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Pathirana, Jayani Chitramali

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CONGENITAL CYTOMEGALOVIRUS IN A HIGH HIV PREVALENT SETTING, SOUTH AFRICA

Jayani Chitramali Pathirana

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

Johannesburg, South Africa 2020

Declaration

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

Signature: 

Date: 15 June 2020

Place: Zurich, Switzerland

Dedication

To the mothers of the babies that participated in this study. This work was possible because of your commitment to the better health of your baby

To my husband, Nicholas, for being by my side from the start

To my family, for your patience, support and love

Publications and presentations arising from this PhD and other research outputs

Publications

1. Pathirana J, Groome M, Dorfman J, Kwatra G, Boppana S, Cutland C, et al. Prevalence of Congenital Cytomegalovirus Infection and Associated Risk of In Utero Human Immunodeficiency Virus (HIV) Acquisition in a High-HIV Prevalence Setting, South Africa. Clin Infect Dis. 2019;69(10):1789–96.

2. Madhi SA, Pathirana J, Baillie V, Cutland C, Adam Y, Izu A, et al. An Observational Pilot Study Evaluating the Utility of Minimally Invasive Tissue Sampling to Determine the Cause of Stillbirths in South African Women. Clin Infect Dis. 2019;69(4):S342–50.

3. Madhi SA, Pathirana J, Baillie V, Izu A, Bassat Q, Blau DM, et al. Unraveling Specific Causes of Neonatal Mortality Using Minimally Invasive Tissue Sampling: An Observational Study. Clin Infect Dis. 2019;69(4):S351–60.

Submitted manuscripts

1. Pathirana J, Texeira L, Munian H, Nakwa F, Mayet I, Maposa I, Groome MJ, Boppana S, Madhi SA. Neurological and Growth Outcomes in South African Children with Congenital Cytomegalovirus: A Cohort Study (under review).

2. Pathirana J, Maposa I, Groome MJ, Madhi SA. Association of Cytomegalovirus Shedding in Women with Congenital and Postnatal Cytomegalovirus Infection in South African Infants.

3. Pathirana J, Kwatra G, Mposula N, Groome MJ, Madhi SA. Effect of congenital cytomegalovirus infection on humoral immune responses to select vaccines administered during infancy.

4. Pathirana J, Baillie V, Izu A, Groome MJ, Nzenze S, Chawana R et al. Association of cytomegalovirus infection and attribution to causes of stillbirth and neonatal deaths in South Africa.

Conference presentations

1. The 7th International Congenital CMV Conference & 17th International CMV Workshop, 07-11 April 2019, Birmingham, Alabama, USA. Prevalence of congenital cytomegalovirus Infection and associated risk of in-utero HIV acquisition in a high HIV prevalence setting, South Africa. Oral presentation

2. The 7th International Congenital CMV Conference & 17th International CMV Workshop, 07-11 April 2019, Birmingham, Alabama, USA. Hearing, neurodevelopment and growth outcomes in children with congenital cytomegalovirus in Soweto, South Africa: A prospective cohort study. Poster presentation

3. The 7th International Congenital CMV Conference & 17th International CMV Workshop, 07-11 April 2019, Birmingham, Alabama, USA. Cytomegalovirus Infection in Stillbirths in a Low-Middle-Income Setting, South Africa. Poster presentation

Other Research Outputs

During the course of my PhD studies I have been involved in a number of additional research activities specific to under five mortality and maternal immunisation. I have been part of The Bill & Melinda Gates Foundation (BMGF) funded pilot study by the Respiratory and Meningeal Pathogens Research Unit on the utility of minimally invasive tissue sampling (MITS), to ascertain the causes of stillbirth, neonatal, and childhood deaths in a low and middle income setting. The pilot study on MITS was the precursor to the Child Health and Mortality Prevention Surveillance (CHAMPS) project currently underway. My role was to conduct the MITS, contribute to project administration and review and revise the manuscripts on the MITS results which have been published [1–3].

I served as a coordinator for The Brighton Collaboration and WHO established Global Alignment of Immunisation safety Assessment in pregnancy (GAIA) network, conducting a systematic review to develop a published case definition for "neonatal death" as an adverse event following maternal immunisation[4]. Additionally I served as a GAIA working group member for the "low birth weight"[5] and "neurodevelopmental delay"[6] case definition working groups.

The WHO, supported by The Bill & Melinda Gates Foundation, established the Maternal Immunization and Antenatal Care Situation Analysis (MIACSA) project to identify the knowledge gaps in maternal immunisation delivery strategies in low and middle income countries[7]. I served as a member of the Expert Advisory Panel contributing to surveys and country visits. This work was conducted to support countries prepare for introduction of new maternal vaccines and analysis of the data is ongoing.

Abstract

Introduction: Prevalence of congenital cytomegalovirus (cCMV) is higher in infants born to women with non-primary than primary cytomegalovirus (CMV) infection and can result in debilitating neurological sequelae.

Objectives: To describe the epidemiology of cCMV in an urban, black South African population in a high human immunodeficiency virus (HIV) prevalence setting in the antiretroviral therapy (ART) era. The prevalence of cCMV in HIV-exposed and HIV-unexposed neonates, the occurrence of symptomatic cCMV at birth and neurological sequelae within 12 months of age were determined. Postnatal CMV (pCMV) acquisition in relation to maternal CMV shedding was assessed and vaccination induced immune responses between cCMV, pCMV and CMV-uninfected infants explored. In stillbirths and neonatal deaths aged below 21 days, the prevalence of cCMV and attribution of CMV to foetal and neonatal demise was explored.

Materials and methods: Neonates born at Chris Hani Baragwanath Academic Hospital in Soweto, between May –December 2015 were screened and confirmed for cCMV by testing saliva and urine samples by polymerase chain reaction assay (PCR) within 21 days of birth. Confirmed cCMV cases were matched by gender, gestation and HIV-exposure to cCMV-uninfected neonatal controls in a 1:2 ratio. Controls tested CMV negative on two independent saliva samples within 21 days of birth. Cases and controls were followed up in a matched cohort study until 12 months of age for neurological sequelae (sensorineural hearing loss and neurodevelopment). Controls had saliva tested at two, six and 12 months of age to identify pCMV acquisition. Mothers of cases and controls had a once off breast milk and vaginal fluid tested by PCR for CMV shedding within 21 days. Whole blood from all infants at seven

months of age was tested for antibodies to the primary series of childhood vaccines, combined Diphtheria-Tetanus-acellular Pertussis-Inactivated Polio-*Haemophilus Influenzae* type b-Hepatitis B (DTaP-IPV-Hib-HBV), by an in-house Luminex multiplex immunoassay. In neonates that died within 21 days of birth and stillbirths identified through a mortality surveillance, minimally invasive tissue sampling tested for CMV by PCR, and with review of antemortem clinical data, cause of death ascertained.

Results: Overall cCMV prevalence was 2.5% (95% Confidence Interval [CI] 1.9–3.2). Prevalence of cCMV was higher in children born to women living with HIV (i.e. HIV-exposed, 5.2%, 95% CI 3.8–6.9) than HIV-unexposed (1.4%, 95% CI 0.9–2.0) neonates. Among all HIV-exposed neonates, those with cCMV were 20-fold (odds ratio [OR] 20.1, 95% CI 6.09–66.46) more likely to be infected with HIV *in-utero* (5/27, 18.5%) than cCMV-uninfected infants (8/716, 1.1%). Symptomatic illness occurred in 7% of cCMV cases. Rate of neurological sequelae within 12 months of age was, however, similar in infants with cCMV (6%) compared to controls without cCMV infection (4%, OR 4.0, 95% CI 0.36-44.11), albeit with wide intervals of statistical uncertainty. Of the cCMV-uninfected, 79.7% acquired CMV postnatally by 12 months of age, which was unrelated to maternal CMV shedding within 21 days postpartum. The immune responses to the primary series DTaP-IPV-Hib-HBV vaccine was similar in cCMV, pCMV and CMV-uninfected infants at seven months of age. CMV was identified in 11.8% of stillbirths and neonatal deaths (<21 days age), however, cCMV was attributed as the cause of death in 3.9% of stillbirths and none of the neonates.

Conclusion: cCMV rates was higher in neonates born to women living with HIV than in HIV-unexposed neonates, even in the era of ART. Vertical HIV transmission was more frequent in neonates with cCMV, suggesting cCMV co-infection may account for a large fraction of the residual maternal to foetal HIV transmission in populations with efficient ART

programs. Neurological sequelae within one year did not differ between cCMV infected and uninfected children. CMV infection was higher in neonates dying within 21 days of birth and stillbirths than in live births; although clinical review of the cases did not attribute CMV to be in the casual pathway of neonatal deaths and in only 3.9% of stillbirths. Follow-up of cCMV-infected infants into childhood is required to determine long term neurological sequelae and whether a universal or targeted cCMV screening programme in newborns would be of value in South African populations.

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Abbreviations

AABR	Automated auditory brainstem response
ART	Antiretroviral therapy
aOR	Adjusted odds ratio
AU	Arbitrary units
ART	Antiretroviral therapy
cCMV	Congenital cytomegalovirus
CCR5	C-C chemokine receptor type 5
CD4	Cluster of differentiation 4
CHBAH	Chris Hani Baragwanath Academic Hospital
CI	Confidence interval
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
dBHL	Decibels hearing level
dBnHL	Decibels normal hearing level
DBS	Dried blood spot
DeCoDe	Determination of Cause of Death
DNA	Deoxyribonucleic acid
DPOAE	Distortion product otoacoustic emissions
FTD	Fast Track Diagnostic
gB	Glycoprotein B
gH/gL	Glycoprotein H and L complex
gM/gN	Glycoprotein M and N complex
H&E	haematoxylin and eosin
HIV	Human immunodeficiency virus
HREC	Human Research Ethics Committee
ICD-10	International Classification of Diseases, 10th revision
ICD-PM	International Classification of Diseases for deaths during the perinatal period
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IQR	Interquartile range
IUGR	Intrauterine growth restriction

kbp	Kilo-base pair
LMIC	Low and middle income countries
NHLS	National Health Laboratory Service (South Africa)
pCMV	Postnatal CMV
OAE	Otoacoustic emissions
OR	Odds ratio
RMPRU	The Respiratory and Meningeal Pathogens Research Unit
SARChI	The South African Research Chairs Initiative
SGA	Small for gestational age
SNHL	Sensorineural hearing loss
WHO	World Health Organisation

1 Introduction and Literature review

Introduction

Congenital cytomegalovirus (cCMV) is the most common congenital infection worldwide[8]. It is the leading cause of non-genetic sensorineural hearing loss (SNHL) in childhood and an important cause of neurodevelopmental delay[9]. Despite being a significant infectious cause of childhood disability, cCMV is often overlooked by health care workers and parents alike. Clinicians may not recognise the value of counselling pregnant women on prevention due to a lack of knowledge on the mode of person to person transmission and risk factors for foetal transmission[10,11]. Surveys of women showed even though cytomegalovirus (CMV) is the most common congenital infection, women are more aware of other congenital conditions and infections such as Human Immunodeficiency Virus (HIV), neural tube defects, foetal alcohol syndrome, Down's syndrome, and toxoplasmosis which are less common than cCMV[11,12]. A contributing factor to the overall lack of awareness is that cCMV is asymptomatic at birth in 85-90% of infants and symptomatic in 10-15% with most symptoms being mild and transient[13]. Both asymptomatic and symptomatic infants with cCMV, however, can go on to develop severe neurological disability later in life, which by then may not be linked to cCMV, unless a stored birth sample of body fluid (saliva, urine or blood) is available for retrospective testing. Studies from high income countries and from a few low income countries indicate cCMV is disproportionately more prevalent in low income populations, in Black than White or Asian races[9,14] and in infants that are HIV-exposed (children born to women living with HIV who are not themselves HIV-infected) or infected with HIV[15]. As such, the population of children most at risk of cCMV and subsequent neurological sequelae are in sub-Saharan Africa. In most low income countries, diagnostics for CMV and most other viral infections are unavailable in the public health sector[16], leaving the most vulnerable and disadvantaged

children without the opportunity for appropriate diagnosis and management and at risk of severe and permanent neurological sequelae. Families of neurologically disabled children are more likely to experience poverty, social discrimination and poor physical and mental health[17]. Thus, there is a great need to determine the epidemiology of cCMV in low income African settings to recognise the importance of early identification of children with and at risk of neurological sequelae and implement public health programmes for diagnosis, long term management and public education.

Literature review

1.1 The virus

Human CMV is a member of the Herpesviridae family belonging to the subfamily of Betaherpesvirinae, with large and linear, double stranded DNA approximately 125–290 kbp in size. The virus is covered by a proteinaceous matrix followed by a lipid envelope containing membrane associated proteins[18].The viral genome is arranged into long and short unique regions coding at least 19 different envelope proteins[19]. Of these, five glycoproteins are essential for virus replication and cell entry which include glycoprotein B (gB), glycoprotein M and N complex (gM/gN) and glycoprotein H and L complex (gH/gL)[19]. Distinct genotypes of CMV have been isolated from different individuals and mixed infection with multiple CMV strains can occur[20]. Human CMV is only capable of replicating in human cells[21] and can effectively spread to any tissue and establish a lifelong infection in the host. Infected cell types include fibroblasts, epithelial cells, endothelial cells, leukocytes, smooth muscle cells, brain stem cells and organ specific cells[18]. Consequently, CMV is highly infectious and can be transmitted through viral shedding in essentially all body fluids including saliva, urine, blood, tears, semen, breast milk and cervical fluid, spreading to all major organs (brain, liver,

lungs, kidneys)[22]. Viral spread can also occur vertically from mother to foetus by infection of the placenta[23,24].

1.2 CMV immunology

Over time, CMV has co-evolved with humans enabling the development of sophisticated immune evasion methods, resulting in the host being unable to clear CMV infection[25,26]. Upon primary infection, the virus first infects monocytes and $CD34^+$ cells, thereafter infecting haematopoietic and endothelial cells allowing systemic spread[18,26]. During viral dissemination, the innate immune system mounts a response which includes production of cytokines, type I interferon and upregulation of costimulatory molecules which are effective in slowing viral spread and concurrently primes a huge adaptive immune response in the immunocompetent host.

The adaptive immune response to CMV elicits robust cellular and humoral immune response[23,27]. This is necessary to maintain CMV latency, prevent cellular damage and viral replication and to enable rapid responses to recurrent CMV infection with new viral strains or reactivation of latent infection. The CMV specific T-cell response is both large in number and long-lasting with both CD4+ and CD8+ T-cells responding to infection[26,27]. Following control of the primary infection, the circulating CMV-specific memory T-cell compartment expands, comprising 10.0% of both CD4+ and CD8+ memory cells in peripheral blood with further increase in recruitment of T-cells with age[26,27]. This has been proposed to contribute to the immunosenescence seen in the elderly who manifest sub-optimal immune response to vaccination and new infections[28]. The humoral immune response to CMV infection target several CMV epitopes including structural, non-structural and envelope proteins; although the mechanism of how this is achieved is still unclear[26]. The antibody responses, which are strain

specific (Immunoglobulin M [IgM] and Immunoglobulin G [IgG]), may play a role in limiting the clinical manifestation of CMV disease and spread of the virus through neutralising activity against gB and gH and against cell entry[27]. Despite the robust human immune response in controlling CMV replication, immunity does not prevent reactivation of latent CMV infection; and immunity to one strain of CMV is not protective against reinfection with a new strain, with multiple strains identified in both adults and newborns[20]. Reactivation and reinfection of CMV is collectively referred to as recurrent or non-primary CMV infection. CMV reactivation is defined as the detection of a viral strain in a previously CMV-infected individual to be indistinguishable from the original CMV strain either by sequencing specific regions of the viral genome or by using a variety of molecular techniques that examine genes known to be polymorphic[29]. Evidence of reinfection includes the presence of new antibody specificities to virally encoded envelope glycoproteins in addition to molecular data indicating the acquisition of a new strain of virus[30]. Complete clearance of the virus is not yet achieved in humans due to the immune evasion strategies used by the virus, which include[25–27]:

1. Interference with the cellular immune responses by:
    a. Evasion of Natural Killer cell recognition
    b. Prevention of antigen presentation to T-cells
2. Blocking of intrinsic cellular defences (apoptosis, interferon production and protein synthesis)
3. Encoding chemokines, chemokine receptors, and cytokine which mimics host interleukins and binds to receptors with immunosuppressive effects

1.3 CMV infection in the immune immature and immunocompromised hosts

CMV replication is controlled by the host immune system and any disturbance to this balance can result in clinical disease from viral reactivation. Population groups particularly vulnerable to developing CMV disease include foetuses, prematurely born neonates, pregnant women, the immunocompromised, geriatrics and sufferers of chronic disease.

1.3.1 CMV infection during pregnancy and foetal CMV transmission

Pregnancy induces an altered immune state with tolerance for the semi-allogenic foetus. Coupled with viral immune evasion mechanisms and immune tolerant conditions, pregnant women are at increased risk of CMV infection, reactivation and/or reinfection with a new viral strain and subsequent transmission to the foetus[31]. *In-utero* transmission of CMV to the foetus is considered to be due to direct infection of the placenta and less commonly, from ascension of the virus from the genital tract, although the exact mechanism is yet to be elucidated[32]. Maternal endometrial basal decidual cells become CMV-infected, although the mechanism through which this occurs is unclear and may be a result of maternal viraemia[24,33]. Foetal infection is facilitated by placental cytotrophoblastic cells which invade the basal decidua and are subsequently infected by CMV where it replicates and spreads to placental trophoblast progenitor cells, syncytiotrophoblasts and endothelial cells which results in local placental inflammation with leukocyte infiltration and cytokine induction leading to placental and foetal damage[34]. The extent of placental inflammation which predisposes to causing foetal damage in unclear, however, the local placental inflammation may contribute to the intra-uterine growth restriction observed in some CMV-infected foetuses[34,35]. cCMV transmission occurs more frequently in mid to late than early gestational age[32]. This is thought to be due partly to the neonatal Fc receptor (FcRN) and Epidermal growth factor receptor (EGFR)

being fully expressed in late gestation, which are utilized by CMV virions as a transport pathway to the placenta in women with weakly neutralising IgG activity against CMV[36]. . Transmission rates are also higher following primary rather than after non-primary maternal infection, indicating that previous maternal immunity has a protective role in prevention of viral transmission[33,37].

Functional immaturity of the foetal immune system, depending on the gestational age at infection and sub-optimal or lack of maternal protective antibody, can result in clinical disease identified either during foetal antenatal scans or at birth. Although the majority of cCMV-infected foetuses are asymptomatic at birth, they may occasionally present with severe disease and neurological impairment[26,33]. Foetuses that become CMV-infected in early pregnancy (<16 weeks) are more likely to have symptomatic disease than those that become infected later in pregnancy due to the immaturity of their immune system[38]. Similarly, preterm and very low birth weight newborns may present with or develop symptomatic cCMV infection as they may have been CMV-infected earlier in pregnancy or have insufficient maternal immunity to control viral replication. These neonates may also acquire CMV postnatally before their immune system has sufficiently matured and are at risk of developing CMV disease[32,39].

1.3.2 Foetal CMV transmission in women living with HIV

Women living with HIV have a higher risk of *in-utero* CMV transmission to their foetus than women without HIV infection[15,40]. This is likely due to poorly controlled CMV replication and dissemination from the depletion of maternal CD4+ cells and dependent neutralising antibody response which has been demonstrated in both humans and rhesus monkey models[41–43]. A further description of CMV infection in the context of HIV-infection and HIV-exposure is provided in section 1.6.6.

1.4 Epidemiology of congenital CMV infection

1.4.1 Maternal CMV infection

The population prevalence of CMV infection is associated with socioeconomic status, living conditions, and differs by race-group, as demonstrated in the US National Health and Nutrition Examination Survey of individuals over six years[44]. The age-adjusted CMV seroprevalence for individuals older than six years was 58.9%. Population groups with lower socioeconomic status (indicated by level of education, household income and type of medical insurance), had significantly higher prevalence of CMV (85.0% in populations having completed 8th grade or less) than higher socioeconomic status groups (46.1% in populations completing college)[44]. In women of reproductive age (12-49 years), the overall CMV IgG seroprevalence was 58.0%[45]. When women were stratified by race, Non-Hispanic Black race had the highest prevalence of CMV (78.0%) compared to Mexican Americans (75.0%) and White Americans (42.0%)[44]. In a Japanese homogenous population, a survey of 7074 pregnant women attending a maternity hospital found a CMV seroprevalence of 69.1% [46], which is within prevalence range found in the various racial groups of American women.

In low and middle income countries (LMIC) and low-income groups within high income countries, the seroprevalence of CMV infection is higher than in high income populations. In a population based study of low to middle socioeconomic status pregnant women in Brazil, high CMV seroprevalence (>96.0%) were found in all ages studied (12-46 years)[47]. A 1992 South African study of 2250 pregnant women attending Johannesburg General Hospital, found an overall CMV seroprevalence of 73.7% in women of Caucasian descent compared to 99.5% in Black-African women, 99.4% in mixed race women and 99.6% in Asian women[48]. In LMIC, over 90.0% of individuals are CMV-infected by adolescence or earlier with individuals living with HIV also at risk of acquiring CMV early[49]. In The Gambia, 86.0% of

children were CMV-infected by one year of age in both urban and rural settings[50]. A study of Malawian children living with HIV found 100% had acquired CMV infection between one and five years of age[51]. Risk factors for early childhood infection include exposure to individuals shedding CMV in body fluids, with the force of infection increasing when living in crowded homes, frequent exposure to other young children, and consumption of CMV-infected breast milk[49,52,53]. In contrast, primary CMV infection usually occurs in adulthood and during pregnancy from exposure to young children in high-income settings, especially if children attend day care or the woman is a day care worker, as well as through sexual activity[54,55].

1.4.2 Congenital cytomegalovirus infection

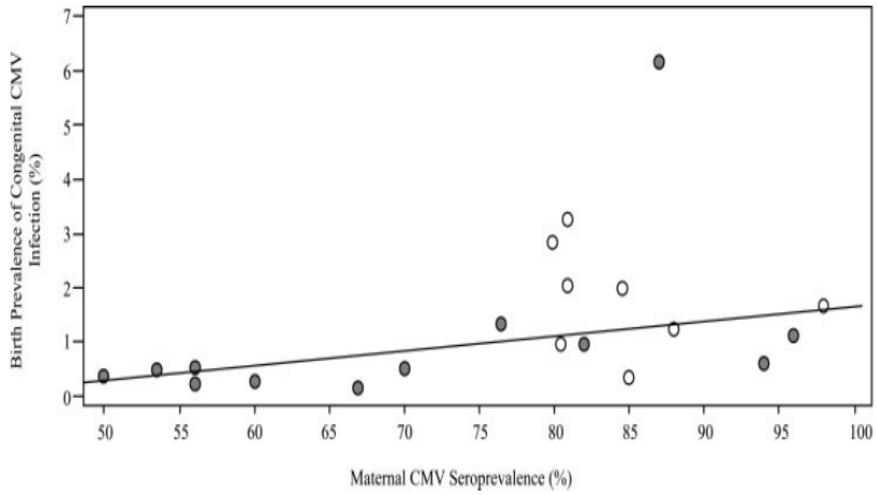
cCMV occurs due to transplacental transmission of CMV to the foetus, with transmission rates varying with gestation and whether infection in the mother was primary or non-primary, with no seasonal variation in rates of infection[56,57].

Gestation at CMV transmission

The rate of intrauterine transmission of CMV is related to gestational age, with significantly higher rates of transmission to the foetus occurring following primary maternal infection in late-stage pregnancy [58–60]. In Italian pregnant women, foetal CMV transmission rate was directly correlated with gestational age at the time of maternal infection (P=0.025); with transmission rates ranging from 8.8%, 19.0%, 30.6%, 34.1% and 40.0% if maternal CMV infection occurred in the pre-conceptional period, peri-conceptional period, first trimester, second trimester and third trimester of pregnancy respectively[60]. Similarly, a retrospective study from 1988 to 2009 of 524 women with primary CMV infection, reported intrauterine transmission rate of 34.5%, 44.1% and 73.3% following CMV infection in the first, second and third trimester, respectively[58,59].

CMV transmission rates between primary and non-primary maternal infection

Transmission rates are higher following primary maternal CMV infection (30 -35%) than with non-primary maternal CMV infection (1.4%)[37], possibly due to the role of pre-existing maternal immunity protecting against transmission. In a study of 123 women identified with primary CMV infection during pregnancy in Belgium, 57.5% transmitted CMV to the foetus[59]. In an Italian study, a foetal infection rate of 24.9% was observed in 241 women with primary CMV infection[60]. The difference in transmission rates between women with pre-conceptual CMV immunity (0.5%) and those with primary infection (20%) during pregnancy has also been reported in Brazilian women who have a high seroprevalence for CMV[61]. Despite the greater rate of cCMV transmission following primary maternal infection, the overall prevalence of cCMV infection increases in settings with high maternal CMV seroprevalence (Figure 1.1, source Kenneson et al [2017])[30,37,62]. This is due to: 1) in high seroprevalence populations, more women are at risk of viral reactivation; 2) the greater reservoir of circulating virus in populations with high CMV seroprevalence results in more frequent exposure to CMV-infected fluids and viral reinfection during pregnancy[37].



Prevalence categorised by ascertainment time: prenatal ascertainment (filled circles) and birth ascertainment (open circles). Line is the linear regression of congenital CMV birth prevalence on maternal CMV seroprevalence

Figure 1.1: Kenneson et al (2007). Review of congenital CMV infection birth prevalence against maternal CMV seroprevalence. Reproduced with permission from John Wiley and Sons (Appendix 8).

Congenital CMV prevalence in different settings

There is a wide variation in the prevalence of cCMV infection between populations as well as variation within a population. Factors contributing to this variation include race, socioeconomic status, and HIV infection and HIV exposure increasingly also being recognised as important risk factors for cCMV infection[9,15,37,49,63].

Global prevalence of cCMV

Kenneson and Cannon (2007) conducted a systematic review and meta-analysis of the global prevalence of cCMV infection based on 27 studies which utilised viral culture methods for CMV infection confirmation[37]. Including studies conducted in LMIC and high income countries (including different sites within a specific population) from 1968 to 2003, they estimated a cCMV prevalence of 0.64% (95% confidence interval [CI] 0.60–0.69). Data from the African continent in this systematic review was, however, limited to a single study from Ivory Coast which reported a cCMV prevalence of 1.4% in women with non-primary CMV infection[64]. The overall prevalence of cCMV in this systematic review of 0.64% may have been an underestimate due to culture rather than PCR being used for detecting CMV infection. Furthermore, the overall prevalence of symptomatic cCMV was 0.07% (range: 0.0-0.22%), albeit with heterogeneity across the studies in the definition of symptomatic infection.

Congenital CMV infection prevalence in high income countries

Several cCMV prevalence studies have been conducted in high-income countries including Australia, several European countries, the USA and Japan. Within the USA, varying prevalence of cCMV has been reported, even when adjusted for risk factors associated with cCMV such as socioeconomic status and race[14]. In a large multicentre study across seven states that screened 100332 newborns, the overall prevalence of cCMV was 0.45% with

significantly higher prevalence in Black infants (0.95%), followed by multiracial infants (0.78%), Hispanic White infants (0.30%), non-Hispanic White infants (0.27%) and Asian infants (0.1%, $p<0.001$)[14]. Similarly, an early study in London, UK, also reported Black infants to have higher prevalence of cCMV, after adjusting for maternal age, socioeconomic status and parity with 0.81%, 0.25% and 0.19% prevalence in Black, White and Asian infants, respectively[65].

Several Western European countries have described the prevalence of cCMV infection including France, Italy, Sweden, Switzerland, Germany, Belgium and The Netherlands. The majority of these studies are on women with primary CMV infection who seroconverted during pregnancy. In France, a cCMV prevalence of 0.37% was found following screening of 11715 newborns, where 52.0% of mothers with cCMV-infected newborns had primary infection and 48.0% had non-primary CMV infection[66]. In The Netherlands, a retrospective study of dried blood spot (DBS, source of blood not specified) testing for cCMV identified 156 cases from 31483 children, i.e. prevalence of 0.5%[67]. Similarly, in Sweden a prevalence of 0.5% was found in a prospective cohort study which screened 16474 infants[68]. In this study, approximately 50.0% of women who transmitted CMV to the foetus had primary infection. An Italian study from 2002-2003, screened 9032 newborns in five regions by testing of DBS (source of blood not specified). Sixteen (0.18%) infants were confirmed as cCMV, of whom 12.5% were born to non-Italian mothers[69]. The CMV seroprevalence was measured in a subset of 1200 women, indicating 79.9% to be seropositive.

In high-income countries, the difference in cCMV prevalence between HIV-exposed and HIV-unexposed neonates has not been well characterised, possibly due to low prevalence of HIV-infection in pregnant women. Also, maternal HIV-infection being associated with increased risk for *in-utero* CMV transmission to the foetus has only recently been

highlighted[70] with most studies on cCMV in high income countries done prior to 2006, before the widespread use of antiretroviral therapy (ART) in pregnant women.

Congenital CMV infection prevalence in low and middle income countries

There are very few well-designed, population based studies on prevalence of cCMV in LMIC despite the likelihood of higher rates in these settings. A 2013 review identified 11 LMIC that reported on cCMV, including from Ivory Coast, The Gambia, China, Korea, Taiwan, Chile, Brazil, Mexico and Panama; with prevalence ranging between 0.6% (Panama) to 6.1% (China)[49]. One of the largest population based screening studies was conducted by Mussi-Pinhata et al (2009) in Brazil, where 12195 newborns were screened for cCMV by DNA detection in saliva and 1% were congenitally infected[71]. This study confirmed that in populations where maternal CMV seroprevalence is close to 100%, the rate of cCMV is higher than found in high income countries (where primary maternal infection is responsible for most cCMV infections). A high rate of cCMV (2.1% of 243) was also reported in newborns from rural India, against a background of 99.0% maternal CMV seroprevalence[72]. In The Gambia, a cCMV prevalence of 5.4% of 741 newborns screened in a rural village in 2002 was found, which was lower than observed in an earlier study done in 1990 that included an urban and rural setting (14.0% of 178 newborns)[50,56].

Congenital CMV prevalence in neonates with HIV exposure and HIV infection

In individuals living with HIV with CD4+ cell counts <100 cells/µl, and those with other immunosuppressive conditions such as solid organ transplantation recipients on immunosuppressive therapy, CMV may cause important opportunistic disease[73]. In such individuals, systemic disease following viral reactivation or reinfection can result in end organ disease (pneumonia, gastrointestinal disease, hepatitis, retinitis, encephalitis) in one or multiple organs[29]. Pregnant women living with HIV who are not on ART or are not

adequately virally suppressed, have higher *in-utero* CMV transmission rates to the foetus than the general population of pregnant women with variation between studies[15,74–76]. Not only does maternal HIV-related immunosuppression facilitate CMV transmission to the foetus due to uncontrolled viral replication, but CMV has also been considered a co-factor of vertical HIV-transmission from mother to foetus *in-utero*[15]. A direct comparison of cCMV transmission between women living with and without HIV infection is yet to be conducted, and the mechanisms underpinning the association of heightened susceptibility to dual *in-utero* HIV and CMV acquisition remains to be elucidated.

Prior to the availability of combination ART, three studies investigated whether the prevalence of cCMV infection in infants with HIV exposure and with HIV infection was higher than in HIV-unexposed infants. One of the first studies was conducted in Texas, USA from 1988-1995[74]. Over the study period, 154 HIV-exposed newborns were screened for cCMV infection by urine culture within three weeks of birth and HIV-infection by blood polymerase chain reaction (PCR). The overall prevalence of cCMV infection was 6.5% (n=10) [74] which was higher than the prevalence in the general neonatal population of 0.45% in the USA[14]. The rate of HIV-infection was 1.9-fold higher in the cCMV-infected (50.0%) than cCMV-uninfected HIV-exposed newborns (3.5%, P=0.008) [74]. Only 17.0% of the mothers were on Zidovudine prophylaxis, with a mean CD4+ cell count of 422 and 427 in the mothers of neonates with HIV infection and HIV exposure respectively[74].

A second American study of HIV-exposed infants from 1990-1997, of whom 52.0% were Black and 30.0% were Hispanic, found a cCMV prevalence of 4.3% in neonates with vertical HIV acquisition and 4.5% in HIV-exposed uninfected neonates[76], which was again higher than the general population prevalence in the USA (0.45%)[14]. When considering maternal

immune status, mothers of infants with cCMV infection had significantly lower mean CD4 counts (371 vs. 507 cells/mm^3, P=0.003).

A study in a low-income, high CMV seroprevalent population in Brazil also investigated the cCMV prevalence between HIV-exposed and HIV-unexposed neonates[77]. In this study, which included 150 mother-newborn dyads with HIV infection and 175 mother-newborn dyads without HIV infection, the prevalence of cCMV was 2.7% and 2.9%, respectively. None of the cCMV-infected neonates were infected with HIV. There was no difference in clinical CMV disease between infants of women with and without advanced HIV-disease and AIDS. The absence of significant difference in cCMV prevalence between HIV-exposed and HIV-unexposed infants was postulated to be due to women living with HIV being relatively immunocompetent (although immune status was not investigated).

Following the introduction of ART to prevent mother-to-child transmission of HIV (PMTCT), two studies described the impact of ART on cCMV infection. In France, a prospective cohort of pregnant women living with HIV and their offspring were enrolled across 96 centres[78]. The observations were stratified by the pre-ART era (1993-1996) and the ART era (2001–2004). The prevalence of cCMV declined from 3.0% in the pre-ART era to 1.5% in the ART era (P<0.001). The prevalence of cCMV infection was 4.7-fold higher among infants who acquired HIV vertically (10.3%) than HIV-exposed uninfected infants (2.2%, P<0 .001), regardless of the ART era. The severity of maternal immunosuppression was associated with cCMV infection, with women with CD4+ cell count <200 cells/mm^3 at delivery more likely to give birth to a newborn with cCMV (P<0.002). Women who initiated ART during the second trimester, were also more likely to give birth to cCMV-infected infant than those initiated on ART during the first trimester, (P<0.007).

A similar study in Los Angeles, USA, however, did not demonstrate any changes in cCMV prevalence (overall 3.6%) in infants born to women living with HIV between the pre-ART and ART eras in a study spanning from 1988 to 2002[79]. There was also no significant association between cCMV infection and infant HIV-status nor with maternal CD4+ cell count. A second study of HIV-exposed neonates in Texas, USA found a cCMV prevalence of 3.0% in 367 neonates tested, of whom none were infected with HIV[75]. The mothers of CMV-infected neonates were, however, more likely to have been first diagnosed with HIV during pregnancy or delivery (80.0% vs. 40.0%, P=0.03) and had higher median HIV viral load (15411 vs. 2209 copies/ml, P=0.02).

Studies of cCMV infection in HIV-exposed infant populations in sub-Saharan Africa have only recently been conducted. A study of Zambian newborns admitted to a referral tertiary neonatal unit from 2012-2013, tested newborn sera, saliva and urine by PCR for cCMV and reported an overall cCMV prevalence of 3.8% (15/395)[80]. Prevalence in HIV-exposed newborns was 11.4% (9/79) and in HIV-unexposed newborns was 2.1% (6/293); with HIV-exposure being an independent risk factor for cCMV infection (odds ratio [OR] 6.7, P=0.001). A Kenyan study of neonates born to women living with HIV on perinatal Zidovudine, identified a cCMV prevalence (based on PCR testing for CMV DNA in cord blood plasma) of 29.0% (4/14) and 2.7% (1/37) in neonates who were perinatally infected with HIV compared to those not infected by HIV respectively[81]. These findings were similar to that observed in Thailand from 1997-2001, where cCMV infection was significantly higher in HIV-exposed neonates who acquired HIV vertically (14.0%) than those who did not (3.0%) [70]. Women in this study were on a trial of either short or long duration of Zidovudine monotherapy during pregnancy.

Although HIV prevalence in South African pregnant women is one of the highest in the world (29.9%)[82], there have only been two published studies that evaluated the association between cCMV and HIV exposure. In an urban Western Cape population, with approximately 50.0% mixed race and 50.0% black Africans, a cCMV prevalence of 2.9% was reported in 748 HIV-exposed neonates[42]. Maternal CD4+ count <200 cells/µl was independently associated with cCMV[42]. The rate of HIV-infection and the clinical outcomes in these infants as well as the prevalence of cCMV infection among HIV-unexposed neonates were not investigated. A study in the rural Eastern Cape of 302 neonates found a cCMV prevalence of 5.96%, and reported no difference in prevalence between HIV-exposed and HIV-unexposed neonates, although specific prevalence rates in each group was not reported[83].

Risk factors for congenital CMV Infection

Several risk factors for cCMV have been identified, most of which are related to maternal social circumstances. Pregnant women living with HIV have an increased risk of *in-utero* transmission of CMV to the foetus, even with pre-conceptual immunity[40,43]. Low maternal CD4+ cell count (<200), high HIV-viral load and the delayed initiation of ART in women living with HIV have been independently associated with cCMV infection, albeit with conflicting findings[15,42,75,78]. Placental CMV infection may also act as a co-factor for HIV transmission to the foetus, whereby placental inflammation and inhibition of local antiviral responses helps placental HIV replication leading to foetal transmission[84].

Women with sexually transmitted infections due to gonorrhoea, trichomoniasis, or bacterial vaginosis also have an increased risk of intrauterine CMV transmission[85]. In The Gambia, placental malaria parasitaemia was associated with cCMV infection, thought to be due to direct inflammatory effect of malaria on the placenta facilitating CMV transmission[56]. High maternal CMV seroprevalence, young maternal age (<25 years), single marital status, black-

race, low parity and low socioeconomic status are also reported risk factors for cCMV, particularly in settings with non-primary maternal CMV infection [37,54,65,66]. In populations where maternal primary CMV infection is the dominant cause of cCMV transmission, however, high socio-economic status and high parity are risk factors for transmission[66,86,87]. In addition, caring for children younger than six years old and living in a crowded household are independently associated with cCMV infection in women with non-primary and primary CMV infection[9,37,54,66].

1.5 Clinical features of maternal CMV and congenital CMV infection

1.5.1 Pathogenesis of symptomatic CMV infection

The pathogenesis of CMV infection and manifestation of disease is thought to occur once a threshold viral load is surpassed eliciting a cycle of events which may result in end-organ disease due to lytic infection[88]. Susceptible cells such as fibroblasts and smooth muscle cells develop a cytomegalic appearance during uncontrolled viral replication and ultimately undergo lysis[19]. The cytopathic effect on infected cells during the early phase of replication is due to an increase in the size of nucleus and a rounding of the cell whilst in the late phase, granular or dense intracytoplasmic and intranuclear inclusion bodies appear with enlargement of the cell due to the increased cell volume[19,89]. Final lysis occurs from disintegration of the nuclear membrane, virus assembly sites displacing normal cellular secretory pathway organelles, and disruption of the plasma membrane[90]. CMV was thus named for its effect on cell morphology.

1.5.2 Clinical features of maternal CMV infection

CMV infection is usually subclinical in healthy women although descriptions of an infectious mononucleosis like syndrome has been described, which include symptoms of fever,

pharyngitis, cervical adenopathy, fatigue, malaise, myalgia, headache, hepatosplenomegaly and rash[86,91]. The manifestation of primary and non-primary CMV infection vary as reported by Nigro et al (2003)[92]. Women with primary infection had significantly higher occurrence of symptoms (59.8%), than women with recurrent infection (19.0%) and non-active infection (i.e. latent, 11.9%, P<0.001); with the occurrence of a flu-like syndrome occurring in 31.4% of women with primary infection. Additionally, women with primary infection showed a significantly increased rate of lymphocytosis (39.2% vs 5.7% or 3.7%, respectively, P < 0.001) and elevated aspartate aminotransferase and/or alanine aminotransferase levels (35.3% vs 3.9% or 0.9%, respectively, P < 0.001). There was no significant difference in clinical manifestations and laboratory abnormalities between women with recurrent infection and those with non-active infection. The manifestation of symptoms, however, vary between studies with 21.0% of French compared to 73.5% of Italian pregnant women manifesting symptoms after primary CMV infection[60,93].

The majority of women in low income populations are likely to have pre-conceptual CMV infection, with reactivation or reinfection of CMV during pregnancy. The extent to which women with non-primary CMV infection experience any symptoms during pregnancy from reactivation or reinfection is unclear. An Israeli and Japanese study reported that 8.3% (1 of 12)[94] and 16.9% (218 of 1287)[95] of women with recurrent CMV infection had flu-like illness or fever respectively whilst data from African populations is lacking.

1.5.3 Symptomatic congenital CMV infection

Signs of cCMV disease can be identified *in-utero* through foetal anomaly scans, which are more common when foetal infection occurs during the first trimester. Antenatal ultrasound imaging techniques have the capability of identifying both cerebral and extra-cerebral signs of cCMV. Cerebral abnormalities include microcephaly, periventricular calcifications or

echogenicity, parenchymal echogenic foci, ventricular dilatation, pseudocysts, abnormal gyral development, and hypoplastic corpus callosum[96]. Extracerebral signs of foetal infection include hyperechogenic bowel, enlargement of the liver and spleen, ascites, hydrops, pericardial effusion, placental enlargement, oligohydramnios and polyhydramnios [18,96,97]. Intrauterine growth restriction (IUGR) may also be noted, although other maternal, placental and foetal causes of IUGR need to be excluded.

In a prospective study of foetuses with cCMV, mainly following maternal primary infection, 30.8% (8/26) had antenatal ultrasound abnormalities[98]. The most frequent finding was abdominal hyperechogenicity (50.0%) followed by IUGR (37.5%) In another study, among 600 foetuses born to women with primary CMV infection, 8.5% had abnormal ultrasound findings, of whom 45.0% had cCMV[99]. In the remaining 92.0%, with normal ultrasound, 24.0% also had cCMV. The most common ultrasound abnormalities observed were hyper-echogenic bowel (30.0%), ventricular dilation (30.0%) and IUGR (13.0%). In the foetuses with ultrasonographic abnormalities, 70.0% were symptomatic at birth or autopsy (elective termination of pregnancy).

Foetuses infected with CMV may also have a higher risk of preterm birth, although this remains uncertain, and preterm birth is not considered a symptom of cCMV. The prevalence of cCMV infection was 2.1% in preterm compared to 1.8% in term Brazilian neonates[100]. In Iowa, USA, the prevalence of cCMV was 0.3% (2/589) in preterm neonates and 0.48% in the general population[101]. In another US population (Alabama), the prevalence of cCMV infection in very low birth weight (VLBW) neonates was 0.4% compared to 0.5% in term infants[102]. The cCMV-infected, VLBW infants had significantly increased rates of SNHL and poor motor development compared to VLBW infants without cCMV[102].

At birth, symptomatic newborns may present with non-specific symptoms such as poor feeding, lethargy and hypo/hyperthermia[18]. More frequent clinical symptoms at birth include petechiae, jaundice and hepatosplenomegaly[13,18,103]. On laboratory investigation, hyperbilirubinaemia (direct/conjugated bilirubin>2 mg/dL) and thrombocytopenia (platelet count <100 000/mm^3) are further indicators of cCMV infection[103]. Central nervous system (CNS) or neurological signs include microcephaly, hearing impairment, chorioretinitis and/or optic atrophy, seizures and hypotonia[9,13,18]. One or more neurological signs may be present at birth, which may result in permanent long-term neurological sequelae. Even in symptomatic infants without CNS involvement at birth, a delayed onset of neurological sequelae can occur[13]. Neurological sequelae include unilateral and/or bilateral SNHL, visual impairment, cognitive impairment and/or developmental delay and motor impairment which includes cerebral palsy[63]. A systematic review and meta-analysis conducted in 2007 estimated permanent neurological sequelae to occur in 40-58% of symptomatic infants[63]. In the symptomatic infants, the prevalence of permanent SNHL was estimated to occur in 27.0-50.0%, cognitive and/or motor impairment in 50.0% and visual impairment in 22.0%[9,13,63].

In neonates with cCMV following maternal primary CMV infection, the majority (85.0-90.0%) are asymptomatic at birth[13]. The remaining infants will manifest clinical symptoms at birth ranging from mild, transient and non-specific findings to multi-organ system involvement and at times death[13]. Until recently, most studies were conducted in infants born to women with primary CMV infection, leading to the perception that symptomatic cCMV almost always occurs following maternal primary CMV infection during pregnancy[104]. This was supported by early studies which indicated symptomatic cCMV to be low (0-1%) in neonates born to women with pre-existing CMV immunity[104–106]. In a more recent study however, symptomatic cCMV occurred at a similar frequency in neonates born to women with pre-conceptual immunity to CMV (17.0%, 8/47) and those born to women with primary

CMV infection (17.0%, 8/47)[103]. Furthermore there was no difference in the occurrence of SNHL, mental retardation, motor impairment, chorioretinitis or seizures when followed through to 43 months of age[103].

A Finnish study of 19 868 neonates screened for cCMV at birth, reported 10.0% (4/40) of cCMV were symptomatic at birth, of whom only one was clinically symptomatic with microcephaly, whilst three had cerebral calcification identified by ultrasound scan[107]. Maternal non-primary CMV infection was the pathogenesis of cCMV in 53.0% of these cases. This study also demonstrated a similar frequency of symptoms in neonates born to women with primary and non-primary CMV infection as two each (10.0%) of the symptomatic neonates were born to women with primary and non-primary infection, and the severity of symptoms was dependent on the gestation at which CMV was transmitted to the foetus. This was one of few studies that included a control group to compare differences in long term outcomes between cCMV and cCMV-uninfected infants, with no difference in SNHL and neurodevelopmental delay through to 18 months of age.

A challenge in accurately quantifying the burden of symptomatic infection at birth is the varying definitions used to define symptomatic infection. Some investigators have defined symptomatic infection based only on clinical symptoms at birth, whilst others include subsequent audiological assessments, cerebral imaging, retinal examination and laboratory tests (conjugated hyperbilirubinaemia, elevated transaminases and thrombocytopenia)[38].

1.5.4 Asymptomatic congenital CMV infection

Infants with cCMV that are asymptomatic at birth, may not be completely free of CMV disease as delayed onset of neurological sequelae can occur later in childhood and adolescence[9,63,108]. Permanent neurological sequelae is estimated to occur in 13.5% of

asymptomatic infants overall[63]. The most common sequelae in infants asymptomatic at birth are SNHL (10.0-12.0%) with other complications occurring at a lower frequency: motor deficits (5.0-7.0%) and chorioretinitis (2.0%)[9,13,63].

The prevalence of permanent neurological sequelae combining SNHL, cognitive/motor impairment and visual impairment in cCMV-infected infants overall regardless of symptoms at birth was estimated at 17.0-20.0% in a systematic review[63]. This might, however, be an underestimate due to the varying follow-up period between the studies. Furthermore, there were no studies from sub-Saharan Africa which reported on sequelae in infants with HIV exposure and HIV infection.

In neonates with asymptomatic cCMV at birth, the most common neurological sequelae is SNHL. In a study of 860 neonates with cCMV, 76.0% (651) were asymptomatic and 24.0% (209) were symptomatic at birth, with symptomatic infection defined as clinically apparent symptoms[109]. SNHL occurred in 7.4% (48) and 40.7% (85) of these children who were asymptomatic and symptomatic at birth, respectively, over a 16 year follow-up period. Of the children with SNHL, delayed onset SNHL occurred in 37.5% and 27.1% of asymptomatic and symptomatic children respectively. Another study by Zavattoni et al. (2014) which followed up a group of asymptomatic cCMV-infected children in Italy, found 5.6% (5/89) of cases had developed neurocognitive impairment over a 6-year follow-up[110].

To investigate whether newborns with asymptomatic cCMV develop neurocognitive impairment at a greater rate than cCMV-uninfected newborns, a study group in Texas, USA, assessed intelligence, receptive and expressive vocabulary and academic achievement between these two groups through to 18 years of age[111]. The asymptomatic cCMV children

without SNHL at the age of two years, were not significantly different in terms of intelligence quotient, vocabulary or academic achievement compared to the cCMV- uninfected children.

CMV infection has also been found to affect growth in children. In asymptomatic Kenyan children with both congenital and postnatally acquired CMV infection detected in blood, higher CMV viral load was associated with lower weight-for-age Z-score (P=0.008) and head circumference-for-age Z-score (P=0.012)[112]. In Zambian HIV-exposed and HIV-unexposed children with CMV infection (congenital or postnatally acquired), CMV-infected children had lower length-for-age by 18 months compared with CMV-uninfected children (P=0.002)[113]. Although the mechanism underlying this observation is yet to be fully understood, it has been postulated that CMV could affect the absorption of nutrients in the gut due to replication in intestinal cells[113]. Additionally, the long-term immune activation caused by CMV may predispose children to other infections which may affect growth. Children infected with CMV (congenital or postnatally acquired) have been found to have more hospital visits and health complaints than CMV-uninfected children in both African and American studies[56,113–115].

As delayed onset of neurological sequelae and possible effects on growth can occur in symptomatic and asymptomatic cCMV newborns, long term monitoring for sequelae is recommended[9,13,116]. In the USA, cCMV related neurological sequelae is the most common cause of birth defects[18,103]. Each year, approximately 8000 children are affected by some form of neurological impairment due to cCMV infection[18], which is higher than the incidence of neurologic disability due to Down's syndrome (4,000/year), foetal alcohol syndrome (5,000/year), or spina bifida (3,500/year)[18].

1.5.5 Congenital CMV infection in infants with HIV infection and HIV-exposure

Coinfection with CMV and HIV can exacerbate HIV-related immune T-cell activation, and increase the reservoir of HIV in the latently infected. In adults, CMV-HIV co-infection has been associated with more rapid progression to acquired immune deficiency syndrome (AIDS)[117].

There are limited (n=10[74–77,118–123]) studies which have investigated the outcomes of cCMV infection in children living with HIV and HIV-exposed uninfected children beyond the neonatal period, mostly conducted in the pre-ART era (Table 1.1). Four[119,121–123] of these were conducted in sub-Saharan Africa, the epi-centre of the HIV pandemic, with neurodevelopment assessed in only one study[113]. There is conflicting evidence whether HIV-CMV co-infection is associated with more rapid HIV-disease progression in children. Kovacs et al. (1990-1997), USA, reported that HIV-exposed infants co-infected with HIV and CMV (either congenital or postnatally acquired), had increased risk of HIV-disease progression and death by 18 months of age compared to children only infected by HIV[76]. Also, children with HIV-CMV coinfection were at increased risk of progressive neurological disease (including HIV related encephalopathy), diminished brain growth and motor impairment than children with HIV-infection alone[76]. Similarly, in an Italian study of children living with HIV between 2-130 months of age, CMV co-infected children (congenital or postnatally acquired) with viraemia had significantly shorter mean survival time (42.5 versus 60 months, $P < 0.01$), lower CD4+ T-cell count and higher mortality rate ($P < 0.0001$)[124].

In contrast, an early study of infants with HIV in Spain (1987-2002), no significant differences in the rate of HIV-disease progression, degree of immunosuppression (absolute and percentage of CD4+ cell count), death or neurological sequelae was observed between

infants with HIV and cCMV coinfection, HIV- and postnatally-acquired CMV infection and children living with HIV but free of CMV infection[118]. In the era of combination ART, a study of Cameroonian children (2007-2013) also did not observe any difference in the rate of death or CD4+ percentage between children who were HIV and CMV (congenital or postnatal) coinfected and children with HIV infection alone[125]. Neurological outcomes were not studied in these children. One of the challenges in comparing these data is differences in diagnostic modalities and populations studied.

With the advent of combination antiretroviral therapy for pregnant women to prevent mother to child transmission of HIV (PMTCT), there is an ever increasing pool of HIV-exposed uninfected children who are at increased risk of mortality, morbidity and slower growth than HIV-unexposed children[126]. The long-term neurological, growth and developmental outcome of these HIV-exposed children and any contributing effect of CMV coinfection on poor outcomes merits investigation. Gompels et al. (2012) reported that HIV-exposed CMV-infected children (congenital or postnatally acquired) had significantly decreased psychomotor skills at 18 months of age even after adjusting for socioeconomic status, maternal education level, duration of breastfeeding and any dietary interventions[113]. A study in the Western Cape, South Africa, found that in 142 HIV-exposed children admitted to hospital with suspected *Pneumocystis* pneumonia (PCP), 26% were more likely to have concurrent CMV pneumonia[127]. Those with concurrent PCP and CMV pneumonia were also more likely to be infected with HIV and have detectable CMV DNA in blood.

With the evidence of the negative effects of CMV infection on the long-term outcomes of children infected with HIV and HIV-exposed children, additional investigations into the effect of cCMV infection on HIV-exposed children in sub-Saharan Africa is warranted.

Table 1.1: Studies evaluating cCMV infection outcomes in infants with HIV exposure and HIV infection

Publication Reference Location Period Design	Relevant aims	Study participants and CMV testing	cCMV prevalence	Maternal ART	Symptomatic infection at birth	Sequelae & Mortality
Gabriel et al. (2007)[118] Spain 1987–2002 Prospective cohort study	Assess the influence of CMV infection in the first year of life and its impact on survival and progression of the disease until 12 years of age	16 HIV-exposed CMV-infected children of whom 6 had congenital CMV infection Urine culture for CMV	Not provided	2% of mothers received ART prenatally. Analysis on cCMV transmission variation by maternal ART not done	50% (3 of 6) with abnormal brain ultrasound: one each with bilateral intraventricular haemorrhage grade I, choroid plexus cysts, and subependimal cysts. One of these children had micro-calcifications in the nucleus caudadus and insula.	No neurological sequelae found By 5 years of age, 20% of children with cCMV died
Doyle et al. (1996)[74] USA 1988-1995 Retrospective data analysis of HIV-exposed infants followed prospectively	Rate of in utero CMV infection in HIV-infected infants. Determine if CMV coinfection affects progression of HIV disease	154 HIV-exposed infants of whom 24 were infected with HIV Urine culture for CMV	6.5% (10/154) cCMV prevalence in HIV-exposed infants of whom 50% (5/10) were HIV-infected The rate of in utero CMV infection was significantly higher in the HIV-infected infants (OR 6.8, 95% CI 1.5-31)	Zidovudine during pregnancy, intrapartum and postnatally in 33 women. Analysis on cCMV transmission variation by maternal ART used not done	10% (1 of 10) with symptomatic cCMV (microcephaly, intracranial calcification, hepatosplenomegaly and thrombocytopenia)	Eight of infants with cCMV had normal audiologic evaluation during the first 24 months of life. One symptomatic at birth HIV & CMV coinfected infant had a mild unilateral hearing loss A trend toward decreased survival in the infants who were coinfected with CMV in early life (P = 0.088, either congenital or perinatal CMV infection) compared to CMV-uninfected infants
Frederick et al. (2012)[120] USA 1988-2002 Prospective cohort study	Examine congenital and perinatal/early postnatal (P/EP) CMV among HIV-exposed infants in the pre- and post-HAART	414 HIV-exposed infants: 201 enrolled from 1988-1996 and 213 enrolled from 1997-2002 Urine and oral swabs for CMV by culture	3.6% (9 of 248) cCMV prevalence which did not differ by infant HIV status	During 1988–1996, 1% of mothers received HAART and 32% received any ART during their pregnancy During 1997–2002, 77% received HAART	22,2% (2 of 9) symptomatic at birth with neither child having had maternal ART: One was HIV-infected with lymphadenopathy & hepatosplenomegaly One was coinfected with congenital toxoplasmosis and	One cCMV symptomatic infant with long-term neurologic impairment (not specified) but not SNHL No deaths

Publication Reference Location Period Design	Relevant aims	Study participants and CMV testing	cCMV prevalence	Maternal ART	Symptomatic infection at birth	Sequelae & Mortality
				and 88% received any ART during their pregnancy. cCMV prevalence did not differ by maternal HAART or any ART	had lymphadenopathy, hepatosplenomegaly, and failure to achieve developmental milestones with HIV/AIDS	
Kovacs et al. (1999)[76] USA 1990-1997 Multicentre prospective study	Examine the association of CMV infection with the progression of HIV disease	600 HIV-exposed infants of whom 93 were HIV-infected and 463 were HIV uninfected. 44 were of indeterminate HIV status. Urine culture for CMV Anti-CMV IgG and IgM antibody PCR in urine for CMV at a single centre	4.3% (2 of 47) cCMV infection in HIV-infected children 4.5% (9 of 200) cCMV infection in HIV-exposed children. No statistical difference in cCMV infection between HIV-infected and HIV-exposed uninfected children	None	Details not provided	By 18 months of age, infants with HIV and CMV coinfections had higher rates of HIV disease progression (70.0% vs. 30.4%, P=0.001) or death (52.5% vs. 21.7%, P=0.008), and impaired brain growth or progressive motor deficits (35.6% vs. 8.7%, P=0.005) than infants infected only with HIV.
Mussi-Pinhata et al. (1998)[77] Brazil 1992-1995 Prospective cohort study	Determine rates of congenital and perinatal CMV among HIV-exposed infants born compared to HIV-unexposed infants from a CMV-immune, low-income population.	150 HIV-exposed and 175 infants HIV-unexposed infants Urine culture and PCR for CMV	2.7% (4 of 150) cCMV prevalence in HIV-exposed infants 2.9% (5 of 175) in HIV-unexposed infants (p = 1.00). 21 HIV-infected infants of whom none had cCMV	Details not provided	50% (2 of 4) cCMV HIV-exposed infants symptomatic (1 with intracranial calcification, 1 with intrauterine growth restriction) 25% (1 of 4) cCMV-infected HIV-unexposed infant symptomatic at birth (intracranial calcifications)	Details not provided

Publication Reference Location Period Design	**Relevant aims**	**Study participants and CMV testing**	**cCMV prevalence**	**Maternal ART**	**Symptomatic infection at birth**	**Sequelae & Mortality**
Gumbo et al. (2014)[119] Zimbabwe 1997-2001 cross-sectional study	Determine whether early (6 week) co-infection with either CMV was associated with increased mortality	135 CMV and HIV co-infected infants at 6 weeks of age Real time PCR on plasma	20% (27 of 135) cCMV prevalence in infants with HIV infection 74% (20 of 27) of cCMV had intra-uterine HIV infection, the remaining with intrapartum HIV	None	Symptomatic infection not assessed	By 6 months of age, infants with cCMV had similar weight and length, but a trend towards lower head circumference Z-scores, compared to CMV-negative infants Mortality only assessed for postnatal CMV
Duryea et al. (2010)[75] USA 1997–2005 Retrospective cohort study	frequency of congenital cytomegalovirus (CMV) infection in infants born to human immunodeficiency virus (HIV)- infected mothers and assess risk factors that may facilitate intrauterine transmission	urine culture for CMV333 HIV-exposed children assessed for cCMV	3% (10 of 333) cCMV prevalence 1% (4 of 333) infants were infected with HIV, and none of these were CMV-infected.	maternal HAART during pregnancy	cCMV-infected infants had significantly lower mean birth weight (2508 vs. 3148 g, P=0.01) and gestational age (37 vs. 39 weeks, P=0.01) 40% (4 of 10) of cCMV infants with suspected symptoms: one with, hepatosplenomegaly, one small for gestational age and microcephaly; one with intrauterine growth restriction; and one with hepatosplenomegaly that did not pass hearing screening	Hearing screening was performed on 280 (84%) of the 333 CMV-screened infants. No SNHL identified Mortality not assessed
Slyker et al. (2009)[122] Kenya 1999-2005 prospective cohort study	Investigate congenital CMV in HIV infected and HIV-exposed children	51 HIV-exposed infants PCR for CMV on cord blood	2.7% (1 of 37) cCMV in HIV-exposed HIV uninfected neonates 29% (4 of 14) cCMV prevalence in HIV-infected neonates	Women received short-course zidovudine for prevention of HIV transmission prenatally. Analysis on cCMV transmission variation by maternal ART not done	Symptomatic infection not assessed	Sequelae not assessed Mortality assessed but not analysed in the context of cCMV

Publication Reference Location Period Design	Relevant aims	Study participants and CMV testing	cCMV prevalence	Maternal ART	Symptomatic infection at birth	Sequelae & Mortality
Chang et al. (2015)[121] Malawi 2004-2010 Retrospective cohort study	Examine the relationship of congenital and postnatal CMV and HIV transmission in a cohort of infants born to mothers with HIV infection mothers. To characterize any effects of CMV infection on the health and growth of these infants	272 plasma samples collected at birth from HIV-exposed infants of whom 30 were from infants with HIV infection CMV PCR on infant plasma and peripheral blood mononuclear cell (PBMC) specimens from birth, 24 weeks, and 48 weeks of age, in order to detect congenital, early postnatal, and late postnatal CMV infection	2.3% (8 of 242) cCMV prevalence in HIV-exposed infants. All eight cases of cCMV were HIV-uninfected until last study visit 10% (3 of 29) cCMV prevalence in infants with HIV infection, significantly higher than in HIV-exposed uninfected infants (P= 0.049)	Three arms of ART prophylaxis in mothers and infants for other study aims Analysis on cCMV transmission variation by maternal ART not done	Details not provided	Details not provided
Adachi et al. (2018)[123] Multi-centre study in Brazil, South Africa, Argentina, and the United States 2004-2018, Prospective cohort study	Compare rates of cCMV among HIV-exposed, uninfected and HIV-infected determining predictors of cCMV; and evaluating mortality rates among infants with cCMV	89 HIV-infected infants, 858 HIV-exposed uninfected infants PCR of urine for CMV	9% (42 of 858) cCMV in HIV-exposed, uninfected infants 23.2% (13 of 56) cCMV prevalence in all infants with HIV infection (aOR 6, 95% CI 3–12.1)	None	Symptomatic infection not assessed	3.1% (2 of 64) deaths among infants with cCMV compared to 2% (19 of 928) in CMV-uninfected. Infant mortality was not associated with cCMV. HIV-exposed infants who died were at increased risk of cCMV

1.5.6 Congenital cytomegalovirus related mortality

The mortality rate in children with cCMV infection has not been thoroughly assessed, despite the high morbidity associated with infection. This evaluation is challenged by not being able to diagnose cCMV retrospectively, unless a stored birth sample of blood, urine, saliva or other tissue is available for testing to determine CMV infection within three weeks of birth. Some longitudinal studies in high income countries have, however, reported considerable mortality rates in cCMV-infected children.

In a cohort of cCMV-infected children in Alabama, USA, 0.5% (2/407) died over a mean follow-up to age 55 months[128]. In an analysis of multiple-cause-of-death data from US death certificates between the years 1990–2006, 777 (0.11%) deaths related to cCMV occurred among 514930 infant deaths; with 41.1% (319) of cCMV associated deaths occurring in neonates[129]. The infant mortality rate associated with cCMV was 8.3 per 1 million infants annually (95% CI 7.65–9.04). In the UK, similar rates of cCMV related infant mortality (0.7[130] to 0.8[131] per 100 000 live births), have been observed. Between 1999-2011 in Australia, a review of national mortality data sets for deaths related to cCMV as an underlying or contributing cause in children and adolescents less than 15 years old was undertaken[132]. Eighty-three deaths associated with cCMV were recorded, with 68.0% occurring in infants with the majority (39.0%) occurring in the neonatal period. The cCMV associated infant mortality rate was 1.6/100,000 live births. These mortality rates are likely an underestimate, as diagnosis of cCMV infection would be biased towards infants born to women with primary CMV infection identified due to maternal seroconversion during pregnancy and those with symptomatic infection at birth. Mortality data from low and middle income countries including sub-Saharan Africa are lacking.

Congenital CMV infection in stillbirths

In 2015 there were 2.6 million stillbirths globally with 98% of these occurring in LMIC[133]. Infections may be responsible for 50% of stillbirths in LMIC whilst in high income countries infections may account for 10-25% of stillbirths[134]. There is a paucity of studies on the contribution of viral infections as a cause of stillbirths in LMIC.

The pathogenesis of stillbirths from infections may be due to multiple processes: 1) Intrauterine foetal demise may be due to maternal systemic illness from the infection causing fever and organ failure[134,135] 2) There may be direct placental infection by the organism resulting in placental destruction and dysfunction[136,137] 3) Foetal infection by placental transmission can result in organ damage and foetal anomalies[138].

Although CMV is the most common congenitally transmitted infection, few studies have investigated the role of CMV as a cause of stillbirths. In the UK between 1988 to 2008 , 0.2% (8/3954) of stillbirths were attributed to cCMV (incidence of 1.1/100,000 births)[139]. The cause of death was attributed to CMV only in cases where the clinical presentation was in keeping with PCR, serology and/or histology results. In an Australian study, DBS obtained from cardiac puncture of stillbirths were tested for CMV by PCR with 9% (7/107) positivity, although none of the stillbirths could be directly attributed to CMV infection[140]. Syridou et al. (2008) in Greece conducted a case-control study where a full autopsy and placental tissue examination was undertaken in stillbirths and investigated for CMV, Parvovirus B19, and Herpes Simplex Virus 1 or 2 infection by molecular assays and histological studies[141]. These foetal deaths were compared to term healthy neonate controls born at the same facility and matched to cases by nationality, maternal age and maternal obstetric history. The detection of CMV was significantly associated with foetal death based on placental examination and histopathological findings. CMV was identified in 16% of placentas of stillbirths compared to

3% of controls (OR 7.5, 95% CI 0.9–63.6, P=0.03)[141]. In another autopsy study of stillbirths, 15% of foetal tissue had CMV DNA detected with 5% in placental, 9% in kidney and 11% in liver tissues[142]. On histopathological examination foetal thrombotic vasculopathy was the only abnormality significantly associated with cCMV compared to cCMV-uninfected stillbirths (P=0.01)[142].

The pathogenesis of intrauterine foetal death due to CMV may be a result of placental infection and damage thus indirectly causing foetal death[137]. Immune dysregulation with placental dysfunction may be the underlying mechanism, as placentas of CMV-infected stillbirths have increased chemokine (Monocyte chemoattractant protein 1, P= 0.001) and cytokine (Tumour Necrosis Factor-alpha, P= 0.007) production compared with placentas of cCMV-uninfected healthy-newborns[137].

Although the stillbirth rate and cCMV infection rate is highest in LMIC, specifically sub-Saharan Africa, the role of CMV infection in the pathogenesis of stillbirth is yet to be characterised.

1.6 Perinatal and postnatal CMV infection

Perinatal CMV infection is defined as CMV infection occurring from 22 weeks (154 days) gestation age onward up to within seven days of birth. Postnatal CMV (pCMV) acquisition is CMV infection acquired any time after birth through contact with maternal cervical fluid during delivery, consumption of CMV-infected breast milk or contact with CMV-infected maternal saliva. Postnatal CMV infection is diagnosed if an infant, negative for CMV infection at birth, has CMV detected in body fluids after 21 days of birth[143].

Most term and preterm infants with pCMV infection are asymptomatic, as maternally transferred CMV IgG antibody provides adequate protection against disease[53]. However, studies have identified a significant risk for low and very low birth-weight premature infants whom acquire CMV postnatally for the development of CMV associated pneumonitis, hepatitis or sepsis-like illness[53]. Sepsis-like symptoms may include the development of classic clinical and laboratory features of cCMV infection such as hepatosplenomegaly, thrombocytopenia and elevated liver enzymes[144,145]. Other concurrent symptoms include apnoea, bradycardia, hepatitis, bowel distension and neutropaenia[53,144]. Severe acquired CMV infection can result in life-threatening disease such as severe pneumonia requiring ventilator support, hepatitis and necrotising enterocolitis[144].

In a systematic review of the role of breast milk in CMV transmission to infants born preterm, CMV was detected in breast milk of 66–96% of CMV IgG seropositive women[145]. Postnatal CMV transmission rates to infants ranged from 5.6–58.6%, occurring between 28-140 days of age[145]. The prevalence of symptomatic disease ranged from 0–34.5% (mean 3.7%) and a sepsis like illness occurred in 0–13.8% (mean 0.7%) of infants[145]. In a large multicentre cohort study of very low birth weight infants in the USA, pCMV infection was associated with bronchopulmonary dysplasia[143]. The damage to lung tissue may either be a direct consequence of CMV infection of cells, or may occur from the immune response to the virus destroying infected cells[146]. The disruption of respiration may also leave the infant at increased risk of exposure to other infections such as can occur through mechanical ventilation[143].

Infants infected with HIV, are also at increased risk of pCMV infection and symptomatic disease. Kovacs et al. (1999) reported that infants living with HIV at six months of age had significantly increased prevalence of CMV infection than HIV-exposed uninfected infants

(39.9% vs 15.3%, P=0.001); with the cumulative percentage of CMV infection at four years of age remaining higher in infants with HIV (80.0% vs 65.0%, P=0.04)[76].

Studies monitoring prematurely born infants with pCMV infection for long-term outcome, indicate that although the majority of infants achieve normal development and intellectual ability[147], some children have manifested lower cognitive outcome than CMV-uninfected controls[148,149]. Audiological outcomes however, have thus far not been found to be different to CMV-uninfected children[144,145]. Data on the outcomes of children with postnatally acquired CMV infection in African children is unknown, although sub-Saharan Africa has the highest preterm and very low birth weight rates globally.

1.7 Immune response of congenital CMV-infected infants to vaccines

The efficacy of infant vaccines in Sub-Saharan Africa has consistently been lower than in high-income countries for a number of vaccines (e.g. measles vaccine, rotavirus vaccine) [150]. Studies in the elderly have shown that the poor immune responses to influenza vaccine may be due to the immune senescence observed in the elderly with latent CMV infection as a result of immune activation and expansion of CD8+CD28- T-cells[151]. Even though congenital and pCMV infection results in an increase in activation and differentiation of T-cell populations, there is limited data on the effects of cCMV on infant immune responses to vaccines. It has been hypothesised that CMV infection, due to large recruitment of T-cells, may modulate the immune responses to vaccines, albeit only investigated in few reported studies[150,152,153].

A review by Falconer[154] surmises that infants infected with CMV (congenital or postnatal) have positive and negative modulated immune responses, depending on immune factors studied: 1) enhanced immune response to measles[153] and 2) depressed immune response to polio vaccines[155], but found no significant differences in responses to *Haemophilus influenzae* type b conjugate or tetanus vaccines[153]. The interaction between CMV and HIV[150] , may also influence immune responses to vaccination. With the high burden of cCMV infection and HIV-exposure in sub-Saharan Africa as well as the reduced efficacy of vaccines in these settings, the effect of both congenital and pCMV infection on infant immune responses to vaccines merit exploration.

1.8 Diagnosis of CMV infection

1.8.1 Diagnosis of maternal CMV infection

As clinical symptoms of CMV infection in healthy women are usually absent or non-specific, the accepted gold standard for diagnosis of maternal infection is through serologic testing for CMV-specific antibodies[96,156]. If a stored sample of maternal blood prior to conception or early gestation is available, primary CMV infection is confirmed when a woman, previously negative for anti-CMV antibody seroconverts to CMV-specific IgM and/or IgG[38]. When maternal serum is unavailable for comparison, the presence of IgM can only partly discriminate between primary and non-primary CMV infection, since the IgM assay has low specificity (false positive results), IgM may persist for several months after primary infection and can also be induced by non-primary infection[157]. Consequently, IgM in combination with IgG avidity is proposed for distinguishing between maternal primary and non-primary CMV infection[158,159].

Antibody avidity is the strength by which antibody binds to antigen, and is an indicator of the maturation of the immune response[160]. Antibodies which are produced during the early phase of primary infection have lower avidity to antigens than antibodies produced following later or non-primary infection. Therefore the presence of CMV-specific IgM combined with low/moderate IgG avidity index can be interpreted as evidence of primary CMV infection[156,158,161]. A high IgG avidity index indicates the absence of an active primary infection or the progression of a recent primary infection[160,162]. If CMV IgM and IgG avidity is determined before 16-18 weeks of gestation, it could potentially identify 100% of women who are at risk of transmitting CMV to the foetus[160]. If serology of primary infection is identified before 12-16 weeks of gestation, this is predictive of a higher risk of symptomatic cCMV in the foetus, even though the risk of transmission to the foetus is lower in early pregnancy, as the foetal immune system is immature with limited maternal protective antibody and a reduced ability to control viral replication[38].

There is an ongoing debate on the value of serologic screening of all pregnant women at risk of primary CMV infection during antenatal care[163]. A number of facilities in high income settings have nevertheless implemented maternal screening programmes, even though there are currently no treatment options for foetal CMV infection except monitoring and elective termination of pregnancy[96,164]. As maternal non-primary CMV infection is more challenging to identify, screening programmes in high CMV seroprevalence settings are not recommended

1.8.2 Diagnosis of foetal CMV infection

Foetal diagnosis of CMV infection is recommended when there is evidence of primary maternal CMV infection and/or when ultrasound abnormalities in keeping with foetal CMV infection are observed[38,96]. Definitive foetal diagnosis is made by testing amniotic fluid, collected through amniocentesis, for CMV DNA using PCR assays which is considered the

gold standard[98,165,166]. Other nucleic acid test assays or viral culture can be done, but the sensitivity of these are inferior to PCR[97]. Sensitivity of amniocentesis is highest (90-95%) if conducted after 20-21 weeks of gestation when foetal urine is present in amniotic fluid[98]. If amniocentesis is performed prior to 20 weeks gestation, the sensitivity drops to as low as 45% as foetal urine production at this stage is not optimal[167].

Methods of predicting perinatal outcomes of foetal infection have been investigated which include quantification of viral load in amniotic fluid and foetal blood, collected by cordocentesis[97]. Viral load in amniotic fluid as a prognostic marker has been inconclusive due to the accumulation of virus in amniotic fluid and increased urine production by the foetus over time[97]. However, foetal thrombocytopenia (<50000-100000/mm^3) and a high viral load (> 4.93 - 4.5 log10 copies/ml) in foetal blood were associated with an increased risk of delivering a symptomatic neonate or leading to termination of pregnancy[97,168,169]. Despite this, cordocentesis to sample foetal blood is no longer routinely conducted due to the risk of miscarriage and foetal death[97].

Ultrasound imaging alone has good prognostic value when the assessor is aware of foetal CMV infection. When ultrasound examination is conducted as part of routine antenatal care, the sensitivity of detecting CMV related abnormalities is low (35%)[99]. Severe brain defects observed on ultrasound in the presence of suspected foetal CMV infection, is predictive of poor outcome and termination of pregnancy should be offered to the parents[168]. However as the onset of brain pathology may occur in late gestation, ultrasound imaging should be offered throughout pregnancy[97].

In LMIC, maternal screening for CMV is not routinely conducted as primary maternal infection is rare. Antenatal ultrasound imaging is also not always available to pregnant

women, unless they are deemed high risk and are referred to facilities where imaging is available. Unless there are overt signs detected during ultrasound imaging, foetal CMV infection is rarely diagnosed in pregnancy in LMIC[9].

1.8.3 Diagnosis of congenital CMV infection

In 2015, an International Congenital Cytomegalovirus Recommendations Group was convened to provide guidance for prevention, diagnosis, and treatment of cCMV[38]. The group recommended universal screening of neonates for cCMV infection to enable identification of infants at risk for sensorineural hearing loss and neurodevelopmental delay to facilitate early detection and intervention and prevent long term disability[38]. The diagnosis of cCMV was recommended to be made by PCR of saliva and/or urine within the first three weeks of life, with saliva being the preferred sample due to ease of collection and high sensitivity (>97.0%) and specificity (99.0%)[38]. Traditionally, the reference method for diagnosis of cCMV was virus isolation from urine, saliva or blood in tissue cultures, with detection of CMV in urine culture considered the gold standard [157]. However implementing culture methods widely for screening or rapid diagnosis was not feasible as viral culture is technically demanding and resource intensive, necessitating alternative laboratory methods for diagnosis[170].

PCR amplification of viral DNA has been found to have high sensitivity (97.6%) for CMV testing in body fluids and tissues[157,171–173]. Additionally, PCR processes can be automated, are technically reproducible, have low operational costs and samples for PCR testing do not require the stringent storage and transport conditions essential for viral culture[170]. Validation studies of the PCR assay in infant blood, saliva and urine with culture indicated that PCR was a reliable method for diagnosis of cCMV infection[38,173–175].

DBS are routinely collected from newborns in high income countries[175]. Studies on the DBS showed there to be low sensitivity for detection of CMV compared to saliva culture (sensitivity of 30.4%, [95% CI 21.5 – 41.0], specificity of 99.9% [95% CI 99.9 – 100]) and thus unsuitable for screening purposes[175]. This may be due to CMV viremia in most congenitally infected infants being absent or below detectable limits[157,170]. Nonetheless, DBS are a valuable source for retrospective diagnosis in infants that are either symptomatic or have developed neurological sequelae in later life, when birth saliva or urine may be unavailable for testing[176].

CMV is shed in urine and saliva in large quantities and for a prolonged duration in children[177,178] and viral loads have also been observed to be higher in younger than older children[177,179]. In addition, collection of urine and saliva do not require invasive methods making them ideal samples for large-scale screening for cCMV. A study comparing the performance of real-time PCR assays of saliva and urine in screening newborns for cCMV found that PCR performed as well as rapid culture of urine or saliva specimens[172] and in some studies was more sensitive than culture[180]. Due to the challenges of collecting urine from young infants using sterile bags, alternative collection methods have been explored. A study comparing urine absorbed in cotton balls placed in the diaper and urine collected in a bag found that CMV was detected in significantly fewer urine samples from cotton balls (54.2%) than in samples collected by bags (95.7%, $p<0.0001$)[181]. A number of studies have also investigated the utility of filter paper placed in the diaper to absorb urine for CMV PCR assays[182,183]. Although preliminary results have shown promise with high sensitivity and specificity, filter paper collection has not been validated with assays or culture of urine collected in bags or saliva.

As saliva is a convenient sample to collect and has high viral titres, saliva PCR assays have increasingly been used in large scale screening studies for cCMV. A landmark study comparing the utility of PCR assays on liquid and dried saliva samples to saliva culture, confirmed that saliva PCR has high sensitivity for detecting cCMV infection [184]. Liquid saliva samples were collected on swabs placed in transport medium and at the same time an additional saliva swab was collected which was allowed to air dry and placed in a dry tube, with both samples subsequently processed for PCR and rapid culture assays. Infants with positive saliva screening results by rapid culture and/or PCR assay were re-tested by repeat rapid culture and PCR assay on additional saliva and urine specimens. The sensitivity and specificity of the liquid-saliva PCR assay were 100% (95% CI 95.8-100) and 99.9% (95% CI 99.9-100) respectively, whilst sensitivity and specificity of the dried-saliva PCR assay were 97.4% (95% CI 90.8-99.7) and 99.9% (95% CI 99.9-100), respectively[184]. There were a number of false positive infants who had initial saliva samples testing positive and subsequent saliva and/or urine samples testing negative for cCMV. This was thought to be due to possible contamination with breast milk or secretions from the genital tract during birth and recommendation for saliva collection following a minimum of one hour after breastfeeding was made[184].

A diagnosis of cCMV infection can only be made if neonatal samples are collected within three weeks of birth, as CMV positivity thereafter cannot be discerned from natal and postnatally acquired CMV [13,18,38,160]. An initial positive CMV screening result should be confirmed by repeating the testing on a subsequent sample of either saliva and/or urine, also obtained within three weeks of birth. Although feasible, large scale CMV infection screening programmes outside of research have not been widely implemented due to a lack of rapid diagnostic tests, high costs and low demand[185].

1.8.4 Newborn audiologic and infection screening for congenital CMV

Universal newborn hearing screening is now widely implemented in high income settings from a previously risk based hearing screening, which was restricted to neonates with low birth weight, family history of hearing loss, confirmed congenital infections, mechanical ventilation and meningitis[128,186]. Universal hearing screening was recommended after the observation that approximately 50.0% of children that developed hearing loss had not been screened at birth[186,187]. Infants with an early diagnosis of hearing loss facilitates early intervention with better language and cognitive outcomes than in infants whom are diagnosed later in life, and as a result most well-resourced countries have implemented such programmes [38,188].

The debate on whether all newborns should receive universal laboratory CMV infection screening or to maintain the status quo of CMV infection screening in infants that fail newborn hearing screening (targeted CMV screening) is ongoing. A study by Fowler et al. (1996) found that universal screening of hearing in neonates will detect less than 50.0% of all SNHL caused by cCMV infection, as most infants with cCMV infection are asymptomatic and likely to have normal hearing at birth, developing problems later in childhood[128]. Infants that pass newborn hearing screening usually do not receive any additional hearing evaluations to identify late-onset hearing loss. Therefore a combined approach of universal hearing screening in neonates, as well as universal screening for cCMV infection, needs to be considered[128]. Cost-benefit analyses have been conducted to compare universal cCMV infection screening over targeted cCMV screening and both approaches were shown to be cost-saving[189]. In LMIC neither universal and targeted screening for cCMV infection nor universal hearing screening is routinely available due to constraints in resources and expertise, with hearing loss usually suspected by the parents later in childhood or at school[190,191].

1.9 Treatment options for congenital CMV infection

1.9.1 Prevention of maternal infection

Prevention of primary maternal infection is mainly limited to behavioural changes brought about by increasing awareness about CMV infection in health care providers and women which has been found to be effective[38,192,193]. Studies have shown that pregnant women are unaware of cCMV infection whilst the knowledge in health care providers is limited[38,194]. Education on behaviour change includes counselling on avoiding contact with body fluids of children by not sharing utensils, good hand hygiene and avoiding contact with saliva[38]. In a randomised controlled trial the effectiveness of hygiene information provided to pregnant women at risk for primary CMV infection, the intervention group, was compared to pregnant women enrolled at delivery who were not provided CMV related information nor tested for CMV serology[195]. In the intervention group fewer women seroconverted, 1.2% (4/331), compared to 7.6% (24/315) in the comparison group (absolute risk reduction=6·4%, 95% CI 3.2–9.6, P<0.001).

There are currently no licensed CMV vaccines which could potentially be the most effective mode of preventing primary and non-primary CMV infection. Several vaccine candidates, have been tested and are still currently under investigation[196]. One of the key challenges to vaccine development has been the limited understanding of maternal immune correlates of protection against foetal transmission, given that pre-existing seroimmunity to CMV only provides partial protection against foetal transmission and disease[196]. Of importance however, is that pre-existing seroimmunity is associated with reduced foetal transmission rates (1.4%) than following primary infection (30.0%); and cCMV may be less frequent and severe in infants born to women with pre-existing immunity[196].

1.9.2 Prevention of foetal CMV infection

Passive immunisation with anti-CMV hyper-immunoglobulin has been tested in pregnant women with primary CMV infection with initial promise of efficacy[197]. To confirm these findings, a phase II double blind, randomised placebo controlled trial of hyper-immune globulin in pregnant women with primary CMV infection to prevent foetal CMV transmission was conducted[198]. In 123 women, the rate of congenital infection was 30.0% in the hyper-immune globulin group and 44% in the placebo group (P=0.13). With these results, the administration of hyperimmune globulin to pregnant women with primary CMV infection to prevent foetal infection is not recommended[38,192,194].

Currently there is insufficient evidence on the efficacy of antivirals in preventing foetal CMV transmission. A study is currently underway to test the efficacy of valaciclovir in prevention of foetal CMV transmission (NCT02351102). Valaciclovir has been found to have no adverse effects on pregnancy, although activity against CMV is weak[38,194].

1.9.3 Treatment of foetal CMV infection

There is currently no effective treatment for foetal CMV infection diagnosed by amniocentesis or cordocentesis. Treatment of foetal infection has been attempted with hyperimmuneglobuin although results have been inconclusive[199]. The use of antivirals effective in neonatal cCMV infection to reduce morbidity and mortality is not licensed for use in pregnancy due to concerns of toxicity[199].

Termination of pregnancy is offered to pregnant women with confirmed foetal infection or ultrasound signs of cerebral and extracerebral involvement[96]. In a study of prenatally diagnosed cCMV infection, 51.0% (28/55) of women elected to terminate the pregnancy on foetal diagnosis although two foetuses were found to be uninfected on autopsy[98]. There is

limited literature on how often termination of pregnancy due to symptomatic foetal infection is offered and taken up in LMIC.

1.9.4 Treatment of neonatal congenital CMV infection

Antivirals, which have proven efficacy against cCMV disease and are licensed for use in neonates, include intravenous ganciclovir or oral valganciclovir. In randomised controlled trials of intravenous ganciclovir, symptomatic neonates with cCMV infection had improvement in hearing parameters between baseline and six months follow-up. Furthermore, those with normal hearing at baseline, remained so at six months of follow-up[200,201]. Six weeks of intravenous ganciclovir at 10-12mg/kg/dose is currently recommended for infants with severe cCMV disease with CNS involvement, retinitis and end-organ disease[202]. Ganciclovir is, however, associated with toxicity of which neutropenia and thrombocytopenia is most common and requires close monitoring during therapy[202].

Oral valganciclovir, the prodrug of ganciclovir, has also been tested in neonates with symptomatic infection. A randomised placebo-controlled trial showed statistically significant improvements in symptomatic neonates treated with six months of valganciclovir compared to neonates treated with six weeks of valganciclovir[203]. The patients on six months of therapy had a three-fold improved hearing or preservation of normal hearing at 12 months and 2.6 fold improvement at 24 months. Neurodevelopmental outcomes were also improved with longer duration of therapy. Although neutropenia was observed more frequently in the six months compared to the six weeks therapy group, the difference was not significant. Valganciclovir is currently recommended for infants with moderate to severe disease at 16 mg/kg per dose orally, twice a day for not longer than six months[38]. The advantage of valganciclovir over ganciclovir is the fact that therapy can be initiated and monitored on an outpatient basis.

Antiviral therapy is currently not recommended for neonates asymptomatic at birth, those with mild disease or isolated SNHL as there is currently no evidence of improved outcome[38,192].

1.10 Economic impact of congenital CMV

The effect of cCMV disease on quality of life and cost to the health system led the Institute of Medicine (IOM) in the USA (1999) to declare development of a CMV vaccine as a highest priority[204]. The IOM estimated that approximately $1.9 billion per year is spent toward medical and educational costs for cCMV-infected children in the USA[9] compared to costs of developing a CMV vaccine of $360 million[185].

A study in The Netherlands estimated the cCMV disease-related healthcare cost in early childhood, based on the average costs for hospital admissions and consultations by healthcare providers[205]. The mean healthcare costs of children with cCMV (€6113, N=133) were higher than children without cCMV (€3570, N=274). The costs of children with permanent disability were twice as higher in children with cCMV (€17205) compared with children without cCMV (€8332). Although these differences were not significant, the higher costs observed in caring for children with cCMV infection warrants investigation of preventive measures[205].

A similar study in the UK aimed to estimate the annual economic burden of managing cCMV and its sequelae for the year 2016 by measuring the direct (costs accrued by the public sector) and indirect (costs accrued personally or by society) costs[206]. An estimated £732 million (Range: £495-£942 million) was spent in managing cCMV in the UK in 2016, with 40.0% of the costs being directly incurred by the public sector and 60.0% being incurred indirectly. The

health economic implications of cCMV in low income countries is under-recognised or unknown as disability specific to cCMV is not identifiable in childhood[207].

1.11 Justification and objectives

There is a paucity of data on the immunology, virology, mortality, disease and disability burden of cCMV infection in LMIC, particularly from populations infected with HIV and HIV-exposed populations in the era of ART. Early detection of SNHL and neurodevelopmental disorders due to cCMV could enable timely intervention to improve quality of life in low income populations at risk of ongoing poverty and poor health from disability.

South Africa remains the most economically unequal country in the world, with over half the population living in poverty (<3$/month) of whom 64.2% are Black[208]. Furthermore, South Africa has one of the highest HIV prevalence's in pregnant women and has implemented a successful PMTCT programme country wide. Thus, there is a large population of children, including an increasing population of HIV-exposed uninfected children, who are at high risk of cCMV and neurological sequelae, but in whom the burden and outcome of cCMV is unknown. With these risk factors there is a need for a discussion on whether cCMV in South African children at risk presents the same public health impact as in high resource countries and the need for a universal or targeted cCMV screening programme in all or only HIV-exposed infants.

The aim of this research was to establish baseline disease epidemiology of cCMV and the effect of HIV-exposure in the era of ART on cCMV prevalence and outcome in a population of urban, middle-low income, black South African children.

The specific research objectives were:

1. To determine the prevalence of and risk factors for congenital CMV in HIV-exposed and HIV-unexposed neonates.
2. To describe hearing, neurodevelopmental and growth outcomes in congenital CMV-infected infants compared to congenital CMV-uninfected matched controls until 12 months of age.
3. To determine the prevalence of postpartum maternal CMV shedding and association with congenital and postnatal CMV infection.
4. To determine the effect of congenital CMV on the humoral responses to the primary series of select vaccines included in the public immunisation program in South Africa.
5. To describe the prevalence and histological findings of congenital CMV infection in stillborn foetuses and neonatal deaths (aged below 21 days), and determine the proportion of foetal and neonatal deaths attributable to congenital CMV.

The reporting format of this Thesis is arranged in chapters. Chapter 2 describes the overall materials and methods common to each objective. Chapters 3 to 7 describes methods, results and discussion pertaining to each objective above and Chapter 8 presents the Thesis Conclusion.

2 Materials and Methods

2.1 Study population and study site

The total population of South Africa is estimated at 58.78 million with close to a third (28.8%) made up of children aged 0-14 years[209]. Black Africans form 80.7% of the population followed by 8.8% Coloured (accepted term for people of mixed-race), 7.9% White and 2.6% Asian/Indian[210]. South Africa is classified as an upper-middle income country by the World Bank[211] and despite the high income classification, South Africa is in the top 25.0% of countries with the highest unemployment rates globally (29.0% unemployment rate)[212]. South Africa is also the most economically unequal country in the world with approximately half (49.2%) of the adult population living below the upper-bound poverty line (<80US$/month) with race being the strongest predictor of poverty[213]. Black Africans, female headed households and children are at highest risk of being poor, living on <US$3.1/day[213]. There is however, reasonable access to health care with public health facilities serving 85.0% of the total population[214].

This study was conducted in Gauteng Province, at the Chris Hani Baragwanath Academic Hospital (CHBAH) in Soweto (South Western Township). Soweto is the largest urban settlement in South Africa, constituting almost exclusively of Black Africans of multiple tribal ethnicities. It is a low-middle income setting with a total population estimated at 1.2 million and a high unemployment rate[215].

Public health care is provided at no cost to all pregnant women and children by the State whilst adults are required to pay a nominal administrative fee for care. The majority of births (95.0%) in Soweto occur at public health facilities and three quarters of these births occur at

CHBAH; the others at one of five midwife-operated units which only attend to uncomplicated, normal vaginal deliveries[216]. CHBAH is a secondary and tertiary care hospital catering for its catchment population and referrals. It has a 3200 bed capacity and facilities include neonatal intensive care. Approximately 35.0% of all births at CHBAH are through caesarean sections and healthy newborns are discharged home 12 hours post-vaginal, or 72 hours post-caesarean delivery[216].

2.1.1 HIV prevalence and testing in pregnant women and infants in South Africa

South Africa has a HIV prevalence of 13.5% in the general population which is the fourth highest HIV/AIDS prevalence rate in the world[209]. The prevalence of HIV infection in pregnant women is also high having stabilised at approximately 30.0% over the past decade, while early vertical HIV transmission rates have declined from 20.9% in 2004 to 1.1% in 2016 due to improved mother to child HIV prevention strategies [217].The intrauterine neonatal HIV infection rate was estimated at 247 infections per 100000 live births in 2016[217]

The South African PMTCT programme was rolled out in 2002 providing single dose Nevirapine to mothers in labour and to the infant within 72 hours of birth[218]. Option B-plus (B+) was adopted in 2015 and continued until November 2019, where pregnant women were initiated on lifelong ART (fixed dose combination therapy [FDC]: Tenofovir, Emtricitabine and Efavirenz), irrespective of $CD4^+$ T-cell count at the time of HIV infection diagnosis with the infant receiving daily Nevirapine through to age six weeks regardless of infant feeding method [219]. The PMTCT programme described in this thesis refers to the Option B+ guidelines in place during the conduct of this research from 2015 to 2019.

National PMTCT guidelines stipulate that all pregnant women are offered HIV testing by rapid antibody tests at their first antenatal visit, and if HIV negative, are offered repeat HIV testing every three months throughout pregnancy, at labour/delivery, at the six-week infant immunisation visit and three monthly throughout breastfeeding[219]. All HIV-exposed neonates have HIV PCR testing at birth and at 10 weeks if HIV PCR negative. Additional HIV antibody/PCR testing (test type dependent on age) is done six weeks post-cessation of breastfeeding and HIV antibody testing at 18 months of age if HIV negative[219].

The neonatal mortality rate in Soweto, based on a longitudinal cohort study from 2011 was estimated to be 22 per 1000 live births (unpublished data from Matflu study)[2],which was higher than the national rate of 14 per 1000 live births estimated for South Africa in 2015[3].

2.2 Study design

This study utilised different study designs and methods to address specific objectives. Procedures and processes common to all objectives will be presented in this chapter. Study design and methods only specific to a single objective will be reported in the relevant chapter.

We undertook a prospective, cross-sectional study at CHBAH from May to December 2016 (registered on ClinicalTrials.gov: NCT03722615). The study was nested within a larger cohort study in which mother-newborn dyads were enrolled antenatally or at birth, since June 2014, to investigate serologic correlates of protection against invasive Group B Streptococcal (GBS) disease (protocol number V98_28OBTP, University of the Witwatersrand Human Research Ethics Committee (Medical [HREC]) no. 140203, ClinicalTrials.gov: NCT02215226). In V98_28OBTP, research staff at antenatal clinic, labour/delivery wards identified and enrolled women interested in participation and collected maternal and infant

contact details, demographic and clinical data, maternal blood, vaginal swab and infant cord blood for laboratory testing. Informed consent for participation in the cCMV study was included in the V98_28OBTP study protocol and informed consent forms.

Inclusion criteria for the cCMV study required mother-newborn dyads to have consented and enrolled in V98_28OBTP, with mothers aged 18 years or older and residing in Soweto.

2.2.1 Participant enrolment and sample collection

Mothers and newborns enrolled in V98_28OBTP were identified during labour and delivery or in the immediate postnatal period in the maternity and neonatal wards of CHBAH between 0700h to 1900h from Monday to Friday. A saliva sample was collected from all enrolled newborns for cCMV testing within 72 hours of birth. Saliva collection took place before breastfeeding or if mothers reported having breastfed, at least one hour after feeding, using a nylon flocked swab (Copan Diagnostics Inc., Murrieta, CA, USA). Swabs were placed in the baby's mouth under the tongue until completely saturated with saliva. Swabs were then allowed to air dry, placed in its dry tube and were stored at 4ºC until transportation to the laboratory, within four hours of collection, for processing and CMV testing.

Mothers of neonates who screened positive for cCMV at birth were contacted and requested to return for a confirmatory test within three weeks of birth. At that visit, additional saliva swabs and urine samples, collected in an adhesive paediatric urine collector, were taken for CMV testing. Neonates with positive repeat samples for CMV were defined as confirmed cCMV cases.

Each confirmed cCMV case was matched to two neonates that screened negative for cCMV at birth based on gestational age (±2 weeks gestational age of case neonate), gender and HIV-exposure and their mothers contacted for consent to participate as cCMV-uninfected controls

for the prospective matched cohort study. The cohort study followed cases and controls until 12 months of age to determine differences in neurological sequelae and growth (Chapter 4). Each control neonate also had a second saliva sample collected within three weeks of birth to confirm they were cCMV-uninfected at the time of enrolment. Serum from women whose infants were confirmed cCMV cases and the subset of women whose infants were enrolled as cCMV-uninfected controls, collected at delivery, was retrieved and tested for anti-CMV IgM and IgG antibodies. Breast milk and a vaginal swab were also collected from these mothers to test for postpartum CMV shedding and described in further detail in Chapter 5.

Maternal and neonatal data were collected by clinical record review. Maternal data included age, HIV status, ART history, mid upper arm circumference (MUAC), past obstetric history and mode of delivery. Neonatal data included gestational age, birth weight, Apgar score, head circumference (measured by study staff), birth HIV PCR results, and history of hospitalisation immediately after birth for any cause. Intergrowth-21 standards[220,221] were used to classify neonates as small for gestational age ([SGA], birth weight below 10th percentile for gestational age[222]) and with microcephaly (head circumference for gestational age below -2 Z-scores[223]). The HIV status of infants was classified as HIV-unexposed (HIV uninfected infants born to HIV uninfected mothers), HIV-exposed (HIV uninfected infants born to mothers with HIV infection) or infants with HIV infection (infants with HIV infection born to mothers living with HIV). Figure 2.1 shows the steps in participant enrolment, age of infants at study visits, study procedures and study objectives during the 12 months follow-up.

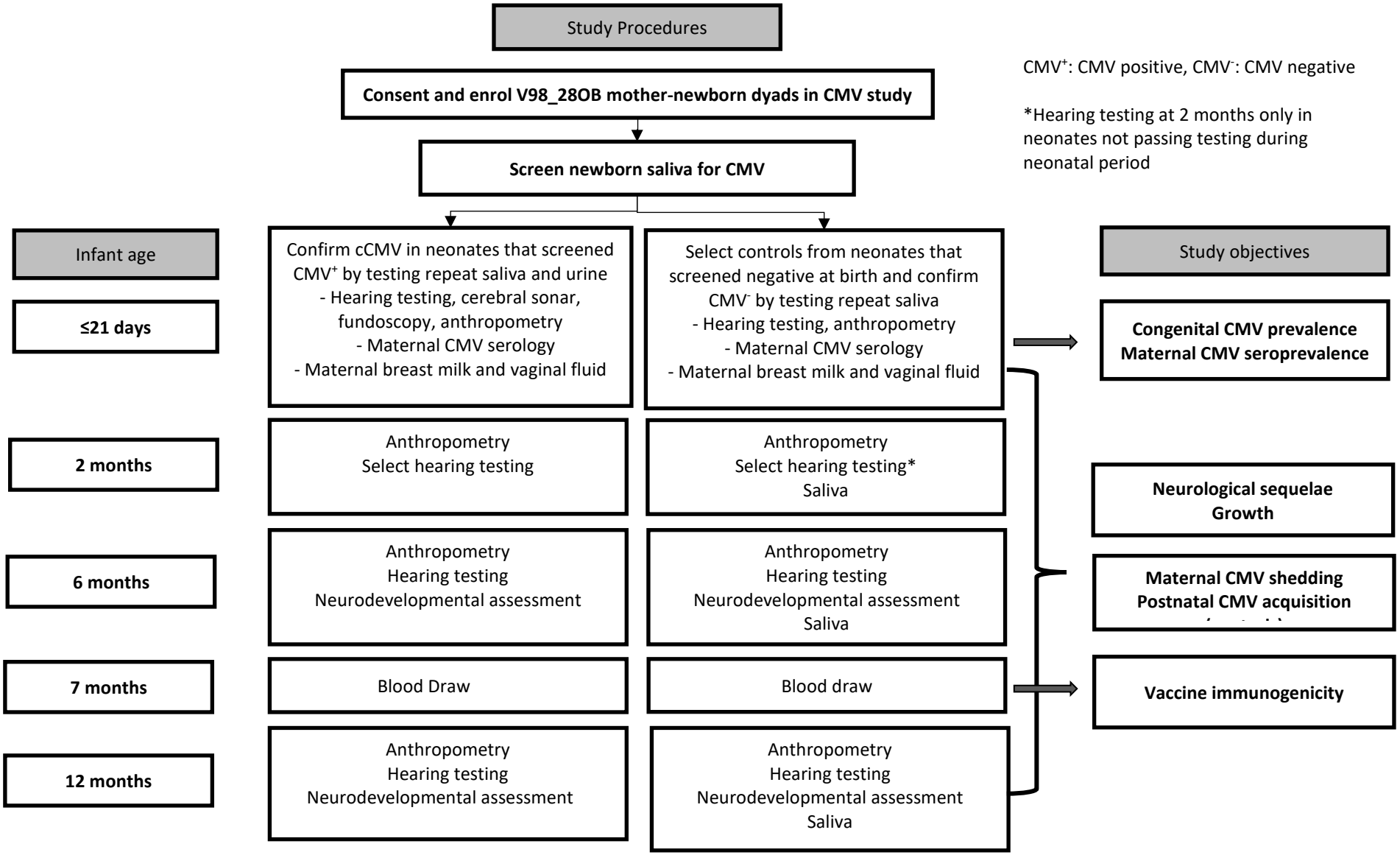


Figure 2.1: Study schema of participant enrolment, study procedures and study objectives

2.2.2 Sample processing and testing

All neonatal and maternal samples were processed and stored at the Respiratory and Meningeal Pathogens Research Unit (RMPRU) laboratory located in CHBAH. Saliva and maternal vaginal swabs were processed and stored at -70°C until testing using the PCR assay as described[175,184]. Urine and breast milk samples were separated into three aliquots and stored at -70°C for PCR testing. DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN) and real time PCR was undertaken using the highly conserved immediate early 2 (IE-2) exon region as the target sequence. CMV DNA detection was performed using the ABI 7500 Real-time PCR System (Applied Biosystems Inc., Foster City, CA). The reaction mixture contained primers at a concentration of 0.9 μM and the probe at 0.2 μM. The primer and probe sequences used were: forward primer GAG CCC GAC TTT ACC ATC CA, reverse primer CAG CCG GCG GTA TCG A and probe VIC - ACC GCA ACA AGA TT. A cycle time cut-off of 38, corresponding to five copies cut off per reaction, was implemented to identify positive and negative samples.

Quantitative serum IgG and IgM immunoassay was done using CMV IgM and IgG ELISA detection kits (Euroimmun) according to the manufacturer's protocol.

Maternal $CD4^+$ T-cell count, HIV viral load and newborn HIV PCR testing was performed at the National Health Laboratory Service (NHLS) as per standard of practice and results extracted from the NHLS corporate data warehouse, a central data repository of all public sector laboratory test results, or from medical records

2.2.3 Sample size calculation

The sample size of infants to be enrolled was determined to allow both a detection of a difference in cCMV prevalence between HIV-exposed and HIV-unexposed newborns, and a difference in neurological sequelae between cCMV cases and controls.

Based on the available literature, prevalence of cCMV in HIV-exposed neonates was anticipated at 2.9%[42] and in unexposed neonates at 1.0%[9]. Thus, a total sample of 1662 neonates with 831 HIV-exposed and 831 unexposed (1:1 ratio) for 80.0% power, was required to detect a 2.9 fold difference in cCMV prevalence between the HIV-exposure groups. These numbers were increased to accommodate the objective of determining neurological sequelae in cCMV-infected infants.

The anticipated prevalence of neurological sequelae in cCMV-uninfected controls with uneventful foetal or perinatal periods was estimated at 1.0%[224]. Prevalence of neurological sequelae in cCMV-infected infants was expected to be higher at approximately 17.0%, which was the lowest estimate (17-20%) selected from a global systematic literature review of cCMV related neurological sequelae (combining SNHL, cognitive/motor impairment and visual impairment) as a result of both primary and non-primary maternal CMV infection and symptomatic and asymptomatic infection at birth[63]. Furthermore, studies have recently demonstrated that neurological sequelae, specifically SNHL, occurs at a similar rate in infants with cCMV as a result of maternal non-primary CMV infection as in maternal primary infection[71,225] therefore the concern of under or overestimating neurological sequelae in our population was minimised. Thus 17% was selected as the most accurate expected prevalence of neurological sequelae (SNHL, neurodevelopmental delay and ophthalmological abnormalities) from the available published literature, given the lack of studies from Africa. At 80.0% power to detect a difference in neurological sequelae of 17-fold (17.0% vs 1.0%)

between cCMV-infected cases and cCMV-uninfected controls, 42 cCMV-infected neonates and 84 cCMV-uninfected neonatal controls (n=126) with an allocation ratio of 1:2 was required, which allowed for an anticipated loss to follow-up of 10.0%. Thus, after adjusting for 10% being non-evaluable CMV saliva screening of 2154 newborns was required. Ranges of sample size estimates for varying CMV prevalence by HIV exposure are given in Appendix 10.

2.3 Ethics statement

The Human Research Ethics Committee of the University of Witwatersrand, Johannesburg, South Africa approved V98_28OBTP (HREC 140203, NCT02215226) where mothers provided written informed consent for cCMV screening their neonates in the maternity wards. An independent Ethics approval (HREC M151161, Appendix 4) was obtained for the cohort study following up cCMV cases and controls until 12 months of age and the associated clinical and laboratory investigation with a separate written maternal informed consent for infant participation in this aspect of the study. The study was additionally registered in clinicaltrial.gov, number NCT03722615.

2.4 Funding

The cCMV prevalence and neurological sequelae study was funded by the South African Medical Research Council and The South African Research Chairs Initiative (SARChI), Grant number: UID 64797.

3 Prevalence of congenital cytomegalovirus infection and associated risk of in-utero HIV acquisition in a high HIV prevalence setting, South Africa

3.1 Introduction

The reported prevalence of cCMV are mostly from Western countries in Europe, USA and South America, with limited population-based studies from sub-Saharan Africa or settings with high HIV prevalence in pregnant women[15].

Early studies show that neonates with HIV infection have significantly higher prevalence of cCMV compared to HIV-exposed uninfected infants (10.3% vs 2.2%, p<0.001)[78]. ART in pregnant women living with HIV was associated with reduced rates of cCMV in HIV-exposed neonates from 3.5% (pre-ART era) to 1.2%[78]. In a study from the Western Cape, South Africa, 2.9% of 748 HIV-exposed neonates of women on prenatal ART were cCMV-infected at birth[42].

Although combination ART has contributed to a dramatic decline in mother-to-newborn HIV transmission rates to <2% [226,] the prevalence of HIV in high burden settings has remained stable over the past decade [217]. In South Africa, the prevalence of HIV-infection in pregnant women has stabilised at approximately 30.0%, while early vertical HIV transmission rates have declined to 1.1% [217].There are no data directly comparing cCMV in HIV-exposed and HIV-unexposed neonates from a high HIV and CMV seroprevalence setting, such as South Africa. The aim of this study was to determine the prevalence of and risk factors for cCMV in HIV-exposed and HIV-unexposed infants in an urban, middle-low income South African

population where women living with HIV infection routinely receive ART (published paper in Appendix 6, co-author approval in Appendix 7 and copyright permission in Appendix 9).

3.2 Methods

As reported in Chapter 2, this was a cross-sectional study conducted from May-December 2016. Briefly, saliva from neonates was screened for cCMV by PCR at birth by study staff. Additional saliva and urine samples were tested within three weeks of birth to confirm CMV positive saliva results. Confirmed cCMV cases were matched to cCMV-uninfected controls and enrolled in a cohort study for neurological sequelae and growth (Chapter 4). HIV PCR testing was done on the whole blood of HIV-exposed neonates as per standard of care by the NHLS. CMV serology (IgM and IgG) of mothers of confirmed cases and controls was tested by ELISA. Maternal and neonatal data were extracted from clinical records.

3.3 Statistical analysis

Prevalence of cCMV, including stratified by maternal HIV status, was calculated by the total number of confirmed cCMV cases and estimated cCMV cases in neonates that did not return for confirmatory testing (calculated by applying the rate of cCMV infection in confirmed cases to those that did not return for confirmatory testing) divided by the total number of neonates screened for cCMV infection at birth.

Maternal demographic and clinical characteristics between confirmed cCMV cases and neonates that screened negative for cCMV at birth were compared by univariate logistic regression to determine odds ratios and P values. Covariates with a P value of 0.2 or less were included in a multivariate regression model by manual backward selection to determine independent maternal risk factors for cCMV and neonatal characteristics, presented as

adjusted odds ratios (aOR) with P values. In HIV-exposed neonates, maternal CD4+ T-cell count, maternal HIV viral load, maternal ART duration, and rate of *in-utero* HIV infection (i.e. tested HIV PCR positive within 48 hours of birth) were analysed by univariate logistic regression. Maternal CMV seroprevalence (anti-CMV IgM and IgG) was determined in mothers of confirmed cases and the subset of cCMV-uninfected controls and compared by Fisher's exact test. Continuous data was analysed using Wilcoxon rank-sum test.

3.4 Results

Between May and December 2016, saliva samples were collected from 2685 neonates at a mean of 17 ± 65 hours after birth, including 828 (30.8%) HIV-exposed and 1856 HIV-unexposed neonates (Figure 3.1). Of the 91 neonates with a positive PCR test for CMV at birth, 62 (68.0%) of mothers consented to confirmatory testing. The mean age at time of confirmatory sample collection was 9.3 ± 6.1 days. There were 46/62 infants (74.2%) confirmed as cCMV cases. In 13/62 (21.0%), the confirmatory saliva and urine were negative for CMV (unconfirmed), and in 3/62 (4.8%), the confirmatory saliva PCR was negative and the urine sample was unavailable for testing (CMV status unknown). Figure 3.1 shows the neonatal CMV infection screening, infection confirmation and enrolment stages. There were no differences in maternal and neonatal characteristics between neonates that had confirmatory testing done and those that did not return for confirmatory testing (n = 30, Table 3.1).

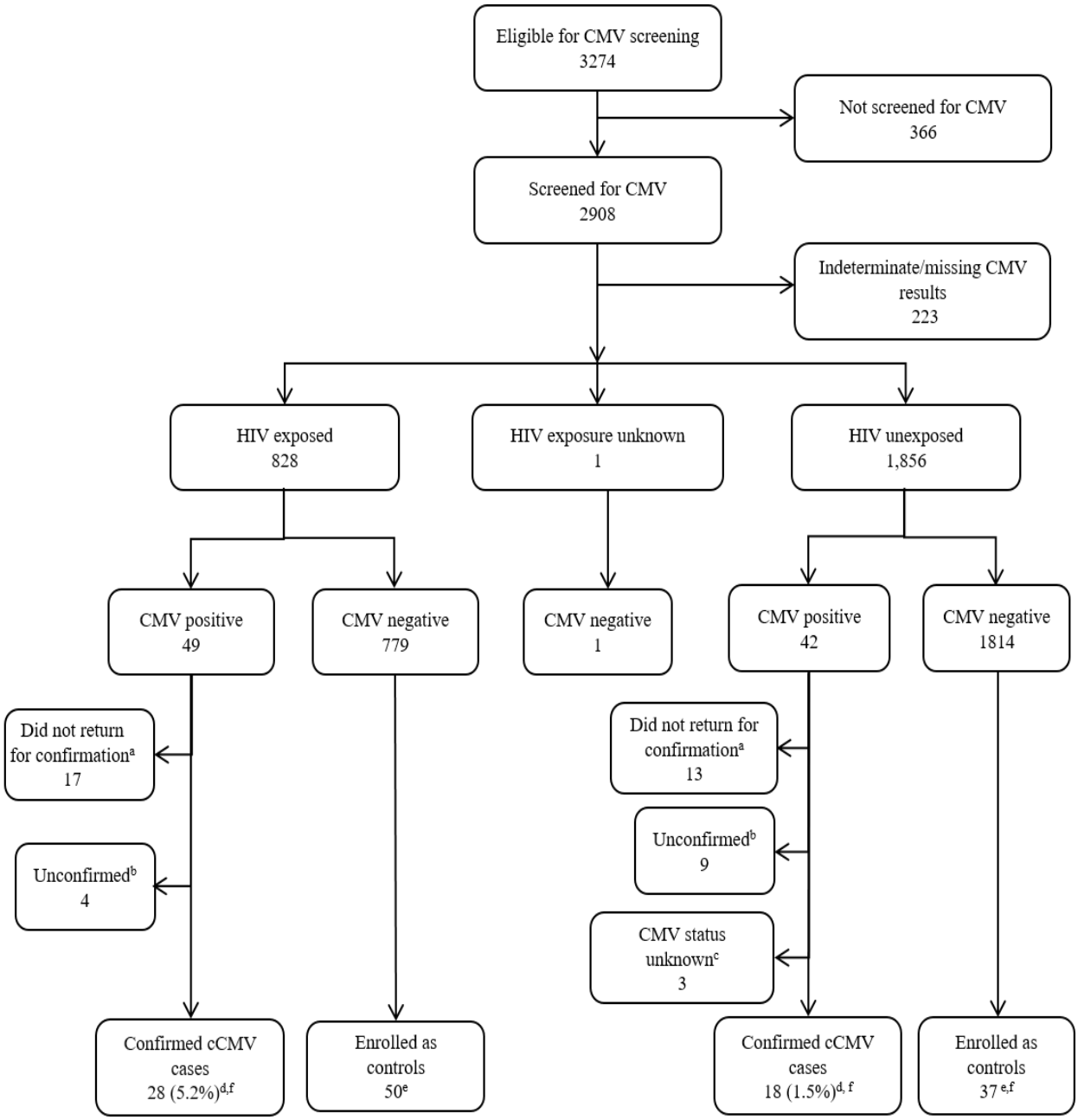
3.4.1 Maternal and Newborn Characteristics and cCMV prevalence

The median maternal age at delivery was 27 years (interquartile range [IQR] 23–32, Table 3.1). Women aged 18–24 years (aOR 2.3, 95% CI 1.05–5.10), women living with HIV (aOR

4.3, 95% CI 2.3–8.0), and women delivering vaginally (aOR 1.9, 95% CI 1.04–3.39) had greater risks of delivering an infant with cCMV (Table 3.1).

The overall prevalence of cCMV was 2.5% (95% CI 1.9–3.2), adjusted for the estimated cCMV rate in participants that did not return for confirmatory testing. The prevalence of cCMV in HIV-exposed neonates was 5.2% (95% CI 3.8–6.9) and in HIV-unexposed neonates was 1.4% (95% CI 0.9–2.0), with HIV-exposed neonates having a 3-fold greater odds of being cCMV-infected than HIV-unexposed neonates (aOR 3.5, 95% CI 1.93–6.41, Figure 3.1 and Table 3.2).

Neonates with cCMV were significantly more likely to be SGA (aOR 2.1, 95% CI 1.01–4.33) and very preterm (<32 weeks gestation, aOR 3.5, 95% CI 1.02–11.96, Table 3.2). There was no significant association of cCMV with gender, Apgar score, or rates of hospitalization immediately after birth (Table 3.2). Birth weight and prematurity were not included in the multivariate regression as these variables were co-linear with SGA.



[a]n=10 declined consent; n=1 died between screening and confirmation, n=18 lost to follow-up

[b]Unconfirmed: screening saliva CMV positive but confirmatory saliva and/or urine negative for CMV

[c]CMV status unknown: screening saliva CMV positive, confirmatory saliva CMV negative and urine sample not available for testing

[d]Prevalence adjusted for neonates that did not return for confirmation (n=17 in HIV-exposed and n=12 in HIV-unexposed)

[e]Controls were CMV negative on saliva on two independent saliva samples collected within 21 days of birth.

[f]Maternal blood of cases and controls tested for CMV IgG and IgM serology

Figure 3.1: Flow chart of neonatal cCMV screening and enrolment

Table 3.1: Comparison of characteristics between infants lost to follow up and infants confirmed for congenital cytomegalovirus

	Total N=92	Neonates lost to follow up n=30	Neonates confirmed for cCMV n=62	Unadjusted OR (95%CI)	p- value
Maternal age (years), n (%)	n=92	n=30	n=62		
18-24	37 (40.2)	11 (36.7)	26 (41.9)	0.58 (0.20, 1.65)	0.305
25-30	29 (31.5)	8 (26.7)	21 (33.9)	0.52 (0.17, 1.60)	0.254
>30	26 (28.3)	11 (36.7)	15 (24.2)	1	
Maternal parity, n (%)	n=91	n=30	n=61		
0	39 (42.9)	15 (50)	24 (39.3)	1.52 (0.51, 4.52)	0.454
1	28 (30.8)	8 (26.7)	20 (32.8)	0.97 (0.29, 3.23)	0.962
≥2	24 (26.4)	7 (23.3)	17 (27.9)	1	
Maternal HIV status, n (%)	n=92	n=30	n=62		
Positive	49 (53.3)	17 (56.7)	32 (51.6)	1.23 (0.51, 2.95)	0.649
Negative	43 (46.7)	13 (43.3)	30 (48.4)	1	
Mode of delivery, n (%)	n=92	n=30	n=62		
Caesarean	54 (58.7)	22 (73.3)	32 (51.6)	2.58 (1.0, 6.68)	0.051
Vaginal	38 (41.3)	8 (26.7)	30 (48.4)	1	
Gender, n (%)	n=92	n=30	n=62		
Male	52 (56.5)	18 (60)	34 (54.8)	1	
Female	40 (43.5)	12 (40)	28 (45.2)	0.81 (0.33, 1.96)	0.640
Gestational age at birth (weeks), n (%)	n=92	n=30	n=62		
<32	3 (3.3)	0	3 (4.8)	-	
32 - <34	5 (5.4)	3 (10)	2 (3.2)	3.13 (0.49, 20.04)	0.228
34 - <37	13 (14.13)	4 (13.3)	9 (14.5)	0.93 (0.26, 3.33)	0.908
≥37	71 (77.2)	23 (76.7)	48 (77.4)	1	
Birth weight (grams), n (%)	n=92	n=30	n=62		
<1500g	4 (4.4)	1 (3.33)	3 (4.84)	0.64 (0.06, 6.48)	0.704
1500g - <2500g	21 (22.8)	6 (20)	15 (24.2)	0.77 (0.26, 2.23)	0.625
≥2500g	67 (72.8)	23 (76.7)	44 (71)	1	

	Total N=92	Neonates lost to follow up n=30	Neonates confirmed for cCMV n=62	Unadjusted OR (95%CI)	p- value
Small for gestational age (SGA)[a], n (%)	n=92	n=30	n=62		
Birth weight for GA<10th percentile	18 (19.6)	5 (16.7)	13 (21)	0.75 (0.24, 2.35)	
Birth weight for GA ≥10th percentile	74 (80.4)	25 (83.3)	49 (79.03)	1	
Head Circumference for gestational age (Z score)	n=92	n=30	n=62		
<-2 Z score for gestational age	2 (2.17)	0	2 (3.23)	-	0.320
≥-2 Z score for gestational age	90 (97.8)	30 (100)	60 (96.8)	-	
5 min APGAR score, n (%)	n=90	**n=30**	**n=60**		
<7	1 (1.1)	0	1 (1.7)	-	0.477
7 -10	89 (98.9)	30 (100)	59 (98.3)	-	
Hospitalisation, n (%)	n=92	n=30	n=62		
Hospitalised at birth	12 (13.0)	2 (6.7)	10 (16.1)	0.37 (0.08, 1.81)	0.221
Not hospitalised	80 (87)	28 (93.3)	52 (83.9)	1	

Abbreviations: OR (Odds ratio); cCMV (congenital cytomegalovirus); CI, confidence interval
[a]Birth weight for GA <10th percentile.

Table 3.2: Maternal characteristics of confirmed cCMV-infected and cCMV-uninfected neonates

	Total N=2639	cCMV-infected N=46	cCMV-uninfected N=2593	OR[a] (95% CI)	P value	aOR[b] (95% CI)	P value
Maternal age in years	n=2631	n=46	n=2585				
Median age (IQR)	27 (23-32)	25 (22-29)	27 (23-32)	-	0.079		
18-24, n (%)	937 (35.1)	19 (41.3)	918 (35.5)	1.8 (0.85, 3.81)	0.123	**2.3 (1.05, 5.10)**	**0.039**
25-30, n (%)	725 (27.6)	16 (34.8)	709 (27.4)	1.9 (0.91, 4.26)	0.087	2.11 (0.96, 4.63)	0.062
>30, n (%)	969 (36.8)	11 (23.9)	958 (37.1)	1		1	
Mode of delivery	n=2632	n=46	n=2586				
Vaginal, n (%)	998 (37.9)	25 (54.4)	973 (37.6)	2.0 (1.1, 3.54)	0.023	**1.9 (1.04, 3.39)**	**0.037**
Caesarean, n (%)	1634 (62.08)	21 (45.7)	1613 (62.4)	1		1	
Parity	n=2627	n=45	n=2582				
0, n (%)	908 (34.6)	17 (37.8)	891 (34.5)	1.4 (0.65, 2.89)	0.403		
1, n (%)	843 (32.1)	16 (35.6)	827 (32.0)	1.4 (0.65, 2.96)	0.389		
≥2, n (%)	876 (33.4)	12 (26.67)	864 (33.5)	1			
Abortion history[c]	n=2615	n=45	n=2570				
None, n (%)	2192 (83.8)	38 (84.4)	2154 (83.8)	1			
Past abortions, n (%)	423 (16.2)	7 (15.6)	416 (16.2)	0.95 (0.42, 2.15)	0.909		
Mid-upper arm circumference	n=2592	n=46	n=2546				
<23cm, n (%)	95 (3.7)	3 (6.5)	92 (3.6)	4.2 (0.94, 19.47)	0.059	3.5 (0.74, 16.07)	0.114
23-26cm, n (%)	567 (21.9)	13 (28.3)	554 (21.8)	3.09 (1.0-9.52)	0.05	2.4 (0.74, 7.48)	0.147
27-32cm, n (%)	1400 (54.0)	26 (56.5)	1374 (54.0)	2.5 (0.86, 7.16)	0.091	2.1 (0.74, 6.23)	0.162
≥33cm, n (%)	530 (20.5)	4 (8.7)	526 (20.7)	1		1	
HIV status	n=2638	n=46	n=2592				
HIV infection, n (%)	807 (30.6)	28 (60.9)	779 (30.1)	3.6 (1.99, 6.58)	<0.001	**4.3 (2.3, 8.0)**	**<0.001**
HIV infected, n (%)	1831 (69.4)	18 (39.1)	1813 (70.0)	1		1	

Values in bold indicate independent risk factors for cCMV infection

Abbreviations: OR (Odds ratio), aOR (Adjusted odds ratio); cCMV (congenital cytomegalovirus); CI (confidence interval), IQR (interquartile range)

[a]ORs are from univariate logistic regression

[b]Covariates with a P value of 0.2 or less in the univariate analysis (OR) were included in a multivariate logistic regression model to determine independent maternal risk factors for cCMV

[c]History of induced or spontaneous abortions

Table 3.3: Characteristics of confirmed cCMV-infected and uninfected neonates

	Total N= 2652	cCMV-infected N=46	cCMV-uninfected N=2606	OR[a] (95% CI)	P value	aOR[b] (95% CI)	P value
Gender	n=2639	n=46	n=2593				
Male, n (%)	1276 (48.4)	21 (45.7)	1255 (48.4)	0.9 (0.50, 1.61)	0.712		
Female, n (%)	1363 (51.6)	25 (54.4)	1338 (51.6)	1			
Gestation (weeks)	n=2638	n=46	n=2592				
<32, n (%)	65 (2.5)	3 (6.5)	62 (2.4)	3.0 (0.90, 10.02)	0.075	**3.5 (1.02,11.96)**	**0.046**
32-<34, n (%)	74 (2.8)	2 (4.4)	72 (2.8)	1.7 (0.41, 7.30)	0.462	1.8 (0.41, 7.70)	0.436
34-<37, n (%)	359 (13.6)	7 (15.2)	352 (13.6)	1.2 (0.54, 2.80)	0.619	1.2 (0.52, 2.71)	0.692
≥37, n (%)	2140 (81.1)	34 (73.9)	2106 (81.3)	1		1	
Birth weight (g)	n=2636	n=46	n=2590				
<1500	36 (1.4)	3 (6.5)	33 (1.3)	6.4 (1.87, 22.02)	0.003		
1500g -<2500	308 (11.7)	11 (23.9)	297 (11.5)	2.6 (1.30, 5.24)	0.007		
≥2500	2292 (87.0)	32 (69.6)	2260 (87.3)	1			
Small for gestation[c]	n=2623	n=46	n=2577				
Yes, n (%)	315 (12.0)	10 (21.7)	305 (11.8)	2.1 (1.02, 4.21)	0.045	**2.1 (1.01, 4.33)**	**0.046**
No, n (%)	2308 (88.0)	36 (78.3)	2272 (88.2)	1		1	
Apgar score, n (%)	n=2576	n=44	n=2532				
<7, n (%)	14 (0.5)	1 (2.3)	13 (0.5)	4.5 (0.58, 35.22)	0.151		
≥7, n (%)	2562 (99.5)	43 (97.7)	2519 (99.5)	1			
HIV exposure, n (%)	n=2639	n=46	n=2592				
HIV-exposed, n (%)	807 (30.6)	28 (60.9)	779 (30.1)	3.62 (1.99, 6.58)	<0.001	**3.5 (1.93, 6.41)**	**<0.001**
HIV-unexposed, n (%)	1831 (69.4)	18 (39.1)	1813 (70.0)	1		1	
Hospitalised at birth, n (%)	n=2639	n=46	n=2593				
Yes, n (%)	292 (11.1)	9 (19.6)	283 (10.9)	1.98 (0.95, 4.16)	0.069		
No, n (%)	2347 (88.9)	37 (80.4)	2310 (89.1)	1			

Values in bold indicate independent risk factors for cCMV infection.
Abbreviations: OR (Odds ratio), aOR (Adjusted odds ratio); cCMV (congenital cytomegalovirus); CI, confidence interval
[a]ORs are from univariate logistic regression.
[b]Covariates with a P value of 0.2 or less in the univariate analysis were included in a multivariate logistic regression model to determine independent maternal risk factors for cCMV.
[c]Birth weight for GA <10th percentile.

Overall mothers of 790 (of 828) HIV-exposed infants were confirmed to have initiated ART at or prior to delivery; including 93.3% (737/790) on combined Tenofovir, Emtricitabine and Efavirenz; 2.0% (16/790) on ART of unknown type; and there were no further details on ART history in the remaining 5.0%. Among all HIV-exposed neonates, 13 (1.8%) were infected with HIV, and those with cCMV were 20-fold (OR 20.1, 95% CI 6.09–66.46) more likely to be infected with HIV *in-utero* (5/27, 18.5%) than cCMV-uninfected infants (8/716, 1.1%, Table 3.3). Of the neonates infected with HIV, the median maternal ART duration at delivery was not significantly different between cCMV-infected (148 days, IQR 89-244) and cCMV-uninfected neonates (173 days, IQR 113-552, Table 3.3). The gestational age at the time of maternal ART initiation and the severity of maternal immunosuppression based on CD4+ T-cell counts and HIV viral loads were not significantly different between cCMV-infected and -uninfected neonates (Table 3.3).

3.4.2 Maternal cytomegalovirus serology

Maternal serum of 122 women were tested for CMV IgG and IgM of whom 41/46 were mothers of confirmed cCMV cases and 81/84 from the subset of mothers of neonates enrolled as cCMV-uninfected controls. All women (100%) were CMV IgG positive, 91.8% were IgM negative and 5.7% (7/122) were both IgM and IgG positive. In those whom were IgM positive, 7.3% (3/41) were mothers of cCMV cases and 4.9% (4/81) were mothers of cCMV-uninfected controls (P=0.561). As IgG avidity testing was not conducted, the type of maternal CMV infection in the IgM positive women could not be determined.

Table 3.4: Characteristics of HIV-exposed, confirmed cCMV-infected and cCMV-uninfected neonates

	Total N=807	cCMV[a] infected n=28	cCMV-uninfected n=779	OR[a] (95% CI)	P value
Infant HIV status	n=743	n=27	n=716		
HIV-exposed infected, n (%)	13 (1.8)	5 (18.5)	8 (1.1)	**20.1 (6.09, 66.46)**	**<0.001**
HIV-exposed uninfected, n (%)	730 (98.3)	22 (81.5)	708 (98.9)	1	
Maternal CD4+ count (cells/mm^3)	n=648	n=19	n=629		
Median (IQR)	432 (277-603)	415 (350-595)	432 (275-603)		0.993
Severe (<200), n (%)	80 (12.4)	3 (15.8)	77 (12.2)	1.6 (0.38, 6.38)	0.537
Advanced (200−<350), n (%)	147 (22.7)	1 (5.3)	146 (23.2)	0.3 (0.03, 2.30)	0.233
Mild (350 - <500), n (%)	175 (27.0)	9 (47.4)	166 (26.4)	2.2 (0.76, 6.21)	0.149
Not significant (≥500), n (%)	246 (38.0)	6 (31.6)	240 (38.2)	1	
Maternal HIV viral load (copies/ml)	n=513	n=3	n=510		
Median (IQR)	40 (0-469)	19 (0-262)	40 (0-470)		0.547
<1000copies/ml, n (%)	401 (78.2)	3 (100)	398 (78.0)		
≥1000copies/ml, n (%)	112 (21.8)	0 (0)	112 (22.0)		
Gestation at ART initiation	n=576	n=23	n=553		
Median ART duration (IQR), days	172 (113-468)	148 (89-244)	173 (113-552)		0.203
Preconception, n (%)	186 (32.3)	5 (21.7)	181 (32.7)	1	
1st trimester, n (%)	49 (8.5)	4 (17.4)	45 (8.1)	3.2 (0.83, 12.47)	0.091
2nd trimester, n (%)	227 (39.4)	8 (34.8)	219 (39.6)	1.3 (0.43, 4.11)	0.629
3rd trimester, n (%)	104 (18.1)	5 (21.7)	99 (17.9)	1.8 (0.52, 6.47)	0.349
Postnatal, n (%)	10 (1.7)	1 (4.4)	9 (1.6)	4.02 (0.42, 38.12)	0.225

Values in bold indicate independent risk factors for cCMV infection.

Abbreviations: ART (antiretroviral therapy), cCMV (congenital CMV), CI (confidence interval), IQR (interquartile range), OR (odds ratio)

[a]ORs are from univariate logistic regression

3.5 Discussion

In this population-based study in Soweto, the overall prevalence of cCMV was 2.5%, which is higher than the rate reported in high income countries (0.2-2.0%) but within the range reported from other LMIC (0.6-6.1%) [49]. The high prevalence in our setting is at least in part due to the greater risk of cCMV in HIV-exposed neonates (5.2%) compared to HIV-unexposed neonates (1.4%). Few other studies have directly compared cCMV infection in HIV-exposed and HIV-unexposed neonates. A study of high-risk neonates admitted to the intensive care unit in a Zambian referral hospital found a high cCMV prevalence of 11.4% (9/79, 95% CI 6.1–20.3) in HIV-exposed infants compared to 2.1% (6/293, 95% CI 0.8–4.6) in HIV-unexposed infants[80]. However, the selected nature of the study patient population makes it difficult to compare findings from that study to our findings. It can be speculated that the increased rate of cCMV in HIV-exposed neonates may be due to more frequent CMV reinfection or reactivation in their mothers, waning of protective immunity or reduced trans-placental transfer of protective antibody. Although the prevalence of cCMV in HIV-exposed infants in our study was higher than previously reported in the Western Cape, South Africa (2.9%), it is possible that differences in the study population such as race, socioeconomic status and living conditions may explain the difference[42].

A strong association of cCMV with *in-utero* transmission of HIV was observed. Among HIV-exposed neonates, 18.5% (5/27) of infants with cCMV were HIV PCR positive at birth, compared to 1.1% (8/716) of cCMV-uninfected neonates. This finding suggests the possibility that cCMV co-infection may account for a large fraction of the residual maternal to foetal HIV transmission in populations with efficient PMTCT programs. There was no significant difference in median ART duration between mothers of HIV and cCMV co-infected neonates and neonates with HIV infection but were cCMV-uninfected, similar to the

findings in the French perinatal cohort[78]. The lack of association however, may be due to insufficient power to make this comparison because of the low rate of *in-utero* HIV infection in our study and the French study. As functional immunity of maternal anti-CMV antibodies was not performed and dysfunction could affect mother to child transmission of both CMV and HIV, cCMV could also be considered a proxy of the maternal immunological imbalance and not only the aetiology of transmission.

A series of possible mechanisms have been proposed in which CMV infection of T-cells potentiates their susceptibility to HIV infection or enhances HIV replication[227]. Johnson et al. (2015) demonstrated that *in-vitro* stimulation of cord blood mononuclear cells by CMV upregulates C-C chemokine receptor type 5 (CCR5) expression levels on memory T-cells, enhancing their susceptibility to HIV infection[228]. Polymorphisms in the Toll-like receptor 9, 1635 locus have been described to be associated with HIV acquisition and progression[229]. Having one or more copies of the 1635A allele was also associated with increased CMV acquisition (congenital or postnatal) in Kenyan infants with HIV infection (42.0%) than those with the 1635GG genotype (11.0%, P=0.03)[229]. This suggests a shared mechanism for HIV and CMV infection in infants. As infants continue to acquire HIV perinatally, even with the success of PMTCT programmes, albeit at much lower risk, further investigation of the pathogenesis of *in-utero* CMV and HIV acquisition in the ART era could help explain the causes for the residual burden of *in-utero* mother-to-newborn HIV transmission.

Maternal $CD4^+$ T-cell counts were not associated with cCMV infection in HIV-exposed neonates in our study. This is in contrast to the study in the Western Cape, where a significantly higher prevalence of cCMV was observed in infants of mothers whose $CD4^+$ T-cell count, mostly measured in the first and second trimester of pregnancy, was <200 cells/μL and the only factor independently associated with cCMV[42]. This lack of association in our

study maybe due to the fact that the majority of women in our study were started on ART earlier in pregnancy with considerable immune recovery by the third trimester and delivery resulting in higher CD4+ T-cell count. Similarly, HIV-viral load tested later in pregnancy was not associated with the risk of CMV transmission from mother to foetus. Since longer duration of ART did not reduce cCMV transmission in our study, other factors may play a role in CMV transmission. These factors include the interaction of HIV and CMV at the maternal-foetal placental interface and the effect of exposure to *in-utero* ART, which may cause a range of phenotypical and functional immunological changes in HIV-exposed foetuses facilitating increased cCMV transmission in women living with HIV infection[230].

Very-preterm birth and SGA was associated with cCMV in our study. Similar findings were reported in a prospective Brazilian study on cCMV prevalence in a high maternal CMV seroprevalence and low-income setting with higher frequency of SGA in neonates with cCMV than those without (26.4% [n=23/87] vs 10.7% [n=852/7960] respectively)[71]. We did not however consider SGA as a symptom of cCMV as we did not systematically collect other maternal factors associated with SGA and preterm-birth such as pregnancy-induced-hypertension, alcohol and smoking practices or chronic maternal conditions other than HIV infection. Considering the impact of low birth weight on morbidity and mortality in African infants[231], the potential association of cCMV contributing to these underlying conditions merits further investigation.

Almost all the women (92.0%) were CMV seroimmune with only anti-CMV IgG detected confirming cCMV infection mostly occurs due to maternal non-primary CMV infection in high CMV seroprevalence settings. In those women with both CMV IgG and IgM detected (7.3%), although there was no statistical significant difference in IgM positivity between those who gave birth to cCMV-infected and cCMV-uninfected neonates, this may have been

due to limited sample size as those who gave birth to cCMV-infected neonates were more frequently IgM positive.

Limitations of our study include a high number of cCMV positive infants at screening that could not be enrolled in the follow-up to confirm cCMV (n=29). However, there were no significant differences in maternal or newborn characteristics between this group and those who returned for follow-up. In addition, screening positive results were not confirmed in follow-up in 13 infants (0.48%). We followed the PCR protocol used in a previous study (Boppana et al. 2011) which evaluated real time PCR on newborn saliva samples in a large prospective newborn CMV screening that included 17327 infants with a sensitivity and specificity of the dried saliva real time PCR determined to be 97.4% and 99.9% respectively.

We report a high prevalence of cCMV in HIV-exposed neonates compared to HIV-unexposed neonates; and an increased rate of *in-utero* HIV infection in HIV-exposed neonates with concomitant cCMV, in the era of universal maternal ART in a population based study in South Africa. The continued risk of cCMV in HIV-exposed infants, could possibly contribute to the higher morbidity and mortality in the first year of life compared to HIV-unexposed infants[126,232]. The impact of cCMV as well as postnatally acquired CMV infection on these infants needs to be determined in natural history studies with monitoring for long-term outcomes.

4 Neurodevelopment, hearing and growth outcomes in African children with congenital cytomegalovirus: a prospective cohort study in Soweto, South Africa

4.1 Introduction

Most cases of cCMV infections are clinically asymptomatic at birth (85-90%)[13], however, permanent neurological sequelae (13.5%) can develop during childhood[63] with SNHL (10%) being the most common[13]. In new-borns with symptomatic cCMV at birth; a higher proportion of them develop CMV associated neurological sequelae (40-58%)[55] including sensorineural hearing loss (SNHL, 27.0-50.0%), cognitive and/or motor deficits (50.0%)[55], and visual impairment (22.0%)[9,13,63].

Most estimates on outcomes of cCMV infection are from high-income settings where cCMV is the result of maternal primary infection. Although most cCMV infection in sub-Saharan African populations are the result of maternal non-primary CMV infection, usually first acquired during childhood[48,113,233], population-based studies estimating neurological sequelae in children of mothers with non-primary CMV infection from African settings are limited[50,56].

Infants with HIV and cCMV co-infection, in the pre-ART era, had accelerated progression of HIV disease, neurological deterioration and increased mortality[76]. With the advent of prevention of mother to child HIV transmission measures through lifelong ART for pregnant women living with HIV, most of their offspring are not infected with HIV. These HIV-

exposed, uninfected children, however, are at increased risk of mortality, growth and developmental deficits compared to HIV-unexposed children[126].

The aim of this study was to compare hearing, neurodevelopmental and growth outcomes in the cohort of cCMV-infected infants (cases) identified in Soweto with a group of cCMV-uninfected infants (matched controls) until 12 months of age. Additionally, the effect of HIV-exposure on hearing, neurodevelopment and growth of infants born to women living with HIV was explored.

4.2 Methods

This was a prospective matched cohort study. Forty-six neonates with confirmed cCMV (cCMV cases) were matched for gender, gestational age (±two weeks) and HIV exposure to 84 cCMV-uninfected neonates (cCMV-uninfected controls) who were confirmed as cCMV-uninfected within 21 days of birth (described in Chapter 2, section 2.2.1). Cases and controls were prospectively followed to assess hearing, neurodevelopment and growth during the first 12 months of life over four planned study visits. Additional assessments were conducted if indicated. Participant enrolment and assessments are shown in Figure 4.1.

As described previously, maternal CMV seroprevalence was determined in mothers of confirmed cases and controls by testing serum collected at delivery for anti-CMV IgG and IgM to determine the proportion of infants born to women with non-primary CMV infection[234].

4.2.1 Anthropometry

Birth weight was extracted from neonatal records. Head circumference, mid-upper arm circumference (MUAC), length (Seca 416 infantometer) and weight (SECA 354 digital baby

scale from zero to six months of age and Seca 876 2-in-1 scale from six to 12 months of age) were measured by the study physician or nurse at each of the study visits which included, within 21 days of birth and at two, six and 12 months of age (Figure 4.1). Anthropometry was converted to standardized Z-scores using the WHO Child Growth Standards[235]. Conversion of anthropometry to Z-scores was not possible for preterm infants at the first visit until corrected age surpassed term gestation.

4.2.2 Clinical Data

Maternal and infant medical records were reviewed for clinical data, HIV PCR results, ART duration, birth weight and any hospital admission and investigation. Symptomatic cCMV infection at birth was defined as one or more findings of jaundice with conjugated hyperbilirubinemia>2mg/dL, thrombocytopenia (platelet count <100 000/mm^3), petechiae, microcephaly, hepatosplenomegaly, seizures and/or chorioretinitis[103]. Platelet counts were conducted only if neonates had clinical findings at birth, thus not all cCMV confirmed neonates had platelet counts.

4.2.3 Ophthalmologic examination

Only cCMV-infected cases had an examination of the retina within 21 days of birth by the ophthalmologist at CHBAH as part of the assessment for symptomatic cCMV. Follow-up care was provided as appropriate if abnormalities were detected. We did not perform ophthalmologic examination in controls as they were assessed to be healthy and CMV-uninfected at birth, and due to limited available resources to undertake such testing in our public-health setting.

4.2.4 Cerebral ultrasonography

Cases of cCMV had cerebral ultrasonography performed by neonatologists within 21 days of birth at CHBAH to describe any cCMV associated lesions as an exploratory objective. Cerebral findings were not included in the definition of symptomatic cCMV. Infants with ultrasound abnormalities were referred to a specialist neonatology clinic for care and management. Cerebral ultrasonography was not conducted in controls as they were required to be cCMV-uninfected and clinically healthy and again to avoid unnecessary investigation in this resource limited setting.

4.2.5 Hearing assessment

Audiologists at CHBAH conducted the hearing assessments on all cases and controls. Within 21 days of birth, infants received hearing screening by automated auditory brainstem response (AABR [Maico MB11 BERAphone®, MAICO Diagnostics GmbH]), with integrated electrodes at an intensity of 35dBnHL. At age six and 12 months, screening included ipsilateral acoustic reflex thresholds, distortion product otoacoustic emissions (DPOAE) and AABR screening. A hearing screening 'pass' during the neonatal period included pass AABR bilaterally. A hearing screening 'pass' at six and 12 months included either: 1) pass AABR bilaterally, or 2) acoustic reflexes present in at least two frequencies in both ears and three screening DPOAEs present at three frequencies in both ears. Diagnostic hearing assessments for children not passing hearing screening (classified as "referred") included otoscopy, tympanometry, electrophysiological measures (i.e. diagnostic auditory brainstem responses, auditory steady state responses, cochlear microphonic testing) with air and bone conduction testing, and diagnostic otoacoustic emissions (OAE) and behavioural audiometric evaluations with air and bone conduction testing as indicated and developmentally appropriate. Audiological management and referral to the paediatric ear nose and throat specialists took place when indicated. SNHL was defined as unilateral SNHL with air and bone conduction

thresholds greater than or equal to *25* decibels hearing level (dBHL) or bilateral SNHL with air and bone conduction thresholds greater than or equal to 25 dBHL in the better ear. Conductive hearing loss (CNHL) was classified when bone conduction thresholds were within normal limits, and air conduction thresholds were raised. Severity of hearing loss was categorized as: mild (26 to 40 dBHL), moderate (>41 to 55dBHL), moderately severe (56 to 70dBHL), severe (71 to 90dBHL), or profound (>90dBHL)[236].

4.2.6 Neurodevelopmental assessment

The Bayley III scales of infant and toddler development was administered to all cases and controls at six and 12 months of age by the study physician. Each neurodevelopmental domain was assessed based on observed responses to a set of tasks presented to the child and scored directly on the following subscales: cognitive scale, language summed scale of receptive and expressive language subscales and motor summed scale of fine and gross-motor subscales. Composite scores were derived from raw scores for cognitive, language, and motor development and scaled to a metric, with a mean of 100, standard deviation of 15, and range of 40 to 160. Performance that was 2.0 or more standard deviations below the mean score in any domain was defined as neurodevelopmental delay[237,238].

Neurological sequelae were defined as a composite of SNHL and neurodevelopmental delay at 12 months of age.

4.2.7 Statistical analysis

The sample size of cases and controls required to detect any difference in neurological sequelae was based on an anticipated neurological sequelae prevalence of 17.0%[63] in cases and 1.0%[224] in controls as described in Chapter 2, section 2.2.3. Allowing for a loss to follow-

up of 10% and 80% power, at least 42 cCMV cases and 84 cCMV-uninfected controls (N=126) with an allocation ratio of 1:2 were required to be enrolled.

Symptomatic cCMV infection in cases was described based on clinical findings at birth and ophthalmologic examination. The difference in symptoms between HIV-exposed and HIV-unexposed cCMV cases were compared by Fisher's exact test. Cerebral ultrasound findings in cCMV cases were described and a comparison made between HIV-exposed and HIV-unexposed cCMV cases using the Fisher's exact test.
Hearing results during the neonatal period, and at six and 12 months of age and neurodevelopmental assessment results using the Bayley III scoring at six and 12 months of age were compared between cCMV cases and cCMV-uninfected controls by conditional logistic regression and odds ratios computed and further analysed stratified by HIV-exposure. Neurodevelopmental analysis was additionally adjusted for comorbidities (one cCMV case with congenital heart disease).

A multivariable linear mixed effects model was used to estimate the differences in anthropometric Z-scores (based on the World Health Organization [WHO] 2006/2007 growth standards) between cCMV cases and cCMV-uninfected controls in general and marginal effects at each study visit in particular with the model adjusted for breastfeeding and HIV infection. In addition, as the analysis considered growth over time, microcephaly present at birth was adjusted for. Individual infant variability in the measurements that were done at different follow-up times was accounted for using random intercept and random slope. For the group matching variable, only a random intercept was assumed. The mean differences between anthropometric Z-scores between HIV-exposed cCMV cases and cCMV-uninfected controls and HIV-unexposed cCMV cases and cCMV-uninfected controls were also analysed. All tests were 2-sided with a significance level of 5%.

4.3 Results

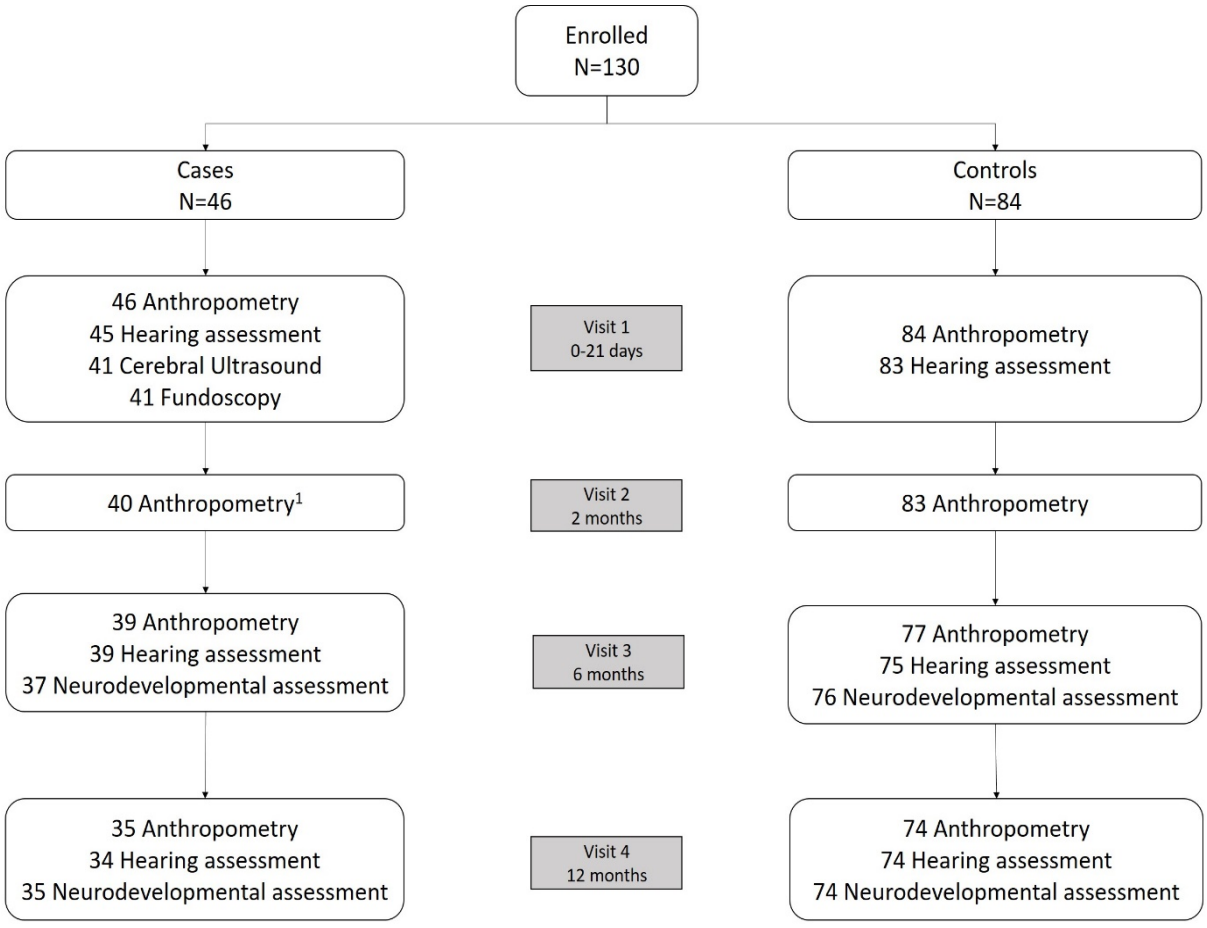
From May to December 2016, 130 infants were enrolled at birth including 46 cCMV cases and 84 cCMV-uninfected controls. Figure 4.1 provides cases and controls assessed at each study visit and Table 4.1 presents baseline characteristics. There were four additional cases identified than the required 42 with follow up offered as duty of care. All infants were black South Africans except for one control infant of mixed race. Of HIV-exposed infants (N=77), five acquired HIV *in-utero* (all also with cCMV) and were initiated on ART. There were 91.8% (112/122) neonates born to women with non-primary CMV infection (only IgG positive as reported in Chapter 3, section 3.4.2). The type of maternal CMV infection was undetermined in 5.7% (7/122) of neonates whose mothers were both IgG and IgM positive. Of these seven women, 7.3% (3/41) were mothers of cCMV cases and 4.9% (4/81) mothers of cCMV-uninfected controls (P=0.561).

At the age of 12 months, 34 (73.9%) cases and 74 (88.1%) controls completed all assessments resulting in 74% power to detect 17.0% difference in neurological sequelae between the groups (assuming a rate of 1.0% in controls). There was no difference in baseline characteristics at birth between infants that dropped out and those remaining in the study.

4.3.1 Symptomatic cCMV infection

Three of 46 (6.5%, 95% CI 1.4-17.9) cCMV cases were symptomatic at birth. One each had microcephaly (also with HIV infection), neonatal jaundice with conjugated hyperbilirubinemia>2mg/dL, (HIV-unexposed) and thrombocytopenia with platelet count <100 000/mm^3 (HIV-unexposed). The type of maternal CMV infection in two of the three symptomatic cases (microcephaly and neonatal jaundice) was confirmed as due to non-

primary CMV infection as serum was only IgG positive. In the case with thrombocytopaenia, the type of maternal CMV infection was undetermined as serum was not available for testing. One (2.2%) asymptomatic cCMV case, co-infected with HIV *in-utero,* died at the age of two months due to meningitis of unknown aetiology. Forty-one cCMV cases had fundoscopic examination during the neonatal period. All had normal examinations except for one with retinopathy of prematurity, born at 28 weeks gestation, which resolved by six months of age. One cCMV case had cardiac surgery for Tetralogy of Fallot at the age of six months. There was no difference in symptoms between HIV-exposed and HIV-unexposed cases.



[1]N=1 died before 2 months age.

Figure 4.1: Flow chart representing cCMV case infants and cCMV-uninfected control children enrolled and assessments at each study visit

Table 4.1: Baseline characteristics cCMV-infected case and cCMV-uninfected control children

	cCMV cases N=46	cCMV-uninfected controls N=84	P value
Female[1], n (%)	21 (45.7)	41 (48.8)	0.730
Birth weight, median (IQR)	2845 (2455-3190)	2903 (2595- 3195)	0.411
Gestational age[1], median (IQR)	38 (36-40)	38 (36-39)	0.897
Small for gestational age, n (%)	10 (21.7)	9 (10.7)	0.100
HIV-exposed[1], n (%)	28 (60.9)	49 (58.3)	0.641
HIV-infection, n (%)	5 (10.9)	0 (0)	**0.002**
Duration of maternal ART prior to delivery, mean days (SD)	317 (462)	531 (816)	0.260
Maternal age at delivery, median (IQR)	25 (22-29)	28 (24-33)	0.021
Breastfeeding, n (%)	35 (76.1)	62 (73.8)	0.775
Mortality, n (%)	1 (2.2)	0 (0)	0.327

Abbreviations: cCMV (congenital CMV), IQR (interquartile range), SD (standard deviation)

[1]Cases and controls were matched on gender, gestational age and HIV-exposure and there are no differences expected in these variables between cases and controls

4.3.2 Cerebral Ultrasound

Forty-one cCMV cases had cerebral ultrasound scans of whom six (14.6%) had abnormalities detected. Abnormalities included three (7.3%) with cysts, two (4.9%) with ventricular dilatation and one (2.4%) with calcifications. There was no significant difference in cerebral abnormalities between HIV-exposed (20.8%) and HIV-unexposed cases (5.9%, P=0.373). The case with microcephaly, could not be assessed successfully due to fusion of the fontanelles. Three cases were initiated on oral valganciclovir (one symptomatic cCMV case with microcephaly and two asymptomatic cCMV cases with abnormal cerebral imaging) at the discretion of the attending neonatologist.

4.3.3 Hearing outcome

Among the 130 infants enrolled, 45 cCMV cases and 83 cCMV-uninfected controls had hearing screening in the neonatal period (Table 4.2). Thirty-nine cases (86.7%) and 76 controls (91.6%) passed hearing screening suggesting intact auditory neural pathways at birth with no significant difference between cases and controls (OR 1.6, 95% CI 0.28-7.99, P=0.631). Of infants that did not pass screening, six (13.3%) were cases and seven (8.4%) were controls. Of these infants that completed repeat testing at two months of age, all had evidence of middle ear dysfunction and SNHL could not be completely assessed.

At six months of age, 38 cases and 75 controls completed hearing assessments of whom 31 (81.6%) cases and 48 (64.0%) controls had normal hearing with no significant difference between cases and controls (OR 0.5, 95% CI 0.21-1.32, P=0.170, Table 4.2). Of the seven (18.4%) cases and 27 controls (36.0%) that did not pass, five (71.4%) cases and 26 (96.3%) controls had evidence of middle ear effusion or dysfunction, detected during hearing assessment and SNHL could not be completely assessed.

At 12 months of age, 34 cases and 74 controls completed hearing assessments of whom 31 (91.2%) cases and 58 (78.4%) controls had normal assessments with no significant difference between cases and controls (OR: 0.5, 95% CI 0.13-1.84, P=0.292, Table 4.2). All three (8.8%) cases with abnormal assessment, had evidence of middle ear dysfunction. Of the 16 (21.6%) controls that did not pass, 12 (75.0%) had evidence of middle ear dysfunction, three (18.9%) did not cooperate with testing on two separate attempts and one had suspected hearing loss of uncertain aetiology who did not return for additional testing.

There was no difference in hearing outcomes between HIV-exposed cCMV cases and HIV-unexposed cCMV cases at both six and 12 months of age.

Table 4.2: Hearing screening results of cCMV-infected case and cCMV-uninfected control children over 12 months

	Total	cCMV-infected cases N=46	cCMV-uninfected controls N=84	Odds Ratio[1] (95% CI)	P value
Newborn screening[2]	n=128	n=45	n=83		
Passed, n (%)	115 (89.8)	39 (86.7)	76 (91.6)	1.5 (0.28, 7.99)	0.631
Referred, n (%)	13 (10.2)	6 (13.3)[3]	7 (8.4)[4]		
6 months screening[5]	n=113	n=38	n=75		
Passed, n (%)	78 (69.0)	31 (81.6)	48 (64.0)	0.5 (0.21, 1.32)	0.170
Referred, n (%)	36 (31.9)	7 (18.4)[6]	27 (36.0)[7]		
12 months screening[5]	n=108	n=34	n=74		
Passed, n (%)	89 (82.4)	31 (91.2)	58 (78.4)	0.5 (0.13, 1.84)	0.292
Referred, n (%)	19 (17.6)	3 (8.8)[8]	16 (21.6)[9]		

Abbreviations: cCMV (congenital CMV), CI (confidence interval)
[1] Odds ratios computed from conditional logistic regression
[2]Newborn screening by Automated Auditory Brainstem Response (AABR)
[3]Two cases dropped out before further testing, 4 cases had evidence of middle ear dysfunction on repeat testing at two months of age
[4]One control dropped out before further testing, 6 controls had evidence of middle ear dysfunction on repeat testing at two months of age.
[5]Six and 12 months screening by ART (acoustic reflex threshold), DPOAE (distortion product optoacoustic emissions), otoscopy and immittance
[6]Five cases had evidence of middle ear effusion or dysfunction, 2 did not complete testing despite repeated attempts
[7]26 had evidence of middle ear effusions or dysfunction, 1 could not complete testing despite repeated attempts.
[8]Three had evidence of middle ear dysfunction
[9]Twelve had evidence of middle ear dysfunction, 3 did not cooperate with testing on two separate attempts and 1 had suspected hearing loss of uncertain aetiology who did not return for additional testing.

4.3.4 Neurodevelopmental Outcome

At age six months, one (2.7%) symptomatic cCMV case (born with microcephaly) and none of the cCMV-uninfected controls, had neurodevelopmental delay (Table 4.3). At 12 months of age two (5.7%) cases (one symptomatic with microcephaly and one asymptomatic), and three (4.1%) controls had neurodevelopmental delay (OR 2.0, 95% CI 0.13-31.99, P=0.958, Table 4.3). The symptomatic cCMV case had delay across all three domains (classified as cerebral palsy), while the asymptomatic case only had motor delay. Of the three controls, two had language delay and one had motor delay. Four (80.0%) of the five children with neurodevelopmental delay were HIV-exposed, although this was not significant (P=0.192).

The mean and range of composite scores, achieved by cases and controls for each domain at six and 12 months of age, are shown in Table 4.4. The odds ratios derived from conditional logistic regression of the composite scores for each domain achieved by cases and controls are also shown in Table 4.4 with no significant differences between the two groups. There was also no significant difference in neurodevelopmental outcome between HIV-exposed cCMV cases and cCMV-uninfected controls and HIV-unexposed cCMV cases and cCMV-uninfected controls at both six and 12 months of age.

A composite of neurodevelopmental delay and SNHL as neurological sequelae were analysed between cases and controls at the age of 12 months. There was no difference in overall neurological sequelae between cases (2/35, 5.7%) and controls (3/74, 4.1%, OR 4.0, 95% CI 0.36-44.11, P=0.258) at 12 months of age.

Table 4.3: Neurodevelopmental delay at visit 3 (6 months) and visit 4 (12 months) in cCMV-infected case and cCMV-uninfected control children

	6 months of age (visit 3)				12 months of age (visit 4)			
	cCMV cases N=37	**cCMV-uninfected controls N=76**	**Odds ratio[1,2] (95% CI)**	**P value**	**cCMV cases N=35**	**cCMV-uninfected controls N=74**	**Odds ratio (95% CI)**	**P value**
Neurodevelopmental delay, n (%)	1 (2.7)	0 (0)	-	1.0	2 (5.7)	3 (4.1)	2.0 (0.13, 31.99)	0.624
No neurodevelopmental delay, n (%)	36 (97.3)	76 (100)			33 (94.3)	71 (95.9)		

Abbreviations: cCMV (congenital CMV), CI (confidence interval)

[1]Adjusted for comorbidities

[2]Neurodevelopmental delay defined as 2.0 or more standard deviations below the mean score in one or more domains

Table 4.4: Comparison of Bayley III neurodevelopmental assessment scores between cCMV-infected case and cCMV-uninfected control children at 6 and 12 months of age

	6 months of age				12 months of age			
Bayley III Domain	**cCMV cases**	**cCMV-uninfected controls**	**Odds ratio[1] (95% CI)**	**P- value**	**cCMV cases**	**cCMV-uninfected controls**	**Odds ratio[1] (95% CI)**	**P value**
Cognition composite	N=37	N=76			N=35	N=74		
Mean (SD)	102.5 (11.3)	105.1 (8.4)	0.98 (0.93, 1.03)	0.459	107 (12.0)	106.1 (8.5)	1.04 (0.99, 1.11)	0.137
Range	55-120	80-115			55-125	80-120		
Language composite	N=37	N=76			N=35	N=74		
Mean (SD)	93.7 (8.7)	96.2 (6.6)	0.97 (0.91, 1.03)	0.327	94.3 (13.5)	94.8 (10.0)	1.0 (0.95, 1.04)	0.924
Range	56-112	77-112			47-118	65-112		
Receptive scaled	N=37	N=76			N=35	N=74		
mean (SD)	9.1 (1.3)	9.5 (1.5)	0.85 (0.61, 1.19)	0.345	9.6 (2.6)	9.6 (2.2)	1.0 (0.80, 1.23)	0.947
Range	5-11	7-12			1-13	4-14		
Expressive scaled	N=37	N=76			N=35	N=74		
mean (SD)	8.8 (2.0)	9.2 (1.3)	0.90 (0.68, 1.19)	0.461	8.4 (2.3)	8.6 (1.57)	0.99 (0.74, 1.32)	0.925
Range	0-13	5-13			1-13	2-11		
Motor composite	N=37	N=76			N=35	N=74		
Mean (SD)	104.2 (13.9)	106.1 (12.0)	0.99 (0.96, 1.03)	0.756	103.1 (14.7)	105.7 (12.1)	0.99 (0.95, 1.04)	0.783
Range	46-121	76-136			46-130	67-130		
Fine motor scaled	N=37	N=76			N=35	N=74		
Mean (SD)	11.3 (2.6)	11.6 (1.9)	0.98 (0.79, 1.20)	0.825	11.2 (2.5)	11.7 (2.3)	0.94 (0.73, 1.20)	0.608
Range	1-15	8-16			1-15	6-19		
Gross motor scaled	N=37	N=76			N=35	N=74		
Mean (SD)	10.0 (2.5)	10.4 (2.8)	0.95 (0.80, 1.14)	0.604	9.7 (2.8)	10.1 (2.9)	0.99 (0.83, 1.19)	0.926
Range	1-13	3-19			1-15	1-16		

Abbreviations: cCMV (congenital CMV), CI (confidence interval), SD (standard deviation)

[1]Odds ratios derived from conditional logistic regression adjusted for comorbidities

4.3.5 Anthropometry

The mean raw anthropometry and standardized Z-scores are shown in Table 4.5. The value of N for the first study visit within 21 days of birth in Table 4.5 varies between mean values and Z-scores, as Z-score conversion of anthropometry is only available for term infants whereas there were nine preterm infants in the study. Over the follow-up period, there was no significance in the mean difference in standardized scores for weight, length, MUAC, head circumference and BMI between cCMV cases and cCMV-uninfected controls after adjusting for breastfeeding and *in-utero* HIV infection. Anthropometry was then analysed for HIV-exposed cCMV cases and controls and HIV-unexposed cCMV-uninfected cases and controls. Table 4.6 shows the mean of the difference in Z-scores for anthropometry at each visit between HIV-exposed cases and controls and between HIV-unexposed cases and controls with confidence intervals. Figure 4.2 shows the trend in the mean difference for each anthropometric Z-score between cases and controls by HIV exposure status over the four study visits. In HIV-exposed children, the mean difference in standardized score for head-circumference was significantly lower at six (P=0.02) and 12 months (P=0.018) of age in HIV-exposed cCMV cases than HIV-exposed cCMV-uninfected controls (Table 4.6 and Figure 4.2A). In HIV-unexposed cases, the mean difference in standardized scores for weight and length at 12 months of age was higher than HIV-unexposed controls at 12 months of age (P=0.013 and 0.043 respectively, Table 4.6 and Figure 4.2B).

Table 4.5: Mean and standardized anthropometry of cCMV-infected case and cCMV-uninfected control children at each visit

	Weight (kg)		Length (cm)		Arm circumference (cm)		Head circumference (cm)		BMI	
	cCMV +	**cCMV-**	**cCMV+**	**cCMV-**	**cCMV+**	**cCMV-**	**cCMV+[1]**	**cCMV-**	**cCMV+**	**cCMV-**
Visit 1	N=46	N=84	N=46	N=84	N=46	N=83	N=46	N=84	N=46	N=84
Mean (SD)	3.0 (0.5)	3.3 (0.6)	49.6 (4.2)	50.9 (3.6)	9.9 (0.8)	10.2 (1.3)	34.7 (1.8)	35.5 (1.8)	14.4 (3.1)	14.7 (2.7)
Range	1140-3770	2.02-4.81	41-61	36.5-58	8.4-11.5	6.8-13.4	28.3-37.7	29.8-38.2	5.9-19.3	8.3-20.0
Visit 1[2]	N=37	N=78	N=37	N=78	NA	NA	N=37	N=78	N=37	N=78
Z-score Mean (SD)	-1.0 (1.0)	-0.9 (1.0)	-0.5 (2.2)	-0.4 (1.5)	NA	NA	-0.13 (1.2)	0.1 (1.2)	-1.0 (1.9)	-0.9 (1.3)
Visit 2	N=40	N=83	N=40	N=83	N=40	N=82	N=40	N=82	N=40	N=83
Mean (SD)	5.2 (0.9)	5.1 (1.0)	58.2 (3.6)	56.9 (3.7)	12.4 (1.1)	12.3 (1.0)	39.4 (2.2)	39.7 (1.8)	15.4 (2.5)	15.3 (3.8)
Range	3.51-7.60	1.08-7.13	51.4-65.3	40.8-68	10.3-15	9.1-15	32.2-46	34.8-45	10.8-21.5	4.9-37.7
Z-score Mean (SD)	-0.6 (1.1)	-0.7 (1.4)	-0.2 (1.5)	-0.8 (1.7)	NA	NA	0.3 (1.8)	0.6 (1.2)	-0.6 (1.2)	-0.3 (2.2)
Visit 3	N=39	N=77	N=39	N=77	N=39	N=77	N=39	N=77	N=39	N=77
Mean (SD)	7.5 (1.3)	7.3 (1.1)	65.9 (2.9)	66.2 (2.6)	13.7 (1.3)	13.6 (1.0)	43.0 (2.1)	43.9 (1.7)	16.3 (2.9)	15.6 (2.4)
Range	5.4-10.9	5.4-9.7	59.3-72.2	59-73	11.4-17.4	11.6-16.5	35.6-48	37.8-48.7	9.9-22.8	9.2-22.0
Z-score Mean (SD)	-0.3 (1.5)	-0.5 (1.1)	-0.4 (1.3)	-0.4 (1.1)	-0.3 (1.2)	-0.4 (0.9)	0.1 (1.6)	0.8 (1.3	-0.1 (1.6)	-0.4 (1.2)
Visit 4	N=35	N=74	N=35	N=74	N=35	N=74	N=35	N=74	N=35	N=74
Mean (SD)	9.2 (1.6)	9.1 (1.3)	72.9 (3.3)	72.9 (4.3)	14.5 (1.3)	14.4 (1.1)	45.6 (2.3)	46.3 (1.7)	16.2 (3.2)	16.2 (3.4)
Range	6.7-13.5	6.7-13.3	66.5-81.5	47.2-79.6	13-19	12-17	37.7-52	42.5-50	9.9-24.1	10.3-38.2
Z-score, Mean (SD)	-0.3 (1.4)	-0.2 (1.5)	-0.8 (1.3)	-0.7 (2.2)	0.1 (1.1)	-0.0 (0.9)	0.1 (1.7)	0.7 (1.4)	0.3 (1.4)	0.3 (1.8)

Abbreviations: cCMV (congenital CMV), SD (standard deviation)

[1]Includes one child with microcephaly [2]In visit 1, N for Z-scores is less than N for mean and range as Z-score conversions for preterm infants was not possible

Table 4.6: Difference in mean anthropometry Z-scores between cCMV-infected case and cCMV-uninfected control children over 12 months by HIV-exposure status

	Visit	WAZ Mean, (95% CI)	P value	LAZ Mean (95% CI)	P value	BMIZ Mean (95% CI)	P value	ACZ Mean (95% CI)	P value	HCZ[1] Mean (95% CI)	P value
Difference between HIV-exposed cases and HIV-exposed controls	**1**[2]	0.4 (-0.35, 1.08)	0.316	0.748 (-0.03, 1.53)	0.060	-0.267 (-1.08, 0.55)	0.519	0.424 (-0.84, 1.68)	0.509	-0.222 (-0.84, 0.39)	0.481
	2[3]	0.1 (-0.49, 0.72)	0.706	0.223 (-0.35, 0.80)	0.447	-0.164 (-0.82, 0.49)	0.622	0.203 (-0.65, 1.06)	0.642	-0.464 (-0.99, 0.06)	0.081
	3[4]	-0.132 (-0.76, 0.50)	0.683	-0.301 (-0.92, 0.31)	0.338	-0.061 (-0.71, 0.59)	0.853	-0.019 (-0.56, 0.52)	0.945	**-0.706 (-1.30, -0.11)**	**0.020**
	4[5]	-0.380 (-1.17, 0.41)	0.344	-0.825 (-1.69, 0.04)	0.061	0.042 (-0.76, 0.84)	0.918	-0.241 (-0.77, 0.29)	0.371	**-0.948 (-1.73, -0.16)**	**0.018**
Difference between HIV-unexposed cases and HIV-unexposed controls	**1**[6]	-0.104 (-0.74, 0.53)	0.749	-0.541 (-1.54, 0.46)	0.289	0.362 (-0.60, 1.32)	0.460	0.201 (-0.99, 1.39)	0.740	0.278 (-0.27, 0.83)	0.317
	2[7]	0.225 (-0.32, 0.77)	0.415	-0.045 (-0.73, 0.64)	0.898	0.361 (-0.27, 0.99)	0.262	0.332 (-0.50, 1.16)	0.434	0.250 (-0.26, 0.76)	0.337
	3[8]	0.554 (-0.01, 1.12)	0.055	0.452 (-0.19, 1.10)	0.170	0.361 (-0.27, 0.99)	0.264	0.463 (-0.10, 1.02)	0.106	0.222 (-0.33, 0.77)	0.427
	4[9]	**0.883 (0.19, 1.58)**	**0.013**	**0.949 (0.03, 1.87)**	**0.043**	0.361 (-0.27, 0.99)	0.462	**0.594 (0.06, 1.13)**	**0.029**	0.194 (-0.45, 0.84)	0.555

Abbreviations: WAZ (weight for age Z-score), LAZ (length for age Z score), BMIZ (Body mass index for age Z-score), ACZ (arm circumference for age Z-score), HCZ (Head circumference for age Z-score), cCMV (congenital CMV), CI (confidence interval)

Values in bold indicate mean difference in anthropometry that was significant between cases and controls

[1]Head circumference analysis adjusted for microcephaly at birth

[2]HIV-unexposed cases = 18, HIV-unexposed controls=35

[3]HIV-unexposed cases = 17, HIV-unexposed controls=34

[4]HIV-unexposed cases = 17, HIV-unexposed controls=34

[5]HIV-unexposed cases = 17, HIV-unexposed controls=33

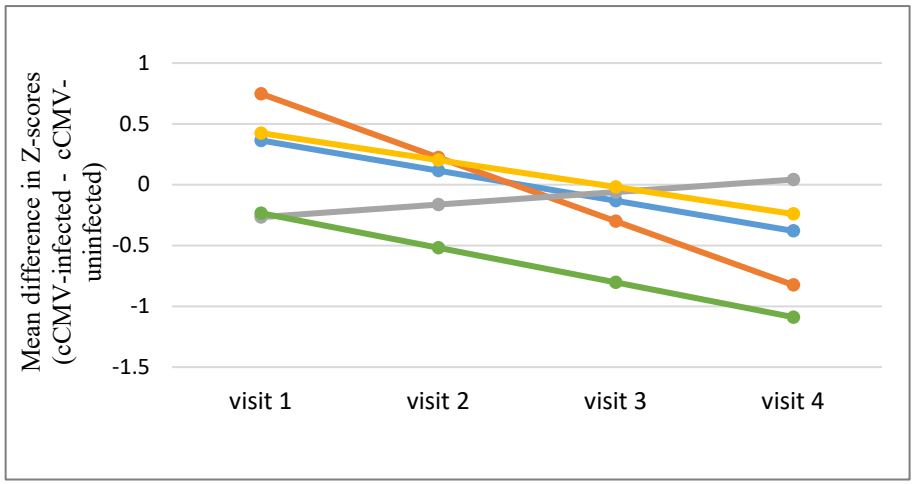
[6]HIV-unexposed cases = 18, HIV-unexposed controls=35,

[7]HIV-unexposed cases = 17, HIV-unexposed controls=34

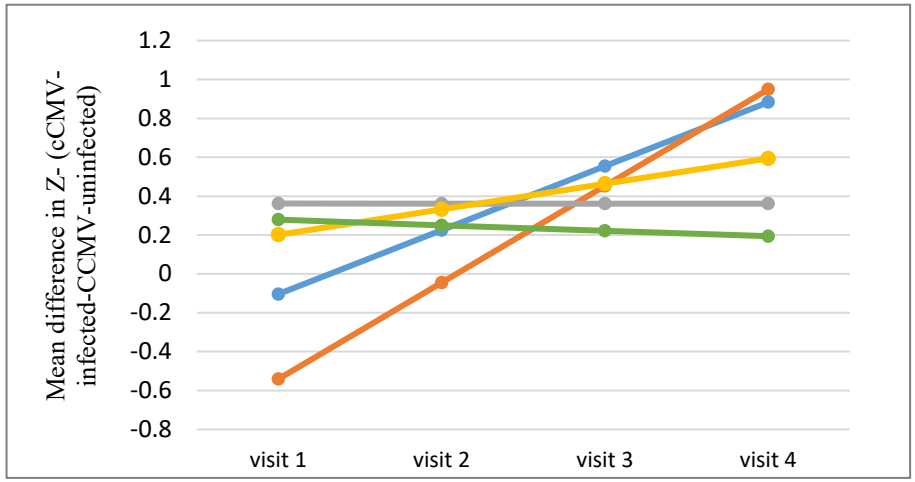
[8]HIV-unexposed cases = 17, HIV-unexposed controls=34

[9]HIV-unexposed cases = 17, HIV-unexposed controls=33

A)



B)



Key

WAZ: Weight for age

LAZ: Length for age

BMIZ: Body mass index for age

ACZ: Arm circumference for age

HCZ: Head circumference for age

cCMV: Congenital CMV

Figure 4.2: Trend in difference in mean anthropometry Z-scores between cCMV case and cCMV-uninfected control children by HIV exposure status A) HIV-exposed cCMV cases and controls B) HIV-unexposed cCMV cases and controls

4.4 Discussion

In this study, clinically symptomatic infection was present in 6.5% of neonates with cCMV, with two-thirds of these confirmed to be as a result of maternal non-primary CMV infection. The prevalence of symptomatic infection is lower than reported in systematic reviews, 10-15%[9,13,63], which were estimates derived from neonatal disease following both primary and non-primary maternal CMV infection, however, the mentioned estimates are contained in the wide confidence interval (1.4-17.9%) calculated in this study. On follow-up to 12 months of age, there was no difference in neurological sequelae and anthropometry between cCMV cases and cCMV-uninfected controls.

The definition of symptomatic infection varies among different studies. We did not consider SGA and presence of cerebral ultrasound abnormalities as symptomatic infection, due to a lack of data on maternal conditions that may have resulted in SGA infants and because we did not investigate other causes for cerebral abnormalities. One symptomatic (microcephaly) cCMV case was co-infected with HIV, and the associated immunosuppression may have contributed to severe CMV disease during the foetal period with brain involvement. The fewer number of symptomatic cases in our study could be due to most cCMV infections following non-primary maternal infection. Underlying differences in population characteristics and other yet to be identified factors may play a role in the frequency and severity of symptomatic cCMV at birth. Additionally, the small sample size may have also prevented a more accurate estimate of symptomatic infection.

None of the cCMV-infected infants who completed assessments had SNHL. Late onset SNHL may manifest after 12 months of age as described by others[71,128,239]. A Brazilian study, also from a high CMV seroimmune population, reported SNHL prevalence of 8.6% (5/58, 95% CI 2.9-19.0) in cCMV-infected children at a median age of 24 months (range 15-50 months)[71]. In

a Japanese study, one (2.3%) of 43 asymptomatic cCMV cases at birth developed SNHL by 12 months of age compared to 12 (70.6%) of 17 symptomatic children[239]. Three children in our study also received oral valganciclovir therapy, which has been shown to improve hearing outcomes[203]. The high prevalence of middle ear disease interfered with testing to reliably document SNHL in both cases and controls.

There was no significant difference in neurodevelopment between cases and controls at 12 months of age. Our findings are similar to prospective studies of asymptomatic cCMV-infected children with follow-up of one to six years reporting a cumulative incidence of neurodevelopmental impairment between 0% to 9.1%[108,240]. In contrast, studies of symptomatic newborns report a prevalence of 30.0-50.0% neurological impairment during childhood[9,63,110]. Some studies have found neurological sequelae to be present only in symptomatic children with obvious brain involvement[241]; however, these were mainly from the Americas and Europe where the epidemiology of cCMV likely differs from our study population. Due to the limited number of symptomatic cCMV in our study population, it was not possible to reliably compare difference in neurodevelopmental outcome between symptomatic and asymptomatic cCMV infection.

Standardized anthropometry was not significantly different between cCMV cases and cCMV-uninfected controls over the 12 months of follow-up. When stratified by HIV-exposure, cCMV-infected, HIV-exposed children had lower standardized head-circumference for-age than HIV-exposed children without cCMV. HIV-unexposed cCMV-infected children however, had higher standardized weight-for-age and length-for-age scores than their controls. Although our numbers for the stratified analysis are low, similar findings have been observed in Zambian HIV-exposed and HIV-unexposed, CMV-infected children[113].

Several mechanisms in which CMV affect growth and development have been proposed which may be both disruptive and beneficial for growth. CMV infects all cell types with the resulting inflammation possibly disrupting cell growth[242], and may affect absorption of nutrients by affecting gut microbiota[113,243], which may be more marked in HIV-exposed children. Conversely, latent herpesvirus infections such as CMV have beneficial immune modulation and outcome in mouse models with resistance to some bacterial pathogens[244]. Whether this occurs in human infants with resulting advantages for growth, requires further study.

Limitations in our study included the higher than anticipated drop-out rate in both cCMV cases and cCMV-uninfected controls which reduced the sample size and may have resulted in missing some cases with neurological sequelae and the lack of a significant difference in neurological sequelae between cases and controls. This study is however the first population based study in a sub-Saharan African setting to assess symptomatic cCMV infection and neurological sequelae within 12 months of age compared to a cCMV-uninfected control group. As there was no difference in baseline characteristics between drop outs and infants remaining in the study there is unlikely to be selection bias. The cerebral ultrasound scans were conducted by different neonatologists which may have led to inter-observer bias in identifying abnormalities.

In conclusion, there was no evidence of a difference in neurological sequelae between cCMV-infected cases and cCMV-uninfected controls by 12 months of age in children from Soweto that completed all investigations. Almost all cCMV infection was confirmed as being the result of maternal non-primary CMV infection. As the study was limited by a loss in sample size, definitive conclusions cannot be drawn on the frequency and severity of neurological sequelae in infants with cCMV from maternal non-primary CMV infection compared to

cCMV from maternal primary infection and needs to be further investigated. The gestational age of foetal CMV infection in women with primary CMV infection can determine whether foetal infection is symptomatic and/or results in permanent neurological sequelae[245]. Determining whether timing of foetal CMV transmission in maternal non-primary CMV infection similarly affects infant outcome may aid in identifying infants at risk of cCMV related morbidity in populations with high CMV seroprevalence. While our study did not show any evidence of poorer outcomes in HIV-exposed cCMV infants at one year of age, this may be a limitation in the sample size given the low frequency of neurological sequelae we observed. Universal screening for cCMV is being advocated in high resource settings as cost-effective enabling early intervention and preventing long-term disability. With the high prevalence of cCMV in HIV-exposed black South African children, long-term follow-up throughout childhood is necessary to determine the burden of late-onset neurological sequelae and the need for a universal or targeted birth cCMV screening program

5 Association of cytomegalovirus shedding in women and congenital and postnatal cytomegalovirus infection in South African infants

5.1 Introduction

Cyclical reactivation of latent CMV infection, and reinfection with new strains is common in populations with a high prevalence of CMV infection, leading to CMV shedding in body fluids[64,178]. The offspring of women shedding CMV in body fluids such as saliva, breast milk and vaginal secretions, are at increased risk for acquiring CMV infection during early infancy[52]. Postnatal acquisition of CMV in infancy is usually asymptomatic however, infants with immunodeficiencies, including those born preterm and if infected with HIV, are at risk of developing active CMV disease (such as pneumonitis, hepatitis or sepsis-like illness) when infected during early infancy[53,76]. There is paucity of data on CMV shedding in African women after delivery of their offspring, and the dynamics of pCMV acquisition in their children[246], including from settings with a high prevalence of women living with HIV[247].

The aim of this study was to determine the prevalence of maternal CMV shedding between three and 21 days of delivery in women living with and without HIV, and the association thereof with congenital (cCMV) and postnatal acquisition of CMV.

5.2 Methods

This study included the birth cohort of mother-newborn dyads with cCMV-infected neonates as cases (n=46) and cCMV-uninfected neonates as controls (n=84) as described in Chapter 2, section 2.3 and Chapter 4, section 4.2.0

5.2.1 Sample collection for maternal CMV shedding and infant postnatal CMV acquisition

Postpartum, self-expressed breast milk (in a sterile bottle) and vaginal fluid swabs (using a nylon Copan™ flocked swab) were collected from the mothers of the cCMV cases and cCMV-uninfected controls by study staff between three and 21 days of the delivery of their infants. Furthermore, saliva was also collected from the cCMV-uninfected controls at two, six and 12 months of age, to test for pCMV acquisition by PCR. The saliva, breast milk and vaginal fluid samples were archived at -70°C until PCR testing for CMV as described in Chapter 2, section 2.2.2.

5.2.2 Statistical analysis

Characteristics of the mother-newborn dyads, including maternal CMV shedding between three and-21 days' postpartum and infant characteristics, were compared between cCMV cases and controls by conditional logistic regression. CMV shedding in women living with HIV infection was compared to HIV uninfected women by logistic regression. In women living with HIV, the effect of ART duration on CMV shedding was compared by the Wilcoxon rank sum test and P values computed. As the effect of HIV and ART duration was a comparison between unmatched women living with and without HIV, a matched analysis was not utilised as when comparing matched infant cases and controls.

Furthermore, we described the rate of pCMV acquisition at two, six and 12 months of age in those infants without cCMV (controls). Thus cCMV-uninfected controls were further categorised into those that acquired CMV postnatally and those that remained CMV-uninfected at 12 months of age. A Cox proportional hazards model was used to determine the association between maternal postpartum CMV shedding, HIV exposure, exclusive breastfeeding since birth and vaginal birth (exposures) on pCMV acquisition in controls at, two, six and 12 months of age. One-way ANOVA test was used to determine if anthropometry between cCMV cases, the controls with pCMV and the controls that remained CMV-uninfected infants differed at 12 months of age.

5.3 Results

We enrolled 130 mother-infant dyads, 46 with cCMV and 84 cCMV-uninfected controls, as previously reported in Chapter 2, section 2.2.1. We collected breast milk and vaginal swabs from 123 and 130 women, respectively between three and 21 days of delivery to determine maternal CMV shedding. Saliva was collected at two, six and 12 months of age from 64 cCMV-uninfected infants to determine the proportion that acquired CMV postnatally and those that remained CMV-uninfected at 12 months of age. The median number of days between delivery and breast milk and vaginal fluid collection was significantly higher (16 vs 8 days) in controls than cases as controls were selected after confirmation of cCMV infection in cases (Table 5.1).

Overall, 74.6% (97/130) of women exclusively breastfed their infants between three and 21 days of age (Table 5.1), with women living with HIV being less likely to breast feed (59.7%, 46/77 vs. 96.2%, 51/53, P<0.001).

5.3.1 Maternal CMV shedding

Overall, 59.2% (77/130) of all women shed CMV either in breast milk (61.0%, 75/123) or vaginal fluid (8.5%, 11/130) between three and 21 days of delivery (Table 5.1). CMV shedding in breast milk was two-fold (OR 2.0, 95% CI 0.83-4.89) higher in women of cCMV cases (68.9%, 31/45) than cCMV-uninfected controls (56.4%, 44/78), albeit not significant (Table 5.1). Also, CMV shedding in vaginal fluid was 5.3-fold (OR 5.3, 95% CI 1.41-20.10) higher in women of cCMV (17.4%, 8/46) cases than cCMV-uninfected controls (3.6%, 3/84). CMV shedding in either breast milk or vaginal fluid was 2.5-fold (95% CI 1.03-5.92) higher in women of cCMV cases (69.6%, 32/46) than cCMV-uninfected controls (53.6%, 45/84). There were nine women who shed CMV in both breast milk and vaginal fluid of whom seven (77.7%) had cCMV-infected neonates (data not shown in tables).

CMV shedding in breast milk was 3.4-fold (95% CI 1.58-7.21) more common in women living with HIV (70.7%, 53/75) than those without HIV (41.7%, 20/48). CMV shedding in vaginal fluid did not differ between women living with (7.8%, 6/77) and without HIV (9.4% 5/53, OR 0.8, 95% CI 0.23-2.81). ART duration data was available for 61 of the 75 women living with HIV. There was no association between maternal ART duration and CMV shedding in breast milk (P=0.450) or vaginal fluid alone (P=0.411) in women living with HIV.

5.3.2 Infant postnatal CMV (pCMV) acquisition

Saliva samples were available for all three post-neonatal time points in 64 cCMV-uninfected controls through to 12 months of age. The pCMV acquisition rate in the cCMV-uninfected controls was 32.8% (21/64), 68.8% (44/64) and 79.7% (51/64) at two, six and 12 months of age, respectively. Risk for infant pCMV acquisition over the 12 month period was not associated with maternal CMV shedding in breast milk (hazard ratio [HR] 1.4, 95% CI 0.76-

2.51, P=0.292) or vaginal fluid (HR 1.4, 95% CI 0.32-6.17, P=0.644) between three and 21 days post-partum. Similarly, pCMV acquisition was not associated with infant HIV-exposure (HR 1.7, 95% CI 0.90-3.33, P=0.103), breastfeeding (HR 2.0, 95% CI 0.96-4.25, P=0.063) or vaginal birth (HR 1.5, 95% CI 0.79-2.70, P=0.227).

There were no differences in anthropometry between children with cCMV, those who acquired CMV postnatally and those who remained CMV-uninfected through to 12 months of age (Table 5.2).

Table 5.1: Maternal characteristics, maternal CMV shedding status and characteristics of cCMV-infected case and cCMV-uninfected control children

	Total N=130	cCMV cases N=46	cCMV-uninfected controls N=84	OR (95% CI)	P value
Median maternal age at delivery (IQR)	25 (22-31)	25 (22-29)	28 (24-33)	0.9 (0.90, 0.99)	0.200
Vaginal birth, n (%)	51/129 (39.5)	25/46 (54.4)	26/83 (31.3)	3.0 (1.33, 6.71)	0.008
Exclusive breastfeeding between 3 and 21 days postpartum, n (%)	97/130 (74.6)	35/46 (76.1)	62/84 (73.8)	1.3 (0.53, 2.94)	0.617
Median days between delivery and breast milk collection (IQR)	15 (9-19)	8 (7-13)	16 (12-19)	0.7 (0.68, 0.81)	<0.001
Median days between delivery and vaginal fluid collection	15 (9-19)	8 (7-13)	16 (13-19)	0.6 (0.52, 0.69)	<0.001
Maternal HIV infection[1]	77/130 (59.2)	28/46 (60.9)	49/84 (58.3)	ND[1]	ND[1]
Maternal CMV shedding between 3- 21 days postpartum[2]	77/130 (59.2)	32/46 (69.6)	45/84 (53.6)	2.5 (1.03, 5.92)	0.042
Breast milk shedding	75/123[3] (61.0)	31/45 (68.9)	44/78 (56.4)	2.0 (0.83, 4.89)	0.122
Vaginal shedding	11/130 (8.5)	8/46 (17.4)	3/84 (3.6)	5.3 (1.41, 20.10)	0.013
Female infants[1], n (%)	62/130 (47.7)	21/46 (45.7)	41/84 (48.8)	ND[1]	ND[1]
Birth weight, median (IQR)	2845 (2520-3190)	2845 (2455-3190)	2903 (2595- 3195)	1.0 (0.9, 1.0)	0.616
Gestational age[1], median (IQR)	38 (36-40)	38 (36-40)	38 (36-39)	ND[1]	ND[1]
HIV-exposed infants[1], n (%)	77/130 (59.2)	28/46 (60.9)	49/84 (58.3)	ND[1]	ND[1]
Infants with HIV infection, n (%)	5/77 (6.5)	5/28 (17.9)	0/49 (0)	64.9 (7.8, 538.31)	<0.001

Abbreviations: cCMV (congenital CMV), IQR (interquartile range), ND (not done)
[1]As cases and controls were matched for maternal HIV infection, gender and gestational age, there was no variation between cCMV cases and controls and comparisons not done
[2]CMV shedding in breast milk and/or vaginal fluid
[3]Seven breast milk samples were unavailable for testing due to spillage

Table 5.2: Comparison of anthropometry at 12 months of age in cCMV-infected, pCMV-infected and CMV-uninfected children

	cCMV N=35	pCMV N=51	CMV-uninfected N=13	P value
Mean WAZ (SD)	-0.3 (1.4)	-0.1 (1.6)	-0.5 (1.1)	0.693
Mean HAZ (SD)	-0.8 (1.3)	-0.4 (2.0)	-1.1 (3.0)	0.451
Mean WHZ (SD)	0.2 (1.4)	0.0 (1.3)	0.8 (4.8)	0.485
Mean HCZ (SD)	0.1 (1.7)	0.8 (1.5)	0.7 (1.3)	0.124
Mean MUACZ (SD)	0.1 (1.1	-0.1 (0.9)	-0.1 (0.9)	0.846

Abbreviations: cCMV (congenital CMV), pCMV (postnatal CMV), WAZ Weight for age Z-score, HAZ length for age Z– score, WHZ weight for height Z-score, HCZ head circumference for age Z-score, MUACZ mid-upper arm circumference for age Z-score, SD (standard deviation)

5.4 Discussion

Close to 60% of the women shed CMV either in breast milk and/or vaginal fluid within 21 days of delivery of their infant, with maternal CMV shedding in breast milk being more common in women living with HIV, even among those on ART. Maternal CMV shedding in vaginal fluid was associated with cCMV in neonates and this association has been previously shown in a study from The Gambia, which additionally demonstrated cCMV to be associated with CMV shedding in colostrum[233]. Our data demonstrated a 5-fold increased risk of cCMV in infants of mothers shedding CMV in vaginal fluid, and there was a trend towards a similar association with CMV shedding in breast milk. Limited power to detect a statistically significant difference in breast milk shedding and cCMV infection may have been a factor due to the high number of mothers of controls also shedding CMV in breast milk, thus a larger sample size would be required to confirm this.

Most studies of healthy pregnant women have not demonstrated a risk of cCMV in infants of mothers shedding CMV in the genital tract [50,248–250]. Vaginal CMV shedding however, may be an indicator of a high rate of viral replication and CMV viraemia with increased risk of foetal CMV transmission. A study of CMV shedding in women living with HIV reported those with detectable CMV viraemia were also more likely to have CMV detected in cervical fluid and breast milk, although the study did not investigate for cCMV[251]. Currently, identifying women at increased risk of CMV transmission to the foetus during pregnancy is limited to those who seroconvert during pregnancy following primary CMV infection and through ultrasound scans of the foetus for secondary prevention. Screening women for vaginal CMV shedding may thus be a plausible investigation in women with non-primary CMV infection to identify those at increased risk of foetal CMV transmission and selecting out which children should be investigated for possible cCMV and followed-up for neurological sequelae. This method may however, not be ideal as a large scale screening method, as vaginal shedding

only occurred in eight of the 46 women with a cCMV-infected neonates thus missing most of the pregnancies at risk of cCMV.

Studies have found a high prevalence of CMV shedding in breast milk in both women living with and without HIV in the era of no or short term use of ART[251]. Barbosa et al. studied CMV shedding in CMV seropositive, HIV uninfected, Brazilian pregnant women during the first, second and third trimester and postpartum period[52]. During the postpartum period, 7.0% of women shed CMV in vaginal secretions and 84.0% in breast milk collected within 30 days of delivery[52], which is comparable to our and others observations[251–253]. This indicates that breast milk and genital tract secretions have the highest rate of CMV shedding. A study in Malawi showed that immune reconstitution secondary to long-term ART during pregnancy (>4 months) and after delivery (>1 year) did not result in a significant decline in CMV shedding in breast milk in women living with HIV, and remained high and stable between one and 12 months after delivery[254]. Similarly, in Kenyan women living with HIV, ART was effective in reducing CMV infection (congenital or postnatal) in their infants but did not reduce CMV shedding in breast milk[255]. In women living with HIV, long-term ART and improved immune function may not have an impact on breast milk shedding and alternative strategies such as a maternal CMV vaccine may need to be explored to prevent early CMV acquisition during infancy. Prevention of early infant CMV acquisition is of importance in preterm, low birth weight and immunocompromised neonates as they may acquire CMV before their immune system has sufficiently matured and are at risk of developing CMV disease[32,39]. Whether a CMV vaccine will raise an adequate immune response in CMV seropositive women and protect against cCMV is, however, uncertain.

As we described, both HIV-exposed and HIV-unexposed children remain at similar risk of pCMV acquisition through infected breast milk during infancy. CMV infection may

negatively affect childhood growth and result in poor health outcomes in preterm and immunosuppressed children. Although there was no difference in growth between cCMV, pCMV and CMV-uninfected infants in our study, a larger sample size is required to confirm this finding.

We did not find an association between infant pCMV acquisition and breastfeeding or maternal postpartum CMV shedding in breast milk or vaginal fluid. This may be due to the large proportion of women that did not breastfeed their infants due to HIV infection in addition to other sources of CMV infection in the household. Most women from our study population live with other small children and return to work within three-four months of delivery placing the infant in day-care or having extended family assisting in caring for their infants including grandparents and older siblings. Crowded living conditions and exposure to other children[54,55] are known risk factors for childhood CMV acquisition [49,52,53].

Limitations of our study include the limited sample size which may have affected the power to detect significant differences. We collected breast milk and vaginal fluid only at a single time point post-partum, whereas serial testing and additional maternal fluids such as saliva and urine may have provided further insight into CMV shedding patterns and differences.

Our data is helpful in understanding CMV shedding patterns in select body fluids in postpartum women and the relationship between maternal CMV shedding and congenital and postnatal acquisition of CMV infection in a maternal population with non-primary CMV infection. In addition, our study confirms that longer duration of ART in women living with HIV may not be effective in reducing CMV shedding, and alternative strategies are required to prevent early pCMV acquisition in their infants if breastfeeding. Studies of immunological

correlates of CMV shedding in pregnant and postpartum women are recommended to better understand congenital and pCMV infection in infants.

6 Effect of congenital cytomegalovirus infection on humoral immune responses to select vaccines administered during infancy

6.1 Introduction

CMV infection in immunocompetent hosts induces virus-specific T lymphocytes that engage as much as 10% of CD8+ CD4+ T-lymphocyte cell memory reservoir [256]. This enables maintenance of CMV to persist in a latent state within the host. CMV infection has been associated with immune senescence in adults[257], and reduced humoral immune response to influenza vaccination in the elderly[151]. There is a paucity of data on the effect of CMV infection, either congenital or postnatal acquired, on immune responses to vaccines given during infancy in children. In The Gambia, infants with congenital or postnatal acquired CMV compared to CMV-uninfected children manifested greater expansion of differentiated CD8+ than CD4+ T-lymphocytes following measles vaccination albeit there being no differences in cytokine responses[153]. The burden of cCMV is highest in low-middle income settings[9]. We reported the rate of cCMV to be 5.2% and 1.4% in infants born to women living with and without HIV respectively, Chapter 3, section 3.4.1[234]. The aim of this exploratory study was to evaluate the effect of cCMV and pCMV infection on humoral immune responses to the primary series of Diphtheria-Tetanus-acellular Pertussis, *Haemophilus influenzae* type b conjugate and Hepatitis-B vaccines (included in a hexavalent formulation containing inactivated polio virus) included in the public immunisation program in South Africa.

6.2 Patients and Methods

The study participants comprised infants born at Chris Hani Baragwanath Academic Hospital (CHBAH) in Soweto with cCMV (cases) and a control group without cCMV (controls). Details of the enrolment of cCMV cases and cCMV-uninfected controls have been provided in Chapter 2, section 2.2.1[234]. The selected controls had repeat saliva samples sent for identification of CMV collected at two, six and 12 months of age to ascertain the rate and timing of pCMV acquisition as described in Chapter 5, section 5.2. Thus infants without cCMV could be further stratified into those that acquired CMV postnatally (pCMV) and those that remained CMV-uninfected through to 12 months of age.

All children received the routine vaccines included in the South African public immunisation program at the RMPRU clinic or local primary health care clinics. Infants received a three dose series of combined Diphtheria-Tetanus-acellular Pertussis-inactivated Polio-*Haemophilus Influenzae* type b- Hepatitis B (DTaP-IPV-Hib-HBV, Hexaxim™, Sanofi Pasteur) vaccine administered at six, 10 and 14 weeks of age. Other vaccines given during early infancy were rotavirus vaccine (Rotarix®, GlaxoSmithKline), pneumococcal conjugate vaccine (Prevenar-13™, Pfizer) and measles vaccine[258], for which we did not analyse for immunogenicity in this study.

6.2.1 Laboratory methods

Venous blood was collected at seven months of age in children with documentation of having received all their scheduled vaccines at the time of sample collection. Blood samples were centrifuged and serum aliquots stored at -70^{0}C before testing.

For the purpose of this study, we measured immune responses to DTaP-Hib-HBV epitopes. Antibody to diphtheria-toxoid (DT), tetanus-toxoid (TT), pertussis-toxoid (PT), filamentous

hemagglutinin (FHA), hepatitis B surface antigen (HBsAg) and polyribosylribitol phosphate (PRP) capsular polysaccharide of Hib were measured by an in-house Luminex multiplex immunoassay as described previously[259]. Coupling of DT (List Biologicals, cat no #151), PT (List Biologicals, cat no #181) FHA (The native Antigen company, cat no: BP-FHA-50), HBsAg (provided by Biovac South Africa), TT (Provided by Biovac South Africa) to microspheres beads (Bio-Rad, USA) was achieved by using a two-step carbodiimide reaction[259] whereas capsular polysaccharide (CPS) antigen for Hib PRP (provided by Biovac South Africa) was coupled to the microsphere beads with the cross-linking agent 4-(4,6-dimethoxy [1,3,5]triazin-2-yl)-4-methyl-morpholinium (DMTMM) as previously described[260].

International Standard for anti-HBsAg immunoglobulin (NIBSC code: 07/164) was used as reference serum for the assay. The reference was further calibrated against the individual international standard reference sera for anti-PT (NIBSC code: 06/140), anti-DT (NIBSC code: 00/496), anti-TT (NIBSC code: 76/589) and anti-PRP (NIBSC code: 96/536). For FHA, the reference was assigned an arbitrary concentration of 100 arbitrary units (AU)/ml. The results are given in international units (IU)/ml for PT, TT, and DT, milli international unit (mIU)/ml for HBsAg, (AU)/ml for FHA and µg/ml for PRP. The lower limit of detection for the assay was 0.0043 AU/ml, 0.0006 IU/ml, 0.2 mIU/ml, 0.0002 IU/ml, 0.0039 AU/ml and 0.008 µg/ml for PT, TT, HBsAg, DT, FHA and PRP, respectively. For statistical analysis, samples with values below these limits were assigned values of half of the lower limit of detection. The optimal serum and secondary antibody dilution was 1:100. Assays were performed in true duplicate and each plate included in-house high and low control sera. Bead fluorescence was read with the Bio-Plex 200 instrument (Bio-Rad) using Bio-Plex manager 6.0 software (Bio-Rad).

The epitope-specific IgG threshold defined as the putative serocorrelate or surrogate for protection were ≥0.1 IU/ml for DT, ≥0.1 IU/ml for TT, ≥10 mIU/ml for HBsAg and ≥0.15mg/ml for PRP (i.e. Hib epitope)[261]. The surrogate for measuring immune responsiveness to PT was defined as ≥5 IU/ml for PT[261]. We did not analyse for any FHA threshold, as pre-vaccination samples were not collected and our assay was not calibrated against the recommended international FHA reference serum.

6.2.2 Statistical Analysis

Infants with *in-utero* HIV-infection (i.e. tested HIV PCR positive within 48 hours of birth) were excluded from the analyses. Analyses included comparing demographic characteristics of cCMV cases to cCMV-uninfected controls, as well as further stratification of the latter group to those with pCMV and those who remained free of CMV infection through to six months of age. To assess immune responses to vaccination and the association with CMV infection, epitope specific IgG, geometric mean concentrations (GMCs) between cCMV cases and controls that did not acquire CMV postnatally remaining CMV-uninfected at six months of age were compared by linear regression for each vaccine antigen adjusting for HIV-exposure. GMCs were also compared between cCMV cases and all cCMV-uninfected controls using mixed effects modelling with the case-control grouping variable assigned as a random effect, and cCMV status and HIV-exposure as fixed effects. The proportion of cases and all cCMV-uninfected controls with IgG titres greater or equal to the putative correlate of protection were compared by conditional logistic regression. The proportion of cases and both pCMV and CMV-uninfected controls with IgG titres greater or equal to the putative correlate of protection were compared by logistic regression analysis, adjusted for HIV-exposure.

6.3 Results

From the cohort of 46 cCMV-infected cases and 84 cCMV-uninfected matched controls enrolled during the neonatal period, three cases were excluded due to *in-utero* HIV infection, ten cases and nine controls were lost to follow-up and one case died before seven months of age; at which point serum samples were collected from 32 cCMV cases, and 75 cCMV-uninfected controls (Table 6.1). Of the cCMV-uninfected controls included, 44 acquired CMV postnatally, 20 remained CMV-uninfected and ten missed having saliva samples collected for CMV acquisition determination at six months of age (Table 6.1). All the infants were black Africans except for one control of mixed African and Asian race. The mean age at administration of the 1st, 2nd and 3rd dose of DTaP-IPV-Hib-HBV were 43, 75 and 105 days respectively; and did not differ between cases and controls (Table 6.1). The mean duration between the 3rd dose of DTaP-IPV-Hib-HBV and blood draw for immunogenicity analysis was 110 days (Table 6.1).

There were no differences in IgG GMCs between cCMV cases and cCMV-uninfected controls for any of the six vaccine antigens (Table 6.2). In the stratified analysis, on comparing cCMV cases to those controls that acquired CMV postnatally and to the controls that remained CMV-uninfected, there was again no difference in IgG GMCs (Table 6.2).

The majority (≥98.7%) of cCMV cases and all cCMV-uninfected controls had seroprotective titres to Hepatitis B, tetanus and diphtheria (Table 6.2). Thus, 100% pCMV and CMV-uninfected controls also had seroprotective concentrations of IgG to Hepatitis B, tetanus and diphtheria. Also, 100% of cCMV cases and cCMV-uninfected controls had IgG concentration ≥5IU/ml to PT (Table 6.2). A lower percentage had seroprotective concentration to Hib (≥0.15µug/ml), albeit being similar between cCMV cases (68.8%) and cCMV-uninfected

controls (74.7%, P=0.168), between cCMV cases and pCMV (73.8%, P=0.611) and between cCMV cases and controls that remained CMV-uninfected (73.7%, P=0.692, Table 6.2).

Furthermore, there was no difference in GMCs or the percentage of children with antibody concentrations above the serocorrelate or surrogate of protection for any of the six epitopes between cCMV cases, all cCMV-uninfected, pCMV-infected and CMV-uninfected controls born to women living with and without HIV (data not shown).

Table 6.1: Demographic characteristics of infants by CMV infection status

	Total N=107	**cCMV Cases N=32**	**cCMV-uninfected controls[1] N=75**	pCMV-infected controls N=44[2]	CMV-uninfected controls N=20[2]
HIV-exposed	58 (54.2)	17 (53.1)	41 (54.7)	25 (56.8)	12 (57.1)
Female infants, n (%)	50 (46.7)	13 (40.6)	37 (49.3)	19 (45.2)	12 (60.0)
Birth weight, g, median (IQR)	2845 (2510-3193)	2840 (2385-3210)	2900 (2610-3185)	2910 (2580-3150)	2915 (2635-3275)
Mean WAZ at 6 of months age (SD)	-0.5 (1.2)	-0.3 (1.5)	-0.5 (1.1)	-0.5 (1.2)	-0.6 (1.1)
Age (days) at vaccination, median (IQR)	N=100[1]	N=30	N=70	N=40	N=17
BCG (birth)	0 (0-2)	0 (0-3)	0 (0-2)	0 (0-1)	0 (0-3)
OPV (birth)	0 (0-3)	0 (0-3)	0 (0-3)	0 (0-2)	1 (0-2)
Hexavalent (1st)	43 (42-47)	43 (42-46)	43 (42-47)	43 (42-45)	43 (42-45)
Rotavirus (6w)	43 (42-46)	43 (42-46)	43 (42-46)	43 (42-45)	43 (42-45)
PCV (6w)	43 (42-47)	43 (42-47)	43 (42-47)	43 (42-45)	43 (42-45)
Hexavalent (2nd)	75 (71-79)	73 (70-78)	75 (71-79)	74 (70-78)	77 (73-84)
Hexavalent (3rd)	105 (100-115)	104 (100-117)	106 (100-114)	105 (99-111)	108 (105-113)
PCV (14w)	106 (100-113)	104 (100-115)	106 (101-111)	105 (98-109)	108 (105-113)
Rotavirus (14w)	106 (100-115)	106 (101-111)	106 (100-115)	107 (100-124)	107 (105-111)
Mean days between 3rd hexavalent dose and blood draw (SD)	110 (46)	106 (32)	111 (51)	114 (90-134)	109 (103-119)

Abbreviations: cCMV (congenital CMV), pCMV (postnatal CMV), IQR (interquartile range), WAZ (weight for age Z-score), BCG (Bacille Calmette-Guerin vaccine), PCV (pneumococcal conjugate vaccine), SD (standard deviation)

[1]Infants enrolled as cCMV-uninfected controls had saliva tested until 12 months of age to identify those that acquired CMV postnatally and those that remained CMV-uninfected

[2]Controls with postnatal CMV (n=44) and those that remained CMV-uninfected (N=20) do not add up to the total number of cCMV-uninfected controls (n=75) as 10 controls did not have postnatal CMV status results available.

Table 6.2: Geometric mean concentrations and proportion with seroprotective antibody levels by CMV infection status of infants following primary series of select vaccinations

Vaccine Antigen	GMC Protective threshold	cCMV cases N=32	cCMV-uninfected controls[1] N=75	Coefficient (95% CI)	P value[2]	pCMV-infected controls N=42	Coefficient (95% CI)	P value[3]	CMV-uninfected controls[4] N=20	Coefficient (95% CI)	P value[5]
HBsAg (mIU/ml)	GMC (95% CI)	551.14 (425.99,713.06)	445.28 (366.71, 540.67)	0.20 (-0.13, 0.53)	0.230	434.34 (336.31,561.09)	-0.23 (-0.59, 0.13)	0.211	407.18 (254.16, 652.35)	0.28 (-0.18, 0.74)	0.229
	≥10 mIU/ml	32/23 (100)	75/75 (100)	-	-	42/42 (100)	-	-	19/19 (100)	-	-
TT (IU/ml)	GMC (95% CI)	0.20 (0.15, 0.28)	0.18 (0.15, 0.22)	0.08 (-0.27, 0.43)	0.656	0.18 (0.14, 0.24)	-0.11 (-0.52, 0.31)	0.608	0.14 (0.11, 0.19)	0.32 (-0.15, 0.79)	0.172
	≥0.01 IU/m	32/32 (100)	75/75 (100)		-	42/42 (100)	-		19/19 (100)	-	-
DT (IU/ml)	GMC (95% CI)	0.17 (0.13-0.22)	0.15 (0.13, 0.17)	0.14 (-0.13, 0.42)	0.303	0.14 (0.12, 0.17)	-0.16 (-0.45,0.14)	0.299	0.15 (0.11, 0.19	0.14 (-0.24, 0.52)	0.462
	≥0.01 IU/ml	32/32 (100)	74/75 (98.7)	0.80 (-0.34, 1.94)	0.168	42/42 (100)	-		19/19 (100)	0.71 (-0.61, 2.03)	0.291
PRP (µg/ml)	GMC (95% CI)	0.25 (0.18,0.34)	0.27 (0.22, 0.34)	-0.11 (-0.48, 0.27)	0.572	0.26 (0.20,0.34)	0.061 (-0.35, 0.47)	0.767	0.24 (0.15, 0.39)	0.0 (-0.54, 0.54)	0.997
	≥0.15 µg/ml	22/32 (68.8)	56/75 (74.7)	-0.74 (-1.79, 0.31)	0.168	31/42 (73.81)	0.265 (-0.76,1.29)	0.611	14/19 (73.7)	-0.26 (-1.53, 1.01)	0.692
PT (IU/ml)	GMC (95% CI)	11.93 (7.62, 18.68)	13.62 (11.34, 16.36)	-0.11 (-0.50, 0.28)	0.568	14.65 (11.50, 18.66)	0.20 (-0.27, 0.68)	0.393	14.06 (11.26, 17.54)	-0.05 (-0.68, 0.59)	0.886
	≥5 IU/ml	32/32 (100)	19/19 (100)			42/42 (100)	-		75/75 (100)		
FHA (AU/ml)[6]	GMC (95% CI)	15.98 (11.33, 22.54)	21.32 (18.13, 25.07)	-0.27 (-0.59, 0.05)	0.099	21.77 (17.92, 26.45)	0.32 (-0.05, 0.68)	0.092	21.89 (18.0, 26.62)	-0.22 (-0.72, 0.27)	0.374

Abbreviations: GMC (Geometric mean concentration), cCMV (congenital CMV), pCMV (postnatal CMV), HBsAg (Hepatitis B surface antigen), PT (Pertussis Toxin), FHA (filamentous haemagglutin), TT (tetanus toxoid), DT (Diphtheria Toxoid), PRP (polyribosylribitol phosphate)

[1]Includes both controls whom acquired postnatal CMV (n=44) and remained CMV-uninfected by 6 months of age (n=20)

[2]Comparison between cCMV cases and cCMV-uninfected controls

[3]Comparison between cCMV cases and controls that acquired CMV postnatally

[4]Only includes controls who remained CMV-uninfected at 6 months of age

[5]Comparison between cCMV cases and controls that remained CMV-uninfected at 6 months of age

[6]Protective thresholds for FHA not compared as pre-vaccination samples were not available for comparison

6.4 Discussion

Neither congenital nor postnatal acquisition of CMV was shown to affect immune responses to vaccine antigens (FHA, PT, HBsAg, TT, DT and PRP) following the primary series of vaccines in infancy in our study. Furthermore, close to 100% of cCMV, cCMV-uninfected, pCMV and CMV-uninfected infants mounted immune responses where the IgG concentration was above the correlate or surrogate of protection to four of the five antigens with an established threshold (HBsAg, TT, DT, and PT). To date, there has been limited studies on the effect of cCMV on immune responses to childhood vaccination compared to a CMV-uninfected control group[150,153,262]. Although our sample size was small, a significant difference in GMCs or in achievement of protective thresholds of antibody responses to vaccination between cCMV-infected and uninfected infants was not observed.

The B-cell responses of infants to cCMV infection, including the production of antibodies against CMV, are similar to the response in adults, but with lower affinity maturation and avidity[23]. While the functional T-cell exhaustion associated with infant CMV infection (congenital or postnatal)[263] could theoretically be associated with attenuated immune responses to vaccines; this was not demonstrated in our study or by other studies in Africa (meningococcal and measles vaccination in Gambian children) and the USA (measles-mumps-rubella vaccine)[150,153,262].

Limitations of this study include that 70% of cCMV-uninfected controls had acquired CMV by six months of age, decreasing the sample size of CMV-uninfected infants for comparison with cases. Also, we did not collect pre-vaccination blood samples to measure passively acquired antibodies and immune responses were only measured approximately three months after the primary series of vaccines, rather than one-month later as is standard practice, hence, we could have missed the peak in IgG concentration. This may also have led to

underestimating the proportion of children with seroprotective IgG titres against Hib for which 68.8-73.7% infants across all groups had IgG concentration above the correlate of protection for invasive Hib disease. This is lower than previously described in a clinical trial of the same vaccine in the same population[264]. The reason for the discrepancy between the studies are unclear, and could be due to differences in the assay used for analysing the serum samples, although we did use the reference serum calibrated against standard reference serums in our study. Furthermore, we did not assess cell-mediated immune responses to measles vaccine, or responses to other vaccines such as pneumococcal conjugate and rotavirus vaccine, which may have given further insight in the overall effect of cCMV on immune responses to childhood vaccines. Other limitations include lack of maternal immunization (HBV, DTaP) data, and overrepresentation of HIV-exposed infants in the analysed sample.

In conclusion, this was an exploratory study suggesting cCMV infection may not affect humoral immune responses to the primary series of DTaP-Hib-HBV vaccination in black-African children who are at high risk for congenital and postnatal acquisition of CMV.

7 Association of cytomegalovirus infection and attribution to causes of stillbirth and neonatal deaths in South Africa

7.1 Introduction

In 2015, 98% of 2.6 million stillbirths were reported in LMIC[133], and 77.0% of 2.4 million neonatal deaths occurred in sub-Saharan Africa and Southern Asia in 2017[265]. Viral infections are increasingly recognised to be associated with foetal and neonatal deaths[139]. CMV is the most common congenital viral infection globally[9]; however, there is limited data on the role of CMV in contributing to stillbirths and neonatal deaths, especially in low-resource settings.

In the USA and UK, infant mortality rates from cCMV were estimated at 0.7-0.8 per 100000 live births[129–131], whilst even higher rates were observed in Australia (1.6/100000 live births), the majority in the neonatal period[132].

Furthermore, in the UK, 0.2% (8/3954) of stillbirths from 1988 to 2008 were attributed to cCMV infection (1.1/100000 births) based on clinical presentation and molecular, serology and/or histology results[139]. Molecular detection of CMV in placental tissue has been positively associated with foetal death[141], and histologically-confirmed foetal thrombotic vasculopathy has also been associated with CMV infection in stillbirths[142].

CMV mortality data from LMIC, including sub-Saharan Africa, where post-mortem examination is not routinely conducted and where polymicrobial infections are common, are lacking.

The aim of this secondary data-analyses was to describe the prevalence and histological findings of CMV infection in stillbirths and neonatal deaths within 21 days of birth (i.e. congenitally infected with CMV) using minimally invasive tissue sampling (MITS); and what proportion of these deaths was attributed to CMV infection.

7.2 Patients and Methods

7.2.1 Study site and population

A prospective, observational study was conducted at CHBAH in Soweto, South Africa from 16 July 2015 to 30 July 2016 to investigate causes of perinatal and paediatric deaths under five years of age as a pilot study preceding the Child Health and Mortality Prevention Surveillance (CHAMPS) currently underway[266]. The study methodology, including tissue collection procedures and laboratory testing has been described[1,2]. A detailed description on the underlying and immediate attributed cause of death (CoD), as adjudicated on by an expert panel, has been published[267].

Stillbirths were defined as absence of any signs of life diagnosed either before delivery, by ultrasonography, or at birth, by the attending clinician/midwife. Assessment of gestational age of the stillbirths was based on the last menstrual period or on ultrasound findings performed as standard of care. Stillbirths with weight ≥1000 grams based on WHO reporting criteria were included[268]. Stillbirths were categorised by timing of foetal death, as antepartum, i.e. foetal death confirmed by sonography prior to the onset of labour; or intrapartum if there was evidence of foetal life during labour by physical examination, cardiotocography or ultrasonography. Study inclusion criteria for neonates included, born alive and birth weight >750 grams. For both stillbirths and neonates, additional inclusion criteria were mother being

resident in Soweto, feasibility of undertaking the MITS within 36 hours after death and parental consent for participation.

7.2.2 Minimally Invasive Tissue Sampling

The MITS was performed by either study doctors or professional nurses with the support of research assistants as published[1,2]. Briefly, standardised procedures on infection prevention to prevent contamination of samples and personal protection was followed by study staff and the corpse decontaminated by washing with water followed by 70% ethanol. In both stillbirths and neonates, tissue was biopsied from the right and left lungs, liver and brain using anatomical markings to locate each organ as described[1,2]. The lungs and liver were biopsied using the Bard Monopty 14-gauge disposable core biopsy needles (Bard Peripheral Vascular Inc., Temple, USA). The brain was biopsied using the Bard Max-core 18-gauge disposable biopsy needle (Bard Peripheral Vascular Inc., Temple, USA). Molecular testing was conducted on lung tissue only whilst lung and liver tissue were cultured, and liver, lung and brain tissues were examined histologically.

Tissue samples for molecular testing were placed in Qiagen lysis buffer and those for histology in 10% neutral buffered formalin. Details of bacterial culture methods are provided elsewhere[1,2]. Additionally, blood and cerebrospinal fluid (CSF) samples were collected for molecular testing and culture. Blood was collected through cardiac puncture or by supraclavicular approach into the left subclavian blood vessel and at least 10ml was placed into an EDTA tube. CSF was collected through a puncture into the cisterna magna posteriorly and placed in a sterile tube. The NHLS in Johannesburg, South Africa, conducted HIV testing on whole blood by PCR (Roche COBAS® TaqMan® HIV-1 Qualitative Test Version 2, Roche Molecular Systems, Inc., Branchburg, NJ). To aid in identifying causes of stillbirths, the placenta was also evaluated. Placental weight was determined at the time of delivery and a

wedge of parenchymal tissue and chorioamniotic membranes placed in a sterile container for bacterial microscopy and culture. The remaining placenta was placed in 10% buffered formalin and transported to Lancet Laboratories (Johannesburg, South Africa). The placenta was macroscopically examined followed by haematoxylin and eosin (H&E) staining using standard protocols[269].

7.2.3 Molecular diagnostic testing

Molecular testing was conducted at the RMPRU as described[2]. The biopsies were macerated in 600µL of sterile saline and mixed with 25mg/mL proteinase K in 20mM Tris-HCl, pH 8.3 stock solution in a 1:1 ratio. The solution was incubated at 55ºC for 15 minutes and vortexed every five minutes. The NucleiSens EasyMag extraction system (BioMerieux, Marcy l'Etoile, France) utilising the Specific B off-board extraction protocol was used to extract DNA from the lung tissue, whole blood and CSF samples according to the manufacturer's instruction.

Fast Track Diagnostic (FTD, Sliema, Malta) multiplexed real-time PCR (RT-PCR) assay kits using the Applied Biosystems 7500® instruments (Applied Biosystems, Foster City, CA) was used to test the DNA samples. DNA from lung and blood samples were tested using the FTD-Neonatal Sepsis kit and CSF samples were tested using the FTD-Neuro-9 meningitis panel which both included probes for CMV amongst other organisms. Cycling conditions were 50ºC for 15 minutes, 95ºC for 10 minutes followed by 40 cycles of 95ºC for eight seconds and then 60ºC for 34 seconds. Each assay supplied with the kits included template and non-template controls as well as external and internal controls. A cycle threshold (Ct) cut-off above 35 was implemented for all FTD kits and only those with Ct<35 were classified as "positive" provided the run passed the controls quality criteria.

7.2.4 Histopathological diagnosis

Tissues from each organ was collected in two sets: one set was evaluated in Johannesburg, South Africa at the NHLS and the second set at the Centres for Disease Control and Prevention, Atlanta, USA. All tissues had H&E staining with special stains dependent on the histological findings. Special stains included Ziehl-Neelsen for mycobacteria, Grocott's methenamine silver and Periodic acid-Schiff for fungi, and Gram's stain for bacteria. Immunohistochemistry was also performed with the choice of antibody guided by the H&E findings, molecular results and microbiology findings.

7.2.5 Determination of Cause of Death (DeCoDE) process

An international panel of medical specialists in pathology, paediatrics, epidemiology, microbiology, obstetrics, infectious disease and international coding and certification convened in South Africa from 26th March to 5th April 2017 to determine the CoD in each case (referred to as the DeCoDe panel) as described previously[2,267]. A decision on the CoD in each case was then made based on the WHO International Classification of Diseases, 10th revision (ICD-10) for deaths during the perinatal period (ICD-PM)[270]. The "underlying cause of death", which initiated the disease process or condition which led to the death, and the "immediate cause of death", which directly caused the death were determined based on the antemortem clinical and MITS information available. For this study, maternal data were only available for stillbirths. The final CoD forms were ICD-10 coded by a medical doctor.

7.2.6 Statistical analysis

Stillbirths and neonatal deaths under 21 days old were analysed separately, each stratified by cCMV infection identified through molecular testing in lung tissue, blood and/or CSF. The analysis is descriptive and P values are reported, only when significant and biologically

plausible, intended to generate hypotheses for future studies and not to draw conclusions on cCMV as a cause of stillbirths or neonatal deaths. For all variables, we calculated descriptive statistics and provided medians with ranges for non-parametric continuous variables, and means and standard deviations for parametric variables. For categorical variables, proportions were provided. For selected variables, differences between cCMV-infected and cCMV-uninfected cases were tested using Wilcoxon rank sum test and t-test for continuous variables, as appropriate, and Fisher's exact test for categorical variables, with a P value ≤0·05 considered significant. Statistical analysis was conducted using STATA software version 13 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LLC).

7.2.7 Ethics approval and funding source

The Human Research Ethics Committee (Medical) (Reference number: 150215, Appendix 5) of the University of the Witwatersrand approved this study.

The study was funded by the Bill & Melinda Gates Foundation (BMGF). BMGF staff did not participate in the study design, data collection, analyses and preparation of manuscripts but did participate in the DeCoDe panel. The interpretation of results and content of the work is the exclusive responsibility of the authors.

7.3 Results

One-hundred and twenty-seven stillbirths and 144 neonatal deaths occurring within 21 days of birth were included in the analyses. The samples and sites available for testing from both stillbirths and neonates are given in Table 7.1.

Table 7.1: Samples available for CMV testing in stillbirths and neonates

Sample and test	**Stillbirths N=127**	cCMV-infected N=15	cCMV-uninfected N=112	**Neonates N=144**	cCMV-infected N=17	cCMV-uninfected N=127
Blood Molecular	**122**	14	108	**133**	9	124
CSF Molecular	**41**	4	37	**132**	6	126
Lung Molecular	**126**	15	111	**143**	15	128
Lung Histology	**104**	11	93	**137**	17	120
Liver Histology	**55**	5	50	**140**	17	123
Brain Histology	**100**	11	89	**140**	17	123
Placenta histology	**96**	11	85	**NA**	NA	NA

7.3.1 Stillbirths

CMV was detected in one or more sites in 11.8% (15/127, 95% CI 6.8-18.7) of stillbirths. The median age of women with stillbirths associated with CMV infection was 31 years and for those without cCMV, 28 years (Table 7.2). The overall prevalence of HIV infection among the women with a stillbirth was 34.6% (similar to the population prevalence of 29.9%[82]), being marginally higher in those where the stillbirth was identified with cCMV (57.1%, 8/14) compared to without cCMV (31.2%, 29/93, P=0.057). Overall, two of 37 (5.4%) stillbirths born to women living with HIV acquired HIV-infection *in-utero*, including one of eight stillbirths with cCMV (12.5%) and one of 29 without cCMV (3.4%). There were no differences in characteristics between stillbirths with and without cCMV (Table 7.2).

The sites of CMV identification in the stillbirths included from the lung (11/15, 66.7%), blood (7/14, 40.0%) and CSF (3/4, 75.0%), including being identified from two or more sites in five (33.3%) of the 15 cases. The majority of stillbirths (66.7%, 73/108), including those with cCMV (66.7%, 10/15) were macerated (Table 7.2).

Histological investigation of stillbirth tissue sampling generally yielded inadequate samples for analyses due to high proportion with tissue autolysis (ranging from 33.3% [brain]-77.8% [liver]), or no targeted tissue being sampled (ranging from 16.7% [liver] to 63.0% [brain], Table 7.3) This limited comprehensive tissue histology examination. Viral cytopathic effects were, however, observed in the lungs of one (of 104 with adequate sample) cCMV-infected stillbirth confirming active CMV disease *in-utero* (Figure 7.1).

Overall, placentas were sent for examination in 75.6% (96/127) of the stillbirths, including eleven (73.3%) of 15 with cCMV (Table 7.4). The placental histology findings were similar between stillbirths with and without cCMV, including presence of cord thrombosis (9.1%

[1/11] and 3.6% [3/83], P=0.397), evidence of chorioamnionitis (27.3% [3/11] and 28.2% [24/85], P=0.99), and macroscopic infarcts (20.0% [2/10] and 28.0% [23/82], P=0.723) respectively. CMV specific viral cytopathic effects were not observed in placental tissues (Table 7.4).

The DeCoDe panel assigned underlying and immediate CoD in all stillbirths, as previously detailed[1] (Table 7.5). Overall, CMV infection was attributed as the immediate CoD in 3.9% (5/127) of the stillbirths, i.e. in 33.3% (n=5/15) of the stillbirths with cCMV. In the five stillbirths with cCMV as the immediate CoD, the underlying attributed CoD were sepsis in all (Table 7.5). The immediate CoD in the remaining 10 cases in which CMV was not considered to be included in the casual pathway were intrauterine hypoxia (60.0%, 6/10), bacterial sepsis (30.0%, 3/10) and congenital disorder (10.0%, 1/10) which were also the underlying CoD. Underlying CoD did not differ between cases with and without cCMV.

Table 7.2: Maternal and stillborn foetal demographic characteristics

Maternal characteristics	Total N=127	cCMV-infected stillbirths N=15	cCMV-uninfected stillbirths N=112
Maternal Age (years)	N=120	N=14	N=106
Median Maternal age (range)	28 (17-42)	31 (20-38)	28 (17-42)
≤25 n (%)	42 (35)	4 (28.6)	38 (35.9)
26-30	33 (27.5)	3 (21.4)	30 (28.3)
≥31	45 (37.5)	7 (50.0)	38 (35.9)
Race			
Black African, n (%)	122/127 (96.1)	15/15 (100)	107/112 (95.5)
HIV infection, n (%)	37/107 (34.6)	8/14 (57.1)	29/93 (31.2)
Syphilis positive on RPR[c], n (%)	6 /102 (4.7)	0/12 (0)	6/90 (6.7)
Pregnancy and labour complications			
Pregnancy induced hypertension	27/127 (21.3)	4/15 (26.7)	23/112 (20.5)
Antepartum haemorrhage	31/127 (24.4)	2/2 (13.3)	29/33 (25.9)
Multiple gestation	7/127 (5.5)	0/15 (0)	7 /112 (6.3)
Caesarean section	33/123 (26.8)	2/14 (14.3)	32/109 (28.4)
Placenta collected	96/127 (75.6)	11/15 (73.3)	85/112 (75.9)
Premature delivery (<37 weeks)	74/108 (68.5)	10/14 (71.4)	64/94 (68.1)
Timing of foetal death			
Antepartum	95/122 (77.9)	11/15 (73.3)	84 (78.5)
Intrapartum	27/122 (22.1)	4/15 (26.7)	23 (21.5)
Stillbirth appearance			
Macerated	73/108 (67.6)	10 (71.4)	63 (67.0)
Fresh	35/108 (32.4)	4 (28.6)	31 (33.0)
Mean stillbirth weight, g (SD)	2124.7 (713.3)	2138.9 (778.9)	2122.9 (708.4)
Stillbirth with HIV infection born to mother living with HIV	2/37 (5.4)	1/84 (12.5)	1/29 (3.4)
Median (IQR) time between birth and MITS (hours)	20.6 (9.6-29.0)	14.3 (2.8-27.3)	21.8 (10.0-29.1

Table 7.3: Histological findings in stillborn foetal tissues

	Total N=127	cCMV-infected N=15	cCMV-uninfected N=112	P value
RIGHT AND LEFT LUNG				
Interstitial pneumonia	3/104 (2.9)	0/11 (0)	3/93 (3.2)	NS
Viral cytopathic effects	1/104 (1.0)	1/11 (9.1)	0/93 (0)	0.029
Inclusions	1/104 (1.0)	0/11 (0)	1/93 (1.1)	NS
Neutrophilic infiltrates in alveolar spaces	1/104 (1.0)	0/11 (0)	1/93 (1.1)	NS
Macrophage cells	2/104 (1.9)	0/11 (0)	2/93 (2.2)	NS
Autolysed	14/23 (60.9)	1/4 (25.0)	13/19 (68.4)	ND
No Tissue	9/23 (39.1)	3/4 (75.0)	6/19 (31.6)	ND
LIVER				
Hepatocellular damage with little inflammation	3/55 (5.5)	0/5 (0)	3/50 (6.0)	NS
Liver necrosis	5/55 (9.1)	0/5 (0)	5/50 (10.0)	NS
Septicaemia	1/55 (1.8)	0/5 (0)	1/50 (2.0)	NS
Autolysis	56/72 (77.8)	5/10 (50.0)	51/62 (82.3)	ND
Necrosis	4/72 (5.6)	3/10 (30.0)	1/62 (1.6)	ND
No tissue	12/72 (16.7)	2/10 (20.0)	10/62 (16.1)	-
BRAIN HISTOLOGY				
Brain lymphohistiocytic infiltrates	5/100 (2.0)	1/11 (9.1)	4/89 (4.5)	NS
Brain perivascular inflammation	1/100 (1.0)	1/11 (9.1)	0/89 (0)	NS
Necrotic	1/27 (3.7)	1/4 (25.0)	15/23 (65.2)	ND
Autolysed	9/27 (33.3)	1/4 (25.0)	8/23 (34.8)	ND
No tissue	17/27 (63.0)	2/4 (50.0)	15/23 (65.22)	ND

NS Non significant

ND Not determined

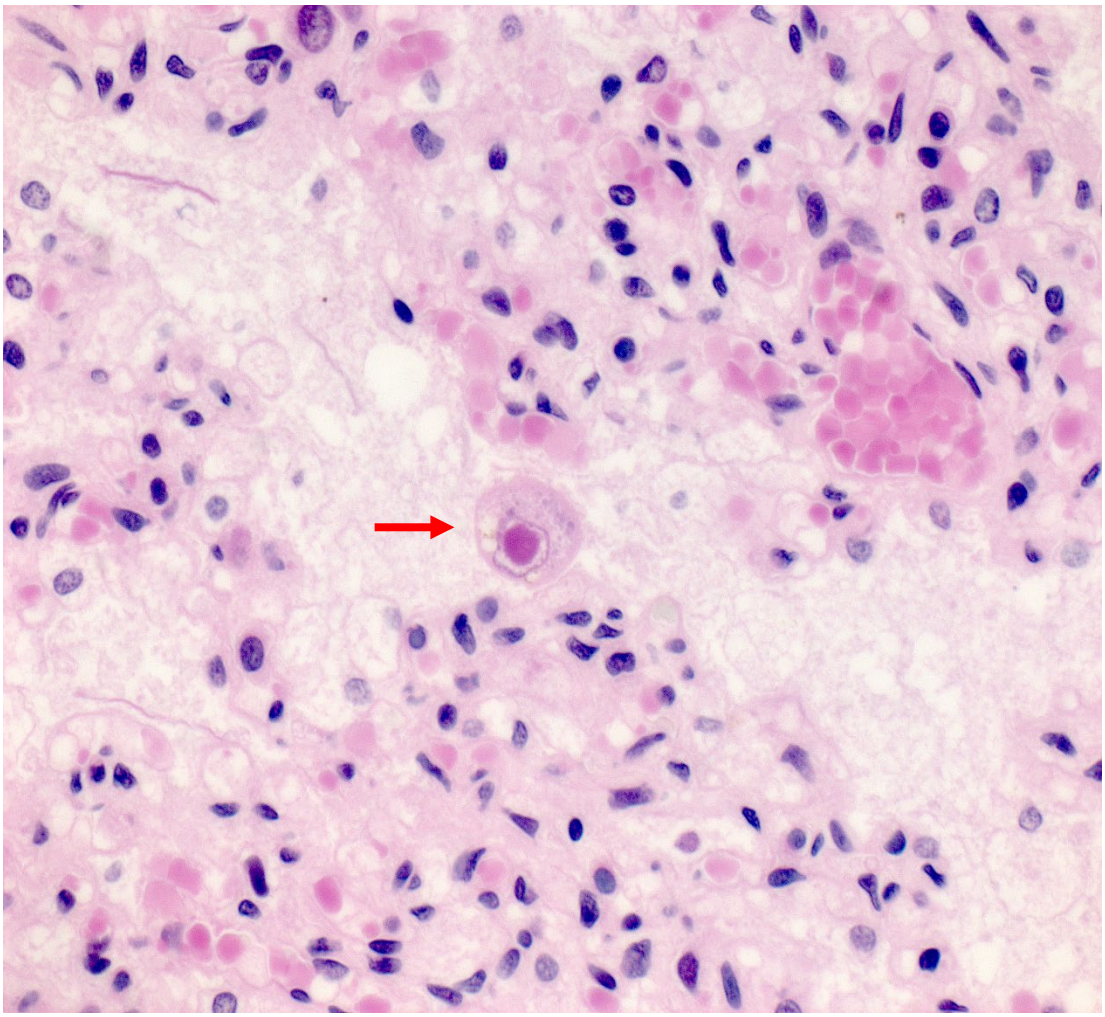


Figure 7.1: Haematoxylin and eosin stain of lung tissue from a stillbirth with disseminated CMV disease. Giant cell with intranuclear and cytoplasmic inclusions (arrow)

Table 7.4: Histological findings in placentas retrieved from stillbirths

PLACENTA HISTOLOGY	Total N=127	cCMV-infected N=15	cCMV-uninfected N=112
Placenta available	N=96	N=11 (73.3)	N=85 (75.9)
Mean Placenta weight, g (SD)	381.5 (142.9)	388.2 (120.4)	380.6 (146.2)
Umbilical cord	N=92	N=11	N=81
Cord insertion: central	41 (44.6)	5 (45.5)	36 (44.4)
Eccentric	49 (53.3)	5 (45.5)	44 (54.3)
Marginal	2 (2.2)	1 (9.1)	1 (1.2)
Number of cord vessels: 3	95 (74.8)	11 (73.3)	84 (75.0)
Cord thrombosis (%)	4/94 (4.3)	1 /11 (9.1)	3/83 (3.6)
Cord knots (%)	4/94 (4.3)	0/11 (0)	4/83 (4.8)
Funisitis (%)	3/96 (3.1)	0/11 (0)	3/85 (3.5)
Placenta membranes			
Membrane Colour: Translucent	78/94 (83.0)	8/10 (80.0)	70/84 (83.3)
Other	16/94 (17.0)	2/10 (20.0)	14/84 (16.7)
Amnion nodosum (%)	1/91 (1.1)	0 (0)	1/81 (1.2)
Chorioamnionitis (%)	27/97 (27.8)	3/11 (27.3)	24/85 (28.2)
Grading			
Grade I (<10 cells/phf)	7/22 (31.8)	1/3 (33.3)	6/19 (31.6)
Grade II (10-20 cells/phf)	9/22 (40.9)	2/3 (66.7)	7/19 (36.8)
Grade III (>30 cells/phf)	6/22 (27.3)	0 (0)	6/19 (31.6)
Placenta parenchyma			
Macroscopic infarct (%)	25/92 (27.20)	2/10 (20.0)	23/82 (28.0)
Colour: Normal	18/87 (20.7)	2/10 (20.0)	16/77 (20.8)
Pale	25/87 (28.7)	3/10 (30.0)	22/77 (28.6)
Congested	44/87 (50.6)	5/10 (50.0)	39/77 (50.7)
Retroplacental hematoma (%)	30/95 (31.6)	2/11 (18.2)	28/84 (33.3)
Placenta cut surface findings			
Infarction (%)	45/91 (49.5)	3/11 (27.3)	42 (52.5)
Intervillous fibrin	11/93 (11.8)	0/10 (0)	11/83 (13.3)
Other chorionic plate abnormalities	3/103 (2.9)	0/12 (0)	3/91 (3.3)
Other decidua basalis abnormalities	3/101 (3.0)	0/12 (0)	3/89 (3.4)

NS Non significant

ND Not determined

Table 7.5: Underlying (in bold) and immediate causes of deaths in stillbirths

CAUSES OF DEATH	Total N=127	CMV-infected N=15	CMV uninfected N=112
Foetal deaths due to sepsis and infection	**N=48 (37.8)**	**N=8 (53.3)**	**N=40 (35.7)**
Bacterial sepsis[1]	38 (29.9)	3 (20.0)	35 (31.3)
Congenital syphilis, unspecified	2 (1.57)	0 (0.00)	2 (1.79)
Other bacterial meningitis	1 (0.79)	0 (0.00)	1 (0.89)
Congenital pneumonia, unspecified	1 (0.79)	0 (0.00)	1 (0.89)
Congenital cytomegalovirus infection	5 (3.94)	5 (33.33)	0 (0.00)
Congenital herpesviral [herpes simplex] infection	1 (0.79)	0 (0.00)	1 (0.89)
Foetal deaths due to complications during pregnancy, labour and delivery	**N=47 (37.0)**	**N=6 (40.0)**	**N=41 (36.6)**
Complications following (induced) termination of pregnancy	1 (0.79)	0 (0.00)	1 (0.89)
Newborn affected by slow intrauterine growth, unspecified	1 (0.79)	0 (0.00)	1 (0.89)
Intrauterine hypoxia[2]	45 (35.4)	6 (40.0)	39 (34.8)
Foetal deaths due to congenital disorders[2]	**N=6 (4.7)**	**N=1 (6.7)**	**N=5 (4.5)**
Unspecified cause of foetal death	**N=26 (20.5)**	**N=0 (0)**	**N=26 (23.2)**
Foetal death of unspecified cause	11 (8.66)	0 (0.00)	11 (9.82)
Inadequate data to determine cause of death	15 (11.81)	0 (0.00)	15 (13.39)

[1]Details of organisms causing bacterial sepsis are given in the published manuscript [1] and were of similar frequencies between cCMV and cCMV-uninfected stillbirths

[2] Details of specific congenital anomalies are given in the published manuscript [1] and were of similar frequencies between cCMV and cCMV-uninfected stillbirths

7.3.2 Neonates

Of the 144 neonatal deaths, 17 (11.8%, 95% CI 7.0-18.2) were identified with cCMV-infection. The median age at hospitalization was zero days of age for neonates who died with or without cCMV, whilst the median age at death was five (range 0-19) and four (range 0-21) days respectively (Table 7.6). The median birth weight was lower in neonatal deaths in whom CMV-infection was identified (1055g, interquartile range [IQR] 780-1370) than those without CMV infection (1255, IQR 920-2365, P=0.027). Forty-seven percent (7/17) of neonates born with CMV-infection were HIV-exposed, and 33.3% (41/127) of those in whom no CMV was identified (P=0.390). Of the HIV-exposed neonates, HIV infection was confirmed in one neonate each with (14.3%, 1/7) and without CMV infection (2.4%, 1/41, Table 7.6).

Among the 17 neonates with cCMV, CMV was identified from the lungs (12/17, 70.6%), blood (9/16, 56.3%) and CSF (6/17, 35.3%); including being detected in two or more sites in 41.2% (7/17) of the cases. The lung and liver histology findings were similar in neonatal deaths associated with and without cCMV (Table 7.7). Non-specific inclusions in lung tissue were observed in one case each of a neonate with (5.9%) and without cCMV infection (0.8%), albeit there not being any other histological evidence of CMV disease (Table 7.7).

CMV disease was not attributed as either the underlying or immediate CoD in any of the 17 neonates identified with cCMV infection (Table 7.8). The most frequent underlying CoD were low birth weight/prematurity complications in neonates with (13/17, 76.5%) and without CMV infection (62/127, 48.8%, P= 0.039). Other underlying CoD in neonates with cCMV are reported in Table 7.8. The immediate CoD in neonates with cCMV were unrelated to CMV with the most frequent identified immediate CoD in neonates with and without cCMV being nosocomial sepsis (6/17, 35.3% and 24/127, 18.9%). Other immediate CoD in neonates with cCMV were due to nosocomial pneumonia (3/17, 17.6%), respiratory distress syndrome

(2/17, 11.8%) and one (5.9%) each of pneumonia, pulmonary mucormycosis, acute renal failure, congenital malformation of the heart and vasculature, nosocomial meningitis and shock (Table 7.8).

Table 7.6: Demographic characteristics of neonatal deaths <21 days by cCMV infection status

	Total N=144	**cCMV-infected N=17**	**cCMV-uninfected N=127**
Median (range) age on admission	0 (0-15)	0 (0-3)	0 (0-15)
Median (range) age at death	4.5 (0-21)	5 (0-19)	4 (0-21)
Male gender (%)	70 (48.6)	8 (47.1)	62 (48.8)
HIV-exposed (%)	48 (33.3)	7 (41.2)	41 (32.2.3)
HIV PCR reactive (%)	2 (1.4)	1 (5.9)	1 (0.81)
Median (IQR) weight (grams, g)	1245 (895-2260)	1055 (780-1370)	1255 (920-2365)
Low birth weight (<2500-1500 g)	116 (81.1)	15 (88.2)	101 (80.2)
Median (IQR) gestational age (GA, weeks)	30 (27-36)	28 (27-30)	30.5 (27-36)
Premature (<37 weeks)	103 (77.4)	14 (93.3)	89 (75.4)
Significant congenital abnormalities (%)	1 (0.8)	0 (0)	1 (0.9)
Median (IQR) time between death and MITS (hours)	24.1 (14.3-37.5)	17.6 (13.9-26.6))	24.4 (14.6-38.9)

Table 7.7: Tissue histology of cCMV-infected and uninfected neonates

	Total N=144	cCMV-infected N=17	cCMV-uninfected N=127
LUNG HISTOLOGY			
Hyaline membranes, n (%)	23/137 (16.8)	4/17 (23.5)	19/120 (15.8)
Interstitial pneumonia, n (%)	20/137 (14.6)	3/17 (17.7)	17/120 (14.2)
Neutrophilic infiltrates in alveolar spaces, n (%)	19/137 (13.9)	3/17 (17.7)	16/120 (13.3)
Diffuse alveolar damage, n (%)	10/137 (7.3)	2/17 (11.8)	8/120 (6.7)
Macrophage cells, n (%)	13/137 (9.5)	2/17 (11.8)	11/120 (9.2)
Intra-alveolar proteinaceous material, n (%)	5/137 (3.7)	2/17 (11.8)	3/120 (2.5)
Non-specific inclusions, n (%)	2/137 (1.5)	1/17 (5.9)	1/120 (0.8)
Acute lung injury with necrosis, n (%)	1/137 (0.7)	0/17 (0)	1/120 (0.8)
No tissue, n (%)	1/7 (14.3)	0/0 (0)	1/7 (14.3)
Autolysis, n (%)	6/7 (0)	0/0 (0)	6/7 (85.7)
LIVER HISTOLOGY			
Necrosis, n (%)	7/140 (5.0)	2/17 (11.8)	5/123 (4.1)
Pigment in Kupffer cells, n (%)	7/140 (5.0)	1/17 (5.9)	6/123 (4.9)
Hepatocellular damage with little inflammation, n (%)	1/140 (0.7)	0/17 (0)	1/123 (0.8)
Septicaemia, n (%)	1/140 (0.7)	0/17 (0)	1/123 (0.8)
Autolysed, n (%)	1/7 (14.3)	0/17 (0)	1/4 (25.0)
No tissue, n (%)	6/7 (85.7)	0/17 (0)	3/4 (75.0)
BRAIN HISTOLOGY			
Brain perivascular inflammation, n (%)	2/140 (1.4)	1/17 (5.9)	1/123 (0.8)
Brain lymphohistiocytic infiltrates, n (%)	23/140 (16.4)	0/17 (0)	23/123 (18.7)
No tissue, n (%)	0/140 (0)	0/17 (0)	4/4 (100)

Table 7.8: Underlying (in bold) and immediate causes of death in cCMV-infected and uninfected neonates

CAUSES OF DEATH	cCMV-infected N=17	cCMV-uninfected N=127	P value
Low birth weight and prematurity, n (%)	**13 (76.5)**	**62 (48.8)**	**0.039**
Nosocomial sepsis, n (%)	6 (35.3)	24 (18.9)	
Nosocomial pneumonia, n (%)	3 (17.6)	12 (9.4)	
Respiratory distress syndrome, n (%)	2 (11.8)	12 (9.4)	
Pneumonia, n (%)	1 (5.9)	2 (1.6)	
Pulmonary mucormycosis, n (%)	1 (5.9)	0	
Other conditions[1], n (%)	0	12 (9.4)	
Infection	**1 (5.9)**	**33 (25.9)**	**0.118**
Acute renal failure, n (%)	1 (5.9))	0	
Sepsis, meningitis and pneumonia[2], n (%)	0	14 (11.0)	
Hypoxic ischemic encephalopathy, n (%)	0 (0)	13 (41.9)	
Other conditions[1], n (%)	0	6 (4.7)	
Congenital malformations, deformations and chromosomal abnormalities, n (%)	**1 (5.9)**	**16 (12.6)**	**0.694**
Congenital malformation of heart and vasculature, n (%)	1 (5.9)	2 (1.6)	
Sepsis and pneumonia[2], n (%)	0	6 (4.7)	
Other conditions[1], n (%)	0	8 (6.3)	
Respiratory and cardiovascular disorders, n (%)	**1 (5.9)**	**4 (3.1)**	**0.471**
Nosocomial meningitis, n (%)	1 (5.9)	0 (0)	
Other conditions, n (%)	0 (0)	4 (3.1)	
Complications of intrapartum events[1]	**0 (0)**	**5 (3.9)**	
Other conditions[1], n (%)	**0**	**5** (3.9)	
Convulsions and disorders of cerebral status, n (%)	**0 (0)**	**1 (0.8)**	**>0.999**
Pneumonia due to Klebsiella pneumoniae/Nosocomial condition, n (%)	0 (0)	1 (0.8)	
Neonatal death of unspecified cause, n (%)	**1 (5.9)**	**0**	
Shock, unspecified, n (%)	1 (5.9)	0	
Other neonatal conditions[1], n (%)	**0 (0)**	**6 (4.7)**	**>0.999**

[1]Details of other conditions are provided in the published manuscript
[2] Includes nosocomial sepsis and pneumonia

7.4 Discussion

The prevalence of cCMV infection in stillbirths (11.8%) and neonates dying within 21 days of birth (11.8%) in this analyses was higher than the prevalence reported in live births (2.5%, 95% CI 1.9-3.2) from the same population[234]. Despite the high prevalence of CMV infection in our study, CMV was only attributed as being in the causal pathway of death in close to 4.0% of stillbirths and none of the neonatal deaths, with alternate underlying and/or immediate CoD attributed to the remaining cases by the DeCoDe panel. This raises the question of the reliability of molecular diagnostics alone in attributing CoD in autopsies and the need for an integrated assessment of post-mortem molecular results with ante mortem history and laboratory data. Nevertheless, the contribution of cCMV (3.9%) was similar to Group B streptococcal sepsis (3.1%, 4/129) as CoD among the stillbirths, as reported in the pilot MITS study[1] and merits further assessment. In the neonates that died, median birth weight was frequently lower in those with cCMV than those without. Low birth weight and prematurity was found to be the leading underlying CoD in neonates overall in this study population as described[2] and, in the study of cCMV in live births, cCMV was also independently associated with SGA and prematurity[234]. It is possible to hypothesise that CMV-infected foetuses may be at risk for preterm birth and being SGA, which in turn is an underlying CoD. The CHAMPS study, currently underway and including multiple study sites will enable exploration of these hypotheses[271].

The difference in cCMV prevalence rates between live births and stillbirths and neonatal deaths may be due to a higher sensitivity of the MITS procedure to detect CMV by sampling multiple sites in the lung, liver, brain, CSF and blood, compared to the method of detection in live births which sampled saliva and/or urine only. Similar high prevalence of cCMV has been reported in stillbirths in other studies[140,142]. Two autopsy studies in Australia identified

CMV infection in 15.0% of 130 stillborn foetuses by molecular testing of placenta, kidney and liver tissue[142] and a second study testing for CMV in cardiac blood identified CMV infection in 9.0% of 107 stillbirths[140] whereas the estimated prevalence of cCMV in live births in Australia is 0.3%[86]. Whether the higher prevalence of cCMV in stillbirths and neonatal deaths is due to an increased risk of mortality in cCMV-infected foetuses and neonates, requires investigation through studies directly comparing CMV infection in live births surviving the neonatal period, stillbirths and neonatal deaths using comparable diagnostic methods such as the additional testing of saliva and urine in the post-mortems.

CMV was detected frequently in the lungs in stillbirths (73.3%) and neonates (70.6%) with disseminated CMV (i.e. two or more sites with CMV detected) that was diagnosed in 33.3% of stillbirths and 41.1% of neonates. Despite the high prevalence of CMV infection in both groups, the histopathological features of CMV associated disease was limited with only one stillbirth having viral cytopathic effects in the lungs and one with liver necrosis and none of the neonatal tissues with CMV associated damage. Similarly, examination of the placentas of stillbirths did not reveal any histological features that could be attributed to CMV infection, although molecular testing of the placenta for CMV and other pathogens was not conducted.

The post-mortem study by Iwasenko et al. (2011) detected CMV infection in 20 (15.3%) of 130 stillbirths by PCR testing of kidney, liver and placenta samples and found two or more sites (disseminated CMV) positive for CMV in four cases (3.1%)[142]. On comparison of PCR to immunohistochemistry, immunohistochemistry was less sensitive than PCR, identifying 75% (15/20) of the CMV-infected foetal tissues. Foetal thrombotic vasculopathy was the only histological finding significantly associated with CMV infection[142]. Another study in Italy of tissue from foetuses with a prenatal diagnosis of cCMV that were terminated or died *in-utero* found CMV antigens in placenta (100%), pancreas (100%), lung (87%), kidney (87%), liver

(71%), brain (55%) and heart (44%) [272]. The organs with the most extensive viral infection and tissue damage was the pancreas (69%), followed by the placenta (52%) and liver (35%)[272]. We hypothesise that the lack of histological evidence of CMV disease in our study, despite the high prevalence rate in stillbirths and neonatal deaths, may be due to CMV transmission being the result of maternal non-primary CMV infection where protective maternal immunity prevented extensive viral infection and tissue damage, although not foetal transmission, as has been perceived previously where clinical symptoms are believed to be less severe in congenital infection following reactivation of the virus during pregnancy[273,274].

Our study has limitations which includes a number of tissue samples obtained by MITS from stillbirths inadvertently being of insufficient quality for examination due to maceration of the foetus. We did not collect urine or saliva samples from the stillbirths or neonatal deaths and were unable to compare CMV detection rates in these samples as have been done for live births. The small number of cases enrolled may also have limited our ability to detect any significant differences between cCMV-infected and cCMV-uninfected groups although the frequency of characteristics such as low birth weight, prematurity and HIV infection was greater in CMV-infected deaths as found in live births.

In conclusion, the prevalence of cCMV in stillbirths and neonatal deaths was higher than in live births, with CMV only being attributed as a significant CoD in stillbirths. Our findings have raised a number of hypotheses to be further investigated on the role of CMV in stillbirths and neonates in the causal pathway to death.

8 Thesis conclusion

There were a number of findings described in this Thesis that contribute to understanding the epidemiology of cCMV in high CMV and HIV seroprevalence settings. The prevalence of cCMV in HIV-exposed neonates in the era of ART was high, whereas the prevalence of cCMV in a comparable group of HIV-unexposed neonates was within the range expected in a middle-low income, black, urban population. Such a comparison has not been previously conducted in sub-Saharan Africa. We observed *in-utero* HIV infection to be higher in cCMV-infected neonates. This raises the question of the residual rate of *in-utero* HIV infection, in the setting of efficient PMTCT programmes, being a consequence of foetal CMV infection. Almost all the cCMV infections were in women with non-primary CMV, confirming that seroimmunity to CMV does not prevent foetal transmission, and, less so in women living with HIV. Well-designed CMV immunology, virology and epidemiology studies of African women living with HIV, their ART regimens and neonatal HIV and cCMV infection rates, are required to better comprehend the symbiotic relationship between CMV and HIV.

The prevalence of symptomatic cCMV disease at birth in neonates was 6.5%, which although lower than reported in other studies (10-15%[9,13,63]), had a CI inclusive of the rate of symptomatic infection reported in these studies (1.4-17.9) which were dominated by cCMV infection as a result of maternal primary CMV. This confirms that populations with high seroimmunity to CMV are not completely protected from symptomatic cCMV at birth by natural immunity. On assessment of neurological sequelae in cCMV cases from birth up to 12 months of age, there was no difference between infants with cCMV and a cCMV-uninfected control group, although this aspect of the study was limited by loss to follow-up and inconclusive hearing assessments in some children. Even with the study limitations, the

observations made raise the hypothesis that African children with cCMV as a result of maternal non-primary CMV, may have lower rates of neurological sequelae than that observed in studies from Europe[108,240], North America[103,275] and Brazil[71,225]. The studies in these locations revealed symptomatic newborn cCMV disease and SNHL to occur at similar frequencies in children born to women with primary CMV and those born to women with non-primary CMV. The follow-up study also monitored growth between children with cCMV, children with postnatally acquired CMV and children without CMV infection. Our numbers were too low to make definitive inferences; however, the results suggested that the combined effect of cCMV and HIV-exposure may impact growth negatively whereas cCMV alone may be beneficial for growth. Investigation of how CMV influences growth *in-utero* and in childhood is warranted.

There is a paucity of follow-up studies that can be compared to the findings in the South African population described in this Thesis. Longitudinal follow-up of a larger study population to at least six years of age is necessary to gain an understanding of the long-term clinical outcomes in children with cCMV, to determine resources required for screening and preventive measures. Ensuring a large enough sample size to account for loss to follow-up will be one of the challenges in this population and strategies to support the maternal caregiver and encourage study attendance need to be considered. Standardised protocols on clinical assessments and definitions of terms such as symptomatic infection and severity of disease for CMV studies are necessary to enable generalisation and comparison of data.

CMV shedding patterns in breast milk and vaginal fluid of women between delivery of their baby and 21 days post-delivery were also explored. CMV shedding in maternal vaginal fluid, although occurring less frequently than shedding in breast milk, was associated with cCMV, similar to a study from The Gambia[233]. CMV shedding in the genital tract of pregnant women

may potentially be a virological marker of CMV transmission to the foetus in the absence of an immunological correlate. Postnatal CMV acquisition by 12 months of age occurred in over half of CMV-uninfected infants and HIV-exposed infants were not at a higher risk of CMV acquisition.

Given the immune modulation observed in cCMV and pCMV-infected infants[150,152,153], vaccine-induced humoral immune responses to HBsAg, TT, DT, PT and FHA were compared between cCMV-infected, pCMV-infected and CMV-uninfected infants. Both congenital and postnatal acquired CMV did not reduce antibody titres to the tested vaccines compared to CMV-uninfected infants. This finding is particularly reassuring in African settings with high CMV seroprevalence, where vaccine efficacy for some vaccines is reportedly lower than in Western populations[150].

The prevalence of cCMV in stillbirths and neonatal deaths aged below 21 days was determined by molecular testing of lung tissue, blood and CSF as part of a wider pilot study investigating the cause of perinatal and under-five death. Additionally, the attribution of CMV as the cause of the perinatal deaths was evaluated by review of antemortem clinical data, laboratory results and post-mortem MITS results by a team of medical specialists. A higher prevalence of cCMV was found in stillbirths and neonatal deaths than found in the live births. The majority of the CMV infection, however, could not be attributed as the direct or underlying cause of death in the stillbirths or neonates. This investigation did raise a number of hypotheses related to CMV infection and low birth weight and prematurity that is being currently explored in the multicentre CHAMPS study in low and middle income African and Asian countries.

The findings from this Thesis give some insights into the epidemiology of cCMV in a high HIV prevalence setting. HIV-exposed neonates had a greater prevalence of cCMV and within the first year of life, cCMV-infected infants had similar neurological sequelae to cCMV-uninfected infants. Universal screening for cCMV is being advocated in high resource settings as cost-effective, by enabling early intervention and preventing onset and progression of cCMV-related disability. Long term follow-up of cCMV-infected children is required to determine the need for universal screening for cCMV in a high CMV seroprevalent South African setting.

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10 Appendices

1. Plagiarism declaration
2. Certificate of final submission
3. Ethics clearance certificate for PhD studies (HREC M151161)
4. Ethics clearance certificate for MITS pilot study (Reference N0. 150215)
5. Paper 1: Pathirana et al (2019)
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10. Turn-it-in report

Appendix 1: Plagiarism declaration

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SENATE PLAGIARISM POLICY: APPENDIX ONE

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Certif

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Abstract and key bibliographic data (i.e. from submission form)

I acknowledge that I am not entitled to the return of the copies of the dissertation or research/project report or thesis or other work I have submitted for the programme.

10. Did your research involve animal experimentation or the use of human subjects, human tissue or other material, or patient records?

☒ Yes
☐ No

If yes, please certify that clearance was obtained from the relevant, approved, University ethics committee:

Clearance number(s): HREC M151161

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14. Candidate:

14.1 The candidate must attach an original "Certificate to Accompany Higher Programmes Research Report" from his/her supervisor(s).

14.2 Is this dissertation or thesis supported by funding from *(please tick):*

☒ DST – NRF (e.g. CoE's; SARCHi Chairs; Innovation; African Origins Platform; Knowledge, Interchange and Collaboration; etc.) [*Please underline the programme that applies]*

☐ DST – CSIR (e.g. NEPTTP e-Research; etc.)

☐ Other (*Please list the full name of the funder*): _____

Signature of candidate:  Date: 15 June 2020

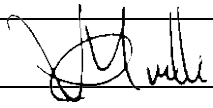
15. Supervisor(s):

I confirm that I provided academic oversight as supervisor during the completion of the degree

Name of supervisor: Shabir A. Madhi

Discipline: Respiratory and Meningeal Pathogens Research Unit

School: Pathology

Signature of Supervisor:  Date: 15 June 2020

I confirm that I provided academic oversight as supervisor during the completion of the degree

Name of second Supervisor (*if more than one*): Michelle J. Groome

Discipline: Respiratory and Meningeal Pathogens Research Unit

School: Pathology

Signature of Supervisor:  Date: 15 June 2020

FOR FACULTY OFFICE USE

- ☐ Retain one unbound copy
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- ☐ Embargo notification attached – if applicable (refer to section 9)
- ☐ Ethics Committee clearance number indicated – if applicable (refer to section 10)
- ☐ Copy of this submission form and attachments included with copies sent to Central Records Office – for forwarding to Library. **Originals placed on student file.**

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Appendix 3: Ethics clearance certificate for PhD studies

R14/49 Dr Jayani Pathirana et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M151161

NAME: (Principal Investigator)	Dr Jayani Pathirana et al
DEPARTMENT:	Respiratory and Meningeal Pathogens Research Unit Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	Congenital Cytomegalovirus (CMV) Infection in a High Human Immunodeficiency Virus (HIV) Prevalent Setting, South Africa
DATE CONSIDERED:	27/11/2015
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Shabir Madhi and Michelle Groome
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	17/02/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004, 10th floor, Senate House/2nd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature

Date 01 March 2016

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH 10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Faculty of Health Sciences, Phillip Tobias Building, Room 304 , 3rd Floor.
Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

31 May 2016

Dr Jayani Pathirana
RMPRU
Chris Hani Baragwanath Academic Hospital
PO Box 90753
Bertsham
2013

Sent by email to: Pathiranaj@rmpru.co.za

Dear Dr Pathirana

Re: **Protocol Ref no: M151161**
Protocol Title: Congenital Cytomegalovirus Infection in a HIV Prevalent Setting, South Africa
Principal Investigator: Dr Jayani Pathirana et al
Protocol Amendment

This letter serves to confirm that the Chairman of Human Research Ethics Committee (Medical) has approved the protocol amendments on the above mentioned study, as detailed in your letter dated 04 May 2016 :
Addition of a hearing assessment during the neonatal period
Inclusion of a new exploratory objective
The addition of breastmilk collection to exploratory objective 5

The following documents were received :

- Patient Information Sheet version 1.32 dated 02 May 2016

Thank you for keeping us informed and updated,

Yours Sincerely,

L.moeng

Mr Lebohang Moeng
Administrative Assistant
Human Research Ethics Committee (Medical)

UNIVERSITY OF THE WITWATERSRAND
OFFICE OF THE DEPUTY VICE CHANCELLOR RESEARCH
31 MAY 2016
RESEARCH OFFICE HREC (Medical)
JOHANNESBURG

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH 10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: PV Tobias Building 3rd Floor. Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za Fax +27 (0)11-717-1265
Email: HREC-Medical.ResearchOffice@wits.ac.za

12 January 2017

Dr Jayani Pathirana
Respiratory and Meningeal Pathogens Research Unit
11th Floor, Central West Wing
Nurses Residence
Chris Hani Baragwanath Academic Hospital
PO Box 90753
Bertsham
2013

Sent by email to: phathiranaj@rmpru.co.za

Dear Dr Phathirana

Re: **Protocol Ref no: M151161**
Protocol Title: Congenital Cytomegalovirus Infection in a high HIV Prevalent Setting, South Africa
Principal Investigator: Dr Jayani Pathirana et al
Protocol Amendment: Request to include a new exploratory objective

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has approved the protocol amendments for the abovementioned study, as detailed in your letter dated 25 September 2016.

The following documents were received

- Cover Letter dated 25 September 2016.
- Study Proposal version 1.3 dated 23 September 2016.
- Participant Information Leaflet version 1.3 dated 23 September 2016.
- English Informed Consent Form for Follow-Up version 1.3 dated 23 September 2016.
- Informed Consent for Transport, Storage and Future use of Samples version 1.3 dated 23 September 2016.
- Participant Information Leaflet version 1.3 dated 23 September 2016. (with track changes)
- English Informed Consent Form for Follow-Up version 1.3 dated 23 September 2016. (with track changes)
- Informed Consent for Transport, Storage and Future use of Samples version 1.3 dated 23 September 2016. (with track changes)

Thank you for keeping us informed and updated,

Yours Sincerely,



.....

Mr Lebohang Moeng

Administrative Assistant

Human Research Ethics Committee (Medical)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

09 May 2017

Dr Jayani Pathirana
Vaccine Preventable Diseases Respiratory and Meningeal Pathogens Research Unit
11th Floor, Central West Wing, Nurses Residence
Chris Hani Baragwanath Hospital
Johannesburg

Sent by email to: pathiranaj@rmpru.co.za

Dear Dr Pathirana

Re: **Protocol Ref No: M151161**
Protocol Title: Congenital Cytomeglovirus (CMV) Infection in a High Human Immunodeficiency Virus (HIV) Prevalent Setting, South Africa
Principal Investigator: Dr.Jayani Pathirana et al
Protocol Amendments

This letter serves to confirm that the Chairman of the Human Research Ethics Committee (Medical) has approved the protocol amendments for the abovementioned protocol, as detailed in your letter dated 25 April 2017.

The following documents were received:

- Cover letter dated 25 April 2017.
- Appendix 7: Follow up ICF - Patient Information Leaflet version 1.4 dated 13 February 2016.
- Request protocol amendments letter dated 22 March 2017.
- Study Protocol version 1.4 dated 22 March 2017.
- English Informed Consent Form for Follow-up version 1.4, dated 13 September 2016.

Thank you for keeping us informed and updated.

Yours Sincerely,

L.moeng

Mr Lebohang Moeng
Administrative Assistant
Human Research Ethics Committee (Medical)

UNIVERSITY OF THE WITWATERSRAND
OFFICE OF THE DEPUTY VICE CHANCELLOR RESEARCH
09 MAY 2017
RESEARCH OFFICE HREC (Medical)
JOHANNESBURG

Appendix 4: Ethics clearance certificate for MITS pilot study (Reference N0. 150215)

University of the Witwatersrand, Johannesburg

Human Research Ethics Committee: (Medical)
FWA Registered No IRB 00001223

SECRETARIAT: Suite 189, Private Bag x2600, Houghton 2041, South Africa Tel: +27-11-274 9200 Fax: +27-11-274 9281

14 May 2015

FAXED & COURIERED

Dr C Cutland

Respiratory and Meningeal Pathogens Research
11th Floor, Central West Wing, Nurses Residence
Chris Hani Baragwanath Hospital
Bertsham
2013

Fax: 086 674 0564

Dear Dr Cutland,

PROTOCOL: POST-MORTEM - MINIMALLY INVASIVE AUTOPSY (MIA) TO ESTABLISH CAUSE-OF-DEATH IN UNDER-5 CHILDREN IN A HIGH HIV-PREVALENCE SETTING

ETHICS REFERENCE NO: 150215

RE : FINAL ETHICS APPROVAL

This is to certify that the above-mentioned trial was reviewed by the University of the Witwatersrand, Human Research Ethics Committee (HREC), and the Protocol Review Committee (PRC) on: 27 February 2015.

The University of the Witwatersrand, Human Research Ethics Committee Approval Granted for the above mentioned study is valid for five years. Where required by Sponsor to have approval on a more frequent basis it remains the responsibility of the Sponsor and Investigator to apply for continuing review and approval, or for the duration of the Trial.

1. THIS APPROVAL IS SUBJECT TO THE FOLLOWING PROVISOS:

* A copy of the MCC Approval and/or MCC Notification letter must be submitted to the Ethics Regulatory Office Secretariat before the study commences / or where an Amendment may be implemented (IF MCC APPROVAL / NOTIFICATION IS APPLICABLE). It remains the responsibility of the Principal Investigator and/or Sponsor to ensure that the relevant approvals are in place.

* The study is conducted according to the protocol submitted to the University of the Witwatersrand, Human Research Ethics Committee. Any amendments to the protocol must first be submitted to the Human Research Ethics Committee for approval.

* During the study, the University of the Witwatersrand, Human Research Ethics Committee is informed immediately of :
  - Any Unexpected Serious Adverse Events or Unexpected Adverse Drug Reactions, which, in the Investigator and/or the Sponsor's opinion are suspected to be related to the study drug. (Refer to POL-IEC–001 and SOP-IEC–005, Item 3.4).
  - Any data received during the trial which, may cast doubt on the validity of the continuation of the study .

* The University of the Witwatersrand, Human Research Ethics Committee is notified of any decision to discontinue the study and the reason stated.

* The Investigators authorised by this approval participate in this study. Additional Investigators shall be submitted to the University of the Witwatersrand, Human Research Ethics Committee for approval prior to their participation in the study.

* In the event of an authorised Investigator ceasing to participate in the study, the University of the Witwatersrand, Human Research Ethics Committee must be informed and the reason for such cessation given.

2. PRINCIPLES OF INFORMED CONSENT:

* The University of the Witwatersrand, Human Research Ethics Committee requires that in all studies, the Principles of Informed Consent are adhered to. This applies to volunteers as well as patients.

3. PROGRESS REPORTS:

* The University of the Witwatersrand, Human Research Ethics Committee requests that the MCC Progress Reports be submitted twice a year either in March and September or six monthly from start of study to the HREC Secretariat Office - 011 274 9281 and a report of the final results, at the conclusion of the study. (IF APPLICABLE)

4. REIMBURSEMENT TO PATIENTS FOR TRANSPORT:

* The Human Research Ethics Committee: (Medical) is in agreement that reimbursement per visit is according to the Medicines Control Council of SA and that reimbursement should be appropriate according to the situation.

5. TRANSPORT AND STORAGE OF BLOOD AND TISSUE SAMPLES IN SOUTH AFRICA:

* If blood specimens are to be stored for future analysis and is planned that such analysis will be done outside Wits, then the blood must be stored at a facility in South Africa agreed with the relevant IRB, with release of sub-samples only once projects have been approved by the local Research Ethics Committee applicable to where the analysis will be done as well as by the Wits Human Research Ethics Committee: (Medical).

6. GENETIC TESTING

* The Human Research Ethics Committee: Medical; will not approve open-ended genetic testing as this does not fit the Human Research Ethics Committee criteria.

7. GOOD CLINICAL PRACTICE

The South African Department of Health, Medicines Control Council requires Good Clinical Practice (GCP) Training for all Investigators in Clinical Trials, and that GCP training be renewed every three (3) years.

As yet, there are no National Guidelines for the content of GCP courses. Until these are available the Wits Human Research Ethics Committee (Medical) will note courses completed by Investigators without approval of the content of the individual courses.

8. THE SUPPORTING APPROVAL DOCUMENTS ARE ATTACHED:

8.1 Ethics Approval Form signed by the Chairperson of the HREC - Kindly return the copy of the Approval Form signed by the Principal Investigator /(s) per fax: 011 274 9281 for our records (this is applicable with the initial Approval).

8.2 Protocol Review Committee Approval Signature page signed by the Acting Chairperson of the PRC.

8.3 List of members present at the HREC meeting held as per INDEPENDENT ETHICS COMMITTEE APPROVAL FORM

9. WE AWAIT YOUR RESPONSES AS REQUESTED: Ensure to have these documents forwarded at the earliest for the HREC records.

* MCC Approval letter and/or letter of Notification before the above study may commence / or where an Amendment may be implemented (IF MCC APPROVAL / NOTIFICATION IS APPLICABLE). It remains the responsibility of the Principal Investigator and/or Sponsor to ensure that the relevant approvals are in place.
* Copy of Independent Ethics Declaration Approval Form signed by the Principal Investigator. (this is applicable with the initial Approval).
* Kindly forward the above to the undersigned at fax: 011 274 9281 at your earliest convenience.

The above has been noted for the Ethics Committee information and records.

KINDLY FORWARD TO THE RELEVANT INVESTIGATORS / CRA / SPONSOR / STUDY CO-ORDINATORS - WHERE APPLICABLE

Regards,

PROF PETER CLEATON-JONES

For and on behalf of the Human Research Ethics Committee: (Medical)

University of the Witwatersrand, Johannesburg

Human Research Ethics Committee: (Medical)
United States FWA Registered No IRB 00001223
South African National Health Research Ethics Council Registration: REC-250208-004

SECRETARIAT: Suite 189, Private Bag x2600, Houghton 2041, South Africa Tel: +27-11-274 9200 Fax: +27-11-274 9281

Dr C Cutland

FAXED & COURIERED

02 December 2015

Respiratory and Meningeal Pathogens Researc
11th Floor, Central West Wing, Nurses Residen
Chris Hani Baragwanath Hospital
Bertsham
2013

Fax: 086 674 0564

Dear Dr Cutland,

PROTOCOL: POST-MORTEM - MINIMALLY INVASIVE AUTOPSY (MIA) TO ESTABLISH CAUSE-OF-DEATH IN UNDER-5 CHILDREN IN A HIGH HIV-PREVALENCE SETTING

ETHICS REFERENCE NO: 150215

RE : APPROVAL FOR PROTOCOL AMENDMENT VERSION 5.0 DATED 12 NOVEMBER 2015

We acknowledge receipt of your letter dated 25 November 2015 with the following documentation pertaining to the above-captioned trial.

Amendment Date:	12-Nov-2015	Amendment Version:	Version 5.0
Amendment Number:		Received Date:	25-Nov-2015

The following has been approved by the Wits Human Research Ethics Committee: (Medical):

* Post mortem study - Protocol Version 5.0 dated 12 November 2015 (tracked and clean)
* Information Leaflet and Informed Consent Form Transport, Storage and Future Use of Samples, Version 2.0 dated 25 November 2015 (tracked and clean)
* Informed Consent - Minimally Invasive Autopsy Study in cause of death in children under-5 (Verbal Autopsy), Version 1.0 dated 12 November 2015 (clean only)
* Previously approved Informed Consent Form, Version 4.0 dated 28 September 2015 (clean only)

The site apply for an ethics amendment to be able to include the following changes:

1. Undertake verbal autopsies at least one month after the death of a child. The verbal autopsy will coincide with the result feedback to the family and a grief counsellor will be present. The verbal autopsy will give the researchers a complete picture of events leading up to the death of a child which are not always captured in the hospital notes. Also, for children that die in the community or on way to hospital without any clinical notes, the verbal autopsy will better inform us on events leading up to the death of the child. The data from the verbal autopsy will complement the clinical notes and laboratory findings to assign a final cause of death to the cases.

2. Take photographs of obvious malformations, enlarged lymph nodes or lesions on the body. This will aid in making the final diagnosis. If photographs of the face are taken, the eyes will be covered so that the child cannot be identified.

3. Allow for genetic testing to confirm genetic abnormalities as well as genetic traits. This would assist us in allocating a cause of death, particularly in still births. At the moment, while we note obvious malformations associated with certain genetic disorders, we are not able to confirm this for a fact. Also, we have had a case in which the attending physicians wanted to confirm a diagnosis of an inborn error of metabolism.

Ethics Approval Date: 02 December 2015

The University of the Witwatersrand, Human Research Ethics Committee Approval Granted for the above mentioned study is valid for five years. Where required by Sponsor to have approval on a more frequent basis it remains the responsibility of the Sponsor and Investigator to apply for continuing review and approval, or for the duration of the Trial.

1. THIS APPROVAL IS SUBJECT TO THE FOLLOWING PROVISOS:

* A copy of the MCC Approval and/or MCC Notification letter must be submitted to the Ethics Regulatory Office Secretariat before the study commences / or where an Amendment may be implemented (IF MCC APPROVAL / NOTIFICATION IS APPLICABLE). It remains the responsibility of the Principal Investigator and/or Sponsor to ensure that the relevant approvals are in place.

* The study is conducted according to the protocol submitted to the University of the Witwatersrand, Human Research Ethics Committee. Any amendments to the protocol must first be submitted to the Human Research Ethics Committee for approval.

* During the study, the University of the Witwatersrand, Human Research Ethics Committee is informed immediately of :

- Any Unexpected Serious Adverse Events or Unexpected Adverse Drug Reactions, which, in the Investigator and/or the Sponsor's opinion are suspected to be related to the study drug. (Refer to POL-IEC-001 and SOP-IEC-005, Item 3.4).
- Any data received during the trial which, may cast doubt on the validity of the continuation of the study .

* The University of the Witwatersrand, Human Research Ethics Committee is notified of any decision to discontinue the study and the reason stated.

* The Investigators authorised by this approval participate in this study. Additional Investigators shall be submitted to the University of the Witwatersrand, Human Research Ethics Committee for approval prior to their participation in the study.

* In the event of an authorised Investigator ceasing to participate in the study, the University of the Witwatersrand Human Research Ethics Committee must be informed and the reason for such cessation given.

2. PRINCIPLES OF INFORMED CONSENT:

* The University of the Witwatersrand, Human Research Ethics Committee requires that in all studies, the Principles of Informed Consent are adhered to. This applies to volunteers as well as patients.

3. PROGRESS REPORTS:

* The University of the Witwatersrand, Human Research Ethics Committee requests that the MCC Progress Reports be submitted twice a year either in March and September or six monthly from start of study to the HREC Secretariat Office - 011 274 9281 and a report of the final results, at the conclusion of the study. (IF APPLICABLE)

4. REIMBURSEMENT TO PATIENTS FOR TRANSPORT:

* The Human Research Ethics Committee: (Medical) is in agreement that reimbursement per visit is according to the Medicines Control Council of SA and that reimbursement should be appropriate according to the situation.

5. TRANSPORT AND STORAGE OF BLOOD AND TISSUE SAMPLES IN SOUTH AFRICA:

* If blood specimens are to be stored for future analysis and is planned that such analysis will be done outside Wits, then the blood must be stored at a facility in South Africa agreed with the relevant IRB, with release of sub-samples only once projects have been approved by the local Research Ethics Committee applicable to where the analysis will be done as well as by the Wits Human Research Ethics Committee: (Medical).

6. GENETIC TESTING

* The Human Research Ethics Committee: Medical; will not approve open-ended genetic testing as this does not fit the Human Research Ethics Committee criteria.

7. GOOD CLINICAL PRACTICE

The South African Department of Health, Medicines Control Council requires Good Clinical Practice (GCP) Training for all Investigators in Clinical Trials, and that GCP training be renewed every three (3) years.

As yet, there are no National Guidelines for the content of GCP courses. Until these are available the Wits Human Research Ethics Committee (Medical) will note courses completed by Investigators without approval of the content of the individual courses.

8. THE SUPPORTING APPROVAL DOCUMENTS ARE ATTACHED:

8.1 Ethics Approval Form signed by the Chairperson of the HREC - Kindly return the copy of the Approval Form signed by the Principal Investigator /(s) per fax: 011 274 9281 for our records (this is only applicable with the Initial Approval).

.

The above has been noted for the Ethics Committee information and records.

. ***KINDLY FORWARD TO THE RELEVANT INVESTIGATORS / CRA / STUDY CO-ORDINATORS***

Regards,

PROF PETER CLEATON-JONES

For and on behalf of the Human Research Ethics Committee: (Medical)

Appendix 5: Paper 1- Pathirana et al (2019)

Clinical Infectious Diseases

MAJOR ARTICLE

Prevalence of Congenital Cytomegalovirus Infection and Associated Risk of In Utero Human Immunodeficiency Virus (HIV) Acquisition in a High-HIV Prevalence Setting, South Africa

Jayani Pathirana,[1,2] Michelle Groome,[1,2] Jeffrey Dorfman,[1,2] Gaurav Kwatra,[1,2] Suresh Boppana,[3,4] Clare Cutland,[1,2] Stephanie Jones,[1,2] and Shabir A. Madhi[1,2]

[1]Medical Research Council, Respiratory and Meningeal Pathogens Research Unit, and [2]Department of Science/National Research Foundation, Vaccine Preventable Diseases, Faculty of Health Science, University of the Witwatersrand, Johannesburg, South Africa; and Departments of [3]Pediatrics and [4]Microbiology, University of Alabama School of Medicine, Birmingham

Background. There is a paucity of data on the burden of congenital cytomegalovirus (cCMV) infections in low- and middle-income countries, including their association with maternal human immunodeficiency virus (HIV) infections. We investigated the prevalence of cCMV in a patient population with a high rate of HIV and antiretroviral therapy (ART) use during pregnancy in Soweto, Johannesburg.

Methods. Saliva from neonates were screened for cytomegalovirus (CMV) infection by polymerase chain reaction (PCR) at birth. Additional saliva and urine samples were tested within 3 weeks of birth to confirm positive saliva results. HIV PCR testing was done on the whole blood of HIV-exposed neonates. Maternal and neonatal data were extracted from clinical records.

Results. Of 2685 neonates screened for cCMV, 828 (31%) were born to HIV-infected women, 95% of whom (790/828) were on ART at delivery. The overall prevalence of cCMV was 2.5% (95% confidence interval [CI] 1.9–3.2), with significantly higher cCMV prevalence in HIV-exposed neonates (5.2%, 95% CI 3.8–6.9) than HIV-unexposed neonates (1.4%, 95% CI 0.9–2.0). The risk of in utero HIV infection was 20-fold greater (odds ratio 20.1, 95% CI 6.09–66.46) in HIV-exposed, cCMV-infected neonates, and this increased risk was not associated with the maternal CD4+ T-cell count or the maternal duration of ART.

Conclusions. The prevalence of cCMV in our setting is substantially higher than the global estimate of 0.64%, partly due to the increased susceptibility for cCMV in HIV-exposed neonates. The significantly increased risk of in utero HIV infection in neonates with cCMV indicates that CMV coinfection plays a major role in the residual burden of in utero HIV transmission, even in the era of ART.

Keywords. congenital cytomegalovirus; HIV infection; risk factors; Africa; prevalence.

Cytomegalovirus (CMV) is the most common cause of congenital infection worldwide. The overall rate of congenital CMV infection (cCMV) is estimated at 0.64% (range 0.2–6.1%), with higher rates reported in low- and middle-income countries [1–3]. In children, cCMV is a leading cause of neurodevelopmental delay and nongenetic sensorineural hearing loss [1, 4–6]. The reported rates of cCMV are mostly from Western countries in Europe, the United States, and South America, with limited population-based studies from sub-Saharan Africa or settings with high human immunodeficiency virus (HIV) prevalences in pregnant women [7].

Early studies have shown that HIV-infected neonates are at significantly higher risk for cCMV compared to HIV-exposed, uninfected infants (10.3% vs 2.2%, $P < .001$) [8]. In the pre-antiretroviral therapy (pre-ART) era, HIV-infected infants with cCMV coinfections have also been shown to have accelerated progressions of HIV disease, neurological deterioration, and increased mortality [9]. In a study from France, the use of highly active ART by HIV-infected pregnant women was associated with reduced rates of cCMV in HIV-exposed neonates, from 3.5% (pre-ART era) to 1.2% [8]. In a study from Cape Town, South Africa, 2.9% of 748 HIV-exposed neonates of women on prenatal ART were CMV infected at birth [10].

Although combination ART has contributed to a dramatic decline in mother-to-newborn HIV-1 transmission rates, to <2% [11], the prevalence of HIV-1 infections in high-burden settings has remained stable over the past decade [12]. In South Africa, the prevalence of HIV infections in pregnant women has stabilized at approximately 30% over the past decade, while early vertical HIV transmission rates have declined to 1.1%

Received 30 October 2018; editorial decision 26 December 2018; accepted 5 January 2019; published online January 7, 2019.

Correspondence: J. Pathirana, Respiratory and Meningeal Pathogens Research Unit, 11th Floor, Central-West Wing, Chris Hani Baragwanath Academic Hospital, Chris Hani Road, Soweto, 2013, South Africa (pathiranaj@rmpru.co.za).

Clinical Infectious Diseases® 2019;69(10):1789–96

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DOI: 10.1093/cid/ciz019

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[12]. There are no data directly comparing cCMV in HIV-exposed and HIV-unexposed neonates from a high HIV-1 and CMV seroprevalence setting, such as South Africa. The aim of this study was to determine the rates and risk factors for cCMV in HIV-exposed and HIV-unexposed infants in an urban, middle- to low-income South African population with a maternal HIV-1 prevalence of 30%, where HIV-infected women routinely receive ART.

METHODS

Study Setting and Population

This study was conducted at the Chris Hani-Baragwanath Academic Hospital in Soweto, South Africa, a low- to middle-income setting. Approximately 75% of all births in Soweto (n = 28 000 annually) occur at this hospital, with most remaining deliveries occurring at 1 of 7 midwife-operated maternity units. Screening for HIV-1 is offered routinely to all pregnant women, including $CD4^+$ T-cell count and HIV-1 viral load quantification, either during antenatal care or labor and delivery. Since 2015, HIV-exposed neonates have been routinely investigated for HIV infection at birth, at 10 weeks of age, and at 6 weeks post–cessation of breastfeeding by polymerase chain reaction (PCR) and at 18 months of age by rapid HIV antibody testing [13]. South Africa implemented Option B+ prevention of mother-to-child HIV-1 transmission (PMTCT) in 2015, initiating HIV-infected pregnant women on lifelong ART (fixed-dose combination therapy of tenofovir, emtricitabine, and efavirenz), irrespective of their $CD4^+$ T-cell count at the time of the HIV-1 infection diagnosis [13].

Study Design

We undertook a prospective, cross-sectional study at the Chris Hani-Baragwanath Academic Hospital from May 2016 to December 2016 (ClinicalTrials.gov: NCT03722615). The study was nested within a larger cohort, in which mother-newborn dyads were enrolled beginning in June 2014 to investigate the serologic correlates of protection against invasive Group B Streptococcal disease (ClinicalTrials.gov: NCT02215226). Inclusion criteria included neonates born to mothers aged 18 years and older, residing in Soweto, who had consented to the parent study. Study participants were identified during labor or the immediate postnatal period, between 0700h to 1900h from Monday to Friday each week. A saliva sample was collected from all enrolled newborns for CMV testing within 72 hours of birth. Saliva collection took place at least 1 hour after feeding, using a nylon flocked swab (Copan Diagnostics Inc., Murrieta, CA). Dried swabs were stored at 4°C until transportation to the laboratory, within 4 hours of collection, for processing and testing.

Mothers of neonates who screened positive for CMV infection at birth were contacted and requested to return for a confirmatory test within 3 weeks of birth. At that visit, saliva and urine samples were collected for CMV testing. Infants with positive repeat samples for CMV were defined as confirmed cCMV cases.

Maternal serum, collected at delivery, was tested for anti-CMV immunoglobin (Ig) M and G antibodies in women whose infants had confirmed cCMV (cases) and a subset of women whose infants were CMV uninfected (controls), and were matched in a 2:1 ratio (based on gender, gestation, and HIV exposure) and included in a longitudinal follow-up study for neurological outcomes, which is ongoing.

Maternal and neonatal data were collected by clinical record review. Maternal data included age, HIV-1 status, ART history, mid upper arm circumference, past obstetric history, and mode of delivery. Neonatal data included gestational age, birth weight, Apgar score, head circumference, birth HIV-1 PCR results, and history of hospitalization immediately after birth for any cause. Intergrowth-21 standards [14, 15] were used to classify neonates that were small for their gestational age (SGA; birth weight below 10th percentile for gestational age [16]) and had microcephaly (head circumference for age less than the 3rd percentile for gestational age [17]).

Sample Processing and Testing

Saliva and urine samples were processed and stored at the Respiratory and Meningeal Pathogens Research Unit laboratory. Saliva swabs were processed and stored at -70°C until testing using the PCR assay, as previously described [18]. Urine samples were separated into 3 aliquots and stored at -70°C for PCR testing. DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN), and real-time PCR was undertaken using the highly conserved, immediate early 2 exon region as the target sequence [19]. A cycle time cut-off of 38, corresponding to 5 copies cut off per reaction, was implemented to identify positive and negative samples. Quantitative serum IgG and IgM immunoassays were done using CMV IgM and IgG enzyme-linked immunosorbent assay detection kits (Euroimmun), according to the manufacturer's protocol.

Maternal $CD4^+$ T-cell count and HIV-1 viral load and newborn HIV-1 PCR testing was performed at the National Health Laboratory Service, as per standard of practice, and results were extracted from the National Health Laboratory Service corporate data warehouse, a central data repository of all public sector laboratory test results, or from medical records.

Statistical Analysis

Anticipating a cCMV rate of 2.9% in HIV-exposed neonates [10], a sample size of 831 HIV-exposed and 831 HIV-unexposed (1:1 ratio) neonates was estimated to provide 80% power to detect a 2.9-fold [10] difference in CMV prevalence between the groups. The number was further increased to 2376 neonates to identify at least 42 confirmed cCMV cases, each matched to 2

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CMV-uninfected controls, to include in the longitudinal study, which was aimed at evaluating neurological sequelae.

The prevalence of cCMV, including data stratified by maternal HIV-1 status, was calculated by the total number of confirmed cCMV cases and estimated cCMV cases in neonates that did not return for confirmatory testing (calculated by applying the rate of cCMV infections in confirmed cases to those that did not return for confirmatory testing), divided by the total number of neonates screened for cCMV infection at birth.

Maternal demographic and clinical characteristics between confirmed cCMV cases and neonates that tested negative for CMV at birth were compared by univariate logistic regression to determine odds ratios and *P* values. Covariates with a *P* value of .2 or less were included in a multivariate regression model by manual backward selection to determine independent maternal risk factors for cCMV and neonatal characteristics, presented as adjusted odds ratios (aOR) with *P* values. In HIV-exposed neonates, maternal $CD4^+$ T-cell count, maternal HIV-1 viral load, maternal ART duration, and rates of in utero HIV-1 infections were analyzed by univariate logistic regression. Maternal CMV sero-prevalence (anti-CMV IgM and IgG) was determined in mothers of confirmed cases and a subset of CMV-uninfected neonates. Continuous data was analyzed using Wilcoxon rank-sum tests.

All data were analyzed using Stata 13.0 (Stata Corp, College Station, TX) and *P* values of <.05 were considered statistically significant.

Study Approval

The Human Research Ethics Committee of the University of Witwatersrand, Johannesburg, South Africa (HREC M151161) approved this study. Mothers provided written informed consent for screening their neonates for CMV, and a separate written consent was obtained for infant participation in the longitudinal study.

RESULTS

Between May and December 2016, saliva samples were collected from 2685 neonates at a mean of 17 ± 65 hours after birth, including 828 (30.8%) HIV-exposed and 1856 HIV-unexposed neonates (Figure 1). Of the 91 neonates with a positive PCR test for CMV at birth, 62 (68%) of mothers consented to confirmatory testing. The mean age at time of confirmatory sample collection was 9.3 ± 6.1 days. There were 46 infants (74%) confirmed as cCMV cases. In 13 (21%), the confirmatory saliva and urine were negative for CMV (unconfirmed), and in 3 (4.8%), the repeat saliva PCR was negative but the urine sample was not available for testing (CMV status unknown; Figure 1). There were no differences in maternal and neonatal characteristics between neonates that had confirmatory testing done and those that did not return for confirmatory testing (n = 29, data not shown).

Maternal and Newborn Characteristics

The median maternal age at delivery was 27.5 years (interquartile range [IQR] 23.1–32.6, Table 1). Women aged 18–24 years (aOR 2.3, 95% confidence interval [CI] 1.05–5.10), HIV-infected women (aOR 4.3, 95% CI 2.3–8.0), and women delivering vaginally (aOR 1.9, 95% CI 1.04–3.39) had greater risks of delivering an infant with cCMV (Table 1).

The overall prevalence of cCMV was 2.5% (95% CI 1.9–3.2), adjusted for the estimated cCMV rate in participants that did not return for confirmatory testing. The prevalence of cCMV was significantly higher in HIV-exposed infants (5.2%, 95% CI 3.8–6.9), compared to HIV-unexposed infants (1.4%, 95% CI 0.9–2.0; aOR 3.5, 95% CI 1.93–6.41; Figure 1; Table 2).

There was a single cCMV-infected neonate with microcephaly (2.2%) and 11 (23.9%) neonates with a history of jaundice at birth, which was not further investigated or treated amongst the confirmed cCMV cases. Neonates with cCMV were significantly more likely to be SGA (aOR 2.1, 95% CI 1.01–4.33) and very preterm (<32 weeks gestation; aOR 3.5, 95% CI 1.02–11.96; Table 2). There was no significant association of cCMV with gender, head circumference at birth, Apgar score, or rates of hospitalization immediately after birth (Table 2).

Overall, 95% (n = 790) of HIV-infected women were on ART at or prior to delivery: 93% (737) of these women were on combined tenofovir, emtricitabine, and efavirenz and the remaining women were on highly active ART, with no further details available. Among all HIV-exposed neonates, 13 (1.75%) were HIV-1 infected and those with cCMV were 20-fold (odds ratio 20.1, 95% CI 6.09–66.46) more likely to be infected with HIV-1 in utero (5/27, 18.5%) than CMV-uninfected infants (8/716, 1.1%; Table 3). Of the HIV-1 infected neonates, the median maternal ART duration at delivery was not significantly different between cCMV-infected (123, IQR 75–156 days) and -uninfected neonates (168, IQR 152–208 days). The gestational age at the time of maternal ART initiation and the severity of maternal immunosuppression based on CD4+ T-cell counts and HIV-1 viral loads were not significantly different between cCMV-infected and -uninfected neonates (Table 3).

Maternal Cytomegalovirus Serology

All women (100%) who were tested for CMV serology were CMV IgG positive, and 92% were IgM negative.

DISCUSSION

In this population-based study in Soweto, the overall prevalence of cCMV was 2.5%, a higher rate than that reported in high-income countries (0.2–2.0%) but within the range of prevalence reported in low- and middle-income countries (0.6–6.1%) [3]. The high prevalence in our setting is, at least in part, due to the greater risk of cCMV in HIV-exposed neonates (5.2%), compared to HIV-unexposed neonates (1.4%). Few other studies have directly compared cCMV infections in HIV-exposed and

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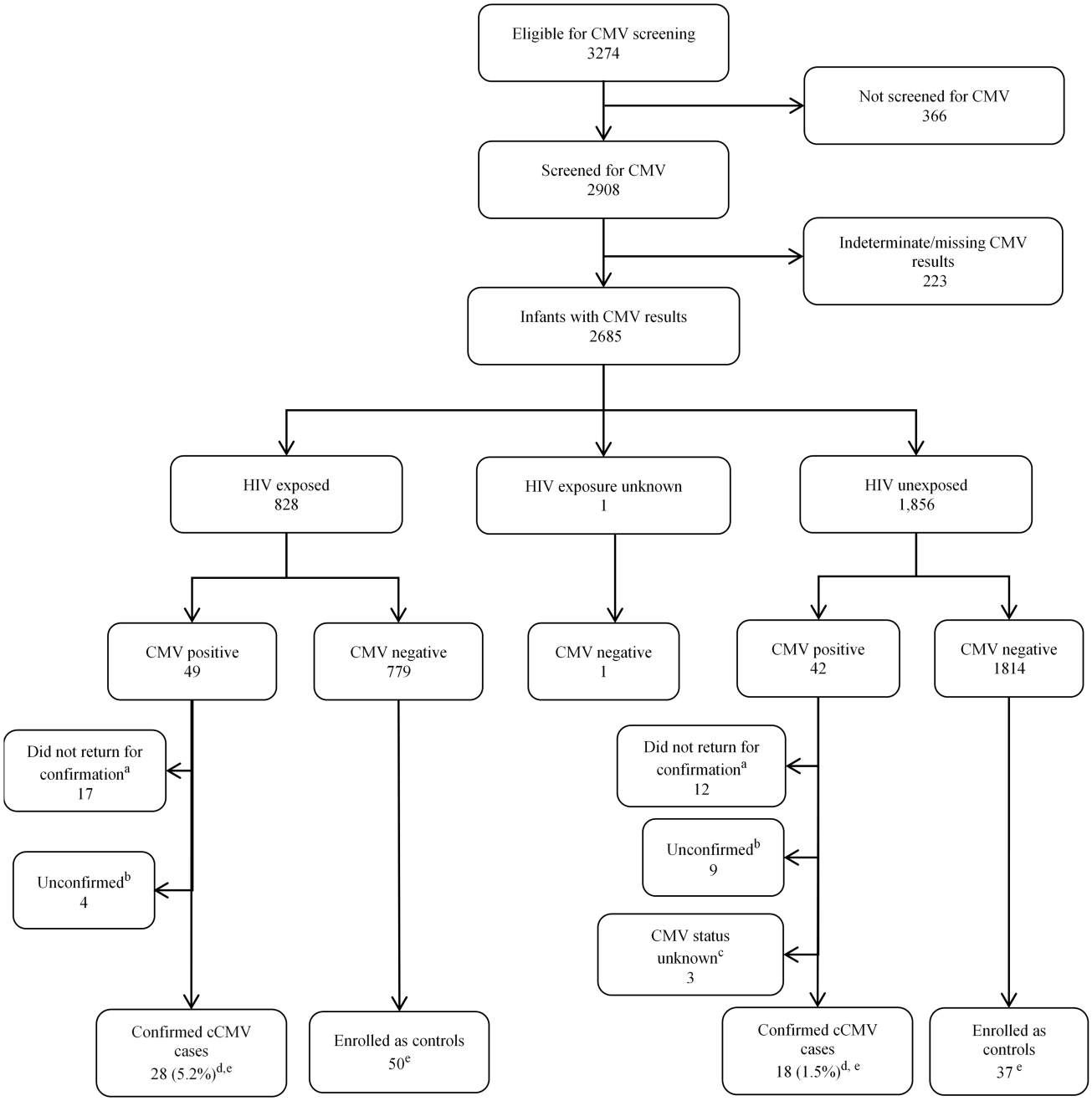


Figure 1. Flow chart. [a]N=10 declined consent; N=1 died between screening and confirmation, N=18 lost to follow up (LTFU). [b]Unconfirrned: screening saliva CMV positive but confirmatory saliva and/or urine negative for CMV. [c]CMV status unknown: screening saliva CMV positive, confirmatory saliva CMV negative and urine sample not available for testing. [d]Prevalence adjusted for neonates that did not return for confirmation. [e]Maternal blood of cases and controls tested for CMV IgG and IgM serology.

-unexposed neonates. A study of high-risk neonates admitted to the intensive care unit in a Zambian referral hospital found a high cCMV prevalence, of 11.4% (9/79; 95% CI 6.1%–20.3%) in HIV-exposed infants, compared to 2.1% (6/293; 95% CI 0.8%–4.6%) in HIV-unexposed infants [20]. However, the selected nature of the study patient population makes it difficult to compare the findings from that study to our findings. It can be speculated that the increased rate of cCMV in HIV-exposed neonates may be due to more frequent CMV reinfection or reactivation in their mothers, the waning of protective immunity, or a reduced trans-placental transfer of protective antibodies. Although the prevalence of cCMV in HIV-exposed infants in our study was higher than previously reported in Cape Town, South Africa (2.9%), it is possible that differences in the study population, such as race,

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Table 1. Maternal Characteristics of Confirmed Congenital Cytomegalovirus-Infected and Cytomegalovirus-Uninfected Infants

	Total (N = 2639)	cCMV-Infected Infants (n = 46)	CMV-Uninfected Infants (n = 2593)	OR[a] (95% CI)	*P* Value	aOR[b] (95% CI)	*P* Value
Maternal age, in years	n = 2631	n = 46	n = 2585				
Median age, n (IQR)	27.5 (23.1–32.6)	25.7 (22.7–29.7)	27.5 (23.1–32.7)	…	.079	…	…
18–24, n (%)	937 (35.61)	19 (41.30)	918 (35.51)	1.8 (.85– 3.81)	.123	**2.3 (1.05–5.10)**	**.039**
25–30, n (%)	725 (27.56)	16 (34.78)	709 (27.43)	1.9 (.91–4.26)	.087	2.11 (.96–4.63)	.062
>30, n (%)	969 (36.83)	11 (23.91)	958 (37.06)	1	…	1	…
Mode of delivery	n = 2632	n = 46	n = 2586				
Vaginal, n (%)	998 (37.92)	25 (54.35)	973 (37.63)	2.0 (1.1–3.54)	.023	**1.9 (1.04–3.39)**	**.037**
Caesarean, n (%)	1634 (62.08)	21 (45.65)	1613 (62.37)	1	…	1	…
Parity	n = 2627	n = 45	n = 2582				
0, n (%)	908 (34.56)	17 (37.78)	891 (34.51)	1.4 (.65–2.89)	.403	…	…
1, n (%)	843 (32.09)	16 (35.56)	827 (32.03)	1.4 (.65–2.96)	.389	…	…
≥2, n (%)	876 (33.35)	12 (26.67)	864 (33.46)	1	…	…	…
Abortion history[c]	n = 2615	n = 45	n = 2570				
None, n (%)	2192 (83.82)	38 (84.44)	2154 (83.81)	1	…	…	…
Past abortions, n (%)	423 (16.18)	7 (15.56)	416 (16.19)	0.95 (.42–2.15)	.909	…	…
Mid-upper arm circumference	n = 2592	n = 46	n = 2546				
<23 cm, n (%)	95 (3.67)	3 (6.52)	92 (3.61)	4.2 (.94–19.47)	.059	3.5 (.74–16.07)	.114
23–26 cm, n (%)	567 (21.88)	13 (28.26)	554 (21.76)	3.09 (1.0–9.52)	.05	2.4 (.74–7.48)	.147
27–32 cm, n (%)	1400 (54.01)	26 (56.52)	1374 (53.97)	2.5 (.86–7.16)	.091	2.1 (.74–6.23)	.162
≥33 cm, n (%)	530 (20.45)	4 (8.7)	526 (20.66)	1	…	1	…
HIV status	n = 2638	n = 46	n = 2592				
Infected, n (%)	807 (30.59)	28 (60.87)	779 (30.05)	3.6 (1.99–6.58)	<.001	**4.3 (2.3–8.0)**	**<.001**
Uninfected, n (%)	1831 (69.41)	18 (39.13)	1813 (69.95)	1	…	1	…

Values in bold indicate independent risk factors for cCMV infection.

Abbreviations: aOR, adjusted odds ratio; cCMV, congenital cytomegalovirus infection; CI, confidence interval; CMV, cytomegalovirus; HIV, human immunodeficiency virus; IQR, interquartile range; OR, odds ratio.

[a]ORs are from univariate logistic regression.

[b]Covariates with a *P* value of .2 or less in the univariate analysis (OR) were included in a multivariate logistic regression model to determine independent maternal risk factors for cCMV.

[c]History of induced or spontaneous abortions.

socioeconomic status, and living conditions, may explain the difference in prevalence [10].

A strong association of cCMV with in utero transmission of HIV-1 was observed. Among HIV-exposed neonates, 18.5% (5/27) of infants with cCMV were HIV-1 PCR–positive at birth, compared to 1% (8/716) of cCMV-uninfected neonates. This finding could be of great importance, as it suggests the possibility that cCMV coinfection may account for a large fraction of the residual maternal to fetal HIV-1 transmission in populations with efficient PMTCT programs. There was no significant difference in ART durations between mothers of HIV-1 and cCMV coinfected neonates and HIV-infected, cCMV-uninfected neonates ($P = .6$), similar to the findings in the French perinatal cohort [8]. The lack of association, however, may be due insufficient power to make this comparison, because of the low rate of in utero HIV-1 infections in our study and the French study.

A series of possible mechanisms have been proposed, in which CMV infections of T-cells potentiate their susceptibility to HIV-1 infection or enhance HIV-1 replication [21]. Johnson et al [19] demonstrated that the in vitro stimulation of cord blood mononuclear cells by CMV upregulates C-C chemokine receptor type 5 (CCR5) expression levels on memory T-cells, enhancing their susceptibility to HIV-1 infection. Polymorphisms in the Toll-like receptor 9, 1635 locus have been described as associated with HIV-1 acquisition and progression [22]. Having 1 or more copies of the 1635A allele was also associated with increased CMV acquisition in HIV-infected Kenyan infants (42%), as compared to infants with the 1635GG genotype (11%; $P = .03$) [22]. This suggests a shared mechanism for HIV-1 and CMV infection in infants. As infants continue to acquire HIV-1 perinatally, even with the success of PMTCT programs, albeit at a much lower rate, further investigation of the pathogenesis of in utero CMV and HIV-1 acquisition in the ART era could help explain the causes for the residual burden of in utero mother-to-newborn HIV-1 acquisition.

Maternal $CD4^+$ T-cell counts were not associated with cCMV infections in HIV-exposed neonates in our study. This is in contrast to the study in Cape Town, where a significantly higher prevalence of cCMV was observed in infants of mothers whose $CD4^+$ T-cell counts, mostly measured in the first and second trimesters of pregnancy, were <200 cells/μL and where the T-cell count was the only factor independently associated with cCMV [23]. This lack of association in our study may be due to the fact that the majority of women in our study were started on ART earlier in pregnancy, with considerable immune recovery by the third trimester

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Table 2. Characteristics of All Confirmed Congenital Cytomegalovirus–Infected and Uninfected Infants

	Total (N = 2652)	cCMV-Infected Infants (n = 46)	CMV-Uninfected Infants (n = 2606)	OR[a] (95% CI)	*P* Value	aOR[b] (95% CI)	*P* Value
Gender	n = 2639	n = 46	n = 2593				
Male, n (%)	1276 (48.35)	21 (45.65)	1255 (48.4)	0.9 (.50–1.61)	.712	…	…
Female, n (%)	1363 (51.6)	25 (54.35)	1338 (51.6)	1	…	…	…
Gestation	n = 2638	n = 46	n = 2592				
<32 weeks, n (%)	65 (2.46)	3 (6.52)	62 (2.39)	3.0 (.90–10.02)	.075	**3.5 (1.02–11.96)**	**.046**
32–<34 weeks, n (%)	74 (2.81)	2 (4.35)	72 (2.78)	1.7 (.41–7.30)	.462	1.8 (.41–7.70)	.436
34–<37 weeks, n (%)	359 (13.61)	7 (15.22)	352 (13.58)	1.2 (.54–2.80)	.619	1.2 (.52–2.71)	.692
≥37 weeks, n (%)	2140 (81.12)	34 (73.91)	2106 (81.25)	1	…	1	…
Birth weight	n = 2636	n = 46	n = 2590				
<1500 g, n (%)	36 (1.37)	3 (6.52)	33 (1.27)	6.4 (1.87–22.02)	.003	…	…
1500–<2500 g, n (%)	308 (11.68)	11 (23.91)	297 (11.47)	2.6 (1.30–5.24)	.007	…	…
≥2500 g, n (%)	2292 (86.95)	32 (69.57)	2260 (87.26)	1	…	…	…
Small for gestation[c]	n = 2623	n = 46	n = 2577				
Yes, n (%)	315 (12.01)	10 (21.74)	305 (11.84)	2.1 (1.02–4.21)	.045	**2.1 (1.01–4.33)**	**.046**
No, n (%)	2308 (87.99)	36 (78.26)	2272 (88.16)	1	…	1	…
Microcephaly[d]	n = 2614	n = 46	n = 2568				
Yes, n (%)	101 (3.86)	3 (6.52)	98 (3.82)	1.758 (0.54–5.77)	.352	…	…
No, n (%)	2513 (96.14)	43 (93.48)	2470 (96.18)	1	…	…	…
Apgar score	n = 2576	n = 44	n = 2532				
<7, n (%)	14 (0.54)	1 (2.27)	13 (0.51)	4.5 (0.58–35.22)	.151	…	…
≥7, n (%)	2562 (99.46)	43 (97.73)	2519 (99.49)	1	…	…	…
HIV exposure	n = 2639	n = 46	n = 2592				
HIV exposed, n (%)	807 (30.59)	28 (60.87)	779 (30.05)	3.62 (1.99–6.58)	<.001	**3.5 (1.93–6.41)**	**<.001**
HIV unexposed, n (%)	1831 (69.41)	18 (39.13)	1813 (69.95)	1	…	1	…
Hospitalized at birth	n = 2639	n = 46	n = 2593				
Yes, n (%)	292 (11.06)	9 (19.57)	283 (10.91)	1.98 (0.95–4.16)	.069	…	…
No, n (%)	2347 (88.94)	37 (80.43)	2310 (89.09)	1	…	…	…

Values in bold indicate independent risk factors for cCMV infection.

Abbreviations: aOR, adjusted odds ratio; cCMV, congenital cytomegalovirus infection; CI, confidence interval; CMV, cytomegalovirus; HIV, human immunodeficiency virus; OR, odds ratio.

[a]ORs are from univariate logistic regression.

[b]Covariates with a *P* value of .2 or less in the univariate analysis were included in a multivariate logistic regression model to determine independent maternal risk factors for cCMV.

[c]Birth weight for GA <10th percentile.

[d]Head circumference for age less than the 3rd percentile for gestational age.

and delivery, resulting in higher CD4+ T-cell counts. Similarly, HIV viral loads tested later in pregnancy were not associated with the risk of CMV transmission from mother to fetus. Since a longer duration of ART did not reduce the risk of cCMV transmission in our study, other factors may play a role in CMV transmission. These factors include the interaction of HIV-1 and CMV at the maternal-fetal placental interface and the effect of exposure to in utero ART, which may cause a range of phenotypical and functional immunological changes in HIV-exposed fetuses, facilitating increased cCMV transmission in HIV-infected women [24].

The majority of cCMV-infected neonates were asymptomatic at birth, as observed in other epidemiological studies in high maternal CMV seroprevalent settings. Congenital CMV infection was associated with SGA and very preterm births in our study. Similar findings were reported in a prospective Brazilian study on cCMV prevalence in a high maternal CMV seroprevalence and low-income setting, with a higher frequency of SGA neonates in those with than without cCMV (26.4% [n = 23/87] vs 10.7% [n = 852/7960], respectively) [4]. We did not, however, define being SGA as a symptom of cCMV, as we did not systematically collect other maternal factors associated with an infant being SGA or having a preterm birth, such as pregnancy-induced hypertension, alcohol and smoking practices, or chronic maternal conditions other than HIV-1 infection. Considering the impact of a low birth weight on the risks of morbidity and mortality in African infants [25], the potential association of cCMV contributing to these underlying conditions merits further investigation.

Limitations of our study include a high number of cCMV-positive infants at screening that could not be enrolled in the follow-up to confirm cCMV (n = 29). However, there were no significant differences in maternal or newborn characteristics between this group and those who returned for follow-up. In addition, positive screening results were not confirmed in follow-up in 13 infants (0.48%). We followed the PCR protocol used in a previous study (Boppana et al [18]), which evaluated real-time PCR on newborn saliva samples in a large, prospective, newborn CMV screening protocol that included 17 327

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Table 3. Characteristics of HIV-exposed, Confirmed Congenital Cytomegalovirus–Infected and Uninfected Infants

	Total (N = 807)	cCMV-Infected Infants[a] (n = 28)	CMV-Uninfected Infants (n = 779)	OR[a] (95% CI)	*P* Value
Infant HIV status	n = 743	n = 27	n = 716		
HIV exposed and infected, n (%)	13 (1.75)	5 (18.52)	8 (1.12)	**20.1 (6.09–66.46)**	**<.001**
HIV exposed but uninfected, n (%)	730 (98.25)	22 (81.48)	708 (98.88)	1	…
Maternal CD4+ count	n = 648	n = 19	n = 629		
Median, n (IQR)	432 (277–603)	415 (350–595)	432 (275–603)	…	.993
Severe: <200 cells/mm^3, n (%)	80 (12.35)	3 (15.79)	77 (12.24)	1.6 (.38–6.38)	.537
Advanced: 200–<350 cells/mm^3, n (%)	147 (22.69)	1 (5.26)	146 (23.21)	0.3 (.03–2.30)	.233
Mild: 350–<500 cells/mm^3	175 (27.01)	9 (47.37)	166 (26.39)	2.2 (.76–6.21)	.149
Not significant: ≥500 cells/mm^3	246 (37.96)	6 (31.58)	240 (38.16)	1	…
Maternal HIV viral load	n = 513	n = 3	n = 510		
Median N (IQR)	40 (0–469)	19 (0–262)	40 (0–470)	…	.547
<1000 copies/ml, n (%)	401 (78.17)	3 (100)	398 (78.04)	…	…
≥1000 copies/ml, n (%)	112 (21.83)	0 (0)	112 (21.96)	…	…
Gestation at ART initiation	n = 576	n = 23	n = 553		
Median ART duration, days (IQR)	172 (113–468)	148 (89–244)	173 (113–552)	…	.203
Preconception, n (%)	186 (32.29)	5 (21.74)	181 (32.73)	1	…
1st trimester, n (%)	49 (8.51)	4 (17.39)	45 (8.14)	3.2 (.83–12.47)	.091
2nd trimester, n (%)	227 (39.41)	8 (34.78)	219 (39.60)	1.3 (.43–4.11)	.629
3rd trimester, n (%)	104 (18.06)	5 (21.74)	99 (17.90)	1.8 (.52–6.47)	.349
Postnatal, n (%)	10 (1.74)	1 (4.35)	9 (1.63)	4.02 (.42–38.12)	.225

Values in bold indicate independent risk factors for cCMV infection.

Abbreviations: ART, antiretroviral therapy; cCMV, congenital cytomegalovirus infection; CI, confidence interval; CMV, cytomegalovirus; HIV, human immunodeficiency virus; IQR, interquartile range; OR, odds ratio.

[a]ORs are from univariate logistic regression.

infants, with the sensitivity and specificity of the dried saliva real-time PCR determined to be 97.4% and 99.9%, respectively.

We report a high prevalence of cCMV in HIV-exposed neonates, compared to HIV-unexposed neonates, and an increased rate of in utero HIV-1 infections in HIV-exposed neonates with concomitant cCMV, in the era of universal maternal ART, in a population-based study in South Africa. The continued risk of cCMV in HIV-exposed infants could possibly contribute to the higher morbidity and mortality rates in the first year of life, compared to HIV-unexposed infants [26, 27]. The impact of cCMV, as well as postnatally-acquired CMV infection, on these infants needs to be determined in natural history studies, with monitoring for long-term outcomes.

Notes

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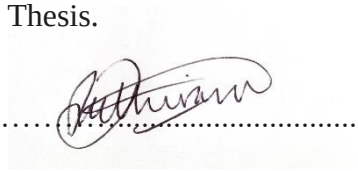
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	Name	**Signature**	**Date**
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2nd Author	Michelle Groome		06 February 2020
3rd Author	Jeffrey Dorfman		05 February 2020
4th Author	Gaurav Kwatra		07/Feb/2020
5th Author	Suresh Boppana		12 February 2020
6th Author	Clare Cutland		5th February 2020
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Appendix 9: Sample size calculation

SAMPLE SIZE CALCULATION

Objective 1

This sample size of objective 2 will detect an approx. ~2.6 fold difference in congenital CMV infection between HIV-exposed and HIV-unexposed infants if in fact the true prevalence of CMV in HIV-exposed is approx. 2.5-2.6%. The true prevalence however is expected to be more in the region of 2.9%

a) Power, fixed = 80%,
b) Alpha level, fixed = 5%
c) Prevalence of congenital CMV in HIV-unexposed neonates assumed, fixed = 1%
d) Prevalence of congenital CMV in HIV-exposed neonates assumed higher than HIV-unexposed => ranging from 2.5% to 3.5%

Power	Prevalence HIV-unexposed (%)	Prevalence HIV-exposed (%)	Fold difference	HIV-exposed (n)	HIV-unexposed (n)	Total population (N)
80%	1%	2.5%	2.5	1199	1199	2398
80%	1%	2.6%	2.6	1083	1083	2166
80%	1%	2.7%	2.7	986	986	1972
80%	1%	2.8%	2.8	903	903	1806
80%	1%	2.9%	2.9	831	831	1662
80%	1%	3.0%	3.0	769	769	1538
80%	1%	3.1%	3.1	714	714	1428
80%	1%	3.2%	3.2	666	666	1332
80%	1%	3.3%	3.3	624	624	1248
80%	1%	3.4%	3.4	586	586	1172
80%	1%	3.5%	3.5	552	552	1104

Objective 2

a) Power, fixed = 80%,
b) Alpha level, fixed = 5%
c) Prevalence Permanent Sequelae in cCMV-uninfected infants assumed, fixed = 1%
d) Prevalence Permanent Sequelae in CMV-infected infants assumed considerably higher than cCMV-uninfected => ranges from 7% to 20%
e) Allocation ratio cCMV-infected: cCMV-uninfected => 1:2

Power	Prevalence Sequelae uninfected (%)	Prevalence Sequelae infected (%)	Fold increase	cCMV-uninfected (n): controls	cCMV-infected (n): Cases	Total population (N)
80%	1.0%	7.0%	7.0	250	125	375
80%	1.0%	8.0%	8.0	205	102	308
80%	1.0%	9.0%	9.0	174	87	261
80%	1.0%	10.0%	10.0	150	75	225
80%	1.0%	11.0%	11.0	132	66	198
80%	1.0%	12.0%	12.0	117	58	175
80%	1.0%	13.0%	13.0	105	52	157
80%	1.0%	14.0%	14.0	96	48	144
80%	1.0%	15.0%	15.0	87	43	130
80%	1.0%	16.0%	16.0	81	40	121
80%	1.0%	17.0%	17.0	75	37	112
80%	1.0%	18.0%	18.0	69	34	103
80%	1.0%	19.0%	19.0	64	32	96
80%	1.0%	20.0%	20.0	60	30	90

Based on the prevalences' of 2.9% congenital CMV in HIV-exposed and 1% in HIV-unexposed and based on the 1:1 allocation ratio of objective 2, the number of CMV-infected babies required is estimated at 37. If we want to ensure 37 cases are attained and we are expecting a 10% loss to follow-up, the total number of cases needed is 42. Of these, approximately 31 congenital CMV cases (~1077 x 0.029) will be HIV-exposed and 11 cases (~1077 x 0.01) will be HIV-unexposed. A total of 2154 neonates will need to be screened to ensure the required number of congenital CMV cases are achieved.

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