CONGENITAL CMV IN A HIGH HIV PREVALENT SETTING, SOUTH AFRICA

PhD Protocol

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SYNOPSIS OF RESEARCH CONTINUED

Introduction

Cytomegalovirus (CMV) infection is the most common congenital infection worldwide and the leading cause of non-genetic sensorineural hearing loss (SNHL) in childhood[1]. Prevalence of congenital CMV infection ranges from 0.6 to 5% dependent on the population studied[1,2]. Up to 17% of all infected newborns are at risk of permanent neurologic sequelae, including SNHL; motor, cognitive and visual impairment; microcephaly; seizures and cerebral palsy[3,4]. Morbidity data on congenital CMV infections has mostly been from high income countries (HIC)[2]. It is necessary to establish whether congenital CMV infection in South African children presents the same public health impact as in high resource countries and determine the effect of HIV on CMV infection.

Aim

The aim of this project is to determine the epidemiology of congenital CMV infection and incidence of neurological sequelae until 12 months age in a high HIV prevalent setting in Soweto, Johannesburg.

Methods

This study will be nested within a larger cohort study of 35000 mother infant dyads to investigate a sero-correlate of protection against invasive GBS disease (HREC 140203) currently underway at Chris Hani Baragwanath Academic Hospital (CHBAH) in Soweto. A cross sectional study design screening 2200 mother-newborn pairs enrolled in V98_28OBTP that consent to this study will be undertaken identifying cases of congenital CMV by saliva PCR. A longitudinal cohort study following up the cases of congenital CMV matched to a CMV negative cohort of newborns will measure the incidence of and risk factors for neurological sequelae until infants are 12 months of age. To measure the association between congenital CMV infection and neonatal death and stillbirths, a longitudinal study on causes of death in under 5 children by Minimally Invasive Tissue Sampling (MITS), which is currently underway (HREC 150215), will include tissue processing for CMV. Maternal CMV seroprevalence will be determined by serological methods as well as the association between vaginal CMV shedding and congenital CMV infection.

These studies will identify CMV seroprevalence in pregnant women which is thought to be high (>70%) and will determine the prevalence of congenital CMV infection in Soweto through universal newborn screening which has only previously been done in symptomatic newborns at CHBAH. This study will also provide the first estimates on the incidence of SNHL and other neurological impairment in CMV infected newborns, adding to the current discussions on increasing resource allocation to universal newborn hearing screening. The measure of the effect of HIV exposure on the prevalence of congenital CMV and the burden of subsequent neurological disability will add to the growing global knowledge on congenital CMV in various settings impacting the prevention, diagnosis and management of infected infants.

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1 Introduction

Cytomegalovirus (CMV) infection is the most common congenital infection worldwide and the leading cause of non-genetic sensorineural hearing loss (SNHL)* in childhood [1]. Global prevalence of congenital CMV infection is estimated at 0.64%, varying across countries (0.2 – 6%) with higher prevalence's in lower and lower- middle income countries (LMIC) [1,2]. Seventeen percent of all infected infants are at risk of permanent neurological disability[3,4]. The impact of congenital CMV infection in neonates is not well recognised as it is mainly asymptomatic at birth with the inability to attribute neurological impairment in children retrospectively to *in-utero* CMV infection.

1.1 Biology and Immunology of CMV

Human CMV is a DNA virus of the Herpesviridae family[5] and viral transmission occurs through body fluids (blood, saliva, breast milk, vaginal fluid, semen and urine) spreading to multiple organs (kidney, liver, lung, brain)[6]. CMV demonstrates strain diversity and mixed infection with multiple strains in an individual is common, whilst immunity to CMV is strain specific[7,8]. Following primary infection, the virus establishes lifelong persistence[9] and is usually asymptomatic in the immunocompetent. Secondary infection occurs with reactivation of a latent virus or infection with a new strain in a previously CMV seropositive person[9]. Strain specific antibodies (IgM and IgG) and a broad T cell response is activated, limiting viral replication and preventing disease, although unable to completely eliminate the virus[10,11].

1.2 Epidemiology of Congenital CMV Infection

Maternal infection: Low socioeconomic status, black race, crowded living conditions and younger age are associated with higher CMV seroprevalence[1,2]. Consequently, maternal seroprevalence may be over 80% in LMIC [12]. A maternal CMV seroprevalence of 86.6% was found in a South African study[13] but is expected to vary with socioeconomic disparity. Primary infection in HIC commonly occurs in adulthood and in women of reproductive age, during

pregnancy[1,14]In lower resource settings CMV infection occurs at young age when exposed to infected maternal saliva, breast milk and because of over-crowded conditions[1,14].

Congenital infection: Viral transmission to the foetus can occur through the maternal-foetal placental interface increasing with gestational age. Foetal effects of infection are more severe if infected in the first 16 weeks of gestation when organs are developing and may lead to abortion and stillbirths[11,15] Transmission rates are higher during primary maternal infection (30 -35%) compared to women with secondary infection (1.4%)[2]. Counterintuitively, there is greater prevalence of congenital CMV in high CMV seroprevalent populations compared to low seroprevalent populations who are at higher risk of primary CMV infection. This is explained by there being more pregnant women at risk of CMV reactivation and reinfection in high seroprevalent populations coupled with other risk factors[2]. A recent systematic review of studies from LMIC, showed population based congenital CMV prevalence ranging from 0.6 – 6.1% (e.g. Panama 0.6%, Gambia 5.4%, China, 6.1%)[12] compared to 0.2-2.0% in HIC (Chile 0.2%, USA 1.2%, Finland 2.0%) [2,4].

Diagnosis of congenital CMV infection: CMV isolation by culture or positive polymerase chain reaction (PCR) test in urine, saliva, blood or tissue taken at biopsy within the first 2 weeks of life is diagnostic of congenital CMV[16]. Acquired CMV during delivery can be detected after 2 weeks incubation and CMV isolation beyond 2 weeks age cannot reliably be concluded as *in utero* infection[16]. CMV PCR in urine and/or saliva is favoured as the standard of diagnosing congenital infection[17].

Clinical significance and long term outcome of congenital CMV: Most newborns with congenital CMV are asymptomatic at birth (85-90%)[18]. Of the 10-15% of symptomatic newborns clinical manifestations range from mild, non-specific findings to multi-organ system involvement[18]. The most frequent signs are petechiae, jaundice and hepatosplenomegaly. Central nervous system (CNS) signs include microcephaly, hearing impairment, chorioretinitis and/or

optic atrophy and seizures [1,5,18]. Of the symptomatic neonates, about 50% are small for gestational age, 33% are born prematurely[18] and 5% die in the neonatal period[18].

Permanent neurological sequelae occurs in 40-58% of symptomatic and 14% of asymptomatic infants at birth[4]. Children with symptomatic birth infection may have multiple neurological sequelae including SNHL (30-60%), cerebral palsy and mental retardation (50-80%) visual impairment (20-35%) and neurodevelopmental behaviour disorders (30-60%)[4,19]. The most common sequela in infants asymptomatic at birth are SNHL (10%) with other complications occurring at a lower frequency: motor deficits (5%) and chorioretinitis (2%)[18].

Effect of HIV: An estimated 3% of congenital CMV infection occurs in HIV exposed infants [1,20] compared to 1% in HIV unexposed infants. HIV infected infants have an even higher prevalence of congenital CMV (10.3%) as found in a French perinatal cohort[21]. In this study, HIV and CMV co-infected newborns had a 3-fold greater risk of symptomatic congenital CMV infection at birth (23%) than HIV exposed but uninfected infants (6.7%)[21]. CMV co-infection may also contribute to HIV disease progression with increased risk of mortality and faster progression of CNS disease in survivors [1,22]. However, the incidence of vertical CMV transmission in HIV infected women decreased following introduction of antiretroviral therapy (ART) during pregnancy [21]. Data on congenital CMV infection among HIV exposed infants in sub-Saharan Africa are limited. A Kenyan study of infants born to mothers on perinatal Zidovudine, identified congenital CMV rates of 29% (4/14) in HIV-infected infants and 2.7% (1/37) in HIV exposed but uninfected infants[23]. Zambian newborns admitted to a referral neonatal unit had a congenital CMV prevalence of 3.8% (15/395) whilst prevalence in HIV exposed newborns was 11.4% (9/79)[24]. Although HIV prevalence in South African pregnant women is one of the highest in the world (29.9%)[25], a single study has evaluated the relationship between congenital CMV and HIV exposure. In Cape Town, Manicklal et al (2013) found a congenital CMV prevalence of 2.9% (22) in 748 HIV exposed babies[26]. Maternal CD4+ count <200 cells/µL was independently

associated with congenital CMV[26]. Clinical outcomes in these infants and prevalence of congenital CMV among HIV unexposed newborns were not investigated.

Perinatal and Postnatal CMV infection: Perinatal CMV infection is defined as CMV infection after 22 completed weeks (154 days) of gestation and seven completed days after birth. Postnatal CMV acquisition is CMV infection acquired any time after birth through consumption of breast milk from CMV seropositive, lactating mothers is common.Most term infants with perinatal CMV infection are asymptomatic, as maternally transferred CMV IgG antibody provides adequate protection against disease [27]. However, studies have identified a significant risk for low-birth-weight (LBW), premature infants for the development of CMV disease such as pneumonia, hepatitis or sepsis-like illness[27]. Data on severity and incidence of disease and long term impact in these infants is currently lacking requiring further study.

Current therapeutic options: Treatment for maternal and newborn CMV infection is limited. Anti-CMV hyperimmunoglobulin is efficacious in preventing transmission from primary maternal infections but its value in secondary infection is uncertain[2]. Some European countries offer universal maternal CMV screening or foetal anomaly ultrasonography to identify ventriculomegaly, intracerebral calcification and microcephaly[28]. Foetal infection is confirmed by amniocentesis, not commonly available in LMIC, and women may be offered termination of pregnancy. Targeted CMV screening in symptomatic newborns and/or fail universal hearing screening occurs in the USA[1]. Screening programmes have not been widely implemented due to lack of awareness, high costs and lack of rapid diagnostic tests[29].

Ganciclovir (GCV) or Valganciclovir is administered to symptomatic CMV infected neonates, associated with improved audiologic outcomes[1]. However GCV's effectiveness in asymptomatic cases has not been fully assessed[1]. Both symptomatic and asymptomatic infants should be monitored regularly for neurological disability.

Economic Impact: The effect of congenital CMV 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[3]. The IOM estimated that approximately $1.9 billion per year is spent toward medical and educational costs for congenitally CMV infected children in the USA[1] compared to costs of developing a CMV vaccine of $360 million[29]. The health economic implications of congenital CMV in low income countries is under-recognised or unknown[30].

Impairment of immune response 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) [31]. It has been postulated that CMV infection, due to its large recruitment of T-cells may have a role to play in modulating immune responses to infant vaccinations but only a few groups thus far have investigated this in children[31–33].

Problem statement Morbidity data on congenital CMV infections has largely been from HIC as a result of maternal primary infection, whereas data from low-income, high secondary infection and high HIV prevalent settings are lacking. In the South African public health sector, only symptomatic newborns have CMV testing whilst newborn hearing screening is conducted in high risk babies born premature, symptomatic or admitted to ICU. Asymptomatic, CMV infected infants at birth are an at risk group of developing disabilities which only become apparent when children are older and interventions less effective. Studies have demonstrated up to 15% of stillbirths have lab confirmed CMV infection[34]. Any association between neonatal deaths and congenital CMV infection is yet to be described.

1.3 Justification for the study

There is no robust data on the immunology, virology, mortality, disease and disability burden from congenital CMV infection in South Africa. The effect of HIV on congenital CMV and subsequent neurological sequelae is also largely unknown in black Africans. Early detection of SNHL and neurodevelopmental disorders could enable timely intervention to improve quality of

life. If sequelae are significantly higher in HIV exposed infants, this study may contribute to discussions towards a targeted screening programme for CMV in HIV exposed infants. With the development of a CMV vaccine in progress[35], it is necessary to establish baseline disease epidemiology determining whether congenital CMV infection in South African children presents the same public health impact as in high resource countries and whether screening programmes or a vaccine is necessary or cost effective in our setting. Most available data suggest that antibody positivity, is not associated with protection from vertical transmission to a foetus or infant or with protection from re-infection. However, some studies suggest that antibody avidity and/or neutralisation activity may be associated with protection from vertical transmission. We wish to explore this further and also assess if either measure is associated with maternal HIV infection.

2 Aim and Objectives

The theme of this project is to determine the epidemiology of congenital CMV infection and incidence of sequelae until 13 months age in a high HIV prevalent setting in Soweto, Johannesburg.

2.1 Primary Objectives:

2. 1. Determine the CMV seroprevalence, antibody avidity and (if possible) anti-CMV neutralising antibody activity in blood plasma of both HIV infected and uninfected pregnant women at the time of delivery and explore associations between these measures and risk of congenital and postnatally acquired CMV infection in the first year of life.To compare the prevalence of congenital CMV infection and incidence of symptomatic infection in HIV-exposed and HIV-unexposed neonates

3. To determine the incidence and risk factors for neurological sequelae in congenitally CMV infected neonates from birth up to 12 months of age compared to a control group of neonates without congenital CMV
4. To determine by minimally invasive tissue sampling (MITS) the association between congenital CMV infection and: 1) Neonatal deaths less than 3 weeks of age and 2) Stillbirths

2.2 Exploratory objectives

5. To explore the association between maternal CMV shedding in vaginal fluid and breast milk and the risk of congenital CMV infection assessed by quantitative CMV PCR.
6. Determine the incidence of postnatal CMV acquisition, in infants not CMV infected at birth, and explore possible risk factors, including maternal HIV status and the magnitude of maternal CMV shedding in vaginal fluid and breast milk.
7. Explore differences in immune responses to the primary and booster series of vaccinations (DTaP-Hib/HepB, PCV rotavirus and measles vaccines) between congenital CMV cases, postnatally acquired CMV cases and non-CMV infected controls at 6, 12 and 13 months of age.

3 Research Methods

3.1 Study Site

This study will be conducted at Chris Hani Baragwanath Academic Hospital (CHBAH) in Soweto, a secondary-tertiary level facility serving a population of 1.2 million. The annual birth cohort in Soweto is approximately 28000, with 75% occurring at CHBAH and the rest at midwife operated units or a nearby district level hospital.

3.2 Overview of study

This will be an epidemiologic cross-sectional study, with a prospective cohort component (refer to Appendix 2 for study schema). Objectives 1, 2 and 5 will be nested within a larger cohort study enrolling mother-infant dyads at birth or antenatally to investigate a sero-correlate of protection against invasive Group B Streptococcal disease in newborns (protocol number V98_28OBTP, HREC no. 140203, Appendix 4). In V98_28OBTP, research staff at labour/delivery wards identifies women enrolled antenatally or consents potentially interested women in labour for participation. Maternal and infant demographic and clinical data, maternal blood, vaginal swab and infant cord blood are collected for laboratory testing. Monitoring for subsequent hospitalization of the infant at CHBAH in the first year of life is undertaken daily in all general paediatric and neonatal wards in an established surveillance known as Babies of Soweto Study (BoSS). As of November 2015, 20 000 mother-newborn dyads are enrolled, and is anticipated to continue until October 2016.

Objective 4 will be nested within a prospective study of cause of death in under-5 children by Minimally Invasive Tissue Sampling (MITS) currently underway at CHBAH (Appendix 13, HREC no. 150215). Stillborn babies are also enrolled into this study.

A summary of objectives, methods and data analysis for this PhD is given in Appendix 3.

3.3 Objective 1, 2and 5 (exploratory) methods

Study design: Cross-sectional study design with consecutive sampling

Study participants: Mothers meeting the inclusion criteria in table 1 and their newborns.

Table 1: Maternal inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
- Enrolled in V98_28OB - Age ≥18y - Residing in Soweto and would be available for study follow-up - Consents to enrol self and baby in study - Baby born alive	- <18 years age - Visiting Soweto - Declines consent

Enrolment will be performed by study staff stationed in labour and delivery around-the-clock. The informed consent form (ICF) used in V98_28OBTP (Appendix 4) also allows collection of data and samples from mothers and newborn to test for CMV.

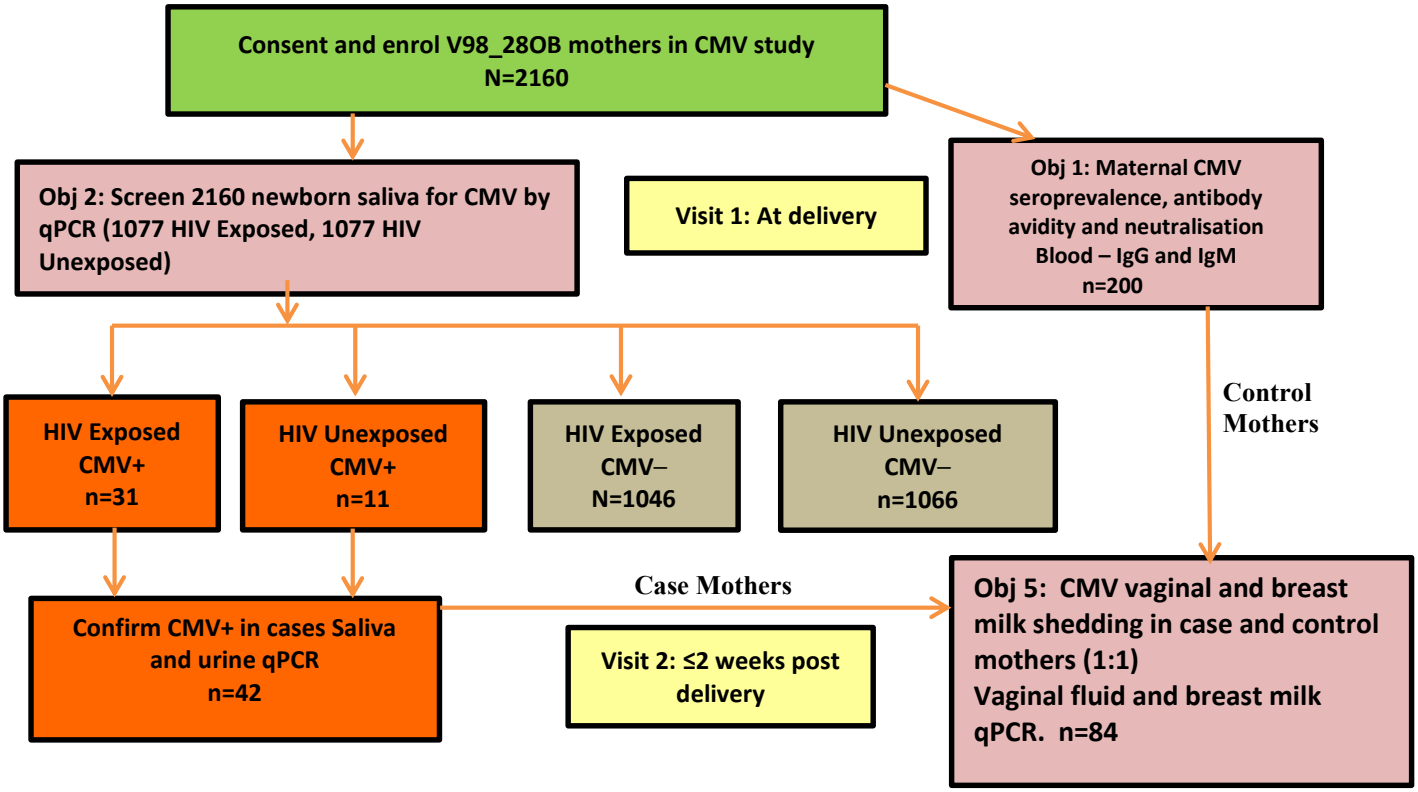
***Data* collection:** Data will be collected by maternal interview and examination of hospital notes of the mother and newborn, recorded onto the V98_28OBTP CRF (Appendix 5) and BoSS CRF (Appendix 6) if symptomatic. Table 2 describes the maternal and newborn data of interest and figure 1 illustrates the data collection process.

Table 2: Maternal and newborn data of interest

Maternal data	**Newborn data**
Demographic: age, race, and contact details	**Demographic:** date of birth, gender, race
Clinical data: antenatal care details, past obstetric history, parity, gestation at delivery, delivery details, co-morbidities, HIV status and CD4 count, ART use	**Clinical:** gestational age at birth, birthweight, head circumference, APGAR scores, HIV exposure, symptoms at birth, discharge or admission details

Newborn saliva samples will be collected by placing an Orocal swab in the mouth for at least 1 minute. All symptomatic neonates for congenital CMV infection (Appendix 11) will also be screened for other congenital infections which include syphilis, toxoplasmosis, rubella and herpes as is the standard of care at CHBAH.

Figure 1: Data collection process for Objectives 1, 2 and 5



All children with any clinical finding are referred to the neonatology department at CHBAH for clinical management as per standard of care. If a newborn PCR indicates CMV infection, the mother will be contacted telephonically to return to the site within two weeks of birth. At this visit the implications of the positive CMV result will be explained and additional consent for the continued follow up of the child (refer to Appendix 7 for ICF and Appendix 8 for schedule of study visits). Should consent be confirmed, a second saliva swab and a urine sample will be collected for PCR to confirm CMV infection. Babies that test CMV negative on both the second saliva swab and urine will not be enrolled as a case as the saliva positivity may be a result of CMV infected breast milk contamination. However, they will be referred for hearing screening as standard of care. Cases testing positive on both saliva and urine or urine alone will continue as cases for objective 3.

Mothers of CMV cases will have a vaginal swab and breast milk collected for quantitative PCR by a research nurse or the investigator. A subset of mothers that tested seropositive for CMV but gave birth to CMV uninfected neonates will also be recalled as controls for the case mothers in a

1:1 ratio, matched by HIV infection, for vaginal fluid and breast milk collection within 2 weeks of delivery.

Sample size: Objective 1: Assuming the estimated CMV prevalence is 86% in pregnant women[13] 200 mothers will be needed to estimate the true value with a desired precision of 5% and confidence interval of 95%.

Objective 2: Prevalence of congenital CMV in HIV exposed neonates is 2.9%[26] and in unexposed neonates is 1.0%[1]. Thus, a total sample of 1662 neonates with 831 HIV exposed and 831 unexposed (1:1 ratio) for 80% power, are required to detect a 2.9 fold difference in CMV prevalence between the 2 groups. This will be increased to 2160 neonates, to accommodate objective 3, to determine clinical sequelae in CMV infected infants (section 3.3.1, Sample size). After adjusting for 10% being non-evaluable, 1077 HIV exposed (providing 31 CMV infected) and 1077 HIV unexposed infants (providing ~11 CMV cases) are required. Ranges of sample size estimates for varying CMV prevalences by HIV exposure are given in Appendix 9.

Objective 5: The number of case mothers tested will be determined by the total number of CMV infected neonates that are identified during screening estimated at 42. HIV infection and age matched control mothers will be allocated in a 1: 1 ratio giving a total sample size of 84.

Laboratory testing: Maternal blood and vaginal swabs, newborn saliva and urine collected will be stored at -70 °C until processed for CMV serology (IgG and IgM in blood) and DNA by PCR assays (vaginal fluid, saliva, urine) at the RMPRU Laboratory or other collaborating laboratory in South Africa. The turnover time for PCR testing is expected to be within 5 working days of sample collection. Refer to Appendix 10 for laboratory methods of sample processing.

Data Analysis: Table 3 describes the planned data analyses, per objective. All analyses will be done on STATA/IC version 13 for Windows, Texas, USA, in consultation with a biostatistician.

Table 3: Data analysis plan

Objective	Data Analysis
1. Determine the seroprevalence of Cytomegalovirus infection (CMV) in HIV infected and uninfected pregnant women attending CHBAH	Table of maternal demographic and clinical characteristics as proportions
	Maternal CMV seroprevalence expressed as a percentage stratified by HIV status (95% confidence interval)
	Chi square test to compare reactivations/recurrent infections determined by IgG avidity and IgM presence compared to latent infection stratified by HIV status
2. Compare the prevalence of and risk factors for congenital CMV infection in HIV exposed and unexposed newborns	Prevalence of congenital CMV infection in HIV exposed and unexposed infants expressed as a percentage with 95% confidence interval
	Multiple regression analysis to adjust for potential confounders: gender, gestational age at birth, birth weight, birth order, symptoms at birth
	In HIV exposed infants, chi square tests for association between: - Congenital CMV infection and maternal ART use - Congenital CMV infection and severity of maternal immunosuppression (CD4+ count levels)
To explore the association between maternal CMV shedding in vaginal fluid and breast milk and the risk of congenital CMV infection assessed by quantitative CMV PCR.	Chi square test for association between vaginal CMV shedding and congenital CMV infection - Stratify by varying ranges of viral shedding (high, moderate, low – quantification to be determined) - Stratify by HIV infection though will have low power for this

Potential challenges: Objective 1, 2 and 5: Mothers may be reluctant to be enrolled in a study for an infection which is unknown to them. To mitigate this, a patient information leaflet will be provided to improve understanding and includes the potential benefit to the infant should he/she

be diagnosed to have congenital CMV (Appendix 7). Training of researchers in effective communication will be conducted.

Objective 5: CMV viral shedding is cyclical, highest during primary infection; reactivation or reinfection which when occurs during pregnancy may be transmitted to the foetus. This study may be limited by the collection of samples after delivery at which point, viral shedding may have ceased since pregnancy.

3.4 Objective 3, 6 (exploratory) and 7 (exploratory) Methods

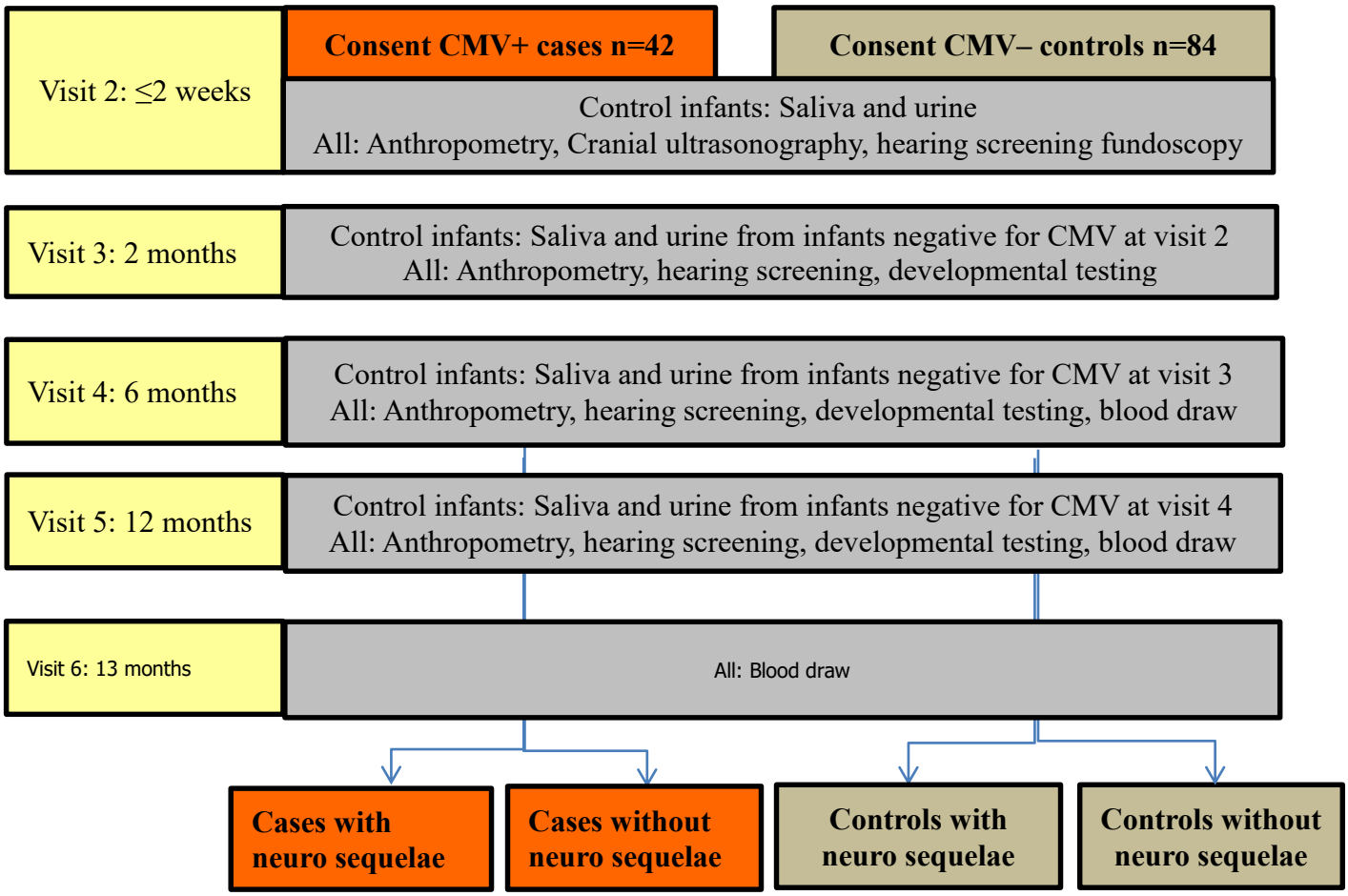
Study design: Prospective longitudinal cohort study of CMV infected and uninfected neonates

Study participants: The cohort will consist of newborn infants identified as laboratory confirmed CMV infected from objective 2 forming the cases and a subset of neonates identified as CMV uninfected at birth chosen as controls. Each case will be matched to 2 controls by age, gestational age at birth (±2 weeks) and HIV exposure. Within 2 weeks of age, cases and controls will be recalled to the site to consent for follow up and initiation of investigations as illustrated in figure 2.

Data collection: Postnatal CMV acquisition: A saliva and urine sample from each control infant will be collected at the 2 weeks visit. At subsequent follow up visits, infant saliva and urine will be collected from those that tested CMV negative at the preceding visit.

Incidence of neurological sequelae: neurological sequelae is one or more of: unilateral or bilateral SNHL, developmental deficit, unilateral or bilateral ocular abnormality, abnormal cerebral imaging, microcephaly, or seizures not related to an acute systemic infection or CNS insult (refer to Appendix 11 for case definition of each sequela).

Figure 2: Schedule of study visits and follow up investigations



Mothers will receive a reminder phone call 1 week prior to each visit. Cranial ultrasonography will be conducted at the neonatology unit and fundoscopic examination at St. John's eye hospital. Hearing screening will be conducted at the audiology department as per standard of care by automated auditory brainstem evoked response audiometry (AABR). Developmental assessment will be performed by myself at the research clinic through a developmental assessment tool (Ages and stages questionnaire or Bayley III scales of infant and toddler development or equivalent). Refer to Appendix 12 for list of follow up data. Standard operating procedures to ensure standardisation of assessments between assessors will be developed.

Blinding of the assessors to the status of CMV infection will be maintained at each assessment. If impairments requiring clinical management are identified, the assessor will be provided with the laboratory data. Any impaired infant will be referred to specialist CHBAH paediatric clinics for clinical management. HIV-exposed infants are tested for HIV-infection by PCR at birth, 6 weeks of age and again at least 3 months post breast-feeding cessation, as per standard of care in South Africa. Should a child test HIV-infected, they will be referred to the HIV clinic at CHBAH.

Infant specific vaccine induced immune responses: Cases and controls identified will be vaccinated as per the immunisation schedule in South Africa with hexavalent DTaPHibHepBIPV at 6, 10 and 14 weeks and PCV and rotavirus vaccine at 6 and 14 weeks of age. Mothers will be encouraged to access vaccines at the vaccination clinic at RMPRU and counselled on ensuring their infants are vaccinated at the scheduled time. At the 3rd study visit when infants are aged 6 months a venous blood sample will be collected by appropriately trained study staff. The volume of blood collected will be not more than 5ml following the recommendations by HREC (medical) on blood volumes from paediatric research participants.

Laboratory Venous blood will be centrifuged and aliquots of serum will be stored at -70°C. Antibody to diphtheria toxoid, tetanus-toxoid, pertussis toxoid, filamentous hemagglutinin (FHA) and hepatitis B surface antigen (HBsAg) will be measured by an in-house Luminex multiplex immunoassay. Immune responses to the primary series of PCV will be measured by a standardized enzyme immunoassay (EIA) to test for vaccine serotype–specific capsular immunoglobulin (Ig) G antibody concentrations and opsonophagocytic assay. Titres of rotavirus-specific IgA will be determined in enzyme-linked immunosorbent assays[36]. Refer to appendix 10 for the serological correlates of protection [37].

Sample size: The anticipated prevalence of neurological sequelae in CMV uninfected controls with uneventful foetal or perinatal periods is estimated at 1%[38]. Prevalence of neurological sequelae in CMV infected infants is expected to be higher at approximately 17%[4]. For 80% power, the difference that could feasibly be detected, is 17-fold i.e. 1% vs 17%. Allowing for a loss to follow up of 10%, 42 CMV infected infants and 84 controls (n=126) with an allocation ratio of 1:2 are required (Appendix 9).

Data Analysis: Table 3 describes the data analysis plan to determine the incidence and risk factors for neurological sequelae in congenital CMV infected infants

Table 3: Data analysis plan

Objective 3 Incidence calculation of one or more neurological sequelae for cases and controls at 2 weeks, 2 months, 6 months and 12 months of age dependent on investigations done at each visit Comparison of proportions with permanent sequelae (hearing impairment and developmental deficit) at each visit between cases and controls using a Z test to compare ratios, including 95% confidence intervals. Adjust for potential confounders (IUGR, symptoms at birth, gender and co-morbidities) by multiple logistic regression analysis to establish factors contributing to neurological sequelae.
Objective 6 Incidence of postnatal acquisition of CMV in newborns will be calculated at 2 months, 6 months and 1 year of chronological age. Incidence of neurological sequelae in postnatally infected infants will be calculated
Objective 7

The magnitude of specific antibody response between cases and controls will be compared using the unpaired t test when data are normally distributed. In the scenario that the distribution is non-normal the Mann-Whitney test will be used instead. Normality will be tested using the Shapiro-Wilk test, where the assumption of non-normal data will be concluded based on a significant result. Frequency plots and P-P plots will also be used to confirm the normality assumption, or not.

Simple correlations will be assessed using Pearson or Spearman correlation in the case of normal or non-normal distribution, respectively. The resulting correlation coefficient will indicate the strength of association, whereas the significance test will provide the evidence of a linear correlation or not in the population.

Potential challenges: To account for loss to follow up and timely completion of investigations, a window period of 30 days is allowed from the date of each scheduled visit in which to perform the investigations. Transport costs will be reimbursed with R150 per scheduled visit and all investigations will be provided at no cost. Neurological sequelae in congenital CMV infected children may manifest beyond 12 months of age and long term follow up to 6 years is recommended[4]. Hence, it is possible that this study may not detect all sequelae by 12 months of age and no differences may be observed between the 2 groups.

Parents may be reluctant to consent to a blood draw at 6 months of age and will be thoroughly counselled on the purpose and processing of the blood samples. The informed consent form has been amended to include the purpose of the blood draw and each mother will be given a new consent form to sign with the option of refusing to participate in the blood draw but continue with the rest of the study procedures.

3.5 Objective 4 Methods

Study design: This study will be nested within a broader study conducting Minimally Invasive Tissue Sampling (MITS) on death of children and stillbirths currently underway in CHBAH,

ongoing until end 2017 (HREC no. 150215), and includes testing for CMV. I perform MITS in this study and will be involved in data analysis as it relates to CMV and neonatal deaths or stillbirths**.**

Study participants: Stillbirths weighing ≥1000g and neonatal deaths weighing ≥750g, less than 3 weeks of age from non-traumatic causes at CHBAH. Parents/guardians provide written consent after death, following grief counselling and provision of an information leaflet.

Data collection: Ante-mortem clinical data will be extracted from patient records in the BoSS CRF (Appendix 6). Core biopsy specimens of lung, liver, kidney, brain tissue, placenta (stillbirths) and fluid specimens of cerebrospinal fluid (CSF) and blood will be sampled from each enrolled case within 72 hours of birth.

Sample size: This will be a descriptive study, and sample size is based on the number of deaths/stillbirths which occur over a18-24 month period. From current experience, we anticipate enrolling approximately 200 stillbirths and 100 neonatal deaths, providing 80% precision to determine that at least 5% of these stillbirths and neonatal deaths are associated with CMV.

Laboratory: Testing and examination for CMV infection by histopathological methods will take place at the National Health Laboratory Services (NHLS). Molecular testing (PCR) will occur at the RMPRU laboratory by in house assays. HIV PCR and antibody ELISA will be done to determine infection and exposure *in utero* as has been approved in the parent protocol (HREC no. 150215). Refer to Appendix 13 for the full tissue sampling protocol of the study.

Data analysis: Descriptive statistics presenting the number of deaths with CMV infection by birthweight, gestational age at birth and age at death in neonates. Multiple regression analysis will adjust for confounding factors such as HIV infection and exposure, prematurity, co-morbidity and co-infection with other pathogens. The odds of CMV disease will be compared to

the prevalence of congenital CMV in the general population, stratified by HIV-exposure status, as measured in Objective 2.

Study challenges: Although the majority (75%) of parents approached thus far, consent to the procedure, enrolment of neonatal deaths has been limited due to cultural practices of burying neonates immediately after death before study staff are able to approach the parents for consent. In contrast, we are enrolling approximately 5 stillbirths per week. This challenge will be mitigated by engaging with attending paediatricians to inform the study team more timeously if deaths occur. MITS in aborted foetuses is not being done in the current study but is an important demographic in describing the outcomes of congenital CMV infection.

4 My role and responsibilities

I am responsible for protocol development and running of procedures specific to this study. I will coordinate the follow-up of infants over 12 months and conduct the developmental assessments. I currently participate in quality control of data in the paediatric (BoSS) surveillance and project administration. For the MITS study, I perform tissue sampling, participate in project administration and will contribute to data analysis as it relates to CMV.

5 Ethics

5.1 Informed consent and confidentiality

The V98_28OBTP (HREC 140203) and MITS (HREC 150215) protocols are approved and currently in progress at CHBAH. Approval for integrating this proposal into these studies and the additional follow up of infants until 12 months of age is sought. Written informed consent from mothers allowing participation will be obtained. Participant contact details and identifiers will be stored independently of source documents, only available to myself and research assistants responsible for interviewing mothers. Transport and incidental costs will be reimbursed at R150

per visit. Infants will benefit from screening and follow up as early intervention will be possible in cases where clinical sequelae are identified within 12 months. Following the conclusion of this study, infants with CMV infection will have hearing monitoring until the age of 6 years by the audiology department.

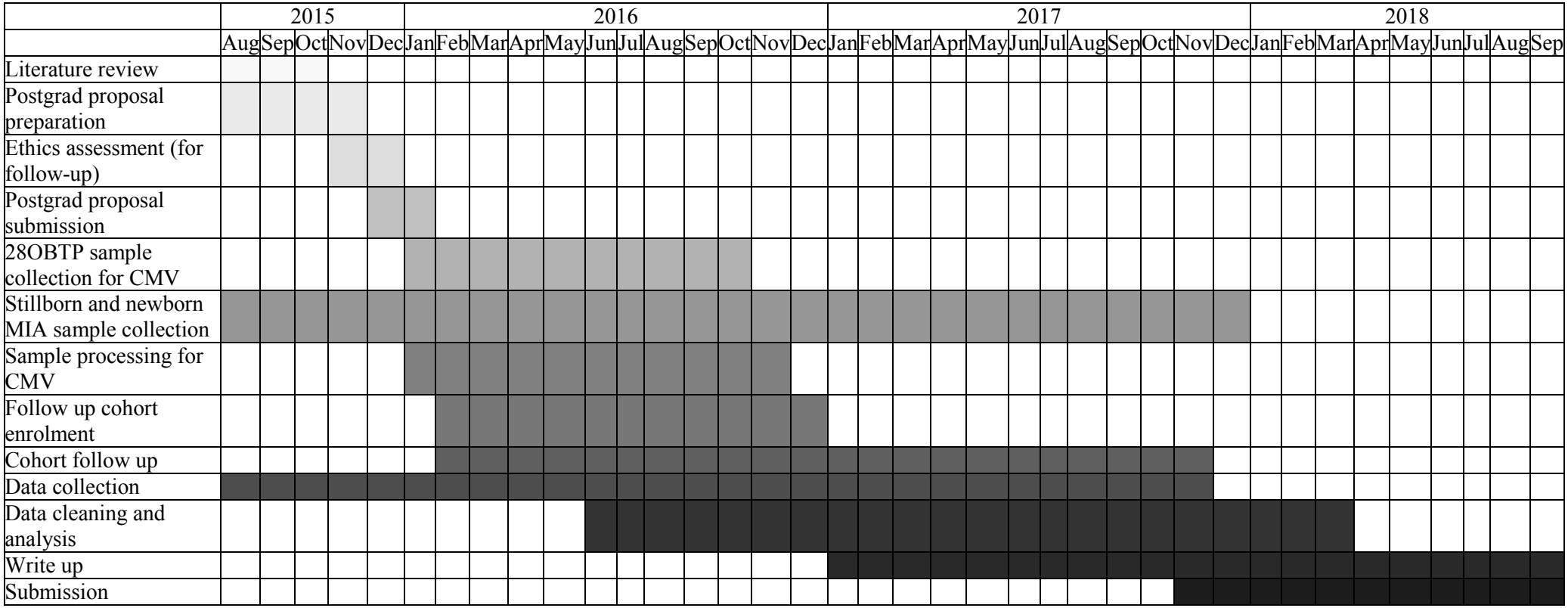
5.2 Results Dissemination

This PhD will be achieved by publication in peer-reviewed journals, where they will also be available to the public (refer to Appendix 14 for proposed manuscripts). Summary results will be reported to the HREC on completion of the study. Results may be presented at local and international scientific meetings.

6 Funding

Funding for the 28OB study is provided by Novartis. Funding specific to this CMV study will be provided by the RMPRU. Refer to Appendix 15 for budget details.

7 Timeline


2015
2016
2017
2018
Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug Sep
Literature review
Postgrad proposal preparation
Ethics assessment (for follow-up)
Postgrad proposal submission
28OBTP sample collection for CMV
Stillborn and newborn MIA sample collection
Sample processing for CMV
Follow up cohort enrolment
Cohort follow up
Data collection
Data cleaning and analysis
Write up
Submission

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Appendices