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Association between head circumference and monocyte function in neonates born to mothers with and without HIV at Kalafong Provincial Tertiary Hospital.

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Declaration

I, Brandon Kleynhans, hereby declare that the dissertation herein submitted to the University of Pretoria for the degree MSc. Human Physiology and the work contained therein is my own original work and has not previously, in its entirety or in part, been submitted to any university for a degree.

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Executive Summary

Current data show that an increasing number of women of reproductive age are living with the human immunodeficiency virus (HIV), particularly in Southern Africa. While antiretroviral therapy (ART) reduces the risk of perinatal transmission of HIV from a mother to her child, this in turn leads to more babies being born exposed to HIV and ART. This presents a stark problem as research suggests that mere exposure to HIV, even if the baby is HIV negative, may be linked to increased rates of suboptimal development, as well as increased morbidity and mortality. For these reasons, it is imperative that our understanding of the subsequent development of children born with exposure to HIV and ART is improved.

Research suggests that children living with HIV, when compared to children not living with HIV, tend to underperform on general cognitive tests, processing speed, and visual-spatial tasks and also tend to show increased risk of developing psychiatric and mental health-related issues. This is a cause for concern as these children approach school-going age and will be expected to successfully integrate into an education environment. This is a particular concern in South Africa, where up to 30% of children born are HIV-exposed-uninfected (HEU).

The role of monocytes and macrophages in the early development of the human brain is an ever-growing field of research. Classically activated macrophages, derived from circulating monocytes, are known to exert neurotoxic effects and, as such, an imbalance of presenting macrophage phenotypes has been associated with a variety of inflammatory conditions. This balance is essential as microglia, in the standard context, contribute to tissue remodelling, repair, and neurogenesis. Less is known about monocyte function and the effect different functional phenotypes might have on the developing brain. The aim of this study was therefore to assess the association between monocyte function and early-life brain development, as reflected by the head circumference (HC).

The results of this study were generated and assessed through two main methods: anthropometric measures and cytokine levels before and after whole blood stimulation (WBS). No differences were observed for HC. However, for the other anthropometric measurements, although no statistically significant differences were observed at birth, notable differences emerged at the 6-month and 12-month time-points. HEU infants were significantly smaller at 12 months of age, showing less optimal growth progression than that of the HIV-unexposed-uninfected (HUU) group at this point. These findings indicate stunting in growth linked to HIV exposure in the HEU infants.

When the cytokine data were considered, the HEU group were more likely to over-express pro-inflammatory cytokines before and after WBS and, notably, under-express anti-inflammatory cytokines. This observation could be as a result of their pre-birth exposure to an inflammatory intra-uterine environment.

Significant positive correlations were observed between HC and the monocyte growth factor, granulocyte monocyte colony stimulating factor (GM-CSF) after stimulation with lipopolysaccharide at birth, as well as tumour necrosis factor-alpha (TNF-α) after stimulation with Polyinosinic:polycytidylic acid at birth. Monocytes are the most important source of TNF-α in humans. Although not providing direct evidence, the positive correlation between these factors and HC indirectly supports the hypothesis that monocytes are important players in brain development.

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As this study considered a relatively small population, these observations are tentative. However, it supports the hypothesis that it would be worth running similar cytokine-based tests in a larger cohort. Such further study may indeed show similar conclusions, in that a pro-inflammatory monocyte profile could be observed in the HEU group, but the converse could of course also be true. Further study would also be necessary to substantiate the link between HC and the observed cytokine data, as the increase in population size may reveal more robust observations. The absence of a significant difference in the HC metric may also be linked to the sample size and is still worth assessing this as part of a larger study, especially since HEU infants tended to fall in the lowest HC quantile and that this difference became more pronounced over time.

Despite the small sample size, this study demonstrated clear differences between the growth trajectory and monocyte profiles of HUU and HEU infants. The importance of further investigation lies in the fact that these observations may lead to an improvement in the understanding of the underlying pathophysiology of stunting in HEU infants. Such information could ultimately lead to interventions that might improve the ante- and postnatal care of HEU infants, which may, in turn, contribute to better outcomes later in life, particularly at school-going age.

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List of Abbreviations

AIDS	Acquired immunodeficiency syndrome
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionate
APGAR	Appearance, Pulse, Grimace, Activity, Respiration
ART	Antiretroviral therapy
ARV	Antiretroviral
BDNF	Brain derived neurotrophic factor
BMI	Body mass index
CD	Cluster of Differentiation
cm	Centimetre
CNS	Central nervous system
DNA	Deoxyribonucleic acid
EAA	Excitatory amino acid
EDC	Electronic data capture
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
etc.	Et cetera
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAD	HIV-associated dementia
HC	Head circumference
HEU	HIV-exposed-uninfected
HIV	Human immunodeficiency virus
HUU	HIV-unexposed-uninfected
ID	Identity code
IFN-ɣ	Interferon gamma
IL	Interleukin
IQ	Intellectual Quotient
IQR	Inter-quartile range
kg	Kilogram
KH	Kalafong Tertiary Provincial Hospital
LBW	Low birth weight
LPS	Lipopolysaccharide
MFI	Mean fluorescent intensity

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MHC	Major histocompatibility complex
mL	Millilitre
MRI	Magnetic resonance imaging
MUAC	Mid-upper arm circumference
NDI	Neurodevelopmental impairment
PAF	Platelet-activating factor
PAMPs	Pathogen-associated molecular patterns
Poly I:C	Polyinosinic:polycytidylic acid
RBC	Red blood cells
RPMI	Roswell Park Memorial Institute
RNA	Ribonucleic acid
TGF-β	Transforming growth factor beta
Th	T-helper
TLR	Toll-like receptors
TNF	Tumour necrosis factor
W4A	Weight for age
W4L	Weight for length
WBS	Whole blood stimulation
WHO	World Health Organization
α	Alpha
μL	Microlitre
°C	Degrees Celsius

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Chapter 1 – Introduction and Literature Review

1.1 General Background

Infection with the human immunodeficiency virus (HIV), while no longer an ever-present threat to most regions, is still to this day a dramatic problem in sub-Saharan Africa(1). In 2020, roughly 37.7 million people worldwide were living with HIV and approximately 1.5 million people were newly infected that year(2). Two thirds of these new infections occurred in the sub-Saharan Africa region, showing that this is the area in the world that is the most affected by HIV(1).

While there is unfortunately no cure for HIV, current treatment has improved dramatically in recent years. Antiretroviral therapy (ART) is currently the standard mode of managing the virus in those infected(3). Antiretroviral therapy is effective in reducing the rate of transmission between partners (horizontal transmission) and, the focus for the purposes of this study, from HIV-infected mothers to their babies (vertical transmission).

Studies show that vertical transmission rates have dropped to below three percent globally(4). This is extremely positive as more children are born uninfected; however, there is still cause for concern. There is still a lot that is unknown about the interaction between ART and pregnancy, particularly when concerned with the subsequent growth and development of the child after birth. Reports have shown that HIV exposed uninfected (HEU) children generally experience more developmental issues and higher morbidity and mortality rates as compared to HIV-unexposed-uninfected (HUU) children(5). The latter is largely attributed to secondary infections(6,7).

There are two main schools of thought as to why these observations have been made with regards to increased morbidity and mortality in HEU children. The first hypothesizes that the antiretroviral (ARV) medications have a direct negative effect on the immune system of the developing foetus, due to the fact that ARVs, such as zidovudine, are known to have toxic effects on mitochondria and can also cross the placental barrier(8). HEU infants typically present with lower counts of red blood cells (RBC), neutrophils, and lymphocytes. This could potentially be explained by the inhibition of haematopoietic progenitor cells, which is an effect observed during treatment with ARVs like zidovudine. It is therefore proposed that ARV medications may have a negative impact on a child's innate immunity(4). The second thought process hypothesizes that the foetus' exposure to the mother's HIV-related antigens, pro-inflammatory cytokines and chemokines, may cause an immune response in the foetus, regardless of ART. This leads to the speculation that the change in the intra-uterine environment of HEU infants may affect their development, growth, and innate immunity after birth, even when not infected with HIV(4).

The innate immune system is the first line of defence for the body against foreign substances and contaminants; as such, components of this system are highly important for the development of young children(9). One such component is monocytes/ macrophages, which are highly versatile and have several roles throughout the body and at different stages of development. They function to react to different environmental stimuli as defensive phagocytic entities(9). One of the most important functions of macrophages is the role that they play in the development of the brain, particularly in the early stages of life. Specifically, a macrophage sub-type located exclusively in the central nervous system (CNS), known as microglia, is thought to be involved in early neurodevelopment by playing a role in determining the circuitry of the brain(10).

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Macrophages are present as two main phenotypes; classically activated (M1) and alternatively activated (M2). Typically, the M1 phenotype exerts pro-inflammatory effects while, conversely, the M2 phenotype is anti-inflammatory in nature. Macrophages are thought to function and fluctuate along a dynamic continuum but the two main phenotypes, M1 and M2, represent two opposite ends of the scale on a highly dynamic state of activation pathways(11). Both of these presenting phenotypes are important for healthy functioning: M1 macrophages function in infection control while the M2 variants contribute to cell repair and tissue remodelling. However, balance is extremely important; an imbalance in presentation of these phenotypic varieties has been linked to various diseases and inflammatory conditions, such as; autoimmune diseases, neurodegenerative disorders, cardiovascular diseases and metabolic diseases(12). The M1 phenotype is typically stimulated by microbial products, such as lipopolysaccharide (LPS), and by pro-inflammatory cytokines that are secreted upon viral infection/exposure (e.g. interferon-gamma [IFN-ɣ] and tumour necrosis factor-alpha [TNF-α]), as well as activation of Toll-like receptor (TLR) signalling pathways(12). The observation that M1 polarized microglia exert cytotoxic effects on neurons and oligodendrocytes is the reason that this study is warranted(12). It is essential to understand how macrophage polarization and function in HEU children affect early development so as to contribute to the continued support of these children through better understanding of the causes behind these observed issues.

1.2 Physiological Background

The human immune system is a complex orchestration of lymphoid tissues, immune cells, and chemicals that interact and execute an immune response in order to protect the body. It has the ability to recognise and destroy harmful pathogens as well as remove abnormal 'self' cells and dead or damaged cells. It has two divisions that help it achieve the abovementioned functions: innate and acquired immunity(9). Acquired immunity is a slower, more specific response to pathogens and is mediated by lymphocyte cells. Once they come into contact with a pathogen, lymphocytes undergo clonal expansion, creating short-lived effector cells, which fight the pathogen, and memory cells, which, on re-exposure, will generate a faster, stronger, and more specific immune response(9).

Innate immunity is less specific but more rapid and is the body's first line of defence against a pathogen. This immunity uses a series of physical and chemical barriers as well as cell-mediated responses. The physical and chemical barriers include skin, mucous linings, and lysozyme secretions(9). Phagocytic cells, namely neutrophils and macrophages, are responsible for the cellular response. The precursors of macrophages are monocytes, which are made in the bone marrow, circulate in the blood, and are transported to the tissues where they mature into tissue macrophages when needed(9). Some macrophages reside in tissue where they patrol the environment, ingesting and digesting dead or damaged cells, while others can be found as stationary, resident bodies(9).

Macrophages are important phagocytic and antigen-presenting cells. They are attracted to a site of injury or infection by chemotaxins (chemical signals released by damaged tissue or bacterial toxins)(9). Once in the tissue, macrophage recognition receptors (e.g. TLRs), bind to pathogen-associated molecular patterns (PAMPs), and initiate phagocytosis as well as release cytokines to attract other immune cells and initiate inflammation(9). Once the pathogen has been ingested and digested, major histocompatibility complex (MHC) II proteins on the cell surface present digested pathogen particles to T-helper cells and a further response is initiated (acquired immunity)(9).

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Traditionally it was thought that macrophages only differentiated from circulating blood monocytes; however, it is now known that some tissue resident macrophages differentiate from embryonic progenitors that seed developing tissues before birth and are maintained by local proliferation in adulthood(13). This pool of resident tissue macrophages can also be replenished from the circulating monocytes available in the blood if the need arises(13).

Macrophage activation pathways and polarisation are controversial and contested. Phenotypically, macrophages vary on what seems to be a spectrum, taking on different roles depending on environmental stimuli, cytokines, modulators, and growth factors. For the purpose of this study, the extremes of activation phenotypes, M1 and M2, are used as they are widely accepted(11).

M1 refers to classically activated and M2 to alternatively activated macrophages. In response to an inflammatory stimulus, macrophages are stimulated by pro-inflammatory mediators such as IFN-ɣ and TNF-α to take on a classical activation state or M1 phenotype(13). After the inflammatory response, macrophages are influenced by interleukin (IL)-4 and IL-13, produced in the T-helper (Th)2 response, and take on an alternative activation state or M2 phenotype(13). M2 macrophages have decreased pro-inflammatory cytokine production and less phagocytic and killing ability. Instead, anti-inflammatory mediators, such as IL-10 and transforming growth factor (TGF)-beta, are produced which aid in controlling the immune response(13). Alternative activation of macrophages plays an important role in wound healing, tissue repair and remodelling, angiogenesis, and tissue patterning(13). M2 macrophages can be further divided into M2a, M2b and M2c functional subsets. M2a and c dampen immune response and promote tissue repair whereas M2b has both pro- and anti-inflammatory functions(10).

These categories of macrophages can be identified by the presence and abundance of certain surface markers. Classically activated macrophages (Cluster of Differentiation [CD]14++CD16-) express high levels of CD14 but no CD16, intermediate macrophages (CD14++CD16+) express high CD14 and low CD16, and non-classically activated macrophages (CD14+CD16++) express low levels of CD14 and high levels of CD16(14).

1.3 Role of Macrophages in Brain Development

Apart from the important role macrophages play in innate and acquired immunity, they also have a distinctly different and significant function in brain development. Microglia manage neuronal cell death, neurogenesis, and synaptic interactions in the brain(15).

There are two sources of CNS macrophages: primitive embryonic haematopoiesis and definitive embryonic haematopoiesis. Primitive embryonic haematopoiesis results from immature progenitor stem cells that are found in the brain early in embryogenesis, arise from the yolk sac, and proliferate locally throughout adulthood(15). Definitive embryonic haematopoiesis results from bone marrow-derived progenitor cells.

Under normal physiological conditions, M0 (resting state) microglia patrol their microenvironment. Injury or infection causes microglia to develop a pro-inflammatory M1 phenotype in order to fight infection or control injury(16). After this inflammatory state, microglia take on an M2 anti-inflammatory phenotype in order to clean up debris and facilitate tissue remodelling and repair(17). Microglial brain-derived neurotrophic factor (BDNF) increases neuronal phosphorylation of kinase receptor, an important mediator of synaptic plasticity, which leads scientists to believe that they play a role in promoting learning-related synapse formation(15). Due to the fact that microglial cells have many important functions in

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the brain, adverse effects may result when there is an imbalance in activation pathways, specifically in the case of over- or prolonged activation of M1. M1 macrophages release pro-inflammatory cytokines, which, if not properly regulated and cleared away, can cause tissue damage(15). The M1 phenotype is stimulated by microbial products (such as LPS)(18) and pro-inflammatory cytokines, such as those classically found during viral exposure (IFN-ɣ and TNF-α), or activation of TLR signalling pathways(12). M1-polarized microglia and/or macrophages exert cytotoxic effects on neurons and oligodendrocytes and may therefore impact on neurological development of the foetal brain.

This impact on the brain has been clearly described in studies that assessed neurological development in infants living with HIV(19). It is well described that HIV infection in infants can present with impaired brain growth and secondary microcephaly. The typical onset is usually noted between two and four months of age(19). This period of post-natal neurological development is known to be particularly vulnerable to excitotoxic neuronal injury because of the active synaptogenesis that takes place at this age which is linked to excitatory amino acid (EAA) receptors(19). The over expression of pro-inflammatory phenotypical perivascular macrophages results in a state of chronic inflammation which can manifest as diffuse microglial activation as well as reactive astrogliosis(19).

There are several inflammatory products of activated microglia which have been shown to act as neuronal toxins, such as TNF-α and platelet-activating factor (PAF)(19). This toxic effect is exacerbated by the blocking of N-methyl-D-aspartate (NMDA)(20) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)(21) glutamate receptors which may then lead to oxidative stress, neuronal injury, and apoptosis(22,23). This is known as glutamate-mediated excitotoxicity as outlined in Figure 1.1 below. TNF-α is a mediator of this excitotoxic mechanism as it functions to block the high-affinity glutamate uptake of astrocytes at the synaptic cleft(24).

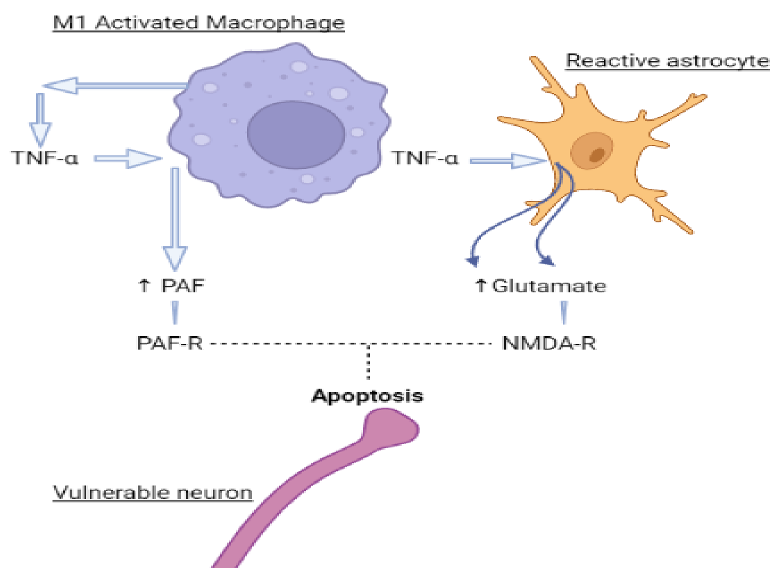

Figure 1.1: Glutamate mediated excitotoxicity. TNF-α release by activated macrophages results in activation of NMDA/AMPA glutamate receptors, leading to neuronal oxidative stress and cell death. High-affinity glutamate uptake receptors on the astrocytes are also downregulated by the increase in TNF-α. This increase may also induce release of PAF, which, through PAF receptors, may act to induce apoptosis (Adapted from source(19)*, created with BioRender.com).*

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This type of excitotoxic neuronal injury may be particularly damaging to the developing brain where extremely active synaptogenesis needs to occur in the early post-natal period. This period of neurological development is particularly dependant on EAA(25). EAA neurotransmitters, such as glutamate, play an extremely important role in synaptogenesis and dendritic differentiation, so over or under production of EAAs is exceedingly important during the two- to four-month age window, where development of the striate cortex is most rapid(26).

1.4 Role of Macrophages in HIV

Macrophages play an important role in the different stages of HIV disease progression(27). HIV targets cells with CD4 receptors and mainly infects CD4+ T cells; however, other immune cells that have low expression of CD4 receptors, such as macrophages, can also be infected(28). Co-receptors, members of the chemokine receptor family, are necessary to induce infection. HIV gains entry into host cells by binding to CD4 receptors and a co-receptor, either CCR5 or CXCR4, fuses with the host cell membrane, and enters the cell. Once inside the host cell, the virus incorporates its viral DNA into the host DNA and uses the cellular machinery to reproduce itself. Later, the host cell bursts, releasing more virus or, alternatively, the virus uses the host membrane as vesicles to bud off(28).

Macrophages have varying lifespans depending on their origin and function. Macrophages derived from blood-circulating monocytes may only survive a few days, whereas resident macrophages, such as microglia in the brain, can survive for weeks, even years(28). HIV infection can be propagated due to the capacity of macrophages to support the HIV replication cycle and their ability to enter almost all tissues and organs in the body(28). This can result in the establishment of HIV reservoirs in long- lived macrophages(29), which poses a problem for virus eradication attempts as the virus can evade innate and acquired immunity. Studies have indicated that, due to these abilities, macrophages are key cells that enable mother-to-child transmission of HIV(28).

Very little is known about the role of macrophages in HEU infants. Since the M1 phenotype is stimulated by microbial products and pro-inflammatory cytokines, such as those classically found during viral exposure(12), and since M1-polarized microglia and/or macrophages exert cytotoxic effects on neurons and oligodendrocytes(30), it is postulated that continuous exposure to HIV, such as found in the foetus or breastfeeding infant of a mother living with HIV, may lead to altered neurological development.

1.5 Neurodevelopmental Impairment in HIV-exposed Infants

Neurodevelopmental impairment (NDI) is of particular concern when infants with a small head circumference (HC) are being considered and it is one of the main reasons for this study. HEU infants have traditionally not been considered to be at increased risk for poor neurodevelopmental outcomes since they are uninfected, and so HIV encephalopathy(19) should not be present. There is, however, a growing literature reporting that post-natal head growth is significantly poorer in HEU children when compared to HUU counterparts(6). It is also well documented that subnormal head size is a predictor for future negative neurodevelopmental outcomes (31–33).

A study based in Zimbabwe observed that head growth in HEU children was significantly stunted as compared to HUU counterparts when followed for the first year of life(6). Similar observations were made by a study conducted in Columbia when the HC of one-month-old infants of HEU status were compared to that of HUU infants and were noted to be significantly

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smaller(34). It has been suggested that these findings may be specific to low-income/developing countries as similar observations have not been made in countries such as the United States of America(35). The impact of the socio-economic environment seems to be supported by another study based in Zimbabwe, in which it was observed that HC and familial low-income background were the most significant risk factors for NDI after adjusting for other factors(31).

These findings were congruent with what was observed during a prospective cohort pilot study, based in Kalafong Hospital (10 Kalafong Road, Atteridgeville, Pretoria, 0008), that was conducted as an initial pilot study to inform the design of the Siyakhula study. The study recruited 40 new mothers who were living with HIV, of which 20 were using ART and 20 were not using ART. A further 20 new mothers without HIV were also recruited. This small feasibility study suggested that the development of HEU infants may be adversely affected by HIV exposure and, furthermore, are vulnerable to detrimental effects of suboptimal nutrition(36).

1.6 Monocyte Function

When macrophage type and function are considered, the importance of monocyte function cannot be overestimated: this is the main focus of this study. Monocytes, which give rise to tissue macrophage populations, are also largely heterogenous in nature(37). Pro-inflammatory monocytes tend to give rise to inflammatory (classically activated) M1 macrophages and dendritic cells, while anti-inflammatory monocytes perform a patrolling function and generally give rise to M2 macrophages(37). Differentiation from monocytes to macrophages with regards to type and function, is outlined in Figure 1.2. The concept of monocyte differentiation is important to note as the pre-natal environment of HEU babies presents the possibility of pro-inflammatory cytokine exposure (38), which may in-turn lead to increased pro-inflammatory monocyte and macrophage populations in the post-natal innate immune system. As mentioned in sections 1.2 and 1.3 above, the importance of the balance between monocyte and macrophage populations can be linked to several factors impacting early neurological development. Studies have shown that pre-natal exposure to inflammatory challenges is a considerable risk factor for the development of cognitive disorders(39). In rodent models, it has been shown that maternal immune activation causes elevated brain immune response to stressors as well as increased susceptibility to cognitive impairment in the resultant offspring later in life(40).

The negative effects of this imbalance in monocyte populations, leading to subsequent imbalance in macrophage populations, could pose quantifiable risks to the normal and healthy neurological development in HEU babies. It is, therefore, extremely important to gain further understanding and insight into this process as it can form an integral component of the effective care and support of these children in their further development.

Whole blood stimulation (WBS) is a useful tool for understanding the monocyte population and their function. Stimulation of these immune cells with an endotoxin, such as LPS, or a TLR agonist, such as Polyinosinic:polycytidylic acid (Poly I:C), results in an increase in production and release of specific cytokines(41). Poly I:C is known to interact with toll-like receptor 3 (TLR3), which is expressed at the endosomal membrane of B-cells, macrophages, and dendritic cells. Poly I:C is structurally similar to double-stranded ribonucleic acid (RNA), which is present in some viruses and is a "natural" stimulant of TLR3. Thus, Poly I:C can be considered a synthetic analogue of double-stranded RNA and is a common tool for scientific research on the immune system(42). Assessing the spectrum of cytokines released, as well as the relative prevalence of pro-inflammatory versus anti-inflammatory cytokines, gives an indication of the monocyte type/function predominant in the sample.

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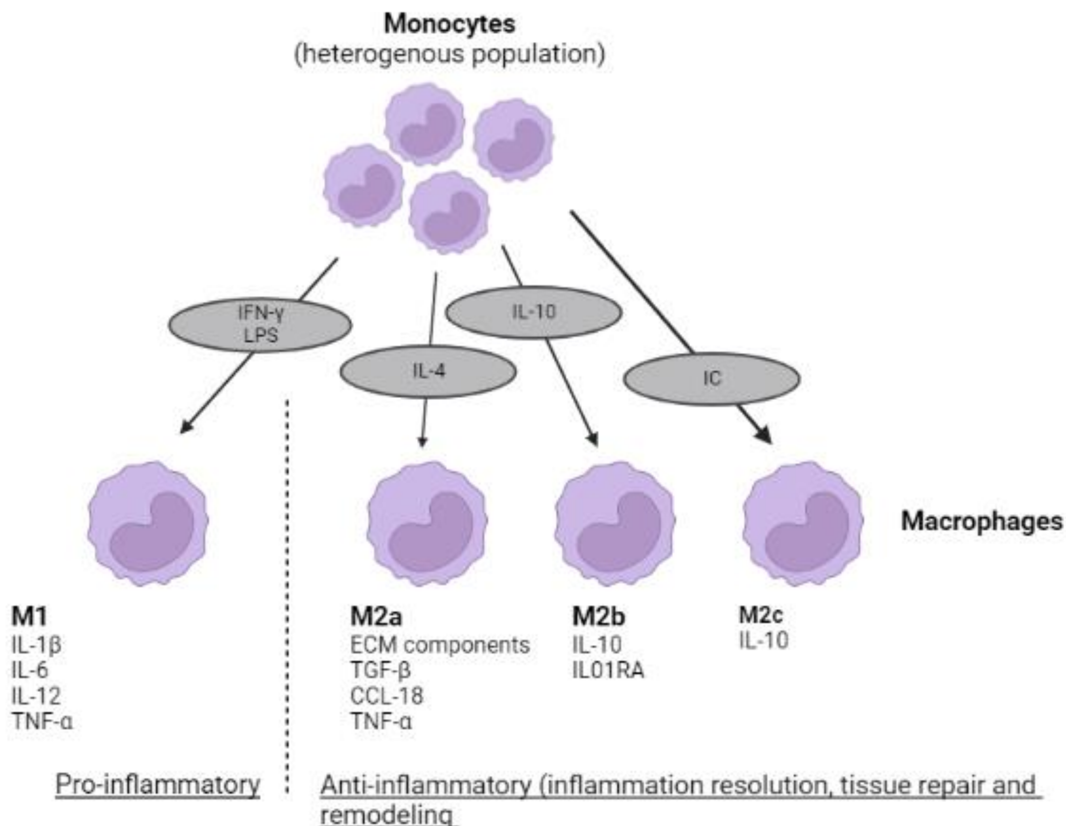

Figure 1.2: Differentiation of monocytes to macrophages and the specific functions and cytokines they produce (Adapted from source(37)*, created with BioRender.com).*

1.7 Cytokines

In order to fully understand the results of the cytokine/chemokine panel produced by the WBS assay, it is important to briefly describe each of the cytokines and chemokines assessed as part of this study:

Interleukin(IL)-2, originally named "T cell growth factor", is a cytokine that is predominantly produced by activated CD4+ and CD8+ T cells(43). The production of IL-2 and expression of the IL-2 receptor is caused by activation of the T cells through the T cell receptor and costimulatory molecules (such as CD28)(43). IL-2 plays an important role in T cell expansion and effector cell development and is linked to initial immune response, an increase in the number of persistent T memory cells, and recall proliferation(44–46). In addition to the important role IL-2 plays in the immediate immune response, the role of IL-2 is equally essential for T memory cell maintenance and development of T regulatory (Treg) cells(43). IL-2 contributes to thymic Treg development and also functions to maintain Treg cells in the peripheral immune compartment(43). Treg cells are essential to maintaining homeostasis of the immune system. As a result, deficiencies in IL-2 have been shown to contribute to autoimmunity(43). Mouse-based studies found that mice deficient in IL-2 displayed lethal autoimmunity which was associated with impaired Treg production(47,48).

IL-2 is also a notable activator of monocytes. Monocytes respond to IL-2 stimulation with anti-microbial and anti-tumoricidal activities, production of cytokines and growth factors, and

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expression of growth factor receptors(49). IL-2 is therefore extremely important for an effective monocyte response to a microbial or viral infection.

IL-4 is a cytokine and a product of CD4+ T cells, specifically the T-helper cell type 2(Th2) phenotype(50). It has also been shown that other cell types have the capability to produce IL-4, such as Natural Killer T cells(51), mast cells(52), and basophils(53). It is important to note that, while IL-4 is a major product of Th2 cells, it is also an inducer of Th2 cell differentiation, which indicates that there may be a positive feedback loop contributing to this aspect of immune response(50). IL-4 has been shown to have a wide range of effects on varying cell types, but is best known as a growth factor for T and B cells(54). It has been shown that monocytes display an M2-like phenotype in response to IL-4 exposure, and thus exert anti-inflammatory effects under these conditions(55).

The cytokine, IL-6, has also been shown to be a product of T cells, as well as of fibroblasts, endothelial cells, monocytes/macrophages, and mast cells, to name just a few(56). IL-6 is not produced in any considerable amounts under normal conditions and production is usually induced by means of viral infection or lipopolysaccharide(LPS)(56). IL-6 has been shown to have an effect on B cells, such as inducing the production of immunoglobulin, and also plays a role in T cell activation. As such, it has been shown that IL-6 contributes to the acute phase immune response to infection through mediating the proliferation of these cells and exerting pro-inflammatory effects(56). The effect that IL-6 has on monocytes is congruent with this, as monocyte/macrophage populations induced by IL-6 display pro-inflammatory properties and contribute to the inflammatory component of the acute phase immune response to a stimulus(57).

IL-8 is a chemokine and is also produced by a wide range of cell types: T cells, monocytes, neutrophils, and endothelial cells, to name a few examples(58). The production of IL-8 is also not expected under normal conditions and is usually as a result of an inflammatory stimulus, such as LPS(58). Similarly to IL-6, IL-8 plays a causal role in the acute phase immune response by means of similar pro-inflammatory activities(58). The effect that IL-8 has on monocyte/macrophage populations is congruently pro-inflammatory: it specifically stimulates the migration of monocytes/macrophages and neutrophils to inflammatory sites. As a result of LPS stimulation, IL-8 has been shown to downregulate aspects of the anti-inflammatory response (such as expression of the IL-4 receptor) and upregulate aspects of the pro-inflammatory response (such as expression of IL-6). It has therefore been suggested that IL-8 acts directly in favour of the pro-inflammatory M1-activated monocytes(59).

IL-10 is a cytokine that is produced by almost all leukocyte cell types, such as: cytotoxic T cells, B cells, dendritic cells, and neutrophils(60). Monocytes and macrophages also produce IL-10 after activation by external mediators, such as LPS(60). IL-10 is extremely influential in the regulation of functions of monocyte/macrophage populations since IL-10 supresses the pro-inflammatory impact of monocytes/macrophages on innate and specific immunity, while enhancing their inhibitory functionality(60). IL-10 directly inhibits the release of pro-inflammatory mediators and enhances the anti-inflammatory response by triggering the release of factors such as soluble TNF-α receptors(60). IL-10 is therefore understood to contribute directly to the anti-inflammatory response and to the anti-inflammatory functionality of resident monocyte/macrophage populations.

Interferon-gamma (IFN-ɣ) is a cytokine produced by CD4+ T helper cells, CD8+ cytotoxic lymphocytes, Natural Killer T cells, dendritic cells, and monocytes/macrophages(61). IFN-ɣ production is regulated by cytokines such as IL-12 and IL-18, which are secreted by

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macrophages. These cytokines serve to link infection to IFN-ɣ production in the innate immune response. Macrophage pathogen recognition induces secretion of IL-12 and chemokines that attract Natural Killer T cells to the site of inflammation(61). In macrophages, the combination of IL-12 and IL-18 further increases the production of IFN-ɣ, which induces direct antimicrobial and antitumour properties in the monocyte/macrophage population and thus contributes to a pro-inflammatory response(61). This substantiates the importance of IFN-ɣ in immune regulation as it is usually only produced under conditions of activation(62), and so is essential to maintaining a pro-inflammatory response to stimuli during inflammation.

Tumour necrosis factor alpha (TNF-α) is a cytokine produced primarily by monocytes/macrophages, but is also produced by other cell types such as T cells, mast cells, and neutrophils(63). TNF-α has been referred to as a sentinel cytokine as it plays an important role in initiating the response to local injury(64). TNF-α is generally understood to be a pro-inflammatory cytokine and, in high concentrations, can lead to excess inflammation and organ damage(64). In the innate immune response, increased levels of TNF-α induce the production of mediators that contribute to inflammation and tissue destruction. As such, TNF-α is at the head of the inflammatory cascade(64). It has also been shown that TNF-α contributes to the pro-inflammatory response by mediating the production and release of IL-10 in monocytes(65).

The final cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF), is produced by haemopoietic cells, such as lymphocytes, and non-haematopoietic cells, such as epithelial cells, though it is usually produced in response to a stimulus (such as LPS)(66). Its principal role is as a growth factor by promoting myeloid cell development and maturation, but GM-CSF also plays a role in inflammation and immunity by exerting effects on cells such as monocytes/macrophages and neutrophils. Monocytes exposed to GM-CSF produce pro-inflammatory cytokines, such as IL-6, and as such it is understood that GM-CSF contributes to the pro-inflammatory functionality of monocytes/macrophages, leading to “M1-like” activation in these populations(66).

1.8 Head Circumference as a Measure of Post-natal Neurological Development

While it is extremely important to quantify and understand the neurological development of HEU infants, this is not always a straightforward endeavour. One such measure of neurological development is brain size, which can be quantified by means of magnetic resonance imaging (MRI). Such scans are unfortunately prohibitively expensive in the developing world and therefore not suitable as a screening tool. Occipitofrontal measurement of HC has therefore traditionally been used as an easy and low-cost analogue for overall brain size(67). Although HC is technically a measure of the skull size it has been shown to correlate well with overall brain volume/mass and is widely used as a proxy/analogue for understanding neural growth and brain size(68,69).

HC has been shown to correlate significantly with brain volume in post-mortem cases(70). Another study, making use of MRI in preterm infants, has found that HC correlates strongly with brain volume at term(67). The same study showed that microcephalic infants, as classified by HC measurement standards, had significantly decreased brain volumes with the most significant difference noted in the deep grey matter(67), with specific reference made to the cortical regions such as the caudate nucleus, putamen, globus pallidus, claustrum and thalamus(19). This relatively simple solution was also used for the purposes of this study since it was based in a government-funded hospital and considered a large number of individuals, making MRI not feasible.

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Currently, the accepted definition of microcephaly is an observed HC > 2 standard deviations below the mean in age- and gender-matched groups(71), i.e.; a HC z-score of -2 or less(72). This definition is recognized and supported by various relevant medical specialists including child neurologists and developmental paediatricians(73,74). A HC measurement that falls below the normal range is considered a risk factor for cognitive impairment and developmental delay(74,75).

The measurement of HC is performed using a non-elastic measuring tape and is to be taken at the largest area of the head (around the back of the head with the tape measure held above the eyebrows and ears)(76). This measurement can then be plotted along the relevant growth chart to quantify the HC and to determine the percentile in which it falls(71). These growth charts are standardised in accordance with the current World Health Organization (WHO) standards. Figures 1.3 and 1.4 show the HC-for-age for boys and girls, respectively.

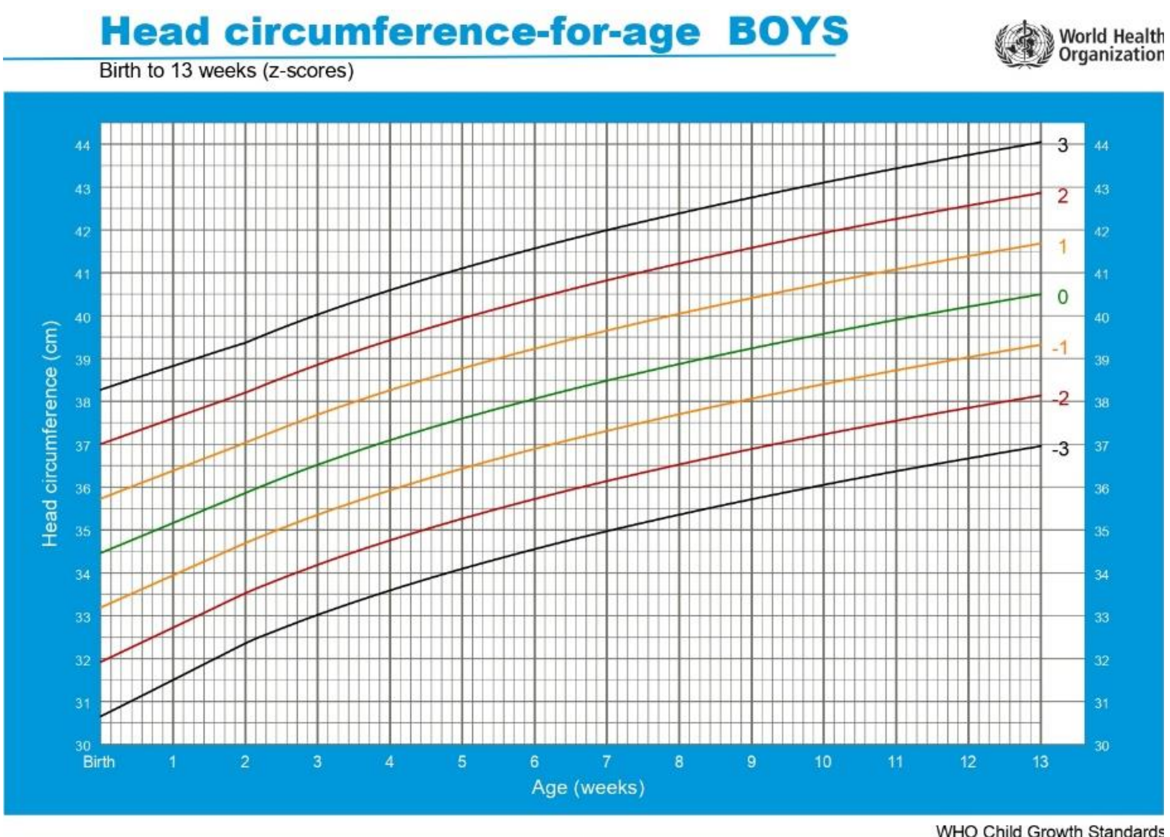

Figure 1.3: WHO growth chart – Head circumference for age pertaining to boys from birth to 13 weeks of age(77).

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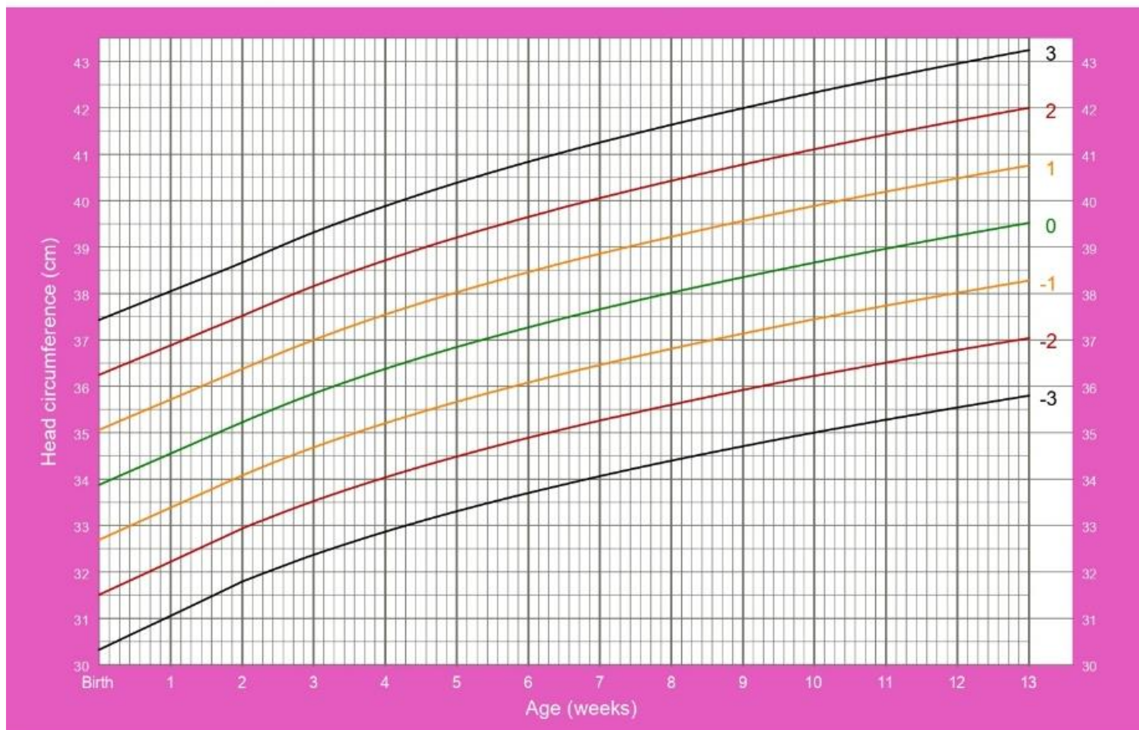

Figure 1.4: WHO growth chart – Head circumference for age pertaining to girls from birth to 13 weeks of age(77).

1.9 Purpose of This Study

These risk factors, namely lower HC in HEU infants coupled with low-income backgrounds, are particularly relevant to the group being considered for this study. In this setting, this study holds particular potential for improving the understanding and future management and treatment for HEU infants as they grow. Lower HC presents a significant risk for suboptimal neurological development, manifesting as poorer Intellectual Quotient (IQ) equivalent, perceptual motor skills, academic achievement, and adaptive behaviour at school age(78). As such, a proactive approach to facilitating the neurological development of at-risk children from an early age may improve the outcomes at school age.

This study also acted as a pilot study for planning the cytokine measurement of the longitudinal Siyakhula study. In analysing a small subset of the overall population of the Siyakhula study, this study provided important data regarding which specific cytokines were detectible under the testing conditions and which were not relevant to the cohort under these conditions. This information will aid the larger Siyakhula study in accuracy and will also help in cost saving, as the cost of running these analyses goes up with each cytokine panel needed.

The aim of this study was to investigate the association between HIV exposure, HC, and monocyte function in HEU and HUU infants. The specific objectives were:

- To investigate whether there was a difference in HC between HEU and HUU infants in the first year of life.
- To characterise and compare monocyte function between HEU and HUU infants in the first year of life by means of whole blood stimulation.
- To determine if there was an association between HC and monocyte function at birth.

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Chapter 2 – Methodology

2.1 Study design

This project formed part of a larger study, Siyakhula, which is a prospective longitudinal cohort study with human immunodeficiency virus (HIV)-exposed and HIV-unexposed groups for comparison. The study recruitment was based in Kalafong Provincial Tertiary Hospital in South-west Tshwane, South Africa. All experimental procedures were carried out in the laboratories of the Department of Immunology at the University of Pretoria, Prinshof Campus. As part of the longitudinal study, a total of 300 participants will be tested. For this study, the MSc candidate was involved in the testing and analysis process for 21 of these samples.

2.2 Sample size

The Siyakhula study has recruited 300 infants (male and female) born to HIV positive (n=150) and HIV negative (n=150) mothers. From this cohort, 21 infants were included in the present study. The infants were randomly selected but had to meet the condition of their anthropometric data and have blood samples collected for all four of the time-points (0 weeks, 10 weeks, six months and 12 months). The breakdown of the infant cohort is as follows:

- HIV-exposed-uninfected (HEU): n=11
- HIV-unexposed-uninfected (HUU): n=10

2.3 Inclusion criteria pertaining to the mothers in Siyakhula

- HIV-positive women with singleton pregnancies
- HIV-negative women with singleton pregnancies
- Eighteen years and older
- Willing and able to give written informed consent on behalf of themselves and their infants

2.4 Exclusion criteria pertaining to the mothers in Siyakhula

- Inability to obtain informed consent
- Multiple pregnancies/gestations
- Maternal hypertension
- Diabetes
- Tuberculosis
- Serious pre-existing medical conditions in mother and/or infant
- Chromosomal or structural abnormalities in the infant
- Maternal antibiotic exposure during labour/delivery and/or postpartum period
- Infant delivery by Caesarean section

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- Mothers who could not commit to follow up appointments

2.5 Data collection

New-born blood collection

All infants whose mothers gave informed consent (*Appendix 1*) were recruited as part of the Siyakhula study and had blood drawn at birth (0 weeks), 10 weeks, six months and 12 months of age by a qualified research nurse. Blood was drawn in vacutainers containing ethylenediaminetetra acetic acid (EDTA) as anti-coagulant. Blood was transported to the Department of Immunology and Whole Blood Stimulation (WBS) testing was performed within four hours of blood collection. From the blood collected, an aliquot (<1 Millilitre [mL]) was taken and processed for plasma isolation. The plasma fraction was stored at -80 degrees Celsius (°C) until use.

Pregnancy data

Medical chart review of the consented mothers provided important data, including:

- Maternal age, weight, and mid-upper arm circumference (MUAC) at the time of giving birth.
- Maternal body mass index (BMI) at the time of giving birth, calculated by dividing the mother's weight (in kilogram) by her height (in centimetres) squared.
- Pregnancy outcomes: gestation length, new-born anthropometry (length, weight, abdominal circumference and head circumference [HC]), Appearance, Pulse, Grimace, Activity, Respiration (APGAR) score (*Appendix 2*).
- Head circumference was measured according to standard guidelines: This is done by looping the measuring tape before slipping it over the infant's head. The measuring tape is placed above the brows, the pinna of the ears, and around the occipital prominence at the back of the skull, ensuring that the tape is flat against the skin. The circumference of the head is recorded.

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2.6 Procedures

HIV status was measured as part of routine care at the first antenatal visit using a standard enzyme-linked immunosorbent assay (ELISA). The methodology for this study is outlined in the flow diagram in Figure 2.1.

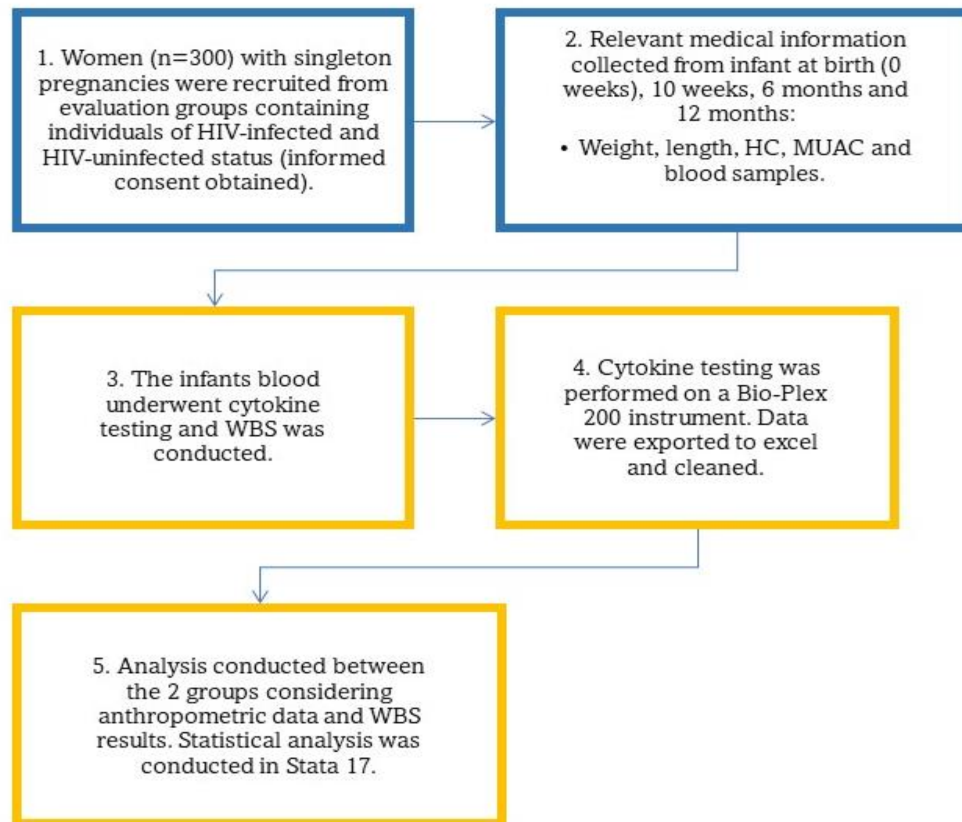

Figure 2.1: Flow diagram of research methodology. Steps 1 and 2 (Blue)were completed as part of the larger Siyakhula study. Steps 3 – 5 (Yellow) were completed as part of the present study.

Abbreviations: Human immunodeficiency virus (HIV); Head circumference (HC); Mid-upper arm circumference (MUAC); Whole blood stimulation (WBS).

2.7 Whole Blood Stimulation

All reagent preparation and whole blood stimulation (WBS) assay setup was conducted in a laminar flow cabinet (Labcon – California, USA) as shown in Image 2.1.

The lipopolysaccharide (LPS) and polyinosinic:polycytidylic acid (Poly I:C) were thawed on ice at least 45 minutes before use. The following steps were followed in order to prepare the LPS for use: 1. 10 microlitres (µL) LPS (InvivoGen – California, USA) was diluted in 2490 µL Roswell Park Memorial Institute medium (RPMI) (Lonza – Basel, Switzerland); 2. The diluted LPS was well mixed by pipetting up and down several times; 3. The prepared LPS was stored on ice. The following steps were then followed in order to prepare the Poly I:C for use: 1. 10 µL Poly I:C (InvivoGen – California, USA) was diluted in 490 µL RPMI; 2. The diluted Poly I:C was mixed well by pipetting, as described above; 3. The prepared Poly I:C was stored on ice.

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For the WBS test, 320 µL of whole blood was transferred to a sterile 1.5 mL Eppendorf tube (Labocare – Secunderabad, India) followed by the addition of 640 µl of RPMI (total volume was 960 µL). The diluted whole blood was then mixed by gently pipetting up and down. Three Eppendorf tubes per subject were labelled with the subject identity code (ID) and respective stimulus (Control, LPS, Poly I:C). Then, 300 µL of diluted whole blood was transferred to each tube, followed by the addition of 50 µL LPS, Poly I:C or RPMI (for the Control) to the respective tubes and the samples were mixed well by gently pipetting up and down. The samples were then incubated at 37°C with five percent carbon dioxide in a humidified incubator (ESCO Technologies – Missouri, USA) for 18 hours (lids were placed loosely on the tubes). The incubator that was used can be seen photographed in Image 2.2 and an example of samples prepared for incubation in Image 2.3.

Following the 18 hour incubation period, the samples were gently mixed by flicking the tubes. The samples were then centrifuged using an Alegra X-12R centrifuge (Beckman Coulter – California, USA) at 302 times gravity (xg) for 10 minutes at 4°C, as seen in Image 2.4.

Finally, the plasma fraction was removed from each sample and stored in a sterile Eppendorf tube, labelled accordingly. These tubes were then stored at -80°C in a Forma 900 series laboratory sample freezer (Thermo Fisher Scientific - Massachusetts, USA), as shown in Image 2.5.

2.8 Cytokine Immunoassay

The MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel (Merck Milliplex – Massachusetts, USA), as seen in Image 2.6, was used for the simultaneous detection of the following biomarkers: interferon-gamma (IFN-ɣ), interleukin (IL)-2, IL-4, IL-10, IL-6, IL-8, tumour necrosis factor (TNF)-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) (as shown outlined in Image 2.7).

Prior to assay, all reagents were allowed to warm to room temperature (20-25°C). The suspension bead array was performed according to the manufacturer's instructions. Briefly, 200 µL of wash buffer was added to each well of the plate being used. The plate was then sealed and mixed on a plate shaker (Stuart Scientific – Staffordshire, UK) for 10 minutes at room temperature (20-25˚C). Next, the wash buffer was discarded and any residual buffer was removed by inverting the plate and tapping several times onto absorbent towels. The plate, provided with the kit, can be seen in Image 2.8 as well as the plate shaker with the aforementioned sample plate in Image 2.9.

Standard, control or sample (25µL) was added into the appropriate wells. Assay buffer was then used for the 0 pg/mL standard. Twenty-five µL of assay buffer was added to the sample wells and 25 µL of serum matrix was added. Twenty-five µL of premixed beads was added to each well before the plate was sealed with a plate sealer, wrapped in foil, and incubated with agitation on a plate shaker (Stuart Scientific – Staffordshire, UK) for two hours at room temperature. Following incubation, the contents of each well was gently removed, and the plate was washed 3 times using a Bio-Plex Pro automatic wash station (Bio-Rad – California, USA), photographed in image 2.10.

Then, 25 µL of detection antibodies was added to each well (note: the antibodies were also allowed to warm to room temperature before use). The plate was sealed, covered in foil, and

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incubated with agitation on a plate shaker for one hour at room temperature (20-25˚C). Following this incubation period 25 µL of streptavidin-phycoerythrin was added to each of the wells. The plate was then sealed again, covered in foil, and incubated with agitation on a plate shaker for 30 minutes at room temperature (20-25˚C). The contents of each well was gently removed and the plate washed three times as described above.

The beads were resuspended by the addition of 150 µL of sheath fluid and the plate was shaken using a plate shaker (Stuart Scientific – Staffordshire, UK) for two minutes. The plate was analysed using a Luminex 200 (Bio Rad – California, USA) with xPONENT Bioplex Management Software 6.0 (Bio Rad – California, USA) used to determine the mean fluorescent intensity (MFI). The MFI for each sample was analysed using a 5-parameter logistic method to calculate the analyte concentrations in the samples. The results are presented as picograms (pg/mL). The Luminex 200 system that was used is shown in Image 2.11.

2.9 Images of workflow

To follow on page 23 to 25.

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Whole Blood Stimulation

Image 2.1: Laminar flow cabinet in which all sterile procedures were performed regarding the Whole Blood Stimulation preparation and process.

Image 2.2: Humidified cell culture incubator (ESCO Technologies – Missouri, USA) which was used to incubate the WBS samples.

Image 2.3: Labelled Eppendorf tubes which were prepared for incubation containing WBS samples.

Image 2.4: Alegra X-12R centrifuge which was used to centrifuge the WBS samples after the incubation step had concluded.

Image 2.5: Forma 900 series freezer (Thermo Fisher Scientific - Massachusetts, USA) used to store the WBS samples once all preparatory steps had been completed until they were needed for testing.

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Cytokine Immunoassay

Image 2.6: Milliplex Panel kit which was being used (prior to opening).

Image 2.7: Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel, here outlining the specific biomarker bead panel included for the purpose of this test.

Image 2.8: The Milliplex sample plate which was included in the kit and was used for the purposes of running this panel.

Image 2.9: Prepared and sealed sample plate here seen photographed on the orbital plate shaker undergoing bead suspension.

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Image 2.10: Bio-Plex Pro Wash Station which was used for plate washing as part of the Immunoassay process.

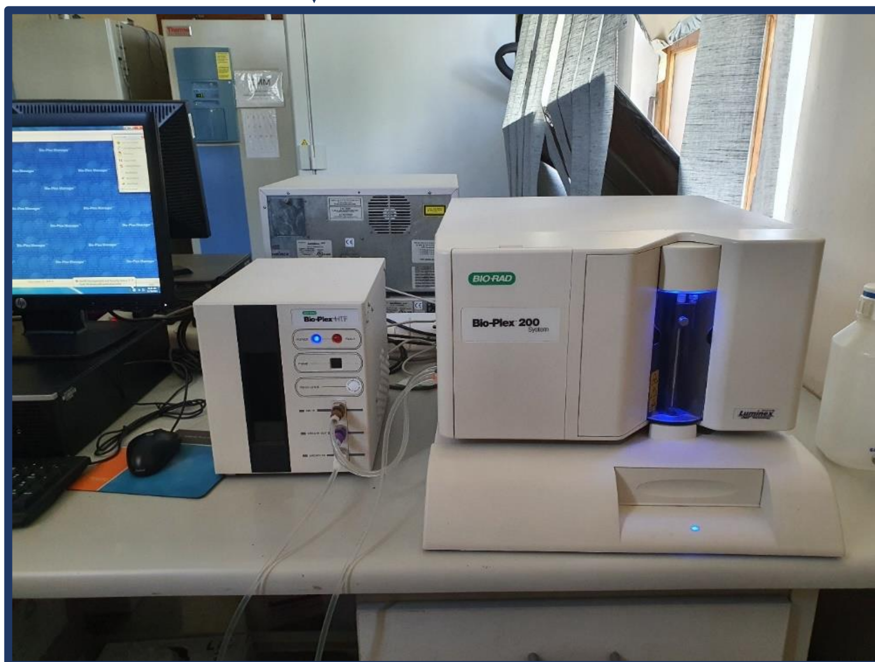

Image 2.11: Bio-Plex 200 system which was used to run the panel.

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2.10 Data Management, Statistical Analysis and Ethical Considerations

Data Management

The anthropometric data pertaining to the mothers and infants recruited as part of the Siyakhula study were collected by a research nurse at the hospital and were then entered and stored securely within a REDCap database. This software is an electronic data capture (EDC) technology specifically meant for the management of clinical research generated data. The data pertaining to the infants included in this study for the specific time-points (0 weeks, 10 weeks, 6 months, and 12 months) were retrieved from this database and compiled into a data-capture spreadsheet for the management of information to be assessed as part of the present study.

Z-scores for the anthropometric data were calculated using the World Health Organization (WHO) Anthro Software version 3.2.2 for Windows. This software includes an anthropometric calculator module which was used to calculate the z-scores for the given data in accordance to WHO standards(79). The calculated z-scores were used to assess and quantify infant growth according to these standards at the given time-points. The z-score is calculated using the following formula(80):

$$\frac{observed\ value - median\ of\ reference\ population}{standard\ deviation}$$

Following the WBS assay and cytokine testing, the generated cytokine results were compiled into this spreadsheet. As a result, one excel spreadsheet was generated which contained all anthropometric and cytokine data pertaining to the 21 infants assessed as part of the current pilot study. The data-capture spreadsheet was then cleaned by means of comparison with a master spreadsheet as well as visual inspection of the data through histograms. Statistical analysis was then performed from the captured data.

Statistical Analysis

Statistical analysis was conducted by means of Stata version 17.0 (StataCorp – Texas, USA). The Stata software was used to generate and summarize the detail of the anthropometric and cytokine concentration data in order to ascertain the medians and inter-quartile ranges (IQR). These statistical measures were used instead of means and standard deviations as the sample size of infants was relatively small for this study and the data were not normally distributed. The study data were also subjected to the following statistical tests:

- Kruskal-Wallis equality of populations test; to assess any differences between groups
- Quantile distributions and Fisher's exact testing; to assess the distribution of individuals throughout the groups and to test for the associations therein
- Categories were generated for z-score values and tested by the Fisher's exact test; in order to assess any differences in the z-score categories across the anthropometric variables between the groups
- Wilcoxon sign-rank test; in order to assess any differences within the groups for the cytokine data
- Spearman correlation test to assess if HC and monocyte function were correlated

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- A p-value of 0.05 was taken as statistically significant but, because the sample size was small and the study was exploratory in nature, all p-values below 0.1 were noted as important.

Ethical Considerations

All relevant study information was given to the mothers of the considered infants by a research nurse and subsequently informed consent was obtained from the mothers on behalf of themselves and their infants. It was ensured that adequate time was given to the mothers to ask any questions and/or to discuss the study with significant others if they so wished before agreeing to take part in the study. Participation in the study was voluntary and participants had the right to refuse to participate in any aspect of the study or to withdraw entirely at any time. Refusal had no negative consequences for the mother or the infant.

The risks related to the collection of anthropometric information and blood samples were minimal, any potential risks were further minimised as the personnel involved in the collection of study data were all experienced in dealing with new mothers and their infants. Every effort was made to ensure the privacy of the individuals involved is protected, clinical data pertaining to the mothers and infants did not contain any personal identifiers.

There was no immediate or direct benefit to the participants of this study however the mothers received some benefit as a result of being part of the study as they were seen by a medical specialist for detailed sonar scans before birth and any identified complications were treated immediately. The infants could also potentially have benefited as there were routinely scheduled visits with a specialist and they received additional screening for growth and brain development indicators. Knowledge gained from this study may provide a better understanding of how maternal HIV status impacts on factors which contribute to the early-life development of these infants and therefore potentially has public health benefits.

This study as well as the larger Siyakhula study have been reviewed and approved by the University of Pretoria Faculty of Health Sciences Research Ethics Committee. The details of the ethical approval certificates are outlined as such:

- Siyakhula study: Ethics reference number 294/2017 (*Appendix 3*)
- MSc. study: Ethics reference number 704/2021 (*Appendix 4*)

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Chapter 3 – Results

3.1 Description of Cohort

In this study, a total of 21 infant samples were analysed. Ten of these infants were born to HIV-uninfected mothers, comprising the HIV-unexposed-uninfected (HUU) cohort and 11 infants were born to mothers living with HIV, making up the HIV-exposed-uninfected (HEU) cohort.

Physical measurements of the included infants were obtained at the four time-points over the course of the first 12 months of life post-partum and were used to compare the physical characteristics and growth progression of these two groups of infants (HUU and HEU). Physical measurements of the mothers were also obtained at birth. The variables measured were:

- Weight (measured in kilograms [kg])
- Length (measured in centimetres [cm])
- Head circumference (measured in centimetres)
- Weight for length (calculated as weight [kg] divided by length [cm]) – represented as W4L
- Weight for age (calculated as weight [kg] divided by age [months]) – represented as W4A
- Mid-upper arm circumference (measured in cm) – represented as MUAC

A summary of the descriptive data pertaining to the mothers of the infants, taken at birth, observed for this study can be seen in Table 3.1 below. Mothers with and without HIV were similar in terms of their anthropometry, but mothers living with HIV were significantly older.

Table 3.1: Descriptive details of the mothers pertaining to the entire cohort.

Statistic	**All mothers - Mean (±std. dev.)**	**HIV+ mothers - Mean (±std. dev.)**	**HIV- mothers - Mean (±std. dev.)**	**P-value**
Age (years)	32.82 (±6.51)	36.84 (±4.29)	28.39 (±5.68)	**0.0025**
Weight (kg)	67.61 (±11.1)	67.53 (±12.53)	67.69(±10.02)	0.9674
MUAC (cm)	28.08 (±2.8)	28.64 (±2.65)	27.46 (±2.96)	0.4813
BMI (kg/cm^2)	26.78 (±3.13)	26.88 (±3.37)	26.66 (±3.07)	0.9233

Abbreviations: Mid-upper arm circumference (MUAC); Body Mass Index (BMI). Standard deviation (std. dev.); kilogram (kg); centimetre (cm).

All infants included in this study were delivered by means of normal vaginal delivery. The gestational ages for the infants are summarized below. A comparison between the difference in gestational ages for the HEU and HUU infants generated a p-value of 0.5688, indicating a high degree of similarity for both groups.

- All mothers (Mean [±std. dev.]) = 39.0 weeks (0.92)
- Mothers living with HIV (Mean [±std. dev.]) = 38.91 weeks (±0.94)
- Mothers living without HIV (Mean [±std. dev.]) = 39.11 weeks (±0.93)

Displayed in Table 3.2 below is a summary of the descriptive anthropometric data of the infants when the entire cohort of 21 infants is considered over the course of the various time points (0 weeks, 10 weeks, 6 months, and 12 months post-partum), providing a broad overview of the

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entire group and the progression of growth on average over the course of the first 12 months of life.

Table 3.2: Anthropometric data of infants considered in this study with all 21 infants included.

	Infant post-partum age [Median (IQR)]			
Statistic	**0 weeks (at birth)**	**10 weeks**	**6 Months**	**12 Months**
Weight (kg)	2.99 (2.76 – 3.28)	5.51 (4.85 – 5.86)	7.63 (7.1 – 8.06)	9.85 (8.7 – 10.2)
Length (cm)	49.0 (47.0 – 50.0)	57 (56.5 – 59.1)	66.4 (63.5 – 68.7)	74.5 (72.6 – 77.2)
HC (cm)	34.0 (33.0 – 35.0)	39.45 (39.0 – 40.2)	43.1 (42.3 – 44.1)	46.0 (45.4 – 47.0)
Weight for Length (kg/cm)	0.60 (0.057 – 0.062)	0.10 (0.09 – 0.103)	0.115 (0.110 – 0.121)	0.13 (0.12 – 0.136)
Weight for age (kg/months)	N/A	2.20 (1.94 – 2.34)	1.27 (1.18 – 1.34)	0.82 (0.73 – 0.85)
MUAC (cm)	N/A	14.25 (13.0 – 15.0)	15.0 (14.0 – 16.0)	15.9 (15.0 – 17.0)

Abbreviations: Interquartile range (IQR); Mid-upper arm circumference (MUAC); Head circumference (HC); kilogram (kg); centimetre (cm); Not applicable (N/A).

Detailed below in Table 3.3 are the observed z-scores pertaining to the measurements for the entire cohort over the progression of the first 12 months of life. These z-scores provide an indication of how this group compares to globally observed normal values, the generated value showing where the observed average is relative to the normal expected value. This is measured in standard deviations, with negative values indicting standard deviations below the expected average and positive values indicating standard deviations above the average, as compared to global WHO standards.

Table 3.3: Z-scores for anthropometric data of infants considered in this study with all 21 infants included.

	Infant post-partum age [Median (IQR)]			
Statistic	**0 weeks (at birth)**	**10 weeks**	**6 Months**	**12 Months**
HC z-score	-0.36 (-0.74 – 0.42)	1.47 (1.15 – 2.46)	0.07 (-0.28 – 1.3)	0.37 (-0.05 – 0.88)
Weight for Length z-score	-0.84 (-1.82 - (-0.24))	0.67 (-0.52 – 1.33)	0.37 (-0.17 – 0.69)	0.38 (-0.53 – 0.84)
Weight for Age z-score	-0.69 (-1.39 - (-0.08))	1.21 (0.1 – 1.77)	0.08 (-0.83 – 0.75)	0.19 (-0.49 – 1.04)
MUAC z-score	N/A*	N/A*	1.01 (0.19 – 1.77)	1.24 (0.65 – 2.13)

**WHO standards for the MUAC measurement begin at 13 weeks of age, as such the observed value at 0- and 10-weeks post-partum has no generated z-scores.*
Abbreviations: Interquartile range (IQR); Mid-upper arm circumference (MUAC); Head circumference (HC); Not applicable (N/A)

3.2 Anthropometric Data

As detailed above for the entire group cohort, the z-scores were then calculated separately for the HEU and HUU groups with regards to HC, W4L, W4A and MUAC. This is to gain a representative understanding of how the observed values compare to the expected values according to WHO standards.

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The observed and calculated values for both HUU and HEU groups can be seen detailed in Table 3.4 and Table 3.5 to follow.

Table 3.4: Summary of anthropometric data: comparison between HUU and HEU infant groups for the 0 week and the 10-week time-points.

Age/Timepoint	Variable	Statistic		
		HUU: Median (IQR)	HEU: Median (IQR)	P-value
0 Weeks	Weight (kg)	3.07 (2.96 - 3.3)	2.91 (2.57 - 3.23)	0.2751
	Length (cm)	48.5 (45.0 - 49.5)	49.0 (48.0 - 50.0)	0.5317
	HC (cm)	34.0 (33.0 - 35.0)	34.0 (32.0 - 35.0)	0.4414
	W4L (kg/cm)	0.06 (0.057 - 0.062)	0.058 (0.052 - 0.062)	0.2110
	W4A (kg/months)	N/A	N/A	N/A
	MUAC (cm)	N/A	N/A	N/A
	HC z-score	0.03 (-0.74 - 0.95)	-0.36 (-1.59 - 0.42)	0.4817
	W4L z-score	-0.37 (-0.72 - [-0.03])	-1.53 (-1.82 - [-0.84])	0.1255
	W4A z-score	-0.47 (-0.76 - 0.1)	-0.93 (-1.55 - [-0.26])	0.4474
	MUAC z-score	N/A	N/A	N/A
10 Weeks	Weight (kg)	5.61 (5.2 - 5.79)	5.47 (4.65 - 5.86)	0.6831
	Length (cm)	56.8 (56.0 - 57.4)	58.15 (56.8 - 60.3)	0.1416
	HC (cm)	39.5 (39.0 - 40.0)	39.0 (39.0 - 42.0)	0.6272
	W4L (kg/cm)	0.1 (0.09 - 0.11)	0.09 (0.08 - 0.1)	0.4142
	W4A (kg/months)	2.24 (2.08 - 2.32)	2.19 (1.86 - 2.34)	0.6831
	MUAC (cm)	14.05 (13.0 - 14.8)	14.25 (12.8 - 15.3)	0.7929
	HC z-score	1.49 (1.15 - 2.29)	1.45 (1.45 - 3.37)	0.4268
	W4L z-score	1.17 (0.86 - 1.62)	-0.05 (-0.66 - 0.67)	**0.0305**
	W4A z-score	1.45 (0.65 - 1.77)	0.98 (-0.13 - 1.4)	0.4624
	MUAC z-score	N/A	N/A	N/A

Abbreviations: Interquartile range (IQR); Kilogram (kg); centimetre (cm); Head circumference (HC); Weight for length (W4L); Weight for age (W4A); Mid-upper arm circumference (MUAC); Not applicable (N/A)

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Table 3.5: Summary of anthropometric data comparison between HUU and HEU infant groups for the 6 month and the 12-month time-points.

Age/Timepoint	Variable	Statistic		
		HUU: Median (IQR)	HEU: Median (IQR)	P-value
6 Months	Weight (kg)	7.78 (7.3 - 8.06)	7.5 (6.6 - 8.23)	0.5495
	Length (cm)	66.65 (65.8 - 68.7)	67.3 (62.1 - 69.0)	0.6727
	HC (cm)	43.45 (42.4 - 44.3)	43.1 (42.3 - 44.0)	0.5495
	W4L (kg/cm)	0.115 (0.112 - 0.119)	0.11 (0.10 - 0.12)	0.8327
	W4A (kg/months)	1.3 (1.22 - 1.34)	1.25 (1.1 - 1.37)	0.5495
	MUAC (cm)	15.4 (14.6 - 16.4)	14.6 (14.0 - 16.0)	0.2178
	HC z-score	0.47 (-0.28 - 1.45)	-0.03 (-0.85 - 0.76)	0.3418
	W4L z-score	0.27 (-0.1 - 0.52)	0.48 (-0.61 - 1.6)	0.7513
	W4A z-score	0.39 (-0.46 - 0.75)	-0.52 (-1.27 - 0.98)	0.5732
	MUAC z-score	1.19 (0.33 - 2.06)	0.36 (-0.22 - 1.77)	0.2313
12 Months	Weight (kg)	10.19 (9.85 - 10.94)	8.89 (8.01 - 9.89)	**0.0183**
	Length (cm)	75.45 (74.2 - 77.1)	73.0 (71.0 - 77.4)	0.5732
	HC (cm)	46.0 (45.8 - 47.0)	46.0 (45.0 - 47.0)	0.4597
	W4L (kg/cm)	0.134 (0.131 - 0.145)	0.121 (0.109 - 0.127)	**0.0346**
	W4A (kg/months)	0.85 (0.82 - 0.91)	0.74 (0.67 - 0.82)	**0.0183**
	MUAC (cm)	16.6 (15.9 - 17.8)	15.4 (14.0 - 17.0)	0.0573
	HC z-score	0.67 (-0.05 - 1.55)	0.26 (-0.52 - 0.88)	0.3072
	W4L z-score	0.67 (0.56 - 1.8)	-0.16 (-1.09 - 0.38)	**0.0167**
	W4A z-score	0.97 (0.19 - 1.41)	-0.32 (-1.45 - 0.81)	**0.0265**
	MUAC z-score	1.76 (1.24 - 2.67)	0.83 (-0.26 - 1.96)	**0.0411**

Abbreviations: Interquartile range (IQR); Kilogram (kg); centimetre (cm); Head circumference (HC); Weight for length (W4L); Weight for age (W4A); Mid-upper arm circumference (MUAC); Not applicable (N/A)

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When the summary of the data in Tables 3.4 and 3.5 is considered, there are no statistically significant differences in the observed measurements between the HUU and HEU groups throughout the 0 weeks, 10 weeks and 6-month time-points, with exception of the weight for length (W4L) z-scores at 10 weeks (p=0.0305). However, there are considerable differences in the physical size of these infants, between groups, when the 12-month time-point is reached: weight (p=0.0183), W4L (p=0.0346) and W4A (p=0.0183), as well as for W4L (p=0.0167), W4A (p=0.0265) and MUAC (p=0.0411).

These observations show that, although these HUU and HEU infants were very similar when measured at birth (0 weeks), 10 weeks and 6 months, there are noteworthy differences at the 12-month time-point when considering the physical size of these infants. The HEU group shows significantly lower values for the aforementioned measurements than that observed in the HUU group and it therefore seems that HUU infants have a more favourable growth progression than their HEU counterparts at 12 months of age.

These observed measurements of anthropometry, and the progression thereof, can be better understood and visualised when represented graphically. Figures 3.1 through to 3.6 show the progression of these two groups when compared to one another.

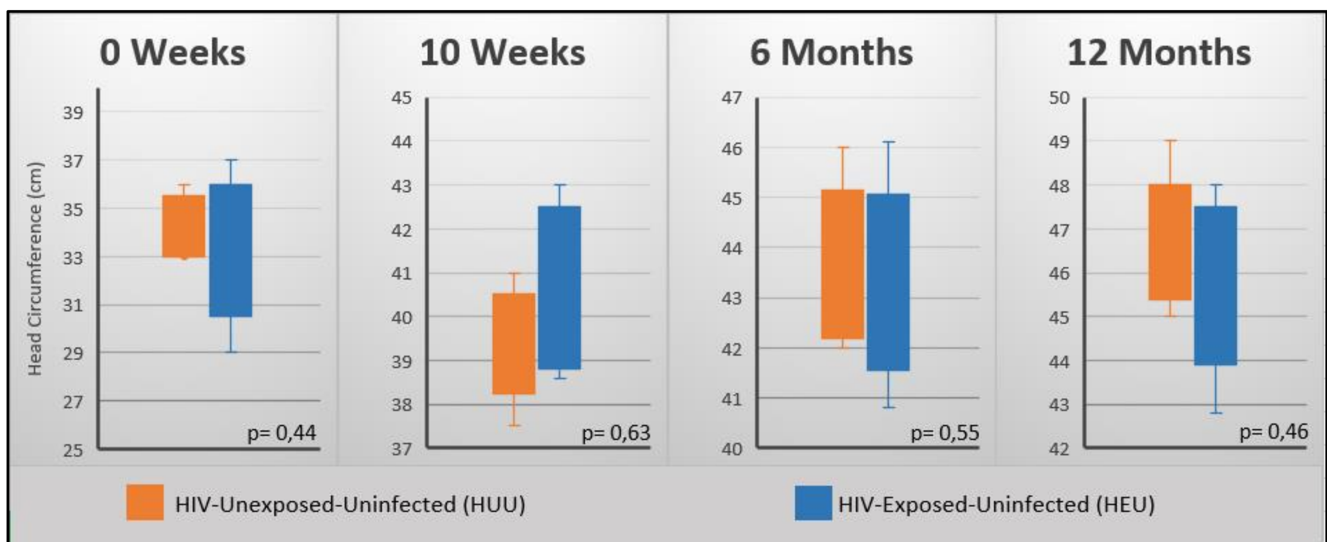

Figure 3.1: Box and whisker plot of infant head circumference and the comparison between HUU and HEU groups at the measured time-points (0 weeks, 10 weeks, 6 months, and 12 months).

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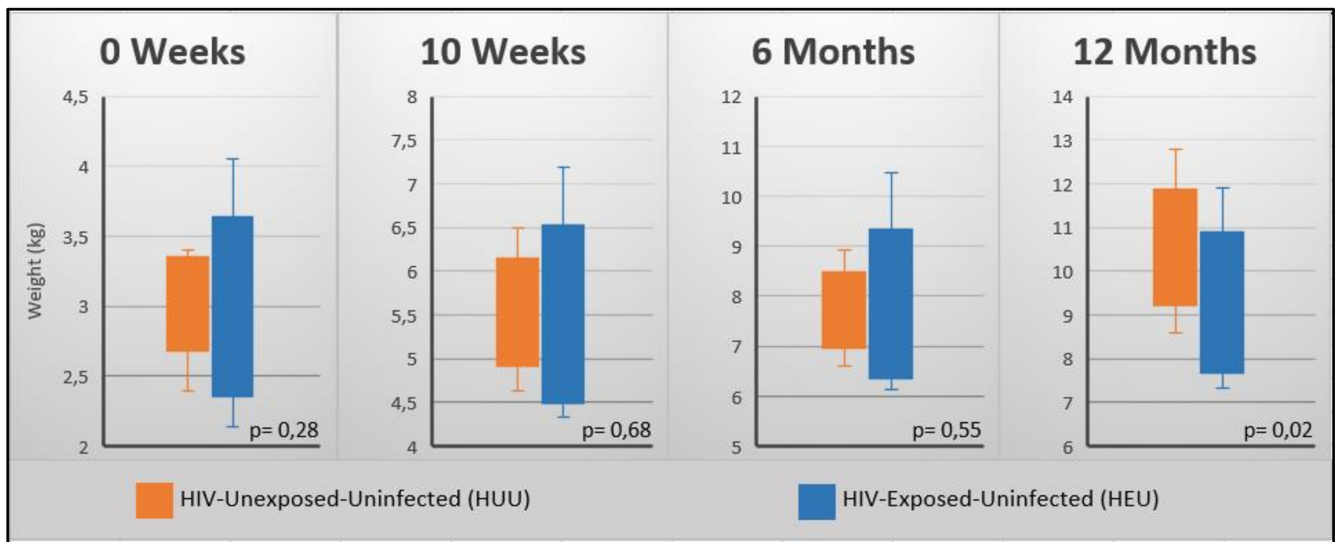

Figure 3.2: Box and whisker plot of infant weight and the comparison between HUU and HEU groups at the measured time-points (0 weeks, 10 weeks, 6 months, and 12 months).

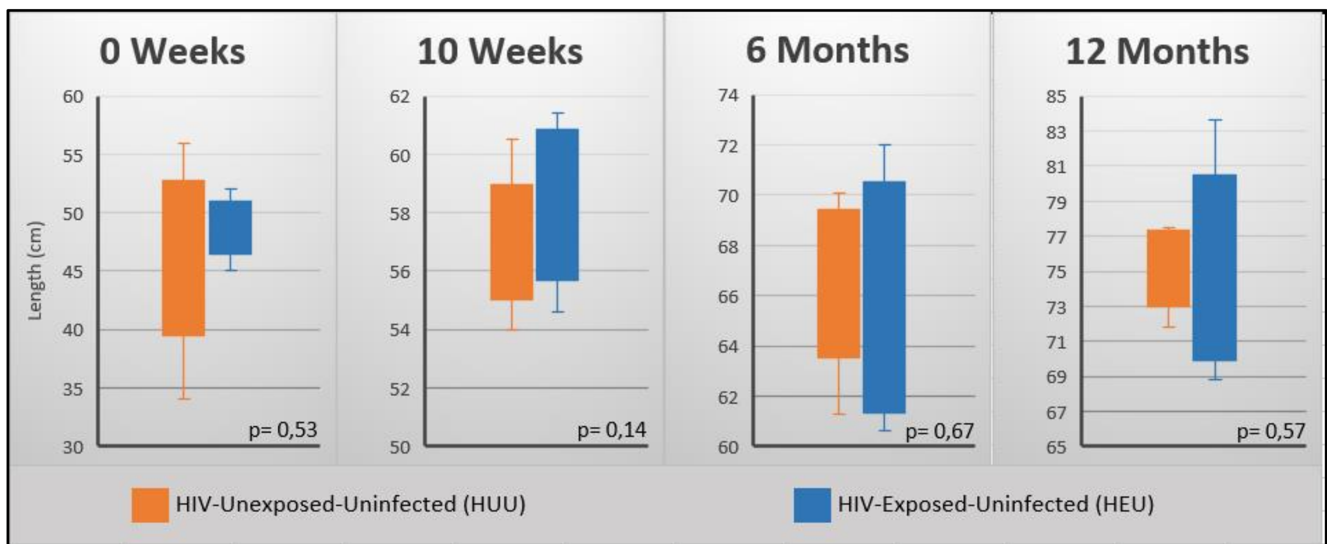

Figure 3.3: Box and whisker plot of infant length and the comparison between HUU and HEU groups at the measured time-points (0 weeks, 10 weeks, 6 months, and 12 months).

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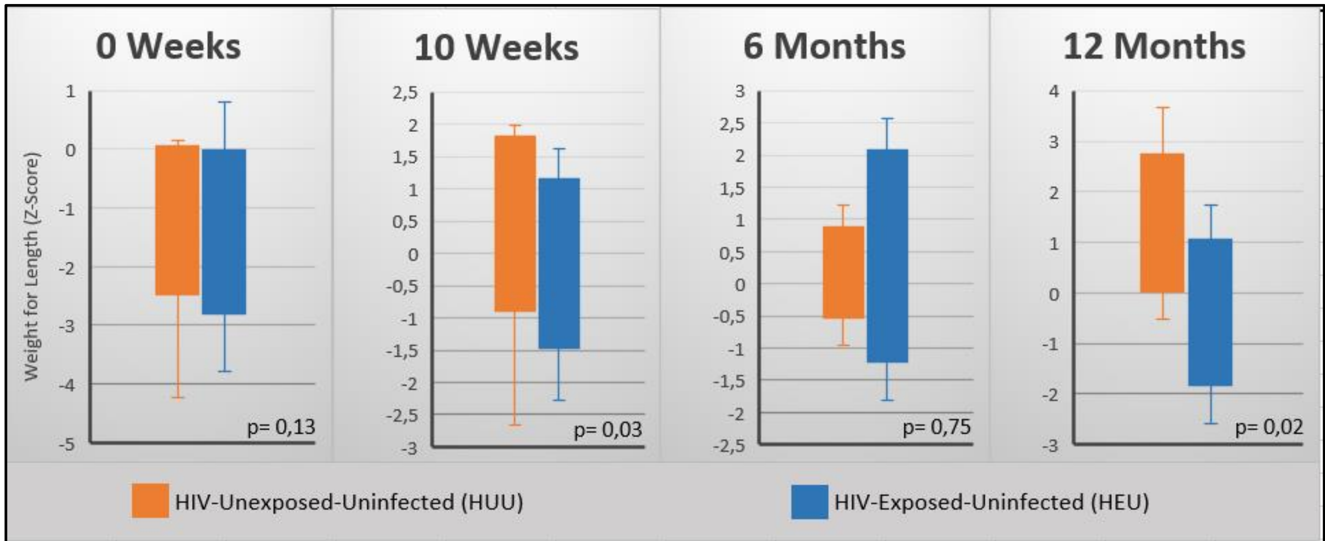

Figure 3.4: Box and whisker plot of infant weight for length z-scores and the comparison between HUU and HEU groups at the measured time-points (0 weeks, 10 weeks, 6 months, and 12 months).

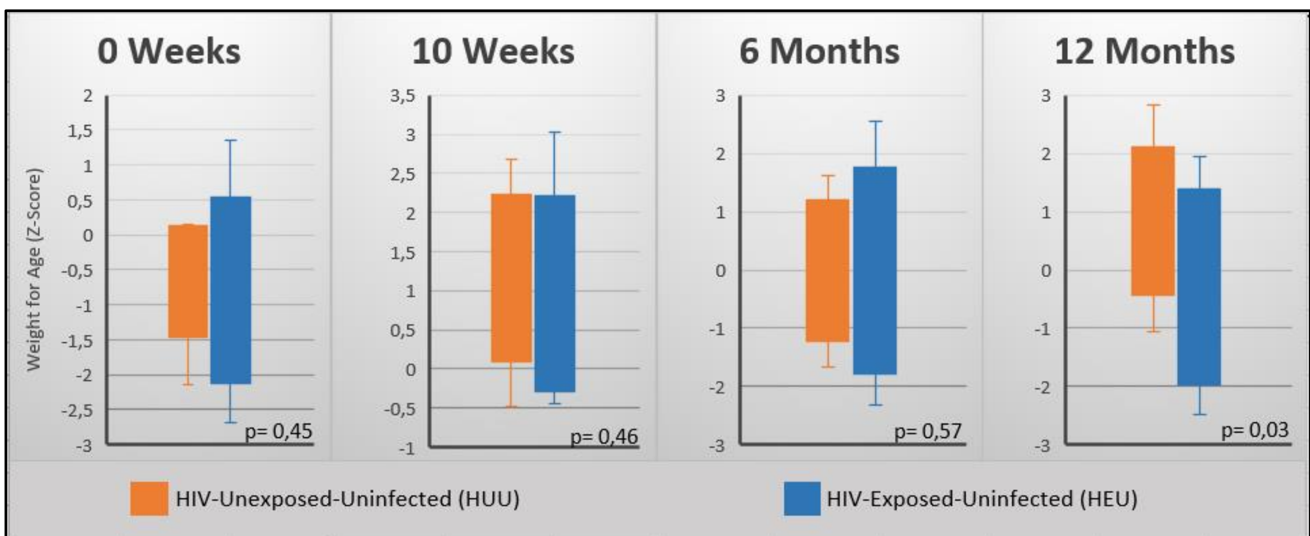

Figure 3.5: Box and whisker plot of infant weight for age z-scores and the comparison between HUU and HEU groups at the measured time-points (0 weeks, 10 weeks, 6 months, and 12 months).

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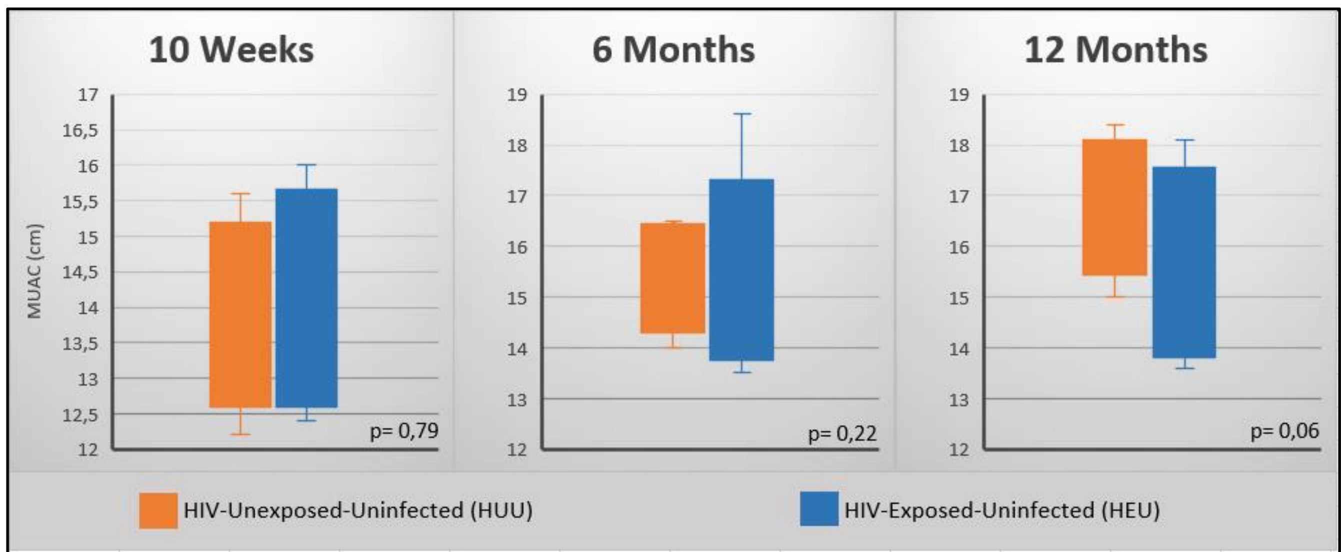

Figure 3.6: Box and whisker plot of infant MUAC and the comparison between HUU and HEU groups at the measured time-points (10 weeks, 6 months, and 12 months).

These graphs depicting the overall summary of the two populations at the various time-points show no real discernible differences between the HUU and HEU groups until the 12-month time-point is analysed. The bar graphs in Figures 3.7 and 3.8 show the progression of the two cohorts over the first 12 months of life for overall weight measurements. While, for the most part, the measured progression seems fairly similar at first, stunting of growth is observed in the HEU group between the 6 month and 12-month observations.

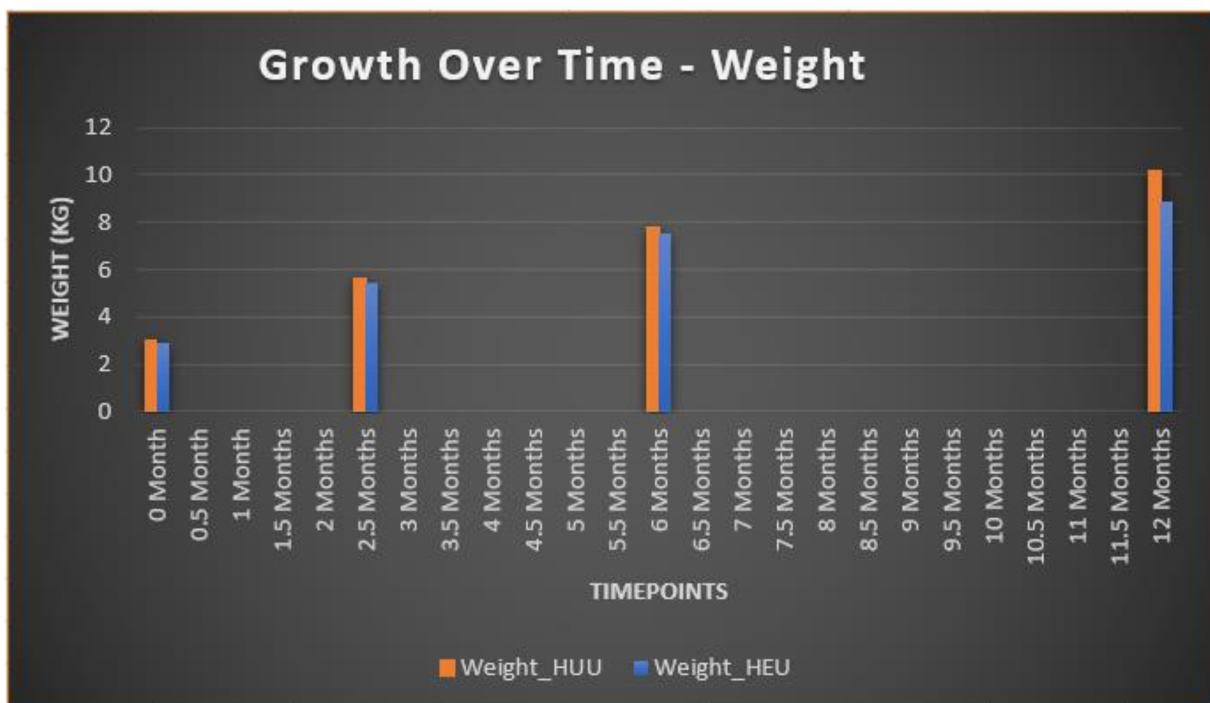

Figure 3.7: Bar-graph depicting growth over time, over the first 12 months of life, for the HUU and HEU groups as measured by weight in kilograms at the 4 time-points.

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Figure 3.8: Bar-graph depicting growth over time, over the first 12 months of life, for the HUU and HEU groups as measured by the weight for length (W4L) metric.

It can be seen in these bar-graphs that the HUU metrics, shown in orange, are generally larger than the HEU metrics, shown in blue. It is worth noting that, at birth, there is no significant difference in measurement between the two groups, however there is greater progression of growth in the HUU group. A similar observation can be noted for the MUAC measurement when compared by means of a bar-graph for the 2 groups, as seen below in Figure 3.9. While the initial measurement, taken at 10 weeks, shows a marginally higher value for the HEU infant cohort, there is a notable difference in the growth progression between the groups. At both the six month and 12-month time-points the HUU group is observed to have overtaken the HEU group. The HUU infants also have greater growth progression when compared to that of the HEU counterparts, who have a more stunted progression. Though there are no statistically significant differences observed in the absolute MUAC measurement, it is worth noting that the difference observed at 12 months is only just below the level of significance, with a p value of 0.0573, and that the MUAC z-score differed significantly at this time point (Table 3.5).

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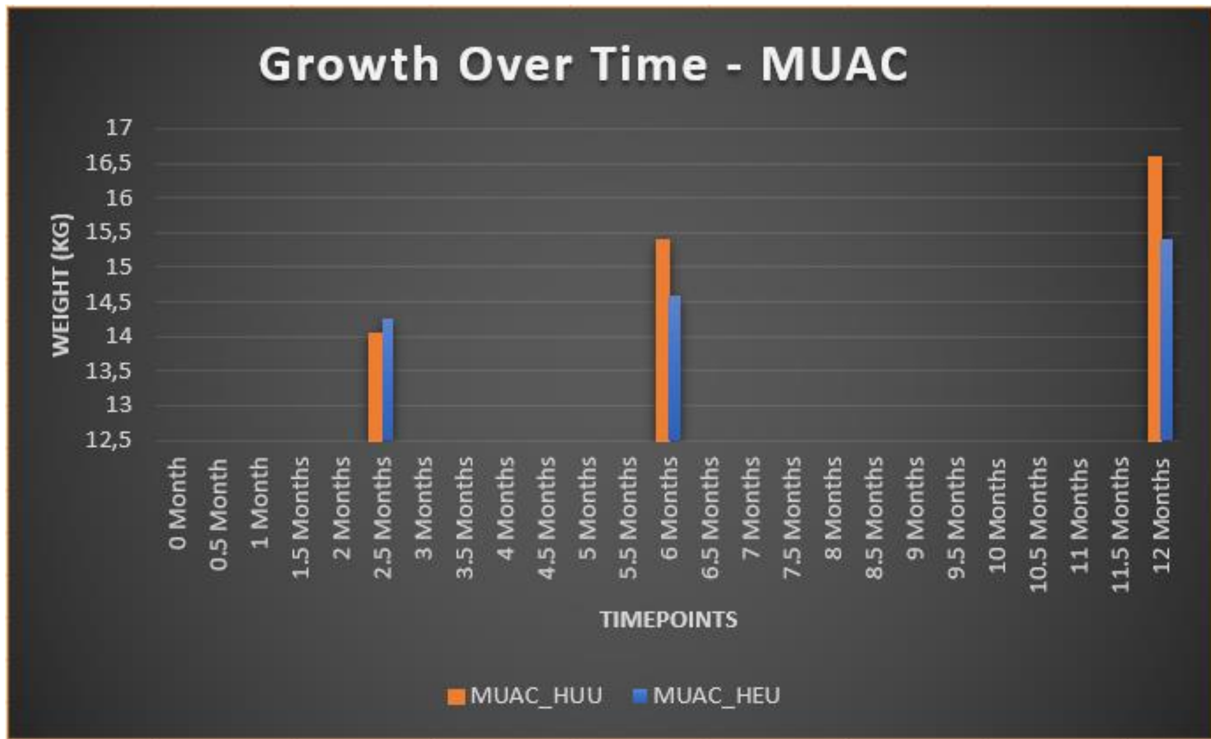

Figure 3.9: Bar-graph depicting growth over time, over the first 12 months of life, for the HUU and HEU groups compared as measured by mid-upper arm circumference (MUAC).

Similar observations were made regarding the HUU and HEU cohorts when analysed according to quantile distributions for the anthropometric measurements. Figure 3.10 shows that the HUU infants were significantly more likely to fall within the upper 50% (quantiles 3 and 4) of W4L, while the HEU infants were significantly more likely to fall within the lower 50% (quantiles 1 and 2) at 12 months.

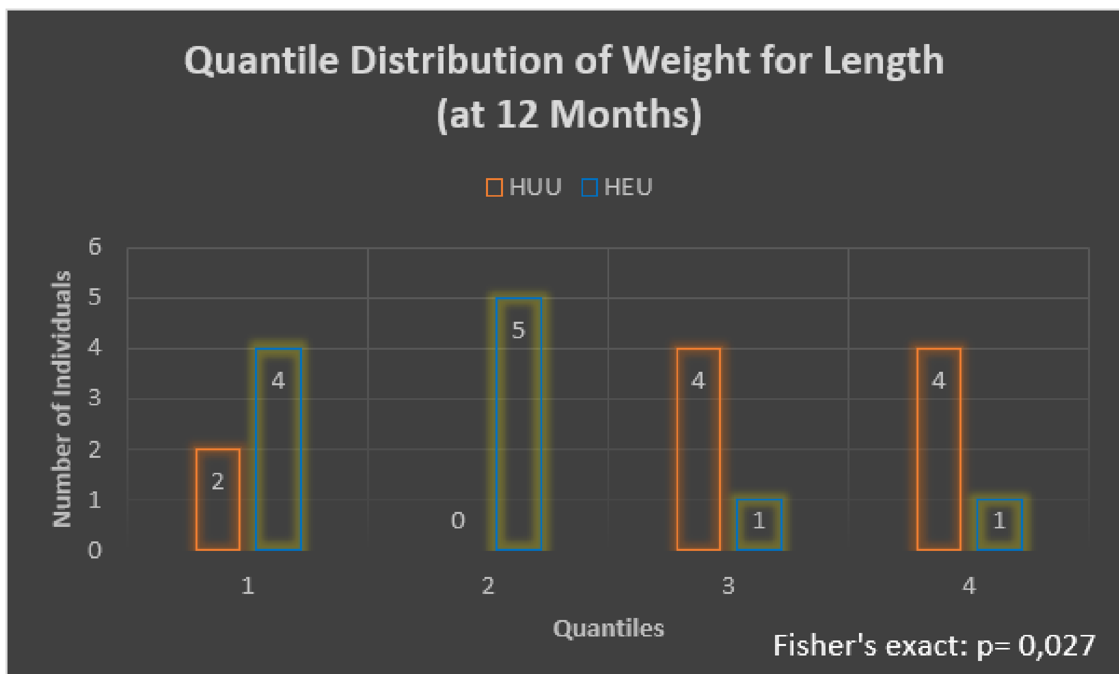

Figure 3.10: Quantile distribution of infants within the HUU and HEU cohorts for the weight for length metric at the 12-month time-point.

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The quantile distributions for the weight difference between 10 weeks and 6 months (Figure 3.11), length difference from 10 weeks to 6 months (Figure 3.12) and the weight difference from 6 months to 12 months of age (Figure 3.13), also show that HUU infants were more likely to fall within the upper 50%, but these differences just missed statistical significance.

The HC metrics showed only slight differences when compared by means of quantile distributions. Figures 3.14 and 3.15 however, below, indicate a tendency for the HEU individuals to fall in the lowest quantile and that this difference became more pronounced over time. Figure 3.16 shows the distribution when the difference between 0 weeks to 10 weeks is considered, showing once again that HEU infants were more likely to have growth in the lowest quantile.

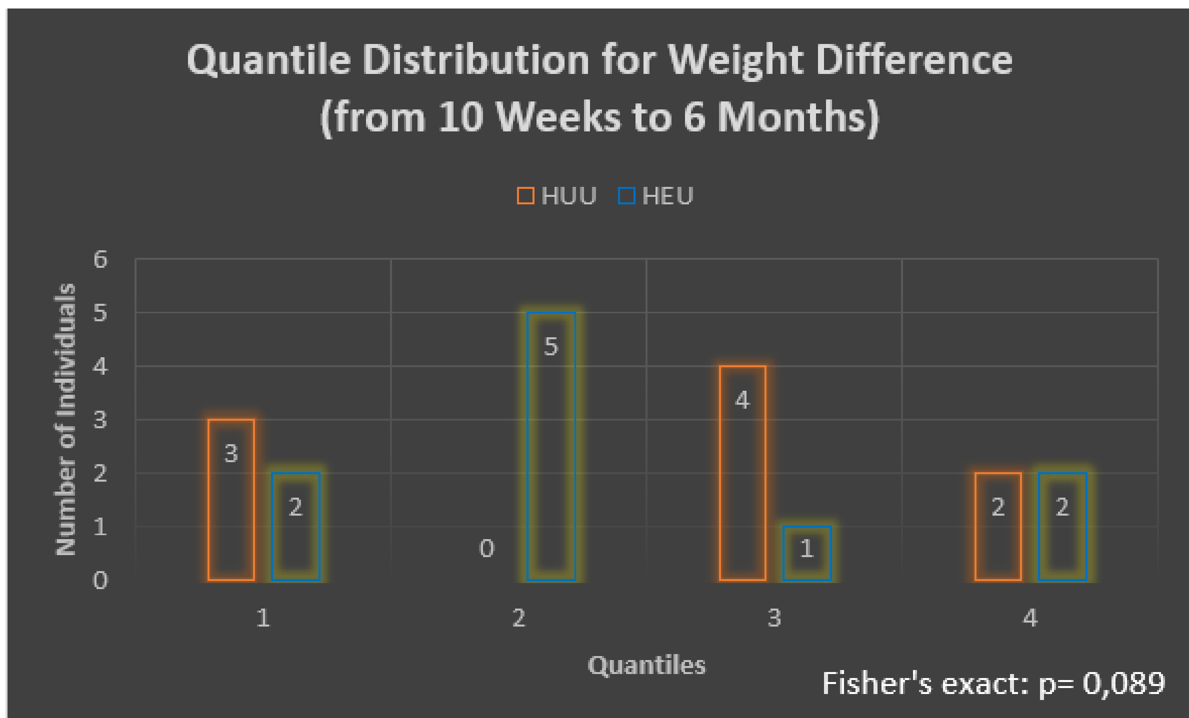

Figure 3.11: Quantile distribution of infants within the HUU and HEU cohorts for the weight difference metric between the 10 week and 6-month time-points, providing an indication of growth in weight (kg) between these observed points.

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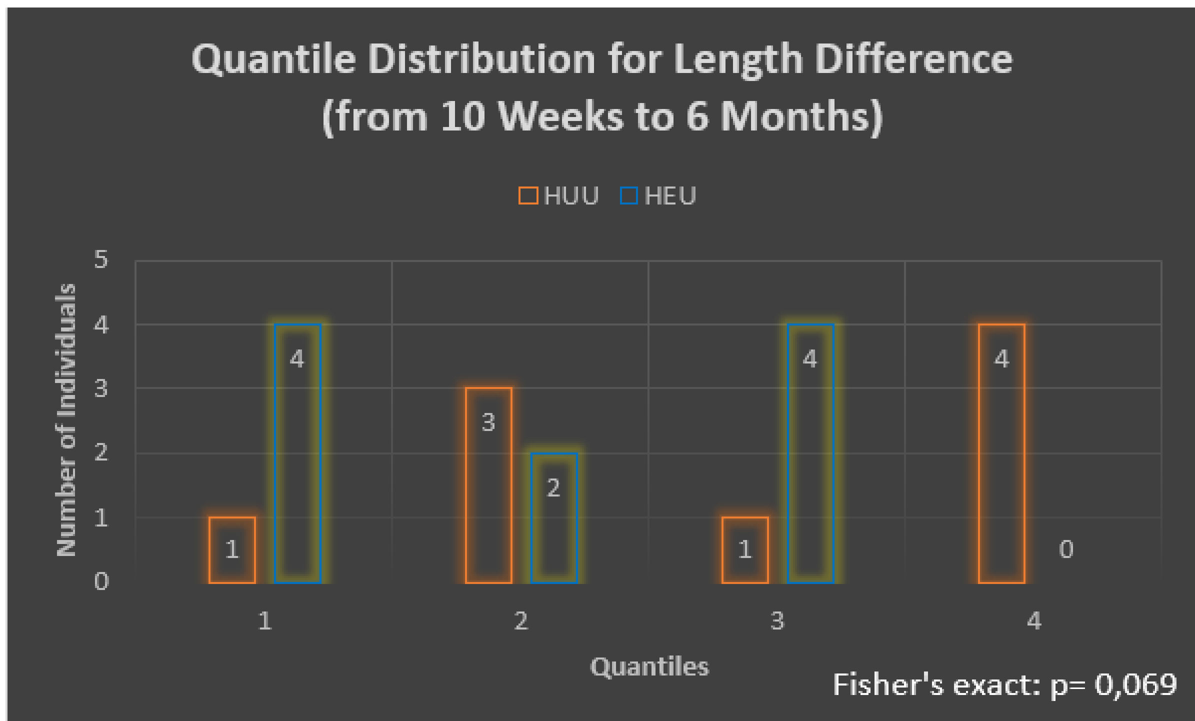

Figure 3.12: Quantile distribution of infants within the HUU and HEU cohorts for the length difference metric between the 10 week and 6-month time-points, providing an indication of growth in length (cm) between these observed points.

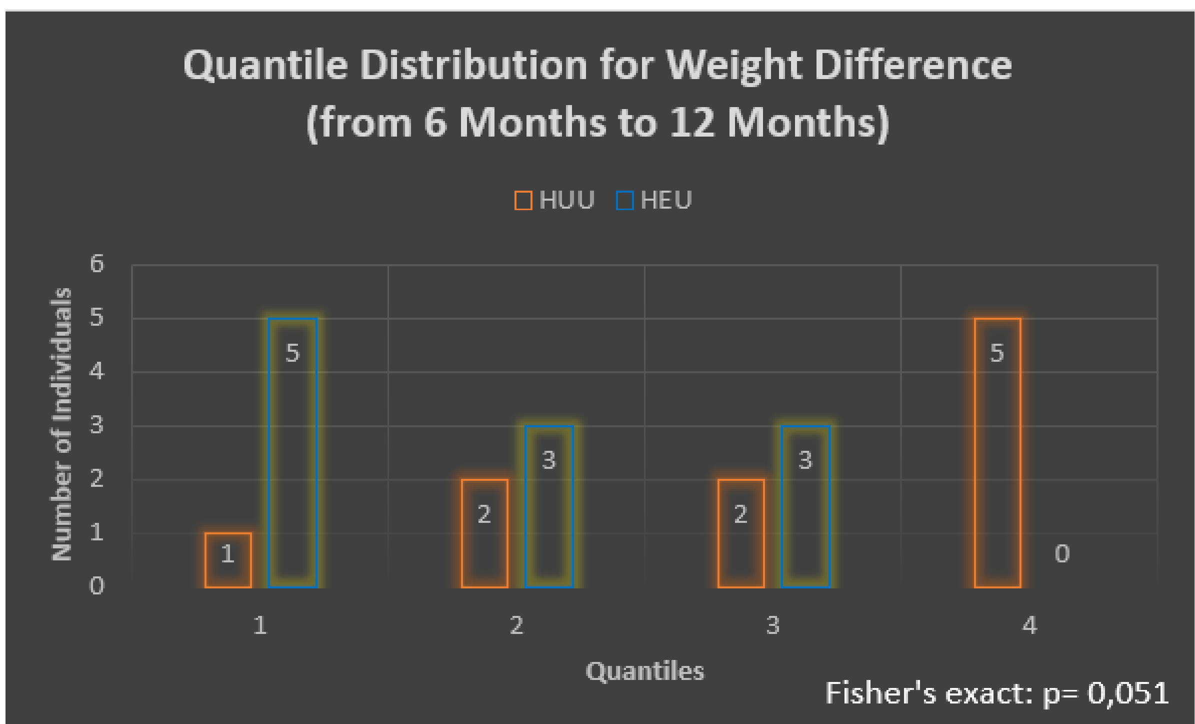

Figure 3.13: Quantile distribution of infants within the HUU and HEU cohorts for the weight difference metric between the 6 month and 12-month time-points, providing an indication of growth in weight (kg) between these observed points.

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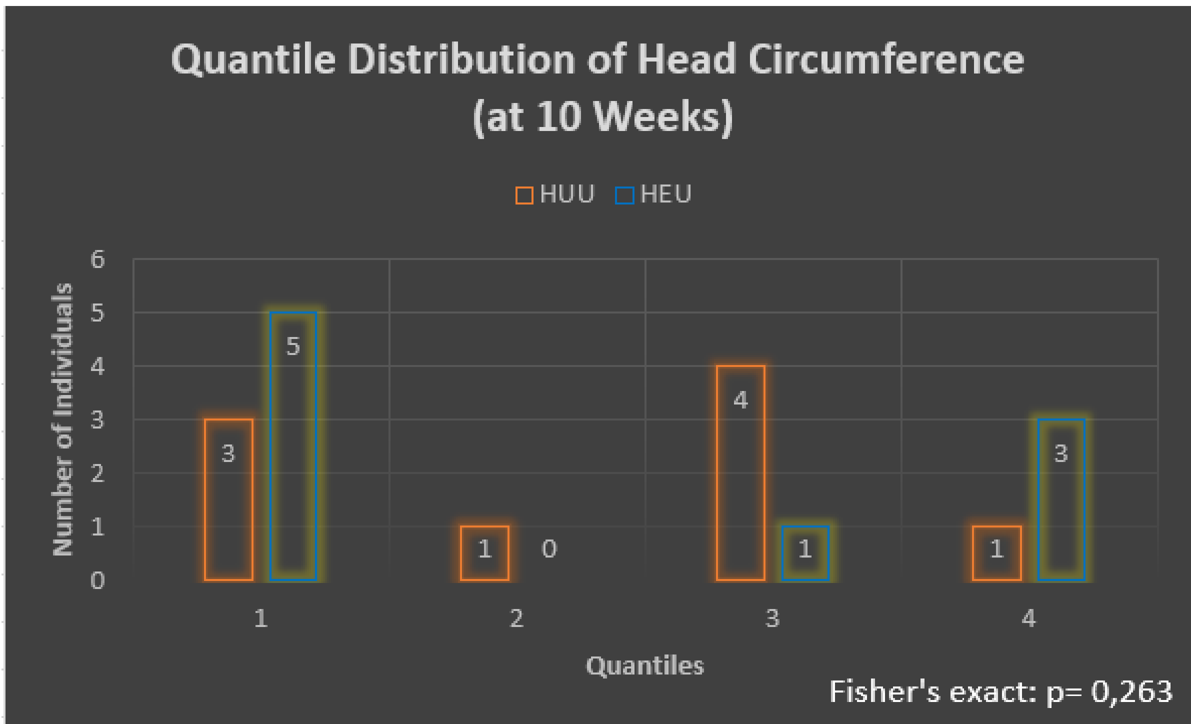

Figure 3.14: Quantile distribution of infants within the HUU and HEU cohorts for the head circumference metric at the 10-week time-point.

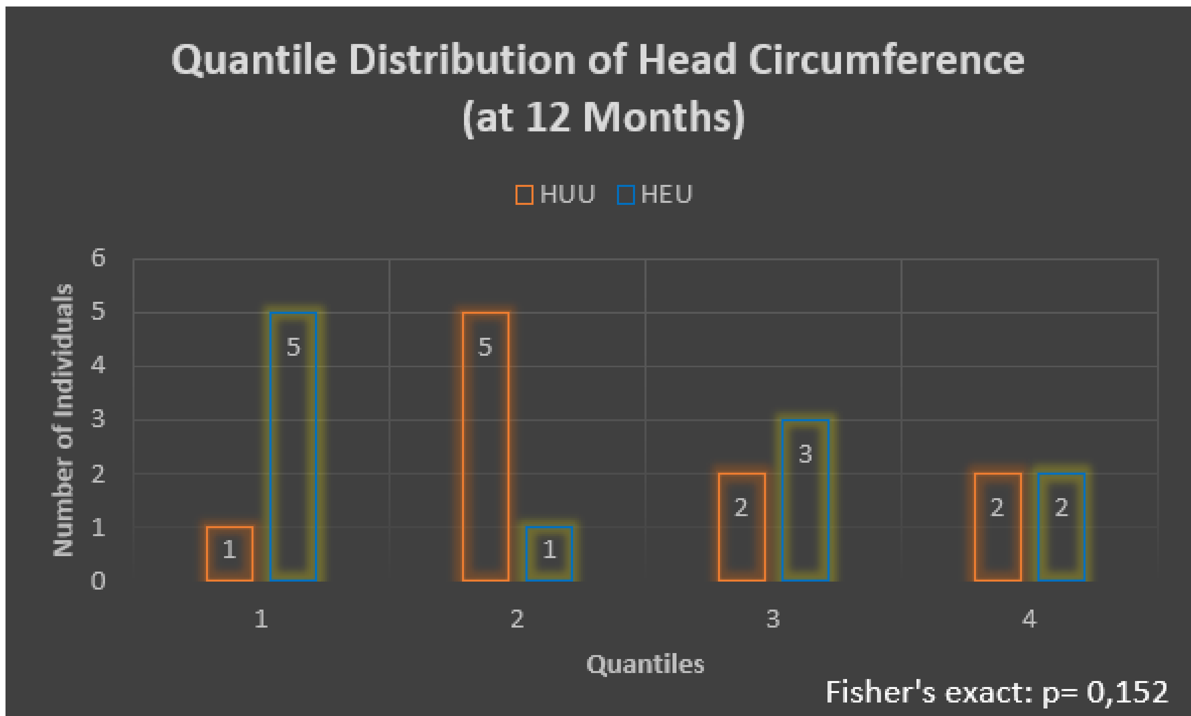

Figure 3.15: Quantile distribution of infants within the HUU and HEU cohorts for the head circumference metric at the 12-month time-point.

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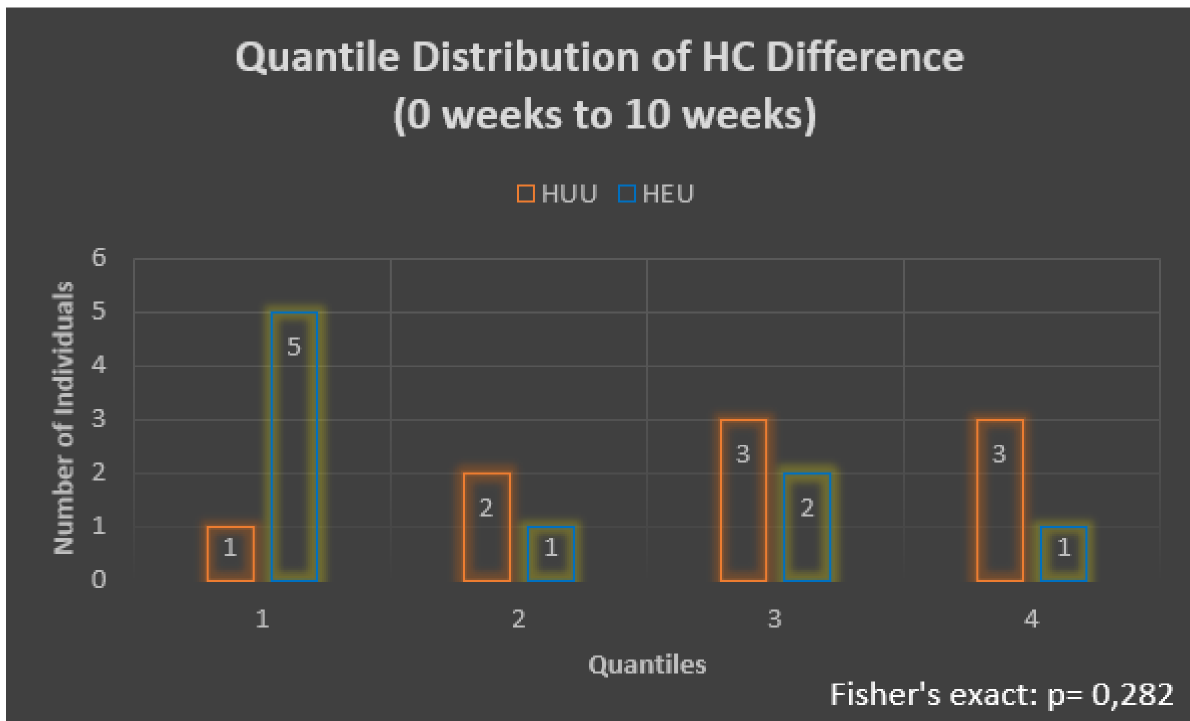

Figure 3.16: Quantile distribution of infants within the HUU and HEU cohorts for the head circumference metric between the 0-week and 10-week time-points, providing an indication of growth in HC between these observed points.

3.3 Cytokine Data

Whole blood stimulation (WBS) and subsequent cytokine testing were conducted for infant blood samples taken at the four time-points: 0 weeks, 10 weeks, 6 months, and 12 months of age. As explained in detail in Chapter 2, the samples were subjected to two types of stimulation in an effort to elicit an immunological response from the monocyte/macrophage population contained therein, namely: Lipopolysaccharide (LPS) simulating an endotoxin insult and Polyinosinic:polycytidylic acid (Poly I:C), which is a toll-like receptor 3 agonist. The results were compared with unstimulated samples from the same participants, serving as the Control.

The Immunoassay used was a Human Cytokine Magnetic Bead panel in which the following eight analytes were assessed:

- Interleukin-2 (IL-2)
- Interleukin-4 (IL-4)
- Interleukin-6 (IL-6)
- Interleukin-8 (IL-8)
- Interleukin-10 (IL-10)
- Interferon-gamma (IFN-ɣ)
- Tumour Necrosis Factor alpha (TNF-α)
- Granulocyte-macrophage colony-stimulating factor (GM-CSF)

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The observed cytokine concentrations for the unstimulated samples (Control), LPS-stimulated (LPS) and Poly I:C (Poly) are described by the median and interquartile ranges in Tables 3.6 – 3.9 below.

It is important to understand the presented values by differentiating between what would be classified as 'high' and what would be classified as 'low'. For instance, an observably high or low concentration seen in a Control test might indicate an inherent tendency to have a more or less inflammatory milieu, respectively. Whereas an observably high or low concentration seen under stimulation would reflect a response to the stimuli. In this assay the LPS tests for a reaction to bacterial pathogens and Poly I:C to viral pathogens, as discussed in Chapter 1.

There appears to be no great observable differences between the HEU and the HUU groups. One noteworthy exception, at the 0-week time-point, is IL-6 when stimulated with Poly I:C. This assessment showed a higher concentration of IL-6 at birth in the HEU infant group than that of the HUU infant group. Another similar exception also was also observed at the 10-week time-point for IL-2, which was observed to show a higher concentration in the HUU group at this point.

The most notable, and only statistically significant, difference is seen for the IL-4 biomarker at the 6-month time-point, with the HEU infant cohort displaying notably lower concentrations for the control, Poly I:C- and LPS-stimulated samples. These observed differences are displayed visually in Figure 3.17.

A similar observation is made when the quantile distribution for the median levels of IL-4 is analysed at the 6-month time-point. Figure 3.18 shows that the HUU were more likely to fall within the upper 50% and the HEU infants the lower 50%. It is also interesting to note that only HEU infants had values in the lowest quantile. None of these differences were, however, observed at the 12-month time-point.

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Table 3.6: Summary of cytokine data comparison between HUU and HEU infant groups for the 0-week time-point.

Age/Timepoint	Cytokine	Statistic			
		Population: Median (IQR) (pg/mL)	HUU: Median (IQR) (pg/mL)	HEU: Median (IQR) (pg/mL)	P-value
0 Weeks	IL8_Control	1260.07 (405.53 - 2340.47)	1266.19 (246.64 - 2340.47)	730.41 (405.53 - 4268.76)	0.9439
	IL10_Control	2.8 (1.37 - 3.2)	3.2 (1.37 - 3.2)	1.75 (1.16 - 3.25)	0.7782
	IL6_Control	3.23 (1.52 - 16.59)	2.78 (1.13 - 3.59)	3.23 (1.52 - 23.81)	0.7782
	TNFa_Control	13.87 (6.96 - 24.36)	18.71 (7.15 - 32.24)	12.93 (5.85 - 21.32)	0.5262
	IFNg_Control	3.97 (3.97 - 3.97)	3.97 (3.97 - 3.97)	3.97 (3.97 - 3.97)	0.9439
	IL2_Control	1.16 (0.65 - 3.24)	1.01 (0.44 - 3.24)	1.28 (0.65 - 3.24)	0.6472
	IL4_Control	4.27 (2.6 - 28.57)	2.95 (0.77 - 20.58)	8.6 (2.6 - 35.01)	0.2178
	GMCSF_Control	1.72 (0.32 - 3.28)	0.78 (0.26 - 2.29)	1.76 (0.42 - 3.28)	0.3600
	IL8_LPS	11728.7 (5055.71 - 15069.24)	8953.49 (4506.61 - 15899.92)	12014.95 (5055.71 - 13062.21)	0.9439
	IL10_LPS	149.78 (22.96 - 417.0)	119.66 (22.96 - 211.29)	190.21 (2.57 - 809.46)	0.6728
	IL6_LPS	410.92 (104.41 - 1088.94)	347.46 (104.41 - 664.61)	851.29 (49.72 - 3559.85)	0.3242
	TNFa_LPS	285.77 (84.22 - 1671.2)	304.26 (129.74 - 1002.62)	282.39 (50.49 - 1842.64)	0.8880
	IFNg_LPS	3.97 (3.97 - 3.97)	3.97 (3.31 - 3.97)	3.97 (3.97 - 3.97)	0.6000
	IL2_LPS	1.19 (0.8 - 3.24)	0.98 (0.8 - 3.24)	1.65 (0.75 - 3.24)	0.6985
	IL4_LPS	11.32 (3.76 - 31.54)	11.78 (2.6 - 19.64)	8.39 (4.27 - 33.68)	0.7782
	GMCSF_LPS	2.63 (1.01 - 3.28)	2.71 (0.62 - 3.28)	2.27 (1.37 - 3.28)	0.5262
	IL8_Poly	1260.53 (379.47 - 5727.8)	921.43 (168.51 - 3142.35)	1971.99 (432.72 - 15554.2)	0.1392
	IL10_Poly	3.2 (1.13 - 3.36)	2.72 (0.95 - 3.2)	3.2 (1.44 - 666.93)	0.1927
	IL6_Poly	3.23 (1.73 - 48.47)	2.56 (1.29 - 3.23)	19.87 (2.25 - 797.0)	0.0783
	TNFa_Poly	11.77 (7.05 - 256.0)	16.43 (7.05 - 26.1)	11.77 (5.67 - 737.88)	0.5732
	IFNg_Poly	3.97 (3.97 - 3.97)	3.97 (3.97 - 3.97)	3.97 (1.96 - 3.97)	0.3787
	IL2_Poly	1.55 (0.49 - 3.24)	1.02 (0.13 - 3.24)	1.59 (1.16 - 3.24)	0.1697
	IL4_Poly	11.87 (2.6 - 28.57)	8.76 (2.6 - 25.29)	18.81 (0.28 - 46.58)	0.7782
	GMCSF_Poly	1.65 (0.45 - 3.28)	0.85 (0.26 - 3.28)	3.28 (0.62 - 5.96)	0.1213

Abbreviations: Lipopolysaccharide (LPS); Polyinosinic:polycytidylic acid (Poly)

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Table 3.7: Summary of cytokine data comparison between HUU and HEU infant groups for the 10-week time-point.

Age/Timepoint	Cytokine	Statistic			
		Population: Median (IQR) (pg/mL)	HUU: Median (IQR) (pg/mL)	HEU: Median (IQR) (pg/mL)	P-value
10 Weeks	IL8_Control	131.44 (22.5 - 443.81)	85.07 (21.19 - 260.35)	443.81 (28.47 - 1283.01)	0.1325
	IL10_Control	3.36 (2.34 - 23.9)	2.83 (1.53 - 5.19)	6.73 (3.2 - 62.49)	0.1480
	IL6_Control	4.41 (0.48 - 47.85)	3.12 (0.29 - 9.49)	4.91 (3.23 - 559.05)	0.1325
	TNFa_Control	19.95 (8.12 - 70.92)	15.44 (10.74 - 23.91)	70.92 (3.36 - 1428.91)	0.2472
	IFNg_Control	3.97 (3.97 - 3.97)	3.97 (1.55 - 3.97)	3.97 (3.97 - 3.97)	0.4179
	IL2_Control	1.13 (0.4 - 3.24)	3.24 (1.23 - 3.24)	0.64 (0.24 - 1.13)	0.0826
	IL4_Control	12.03 (2.6 - 43.2)	16.06 (2.7 - 103.69)	12.03 (2.6 - 43.2)	0.6025
	GMCSF_Control	1.01 (0.44 - 3.28)	1.86 (0.38 - 3.28)	1.01 (0.52 - 3.28)	0.8170
	IL8_LPS	4116.46 (861.26 - 13004.76)	5219.15 (1690.48 - 10466.98)	4018.3 (861.26 - 13004.76)	0.8170
	IL10_LPS	27.85 (12.05 - 157.96)	24.16 (0.47 - 45.33)	155.17 (24.7 - 272.46)	0.2472
	IL6_LPS	728.43 (258.25 - 1512.43)	650.34 (191.78 - 1176.13)	1131.28 (434.17 - 5095.25)	0.6434
	TNFa_LPS	352.7 (47.0 - 1743.49)	285.08 (165.83 - 440.45)	464.9 (5.46 - 4050.4)	0.9079
	IFNg_LPS	1.27 (0.54 - 3.97)	1.77 (0.72 - 3.88)	1.13 (0.38 - 3.97)	0.7285
	IL2_LPS	3.24 (1.02 - 3.24)	3.24 (1.42 - 3.24)	2.53 (0.64 - 3.24)	0.4179
	IL4_LPS	5.04 (2.8 - 47.58)	20.9 (3.28 - 106.03)	4.92 (2.8 - 42.33)	0.5245
	GMCSF_LPS	3.28 (1.01 - 4.26)	3.28 (1.23 - 5.69)	1.75 (0.72 - 4.26)	0.5245
	IL8_Poly	107.6 (23.02 - 533.22)	101.79 (37.9 - 145.98)	238.07 (15.73 - 8451.04)	0.8170
	IL10_Poly	6.14 (1.92 - 11.7)	2.22 (1.11 - 7.25)	8.29 (3.13 - 18.14)	0.1649
	IL6_Poly	4.65 (2.15 - 28.01)	6.36 (1.57 - 14.32)	4.14 (3.23 - 208.39)	0.5628
	TNFa_Poly	20.97 (12.52 - 114.66)	17.59 (11.55 - 56.2)	27.29 (12.52 - 378.64)	0.3545
	IFNg_Poly	3.97 (0.9 - 3.97)	3.31 (1.4 - 7.27)	3.97 (0.9 - 3.97)	0.7285
	IL2_Poly	1.0 (0.49 - 3.24)	2.63 (0.55 - 3.24)	0.81 (0.49 - 3.24)	0.3854
	IL4_Poly	14.44 (4.92 - 38.86)	26.65 (4.98 - 124.93)	8.86 (1.6 - 31.97)	0.1325
	GMCSF_Poly	2.46 (0.93 - 3.28)	2.87 (1.28 - 3.28)	2.17 (0.44 - 3.28)	0.4875

Abbreviations: Lipopolysaccharide (LPS); Polyinosinic:polycytidylic acid (Poly)

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Table 3.8: Summary of cytokine data comparison between HUU and HEU infant groups for the 6-month time-point.

Age/Timepoint	Cytokine	Statistic			
		Population: Median (IQR) (pg/mL)	HUU: Median (IQR) (pg/mL)	HEU: Median (IQR) (pg/mL)	P-value
6 Months	IL8_Control	383.77 (122.39 - 4737.01)	284.99 (92.53 - 1120.32)	502.98 (232.11 - 11369.94)	0.2050
	IL10_Control	5.32 (2.34 - 11.21)	4.69 (2.34 - 9.74)	5.41 (2.09 - 177.96)	0.5495
	IL6_Control	24.64 (3.83 - 133.19)	21.95 (8.45 - 133.19)	38.55 (1.46 - 7053.92)	1.000
	TNFa_Control	36.89 (15.88 - 215.39)	31.04 (15.88 - 171.4)	40.04 (14.85 - 3128.88)	0.4813
	IFNg_Control	3.97 (1.82 - 3.97)	3.49 (1.51 - 3.97)	3.97 (1.96 - 3.97)	0.5974
	IL2_Control	1.47 (0.52 - 3.24)	1.38 (0.47 - 3.24)	1.47 (0.52 - 3.24)	0.7782
	IL4_Control	82.53 (14.07 - 319.59)	261.0 (67.39 - 337.71)	14.07 (2.6 - 252.94)	**0.0346**
	GMCSF_Control	3.28 (1.01 - 3.28)	2.83 (1.41 - 3.83)	3.28 (0.83 - 3.63)	0.8053
	IL8_LPS	10648.46 (5673.55 - 12447.65)	8837.16 (1950.57 - 11987.39)	11843.73 (9812.64 - 15727.71)	0.0671
	IL10_LPS	60.27 (12.42 - 259.71)	56.01 (12.42 - 161.11)	75.83 (12.42 - 287.93)	0.9159
	IL6_LPS	1077.28 (305.44 - 9848.67)	1449.88 (267.51 - 6013.35)	1077.28 (305.44 - 10557.34)	0.3600
	TNFa_LPS	1300.82 (542.25 - 5604.61)	1614.54 (542.25 - 5604.61)	1300.82 (453.55 - 6420.08)	0.9439
	IFNg_LPS	3.97 (1.82 - 3.97)	3.02 (0.9 - 3.97)	3.97 (2.24 - 3.97)	0.4813
	IL2_LPS	1.05 (0.65 - 3.24)	1.44 (0.65 - 3.24)	0.98 (0.52 - 3.24)	1.000
	IL4_LPS	72.01 (31.95 - 333.09)	252.56 (66.23 - 562.65)	31.95 (3.29 - 203.73)	**0.0242**
	GMCSF_LPS	3.28 (1.55 - 4.01)	2.12 (1.55 - 3.28)	3.28 (1.01 - 4.06)	0.5262
	IL8_Poly	680.19 (168.04 - 9118.38)	324.86 (116.21 - 3586.01)	884.67 (168.04 - 11578.35)	0.4813
	IL10_Poly	7.29 (3.05 - 10.72)	7.43 (3.05 - 10.4)	7.02 (3.02 - 195.65)	0.9719
	IL6_Poly	50.35 (5.3 - 180.32)	50.1 (8.71 - 116.76)	50.35 (1.15 - 6877.61)	0.8880
	TNFa_Poly	67.49 (18.44 - 308.62)	132.61 (17.33 - 308.62)	42.79 (18.44 - 2443.58)	0.9439
	IFNg_Poly	3.97 (1.51 - 4.63)	3.97 (1.51 - 4.88)	3.31 (1.51 - 3.97)	0.4181
	IL2_Poly	1.19 (0.64 - 3.24)	1.13 (0.35 - 3.24)	3.24 (0.74 - 3.24)	0.4181
	IL4_Poly	78.95 (16.85 - 321.54)	234.14 (49.34 - 407.39)	16.85 (0.28 - 278.25)	**0.0290**
	GMCSF_Poly	3.28 (1.82 - 3.28)	2.83 (1.51 - 3.28)	3.28 (2.46 - 3.28)	0.6472

Abbreviations: Lipopolysaccharide (LPS); Polyinosinic:polycytidylic acid (Poly)

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Table 3.9: Summary of cytokine data comparison between HUU and HEU infant groups for the 12-month time-point.

Age/Timepoint	Cytokine	Statistic			
		Population: Median (IQR) (pg/mL)	HUU: Median (IQR) (pg/mL)	HEU: Median (IQR) (pg/mL)	P-value
12 Months	IL8_Control	129.26 (41.74 - 309.52)	208.48 (69.06 - 367.07)	123.89 (27.09 - 278.03)	0.3981
	IL10_Control	2.91 (1.75 - 4.77)	2.73 (1.44 - 7.57)	2.91 (1.75 - 4.77)	1.000
	IL6_Control	10.44 (5.25 - 23.1)	11.39 (7.65 - 18.82)	9.62 (4.12 - 32.76)	0.6727
	TNFa_Control	19.4 (15.45 - 26.81)	18.12 (15.28 - 19.95)	22.61 (15.71 - 51.33)	0.2453
	IFNg_Control	1.96 (0.9 - 3.03)	1.62 (0.9 - 3.03)	1.96 (0.9 - 3.97)	0.7513
	IL2_Control	0.97 (0.55 - 3.24)	1.01 (0.75 - 3.24)	0.97 (0.3 - 3.24)	0.5495
	IL4_Control	161.06 (54.64 - 442.85)	176.63 (115.94 - 442.85)	155.07 (12.23 - 645.11)	0.4386
	GMCSF_Control	1.75 (0.91 - 3.28)	2.52 (0.91 - 3.28)	1.14 (0.81 - 3.28)	0.3600
	IL8_LPS	8139.76 (3098.53 - 11731.13)	8843.13 (3811.39 - 11304.15)	7629.26 (924.04 - 11900.97)	0.7248
	IL10_LPS	114.04 (29.36 - 160.76)	90.53 (29.36 - 139.75)	114.04 (22.12 - 217.24)	0.5732
	IL6_LPS	1629.87 (427.24 - 4716.64)	2632.44 (427.24 - 5772.16)	1629.87 (200.13 - 2922.31)	0.6221
	TNFa_LPS	1843.18 (512.16 - 5075.55)	2304.01 (360.75 - 5513.74)	1843.18 (573.13 - 4875.88)	0.7248
	IFNg_LPS	2.98 (1.88 - 4.16)	3.31 (1.96 - 4.88)	2.98 (1.13 - 3.97)	0.3600
	IL2_LPS	0.86 (0.32 - 3.24)	0.81 (0.31 - 3.24)	1.05 (0.32 - 3.24)	0.6472
	IL4_LPS	141.73 (38.86 - 424.51)	184.64 (110.16 - 424.51)	80.11 (2.8 - 532.57)	0.2050
	GMCSF_LPS	3.0 (1.41 - 4.05)	2.76 (2.06 - 5.11)	3.0 (1.21 - 3.28)	0.4597
	IL8_Poly	165.05 (38.72 - 314.38)	299.86 (51.41 - 316.16)	105.85 (35.87 - 264.48)	0.4386
	IL10_Poly	3.2 (2.24 - 7.09)	3.17 (1.44 - 7.93)	3.2 (2.24 - 7.09)	0.7782
	IL6_Poly	10.06 (3.23 - 35.45)	8.61 (3.23 - 16.89)	10.85 (3.23 - 38.06)	0.8053
	TNFa_Poly	18.18 (15.79 - 28.39)	17.77 (13.98 - 24.94)	19.0 (16.31 - 54.37)	0.3981
	IFNg_Poly	3.97 (1.82 - 3.97)	3.97 (2.64 - 3.97)	1.96 (0.54 - 3.97)	0.0783
	IL2_Poly	1.06 (0.63 - 3.24)	1.02 (0.56 - 3.24)	1.06 (0.63 - 3.24)	0.6472
	IL4_Poly	156.38 (32.7 - 445.01)	168.51 (32.7 - 445.01)	140.7 (16.85 - 590.74)	0.7248
	GMCSF_Poly	2.48 (1.14 - 3.28)	2.31 (1.14 - 3.28)	2.48 (0.72 - 3.28)	0.6472

Abbreviations: Lipopolysaccharide (LPS); Polyinosinic:polycytidylic acid (Poly)

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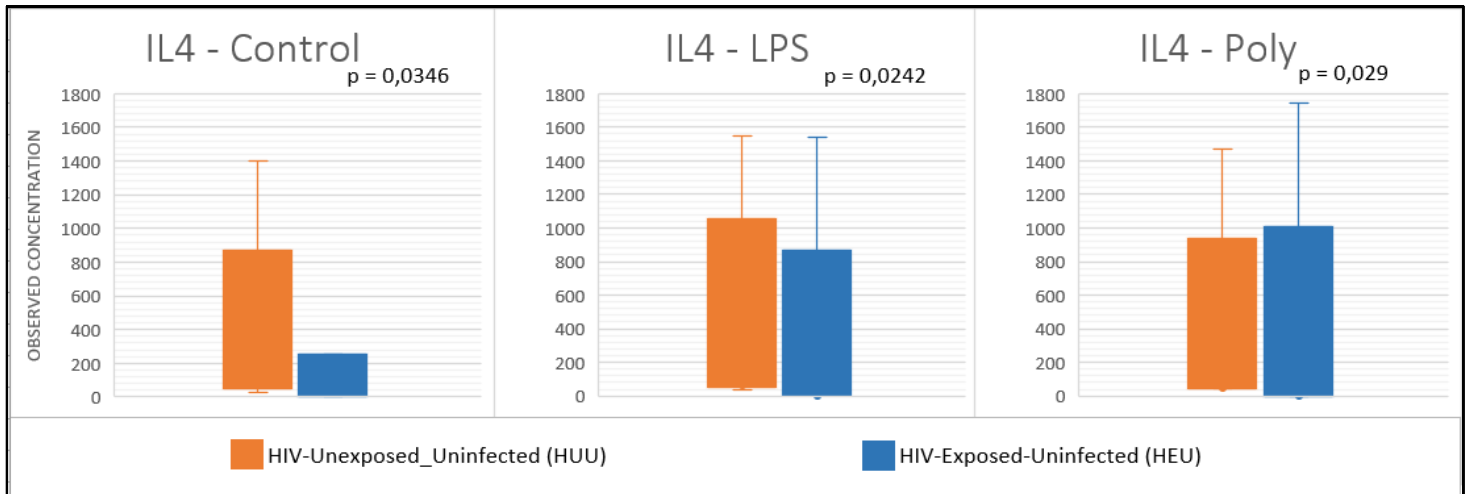

Figure 3.17: Box and whisker plot displaying the observed differences in concentration for the IL-4 cytokine at the 6-month time-point for the Control, LPS and Poly I:C tests.

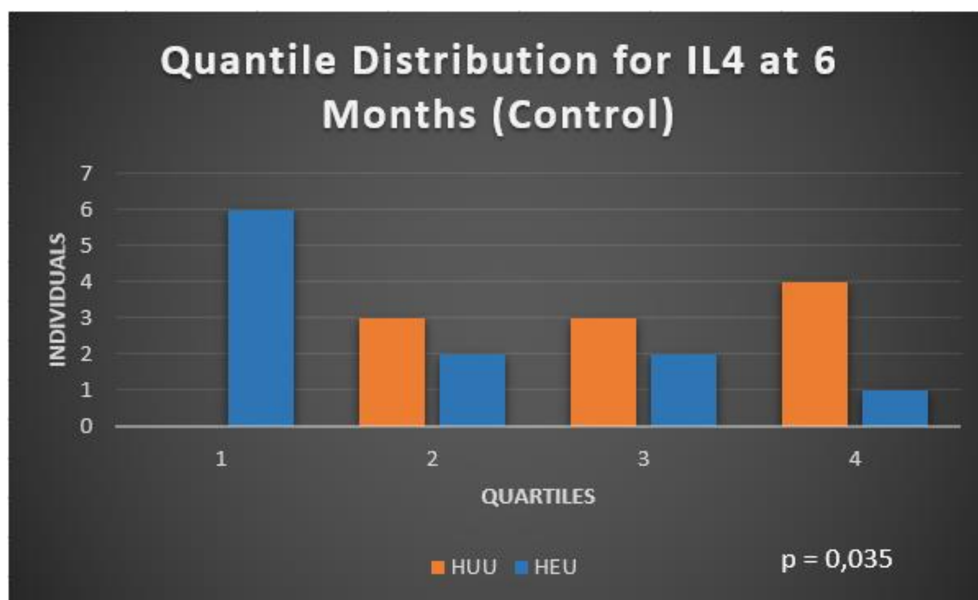

Figure 3.18: Quantile distribution of IL-4 in infants within the HUU and HEU cohorts at 6 months of age for the Control test.

Though not strictly statistically significant, there is an apparent difference at the 10-week time-point for the concentrations of TNF-α between the groups. For this immunological biomarker, the HEU group displayed higher concentrations when compared to that of the HUU cohort, as can be seen from the quartile distribution depicted below in Figure 3.19. This specific difference is highlighted as it is the only observed cytokine quantile distribution, other than for IL-4 above, that has a calculated p-value below the significance threshold of 0.1.

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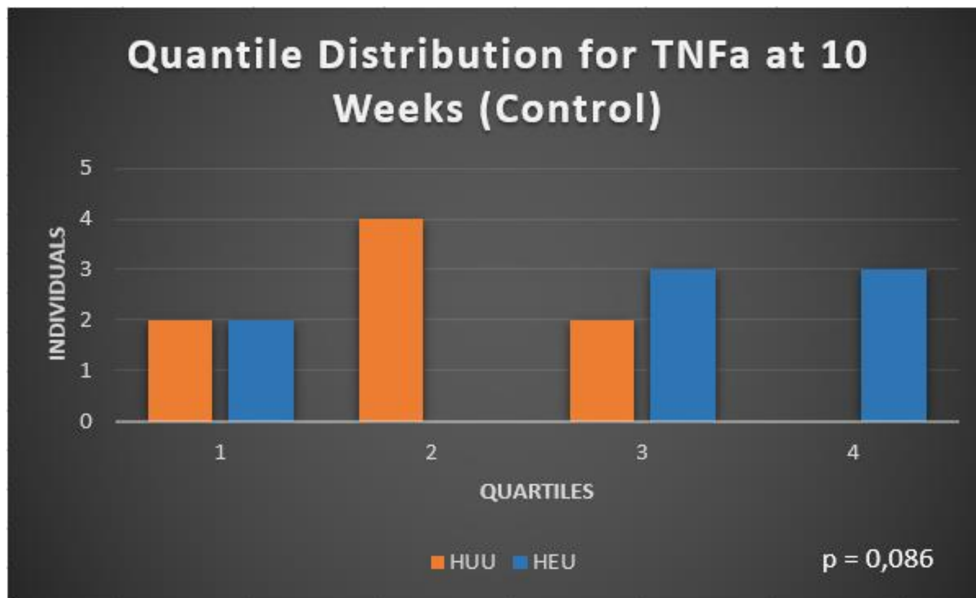

Figure 3.19: Quantile distribution of TNF-α in infants within the HUU and at 10 weeks of age for the Control test.

Though there are not many stand-out differences between the HEU and HUU cohorts for the cytokines when this small group was considered, these quantile distributions provide an interesting insight. The HUU infants had higher concentrations of IL-4, while the HEU infants had higher concentrations of TNF-α. This is noteworthy as IL-4 is linked to the anti-inflammatory functionality of monocytes, while TNF-α is associated with the pro-inflammatory activation of monocytes. This suggests some agreement in these observations with the postulation that HIV exposure tends to lead to pro-inflammatory monocyte activation, and a possible imbalance therein. This observation is further supported by the higher (although not significantly so) levels of the pro-inflammatory cytokine, IL-6, and the chemokine, IL-8, observed in the HEU group at 0 weeks upon Poly I:C and 6 months upon LPS stimulation, respectively. In contrast, it should also be noted that the HUU group had higher levels of IL-2 at 10 weeks in the unstimulated samples, and higher levels of IFN-ɣ at 12 months upon Poly I:C stimulation.

Noteworthy observations can also be made when considering an analysis of the differences between the cytokine concentrations at different time-points within the cohorts. This can provide a broad overview of the progression of concentration of these cytokines within the group and how this might correspond to what is observed in the other group (HUU vs. HUU). Of the entire analysis, there were several time-point comparisons that differed significantly (p-value <0.05) or almost significantly (p-value >0.05 but <0.1). These can be seen outlined in Table 3.10 for the HUU group and Table 3.11 for the HEU group.

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Table 3.10: Observed differences of cytokine concentrations within the HUU cohort for significant comparisons at different time-points.

HIV-Unexposed-Uninfected						
Cytokine	Timepoint 1	Median (IQR) (pg/mL)	Timepoint 2	Median (IQR) (pg/mL)	P-value	Progression of Concentration
IL-8	Control-0 Weeks	1266.19 (246.64 - 2340.47)	Control-10 Weeks	85.07 (21.19 - 260.35)	**0,0357**	Decrease
	Poly-0 Weeks	921.43 (168.51 - 3142.35)	Poly-10 Weeks	101.79 (37.9 - 145.98)	0,0687	Decrease
	Control-0 Weeks	1266.19 (246.64 - 2340.47)	Control-12 Months	208.48 (69.06 - 367.07)	**0,0166**	Decrease
IL-6	Control-10 Weeks	3.12 (0.29 - 9.49)	Control-6 Months	21.95 (8.45 - 133.19)	0,0687	Increase
	Poly-10 Weeks	6.36 (1.57 - 14.32)	Poly-6 Months	50.1 (8.71 - 116.76)	0,0687	Increase
	Poly-6 Months	50.1 (8.71 - 116.76)	Poly-12 Months	8.61 (3.23 - 16.89)	0,0593	Decrease
IL-4	Control-10 Weeks	16.06 (2.7 - 103.69)	Control-6 Months	261.0 (67.39 - 337.71)	**0,0499**	Increase
	LPS-10 Weeks	20.9 (3.28 - 106.03)	LPS-6 Months	252.56 (66.23 - 562.65)	**0,0357**	Increase
	Poly-10 Weeks	26.65 (4.98 - 124.93)	Poly-6 Months	234.14 (49.34 - 407.39)	0,0687	Increase
	Control-0 Weeks	2.95 (0.77 - 20.58)	Control-12 Months	176.63 (115.94 - 442.85)	**0,0262**	Increase
	LPS-0 Weeks	11.78 (2.6 - 19.64)	LPS-12 Months	184.64 (110.16 - 424.51)	0,0593	Increase
	Poly-0 Weeks	8.76 (2.6 - 25.29)	Poly-12 Months	168.51 (32.7 - 445.01)	0,0745	Increase
TNF-α	Control-6 Months	31.04 (15.88 - 171.4)	Control-12 Months	18.12 (15.28 - 19.95)	**0,0469**	Decrease
GMCSF	Control-0 Weeks	0.78 (0.26 - 2.29)	Control-12 Months	2.52 (0.91 - 3.28)	**0,0402**	Increase

Abbreviations: Lipopolysaccharide (LPS); Polyinosinic:polycytidylic acid (Poly)

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Table 3.11: Observed differences of cytokine concentrations within the HEU cohort for significant comparisons at different time-points.

HIV-Exposed-Uninfected						
Cytokine	Timepoint 1	Median (IQR) (pg/mL)	Timepoint 2	Median (IQR) (pg/mL)	P-value	Progression of Concentration
IL-8	LPS-10 Weeks	4018.3 (861.26 - 13004.76)	LPS-6 Months	11843.73 (9812.64 - 15727.71)	**0,028**	Increase
	Control-6 Months	502.98 (232.11 - 11369.94)	Control-12 Months	123.89 (27.09 - 278.03)	0,0505	Decrease
	LPS-6 Months	11843.73 (9812.64 - 15727.71)	LPS-12 Months	7629.26 (924.04 - 11900.97)	**0,0033**	Decrease
	Poly-6 Months	884.67 (168.04 - 11578.35)	Poly-12 Months	105.85 (35.87 - 264.48)	0,0505	Decrease
	Control-0 Weeks	730.41 (405.53 - 4268.76)	Control-12 Months	123.89 (27.09 - 278.03)	**0,0166**	Decrease
	Poly-0 Weeks	1971.99 (432.72 - 15554.2)	Poly-12 Months	105.85 (35.87 - 264.48)	**0,0262**	Decrease
IL-6	Control-6 Months	38.55 (1.46 - 7053.92)	Control-12 Months	9.62 (4.12 - 32.76)	0,0619	Decrease
	Poly-6 Months	50.35 (1.15 - 6877.61)	Poly-12 Months	10.85 (3.23 - 38.06)	0,0619	Decrease
IFN-γ	Control-0 Weeks	3.97 (3.97 - 3.97)	Control-12 Months	1.96 (0.9 - 3.97)	**0,0285**	Decrease
IL-2	Control-0 Weeks	1.28 (0.65 - 3.24)	Control-12 Months	0.97 (0.3 - 3.24)	**0,0103**	Decrease
	Poly-0 Weeks	1.59 (1.16 - 3.24)	Poly-12 Months	1.06 (0.63 - 3.24)	0,0989	Decrease
IL-4	Control-0 Weeks	8.6 (2.6 - 35.01)	Control-12 Months	155.07 (12.23 - 645.11)	0,0593	Increase
	Poly-0 Weeks	18.81 (0.28 - 46.58)	Poly-12 Months	140.7 (16.85 - 590.74)	**0,0262**	Increase

Abbreviations: Lipopolysaccharide (LPS); Polyinosinic:polycytidylic acid (Poly)

The progression of the various cytokines observed within the groups seems to be in agreement with what has been shown by the data thus far. The HUU group shows a greater increase in the cytokines associated with anti-inflammatory monocyte activation, such as IL-4, and a decrease in the pro-inflammatory cytokines. Specifically, IL-8 shows a continuous decrease while IL-6 and TNF-α show a decrease between 6 and 12 months. The pattern observed in the HEU groups is more complicated to interpret. While IL-6, IL-8, and IL-2 levels decrease between 0, 6 and 12 months in the Control and stimulated samples, IL-8 increases in the LPS-stimulated sample between 6 and 12 months. In tandem, there is an increase in IL-4 levels between 0 and 12 months. This points towards an imbalance in monocyte activation between the HUU and HEU groups, with the HEU group showing a tendency towards pro-inflammatory activation, but also seemingly an attempt to correct the imbalance.

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3.4 Head Circumference and Monocyte Function

When analysing the correlation between HC and monocyte function, two cytokines showed a significant correlation with HC at the 0-week time-point: GM-CSF when stimulated with LPS and TNF-α when stimulated with Poly I:C. These correlations are shown visually in the scatter-plot graphs of Figures 3.20 and 3.21.

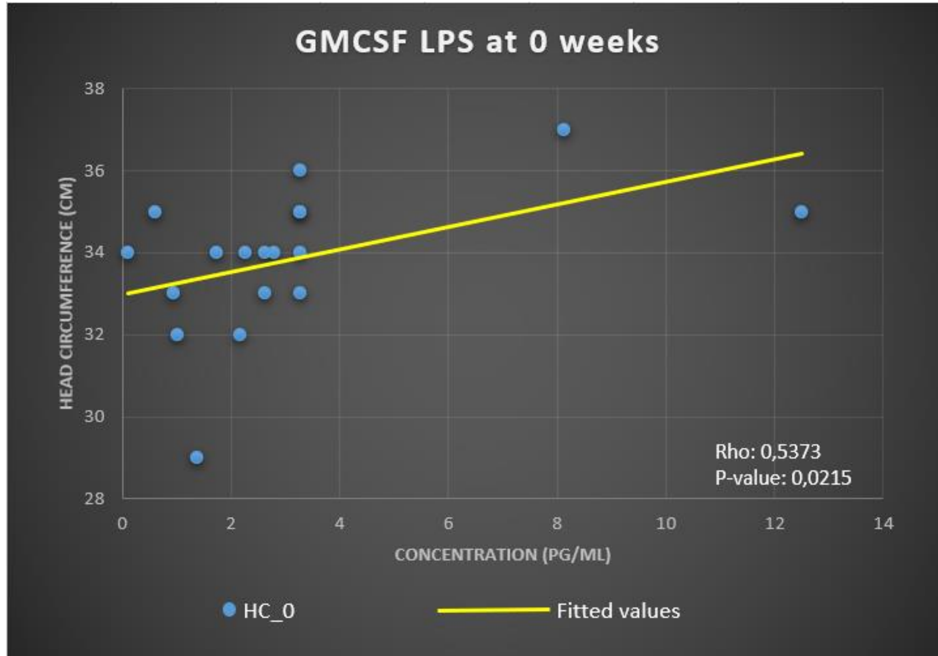

Figure 3.20: Scatter-plot graph showing the degree of correlation between GM-CSF concentration (stimulated with LPS) and head circumference at 0-weeks.

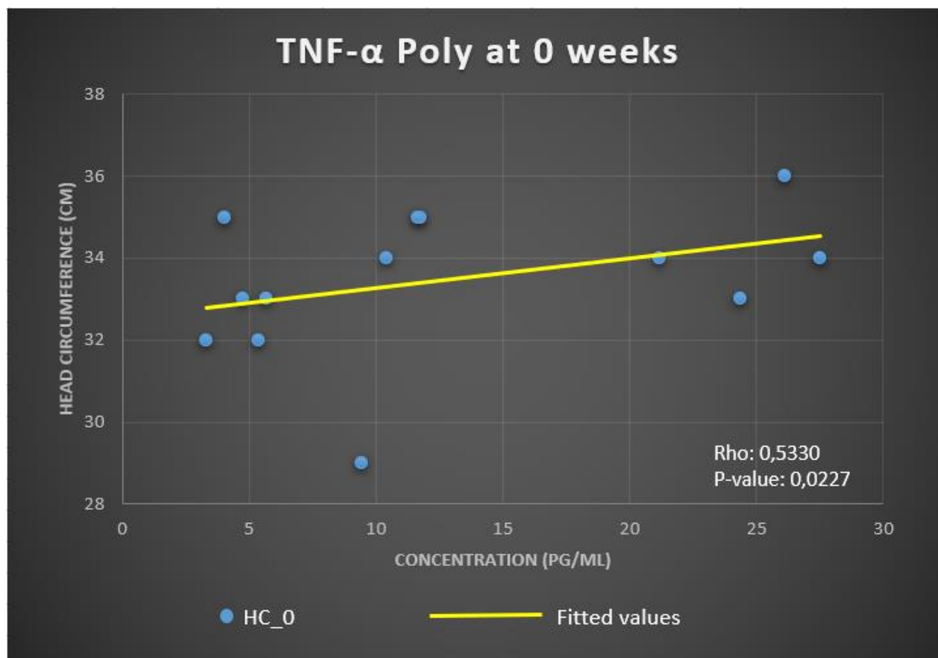

Figure 3.21: Scatter-plot graph showing the degree of correlation between TNF-α concentration (stimulated with poly) and head circumference at 0-weeks.

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One other observation that is worth mentioning here, although it just missed statistical significance, is the TNF-α concentration observed with LPS stimulation at the 0-week time-point, shown in Figure 3.22. No other significant or near significant correlations were noted at any of the other time points.

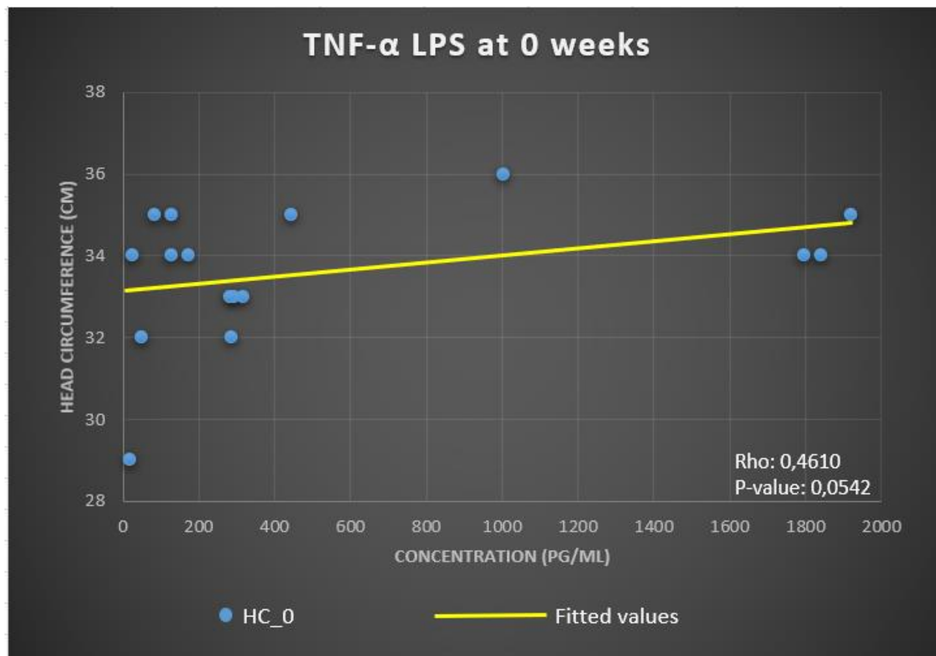

Figure 3.22: Scatter-plot graph showing the degree of correlation between TNF-α concentration (stimulated with LPS) and head circumference at 0-weeks.

The significant correlations observed are of particular interest since GM-CSF is a monocyte growth factor. In addition, monocytes are the most important source of TNF-α in humans(65). The observed correlation between these factors and HC indirectly supports the hypothesis that monocytes are important players in brain development. Further testing in a larger cohort will be needed to further explore the importance of this finding.

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Chapter 4 – Discussion and Conclusion

4.1 Anthropometric Data

The high number of HIV-exposed but uninfected (HEU) infants born annually, especially in sub-Saharan Africa, coupled with concerns about increased morbidity and mortality in this population, motivated this study of innate immune responses(5,81,82). One major concern is the occurrence of microcephaly and associated neurodevelopmental delay, which could significantly impact on scholastic performance(78). Given the important role of innate immune cells, especially monocytes, in brain development, this study specifically aimed to investigate the association between HIV exposure, monocyte function, and head circumference (HC), as a proxy for brain mass, in HEU and HIV-unexposed uninfected (HUU) infants.

When the anthropometric measurements of the HUU and the HEU groups were considered, several noteworthy observations were made; the first of which is aligned to the first objective of this study: "To investigate whether there is a difference in HC at birth between HEU and HUU neonates". HC is an important measurement for neurological development, which has been discussed in depth in Chapter 1, and was expected to differ between the HEU and HUU groups. Previous studies have shown that, on average, the HC for HEU infants is smaller than HUU infants. A study based in Kenya found that their HEU infant cohort had lower mean HC than the HUU infant cohort, and a higher prevalence of microcephaly. This Kenyan study followed 456 HEU infants and 2001 HUU infants, and the measurements were obtained in primary healthcare clinics during the routine six-week and nine-month immunizations(83). Another study, based in South Africa, indicated that HEU infants had, on average, a smaller HC than HUU infants, and also demonstrated an increased risk of stunting in the HEU cohort. This study was a small feasibility pilot study which analysed 33 HEU infants and 22 HUU infants(36) and served as a pilot for the ongoing Siyakhula study.

In contrast with the Kenyan study, as well as what had been observed in the pilot study on which this study was based, no observable difference in HC was seen between the two groups of infants. It is, however, critical to remember that this was assessed in a relatively small population (n=21) in the current study and the same observation should not necessarily be expected when assessing HC in the larger Siyakhula group (n=300). This is supported by the fact that HEU infants tended to be increasingly likely to have a HC in the lower quartile over time.

Methodological differences between the pilot and current study that could partly explain the disparate HC findings include the strict exclusion of mothers with co-morbidities as well as accurate assessment of gestational age early in pregnancy in the current study. Since z-score calculations correct for gestational age, it is possible that many of the infants in the pilot study were in fact premature and that their HC had therefore been underestimated.

The inclusion of z-scores in the current study is a significant strength and provides an important insight into how the observed measurements in these groups compare to expected WHO standards(77). Both infant groups had z-scores within acceptable limits as microcephaly would only be considered with an observed z-score of lower than -2. The growth progression would also appear to be proportional as microcephaly was not observed at any time point, and no statistically significant differences were observed between the groups at the further time-points.

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It is not only the HC, but the overall size, that is expected to differ between HEU and HUU infants at birth. Previous studies have shown that HEU infants are, overall, smaller than their HUU counterparts at birth(84) and are also more likely to exhibit growth stunting(81,85,86). A prospective study on infants in Uganda, following 170 HEU infants, noted significant growth faltering which was associated with lower attainment of developmental milestones at 18 months of age(81). A cross-sectional population-based survey done in Botswana, examining 396 HEU children and 1109 HUU children younger than five years of age, found that HEU children were at increased risk of stunting compared to their HUU counterparts(85). A Zambian-based study, which recruited 279 HUU children and 111 HEU children, also found that anthropometric measurements for HEU children were significantly lower than that of the HUU group(86).

In the current study, important variables that aided comparison of infant size are the weight for length (W4L) and weight for age (W4A) metrics. These are also age corrected and used to calculate z-scores in order to gain a more representative understanding of the observed measurements for the HEU and HUU groups.

At the 10-week time-point, there was a statistically significant difference observed for the calculated z-scores for the W4L metric when the measurements of the HUU and HEU infants were compared. This difference indicates that the infants of the HEU cohort were significantly smaller than that of the HUU cohort. This was the only statistically significant observation to note for the first three time-points.

Throughout the 0-week, 10-week and six-month time-points, the observed differences in anthropometric measurements between the two groups were marginal. However, when the 12-month time-point was considered, there were a large number of statistically significant differences in the over-all size of the HEU and HUU infants.

Overall, the HUU infants had more optimal progression, showing a better growth rate, especially from six to 12 months, than their HEU counterparts. This was also seen in the quantile distributions wherein HUU infants tended to fall within the higher two quantiles, and so in the upper 50% of growth difference between 10 weeks and six months as well as between six months and 12 months.

The observation that HUU infants had more desirable growth could be explained by many factors. One such factor could be complimentary feeding by the mothers(87), in other words when infants are not only fed by means of breast milk but also with solid food. In some cases, for instance for cultural reasons, mothers elect to supplement the breast milk regimen with other foods which might impact the growth of the infants, either positively or negatively. Another factor which could explain this is maternal nutritional status. Maternal reports of food insecurity have been associated with an increased risk of infant growth stunting at birth, especially in HEU infants.(36)As the exact diet of each infant was not recorded as part of this study, it is not possible to know if this was indeed a factor that could explain the difference in growth between the groups. This observation could, however, also support a tentative link between HIV exposure and a stunted growth, especially as the infants progress past the first six months of life. As the growth of infants is expected to become more exponential than linear after the first 12-24 months, it would be worth following these HUU and HEU groups to ascertain whether this stunting remains a factor or if it becomes less pronounced as time goes by. These differences in size and progression will also be better understood when analysed using a larger sample size, such as in the Siyakhula study.

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This observed pattern of growth stunting is important to understand, since it is a factor that has been commonly associated with HEU populations in African-based studies(85,88,89). It has been shown that HEU children have poorer early-life growth progression than HUU children, and that this tends to persist into later growth(90). In cases of low birth weight (LBW) infants, where HIV exposure is not a factor, it is expected that there will be a post-natal period of growth catch-up(91). This tends not to occur in HEU infant cohorts. In fact, HEU infant groups show a significantly impaired early-life growth progression(85,88), and tend not to experience post-natal catch-up growth when born with LBW(90,92). Figure 4.1 graphically shows an expectation of the projected pre- and post-natal growth patterns in cases of HUU and HEU infants born with normal weight, LBW infants without HIV-exposure, and LBW infants with HIV-exposure.

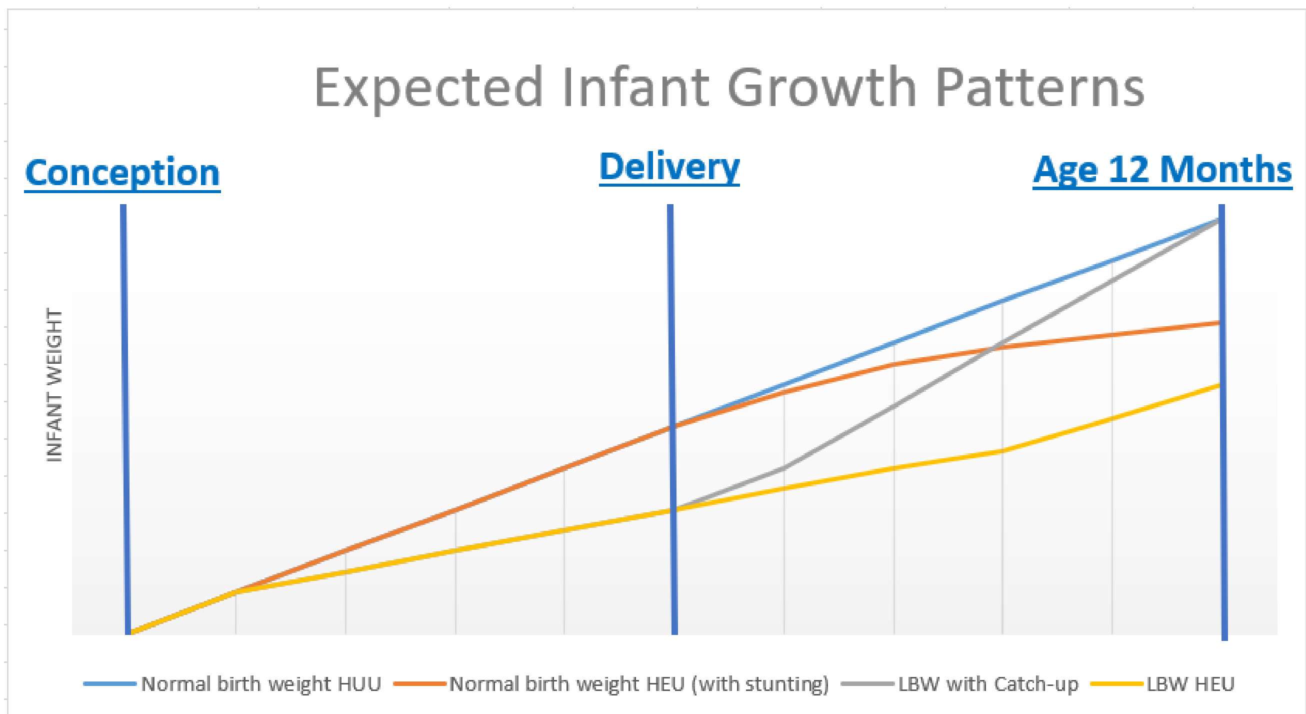

Figure 4.1:Expected pre- and post-natal growth patterns from conception until 12 months of age (post-partum)(88,90–92).

Insufficient nutrition in the diet during pregnancy has been linked to infants being born with LBW, particularly in developing countries(93,94). LBW poses a significant additional risk to infants as it has been shown that LBW is significantly associated with adverse early-life outcomes, such as increased morbidity and mortality, as well as poor early school performance(95). Pre-natal growth stunting is a particularly concerning issue in the case of mothers living with HIV who may not be able, for socioeconomic reasons, to maintain optimum nutrition during pregnancy. This issue is compounded because the LBW HEU infants do not seem to undergo the same post-natal catch-up growth as HUU LBW infants. It has been observed in a Zambian-based study that post-natal interventions may not be sufficient for ensuring optimum growth in LBW HEU infants(92). This randomized controlled trial studied 125 HEU and 382 HUU children and provided them with micro-nutrient fortified complementary/replacement food from the ages of 6 to 18 months. The study observed that post-natal malnutrition may not be contributing to growth stunting in HEU infants, and that

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factors earlier in life likely had an irreversible effect(92). Another Zambian-based longitudinal study, which analysed children from infancy to school-age, substantiated this observation(90). This study used data from two cohort studies in Lusaka, Zambia, and followed a total of 787 HUU and 365 HEU infants. This study concluded that interventions aimed at improving growth of HEU children should be targeted at pregnant women rather than at the post-natal stage(90).

It is, however, encouraging to note the study based in Botswana, mentioned above. This study substantiated the observation of HEU growth stunting but also demonstrated that antenatal interventions undertaken to increase birth weight and support optimal growth may significantly reduce the risk of stunting in HEU infants(85). An essential pre-natal intervention would be to ensure the proper nutritional intake for the mother, since the nutritional status of the mother has a major impact on the birth weight of the infant(93,94). Once the infant is born it essential to properly manage the post-natal nutritional intake, especially in cases of LBW HEU infants. Whether of normal birth weight or LBW, it is important that breastfeeding continues and nutritious complementary foods are provided during the first two years of life(85). A prospective cohort study, based in Kenya, found that children who were breastfed throughout a 6-month follow up period had significantly better growth outcomes when compare to children to were breastfed for only 3 months of the follow up period(96). There is also evidence to suggest that continued breastfeeding during the first two years of life may positively contribute to growth outcomes in HEU children(97). These findings provide positive insights into the issue of HEU infant growth stunting and show that there are means to improve the outlook for the future growth of these infants.

It is also worth noting the studies based in developed countries have not reported this increased risk of stunting(35). This could therefore substantiate the hypothesis that optimal nutrition during pregnancy and early childhood might offset some of the growth stunting observed in HEU populations in the developing world.

4.2 Cytokine Data

In the current study, for the purposes of objectives two and three, a fairly large range of different cytokines were assessed in the immunoassay panel. One reason for this was for this study to act as a 'pilot' for the cytokine testing portion of the larger Siyakhula study in order to gain a preliminary understanding of which cytokines, both pro- and anti-inflammatory, might be relevant in this type of cohort. The intent is for the observed cytokine concentrations to provide an indication of the function of the different types of monocytes found within the blood samples of the HUU and HEU infants over the considered time-points. The balance of monocytes present, whether tending towards pro-inflammatory or anti-inflammatory profiles, may provide valuable insight into observed growth patterns in the cohort when analysed in conjunction to the anthropometric data.

An imbalance in the monocyte profile may also indicate possible problems for anti-viral and anti-bacterial responses in the HEU infants. The HEU infants' tendency towards a pro-inflammatory response may result in an over-reaction by the immune system when a real-world viral or bacterial infection is faced by the infants. The balance of this immune response is integral to ensuring that no damage is inadvertently done to healthy cells, and so an unbalanced response could result in further harm being done over and above the already present risks involved.

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The specific cytokines and chemokines assessed as part of this study, which have been discussed in detail in Chapter 1, are outlined as follows:

- Interleukin-2 (IL-2)
- Interleukin-4 (IL-4)
- Interleukin-6 (IL-6)
- Interleukin-8 (IL-8)
- Interleukin-10 (IL-10)
- Interferon-gamma (IFN-ɣ)
- Tumour Necrosis Factor alpha (TNF-α)
- Granulocyte-macrophage colony-stimulating factor (GM-CSF)

For the purposes of discussion, any comparison showing a calculated p-value of less that 0.10 was noted due to the small sample size of the cohorts assessed as these observed differences may indicate possibly more significant observations that may arise when a larger cohort is assessed, such as in the Siyakhula study (n=300).

For the first time-point, taken at 0 weeks, the cytokine responses in the two groups were very similar to one another. One noteworthy exception at this time-point was IL-6 when stimulated with Poly I:C. This assessment showed a higher expressed concentration at birth in the HEU cohort when compared to the HUU counterparts. IL-6 is generally linked to pro-inflammatory functionality as triggered by Toll-like receptor (TLR)-linked immune signalling cascades, similar to that of TNF-α(98). This may indicate a tendency towards a monocyte functional imbalance, with higher pro-inflammatory responses being noted in the HEU infants.

At the 10-week time-point, the comparisons across the various cytokines also showed very similar observed concentrations in both HEU and HUU infants. One notable exception was IL-2. Interestingly, this was observed under the Control parameters, so there was no stimulating agent present for this observation. The concentration of IL-2 was higher in the HUU infant group. As mentioned above, IL-2 has many functions. Firstly, IL-2 is necessary for the activation of T cells. Secondly, it is necessary for the differentiation of CD4+ T cells into their respective effector subsets(99). Thirdly, it is associated with anti-inflammatory function and plays an important role in immune tolerance, primarily through promoting development of regulatory T cells while supressing activated T cells that might otherwise be primed to attack healthy normal cells(99). This latter function aids in the general suppression of auto-immune dysfunction. It is worth noting that this difference in concentration, tending towards a more 'helpful' anti-inflammatory balance is observed in the HUU group, whereas the HEU group showed a lower concentration of this cytokine at the same point. IL-2 is particularly critical to maintaining immune balance as low levels of IL-2 could lead to under-activation of T cells, required for a healthy immune response, but also to impaired immune regulation through suboptimal stimulation of regulatory T cells(100).

It is also worth noting the quantile distribution observed for TNF-α at the 10-week time-point, also under the control test parameters. This distribution showed that HEU infants tended to fall within the upper two quantiles while HUU infants fell within the lower two quartiles. TNF-α is associated with pro-inflammatory functionality(21), so this observation substantiates the

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argument that an imbalance towards a more pro-inflammatory profile can be seen in the HEU infant group.

At the six-month time-point, the first real statistically significant difference emerged between the HUU and HEU groups. There was a significant difference in the levels of IL-4 seen between the two cohorts for the control, LPS, and Poly I:C tests, with higher levels in the HUU infant group. IL-4 is largely associated with the anti-inflammatory functionality of monocytes(101). This supports the thinking that HEU infants may have a tendency towards a pro-inflammatory imbalance within the monocyte/macrophage population.

At 12 months there was, again, not much of a difference between the two groups, with one notable exception: IFN-ɣ was expressed at higher concentrations in the HUU group for the Poly I:C test. This cytokine is linked to pro-inflammatory functionality(102) and hence further substantiates the presence of an imbalance in HEU infants as Poly I:C simulates viral infection, and IFN-ɣ release is generally expected to be increased in a normal response to a viral stimulus(62). IFN-ɣ is extremely important in driving cellular immunity, as discussed above, and plays an essential role in organizing several protective functions which heighten immune responses to infections(102). This could be linked to the change in cytokine expression over time, as this specific difference between the HEU and HUU groups is only observed at the 12-month time-point.

Though the data may not indicate a stark difference between the groups, there does seem to be a noticeable imbalance when the HEU infants were compared to their HUU counterparts. This balance is crucially important in the innate immune systems of infants since properly regulated levels of both pro- and anti-inflammatory cytokine and chemokine levels are required in order for the immune system to function properly(4,38,39,103). This imbalance may be explained by the 'memory' aspect of the progenitor cells that give rise to the macrophage/monocyte population, which could last for several years, and so could maintain such an imbalance due to the fact that the HEU infant would have been exposed to a pro-inflammatory intra-uterine environment before birth. Although the infant has tested negative for HIV themselves, the fact that they have been exposed to HIV proteins may lead to this tendency towards an imbalanced monocyte population(82). This unbalanced monocyte profile may lead to improper immune responses to viral and bacterial insults later in life. This could be detrimental as an under-reaction might not adequately protect the individual from the immune threat and an over-reaction may result in more harm being done to healthy cells by the improperly regulated immune response.

This observation is extremely relevant as it speaks to one of the important reasons for this study. Generally, it is assumed that if an infant is HIV-uninfected, the post-partum care regime should be similar to that of an HIV-unexposed infant. The observation that HIV exposure might cause an imbalanced monocyte profile may indicate that extra consideration is necessary in order to properly manage the early-life care of these individuals, especially if aspects such as sub-optimal growth progression is noted and could be linked to this phenomenon(78,104). Other studies have noted significant differences at school age for children for which HIV exposure is a factor, with significant differences noted in intellectual quotient tests, perceptual motor skills, academic achievement and adaptive behaviour(78). As such, it would stand to benefit these individuals greatly if a more active care and developmental regime could be implemented immediately post-partum, instead of a more reactionary development program being instituted when developmental stunting may be noted closer to school-going age.

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4.3 Head Circumference and Monocyte Function

In order to address objective three for this study, "To determine if there is an association between HC and monocyte function at birth", correlations between HC and the cytokine/chemokine panel results were analysed. The significant correlations observed between stimulated concentrations of GM-CSF and TNF-α, and HC at the 0-week timepoint are noteworthy.

These positive correlation with the GM-CSF showed that, in this cohort, infants with larger HC had higher levels of this growth factor. This is particularly relevant as GM-CSF plays an important role in the proliferation of monocytes in the healthy immune system and also contributes to the pro-inflammatory functionality in monocytes/macrophages in the event of infection(66). TNF-α is just as noteworthy due to the fact that TNF-α production is directly dependant on optimum levels of monocytes as monocytes are the primary producers of this cytokine(64). As both of these significantly correlated observations are regarding cytokines that are directly linked to monocytes/macrophages, these results are relevant to this study and to the larger Siyakhula study.

The correlations outlined in Chapter three seem to support the hypothesis that a balanced monocyte profile is an important aspect of early neurological development. It is likely that the small number of significant correlations found in this study is linked to the small cohort size assessed; however, the results found by this study indicate that further study in a larger cohort is needed to further explore this finding.

4.4 Conclusions

- In this cohort of 21 HEU and HUU infants, no statistically significant differences were observed in the HC at birth between the two groups. This is highly likely to be linked to the small sample size of infants included and so this metric should be further assessed with the larger cohort of the Siyakhula study as previous studies have shown a link between HIV exposure and smaller HC at birth. It should also be noted that HEU infants tended to be increasingly likely to have a HC in the lower quartile over time.

- When the overall anthropometric data were assessed, a link was observed between sub-optimal growth progression at the 12-month time-point and HIV exposure in the infant cohort. Though the infants seemed similar at birth, the HEU infants showed a lower level of growth progression from the six-month to the 12-month time-point, exhibiting statistically significant differences for overall size metrics at 12 months of age, indicative of growth stunting.

- When the monocyte function was characterised by means of WBS testing and compared, there were important differences between the HUU and HEU groups. The HEU infant cohort showed a tendency towards overexpression of pro-inflammatory cytokines and a tendency towards under-expression of anti-inflammatory cytokines. This is congruent with what has been shown in previous studies and may indicate a link between a pro-inflammatory shifted monocyte population and HIV exposure in these infants, possibly secondary to their intrauterine environment. The WBS assay is an important tool in this regard as one of its main advantages is to simulate the immune environment in circulation, and so other blood-based cell types may be present in the sample which also produce cytokines, chemokines,

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and growth factors. The specific relevance of the inflammatory shift observed through WBS in this cohort and the possible link to monocyte function has been outlined in previous literature(105).

- Very few significant correlations could be drawn between monocyte function and HC, however, the correlations that were observed are notable. The correlations shown between HC and monocyte response to stimulus tentatively supports the hypothesis that monocyte function is linked to HC at birth, and therefore to neurological development. This is particularly relevant to HEU infants as the results of this study have shown that they are more likely to display imbalanced monocyte function, possibly as a result of intrauterine HIV exposure. Further study is recommended to assess this factor in-depth in a larger cohort.

- The overall purpose of this study was to contribute to the improved post-partum care of HEU infants. The tentative associations shown by this small data subset, especially if backed-up by the larger Siyakhula study, may indicate a need for active steps to be taken for infants born to mothers with HIV, even if the infant has tested negative for HIV. Previous literature has shown links between maternal HIV exposure and associated factors such as antiretroviral (ARV) medications, and increased levels of morbidity, with an especially increased likelihood of learning disabilities and stunting to be expected later in life for the HEU individuals. A better understanding of the factors that may contribute to these adverse outcomes may go a long way towards developing a higher standard of care to meet the specific needs of these infants both antenatally and as they progress later in life.

4.5 Strengths of the study

- The data gathered and assessed as part of this study are prospective and form part of a much larger, longitudinal study (Siyakhula).

- All laboratory-based tests were closely supervised by an Extraordinary Professor (Prof. H Steel), who has many years of experience in this line of lab assessment. Prof. Steel did the pipetting of all the samples and reagents in order to maintain congruency and accuracy across all samples assessed.

- All samples run per assay kit were thawed from -80˚C only once and were tested immediately on the same day.

- A single reagent batch was used for all cytokines, thereby excluding variability introduced by different batches.

- All infant blood samples acquired from the hospital were taken by a registered paediatric nurse.

- This study functions as a pilot study in order to contribute understanding towards the assessments and analysis of the larger Siyakhula study.

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4.6 Weaknesses of the study

- For the purposes of this study, a relatively small sample of only 21 infants was assessed.
- The study only assessed the infants within the first 12 months post-partum, further follow-up may yield different results. This aspect will be covered by the Siyakhula study.
- There was no assessment of the dietary components for these infants, so aspects such as possible complimentary feeding by the mothers could not be accounted for.

4.7 Recommendations for future study

- Though there were no statistically significant differences between the HEU and HUU groups at birth noted for this study, this may be due to the sample size. It is recommended that the entire Siyakhula cohort be assessed for anthropometric differences at birth as there will likely be different observations to be made.
- As observable differences emerged in the anthropometric data between the HEU and HUU groups between the six-month and 12-month time-point, follow-up after the 12-month time-point should be undertaken to assesses whether this trend continues. This will likely also be revealed in more depth as part of the Siyakhula study as there will be 18-month and 24-month time-point follow ups with these infants.
- A dietary assessment component may provide a valuable addition to this data analysis. It would give good insight into other factors that may affect the growth of these infants over and above the anthropometric and cytokine data assessed as part of the Siyakhula study. Aspects such as complimentary feeding may impact the growth of the infants, either positively or negatively.

The differences that have been observed between the HUU and HEU groups as part of this study are particularly relevant as these may be linked to the relatively higher levels of morbidity and mortality described in HEU children in the developing world. The analysis and understanding of these differences and imbalances, seen in HEU individuals, is crucially important as there is a need for improved future management and treatment of HEU infants, even though they are shown to be HIV-uninfected. These observations also fulfilled the purpose of a pilot study to inform the larger Siyakhula study: the cytokine data pertaining to this subset will aid in the accurate selection of the cytokine panel for the Siyakhula study. Overall, the characterisation and analysis of the monocyte function in HEU neonates should lead to a better understanding of the cytokine imbalance that may be resultant from intrauterine HIV exposure.

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Appendices

Appendix 1: Informed Consent Documentation

PATIENT / PARTICIPANT'S INFORMATION LEAFLET & INFORMED CONSENT FORM FOR A NON-INTERVENTION STUDY

STUDY TITLE: Assessment of factors impacting on foetal and infant immunity, growth, and neurodevelopment in HIV- and antiretroviral-exposed uninfected children

SPONSOR: The International AIDS Society

Principal Investigator: Prof Ute Feucht

Institution: University of Pretoria

MRC

DAYTIME AND AFTER HOURS TELEPHONE NUMBER(S):

Daytime numbers: 012 373 1082

Afterhours: 083 368 4995

DATE AND TIME OF FIRST INFORMED CONSENT DISCUSSION:

dd	**mmm**	**ivy**

:
Time

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Dear Patient

Dear Ms. / Mrs. ....

1) INTRODUCTION

We invite you to participate in a research study. We are doing research on factors that may influence the immune system (these are the cells of the body that fight infection), growth and development of children born to HIV negative women compared to HIV positive women. I am going to give you information about the study and invite you to be part of this research. If there is anything that you do not understand please ask me to explain. You should not agree to take part unless you are completely happy about all the procedures involved.

2) THE NATURE AND PURPOSE OF THIS STUDY

The aim of the research is to understand how mother's HIV infection influences the growth of the foetus (unborn baby) during pregnancy compared to HIV negative women. We also want to follow up your baby after birth to learn about the immune function, growth and brain development of babies from both HIV negative and HIV positive mothers.

3) EXPLANATION OF PROCEDURES TO BE FOLLOWED

We are inviting all women from the Southwest Tshwane, with a pregnancy before 22 weeks, to participate in the research. We are looking for HIV negative and HIV positive women on treatment who are able to follow up at the clinic with their babies for 2 years after delivery. We will pay for your transportation to the clinic for the study.

If you agree to participate in the research, we will ask you to come for 3 visits for a sonar to Kalafong Hospital during your pregnancy. You will deliver your baby at Kalafong or Pretoria West Hospital. After delivery, we will ask to see you and your baby for 8 visits at Kalafong Hospital until the child is 2 years old. The following procedures will be done during pregnancy, delivery and after delivery.

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3.1 The procedures for the mother

3.1.1 During pregnancy

- We routinely do one ultrasound (sonar) to see how far pregnant you are. More sonars are done if there are problems with the pregnancy. In this research, you will have a total of 3 sonars to look at any abnormalities and to see how the baby is growing.
- We will ask you questions about your health and social circumstances.
- The routine antenatal care clinical examinations and tests will be done as always.
- A small amount of blood, 30 millilitres (about 2 tablespoons), will be collected from your arm with a syringe, at 28 and 36 weeks. The blood will be sent for tests to look for markers of inflammation and other related biological factors. If you are HIV infected, blood will also be sent for antiretroviral drug levels – this is to see how much medicine is in your blood.
- We will also take a vaginal swab at 36 weeks. This sample will be tested to look for markers of inflammation and infections and other biological markers important for your health
- An oral glucose tolerance test (a test to look for abnormal blood sugar levels) is usually done in patients who have a high risk of diabetes. In this study, we will do this test in all women because, if the mother is diabetic, this can affect the growth of the unborn baby.

3.1.2 At delivery

- At delivery or just after birth, we will collect another 30 millilitres (about 2 tablespoons), of blood to look for markers of inflammation and other biological factors. In women who are HIV-infected, we will also test the amount of virus in your blood.
- After your baby is born we will use a small needle to take blood from the umbilical cord to test for inflammation and other related biological factors important for the development of the baby, such as infection markers and growth factors.
- We will also take a small piece of the placenta after delivery and we will test factors that are important for the development of the baby.
- Before you leave the hospital, we will ask you to express some breast milk (about one tablespoon) so that we can measure substances in the breast milk that are important for the newborn.
- We will also ask you to give a stool sample if at all possible.

3.1.3 The next two years

- We will ask you to come to Kalafong Hospital with your baby for 8 visits when the baby is 6, 10 and 14 weeks, and 6, 9, 12, 18 and 24 months old. These visits are part of the routine follow-up care for you and your baby and will replace your usual clinic visits.
- At these visits, we will ask you to answer some questions about you and your baby's health, diet and how you feed your baby.
- We will take 30 millilitres (about 2 tablespoons) of blood to look for markers of inflammation and infections and other related biological markers important for your health.
- At each visit, we will also ask you to express some breastmilk (about 6 tablespoons) so that we can measure substances in the breast milk that are important for the development of your baby.
- We will ask for a stool sample at 6 weeks and again at 3, 6 and 12 months. We will provide you with a container so you can do this at home, if you so prefer.

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3.2 The procedures for the child

3.2.1 Newborn

- The routine measurements of the newborn, such as length, weight and the size of the head, will be taken.
- In addition, we will collect stool from the newborn to look at the organisms in the stool.
- For babies born to HIV infected mothers, 5 millilitres (one teaspoon) of blood will be taken on the baby for HIV birth PCR test as part of routine newborn care.

3.2.2 Child visits

- The child visits will be when the baby is 6, 10 and 14 weeks, and 6, 9, 12, 18 and 24 months old.
- At these visits we will weigh and measure your baby's length and head size and to look at his or her Road to Health chart.
- We will do an assessment of your child's brain development at these time points by looking if he or she can do the usual things expected of a child at that age.
- At these visits, we will take 10 millilitres (about 2 teaspoons) of blood from your baby to check for low iron levels (anaemia) and to look at biological factors important for the growth and health of the baby.
- We will collect stool from your child at 6 weeks and 3, 6 and 12 months.
- If there are any problems with the child's development or anaemia, your child will be referred for further care.
- We will also offer the childhood immunisations at all time points as required by the national immunisation programme and this will replace your regular clinic visits.

3.3 Testing of samples

Most of the tests will be done at the Department of Immunology at the University of Pretoria. We will also send a small amount of blood, vaginal swab, breastmilk, placenta and stool overseas for testing at the Department of Health Sciences at Carleton University in Canada. We also ask your permission to store all the left-over samples that we have collected for future testing. We will first get approval from the Faculty of Health Sciences Research Ethics Committee, University of Pretoria and the Research Ethics Board at Carleton University before doing any more tests on these samples.

4) RISK AND DISCOMFORT INVOLVED.

The main inconvenience for you will be your doctor visits will be longer than usual. There is only minimal risk or possible discomfort involved with providing blood, breast milk or stool samples, or having the vaginal swab, or measuring your child's growth and development. Taking blood can sometimes be painful, could make you feel faint, and could cause bruising afterwards.

5) POSSIBLE BENEFITS OF THIS STUDY.

The benefits during pregnancy; you will be seen by a specialist and you will have detailed sonars by a skilled specialist in this field. If there are any complications, you will receive treatment immediately.

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The benefits for your baby are that a specialist will do the routine visits. Your child will receive additional screening for growth and brain development. We will be able to diagnose anaemia and any problems with development early and your child can get treatment. Your child will also get all required immunisations, which means that your child will not have to go to the clinic as well.

6) VOLUNTARY PARTICIPATION

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. Whether you choose to participate or not, all the necessary services at this clinic or hospital will continue and nothing will change. If you choose not to participate in this research project you will be offered the treatment that is routinely offered in this clinic or hospital. You are allowed to withdraw from the study at any time. Any information or samples we collect from you as part of the study before you withdraw will remain part of the study. There will be no further information or samples collected from you once you withdraw from the study.

7) I understand that if I and my baby do not want to participate in this study, I will still receive standard treatment for my illness.

8) I may at any time withdraw from this study.

9) REIMBURSEMENTS

There are no direct financial benefits to you, but we will give you money to pay for your transport to the hospital during pregnancy and for the follow-up visits. The amount will be based on the distance you stay from the clinic.

10) HAS THE STUDY RECEIVED ETHICAL APPROVAL?

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 3563084 / 012 3563085 and written approval has been granted by that committee. This protocol was also submitted to the Carleton University Research Ethics Board, and written approval has been granted. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2013), which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

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11) INFORMATION If you have any questions concerning this study, you should contact:

1. Dr Felicia Molokoane: 083 368 4995
2. Prof Mphele Mulaudzi: 083 258 8705
3. Prof Ute Feucht: 072 428 0465

12) CONFIDENTIALITY

The information that we collect from this research project will be kept confidential. Participants will be identified for study purposes with a unique study number. Your personal identifying information will not be connected to the information collected for this research study. Information collected about you and your baby during the research will be stored safely and will only be available to the approved researchers.

13) CONSENT TO PARTICIPATE IN THIS STUDY

I have read or had read to me in a language that I understand the above information before signing this consent form. The content and meaning of this information have been explained to me. I have been given the opportunity to ask questions and am satisfied that they have been answered satisfactorily. I understand that if I do not participate it will not alter my management in any way. I hereby volunteer to take part in this study.

I have received a signed copy of this informed consent agreement.

....

Patient name Date

....

Patient signature Date

....

Investigator's name Date

....

Investigator's signature Date

....

Witness name and signature Date

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VERBAL PATIENT INFORMED CONSENT **(applicable when patients cannot read or write)**

I, the undersigned, Dr ………………………………………..…, have read and have explained fully to the patient, named ……………….. and/or his/her relative, the patient information leaflet, which has indicated the nature and purpose of the study in which I have asked the patient to participate. The explanation I have given has mentioned both the possible risks and benefits of the study and the alternative treatments available for his/her illness. The patient indicated that he/she understands that he/she will be free to withdraw from the study at any time for any reason and without jeopardizing his/her treatment.

I hereby certify that the patient has agreed to participate in this study.

Patient's Name ______________________

(Please print)

Patient's Signature ____________________ Date _____________

Investigator's Name ______________________

(Please print)

Investigator's Signature ______________________ Date _____________

Witness's Name ________________ Witness's Signature _____ Date _____________

(Please print)

(Witness - sign that he/she has witnessed the process of informed consent)

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Appendix 2: Anthropometric Measurements Guidelines

Infant weight

1. Have the mother remove all of the infant's clothes, including the diaper. The infant needs to be naked during the weight assessment.

2. Ensure the scale is on a flat and level surface. Turn on the scale. Tare the scale. If it is cold, a thin blanket can be placed on the scale, but the scale must be tared *after* the blanket is placed on the scale.

3. Place the infant on the scale and wait for him/her to stop moving. Record the weight of the infant to the nearest 10 g.

Crown-heel length

1. Lay the infant on his/her back with legs extended. The infant's shoulders and hips should be aligned at right angles to the long axis of the body. Gentle pressure can be applied on the knees to straighten the legs.

2. Position the infant's head in the Frankfort Plane relative to the extended torso (i.e.: such that a vertical line from the ear canal to the lower border of the eye socket is perpendicular to the table upon which the infant is lying). To keep the infant's head in the correct position, an assistant can cup his/her hands over the infant's ears.

3. Ideally, place a board against the feet of the infant (with extended legs) and measure the distance from the vertex (top of head) to the heel of the right foot. If a board is not available, measure the distance from the vertex directly to the heel. Read the measurement as soon as possible after the footboard/legs have been positioned.

4. Record the crown-heel length to the to the nearest 1 mm and the last completed unit of measure (not the nearest unit). For example, if the length measurement value lies between 55.6 and 55.7 cm, the value to be recorded is 55.6.

Head circumference

1. Have the mother remove all headbands or hairpins from the infant's head/hair.

2. Loop the measuring tape before slipping it over the infant's head.

3. Wrap the measuring tape around the infant's head, placing it above the brows, the pinna of the ears, and around the occipital prominence at the back of the skull. Ensure that the tape is flat against the skin (the tape may have to be pulled tightly to flatten the infant's hair).

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4. Record the circumference to the nearest 1 mm and the last completed unit of measure (not the nearest unit). For example, if the head circumference measurement value lies between 34.2 and 34.3 cm, the value to be recorded is 34.2.

Abdominal circumference

1. Measure the infant's abdomen at the point of greatest girth.

2. Wrap a measuring tape around the infant's abdomen at the umbilicus. Ensure that the tape is flat against the skin.

3. Record the circumference to the to the nearest 1 mm and the last completed unit of measure.

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Appendix 3: Ethical Clearance Letter – Siyakhula Study

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

27/07/2017

Approval Certificate
New Application

Ethics Reference No: 294/2017

Title: Assessment of factors impacting on foetal and infant immunity, growth, and neurodevelopment in HIV- and antiretroviral-exposed uninfected children [Umbrella study]

Dear Prof Ute Feucht

The **New Application** as supported by documents specified in your cover letter dated 19/07/2017 for your research received on the 21/07/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 26/07/2017.

Please note the following about your ethics approval:
- Ethics Approval is valid for 5 years
- Please remember to use your protocol number (**294/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

Additional Conditions:
- Approval is conditional upon the Research Ethics Committee receiving permissions from the CEO of Tshwane Municipal Health Services, District Health Services as well as Kalafong Hospital.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

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Appendix 4: Ethical Clearance Letter – MSc. Study

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Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

Faculty of Health Sciences **Research Ethics Committee**

17 March 2022

Approval Certificate
New Application

Dear Mr B Kleynhans

Ethics Reference No.: 704/2021
Title: Association between head circumference and monocyte function in neonates born to mothers with and without HIV at Kalafong Provincial Tertiary Hospital

The **New Application** as supported by documents received between 2022-02-14 and 2022-03-16 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-03-16 as resolved by its quorate meeting.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-03-17.
- Please remember to use your protocol number (704/2021) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers

MBChB, MMed (Int), MPharmMed, PhD

Deputy Chairperson *of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

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