The association of maternal HIV status during pregnancy on longitudinal neuro-immune regulation, and neurodevelopment in HIV-exposed uninfected children.

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DECLARATION

I, Tatum Sevenoaks, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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ABSTRACT

Introduction: It has long been established that the Human Immunodeficiency Virus (HIV) and its effects, such as its impact on the immune system and then the numerous consequences on other biological systems including the central nervous system (CNS), have had a significant effect worldwide. This is particularly relevant in South Africa, where the prevalence of adults living with HIV remains high. However, with the improved access to antiretroviral therapy (ART), more children are now being born uninfected with HIV while still being exposed to the virus *in utero*. Exposure to HIV *in utero* may still negatively affect the developing brain of these children. However, the biological mechanisms involved in the neurodevelopmental outcomes in HIV-exposed uninfected (HEU) children are still largely unknown. Evidence from clinical studies showed that HEU children have an altered immune regulation compared to their unexposed counterparts, and this is hypothesised to play a role in neurodevelopmental outcomes in HEU children. The aim of this study was to evaluate the longitudinal relationship between the inflammatory environment of pregnant mothers living with HIV on ART and their children and the association of the inflammatory environment with neurodevelopmental outcomes in HEU children.

Methods: This study was performed in a sub-sample of the Drakenstein Child Health Study (DCHS), a South African birth cohort of 1137 mother-infant pairs. This sub-study included mothers at ≈26 weeks gestation (n=267), their infants at 6-10 weeks (n=222) and children at 24-28 months (n=267). Maternal HIV status was determined at ≈26 weeks gestation. This sub-study included n=190 HIV negative mother-infant pairs and n=77 HIV positive mother-infant pairs. Serum inflammatory markers (Granulocyte-macrophage colony-stimulating factor (GM CSF), Interferon-γ (IFN-γ), Interleukin IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, tumour necrosis factor-α (TNF-α), neutrophil gelatinase-associated lipocalin (NGAL/Lcn2) and metalloproteinase-9 (MMP-9)) were

analyzed in all study participants with a multiplex bead array and ELISA. The Bayley Scales of Infant and Toddler Development (Bayley-III) were used to assess the neurodevelopmental domains: cognitive, motor, language, social-emotional behaviour and adaptive behaviour at 24-28 months of age.

Results: Mothers living with HIV on ART had significantly lower levels of the inflammatory markers GM-CSF and MMP9 compared to mothers without HIV. Serum levels of inflammatory markers IFN-γ and IL-1β were significantly lower in HEU infants at 6-10 weeks compared to HIV-unexposed uninfected (HUU) infants. At 24-28 months of age, HEU children also proved to have significantly lower serum levels of the inflammatory markers IFN-γ, IL-1β, IL-2 and IL-4 compared with HUU children. Increased levels of the inflammatory markers; GM-CSF, IFN-γ, IL-10, IL-12p70, IL-1β, IL-2, IL-4, IL-6 and Lcn2 in HEU infants at 6-10 weeks of age was associated with impaired motor neurodevelopment at 24-28 months of age.

Conclusion: This is the first study to evaluate the longitudinal associations of immune markers with neurodevelopment in HEU children. The results show that maternal HIV infection was associated with lower levels of inflammatory markers in mothers and their children. Our results further indicate that an altered immune system in HEU infants, specifically at the earliest stages of life, was associated with impaired motor function at 2 years of age. These findings may provide further insights into the involvement of immune regulation, linking maternal HIV status and neurodevelopment in South African HEU children.

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LIST OF ABBREVIATIONS

ACTH Adrenocorticotropic hormone

ART Antiretroviral therapy

ARV Antiretroviral

BINS Bayley Infant Neurodevelopmental Screener

Bayley-III Bayley Scales of Infant and Toddler Development

CMV Cytomegalovirus

CNS Central Nervous System

CRH Corticotropin-releasing hormone

CRP C-reactive protein

DASII Development Assessment scale for Indian Infants

DCHS Drakenstein Child Health Study

DTI Diffusion Tensor Imaging

FcRN Neonatal Fc Receptor

FSDQ Full-Scale Developmental Quotient

GP120 Envelope glycoprotein

GM-CSF Granulocyte-macrophage colony-stimulating factor

HAD HIV Associated Dementia

HAND HIV Associated Neurocognitive Disorders

HIV Human Immunodeficiency Virus

HEU HIV-exposed uninfected

HPA Hypothalamic-pituitary adrenal

HUU HIV-unexposed uninfected

IFN Interferon

IgG Immunoglobulin G

IL Interleukin

Lcn Lipocalin

MIA Maternal immune activation

MMP9 Metalloproteinase-9

MRI Magnetic resonance imaging

NGAL neutrophil gelatinase-associated lipocalin

PMTCT Prevention of mother to child transmission

SASH South African Stress and Health Study

TNF Tumor Necrosis Factor

SYN Syncytiotrophoblasts

ZDV Zidovudine

INTRODUCTION

It has long been established that the Human Immunodeficiency Virus (HIV) and its effects, such as its impact on the immune system and the numerous consequences on other biological systems including the central nervous system (CNS), have had a significant impact worldwide. This is relevant particularly in South Africa, where the prevalence rate of adults living with HIV is still at 18.9% (1). Globally South Africa has the biggest and most high-profile HIV epidemic, with an estimated 7.2 million people living with HIV in 2017 (1). However, with the increased use of antiretroviral therapy (ART) more children are now born uninfected with HIV but still being exposed *in utero* to the virus. In 2018, UNAIDS reported that 4.4 million people were receiving treatment in South Africa; this is more than half (61%) of individuals infected with HIV (2). HIV infection amongst South African children between the ages of 0 and 4 has declined from 26,000 in 2010 to 13,000 in 2017 (1). This is primarily due to prevention of mother to child transmission (PMTCT) programs; where the rate of transmission is 1.3% in 2017, which is down from 3.6% in 2011 (3), as well as the fact that there is an estimated 53 000 children in South Africa who have averted infection with HIV due to PMTCT, compared to only 18 000 children in 2007 (2). With the decline in children living with HIV there is now a drastic increase in the number of children between the ages of 0 and 14 who are exposed but uninfected with HIV. However, exposure *in utero* may still negatively affect the health of these children. Numerous studies have so far been conducted to evaluate the effect of HIV exposure *in utero* on these children including assessing their neurodevelopmental outcome (4). Unfortunately, the biological mechanisms involved in the neurodevelopmental outcomes in HIV-exposed uninfected (HEU) children are still largely unknown.

Recently the role of the immune system in neurodevelopment and neuropsychiatric disorders has become a growing focus in scientific research. An increasing number of studies suggest that maternal immune activation (MIA) and increased levels of certain inflammatory markers during pregnancy can contribute to impaired brain development of

the foetus (5–11). Moreover, studies have shown that increased levels of inflammatory cytokines, such as Interleukin-6 (IL-6), IL-1β and Tumour necrosis factor-α (TNF-α) are associated with numerous neuropsychiatric disorders, such as schizophrenia and autism spectrum disorder (12–15). It is well known that HIV has a significant impact on the immune system. Therefore, the relationship between this infamous disease and its potential effect on neurodevelopment via the immune system is a crucial area of investigation.

HIV has a significant impact on the immune system of the mother during pregnancy; this in turn may impact the development of the foetus. According to studies previously conducted, HIV impacts the immune system by creating a pro-inflammatory environment (16,17), which is characterised predominantly by increased levels of certain cytokines, such as IL-1, IL-6 and TNF-α, and other inflammatory markers, such as metalloproteinase 9 (MMP9) and lipocalin 2 (Lcn2). Peripheral immune activation during pregnancy is a normal occurrence at certain stages of the pregnancy such as in the first and third trimester (18,19). However, with HIV infection inflammatory markers are increased even further (20). This is supported in a study by Richardson *et al* (21), who reported that a number of cytokines including IL-1, IL-4, IL-8, IL-10, IFN-γ and TNF-α were increased in pregnant women living with HIV compared to uninfected pregnant women. Many of the important inflammatory cytokines that play a role in immune regulation during pregnancy have also shown to be deregulated with HIV infection. These particular cytokines go on to contribute to the elevated inflammatory environment expressed by the mothers. Furthermore, whether or not the immune system of the children is similar to that of their mothers is a critical area for further exploration, including potential mechanisms that underlie the concept of "intergenerational transmission". Additionally, the transfer of inflammatory markers from the pregnant mother to the foetus is also poorly understood; it is uncertain if specific cytokines transfer directly through the placenta to the developing foetus or whether the mothers' immune system "primes" the immune system of the

foetus. In addition, whether or not the altered immune system of the foetus persists once the child is born and begins to age is also uncertain. Studies conducted so far, which evaluate the immune system of HEU, express the vulnerability of their immune system (22). A comprehensive review by Abu-Raya *et al* indicates that HEU children have an increase in innate immune cytokine production (23).

A number of studies have shown that MIA has an impact on the neurodevelopment of the foetus through increased levels of inflammatory cytokines (5–7,10); and may in turn lead to the development of neuropsychiatric disorders (9). The cause behind MIA in these studies varied, however the fact that there is a resulting increased level of certain inflammatory markers allows the connection between HIV, the immune system and neurodevelopment to be made.

A recent meta-analysis by McHenry *et al* (4), which included 45 studies assessing neurodevelopment in HEU children, found that HEU children did have lower cognitive and motor scores than their HUU counterparts. However, many of the studies are limited to the evaluation of cognitive abilities of the children at a very young age, therefore whether these cognitive deficits persist later on in life is still unknown.

Given the gaps outlined in the literature, the aims of the study are as follows: firstly, to investigate the impact of HIV on the inflammatory environment of the mothers during pregnancy. Secondly, to investigate the inflammatory environment of HEU children. Lastly, to assess whether adaptations in the inflammatory environment of the mothers and children is associated with impaired neurodevelopment of the children. Mother-infant pairs present within the Drakenstein Child Health Study (DCHS), the parent study, will aid in achieving these aims (24,25).

The significance of the current study lies in the gap in knowledge of the link between maternal HIV infection, the inflammatory environment of both the mother and infant and the neurodevelopment of the HEU children. By identifying changes in particular inflammatory markers as a result of HIV exposure and determining how these inflammatory markers play a role in neurodevelopment it may be possible to mitigate these neurodevelopmental disorders. Particularly in South Africa, where HIV is still rife and the numbers of HEU children are increasing, the demand to ensure that these children do not become susceptible to neurodevelopmental disorders in the future is critical.

The thesis will comprise four further chapters, as follows:
The first chapter will contain a literature review, where the impact of HIV on the immune system will be assessed, in particular the inflammatory environment, of both the mothers during pregnancy and the children. Following that, the impact of MIA on the neurodevelopment of the children will be examined, as well as the impact of HIV on the neurodevelopment of the children. The chapter will conclude with an outline of the gaps present within the literature and the hypotheses of the study.

The second chapter describes the methods and materials used in this study. This section includes the characteristics of the study population, the recruitment criteria, the study design, research procedures and data collection. An outline of the statistical procedures used for analysing the results will also be included as well as an ethical statement.

The third chapter will outline the results of the study. The fourth and final chapter will be a discussion to summarise these results and place them into context with the themes covered in the literature review. In addition, limitations will be discussed and future research prospects will be suggested, which could build on this study and our understanding of the link between HIV, the immune system and neurodevelopment in HEU children.

1. LITERATURE REVIEW

This literature review aims to highlight the gaps of knowledge in scientific research regarding the impact of HIV on the inflammatory environment of pregnant mothers and their children, as well as the association between adaptations in the inflammatory environment with the neurodevelopment of the children.

1.1 The impact of HIV on the inflammatory environment of the mother during pregnancy

The immune system plays a critical role during pregnancy and the development of the foetus *in utero.* This section aims to assess the impact that HIV has on serum levels of inflammatory markers during pregnancy and how this consequently impacts the immune system of the foetus through intergenerational transmission.

Immune regulation during healthy pregnancy without comorbidities

The first trimester is a crucial period that oversees fundamental elements of both placental and foetal development; the placenta provides a powerful barrier protecting the foetus from infectious pathogens. The placenta is made up of chorionic villi (foetal capillaries and villous stroma) and trophoblast cells (26). The villi are covered with multinucleated fused syncytiotrophoblasts (SYN), which make direct contact with maternal blood (27). When SYNs are differentiated they become highly resistant to viral infection (28). The mothers' immune system provides further protection to the foetus; SYNs express neonatal Fc receptors (FcRn) that transfer maternal immunoglobulin G (IgG) antibodies to the foetus (29).

It has been shown that HIV affects the transfer of antibodies to the foetus. For example, if the mother has increased serum IgG levels, placental transfer of antibodies is impaired (30,31).

Trophoblast cells are immune cells within the placenta responsible for secreting cytokines. Along with other immune cells, cytokines secreted by the trophoblast cells in the placenta play a role in regulating the immune response during pregnancy. Cytokines are multifunctional pleiotropic proteins, which play a critical role in cell-to-cell communication and activation in a variety of physiological and pathological processes, particularly in regards to the immune response (32). During healthy pregnancy without comorbidities, the first trimester is postulated to provide a pro-inflammatory response in the mother as her body struggles to adapt to the presence of the foetus; however the second trimester highlights the induction of an anti-inflammatory state (33,34). Renewed inflammation occurs in the third trimester as the mother prepares to deliver the baby. Pro-inflammatory cytokines, including Interleukin-6 (IL-6) and Tumour Necrosis Factor-α (TNF-α) are found to be elevated during the onset of labour, however these same cytokines in excess can detriment the labour process (19). Anti-inflammatory cytokines such as IL-10 and IL-4 are also modulated in healthy pregnancy and have shown to be decreased as a result of maternal infection consequentially causing adverse pregnancy outcomes (34).

Role of inflammatory markers in HIV infection

Certain cytokines released by trophoblasts have the ability to up-regulate HIV expression and replication in infected cells (26). A high level of HIV transcripts is associated with elevated expression of inflammatory cytokines (26,35). HIV infection has also been associated with an increased risk for placental membrane inflammatory lesions (36). Studies conducted so far have predominantly focused on three pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α, during pregnancy (21,35,37). These studies have expressed that these pro-inflammatory cytokines are significantly involved in immune regulation during pregnancy and are deregulated with HIV infection and in fact can enhance HIV replication (35). It is important to

note that a chronic inflammatory state present with HIV infection persists even when treated successfully with ART in the majority of studies conducted so far (17). An increase in inflammatory cytokines during pregnancy, which in turn is exacerbated due to infection with HIV, may impact the developing foetus.

The pro-inflammatory cytokine TNF-α plays a critical role in pregnancy; it is associated with the inflammatory mechanisms that underlie implantation, placentation and pregnancy outcome as a whole (18). An increase in TNF-α has been associated with numerous adverse pregnancy outcomes including miscarriages, pre-eclampsia and preterm birth (18). Studies have shown that TNF-α is significantly increased during pregnancy in mothers living with HIV on ART who were both virologically unsuppressed (35,38) and suppressed (39). Another study conducted by Mikyas *et al* (20), reported that serum levels of TNF-α were significantly elevated during all time points throughout the three trimesters in pregnant women living with HIV with 67% of women treated with ART in comparison to uninfected pregnant women. It is unclear in this study whether these women were virologically suppressed.

Elevated levels of IL-6 are observed in many chronic inflammatory and autoimmune disorders, including HIV, and often serves as a marker for the systemic activation of pro-inflammatory cytokines. IL-6 along with TNF-α has also been shown to be increased during healthy pregnancy (40), and then furthermore with HIV infection (35,41). IL-1β is another pro-inflammatory cytokine that has shown to be increased in placental cells of mothers living with HIV not treated with ART who were both virologically suppressed and unsuppressed, whereas secretion of IL-1β by trophoblast cells in uninfected mothers remained low and stable (41). Other cytokines that have shown to be increased inpregnant mothers living with HIV on ART who were virologically suppressed are IL-8 and interferon-γ (IFN-γ) (21,39).

Contrastingly, a study by Maharaj *et al* (42), evaluating cytokine levels in pregnant women living with HIV and uninfected pregnant women with and without pre-eclampsia reported lower levels of the inflammatory cytokines TNF-α, IL-2 and IL-6 in mothers living with HIV compared with uninfected mothers. The women living with HIV were all treated with highly active antiretroviral therapy (HAART), which may have had an impact on the results. A study by Moussa *et al* (37), also found decreased levels of IL-6 in mothers living with HIV on ART. It is unclear in this study whether mothers were virologically suppressed.

Further, pregnancy also has an effect on the pathology of HIV infection, for example a study by Sutton *et al* (43), revealed that pregnancy down-regulates the production of IL-2 in pregnant mothers living with HIV on ART who were virologically unsuppressed compared to mothers who are uninfected. This was not seen in the comparison of non-pregnant individuals living with HIV. The anti-inflammatory cytokine, IL-10, has been shown to be secreted at higher concentrations in mothers living with HIV by Hofbauer cells and additionally has the ability to inhibit HIV replication *in vitro* (44). Notably, many of the studies in this section differ in regards to whether or not the mothers were treated with ART and whether they were virologically suppressed. These factors most likely impact the inflammatory environment of the mothers and outline a gap in the literature. Another factor to take into consideration is that the majority of the studies assessed have been conducted in western countries; this is important, as different countries possess different subtypes or clades of HIV infection, which can present different inflammatory profiles (45). The predominant subtype in South Africa is Clade C (46), which tends to present a more anti-inflammatory profile (47,48).

Intergenerational transmission

The transfer of inflammatory mediators to the foetus is less well understood. Intergenerational transmission refers to the concept that stress exposure in the mother has a direct impact on the children via changes to gametes and the gestational uterine environment (49). "Stress" can encompass numerous examples including HIV infection. The changes imposed on the gametes and the gestational uterine environment involves changes to the immune system and in the case of HIV result in an elevated inflammatory environment (44). Penetration of HIV viral particles, such as envelope glycoprotein (GP120), across the placental barrier even in the absence of foetal infection has been supported with experimental results (50,51). A review by Abu-Raya *et al* substantiates the alteration that HIV has on the transfer of maternal immune factors to the infants and underlies the importance of investigating further the mechanisms behind this (52).

Alterations that HIV poses on the transfer of maternal immune factors include immune activation in the pregnant mother living with HIV and its association with the production of inflammatory cytokines at the maternofoetal interface, which may lead to inflammatory responses in the infant (53). An extensive review of the placenta by Pasca and Penn posits that the placenta has the ability to produce its own array of cytokines that have the ability to enter the foetal circulation and thereby modulate foetal neurodevelopment (54). Morelli *et al* hypothesised that exposure of the foetus *in utero* to inflammatory cytokines, elicited by the mothers immune response, programs the immune system of the foetus; resulting in a "pro-inflammatory phenotype" that may persist into adulthood and further be exacerbated by any sub-sequential exposure to an immune stimulus (10). Kakkar *et al* also puts forward this "two-hit" hypothesis, which elucidates that HEU infants may have an aberrant developmental outcome as a result of both a transferred state of immunodeficiency and a decreased placental transfer of protective

maternal antibodies due to HIV exposure *in utero* (55). This underlines the need to explore the extent to which the mothers' immune system influences and predicts the immune system of their children and how this impacts their development.

However, it is important to note that there are many other aspects that determine the outcome of the children's immune system, including maternal antibodies, maternal diet and micronutrients and the maternal microbiome, to name a few (29). It is important to note that priming of foetal development *in utero* may in turn lead the children to respond inappropriately to the environment once born due to the fact that it is different to the environment for which it was prepared for *in utero* (8). Miyamoto *et al* suggests that the first postnatal year of life is the key period for programming the immune system of the children (56). This is supported by Prendergast *et al,* which stated that HEU infants have a sustained inflammatory phenotype until at least 6 months of age (57). A study conducted by Zaretsky *et al* reported that there is a bidirectional transfer of IL-6 across the placenta, indicating that the immune response of the mother does transfer to the foetus (58). Borges-Almeida *et al* also indicated a high correlation of cytokine concentrations between the HEU infants and the mothers (59).

On the other hand, a study by Aaltonen *et al* reported no transfer of cytokines from the mother to the foetus or vice versa, but did find an endogenous release of IL-6 believed to be of foetal origin (60). Furthermore, a study by Lopez *et al* showed that there was no difference in foetal levels of IL-6 from mothers both living with HIV and uninfected, suggesting that an increase in inflammatory markers in women living with HIV may be an immune response isolated to the mother (61). Ultimately, the exact mechanisms behind the relationship of the mothers' immune system to that of the foetus need further investigation.

1.2 The inflammatory environment of HIV-exposed uninfected (HEU) children

Although HEU children are considered in the most part to be healthy, more research is proving that exposure to HIV *in utero* has an effect on their immune system (62), as hypothesised above through the concept of intergenerational transmission. Numerous studies so far have shown that there is an alteration in the immune system of the HEU children, particularly in the immune cell subsets, CD4 and CD8, which have both been shown to be decreased in HEU infants (55,63,64). HIV exposure during pregnancy has also been associated with quantitative and qualitative changes in dendritic cells, which could also play a role in the abnormally increased T-cell activation (51). In addition, HEU children also show phenotypical differences; expressing a more antigen-experienced cellular phenotype, with expanded memory T-cell subsets and heightened immune activation with increased apoptosis (22). Despite numerous studies assessing the immune cell subsets in HEU children, fewer studies have investigated inflammatory markers, highlighting a gap in the literature.

Expression of inflammatory markers in HEU compared to HUU children

A study by Reikie *et al* that looked at cytokine production in monocytes stimulated with pathogen associated molecular patterns (PAMP) reported an enhanced pro-inflammatory response of monocytes in HEU infants versus HUU infants; mononuclear cells from HEU infants produced more cytokine on a per-cell basis compared to their HUU counterparts; IL-12, IL-6 and TNF-α were all elevated in HEU infants (65). However, by 12 months of age any difference in reactivity between HEU and HUU mononuclear cells had disappeared completely (65). Contrastingly, Chougnet *et al* reported decreased levels of IL-12 in cord blood of HEU infants from mothers who were virologically unsuppressed compared to HUU infants (66). Cord blood IL-12 was correlated with maternal IL-12 implying that exposure to HIV *in utero* alters the quality of T-cell response and impacts foetal

immune development (66). Notably, mothers in these studies were treated with ART.

Significantly increased levels of IL-8 and IL-1β were detected in the plasma of HEU infants with virologically suppressed mothers compared with HUU infants in a study conducted in the Netherlands by Bunders *et al* (62). Increased expression of inflammatory response-related chemokine receptors in HEU infants were also accounted for in the same study (62). A study by Miyamoto *et al* reported significantly increased levels of IL-4 in HEU children between the ages of 6 and 12 years with mothers who were virologically unsuppressed; and therefore concluded that HEU children are not significantly immune compromised (56). Another study by Borges-Almeida *et al*, however, reported decreased levels of IL-4 in HEU infants with mothers who were virologically unsuppressed (59). Increased levels of IFN-γ were also found in the same study. Similarly a study by Hygino *et al* also revealed significantly increased levels of IFN-γ as well as TNF-α in the cord blood of HEU infants with mothers who were both virologically suppressed and unsuppressed compared with HUU infants, they also found reduced levels of IL-10 in the HEU infants (67). Contrastingly, a study by Kuhn *et al* found significantly increased levels of IL-10 in HEU infants; although increased levels subsided by 6 months of age, however mothers in this study were not treated with ART unlike in the other studies mentioned (68).

A study by Clerici *et al* showed that HEU infants expressed significantly increased levels of IL-7 compared to HUU infants at birth and later on, suggesting that changes in the immune system of HEU infants persist later on in life (50). Prendergast *et al* did not find a significant difference between cytokine levels of HEU and HUU infants but did find increased levels of C-reactive protein (CRP) in HEU infants, indicating that they still possess an elevated inflammatory

environment, mothers in this study were not on ART and were virologically suppressed (57). Additionally, peripheral blood cells of HEU infants were reported to proliferate and produce IL-2 after exposure to HIV (69). The variation in findings between the studies may be due to a number of factors including a difference in the age of the infants/children, their socioeconomic background, the subtype of HIV to which they were exposed, maternal HIV load, ART exposure, maternal microbiome and whether they had any other infections as well as differences in the number of participants in the relative studies. The variation in findings from the different studies included further indicates the complexity of the immune system in relation to HIV exposure.

Mortality and morbidity in HEU children

A number of studies evaluated the mortality and morbidity of HEU children and found that they do have an increased mortality rate and are more likely to contract infections (70–72). For example, Slogrove *et al* reported that twice as many HEU infants had at least one infection associated with hospitalization in the first year of life compared to HUU infants (71). Studies by Evans *et al* also support an increase in mortality and morbidity in HEU children compared with HUU children suggesting an impaired ability to control the infection (72,73). Furthermore, one study, which looked at Cytomegalovirus (CMV) acquisition and inflammation in HEU children, also reported a pro-inflammatory environment and higher CMV load in HEU children and an overall increase in mortality (73). Dirajilal-Fargo *et al*, also expressed that increased IL-6 at birth may contribute to increased HEU morbidity (74). On the other hand, studies have shown that inflammatory markers were not associated with mortality and morbidity in HEU children (57). The majority of studies evaluating mortality and morbidity in HEU children found that increased risk of contracting other infections led to the death of these children, this highlights the fact that the immune system of HEU children is most likely compromised.

Exposure to ARVs in HEU children

It is important to note that HEU children are not only exposed *in utero* to HIV but also to ARVs, which may further contribute to adverse pregnancy outcomes (75). Certain ARVs such as Zidovudine (ZDV) have been implicated in mitochondrial toxicity due to inhibition of host cell gamma-polymerase and accumulation of somatic mitochondrial DNA mutations; which impact the development of the HEU children's immune system (76). A study by Cassidy *et* al, in Botswana showed that exposure to efavirenz-based ART was associated with lower receptive language scores at 2 years of age as compare with HEU children with non-efavirenxz-based ART (77). Other studies, however, have found no impact of ART exposure *in utero* on neurodevelopmental outcomes in HEU children at 2 years of age (78,79). Ultimately, the impact of ART on the inflammatory environment and development of HEU children remains uncertain, particularly when these children have also been exposed to HIV *in utero*. However, the impact of ARVs is beyond the scope of this study, due to the fact that all mothers in this study were on ARVs as per standard treatment guidelines.

1.3 The impact of maternal immune activation (MIA) on the neurodevelopment of their children.

The impact of the immune system on the developing nervous system is an important emerging area of interest. Deregulation within the immune system may lead to neurodevelopmental or psychiatric disorders and visa versa; as suggested by Damman *et al* (80). Cytokines present in the amniotic fluid and foetal circulation, representing a foetal inflammatory response may increase the risk of injury to the developing brain. The studies evaluated in this section investigated MIA (not necessarily due to HIV) in relation to neurodevelopmental issues.

Impact of cytokines on glial cells

Increased levels of inflammatory cytokines are suggested to have an impact on glial cells, such as astrocytes and microglia, which play a role in neurodevelopment (11,81). Astrocytes are important in neuronal development and repair, as well as synapse formation (82). Microglia are the immune cells of the CNS and have an ability to produce cytokines; they react to insult by transforming into amoeboid microglia that proceed to synthesize large amounts of inflammatory cytokines (5). The exposure of the foetus to infection resulting in amoeboid microglia can persist into postnatal life, exposing the brain of the post-natal child to the overexpression of inflammatory cytokines (5), particularly IL-1, IL-6 and TNF-α, these cytokines divert stem cells from neuronal differentiation and in turn increases apoptosis in neuronal and non-neuronal cells (6). IL-1, IL-6 and TNF-α are pleiotropic cytokines heavily involved in the CNS. TNF-α has been shown to influence neuronal progenitor cell proliferation and differentiation. IL-1 exerts functions in the CNS such as regulation of temperature, food intake and neuroendocrine function. IL-6 co-stimulates the immune response by synergizing with TNF-α and IL-1 to result in B-cell replication, differentiation and immunoglobin production (83). IL-6 also plays a role in the hypothalamic-pituitary adrenal (HPA) axis function, reduction of food intake, induction of fever and neuronal growth (83).

Additionally, cytokines are involved in most stages of neurogenesis including formation of the neural tube, synapses and dorsoventral domains, anteroposterior regionalization, specification of neuronal and glial phenotypes as well as the expression of neurotransmitters and receptors (7). It is even suggested that low levels of circulating inflammatory cytokines have the ability to influence complex brain functions (84). A review by Bilbo and Schwarz describes how increased cytokine exposure during critical periods of neurodevelopment may act as a "vulnerability" factor for psychiatric conditions later in life by sensitizing neural

substrates and altering the way the brain responds to further immune stimuli (85). It is also hypothesised that MIA may potentiate the effects of "risk" genes resulting in neuropsychiatric disorders (9). The CNS itself is also capable of producing cytokines during the inflammatory response; with the expression of IL-1, IL-6 and TNF-α being most prominent in the hypothalamus and hippocampus and less so in the cortex and brain stem regions (86). A profound example of how cytokines can impact brain function is through the expression of "sickness behaviour" outlined in a review by Dantzer *et al* where he explains how cytokines, particularly IL-1, TNF-α and IL-6, induce not only symptoms of sickness in response to infection but also major depressive disorders in patients with no previous history of mental health disorders (87). Overall the role of cytokines in the CNS and brain function and development is vast and complex; significant increases or decreases in levels of cytokines may therefore negatively impact important brain function.

Impact of cytokines on the hypothalamic-pituitary adrenal (HPA) axis

As well as the effect of MIA on cytokine levels, numerous other physiological effects may occur. Ratnayake *et al* proposes that an alternative mechanism by which an induction of pro-inflammatory cytokines can result in neurodevelopmental issues is through the HPA axis rather than through glial cells (11). An increase in cytokine levels, particularly IL-1 (6), can activate the HPA axis, responsible for the generalised stress response (88). Activation of the HPA axis results in the release of numerous glucocorticoids that may modify foetal brain development and permanently alter the function of the HPA axis in postnatal life (88). Excessive release of the hormones adrenocorticotropic hormone (ACTH) and corticotropin-releasing hormone (CRH) can be detrimental to foetal development (2). It is important to note that dysfunction with the HPA axis is hypothesised to play a critical role in numerous psychological diseases, including depression (89).

Impact of cytokines on neurotransmitter systems

Cytokines further impact neurotransmitter systems in the developing brain, altering their concentration or availability (90). For instance cytokine elevation due to inflammation can result in decreased survival of foetal serotonin neurons in the rostral raphe and increased expression of gamma-aminobutyric acid receptors in the amygdala (91). In addition, placental inflammation is also associated with disruptions to the tryptophan pathway in the placenta, which may have a corresponding effect on the production of serotonin and neural development (8). Furthermore, MIA can result in the production of reactive oxygen species (ROS) leading to oxidative stress that may induce neurotoxicity (92). Pro-inflammatory cytokines also trigger the induction of metallothionein, which may lead to zinc deficiency that can also contribute to poor developmental outcomes (92). Neurotransmitters are crucial in healthy brain function; it is well established that altered levels of neurotransmitters are present in numerous neuropsychological disorders for example individuals with depression often have alterations in serotonin, norepinephrine and dopamine (93).

It is important to note that specific regions of the brain may be relevant to distinct neuropsychiatric disorders. A review by Estes *et al* also states the importance of timing in MIA; infection during early versus late gestational stages causes distinct foetal brain cytokine responses (12). Whether this in-turn leads to distinct neuropsychiatric disorders is still unclear. However, around the time of birth the neurodevelopmental process is particularly active within the portal and temporal cortices that go on to impact complex cognitive and social functions in the children, such as cell migration, organization and synaptic maturation (15,94). It is important to note that HIV affects the mother's immune system and therefore the immune system of the foetus throughout the entire gestational period, making it a marked immune insult.

Role of MIA and cytokine expression in neurodevelopmental and psychiatric disorders

A number of studies have been conducted using animal models to assess the impact of MIA on the neurodevelopment of the foetus, in stark contrast to the limited number of studies conducted in humans. It is crucial that more human based studies are done, because the relationship between the inflammatory environment of the mother to that of the foetus differs between species. A number of studies in animal models have highlighted a relationship between MIA and the development of neuropsychiatric disorders such as Schizophrenia and Autism Spectrum Disorder in adult offspring (13,95). However MIA has not been directly linked with specific neuropsychiatric disorders in humans but instead is hypothesised to prime the immune system of infants so that they are more susceptible to triggers that may cause the disorders later in life (12).

One study conducted by Yoon *et al* did however report an association of higher concentrations of TNF-α, IL-1β and IL-6 with white matter lesions and cerebral palsy in newborns by measuring the amniotic fluid concentration of the respective cytokines (96). Furthermore, an epidemiological study conducted by Brown *et al* examined the role of prenatal infection in the development of Schizophrenia (14). In this study it was shown that an elevation in maternal levels of IL-8 during the second and third trimester was associated with increased risk of children developing Schizophrenia (14). It was also noted at the time of birth that TNF-α was significantly increased amongst mothers of children with psychosis (14). They did not find, however, an association between IL-1β, IL-6 and IL-2 with the development of neuropsychiatric disorders (14). As mentioned previously, the inflammatory markers IL-6, TNF-α and IL-1β, which have shown in previous studies to be elevated with HIV infection, also play a critical role in cognitive function.

Normal physiological levels of IL-1β, IL-6 and TNF-α play an important role in maintaining synaptic plasticity mechanisms in the brain, particularly memory function (97). Increased levels of IL-1β in the brain on the other hand are strongly correlated with memory impairment (97). Heightened levels of IL-1β have been reported in numerous neuropsychiatric disorders including AIDS related dementia and chronic inflammatory diseases such as Alzheimer's Disease (98,99). IL-6 is expressed in the CNS under both pathological and normal conditions, however levels remain low in the intact brain (100). A study by Rudolph *et al* showed that maternal IL-6 levels (indicating maternal inflammation) during pregnancy are associated with differences in functional brain networks, specifically the sub-cortical, salience and dorsal attention systems in the newborns and then negatively associated with working memory in the children at 2 years of age (101). This is of interest, since working memory is a core component of executive functioning that is deregulated in neuropsychiatric disorders such as autism spectrum disorders and schizophrenia.

IFN-γ has been associated with CNS disease states such as multiple sclerosis and AIDS dementia complex (82). In addition, infection in the third trimester is linked with increased risk of developing autism spectrum disorder (15). A recent study by Graham *et al* is one of the first studies in humans that reported association of elevations of a specific mediator of maternal inflammation, IL-6, with the newborn brain (91). In summary, they found that heightened IL-6 levels in the mother correlated to an altered balance between the impulsivity and regulatory capacity of the newborn through alterations in the developing amygdala (91). The fact that they studied the brain shortly after birth also reduces potential confounding environmental impact (91). TNF-α has been shown to cross the blood brain barrier and reduce cerebral oxygen uptake resulting in intracranial pressure, making it a potent cytotoxic agent in neural tissue (102). In addition, substantial evidence is

present for microglial activation in the post-mortem brains of patients that suffered from a mental illness (103–105). This is of importance as we highlighted earlier that activated microglia are critical in the inflammatory process and play a role in secreting pro-inflammatory cytokines. In summary, this evidence supports the role of MIA and a pro-inflammatory environment in the development of neuropsychiatric disorders.

1.4 The impact of maternal HIV on the neurodevelopment of HEU children

This section will focus on studies specifically looking at maternal HIV infection and the neurodevelopment of the uninfected children. Cognitive decline in infants living with HIV has convincingly been associated with increased early HIV viral load and can therefore be linked with HIV infection (106). Maternal inflammation has been strongly associated with risk of preterm birth (34), which is also fairly common in mothers living with HIV (52,61). Preterm birth has, in turn, been associated with neurocognitive impairments (102). However, it is also hypothesised that detectable developmental delays among HEU children may, in part be a result of the environment and not of any direct biological consequence due to being exposed to HIV *in utero* (70). In the majority of studies conducted on HEU children, the primary measure of neurodevelopment of the children is through the Bayley Scales of Infant and Toddler Development (Bayley-III); this assessment is designed to measure developmental function through five different scales: Cognitive, Language, Motor, Social-Emotional and Adaptive Behaviour (107). It is important to note that the impact of HIV extends far beyond just the neurodevelopment of HEU children into areas such as physical along with mental health, education and exposure to stigma and household environment, which in many countries coincides with exposure to poverty (108). These factors most likely influence the neurodevelopment of HEU perhaps even more so than HIV exposure *in utero* and therefore need to be taken into careful consideration when conducting analyses.

Studies investigating neurodevelopment in HEU children using the Bayley-III

Neurodevelopment in HEU children has been assessed in numerous studies, with a number of recently published reviews. The most extensive and up–to-date meta-analysis by McHenry *et al* looked at 45 studies where the neurodevelopment of the HEU children in the various studies were assessed using the Bayley-III and found that HEU children had over-all lower cognitive and motor scores compared to HUU children (4). Another review by Le Doaré *et al* established that HEU children along with children living with HIV also demonstrated greater neurodevelopmental delay compared with HUU children, particularly when it came to language and adaptive behaviour below the age of 5 (109). Unfortunately, a lot of the studies conducted so far, including the ones evaluated within the above reviews do not have a control group of HUU children to compare results with. It is important to note that the majority of studies conducted so far have found impaired neurodevelopment in HEU children compared to HUU children.

A study conducted by Le Roux *et al* in South Africa found increased odds of cognitive and motor delay in HEU infants compared to HUU infants (110). However, they did not find a delay in language scores and any neurodevelopmental impairment observed was not severe (110). Another study by Van Rie *et al* assessed neurodevelopment in HEU children in Kinshasa and found moderate to severe delay in motor and language development in a substantial amount of HEU children (111). It was also highlighted that the living conditions of these children played a critical role in their neurodevelopmental outcome (111). Furthermore, a study in China reported significantly lower composite scores, particularly cognitive and adaptive behaviour scores, in the Bayley-III assessment in HEU children compared to HUU children (112). Moreover, older HEU children (30-

35 months) had the lowest neurodevelopmental level in comparison to younger HEU children in the same study (112).

On the other hand, some studies did not find a significant difference in neurodevelopmental outcomes between HEU and HUU children using the Bayley-III. A study by Alimenti *et al* found that perinatal HAART-exposed HEU children had lower adaptive behavioural outcomes in comparison to HUU, however this significance was lost after controlling for substance abuse (113). Furthermore, a study by Chaudhury *et al* found HEU children at 24 months performed equally well on neurodevelopmental assessments in comparison to HUU children (114). Springer *et al* performed a study comparing neurodevelopment using the Bayley-III scale and Alarm Distress Baby Scale (ADBS), between HEU and HUU infants in a peri-urban area of South Africa (115). They found the cognitive, language and motor scores were within average range and only found minor differences in vocalization and social withdrawal between the two groups indicating no significant difference. As mentioned previously there are numerous factors that play a role in the neurodevelopment of HEU children; discrepancies between these studies may be due to ART exposure, stigma and socio-economic status.

Studies investigating neurodevelopmental impairment in HEU children using alternative methods to the Bayley-III

Alternatively, many studies examined the neurodevelopment of HEU children through numerous other methods than the Bayley-III. A systematic review conducted by Sherr *et al*, which assessed neurodevelopment in HEU children compared to HUU children concluded that HEU children are worthy of concern and points out, albeit tentatively, that HEU children are functioning below HUU children on numerous developmental, behavioural and cognitive parameters (116). This

review included studies that assessed neurodevelopment through both the Bayley-III and other standardised neurodevelopmental assessments.

A study by Kerr *et al* demonstrated that HEU children have modest but significant reductions in verbal IQ and full-scale IQ and Stanford-Binet Bead Memory scores (which assesses non-verbal fluid reasoning) compared with HUU children (117). They did however find no difference in performance IQ, sentence, digit or object memory (117). A study by Tran *et al* looked at white matter microstructural integrity in the brains of HEU newborns compared with HUU newborns and found higher fractional anisotropy in the middle cerebellar peduncles of HEU newborns compared with HUU newborns using Diffusion Tensor Imaging (DTI) (118). DTI gives insight into white matter microstructure and organization by tracking the diffusion of water molecules through white matter tracts of the brain; indicating a compromise in white matter microstructural integrity in HEU newborns (118). Cerebral changes are common in the last trimester; therefore, structural alterations that occur at this time may result in motor and cognitive deficits later in life (118). A study looking at academic achievement scores of children living with HIV and HEU children reported that the global and cognitive index scores of both groups were in the low average to average range of intellectual functioning compared to normative means across academic domains (119).

Studies that found no difference in neurodevelopment between HEU and HUU children include a study conducted by Rajan *et al* on a cohort of HEU children in Dehli, which found that no neurodevelopmental impairment in HEU children using the Development Assessment scale for Indian Infants (DASII) (120). However neurodevelopmental impairment was associated with socioeconomic status and presence of wasting in both HEU and HIV infected children (120). The authors concluded that HEU children will develop normally with sufficient care, whereas

children living with HIV will continue to have delayed development despite optimum care. A study measuring the IQ between HUU and HEU children found that they did not differ in IQ scores (121). Additionally, no significant differences were detected in brain volume and DTI metrics between HUU and HEU children (121). Another study by Kandawasvika *et al* examining neurodevelopmental impairment between HEU, HUU and children living with HIV using the Bayley Infant Neurodevelopmental Screener (BINS) found that the percentage of children with a high risk for impaired neurodevelopment was no different between HEU and HUU but both these groups had significantly less chance of neurodevelopmental impairment compared to children living with HIV (122). They did however find an increase in neurodevelopmental impairment from 3 months to 9 months despite being infected, exposed or unexposed to HIV (122). Springer *et al* performed a pilot study in South Africa to assess the neurodevelopment of HEU children in comparison to HUU children using the Griffiths Mental Developmental Scales (GMDS), which measures locomotor, personal/social, hearing and speech, eye and hand coordination and performance (123). In this study they found no significant differences between the groups apart from the personal/social subscale where the HEU children performed significantly more poorly. The use of these different studies highlight important gaps in the literature and indicate that there may be differences in IQ as well as brain connectivity and structure between HEU and HUU children.

Studies evaluating the impact of ARVs on the neurodevelopment of HEU children

ARVs have had a massive impact on reducing the vertical transmission of HIV to the infants and more common than not pregnant mothers are now being treated with ART. The effect of ART on the children is therefore, in addition to HIV exposure, not fully explored. However, the majority of studies conducted so far have not indicated any significant negative impacts. The following studies looked

specifically at the impact that ART has on the neurodevelopment of HEU children. A study assessing the impact of the ARV Zidovudine (ZDV) in comparison to a placebo in pregnant mothers yielded results reflecting that there was no significant difference in cognitive function between the children of the two groups (treatment with ZDV vs. treatment with a placebo), suggesting that ARVs don't significantly impact neurodevelopment (124). It is important to note that this study assessed cognitive outcome in the same children every year up until the age of 4 (with intention to continue to age 21, and has reported no significant health or cognitive impairments so far (124). A European collaborative study also agrees that ART exposure in HEU children does not have a negative impact on their neurodevelopment, but did find a correlation of ART with premature birth (125). Another study by Williams *et al* assessing neurodevelopment also found no significant differences between HEU and HUU children at 2 years of age, only slightly higher scores for HEU children were observed using the Bayley-III scale but no difference was seen on the Mental Developmental Index and Psychomotor Developmental Index scores (126).

A study by Ngoma *et al* assessing the neurodevelopment of HEU compared to HUU infants exposed to ART *in utero* and then later on through breastfeeding also established no cognitive or language impairment, using the Capute Full-Scale Developmental Quotient (FSDQ) (127). They did however find that a lower FSDQ score, indicating neurodevelopmental impairment, was associated with lower birth weight and lower family income (127). A study conducted on 5-13 year old HEU children who had *in utero* or neonatal exposure to ART found that their cognitive and academic outcomes, assessed using the Weschsler intelligence and academic scales, were slightly below average but not significant; this indicated that there is no latent onset of neurodevelopmental impairment in children (128). No individual ARV drug was associated with neurodevelopmental impairment in this study,

supporting the safety of ART (128). A longitudinal study by Smith *et al* assessed verbal, performance and full-scale IQ as well as language in HEU children exposed to ART (129). At 3.5 and 5.5 years of age they found that at both ages the scores were within the normal range. However despite their average scores in these cognitive developmental aspects their scores in adaptive function, including Adaptive Behaviour Composite and Socialisation scores, were significantly lower than average (129).

Sirois *et al* conducted a study on infants ranging from 9-15 months, which all completed the Bayley-III and found that not one parameter of this assessment was associated with ART exposure, except for the individual drug Atazanir which was associated with language delay, overall this study supported the safety of ART (130). One other study also found an association of the ARV drug Atazanir with language deficits in 1-year old infants. However, no individual ARV drug was associated with language deficits in 2 year-olds in the same study (131). A review by Heidari *et al* on ARVs and their effects on HEU children concluded that the benefits that ARVs have on the health and survival of the mother far outweighs any potential adverse effects identified to date (132). It is important to note that these studies may not be taking into consideration the impact of HIV exposure *in utero* rather than ARV exposure into their assessment on neurodevelopment of HEU children, however it is important to highlight their potential role.

1.5 Hypotheses and aims

Based on the literature review, 3 main gaps were identified. Firstly, more insight is needed surrounding the impact of HIV infection on the immune system of HIV positive pregnant mothers. Secondly, the immune system and serum levels of inflammatory markers of HEU children need further investigation. Finally, it is critical to further evaluate the association of inflammatory markers with

neurodevelopment in this population. Ultimately, the aim of this study was to delineate the relationship between the immune system of pregnant mothers living with HIV and their children, as well as the association between inflammatory markers and neurodevelopment in HEU children.

The hypotheses were that:

1. Pregnant mothers living with HIV would show increased levels of inflammatory markers compared to uninfected mothers.
2. HEU infants and children will have increased levels of inflammatory markers compared to HUU infants and children, suggesting that the mother's immune system predicts the immune system of their children
3. Increased serum levels of inflammatory markers in HEU children will be associated with impaired neurodevelopment compared to HUU children.

2. METHODS

2.1. Characteristics of the study population

Recruitment for the parent study: the Drakenstein Child Health Study (DCHS) took place from 2012 to 2015 in the Drakenstein peri-urban region of the Western Cape, South Africa. Like many peri-urban regions in South Africa it is characterized by a high prevalence of risk factors for poor child health such as HIV. Whilst there is a high prevalence of disease, there is access to well-established free primary health care services including 23 clinics and 1 hospital (25). Two of the primary health care clinics, TC Newman clinic and Mbekweni clinic, were used to recruit participants for the DCHS. Obstetric care was provided at Paarl Hospital where all births took place. In summary, these characteristics were ideal for recruitment of pregnant mothers with and without HIV infection as well as HEU children.

2.2. Recruitment criteria

In the DCHS n=1137 pregnant women between 20 and 28-weeks' gestation were recruited by the DCHS staff at an initial antenatal visit as part of the parent study. Pregnant women were successfully enrolled if they were at least 18 years of age, planned to live in the region for at least 1 year and provided written signed consent (24,25). Out of the women recruited for the DCHS a sub-sample of 267 women were selected based on availability of follow-up serum samples and their HIV status that reflects the whole DCHS sample. As this study is nested in the DCHS (24) the time points at which the serum samples were taken (26 weeks gestation for the mothers and 6-10 weeks and 24-28 months for the children) was already established. These time points are advantageous as it allows the evaluation of neurodevelopment longitudinally up up to 2 years of age, which is suggested to be a crucial time period in neurodevelopment. Study numbers for this sub-sample are represented in Tables 1, 2 and 3.

2.3. Study measures, Design, Research Procedures and Data Collection

Blood sample collection and processing

Blood serum samples were taken at 26 weeks gestation for mothers and at 6-10 weeks and 24-28 months for children as outlined in the DCHS (3). Bloods were collected in serum tubes and allowed to clot for 30-60 minutes. Samples were transported on ice and processed in a central research laboratory. The samples were subject to 2 freeze/thaw cycles, which have no effect on cytokine levels (133). There was no reported problem with the collection, transportation and processing of blood samples.

Laboratory analysis of cytokines

For this thesis serum inflammatory markers, Lcn2 and MMP9, were quantified using commercially available ELISA kits (R&D systems) (134,135) in serum samples from the mothers and children. In addition, a premixed 13-plex – Immunology Multiplex Assay, known as MILLIPLEX® (136) was used to analyse other pro-inflammatory markers (GM-CSF, INF-γ, IL-1β, IL-2, IL-5, IL-6, IL-7, IL-8 and TNF-α) and anti-inflammatory markers (IL-4, IL-10, IL-12 p70 and IL-13) on the same blood samples, using the Luminex® immunoassay reader. All samples were tested in duplicate. There were no reported problems with the testing standards and calibration of the immunoassay reader.

Neurodevelopmental assessment

Neurodevelopment of the children at 24-28 months was assessed with the Bayley Scales of Infant and Toddler Development (Bayley-III) assessment (107,137). Trained assessors involved in the DCHS captured this existing data, which was then used in data analysis for this thesis. The Bayley-III assessment is a well-validated tool that encompasses numerous scales to test for cognitive, language, motor, social-emotional and adaptive behaviour impairments from 1-42 months (107). A

trained physiotherapist and a trained occupational therapist were blinded to maternal HIV status and performed the Bayley-III testing. Training was performed in accordance with the Bayley-III manual by a pediatric neurodevelopmental specialist who periodically monitored the assessors throughout the testing period to validate standardized data collection across sites and ensure agreement between assessors on both administration and scoring. The assessors alternated between the TC Newman and Mbekweni clinics and assessed equal number of children at each site. Assessments were performed with prompts in the child's preferred language. Neurodevelopmental assessments were not conducted on children when they had a fever, these children were then asked to return when possible. External scoring quality control checks were also performed centrally before data capture. For the current study standardized composite scores were used. Composite scores were generated for each cognitive, language, motor, social-emotional and adaptive behaviour domains, with a mean of 100 and standard deviation of 15, using normative United States data. The use of these composite scores has been validated in the South African setting (138,139). There were no adaptations to the social-emotional and adaptive behaviour domains.

Variable measures and collection

All variable measures and covariates mentioned in this section were captured by study staff involved in the DCHS and were used in the data analysis conducted in this study. Covariates included data on maternal demographics and health. This included residential area, education, HIV status, smoking, alcohol use, age and socioeconomical status, this data was obtained at enrolment. Maternal body mass index (BMI) was determined at 6–10 weeks postpartum. Data for mode of delivery, gestational age, infant sex, birth weight and length were obtained at the time of delivery at Paarl Hospital, where all births took place. Gestational age was calculated using the best estimated delivery date based on the last menstrual

period, antenatal ultrasound, or symphysis-fundal height. Prematurity was defined at less than 37 weeks gestation. Maternal CD4 cell count and viral load during pregnancy were measured, with the result closest to 26 weeks gestation taken to coincide with the immune variables. Viral load was categorized as below the detectable limit with <40 copies/ml, detectable with 40-1000 copies/ml and unsuppressed with >1000 copies/ml. All mothers living with HIV received antiretroviral therapy (ART) during pregnancy according to PMTCT guidelines at the time. Maternal ART initiation was categorized as 'before pregnancy' or 'during pregnancy'. All HEU infants received prophylaxis (nevirapine alone or combined with zidovudine) from birth. (24,25).

Information on breast-feeding practice and the presence of a fever were obtained at infant follow-up visits at 4-12 weeks, and 20-28 months of age. Breastfeeding was categorized as breastfeeding only vs. formula, solids feeding or their combination with breastfeeding. None of the children were breast fed at 24-28 months of age (24,25).

Socioeconomic status was determined via a sociodemographic questionnaire that was adapted from items used in the South African Stress and Health Study (SASH) (140). Mothers self-reported employment status, education level, asset ownership, household income and clinic during an antenatal study visit between 28 and 32 weeks gestation (24,25).

Maternal alcohol use during pregnancy was assessed using the Alcohol, Smoking, and Substance Involvement Screening Test; mothers were classified with either moderate-severe alcohol exposure vs. un-exposed. Infants and children were classified as being alcohol-exposed in utero if their mother was classified with moderate-severe alcohol exposure (24).

Smoking was determined through the measure of cotinine levels in the blood using the IMMULITE 1000 nicotine metabolite kit (141). An individual was considered a non-smoker if cotinine levels were <10ng/ml, a passive smoker with levels between 10-500ng/ml and an active smoker with levels >500ng/ml (25). This data is summarized in Table 1.

2.4. Data Safety and Monitoring

Criteria for adverse events and potential harms were explicated in the parent study, which has been approved by the UCT HREC (HREC 401/2009 and HREC 648/2018). Expected adverse events in human subjects was not relevant for this sub-study.

2.5. Statistical Analysis

All analyses were conducted using SPSS (version 25, IBM, USA). P-values were considered statistically significant for all analyses at a value of less than 0.05. Because of the skewed distribution, all markers were ln-transformed and used for further statistical analyses. As some covariates had missing data, we imputed values estimated from all predictor variables. Statistical analyses were performed on five imputed datasets. Variables with missing values were: delivery method (n=17), smoking status (n=8), alcohol use (n=14), socioeconomic status (n=5), BMI at 6 weeks (n=51), breastfeeding at 6-10 weeks (n=20) and finally birth weight (n=2). Data were complete for all other covariates. First, unpaired *t*-tests were used to explore the differences of the inflammatory markers in mothers during pregnancy, infants and children according to maternal HIV status. Linear regression analyses were subsequently performed with the respective markers as dependent variables, maternal HIV status as predictor and the covariates as previously described and was adjusted for multiple comparisons. Pearson's correlations were

used to explore the associations between the inflammatory markers at each time point with neurodevelopment measures at 24-28 months of age. Regression analyses were further performed on significant findings with the respective inflammatory markers as dependent variables, neurodevelopment measure as predictor and controlled for by covariates and adjusted for multiple comparisons. The Benjamini-Hochberg procedure was used to control for the false discovery rate due to multiple testing (142). This procedure was used because it is less conservative than the Bonferroni correction method and is also more appropriate to control for false discovery rate. The Benjamini-Hochberg method p-values are ordered by ascending values according to the number of tests that were performed and accepted or rejected based on the adjusted p-values. Each individual p-value is compared to its Benjamini-Hochberg critical value $(i/m)Q$, where i is the rank, m is the total number of tests, and Q is the false discovery rate. The largest p-value that has $p<(i/m)Q$ is accepted as significant and all the other subsequent smaller p-values in the order. According to the Benjamini-Hochberg some of the p-values were considered as false-positives. However, these findings cannot be ignored and should only be interpreted with caution.

2.6. Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki (World Medical, 2000) and approved by the Human Ethics Research Committee of the Faculty of Health Sciences at the University of Cape Town (HREC 401/2009 and HREC 648/2018). The study was part of a parent study known as the Drakenstein Child Health Study (DCHS). In the parent study written informed consent was obtained from the mothers (older than or equal to 18 years), in their preferred language, on behalf of herself and her infant. Mothers were then given copies of their consent forms. All paper-based records of participant information are stored in a secure location and are only accessible to study staff involved in the DCHS. A

copy of each consent form is locked in a fireproof filing cabinet, which is only accessible to study staff. Study staff of the proposed study have password regulated access to computer data involving participant information. Additionally, all participants have been allocated anonymous numeric codes, and the actual identities of mother-infant pairs was not available to the student investigator. Thus, participants' identities have been kept confidential.

3. RESULTS

3.1. Participants

Table 1 presents demographic data for the mothers living with HIV and uninfected mothers included in the study. There was a significant difference in the site where the women were recruited (*p<0.001*), with more women living with HIV being recruited from Mbekweni and more HIV uninfected women being recruited from TC Newman. Additionally, there were significantly more women living with HIV with moderate-severe alcohol exposure compare to uninfected women (*p=0.032*).

Table 1: Descriptive demographic characteristics for mothers living with HIV and uninfected mothers

MOTHERS	Living with HIV	Uninfected	P-value
N (%)	77 (28.8)	190 (71.2)	0
Age, mean (SD)	29 (5.43)	26 (5.87)	0.377
Site, N (%)			**0.000**
Mbekweni	70 (90.9)	70 (36.8)	
TC Newman	7 (9.1)	120 (63.2)	
Education, N (%)			0.289
Primary	8 (10.4)	14 (7.4)	
Some secondary	50 (64.9)	108 (56.8)	
Secondary	17 (22.1)	56 (29.5)	
Tertiary	2 (2.6)	12 (6.3)	
Married, N (%)			0.933
Married/cohabitating	31 (39.2)	76 (39.8)	
Other	48 (60.8)	115 (60.2)	
BMI, mean (SD)	28.81 (6.60)	26.23 (6.06)	0.551
Viral load during pregnancy, N (%)			-
Below detectable limit (<40 copies/mL)	40 (78.4)	-	
Detectable (≥40-1000 copies/mL)	5 (9.8)	-	
Unsuppressed (>1000 copies/mL)	6 (10.3)	-	
CD4+ count during pregnancy, median (range)	453.50 (339.25-628)		
Antiretroviral regimen during pregnancy, N (%)			-
Prevention of mother-to-child transmission prophylaxis (zidovudine)	11 (14.3)	-	
First-line triple therapy	64 (83.1)	-	
Second-line or third-line therapy	2 (2.60)	-	

Initiation of antiretroviral treatment, N (%)			-
Before pregnancy	32 (41.6)	-	
During pregnancy	45 (58.4)	-	
Nicotine Users (cotinine levels ng/ml), N (%)			0.235
Non-smoker (<10ng/ml)	21 (28.4)	37 (20.0)	
Passive smoker (10-500 ng/ml)	31 (41.9)	76 (41.1)	
Active smoker (>500ng/ml)	22 (29.7)	72 (38.5)	
Alcohol Use, N (%)			**0.032**
Unexposed	61 (89.7)	179 (96.8)	
Moderate-severe exposure	7 (10.3)	6 (3.2)	
Illness during pregnancy, N (%)	11 (13.9)	17 (8.9)	0.213
Social-economic standard (SES), N (%)			0.475
Lowest SES	25 (32.5)	43 (23.2)	
Low-moderate SES	17 (22.1)	49 (26.5)	
Moderate-high SES	20 (26.0)	55 (29.7)	
High SES	15 (19.5)	38 (20.5)	
Baby Sex, N (%)			0.813
Male	30 (39.0)	77 (40.5)	
Female	47 (61.0)	113 (59.5)	
Premature Birth			0.464
<37 weeks, N (%)	69 (89.6)	164 (86.3)	
>= 37 weeks, N (%)	8 (10.4)	26 (13.7)	
Birth weight, mean (SD)	3.02 (0.52)	3.02 (0.59)	0.202
Delivery Method, N (%)			0.052
Vaginal	48 (69.6)	151 (83.4)	
Elective Cesarean	6 (8.7)	9 (5.0)	
Emergency Cesarean	15 (21.7)	21 (11.6)	

***Significant difference between groups (P<0.05), SD = Standard deviation, N = numbers**

Table 2 presents data for the HUU and HEU infants at 6-10 weeks included in the study. There was a significant difference between HUU and HEU infants who were breastfed at 6-10 weeks (*p=0.032*), with significantly more HUU infants being breastfed compared to HEU infants.

Table 2: Descriptive demographic characteristics for HEU and HUU infants at 6-10 weeks

INFANTS 6 WEEKS	HEU	HUU	P-value
N (%)	63 (71.6)	159 (28.4)	
Age (weeks), mean (SD)	8.1 (1.5)	7.9 (1.6)	0.277
Weight, mean (SD)	4.91 (0.70)	4.83 (0.79)	0.505
Length, mean (SD)	54.75 (2.66)	54.67 (2.90)	0.370
Head circumference, mean (SD)	38.86 (1.54)	38.61 (1.81)	0.197
Breast-fed, N (%)	30 (45.5)	110 (60.8)	**0.032**
Fever, N (%)	0 (0.0)	2 (1.1)	0.366

***Significant difference between groups (P<0.05), SD = Standard deviation, N = numbers**

Table 3 presents data for the HUU and HEU children at 24-28 months, where no significant differences were found between the two groups.

Table 3: Descriptive demographic characteristics for HEU and HUU children at 24-28 months

INFANTS 24 MONTHS	HEU	HUU	P-value
N (%)	77 (28.8)	190 (71.2)	
Age (months), mean (SD)	27.3 (4.0)	27.2 (3.5)	0.331
Weight, mean (SD)	11.98 (1.88)	11.61 (1.63)	0.249
Length, mean (SD)	83.40 (3.65)	83.73 (3.54)	0.889
Head circumference, mean (SD)	48.43 (2.15)	47.96 (1.89)	0.858
Breast-fed, N (%)	0	0	N/A
Fever, N (%)	5 (6.6)	16 (8.4)	0.582

***Significant difference between groups (P<0.05), SD = Standard deviation, N = numbers**

3.2. Inflammatory markers of mothers, their infants and children according to maternal HIV status

Figure 1 presents the serum levels of the inflammatory markers GM-CSF (p=0.003) and MMP9 (p<0.001), which both proved to be significantly decreased in mothers living with HIV compared to uninfected mothers. The inflammatory markers IL-1β (*p=0.021*) and IL-4 (*p=0.019*) were also shown to be significantly decreased in mothers living with HIV initially, however this significance did not survive the corrections for multiple comparisons. *Appendix 1* presents the ln-transformed values for all the inflammatory markers for the mothers living with HIV and uninfected mothers.

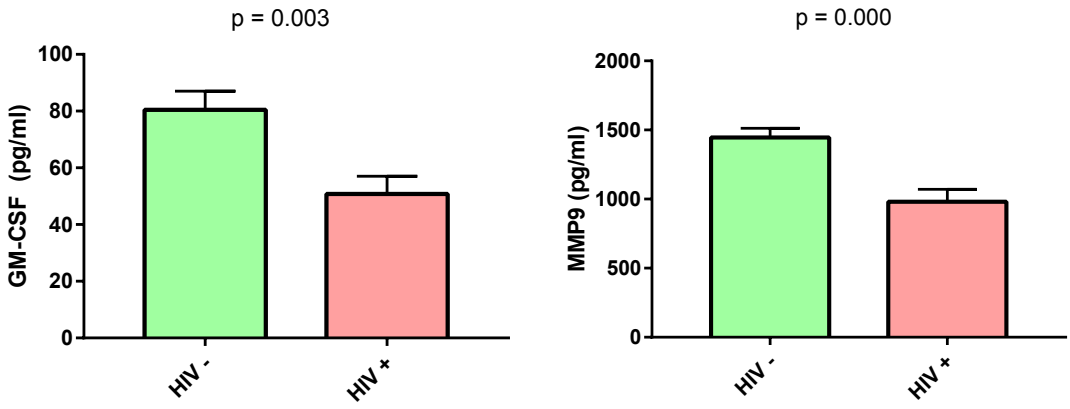

Figure 1: Serum levels (pg/ml) of inflammatory markers GM-CSF and MMP9 in mothers living with HIV and uninfected mothers

Figure 2 presents the serum levels of the inflammatory markers IFN-γ (*p=0.006*) and IL-1β (*p=0.006*), which both proved to be significantly decreased in HEU infants compared to HUU infants at 6-10 weeks of age. The inflammatory markers IL-12p70 and IL-4 did initially show to be significantly decreased in HEU infants but did not remain so after corrections for multiple comparisons. *Appendix 2* presents the ln-transformed values for all the inflammatory markers for HEU and HUU infants at 6-10 weeks of age.

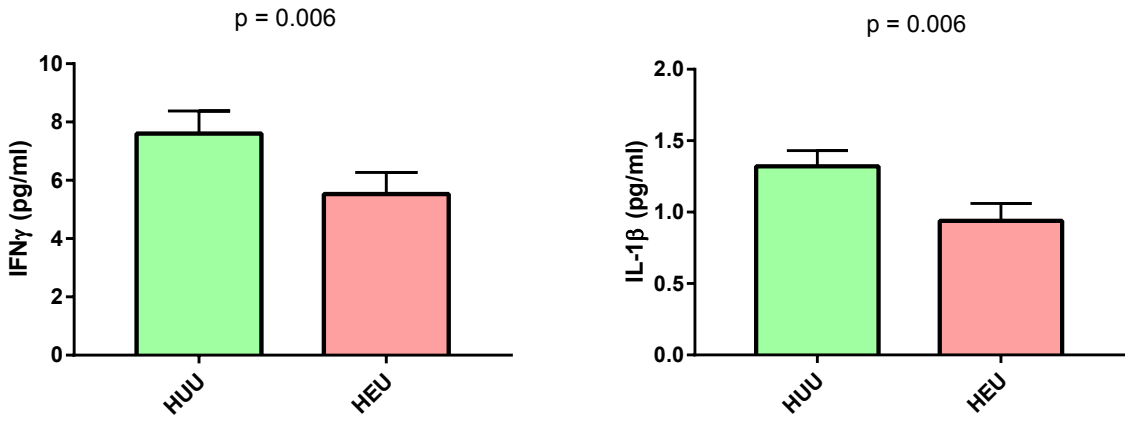

Figure 2: Serum levels (pg/ml) of inflammatory markers IFN--γ and IL-1β in HEU and HUU infants at 6-10 weeks

Figure 3 presents the serum levels of inflammatory markers for HEU and HUU children at 24-28 months; the inflammatory markers IFN-γ (*p=0.005*), IL-1β (*p<0.001*), IL-2 (*p=0.004*) and IL-4 (*p=0.013*) were all shown to be significantly decreased in HEU children compared with HUU children after corrections for multiple comparisons. *Appendix 3* presents the ln-transformed values for all the inflammatory markers for HEU and HUU children at 24-28 months.

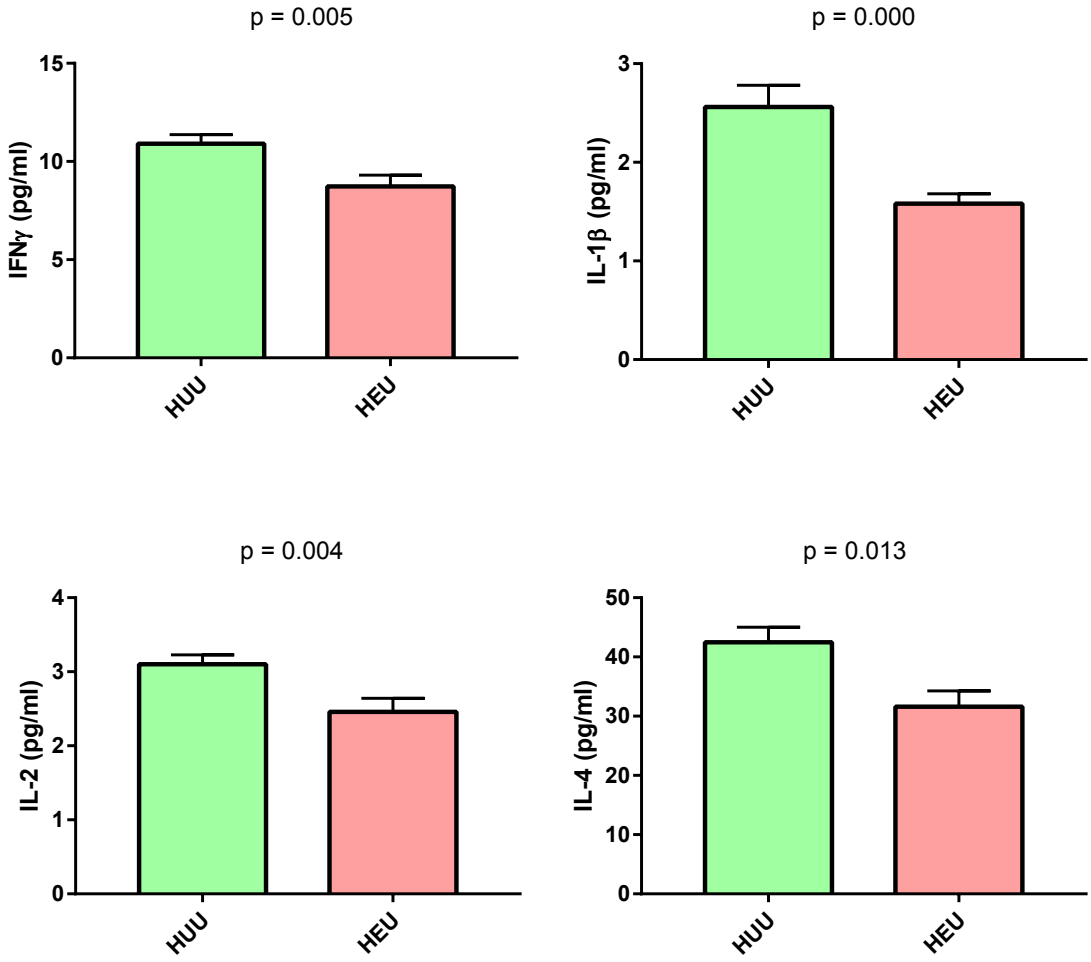

Figure 3: Serum levels (pg/ml) of inflammatory markers IFN-γ , IL-1β, IL-2 and IL-4 in HEU and HUU children at 24-28 month

Table 4 shows the linear regression analysis for differences in inflammatory markers between mothers living with HIV and uninfected mothers and their HEU and HUU infants and children, corrected for potential covariates. Our results show that GM-CSF (*p=0.016*) and MMP9 (*p=0.034*) remained significantly decreased in mothers living with HIV after correcting for covariates. IFN-γ (*p=0.001*)remained significantly decreased in HEU infants, however IL-1β (*p=0.120*) was not significant after controlling for all covariates. Our results further show HEU children had decreased IFN-γ (*p=0.019*), IL-1β (*p=0.001*), IL-2 (*p=0.035*) and IL-4 (*p=0.017*) levels after corrections for covariates were made.

Table 4: Linear regression analyses of identified markers that were found to be significantly different between mothers living with HIV and uninfected mothers, their infants (6-10 weeks) and children (24-28 months) after adjustments for covariates

	B (SE)	β	p
Mothers*			
GM-CSF	-0.303 (0.15)	-0.150	0.016
MMP9	-0.243 (0.11)	-0.150	0.034
Infants 6-10 weeks#			
IFN-γ	-0.544 (0.17)	-0.251	0.001
IL-1β	-0.201 (0.16)	-0.101	0.120
Children 24-28 months†			
IFN-γ	-0.237 (0.10)	-0.180	0.019
IL-1β	-0.391 (0.12)	-0.242	0.001
IL-2	-0.204 (0.11)	-0.130	0.035
IL-4	-0.350 (0.161)	-0.158	0.017

*** covariates**: residential area, education, smoking, alcohol use, age, socioeconomic status and body mass index (BMI) at 6–10 weeks postpartum. **#covariates**: Residential area, fever at time of visit, maternal smoking during pregnancy, maternal alcohol use during pregnancy, gestational age, maternal socioeconomic status and body mass index (BMI) at 6–10 weeks postpartum, premature birth, birth weight, infant sex and breastfeeding at 6-10 weeks. **†covariates**: Residential area, fever at time of visit, maternal smoking during pregnancy, maternal alcohol use during pregnancy, premature birth, maternal socioeconomic status and body mass index (BMI) at 6–10 weeks postpartum, gestational age, birth weight and infant sex.

3.3. Association of inflammatory markers with neurodevelopment

Table 5 presents correlation analysis of inflammatory markers and neurodevelopment measures carried out on uninfected mothers. No inflammatory markers proved to be significantly associated with any of the neurodevelopment measures once corrections for multiple comparisons were applied. However, the inflammatory marker IL-8 (*p=0.029, r=0.164*) did initially show to be significantly associated with the cognitive neurodevelopment measure.

Table 5: Correlation analysis between inflammatory markers in uninfected mothers with neurodevelopment measures in their children at 24-28 months

		GM-CSF	IFN-γ	IL-10	IL-12p70	IL-13	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	TNF-α	Lcn2	MMP9
Cognitive	*r*	-.066	-.002	-.030	-.051	.048	-.017	-.031	.035	-.016	.016	-.072	**.164***	.039	.003	.054
	p-value	.383	.980	.687	.500	.520	.817	.676	.638	.828	.834	.336	**.029**	.609	.969	.477
Language	*r*	-.076	-.051	-.020	-.093	.043	-.033	-.017	-.004	.063	.002	.011	.123	.014	-.038	-.045
	p-value	.326	.512	.800	.228	.580	.667	.828	.961	.415	.983	.887	.110	.855	.627	.564
Motor	*r*	-.036	.026	-.004	-.034	.003	.060	.000	.076	.001	-.011	.023	.112	.058	-.030	-.014
	p-value	.635	.727	.955	.650	.968	.432	.996	.315	.986	.888	.764	.138	.441	.693	.853
Socio-emotional	*r*	-.046	.021	.054	.044	.008	.028	.024	.035	.041	.059	-.032	-.009	.050	-.051	-.048
	p-value	.541	.779	.475	.558	.918	.710	.745	.646	.583	.435	.667	.900	.502	.496	.521
Adaptive Behaviour	*r*	-.120	-.087	-.052	-.125	-.034	-.001	-.081	-.004	.009	-.024	-.080	.071	.040	-.053	-.088
	p-value	.110	.249	.492	.095	.653	.992	.279	.963	.908	.751	.288	.346	.598	.481	.240

***p<0.05, **p<0.01**

Table 6 presents correlation analysis of inflammatory markers and neurodevelopment measures in mothers living with HIV. Inmothers living with HIV, IFN-γ (*p=0.014, r=-0.295*), IL-10 (*p=0.036, -0.253*), IL-12p70 (*p=0.030, r=-0.262)* and IL-7 (*p=0.041, r=-0.246*) did prove initially to be associated with the language neurodevelopment measure prior to corrections. TNF-α (*p=0.014, r=-0.288*) also showed to be significantly associated with the cognitive measure prior to corrections. The socio-emotional measure was initially associated with the inflammatory markers IL-10 (*p=0.014, r=-0.295*), IL-12p70 (*p=0.026, r=-0.262*) and IL-6 (*p=0.042, r=-0.240*). However, all significance was lost after correcting for multiple comparisons.

Table 6: Correlation analysis between inflammatory markers in mothers living with HIV with neurodevelopment measures in their children at 24-28 months

		GM-CSF	IFN-γ	IL-10	IL-12p70	IL-13	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	TNF-α	Lcn2	MMP9
Cognitive	*r*	.085	-.131	-.069	-.045	-.051	-.023	-.086	.049	-.128	-.144	-.226	-.071	**-.288***	-.016	-.122
	p-value	.477	.272	.566	.705	.669	.850	.475	.684	.283	.227	.057	.556	**.014**	.892	.307
Language	*r*	-.142	**-.295***	**-.253***	**-.262***	-.174	-.223	-.232	-.208	-.211	-.204	**-.246***	-.128	-.206	.081	.045
	p-value	.245	**.014**	**.036**	**.030**	.153	.066	.056	.086	.081	.093	**.041**	.295	.089	.509	.711
Motor	*r*	.031	-.166	-.147	-.164	-.082	-.133	-.085	-.123	-.134	.012	-.109	-.029	-.138	-.036	-.068
	p-value	.807	.182	.240	.189	.511	.288	.496	.325	.283	.923	.383	.815	.269	.775	.589
Socio-emotional	*r*	.042	-.182	**-.270***	**-.262***	-.087	-.214	-.186	-.186	-.120	**-.240***	-.167	-.210	-.103	.011	-.173
	p-value	.729	.126	**.022**	**.026**	.467	.071	.118	.117	.317	**.042**	.161	.077	.388	.929	.147
Adaptive Behaviour	*r*	.043	-.212	-.184	-.204	-.181	-.147	-.225	-.177	-.138	-.200	-.213	-.109	-.169	.084	-.019
	p-value	.719	.074	.121	.085	.129	.218	.058	.136	.247	.092	.073	.364	.155	.481	.875

***p<0.05,**p<0.01**

Table 7 presents correlation analysis of inflammatory markers and neurodevelopment measures in HUU infants at 6-10 weeks of age. No significance was found with any of the inflammatory markers after corrections. However, the cognitive measure was initially associated with the inflammatory marker IL-1β (*p=0.042, r=-0.169*). The language measure was also initially significantly associated with the inflammatory markers IL-1β (*0.004, r=-0.245*), IL-2 (*p=0.037, r=-0.178*), IL-7 (*p=0.029, r=-0.186*) and MMP9 (*p=0.038, r=-0.178*).

Table 7: Correlation analysis between inflammatory markers in HUU infants at 6-10 weeks with neurodevelopment measures at 24-28 months

		GM-CSF	IFN-γ	IL-10	IL-12p70	IL-13	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	TNF-α	Lcn2	MMP9
Cognitive	*r*	-.095	-.097	-.058	-.127	-.145	**-.169***	-.120	-.048	-.075	-.096	-.080	-.058	-.048	-.064	-.067
	p-value	.253	.243	.485	.128	.081	**.042**	.149	.564	.370	.251	.341	.490	.568	.446	.424
Language	*r*	-.100	-.115	-.051	-.165	-.090	**-.245****	**-.178***	-.066	-.140	-.150	**-.186***	-.036	-.069	-.137	**-.178***
	p-value	.244	.181	.552	.053	.296	**.004**	**.037**	.443	.102	.080	**.029**	.679	.420	.112	**.038**
Motor	*r*	-.044	-.018	-.048	-.140	-.095	-.105	-.148	-.072	-.131	-.077	-.096	.011	.010	-.062	-.117
	p-value	.600	.829	.569	.093	.257	.209	.075	.393	.117	.358	.252	.896	.904	.462	.164
Socio-emotional	*r*	-.085	.020	-.113	-.094	.004	.016	-.044	.005	-.027	-.059	-.041	-.038	.068	.010	.018
	p-value	.307	.807	.175	.257	.961	.853	.597	.951	.748	.478	.628	.646	.416	.903	.828
Adaptive Behaviour	*r*	-.022	-.006	-.063	-.110	-.116	-.073	-.124	-.019	-.074	-.061	-.089	.018	-.037	.007	-.103
	p-value	.796	.939	.450	.187	.162	.381	.137	.824	.378	.468	.289	.831	.657	.934	.217

***p<0.05, **p<0.01**

Table 8 presents correlation analysis of inflammatory markers and neurodevelopment measures in HEU infants at 6-10 weeks of age. The majority of inflammatory markers GM-CSF (*p0.022, r=10.309*), IFN-γ (*p=0.011, r=-0.339*), IL-10 (*p=0.001, r=-0.451*), IL-12p70 (*p=0.004, r=-0.379*), IL-1β (*p=0.000, r=-0.491*), IL-2 (*p=0.022, r=-0.308*), IL-4 (*p=0.002, r=-0.418*), IL-6 and Lcn2 (*p=0.004, r=-0.383*) were found to have a significant association with the motor neurodevelopment measure even after correction for multiple comparisons. MMP9 (*p=0.034, r=-0.289*) also did initially show to be associated with this measure prior to corrections. The language measure was also initially associated with MMP9 (*p=0.022, r=-0.305*). IL-1β (*p=0.008, r=-0.342*) further proved to be associated with the socio-emotional measure prior to corrections.

Table 8: Correlation analysis between inflammatory markers in HEU infants at 6-10 weeks with neurodevelopment measures at 24-28 months

		GM-CSF	IFN-γ	IL-10	IL-12p70	IL-13	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	TNF-α	Lcn2	MMP9
Cognitive	*r*	.005	-.178	-.136	-.089	-.091	-.207	-.126	-.164	-.010	-.181	-.238	-.225	-.178	-.153	-.186
	p-value	.971	.178	.305	.501	.492	.116	.343	.214	.938	.171	.070	.086	.177	.251	.162
Language	*r*	.081	-.106	-.116	-.055	-.025	-.199	.007	-.182	-.070	-.165	-.061	-.221	-.152	-.169	**-.305***
	p-value	.548	.430	.390	.684	.855	.138	.958	.177	.607	.220	.654	.098	.260	.214	**.022**
Motor	*r*	**-.309*#**	**-.339*#**	**-.451**#**	**-.379**#**	-.243	**-.491**#**	**-.308*#**	**-.418**#**	-.237	**-.335*#**	-.239	-.003	-.071	**-.383**#**	**-.289***
	p-value	**.022**	**.011**	**.001**	**.004**	.074	**.000**	**.022**	**.002**	.082	**.012**	.079	.981	.607	**.004**	**.034**
Socio-emotional	*r*	-.043	-.091	-.126	-.096	-.047	**-.342****	-.108	-.254	-.112	.022	.003	.040	-.057	-.156	-.249
	p-value	.744	.493	.342	.469	.725	**.008**	.415	.052	.398	.870	.984	.761	.667	.241	.060
Adaptive Behaviour	*r*	.016	-.050	-.018	-.021	-.094	-.192	-.055	-.173	-.154	-.089	-.062	-.093	-.057	-.165	-.224
	p-value	.907	.704	.891	.877	.481	.145	.680	.191	.244	.504	.643	.484	.671	.216	.091

***p<0.05, **p<0.01. # Remained significant after correcting for multiple comparisons.**

Table 9 presents the correlation analysis of inflammatory markers and neurodevelopment measures of HUU children at 24-28 months. No inflammatory markers proved to have a significant association after corrections on neurodevelopment measures. However, it was shown that impairment in the language neurodevelopment measure was initially associated with GM-CSF (*p=0.034, r=0.162*).

Table 9: Correlation analysis between inflammatory markers in HUU children with neurodevelopment measures at 24-28 months

		GM-CSF	IFN-γ	IL-10	IL-12p70	IL-13	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	TNF-α	Lcn2	MMP9
Cognitive	*r*	.034	-.100	.007	.010	-.071	-.078	-.028	.067	-.060	-.106	-.001	-.023	-.037	-.030	.034
	p-value	.651	.184	.929	.892	.347	.299	.708	.376	.427	.157	.990	.759	.625	.688	.654
Language	*r*	**.162***	-.061	-.001	-.006	-.101	-.057	-.017	.077	-.040	**-.184***	-.051	-.077	-.089	.083	.054
	p-value	**.034**	.426	.989	.938	.191	.464	.829	.320	.601	**.016**	.507	.315	.248	.282	.482
Motor	*r*	**.211****	-.055	.025	.067	.015	-.029	-.017	.090	.002	-.076	-.003	-.102	-.091	-.017	.002
	p-value	**.005**	.470	.746	.378	.846	.699	.819	.235	.980	.315	.965	.179	.230	.819	.977
Socio-emotional	*r*	.081	-.062	.041	.006	-.075	-.063	-.054	-.033	-.046	-.137	-.073	-.065	-.014	-.077	-.104
	p-value	.283	.412	.589	.934	.321	.402	.472	.663	.540	.068	.332	.388	.856	.307	.166
Adaptive Behaviour	*r*	**.153***	-.084	-.023	.002	-.039	-.060	-.075	.010	-.018	**-.195****	-.099	-.069	-.139	.020	-.071
	p-value	**.041**	.262	.759	.980	.601	.426	.316	.893	.806	**.009**	.188	.361	.063	.791	.347

***p<0.05, **p<0.01**

Table 10 presents correlation analysis of inflammatory markers and neurodevelopment measures of HEU children at 24-28 months. No inflammatory markers were significantly associated with neurodevelopment measures after corrections for multiple comparisons. However, IL-10 (*p=0.010, r=-0.303*) did initially have an association with the cognitive neurodevelopment measure. The language neurodevelopment measure was also initially associated with the inflammatory markers IFN-γ (*p=0.011, r=-0.307*), IL-10 (*p=0.008, r=-0.319*), IL-12p70 (*p=0.019, r=-0.284*), IL-1β (*p=0.048, r=-0.241*) and IL-2 (*p=0.037, r=-0.253*).

Table 10: Correlation analysis between inflammatory markers in HEU children with neurodevelopment measures at 24-28 months

		GM-CSF	IFN-γ	IL-10	IL-12p70	IL-13	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-7	IL-8	TNF-α	Lcn2	MMP9
Cognitive	*r*	-.032	-.191	**-.303***	-.166	.018	-.148	-.148	-.118	.013	-.106	-.177	.010	-.054	.207	-.007
	p-value	.793	.111	**.010**	.166	.884	.217	.219	.329	.916	.381	.139	.933	.654	.081	.956
Language	*r*	-.234	**-.307***	**-.319****	**-.284***	.029	**-.241***	**-.253***	-.217	-.049	-.012	-.140	-.025	-.083	.159	-.001
	p-value	.055	**.011**	**.008**	**.019**	.817	**.048**	**.037**	.076	.690	.922	.255	.842	.503	.193	.991
Motor	*r*	-.050	-.073	-.215	-.143	.089	-.124	-.011	-.154	.100	.091	-.028	.105	-.027	.112	.023
	p-value	.690	.562	.086	.256	.480	.326	.934	.221	.429	.473	.825	.405	.830	.371	.856
Socio-emotional	*r*	-.011	-.060	-.042	-.093	.148	-.143	-.018	-.088	.081	.185	.018	.124	.025	.009	-.152
	p-value	.929	.618	.730	.439	.218	.235	.882	.463	.505	.123	.879	.305	.836	.940	.202
Adaptive Behaviour	*r*	-.134	-.224	-.216	-.151	.078	-.152	-.151	-.184	.082	.031	-.181	.079	-.183	.060	.008
	p-value	.264	.061	.070	.209	.516	.205	.208	.125	.495	.801	.131	.515	.126	.614	.944

p<0.05,p<0.01**

Table 11 shows the linear regression analysis for the motor neurodevelopment measure with inflammatory markers for infants at 6-10 weeks, which proved to be significant as indicated previously in *Table 8*. The inflammatory markers GM-CSF, IFN-γ, IL-10, IL-12p70, IL-1β, IL-2, IL-4, IL-6 and Lcn2 all proved to be significantly associated with impaired motor function ($p<0.05$).

Table 11: Linear regression analysis of motor functioning with inflammatory markers in infants at 6-10 weeks after adjustments for covariates

	B (SE)	β	p
GM-CSF	-0.018 (0.01)	-0.193	0.009
IFN-γ	-0.009 (0.01)	-0.121	0.003
IL-10	-0.11 (0.01)	-0.151	0.001
IL-12p70	-0.017 (0.01)	-0.226	0.002
IL-1β	-0.014 (0.01)	-0.203	0.003
IL-2	-0.015 (0.01)	-0.203	0.005
IL-4	-0.016 (0.01)	-0.164	0.031
IL-6	-0.017 (0.01)	-0.174	0.017
Lcn2	-0.006 (0.01)	-0.144	0.010

[#]**covariates**: Residential area, fever at time of visit, maternal smoking during pregnancy, maternal alcohol use during pregnancy, gestational age, maternal socioeconomic status and body mass index (BMI) at 6–10 weeks postpartum, gestational age, birth weight, infant sex and breastfeeding.

4. DISCUSSION

The aim of this thesis was to delineate the relationship between the inflammatory environment of mothers living with HIV on antiretroviral therapy (ART) and their children, and the association between inflammatory markers and neurodevelopment in HEU children.

There were three main findings. Firstly, contrary to our first hypothesis we found that mothers living with HIV had decreased levels of the inflammatory markers GM-CSF and MMP9 compared to uninfected mothers. Secondly, also contrary to our hypothesis, we found that the inflammatory markers IFN-γ and IL-1β were decreased in HEU infants at 6-10 weeks compared to HUU infants. At 24-28 months serum levels of the inflammatory markers IFN-γ, IL-1β, IL-2 and IL-4 were found to be significantly decreased in HEU children compared with HUU children. Despite HEU children having reduced serum levels of inflammatory markers compared to HUU children the results still suggest that the altered inflammatory environment of the mothers living with HIV may predict the inflammatory environment of their children longitudinally. Finally, in line with our third hypothesis, we found that there was an association between increased inflammatory markers in HEU infants at 6-10 weeks and impaired motor neurodevelopment at 24-28 months.

The first finding that mothers living with HIV had significantly decreased serum levels of inflammatory markers is not consistent with the literature outlined in the literature review. It was expected that mothers living with HIV would present an elevated inflammatory environment rather than an inhibitory inflammatory environment. Most studies conducted so far indicated that three pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α,

which play an important role in immune regulation during pregnancy, proved to be elevated in pregnant women living with HIV (35,41). One potential explanation for the findings in this study may possibly be explained by the subtype or clade of HIV present within our infected mothers. The subtypes of HIV are believed to represent different lineages of HIV and have been associated with different geographical regions (143). The mothers included in this study are of the clade C subtype. Clade C is the clade most prevalent within South Africa (143). Clade C tends to present a more immunosuppressive profile in comparison to other clades such as Clade B, which has shown to have a pro-inflammatory affect and result in increased neuroinflammation due to the presence of the Tat protein (47,48). The Clade C subtype affects the Tat protein sequence; there are polymorphisms that affect specific sites on the Tat protein and consequently affect its biological functions. These polymorphisms include a serine substitution at residue 31 (C31S) and a serine substitution at residue 57 (R57S) (48). This therefore may explain the inconsistency when comparing our findings to the literature, as most of the literature looks at studies outside of South Africa, where the clades present a more inflammatory profile compared to clade C present within this study. It is also important to note that Clade C, in comparison to other clades, has been reported to have a greater association with HIV associated dementia (HAD) and HIV associated neurocognitive disorders (HAND) in South Africa (144).

Furthermore, the fact that all mothers living with HIV included in this study were on ART may also have had an impact on the mothers' immune system and resulted in the inhibitory inflammatory environment reported in our study. The ARVs given to the mothers living with HIV may have

allowed their immune system to combat the elevation in inflammatory markers caused by the virus resulting in the significantly decreased serum levels of inflammatory markers found in the mothers in this study. This theory is supported in a review conducted by Hileman *et al*, which also suggests that ART drastically reduces systemic inflammation and immune activation (145). It may be important to note that the immune response can be linked to ethnicity as well as pregnancy outcome. For example, a study by Velez *et al* indicated significant differences in the cytokine levels between African Americans and Caucasians (146). IL-1β was shown to be co-regulated with several other cytokines in the amniotic fluid of African American women with pre-term labour, however this was not found in Caucasian women, indicating a difference in the underlying process leading to pre-term labour (146).

Due to the fact that we found inflammatory markers significantly decreased in HEU infants and children, it is fair to suggest that the mothers' inflammatory environment may have had a longitudinal prediction on the inflammatory environment of the children, however further statistical analysis is needed to confirm this. HEU infants and children at 6-10 weeks and 24-28 months presented significantly reduced serum levels of inflammatory markers, similar to that of their mothers. This suggests that the inhibitory immune environment in the mothers living with HIV may have impacted the immune environment of their children, which presented itself more acutely later on in life. This supports the concept presented by Clerici *et al*, which suggests that changes in the immune system of HEU children persist to later on in life (50). Additionally, studies have indicated that the first period of postnatal life up until 6 months is key in the programming of the immune system

(56,57). Furthermore, it is important to recall that a study by Kakkar *et al* reported that HEU infants might have a poorer developmental outcome as a result of a transferred state of immunodeficiency (55).

However, it may also be possible that the significantly decreased levels of inflammatory markers in children at 24-28 months, is due to their immune system attempting to regulate itself as a result of exposure to viral particles. Suggesting that the inflammatory environment of the children is independent to that of their mothers. However, this is unlikely as the majority of mothers were virologically suppressed and therefor the exposure of the fetus *in utero* to viral particles would be limited. Notably, the mothers living with HIV had lower GM-CSF levels. GM-CSF plays an important function in the survival and activation of mature myeloid cells and contributes to the maintenance of the innate immune system (147). Animal studies have indicated that a reduction in GM-CSF lead to an impaired development of the immune system and a reduced immune response (148–150). It is possible; therefore, that lower GM-CSF during pregnancy in mothers living with HIV can contribute to the altered inflammatory environment in the HEU children, which is reflected by a reduction in inflammatory markers, IFN-γ, IL-1β, IL-2 and IL-4 in infants at 6-10 weeks and children at 24-28 months.

The decreased serum levels of inflammatory markers in the HEU infants and children at 6-10 weeks and 24-28 months is contrary to what is indicated in the majority of the literature that explores inflammatory markers in HEU children (21,56,74). For example, unlike in this study Bunders *et al* (62), found increased levels of IL-1β in HEU infants. Additionally, a study by Reikie *et al* (65), also reported a pro-inflammatory

environment in HEU children. However, there was literature that indicated that the immune system of HEU children did not differ to that of HUU children (57). Furthermore, some studies did find reduced levels of inflammatory markers, IL-4, IL-7 and IL-12 in HEU infants. It is also worth mentioning that other immunological factors, such as antibodies, cross the placenta during pregnancy, which may have impacted the immune system of the children later in life.

When looking at the linear regression analysis and the relationship of the inflammatory markers with the neurodevelopment measures, the study groups were assessed individually or within-group. In the case of HEU infants at 6-10 weeks, we found that impaired motor neurodevelopment at 24-28 months was significantly associated with an increase in serum levels of the majority of inflammatory markers. This is most likely due to the fact that the immune system of the HEU infants is more sensitive and therefore more susceptible to changes in levels of inflammatory markers. The fact that the HEU infants were recently exposed to the HIV virus *in utero* explains the sensitivity of their immune system as the pathology of the virus is still impacting the immune system and therefore the neurodevelopment of these infants. This results in these infants having a lower threshold when it comes to dealing with fluctuations in inflammatory markers, unlike HUU infants. This is supported by the concept presented by Pollmacher *et al* that even low levels of cytokines can influence complex brain functions (84). In addition, a study conducted by Gilman *et al*, indicated that decreased serum levels of IL-8 was associated with higher risks of neurological abnormalities in children at 4 years of age (151).

On the other hand, the immune system of HUU infants are assumed to be functioning under typical physiological conditions and therefore may be able to adapt more easily to fluctuations in inflammatory markers. Previous findings in animal models also suggest that maternal immune compromise affects neurodevelopment via changes in foetal inflammatory markers (28). Ultimately, this suggests that the time-point of 6-10 weeks is crucial to neurodevelopment, particularly motor neurodevelopment. This is supported by Black and Merseth (94), who highlight the importance of the neurodevelopmental process around the time of birth. It is important to mention that, although the literature is not entirely consistent; evidence from most recent studies relevant to HEU children did indicate impaired motor neurodevelopment (4).

As outlined earlier in the literature review, inflammatory markers and cytokines can impact neurodevelopment through numerous systems, including glial cells, the HPA axis and neurotransmitter systems, at various time points *in utero* and in the early stages of life. The fact that HEU children are being exposed to HIV *in utero* and are subject to its consequences on their inflammatory environment strongly supports the idea that HEU children will have a sensitive immune system. This theory is corroborated by the review conducted by Abu-Raya *et al*, which highlights that both HIV exposure and ARV exposure impacts the immune system of the children (23). On the other hand, the immune system of HUU infants' functions at normal physiological conditions and therefore can adapt much more easily to fluctuations in inflammatory markers.

It is important to note that impairment in the language neurodevelopment measure in HEU children was also found to be initially

associated with increased serum levels of IFN-γ, IL-10, IL-12p70 and IL-7 in the mothers living with HIV. However, this was not found to be significant after correction for multiple comparisons. Additionally, the same association was found in HEU children at 24-28 months, which also did not survive corrections for multiple comparisons. It is still important to mention though as many studies previously found that language was impacted significantly by exposure to HIV, as reported in the review conducted by Le Doaré *et al* (109). Furthermore, another recent study conducted on a subsample from the DCHS by Wedderburn *et al* (152), found significantly lower language scores in HEU children compared with HUU children, which further supports these findings.

Several limitations of this study deserve emphasis. Firstly, although we found the inflammatory environment of the mothers to be similar to that of their children, the mechanism behind intergenerational transmission is still uncertain. Secondly, despite controlling for an array of covariates, it is still possible that other factors may have an influence on the neurodevelopment of the children. For example, many studies have shown that prenatal stress and mental illness in mothers may impact the neurodevelopment of their children (153). This was not accounted for in our study. Thirdly, different types of ARVs were not controlled for. As mentioned earlier, ARVs most likely impacted the immune system of the mothers resulting in an inhibitory inflammatory environment. Finally, this study was isolated to a specific subset of the population in South Africa, where all mothers in this study came from a poorer socioeconomic background. Low to middle income (LMIC) studies conducted has shown that this has an impact on the neurodevelopment of the children (154). Although there is evidence in this study to prove that motor and possibly

language neurodevelopment measures are affected, the actual neurobiological mechanism of these dysfunctions still remains inscrutable. This is due to the fact that the Bayley-III used to measure the neurodevelopment of the children encompasses a more general rather than targeted approach.

Given the limitations outlined in this study, a number of future research directions can be suggested. Firstly, future studies should include a more diverse subset of the population from different socioeconomic backgrounds. Secondly, controlling for different types of ARVs would also be beneficial to try and distinguish whether or not adaptations to the immune system of the mothers living with HIV and their children are due to infection or ARV exposure. Thirdly, in order to identify specific domain regions in the brain that are being impacted and causing impaired motor neurodevelopment, future studies should consider using a more targeted neuropsychological assessment. For example, one could assess magnetic resonance imaging (MRI) scans of infants and children to evaluate which particular regions of the brain are being affected. Assessments within the National Institutes of Health Toolbox, which includes over 100 stand-alone measures that assess cognition, emotion, motor and sensation, could also be used to monitor neurodevelopment (155). Finally, although this study did look at two time-points in HEU children, which is quite extensive considering current studies conducted on HEU children, it would be beneficial to include more time-points. For example, the cord bloods of the HEU infants could be evaluated, and serums samples of the children at 12 months of age and then later on in life as the children develop through school age towards adolescence. Lastly, longitudinal analysis could be conducted on the serum levels of inflammatory markers

in order to get more clarity on whether or not the mothers' immune system did indeed predict the immune system of their children.

5. CONCLUSION

This study shows that maternal HIV infection is associated with immune regulation, with results indicating lower serum levels of inflammatory markers in mothers living with HIV on ART and their HEU children. The results further indicated that an altered immune system in the early stages of life of HEU infants is associated with impaired motor neurodevelopment of the children later in life. The study also presented the likelihood that the mother's immune system did predict that of their childs' longitudinally. This highlights the need to intervene at an early stage in the development of HEU children to prevent neurodevelopment impairment later on in life.

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7. Appendix

Appendix 1: Comparison of mean ln-transformed values of inflammatory markers between mothers living with HIV and uninfected mothers

	Mean (SD)					
	HIV- (n=190)	HIV+ (n=77)	t	95% CI		p-value
GM-CSF	4.0138 (0.90)	3.6475 (0.85)	3.052	.12994	.60256	**.003**[#]
IFN-γ	2.0926 (0.68)	1.9633 (0.83)	1.315	-.06431	.32308	.190
IL-10	2.6231 (0.97)	2.4113 (1.22)	1.493	-.06750	.49098	.137
IL-12p70	1.3389 (0.75)	1.1674 (0.89)	1.608	-.03855	.38165	.109
IL-13	1.8012 (1.00)	1.5341 (1.07)	1.937	-.00437	.53864	.054
IL-1β	.4600 (0.70)	.2282 (0.83)	2.316	.03473	.42891	**.021**
IL-2	.8579 (0.91)	.6429 (1.09)	1.653	-.04117	.47115	.100
IL-4	3.4384 (1.01)	3.0900 (1.26)	2.370	.05894	.63777	**.019**
IL-5	.9154 (0.76)	.8691 (0.86)	.432	-.16463	.25721	.666
IL-6	1.0005 (0.96)	.9871 (1.06)	.100	-.24988	.27664	.920
IL-7	2.4079 (0.55)	2.3732 (0.70)	.431	-.12398	.19345	.667
IL-8	1.6418 (0.93)	1.6635 (0.91)	-.173	-.26804	.22465	.862
TNF-α	1.7535 (0.54)	1.8991 (0.62)	-1.923	-.29474	.00351	.056
Lcn2	5.1494 (0.60)	5.0213 (0.61)	1.566	-.03297	.28903	.119
MMP9	7.0693 (0.68)	6.6142 (0.75)	4.824	.26937	.64087	**.000**[#]

Appendix 2: Comparison of mean ln-transformed values of inflammatory markers between HEU and HUU children at 6-10 weeks

	Mean (SD)						
	HUU (n=155)		HEU (n=61)		t	95% CI	p-value
GM CSF	2.5480	(0.98)	2.2258	(1.50)	1.865	-.01839	.66284	.064
IFN-γ	1.6093	(0.92)	1.2071	(1.10)	2.757	.11462	.68962	**.006**[#]
IL-10	2.5587	(0.89)	2.4162	(0.91)	1.057	-.12311	.40811	.292
IL-12p70	0.7451	(0.92)	0.4567	(1.07)	2.000	.00424	.57254	**.047**
IL-13	0.9104	(1.47)	1.0524	(1.57)	-.632	-.58538	.30129	.528
IL-1β	-0.0940	(0.88)	-0.4650	(0.91)	2.771	.10712	.63484	**.006**[#]
IL-2	0.0810	(0.92)	-0.1400	(1.00)	1.566	-.05721	.49921	.119
IL-4	2.6402	(1.22)	2.2595	(1.28)	2.048	.01432	.74702	**.042**
IL-5	0.2544	(0.96)	0.3970	(0.86)	-1.022	-.41763	.13244	.308
IL-6	0.6139	(1.20)	0.4270	(1.36)	.994	-.18353	.55732	.321
IL-7	1.7684	(0.72)	1.8026	(0.81)	-.305	-.25545	.18706	.761
IL-8	2.3926	(0.10)	2.3708	(1.11)	.140	-.28357	.32706	.889
TNF-α	2.9360	(0.55)	3.0252	(0.82)	-.928	-.27846	.10017	.354
Lcn2	4.6456	(0.53)	4.5154	(0.58)	1.573	-.03293	.29325	.117
MMP9	6.1367	(0.72)	6.0666	(0.87)	.603	-.15892	.29914	.547

Appendix 3: Comparison of mean ln-transformed values of inflammatory markers between HEU and HUU children at 24-28 months

	Mean (SD)							
	HUU (n=190)		HEU (n=76)		t	95% CI		p-value
GM CSF	4.7496	(0.81)	4.5983	(0.95)	1.305	-.07696	.37960	.193
IFN-γ	2.2322	(0.58)	2.0086	(0.57)	2.851	.06914	.37790	**.005#**
IL-10	2.9874	(0.66)	2.8362	(0.67)	1.677	-.02630	.32879	.095
IL-12p70	1.4775	(0.61)	1.2868	(0.64)	2.278	.02585	.35558	.024
IL-13	2.3888	(1.07)	2.2004	(0.97)	1.336	-.08918	.46598	.183
IL-1β	0.6314	(0.76)	0.2678	(0.67)	3.664	.16822	.55902	**.000#**
IL-2	0.9467	(0.66)	0.6757	(0.73)	2.924	.08852	.45336	**.004#**
IL-4	3.4085	(0.93)	3.0853	(0.99)	2.510	.06961	.57668	**.013#**
IL-5	1.3115	(0.79)	1.1957	(0.70)	1.114	-.08879	.32024	.266
IL-6	1.2464	(0.85)	1.1622	(0.90)	.717	-.14731	.31587	.474
IL-7	2.3037	(0.51)	2.2253	(0.54)	1.108	-.06084	.21756	.269
IL-8	2.5692	(1.12)	2.4684	(1.06)	.675	-.19329	.39485	.500
TNF-α	2.5764	(0.66)	2.5338	(0.74)	.460	-.13998	.22527	.646
Lcn2	5.1152	(0.58)	5.0838	(0.57)	.404	-.12152	.18431	.686
MMP9	6.6942	(0.61)	6.6613	(0.58)	.404	-.12731	.19309	.686