The birth prevalence of congenital CMV infection in HIV-exposed newborns in Cape Town, South Africa – A pilot study. The "CYPREHEN" (CYtomegalovirus PREvalence in HIV-Exposed Newborns) study.

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PURPOSE OF DISSERTATION:

This dissertation is being submitted for the Master of Public Health degree. The research study and dissertation comprise 50% of the degree. The journal article manuscript format has been used with the inclusion of the manuscript that was published in Clinical Infectious Diseases Journal in May 2014, Vol 58, Issue 10, pages 467-72 with doi: 10.1093/cid/ciu096.
UKZN Biomedical Research Ethics Committee approval date and number: 19 June 2014, REF: BE124/14.

Abstract

Background

Congenital cytomegalovirus infection (CMV) is a leading non-genetic cause of sensorineural hearing loss worldwide. The birth prevalence of congenital CMV infection correlates positively with the level of CMV seroimmunity in the adult population. In addition, women infected with Human Immunodeficiency Virus (HIV) constitute a special at risk subpopulation for the intrauterine transmission of CMV. Despite a high prevalence of both HIV and CMV, the birth prevalence of congenital CMV infection has not been assessed in sub-Saharan Africa.

Purpose

The purpose of the study was to determine the birth prevalence of congenital CMV infection among HIV-exposed newborns born in a public sector hospital in the Western Cape in 2012, during the era of prenatal antiretroviral therapy.

Objectives

The objectives of this study were:

- To determine the prevalence of congenital CMV infection among HIV-exposed newborns;
- To assess the predictors of congenital CMV infection transmission among HIV-infected women; and
- To inform the design of an analytic study to determine if newborn CMV screening should be implemented in this population.

Study design

An observational descriptive cross-sectional study design was used.

Settings

The study was conducted at Mowbray Maternity Hospital (MMH), which serves the Cape Town Metropole area.

Study population

The study population comprised infants born to HIV-infected mothers delivering at MMH.

Study sample

Non-probability convenience sampling was used to enroll 750 newborns.

Methods

HIV-infected mothers were recruited in the immediate postnatal period at a referral maternity hospital between April and October 2012. Maternal and infant clinical data and newborn oral swabs (saliva) were collected. Saliva was assayed by real-time PCR for CMV. Data were analysed using univariate and multivariate logistic regression analyses to determine specific demographic, maternal and newborn characteristics associated with congenital CMV infection.

Results

CMV was detected in 22/748 newborn oral swabs (2.9%; 95% Confidence Interval (CI), 1. 9%-4.4%). Maternal CD4 count less than 200 cells/μL during pregnancy was independently associated with congenital CMV infection (adjusted Odds Ratio (aOR) 2.9; 95% CI, 1.2-7.3). A negative correlation between CMV viral load in saliva and maternal CD4 count was observed ($r = -0.495$, $n = 22$, $p = 0.019$).

Conclusions

The birth prevalence of congenital CMV infection was high despite prenatal ARV prophylaxis, and was associated with advanced maternal immunosuppression.

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Declaration

I, Sheetal Manicklal, declare that:

I. The research reported in this dissertation, except where otherwise indicated, and is my original research.

II. This dissertation has not been submitted for any degree or examination at any other university.

III. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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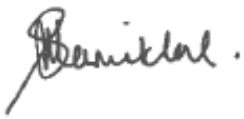

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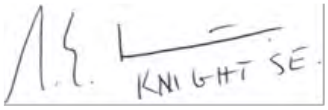

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Acronyms and Abbreviations

AIDS ..... Acquired Immunodeficiency Syndrome
ARV ..... Antiretroviral therapy
AZT ..... Zidovudine
CMV ..... Cytomegalovirus
HAART ..... Highly Active Antiretroviral Therapy
HBV ..... Hepatitis B virus
HIV ..... Human Immunodeficiency Virus
MMH ..... Mowbray Maternity Hospital
MTCT ..... Mother-to-child transmission
NICU ..... Neonatal Intensive Care Unit
PCR ..... Polymerase chain reaction
SNHL ..... Sensorineural hearing loss
UK ..... United Kingdom
UKZN ..... University of KwaZulu-Natal
US ..... United States of America

Chapter One: Introduction

This chapter presents a concise background of the research problem in order to contextualize the research aims. It also details the format of the dissertation.

Cytomegalovirus (CMV) is a common viral infection of humans, but is “not any the less challenging through its ubiquity” (1). The role of CMV in producing morbidity and mortality in persons with impaired immunity, such as patients with Acquired Immune Deficiency Syndrome (AIDS) and transplant recipients, is widely appreciated (2, 3). However, the fact that CMV is also a leading cause of congenital infections worldwide and an important cause of sensorineural deafness and neurodevelopmental delay is barely recognized (4-7).

The birth prevalence of congenital CMV infection correlates with the level of CMV seroimmunity[a] in the adult population, and is higher in populations with a high prevalence of CMV (8, 9). Not only is the birth prevalence of congenital CMV infection higher in women with pre-existing CMV immunity, but such immunity also fails to protect against symptoms at birth and sequelae in the infant (10-14). Of salient concern, hearing loss occurs in 10%-15% of infected infants (14-16).

In South Africa, persistent infection with CMV in the antenatal population is nearly universal (17). In addition, the maternal HIV seroprevalence is among the highest in the world, with 30% of antenatal clinic attendees on average seropositive for HIV (18). Available data indicate that HIV-infected women constitute a special at risk subpopulation for the intrauterine transmission of CMV (19-21). Despite a high prevalence of both HIV and CMV in sub-Saharan Africa (22, 23), the birth

[a] Pre-existing immunity to CMV, which is a marker of past and therefore persistent infection with CMV.

prevalence of congenital CMV infection has not been assessed in a large sample of HIV-exposed newborns in this region. In addition, several factors may influence the transmission risk of congenital CMV infection in HIV-infected women, particularly the use of antiretroviral therapy (ART)[b] and maternal immune status as measured by CD4 cell counts (20), although these have not been systematically assessed in such women.

In summary, congenital CMV infection and its associated sequelae is a well described problem affecting young children in upper income countries, however there is sparse data from high HIV prevalence settings where the burden of infection and disease is likely to be substantial. As such, the purpose of this study was to assess the birth prevalence of congenital CMV infection among HIV-exposed newborns born in a public sector hospital in the Western Cape during the era of prenatal antiretroviral therapy[c] in 2012. The secondary objectives were to investigate the predictors of congenital CMV transmission among HIV-infected women, as well as to inform the design of an analytic study to determine if HIV exposed infants should be considered for newborn CMV screening in this population.

Chapter One presents a concise background of the research problem in order to contextualize the research aims. In Chapter Two a literature review is presented, describing the global epidemiology of congenital CMV infection and paucity of African data. This is followed by the journal article manuscript in Chapter Three. Chapter Four then provides additional results from analyses undertaken on the dataset that were not included in the journal article, as well a systematic discussion of the study limitations and additional recommendations. Next, Part E contains all

[b] ART encompasses monodrug, dual-drug as well as triple antiretroviral therapy (HAART) used in HIV-infected pregnant women for the prevention of mother to child transmission (PMTCT) of HIV.
[c] WHO PMTCT option A was being implemented at the time of the study.

appendices, namely the research protocol, University of KwaZulu-Natal (UKZN) research ethics approval, acknowledgement of registration for the degree, acknowledgement of author roles and contributions, and the instructions to authors for the Clinical Infectious Diseases Journal. Lastly, the bibliography for all chapters is presented in Part F.

Chapter Two: Literature review

This chapter begins with a basic background of key aspects of the virology and immunology of CMV, together with a brief overview of congenital CMV infection. This background information will set the scene for a discussion of the global epidemiology and disease burden of congenital CMV infection, which will serve to underscore the importance of congenital CMV infection in high seroprevalence settings[d] as well as in HIV infected women. Next, approaches to preventing the burden of congenital CMV infection will be examined with emphasis on the challenge posed by maternal non-primary infection[e]. Finally, an introduction to the laboratory diagnosis of congenital CMV infection will be provided, focusing on the latest technologies. The chapter will close with a summary of the literature review that contextualizes congenital CMV infection as an important cause of hearing loss worldwide, and underscores the need for research in South Africa.

Data for the literature review were identified by searching PubMed to identify full-length articles as well as abstracts published in English between January 1, 1980 and June 30, 2014. The following search terms were used: congenital cytomegalovirus AND (HIV OR epidemiology OR risk factors OR diagnosis). This search gave 1174 results, and after review of titles, 160 abstracts remained. After review of the 1174 titles and abstracts, the full text of the 40 relevant articles were reviewed. Appropriate citations from these articles were also reviewed. Relevant

[d] These refer to populations where > 90% of the adult population have had past infection with CMV and are thus persistently infected and CMV seropositive. Such populations are typically present in low and middle income countries.

[e] This refers to active CMV infection in persons who are already CMV seropositive as a result of past infection with CMV. This may occur as the result of reactivation of latent (endogenous) virus or re-infection with new strains (exogenous) of CMV.

textbook sections on congenital CMV infection were also read for background information and a general overview.

2.1 Background

CMV belongs to the herpesviridae family of viruses which it shares with herpes simplex virus and varicella zoster virus, the causative agents of cold sores and chickenpox, respectively (24). Unlike these viruses, infection with CMV is usually asymptomatic in healthy individuals (25) which possibly accounts for the low awareness of the existence or importance of this pathogen globally (6, 26, 27).

A common feature of all herpesviral infections is the establishment of a lifelong or persistent virus infection following primary infection[f] (24). In general, viral infections may assume different patterns of persistence within an individual, mainly those of latency and chronic infection. In chronic infections such as HIV and Hepatitis B Virus (HBV), there is continuous viral production throughout the period of infection, which may be detected at any time in the blood or body fluids of infected individuals. In comparison, upon primary infection with CMV, there is an initial period of active virus production for a few months followed by the establishment of latency during which the virus remains dormant[g] in cells of the monocyte-macrophage lineage, as well as the immuneprivileged sites of the kidney, mammary and salivary glands (24). Reactivation from latency and virus production only occurs periodically with virus shedding in blood (viraemia), urine (viruria), breast milk, saliva and genital tract secretions (24, 28). As in primary infection, such virus excretion is usually of little clinical consequence in healthy persons but instead serves as a mode of virus transmission through close contact,

[f] The initial or first time a seronegative person is infected with CMV.
[g] Absence of detectable virus production or excretion by conventional techniques in an infected person.

breastfeeding, sexual contact, and less commonly by blood and organ donation (29-31).

Following primary infection with CMV, an individual mounts an immunological response to the virus within weeks to months. The antibody and T cell immune response to the virus serve as markers of infection even during periods of absent virus excretion (latency). The antibody response detectable in blood is the easily measurable component of the immune response and its presence or absence is termed seropositivity (seroimmunity) or seronegativity, respectively. The appearance of antibodies in the blood of a seronegative person is known as seroconversion.[h] At a population level, the proportion of seropositive individuals is termed seroprevalence.

Despite the presence of a robust immune response to CMV characterized by high levels of antibodies and around 10% of the total peripheral T cell compartment dedicated to CMV in healthy individuals, CMV is still able to reactivate from latency (32, 33). It is thought however that pre-existing immunity serves to limits the magnitude and duration of reactivation in healthy individuals. The T cells in particular play a crucial role in keeping the virus in a quiescent state for most of its period in the host, as well as in preventing uncontrolled replication of the virus during periods of active infection[i] (32, 34). The importance of intact T cell immunosurveillance is evidenced by the production of clinical CMV disease in immunocompromised individuals, notably persons with advanced HIV and transplant recipients (2, 3, 35). As the developing foetus also has a lower capacity

[h] Transition of a seronegative person to being seropositive.
[i] Primary infection, re-infection or reactivation.

for immunoprotection (36), it represents an additional vulnerable host for CMV infection and disease (37).

2.1.1 Congenital CMV infection

Infection with CMV in utero is known as congenital CMV infection, and is distinguished from CMV acquired during birth and postnatally which are termed perinatal and postnatal CMV infection respectively. Transplacental transmission of CMV leading to congenital infection may occur at any stage of pregnancy, and is thought to follow CMV viraemia produced during active CMV infection in the mother (38, 39). Apart from reactivation of endogenous virus, CMV seropositive mothers can be re-infected with new strains (genotypes) of CMV (40). Reactivation of CMV and re-infection with different strains are known as non-primary CMV infection, and are differentiated from primary infection on the basis that the former occurs in persons with pre-existing immunity. Virus strains are told apart by molecular sequencing, which has in recent years revealed a very high diversity of CMV within and between individuals (41, 42). These insights suggest a role for different virus genotypes in CMV transmission and possibly disease (43). As new CMV strains can evade an individual's pre-existing T cell repertoire (33, 44), re-infection events may produce higher levels of viraemia in the early stages of infection compared with reactivation of latent virus strains. CMV re-infection may therefore have implications for transplacental virus transmission in pregnant women (45, 46).

Intrauterine infection leads to symptoms at birth in around 10% of infected infants (16). Owing to the broad tissue tropism of CMV, the clinical spectrum of symptomatic congenital CMV infection is wide (Table 1), ranging from isolated disease through a variable combination of symptoms to disseminated life-threatening neonatal infection (16, 24, 47). Although the majority of intrauterine

infections are clinically silent in the newborn period, around 10-15% of these asymptomatic newborns are at risk of permanent neurological sequelae, the majority of which are detectable in the first two years of life (14-16, 48). Overall sensorineural hearing loss is the most frequently occurring sequelae of congenital CMV infection (16).

Table 1. Spectrum of clinical outcomes following congenital CMV infection, according to type of clinical presentation at birth asymptomatic versus symptomatic.

	Asymptomatic	Symptomatic
Proportion of infected infants	90%	10%
Newborn signs and symptoms		
Transient manifestations		Jaundice, hepatosplenomegaly, petechial or purpuric rash
Acute CNS manifestations		Microcephaly, seizures, poor feeding, lethargy, hypotonia
Laboratory findings		Anaemia (Hb < 11g/dL), raised ALT (> 80 IU/L), direct bilirubin > 2g/dL, thrombocytopenia (< 100 000/mm^3), elevated CSF protein or lymphocytes
Risk of permanent sequelae	14%	40-58%
Long term sequelae		
Sensory	Sensorineural hearing loss (uni- or bilateral)	
Cognitive		Intellectual disability, attention deficit disorder
Motor		Spastic diplegia, seizure disorder

Abbreviations: CNS, central nervous system; Hb, haemoglobin; ALT, alanine transaminase; CSF, cerebrospinal fluid.
(Source: (8, 16, 24)).

2.2 Epidemiology and disease burden of congenital CMV infection

CMV is a ubiquitous viral infection of humans. Although CMV has a global distribution, substantial differences exist in the proportion of seropositive adults within and between populations owing to varying rates of CMV acquisition, leading to the recognition of low and high CMV seroprevalence settings (49). The rate of CMV acquisition in a population increases with age, and correlates inversely with socio-economic status and levels of public health standards (24, 49). Therefore, in industrialized countries, where primary infection occurs frequently later in life (adolescence and adulthood), 50-70% of women of childbearing age are CMV seropositive (50). These are considered low seroprevalence settings. In comparison, in low and middle income countries, including South Africa, where breastfeeding as well conditions of overcrowding are common, CMV infection occurs rapidly in infancy and childhood, with the majority of adult women seropositive for CMV (17, 49).

2.2.1 Birth prevalence of congenital CMV infection

As with the seroprevalence of CMV, the birth prevalence of congenital CMV infection exhibits substantial worldwide variation (Table 2), with rates overall of 0.6-0.7% in industrialized (low seroprevalence) settings compared with 1-5% in low and middle income (high seroprevalence) settings (8, 9, 51-53). Kenneson *et al*. tested the hypothesis that the birth prevalence of congenital CMV infection is linked to maternal seroprevalence by performing linear regression analysis of 20 study populations (8). They found an increase in the birth prevalence of congenital CMV infection of 2.6 per 1000 live births for every 10% increase in maternal seroprevalence level (Figure 1). In addition, maternal seroprevalence explained 30% of the variability in congenital CMV infection birth prevalence.

Table 2. Birth prevalence of congenital CMV infection based on studies of unselected infants identified by culture/PCR of urine/saliva.

	Country	Birth years	Sample size	Birth prevalence (%) (95% CI)
Upper income countries (0.2-1%)				
	Austria	1993	3/1693	0.2 (0.1-0.5)
	Canada	1973-1976	64/15212	0.4 (0.3-0.5)
	Denmark	1974-1977	12/3060	0.4 (0.2-0.7)
	Finland	1972	3/148	2.0 (0.1-5.8)
	Italy	1994-1995	6/1268	0.5 (0.2-1.0)
	Japan	1997-2002	37/11938	0.3 (0.2-0.4)
	Korea	1989-1991	6/514	1.2 (0.6-2.5)
	Sweden	1978-1986	76/16474	0.5 (0.4-0.6)
	Slovenia		4/2841	0.1 (0.1-0.4)
	UK	1979-1982	42/14200	0.3 (0.2-0.4)
	US-AL	1980-1990	52/9892	0.5 (0.4-0.7)
			215/17163	1.2 (1.1-1.4)
Low and middle income countries (1-2%)				
	Chile	1989-1994	12/658	1.8 (1.0-3.2)
	China	pre-1996	18/1000	1.8 (1.1-2.8)
	Brazil	2003-2009	121/12195	1.0 (0.8-1.2)
	Mexico	2001	5/560	0.9 (0.4-2.1)
	Panama	2003-2004	2/317	0.6 (0.2-2.5)
	India		9/423	2.1 (1.1-4.0)
Sub-Saharan Africa (>2%)				
	Gambia	pre-1991*	25/184	13.6 (9.4-19.0)
	Gambia	2002-2005§	40/741	5.4 (4.0-7.3)
	Ivory Coast	pre-1978	28/2032	1.4 (1.0-2.0)
	Nigeria	2013	10/275	3.6 (1.8-6.6)

* Live born infants recruited from rural and urban Gambia.
§ Conducted in a well-baby nursery.
(Source: Adapted from Kenneson 2007, (8, 9, 51-53))

Indeed, there has been a growing appreciation in recent years of the contribution of non-primary maternal CMV to the incidence of congenital CMV infection (8, 13, 14, 54). Wang *et al*. recently estimated the proportion of congenital CMV infection that was due to primary versus non-primary maternal infection during the period 1988-1994 in the United States (US), where the annual incidence of congenital CMV infection is in excess of 30 000 cases (54). Using population based data on the seroprevalence of CMV and the rates of primary infection in pregnancy, they estimated that three-quarters of congenital CMV infections in the US are due to non-primary maternal infection, with substantial variation in the attributable fraction by race/ethnicity and age.

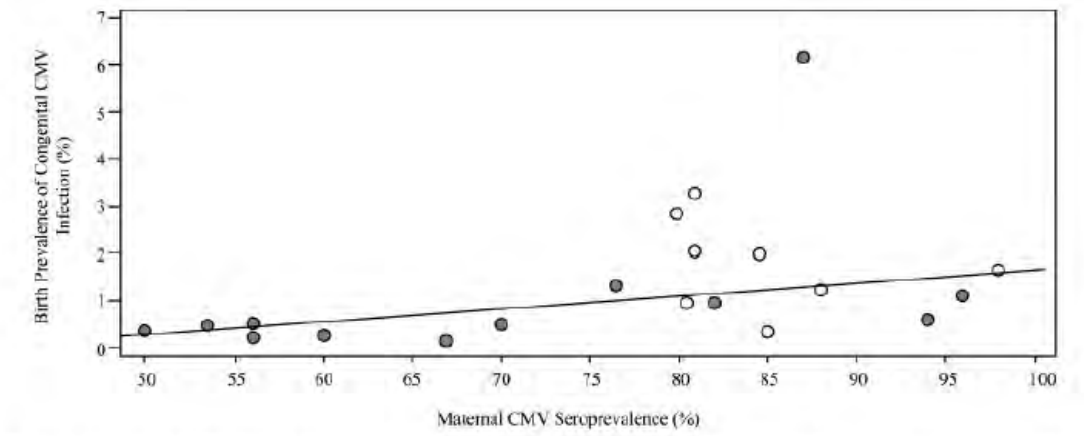

Figure 1. Linear regression showing association between maternal CMV seroprevalence and congenital CMV infection birth prevalence.
(Source: Kenneson 2007, (8))

The contribution of non-primary maternal infection to the majority of congenital CMV infections appears paradoxical given that maternal immunity offers considerable protection against congenital CMV infection at the individual level (14, 55). In women with pre-existing immunity (seropositive or seroimmune women), the risk of vertical transmission to the foetus is 1-2%, compared with 30-50% in women who experience a primary CMV infection during pregnancy (8). However,

congenital CMV infection birth prevalence at the population level depends not only on the individual transmission risk, but also the size of the at-risk population. All women with prior CMV infection are at risk, whereas only a fraction of seronegative women are infected with CMV during pregnancy and therefore at risk of transmitting the virus to their infants.

The following hypothetical scenario illustrates the role of non-primary maternal CMV in driving the birth prevalence of congenital CMV infection as a population level phenomenon. In seronegative women of upper income settings, the risk of primary CMV infection in pregnancy is 1-7% (56, 57). Assuming a 2% risk of primary infection and 35% risk of vertical transmission upon primary infection, the combined probability of congenital CMV infection in seronegative women is 0.7% (7 per 1000 deliveries). In seropositive women on the other hand, the frequency of CMV transmission is 1-2% (8), leading to 10 to 20 vertical infections per 1000 deliveries. Based on these assumptions, if the CMV seroprevalence in a high income population were 50% with a 1% transmission risk in seropositive women, the birth prevalence of congenital CMV infection would be around 8.5 per 1000 deliveries, with 5 infections (59%) or nearly two-thirds attributable to non-primary maternal infection. If the seroprevalence in a high income population increased to 70%, the birth prevalence of congenital CMV infection would be 9.1 per 1000 deliveries, with 7 infections (77%) attributable to non-primary maternal infection.

2.2.2 Congenital CMV disease burden

Congenital CMV infection is a leading cause of childhood hearing loss and important cause of mental impairment in high income countries. In the US, congenital CMV infection accounts for a fifth to a quarter of cases of hearing loss at birth and four years of age respectively, second only to genetic causes (Figure 2)

(7).

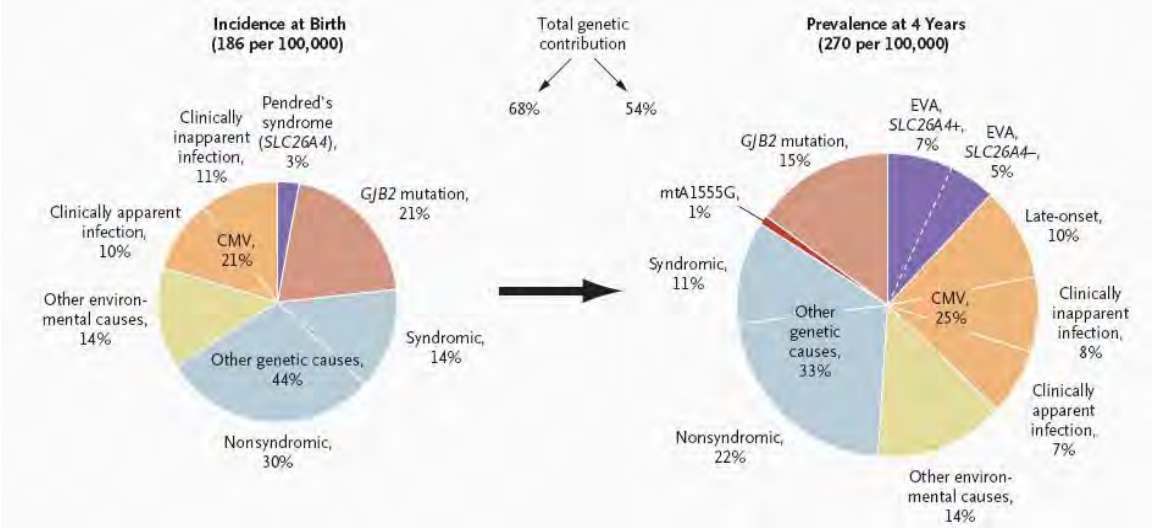

Figure 2. Estimates of causes of deafness at birth and at four years in the United States.

(Source: Morton 2006, (7))

Despite a higher birth prevalence of congenital CMV infection in highly seropositive populations, the findings of earlier studies of congenital CMV infection among infants of women with pre-existing immunity led to a dogma that 'maternal immunity is protective against congenital CMV disease' (58-60). Stagno *et al*. studied the effects of maternal primary versus non-primary CMV on symptomatic disease at birth in the 1970s and 1980s (59). Of the 32 cases of congenital CMV infection studied, an equal proportion were due to maternal primary infection and non-primary infection, however clinically apparent disease was more common in the primary infection group. The authors pooled the data with cases of congenital CMV infection identified at other prenatal clinics, and concluded that "congenital cytomegalovirus infection resulting from primary maternal infection is more likely to be serious than that resulting from recurrent infection" (59). Fowler *et al*.

subsequently reported on a cohort of 197 newborns with congenital CMV infection born in the US between 1972 and 1990, 125 of whom were born to women with a primary infection and 64 of whom were born to women with non-primary infection (60). They found that the frequency of one or more sequelae was 25% in the primary infection group of infants compared with 8% in the non-primary infection group. In addition, mental impairment, bilateral hearing loss and multiple sequelae were restricted to the primary infection group. Only a slightly lower proportion of infants (83% vs 94%) were identified by screening (versus referral) in the primary infection group compared with the non-primary infection group. They concluded that “pre-existing maternal antibody to CMV protects the foetus and lessens the severity of the sequelae of congenital CMV infection” (60).

The prevailing view among public health officials has therefore long been that congenital CMV infections in seropositive women are mild or inconsequential. Given that there is no active screening for congenital CMV infection in most parts of the world, most infants are subclinically infected and the symptoms of CMV infection in the newborn are non-specific, such a dogma would be difficult to disprove in the absence of systematic studies. This has perpetuated a focus on strategies that target primary CMV infection in high income countries, and has led to continued inattention to congenital CMV infection in low and middle income countries.

In recent decades there has been a growing body of evidence to counter this view (11, 13, 14, 61). Ahlfors *et al*. documented the findings from a prospective long term study of 76 infants with congenital CMV infection in Sweden identified by population screening of 16 474 newborns between 1977-1985 (61). Of the 80% (62/76) of infected newborns in whom type of maternal infection was determined, 48% (30/62) were attributable to primary maternal infection and 52% (32/62) to

non-primary infection. At seven years of follow up the frequency of neurological sequelae was 18% (5/27) in the primary infection group and 26% (6/23) in the non-primary infection group. Furthermore, a pooled analysis of data from this Swedish study combined with a large prospective study of childhood outcomes[j] in the United Kingdom was recently reported by Townsend *et al*. (Table 3) (13). The authors stated that their analysis "highlights the contribution of non-primary maternal infection to the burden of congenital CMV disease in childhood, even in countries where maternal seroprevalence is relatively low" (13).

Table 3. Frequency of congenital CMV infection outcomes by type of maternal infection combining data from population based studies in the UK and Sweden.

	Symptomatic infection at birth			Moderate/severe outcomes		
	no.	n	%	no.	n	%
Type of maternal infection						
Primary	8	82	9,8	5	73	6,9
Non-primary	6	45	13,3	9	39	23,1
Not known	5	49	10,2	2	42	4,8
Total	19	176	10,8	16	154	10,4

Abbreviations: no., number with outcome; n, number of infants with congenital CMV infection in that category.
(*Source: Adapted from Townsend 2013, (13)*)

In the US, Ross *et al*. examined hearing outcomes in a cohort of 300 infants with congenital CMV infection, identified by newborn virologic screening, by type of maternal infection (11). They documented similar rates of hearing loss of 10% and 11% in infants born to women with non-primary and primary infection, respectively. Although progressive and severe/profound hearing loss was more common in the

[j] Assessments included neurologic, audiologic, and ophthalmologic status and/or development at least up to age 5 years.

primary infection group, the occurrence of bilateral and high frequency hearing loss was not different between the two groups. In support of these findings, de Vries *et al*. recently conducted the first meta-analysis of hearing loss by type of maternal infection pooling data from seven prospective studies that identified congenital CMV infection by screening of newborns. The pooled estimate of hearing loss among infants with congenital CMV infection was 13% (50/385, 95% (Confidence Interval (CI) 10%–16%) in the primary infection group and 11% (28/253; 95% CI, 7%–15%) in the non-primary infection group. Of note, these data are consistent with recent findings from large newborn CMV screening studies in India and Brazil where maternal CMV seropositivity is nearly universal (12). In Brazil, 9.8% (95% CI, 5.1-16.7%) of congenital CMV infected infants developed hearing loss (12), whereas unilateral or bilateral sensorineural hearing loss (SNHL) was observed in 2/20 (10%) children with congenital CMV infection identified between 2010 and 2012 in New Delhi, India (personal communication, Dr Suresh B Boppana).

Apart from the type of maternal infection, the frequency of congenital CMV infection sequelae has also been examined in relation to the type of newborn infection, symptomatic versus asymptomatic. Townsend *et al*. in their pooled analysis of data from the United Kingdom (UK) and Sweden reported that 42% of symptomatic (8/19) compared with 14% (19/135) of asymptomatic infants developed sequelae (13). In a recent systematic review of studies which included populations of low socioeconomic status and avoided studies with an overrepresentation of maternal primary infection, Dollard *et al*. showed that the overall prevalence of symptomatic infection[k] was 13%, with a 40-58% risk of

[k] Clinical indications of CMV infection in newborns is known as cytomegalovirus inclusion disease and was defined as the presence of one or more of the following symptoms: petechiae, jaundice with associated hyperbilirubinaemia, hepatosplenomegaly, thrombocytopaenia, chorioretinitis, seizures, microcephaly, intracranial calcifications or foetal hydrops (excluded intrauterine growth restriction).

sequelae[l] in symptomatic infants and a 13% risk of sequelae in asymptomatic infants (16). Owing to the preponderance of asymptomatic congenital CMV infections and the notable frequency of sequelae in this group, Dollard *et al*. estimated that asymptomatic congenital CMV infection contributes a greater burden of cases of permanent sequelae as compared with symptomatic congenital CMV infection (Dollard, Grosse *et al*. 2007) (Table 4).

Table 4. Estimates of long-term sequelae in infants with congenital CMV infection according to type of infant infection.

	Symptomatic	Asymptomatic
Number of infants	127 (12.7%)	873 (87.3%)
Deaths	5	0
Survivors	122	873
Number with permanent sequelae	50-70 (40-58%)	118 (13.5%)
Conclusion	17-20% of the 1000 infected infants will have permanent sequelae; 1/3 from the symptomatic group and 2/3 from the asymptomatic group	

(*Source: Dollard 2007, (16)*)

2.2.3 Role of HIV in congenital CMV infection birth prevalence

Studies conducted in Europe and the Americas suggest an increased risk of intrauterine CMV transmission in mothers infected with both HIV and CMV (Table 5) (19-21). By impairing cellular immunity, maternal HIV may increase the frequency or magnitude of CMV reactivation/re-infection in the mother, thus predisposing to intrauterine transmission of CMV. HIV infection of the foetus may

[l] Hearing loss, cognitive deficit, or motor deficit.

also increase its susceptibility to CMV infection in utero. As HIV infected mothers are not a homogenous population, the risk of congenital CMV infection is not evenly distributed among these women. Indeed, the birth prevalence among infants born to HIV-infected women has been shown to vary according to maternal characteristics such as CD4 count, antiretroviral therapy (ART) use and infant HIV infection status. Infant HIV infection, as well as maternal HIV stage, CD4 count, and ART/highly active antiretroviral therapy (HAART)[m] use may serve as markers of maternal immune status during pregnancy and therefore of the risk for congenital CMV infection.

Among infants infected with HIV, the birth prevalence of congenital CMV infection ranges from 4.3% to 29% (19, 20). In a retrospective case control study of congenital CMV infection and maternal HIV in a Thai clinical trial of zidovudine prophylaxis, infants with in utero HIV were found to have an eight times increased odds of congenital CMV infection compared with HIV exposed but uninfected controls (62), suggesting that congenital CMV infection was a risk factor for perinatal HIV in the pre-HAART era (or vice versa). In contrast, in HIV-exposed but uninfected newborns the birth prevalence of congenital CMV infection varies from 2.2% to 6.3%, with rates at the lower end of the range in post- compared with pre-HAART era (Table 5) (19, 20).

Studies in Europe (Table 5) have shown overall a reduction in congenital CMV infection birth prevalence over time and with increasing maternal HAART use, however rates of congenital CMV infection in HIV exposed infants have persisted above those of the general population in these countries (20, 63, 64). Furthermore,

[m] Congenital CMV infection rates in the context of HIV-infected pregnant women span two time periods, the era preceding and following the introduction of ART/HAART for the prevention of mother-to-child-transmission of HIV and restoration of maternal immunity and health. Highly active antiretroviral therapy refers to the era of triple antiretroviral therapy that began in the early 2000's.

data from France, which represents the largest prospective study of maternal HIV and congenital CMV infection, has also demonstrated an association between congenital CMV infection and infant HIV infection, as well as maternal CD4 count < 200 cells/μL close to delivery (20). The French Perinatal Cohort authors also examined the impact of duration and type of antiretroviral therapy on congenital CMV infection birth prevalence (20). They documented an increased risk of congenital CMV infection among mothers initiating ARV therapy in the second trimester compared with earlier in pregnancy, and suggested that earlier initiation of HAART may protect against CMV reactivation for a longer duration of the pregnancy. No difference was found in the risk of congenital CMV transmission by type of ARV therapy[n].

[n] Categories of ARV therapy in this study were HAART, two nucleoside reverse transcriptase inhibitors (NRTI), monodrug therapy and none.

Table 5. Birth prevalence of congenital CMV infection among HIV exposed infants (HIV-E), including HIV exposed uninfected infants (HIV-EU) and HIV exposed infected infants (HIV-EI) from studies in Europe, the United States and Brazil.

Author and year of publication	Country and year of study	Study population	CMV test method	n (infants)	HIV-E		HIV-EU		HIV-EI		Comment
					n	%	n	%	n	%	
Guibert 2009	France 1993-2004	HIV infected pregnant women and their children enrolled at 96 centers throughout France enrolled in EPF cohort.	urine culture/PCR	4797	111/4797	2.3%	94/4302	2.2%	13/126	10.3%	Prevalence of congenital CMV decreased over time with increasing use of HAART in the cohort. Congenital CMV transmission was associated with delivery period, maternal age, time at ARV initiation, maternal CD4 < 200 cells/μL close to delivery.
Barbi 2006	Italy 2000-2005	Retrospective survey of cohort of consecutive HIV infected pregnant women on ART/HAART.	saliva PCR/ culture	303	9/303	3.0%	9/301	3.0%			Congenital CMV infection prevalence was about 10 times higher than in the open Italian population (0.2%) but lower than that found in a previous Italian study on babies born to HIV infected mothers (5.7%). The lower rate of transmission may have been due to the reduction of CMV reactivation caused by ART/HAART. HIV transmission risk was 0.6%.
Gabriel 2005	Spain 1987-2003	Prospective cohort of consecutive HIV infected women and their children.	urine culture	257	12/257	4.6%	6/234	2.6%	6/23	26%	Before 1997 the congenital CMV infection prevalence among HIV exposed infants was 9.2 % vs 1.3% in the second period ($p < 0.01$), suggesting an association with study period. In infants born to HIV-infected women without zidovudine therapy the prevalence was 6.3 % compared with 3.1 % in the group with zidovudine therapy ($p > 0.05$).
Kovacs 1999	USA, California pre 1996	HIV infected women and their children recruited from 5 clinical centres in the US (P2C2 HIV study group).	urine culture/PCR	440	11/247	4.5%	9/200	4.5%	2/47	4.3%	No significant difference in frequency of congenital CMV infection in HIV infected and uninfected infants.
Frederick 2012	USA, California 1998-2002	Los Angeles County referral site for HIV infected women and their children.	urine/ saliva culture		248	3.6%					Prevalence of congenital CMV infection did not differ over the two time periods (1988-1996 vs 1997-2002) by infant HIV status, maternal HAART/ART use, maternal CD4 count or demographic/birth characteristics.
Doyle 1996	USA, Texas 1988-1995	HIV infected women and their infants.	urine culture	206	10/154	6.5%	5/130	3.8%	5/24	21%	Rate of in utero HIV infection was significantly higher in HIV infected infants.
Duryea 2010	USA, Texas 1997-2005	Retrospective cohort of HIV infected women delivering at a large urban county hospital.	urine culture	333	10/333	3%	10/329	3.0%	4		HAART was available for these women during the entire study period; HAART use was higher among mothers of CMV uninfected infants. CD4 < 200 cells/μL was more common among mothers of CMV infected infants. HIV mother-to-child transmission risk was 1%.
Mussi-Pinhata 1998	Brazil 1992-1995	Consecutive deliveries of low income population of CMV seropositive women at a University Hospital. Ninety one percent were asymptomatic HIV carriers and none had late stage HIV infection.	urine culture/PCR	325			4/150	2.7%			No difference in transmission risk between HIV exposed and HIV unexposed infants. Congenital CMV infection birth prevalence was 2.9% (5/175) among HIV unexposed infants. None of the 21 HIV infected infants had congenital CMV infection.
Slyker 2009	Kenya 1999-2003	HIV infected pregnant women and their children enrolled in a cohort to study acute CMV infection.	cord blood PCR				20	6.3%	15	29%	
Mwaanza 2013	Zambia 2012-2013	HIV exposed infants admitted to NICU.	urine PCR	395	9/79	11.4%					HIV exposed and HIV unexposed infants were studied. No data was provided on infant HIV infection rates or maternal CD4 counts.

Abbreviations: EPF, French Perinatal Cohort; ART/HAART, antiretroviral therapy or highly active antiretroviral therapy; USA, United States of America; P2C2, Pediatric Pulmonary and Cardiovascular Complications; NICU, neonatal intensive care unit.
(Source: Adapted from Duryea 2010, (19). Other references referred to in the table are Guibert 2009 (20); Barbi 2006 (64); Gabriel 2005 (63); Kovacs 1999 (65); Frederick 2012 (21); Doyle 1996 (66); Duryea 2010 (19); Mussi-Pinhata 1998 (67); Slyker 2009 (68); Mwaanza 2014 (69))

In the US, studies from Texas mirror the data from Europe showing an association of congenital CMV infection with infant HIV infection, and a reduction in congenital CMV infection birth prevalence in post-HAART era (19, 66). Duryea *et al*. also reported an inverse trend between categories of maternal CD4 counts and congenital CMV infection prevalence in HIV-exposed uninfected newborns born to women using prenatal antiretroviral therapy in the US (19). Data from California, on the other hand, show no change in congenital CMV infection birth prevalence among infants of HIV infected women from pre- to post HAART era and no relationship of congenital CMV transmission with infant HIV infection, although the risk of congenital CMV infection in HIV infected women is higher than the general population as in studies from other industrialized countries (21, 65).

In low and middle income countries and high seroprevalence settings, data on congenital CMV infection in infants born to HIV-infected women is sparse. In the pre-HAART era in Brazil, a study of a selected population of asymptomatic HIV infected women and a control group of HIV uninfected women showed no association of maternal HIV with congenital CMV infection (67). This study suggests that in highly seroimmune populations, maternal HIV may not increase the risk for congenital CMV infection above that of the general population if maternal health (and by extension, maternal immunity) is intact. Sub-Saharan Africa bears the burden of the HIV epidemic with young women of child bearing age disproportionately affected (18, 22). However, there is no population based data on congenital CMV infection in HIV infected women in this region of the world (70). In a recent cross-sectional survey of neonates admitted to a neonatal intensive care unit (NICU) in Zambia, Mwaanza *et al*. demonstrated that maternal HIV increased the risk of congenital CMV infection (OR 6.7; 95% CI, 2.1-20.9), however no data was reported on maternal CD4 count, ART/HAART use or infant HIV status (69). An important drawback of the sample based estimates from this study relates to the fact that case ascertainment in NICUs may incorrectly

represent the burden of CMV in the newborn period. Lanzieri *et al*. recently assessed the burden of congenital CMV infection in infants admitted to NICUs in California for the period 2005 to 2010 (71). The authors noted that they were only able to identify 5% of the expected number of infants with symptomatic congenital CMV infection for that period.

Overall these data show that maternal ART/HAART use alters the natural history of HIV in pregnant women, and may therefore alter the natural history of CMV in the mother as well as of congenital CMV infection, in a beneficial way (72). While there is a tendency for ART/HAART to lower the transmission of congenital CMV infection, most studies show that congenital CMV birth prevalence in HIV exposed infants does however remain higher than that of the general population, suggesting a residual excess risk even in women on ART/HAART. As none of the studies in HIV infected women included a contemporaneous comparison group of HIV uninfected women, except small studies of selected populations from Brazil and Zambia which did not report on ART/HAART use (67, 69), the impact of maternal ART/HAART on congenital CMV infection birth prevalence is inconclusive. With increasing rollout of ART/HAART in sub-Saharan Africa in the last decade (73), a careful assessment of the risk of congenital CMV infection as well as the burden of congenital CMV infection associated sequelae in infants born to HIV infected women is warranted.

A sizeable portion of HIV infections in sub-Saharan Africa are concentrated in South Africa and disproportionately affect young women of childbearing age (18). In recent years, the South African HIV epidemic has stabilized, with a national adult HIV prevalence of 17.3% (95% CI, 16.6%-18.1%) in 2011 (18). In addition, strategies to prevent mother-to-child transmission (MTCT) of HIV, especially rollout of ART, has resulted in a successful reduction of HIV perinatal transmission rates to under four percent (74). Despite these advances, the antenatal HIV prevalence

in South Africa remains alarming, ranging from 17% (95% CI, 14.3%-20%) to 37.4% (95% CI, 35.8%-39%) in 2011 (18).

With an annual birth cohort in South Africa of approximately one million infants, nearly 300 000 infants are born HIV-exposed each year. Assuming a 1%, 3% and 10% risk of CMV transmission in HIV unexposed (n=700 000), HIV exposed uninfected (n=294 000) and HIV infected (n=6 000) newborns respectively, we estimated that around 1642 newborns per 100 000 deliveries would be born congenitally infected with CMV (annual excess of 6420 infected newborns due to maternal HIV) (Table 6). Based on the rates of sequelae from population based estimates in the US and UK, approximately 273 infants per 100 000 live born infants in South Africa would develop hearing and mental deficits. Furthermore, South Africa is likely to have roughly 2.5 times the rate of congenital CMV infections and sequelae per capita as compared to the US.

The above analysis ignored the potential additional risk of congenital CMV infection associated sequelae that may occur in HIV exposed infants. In pre-HAART era, HIV exposure in the absence of infection was shown to produce immune defects, as well as significantly higher rates of childhood morbidity compared with unexposed infants (75-77). It is plausible that intrauterine CMV infection may have a worse clinical course in HIV exposed but uninfected infants in Africa even in the HAART era. As a result, South Africa potentially faces a unique burden of congenital CMV infection. In spite of this, there are no reliable data on the birth prevalence of congenital CMV infection in general, or in the subpopulation of HIV-exposed infants in South Africa.

Table 6. Estimated prevalence of congenital CMV infection associated sequelae in South Africa and the United States.

	United States	South Africa		
		HIV unexposed	HIV exposed uninfected	HIV exposed infected*
Annual birth rate	4 000 000	700 000	294 000	6 000
Birth prevalence of congenital CMV infection	1%	1%	3%	10%
Annual cases of congenital CMV infection	40 000	7000	8820	600
Total annual cases of congenital CMV infection	40 000			16 420
Total symptomatic at birth (10% of infected newborns)	4000			1642
Permanent sequelae among symptomatic (40%)	1600			657
Total asymptomatic at birth (90% of infected newborns)	36 000			14 778
Permanent sequelae among asymptomatic (14%)	5040			2069
Total infants with permanent sequelae	6640			2726
Per 100 000 capita§	2.1			5.2
Per 100 000 deliveries	166			273

* Assuming an HIV mother-to-child transmission rate of 2% in South Africa.
§ Based on a population size in the United States of 316 000 000 persons and in South Africa of 52 000 000 persons.

2.2.4 Other risk factors for congenital CMV infection

Apart from maternal non-primary CMV infection driving a higher birth prevalence of congenital CMV infection at a population level, and the impact of maternal HIV on congenital CMV infection described above, other risk factors for congenital CMV infection have also been identified mainly from data in upper income settings. These include preterm birth, admission to NICU, Black race, low socioeconomic status, young maternal age and CMV re-infection in pregnancy (8, 46). Factors that may be relevant to high seroprevalence settings, including South Africa, are discussed here.

2.2.4.1 Young maternal age

Kenneson *et al*. summarized data on maternal age and congenital CMV transmission from studies in the US and Korea that included data on maternal seroprevalence (8). They found an inverse relationship between maternal age and birth prevalence of congenital CMV infection. However, after adjusting for maternal seroprevalence, the association was no longer significant suggesting that maternal seroprevalence was a confounding factor. In a separate large US study that included an urban population of predominantly low income, non-white mothers delivering at a public hospital between 1980 and 1990, young maternal age (<20 years) was associated with a nearly five times increased odds (adjusted prevalence odds ratio (aPOR) 4.8; 95% CI, 2.6-8.9) of congenital CMV infection, compared with mothers aged 30 years and older (78). The authors suggested that younger women may have more frequent exposure to CMV through sexual activity, or for biological reasons may have poorer control of virus excretion, which places them at an increased risk of congenital CMV infection.

2.2.4.2 Maternal CMV re-infection

Re-infection with a new strain of CMV during pregnancy may carry a higher risk of virus transmission in seropositive women than reactivation of endogenous virus. Yamamoto *et al*. recently conducted a case control study of CMV re-infection in Brazil and demonstrated that maternal re-infection during pregnancy was more frequent among infants with congenital CMV infection than uninfected control newborns (OR 4.4; 95% CI 1.9-10.2) (46). In high seroprevalence settings, the number of people shedding virus and accordingly the circulating pool of virus and force of infection is greater than in low seroprevalence settings (14), increasing the likelihood of a re-infection event occurring during pregnancy.

2.2.4.3 Preterm birth and NICU admission

In a highly seroimmune population in Brazil, preterm birth was not associated with the birth prevalence of congenital CMV infection. In this study, the prevalence of congenital CMV infection was 2.1% (95% CI, 0.8%-4.7%) among a sample of 289 consecutive born preterm infants, and 1.8% (95% CI, 0.5%-5.7%) among 163 consecutive born term infants as assessed by urine culture or polymerase chain reaction (PCR) (79). Santos *et al*. also determined the frequency of congenital CMV infection among newborns from a NICU in Brazil, and found a prevalence of 6.2% (20/292) by urine PCR screening (80). A study of selected neonates admitted to NICU in Hungary also reported similar congenital CMV infection frequencies of 16.7% (6/36) and 14.7% (5/34) in pre-term and term newborns, respectively (81).

2.3 Prevention of the burden of congenital CMV infection associated sequelae

From a theoretical perspective, the disease burden from congenital CMV infection could be reduced by intervening at different stages of the natural history of this infection. This could be accomplished firstly by preventing in utero transmission of the virus (primary prevention). If this fails, steps can be taken to limit the effects of the infection in the newborn (secondary prevention).

Currently, there are no effective biomedical strategies available for the prevention of in utero CMV transmission. Boosting of maternal immunity in seronegative women with primary infection by the administration of CMV hyperimmune globulin (HIG) was recently shown in a randomized controlled trial to be ineffective (82). Efforts to develop an anti-CMV vaccine continue, with increasing recognition that to reduce the burden of congenital CMV disease an effective vaccine would need to induce protective immune responses to protect seronegative women against primary CMV infection, as well as to protect seropositive women who already have natural immunity to CMV. However, as the immunological correlates of protection against CMV transmission in seronegative and seropositive women remain elusive, vaccine design is challenging (36).

In the absence of effective immunisation strategies, the restriction of maternal CMV infection relies predominantly on behavioural measures to limit CMV infection during pregnancy (primary infection in seronegative women, and re-infection in seropositive women) such as frequent handwashing, and possibly safe sexual practices (83, 84). However, the effectiveness of these strategies have not been evaluated in high seroprevalence settings.

Secondary prevention requires the prompt identification of congenital CMV infection by virologic screening in the newborn period, coupled with the deployment of measures that can ameliorate sequelae in the infected newborn, such as the use of antiviral therapy[o] and regular developmental and auditory assessments (85, 86). Because symptoms of congenital CMV infection are non-specific, and the vast majority of infections and associated sequelae occur in asymptomatic infants (16), a substantial reduction of the disease burden from this infection requires a strategy of universal or targeted newborn CMV screening. The cost-effectiveness of universal newborn CMV screening is currently debatable and context-specific (87, 88). While targeted screening approaches generally have greater appeal in developing settings, these require the prior identification of subpopulations of mothers or infants at increased risk for in utero CMV transmission.

2.4 Laboratory considerations

The diagnosis of congenital CMV infection relies on the detection of CMV in clinical specimens (urine or saliva) within the first 14 days of postnatal life (89, 90). This cut off time period is required to differentiate between infections acquired in utero compared with those acquired during passage through the birth canal and postnatally (38), as the incubation period from inoculation of the virus to peripheral excretion is 2 to 8 weeks (89). The usual laboratory methods for the identification of CMV in newborn specimens are growth of whole virus in cell culture, or detection of virus DNA by PCR. While most clinical laboratories are experienced in congenital CMV infection diagnosis using urine, obtaining urine samples from infants is often a difficult task, and may require the use of invasive techniques.

[o] Antiviral therapy is currently only recommended for symptomatic infants.

In recent years, the use of saliva for congenital CMV infection diagnosis has been receiving increasing attention. This is not unintuitive given that the virus itself was initially discovered in the salivary glands of infants with disseminated congenital CMV disease during post-mortem studies in the 1950s (91). Oral swabs of the inner cheeks can be obtained noninvasively and without technical expertise, making saliva by far a more practical specimen than urine (90, 92). The virus is shed in high concentrations in both urine and saliva, and there is a high agreement of virus detection between the two sample types when either culture or PCR are used (92-94).

Saliva screening of congenital CMV infection using a low cost rapid in house real time PCR assay was recently evaluated in a multi-centre prospective study of 34 989 newborns in the USA (90). The authors reported a high sensitivity (100%, 95% CI 95.8%-100%) and specificity (99.9%, 95% CI 99.9%-100%) of the PCR assay compared with culture of saliva, and highlighted the utility of saliva PCR for mass newborn screening. As the high cost of PCR using commercial CMV assays is often seen as a barrier to newborn screening in low and middle income countries, the widespread availability of such cheaper technologies are urgently needed to better inform cost-effectiveness analyses.

2.5 Summary

Congenital CMV infection is a common cause of congenital infections in all parts of the world, with higher birth prevalences observed in high seroprevalence settings. Seeing as firstly the transmission risk for congenital CMV infection is higher in CMV seropositive compared with CMV seronegative women, secondly most women are seropositive for CMV and thirdly, sequelae in seropositive women occur at a similar rate to seronegative women, maternal non-primary CMV infection is the major source of congenital CMV infection and sequelae worldwide. That is,

contrary to the commonly held view, maternal pre-existing antibody neither protects against in utero CMV transmission or disease in infected infants when a population perspective is taken. In addition, a substantial burden of damaging neurological disease, particularly hearing loss, occurs among infants with no clinical signs at birth and who can, therefore, only be identified by newborn virologic screening.

In the US, congenital CMV infection is the second leading cause of childhood hearing loss after genetic defects (7). This highlights the potentially preventable burden of hearing loss due to congenital CMV infection in industrialized settings. In the absence of early detection of hearing impairment, speech, language, and social impairment occur in a significant number of infected children, with an estimated annual cost of $1-$2 billion in the US (83). Importantly, even unilateral and milder forms of hearing impairment are harmful to cognitive, psychological and socio-emotional development (95-99). In view of these adverse consequences, early hearing impairment detection and intervention (EDHI) is gaining increasing recognition as a cost-effective strategy (95). But, childhood disabilities have not been regarded as a major public health priority in many parts of the world, and data on the burden of childhood hearing loss are scarcely available from low and middle income countries (96). In addition, data from developed countries suggest a sizeable burden of congenital CMV disease in low and middle income countries (13, 14), although there are few studies documenting hearing outcomes associated with congenital CMV infection in these settings. With emerging reports from large newborn CMV screening studies in Brazil and India confirming a role for congenital CMV infection as a significant cause of SNHL in high seroprevalence settings (12), studies in Africa are even more pressing.

Sub-Saharan Africa may have an additional burden (above that resulting from a high CMV seroprevalence) of congenital CMV infection due to the high burden of HIV in this region of the world. Despite this, there is no reliable data on congenital

CMV infection birth prevalence in African HIV exposed newborns (9, 70). With a large proportion of pregnant women living with HIV in South Africa, determining the birth prevalence of congenital CMV infection is timely and of high relevance in this country.

Chapter Three: Journal article manuscript

This section presents a portable document format version of the journal article that was published in the Clinical Infectious Diseases Journal encapsulating the key findings from this study[p,q]. The manuscript was received by the Journal on 19 September 2013, accepted on 10 February 2014 and first published online on 23 February 2014. The article begins with a structured abstract, followed by four major sections: background, methods, results and discussion. Author contributions, as well as a timeline of the research process and an expanded discussion of the study limitations are presented later in Chapter Four.

[p] The article uses American English spelling and the rest of the dissertation uses British English spelling.

[q] "Congenital cytomegalovirus" and "congenital CMV" in the manuscript refer to intrauterine infection with CMV, and not CMV disease. There was no outcome data collected as part of this study.

Birth Prevalence of Congenital Cytomegalovirus Among Infants of HIV-Infected Women on Prenatal Antiretroviral Prophylaxis in South Africa

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Background. A high rate of congenital cytomegalovirus (CMV) has been documented in human immunodeficiency virus (HIV)–exposed infants in industrialized settings, both in the pre– and post–highly active antiretroviral therapy (HAART) era. Only limited data on the birth prevalence of congenital CMV among infants of HIV-infected women on prenatal antiretroviral (ARV) prophylaxis are available from sub-Saharan Africa, despite a high prevalence of both infections. We evaluated the prevalence of congenital CMV in HIV-exposed infants in the Western Cape, South Africa.

Methods. HIV-infected mothers were recruited in the immediate postnatal period at a referral maternity hospital between April and October 2012. Maternal and infant clinical data and newborn saliva swabs were collected. Saliva swabs were assayed by real-time polymerase chain reaction for CMV. Data were analyzed using univariate and multivariate logistic regression analyses to determine specific demographic, maternal, and newborn characteristics associated with congenital CMV.

Results. CMV was detected in 22 of 748 newborn saliva swabs (2.9%; 95% confidence interval [CI], 1.9%–4.4%). Overall, 96% of mothers used prenatal ARV prophylaxis (prenatal zidovudine, 43.9%; HAART, 52.1%). Maternal age, gestational age, prematurity (<37 weeks' gestation), type of ARV prophylaxis, length of ARV prophylaxis, birth weight, small for gestational age, and infant feeding choice were not significantly different between CMV-infected and -uninfected infants. Maternal CD4 count <200 cells/μL during pregnancy was independently associated with congenital CMV (adjusted odds ratio, 2.9; 95% CI, 1.2–7.3). A negative correlation between CMV load in saliva and maternal CD4 count was observed ($r = -0.495$, n = 22, $P = .019$).

Conclusions. The birth prevalence of congenital CMV was high despite prenatal ARV prophylaxis, and was associated with advanced maternal immunosuppression.

Keywords. HIV; congenital CMV; prevalence; antiretroviral prophylaxis; South Africa.

Cytomegalovirus (CMV) is a leading cause of congenital infections worldwide and a leading nongenetic cause of childhood hearing loss in the post–rubella vaccination era. The birth prevalence of congenital CMV in a population is associated with the proportion of mothers who are seropositive for CMV [1]. In developing country settings with near-universal CMV seroimmunity, congenital CMV rates of 1%–5% have been reported, compared with rates of 0.6%–0.7% in industrialized nations [2–4].

Human immunodeficiency virus (HIV)–infected mothers constitute a special subpopulation, in whom an increased frequency of in utero CMV transmission

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has been consistently documented in countries in Europe and the Americas. The birth prevalence of congenital CMV in these settings ranges from 4%–26% among HIV infected newborns, to 1.2%–5% in HIV-exposed but -uninfected (HIV-EU) infants [5–7]. Maternal risk factors associated with in utero CMV transmission have not been systematically assessed, although an association with advanced maternal immunosuppression (CD4 <200 cells/μL), both in HIV-infected and -uninfected infants, was documented in the French Perinatal Cohort (FPC) [6]. HIV-infected and -exposed infants who acquire CMV in the first 18 months of life have a higher risk of neurological morbidity [8, 9].

The impact of prenatal antiretroviral (ARV) prophylaxis, either prenatal zidovudine (ZDV) or highly active antiretroviral therapy (HAART), on congenital CMV transmission in HIV-infected women is unclear. In the FPC, a reduction in congenital CMV transmission rates to 1.2% in the HAART era, from 3.5% in the pre-HAART era, was observed among HIV-EU neonates [6]. However, congenital CMV transmission rates remained constant over time in 2 consecutive HIV-exposed birth cohorts in the United States, despite increasing use of maternal prenatal HAART [10].

Data on congenital CMV infection among HIV-exposed infants in sub-Saharan Africa is limited. A study in Kenya, conducted on a small sample of infants born to HIV-infected women who used perinatal ZDV, reported congenital CMV rates of 29% (n = 15) and 6.3% (n = 20) in HIV-infected and -uninfected newborns, respectively [11]. In addition, a recent study of high-risk newborns admitted to a referral neonatal unit in Zambia documented a birth prevalence of congenital CMV of 3.8% (15/395) overall, and 11.4% (9/79) in those infants exposed to maternal HIV [12]. As the HIV epidemic in sub-Saharan Africa disproportionately affects women of childbearing age, and antenatal HIV seroprevalence rates are stabilizing at alarming proportions in many countries, the sparsity of data on congenital CMV in these populations is concerning [13]. We evaluated the prevalence of congenital CMV in a large sample of HIV-exposed newborns in South Africa.

METHODS

Study Population

HIV-exposed newborns were recruited from the postnatal wards of Mowbray Maternity Hospital (MMH), a secondary-level referral hospital in the Western Cape Province of South Africa between April and October 2012. MMH serves the local Mowbray area, in addition to surrounding Midwife Obstetric Units offering subsidized healthcare for pregnant mothers and their babies in the region. Approximately a third of live-born infants (11 000/35 000) in 2012 in the Metropole West region of the Western Cape were born at MMH. Of the 11 000 babies born at MMH, approximately 13% are HIV exposed. Approximately 95% of patients seen at MMH do not have access to private healthcare facilities. Overall, the patient population is representative of the general Western Cape population, consisting of approximately 50% mixed race and 50% indigenous black African as well as an increasing number of African migrants/refugees. Maternal HIV status is ascertained prenatally by a rapid HIV test.

Study Design and Data Collection

The study was carried out as an unlinked anonymous cross-sectional survey with convenience sampling. Mothers were eligible for the study if they were known to be HIV-infected, 18 years and older, within 14 days after delivery, and living in the greater Cape Town area and had given written informed consent. Eligible mothers were approached during Monday through Friday for participation in the study. Maternal age, CD4 count, date of CD4 count, type of prenatal ARV prophylaxis (none, intrapartum ZDV and single-dose nevirapine in labor only, prenatal ZDV, HAART) and date of commencement of ARV, infant feeding choice, and infant gestational age and birth weight were recorded. Infants with birth weights <10th percentile for the gestational age were considered small for gestational age. A saliva swab in viral transport medium was collected from enrolled newborns; this was done immediately before the next feed in breastfed infants. There were no follow-up visits and mothers were not informed of the infant's CMV status.

Testing of Samples for CMV

Saliva swabs were stored at −80°C at a regional laboratory in Cape Town. After the completion of study enrollment, samples were shipped to the University of Alabama at Birmingham (UAB) for CMV testing. The newborn saliva swabs were processed and tested for CMV using a real-time polymerase chain reaction (PCR) assay described previously [14]. The PCR positive samples were also tested by the rapid culture method to confirm the PCR result.

Ethical Considerations

Ethical approval was obtained from the University of Cape Town Health Sciences Faculty Human Research Ethics Committee, MMH Ethics Committee, and the Institutional Review Board for Human Use of UAB. Written informed consent was obtained from HIV-infected mothers prior to enrolling infants in the study.

Statistical Analysis

The demographic, maternal, and newborn characteristics were compared between CMV-infected and -uninfected infants, and statistical significance was determined using χ^2 test, Fisher exact test, or student t test as appropriate. Crude odds ratios (ORs) were calculated from 2×2 tables to determine the association

of various factors with an increased risk for CMV transmission. Logistic regression analyses were performed to determine covariates that were independently associated with intrauterine transmission of CMV. Maternal age, birth weight, gestational age, length of ARV prophylaxis <120 days, and CD4 count <200 cells/μL were included in the logistic regression model. Adjusted odds ratios (aORs) were calculated and 95% confidence intervals (CIs) were determined using the parameter estimates and their respective standard errors. All statistical analyses were performed using the SPSS version 21 statistical package (IBM Corp, Armonk, New York).

RESULTS

Subjects and Specimens

A total of 833 HIV-infected mothers delivered 831 live-born infants during the study period (April to October 2012), and 757 of those mothers were approached for participation in the study during weekdays, of which 737 mothers consented for participation. An additional 11 babies were enrolled during the training component of the study in March 2012. Therefore, 90.9% (757/833) of eligible mothers were approached for participation in the study and most women (97.4% [737/757]) agreed to participate. The median age at the collection of saliva specimens was 1.0 day (interquartile range [IQR], 1.0–2.0 days).

Birth Prevalence of Congenital CMV

Of the 748 HIV-exposed newborns screened for congenital CMV by real-time PCR of saliva, 22 infants were positive, giving a prevalence of 2.9% (95% CI, 1.9%–4.4%). Twenty of the 22 PCR-positive saliva specimens were also positive by rapid culture for CMV.

Factors Associated With CMV Transmission

Overall, 96% of mothers used prenatal ARV prophylaxis (prenatal ZDV, 43.9%; HAART, 52.1%). The median length of ARV prophylaxis was 130 days (IQR, 95–165 days) for the women receiving prenatal ZDV (n = 327) and 167 days (IQR, 101–829 days) days for the group on HAART (n = 390). Of the 746 mothers with known CD4 counts, the timing of CD4 counts was available for 731 mothers. Of those, 721 mothers had CD4 counts obtained during the first or second trimesters of pregnancy at a median of 18 weeks (IQR, 13–24 weeks) of gestation, and the remaining 10 women had CD4 counts obtained prior to conception at a median of 29 weeks (IQR, 12–40 weeks) preconception. Maternal age, gestational age, prematurity (<37 weeks' gestation), type of ARV prophylaxis, length of ARV prophylaxis, birth weight, small for gestational age, and infant feeding choice were not significantly different between CMV-infected and uninfected infants (Table 1). Significantly more mothers with CD4 counts <200 cells/μL had babies with congenital CMV (8/126 [6.3%]) compared with

Table 1. Comparison of Demographic, Maternal, and Newborn Characteristics Between Cytomegalovirus-Infected and -Uninfected Newborns Exposed to HIV

Finding	CMV-Infected Infants (n = 22)	CMV-Uninfected Infants (n = 726)	OR (95% CI)	*P* Value
Maternal age, mean ± SD	27.1 ± 5.1	28.5 ± 5.3	0.95 (.9–1.0)	.23
Length of ARV prophylaxis, d, mean ± SD	344 ± 526	383 ± 652	0.99 (.9–1.0)	.78
Maternal CD4 count, mean ± SD	312 ± 211	395 ± 205	0.99 (.9–1.0)	.06
Gestational age, wk, mean ± SD	36.8 ± 2.7	37.5 ± 1.8	0.86 (.7–1.0)	.25
Birth weight, kg, mean ± SD	2.8 ± 0.7	3.0 ± 0.6	0.99 (.9–1.0)	.06
Type of ARV prophylaxis				.89
None	1 (4.5)	15 (2.1)		
Intrapartum ZDV/sdNVP	0 (0)	10 (1.4)		
Prenatal ZDV	9 (40.9)	319 (43.9)		
HAART	12 (54.5)	378 (52.1)		
Length of ARV prophylaxis <120 d	11 (50)	262 (36)	1.8 (.8–4.4)	.18
Maternal CD4 count <200 cells/μL	8 (36.4)	118 (16.3)	2.9 (1.2–7.0)	.01
Prematurity (<37 wk)	5 (22.7)	104 (17)	1.7 (.6–4.6)	.35
Small for gestational age	2 (9.1)	67 (9.3)	1.0 (.2–4.4)	.98
Infant feeding choice				.69
Breastfeeding	14 (63.6)	476 (65.6)		
Formula	7 (31.8)	236 (32.5)		

Data are presented as No. positive (%) unless otherwise specified.

Abbreviations: ARV, antiretroviral; CI, confidence interval; CMV, cytomegalovirus; HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; OR, odds ratio; sdNVP, single-dose nevirapine; SD, standard deviation; ZDV, zidovudine.

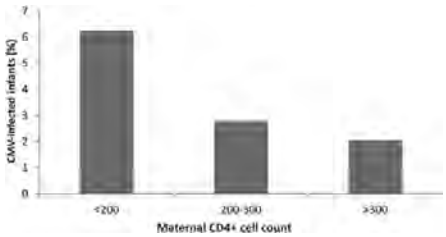

Figure 1. The proportion of human immunodeficiency virus–exposed infants with congenital cytomegalovirus (CMV) infection according to maternal CD4 counts was analyzed using χ^2 for trend analysis ($P < .005$). Ten of the 475 (2.1%) infants born to mothers with CD4 count >300 cells/μL had congenital CMV, whereas 4 of 145 (2.8%) and 8 of 126 (6.3%) with maternal CD4 counts between 200 and 300 cells/μL and <200 cells/μL, respectively, had congenital CMV.

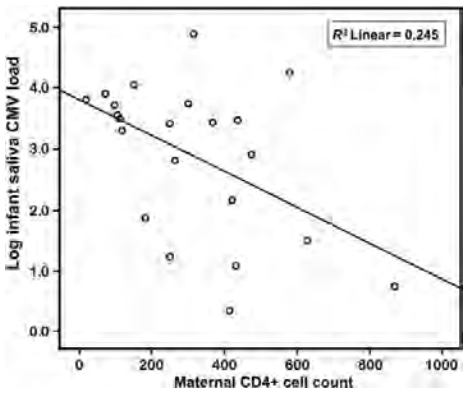

Figure 2. Scatterplot summarizing the relationship between maternal CD4 count and infant saliva cytomegalovirus (CMV) load. A negative correlation existed between the 2 variables ($r = -0.495$, n = 22, $P = .019$).

mothers with CD4 counts >200 cells/μL (14/620 [2.3%], $P = .01$) (Table 1). A significant association between maternal CD4 counts and intrauterine transmission of CMV was observed when the data were analyzed using χ^2 for trend ($P < .005$). Ten of the 475 (2.1%) infants born to mothers with CD4 count >300 cells/μL had congenital CMV, whereas 4 of 145 (2.8%) and 8 of 126 (6.3%) with maternal CD4 counts between 200 and 300 cells/μL and <200 cells/μL, respectively, had congenital CMV (Figure 1). As shown in Table 2, maternal CD4 count <200 cells/μL was the only factor that was independently associated with the risk for congenital CMV (aOR, 2.9; 95% CI, 1.2–7.3).

Viral Load

The geometric mean saliva CMV load among infected infants was 776.3 copies/mL (95% CI, 234.4–2570.4 copies/mL). A negative correlation between CMV viral load in saliva and maternal CD4 counts was observed ($r = -0.495$, n = 22, $P = .019$; Figure 2).

DISCUSSION

We evaluated the prevalence of congenital CMV among infants born to HIV-infected mothers in the Western Cape. This is the first study to document congenital CMV prevalence among a large sample of HIV-exposed infants in sub-Saharan Africa, and the first report of congenital CMV in South Africa. Despite universal maternal ARV prophylaxis, the prevalence of congenital CMV in this study was high compared with the rate in the general population documented in newborn CMV screening studies in the United States, Europe, and South America [1, 15]. In addition, the overall rate of congenital CMV in this study was consistent with that reported for HIV-EU infants in these countries [6, 7, 10].

We observed a significantly higher prevalence of congenital CMV among infants of mothers whose CD4 count during pregnancy was <200 cells/μL. On logistic regression analysis, this was the only factor independently associated with congenital CMV in the study population. Maternal immunosuppression close to delivery was also reported as an independent predictor of congenital CMV in the FPC [6]. In addition, we found an inverse relationship between various categories of maternal CD4 count and congenital CMV infection prevalence, further supporting the role of maternal immunity in CMV transmission (Figure 1). A similar, although nonsignificant, trend was recently documented in HIV-EU newborns of mothers on antenatal antiretroviral therapy (ART) in the United States [7]. In mothers with CD4 counts >200 cells/μL, the rate of congenital CMV infection in our study remained elevated in relation to populations with high CMV seroimmunity [2, 15].

The mechanisms by which maternal immunosuppression is linked to congenital CMV transmission in HIV-exposed newborns have not been elucidated. HIV-infected individuals are often CMV seropositive; therefore, it is plausible that impaired

Table 2. Logistic Regression Analysis to Determine Risk Factors for Congenital Cytomegalovirus Infection in HIV-Exposed Infants

Risk Factor	Adusted OR (95% CI)	*P* Value
Maternal age	0.93 (.8–1.0)	.14
Birth weight	0.99 (.9–1.0)	.41
Gestational age	0.86 (.7–1.1)	.52
Maternal CD4 count <200 cells/μL	2.9 (1.2–7.3)	.02
Length of ARV prophylaxis <120 d	1.6 (.7–3.9)	.29

Abbreviations: ARV, antiretroviral; CI, confidence interval; HIV, human immunodeficiency virus; OR, odds ratio.

maternal immunity could lead to more frequent reactivation or reinfection with CMV, or higher levels of CMV viremia [16]. HIV viremia during pregnancy, prior to the initiation of ART, or as a result of incomplete virological suppression in mothers on treatment, could mediate congenital CMV transmission by potentiating CMV replication [17], or leading to vertical transmission of HIV [18]. Although low rates of mother-to-child transmission (MTCT) of HIV (2%–3%) have been reported in the era of prenatal ARV prophylaxis in South Africa [19], ongoing HIV transmission could be sufficient to drive an excess risk of congenital CMV among HIV-exposed infants [5, 6]. As maternal CD4 count could reflect the ability to control CMV infection, the level of maternal HIV viremia, and the risk of MTCT of HIV, it may be a good overall predictor of congenital CMV transmission in HIV-infected women.

CMV transmission rates in this study did not differ between mothers using prenatal ZDV prophylaxis and mothers on HAART. A lack of association between type of prenatal ARV prophylaxis and prevalence of congenital CMV was previously documented in cohorts of HIV-exposed infants in the United States and Europe [6, 10].

Maternal CD4 count correlated inversely with CMV load in saliva of newborns with congenital CMV. This suggests that impaired maternal immunity may have resulted not only in increased CMV transmission to the fetus but also increased CMV replication in infected fetuses (Figure 2).

There are several limitations to this study. The background prevalence of congenital CMV in the general population and in the pre-ARV era in South Africa is not known. Therefore, it is not possible to determine whether the birth prevalence we observed is higher than expected for the general population or to delineate the impact of maternal ARV prophylaxis on congenital CMV prevalence. The anonymous unlinked design of this study precluded ascertainment of infants' HIV infection status, and clinical and follow-up assessments of CMV-infected infants. In addition, maternal viral load data were not routinely available, and thus not collected. Both maternal HIV load and infant HIV infection are possible mediators or confounders of the relationships we observed between congenital CMV transmission and maternal CD4 count. Furthermore, maternal prenatal CD4 counts were only obtained at the start of ARV prophylaxis and serial measurements were not available, making it difficult to assess maternal immune status later in pregnancy. The absence of demographic characteristics, such as race, education, and socioeconomic status, which could also have played a role in intrauterine CMV transmission, was an additional limitation. Although the storage of specimens for several months prior to testing may have affected the saliva real-time PCR results, this is unlikely as the specimens were kept frozen at −80°C for the study duration, and shipped on dry ice. Storage of specimens could have affected the results of the rapid culture assay, and this could explain the failure to culture 2 of the PCR-positive specimens. On the other hand, the results from our ongoing multicenter CMV screening study suggest that saliva real-time PCR assay is more sensitive than the rapid culture [20], which could be the basis for the discrepant culture and PCR results. Although it is not possible to exclude breast milk contamination of samples, this is unlikely given that our specimen collection method allowed an interval of at least 1–2 hours between infants' last exposure to breast milk and saliva swab collection.

The link between maternal CD4 count and congenital CMV transmission, shown here and in upper income countries, suggests that early initiation of combination ART in HIV-infected women of child-bearing age, prior to becoming immunocompromised, could lower the risk of congenital CMV in their infants. This has important implications for countries with high maternal HIV prevalence.

In South Africa, intensification of the adult HIV program in recent years [21] can be expected to continue to reduce the rates of immunosuppression among women prior to conception. In addition, recent implementation of World Health Organization option B guidelines for prevention of MTCT will impact pregnant women across all CD4 count categories. Therefore, the birth prevalence of congenital CMV in HIV-exposed infants in this population should be reevaluated, and the transmission rate in HIV-uninfected mothers should also be determined. In addition, systematic studies are needed to investigate the risk factors, including immunological and virological markers, associated with congenital CMV transmission in settings with a high burden of HIV. Furthermore, the burden of congenital CMV-induced hearing loss in this population, as well as the impact of congenital CMV infection on morbidity, growth, and development in HIV-exposed infants, should be evaluated. Finally, the validity of saliva real-time PCR for newborn CMV screening in populations where breastfeeding is common should be formally assessed.

In summary, the findings of our study demonstrate a high rate of congenital CMV in the era of prenatal ART in this South African population of HIV-exposed newborns, and an association of CMV transmission with advanced maternal immunosuppression.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Chapter Four: Additional Results, Discussion, Limitations and Recommendations

This chapter details additional analyses and discussion that is intended to supplement the journal article manuscript presented in Chapter Three. First, the findings from additional data analyses that were undertaken on the dataset but not included in the journal article will be presented. This will be followed by a critical review of the potential random and systematic sources of error in this study. The chapter will close with suggestions for how the study could have been conducted under ideal conditions.

4.1 Additional Results

The study sample comprised HIV infected women on different prenatal prophylaxis regimens, predominantly HAART and prenatal zidovudine (AZT). Although the risk of congenital CMV transmission was not associated with antiretroviral (ARV) regimen in this study, further analyses were undertaken for each of the ARV subgroups to determine if there were predictors of CMV transmission identifiable within these groups. This could provide insight into risk factors for congenital CMV infection within treatment subgroups in settings deploying WHO option A for the prevention of MTCT of HIV.

4.1.1 Statistical analysis[r]

All statistical analysis was undertaken using SPSS version 21 and Stata/IC 13.0 statistical packages. The dataset for the whole population was split into two files, one for mothers on AZT, and one for mothers on HAART.

[r] I conducted all analyses in this section independently.

4.1.1.1 Descriptive statistics

Means and standards deviation were computed for each of the continuous variables (maternal age, length of ARV prophylaxis, maternal CD4 count, gestational age and birth weight) for the AZT and HAART groups, as well as within AZT and HAART groups using infant CMV status as a grouping variable. Similarly for the categorical variables (length of ARV prophylaxis <120 days, maternal CD4 count <200, prematurity <37 weeks and infant feeding choice), crosstabulations were performed for each prophylaxis group as well as within prophylaxis groups according to infant CMV status.

4.1.1.2 Analytical statistics

The AZT and HAART groups were compared on each of the continuous (means) and categorical variables (proportions), using the unpaired t test and Chi square test respectively. All other analyses were intrasubgroup. The Mann-Whitney U test was used to compare the distribution of the continuous variables in CMV infected versus uninfected infants within each of the prophylaxis groups. The Chi square or Fisher's exact tests were used to test the statistical significance of the association between each of the categorical variables and congenital CMV infection within each of the prophylaxis groups. Bivariable logistic regression was used to compute the odds ratios with confidence intervals for the association of each continuous and categorical variable with congenital CMV infection. Finally, multivariable logistic regression was done to test the association of multiple prognostic factors with congenital CMV infection.

4.1.2 Results

Of the total study sample, 328 mothers used AZT during pregnancy for prevention

of MTCT of HIV, while 390 mothers were on HAART. Maternal CD4 count, length of ARV prophylaxis and birth weight were significantly different between the two groups (Table 7), with length of ARV prophylaxis <120 days more common in the prenatal AZT group and maternal CD4 count <200 more common in the HAART group.

In the AZT group, length of ARV prophylaxis, gestational age and birth weight were significantly different between CMV infected and uninfected infants, with a near significant association for maternal CD4 count (Table 8). In the multivariable logistic regression, birth weight was an independent predictor of congenital CMV infection among mothers on prenatal AZT, with length of ARV prophylaxis <120 days and maternal CD4 count approaching significance (Table 9). In the HAART group, none of the variables were associated with congenital CMV infection in bivariate (Table 10) or multivariable analyses (Table 11).

4.1.3 Discussion

Mothers enrolled into the study were subject to WHO Option A Guidelines for prevention of MTCT of HIV, which stratifies antiretroviral therapy regimens according to maternal CD4 count. Specifically, women with a favourable immunological profile (CD4 >350 cells/μL) were given AZT monotherapy from 12 weeks gestation, whereas women with a CD4 count <350 cells/μL were placed on triple antiretroviral prophylaxis. In this study, there was roughly an equal split of women in each of these prophylaxis groups, providing an opportunity for subgroup analysis. Although these analyses were not expected to produce robust estimates, they could provide early insights into potentially important associations with congenital CMV infection in women on antiretrovirals in the African context, given that this was the first study of congenital CMV infection in this setting.

The higher frequency of length of ARV prophylaxis <120 days and maternal CD4 count < 200 cells/μL in the prenatal AZT group and HAART group, respectively reflected implementation of WHO Option A Guidelines. The less favourable baseline immunological status in mothers in the HAART group which would have selected these women for HAART, whereas the shorter duration of ARV use in the prenatal AZT group is consistent with the fact that prenatal AZT use was limited to pregnancy, whereas HAART use commenced in some mothers prior to pregnancy. Despite these differences, the frequency of congenital CMV infection was similar between the two groups, with 2.7% (9/328; 95% CI, 1.3%-5.1%) of infants infected with congenital CMV in the AZT group compared with 3.1% (12/390; 95% CI, 1.6%-5.3%) in the HAART group. This is expected as the longer duration of HAART coupled with its efficacy would have improved the immunological status of mothers in the HAART group possibly to approximate those in the AZT group. However, serial CD4 count measurements were not available in pregnancy and only a snapshot of mostly pre-ARV maternal immune status was measured in this study.

In each of the subgroups, the expected direction of association with congenital CMV infection based on biological plausibility and the existing literature was protective (OR <1) for each of the continuous variables, and harmful (OR >1) for each of the categorical variables. In the bivariate analyses, the direction of the ORs were consistent with those expected in the AZT group, but this was not the case for the HAART group. Birth weight was independently associated with congenital CMV infection among mothers on prenatal AZT, and there was also a suggestion that length of AZT prophylaxis during pregnancy could impact congenital CMV transmission. The possible association between duration of maternal prenatal AZT and congenital CMV infection suggests that early initiation of AZT in pregnant HIV-infected women, who do not require combination antiretroviral therapy for their own health, could reduce in utero CMV transmission among such women. Since low

pre-treatment CD4 count also tended to predict intrauterine CMV transmission in this group, it is plausible AZT directly affected CD4 cell immunity. Increasing duration of maternal AZT could have hastened immunological recovery (100) or prevented CD4 cell decline (101), and thereby reduced the frequency of active CMV infection during pregnancy. Furthermore, lengthened maternal AZT use could have suppressed maternal HIV viral level to a greater extent (102, 103) and consequently reduced the risk of CMV transmission by the mechanisms mentioned above. In the Thai Perinatal HIV Prevention trial, the rate of in utero HIV transmission was 5.1% in mothers using prenatal AZT from 35 weeks gestation in comparison to 1.6% in those on prenatal AZT from 28 gestational weeks (104). It is therefore also possible that the protective effect of increasing exposure to zidovudine may have been due to a reduction in MTCT of HIV. In the group of women on triple ARVs, none of the factors examined were associated with CMV transmission.

Table 7. Comparison of demographic, maternal and newborn characteristics between infants born to women in the AZT and HAART groups.

Finding	AZT group (n=328)	HAART group (n=390)	P value
	Mean ± SD		
Maternal age	27.8 ± 6.7	28.1 ± 6.3	0.54
Length of ARV prophylaxis (days)	129 ± 51	594 ± 821	<0.001
Maternal CD4 count	507 ± 199	298 ± 159	<0.001
Gestational age (weeks)	37.5 ± 1.8	37.5 ± 1.7	1.0
Birth weight (kg)	3.1 ± 0.6	3.0 ± 0.6	0.03
	Positive (%)		
Length of ARV prophylaxis <120 days	143 (44)	130 (33)	0.004
Maternal CD4 count <200	17 (5.2)	104 (27)	<0.001
Prematurity (<37 weeks)	44 (13)	58 (15)	0.54
Infant feeding choice			<0.001
Breastfeeding	241 (73)	228 (58)	
Formula	87 (27)	162 (42)	

Table 8. Comparison of demographic, maternal and newborn characteristics between CMV-infected and uninfected newborns born to mothers on prenatal AZT.

Finding	CMV infected infants (n=9)	CMV uninfected infants (n=319)	OR (95%CI)	P value
		Mean ± SD		
Maternal age	29.2 ± 5.3	27.7 ± 6.8	1.0 (0.9-1.2)	0.57
Length of ARV prophylaxis (days)	85 ± 53.5	130 ± 50	0.98 (0.97-0.99)	0.01
Maternal CD4 count	445 ± 239	508 ± 197	0.99 (0.9-1.0)	0.46
Gestational age (weeks)	36.6 ± 2.6	37.5 ± 1.7	0.8 (0.6-1.1)	0.03
Birth weight (kg)	2.6 ± 0.6	3.1 ± 0.6	0.99 (0.9-1.0)	0.01
		Positive (%)		
Length of ARV prophylaxis <120 days	8 (89)	135 (42)	10.8 (1.3-7.7)	0.01
Maternal CD4 count <200	2 (22)	15 (5)	5.8 (1.1-30.2)	0.07
Prematurity (<37 weeks)	3 (33)	41 (13)	3.4 (0.8-14)	0.11
Infant feeding choice				0.11
Breastfeeding	6 (66)	235 (74)		
Formula	2 (22)	79 (25)		

Table 9. Logistic regression analysis to determine risk factors for congenital CMV infection in HIV-exposed infants born to mothers on prenatal AZT.

Risk factor	aOR (95% CI)	P value
Maternal age	1.1 (0.9-1.2)	0.36
Birth weight	0.99 (0.9 - 1.0)	0.04
Gestational age	1.2 (0.8-1.7)	0.41
Maternal CD4 count <200	4.8 (0.8-28.9)	0.09
Length of ARV prophylaxis <120 days	7.8 (0.9 – 66.7)	0.06

Table 10. Comparison of demographic, maternal and newborn characteristics between CMV-infected and uninfected newborns born to mothers on HAART.

Finding	CMV infected infants (n=12)	CMV uninfected infants (n=378)	OR (95%CI)	P value
	Mean ± SD			
Maternal age	27.0 ± 3.6	28.2 ± 6.4	0.97 (0.9-1.1)	0.3
Length of ARV prophylaxis (days)	538 ± 638	596 ± 826	1.0 (0.99-1.0)	0.7
Maternal CD4 count	222 ± 136	301 ± 159	0.99 (0.9-1.0)	0.1
Gestational age (weeks)	37.7 ± 1.0	37.5 ± 1.7	1.1 (0.7-1.6)	0.8
Birth weight (kg)	3.1 ± 0.5	3.0 ± 0.6	1.0 (0.99-1.0)	0.6
	Positive (%)			
Length of ARV prophylaxis <120 days	3 (25)	127 (34)	0.7 (0.2-2.5)	0.76
Maternal CD4 count <200	5 (41.7)	99 (26.2)	2.0 (0.6-6.5)	0.32
Prematurity (<37 weeks)	1 (8.3)	57 (15.3)	0.5 (0.06-3.9)	1.0
Infant feeding choice				0.91
Breastfeeding	7 (58.3)	221 (58.5)		
Formula	5 (41.9)	151 (39.9)		

Table 11. Logistic regression analysis to determine risk factors for congenital CMV infection in HIV-exposed infants born to mothers on HAART.

Risk factor	aOR (95% CI)	P value
Maternal age	0.97 (0.89-1.05)	0.47
Birth weight	1.0 (0.99 - 1.0)	0.71
Gestational age	1.02 (0.64-1.64)	0.92
Maternal CD4 count <200	2.2 (0.67-7.3)	0.19
Length of ARV prophylaxis <120 days	0.56 (0.15 – 2.2)	0.40

4.1.4 Limitations

In general, an observed epidemiological association may be due to a true causal association between the exposure and outcome, error or a variable combination of true association and error. Sources of error include random error, bias and confounding. In the subgroup analyses presented in this chapter, random error is of particular salience. The subgroup analyses lacked an a priori hypothesis and were not powered to detect statistically significant associations. Breaking down the dataset into smaller subgroups led to small sample sizes and increased imprecision of the estimates. It is not known whether the wide confidence intervals for the odds ratios are due to the absence of a true association, or the small numbers in the subgroups as standard error is inversely related to sample size. Lack of stability in the sample based estimates may also have accounted for the unexpected associations in the HAART group. The findings of the subgroup analyses are therefore inconclusive.

4.2 Additional discussion

A brief overview of study limitations was provided in the journal article manuscript in Chapter Three. This section extends the discussion of the study limitations, with a critical examination of random and systematic sources of error and confounding as well as the measures to reduce these. Table 12 summarizes the discussion of the study limitations.

4.2.1 Random sampling error

The study sample size was determined in advance, assuming a congenital CMV infection birth prevalence of 2% among infants born to HIV infected women, with a desired margin of error of 1%. In addition, confidence intervals and p values were provided with the sample based measures of association to determine the association between different variables at the population level.

4.2.2 Random information error

Random information error could have occurred during the measurement of each of the study variables that were already present in the medical records at the time of the study. Specifically, there may have been random error in extraction and interpretation of data by the research assistant. Training and supervision of the research assistant by specialist neonatologists could be expected to reduce this source of random error. In addition, random error could have occurred further upstream during laboratory and clinical measurement of the variables recorded in the medical charts. This is unlikely as validated instruments and established procedures are used for clinical measures such as birth weight and gestational age. Furthermore, validated sensitive and specific laboratory test platforms are used for the measurement of CD4 cell counts with strict quality assurance

procedures. Although these procedures protect against random error, the study findings were limited by the fact that data in the medical records were not independently verified by repeat measurements, and the accuracy of the extracted data was not independently verified by a third investigator. Random error during CMV testing was guarded against by the use of two validated CMV assays and duplicate PCR testing. Furthermore, all CMV testing were performed by trained laboratory personnel.

4.2.3 Selection bias

The study base comprised all HIV-infected women delivering at MMH during the study period. Convenience sampling (restriction of recruitment to weekday working hours) introduced the risk of sampling bias. However, the research assistant identified all HIV infected women in the postnatal wards each weekday, and it is unlikely that weekend deliveries differed systematically from weekday deliveries as parturition is an unplanned event. Overall, 90.9% (757/833) of eligible mothers were approached for participation in the study and most women (97.4%, 737/757) agreed to participate. Given the high coverage of the study base and high participation rates, enrolled mothers were likely representative of all HIV-infected mothers delivering at MMH during the study period.

4.2.4 Information bias

Information bias results from a differential accuracy of information between comparison groups, in this case between CMV infected and uninfected infants. This is unlikely as data on all variables were collected from the same information source using standardized data forms.

The demographic, clinical and newborn data were largely abstracted from existing antenatal records collected as part of routine clinical care, reducing the potential for recall bias. Furthermore, recording bias was unlikely as the research assistant was

blinded to possible study hypotheses. In addition, clinical data for the study were collected prior to identification of CMV infected and uninfected infants, virtually eliminating the potential for recall or recording bias.

CMV testing was conducted by investigators at a distant site independent of clinical data, thereby eliminating detection or diagnostic bias. A validated saliva PCR test was performed by qualified laboratory personnel with confirmatory testing using virus culture as a second detection platform.

4.2.5 Confounders

Apart from chance and bias, the detection of associations in the study or the incorrect estimation of effect sizes could have occurred as a result of a differential distribution of risk factors in the study base. Relevant confounding variables were dealt with in the analysis phase of the study by logistic regression. In addition, where appropriate, categorization of continuous variables was avoided. However, residual confounding from unmeasured confounders, unknown confounders and confounder mismeasurement could not be excluded.
In particular, maternal HIV viral load and infant HIV infection are possible unmeasured mediators or positive confounders of the association we observed between maternal CD4 count and congenital CMV transmission. Women with low CD4 counts may be more likely to have a high HIV viral load or to transmit HIV to their infants. High HIV loads may drive higher CMV levels due to biological interaction and thus predispose to congenital CMV infection. Furthermore, a link between infant HIV infection and congenital CMV infection has been suggested by data from upper income countries (Table 5). Other potential confounding variables include maternal education and socioeconomic status, which were not measured in this study.

In the overall study sample, the direction of association of each of the variables of interest with congenital CMV infection was consistent with the existing literature,

with a protective effect estimate noted for each of the continuous variables and an increased risk noted for each of the categorical variables (Tables 1 and 2 in the journal article manuscript). This seems to suggest that the study findings were valid overall. This study was powered to determine congenital CMV infection birth prevalence, and not the association of any predictor variable with congenital CMV transmission. Therefore, nonsignificant associations are difficult to interpret and may have been due to a lack of statistical power.

Table 12. Threats to internal validity and measures to address these.

	Potential source	Measures to reduce	Residual source
Random sampling error	Random sampling from study base	Sample size was calculated in advance to achieve precision of 1%. Confidence intervals and p values were provided with sample based estimates.	
Random measurement error	Measurement of study variables in medical records	Established clinical procedures were used and quality assurance of laboratory (CD4 count) tests are routine practice.	Repeated measurements for research purposes were not done.
	Abstraction of study data from medical records	Training and supervision of the research assistant was ensured.	Independent verification of abstracted data was not done.
	Laboratory testing of study specimens	Testing was undertaken by trained personnel using two validated assays with duplicate PCR testing.	
Systematic sampling error (selection bias)	Non random sampling scheme (convenience sampling)	Eligible population comprised consecutive deliveries, with weekends excluded. Weekend deliveries should not differ from weekday deliveries.	
	Non-participation bias	High participation rates were achieved.	
Systematic measurement error (information bias)	Interviewer bias, recall/reporting bias and recording bias	Not applicable as possible study hypotheses were not revealed to the research assistant or participants. Infant CMV status was not known at time of data collection and medical records were the major data source.	
	Diagnostic suspicion bias	CMV testing was conducted at distant site by blinded personnel.	
	Laboratory tests	An accurate validated saliva PCR test was used. Confirmatory testing was done using an established test method (virus culture).	Freezing and storage of specimens could have affected validity of PCR and culture results.
		Standardized procedure used for infant swab collection, with waiting period in breastfeeding women.	Breast milk contamination of samples could have generated false positive results.
Confounding	Uneven distribution of risk factors in the study base	Multivariable modelling (logistic regression) was used.	Residual confounding could have occurred due to unknown or unmeasured confounders and confounder mismeasurement.

4.3 Additional recommendations

If we had an opportunity to repeat the study with the primary objective of obtaining a prevalence estimate under the conditions of unlimited resources, a few aspects of the study would have been approached differently.

In order to reduce selection bias and improve external validity, we would have extended the working hours of the research assistant to include weekends. This would have provided an opportunity to invite all HIV infected women to participate in the study. Furthermore, reasons for non-participation and baseline information would have been documented on mothers declining participation using a rapid questionnaire. This would have allowed for the evaluation of non-participation bias.

To reduce information bias and improve the breadth of information generated from the study, we would have carried out the study with personal identifying information and undertook local CMV testing. All data would have been collected as part of study procedures and verified against antenatal and neonatal records. We would have set up and validated the UAB developed saliva PCR assay in the Virology Laboratory at Groote Schuur Hospital. All saliva specimens would have been collected in duplicate for confirmatory testing in the US. In addition, a urine specimen would have been collected from all infants to compare sensitivity and specificity of CMV detection in saliva and urine under local conditions. Furthermore, besides using the UAB developed saliva assay, we would have repeated all tests using an existing commercial assay to compare the performance of the two assays. By running the tests locally, we would have been able to follow up all infants with confirmed congenital CMV infection in real time, with a systematic assessment of clinical features including newborn hearing screening in the newborn period as well as serially in the first two years of life.

Finally, to improve the quality of information about predictor variables, it would have been preferable to have enrolled mothers at their first antenatal visit and collected baseline and serial demographic, clinical, immunological and virologic data (HIV and CMV) on enrolled mothers. We would also have assessed infant HIV status at birth and six weeks of age to test the association of in utero HIV with in utero CMV.

Part E: Annexures

I. Research protocol

Title of Research Study

The birth prevalence of congenital CMV in HIV-exposed newborns in Cape Town, South Africa – A pilot study. The "CYPREHEN" (CYtomegalovirus PREvalence in HIV-Exposed Newborns) study.

STUDENT:

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CO-INVESTIGATORS:

Name: Dr A.M. Van Niekerk
Department: Division of Neonatal Medicine, School of Child and Adolescent Health
Role: Principal Investigator, training and supervising the Research Assistant in the collection of samples and obtaining informed consent from patients, contributing intellectual skill including participating in the design and analysis of the study

Name: Dr S.M. Kroon
Department: Division of Neonatal Medicine, School of Child and Adolescent Health
Role: Training and supervising the Research Assistant in the collection of samples and obtaining informed consent from patients, contributing intellectual skill including participating in the design and analysis of the study

Name: Dr M. Hsiao
Department: Division of Medical Virology, Department of Clinical Laboratory Sciences
Role: Contributing intellectual skill including participating in the design and analysis of the study

Purpose of Protocol: This protocol is being submitted for the Master of Public Health degree. The research component comprises 50% of the degree. The protocol will be submitted for ethical review to the Biomedical Research Ethics Committee for expedited ethical approval.

November 19, 2013

RE: Request for retrospective registration of research study

Dear Prof Mars, Chair, School of Nursing and Public Health Research Committee

Please consider the enclosed protocol, "The birth prevalence of congenital CMV in HIV-exposed newborns in Cape Town, South Africa – A pilot study. The "CYPREHEN" (CYtomegalovirus PRevalence in HIV-Exposed Newborns) study." for retrospective registration for the Master of Public Health degree at UKZN.

I developed the protocol in my third year of registrar training (2011) in Medical Virology at the University of Cape Town. As I had an MMed project underway at that stage, this study was not registered for degree purposes. Ethical approval for the study was obtained from the Faculty of Health Sciences Research Ethics Committee at UCT, HREC Ref 444/2011, and the Institutional Review Board at the University of Alabama.

The enrollment phase was conducted from April to November, 2012 and the samples were subsequently shipped to the collaborating microbiology laboratory at UAB. I deregistered from the Medical Virology program at the end of 2012 and relocated to Durban, where I have since completed two semesters of coursework for the MPH. During this time, I received the study results from UAB, undertook the data analysis and have prepared a manuscript with the potential for peer-review and publication.

I confirm that all study co-investigators agree to the use of this data for degree purposes at UKZN.

Sincerely,
Sheetal Manicklal

CO-INVESTIGATORS

Name: Dr AM van Niekerk
Department: Division of Neonatal Medicine, School of Child and Adolescent Health, University of Cape Town
Role: Principal Investigator, training and supervising the Research Assistant in the collection of samples and obtaining informed consent from patients; contributing intellectual skill including participating in the design and analysis of the study

Signature:

Name: Dr Marvin Hsiao
Department: Division of Medical Virology, Department of Clinical Laboratory Sciences, University of Cape Town
Role: Supervisor; contributing intellectual skill including participating in the design and analysis of the study

Signature: 

Name: Dr SM Kroon
Department: Division of Neonatal Medicine, School of Child and Adolescent Health, University of Cape Town
Role: Training and supervising the Research Assistant in the collection of samples and obtaining informed consent from patients; contributing intellectual skill including participating in the design and analysis of the study

Signature:

CO-INVESTIGATORS

Name: Dr AM van Niekerk
Department: Division of Neonatal Medicine, School of Child and Adolescent Health, University of Cape Town
Role: Principal Investigator, training and supervising the Research Assistant in the collection of samples and obtaining informed consent from patients; contributing intellectual skill including participating in the design and analysis of the study

Signature: 

Name: Dr Marvin Hsiao
Department: Division of Medical Virology, Department of Clinical Laboratory Sciences, University of Cape Town
Role: Supervisor; contributing intellectual skill including participating in the design and analysis of the study

Signature:

Name: Dr SM Kroon
Department: Division of Neonatal Medicine, School of Child and Adolescent Health, University of Cape Town
Role: Training and supervising the Research Assistant in the collection of samples and obtaining informed consent from patients; contributing intellectual skill including participating in the design and analysis of the study

Signature: 

v

UAB MEDICINE
PEDIATRICS

Children's of Alabama

December 6, 2013

Prof Mars
Chair, School of Nursing and Public Health Research Committee

RE: Request for retrospective registration of research study

Dear Prof Mars,

I am in agreement with Sheetal Manicklal's request to register the protocol, "The birth prevalence of congenital CMV in HIV-exposed newborns in Cape Town, South Africa – A pilot study. The "CYPREHEN" (CYtomegalovirus PRevalence in HIV-Exposed Newborns) study" for the Master of Public Health degree at UKZN.

Sheetal developed and completed the project during her third year of registrar training (2011) in Medical Virology at the University of Cape Town. I also agree to continue to be involved in the data analysis and future work on CMV at UKZN as needed.

Sincerely,

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Abstract

Background

Congenital cytomegalovirus (CMV) is the leading non-genetic cause of sensorineural hearing loss worldwide, in the post-rubella vaccination era. The birth prevalence of congenital CMV correlates positively with the level of CMV seroimmunity in the adult population, and is higher in highly seroimmune populations. In addition, women infected with Human Immunodeficiency Virus (HIV) constitute a special at risk subpopulation for the intrauterine transmission of CMV. Despite a high prevalence of both HIV and CMV, the birth prevalence of congenital CMV has not been assessed in sub-Saharan Africa.

Purpose

The purpose of the study is to determine the birth prevalence of congenital CMV among HIV-exposed newborns born in a public sector hospital in the Western Cape in 2012, during the era of prenatal antiretroviral therapy.

Objectives

The objectives of this study are:

- To determine the prevalence of congenital CMV among HIV-exposed newborns;
- To assess the predictors of congenital CMV transmission among HIV-infected women; and
- To inform the design of an analytic study to determine if newborn CMV screening should be implemented in this population.

Study design

An observational descriptive cross-sectional study design is used.

Settings

The study will be conducted at Mowbray Maternity Hospital, which serves the Cape Town Metropole area.

Study population

The study population will comprise infants born to HIV-infected mothers delivering at MMH.

Study sample

Non-probability convenience sampling will be used to enroll 750 newborns.

Data collection

A trained research assistant will extract data from the medical records of participants using a standardized data collection sheet, and will collect an infant saliva swab in viral transport medium.

Statistical methods

The birth prevalence will be calculated as the proportion of enrolled infants that test positive for CMV. Associations between clinical variables and congenital CMV infection will be tested.

Abstract word count: 300 words

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Name: Sheetal Surname: Manicklal

Student Number: 200266595 Year of Study: 2nd

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Manicklal. 13th Feb 2014

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Table of Contents

1 Introduction

Cytomegalovirus (CMV) is a common viral infection of humans. As a virus that is highly adapted to its host, infection of immune competent persons leads to lifelong persistence and is usually clinically inapparent. In contrast, the role of CMV in producing morbidity and mortality in hosts with impaired immunity is well established. However, the fact that CMV is also a leading cause of congenital infections worldwide is barely recognized.

The birth prevalence of congenital CMV correlates with the level of CMV seroimmunity in the adult population, and is higher in highly CMV seroimmune populations. Not only is the prevalence of congenital CMV higher in women with pre-existing CMV immunity, but such immunity fails to protect against symptoms at birth and sequelae in the infant. Of salient concern, hearing loss occurs in around one to 10 infants infected with CMV in utero.

Infection with CMV in the adult population in South Africa is nearly universal. In addition, the maternal HIV seroprevalence is among the highest in the world, with an average of 30% of antenatal clinic attendees seropositive for HIV. HIV-infected women constitute a special at risk subpopulation for the intrauterine transmission of CMV. Despite a high prevalence of both HIV and CMV in sub-Saharan Africa, the birth prevalence of congenital CMV has not been assessed in a large sample of HIV-exposed newborns. Furthermore, several factors may influence the transmission risk in HIV-infected women, especially maternal antiretroviral therapy (ART), although these have not been systematically assessed.

This study will contribute important preliminary knowledge on the frequency of congenital CMV infection among infants of HIV-infected women on prenatal ART. This information will help design a study to investigate whether maternal HIV infection is a risk factor for intrauterine CMV transmission in the ART era, in order to determine whether HIV exposed infants should be considered for newborn CMV screening.

2 Literature Review

2.1 Congenital CMV infection – a global public health problem

Cytomegalovirus (CMV) is an important cause of congenital infections worldwide. In industrialized countries, following successful strategies to eliminate rubella, CMV has emerged as the commonest non-genetic cause of hearing loss and neurodevelopmental delay in childhood.[1]

Infections with CMV have a global distribution, and nearly all persons will be infected at some point during their life. The rate of CMV acquisition in a population increases with age, and correlates inversely with socio-economic status and levels of public health standards.[2] Therefore, in industrialized countries, where primary infection occurs mainly in adolescence and adulthood, 30-50% of women of childbearing age seronegative.[3] In comparison, in low and middle income countries, CMV infection usually occurs in infancy and childhood, with fewer than 10% of adult women being seronegative.[3] Since the birth prevalence of congenital CMV correlates positively with the level of seroimmunity in these populations, it averages 0.6% in high resource settings compared with 0.9% to 2.1% in poorer countries.[4]

Intrauterine transmission of CMV may occur during any trimester of pregnancy. Recent data of maternal primary CMV infection shows a higher transmission risk in later gestation, while timing of transmission remains unknown in nonprimary infection. Overall, maternal primary infection leads to fetal infection in 30 to 35% of cases and nonprimary infection carries a transmission risk of 1.4%.[5] Owing to the substantially higher risk of CMV transmission following maternal primary infection, and the dogma that maternal immunity protects against congenital CMV disease, focus has largely centered on CMV infection in seronegative mothers. Accordingly, the natural history of congenital CMV in industrialized countries has been extensively studied, with historically little attention to developing world settings.

In developed countries, congenital CMV accounts for a fifth to a quarter of cases of hearing loss at birth and 4 years of age, respectively.[6] In the absence of early detection of hearing impairment, speech, language, and social impairment occurs in a significant number of children. This places a burden on medical care resources.[7] In recent decades, evidence of severe disease and sequelae in newborns of mothers with pre-existing CMV seroimmunity has been growing,[8-11] highlighting the

importance of investigating the natural history of congenital CMV infection in these regions. Of particular importance, recent data from a newborn screening study in Brazil has revealed that 9.8% (95% CI: 5.1-16.7%) of congenital CMV infected infants develop hearing loss, not different to the rates reported in developed countries, where the frequency of hearing loss ranges from 7 to 23%.[11, 12]

2.2 The role of maternal HIV infection in congenital CMV

Apart from maternal nonprimary CMV driving a higher birth prevalence of congenital CMV in developing world settings, data suggest that additional risk factors could exist. Maternal malaria and HIV infection, both of which are common in these regions, could additionally increase the risk of congenital CMV. Recently, a CMV birth prevalence of 5.4%(40/741) was documented in an ongoing birth cohort study in peri-urban West Africa between January 2002 and January 2005, and maternal malaria infection was identified as a risk factor for the vertical transmission of CMV.[13]

Data from Europe and the Americas suggest an increased risk of intrauterine CMV transmission in mothers infected with both HIV and CMV.[14] Among newborns living with HIV, the birth prevalence of congenital CMV ranges from 6% to 21%.[14] In one study, infants with in utero HIV were found to have an eight times increased odds of congenital CMV compared with HIV exposed but uninfected infants.[15] Furthermore, even among HIV-exposed but uninfected newborns, the birth prevalence of congenital CMV ranges from 1.5% to 3% in pre- and post- HAART[a] era, substantially higher than among HIV-unexposed newborns.[14, 16]

CMV may act as a co-factor for infant HIV disease progression. Poorer congenital CMV and HIV related clinical outcomes in co-infected infants may be due to the neuro-invasive and immunosuppressive properties common to both pathogens.[17, 18] In these infants, the risk for neurological morbidity and infant mortality is increased.[19] Although the clinical course of congenital CMV-related hearing loss in HIV-exposed

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[a] The term HAART is used here to refer to the era of combination antiretroviral therapy that began in the early 2000's.

infants has not been described, HIV exposed infants are relatively immunosuppressed compared with their HIV unexposed counterparts, and may have a poorer prognosis.

2.3 Predictors of congenital CMV infection in highly CMV seroimmune populations and HIV-infected women

In order to design appropriate preventive strategies to reduce the birth prevalence of congenital CMV and burden of congenital CMV associated disease, it is important to determine clinical, immunological and virological predictors of congenital CMV infection. The following factors have been examined in the context of highly CMV seroimmune maternal populations, and HIV-infected women.

- **Maternal age**

Young maternal age (<20 years) was associated with a nearly five times increased odds (aPOR 4.8, CI: 2.6-8.9) of congenital CMV, compared with mothers aged 30 years and greater, in an urban population of predominantly low income, non-white mothers delivering at a public hospital in the USA between 1980 and 1990.[20] Among young women less than 20 years, the birth prevalence of congenital CMV was 2.35% (95% CI 1.91 – 2.87). The risk of congenital CMV remained elevated for mothers between the ages of 20 and 25 years (aPOR 2.7, 95% CI: 1.4-5.1), but not those over the age of 25 years.[20] In another study based in the USA of 3461 women whose infants were screened for congenital CMV, maternal age above 25 years resulted in an 80% reduction of congenital CMV infection.[21]

- **Prematurity**

Prematurity and low birth weight have been documented in around 30% of infants with symptomatic congenital CMV. In addition, a significantly lower mean birth weight (deficit of 163 grams) was reported among asymptomatic infants from a low income population in New York following maternal primary infection.[22] On the other hand, in a highly seroimmune population in Brazil, prematurity was not associated with the birth prevalence of congenital CMV. The prevalence of congenital CMV was 2.1% (95% CI, 0.84 to 4.68) among preterm infants, and 1.8% (95% CI, 0.48 to 5.74) among term infants.[23]

- **Maternal CD4 count**

HIV-infected individuals are often CMV seropositive, therefore it is plausible that impaired maternal immunity could lead to more frequent reactivation or re-infection with CMV, or higher levels of CMV viremia. Maternal immunosuppression (maternal CD4 count < 200 cells/uL) close to delivery was reported as an independent predictor of congenital CMV in the FPC.[16] In addition, an inverse, although non-significant, trend was documented between increasing maternal CD4 counts and congenital CMV infection prevalence in HIV-exposed uninfected newborns born to mothers on antenatal antiretroviral therapy in the USA.[14]

- **Type and length of antiretroviral prophylaxis**

Early initiation of combination antiretroviral therapy is known to dramatically reduce CMV blood DNA levels, and reconstitute humoral immunity to CMV.[24] This may in turn reduce the frequency of nonprimary CMV infection and accordingly the risk of vertical CMV transmission.

The FPC authors examined the impact of duration and type of antiretroviral therapy on congenital CMV birth prevalence.[16] They documented a higher birth prevalence of congenital CMV among mothers initiating ARV therapy in the second trimester compared with the those initiating ARVs in the first trimester of pregnancy, and suggested that earlier initiation of HAART may protect against CMV reactivation for a longer duration of the pregnancy. Although congenital CMV transmission risk did not differ significantly by type of ARV therapy (HAART, two NRTIs, monodrug therapy, none), the authors noted that mothers who received NRTI monotherapy, according to French guidelines, were more likely to have a favourable immunological and virological status, and therefore to be at lower risk of intrauterine CMV transmission.

2.4 Regional relevance

Sub-Saharan Africa has the highest burden of HIV, with most infections occurring in South Africa and disproportionately affecting young women of childbearing age. In recent years, the South African HIV epidemic has stabilized, with a national adult HIV prevalence of 17.3% (95% CI 16.6 - 18.1%) in 2011, and a reduction in vertical transmission rates to under 3%. However, the antenatal HIV prevalence remains alarming, ranging from 17% (95% CI 14.3 – 20%) to 37.4% (95% CI 35.8 – 39%) in

2011.[25] With an annual birth cohort of 1 million infants, nearly 300 000 children are born HIV-exposed each year. Taking into account data on disease frequency from developed countries, South Africa potentially faces a unique burden of congenital CMV disease. In spite of this, there are no reliable data on the birth prevalence of congenital CMV in general, and particularly in the subgroup of HIV-exposed infants.

2.5 Laboratory considerations

2.5.1 Screening for congenital CMV

The diagnosis of congenital CMV relies on the detection of viable virus by cell culture, or viral genomes by PCR in clinical specimens (urine or saliva) within the first 14 days of postnatal life. While most laboratories are experienced with congenital CMV diagnosis in urine, obtaining urine samples from infants is often difficult, requiring invasive techniques. Saliva swabs, on the other hand, are noninvasive and non-technical, and there is a high agreement of virus detection between the two sample types when either culture or PCR are used.[26, 27] In addition, saliva screening of newborns for congenital CMV was recently validated in a large, multi-center prospective study in the USA.[28]

3 Purpose of the Study

The purpose of the study is to determine the birth prevalence of congenital CMV among HIV-exposed newborns born in a public sector hospital in the Western Cape in 2012, during the era of prenatal antiretroviral therapy.

4 Specific Objectives

The objectives of this study are:

- To determine the prevalence of congenital CMV among HIV-exposed newborns;
- To assess the predictors of congenital CMV transmission among HIV-infected women; and
- To inform the design of an analytic study to determine if newborn CMV screening should be implemented in this population.

5 Type of Research

This is epidemiological research.

6 Definitions

Cytomegalovirus	Betaherpesvirus (HHV-5) which is ubiquitous in human populations. Most infections occur in childhood in low and middle income countries. Infections are asymptomatic in the healthy host, but can be severe in hosts with compromised immunity such as the fetus and persons with advanced HIV infection.
Maternal primary CMV infection	Initial infection with CMV during pregnancy. This can be detected as CMV seroconversion in the laboratory.
Maternal nonprimary CMV infection	Reactivation of endogenous virus or re-infection with exogenous virus during pregnancy (see below).
CMV reactivation	Following initial or primary infection, CMV assumes a latent state in the host, where it is under tight immune control. However, the virus reactivates periodically and may be shed in the blood and bodily fluids.
CMV re-infection	New infection with CMV in a person who has already experienced an initial infection. This infection would be with a different CMV strain to the original infecting virus. In this way, an accumulation of CMV strains can occur in a single host.

7 Research Methods

This study will draw on quantitative data sources and research methods. All data will be measured using objective instruments, thereby increasing the reliability and accuracy of the findings.

7.1 Study setting

The study will be conducted at MMH which serves the Cape Town Metropole area.

7.2 Study design

An observational descriptive cross-sectional study will be used.

7.3 Target population

HIV-exposed newborns in the Western Cape

7.4 Study population

The study population will comprise infants born to HIV-infected women delivering at MMH.

7.4.1 Inclusion/Exclusion criteria

The inclusion criteria for mothers are as follows:

- Known HIV-infected;
- Age 18 years and older;
- Less than 14 days post-delivery;
- Residing in the greater Cape Town area; and
- Willing to give written informed consent.

7.5 Study sample

7.5.1 Method of selecting sample

A non-probability convenience sampling strategy will be used.

7.5.2 Size of sample

A sample of 750 newborns is required to demonstrate a congenital CMV birth prevalence of 2%, with a precision of 1%, assuming a 95% confidence interval.

7.6 Data sources

7.6.1 Measurement instruments/Data collection techniques

Demographic and clinical data will be abstracted from antenatal chart records using a standardized data collection sheet. In addition, an infant saliva swab in viral transport medium will be collected at least two hours before breastfeeding, for CMV PCR testing.

7.7 Measures to ensure validity

7.7.1 Selection bias

Although we will use non-probability convenience sampling, most of the HIV-infected low-risk mothers delivering at MMH during the study period will be approached for participation, as the research assistant will identify all HIV-exposed deliveries each week day. Assuming good participation rates, this will help to ensure that the sample is representative of the population of HIV-infected women delivering at MMH.

7.7.2 Information bias

Information bias will be reduced by training the research assistant on the study procedures. The completeness of data collection will be ensured by using a standardized data collection sheet to collect data on demographic and clinical characteristics for all enrolled newborns. This data will be retrieved from maternity hospital records, thereby eliminating reliance on recall. Maternal HIV status will be determined on the basis of antenatal records which is also thought to be highly accurate, and will be indirectly verified during the consent process.

By standardizing the timing of infant saliva swab collection, the likelihood of false positive results from breast milk contamination of saliva samples will be reduced. In addition, accuracy of the laboratory results will be enhanced by using a validated highly sensitive and specific screening assay, and a confirmatory test.

7.7.3 External validity/Generalizability

Mowbray Maternity Hospital is a referral hospital for surrounding Midwife Obstetric Units in the Metropole West region of the Western Cape. However, MMH also provides subsidized healthcare for pregnant women in the local Mowbray area, and we will restrict the study population to these low risk deliveries only. Overall, the patient population is representative of the general Western Cape population consisting of approximately 50% mixed race and 50% indigenous black African as well as an increasing number of African migrants/refugees.

7.8 List of variables

The variables for which data will be collected are outlined in Table 1a. The study identifiers will be a numerical code (CODE), and the date of specimen collection (DATESPEC).

Table 1a. Study variables according to category

CATEGORY	VARIABLE	VARIABLE NAME	TYPE	UNIVARIATE ANALYSIS	DEPENDENT/ INDEPENDENT
Maternal	Maternal age	MOMAGE	Continuous	Mean, SD	Independent
	Maternal ARV regimen - AZT and NVP in labour only - AZT during pregnancy - HAART - None	PROPHLY	Categorical	Proportions	Independent
	Date of initiation of ARV	STARTPROP	Continuous		
	Maternal CD4 count	CD4STAT	Continuous	Mean, SD	Independent
	Date of CD4 count	DATECD4	Continuous		
Infant	Infant date of birth	DOB	Continuous		
	Infant gestational age	GESTAGE	Continuous	Mean, SD	Independent
	Infant birth weight	BIRTHWT	Continuous	Mean, SD	Independent
	Infant feeding choice	FEED	Categorical	Proportions	Independent
	Saliva CMV PCR result	SALPCR	Categorical	Proportion	Dependent
	Saliva CMV viral load	SALVL	Continuous	Geometric	Dependent

Table 1b. New variables to be generated during data analysis

NEW VARIABLE	VARIABLE NAME	FORMULA/CUTOFF	TYPE	UNIVARIATE ANALYSIS	DEPENDENT/ INDEPENDENT
Days post-delivery	DPOSTDEL	DATESPEC – DOB	Continuous	Mean, SD	
Length of ARV prophylaxis	DURARV	DOB – DATECD4	Continuous	Mean, SD	Independent
Gestational age at CD4 count	GACD4STAT	[GESTAGE*7 – (DOB – DATECD4)]/7	Continuous	Mean, SD	
Maternal age less than 20	MOMAGELT20	20 years	Categorical	Proportions	Independent
Birth weight less than 2500	BWLT2500	2500 grams	Categorical	Proportions	Independent
CD4 count less than 200	CD4LT200	200 cells/uL	Categorical	Proportions	Independent
Prematurity	GALT37	37 weeks	Categorical	Proportions	Independent
Length of ARV prophylaxis less than 120	DURARVLT120	120 days	Categorical	Proportions	Independent

7.9 Plan for data collection

7.9.1 Recruitment of participants

A trained research assistant (RA) will identify potential participants daily from Monday to Friday in the postnatal wards of MMH, based on hospital records of maternal HIV status. The purpose of the study will be explained to HIV-infected mothers and written informed consent (Addendum 13.2) will be obtained from those who are willing to have their infant participate. Additional consent will be taken from mothers who agree to storage and future testing of their child's specimen.

7.9.2 Data and specimen collection

Enrolled infants will be assigned a unique sequential numerical code that, together with their date of enrollment, will serve as an identifier for the study. The RA will keep a written record of all participants on a clinic enrollment register, and will use a data collection sheet (Addendum 13.3) to retrieve relevant demographic and clinical data from the mother's folder.

The RA will swab the buccal mucosa and gums of the infant for several seconds with a sterile cotton swab, around one to two hours prior to the next feed. The tip of the swab will be placed in a cryovial, containing 2.0 mL of viral transport medium, (VTM), which will be labeled with the unique participant code and date of collection. A duplicate, "paired" sample will be collected for the first 250 newborns of mothers who give consent to storage and future testing of the infant's specimen. The specimen will be placed individually with the data collection sheet in a plastic bag and kept in the refrigerator at 4^{o}C. Specimens will be transported weekly to the National Health Laboratory Service Virology Laboratory at Groote Schuur Hospital.

7.9.3 Specimen storage and shipment

In the laboratory, specimens will be recorded on a laboratory study register upon receipt, noting the specimen (participant) code and date of delivery. The specimens will be stored at -80^{o}C.

Once 750 specimens have been collected, they will be packaged and labeled in a box for biohazard substances, according to the triple packaging system (category B infectious substances) in order to comply with the International Air Transport

Association Dangerous Goods Regulations. The specimens will be transported frozen to the University of Alabama Microbiology Laboratory for CMV PCR testing.

7.10 Plan for data handling and processing

The clinic enrollment register will be stored in a locked cupboard at the end of each day of enrollment, and consent forms will be handed to the Principal Investigator for safekeeping.

Information from the data collection sheet will be captured on a password protected MS Excel spreadsheet by the project coordinator. Hard copies of the data collection sheet and the laboratory study register will be filed in a locked cupboard in the Department of Virology. No specimens will be registered on the laboratory information system.

The CMV PCR and genotyping results will be provided to the project co-coordinator through electronic communication, and will be recorded on the electronic database. The database will then be shared with all collaborating parties for the purpose of the analysis.

7.11 Statistical methods

7.11.1 Descriptive statistics

The birth prevalence will be calculated as the proportion of total participants that test positive. A 95% confidence interval for the prevalence will be calculated.

7.11.2 Analytic statistics

Exploratory analysis for associations between clinical and demographic variables and congenital CMV infection will be undertaken based on previously described predictors of congenital CMV (see Section 2.3 Predictors of Congenital CMV Infection in Highly CMV Seroimmune Populations and HIV-infected Women), however the study is not powered to identify such predictors or outcomes. A description of this analysis is provided below.

7.11.2.1 Generation of new variables (Table 1b):

The following calculations will be performed in MS Excel:

- The infant date of birth will be subtracted from the date of specimen collection to determine how many days post-delivery (DPOSTDEL) the saliva sample was collected. The date maternal ARVs was commenced will be subtracted from the date of delivery to determine the number of days prior to delivery the mother was on antiretrovirals (DURARV); and
- The date of CD4 count will be subtracted from the infant date of birth to determine, first, the number of days prior to delivery that the CD4 count was taken. This will in turn be subtracted from the gestational age (converted to days), to determine the number of days since conception that the CD4 count was taken. This will then be converted to weeks to determine the gestational age at which the CD4 count was taken (GACD4STAT).

The excel file will then be transferred to SPSS v. 21 statistical package (IBM Corp., Armonk, NY, USA).

Continuous variables will be categorized based on pre-established clinically significant cutoffs. This will generate the following independent variables:

- “Maternal age less than 20” (MOMAGELT20);
- “Birth weight < 2500” (BWLT2500);
- “CD4 count < 200” (CD4LT200);
- “Prematurity” (PREMLT37); and
- “Length of ARV prophylaxis < 120” (PROPHYLCAT).

7.11.2.2 Univariate analysis

Univariate analysis will be performed as illustrated in Tables 1a and Table 1b. Test for normality will be performed, and summary statistics (mean and SD, or median and IQR) will be chosen accordingly.

7.11.2.3 Bivariate analysis

The study variables will be assessed for normality. Depending on whether the study variables are normally distributed, or not, the t test or Mann Whitney U test, respectively, will be used to explore associations between maternal age, maternal CD4 count, gestational age, birth weight and length of ARV prophylaxis (independent continuous variables) and infant CMV status (SALPCR) (dependent variable).

Crude odds ratios will be calculated from 2 x 2 tables to determine the association of the generated categorical variables (Table 1b), as well as between infant feeding choice (to examine possible contamination of infant saliva swabs by CMV shed in breast milk), and intrauterine CMV transmission. Fischer exact and Chi Square test will be used to explore associations between these categorical variables. Ninety five percent confidence intervals for measures of effect will be presented.

Simple linear regression will be used to determine whether maternal age, maternal CD4 count, infant gestational age, infant birth weight and length of prophylaxis predict infant CMV viral load. Based on this it will be decided if a multivariable linear regression model should be developed. Diagnostic tests will be run to ensure that linear regression assumptions are not violated.

7.11.2.4 Multivariable analysis

Each predictor will be tested bivariately against infant CMV status using a logistic regression. Variables significant at the $p<0.2$ will be selected for entry into the final multivariable model. Multivariable model fit will be assessed using the Hosmer and Lemeshow's goodness of fit statistic. The link test will also be performed to check model specification. A receiver operating characteristic (ROC) curve will also be produced and assessed. This is a plot of specificity versus 1-sensitivity generated by varying the cut-point. Multicollinearity between predictors will also be assessed as this can erroneously affect the coefficient values. This will be assessed using the variance inflation factor (VIF). As a general rule of thumb, the VIF should not exceed 10, though some literature suggests this cut-off should be lower i.e. 5.

7.12 Limitations

The study has the following limitations:

- The use of a convenience sampling approach could introduce selection bias if participation rates are low.
- The anonymous unlinked design of the study precludes clinical and follow-up assessments of CMV-infected infants.
- If the expected birth prevalence of 2% holds true, it will be difficult to attain statistical significance with respect to the analytical component.
- The assessment of predictors is limited as data on maternal social and demographic characteristics, maternal HIV viral load as well as infant 6 week PCR are not available.
- The storage of specimens for several months prior to shipping and testing could affect the validity of the results.
- The PCR assay has not been validated for congenital CMV detection in a populations with high rates of breastfeeding.

8 Ethical Considerations

8.1 Institutional Ethical Review Board

The protocol for this study will be submitted to the University of Cape Town (UCT) Health Sciences Faculty Human Research Ethics Committee (HREC) (Addendum 11.3). The study has been designed in keeping with the Declaration of Helsinki (Seoul version 2008).

8.2 Rationale for anonymous unlinked testing

Current standard of care in South Africa regarding congenital CMV is no routine testing. Only babies with signs or symptoms of congenital infection are tested and considered for treatment.

Infants with signs or symptoms will still be tested and managed following existing protocols independent of this study.

Both the anonymity and unlinking of specimens is therefore ethically justifiable.

8.2.1 Future testing of stored specimens

The use of stored sample for additional study outside this protocol will require further ethical clearance from both ethics committees (UCT and UAB).

8.3 Permissions

Permission for conducting the study at MMH will be sought from MMH Ethics Committee.

8.4 Informed consent and participant information

Only mothers who provide full written informed consent will be enrolled in this study. Written information about the study, nature of the child's involvement and potential risks and benefits will be provided in a language that she understands (verbal translations in Xhosa and Afrikaans will be available), and she will be allowed sufficient opportunity to consider participation, in the absence of coercion. The

informed consent sheet will explain the voluntary nature of participation, and that mothers are free to decline participation without any prejudice.

9 Work Plan

9.1 Budget

The study budget is presented in Table 2. A grant application will be submitted to the School of Child and Adolescent Health at the Faculty of Health Sciences, Red Cross Children's Hospital, and other sources will be approached for the required funds.

Table 2. Study budget

REQUIREMENTS	UNIT COST	TOTAL COST
Collection items for hospital		
3mL VTM cryovials	758. 00 per 300	1 500.00
Laboratory testing items	N/A	
Clinic research assistant	40.00 per hour	14 400.00
Overseas shipment of specimens		10 000.00
TOTAL		25 900.00

9.2 Study Period

The study will be carried out over a 6 to 9 month period, depending on the accrual rate, starting in April 2012.

10 Acknowledgments

I would like to extend my gratitude to Drs. Anika Van Niekerk, Max Kroon and Marvin Hsiao of the University of Cape Town for supporting this endeavour. I am indebted to Dr. Suresh Boppana for agreeing to collaborate on the study and for his guidance on the manuscript. Sincere thanks also go to Dr. Stephen Knight for supervising this work, and Professor Benn Sartorius for helpful advice on the statistical analysis. Lastly, I would like to thank the reviewers for their valuable comments that have greatly improved the protocol.

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12 List of Tables

13 Acronyms and Abbreviations

AZT	Azidothymidine
ARV	Antiretroviral therapy
FPC	French Perinatal Cohort
HIV	Human Immunodeficiency Virus
MMH	Mowbray Maternity Hospital
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NVP	Nevirapine
PCR	Polymerase chain reaction
PMTCT	Prevention of mother to child transmission of HIV
UAB	University of Alabama at Birmingham
UCT	University of Cape Town
USA	United States of America

14 Addenda

14.1 Application for expedited ethics approval

February 14, 2014

Professor Douglas R Wassenaar
Chair of the UKZN Biomedical Research Ethics Committee
School of Applied Human Sciences
UKZN
Private Bag X01
SCOTTSVILLE
3209
KwaZulu-Natal

RE: Request for expedited approval of Master of Public Health research study

Dear Professor Wassenaar,

Please consider the enclosed protocol, “The birth prevalence of congenital CMV in HIV-exposed newborns in Cape Town, South Africa – A pilot study. The “CYPREHEN” (CYtomegalovirus PREvalence in HIV-Exposed Newborns) study.” for expedited review at the Biomedical Research Ethics Committee of UKZN.

I developed the protocol in my third year of registrar training (2011) in Medical Virology at the University of Cape Town (UCT). As I had a MMed project underway at that stage, this study was not registered for degree purposes. Ethical approval for the study was obtained from the Faculty of Health Sciences Research Ethics Committee at UCT, HREC Ref 444/2011, and the Institutional Review Board at the University of Alabama at Birmingham (UAB).

The enrollment phase was conducted from April to October 2012 and the samples were subsequently shipped to the collaborating microbiology laboratory at UAB. I deregistered from the Medical Virology program at the end of 2012 and relocated to

Durban, where I have since completed two semesters of coursework for the MPH. During this time, I received the study results from UAB, undertook the data analysis and have prepared a manuscript with the potential for peer-review and publication, listing both UCT Division of Medical Virology and UKZN Discipline of Public Health Medicine as my affiliations.

All investigators have approved of the use of this data towards my MPH at UKZN. In addition, the protocol has been internally reviewed in the Discipline of Public Health Medicine for scientific integrity, and the study has been approved by Prof. Mars, Chair, School of Nursing and Public Health Research Committee for retrospective registration.

Sincerely,

Sheetal Manicklal

Discipline of Public Health Medicine
School of Nursing and Public Health
University of KwaZulu-Natal
Durban, 4041
+27721478413 – tele
smanicklal@gmail.com

UKZN BIOMEDICAL RESEARCH ETHICS COMMITTEE

APPLICATION FOR EXPEDITED ETHICS APPROVAL

This protocol has full approval from the UCT Ethics Committee. It has been approved by the School of Nursing and Public Health as a Master of Public Health research project.

Hence the request for an expedited approval from BREC.

Dr Stephen Knight (local supervisor of MPH research)

1. ADMINISTRATIVE DETAILS

NAME: PI - Prof/Dr/Mr/Mrs/Miss/Ms	Dr. Anika Van Niekerk
Gender:	Female
Race:	White
NAME: Co-investigator- Prof/Dr/Mr/Mrs/Miss/Ms	Drs. S Manicklal, M Hsiao, SM Kroon and Prof. SB Boppana
Professional status (if student, year of study)	Graduate Student (MPH) – 2nd year
UKZN College	Health Sciences
UKZN Discipline	Public Health Medicine
Hospital / Institution where employed	n/a
Full Postal address	44 Chatsworth Main Road, Umhlatuzana, 4092
Contact telephone and fax numbers	Cell: 0721478413

	Fax: 0865676692
Email Address	smanicklal@gmail.com
Full time/part time employment	Not employed
Current HPCSA Number (or equivalent statutory health council registration no. as appropriate) - if registration is pending, submit proof of application.	MP0632317
Title of Study	The birth prevalence of congenital CMV in HIV-exposed newborns in Cape Town, South Africa – A pilot study. The "CYPREHEN" (CYtomegalovirus PREvalence in HIV-Exposed Newborns) study.

2. Will there be direct participant contact? **YES***

   If YES, please explain and attach Informed Consent and Information Sheets

   A saliva swab will be obtained from infants of mothers who agree to participate in the study. This is a non-invasive sample, and will be collected by a trained research assistant under the supervision of the PI, who is a neonatologist.

3. **Where will the Research be carried out? (Hospital, clinic etc).**

   Mowbray Maternity Hospital.

4. Is this a retrospective study? **NO**

   (a) Tick type of study:

   Previously conducted cross sectional study – for retrospective registration

5. Will participants' confidentiality be maintained? **YES**

   Explain:

This is an anonymous unlinked study. No personal identifying information will be used in the study. Instead, participants will be assigned a sequential numerical code.

6. Will Informed Consent be obtained? **YES**

Explain:

Written information about the study, nature of the child's involvement and potential risks and benefits will be provided in a language that the child's mother understands (verbal translations in Xhosa and Afrikaans will be available), and she will be allowed sufficient opportunity to consider participation, in the absence of coercion. The informed consent sheet will explain the voluntary nature of participation, and that mothers are free to decline participation without any prejudice. Only mothers who provide full written informed consent will be enrolled in this study.

7. Is this project intended to produce any information of diagnostic significance to the patient? **YES**

If yes, will such information be forwarded to the patient's physician? **NO**

No. The diagnostic information is not current standard of care in South Africa. That the test results will not be provided to mothers will be explained during the informed consent process.

8. Proof of concept

Congenital CMV is a leading cause of childhood hearing loss globally. Studies from Europe and the Americas have consistently demonstrated an increased birth prevalence of congenital CMV infection among infants of HIV infected mothers, ranging from 4.5% to 26%. In addition, detection of congenital CMV infection by newborn saliva PCR screening has shown to be reliable and accurate. Despite this, congenital CMV birth prevalence data is sparse among

HIV exposed infants in sub-Saharan Africa, and has not been documented in South Africa.

9. Motivation for (justify) expedited review:

This is a request for retrospective approval of a minimal risk anonymous unlinked cross-sectional study. No invasive procedures were undertaken. Furthermore, there were no anticipated risks to the newborn as the collection of a saliva swab was painless, quick and noninvasive.

10. Has an application for funds been made to other sources to support this project?
Yes: ✓

If Yes, state name/s of funding agency and amount requested:

- School of Child and Adolescent Health, Department of Paediatrics, Red Cross Children's Hospital (UCT affiliated) - R15 000
- Poliomyelitis Research Foundation – R120 000 (awarded R60 000)

Please indicate whether review fees have been paid for this project?

YES	**NO** ✓	

11. I certify that all information provided above is correct and that it will apply throughout the performance of the proposed research and that I shall be responsible for the safeguarding of the confidentiality of human subjects information involved.

I agree to comply with the UKZN Biomedical Research Ethics Committee's Terms of reference and the SA Department of Health (2004) *Ethics in health research: Principles. Structures and processes,* and, if applicable, the SA Department of

Health (2006) *South African good clinical practice guidelines.* All are available at http://research.ukzn.ac.za/ResearchEthics11415.aspx

Signature of Researcher:  **Date:** 12 Feb 2014

Signatures of co-workers/co-investigators:

Please see letters inserted in the study proposal.

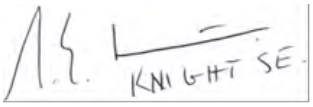
SIGNATURE OF HEAD OF DEPARTMENT:

.....

FOR DEGREE PURPOSES ONLY: (attach approval letter from Postgraduate Education Committee)

Degree: Master's in Public Health Student No: 200266595

Supervisor Name:



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11 February 2014

14.2 Informed consent and participant information sheet

CYPREHEN STUDY Information sheet and Consent

Cytomegalovirus (CMV) infection is very common in children and adults in South Africa, and usually causes no harm if the immune system is intact. CMV is also one of the most common infections a mother can pass to her baby during pregnancy – this is called congenital CMV infection. Worldwide it is thought that one in every hundred babies is born with this infection. Babies with this infection are usually well at birth, but up to one in five infected babies may develop problems (mainly hearing loss) later in early childhood. In other countries, it has been shown that mothers who have HIV are at increased risk of transmitting CMV to their babies during pregnancy. In South Africa, we do not normally look for CMV infection and therefore we don't have information to show whether this is true.

We would like to do a study in partnership with researchers in Alabama (United States). We would like to test 750 babies born to mothers with HIV at Mowbray Maternity Hospital to see how many babies have been infected with CMV during pregnancy. This will be done done by collecting a saliva sample from baby in the first two weeks of life, which will be tested in the laboratory for CMV. Recent studies show that often more than one strain of virus is passed to the baby in utero. We would like to determine whether this is also the case here in South Africa. Therefore, if the result is positive, we will do another test to determine the strain/s of the virus in the saliva sample.

By doing this pilot study we hope to better understand how common congenital CMV infection is in HIV exposed babies in the Western Cape.We are asking you to allow your baby to take part in this study because your HIV status is known. This study is voluntary and the doctors will provide the same care to you and your baby whether you join the study or not.

Description of procedure
If you allow your baby to take part in this study, the research assistant (person who is discussing this study with you) will take either one or two samples of saliva from your baby's mouth using a soft cotton swab. This will be done by rolling the cotton-tipped swab in the inside of the baby's cheek.

She will also collect a few details about your baby (birth weight, gestational age) and you (age, CD4 count, drugs used to prevent HIV transmission to your baby, feeding choice). We will **not** collect any information that will enable us to identify you or your baby. The samples will be labeled with a code number which will not be linked to your's or your baby's clinic folder number, or name. Therefore it will not be possible for you or anyone to know your baby's result. No additional visit will be necessary for this study.

The swab from your baby will be kept in the Groote Schuur Hospital Virology lab in South Africa until enough samples are collected to be shipped to a laboratory in Birmingham (Alabama) for testing. If a second sample was taken at the same time as the first one, this sample will be tested at our laboratory in Groote Schuur Hospital (GSH). This result will be compared with the result from the laboratory at the University of Alabama, in order to evaluate the performance of our CMV test at the GSH laboratory. The samples will not be stored for future use. The samples will be destroyed once the study and the testing has been completed at both labs. You will not be paid for any technology or medicine developed as a result of the study.

Sample collection
Collection of the sample is a painless and will not cause any discomfort to your baby.

Potential Benefit
Information obtained from this study may enable us to plan future research studies in order to evaluate the long-term complications in infected babies. This will help us determine whether this infection should be looked for in all babies exposed to HIV in our setting. This may improve the care to children in South Africa.

If you have any questions, please feel free to contact the project co-coordinator (details below)

PROJECT COORDINATOR: Dr Sheetal Manicklal	Contact numbers 0721478413/0214045302	Department of Virology, Groote Schuur Hospital

Consent form for mothers

YOUR SIGNATURE INDICATES THAT:

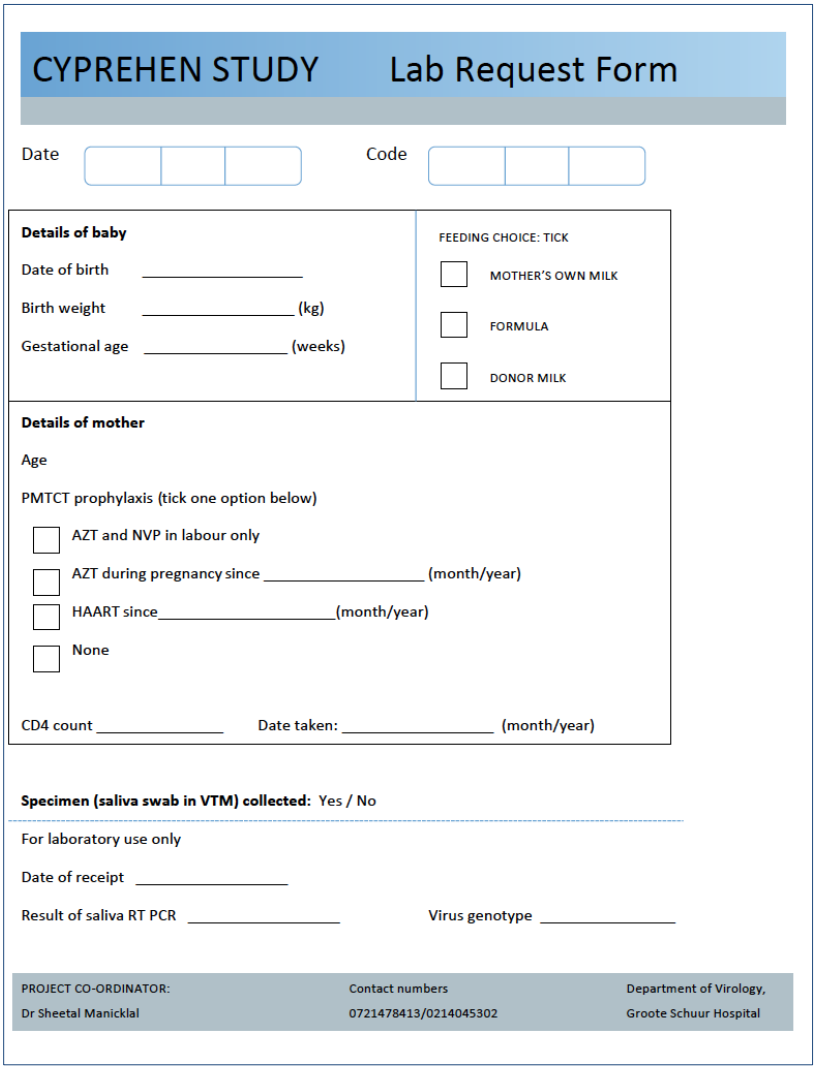
- YOU HAVE READ AND UNDERSTOOD THE INFORMATION SHEET
- YOU HAVE DISCUSSED THIS STUDY WITH THE PERSON OBTAINING CONSENT
- YOU HAVE DECIDED TO TAKE PART IN THE STUDY BASED ON THE INFORMATION PROVIDED
- A COPY OF THIS FORM HAS BEEN GIVEN TO YOU

Signature of mother	Date
Signature of witness if applicable	Date
Full name of witness	

Signature of person who obtained Informed consent	Date
Full name of person who obtained Informed consent	Date

PROJECT COORDINATOR: Dr Sheetal Manicklal | Contact numbers 0721478413/0214045302 | Department of Virology, Groote Schuur Hospital

14.3 Data collection sheet



CYPREHEN STUDY Lab Request Form

Date | | | | Code | | | |

Details of baby

Date of birth _____

Birth weight _____ (kg)

Gestational age _____ (weeks)

FEEDING CHOICE: TICK

☐ MOTHER'S OWN MILK

☐ FORMULA

☐ DONOR MILK

Details of mother

Age

PMTCT prophylaxis (tick one option below)

☐ AZT and NVP in labour only

☐ AZT during pregnancy since _____ (month/year)

☐ HAART since_____(month/year)

☐ None

CD4 count _____ Date taken: _____ (month/year)

Specimen (saliva swab in VTM) collected: Yes / No

For laboratory use only

Date of receipt _____

Result of saliva RT PCR _____ Virus genotype _____

PROJECT CO-ORDINATOR:	Contact numbers	Department of Virology,
Dr Sheetal Manicklal	0721478413/0214045302	Groote Schuur Hospital

14.4 UCT ethical approval

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Faculty of Health Sciences Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
e-mail: sumayah.ariefdien@uct.ac.za

04 November 2011

HREC REF: 444/2011

Dr A van Niekerk
Neonatal Nursery
Mowbray Maternity Hospital
Hornsley Road
Mowbray 7700

Dear Dr van Niekerk

PROJECT TITLE: THE CYPREHEN (CYTOMEGALOVIRUS PREVALENCE IN HIV-EXPOSED NEWBORNS) PILOT STUDY

Thank you for your response to the issues raised.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study.

Approval is granted for one year till the 28 November 2012.

Please submit a progress form, using the standardised Annual Report Form (FHS016), if the study continues beyond the approval period. Please submit a Standard Closure form (FHS010)if the study is completed within the approval period.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

sAriefdien

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

sAriefdien

14.5 MMH ethical approval

COMPONENT: Department of Obstetrics
REFERENCE: Research
ENQUIRIES: Prof. Sue Fawcus
DATE: 23 November 2011

Dear Dr. Van Niekerk

Re: Research Study - The cytomegalovirus prevalence in HIV exposed newborns pilot studies.

Permission is granted by the MMH research committee to conduct your research at this institution.

We will be interested to have a presentation of study results after completion.

Thank You

SRfawcus

Professor S.R. Fawcus (MBBS FRCOG)
Head Obstetric MMH
Associate/Professor
Department: Obstetrics/ Gynaecology
University of Cape Town

13 Hornsey Road, Mowbray, 7700
tel: +27 21 659 5578/9 fax: +27 21 658 2991
Private Bag X1, Mowbray, 7705
Email: sfawcus@pgwc.gov.za

II. UKZN research ethics approval

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

19 June 2014

Dr Sheetal Manicklal
44 Chatsworth Main Road
Umhlatuzana
4092
smanicklal@gmail.com

Dear Dr Manicklal

PROTOCOL: The birth prevalence of congenital CMV in HIV-exposed newborns in Cape Town, South Africa - A pilot study. The "CYPREHEN" Cytomegalovirus PREvalence in HIV exposed Newborns) study. REF: BE124/14

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 17 March 2014.

The study was provisionally approved pending appropriate responses to queries raised. Your responses received on 03 June 2014 to queries raised on 15 May 2014 have been noted by a sub-committee of the Biomedical Research Ethics Committee. The conditions have now been met and the study is given full ethics approval and may begin as from 19 June 2014.

This approval is valid for one year from **19 June 2014.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2004), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be **RATIFIED** by a full Committee at its meeting taking place on **08 July 2014.**

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincerely

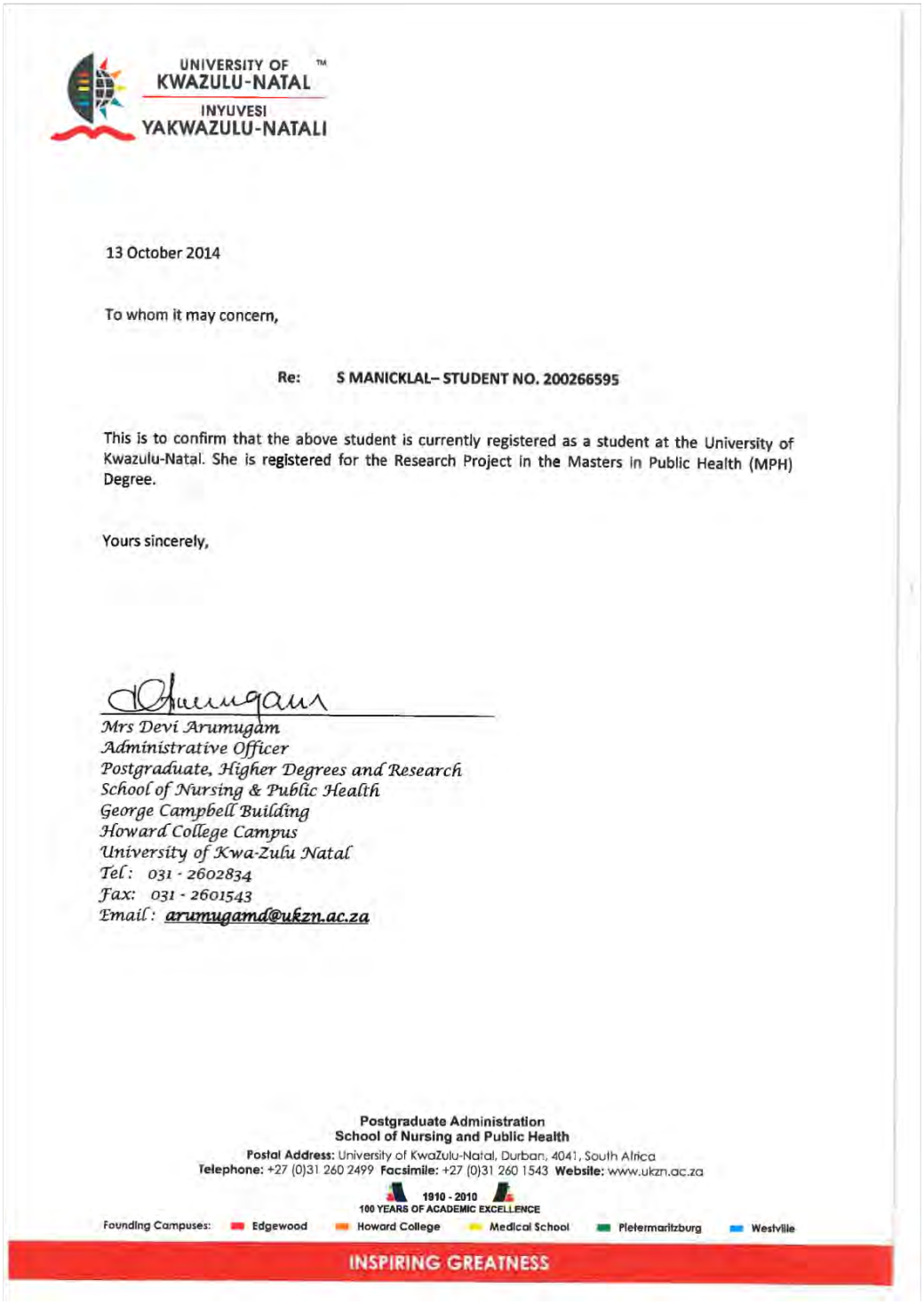
Professor D.R Wassenaar
Chair: Biomedical Research Ethics Committee

Professor D Wassenaar (Chair)
Biomedical Research Ethics Committee
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban, 4000, South Africa
Telephone: +27 (0)31 260 2384 **Facsimile:** +27 (0)31 260 4609 **Email:** brec@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

INSPIRING GREATNESS

III. Acknowledgement of degree registration



UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

13 October 2014

To whom it may concern,

Re: S MANICKLAL– STUDENT NO. 200266595

This is to confirm that the above student is currently registered as a student at the University of Kwazulu-Natal. She is registered for the Research Project in the Masters in Public Health (MPH) Degree.

Yours sincerely,

Mrs Devi Arumugam
Administrative Officer
Postgraduate, Higher Degrees and Research
School of Nursing & Public Health
George Campbell Building
Howard College Campus
University of Kwa-Zulu Natal
Tel: 031 - 2602834
Fax: 031 - 2601543
Email: arumugamd@ukzn.ac.za

Postgraduate Administration
School of Nursing and Public Health
Postal Address: University of KwaZulu-Natal, Durban, 4041, South Africa
Telephone: +27 (0)31 260 2499 **Facsimile:** +27 (0)31 260 1543 **Website:** www.ukzn.ac.za

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INSPIRING GREATNESS

IV. Acknowledgement of role played by student in research study

This section presents the author contributions to the manuscript, followed by a tabulated outline of the research process (Table 13).

Sheetal Manicklal (SM) developed the concept and designed the study. SM, Suresh B Boppana (SBB) and Zdenek Novak (ZN) analysed the data. The initial draft of the manuscript was prepared by SM and SBB. Anika M van Niekerk (AMN) and Stuart M Kroon (SMK) participated in the study design, supervision of the research assistant and with the collection of specimens. Nei-yaun Hsiao (NYH) participated in the collection, storage, maintenance and transport of specimens. Cecelia Hutto (CH) participated in the study design and preparation of the manuscript. Sunil K Pati (SKP) and Nazma Chowdhury (NC) were responsible for testing specimens for CMV DNA and for genotyping. All authors contributed to the manuscript and approved the final manuscript.

Table 13. Timelines of research activities from protocol development to manuscript publication.

Approx. date	Stage of research
Sep-11	The research protocol for data collection at MMH was developed (SM) (not for MMed or other degree purposes at UCT). The study was powered for prevalence. The research protocol was reviewed by the research team comprising investigators at UCT and UAB and was submitted to UCT HREC.
Nov-11	UCT HREC and MMH ethical approvals for the study were granted to the PI (AMN).
Mar-12	A UAB-UCT material transfer agreement was signed for transport and testing of specimens from South Africa at UAB. A memorandum of agreement was concluded outlining research roles and responsibilities for AMN, SBB, SMK, NYH and SM. A research assistant was appointed and trained for data and specimen collection (AMN and SMK).
Apr-12 to Nov-12	Specimens were collected from MMH and stored at the NHLS Virology laboratory.
Sep-12 to Nov-12	Study data were captured and data entries were rechecked (SM).
Jan-13	Study specimens were shipped to UAB for testing (NYH).
Feb-13	Study results were made available by the UAB laboratory.
Jun-13	Data analysis and manuscript preparation were undertaken (SM under guidance of SBB). SM produced and circulated first draft of manuscript to AMN, SMK and NYH for feedback and comments.
Sep-13	The first version of the manuscript was submitted to CID (SM).
Dec-13	The research protocol was rewritten for the Master of Public Health degree at UKZN (SM, under guidance of SK as primary supervisor). This was reviewed internally and the recommendation of including a secondary objective on predictors was incorporated into the protocol. This was consistent with requirements for Master's level research.

	Approval for the use of data collected at UCT towards the Master of Public Health degree at UKZN was sought from UAB, UCT and Prof Mars at UKZN. Approval was granted by all three institutions.
Feb-14	The Master of Public Health protocol was submitted to UKZN BREC. The manuscript was accepted by CID and published electronically.
Jun-14	UKZN BREC granted approval of the study.
Jul-14	The School of Nursing and Public Health board granted approval for retrospective registration of the study.
Aug-14	The College Academic Affairs Board granted approval for retrospective registration of the study.

Abbreviations: MMH, Mowbray Maternity Hospital in Western Cape; UCT, University of Cape Town; HREC, Human Research Ethics Committee; UAB, University of Alabama at Birmingham; UKZN, University of KwaZulu-Natal; BREC, Biomedical Research Ethics Committee; PI, principal investigator; NHLS, National Health Laboratory Service; AMN, Anika M van Niekerk; SM, Sheetal Manicklal; SMK, Stuart M Kroon; NYH, Nie-yaun Hsiao; SK, Stephen Knight; SBB, Suresh B Boppana; CID, Clinical Infectious Diseases Journal.

V. Instructions to authors for the Clinical Infectious Diseases Journal

The manuscript preparation instructions for authors as outlined on the Clinical Infectious Diseases Journal website (105) are presented in this section.

Submission

Please read these instructions carefully and follow them closely to ensure that the review and publication of your paper is as efficient and quick as possible. The Editors reserve the right to return manuscripts that are not in accordance with these instructions.

All material to be considered for publication in *Clinical Infectious Diseases* should be submitted in electronic form via the journal's online submission system at http://www.editorialmanager.com/cid/. Once you have prepared your manuscript according to the instructions below, instructions on how to submit your manuscript online can be found by clicking here.

Supporting Documents

All submitted manuscripts should include the following supporting documents:

Cover Letter

The cover letter must include the completed contact information [addresses, telephone and fax numbers, and e-mail] for both the corresponding author and an alternate author who can be contacted if the corresponding author is unavailable. The letter should warrant that all authors have seen and approved the manuscript, contributed significantly to the work, and also that the manuscript has not been previously published nor is not being considered for publication elsewhere. Authors are asked to provide names and contact information for 4 potential unbiased reviewers. They may also note the names of individuals whom they do not want to review their manuscript. Authors also should state in their cover letter whether they would bear the cost of reproducing their colour figures or whether they prefer to have them published in black and white at no additional cost.

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Article type

Major articles report clinically relevant investigations or observations within *CID*s scope of interests.

Format guide:
• Word limit: 3000 words (excluding the abstract and references).
• Key points should be summarized on the title page in 40-words or less.
• References: 40 or less.
• Abstract: Up to 250 words, structured using the headings Background, Methods, Results and Conclusions.
• Tables/Figures: Data in the text should not be repeated extensively in tables or figures.

Manuscript format and structure

Please refer to a recent issue of *Clinical Infectious Diseases* for guidance on style and layout of articles. Also refer to the Article type section for guidance on relevant information for each article type.

File Formats

The preferred format for submitting manuscripts online is Microsoft Word (.doc files). PDF files are not acceptable for submission.

File Contents

Manuscript .doc submissions are preferred as a single file, except for figures, which can be uploaded separately. You must also submit a cover letter in a second file, in the same format as your main file. Videos must be submitted in the MPEG or Quicktime format. For each video, please submit a still image captured from the MPEG or Quicktime file; this image will appear as a printable figure with the article. A video must have a legend that will appear with the still image. If you wish to submit a video, please consult with the *CID* editorial office for further details.

Manuscript Preparation

Manuscripts should be double-spaced throughout, including the references and the table and figure legends, with 1-inch margins on each side. All pages, except for the figures, should be numbered in the lower right-hand corner of the page, with the title page as page 1. The recommended layout is as follows: title page, abstract, text, acknowledgments, references, tables, figure legends, and figures.

Title Page

All manuscripts, including Correspondence, should have a title page that includes the following information:

1. A concise, informative title
2. The names and affiliations of all authors. The first name, initial(s), and surname of each author should be followed by his or her department, institution, city, and country.
3. Up to 5 keywords
4. A running title of no more than 40 characters and spaces
5. The complete contact information for both the corresponding and alternate corresponding authors.
6. Major Articles, Reviews, and Viewpoints should also include a 40-word summary of the article's main point.

It is editorial policy to list only one author for correspondence. We do not accept co-first authors, nor co-corresponding authors. However, it is acceptable to state that "author X, author Y, etc. contributed equally to this manuscript."

Any changes of address may be given next to the Affiliations or in the Acknowledgments. Any deletions or additions to the author list after submission of the paper must be submitted in writing, and signed by all authors.

Abstract

The second page of the manuscript should contain the Abstract. Please refer to the Article Type for Abstract formats. The Abstract should be comprehensible to readers before they have read the paper and should not contain reference citations.

Abbreviations

Non-standard abbreviations should be kept to a minimum. They should be defined at the first occurrence and introduced only where multiple use is made.

Text

Authors are encouraged to follow the *Uniform Requirements for Manuscripts Submitted to Biomedical Journals*. They should strive for a concise article without excessive detail (word limits are specified under Categories of Articles. All but the shortest articles should have subheadings.

Funding

Details of all funding sources for the work in question should be given in a separate section entitled “Funding.” This should appear before the “Acknowledgment” section.

The following rules should be followed:

• The sentence should begin: “This work was supported by …”
• The full official funding agency name should be given, ie “the National Cancer Institute at the National Institutes of Health” or simply “National Institutes of Health” not “NCI” (one of the 27 subinstitutions) or “NCI at NIH.” Please see here for a full RIN-approved list of UK funding agencies.
• Grant numbers should be complete and accurate and provided in brackets as follows: “[grant number ABX CDXXXXXX]”
• Multiple grant numbers should be separated by a comma as follows: “[grant numbers ABX CDXXXXXX, EFX GHXXXXXX]”
• Agencies should be separated by a semi-colon (plus “and” before the last funding agency)
• Where individuals need to be specified for certain sources of funding the following text should be added after the relevant agency or grant number “to [author initials].”

An example is given here: “This work was supported by the National Institutes of Health [P50 CA098252 and CA118790 to R.B.S.R.] and the Alcohol & Education Research Council [HFY GR667789].”

Conflict of Interest

Further guidance on Conflict of Interests is available here.

Acknowledgments

Personal acknowledgment should precede those of institutions of agencies. Any substantial assistance in preparing the manuscript—for example, in data retrieval or statistical analysis—other than by an author should be stated.

Please note that acknowledgment of funding bodies and declarations regarding conflicts of interest should be given in separate Funding and Conflicts of Interest sections, respectively.

Further guidance on Conflict of Interests is available here.

References

EndNote and Reference Manager are software programs for publishing and managing references/bibliographies, which are available from Thomson Reuters. If you use EndNote or Reference Manager to facilitate referencing citations, this journal's style is available for use. The EndNote program and relevant information can be found here: http://www.endnote.com/support/enstyles.asp. Please follow the instructions on this page regarding purchasing, downloading, and using the software.

CID reference style is based on the *Uniform Requirements for Manuscripts Submitted to Biomedical Journals*.

Names of journals are abbreviated according to the List of Journals Indexed for Medline. Titles of journals not listed in *Medline* should be spelled out in full. References should be numbered consecutively as they appear in the text, with the numbers in brackets on the text line (e.g., [3, 7–9, 57]). References first cited in tables or figures should be in sequence with those in the text; for example, if table 1 is mentioned in the text after reference [8], the next new reference cited in table 1 will be reference [9]. Unpublished data should be cited in the text as (unpublished data), but not included in the references list. References to manuscripts submitted, but not yet accepted, should be cited in the txt as (B Jones and L Smith, manuscript in preparation) and should not be included in the reference list. Citations of submitted manuscripts should include all authors involved. For

references with >6 authors, the first 3 authors should be listed, followed by *et al.* Reference to a doctoral dissertation should include the author, title, institution, location, year, and publication information, if published. For online resources, a URL and date accessed should be included. Accuracy of references is the responsibility of the authors.

The citation of journals, books, multi-author books, and articles published online should conform to the following examples:

- Gorecki DC, Monaco AP, Derry JMJ, Walker AP, Barnard, EA, Barnard, PJ. Expression of four alternative dystrophin transcripts in brain regions regulated by different promoters. Hum Mol Genet, **1995**; 155: 505-511.
- Francis V, Bastin M. (2000) Gene targeting in rat embryo fibroblasts promoted by the polyomavirus large T antigen. Nucleic Acids Res [in press].
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- Public Health Service Task Force. Recommendations for the use of antiretroviral drugs in pregnant HIV-1 infected women for maternal health and interventions to reduce perinatal HIV-1 transmission in the United States. Available at: http://www.aidsinfo.nih.org. Accessed 24 April 2002.
- Lyon DJ, Cheng AFB, Norrby SR. Mechanisms of cefotaxime resistance in blood culture isolates of Enterobacter high prevalence of extended-spectrum β-lactamases [abstract C43]. In: Program and abstracts of the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy (San Francisco). Washington, DC: American Society for Microbiology, 1995:47.

Tables

All tables should be on separate pages and accompanied by a title, and footnotes where necessary. The tables should be numbered consecutively using Arabic numerals. Units in which results are expressed should be given in parentheses at the top of each column and not repeated in each line of the table. Ditto signs are not used. Avoid overcrowding the tables and excessive words. The format of tables should be in keeping with that normally used by the journal; in particular, vertical lines, coloured texts, and shading should not be used. Please be certain that the data given in tables are correct.

In a footnote to the table, all abbreviations used should be defined, unless

otherwise defined in the text, excluding units of measure. Footnotes and accompanying explanatory material should be kept to a minimum. Footnotes should be placed below the table and designated by superscript lowercase letters (listed in order of location when the table is read horizontally). Each column must have a heading describing the data below, and units of measure must be clearly indicated for all data.

For further details on table formatting, please click here.

Figure Legends

These should be on a separate, numbered manuscript sheet. Define all symbols and abbreviations used in the figure. Figures and legends should be intelligible without reading the text of the manuscript.

Part F: Bibliography

1. Pagano JS. Perspective on Cytomegalovirus. Plotkin SA, Michelson S, Pagano JS, Rapp F, editors. In: CMV: Pathogenesis and Prevention of Human Infection. New York: Alan R. Liss, Inc.; 1984.
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9. Lanzieri TM, Dollard SC, Bialek SR, Grosse SD. Systematic review of the birth prevalence of congenital cytomegalovirus infection in developing countries. Int J Infect Dis. 2014 May;22:44-8.
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12. Yamamoto AY, Mussi-Pinhata MM, Isaac Mde L, Amaral FR, Carvalheiro CG, Aragon DC, *et al*. Congenital cytomegalovirus infection as a cause of sensorineural hearing loss in a highly immune population. Pediatr Infect Dis J. 2011 Dec;30(12):1043-6.

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