.; Van Zyl, G.U.; Mellors, J.W.; Glashoff, R.H. *Characterizing the inflammatory cytokine profile and latent HIV reservoir in early treated and long-term suppressed HIV infected children – The surfacing insights into HIV persistence.* **South African Immunology Society (SAIS), August 2017, Cape Town, South Africa** (Oral and Poster Presentation)

9. Naidoo, S.; Katusiime, M.G.; Cotton, M.F.; Van Zyl, G.U.; Glashoff, R.H. *Upregulation of Chronic Inflammatory Cytokines Despite Early Treatment and Virological Suppression in Children: A Role in HIV Persistence?* **International AIDS Society (IAS), July 2017, Paris, France** (Poster)

10. Naidoo, S., Sobolewski, M.D.; Simmons, T.; Cyktor, J.C.; Tosiano, M.A.; Katusiime, M.; Van Zyl, G.U.; Cotton, M.F.; Glashoff, R.H.; Mellors, J.W. *Detection of Inducible HIV in Children on ART Despite Low HIV-1 DNA*. Presented at: **Conference on Retroviruses and Opportunistic Infections (CROI), February 2017, Seattle, Washington (Poster)**

11. Innes, S.; Glashoff, R.H.; Naidoo, S.; Otwombe, K.N.; Laughton, B.; Karris, M.; Cotton, M.F.; Browne, S.H. *sVCAM and MCP-1 Involved in Premature Arterial Wall Stiffness in Prepubertal Children:* Presented at: **Conference on Retroviruses and Opportunistic Infections (CROI), February 2017, Seattle, Washington** (Poster)

International Training

April – September 2016

Collaborative scholar exchange programme – **University of Pittsburgh, Pennsylvania, USA** under the supervision of **Professor John Mellors** *(Infectious Diseases and Microbiology Chief, Division of Infectious Diseases, Endowed Chair, Global Elimination of HIV and AIDS)*

Received training in both molecular and virus isolation/ techniques (Quantitative Virus Outgrowth Assay (QVOA), Total Virus Recovery Assay (TVR), *integrase* Single Copy Assay (iSCA) using RT-PCR.

Outcomes of this training as lead to poster presentation at the Conference on Retroviruses and Opportunistic Infections **(CROI 2017), Seattle, Washington** as well as the Young Investigator's Scholarship Award

List of Media Mentions Related to this Doctoral Research

1. https://www.geekyreality.com/blog/stemstories-shalena-phd-candidate-south-africa/
2. https://www.cnbcafrica.com/videos/2018/12/03/women-scientists-given-platform-to-provide-solutions-to-africas-battle-with-hiv-aids/
3. https://www.timeslive.co.za/news/sci-tech/2018-12-10-sa-women-scientists-honoured-in-kenya-for-pioneering-research/
4. https://www.sowetanlive.co.za/news/south-africa/2018-12-10-sa-women-scientists-honoured-in-kenya-for-pioneering-research/
5. https://www.iol.co.za/news/south-africa/8-female-sa-scientists-among-14-awarded-fellowships-by-loreal-foundation-18422680

CHAPTER 1:

INTRODUCTION

1.1 Background

The Human Immunodeficiency Virus 1 (HIV-1) remains a profound health care challenge in South Africa with just under 20 percent of the population (8.2 million) infected with the virus (mid-year population statistics, 2021). Despite the implementation of highly effective combination antiretroviral therapy (cART) programmes, children remain a vulnerable population whose reliance on lifelong therapy is accompanied by several immune abnormalities (Tobin and Aldrovandi, 2013).

The use of highly active ART (HAART) has shown to effectively suppress the virus to below detection levels (<40 copies/ml) allowing HIV-1 infected individuals to control their infection and live long and productive lives (Lewin et al., 2011).

Following the publicity surrounding the "Berlin Patient", the first functional cure to be achieved (Hütter et al., 2009) as well as the "Mississippi Baby" (Persaud et al., 2013) the HIV research agenda has shifted towards eradication and a deeper understanding of remission and post-treatment control.

More recently, a perinatally HIV-1 infected South African child born in 2007, enrolled in the Children with HIV Early Anti-Retroviral (CHER) Randomised Control Trial and randomised to the immediate, time-limited 40 weeks cART study arm, sustained long-term virological control for over 9.5 years following cART cessation. This infant was one of 227 early treated children and is currently the only child that has been able to establish long-term virological control following cART interruption. The viral load as well as HIV-1 cell-associated deoxyribonucleic acid (DNA) (CAD) levels of the child remain low (6.6 copies/ml and 5 copies per million peripheral blood mononuclear cells (PBMCs) and further prompts the need to evaluate host characteristics that allow for virological control in the context of early cART (Violari et al., 2019).

In the absence of an effective vaccine, there is currently a strong emphasis on HIV-1 cure – i.e., implementation of strategies that would eliminate all traces of the virus from the infected host. Although cART leads to viral suppression, unless a person infected with HIV-1 is treated at the time of acquisition, the virus is still able to establish latent reservoirs (replication-competent but silent viral populations) within host cells and this is considered the major barrier to achieving cure (Castro-Gonzalez, Colomer-Lluch and Serra-Moreno, 2018).

HIV-1 reservoirs, consisting of intact integrated HIV-1 genomes, are also considered a major source of viral rebound (release of infective virus upon activation) following treatment failure or interruption (Chun et al., 1998; Eisele & Siliciano, 2012). Latent reservoirs are established during the first weeks of infection (Svicher et al., 2014; Chun et al., 1998; Schacker et al., 2001; Archin et al., 2012) and can be localised in various organs and cell types including the thymus (Fabre-Mersseman et al., 2011; Scinicariello et al., 2010), gastrointestinal tract (GIT) and brain (Chun et al., 2008), perivascular macrophages, parenchymal microglia and astrocytes (Thompson et al., 2011). Investigations evaluating the long-term decay or kinetics of HIV-1 reservoirs show that infected cells could persist for as long as 60 years. Various mechanisms for this on-going persistence

include constant replenishing of the reservoir through cell to cell spread, cell-associated replication or possible residual replication in mucosal associated lymphoid tissues (MALT) (Klatt et al., 2013). Modelling studies suggest the production of intermittent viral blips is indicative of replenishing of the latent reservoir through homeostatic proliferation and/or de novo infection and occurs by the activation of latently infected cells that can retain persistent low-level viral infection (Rong and Perelson, 2009)

Studies evaluating the timing of appropriate cART initiation in relation to the HIV-1 reservoir size have shown varying outcomes (Lori et al., 1999; Zhang et al., 1999; Blankson et al., 2000; Jain et al., 2013). Most studies have shown positive correlation with early cART initiation and a diminished HIV-1 reservoir size. Strain, et al., (2005) demonstrated that initiation of cART within the first 6 months of infection was associated with substantially decreased cell-associated HIV-1 infectivity. Another study assessing early versus late treatment found that HIV-1 persistence remained relatively stable during therapy. It was however, further suggested that the delay of cART in patients may lead to a permanently enlarged HIV-1 reservoir resulting in a reduced ability to later clear the latent reservoir (Jain et al., 2013).

In new-born children, the timing of cART has an impact on reservoir establishment – as evidenced by more rapid and elevated viral rebound when treatment is interrupted in those receiving delayed treatment (Goulder, Lewin and Leitman, 2016a). The length of time from initial infection to initiation of therapy should ideally be as short as possible to reduce dissemination of the virus and establishment of an expanded reservoir. Latency in HIV-1 infection may be an evolutionary adaptation due to low penetrance of transmitting virus – most HIV-1 infections being initiated by a single founder virus. In the absence of suitable target cells (primarily activated and replicating CD4+ T-cells co-expressing C-C chemokine receptor type 5 (CCR5)), the virus may establish latency in other available cells, allowing for replication when such a cell is activated or divides (Castro-Gonzalez, Colomer-Lluch and Serra-Moreno, 2018).

Suggested strategies for reservoir depletion require multiple approaches since HIV-1 latency is itself multifactorial. The impact and timing of early cART is therefore significant when considering eradication strategies (Archin and Margolis, 2014). Furthermore, measurement of the HIV-1 latent reservoirs is important for understanding and then developing strategies for cure. Data generated from the Virological and Immunological studies in CONtrollers after Treatment Interruption (VISCONTI) cohort, showed that early cART initiation as well as prolonged therapy during primary infection resulted in a lower pool of cellular HIV-1 CAD (Sáez-Cirión et al., 2013). A study conducted by Persaud et al., showed a reservoir size below detection limits of the endpoint-limiting dilution cultures for measuring replication-competent virus assay at 48 weeks in more than 50% of children initiated on cART at a median of 8 weeks of age (Persaud et al., 2012). Another study conducted in the United States of America (USA) showed that five adolescents given suppressive cART at less than two months of age, had smaller latent reservoirs with no infectious HIV-1 recovery when compared to adolescents who received treatment later (Luzuriaga et al., 2014).

Immune factors are considered critically important for the establishment and maintenance of HIV-1 latent reservoirs and for determining whether a reservoir is established and maintained, but these factors are not well characterised, particularly in children. Foetal development is characterised by immune tolerance or

immunosuppression, which gradually switches to a more reactive pro-inflammatory status after birth. Although the length of time that a child may have been infected pre-therapy remains a key factor, the immune status pre-therapy (and post-therapy) may be as important in promoting the establishment and maintenance of the latent reservoir.

Studies have well-documented improved immunological outcomes with early cART initiation and subsequent virological suppression. Improved CD4+ T-cell numbers and decreased levels of immune activation are key features of improved outcomes following therapy (Cotton et al., 2013; Kabue et al., 2012; Sáez-Cirión et al., 2013; Violari et al., 2008; Jain et al., 2013; Koblavi-Dème et al., 2003; Resino et al., 2003). The CHER trial conducted in 2004 showed significantly better clinical as well as immunological outcomes in children treated early as opposed to those with deferred cART (Cotton et al., 2013a). Other key studies also show that ART-mediated virological suppression reduces the activation status of both CD4+ and CD8+ T-cells (Hunt et al., 2003; Robbins et al., 2009; Jain et al., 2013). Another study investigating the effects of early initiation of cART showed a normalisation in the levels of the monocyte and macrophage activation marker - soluble CD163 (sCD163) (Burdo et al., 2011).

To structure appropriate interventions or mechanisms for the clearance of persisting HIV-1 infection, it is important to understand the hosts' immunological environment during early treatment and viral suppression. Furthermore, it is also important to understand the role of immunological responses to HIV-1 persistence. Recently it has been proposed that latency/reservoir establishment may be an adaptation of the virus to overcome existing host barriers to establishing successful infection. If the virus can establish latency early, it has backup capacity to establish infection within the host if early host defences are effective. Should early replication and spread be effectively controlled, the size of the reservoir may be related to host immunity as much as to viral factors.

Despite positive immunological outcomes following early therapy as well as virological suppression due to highly effective cART, immune abnormalities including CD4+ and CD8+ T cell activation or dysfunction may still occur and CD4+ T cell populations do not always reach normal levels when compared to uninfected counterparts, even after years of suppressive therapy (Corbeau and Reynes, 2011; Klatt et al., 2013; Rajasuriar, Wright and Lewin, 2015). The ability of early cART to prevent these potentially irreversible outcomes remain unclear and studies have underlined a number of immune abnormalities that persist despite many years of viral suppression (Valdez et al., 2002; Aiuti and Mezzaroma, 2006; Kelley et al., 2009). When compared to uninfected individuals, HIV-1 infected persons may also display a reduced function of the adaptive immune compartment (Lange et al., 2002; Lederman et al., 2011).

T-cell populations expressing high levels of key activation markers such as Human leukocyte antigen-DR (HLA-DR) and CD38 during untreated HIV-1 serve as highly significant prognosticators for disease progression. However, the relevance of these immunological markers during treatment remains relatively undefined. Some studies suggest at least some level of predictive significance (Hunt et al., 2011; Kaplan et al., 2011; Klatt et al., 2013). Other anomalies of immune markers characterised during effective cART treatment include decreased levels of CD28, and increased levels of CD57 and programmed cell death protein 1 (PD-1).

These described markers relate to an immunological profile defined by T-cell senescence and dysfunction (Appay and Sauce, 2008; Kaplan et al., 2011; Tassiopoulos et al., 2012; Hatano et al., 2013; Sauce, Elbim and Appay, 2013).

According to the opinion of Klatt et al.: immune aberrations and chronic inflammation may be the cause of HIV-1 persistence in the context of cART. Immune dysfunction may increase viral production and generate new target cells that can activate and infect resting cells. These events may influence the migratory patterns of susceptible target cells, result in the increased proliferation of infected cells and therefore prevent HIV-specific clearance (Klatt et al., 2013).

Most recent work investigating HIV-1 latent reservoirs is predominantly focussed on CD4+ T cells. However, these cells do not serve as the only targets for the HIV-1 virus. Monocytes/macrophages are also susceptible to HIV-1 infection possibly due to the expression of the CCR5 receptor (Tuttle et al., 1998). Hearps et al., showed the persistence of monocyte dysfunction, such as decreased phagocytic capabilities, despite continuous cART (Hearps et al., 2012). Other studies investigating monocyte activation showed an increase in sCD163 in patients on cART (Burdo, Lentz, et al., 2011; Burdo, Lo, et al., 2011).

Macrophages can be found in all tissues and body compartments including the gut-associated lymphoid tissue (GALT) and the brain. Traditionally, monocytes are not considered long-lived viral reservoirs due to their characteristic of a short life span in the bloodstream. However, susceptible cell populations may represent an important route for the establishment of inherent reservoirs cells as these cells migrate into tissues and differentiate into macrophages. Macrophages are therefore important HIV-1 reservoirs because of their longevity and ability to reside in multiple tissue compartments. Macrophages can live for several weeks to years, depending on the tissue. Professional tissue-resident macrophages (e.g., Kupffer cells) have stem cell-like properties, which allows for replenishment. If HIV-1 infects such cells, or monocyte-derived macrophages that acquire stem like properties, these cells could provide a source of reservoir for viral replenishment for long periods of time (Kruize and Kootstra, 2019).

Microglial cells are the most long-lived macrophage cell types. These cells harbour HIV-1 for long periods and may be responsible for a slower second-phase decline in plasma viremia seen in patients on therapy (Watters et al., 2013).

HIV-1 enters the central nervous system (CNS) within 14 days of primary infection (Sailasuta et al., 2012). It has been suggested that the virus enters the CNS mainly through infected monocytes with the intent to become brain-resident macrophages or perivascular macrophages soon after peripheral infection of circulating CD4+ T-cells and monocytes. Studies show monocytes to be less susceptible to cytopathic effects of HIV-1 compared to CD4+ T-cells (Crowe et al., 1987; Olivetta and Federico, 2006; Cummins and Badley, 2013). Peripheral monocytes therefore serve an important role in channelling HIV-1 into the central nervous system (CNS) during acute or chronic infection (Liu et al., 2000). These cells may therefore result in long-lived reservoirs within the CNS. Poor neurocognitive function in HIV-1 infected children may be explained by HIV-1 entry into the CNS prior to cART. A number of studies have supported good neurocognitive functioning in treated patients due to improved penetration of ARVs into the CNS (Smurzynski et al., 2011).

When HIV-1 enters the brain it initiates events that result in cognitive-, motor-, as well as behavioural deficits (Williams et al., 2014; Ances & Ellis 2007; Kaul et al., 2005; Heaton et al., 2011). It has been hypothesised that HIV-1 associated neurocognitive disorders (HAND) occur due to the transmigration of the CD14+CD16+ monocyte sub-population across the blood-brain barrier and into the CNS (Burdo, Lackner and Williams, 2013; Williams et al., 2013, 2014). Subsequent to this process, viral infection of the brain occurs mediated through interactions between monocytes, blood-brain barrier (BBB) cells as well as chemotactic gradients that channel leucocytes into the CNS. Despite effective cART, monocytes are believed to enter into the CNS of HIV-1 infected individuals resulting in low-level on-going, chronic neuroinflammation (Williams et al., 2014).

C–C motif chemokine ligand 2 (CCL2) and C-X-C motif chemokine 12 (CXCL12), monocyte chemokines, have been implicated in the pathogenesis of HIV-1 associated neuropathogenesis. During cART and even during viral suppression, CCL2 levels have been documented to remain relatively high in cerebrospinal fluid (CSF) of HIV-1 infected individuals when compared with negative controls. Williams et al., (2014) therefore suggests, that despite cART, HIV-1 infected individuals experience an influx of CCL2-mediated monocyte influx into the CNS which increases the risk for HAND despite the presentation of cognitive deficits (Chang et al., 2004; Letendre et al., 2011; Kamat et al., 2012).

Understanding the pathways and mechanisms that lead to monocyte activation and the subsequent contribution to neuroinflammation serve important when addressing HIV-1 associated cognitive disorders that may occur despite cART and viral suppression.

Perinatally HIV-1 infected children (PHIV) present an opportunity for investigating HIV-1 cure strategies due to (1) known timing of infection, (2) feasibility of initiating therapy early before establishment of the latent reservoir, (3) early cART initiation can improve the time to viral suppression, and (4) early-life immune ontogeny that favours a tolerogenic environment. However, further knowledge is required to understand immunological mechanisms that drive HIV-1 persistence and neurodevelopmental outcomes in the context of long-term viral suppression from a longitudinal perspective i.e., from before therapy initiation to pre-adolescence. An overview of the immunological landscape may provide pertinent information to further understand chronic diseases that may surface as the child approaches adolescence where adherence, drug resistance and sexual activity comes into play.

1.2 Study Cohort

Study participants originating from the randomised CHER trial, between 2005 and 2011 were selected for this investigation. The trial was conducted in Soweto and Cape Town, South Africa and evaluated the clinical, virological and immunological outcomes of early limited and deferred cART and has influenced ART guidelines worldwide in favour of early treatment (Violari, et al., 2008; Cotton et al., 2013a). HIV-1 positive infants, confirmed by a positive PCR test for HIV-1 DNA and plasma HIV-1 RNA levels on PCR >1000 copies/ml, were enrolled between 6 and 12 weeks of age. Infants with a CD4 percentage greater than 24% were included in the trial.

This investigation served as a follow-up on CHER participants from Cape Town. The Post-CHER study or "Reservoir Study" was approved by the Health Research Ethics Committee of Stellenbosch University (Ethics Reference No: M14/07/029 – Appendix A). All participants were consented, and blood was taken for PBMC extraction (stored in cryovials in liquid nitrogen (LN_2) and plasma (stored in plastic Eppendorf tubes at -80°C) storage. Early collected samples (PBMCs and plasma) were retrieved from Clinical Laboratory Services (CLS)™ storage repository in Cape Town, South Africa.

First line cART comprised lopinavir-ritonavir, zidovudine and lamivudine at ART initiation and re-initiation following therapy interruption. The primary endpoint of the original study was time-to-failure of first-line ART (immunological/clinical/virological) or death. Comparisons were by intent-to-treat, using time-to-event methods. Defined parameters for failure included: (1) failure to achieve a CD4 percentage of 20% or more following 24 weeks of ART or a decrease in CD4 percentage to less than 20% after 24 weeks of ART defined as "immunologic failure", (2) Centers for Disease Control and Prevention (CDC) stage B or stage C events defined as "clinical failure", (3) drug toxicity requiring more than one drug replacement of the same class or (4) a required switch to a new drug class or (5) requiring the permanent termination of treatment – defined as "regimen-limiting toxicity failure".

Initiation or re-initiation of therapy following interruption were based on CDC stage C or severe stage B events including lymphoid interstitial pneumonitis, bronchiectasis, nephropathy, cardiomyopathy, and failure to thrive.

The CHER Randomised Controlled Trial implemented three study arms: Arm 1 (deferred ART), Arm 2 (early ART initiation for 40 weeks followed by interruption) and Arm 3 (early ART initiation for 96 weeks followed by interruption). Children were randomised into one of listed study arms.

Recruitment of the post-CHER follow-up study commenced on the 1st of November 2014 (median age of 8 years; IQR: 8.4-9.0 years) and continued over a five-year period, until December 2019. A total of 88 post-CHER children were followed up (23%) of the total clinical trial enrolment (n=377). Additional age- and demographically matched HIV Exposed Uninfected (HEU) and HIV Unexposed Uninfected (HUU) controls were also recruited. These controls originated from the same community and were generally considered healthy.

Children within the post-CHER follow-up also participated in a neuro-imaging/neurocognitive study **(**Ethics Reference No: N05/05/092 and M11/10/042) which will continued until the age of nine years and subsequently be expanded to include a sub-study for an additional 3 years. Neurocognitive outcomes were correlated with immunological and HIV-1 reservoir measurements.

Researchers and all laboratory staff were blinded to the identity of the study participants. All participants including controls were allocated a PID number which was allocated at the start of the trial. All samples were labelled with sample codes.

1.3 Study Rationale

Perinatally HIV-1 infected (PHIV) children are of significant interest when considering "cure" and remission strategies in the context of early therapy (Goulder et al., 2016). Delineating and understanding the immunological mechanisms that can potentially drive HIV-1 persistence and other chronic diseases is important in these children approaching adolescence. Longitudinal studies evaluating the relationship between immune status, the HIV-1 latent reservoir and neurocognitive outcomes are limited, and inadequately studied, specifically within the South African subtype C context.

1.4 Overarching Research Hypothesis

Inflammation and immune abnormalities persist after long-term cART therapy in virologically suppressed PHIV children and both immune and viral status before the initiation of therapy are strong predictors of HIV-1 latent reservoir size and neurocognitive outcomes later in life.

1.5 Study Aims and Objectives

Study Aim

To longitudinally investigate and characterize host immune status before and after early initiated, delayed and interrupted cART in relation to HIV-1 latent reservoir size and neurocognitive outcomes in perinatally HIV-1 infected children.

Study Objectives:

1. To extensively quantify and profile soluble immune biomarkers (cytokines, chemokines and receptor antagonists) related to T-cell, monocyte/macrophage activation and gut permeability/damage and to determine their relationship to HIV-1 reservoir biomarkers;
2. To evaluate monocyte subset distribution for activation and homing propensity;
3. To optimise and implement Total Virus Recovery (TVR) and Infectious Virus Recovery (IVR) assays for the quantification of viable latent HIV-1 reservoir in a subset of children who received early treatment and display virological suppression; and
4. To determine whether specific immunological markers of inflammation could serve as early predictors for neurocognitive outcomes in HIV-1 infected children

1.6 Overview of Study Design

This investigation adopted both a longitudinal and cross-sectional study design dependent on the research question and availability of samples. Objectives 1 and 2 consisted of both longitudinal (soluble immune biomarker profiling) and cross-sectional (monocyte phenotyping at 8 years of age) approach. Objective 3 was a cross-sectional study completed on a subset of post-CHER participants and Objective 4 was a longitudinal study focused on predictive analysis.

Figure 1.1 provides an overview of the relative study design assigned to each objective.

1.7 Overview of Dissertation Chapters

This dissertation is structured into eight Chapters. **Chapter 1** (Introduction) provides an overview of the research conducted and includes a brief background to the field of study, a description of the study cohort, the study rationale, the overarching hypothesis, study aims and objectives and a snapshot of the study design.

Chapter 2 (Literature Review) provides a more extensive overview of the literature presented within the field with a focus on HIV pathogenesis, HIV-1 persistence in perinatally infected children and immunological abnormalities in children with long-term viral suppression.

Chapters 3, 4, 5, 6 and 7 present the key findings of this research and is written in publication format which includes sections comprising of an abstract, introduction, materials and methods, results, discussion and conclusions. The contributions of each co-author are highlighted in the beginning of each chapter.

Chapter 3, titled: "Evidence of persisting chronic inflammation despite early therapy in perinatally HIV-1 infected children at 8 years of age: association with gut permeability*",* presents key evidence for chronic inflammation evidently associated with gut permeability in perinatally HIV-1 infected children following long-term suppressive therapy.

Chapter 4, titled: "Elevated IL-18 is associated with HIV-1 cell-associated DNA in pre-adolescent children: relationship with other key innate biomarkers", highlights IL-18 as a key inflammasome-mediated immune biomarker that holds the potential to serve as a surrogate biomarker of the HIV-1 latent reservoir. We also highlight the potential of IL-18 to provide insights into immune dysfunction and regulation. As cART never normalizes the levels of IL-18, the immune biomarker may be utilized to measure treatment success as well as overall immune health. This marker could also provide an indication of which patients will require added vigilance after ART initiation.

Chapter 5, titled: "Longitudinal impact of therapy regimen and clinical parameters on the expression patterns of soluble immune biomarkers in perinatally HIV-infected children in South Africa", documents the longitudinal interplay between of immune status and clinical parameters in relation to various therapy regimens. Extensive predictive analysis of biomarkers indicates a complex interplay between cytokines, chemokines and growth factors that shape the landscape for disease progression.

Chapter 6, titled: "Evaluation and Implementation of a Viral Outgrowth Assay (VOA) for the Measurement of HIV-1 Latency in Early-Treated and Long-term Suppressed Perinatally Infected Children", focuses on the in-depth methodology implemented for the attempted measure of the HIV-1 latent reservoir in a subset of ten (10) long-term virological, suppressed children at 8 years of age. In this Chapter, we also provide an overview of the novel *integrase* single-copy assay (iSCA) designed and implemented to measure HIV-1 ribonucleic acid (RNA) in cell culture supernatant fluid.

Chapter 7, titled: "Delineating the longitudinal relationship between antiretroviral treatment regimens and HIV-1 associated inflammation on neurocognitive outcomes in perinatally infected (PHIV) children in South Africa", provides key findings following an in-depth analysis of neurocognitive outcomes in children from 11

months to 9 years of age and followed by an assessment of whether immunological, virological as well as clinical immune biomarkers can serve as predictors for these neurocognitive outcomes.

Finally, **Chapter 8** provides an overview of the key findings, conclusions, limitations of this research as well as insights into the future direction of this investigation.

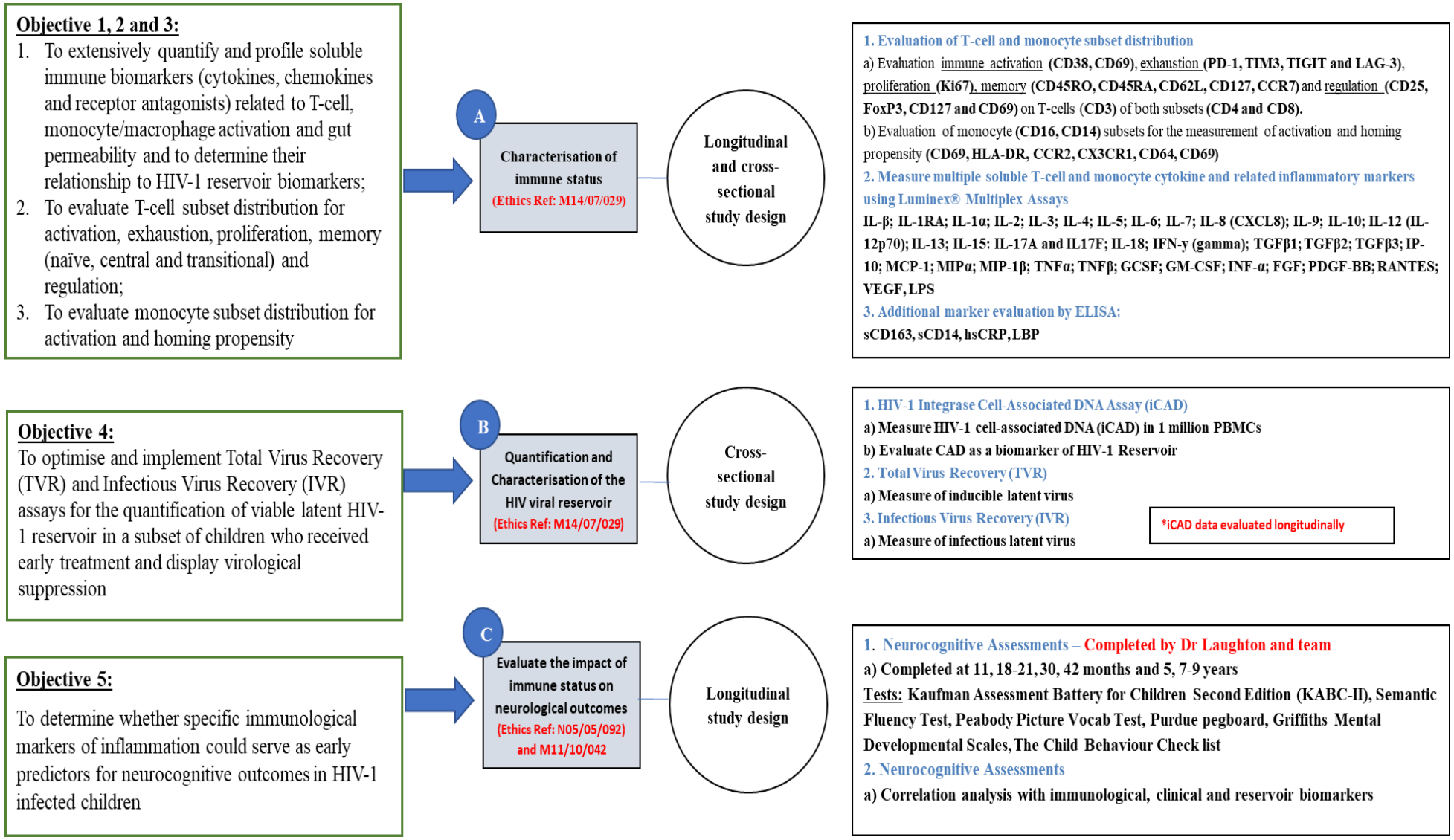


Figure 1.1: *Overview of study design implemented for doctoral research evaluating the impact of immune status on the HIV-1 latent reservoir and neurocognitive developmental outcomes in virologically suppressed HIV-1 infected children.*

CHAPTER 2:
LITERATURE REVIEW

2.1 The Epidemiology of HIV – Global, Sub-Saharan Africa and South Africa

HIV-1, the virus responsible for the HIV global epidemic originated from zoonotic infections with Simian Immunodeficiency Virus (SIV) from African primates. HIV-1 is now known to have been transmitted from African Ape populations during the late 1950s, whereas the related HIV-2 was derived from sooty mangabey monkeys (Sharp and Hahn, 2011). HIV-1 is classified into four groups (M, N, O and P) with M being the most widespread and known to be the cause of the global HIV-1 pandemic. Groups M, N and O are known to have been transmitted from chimpanzees and P from gorillas. Groups N, O and P are constrained to West Africa (Sharp and Hahn, 2011; Maartens, Celum and Lewin, 2014). Group M consists of nine subtypes i.e., A-D, F-H, J and K, with subtype C being the most prevalent, dominating approximately 48% of global HIV-1 infections in 2007 and also infections in Southern Africa, Indian and Ethiopia (Hemelaar et al., 2011; Wilkinson, Engelbrecht and De Oliveira, 2015; Gartner et al., 2020). Western Europe, the USA and Australia have an HIV-1 subtype B predominance, however recombinant subtypes are circulating more commonly worldwide (Hemelaar et al., 2011).

The HIV Epidemic

Just under 40 years has passed since the discovery of HIV and the disease remains one of the most devastating epidemics encountered by mankind. The current global Coronavirus Disease 2019 (COVID-19) pandemic has adversely impacted on measures to curb HIV-1 transmission and spread. Approximately 79 million people have been infected with HIV-1 and just over 36 million people have died due to the acquired immunodeficiency syndrome (AIDS)-related illnesses since the start of the epidemic. In 2020, an estimated 38 million people were living with HIV-1 of which 1.5 million individuals were considered newly infected (UNAIDS, 2021)

Of those living with HIV-1 in 2020, 1.7 million were children younger than 14 years of age. Women and children remain the most vulnerable populations affected by the HIV pandemic and account for 53% of people living with HIV-1, globally. Sub-Saharan Africa accounts for approximately 67% (two-thirds) of people living with HIV-1 with countries Eswatini (Swaziland), Lesotho, Botswana and South Africa holding the highest prevalence rates of 27%, 22.8%, 20.7% and 19% respectively (Barr *et al.*, 2021).

South Africa carries the largest HIV-1 epidemic burden, accounting for about a third of all new HIV infections in southern Africa and bears one of the most highly profiled HIV-1 prevalence rates in the world with an estimated 7.5 million people living with the virus in 2020 with an adult (age 15-49 years) prevalence rate of 19% (Barr *et al.*, 2021).

HIV-1 Transmission

HIV-1 viral load is the most significant factor for the increased risk of sexual transmission of HIV-1. A 2-4 times increased risk for transmission exists for every 1 $\log_{10}$ increase in viral load (Quinn et al., 2000). During the acute phase of HIV-1 infection individuals generally have very high plasma HIV-1 RNA load, which is

considered a significant driver of HIV-1 transmission and the epidemic itself (Cohen et al., 2011). The HIV-1 viral load contained in semen and the endocervix can serve as good predictors of sexual transmission (Baeten et al., 2011) along with other factors associated with sexually transmitted infections such as genital ulcers, herpes simplex type-2 infection and bacterial vaginosis. Pregnancy and anal intercourse are also considered high risk factors for HIV-1 transmission (Røttingen et al., 2001; Atashili et al., 2008; Glynn et al., 2009; Mugo et al., 2011; Maartens et al., 2014).

HIV-1 Transmission in Children

Due to prevention of mother-to-child (PMTCT) programs along with increasing coverage of cART, HIV/AIDS related morbidities and mortalities have decreased significantly in children over the past decade (Teshale et al., 2021).

Effective PMTCT programs are powerful interventions for reducing new childhood HIV infections and has prevented roughly 1.4 million infections in children. PMTCT programs have also been a significant contributor to eliminating new HIV-1 infections in low- and middle-income settings such as South Africa (Teshale et al., 2021). Interventions have included the use of ART drug combinations during pregnancy and delivery, neonatal prophylactic medications, elective caesarean delivery methods and monitoring of breastfeeding practices (Kourtis and Bulterys, 2010). The administration of antiviral drugs to new-borns within the first few hours of birth, as a post viral exposure prophylaxis, assists in protecting against cell-free or cell-associated virus that might have entered the infants' systemic circulation through maternal-foetal transfusion or virus that might have been swallowed by the infant whilst passing through the birth canal (Mandelbrot et al., 1999; Kourtis et al., 2006; Kourtis and Bulterys, 2010).

The early initiation of cART in women living with HIV-1 (WLHIV) can actively suppress the viral load and reduce mother-to-child transmission (MTCT) of HIV-1 to unborn infants by 20% for each additional week of ART taken during the antenatal period (Patel et al., 2007; Townsend et al., 2008; Fitzgerald et al., 2010; Teshale et al., 2021).

In 2017, approximately 50% of the 180,000 new HIV infections documented in children were due to breastfeeding practices, highlighting the importance of cART adherence education in PMTCT programs. The risk of MTCT ranges from 15-45% in the absence of any intervention (5–10% during pregnancy, 10–20% during childbirth, and 10–20% via mixed infant feeding) (Teshale et al., 2021). Transmission rates lower than 1-2% have been achieved even in resource limited settings (Cooper et al., 2002; Lallemant et al., 2004; Kourtis and Bulterys, 2010; Zhou et al., 2010).

In the classical early events of the HIV-1 life cycle, the reverse transcription of HIV-1 viral ribonucleic acid (RNA) and the subsequent integration of reverse transcripts occur. Reverse transcription occurs in the cytoplasm followed by translocation of complementary DNA (cDNA) to the nucleus which give rise to two types of cDNA i.e., linear and circular. Linear cDNA is integrated into host DNA and is transcribed into viral mRNA used in the production of viral progeny. Circular forms of viral DNA contain one or two copies of the long terminal repeat (LTR) region, accumulate in the infected cells because of failed integration processes

(Hamid, Kim and Shin, 2017). Unintegrated viral sequences have been used as a measure for determining exposure to HIV-1.

In approximately 18% of uninfected infants born to HIV-1 infected mothers, the presence of unintegrated virus i.e., polymerase chain reaction (PCR) detection of viral LTR indication of a partially reverse-transcribed HIV-1 genome, instead of gag detection in PBMCs was observed (Lee FK, Scinicariello F, Ou CY, et al. Partially reverse transcribed HIV-1 genome in uninfected HIV-exposed infants. Presented at the 11th Conference on Retroviruses and Opportunistic Infections. San Francisco (CA), February 8–12, 2004.). Although this viral intermediatory is unintegrated, it is considered biologically active and will decay over time in the absence of appropriate activation (Zack et al., 1992). Therefore, HIV-1 may enter the systemic circulation of the foetus or infant but in the absence of lymphocyte activation, integration of the virus and establishment of infection may not occur until later or not at all. If HIV-1 cannot complete its reverse transcription cycle until cell activation occurs, then viral entry might not equal transmission, which questionably occurs only at the time of viral integration and therefore establishment of infection occurs. Accepting this hypothesis, most instances of MTCT must occur around the time of birth, when extensive lymphocyte activation starts to occur, even though viral entry could have occurred days or weeks earlier (Kourtis and Bulterys, 2010).

Although the mechanisms of MTCT have not been fully elucidated and a lot remains unclear, it is considered multifactorial with several host (maternal and/or infant) and viral factors playing a role. In addition, the timing and mechanisms of HIV-1 transmission have been important modalities considered for prevention strategies as described above (Kourtis and Bulterys, 2010). The risk of HIV-1 transmission is significantly greater for mothers with increased HIV-1 viral loads (key driver of horizontal transmission) as well as with clinical (co-infections, maternal age, length of gestation) and immunological markers (CD4 count) of advanced disease (Mofenson and McIntyre, 2000; Thorne and Newell, 2005).

Figure 2.1 provides a summary of factors that may contribute to MTCT of HIV-1 as adapted from Kourtis and Bulterys, (2010) and Ellington, King and Kourtis, (2011).

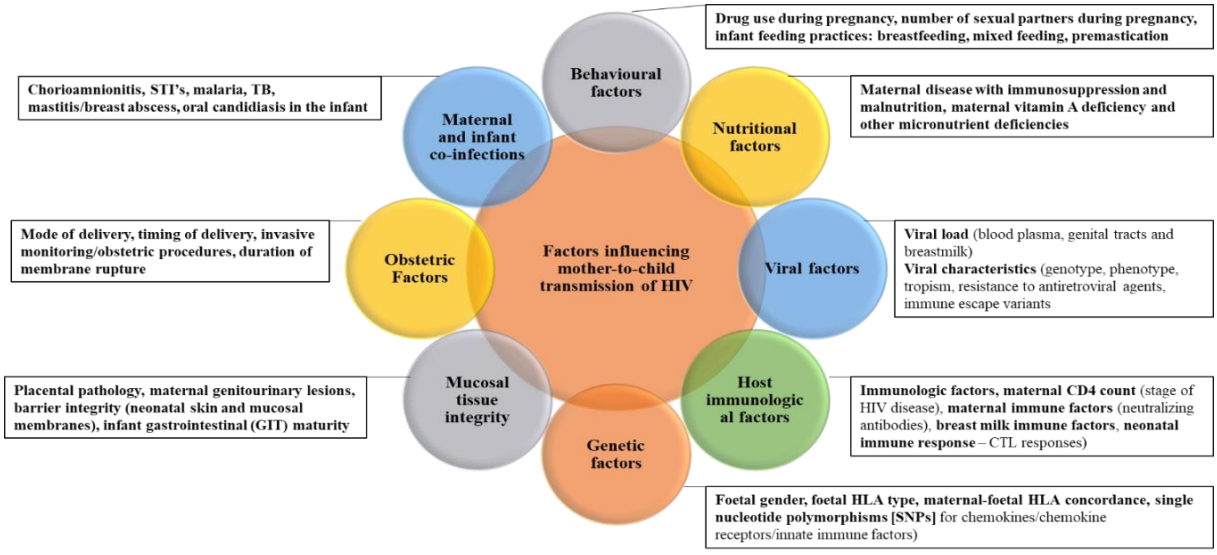


Figure 2.1: *Factors that influence MTCT of HIV adapted from (Kourtis and Bulterys, 2010; Ellington, King and Kourtis, 2011).*

2.2 HIV-1 Pathogenesis

Pathogenesis following HIV-1 infection is considered complex with most of the crucial events i.e., virus transmission and CD4+ T-cell destruction, occurring in mucosal tissues. The GIT is considered as a key site of virus-induced pathology, as this is where massive CD4+ memory T-cell depletion occurs (Lackner et al., 2012).

Insights into HIV-1 disease progression, specifically early pathogenic events, has been acquired through the study of its natural host, the non-human primate model who carries SIV, a close relative of HIV-1 (Lackner, Lederman and Rodriguez, 2012). The more susceptible primate models, rhesus macaques, are also used in the study of HIV-1 disease progression (Thippeshappa et. al., 2020).

HIV-1 life cycle

Activated memory CD4+ T lymphocytes are the main target cells of HIV. The virus enters these cells through the interaction with the CD4 receptor, and the chemokine coreceptors, CCR5 or C-X-C chemokine receptor type 4 a (CXCR4). Other cells expressing CD4+ and chemokine coreceptors include resting and naive CD4+ T-cells, and certain sub-populations of monocytes, macrophages and dendritic cells (DCs) (Maartens et al., 2014). Viral replication can either be limited or promoted depending on the interaction of host proteins with HIV proteins or HIV-1 DNA. Some of these host proteins include apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like-3G (APOBEC-3G), a RNA editing protein that targets the HIV-1 accessory protein vif (Sheehy et al., 2003), tripartite motif (TRIM-5α) which targets the viral capsid protein (Stremlau et al., 2004), Tetherin (BST-2) an immunomodulatory membrane protein that inhibits virus budding by targeting the vpu viral protein (Neil et al., 2008) and sterile α motif histidine aspartic acid domain-containing protein (SAMHD1) which is involved in restricting efficient reverse transcription of HIV-1 by targeting the viral vpx protein (Hrecka et al., 2011; Laguette et al., 2011).

Transmission of one founder virus across the mucosal membrane usually establishes infection. This transmitted founder virus has a unique phenotype and specificity for coreceptor CCR5 for entry into the host (Keele et al., 2008). Following transmission of the founder virus, HIV-1 replication rapidly increases which is followed by a significant increase in the production of inflammatory cytokines and chemokines (Stacey et al., 2009).

Figure 2.2 below adapted from Maartens, Celum and Lewin, (2014) and originally from Walker et al., (2014) provides a snapshot of the key stages of the HIV-1 life cycle from entry into the host cell to viral release and maturation. ART regimes, discussed below, have been designed to target various points of this life cycle.

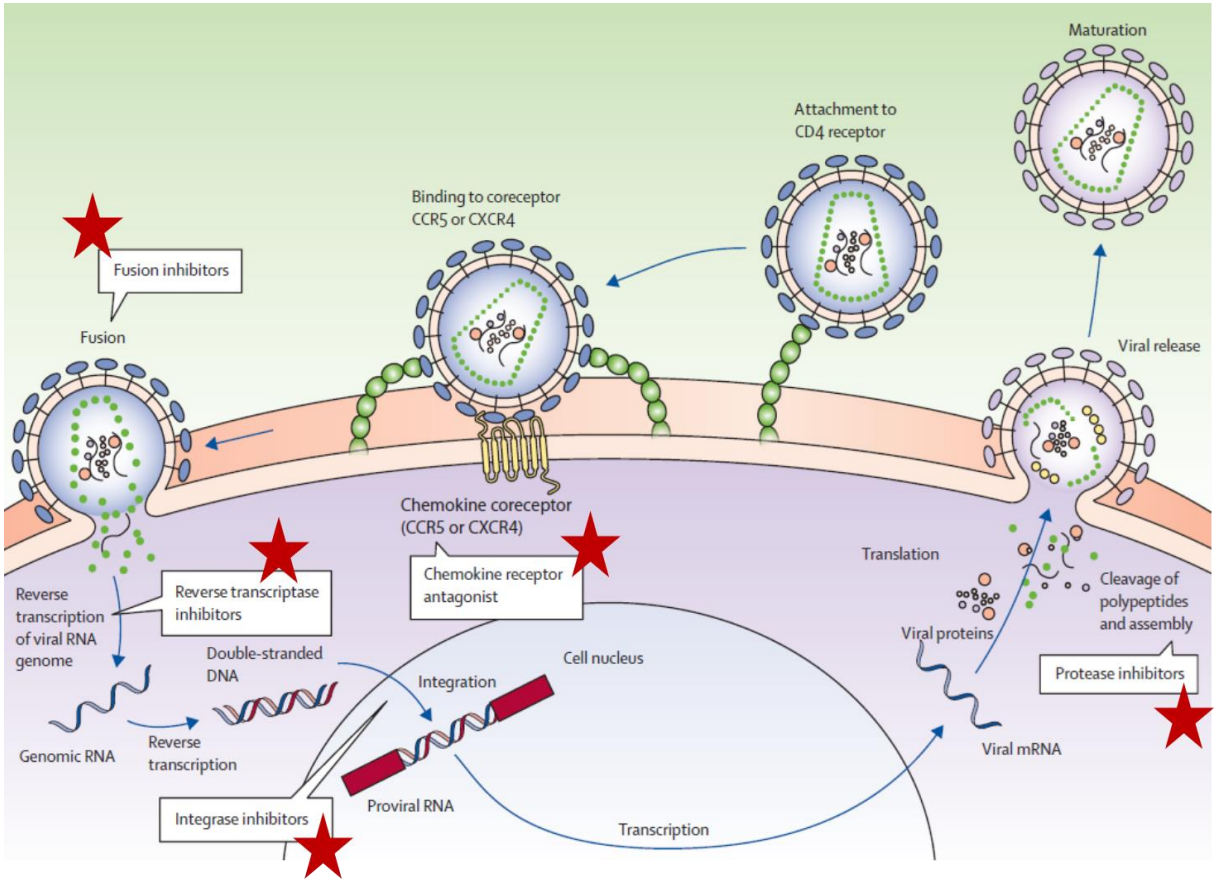


Figure 2.2: *HIV-1 life cycle. Adapted from Maartens, Celum and Lewin, (2014) and originally from Walker et al., (2014). ART targets various stages of the HIV-1 life cycle and are categorised into various drug classes i.e., fusion inhibitors, reverse transcriptase inhibitors, integrase inhibitors, chemokine receptor antagonist and protease inhibitors (depicted by red symbols).*

Host immune response to HIV-1

Innate and adaptive immune responses act to decrease the viral load and allow for a viral setpoint to be established. Soon after infection, infected cells are killed through the mounting of a potent adaptive immune response mediated by HIV-specific CD8+ T-cell responses (Figure 2.3). This powerful adaptive immune response to HIV-1 infectivity selects for the emergence of mutations in key epitopes, often leading to immune escape (Goonetilleke et al., 2009).

From an immunogenetic point, certain host human leukocyte antigen (HLA) types are associated with a more effective immune response e.g., the HLA-B27 allele where individuals infected with subtype B are able to mount a more robust HIV-specific T-cell response with higher avidity, polyfunctionality and proliferative capacity (Almeida et al., 2007). Following infection, HIV-1 neutralising antibodies start increasing at around three months which then select for viral escape mutants (Richman et al., 2003). It has been documented that only 20% of HIV infected individuals are able to produce broadly neutralising antibodies (bNAbs) which are able to neutralise various HIV-1 subtypes (Walker et al., 2011). These bNAbs are the current focus of vaccine research and immune therapeutic design strategies due to their ability to develop a high frequency of somatic mutations that usually develop over a number of years during the course of infection (Jardine et al., 2013; Liao et al., 2013).

Other immunological factors subsequent to HIV-1 infection include the loss of effector functions of HIV-specific T-cells as well as the exhaustion of HIV-specific and total T-cells which is characterised by an increase in the PD-1, Tim-2 and Lag-3 (Trautmann et al., 2006). Natural killer (NK) cells form part of the innate immune response and play a significant role in controlling viral replication. Viral escape mutants can also emerge causing a decrease or an ineffective response from these NK cells (Alter et al., 2011; Elemans et al., 2017).

The progressive depletion of CD4+ T-cells is considered a key characteristic of HIV disease. The destruction of these CD4+ T-cells is not only due to direct infection with HIV-1, but by bystander effects of syncytia development, immune activation, proliferation, exhaustion and activation-induced apoptosis (Cooper et al., 2013). During the acute infection stage, a temporary reduction in circulating CD4+ T-cells occurs which then precedes recovery to almost normal levels, followed by a slow decrease of about 50-100 cells per µl (Maartens et al., 2014). Further depletion of both CD4+ and CD8+ naïve T-cells results from the damage of the fibroblastic reticular cell networks, and ,decreased expression of IL-7, a key T-cell survival factor, in the lymphoid tissue (Zeng et al., 2012).

The majority of T-cell homeostasis dysfunction happens during early infection and in the GIT. The GIT is the location of a catastrophic depletion of activated memory CD4+ T-cells. ART has minimal effect on the recovery of the loss of these activated CD4+ T-cells (Mehandru et al., 2007). Several changes in T-cell subsets occur in addition to the loss of total CD4+ T-cells during HIV-1 infection. A significant loss in memory T cell populations occur thereby eliminating the ability of the host to mount an effective defence against pathogens for which the effective memory response would be highly effective (Yates et al., 2007). A preferential loss of T helper 17 (Th17) cells, which are important components in mucosal immunity and barrier defence, along with mucosal-associated invariant T (MAIT) cells which exhibit effector-like function in their response to microbial pathogens, has been documented (Cosgrove et al., 2013). T follicular helper (TFh) cells are specialised subsets of CD4+ T cells and play a crucial role in providing protective immunity in supporting B cells produce antibodies against pathogens. These cells are considered highly permissive to HIV-1 infectivity and have been implicated in HIV-1 persistence by supporting viral replication during treated infection and are known to serve as significant cellular reservoirs of HIV-1 DNA (Thornhill et al., 2017)

Due to the significant loss of important lymphoid cells in the GIT, enterocyte apoptosis and subsequent compromised GIT integrity, HIV infected individuals develop “leaky gut” syndrome. Gut permeability is further characterised by increased concentration of microbial products such as lipopolysaccharides (LPS) in blood plasma due to translocation of microbial products as a result of gaps within the tight junctions of the intestinal epithelial barrier. Microbial translocation has been implicated in being the cause of systemic immune activation and persistent inflammation in a chronic HIV-1 disease (Brenchley et al., 2006a).

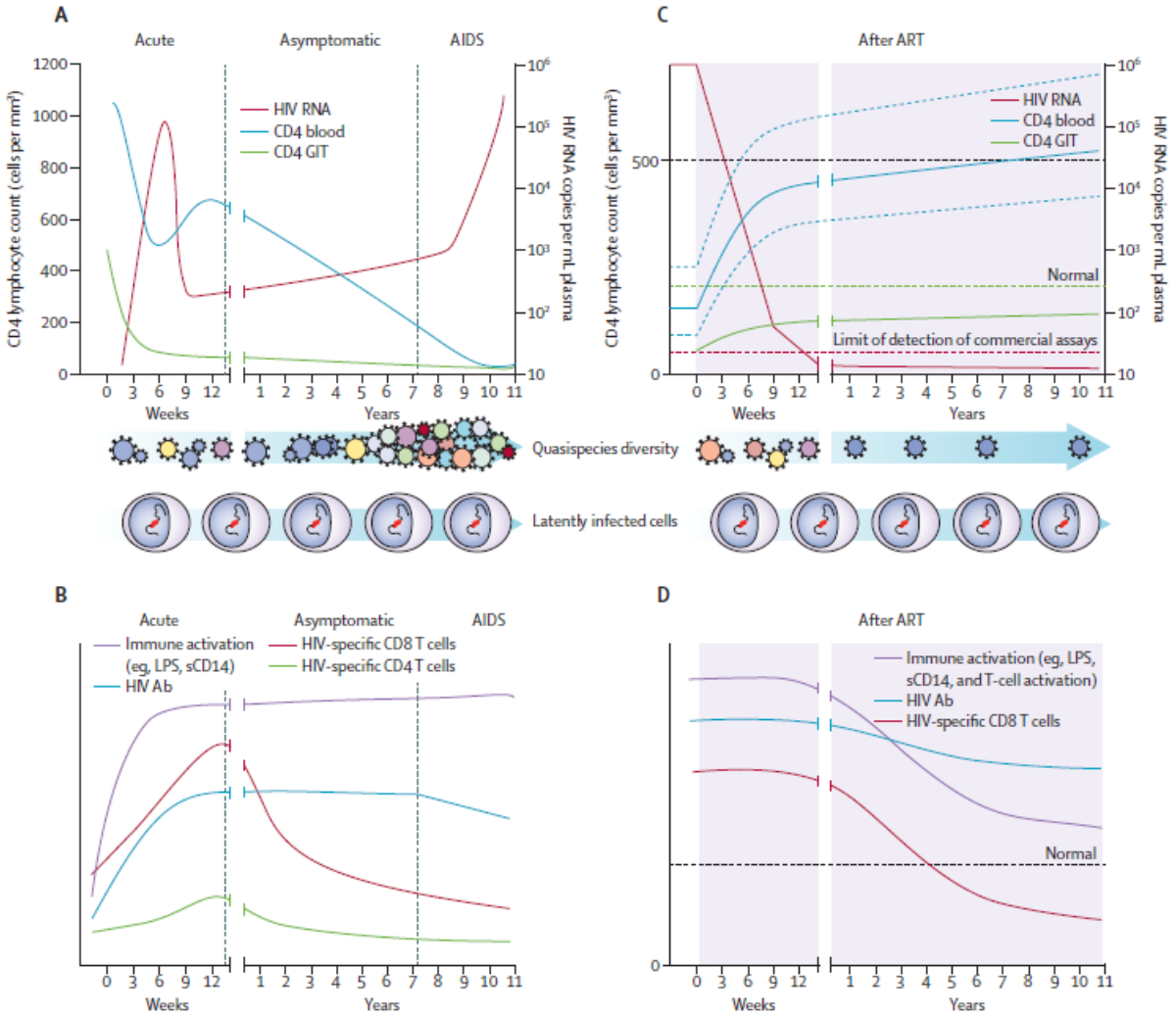


Figure 2.3: *Progression of clinical, virological and immunological parameters during untreated and treated HIV-1 infection as adapted from Maartens, Celum and Lewin, 2014.*
(A) During untreated infection a progressive loss of CD4+ T-cells in the GIT (depicted in green) and blood (depicted in blue) and an increase in HIV-1 RNA in the acute phase after which a viral set-point is reached and rapid increase during the progression to AIDS (red). (B) During the acute phase of HIV-1 infection, a significant increase in markers of immune activation (IL-6, LPS and sCD14) is observed along with the production of non-neutralising antibodies and a temporal decrease in HIV-specific CD4+ and CD8+ T-cells in the blood. (C) Following ART initiation, HIV RNA (red) in the blood decreases significantly to undetectable levels, CD4+ T-cells start increasing in the blood (blue), however, CD4+ T-cells in the GIT do not reach normality and remains reduced (green). (D) Following ART and decreased HIV RNA levels, HIV-specific T-cells decrease in addition. HIV antibodies persist over time in all individuals. Immune activation decreases following therapy but does not reach normal levels when compared to uninfected counterparts.

Immune activation

An increase in immune activation is a key characteristic following HIV-1 infection. Immune activation induced by the virus affects both innate and adaptive immune compartments and results in defects in coagulation (Lichtfuss et al., 2011).

Pathogen-associated molecular patterns (PAMPs) are recognised by specific receptors of the hosts' innate immune system. These include TLRs, C-type lectin receptors, retinoic acid-inducible gene I-like receptors and leucine-rich repeat (NLR) containing NLR proteins (Guo et al., 2014). The key drivers of immune activation include the direct effects of HIV-1 as a ligand for Toll-like receptors (TLR) 7 and 8 which are abundantly expressed on plasmacytoid dendritic cells (pDCs) which then lead to the production of interferon (IFN)-α

(Meier et al., 2009). LPS elevation triggered by microbial translocation is a powerful activator of TLR 4 and leads to the production of proinflammatory cytokines including interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) (Browne, 2020). IL-1β, a proinflammatory cytokine, plays an important role in driving inflammation through a binding mechanism with the IL-1 receptor. This binding induces the production of more proinflammatory cytokines. The production of inflammatory cytokines is strictly controlled by (1) the induction of pro-IL-1β gene expression and (2) caspase-1-mediated pro-IL-1β cleavage. Inflammasome complexes of the innate immune system regulate the cleavage of pro-IL-1β through the assembly into multicomponent proteins. Several inflammasome complexes have been described (i.e., NLRP1, NLRP3 most extensively described), NLRC4, NLRP6, and NLRP12 and the pyrin and HIN200 domain-containing Interferon-inducible protein (AIM2) (Guo et al., 2014)

Other important drivers of immune activation during HIV-1 infection include co-infections. Cytomegalovirus (CMV) infections induce the increase of specific T-cells and result in the reduction of Th17 and T regulatory cells (Tregs) in the GIT (Prendergast et al., 2010).

HIV-1 Pathogenesis in children compared to adults

In early life, the immune system of an infant is considered relatively immature with limited pathogenic memory in the adaptive immune compartment. The immune system develops over time as more antigen exposure occurs and T- and B-cell populations expand. However, the innate immune system can mount a rapid and broadly antigen-nonspecific response following the introduction of pathogens. Important cells of the innate immune system include DCs, NK cells, neutrophils, monocyte/macrophages, humoral proteins (complement, acute proteins), cytokines and chemokines. In early life, an innate response is favoured along with a T helper (Th)-2 type (anti-inflammatory/tolerogenic) response (Tiemessen and Kuhn, 2006).

The mechanisms, both virological and immunological, of HIV-1 disease progression in children are different to those of adults (Goulder et al., 2016b). In the absence of cART, more than 50% of children would die by the age of 2 years compared to adults where the survival time is approximately 11 years (Babiker et al., 2000; Newell et al., 2004). In ART-naïve adults, a viral set-point is achieved after 6 weeks compared to ART-naïve children in whom which a viral set point is reached after 5 years (Goulder et al., 2016a) (Figure 2.4).

Non-progressing adults or “elite controllers”, defined as ART-naïve individuals infected with HIV-1 and able to maintain normal CD4+ counts and immune function without progression to AIDS, are characterised by having strong HIV-specific CD8+ T-cell responses and protective Major Histocompatibility Complex (MHC) class I alleles, HLA-B27 and HLA-B57 (Kaslow et al., 1996; Kiepiela et al., 2007; Pereyra et al., 2010). Through this mechanism, effective suppression of HIV-1 viremia to undetectable levels allows for high CD4+ T-cells to be preserved. However, in non-progressing HIV-1 infected children, characterised as older than 5 years of age who sustain absolute CD4+ counts in the normal range for age-matched counterparts, HLA-B alleles do not have as strong an influence on HIV-1 disease outcomes as in adult populations (Adland et al., 2015). Therefore, non-progressing infection in adults is characterised by low or undetectable viral loads and low levels of immune activation compared to non-progressing HIV-1 infected children, where infection is characterised by high viral loads (~30, 000 copies/ml) and low levels of immune activation (Figure 2.4). The

prevalence of "elite controllers" in paediatric populations is around 5-10% compared to adults which is around 0.3% (Blanche, 1997; Paul et al., 2005; Pereyra et al., 2008; Goulder et al., 2016a).

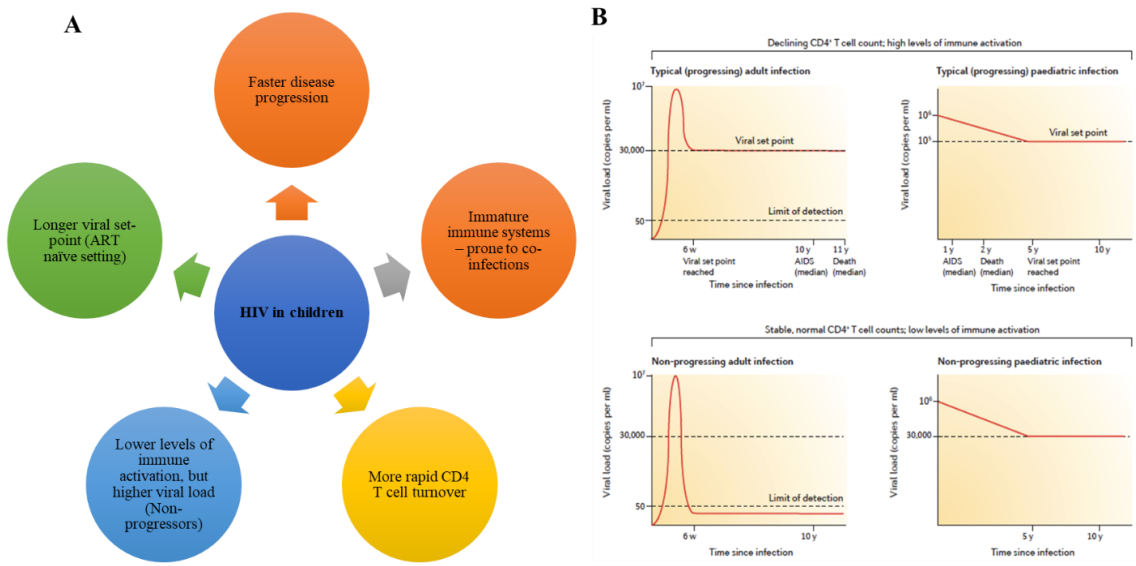


Figure 2.4: *Key features of HIV-1 pathogenesis in children compared to adults (Goulder et al., 2016a). (A) Clinical factors that define HIV-1 infection in children compared to adults. (B) Viral load changes following HIV-1 infection in children and adults. Progressing adult and paediatric infection (top) and non-progressing adult and paediatric infection (bottom).*

2.3 HIV-1 Treatment

cART regimes were developed during the late 1990s and were able to effectively suppress viral replication transforming HIV-1 from a disease with high mortality to a manageable chronic disease. Over 25 licensed antiviral drugs have been developed to block various stages of HIV-1 replication (Figure 2.3).

The accessibility to improved antiviral drugs in both low- and middle-income countries is considered a key milestone in the history of HIV disease (Simelela and Venter, 2014). The efficacy of cART has significantly decreased the morbidity and mortality rates related to HIV infection over the last 18 years (Mocroft et al., 2003; Braitstein et al., 2006; Who & Unicef 2011; Simelela & Venter 2014). Treatment regimens have become simpler, less toxic, have a lower pill burden and dosed less frequently than the initially developed protease inhibitor based regimens (Palella et al., 1998; Palmisano and Vella, 2011).

The use of cART has shown to effectively supress HIV-1 to below detection levels (<40 copies/ml in the vast majority of patients), allowing infected individuals to control their infection and live long and productive lives (Lewin et al., 2011). Standard cART regimens combine two nucleoside reverse transcriptase inhibitors (emtricitabine or lamivudine together with one of abacavir, tenofovir, or zidovudine) with a non-nucleoside reverse transcriptase inhibitor, protease inhibitor, or integrase inhibitor (Maartens et al., 2014).

Following the initiation of ART, plasma HIV-1 viral load levels decrease to concentrations below the lower limit of routine laboratory detection, usually within 3 months (Figure 2). However, the recovery of CD4+ T-cells in HIV infected individuals may differ. The factors associated with poor CD4+ T-cell recovery include host factors (age, lower CD4 nadir, HLA type), viral factors (co-receptor preferences, co-infections) and

immunological factors such as lower thymic outputs, poor bone marrow function, increased immune activation, proliferation, exhaustion and increased apoptosis (Rajasuriar et al., 2011; Maartens et al., 2014).

HIV-1 treatment regimens in children

The rapid scale-up of cART in resource-limited settings globally has allowed for the successful enrolment of millions of HIV-1 infected children into clinical care and treatment programmes. cART has been beneficial in improving the overall virological and immunological parameters in HIV-1 infected children and largely reduced the mortality, morbidity and comorbidity incidences (Johnson et al., 2008; Sutcliffe et al., 2008; Makatini et al., 2021). In 2018, 260, 000 children (0-14 years) were living with HIV-1 of which only 63% were documented to be on treatment (Hackett et al., 2021).

Current improved guidelines for the treatment of HIV-1 infected children include: The provision of cART for all children regardless of the CD4 count or clinical staging, immediate initiation of infant cART following the first positive HIV-1 PCR whilst awaiting confirmatory test results, the use of a follow-up HIV-1 PCR confirming the HIV-1 PCR positive test; HIV-1 viral loads no longer used for the baseline assessment of cART initiation in children (Department of Health National Consolidated Guidelines for the Prevention of Mother-to-Child Transmission of HIV (PMTCT) and the Management of HIV in Children, Adolescents and Adults).

The World Health Organization (WHO) has recommended the use of lopinavir/ritonavir (LPV/r)-based first-line ART for children under 3 years of age since 2010. The use of nonnucleoside reverse transcriptase inhibitors (NNRTIs), such as nevirapine (NVP) and efavirenz (EFV), are common (Hackett et al., 2021).

A number of challenges exist for the provision of cART in HIV-1 infected children outside of a clinical trial in South Africa. Reasons for poor uptake of cART in children include unpalatable formulations for children, the significantly higher cost of protease inhibitors, and the limited number of well-trained clinicians in paediatric (Hackett et al., 2021).

We have previously alluded to the benefits of early cART initiation in improving virological and immunological outcomes as well as playing a role decreasing the size of the seeding HIV-1 reservoir (Bachmann et al., 2019). cART can decrease the HIV-1 viral loads to below the levels of detection. Due to this, the HIV research agenda has shifted towards HIV-1 “cure”, complete elimination of the virus from the host, thereby ending the requirement for lifelong cART.

2.4 Barriers to HIV Cure: Establishment of the Latent Reservoirs

How is the latent reservoir established?

It is well documented that activated CD4+ T-cells, expressing increased amounts of the CD4+ receptor and chemokine co-receptors, serve as the main target cells of HIV-1. Infection of these CD4+ T-cells is followed by viral replication and subsequent integration of viral DNA into the host genome (Sonza et al., 2001; Chomont et al., 2009). When the provirus integrates into long-living resting memory cells, it is able to survive without viral replication - this is termed viral latency and allows for preserving HIV-1 reservoirs of replication-competent virus despite suppression with cART. Most latently infected cells contain a single copy of HIV-1

DNA that is stably integrated into the host's genome and is considered transcriptionally silent. These HIV-1 proviruses are also capable of resuming replication following the discontinuation of ART (Josefsson et al., 2011, 2013; Barton, Winckelmann and Palmer, 2016). HIV-1 latent reservoirs are established during the first week of infection (Chun et al., 1998; Schacker et al., 2001; N. M. Archin et al., 2012; Svicher et al., 2014) and can also be localised in various anatomical regions, organs and cell types including the thymus (Scinicariello et al., 2010; Fabre-Mersseman et al., 2011), GIT and brain (Chun et al., 2008), perivascular macrophages, parenchymal microglia and astrocytes (Thompson et al., 2011).

The generation and preservation of a silent provirus in resting memory CD4+ T-cells is well-characterised and theoretically the most vital mechanism for HIV-1 persistence (Finzi et al., 1997; Wong et al., 1997; Chun et al., 1997). However, more recent studies show that latency, a stochastic event, can be established and may persist in activated and resting CD4+ T-cells (Chavez et al., 2015; Barton et al., 2016). Researchers have also demonstrated HIV-1 persistence in three key memory T-cell subsets, each with specific functional and survival capabilities. These include central memory (Tcm) (CD45RA-CCR7+CD27+), transitional memory (Ttm) (CD45RA-CCR7-CD27+) and effector memory (Tem) (CD45RA-CCR7-CD27-) CD4+ T-cells (Chomont et al., 2009; Klatt et al., 2013). T stem cell memory (Tscm), which account for 2-4% of circulating lymphocytes, are also considered robust HIV-1 reservoirs, as they are relatively stable and preserved through "homeostatic self-renewal" (Gattinoni et al., 2011; Buzon et al., 2014a). In addition, a fairly recent study by Banga et al., (2016) showed that TFh expressing cell surface receptors, C-X-C chemokine receptor type 5 (CXCR5) and PD-1, are a key source of replication-competent HIV-1 virus in treated aviraemic patients.

Latent HIV-1 reservoirs are undetected by the hosts' immune system as the absence of antigenic expression circumvents any detection. Reservoirs persist despite long-term therapy and are therefore considered key barriers to eradication and cure (Chun and Fauci, 2012; Eisele and Siliciano, 2012). Investigations evaluating the long-term decay or kinetics of HIV-1 reservoirs show that infected cells could persist for as long as 60 years. Various mechanisms for this on-going persistence include constant replenishing of the reservoir through cell-to-cell spread, cell-associated replication or possible residual replication in MALT (Klatt et al., 2013). Modelling studies investigated by Rong & Perelson (2009) suggest the production of intermittent viral blips generated whilst replenishing the latent reservoir through homeostatic proliferation and/or de novo infection measures occur by the activation of latently infected cells that can retain persisting virus.

Studies evaluating the role of appropriate timing of cART initiation in relation to the HIV-1 reservoir size have shown varying outcomes (Lori et al., 1999; Zhang et al., 1999; Blankson et al., 2000; Jain et al., 2013). Most studies report a positive correlation between early cART initiation and a diminished HIV-1 reservoir size. Strain et al., (2005) demonstrated that initiation of ART within the first 6 months of infection was associated with substantially decreased cell-associated HIV-1 infectivity. Another study assessing early versus late treatment found that HIV-1 persistence remained relatively stable during therapy. It was however, suggested that the delay of cART in patients may lead to a permanently enlarged HIV-1 reservoir resulting in a reduced ability to clear the latent reservoir (Jain et al., 2013).

Data generated from the VISCONTI cohort, showed that early ART initiation as well as prolonged therapy during primary infection resulted in a lower pool of HIV-1 CAD (Sáez-Cirión et al., 2013). A study conducted by Persaud et al., (2012) showed a reservoir size below detection limits of the assay at 48 weeks in more than 50% of children given cART at a median of 8 weeks of age. Another study conducted in the USA showed that five adolescents given suppressive cART within two months of age, had smaller latent reservoirs with no infectious HIV-1 recovery when compared to adolescents who received treatment later (Luzuriaga et al., 2014). Children who had early therapy initiation, showed notably lower levels of both HIV-1 cell-associated DNA and RNA (Luzuriaga et al., 2014; Ananworanich et al., 2014; Martinez-Bonet et al., 2015). In the CHER cohort, children in which cART was initiated before 2 months of age, showed decreased frequency of HIV-1 infected CD4+ T-cells at 7-8 years of age compared with those children who received therapy later (Van Zyl et al., 2015).

Reservoir establishment in early life HIV-1 infection (perinatal)

The pathogenesis of HIV-1 infection and establishment of latent reservoirs are seemingly different in children than in adults and further highlights the need for further defining of various immunological mechanisms in paediatric HIV-1 infection. Under optimal circumstances, therapy in HIV-1 infected children can be initiated earlier than in adults. Following the discontinuation of cART, early treated HIV infected children showed improved control of viral replication and therefore serve as good candidates for evaluating HIV remission or eradication strategies (Goulder et al., 2016a). HIV-1 CAD levels have been shown to decline over a longer period than when compared to adults that seem to reach a plateau at approximately 4 years of infection (Besson et al., 2014). Studies have also indicated a shorter half-life of latently infected resting CD4+ T-cells in children compared to adults (Goulder et al., 2016a). Other factors that may contribute to the significance of evaluating early treated children as “cure” candidates include a tolerogenic environment (Th17 bias) in early life that favours low levels of immune activation and a high proportion of naïve CD4+ T-cells that are present (Mold et al., 2008; Bunders et al., 2012). In addition, children who have been infected in utero display HIV-specific CD8+ T-cell responses that are present in early infancy; however, early responses may not have generated a memory response. Early treated HIV-1 infected children, therefore, serve as relevant study candidates for a potential T-cell based vaccine (Luzuriaga et al., 1995; Thobakgale et al., 2007; Gray et al., 2014; Goulder et al., 2016a).

Suggested strategies for reservoir depletion require multiple approaches since HIV-1 latency is itself multifactorial. The impact and timing of early ART treatment is therefore significant when considering eradication strategies (Archin and Margolis, 2014). Furthermore, the measurement and composition of the HIV-1 latent reservoirs are significantly important for understanding and then developing strategies for cure.

Methods for the quantification and characterisation of the latent reservoir

The gold standard for measuring the occurrence of replication-competent proviruses contained within resting CD4+ T -cells utilizes Quantitative Viral Outgrowth Assays (QVOAs) developed in the mid-1990s (Finzi et al., 1997; Finzi et al., 1999; Siliciano & Siliciano 2005). These QVOAs essentially involve the use of a strong T-cell mitogen, phytohemagglutinin (PHA), which aims to activate resting CD4+ T-cells. The stimulated cells

are thus induced to promote expression of viral genes that ultimately leads to release of HIV-1 from latent infected cells. Following the development of QVOA, simpler measures for the latent reservoir have been developed of which many are PCR-based, including the CAD assay – a molecular biomarker of HIV-1 persistence (Rouzioux et al., 2014; O'Doherty et al., 2002; Strain et al., 2013).

Correlation studies between PCR- and culture-based assays have shown several discrepancies. This is primarily due to the nature of proviruses contained within CD4+ resting cells being of heterogeneous nature (Eriksson et al., 2013; Bruner et al., 2015; Ho et al., 2013). Recent investigations highlight the challenge of available assays in providing an appropriate and accurate measurement of the reservoir size. Additional hurdles include the high cost of viral recovery assays as well as the large volume of sample required for processing (Bruner et al., 2015). These incongruities in measuring the reservoir therefore serve as major barriers to clinical trials aiming to investigate and subsequently understand the efficacy of HIV-1 eradication strategies.

Measuring the HIV-1 latent reservoir in children with virological suppression

In the context of PHIV, the long-term effects of HIV-1 persistence comes with challenges: (1) Lifelong cART adherence poses challenges (2) increased risk for the development of co-morbidities and illnesses associated with chronic inflammation and immune senescence. Measuring HIV-1 latency in virologically suppressed children also poses challenges due to the limited biomarkers of HIV-1 reservoir size, low HIV-1 diversity and it has been shown that HIV-1 reservoir persistence occurs by the mechanisms of clonal expansion (Katusiime et al., 2021).

QVOA, the gold standard approach to measuring HIV-1 replication competent virus, requires a large sample volume input (as described above) and may therefore not always be feasible for paediatric cohorts' part of large-scale studies. Total HIV-1 CAD, a quantitative PCR (qPCR) is considered a putative molecular biomarker of HIV-1 persistence and often utilised within the field (Henrich et al., 2012; Strain et al., 2013b; Kiselinova et al., 2014; De Oliveira, Gianella, et al., 2015) and have been utilised in a number of cohorts to measure the HIV-1 latent reservoir following early/immediate initiation of ART (Nancie M. Archin et al., 2012).

qPCR approaches are considered favourable in that they are highly sensitive, have a relatively high-throughput, are cost-effective and require low sample volumes. However, qPCR approaches may not provide an accurate measure of the HIV-1 latent reservoir given its inability to distinguish between genetically defective or intact proviruses and therefore generally overestimates (by 300 fold) the size of the HIV-1 latent reservoir (Ho, Shan, Hosmane, Wang, Laskey, Daniel I S Rosenbloom, et al., 2013).

Alternative options to the QVOA or assays used in conjunction with QVOA include the Intact Proviral Detection Assay (IPDA) (Bruner et al., 2019) which utilises droplet digital PCR to target two highly conserved regions of the HIV-1 viral genome wand allows for the concurrent quantification and discernment of both intact and defective viral genomes and tat/rev induced limiting dilution assay (TILDA) which quantifies the inducible replication competent HIV-1 reservoir within a short time (Procopio et al., 2015). TILDA relies on

the measurement of tat/rev transcripts from cells plated in limiting dilution following activation with Phorbol 12-myristate 13-acetate (PMA) and ionomycin.

2.5 HIV-1 persistence in children during virological suppression

Residual Immune dysfunction under long-term suppressive therapy

Even with early long-term suppressive therapy leading to undetectable viral loads, decreased reservoir size and reconstituted CD4 counts, the immune system, both innate and adaptive, remains in a state of dysregulation and imbalance (Cassol et al., 2010; Gaardbo et al., 2012; Mendez-Lagares et al., 2012; Wilson and Sereti, 2013; Muenchhoff et al., 2014; Hunt et al., 2016; Alvarez et al., 2017). The extent to which cART can fully normalise functional immunity remains unclear. Chronic inflammation as a result of CD4+ T-cell activation and destruction, is considered a hallmark in the pathogenesis and disease progression of HIV-1 infection leading to a number of non-AIDS defining morbidities including cardiovascular, metabolic and neurologic disease outcomes in both children and adult populations (Lichtfuss et al., 2011). Key factors such as low-level viremia (often appearing as viral blips in patients with undetectable viral loads), chronic co-infections such as CMV and Epstein-Barr virus (EBV) as well as microbial translocation due to gut epithelial integrity loss, serve as key drivers of these resultant morbidities (Estes et al., 2010; Plaeger et al., 2011; Fitzgerald et al., 2013).

Microbial translocation has been documented to play a significant role in defining the persistence of T-cell as well as monocyte/macrophage activation in both adults and children. Microbial translocation has been highlighted as one of the major contributors to chronic immune activation in the setting of HIV-1 infection (Estes et al., 2010; Sandler and Douek, 2012; Fitzgerald et al., 2013; Marchetti et al., 2013; Pasternak and Berkhout, 2016). Microbial translocation has been linked to the dramatic depletion of CCR5 expressing CD4+ T-cells in the GALT during early stages of infection. Inflammatory processes have been implicated in damage to the structural integrity of the gut epithelium, which in turn permits translocation of gut components into the bloodstream. In HIV-1 infected infants, the intestinal epithelium is more permissive to antigen than adults and consists of a profusion of memory CD4+CCR5+ T-cells resulting in more dramatic gut damage. These phenomena can be further exacerbated by low birth weight and the composition of gut flora (Wallet et al., 2010; Papasavvas et al., 2011; Tobin and Aldrovandi, 2013; Roider et al., 2016). The resultant gut damage and its implications in persisting immune dysregulation in children on early, long-term suppressive therapy requires further delineation.

Other features of immune dysregulation despite cART and CD4+ T-cell reconstitution include the incomplete restoration of pre-existing memory CD4+ T-cells for the clearance of common pathogens (Riou et al., 2015), immune exhaustion (Nakanjako et al., 2013), premature immune ageing (Appay and Rowland-Jones, 2002), impaired T-cell proliferation (Nakanjako et al., 2013) as well as low antigen-specific CD4+ T-cell immune responses (Nabatanzi et al., 2014).

The majority of studies documenting immune dysregulation have been focused on adult cohorts. The extent of HIV-related chronic inflammation and the effects thereof is much more poorly described and understood in infected children on long-term cART, specifically within regions of high disease burden such as South Africa.

There is great need for delineating immune dysregulation that may exist in children infected with HIV-1 at birth, initiated with early therapy and who remain virologically suppressed over the long-term. There is, for example, a clear link between persistent inflammation and immune activation and the risk of development of cardiovascular disorders. Approaches which may address ongoing aberrant inflammatory processes requires novel and informed approaches.

It is also important to understand the role of immunological responses to HIV-1 persistence. Recently it has been proposed that latency/reservoir establishment may be an adaptation of the virus to overcome host barriers to establishing successful infection (Barton et al., 2016). If the virus is able to establish latency early, it has another chance to establish infection within the host should initial target cell infection be unproductive. As mentioned above, the host cell possesses an array or antiviral factors, and when combined with early innate immune effector mechanisms, can block active viral replication. The reservoir provides an infection restart source. For this reason, the size of the reservoir may be related to host immune factors, as much as to viral factors.

Studies have well-documented improved immunological outcomes with early cART initiation and subsequent virological suppression. Improved CD4+ T-cell numbers and decreased levels of immune activation are key features of improved outcomes following therapy (Cotton et al.; 2013; Koblavi-Dème et al., 2003; Resino et al., 2003; Kabue et al., 2012; Sáez-Cirión et al., 2013). The CHER trial showed significantly better clinical as well as immunological outcomes in children treated early as opposed to those with deferred cART therapy (Cotton et al., 2013a). Other key studies also show that ART-mediated virological suppression reduces the activation status of both CD4+ and CD8+ T-cells (Hunt et al., 2003; Robbins et al., 2009; Jain et al., 2013). Another study investigating the effects of early initiation of ART showed a normalisation in the levels of monocyte and macrophage activation marker sCD163 (Burdo et al., 2011). However despite positive immunological outcomes following early therapy as well as virological suppression, immune abnormalities including CD4+ and CD8+ T-cell activation or dysfunction may still occur and CD4+ T-cell populations do not always reach normal levels even after years of suppressive therapy (Corbeau and Reynes, 2011; Klatt et al., 2013; Rajasuriar et al., 2015). The ability of early cART to prevent these potentially irreversible outcomes remains unclear and studies have underlined a number of immune abnormalities that persist despite many years of viral suppression (Valdez et al., 2002; Aiuti and Mezzaroma, 2006; Kelley et al., 2009). When compared to uninfected individuals, HIV-1 infected persons may display a reduced function of the adaptive immune compartment despite effective virological control (Lange et al., 2002; Lederman et al., 2011).

T-cell populations expressing high levels of key activation markers such as HLA-DR and CD38 during untreated HIV-1 serve as highly significant prognosticators for disease progression. However, the relevance of these immunological markers during treatment remains relatively undefined. Some studies suggest some level of predictive significance (Hunt et al., 2011; Kaplan et al., 2011; Klatt et al., 2013). Other anomalies of immune markers characterised during effective cART treatment include decreased levels of CD28, and increased levels of CD57 and PD-1. These described markers relate to a predominant immunological profile

of T-cell senescence and dysfunction (Appay and Sauce, 2008; Kaplan et al., 2011; Tassiopoulos et al., 2012; Hatano et al., 2013; Sauce et al., 2013).

Most recent work investigating HIV-1 latent reservoirs is predominantly focused on peripheral blood CD4+ T-cells. However, these cells do not serve as the only targets for HIV-1. Monocytes and especially macrophages are susceptible to HIV-1 infection due to the expression of the CCR5 receptor (Tuttle et al., 1998). Macrophages can be found in all tissues and body compartments including the GALT and the brain and they are able to support active viral replication (Honeycutt et al., 2016). Traditionally, monocytes are not considered long-lived viral reservoirs due to their characteristically short life span in the bloodstream. However, susceptible cell populations may represent an important route for the establishment of inherent reservoirs cells as these cells migrate into tissues and differentiate into macrophages. Macrophages, therefore, represent important HIV-1 reservoirs because of their longevity (several weeks to years) and ability to reside in multiple tissue compartments. Microglia are the most long-lived macrophage cell types. These cells harbour HIV-1 for long periods and may be responsible for a slower second-phase decline in plasma viremia seen in patients on therapy (Watters et al., 2013).

Due to the limitations in accessing tissue macrophages, much work has focused on monocytes. Hearps et al., (2012) showed the persistence of monocyte dysfunction, such as decreased phagocytic capabilities, despite continuous ART. Other studies investigating monocyte activation showed a sustained increase level of sCD163 in patients on combination therapy (Burdo et al., 2011a; Burdo et al., 2011b).

Soluble immune biomarkers including cytokines, chemokines and growth factors are diverse and pleiotropic in nature. These immune biomarkers play an important role in maintaining the homeostasis of the immune system and has attracted much attention as diagnostic biomarkers for infectious and inflammatory diseases in recent years (Hamidzada and Guzzo, 2019). There is major dysregulation in the production of related cytokines and chemokines during HIV-1 infection (Biggs et al., 1995; Kedzierska et al., 2000; Kedzierska and Crowe, 2001). A number of studies have acknowledged the importance of these soluble immune biomarkers in shaping the immune response during the progression of HIV-1 infection, several of which play an important role in HIV-1 control (Hamidzada and Guzzo, 2019). Research studies into HIV-1 progressors and non-progressors highlight significant differences in cytokine production and function between these two groups and therefore suggests an important role of these immune biomarkers towards viral control (Reuter et al., 2012).

In early research, it was well documented that HIV-1 resulted in a predominant switch to Th2 (anti-inflammatory) cytokines rather than a Th1 (pro-inflammatory) cytokine milieu. Th2 cytokines, including IL-1, IL-4, IL-6, IL-8 and tumour necrosis factor (TNF)-α were observed to be elevated in comparison to IL-2 and IFN-γ which are Th1 cytokines and have been documented to be much lower during HIV-1 infection (Esser et al., 1991; Clerici and Shearer, 1993). More recent research is indicative of an adaptation of the immune system to ongoing infection and the Th1-Th2 switch in cART treated patients is less clear. In HIV-1 infected children there is a predominance of Th2 and Th17 responses compared to adults. Th2 responses are usually observed in untreated patients due to the selective depletion of Th1 memory cells. Under cART, the

switch, if still observed may reflect the exhaustion of Th1 cells due to ongoing inflammation and immune exhaustion.

The degree of T-cell activation and monocyte or macrophage differentiation determines the level of susceptibility of cells to HIV-1 infection. It is therefore important to highlight the role of cytokines, chemokines and immune growth factors that contribute to facilitating the activation and differentiation of the cells of the immune system. Furthermore, the function of these immune molecules suggests its pertinent role in modulating both HIV-1 infection and replication (Zack et al., 1990; Sonza et al., 1996).

The immunological impact of HIV-1 is said to be either inhibitory, stimulatory, or bifunctional (both inhibitory and stimulatory). This implies that HIV-1 pathogenesis (infection and replication) is under the continuous regulation of a complex cytokine network produced by a variety of cells (Kedzierska and Crowe, 2001; Hamidzada and Guzzo, 2019).

The most frequently investigated cytokines in both the HIV-1 and uninfected groups include IL-6, IL-10, IFN-γ and TNF-α (Hamidzada and Guzzo, 2019). The latter cytokines are known to play a key role in infectious and inflammatory pathways and have the potential of being included as diagnostic and drug targets (Decker et al., 2017). In more recent investigations, other soluble immune biomarkers have surfaced as pertinent role players in HIV-1 pathogenesis. These include IL-18, sCD163 and sCD14.

Cytokines such as TNF-α, TNF-β, IL-1 and IL-6 have been documented to play a role in stimulating HIV-1 replication in T-cells and macrophages. IL-2, IL-7 and IL-15 have functions in upregulating HIV-1 in T-cells and IL-10, IL-13, IFN-α and IFN-β play a role in inhibiting HIV-1 replication in T-cells and monocyte derived macrophages. IL-4, IL-6, IFN-γ and granulocyte-macrophage colony-stimulating factor (GMCSF), display both an inhibitory and stimulatory effect on HIV-1 (Kedzierska et al., 2000; Kedzierska and Crowe, 2001).

The key immune molecules involved in inhibiting HIV-1 replication in macrophages include those within the β-chemokine groups whereas α-chemokines play a role in suppressing infection of T-tropic strains of HIV-1. Examples of these chemokines include CXCR4, macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES (Mackewicz et al., 1997; Kedzierska et al., 2000; Kedzierska and Crowe, 2001).

The antiviral properties of cytokines can be produced through direct antiviral activity of a specific cytokine, but can also be produced indirectly, through the suppression of other cytokines that behave to enhance HIV-1 replication (Kedzierska and Crowe, 2001). Within the interleukin family of cytokines, several cytokines have been established to play a critical role in inhibiting HIV-1 replication (e.g., IL-21, IL-27, IL-32) (Imamichi et al., 2008; Nold et al., 2008; Greenwell-Wild et al., 2009; Adoro et al., 2015; Hamidzada and Guzzo, 2019).

Knowledge of the interplay of these soluble immune biomarkers provides insight into the complexity of the immune system and can therefore provide an understanding of its response to HIV infection as well as the effects of treatment regimend. In addition, evaluating these complex networks can also provide an overview of the immune response to other persisting co-morbidities such as early gut damage.

The long-term predictive and pathophysiological role of soluble immune biomarkers in HIV infection particularly within perinatally infected children from birth to pre-adolescence, remains unclear. These

immunological profiles can provide key insights into either clinical improvement of disease or progression leading to morbidities that require clinical surveillance and follow-up.

On the other hand, the efficiency of ART may be influenced by the imbalance of pro- and anti-inflammatory soluble immune biomarkers which contribute to regulating the immune response. Therefore, evaluating the levels of these cytokines may provide a good picture of the rate of viral replication and the resultant damage or restoration of the immune system during the implementation of ART regimens including the delay and interruption of therapy (Gaardbo et al., 2012; Osuji et al., 2018).

By fully identifying and characterizing the longitudinal pleiotropic role of immune biomarkers in relation to clinical outcomes in HIV infected children, we can provide critical information to understanding disease progression as well as HIV control or replication. Furthermore, this information provides insights into how therapeutic targets may be structured.

2.6 HIV-1 and the CNS

Immune status and Neurocognitive Outcomes in HIV-1 Infected children

HIV enters the CNS within 14 days of primary infection (Sailasuta et al., 2012). It has been suggested that this entry is mainly through infected monocytes with the intent to become brain-resident macrophages or perivascular macrophages soon after peripheral infection of circulating CD4+ T-cells and monocytes. Studies show monocytes to be less susceptible to cytopathic effects of HIV compared to CD4+ T-cells (Crowe et al., 1987; Olivetta and Federico, 2006; Cummins and Badley, 2013). Peripheral monocytes, therefore, serve an important role in channelling HIV into the CNS during acute or chronic infection (Liu et al., 2000). These cells may therefore result in long-lived reservoirs within the CNS. Poor neurocognitive function in HIV-infected children may be explained by HIV entry into the CNS prior to ART. A number of studies have supported good neurocognitive functioning in treated patients due to improved penetration of ART into the CNS (Smurzynski et al., 2011).

When HIV enters the brain it initiates events that result in cognitive, motor, as well as behavioural deficits (Kaul et al., 2005; Ances and Ellis, 2007; Heaton et al., 2011; Williams et al., 2014). It has been hypothesised that HAND occurs due to the transmigration of the CD14+CD16+ monocyte sub-population across the blood-brain barrier and into the CNS. This is followed by viral infection of microglia cells of the brain, which is mediated through interactions between monocytes, BBB cells, as well as chemotactic gradients that channel leucocytes into the CNS. Despite effective ART, monocytes are believed to enter into the CNS of HIV infected individuals resulting in low level on-going, chronic neuroinflammation (Williams et al., 2014).

CCL2 and CXCL12, two monocyte chemokines, have been implicated in the pathogenesis of HIV-associated neuropathogenesis. During cART and even during viral suppression, CCL2 levels have been documented to remain relatively high in the CSF of HIV infected individuals when compared with negative controls. Williams et al., (2014) therefore suggest, that despite cART, HIV infected individuals experience CCL2-mediated monocyte influx into the CNS which increases the risk for HAND despite the presentation of cognitive deficits (Chang et al., 2004; Letendre, 2011; Kamat et al., 2012; Williams et al., 2014).

Understanding the pathways and mechanisms that lead to monocyte activation and the subsequent contribution to neuroinflammation serve important when addressing HIV-associated cognitive disorders that may occur despite antiretroviral therapy and viral suppression.

2.7 Identified Knowledge Gaps

Children remain one of the most vulnerable populations affected by the HIV pandemic. ART has drastically reduced the rates of MTCT, and the number of HIV infected children has been significantly reduced, however MTCT still occurs, and the long-term impact of HIV in recently infected as well as older children on suppressive therapy remains understudied.

Although significant progress has been made to understand HIV-1 pathogenesis, long-term treatment effects and persistence in PHIV children, further knowledge is required to fully understand the immunological landscape that develops from time of infection and the unresolved immune dysfunction that persists in the context of long-term suppressive ART. Chronic immune abnormality is linked to the development of a number of disorders such as cardiovascular disease and metabolic syndrome often linked to inflammation and is less understood in PHIV approaching adolescence where adherence to ART is compromised.

Although children with PHIV have shown great potential for cure and remission studies, the development of the latent reservoir remains a challenge. Research is needed to address both immune dysfunction and HIV-1 latency.

Detailed longitudinal studies evaluating immunological outcomes along with clinical, virological and neurological determinants are lacking in children and would provide greater insight to forecasting health outcomes as children infected with HIV progress through the disease. Longitudinal immune profiling assessments of well-described cohorts such as the CHER cohort provide opportunities to ascertain how immune dysfunction manifest. Large cohort studies with age and demographically matched controls including HIV exposed uninfected (HEU) children, a significant expanding population within our setting, are important for evaluating change in immune status over time matched with changes to virus.

Insights and further identification of immune biomarkers that can predict the latent reservoir or provide an indication of the number of cells harbouring HIV-1 DNA is required, specifically in high disease burden settings where resources are limited, and high-cost laboratory techniques cannot always be implemented. Immune biomarkers hold significant potential to pinpoint important pathways to explore further.

More work is required to fully understand the immune dysregulation in children on continuous cART within our setting, which may also inform novel therapeutic approaches to minimize some the negative immunological aspects of long-term therapy.

Cohort studies that build on existing knowledge and run parallel studies examining clinical aspects of long-term suppressive ART such as neurocognitive and cardiovascular functioning are important for understanding the role and impact of immune function and dysregulation on these outcomes.

CHAPTER 3:

EVIDENCE OF PERSISTING CHRONIC INFLAMMATION DESPITE EARLY, SUPPRESSIVE THERAPY IN PERINATALLY HIV-1 INFECTED CHILDREN AT 8 YEARS OF AGE: AN ASSOCIATION WITH GUT PERMEABILITY

SUMMARY OF KEY FINDINGS – CHAPTER 3

- HIV+ children showed significantly higher levels of biomarkers associated with generalised chronic inflammation, particularly those associated with myeloid cell (monocyte/macrophage and dendritic cell) activation compared to HEU and HUU controls at 8 years of age.
- Multivariate cytokine and chemokine biomarker measurements show elevated levels of biomarkers associated with **chronic inflammation** (hsCRP, MIP-1β, IL-1α, IFN-α, CD40L, TGFβ2, VEGF) **macrophage activation** (sCD14, sCD163, IL-18, IL-17F) and **gut damage** (I-FABP, s100A8/A9, LBP) in HIV infected (HIV+) children
- Increased expression of novel plasma biomarkers indicative of direct epithelial damage (**I-FABP**) and intestinal inflammation (**MAdCAM-1**) provide further evidence of microbial translocation in HIV+ children and a chronic inflammatory profile
- Significantly **diminished expression of selected T-cell mediated plasma immune biomarkers** (GCSF, IL-3, RANTES, IFN-γ, IL-17A) in HIV-1 infected children at 8 years of age
- Decreased M1 macrophage-associated cytokine expression and expansion of classical and non-classical monocyte phenotype (**M1/M2 switch**) was observed.
- **Monocyte subset distribution was altered in the HIV+ group**, with a significantly increased proportion of a classical monocyte subset (CD14++CD16-) compared to the HUU group (p=0.032) but not the HEU group.
- A negative association between **LBP** (and other immune biomarkers) and **CD4 nadir**, highlights importance in minimising early pathology to mitigate the impact on inflammatory status years later.
- ***Conclusion**: Early interventions to minimise monocyte/macrophage activation, general inflammation and gut damage will be of importance to ensure a less aberrant immune response when approaching adolescence.*

ABSTRACT

Background: The extent to which cART can fully normalise functional immunity remains unclear. Following PHIV, South African adolescents display evidence of multi organ/system impairment attributed to long-term ART exposure. This impairment is related to long-term immune suppression and residual impact of HIV-1 infection itself. The extent of HIV-1 related chronic inflammation and its effects is less well described and understood in children with PHIV on long-term ART than in adults, particularly in developing countries.

Methods: A cross-sectional study at a median pre-adolescent age of 8 years (IQR; 8.4-9.0 years), was employed to assess wide-ranging immunological parameters as part of a follow-up extended study in HIV+ children (n=88). An extensive panel of soluble inflammatory/immune biomarkers was evaluated by Luminex® Multiplex assays and Enzyme-Linked ImmunoSorbent Assays (ELISAs). A subset of participants (n=64) was evaluated for the frequency and distribution of monocyte subsets by multiparameter flow cytometry. Age and community-matched controls consisting of HEU as well as HUU children were included in this investigation and evaluated for the same immune parameters (n=32 and 41, respectively). HIV-1 CAD was also quantified at two time points in a subset (n=32) of the HIV+ children.

Results: A total of 161 samples were quantified for immune biomarkers, 138 for intestinal fatty-acid binding protein (I-FABP), mucosal vascular addressin cell adhesion molecule 1 (MAdCAM-1) and s100A8/A9 and 64 samples were assessed for monocyte subset distribution. HIV+ children initiated cART at a median age of 6 weeks (2.1 months) of which 97% sustained virological control following therapy. The median viral load at therapy initiation was 738,501 copies/ml and the median CD4% at baseline was 36.9%. At 8 years of age, there were no significant differences in CD4% between the HIV+ group and the HEU and HUU groups ($p=0.261$). All children, except five showed undetectable viral loads.

HIV+ children showed significantly higher levels of biomarkers associated with generalised chronic inflammation, particularly those associated with myeloid cell (monocyte/macrophage and dendritic cell) activation compared to HEU and HUU controls at 8 years of age. These included highly sensitive C-reactive protein (hsCRP) ($p=0.01$), macrophage inflammatory protein 1 beta (MIP-1β) ($p=0.03$), IL-1α ($p<0.001$), INF-α ($p<0.001$), CD40L ($p<0.001$), transforming growth factor beta 2 (TGFβ2) ($p=0.02$), vascular endothelial growth factor (VEGF) ($p<0.00001$), sCD14, sCD163, IL-18, IL-17F ($p<0.001$) and platelet derived growth factor BB (PDGF-BB) ($p<0.00001$). We also observed a significant elevation of soluble calcium binding alarmin protein involved in the regulation of the inflammatory process and immune response, s100A8/A9, in the HIV+ group compared to the two study control groups ($p<0.001$). Within the HIV+ group, significant elevation of biomarkers associated with gut epithelial damage/microbial translocation was observed. Significant ($p<0.01$) elevation in the HIV+ group for lipopolysaccharide binding protein (LBP) and IFABP were noted. Spearman correlation analysis indicates a highly significant positive association between LBP and sCD14, sCD163, IL-18 and IL-17F in the HIV+ group ($p<0.01$). MAdCAM-1 measured significantly ($p=0.011$) higher in the HIV+ group (median:115.3 ng/ml) followed by the HEU group (median: 103.2 ng/ml), with HUU having the lowest MAdCAM-1 levels (median: 97.00 ng/ml).

The HIV+ group showed significantly lower plasma levels of regulatory, homeostatic and T-cell associated biomarkers, IL-3, Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES) (CCL5), granulocyte-colony stimulating factor (GCSF), INF-γ, TGFβ$_3$ and IL-17A) when compared to both HEU and HUU groups ($p<0.01$). We observed a significantly ($p<0.01$) decreased IL-1β, TNF-α and IL-6 in the HIV+ group compared to the other control groups. Monocyte subset distribution was altered in the HIV+ group, with a significantly increased proportion of a classical monocyte subset (CD14++CD16-) compared to the HUU group ($p=0.032$) but not the HEU group. We also observed a negative association ($r = -0.24$; $p=0.03$) between LBP and CD4 nadir.

Conclusions: We observed several significant differences in both innate and adaptive immune plasma biomarkers in HIV+ children at pre-adolescent years when compared to study controls even after early and long-term suppressive ART. Persisting inflammation and immune dysregulation associated with gut damage serves as a key finding in this investigation along with dysregulation of monocyte/macrophage pathways. These findings prompt further and more detailed immune profiling and clinical follow-up to establish the severity of morbidity outcomes that may progress from this immune dysregulation in children with PHIV. There is significant importance in minimising early pathology to mitigate the impact on inflammatory status years later.

3.1 Introduction

Children remain the most vulnerable population affected by the HIV-1 pandemic and their disease pathogenesis is different and more rapid than in adults (Goulder, Lewin and Leitman, 2016b; Roider, Muenchhoff and Goulder, 2016). The efficacy of cART has significantly decreased the morbidity and mortality rates in HIV-infected (HIV+) adults and children over the last 20 years (Mocroft et al., 2003; Braitstein et al., 2006; World Health Organization (WHO) et al., 2010; Simelela and Venter, 2014).

Combination ART effectively supresses HIV-1 replication to below detection levels (<40 copies/ml) allowing individuals to control their infection and live long and productive lives (Lewin et al., 2011). Therapy has become simpler and less toxic, converting HIV-1 into a chronic and manageable disease (Palella et al., 1998; Palmisano and Vella, 2011). Early cART initiation significantly improves clinical and immunological outcomes in children and adults (Lori et al., 1999; Zhang et al., 1999; Blankson et al., 2000; Strain et al., 2005; Violari, Mark F Cotton, et al., 2008; Jain et al., 2013). Early cART also limits the size of the seeding HIV-1 latent reservoir and substantially decreases cell-associated HIV-1 infectivity (Jain et al., 2013; Ananworanich et al., 2014; Luzuriaga, Tabak, Garber, Chen, Mcmanus, et al., 2014; Ananworanich and Mellors, 2015).

Despite early long-term suppressive therapy, smaller viral reservoirs and effectively reconstituted CD4 counts, both innate and adaptive immune systems remain dysregulated and imbalanced (Cassol et al., 2010; Gaardbo et al., 2012; Mendez-Lagares et al., 2012; Wilson and Sereti, 2013; Muenchhoff, Prendergast and Goulder, 2014; Hunt, Lee and Siedner, 2016; Alvarez et al., 2017). The extent to which cART can fully normalise functional immunity remains unclear. Chronic inflammation from CD4+ T cell activation and destruction is considered a hallmark of the pathogenesis and disease progression of HIV-1 infection, leading to non-AIDS defining morbidities including cardiovascular, metabolic and neurologic disease outcomes in the HIV+ population (Lichtfuss et al., 2011). Following PHIV, South African adolescents have shown evidence of multisystem impairment attributed to long-term ART exposure, immune suppression and the effects of HIV-1 infectivity (Frigati et al., 2019). Other key factors such as low-level viremia (often appearing as viral blips in those with undetectable viral loads), chronic co-infections such as CMV and EBV as well as microbial translocation from the gastrointestinal tract serve as significant drivers of ongoing immune dysregulation (Estes et al., 2010; Plaeger et al., 2011; Fitzgerald et al., 2013).

HIV-1 infection leads to persistent immune activation and ultimately chronic inflammation, even during cART and subsequent viral suppression. Chronic immune activation is associated with overall immune dysfunction, premature ageing and faster disease progression to AIDS (Salazar-Gonzalez et al., 1998; Hunt et al., 2008; Kuller et al., 2008; Rodger et al., 2009; Lichtfuss et al., 2011; Klatt, Funderburg and Brenchley, 2013; Seddiki, Brezar and Draenert, 2014; Hong et al., 2016). Despite cART improving the quality of life for infected patients, life expectancy remains reduced compared to HIV-1 uninfected counterparts.

Early insults to the immune system following infection with HIV-1 leads to a rapid depletion of CD4+ T cells in the mucosal tissues where a concentrated number of activated memory CD4+ T cells resides and serve as one of the important targets of the virus (Douek, Picker and Koup, 2003; Brenchley et al., 2006b; Klatt, Funderburg and Brenchley, 2013). The mechanisms contributing to persistent immune activation during

infection is considered multifaceted and complex. Although viral replication contributes to persistent immune activation, the extent to which the immune system is activated during chronic HIV-1 infection, cannot solely be attributed to the virus itself (Appay and Sauce, 2008).

Residual immune activation in treated HIV-1 has several causes, a key contributor is unresolved gut epithelial damage. Microbial translocation plays a significant role in defining the persistence of T-cell as well as monocyte/macrophage activation in both adults and children. Microbial translocation has been highlighted as one of the major contributors to chronic immune activation in the setting of HIV-1 infection (Estes et al., 2010; Sandler and Douek, 2012; Fitzgerald et al., 2013; Marchetti, Tincati and Silvestri, 2013a; Pasternak and Berkhout, 2016) and has been linked to the dramatic depletion of CCR5 expressing CD4+ T-cells in the GALT during early stages of infection. Inflammatory processes are implicated in damage to the structural integrity of the gut epithelium, which in turn permits translocation of gut components into the bloodstream. In HIV+ infants, the intestinal epithelium is more permissive to antigen than in adults and it has a profusion of memory CD4+CCR5+ T cells resulting in more dramatic gut damage. These phenomena can be further exacerbated by low birth weight and the composition of gut flora (Wallet et al., 2010; Papasavvas et al., 2011; Tobin and Aldrovandi, 2013; Roider, Muenchhoff and Goulder, 2016). The resultant gut damage and its implications in persisting immune dysregulation in infected children on early, long-term suppressive therapy requires further delineation particularly within our setting of high disease and social burden impacting cART adherence.

Other features of immune dysregulation despite ongoing cART and CD4+ T cell reconstitution, include the incomplete restoration of pre-existing memory CD4+ T cells for the clearance of common pathogens (Riou et al., 2015), immune exhaustion (Nakanjako et al., 2013), premature immune ageing (Appay and Rowland-Jones, 2002), impaired T-cell proliferation (Nakanjako et al., 2013) as well as low antigen-specific CD4+ T cell immune responses (Nabatanzi et al., 2014). In addition to CD4+ and CD8+ T cell loss, an altered NK cell functionality, decreased cytokine production, increased cytotoxicity as well as impaired homing of cells of the immune system are observed (Alter et al., 2004; Klatt et al., 2013; Klatt, Funderburg and Brenchley, 2013).

The majority of studies documenting immune dysregulation and the causes thereof have focused on adults. The extent of HIV-1 related chronic inflammation and its effects is more poorly described and understood in children with PHIV on long-term ART, specifically within regions of high disease burden such as South Africa. There is great need for delineating immune dysregulation that may exist in children with PHIV initiated on early ART and who remain virologically suppressed over the long-term. There is, for example, a clear link between persistent inflammation and immune activation and the risk of cardiovascular disorders. Strategies which may address ongoing aberrant inflammatory processes require novel and informed approaches.

This study aimed to evaluate immunological factors in chronic inflammation with a focus on microbial translocation and resultant gut damage as a key driver in children who acquired HIV-1 through vertical transmission and are on early, long-term and suppressive ART.

Research hypothesis: Virological suppression following early suppressive cART does not fully restore immune dysfunction following HIV-1 infectivity. Chronic inflammation may be largely driven by innate immune mechanisms triggered by early gut damage.

3.2 Materials and Methods

3.2.1 Study design

A cross-sectional study design, at a median pre-adolescent age of 8 years (IQR; 8.4-9.0 years), was employed to assess wide-ranging immunological parameters as part of a follow-up extended study in HIV+ children. An extensive panel of soluble immune biomarkers was evaluated and in addition, a subset of participants was evaluated for the frequency and distribution of monocyte subsets.

Age and community-matched controls consisting of HEU as well as HUU children were included in this investigation and evaluated for the same immune parameters.

Lastly, HIV-1 CAD was quantified at two time points in a smaller subset (n=32) of the HIV+ children, i.e., at baseline (closest time to therapy initiation) and follow-up (8 years of age).

3.2.2 Study participants

Study participants originated from the well-characterised randomised controlled CHER trial, between 2005 and 2011. All participants were consented (the legal guardian or caregiver of the participating child provided relevant consent – Appendix 2), and blood was collected for isolation of PBMC (stored in cryovials and placed in LN_2) and plasma (stored in polypropylene Eppendorf's at -80°C) storage. Early collected samples (PBMCs and plasma) were retrieved from Clinical Laboratory Services (CLS)™ in Cape Town, South Africa, storage repository.

First line cART comprised of lopinavir-ritonavir, zidovudine and lamivudine at ART initiation and re-initiation following therapy interruption. The primary endpoint of the original study was time-to-failure of first-line ART (immunological/clinical/virological) or death. Comparisons were by intent-to-treat, using time-to-event methods.

The original CHER randomised control trial implemented three study arms: Arm 1 (deferred ART – ART-Def), Arm 2 (early ART initiation for 40 weeks followed by interruption -ART-40W) and Arm 3 (early ART initiation for 96 weeks followed by interruption – ART-90W).

3.2.3 Routine testing

HIV-1 viral load

Viral loads were quantified by an accredited routine diagnostic laboratory, the National Health Laboratory Services (NHLS), Tygerberg Cape Town, South Africa.

HIV-1 plasma RNA levels were first measured using the Roche Amplicor HIV monitoring assay (version 1.0) with a lower limit of detection (LOD) of 400 copies/ml. Subsequently, an ultrasensitive Roche Amplicor HIV Monitor assay was implemented with a LOD of 50 copies/ml. Following the CHER trial in August 2011, the Abbott Diagnostics Realtime HIV-1 assay was implemented with an LOD of 150 copies/ml for 200 µl plasma sample input or 40 copies/ml for 1.0 ml input. The Roche CAP/CTM version 2.0 assay with an LOD of

100 copies/ml for 200 µl plasma sample input or 20 copies/ml for 1 ml input was used to quantify HIV-1 RNA in follow-up CHER children from December 2014.

Lymphocyte count

Routine lymphocyte analyses (CD4 and CD8 counts, percentages, and CD4:CD8 ratios) were routinely completed for all study participants including control samples at each follow-up visit. Samples were processed using the MultiTest™ (BD Bioscience, USA) monoclonal antibody mixture (CD3-FITC/CD4-APC/CD8-PE/CD45-PerCP). Samples were subsequently analysed on a flow cytometer (FACSCanto, Becton Dickinson) using the automated BD™ clinical software.

3.2.4 Blood plasma immune biomarker measurements

An extensive panel of 44 soluble biomarkers were assessed in the 3 study groups. These included IL-17F, IL-1β, IL-1Ra, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (CXCL8), IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A, IP-10 (CXCL10), IFN-γ, macrophage chemoattractant protein 1 (MCP-1 or CCL2), MIP-1α (CCL3), MIP-1β (CCL4), TNF-α, TNF-β, GCSF, GMCSF, CD40L, VEGF, INF-α, fibroblast growth factor (FGF), RANTES, (CCL5) PDGF-BB, TGFβ1,2 and 3, sCD14, sCD163, IL-18, hsCRP, LBP, I-FABP, MAdCAM-1 and s100A8/A9), which were measured using (a) validated Hycult® Biotech Enzyme Linked ImmunoSorbent Assays (ELISA) [for hsCRP, LBP and I-FABP measurements]; and (b). Luminex® Multiplex assays from R&D Systems [for sCD14, sCD163, IL-18, MAdCAM-1 and s100A8/A9] and Milliplex®MAP (Merck) [for all remaining markers].

A classical Sandwich ELISA method was applied to samples for measurement of hsCRP, LBP and I-FABP. Standards were prepared in duplicate. Due to limited sample volumes, single measures were taken. The assay and measurements were performed as per the manufacturer's protocol guidelines with an overnight sample thawing at 4°C.

Samples were pre-diluted using a 1:1 ratio (50 µl plasma to 50 µl sample dilution buffer) in a separate microtiter plate. Following dilution and standard preparation, 100 µl of each standard, sample and control was aliquoted into the pre-coated ELISA plate wells.

Absorbance values were measured at 450 nm using the iMark Microplate reader (Bio-Rad). To convert the absorbance values obtained to concentrations, the blank standard was averaged and subtracted from each absorbance value, correcting for background interference. The mean standard concentration absorbance values were plotted on the y-axis against the log standard concentrations to generate a standard curve. The standard curves for each plate were used to calculate the appropriate concentration of biomarker in each sample.

Similarly, Luminex® Multiplex assays were customized with analytes combined for maximum stability and least cross-reactivity. Commercial assays (except R&D Systems) included quality controls for monitoring the successful implementation of the assay. Reagent preparation, sample dilution and assay procedures were carried out as per the manufacturer's guidelines and protocols contained within the kits. Samples were thawed overnight at 4°C and allowed to reach room temperature before sample preparation began.

Depending on the number of analytes measured in each kit, standard curves for each analyte was generated using known concentration standards of the cytokine/chemokine levels evaluated and provided in the selected kits. Plasma cytokine levels were determined using a multiplex array reader from Luminex® Instrumentation System (Bio-Plex II Workstation from Bio-Rad®, California, USA). Plasma cytokine/chemokine concentrations were calculated as the average of two independent measures using Bioplex® Manager Software (California, USA). Standard curves were configured as a five-parameter logistic curve via computer software during plate reading.

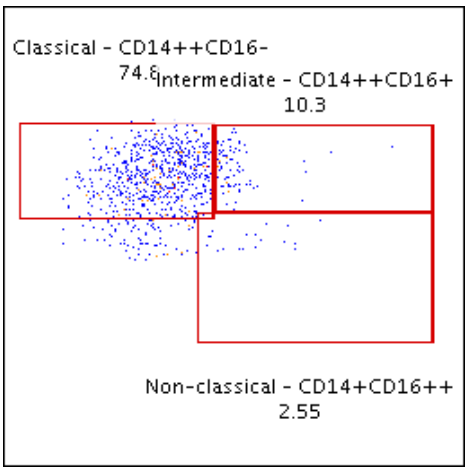
Rationale for biomarker selection*:* A broad range of plasma immune biomarkers were selected to understand both innate and adaptive immune compartments. These biomarkers have been previously documented in the literally when evaluating immune health and mechanism of HIV-1 infected populations. Majority of these have shown to be aberrant or abnormally expressed in the context of HIV infection. Biomarkers have also been selected to investigate gut permeability/integrity within our cohort. In addition, we aimed to include novel biomarkers i.e. MAdCAM-1, s100A8/A9 and I-FABP that have not been extensively studied within PHIV children. Furthermore, biomarkers available on commercial multiplex platforms together with markers of interested were assessed additionally.

3.2.5 Cellular immune biomarker measurements

PBMCs were isolated by Ficoll-Hypaque density gradient according to standard isolation procedures from approximately 35ml of EDTA whole blood and stored in liquid nitrogen until immunophenotype processing.

Using multi-parameter flow cytometry on a subgroup of samples, monocyte profiling for this study was achieved by evaluating monocyte subset distribution of classical (CD14+CD16-), intermediate ($CD14^{hi}$CD16+) and non-classical ($CD14^{low}$CD16+) subsets. The monoclonal antibodies (mAb) used to identify these subsets included CD3-BV510, CD14-PerCP and CD16-PECy7 (Becton Dickinson, San Jose, California, USA). A viability stain-APC-H7 (Becton Dickinson, San Jose, California, USA) was also used for the exclusion of dead or apoptosed cells. Appropriate titration analysis was implemented to determine volume of monoclonal antibody for maximum binding and fluorescence. An average of 30 000 events were acquired using BD® LSRII (BD Bioscience, US) instrumentation and BD FACSDiva® software.

Gating strategies included selection of live cells of monocyte population using forward and side scatter properties followed by analysis of monocyte subpopulation post-acquisition performed using FlowJo™ (LLC, USA) version 10 software from BD® Lifesciences, San Jose, California, USA.



Following a thaw (at 37°C) and wash procedure (Roswell Park Memorial Institute Medium (RPMI) with 10% foetal bovine serum (FBS)), PBMCs were incubated for 20 minutes at room temperature, washed with stain buffer, fixed (4% paraformaldehyde, BD Bioscience, US).

3.2.6 Assessment of integrated cell-associated HIV DNA (iCAD)

A sensitive qPCR adapted for HIV-1 subtype C was implemented for the measurement of HIV-1 total DNA. This assay targets a region in HIV-1 integrase with a detection limit of 3 copies/reaction as previously described (van Zyl et al., 2015; Hong et al., 2016; Veldsman et al., 2018). The novel iCAD assay was done on a subset of participants where early (baseline) stored samples were available.

For the preparation of integrase HIV-1 subtype C DNA integrase standard for iCAD, a set of primers i.e.,

Forward primer: 5'-CCCTACAATCCCCAAAGTCA-3'

Reverse primer: 5'-CACAATCATCACCTGCCATC-3', were designed to amplify a 418bp fragment from the HIV-1 subtype C integrase gene and was used to generate amplicons from the pMJ4 plasmid (HIV-1 subtype C clone).

3.2.7 Statistical Analysis

Numerical variables measured for each of the groups were analysed using TIBC® Statistica™ version 13.3 and GraphPad Prism version 8 software. A Shapiro-Wilke's normality test was done to test the data for normality. Non-parametric statistical tests were used to analyse data that were not normally distributed or values that fell outside the normal range. No outliers were excluded in the analysis. Extreme values were transformed by use of winsorisation of statistics to limit the effect of spurious outliers and to retain the power of sampling size.

A non-parametric one-way analysis of variance (ANOVA) Kruskal-Wallis test was applied for the comparison of the three study groups which was followed by a post-hoc Fisher's Least Significant Difference (LSD) to further identify and verify the significant differences between the groups. Correlations between soluble biomarkers, clinical parameters and other laboratory measurements (cellular markers and iCAD measurements) were performed by Spearman's correlation rank test (verified additionally with a Pearson's correlation rank test) and linear regression analysis. *P* values less than 0.05 were considered statistically significant.

Note: Biomarkers for the HIV+ group was first analysed per therapy arm as per the original grouping of the cohort at the start of the clinical trial (interruption vs uninterrupted and delayed vs early therapy) before they were grouped as a single group (i.e., HIV+) for subsequent cross-sectional analyses in this study. Early interruption of therapy, at either 40 or 96 weeks, had no significant difference on plasma biomarker expression at 8 years of age. When evaluating delayed vs early therapy initiation, we observed a significant difference only for biomarkers MIP-1β (p=0.01), IL-5 (p=0.01), IL-4 (p=0.03) and IL-1RA (p=0.02), where the delayed therapy arm had lower levels.

All participants that were recruited for the follow-up study i.e. Post-CHER follow-up with sample availability were assessed in this research study. No power calculations to determine appropriate sample size were initiated other than that of the original clinical trial. This investigation aimed to take a descriptive approach of which appropriate statistical tools were engaged for already established sample numbers.

3.3 Results

3.3.1 Demographic and clinical parameters of study cohort at baseline and follow-up

Cytokine and chemokine plasma biomarkers were quantified in one hundred and sixty-one samples (88 HIV+, 32 HEU and 41 HUU). Biomarker analysis for I-FABP, MAdCAM-1 and s100A8/A9 was completed for 138 samples (77 HIV+, 27 HEU and 34 HUU). A subset of the cohort (29 HIV+, 18 HEU and 17 HUU) was evaluated for cellular immune markers of monocyte subset distribution.

Combination ART within the HIV+ group was initiated at a median age of 2.1 months with sustained viral suppression for 97% of the cohort at 8 years of age. Median pre-therapy viral load at cART initiation was 738,501 copies/ml. Median CD4-percentage at baseline was 36.9% (range: 23.1-57.1%). At 8 years of age, there were no significant differences between the CD4-percentage of the HIV+ (38.3%) and control groups (40.0%) (p=0.261). All children except five (range: 502-26,576 copies/ml) showed undetectable viral loads at the follow-up sampling (Summary in Table 3.1).

Table 3.1: Demographic and clinical parameters of study cohort investigated

	HIV+ group	HIV Uninfected Controls		Kruskal-Wallis p-value
		HEU	HUU	
n	88	32	41	N/A
Demographic Factors				
Age, years (median, IQR)	8.0 yrs. (6.0-9.0 yrs.)	9.0 yrs. (8.0-11.0 yrs.)	10.0 yrs. (9.0-11.0 yrs.)	0.062
Male, sex (n) (%)	43 (48.9%)	18 (56.3%)	23 (56.1%)	0.057
Baseline HIV Treatment and CD4 Outcomes				
Months to therapy initiation, median (IQR)	2.1 months (1.8-2.8 months)	-	-	N/A
Viral load at therapy initiation (median, IQR)	738,501 copies/ml (373,000-750,001 copies/ml)	-	-	N/A
$CD4^+$ cell count, median (IQR)	1742.6 cells/µl (1207.0-2367.5 cells/µl)	-	-	N/A
$CD4^+$ percent, median (IQR)	32.5% (25.9-40.0%)	-	-	N/A
HIV-1 iCAD (subset of n=40), median (IQR)	1,470.1 copies/10^6 PBMCs (774.0-2, 888.7 copies/10^6 PBMCs)	-	-	N/A
8-year Follow-up parameters				
$CD4^+$ cell count, median (IQR)	1, 076.6 (805.3-1,251.0)	926.8 (813.3-1,061.5)	1, 007.33 (815.0-1,151.0)	0.26
CD4 percent, median (IQR)	38.4 (34.0-43.0)	38.4 (34.0-42)	41.5 (37.0-49.0)	0.22
$CD8^+$ cell count, median (IQR)	960.8 (621.8-1,165.9)	636.0 (528.5-790.8)	593.8 (456.0-1,791.5)	**<0.01**
CD8 percent, median (IQR)	33.6 (28.8-39.0)	26.9 (24.8-30.0)	24.6 (22.0-26.0)	**<0.01**
CD4/CD8 Ratio, median (IQR)	1.24 (0.87-1.50)	1.54 (1.1-1.8)	1.74 (1.4-1.9)	**<0.01**
HIV-1 iCAD (subset of n=40), median (IQR)	20.3 copies/10^6 PBMCs (6.4-37.6 copies/10^6 PBMCs)	-	-	N/A

*IQR = Interquartile Range; HEU = HIV exposed uninfected; HUU = HIV unexposed uninfected; iCAD =integrated cell-associated DNA; PBMCs= peripheral blood mononuclear cells

***Lymphocyte reference ranges**

Reference: (Lawrie *et al.*, 2015)			
Age range	**0-3 months**	**1-2 years**	**6-12 years**
CD4 T lymphocytes (cells/µL)	1520-5160	1374-3928	568-2013
CD8 T lymphocytes (cells/µL)	428-2478	669-3247	340-121-
CD4:CD ratio	1.29-5.29	0.78-3.88	0.95-2.47
Reference: (Payne *et al.*, 2020)			
Age range	**1-2 months**	**15-24 months**	**5-10 years**
CD8 (%)	10.7-28.9	13.8-34.9	15.1-34.8
Reference: (Shearer *et al.*, 2003)			
Age range	**0-3 months**	**1-2 years**	**6-12 years**
CD4 (%)	35-64	32-51	31-47

3.3.2 Multivariate cytokine and chemokine biomarker measurements show elevated levels of biomarkers associated with chronic inflammation, macrophage activation and gut damage in HIV infected children

Systemic levels of 40 biomarkers inclusive of cytokines, chemokines, and soluble cytokine receptors by Luminex® Multiplex Assays and ELISAs were measured in all study groups. An online tool for the visualisation of clustering of multivariant data, ClustVis®, was used to provide a visual snapshot of biomarker levels and clustering in each study group (Figure 3.1).

Analysis of the multivariate cluster heat map (Figure 3.1) shows a tendency of the HIV+ group to have elevated expression of biomarkers associated with chronic inflammation (IL-2, CD40L, VEGF, IL-18, IL-10, sCD163, hsCRP) and microbial translocation (LBP) compared to the HEU and HUU groups. In addition, HIV+ children showed elevated expression of plasma biomarkers associated with monocyte/macrophage activation (e.g., sCD14, sCD163, IL-18). Conversely, the HIV+ group displayed low levels of biomarkers associated with T-cell immunity (e.g., IFN-γ and TNF-α). Figure 3.1 also reveals major clustering of elevated biomarkers associated with T cell activation within the HEU group. Although HEU is not the focus point for this research, the distinct differences between the three study groups are noteworthy. The HUU group had consistently low biomarker expression (except for IL-1RA). Detailed statistical analysis is described in section 3.3.3 below.

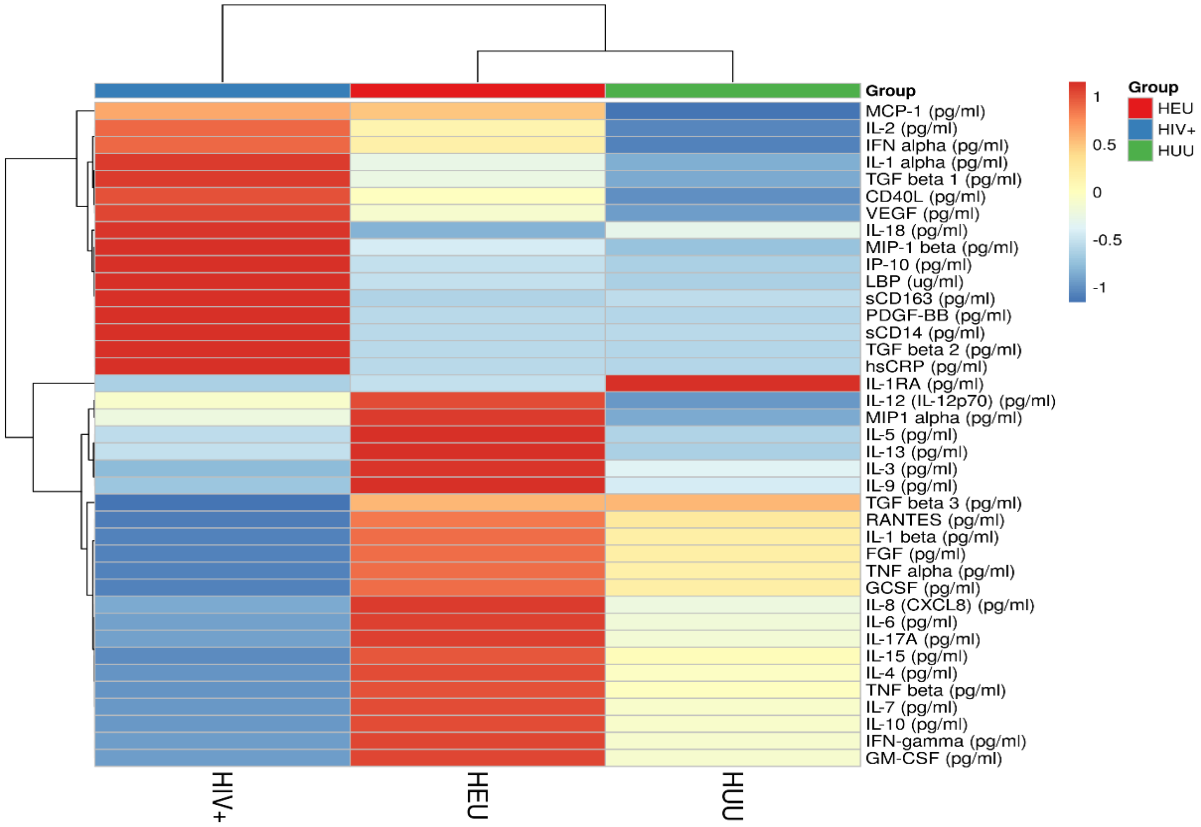


Figure 3.1: *Select biomarker clustering analysis between the three study groups.*
Forty biomarkers were measured in blood plasma from HIV+ children at a median age of 8 years and in controls (HEU and HUU). Columns with similar annotations were collapsed by calculating the median of each biomarker in each group. Rows were centred and a unit variance of scaling was applied to each row. Rows and columns were clustered using correlation distance and average linkage. A colour scale ranging from 1 to minus 1 (red-blue) denotes the relative biomarker expression with red indicating elevated biomarker expression.

3.3.3 Significant elevation in plasma biomarkers associated with chronic inflammation and monocyte/dendritic cell activation

HIV+ children show a significant increase in biomarkers associated with generalized chronic inflammation (hsCRP), monocyte/macrophage and DC activation compared to HEU and HUU controls, both of which show similar levels of these associated biomarkers ($p>0.05$).

Kruskal-Wallis test by ranks comparing all three study groups indicate significant differences (with the HIV-infected group showing significantly higher levels) for biomarkers **hsCRP** ($p=0.01$), **MIP-1β** ($p=0.03$), **IL-1α** ($p<0.001$), **INF-α** ($p<0.001$), **CD40L** ($p<0.001$), **TGFβ2** ($p=0.02$), **VEGF** ($p<0.00001$) and **PDGF-BB** ($p<0.00001$). These data are depicted in Figure 3.2 and a summary of statistics for each biomarker is listed in Table 3.2 below.

In addition, we also observed a significant elevation of soluble calcium binding protein involved in the regulation of the inflammatory process and immune response, **s100A8/A9**, in the HIV+ group compared to the two study control groups ($p<0.001$). Graphical representation and statistical summaries are provided in Table 3.2 and Figure 3.3.

Table 3.2: Statistical summary including medians, IQR and p-values for hsCRP, IL-1α, IFN-α, CD40L, PDGF-BB, VEGF and s100A9 for HIV+, HEU and HUU children at 8 years of age

Biomarker	**HIV+**	**HEU**	**HUU**	**Kruskal-Wallis p-value**
	n= 85	n=30	n=36	
hsCRP (ng/ml); median; (IQR)	1,004.0 (282.0-3, 000.0)	389.0 (353.0-447.8)	378.5 (343.8-655.0)	**0.01**
IL-1α (pg/ml); median; (IQR)	24.9 (3.0-62.6)	9.2 (2.5-15.9)	2.5 (0.0-15.5)	**0.03**
IFN-α (pg/ml); median; (IQR)	17.5 (8.3-43.2)	72.0 (32.5-95.9)	2.5 (0.0-15.5)	**<0.001**
CD40L (pg/ml); median; (IQR)	1,465.9 (916.2-2, 204.4)	1,066.5 (247.5-1, 549.7)	667.8 (334.2-1, 220.5)	**<0.001**
PDGF-BB (pg/ml); median; (IQR)	1,868.6 (1, 339.2-3, 318.5)	33.9 (23.1-44.0)	29.0 (16.5-39.1)	**<0.00001**
VEGF (pg/ml); median; (IQR)	256.8 (184.2-353.1)	184.8 (107.6-226.8)	132.9 (76.9-212.9)	**<0.00001**
s100A9 (pg/ml); median; (IQR)	36,380.0 (2, 744.9-36, 380.0)	1,965.4 (746.1-5, 113.9)	3,279.5 (1, 368.9-4, 301.3)	**<0.001**

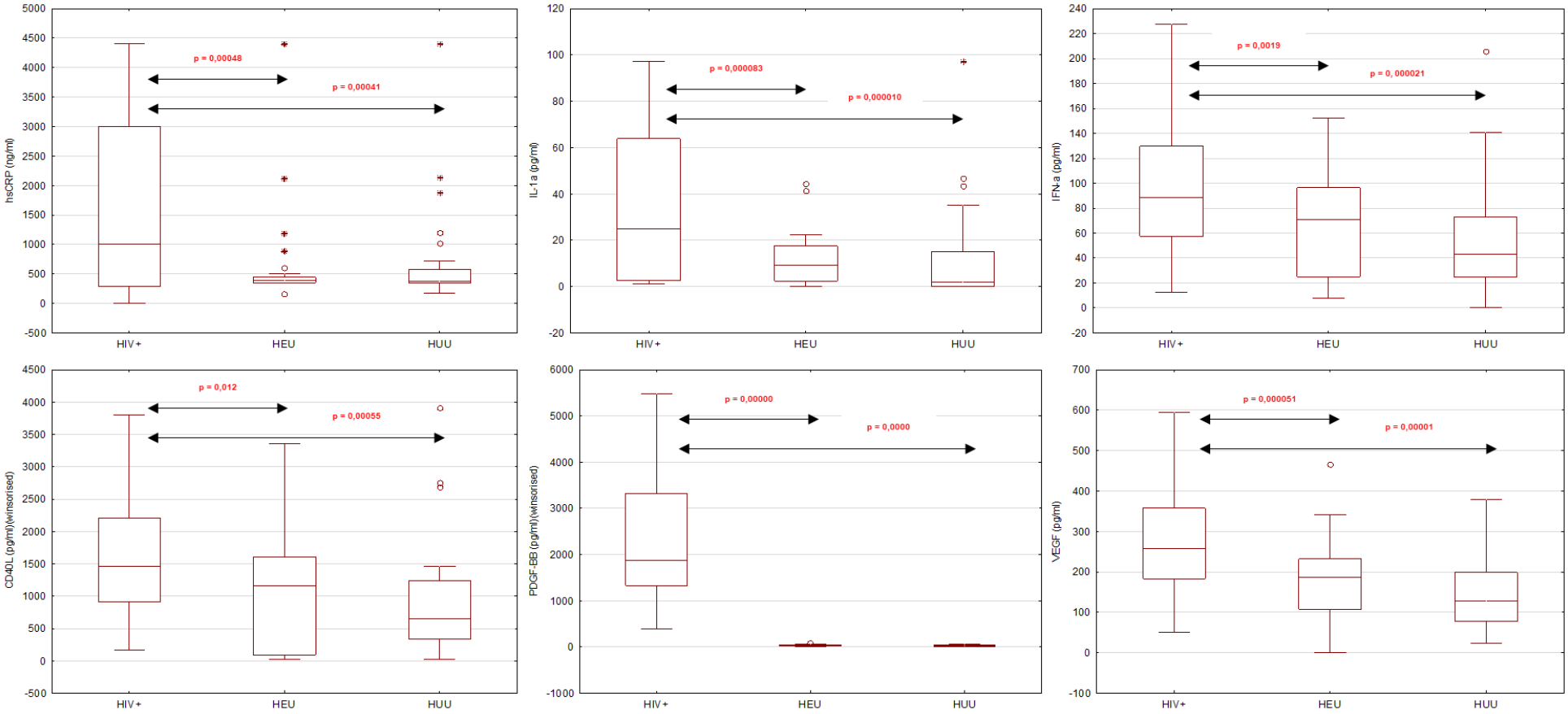


Figure 3.2: *A one-way ANOVA (also known as the Kruskal-Wallis test by ranks) comparing the differences that exist between the groups was implemented followed by a Fisher's Least Significant Difference (LSD) test to evaluate each of the study groups in comparison to each to each other.*
Graphical representation of Box and Whisker plots depicting median concentration, 25-75% range within the standard deviation (SD) within non-outlier range for biomarkers hsCRP, IL-1α, INF-α, CD40L, PDGF-BB and VEGF in all three study groups (HIV+, HEU and HUU). Outliers are indicated with circles and extreme outliers with an Asterix (*). Each biomarker represented show significantly elevated levels in the HIV+ group compared to the HEU and HUU groups respectively. Only p-values that indicate significant probability differences ($p<0.05$) are shown in each of the diagrams. No significant difference ($p>0.05$) between HEU and HUU biomarker levels was noted herewith.

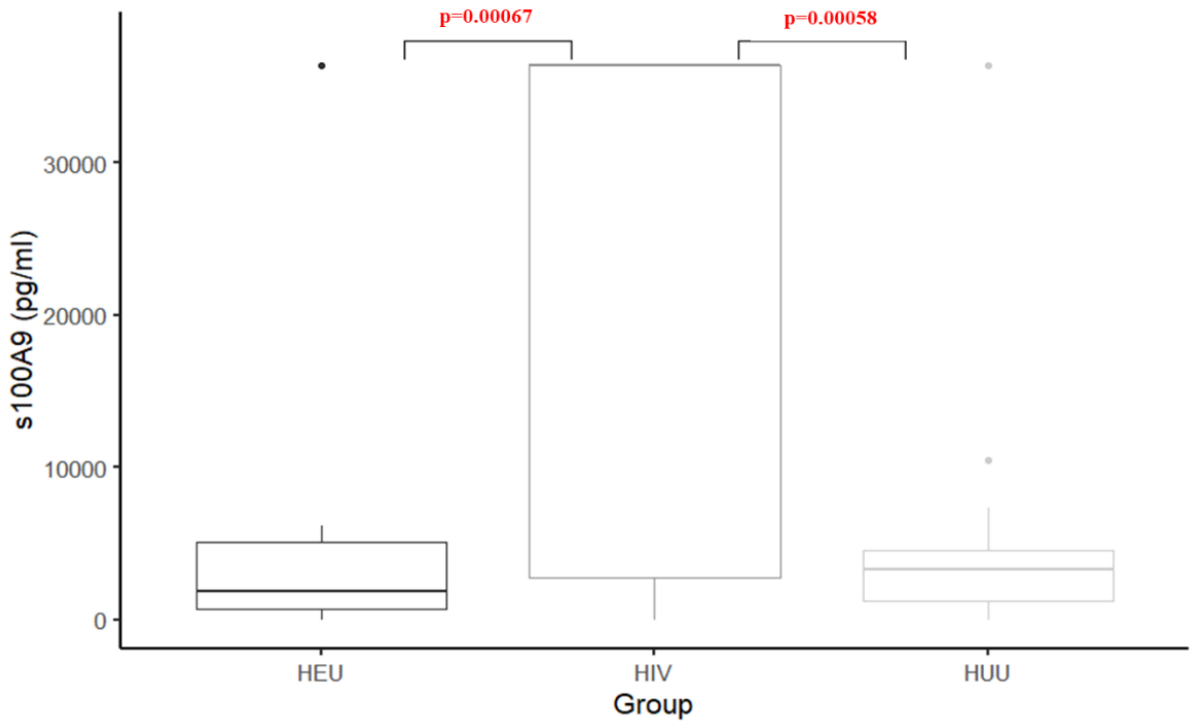


Figure 3.3: *Boxplot (with data point scatter) depicting s100A9 plasma concentrations shown for all three study groups, HIV+ (n=77), HEU (n=33) and HUU (n=39).*
Extreme outliers have been excluded in this analysis. Graphical representation shows IQR and medians for each study group. A significant difference is noted between the HIV+ group and the HEU (p=0.00067) and HUU group (p=0.00058). The interaction between all three study groups is statistically significant with a Kruskal Wallis p-value of <0.001.

3.3.4 Significant elevation of plasma biomarkers associated with gut damage and monocyte/ macrophage activation

Within the HIV+ group, significant elevation of biomarkers associated with gut damage/microbial translocation was observed. Kruskal-Wallis test by ranks comparing the difference between all study groups indicate significant ($p<0.01$) elevation in the HIV+ group for biomarkers **LBP, sCD14, sCD163, IL-18 and IL-17F**. Statistical parameters and graphical representation are depicted in Figure 3.4 and Table 3.3 below.

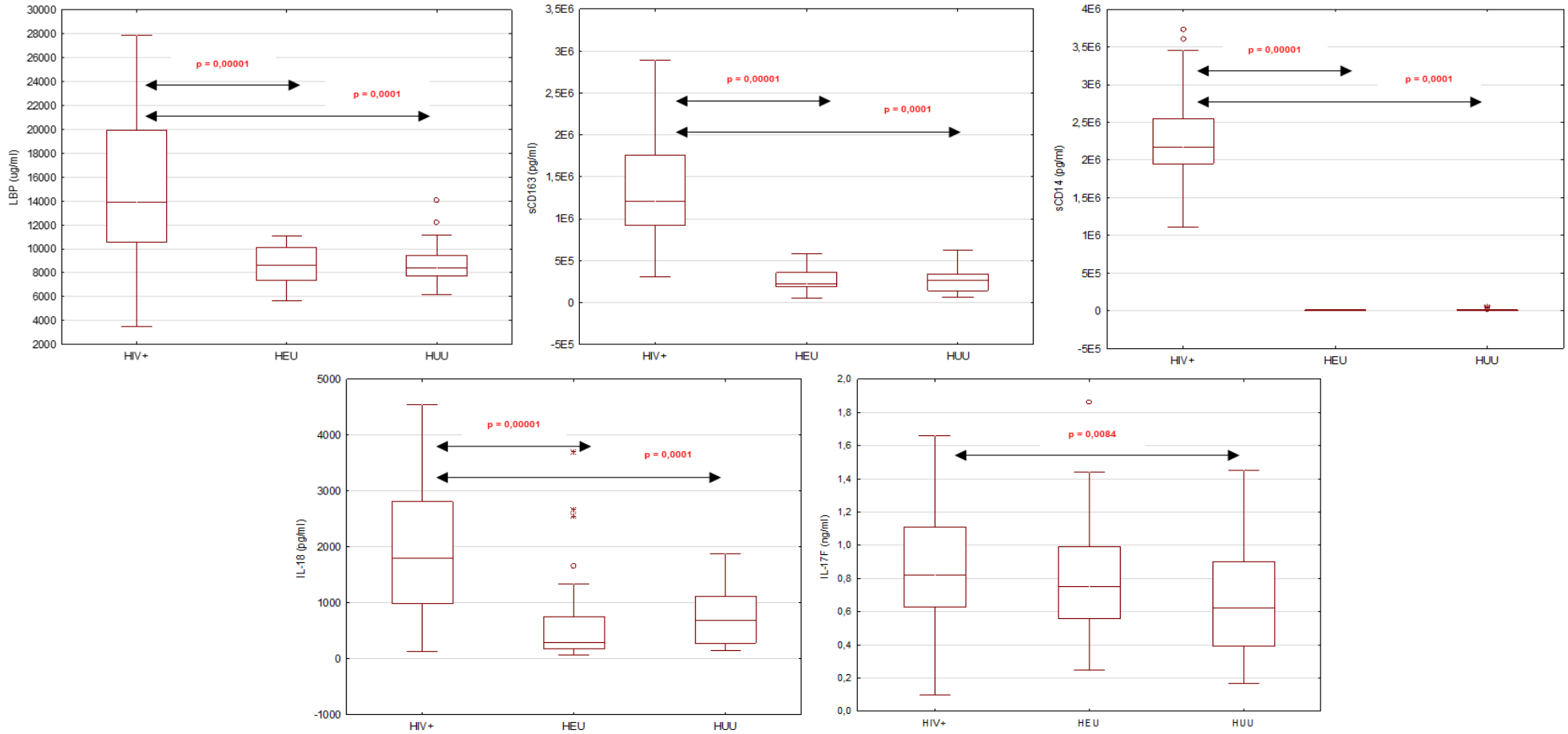


Figure 3.4: *A one-way ANOVA (also known as the Kruskal-Wallis test by ranks) comparing the differences that exist between the groups was implemented followed by a Fisher's Least Significant Difference (LSD) test to evaluate each of the study groups in comparison to each to each other.*
Graphical representation of Box and Whisker plots depicting median concentration, 25-75% range within the standard deviation (SD) within non-outlier range for biomarkers associated with gut damage including: LBP, sCD163, sCD14, IL-18 and IL-17F in the three study groups (HIV+, HEU and HUU). Outliers are shown in circles and extreme outliers with an Asterix (*). Each biomarker represented show significantly elevated levels within the HIV infected group compared to the HEU and HUU groups except for IL-17F. Only p-values that indicate significant probability differences ($p<0.05$) are shown in each of the diagrams. No significant difference ($p>0.05$) between HEU and HUU biomarker levels was noted for any the biomarkers associated with gut damage as represented.

Table 3.3: Statistical summary including medians, IQR and p-values for LBP, sCD14, sCD163, IL-18 and IL-17F

Biomarker	**HIV+**	**HEU**	**HUU**	**Kruskal-Wallis p-value**
	n= 85	n=30	n=36	
LBP (µg/ml); median; (IQR)	13,925.0 (10,562.0-19,921.0)	8,608.0 (7,574.5-20,107.0)	8,189.0 (7,533.3-8,894.9)	**<0.01**
sCD14 (pg/ml); median; (IQR)	2,172,950.0 (1,961,825.0-2,548,475.0)	8,091.7 (6,773.8-9,514.6)	7,789.0 (6,648.0-1,1036.9)	**<0.01**
sCD163 (pg/ml); median; (IQR)	1,209,150.0 (922,492.4-1,751,950.0)	220,313.8 (180,754.6-362,861.0)	261,001.3 (141,729.7-334,731.4)	**<0.01**
IL-18 (pg/ml); median; (IQR)	1,805.5 (999.9-2,781.9)	259.5 (179.4-680.8)	687.5 (294.6-1,090.5)	**<0.01**
IL-17F (pg/ml); median; (IQR)	0.8 (0.6-1.1)	0.8 (0.5-1.0)	0.6 (0.4-0.8)	**0.02**

Through multidimensional scaling (MDS) plot analysis of all plasma biomarkers, we show a degree of inter-relatedness between those associated with gut damage (Figure 3.5). In addition, Spearman correlation analysis indicates a highly significant positive association between LBP and sCD14, sCD163, IL-18 and IL-17F in the HIV+ group (Table 3.4). Furthermore, when evaluating clinical parameters that may be associated with these biomarkers of gut damage, we observed a significant negative correlation between LBP and CD4 nadir as well as CD4 and CD8 cell counts at 8 years of age. We also showed significant correlations between both sCD163 and IL-17F and CD4:CD8 ratios (Table 3.4). No association was found with treatment interruption, time to ART initiation or viral load at the time of therapy initiation.

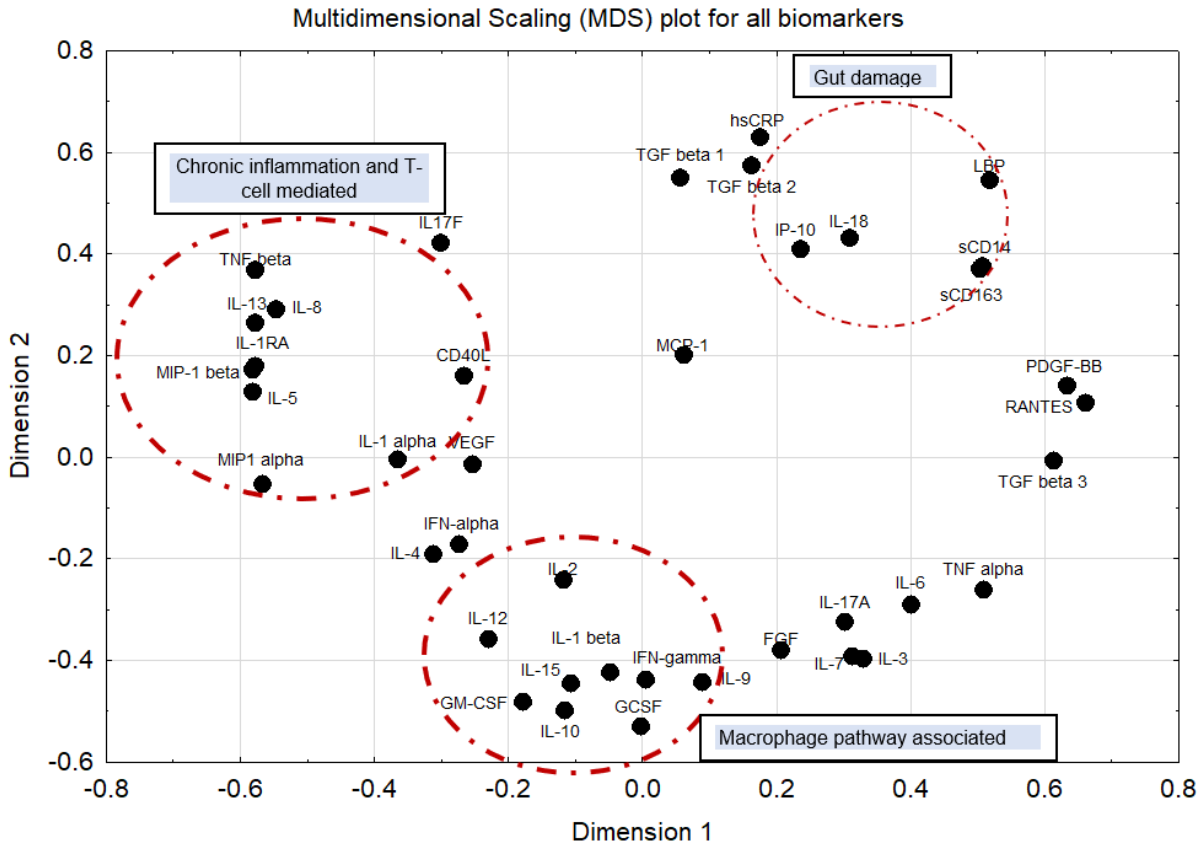


Figure 3.5: *Multidimensional Scaling (MDS) visual plots provide a representation of individual cases within a data set that have a degree of similarity displayed within a distance matrix.*
MDS visual plot for classified Dimension 1 and 2 biomarkers depicted here show inter-relatedness between markers associated with gut damage (LBP, sCD14, sCD163, IL-18). Two additional clustering of biomarkers associated with chronic inflammation, T-cell mediated immunity and macrophage pathways exist within this plot.

Table 3.4*:* Summary of correlations between LBP, sCD163 and IL-17F with biomarkers associated with gut damage as well as monocyte/macrophage activation
Clinical parameters including baseline CD4 count, CD4 and CD8 counts at 8 years of age show significant correlations with LBP, sCD163, IL-17F. Spearman rho depicting the relationship between Variables 1 and 2 and the relative Spearman probabilities is indicated in the table below.

Variable 1	Variable 2	Spearman rho (r)	Spearman p-value
LBP	sCD14	+0.59	**<0.01**
	sCD163	+0.54	**<0.01**
	IL-18	+0.39	**<0.01**
	hsCRP	+0.40	**<0.01**
	CD40L	+0.23	**<0.01**
Clinical parameters			
LBP	Baseline CD4 count	-0.24	**0.03**
	CD4 count at 8 years	-0.26	**0.02**
	CD8 count at 8 years	-0.23	**0.04**
sCD163	CD4:CD8 ratio at 8 years	-0.27	**0.02**
	CD4 percentage at 8 years	+0.25	**0.03**
	CD8 percentage at 8years	+0.24	**0.04**
IL-17F	CD4:CD8 at 8 years	+0.24	**0.03**
	CD4 percentage at 8 years	+0.35	**<0.01**

3.3.5 Increased expression of novel plasma biomarkers indicative of direct epithelial damage (I-FABP) and intestinal inflammation (MAdCAM-1) provide further evidence of microbial translocation in HIV infected children

Within the HIV+ group, we observed a higher plasma concentration of I-FABP (median: 395 pg/ml) compared to the HEU (median: 337.3 pg/ml) and HUU (median: 283.4 pg/ml) control groups. A significant difference was noted between the HIV+ and HUU group (p=0.038) for I-FABP as shown in Figure 3.6a below. Evaluating the interaction between all three groups via a multiple comparison analysis, we noted a Kruskal-Wallis p-value of 0.027. Overall, the HUU children showed a lower level of I-FABP compared to HIV+ and HEU controls. The HEU control group had higher median I-FABP levels, but these were not significant as determined in Dunn's post-test (p=0.319).

MAdCAM-1 measured higher in the HIV+ group (median:115.3 ng/ml) followed by the HEU group (median: 103.2 ng/ml), with HUU having the lowest MAdCAM-1 levels (median: 97.00 ng/ml) as shown in Figure 3.6b below. MAdCAM-1 was significantly elevated in the HIV+ group as compared to the HUU control group, per Dunn's post hoc test (p=0.011). No significant difference was observed between the HIV+ and HEU groups or between HEU and HUU (p=0.168 and p=0.272 respectively).

A summary of statistical parameters for I-FABP and MAdCAM-1 is listed in Table 3.5 below.

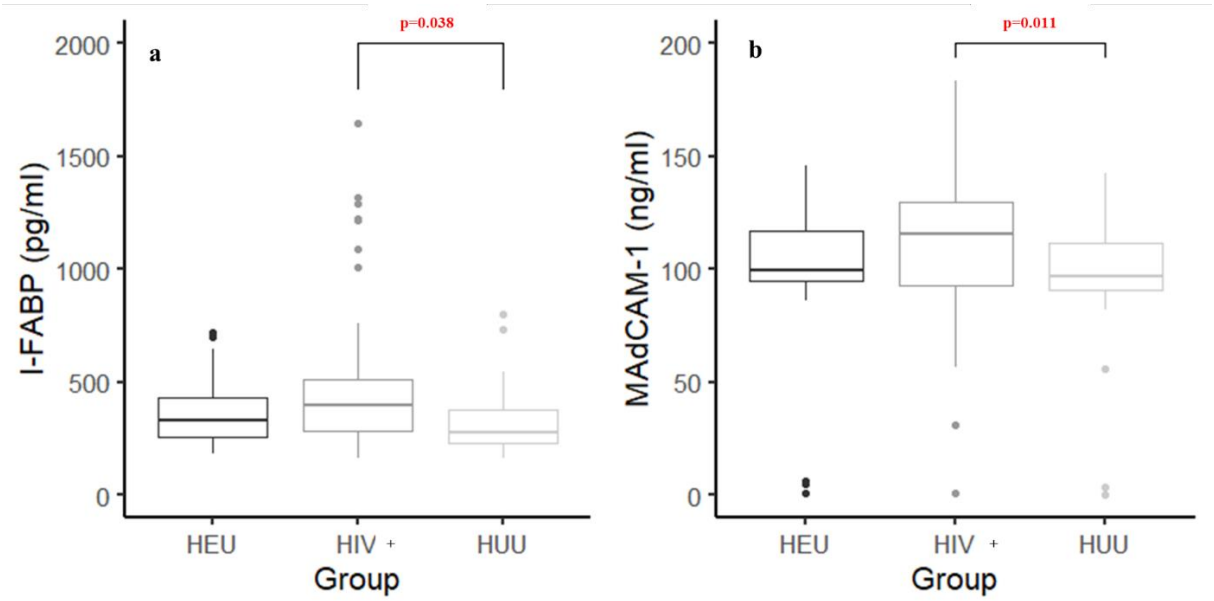


Figure 3.6a and b: *Boxplot representation (with data point scatter) depicting I-FABP (a) and MAdCAM-1 (b)plasma concentrations for all three study groups, HIV+ (n=77), HEU (n=33) and HUU (n=39).*
Extreme outliers have been excluded in this analysis. Graphical representation shows IQR and medians for each of the study group. A significant difference is noted between the HIV+ (median = 395.3pg/ml) and HUU (median: 283.4pg/ml) groups (p=0.038) for I-FABP. The interaction between all three study groups is statistically significant with a Kruskal Wallis p-value of 0.027. For MAdCAM-1, a significant difference is noted between the HIV+ (median = 115.34 ng/ml) and HUU (median: 97.0 ng/ml) groups (p=0.011). No statistical difference was observed between the HIV+ and the HEU (median: 103.2pg/ml) group (p=0.18) or HEU and HUU controls (p=0.272). The interaction between all three study groups is statistically significant with a Kruskal Wallis p-value of 0.009.

Table 3.5: Statistical summary including medians, IQR and p-values for I-FABP and MAdCAM-1

Biomarker	**HIV+**	**HEU**	**HUU**	**Kruskal-Wallis p-value**
	n= 77	n=33	n=39	
I-FABP (pg/ml); median; (IQR)	395.3 (288.1-530.4)	337.3 (257.6-445.1)	283.4 (231.5-420.4)	**0.027**
MAdCAM-1 (ng/ml); median; (IQR)	115.3 (92.6-129.6)	103.2 (94.4-118.8)	97.0 (91.0-111.3)	**0.009**

Spearman correlation analysis show a weak positive correlation of I-FABP to IL-18 and sCD14 (r=0.21 and r=0.20) respectively. Correlation scatter plots for these analyses are shown in Figure 3.7a and b below.

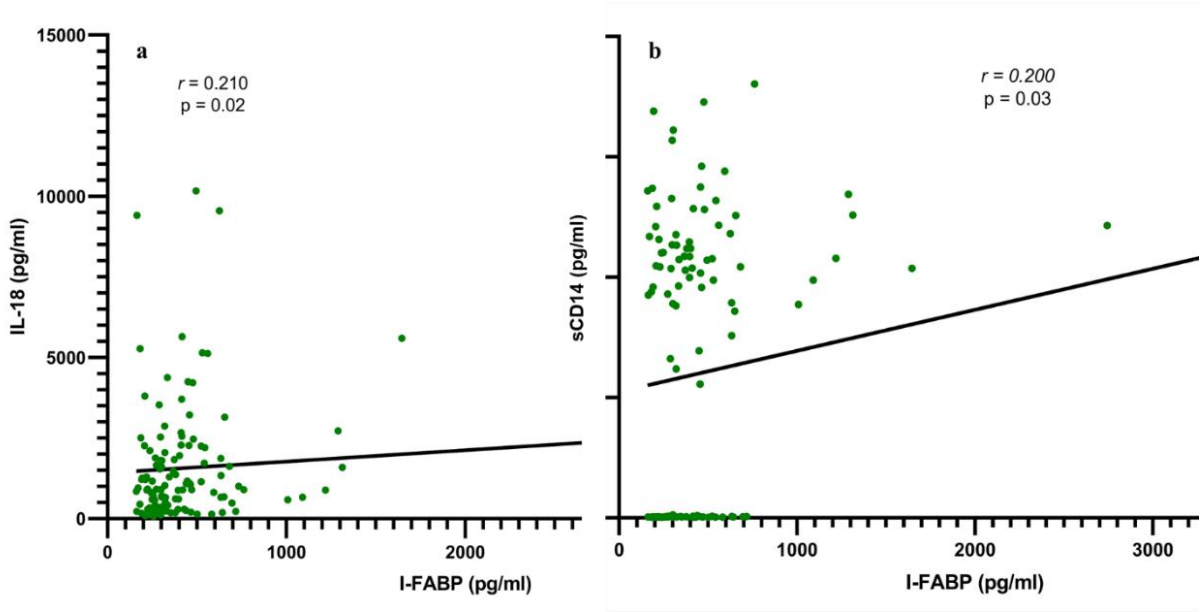


Figure 3.7: *Spearman correlation scatter plots for I-FABP with IL-18 (a) and sCD14 (b).*
Statistically significant positive correlations were found between I-FABP and IL-18 (a) and sCD14 (b) with significant p-values of 0.02 and 0.03 respectively.

Correlation analysis between MAdCAM-1 and other biomarkers were greater than those observed for I-FABP. MAdCAM-1 showed a significant correlation with several markers including the surrogate microbial translocation and immune activation markers sCD14 (r=0.23; p=0.012) and sCD163 (r=0.31; p=0.006). Both s100A8 and s100A9 were significantly correlated to MAdCAM-1, both having identical correlations (r=0.27 and p<0.001).

A trend was observed between LBP and MAdCAM-1 with a p-value of 0.053 which further supports the associations between MAdCAM-1 and microbial translocation (*data not shown)*. As the soluble calprotectin monomers were associated with MAdCAM-1, it follows that s100A8/A9 is likely being produced in the intestinal tract where inflammation is taking place. No significant relationship was observed between MAdCAM-1 and I-FABP itself (p=0.059, r=0.16). Refer to Figure 3.8 below for correlation analysis.

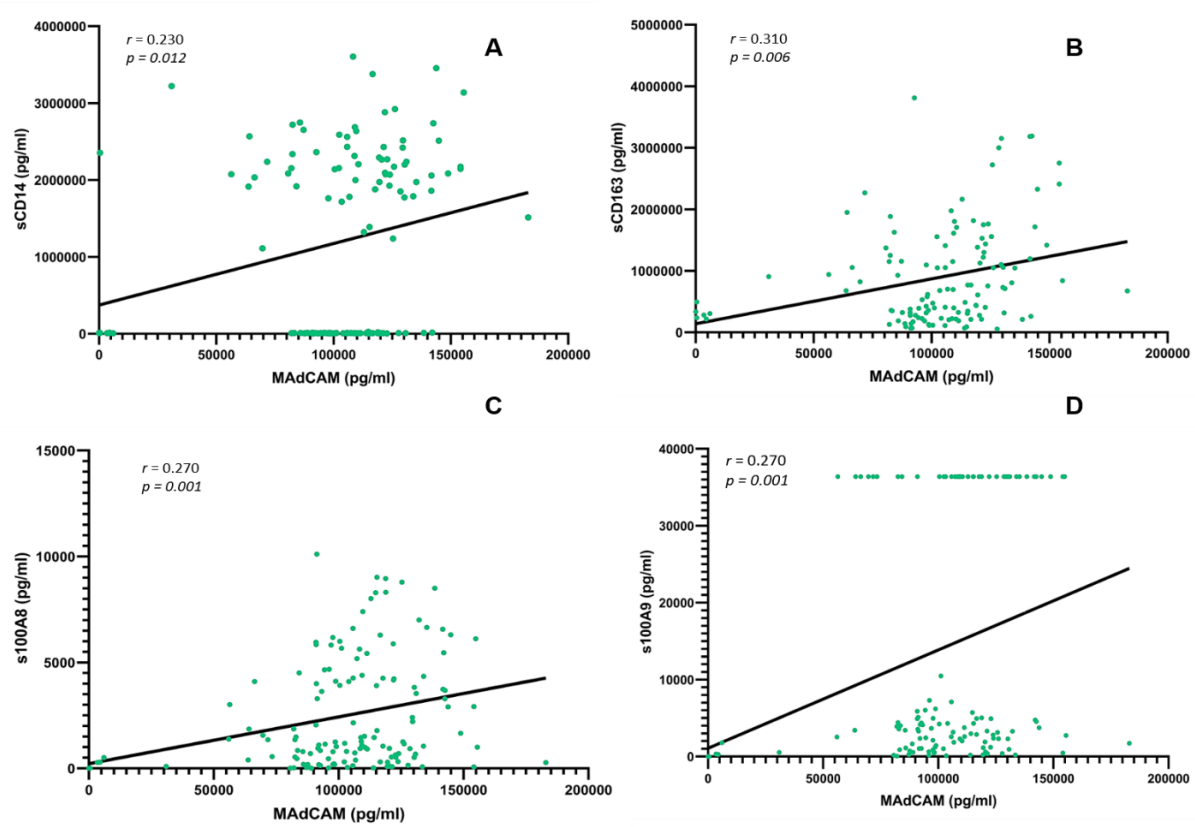


Figure 3.8: *Spearman correlation scatter plots for MAdCAM-1 and other biomarkers of interest. Statistically significant positive correlations are shown for (A) sCD14 (p=0.012), (B) sCD163 (p=0.006), (C) s100A8 (p =0.001) and (D) s100A9 (p =0.001). sCD163 displayed the strongest correlation to MAdCAM-1 (r= 0.31; p=0.006).*

s100A9 was found to have several significant correlations. Weakly positive relations were observed between s100A9 and sCD14 (r = 0.28; p = 0.002), sCD163 (r = 0.38; p <0.001), LBP (r = 0.27; p= 0.002) and MAdCAM-1 (r = 0.27; p =0.001) whilst stronger correlations were found between s100A9 and IL-18 (r = 0.53 and p <0.001). Refer to Figure 3.9 below.

The p-values for s100A9 correlations were all largely confident, as they were near indistinguishable from zero (indicated by p-values <0.05 and <0.001 statistically). The results obtained for s100A9 provides further evidence of current inflammation, the localized property of this marker suggests gastrointestinal inflammation together with its correlation to MAdCAM-1, a novel gastrointestinal inflammatory marker.

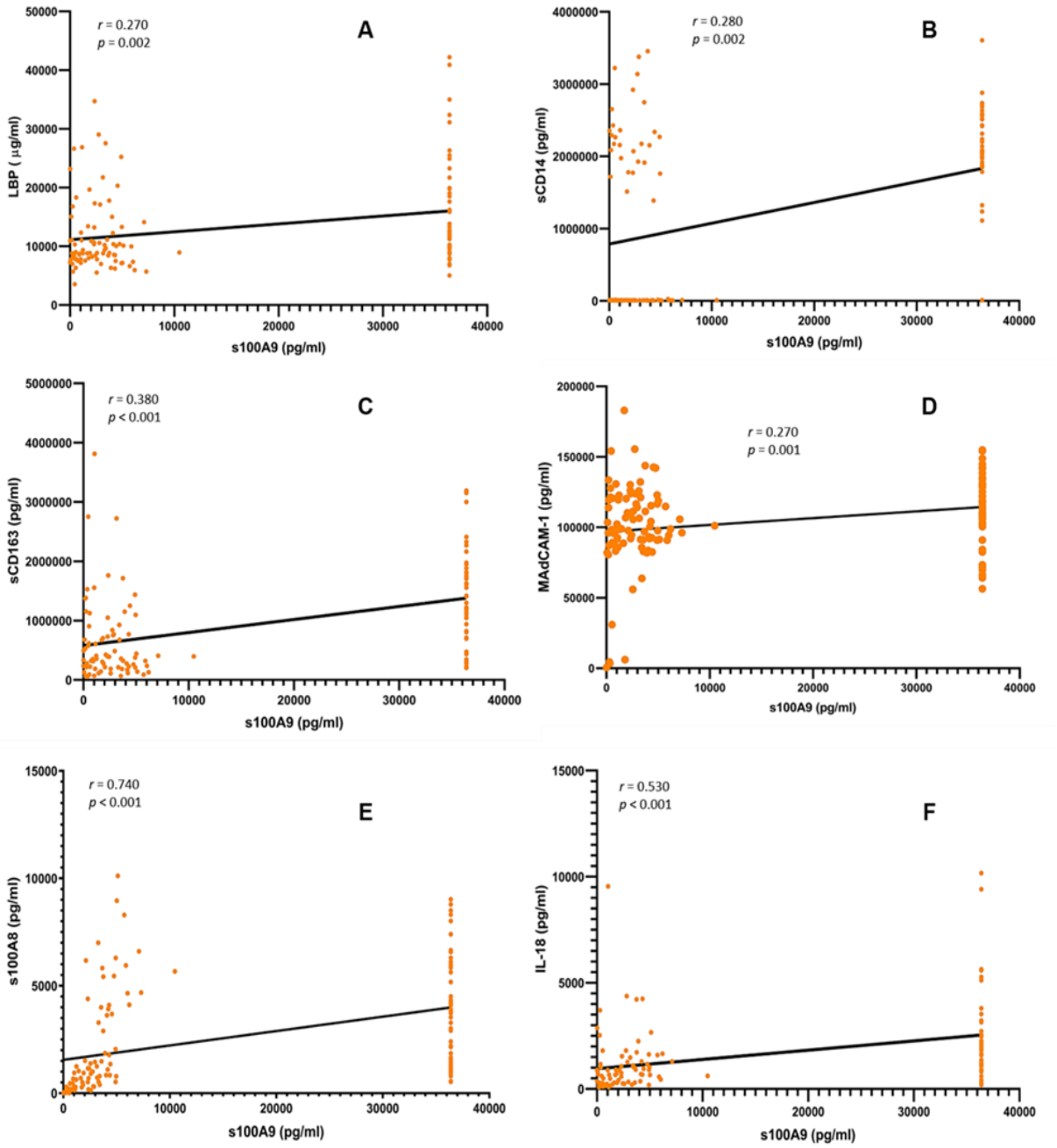


Figure 3.9: *Spearman correlation scatter plots for s100A9 and other biomarkers of interest.*
All statistically significant correlations are shown for s100A9. (A) depicts a weakly positive correlation with LBP (r = 0.270; p= 0.002), (B) s100A9 correlated to sCD14 (r = 0.280; p= 0.002), (C) sCD163 correlated to s100A9 (r = 0.380; p <0.001). (D) MAdCAM-1 correlated to s100A9 (r =0.280; p= 0.001), (E) depicts a strong correlation between s100A8 and s100A9 (r =0.740; p <0.001) and (F) modest correlation between IL-18 and s100A9 (r = 0.530; p <0.001).

3.3.6 Significantly diminished expression of selected T-cell mediated plasma immune biomarkers in HIV-1 infected children at 8 years of age

Evaluating the expression of cytokine biomarkers associated with T-cell mediated immunity, we observed that the HIV+ group showed significantly lower plasma levels of IL-3, RANTES, GCSF, INF-γ, TGFβ$_3$ and IL-17A when compared to both HEU and HUU groups (Figure 3.10). Compared to HUU, the HEU group showed significantly elevated levels of chemokine, RANTES (p = 0.0047) and cytokine IL-17A (p = 0.012).

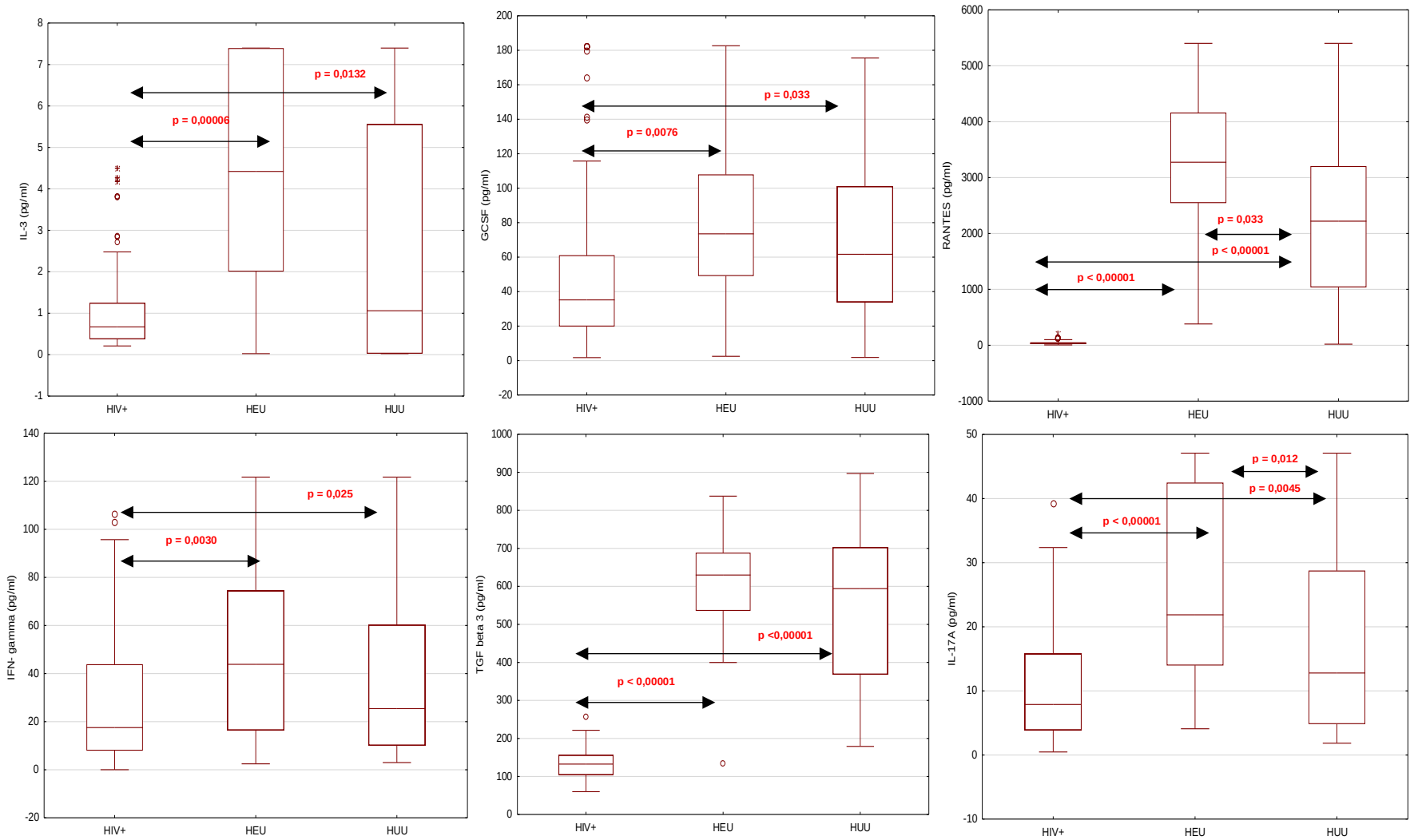


Figure 3.10: *Box and Whisker plots depicting median concentration, 25-75% range within the standard deviation (SD) within non-outlier range for T-cell mediator biomarkers: IL-3, GCSF, RANTES, IFN-γ, TGFβ3 and IL-17A in the three study groups (HIV+, HEU and HU).*
Outliers are shown in circles and extreme outliers with an Asterix (*). Each biomarker represented show significantly decreased levels in the HIV infected group compared to the HEU and HUU groups. Only p-values that indicate significant probability differences ($p<0.05$) are shown in each of the diagrams. Significant differences ($p>0.05$) between HEU and HUU biomarker levels was noted for RANTES and IL-17A.

A summary of all statistical parameters is tabulated in Table 3.6 below.

Table 3.6: Statistical summary including medians, IQR and p-values for Il-3, GCSF, RANTES, IFN-γ, TGFβ$_3$ and IL-17A

Biomarker	**HIV+**	**HEU**	**HUU**	**Kruskal-Wallis p-value**
	n= 88	n=32	n=41	
IL-3 (pg/ml); median; (IQR)	0.7 (0.4-1.3)	4.0 (2.1-8.5)	1.5 (0.0-7.5)	**<0.01**
GCSF (pg/ml); median; (IQR)	35.2 (20.2-60.9)	75.3 (50.3-109.2)	61.0 (34.6-101.0)	**<0.01**
RANTES (pg/ml); median; (IQR)	30.9 (20.5-53.7)	3,235.5 (2,469.3-4,020.6)	2,277.6 (1,087.5-3,205.0)	**<0.01**
IFN-γ (pg/ml); median; (IQR)	17.5 (8.3-43.2)	39.5 (18.7-69.3)	26.9 (10.1-60.5)	**<0.01**
TGFβ$_3$ (pg/ml); median; (IQR)	132.7 (106.4-156.4)	598.7 (526.1-681.8)	598.7 (370.0-694.8)	**<0.01**
IL-17A (pg/ml); median; (IQR)	7.9 (3.9-15.6)	20.3 (11.4-41.7)	12.9 (4.9-28.1)	**<0.01**

GCSF *= granulocyte-colony stimulating factor;* ***RANTES*** *= Regulated on Activation, Normal* T-cell *Expressed and Secreted;* ***IFN-γ*** *= Interferon gamma;* ***TGFβ$_3$*** *= Transforming growth factor beta-3*

3.3.7 Decreased M1 macrophage-associated cytokine expression and expansion of classical and non-classical monocyte phenotype (M1/M2 switch)

We observed a significant (p<0.01) decrease in IL-1β, TNF-α and IL-6 in the HIV+ group compared to the other control groups as tabulated in Table 3.7 and depicted in Figure 3.11 below. Another interesting observation is the HEU group displaying elevated levels of these biomarkers compared to HIV+ and HUU groups.

Due to this finding, monocyte subset distribution in a subgroup of participants from all three study groups showed a significantly increased proportion of a classical monocyte subset (CD14++CD16-) in the HIV+ group compared to the HUU group (p=0.032). Refer to Figure 3.12 below.

Table 3.7: Statistical summary including medians, IQR and p-values for IL-1β, TNF- α and IL-6

Biomarker	**HIV+**	**HEU**	**HUU**	**Kruskal-Wallis p-value**
	n= 88	n=32	n=41	
IL-1β (pg/ml); median; (IQR)	6.6 (2.3-12.3)	13.6 (6.6-19.2)	11.2 (6.8-18.2)	**<0.01**
TNF-α (pg/ml); median; (IQR)	11.0 (9.0-14.7)	35.1 (25.7-61.3)	26.5 (16.2-37.1)	**<0.01**
IL-6 (pg/ml); median; (IQR)	1.3 (1.3-16.8)	6.6 (2.8-24.1)	3.3 (1.3-6.0)	**<0.01**

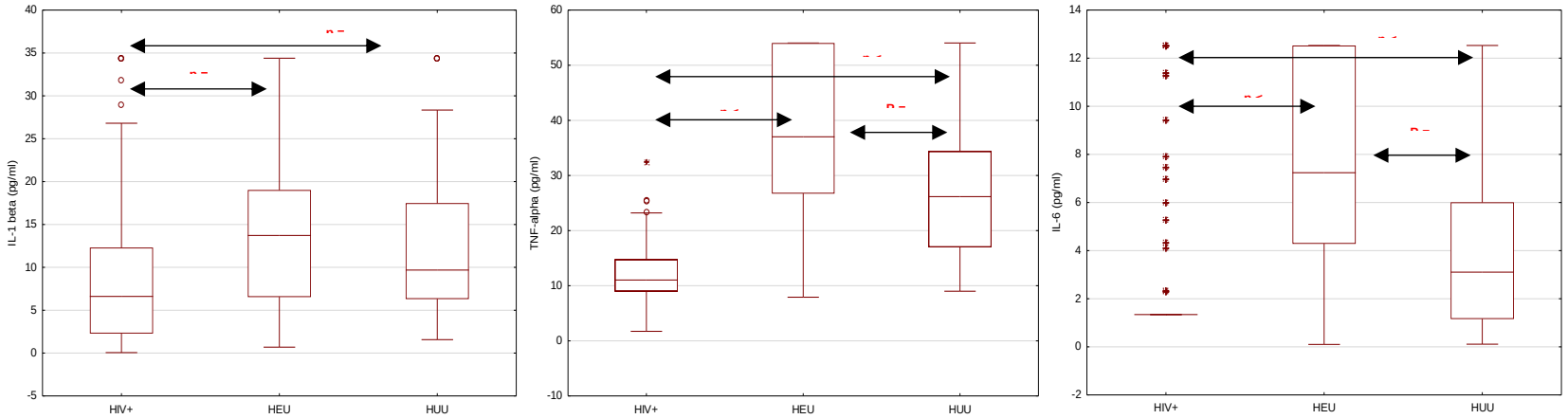


Figure 3.11: *Box and Whisker plots depicting median concentration, 25-75% range within the SD (non-outlier range) for biomarkers associated with M1 cytokines: IL-1β, TNFα and IL-6 in the three study groups (HIV+, HEU and HUU).*
Outliers are shown in circles and extreme outliers with an Asterix (*). Each biomarker represented show significantly decreased levels in the HIV+ group compared to the HEU and HUU groups. Significant differences ($p<0.05$) between HEU and HUU biomarker levels was noted for TNFα and IL-6.

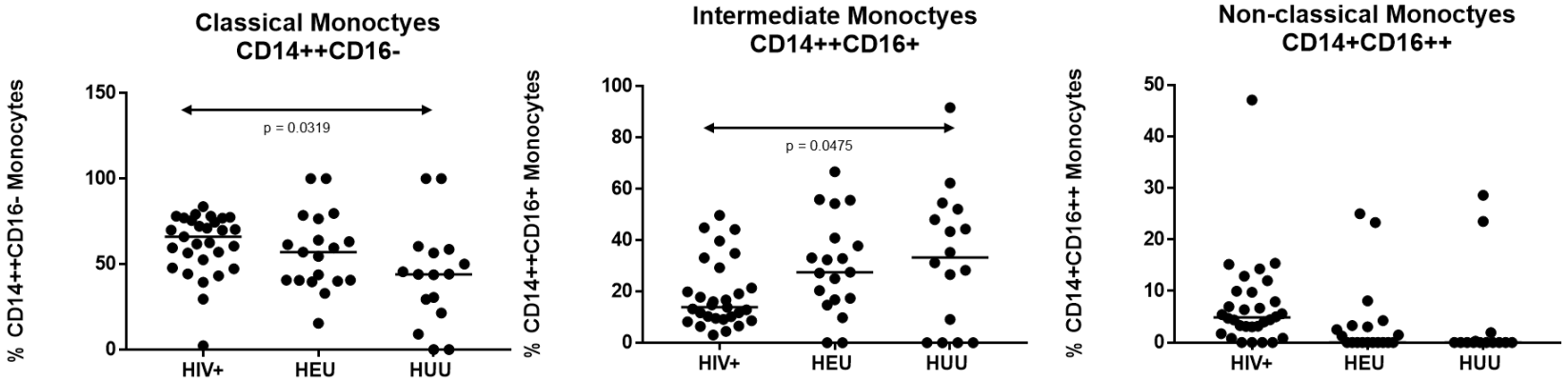


Figure 3.12*: Monocyte subset profile scatter plots. Multiparameter flow cytometry was employed for evaluating monocyte profiles (classical [$CD14^{hi}$], intermediate [$CD16^{lo}$] and non-classical [$CD14^{lo}CD16^{hi}$]) in all three study groups.*
Medians representing each subset are shown. Only significant probabilities that exist between the groups are indicated. HIV+ children display significantly higher levels of $CD14^{hi}$ monocytes than HUU children. No significant difference is observed between the HIV+, HEU and HUU groups. Monocytes representing an intermediate profile (CD16low) was significantly decreased in the HIV+ group ($p=0.048$) compared to HUU. No significant difference ($p=0.32$) in the expression of $CD16^{hi}$ monocyte populations exist between the three study groups.

3.4 Discussion

Limited detailed immunological data exist for long-term virologically suppressed children with PHIV as they approach adolescence. Investigating persisting immune dysregulation provides both a retrospective view of the impact of early cART and its relationship to HIV-1 pathogenesis in children. Prospectively, the impact of these immunological outcomes or snapshots may provide valuable insights into morbidities associated with HIV-1 disease progression in a setting of viral suppression as these children progress into adolescence. With increasing focus on HIV-1 remission, especially after early cART, knowledge of the inflammation status may become increasingly important and may influence candidate selection for these emerging therapies.

Our main hypothesis was that persisting immune dysregulation still exists despite early initiated ART, long-term viral suppression, low HIV-1 cell infectivity, reconstituted CD4 counts and good clinical follow-up and that this dysregulation might contribute to HIV-1 persistence and other AIDS defining morbidities as these children progress as adolescents. Our model is that early damage impacts on subsequent inflammation through gat damage. Low nadir CD4 predicts increased cytokine levels. Multiple cytokines correlate with gut damage. Therefore, the extent of damage may be related to impact of acute infection.

Persistent immune aberrations were found following early cART in the setting of reconstituted CD4 counts in HIV+ children at 8 years of age relative to their age and demographically (age, race and geographical) matched counterparts. A fundamental confirmatory finding was the incomplete restoration of both innate and adaptive immune components as presented in a systematic review conducted in 2015 (Montagnani et al., 2015).

Gut damage in the context of suppressive long-term therapy has been documented in both adult and paediatric HIV+ populations (Guadalupe et al., 2003, 2006; Redd et al., 2009). Our findings support persistent gut damage through elevation of biomarkers (I-FABP, MAdCAM-1, s100A8/A9, LBP, sCD14, sCD163, IL-18 and IL-17F) indirectly associated with gut damage. We also show that these biomarkers have some inter-relatedness. Although many studies have implicated microbial translocation as a driver of immune dysregulation in HIV+ children (Pilakka-Kanthikeel et al., 2012, 2014; Prendergast et al., 2017; Lu et al., 2018), these studies are not consistent in the use of biomarker or quantification assays. Biomarkers measured in our study included LBP (which binds directly to LPS), and is produced by many cells of the innate immune response to bacterial infection (Villar et al., 2009). This LPS-LBP complex is then bound by CD14 expressed on monocytes (bound to TLR4) which results in monocyte activation and secretion of sCD14 (Lichtfuss et al., 2011). Our method of quantifying microbial translocation therefore provides an indirect (or proxy) measurement. Other studies have measured the role of gut translocation by measuring plasma LPS, DNA sequences encoding bacterial 16S ribosomal RNA (Jiang *et al.*, 2009; Pilakka-Kanthikeel *et al.*, 2014), I-FABP (Prendergast et al., 2017) and endotoxin core antibodies (EndoCAb) (Brenchley et al., 2006b; Sandler et al., 2011). We explored the levels of S100A9 and MAdCAM-1 as additional biomarkers of gut integrity which have not been extensively studied and are therefore considered novel.

Our findings of significantly elevated levels of sCD14 and sCD163 is consistent with a recent observation showing abnormal levels of these biomarkers despite 8 years of suppressive cART out of a total of 92 biomarkers of inflammation measured (Babu, et al. 2019). Although this study recruited adults, sCD14 and

sCD163 are important biomarkers in monocyte/macrophage activation and are implicated in immune ageing, cardiovascular and neurological disease outcomes (Babu, et al. 2018, Burdo, Lentz, et al., 2011; Burdo, Lo, et al., 2011; Subramanian et al., 2012; Stilund et al., 2015). Furthermore, sCD163 may play a pertinent role in disease progression as shown by its relationship to clinical parameters such as CD4+ and CD8+ T cell counts in HIV+ children at 8 years.

Despite varying methods for the quantification of microbial translocation, most studies allude to its role in mitigating immune aberrations. However, the role of microbial translocation as a key driver of immune activation and disease progression in HIV-1, remains somewhat controversial (Brenchley et al., 2006b; Jiang et al., 2009; Redd et al., 2009; Lichtfuss et al., 2011; Shan and Siliciano, 2013; F. et al., 2017). A recent Ugandan study included 142 HIV + children recruited on the CHAPAS-3 trial of which 15% included children on cART for approximately 4 years. Microbial translocation was measured using a qPCR panel targeting bacterial species involved in translocation in blood plasma. Parameters related to immune activation and inflammation (both cellular and systemic) were also measured. In this study plasma bacterial DNA was detected at very low levels with minimal differences between HIV+ and uninfected children or between ART groups. Where bacterial DNA was present there was no association with the levels of immune activation. The study concluded that within Ugandan setting, microbial translocation may not drive HIV itself (F. et al., 2017). Another study conducted in treatment-naïve, HIV+ children did show an association between microbial translocation and immune activation (Pilakka-Kanthikeel et al., 2014).

Absence of blood bacterial detection does not necessarily imply that there is no gut translocation. The vasculature that drains from the GIT accesses the liver first, and liver macrophages or Kupffer cells are likely to phagocytose the bacterial components themselves. There would therefore be macrophage activation and local inflammation manifested in systemic changes in biomarkers levels, even without bacterial presence. We are therefore not of the opinion that gut damage alone is involved, however markers, S100A9 and I-FABP, show some damage and is further evidenced by the higher associated markers such as LBP. The damage itself may stimulate inflammation – as damage-associated molecular patterns (DAMPs) could also be driving some of the inflammation. Additional damage caused to lymphoid tissue structures that may also contribute to this inflammation.

In our study, we observed a relationship between markers of gut translocation and monocyte activation implicated in the gut damage (sCD14, sCD163, IL18), however we found no increase in the classical T cell markers of immune activation. Both plasma-derived and T cell activation markers were significantly decreased in our HIV+ population. Interestingly, the plasma biomarkers that were significantly elevated within the HIV+ group were mainly associated with DC and monocyte activation (MIP-1β, IL-1α, INF-α, IL-2, TGFβ1,2). It seems likely that monocytes/macrophages are key contributors to inflammation related to microbial translocation in the paediatric HIV setting.

These findings are supported by a 2010 study conducted in USA infants showing that microbial translocation was associated with persistent monocyte/macrophage activation independent of viral replication or T-cell activation (Wallet et al., 2010). Although this study evaluated LPS and EndoCAb as measurements of

microbial translocation the investigators showed that in the context of paediatric HIV-1 infection, T cell activation and CD4+ T cell loss were correlated to viral replication that was dependent on the timing of infection. However, macrophage activation is related to both microbial translocation and the effects of HIV despite undetectable viral loads (Wallet et al., 2010).

Furthermore, systemic immune activation in T cell versus monocyte/macrophage compartments can be dissociated based on distinct mechanisms *(Wallet et al., 2010). "While viral replication is the principal factor driving lymphocyte activation, microbial translocation is also implicated in systemic* T-cell *activation in some, but not all natural history studies of HIV-1 infection"* (Brenchley et al., 2006b; Marchetti et al., 2008; Redd et al., 2009). In addition, we observed a distinct pattern depicting an apparent M1/M2 macrophage switch as evidenced by decreased levels of IL-1β, TNFα, IL-6 with increased levels of IL-1α in HIV+ children. In our study we also observed an expansion of the classical monocyte subset in HIV+ and HEU children compared to HUU children. This is different when compared to HIV infected adults during treatment

Therefore, in keeping with the findings and opinions of Wallet et al., 2010, our study of perinatally-infected children found no direct association between microbial translocation and surrogate biomarkers of T cell activation. We did observe elevated levels of immune exhaustion. This has been described in other studies. Ongoing viral replication coupled with microbial translocation may result in elevated exhaustion of T cells as a protective mechanism, which would explain the seemingly anomalous finding of low T cell activation. Our findings further highlight the complexity of the relationship that exists between the causes and consequences of immune activation.

In addition, thymic output studies within this cohort of the CHER trial showed that following cART, reconstitution of CD4+ T cells and increased TRECs were observed (Payne et al., 2015; Lewis et al., 2017). This further indicates that thymic output serves as contributor to post-ART lymphocyte reconstitution but is independent of microbial translocation (Douek et al., 1998, 2000; JW, 2003; Yin et al., 2008).

Based on our observation, cART fails to attenuate microbial translocation in HIV+ children unlike adults (Schacker et al., 2002; Brenchley et al., 2006b). Although the cross-sectional study design is a limitation of our study, we have shown immune abnormalities that persists after 8 years in HIV+ children.

The mechanistic link between microbial translocation and monocyte/macrophage activation may be augmented by HIV-1 serving as a co-factor for LPS-induced inflammation. HIV-1 may directly activate monocytes or sensitize macrophages to activation with TLR ligands which include LPS (Brown et al., 2008; Lee et al., 2008; Lester et al., 2008; Jiang et al., 2009; Wallet et al., 2010). Macrophages are considered good targets for HIV-1 infectivity (McElrath, Pruett and Cohn, 1989; Wallet et al., 2010)

The extent of GALT depletion is linked to gut damage. As nadir CD4 is a proxy for extent of GALT depletion, it should reflect level of gut damage too (addressed later). Our model holds that our patients with more extensive early pathology have persisting elevated inflammatory markers at 8 years of age/preadolescence.

The effects of microbial translocation and subsequent HIV pathogenesis in children may be different than in adults. This may be related to differences in gut flora during early developmental stages (Cohen et al., 1995;

Qing, Howlett and Bortolussi, 1996). Systemic activation of monocytes and macrophages may contribute to other AIDS morbidities such as neurocognitive related outcomes, cardiovascular events and dementia (Ancuta et al., 2008; Crowe et al., 2010; Funderburg et al., 2010; Alfano et al., 2013)

Another key observation of our study is that decreased T-cell mediated cytokine biomarker IL-3, RANTES, GCSF, INFγ, TGFβ1, IL-17A. This would appear to confirm that exhausted T cells are non-responsive and essentially shut-down cytokine production.

3.5 Conclusion

Overall, the results of this study showed several significant immunological differences that exist within both innate and adaptive immune compartments in HIV+ children at pre-adolescent years when compared to study controls even after early and long-term suppressive ART. Persisting inflammation and immune dysregulation associated with gut damage serves as a key finding in this investigation along with dysregulation of monocyte/macrophage pathways. These findings prompt further and more detailed immune profiling and clinical follow-up to establish the severity of morbidity outcomes that may progress from this immune dysregulation in HIV infected children.

Considering that perinatally-infected neonates will likely survive multiple decades with ART, the implications of persistent microbial translocation and monocyte/macrophage activation should be a major focus of investigation in coming years. Interventions to address early gut damage/permeability in PHIV children due to HIV-1 infection would serve beneficial in mitigating some immune inflammation and elevate early onset of chronic inflammation.

The findings of this study lead to further investigations as discussed in Chapters 4 and 5 of this dissertation. Further to this, we pose the question: *Due to importance of viral reservoir in cure research, do the findings of this research prompt an investigation into whether any of the markers are associated with measures of the reservoir (and gut damage)?*

CHAPTER 4:

ELEVATED IL-18 IS ASSOCIATED WITH HIV-1 CELL-ASSOCIATED DNA IN PRE-ADOLESCENT CHILDREN: RELATIONSHIP WITH OTHER KEY INNATE BIOMARKERS

SUMMARY OF KEY FINDINGS – CHAPTER 4

- Highly significant increase in IL-18 expression from baseline (before therapy initiation) to 8 years of age in HIV+ group
- IL-18 remains significantly elevated at 8 years of age despite therapy regime in HIV-1 infected children when compared to age-matched controls
- Therapy interruption showed no significant difference in IL-18 expression in HIV-infected children at 8 years of age
- IL-18 shows significant association with HIV-1 cell-associated DNA at baseline (closest time to therapy initiation) and 8 years of age
- IL-18 displayed significant associations with other important immune biomarkers including sCD14, sCD163, IL-1RA, LBP and INF-α
- IL-18 showed significant associations with clinical parameters including the CD4 count at baseline, CD4:CD8 ratio at 8 years and absolute and percentage CD8 at 8 years

- ***Conclusion****: IL-18, a potent pro-inflammatory cytokine, may serve as a putative biomarker for measures of the HIV-1 latent reservoir. The associations with IL-18 with other important markers such as sCD14, sCD163, IL-1RA, LBP and INF-α may provide a more important biosignature for the evaluation of HIV-1 persistence within our population*

ABSTRACT

Background: The role and function of cytokines and chemokines in HIV-1 disease progression have been exhaustively described in adult populations but remains less well studied in chronically HIV-1 infected children. The impact of soluble immune mediators on the latent HIV-1 reservoir as well as other clinical parameters defining chronic disease status, is also yet to be investigated. IL-18 is a powerful pleiotropic pro-inflammatory cytokine with multifunctional capabilities. Mechanisms of IL-18-driven pathogenesis in HIV-1 paediatric populations is yet to be elucidated. This sub-study aimed to determine the possible associations between the concentration of blood plasma IL-18 (and other cytokines/chemokines) and HIV-1 cell infectivity (as the measurement of integrated HIV-1 cell associated DNA (iCAD).

Methods: A longitudinal cohort study was implemented to investigate the relationship between immunological (IL-18), clinical (CD4, CD8 counts) and virological factors (viral load, HIV-1 CAD) in PHIV children from birth to 8 years of age. Study participants originated from the CHER trial and randomised into early, delayed and timed therapy interruption. Age and community-matched controls consisting of HEU as well as HIV unexposed uninfected (HU) children were included at 8 years of age. A panel of plasma biomarkers, inclusive of IL-18 was measured using validated ELISA and Luminex® Multiplex assays. A subset (n=32) of PHIV children was quantified for HIV-1 CAD at two time points – baseline (closest time to therapy initiation) and at 8 years of age. A sensitive qPCR adapted for HIV-1 subtype C was implemented for the measurement of HIV-1 total DNA in PBMCs aimed at targeting a region in HIV-1 integrase with a detection limit of 3 copies/reaction.

Results: A total of 141 (76 PHIV, 29 HEU and 36 HU) participants were studied. PHIV children showed a significantly ($p<0.0005$) higher (4-9-fold) level of IL-18 at 8 years of age compared to baseline (chronic HIV infection). Following 2 years of ART, IL-18 levels were similar to that of baseline in all study groups. At 8 years of age, PHIV children showed significantly higher levels of IL-18 (median: 2075.6 pg/ml) when compared to HEU (median: 678.6; $p<0.0005$) and HU (median: 752.6 pg/ml; $p<0.0005$). Early therapy interruption had no impact on IL-18 expression ($p=0.47$).

IL-18 significantly correlated with HIV-1 CAD at baseline ($r = +0.35$; $p=0.04$) and at the 8-year follow-up ($r = +0.38$; $p=0.02$) as well as key immune biomarkers specifically related to innate IL-1 family of immune biomarkers including IL-1RA ($r = +0.33$; $p<0.01$), IL-1α ($r = +0.22$; $p<0.01$), IL-1β ($r = +0.26$; $p<0.01$), and IL-1RA ($r = +0.32$; $p<0.01$). In addition, IL-18 significantly correlated with biomarkers of monocyte/macrophage activation and gut damage, including: sCD14 ($r = +0.50$; $p<0.01$), sCD163 ($r = +0.40$; $p<0.01$), LBP ($r = +0.26$; $p<0.01$) and MIP-1β ($r = +0.19$; $p=0.02$). IL-18 showed significant negative associations ($p<0.01$) with CD4 % at baseline, longitudinal CD4 count and CD4:CD8 ratio at 8 years ($r = +0.35$; $p=0.04$).

Conclusions: We observed the potential of IL-18 as an important marker providing insights into immune dysfunction and inflammation, and as having an association to HIV-1 cell infectivity. Measuring IL-18 in plasma can also be used a proxy for deviant immune activation that is considered as a correlate of clinical progression in HIV-1 infection. This marker could also provide an indication of which patients will require

added vigilance after ART initiation. Our data implies that IL-18 levels may be related to early stages of reservoir establishment and may also be indicative of reservoir persistence. We alluded to HIV-1 reservoir cells sometimes permitting limited transcription and translation of the proviral DNA, which may trigger the PAMPs that stimulate IL-18 release via inflammasome activation. The continued presence of LBP (shown previously) ties in with this hypothesis – as TLR stimulation via LPS together with other PRR stimulation ties in with inflammasome function.

4.1 Introduction

The ongoing impact of the global HIV-1 pandemic requires continued pathogenicity studies that will provide insight into newer therapeutic approaches for improved clinical outcomes and possible elimination or cure (Lewin et al., 2011; Goulder, Lewin and Leitman, 2016.)

Evaluation of immune status and pathogenic features of children born infected with HIV-1 subtype C and initiated on early long-term suppressive cART for an extended period requires further delineation in our setting, specifically as they approach adolescence with a chronic infection status (Mofenson and Cotton, 2013). With emerging data suggesting the need for more advanced clinical follow-up in this population, there is great need for improved understanding of cART effects, long-term disease progression and immune health (Barlow-Mosha et al., 2013). Furthermore, understanding the interplay between the virus and the immune system may provide further insight into the mechanisms of HIV-1 cell infectivity and persistence in children on long-term therapy.

Immune biomarkers can provide insight into the mechanisms of HIV-1 latency (establishment of the latent reservoir), pathogenesis and the effects of cART within the infected paediatric and adolescent populations (Nixon and Landay, 2010; Justice et al., 2018). More importantly, immune biomarkers can provide tools to identify specific patients for further evaluation in HIV-1 remission studies.

It is well documented that HIV-1 infection is accompanied by immune dysregulation that never reaches normality despite early initiated, long-term cART (Douek, 2013; Bordoni et al., 2020). Chronic inflammation is a hallmark consequence of HIV-1 infection and leads to the development of opportunistic infections (Zhang et al., 2003). Mounting an effective immune response to viral infection requires both innate and adaptive interactions with various cell components including T cells, DCs, macrophages and natural killer (NK) cells.

Cytokines are very important immune molecules that allow for the communication and interaction of these cell types in order to respond effectively to infection (Torre and Pugliese, 2006). One of the key factors contributing to abnormal immunity in HIV-1 patients, includes a dysfunctional cytokine profile.

Cytokine and chemokine biomarkers circulating in blood plasma can provide valuable information into understanding the relationship between disease progression and clinical outcomes (Strimbu and Tavel, 2010). Variations in the expression of cytokines in HIV-1 infection can impact the functioning of the immune system as well as implicate and influence the course of disease progression by promoting or suppressing HIV-1 replication (French et al., 2015). Furthermore, immune biomarkers could lead the way for the development of screening algorithms for the identification of patients that may experience clinical failure (Yasuda, Nakanishi and Tsutsui, 2019).

The role and function of cytokines and chemokines in HIV-1 disease progression have been exhaustively described in adult populations but remains less well studied in chronically HIV-1 infected children (Pallikkuth et al., 2016). Furthermore, the impact of soluble immune mediators on the latent HIV-1 reservoir as well as other clinical parameters defining chronic disease status, is also yet to be investigated.

IL-18, first referred to as the IFN-γ inducing factor, is a powerful pleiotropic pro-inflammatory cytokine with multifunctional capabilities. This cytokine belongs to the IL-1 family of cytokines which includes the IL-1 subfamily (IL-1α, IL-1β, IL-1RA and IL-33), IL-18 subfamily (IL-18 and IL-37) and the IL-36 subfamily (IL-36α, β, γ, IL-36RA and IL-38) (Dinarello, 2018). IL-18 shares a number of key features with IL-1β, with both cytokines being potent inflammasome-associated mediators of inflammation. IL-18 is produced by activated macrophages, conventional/myeloid DCs, keratinocytes as well as epithelial cells of the entire GIT. In addition, IL-18 genes have also been documented to be expressed by certain brain neurons in the presence of infection (Dinarello et al., 2013). IL-18 is produced as an inactive 24kD precursor protein containing 192 amino acids which is then cleaved in the cytoplasm on the carboxyl side of its aspartate 36 by the IL-1β converting enzyme into a mature biologically active 18kD cytokine (Yasuda, Nakanishi and Tsutsui, 2019).

Cytokines IL-1β and IL-18 are also involved in the differentiation of naïve CD4+ T cells to Th1 and Th17 profiles, thereby enhancing immune activation and further promoting viral replication since these cells are key HIV-1 targets (Feria et al., 2018). It has also been documented that due to the pleiotropic nature of IL-18, dysregulation or over production of its synthesis leads to the expansion of HIV-1 target cells and increase in the HIV-1 viral load thereby further implicating their crucial involvement in HIV-1 pathogenesis (Wiercinska-Drapalo et al., 2004). Other studies have shown IL-18 and its ability to inhibit HIV-1 replication in PBMCs (Choi, Dinarello and Shapiro, 2001; Stylianou et al., 2003), but conversely been reported to also enhance HIV-1 replication in monocytic and T cell lines in other studies (Shapiro et al., 1998; Klein et al., 2000).

The release of IL-1β and IL-18 succeeding the activation of the inflammasome is important for stimulating the cells of the innate immune system which also influences the mechanisms of the adaptive immune response. These adaptive immune responses include the role of IL-18 in influencing Th1 responses as well as the release of IFN-γ from CD8+ T cells (Dostert, Ludigs and Guarda, 2013; Leal, Reis and Pontillo, 2020).

Biologically active IL-18, tightly controlled by antagonist IL-18 binding protein (IL-18BP), exerts its effect on target cells via the IL-18 receptor (IL-18R) which is expressed on a number of immune cells including vascular endothelial cells, NK, NKT cells, Th1 CD4+ T cells and DCs (Dinarello et al., 2013).

The inflammatory response induced by HIV-1 leads to the formation of molecular inflammasome complexes such as NLRP1, NLRP3, NLRC4m and Pyrin. These complexes are also involved in the regulation and maturation of cytokines within the IL-1 family of which IL-18 and IL-1β form part of and also results in the activation of Caspase-1 (Feria et al., 2018). Higher expression of IL-1β, IL-18 and Caspase-1 genes in GALT biopsies and PBMCs stimulated with inflammasome agonists was observed in HIV-1 progressors when compared to HIV-1 controllers. The elevated concentration of IL-18 within blood plasma also served as an indicator of baseline inflammation (Feria et al., 2018.)

Measures of the HIV-1 latent reservoir and persistence require easily measurable biomarkers (cell communicators such as cytokines) as determinants and/or predictors of cellular infection. This sub-study aimed to determine the possible associations between the concentration of blood plasma IL-18 and HIV-1 cell infectivity (as the measurement of integrated HIV-1 CAD). Moreover, we attempted to investigate the relationship between IL-18 levels, antiviral treatment and clinical parameters in our HIV-1 infected subjects.

We posed the question of whether IL-18 could represent a putative biomarker for the characterisation of HIV-1 persistence. In addition, we evaluated whether IL-18 could provide a snapshot view on overall immune health that may predict possible clinical failure as well as a possible criterion for inclusion in HIV-1 remission studies in HIV-infected children of the randomised CHER trial.

4.2 Materials and Methods

4.2.1 Study Design and Selected Participants

This was a longitudinal cohort study for the assessment of various immunological outcomes as part of a follow-up investigation of HIV+ (infected) children. Study participants originated from the randomised CHER trial as described previously (Chapter 3).

For the purpose of this study component, we have evaluated the post-CHER study cohort follow-up following regrouping of the original study arms as described above based on the following therapy descriptions: ***Group 1*** (delayed followed by continuous therapy), ***Group 2*** (early continuous therapy), ***Group 3*** (early initiated therapy followed by interruption at 40 weeks) and ***Group 4*** (early initiated therapy followed by interruption at 90 weeks). Clinical details of these groups are documented in Table 4.1 below. Early baseline time points did not include a control group due to inability to access samples.

At the time of follow-up sampling, the HIV-1 infected children were between the ages of 7 and 11 years (median of 8 years). Age and community-matched controls consisting of HEU as well as HUU children were included in this investigation. A subset (n=32) of the HIV-infected children was quantified for iCAD at two time points – baseline (closest time to therapy initiation) and follow-up (8 years of age).

4.2.2 Routine Testing

HIV Viral Load

HIV-1 viral loads were measured in an accredited routine laboratory, the National Health Laboratory Service (NHLS), Tygerberg Cape Town, South Africa and described previously (Chapter 3)

Lymphocyte Count

Described previously (Chapter 3).

4.3.2 Plasma Biomarker Measurements

A panel of 43 plasma biomarkers as described in Chapter 3, excluding biomarkers I-FABP, MAdCAM-1 and s100A8/A9 were assessed in this research component.

4.2.4 Assessment of HIV-1 Cell-Associated DNA

A sensitive qPCR adapted for HIV-1 subtype C was implemented for the measurement of HIV-1 total DNA (also described in Chapter 3). This assay targets a region in HIV-1 integrase and has a detection limit of 3 copies/reaction as previously described (van Zyl et al., 2015; Hong et al., 2016; Veldsman et al., 2018).

4.2.5 Statistical Analysis

Numerical variables measured for each of the groups were analysed using TIBC® Statistica™ version 13.3 software. Testing for normality using normality probability plots was done for all variables. All values that were outside the normality ranges were included by use of winsorisation of outliers to bring all data points within range for analysis. A nonparametric one-way analysis of variance (ANOVA) Kruskal-Wallis test was used. Correlations between clinical parameters and laboratory measurements (plasma biomarkers, cellular markers and iCAD measurements) were performed by Spearman correlation tests and linear regression analysis assuming non-parametric distribution. *P*-values less than 0.05 were considered statistically significant.

4.3 Results

At the follow up visit of (median 8 years of age), we investigated a total of 141 (76 HIV-1 infected, 29 HEU and 36 HUU) participants for this sub-study. The baseline and HIV related clinical parameters of the HIV-1 infected study participants are listed in Table 4.1 below and 8-year follow-up parameters in summarised in Table 4.2.

4.3.1 Highly significant increase in IL-18 expression from baseline (before therapy initiation) to 8 years of age in HIV+ group

Following group comparative analysis (simple T-test analysis), we compared the differences in IL-18 levels at baseline (before therapy initiation) compared to the levels at 8 years of age following long-term suppressive therapy. The levels of the control groups i.e., HEU and HUU were also compared.

Figure 4.1 below provides a Box and Whisker plot displaying the medians, IQRs and outliers for each group at baseline and 8 years of age. A summary of the statistical parameters is tabulated in Table 4.3.

Evaluating the IL-18 levels at three time points across all therapy groups, we find a similar trend across the groups with a significant spike in IL-18 levels at 8 years of age (Figure 4.2)

Table 4.1: Summary of clinical parameters for the HIV+ children in classified into Group 1, 2 3 and 4

	Group 1 Delayed Continuous Therapy	**Group 2 Early Continuous Therapy**	**Group 3 Early Therapy Interrupted at 40 weeks**	**Group 4 Early Therapy Interrupted at 90 weeks**
n	**10**	**23**	**27**	**16**
Gender (Number of Females); percentage of total	5 (50%)	9 (39%)	14 (52%)	11 (69%)
Birth weight (grams); Median (range)	3215 (2640-3640)	2900 (2000-3780)	3200 (2400-4200)	2900 (2188-3500)
Age cART first initiated (months); median (range)	9 (5.6-17.7)	2 (1.4-5.4)	2 (1.4-2.8)	2 (1.6-2.8)
Pre-treatment log10 HIV-1 RNA load; median (range)	5.8 (5.0-6.0)	6.0 (5.0-6.0)	5.9 (3.8-5.9)	5.9 (4.2-5.9)
Time to viral load suppression (days); median (range)	336.6 (30.0-839.0)	168.0 (161.0-1931.0)	172.0 (164.0-1414.0)	168.5 (162.0-1404.0)
Length of therapy interruption (months); median (range)	-	-	7.47 (1.33-92.87)	9.57 (3.82-81.90)
Baseline CD4%; median (range)	32.9 (25.4-52.8)	28.5 (8.9-49.0)	35.2 (9.88-49.7)	35.6 (23.1-57.1)
Baseline absolute CD4 count cells/microliter; median (range)	1742.5 (1150-3731)	1430.0 (340-3828)	1823.0 (284-5044)	1868.5 (829-4272)
Absolute CD4 count nadir cells/microliter; median (range) at 8 years of age	926.5 (568-2731)	1048 (323-2562)	1037 (446-1483)	930 (563-1438)
CD4% median (range) at 8 years of age	34 (31-54)	38 (21-56)	41 (28-51)	36.5 (27-47)
Absolute CD8 median (range) at 8 years of age	967 (475-1767)	745 (357-2894)	713 (474-1300)	993 (303-1605)
CD8% median (range) at 8 years of age	31 (22-41)	33 (20-52)	30 (19-47)	38.5 (18-51)
CD4:CD8 ratio median(range) at 8 years of age	1.18 (0.77-1.99)	1.19 (0.45-2.81)	1.36 (0.62-2.25)	0.99 (0.59-2.65)

Table 4.2: Summary of participant (HIV+, HEU and HUU) clinical characteristics at 8 years of age

	HIV +	**HEU**	**HUU**	**Kruskal-Wallis p-value**
n	**76**	**29**	**36**	
Absolute CD4 count nadir cells/µl median (range)	998.5 (323-2731)	948 (501-1332)	933 (339-2473)	0.26
CD4% (range) at 8 years of age	38 (21-56)	40.0 (27-52)	40.5 (28-57)	0.22
Absolute CD8 (range) at 8 years of age	3975 (303-2894)	624 (311-1021)	596 (174-981)	**<0.01**
CD8% (range) at 8 years of age	31.5 (18-52)	26.0 (18-38)	24.5 (18-36)	**<0.01**
CD4:CD8 ratio at 8 years of age	1.18 (0.45-2.81)	1.57 (0.81-2.36)	1.66 (0.8-3.0)	**<0.01**

***Lymphocyte reference ranges**

Reference: (Lawrie *et al.*, 2015)			
Age range	**0-3 months**	**1-2 years**	**6-12 years**
CD4 T lymphocytes (cells/µL)	1520-5160	1374-3928	568-2013
CD8 T lymphocytes (cells/µL)	428-2478	669-3247	340-121-
CD4:CD ratio	1.29-5.29	0.78-3.88	0.95-2.47
Reference: (Payne *et al.*, 2020)			
Age range	**1-2 months**	**15-24 months**	**5-10 years**
CD8 (%)	10.7-28.9	13.8-34.9	15.1-34.8
Reference: (Shearer *et al.*, 2003)			
Age range	**0-3 months**	**1-2 years**	**6-12 years**
CD4 (%)	35-64	32-51	31-47

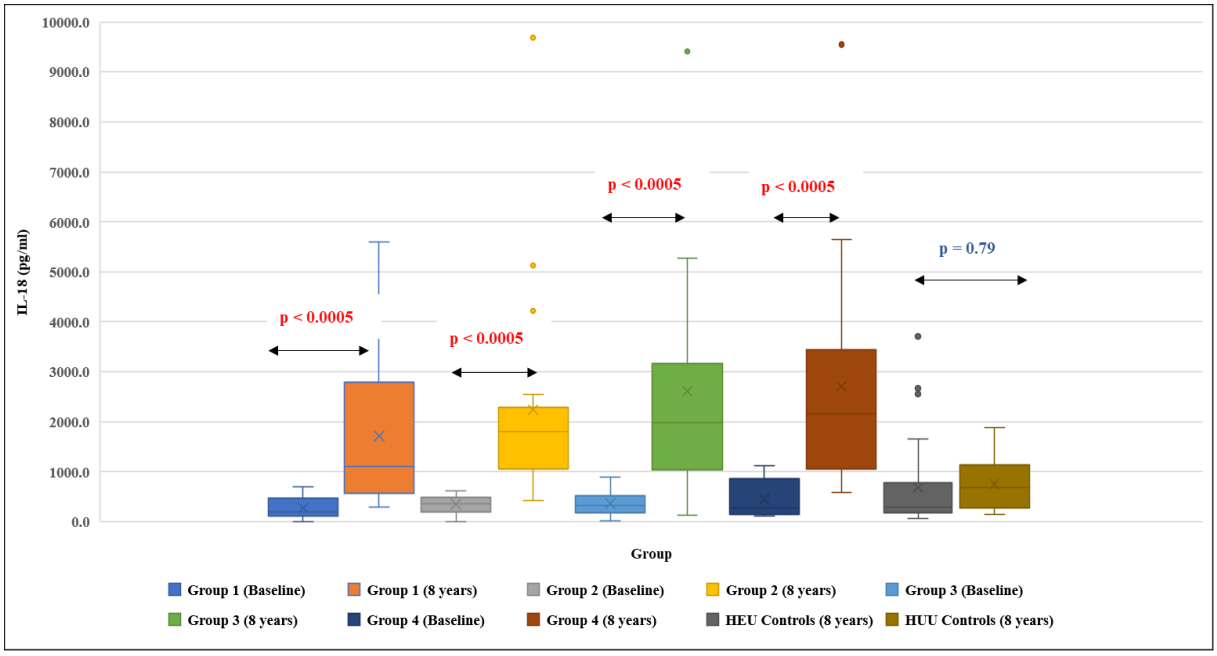


Figure 4.1: *Box and Whisker plot displaying medians, IQRs, outliers with 95% confidence intervals for Groups 1 to 4 at baseline (before therapy initiation) and 8 years of age following a group comparison analysis.*
IL-18 levels for ***Group 1*** *(delayed therapy initiation),* ***Group 2*** *(early continuous therapy),* ***Group 3*** *(interrupted at 40 weeks) and* ***Group 4*** *(interrupted at 90 weeks) are presented along with HEU and HUU controls. A significant difference between the time point baseline (before therapy initiation) and 8 years of age is noted with a 4-9-fold increase from baseline to 8 years for all therapy groups (p<0.0005). Levels of IL-18 in both control groups were observed to be similar (p=0.79) and significantly lower compared to IL-18 levels at 8 years of each for each of the HIV+ groups.*

Table 4.3: Summary of statistical parameters for IL-18 levels from baseline to 8 years of age as graphically represented in Figure 1 above

	Group 1	**Group 2**	**Group 3**	**Group 4**	**HEU**	**HUU**
n	**10**	**14**	**9**	**7**	**-**	**-**
IL-18 (pg/ml); median (IQR) at baseline	185.7 (143.1-402.1)	348.5 (213.1-480.1)	330.6 (239.6-382.9)	272.3 (156.4-652.5)	-	-
n	**10**	**23**	**27**	**16**	**29**	**36**
IL-18 (pg/ml); median (IQR) at 8 years of age	1,104.3 (661.7-2,435.1)	1,803.2 (1,132.5-2,265.4)	1,975.4 (1,048.6-3,155.9)	2,157.8 (1,226.1-3,245.7)	294.5 (179.4-748.0)	680.6 (287.5-1,103.8)
p-value	**p<0.0005**	**p<0.0005**	**p<0.0005**	**p<0.0005**	**p=0.79**	

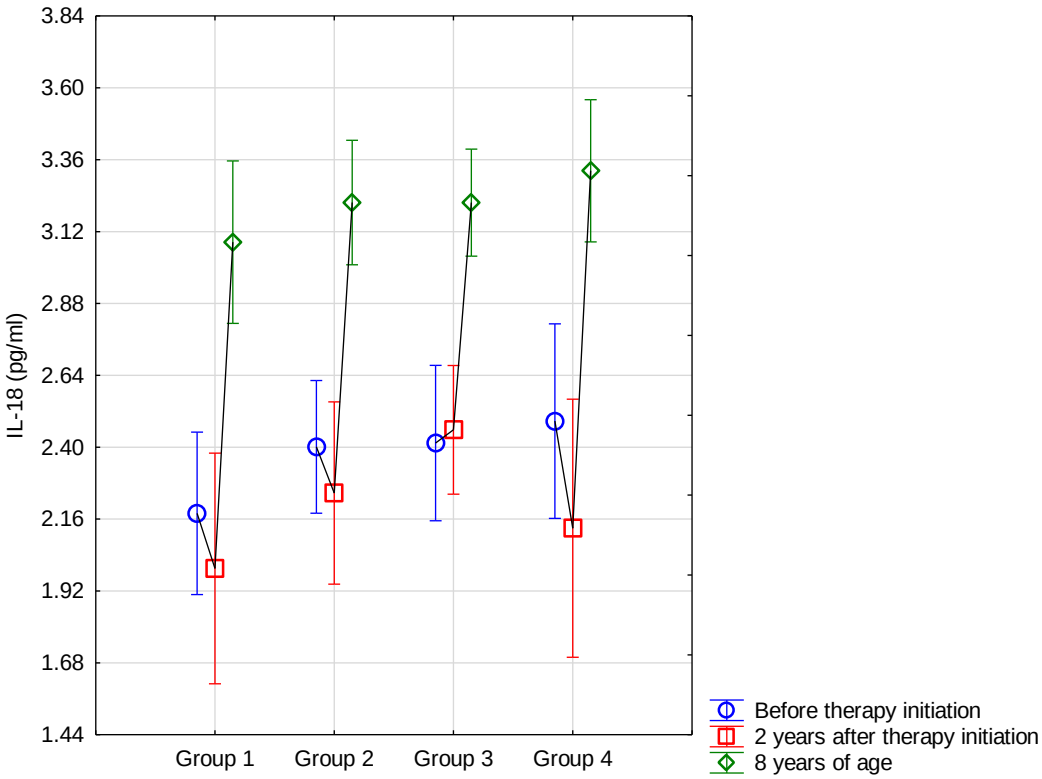


***Figure 4.2**: Vertical bar graph with 95% confidence intervals depicting log median IL-18 levels (pg/ml) at three time points; before therapy initiation, 2 years after therapy initiation and 8 years of age for each therapy arm as shown on the x-axis of the graph.*

4.3.2 IL-18 remains significantly elevated at 8 years of age despite therapy regime in HIV-1 infected children when compared to age-matched controls

When investigating the IL-18 levels in each HIV+ therapy group, compared to HIV-1 negative controls (both HEU and HUU), we found that IL-18 levels were significantly higher in all therapy groups compared to both HIV-1 negative control groups. At 8 years of age, the delayed therapy group had non-significantly lower levels of IL-18 compared to the other groups. No significant difference was noted between the HEU and HUU control groups (Table 4.4).

Table 4.4: Summary of statistical parameters (mean, standard error and medians) for IL-18 levels for each of the six study groups evaluated at 8 years of age as graphically represented in Figure 3 above

Group	n	IL-18 (pg/ml); mean at 8 years	IL-18 (pg/ml); Standard Error of the mean (SEM)	IL-18 (pg/ml); median (range) at 8 years
Group 1	10	1609.4	344.1	1247.7 (495.1-5591.6)
Group 2	14	2022.8	232.0	1807.7 (417.6-4216.7)
Group 3	9	2140.7	205.7	1864.8 (610.9-1016.8)
Group 4	7	2325.7	272.0	2707.2 (1316.6-9546.5)
HEU controls	29	678.6	202.1	295.4 (69.9-2705.9)
HUU controls	36	752.4	181.4	680.6 (137.5-1879.6)

	p-value matrix comparing significance amongst study groups including controls					
	Group 1	**Group 2**	**Group 3**	**Group 4**	**HEU controls**	**HUU controls**
Group 1	-	0.32	0.19	0.10	**0.02**	**0.03**
Group 2	0.32	-	0.70	0.40	**0.00**	**0.00**
Group 3	0.19	0.70	-	0.59	**0.00**	**0.00**
Group 4	0.10	0.40	0.59	-	**0.00**	**0.00**
HEU controls	**0.02**	**0.00**	**0.00**	**0.00**	-	0.79
HUU controls	**0.03**	**0.00**	**0.00**	**0.00**	0.79	-

**Summary matrix of p-values indicating significance between each of the six groups evaluated for this investigation*
**Group 1 (delayed therapy initiation), Group 2 (early continuous therapy), Group 3 (interrupted at 40 weeks) and Group 4 (interrupted at 90 weeks)*

4.3.3 Therapy interruption showed no significant difference in IL-18 expression in HIV-1 infected children at 8 years of age

We evaluated the effect of therapy interruption (Group 3 and 4) on IL-18 levels in blood plasma in HIV+ children at 8 years of age by use of a nonparametric Mann-Whitney U statistical test. Although IL-18 levels were higher in the interrupted group compared to the other groups, this did not reach statistical significance ($p=0.47$). We therefore conclude that interruption of therapy does not influence the levels of IL-18 in HIV+ children by the time they reach 8 years of age following long-term suppressive therapy. Nonparametric testing indicated a Mann-Whitney U test p-value of 0.47 as indicated in Figure 4.3 below.

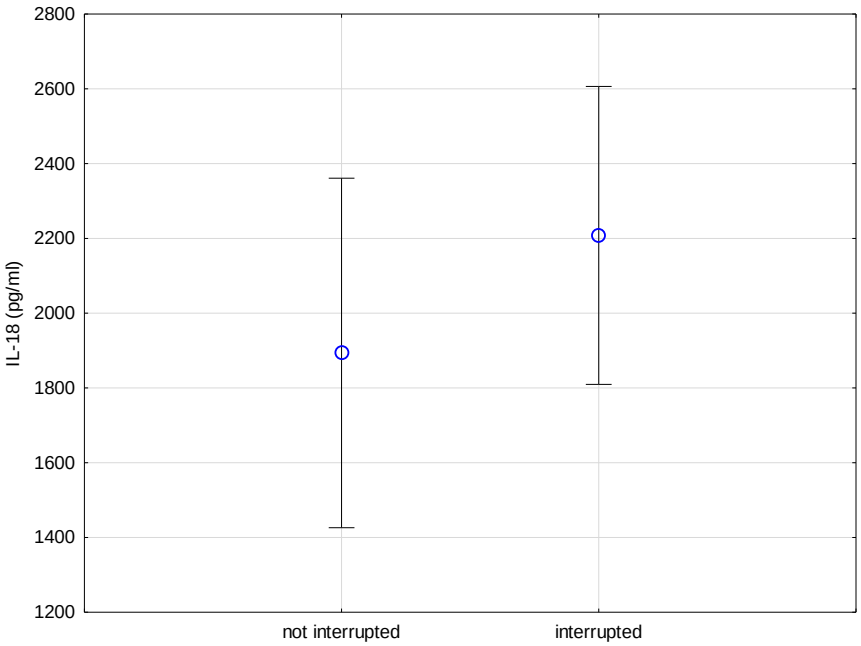


Figure 4.3: *Vertical bar graph depicting mean levels of IL-18 in blood plasma at 8 years of age in HIV+ children with (n=44) and without interruption (n=32).*
Mean IL-18 levels for the interrupted group was 2208.0 pg/ml (SEM = 200.0) and the group not interrupted had an IL-18 mean level of 1898.6 pg/ml (Std Err. 234.5). No significant difference is noted between the two groups (p=0.47).

In summary thus far, we have shown that HIV+ children display a significant increase in IL-18 levels from baseline (before therapy initiation) to 8 years of age (Figure 4.1). When evaluating an intermediate time point i.e., 2 years after therapy initiation (Figure 4.2) for all groups, we show that levels either decrease minimally or remain the same. Therapy interruption did not impact the levels of IL-18 at baseline or 8 years of age (Figure 4.3). When compared to HIV-1 uninfected controls, HEU and HUU, we observed significantly lower levels in both control groups compared to HIV+ children irrespective of therapy grouping. Within the control group we noted no significant differences between HEU and HUU children at 8 years of age.

Thus far we have evaluated and presented IL-18 levels based on their respective stratified grouping.

Following on the above, the analysis to follow will include HIV+ children as a group inclusive of all therapy criteria. A cross-sectional analysis at 8 years of age is a comparative analysis inclusive of HIV-1 uninfected controls (HEU and HUU) as presented in Figure 4.4 below. As a limitation to this study, we cannot present data of HIV-1 negative controls at earlier time points and therefore cannot draw inferences on the normality of the levels of IL-18 in HIV infected children compared to HIV-1 negative controls during early infancy.

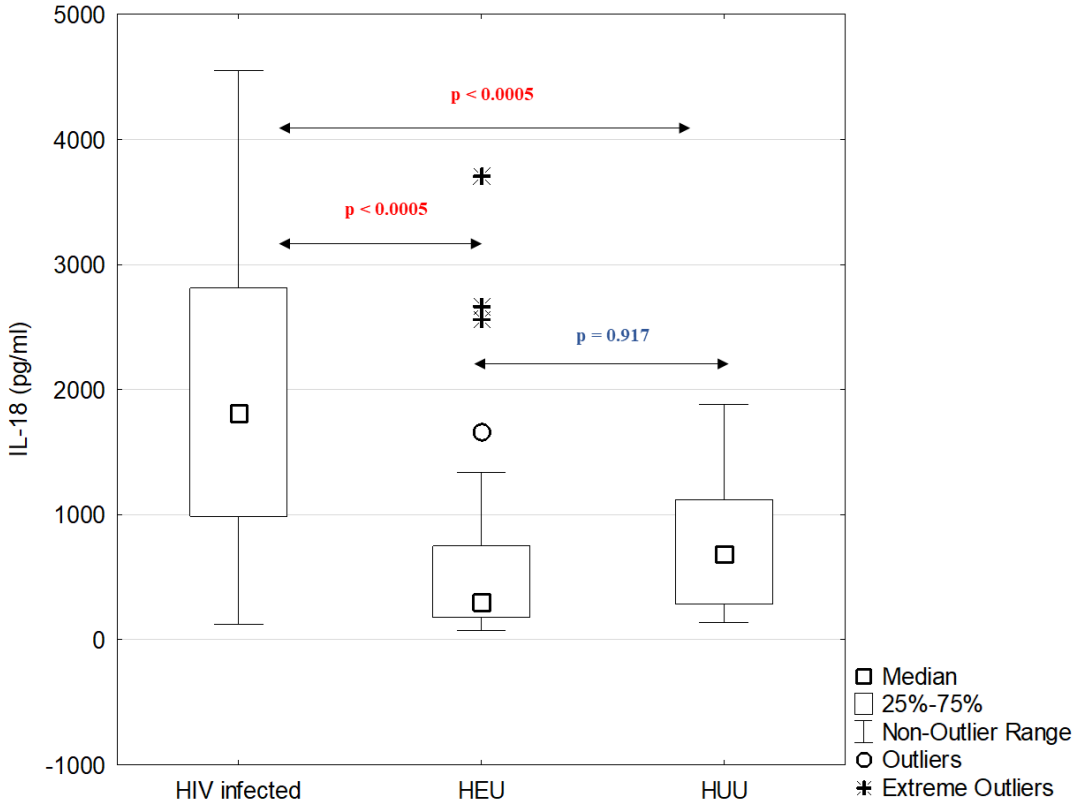


Figure 4.4: *Box and Whisker plot representing IL-18 levels at 8 years of age for three study groups HIV-1 infected, HEU and HUU children.*
The medians for the HIV-1 infected, HEU and HUU are indicated on the graphical representation. Outliers and the extreme outliers are noted in the HEU group and specific IL-18 concentration indicated. Table 4.5 below provides a summary of the statistical data.

Table 4.5: Summary of statistical parameters for IL-18 levels depicted in Figure 5 for grouped HIV infected children at 8 years of age compared to the HEU and HUU groups respectively

Group	n	IL-18 (pg/ml); mean at 8 years	IL-18 (pg/ml); Standard Deviation	IL-18 (pg/ml); median (range) at 8 years	p-value (HIV+ vs HEU)	p-value (HIV+ vs HUU)	p-value (HEU vs HUU)
HIV+	76	2075.6	1327.2	1805.49 (124.4-10168.0)	**p<0.0005**	**p<0.0005**	p =0.917
HEU	29	678.6	893.7	295.4 (70.0-3705.9)			
HUU	36	752..4	500.1	680.6 (137.5-1879.6)			

4.3.4 IL-18 shows significant association with HIV-1 cell-associated DNA at baseline (closest time to therapy initiation) and 8 years of age

A subset of HIV+ children in Groups 1 to 4 were longitudinally measured for HIV-1 cell associated infectivity or iCAD. In summary, we observed a significant decrease in iCAD levels following therapy initiation in each of the HIV+ study groups (Figure 4.5 and Table 4.6). Using both Spearman and Pearson correlation analysis,

we correlated available iCAD levels with IL-18 levels at baseline and 8 years of age. Correlation scatter plots are represented in Figure 4.6 below.

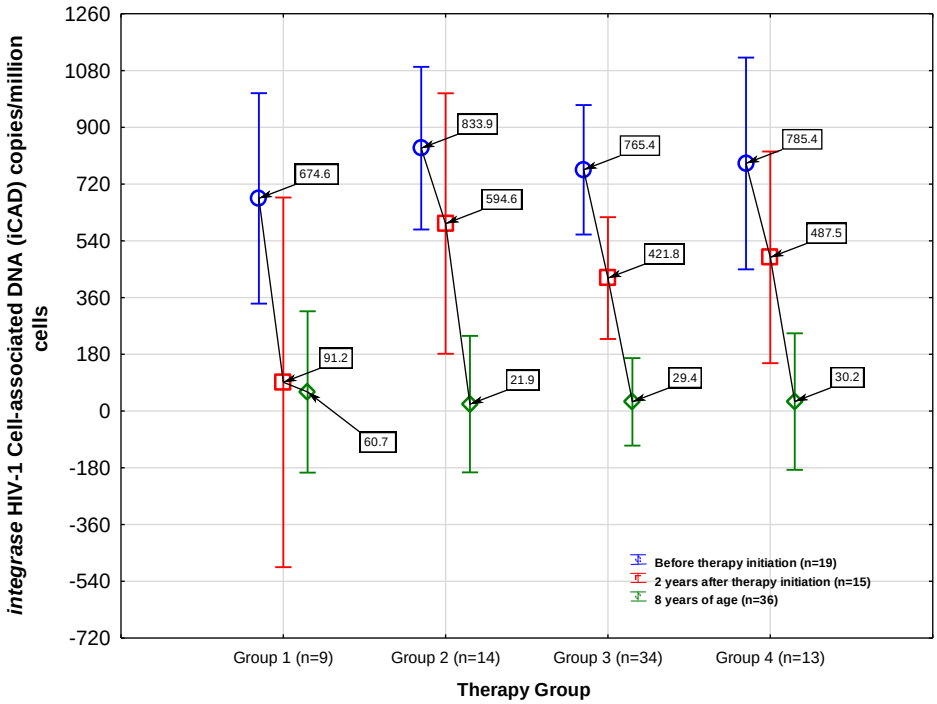


Figure 4.5: *Vertical bar graph denoting 95% confidence intervals and relative mean levels of HIV-1 iCAD (copies/million cells) for each therapy arm (Group 1-4) at three time points; before therapy initiation, 2 years after therapy initiation and 8 years of age.*
Mean levels are indicated on the vertical bar graph. A significant decrease in HIV-1 iCAD levels were noted for all therapy groups over the three time points measured. Statistical summary for Figure 4.5 is listed in Table 4.6 below - a matrix of p-values tested amongst the 12 data set variables.

Table 4.6: p-value Matrix representing the significant differences between the variables measured in Figure 6

			1	2	3	4	5	6	7	8	9	10	11	12
1	Group 1	Before therapy initiation		0.08	**0.00**	0.45	0.76	**0.00**	0.64	0.19	**0.00**	0.64	0.43	**0.00**
2	Group 1	2 years after therapy initiation	0.08		0.92	**0.02**	0.17	0.83	**0.03**	0.29	0.84	**0.04**	0.25	0.85
3	Group 1	8 years of age	**0.00**	0.92		**0.00**	**0.03**	0.82	**0.00**	**0.03**	0.83	**0.00**	0.05	0.86
4	Group 2	Before therapy initiation	0.45	**0.02**	**0.00**		0.32	**0.00**	0.68	**0.01**	**0.00**	0.82	0.11	**0.00**
5	Group 2	2 years after therapy initiation	0.76	0.17	**0.03**	0.32		**0.01**	0.46	0.45	**0.01**	0.48	0.69	**0.02**
6	Group 2	8 years of age	**0.00**	0.83	0.82	**0.00**	**0.01**		**0.00**	**0.01**	0.95	**0.00**	**0.02**	0.96
7	Group 3	Before therapy initiation	0.64	**0.03**	**0.00**	0.68	0.46	**0.00**		**0.02**	**0.00**	0.92	0.16	**0.00**
8	Group 3	2 years after therapy initiation	0.19	0.29	**0.03**	**0.01**	0.45	**0.01**	**0.02**		**0.00**	0.07	0.74	**0.01**
9	Group 3	8 years of age	**0.00**	0.84	0.83	**0.00**	**0.01**	0.95	**0.00**	**0.00**		**0.00**	**0.01**	0.99
10	Group 4	Before therapy initiation	0.64	**0.04**	**0.00**	0.82	0.48	**0.00**	0.92	0.07	**0.00**		0.21	**0.00**
11	Group 4	2 years after therapy initiation	0.43	0.25	0.05	0.11	0.69	**0.02**	0.16	0.74	0.01	0.21		**0.02**

12	Group 4	8 years of age	**0.00**	0.85	0.86	**0.00**	**0.02**	0.96	**0.00**	**0.01**	0.99	**0.00**	**0.02**	

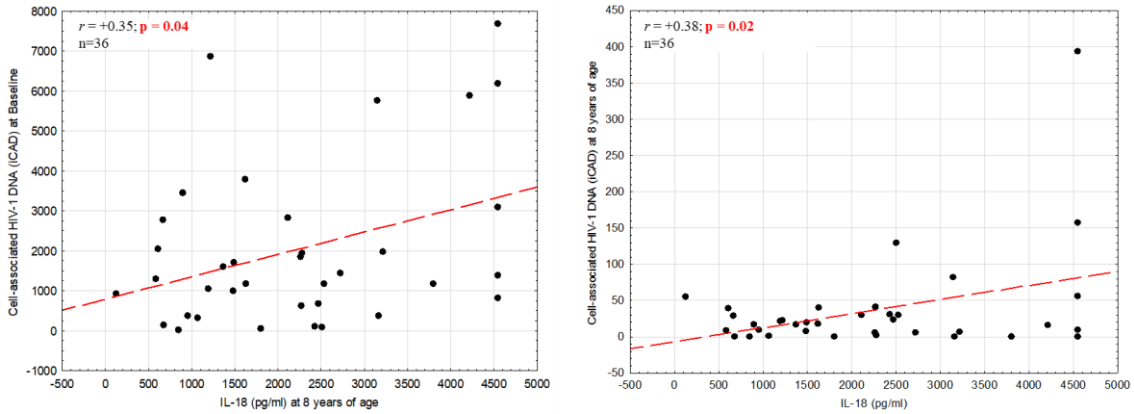


Figure 4.6: *Correlation scatterplots depicting the relationship of iCAD measures at baseline (left) and 8 years of age (right) with IL-18 levels at 8 years of age.*
We observed a significant positive correlation between the two variables, baseline and at 8 years respectively (r = +0.35; p=0.04 and r = +0.38; p=0.02).

4.3.4 IL-18 displayed significant associations with other important immune biomarkers including sCD14, sCD163, IL-1RA, LBP and INF-α

We evaluated the relationship of IL-18 with other immune biomarkers of interest. A total of 39 biomarkers were correlated with each other by use of Pearson Correlation analysis. We noted a significant positive relationship with important biomarkers such as sCD14, sCD163, IL-1RA, LBP, IL-1β, INF-α and IL-1α. A significant negative relationship was observed for TGFβ, RANTES, TNFα, IL-6 and IL-17A. We present this data by use of a correlation ranking analysis plot as depicted in Figure 4.7 below. Statistical correlations (*r* coefficients and p-values) are summarised in Table 4.7 below.

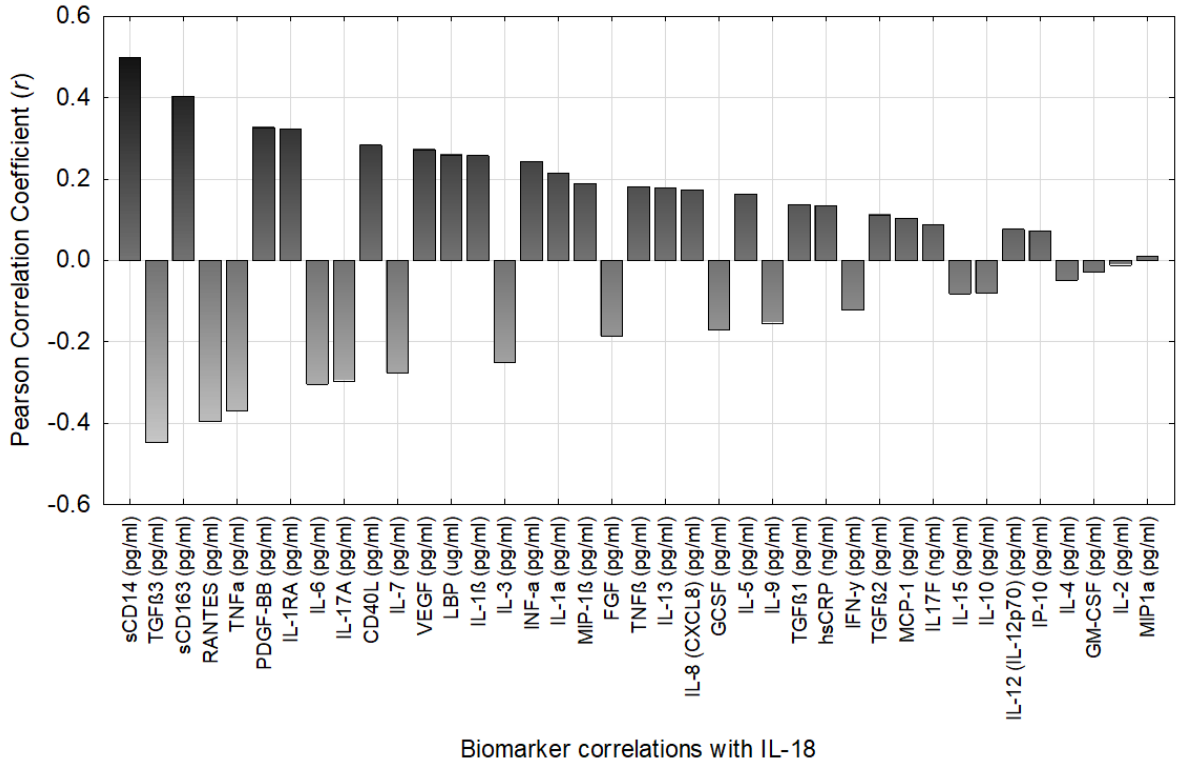


Figure 4.7: *Correlation ranking presentation depicting the relationship of IL-18 with other immune biomarkers. The top half of the correlation ranking plot represents the biomarkers associated with a positive Pearson Coefficient whereas the bottom half of the graphic represents biomarkers that have a negative Pearson Coefficient or correlation with IL-18. Correlated biomarkers are arranged in order of most significant (probable) relationship from left to right.*

Table 4.7 below provides a statistical summary of the correlations of specific biomarkers strongly associated with IL-18.

Table 4.7: Summary of statistical outcomes (*r* coefficients and p-values) of correlations of IL-18 and other immune biomarkers of interest

Biomarker correlated with IL-18	Pearson Coefficient (*r*)	p-value	Number of cases (n)
sCD14	+0.50	**<0.01**	140
TGFβ3	-0.45	**<0.01**	140
sCD163	+0.40	**<0.01**	141
RANTES	-0.40	**<0.01**	141
TNFα	-0.37	**<0.01**	141
PDGFBB	+0.33	**<0.01**	140
IL-1RA	+0.32	**<0.01**	141
IL-6	-0.30	**<0.01**	141
IL-17A	-0.30	**<0.01**	141
CD40L	+0.28	**<0.01**	140
IL-7	-0.28	**<0.01**	141
VEGF	+0.27	**<0.01**	141
LBP	0.26	**<0.01**	135
IL-1β	+0.26	**<0.01**	141
IL-3	-0.25	**<0.01**	140
INF-α	+0.22	**<0.01**	140
IL-1α	+0.22	**<0.01**	140
MIP-1β	+0.19	**0.02**	141
FGF	-0.19	**0.03**	140
TNF-β	+0.18	**0.03**	140
IL-8	+0.17	**0.04**	141

4.3.5 IL-18 show d significant associations with clinical parameters including the CD4 count at baseline, CD4:CD8 ratio at 8 years and absolute and percentage CD8 at 8 years

Following correlation analysis (Pearson and Spearman) with a number of clinical parameters at baseline and 8 years of age, the most significant relationships (possible predictors) of IL-18 levels were CD4, CD8 counts as well as CD4:CD8 ratios at baseline and 8 years of age. Correlation scatter plots are represented in Figure 4.8 below and statistical values (*r* coefficients and p-values) are summarised in Table 4.8.

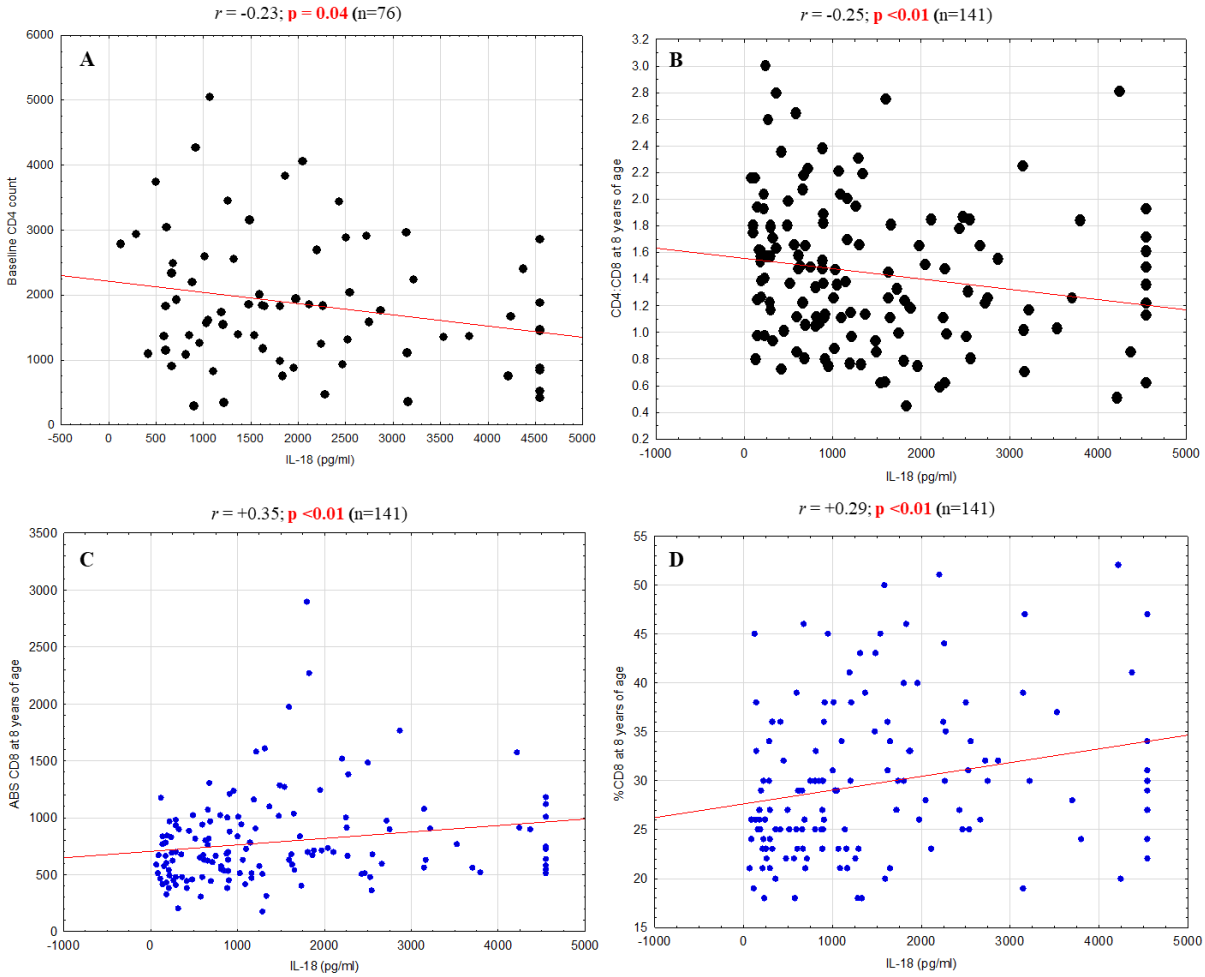


Figure 4.8: *Correlation scatterplots depicting the relationship of IL-18 measures at baseline (left) and clinical parameters.*
We observed a significant negative correlation between IL-18 and the CD4 count at baseline (r = -0.23; p=0.04) as well as the CD4:CD8 ratio at 8 years of age (r = -0.25; p<0.01). A significant positive correlation was observed between IL-18 and CD8 count and percentage at 8 years of age respectively (r =+0.35; p<0.01 and r = +0.29; p<0.01).

Table 4.8: Summary of statistical outcomes (*r* coefficients and p-values) of correlations of IL-18 and clinical parameters

Clinical parameter correlated with IL-18	Pearson Coefficient (*r*)	p-value	Number of cases (n)
Baseline CD4 count	-0.23	**0.04**	76
CD4:CD8 ratio at 8 years	-0.25	**<0.01**	141
CD8 count at 8 years	+0.35	**<0.01**	141
%CD8 at 8 years	+0.29	**<0.01**	141

4.4 Discussion

Understanding HIV-1 pathogenesis and persistence in long-term virologically suppressed HIV-1 infected children as they approach adolescence, is central for the effective clinical management of HIV-1 associated co-morbidities. In addition, describing the key immunological mechanisms with virological interplay could provide important insights to initiating immune therapeutic approaches for targeted cure strategies in HIV-infected children in remission. Furthermore, a deeper understanding of immune biomarkers that contribute to driving HIV-1 pathogenesis such as cytokines and chemokines, can assist in the identification of critical components of the immune system for protective immunity.

In this follow-up sub-study of the CHER cohort, we evaluated a range of cytokines and chemokines and their relevant relationship to HIV-1 pathogenesis and persistence. IL-18 has emerged as a pertinent biomarker displaying relationships with HIV-1 reservoir persistence, inflammatory markers of gut damage and may provide insight into the mechanistic link in these pathways, and to overall immune health in HIV-1 infected children on long-term therapy.

To our knowledge, this is the first study that documents the role of IL-18 and its relationship to total HIV cell-associated DNA, a putative marker of HIV-1 latency, as well other immune and clinical parameters in HIV-1 infected pre-adolescent children in South Africa.

The results presented in this study confirms other reports documenting a significant increase in plasma levels of IL-18 in both paediatric and adult HIV-infected subjects compared to uninfected, healthy controls (Torre et al., 2000; Ahmad et al., 2002; Stylianou et al., 2003; Song et al., 2006; Torre and Pugliese, 2006; Iannello, Boulassel, et al., 2010).

We have demonstrated that IL-18 levels did not reach normality when compared to age and population-matched HIV-1 uninfected controls at 8 years of age. This was in spite of long-term viral suppression, reconstituted CD4 counts, and low HIV-1 cell infectivity. It is also worth noting, that although a decrease in IL-18 levels was observed following ART initiation in all therapy groups, there was a significant increase in levels years later irrespective of therapy group or interruption. Other studies have implicated anti-HIV-1 drugs such as protease inhibitors as inducers of proinflammatory cytokines such as TNF-α, IL-6 and IL-1β (Chen et al., 2009; Iannello, Boulassel, et al., 2010). As IL-18 and IL-1β show similar mechanisms and a significant relationship, it is possible likely that long-term exposure to components of HIV-1 therapy contributed to the significant production of IL-18 from macrophages in our HIV-infected pre-adolescents. Additional studies have shown that nucleoside reverse transcript inhibitors (NRTIs) may also result in oxidative stress leading to the depletion of mitochondrial DNA and defects in the electron transport chain. This kind of stress has been documented to activate the NLRP3 inflammasome (Torres and Lewis, 2014).

Another hypothesis that may be considered is that it may be more related to limited transcription and translation of proviral HIV-1 DNA, allowing intracellular cell stimulation via inflammasome mechanisms. Inflammasome activation is often associated with pyroptosis – death of the host cell which could be linked to gradual HIV-1 CAD decrease in addition.

As mentioned, there are limited studies evaluating cytokine levels and their relationship to HIV-1 persistence in children in South Africa. We have observed significantly elevated levels of plasma IL-18 in our cohort that is more comparable to chronically infected adults in both non-resource limited (Stylianou et al., 2003; Wiercinska-Drapalo et al., 2004; Iannello, Boulassel, et al., 2010) and resource-limited settings (Song et al., 2006; Balagopal et al., 2016). These highly elevated levels observed in our cohort is also comparable to untreated adults (Torre et al., 2000). This is of concern for premature immune ageing and dysfunction for children within our cohort. As all of our participants contracted HIV-1 either *in utero* or at the time of birth, it is important to note the findings of a Chinese study who documented IL-18 levels to be significantly higher in children who contracted HIV-1 through mother-to-child-transmission and children with lower CD4+ T cell

counts compared to adults where CD4+ T cell count did not influence the expression of IL-18 in blood plasma (Jin et al., 2009). Unlike this latter study that did not find a relationship between CD4+ T cell count and IL-18, our observation in children show a significant relationship thus alluding to a different mechanism of pathogenesis in children and adults. Furthermore, another study in adults have shown a positive correlation between plasma IL-18 levels and HIV-1 viral load, but not between the cytokine and CD4+ and CD8+ T cell counts or ratios despite IL-18 concentrations being significantly elevated in late-stage symptomatic patients not receiving therapy (Wiercinska-Drapalo et al., 2004).

In a multi-country case-control study aimed at the continuous evaluation of key cytokines including IL-18 and IFN-γ, before and after the initiation of ART and clinical failure, researchers showed that high concentrations of IL-18 remained strongly associated with clinical failure along with sCD14 after adjusting for age, sex, country, treatment, baseline body mass index (BMI), CD4 count, HIV-1 RNA and Mycobacterium tuberculosis. In a secondary analysis, adjusting for virological suppression, researchers showed that continuous elevation of IL-18 was associated with clinical failure, even during virological suppression following treatment (Balagopal et al., 2016). In our study, IL-18 and sCD14 displayed the most significant positive association when ranked against other cytokines of interest. Research of (Balagopal et al., 2016) also observed that continuous elevation of IFN-γ was associated with a lower likelihood and protection from clinical failure. When observing the latter cytokine within our cohort at the 8-year time point, we observed that IFN-γ was significantly lower in the subjects compared to HIV-uninfected controls – implying good prognostic outcome according to this model. Significant data generated from a multi-country study evaluating IL-18 along with other immune biomarkers recommended that measures of IL-18, sCD14 and IFN-γ be applied for the appropriate identification of ART-naïve persons for whom ART initiation alone may not be adequate to prevent HIV-1 complications (Balagopal et al., 2016).

IL-18 and sCD14 are key markers of monocyte activity. As previously mentioned, the release of IL-18 is the result of stimulation of the inflammasome involved in innate immune signalling. It is important to note that HIV-1 virions are powerful triggers of the inflammasome in circulating monocytes (Iannello, Samarani, et al., 2010; Chattergoon et al., 2014). Monocyte activation and their release of sCD14 have been increasingly documented as a key component of the immune activation phenotype that drives AIDS pathogenesis. In addition, the role of monocytes and macrophages in HIV-1 latency has received much attention.

IL-18 at basal physiological levels in circulation and in tissue rich compartments such as the GIT is important for the maintenance of the intestinal barrier. Whilst basal levels of this cytokine are required for regular functioning of the gut, abnormally higher levels can result in morbidity. Studies have documented a correlation of intestinal permeability and microbial translocation with IL-18 associated immune activation (Allam, Samarani and Ahmad, 2013). A Swedish study showed that HIV-1, along with ART induced the production of IL-18 and inhibited its antagonist, IL-18BP in intestinal cells. Excessive IL-18 levels resulted in intestinal permeability leading to cell death by the activation of caspase-1 and caspase-3 (Allam et al., 2018).

Unpublished data of the cohort we are investigating has observed a large degree of persistent gut damage by noting significantly elevated levels of both direct and indirect biomarkers associated with gut damage. In

addition, we documented that this finding may be a large contributor to chronic inflammation observed within our cohort. LPS initiated gut damage results in an increase in the levels of IL-18. LPS is known to bind to TLR4 resulting in the production of IL-18 via activation of caspase-1 (Yasuda, Nakanishi and Tsutsui, 2019). Studies have documented that upon stimulation with LPS, DCs and macrophages increase the transcription of pro-IL-18 mRNA which is then followed by an elevation in pro-IL-18 levels which is then processed by caspase-1 and leads to the secretion of mature IL-18.

As presented in our results, we show a highly significant association of IL-18 to key immune biomarkers associated with gut damage and macrophage activation. These include sCD14, sCD163, LBP and MIP-1β. Could the evidence of severe gut translocation be fuelling the production of IL-18 in our cohort and in turn leading to low levels of viral replication evidenced by the IL-18 association with total HIV-CAD?

IL-18 has high pleiotropic activity that is dependent on its cytokine environment as reviewed by Yasuda, Nakanishi and Tsutsui, 2019. These include activity on T cells, NKT cells as well as mast cells. Providing additional evidence to IL-18 increase due to microbial translocation we see highly significant and strong associations with IL-4, IL-9, IL-13 and CD40L cytokines which highlight a heightened response to extracellular microbes.

Low-grade replication of the residual virus in sanctuary sites such as the gut can very likely be the main driver of inflammation. This may be partly due to increased IL-18 production and other proinflammatory cytokines resulting in a drive of HIV-1 replication, a defective intestinal barrier, microbial translocation and detrimental chronic low-grade inflammation (Shapiro et al., 1998; Klein et al., 2000; Nowarski et al., 2015). These morbidity outcomes related to low grade chronic inflammation, is likely to lead to immunosenescence, premature aging, cardiovascular disease onset and other metabolic diseases (D'Arminio Monforte, 2004; Villarroya, Domingo and Giralt, 2007; Deeks, 2011).

TGFβ and IL-10 are known to decrease the production of IL-18 in HIV-infected macrophages (Samarani et al., 2016). In our study, we have noted an inverse relationship between IL-18 and TGFβ, however no significant correlation was noted for IL-10. Other adult studies evaluating HIV patients with significantly elevated levels of TGFβ found low production levels of IL-18 from monocytes in peripheral blood (Ahmad et al., 2002).

We have documented a significant association of IL-18 to total HIV-1 iCAD levels at two significant time points, i.e., baseline (before therapy initiation) and at 8 years of age. Despite the rapid decline of HIV-1 CAD to relatively low levels at 8 years of age coupled with viral suppression, a persistently high concentration of IL-18 is noted. This may suggest that undetectable low levels of viral replication may be inducing the release of this proinflammatory cytokine (Torres and Lewis, 2014; Samarani et al., 2016)

It has been documented, that under usual physiological conditions, most of IL-18 in circulation is bound to IL-18BP and is therefore rendered as inactive. The context of HIV-1 infection and more so in chronic infection, an imbalance of the IL-18 antagonist exists. The increase in IL-18 production results in an increase in biological activity that contributes to the pathogenesis of disease (Samarani et al., 2016). This imbalance stimulates HIV infection, induces the expression of CXCR4, results in the attenuation of anti-viral immunity through

dampening of Th1 responses and contributes to inflammation thereby contributing to a the association with HIV-1 CAD (Samarani et al., 2016).

In addition, Song et al., 2006 has noted that IL-18 concentrations in circulation were shown to positively correlate with cell free viral DNA and negatively with CD4+ T cell counts as per our finding. Wiercinska-Drapalo et al., 2004 also observed a positive correlation of IL-18 with HIV-1 viral load. This observation is of special interest, since we know that HIV-1 replication may be stimulated by various host factors, including selected cytokines. HIV-inducing cytokines include TNF-α, TNF-β, IL-1 and IL-6 which stimulate HIV-1 replication in T cells and monocyte derived macrophages.

Wiercinska-Drapalo et al., (2004) showed that excessive production of IL-18 in HIV-1 infected patients with peak values observed in the late stages of disease. According to their study, chronic excessive production of IL-18 may promote viral replication and disease progression in advanced staged HIV-1 infected patients.

Therefore, the monitoring of IL-18 may be helpful during the early stages of HIV-1 infection, with promotion of vigorous T-cell mediated immunity. During the late stages of the disease, especially when IFN-γ and IL-12 production is diminished, Th2 related immune response and persistent viral replication occur (Meyaard et al., 1997; Wiercinska-Drapalo et al., 2004).

We observed a highly significant inverse relationship between IL-18 and IL-6 as well as with IL-18 and TNF-α. This observation supports a previously described hypothesis that IL-6 and TNF-α serve as regulators of IL-18 production (Shapiro et al., 1998; Amirayan-Chevillard et al., 2000).

IL-18 is also known for its role in hypercoagulability and artherogenesis (Badimon, 2012). Data presented at the Conference on Retroviruses and Opportunistic Infections (CROI) in 2017, provided cross-sectional evidence in this particular cohort that strongly suggested increased prevalence of vascular disease in HIV-infected children on ART. Biomarkers, sVCAM and MCP-1 were implicated in the premature arterial-wall stiffness in children of 8 years of age (Innes et al., 2017). In our analysis, we also show a strong positive correlation between IL-18 and MCP-1 as well as signal protein, VEGF. As this finding does not particular provide sufficient evidence of IL-18 and its role in cardiovascular disease in HIV-infected children, it may be a possible biomarker that could provide insight into early disease onset. It is also known that mature forms of IL-18 is expressed in mononuclear phagocytes and that these cytokine receptors are expressed in atheroma-associated vascular endothelial and smooth muscle cells in the atherosclerotic plaques (Badimon, 2012; Samarani et al., 2016).

An important intention of this research is to also understand the interplay between immunological dysfunction and neurological and cognitive outcomes. Considering the pre-adolescent spike of IL-18 in our cohort, it would be appropriate to consider the psychosocial and physiological changes that may occur in response to stressors. Activation and shifts in the hypothalamus pituitary-adrenal (HPA) axis by physical, mental and psychosocial stress lead to the release of adrenocorticotropic hormone (ACTH) which has interestingly been shown to induce the production of IL-18 and cortisol from cells of the adrenal cortex (Sekiyama et al., 2005; Sugama and Conti, 2008; Romeo, 2013). In addition to maintaining the intestinal barrier functions at physiological level, IL-18

also maintains the blood-brain barrier function and has receptors that are expressed in different parts of the brain (Alboni et al., 2010). At higher concentrations of IL-18, these barriers are considered compromised (Samarani et al., 2016) and it increases the susceptibility of the blood-brain barrier to toxic substances that are present in circulation (Alboni et al., 2010). This is of notable importance when considering the role of HIV-1 sanctuary sites, such as the brain and the relating outcomes for neurocognitive development in these children. It would be of significance to correlate IL-18 with neurocognitive outcomes within our cohort of HIV-infected pre-adolescents considering that studies have shown an association between elevated IL-18 levels and cognitive decline (von Giesen et al., 2004; Liu and Chan, 2014).

It is clear that the role of IL-18 in HIV-1 pathogenesis expands through multiple facets resulting in dysregulation and consequent morbidities.

We have described a potential role of a potent proinflammatory cytokine, IL-18 in HIV-1 pathogenesis, however further research is required to understand its mechanism in HIV-1 persistence and onset of clinical failure. IL-18 was only immune biomarker of a whole range of markers to associate significantly with iCAD at two time points. This needs further investigation in future. It would also be of value to measure the level and functionality of IL-18BP. The IL-18 antagonist is produced constitutively as well as in response to the increase in IL-18 levels. It serves as a negative feedback mechanism for control of inflammation. The imbalance in the production of IL-18 and its antagonist has been described in many chronic inflammatory diseases in humans (Samarani et al., 2016).

Therapeutic options and challenges for correcting this imbalance have been previously explored. Aiming to attenuate chronic inflammation in HIV-infected children at a relatively young age, would prove beneficial for overall health and quality of life. As IL-18 is a reasonably potent driver of inflammation, aiming to decrease its effects may be a rational therapeutic option. Plasma levels of IL-18 may present a necessary proxy biomarker for inflammation as well as for overall immune activation status and in addition allow for the sifting of selecting appropriate participants for remission studies.

4.5 Conclusion

Within our investigation into understanding immune mechanisms related to HIV-1 persistence in South African children, we observed the potential of IL-18 to provide insights into immune dysfunction, regulation and having an association to HIV-1 cell infectivity. This therefore suggests that IL-18 may be used as a marker to measure treatment success as well as overall immune health. Measuring IL-18 in plasma can also be used as a proxy for deviant immune activation that is considered as a correlate of clinical progression in HIV-1 infection.

We identify IL-18 and other immune biomarkers such as sCD14, sCD163, IL-1RA, LBP and INF-α as a possible biosignature to predict early clinical outcomes as well as HIV-1 persistence outcomes. This biomarker signature could also provide an indication of which patients will require added vigilance after ART initiation.

CHAPTER 5:

LONGITUDINAL IMPACT OF THERAPY REGIMEN AND CLINICAL PARAMETERS ON THE EXPRESSION PATTERNS OF SOLUBLE IMMUNE BIOMARKERS IN PERINATALLY HIV-INFECTED CHILDREN IN SOUTH AFRICA

SUMMARY OF KEY FINDINGS – CHAPTER 5

- Therapy regimen (delayed, early or interrupted) had minimal impact on the longitudinal expression patters formost soluble immune biomarkers measured.
- Significant differences in biomarker expression patterns were observed for proinflammatory biomarkers: **IL-17F, IL-2, IL-4, IL-7, IL-13, IFN-γ, TNFβ, CD40L, IFNα, PDGF, TGFβ$_3$ and LBP** specifically for HIV+ children subjected to early continuous therapy (Group 2) – this may primarily be due to early clinical and virological factors such as lower birthweights, gestation, lower CD4 counts, and higher HIV-1 cell infectivity at birth.
- Therapy interruption at either 40 or 96 weeks had minimal impact on the longitudinal expression of key biomarkers including **IL-1RA, IL-1α, IL-6, IP-10, MCP-1, MIP-1α, MIP-1β, TNFα, RANTES, sCD14, sCD163, IL-18 and LBP.**
- Following therapy initiation, minimal to no impact was observed for specific biomarkers: **IL-1α, MIP-1α, MIP-1β, CD40L, sCD14, sCD163 and IL-18** followed by a spike at 8 years of age for the same biomarkers despite viral suppression and CD4 T cell reconstitution.
- Levels of **IL-3, MCP-1, TNFα, hsCRP and LBP** remained the same for each time point measured i.e., before therapy initiation, 2 years after therapy and at 8 years of age across all therapy groups.
- Following 2 years of therapy initiation, **IL-1RA, IL-5, IL-6, IL-8 and TNFβ** significantly increased and then decreased to baseline levels by 8 years of age.
- **IL-4, IL-10, IP-10 and RANTES** declined consistently for each timepoint measured.
- Principal component analysis indicated that about 36% of the soluble biomarkers measure including: **IL-15, TNFβ, GCSF, IL-1RA, IL-5, IL-4, IL-8, IL-10 and RANTES** contributed to the largest parameter change across all treatment groups. Before therapy initiation, the key clinical predictors of plasma biomarker expression include **(1) time to therapy initiation, (2) time to viral suppression, (3) longitudinal CD4% and absolute count and (4) CD4 and CD8% and absolute counts at birth.**
- ***Conclusion:*** *Extensive predictive analysis of biomarkers over time indicates a complex interplay between cytokines, chemokines, growth factors and therapy regimen that shape the landscape for disease progression and would serve pertinent when understanding the course of HIV-1 persistence over time.*

ABSTRACT

Background: The immunological impact of HIV-1 is said to be either inhibitory, stimulatory, or bifunctional. This implies that HIV-1 pathogenesis (infection and replication) is under the continuous regulation of a complex cytokine network or immune modulation. The dysregulated expression of a number of soluble immune modulators contributes to the landscape of immunological abnormalities in both adult and paediatric populations further influencing comorbidities in HIV-1 infection. Changes in the levels of these immune modulators can be influenced by treatment, clinical parameters, virological outcomes and even by each other. The longitudinal cytokine and chemokine patterns in PHIV children from birth to pre-adolescent years is yet to be delineated.

Methods: A longitudinal study design was employed to assess specific plasma immune biomarkers across four therapy groups i.e., Group 1 (delayed therapy), Group 2 (early continuous therapy), Group 3 (early therapy interrupted at 40 weeks) and Group 5 (early therapy interrupted at 90 weeks). An extensive panel of 40 plasma immune biomarkers were measured by Luminex® Multiplex Assay and ELISAs across three specific key time points, i.e., before therapy initiation, 2 years after therapy initiation and at 8 years of age. This study is primarily focused on the PHIV children from the CHER trial. No early age and community matched controls could be employed for this study and therefore serves as a significant limitation. A subset (n=32) of PHIV children was used to quantify cell-associated HIV-1 DNA (CAD) at various time points including measurements done in parallel to immunological time points.

Results: Significant differences in biomarker expression patterns were observed for proinflammatory biomarkers: IL-17F, IL-2, IL-4, IL-7, IL-13, IFN-γ, TNFβ, CD40L, IFNα, PDGF, $TGF\beta_3$ and LBP specifically for HIV+ children subjected to early continuous therapy (Group 2) – this may primarily be due to their early clinical and virological factors such as lower birthweights, gestation, lower CD4 counts, and higher HIV-1 cell infectivity at birth. Following 2 years of therapy initiation, IL-1RA, IL-5, IL-6, IL-8 and TNFβ significantly increased and then decreased to baseline levels by 8 years of age. IL-4, IL-10, IP-10 and RANTES declined consistently for each timepoint measured. Principal component analysis indicated that about 36% of the soluble biomarkers measure including: IL-15, TNFβ, GCSF, IL-1RA, IL-5, IL-4, IL-8, IL-10 and RANTES contributed to the largest parameter change over time across all treatment groups. The key early clinical predictors of plasma biomarker expression include (1) time to therapy initiation, (2) time to viral suppression, (3) longitudinal CD4% and absolute count and (4) CD4 and CD8% and absolute counts at birth.

Conclusion: A strong interplay exists between various immune modulators (cytokine and chemokines) in both innate and adaptive immune compartments that shapes the landscape for disease progression. This interplay is further influenced by the early clinical milieu and virological outcomes. Extensive composite cytokine and chemokine data is useful for assessing associations with parameters such as iCAD and other immune subsets

5.1 Introduction

In Chapter 3 we evaluated immunological biomarkers cross-sectionally at a pre-adolescent age of 8 years providing strong evidence of chronic inflammation driven by monocyte/macrophage and DC compartments. We have shown in the preceding Chapter, Chapter 4, that IL-18 showed associations with HIV-1 cell infectivity and displayed an interplay with other immune biomarkers providing valuable insight into the potential of IL-18 in providing a snapshot of overall immune health.

The next step was to evaluate longitudinal cytokine changes within PHIV children in order to understand if distinct patterns or certain immune markers rise or decrease overtime.

It is well-known that HIV-1 infection triggers immune activation which can persist even under suppressive cART. Persisting immune activation is considered one of the significant challenging facets of HIV-1 pathogenesis of which the causes and mechanisms are not well known (Ambrosioni et al., 2014; Archin et al., 2014; Garbelli et al., 2017; Mercurio et al., 2021). A number of contributing factors have been hypothesised to be contributory to this residual immune activation and includes general immune dysfunction (Wada et al., 2015; Brites-Alves et al., 2018), microbial translocation (Marchetti, Tincati and Silvestri, 2013b; De Voeght et al., 2017; Ortiz et al., 2019), persisting opportunistic infections (Achappa, Unnikrishnan and Venugopal, 2012; Beltrán et al., 2015), low-level HIV-1 viral replication and the presence of viral proteins in various physiological compartments (Edén et al., 2010; Hladnik et al., 2017; Collini et al., 2018; Henderson et al., 2019; Imamichi et al., 2020; Mercurio et al., 2021). Furthermore, long-term cART has also been implicated in inducing a proinflammatory environment (Mercurio et al., 2021).

Soluble immune biomarkers inclusive of cytokines, chemokines and growth factors, are diverse and pleiotropic in nature and play an important role in maintaining the homeostasis of the immune system. Soluble immune biomarkers attracted much attention as diagnostic biomarkers for infectious and inflammatory diseases in recent years (Hamidzada and Guzzo, 2019).

The degree of T cell activation and monocyte or macrophage differentiation determines the level of susceptibility of cells to HIV-1 infection. It is therefore important to highlight the role of cytokines, chemokines and immune growth factors that contribute to facilitating the change in activation and differentiation of the cells of the immune system under HIV-1 infection. Furthermore, the function of these immune molecules suggests its pertinent role in modulating both HIV-1 infection and replication (Zack et al., 1990; Sonza et al., 1996).

The immunological impact of HIV-1 is said to be either inhibitory, stimulatory, or bifunctional (both inhibitory and stimulatory). This implies that HIV-1 pathogenesis (infection and replication) is under the continuous regulation of a complex cytokine network produced by a variety of cells (Kedzierska and Crowe, 2001; Hamidzada and Guzzo, 2019).

The dysregulated expression of a number of soluble immune modulators contributes to the landscape of immunological abnormalities in both adult and paediatric populations further influencing comorbidities in HIV-1 infection (Osuji et al., 2018).

HIV-associated immune deficiency leads to a progressive decline in the number of CD4+ T lymphocytes resulting in defective immunological functions in both the T cell and macrophage components of the immune system. In addition, there is major dysregulation in the production of related cytokines and chemokines (Biggs et al., 1995; Kedzierska et al., 2000; Kedzierska and Crowe, 2001). A number of studies have acknowledged the importance of these soluble immune biomarkers in shaping the immune response during the progression of HIV-1 infection, several of which play an important role in HIV-1 control (Hamidzada and Guzzo, 2019). Research studies into HIV-1 progressors and non-progressors highlight significant differences in cytokine production and function between these two groups and therefore suggest an important role of these immune biomarkers towards viral control (Reuter, Pombo and Betts, 2012).

The most frequently investigated cytokines in both the HIV-1 and uninfected groups includes IL-6, IL-10, IFN-γ and TNF-α (Hamidzada and Guzzo, 2019). The latter cytokines are known to play a key role in infectious and inflammatory pathways and have the potential of being included as diagnostic targets and drug targets (Decker, Grobusch and Ritz, 2017). In more recent investigations, other soluble immune biomarkers have surfaced as pertinent role players in HIV-1 pathogenesis. These include IL-18, soluble CD163 and soluble CD14.

Cytokines such as TNF-α, TNF-β, IL-1 and IL-6 have been documented to play a role in stimulating HIV-1 replication in T cells and macrophages. IL-2, IL-7 and IL-15 have functions in upregulating HIV-1 in T cells and IL-10, IL-13, IFN-α and IFN-β play a role in inhibiting HIV-1 replication in T cells and monocyte derived macrophages. IL-4, IL-6, IFN-γ and granulocyte-macrophage colony-stimulating factor (GMCSF), display both an inhibitory and stimulatory effect on HIV-1 (Kedzierska et al., 2000; Kedzierska and Crowe, 2001).

The key immune molecules involved in inhibiting HIV-1 replication in macrophages include those within the β-chemokine groups whereas α-chemokines play a role in suppressing infection of T-tropic strains of HIV-1. Examples of these chemokines include CXCR4, MIP-1α, MIP-1β and RANTES (Mackewicz et al., 1997; Kedzierska et al., 2000; Kedzierska and Crowe, 2001).

Knowledge of the interplay of these soluble immune biomarkers provides insight into the complexity of the immune system and can therefore provide an understanding of its response to HIV-1 infection as well as the effects of treatment regimes. In addition, evaluating these complex networks can also provide an overview of the immune response to other persisting co-morbidities such as early gut damage.

The long-term predictive and pathophysiological role of soluble immune biomarkers in HIV infection particularly within perinatally infected children from birth to pre-adolescence, remains unclear. These immunological profiles can provide key insights into either clinical improvement of disease or progression leading to morbidities that require clinical surveillance and follow-up.

On the other hand, the efficiency of ART may be influenced by the imbalance of pro- and anti-inflammatory soluble immune biomarkers which contribute to regulating the immune response. Therefore, evaluating the levels of these cytokines may provide a good picture of the rate of viral replication and the resultant damage

or restoration of the immune system during the implementation of ART regimens including the delay and interruption of therapy (Gaardbo et al., 2012; Osuji et al., 2018).

By fully identifying and characterising the longitudinal pleiotropic role of immune biomarkers in relation to clinical outcomes in HIV infected children, we can provide critical information to understanding disease progression as well as HIV control or replication. Furthermore, this information provides insights into how therapeutic targets may be structured.

Extensive evaluation of the cytokine profile in children with HIV-1 infection is limited (Foldi et al., 2017; Munhoz et al., 2019). The aim of this study was to extensively investigate and profile the impact of both primary and chronic HIV-1 infection in children from birth to 8 years of age with regards to the inflammatory and regulatory milieu of cytokines, chemokines, and growth factors. In addition, we aimed to evaluate the impact of early, delayed, and interrupted ART on restoring a healthy immune profile by pre-adolescent age.

Furthermore, this research also aimed to know whether monitoring the production of soluble immune biomarkers along with CD4+ T cell counts, and viral load could provide additional beneficial information in children with HIV-1 infection on ART. We attempted to identify immune biomarker profiles that are specific to either clinical improvement or disease progression, so that immunological manipulation towards an advantageous profile might be endeavoured.

Research hypothesis: Cytokine patterns change over the course of HIV-1 infection and is dependant on early clinical, virological and immune status at start of infection. This pattern is different in PHIV children compared to HIV-1 infected adults.

5.2 Materials and Methods

5.2.1 Study design

A longitudinal study design was employed to assess specific plasma immune biomarkers across four therapy groups i.e., Groups 1-4 as previously described. An extensive panel of plasma immune biomarkers were measured across three specific key time points, i.e., before therapy initiation, 2 years after therapy initiation and at 8 years of age. These time points were selected based the availability of samples as well as for the design of further predictive analysis of biomarkers with clinical, neurological, and virological parameters.

This study is primarily focused on the PHIV children from CHER trial. No early age and community matched controls could be employed for this study and therefore serves as a significant limitation.

Lastly, a subset (n=32) of PHIV children was used to quantify cell-associated HIV-1 DNA (CAD) at various time points including measurements done in parallel to immunological time points.

5.2.2 Study participants

Study participants originated from the CHER trial as previously described (Chapter 3)

5.2.3 Routine testing

HIV-1 viral load

Viral load testing was measured within an accredited routine laboratory, the National Health Laboratory Service (NHLS), Tygerberg Cape Town, South Africa as previously described.

Lymphocyte count

Routine lymphocyte analyses (CD4 and CD8 counts, percentages, and CD4:CD8 ratios) were routinely completed for all study participants including control samples at each follow-up visit as previously described.

5.2.4 Blood plasma immune biomarker measurements

An extensive panel of 40 plasma biomarkers as previously listed in Chapter 3 (excluding I-FABP, MAdCAM-1, s100A8/A9) was evaluated using ELISA and Luminex® Multiplex Assays.

5.2.5 Assessment of integrated cell-associated HIV DNA (iCAD)

A sensitive quantitative PCR adapted for HIV-1 subtype C was implemented for the measurement of HIV-1 total DNA as described in Chapter 4 (van Zyl et al., 2015; Hong et al., 2016; Veldsman et al., 2018).

5.2.6 Statistical Analysis

Quantitative variables measured for each of the groups were analysed using TIBC® Statistica™ version 13.3 and GraphPad Prism version 8 software. Data was subjected to normality testing (Shapiro-Wilke's normality test). All values outside of the normal range, a rejected null hypothesis of normally distributed data, justified the use of non-parametric tests for statistical analysis. No outliers were excluded in the analysis. Extreme values were transformed by use of winsorisation or log transformation of statistics to limit the effect of spurious outliers and retain the power of sampling size.

A non-parametric one-way analysis of variance (ANOVA) Kruskal-Wallis test was implemented for the comparison of the three study groups which was followed by a post-hoc Fisher's Least Significant Difference (LSD) to identify and verify the significant differences between the groups. Correlations between biomarkers, clinical parameters, and laboratory measurements (plasma biomarkers, cellular markers and iCAD measurements) were performed by Spearman's correlation rank test (verified additionally with a Pearson's correlation rank test) and linear regression analysis. *P* values less than 0.05 were considered statistically significant.

5.3 Results

5.3.1 Sampling Outline

For this descriptive study, a total of 74 HIV+ CHER children were longitudinally evaluated for an extensive analysis of soluble immune biomarkers over three time points 1) before therapy initiation, 2) two years after therapy initiation and 3) at 8 years of age.

Clinical birth and follow-up parameters including HIV-1 viral load and CD4+ and CD8+ T cell counts were collected and recorded longitudinally. In addition, a subset (n=32) of HIV+ participants were evaluated for HIV-1 CAD levels.

HIV+ study participants were evaluated based on four therapy groupings as described previously and adapted from the CHER randomised control trial.

Figure 5.1 below presents a summary flow diagram of the sampling process for this study.

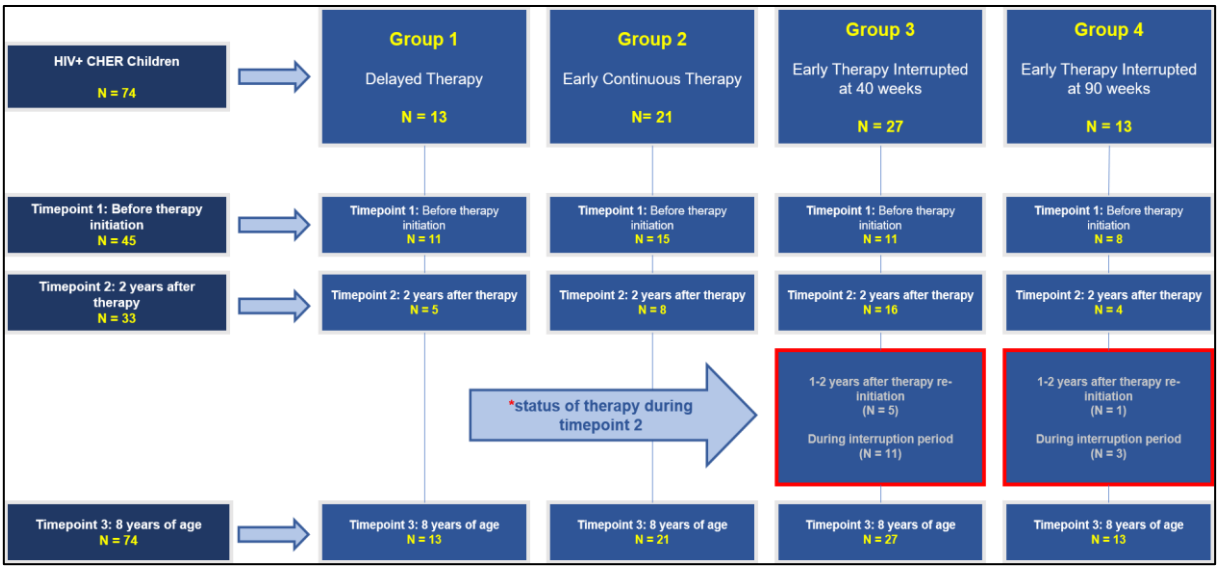


Figure 5.1: *Summary of participant sampling process for longitudinal soluble immune biomarker study. A total of 74 HIV+ CHER participants characterised into four therapy groups, Group 1 (n = 13), Group 2 (n = 21), Group 3 (n = 27) and Group 4 (n = 13) were measured at three time points (before therapy initiation, two years after therapy initiation and 8 years of age). Groups 3 and 4 were subjected to therapy interruption at 40 and 90 weeks of which 69% and 75% of these samples were measured at the second time point were during the interruption period.*

5.3.2 Demographic and clinical parameters of study cohort for longitudinal follow-up of soluble immune biomarkers

Table 5.1 provides an overview of the demographic and clinical parameters measured at baseline (before therapy initiation) and at 8 years of age, the last pre-adolescent time point evaluated for this research component.

Table 5.1: Summary of demographic, clinical, virological, and immunological parameters of study cohort (Groups 1 – 4)

	Group 1	**Group 2**	**Group 3**	**Group 4**
	Delayed Therapy	**Early Continuous Therapy**	**Early Therapy Interrupted at 40 weeks**	**Early Therapy Interrupted at 90 weeks**
n	**13**	**21**	**27**	**13**
Demographic and Birth Parameters				
Male, sex (n) (%)	6 (46.2%)	11 (52.4%)	11 (40.7%)	3 (23.1%)
Gestation (weeks), median; (range)	40 weeks (37.0 - 42.0 weeks)	40 weeks (30.0 - 41.0 weeks)	40 weeks (36.0 - 42.0 weeks)	40 weeks (32.0 - 40.0 weeks)
Birth weight (grams), median; (range)	3,227.5 g (2,640.0 - 3,640.0g)	2,850.0 g (2,000.0 - 3,780.0g)	3,160.0 g (2,400.0 - 4,200.0g)	3,000 g (2,188.0 - 3,500.0g)
Birth height (centimetres), median; (range)	34.0 cm (32.0 - 37.0 cm)	33.0 cm (30.0 - 38.5 cm)	34.0 cm (31.0 - 37.5 cm)	33.0 cm (30.5 - 36.0 cm)
Baseline HIV and Immunological Parameters				
Pre-treatment log10 HIV-1 RNA load, median; (range)	5.8 (5.0 - 6.0)	6.0 (5.0 - 6.0)	5.9 (3.8 - 5.9)	5.9 (4.2 - 5.9)
Time to therapy initiation (weeks), median; (range)	9.0 weeks (6.0 -18.0 weeks)	2.0 weeks (2.0 - 5.0 weeks)	2.0 weeks (1.0 - 3.0 weeks)	2.0 weeks (2.0 - 3.0 weeks)
Time to viral suppression (days), median; (range)	181.0 days (1.0 – 839.0 days)	168.0 days (161.0 – 505.0 days)	280.0 days (164.0 – 1,455.0 days)	169.0 days (167.0 – 1,404.0 days)
CD4+ cell count at birth (cells/microliter), median; (range)	1,759.0 cells/µl (1,150 - 2,731.0 cells/µl)	1,480.0 cells/µl (146.0 – 3,828.0 cells/µl)	1,573.0 cells/µl (294.0 – 5,044.0 cells/µl)	1,851.0 cells/µl (829.0 – 4,272.0 cells/µl)
CD4+ percent at birth, median; (range)	36.6 % (25.4 – 52.8 %)	28.5 % (17.1 – 53.9 %)	36.7 % (11.2 – 50.0 %)	36.0 % (27.9 – 57.1 %)
CD8+ cell count at birth (cells/microliter), median; (range)	830.5 cells/µl (236.0 – 982.0 cells/µl)	819.0 cells/µl (278.0 – 947.0 cells/µl)	858.0 cells/µl (522.0 – 999.0 cells/µl)	**Data not available*
CD8+ percent at birth, median; (range)	20.8 % (14.8 – 39.3 %)	25.8 % (11.2 – 41.8 %)	20.8 % (11.5 – 43.6 %)	**Data not available*
Interruption time (days), median; (range)	*N/A*	*N/A*	208.5 days (40.0 – 2,786 days)	318.0 days (133.0 – 2,457 days)
8-year Follow-up parameters				
CD4+ cell count at 8 years (cells/microliter), median; (range)	930.0 cells/µl (568.0 - 2,731.0 cells/µl)	1,015.0 cells/µl (323.0 - 2,277.0 cells/µl)	979.0 cells/µl (446.0 - 2,063.0 cells/µl)	929.0 cells/µl (563.0 – 1,438.0 cells/µl)
CD4+ percent at 8 years, median; (range)	38.0 % (31.0 – 54.0 %)	37.0 % (21.0 – 49.0 %)	41.0 % (28.0 – 51.0 %)	37.0 % (27.0 – 47.0 %)
CD8+ cell count at 8 years (cells/microliter), median; (range)	894.0 cells/µl (472.0 – 1,767.0 cells/µl)	1,000.0 cells/µl (357.0 – 2,894.0 cells/µl)	724.0 cells/µl (474.0 – 1,416.0 cells/µl)	1,116.0 cells/µl (303.0 – 1,605.0 cells/µl)
CD8 percent, median (IQR) CD8+ percent at 8 years, median; (range)	30.0 % (22.0 – 41.0 %)	34.0 % (22.0 – 53.0 %)	30.0 % (22.0 – 47.0 %)	39.0 % (18.0 – 51.0 %)
CD4/CD8 ratio at 8 years, median; (range)	1.23 (0.77 – 1.99)	1.17 (0.45 – 2.23)	1.31 (0.62 – 2.21)	0.97 (0.59 – 2.65)
HIV-1 RNA Viral Load at 8 years (copies/ml); % Undetected	**85%** (11 Undetected) 1 (<40 copies/ml) 1 (42 copies/ml)	**81%** (17 Undetected) 2 (<40 copies/ml) 2 (>100 copies/ml)	**78%** (21 Undetected) 1 (<40 copies/ml) 3 (>500 copies/ml) 1 (19, 549 copies/ml) 1 (60 copies/ml)	**92%** (12 Undetected) 1 (26, 576 copies/ml)

***Lymphocyte reference ranges**

Reference: (Lawrie *et al.*, 2015)			
Age range	**0-3 months**	**1-2 years**	**6-12 years**
CD4 T lymphocytes (cells/µL)	1520-5160	1374-3928	568-2013
CD8 T lymphocytes (cells/µL)	428-2478	669-3247	340-121-
CD4:CD ratio	1.29-5.29	0.78-3.88	0.95-2.47
Reference: (Payne *et al.*, 2020)			
Age range	**1-2 months**	**15-24 months**	**5-10 years**
CD8 (%)	10.7-28.9	13.8-34.9	15.1-34.8
Reference: (Shearer *et al.*, 2003)			
Age range	**0-3 months**	**1-2 years**	**6-12 years**
CD4 (%)	35-64	32-51	31-47

5.3.3 Longitudinal CD4 percentage pattern from baseline (before therapy initiation) to 8 years of age

Figure 5.2 below displays the longitudinal pattern for CD4+ T cell percentages in each of the four therapy groups over the three time points measured. Significant differences exist between the four groups as well as over time ($p<0.01$). Whilst we observed that Groups 1 and 2 show similar patterns of CD4+ T cell percentage increase over time, the patterns are seemingly different for the two interrupted groups where a lag in CD4+ T cell percentage reconstitution is observed. However, at the 8-year time point, all therapy groups have CD4 percentage above 35% with Group 3 displaying the highest percentage of 41%.

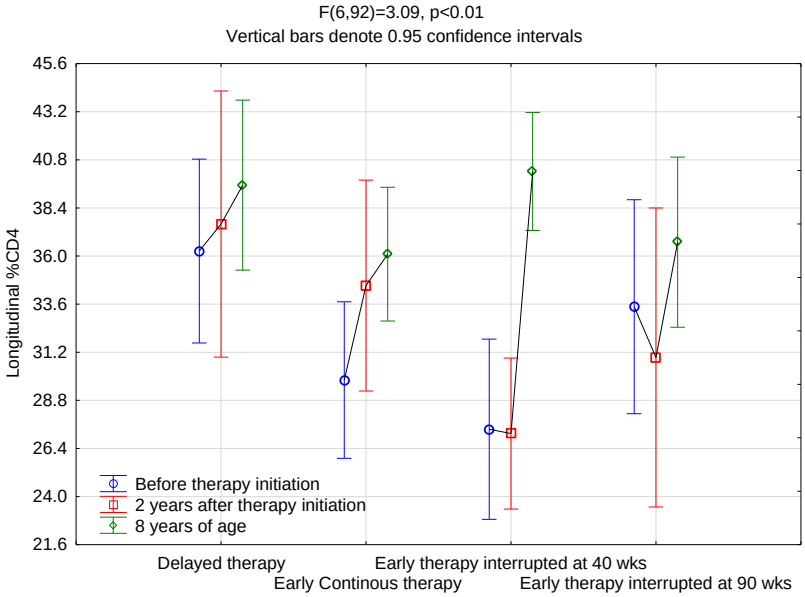


Figure 5.2: *Vertical bar graphs with 95% confidence intervals depicting the mean longitudinal CD4+T cell percentage across three measured time points for each study group.*
The overall interaction between the groups over time depicts significant differences ($F=3.09$; $p<0.01$). Groups 1 and 2 showed a steady increase in CD4 percentage T cell levels whilst the two interrupted groups (Group 3 and 4) displayed a noticeable lag in CD4 percentage increase following therapy initiation (note: this may be due to the interruption period which some children were subjected to in this time point).

The statistical probabilities between each therapy group at the relative time point status is summarised in Table 5.2.

5.3.4 Longitudinal HIV-1 iCAD measurements show significant decline over time for each study group evaluated.

Longitudinal HIV-1 CAD measurements were completed for a subset of HIV+ participants depending on the availability of samples. A total of 36 samples were evaluated across the four study groups in this research study component. Before therapy was initiated, children in Group 2 (subjected to early continuous therapy) presented with the highest levels of HIV-1 CAD (median = 3,795.1 copies/10^6 PBMCs) compared to the other three study groups. Group 1 (subjected to delayed therapy) displayed the lowest levels of HIV-1 CAD (median = 99.2 copies/10^6 PBMCs) before therapy was initiated compared to the other study groups.

All groups presented with a significant decrease in HIV-1 CAD levels over time and at 8 years of age all study groups displayed median levels well below 25 copies/10^6 PBMCs.

Table 5.2: Summary of statistical probabilities for Post Hoc tests implemented for % CD4 measurements

Probabilities for Post Hoc Tests (CD4 %) – Figure 5.2													
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.72	0.23	**0.04**	0.63	0.96	**0.01**	**0.00**	0.15	0.44	0.23	0.89
2	Delayed therapy	2 years after therapy initiation	0.72	-	0.60	0.05	0.47	0.69	**0.01**	**0.01**	0.48	0.34	0.19	0.82
3	Delayed therapy	8 years of age	0.23	0.60	-	**0.00**	0.14	0.21	**0.00**	**0.00**	0.79	0.08	0.05	0.35
4	Early Continuous therapy	Before therapy initiation	**0.04**	0.05	**0.00**	-	0.13	**0.01**	0.42	0.33	**0.00**	0.28	0.79	**0.02**
5	Early Continuous therapy	2 years after therapy initiation	0.63	0.47	0.14	0.13	-	0.59	**0.04**	**0.03**	0.06	0.78	0.44	0.53
6	Early Continuous therapy	8 years of age	0.96	0.69	0.21	**0.01**	0.59	-	**0.00**	**0.00**	0.07	0.41	0.21	0.83
7	Early therapy interrupted at 40 wks.	Before therapy initiation	**0.01**	**0.01**	**0.00**	0.42	**0.04**	**0.00**	-	0.94	**0.00**	0.09	0.42	**0.00**
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	**0.00**	**0.01**	**0.00**	0.33	**0.03**	**0.00**	0.94	-	**0.00**	0.06	0.37	**0.00**
9	Early therapy interrupted at 40 wks.	8 years of age	0.15	0.48	0.79	**0.00**	0.06	0.07	**0.00**	**0.00**	-	**0.03**	**0.02**	0.18
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.44	0.34	0.08	0.28	0.78	0.41	0.09	0.06	**0.03**	-	0.57	0.29
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.23	0.19	0.05	0.79	0.44	0.21	0.42	0.37	**0.02**	0.57	-	0.16
12	Early therapy interrupted at 90 wks.	8 years of age	0.89	0.82	0.35	**0.02**	0.53	0.83	**0.00**	**0.00**	0.18	0.29	0.16	-

HIV-1 CAD kinetic studies for the same cohort have recently been published. In summary, kinetic studies concluded that following six months of continuous suppressive therapy, initiated within the first few weeks of life, HIV-1 CAD decreased significantly over time compared to groups who initiated therapy later.

Furthermore, following multivariate analyses, HIV-1 CAD and the change in its levels during interruption were observed to be independent predictors of slower HIV-1 DNA decay (Veldsman et al., 2019).

Table 5.3 below provides a summary of the median HIV-1 CAD levels including ranges measured over five consecutive time points. Three of these time points were also analysed in parallel measurements of soluble immune biomarkers measured over time to evaluate a possible relationship between virological and immunological parameters in our cohort.

Figure 5.3 below displays the longitudinal pattern for HIV-1 CAD levels in each of the four therapy groups over the three time points measured. Although the general pattern of a continuous decrease in HIV-1 CAD over time can be observed across all four therapy groups, significant differences exist at various observation points between the four groups. The probabilities for each of the Post Hoc tests implemented are summarised in Table 5.4.

Table 5.3: Summary of median values and ranges of HIV-1 CAD levels (copies/10^6 PBMCs) for therapy Groups 1 to 4 at five measured time points (before therapy initiation, 1, 2 and 3 years after therapy initiation and 8 years of age)

	Before Therapy Initiation	**1 year after therapy initiation**	**2 years after therapy initiation**	**3 years after therapy initiation**	**8 years of age**
Group 1: Delayed Therapy (N = 5)					
n	**3**	**3**	**1**	**2**	**5**
HIV-1 CAD Median (copies/10^6 PBMCs)	99.2	245.5	6.7	122.9	21.2
HIV-1 CAD Range (copies/10^6 PBMCs)	13.2 – 100,080.8	137.1 – 628.5	N/A	0.2 – 245.5	0.4 – 247.6
Group 2: Early Continuous Therapy (N = 7)					
n	**7**	**2**	**2**	**3**	**7**
HIV-1 CAD Median (copies/10^6 PBMCs)	3,795.1	41.9	609.6	16.5	17.8
HIV-1 CAD Range (copies/10^6 PBMCs)	48.0 – 5,884.0	0.0 – 83.8	333.9 – 885.3	0.0 – 171.0	0.0 – 40.1
Group 3: Early Therapy Interrupted at 40 weeks (N= 17)					
n	**8**	**7**	**9**	**8**	**17**
HIV-1 CAD Median (copies/10^6 PBMCs)	1,150.9	324.5	300.0	122.7	23.2
HIV-1 CAD Range (copies/10^6 PBMCs)	146.7 – 6,180.7	41.9 – 1,446.6	0.0 – 2,514.8	0.0 – 423.6	0.0 – 109.0
Group 4: Early Therapy Interrupted at 90 weeks (N = 7)					
n	**3**	**1**	**3**	**5**	**7**
HIV-1 CAD Median (copies/10^6 PBMCs)	1,384.2	1, 232.3	261.0	178.7	9.4
HIV-1 CAD Range (copies/10^6 PBMCs)	289.1 – 2,819.2	N/A	132.4 – 1,299.2	76.5 – 542.9	5.5 – 129.6

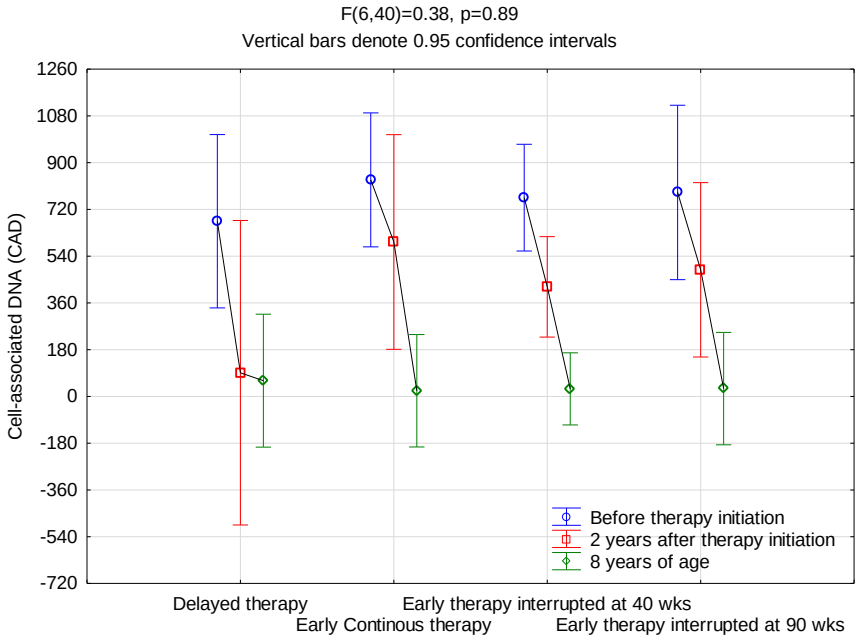


Figure 5.3: *Vertical bar graph with 95% confidence intervals depicting the longitudinal mean HIV-1 CAD levels (copies/10^6 PBMCs) across three measured time points for each study group.*
The general trend for each group displays an overall decrease in HIV-1 CAD over time.

Table 5.4: Summary of statistical probabilities for Post Hoc tests implemented for HIV-1 CAD measurements for the therapy study groups (Groups 1 – 4) over the three time points evaluated (before therapy initiation, 2 years after therapy initiation, 8 years of age)

Probabilities for Post Hoc Test (HIV-1 CAD copies/10^6 PBMCs) – Figure 5													
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.08	**0.00**	0.45	0.76	**0.00**	0.64	0.19	**0.00**	0.64	0.43	**0.00**
2	Delayed therapy	2 years after therapy initiation	0.08	-	0.92	0.02	0.17	0.83	**0.03**	0.29	0.84	**0.04**	0.25	0.85
3	Delayed therapy	8 years of age	**0.00**	0.92	-	**0.00**	**0.03**	0.82	**0.00**	**0.03**	0.83	**0.00**	0.05	0.86
4	Early Continuous therapy	Before therapy initiation	0.45	**0.02**	**0.00**	-	0.32	**0.00**	0.68	**0.01**	**0.00**	0.82	0.11	**0.00**
5	Early Continuous therapy	2 years after therapy initiation	0.76	0.17	**0.03**	0.32	-	**0.01**	0.46	0.45	**0.01**	0.48	0.69	**0.02**
6	Early Continuous therapy	8 years of age	**0.00**	0.83	0.82	**0.00**	**0.01**	-	**0.00**	**0.01**	0.95	**0.00**	**0.02**	0.96
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.64	**0.03**	**0.00**	0.68	0.46	**0.00**	-	**0.02**	**0.00**	0.92	0.16	**0.00**
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.19	0.29	**0.03**	**0.01**	0.45	**0.01**	**0.02**	-	**0.00**	0.07	0.74	**0.01**
9	Early therapy interrupted at 40 wks.	8 years of age	**0.00**	0.84	0.83	**0.00**	**0.01**	0.95	**0.00**	**0.00**	-	**0.00**	**0.01**	0.99
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.64	**0.04**	**0.00**	0.82	0.48	**0.00**	0.92	0.07	**0.00**	-	0.21	**0.00**

11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.43	0.25	0.05	0.11	0.69	**0.02**	0.16	0.74	**0.01**	0.21	-	**0.02**
12	Early therapy interrupted at 90 wks.	8 years of age	**0.00**	0.85	0.86	**0.00**	**0.02**	0.96	**0.00**	**0.01**	0.99	**0.00**	**0.02**	-

Multivariate analysis using a mixed model approach was employed to measure the interdependence and change in soluble immune biomarker levels in each therapy group across the three time points of interest. The soluble immune biomarkers were formerly log transformed to allow for the data to be more interpretable (enhance normality distribution) and meet the appropriate assumptions of inferential statistics.

5.3.5 Longitudinal evaluation of plasma immune biomarkers: Therapy grouping had minimal impact on the longitudinal expression on most soluble immune biomarkers measured.

Of the 40 soluble immune biomarkers measured longitudinally, we observed that the effect of therapy regimen (delayed, early or interrupted) was minimal when evaluated as a composite without the time point effect.

Significant differences across the therapy groups were, however, noted for specific immune biomarkers including IL-17F, IL-2, IL-4, IL-7, IL-13, IFN-γ, TNF-β, CD40L, IFN-α, PDGF, $TGF\beta_3$ and LBP. These differences were very specific to Group 2, given early continuous therapy, where levels of these biomarkers were elevated compared to the other therapy groups and significantly higher ($p<0.05$) compared to Group 4 (early therapy interrupted at 90 weeks) that displayed significantly lower levels of these soluble immune biomarkers.

We have graphically presented (Figures 5.4a-f) these differences for key biomarkers including IL-2 ($p=0.03$), IL-4 ($p=0.01$), IFN-γ ($p=0.04$), TNFβ ($p=0.02$), IFNα ($p=0.03$) and $TGF\beta_3$ ($p=0.01$) below. A summary of all statistical data parameters including means, standard deviation (SD) and probabilities is presented in Table 5.5.

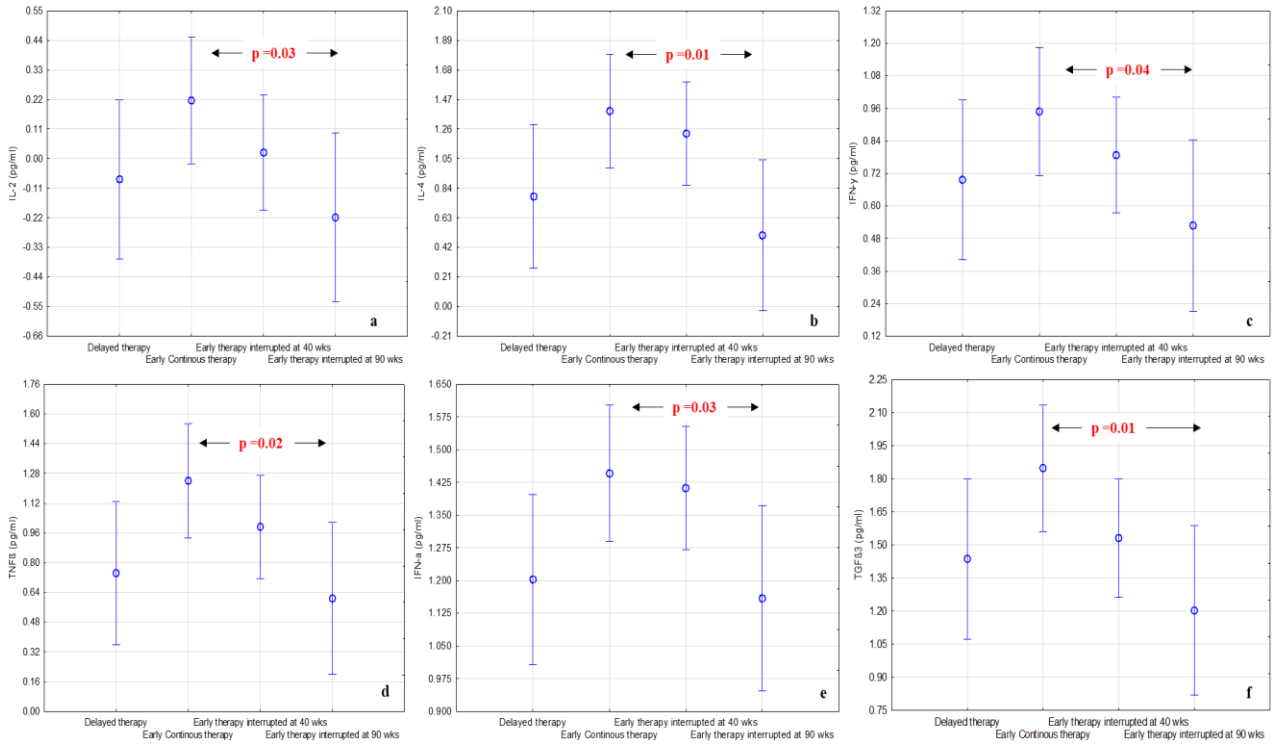


Figure 5.4a-f: *Vertical bar graph with 95% confidence intervals depicting composite log10 mean levels for IL-2, IL-4, IFN-γ, TNF-β, IFN-α and TGFβ$_3$ (pg/ml).*
Group 2, subjected to early continuous therapy, showed increased levels of these specific plasma cytokines compared to the other three groups. Group 4 initiated on early therapy and interrupted at 90 weeks displayed significantly lower levels of these plasma immune biomarkers compared to the other therapy groups and significantly lower levels when compared to Group 2.

Table 5.5: Summary of statistics (n, mean, SD and probabilities) for IL-2, IL-4, IFN-γ, TNF-β, IFN-α, TGFβ$_3$ (pg/ml) and LBP levels (µg/ml) measures for each therapy grouping without the time point effect

	n	Mean	SD	Probabilities (p-values)			
				Delayed therapy	Early continuous therapy	Early therapy interrupted at 40 weeks	Early therapy interrupted at 90 weeks
IL-2 (pg/ml)							
Delayed therapy	28	0.03	0.74	-	0.13	0.59	0.52
Early continuous therapy	43	0.34	0.81	0.13	-	0.23	**0.03**
Early therapy interrupted at 40 weeks	53	0.18	0.76	0.59	0.23	-	0.21
Early therapy interrupted at 90 weeks	25	0.05	0.93	0.52	**0.03**	0.21	-

Table 5.5 (continued)

IL-4 (pg/ml)							
Delayed therapy	28	0.63	1.22	-	0.07	0.16	0.46
Early continuous therapy	43	1.31	0.93	0.07	-	0.56	**0.01**
Early therapy interrupted at 40 weeks	53	1.05	1.24	0.16	0.56	-	**0.03**
Early therapy interrupted at 90 weeks	25	0.60	1.26	0.46	**0.01**	**0.03**	-
IFN- γ (pg/ml)							
Delayed therapy	28	0.80	0.56	-	0.19	0.62	0.44
Early continuous therapy	43	1.02	0.61	0.19	-	0.32	**0.04**

Early therapy interrupted at 40 weeks	53	0.90	0.57	0.62	0.32	-	0.18
Early therapy interrupted at 90 weeks	25	0.62	1.18	0.44	**0.04**	0.18	-
TNF-β (pg/ml)							
Delayed therapy	28	0.65	0.92	-	0.05	0.30	0.63
Early continuous therapy	43	1.21	0.98	0,05.	-	0.24	**0.02**
Early therapy interrupted at 40 weeks	53	1.10	0.91	0.30	0.24	-	0.13
Early therapy interrupted at 90 weeks	25	0.75	0.84	0.63	**0.02**	0.13	-
IFN-α (pg/ml)							
Delayed therapy	28	1.34	0.73	-	0.06	0.09	0.76
Early continuous therapy	43	1.59	0.60	0.06	-	0.75	**0.03**
Early therapy interrupted at 40 weeks	53	1.54	0.60	0.09	0.75	-	0.05
Early therapy interrupted at 90 weeks	25	1.47	0.79	0.76	**0.03**	0.05	-
TGFβ3 (pg/ml)							
Delayed therapy	25	1.51	1.07	-	0.08	0.67	0.38
Early continuous therapy	40	1.84	0.97	0.08	-	0.11	**0.01**
Early therapy interrupted at 40 weeks	48	1.77	0.95	0.67	0.11	-	0.16
Early therapy interrupted at 90 weeks	25	1.41	1.07	0.38	**0.01**	0.16	-
LBP (µg/ml)							
Delayed therapy	28	4.26	0.23	-	0.91	0.29	0.07
Early continuous therapy	43	4.27	0.28	0.91	-	0.18	**0.04**
Early therapy interrupted at 40 weeks	52	4.17	0.33	0.29	0.18	-	0.29
Early therapy interrupted at 90 weeks	25	4.08	0.26	0.07	**0.04**	0.29	-

The effect of therapy interruption at (40 or 90 weeks) had limited impact on the longitudinal expression of most levels of plasma immune biomarkers including key cytokines and chemokines such as IL-1RA, IL-1α, IL-6, IP-10, MCP-1, MIP-1α, MIP-1β, TNFα, RANTES, sCD14, sCD163, IL-18 and hsCRP.

When evaluating the composite effect of therapy interruption in Groups 3 (interrupted at 40 weeks) and 4 (interrupted at 90 weeks), we observed no significant differences ($p>0.05$) in the longitudinal levels of expression for several key immune biomarkers.

A summary of the statistical parameters for IL-RA, IL-1α, IL-6, IP-10, MCP-1, MIP-1α, MIP-1β, TNFα, RANTES, sCD14, sCD163, IL-18 (pg/ml) and hsCRP (ng/ml) is presented in Appendix C.

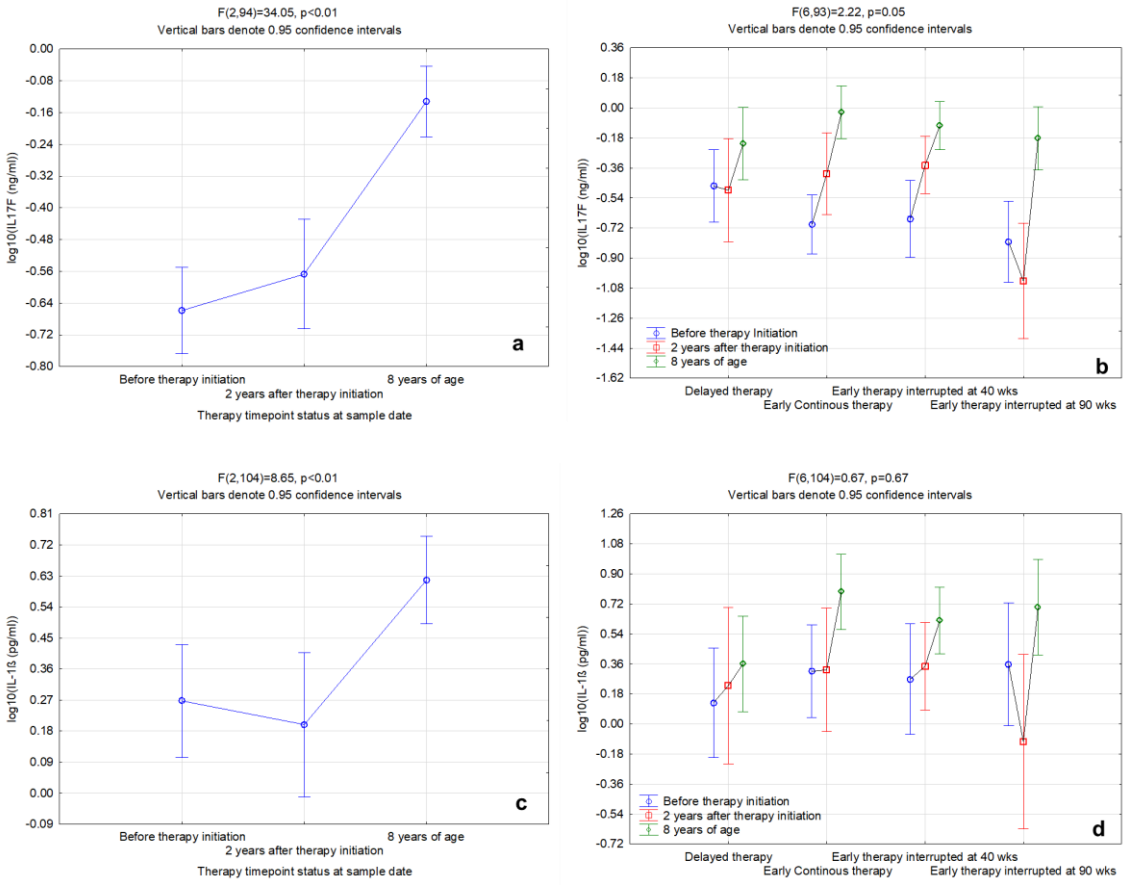
Following therapy initiation, minimal to no impact was observed for specific biomarkers followed by a significant spike at 8 years of age for the same biomarkers despite viral suppression and CD4 T cell reconstitution.

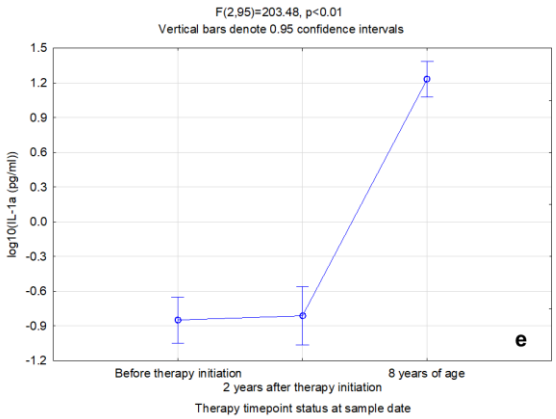
When evaluating the levels of soluble immune biomarkers over time following therapy initiation in all four therapy groups, we observed a continuous elevation of specific plasma biomarkers including IL-17F, IL-1β, IL-1α, IL-2, IL-12, IL-13, IL-17A, IFN-γ, MIP-1α, MIP-1β, GCSF, CD40L, VEGF, FGF, PDGF, $TGF\beta_1$, $TGF\beta_2$, $TGF\beta_3$, sCD14, sCD163 and IL-18.

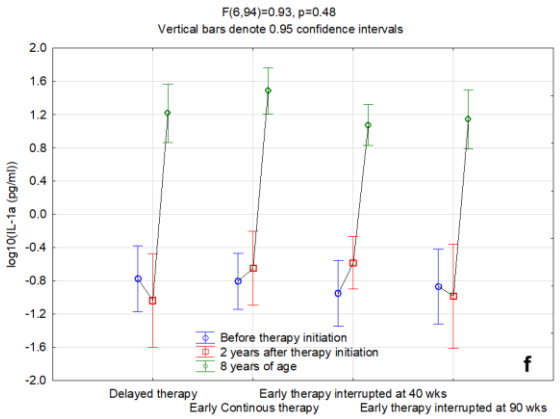
In addition, we observed a significant spike at 8 years of age in the levels of IL-1α, MIP-1α. MIP-1β, CD40L, sCD14, sCD163 and IL-18 despite viral suppression, reconstituted CD4 counts and low HIV-1 CAD. When evaluating these biomarkers in relation to age-matched controls in our cross-sectional study (see Chapter 3), these biomarkers (except for MIP-1α) were significantly higher in comparison.

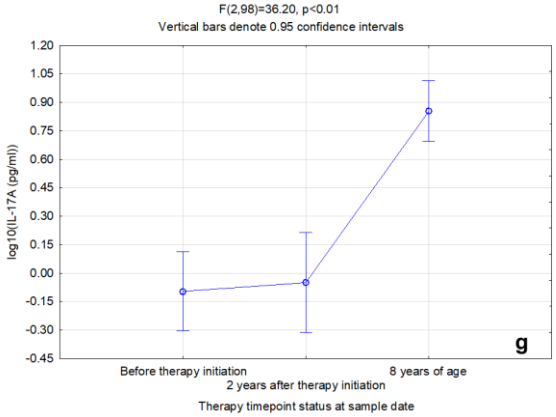
These increasing patterns from baseline to 8 years of age are relatively consistent for each of the study groups evaluated.

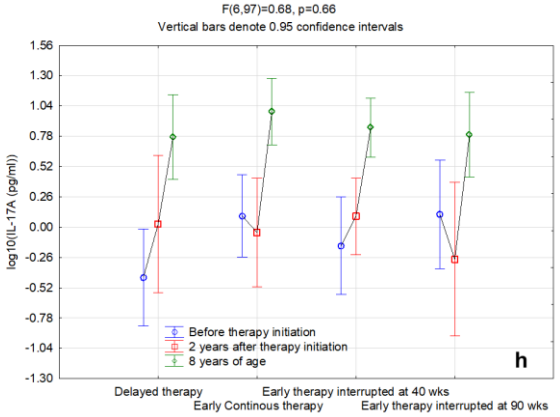
Graphical representations of these plasma immune biomarkers are selectively (IL-17F, IL-1β, IL-1α, IL-17A, IFN-γ, MIP-1α, MIP-1β, CD40L, sCD14, sCD163 and IL-18) shown in Figures 5.5a-v below. Figures on the left display composite biomarker levels at specific time points (longitudinal time snapshot) and figures on the right display both time point and therapy group patterns. The probability data for each of these immune biomarkers is listed in Appendix D.

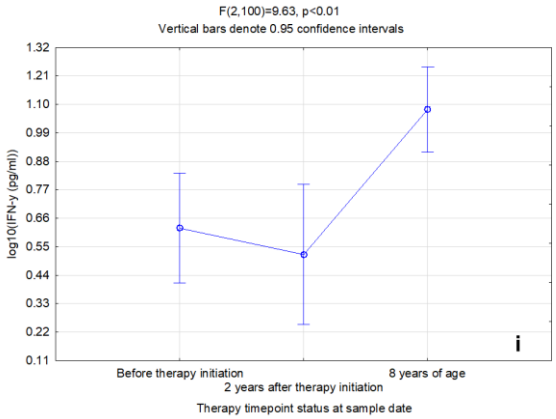


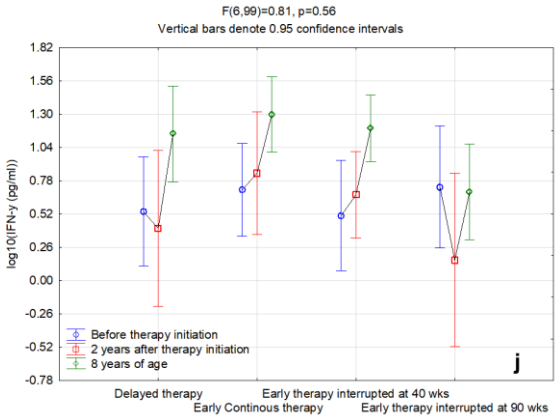


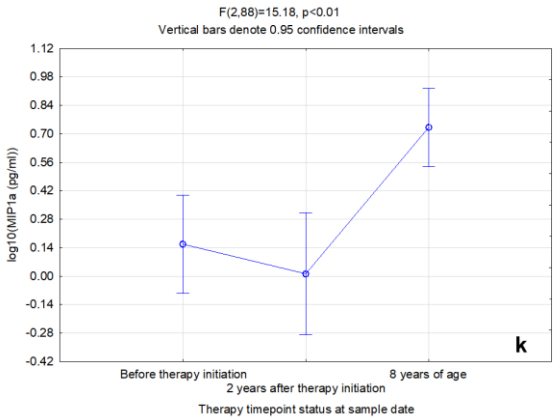


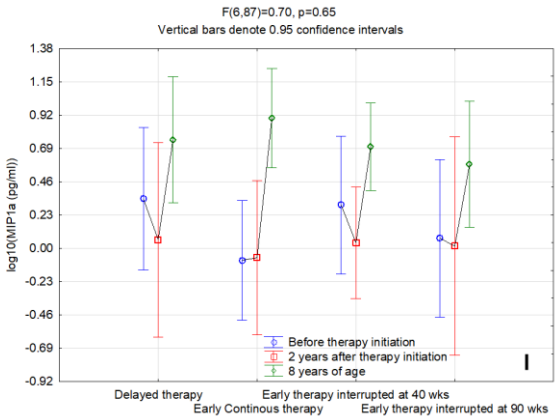


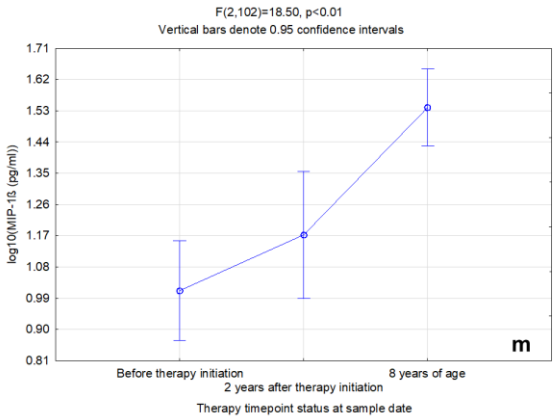


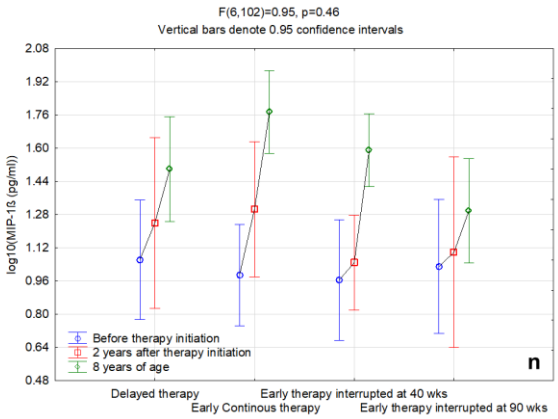


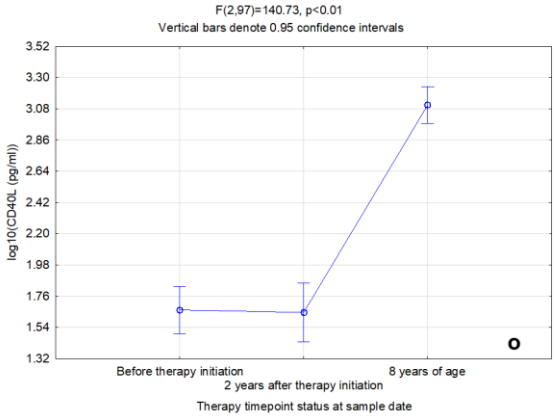


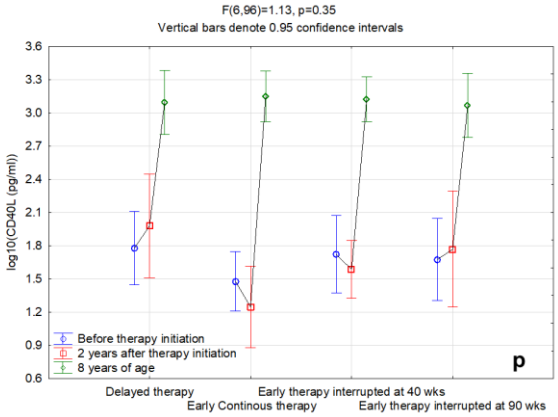


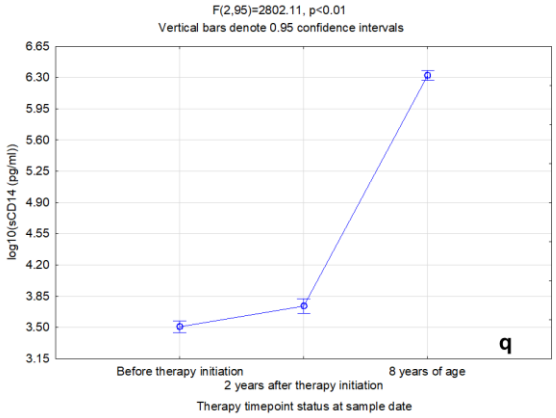


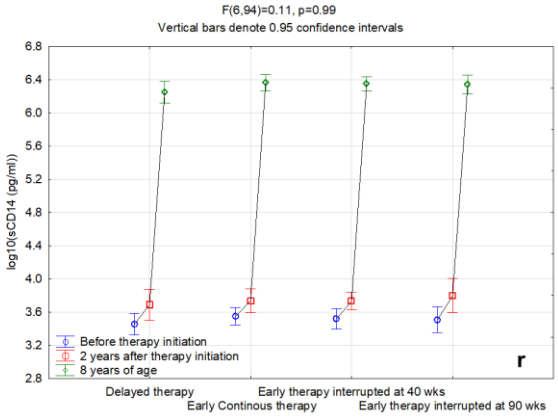


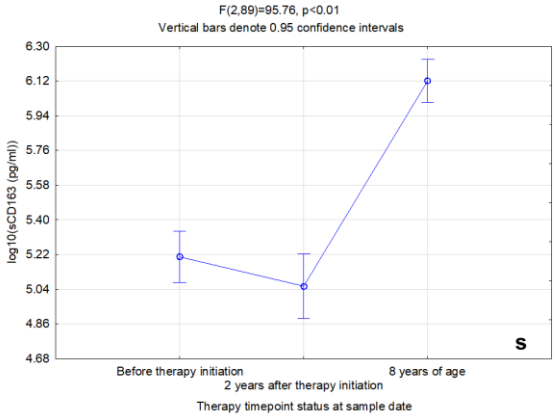


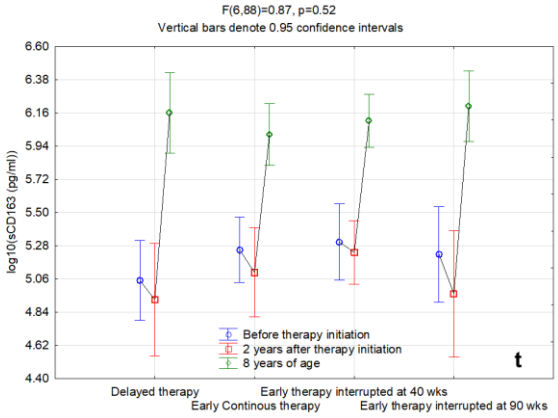












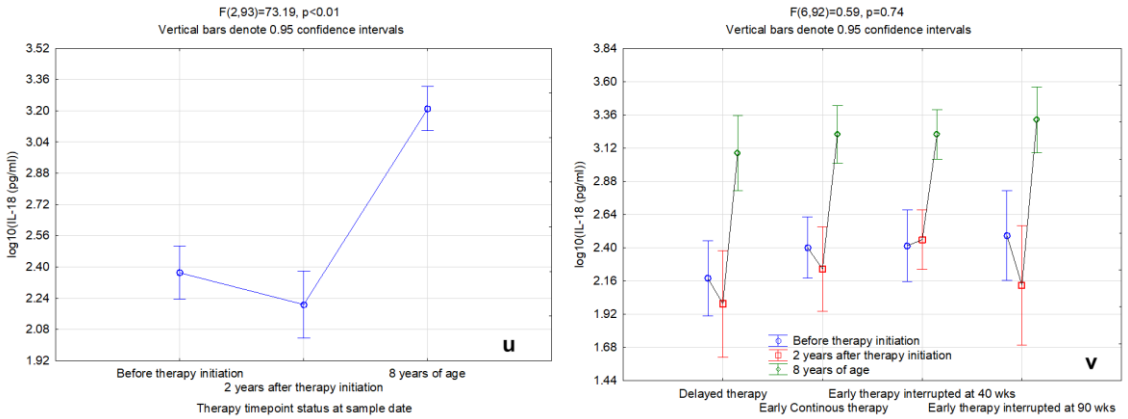


Figure 5.5a-v: *Vertical bar graphs with 95% confidence intervals depicting log10 mean IL-17F (ng/ml), IL-1β, IL-1α, IL-17A, IFN-y, MIP-1α, MIP-1β, CD40L, sCD14, sCD163 and IL-18 (pg/ml).*
The left side of the graph represents composite log10 mean biomarker levels measured at specific time points without the therapy effect interaction. The right side of the graph represents log10 mean biomarker levels for each therapy group measured at three time points.

Statistical summaries for IL-17F (ng/ml), IL-1β, IL-1α, IL-17A, IFN-y, MIP-1α, MIP-1β, CD40L, sCD14, sCD163 and IL-18 (pg/ml) measures at each time point and for each therapy group is summarised in Tables 5.5 and 5.7 below.

Table 5.6: Summary of statistical data (n, mean, SD and probabilities) for IL-17F (ng/ml), IL-1β, IL-1α, IL-17A, IFN-y, MIP-1α, MIP-1β, CD40L, sCD14, sCD163 and IL-18 (pg/ml) measures for each time point

	n	Mean	SD	Probabilities (p-values)		
				Before therapy initiation	2 years after therapy initiation	8 years of age
IL-17F (ng/ml)						
Before therapy initiation	42	-0.65	0.49	-	0.29	**0.00**
2 years after therapy initiation	33	-0.47	0.41	0.29	-	**0.00**
8 years of age	65	-0.12	0.21	**0.00**	**0.00**	-
IL-1β						
Before therapy initiation	42	0.27	0.39	-	0.61	**0.00**
2 years after therapy initiation	33	0.27	0.42	0.61	-	**0.00**
8 years of age	74	0.64	0.62	**0.00**	**0.00**	-

Table 5.6 (continued)

IL-1α						
Before therapy initiation	42	-0.86	0.38	-	0.82	**0.00**
2 years after therapy initiation	33	-0.70	0.58	0.82	-	**0.00**
8 years of age	74	1.23	0.76	**0.00**	**0.00**	-
IL-17A						
Before therapy initiation	42	-0.10	0.81	-	0.78	**0.00**
2 years after therapy initiation	33	0.01	0.86	0.78	-	**0.00**
8 years of age	74	0.7	0.42	**0.00**	**0.00**	-
IFN-γ						
Before therapy initiation	42	0.62	0.51	-	0.55	**0.00**
2 years after therapy initiation	33	0.61	0.58	0.55	-	**0.00**
8 years of age	74	1.12	0.80	**0.00**	**0.00**	-
MIP-1α						
Before therapy initiation	42	0.09	0.73	-	0.42	**0.00**
2 years after therapy initiation	33	0.10	0.92	0.42	-	**0.00**
8 years of age	74	0.74	0.76	**0.00**	**0.00**	-
MIP-1β						
Before therapy initiation	42	1.01	0.41	-	0.17	**0.00**
2 years after therapy initiation	33	1.15	0.29	0.17	-	**0.00**
8 years of age	74	1.57	0.54	**0.00**	**0.00**	-
CD40L						
Before therapy initiation	42	1.64	0.59	-	0.89	**0.00**
2 years after therapy initiation	33	1.57	0.83	0.89	-	**0.00**
8 years of age	74	3.11	0.26	**0.00**	**0.00**	-
sCD14						
Before therapy initiation	43	3.52	0.28	-	**0.00**	**0.00**
2 years after therapy initiation	33	3.74	0.14	**0.00**	-	**0.00**
8 years of age	63	6.33	0.16	**0.00**	**0.00**	-
sCD163						
Before therapy initiation	43	5.21	0.65	-	0.14	**0.00**
2 years after therapy initiation	33	5.12	0.36	0.14	-	**0.00**
8 years of age	63	6.10	0.22	**0.00**	**0.00**	-
IL-18						
Before therapy initiation	43	2.37	0.54	-	0.14	**0.00**
2 years after therapy initiation	33	2.28	0.39	0.14	-	**0.00**
8 years of age	63	3.21	0.37	**0.00**	**0.00**	-

Table 5.7: Summary of statistical data (n, mean, SD and probabilities) for IL-17F (ng/ml), IL-1β, IL-1α, IL-17A, IFN-γ, MIP-1α, MIP-1β, CD40L, sCD14, sCD163 and IL-18 (pg/ml) measures for each therapy group without the time point effect

	n	Mean	SD	Probabilities (p-values)			
				Delayed therapy	Early continuous therapy	Early therapy interrupted at 40 weeks	Early therapy interrupted at 90 weeks
IL-17F (ng/ml)							
Delayed therapy	25	-0.39	0.41	-	0.85	0.83	**0.01**
Early continuous therapy	42	-0.39	0.49	0.85	-	0.99	**0.00**
Early therapy interrupted at 40 weeks	48	-0.29	0.35	0.83	0.99	-	**0.00**
Early therapy interrupted at 90 weeks	25	-0.51	0.47	**0.01**	**0.00**	**0.00**	-
IL-1β (pg/ml)							
Delayed therapy	28	0.25	0.48	-	0.09	0.20	0.63
Early continuous therapy	43	0.55	0.47	0.09	-	0.57	0.28
Early therapy interrupted at 40 weeks	53	0.47	0.58	0.20	0.57	-	0.51
Early therapy interrupted at 90 weeks	25	0.46	0.67	0.63	0.28	0.51	-
IL-1α (pg/ml)							
Delayed therapy	28	0.12	1.17	-	0.27	0.80	0.86
Early continuous therapy	43	0.34	1.25	0.27	-	0.30	0.20
Early therapy interrupted at 40 weeks	53	0.19	1.15	0.80	0.30	-	0.66
Early therapy interrupted at 90 weeks	25	0.15	1.27	0.86	0.20	0.66	-
IL-17A (pg/ml)							
Delayed therapy	28	0.20	0.81	-	0.24	0.44	0.69
Early continuous therapy	43	0.51	0.86	0.24	-	0.60	0.48
Early therapy interrupted at 40 weeks	53	0.43	0.75	0.44	0.60	-	0.78
Early therapy interrupted at 90 weeks	25	0.40	0.80	0.69	0.48	0.78	-
IFN-γ (pg/ml)							
Delayed therapy	28	0.80	0.56	-	0.19	0.62	0.44
Early continuous therapy	43	1.02	0.61	0.19	-	0.32	**0.04**
Early therapy interrupted at 40 weeks	53	0.90	0.58	0.62	0.32	-	0.18
Early therapy interrupted at 90 weeks	25	0.62	1.18	0.44	**0.04**	0.18	-
MIP-1α (pg/ml)							
Delayed therapy	28	0.5	0.97	-	0.59	0.88	0.56
Early continuous therapy	43	0.41	0.76	0.59	-	0.64	0.91
Early therapy interrupted at 40 weeks	53	0.42	0.80	0.88	0.64	-	0.61
Early therapy interrupted at 90 weeks	25	0.31	0.99	0.56	0.91	0.61	-

Table 5.7 (continued)

MIP-1β (pg/ml)							
Delayed therapy	28	1.30	0.41	-	0.48	0.58	0.38
Early continuous therapy	43	1.43	0.57	0.48	-	0.14	0.11
Early therapy interrupted at 40 weeks	53	1.31	0.51	0.58	0.14	-	0.64
Early therapy interrupted at 90 weeks	25	1.18	0.58	0.38	0.11	0.64	-
sCD40L (pg/ml)							
Delayed therapy	28	2.42	0.80	-	**0.03**	0.34	0.52
Early continuous therapy	44	2.23	1.01	**0.03**	-	0.15	0.18
Early therapy interrupted at 40 weeks	52	2.40	0.99	0.34	0.15	-	0.86
Early therapy interrupted at 90 weeks	25	2.41	0.75	0.52	0.18	0.86	-
sCD14 (pg/ml)							
Delayed therapy	25	4.62	1.39	-	0.14	0.21	0.20
Early continuous therapy	40	4.78	1.39	0.14	-	0.72	0.98
Early therapy interrupted at 40 weeks	50	4.89	1.37	0.21	0.72	-	0.78
Early therapy interrupted at 90 weeks	24	5.09	1.40	0.20	0.98	0.78	-
sCD163 (pg/ml)							
Delayed therapy	25	5.46	0.75	-	0.53	0.17	0.56
Early continuous therapy	40	5.54	0.60	0.53	-	0.39	0.97
Early therapy interrupted at 40 weeks	50	5.65	0.59	0.17	0.39	-	0.50
Early therapy interrupted at 90 weeks	24	5.70	0.63	0.56	0.97	0.50	-
IL-18 (pg/ml)							
Delayed therapy	25	2.50	0.68	-	0.11	**0.02**	0.12
Early continuous therapy	40	2.72	0.60	0.11	-	0.47	0.85
Early therapy interrupted at 40 weeks	50	2.80	0.59	**0.02**	0.47	-	0.69
Early therapy interrupted at 90 weeks	24	2.88	0.61	0.12	0.85	0.69	-

Levels of IL-3, MCP-1, TNFα, hsCRP and LBP remained the same for each time point measured i.e., before therapy initiation, 2 years after therapy and at 8 years of age across all therapy groups.

We observed no significant change in the levels of IL-3, MCP-1, TNFα, hsCRP and LBP from baseline to 8 years of age. This pattern was similar across all therapy groups except for LBP of which some significant differences were noted between the therapy groups particularly in Group 2. It is also worth noting that in our cross-sectional study (Chapter 3), we observed significantly elevated levels of hsCRP and LBP in the HIV+ children compared to HIV-1 negative controls at 8 years of age. No difference was noted for MCP-1 between HIV+ children and HIV-1 controls and significantly decreased levels of IL-3 and TNFα was observed for HIV+ children compared to HIV-1 negative controls at 8 years of age.

The probability data for IL-3, MCP-1, TNFα, hsCRP and LBP is listed in Appendix E

Statistical summaries for IL-3, MCP-1, TNF-α, hsCRP and LBP measures at each time point and for each therapy group is summarised in Tables 5.8 and 5.9 below.

Table 5.8: Summary of statistical data (n, mean, SD and probabilities) for IL-3, MCP-1, TNFα (pg/ml), hsCRP (ng/ml) and LBP (µg/ml) measures for each time point

	n	Mean	SD	Probabilities (p-values)		
				Before therapy initiation	Before therapy initiation	Before therapy initiation
IL-3 (pg/ml)						
Before therapy initiation	42	-0.13	0.71	-	0.70	0.91
2 years after therapy initiation	33	-0.12	0.85	0.70	-	0.74
8 years of age	74	-0.14	0.38	0.91	0.74	-
MCP-1 (pg/ml)						
Before therapy initiation	42	2.17	0.60	-	0.92	0.55
2 years after therapy initiation	33	2.19	0.28	0.92	-	0.54
8 years of age	74	2.14	0.33	0.55	0.54	-
TNFα (pg/ml)						
Before therapy initiation	42	1.16	0.47	-	0.49	0.06
2 years after therapy initiation	33	1.15	0.21	0.49	-	0.40
8 years of age	74	1.06	0.19	0.06	0.40	-

Table 5.8 (continued)

hsCRP (ng/ml)						
Before therapy initiation	44	2.94	0.70	-	0.41	0.45
2 years after therapy initiation	33	3.15	0.71	0.41	-	0.13
8 years of age	71	2.84	0.91	0.45	0.13	-
LBP (µg/ml)						
Before therapy initiation	44	4.27	0.38	-	0.39	0.13
2 years after therapy initiation	33	4.19	0.30	0.39	-	0.75
8 years of age	71	4.17	0.21	0.13	0.75	-

Table 5.9: Summary of statistical (n, mean, SD and probabilities) for IL-3, MCP-1, TNFα (pg/ml), hsCRP (ng/ml) and LBP (µg/ml) measures for each therapy group without the time point effect

	n	**Mean**	**SD**	**Probabilities (p-values)**			
				Delayed therapy	**Early continuous therapy**	**Early therapy interrupted at 40 weeks**	**Early therapy interrupted at 90 weeks**
IL-3 (pg/ml)							
Delayed therapy	28	-0.17	0.50	-	0.97	0.61	0.98
Early continuous therapy	43	-0.12	0.60	0.97	-	0.60	0.99
Early therapy interrupted at 40 weeks	53	-0.18	0.64	0.61	0.60	-	0.65
Early therapy interrupted at 90 weeks	25	-0.04	0.67	0.98	0.99	0.65	-
MCP-1 (pg/ml)							
Delayed therapy	28	2.22	0.49	-	0.29	0.84	0.39
Early continuous therapy	43	2.13	0.45	0.29	-	0.31	0.95
Early therapy interrupted at 40 weeks	53	2.18	0.32	0.84	0.31	-	0.43
Early therapy interrupted at 90 weeks	25	2.11	0.45	0.39	0.95	0.43	-
TNF-α (pg/ml)							
Delayed therapy	28	1.10	0.35	-	0.89	0.52	0.70
Early continuous therapy	43	1.10	0.37	0.89	-	0.37	0.78
Early therapy interrupted at 40 weeks	53	1.13	0.18	0.52	0.37	-	0.30
Early therapy interrupted at 90 weeks	25	1.08	0.32	0.70	0.78	0.30	-
hsCRP (ng/ml)							
Delayed therapy	28	2.86	0.83	-	0.99	0.53	0.53
Early continuous therapy	43	2.83	0.83	0.99	-	0.48	0.50
Early therapy interrupted at 40 weeks	52	3.06	0.74	0.53	0.48	-	0.91
Early therapy interrupted at 90 weeks	25	2.96	0.91	0.53	0.50	0.91	-

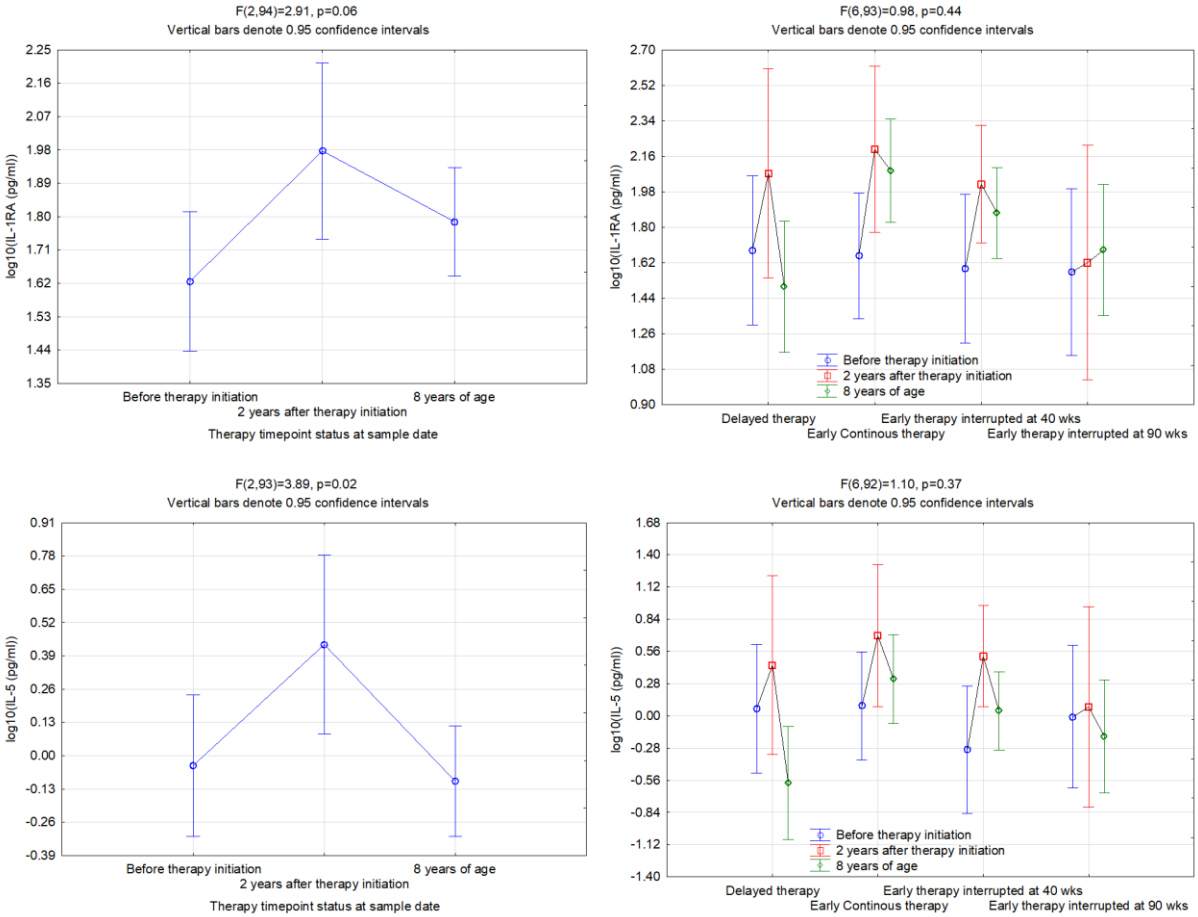
Table 5.9 (continued)

LBP (µg/ml)							
Delayed therapy	28	4.26	0.23	-	0.91	0.29	0.07
Early continuous therapy	43	4.27	0.28	0.91	-	0.18	**0.04**
Early therapy interrupted at 40 weeks	52	4.17	0.33	0.29	0.18	-	0.29
Early therapy interrupted at 90 weeks	25	4.09	0.26	0.07	**0.04**	0.29	-

Following 2 years of therapy initiation, IL-1RA, IL-5, IL-6, IL-8 and TNFβ significantly increases and the then decreased by 8 years of age.

In Figures 5.6, we observed an interesting pattern of a spike in biomarker levels for IL-1RA, IL-5, IL-6, IL-8 and TNFβ following therapy initiation and a decrease at viral suppression and immune reconstitution at 8 years of age. This pattern was consistent across all study groups evaluated. Following long-term therapy, the levels of these biomarkers decrease close to baseline levels. Evaluating these biomarkers to 8-year-old HIV- controls in our cross-sectional analysis (Chapter 3), we observed that only IL-1RA and IL-8 levels restore to levels equivalent to HIV negative controls, whereas IL-5 and TNFβ are still significantly higher compared to HUU controls and IL-6 levels are significantly lower compared to the same group.

The probability data for IL-1RA, IL-5, IL-6 and TNFβ is listed in Appendix F



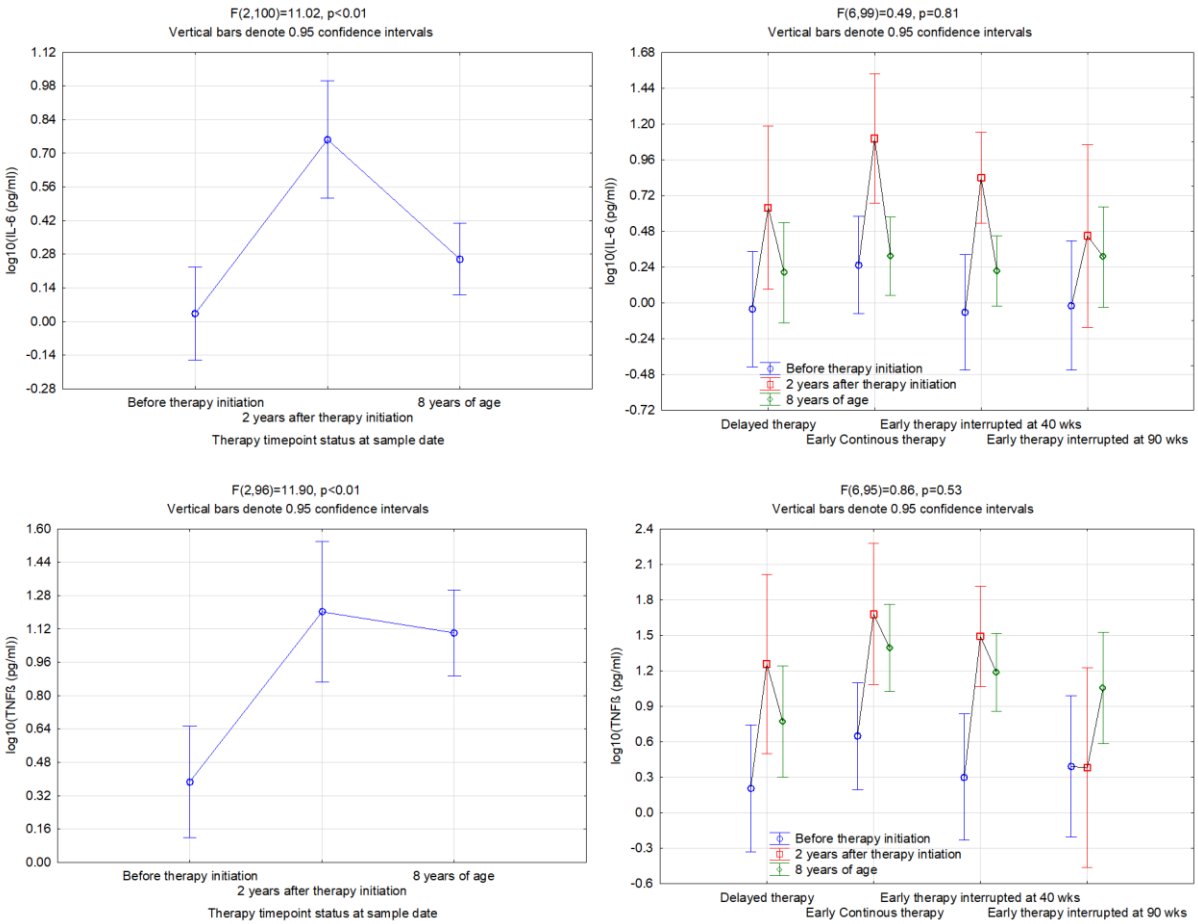


Figure 5.6: *Vertical bar graphs with 95% confidence intervals depicting log10 mean IL-1RA, IL-5, IL-6 and TNFβ (pg/ml).*
Left side of the graph represents composite log10 mean biomarker levels measured at specific time points without the therapy effect interaction. Right side of the graph represents log10 mean biomarker levels for each therapy group measured at three time points.

Statistical summaries for IL-1RA, IL-5, IL-6 and TNFβ measures at each time point and for each therapy group is summarised in Tables 5.10 and 5.11 below.

Table 5.10: Summary of statistical data (n, mean, SD and probabilities) for IL-1RA, IL-5, IL-6 and TNFβ (pg/ml)

	n	Mean	SD	Probabilities (p-values)		
				Before therapy initiation	Before therapy initiation	Before therapy initiation
IL-1RA (pg/ml)						
Before therapy initiation	42	1.62	0.54	-	**0.02**	0.15
2 years after therapy initiation	33	2.05	0.44	**0.02**	-	0.15
8 years of age	74	1.84	0.70	0.15	0.15	-
IL-5 (pg/ml)						
Before therapy initiation	42	-0.05	0.65	-	**0.03**	0.70
2 years after therapy initiation	33	0.54	0.40	**0.03**	-	**0.01**
8 years of age	74	-0.03	1.14	0.70	**0.01**	-

Table 5.10 (continued)

IL-6 (pg/ml)						
Before therapy initiation	42	0.05	0.88	-	**0.00**	0.06
2 years after therapy initiation	33	0.82	0.69	**0.00**	-	**0.00**
8 years of age	74	0.26	0.31	0.06	**0.00**	-
TNFβ (pg/ml)						
Before therapy initiation	42	0.41	0.96	-	**0.00**	**0.00**
2 years after therapy initiation	33	1.38	0.66	**0.00**	-	0.60
8 years of age	74	1.15	0.89	**0.00**	0.60	-

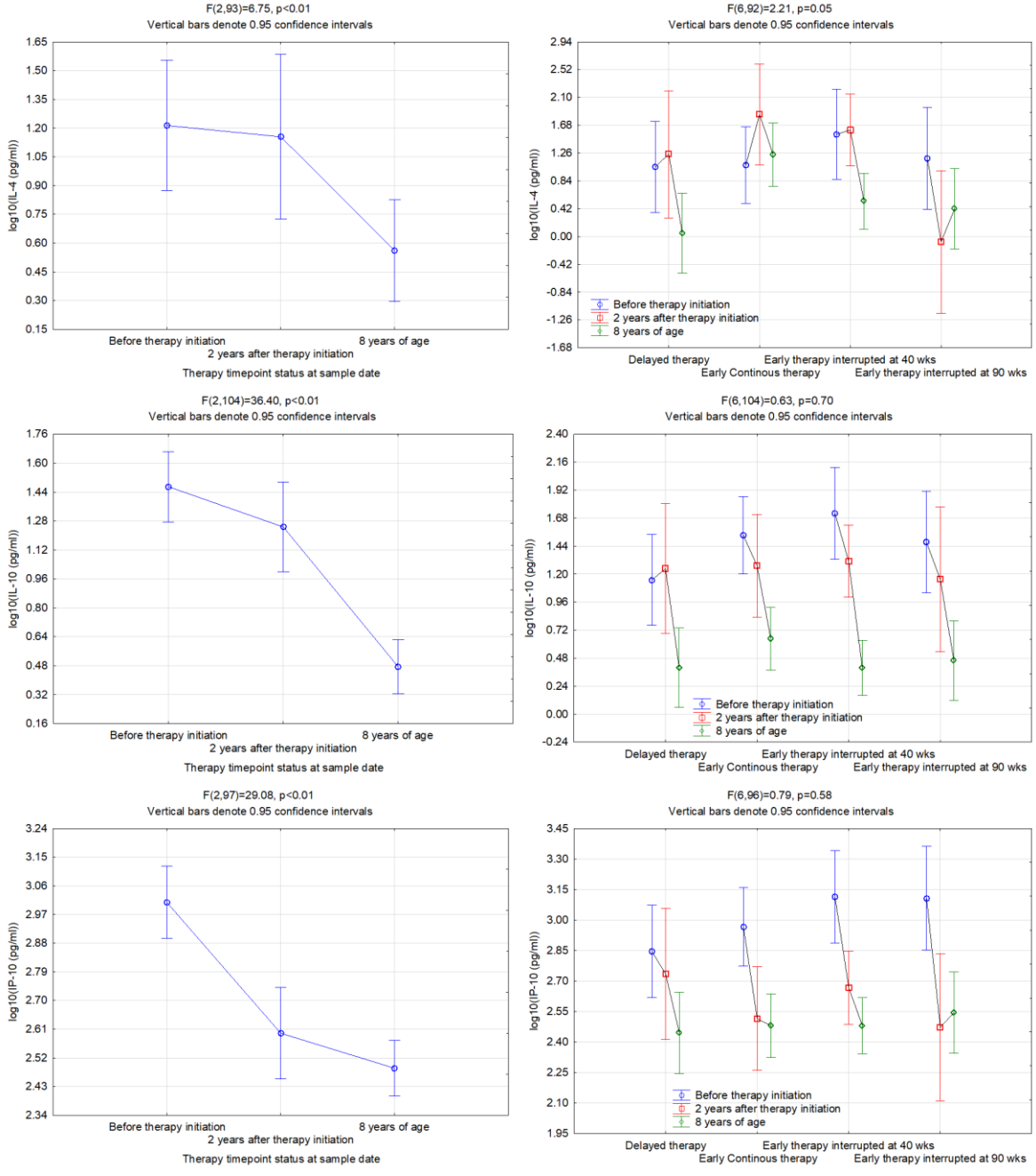
Table 5.11: Summary of statistical (n, mean, SD and probabilities) for measures for IL-1RA, IL-5, IL-6 and TNFβ (pg/ml) each therapy group without the time point effect

	n	**Mean**	**SD**	**Probabilities (p-values)**			
				Delayed therapy	**Early continuous therapy**	**Early therapy interrupted at 40 weeks**	**Early therapy interrupted at 90 weeks**
IL-1RA (pg/ml)							
Delayed therapy	28	1.67	0.52	-	0.20	0.66	0.53
Early continuous therapy	43	1.97	0.64	0.20	-	0.31	0.06
Early therapy interrupted at 40 weeks	53	1.86	0.63	0.66	0.31	-	0.26
Early therapy interrupted at 90 weeks	25	1.65	0.62	0.53	0.06	0.26	-
IL-5 (pg/ml)							
Delayed therapy	28	-0.18	0.89	-	0.14	0.65	0.97
Early continuous therapy	43	0.33	0.92	0.14	-	0.21	0.14
Early therapy interrupted at 40 weeks	53	0.11	0.90	0.65	0.21	-	0.63
Early therapy interrupted at 90 weeks	25	-0.07	0.96	0.97	0.14	0.63	-
IL-6 (pg/ml)							
Delayed therapy	28	0.19	0.69	-	0.09	0.70	0.92
Early continuous therapy	43	0.44	0.64	0.09	-	0.12	0.09
Early therapy interrupted at 40 weeks	53	0.35	0.67	0.70	0.12	-	0.63
Early therapy interrupted at 90 weeks	25	0.23	0.67	0.92	0.09	0.63	-
TNFβ (pg/ml)							
Delayed therapy	28	0.65	0.92	-	0.05	0.30	0.63
Early continuous therapy	43	1.21	0.98	0.05	-	0.24	**0.02**
Early therapy interrupted at 40 weeks	53	1.10	0.91	0.30	0.24	-	0.13
Early therapy interrupted at 90 weeks	25	0.75	0.84	0.63	**0.02**	0.13	-

IL-4, IL-10, IP-10 and RANTES declined consistently for each timepoint measured.

In Figure 5.7, we show a constant decline in biomarkers IL-4, IL-10, IP-10 and RANTES over time. This pattern is consistent across all the therapy groups studied except for IL-4 where a lag in the decrease is observed. Evaluating our cross-sectional data at 8 years of age compared to HIV negative controls, we observed that levels for IL-4, IL-10 and IP-10 was restored to that of the HUU group (HEUs displayed higher levels), however the same was not true for RANTES, were a significantly lower level was noted for HIV+ children compared to both HEU and HUU children at 8 years of age.

The probability data for IL-4, IL-10, IP-10 and RANTES is listed in Appendix G



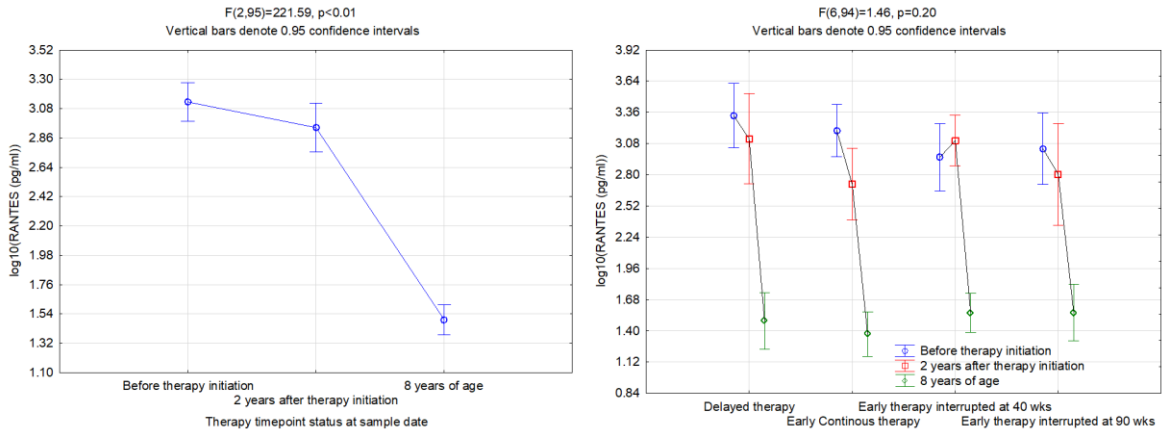


Figure 5.7: *Vertical bar graphs with 95% confidence intervals depicting log10 mean IL-4, IL-10, IP-10, TNFα and RANTES (pg/ml).*
Left side of the graph represents composite log10 mean biomarker levels measured at specific time points without the therapy effect interaction. Right side of the graph represents log10 mean biomarker levels for each therapy group measured at three time points.

Statistical summaries for IL-4, IL-10, IP-10, TNFα and RANTES measures at each time point and for each therapy group is summarised in Tables 5.12 and 5.13 below.

Table 5.12: Summary of statistical data (n, mean, SD and probabilities) for IL-4, IL-10, IP-10 and RANTES (pg/ml)

	n	Mean	SD	Probabilities (p-values)		
				Before therapy initiation	Before therapy initiation	Before therapy initiation
IL-4 (pg/ml)						
Before therapy initiation	42	1.18	0.64	-	0.82	**0.00**
2 years after therapy initiation	33	1.47	0.91	0.82	-	**0.01**
8 years of age	74	0.63	1.41	**0.00**	**0.01**	-
IL-10 (pg/ml)						
Before therapy initiation	42	1.48	0.62	-	0.16	**0.00**
2 years after therapy initiation	33	1.27	0.29	0.16	-	**0.00**
8 years of age	74	0.48	0.71	**0.00**	**0.00**	-
IP-10 (pg/ml)						
Before therapy initiation	42	3.00	0.49	-	**0.00**	**0.00**
2 years after therapy initiation	33	2.62	0.32	**0.00**	-	0.18
8 years of age	74	2.49	0.29	**0.00**	0.18	-
RANTES (pg/ml)						
Before therapy initiation	42	3.14	0.64	-	0.09	**0.00**
2 years after therapy initiation	33	2.98	0.51	0.09	-	**0.00**
8 years of age	74	1.50	0.31	**0.00**	**0.00**	-

Table 5.13: Summary of statistical data (n, mean, SD and probabilities) for measures of IL-4, IL-10, IP-10 and RANTES for each therapy group without the time point effect

	n	Mean	SD	Probabilities (p-values)			
				Delayed therapy	Early continuous therapy	Early therapy interrupted at 40 weeks	Early therapy interrupted at 90 weeks
IL-4 (pg/ml)							
Delayed therapy	28	0.63	1.22	-	0.07	0.16	0.46
Early continuous therapy	43	1.31	0.93	0.07	-	0.56	**0.01**
Early therapy interrupted at 40 weeks	53	1.04	1.24	0.16	0.56	-	**0.03**
Early therapy interrupted at 90 weeks	25	0.60	1.26	0.46	**0.01**	**0.03**	-
IL-10 (pg/ml)							
Delayed therapy	28	0.82	0.70	-	0.19	0.19	0.61
Early continuous therapy	43	1.05	0.73	0.19	-	0.96	0.49
Early therapy interrupted at 40 weeks	53	0.92	0.80	0.19	0.96	-	0.50
Early therapy interrupted at 90 weeks	25	0.89	0.85	0.61	0.49	0.50	-
IP-10 (pg/ml)							
Delayed therapy	28	2.63	0.47	-	0.84	0.43	0.78
Early continuous therapy	43	2.65	0.46	0.84	-	0.26	0.62
Early therapy interrupted at 40 weeks	53	2.66	0.38	0.43	0.26	-	0.67
Early therapy interrupted at 90 weeks	25	2.71	0.39	0.78	0.62	0.67	-
RANTES (pg/ml)							
Delayed therapy	28	2.43	0.94	-	0.10	0.42	0.24
Early continuous therapy	44	2.24	0.98	0.10	-	0.32	0.78
Early therapy interrupted at 40 weeks	52	2.28	0.87	0.42	0.32	-	0.58
Early therapy interrupted at 90 weeks	25	2.23	0.91	0.24	0.78	0.58	-

Changes in soluble immune biomarker levels from baseline (before therapy initiation) to 8 years of age

A principal component (PC) analysis was implemented provide an analysis and visual of the changes that occur in immune biomarkers over time for each therapy group. The PC plot in Figure 5.8 below indicates that about 36% of the soluble biomarkers measured which includes IL-15, TNFβ, GCSF, IL-1RA, IL-5, IL-4, IL-8, IL-10 and RANTES contributes to the largest parameter change across all treatment groups. Immune biomarkers including TGFβ1, PDGF-BB, sCD14, CD40L, sCD163, IL-18 and IL-1α in the PC2 region (left of Figure 5.8) accounts for 18% of the major changes that occur across all groups over time.

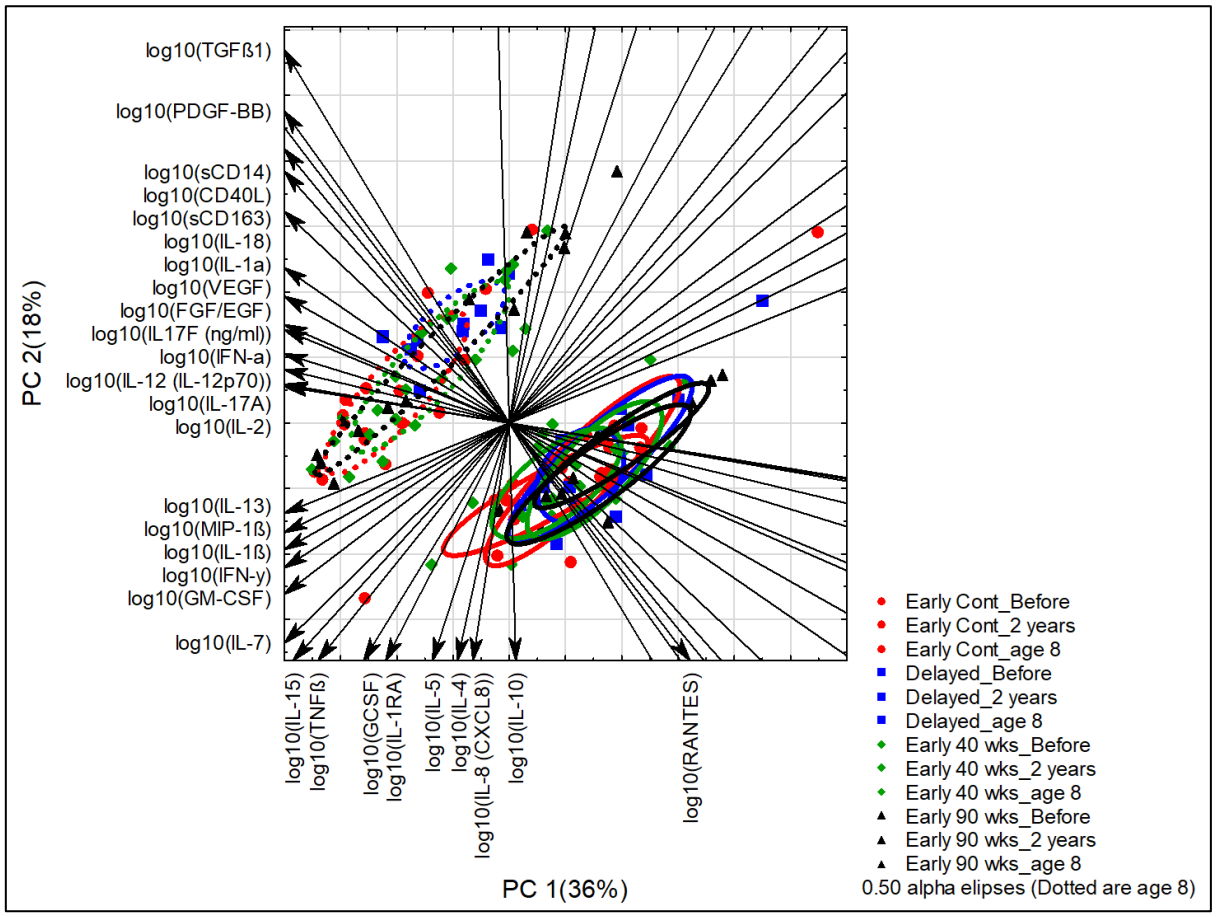


Figure 5.8: *Principal Component Analysis (PCA) depicting the change over time in soluble immune biomarker levels for each therapy group.*
The PC plot indicates that 36% of the soluble biomarkers measured which includes IL-15, TNFβ, GCSF, IL-1RA, IL-5, IL-4, IL-8, IL-10 and RANTES contributes to the largest parameter change across all treatment groups. Immune biomarkers including TGFβ1, PDGF-BB, sCD14, CD40L, sCD163, IL-18 and IL-1α in the PC2 region (left) accounts for 18% of the major changes that occur across all groups over time.

Extensive predictive analysis show that key immune biomarkers measured at baseline (before therapy initiation) can predict the levels of immune biomarkers at 8 years of age indicating a complex interplay between immune variables.

Multivariate regression analysis shows a significant relationship between baseline/early (before therapy initiation) immune biomarker levels and expression levels of immune biomarkers after 8 years following virological suppression.

A heat map visual of the relationship between immune biomarkers at baseline and 8 years of age is presented in Figure 5.9 and relative Spearman correlation analysis is presented in Table 5.14 below

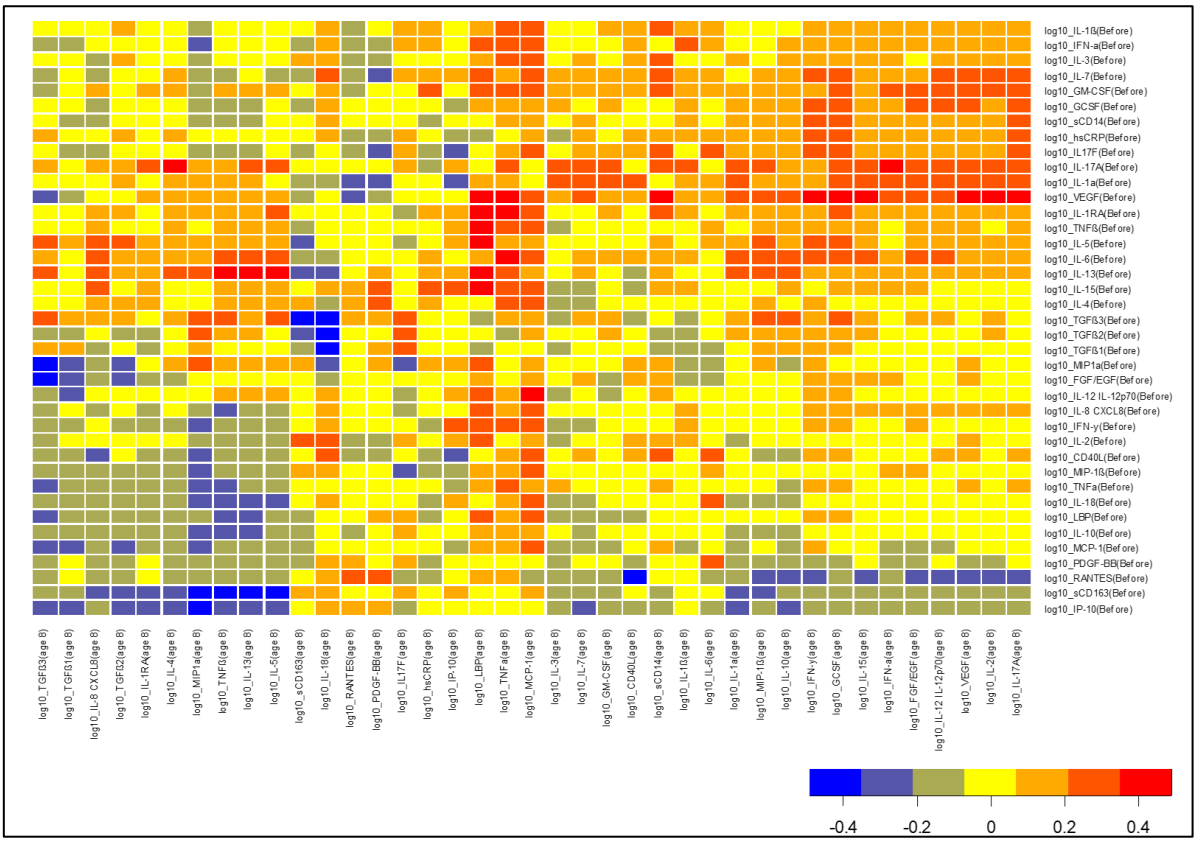


Figure 5.9: *Heat map visual of all soluble immune biomarkers measured at baseline and its relative correlation with biomarkers at 8 years of age to determine which early biomarkers may predict biomarker levels later.*
Heat map scale depicted by the R coefficient ranges from blue (indicative of a strong negative association) to red (indicative of a strong positive association). Yellow squares/scales would indicate no association. Parameters indicated on the y-axis are immune biomarkers measured at baseline (before therapy) and parameters indicated on the x-axis are immune biomarkers measured at 8 years of age.

Table 5.14: Summary of all statistically significant Spearman correlation coefficients (*r*) as well as probabilities following correlation analysis of soluble immune biomarkers at baseline (before therapy initiation) and 8 years of age

Baseline soluble Immune Biomarkers	8-year soluble Immune Biomarkers	Spearman Correlation (*r* Coefficient)	Spearman Correlation (p-value)	n
IL-1RA (Before)	IL-5 (age 8)	+0.35	**0.03**	42
	LBP (age 8)	+0.37	**0.02**	40
IL-1α (Before)	IL-1α (age 8)	+0.32	**0.04**	42
	RANTES (age 8)	-0.34	**0.03**	42
	TGFβ1 (age 8)	+0.36	**0.04**	34
	TGFβ2 (age 8)	+0.39	**0.02**	34
	TGFβ1 (age 8)	+0.37	**0.03**	34
IL-4 (Before)	PDGF-BB (age 8)	+0.32	**0.04**	42
IL-5 (Before)	LBP (age 8)	+0.39	**0.01**	40
IL-6 (Before)	IL-5 (age 8)	+0.33	**0.03**	42
	TNFα (age 8)	+0.34	**0.03**	42
	TNFβ (age 8)	+0.33	**0.04**	42
IL-7 (Before)	MCP-1 (age 8)	+0.36	**0.02**	42
	LBP (age 8)	+0.33	**0.04**	40
IL-8 CXCL8 (Before)	LBP (age 8)	+0.35	**0.03**	40
IL-10 (Before)	IL17F (age 8)	+0.38	**0.03**	35

Table 5.14 (continued)

IL-12p70 (Before)	MCP-1 (age 8)	+0.36	**0.02**	42
IL-13 (Before)	IL-5 (age 8)	+0.39	**0.01**	42
	IL-13 (age 8)	+0.32	**0.04**	42
	TNFβ (age 8)	+0.33	**0.03**	42
	LBP (age 8)	+0.41	**<0.01**	40
	LBP (age 8)	+0.32	**0.04**	40
IL-17A (Before)	IL-1RA (age 8)	+0.31	**0.04**	42
	IL-1α (age 8)	+0.32	**0.04**	42
	IL-4 (age 8)	+0.39	**0.01**	42
	IL-5 (age 8)	+0.32	**0.04**	42
	IL-15 (age 8)	+0.33	**0.03**	42
	MIP-1β (age 8)	+0.36	**0.02**	42
	IFN-α (age 8)	+0.33	**0.03**	42
	FGF/EGF (age 8)	+0.34	**0.03**	42
IP-10 (Before)	IL-2 (age 8)	-0.36	**0.02**	42
	IL-7 (age 8)	-0.38	**0.01**	42
	IL-12p70 (age 8)	-0.33	**0.03**	42
	IL-17A (age 8)	-0.33	**0.03**	42
	TGFβ1 (age 8)	-0.53	**<0.01**	34
	TGFβ2 (age 8)	-0.54	**<0.01**	34
	TGFβ3 (age 8)	-0.52	**<0.01**	34
IFN-y (Before)	IP-10 (age 8)	+0.32	**0.04**	42
MCP-1 (Before)	IL-7 (age 8)	-0.33	**0.03**	42
	MCP-1 (age 8)	+0.41	**<0.01**	42
	TGFβ3 (age 8)	-0.45	**<0.01**	34
MIP-1α (Before)	TGFβ3 (age 8)	-0.46	**<0.01**	34
TNFα (Before)	TGFβ3 (age 8)	-0.50	**<0.01**	34
	LBP (age 8)	+0.40	**0.01**	40
IFN-α (Before)	MCP-1 (age 8)	+0.31	**0.04**	42
RANTES (Before)	CD40L (age 8)	-0.37	**0.02**	42
TGFβ2 (Before)	IL-18 (age 8)	-0.43	**0.01**	34
	IL-1β (age 8)	-0.34	**0.03**	42
	IL-18 (age 8)	-0.52	**<0.01**	34
TGFβ3 (Before)	IL17F (age 8)	+0.36	**0.03**	35
	sCD163 (age 8)	-0.42	**0.01**	34
sCD163 (Before)	IL-4 (age 8)	-0.31	**0.04**	43
	IL-5 (age 8)	-0.35	**0.02**	43
	IL-8 CXCL8 (age 8)	-0.31	**0.04**	43
	MIP1α (age 8)	-0.36	**0.02**	43
	TGFβ2 (age 8)	-0.38	**0.02**	35
IL-18 (Before)	MCP-1 (age 8)	+0.35	**0.02**	43

We evaluated the effect of early and late clinical parameters including time to therapy initiation, time to viral suppression, interruption time, birth weight and height, gestation time, CD4 and CD8 count and percentage at

birth, CD4 and CD8 count and percentage at 8 years of age as well as the CD4:CD8 ratio at 8 years of age on the levels of plasma immune biomarkers at various time points (before therapy initiation, 2 years after therapy initiation and at 8 years of age) – Figures 5.11,5.12 and 5.13 provide correlation heat map diagrams showing associations at the three latter described time points. Spearman correlation rankings were completed between each clinical parameter and plasma immune biomarker and tabulated (with *r* coefficients and p-values) in Tables 5.15, 5.16 and 5.17 respectively. We found that time to therapy initiation, time to viral suppression, CD4 and CD8 percentages were strong predictors of immune biomarkers at all time points measured.

At baseline (before therapy initiation) the key clinical predictors of plasma biomarker expression include (1) time to therapy initiation, (2) time to viral suppression, (3) longitudinal CD4% and absolute count and (4) CD4 and CD8% and absolute counts at birth.

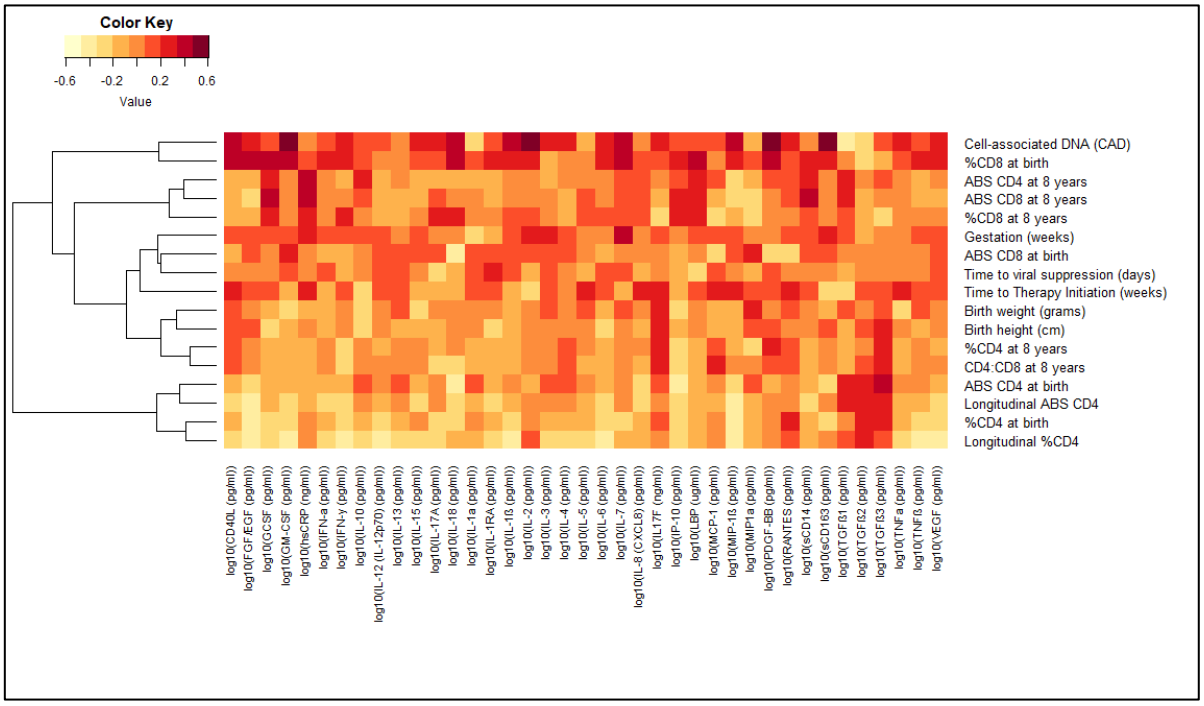


Figure 5.10: *Correlation heat map of clinical parameters and plasma immune biomarkers at baseline (before therapy initiation).*
Colour scaling ranging from a highly negative correlation in yellow (left) to a highly positive correlation in dark red (right) depicts the Pearson Correlation Coefficient (R). The R coefficients and probabilities for both Pearson and Spearman correlations are listed in the table below.

Table 5.15: Summary of all statistically significant Spearman correlation coefficients (*r*) as well as probabilities following correlation analysis of early clinical parameters and baseline (before therapy initiation) plasma immune biomarkers at 8 years of age

Clinical Parameter	Immune biomarker levels before therapy initiation	Spearman Correlation (*r* Coefficient)	Spearman Correlation (p-value)	n
Time to Therapy Initiation (weeks)	IL17F (ng/ml))	+0.34	**0.03**	41
	IL-2 (pg/ml))	-0.33	**0.04**	41
Time to viral suppression (days)	LBP (ug/ml))	-0.31	**0.04**	44
Gestation (weeks)	(IL-7 (pg/ml))	+0.36	**0.02**	40
	sCD163 (pg/ml))	+0.32	**0.04**	41
Longitudinal %CD4	IL-1ß (pg/ml))	-0.38	**0.01**	42
	IL-6 (pg/ml))	-0.35	**0.03**	42
	IL-12 (IL-12p70) (pg/ml))	-0.34	**0.03**	42
	IL-17A (pg/ml))	-0.32	**0.04**	42
	IFN-y (pg/ml))	-0.38	**0.01**	42
	MIP-1ß (pg/ml))	-0.46	**<0.01**	42
	TNFβ (pg/ml))	-0.37	**0.02**	42
	GM-CSF (pg/ml))	-0.44	**<0.01**	42
	CD40L (pg/ml))	-0.32	**0.04**	42
	VEGF (pg/ml))	-0.37	**0.02**	42
	FGF/EGF (pg/ml))	-0.37	**0.02**	42
	IP-10 (pg/ml))	-0.34	**0.03**	42
	MIP-1ß (pg/ml))	-0.39	**0.01**	42
	FGF/EGF (pg/ml))	-0.42	**<0.01**	42
%CD8 at birth	IL-7 (pg/ml))	+0.44	**0.02**	30
	GCSF (pg/ml))	+0.37	**0.04**	30
	CD40L (pg/ml))	+0.39	**0.03**	30
	FGF/EGF (pg/ml))	+0.41	**0.02**	30
	IL-18 (pg/ml))	+0.46	**<0.01**	32
	LBP (ug/ml))	+0.46	**<0.01**	32
ABS CD8 at birth	IL-18 (pg/ml))	-0.36	**0.04**	32
%CD4 at birth	IFN-y (pg/ml))	-0.33	**0.03**	42
	MIP-1ß (pg/ml))	-0.37	**0.02**	42
	FGF/EGF (pg/ml))	-0.34	**0.03**	42
	IP-10 (pg/ml))	-0.37	**0.01**	42
	IL-18 (pg/ml))	-0.37	**0.02**	43

Following the 2 years of therapy the key clinical predictors of plasma biomarker expression include (1) time to therapy initiation, (2) time to viral suppression, (3) birth weight (4) gestation, (5) longitudinal CD4% and absolute count and (6) CD4 and CD8% and absolute counts at birth.

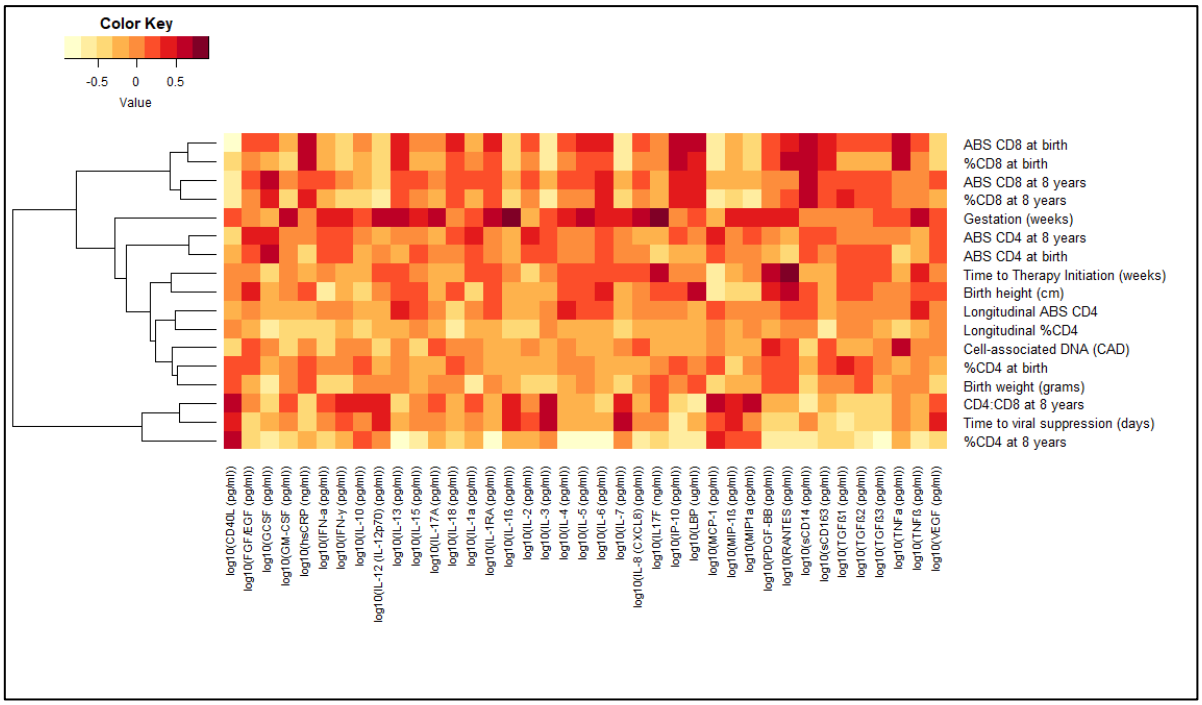


Figure 5.11: *Correlation heat map of clinical parameters and plasma immune biomarker levels following two years of therapy initiation.*
Colour scaling ranging from a highly negative correlation in yellow (left) to a highly positive correlation in dark red (right) depicts the Pearson Correlation Coefficient (R). The R coefficients and probabilities for both Pearson and Spearman correlations are listed in the table below.

Table 5.16: Summary of all statistically significant Spearman correlation coefficients (*r)* as well as probabilities following correlation analysis of early clinical parameters and plasma immune biomarker levels following two years of therapy initiation

Clinical Parameter	Immune biomarker levels at 8 years of age	Spearman Correlation (*r* Coefficient)	Spearman Correlation (p-value)	n
Time to Therapy Initiation (weeks)	IL17F (ng/ml))	+0.59	**0.04**	12
	RANTES (pg/ml))	+0.73	**<0.01**	12
	PDGF-BB (pg/ml))	+0.66	**0.02**	12
Time to viral suppression (days)	IL-3 (pg/ml))	+0.64	**0.03**	11
Gestation (weeks)	IL17F (ng/ml))	+0.72	**0.01**	11
	IL-1ß (pg/ml))	+0.73	**0.01**	11
	IL-1RA (pg/ml))	+0.65	**0.03**	11
	IL-5 (pg/ml))	+0.65	**0.03**	11
	IL-8 (CXCL8) (pg/ml))	+0.66	**0.03**	11
	TNFβ (pg/ml))	+0.67	**0.02**	11
	GM-CSF (pg/ml))	+0.67	**0.02**	11
Longitudinal %CD4	IL-7 (pg/ml))	-0.45	**<0.01**	33
	IL-10 (pg/ml))	-0.47	**<0.01**	33
	TNFα (pg/ml))	-0.51	**<0.01**	33
	GCSF (pg/ml))	-0.53	**<0.01**	33
	GM-CSF (pg/ml))	-0.39	**0.02**	33
	sCD163 (pg/ml))	-0.57	**<0.01**	33
	IL-18 (pg/ml))	-0.59	**<0.01**	33
	hsCRP (ng/ml))	-0.38	**0.03**	33

At 8 years of age the key clinical predictors of plasma biomarker expression include (1) time to therapy initiation, (2) time to viral suppression, (3) birth weight (4) gestation, (5) longitudinal CD4% and absolute count and (6) CD4 and CD8% and absolute counts at birth.

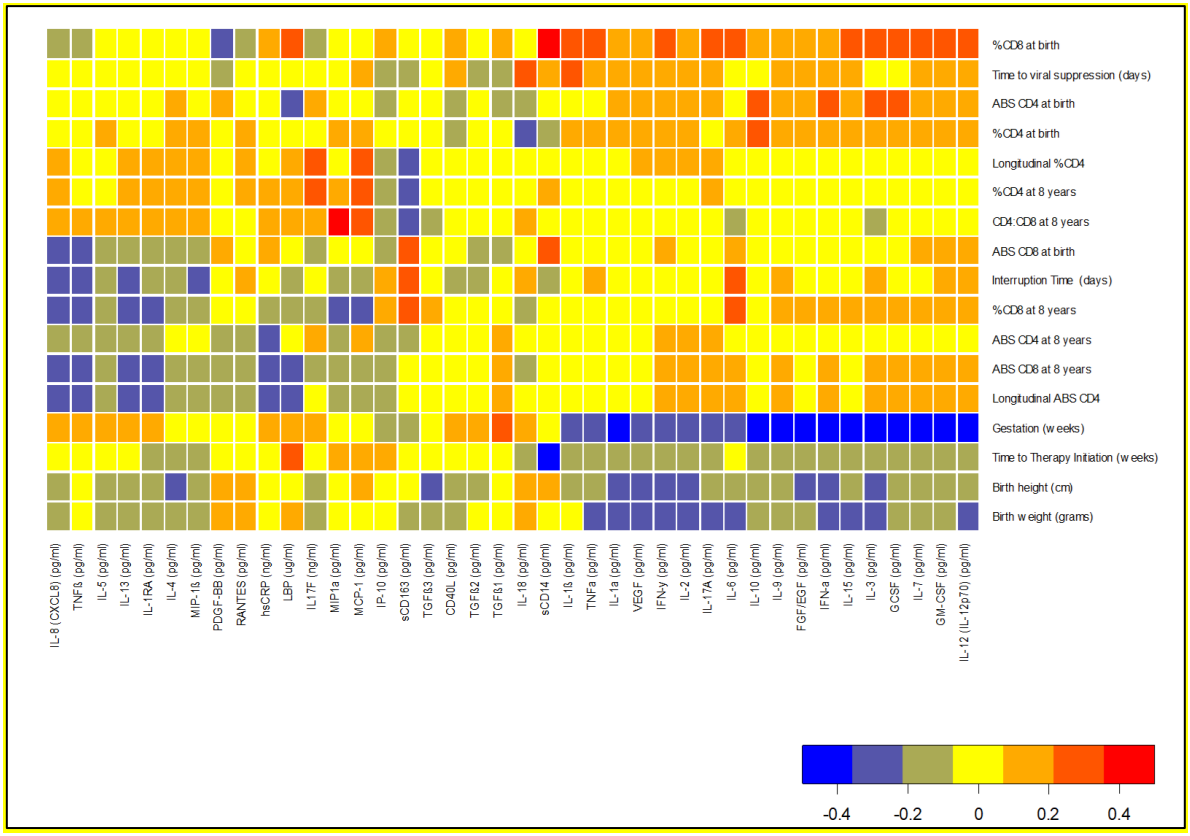


Figure 5.12: *Correlation heat map of early clinical parameters and plasma immune biomarkers at 8 years of age. Colour scaling ranging from to a highly negative correlation in blue (left) to a highly positive correlation in red (right) depicts the Pearson Correlation Coefficient (R). R coefficients and probabilities for both Pearson and Spearman correlations are listed in the table below.*

Table 5.17: Summary of statistically significant Pearson and Spearman correlation coefficients (*r)* as well as probabilities following correlation analysis of early clinical parameters and plasma immune biomarkers at 8 years of age

Clinical Parameter	Immune biomarker levels at 8 years of age	Spearman Correlation (*r* Coefficient)	Spearman Correlation (p-value)	n
Time to Therapy Initiation (weeks)	LBP (ug/ml)	+0.33	**<0.01**	67
Time to viral suppression (days)	IL-7 (pg/ml)	+0.25	**0.03**	74
	IL-12 (IL-12p70) (pg/ml)	+0.24	**0.04**	74
	IL-17A (pg/ml)	+0.26	**0.02**	74
	IFN-y (pg/ml)	+0.27	**0.02**	74
	TNFα (pg/ml)	+0.25	**0.03**	74
Birth weight (grams)	IL-2 (pg/ml)	-0.25	**0.03**	73
	IL-6 (pg/ml)	-0.25	**0.03**	73
	IL-17A (pg/ml)	-0.24	**0.04**	73
	RANTES (pg/ml)	+0.24	**0.04**	73
Gestation (weeks)	IL-6 (pg/ml)	-0.26	**0.03**	69
	IFN-a (pg/ml)	-0.25	**0.03**	69
	FGF/EGF (pg/ml)	-0.25	**0.04**	69
Longitudinal %CD4	IL17F (ng/ml)	+0.32	**0.01**	65
	MCP-1 (pg/ml)	+0.32	**<0.01**	74
	sCD163 (pg/ml)	-0.30	**0.02**	63
Longitudinal ABS CD4	IL-1RA (pg/ml)	-0.24	**0.04**	74
	IL-5 (pg/ml)	-0.24	**0.04**	74
	IL-8 (CXCL8) (pg/ml)	-0.34	**<0.01**	74
	IL-13 (pg/ml)	-0.28	**0.02**	74
	MIP1a (pg/ml)	-0.23	**0.04**	74
	TNFβ (pg/ml)	-0.29	**0.01**	74
	LBP (ug/ml)	-0.25	**0.03**	71
%CD8 at birth	IL-6 (pg/ml)	+0.39	**<0.01**	55
	IL-7 (pg/ml)	+0.29	**0.03**	55
	GCSF (pg/ml)	+0.30	**0.02**	55
	sCD14 (pg/ml)	+0.36	**0.02**	46
ABS CD8 at birth	sCD163 (pg/ml)	+0.37	**0.01**	46
	hsCRP (ng/ml)	+0.28	**0.04**	53
ABS CD4 at birth	CD40L (pg/ml)	-0.24	**0.04**	72
	IL-18 (pg/ml)	-0.26	**0.04**	61
	LBP (ug/ml)	-0.25	**0.04**	69
%CD4 at 8 years	IL17F (ng/ml)	+0.32	**<0.01**	63
	MCP-1 (pg/ml)	+0.32	**<0.01**	72
	sCD163 (pg/ml)	-0.27	**0.04**	61
ABS CD8 at 8 years	IL-8 (CXCL8) (pg/ml)	-0.34	**<0.01**	72
	IL-13 (pg/ml)	-0.26	**0.03**	72
	TNFβ (pg/ml)	-0.26	**0.03**	72
	hsCRP (ng/ml)	-0.26	**0.03**	69
%CD8 at 8 years	IL-8 (CXCL8) (pg/ml)	-0.27	**0.02**	72
	MCP-1 (pg/ml)	-0.28	**0.02**	72
CD4:CD8 at 8 years	MCP-1 (pg/ml)	+0.35	**<0.01**	72
	sCD163 (pg/ml)	-0.28	**0.03**	61

5.4 Discussion

The effect of HIV-1 on the maturing immune system in particular, the interplay of soluble immune biomarkers is not well understood in children with PHIV subjected to early long-term antiretroviral treatment with interruption.

To our knowledge, this is the first extensive study evaluating the longitudinal outcome of soluble immune biomarkers in PHIV South African children from infancy to pre-adolescence. Knowledge obtained from exploring these immune biomarkers over time in relation to therapy regimes such as delayed, early continuous, and interrupted treatment is significant for remission and immune therapy strategies in the field towards HIV cure.

Overall, our findings show a complex interplay of these soluble biomarkers over time that is governed by factors such as clinical outcomes at birth, age, virological parameters as well as morbidities acquired during the course of HIV-1 infection.

When evaluating the levels of the 40 soluble biomarkers, we observed, following composite data analysis, that therapy grouping had minimal impact on the levels of majority of these soluble immune biomarkers. However, significant differences were observed for those that received early, continuous therapy particularly with an elevation in IL-17F, IL-2, IL-4, IL-7, IL-13, IFN-γ, TNFβ, sCD40L, IFN-α, PDGF, $TGF\beta_3$ and LBP levels. Most of the latter biomarkers function as pro-inflammatory biomarkers. When evaluating early clinical parameters of this group, it is worth noting that these children had decreased gestation time, lower birthweights, lower CD4 T cell counts and higher levels of HIV-1 CAD before therapy initiation.

In a recent Brazilian study assessing adolescents and young adults who acquired vertical HIV-1 infection in the 1990s and received cART relatively late, young adults on ART for approximately 16 years and had detectable viral loads, produced less IFN-γ, IL-10, IL-13, IL-17A, IL-5 and IL-6 than the HIV-1 positive group with undetectable viral loads (Munhoz et al., 2019). In the same study, young adults with undetectable viral loads on therapy for approximately 14 years, had increased levels of ITAC, IL-4 and IL-23 than age matched HIV negative controls. In summary, this study observed a pervasive decrease in cytokine production in young adults with detectable viral loads – i.e., a shift from Th1 responses to Th2 responses. This Th1-Th2 imbalance contributes to HIV-1 associated immune dysregulation and suggests that a Th1 to Th2 dominance is linked with resistance to HIV-1 infection and/or the progression to AIDS as hypothesised by (Clerici and Shearer, 1993). The latter study based its conclusion on the findings that progression to AIDS was characterised by a loss of IL-2 and IFN-γ production and a related increase in IL-4 and IL-10 levels.

Other studies have documented an association of lowered IL-10 expression with a depletion of regulatory T cells specific to gut-associated lymphoid tissues (GALT). HIV infection is known to destroy Th17 associated cells in the GALT and contributes to a decrease in IL-17A expression and subsequent increase in T cell activation leading to increased microbial translocation (Reuter, Pombo and Betts, 2012).

In the Brazilian study mentioned above, HIV infected young adults with undetectable viral loads also produced IL-17A levels that was comparable to the control group and that was significantly higher than the group of

infected adults with detectable viral loads. A relationship of IL-17A to IL-23 could indicate an attempt of the immune system to return to homeostasis (Fernandes et al., 2017; Munhoz et al., 2019).

Despite a gap in the current knowledge of cytokine expression in both healthy and vertically HIV infected children, age has been documented to play a role in the expression patterns of children (Decker, Grobusch and Ritz, 2017). Although the expression frequency of IFN-γ and TNFα is generally detected in differentiating frequency, a number of studies have documented an age-associated increase in these biomarkers (Härtel et al., 2005; Yerkovich et al., 2007; Djuardi et al., 2016; Decker, Grobusch and Ritz, 2017).

Immune maturation in the first five years of life is expected to be most prominent. However, a study evaluating the first five years of life of healthy children, showed no association of any of the immune biomarkers investigated. The average concentration of all cytokines measured were documented to be below 10pg/ml (Berdat et al., 2003).

In healthy children, pro-inflammatory cytokine, IL-1RA and IL-6 showed a bimodal (having one peak) pattern (Sack et al., 1998).

In addition to age, demographical location may also play a role in cytokine release. In four cohorts from South Africa, Belgium, Ecuador and Canada, Smolen et. al. documented innate immune responses in early life. Following PRR stimulation, South African children secreted significantly less cytokines compared to the other three cohorts (Smolen et al., 2014). On a single-cell level, a reduction in intracellular cytokine production in monocytes and dendritic cells. In addition, polyfunctionality in each cell compartment was lowered in South African children. This decreased cytokine responses may provide challenges in terms of vaccine responses and increased risks for diseases. Further studies are therefore required.

Planned treatment interruptions have received deliberating reviews in both adult and paediatric cases. Two key clinical trials commissioning planned treatment interruption in children included the Paediatric European Network for Treatment of AIDS (PENTA) II (Castro, 2010) and the CHER trial (Cotton et al., 2013a). Both clinical trials reported no serious clinical or immunological outcomes following structured and carefully monitored treatment interruption. The PENTA trial showed improved CD4 T cell recovery following therapy re-initiation in children but not in adults (Bunupuradah et al., 2013). Adult interruption studies have not shown positive outcomes with increased incidence of infection and mortality documented in a number of studies following re-initiation of therapy and is therefore not recommended (Ananworanich et al., 2006; Danel et al., 2006; Marchou et al., 2007; Palmisano et al., 2007; Latif et al., 2008). Both the PENTA II and CHER trials therefore imply that treatment interruption may therefore be more appropriate for children than adults due to differing immunological landscape of children compared to adults. In the early life stages of children, mild immunosuppression is staged however, due to greater functional thymus activity in children, following the initiation of therapy, children exert greater immune reconstitution compared to adults (Franco et al., 2000; De Rossi et al., 2002; Sabin, 2008; Lewis et al., 2017).

In 2019, the European Pregnancy and Paediatric HIV Cohort Collaboration (EPPIC) study assessed parameters of immune recovery following ART interruption in children and adolescents with HIV infection in Europe and

Thailand (Galli et al., 2019). The review evaluated pooled data from 19 HIV cohorts in 17 countries. An additional aim to this study was to understand the factors that can predict improved immunological recovery following re-initiation of ART. Key findings of this study indicated that immunological recovery after a planned treatment interruption is more efficient in younger children with higher CD4 percentages at the time of interruption. Factors such as younger age and higher CD4 nadir served as good predictors of adequate immune reconstitution (Galli et al., 2019). Treatment interruption strategies have gained much attention over the last few years and may be implemented to better understand the efficacy of interventions that aims to achieve sustained virological remission in the absence of ART (Lau et al., 2019).

The importance of monitoring biomarkers of inflammation in interruption studies have been highlighted in studies of the SMART trial (Kuller et al., 2008; Baker et al., 2011).

5.5 Conclusion and Future Perspectives

Extensive longitudinal analysis of immune modulators (biomarkers) indicates a complex interplay between cytokines, chemokines and growth factors that shape the landscape for disease progression. While for some immune biomarkers, cART may contribute to circumventing further immune activation and inflammation, it may not on its own prevent long-term immune dysregulation. Other factors that contribute to cytokine and chemokine patterns in PHIV children relates to early clinical outcomes such as time to therapy initiation, time to reach virological suppression and CD4 and CD8 percentages. Extensive composite cytokine and chemokine data is also useful for assessing associations with parameters such as iCAD and changes in immune subsets.

CHAPTER 6:

EVALUATION AND IMPLEMENTATION OF A VIRAL OUTGROWTH ASSAY (VOA) FOR THE MEASUREMENT OF HIV-1 LATENCY IN EARLY-TREATED AND LONG-TERM SUPPRESSED PERINATALLY INFECTED CHILDREN

SUMMARY OF KEY FINDINGS – CHAPTER 6

- Of the 10 early treated children with long-term virological suppression, none had detectable replication competent virus by Q-VOA at 7-8 years.
- However, despite little HIV-1 cell-associated DNA (iCAD), we detected inducible virus using a highly sensitive single-copy assay for HIV-1 RNA (iSCA).
- A key limitation to this study is the restricted blood volume available from children which limit sensitivity to detect inducible virus.
- More sensitive molecular assays such as iSCA (also excludes HIV-1 DNA contamination), may be a more sensitive means of detecting inducible virion production in cell culture supernatants from persistent HIV reservoirs in children.

- ***Conclusion****: Although the QVOA is considered the gold standard to measuring the replication competent HIV-1 reservoir, we were unable to adequately implement this assay within our virologically suppressed paediatric population due to low sample acquisition and input. In addition, this assay is expensive and unsuitable for our resource-limited setting. Measurement of HIV-1 specific antibody concentrations have been shown to be associated with HIV-1 viral persistence and may provide a good estimate of the residual reservoir in virologically suppressed children. Furthermore, HIV-1 antibody measures may also provide a means for the screening of children on suppressive cART for HIV-1 remission studies.*

ABSTRACT

Background: Early cART initiation in infants reduces the number of HIV-1 infected cells and may delay viral rebound after cART interruption. It is unknown whether the few HIV-1 infected cells that persist on long-term ART harbour proviruses capable of virion production. This study was undertaken to evaluate how often HIV-1 is inducible from CD4+ T lymphocytes in early ART treated children.

Methods: Stored PBMC samples were analysed from post-CHER participants who began cART at <1 year of age and sustained suppression of viremia through to time of sampling at age 7-8. Total HIV-1 DNA in PBMC was quantified by qPCR targeting a conserved region in integrase. Total inducible virus recovery (TVR) assays were performed on purified CD4+ T cells stimulated with PMA and ionomycin for 7 days. Virus outgrowth assays (VOA) were also performed with purified CD4+ T cells, activated with phytohemagglutinin and co-cultured for 28 days with irradiated HIV-1 negative feeder cells and CD8-depleted blasts. Depending on CD4+ T cell recovery, replicates of $1x10^6$ CD4+ T cells were seeded for both assays. Supernatants were collected on day 8 for the TVR assay and days 7, 14, 21 and 28 for VOA. HIV-1 RNA was measured in cell culture supernatants using a HIV-1 qPCR assay targeting *integrase* Single Copy Assay (iSCA) with single-copy sensitivity. VOA supernatants were also assayed for HIV-1 p24 antigen by EIA.

Results: Samples from 10 children (6 females, 4 males) were studied (median cART initiation age: 6 months) after 7-8 years of viremia suppression. Five participants had pre-ART HIV RNA >750,000 copies/ml and 5 had a median pre-ART HIV RNA of 635,000 copies/ml. The median CD4 percentage at the age of 7-8 years was 39%. Median cell associated HIV-1 DNA was 38 copies per million cells (range: 4.5-186). Two of 10 children had inducible HIV-1 RNA by TVR assay detected by iSCA at 2267 and 24 copies/ml, respectively. All VOA supernatants collected were negative for HIV-1 p24 antigen, but 4 participants had detectable HIV-1 RNA at day 21 (range: 9-697 copies/ml). No-RT controls excluded HIV-1 DNA contamination.

Conclusion: Children initiated on cART early did not have infectious virus that could be readily isolated in VOAs. However, inducible virion production was detected in 4 of 10 children using a highly sensitive HIV-1 RNA assay. This assay may be a sensitive biomarker for virion production in latent HIV reservoirs from children from whom only small sample volumes are feasible.

6.1 Introduction

The majority of paediatric HIV-1 infections are acquired perinatally, thereby giving rise to prevention of PMTC programmes contributing to the decline in the number of children born to mothers living with HIV (MLWH) from 28 000 in 2010 to 14 000 in 2018 (Teshale et al., 2021).

The accessibility to improved antiviral drugs in both low- and middle-income countries is considered a key milestone in the history of HIV disease (Simelela and Venter, 2014). The efficacy of combination ART (cART) has significantly decreased the morbidity and mortality rates related to HIV infection over the last 18 years (Mocroft et al. 2003; Braitstein et al. 2006; Who & Unicef 2011; Simelela & Venter 2014). Treatment regimens have become simpler and less toxic and have converted HIV infection into a chronic and manageable condition (Palella et al., 1998; Palmisano and Vella, 2011).

The use of cART has shown to effectively supress the HI virus to below detection levels (<40 copies/ml) allowing infected individuals to control their infection and live long and productive lives (Lewin et al. 2011). However, despite the high efficacy of cART in decreasing viral loads to undetectable levels, it is not curative.

Following the publicity of the "Berlin Patient" (Hütter et al. 2009) and more recently the "London patient", the only two cases of "HIV cure" documented to date (Scarborough, Goguen and Gatignol, 2019) as well a few cases of functional cure in early treated children such as the case of the "Mississippi Baby" (Persaud et al. 2013), the HIV research agenda has been invigorated in a new quest for eradication and cure strategies.

It is well documented that activated CD4+ T cells, expressing increased amounts of the CD4 receptor, serve as the main target cells of the HIV-1 virus. Infection of these CD4+ T cells is followed by viral replication and subsequent integration of viral DNA into the host genome (Sonza et al. 2001; Chomont et al. 2009). When the provirus integrates into long-living resting memory cells, it can survive without viral replication - this is termed viral latency and allows for preserving HIV-1 reservoirs of replication-competent virus despite suppression with cART.

Most latently infected cells contain a single copy of HIV-1 DNA that is stably integrated into the host's genome and is considered transcriptionally silent. These HIV-1 proviruses are also capable of resuming replication following the discontinuation of ART (Josefsson et al., 2011, 2013; Barton, Winckelmann and Palmer, 2016).

HIV-1 latent reservoirs are established during the first week of infection (Chun et al., 1998; Schacker et al., 2001; N. M. Archin et al., 2012; Svicher et al., 2014) and can also be localised in various anatomical regions, organs and cell types including the thymus (Scinicariello et al., 2010; Fabre-Mersseman et al., 2011), gastrointestinal tract (GIT) and brain (Chun et al. 2008), perivascular macrophages, parenchymal microglia and astrocytes (Thompson et al. 2011).

The generation and preservation of a silent provirus in resting memory CD4+ T cells is well-characterised and theoretically the most vital mechanism for HIV-1 persistence (Finzi et al. 1997; Wong et al. 1997; Chun et al. 1997). However, more recent studies show that latency, a stochastic event, can be established and may persist in activated and resting CD4+ T cells (Chavez, Calvanese and Verdin, 2015; Barton, Winckelmann and Palmer, 2016).

Researchers have also demonstrated HIV persistence in three key memory T cell subsets, each with specific functional and survival capabilities. These include central memory (Tcm) (CD45RA-CCR7+CD27+), transitional memory (Ttm) (CD45RA-CCR7-CD27+) and effector memory (Tem) (CD45RA-CCR7-CD27-) CD4+ T cells (Chomont et al. 2009; Klatt et al. 2013). T stem cell memory (Tscm), which account for 2-4% of circulating lymphocytes, are considered robust HIV-1 reservoirs, relatively stable and preserved through "homeostatic self-renewal" (Gattinoni et al. 2011; Buzon et al. 2014a). In addition, a study by Banga et al. (2016) showed that T follicular helper cells (Tfh) expressing cell surface receptors, CXCR5 and programmed cell death (PD-1), are a key source of replication-competent HIV-1 virus in treated avireamic patients.

Latent HIV reservoirs are undetected by the hosts' immune system, persist despite long-term therapy and are therefore considered as one of the key barriers to eradication and cure (Chun and Fauci, 2012; Eisele and Siliciano, 2012). Investigations evaluating the long-term decay or kinetics of HIV-1 reservoirs show that infected cells could persist for as long as 60 years. Various mechanisms for this on-going persistence include constant replenishing of the reservoir through cell-to-cell spread, cell-associated replication or possible residual replication in MALT (Klatt et al. 2013). Modelling studies investigated by Rong & Perelson (2009) suggest the production of intermittent viral blips generated whilst replenishing the latent reservoir through homeostatic proliferation and/or de novo infection measures occur by the activation of latently infected cells that can retain persistent low-level viraemia.

Studies evaluating the role of appropriate timing of ART initiation in relation to the HIV-1 reservoir size have shown varying outcomes (Lori et al. 1999; Zhang et al. 1999; Blankson et al. 2000; Jain et al. 2013). Most studies report a positive correlation between early ART initiation and a diminished HIV-1 reservoir size. Strain et al. (2005) demonstrated that initiation of ART within the first 6 months of infection was associated with substantially decreased cell-associated HIV infectivity. Another study assessing early versus late treatment found that HIV-1 persistence remained relatively stable during therapy. It was however, suggested that the delay of cART in patients may lead to a permanently enlarged HIV reservoir resulting a reduced ability to clear the latent reservoir (Jain et al. 2013).

Data generated from the VISCONTI cohort, showed that early cART initiation as well as prolonged therapy during primary infection resulted in a lower pool of HIV-1 CAD (Sáez-Cirión et al. 2013). A study conducted by Persaud et al. (2012) showed a reservoir size below detection limits of the assay at 48 weeks in more than 50% of children given cART at a median of 8 weeks of age. Another study conducted in the USA showed that five adolescents given suppressive ART within two months of age, had smaller latent reservoirs with no infectious HIV recovery when compared to adolescents who received treatment later (Luzuriaga et al. 2014).

Children who had early therapy initiation, showed notably lower levels of both HIV-1 CAD and RNA (Luzuriaga et al. 2014; Ananworanich et al. 2014; Martinez-Bonet et al. 2015). In the CHER cohort, children in which ART was initiated before 2 months of age, showed decreased frequency of HIV-1 infected CD4+ T cells at 7-8 years of age compared with those children who received therapy later (Van Zyl et al. 2015).

HIV-1 latent reservoirs, the major barriers to cure are therefore the main targets for both functional and sterilizing cures. The latter refers to a complete elimination of the latent reservoir such as that described in the

"Berlin" and "London" patients. The complete suppression or silencing of the HIV-1 latent reservoir by sustaining virological control without ARVs is considered a functional cure and is also referred to as HIV remission (Thomas et al., 2020).

Prominent cases of HIV-1 remission have been documented in children including the "Mississippi baby" initiated on intense ART within 30 hours of birth and continued for 18 months after which therapy was terminated. Viral loads remained undetected for 27 months following the cessation of therapy. There was no evidence of viral rebound, the detection of HIV-1 RNA I blood plasma or the presence of HIV-1 antibodies (Persaud et al., 2013). Due to the detection of low levels of proviral HIV-1 DNA the case was considered as remission or an achievement of a functional cure. HIV-1 remission was held for just over two years when a HIV-1 RNA level of just under 17 000 copies/ml was detected. A hypothesis explaining the unfortunate viral rebound put forward by Robert F. Siliciano alludes to initiating intense ART before the infant produced a sufficient pool of memory T cells which serve as important viral reservoirs. If treatment was initiated before the development of these immune cells, there may be no target "hiding" cells for the virus to remain latent (Check Hayden, 2013). Other suggestions for viral rebound following remission include a limited adaptive immune response with viral rebound increasing rapidly after CD4+ T cell decline (Luzuriaga et al., 2015) and what appeared as remission was essentially a slow or delay in viral rebound due to a limited reservoir size (Hill et al., 2016).

The second case of HIV-1 remission was observed a French girl initiated on ART at three months of age of which cessation of therapy occurred between five and seven years of age. Virological control with undetected HIV-1 RNA was maintained for over 12 years (Frange et al., 2016).

A South African child originally enrolled into the CHER trial in 2004 (Violari, et al., 2008; Cotton et al., 2013b) represents the third case of HIV-1 remission described in children. Therapy cessation occurred just under 1 year of age (50 weeks) and has been able to maintain long-term virological control for over 10 years. At 9.5 years of age, researchers detected HIV-1 plasma RNA level of 6.6 copies/ml, a HIV-1 cell-associated DNA level of 5 copies per million PBMCs and undetected replication-competent virus. Other immunological assays concluded high CD4:CD8 ratios, decreased T cell activation as well as a weak HIV-specific CD4+T cell and antibody response (Violari et al., 2019).

These cases of post-treatment control, although more extensively described in adult populations (Sáez-Cirión et al., 2013b; Maggiolo et al., 2018) than paediatric populations may provide pertinent insights into the crucial host determinants for controlling HIV-1 replication, halt HIV persistence and inform various strategies for remission and cure.

The pathogenesis of HIV-1 infection and establishment of latent reservoirs are seemingly different in children than in adults and further highlights the need for further defining of various immunological mechanisms in paediatric HIV infection. Under optimal circumstances, therapy in HIV-1 infected children can be initiated earlier than in adults. Following the discontinuation of ART, early treated HIV-1 infected children showed improved control of viral replication and therefore serve as good candidates for evaluating HIV remission or eradication strategies (Goulder, Lewin and Leitman, 2016a). Cell-associated HIV-1 DNA levels have been

shown to decline over a longer period than when compared to adults that seem to reach a plateau at approximately 4 years of infection (Besson et al., 2014). Studies have also indicated a shorter half-life of latently infected resting CD4+ T cells in children compared to adults (Goulder, Lewin and Leitman, 2016a). Other factors that may contribute to the significance of evaluating early treated children as "cure" candidates include a tolerogenic environment (Th17 bias) in early life that favours low levels of immune activation and a high proportion of naïve CD4+ T cells that are present (Mold et al., 2008; Bunders et al., 2012). In addition, children who have been HIV-1 infected in utero display HIV-specific CD8+ T cell responses that are present in early infancy; however, early responses may not have generated a memory response. Early treated HIV infected children, therefore, serve as relevant study candidates for a potential T cell based vaccine (Luzuriaga et al., 1995; Thobakgale et al., 2007; Gray et al., 2014; Goulder, Lewin and Leitman, 2016a).

Suggested strategies for reservoir depletion require multiple approaches since HIV-1 latency is itself multifactorial. The impact and timing of early ART treatment is therefore significant when considering eradication strategies (Archin and Margolis, 2014). Furthermore, the measurement and composition of the HIV latent reservoirs are significantly important for understanding and then developing strategies for cure.

The gold standard for measuring the occurrence of replication-competent proviruses contained within resting CD4+ T cells utilises viral outgrowth assays (VOAs) developed in the mid-1990s (Finzi et al. 1997; Finzi et al. 1999; Siliciano & Siliciano 2005). These VOAs essentially involve the use of a strong T cell mitogen, phytohaemagglutinin (PHA), which aims to activate resting CD4+ T cells. The stimulated cells are thus induced to promote expression of viral genes that ultimately leads to release of HIV-1 from latent infected cells. Following the development of VOAs, simpler measures for the latent reservoir have been developed of which many are PCR based, including the CAD assay (Rouzioux et al. 2014; O'Doherty et al. 2002; Strain et al. 2013).

Correlation studies between PCR and culture-based assays have shown several discrepancies. This is primarily due to the nature of proviruses contained within CD4+ resting cells being of heterogeneous nature (Eriksson et al. 2013; Bruner et al. 2015; Ho et al. 2013). Recent investigations highlight the challenge of available assays in providing an appropriate and accurate measurement of the reservoir size. Additional hurdles include the high cost of viral recovery assays as well as the large volume of sample required for processing (Bruner et al. 2015). These incongruities in measuring the reservoir therefore serve as major barriers to clinical trials aiming to investigate and subsequently understand the efficacy of HIV-1 eradication strategies.

In order to structure appropriate interventions or mechanisms for clearance of persisting HIV-1 infection, it is of importance to understand the hosts' immunological environment during early treatment and viral suppression. Furthermore, it is also important to understand the role of immunological responses to HIV persistence. Recently it has been proposed that latency/reservoir establishment may be an adaptation of the virus to overcome host barriers to establishing successful infection (Barton, Winckelmann and Palmer, 2016). If the virus is able to establish latency early, it has another chance to establish infection within the host should initial target cell infection be unproductive. For this reason, the size of the reservoir may be related to host immune factors, as much as to viral factors.

Studies have well-documented improved immunological outcomes with early ART initiation and subsequent virological suppression. Improved CD4+ T cell numbers and decreased levels of immune activation are key features of improved outcomes following therapy ('Cotton, Violari et al. 2013.pdf', no date; Koblavi-Dème et al., 2003; Resino et al., 2003; Kabue et al., 2012; Sáez-Cirión et al., 2013a). The CHER trial conducted in 2007 showed significantly better clinical as well as immunological outcomes in children treated early as opposed to those with deferred ART therapy (Cotton et al., 2013a). Other key studies also show that ART-mediated virological suppression reduces the activation status of both CD4+ and CD8+ T cells (Hunt et al., 2003; Robbins et al., 2009; Jain et al., 2013). Another study investigating the effects of early initiation of ART showed a normalisation in the levels of monocyte and macrophage activation marker - soluble CD163 (sCD163) (Burdo et al. 2011). However despite positive immunological outcomes following early therapy as well as virological suppression, immune abnormalities including CD4+ and CD8+ T cell activation or dysfunction may still occur and CD4+ T cell populations do not always reach normal levels even after years of suppressive therapy (Corbeau and Reynes, 2011; Klatt et al., 2013; Rajasuriar, Wright and Lewin, 2015). The ability of early ART to prevent these potentially irreversible outcomes remains unclear and studies have underlined a number of immune abnormalities that persist despite many years of viral suppression (Valdez et al., 2002; Aiuti and Mezzaroma, 2006; Kelley et al., 2009). When compared to uninfected individuals, HIV infected persons may display a reduced function of the adaptive immune compartment despite effective virological control (Lange et al., 2002; Lederman et al., 2011).

T-cell populations expressing high levels of key activation markers such as HLA-DR and CD38 during untreated HIV serve as highly significant prognosticators for disease progression. However, the relevance of these immunological markers during treatment remains relatively undefined. Some studies suggest some level of predictive significance (Hunt et al., 2011; Kaplan et al., 2011; Klatt et al., 2013). Other anomalies of immune markers characterised during effective ART treatment include decreased levels of CD28, and increased levels of CD57 and PD-1. These described markers relate to an immunological profile defined by T-cell senescence and dysfunction (Appay and Sauce, 2008; Kaplan et al., 2011; Tassiopoulos et al., 2012; Hatano et al., 2013; Sauce, Elbim and Appay, 2013).

6.2 Study Objective

To implement Total Virus Recovery (TVR) and Infectious Virus Recovery (IVR) or VOA assays for the quantification of inducible and infectious latent HIV-1 in children receiving early, continuous treatment and display virological suppression.

6.3 Materials and Methods

6.3.1 Study Design

This study involved the implementation of a culture-based reservoir detection assay followed by a cross-sectional evaluation. The validity of the QVOA utilising two key assay components i.e., Total Inducible Virus Recovery (TVR) and Infectious Virus Recovery (IVR) assays on a subset of paediatric samples was explored. Figure 6.1 below provides a snapshot of the study design implemented which will be further elaborated on.

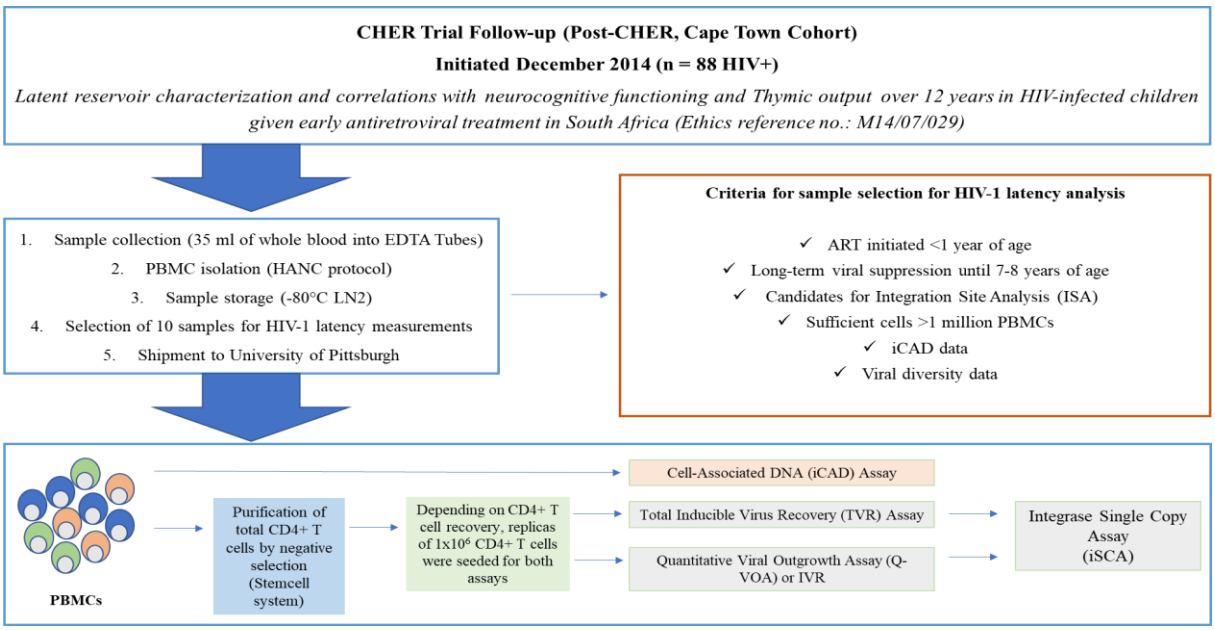


Figure 6.1: *Schematic summary of the study design and methodologies implemented for the study of HIV-1 latency in HIV-1 suppressed children of the post-CHER cohort.*
Of the 88 children followed-up, ten were selected based on specific criteria as described above. Isolated PBMCs were quantified for iCAD, and some were purified for total CD4+ T-cells *and then subjected to the TVR and QVOA/TVR assays. The iSCA assay was implemented to measure HIV-1 RNA copies as an output measure for TVR and QVOA/TVR.*

Clinical and immunological data including therapy regimen, longitudinal viral load and lymphocyte counts were collected for each of the study samples.

This sub-study was ethically approved under the Stellenbosch University Health Research Ethics Committee (Ethics reference no.: M14/07/029) as part of the larger cohort follow-up study designed to evaluate the relationship between HIV-1 latency and neurodevelopmental outcomes.

6.3.2 Study Participants and Sample Collection

Study participants originated from CHER trial. The current study served as a sub-study for the follow-up on CHER participants from the Cape Town cohort. All participants were consented, and 35ml of blood was drawn into Ethylenediaminetetraacetic acid (EDTA) tubes by an experienced research nurse for peripheral blood mononuclear cell (PBMC) extraction (described below). PBMC buffy coats were collected in cryovials at 2.5 million cells/ml and stored in liquid nitrogen (LN_2) for at -80°C.

6.3.3 Sample Selection Criteria

The following selection criteria was considered for the ten samples for which TVR and QVOA/IVR was implemented:

i. ART initiated within less than 1 year of life
ii. Virological suppression at 7-8 years of age (time of sampling)
iii. Sufficient PBMCs available for experiment (>1 million PBMCs)
iv. iCAD data generated
v. Low virological diversity
vi. Appropriate candidate for ISA

6.3.4 Laboratory Assays

HIV-1 plasma viral load detection

Viral load testing was measured within a South African National Accreditation System (SANAS) accredited routine laboratory, the National Health Laboratory Services (NHLS), Tygerberg Cape Town, South Africa as previously described in previous Chapters.

PBMC isolation

The procedure for PBMC isolation was done in a Biosafety Level 2 laboratory. Collected whole blood was transferred from EDTA tubes to 50ml Falcon tubes and centrifuged Rotanta 460 R, Hettich, USA) at 967 x g for approximately 10 minutes with the brake. Blood plasma supernatant (top layer) was removed and placed into Eppendorf tubes for routine viral load and the rest stored at -80°C for immunological studies.

The plasma supernatant was then replaced with an equal amount of 1X phosphate-buffered saline (PBS) (Lonza, Switzerland). PBS and the remaining blood products were thoroughly mixed, reconstituted and then carefully layered onto approximately 6-7ml of Histopaque Ficoll (Sigma-Aldrich, USA) which served as a density gradient cell separation medium. The layered blood on Histopaque Ficoll was then centrifuged for 30 minutes at 434 x g, without a brake or acceleration.

Following centrifugation, the buffy coat, rich in white blood cells (PBMCs) was carefully removed by use of a bulb pipette and placed in a sterile 50ml Falcon tube. 1X PBS was added to the maximum volume allowed in the tube and washed during a centrifugation step of 278 x g for 15 minutes (no brake or acceleration added). Following this wash step, the PBS supernatant was carefully decanted and the remaining pellet re-suspended and topped with 1X PBS for another round of washing. Following two wash steps, PBMC cells were resuspended in 15-20ml of 1X PBS for cell counting. Trypan Blue (Bio-Rad Laboratories, USA) was used to stain the cells to count the cells using a TC20™ Automated cell counter (Bio-Rad Laboratories, USA). At this step, 50µl of Trypan Blue was mixed with 50 µl of PBMCs of which 10 µl of this mixture was pipetted onto both sides of a counter slide and placed into the cell counter. The lower gate was set to 6 µM and the upper gate set to 17 µM. A total of four counts were obtained (two on each of the slide) and the average cell count was used to calculate volume of cryopreservation media required. Cryopreservation or freezing media consisted of 10% dimethyl sulfoxide (DMSO) in foetal bovine serum (FBS).

The PBMCs were then centrifuged at 275 x g for ten minutes. After centrifugation, the PBS supernatant was decanted, and the pellet reconstituted in the remaining PBS. The freezing media was added to the cells in a drop wise manner while swirling the tube to ensure that the cells were evenly suspended into the media.

One millilitre aliquots of 2.5 million cells were added to cryovials and stored in a Mr Frosty overnight at -80˚C. Cryovials were then then transferred to LN2 for long term storage.

Assessment of integrase cell associated total HIV-1 DNA (iCAD)

Full assessment and quantification of total HIV-1 DNA was conducted within our research group by MSc Candidate, Ms Kirsten Veldsman and were based on previously described protocols adapted for HIV-1 subtype C (van Zyl et al., 2015; Hong et al., 2016; Veldsman et al., 2018).

In summary, HIV-1 total DNA was extracted from stored PBMC samples following thawing, washing, splitting appropriate cell numbers (800 000 to 1 000 000 cells) and DNA extraction methods. The DNA concentration following extraction methods was measured by use of a Nanodrop 1000 spectrophotometer. A final concentration of 120 ng/µl was ensured with appropriate dilutions to avoid over saturation of the assay that may result in inhibition activity due to the nature of genomic DNA.

DNA extraction was then followed by the implementation of a sensitive in-house quantitative real time quantitative PCR (qRT-PCR) adapted for HIV-1 subtype C for the measurement of HIV-1 total DNA. This assay was aimed at targeting a region in HIV-1 integrase with a ultrasensitive assay detection limit of 3 copies per million cells as previously described (Hong et al., 2016). Two standards are employed in this protocol: (1) an HIV-1 integrase DNA standard for the quantification of cell-associated DNA and (2) a CCR5 standard for the quantification of the total amount of cells tested in each sample due to each human PBMCs contains two copies of the gene.

6.3.5 Measuring the HIV-1 Latent Reservoir via VOAs

We explored three key experiments for measuring of the HIV-1 latent reservoir in a HIV-1 virologically suppressed paediatric population:

i. **Mellors Laboratory Total Inducible Virus Recovery (TVR) Assay** for measuring the inducible HIV-1 viral reservoir following stimulation of CD4+ T cells with Phorbol 12-myristate 13-acetate (PMA) and ionomycin.

ii. **Siliciano (adapted) Infectious Virus Recovery (IVR) Assay or QVOA** for measuring recovered infectious HIV-1 latent virus following the activation of CD4+ T cells with phytohemagglutinin (PHA) and co-culturing with irradiated HIV-1 feeder cells and CD8-depleted blasts (method adapted Siliciano and Siliciano, 2005).

iii. **Mellors Laboratory Sensitive Integrase Single-Copy HIV-1 RNA (iSCA) Assay** for measuring the HIV-1 RNA in cell culture supernatants in experiments I and II above.

Figure 6.2 below provides a schematic summary of the experimental assays employed for the evaluation of the HIV-1 latent reservoir in paediatric samples.

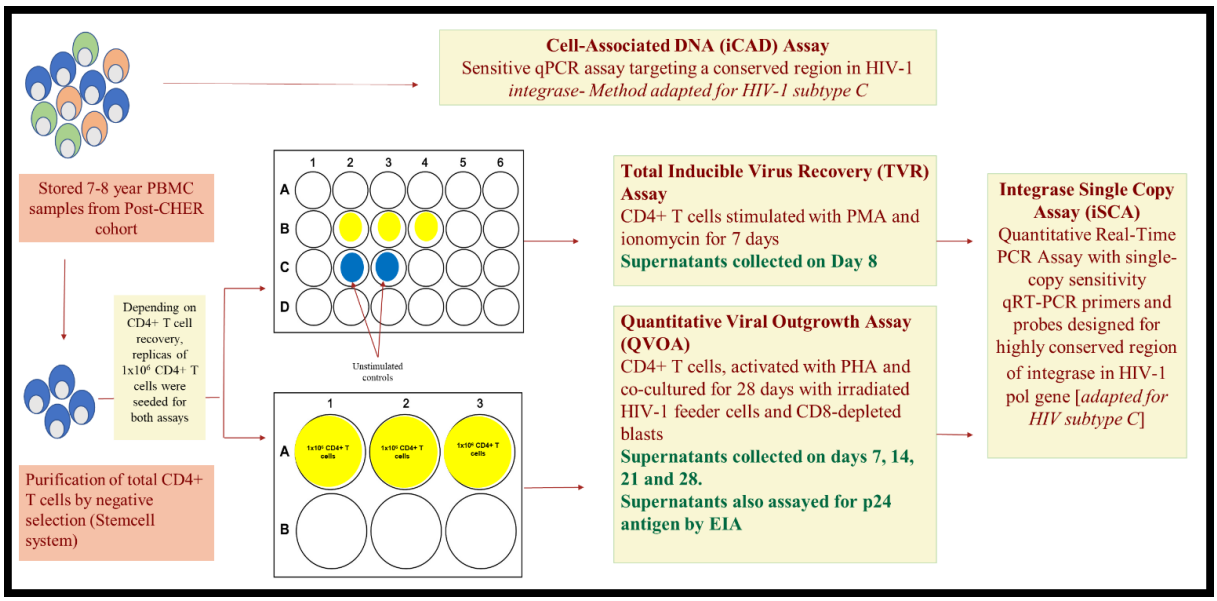


Figure 6.2: *Schematic summary of the assays implemented to evaluate the HIV-1 latent reservoir in a subset of samples from the post-CHER cohort.*
PBMCs samples were purified for total CD4+ T-cells *through negative selection. Following purification, total CD4+* T-cells *were seeded into 24-well plates following PMA and ionomycin stimulation and 6-well plates (1x10^6 CD4+* T-cells *each) following activation with PHA for measuring inducible and infectious, replication-competent virus respectively. Supernatants were collected following completion of culturing methods (described in detail below) and assessed for HIV-1 RNA levels by use of a highly sensitive iSCA method. *Not included in this figure is the HIV-1 p24 ELISA implemented at days 7, 14, 21 and 28 for the measure of HIV-1 p24 antigen levels in cell culture supernatants.*

Total Inducible Virus Recovery (TVR) Assay

Reagents and materials

- **Complete PBMC Media (CPM):**
  - 500 ml phenol red-free Roswell Park Memorial Institute Medium (RPMI) 1640 (Lonza cat:12-918F)
  - 10% heat inactivated FBS (Fisher cat: SH30070.03)
  - 3 ml Penicillin/Streptomycin (Invitrogen cat: 15140122) -Pen/Strep
  - 5 ml 200mM L-Glutamine (Lonza cat: 17-605E)

  ***Note:** Phenol red-free media is required if viral RNA is to be quantified in supernatants using the Roche Taqman 2.0 automated RNA extraction/qRT-PCR system.*
- **Wash Media (WM):** PBS + 2% FBS (StemCell cat: 07905)
- **Ficoll-Paque Plus** (Fisher cat: 45-001-749)
- **RoboSep Buffer** (StemCell cat: 20104) – *brought to room temperature for purification of total CD4+* T-cells
- **EasySep Custom Human Resting CD4+ T cell Enrichment Cocktail** (StemCell cat: 19052)
- **Easy 50 EasySep Magnet** (StemCell Technologies cat: 18002)

Purification of Total CD4+ T cells

**[protocol adaption for paediatric samples - due to very limited recovery of resting CD4+ T-cells from frozen paediatric PBMC samples, the TVR assay was followed through with the recovery of total CD4+ T-cells instead]*

PBMCs samples were appropriately thawed by placing 3-4 cryovials containing $2.5x10^6$ cells/ml each into a 37°C bead bath. Cells were then transferred into 15ml conical tubes and washed (wash media added followed by centrifugation at 400 x g at room temperature (RT) for 10 minutes). PBMCs are counted in Trypan blue. The total PBMCs were pelleted and resuspended at 5×10^7 cells/ml at RT in RoboSep buffer.

For a hypothetical yield of 300 million PBMCs, resuspended in 6 ml of RoboSep buffer:

3.0×10^8 cells in 6 ml of RoboSep buffer = 5×10^7 cells/ml

The cells were resuspended in the 6ml of RoboSep buffer was then transferred to a sterile 50ml conical tube after which enrichment cocktail was added at 50µl per ml of cells (a volume of 6ml would therefore require 300µl of enrichment cocktail). Cells with enrichment cocktail was adequately mixed with a pipette and incubated at RT for 10 minutes. To ensure uniform distribution, EasySep magnetic particles were vortexed for 30 seconds and then added to the cells at concentration of 100 µl per ml (a volume of 6ml would therefore require 600µl of magnetic bead particles), mixed gently with a pipette and then incubated at RT for 5 minutes.

The cells were then placed onto Easy 50 EasySep magnet for 10 minutes at RT. Total CD4+ T cells were contained via negative selection. The enriched cell suspension was carefully removed with a pipette and placed into a 15 ml conical tube. The enriched cells were then centrifuged at RT for 10 minutes at 400 x g. Following centrifugation, the cell pellet is resuspended in CPM and counted 1:100 in trypan blue.

Plating of Total CD4+ T cells

For this TVR assay, cells were plated in 800µl of CPM with 300nM efavirenz (EFV) and 300nM raltegravir (RAL) into a 48-well plat at 1 million cells per well.

Following centrifugation at 400 x g for 10 minutes, total CD4+ T cells were resuspended in 10ml of the CPM+EFV+RAL media at a concentration of 1 million cells per ml. Eight hundred microlitres of cells were placed in wells (dependent on the number of cells obtained). Standard protocol allows for 6 untreated controls and 6 for PMA/ionomycin treated wells. The number of wells differed for each patient studied which was dependent on the total CD4+ T cells recovery yield. Figure 6.3 below provides and overview of the plating of total CD4+ T cells for the TVR assay.

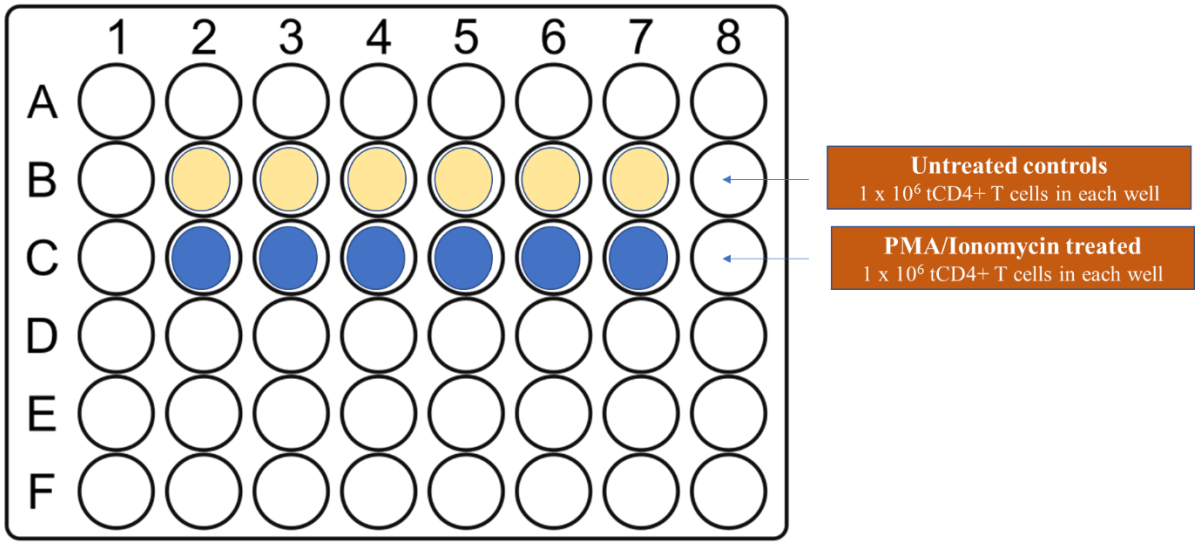


Figure 6.3: *Graphical representation of plating for TVR assay (measure of the inducible latent reservoir). The yellow cells from well B2 to B7 represent the untreated control cells and the blue cells from well C2 to C7 represent the PMA/Ionomycin treated cells. Each well would typically contain 1 x 10^6 total CD4+* T-cells.

The plate was then placed in a 37°C incubator overnight.

Treatment of cells for TVR

To prepare the CPM+EFV+RAL media for the treated wells. Ten microlitres of 3mM EFV stock was added to 290µl of CPM media in an Eppendorf tube. For RAL, 6ul of a 1mM stock solution was added to 20ml of media. Therefore, to make a 100nM EFV + 300nM RAL, 20µl of made-up EFV and 6µl of made-up RAL was added to 20ml of CPM media.

To prepare PMA for treatment from a 1mg/ml stock in DMSO, the following dilutions were carried out:

Step 1: 1mg/ml PMA stock was diluted 1:200 in media+EFV+RAL to a total volume of 500µl
i.e., 2.5µl of 1mg/ml PMA into 497.5µl of media = 5µg/ml PMA with 0.5% DMSO

Step 2: Above stock was then diluted 1:20 in media+EFV+RAL to a total volume of 6ml
i.e., 300µl of 5µg/ml into 5.5 ml of media = 250ng/ml, 0.025% DMSO

Step 3: There is a 1:5 dilution when above stocks are added to the wells with 800µl of media
i.e., 200µl of 250ng/ml PMA into 800µl media in well = 50ng/ml PMA, 0.005% DMSO

To prepare Ionomycin for treatment wells – stock concentration is 10mM in DMSO = 7 470µg/ml:

Step 1: Dilute the stock solution 1:10 in media+EFV+RAL to a total volume of 35µl
i.e., 3.5µl of 7 470µg/ml ionomycin into 31.5µl of media = 747µg/ml Ionomycin, 10% DMSO

Step 2: Dilute the above stock solution 1:10 in media+EFV+RAL to a total volume of 300µl
i.e., 30µl of 747µg/ml ionomycin into 270µl of media = 74.7µg/ml Ionomycin, 1% DMSO

Step 3: Dilute the above stock solution 1:30 in media+EFV+RAL to a total volume of 6ml
i.e., 200µl of 74.7µg/ml ionomycin into 5.5ml of media = 2.49µg/ml Ionomycin, 0.03% DMSO

Step 4: There is a 1:5 dilution when the above stocks are added to the treated well.
i.e., 200µl of 2.49µg/ml ionomycin into 800µl of well media = 0.498µg/ml Ionomycin, 0.006% DMSO

Collection of TVR Cell Culture Supernatants

Following 7 days of culturing, the pooling of supernatants occurs on Day 8. Eight hundred microlitres of supernatants from the untreated control wells and the PMA/Ionomycin treated wells were pooled into two separate 15ml conical tubes. The conical tubes were then centrifuged at 500 x g for 5 mins after which the supernatants were then transferred into 1.2 ml screw-top tubes and stored at -80°C.

Measurement of HIV-1 RNA in Supernatants

The original protocol aimed to measure HIV-1 RNA in supernatants following the TVR assay for inducible latent reservoir measurement by using the COBAS Ampliprep/TaqMan HIV-1 Text, v2.0. An adaption to this protocol was the implementation of a highly sensitive iSCA assay which is described in more detail below.

Infectious Virus Recovery Assay or QVOA

The gold standard for measuring the HIV-1 latent reservoir as designed by the Siliciano lab (Siliciano and Siliciano, 2005) was implemented on a sub-set of our paediatric samples with some modifications as detailed below.

Reagents – Culturing Media

WM – PBS with 2% FBS

B-cell Media – RPMI-1640, 1 x Glutamaz, 10% FBS and Penicillin/Streptomycin

Super T- Cell Media (STCM) – RPMI-1640, 1 x Glutamax, 10% FBS and Pen/Strep, 100U/ml rh-IL-2 (38.5µl) and 2% T-cell Growth Factor (TCGF)

Preparation of T-cell Growth Factor (TCGF) for STCM

TCGF is an important component of the STCM required to perform an effective virus recovery. The proliferation of T cells is important and is provided for by signalling molecules such as growth factors. IL-2 and IL-15 are also considered important growth factors required for the stimulation, activation and proliferation of T cells.

The media used for the preparation of the TCGF includes the WM made up of PBS, 2%FBS, 0.1% dextrose, 1 x pen/strep and 12mM HEPES buffer at a pH of 7.2 and the cell culturing media composed of RPMI-1640 with Glutamax, 2.5% heat-inactivated AB type human serum, 20µg/ml streptomycin and 20U/ml penicillin (1x pen/strep).

PBMC isolation procedures was initiated on two leukopaks obtained from two different HIV-1 negative donors via the New York Blood Bank. The leukopak was diluted 1:1 in WM. The careful layering of 12.5 ml of diluted product onto 15 ml of Ficoll in eight 50ml conical tubes was completed per donor. A total of 16 tubes were layered.

The layered tubes were then centrifuged at 470 x g for 40 minutes at RT with the brake off. With the use of a 10 ml pipet, the buffy coats were removed and placed into fresh 50 ml conical tubes – each donor had eight tubes each and these were combined into four tubes. WM was added to each of the tubes containing the PBMCs

to a final volume of 45ml and centrifuged at 470 x g for 15 minutes at room temperature. Supernatants were then discarded, and the same wash procedure carried out. After the third wash cycle, the pellets were combined (2 conical tubes per donor). WM was added again to a final volume of 45 ml and centrifuged for another 10 minutes at 470 x g. Following this, the supernatants were discarded an all the pellets were combined in 35 ml the cell culture media described above. Five millilitres (5ml) of cells were added to about 6 T150 cell culture flasks with an additional 70 ml of cell culture media added to reach a total volume of 75ml. The flasks were incubated at 37°C, 5% CO_2 overnight.

Following overnight incubation, PHA (2µg/ml) and PMA (5ng/ml) was added to each of the flasks to activate cells and incubated for another 4 hours at 37°C with 5% CO_2. Dilutions for the appropriate concentrations were carried out as follows:

PHA was at a concentration of 150ug/ml in PBS: needed a 75-fold dilution therefore added 1ml to 75ml
PMA was at a concentration of mg/ml in DMSO:
First dilution, 1:200 in DMSO: add 2.5ul to 497.5ul DMSO for 5ug/ml
Need to dilute 1:1000: therefore, added 75uL per 75ml

After the 4-hour incubation step, supernatants from the activated cells were gently discarded from each flask and the settled PBMCs rinsed three times with 15ml of warm WM. Seventy millilitres (70ml) of fresh media [RPMI + 2.5% heat-inactivated human AB serum + 1x penicillin-streptomycin] was added to each flask and incubated for 40 hours at 37°C, 5% CO_2.

Following incubation culture supernatants were collected from the activated PBMCs and transferred to fresh 50ml conical tubes. The tubes were centrifuged at 470 x g for 10 mins to remove the cell debris. With the use of a 0.45µm Nalgene filter unit, the culture supernatants were filtered. The filtered culture medium is the TCGF. Ten millilitres (10ml) of TCG aliquots were made and stored at -20°C.

To test the proliferation capacity of the isolated and activated PBMCs as well as to determine to the ideal amount of TCGF to be added to the cell culture media when implementing the IVR/QVOA experiment, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) Cell Proliferation Assay Kit (Abcam®, Cambridge, UK) was used. Testing of the proliferative capacity of PBMCs was doing using 1%, 2%, 4% and 8% of TCGF in BCM with a previous TCGF as a control. The MTS kit utilises a colorimetric analysis that measures the intensity of light absorbed by a substance within a visible region of 400 to 800 nm.

When the MTS reagent is added, cell proliferation can be measured based on the reduction of MTS tetrazolium compound by viable cells to generate a coloured formazan product that is soluble in cell culture media.

Formazan, produced by viable cells, can be quantified by measuring the absorbance at an optical density (OD) of 490-500 nm. Usually, no more than 2% of TCGF is required to stimulate maximal cell proliferation (Laird et al., 2013a; Wang et al., 2018). Higher concentrations of TCGF can result in CD8+ T cell activation and proliferation and trigger the production of cytokines and chemokines and inhibit viral replication in the IVR/QVOA (Laird et al., 2016).

To evaluate viability of the TCGF, approximately 35-40ml of blood was obtained from a HIV-1 negative donor followed by isolation of PBMCs and a cell count. Depending on the average of the live cell count, appropriate media (RPMI 1640 medium with L-Glutamine and phenol red (Lonza Group Ltd), add 20% HI & GI FBS (Thermo Fisher Scientific) and 100 U/ml Gibco™ Recombinant human IL-2 (Thermo Fisher Scientific, Waltham, USA) was added to the cells to provide a final concentration of 0.50 x 10^6 cells/ml. Various concentrations (1-8% as described above) were plated in triplicate to a final volume of 100µl per well using a 96 well TC-treated microplate (Corning® Incorporated, New York, USA). The cells were incubated for three days at 37°C, 5% CO2 in a Nuaire NU-5510E (Nuaire) incubator.

Figure 6.4 below provides a graphical representation of the plate layout for measuring TCGF proliferative capacity for the IVR/QVOA experiment.

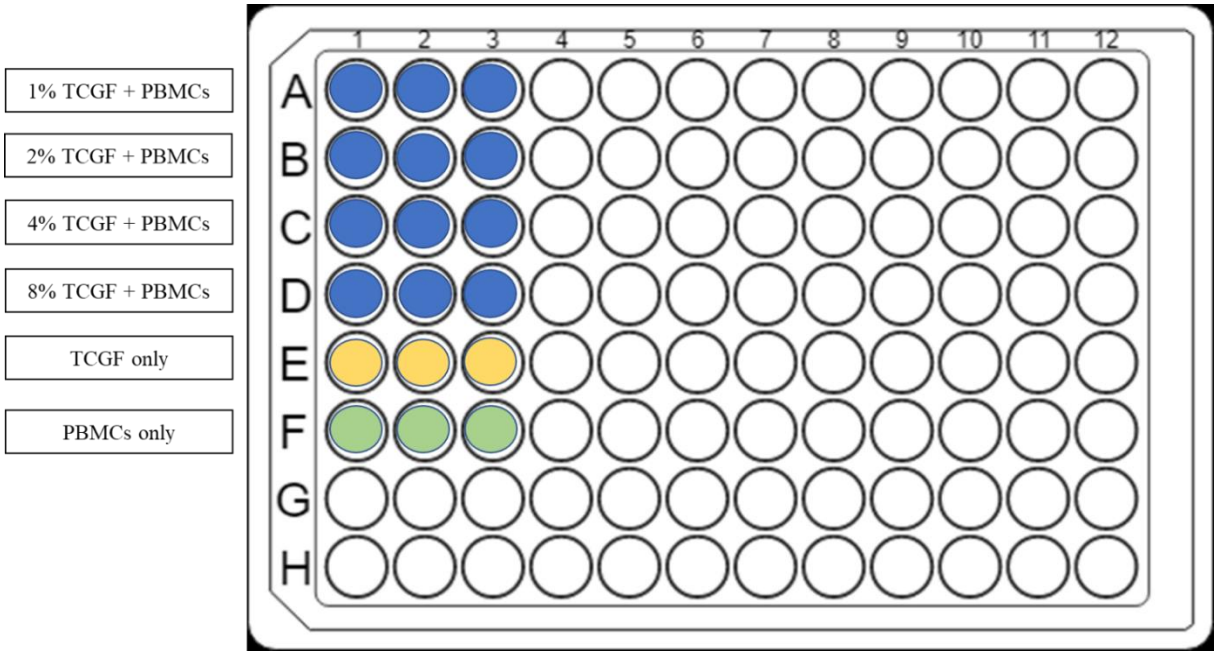


Figure 6.4: *Graphical representation of plating (TC-treated 96 well plate) method for measuring appropriate TCGF concentration needed for maximum proliferation of cells when implementing the IVR/QVOA experiment.*
TCGF concentrations were plated at 1%, 2%, 4% and 8% with PBMCs in triplicate (wells A-D). TCGF without T-cells and PBMCs were plated as controls, also in triplicate.

Following Day 3 of the incubation, 20 µl of the MTS reagent was added to each of the wells. The cells were incubated for an additional 2 hours at 37°C, 5% CO_2. The absorbance was measured using the EZ-Read 400 microplate reader (Biochrom Ltd., Cambridge, UK) at OD 492 nm and the OD was obtained using Galapagos software version 1.1.0.0 (Biochrom Ltd., Cambridge, UK). The concentration of TCGF with the highest OD at 492nm was considered the most appropriate concentration to be used in the assay.

Following media preparation, the adapted IVR/VOA procedure for post-CHER samples is summarised in Figure 6.5

Adapted IVR/QVOA Procedure Implemented on post-CHER samples

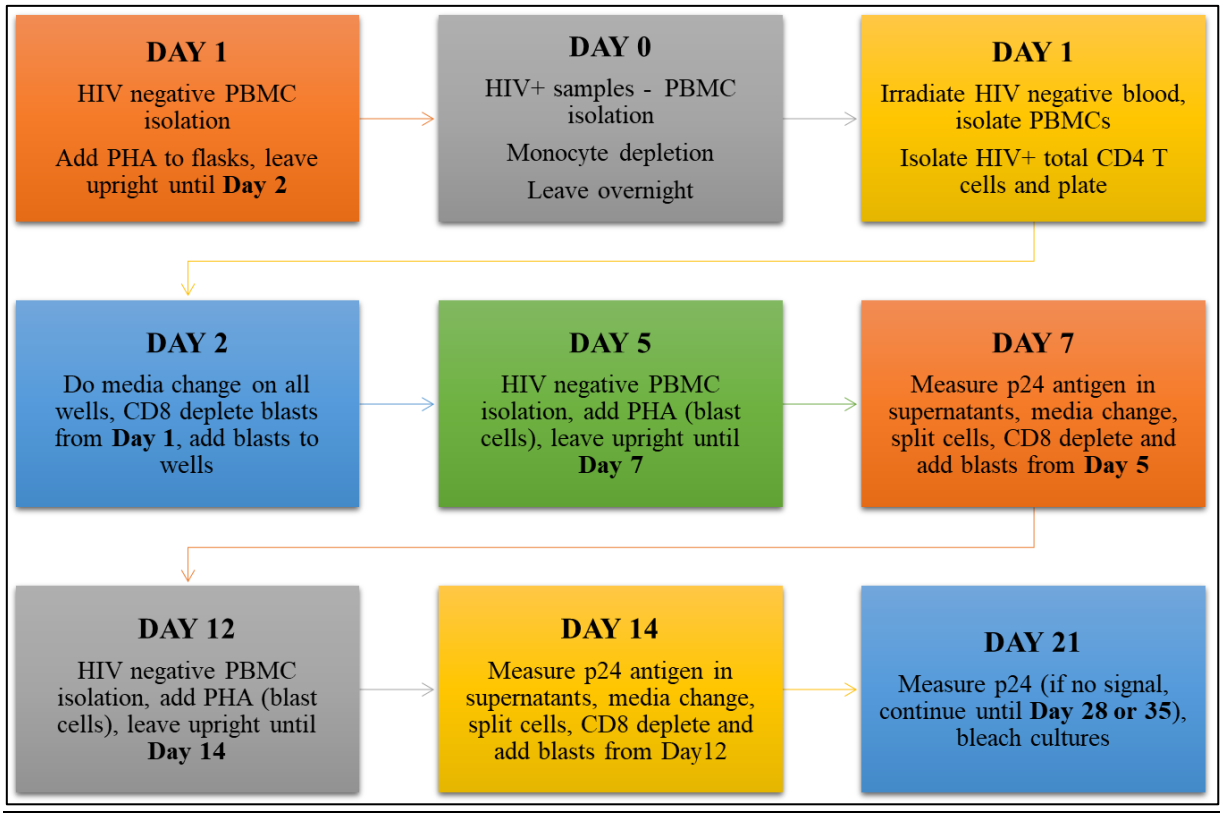


Figure 6.5: *General outline of the IVR/QVOA experiment carried out on post-CHER paediatric samples at 7-8 years of age.*
Typically, the QVOA assay is carried out until Day 21 (three rounds of viral replication). With some samples, additional rounds were implemented in the event that a signal (HIV-1 p24 antigen expression) was not reached.

On **Day 1**, preparation of the CD4+lymphoblasts was carried out following blood collection (120ml) from HIV-1 negative donors. Blood was diluted with 100-120ml of WM (PBS with 2%FBS) and carefully layered (30ml) into 15 ml of Ficoll in six to eight 50ml conical tubes. Samples were then centrifuged at 400 x g for 40 minutes at RT with no brake. Using a 10ml pipet, the buffy coats containing PBMCs were carefully removed and placed into a fresh 50ml conical tube. To wash the cells, WM was added to each of the tubes containing the PBMCs to a final volume of 45ml and centrifuged at 400 x g for 10 minutes at RT. Following the first wash step, the supernatant was discarded, and the cells resuspended in remaining media, topped with WM to 45ml and the wash cycle repeated. Following the final wash step, WM was completely removed, and pellets were resuspended in 60ml of STCM, with PHA to reach a final concentration of 0.5µg/ml. Cells were then added to two T75 flasks (30ml in each flask) and incubated in an upright position at 37°C, 5% CO2 until **Day 2**.

On **Day 0**, we proceeded deplete monocytes within the frozen PBMC samples [*protocol adaption: HIV+ samples would be freshly isolated for PBMCs following the PBMC isolation technique above].* Post-CHER samples were retrieved from LN_2 storage and placed in a 37°C bead bath along with STCM and WM media. Once thawed, the PBMCs were transferred into sterile conical tubes and carefully topped with warm WM. PBMCs were centrifuged at 400 x g for 10 minutes with no break. The supernatant was discarded and following

two cycles of washing, the pellets were resuspended in 30ml of BCM. Cells were then added to a T75 flask and incubated at 37°C, 5% CO2 overnight to allow for the monocytes to adhere to the bottom of the flask.

The first part of **Day 1** of the IVR/QVOA procedure, we prepared irradiated PBMC feeder cells. One hundred and twenty millilitres of blood were collected from a HIV-1 negative donor and transferred to a three 50ml conical tubes. Cells were inactivated following γ-irradiation with 500R in a caesium source irradiator. Following the irradiation procedure, PBMCs were isolated as previously described. Cells were then resuspended in STCM at a concentration of $2.5x10^6$ cells/ml. PHA was added to the cells at a final concentration of 1µg/ml and immediately placed on ice to prevent the macrophages from adhering to the walls of the conical tube until ready for plating.

The second part of Day 1 of the IVR/QVOA procedure is to isolate and purify the total CD4+ T cells from the samples from Day 0. Cells were decanted and centrifuged. Total PBMCs were then resuspended at 5×10^7 cells/ml at RT in RoboSep buffer. For a hypothetical yield of 300 million PBMCs, cells were resuspended in 6ml of RoboSep buffer.

The 6ml of cells in RoboSep buffer was then transferred to a new 50ml conical tube of which enrichment cocktail at a concentration of 50µl per ml of cells (i.e., 300µl for 6ml of cells) was added, mixed and allowed to incubate at RT for 10 minutes. EasySep magnetic particles were vortexed to ensure uniform distribution of cells and added to cells at a concentration of 100µl per ml of cells (i.e., 600µl for cells in 6ml). Magnetic particles were then incubated at RT for 5 minutes, gently mixed using a pipette and placed on the Easy 50 magnet for negative selection of total CD4+ T cells. Cells were incubated for 10 minutes at RT on the magnet. The enriched cell suspension was then carefully removed using a pipette and transferred to a 15 ml falcon tube. The cell suspension containing the selected total CD4+ T cells were then centrifuged at RT for 10 minutes at 400 x g for pelleting of cells. Following centrifugation, cells were then resuspended in STCM and counted (1:10 dilution) in Trypan Blue using a manual counting system. Following the cell count, cells were then resuspended at a concentration of 1×10^6 cells/ml in STCM to be plated in 6 well plates.

[protocol adaptation: when conducting the traditional QVOA assay, cells are serially diluted from 1 x 10^6 cells/ml to 320 cells/ml, due to limited number of PBMCs isolated in this paediatric cohort, followed by isolation of total CD4+ T-cells *(30% of PBMCs), we eliminated serial dilutions and proceeded with plating 1 x 10^6 cells per well depending on the concentration of total* T-cells *isolated]*

One million total CD4 T cells were plated in every second well (cells were not placed in adjacent wells) with a total volume of 8ml (i.e., 1 ml of resuspended cells + 4ml of 1×10^7 irradiated PBMCs+PHA + 3ml of STCM). Plates were then wrapped in foil to prevent condensation and incubated at 37°C until **Day 2**.

Changing of the media (to remove toxic PHA from culture) and CD8 depletion of lymphoblasts took place on **Day 2.** Using a sterile pipette tip for each well, we carefully removed 6ml of media from each PHA-containing well. Careful attention was taken to not disturb or mix the cells at the bottom of the plate wells. The same volume was then replaced with warm STCM, and cells were placed back in the incubator for two hours to allow them to settle. This initiates the washing procedure.

Whilst the cells are incubating, CD8 depletion of the lymphoblasts was initiated. Cells were removed from the incubator from Day 1, counted and aliquoted the necessary amount of blasts needed for the CD8 depletion. The cell pellet was resuspended in the appropriate amount of CD8 depletion beads (BD Biosciences, USA) (50µl per 10 million lymphoblasts) and incubated for 30 minutes at RT. An 8-10-fold excess of iMAG™ Buffer (BD Biosciences, USA) was added and placed on the magnet for 10-15 minutes. CD8 T cells remained at the bottom of the magnetic and the supernatant was carefully removed and placed into a new fresh tube. To improve yield, the beads were washed again with iMAG™ Buffer (BD Biosciences) and placed on the magnetic for another 10-15 minutes followed by the removal of the supernatant. The CD8 depleted cells were then pelleted and resuspended in STCM followed by a manual cell count as previously described. Cells were resuspended at a concentration of 0.67 x 10^6 cells/ml.

Following the two-hour incubation allowed for the cells to settle, 6ml of the supernatant was removed and replaced with CD8 depleted lymphoblasts i.e., 4 X 10^6 lymphoblasts in 6ml of STCM. Plates were then returned to the incubator and left to culture until **Day 7** at 37°C

[protocol adaptation: when conducting the traditional QVOA assay, a media change is done at Day 5 – this step was eliminated in this protocol]

On **Day 5** PBMCs from HIV negative donors are prepared for blasts to be added for **Day 7** as described above.

Day 7 consisted of two parts: (I) The CD8 depletion of the lymphoblasts collected on **Day 5** and (II) Splitting of cells plated on **Day 2** and the addition of fresh lymphoblasts. The same procedure for CD8 depletion was followed as previously described, however following depletion and counting, lymphoblasts needed to be resuspended at a concentration of 1 x 10^6 cells/ml in STCM.

For the splitting of cells incubated on **Day 2.** First 1.8ml of the supernatant of each well was collected in Eppendorf tubes and stored at -80 °C for HIV-1 RNA quantification and 200µl of supernatant was used for HIV-1 p24 antigen detection by ELISA (described below). The rest of the contents of the wells were then gently mixed and a further 1ml of media was removed and discarded into a bleach bucket. Fresh lymphoblasts were added at a concentration of 4 x10^6 blasts in 4ml of STCM.

On **Day 12,** fresh lymphoblasts were prepared from a HIV negative donor as previously described on **Day 1** and added to the cell culture media on **Day 14**.

On **Day 14** splitting of cells, the same procedure for splitting of cells were completed (1.8ml of supernatant stored for HIV-1 RNA analysis and 200µl used for HIV-1 p24 antigen quantification) followed by replacement of media with fresh lymphoblasts subjected to CD8 depletion and left for incubation until Day 21.

[protocol adaptation: Generally, a HIV-1 p24 antigen ELISA signal is detected on Day 14. When no signal is detected, the culture was left for another 7 days until Day 21. For the evaluation of VOA in paediatric samples, the cell culture was extended to Day 28 – this implies that on Day 12 and 19 fresh lymphoblasts were prepared and incubated for addition following splitting on Day 14 and 21. On day 28, cells were just split, and no additional blasts added. The VOA procedure was then suspended and discarded in bleaching solution]

Measuring TVR and IVR signals

The gold standard output measurement for measuring replication component HIV-1 latent virus following implementation of the VOA is a strong HIV-1 p24 antigen signal measured on Day 7, 14 and 21. For this research study the implementation of a highly sensitive assay for the measurement of HIV-1 RNA in cell culture supernatants. A description of each of these output signals as presented in Figure 6.6 below is described.

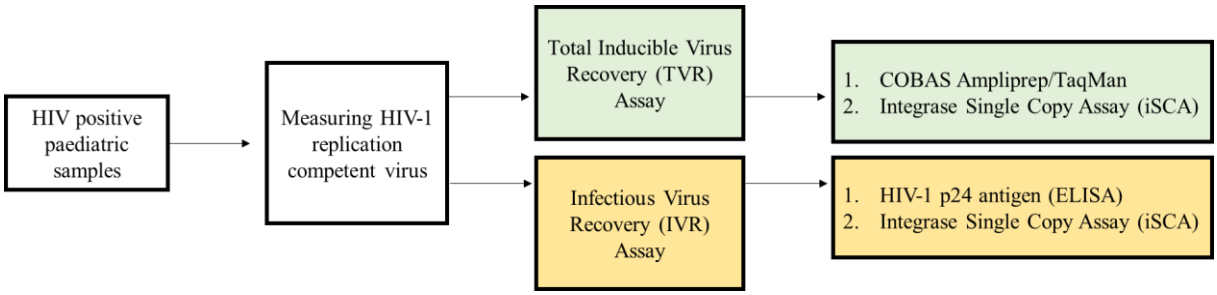


Figure 6.6: *Summary of assays to measure the output signals following the implementation of TVR and IVR assays.*

HIV-1 p24 Assay

The HIV-1 p24 ELISA kit (PerkinElmer®, USA) was used to measure the expression of HIV-1 p24 antigen levels in cell culture supernatants following the TVR and IVR (days 7, 14, 21 and 28) assays. Briefly, before beginning the ELISA assay, all reagents were brough to room temperature (between 15-30°C) as per the manufacturer's guidelines. Each plate included a substrate blank, three negative controls and two 100 pg/ml positive control wells. The positive control for the assay was prepared by diluting the 200 ng/ml concentrate provided in the kit to a working concentration of100 pg/ml with cell culture media. The negative control used was the STCM. The ELISA reaction plate was coated with Triton X-100 except for the substrate blank well. Two hundred microliters (200µl) of STCM were added to the appropriate wells as outlined in Figure 6.7 below, the same volume of the positive control of appropriate concentration was added to the well as well 200µl of the samples (cell culture supernatant) added in triplicate. Wells were placed on a shaker for mixing and then incubated for 2 hours at 37°C.

Following the 2-hour incubation, the plates were washed six times using 300µl of 1X of the diluted wash buffer provided in the kit. Plate was tapped on tissue paper to remove excess buffer. For the next step, we added 100µl of a detection antibody (for the capture of any p24 antigen present in the cell culture supernatant and form a complex) contained in the kit to all the wells except for the substrate blank. The plate was then sealed and incubated for 1 hour at 37°C. As described previously the plate was then washed which was then followed by the addition of 100µl of streptavidin-horseradish peroxidase (HRP) conjugate. The plate was sealed and incubated for 30 minutes at room temperature. The resulting complex formation was then detected following the addition of 100µl of ortho-phenylenediamine-HCl (OPD) substrate solution and incubation for 30 minutes at room temperature in the dark. This step allows for a colour reaction to take place. A colour change of yellow is relative to the amount of HIV-1 p24 antibody present in the sample and captured by the assay. Following the final incubation step, 100µl of a "Stop" solution was added to all the wells. The ELISA reaction plate was then read using a Bio-Rad® (USA), microtiter plate reader at 490nm. Readings were taken with a reference filter greater than 600nm.

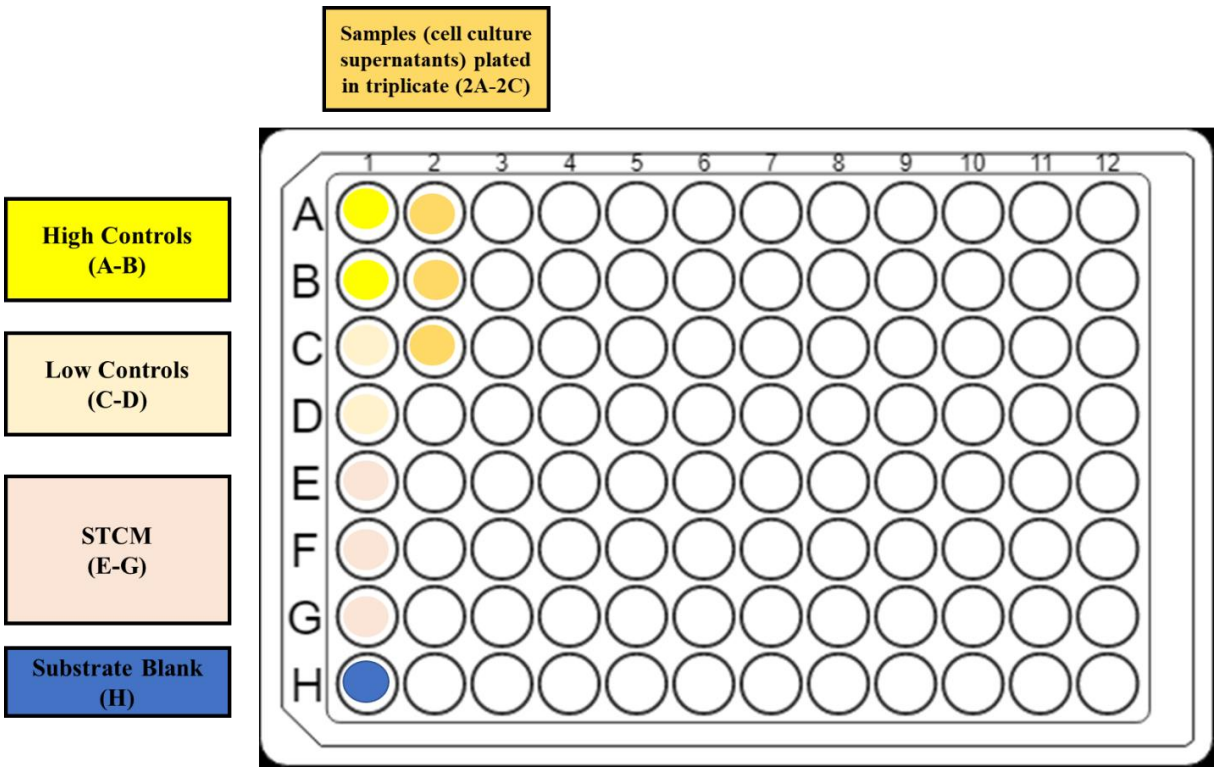


Figure 6.7: *HIV-1 p24 antigen detection ELISA plate layout.*

Integrase Single Copy Assay (iSCA) for the measurement of HIV-1 RNA in cell culture supernatants

The first generation two-step RT-qPCR single copy assays targeted the HIV-1 *gag* region (gSCA) of the HIV-1 viral genome (Palmer et al., 2003). Following the development of gSCA assay, the next generation of the single copy qRT-PCR assay improved the detection capabilities of the previous assay by targeting the highly conserved region of the integrase in the HIV-1 pol gene (iSCA). The latter described assay also improved the nucleic assay recovery from plasma (Cillo et al., 2014)

To measure the HIV-1 RNA levels in cell culture supernatants for both TVR and IVR assays following stimulation procedures, we implemented an optimized inhouse highly sensitive single-copy assay targeting the *integrase* region (iSCA) of the HIV-1 viral genome following adaptations to the assay development by Cillo et al., 2014 and will be referred to iSCA v1.0 given that adaptions to this assay were further developed and published by Tosiano et al., 2019.

This method was optimized for HIV-1 Sub-type C and served significant to this evaluation of the assay given that the QVOA requires a large sample input for the quantification of HIV-1 replication-competent virus for which paediatric sampling is limited and therefore impacts the sensitivity of the QVOA. In order to obtain a positive signal for the HIV-1 p24 ELISA assay, cell culture supernatants would require 100 000 HIV-1 RNA copies. Therefore, implementation of iSCA v1.0 provided an indication of the HIV-1 RNA release following rounds of latent virus activation stimulation of the total CD4 T cells.

It is important to note that measuring HIV-1 RNA release following activation of latent CD4+ T cells does not indicate that the isolated HIV-1 virus is replication-competent buy may be inducible and defective given that

clones with intact or even partially intact proviruses may release a defective HIV-1 RNA copies (Imamichi et al., 2020).

For the implementation of the iSCA (version 1) the following materials and reagents were utilized:

Materials

- Eppendorf 2.0 ml Safe-Lock tubes (Eppendorf® 022600044)
- 15 ml Falcon Polyproplylene Centrifuge Tubes (352097)
- Genemate® Microcentrifuge Tubes 1.7ml (GeneMate® No. C-3262-1)
- Genemate® Pipet Tips 20, 200, 1000µl (P-1237-20; P-1237-200; P-1237-1000)
- LightCycler 480 Multiwell plate 96 white + Sealers (ROCHE 04729692001)
- Plate Seals for cDNA Synthesis Bio-Rad™ MicroSeal 'B' Seal (MSB1001)
- Rainin Tip Packages (RAININ GP-L10F)
- Sarstedt 2ml microtubes (ref 72.694.106)
- Reagent Reservoirs 25ml (ThermoScientific™ no. 8093-11)
- Reagent Reservoirs with dividers 25ml (ThermoScientific™ no. 8095)
- Transfer Pipettes Sterile Extended Fine Tip (Samo Scientific™ 231-20S)

RT-qPCR reagents

- In-house Buffer A (100 mM Tris-HCl, 500 mM M KCl, 0.2% Tween, and molecular grade water)
- $CaCl_2$ (100mM, made from 1M $CaCl_2$ Sigma® 21115-100ml)
- dNTPs (4x1ml, Bioline® BIO-39049)
- DTT, 100mM (comes with Stratagene RT)
- EDTA (0.5M, pH 8.0 Ambion® AM926G)
- Ethanol 200 Proof (Decon 2716)
- Glycogen (20mg/ml, ROCHE 10901393001)
- Guanidinium HCl (8M, SIGMA G9284)
- Guanidinium Isothiocyanate (6M, 50ml, SIGMA 50983)
- Isopropanol 100% (SIGMA I9516)
- KCl (1M Tris pH 8.0, SIGMA 60142-500ML-F)
- Low-copy and negative control VQA plasma standard (0, 5, and 50 cps/ml DAIDS Virology Quality Assurance at Rush University)
- $MgCl_2$ (50mM, ROCHE or APPLIED BIOSYSTEMS)
- Proteinase-K (20mg/ml, APPLIED BIOSYSTEMS AM2548)
- Random Hexamers (500 µg/ml, PROMEGA C1181)
- RCAS Internal Standard (received from NCI, Frederick, Maryland)

- Recombinant RNAsin (10000 U, PROMEGA N2115)
- 2x Roche MasterMix LC480 (10x5ml, ROCHE 4887301001)
- RPMI (LONZA 12-702F)
- Stratagene AffinityScript Reverse Transcriptase (2000U/uL, AGILENT 600107)
- Tris-HCL (5mM, made from 1M, pH 7.6 Tris-HCl 1M, pH 7.6 SIGMA)
- Tween (100%, SIGMA P9416-100ml)
- Ultra-Pure Distilled Water DNAse, RNAse, Free (Gibco 10977)

Standards and Controls

Plasma from a viraemic HIV-1 positive patient with a previously quantified HIV-1 RNA level and a matched integrase sequence to the primers and probes for sub-type C previously designed was used. These aliquots were collected and stored at -80°C. For the low-copy number standards of 20, 5, 4, 3 1, 0.3 and 0.1 cps/ml, HIV-1 RNA plasma from a patient was diluted with SeraCon Matribase negative Diluent (catalog number 1800-0005, SeraCare) and filtered with an EMD Millipore Stericup sterile vacuum filter unit (0.45-µm HV Durapore membrane). Aliquots of 1.8ml of these low copy number HIV-1 RNA plasma standards were stored at -80°C until use.

RCAS internal control for HIV-1 RNA recovery

A replication-competent avian leukosis virus (ALV) long terminal repeat (LTR) with a splice adaptor (RCAS) was used as an internal control in this study as well as previously published sing-copy assay (Palmer et al., 2003; Cillo and Mellors, 2016; Gay et al., 2017; Gandhi et al., 2018; Tosiano et al., 2019). RCAS internal controls were sourced from the HIV Dynamics and Replication Program (HIV DRP) at the National Cancer Institute (NCI) and consisted of virions of 1.2×10^6 copies which was spiked into each of the samples (plasma and cell culture supernatants) to evaluate and validate viral RNA recovery and amplification. The number of virions present in the RCAS spikes was previously confirmed by performing a qRT-PCR on serial dilutions of cell culture supernatants from RCAS plasmid-transfected DF-1 cells (Gay et al., 2017; Gandhi et al., 2018). A RCAS recovery rate of above 10% for within-run plasma standards (5 and 10 cps/ml) were considered a successful and adequate RNA recovery (Tosiano et al., 2019).

HIV-1 RNA Extraction Procedure

Stored cell culture supernatants and HIV-1 plama were removed from the -80°C freezer and defrosted using a water bath set at room temperature. Whilst the samples were thawing centrifuges were pre-cooled to 4°C, the bead bath was set to 42°C and 70% EtOH was cooled to -20°C. All standards and controls the RCAS spike was kept on dry ice until use. The HIV-1 RNA extraction procedure took place in a “clean room” - limited to contamination.

Firstly, three aliquots of the RCAS spike were pooled and 200µl of the spike was added to each patient sample tube. The RCAS spike was then stored in 4°C refrigerator until it was needed. Samples were then centrifuged for 15 minutes at 3000 x g at 4°C in a Sorvall ™ Legend 21R centrifuge to remove lipids, cellular debris, and

fibrinogen. Once the samples were subjected to centrifugation, the standards, and controls (QA) samples were thawed at room temperature in a water rack. Once thawed the QA samples were placed in 2ml snap-cap tubes. Two-hundred microliters (200µl) of the RCAS spike was then added to each of the QA tubes.

Once cell culture supernatants completed the pre-spin, they were transferred to 2ml snap-cap tubes and labelled accordingly. All samples including the QAs were then centrifuged at 21, 000 x g for 2 hours at 4° C to pellet the virions. Using a sterile 100µl pipette, the supernatants were removed using a clean tip each time. One hundred microliters (100µl) of 3M GuHCl mix with fresh Protease K (50µl of Protease K was added to 1ml of 3MGuHCL mix) was added to to each tube immediately following aspiration of the supernatant. Samples were then vortexed thoroughly to resuspend the pellet. To collect all, samples were "flash-spun" using a tabletop centrifuge.

All tubes were then placed in a 42°C water bath and incubated for 1 hour with an interval vortex at 30 minutes.

Following the 1 hour incubation, a vortex and "flash-spin" was done which was followed by the addition of 400µl of the GuSCN mix with fresh glycogen to the 2ml snap cap tubes which was then vortexed for 10 seconds. Five-hundred microliters of room temperature 100% isopropanol was then added to the tubes and vortexed for another 10 seconds after which samples were then centrifuged at 21,000 x g for 20 minutes at room temperature.

Following centrifugation, the supernatant aspirated and 750µl of 70% molecular grade ethanol was added to the glycogen pellet which was then vortexed to free the pellet from the bottom. Samples were then stored overnight or a maximum of two weeks in ethanol at -80°C until further processing.

The real-time (RT), no reverse transcriptase (NRT) and 5 mM Tris-HCl, 100 mM DTT, 10 000 units/ml recombinant RNasin ribonuclease inhibitor (TDR) mixes were prepared and kept on ice or a cold block.

RT Master Mixes for cDNA synthesis

Reagents	**Final Concentration**	**Volume/Reaction (µl)**	**RT Cocktail 94 x Reactions**	**No-RT Control 14 x Reactions**
Molecular Grade Water		8.1	761.4	114.8
25 mM $MgCl_2$		6.0	564	84
25mM dNTPs (Bioline)	0.5 mM	0.6	56.4	8.4
100 mM DTT	1 mM	0.2	18.8	2.8
Random Hexamers (*add 160 ul of water to make*)	0.15µg/rxn	1.5	141	21
10 x Taqman Buffer A	1 x	3.0	282	42
Rnasin (40 U/µl)	20 U	0.5	47	7
Strategene RT (200 U/µl)	20 U	0.1	9.4	No RT added
Total		**20.0**	**1880**	**280**
Sample RNA		10.0		
Total Volume		30.0		

Samples were centrifuged at 21, 000 x g for 15 minutes at room temperature. During the centrifugation, the plates were preloaded with RT, NRT and H2O as outlined in Table 6.1 below.

Table 6.1: RT Reaction Mix for Each cDNA well

Rt reaction mix							
	Volumes in µl						
	H_2o	**Extracted RNA**	**Diluted HIV transcripts**	**RCAS 7500 transcripts**	**RT cocktail**	**No-T cocktail**	**Total vol.**
NRT-HIV	5	5	X	X	X	20	30
Sample-HIV	X	10	X	X	20	X	30
NTV-HIV	10	X	X	X	20	X	30
HIV ISCA standard curve	X	X	10	X	20	X	30
NTC-RCAS	10	X	X	X	20	X	30
7500 RCAS	5	X	X	5	20	X	30
Sample-RCAS	5	5	X	X	20	X	30

Following the centrifugation, the ethanol was aspirated completely and "flash-spun" for 30 seconds, to collect the residual ethanol which was also carefully aspirated. The sample tube caps were then open and air dried for 20 minutes under a sterile hood until the pellet became clear and no liquid was present. Whilst the pellet was drying, TDR was aliquoted into tubes for the HIV standard cure and stored at room temperature.

The sample pellets were then resuspended in 55µl of the TDR. To dissolve the pellet, samples were vortexed and placed on ice or a cooling block.

RT Reaction Mix and Standard Curve Preparation (Performed in clean room)

HIV-1 RNA transcript

HIV-1 RNA transcripts used in the generation of the standard curve were previously developed (Wain-Hobson et al., 1991; Cillo et al., 2013, 2014; Hong et al., 2016; Tosiano et al., 2019).

Essentially, HIV-1 RNA transcripts were transcribed *in vitro* from a T7 expression plasmid containing a 1.7-kb insert containing the 3' end of the pol (bases 4186 to 5913 of the HIV-1 reference sequence HXB2) generated by standard PCR from the plasmid xxLAI (GenBank ascension no. K02013) using the forward primer 5'-AACAAGTAGATAAATTAGTCA-3' and reverse primer 5'-TTACAATAGCAAGTGGTA-3' at 600nM each. Transcripts were purified, and unincorporated nucleotides were removed as previously described.

A standard curve was generated for each assay completed by using a 3-fold serial dilution of the HIV-1 RNA transcripts that was characterised by an OD of 260nm and serial endpoint dilution to 1 copy per qRT-PCR (Cillo et al., 2014; Tosiano et al., 2019).

Dilution for HIV-1 RNA transcript

The HIV-1 RNA iSCA Transcript Stock consists of 1 x 10^3 HIV-1 RNA copies/ul which equated to 1 x 10^4 copies/10 ul

RCAS Transcript Stock = 7500 copies/10 ul (5 ul directly added to plate)

Transcripts on plate = copies / reaction (10 ul HIV or 5 ul RCAS loaded onto plate)

900 HIV (893.3)	300 HIV (297.8)	100 HIV (99.3)	30 HIV (33.1)	10 HIV (11.0)	3 HIV (3.7)
6.7µl stock into 68.3µl TDR	25µl prior into 50µl TDR	25µl prior into 50µl TDR	25µl prior into 50µl TDR	25µl prior into 50µl TDR	25µl prior into 50µl TDR

* 1200µl of TDR consisted of 1, 158µl of Tris buffer, 12µl of DTT and 30µl of Rnasin

Plating for cDNA synthesis reaction

Five microliters (5µl) of molecular grade water were added to internal standard, NRT and RCAS transcript wells with 5µl of sample or control added to them. Twenty microliters (20µl) of the RT cocktail were added to appropriate wells (all wells except NRT wells). Twenty microliters (20µl) of the No RT cocktail was added to the NRT wells on the plate, 10µl of diluted HIV-1 RNA transcripts added to the wells for the standard curves, 5µl of diluted RCAS 7500 transcripts added to the wells for the standard curves, 10µl of extracted RNA samples were added in triplicate to appropriate wells followed by the addition of 5µl of extracted RNA samples to the internal standard wells, in duplicate and the NRT wells (one per sample) to be run with the RCAS primers and probes.

The plate was then sealed with Bio-Rad® covers for the RT step. To ensure appropriate mixing, the plate was spun briefly in a centrifuge and placed in the thermocycler for cDNA synthesis using the following program settings for iSCA cDNA:

Step 1: 25° C for 15 minutes

Step 2: 42° C for 40 minutes

Step 3: 85° C for 10 minutes

Step 4: 25° C for 30 minutes

Step 5: 4° C hold

Real Time PCR Reaction

Following the RT run, the seal was gently removed and 20µl of the PCR mixes added to the appropriate wells as outlined in Table 6.2 below

Table 6.2: Roche PCR Master Mix for Real Time PCR

Reagents	Final Conc.	Vol/Rxn (µL)	HIV Rxn 80 Rxns	RCAS Rxn 34 x Rxn
2x Roche Master Mix	1.0 x	19.5	1560	657.9
*Forward Integrase Primer	400 nM (600nM RCAS)	0.2 (0.3)	16	10.2
* Reverse Integrase Primer	400 nM (600nM RCAS)	0.2 (0.3)	16	10.2
*Probe	200 nM (100nM RCAS)	0.1 (0.05)	8	1.7
Sample RNA		10.0	1444	
Total Cocktail		20.0	1600	680
Sample RNA/RT Mix		30.0		
Total Volume		**50.0**		

Probe and Primer Nucleotide Sequences for HIV and RCAS

HIV-1Forward Primer 5'-CATGTTTTCAGCATTATCAGAAGGA-3'
HIV-1 Reverse Primer 5'-TGCTTGATGTCCCCCCACT-3'

HIV-1 Probe 5'FAM-CCACCCCACAAGATTTAAACACCATGCTAA-TAMRA 3'

RCAS Forward Primer 5'-GTCAATAGAGAGAGGGATGGACAAA-3'

RCAS Reverse Primer 5'-TCCACAAGTGTAGCAGAGCCC-3'

RCAS Probe 5'FAM-TGGGTCGGGTGGTCGTGCC-TAMRA 3'

The platen (as the layout depicted in Table 6.3) was then sealed with Roche optical cover and run on the Roche Lightcycler 480 following a brief spin and the following program settings:

Step 1: 95° C for 10 minutes

Step 2: 95° C for 15 seconds .

Step 3: 60° C for 1 minute

Repeat steps 2 – 3 for 45 cycles

Following completion of the run the data was analysed via LightCycler 480 SW 1.5 software.

Table 6.3: Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	VQA 0 NRT	VQA 5 NRT	VQA 5 NRT	VQA 50 NRT	VQA 50 NRT	PQA 5 NRT	PQA 20 NRT	FN-63 NRT	N/A NRT	N/A NRT	N/A NRT	N/A NRT
B												
C	VQA 0	VQA 5	VQA 5	VQA 50	VQA 50	PQA 5	PQA 20	FN-63	N/A	N/A	N/A	N/A
D												
E												
F	3 HIV	3 HIV	3 HIV	3 HIV	10 HIV	10 HIV	10 HIV	10 HIV	30 HIV	30 HIV	30 HIV	30 HIV
G	100 HIV	100 HIV	300 HIV	300 HIV	900 HIV	900 HIV	HIV NTC	HIV NTC	RCAS NTC	RCAS NTC	7500 RCAS Std	7500 RCAS Std
H	VQA 0 RCAS	VQA 5 RCAS	VQA 5 RCAS	VQA 50 RCAS	VQA 50 RCAS	PQA 5 RCAS	PQA 20 RCAS	FN-63 RCAS	N/A RCAS	N/A RCAS	N/A RCAS	N/A RCAS

6.4 Results

Participant selection and characteristics

Ten post-CHER participants were selected for this research study for the evaluation and implementation of virus recovery assays.

A summary of the clinical parameters of the selected participants is listed in Table 6.4 below:

Total CD4 isolation and plating of cells for IVR and TVR assays

HIV-1 CAD for each of the selected samples was quantified previously and listed in the table below (Table 6.5). The total number of PBMCs following the freeze-thaw cycle is listed in Table 6.2 below including the total CD4 T cells isolated. In addition, the number of replicas of 1 $x10^6$ cells/ml plated for each IVR and TVR (including vehicle controls (VCs) is summarised. Samples with low PBMCs following thawing did not have any VC and prioritised for stimulation with PMA and Ionomycin (P/I).

Following the thawing procedure, the median percentage recovery of PBMCs was 78.5% (range: 48-108%) and the recovery of total CD4s was 21.5% (range: 12-30%).

Cell viability measures by CellTiter-Glo® 3D Cell Viability Assay and TVR TaqMan® viral load

Cells subjected to PMI and ionomycin stimulation as well as total CD4 T cells serving as vehicle controls were measured for sustained viability following plating for cell culture isolation.

Cell viability was determined for five participant vehicle control samples and seven participant stimulated cell samples. Cell viability measured as exhibited luminescence or fluorescence (FL) ranged from 2.3 e^6 to 3.9 e^7 with relatively high cell viability for stimulated total CD4 T cells as summarised in Table 6.6 below.

Following the seven-day TVR culturing of stimulated total CD4 T cells as a measure of inducible virus, culture supernatants were quantified for HIV-1viral loads by use of the TaqMan® platform. TaqMan® viral loads were measured for five TVR participant vehicle control samples which ranged from two target not detected (TND) or non-detectable viral load to 476 copies of HIV-1 RNA in the VC sample for PID 340116. TaqMan® HIV-1 viral loads for the stimulated cells were measured for seven study participants and ranged from <20 copies/ml to 1328 copies/ml with one sample displaying an “invalid” result. These results are tabulated in Table 6.6 below.

Table 6.4: Summary of clinical, immunological and virological parameters of participants selected for latent viral outgrowth measurements

PID	Gender	Mode of Delivery	Birth Weight (grams)	CD4 count at birth (percentage)	CD4 count at birth (abs)	~Age of ART initiation (months)	Viral load pre-ART (copies/ml)	Longitudinal viral load	Viral load at 8 years (copies/ml)	CD4 count at 8 years (percentage)	CD4 count at 8 years (abs)
337286	Male	C-section	3260	33.5	2587	2	>750, 000	1 viraemic episode 6 months after cART	Not detected	29	884
334436	Female	NVD	3240	47.5	1927	9,3	>750, 000	Suppressed	Not detected	54	817
340116	Female	Unknown	Unknown	52.8	3731	11,1	696, 000	Poorly suppressed until after 2 years on cART	42	47	2029
339266	Female	NVD	3020	51.3	1759	9,2	635, 000	Suppressed	Not detected	50	2731
335836	Male	NVD	3050	25.4	1150	2,2	>750, 000	1 viraemic episode at 2 years on cART	Not detected	36	584
341862	Female	NVD	2800			12,6	654, 000	Suppressed		33	
335106	Female	NVD	3780	49.0	3828	2,8	>750, 000	Suppressed	Not detected	46	795
360806	Female	NVD	2500			10	>750, 000	Suppressed		35	
332406	Male	NVD	3600	33.1	2039	5,1	277, 000	Suppressed	Not detected	31	659
338206	Male	NVD	3380	29.4	1446	1,8	510, 000	1 viraemic episode	Not detected	39	880

Table 6.5: Summary of HIV-1 CAD results, PBMCs isolated for IVR and TVR inputs, total CD4 T cells recovered from PBMCs and total CD4 T cell count inputs for IVR and TVR assays

PID	Year 7-8 on cART Total Copies HIV DNA (per 10^6 PBMC)	No. of PBMC selected for processing (x 10^6)	PBMC total after thawing (x 10^6) (% recovery)	tCD4 isolated (x 10^6) (% recovery)	IVR (1 x 10^6 Reps)	TVR (1 x 10^6 per well)
337286	32.8	32,5	23,6 (73%)	7,1 (30%)	4	2 (P/I) 1 (VC)
334436	86.3	22,5	17,3 (77%)	4,3 (25%)	2	2 (P/I) No VC
340116	181.5	22,5	24,3 (108%)	6,7 (28%)	3	2 (P/I) 1 (VC)
339266	46.7	20,0	20,5 (103%)	5,1 (25%)	2	2 (P/I) 1 (VC)
335836	11.9	32,5	18,0 (55%)	2,1 (12%)	1	1 (P/I) No VC
341862	42.3	32,5	31 (95%)	5,8 (19%)	3	2 (P/I) 1 (VC)
335106	4.5	25,0	20,0 (80%)	4,8 (24%)	2	2 (P/I) 1 (VC)
360806	186.2	25,0	12,0 (48%)	1,5 (13%)	1	1 (P/I) No VC
332406	23.6	17,5	14,0 (80%)	1,8 (13%)	1	1 (P/I) No VC
338206	21.3	17,5	11,0 (63%)	1,8 (16%)	1	1 (P/I) No VC

IVR = infectious virus recovery, **TVR = total inducible virus recovery, **P/I** = PMA and ionomycin stimulation, **VC** = unstimulated vehicle controls*

Table 6.6: Summary of cell viability measures and HIV-1 viral loads measured by TaqMan® assay for both vehicle controls (VC) and stimulated (P/I) samples

PID	Year 7-8 on cART Total Copies HIV DNA (per 10^6 PBMC)	Cell Viability/Glow Assay -FL (VC)	Cell Viability/Glow Assay- FL (P/I)	TVR TaqMan® Viral load (V/C)	TVR TaqMan® Viral load (P/I)
337286	32.8	$3{,}6e^6$	$3{,}9e^7$	150 copies/ml	148 copies/ml
334436	86.3	-	$1{,}7e^7$	-	*Invalid*
340116	181.5	$2{,}3e^6$	$2{,}1e^7$	476 copies/ml	794 copies/ml
339266	46.7	$3{,}3e^6$	$1{,}0e^7$	TND	1328 copies/ml
335836	11.9	-	$7{,}0e^6$	-	142 copies/ml
341862	42.3	$2{,}7e^6$	$1{,}1e^7$	<20 copies/ml	40 copies/ml
335106	4.5	$2{,}3e^6$	$1{,}1e^7$	TND	<20 copies/ml
360806	186.2	-	*Collected cells*	-	-
332406	23.6	-	*Collected cells*	-	-
338206	21.3	-	*Collected cells*	-	-

** **TVR** = total inducible virus recovery, **P/I** = PMA and ionomycin stimulation, **VC** = unstimulated vehicle controls, **TND** = target not detected*

p24 Antigen ELISA measurements of cell culture supernatants following IVR assay at days 7, 14, 21 and 28

Following implementation of the IVR assay (or VOA), the levels of p24 antigen expressed in cell culture supernatants were measured as an output for determining the presence of HIV-1 replication-competent latent virus. ELISAs for p24 antigen was implemented on days 7, 14, 21 and 28. A positive p24 ELISA requires 100, 000 HIV-1 RNA copies to provide a positive signal. The results for p24 antigen measurements are tabulated in Table 6.7 below and depicts a negative finding for all participants.

Table 6.7: p24 antigen measured in cell culture supernatants on days 7, 14, 21 and 28 of IVR (VOA) assay

PID	Year 7-8 on cART Total Copies HIV DNA (per 10^6 PBMC)	p24 antigen Day 7	p24 antigen Day 14	p24 antigen Day 21	p24 antigen Day 28
337286	32.8	NEG	NEG	NEG	NEG
334436	86.3	NEG	NEG	NEG	NEG
340116	181.5	NEG	NEG	NEG	NEG
339266	46.7	NEG	NEG	NEG	NEG
335836	11.9	NEG	NEG	NEG	NEG
341862	42.3	NEG	NEG	NEG	NEG
335106	4.5	NEG	NEG	NEG	NEG
360806	186.2	NEG	NEG	NEG	NEG
332406	23.6	NEG	NEG	NEG	NEG
338206	21.3	NEG	NEG	NEG	NEG

HIV-1 RNA expression in IVR (VOA) cell culture supernatants measured on days 7, 14, 21 and 28 using the novel integrase single copy assay (iSCA)

As described the implementation of the iSCA assay was implemented to determine HIV-1 RNA expression (ramp-up) in cell-culture supernatants. Table 6.8 provides a summary of the HIV-1 RNA copies per ml following qPCR iSCA

Table 6.8: HIV-1 RNA copies/ml measured at Days 7, 14, 21 and 28 in cell-culture supernatants

PID	iSCA HIV-1 RNA copies/ml Day 7	iSCA HIV-1 RNA copies/ml Day 14	iSCA HIV-1 RNA copies/ml Day 21	iSCA HIV-1 RNA copies/ml Day 28
337286	-	-	<1,3	<1,3
334436	47,9	41,9	10,5	<2,6
340116	1,7	<1,7	696,7	134,4
339266	36,7	<2,6	8,8	7,9
335836	-	-	<5,2	<5,2
341862	-	-	<1,7	<1,7
335106	-	-	<2,6	<2,6
360806	-	<4,6	10,5	<5,2
332406	-	<4,6	<5,2	<4,6
338206	-	<4,6	<5,2	<4,6

Comparative sub-set TVR analysis of TaqMan® HIV-1 viral load measurement versus iSCA HIV-1 RNA viral load measurements

We compared TaqMan® HIV-1 RNA viral load measurements with that of the qPCR iSCA method in cell culture supernatants. Results are summarised in Table 6.9 below. We show that TaqMan® overestimates HIV-1 RNA copies, whereas iSCA provides a more accurate measurement as it excludes HIV-1 DNA contaminants.

Table 6.9: Comparative analysis of HIV-1 RNA copies measured in cell culture supernatants with COBAS AmpliPrep/COBAS TaqMan® and the more sensitive qPCR iSCA method

PID	TVR TaqMan® Viral load (copies/ml) (P/I)	TVR iSCA HIV-1 RNA (copies/ml)
337286	148 copies/ml	<2,8
334436	Invalid	2266,9
340116	794 copies/ml	<6,1
339266	1328 copies/ml	<5,2
335836	142 copies/ml	-
341862	40 copies/ml	<2,8
335106	<20 copies/ml	<2,8

6.5 Discussion

The aim of this research component was to explore the implementation of cell-culture based assays for the quantification of latent HIV-1 replication-competent virus in ten long-term virologically suppressed PHIV children (7-8 years of age) initiated on ART within 6 months of life.

Of the ten early treated children, we were unable to detect replication-competent virus by the gold standard assay, QVOA, as depicted by negative p24 cell culture wells following 28 days of culturing. However, despite low HIV-1 iCAD, virological suppression and reconstituted CD4 T cell counts, we were able to detect inducible virus in cell culture supernatants using a novel highly sensitive RT-qPCR assay (iSCA) for the detection of HIV-1 RNA copies/ml.

Implementation of QVOA has been extensively and successfully implemented for adult populations and although some studies have implemented QVOA in paediatric participants, very few of these were within children with long-term virological suppression and low copies of cell-associated HIV-1 DNA.

In contrast to our study, Persaud et al., 2000 were able to successfully demonstrate a stable replication-competent HIV-1 reservoir in resting CD4 T lymphocytes in children between the ages 0.25 to 13.8 years of age with PHIV. The plasma viral load levels of these participants however ranged from <400 to 750,000 HIV-1 RNA copies/ml. Of the 21 participants subjected the QVOA, 18 children were found to have replication-competent HIV-1. In addition, these researchers were able to show a direct correlation between the frequency of latently infected resting CD4+ T cells and plasma virus levels, suggesting a pre-integration state of latency in these cells in children with ongoing viral latency (Persaud et al., 2000). In the same study, replication-competent HIV-1 was isolated from seven children who displayed undetectable viral loads and viral

suppression following 1-3 years of cART. These children showed limited HIV-1 reservoir decay suggesting a more stable reservoir within the post-integration stage of latency.

In another study conducted in 14 children subjected to cART initiation at 2 months of age, infectious virus was detectable in 60% by QVOA following 1.8 years of therapy (Persaud et al., 2012b). In addition, researchers found that the size of the latent reservoir was associated with the time to achieve the first undetectable viral load highlighting further the role of early cART in curtailing the size of the latent reservoir.

Rainwater-Lovett et al., 2017 however, showed no inducible HIV-1 by QVOA in 11 PHIV children initiated on ART within 8.9 week of age with virological suppression sustained for 11 years. In addition, researchers demonstrated defective non-induced proviral genomes (NIPG) which they hypothesise is likely due to do early cART initiation and long-term virological suppression thwarting the persistence of intact proviral genomes (Rainwater-Lovett et al., 2017). Further demonstrating the same finding, is a more recent study by Dhummakupt et al., 2020 who compared the inducibility of HIV-1 viral reservoirs between perinatal and adult infection and found fewer replication-competent proviruses persisting in children on long-term cART.

Similarly, published data within our research group from the same CHER children assessed for QVOA were evaluated for proviral sequences by nearly full-length proviral amplification and sequencing (NFL-PAS) at the same time point. This research showed a low frequency of intact proviruses compared to reported HIV-1 subtype B infected adults treated early. Cells that harbour intact HIV-1 proviruses are considered rare within the context of long-term suppression and early cART and therefore would require a large number of cells to quantify the HIV-1 latent reservoir (Katusiime et al., 2020).

In itself, the QVOA requires a significantly large sample volume (120 -180ml) from respective donors in order to successfully quantify the HIV-1 latent reservoir. Following the mitogenic reactivation of resting CD4+ T cells by limiting dilution, maximum likelihood statistics are then used to estimate the amount of reactivated virus as IUPM (Tae Wook Chun et al., 1997; Siliciano and Siliciano, 2005; Eriksson et al., 2013; Rosenbloom et al., 2015). As mentioned, QVOA is considered the gold standard method compared to other molecular based techniques due to the high threshold requirements for a positive p24 ELISA signal (100, 000 HIV-1 RNA copies required) thereby limiting the over estimation of the HIV-1 latent reservoir size (Siliciano and Siliciano, 2005).

A key limitation to this research was therefore the restricted blood volume available from children and the amount of stored PBMC material for QVOA assays which limited the sensitivity to detect inducible replication component virus in virologically suppressed children with low iCAD. The requirement for large blood volumes, is considered one of the drawbacks of QVOA. Other limitations for the implementation of this technique includes it being time consuming (requires three weeks of culturing for virus outgrowth), labor-intensive and expensive. In addition, QVOA underestimates the size of the HIV-1 latent reservoir by 25-fold in adults treated within the acute infection phase and 27-fold in those treated during the chronic infection phase. This is due to not all genetically intact proviruses being sufficiently reactivated in vitro and may therefore require multiple rounds of stimulation (Bruner et al., 2016; Hosmane et al., 2017). QVOA is therefore not considered suitable for large-scale studies.

Subsequent to implementation of the QVOA assay within our cohort, improvements to the QVOA were followed within our research laboratory and incorporated the use of MOLT4/CCR5 cells lines as designed by Laird et al., 2013b. This improved adaptation to QVOA allows for a more rapid measurement of the number of latently infected CD4+ T cells. MOLT4/CCR5 T cells that are highly permissive to HIV-1 replication and used to propagate the HIV-1 virus instead of the significant amount of donor lymphoblasts cells used in the original QVOA (Laird et al., 2013b; Bruner, Hosmane and Siliciano, 2015b). These MOLT4/CCR5 cells are also only added once following activation thereby reducing labor intensity. The improved assay also replaces the p24 antigen ELISA assay with a more sensitive RT-PCR assay used to quantify the HIV-1 viral RNA copies. This adaptation of including a RT-PCR assay is similar to our novel iSCA assay implemented to measure inducible HIV-1 virus. The highly sensitive iSCA assay excluded HIV-1 DNA contamination and provides a more sensitive means for detecting inducible virion production in QVOA cell culture supernatants from persisting HIV-1 reservoirs in children. This iSCA method has since been further optimised and published (Tosiano et al., 2019).

No significant differences were observed between the gold standard QVOA and the MOLT4/CCR5 VOA and therefore offers great potential for improving the gold standard QVOA method given its ability to offer rapid, more sensitive, is cost-efficient, consumes less cells and is less labour intensive and therefore considered more suitable for large-scale clinical trials (Laird et al., 2013b; Hodel et al., 2016). Drawbacks of the MOLT4/CCR5 assay, however, include its underestimation of the size of the true latent reservoir.

More recent comparative analysis for determining target cell populations that are most sensitive for detecting inducible viral outgrowth include evaluating MOLT4-R5, SupT1-R5 and allogeneic blasts (Enick et al., 2021). This study compared these cell populations across 15 individuals on suppressive cART. Researchers found that the MOLT4-R5 method yielded a higher proportion (42%) of p24 antigen positive wells than both the SupT1-R5 and allogeneic blasts. In addition, 5 of the 7 QVOA that were negative for p24 antigen signal showed viral outgrowth by HIV-1 RNA quantification that were greater than 10-fold higher within 7 days. Therefore, this comparative analysis further revealed the potential for underestimation of the latent, inducible reservoir by QVOA. Enick et al., 2021 therefore suggested the use of MOLT4-R5 cells in in tandem with both p24 ELISA and HIV-1 RNA RT-qPCR methods to detect viral outgrowth.

The strengths of qPCR approaches to measuring viral outgrowth include being highly sensitive, inexpensive, requiring low sample volumes and having high-throughput potential. Yet, the majority of proviral HIV-1 is replication defective especially when on suppressive cART. Total HIV-1 DNA assays are unable to distinguish between defective and intact provirus and therefore overestimate the true size of the reservoir by more than 300-fold (Ho, Shan, Hosmane, Wang, Laskey, Daniel I.S. Rosenbloom, et al., 2013).

A more recently developed novel assay either used in place of QVOA or in conjunction with it, is the Intact Proviral Detection Assay (IPDA) (Bruner et al., 2019). This assay utilises droplet digital PCR to target two highly conserved regions of the HIV-1 viral genome which allows for the concurrent quantification and discernment of both intact and defective viral genomes (Bruner et al., 2019). Following the IPDA assay implemented at two years on cART, early treated children (within 2 weeks of life) indicated that intact

proviruses decayed significantly faster than defective genomes during the first 24 weeks on ART (Garcia-Broncano et al., 2019).

The tat/rev induced limiting dilution assay (TILDA) has also been utilised for the measurement of the latent reservoir which quantifies the inducible replication-competent HIV-1 reservoir within 3 days (Procopio et al., 2015). TILDA relies on the measurement of tat/rev transcripts from cells plated in limiting dilution following activation with PMA and ionomycin. Although TILDA correlated relatively well to HIV-1 DNA and measures of the latent reservoir, this assay does not correlate well with QVOA and is susceptible to overestimating (48-fold greater than detected in adult infections) (Ho, Shan, Hosmane, Wang, Laskey, Daniel I S Rosenbloom, et al., 2013; Procopio et al., 2015; Dhummakupt et al., 2020). Due to its small sample volume input, TILDA is suitable for paediatric studies and large-scale clinical trials.

Lastly, whilst several assays as mentioned above have been developed for the measurement and characterisation of the HIV-1 replication-competent reservoir, the application of these assays in children within resource-limited settings is limited. This is largely due to these assays being expensive, requiring large sample volumes and is considered labour intensive. Measurements of HIV-1 specific antibody concentrations has been shown to be associated with HIV-1 viral persistence and may be used for the estimation of the size of the residual latent reservoir in children on suppressive ART (Palma et al., 2020). In addition, measurements of HIV-1 specific antibodies are also useful for the screening of children on suppressive cART for the enrolment into possible vaccine trials or HIV-1 remission strategies.

6.6 Conclusion

Children initiated on cART early did not have infectious replication-competent virus that could be readily isolated following the viral outgrowth assays. However, inducible virion production was detected in 4 of 10 children using a highly sensitive HIV-1 RNA assay. This assay may be a sensitive biomarker for virion production in latent HIV-1 reservoirs from children from whom only small sample volumes are feasible.

The ability to detect inducible viral HIV-1 RNA provides further knowledge to HIV-1 persistence in children on long-term suppressive therapy along with measures of HIV-1 cell infectivity (iCAD). Techniques such QVOA and others are important in cure-based research, as accurate determination of true replication-competent latent reservoir HIV-1 is the cornerstone of assessing effectiveness of any approach in eliminating all traces of the virus.

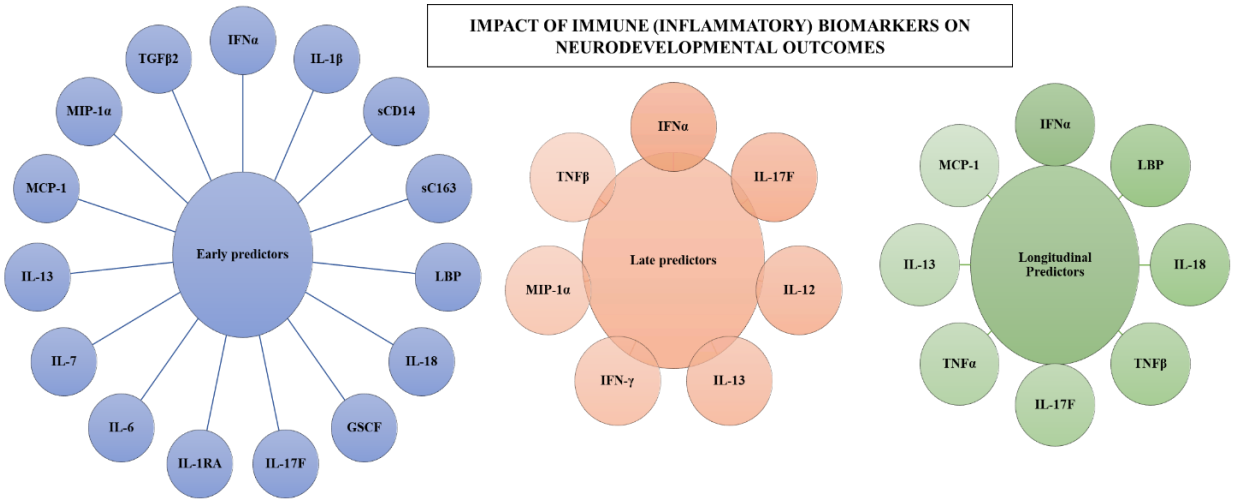
CHAPTER 7:

DELINEATING THE LONGITUDINAL RELATIONSHIP BETWEEN ANTIRETROVIRAL THERAPY (ART) AND HIV-1 ASSOCIATED INFLAMMATION ON NEUROCOGNITIVE OUTCOMES IN PERINATALLY INFECTED (PHIV) CHILDREN IN SOUTH AFRICA

SUMMARY OF KEY FINDINGS – CHAPTER 7

Therapy regimen and neurodevelopmental outcomes	•**Deferred therapy had lower neurodevelopmental scores from 11 to 42 months** •Delaying ART results in initial developmental delay – recovery after 60 months •**Therapy interruption had minimal impact on overall neurodevelopmental outcomes**
Early (ART naïve) immune biomarkers of neurodevelopmental outcomes	•**Significant pos associations:** IL-1β, G-CSF, sCD14, sCD163, IL-18 and LBP (neuroprotective mechanism) •**Significant neg associations:** IL-17F, IL-1RA, IL-6, IL-7, IL-13, MCP-1, MIP-1$^{\alpha}$, IFN-α and TGFβ
Associated change in biomarker levels with change in neurodevelopmental outcomes	•**Significant pos associations:** IL-18 (Baseline to 8 years) and VMI scores (5 to 9 years) •**Significant neg associations**: MCP-1, TNFα, IL-18 and LBP
Pre-adolescent (8 years) biomarker association with pre-adolescent neurodevelopmental outcomes	•**Significant pos association**: IFABP at 8 years and VMI Standard and Raw scores at 9 years of age •**Significant neg association**: IL-17F, IL-12, IL-13, IFN-γ, MIP-1$^{\alpha}$, TNFβ and IFN-α
Clinical predictors of neurodevelopmental outcomes	•**Significant + associations**: Time to viral suppression, gestation time, CD8 T cell count at birth •**Significant neg association**: Time to therapy initiation and CD4 T cell count at birth
Virological predictors of neurodevelopmental outcomes	•Baseline HIV-1 CAD associated with General Griffiths and Locomotor test scores (pos association)
CMV Associations (before therapy initiation)	•App. 24% of children were positive for CMV (10 detected) •CMV pos group had lower %CD4 counts and higher %CD8 counts at birth •CMV pos group had significantly increased levels of IL-12, IP-10, sCD163, IL-18 and LBP
CMV Associations (8 years of age)	•At 8 years of age, app. 90% of children had CMV exposure •CMV viral loads were significantly associated with IL-1β (-), hsCRP (+) and LBP (+) •CMV has no association to neurodevelopmental outcomes at 8 years of age

***Conclusion**: We have shown significant longitudinal associations between immunological, virological and clinical parameters and neurocognitive outcomes in PHIV children. Exploring the relationship between the gut and brain i.e. the gut-brain axis in early life will be of value when investigating adequate brain function in children.*



Explanation of the schematic above: *A summary of immune biomarkers that have associations with neurocognitive outcomes at baseline (early predictors), 8 years of age (late predictors) and biomarkers that are able to longitudinally predicate neurocognitive outcomes during the course of HIV-1 infection. Consistent biomarkers that span early life and pre-adolescent years include INFα, LBP, IL-18, TNFβ, IL-17F, TNFα, IL-13 and MCP-1 – majority of these biomarkers mainly play a role in monocyte/macrophage activation and migration between the periphery and the brain as well as the gut.*

ABSTRACT

Background: The complete characterisation of the neuropathology of HIV-associated neurocognitive disorders (HAND) in both adults and children during viral suppression still requires further delineation as HIV-1 infection is confirmed earlier and treatment options improve. Clinical and immunological biomarker associations with neurological outcomes in children with PHIV are limited. Not only do current studies not examine a wide range of clinical and immunological biomarkers and their resultant relationship to neurodevelopment, but also do not evaluate the change in these biomarker levels longitudinally in relation to disease progression, different therapy regimes and long-term virological suppression.

Methods: This research study employs a mixed methodology, longitudinal and cross-sectional, study design for investigating the impact of clinical, immunological, and virological parameters on longitudinal neurodevelopmental outcomes in PHIV children in Cape Town, South Africa. Retrospective samples were analysed following sample collection at the start of the CHER trial in 2004. Neurocognitive assessments included the Griffiths Mental Development Scales (GMDS) administered at 11, 18, 30, 42 and 60 months of age and included assessments of locomotor, personal-social, hearing and language, eye-hand co-ordination and performance. The Beery-Buktenica Development Test for Visual-Motor Integration (Beery-VMI) was implemented at 5, 7 and 9 years of age. A total of 40 Immunological plasma marker levels were measured by Luminex® Multiplex Assays and ELISA. A sensitive qPCR adapted for HIV-1 subtype C was implemented for the measurement of HIV-1 total DNA. This assay was aimed at targeting a region in HIV-1 integrase.

Results: In early neurodevelopmental tests related to Locomotor and General Griffiths Test Scores, we observed significant positive correlations between pro-inflammatory immune biomarkers such as IL-1β, GCSF, sCD14, sCD163, IL-18 and LBP. These biomarkers have primary functions in monocyte/macrophage

activation and responding to bacterial infections. Negative correlations between early immune biomarkers and early neurodevelopmental outcomes were also observed which may be indicative of protective immune response to preserving the CNS. Proinflammatory biomarkers associated monocyte/macrophage function such as IL-1RA, MCP-1 and MIP-1α were negatively associated with Locomotor and General Griffiths Test scores. Anti-inflammatory cytokines including IL-13, IFN-α as well pleiotropic cytokines including IL-6 and TGF-β2 were also negatively associated with both early and late neurodevelopmental parameters. IL-7, required in early T cell development and IL-17F, produced by T-cells and macrophages displayed negative associations with early neurodevelopmental scores. The latter cytokines were the only T-cell related cytokines measured in early life and before therapy initiation that had an association of neurodevelopmental outcomes. Important early clinical parameters showed significant associations with both early and late neurodevelopmental outcomes. These included time to viral suppression (r=0.34; p=0.03), gestation (r=0.33; p=0.03), % CD8 at birth (r=0.41; p=0.03), Abs CD8 count at birth (r=0.40; p=0.02), time to therapy initiation (r= -0.41; p<0.01) and %CD4 at birth (r = -0.36; p=0.02).

Conclusion: Neurocognitive outcomes can be predicted by early immunological, virological and clinical parameters. Early initiation of cART is significantly import for mitigating immune activation and inflammatory responses which significantly impact the neuro-immune relationship.

7.1 Introduction

Early initiation of cART has shown much benefit in improving the overall survival and quality of life in HIV-1 infected populations. Co-morbidities however remain a significant growing health challenge for people living with HIV (PLWH). Neurological deficits constitute one such challenge with significantly high prevalence, and therefore requires detailed attention and investigation (McGrath et al., 2006; Malee et al., 2011; Cross, Combrinck and Joska, 2013; Lentoor, 2020).

Given the importance of neurocognitive health in the individuals ability to live and function appropriately in society, the complete characterisation of the neuropathology of HAND in both adults and children during viral suppression still requires further delineation as HIV infection is confirmed earlier and treatment options improve (Gelman 2015).

Prevalence, risk factors and diagnosis for HIV Associated Neurocognitive Disease (HAND)

With the variability in study population characteristics and risk profiles in mind, HAND affects approximately 18-69% of adults living with HIV-1 even with viral suppression with cART (Robertson et al., 2007; Heaton et al., 2010; Simioni et al., 2010; Cystique and Brew, 2011; Sacktor et al., 2016; dos Santos-Silva et al., 2017).

In children, neurological disease is three times more prevalent compared to adults with 75% of cases with neurological dysfunction occurring within the first two years of life (Blanche, 1997; Chase et al., 2000; Tardieu et al., 2000; Walker et al., 2013). During the first year of infection, the incidence of clinical CNS disease is significantly higher in children (approximately 10%) than in adults (0.3%).

Due to the vulnerability of a developing brain, children with PHIV present with more frequent CNS opportunistic infections as a result of HIV-1 immunosuppression compared to adults (Crowell et al., 2014; Lentoor, 2020). In a South African study cortical and subcortical structural impairment in children with PHIV was documented to be the major neurological manifestation associated with long-term neurocognitive impairments (Knox et al., 2018).

In the absence of cART, between 20-50% of children with PHIV will develop HIV-associated encephalopathy (HIVE) due to the complications associated with uncontrolled HIV-1 infection. HIVE is strongly associated with reduced brain volume and chronic immune activation and inflammation. However, even in the setting of early cART, sustained virological control and good clinical follow-up, children with PHIV infection still show compromised and worsened neurodevelopmental outcomes compared to their uninfected counterparts (Boivin et al., 2020; Kapetanovic et al., 2021).

Three categories of HAND have been defined in the general HIV population (Antinori et al., 2007) in order of increasing cognitive and functional severity: (1) asymptomatic neurocognitive impairment (ANI), (2) mild neurocognitive disorders (MND) and (3) HIV-associated dementia (HAD).

The prevalence of HAD, the most severe form of HAND and a key driver of HIV-associated mortality, has significantly decreased from approximately 20% to 2-4% due to the efficacy of cART implementation and subsequent viral suppression in PLWH (Heaton et al., 2010). The incidence of ANI and MND remain high in

virologically suppressed individuals leading to deficiencies in memory, learning and attention functions (Cysique, Maruff and Brew, 2004; Robertson et al., 2004; Heaton et al., 2010, 2011; Harezlak et al., 2011). In addition, the presence of ANI has been associated with an increased risk of more severe HAND morbidities (Grant et al., 2014) further highlighting the societal implications and financial burden of HAND in PLWH (Angelovich et al., 2020) specifically in high disease burden settings such as South Africa.

There are many risk factors known to be associated with HAND outcomes in PLWH. These include CD4+ T cell counts at the time of diagnosis as well as nadir T cell counts, the duration of HIV-1 infectivity, age, co-infections such as CMV, the presence of co-morbidities such as diabetes, hypertension and dyslipidaemia and substance abuse (Saylor et al., 2016).

In addition, persistent inflammation and immune activation play a highly significant role in the pathophysiology of HAND (Angelovich et al., 2015) and serve as the key focus of this research.

In the case of virologically suppressed older PLWH, increased HIV-1 DNA in PBMCs has shown significant correlation with neurocognitive impairment highlighting the relevance of persisting HIV-1 latent reservoirs in neuropathology (De Oliveira, Murrel, et al., 2015). Limited knowledge is available on the role of HIV-1 latent reservoirs and their association with neurocognitive outcomes in PHIV.

Other risk factors highly associated with HAND and briefly alluded to above, include lipid dysfunction (Fogel et al., 2015) which has been described in older populations of PLWH. Very little is understood about the association of HAND and the increasing prevalence of early onset of cardiovascular disease in children with PHIV. Furthermore, observations in adult HIV-1 infected populations have indicated a strong association between HAND and a number of other HIV-related co-morbidities including hypertension and increased blood cholesterol (Wright et al., 2010; Guaraldi et al., 2011). HAND has also been associated with CSF dyslipidaemia and kidney function (Bandaru et al., 2013; Yuen et al., 2017). Very little is understood about the association of HAND and the increasing prevalence of early onset of cardiovascular disease in children with PHIV.

CMV as well as Hepatitis C are common co-infections in PLWH. Recent data have shown an association between anti-CMV IgG levels and neurocognitive impairment. Similarly, HIV-Hepatitis C co-infection has shown a twofold increased risk of cognitive impairment compared to HIV-1 infection alone (Ciccarelli et al., 2013; Brunt et al., 2016; Letendre et al., 2018). The role of CMV and Hepatitis C in neurocognitive impairment in virological suppressed PHIV children requires more detailed investigation.

Diagnosis of HAND in adults is achieved by both clinical neurological assessments and neuropsychological examinations. These assessments are followed by magnetic resonance imaging (MRI) of the brain and CSF analysis. In 2007, the National Institute of Mental Health along with the National Institute of Neurological Disease and Stroke proposed the “Frascati” criteria for the assessment of neurocognitive function by scoring motor skills, sensory-perceptual capacity, speed of information processing, memory, abstraction and executive function, attention and working memory, verbal and language skills and whether assistance is needed to carry out daily activities (Antinori et al., 2007; Angelovich et al., 2020). The mentioned scores are then compared

to the scores of age- and education-matched neurologically unimpaired individuals. When an impairment of two or more of the evaluated scores occur, a case of HAND is diagnosed.

However, for HIV-1 infected children, particularly in South Africa, diagnosis of HIVE and HAND is made by use of developmental abilities on implemented neurocognitive tests. The use of MRIs is not considered standard practice in this setting and is mainly implemented within a clinical trial scenario.

HIV-1 infection of the CNS

Within days of primary infection, HIV-1, a neurotropic virus, can enter the CNS resulting in HIV-related neurological manifestations as described above. The virus does not directly infect the neurons of the CNS but infects the glial cells that support and protect these neurons. In addition, HIV-1 results in inflammation that leads to further damaging of the brain and spinal cord (Vincenti, Ilena and Merkler, 2021).

Immune surveillance and maintenance of homeostatic conditions of the CNS is arbitrated by both innate immune cells and specific subsets of T cells (Vincenti, Ilena and Merkler, 2021). This neurotropic virus can deflect CNS barriers through either transcellular, paracellular mechanisms or by “Trojan Horse” infection of the CNS infiltrating leukocytes and leading to the crossing over of CD14+CD16+ HIV infected peripheral blood monocytes and T cells into the BBB (Fischer-Smith et al., 2001; Honeycutt et al., 2018).

HIV-1 infected monocytes and T cells migrate to the BBB to seek perivascular macrophages, astrocytes, and microglia. Viral persistence may therefore drive the activation of microglia and astrocytes thereby inducing pro-inflammatory cytokine production, altered glutamate uptake and altered metabolism which further drives neuronal injury and degradation contributing to the BBB damage, brain atrophy and ultimately HAND (Angelovich et al., 2020). Current theories consider BBB damage as a result of CNS infection. To a certain degree, the opposite may be true as systemic infection may also directly damage the BBB. During acute HIV infection, HIV-1 RNA is detected in CSF as early as 8 days following infection and is likely to continue throughout natural infection in response to chemokines produced by microglia and astrocytes (Persidsky et al., 1999; Valcour et al., 2012).

Preceding transmigration to the CNS, monocytes mature into perivascular macrophages, which may support HIV-1 replication and mediate infectivity of the surrounding cells such as macrophages that express CD4 receptors and microglia (Fischer-Smith et al., 2001; Kim et al., 2006; Thompson et al., 2011; Burdo, Lackner and Williams, 2013). In addition, perivascular macrophages may also harbour latent HIV-1 reservoirs (Kim et al., 2006). Although HIV-infected T cells and cell-free virus may also enter the CNS, possibly due to the breakdown of the tight junctions of the BBB, macrophage-driven viral replication is considered to be the primary source of HIV-1 replication in the CNS of patients at least those with severe HAND (Schnell et al., 2009; Strazza et al., 2011).

After CNS infection, HIV-1 viral proteins (i.e., gp120, nef, vpr and tat) contribute to activating nearby macrophages, microglia, and astrocytes. Following the activation of these cells, a host of cytokines and chemokines are released that drive the process of localized neurological inflammation of which multiple sites of the CNS is targeted (Shah and Kumar, 2010; Shah, Singh, et al., 2011; Shah, Verma, et al., 2011; Bagashev

and Sawaya, 2013; Sami Saribas et al., 2017). The release of cytokines and chemokines have the potential to activate and result in damage of the BBB. The resultant damage may further enhance the permeability and recruitment of monocytes. This mechanism is mainly driven by MCP-1, CD14+CD16+ monocytes and CCL2. Further enhancement of HIV-1 replication in the CNS can also result from cellular activation (Conant et al., 1998; Williams et al., 2009, 2013, 2014; Brown et al., 2011).

A robust innate immune response present at the CNS barrier, consisting of specialized macrophages such as meningeal, perivascular and choroid plexus macrophages which are collectively called CNS associated macrophages (CAMs), prevents viral spread into the sub-adjacent parenchyma cells (Kierdorf et al., 2019).

During an early pathogen invasion, CAMs and associated CNS-resident microglia, initiates the release of pro-inflammatory cytokines and chemokines (Jordão et al., 2019; Van Hove et al., 2019). An initiation of an inflammatory response by CAMs triggers the recruitment of additional immune cells including T-cells, neutrophils, and monocytes.

Long-lived meningeal macrophages survey their environment and support a robust inflammatory response. These cells are also considered tissue-resident and are not replenished by blood monocytes in healthy CNS. More importantly, these long-lived meningeal macrophages may also harbour HIV-1 latent virus (Lamers et al., 2011; Goldmann et al., 2016).

HAND pathophysiology in the context of HIV-1 viral suppression

In the past, HIV-1 viremia, low CD4 T cells counts and a high viral load in the CSF was significantly associated with severe HAND outcomes. However, in the setting of virological suppression, HAND morbidities are more likely to be related to other factors indicating a fundamental and distinct pathology of the CNS. Limitations to fully understanding the pathophysiology of the disease attributes to the inability to conduct CNS biopsies from virologically suppressed individuals. Most evidence describing existing pathology in the CNS of virologically suppressed individuals stems from peripheral indicators of CNS disease or brain imaging data (Angelovich et al., 2020).

Even with cART, individuals with HAND show CNS pathologies indicative of degradation of cortical grey matter, dysfunction of the BBB (Chaganti et al., 2019), persistent reduction of brain volume (Cardenas. et al., 2009; Küper et al., 2011), continual activation of the microglia (Garveya et al., 2014; Rubin et al., 2018) and an increase in cerebral metabolites as indication of continuing neuronal damage (Young et al., 2014).

Other associations of cognitive decline include elevation of CSF neurofilament light chain which is indicative of axonal damage (Krut et al., 2014; Sailasuta et al., 2016; Sun et al., 2017). Osteopontin, a pro-inflammatory bone matrix protein biomarker has also shown elevation in virologically suppressed patients diagnosed with HAND (Brown et al., 2011).

In addition, chronic cellular activation in the blood periphery may also be contributory to HAND in virologically suppressed individuals. Studies have indicated an increase in the number of activated macrophages in the CNS when compared to neurologically unimpaired individuals (Tavazzi et al., 2014). Levels of monocyte chemoattractant protein-1 (MCP-1) and C-X-C motif chemokine ligand 10 (CXCL10),

produced by activated monocytes and macrophages are higher in suppressed PLWH and have been documented to be significantly increased in suppressed individuals with HAND (Letendre, 2011). Soluble CD14, an indicator of monocyte activation was also significantly elevated in virologically suppressed individuals with HAND and has also been associated with deteriorated neurological outcomes in unsuppressed HIV infected individuals (Edén et al., 2007; Spudich et al., 2011; Kamat et al., 2012; Yilmaz et al., 2013). Other pro-inflammatory cytokines such as TNF, IL-6 and IL-27 have shown to have associations with individuals with HAD or MND compared to neurologically unimpaired individuals (Venkatachari et al., 2017). TNF-α has also been shown to be elevated following its release in the case of astrocyte dysfunction that results in the improper buffering of glutamate and the dysregulation of BBB permeability.

A recent cross-sectional South African study investigating the relationship between clinical immune status and neurocognitive function in children with PHIV found a significant correlation between CD4+ T cells and neurocognitive function scores related to working memory, processing speed and perceptual reasoning (Lentoor, 2020). Another study showed an inability to improve neurocognitive function at 5 years of age despite early ART initiation (Milligan and Cockcroft, 2017). Furthermore, a study conducted in India, provided significant evidence indicating severe neurocognitive impairment despite long-term ART, viral suppression and normal CD4+ T cell counts (Ravindran, Rani and Priya, 2014).

Pinpointing the potential mechanisms driving HAND outcomes in ART suppressed vulnerable populations such as HIV infected children in South Africa, can provide important insights to biological or therapeutic targets that may be used to improve the quality of neurological outcomes.

Key Mechanisms driving HAND during virological suppression

Mechanisms that drive HAND during long-term virological suppression include low-level viral replication, systemic inflammation driven by gut damage (“leaky gut” syndrome) and possibly epigenetic changes (Angelovich et al., 2020). It is also important to note that not all PLWH present with HAND outcomes implying a possible role for special host factors in disease manifestations. Therefore, in the setting of virological suppression, the clinical factors presented above may provide some diagnostic value.

Even in the presence of effective ART, there is a need for early identification of neurocognitive deficits in children to mitigate for better outcomes through mental rehabilitation interventions. Microbial infections that are not associated with the CNS can have an important impact on the CNS. In addition, the immune system plays a significant and important role that serves as both protective and pathogenic (Hong and Banks, 2015). Challenges of early ART relates to the poor ability of these drugs to cross the BBB and target virus contained with the CNS.

Current treatment with cART including abacavir, lamivudine, nevirapine, lopinavir, dolutegravir and additional integrase inhibitors have shown evidence of improved neurocognitive outcomes indicating a capacity to penetrate the CNS well (Cysique et al., 2009). In addition, modern ARVs with penetrative capabilities have shown increased suppression of HIV RNA within the CNS (Carvalhal et al., 2016).

Data generated from a sub-study of the Strategic Timing of AntiRetroviral Treatment (START) have however shown no neurocognitive advantage of immediate versus delayed initiation of ART specifically in individuals with T cell counts above 500 cells/µl (Wright et al., 2018).

Biomarkers of HIV-1 CNS infection and HAND

The identification of biomarkers that may predict and provide further insight to neurocognitive impairment in vulnerable populations is considered a key research focus. Methods to reliably predict HAND before onset, then accurately diagnose cognitive impairment and screen for changes in cognitive performance over time is much needed. Highly efficient biomarkers are characterised by being readily available, affordable, and consistently reliable. Current well-established plasma and CSF biomarkers of inflammation include sCD14, osteopontin (Brown et al., 2011), TNF-α, IFN-γ, IL-1β, IL-6, S100β, TGF-β (Lyons et al., 2011; Abassi et al., 2017). These markers however are good predictors of HAND in viraemic or ART naïve individuals but not predictive of HAND in virologically suppressed individuals (Lyons et al., 2011; Kamat et al., 2012). In addition, HIV-1 viremia such as surrogate markers of nadir CD4+ T cell counts are also considered good predictors of neurocognitive dysfunction in non-virologically suppressed individuals.

Other studies have also alluded to the clinical benefit of biomarkers relating monocyte/macrophage (e.g. sCD163) and endothelial activation (e.g. sE-selectin, endothelin-1,VEGF) in predicting neurodevelopmental outcomes in perinatally HIV infected children (Kapetanovic et al., 2010, 2014a; Ananworanich et al., 2015; Benki-Nugent et al., 2019). Biomarkers such as CRP and IL-6 have been shown to have negative associations with neurocognitive outcomes such as deficits in processing speed even in children with virological suppression (Kapetanovic et al., 2014a). VEGF, an angiogenesis promoting factor in the CNS, as well as MIP-1β, a competitive CCR5 receptor ligand, indicated a likely relationship with neurocognitive outcomes relating to attention and impulsivity disorders (Carmeliet et al., 1996; Ferrara et al., 1996; Jin, Mao and Greenberg, 2000; Storkebaum et al., 2005; Obuku et al., 2016; Kapetanovic et al., 2021).

A recent longitudinal retrospective cohort study conducted in sub-Saharan Africa (South Africa, Malawi, Uganda and Zimbabwe) investigated the relationship between plasma immune biomarkers and neurodevelopmental outcomes in PHIV infection with controlled viremia initiated on ART at <3 years of age (Kapetanovic et al., 2021). In the latter study, 23 plasma immune biomarkers were assessed at two time points in relation to neurodevelopmental assessments at 5 and 11 years of age. Conclusions of this study highlighted an importance of endothelial cell activation biomarkers including sICAM-1, sVCAM-1 and sCD163, a biomarker of monocyte/macrophage in its role in neurodevelopment in children.

Clinical and immunological biomarker associations with neurological outcomes in children with PHIV are limited. Not only do current studies not examine a wide range of clinical and immunological biomarkers and their resultant relationship to neurodevelopment, but also do not evaluate the change in these biomarker levels longitudinally in relation to disease progression, different therapy regimes and long-term virological suppression.

Within this study cohort of PHIV children originating from CHER trial, extensive neurodevelopmental and neuroimaging outcomes have been published (summarized in Table 7.1) highlighting neurological issues that appear in early life and persist long-term despite early ART and subsequent virological suppression. A missing key component to understanding HIV-1 neurological pathogenesis in children with PHIV, is the immunological mechanism that may be contributory to these neurological outcomes and is therefore the objective of this research.

Table 7.1: Key published findings on neurodevelopmental and brain imaging outcomes in children of the CHER trial

Age evaluated	Key Findings	Reference
11 months of age (64 on early ART initiation; 26 on deferred ART)	All GMDS scores lower in deferred therapy arm and All General Griffiths and Locomotor scores were significantly lower	(Laughton et al., 2012)
3 years of age (10 deferred therapy; 34 early therapy)	White matter signal abnormalities – initiating ART by 8 weeks may be too late to prevent HIV from entering the CNS	(Ackermann et al., 2014)
5 years of age (38 HIV **positive** children of which 26 initiated ART at/ before 12 weeks; 15 HIV **negative** Controls	HIV-infected children initiating ART after 12 weeks had lower creatine, choline, and glutamate than those initiating ART at/before 12 weeks. CD4:CD8 ratio at baseline correlated with NAA and choline at 5 years, irrespective of treatment regimen and ART interruption. Despite early ART, low baseline CD4:CD8 ratio predicts brain metabolite levels in later childhood.	(Mbugua et al., 2016)
5 years of age (39 HIV+ and 13 HIV- Controls)	HIV+ children at 5 years of age have white matter abnormalities despite early ART, suggesting that early ART does not fully protect the white matter from either peripartum or in utero infection	(Ackermann et al., 2016)
5 years of age (28 deferred therapy; 35 interrupted at 40 wks; 33 interrupted at 96 weeks; 34 HEU and 39 HUU)	GMDS scores similar between the 5 groups, however visual perception scores were significantly lower in HIV+ children compared to controls	(Laughton et al., 2018)
7 years of age (65 HIV+, 46 HEU and HUU controls)	Damage to white matter integrity that persists from 5 years and unresolved at 7 years despite early ART and viral suppression	(Jankiewicz et al., 2017)
7 years of age (61 HIV+ and 42 HIV- Controls)	HIV+ children showed reduced gyrification compared to controls in bilateral medial parietal regions as well as reduced volumes of right putamen, left hippocampus and global white and gray matter and thicker cortex in small lateral occipital regions.	(Nwosu et al., 2018)
11 years of age (76 HIV+, 30 HEU and 30 HUU)	Elevated choline levels in brain Gray matter – indicative of inflammation. Reduced NAA in white matter components of PHIV children and HEU indicative of possible axonal damage	(Graham et al., 2020)

**GMDS – Griffiths Mental Development Scale; NAA – N-acetyl-aspartate; HEU – HIV exposed uninfected; HUU – HIV unexposed*

This research explores the implications of various clinical, virological, and soluble plasma immune biomarkers in association with neurodevelopmental outcomes in children with PHIV within a South African context. In addition, we evaluated the longitudinal changes that occurred from the closest time to ART initiation to 8 years of age when subjected to various cART regimens.

7.2 Materials and Methods

7.2.1 Study Design

This research study employs a mixed methodology, longitudinal and cross-sectional, study design for investigating the impact of clinical, immunological, and virological parameters on longitudinal neurodevelopmental outcomes in PHIV children in Cape Town, South Africa. Retrospective samples were analysed following sample collection at the start of the CHER trial in 2004. Follow-up of the Cape Town cohort conducted at the Family Centre for Research with Ubuntu (FAM-CRU) commenced in December 2014 when the CHER cohort was 7-8 years of age.

Treatment regimens (delayed therapy, early continuous therapy, early therapy interrupted at 40 and 96 weeks of age) were considered for each of the parameters listed above. This research study gathers the findings of key two sub-studies (immunological and neurodevelopmental) of the larger cohort study ethically approved under Stellenbosch University Health Research Ethics Committee (Ethics reference no.: M14/07/029).

Complete neurocognitive assessments were carried out by Prof Barbara Laughton, a Developmental Paediatrician at Stellenbosch University and Tygerberg Children's Hospital. For this study, primarily focused on immunological associations, only the neurocognitive developmental outcomes that showed significant variation amongst the study groups were included for correlation analysis.

Figure 7.1 below provides an overview of the cumulative study design incorporating immunological and neurodevelopmental assessments.

The Griffiths Mental Development Scales (GMDS) was administered at 11, 18, 30, 42 and 60 months of age (Griffiths, 1997; Laughton et al., 2018). GMDS assesses five subscales including locomotor, personal-social, hearing and language, eye-hand coordination, and performance. A General Griffiths score is generated accordingly. The Beery-Buktenica Development Test for Visual-Motor Integration (Beery-VMI) was implemented at 5, 7 and 9 years of age (Beery KE, Beery NA. Beery VMI: The Beery-Buktenica Developmental Test of Visual-Motor Integration. Administration, scoring and teaching manual. 6th ed. 6th ed. San Antonio TX: Pearson; 2010).

The VMI raw scores and standard test scores have been included for association analysis in this investigation

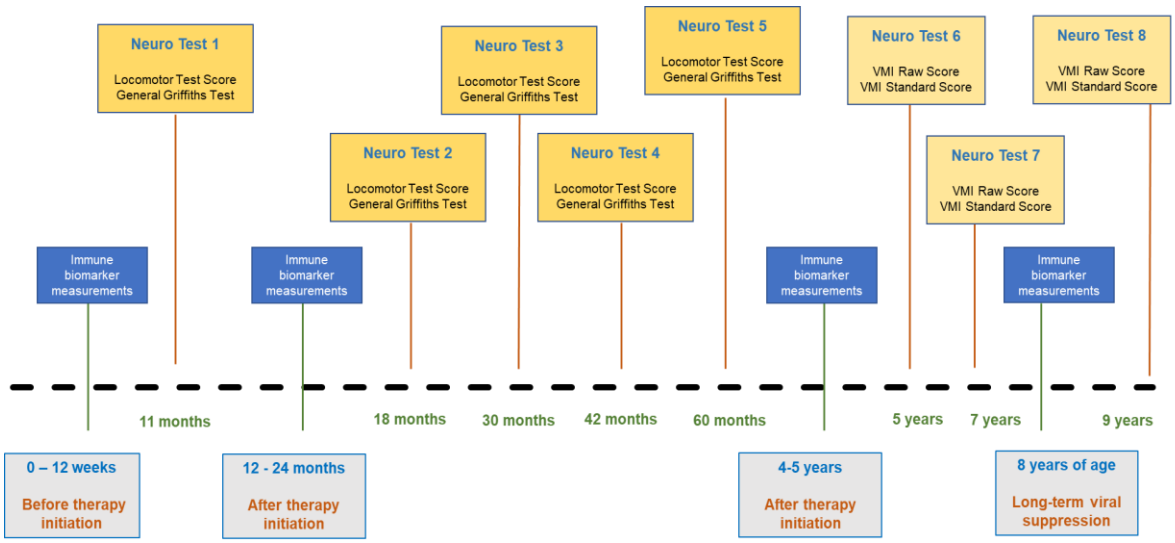


***Figure 7.1**: Overview of the immunological and neurocognitive outcome data collection timeline. Quantification of plasma biomarkers (a total of 44 biomarkers) levels were measured within the first 12 weeks of life (before the initiation of therapy), 12-24 months after therapy was initiated, and at 4-5 years and 8 years of age (at long-term viral suppression). General Griffiths test scores including the GMDS sub-scale locomotor test score was measured as 11, 18, 30, 42 and 60 months of age. VMI raw score and VMI standard score was measured at 5, 7 and 9 years of age.*

7.2.2 Study Participants

Study participants originated from CHER trial as previously described.

The CHER randomised control trial included three study arms: Arm 1 (delayed ART), Arm 2 (early ART initiation for 40 weeks followed by interruption) and Arm 3 (early ART initiation for 96 weeks followed by interruption). Participants on the early ART arms interrupted ART and restarted once immunological or clinical criteria were met. After the follow-up of the cohort, we have further stratified the CHER study arms to include an evaluation of a study group that was subjected to early, continuous therapy. Therefore, four treatment groups were evaluated: Group 1 (delayed therapy), Group 2 (early and continuous therapy), Group 3 (early therapy interrupted at 40 weeks) and Group 4 (early therapy interrupted at 96 weeks). In addition, control groups including HIV exposed uninfected (HEU) and HIV unexposed uninfected (HUU) groups were evaluated and compared to the HIV+ group. The controls were labelled as Group 5 (HEU) and Group 6 (HUU) respectively. Although control groups were available for both early and late evaluations of neurocognitive outcomes, no control samples were available for early measurements of plasma immune biomarkers. Controls samples for the latter were only measured at the 8-year time point.

7.2.3 Routine Testing

HIV-1 Viral Load

Viral load was quantified within a South African National Accreditation System (SANAS) accredited routine Medical Virology laboratory, at the National Health Laboratory Service (NHLS), Tygerberg Cape Town, South Africa as previously described in preceding Chapters.

Lymphocyte Count

Routine lymphocyte analyses (CD4 and CD8 counts, percentages, and CD4:CD8 ratios) were completed for all study participants and control samples at each follow-up visit as previously described in preceding Chapters.

7.2.4 Measurement of Plasma Immune Biomarkers

An extensive panel of 40 plasma biomarkers were measured using ELISA and Luminex® Multiplex assays as previously described in preceding Chapters.

7.2.5 Assessment of integrated HIV-1 Cell-associated DNA (iCAD)

A sensitive quantitative polymerase chain reaction (PCR) adapted for HIV-1 subtype C was implemented for the measurement of HIV-1 total DNA. This assay was aimed at targeting a region in HIV-1 integrase with a detection limit of 3 copies/reaction as previously described (van Zyl et al., 2015; Hong et al., 2016; Veldsman et al., 2018) as described in preceding Chapters.

7.2.6 CMV Evaluation

CMV viral loads were evaluated at baseline (a median age of 7.8 weeks of age) and again at 8 years of age. Early CMV viraemia was determined by Roche™ Cobas® AmpliPrep/Cobas® TaqMan (CAP/CTM, Roche Molecular Diagnostics, Branchburg, New Jersey) (Hsiao et al., 2014).

CMV quantification at the 8-year time point was determined by ELISA for the detection and quantification of CMV-specific IgG antibodies. A quantitative PCR was optimized for the detection of active CMV infection in blood plasma.

Detection of anti-CMV IgG by ELISA

The Euroimmun™ Anti-CMV ELISA kit (Euroimmun™, Germany) was used to quantify IgG antibodies in plasma. The aliquoted samples and kit reagents were brought to room temperature (18^{o}C to 25^{o}C) before analysis.

In the first step, 10 µl of sample was diluted with 1 ml of sample buffer and then pipetted into the individual microplate wells and were then incubated at room temperature for 30 minutes. Each microplate well was coated with CMV antigens, and the incubation period allowed CMV antibodies, if present, to bind to the CMV antigens. A wash step followed the incubation period; each microplate was washed three times with 450 µl wash buffer using the PW 40 automatic washer (BioRad™, Hercules). This was followed by the addition of 100 µl enzyme conjugate (peroxidase-labelled anti-human IgG) into each individual microplate well. The plate was once again incubated at room temperature for 30 minutes and was followed with a second wash step. Thereafter, 100 µl chromogen/substrate (TMB/H_2O_2) solution was added into each individual microplate well and incubated at room temperature for 15 minutes. Colour changes in the individual wells could be observed during this incubation step. Lastly, 100 µl stop solution (sulphuric acid) was added to each individual microplate well. The colour intensity was then measured using the EZ Read 400 microplate reader (Biochrom™, UK) within 10 minutes of adding the stop solution at a wavelength of 450 nm with a reference wavelength of 620 nm.

Each ELISA run included a positive control, a negative control and three calibrators. Both the positive and the negative controls served as an internal control for the reliability of the test. The three calibrators served as the standards and were used to determine the standard curve. The validity of the test was confirmed when the OD values of the calibrators were within the following reference limits: calibrator 1 (200 RU/ml) with reference value of 1.339; calibrator 2 (20 RU/ml) with reference value of 0.278 and calibrator 3 (2 RU/ml) with reference value of 0.027. The test was deemed reliable if the positive control's concentration fell between 71-133 and if the negative control's concentration, between 0-15.

The standard curve was determined by using point-to-point plotting of the extinction values for the three calibrators against the corresponding units. The linear equation ($y = 0.0075x + 0.1542$) derived from the standard curve was then used to calculate the antibody concentrations based on the OD values. The LOD of the Anti-CMV ELISA kit is 0.4 RU/ml.

Quantification of extracted DNA using TaqMan PCR assay

Quantitative real-time PCR with a mCMV internal control was performed as previously described with minor modifications (Preiser et al, 2003).

The modifications include the changes in primer and probe concentrations as described above. The PCR reaction was performed in a 96 well PCR plate and contained 20 µl of the PCR master mix and 5 µl of the DNA template. Once the master mix was added to the PCR plates, the standards were pipetted in duplicate as well as the positive control, negative controls, and no template control. The samples were however added in singlet. Thereafter, the PCR plate was sealed on the PX1 PCR Plate Sealer (BioRad™, Hercules) by using the following settings: a temperature of 175˚C with a sealing time of 20 seconds. After sealing, the PCR plate was examined for any air bubbles, if air bubbles were present, they were removed by flicking the bottom of the PCR plate with index and thumb finger. Lastly, the PCR plate was briefly centrifuged on the Mini Plate Spinner (Labnet™, Edison).

7.2.7 Evaluation of neurodevelopmental outcomes

Neurocognitive assessments were carried out by Dr Barbara Laughton, a Developmental Paediatrician at Stellenbosch University and Tygerberg Children's.

Two sets of measurements were employed for the evaluation of neurodevelopmental outcomes in our cohort:

1. The GMDS
2. The Beery-VMI

Griffiths Mental Developmental Scale

The GMDS is a valuable available test for the assessment of overall development of young children. The GMDS was developed in the UK in 1954 by Ruth Griffiths. Griffiths's purpose was to develop an instrument that contained a comparative profile of abilities across various domains of development, and which would facilitate early diagnosis of deficits in child development. Two newer versions of the tests exist for infants (aged 0-2 years) and young children (2-8 years of age) (Griffiths, 1997). Although standardised in the UK, the

GMDS is widely used internationally and is especially widespread in South Africa with many test items introduced by psychologists at Nelson Mandela Metropolitan University (Luiz, Oelofsen, Stewart & Michell, 1995). In South Africa, testing and assessment have been heavily criticised as possessing limited value for culturally diverse populations (Foxcroft, 1997; Nzimande, 1995; Sehlapelo & Terre Blanche, 1996), with the 2006 version much improved.

This investigation employed and adapted GMDS at 11, 18, 30, 42 and 60 months of age and assessed five subscales at 0-2 years with a six added 2 – 8 years:

1. Locomotor
   (*Parameter analysed for association with immunological outcomes*)
2. Personal-Social
3. Hearing and Language
4. Eye-hand coordination
5. Performance (visual motor abilities)
6. Practical Reasoning

The Locomotor Scale measures developing gross motor skills important for an upright posture, walking, running, balance and climbing. It allows for the observation of physical weakness or disability or defects of movement.

A global score, the General Griffiths, is also calculated. Raw scores are converted into quotients, derived from norms of healthy British children, with a mean of 100 and standard deviation of 16.

Children enrolled for neurodevelopmental assessment were assessed in their home language using standardised translations. HIV negative controls were used for comparative standards for neurodevelopmental and neurocognitive outcomes and originated from similar neighbourhoods.

Beery-Buktenica Developmental Test of Visual Motor Integration

The Beery-Buktenica Development Test for Visual-Motor Integration (Beery-VMI) is considered an internationally recognised test for the appropriate screening of possible deficits that may exist leading to learning, neuropsychological and behavioural challenges. The test can be implemented for ages from 2 to 18 years of age. Children are asked to copy geometric diagrams in a pencil and paper-based test.

In this investigation, the Beery-VMI was implemented at 5, 7 and 9 years of age. The VMI raw scores are calculated, and age adjusted standard test scores are determined using USA norms have been included for association analysis in this investigation.

7.2.8 Statistical Analysis

Quantitative variables measured for each of the groups were analysed using TIBC® Statistica™ version 13.3 and GraphPad Prism version 8 software. Data was subjected to normality testing (Shapiro-Wilke's normality test). All values outside of the normal range, a rejected null hypothesis of normally distributed data, justified the use of non-parametric tests for statistical analysis. No outliers were excluded in the analysis. Extreme

values were transformed by use of winsorisation or log transformation of statistics to limit the effect of spurious outliers and retain the power of sampling size.

A non-parametric one-way analysis of variance (ANOVA) Kruskal-Wallis test was implemented for the comparison of the three study groups which was followed by a post-hoc Fisher's Least Significant Difference (LSD) to identify and verify the significant differences between the groups. Correlations between biomarkers, clinical parameters, and laboratory measurements (plasma biomarkers, cellular markers and iCAD measurements) were performed by Spearman's correlation rank test (verified additionally with a Pearson's correlation rank test) and linear regression analysis. *P* values less than 0.05 were considered statistically significant.

Neurodevelopmental measures and biomarker levels at each time point and the resultant changes between the time points were summarized. Biomarker data were log10-transformed and standardized (mean=0, SD=1) to put all measures on the same scale prior to conducting factor analyses.

Multivariable linear regression models were fit to assess associations between the clinical, virological, immunological, and neurodevelopmental outcomes. We report regression coefficient estimates and 95% confidence intervals, highlighting estimates whose 95% confidence interval did not contain zero. We used a case analysis approach to handle data. Participants that were missing any biomarker measurement at a given time point were excluded from the factor analyses and regression models corresponding to that time point.

7.3 Results

7.3.1 Sampling Outline

Longitudinal study design: Neurodevelopmental Outcomes

A total of 139 children including HEU and HUU controls were evaluated for neurodevelopmental outcomes in the following grouping sample numbers: Group 1 (n=13), Group 2 (n=27), Group 3 (n=31), Group 4 (n=16), Group 5 (n=22) and Group 6 (n=30). Longitudinal developmental outcomes were captured for locomotor and general GDMS scores at 11, 18, 30, 42 and 60 months and Beery-VMI and standard test scores at 5, 7 and 9 years of age.

Cross-sectional study design: Correlation analysis of baseline (before therapy initiation) clinical, virological (HIV-1 iCAD) and immunological parameters with neurodevelopmental outcomes from 11 months to 9 years of age (HIV+ children only)

A total of 45 HIV+ children were subjected to correlation analysis for clinical parameters (time to therapy initiation, time to viral suppression, birth weight, gestation, birth height, longitudinal %CD4 and Abs CD4, %CD4 and Abs CD4 at birth, %CD8 and Abs CD8 at birth), virological parameters (a subset of n=19 HIV+ participants included in HIV-1 iCAD measurements) and immunological parameters/immune biomarkers.

Longitudinal study design: Correlation analysis of clinical, virological, and immunological parameters with neurodevelopmental outcomes (HIV+ children only)

A total of 69 HIV+ children (Group 1; n=13, Group 2; n=18, Group 3; n=25, Group 4; n=13) were longitudinally evaluated following therapy stratification (before therapy initiation, 1 year after therapy initiation, 2-3 years after therapy initiation and 8 years of age) for associations between HIV-1 iCAD, immune biomarkers and neurodevelopmental outcomes.

Cross-sectional study design: Correlation analysis of clinical, virological, and immunological of HIV+, HEU and HUU children at 8 years of age with neurodevelopmental outcomes at 5, 7 and 9 years.

A total of 137 children across six groups including two HIV- control groups (Group 1; n=13, Group 2; n= 25, Group 3, n=31, Group 4; n= 16, Group 5; n= 22 and Group 6; n=30) were cross-sectionally evaluated for clinical, virological, and immunological biomarker expression at a pre-adolescent median age of 8 years. Association analyses were conducted between the latter parameters and neurodevelopmental outcomes via the Beery-VMI test scores at 5, 7 and 9 years of age following long-term virological suppression, reconstituted CD4 counts, and low HIV-1 cell infectivity.

7.3.2 Demographic and clinical parameters of study cohort

Table 7.2 provides an overview of the demographic and clinical parameters measured at baseline (before therapy initiation) and at 8 years of age, the last pre-adolescent time point evaluated for this research component specific for the HIV+ CHER cohort.

Table 7.2: Summary of demographic, clinical, virological, and immunological parameters of study cohort (Groups 1 – 4)

	Group 1	**Group 2**	**Group 3**	**Group 4**
	Delayed Therapy	**Early Continuous Therapy**	**Early Therapy Interrupted at 40 weeks**	**Early Therapy Interrupted at 90 weeks**
n	**13**	**21**	**27**	**13**
Demographic and Birth Parameters				
Male, sex (n) (%)	6 (46.2%)	11 (52.4%)	11 (40.7%)	3 (23.1%)
Gestation (weeks), median; (range)	40 weeks (37.0 - 42.0 weeks)	40 weeks (30.0 - 41.0 weeks)	40 weeks (36.0 - 42.0 weeks)	40 weeks (32.0 - 40.0 weeks)
Birth weight (grams), median; (range)	3,227.5 g (2,640.0 - 3,640.0 g)	2,850.0 g (2,000.0 - 3,780.0 g)	3,160.0 g (2,400.0 - 4,200.0 g)	3,000 g (2,188.0 - 3,500.0 g)
Birth height (centimetres), median; (range)	34.0 cm (32.0 - 37.0 cm)	33.0 cm (30.0 - 38.5 cm)	34.0 cm (31.0 - 37.5 cm)	33.0 cm (30.5 - 36.0 cm)

Table 7.2 (continued)

Baseline HIV and Immunological Parameters				
Pre-treatment log10 HIV-1 RNA load, median; (range)	5.8 (5.0 - 6.0)	6.0 (5.0 - 6.0)	5.9 (3.8 - 5.9)	5.9 (4.2 - 5.9)
Time to therapy initiation (weeks), median; (range)	9.0 weeks (6.0 -18.0 weeks)	2.0 weeks (2.0 - 5.0 weeks)	2.0 weeks (1.0 - 3.0 weeks)	2.0 weeks (2.0 - 3.0 weeks)
Time to viral suppression (days), median; (range)	181.0 days (1.0 – 839.0 days)	168.0 days (161.0 – 505.0 days)	280.0 days (164.0 – 1,455.0 days)	169.0 days (167.0 – 1,404.0 days)
CD4+ cell count at birth (cells/µl), median; (range)	1,759.0 cells/µl (1,150 - 2,731.0 cells/µl)	1,480.0 cells/µl (146.0 – 3,828.0 cells/µl)	1,573.0 cells/µl (294.0 – 5,044.0 cells/µl)	1,851.0 cells/µl (829.0 – 4,272.0 cells/µl)
CD4+ percent at birth, median; (range)	36.6% (25.4 – 52.8%)	28.5% (17.1 – 53.9%)	36.7% (11.2 – 50.0%)	36.0% (27.9 – 57.1%)
CD8+ cell count at birth (cells/microliter), median; (range)	830.5 cells/µl (236.0 – 982.0 cells/µl)	819.0 cells/µl (278.0 – 947.0 cells/µl)	858.0 cells/µl (522.0 – 999.0 cells/µl)	**Data not available*
CD8+ percent at birth, median; (range)	20.8% (14.8 – 39.3%)	25.8% (11.2 – 41.8%)	20.8% (11.5 – 43.6%)	**Data not available*
Interruption time (days), median; (range)	*N/A*	*N/A*	208.5 days (40.0 – 2,786 days)	318.0 days (133.0 – 2,457 days)
8-year Follow-up parameters				
CD4+ cell count at 8 years (cells/microliter), median; (range)	930.0 cells/µl (568.0 - 2,731.0 cells/µl)	1,015.0 cells/µl (323.0 - 2,277.0 cells/µl)	979.0 cells/µl (446.0 - 2,063.0 cells/µl)	929.0 cells/µl (563.0 – 1,438.0 cells/µl)
CD4+ percent at 8 years, median; (range)	38.0 % (31.0 – 54.0 %)	37.0 % (21.0 – 49.0 %)	41.0 % (28.0 – 51.0 %)	37.0 % (27.0 – 47.0 %)
CD8+ cell count at 8 years (cells/microliter), median; (range)	894.0 cells/µl (472.0 – 1,767.0 cells/µl)	1,000.0 cells/µl (357.0 – 2,894.0 cells/µl)	724.0 cells/µl (474.0 – 1,416.0 cells/µl)	1,116.0 cells/µl (303.0 – 1,605.0 cells/µl)
CD8 percent, median (IQR) CD8+ percent at 8 years, median; (range)	30.0 % (22.0 – 41.0 %)	34.0 % (22.0 – 53.0 %)	30.0 % (22.0 – 47.0 %)	39.0 % (18.0 – 51.0 %)
CD4/CD8 ratio at 8 years, median; (range)	1.23 (0.77 – 1.99)	1.17 (0.45 – 2.23)	1.31 (0.62 – 2.21)	0.97 (0.59 – 2.65)
HIV-1 RNA Viral Load at 8 years (copies/ml); % Undetected	**85%** (11 Undetected) 1 (<40 copies/ml) 1 (42 copies/ml)	**81%** (17 Undetected) 2 (<40 copies/ml) 2 (>100 copies/ml)	**78%** (21 Undetected) 1 (<40 copies/ml) 3 (>500 copies/ml) 1 (19, 549 copies/ml) 1 (60 copies/ml)	**92%** (12 Undetected) 1 (26, 576 copies/ml)

Summary of demographic and clinical parameters: Before therapy initiation there were equal male to female ratios within the study population. Study groups had similar gestation times, birth weights and log10 HIV-1 RNA loads. Group 3 displayed a longer time to viral suppression. CD4 percentage was similar across groups except for Group 2 were they displayed a lower percentage. At 8 years of age, all groups displayed the

same CD4 counts and percentages, CD8 counts were lower in Group 3, however the same for CD8%. More children within Group 3 displayed detectable viral loads at 8 years of age compared to the other groups.

Table 7.3 provides demographics and clinical parameters of the HEU and HUU control groups analysed cross-sectionally at 8 years of age. Kruskal-Wallis p-values are presented for comparative analysis of parameters between three study groups (HIV+, HEU and HUU).

Table 7.3: Summary of demographics, clinical and virological parameters across three study groups (HIV+, HEU and HUU) at a median age of 8 years. A Kruskal-Wallis p-value is presented for comparative analysis amongst the three groups

	HIV+	**HIV Uninfected Controls**		**Kruskal-Wallis p-value**
		HEU	**HUU**	
n	88	32	41	
Demographic Factors				
Age, years (median, range)	8.0 yrs (6.0-9.0 yrs)	9.0 yrs (8.0-11.0 yrs)	10.0 yrs (9.0-11.0 yrs)	0.062
Male, sex (n) (%)	43 (48.9%)	18 (56.3%)	23 (56.1%)	0.051
Baseline HIV Treatment and CD4 Outcomes				
Months to therapy initiation, median (IQR)	2.1 months (1.8-2.8 months)	-	-	N/A
Viral load at initiation (median)	738,501 copies/ml	-	-	N/A
CD4+ cell count, median (IQR)	1742.6 cells/µl (1207.0-2367.5 cells/µl)	-	-	N/A
CD4 percent, median (IQR)	32.5% (25.9-40.0%)	-	-	N/A
HIV cell-associated DNA (CAD) (subset of n=40), median (IQR)	1, 470.1 copies/10^6 PBMCs (774.0-2, 888.7 copies/10^6 PBMCs)	-	-	N/A
8-year Follow-up parameters				
CD4+ cell count, median (IQR)	1, 076.6	926.8	1, 007.33	0.26
CD4 percent, median (IQR)	38.4	38.4	41.5	0.22
CD8+ cell count, median (IQR)	960.8	636.0	593.8	**<0,01**
CD8 percent, median (IQR)	33.6	26.9	24.6	**<0,01**
CD4/CD8 Ratio, median (IQR)	1.24	1.54	1.74	**<0,01**
HIV cell-associated DNA (CAD) (subset of	20.3 copies/10^6 PBMCs	-	-	N/A

n=40), median (IQR)	(6.4-37.6 copies/10^6 PBMCs)			

***Lymphocyte reference ranges**

Reference: (Lawrie *et al.*, 2015)			
Age range	**0-3 months**	**1-2 years**	**6-12 years**
CD4 T lymphocytes (cells/µL)	1520-5160	1374-3928	568-2013
CD8 T lymphocytes (cells/µL)	428-2478	669-3247	340-121-
CD4:CD ratio	1.29-5.29	0.78-3.88	0.95-2.47
Reference: (Payne *et al.*, 2020)			
Age range	**1-2 months**	**15-24 months**	**5-10 years**
CD8 (%)	10.7-28.9	13.8-34.9	15.1-34.8
Reference: (Shearer *et al.*, 2003)			
Age range	**0-3 months**	**1-2 years**	**6-12 years**
CD4 (%)	35-64	32-51	31-47

7.3.3 Longitudinal CD4 percentage pattern for HIV+ children from baseline (before therapy initiation) to 8 years of age for PHIV children

CD4 percentages were measured every three months following recruitment and initiation of the CHER trial in 2004 until 2011. We have documented the CD4 T cell percentages at the closest time to which the soluble immune biomarkers were measured.

Longitudinal CD4 percentage data for HIV+ children from baseline to 8 years of age has been described in Chapter 5.

In summary, significant differences in CD4 percentage between the four groups as well as over time ($p<0.01$). Whilst we observed that Groups 1 and 2 show similar patterns of CD4 percentage increase over time, the patterns are seemingly different for the two interrupted groups where a lag in CD4 percentage T cell reconstitution is observed. However, at the 8-year time point, all therapy groups have CD4 percentage above 35% with Group 3 displaying the highest percentage of 41%.

7.3.4 Longitudinal HIV-1 Cell-Associated DNA (CAD) measurements show significant decline over time for each study group evaluated.

Longitudinal HIV-1 CAD measurements were completed for a subset of HIV+ participants depending on the availability of samples. A total of 36 samples were evaluated across the four study groups. Longitudinal data for HIV-1 CAD has been previously presented (Chapter 5).

In summary, before therapy was initiated, children in Group 2 (early continuous therapy) presented with the highest levels of HIV-1 CAD (median = 3, 795.1 copies/10^6 PBMCs) compared to the other three study groups. Group 1 (delayed therapy) displayed the lowest levels of HIV-1 CAD (median = 99.2 copies/10^6 PBMCs) before therapy was initiated compared to the other study groups.

All groups presented with a significant decrease in HIV-1 CAD levels over time and at 8 years of age all study groups displayed median levels well below 25 copies/10^6 PBMCs.

HIV-1 CAD kinetic studies for the same cohort have recently been published (Veldsman et al., 2019). In summary, kinetic studies concluded that following six months of continuous suppressive therapy, initiated within the first few weeks of life, HIV-1 CAD decreased significantly over time compared to groups who initiated therapy later. Furthermore, following multivariate analyses, HIV-1 CAD and the change in its levels during interruption were observed to be independent predictors of slower HIV-1 DNA decay (Veldsman et al., 2019).

7.3.5 Neurodevelopmental Outcomes

Variation over time of GDMS sub-scale 1, Locomotor scores, were evaluated at 11, 18, 30, 42 and 60 months of age (Test 1-5) in six study groups as outlined in Figure 7.2 and Table 7.4 below which also depict a graphical trend in Locomotor scores over time and the relevant statistical parameters.

Group 1 randomised into the delayed therapy arm, displayed the lowest mean Locomotor scores at 11 (mean: 83.5; SD: 17.6), 18 (mean: 87.8; SD: 20.0) and 30 (mean: 83.7; SD: 22.1) months of age. At 11 months of age, the mean scores of Group 1 were also significantly lower than Group 3 (mean: $p<0.01$), Group 4 ($p=0.01$) and Group 5 ($p<0.01$). At 18 months of age, the mean Locomotor scores for Group 1 were also significantly lower than Group 3 ($p=0.04$) and Group 6 ($p=0.02$). At the same time point, Group 2, randomised to early continuous therapy, had a significantly lower mean Locomotor score (mean: 89.1; SD: 19.0) compared to Group 3 ($p=0.03$) and Group 6 ($p=0.01$) depicting mean scores of 98.4 (SD: 14.9) and 101.8 (SD: 6.8) respectively.

At the 30 months testing time point, Group 1 still presented with the lowest mean Locomotor test score of 83.97 (SD: 22.1). Interestingly, Groups 1 to 5 depicted similar mean scores, but significantly lower ($p<0.01$ for Groups 1-4 and $p=0.02$ compared to Group 5) than the HUU group (Group 6) who had a mean score of 104.4 (SD: 86.9).

At 42 months, the delayed therapy group still had the lowest mean Locomotor test score (mean: 86.9; SD: 17.4), which was significantly lower than any of the other groups. Group 3 displayed a decreased test score (mean: 87.4; SD: 14.2) which was significantly lower ($p=0.03$) than Group 2 with a mean score of 96.5; SD: 11.7.

At 60 months of age, no differences between the mean Locomotor test scores were noted with all groups displaying a similar score ($p>0.05$).

From the graphical representation below (Figure 7.2) all six study groups evaluated displayed variation in scores over time. At 2 years of age, similarity in test scores were reached indicating normalisation ability of the HIV infected group, possibly due to the impact of therapy.

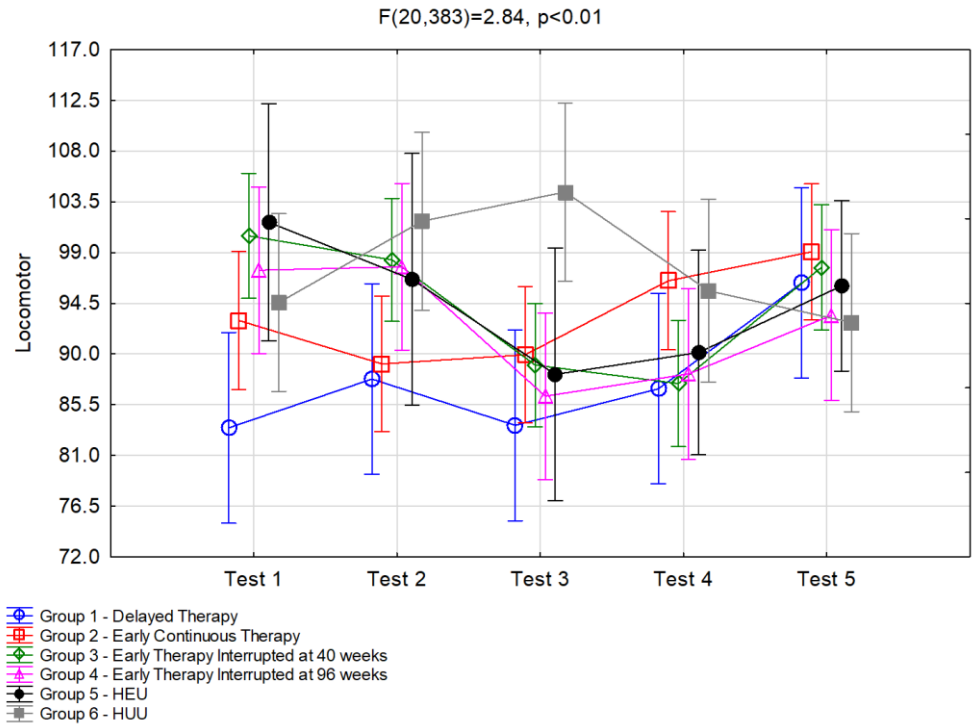


Figure 7.2: *Vertical bar graphs denoting 95% confidence intervals with mean locomotor scores and relevant standard deviation (SD) for each study group (Group 1 – 6) measured at 11 months (Test 1), 18 months (Test 2), 30 months (Test 3), 42 months (Test 4) and 60 months (Test 5) of age.*
Groups are distinguished by colour coding (blue, red, green, purple, black, and grey).

Below we present the findings of the GDMS overall General Griffiths test scores evaluated at 11, 18, 30, 42 and 60 months of age (Test 1 to 5) across all six study groups.

The overall interaction of the groups measured over time is significantly different as depicted in Figure 7.2 ($p<0.01$). In addition, evaluating the overall trend of the General Griffiths score, we observed a continuous decline in the scores from Test 1 to 5 in all groups, however the scores of the HUU groups remain relatively higher than the other groups over time.

At 11 months of age, the Group 1, with delayed therapy, had a lower mean General Griffiths score (mean: 98.8 ±15.9) compared to the other groups. This score was significantly lower ($p=0.01$) than Group 3 (interrupted at 40 weeks) who had the highest score (mean: 107.1; SD: 9.9). Group 2 (mean: 101.6; SD: 11.6) was significantly lower than Group 3 ($p=0.03$).

At 18 months of age, Group 1 remained the group with the lowest mean General Griffiths test score compared to the other groups (mean: 88.3; SD: 15.5). This test score was significantly lower than Group 3 ($p=0.02$), Group 5 ($p=0.03$) and Group 6 ($p<0.01$) who had mean test scores of 96.1 (SD: 11.2), 100.8 (SD: 2.9) and 100.0 (SD: 7.6) respectively. Group 2 displayed the second lowest test score that was significantly lower than Group 3 ($p=0.04$) and Group 6 ($p<0.01$). The two controls groups, HEU and HUU displayed the highest scores at this time point.

Table 7.4: Summary of statistics (N, mean, SD, SE, Lower CL, Upper CL and p-values) for locomotor test scores at 11, 18, 30, 42 and 60 months of age

Locomotor Test Scores at 11 months (Test 1)												
	n	Mean Locomotor Test Score	SD	SE	Lower CL	Upper CL	Probabilities (P-values)					
							Delayed therapy	Early continuous therapy	Early therapy interrupted at 40 weeks	Early therapy interrupted at 96 weeks	HEU	HUU
Delayed therapy	12	83.5	17.6	4.3	75.0	91.9	-	0.07	**<0.01**	**0.01**	**<0.01**	0.06
Early continuous therapy	23	93.0	15.3	3.1	86.6	99.1	0.07	-	0.07	0.36	0.16	0.75
Early therapy interrupted at 40 weeks	28	100.5	12.0	2.8	94.9	106.0	**<0.01**	0.07	-	0.52	0.84	0.23
Early therapy interrupted at 96 weeks	16	97.4	17.1	3.8	90.0	104.8	**0.01**	0.36	0.52	-	0.52	0.60
HEU	7	101.7	23.2	5.4	91.2	112.2	**<0.01**	0.16	0.84	0.52	-	0.29
HU	14	94.6	11.8	4.0	86.7	102.4	0.06	0.75	0.23	0.60	0.29	-
Locomotor Test Scores at 18 months (Test 2)												
Delayed therapy	12	87.8	20.0	4.3	79.3	96.2	-	0.80	**0.04**	0.08	0.21	**0.02**
Early continuous therapy	24	89.1	19.0	3.1	83.1	95.2	0.80	-	**0.03**	0.08	0.25	**0.01**
Early therapy interrupted at 40 weeks	29	98.4	14.9	2.8	92.9	103.8	**0.04**	**0.03**	-	0.90	0.79	0.48
Early therapy interrupted at 96 weeks	16	97.8	17.1	3.8	90.4	105.1	0.08	0.08	0.90	-	0.87	0.46
HEU	6	96.6	12.2	5.7	85.5	107.8	0.21	0.25	0.79	0.87	-	0.46
HU	14	101.8	6.8	4.0	93.9	109.7	**0.02**	**0.01**	0.48	0.46	0.46	-
Locomotor Test Scores at 30 months (Test 3)												
Delayed therapy	12	83.7	22.1	4.3	75.2	92.1	-	0.24	0.30	0.65	0.53	**<0.01**
Early continuous therapy	24	89.9	12.3	3.1	83.9	96.0	0.24	-	0.82	0.45	0.79	**<0.01**

Early therapy interrupted at 40 weeks	29	89.0	14.5	2.8	83.6	94.5	0.30	0.82	-	0.55	0.90	**<0.01**
Early therapy interrupted at 96 weeks	16	86.3	14.4	3.8	78.9	93.7	0.65	0.45	0.55	-	0.78	**<0.01**
HEU	6	88.2	14.0	5.7	77.0	99.4	0.53	0.79	0.90	0.78	-	**0.02**
HU	14	104.4	16.9	4.0	96.5	112.3	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**0.02**	-
Locomotor Test Scores at 42 months (Test 4)												
Delayed therapy	12	86.9	17.4	4.3	78.5	95.3	-	0.07	0.93	0.82	0.61	0.14
Early continuous therapy	23	96.5	11.7	3.1	90.4	102.6	0.07	-	**0.03**	0.10	0.25	0.86
Early therapy interrupted at 40 weeks	27	87.4	14.2	2.9	81.8	93.0	0.93	**0.03**	-	0.86	0.61	0.10
Early therapy interrupted at 96 weeks	15	88.2	12.5	3.8	80.7	95.8	0.82	0.10	0.86	-	0.75	0.19
HEU	10	90.1	11.7	4.6	81.1	99.2	0.61	0.25	0.61	0.75	-	0.38
HU	13	95.6	15.2	4.1	87.5	103.7	0.14	0.86	0.10	0.19	0.38	-
Locomotor Test Scores at 60 months (Test 5)												
Delayed therapy	12	96.3	17.0	4.3	87.9	104.7	-	0.60	0.79	0.62	0.96	0.55
Early continuous therapy	24	99.1	13.7	3.1	93.0	105.1	0.60	-	0.74	0.25	0.54	0.22
Early therapy interrupted at 40 weeks	28	97.7	15.8	2.8	92.2	103.2	0.79	0.74	-	0.37	0.73	0.32
Early therapy interrupted at 96 weeks	15	93.4	13.6	3.8	85.9	101.0	0.62	0.25	0.37	-	0.63	0.91
HEU	15	96.0	12.8	3.8	88.5	103.6	0.96	0.54	0.73	0.63	-	0.56
HU	14	92.8	13.9	4.0	84.9	100.7	0.55	0.22	0.32	0.91	0.56	-

At the 30-month time point (Test 3), Groups 1-5 displayed relatively similar mean General Griffiths test scores. These scores were all significantly lower ($p<0.01$) than Group 6 (mean: 97.4; SD: 9.1). Similarly at 42 months (Test 4), Group 1 displayed the lowest mean test score (mean: 80.8; SD: 6.9) which was significantly lower ($p<0.01$) than Group 6 (mean: 90.2; SD: 8.2). Group 3 with a mean of 83.0 (SD: 6.3) also had a significantly lower score than Group 6 ($p=0.01$).

As with the Locomotor Test scores presented above, for the General Griffiths test scores, all Groups presented with similar mean scores with no significant differences between them.

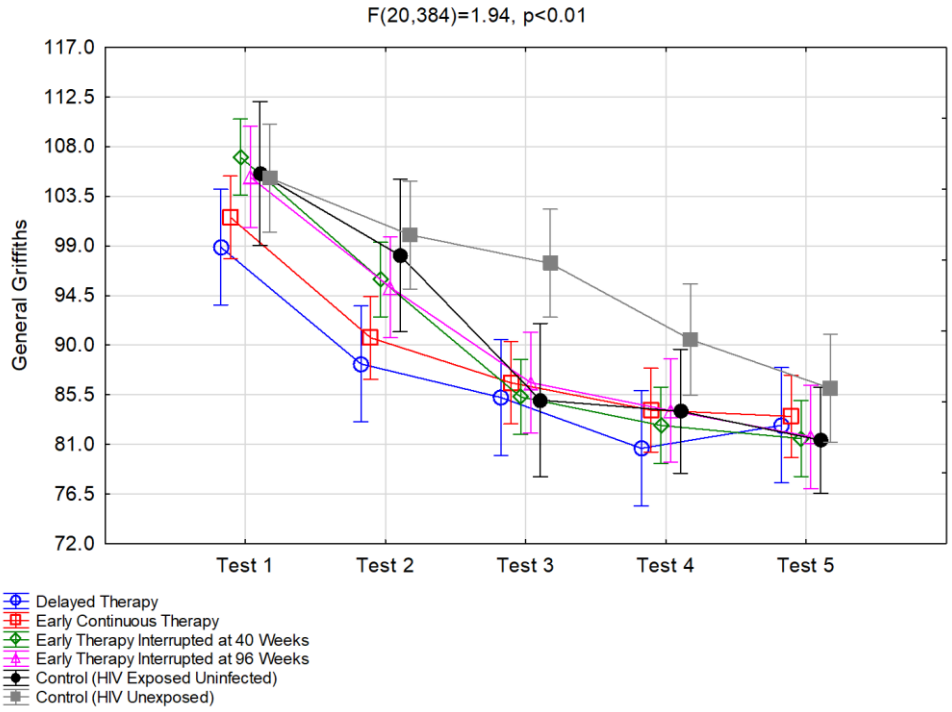


Figure 7.3: *Vertical bar graphs denoting 95% confidence intervals with mean General Griffiths test scores and relevant standard deviation (SD) for each study group (Group 1 – 6) measured at 11 months (Test 1), 18 months (Test 2), 30 months (Test 3), 42 months (Test 4) and 60 months (Test 5) of age.*
Groups are distinguished by colour coding (blue, red, green, purple, black, and grey).

Table 7.5: Summary of statistics (N, mean, SD, SE, Lower CL, Upper CL and p-values) for General Griffiths test scores at 11, 18, 30, 42 and 60 months of age

							Probabilities (P-values)					
	n	**Mean General Griffiths Test Score**	**SD**	**SE**	**Lower CL**	**Upper CL**	**Delayed therapy**	**Early continuous therapy**	**Early therapy interrupted at 40 weeks**	**Early therapy interrupted at 96 weeks**	**HEU**	**HUU**
General Griffiths Test Scores at 11 months (Test 1)												
Delayed therapy	12	98.8	15.9	4.3	75.0	91.9	-	0.41	**0.01**	0.07	0.12	0.09
Early continuous therapy	24	101.6	11.6	3.1	86.9	99.1	0.41	-	**0.03**	0.22	0.30	0.26
Early therapy interrupted at 40 weeks	28	107.1	9.9	2.8	94.9	106.0	**0.01**	**0.03**	-	0.53	0.69	0.53
Early therapy interrupted at 96 weeks	16	105.3	12.6	3.8	90.0	104.8	0.07	0.22	0.53	-	0.94	0.97
HEU	7	106.3	13.9	5.4	91.2	112.2	0.12	0.30	0.69	0.94	-	0.92
HU	14	105.1	12.2	4.0	86.7	102.5	0.09	0.26	0.53	0.97	0.92	-
General Griffiths Test Scores at 18 months (Test 2)												
Delayed therapy	12	88.3	15.5	4.3	79.3	96.2	-	0.47	**0.02**	0.05	**0.03**	**<0.01**
Early continuous therapy	24	90.7	12.5	3.1	83.1	95.2	0.47	-	**0.04**	0.13	0.06	**<0.01**
Early therapy interrupted at 40 weeks	29	96.1	11.2	2.8	92.9	103.8	**0.02**	**0.04**	-	0.81	0.58	0.18
Early therapy interrupted at 96 weeks	16	95.3	10.5	3.8	90.4	105.1	0.05	0.13	0.81	-	0.49	0.16
HEU	6	100.8	2.9	5.7	85.5	107.8	**0.03**	0.06	0.58	0.49	-	0.67
HU	14	100.0	7.6	4.0	93.9	109.7	**<0.01**	**<0.01**	0.18	0.16	0.67	-
General Griffiths Test Scores at 30 months (Test 3)												
Delayed therapy	12	85.2	6.3	4.3	75.2	92.1	-	0.68	0.99	0.70	0.96	**<0.01**
Early continuous therapy	24	86.6	6.0	3.1	83.9	96.0	0.68	-	0.61	1.00	0.70	**<0.01**

Early therapy interrupted at 40 weeks	29	85.5	6.8	2.8	83.6	94.5	0.99	0.61	-	0.65	0.94	**<0.01**
Early therapy interrupted at 96 weeks	16	86.6	7.5	3.8	78.9	93.7	0.70	1.00	0.65	-	0.71	**<0.01**
HEU	6	85.8	7.6	5.7	77.0	99.4	0.96	0.70	0.94	0.71	-	**<0.01**
HU	14	97.4	9.1	4.0	96.5	112.3	**<0.01**	**<0.01**	**<0.01**	**<0.01**	**<0.01**	-
General Griffiths Test Scores at 42 months (Test 4)												
Delayed therapy	12	80.8	6.9	4.3	78.5	95.3	-	0.29	0.52	0.34	0.39	**<0.01**
Early continuous therapy	23	84.4	6.4	3.1	90.4	102.6	0.29	-	0.60	0.99	0.97	0.05
Early therapy interrupted at 40 weeks	27	83.0	6.3	2.9	81.8	93.0	0.52	0.60	-	0.65	0.70	**0.01**
Early therapy interrupted at 96 weeks	15	84.1	10.1	3.8	80.7	95.8	0.34	0.99	0.65	-	0.99	0.07
HEU	10	83.8	9.6	4.6	81.1	99.2	0.39	0.97	0.70	0.99	-	0.09
HU	13	90.2	8.2	4.1	87.5	103.7	**<0.01**	0.05	**0.01**	0.07	0.09	-
General Griffiths Test Scores at 60 months (Test 5)												
Delayed therapy	12	82.8	6.2	4.3	87.9	104.7	-	0.81	0.71	0.77	0.71	0.36
Early continuous therapy	24	83.5	6.6	3.1	93.0	105.1	0.81	-	0.44	0.55	0.49	0.42
Early therapy interrupted at 40 weeks	28	81.6	6.5	2.8	92.2	103.2	0.71	0.44	-	0.96	0.96	0.13
Early therapy interrupted at 96 weeks	15	81.7	7.3	3.8	85.9	101.0	0.77	0.55	0.96	-	0.93	0.20
HEU	14	81.5	9.7	3.8	88.5	103.6	0.71	0.49	0.96	0.93	-	0.18
HU	14	86.1	5.6	4.0	84.9	100.7	0.36	0.42	0.13	0.20	0.18	-

We explored the contributory effect of therapy interruption at 40 and 96 weeks with the uninterrupted group for both the Locomotor and General Griffiths test scores measured at 11, 18, 30, 42 and 60 months of age.

We present this data in Figures 7.4a and b below. The statistical summary for each time point is tabulated below (Table 7.6).

At 11 months of age, the uninterrupted group (representing both delayed and continuous therapy groups), displayed a significantly lower ($p<0.01$) mean Locomotor test score (mean: 89.7; SD: 16.5) compared to the group that was randomised to therapy interruption at 40 weeks (mean: 100.01; SD: 12.0). At 18 months of age, a similar trend was noted ($p<0.01$).

Subsequently to 18 months, no significant differences between the interrupted and uninterrupted groups at the 30-, 42- and 60-months testing time points ($p>0.05$).

A similar trend was observed for the Griffiths General test score when comparing the interrupted and uninterrupted groups at 11, 18, 30, 42 and 60 months of age. At the 11- and 18-months testing time point, the uninterrupted group had significantly lower ($p<0.01$) mean test scores (mean: 100.7; SD: 13.0 and mean: 89.0; SD: 13.4 respectively) compared to the group interrupted at 40 weeks (mean: 107.1; SD: 9.9 and mean: 96.1; SD: 11.2 respectively).

Similar to the Locomotor test scores, no significant differences between the interrupted and uninterrupted groups at subsequent testing points.

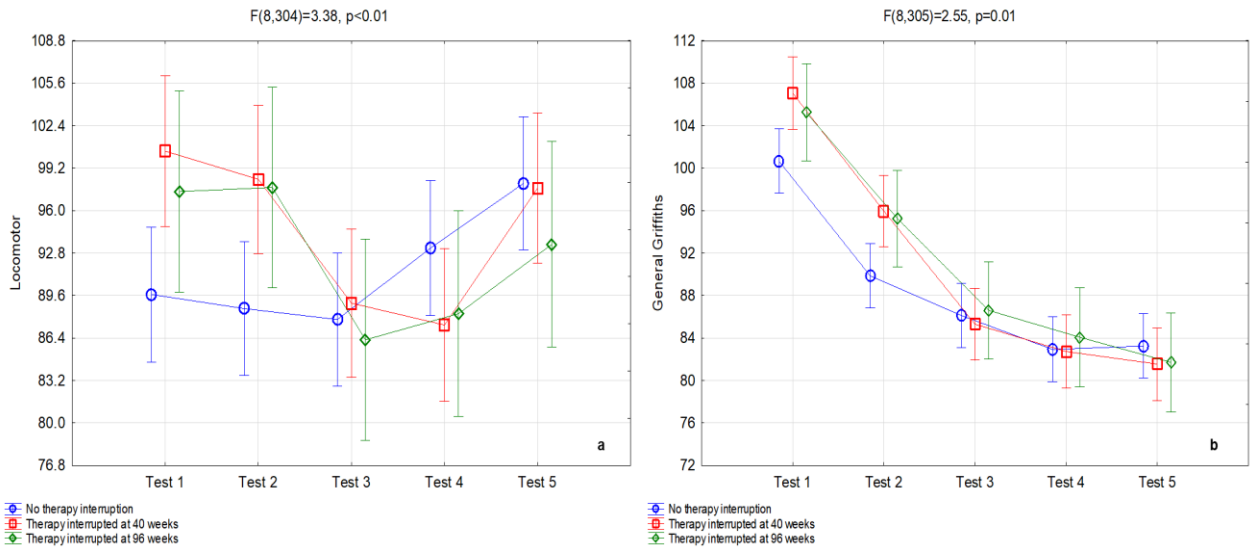


Figure 7.4a and b: *Vertical bar graphs denoting 95% confidence intervals with mean locomotor test scores (Figure 3a - Left) and mean General Griffiths test scores (Figure 3b – Right).*
The relevant SD for each group (no therapy interruption, therapy interrupted at 40 and 96 weeks) measured at 11 months (Test 1), 18 months (Test 2), 30 months (Test 3), 42 months (Test 4) and 60 months (Test 5) of age. Groups are distinguished by colour coding (blue – no therapy interruption, red – therapy interrupted at 40 weeks and green – therapy interrupted at 96 weeks).

Table 7.6: Summary of statistics (N, mean, SD, SE, Lower CL, Upper CL and p-values) for locomotor and General Griffiths test scores at 11, 18, 30, 42 and 60 months of age for interrupted and uninterrupted therapy groups

	n	Mean Locomotor Test Score	SD	SE	Lower CL	Upper CL	Probabilities (P-values)		
							No therapy interruption	Therapy interrupted at 40 weeks	Therapy interrupted at 96 weeks
Locomotor Test Scores at 11 months (Test 1)									
No therapy interruption	35	89.7	16.5	2.6	84.6	94.8	-	**<0.01**	0.09
Therapy interrupted at 40 weeks	28	100.1	12.0	2.9	94.8	106.2	**<0.01**	-	0.53
Therapy interrupted at 96 weeks	16	97.4	17.1	3.8	89.9	105.0	0.09	0.53	-
Locomotor Test Scores at 18 months (Test 2)									
No therapy interruption	36	88.8	19.0	2.6	83.6	93.6	-	**0.01**	0.05
Therapy interrupted at 40 weeks	29	98.6	14.9	2.8	92.8	104.0	**0.01**	-	0.90
Therapy interrupted at 96 weeks	16	97.8	17.1	3.8	90.2	105.3	0.05	0.90	-
Locomotor Test Scores at 30 months (Test 3)									
No therapy interruption	36	88.0	16.1	2.6	82.8	92.8	-	0.75	0.74
Therapy interrupted at 40 weeks	29	89.3	14.5	2.8	83.4	94.6	0.75	-	0.56
Therapy interrupted at 96 weeks	16	86.3	14.4	3.8	78.7	93.8	0.74	0.56	-
Locomotor Test Scores at 42 months (Test 4)									
No therapy interruption	35	93.7	14.5	2.6	88.1	98.3	-	0.14	0.29
Therapy interrupted at 40 weeks	27	87.5	14.2	2.9	81.6	93.1	0.14	-	0.86
Therapy interrupted at 96 weeks	15	88.3	12.5	3.9	80.5	96.0	0.29	0.86	-
Locomotor Test Scores at 60 months (Test 5)									
No therapy interruption	36	98.2	14.7	2.6	93.0	103.1	-	0.93	0.33
Therapy interrupted at 40 weeks	28	97.6	15.8	2.9	92.0	103.4	0.93	-	0.38
Therapy interrupted at 96 weeks	15	93.6	13.6	3.9	85.7	101.2	0.33	0.38	-
General Griffiths Test Scores at 11 months (Test 1)									

No therapy interruption	36	100.7	13.0	1.5	97.6	103.7	-	**<0.01**	0.10
Therapy interrupted at 40 weeks	28	107.1	9.9	1.7	103.6	110.5	**<0.01**	-	0.53
Therapy interrupted at 96 weeks	16	105.3	12.6	2.3	100.7	109.8	0.10	0.53	-
General Griffiths Test Scores at 18 months (Test 2)									
No therapy interruption	36	89.9	13.4	1.5	86.8	92.9	-	**<0.01**	0.05
Therapy interrupted at 40 weeks	29	96.1	11.2	1.7	92.6	99.3	**<0.01**	-	0.81
Therapy interrupted at 96 weeks	16	95.3	10.5	2.3	90.7	99.8	0.05	0.81	-
General Griffiths Test Scores at 30 months (Test 3)									
No therapy interruption	36	86.1	6.0	1.5	83.1	89.2	-	0.72	0.86
Therapy interrupted at 40 weeks	29	85.5	6.8	1.7	81.9	88.7	0.72	-	0.65
Therapy interrupted at 96 weeks	16	86.6	7.5	2.3	82.0	91.2	0.86	0.65	-
General Griffiths Test Scores at 42 months (Test 4)									
No therapy interruption	35	83.2	6.7	1.6	79.9	86.0	-	0.94	0.69
Therapy interrupted at 40 weeks	27	83.0	6.3	1.8	79.3	86.2	0.94	-	0.65
Therapy interrupted at 96 weeks	15	84.1	10.1	2.4	79.4	88.7	0.69	0.65	-
General Griffiths Test Scores at 60 months (Test 5)3									
No therapy interruption	36	83.	6.4	1.5	80.2	86.3	-	0.46	0.59
Therapy interrupted at 40 weeks	28	81.6	6.5	1.7	78.1	85.0	0.46	-	0.96
Therapy interrupted at 96 weeks	15	81.7	7.3	2.4	77.0	86.4	0.59	0.96	-

The Beery-VMI was measured at 5, 7 and 9 years of age in all therapy groups (Group 1 to 6). From this test, a VMI raw score and VMI standard test score was generated.

Figures 7.5 and 7.6 below presents the findings of these test scores for each of the study groups including the HEU and HU controls.

Evaluating the overall VMI Raw scores between the study groups at 5, 7 and 9 years of age. We observe no significant difference in the F-test trend (F=0.94l p=0.60). When evaluating the individual statistical differences between the group over time, all group scores increased from 5 to 9 years, but no difference was found between the groups as tabulated in Table 7.7 below.

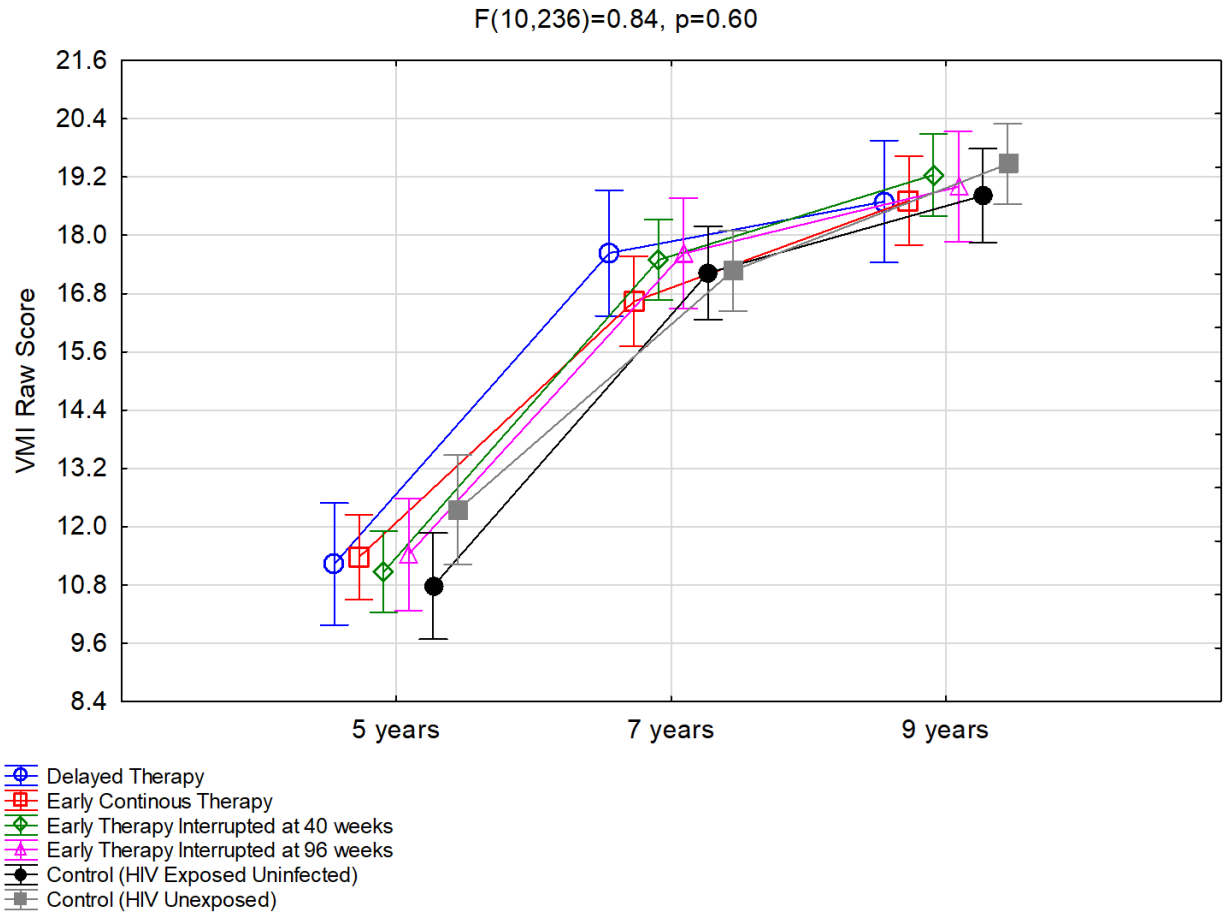


Figure 7.5: *Vertical bar graphs denoting 95% confidence intervals with mean VMI Raw scores and relevant standard deviation (SD) for each study group (Group 1 – 6) measured at 5, 7 and 9 years of age.*
Groups are distinguished by colour coding (blue, red, green, purple, black, and grey).

Table 7.7: Summary of statistics (N, mean, SD, SE, Lower CL, Upper CL and p-values) VMI Raw test scores at 5, 7 and 9 years of age

VMI Raw Score at 5 years												
	n	**Mean VMI Raw Test Score**	**SD**	**SE**	**Lower CL**	**Upper CL**	**Probabilities (P-values)**					
							Delayed therapy	**Early continuous therapy**	**Early therapy interrupted at 40 weeks**	**Early therapy interrupted at 96 weeks**	**HEU**	**HU**
Delayed therapy	13	11.2	2.4	0.6	10.0	12.5	-	0.86	0.84	0.82	0.59	0.19
Early continuous therapy	27	11.4	2.1	0.4	10.5	12.2	0.86	-	0.63	0.94	0.40	0.18
Early therapy interrupted at 40 weeks	29	11.0	1.7	0.4	10.2	11.9	0.84	0.63	-	0.63	0.67	0.08
Early therapy interrupted at 96 weeks	15	11.4	3.1	0.6	10.3	12.6	0.82	0.94	0.63	-	0.42	0.26
HEU	16	10.7	2.5	0.6	9.7	11.9	0.59	0.40	0.67	0.42	-	0.05
HU	14	12.6	1.6	0.6	11.2	13.5	0.19	0.18	0.08	0.26	0.05	-
VMI Raw Score at 7 years												
Delayed therapy	12	17.6	2.8	0.7	16.3	18.9	-	0.22	0.86	0.99	0.62	0.64
Early continuous therapy	23	16.6	3.0	0.5	15.7	17.6	0.22	-	0.18	0.19	0.39	0.32
Early therapy interrupted at 40 weeks	29	17.4	2.2	0.4	16.7	18.3	0.86	0.18	-	0.86	0.68	0.70
Early therapy interrupted at 96 weeks	16	17.6	2.8	0.6	16.5	18.8	0.99	0.19	0.86	-	0.60	0.62
HEU	22	17.2	2.0	0.5	16.3	18.2	0.62	0.39	0.68	0.60	-	0.95
HU	30	17.3	1.43	0.4	16.4	18.1	0.64	0.32	0.70	0.62	0.95	-
VMI Raw Score at 9 years												

Delayed therapy	13	18.	1.8	0.6	17.4	19.9	-	0.98	0.47	0.72	0.88	0.31
Early continuous therapy	24	18.8	2.3	0.5	17.8	19.6	0.98	-	0.40	0.70	0.88	0.23
Early therapy interrupted at 40 weeks	28	19.2	2.0	0.4	18.4	20.1	0.47	0.40	-	0.73	0.51	0.71
Early therapy interrupted at 96 weeks	16	19.0	2.5	0.6	17.9	20.1	0.72	0.70	0.73	-	0.81	0.51
HEU	22	18.8	2.6	0.5	17.9	19.8	0.88	0.88	0.51	0.81	-	0.32
HU	30	19.5	2.6	0.4	18.6	20.3	0.31	0.23	0.71	0.51	0.32	-

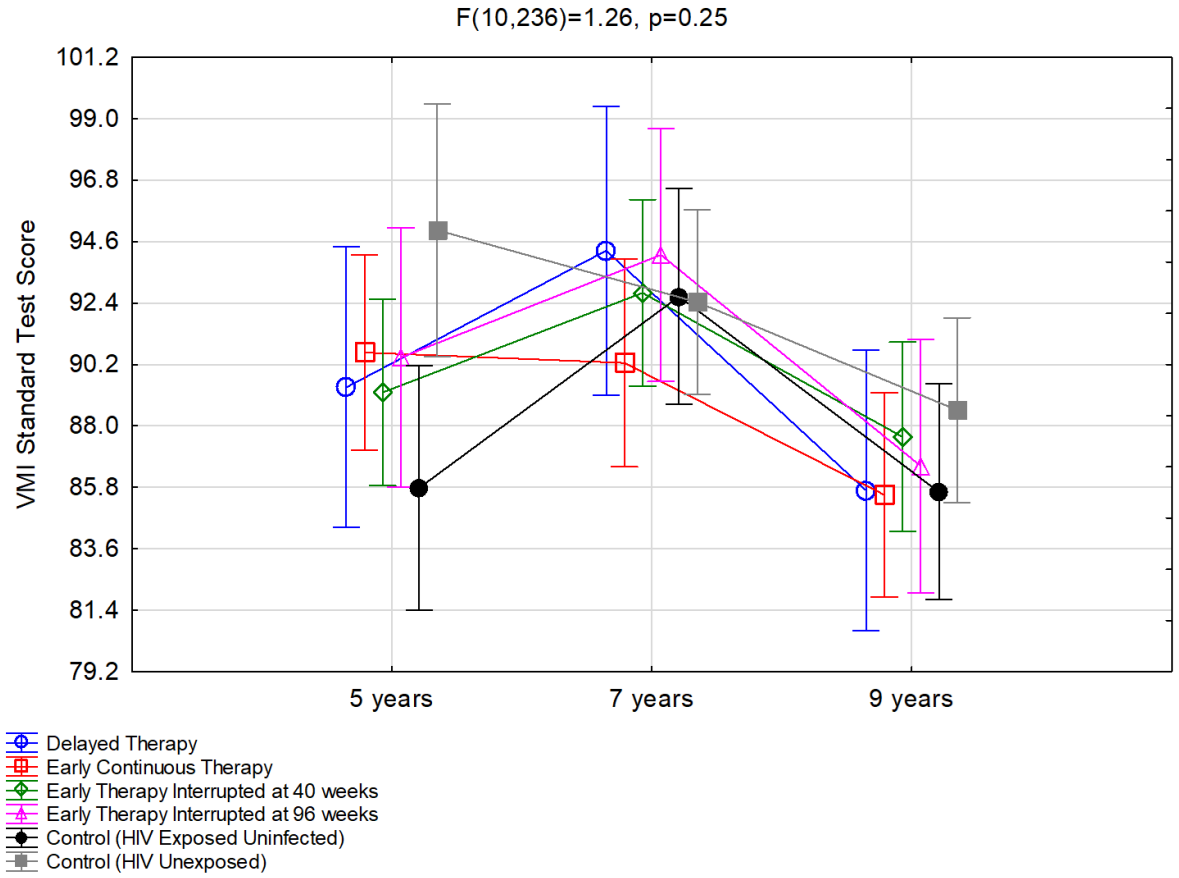


Figure 7.6: *Vertical bar graphs denoting 95% confidence intervals with mean VMI Standard test scores and relevant standard deviation (SD) for each study group (Group 1 – 6) measured at 5, 7 and 9 years of age.*
Groups are distinguished by colour coding (blue, red, green, purple, black, and grey).

Evaluation of the VMI Standard Test scores at 5, 7 and 9 years of age we observed no significant difference between the overall time-group effect (F test = 1.26; p=0.25). However, at 5 years of age, the HEU mean VMI Standard Test score (mean:85.5; SD: 9.5) was significantly lower ($p<0.01$) than the HU score (mean: 95.8' SD: 7.0). These differences subsided at subsequent time points.

From the graphical representation (Figure 7.6) we observed a continual decrease from 5 to 9 years of age of the VMI Standard Test Score for HUU children compared to the other groups who showed a pattern of score increase from 5 to 7 years and then a decrease from 7 to 9 years of age.

Table 7.8: Summary of statistics (N, mean, SD, SE, Lower CL, Upper CL and p-values) VMI Standard test scores at 5, 7 and 9 years of age

							Probabilities (P-values)					
	n	Mean VMI Standard Test Score	SD	SE	Lower CL	Upper CL	Delayed therapy	Early continuous therapy	Early therapy interrupted at 40 weeks	Early therapy interrupted at 96 weeks	HEU	HUU
VMI Standard Test Score at 5 years												
Delayed therapy	13	89.4	11.0	2.6	84.4	94.4	-	0.69	0.95	0.76	0.29	0.10
Early continuous therapy	27	90.6	8.9	1.8	87.1	94.1	0.69	-	0.56	0.95	0.09	0.13
Early therapy interrupted at 40 weeks	29	89.0	7.2	1.7	85.9	92.5	0.95	0.56	-	0.67	0.22	0.04
Early therapy interrupted at 96 weeks	15	90.3	13.1	2.4	85.8	95.1	0.76	0.95	0.67	-	0.15	0.17
HEU	16	85.5	9.5	2.2	81.4	90.2	0.29	0.09	0.22	0.15	-	**<0.01**
HU	14	95.8	7.0	2.3	90.5	99.5	0.10	0.13	0.04	0.17	**<0.01**	-
VMI Standard Test Score at 7 years												
Delayed therapy	12	94.1	10.7	2.6	89.1	99.5	-	0.22	0.63	0.97	0.62	0.56
Early continuous therapy	23	90.0	11.7	1.9	86.5	94.0	0.22	-	0.32	0.19	0.38	0.39
Early therapy interrupted at 40 weeks	29	92.7	8.7	1.7	89.4	96.1	0.63	0.32	-	0.63	0.96	0.89
Early therapy interrupted at 96 weeks	16	94.1	10.8	2.3	89.6	98.7	0.97	0.19	0.63	-	0.62	0.55
HEU	22	92.6	7.3	2.0	88.8	96.5	0.62	0.38	0.96	0.62	-	0.94
HU	30	92.4	5.5	1.7	89.1	95.7	0.56	0.39	0.89	0.55	0.94	-
VMI Standard Test Score at 9 years												
Delayed therapy	13	85.7	7.1	2.6	80.7	90.7	-	0.96	0.53	0.80	0.99	0.35

Early continuous therapy	24	85.7	9.0	1.9	81.9	89.2	0.96	-	0.41	0.73	0.97	0.23
Early therapy interrupted at 40 weeks	28	87.5	7.9	1.7	84.2	91.0	0.53	0.41	-	0.72	0.45	0.69
Early therapy interrupted at 96 weeks	16	86.6	10.4	2.3	82.0	91.1	0.80	0.73	0.72	-	0.76	0.48
HEU	22	85.6	10.7	2.0	81.8	89.5	0.99	0.97	0.45	0.76	-	0.26
HU	30	88.6	10.2	1.7	85.3	91.9	0.35	0.23	0.69	0.48	0.26	-

We evaluated the effect of treatment interruption on the VMI Raw Score and VMI Standard Test Score at 5-, 7- and 9-years age. For both neurodevelopmental parameters, no significant differences between the uninterrupted and interrupted (at 40 or 96 weeks) was observed ($p>0.05$). All groups followed a very similar patterns in test scores (both VMI Raw and Standard Scores) across time.

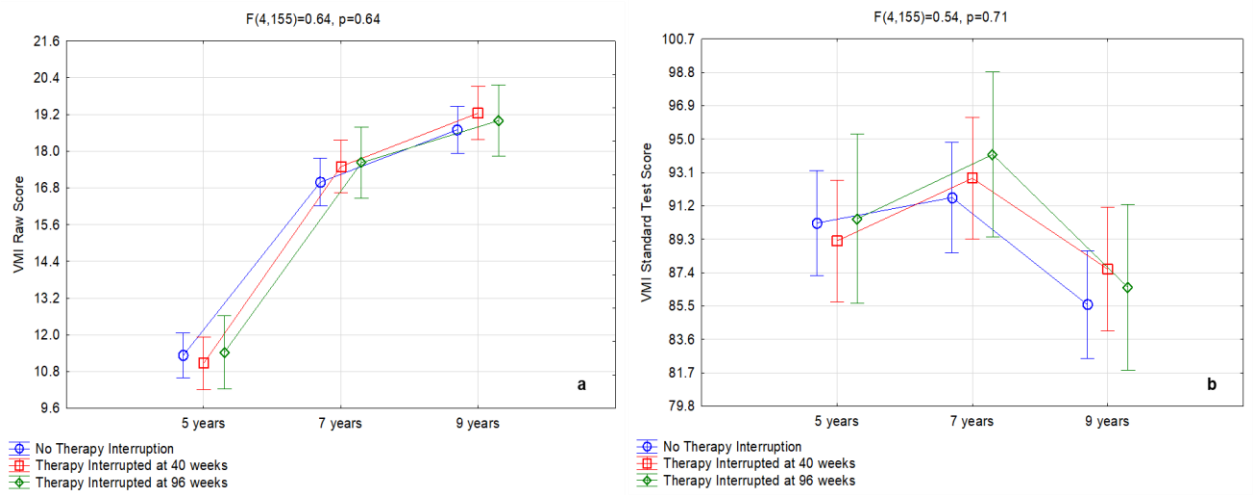


Figure 7.7: *Vertical bar graphs denoting 95% confidence intervals with mean VMI Raw scores (Figure 7a – Left) and mean VMI Standard test scores (Figure 7b – Right)*
The relevant standard deviation (SD) for each group (no therapy interruption and therapy interrupted at 40 and 96 weeks) measured at 5,7 and 9 years of age. Groups are distinguished by colour coding (blue – no therapy interruption, red – therapy interrupted at 40 weeks and green – therapy interrupted at 96 weeks).

Table 7.9: Summary of statistics (N, mean, SD, SE, Lower CL, Upper CL and p-values) of VMI Raw Scores and Standard Test Scores for uninterrupted and interrupted at 40 and 96 weeks of age groups

VMI Raw Score at 5 years									
	n	**Mean VMI Raw Test Score**	**SD**	**SE**	**Lower CL**	**Upper CL**	**Probabilities (P-values)**		
							No therapy interruption	**Therapy interrupted at 40 weeks**	**Therapy interrupted at 96 weeks**
No therapy interruption	40	11.3	2.1	11.3	0.4	10.6	-	0.66	0.88
Therapy interrupted at 40 weeks	29	11.0	1.7	11.1	0.4	10.2	0.66	-	0.63
Therapy interrupted at 96 weeks	15	11.4	3.1	11.4	0.6	10.2	0.88	0.63	-
VMI Raw Score at 7 years									
No therapy interruption	35	16.9	2.9	17.0	0.4	16.2	-	0.38	0.37
Therapy interrupted at 40 weeks	29	17.4	2.2	17.5	0.4	16.6	0.38	-	0.87
Therapy interrupted at 96 weeks	16	17.6	2.8	17.6	0.6	16.5	0.37	0.87	-
VMI Raw Score at 9 years									
No therapy interruption	37	18.7	2.2	18.7	0.4	17.9	-	0.35	0.67
Therapy interrupted at 40 weeks	28	19.2	2.0	19.2	0.4	18.4	0.35	-	0.74
Therapy interrupted at 96 weeks	16	19.0	2.5	19.0	0.6	17.8	0.67	0.74	-
VMI Standard Score at 5 years									
No therapy interruption	40	90.2	9.5	90.2	1.5	87.2	-	0.66	0.93
Therapy interrupted at 40 weeks	29	89.0	7.2	89.2	1.8	85.7	0.66	-	0.68
Therapy interrupted at 96 weeks	15	90.3	13.1	90.5	2.4	85.7	0.93	0.68	-

VMI Standard Score at 7 years									
No therapy interruption	35	91.4	11.4	91.7	1.6	88.5	-	0.64	0.39
Therapy interrupted at 40 weeks	29	92.7	8.7	92.8	1.6	89.3	0.64	-	0.65
Therapy interrupted at 96 weeks	16	94.1	10.8	94.1	2.4	89.4	0.39	0.65	-
VMI Standard Score at 9 years									
No therapy interruption	37	85.7	8.3	85.6	1.6	82.5	-	0.39	0.73
Therapy interrupted at 40 weeks	28	87.5	7.9	87.6	1.8	84.1	0.39	-	0.73
Therapy interrupted at 96 weeks	16	86.6	10.4	86.6	2.4	81.9	0.73	0.73	-

7.3.6 Correlation analysis of immune biomarkers at baseline (before therapy initiation) and HIV-1 DNA (a surrogate marker of HIV-1 reservoir) with neurocognitive outcomes (Test 1 – 8) in HIV+ CHER children

Evaluating early immune biomarker (cytokines, chemokines and relative receptors) levels as potential predictors for neurodevelopmental outcomes (11 months to 9 years) showed several significant associations as graphically shown in the heat map below (Figure 7.8). These associations are also tabulated in Table 7.10.

In early neurodevelopmental tests related to Locomotor and General Griffiths Test Scores, we observed significant positive correlations between pro-inflammatory immune biomarkers such as IL-1β, GCSF, sCD14, sCD163, IL-18 and LBP. These biomarkers have primary functions in monocyte/macrophage activation and responding to bacterial infections.

Negative correlations between early immune biomarkers and early neurodevelopmental outcomes were also observed. Proinflammatory biomarkers associated monocyte/macrophage function such as IL-1RA, MCP-1 and MIP-1α were negatively associated with Locomotor and General Griffiths Test scores. Anti-inflammatory cytokines including IL-13, IFN-α as well pleiotropic cytokines including IL-6 and TGF-β2 were also negatively associated with both early and late neurodevelopmental parameters. IL-7, required in early T cell development and IL-17F, produced by T-cells and macrophages displayed negative associations with early neurodevelopmental scores. The latter cytokines were the only T-cell related cytokines measured in early life and before therapy initiation that had an association of neurodevelopmental outcomes.

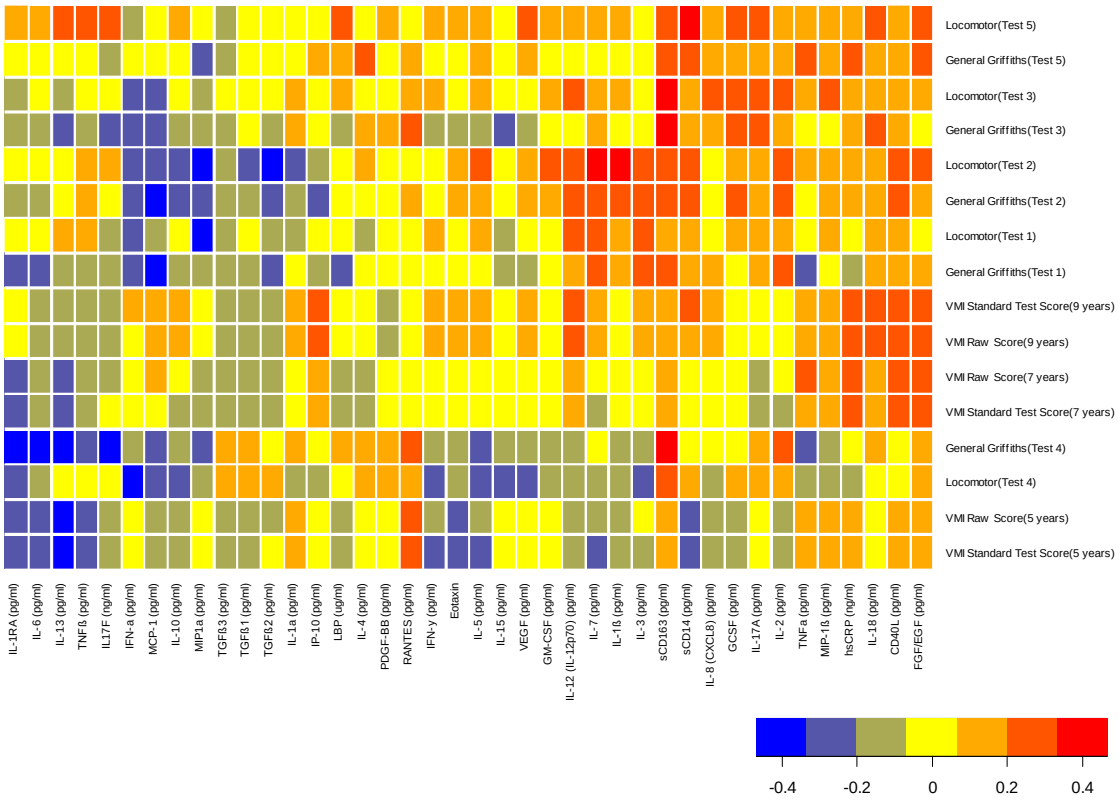


***Figure 7.8**: Correlation heat map representation of immune biomarkers measured at baseline (before therapy initiation) and neurocognitive scores from 11 months to 9 years of age.*
Colour scale ranging from blue (negative) to red (positive) depicts the Pearson Correlation Coefficient (R). Probabilities for both Pearson and Spearman correlations are listed in Table 15 below.

Table 7.10: Summary of Pearson and Spearman correlation coefficients (*r*) as well as probabilities following predication analysis of immune biomarkers measured at baseline (before therapy initiation) and neurocognitive outcomes (Test 1 – 8)

Immune Biomarker (baseline)	Neurocognitive measurement	Pearson Correlation (*r* -Coefficient)	Pearson Correlation (p-value)	Spearman Correlation (*r* -Coefficient)	Spearman Correlation (p-value)
IL-17F	General Griffiths (Test 4)	-0.34	**0.04**	-0.34	**0.04**
IL-1β	Locomotor (Test 2)	+0.35	**0.03**	+0.39	**0.01**
IL-1RA	Locomotor (Test 4)	-0.46	**<0.01**	-0.50	**<0.01**
IL-1RA	VMI Score (5 years)	-0.29	**0.06**	-0.31	**0.04**
IL-6	General Griffiths (Test 4)	-0.44	**<0.01**	-0.42	**<0.01**
IL-7	Locomotor (Test 2)	-0.33	**0.03**	-0.39	**0.01**
IL-13	General Griffiths (Test 4)	-0.35	**0.03**	-0.30	**0.06**
IL-13	VMI Raw Score (5 years)	-0.33	**0.03**	+0.26	0.10
IL-13	VMI Standard Score (5 years)	-0.34	**0.03**	-0.31	**0.05**
MCP-1	General Griffiths (Test 1)	-0.35	**0.02**	-0.29	**0.07**
MCP-1	General Griffiths (Test 2)	-0.35	**0.03**	-0.05	0.75
MIP-1α	Locomotor (Test 1)	-0.36	**0.02**	-0.22	0.18
MIP-1α	Locomotor (Test)	-0.35	**0.03**	-0.13	0.41
GCSF	Locomotor (Test 3)	+0.33	**0.04**	+0.28	0.08
IFNα	Locomotor (Test 4)	-0.37	**0.02**	-0.17	0.29
TGF-β2	Locomotor (Test 2)	-0.34	**0.04**	-0.42	**<0.01**
sCD14	Locomotor (Test 5)	+0.41	**<0.01**	+0.34	**0.03**
sCD163	Locomotor (Test 3)	+0.41	**<0.01**	+0.36	**0.02**
sCD163	General Griffiths (Test 3)	+0.39	**0.01**	+0.45	**<0.01**
sCD163	General Griffiths (Test 4)	+0.47	**<0.01**	+0.56	**<0.01**
IL-18	General Griffiths (Test 3)	+0.23	0.16	+0.36	**0.02**
IL-18	Locomotor (Test 5)	+0.20	0.20	+0.34	**0.03**
LBP	Locomotor (Test 5)	+0.33	**0.03**	+0.32	**0.04**

When evaluating the role of HIV-infectivity by levels of HIV-1 iCAD in PBMCs – we found an interesting and unexpected positive correlation between HIV-1 iCAD and early neurodevelopmental outcomes including the General Griffiths test score at the 30 months and Locomotor test scores at the 60 months testing time points. It is important to note however, that a significant association between HIV-1 iCAD and neurodevelopment was only observed for the Spearman correlation analysis and not for the Pearson correlation.

Table 7.11: Summary of Pearson and Spearman correlation coefficients (R) as well as probabilities following predication analysis of HIV-1 cell-associated DNA measured at baseline (before therapy initiation) and neurocognitive outcomes (Test 1 – 8)

	Neurocognitive measurement	Pearson Correlation (*r* -Coefficient)	Pearson Correlation (p-value)	Spearman Correlation (*r* -Coefficient)	Spearman Correlation (p-value)
Cell-associated DNA (CAD)	Locomotor (Test 1)	-0.15	0.55	+0.13	0.60
	General Griffiths (Test 1)	-0.28	0.25	+0.18	0.46
	Locomotor (Test 2)	**+0.14**	**0.58**	**+0.42**	**0.07**
	General Griffiths (Test 2)	+0.22	0.36	+0.34	0.16
	Locomotor (Test 3)	-0.19	0.44	+0.28	0.24
	General Griffiths (Test 3)	**+0.03**	**0.91**	**+0.54**	**0.02**
	Locomotor (Test 4)	-0.08	0.76	+0.12	0.63
	General Griffiths (Test 4)	-0.25	0.31	+0.46	0.06
	Locomotor (Test 5)	**+0.30**	**0.21**	**+0.49**	**0.03**
	General Griffiths (Test 5)	**+0.13**	**0.60**	**+0.40**	**0.09**
	VMI Raw Score (5 years)	+0.22	0.37	+0.25	0.30
	VMI Standard Test Score (5 years)	+0.19	0.44	+0.20	0.40
	VMI Raw Score (7 years)	+0.00	0.99	+0.13	0.60
	VMI Standard Test Score (7 years)	-0.03	0.91	+0.13	0.60
	VMI Raw Score (9 years)	+0.07	0.80	+0.21	0.42
	VMI Standard Test Score (9 years)	+0.07	0.79	+0.20	0.45

7.3.7 Baseline clinical parameters that predict neurocognitive outcomes (Test 1 – 8) in HIV+ CHER children

Figure 7.9 and Table 7.12 display a graphical representation of the associations between the early clinical parameters and the early and late neurodevelopmental outcomes.

Important early clinical parameters showed significant associations with both early and late neurodevelopmental outcomes. These included time to viral suppression (r=0.34; p=0.03), gestation (r=0.33; p=0.03), % CD8 at birth (r=0.41; p=0.03), Abs CD8 count at birth (r=0.40; p=0.02), time to therapy initiation (r= -0.41; p<0.01) and %CD4 at birth (r = -0.36; p=0.02).

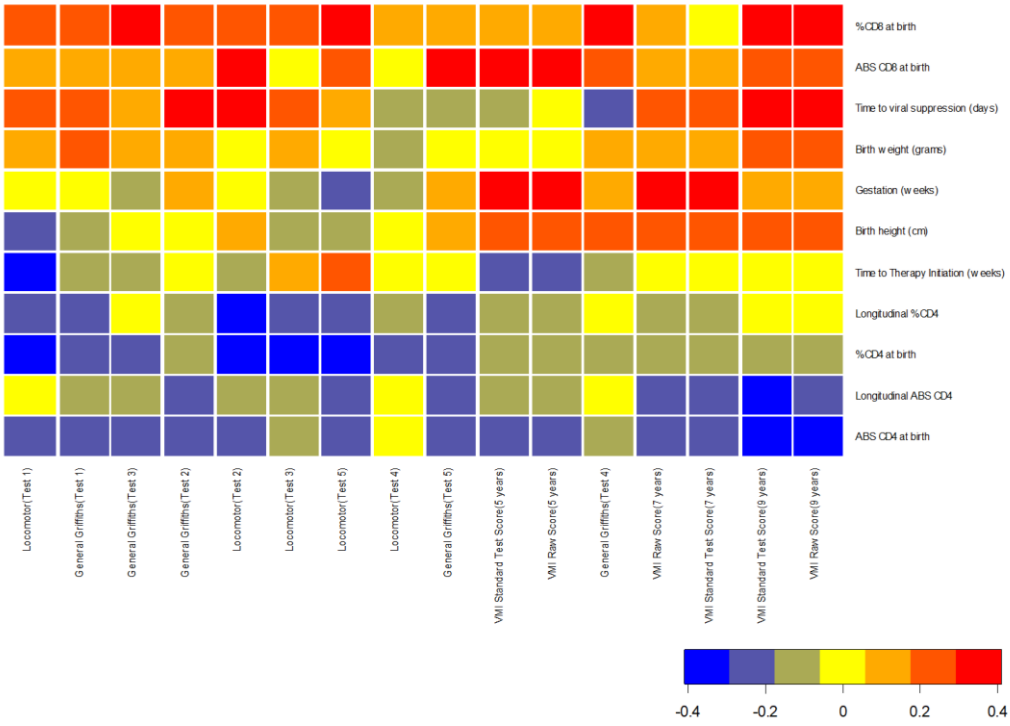


***Figure 7.9**: Correlation heat map representation of clinical parameters measured at baseline (before therapy initiation) and neurocognitive scores from 11 months to 9 years of age.*
Colour scale ranging from blue (negative) to red (positive) depicts the Pearson Correlation Coefficient (R). Probabilities for both Pearson and Spearman correlations are listed in Table 3 below.

Table 7.12: Summary of Pearson and Spearman correlation coefficients (R) as well as probabilities following predication analysis of clinical parameters measured at baseline (before therapy initiation) and neurocognitive outcomes (Test 1 – 8)

Clinical Parameter	Neurocognitive measurement	Pearson Correlation (*r* -Coefficient)	Pearson Correlation (p-value)	Spearman Correlation (*r* -Coefficient)	Spearman Correlation (p-value)
Time to therapy initiation (weeks)	Locomotor (Test 1)	-0.41	**<0.01**	-0.30	**0.06**
Time to viral suppression	Locomotor (Test 2)	+0.34	**0.03**	+0.27	**0.08**
Time to viral suppression	General Griffiths (Test 4)	+0.33	**0.03**	+0.30	**0.06**
Time to viral suppression	VMI Raw Score (9 years)	+0.34	**0.03**	+0.35	**0.02**
Time to viral suppression	VMI Standard Score (9 years)	+0.34	**0.03**	+0.34	**0.03**
Gestation	VMI Raw Score (5 years)	+0.33	**0.03**	+0.18	0.26
Gestation	VMI Raw Score (7 years)	+0.37	**0.02**	+0.20	0.22
Gestation	VMI Standard Score (7 years)	+0.40	**0.01**	+0.21	0.18
% CD4 at birth	Locomotor (Test 1)	-0.36	**0.02**	-0.44	**<0.01**
% CD4 at birth	Locomotor (Test 2)	-0.19	**0.23**	-0.32	**0.04**
% CD4 at birth	Locomotor (Test 3)	-0.33	**0.03**	-0.36	**0.02**
% CD4 at birth	VMI Standard Score (9 years)	-0.33	**0.03**	-0.23	0.16
%CD8 at birth	General Griffiths (Test 4)	+0.41	**0.03**	+0.40	**0.03**
%CD8 at birth	Locomotor (Test 5)	+0.40	**0.02**	+0.54	**<0.01**
ABS CD8 at birth	Locomotor (Test 2)	+0.40	**0.02**	+0.39	**0.03**
ABS CD8 at birth	VMI Raw Score (5 years)	+0.41	**0.02**	+0.38	**0.03**
ABS CD8 at birth	VMI Standard Score (5 years)	+0.36	**0.04**	+0.32	**0.07**

7.3.8 Change in biomarker expression correlation with change in neurocognitive outcomes over time.

Figure 7.10 and Table 7.13 display a graphical representation in the form of a heat map, of the associations between the early clinical parameters and the early and late neurodevelopmental outcomes. Here we calculated the change differences between both the biomarker expression levels and early and late neurodevelopmental outcomes.

The change in biomarker expression levels, measured as the difference between the last time point measured (i.e., 8 years of age) and the earliest time point (i.e., before therapy initiation) were correlated with the changes in neurodevelopmental scores from Test 1 to Test 5 for Locomotor and General Griffiths test scores and 5 years to 9 years for the VMI Raw and Standard Test scores.

For these measured changes over time for both immunological and neurodevelopmental outcomes, we observed associations with biomarkers IL-18, MCP-1 (r= -0.40; p=0.01), TNFα (r= -0.41; p<0.01) and LBP (r= -0.34; p=0.03). Interestingly, IL-18 showed both and negative association. Positive associations were observed between IL-18 and the change in VMI raw (r=0.49; p<0.01) and the change in VMI Standard test scores (r=0.50; p<0.01) whereas a negative association was observed for the change in General Griffiths test scores (r=0.39; p=0.03).

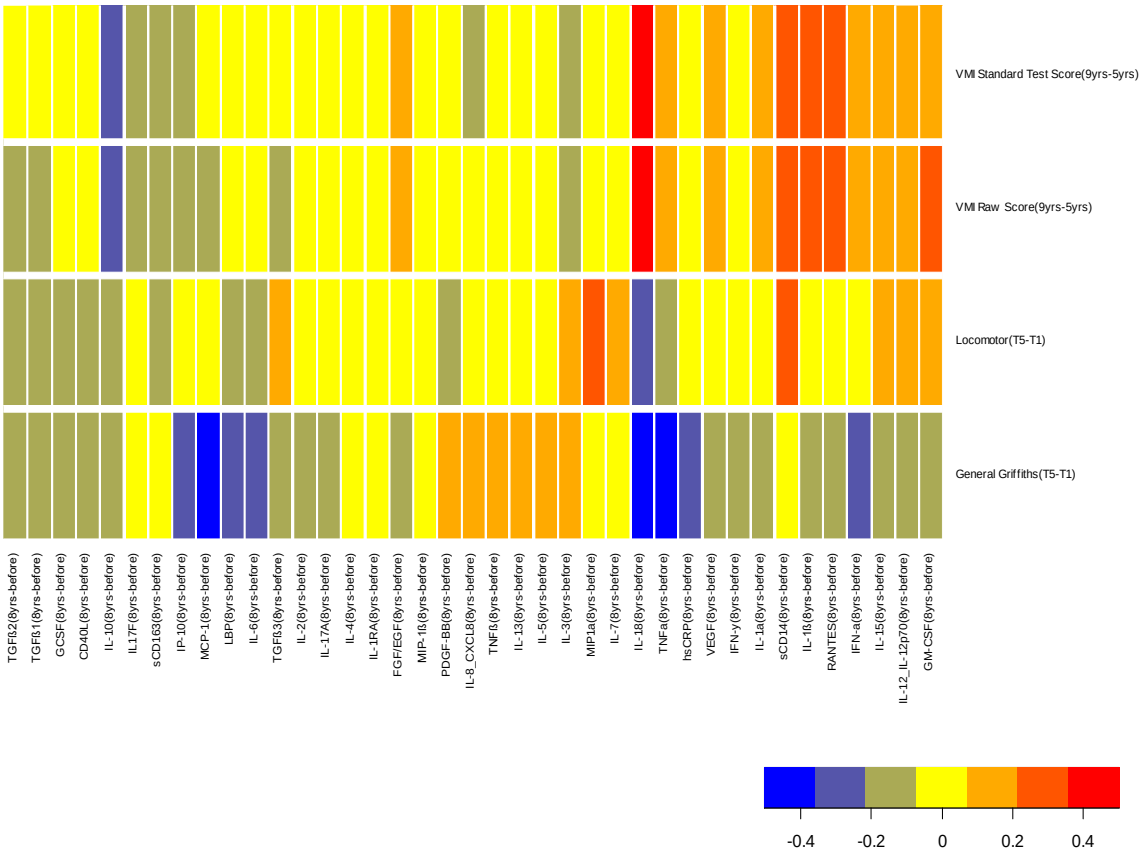


***Figure 7.10**: Correlation heat map representation of change in biomarker levels from baseline to 8 years of age versus the change in neurocognitive outcomes from 11 months to 9 years of age.*
Colour scale ranging from blue (negative) to red (positive) depicts the Pearson Correlation Coefficient (R). Probabilities for both Pearson and Spearman correlations are listed in Table 7.17 below.

Table 7.13: Summary of Pearson and Spearman correlation coefficients (R) as well as probabilities following correlation analysis of change in biomarker levels from baseline to 8 years of age versus the change in neurocognitive outcomes from 11 months to 9 years of age

Immune Biomarker (change over time)	Neurocognitive measurement (change over time)	Pearson Correlation (*r*-Coefficient)	Pearson Correlation (p-value)	Spearman Correlation (*r*-Coefficient)	Spearman Correlation (p-value)
MCP-1 (8yrs-before)	General Griffiths(T5-T1)	-0.40	**0.01**	-0.17	0.29
TNFα (8yrs-before)	General Griffiths(T5-T1)	-0.41	**<0.01**	-0.32	**0.05**
IL-18 (8yrs-before)	General Griffiths(T5-T1)	-0.39	**0.03**	-0.49	**<0.01**
IL-18 (8yrs-before)	VMI Raw Score(9yrs-5yrs)	+0.49	**<0.01**	+0.53	**<0.01**
IL-18 (8yrs-before)	VMI Standard Test Score(9yrs-5yrs)	+0.50	**<0.01**	+0.51	**<0.01**
LBP (8yrs-before)	General Griffiths(T5-T1)	-0.34	**0.03**	-0.37	**0.02**

7.3.9 Correlation analysis following long-term viral suppression – Immune biomarker expression and HIV-1 DNA levels at 8 years of age vs neurocognitive outcomes at ages 5, 7 and 9 years of age

Lastly, by implementation of cross-sectional study design analysis, we evaluated possible associations between plasma biomarker levels at 8 years of age (with additional biomarkers included i.e., MAdCAM, s100A8, s100A9 and i-FABP) and late neurodevelopmental outcomes (i.e., VMI Raw and VMI Standard Test scores). We also evaluated a possible association between HIV-1 cell infectivity (i.e., the levels of iCAD) with neurodevelopmental outcomes.

Figure 7.11 and Table 7.14 displays a graphical representation in the form of a heat map and the relevant statistical associations.

Biomarker levels at 8 years of age showed significant negative associations with IL-17F (*r*= -0.20; p=0.02), IL-12 (*r*= -0.19; p=0.04), IL-13 (*r*= - 0.19; p=0.03), IFNγ (*r*= -0.20; p=0.02), MIP-1α (*r*= 0.20; p=0.02), TNFβ (*r*= -0.18; p=0.04) and IFN-α (*r*= -0.80; p=0.04). Interestingly, biomarkers with strong negative associations at baseline including IL-17F, IL-13, MIP-1α and IFN-α remained associated with neurodevelopmental outcomes at 8 years of age. IL-12 (produced by macrophages), IFN-γ (secreted by activated T cells) and TNFβ (produced by activated T-cells) all of which are potent pro-inflammatory cytokines, had no baseline association to neurodevelopmental outcomes.

A positive association was only observed for I-FABP (*r*=0.23; p=0.01) and the VMI Raw and Standard Scores.

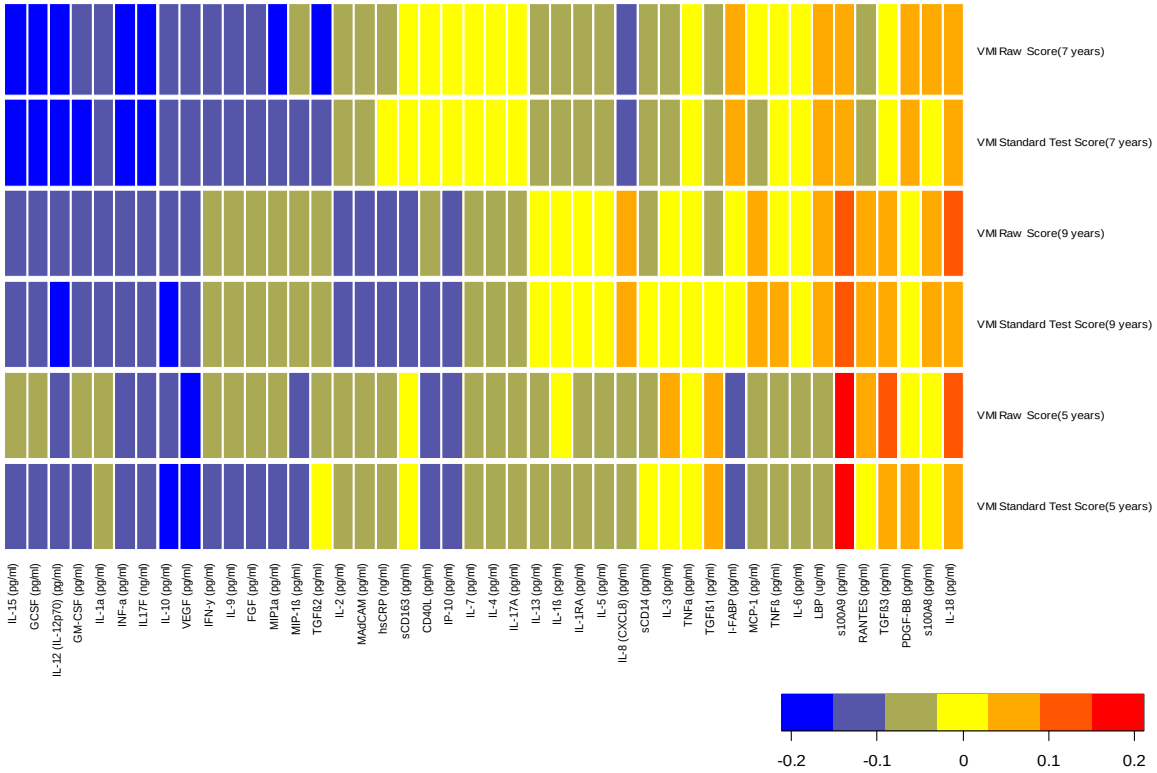


***Figure 7.11**: Correlation heat map representation immune plasma biomarker levels at 8 years of age versus neurocognitive parameters at 5, 7 and 9 years of age.*
Colour scale ranging from blue (negative) to red (positive) depicts the Pearson Correlation Coefficient (R). Probabilities for both Pearson and Spearman correlations are listed in Table 5 below.

Table 7.14: Summary of Pearson and Spearman correlation coefficients (R) as well as probabilities following correlation analysis of immune biomarker levels at 8 years of age versus neurocognitive outcomes at 5, 7 and 9 years of age

Immune Biomarker (8 years of age)	Neurocognitive measurement (Test 6, 7 and 8)	Pearson Correlation (*r*- Coefficient)	Pearson Correlation (p-value)	Spearman Correlation (*r*- Coefficient)	Spearman Correlation (p-value)
IL-17F (ng/ml)	VMI Raw Score (7 years)	-0.20	**0.02**	-0.15	**0.09**
IL-17F (ng/ml)	VMI Standard Test Score (7 years)	-0.21	**0.02**	-0.16	**0.07**
IL-12 (IL-12p70) (pg/ml)	VMI Raw Score (5 years)	-0.09	0.32	-0.19	**0.04**
IL-12 (IL-12p70) (pg/ml)	VMI Standard Test Score (5 years)	-0.12	0.22	-0.23	**0.02**
IL-13 (pg/ml)	VMI Raw Score (7 years)	-0.07	0.46	-0.19	**0.03**
IL-13 (pg/ml)	VMI Standard Test Score (7 years)	-0.07	0.45	-0.20	**0.02**
IFN-γ (pg/ml)	VMI Standard Test Score (5 years)	-0.11	0.26	-0.20	**0.04**
MIP-1α (pg/ml)	VMI Raw Score (7 years)	-0.15	**0.08**	-0.20	**0.02**
MIP1α (pg/ml)	VMI Standard Test Score (7 years)	-0.13	0.14	-0.20	**0.02**
TNF-ß (pg/ml)	VMI Standard Test Score (7 years)	-0.01	0.92	-0.18	**0.04**
INF-α (pg/ml)	VMI Standard Test Score (7 years)	-0.18	**0.04**	-0.14	0.12
I-FABP (pg/ml)	VMI Raw Score (9 years)	+0.00	0.97	+0.23	**0.01**
I-FABP (pg/ml)	VMI Standard Test Score (9 years)	+0.01	0.93	+0.23	**0.01**

Table 7.15: Summary of Pearson and Spearman correlation coefficients (R) as well as probabilities following predication analysis of HIV-1 cell-associated DNA measured at 8 years of age and neurocognitive outcomes measured at 5, 7 and 9 years of age

	Neurocognitive measurement	Pearson Correlation (*r*-Coefficient)	Pearson Correlation (p-value)	Spearman Correlation (*r*-Coefficient)	Spearman Correlation (p-value)
Cell-associated DNA (CAD)	VMI Raw Score (5 years)	+0.18	0.31	+0.24	0.16
	VMI Standard Test Score (5 years)	+0.13	0.45	+0.21	0.24
	VMI Raw Score (7 years)	+0.03	0.87	+0.22	0.23
	VMI Standard Test Score (7 years)	+0.04	0.83	+0.23	0.21
	VMI Raw Score (9 years)	-0.02	0.90	-0.07	0.69
	VMI Standard Test Score (9 years)	-0.02	0.92	-0.07	0.71

7.3.10 Associations between cytomegalovirus (CMV) concentrations at baseline and 8 years of age, immune biomarkers and neurodevelopmental outcomes

The role of CMV, measured at baseline and 8 years of age, on neurocognitive outcomes, HIV-1 CAD, clinical and immune biomarker parameters was evaluated in a subset of HIV+ children (n=41).

CMV findings at baseline, before therapy initiation

Of the 41 PHIV children included in this CMV analysis, approximately 24.0% (n=10) children displayed detectable CMV viral load ranging from 150 to 3,519 copies/ml (median: 150 copies/ml).

HIV+ children were categorized into CMV “detected” and “undetected”. We compared various clinical, immunological, virological and neurocognitive scores between the detected and undetected groups.

We observed that the CMV detected group displayed a longer time to viral suppression (mean: 439.6 days vs 325.87 days) compared to the CMV undetected group ($p<0.05$). The percentage CD4 at birth was significantly ($p<0.01$) lower in the detected group compared to the undetected group (mean: 22.7% vs 34.8%). When evaluating the longitudinal %CD4 T cell count, we observed that the CMV detected group at birth displayed a significantly lower ($p<0.01$) count over time compared to the undetected group (mean: 23.1% vs 33.5%). Furthermore, the %CD8 T cell count at birth was observed to be significantly higher ($p<0.05$) in the CMV detected group (mean: 31.2% vs 33.5%).

Figures 7.12a – d below provides a graphical representation of the clinical findings when comparing the CMV detected and undetected groups at birth.

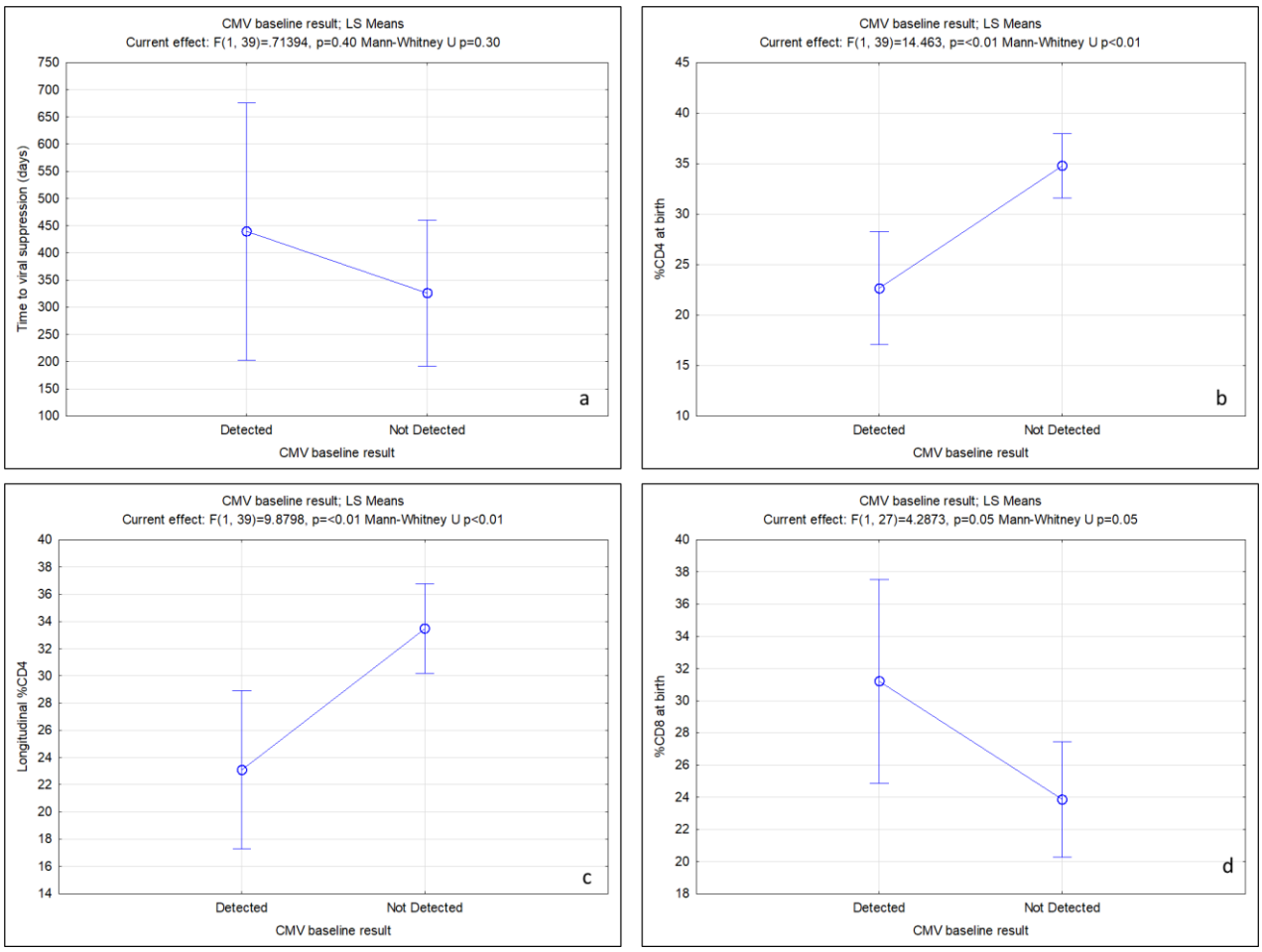


Figure 7.12 a – d: *Vertical bar graph with 95% confidence intervals depicting the mean values of clinical parameters (a) time to viral suppression (b) % CD4 T-cell count at birth (c) longitudinal %CD4 T-cell counts and (d) %CD8 T-cell counts at birth for CMV detected and undetected groups evaluated at birth.*

We evaluated the level of immune biomarker expression in plasma at baseline for the CMV detected and undetected groups.

We observed significantly higher ($p<0.05$) expression for plasma immune biomarkers **IL-12** (mean: 4.2 vs 2.0 pg/ml), **IP-10** (mean: 1806.3 vs 1410.6 pg/ml), **GM-CSF** (mean: 2.9 vs 1.85 pg/ml), **TGF-β2** (mean: 908.7 vs 719.9 pg/ml), **sCD163** (mean: 485180.5 vs 245227.3 pg/ml), **IL-18** (mean: 456.4 vs 338.9 pg/ml) and **LBP** (38812.89 vs 22485.52 µg/ml).

Conversely, we observed significantly lower levels of **RANTES** in the CMV detected group vs the undetected group (mean: 1996.8 vs 3816.2 pg/ml).

Figures 7.13a – h below provides a graphical representation of the immunological biomarker findings when comparing the CMV detected and undetected groups at birth.

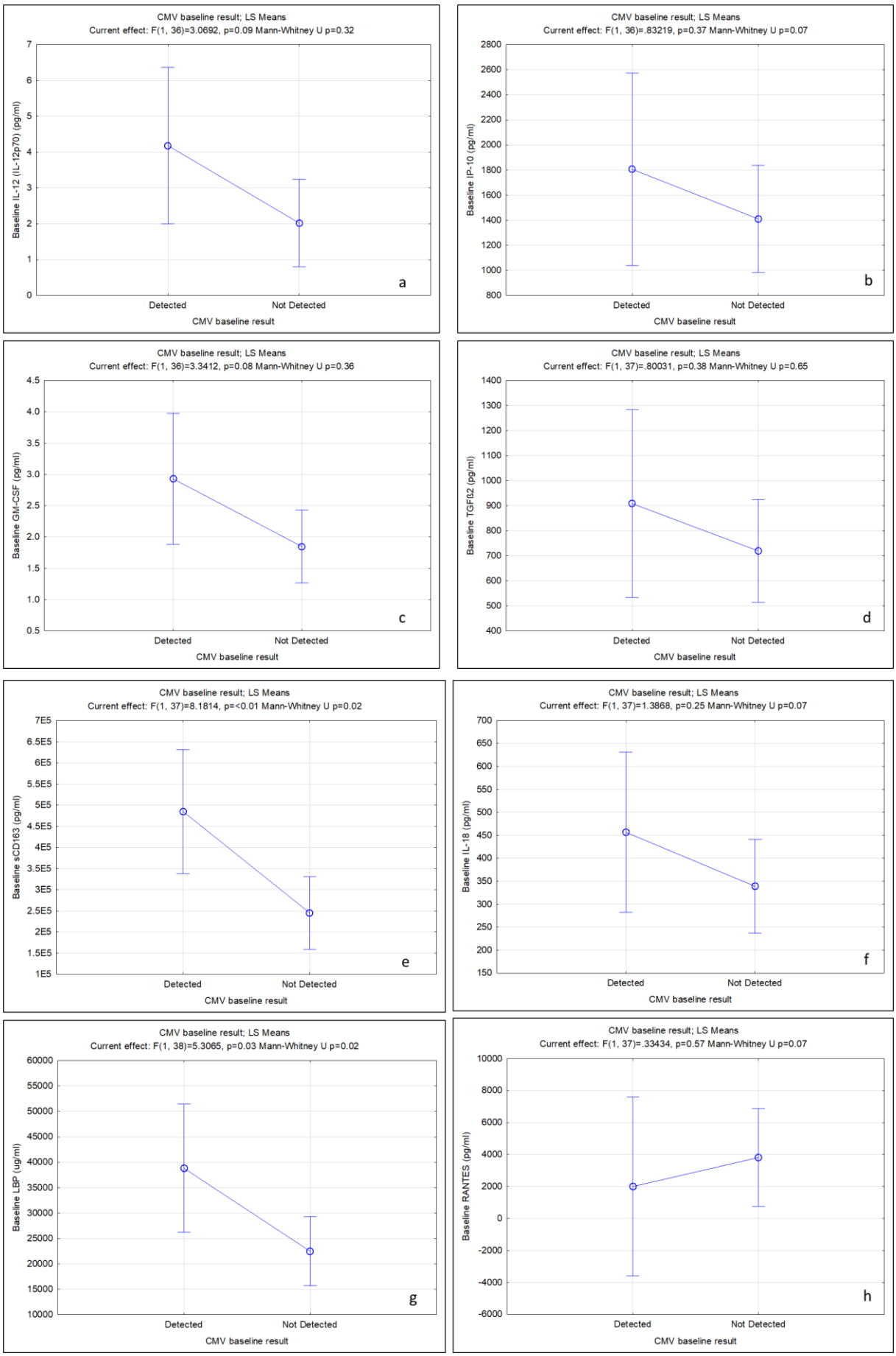


Figure 7.13 a – h: *Vertical bar graph with 95% confidence intervals depicting the mean levels of plasma immune biomarkers measured at birth (a) IL-12, (b) IP-10, (c) GM-CSF, (d) TGF-β2, (e) sCD163, (f) IL-18, (g) LBP and (h) RANTES for CMV detected and undetected groups at birth*

Interestingly, we observed significantly higher Locomotor and General Griffiths scores (Test 3 and 5) in the CMV detected group compared to the CMV undetected group.

We present these findings in Figures 7.14 a-c below.

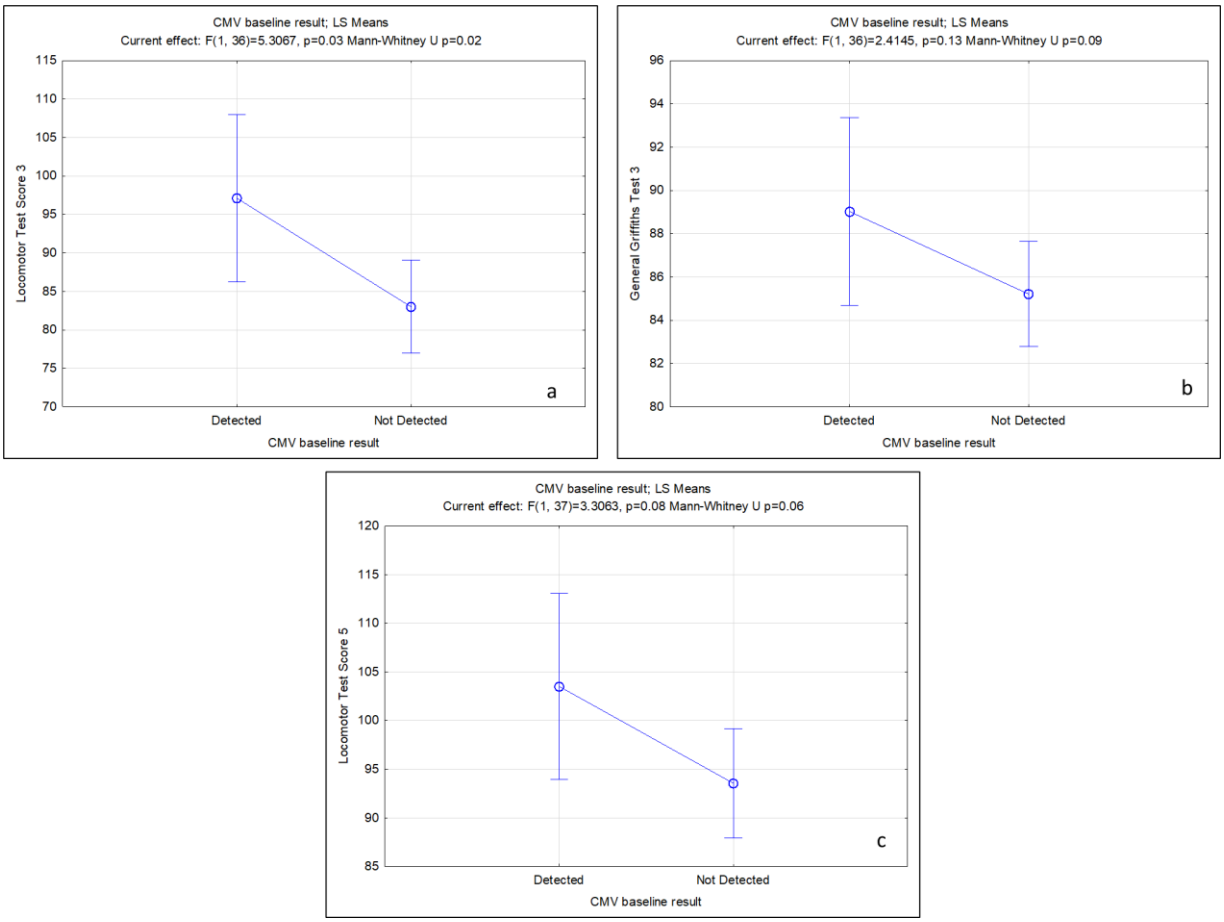


Figure 7.14 a – c: *Vertical bar graph with 95% confidence intervals mean Locomotor Test 3 and 5 and General Griffiths Test 5 scores for CMV detected and undetected groups at birth*

We observed no significant differences for HIV-1 CAD at baseline between the CMV detected and undetected groups.

CMV findings at 8 years of age

At 8 years of age, we included 137 participants for the evaluation of CMV viral load. A summary of these findings is tabulated below.

Table 7.16: Summary of CMV findings at 8 years of age

	HIV+	HEU	HUU
n	85	22	30
Positive CMV	44	13	17
Negative CMV	5	1	1
No result	36	8	12
% CMV positive	89.9%	92.0%	94.4%
Median CMV (copies/ml)	118.2	111.6	115.7
Range CMV copies/ml	16.5 – 114, 388.0	52.7 – 246.8	53.1 – 173.4

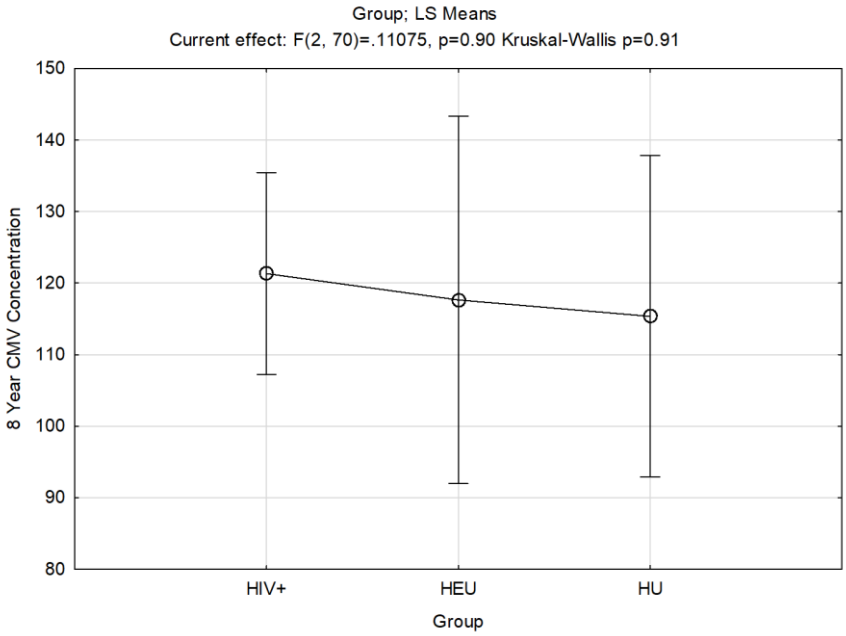


Figure 7.17: *Vertical bar graphs denoting 95% confidence intervals with least significant (LS) mean values for CMV concentration (copes/ml) in three study groups (HIV+, HEU and HU).*
No significant differences between all three study groups were observed at 8 years of age as depicted by the Kruskal-Wallis p-value of 0.91.

Table 7.17: Summary of statistics for group comparative analysis of CMV concentration at 8 years of age for HIV+, HEU and HU children

	n	Mean CMV concentration	SD	SE	Lower CL (-95%)	Upper CL (+95%)	Probabilities (P-values)		
							HIV+	HEU	HUU
HIV+	43	121.33	49.38	7.08	107.21	135.45	-	0.80	0.65
HEU	13	117.65	53.93	12.88	91.97	143.33	0.80	-	0.89
HUU	17	115.35	29.09	11.26	92.89	137.81	0.65	0.89	-

At this time point, we observed no significant associations between CMV levels and neurodevelopmental outcomes.

We did however find associations between CMV viral load and immune biomarkers as tabulated below.

Table 7.18: Summary of Spearman correlation coefficients (R) as well as probabilities following predication analysis CMV analysis at 8 years of age and biomarker levels at 8 years of age

Immune biomarker levels at 8 years	Spearman Correlation (*r*-Coefficient)	Spearman Correlation (p-value)
IL-1β	-0.30	**<0.01**
hsCRP	+0.24	**0.04**
LBP	+0.27	**0.02**

7.4 Discussion

The primary aim of this investigation was to evaluate the long-term role of ART (delayed, early and interrupted) and HIV-1 associated inflammation on neurocognitive outcomes in PHIV children in South Africa. In addition, we also investigated the relationship between early (before therapy initiation) and late (pre-adolescent age) clinical and virological factors that may influence neurocognitive outcomes. Our study population included well-characterised and clinically monitored children from the randomised-control CHER trial. To our knowledge, we have presented the most comprehensive descriptive longitudinal data evaluating the interplay of the immune system, clinical and virological outcomes on the neurocognitive performance in PHIV children in South Africa.

Neurodevelopmental test scores were selected and analysed based on previously administered full panel assessments. We observed that PHIV children randomised to the delayed therapy arm presented with significantly lower Locomotor and General Griffiths scores from Test 1 (11 months) to Test 4 (42 months). At Test 5 (60 months), there were no significant differences between all five study groups implying adequate recovery of neurocognitive performance in PHIV children subjected to delayed therapy. In addition, neurodevelopmental tests implemented at 5, 7 and 9 years of age i.e., the Beery-VMI, showed no significant differences between any of the HIV+ groups including that of the delayed therapy arm. Several other studies, including similar analysis within this cohort have pronounced the benefit of early therapy initiation for improved neurodevelopmental outcomes (Van Rie et al., 2009; Laughton et al., 2012, 2018; Lowick, Sawry and Meyers, 2012; Puthanakit et al., 2012, 2013; Brahmbhatt et al., 2017; Gómez et al., 2018). Another study conducted within our research group evaluated neurodevelopmental outcomes in infants initiated on ART within 21 days of life. In this study of 29 infants, preliminary findings suggested normal neurodevelopmental scores in PHIV infants who started ART at a median of 6 days of age (Laughton et al., 2019).

It is however worth exploring the possible circumstances that may have contributed to the relative recovery of children subjected to delayed therapy. Improvements in neurodevelopmental parameters may be attributed to the cohort having access to superior clinical care and adequate follow-up allowing for this recovery to match those of HIV uninfected counterparts. Another study evaluating the neurodevelopmental trajectory of HIV infected children conducted in the Democratic Republic of Congo, observed that children accessing care early in the stage of HIV disease showed improved motor development along with other cognitive parameters (Van Rie et al., 2009). Another circumstance to consider is the contribution of the "survivor effect" within the delayed therapy arm following early deaths. Other considerations include the use of neurodevelopmental tests such as the Beery-VMI that may not be sufficiently sensitive to measure specific differences that may exist in young children (Laughton et al., 2012, 2018). In addition, along with the relatively small sample size of our study population that may not be sufficiently powered to detect greater differences between the groups, some of the participants in our control group for longitudinal neurodevelopmental assessments had to be replaced following low retention rates due to various reasons. Recruitment of new control subjects at various time points of assessments may not be subjected to the same clinical care and follow-up of other participants allowing for the confounding effects of our comparative analysis.

When investigating the effect of therapy interruption on neurodevelopment in PHIV children, we observed that the uninterrupted therapy group (consisting of the delayed and early randomised therapy groups) had lower Locomotor scores from 11 to 18 months. Therapy interruption had no significant impact on neurodevelopment tests implemented at 5, 7 and 9 years of age. Planned therapy interruption at either 40 or 96 weeks had limited impact on neurodevelopmental test scores across all time points measured. In previous unpublished reports of this research study, we also found limited longitudinal immunological differences within the interruption groups compared to the uninterrupted groups. These findings indicate that limited ART interruption under cautious guidance in asymptomatic children does not adversely affect their relative neurodevelopmental outcomes. In addition, the toxicity of ART and its role in neurocognitive impairments has long been hypothesised to play a role in HIV-related neurocognitive impairments despite immunological recovery following treatment initiation. With the use of non-nucleoside reverse transcriptase inhibitor, efavirenz, associations with increasing prevalence of neuropsychiatric symptoms have been documented following planned treatment interruptions, where symptoms were deemed reversible (Church et al., 2001; Tamula et al., 2003; Price and Spudich, 2008; Blokhuis et al., 2016). Furthermore, these findings support the clinical, immunological and virological findings from the larger CHER cohort that early time-limited ART seems relatively safe when planned appropriately under careful clinical guidance (Violari, Mark F Cotton, et al., 2008; Cotton et al., 2013a; Laughton et al., 2018).

Recent brain imaging investigations into the CHER cohort aimed to evaluate the effect of ART interruption on brain cortical thickness and folding at 5 years of age. The findings, following magnetic resonance (MRI) scans showed that children subjected to treatment interruption had thicker cortex than HIV negative controls in the left frontal and right insular regions. Children randomised to continuous therapy showed no difference to age-matched controls. Additional findings indicated that PHIV children randomised to both interrupted and continuous ART displayed region-specific alterations in local gyrification indices when compared to uninfected controls. Researchers concluded that cortical folding appears more sensitive than cortical thickness to early events such as early ART and subsequent interruption (Nwosu et al., 2021).

Compared to studies evaluating neurocognitive dysfunction in HIV infected adults, we show that neurocognitive impairment occurs in early stages of life in PHIV children and that these impairments eventually resolve over time. Further studies are required to evaluate whether adequate neurocognitive function exists into adolescence – a time where social stigmatism, adherence issues and other co-morbidities emerge.

Soluble immune biomarkers have gained interest in their clinical utility for predicting neurodevelopmental outcomes in children with PHIV (Kapetanovic et al., 2010, 2014b; Ananworanich et al., 2015; Benki-Nugent et al., 2019).

We interestingly observed a significant positive relationship between early (before therapy initiation) plasma immune biomarkers, IL-1β, G-CSF, sCD14, sCD163, IL-18 and LBP and various neurocognitive test scores. These biomarkers have been associated with monocyte/macrophage activation and microbial translocation and appears to be the driver of chronic inflammation in later pre-adolescent years (Chapter 3). However, before the initiation of therapy and the absence of viral suppression, elevated levels of these biomarkers have an

association with improved neurocognitive outcomes. Although the role of monocyte/macrophage activation is less clearly understood in untreated children, our findings are consistent with that of a Kenyan study (Benki-Nugent et al., 2019) that observed elevated levels of sCD163 that had a strong association to neurodevelopmental outcomes in untreated infants. The immunological mechanism hypothesised for the increase in sCD163 is the shedding of this scavenger receptor by peripheral monocyte subsets such as CD14+CD16+, that have newly migrated from the bone marrow with the function of reducing the T-cell activation induced by HIV and reducing the inflammatory response in the brain (Burdo et al., 2010; Burdo, Lentz, et al., 2011; Benki-Nugent et al., 2019). These increased levels of plasma sCD163 together with the harmful expansion of specific monocyte subsets may therefore offer a mechanism of protecting the brain from inflammatory injury. This mechanism has been documented and explained in simian immunodeficiency virus (SIV)-infected macaques (Burdo, Lentz, et al., 2011; Burdo, Lackner and Williams, 2013).

When evaluating longitudinal levels of sCD163 together with IL-1β, sCD14, IL-18 and LBP (Chapter 5) we found that the levels of these biomarkers increased over time in PHIV children. Similarly, in SIV infection, monocyte turnover seemed to have remained abnormally high therefore suggesting a disruption of normal monocyte activity due to HIV/SIV infection (Sugimoto et al., 2017). In addition, higher sCD163 levels in acute phase HIV infected adults along with greater CNS outcomes have also been documented compared to Fiebig stage III adults who had deteriorated CNS outcomes (D'Antoni et al., 2018).

Furthermore, other researchers evaluating neurocognitive scores have also speculated protective immune mechanisms at play in long-term untreated HIV infected children (Ananworanich et al., 2015). The latter study observed increased levels of activated monocytes/macrophages following ART initiation along with higher neurocognitive scores.

Although the Kenyan study alluded to above did not find similar trends in sCD14, the study also did not expand on plasma biomarkers of monocyte/macrophages or biomarkers of microbial translocation.

We have previously described chronic immune activation at 8 years of age (Chapter 3) in this cohort. Our research showed strong evidence of monocyte/macrophage activation associated gut damage as a key driver of this described chronic immune activation with significantly elevated levels of sCD14, sCD163, LBP, IL-18 and IL-17F amongst other plasma immune biomarkers. In this study component, we observed significant associations of MCP-1, TNF-α, IL-18 and LBP with neurodevelopmental outcomes at 7 and 9 years of age. These findings allude the prominent role of the gut-brain axis that has received much attention in the field of precision medicine.

CMV co-infection is one of the most common AIDS-defining diseases acting as a significant cofactor for HIV disease progression and exacerbating both gastrointestinal as well as CNS disease (Drew, 1988; Doyle, Atkins and Rivera-Matos, 1996; Kovacs et al., 1999; Guibert et al., 2009). We observed a relatively low CMV prevalence within our cohort when compared to other African cohorts (Bates and Brantsaeter, 2016; Kfutwah et al., 2017).

When evaluating early immunological, clinical and neurological parameters within the CMV detected group of PHIV children in our study, we found significant differences in several parameters. Infants with detected CMV viral load at baseline (before therapy initiation) displayed lower %CD4 T cell counts at birth and higher %CD8 T cell counts. These infants also had a longer time to viral suppression. Dysregulation as a result of HIV infection compromises both innate and adaptive immune responses and also compromises the integrity of mucosal sites. The impact of HIV infectivity on infant early life is also dependant on maternal health. Low CD4 counts at birth may therefore enable early CMV acquisition and impair the containment of CMV (Muenchhoff, Prendergast J. and Goulder, 2014; Ruck et al., 2016; Slyker, 2016).

A significantly higher level of plasma biomarkers of inflammation including IL-12, IP-10, GM-CSF, TGF-β2, sCD163, IL-18 and LBP was observed in the CMV detected group. These immune biomarkers function in a pro-inflammatory response, involved in monocyte/macrophage activation and interestingly upregulated in microbial translocation and previously described. CMV infection is known to induce the expression of pro-inflammatory cytokine, IL-6 (Iwamoto and Konicek, 1997). We did not particularly observe this elevation in the CMV detected group this, but rather increased expression of other pro-inflammatory cytokines as indicated. Studies have shown associations of CMV IgG levels with IL-6 and sCD14 levels in blood plasma of CMV co-infected women indicating a possible relationship with the ability of CMV to increase gut microbial translocation and monocyte activation (Iwamoto and Konicek, 1997; Lurain et al., 2016). The mechanism of increasing gut microbial translocation and monocyte activation may also fuel persistent inflammation and drive other non-AIDS defining diseases such as cardiovascular and metabolic diseases as adolescence approaches (Kuller et al., 2008; Sandler et al., 2011; Tenorio et al., 2014).

Interestingly, despite a significant association between CMV detection and immune biomarkers of inflammation and microbial translocation, the CMV positive group, at the earliest time point displayed higher Locomotor and General Griffiths test scores. Therefore, the higher the CMV viral load, the better the neurodevelopmental score. Whilst some studies have implicated poor neurodevelopmental outcomes including hearing loss in infants co-infected with CMV, others have found no relationship between neurodevelopmental outcomes and CMV in PHIV and HEU children (Kapetanovic et al., 2012; Slyker, 2016; Moraka et al., 2020; Pathirana et al., 2020). We hypothesise a similar mechanism of protecting the brain via the BBB from inflammatory components as described earlier for similar inflammatory biomarkers associated with CMV infection.

Whilst some early immune biomarkers as highlighted above have a mechanistic protection of the BBB response, others have an inverse effect on neurodevelopmental outcomes. We observed strong negative associations with baseline plasma levels of IL-17F, IL-1RA, IL-6, IL-7, IL-13, MCP-1, MIP-1α, IFN-α, TGF-β2 and poor neurodevelopmental scores at various time point measurements. Properties of these cytokines and chemokines include both pro-and anti-inflammatory response functions. Other cytokines such as IL-7 and TGF-β2 function in maintaining immune homeostasis. Overproduction and/or dysregulation of these immune mediators due to HIV-1 infection leads to neurodevelopmental impairment.

Inflammation has increasingly been implicated as a key contributor to CNS dysfunction especially on the developing brain that is particularly vulnerable to inflammatory insults *in utero*, infancy and early childhood. The effects of these early insults have significant potential to cause long-term neurodevelopmental disorders (Deverman and Patterson, 2009; Spann et al., 2018).

HIV replication at the early stages of disease triggers the onset of immune activation and inflammation. HIV-associated inflammation may result in an increase in the permeability of the BBB thereby promoting the recruitment of additional target cells from the periphery (Andersson et al., 2001; Spudich and González-Scarano, 2012). This may reflect the need for a balance between effective immune activation necessary to mount an immune response before ART and damaging activation that remains after the initiation of ART.

IL-6, a pleiotropic cytokine (both pro- and anti-inflammatory), has been extensively studied and has shown strong relationships to poor neurocognitive outcomes in both adults and children. IL-6 has been known to have direct neurotoxicity effects as well as amplifying abilities to enhance viral relation and further promote immune activation (Rao, Ruiz and Prasad, 2014). Furthermore, IL-6 expression results in neuronal dysfunction by causing cytoplasmic vacuoles within neurons.

Neuroinflammation leading to neurocognitive dysfunction is further driven by chemokines such as IL-8, IP-10, MIP-1α, MIP-1β, MCP-1 and RANTES (González-Scarano and Martín-García, 2005; Yang et al., 2009). In this research study, we observed a prominent role of chemokines MIP-1α, MIP-1β and MCP-1 in driving neurocognitive dysfunction.

Altogether, these studies of adults, older children, and infants suggest that timing of infection and initiation of ART with respect to individual immune maturation may both influence the balance between damaging and protective immune function. Uniquely for PHIV children, development of both the immune system and the CNS for a large part occurs during exposure to HIV and cART, therefore the consequences of HIV-related immune activation and cerebral changes may well differ from those in adults (Bilbo and Schwarz, 2012).

Viral suppression on cART evidently does not fully protect against cerebral injury, but knowledge concerning the pharmacokinetics and toxicity of cART in the paediatric CNS is lacking. Even in the presence of effective ART, there is a need for early identification of neurocognitive deficits in children to mitigate for better outcomes through mental rehabilitation interventions. Microbial infections that are not associated with the CNS can have an important impact on the CNS. In addition, the immune system plays a significant and important role that serves as both protective and pathogenic (Hong and Banks, 2015). Challenges of early ART relates to the poor ability of these drugs to cross the BBB and target virus contained with the CNS.

Current treatment with antiretrovirals including abacavir, lamivudine, nevirapine, lopinavir, dolutegravir and additional integrase inhibitors have shown evidence of improved neurocognitive outcomes indicating a capacity to penetrate the CNS well (Cysique et al., 2009). In addition, modern ARVs with penetrative capabilities have shown increased suppression of HIV RNA within the CNS (Carvalhal et al., 2016).

Data generated from a sub-study of the START study have however shown no neurocognitive advantage of immediate versus delayed initiation of ARVs specifically in individuals with T cell counts above 500 cells/µl (Wright et al., 2018)

Increasing evidence implies significant roles for ongoing neuroinflammation, vascular dysfunction and hypercoagulability induced by HIV in the setting of viral suppression. Whilst we have highlighted the role of HIV-associated inflammation on early paediatric brain development, the underlying mechanisms driving CNS pathology in children remain unclear. The role of immune activation together with coagulation has been suggested has key drivers of poor neurodevelopment in PHIV children. Additional data within this cohort evaluated the levels of soluble chemokines sICAM-1, sVCAM-1 and MCP-1 in 136 children (HIV+ and negative controls) at a median age of 7.8 years in relation to pulse wave velocity (PWV) measures (a sophisticated measure of arterial wall stiffness). The findings confirmed a relationship between the PWV scores and an increase in sVCAM-1 and MCP-1 implying a significant involvement of these chemokines in the mechanism of premature vascular disease in these children (Innes et al., 2017). Other markers of endothelial activation along with a pro-angulation state indicated by an increase in plasma D-dimer, fibrinogen may also facilitate the adhesion and migration of immune cells to the brain (Pontrelli et al., 2010; Miller et al., 2012; Sainz et al., 2014).

It is also important to note that our study primarily focused on plasma immune biomarkers. In adult studies, CSF immune biomarkers have shown a stronger relationship to neurocognitive outcomes (Anderson et al., 2017). CSF studies in children are however limited. MCP-1 and IP-10 levels in CSF have been associated with inadequate cognitive function (Yuan et al., 2013; Thames et al., 2015). The relationship between plasma (indicative of systemic inflammation) and CSF (inflammatory status within the brain itself) biomarkers is less clear and requires further investigation specifically in paediatric cohorts.

Limitations to this study include the lack of early control samples for immune biomarker analysis and appropriate comparison between PHIV and uninfected children. In addition, further stratification of study arms limits the power of our analysis. Studies evaluating the relationships between the immune system and neurodevelopmental outcomes may differ with regards to factors such as the selection of neurocognitive assessments utilized within cohorts, clinical staging of disease, timing of ART initiation, social demographic profile of the cohort, HIV sub-type and the research study design.

Neurocognitive assessments are considered subjective in its administration and may differ between administrators as well as between various populations. Neuroimaging on the other hand is less susceptible to bias representation and may provide further evidence for the neuro-inflammatory mechanism driving neuropathology in PHIV children.

An approach that is based on factor analysis may provide an appropriate tool for the identification of PHIV children at high risk for poor neurodevelopmental outcomes. In addition, factor-based tools may also provide a deeper understanding of the underlying mechanisms driving neurodevelopmental dysfunction and therefore drive the identification of possible therapeutic interventions. Our data suggests a strong link between HIV driven inflammation and decreased neurodevelopmental outcomes. There is further need to study this

population from a multidisciplinary perspective encompassing neuroimaging, cardiovascular monitoring and virological mechanism in playing a role in neurodevelopmental outcomes.

We have alluded to and provided evidence for the significant value of combining neuropsychological assessments, multimodal neuroimaging and laboratory evaluation of inflammation and neurodegenerative biomarkers to improve our understanding of the pathogenesis of HIV on the developing nervous system as well as treatment and prevention strategies.

Additional longitudinal research is important and crucial in order to observe the long-term consequences of CNS exposure to HIV and ART in combination with other HIV co-morbidities in PHIV children as they survive into adulthood.

HEU children, although not the primary focus of this research study, represent an important rapidly growing population. We have shown some evidence of lingering immune dysfunction beyond infancy and that may have a role in neurodevelopmental outcomes. Further evidence is provided in other studies evaluating HEU children in South Africa. Wedderburn et. al. (2019) observed receptive and expressive language delays in HEU infants at 2 years of age further concluding the need for long-term investigations into understanding neurodevelopmental outcomes in this vulnerable population (Wedderburn *et al.*, 2019). Delineating HEU neurodevelopmental outcomes in the context of maternal HIV-1 infection will provide valuable insight into factors influencing brain development in infants exposed to maternal HIV infection. HIV+ mothers who have part of their pregnancy without treatment are more likely to have infants with residual immune defects. When pregnancy occurs during the time of viral suppression and proper therapy adherence, exposure to the virus is limited if not absent. Distinguishing and appropriately studying these two HEU infant groups would provide greater insight to the mechanisms contributing to inadequate brain development.

7.5 Conclusion

This research aimed to delineate the longitudinal relationship between cART and HIV-1 associated inflammation. Both these parameters, along with early clinical parameters such as CD4 and time to viral suppression, provide good predictors for neurocognitive outcomes in children with PHIV. This study was able to provide important insights into immune biomarkers of inflammation that may serve as early predictors of neurocognitive outcomes. This data also highlights the important role of the early immune response to HIV-1 in early and late neurodevelopment and provide a foundation for future research. There is also a role to further explore the gut-brain axis relationship further within this population.

CHAPTER 8:

CONCLUSION

The research undertaken for this dissertation aimed to describe the immunological landscape of perinatally PHIV children subjected to early initiated, delayed and interrupted long-term combination cART. The relative importance and relatedness of a number of markers were assessed. The immunological parameters were then also related to measures of the HIV-1 latent reservoir and neurocognitive outcomes.

By investigating the CHER randomised control trial cohort, we were able to provide important insights into immunological mechanisms that contribute to driving HIV-1 persistence during long-term suppressive therapy. This research also provides important knowledge to further exploring PHIV children as potential "cure" and remission candidates.

Despite early initiated and long-term suppressive therapy, children with PHIV show persisting immune dysfunction that does not fully normalise when compared to their demographically matched pre-adolescent (8 years of age) uninfected counterparts. This abnormality persists despite good clinical follow-up, well-reconstituted CD4 counts, and low HIV-1 cell infectivity. Several significant immunological differences that exist within both innate and adaptive immune compartments of children with PHIV have been observed in this study.

Through multivariate cytokine and chemokine measurements, we showed elevated levels of immune biomarkers associated with persisting chronic inflammation (hsCRP, MIP-1β, IL-1α, IFN-α, CD40L, VEGG, PDGF-BB, TGFβ2), macrophage and dendritic cell activation (sCD14, sCD163, IL-18, IL-17F) and unresolved gut damage at a critical pre-adolescent age where cART adherence and the emergence of non-infectious co-morbidities exists. Increased expression of novel plasma biomarkers (MAdCAM-1 and S001A8/9), indicative of direct epithelial damage and intestinal inflammation, provided further evidence of microbial translocation in HIV-1 infected children.

Furthermore, we have shown an interesting monocyte/macrophage profile defined by a decrease in macrophage-associated cytokine expression and expansion of classical and non-classical monocyte phenotype. The alteration to this myeloid compartment may also steer the altered cytokine profile observed. This finding further highlights a chronic inflammatory profile driven by the innate immune system triggered by microbial translocation.

Significantly diminished expression of specific T cell mediated immune biomarkers (IL-3, RANTES, IFN-γ, IL-17A) in PHIV at 8 years of age highlights further dysfunction and prompts further exploration of HIV-1 inflicted premature immune exhaustion.

Following extensive biomarker profiling, we were able to identify the potential of IL-18, a potent inflammatory cytokine, to provide insights into immune dysfunction, regulation and HIV-1 cell infectivity and/or HIV-1 latency. In addition, we observed the ability of IL-18 to serve as a proxy marker for measuring treatment success as well as overall immune health.

Extensive predictive analysis of immune biomarkers indicates a complex interplay between cytokines, chemokines and growth factors that shape the landscape for disease progression during HIV-1 infection in children. Evaluating the impact of therapy regimens and clinical parameters on the expression patterns of soluble immune biomarkers within our cohort, we were able to conclude that clinical and virological parameters such as lower birth weight, lower CD4 counts and the degree of HIV-1 cell infectivity at the time of birth, has a greater impact on the expression of soluble inflammatory parameters than therapy regimen (i.e., delayed, early or interruption). Where the initiation of cART played a role in lowering some immune parameters associated with activation and inflammation, others were minimally impacted further alluding to the inability of cART to reverse immune dysregulation due to HIV-1 infection the significant need for immune therapeutic approaches to enhance immune health.

Furthermore, the study confirmed that that the sooner cART is initiated, and the sooner virological suppression is achieved and also the better the immunological outcomes will be several years later.

This research also investigated the feasibility of implementing a cell-culture based assay for measuring the viable viral reservoir, the quantitative viral outgrowth assay (QVOA), in long-term suppressed HIV-1 infected children. Children initiated on cART early did not have infectious virus that could be readily isolated in viral outgrowth assays due to low cell input. Although the gold-standard QVOA for recovering infectious virus appeared unsuited for paediatric populations, inducible virion production was detected in 4 of the 10 participants selected using a highly sensitive HIV-1 single copy RNA assay. This assay may be a more sensitive screen (and indirect measure) of virion production in latent HIV-1 reservoirs from children with small sample volumes.

We also investigated the interplay of the immune system along with clinical and virological outcomes on the neurocognitive performance in PHIV children in South Africa. To our knowledge, this was the most comprehensive descriptive longitudinal study evaluating this interplay within this setting. We identified immune parameters that serve as early and late predictors of neurocognitive outcomes. Whilst some early immune markers of inflammation result in specifically poorer neurocognitive parameters, others engage in possible neuroprotective mechanisms of the blood brain barrier thereby decreasing the impact of neuroinflammatory conditions. Time to therapy initiation and viral suppression as well as CD4 count at birth, were found to have significant associations with neurocognitive outcomes in our cohort. Again, this research highlights a role for addressing early immune insults with immune therapeutic approaches for improving long-term and overall neurocognitive performance in children with PHIV.

An important finding was the association between biomarkers of gut damage and neurocognitive outcomes, highlighting the need to further explore the gut-brain axis in the context of neuroinflammation in children with PHIV within our setting.

Children of the randomised CHER trial serve a unique and highly significant population for scientific investigation. This study group represents the largest well-characterised cohort worldwide after receiving early cART that is still under clinical care and is therefore considered an important strength of this research

Additional strengths of this research include its longitudinal (limited in the field) and cross-sectional study design also encompassing HIV-1 uninfected study controls of both HIV-1 exposed uninfected (HEU) and HIV-1 unexposed uninfected (HUU) controls. HEU children represent a largely expanding population that require longitudinal immunological delineation.

Although this research was primarily focused on describing the immunological landscape of PHIV children from the closest time to birth to pre-adolescence, it drew on a multidisciplinary approach to understanding HIV-1 pathogenesis and persistence in other contexts such as neurocognitive assessments.

Research Novelty/Contribution to New Knowledge

This doctoral research has provided five key novel aspects to the field of paediatric HIV-1 immunology:

1. PHIV children display elements of chronic inflammation at pre-adolescent (8 years of age) that appears largely driven by myeloid (innate) pathways stimulated by early, cART irreversible microbial translocation related to gut damage)
2. Novel immune biomarkers of intestinal inflammation, MAdCAM-1 and s100A8/A9, here first described in a paediatric cohort of PHIV children are associated with other gut epithelial and translocation markers.
3. IL-18 was identified as a putative marker, of HIV-1 cell infectivity, overall immune health and an identification tool for patients which may require added vigilance following cART.
4. QVOA was implemented and a novel highly sensitive single copy HIV-1 RNA assay was developed and optimized to measure HIV-1 RNA copies expressed in cell culture supernatants.
5. Extensive longitudinal evaluating the neuro-immune interplay in children PHIV was performed, which yielded key immune and gut damage associations with specific neurocognitive measures.

Study Limitations

A key limitation of this study is the lack of access to early HIV-1 uninfected control samples for adequate immunological comparison. Due to this, we are unable to compare the extent of early immunological HIV-1 damage within our cohort. Other limitations to this research include the ART treatment groups being incomparable and may not represent a completely randomised cohort. Group 2, subjected to early, continuous therapy displayed lower birth weights and lower CD4 T cells counts at baseline.

Following the stratification of our HIV-1 infected group into various treatment arms, the statistical power of our analysis was significantly decreased compromising the relative conclusions drafted for this investigation.

Between the associations observed, it is a relatively complex task having to establish a cause or effect with the associations observed. Further investigations are required in this regard. Due the nature and study design of this research, a number of challenges exist when interpreting the significance of immune biomarkers within a complex and developing immunological landscape.

When attempting to quantify replication competent latent reservoirs, we were limited by the small sample volumes obtained from children. In addition, working with cryopreserved material rather than fresh samples influenced experimental design such as the purification of total and resting CD4 T cells.

PBMC sample material along with blood plasma were the most utilised for understanding our research questions on the interplay of the immune system, virus and brain. However, these samples only provide a snapshot and may not always be a true reflection of components or signals in the gut and brain. Access to imaging and biological tissue samples would be more applicable when attempting to understand organ-specific pathologies related to HIV-1 infection.

When understanding immune ontogeny in children, maternal health as well as nutritional status is of significance. One of the limitations and possible confounders of this research study was the inability to have access to maternal health status or nutritional information which could have provided further insights biomarker levels and associations.

Future Direction

The findings reported in this thesis have highlighted some key features of immune abnormalities persisting after early initiated long-term ART in paediatric populations. These findings prompt further and more detailed immune profiling and clinical follow-up to establish the severity of morbidity outcomes that may progress from this immune dysregulation in children with PHIV. An assessment of HIV-1 specific memory immune responses under virological suppression would enhance the overall picture. In addition, mechanistic exploration of pathways such as the IL-18 and its relationship to viral reservoir and gut epithelial damage needs interrogation.

More functional studies to be implemented would further validate some of the immune abnormalities observed within our cohort. In addition, comparative analysis of other cohorts subjected to even earlier treatment regimens would be of interest.

Some biomarker signatures could provide important tools for monitoring overall immune health of PHIV children within a clinical setting.

The CHER cohort presents the world's most well-described PHIV cohort and remains part of a clinical follow-up until adolescence. Therefore, evaluation of the persistence of any of the key associations through and beyond adolescence will aid in building a lifelong profile of immune changes in the MTCT-infected children.

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APPENDICES

APPENDIX A: ETHICS APPROVAL FOR POST-CHER (RESERVOIR STUDY)

UNIVERSITEIT•STELLENBOSCH•UNIVERSITY
jou kennisvennoot • your knowledge partner

16 September 2014

MAILED

Prof MF Cotton

KID-CRU, Ward J8
Tygerberg Academic Hospital
Francie van Zijl Drive
Parow Valley
7500

Fax: (021) 938-4153

Sender:
MRS M.A. DAVIDS
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Dear Prof Cotton

PROTOCOL: RESERVOIR STUDY
Latent reservoir characterization and correlations with neurocognitive functioning and thymic output over 12 years in HIV-infected children given early antiretroviral treatment in South Africa

ETHICS REFERENCE NO: M14/07/029

At a meeting of the Committee for Pharmaceutical Trials, held on 06 August 2014, the above project was approved on condition that further information that was required, be submitted. This information has now been provided and the study is now finally approved, as of the above date.

Kindly note that the Letter of Approval from the Medicine's Control Council of SA's, which includes the Investigator's participation in the study, is imperative before the commencement of the study. Please forward a copy of the Letter of Approval to the undersigned if it has not already been submitted with the documentation.

The following documents were reviewed and approved by the Committee:

Protocol:	Submitted Version	Date submitted	Approved Version	Date of Approval
	Protocol version 1.0	07 Jul 2014	Version 1.0 dated 03 July 2014	06 Aug 2014

Patient Forms:	Document Date	Version	Description
		Version 1.0	Kid-Cru Participant payment structure, Version 1.0 dated July 2014
		Version 1.0	Reservoir Study Final Site specific English ICF (post CHER) version 1.0 dated 03 July 2014
			Reservoir Study Site specific English ICF (Babies) version 1.0 dated 03 July 2014

Other Documentation:	Document Date	Description

APPENDIX B: PARTICIPANT CONSENT FORMS

PARTICIPANT INFORMATION LEAFLET AND INFORMED CONSENT FORM

Title of the study: Latent reservoir characterization and correlations with neurocognitive functioning and thymic output over 12 years in HIV-infected children given early antiretroviral treatment in South Africa

Short title of the study: Reservoir Study

Principal Investigator:
Name: Prof M F Cotton

Address: FAMCRU ward J8, Tygerberg Children's Hospital
Francie van Zijl Drive, Tygerberg 7505

Contact no: 021 9384295

This information is provided to help you decide whether you will allow your child to take part in this clinical research study and whether to have a video recording done as part of this clinical research study. Please read this form carefully and ask the study doctor or the study staff to explain any words or procedures you do not understand.

Dear Parent
Good day, my name is _____ and I am a _____ at FAMCRU. I would like to invite your child to take part in the research study called "Reservoir Study". Before agreeing to participate in this study, it is important that you fully understand what is involved. If you have any questions, which are not fully explained in this leaflet and consent form, do not hesitate to ask the doctor in charge of the study or the study staff at any time.
If you agree and allow your child to take part in this study, you will be asked to sign and date this consent form. You will get a copy of the signed consent form to keep. You should only sign the consent form if all details of the study are completely clear to you, if you are willing to let your child be part of the study and if you completely understand your child's rights as a participant in this study. If you want to withdraw, you can do so at any stage without any consequences.

WHY IS THIS STUDY BEING DONE?

In this study we would like to measure the HIV virus that hides in the cells of a person's body and how Antiretroviral Treatment can affect this. This hidden virus is called the reservoir. We would like to investigate how the viral reservoir affects a child's health and brain development and function. The viruses that are in hiding and those that are free in the blood could have many differences. These different viruses may also change over time. Therefore, we will investigate how these viruses change over time and study the reservoir cells to find out where in the cells different HIV viruses hide. We are hoping that by measuring this reservoir in children, we will be able to discover even better ways to treat children will HIV.

HOW MANY CHILDREN WILL TAKE PART AND HOW LONG WILL MY CHILD BE IN THIS STUDY?

Approximately 177 children from Cape Town will participate in the study including both HIV-infected and HIV-uninfected children. Most of the children have been on the CHER study, but there will also be babies enrolled onto this study. For the children who were on the CHER study this study will end at 12 years old.

STUDY PROCEDURES

Study visits:

- Your child will need to come to the clinic and continue on the other studies' appointments as they have done before. For HIV-infected children this is every 3 months and for HIV-uninfected children this will be twice a year.
- At the study visit, you will be asked about your child's previous illnesses, any hospitalisations and any medications.
- Your child will see a doctor and have a general check-up and if your child is HIV-positive he/she will receive treatment for this
- For HIV-infected children we will take blood samples twice in the first year and then once a year, with a total of approximately 40 millilitres (just a bit less than 3 tablespoons) per year. Some of the blood is important for health care for children with HIV and the other will be stored and analysed for research on the HIV virus.

- For HIV-uninfected children we will take blood samples once a year with a total of 4ml (less than one teaspoon) per year.
- We will put anaesthetic cream on your child's skin when we take blood samples to decrease the pain.
- We would also like to do research on any blood samples that are left over and in storage from the CHER study.

Between 11 and 11 ½ years of age your child will need to come for two visits:

- Tests of brain function will be done. These tests are to find out whether your child is able to do the same things that other children of the same age are able to do. (These tests are the same that we do on the CHER study at 7 and 9 years of age)
- These tests will be done over two mornings quite close to each other
- With your permission, a video recording of your child doing the brain function tests may be done. This video is to check that the tests are done correctly and to train other team members.
- While your child is doing the brain function tests, we will ask you questions about your child's behaviour.

Caregiver health questionnaire

You will be asked to answer a list of questions with the help of a trained staff member. The questionnaire contains questions on the physical and emotional health and social status of yourself, the mother/father and/ or caregiver of your child.

We may contact you to remind you of appointments or to change appointments if needed. We may record your contact details and the contact details of close relatives/friends. We may possibly use these contact details to remind you of appointments or to reschedule appointments.

We will try our best to schedule appointments so that your child does not miss too many important school days.

WHY IS A VIDEO RECORDING BEING DONE?

A video recording of the brain function test may be done. The reason for the recording is to check that the tests are done correctly and to teach other team members the correct way of doing the tests. A different tester can also watch the video and see if they can give your child the same scores for the test.

WHAT ARE THE RISKS TO MY CHILD WHEN HE/SHE PARTICIPATES IN THE STUDY?

Your child may experience pain or discomfort from having blood samples taken. The usual risks of blood-taking are:

- Pain at the site where the needle goes in.
- Bruising around the site.
- There is a small chance of skin infection at the site.

If your child is not able to do some of the brain function tests, he or she may begin to feel worried or nervous. We will reassure him or her that all we need is for them to do their best, and some of the tests are difficult for all children. We will not pressurise your child to continue if he or she feels uncertain.

STUDY RESULTS:

We will combine all the findings of this study with the other studies that your child has been on as well as any future studies that your child may enroll on. We may also compare these results with similar studies done at different places in the world.

WHAT ABOUT CONFIDENTIALITY?

All medical information collected during the study will be treated as confidential and will be available only to staff members involved with this study who are directly involved in your child's care. Any information that is used for research or publication purposes will be kept confidential and will not have your or your child's name recorded on it.

If you give permission for the session to be taped, the video recordings will be kept in a safe place and only study team members will be allowed to see the recording. The video tapes will be kept for at least 2 years if the results are not published. If the results are published, the video tapes may be kept for longer.

It is possible that the regulatory authorities, such as the Ethics Committee, U.S. National Institutes of Health (NIH), the U.S. Office of Human Research Protections (OHRP), Fam-Cru, the National Health Research Ethics Council and study staff, may want to review the study may want to review the study documents at a later stage in which case every effort will be made to protect your child's confidentiality.

YOUR CHILD'S PARTICIPATION IS VOLUNTARY

Your child's participation in the study is voluntary. You can refuse to let him/her participate or stop his/her participation at any time that you choose.
If you decide not to do the video recording, it will not affect your child's participation in the brain function tests for the study.

Your child's withdrawal from the study will not affect his/her access to other medical care.

ARE THERE POTENTIAL BENEFITS FOR MY CHILD FOR BEING IN THE STUDY?

There are no direct benefits for participating in the study. If anything is detected, your child will be referred to appropriate services.
If the tests show that your child has problems that may improve with help, we will discuss this with you. If you agree, we will then refer your child to other doctors, therapists or psychologists.

WHAT HAPPENS IF MY CHILD IS INJURED?

A study-related injury or illness is one that occurs as a direct result of the study-specific procedures. If your child is injured as a result of being in this study, the study sites will give your child immediate necessary treatment for the injuries without any cost to you. The study will not pay to treat a medical condition or disease you had before joining this study or expenses for injury, treatment, or hospitalization your baby may require that are not the result of participation in the study.

WHAT ARE THE COSTS TO ME?

The study will be done at no cost to you. Neither you nor your child's medical scheme will be expected to pay for the study related visit or study procedures.

WILL I RECEIVE ANY PAYMENT FOR MY CHILD'S PARTICIPATION?

You will not receive payment for your child's participation in the study, although you will receive money for transport expenses to the clinic for each scheduled study visit.

ETHICAL APPROVAL

- This clinical study protocol has been submitted to the Health Research Ethics Committee (HREC) at the University of Stellenbosch and has received approval.
- The study has been structured in accordance with the Declaration of Helsinki (last updated October 2008), which deals with recommendations guiding doctors in biomedical research involving human participant.
- I do not have any financial or personal interest with this organization that may bias my actions.

WHAT DO I DO IF I HAVE QUESTIONS OR PROBLEMS?

If at any time you have any questions about the study, please do not hesitate to contact a doctor in charge of the study or the study staff.

- Drs E Dobbels: P Zuidewind, S Innes, R Hickman
  021-9384295 (during office hours)

The 24-hour telephone number that you can reach the doctor in charge, or another authorized person is: 084 6410735

If you want any information regarding your child's rights as a research participant, or have complaints regarding this research study, you may contact:

The Chairperson: Health Research Ethics Committee
Faculty of Health Sciences, Stellenbosch University
Phone no: 021 9389207

This independent committee is established to help protect the rights of research participants and gave written approval for the study protocol.

If you have questions about this trial, you should first discuss them with your doctor or the ethics committee.

REFERRAL OPTION

Please indicate below, whether you want the study team to refer your child for help if we notice that your child has problems on the brain function testing or behaviour questionnaire.

□ **YES**, I want you to refer my child for further help if problems are identified on the brain function testing or behaviour questionnaire.

□ **NO**, I do not want you to refer my child for further help if problems are identified on the brain function testing or behaviour questionnaire.

Reservoir Study
INFORMED CONSENT SIGNATURE PAGE

Name of participant: _____

To parents/legal guardians

Have you read this information sheet about this study, or has someone read it to you and to your satisfaction?	Yes	☐	No	☐
Have you had an opportunity to ask questions and discuss this study?	Yes	☐	No	☐
Have you received satisfactory answers to all your questions?	Yes	☐	No	☐
Have you received enough information about this study	Yes	☐	No	☐
Do you understand that you are free to withdraw your child from this study at any time?	Yes	☐	No	☐
Do you agree to let your child take part in this study?	Yes	☐	No	☐
Do you consent to having a video recording of the testing done? (Participation in the study will not be affected even if you decline permission to record the video.)	Yes	☐	No	☐
Do you consent to completing the Caregiver's Health questionnaire? (Participation in the study will not be affected even if you decline)	Yes	☐	No	☐

Parent/legal guardian:

Name	Signature	Date	Time
_____	_____	_____	☐☐☐☐

Witness (if applicable):

Name	Signature	Date	Time
_____	_____	_____	☐☐☐☐

Person conducting informed consent process:
I am satisfied that the parent/legal understands what the consent form is about, and that his/her questions have been answered.

Name	Signature	Date	Time
_____	_____	_____	☐☐☐☐

APPENDIX C: CHAPTER 5 RESULTS -STATISTICAL PARAMETERS FOR IL-RA, IL-1α, IL-6, IP-10, MCP-1, MIP-1α, MIP-1β, TNFα, RANTES, SCD14, SCD163, IL-18 AND HSCRP

	n	Mean	SD	Probabilities (p-values)			
				Delayed therapy	Early continuous therapy	Early therapy interrupted at 40 weeks	Early therapy interrupted at 90 weeks
IL-1RA (pg/ml)							
Delayed therapy	28	1.67	0.52	-	0.20	0.66	0.53
Early continuous therapy	43	1.97	0.64	0.20	-	0.31	0.06
Early therapy interrupted at 40 weeks	53	1.86	0.63	0.66	0.31	-	0.26
Early therapy interrupted at 90 weeks	25	1.65	0.62	0.53	0.06	0.26	-
IL-1α (pg/ml)							
Delayed therapy	28	0.12	1.17	-	0.27	0.80	0.86
Early continuous therapy	43	0.34	1.25	0.27	-	0.30	0.20
Early therapy interrupted at 40 weeks	53	0.19	1.15	0.80	0.30	-	0.66
Early therapy interrupted at 90 weeks	25	0.15	1.27	0.86	0.20	0.66	-
IL-6 (pg/ml)							
Delayed therapy	28	0.19	0.69	-	0.09	0.70	0.92
Early continuous therapy	43	0.44	0.64	0.09	-	0.12	0.09
Early therapy interrupted at 40 weeks	53	0.35	0.67	0.70	0.12	-	0.63
Early therapy interrupted at 90 weeks	25	0.23	0.67	0.92	0.09	0.63	-
IP-10 (pg/ml)							
Delayed therapy	28	2.63	0.47	-	0.84	0.43	0.78
Early continuous therapy	43	2.65	0.46	0.84	-	0.26	0.62
Early therapy interrupted at 40 weeks	53	2.66	0.38	0.43	0.26	-	0.67
Early therapy interrupted at 90 weeks	25	2.71	0.39	0.78	0.62	0.67	-
MCP-1 (pg/ml)							
Delayed therapy	28	2.22	0.49	-	0.29	0.84	0.39
Early continuous therapy	43	2.13	0.45	0.29	-	0.31	0.95
Early therapy interrupted at 40 weeks	53	2.18	0.32	0.84	0.31	-	0.43
Early therapy interrupted at 90 weeks	25	2.11	0.45	0.39	0.95	0.43	
MIP-1α (pg/ml)							
Delayed therapy	28	0.50	0.97	-	0.59	0.88	0.56
Early continuous therapy	43	0.41	0.76	0.59	-	0.64	0.91
Early therapy interrupted at 40 weeks	53	0.42	0.80	0.88	0.64	-	0.61
Early therapy interrupted at 90 weeks	25	0.31	0.99	0.56	0.91	0.61	-
MIP-1β (pg/ml)							
Delayed therapy	28	1.30	0.41	-	0.48	0.58	0.38
Early continuous therapy	43	1.43	0.57	0.48	-	0.14	0.11
Early therapy interrupted at 40 weeks	53	1.31	0.51	0.58	0.14	-	0.64
Early therapy interrupted at 90 weeks	25	1.18	0.58	0.38	0.11	0.64	-
TNF-α (pg/ml)							
Delayed therapy	28	1.10	0.35	-	0.89	0.52	0.70
Early continuous therapy	43	1.10	0.37	0.89	-	0.37	0.78
Early therapy interrupted at 40 weeks	53	1.13	0.18	0.52	0.37	-	0.30
Early therapy interrupted at 90 weeks	25	1.08	0.32	0.70	0.78	0.30	-
RANTES (pg/ml)							
Delayed therapy	28	2.43	0.95	-	0.10	0.42	0.24

Early continuous therapy	44	2.24	0.98	0.10	-	0.32	0.78
Early therapy interrupted at 40 weeks	52	2.28	0.87	0.42	0.32	-	0.58
Early therapy interrupted at 90 weeks	25	2.23	0.91	0.24	0.78	0.58	-
sCD14 (pg/ml)							
Delayed therapy	25	4.62	1.39	-	0.14	0.21	0.20
Early continuous therapy	40	4.78	1.39	0.14	-	0.72	0.98
Early therapy interrupted at 40 weeks	50	4.89	1.37	0.21	0.72	-	0.78
Early therapy interrupted at 90 weeks	24	5.09	1.40	0.20	0.98	0.78	-
sCD163 (pg/ml)							
Delayed therapy	25	5.46	0.75	-	0.53	0.17	0.56
Early continuous therapy	40	5.54	0.60	0.53	-	0.39	0.97
Early therapy interrupted at 40 weeks	50	5.65	0.59	0.17	0.39	-	0.50
Early therapy interrupted at 90 weeks	24	5.70	0.63	0.56	0.97	0.50	
IL-18 (pg/ml)							
Delayed therapy	25	2.49	0.68	-	0.11	**0.02**	0.12
Early continuous therapy	40	2.72	0.60	0.11	-	0.47	0.85
Early therapy interrupted at 40 weeks	50	2.80	0.59	**0.02**	0.47	-	0.69
Early therapy interrupted at 90 weeks	24	2.88	0.61	0.12	0.85	0.69	-
hsCRP (ng/ml)							
Delayed therapy	28	2.86	0.83	-	0.99	0.53	0.53
Early continuous therapy	43	2.83	0.83	0.99	-	0.48	0.50
Early therapy interrupted at 40 weeks	52	3.06	0.74	0.53	0.48	-	0.91
Early therapy interrupted at 90 weeks	25	2.96	0.91	0.53	0.50	0.91	-

APPENDIX D: CHAPTER 5 RESULTS – PROBABILITY (P-VALUES) PARAMETERS FOR IL-17F, IL-1β, IL-1α, IL-17A, IFN-γ, MIP 1α, MIP-1β, CD40L, SCD14, SCD163 AND IL-18

Probabilities for Post Hoc Tests (IL-1β)

	Therapy Grouping	Therapy time point status at sample date	1	2	3	4	5	6	7	8	9	10	11	12
1	Delayed therapy	Before therapy initiation	-	0.72	0.29	0.39	0.43	**0.00**	0.55	0.31	**0.01**	0.36	0.46	**0.01**
2	Delayed therapy	2 years after therapy initiation	0.72	-	0.64	0.76	0.76	**0.03**	0.90	0.67	0.13	0.67	0.35	0.09
3	Delayed therapy	8 years of age	0.29	0.64	-	0.83	0.88	**0.02**	0.68	0.94	0.14	0.99	0.13	0.10
4	Early Continuous therapy	Before therapy initiation	0.39	0.76	0.83	-	0.97	**0.01**	0.83	0.87	0.08	0.86	0.16	0.06
5	Early Continuous therapy	2 years after therapy initiation	0.43	0.76	0.88	0.97	-	**0.03**	0.82	0.93	0.17	0.90	0.19	0.12
6	Early Continuous therapy	8 years of age	**0.00**	**0.03**	**0.02**	**0.01**	**0.03**	-	**0.01**	**0.01**	0.26	0.05	**0.00**	0.61
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.55	0.90	0.68	0.83	0.82	**0.01**	-	0.72	0.07	0.72	0.24	0.05
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.31	0.67	0.94	0.87	0.93	**0.01**	0.72	-	0.10	0.96	0.13	0.07
9	Early therapy interrupted at 40 wks.	8 years of age	**0.01**	0.13	0.14	0.08	0.17	0.26	0.07	0.10	-	0.22	**0.01**	0.65
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.36	0.67	0.99	0.86	0.90	0.05	0.72	0.96	0.22	-	0.16	0.15
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.46	0.35	0.13	0.16	0.19	**0.00**	0.24	0.13	**0.01**	0.16	-	**0.01**
12	Early therapy interrupted at 90 wks.	8 years of age	**0.01**	0.09	0.10	0.06	0.12	0.61	0.05	0.07	0.65	0.15	**0.01**	-

Probabilities for Post Hoc Tests (IL-1α)

	Therapy Grouping	Therapy time point status at sample date	1	2	3	4	5	6	7	8	9	10	11	12
1	Delayed therapy	Before therapy initiation	-	0.43	**0.00**	0.91	0.68	**0.00**	0.54	0.46	**0.00**	0.76	0.58	**0.00**
2	Delayed therapy	2 years after therapy initiation	0.43	-	**0.00**	0.49	0.29	**0.00**	0.80	0.17	**0.00**	0.64	0.90	**0.00**
3	Delayed therapy	8 years of age	**0.00**	**0.00**	-	**0.00**	**0.00**	0.24	**0.00**	**0.00**	0.51	**0.00**	**0.00**	0.76
4	Early Continuous therapy	Before therapy initiation	0.91	0.49	**0.00**	-	0.56	**0.00**	0.58	0.35	**0.00**	0.82	0.62	**0.00**

5	Early Continuous therapy	2 years after therapy initiation	0.68	0.29	**0.00**	0.56	-	**0.00**	0.32	0.81	**0.00**	0.49	0.39	**0.00**
6	Early Continuous therapy	8 years of age	**0.00**	**0.00**	0.24	**0.00**	**0.00**	-	**0.00**	**0.00**	**0.03**	**0.00**	**0.00**	0.13
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.54	0.80	**0.00**	0.58	0.32	**0.00**	-	0.13	**0.00**	0.79	0.93	**0.00**
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.46	0.17	**0.00**	0.35	0.81	**0.00**	0.13	-	**0.00**	0.31	0.26	**0.00**
9	Early therapy interrupted at 40 wks.	8 years of age	**0.00**	**0.00**	0.51	**0.00**	**0.00**	**0.03**	**0.00**	**0.00**	-	**0.00**	**0.00**	0.75
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.76	0.64	**0.00**	0.82	0.49	**0.00**	0.79	0.31	**0.00**	-	0.77	**0.00**
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.58	0.90	**0.00**	0.62	0.39	**0.00**	0.93	0.26	**0.00**	0.77	-	**0.00**
12	Early therapy interrupted at 90 wks.	8 years of age	**0.00**	**0.00**	0.76	**0.00**	**0.00**	0.13	**0.00**	**0.00**	0.75	**0.00**	**0.00**	-
Probabilities for Post Hoc Tests (IFN-γ)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.72	**0.03**	0.55	0.36	**0.00**	0.92	0.63	**0.01**	0.55	0.35	0.59
2	Delayed therapy	2 years after therapy initiation	0.72	-	**0.04**	0.40	0.27	**0.01**	0.79	0.46	**0.02**	0.41	0.59	0.43
3	Delayed therapy	8 years of age	**0.03**	**0.04**	-	0.10	0.32	0.53	**0.03**	0.06	0.85	0.18	**0.01**	0.09
4	Early Continuous therapy	Before therapy initiation	0.55	0.40	0.10	-	0.66	**0.01**	0.48	0.88	**0.03**	0.94	0.16	0.95
5	Early Continuous therapy	2 years after therapy initiation	0.36	0.27	0.32	0.66	-	0.10	0.31	0.57	0.21	0.75	0.11	0.63
6	Early Continuous therapy	8 years of age	**0.00**	**0.01**	0.53	**0.01**	0.10	-	**0.00**	**0.01**	0.58	0.05	**0.00**	**0.01**
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.92	0.79	**0.03**	0.48	0.31	**0.00**	-	0.55	**0.01**	0.49	0.39	0.52
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.63	0.46	0.06	0.88	0.57	**0.01**	0.55	-	**0.01**	0.84	0.19	0.93
9	Early therapy interrupted at 40 wks.	8 years of age	**0.01**	**0.02**	0.85	**0.03**	0.21	0.58	**0.01**	**0.01**	-	0.10	**0.01**	**0.03**
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.55	0.41	0.18	0.94	0.75	0.05	0.49	0.84	0.10	-	0.17	0.90

11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.35	0.59	**0.01**	0.16	0.11	**0.00**	0.39	0.19	**0.01**	0.17	-	0.17
12	Early therapy interrupted at 90 wks.	8 years of age	0.59	0.43	0.09	0.95	0.63	**0.01**	0.52	0.93	**0.03**	0.90	0.17	-
Probabilities for Post Hoc Tests (CD40L)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.47	**0.00**	0.17	**0.04**	**0.00**	0.82	0.37	**0.00**	0.69	0.98	**0.00**
2	Delayed therapy	2 years after therapy initiation	0.47	-	**0.00**	0.07	**0.02**	**0.00**	0.39	0.15	**0.00**	0.32	0.55	**0.00**
3	Delayed therapy	8 years of age	**0.00**	**0.00**	-	**0.00**	**0.00**	0.77	**0.00**	**0.00**	0.87	**0.00**	**0.00**	0.90
4	Early Continuous therapy	Before therapy initiation	0.17	0.07	**0.00**	-	0.31	**0.00**	0.28	0.57	**0.00**	0.40	0.33	**0.00**
5	Early Continuous therapy	2 years after therapy initiation	**0.04**	**0.02**	**0.00**	0.31	-	**0.00**	0.07	0.14	**0.00**	0.11	0.11	**0.00**
6	Early Continuous therapy	8 years of age	**0.00**	**0.00**	0.77	**0.00**	**0.00**	-	**0.00**	**0.00**	0.87	**0.00**	**0.00**	0.67
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.82	0.39	**0.00**	0.28	0.07	**0.00**	-	0.52	**0.00**	0.86	0.89	**0.00**
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.37	0.15	**0.00**	0.57	0.14	**0.00**	0.52	-	**0.00**	0.70	0.54	**0.00**
9	Early therapy interrupted at 40 wks.	8 years of age	**0.00**	**0.00**	0.87	**0.00**	**0.00**	0.87	**0.00**	**0.00**	-	**0.00**	**0.00**	0.76
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.69	0.32	**0.00**	0.40	0.11	**0.00**	0.86	0.70	**0.00**	-	0.77	**0.00**
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.98	0.55	**0.00**	0.33	0.11	**0.00**	0.89	0.54	**0.00**	0.77	-	**0.00**
12	Early therapy interrupted at 90 wks.	8 years of age	**0.00**	**0.00**	0.90	**0.00**	**0.00**	0.67	**0.00**	**0.00**	0.76	**0.00**	**0.00**	-
Probabilities for Post Hoc Tests (sCD14)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	**0.04**	**0.00**	0.26	**0.00**	**0.00**	0.47	**0.00**	**0.00**	0.60	**0.01**	**0.00**
2	Delayed therapy	2 years after therapy initiation	**0.04**	-	**0.00**	0.20	0.68	**0.00**	0.13	0.69	**0.00**	0.14	0.44	**0.00**
3	Delayed therapy	8 years of age	**0.00**	**0.00**	-	**0.00**	**0.00**	0.15	**0.00**	**0.00**	0.19	**0.00**	**0.00**	0.27

4	Early Continuous therapy	Before therapy initiation	0.26	0.20	**0.00**	-	**0.04**	**0.00**	0.72	**0.02**	**0.00**	0.66	**0.04**	**0.00**
5	Early Continuous therapy	2 years after therapy initiation	**0.00**	0.68	**0.00**	**0.04**	-	**0.00**	**0.03**	0.95	**0.00**	**0.03**	0.64	**0.00**
6	Early Continuous therapy	8 years of age	**0.00**	**0.00**	0.15	**0.00**	**0.00**	-	**0.00**	**0.00**	0.80	**0.00**	**0.00**	0.77
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.47	0.13	**0.00**	0.72	**0.03**	**0.00**	-	**0.01**	**0.00**	0.91	**0.02**	**0.00**
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	**0.00**	0.69	**0.00**	**0.02**	0.95	**0.00**	**0.01**	-	**0.00**	**0.02**	0.57	**0.00**
9	Early therapy interrupted at 40 wks.	8 years of age	**0.00**	**0.00**	0.19	**0.00**	**0.00**	0.80	**0.00**	**0.00**	-	**0.00**	**0.00**	0.93
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.60	0.14	**0.00**	0.66	**0.03**	**0.00**	0.91	**0.02**	**0.00**	-	**0.03**	**0.00**
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	**0.01**	0.44	**0.00**	**0.04**	0.64	**0.00**	**0.02**	0.57	**0.00**	**0.03**	-	**0.00**
12	Early therapy interrupted at 90 wks.	8 years of age	**0.00**	**0.00**	0.27	**0.00**	**0.00**	0.77	**0.00**	**0.00**	0.93	**0.00**	**0.00**	-
Probabilities for Post Hoc Tests (sCD163)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.56	**0.00**	0.24	0.79	**0.00**	0.17	0.28	**0.00**	0.41	0.72	**0.00**
2	Delayed therapy	2 years after therapy initiation	0.56	-	**0.00**	0.13	0.46	**0.00**	0.10	0.15	**0.00**	0.23	0.89	**0.00**
3	Delayed therapy	8 years of age	**0.00**	**0.00**	-	**0.00**	**0.00**	0.40	**0.00**	**0.00**	0.73	**0.00**	**0.00**	0.81
4	Early Continuous therapy	Before therapy initiation	0.24	0.13	**0.00**	-	0.40	**0.00**	0.76	0.91	**0.00**	0.88	0.22	**0.00**
5	Early Continuous therapy	2 years after therapy initiation	0.79	0.46	**0.00**	0.40	-	**0.00**	0.30	0.47	**0.00**	0.58	0.59	**0.00**
6	Early Continuous therapy	8 years of age	**0.00**	**0.00**	0.40	**0.00**	**0.00**	-	**0.00**	**0.00**	0.51	**0.00**	**0.00**	0.23
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.17	0.10	**0.00**	0.76	0.30	**0.00**	-	0.66	**0.00**	0.69	0.17	**0.00**
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.28	0.15	**0.00**	0.91	0.47	**0.00**	0.66	-	**0.00**	0.95	0.25	**0.00**
9	Early therapy interrupted at 40 wks.	8 years of age	**0.00**	**0.00**	0.73	**0.00**	**0.00**	0.51	**0.00**	**0.00**	-	**0.00**	**0.00**	0.50

10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.41	0.23	**0.00**	0.88	0.58	**0.00**	0.69	0.95	**0.00**	-	0.32	**0.00**
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.72	0.89	**0.00**	0.22	0.59	**0.00**	0.17	0.25	**0.00**	0.32	-	**0.00**
12	Early therapy interrupted at 90 wks.	8 years of age	**0.00**	**0.00**	0.81	**0.00**	**0.00**	0.23	**0.00**	**0.00**	0.50	**0.00**	**0.00**	-
Probabilities for Post Hoc Tests (IL-18)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.43	**0.00**	0.21	0.74	**0.00**	0.22	0.11	**0.00**	0.15	0.85	**0.00**
2	Delayed therapy	2 years after therapy initiation	0.43	-	**0.00**	0.07	0.31	**0.00**	0.08	0.04	**0.00**	0.06	0.65	**0.00**
3	Delayed therapy	8 years of age	**0.00**	**0.00**	-	**0.00**	**0.00**	0.44	**0.00**	**0.00**	0.42	**0.01**	**0.00**	0.19
4	Early Continuous therapy	Before therapy initiation	0.21	0.07	**0.00**	-	0.41	**0.00**	0.94	0.72	**0.00**	0.67	0.27	**0.00**
5	Early Continuous therapy	2 years after therapy initiation	0.74	0.31	**0.00**	0.41	-	**0.00**	0.41	0.26	**0.00**	0.29	0.66	**0.00**
6	Early Continuous therapy	8 years of age	**0.00**	**0.00**	0.44	**0.00**	**0.00**	-	**0.00**	**0.00**	1.00	**0.00**	**0.00**	0.51
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.22	0.08	**0.00**	0.94	0.41	**0.00**	-	0.79	**0.00**	0.73	0.27	**0.00**
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.11	**0.04**	**0.00**	0.72	0.26	**0.00**	0.79	-	**0.00**	0.88	0.18	**0.00**
9	Early therapy interrupted at 40 wks.	8 years of age	**0.00**	**0.00**	0.42	**0.00**	**0.00**	1.00	**0.00**	**0.00**	-	**0.00**	**0.00**	0.48
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.15	0.06	**0.01**	0.67	0.29	**0.00**	0.73	0.88	**0.00**	-	0.19	**0.00**
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.85	0.65	**0.00**	0.27	0.66	**0.00**	0.27	0.18	**0.00**	0.19	-	**0.00**
12	Early therapy interrupted at 90 wks.	8 years of age	**0.00**	**0.00**	0.19	**0.00**	**0.00**	0.51	**0.00**	**0.00**	0.48	**0.00**	**0.00**	-
Probabilities for Post Hoc Tests (MIP-1α)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.45	0.14	0.19	0.27	0.07	0.90	0.34	0.22	0.46	0.47	0.47
2	Delayed therapy	2 years after therapy initiation	0.45	-	0.06	0.72	0.78	**0.03**	0.56	0.96	0.09	0.98	0.93	0.20
3	Delayed therapy	8 years of age	0.14	0.06	-	**0.01**	**0.02**	0.60	0.17	**0.02**	0.86	0.06	0.10	0.59

4	Early Continuous therapy	Before therapy initiation	0.19	0.72	**0.01**	-	0.96	**0.00**	0.23	0.67	**0.00**	0.66	0.82	**0.03**
5	Early Continuous therapy	2 years after therapy initiation	0.27	0.78	**0.02**	0.96	-	**0.00**	0.31	0.76	**0.01**	0.73	0.86	0.07
6	Early Continuous therapy	8 years of age	0.07	**0.03**	0.60	**0.00**	**0.00**	-	0.05	**0.00**	0.40	**0.01**	**0.04**	0.26
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.90	0.56	0.17	0.23	0.31	0.05	-	0.35	0.12	0.53	0.53	0.39
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.34	0.96	**0.02**	0.67	0.76	**0.00**	0.35	-	**0.00**	0.93	0.96	0.07
9	Early therapy interrupted at 40 wks.	8 years of age	0.22	0.09	0.86	**0.00**	**0.01**	0.40	0.12	**0.00**	-	0.05	0.10	0.65
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.46	0.98	0.06	0.66	0.73	**0.01**	0.53	0.93	0.05	-	0.90	0.09
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.47	0.93	0.10	0.82	0.86	**0.04**	0.53	0.96	0.10	0.90	-	0.16
12	Early therapy interrupted at 90 wks.	8 years of age	0.47	0.20	0.59	**0.03**	0.07	0.26	0.39	0.07	0.65	0.09	0.16	-

APPENDIX E: CHAPTER 5 RESULTS – PROBABILITY PARAMETERS (P-VALUES) FOR IL-3, MCP-1, TNFα, HSCRP AND LBP

Probabilities for Post Hoc Tests (IL-3)													
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.67	0.56	0.82	0.75	0.97	0.28	0.66	0.80	0.41	0.27	0.58
2	Delayed therapy	2 years after therapy initiation	0.67	-	0.36	0.79	0.51	0.66	0.19	0.92	0.51	0.78	0.19	1.00
3	Delayed therapy	8 years of age	0.56	0.36	-	0.38	0.84	0.47	0.56	0.26	0.66	0.16	0.47	0.23
4	Early Continuous therapy	Before therapy initiation	0.82	0.79	0.38	-	0.58	0.82	0.16	0.82	0.57	0.50	0.19	0.72
5	Early Continuous therapy	2 years after therapy initiation	0.75	0.51	0.84	0.58	-	0.69	0.48	0.45	0.89	0.28	0.41	0.40
6	Early Continuous therapy	8 years of age	0.97	0.66	0.47	0.82	0.69	-	0.19	0.62	0.71	0.37	0.22	0.54
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.28	0.19	0.56	0.16	0.48	0.19	-	0.10	0.28	0.07	0.78	0.09
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.66	0.92	0.26	0.82	0.45	0.62	0.10	-	0.38	0.62	0.14	0.88
9	Early therapy interrupted at 40 wks.	8 years of age	0.80	0.51	0.66	0.57	0.89	0.71	0.28	0.38	-	0.23	0.30	0.33
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.41	0.78	0.16	0.50	0.28	0.37	0.07	0.62	0.23	-	0.09	0.72
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.27	0.19	0.47	0.19	0.41	0.22	0.78	0.14	0.30	0.09	-	0.12
12	Early therapy interrupted at 90 wks.	8 years of age	0.58	1.00	0.23	0.72	0.40	0.54	0.09	0.88	0.33	0.72	0.12	S -
Probabilities for Post Hoc Tests (MCP-1)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.44	0.91	0.61	0.70	0.69	0.36	0.96	0.70	0.65	0.87	0.67
2	Delayed therapy	2 years after therapy initiation	0.44	-	0.47	0.25	0.31	0.28	0.96	0.43	0.27	0.29	0.47	0.28
3	Delayed therapy	8 years of age	0.91	0.47	-	0.51	0.62	0.59	0.38	0.87	0.59	0.58	0.81	0.58
4	Early Continuous therapy	Before therapy initiation	0.61	0.25	0.51	-	0.94	0.84	0.13	0.60	0.84	1.00	0.84	0.93

5	Early Continuous therapy	2 years after therapy initiation	0.70	0.31	0.62	0.94	-	0.94	0.20	0.70	0.92	0.95	0.89	0.99
6	Early Continuous therapy	8 years of age	0.69	0.28	0.59	0.84	0.94	-	0.14	0.69	0.97	0.89	0.92	0.95
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.36	0.96	0.38	0.13	0.20	0.14	-	0.24	0.10	0.19	0.38	0.16
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.96	0.43	0.87	0.60	0.70	0.69	0.24	-	0.65	0.66	0.89	0.67
9	Early therapy interrupted at 40 wks.	8 years of age	0.70	0.27	0.59	0.84	0.92	0.97	0.10	0.65	-	0.87	0.93	0.92
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.65	0.29	0.58	1.00	0.95	0.89	0.19	0.66	0.87	-	0.85	0.93
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.87	0.47	0.81	0.84	0.89	0.92	0.38	0.89	0.93	0.85	-	0.88
12	Early therapy interrupted at 90 wks.	8 years of age	0.67	0.28	0.58	0.93	0.99	0.95	0.16	0.67	0.92	0.93	0.88	-
Probabilities for Post Hoc Tests (TNF-α)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.81	0.21	0.65	0.57	0.46	0.59	0.83	0.27	0.75	0.42	0.39
2	Delayed therapy	2 years after therapy initiation	0.81	-	0.46	0.90	0.80	0.75	0.51	0.68	0.55	0.96	0.59	0.65
3	Delayed therapy	8 years of age	0.21	0.46	-	0.43	0.62	0.55	0.08	0.12	0.81	0.45	0.98	0.73
4	Early Continuous therapy	Before therapy initiation	0.65	0.90	0.43	-	0.85	0.77	0.30	0.45	0.49	0.94	0.60	0.66
5	Early Continuous therapy	2 years after therapy initiation	0.57	0.80	0.62	0.85	-	0.98	0.28	0.41	0.72	0.81	0.73	0.84
6	Early Continuous therapy	8 years of age	0.46	0.75	0.55	0.77	0.98	-	0.18	0.27	0.65	0.75	0.71	0.82
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.59	0.51	0.08	0.30	0.28	0.18	-	0.69	0.07	0.41	0.22	0.16
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.83	0.68	0.12	0.45	0.41	0.27	0.69	-	0.09	0.58	0.31	0.23
9	Early therapy interrupted at 40 wks.	8 years of age	0.27	0.55	0.81	0.49	0.72	0.65	0.07	0.09	-	0.52	0.90	0.87
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.75	0.96	0.45	0.94	0.81	0.75	0.41	0.58	0.52	-	0.58	0.62

11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.42	0.59	0.98	0.60	0.73	0.71	0.22	0.31	0.90	0.58	-	0.82
12	Early therapy interrupted at 90 wks.	8 years of age	0.39	0.65	0.73	0.66	0.84	0.82	0.16	0.23	0.87	0.62	0.82	-
Probabilities for Post Hoc Tests (hsCRP)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.91	0.61	0.91	0.91	0.36	0.63	0.37	0.76	0.80	0.47	0.65
2	Delayed therapy	2 years after therapy initiation	0.91	-	0.78	0.84	0.85	0.56	0.78	0.43	0.73	0.76	0.48	0.81
3	Delayed therapy	8 years of age	0.61	0.78	-	0.53	0.58	0.69	0.98	0.15	0.37	0.48	0.28	0.97
4	Early Continuous therapy	Before therapy initiation	0.91	0.84	0.53	-	0.99	0.23	0.53	0.39	0.84	0.87	0.50	0.54
5	Early Continuous therapy	2 years after therapy initiation	0.91	0.85	0.58	0.99	-	0.33	0.58	0.49	0.88	0.89	0.55	0.60
6	Early Continuous therapy	8 years of age	0.36	0.56	0.69	0.23	0.33	-	0.72	0.04	0.13	0.27	0.16	0.66
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.63	0.78	0.98	0.53	0.58	0.72	-	0.15	0.37	0.49	0.29	0.96
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.37	0.43	0.15	0.39	0.49	0.04	0.15	-	0.43	0.59	0.90	0.15
9	Early therapy interrupted at 40 wks.	8 years of age	0.76	0.73	0.37	0.84	0.88	0.13	0.37	0.43	-	0.99	0.56	0.38
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.80	0.76	0.48	0.87	0.89	0.27	0.49	0.59	0.99	-	0.62	0.48
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.47	0.48	0.28	0.50	0.55	0.16	0.29	0.90	0.56	0.62	-	0.27
12	Early therapy interrupted at 90 wks.	8 years of age	0.65	0.81	0.97	0.54	0.60	0.66	0.96	0.15	0.38	0.48	0.27	-
Probabilities for Post Hoc Tests (LBP)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.93	0.96	0.31	0.97	0.44	0.48	0.42	0.42	0.44	0.16	0.08
2	Delayed therapy	2 years after therapy initiation	0.93	-	0.90	0.48	0.96	0.49	0.51	0.48	0.48	0.47	0.19	0.14
3	Delayed therapy	8 years of age	0.96	0.90	-	0.28	0.94	0.45	0.49	0.44	0.44	0.46	0.17	0.08

4	Early Continuous therapy	Before therapy initiation	0.31	0.48	0.28	-	0.34	**0.02**	0.08	0.05	**0.03**	0.08	**0.03**	**0.00**
5	Early Continuous therapy	2 years after therapy initiation	0.97	0.96	0.94	0.34	-	0.41	0.48	0.44	0.44	0.45	0.17	0.10
6	Early Continuous therapy	8 years of age	0.44	0.49	0.45	**0.02**	0.41	-	0.97	0.96	0.99	0.88	0.34	0.24
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.48	0.51	0.49	0.08	0.48	0.97	-	0.99	0.96	0.92	0.39	0.33
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.42	0.48	0.44	0.05	0.44	0.96	0.99	-	0.94	0.92	0.37	0.28
9	Early therapy interrupted at 40 wks.	8 years of age	0.42	0.48	0.44	**0.03**	0.44	0.99	0.96	0.94	-	0.87	0.33	0.21
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.44	0.47	0.46	0.08	0.45	0.88	0.92	0.92	0.87	-	0.44	0.35
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.16	0.19	0.17	**0.03**	0.17	0.34	0.39	0.37	0.33	0.44	-	0.86
12	Early therapy interrupted at 90 wks.	8 years of age	0.08	0.14	0.08	**0.00**	0.10	0.24	0.33	0.28	0.21	0.35	0.86	-

APPENDIX F: CHAPTER 5 RESULTS - PROBABILITY PARAMETERS (P-VALUES) FOR IL-1RA, IL-5, IL-6 AND TNFβ

Probabilities for Post Hoc Tests (IL-1RA)													
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.21	0.43	0.91	0.08	0.08	0.73	0.17	0.40	0.70	0.86	0.99
2	Delayed therapy	2 years after therapy initiation	0.21	-	0.06	0.18	0.72	0.96	0.14	0.86	0.49	0.15	0.26	0.22
3	Delayed therapy	8 years of age	0.43	0.06	-	0.51	0.01	0.01	0.72	0.02	0.07	0.79	0.73	0.44
4	Early Continuous therapy	Before therapy initiation	0.91	0.18	0.51	-	**0.04**	**0.03**	0.79	0.10	0.28	0.76	0.92	0.90
5	Early Continuous therapy	2 years after therapy initiation	0.08	0.72	**0.01**	**0.04**	-	0.65	**0.04**	0.50	0.19	**0.04**	0.12	0.06
6	Early Continuous therapy	8 years of age	0.08	0.96	**0.01**	**0.03**	0.65	-	**0.03**	0.73	0.22	**0.04**	0.16	0.06
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.73	0.14	0.72	0.79	**0.04**	**0.03**	-	0.06	0.18	0.95	0.93	0.71
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.17	0.86	**0.02**	0.10	0.50	0.73	0.06	-	0.41	0.09	0.24	0.14
9	Early therapy interrupted at 40 wks.	8 years of age	0.40	0.49	0.07	0.28	0.19	0.22	0.18	0.41	-	0.22	0.44	0.36
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.70	0.15	0.79	0.76	**0.04**	**0.04**	0.95	0.09	0.22	-	0.89	0.66
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.86	0.26	0.73	0.92	0.12	0.16	0.93	0.24	0.44	0.89	-	0.85
12	Early therapy interrupted at 90 wks.	8 years of age	0.99	0.22	0.44	0.90	0.06	0.06	0.71	0.14	0.36	0.66	0.85	-
Probabilities for Post Hoc Tests (IL-5)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.40	0.06	0.94	0.13	0.45	0.37	0.20	0.96	0.87	0.97	0.52
2	Delayed therapy	2 years after therapy initiation	0.40	-	**0.02**	0.44	0.61	0.78	0.13	0.86	0.36	0.37	0.54	0.18
3	Delayed therapy	8 years of age	0.06	**0.02**	-	0.05	**0.00**	**0.01**	0.44	**0.00**	**0.04**	0.15	0.19	0.25
4	Early Continuous therapy	Before therapy initiation	0.94	0.44	0.05	-	0.10	0.41	0.30	0.19	0.88	0.81	0.98	0.44

5	Early Continuous therapy	2 years after therapy initiation	0.13	0.61	**0.00**	0.10	-	0.27	**0.02**	0.64	0.07	0.11	0.25	**0.03**
6	Early Continuous therapy	8 years of age	0.45	0.78	**0.01**	0.41	0.27	-	0.07	0.50	0.29	0.38	0.62	0.12
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.37	0.13	0.44	0.30	**0.02**	0.07	-	**0.02**	0.27	0.50	0.48	0.76
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.20	0.86	**0.00**	0.19	0.64	0.50	**0.02**	-	0.07	0.17	0.37	**0.04**
9	Early therapy interrupted at 40 wks.	8 years of age	0.96	0.36	**0.04**	0.88	0.07	0.29	0.27	0.07	-	0.89	0.94	0.46
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.87	0.37	0.15	0.81	0.11	0.38	0.50	0.17	0.89	-	0.87	0.64
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.97	0.54	0.19	0.98	0.25	0.62	0.48	0.37	0.94	0.87	-	0.59
12	Early therapy interrupted at 90 wks.	8 years of age	0.52	0.18	0.25	0.44	**0.03**	0.12	0.76	**0.04**	0.46	0.64	0.59	-
Probabilities for Post Hoc Tests (IL-6)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	**0.04**	0.33	0.25	**0.00**	0.13	0.94	**0.00**	0.26	0.93	0.18	0.18
2	Delayed therapy	2 years after therapy initiation	**0.04**	-	0.17	0.23	0.19	0.29	**0.04**	0.53	0.16	0.07	0.65	0.31
3	Delayed therapy	8 years of age	0.33	0.17	-	0.83	**0.00**	0.61	0.31	**0.01**	0.95	0.43	0.49	0.66
4	Early Continuous therapy	Before therapy initiation	0.25	0.23	0.83	-	**0.00**	0.77	0.22	**0.01**	0.85	0.32	0.58	0.82
5	Early Continuous therapy	2 years after therapy initiation	**0.00**	0.19	**0.00**	**0.00**	-	**0.00**	**0.00**	0.33	**0.00**	**0.00**	0.09	**0.00**
6	Early Continuous therapy	8 years of age	0.13	0.29	0.61	0.77	**0.00**	-	0.11	**0.01**	0.58	0.20	0.69	0.98
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.94	**0.04**	0.31	0.22	**0.00**	0.11	-	**0.00**	0.22	0.87	0.16	0.15
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	**0.00**	0.53	**0.01**	**0.01**	0.33	**0.01**	**0.00**	-	**0.00**	**0.00**	0.26	**0.02**
9	Early therapy interrupted at 40 wks.	8 years of age	0.26	0.16	0.95	0.85	**0.00**	0.58	0.22	**0.00**	-	0.35	0.48	0.65
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.93	0.07	0.43	0.32	**0.00**	0.20	0.87	**0.00**	0.35	-	0.22	0.23

11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.18	0.65	0.49	0.58	0.09	0.69	0.16	0.26	0.48	0.22	-	0.68
12	Early therapy interrupted at 90 wks.	8 years of age	0.18	0.31	0.66	0.82	**0.00**	0.98	0.15	**0.02**	0.65	0.23	0.68	-
Probabilities for Post Hoc Tests (TNFβ)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	**0.02**	0.09	0.21	**0.00**	**0.00**	0.81	**0.00**	**0.00**	0.65	0.73	**0.02**
2	Delayed therapy	2 years after therapy initiation	**0.02**	-	0.26	0.17	0.39	0.75	**0.04**	0.59	0.86	0.08	0.13	0.65
3	Delayed therapy	8 years of age	0.09	0.26	-	0.71	**0.02**	**0.04**	0.19	**0.03**	0.15	0.33	0.43	0.40
4	Early Continuous therapy	Before therapy initiation	0.21	0.17	0.71	-	**0.01**	**0.01**	0.33	**0.01**	0.06	0.50	0.58	0.22
5	Early Continuous therapy	2 years after therapy initiation	**0.00**	0.39	**0.02**	**0.01**	-	0.40	**0.00**	0.61	0.15	**0.00**	**0.01**	0.11
6	Early Continuous therapy	8 years of age	**0.00**	0.75	**0.04**	**0.01**	0.40	-	**0.00**	0.73	0.41	**0.01**	**0.03**	0.27
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.81	**0.04**	0.19	0.33	**0.00**	**0.00**	-	**0.00**	**0.00**	0.82	0.87	**0.04**
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	**0.00**	0.59	**0.03**	**0.01**	0.61	0.73	**0.00**	-	0.23	**0.00**	**0.02**	0.17
9	Early therapy interrupted at 40 wks.	8 years of age	**0.00**	0.86	0.15	0.06	0.15	0.41	**0.00**	0.23	-	**0.02**	0.08	0.65
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.65	0.08	0.33	0.50	**0.00**	**0.01**	0.82	**0.00**	**0.02**	-	0.98	0.07
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.73	0.13	0.43	0.58	**0.01**	**0.03**	0.87	**0.02**	0.08	0.98	-	0.15
12	Early therapy interrupted at 90 wks.	8 years of age	**0.02**	0.65	0.40	0.22	0.11	0.27	**0.04**	0.17	0.65	0.07	0.15	-

APPENDIX G: CHAPTER 5 RESULTS - PROBABILITY PARAMETERS (P-VALUES) FOR IL-4, IL-10, IP-10 and RANTES

Probabilities for Post Hoc Tests (IL-4)													
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.73	**0.02**	0.95	0.13	0.66	0.32	0.21	0.21	0.81	0.08	0.17
2	Delayed therapy	2 years after therapy initiation	0.73	-	**0.03**	0.77	0.33	0.99	0.62	0.51	0.18	0.92	0.07	0.15
3	Delayed therapy	8 years of age	**0.02**	**0.03**	-	**0.02**	**0.00**	**0.00**	**0.00**	**0.00**	0.20	0.02	0.83	0.40
4	Early Continuous therapy	Before therapy initiation	0.95	0.77	**0.02**	-	0.10	0.65	0.31	0.19	0.13	0.84	0.06	0.12
5	Early Continuous therapy	2 years after therapy initiation	0.13	0.33	**0.00**	0.10	-	0.15	0.56	0.62	**0.00**	0.22	**0.00**	**0.00**
6	Early Continuous therapy	8 years of age	0.66	0.99	**0.00**	0.65	0.15	-	0.47	0.31	**0.03**	0.90	**0.03**	**0.04**
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.32	0.62	**0.00**	0.31	0.56	0.47	-	0.87	**0.01**	0.48	**0.01**	**0.02**
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.21	0.51	**0.00**	0.19	0.62	0.31	0.87	-	**0.00**	0.37	**0.01**	**0.00**
9	Early therapy interrupted at 40 wks.	8 years of age	0.21	0.18	0.20	0.13	**0.00**	**0.03**	**0.01**	**0.00**	-	0.15	0.29	0.76
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.81	0.92	0.02	0.84	0.22	0.90	0.48	0.37	0.15	-	0.06	0.09
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.08	0.07	0.83	0.06	**0.00**	**0.03**	**0.01**	**0.01**	0.29	0.06	-	0.40
12	Early therapy interrupted at 90 wks.	8 years of age	0.17	0.15	0.40	0.12	**0.00**	**0.04**	**0.02**	**0.00**	0.76	0.09	0.40	-
Probabilities for Post Hoc Tests (IL-10)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.77	**0.01**	0.14	0.68	**0.04**	**0.04**	0.52	**0.00**	0.27	0.99	0.01
2	Delayed therapy	2 years after therapy initiation	0.77	-	**0.01**	0.38	0.95	0.06	0.17	0.85	**0.01**	0.53	0.83	**0.02**
3	Delayed therapy	8 years of age	**0.01**	**0.01**	-	**0.00**	**0.00**	0.26	**0.00**	**0.00**	1.00	**0.00**	**0.04**	0.81
4	Early Continuous therapy	Before therapy initiation	0.14	0.38	**0.00**	-	0.35	**0.00**	0.47	0.33	**0.00**	0.83	0.29	**0.00**

5	Early Continuous therapy	2 years after therapy initiation	0.68	0.95	**0.00**	0.35	-	**0.02**	0.13	0.89	**0.00**	0.52	0.76	**0.00**
6	Early Continuous therapy	8 years of age	**0.04**	0.06	0.26	**0.00**	**0.02**	-	**0.00**	**0.00**	0.17	**0.00**	0.14	0.39
7	Early therapy interrupted at 40 wks.	Before therapy initiation	**0.04**	0.17	**0.00**	0.47	0.13	**0.00**	-	0.11	**0.00**	0.41	0.13	**0.00**
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.52	0.85	**0.00**	0.33	0.89	**0.00**	0.11	-	**0.00**	0.55	0.66	**0.00**
9	Early therapy interrupted at 40 wks.	8 years of age	**0.00**	**0.01**	1.00	**0.00**	**0.00**	0.17	**0.00**	**0.00**	-	**0.00**	**0.03**	0.77
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.27	0.53	**0.00**	0.83	0.52	**0.00**	0.41	0.55	**0.00**	-	0.41	**0.00**
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.99	0.83	**0.04**	0.29	0.76	0.14	0.13	0.66	**0.03**	0.41	-	0.05
12	Early therapy interrupted at 90 wks.	8 years of age	**0.01**	**0.02**	0.81	**0.00**	**0.00**	0.39	**0.00**	**0.00**	0.77	**0.00**	0.05	-

Probabilities for Post Hoc Tests (IP-10)

	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.56	**0.01**	0.43	0.06	**0.01**	0.10	0.23	**0.01**	0.13	0.09	0.05
2	Delayed therapy	2 years after therapy initiation	0.56	-	0.12	0.23	0.29	0.16	0.06	0.72	0.15	0.08	0.29	0.33
3	Delayed therapy	8 years of age	**0.01**	0.12	-	**0.00**	0.67	0.78	**0.00**	0.10	0.78	**0.00**	0.89	0.48
4	Early Continuous therapy	Before therapy initiation	0.43	0.23	**0.00**	-	**0.00**	**0.00**	0.33	**0.03**	**0.00**	0.39	**0.02**	**0.00**
5	Early Continuous therapy	2 years after therapy initiation	0.06	0.29	0.67	**0.00**	-	0.81	**0.00**	0.34	0.81	**0.00**	0.85	0.85
6	Early Continuous therapy	8 years of age	**0.01**	0.16	0.78	**0.00**	0.81	-	**0.00**	0.13	1.00	**0.00**	0.97	0.61
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.10	0.06	**0.00**	0.33	**0.00**	**0.00**	-	**0.00**	**0.00**	0.97	**0.00**	**0.00**
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.23	0.72	0.10	**0.03**	0.34	0.13	**0.00**	-	0.09	**0.01**	0.34	0.37
9	Early therapy interrupted at 40 wks.	8 years of age	**0.01**	0.15	0.78	**0.00**	0.81	1.00	**0.00**	0.09	-	**0.00**	0.97	0.59
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.13	0.08	**0.00**	0.39	**0.00**	**0.00**	0.97	**0.01**	**0.00**	-	**0.00**	**0.00**

11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.09	0.29	0.89	**0.02**	0.85	0.97	**0.00**	0.34	0.97	**0.00**	-	0.72
12	Early therapy interrupted at 90 wks.	8 years of age	0.05	0.33	0.48	**0.00**	0.85	0.61	**0.00**	0.37	0.59	**0.00**	0.72	-
Probabilities for Post Hoc Tests (RANTES)														
	Therapy Grouping	**Therapy time point status at sample date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1	Delayed therapy	Before therapy initiation	-	0.39	**0.00**	0.49	**0.01**	**0.00**	0.08	0.23	**0.00**	0.18	0.06	**0.00**
2	Delayed therapy	2 years after therapy initiation	0.39	-	**0.00**	0.75	0.12	**0.00**	0.52	0.95	**0.00**	0.74	0.30	**0.00**
3	Delayed therapy	8 years of age	**0.00**	**0.00**	-	**0.00**	**0.00**	0.47	**0.00**	**0.00**	0.65	**0.00**	**0.00**	0.69
4	Early Continuous therapy	Before therapy initiation	0.49	0.75	**0.00**	-	**0.01**	**0.00**	0.21	0.59	**0.00**	0.42	0.13	**0.00**
5	Early Continuous therapy	2 years after therapy initiation	**0.01**	0.12	**0.00**	**0.01**	-	**0.00**	0.28	0.05	**0.00**	0.17	0.75	**0.00**
6	Early Continuous therapy	8 years of age	**0.00**	**0.00**	0.47	**0.00**	**0.00**	-	**0.00**	**0.00**	0.16	**0.00**	**0.00**	0.24
7	Early therapy interrupted at 40 wks.	Before therapy initiation	0.08	0.52	**0.00**	0.21	0.28	**0.00**	-	0.41	**0.00**	0.73	0.58	**0.00**
8	Early therapy interrupted at 40 wks.	2 years after therapy initiation	0.23	0.95	**0.00**	0.59	0.05	**0.00**	0.41	-	**0.00**	0.72	0.24	**0.00**
9	Early therapy interrupted at 40 wks.	8 years of age	**0.00**	**0.00**	0.65	**0.00**	**0.00**	0.16	**0.00**	**0.00**	-	**0.00**	**0.00**	1.00
10	Early therapy interrupted at 90 wks.	Before therapy initiation	0.18	0.74	**0.00**	0.42	0.17	**0.00**	0.73	0.72	**0.00**	-	0.41	**0.00**
11	Early therapy interrupted at 90 wks.	2 years after therapy initiation	0.06	0.30	**0.00**	0.13	0.75	**0.00**	0.58	0.24	**0.00**	0.41	-	**0.00**
12	Early therapy interrupted at 90 wks.	8 years of age	**0.00**	**0.00**	0.69	**0.00**	**0.00**	0.24	**0.00**	**0.00**	1.00	**0.00**	**0.00**	-