Association between head circumference and monocyte phenotype in neonates exposed and unexposed to HIV at Kalafong Provincial Tertiary Hospital

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DECLARATION

I, Iwan Sipsma, hereby declare that this work, which I submit for a Master's degree in Human Physiology to the University of Pretoria, is my own original work and has not previously, in its entirety or in part, been submitted by me for a degree at this or any other tertiary institution. Where another person's work has been used, it has been properly acknowledged and referenced. Procedures were carried out in accordance with the ethical rules prescribed by the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria.

Signature: 

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EXECUTIVE SUMMARY

With the increasing number of women of reproductive age living with the human immunodeficiency virus (HIV) there is an increase in the number of children being born exposed to HIV. Regardless of whether the children are infected with HIV or merely exposed to the virus, concerns have been raised about how the children might be affected in terms of growth, development, and immune function.

It is well documented that children living with HIV do not perform as well as children not living with HIV on general cognitive tests, processing speed, and visual-spatial tasks, and are at much higher risk for psychiatric and mental health issues. One would expect that HIV-exposed-uninfected (HEU) children would fare as well as HIV-unexposed-uninfected (HUU) children; however, research shows that HEU children not only have immune dysfunction, as well as higher morbidity and mortality than their HUU counterparts, but also worse performance on certain neurodevelopmental tests by a small, but statistically significant, margin. This is cause for concern, since as many as 30% of children in some sub-Saharan countries, such as South Africa, are HEU. As these children enter school, they may be at risk of learning difficulties.

The role of monocytes/macrophages in the development of the brain is a growing field of research. Macrophages in the brain, called microglia, assist in tissue remodelling, repair, and neurogenesis. An imbalance between macrophage phenotypes has been associated with various neurological diseases and inflammatory conditions, since classically activated microglia and/or macrophages are known to exert cytotoxic effects on neurons and oligodendrocytes. Macrophages of different activation profiles are linked to monocyte polarization in blood. This study therefore set out to characterise and compare the monocyte phenotypes of HEU and HUU children in blood and investigated the association between HIV exposure, infant growth, including brain size as measured by head circumference (HC), and patterns of monocyte polarisation.

For this study, 23 mothers living with and 19 mothers not living with HIV were randomly selected. The mothers were similar in terms of age, anthropometry, and monocyte phenotype percentages. The median gestational age in weeks at birth

for the HUU group was slightly longer (39 [IQR 38 – 39] versus 38 [IQR 38 – 39] for the HEU group); but the difference was not statistically significant.

At birth, the weight, height, and HC of the groups were similar, as were the z-scores for weight-for-length (WLZ), length-for-age (LAZ), weight-for-age (WAZ), body mass index (BMI) for-age (BAZ), and HC-for-age (HCZ). At the 10 week timepoint, HUU infants had a higher BMI, WLZ, and BAZ than HEU infants, and they were still heavier at the six months follow-up visit, as measured by WLZ. Lastly, at the 12 month follow-up visit, the BMI, WLZ, and BAZ were significantly higher, while LAZ was lower, in the HUU group. Importantly, for all the z-scores that differed significantly, HEU infants had negative values, while HUU infants had positive values. The negative values were, however, not smaller than -2, which would have meant that they were underweight according to the World Health Organisation's growth standards.

With regards to the monocyte subsets, HEU infants had a significantly higher proportion of intermediate monocyte (IM) at birth, statistical significance set at 10%. No other statistically significant differences were seen with regards to the other monocyte phenotype subgroups or at any of the other time points.

No correlation was found between monocyte polarisation and HC. This study therefore did not show that HIV-exposure affected the HC in this small group of infants. In future studies, more precise measurements for anthropometric data might reflect different results and the connection between other neurotropic viruses, such as herpes simplex virus and enteroviruses, with HIV-exposure could be looked at for an enhanced understanding of HEU infants' neurodevelopment.

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
APGAR	Appearance, Pulse, Grimace, Activity and Respiration
ART	Antiretroviral therapy
ARV	Antiretroviral
BAZ	BMI for age z-score
BMI	Body mass index
BSA	Sigma buffer
BSID-III	Bayley Scales of Infant and Toddler Development-3rd Edition
cART	Combination antiretroviral therapy
C	Celsius
CCR	C-C Motif chemokine receptor
CD	Cluster of differentiation
CM	Classical monocyte
cm	Centimetre
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
dNTP	Deoxynucleotide triphosphates
ECD	Electron coupled dye
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunoassay
FcR	Fragment crystallizable receptor
FcgR	Fc gamma receptor
FCS	Flow cytometry standard
FDC	Fixed-dose combination
FITC	Fluorescein isothiocyanate
FSC-A	Forward scatter area
FSC-H	Forward scatter height
GA	Gestational age
g-Force	Gravitational force
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorder
HC	Head circumference

HCZ	Head circumference z-score
HCAZ	Head circumference for age z-score
HEI	HIV-exposed-infected
HEU	HIV-exposed-uninfected
HIV	Human immunodeficiency virus
HIVE	HIV-associated encephalopathy
HLA-DR	Human leukocyte antigen-DR locus
HUU	HIV-unexposed-uninfected
IQR	Inter-quartile range
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IM	Intermediate monocyte
KH	Kalafong Provincial Tertiary Hospital
kg	Kilogram
LAZ	Length for age z-score
LLE	Late language emergence
LPS	Lipopolysaccharide
M1	Classically activated macrophage
M2	Alternatively activated macrophage
MFI	Median fluorescence intensity
moDCs	Monocyte derived dendritic cells
MHC	Major-histocompatibility complex
MRI	Magnetic resonance imaging
MUAC	Mid-upper arm circumference
MUACZ	MUAC z-score
m	Metres
mL	Millilitres
nm	Nanometres
NCM	Non-classical monocyte
NCs	Neurologic conditions
NFL	Neurofilament
Nr4a1	Nuclear receptor subfamily 4 group A member 1
PBS	Phosphate-buffered saline
pH	Potential of hydrogen

PSGL-1	P-selectin glycoprotein ligand-1
qPCR	Quantitative polymerase chain reaction
QC	Quality control
ROS	Reactive oxygen species
SAMHD1	Sterile Alpha Motif Histidine-Aspartic acid domain containing protein 1
SSC-A	Side scatter area
SLAN	6-sulfo LacNAc
TB	Tuberculosis
T-cells	T-lymphocytes
TNF	Tumour necrosis factor
TLR	Toll-like receptors
tSNE	t-Distributed Stochastic Neighbour Embedding
VL	Viral load
WAZ	Weight for age z-score
WHO	World Health Organisation
WLZ	Weight for length z-score
α	Alpha
µm	Micrometres
µL	Microliters
µg/µL	Micrograms per microliter
%	Percentage
_0	Birth
_10	10 weeks
_6	6 months
_12	12 months

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CHAPTER 1

INTRODUCTION:

Literature Review, Aim and Objectives

1. INTRODUCTION

1.1 BACKGROUND

In 2020, there were approximately 37.7 million people living with the human immunodeficiency virus (HIV) worldwide, of whom 25.4 million live in the World Health Organisation (WHO) African region[1-5]. HIV is a major public health issue as it has caused approximately 36.3 million deaths so far, with between 480 000-1.0 million of those occurring in 2020 alone[1]. Two-thirds of newly infected people come from sub-Saharan Africa, which is the most affected region in terms of incidence and prevalence[1-2].

There is currently no cure for HIV; however, antiretroviral (ARV) medication is available to manage the virus. ARV medication reduces transmission from the infected to the uninfected partner (horizontal transmission) as well as from the mother to the child (vertical transmission), so that those living with the virus or at high risk of contracting it, have the best chance of living a normal healthy life.

Of the estimated 7.8 million people living with HIV in South Africa, ±4,8 million are women of reproductive age (15 years and older) [3]. This has major health implications for the generation of children born to these women. Since the introduction of antiretroviral therapy (ART) as a main treatment plan for people living with HIV, particularly pregnant women, the vertical transmission rate of HIV from mother to child has dropped below 1%[6].

While this is promising, there is still much that is unknown about the effects of ARV medication and maternal HIV exposure on the growth and development of a child both before and after birth. HIV-exposed-uninfected (HEU) children have been shown to have slower levels of growth and development than their HIV-unexposed-uninfected (HUU) counterparts[1,4,7-8]. Some studies have also reported higher morbidity and mortality in HEU infants, mostly secondary to infections, although the exact reasons are still unknown[9]. In 2007 Mussi-Pinhata *et al.* concluded that 61% of HEU infants had at least one infection (mostly a lower respiratory tract infection[10]) in the first six months of life[11-14]. These HEU infants had lower amplified antibody reactions to vaccines[11,15] likely secondary to reduced transplacental transfer of maternal antibodies[15].

The flow diagrams presented in Figure 1.1.1 and Figure 1.1.2 below demonstrate how children are classified as HEU and HUU children[16-17].

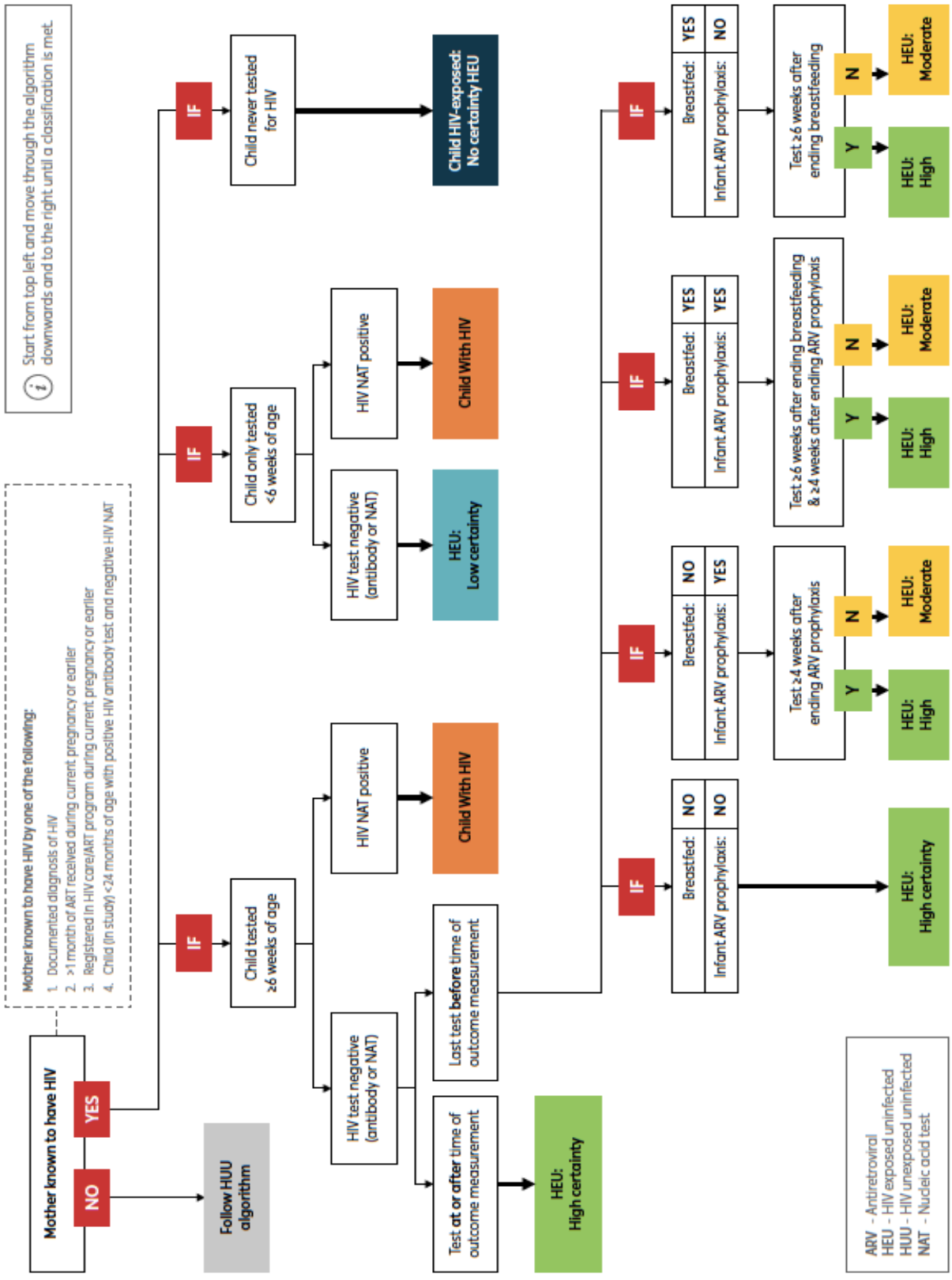

Figure 1.1.1: Algorithm for classification of children who are *in utero* HIV exposed and uninfected[16].

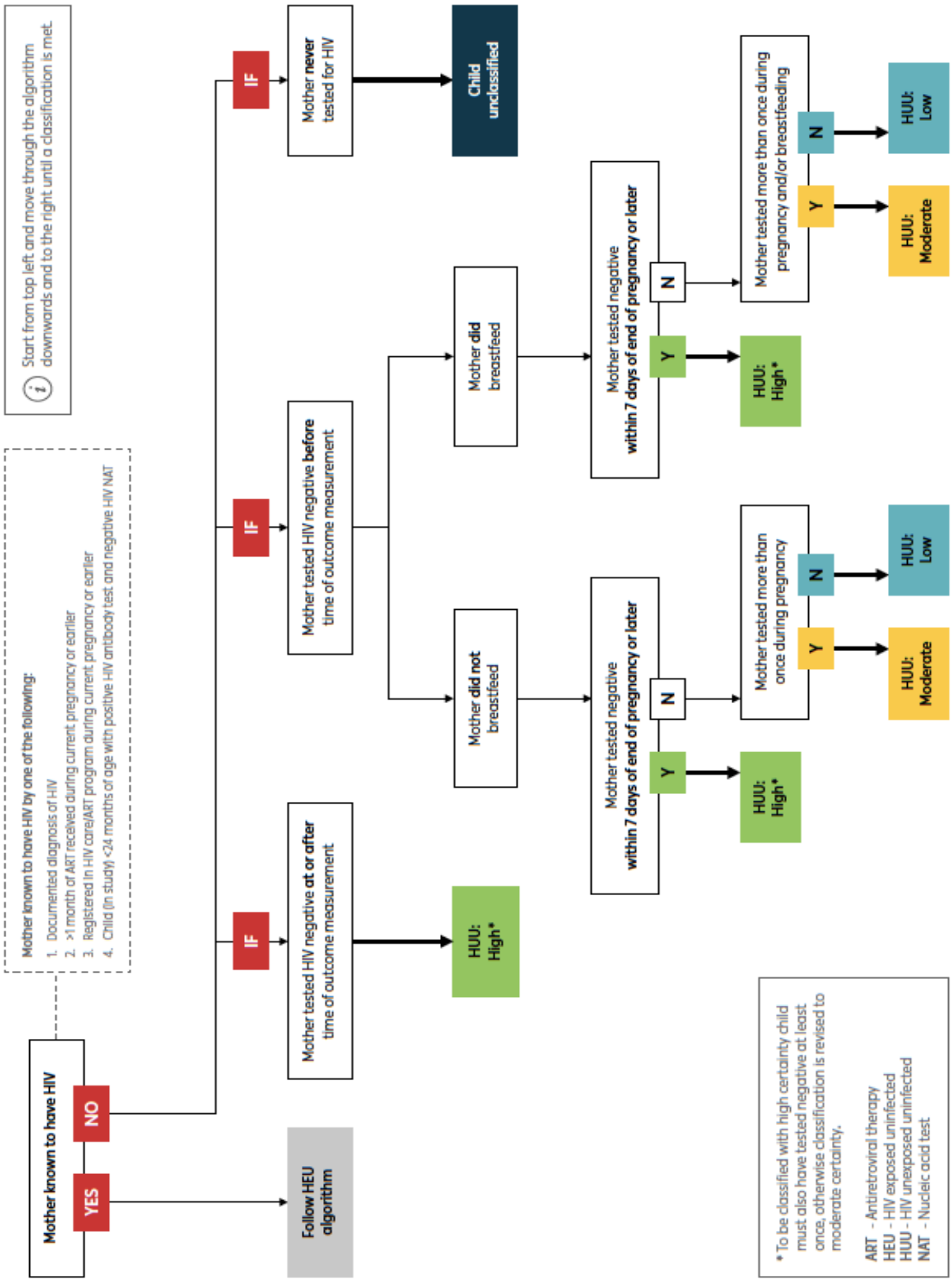

Figure 1.1.2: Algorithm for classification of children who are *in utero* HIV unexposed and uninfected[17].

The deleterious neurotrophic effect of HIV infection on the developing brain, with subsequent microcephaly, cognitive, motor, and behavioural abnormalities, is well established[7,18-19]. Mental and motor impairments seem to be associated with the severity of HIV infection and are more likely to occur in children living with HIV who develop a serious acquired immunodeficiency syndrome (AIDS)-defining illness in the first two years of life[20]. Interestingly, studies from developing countries[21-22] have reported specific neurodevelopmental delays in HEU infants, such as lower brain volumes, even though these infants have only been exposed to HIV and are themselves HIV uninfected. It is still unclear what the potential pathological mechanisms underlying such delays in HEU infants could be[21].

Microcephaly at birth has been associated with impaired neurodevelopment in infants living with and exposed to HIV[23-24]. This is not surprising seeing that head size is directly related to brain size[9,25]. Head size is measured routinely at birth and is recorded as the head circumference (HC).

There are two main areas of research into the possible underlying causes of the range of aberrations observed in HEU children. The first line of investigation hypothesizes that ARV medication influences the foetus' developing immune system since the ARV medication, such as zidovudine, which is known to have toxic effects on mitochondria, can cross the placenta[26]. Studies have shown that exposure to zidovudine inhibits haematopoietic progenitor cells, which could explain associated decreased red blood cell, neutrophil, and lymphocyte counts observed in HEU children. ARV medication could therefore potentially impair HEU children's innate and adaptive immunity[1].

The other hypothesis is that exposure of the foetus to the mother's HIV- or non-HIV- related antigens and pro-inflammatory cytokines and chemokines, may cause a hypo- or hyper-immune response in the foetus. In either case, it is speculated that a change in the intra-uterine environment of HEU children, despite not being infected with the virus, might affect their growth, development, and immunity[1].

In contrast to T-lymphocytes (T-cells), monocyte-macrophage lineage cells show a moderate resistance to HIV infection due to a restriction factor called Sterile Alpha Motif Histidine-Aspartic acid domain containing protein 1 (SAMHD1) [27-29]. SAHMD1 is critical for maintaining homeostatic equilibrium of deoxynucleotide

triphosphates (dNTPs) [28] and is also an effector for innate immunity[28]. Monocytes, macrophages, and dendritic cells all actively express high levels of SAHMD1 that contributes to the maintenance of dNTP levels[28]. Interestingly enough, however, it has been demonstrated that, in adults, HIV does not infect neurons, but rather brain mononuclear phagocytes (microglia and perivascular macrophages) [27,30]. HIV encephalitis has been associated with the number of stimulated brain mononuclear macrophages, but not with the number of infected cells or the quantity of HIV[27,31-32].

Nearby 160 00 infants are newly infected with HIV yearly, and an estimated 1.7 million children are living with HIV world-wide[33]. The innate immune response of infants, especially against HIV-infection, is critical. Adaptive immunity in infants takes time to develop and to be established, and their maternal antibodies provide only limited antiviral activity[33]. A study conducted by Bortlik *et al.* (2021) followed 1 338 infants, of whom 178 were HUU, 712 were HEU, 369 were living with HIV, and 79 were perinatally exposed to HIV but with an unclassified status[34]. They analysed gene expression of SAMHD1 (and others) by means of quantitative polymerase chain reaction (qPCR) of cluster of differentiation 4 (CD4+) T-cells, while CD4+ T-cell activation was analysed by flow cytometry[34]. Their results showed that CD4+ T-cells with reduced amounts of SAHMD1 were more susceptible to an HIV infection[34]. SAHMD1 expression therefore seems to protect cells from HIV infection as it restricts the dNTPs available to HIV[34].

Toll-like receptors (TLRs) (expressed by all monocyte phenotypes namely: classical monocytes [CM], intermediate monocytes [IM], and nonclassical monocytes [NCM]) are responsible for the innate recognition of viral, bacterial, and fungal infections[27,34]. Pathogens that enter the central nervous system (CNS) are counteracted by microglial cells through activation of their TLRs that initiate an innate and, secondarily, an adaptive immune response[27,35]. Disproportionate TLR activation of brain microglial cells is the likely cause of CNS diseases, such as HIV encephalitis. In addition, persistent pro-inflammatory reactions caused by monocytes' response to HIV infection might lead to other neurocognitive disorders, such as neuronal damage[27,36-38].

1.2 MACROPHAGE/MONOCYTE POLARISATION

The innate immune system is a person's first line of defence against harm from foreign substances. Components of the innate immune system play important roles in the growth and development of a child[39]. One such component of interest is monocytes and macrophages. Macrophages play many roles throughout the body at different stages of development and in response to different environmental stimuli[39]. Apart from their role as defensive phagocytic entities, they also enable wound healing and tissue repair. Their more recently discovered function and, some would argue the most interesting, is their role in the developing brain. Microglia (a macrophage sub-type in the CNS) are thought to be involved in developing and determining the circuitry of the brain[40]. Damage to neurons can activate microglia to produce pro-inflammatory cytokines[41]. Figure 1.2.1 below, shows the "resting" state of microglia (M0) [41] as well as their activated phenotypes.

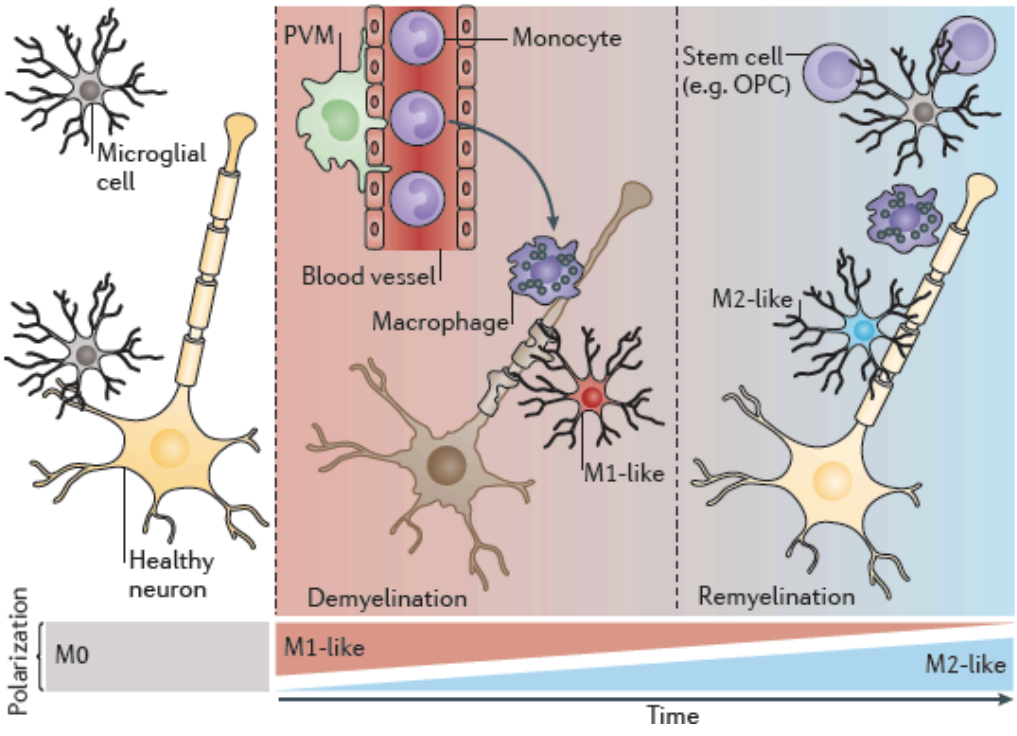

Figure 1.2.1: Functional reprogramming of microglia and macrophages in response to brain injury[41].

Macrophages can either originate from embryonic progenitors, including the yolk sac, or develop from blood monocytes[42-43]. According to the simplified classification of macrophages, there are two main phenotypes: classically polarised monocytes lead to M1 macrophages, which are pro-inflammatory in nature, and alternatively polarised monocytes lead to M2 macrophages, which are anti-inflammatory in nature[42]. While macrophage function is thought to vary along a continuum, these phenotypes represent two extremes of a dynamic state of activation pathways[44]. New evidence suggests that monocytes and macrophages remember memories of past infections, keeping them alert to react in case of re-exposure[42]. This innate immune recollection is made-up of another excitable-receptive phenotype named *trained innate immunity*[42].

Both phenotypes serve important functions of infection control (M1) and tissue repair/remodelling (M2) but, when not regulated, can have adverse or even pathological results. An imbalance between phenotypes has been associated with various diseases or inflammatory conditions, such as neurodegenerative diseases, tumours, and atherosclerotic plaques[45]. The M1 phenotype is stimulated by microbial products (such as lipopolysaccharide [LPS]) and pro-inflammatory cytokines, such as those classically found during viral exposure such as interferon-gamma (IFN-γ) and tumour necrosis factor (TNF), or activation of TLR signalling pathways[45]. M1-polarized microglia and/or macrophages exert cytotoxic effects on neurons and oligodendrocytes; thus, warranting this study and those like it, in understanding how macrophage function is altered in HEU children when compared to HUU children[45].

1.3 MONOCYTES

Monocytes and their progeny form part of the innate immune response that make up the initial line of defence[46]. Monocytes are released into the blood circulation after they had matured in the bone marrow. Monocytes originating from bone-marrow leukocytes that disseminate in blood can segregate into monocyte-derived dendritic cells (moDCs) and monocyte-derived macrophages that form the link between the adaptive and innate immune response[47-48]. These highly plastic cells are identified based on their expression of CD14 and CD16[47]. CD14 is a co-

receptor for TLR4 and facilitates LPS signalling, whereas CD16 is a low-affinity receptor for monomeric immunoglobulin (Ig) G, called Fc gamma receptor (FcgR) IIIa[47]. Phenotyping by means of flow cytometry differentiates between three subsets of monocytes: CM ($CD14^{++}$ and $CD16^{-}$), IM ($CD14^{+}$ and $CD16^{+}$), and NCM ($CD14^{+}$ and $CD16^{++}$)[47,49-50].

The released flowing monocytes from bone marrow are known to be $CD14^{+}$ CM[48]. CM gradually evolve into NCM ($CD14^{+}$ [or $CD14^{dim}$] $CD16^{++}$) through the intermediate step of $CD14^{+}CD16^{+}$ [48], although the evolution of CM to IM is still not fully understood. Table 1.3.1 below shows a comparison of the different subsets of monocytes.

Table 1.3.1: Comparison of monocyte subsets[47,51]

	Classical Monocytes	**Intermediate Monocytes**	**Non-classical Monocytes**
Abundance	80-95%	2-8%	2-11%
Phenotype and surface markers	$CD14^{++}$ and $CD16^{-}$	$CD14^{+}$ and $CD16^{+}$	$CD14^{+}$ and $CD16^{++}$
Pro-/Anti-inflammatory	Pro-inflammatory	Pro-inflammatory	Anti-inflammatory
Phagocytotic functioning	+	+	-
Migration	CCR2 dependent migration	CCR2 dependent migration	Mobile and guard the endothelium
Functions originated from phenotype	Phagocytic Scavenger cells	Production of ROS, antigen presentation, stimulation of T cells, angiogenesis, and inflammatory response	Involved in antigen presentation and T cell stimulation

Abbreviations: CD (cluster of differentiation), CCR (C-C Motif Chemokine Receptor), ROS (Reactive Oxygen Species).

Of the monocyte subsets, CM encompass around 80-95% of all flowing monocytes (Table 1.3.1) [47,52]. These CM are known to be exceedingly phagocytic and are thus essential scavenger cells[47]. CM respond to an infection, inflammation, or injury by being released into the blood circulation using a chemokine receptor C-C Motif Chemokine Receptor 2 (CCR2)-dependent method and travel to the site of importance by means of a chemokine gradient[53-54]. For instance, when a bacterial infection occurs, CM travel to the infected site and phagocytose the pathogens; this phagocytotic reaction releases a distinctive collection of chemokines that in turn employ other immune cells with class II major-histocompatibility complex (MHC) [53,55]. Supplementary markers of CM, such as CD36, CD64, and CCR2 participate in anti-microbial responses; for instance: phagocytosis, migration, and adhesion to the endothelium[48].

The non-classical subset of monocytes encompasses about 2-11% of the flowing monocytes[47]. NCM are progenies of CM that reverted back to the bone marrow and are seasoned into NCM under the regulation of nuclear receptor subfamily 4 group A member 1 (Nr4a1), also known as Nur77[53]. Currently, there are contradictory thoughts as to the function of NCM[53]. The contradiction centres around whether NCM release pro-inflammatory or anti-inflammatory cytokines[53]. These contradictions are mainly due to the challenge of differentiating between non-classical monocytic cell surface markers and IM[53]. IM are in fact intermediate due to the expression of high levels of MHC class II molecules participating in antigen presentation[56]. To further shed light on these contradictions between NCM and IM, 6-sulfo LacNAc (SLAN) markers (sugar structures connected to cell surface protein P-selectin glycoprotein ligands [PSGL-1] [57]) were used and found that IM are $CD16^+$ $SLAN^-$ and that NCM are in fact $CD16^+$ but $SLAN^+$ [56]. Studies in humans have demonstrated that NCM recognise viruses and nucleic acids using TLR7 signalling and, in turn, commence the innate immune response by discharging cytokines[53].

The main characteristic of IM is the high expression of CCR5 and human leucocyte antigen – DR locus (HLA-DR) molecular markers that are involved in trans-endothelial migration and antigen presentation[48]. $SLAN^+$ populaces are divisions of NCM that express high levels of CX_3CR1 and focus on trans-endothelial

migration, anti-viral responses, and fragment crystallizable receptor (FcR)-mediated phagocytosis[48].

Monocyte and macrophage are essential for the progression of an inflammatory cascade that is swiftly inducted after an injury. They are also critical for successful tissue repair after the inflammatory response[58-59]. Circulating in the bloodstream, monocytes are employed to sites of inflammation where their functions include: re-establishment of tissue integrity, unblocking of cellular debris, and advancement of angiogenesis and arteriogenesis (separate processes that control the post-injury restoration of muscle injury) [58-64]. As aforementioned, M1 macrophages are essential in the production inflammatory cytokines[58,65], while M2 (alternatively activated) macrophages have pro-reformative behaviours such as: extracellular matrix restoration[58,66-67], production of anti-inflammatory cytokines[58,65], resolution of inflammation[58,68], and angiogenesis[58,69-70]. Conditional to the severity of nerve damage, circulating blood monocytes are involved in the unprompted recovery of these damaged nerve cells[71-72]. NCM's anti-inflammatory cytokine production aids in useful recovery and resolves the initial inflammatory response[71-72]. With a CNS injury, high concentrations of NCM will be needed to resolve such an injury[71]. In a study, tissue repair and remodelling of a spinal cord injury and the restoration of the CNS were observed, and it was perceived that directly after the injury M1 macrophages were recruited to the site of injury, and small numbers of M2 macrophages were observed[68, 71]. The anti-inflammatory response from the M2 macrophages and NCMs promotes the survival of neural cells[68, 71].

1.4 HEAD CIRCUMFERENCE

The estimation of brain size, neurocognitive development, and total grey matter volume can be done through measurement of the HC[73]. Procedural anthropometric measurements are especially performed in paediatric settings and used world-wide as age-related and health-related indicators[73-74]. Stages of prenatal and early development as well as maturation of a child have been associated with brain growth. Head size reflects intracranial volume[73,75-76], and a strong association has been found between HC and total brain volume in children up to the age of 6 years[73,77]. The strength of this association, however, declines for children aged 12

years and older[73,78]. Since HC measures a single dimension, it can of course not provide a comprehensive understanding of neurocognitive development; yet it remains a good proxy measurement for brain size[73,77,79].

1.5 PAST RESULTS

In order to demonstrate proof of concept for this study, a pilot study was conducted in the Department of Immunology, titled: **Testing the association between macrophage activation pathways and head circumference in HIV exposed and unexposed children born at Kalafong Hospital.** The pilot study was conducted in 2017 at Kalafong Provincial Tertiary Hospital (KH), South Africa, and forms the basis for this project. With the pilot study, 55 pregnant mothers were recruited: 33 mothers were living with HIV; of whom 29 were on ARV medication at the time the study was conducted. Of the infected mothers, 28 were on a fixed-dose combination (FDC) of efavirenz, tenofovir disoproxil fumarate and emtricitabine, and one mother was on lamivudine, zidovudine, and ritonavir-booster lopinavir. The four residual mothers were not on ART. Anthropometric measurements (weight, length, mid upper-arm circumference [MUAC], abdominal circumference, and HC) were taken at birth and 10-12 weeks. Blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes for flow cytometric phenotyping of monocyte subsets by means of assessing CD14 and CD16 expression. Monocyte activation was determined by the co-expression of CCR2 on the monocyte subsets. The responsibility of CCR2 is that of transmigration of monocytes in response to inflammatory circumstances, especially inflammatory diseases of the CNS such as: Alzheimer's disease, multiple sclerosis, and ischemic stroke[80].

In Table 1.5.1 below, a brief report taken from the results of the pilot study shows the infant anthropometric measurements at birth and 10-12 weeks according to the HIV exposure status of the infants[81].

Table 1.5.1: Pilot study: Infant anthropometric measurements at birth and 10-12 weeks according to their exposure status.

Measurement	Time point	HIV Exposed (n=33)		Unexposed (n=22)		p-value
		Median	IQR	Median	IQR	
Weight (kg)	Birth	2,910	2,640 – 3,260	3,160	2,710 – 3,480	0.2791
	10-12 weeks	5,500	5,100 – 5,800	5,925	4,900 – 6,300	0.1696
Length (cm)	Birth	46.5	45.5 – 48.0	47.5	44.5 – 50.0	0.4298
	10-12 weeks	57.5	56.50 – 58.0	57.5	53.80 – 59.50	1.0000
MUAC (cm)	Birth	10.5	10.0 – 11.0	11.0	10.0 – 12.0	0.1059
	10-12 weeks	13.0	12.0 – 14.0	13.5	12.0 – 14.0	0.3094
Abdominal circumference (cm)	Birth	30.0	27.0 – 31.0	30.0	27.0 – 32.0	0.3225
Head circumference (cm)	Birth	33.0	31.5 – 33.0	33.25	33.0 – 35.0	***0.0054**
	10-12 weeks	38.0	37.0 – 38.0	38.0	38.0 – 39.0	0.0770
APGAR score (/10)	1 min	9	8 – 9	9	8 – 9	0.9159
	5 min	9	9 – 9	9	9 – 10	0.6920

In Figure 1.5.1, a visual representation of a box-and-whisker diagram illustrates the association between HIV exposure status and HC in the infant groups in the pilot study. In Figure 1.5.1, group 0 is the HUU infant group and group 1 is the HEU infant group. From the figure, it is well-illustrated that HEU infants had smaller median HC than their HUU counterparts, and that the difference was statistically significant, i.e. p=0.0054.

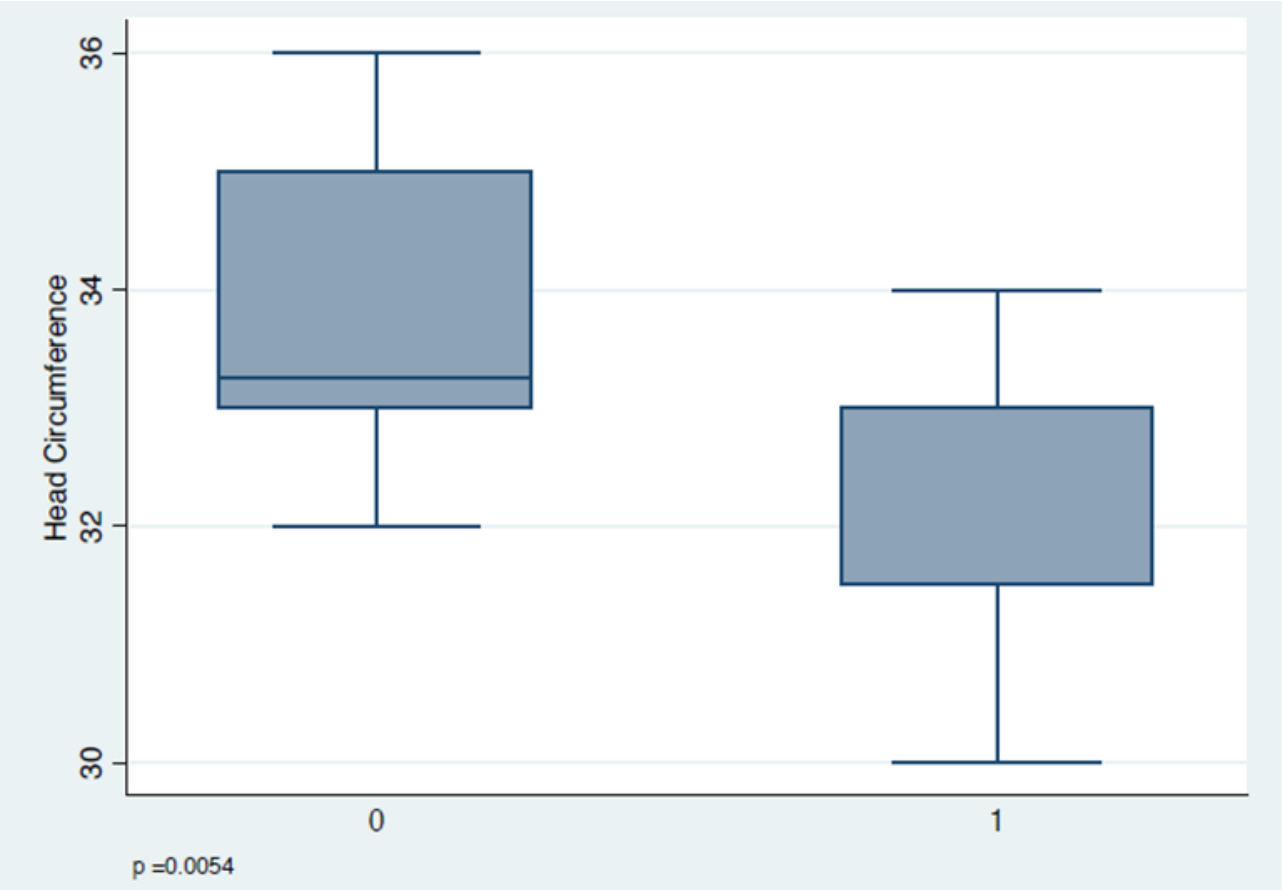

Figure 1.5.1: Pilot study: Infant head circumference according to HIV exposure status.

Table 1.5.2 compares the results of the immunological biomarkers at birth and 10-12 weeks in the HEU and HUU infants. The difference in expression of CD16+ on monocytes was significantly different between the two groups, with HEU infants having lower median levels at 10-12 weeks after birth (p=0.0275). On the other hand, HEU infants had significantly higher levels of expression of CD14+/CCR2+ on monocytes (p=0.0005) at birth.

Table 1.5.2: Pilot study: Infant immunological biomarker results at birth and 10-12 weeks according to their HIV exposure status.

Biomarkers	Time point	HIV Exposed (n=33)		HIV Unexposed (n=22)		p-value
		Median	IQR	Median	IQR	
Total CD16+	Birth	49.09	40.17 – 56.45	45.97	38.92 – 55.36	0.5306
	10-12 weeks	11.59	8.13 – 17.51	21.4	13.01 – 27.12	***0.0275**
Total CD14+	Birth	9.18	6.64 – 11.87	10.51	8.72 – 12.21	0.2462
	10-12 weeks	6.39	5.14 – 8.78	7.24	5.0 – 9.53	1.0000
Total CCR2+	Birth	11.08	8.92 – 13.98	10.415	8.80 – 12.55	0.7311
	10-12 weeks	11.02	8.80 – 13.60	12.48	9.81 – 13.52	0.6400
% Viability	Birth	96.57	93.58 – 97.64	96.96	90.21 – 98.04	0.9179
	10-12 weeks	94.1	92.59 – 95.84	94.59	92.0 – 96.92	0.9467
CD16+/CCR2+	Birth	0.36	0.17 – 0.80	0.39	0.14 – 0.93	0.7966
	10-12 weeks	1.18	0.80 – 1.91	0.72	0.54 – 1.63	0.2164
CD14+/CCR2+	Birth	87.25	84.65 – 89.71	80.47	73.57 – 84.66	***0.0005**
	10-12 weeks	81.48	75.27 – 82.90	81.11	76.15 – 86.54	0.5930
CD14+/CD16+	Birth	1.41	0.85 – 2.25	1.89	1.16 – 2.72	0.1747
	10-12 weeks	2.08	1.45 – 2.40	1.81	1.54 – 1.92	0.4260
CD14+/CD16-	Birth	7.6	5.51 – 8.99	8.325	5.3 – 10.27	0.4601
	10-12 weeks	5.46	4.29 – 6.38	6.93	4.43 – 8.10	0.1779
CD14-/CD16+	Birth	51.15	42.78 – 56.96	46.8	40.25 – 53.02	0.3903
	10-12 weeks	19.98	13.50 – 23.83	26.27	16.82 – 30.90	0.1416

Figure 1.5.2 below, a box-and-whisker plot shows the concurrent CD14 and CCR2 expression on monocytes in the HEU and HUU infant groups in the pilot study: 0 denotes the unexposed infant group whilst 1 denotes the exposed infant group. From the figure, it is clear that in the HEU group the concurrent expression of CD14+/CCR2+ was higher than in the HUU group, suggesting higher levels of inflammatory macrophages in the HEU group.

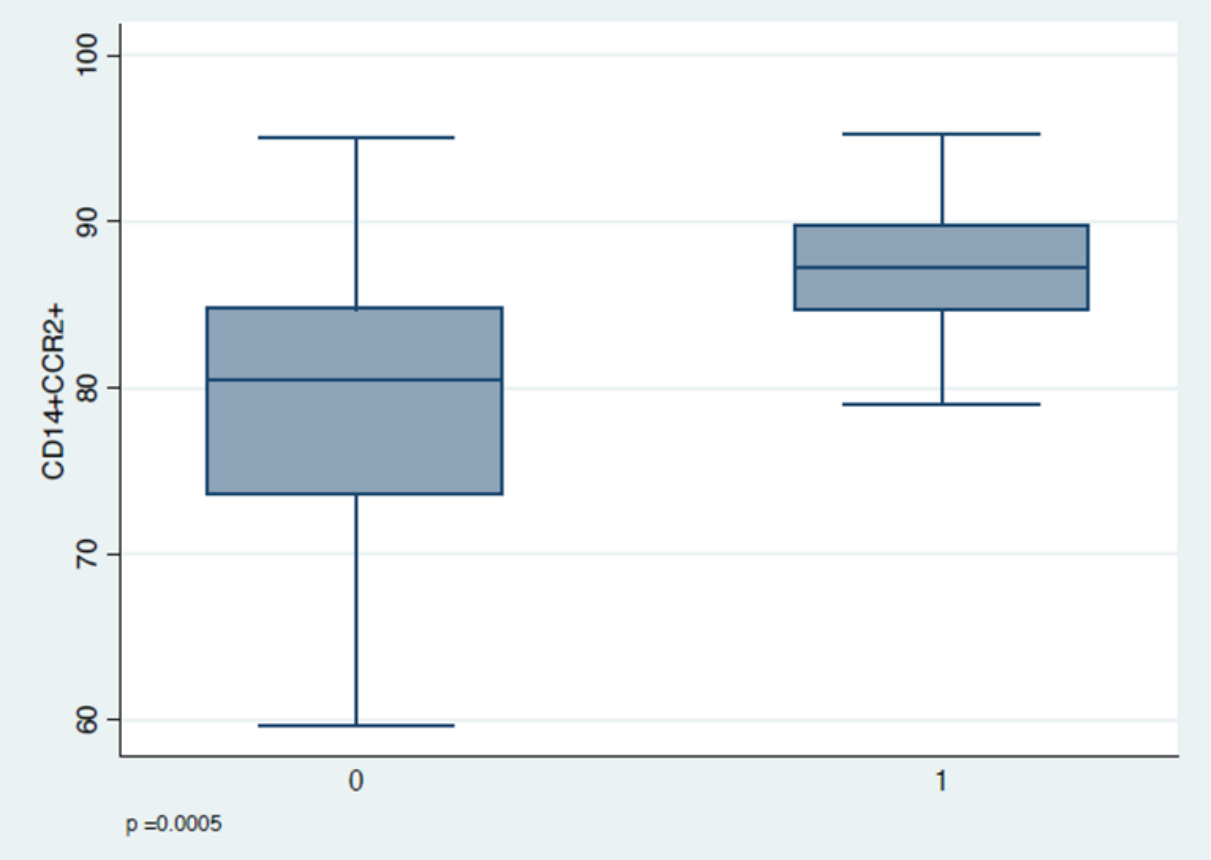

Figure 1.5.2: Pilot study: Concurrent CD14 and CCR2 expression on blood monocytes according to HIV exposure status.

The results from the pilot study confirmed the need for a larger study. Hence, the Siyakhula project is currently being conducted and is the larger, prospective study on which the current study is based. The Siyakhula project has two main objectives. Firstly, to grasp how *in utero* and early postnatal environments, altered by the mothers' HIV status, influence the children's growth, and potentially distort their immune expansion and their cognitive development, regardless of their own

HIV status. The second objective is to understand how maternal breast milk and breastfeeding habits could change the relationship between *in utero* HIV exposure and infant outcomes. The Siyakhula project enrolled 300 pregnant mothers, of whom 150 are living with HIV and 150 are HIV-uninfected. The specific focus of this current study is to assess the relationship between infant HC and monocyte polarisation in a subgroup of these 300 women.

1.6 PURPOSE OF THE STUDY

1.6.1 AIM

The aim of the study was to investigate whether there is an association between HIV exposure, anthropometry, including HC, and patterns of monocyte polarisation in infants born at KH.

1.6.2 OBJECTIVES

- To investigate whether there is a difference in anthropometry at birth, 10 weeks, six months, and 12 months between HEU and HUU infants by means of anthropometric data.
- To characterise and compare the monocyte phenotypes between HEU and HUU infants at birth, 10 weeks, and six months using flow cytometry.
- To determine if there is an association between HC and monocyte phenotype at birth, 10 week, and six months.
- To explore associations and correlations between anthropometric measures (apart from HC) and monocyte phenotypes.

CHAPTER 2

MATERIALS AND METHODOLOGY

2.1. STUDY DESIGN

This project forms part of the larger Siyakhula study which is a prospective, longitudinal cohort study with a human immunodeficiency virus (HIV)-exposed group and an HIV-unexposed comparison group.

2.2. SETTING

Pregnant females from both evaluation groups, living with and not living with HIV, with singleton pregnancies, were recruited from antenatal clinics in Southwest Tshwane, South Africa. The geographical area has roughly 10,000 births/year from 14 antenatal clinics in South-west Tshwane. All experiments were carried out in the laboratories of the Immunology department of the University of Pretoria, Prinshof campus. Three hundred women were recruited, 150 in each evaluation group.

2.3 ETHICAL CONSIDERATIONS

Ethics approval had been obtained for the pilot study as well as the prospective longitudinal cohort (Siyakhula) study from the Research Ethics Committee of the Faculty of Health Sciences at the University of Pretoria (185/2016, 294/2017) *(Appendix 1 and 2).* Written informed consent was obtained from all the mothers on behalf of themselves and their infants *(Appendix 3).*

In addition, the current study was granted ethics approval (UP REC reference number 510/2021) from the Research Ethics Committee, Faculty of Health Science, University of Pretoria.

Participation in both above-mentioned studies was completely voluntary, and participants' anonymity was maintained by assigning unique numbers to each participant sample.

2.4 SAMPLE SIZE AND COLLECTION

The Siyakhula study recruited 300 pregnant women with singleton births (both male and female infants). The sample size was based on the feasibility of recruiting pregnant women during a 12-month period.

2.4.1 Inclusion criteria

- <28 weeks pregnant
- Known HIV status
- Eighteen years and older
- Willing and able to give written informed consent on behalf of themselves and their infants

2.4.2 Exclusion criteria

- 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
- Mothers who could not commit to follow up appointments

2.5 BLOOD COLLECTION

All mothers had blood drawn by a research nurse at 28- and 36-weeks' gestation as well as at birth. Infants had blood drawn at birth, at six, 10, and 14 weeks, and again at six and 12 months. Blood was transported to the Department of Immunology and flow cytometry was performed in real time. From this collected blood, an aliquot (<1 millilitre [mL]) was taken and plasma isolated and stored at -80°Celsius (C) for future testing of a panel of inflammatory cytokines and chemokines. Flow cytometry was performed on whole blood within four hours of the blood draw. (*Appendix 3)*

2.6 DATA COLLECTION

2.6.1 Collection of metadata

2.6.1.1 Pregnancy data

Retrospective medical chart review from the pregnancies of the consented mothers provided important data such as: *(Appendix 4*)

- *Maternal age.*
- *Maternal HIV status* - measured as part of routine care at the first antenatal visit using standard ELISA assays.
- *Maternal anthropometry*: length, weight, mid upper-arm circumference (MUAC). Maternal body mass index (BMI) was calculated by dividing the mother's weight (in kilogram [kg]) by her height (in centimetre [cm]) squared.
- *Pregnancy outcomes*: mode of delivery, gestational age, new-born gender and anthropometry (length, weight, and head circumference [HC]) *(Appendix 5).*
    - *HC was measured according to standard guidelines*: Firstly, the measuring tape was looped before it was slipped over the infant's head. The tape was then placed above the brows, the pinna of the ears, and around the occipital prominence at the back of the skull. Then it was ensured that the tape was flat against the skin. The circumference was recorded.

2.7 EQUIPMENT

- Multi-parameter flow cytometry CytoFlex flow cytometer (Beckman Coulter, CA, USA)

2.8 PROCEDURE

Participants used for the flow cytometry part for this study were randomly selected from participants with the most complete data set for all the time points, namely: Mother at 28 weeks, mother at birth, baby at birth, baby at 10 weeks, baby at six months, and baby at 12 months.

2.8.1 Flow cytometry procedure

2.8.1.1 Daily quality control

The CytoFLEX flow cytometer (Beckman Coulter) has a preloaded quality control (QC) component included in the CytEx software (Beckman Coulter). CytoFLEX Daily QC Fluorospheres (Beckman Coulter) is a suspension of fluorescent microspheres with a uniform size (~3 micrometre [µm]) and fluorescence intensity (fluorescence emission of 410 nanometres [nm] to 800 nm when excited at 405 nm, 488 nm, or 635 nm). The fluorospheres allow for verification of the optical and fluidics systems' performance. Each lot number of Daily QC beads will have lot-specific targets per channel. The instrument automatically adjusts its gains to reach these targets. It is a completely locked QC component, and the operator cannot make any adjustments. Statistics assessed are laser power for all three lasers, laser delay, gains, targets, and robust coefficient of variation. All parameters are monitored automatically via Levy-Jennings graphs[82].

The CytoFLEX Daily QC Fluorospheres (Beckman Coulter) were mixed vigorously by vortexing (Velp Scientifica SRL, Usmate Velate, Italy) for a few seconds prior to use. Three drops were added to one mL of deionised water and mixed by vortexing (Velp Scientifica SRL) again for a few seconds. The prepared suspension was used for up to five days after dilution while stored at two to eight °C[82].

Adjustments to gains were made automatically to the Daily QC component on the CytoFLEX, in order to reach the median fluorescence intensity (MFI) targets +/- 20%, prescribed for the beads. Any adjustments to the gain were imported with the recommended settings, thereby standardizing the MFI of the populations per run.

2.8.1.2 Titration of Monocyte panel monoclonal antibodies

Titration experiments of single monoclonal antibodies were performed prior to setting up the single colour compensation for this panel on the system. This was done in order to determine the optimal volume of each individual antibody required that will give the highest discrimination of positive cells from negative cells as well as to prevent wastage of reagents.

A doubling dilution starting with twice the recommended volume was set-up for each marker. A set of flow tubes was labelled for each antibody per volume of antibody to be added. Fifty microliters (µL) of whole blood collected in an ethylenediamine tetra-acetic acid (EDTA) collection tube from a healthy donor was added to all the tubes. The fluorochrome-conjugated antibodies listed in Table 2.7.10 (Beckman Coulter & Biocom Africa (Pty) Ltd., Pretoria, South Africa) were added to each corresponding tube in the predetermined volumes specified. Samples were processed as described below and analysed on the CytoFLEX flow cytometer (Beckman Coulter).

2.8.1.3 Colour compensation set-up

Before commencing with sample testing, colour compensation set-up was performed using VersaComp Antibody Capture Bead Kit (Beckman Coulter). One drop of negative beads and one drop of positive beads were added to each test tube containing a fluorochrome-conjugated antibody included in the antibody panel for monocyte activation. The test tubes were mixed by vortexing (Velp Scientifica SRL, Usmate Velate, Italy) for ten seconds followed by incubation at room temperature in the dark for 20 minutes.

After incubation, one mL of Phosphate-buffered saline (PBS; pH7.4) (Merck, Darmstadt, Germany) was added to each test tube. The test tubes were mixed by vortexing (Velp Scientifica SRL) for ten seconds followed by centrifugation at 300 x gravitational force (g) for five minutes. The supernatant was decanted, and the pellet resuspended in 600 µL of PBS (Merck). This procedure was performed again when required. An acquisition protocol for this panel was created, including all histograms, gating logic, and statistics.

In Table 2.8.1 below the antibodies, volumes, and concentrations used for the peripheral whole blood staining are shown.

Table 2.8.1: Antibodies, volumes, and concentrations used for peripheral whole blood staining.

Antibody	Volume used per sample	Concentration used per sample
CCR2-FITC (Milytec)	10 µL	100 µg/µL
CD14-ECD (Beckman Coulter)	5 µL	1250 µg/µL
CD16-Krome Orange (Beckman Coulter)	2.5 µL	20 000 µg/µL

Abbreviations: ECD (Electron coupled dye), FITC (Fluorescein isothiocyanate), µL (microlitre), µg/µL (microgram/microlitre).

Peripheral whole blood was stained with cluster of differentiation (CD)14, CD16, and CCR2 monoclonal antibodies in the following way:

First, 50 µL EDTA blood was added to a blue flow tube (Labucon) for monocytes. Then two mL Versalyse (Beckman Coulter) was added to the monocyte tube, the tube was capped, and vortexed (Vortex-e genie – Scientific Industries) for 10 seconds and incubated in the dark for 15 minutes. After the 15 minutes, the monocyte tube was centrifuged (Allegra X-12R Centrifuge) at 500 x g for five minutes and the supernatant was decanted. After the decantation, the monocyte tube cell pellet was gently vortexed, and the volume indicated on the tube of the antibody (CCR2 – Fluorescein isothiocyanate [FITC]; CD14 – electron coupled dye [ECD]; CD16 – Krome Orange) then the cocktail was aliquoted to the bottom of the tube. Next, five µL Super Bright (Invitrogen 0.5 mL) complete staining buffer was added to the monocyte tube. Then the monocyte tube was vortexed for ten seconds and the tubes were incubated in the dark for 15 minutes. After the incubation, two mL of 2% BSA (Sigma) staining buffer was added to the monocyte tube, the tube was capped, inverted a few times, and allowed to stand for five minutes. The tubes were then centrifuged at 500 x g for five minutes and the supernatant was decanted. The monocyte tube pellet was gently vortexed again and 500 µL PBS containing 0.1% formaldehyde (Beckman Coulter) was added. Samples were stored in the dark until acquisition and analysis on the flow cytometer[83].

2.8.1.4 Monocyte gating strategy

Data Clean-up:

Acquisition flow cytometry standard (FCS) files were uploaded to Kaluza software (Beckman Coulter). All file parameters were checked to ensure they are the same. The compensation matrix calculated previously was imported into the protocol for each file. A dot plot was created with forward scatter area (FSC-A) versus Time (Figure 2.8.1 Panel 1). Only sections where acquisition occurred continuously were included in the added Time gate. A second dot plot with FSC height (FSC-H) versus FSC-A gated on Time were added to exclude doublets (Figure 2.8.1 Panel 2). A gate called Singlets was drawn to include single events. A third dot plot gated on the Singlets gate was created with FSC-A versus side scatter area (SSC-A) and a gate labelled Cells were drawn around all the cells excluding debris, as seen in Figure 2.8.1 Panel 3. Another dot plot was added with cluster of differentiation (CD)14 versus CD16 gated on the Cells gate. In this plot a gate labelled CD14+CD16+ was drawn to include all three monocyte phenotypes as shown in Figure 2.8.1 Panel 4 below.

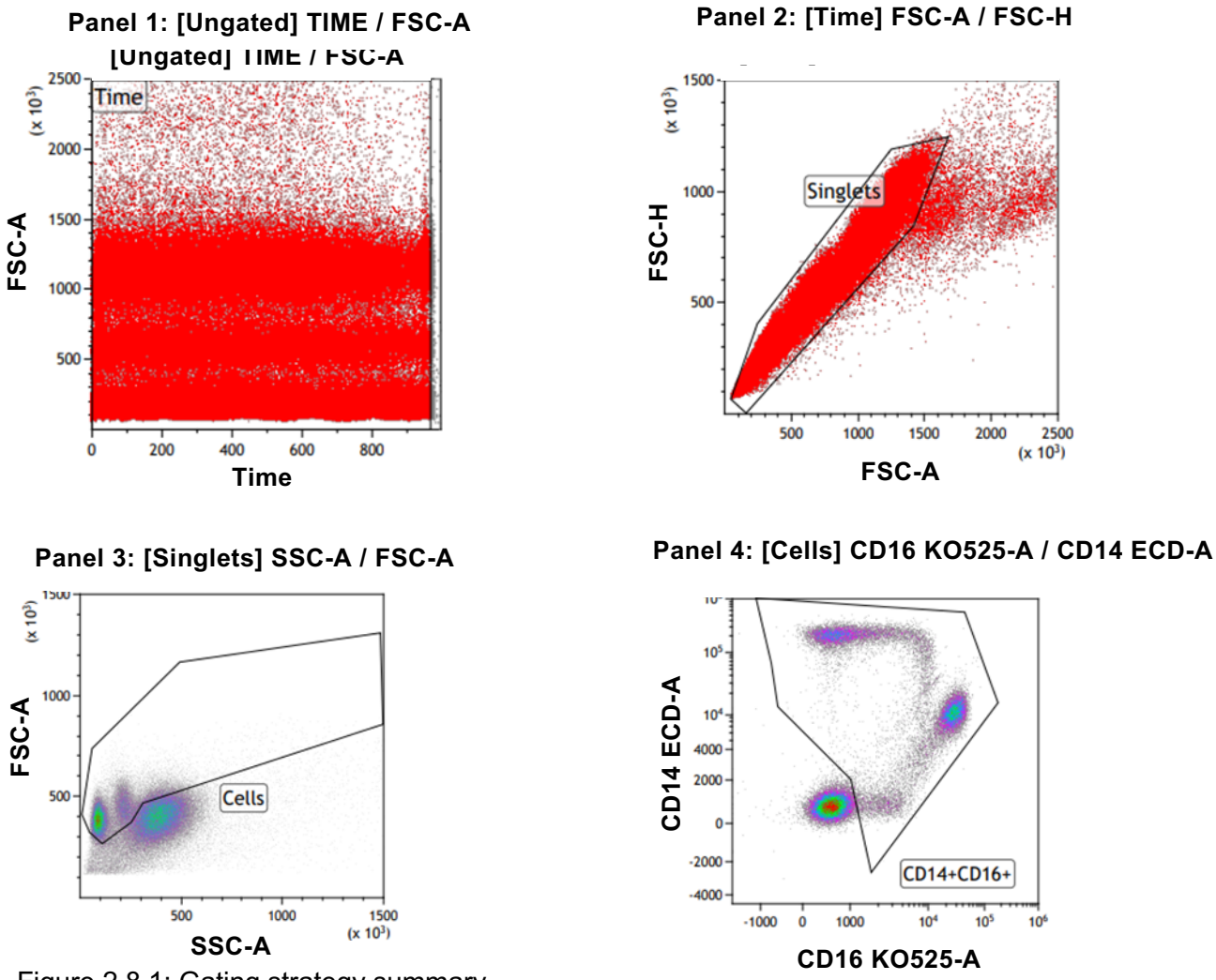

Figure 2.8.1: Gating strategy summary.

2.8.2 Cytobank analysis procedure

The newly gated FCS files were uploaded to the Cytobank software (Beckman Coulter) for further advanced analysis. The CD14+CD16+ gate was used for uploading the files. Cytobank consists of various dimension reduction and clustering algorithm programs. The t-distributed Stochastic Neighbour Embedding (tSNE) algorithm, a non-linear dimensionality reduction algorithm was used for this analysis. Dimension reduction analysis gives a visual overview of the data allowing rapid exploratory data analysis. The Cytobank software also allows performance of statistical analysis of the resulting data. The Kruskal-Wallis significance test together with the Bonferroni correction method were used to determine statistical significance of marker expression between the two groups. Examples of the

Cytobank algorithms used and statistical analysis data are shown in Figure 2.8.2 Panel A below[84-85]. The figure below shows ungated expression of markers CD14, CD16, and CCR2. Strong positive expression of these markers is indicated by dark-red, positive expression as red, and negative expressions as blue. In Figure 2.8.2 Panel B, an example of the statistical analysis of the resulting data can be observed, where for this example statistically significant differences can be seen between the HEU and HUU infants for the number of CD14 expressing monocytes (p=0.0238).

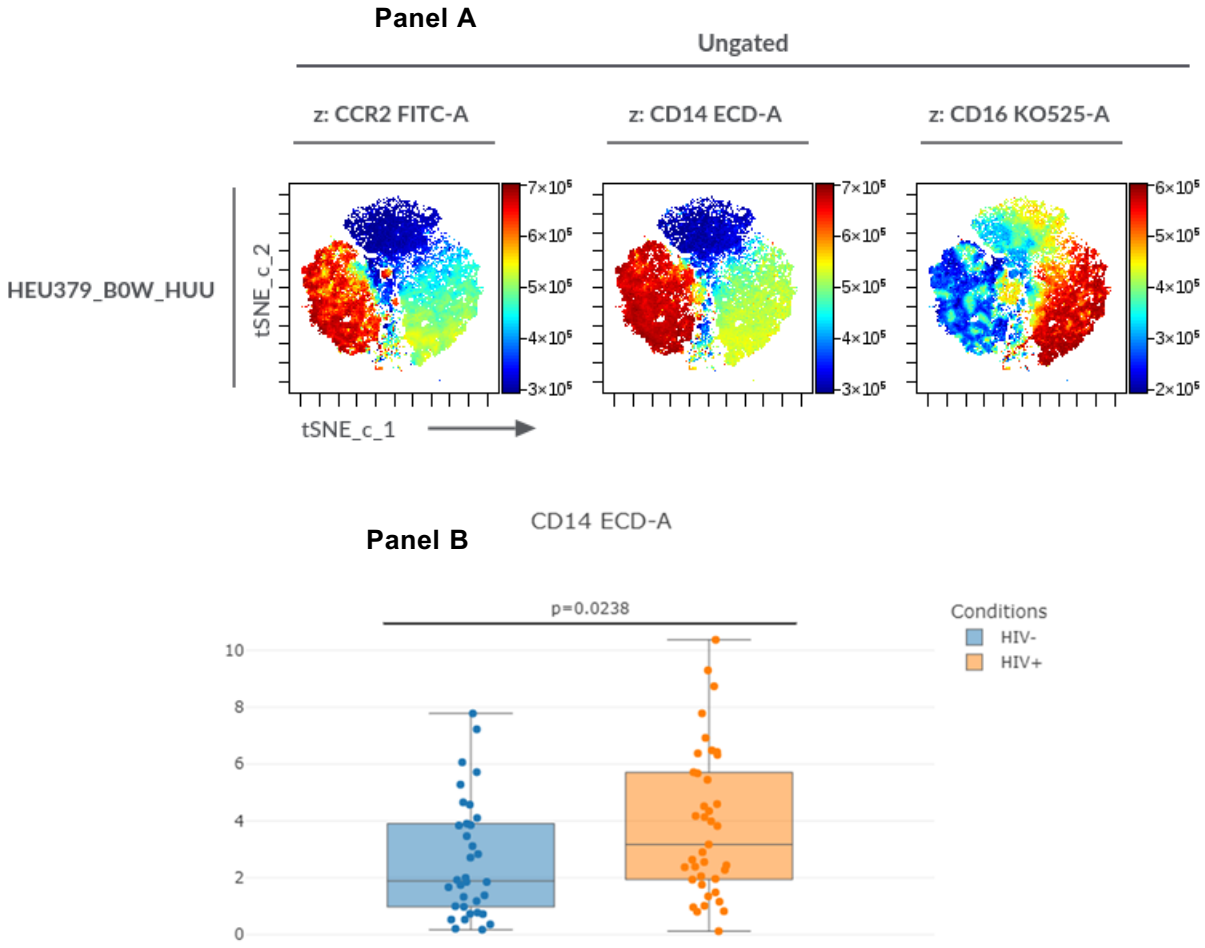

Figure 2.8.2: Examples of Cytobank tSNE dot plots (A) and statistical analysis results (B).

2.8.3 Data cleaning

After the collection of anthropometric data and flow cytometric data, the data were entered into two separate Microsoft Excel spreadsheets. Both spreadsheets were checked for outliers and possible incorrect entries using histograms.

2.8.4 Statistical analysis procedure

Z-score calculations were conducted using WHO's ANTHRO program version 3.2.2 (Department of Nutrition, WHO – Geneva, Switzerland). Preparation for the z-score calculations were conducted using Microsoft Excel version 16.69.1 (Microsoft 365) *(Appendix 6).*

Statistical analysis was conducted using STATA version 17.0 (Stata Corp – Texas, USA). The medians and inter-quartile ranges (IQR) of the anthropometric and expression concentration data were calculated to describe the sample. The following analytical tests were performed:

- Kruskal-Wallis equality of populations test; to assess any differences in continuous variables between groups.
- Quantile distributions and Fisher's exact testing; test for associations between categorial variables.
- Categories were generated for z-score values and tested by the Fisher's exact test to assess any differences in the z-score categories across the anthropometric variables between the groups.
- The Wilcoxon sign-rank test was used to assess any differences within the groups.

A p-value of 10% was considered significant, due to the exploratory nature of this study. Graphs were constructed using Microsoft Excel.

CHAPTER 3

RESULTS

3.1 DESCRIPTION OF THE MOTHERS

For this current study, 42 randomly selected mothers and their infants were included: 23 mothers living with human immunodeficiency virus (HIV) and their HIV-exposed uninfected (HEU) infants and 19 mothers not living with HIV and their HIV-unexposed uninfected (HUU) infants. As can be seen in Table 3.1.1, the mothers living with HIV were older: median age (years) of 38 (inter-quartile range [IQR] 33 – 40), whereas the median age of the HIV-uninfected mothers was 34.5 and (IQR 29 – 38), but this difference was not statistically significant. They also had a slightly higher weight and mid-upper arm circumference (MUAC) at the time of delivery than the uninfected mothers, but these differences were also not statistically significant. Given the standard definition for overweight during pregnancy of MUAC >29.9 cm, 18 of the 38 mothers with MUAC recorded, i.e., 47.7% were overweight[86]. The proportion of overweight mothers did not differ between groups: 10/21 (47.6%) of mothers living with HIV versus 8/17 (47.1%) of mothers not living with HIV (p=0.973).

The cluster of differentiation 4 (CD4) count is an indication of the degree of immune suppression during HIV infection and is classified as normal in South Africa from 332 to 1642 cells per microliter (µL) of blood, low if between 200 and 332 cells/µL, and very low if there are equal to or fewer than 200 cells/µL[87]. The median CD4 count of the 16 mothers with such information available at the time of delivery, was 474.5 (IQR 332 – 778.5) cells/µL. Only one of these mothers had a CD4 count <200 cells/µL. Other information of importance for the mothers living with HIV is their HIV viral load (VL) values. The VL is considered very high if there are more than 100 000 copies per millilitre (mL) of blood, high if >1 000 copies/mL, intermediate if the value is more than 50, but less than or equal to 1 000 copies/mL, and suppressed when ≤50 copies/mL. The median VL of the 19 mothers with this information available at delivery was 9 (IQR 0 – 20) copies/mL and only three mothers had a VL >50 copies/mL. The VL in these three mothers was 5 991, 7 877, and 29 305 copies/mL respectively, therefore in the high VL range.

Due to the strict entry criteria of the study, none of the mothers had any health issues, including: epilepsy, hypertension, cardiac diseases, tuberculosis (TB) or other pulmonary diseases or any cancers. According to self-report, none of the

mothers used tobacco products, alcohol, or illegal substances. All of the mothers had singleton pregnancies and had normal vaginal deliveries (no caesarean sections).

The phenotypic classification of monocytes (classical [CM], intermediate [IM], and non-classical [NCM]) and C-C Motif chemokine receptor 2 (CCR2) expression were similar between the two maternal groups. It is interesting to note, however, that even though no statistically significant differences were found between the two mother groups at birth, the proportion of CM in the HIV-uninfected mothers was slightly higher, whereas the proportion of IM and NCM was slightly elevated in the mothers living with HIV. The same pattern emerged when looking at CCR2 expression on the monocytes, where the median expression of CCR2 for CM (CCR2+CM) was higher in the HIV-uninfected mothers and the median expression of CCR2 on IM (CCR2+IM) and CCR2 on NCM (CCR2+NCM) was higher for the mothers living with HIV.

Table 3.1.1: Comparison of demographic, anthropometric, and flow cytometric results of mothers with and without HIV at the time of delivery.

Variable	HIV uninfected Median (IQR)	HIV infected Median (IQR)	Probability
Age (years)	n=19 34.50 (29.00 – 38.00)	n=23 38.00 (33.00 – 40.00)	0.1678
Weight (kg)*	n=18 70.10 (60.00 – 82.00)	n=20 71.05 (66.20 – 80.65)	0.2595
Height (m)*	n=16 1.60 (1.53 – 1.63)	n=19 1.59 (1.56 – 1.66)	0.3205
MUAC*	n=17 28.70 (26.50 – 32.90)	n=19 29.70 (27.00 – 32.00)	0.9766
BMI*	n=17 28.92 (23.40 – 31.95)	n=19 29.17 (25.44 – 30.12)	0.9736
CM (%)*	n=16 77.16 (58.32 – 83.64)	n=13 71.85 (66.33 – 76.93)	0.4752
IM (%)*	n=15 3.57 (2.02 – 11.08)	n=13 6.74 (3.28 – 17.17)	0.2442
NCM (%)*	n=14 11.68 (9.23 – 16.45)	n=12 14.76 (9.89 – 22.08)	0.1419
CCR2+CM (%)*	n = 16 70.94 (56.07 – 79.52)	n=13 65.74 (61.79 – 71.25)	0.3941
CCR2+IM (%)*	n=15 3.57 (2.02 – 11.08)	n=13 6.74 (3.28 – 17.17)	0.2442
CCR2+NCM (%)*	n=14 0.34 (0.13 – 0.65)	n=8 0.39 (0.37 – 0.43)	0.5623

Abbreviations: kg (kilogram), m (meter), MUAC (mid-upper arm circumference), BMI (body mass index), CM (classical monocyte), IM (intermediate monocyte), NCM (non-classical monocyte), CCR2 (C-C Motif chemokine receptor 2), HIV (human immunodeficiency virus).
*Data were not available for all the participants.

3.2 DESCRTIPTION OF THE INFANTS

For the study, the main focus was on the infants born to mothers living with or without HIV. A total of 19 HUU infants and 23 HEU infants were included, however not all the infants had all the available data needed for the anthropometric z-score calculations and monocyte phenotype percentages.

According to the World Health Organisation (WHO), the standards of weight, length and HC adapted to z-scores are as follows: WAZ is considered underweight if WAZ is < -2 z-score; LAZ is underdeveloped if LAZ is < -2 z-score; WLZ undersized if WLZ is < -2 z-score; and HCZ is classified as macrocephaly if HCZ is > +2 z-score and microcephaly if HCZ is < -2 z-score[86].

The median gestational age (GA) in weeks at birth for the HUU group was slightly longer: 39 (IQR 38 - 39) and 38 (IQR 38 - 39) for the HEU group; but the difference was not statistically significant. At birth, the weight, height, and head circumference (HC) of the groups were similar (Table 3.2.1). There was also no difference in the z-scores for weight-for-length (WLZ), length-for-age (LAZ), weight-for-age (WAZ), body mass index (BMI) for-age (BAZ), and HC-for-age (HCZ) for the infant groups at birth.

With regards to the monocyte phenotype, statistically significant differences (when setting the level of significance at 10%) were observed in the IM subgroup between the infant groups at birth: the HEU infant group had higher median percentages compared to the HUU infants. The expression of CCR2 on the IM subgroup was also higher in HEU than in HUU infants, but this difference was not statistically significant, as shown in Table 3.2.1. Figure 3.2.1 below shows t-distributed Stochastic Neighbour Embedding (tSNE) dimension reduction dot plots of the monocyte phenotypes for a representative HEU and HUU infant at birth, also showing a higher percentage of the IM phenotype, as demonstrated by increased CD16 expression, in the HEU infant.

Table 3.2.1: Comparison of anthropometric and flow cytometric results of HUU and HEU infants at birth (0 weeks).

Variable	HUU Median (IQR)	HEU Median (IQR)	Probability
GA (weeks)*	n=18 39.00 (38.00 – 40.00)	n=22 38.00 (38.00 – 39.00)	0.4195
Weight (kg)	n=19 2.93 (2.74 – 3.39)	n=23 2.90 (2.69 – 3.19)	0.3510
Length (cm)*	n=19 50.00 (49.00 – 52.00)	n=20 49.00 (48.00 – 51.00)	0.2141
HC (cm)*	n=18 34.00 (34.00 – 35.00)	n=22 34.00 (33.00 – 35.00)	0.4115
BMI*	n=19 11.74 (11.20 – 12.28)	n=20 11.18 (10.60 – 12.66)	0.3087
WLZ*	n=19 -1.79 (-2.00 – (-0.60))	n=23 -1.75 (-3.12 – (-0.45))	0.5626
LAZ*	n=19 0.26 (-0.08 – 1.53)	n=20 -0.08 (-1.00 – 0.79)	0.2364
WAZ*	n=19 -0.80 (-1.18 – 0.32)	n=23 -0.80 (-1.46 – (-0.33))	0.3718
BAZ*	n=19 -1.43 (-1.93 – (-0.93))	n=20 -1.94 (-2.54 – (-0.61))	0.3202
HCZ*	n=18 0.1 (-0.36 – 0.95)	n=22 -0.13 (-1.15 – 0.95)	0.4445
CM (%)*	n=16 87.42 (81.03 – 89.94)	n=12 83.31 (76.17 – 88.52)	0.2617
IM (%)*	n=15 2.09 (0.71 – 4.58)	n=13 3.76 (3.15 – 4.79)	**0.0990**
NCM (%)*	n=13 5.35 (4.44 – 6.62)	n=12 5.95 (3.42 – 8.61)	0.6254
CCR2+CM (%)*	n=15 77.57 (67.17 – 82.95)	n=13 77.04 (67.50 – 81.00)	0.5935
CCR2+IM (%)*	n=14 2.56 (0.90 – 4.58)	n=13 3.76 (3.15 – 4.79)	0.1585

Abbreviations: GA (gestational age), kg (kilogram), cm (centimetre), HC (head circumference), BMI (body mass index), WLZ (weight for length z-score), LAZ (Length for age z-score), WAZ (weight for age z-score), BAZ (BMI for age z-score), HCZ (HC for age z-score), % (percentage), CM (classical monocyte), IM (intermediate phenotype), NCM (non-classical monocyte), CCR2 (C-C Motif chemokine receptor), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected).
*Data were not available for all the participants.

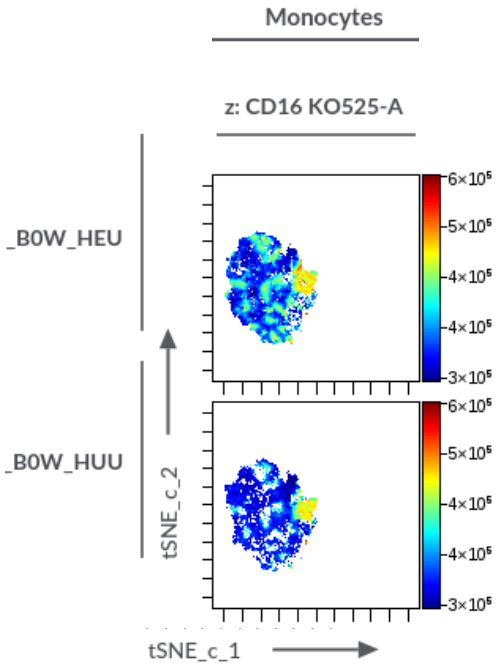

Figure 3.2.1: tSNE dimension reduction diagram for an HEU and HUU infant at birth (0 weeks). Abbreviations: B0W (Infant at birth), CD (cluster of differentiation), HEU (HIV-exposed uninfected), HUU (HIV-unexposed uninfected), tSNE (t-distributed Stochastic Neighbour Embedding.

For the infants' follow-up visit at 10 weeks (Table 3.2.2), significant differences started to emerge in the anthropometric data of the HUU and HEU groups. The HUU infants had significantly higher median values for BMI, WLZ, and BAZ than the HEU infants. In Figure 3.2.2 below, a box-and-whisker plot shows the significant difference of the HUU and HEU infants' BMI at 10 weeks of age. No other measurements, including HC, were significantly different between the groups. None of the differences observed in the monocyte phenotypes at birth were evident at 10 weeks (Table 3.2.2).

Table 3.2.2: Comparison of anthropometric and flow cytometric results of HUU and HEU infants at 10 weeks.

Variable	HUU Median (IQR)	HEU Median (IQR)	Probability
Weight (kg)*	n=14 5.15 (4.92 – 5.79)	n=18 5.21 (5.07 – 5.59)	0.8414
Length (cm)*	n=14 56.20 (54.60 – 58.10)	n=18 56.90 (55.80 – 58.60)	0.2980
HC*	n=14 39.70 (39.00 – 40.20)	n=18 39.85 (38.90 – 40.40)	0.9999
MUAC*	n=14 13.80 (13.20 – 14.40)	n=16 13.75 (12.90 – 14.00)	0.3458
BMI*	n=14 16.29 (16.05 – 17.81)	n=18 15.77 (14.76 – 16.90)	***0.0716**
WLZ*	n= 1.17 (0.27 – 1.55)	n=18 -0.09 (-0.88 – 0.87)	***0.0138**
LAZ*	n=14 -0.85 (-1.25 – 0.11)	n=18 -0.11 (-0.73 – 0.55)	0.1185
WAZ*	n=14 0.08 (-0.56 – 0.88)	n=18 -0.07 (-0.94 – 0.22)	0.6742
BAZ*	n=14 0.64 (-0.06 – 1.21)	n=18 -0.17 (-0.89 – 0.60)	***0.0889**
HCZ*	n=14 1.26 (0.22 – 1.45)	n=18 0.80 (0.29 – 1.63)	0.9840
MUACZ*	n=14 -0.45 (-1.14 – 0.38)	n=16 -0.64 (-0.89 – (-0.03))	0.7000
CM (%)*	n=14 68.30 (63.48 – 75.30)	n=14 72.68 (57.91 – 75.48)	0.8843
IM (%)*	n=14 1.75 (0.61 – 2.16)	n=14 1.05 (0.58 – 1.68)	0.2071
NCM (%)*	n=13 30.07 (21.27 – 34.15)	n=13 25.87 (23.04 – 33.37)	0.8278
CCR2+CM (%)*	n=14 61.51 (56.86 – 69.98)	n=8 70.50 (68.99 – 71.35)	0.2183
CCR2+IM (%)*	n=14 1.75 (0.60 – 2.16)	n=14 1.05 (0.58 – 1.68)	0.2071

Abbreviations: kg (kilogram), cm (centimetre), HC (head circumference), BMI (body mass index), WLZ (weight for length z-score), LAZ (Length for age z-score), WAZ (weight for age z-score), BAZ (BMI for age z-score), HCZ (HC for age z-score), % (percentage), CM (classical monocyte), IM (intermediate phenotype), NCM (non-classical monocyte), CCR2 (C-C Motif chemokine receptor 2), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected).
*Data were not available for all the participants.

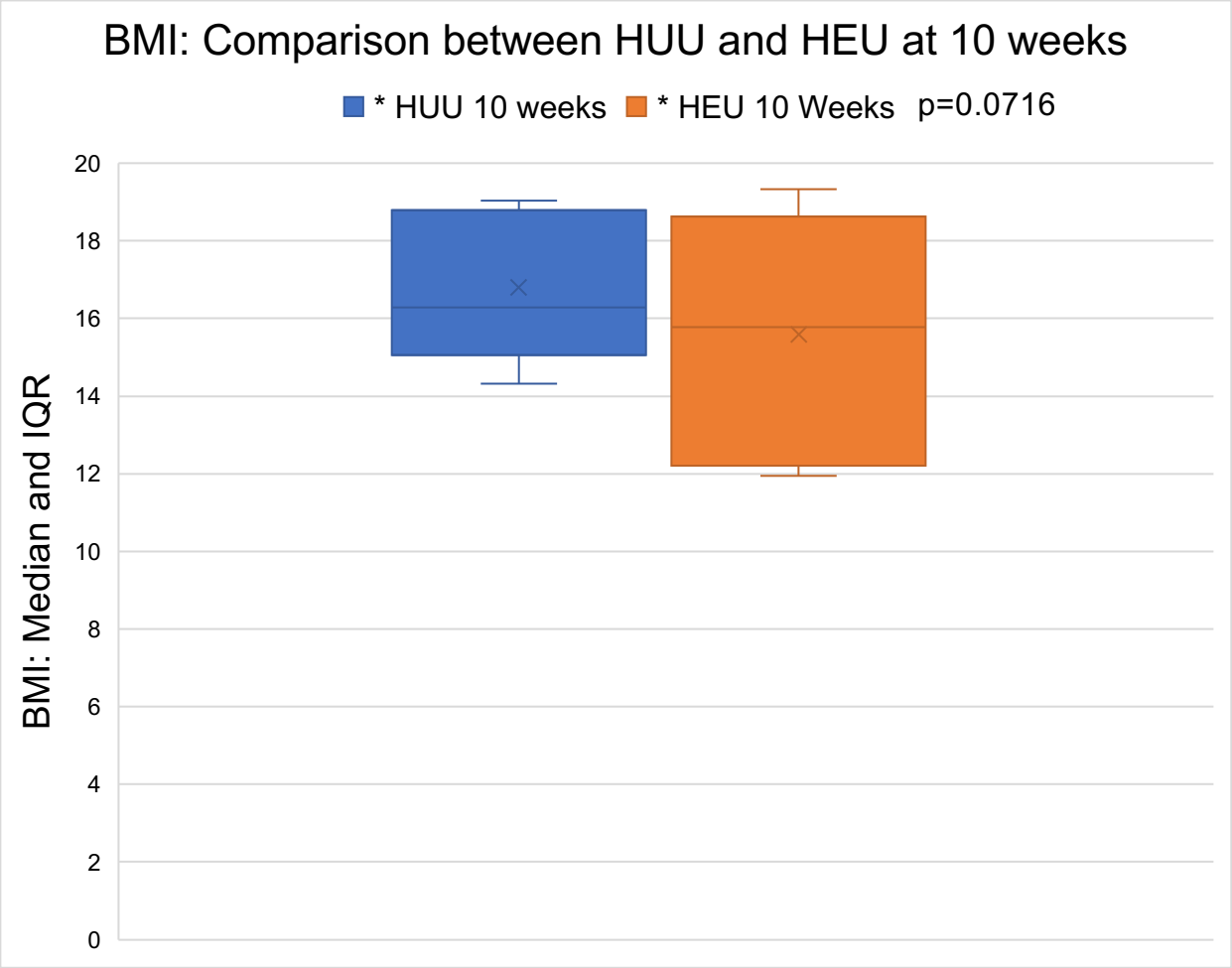

Figure 3.2.2: BMI comparison between HUU and HEU at 10 weeks.
Abbreviations: BMI (body mass index), IQR (inter-quartile range), HEU (HIV-exposed uninfected), HUU (HIV-unexposed uninfected).

Figure 3.2.3, below, shows tSNE dimension reduction dot plots of the monocyte phenotypes for a representative HEU and HUU infant at 10 weeks, showing similar patterns in the IM phenotype between the two groups.

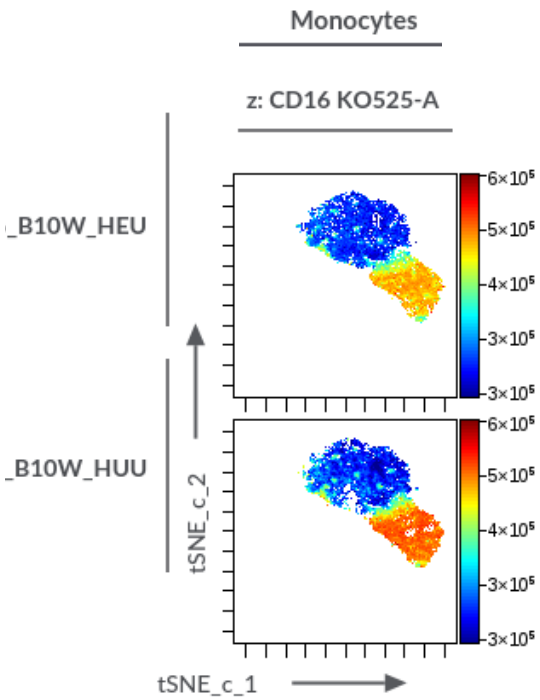

Figure 3.2.3: tSNE dimension reduction diagram for an HEU and HUU infant at 10 weeks. Abbreviations: B10W (Infant at 10 weeks), CD (cluster of differentiation), HEU (HIV-exposed uninfected), HUU (HIV-unexposed uninfected), tSNE (t-distributed Stochastic Neighbour Embedding.

No significant differences were observed between the monocyte subsets at six months, as seen in Table 3.2.3 below. Interestingly, at the infants' six-month follow-up visit, while no significant differences were seen for the anthropometric data (length, HC and MUAC), HUU infants were statistically significantly heavier and had higher WLZs, although this difference was not as pronounced as observed at 10 weeks. Apart from LAZ (which was slightly but not significantly higher), all the other z-scores (WAZ, BAZ, HCZ and, MUAC-for-age z-scores [MUACZ]) were slightly, but not significantly, lower for the HEU infants when compared to their HUU counterparts.

These observations are supported by the box-and-whisker plot (Figure 3.2.4) and tSNE dot plots (Figure 3.2.5) below. For Figure 3.2.4 it demonstrates significant differences between HUU and HEU infants' weight at six months. In this figure, it is clear that the median weight for the HUU infant group is higher than that of the HEU group, albeit at a significance level of 10%.

Table 3.2.3: Comparison of anthropometric and flow cytometric results of HUU and HEU infants at six months.

Variable	HUU Median (IQR)	HEU Median (IQR)	Probability
Weight (kg)*	n=16 7.42 (6.96 – 8.30)	n=21 7.10 (6.43 – 7.92)	***0.0650**
Length (cm)*	n=16 65.20 (63.20 – 69.00)	n=21 65.40 (63.40 – 67.00)	0.6766
HC*	n=16 44.20 (43.00 – 45.00)	n=21 43.90 (42.50 – 44.50)	0.2351
MUAC*	n=16 15.00 (14.00 – 16.00)	n=21 14.30 (14.00 – 15.00)	0.1122
BMI*	n=16 17.08 (16.12 – 18.74)	n=21 16.16 (15.34 – 17.72)	0.1197
WLZ*	n=16 0.00 (-0.73 – 1.23)	n=21 -0.79 (-1.01 – 0.34)	***0.0803**
LAZ*	n=16 -0.93 (-1.44 – 1.52)	n=21 -0.17 (-1.33 – 0.63)	0.8487
WAZ*	n=16 0.33 (-1.02 – 0.78)	n=21 -0.15 (-1.30 – 0.25)	0.1227
BAZ*	n=16 -0.15 (-0.87 – 1.14)	n=21 -0.52 (-1.15 – 0.35)	0.2155
HCZ*	n=16 1.04 (0.64 – 2.14)	n=21 0.54 (-0.22 – 1.66)	0.1090
MUACZ*	n=16 1.01 (0.22 – 1.77)	n=21 0.51 (-0.04 – 1.07)	0.2596
CM (%)*	n=15 63.83 (57.58 – 75.38)	n=15 65.69 (61.99 – 73.91)	0.4070
IM (%)*	n=13 1.85 (0.86 – 3.57)	n=14 1.54 (1.13 – 1.85)	0.5037
NCM (%)*	n=15 30.95 (19.03 – 36.95)	n=16 27.81 (21.91 – 34.35)	0.5606
CCR2+CM (%)*	n=14 60.04 (53.34 – 66.02)	n=14 60.47 (57.75 – 64.63)	0.9227
CCR2+IM (%)*	n=13 1.85 (0.86 – 3.57)	n=14 1.54 (1.13 – 1.85)	0.5037
CCR2+NCM (%)*	n=14 0.95 (0.86 – 1.34)	n=16 0.92 (0.36 – 1.76)	0.7925

Abbreviations: kg (kilogram), cm (centimetre), HC (head circumference), MUAC (mid-upper arm circumference), BMI (body mass index), WLZ (weight for length z-score), LAZ (Length for age z-score), WAZ (weight for age z-score), BAZ (BMI for age z-score), HCZ (HC for age z-score), % (percentage), CM (classical monocyte), IM (intermediate phenotype), NCM (non-classical monocyte), CCR2 (C-C Motif chemokine receptor), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected).

*Data were not available for all the participants

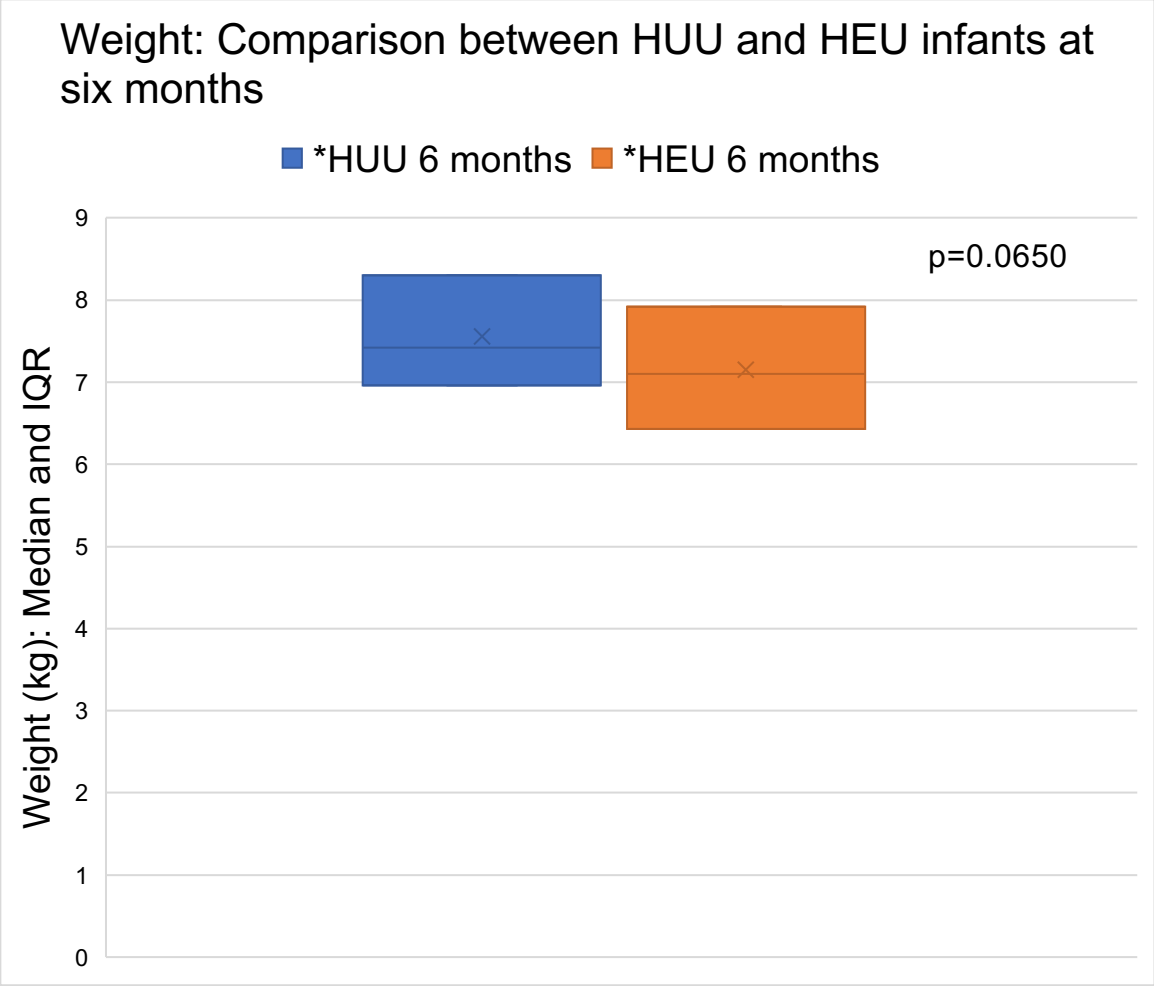

Figure 3.2.4: Weight comparison between HUU and HEU at six months.
Abbreviations: IQR (inter-quartile range), HEU (HIV-exposed uninfected), HUU (HIV-unexposed uninfected), kg (kilogram).

For Figure 3.2.5 a tSNE dimension reduction dot plot of the monocyte phenotypes for a representative HEU and HUU infant at six months, showing similar patterns in the IM phenotype between the two groups, similar to that at 10 weeks.

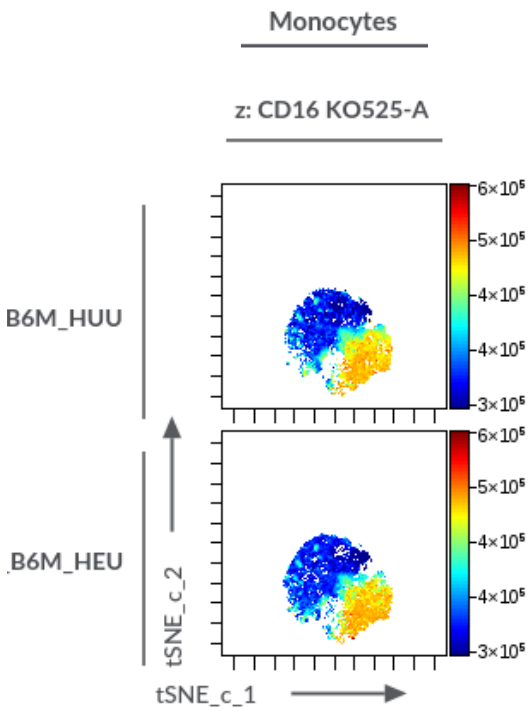

Figure 3.2.5: tSNE dimension reduction diagram for an HEU and HUU infant at six months. Abbreviations: B6M (Infant at six months), CD (cluster of differentiation), HEU (HIV-exposed uninfected), HUU (HIV-unexposed uninfected), tSNE (t-distributed Stochastic Neighbour Embedding.

Lastly, the 12-month follow-up visit's anthropometric data, as seen in Table 3.2.4 below, showed that HUU infants were slightly, but not significantly, shorter than HEU infants. Even though the HUU infants were slightly shorter, they were slightly heavier than their HEU counterparts. This is reflected in the significantly higher BMI, BAZ and WLZ, and lower LAZ observed in the HUU group. An interesting pattern to note for the z-score values is that for WLZ, WAZ, and BAZ, HEU infants' median values were in the negative range at all timepoints. While the z-scores were not lower than -2, which would have signified that these infants were underweight according to the WHO standards, HEU had persistently lower values than the HUU infants.

No data were available for the infant groups' monocyte subsets at the 12-month timepoint.

Table 3.2.4: Comparison of anthropometric results of HUU and HEU infants 12 months.

Variable	HUU Median (IQR)	HEU Median (IQR)	Probability
Weight (kg)*	n=14 9.36 (8.49 – 10.52)	n=18 8.91 (8.54 – 9.97)	0.6889
Length (cm)*	n=14 74.20 (71.50 – 75.60)	n=18 75.50 (73.8 – 77.40)	0.1233
HC*	n=14 46.80 (46.00 – 48.00)	n=18 47.35 (46.00 – 48.20)	0.9681
MUAC*	n=14 16.40 (14.90 – 17.00)	n=18 15.00 (14.60 – 16.00)	0.3267
BMI*	n=14 17.30 (16.19 – 18.74)	n=18 15.99 (14.64 – 17.06)	***0.0547**
WLZ*	n=14 0.10 (-0.47 – 1.32)	n=18 -0.52 (-1.16 – 0.30)	***0.0927**
LAZ*	n=14 0.06 (-0.80 – 0.30)	n=18 0.40 (-0.46 – 0.85)	***0.0901**
WAZ*	n=14 -0.16 (-0.93 – 1.11)	n=18 -0.42 (-0.93 – 0.50)	0.7696
BAZ*	n=14 0.36 (-0.49 – 1.36)	n=18 -0.39 (-1.34 – 0.07)	***0.0753**
HCZ*	n=14 0.87 (0.63 – 1.56)	n=18 0.86 (0.18 – 2.10)	0.8342
MUACZ*	n=14 1.71 (0.27 – 2.15)	n=18 0.33 (-0.03 – 1.18)	0.3572

Abbreviations: kg (kilogram), cm (centimetre), HC (head circumference), MUAC (mid-upper arm circumference), BMI (body mass index), WLZ (weight for length z-score), LAZ (Length for age z-score), WAZ (weight for age z-score), BAZ (BMI for age z-score), HCZ (HC for age z-score), MUACZ (MUAC for age z-score), % (percentage), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected).
*Data were not available for all the participants.

Figure 3.2.6 below depicts the growth, indicated by BMI, of the two infant groups over the 12-month period. While the HUU group had persistently higher BMIs at all time points, the difference was only statistically different at 10 weeks and 12 months.

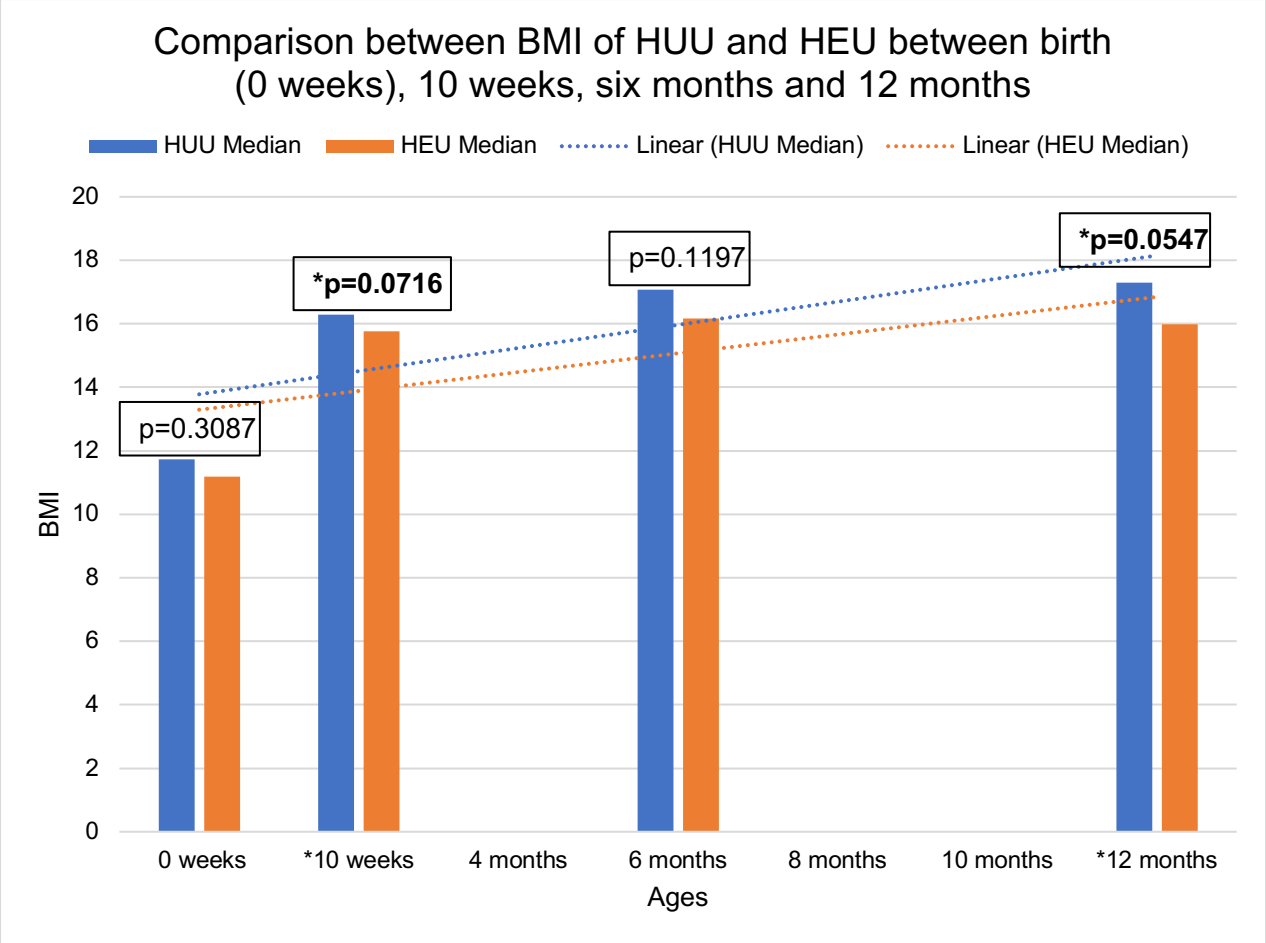

Figure 3.2.6: Comparison between infants' (HUU and HEU) BMI at different ages.
Abbreviations: BMI (body mass index), HEU (HIV-unexposed uninfected), HUU (HIV-unexposed uninfected).

In Figure 3.2.7 below, a comparison of the two infant groups' weight over the 12-month observation period is shown. Again, the HUU infants were slightly heavier at birth and, even though the HEU infant group had overtaken them at 10 weeks, HUU infants surpassed them again at six and 12 months, with the difference at six months being statistically significant.

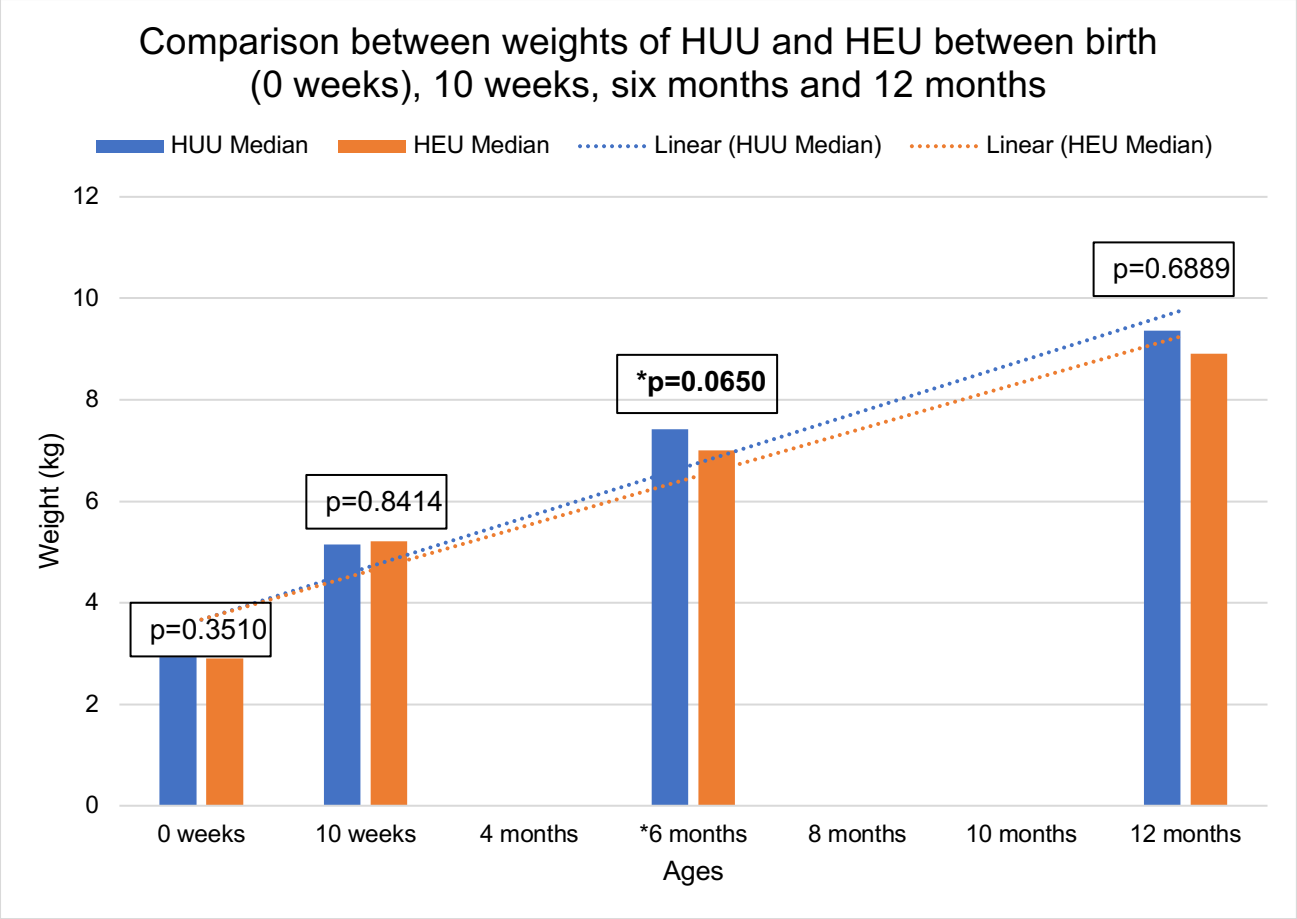

Figure 3.2.7: Comparison between infants' (HUU and HEU) weight at different ages.
Abbreviations: HEU (HIV-exposed uninfected), HUU (HIV-unexposed uninfected), kg (kilogram).

3.3 COMPARISON OF CHANGES IN THE ANTHROPOMETRIC MEASUREMENTS FOR HEU AND HUU INFANTS

In next set of results, a comparison was made between the two groups with regards to the differences over time in specific anthropometric data. Although no significant differences were seen in the change in weight, length, or HC between the infant groups between 0 weeks and 10 weeks (as seen in Table 3.3.1), nor any significant differences in the change in weight, length, HC, and MUAC between 10 weeks and six months (Table 3.3.2), significant differences emerged in the change in weight, length, HC, and MUAC between six months and 12 months (Table 3.3.3). The HEU group had a larger increase in the change in median length as well as HC. These significant differences show that the HEU infant group had a larger growth increase between six months of age and 12 months of age. No significant differences were observed in any of the anthropometric data between 10 weeks and 12 months *(Appendix 7)*.

Table 3.3.1: Comparison of anthropometric results of HUU and HEU infants between birth and 10 weeks of age.

Variable	HUU Median (IQR)	HEU Median (IQR)	Probability
Weight diff (kg)	2.30 (2.03 – 2.54)	2.36 (1.95 – 2.70)	0.9093
Length diff (cm)	7.10 (4.90 – 7.80)	6.70 (6.00 – 9.80)	0.2750
HC diff (cm)	5.00 (4.5 – 7.00)	6.00 (5.00 – 6.80)	0.6597

Abbreviations: kg (kilogram), diff (difference), cm (centimetre), HC (head circumference), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected).

Table 3.3.2: Comparison of anthropometric results of HUU and HEU infants between 10 weeks and six months of age.

Variable	HUU Median (IQR)	HEU Median (IQR)	Probability
Weight diff (kg)	2.13 (1.85 – 2.63)	1.85 (1.53 – 2.45)	0.2585
Length diff (cm)	10.20 (8.30 – 10.90)	7.70 (6.00 – 10.40)	0.1547
HC diff (cm)	4.40 (3.50 – 5.40)	4.20 (3.80 – 4.30)	0.2954
MUAC diff (cm)	1.20 (0.80 – 1.80)	1.00 (0.30 – 2.10)	0.5349

Abbreviations: kg (kilogram), diff (difference), cm (centimetre), HC (head circumference), MUAC (mid-upper arm circumference), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected).

Table 3.3.3: Comparison of anthropometric results of HUU and HEU infants between six months and 12 months of age.

Variable	HUU Median (IQR)	HEU Median (IQR)	Probability
Weight diff (kg)	1.73 (1.28 – 2.20)	1.92 (1.58 – 2.13)	0.4118
Length diff (cm)	6.00 (5.30 – 8.40)	9.60 (8.40 – 11.00)	***0.0146**
HC diff (cm)	3.00 (2.40 – 3.20)	3.50 (2.80 – 3.80)	***0.0432**
MUAC diff (cm)	0.60 (0.00 – 1.30)	0.80 (-0.40 – 2.30)	0.9681

Abbreviations: kg (kilogram), diff (difference), cm (centimetre), HC (head circumference), MUAC (mid-upper arm circumference), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected).

Figure 3.3.1 below demonstrates the difference in HC between HUU and HEU infants between six months and 12 months. It is observed that the HEU group had a greater increase in HC between six months and 12 months.

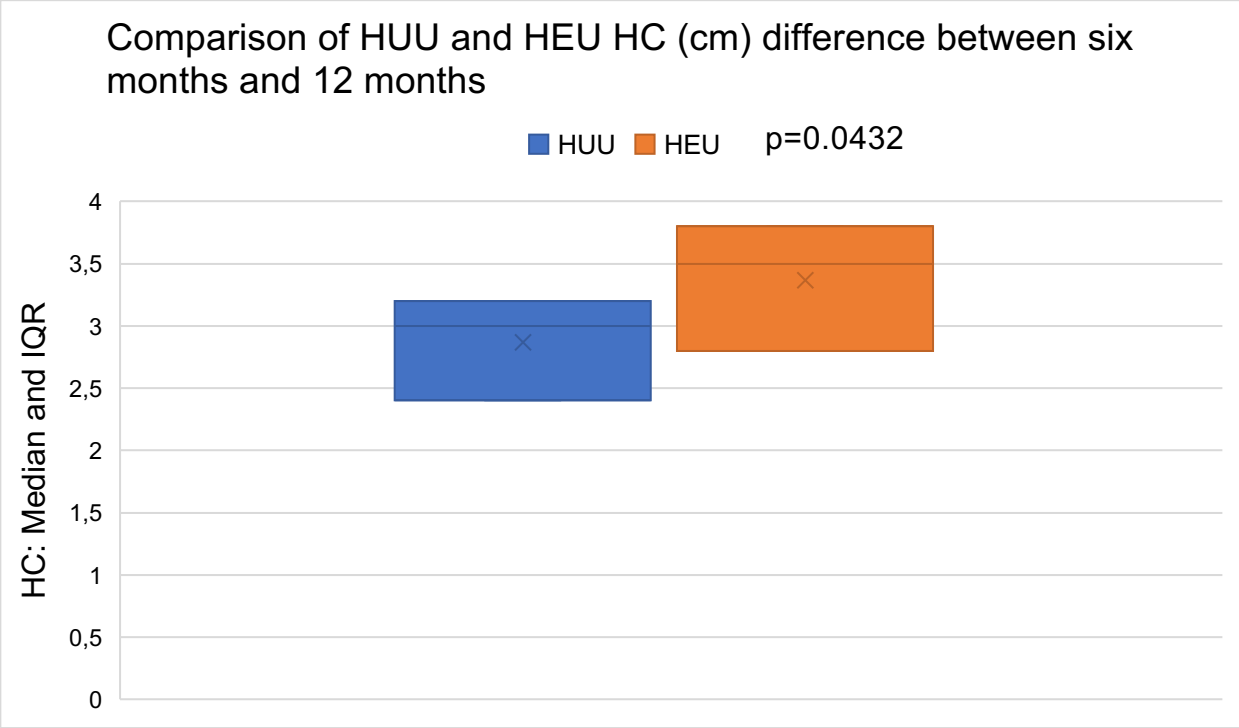

Figure 3.3.1: HC difference at six months and 12 months between HUU and HEU infants.
Abbreviations: cm (centimetre), HC (head circumference), HEU (HIV-exposed uninfected), HUU (HIV-unexposed uninfected), IQR (inter-quartile range).

3.4 COMPARISON OF CHANGES IN MONOCYTE PHENOTYPE PERCENTAGES FOR HEU AND HUU INFANTS

In Tables 3.4.1 and 3.4.2, results were generated using the Wilcoxon-signed rank test to compare the changes in the monocyte phenotype percentages within the HUU and HEU groups between birth and 10 weeks and then again between 10 weeks and six months.

In Table 3.4.1, it is noticeable that the median for the CM for the HUU as well as for the HEU infants decreased significantly from birth to the 10-week timepoint. For the IM phenotype, there was only significant differences for the HEU infants from birth up until 10 weeks, in which time the percentage decreased. Interestingly, for the NCM, the percentages drastically increased in both infant groups from birth

to 10 weeks. There was a significant decrease in the CCR2+CM in the HUU infants and the CCR2+IM in the HEU infants from birth to 10 weeks.

Table 3.4.1: Comparison of phenotype percentages between birth and 10 weeks for infants HUU and HEU.

Variable (%)	HIV status	Median (IQR) 0 weeks	Median (IQR) 10 weeks	Probability (p ≤ 0.1)
CM	HUU	87.42 (81.03 – 89.94)	68.30 (63.48 – 75.30)	***0.0005**
	HEU	83.31 (76.17 – 88.52)	72.68 (57.91 – 75.48)	***0.0390**
IM	HUU	2.09 (0.71 – 4.58)	1.75 (0.61 – 2.16)	0.2402
	HEU	3.76 (3.15 – 4.79)	1.05 (0.58 – 1.68)	***0.0039**
NCM	HUU	5.35 (4.44 – 6.62)	30.07 (21.27 – 34.15)	***0.0020**
	HEU	5.95 (3.42 – 8.61)	25.87 (23.04 – 33.37)	***0.0039**
CCR2+CM	HUU	77.57 (67.17 – 82.95)	61.51 (56.86 – 69.98)	***0.0068**
	HEU	77.04 (67.50 – 81.00)	70.50 (68.99 – 71.35)	0.5625
CCR2+IM	HUU	2.56 (0.90 – 4.58)	1.75 (0.60 – 2.16)	0.2402
	HEU	3.76 (3.15 – 4.79)	1.05 (0.58 – 1.68)	***0.0039**

Abbreviations: % (percentage), IQR (inter-quartile range), CM (classical monocyte), IM (intermediate phenotype), NCM (non-classical monocyte), CCR2 (C-C Motif chemokine receptor 2), HIV (human immunodeficiency virus), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected).

In Table 3.4.2, the changes from 10 weeks to six months for the monocyte phenotypes are shown. The only significant differences between the two timepoints are that the CM and CCR2+CM further decreased in the HEU infants.

Table 3.4.2: Comparison of phenotype percentages between 10 weeks and six months for infants.

Variable (%)	HIV status	Median (IQR) 10 weeks	Median (IQR) 6 months	Probability (p ≤ 0.1)
CM	HUU	68.30 (63.48 – 75.30)	63.83 (57.58 – 75.38)	0.1294
	HEU	72.68 (57.91 – 75.48)	65.69 (61.99 – 73.91)	***0.0273**
IM	HUU	1.75 (0.61 – 2.16)	1.85 (0.86 – 3.57)	0.5566
	HEU	1.05 (0.58 – 1.68)	1.54 (1.13 – 1.85)	0.3223
NCM	HUU	30.07 (21.27 – 34.15)	30.95 (19.03 – 36.95)	0.7646
	HEU	25.87 (23.04 – 33.37)	27.81 (21.91 – 34.35)	0.5566
CCR2+CM	HUU	61.51 (56.86 – 69.98)	60.04 (53.34 – 66.02)	0.7646
	HEU	70.50 (68.99 – 71.35)	60.47 (57.75 – 64.63)	***0.0156**
CCR2+IM	HUU	1.75 (0.60 – 2.16)	1.85 (0.86 – 3.57)	0.5566
	HEU	1.05 (0.58 – 1.68)	1.54 (1.13 – 1.85)	0.3223

Abbreviations: % (percentage), IQR (inter-quartile range), CM (classical monocyte), IM (intermediate phenotype), NCM (non-classical monocyte), CCR2 (C-C Motif chemokine receptor 2), HIV (human immunodeficiency virus), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected).

3.5 STATISTICAL ANALYSIS OF MONOCYTE PHENOTYPE PERCENTAGES AND ANTHROPOMETRIC Z-SCORES

The next results are the Kruskal-Wallis H test for the association of monocyte phenotype percentages at different ages with the anthropometric z-scores for both infant groups combined. The results of the z-score cut-off at -2 are only shown in the appendices since so few infants had values below this value.

Firstly in Table 3.5.1, the association between the monocyte phenotype percentages at birth and 10 weeks, and HCZ was tested. HCZ was categorised into a binary variable according to z-score values less than -1 and greater or equal to -1. At birth, 9 infants were in the lower category (i.e., HCZ <-1) with three of these infants belonging to the HEU group. No significant associations were seen at birth or at 10 weeks.

Table 3.5.1: Association between infant monocyte phenotype percentages and head circumference (HC) for age z-scores (HCZ) at different ages.

Variable (%)	HCZ <-1 Median (IQR)	HCZ ≥-1 Median (IQR)	Probability
CM_0*	n=9 89.93 (89.93 – 89.93)	n=30 85.41 (78.81 – 89.16)	0.3317
IM_0*	n=9 4.29 (4.29 – 4.29)	n=30 3.21 (1.51 – 5.67)	0.4881
NCM_0*	n=9 4.20 (4.20 – 4.20)	n=30 5.24 (3.73 – 7.13)	0.5465
CCR2+CM_0*	n=9 85.78 (85.78 – 85.78)	n=30 77.00 (67.13 – 81.79)	0.1272
CCR2+IM_0*	n=9 4.30 (4.30 – 4.30)	n=30 3.28 (1.54 – 4.75)	0.5156
CM_10*	n=18 81.03 (81.03 – 81.03)	n=12 84.23 (78.35 – 88.79)	0.2571
IM_10*	n=18 1.75 (1.75 – 1.75)	n=22 1.22 (0.60 – 2.08)	0.2821

Abbreviations: _0 (birth), _10 (10 weeks), % (percentage), CM (classical monocyte), CCR2 (C-C Motif chemokine receptor 2), HC (head circumference), HCZ (HC-for-age z-score), IM (intermediate monocyte), NCM (non-classical monocyte).
*Data were not available for all the participants.

Next, the association between the monocyte phenotype percentages at birth, 10 weeks and six months, and BAZ was tested. BAZ was categorised into a binary variable according to z-score values less than -1 and greater or equal to -1. At birth, 24 infants were in the lower category (i.e., BAZ <-1) with 8/24 being in the HEU group. This number decreased to 5 infants (in the HEU group) at 10 weeks, and 7 infants in the HEU group at six months.

As seen in Table 3.5.2, significant associations could be found for the monocyte subsets at birth, where the group with higher BAZ (i.e. BAZ ≥-1) had higher median CCR2+CM proportions. No significant associations could be found for the monocyte subsets at 10 weeks or six months.

Table 3.5.2: Association between infant monocyte phenotype percentages and BMI for age z-scores (BAZ) at different ages.

Variable (%)	BAZ <-1 Median (IQR)	BAZ ≥-1 Median (IQR)	Probability
CM_0*	n=24 83.39 (78.35 – 89.94)	n=14 86.63 (81.99 – 89.52)	0.3771
IM_0*	n=24 3.76 (1.54 – 4.90)	n=14 2.84 (1.48 – 4.58)	0.8486
NCM_0*	n=24 4.00 (2.71 – 7.04)	n=13 5.34 (4.77 – 6.98)	0.4008
CCR2+CM_0*	n=24 71.26 (64.73 – 81.76)	n=15 79.34 (67.17 – 82.23)	***0.0748**
CCR2+IM_0*	n=24 3.80 (3.28 – 4.90)	n=14 2.84 (1.48 – 4.58)	0.6892
CM_10*	n=5 83.22 (83.22 – 83.22)	n=26 84.23 (78.35 – 88.79)	0.8738
IM_10*	n=5 1.65 (1.65 – 1.65)	n=26 1.22 (0.60 – 2.08)	0.5355
CM_6*	n=7 65.69 (65.69 – 65.69)	n=26 63.99 (59.78 – 75.38)	0.8028
IM_6*	n=7 1.23 (1.23 – 1.23)	n=26 1.64 (0.83 – 2.89)	0.3743
NCM_6*	n=7 28.28 (28.28 – 28.28)	n=26 30.27 (19.03 – 36.11)	0.7369
CCR2+CM_6*	n=7 54.14 (54.14 – 54.14)	n=26 59.81 (55.53 – 64.74)	0.5246
CCR2+IM_6*	n=7 1.23 (1.23 – 1.23)	n=26 1.64 (0.83 – 2.89)	0.3743
CCR2+NCM_6*	n=7 0.41 (0.41 – 0.41)	n=26 0.95 (0.52 – 1.41)	0.3507

Abbreviations: _0 (birth), _10 (10 weeks), _6 (six months), % (percentage), BMI (body mass index), BAZ (BMI-for-age z-score, CM (classical monocyte), CCR2 (C-C Motif chemokine receptor 2), IM (intermediate phenotype), NCM (non-classical monocyte).
*Data were not available for all the participants.

The last association tested was for the infant groups' monocyte phenotype percentages at birth, 10 weeks and six months, with WAZ. WAZ was also categorised into a binary variable according to z-score values less than -1 and greater or equal to 1. At birth, 17 infants were in the lower category (i.e., WAZ <-1) with 10/17 being in the HEU group, but this number decreased to 4/6 infants (all in the HEU group) at 10 weeks, and 7/11 at six months.

As seen in Table 3.5.3, significant associations could be found for the IM, CCR2+CM, and CCR2+IM monocyte subsets at birth. For the IM and CCR2+IM the proportions were higher for the group with lower WAZ, and for CCR2+CM the

proportions were higher for the group with higher WAZ. No other significant associations could be found for 10 weeks and six months.

Table 3.5.3: Association between infant monocyte phenotype percentages and weight for age z-scores (WAZ) at different ages.

Variable (%)	WAZ <-1 Median (IQR)	WAZ ≥-1 Median (IQR)	Probability
CM_0*	n=17 83.22 (81.03 – 89.94)	n=24 85.40 (78.81 – 89.16)	0.2617
IM_0*	n=17 4.30 (3.65 – 4.91)	n=24 3.14 (1.51 – 4.67)	***0.0877**
NCM_0*	n=17 4.20 (3.79 – 7.04)	n=24 5.41 (3.73 – 8.31)	0.1005
CCR2+CM_0*	n=17 70.37 (68.04 – 85.78)	n=24 77.61 (67.13 – 81.79)	***0.0510**
CCR2+IM_0*	n=17 4.30 (3.65 – 4.90)	n=24 3.15 (1.54 – 4.75)	***0.0274**
CM_10*	n=6 88.19 (84.81 – 89.94)	n=25 83.73 (78.39 – 88.52)	0.8958
CCR2+IM_10*	n=6 1.65 (1.65 – 1.65)	n=25 1.22 (0.60 – 2.08)	0.5485
CM_6*	n=11 70.22 (66.53 – 73.91)	n=23 64.15 (59.78 – 75.38)	0.5485
IM_6*	n=11 1.56 (1.34 – 1.77)	n=23 1.58 (0.87 – 2.73)	0.3583
NCM_6*	n=11 24.67 (21.99 – 27.34)	n=23 29.61 (20.29 – 35.69)	0.2701

Abbreviations: _0 (birth), _10 (10 weeks), _6 (six months), % (percentage), CCR2 (C-C Motif chemokine receptor 2), CM (classical monocyte), IM (intermediate phenotype), NCM (non-classical monocyte).
*Data were not available for all the participants.

Additional tables *(Appendix 8)* show the associations between monocyte phenotype percentages at different timepoints (birth, 10 weeks, and six months) and HCZ, BAZ, WAZ, and LAZ, all categorized into a binary variable according to z-score values less than -2 and greater or equal to -2.

Appendix 9 shows the associations between mothers' monocyte phenotype percentages at their infants' birth with BAZ and WAZ, with neither having any significant associations.

3.6 CORRELATIONS OF GA WITH MONOCYTE PHENOTYPE PERCENTAGES

In Table 3.6.1, correlations between GA and monocyte phenotype percentages were evaluated by means of a Spearman correlation test. No significant correlations were seen for any of the monocyte phenotypes and GA. As expected, however, negative correlations were seen between CM and the other monocyte subsets – CM and IM (rho=-0.5910; p=0.0061), CM and NCM (rho=-0.5368; p=0.0148) – and positive correlations between specific monocyte subsets and their activated phenotype – CM and CCR2CM (rho=0.7594; p=0.0001), IM and CCR2IM (rho=1.0000; p= <0.0001) – while IM and its activated phenotype were both negatively correlated with CM and CCR2+CM: IM and CCR2+CM (rho=-0.5053; p=0.0231), CCR2+CM and CCR2IM (rho=-0.5053; p=0.0231), CCR2+IM and CM (rho=-0.5910; p=0.0061). Finally, NCM was also negatively correlated with the activated phenotype of CM (rho=-0.5368; p=0.0148). In Table 3.6.1, red designates correlations where the p-values were significant, orange designates where the correlations were close to significant p-values, green designates correlations that had p-values lower than one but not significant, and blue shows no correlation .

Table 3.6.1: Correlation between monocyte phenotype percentages and gestational age (GA) in infants.

	GA	CM	IM	NCM	CCR2+CM	CCR2+IM
GA	1.0000 20					
CM	-0.0046 20 0.9846	1.0000 20				
IM	0.0331 20 0.8898	-0.5910 20 ***0.0061**	1.0000 20			
NCM	0.4280 20 0.0598	-0.5368 20 ***0.0148**	0.2421 20 0.3038	1.0000 20		
CCR2+CM	0.0870 20 0.7154	0.7594 20 ***0.0001**	-0.5053 20 ***0.0231**	-0.2346 20 0.3195	1.0000 20	
CCR2+IM	0.0331 20 0.8898	-0.5910 20 ***0.0061**	1.0000 20 ***<0.0001**	0.2421 20 ***0.0231**	-0.5053 20 ***0.0231**	1.0000 20

Abbreviations: GA (gestational age), CM (classical monocyte), IM (intermediate phenotype), NCM (non-classical monocyte), CCR2 (C-C Motif chemokine receptor 2).
Description: First row designates the rho-values, second row designates the number of infants (n), and third row designates the p-value

3.7 CORRELATIONS BETWEEN HC AND MONOCYTE PHENOTYPE PERCENTAGES

Correlations between HC and monocyte phenotype percentages were evaluated by the same correlation test as in Table 3.6.1 (see Appendix 10). No significant correlations were seen for any of the monocyte phenotypes and HC.

CHAPTER 4

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 DISCUSSION

It has long been known that human immunodeficiency virus (HIV) impacts on the neurological function of people living with the disease. As a paradigmatic case, HIV-associated dementia (HAD) is a severe case of HIV-associated neurocognitive disorders (HAND) [88]. Literature suggests that HAND influences around 50% of adults who are living with HIV[88]. A retrospective cross-sectional study of 48 adults living with HIV, conducted by McGuire *et al* (2015), assessed central and peripheral markers of neurodegeneration and monocyte activation in HAND. Neurofilament subunits (NFL) have been demonstrated to be responsive markers of neuronal damage in numerous neurodegenerative diseases such as HAD[89]. The researchers quantified NFL and monocyte activation markers (cluster of differentiation [CD]14 and CD163) in cerebrospinal fluid (CSF) and plasma samples[89]. Patients living with HIV and HAD expressed higher CSF NFL levels than patients with HAD but who were HIV-negative. They found a significant positive correlation between CSF NFL and CD14, suggesting that monocytes were activated in the CNS and correlated with neuronal injuries at different stages of HAND, such as HAD[89].

A study conducted in Mozambique in 2019 by Chaúque *et al.* looked at associations between cognitive defects and HIV-associated encephalopathy (HIVE) in infants living with HIV[90]. The study assessed 27 infants with confirmed HIV infection aged <12 months, 7 patients (26%) of whom were classified as HIVE+ had delay in at least one parameter[90]. HIVE in children is defined by the World Health Organisation (WHO) when one of the following clinical parameters progress over two months in the absence of another disease: a) loss of, or failure to reach developmental milestones: b) symmetrical motor deficit supplemented by two or more of the following: ataxia, paresis, gait disturbances, or pathological reflexes; or c) impaired brain growth as indicated by small/stagnated head circumference (HC) [90-91]. Infants living with HIV and with HIVE thus have suboptimal neurological development and need holistic care that includes occupational and physical therapy, as well as antiretroviral therapy (ART) [90].

It has also been hypothesised that exposure to HIV antigens *in utero* could possibly also cause neurodevelopmental delay in children who remain uninfected

through processes such as alterations in microstructural integrity of the brain's white matter[92-93]. The literature is, however, conflicting on this issue.

For instance, a study conducted by Rice *et al.* (2013) investigated the late language emergence (LLE) in HIV-exposed uninfected (HEU) toddlers in the United States of America[94]. They defined LLE for one-year-olds with a caregiver-report score ≤10th percentile in any of the four domains of the MacArthur-Bates Communicative Development Inventory and for two-year-olds a standard deviation of ≥1 below age-specific norms for the Ages and Stages Questionnaire[94]. In their study, they assessed 792 one- and two-year olds and conducted 1 129 language assessments, and identified that 26% of the HEU one-year-olds and 23% of the HEU two-year-olds had LLE[94].

A study by Strehlau *et al.* (2020), however, looked at 70 HEU new-born infants from Johannesburg in South Africa, of whom 49 were assessed using Bayley Scales of Infant and Toddler Development-3rd Edition (BSID-III) (a formal developmental assessment tool for the diagnosis of developmental delays in early childhood[95]) [93]. In their study, none of the HEU infants were categorized as having any developmental or neurodevelopmental impediments; in fact, their results showed that HEU infants at 12 months of age had higher motor composite, language and cognitive scores than described for the norm-referenced population[93].

In a meta-analysis, McHenry *et al.* (2018) from the Department of Pediatrics at Indiana University in the United States, looked at neurodevelopment of HEU and HIV-uninfected unexposed (HUU) children and concluded that children living with HIV and HEU children had inferior neurodevelopment compared to HUU children. Importantly, they also stated that the results should be supported by more comprehensive research since, when assessing neurodevelopment in children, a vast number of factors should be taken into consideration such as: maternal mental health, nutritional status, socioeconomic status, prematurity, and maternal education[96].

Infection with HIV is also known to impede childhood growth. In a study conducted by Omoni *et al.* (2017), assessed 14 110 infants part of the Zimbabwean Vitamin A for mothers and babies trial[97]. In the study, anthropometric measurements were

taken at birth through to 12-24 months of age and compared between 5 groups namely: infants who had been infected with HIV *in* utero (n=382), infected with HIV postnatally (n=262), or infected with HIV intrapartum (n=505); 3120 HEU infants; and 9210 HUU infants (as the control group) [97]. Infants living with HIV, postnatally, and intrapartum infants had significantly lower length-for-age z-scores (LAZ) and weight-for-age z-scores (WAZ) throughout the first 24 months of age compared to the HEU and HUU infants[97]. Their results concluded that infants living with HIV had elevated rates of growth failure during the first 24 months of age, and that HEU infants had inferior growth rates compared to their HUU counterparts[97].

In contrast, a South African study conducted in 2014 by Ramokolo *et al.* reported that living with HIV and not just the exposure to HIV, affected weight and length[98]. They assessed 65 infants living with HIV, 502 HEU, and 216 HUU infants between the ages of three weeks and 24 weeks. They looked at the infants' weight-velocity z-scores and length-velocity z-scores, and found lower z-scores for infants living with HIV than infants who were HEU and HUU[98]. They also found no differences for the mean LAZ, WAZ, and weight-for-length z-score (WLZ) between the HEU and HUU infants at any of the timepoints (three weeks and 24 weeks) [98].

Nutrition also plays an important role in any infant's growth, a study by Arpadi *et al.* (2000) from the Department of Pediatrics and HIV Centre at the Columbia University, assessed the relationship of HIV replication, growth of children, and energy balance of children with HIV-associated growth failure[99]. They assessed 16 children living with HIV (mean age of 8.3 years) having growth failure (defined as a 12 month height velocity ≤ 5th percentile for age), and 26 children living with HIV (mean age of 6.5 years) without any traces of growth failure[99]. The childrens' energy intake was assessed by measuring a repeated 24 hour dietary recall, total energy expenditure by the doubly labelled water method, and resting energy expenditure by indirect calorimetry[99]. Their results showed that the children living with HIV and having growth failure had a mean daily energy shortage of 674 kilojoules per day, whereas their counterparts had a mean daily energy excess of 1448 kilojoules per day[99]. Their findings propose that HIV-associated growth failure might be a result of chronic low-grade undernutrition, but that undernutrition is not the only cause of growth irregularities in children living with HIV[99].

In addition to suboptimal nutrition, HEU infants potentially encounter multiple threats that might impede their growth and development, such as HIV and antiretroviral (ARV) drug exposure[89,100], economic disadvantages (for example impoverishment) [100], social drawbacks (for example humiliation) [101], and environmental risks (for example hazardous drinking water and poor hygiene) [102].

In a study conducted in Latin America and the Caribbean by Spaulding *et al.* from 2002 to 2009, the neurological differences for HEU infants were examined with regards to their exposure to ARV drugs during pregnancies[103]. In their study, 1400 HEU infants were evaluated, with no HUU control infants. Neurologic conditions (NCs) were assessed by looking at the association of covariates using bivariable and logistic regression analysis. In total 105 infants were reported to have microcephaly (described as having a HC z-score less than -2) and 33 had a specific neurological diagnosis (hypertonia, hypotonia, hypoxia, and neonatal seizures) [103]. No specific ARV (ARVs used: lamivudine, lopinavir/ritonavir, zidovudine, and nelfinavir) was associated with the risk of microcephaly or NCs[103-104], but HEU infants exposed to combination ART (cART) had an increased risk of microcephaly[103]. In addition, male HEU infants, infants with lower birth weights, infant infections, maternal infections and lower Appearance, Pulse, Grimace, Activity and Respiration (APGAR) scores were associated with a higher likelihood of NCs[103].

This current study set out to investigate whether there was an association between HIV exposure, infant growth as measure by anthropometric data, including HC, and patterns of monocyte polarisation in infants born at Kalafong Provincial Tertiary Hospital (KH) in Pretoria, South Africa. The study assessed 19 infants who were HUU and 23 infants who were HEU. To minimise the risk of confounding by these variables, in the current study, HEU and HUU infants were enrolled from the same geographic location and socio-economic environment[105].

Maternal and infant monocytes were examined using a flow cytometric procedure to compare the monocyte phenotypes at the different timepoints (birth, 10 weeks, six months, and 12 months). Statistical analysis was used to determine any associations between the infant groups' monocyte phenotypes and HC as well as any associations and correlations between other anthropometric data and

monocyte phenotypes. Four objectives were explored in this study and will be discussed in turn.

4.1.1 THE FIRST OBJECTIVE: TO INVESTIGATE WHETHER THERE IS A DIFFERENCE IN HC AT BIRTH, 10 WEEKS, SIX MONTHS, AND 12 MONTHS BETWEEN HEU AND HUU INFANTS BY MEANS OF ANTHROPOMETRIC DATA.

In this study, no significant differences were observed between the z-scores for infants' weight, length, HC or, body mass index (BMI) at birth. However, for all the subsequent timepoints, the HEU infants were smaller than HUU infants with significantly lower WLZ at 10 weeks, six months and 12 months, and lower BMI-for-age z-scores (BAZ) at 10 weeks and 12 months. The LAZ for the HEU infants was higher than their HUU counterparts at 12 months.

In a study conducted in 2020 in KwaDukuza in South Africa on 392 women (56.4% not living with HIV and 43.6% living with HIV and using ART), no significant differences were seen in the new-borns (no follow-up visits were assessed) in terms of birthweight, birth length, or HC[106]. These results resemble the results in the current study at birth. Their study sample had also excluded mothers with other comorbidities (smokers and drug users) [106]. The same results were reported by a study from India in the Pune region in 2011, that assessed 342 women, 62% of whom were living with HIV[107].

In the current study, growth differences only emerged during follow-up. This is in contrast to what other researchers have found. For instance, a study looking at the growth of HEU infants in Southwest China found that the mean WAZ from birth to the first 12 months of life for the HEU infants was significantly smaller than their HUU counterparts[108]. A Zambian longitudinal analysis study also assessed growth differences between HEU and HUU infants up to school age[109]. The results showed that HEU children had lower WAZ, LAZ and BAZ than HUU children, from birth and the early on follow-up visits (between 1 and 6 weeks) up to school age (between 18 months and around 7.5 years) [109].

Similarly, a study conducted in Tanzania in 2012 found that HEU infants' LAZ was lower than HUU infants at three and six months of age[110]. Of the 114 infants (44 HEU and 70 HUU) in their study the HEU infants were underweight and had

stunted growth (significantly lower LAZ at two months and significantly lower WAZ at two, three, and six months) as defined by the WHO's Anthro programme, where stunted growth is defined as LAZ, WAZ, and WLZ less than -2; compared to the HUU infants for the first six months after birth[110]. While the higher likelihood of stunted growth in HEU infants could be secondary to inadequate nutrition, HIV-exposure was an independent risk factor in this study[110]. This finding is very relevant for any health care programme, since stunting is associated with inferior mental and psychomotor development[110-111].

Other studies have, however, shown that, while HEU children might be smaller at birth, they tend to catch up with the HUU counterparts in the first year of life. For instance, a study conducted by Chalashika *et al.* (2017) looked at the birthweight of 413 infants (154 HEU and 259 HUU) in Botswana. The results (using anthropometric z-scores) showed that HEU infants were more likely to be underweight than their HUU counterparts at birth[111] but had caught up by three months with the HUU infants in terms of their length and weight[111]. These results are similar to other studies, such as the study conducted by Bailey *et* al on the growth of children in the Democratic Republic of Congo[112]. They assessed 68 children living with HIV, 190 HEU, and 256 HUU children from birth up to 20 months of age[112]. Comparing the children's anthropometric data (LAZ, WAZ, and WLZ) with the National Centre for Health Statistics, they found that the mean WAZ and WLZ in children living with HIV were lower at birth and onward (indicating they were lighter and more wasted) than the HEU and HUU children. The HEU children were lighter than the HUU children up to three months, but had caught up by three months in terms of their weight and length and it remained unchanged up to 20 months[112]. In the current study, HEU infants also displayed greater length and HC differences between six and 12 months than HUU infants, suggestive of "catch-up" growth.

Interestingly, however, the current study did not find any association between HIV exposure and HC at any of the time points investigated. Evans *et al.* (2016) showed that children living with HIV have an overall smaller HC when compared to children not living with HIV and that the HC of HEU was smaller than HUU infants[9]. In a study conducted in Kenya, Neary *et al.* (2022) found that the mean HC for age z-scores (HCAZ) was similar between HEU and HUU up to six weeks

of age, but that a significantly lower HCAZ could be seen for the HEU infants at nine months of age[113]. These differences were mirrored by significant differences in the infants' weight and length at nine months[113]. The findings of their study are different from the HC findings in this current study, but agree with the BMI findings.

A greater risk of premature delivery in women living with HIV poses an additional risk to the optimal development of infants[114]. In a cross-sectional study conducted in Johannesburg, South Africa, 30 HEU and 30 HUU infants aged between 16 days and six months, all infants born premature at 28-37 weeks gestational age, were evaluated using BSID-III[114]. Their results showed that more HUU infants exhibited lower developmental scores in gross motor and expressive language scales than their HEU counterparts all born prematurely[114]. Another study corroborate that infants born premature and living with HIV have decreased neurodevelopmental rates than infants that are HEU and HUU[115]. Neonatal complications such as neonatal jaundice and meningitis was also more apparent in HUU infants than the HEU infants at birth[114]. It is important to note that the current study had no premature infants and no tests were conducted to indicate lower developmental between the infant groups.

A study conducted in Botswana examined the associations between HIV-exposure and cytomegalovirus (CMV) infection, and growth and neurodevelopment of infants over a 24-month period[116]. Of the 317 infants, 178 were HUU and 139 were HEU; all infants tested positive for anti-CMV immunoglobulin (Ig) G by means of enzyme-linked immunoassay (ELISA) on stored plasma samples. While CMV infection was not associated with WAZ, WLZ, or LAZ at 24 months, CMV-positive HUU infants (but nor CMV-positive HEU infants) had a smaller HCAZ at the 24 month timepoint. No negative neurodevelopmental outcomes (using BSID-III developmental assessment) were seen in either group at 24 months[116]. While CMV testing was not included in the current study, it seems that CMV-positive infants showed no negative neurodevelopment despite their HIV-exposure status.

Another factor to consider is whether infants' birth weight, length, and HC do affect their cognitive functioning. In a study conducted in 2010 in South India, Veena *et al.* tested 505 full-term children with a mean age of 9.7 years. They adjusted for age, BMI, time of testing, sex, height, parent's education, socioeconomic status,

as well as gestation and maternal age using multiple linear regression. They also tested learning ability, long-term memory storage and memory retrieval using the Atlantis score together with Kohs' block design score for visuospatial abilities[117]. The associations were adjusted for the children's' current HC. The study found that the Atlantis score (learning ability/long-term storage and retrieval) rose by 0.1 SD per SD increase in new-born weight and head circumference respectively ($p<0.05$ for all) and Kohs' block design score (visuo-spatial ability) increased by 0.1 SD per SD increase in birthweight ($p<0.05$). They found no associations with measures of short-term memory, fluid reasoning, verbal abilities, attention, and concentration, however. They concluded that children who had larger HC at birth with larger birth weights had better childhood cognitive abilities, specifically visuospatial ability, and long-term memory storage and retrieval[117]. The above study assessed healthy children not living with HIV, but no similar studies could be found in HEU children.

4.1.2 THE SECOND OBJECTIVE: TO CHARACTERISE AND COMPARE THE MONOCYTE PHENOTYPES BETWEEN HEU AND HUU INFANTS AT BIRTH, 10 WEEKS, AND SIX MONTHS USING FLOW CYTOMETRY.

Monocytes are multifunctional cells with effects ranging from immune defence to tissue repair[117]. In this study, subcategories of monocyte phenotypes - classical monocytes (CM), intermediate monocytes (IM), and non-classical monocytes (NCM) - were compared between HEU and HUU infants.

Looking at the monocyte phenotype percentages at each separate timepoint, the HEU group had significantly higher median percentages of IM at birth compared with their HUU counterparts. No differences were evident at 10 weeks and six months. The higher proportion for the IM phenotype within the HEU group at birth could be explained in the context of exposure to HIV antigens, since IM are responsible for the production of reactive oxidant species (ROS), antigen presentation, stimulation of T cells, angiogenesis, and inflammation. Since HEU children would have had exposure to HIV antigens *in utero* and HUU did not, this could be a plausible explanation of this difference.

Significant differences were also seen within the subgroups between birth and 10 weeks: both groups had decreases in CM (with the HUU group also having a decrease in CCR2+CM) and increases in NCM, while HEU infants also had a decrease in IM as well as its activated phenotype. Between 10 weeks and 6 months, HEU infants had a further decrease in CM and its activated phenotype. It is interesting to note that the proportions of the three phenotypes observed at birth resembled those of adults (80-95% CM; 2-8% IM; 2-11% NCM), probably reflecting the maternal milieu. After birth, the phenotype proportions changed in both groups with a much larger proportion of NCM than usually seen in adults. Roughly, the phenotype proportions were 70% CM; 1% IM; and 28% NCM. Discrepancies in monocyte subset proportions between children and adult proportions have been described. The distribution of subsets differs significantly in the first six months of life: CM peak in cord blood, whereas IM and NCM populations peak in new borns and then decrease until pre-adolescence[118].

There is an increasing number of studies evaluating monocyte activation with regards to HIV exposure in infants. A study assessing monocyte activation in Brazilian mothers and their infants (86 HEU and 88 HUU) found that HEU infants had higher quantities of monocyte activation (quantified using ELISA) and inflammation than their HUU counterparts at birth and 6 months of age[119]. Inflammatory markers used for the study were either drivers of immune dysregulation or they correlated with non-acquired immunodeficiency syndrome (AIDS) comorbidities[119]. The inflammatory markers, tested by means of ELISA, included: D-dimer, interleukin (IL)- 6, and tumour necrosis factor alpha (TNFα)[119]. These results complement the ones from the current study in that IM secrete inflammatory cytokines such as IL-6, IL-1β, and mainly TNF-α[120].

In contrast, Reikie *et al.* (2014) showed in a study conducted in Cape Town, South Africa, that cytokine production of monocytes (tested by multiparameter flow cytometry) were comparable between 27 HEU and 28 HUU infants by 12 months of age[121]. In addition, their plasmacytoid dendritic cells and classical dendritic cells (immune cells that mainly secrete interferon [IFN]) were comparable[121]. Captivatingly, their research findings showed that the monocyte production was not different between HEU and HUU infants in the first six weeks after birth[121]. This is slightly in contrast to the findings of the current study, as significant

differences were seen between the two infant groups' IM at birth. It should, however, be kept in mind that the methodology used in the latter study was different from the one used in the current study.

Some studies have also assessed the role of monocytes in the pathogenesis of HAND, since it is caused by the release of soluble viral and cellular neurotoxins that can damage spectator cells in the brain[122]. A study led by Veenstra *et al.* (2018), from the Department of Pathology at the Albert Einstein College of Medicine in New York, looked at the association between CCR2 expression (using flow cytometry) on peripheral blood cluster of differentiation (CD14+CD16+[IM]) monocytes and neuronal damage of 45 individuals (18 years and older) living with HIV[123]. They found that amplified expression of CCR2 on IM was associated with neuronal damage[123].

Another study by Zenón *et al.* (2015), from the Department of Microbiology at University of Puerto Rico in San Juan, Puerto Rico have reported that elevated CCR2 expression was not associated with comorbid ailments such as liver infections, diabetes, or substance use[122]. Therefore the research done in this abovementioned study propose that the increase levels of CCR2 may be associated with risks of neurocognitive discrepancies due to HIV infection[122].

While the study conducted by Veenstra *et al.* differed in important ways from the current study, in that children exposed to HEU rather than adults living with HIV were evaluated, it is interesting to note that HEU children had (non-significantly) higher proportions of CCR2+IM at birth, and had a significant decrease in this proportion from a median of 3.76 to 1.05 between birth and 10 weeks[123]. This therefore seems to have been a correction in the activation of the IM phenotype after birth, likely because of the reduction in HIV antigen exposure. It is hoped that this signals a reduced risk of neuronal damage in HEU children[123].

4.1.3 THE THIRD OBJECTIVE: TO DETERMINE IF THERE IS AN ASSOCIATION BETWEEN HC AND MONOCYTE PHENOTYPES AT BIRTH, 10 WEEKS, AND 6 MONTHS.

No significant associations could be detected at birth up until six months of age between HC and monocyte phenotypes in either HEU or HUU. This was also true when looking at activation of the various monocyte subsets, as detected by CCR2 expression.

Research performed by Williams *et al.* (2014), form the Department of Pathology at the Albert Einstein College in New York, found that CCR2 expression on IM through an *in* vitro model are the perfect peripheral blood biomarkers for detecting HAND since it is an effective monocyte chemoattractant that is increased in the brain during HIV infection[124]. The more matured the IMs are the more the monocyte subset is involved in HAND by passing the virus into the CNS and spreading small quantities of neuroinflammation[123,125]. An augmented risk of expansion of HAND can be due to IM that are exceedingly prone to infection with HIV due to high levels of CCR5 expression, and act as a peripheral virus-related tank[124-126]. Even though Williams *et al.* determined that CCR2+IM are perfect biomarkers for determining HAND[125], this study could not find any association with HC in HEU infants. This could either be because HEU infants do not have HAND, or because HC is too crude a marker for neuronal damage.

Interestingly, however, a study conducted by White *et al.* (2020) in Pretoria, South Africa, did report lower HC and raised CCR2 on monocytes at birth in HEU infants compared to their HUU counterparts[127]. Their study focused on *in utero* HIV exposure and the influence of the nutritional environment of infants' development and immune outcomes. The results of their study showed that HEU infants had lower HC at birth and also smaller birth weight, length, BMI and HC compared to the HUU infant group[127]. Further results of their study showed that HEU infants had amplified CCR2 expression specifically on the IM at birth and 12 weeks[127]. This study's results differed from the current study in a number of important aspects: no difference for HC in HEU and HUU infants were found, the HEU infants did not have raised CCR2 expression for any of the monocyte subsets at birth; HEU infants' anthropometric results (weight, length, and BMI) were comparable to their counterparts at birth; and the HEU infants had higher (non-significantly)

proportions of CCR2+IM at birth but the proportions were lower than the HUU infants at 10 weeks.

It seems reasonable to assume that neuroinflammation and neuropsychological damage in adults that is induced by HIV-infection are associated with amplified CCR2 expression and amplified monocyte subgroup recruitment across the blood-brain barrier[127]. Unfortunately, it is still not clear whether amplified CCR2 expression of monocyte subgroups in HEU children may have consequences for their neurodevelopment[127].

4.1.4 THE FOURTH OBJECTIVE: TO EXPLORE ASSOCIATIONS AND CORRELATIONS BETWEEN ANTHROPOMETRIC MEASURES (APART FROM HC) AND MONOCYTE PHENOTYPES.

For the current study the association between BAZ and WAZ and monocyte phenotypes were tested. When categorised into binary variables according to z-score values less than and greater or equal to -1, infants in the higher BAZ category had higher median values for CCR2+CM at birth and infants in the lower category of WAZ had higher median values for IM and CCR2+IM at birth.

As discussed previously the HEU infants' IM phenotype proportions at birth were higher than their counterparts and could be because of the exposure to HIV antigens, and IM is responsible for an inflammatory response. This exposure could explain why the median WAZ values for the lower category is elevated than the higher category indicating the lower weights for the HEU infants at birth.

Limited research on the associations and correlations between anthropometric measurements and monocyte phenotypes have been conducted. A study at KH in Pretoria, South Africa, which also served as the pilot study for this current study, assessed 20 mothers living with HIV and on ARV, 20 mothers living with HIV and not on ARV, and 20 mothers not living with HIV, to compare infants' growth, neurodevelopment, and immune development in early life between HEU and HUU[127]. They also assessed monocyte subsets (CD14, CD16, and CCR2) using flow cytometry at birth and 12 weeks[127]. The study showed lower HC and elevated CCR2+CM and CCR2+IM at birth and 12 weeks postpartum, respectively, in HEU

compared to HUU infants[127]. Unfortunately, this study did not formally explore associations and correlations between the monocyte subsets and infant growth.

4.2 CONCLUSION

Looking at the 19 HUU and 23 HEU infants over a course of 12 months, small but significant differences were detected in terms of their weight and BMI with the HEU infants being smaller. These findings correspond with some past studies but conflict with others. No significant differences were, however, seen between the infant groups' HC. For the monocytes, statistically significant differences were seen between the infant groups where the HEU infants had higher proportions of IM at birth. In addition, while both HEU and HUU infants had decreased CM and increased NCM between birth and 10 weeks, only HEU infants had decreased IM and a further decrease in CM between 10 weeks and six months. The change in the IM proportions in HEU infants might indicate less antigen exposure after birth. No correlations were found between the monocyte polarisation and HC for the infants.

When comparing the results of this study, it is perplexing that the results do not correspond to the initial pilot study's results. A number of factors might be the cause of that. Importantly, in contrast to the pilot study, mothers who had any comorbidities, such as maternal hypertension, diabetes, tuberculosis, or other serious pre-existing medical conditions, were excluded in the current study. It is also possible that their socio-economic status might have been different to that of the population included in the pilot study, although this has not been formally investigated. Also, the infants of this current study had no chromosomal or structural abnormalities, or exposure to maternal antibiotic usage, during labour or delivery. Overall, both the infants of this study and their mothers were overall healthier than the dyads included in the pilot study

4.3 LIMITATIONS AND STRENGTHS

The major limitation of the study is the small sample size (n=42). Not all the infants who were part of the study had data available for all timepoints, as some

participants moved and discontinued with the study. Anthropometric measurements were taken by a few clinical nurses, and inter-user variability and human error might have interfered with precise measurements. More accurate measurements for length and HCs might have detected differences that were not evident in the current study. The study is still ongoing and no long-term data are currently available to see if differences might emerge in infants older than 12 months of age. Additional data, such as information about infant feeding, maternal nutrition, and breast milk composition, that were collected during the study, have not yet been analysed and could not be used as variables in the current study. Finally, the monocyte markers used in the current study could have been expanded with new markers, such as SLAN, to allow for more precise classification of subsets.

On the positive side, the study recruited mothers living with and without HIV from the same geographic area and with similar socio-economic circumstances in order to reduce confounding. Early antenatal ultrasound allowed for accurate gestational age calculation and hence prevented problems of confusing prematurity with growth restriction, which has been a common problem in previous studies.

4.4 RECOMMENDATIONS

For this study, only centimetres (cm) were used as the HC value. Since this is a very crude assessment, it is critical that future studies should use millimetres (mm) for more accurate measurement. Other viral infections, such as herpes simplex virus and enteroviruses, that might infect infants at birth shows similar growth defects as HEU infants. Looking at the mothers' mental health and education is another factor to consider when understanding the neurodevelopment of children.

Although HC measurement is a good and safe proxy for a prediction of an infant's brain growth, it is not accurate enough to approximate neurocognitive development. Neurodevelopmental tests, such as the BSID-III and brain imaging scans (Magnetic resonance Imaging [MRI]) would be better dimensions to fully understand the neurodevelopment of these infants.

The growth and development of HEU infants should be monitored for longer than 12 months, with a larger sample size, to gain adequate data and information. Monocytes are important for immune response and tissue repair, but other immune active cells could shed more light on why HEU infants have lower neurodevelopmental outcomes than HUU infants. Sterile Alpha Motif Histidine-Aspartic acid domain containing protein 1 (SAMHD-1), an important restriction factor for homeostasis of deoxynucleotide triphosphates (dNTPs) could be looked at for future studies as it is also an effector of innate immunity. Toll-like receptors (TLRs) recognise chronic inflammation, a key characteristic of HIV infection, could also aid in understanding how chronic inflammation might impact the development of HEU infants. Other inflammatory markers secreted by monocytes, such as IL IL-6, IL-4, and IL-1β, TNFα, and IFN, might also give a more comprehensive understanding of the role of monocytes in the context of HIV exposure.

More varied populations could be considered for future studies to understand how different populations (different socio-economic and geographic populations) grow and develop to aid in better understanding the development of HEU infants in different contexts. Mothers with comorbidities, such as: diabetes, tuberculosis (TB), active smoking, alcohol abuse, and recreational drug usage, could also be examined in future studies, as it is known that these comorbidities have negative effects on the growth and development of infants.

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ETHICS

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

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**

28 October 2021

Approval Certificate
New Application

Dear Mr I Sipsma

Ethics Reference No.: 550/2021
Title: Association between head circumference and monocyte phenotype in neonates exposed and unexposed to HIV at Kalafong Provincial Tertiary Hospital

The **New Application** as supported by documents received between 2021-09-27 and 2021-10-27 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2021-10-27 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 2022-10-28.
- Please remember to use your protocol number (550/2021) 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)

Research Ethics Committee
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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

PERMISSION LETTER

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

MSc Committee
School of Medicine
Faculty of Health Sciences

14 September 2021

Prof TM Rossouw
Department of Immunology
Faculty of Health Sciences

Dear Prof,

Mr I Sipsma, Student no 14067511

Please receive the following comments with reference to the MSc Committee submission of the above mentioned student:

Student name	Mr I Sipsma	**Student number**	14067511
Name of study leader	Prof TM Rossouw		
Department	Immunology		
Title of MSc	Association between head circumference and monocyte phenotype in neonates exposed and unexposed to HIV at Kalafong Provincial Tertiary Hospital		
Date of first submission	25 August 2021		
Comments to study leader August 2021	• Please submit CV's of the student and both supervisors. • Please submit a funding letter • Revise the document to reflect clearly what still needs to be done and what has already been done (check tenses etc. throughout document) • Include a flow diagram clearly showing what the student will be doing • Indicate how many samples will be done by the student • The title infers that there already is an association. Please consider revising • Remove citations from executive summary • Check grammar throughout the document i.e. "dropped below 1% or less". It can't be less than below 1%. • Ensure that all facts in the literature review have citations • Figures need legends. Also check the numbering carefully of both figures and tables • Include the "how" in objective two (how will this be achieved?) • Expand on flow cytometry methodology e.g. antibody concentrations not volumes etc. • Include a comprehensive budget that includes antibody and flow cytometry costs • Revise timeline as this indicates only four months before submission • Check reference list thoroughly		

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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

September 2021	• Thank you for submitting the revised protocol and requested documents. • Kindly ensure that the following is addressed before submission to ethics committee: • **Please include a data capturing sheet.** • **State how objective 3 will be achieved. This should be clearly described in methodology.** • **Expand the data management section – include statement that the metadata will be submitted to UP Research Data Repository system.** • **Please clarify how many samples will be analysed by the candidate using flow cytometry.**
Decision	This protocol has been provisionally approved. Please submit the revised protocol to ethics, and supply the MSc committee with proof of acceptance. The internal and external examiners can be nominated and submitted to the MSc Committee six months prior to submission of the dissertation. Please ensure that the CV of the examiners includes: supervision, examination and publication records.

Yours sincerely

Prof Marleen Kock
Chair: MSc Committee

APPENDIX 1

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 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 22/04/2017.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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Faculty of Health Sciences Research Ethics Committee

26/05/2016

Approval Certificate
New Application

Ethics Reference No.: 185/2016

Title: The impact of maternal HIV status on breast milk composition and its relationship with child development and health – a pilot study

Dear Prof T. Rossouw

The **New Application** as supported by documents specified in your cover letter dated 24/05/2016 for your research received on the 24/05/2016, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 25/05/2016.

Please note the following about your ethics approval:

Ethics Approval is valid for 5 years

- Please remember to use your protocol number (**185/2016**) 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.

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 2004 (Department of Health).

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APPENDIX 2

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 3

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**

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.

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.

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.

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.

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

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)

APPENDIX 4

Maternal and infant postpartum questionnaire

BREASTFEEDING

1. Did you ever breastfeed or try to breastfeed your baby, even if only for a single feed?
   - ☐ Yes → Skip to question 3
   - ☐ No
   - ☐ Prefer not to answer → Skip to question 5

2. If no, why was this? Select all that apply
   - ☐ Personal choice → Skip to question 10
   - ☐ Personal circumstances (e.g., other demands, return to work) → Skip to question 10
   - ☐ You were unwell → Skip to question 10
   - ☐ Baby was too small or unwell→ Skip to question 10
   - ☐ Didn't think you had enough milk → Skip to question 10
   - ☐ Lack of support/resources → Skip to question 10
   - ☐ Other reason please specify:_____ → Skip to question 10
   - ☐ Prefer not to answer → Skip to question 12

3. How soon after birth was your baby first put to the breast? ***(SKIP IF NO TO question 1)***
   _____ minutes or _____ hours after birth
   - ☐ Never (baby was fed pumped milk)
   - ☐ Prefer not to answer

4. Has your baby ever been fed breast milk from a bottle?
   - ☐ Yes
   - ☐ No
   - ☐ Prefer not to answer

5. Are you currently breastfeeding your baby or giving your baby expressed breast milk?
   - ☐ Yes
   - ☐ No → Skip to question 7
   - ☐ Prefer not to answer → Skip to question 12

   If yes, is your baby currently receiving breast milk only?
   - ☐ Yes → Skip to question 12
   - ☐ No, my baby receives both breast milk and formula
   - ☐ Prefer not to answer

6. Which scenario best describes your baby's feeding?
   - ☐ My baby receives infant formula 80-100% of the time.
   - ☐ My baby receives breast milk 80-100% of the time.
   - ☐ My baby receives both breast milk and formula equally.
   - ☐ Prefer not to answer

7. How old was your baby when you stopped breastfeeding?
   _____ days or _____weeks
   - ☐ Prefer not to answer

8. How old was your baby when you introduced formula?
   _____ days or _____weeks
   - ☐ Prefer not to answer

9. What was the main reason for introducing formula?
   - ☐ Breastfeeding took too long or was too tiring
   - ☐ Needed to return to work
   - ☐ Convenience or to allow others to feed
   - ☐ To try and get baby to sleep through the night
   - ☐ Insufficient milk to satisfy the baby
   - ☐ Baby wouldn't suck because unwell or low birth weight
   - ☐ Baby wouldn't suck for no apparent reason
   - ☐ Baby irritable or colicky
   - ☐ Baby not gaining weight
   - ☐ Painful breasts or sore nipples
   - ☐Mastitis or breast abscess
   - ☐ Milk dried up
   - ☐ The right time/age to change
   - ☐ Other reason → (Please specify:_____)
   - ☐ Prefer not to answer

10. What type of formula do you usually feed your baby?
   - ☐ Cow's milk-based formula
   - ☐ Lactose-free cow's milk-based formula
   - ☐ Soy-based formula
   - ☐ Other → (Please specify:_____)
   - ☐ Prefer not to answer

What is the specific brand and type of formula that you usually feed your baby? Indicate all that apply

_____

11. What form of formula do you usually use?
- ☐ Liquid ready-to-use
- ☐ Liquid concentrate (add water)
- ☐ Powder concentrate (add water)
- ☐ Prefer not to answer

12. Has your baby had any liquids other than breast milk or formula since his/her birth (even if it was a temporary supplement)? Other liquids include water, glucose water, evaporated milks, goat's milk, cow's milk or any other drink.
- ☐ Yes → if yes, please specify: _____
- ☐ No
- ☐ Prefer not to answer

13. Does your baby receive any vitamins or supplement drops?
- ☐ Yes
- ☐ No
- ☐ Prefer not to answer

14. If yes, which of the following? How often are you giving the vitamins or supplements?
- ☐ Vitamin D drops _____ times per (day, week, month)
- ☐ Other (Please specify:_____) _____ times per (day, week, month)
- ☐ Prefer not to answer

15. Has your baby ever taken any prescribed medications?
- ☐ Yes → If yes, please specify: _____
- ☐ No
- ☐ Prefer not to answer

MOTHER'S DIETARY SUPPLEMENTS

Now we would like to ask some general questions about your health and lifestyle since your baby was born

16. How would you currently rate your general health?
- ☐ Excellent
- ☐ Very good
- ☐ Good
- ☐ Fair
- ☐ Poor
- ☐ Prefer not to answer

17. What is your current weight?

_____ pounds or _____kilograms

☐ Prefer not to answer

18. Since your baby was born, have you taken any vitamins, minerals or other dietary supplements?

☐ Yes

☐ No → Skip to question 19

☐ Prefer not to say → Skip to question 19

	How often have you used this since your baby was born?
Prenatal vitamin	☐ Never ☐ Less than 1 per month ☐ 1-3 days per month ☐ 1-3 days per week ☐ 4-6 days per week ☐ Every day ☐ Prefer not to say If Yes, please list the brand name and specific type: _____
Multivitamin (not pregnancy specific)	☐ Never ☐ Less than 1 per month ☐ 1-3 days per month ☐ 1-3 days per week ☐ 4-6 days per week ☐ Every day ☐ Prefer not to say If Yes, please list the brand name and specific type: _____ Does your multivitamin usually contain minerals (such as iron, zinc, etc.)? ☐ Yes ☐ No ☐ Prefer not to say
Folic acid or folate (NOT as part of a multivitamin or prenatal multivitamin)	☐ Never ☐ Less than 1 per month ☐ 1-3 days per month

	☐ 1-3 days per week ☐ 4-6 days per week ☐ Every day ☐ Prefer not to say ☐ less than 400 mcg (0.4mg) ☐ 400-799 mcg ☐ 800-999 mcg ☐ 1000 (1mg) or more ☐ Don't know ☐ Prefer not to say
Iron (NOT as part of a multivitamin or prenatal multivitamin)	☐ Never ☐ Less than 1 per month ☐ 1-3 days per month ☐1-3 days per week ☐ 4-6 days per week ☐ Every day ☐ Prefer not to say ☐ Less than 10 mg ☐ 10-14 mg ☐ 15-39 mg ☐ 40 mg or more ☐ Don't know ☐ Prefer not to say
Calcium supplements or calcium containing antacids (NOT as part of a multivitamin or prenatal multivitamin)	☐ Never ☐ Less than 1 per month ☐ 1-3 days per month ☐ 1-3 days per week ☐ 4-6 days per week ☐ Every day ☐ Prefer not to say ☐ less than 500 mg ☐ 500-599 mg ☐ 600-999 mg ☐ 1000 or more ☐ Don't know ☐ Prefer not to say

Vitamin C (NOT as part of a multivitamin or prenatal multivitamin)	☐ Never ☐ Less than 1 per month ☐ 1-3 days per month ☐ 1-3 days per week ☐ 4-6 days per week ☐ Every day ☐ Prefer not to say ☐ less than 400 IU ☐ 400-799 IU ☐ 800-999 IU ☐ 1000 IU or more ☐ Don't know ☐ Prefer not to say
Zinc (NOT as part of a multivitamin or prenatal multivitamin)	☐ Never ☐ Less than 1 per month ☐ 1-3 days per month ☐ 1-3 days per week ☐ 4-6 days per week ☐ Every day ☐ Prefer not to say ☐ less than 10 mg ☐ 10-14 mg ☐ 15-39 mg ☐ 40 mg or more ☐ Don't know ☐ Prefer not to say
Vitamin D on its own or as part of a calcium supplement (NOT as part of a multivitamin or prenatal multivitamin)	☐ Never ☐ Less than 1 per month ☐ 1-3 days per month ☐ 1-3 days per week ☐ 4-6 days per week ☐ Every day ☐ Prefer not to say ☐ less than 400 IU ☐ 400-599 IU ☐ 600-999 IU

	☐ 1000 IU or more ☐ Don't know ☐ Prefer not to say
Probiotics such as acidophilus in supplement form only (not in food)	☐ Never ☐ Less than 1 per month ☐ 1-3 days per month ☐ 1-3 days per week ☐ 4-6 days per week ☐ Every day ☐ Prefer not to say If Yes, please list the brand name and specific type: _____
Cod liver oil	☐ Never ☐ Less than 1 per month ☐ 1-3 days per month ☐ 1-3 days per week ☐ 4-6 days per week ☐ Every day ☐ Prefer not to say
Other Fish oil or Omega 3 fatty acids	☐ Never ☐ Less than 1 per month ☐ 1-3 days per month ☐ 1-3 days per week ☐ 4-6 days per week ☐ Every day ☐ Prefer not to say
Other, Please specify: _____	☐ Never ☐ Less than 1 per month ☐ 1-3 days per month ☐ 1-3 days per week ☐ 4-6 days per week ☐ Every day ☐ Prefer not to say

FOOD SECURITY

The following questions ask about your access to food over the past 12 months.

19. Which of the following statements best describes the food eaten in your household in the past 12 months?

- ☐ You and other household members always had enough of the kinds of food you wanted to eat.
- ☐ You and other household members had enough to eat, but not always the kinds of food you wanted.
- ☐ Sometimes you and other household members did not have enough to eat.
- ☐ Often you and other household members didn't have enough to eat.
- ☐ Don't know
- ☐ Prefer not to answer

The following statements may be used to describe the food situation for a household. Please indicate if the statement was often true, sometimes true, or never true for you and other household members in the past 12 months.

20. You and other household members worried that food would run out before you got money to buy more. Was that often true, sometimes true, or never true in the past 12 months?

- ☐ Often true
- ☐ Sometimes true
- ☐ Never true
- ☐ Don't know
- ☐ Prefer not to answer

21. The food that you and other household members bought just didn't last, and there wasn't any money to get more. Was that often true, sometimes true, or never true in the past 12 months?

- ☐ Often true
- ☐ Sometimes true
- ☐ Never true
- ☐ Don't know
- ☐ Prefer not to answer

22. You and other household members couldn't afford to eat balanced meals. In the past 12 months was that often true, sometimes true, or never true?

- ☐ Often true
- ☐ Sometimes true
- ☐ Never true
- ☐ Don't know
- ☐ Prefer not to answer

If the participant responds "often true" or "sometimes true" to ANY ONE of question 20-22 OR "Sometimes" or "Often" to question 19, then continue to question 23; otherwise, skip to the next section (question 29).

The following questions are about the food situation in the past 12 months for you or any other adults in your household.

23. In the past 12 months, did you or other adults in your household ever cut the size of your meals or skip meals because there wasn't enough money for food?

- ☐ Yes
- ☐ No → Skip to question 27
- ☐ Don't know
- ☐ Prefer not to answer

24. How often did this happen?

- ☐ Almost every month
- ☐ Some months but not every month
- ☐ Only 1 or 2 months
- ☐ Don't know
- ☐ Prefer not to answer

25. In the past 12 months, did you personally ever eat less than you felt you should have because there wasn't enough money to buy food?

- ☐ Yes
- ☐ No
- ☐ Don't know
- ☐ Prefer not to answer

26. In the past 12 months, did you personally lose weight because you didn't have enough money for food?

- ☐ Yes
- ☐ No
- ☐ Don't know
- ☐ Prefer not to answer

If the participant responded "yes" to question 23, 25 or 26, continue to 27; otherwise, skip to the next section (question 29).

27. In the past 12 months, did you or other adults in your household ever not eat for a whole day because there wasn't enough money for food?

- ☐ Yes
- ☐ No
- ☐ Don't know

☐ Prefer not to answer

28. How often did this happen?
- ☐ Almost every month
- ☐ Some months but not every month
- ☐ Only 1 or 2 months
- ☐ Don't know
- ☐ Prefer not to answer

SMOKING AND ALCOHOL

29. How many cigarettes do you smoke each day now?
_____ number of cigarettes
- ☐ Do not smoke → **Skip to question 31**
- ☐ Prefer not to answer

30. Do you smoke inside your home?
- ☐ Yes
- ☐ No
- ☐ Prefer not to answer

31. Does any member of your household smoke cigarettes (even if not inside your home)?
- ☐ Yes
- ☐ No
- ☐ Prefer not to answer

32. How often are you usually exposed to other people's tobacco smoke inside your home?
- ☐ Every day
- ☐ Almost every day
- ☐ At least once a week
- ☐ At least once a month
- ☐ Less than once a month
- ☐ Never
- ☐ Don't know
- ☐ Prefer not to answer

33. During leisure time outside of your home, how often are you usually exposed to other people's tobacco smoke?
- ☐ Every day
- ☐ Almost every day
- ☐ At least once a week
- ☐ At least once a month
- ☐ Less than once a month

- ◻ Never
- ◻ Don't know
- ◻ Prefer not to answer

34. Since your baby was born, how often do you drink alcohol?
    - ◻ 6 to 7 times a week
    - ◻ 4 to 5 times a week
    - ◻ 2 to 3 times a week
    - ◻ Once a week
    - ◻ 2 to 3 times a month → skip to next section
    - ◻ About once a month → skip to next section
    - ◻ Less than monthly → skip to next section
    - ◻ Never → skip to next section
    - ◻ Don't know → skip to next section
    - ◻ Prefer not to answer → skip to next section

35. Since your baby was born, how often do you have four or more alcoholic drinks at the same sitting or occasion?
    - ◻ 6 to 7 times a week
    - ◻ 4 to 5 times a week
    - ◻ 2 to 3 times a week
    - ◻ Once a week
    - ◻ 2 to 3 times a month
    - ◻ About once a month
    - ◻ 6 to 11 times a year
    - ◻ 1 to 5 times a year
    - ◻ Never
    - ◻ Don't know
    - ◻ Prefer not to answer

DIETARY RECALL

Please write today's date (day/month/year) _____

What day of the week does this recall refer to? ☐Sunday ☐Monday ☐Tuesday ☐Wednesday ☐Thursday ☐Friday ☐Saturday

Is this a typical day of food and drink for you? ☐YES ☐NO If NO, why not? _____

Please recall what you ate in the last 24 hours, including all food, drink and snacks.

Details of food and drink List all foods and beverages for every meal and snack during the 24-hour period, including water, coffee, tea, and any vitamins and supplements taken	**Portion size** How many pieces, slices, servings, packages, grams, kilograms, cups, teaspoons, tablespoons or millilitres?	**How was it prepared?** Was it baked, boiled, broiled, steamed, fried, or raw?	**Was anything added to it?**
Example: tea	*1 cup*	*boiled*	*1 teaspoon sugar*
Example: white bread	*1 slice*	*toasted*	*1 teaspoon butter*
Breakfast			
Mid-morning snack			
Lunch			
Afternoon snack			
Dinner			
Evening snack			

END OF QUESTIONNAIRE

Thank you for completing this questionnaire. We really appreciate your participation in our study.

APPENDIX 5

Anthropometric measurements for maternal HIV, breast milk composition, and child development and health study

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).

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.

APPENDIX 6

Correcting Age for Pre-term babies.

Calculations for gestational age (GA):

- Needed: Estimated Date of Delivery (EDD) from 22 week SONAR and infant's date of birth (DOB)
- GA_Days = DOB – EDD (positive or negative answers) + 280

Age corrections:

- GA (weeks) = weeks x 7
- Prematurity (Prem) = 280 – GA_Days
- Corrected Age (CA_Days) = GA_D – Prem
- CA_Months = CA_D / 365x12

Postnatal Visits calculations – 10 weeks, six months and 12 months:

- Needed: DOB and Date of Visit (DOV)
- DOV – DOB = Days (positive answer)
- Years = Days / 365
- Months = Years x 12

APPENDIX 7

Additional Table: Comparison of anthropometric results of HUU and HEU infants between 10 weeks and 12 months old.

Variable	HUU Median (IQR)	HEU Median (IQR)	Probability
Weight diff (kg)	3.75 (3.59 – 4.54)	3.92 (3.26 – 4.73)	0.6783
Length diff (cm)	16.50 (15.15 – 18.55)	17.50 (15.30 – 19.40)	0.3932
HC diff	6.55 (6.25 – 8.70)	7.00 (6.50 – 7.90)	0.9417
MUAC diff	2.15 (1.05 – 2.85)	2.00 (1.00 – 3.30)	0.7857

Abbreviations: kg (kilogram), diff (difference), cm (centimetre), HC (head circumference), MUAC (mid-upper arm circumference), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected).

APPENDIX 8

For the monocyte phenotype percentages and HCZ, 24 infants at birth were in the higher category (i.e., HCZ ≥-2), no significant associations were found. Looking at the monocyte phenotype percentages and BAZ, 14 infants at birth were in the higher category (i.e., BAZ ≥-2), no significant associations were found, apart from the CM at 10 weeks where the group with lower BAZ had lower proportions than the higher category. Next, looking at the monocyte phenotype percentages and WAZ, 24 infants were in the higher category (i.e., WAZ ≥-2). No significant associations where found, except for the CM at 10 weeks where the group with higher WAZ had significantly lower proportions. Lastly, looking at the comparison of the infants' monocyte phenotype percentages at birth, 10 weeks, and six months with LAZ. At birth, 23 infants were in the higher category (i.e., LAZ ≥-2). No significant associations could be found for the monocyte subsets at birth, 10 weeks, or six months.

Additional Table: Association between infant monocyte phenotype percentages and head circumference (HC) for age z-scores (HCZ) at different ages.

Variable (%)	HCZ <-2 Median (IQR)	HCZ ≥-2 Median (IQR)	Probability
CM_0*	n=24 85.41 (78.81 – 89.16)	n=1 89.93 (89.93 – 89.93)	0.3317
IM_0*	n=24 3.21 (1.51 – 5.67)	n=1 4.29 (4.29 – 4.29)	0.4881
NCM_0*	n=22 5.24 (3.73 – 7.13)	n=1 4.20 (4.20 – 4.20)	0.5465
CCR2CM_0*	n=24 77.00 (67.13 – 81.79)	n=1 85.78 (85.78 – 85.78)	0.1272
CCR2IM_0*	n=23 3.28 (1.54 – 4.75)	n=1 4.30 (4.30 – 4.30)	0.5156
CM_10*	n=19 84.23 (78.35 – 88.79)	n=1 81.03 (81.03 – 81.03)	0.2571
IM_10*	n=23 1.22 (0.60 – 2.08)	n=1 1.75 (1.75 – 1.75)	0.2821

Abbreviations: _0 (birth), _10 (10 weeks old), % (percentage), CM (classical monocyte), CCR2 (C-C Motif chemokine receptor 2), HC (head circumference), HCZ (HC-for-age z-score), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected), IM (intermediate phenotype), NCM (non-classical monocyte).
*Data were not available for all the participants.

Additional Table: Association between infant monocyte phenotype percentages and BMI for age z-scores (BAZ) at different ages.

Variable (%)	BAZ <-2 Median (IQR)	BAZ ≥2 Median (IQR)	Probability
CM_0*	n=14 86.63 (81.99 – 89.52)	n=11 83.39 (78.35 – 89.94)	0.7016
IM_0*	n=14 2.84 (1.48 – 4.58)	n=11 3.76 (1.54 – 4.90)	0.3244
NCM_0*	n=13 5.34 (4.77 – 6.98)	n=10 4.00 (2.71 – 7.04)	0.2643
CCR2CM_0*	n=15 79.34 (67.17 – 82.23)	n=10 71.26 (64.73 – 81.76)	0.3748
CCR2IM_0*	n=14 2.84 (1.48 – 4.58)	n=10 3.80 (3.28 – 4.90)	0.1432
CM_10*	n=19 84.23 (78.35 – 88.79)	n=1 83.22 (83.22 – 83.22)	***0.0966**
IM_10*	n=23 1.22 (0.60 – 2.08)	n=1 1.65 (1.65 – 1.65)	0.8284
CM_6*	n=26 63.99 (59.78 – 75.38)	n=1 65.69 (65.69 – 65.69)	0.7001
IM_6*	n=23 1.64 (0.83 – 2.89)	n=1 1.23 (1.23 – 1.23)	0.6131
NCM_6*	n=27 30.27 (19.03 – 36.11)	n=1 28.28 (28.28 – 28.28)	0.8527
CCR2CM_6*	n=24 59.81 (55.53 – 64.74)	n=1 54.14 (54.14 – 54.14)	0.4054
CCR2IM_6*	n=23 1.64 (0.83 – 2.89)	n=1 1.23 (1.23 – 1.23)	0.6131
CCR2NCM_6*	n=26 0.95 (0.52 – 1.41)	n=1 0.41 (0.41 – 0.41)	0.3044

Abbreviations: _0 (birth), _10 (10 weeks old), _6 (six months old), % (percentage), BMI (body mass index), BAZ (BMI-for-age z-score, CM (classical monocyte), CCR2 (C-C Motif chemokine receptor 2), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected), IM (intermediate phenotype), NCM (non-classical monocyte).

*Data were not available for all the participants.

Additional Table: Association between infant monocyte phenotype percentages and Weight for age z-scores (WAZ) at different ages.

Variable (%)	WAZ <-2 Median (IQR)	WAZ ≥2 Median (IQR)	Probability
CM_0*	n=24 85.40 (78.81 – 89.16)	n=3 83.22 (81.03 – 89.94)	0.8774
IM_0*	n=24 3.14 (1.51 – 4.67)	n=3 4.30 (3.65 – 4.91)	0.2472
NCM_0*	n=22 5.41 (3.73 – 8.31)	n=3 4.20 (3.79 – 7.04)	0.6158
CCR2CM_0*	n=24 77.61 (67.13 – 81.79)	n=3 70.37 (68.04 – 85.78)	0.8170
CCR2IM_0*	n=23 3.15 (1.54 – 4.75)	n=3 4.30 (3.65 – 4.90)	0.2786
CM_10*	n=20 83.73 (78.39 – 88.52)	n=3 88.19 (84.81 – 89.94)	***0.0966**
CCR2IM_10*	n=23 1.22 (0.60 – 2.08)	n=1 1.65 (1.65 – 1.65)	0.8284
IM_6*	n=24 1.58 (0.87 – 2.73)	n=2 1.56 (1.34 – 1.77)	0.1900

Abbreviations: _0 (birth), _10 (10 weeks old), _6 (six months old), % (percentage), CCR2 (C-C Motif chemokine receptor 2), CM (classical monocyte), IM (intermediate phenotype), NCM (non-classical monocyte), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected).
*Data were not available for all the participants.

Additional Table: Association between infant monocyte phenotype percentages and Length for age z-scores (LAZ) at different ages.

Variable (%)	LAZ <-2 Median (IQR)	LAZ ≥2 Median (IQR)	Probability
CM_0*	n=23 85.83 (81.03 – 89.52)	n=2 84.56 (79.19 – 89.94)	0.9999
IM_0*	n=23 3.15 (1.48 – 4.75)	n=2 4.44 (4.30 – 4.58)	0.2705
NCM_0*	n=22 5.24 (3.73 – 7.04)	n=1 4.20 (4.20 – 4.20)	0.5465
CCR2CM_0*	n=23 77.04 (67.17 – 81.82)	n=2 65.53 (45.28 – 85.78)	0.9202
CCR2IM_0*	n=22 3.22 (1.54 – 4.75)	n=2 4.44 (4.30 – 4.58)	0.2963
CM_10*	n=18 83.73 (78.35 – 88.25)	n=2 84.56 (79.19 – 89.94)	0.5127
IM_10*	n=21 1.33 (0.90 – 1.99)	n=3 0.91 (0.91 – 3.83)	0.7600
NCM_10*	n=20 28.83 (24.14 – 34.15)	n=2 29.81 (18.43 – 41.18)	0.9091
CCR2CM_10*	n=16 64.18 (58.12 – 70.88)	n=2 58.81 (42.10 – 75.53)	0.9999
CCR2IM_10*	n=21 1.33 (0.90 – 1.99)	n=3 0.91 (0.91 – 3.83)	0.7600
CM_6*	n=25 63.92 (59.78 – 75.38)	n=2 69.49 (67.53 – 71.44)	0.3085
IM_6*	n=22 1.71 (0.99 – 2.89)	n=2 0.89 (0.38 – 1.40)	0.2101
NCM_6*	n=26 31.07 (19.03 – 36.11)	n=2 22.67 (21.83 – 23.50)	0.4754
CCR2CM_6*	n=23 59.58 (53.66 – 64.84)	n=2 60.89 (59.02 – 62.76)	0.7638
CCR2IM_6*	n=22 1.71 (0.89 – 2.89)	n=2 0.89 (0.38 – 1.40)	0.2101
CCR2NCM_6*	n=25 0.95 (0.71 – 1.41)	n=2 0.48 (0.44 – 0.52)	0.2288

Abbreviations: _0 (birth), _10 (10 weeks old), _6 (6 months old), % (percentage), CM (classical monocyte), CCR2 (C-C Motif chemokine receptor 2), HUU (HIV-unexposed uninfected), HEU (HIV-exposed uninfected), IM (intermediate phenotype), LAZ (length-for-age z-score), NCM (non-classical monocyte).

*Data were not available for all the participants.

APPENDIX 9

Additional Table: Association of mothers' monocyte phenotype percentages and BMI for age z-score (BAZ) at birth.

Variability (%)	BAZ <-2 Median (IQR)	BAZ ≥2 Median (IQR)	Probability
CM_0	72.11 (52.14 – 79.27)	76.43 (62.60 – 83.64)	0.7426
IM_0	6.60 (3.48 – 15.13)	6.62 (3.28 – 9.96)	0.1928
CCR2CM_0	64. 85 (50.32 – 76.02)	69.43 (60.12 – 78.69)	0.5165
CCR2IM_0	3.66 (2.02 – 12.04)	6.67 (3.48 – 15.13)	0.6572
CCR2NCM_0	0.37 (0.20 – 0.65)	0.43 (0.32 – 0.51)	0.7257

Abbreviations: _0 (birth), _10 (10 weeks old), _6 (6 months old), % (percentage), _0 (birth), % (percentage), BMI (body-mass index), BAZ (BMI-for-age z-score), CCR2 (C-C Motif chemokine receptor 2), CM (classical monocyte), IM (intermediate phenotype), IQR (inter-quartile range).
*Data were not available for all the participants.

Additional Table: Association of mothers' monocyte phenotype percentages and weight for age z-score (WAZ) at birth.

Variability (%)	WAZ <-2 Median (IQR)	WAZ ≥2 Median (IQR)	Probability
CM_0	72.09 (62.60 – 78.55)	77. 16 (76.43 – 91.78)	0.1694
IM_0	6.67 (2.20 – 13.58)	3.48 (0.84 – 3.66)	0.2170
CCR2CM_0	65.77 (60.11 – 74.98)	75.59 (69.43 – 86.84)	0.1103
CCR2IM_0	6.60 (2.37 – 12.04)	2.16 (0.84 – 3.48)	0.1949
CCR2NCM_0	0.39 (0.32 – 0.65)	0.32 (0.13 – 0.51)	0.8106

Abbreviations: _0 (birth), _10 (10 weeks old), _6 (6 months old), % (percentage), _0 (birth), % (percentage), CCR2 (C-C Motif chemokine receptor 2), CM (classical monocyte), IM (intermediate phenotype), IQR (inter-quartile range), WAZ (weight-for-age z-score).
*Data were not available for all the participants.

APPENDIX 10

Positive correlations were seen between CM and CCR2CM (rho= 0.7789; p=0.0008). The only other monocyte subset with a positive correlation was between IM and CCR2IM (rho=1.0000; p=<0.0001). In the Additional Table red designates where the p-values were significant.

Additional Table: Correlation between monocyte phenotype percentages and head circumference (HC) of infants.

	HC	CM	IM	NCM	CCR2CM	CCR2IM
HC	1.0000 20					
CM	-0.0779 20 1.0000	1.0000 20				
IM	-0.1766 20 1.0000	-0.4962 20 0.3908	1.0000 20			
NCM	0.5337 20 0.2305	-0.5143 20 0.3052	0.1023 20 1.0000	1.0000 20		
CCR2CM	-0.0841 20 1.0000	0.7789 20 ***0.0008**	-0.4075 20 1.0000	-0.2797 20 1.0000	1.0000 20	
CCR2IM	-0.1766 20 1.0000	-0.4962 20 0.3908	1.0000 20 ***<0.0001**	0.1023 20 1.0000	-0.4075 20 1.0000	1.0000 20

Abbreviations: CM (classical monocyte), HC (head circumference), IM (intermediate phenotype), NCM (non-classical monocyte), CCR2 (C-C Motif chemokine receptor 2).
Description: First row designates rho-values, second row designates amount of infants (n), and third row designates p-value