Role of antiretroviral therapy exposure host genetics on cytomegalovirus infection status and association with gut microbiome profiles among pregnant black African women

by

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1. (Status: *published*) **Doreen Mhandire**, Sarah Rowland-Jones, Kudakwashe Mhandire, Mamadou Kaba, Collet Dandara (2019). **A review on the epidemiology of Cytomegalovirus infection among pregnant women in Africa.** Journal of Infection in Developing Countries 13:865-876. doi: 10.3855/jidc.11373

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3. (Status: *in press*) **Doreen Mhandire**, Gene Morse, Charles Maponga, Collet Dandara. **Plasma efavirenz concentration inversely correlates with increased risk of Cytomegalovirus infection in HIV infected pregnant women**. *South African Medical Journal*

4. (Status: *accepted for publication in BMC Medical Genetics)* **Doreen Mhandire**, Kudakwashe Mhandire, Mulalo Magadze, Collet Dandara. **Toll like receptor and Interleukin-6 genetic polymorphisms influence CMV infection: Implication on Reactivation in Pregnancy.**

5. (Status: *manuscript in preparation for submission*) **Doreen Mhandire**, Harris Onywera, Mamadou Kaba, Collet Dandara. **Comparative diversity of gut microbiota in pregnant women: effects of cytomegalovirus infection.**

Abstract

Cytomegalovirus (CMV) is an important antenatal infection that is prevalent in the developing world. The disabling and potentially fatal effects of CMV acquisition or reactivation during pregnancy on the developing foetus and or neonate are known but, factors predisposing pregnant women to CMV are not well studied. CMV has a wide host cell tropism that includes gut epithelial cells. CMV infection in the gut epithelial cells results in a leaky gut and potential gut microbial dysbiosis. In this study, we set out to determine the prevalence of CMV infection as well as factors associated with CMV reactivation in a cohort of pregnant Zimbabwean women. We also aimed to determine the role of CMV infection and CMV susceptibility host genetics on gut bacterial profiles.

Seroprevalence of CMV was determined using the enzyme-linked immunosorbent assay. A high prevalence of previous exposure to CMV, as denoted by the presence of anti-CMV IgG antibodies in participants' sera, was observed. Anti-CMV IgM antibodies that denote active CMV infection were detected in the sera of 4.6% (n=35/524) study participants. Prevalence of CMV was also determined using real time PCR, CMV reactivation was higher (6.7%) when using PCR than when using immunological assays (4.6%). The presence of CMV DNA was significantly associated with HIV positivity (p=0.04). PCR is the gold standard for CMV diagnosis, thus, CMV DNA positivity was used to denote CMV infection status in this thesis.

The second objective was to determine if the differential effect of CMV acquisition or reactivation among HIV infected participants was due to variability in plasma efavirenz containing antiretroviral therapy (ART) exposure. Efavirenz (EFV) plasma concentrations were determined using high performance liquid chromatography (HPLC). Single nucleotide polymorphisms (SNPs) in the *CYP2B6* gene, which encodes the main EFV metabolizing enzyme were genotyped. Carriers of *CYP2B6* poor metaboliser (PM) genotypes (*c.516T/T* and *c.983T/C*) had significantly higher mean plasma EFV concentration compared to carriers of *CYP2B6* fast metabolizer genotypes (i.e., *c.516G/G* and *c.983T/T*). CYP2B6 PM genotype carriers were significantly less likely to be positive for CMV DNA when compared with fast metabolizer genotype carriers (p<0.001 for both SNPs).

Considering the role of the immune system in keeping latent CMV infection in check, the third objective was to determine the role of SNPs in genes coding for proteins involved in response against CMV. Twenty SNPs in 10 genes (*TLR2, TLR4, TLR7, TLR9, IL6, IL6R, IL10, IL28B, IFNAR1, IL1A*) were characterized and the following were found to be significantly associated

with increased risk of CMV infection; *TLR2* rs1816702T>C (p=0.002), *TLR7* rs179008A>C (p<0.001), *TLR9* rs352139T>C (p=0.003). In contrast, presence of the *IL6* rs10499563T>C polymorphism was inversely correlated with CMV infection (p=0.002). The reported genetic variants are reported to modulate proteins involved in immune responses against viral infections, thus, their association with susceptibility to CMV infection. Such findings may assist in the designing of a much-needed candidate CMV vaccine.

Lastly, we set out to determine the possible role of CMV infection in shaping gut microbiota profiles. We report on a significant difference (p=0.001) in the beta diversity of gut bacterial profiles between HIV- and age-matched CMV-infected (cases) and CMV-uninfected (controls) participants. Using linear discriminant analysis (LDA) effect size (LefSe), significant differences in the relative abundance of specific bacterial taxa were observed between cases and controls (p<0.05, LDA>2). Significantly lower abundance of *Lactobacillus reuteri and Roseburia,* genera associated with lower microbial translocation was observed in cases than controls. Lower relative abundance of *Lactobacillus* and *Roseburia,* is consistent with microbial translocation and heightened inflammation, respectively, hence higher likelihood of microbial translocation and inflammation occurring in cases than controls. Furthermore, *Prevotella copri*, a species that has been association with cytokine release and chronic inflammation was significantly more abundant in cases than controls. CMV is a known chronic inflammatory condition, and this study provides further confirmation through the higher relative abundance of *P.copri* in cases than controls. Biomarker identification has proven to be a successful means of translating molecular data into clinical practice, such as vaccine development in the case of CMV infection.

Overall, this study reports the possible interaction of various host factors in facilitating CMV acquisition or reactivation during pregnancy. In the setting of HIV-CMV coinfection, our findings emphasise on the need for genotype guided drug dosage to achieve therapeutic EFV so as to maintain the balance between host and coinfecting microbes in HIV management. Comprehensive genotype guided drug dosage, if taken as a once-off test should be affordable especially in resource-limited settings. This is particularly important in pregnant women who are at a risk of vertically transmitting infection to the immunologically immature foetus and or neonate. Data from this study may assist in curbing the host associated challenges in designing an effective CMV vaccine. Moreover, the biomarkers reported may assist in diagnosis and management of potential CMV acquisition or reactivation during pregnancy. However, bigger

prospective, functional studies would be needed to confirm the exact roles of the biomarkers identified in this study in the diagnosis, prognosis and therapeutics of CMV infection.

I dedicate this work to

- The late ***Professor Babill Stray Pedersen*** – thank you for the opportunity to tap my real potential and reach the highest level of learning.

- My departed friend and grandmother ***Nzarwo Shava*** – this is how far your little girl has come

  REST IN POWER MIGHTY WOMEN!!

In the fulness of time, God makes all things beautiful…

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Michelle and Tatenda, Cape Town would get really lonely but you always came through!

My sisters Esther and Winnie Duri, thank you for taking care of my children while I was away studying.

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To my all-weather friend, confidante and husband Kuda, you are the real MVP! thank you for your unwavering support and for taking care of our kids while I was away…. You complete my world, I LOVE YOU. To Shona and Kala…. you make my heart full, keep shining!

Table of Contents

List of Figures

List of Abbreviations and Acronyms

16S rRNA – 16S ribosomal ribonucleic acid

3TC - lamivudine

AJOL – Africa journal online

ART – anti-retroviral therapy

ASV – amplicon sequence variant

BMI – body mass index

cART – combination anti-retroviral therapy

cCMV - congenital cytomegalovirus

CD4+ - cluster of differentiation 4

CD8+ - cluster of differentiation 8

CI – confidence interval

CMV - cytomegalovirus

CPQA – clinical pharmacology quality assurance

CYP2B6 – cytochrome P450 2B6

DADA - divisive amplicon denoising algorithm

DNA – deoxyribonucleic acid

dsDNA – double stranded deoxyribonucleic acid

E - early

EBV – epstein barr virus

EFV - efavirenz

ELISA – enzyme linked immunosorbent assay

FTC - emtricitabine

g – glycoprotein (gB, gH, gL, gM)

HHV – human herpes virus

HIG – human immunoglobulin

HIV - human immunodeficiency virus

HSV – herpes simplex virus

IBD – inflammatory bowel disease

IE – immediate early

IFNAR1 – interferon-alpha/beta receptor

IFNα – interferon alpha

IFNβ – interferon beta

IgG - immunoglobulin G

IgM - immunoglobulin M

IL - interleukin

IQR – interquartile range

JREC – joint research ethics committee

KIR – killer cell immunoglobulin-like receptor

L - late

LDA - linear discriminant analysis

LefSe - linear discriminant analysis effect size

mg - milligram

MHC – major histocompatibility complex

mL - millilitre

MRCZ – medical research council of Zimbabwe

MTCT – mother to child transmission

NaOH – sodium hydroxide

NFκ-β – nuclear factor kappa beta

Ng - nanogram

NK – natural killer

nM - nanomolar

NNRTI – non-nucleoside reverse transcriptase inhibitor

NRTI – nucleoside reverse transcriptase inhibitor

OR – odds ratio

PAMP – pathogen associated molecular pattern

PCR – polymerase chain reaction

PD – phylogenetic diversity

PERMANOVA - permutation multivariate analysis of variance

pM - picomolar

PMTCT – prevention of mother to child transmission

PRR – pattern recognition receptor

QIIME – quantitative insights into microbial ecology

qPCR – quantitative polymerase chain reaction

REDCap – research electronic data capture

Sd – standard deviation

SNP – single nucleotide polymorphism

TDF – tenofovir disoproxil fumarate

TENOLAM-E – tenofovir, lamivudine and efavirenz

TLR – toll like receptor

TNF – tumor necrosis factor

USD – United States dollar

UZ-CHS BC – University of Zimbabwe – College of Health Sciences birth cohort

UZ-IPSL – University of Zimbabwe- International pharmacology specialty laboratory

VZV – varicella-zoster virus

1 Chapter 1: Introduction and Literature Review

Synopsis

Chapter 1 consists of three parts which are as follows:

(i) An overview of the global prevalence, structure, life cycle, pathophysiology, immunity elicited, as well as treatment of cytomegalovirus (CMV), from section 1.1 to section 1.5

(ii) A published review on the epidemiology of CMV infection among pregnant women in Africa. *The review was published in the Journal of Infection in Developing Countries* (available here; https://jidc.org/index.php/journal/article/view/11373/2135). This part makes up section 1.6

(iii) This final section (section 1.7 to 1.7.2) of chapter 1 provides the aims, rationale to the concept and the specific objectives that were developed and executed in order to address the thesis aims.

1.1 Overview and Background

Cytomegalovirus (CMV) is a common infection which is almost ubiquitous in the developing world. CMV infection may occur in people without prior exposure to the virus, resulting in primary infection and may also reactivate in those with latent infection or may be reinfected with a different strain of CMV (Itell, Nelson, Martinez, & Permar, 2017). All three instances CMV infection usually remain asymptomatic and subclinical, mainly because the immune system keeps viral replication under control. However, in patients who fail to mount adequate immunity, CMV may be uncontrolled and lead to high viral loads which are associated with viral shedding, dissemination to multiple organs and diseases such as pneumonitis, retinitis, hepatitis or gastroenteritis (Emery, 2001; Zuhair et al., 2019). CMV therefore becomes an important pathogen in individuals with immature or compromised immunity such as the unborn child, pregnant women, HIV infected and allograft recipients. CMV may have potentially fatal effects in the setting of compromised or immature immunity.

When any of the three types of CMV infections occur in pregnancy, an antenatal infection results which can potentially be transmitted to the developing foetus, during parturition and within the first 21 days post-natal, resulting in congenital CMV infection (cCMV) (Pass & Anderson, 2014). In the ART era, which has resulted in a massive decrease in vertical transmission of HIV, cCMV has become the most common vertically transmitted pathogen, impacting approximately one million newborns annually, globally (Dollard, Grosse, & Ross, 2007; Kenneson & Cannon, 2007). cCMV can occur as a result of any of the three types of CMV infection in pregnancy, with viraemia leading to involvement of the placenta then the foetus. cCMV has resulted in a global burden of permanent sequelae such as sensorineural hearing loss, growth restriction and intellectual disability (Lanzieri et al., 2017). Moreover,

cCMV has recently been linked to an increased risk of lymphoblastic leukemia and chronic conditions such as glioblastoma (Francis et al., 2017).

This thesis is focused on the prevalence of CMV factors that may be associated with acquisition of CMV during pregnancy.

1.2 Global burden of CMV

CMV infection is transmitted from person to person through contact with infectious mucosal body fluids such as blood, urine, saliva, breast milk, genital secretions and blood products. CMV is also transmitted during solid organ transplantation or intrauterine from mother to foetus during pregnancy (Forbes, 1989; Numazaki, Chiba, & Asanuma, 2001). As a result, CMV is one of the most successful human pathogens as it can be transmitted both horizontally and vertically. CMV infection status is generally determined by detection of anti-CMV immunoglobulin G (IgG) and immunoglobulin M (IgM) in serum. Detection of CMV DNA by polymerase chain reaction (PCR), the gold standard is a more reliable method and gives a better reflection of the presence of the virus. However, routine diagnosis of CMV is done by detection of anti-CMV antibodies as it is a more accessible method.

Globally, between 40 and over 90% of the general population are infected with CMV, with prevalence increasing as we move from developed to developing world (Zuhair et al., 2019). In the developed world, seroprevalence ranges from 40-70% and tends to increase with increasing age (Cannon & Davis, 2005; Zuhair et al., 2019). In the developing world, most seroconversion occurs during childhood, resulting in adult seroprevalence of 80 to over 90% (Cannon & Davis, 2005). Similarly, seroprevalence of CMV in women of child bearing age follows the same trend, with higher rates in the developing world compared to the developed world, as shown in Figure 1.1.

The epidemiology of maternal CMV has a complex relationship with that of cCMV since maternal infection sustains cCMV. The vertical transmission rates of CMV are 1-5% and 0.6-0.7% in high seroprevalence and low prevalence settings, respectively (Dollard et al., 2007; Kenneson & Cannon, 2007). The global mortality rate of cCMV is approximately 30% (Zuhair et al., 2019). However, a considerable number of cCMV are likely missed due to the asymptomatic nature of cCMV. Significant differences in seroprevalence exist between populations, which correlate closely to socioeconomic status, race and variations in burden of immunodeficiency diseases, among other factors.

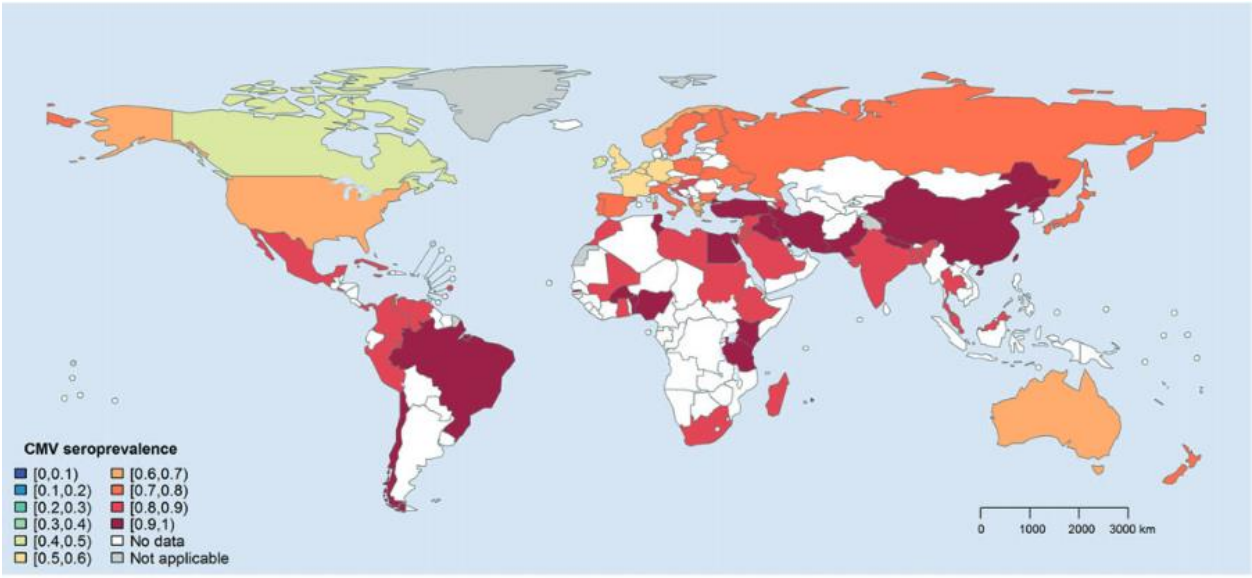

Figure 1.1. Global CMV seroprevalence rates in women of child bearing age (Adapted from Zuhair et al., 2019)

1.3 CMV structure, life cycle and pathogenesis

Cytomegalovirus (CMV) is a member of the human herpesvirus family that consists of 8 viruses: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), CMV, human herpes virus 6 A and B (HHV-6A and B), human herpes virus 7 (HHV-7), and human herpes virus 8 (HHV-8) (McGeoch, Cook,

Dolan, Jamieson, & Telford, 1995). The Herpesviridae family can be divided into three subfamilies (herpesvirinae): alpha, beta and gamma. CMV belongs to the beta (β) family of herpesviruses, which infect organisms in a species-specific manner (McGeoch et al., 1995; Tomtishen III, 2012).

CMV is a typical herpes virion which is approximately 200-300nm in diameter and contains three identifiable regions, the capsid 100nm in diameter embedded in a proteinaceous matrix (the tegument), which is surrounded by a lipid envelope (Shenk & Stinski, 2008)(Figure 1.2). The capsid exhibits icosahedral symmetry with 162 capsomers enclosing the large double stranded DNA genome (Chen, Jiang, Lee, Liu, & Zhou, 1999; Shenk & Stinski, 2008). CMV represents the largest genome of the characterised human herpesvirus family to date, with a genome of 236 kbp (Cha et al., 1996; Riley, 1997; Stern-Ginossar et al., 2012). The CMV genome is estimated to contain ~192 open reading frames encoding more than 80 viral proteins, including glycoproteins which are located in the tegument (Cha et al., 1996; Chee et al., 1990; Gibson, 2008). These include glycoprotein B (gB), gH, gL, gM, gN and gO which serves various functions including adsorption to host cells (Varnum et al., 2004). The genome is packaged in an icosahedral capsid surrounded by a lipid envelope. Viral phosphoproteins are found in the tegument, a space between the nucleocapsid and the envelope.

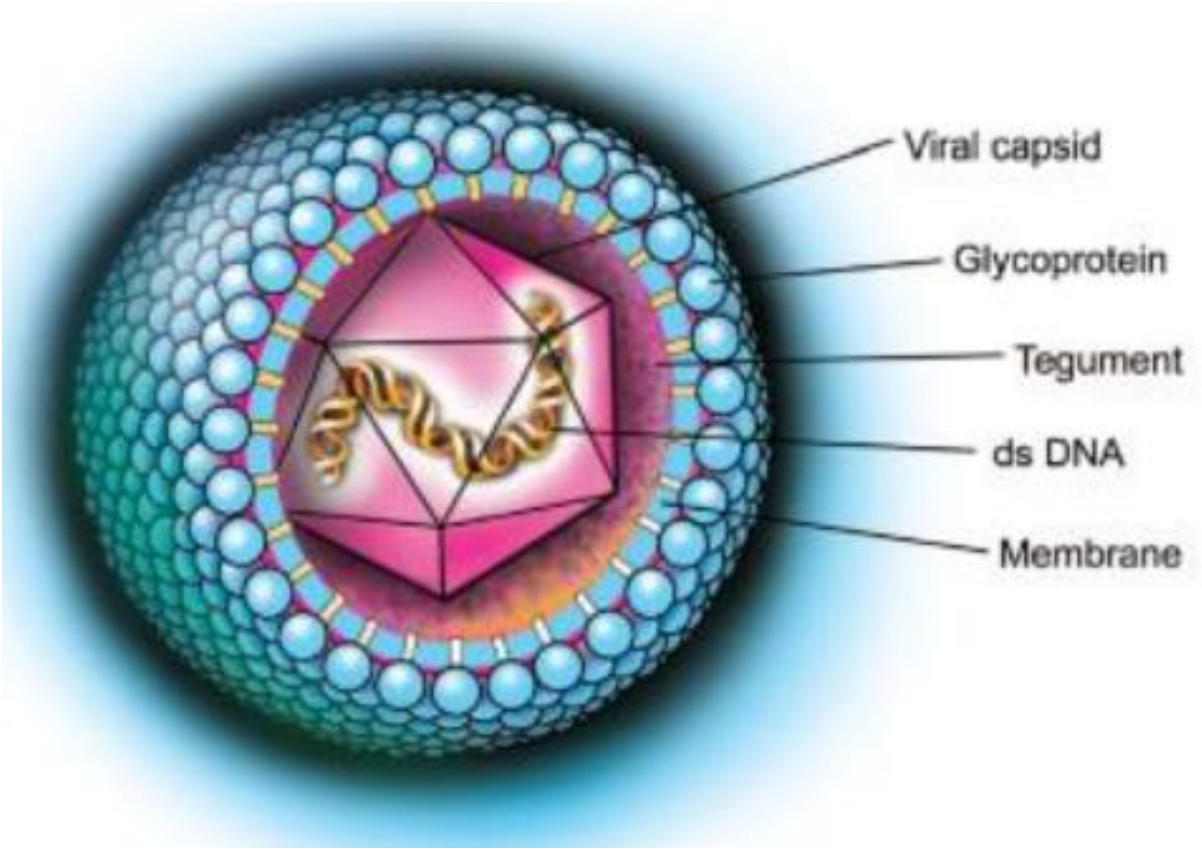

Figure 1.2. CMV virion structure and structural components (https://pedclerk.bsd.uchicago.edu/page/cytomegalovirus-cmv).

CMV effectively penetrates virtually all types of cells, including epithelial cells of gland and mucosal tissue, smooth muscle cells, fibroblasts, macrophages, dendritic cells, neurons, hepatocytes and vascular endothelial cells (Sinzger, Digel, & Jahn, 2008). This penetration occurs through endocytosis and fusion at low-pH in epithelial and endothelial cells while it is accomplished by a pH-independent fusion (Ryckman, Jarvis, Drummond, Nelson, & Johnson, 2006). The receptor-mediated endocytosis is triggered by virally encoded glycoproteins particularly gB and gH, binding to target cellular receptors (Vanarsdall & Johnson, 2012). As

the virus penetrates the cell, viral proteins regulate cell-signaling pathways and cellular metabolism to support viral replication and immune evasion.

After membrane fusion, the viral capsid loses its tegument layer (Feng, Schröer, Yu, & Shenk, 2006). The released tegument proteins facilitate the migration of the viral capsid towards the nucleus, via microtubules, the release of the viral genome from the capsid, and activation of viral gene expression (Compton & Feire, 2007). Viral genome replication can now occur in the new host cell. Once within the nucleus the viral DNA genome circularises by fusion of double stranded DNA termini. The circular DNA becomes a template for DNA replication forming large interlinked DNA copies. After circularisation, a tegument protein and cell proteins bind to DNA in order to initiate transcription signaling CMV lytic infection which is characterised by a finely-regulated cascade of gene expression separated into: (i) immediate early (IE) genes that encode proteins responsible for initiating and terminating each of the subsequent steps in replication. (ii) early (E) encode proteins that are responsible for viral DNA replication. Both IE and E proteins contribute to a cellular environment which is optimal for viral gene expression and viral genome synthesis (Fields, Knipe, & Howley, 2007; Fortunato & Spector, 1999). Late (L) genes encode viral structural proteins responsible for virion assembly and maturation. Late genes are expressed from around 48 hours post infection, and their expression begins the production of infectious virions (Fields et al., 2007).

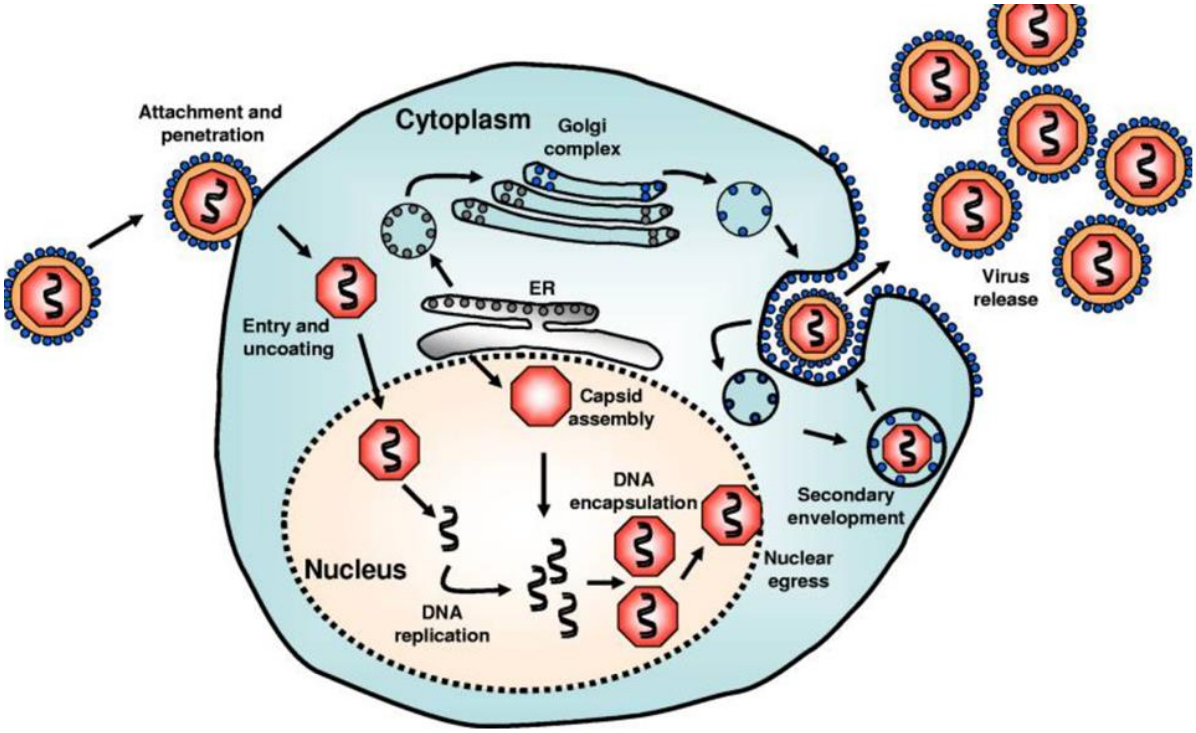

Figure 1.3. Overview of the CMV life cycle (Crough & Khanna, 2009).

In the late stages of replication, the capsid is assembled in the nucleus. Nucleocapsid particles accumulate in inclusions that represent one of the cytopathic effects of CMV infected cells (intracellular inclusions) (Griffiths, Baraniak, & Reeves, 2015). CMV associated diseases occur when the immune system loses control of either primary or reactivated viral infection. During the lytic cycle, viral replication disrupts the cytoskeleton, causing massive cell enlargement (Griffiths et al., 2015; Klemola, Von Essen, Henle, & Henle, 1970). The replication continues for several days until cell lysis occurs. The wide cell tropism of CMV has resulted in CMV being implicated in multiple organ-specific damages such as CMV colitis, hepatitis, pneumonitis, retinitis (Sinzger & Jahn, 1996). Viral replication and load are important factors in pathogenesis, with CMV viral load being directly associated with disease (Cope et al., 1997; McBride et al., 2019; Regoes et al., 2006). A threshold relationship between CMV viraemia and disease has been described in renal and liver transplant patients where the quantity of CMV in serum was directly linked to the risk of CMV recurrence,

pathogenicity and mortality (Humar, Kumar, Boivin, & Caliendo, 2002; McBride et al., 2019).

CMV infected immunocompromised patients present with protracted fever, fatigue or malaise and increased white blood cell count (Britt, 2008). Immunocompetent hosts are usually asymptomatic but may present with a self-resolving mononucleosis syndrome. CMV can also cause organ specific damage such as CMV colitis, granulomatous hepatitis and focal neurological deficits (Poole, Wills, & Sinclair, 2014). cCMV infection has been associated with cerebral calcifications which result in neural and developmental disorders (Cheeran, Lokensgard, & Schleiss, 2009).

1.4 Immune response to CMV infection

The outcome of viral infections is determined by tropism and virulence of the virus, its ability to manipulate the immune system, and, notably, the effectiveness of the host's immune response in retaining the virus. Relationship between CMV and host immunity, including recognition, priming, and the subsequent host response, is a major determinant of CMV pathogenesis. CMV infection triggers forceful immune reaction in the human body, initially by innate natural killer (NK) cells, followed by adaptive CD4+ and CD8+ T cells and B cell high avidity neutralizing antibodies (Rook, 1988). Figure 1.4 illustrates the different types of immunity mounted against CMV as well as some of the immune cells involved.

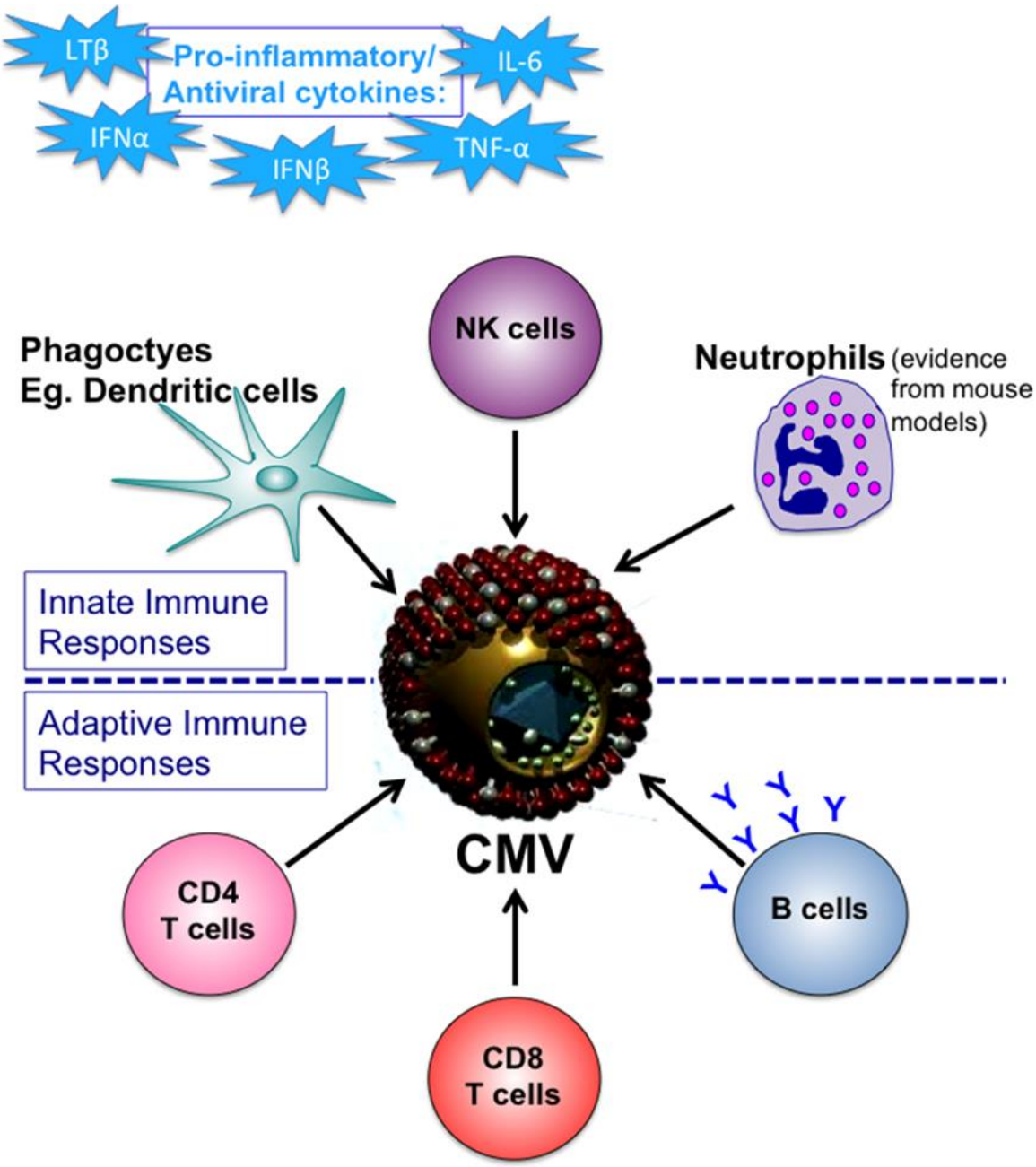

Figure 1.4. Immunity against CMV (https://www.immunology.org/public-information/bitesized-immunology/pathogens-and-disease/human-cytomegalovirus-hcmv)

1.4.1 Innate immunity

The innate immunity is normally the first line of cellular defense, which afterwards has a cross interaction priming the adaptive immunity response. Innate immunity does not confer long term immunity against pathogens but it is the dominant immune system of host defense

in initial response, hence plays a big role in determination of the strength of subsequent immune responses (La Rosa & Diamond, 2012). The innate immunity relies on evolutionarily ancient germ line encoded receptors, pattern-recognition receptors (PRRs) that recognize highly conserved microbial molecular structures, known as pathogen associated molecular patterns (PAMPs). The PRRs that play a role in immunity against CMV are the toll like receptors (TLRs) (Compton et al., 2003).

TLRs are generally expressed on almost all the immune effector cells; macrophages, mast cells, neutrophils, dendritic cells, basophils, eosinophils and natural killer cells (Botos, Segal, & Davies, 2011). Attachment and binding of viral glycoproteins, particularly gB and gH to host-cell toll-like receptors (TLR) activate transcription factors, which are the first defense mechanism of the host cell (e.g. nuclear factor kappa (NFκ-B) (Boehme, Guerrero, & Compton, 2006; Powers, DeFilippis, Malouli, & Früh, 2008). These factors induce inflammatory cytokines and interferon-stimulated genes, such as tumor necrosis factor (*TNF-α*), interleukin (*IL*)-*1*, *IL-6*, *IL-8*, *IL12* and *IL-18* to inhibit viral replication and initiate adaptive immune responses (Kogut, Chiang, Swaggerty, Pevzner, & Zhou, 2012; La Rosa & Diamond, 2012).

1.4.2 Adaptive immune response

Adaptive immune response is closely related to the innate immune response and the pathway of communication is one target of HCMV evasion. Adaptive immunity is classified into humoral and cellular immunity. Humoral immunity is mediated by B-lymphocytes, which secrete antibodies. Antibodies are produced to target viral phosphoproteins and glycoproteins. HCMV specific antibodies have neutralizing activity and complement fixing activity that is important to disrupt viral replication (Gerna et al., 2008; Macagno et al., 2010). T-lymphocytes mediate cellular immunity, and consist of CD8+ cytotoxic T cells and CD4+ helper T cells. CD8+ T cells are able to recognise and destroy infected cells via recognition of

viral peptides presented via MHC-I molecules on infected cell surface while CD4+ helper T cells are mediated by expression of MHC-II molecules (Hertoghs et al., 2010; Sylwester et al., 2005).

The capacity of CMV to establish latency and to co-exist with the host may be the result of multiple immune evasion strategies. The large genome of CMV codes for various proteins which aid in immune evasion thereby facilitating latency and co-evolution with the human host, following a primary infection. Viral proteins such as UL16 and UL18 are responsible for helping the virus to avoid apoptotic signaling and NK response (Jackson, Mason, & Wills, 2011; Noriega, Redmann, Gardner, & Tortorella, 2012). CMV has also been shown to downregulate both MHC class I and II expression in infected cells and to interfere with MHC class II expression by several mechanisms (Jackson et al., 2011; Tortorella, Gewurz, Furman, Schust, & Ploegh, 2000).

1.5 Treatment and vaccine against CMV

1.5.1 Antiviral treatment

Several anti-viral drugs such as ganciclovir, valganciclovir, foscarnet and cidofovir are available for the treatment of CMV. Ganciclovir and its prodrug valganciclovir are nucleoside analogues which inhibit viral DNA polymerase are the currently approved drugs for first line treatment of CMV (Freitas, Smee, Chernow, Boehme, & Matthews, 1985; Martin, Dvorak, Smee, Matthews, & Verheyden, 1983). In addition to reported viral resistance after a few months of therapy, ganciclovir has been associated with neutropenia, thrombocytopenia and putative long term infertility (Faqi, Klug, Merker, & Chahoud, 1997; Steininger, 2007). Valganciclovir has therefore become a drug of choice for cCMV disease (Kimberlin et al., 2015). The second-line regimen for CMV treatment comprises DNA polymerase inhibitors foscarnet and cidofovir, UL97 kinase inhibitor maribavir and nucleocapsid tegumentation

inhibitor leflunomide (Chacko & John, 2012; Tan, 2014). Letermovir, a viral terminase inhibitor is used to prevent infection and disease in CMV seropositive patients (Foolad, Aitken, & Chemaly, 2018; Verghese & Schleiss, 2013). None of the CMV treatment drugs are licensed for use during pregnancy as cidofovir and foscarnet are nephrotoxic and likely to have teratogenic effects on the foetus. Ganciclovir has been shown to affect development of sexual organs in animal studies (Rawlinson et al., 2017; Tan, 2014) and similar results are anticipated for a developing foetus, hence ganciclovir use during pregnancy is prohibited. Safety of use during pregnancy has not been assessed in the other CMV treatment drugs. Furthermore, the high costs of anti-CMV drugs are inhibiting and makes CMV treatment highly inaccessible in the developing world.

The Food and Drug Administration of the USA has also approved CMV human immunoglobulin (HIG) therapy for prophylaxis of CMV infection. CMV HIG is a high titre of plasma derived anti-CMV antibodies collected from donors with high antibody levels. For prevention of cCMV, CMV HIG has mostly been used as an intervention in cases where maternal ante-natal seroconversion has been confirmed. Nigro et al. (Nigro et al., 2012) conducted a nonrandomized clinical trial among women with known recent CMV primary infection divided into two groups, one that received CMV HIG and the second group which did not receive CMN HIG. After administration of HIG before 21 weeks of gestation, it was observed that there was a significant difference in cCMV infection with infants in the HIG arm having less infection (16%) compared to 40% in the control group, suggesting a protective effect of CMV HIG. However, another study in Italian pregnant women did not show any differences (Revello et al., 2014). Findings from clinical trials investigating the utility of HIG in treatment of CMV have been inconclusive and unsatisfactory hence, no recommendation of HIG in clinical management of CMV to date (Khalil, Jones, & Ville, 2017).

1.5.2 Vaccine against CMV

Owing to the significant morbidity, mortality and sequelae associated with CMV, the American Institute of Medicine has prioritised the development of CMV vaccine (Medicine, 1999) while the Centre for Disease Control has assigned it second highest priority after HIV. Since then, a potent vaccine for CMV infection has not been found. Over the years, the need for CMV vaccine has been overtaken by other viruses such as HIV, HPV and the flu virus. However, CMV infection is still a public health challenge especially during pregnancy where there is risk of vertical transmission with potentially fatal effects.

In a clinical trial, a candidate vaccine which was live attenuated Towne strain of CMV had low immunogenicity and was unable to protect against reactivation of infection (Cheeran et al., 2009; Plotkin et al., 1994). The ability of CMV to superinfect an already persistently infected host complicates the development of an effective vaccine. In addition, considering the multi-tier arsenal used by CMV to evade host immune defenses, a vaccine that incorporates different viral immunogens which can elicit a robust, adequate immune response is needed.

The first phase II randomized, double blinded, placebo-controlled clinical trial was a recombinant CMV gB vaccine and MF59 adjuvant (Pass et al., 2009). The vaccine protected 50% of seronegative women against primary infection. Trials for CMV vaccine have been ongoing but none has shown complete potency against CMV. As a result, there currently is no CMV vaccine in use.

1.6 Epidemiology of Cytomegalovirus among pregnant women in Africa

Based on published review; **Doreen Mhandire**, Sarah Rowland-Jones, Kudakwashe Mhandire, Mamadou Kaba, Collet Dandara (2019). **A review on the epidemiology of Cytomegalovirus infection among pregnant women in Africa.** Journal of Infection in Developing Countries 13:865-876. doi: 10.3855/jidc.11373

Abstract

Vertical transmission of Cytomegalovirus (CMV), resulting in congenital CMV (cCMV) infection could have disabling and potentially fatal effects on the foetus or neonate. Although primary infection probably has a higher risk of leading to cCMV, in highly seropositive populations, a significant risk of vertical transmission is thought to be due to CMV reactivation and or reinfection during pregnancy. In this narrative review, we summarise the prevalence of CMV infection and associated risk factors among pregnant African women, in a setting where primary CMV infection usually occurs during infancy. A systematic search of literature published between January 2000 and January 2019, retrieved on five bibliographic databases was performed. Search for relevant articles was performed using the following keywords: cytomegalovirus, CMV, infection, antenatal infections, pregnancy, pregnant women, gravidity, developing countries and Africa, with appropriate qualifiers such as OR, AND. Systematic searching retrieved 11 relevant original research papers. Prevalence of anti-CMV IgG and IgM antibodies ranged from 60-100% and 0-15.5%, respectively. Prevalence of CMV DNA ranged from 0-29%, depending on the specimen used. However, there was no geographic trend for CMV seroprevalence or CMV DNA prevalence across the African continent. Overall, a substantial percentage of women of reproductive-age were CMV seronegative and at risk of primary infection. Association of sociodemographic factors with CMV infection were inconsistent across all reviewed studies. The limited data and

inconsistency of findings from the few studies carried out in Africa calls for prospective studies comparing prevalence and outcomes of cCMV in infants born to women with both primary and reactivated CMV in Africa.

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Review

Epidemiology of Cytomegalovirus among pregnant women in Africa

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Abstract

Introduction: Vertical transmission of Cytomegalovirus (CMV), resulting in congenital CMV (cCMV) infection could have disabling and potentially fatal effects on the foetus or neonate. Although primary infection probably has a higher risk of leading to cCMV, in highly seropositive populations, a significant risk of vertical transmission is thought to be due to CMV reactivation and or reinfection during pregnancy. In this narrative review, we summarise the prevalence of CMV infection and associated risk factors among pregnant African women, in a setting where primary CMV infection usually occurs during infancy.

Methodology: A systematic search of literature published between January 2000 and January 2019, retrieved on five bibliographic databases was performed. Search for relevant articles was performed using the following keywords: cytomegalovirus, CMV, infection, antenatal infections, pregnancy, pregnant women, gravidity, developing countries and Africa, with appropriate qualifiers such as OR, AND.

Results: Systematic searching retrieved 11 relevant original research papers. Prevalence of anti-CMV IgG and IgM antibodies ranged from 60-100% and 0-15.5%, respectively. Prevalence of CMV DNA ranged from 0-29%, depending on the specimen used. However, there was no geographic trend for CMV seroprevalence or CMV DNA prevalence across the African continent. Overall, a substantial percentage of women of reproductive-age were CMV seronegative and at risk of primary infection. Associations of sociodemographic factors with CMV infection were inconsistent across all reviewed studies.

Conclusions: The limited data and inconsistency of findings from the few studies carried out in Africa calls for prospective studies comparing prevalence and outcomes of cCMV in infants born to women with both primary and reactivated CMV in Africa.

Key words: cytomegalovirus; prevalence; risk factors; pregnant; pregnancy.

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Introduction

Cytomegalovirus (CMV) is the second most common cause of congenital viral infections in the developing countries, after HIV [1]. Congenital CMV (cCMV) occurs when there is transmission of CMV from the mother to the infant *in utero* or perinatally. Congenital CMV is diagnosed by the detection of CMV DNA in any of the infant's body fluids within the first 21 days of life [2]. Because CMV infection or reactivation takes advantage of decreased or compromised immunity, pregnant women are particularly at risk of CMV reactivation due to the immune down-regulation which occurs in pregnancy. Pregnancy has been described as an immunological condition which presents multiple challenges in diagnosis, prevention and management of infectious diseases [3].

Maternal immunity acquired against CMV prior to conception does not confer complete protection to the developing foetus, as is the case with the other antenatal and perinatal congenital infections such as toxoplasmosis and rubella. As a result, vertical transmission of CMV can occur in both primary and non-primary maternal infections with rates of 30% and 2%, respectively [4–5]. It was previously believed that vertical transmission of CMV and severe neonatal symptoms of cCMV only occur in infants born to mothers with primary infection in pregnancy. However, over 60% of the infants infected *in utero* with CMV are born to mothers with preconceptional immunity who have secondary infection in pregnancy, and more and more studies show severe sequelae in these infants. We therefore conclude that congenital CMV may be a significant problem even in children born to mothers with pre-pregnancy immunity [6–10]. Because cCMV

prevalence (~3%) is higer in resource-limited settings where previous maternal infection is widespread, the majority of cCMV cases are a result of non-primary maternal infections compared with the developed world which has cCMV prevalence of ~0.3% [11–12]. The role of non-primary CMV infection in cCMV has been shown in Brazil where a maternal CMV seropositivity of ≥ 97% resulted in more cases of cCMV compared to primary maternal infection [13–14]. Similar data are lacking in Africa despite a high seroprevalence of CMV and a likely growing burden of children with cCMV associated sequelae. This may justify the use of invasive methods for the detection of possible fetal infection even in cases of secondary CMV infection. This also brings in an additional problem, when considering the need for immunisation strategies against CMV, as current vaccine development is primarily aimed at seronegative women to prevent primary infection. Furthermore, CMV infection during pregnancy increases the risk of spontaneous abortions [15].

Identification and treatment of cCMV is complicated as only 10–15% of infected infants present with symptoms at birth, and current guidelines recommend giving antiviral therapy only to symptomatic infants. However, as many as 15%–25% of asymptomatic infants go on to develop CMV-associated sequelae, particularly hearing loss [9,16]. Treatment of cCMV, especially in Africa, is further complicated by the high cost of antiviral drugs. The recommended 16 mg/kg twice daily dose of valganciclovir for six months costs approximately USD1820 [17–18]. This cost is out of reach for most people in developing countries who survive on less than USD1 per day and whose governments are already battling to sustain HIV antiretroviral therapy programs [19–20]. Moreover, in some cases, resistance against the widely used ganciclovir has been reported [21]. Further, the asymptomatic viral shedding of CMV sustains lack of recognition and underdiagnosis of the infection and risks of onward transmission.

Currently, there are no ideal safe and effective interventions to control CMV infection in pregnancy, as animal studies have shown teratogenicity associated with the principal anti-CMV agent, valganciclovir, when used in early pregnancy. There are currently no data on the teratogenicity of valganciclovir in humans [22]. In the absence of an effective vaccine, timely and robust diagnosis of maternal CMV infection and identification of mothers at highest risk of transmitting CMV becomes a priority so that infants with cCMV infection can be diagnosed and treated.

The prevalence of CMV differs among populations and regions, with Africa being one of the regions with the highest prevalence: Africa (> 95%), South America (> 95%), Asia (81-95%), North America (≤ 70%), Australia (≤ 65%), Europe (≤ 60%) [23–24]. Surprisingly, despite the high prevalence reported in the few studies done in Africa, most African countries lack CMV prevalence reports, especially in pregnant women. In addition, while identification of factors influencing the vertical transmission of CMV is critical in curbing new infections, very little is documented on these factors. As the world focuses on the WHO-led 90-90-90 targets for HIV diagnosis, treatment and control [25], important co-infections such as CMV are neglected. In view of the potentially disabling and/or fatal effects of congenital CMV infection, we summarise the prevalence and associated risk factors of CMV infection among pregnant women in Africa.

Methodology

We conducted a systematic literature search for this narrative review which summarises the seroprevalence/prevalence of CMV infection and associated risk factors among pregnant African women.

Definition of CMV infection

In this review, the following terms are used and should be defined to understand the different types of CMV infection status [26]. CMV seropositive refers to presence of either CMV IgM or IgG antibodies in either serum or plasma. CMV IgG seropositivity is used as a marker of exposure to CMV at some point during an individual's lifetime. CMV IgM seropositivity is a marker of CMV infection in a previously CMV seronegative individual (primary infection) or CMV reactivation/reinfection in a CMV IgG seropositive individual (secondary infection). CMV IgG antibody avidity is the aggregate strength with which CMV IgG antibodies bind to the CMV antigens. IgG antibody avidity gives a reflection of how long antibodies have been in circulation, following exposure to an antigen. The longer the antibodies would have been in circulation, the higher the avidity and *vice versa* [27]. Therefore, low avidity antibodies are a better marker of recent and/or active (≤ 6 weeks) infection, following a positive CMV IgM test [28]. CMV DNAemia is defined as the presence of CMV DNA in blood, while CMV shedding is the presence of CMV DNA in any other body fluids.

Study selection

Literature for this review was accessed from PubMed/ Medline, Scopus, EBSCOHost, African Journals Online (AJOL) and Institute for Scientific Information (ISI) web of knowledge databases. The search terms were as follows: (cytomegalovirus OR CMV OR CMV infection) AND (antenatal infections) AND (pregnancy OR pregnant women OR gravidity) AND (Africa OR developing countries). The search strategy was discussed by all authors, adjusted to ensure maximum relevance and read by DM. The identified papers were then read by DM and results were recorded in Table 1, in which we entered details about the study population, method of CMV diagnosis, country and province/city of study as well as CMV seroprevalence/prevalence. We then compared the details of included papers for commonalities.

Results

The search strategy, summarised in Figure 1 yielded 78 articles, whose abstracts were all reviewed. Sixty-four abstracts were excluded for one or more of the following reasons: were not carried out in Africans/African countries; did not report on seroprevalence or prevalence of CMV. Three of the remaining 14 full text articles were excluded because the studies did not focus on pregnant women. Only full text articles published in English were included in this review. An article publication date range of January 2000 to January 2019 was selected to enable a sample of studies large enough to inform meaningful discussion and recent enough that it reflects current practice. We accessed 11 full articles reporting the prevalence of CMV in pregnancy in Africa. Of the 11 studies, two were conducted in North Africa (both in

Figure 1. Flow diagram of the article searches yielded, excluded and reviewed for the purpose of this review.

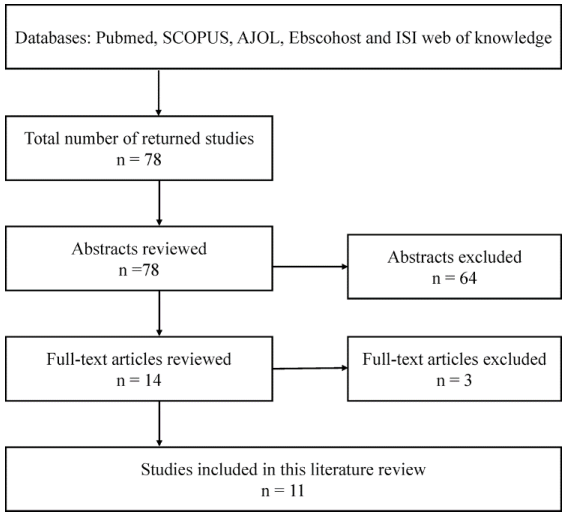

Table 1. Summary of findings from the studies reviewed.

Region and country	*Study population	CMV status			Reference
		DNA (n)	IgM %	IgG %	
East Africa					
Kenya	260 women at Thika hospital	ND	8	77	[36]
Ethiopia	200 women in Addis Ababa	ND	16	89	[35]
Sudan	231 women in El-Rahad	ND	3	72	[37]
Tanzania	261 women in Mwanza	ND	0.4	74	[38]
North Africa					
Egypt	546 women in Ismailia	ND	7	100	[30]
Egypt	62 women (50 with a history of recurrent spontaneous abortion (cases), 12 with no history of spontaneous abortion (controls) in Mansoura)	12 in cases 0 in controls	ND	ND	[29]
West Africa					
Ghana	72 women in Jomoro	ND	0	100	[32]
Nigeria	174 women in Osogbo	ND		60	[33]
Gambia	169 HIV-infected women in Sukutu	Vaginal swab – 24 Colostrum – 29 Saliva – 13 Urine – 1 Plasma – 2		100	[31]
Nigeria	180 women in Kano	ND	ND	91	[34]
Southern Africa					
Malawi	81 HIV-infected ART-naïve women in Blantyre	ND	ND	100	[39]

* - all participants were pregnant and in the reproductive age (18-45 years), ND - not done, DNA – CMV DNA detected in any type of body fluid, IgM – anti-CMV IgM antibodies detected in serum, IgG – anti-CMV IgG antibodies detected in serum.

Egypt [29,30]), four in West Africa (one in each of Gambia [31] and Ghana [32], two in Nigeria [33,34]), four in East Africa (one in each of the following countries; Ethiopia [35], Kenya [36], Sudan [37] and Tanzania [38]); and one in Southern Africa (Malawi) [39].

Prevalence of Cytomegalovirus in pregnancy

All studies reported CMV IgG seroprevalence, with five concurrently reporting CMV IgM results. Prevalence of anti-CMV IgG seropositivity during pregnancy in Africa ranged from 60% to 100%. Four of the 11 studies (conducted in Gambia, Egypt, Malawi and Ghana) reported 100% CMV IgG seroprevalence, demonstrating that the entirety of the study participants in these studies had been exposed to CMV in their lifetime. This is in keeping with studies in Gambian infants which showed that 100% of children acquired CMV infection by the age of eighteen months [10]. Two of the 11 studies reviewed reported on the prevalence of CMV DNA. Table 1 presents an overview of all the studies included in this review as well as CMV prevalence.

Factors associated with acquisition of CMV in African pregnant women

HIV co-infection

Two studies from Gambia and Malawi enrolled HIV-infected women only. The Kenyan study enrolled both HIV-infected and HIV- uninfected women in order to investigate HIV as a risk factor for CMV infection in Kenyan pregnant women. The Kenyan study reported no significant association between HIV status and CMV infection [36]. The remaining eight studies did not consider HIV as a risk factor for CMV infection or did not have data on the HIV status of participants despite the known biological interactions between HIV and CMV.

Socio-economic status

None of the reviewed studies considered socio-economic status as a correlate of CMV acquisition. However, several factors can be considered markers of socio-economic status, among them, place of residence, employment status, income and level of education [40]. The study carried out in Kenya reported on a significant association between risk of CMV infection and low levels of education (Odds ratio (OR) = 3.8, 95% CI = 3.023–6.96, $p < 0.001$) [36]. Hamid *et al.* [34] did not find any significant association between occupation and CMV seropositivity in Nigerian pregnant women. Contrary to findings from the Nigerian study, a study among Sudanese women [37], where an association between illiteracy and CMV IgG seropositivity ($p < 0.05$) was reported. However, the study carried out among Sudanese women did not report odds ratios or 95% confidence intervals. A study done on a Tanzanian cohort reported urban residence to be a risk factor for CMV IgG seropositivity (OR = 6.329, 95% CI 2.885–13.887, $p < 0.001$) [38].

Maternal age and parity

Three of the 11 studies in this review investigated age as a risk factor for CMV acquisition in pregnancy. Zaki and Goda [29] reported a significantly ($p < 0.001$) higher median age among IgG seropositive (median = 25, IQR 19–27) compared to that of IgG seronegative (median = 20, IQR 18–20) Egyptian women. Among Tanzanian women, a one-year age increase resulted in a 0.3% (95% CI 0.13–0.47, $p = 0.001$) increase in seroprevalence while at the same time the risk of being CMV IgG positive increased by 24% [38]. Hamdan *et al.* [37] observed that advanced maternal age was associated with a higher risk of CMV seropositivity in Sudanese pregnant women. However, the significance was lost after multivariate analysis [37], suggesting that the influence of maternal age on CMV acquisition could perhaps be marginal. Studies done in Kenyan and Nigerian women did not report any association between maternal age and risk of CMV seropositivity [34,36]. Higher parity was found to be significantly associated with a higher risk of CMV infection in the studies carried out in Tanzanian (OR = 2.9, 95% CI 1.6-5.4, $p < 0.001$), Kenyan (OR = 3.8, 95% CI 3-7, $p < 0.0001$) and Sudanese (OR = 15, 95% CI 2-123, $p = 0.01$) pregnant women [34,36–38].

Blood transfusion

Two of the 11 studies investigated history of blood transfusion as a risk factor for CMV seropositivity [33,36]. There was a significant difference ($p < 0.05$) in CMV IgG seropositivity between women who previously had a blood transfusion (93%) compared to women without a transfusion history (82%) among Nigerian women [34]. This finding was confirmed by observations in the Kenyan cohort, where history of blood transfusion was associated with both CMV IgG and IgM antibody seropositivity [36].

Recurrent spontaneous abortion

Four of the 11 studies investigated CMV seropositivity as a risk factor for history of recurrent spontaneous abortion [29,35,37-38]. CMV seropositivity was reported to be significantly

associated with history of spontaneous abortion in the Tanzanian study, both in univariate (OR = 5.6, 95% CI 1.3-24.2, p < 0.02) and multivariate analysis (OR = 5.2, 95% CI 1.1-24.4, p = 0.038) [38]. In addition to a significant association between IgG seropositivity and history of spontaneous abortion in the Tanzanian cohort, the only woman who was CMV IgM seropositive had adverse birth outcomes with a low birth weight baby who also had microcephaly and *spina bifida,* consistent with a cCMV diagnosis [38]. In contrast, Yeshwondm *et al*. [35], Hamdan *et al.* [37] as well as Zaki and Goda (29) reported no significant association between history of spontaneous abortion and CMV infection in Ethiopian, Sudanese and Egyptian pregnant women, respectively.

Discussion

Seroprevalence of CMV

The prevalence of CMV observed in African countries is generally higher than the average 40% to 70% reported in the developed world [41]. The seroprevalence estimates reported in all the studies reviewed here were obtained using enzyme linked immunosorbent assays (ELISA) and therefore can be regarded as relatively comparable. Of all the methods used to detect presence of antibodies, ELISA has been found to be the most superior and reliable [26]. However, detection of CMV DNA by polymerase chain reaction (PCR), the gold standard for detection of CMV in body fluids, provides a better reflection of the presence of actively replicating virus. CMV PCR compared to immunoglobulin assays offers superior specificity (100% *vs* 96%) and sensitivity (100% *vs* 22%) [42]. However, PCR may result in false positives for active CMV infection as it can also detect genetic material from latent viruses [43]. PCR is a relatively expensive assay that requires use of commercial kits and specialist equipment; hence, access to the technique in the African setting is limited. Thus, serology remains the method of choice. As such, seroprevalence is the main tool for CMV surveillance on the continent, hence forms the main focus of this review.

Exposure to CMV among African pregnant women in the reviewed studies was very high, but appears to vary across the continent. The prevalence of active infection, denoted by presence of IgM antibodies (0-15.5%), was much lower than that of IgG antibodies (60-100%) [29–39]. However, the seroprevalence of both CMV IgG and IgM antibodies did not show any geographic trends across the African continent. This observation probably rules out a possible role for geographical hotspots in CMV acquisition. The discrepancy between high CMV IgG seropositivity and low CMV IgM seropositivity suggests that the majority of the congenital CMV cases in high CMV prevalence settings in the reviewed studies are consequent of non-primary infection [44]. Thus, CMV antibody avidity, an additional measurement meant to detect IgG positive individuals in whom CMV is likely to have been or is reactivated is critical in clinical decision making when serology is the only available method of diagnosis.

Two studies conducted in Egypt and Kenya reported avidity results and noticeably there were discrepancies between IgM seropositivity and low IgG avidity in both studies [30,36]. In the Egyptian study, 40% tested positive for IgM antibodies, but none of the participants that tested positive for CMV IgM antibodies had IgG antibodies of low avidity. This could suggest that CMV IgM antibodies persist in the circulation for longer than 6 weeks and hence may not be a good marker of current CMV infection [30]. On the other hand, the Kenyan study reported a low avidity IgG antibodies prevalence of 5%, which was less than the 8% seroprevalence of IgM antibodies [36]. Such discrepancies between IgM and IgG avidity test outcomes highlight some of the challenges faced in detection of potentially vertically transmissible CMV infections during pregnancy. Despite IgG avidity being the readily available method for ascertaining recent/current infection in Africa, a previous study in Italian women found vertical transmission of CMV to be 4% and 2% among women with intermediate and high anti-CMV IgG antibody avidity, respectively [45]. Findings from the Italian study point out to potential misdiagnosis of women who are likely to transmit CMV to their offspring using serological tests. Therefore, determination of CMV DNA in blood (DNAemia) and that shed into other body fluids (viral shedding) presents a much more relevant and reliable test for active infection and potential vertical transmission in pregnant women.

In addition to CMV serology, one study reported CMV DNAemia as well as CMV shedding in various specimen types in pregnant women in the Gambia [31]. While CMV seropositivity was 100% in the Gambian population, CMV DNA prevalence was much lower in the different sample types tested: 29% in colostrum, 14% in vaginal swabs, 13% in saliva, 2% in plasma and 1% in urine [31]. It would have been interesting to compare the CMV DNA prevalence results with CMV avidity results to determine the concordance of the results. None of the studies included in this review provided such information. The 29% percent CMV DNA detection in colostrum suggests a huge

contribution of breastfeeding to early vertical transmission of CMV [31].

Studies from Kenya, Sudan and one of the two from Nigeria report the lowest CMV seroprevalence frequencies (< 80%) [34,36–37], while the rest of the studies reviewed reported frequencies greater than 90% [29–33,35,38–39]. The differences in the prevalence rate across the African populations could be due to differences in other suggested or reported risk factors for CMV infection as well as study designs.

Factors associated with acquisition of CMV in African pregnant women

HIV co-infection

Despite the reported association between CMV and HIV pathogenesis, there is a research gap on studies comparing prevalence of CMV between HIV-infected and HIV-uninfected pregnant women in African populations. For example, the 100% CMV IgG seroprevalence reported in the only two studies included in this review that considered CMV/HIV coinfection may not be representative of the general Malawian and Gambian populations, but rather influenced by the HIV co-infection [31,39].

CMV, like other herpesviruses, may lie latent after initial infection if viral replication is significantly suppressed by T-lymphocytes [46]. CMV is reactivated later when the immune system fails to mount an adequate cell-mediated immune response to contain CMV replication, resulting in viral shedding. Consequently, the immune-compromised HIV-infected individuals are at a greater risk of CMV reactivation compared to HIV-uninfected individuals [47]. As a result, the risk of CMV reactivation is even higher in HIV-infected pregnant women whose already HIV compromised immunity is further down-regulated in pregnancy [3]. Some studies have observed that virtually all HIV-infected women are CMV IgG positive. Furthermore, CMV-specific IgG avidity results from these studies suggest reactivation of subclinical infection [48–49. The dynamics of HIV and CMV coinfection are not fully understood but the two have been found to co-activate each other *in vitro* [50]. Both CMV and HIV preferentially replicate in an activated environment, and there was a linear relationship between CMV and HIV viral load in the Kenyan infant study [51]. The relatively high prevalence of CMV in Africa could in part be driven by the HIV burden in the region [52]. Interestingly, one of the studies carried out among Nigerians showed a trend towards increased risk of being CMV IgG positive with having more than one sexual partner [34], a trend similar to what has been observed in risk of HIV infection [53]. *Per contra* , Barbosa *et al.* did not find any relationship between number of sexual partners and CMV viral shedding among Brazilian women [6].

CMV infection is an independent predictor of morbidity and mortality in HIV-infected individuals [54,55]. In a study carried out among HIV-infected infants in Kenya, CMV-induced increase in T-cell activation and apoptosis was hypothesised to contribute to rapid HIV disease progression in coinfected infants, suggesting a possible role of CMV in driving HIV-associated morbidity and mortality [56]. In another study carried out to determine the relationship between CMV DNAemia and maternal-infant mortality, HIV infected infants born to CMV DNAemic women had a four-fold increased risk of mortality within two years *post-partum*. The finding was independent of markers of HIV disease progression such as CD4+ T cell count and HIV viral load [57]. The discordance of CMV seroprevalence and HIV infection has also been shown in the Gambian population where a relatively high prevalence of cCMV (5.4%) was reported among HIV unexposed infants [10]. It is worth noting that the Gambian study enrolled only healthy infants and excluded premature or low birth weight infants who would been at a higher risk of cCMV. The finding emphasises the potential burden of cCMV independent of and in addition to the high HIV burden in Africa.

In addition to HIV, inflammatory bowel disease (IBD) and colitis have been associated with a higher risk of CMV acquisition [58]. However, none of the studies reviewed here investigated or reported either IBD or colitis as a risk factor for CMV infection.

Socio-economic status

Since CMV infection is transmitted through contact with infected body fluids, hygiene and hence socioeconomic status are important risk factors for CMV acquisition. The influence of education/literacy on CMV acquisition could be explained by the direct relationship between education and income which determines living conditions [59–60]. A study in Tanzania found urban residence to be a risk factor for CMV seropositivity when compared to rural residence [38]. The finding may be explained by the greater population densities in urban areas compared to rural areas, especially given that the study was conducted at a public medical facility, where there would be more human contact and an increased risk of CMV transmission.

Maternal age and parity

The association of CMV seroprevalence and maternal age has been attributed to hygiene habits as well as contact with young children who may be at a higher risk of shedding CMV. A study by Pass *et al.* reported higher rates of CMV infection among parents of children who attended day care [61]. The children in day care are exposed to CMV through interaction with their peers, hence increase risk of CMV exposure. The Tanzanian study reviewed here reported a significant association between younger maternal age and increased risk of CMV seropositivity. They argued that the older mothers have more household experience and are more aware of their surroundings, hence develop better hygiene habits which reduce risk of CMV acquisition or transmission [38,62]. Findings from the Tanzanian study are supported by findings from an earlier study where younger maternal age was significantly associated with poor hygiene and increased risk of CMV infection [63–64].

Parity is defined as the number of term pregnancies a woman has had, regardless of whether they led to spontaneous abortion, stillbirth or livebirth. In the Kenyan [36] study reviewed, high parity was defined as having more than four children prior to the current pregnancy, while in the Sudanese [37] study, it was defined as having more than five children prior to the current pregnancy. Both the Kenyan and Sudanese studies reported a significant association between higher parity and risk of anti-CMV IgG seropositivity. Other studies have however reported conflicting findings, likewise, with the speculation that women with lower parity would be younger and less experienced, hence at a greater risk of CMV acquisition and transmission [63]. Thus, the role of parity in CMV acquisition remains contentious with need for further research, perhaps using CMV DNA and IgM seropositivity instead of the largely ubiquitous IgG seropositivity.

Blood transfusion

CMV infection can be transmitted through transfusion of CMV-infected blood [65]. It is possible that some participants could have received CMV-infected transfused blood, perhaps due to lack of CMV screening in the blood products intended for transfusion. CMV acquired through blood transfusion could be reactivated in settings of compromised immunity and pregnancy, thus increasing the risk of vertical transmission. Presence of anti-CMV antibodies in transfused blood does not necessarily reflect the virological status of the donor, hence is not an accurate proxy for the risk of transmission. Thus, more definitive diagnostic tests such as CMV DNA PCR are critical in making a reliable conclusion on the safety of blood intended for transfusion. Unfortunately, in Africa, serology remains the method of choice for CMV screening in blood products [26]. Given the discrepancies in seroprevalence, avidity and CMV DNA status reported and discussed in earlier sections, pregnant women may still be receiving CMV contaminated blood through transfusion.

Recurrent spontaneous abortion

Recurrent spontaneous abortion is defined as three or more consecutive pregnancy losses. Evidence has shown that potentially preventable infections may account for up to 66% of spontaneous abortions [15,66]. Infections such as *Mycoplasma* hominis, herpes simplex virus type 2 as well as CMV have been found to lead to inflammatory processes which result in spontaneous abortion [66–67]. In addition to CMV inducing inflammatory processes that increase apoptosis in trophoblast cells during pregnancy, CMV also activates TNF-α, leading to cell death [15]. Furthermore, CMV has also been shown to disturb normal physiology of placental cells, resulting in placental dysfunction and increased risk of spontaneous abortion or still birth [66,68]. The non-significant associations of CMV with spontaneous abortion reported in the Sudanese study could be attributed to an overrepresentation (80% vs 20%) of women without a history of recurrent spontaneous abortion in the study cohort, therefore minimising the effect of CMV on underrepresented women.

Other possible factors associated with CMV infection but not evaluated in African studies

Genetic predisposition

Polymorphisms in host genes encoding factors involved in innate immunity have been found to influence CMV acquisition [69]. Genes coding for TLR as well as NK cell surface receptors have been implicated.

TLRs are protein recognition receptors which sense the presence of pathogen associated molecular patterns to mediate and trigger an immune response, particularly nuclear factor-kappa B (NF-κB) activation and cytokine secretion [70]. TLR2, 4 and 9 are the major TLRs involved in immune responses against dsDNA viruses such as CMV [71]. On the *TLR2* gene, carriage of the C allele on the 1350T>C polymorphic site and that of the A allele on the 2258G>A polymorphic site were associated with increased susceptibility to cCMV

infection among Japanese children [72]. Two polymorphisms on the *TLR4* gene (896A >G and 1196 C>T) were significantly associated with the risk factors for invasive aspergillosis that included CMV seropositivity [73]. A study performed among Polish foetuses and neonates reported increased risk of infection with 1486 T>C and 2848G>A SNPs on the *TLR9* gene [74]. Polymorphisms that are associated with increased susceptibility to CMV infection involve a nucleotide change which results in a less potent protein being produced [72]. In contrast, Jablonska *et al.* reported no association between polymorphism in the *TLR2* (1350T>C, 2258G>A) and *TLR4* (896A>G and 1196C>T) genes and risk of cCMV infection among infants, but reported a decreased CMV infection risk in adults carrying the *TLR2* 2258A allele [75]. Polymorphisms in the *TLR* genes that confer protection against CMV infection encode potent and relatively highly functional TLR proteins.

NK cells expressing the human leukocyte antigen (HLA) E-binding receptor NKG2C have been found to be particularly important for CMV control [76–77]. A deletion in *NKG2C*, a gene that encodes for the NKG2C receptor protein has been associated with decreased absolute number of $NKG2C^+$ NK cells, with a decreased expression of NKG2C receptors on the NK cell surface as well as lower activation and degranulation of $NKG2C^+$ NK cells. Studies have found transplant patients who are heterozygous or homozygous for the deletion to be at an increased risk of CMV reactivation and symptomatic CMV disease after immunosuppressive therapy [78]. It is important to note that the allele and genotype frequencies of the *NKG2C* deletion differ across populations [79] and so findings may not be generalised. Genetic findings should therefore be population-specific especially considering the genetic differences between Africans and other world populations, and the extensive genetic diversity within Africa. Of note is the high prevalence of the *NKG2C* deletion among African population compared with other populations. The *NKG2C* genotype frequencies further demonstrate a striking difference between East and West Africans [79].

Variation in genes encoding human leukocyte antigen (*HLA*) and killer cell immunoglobulin like (*KIR*) receptors have also been widely implicated in susceptibility or resistance to infection and disease progression of various viruses due to their critical role in immune regulation [80]. Considering that CMV has an extensive repertoire of mechanisms for immune evasion, polymorphisms in genes encoding proteins involved in the immune cascade such as IL, IFN and TNFα may play a role CMV infection [81]. However, relatively little is known about genetic predisposition to CMV infection and transmission, particularly in the African setting, yet knowledge of such genetic determinants may yield novel therapeutic targets for CMV.

Gut microbiome profiles

Gut microbiome profiles shape the microbial and immunological environment of the gut, hence indirectly protect against pathogenic infections and influence immune responses [82–83]. An altered microbiome has been associated with an increased risk of disease acquisition, with some bacterial species being protective against infection while others have a more pro-inflammatory effect. Gut microbiome dysbiosis in pregnancy may weaken immunity, thereby facilitating antenatal infections such as CMV which place the developing foetus at risk [84–85]. In addition to the possible pregnancy induced microbiome dysbiosis, CMV replication in the gut epithelia has been associated with disruption of the tight junctions of the gut epithelia which results in increased intestinal permeability [86]. This effect of CMV on the intestinal epithelia has been found to be partly mediated by the CMV-induced proinflammatory cytokine IL-6. The effect of CMV on the integrity of the gut epithelia has been described in HIV-coinfected individuals [87], leading to disruption of the gut barrier and microbial translocation, which in turn fuel immune activation and inflammation and result in poorer outcomes [83]. CMV, independent of HIV, results in the tight junction disruption as treatment with letermovir, an anti-CMV drug, dampens the effects of CMV on the gut by restoring the epithelia [87]. As such, CMV infection may be associated with certain aberrations in gut microbiome profiles [88]. A recent longitudinal study found that gut colonisation by *Staphylococcus* aureus was protective against early acquisition of CMV infection [89]. Barton *et al.* reported that latent CMV infection confers protection against symbiotic bacterial infection [90], further emphasizing the possible role of CMV on the normal gut flora. However, none of the studies reviewed here investigated possible relationships between CMV infection and gut microbiome profiles, so this represents an important area for future research.

Immune activation

Immune activation is characterised by chronic inflammation and stimulation of immune cells, which in turn secrete inflammatory factors such as cytokines

and chemokines in response to infection or immune dysregulation [91]. In addition, there is sustained immune cell proliferation which exhausts naïve immune cells. This immune environment facilitates inflammation and viral replication in host cells, hence increasing chances of viral shedding into the mucosa and body fluids. CMV has been described as a disease of inflammation in pregnancy [92], but the dynamics of immune activation, CMV DNAemia and CMV acquisition/transmission are unknown. In the study carried out among Kenyan HIV-infected infants, mentioned earlier in this review, the CMV-induced increase in T-cell activation and apoptosis observed could potentially be a marker of CMV-induced immune activation [51]. Also, since CMV has been found to have a synergistic intracellular co-activation with HIV, it is possible that CMV is also sustained by chronic immune activation [47]. The ability of CMV to evade host innate immunity may also implicate CMV infection in chronic immune activation. Interestingly, chronic immune activation is associated with gut microbiome dysbiosis [85]. However, not much work has been done into investigating the role of chronic immune activation in sustaining CMV infection.

Conclusion

The risks of CMV infection and reactivation are determined by a variety of host, viral and environmental factors. However there still is a knowledge gap on the extent to which these various factors are operating in Africa, especially compared with the developed world, yet Africa is one of the regions with the greatest CMV burden. In the absence of a vaccine, it would be worthwhile to develop more rigorous and sensitive diagnostic tools, especially targeting women in the reproductive-age group, who have the greatest potential risks for CMV acquisition and reactivation with the consequent risk of vertical transmission. The initiation of CMV education to women by the American Medical Society resulted in a drastic decrease in CMV prevalence [93]. It might therefore also be valuable to educate African women on the possible effects of maternal and congenital CMV infection and the necessary steps to avoid acquisition and transmission to the fetus and neonate, wherever and whenever possible.

Most of the studies investigating the role of HIV in CMV infection were carried out prior to the roll-out of the universal Option B+ (lifelong ART regardless of CD4+ Tcell count) by the WHO. The role of ART in CMV reactivation and risk of cCMV in HIV exposed children remains unclear. Studies on the potential role of ART in reducing risk of cCMV among HIV exposed infants are especially needed in Africa where there is a high burden of HIV. High birth rates of HIV exposed children who have been described to be more prone to disease and disability compared to their HIV-uninfected counterparts also necessitates these studies. This is further emphasized by several studies which have reported cCMV rates of between three and 11% in HIV exposed infants, potentially contributing to hearing loss and other disabilities in HIV-exposed children. As reviewed by Manickal *et al*. [1], in South Africa alone, over 18,000 infants are estimated to be born with cCMV infection each year [1]. In addition to the HIV burden, a study by Slyker *et al*. reported a discrepancy in the rate of CMV DNA detection in plasma, cervical secretions and breast milk samples [94]. This points to the possibility of missing potentially transmitting mothers using only the widely-used plasma samples. Hence, there is need for more studies exploring methods of detecting CMV in different body compartments in the pregnant women and determining their predictive value for vertical transmission, so as to curb the burden of cCMV and its disabling effects for African children.

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1.7 Aims

Set on the given background, this thesis therefore aimed to determine the prevalence of CMV infection as well as factors associated with CMV reactivation in a cohort of pregnant Zimbabwean women. Furthermore, we aimed to determine the role of CMV infection and CMV susceptibility host genetics on gut bacterial profiles

1.8 Rationale

Although most CMV infections are asymptomatic, certain patient groups such as pregnant women, HIV infected individuals and infants are at risk of develop serious illnesses. CMV infection during pregnancy may be transmitted to the developing foetus and early neonate resulting in congenital CMV. The consequences of cCMV include mental and developmental disorders as well as sensorineural hearing loss for which CMV is the leading non-genetic cause. In addition to vertical transmission, CMV, infection of gut epithelial cells can result in a ‘leaky gut’ which is characteristic of gut bacterial dysbiosis. Gut microbiota plays a significant role in maternal immunity as well as the immunity and neurodevelopment of the foetus via the gut-brain axis. Maintenance of a healthy eubiotic gut microbiota is therefore essential in pregnancy

Prevalence of both maternal CMV and cCMV is higher in the developing world compared to the developed word. In addition to unavailability of an effective vaccine, treatment of both maternal and congenital CMV is complicated by the largely asymptomatic nature of the infection. Furthermore, costs of the recommended drugs for the treatment of CMV make them highly inaccessible to the developing world. These factors together with the high birth rate in the developing world warrant an investigation into the possible factors that may predispose pregnant women to CMV infection. Knowledge of predisposing factors and effects of CMV

infection may allow identification and prioritization of women in need of intervention to curb antenatal CMV infection and its potential effects.

1.9 Specific Objectives

To accomplish the above aims, participants meeting inclusion criteria were recruited so as to achieve the objectives outlined below:

1. Determination of the seroprevalence of CMV infection in HIV-infected and HIV-uninfected pregnant women.

2. Investigation of possible effects of efavirenz (as a proxy for antiretroviral therapy) exposure on acquisition of CMV during pregnancy.

3. Determination of the role of polymorphisms in genes which encode pattern recognition proteins (TLRs 2,4,7 and 9) and immune cascade proteins (IL-1A, IL-6, IL-6R, IL-10, IL-28B, IFNAR1) involved in response to CMV infection.

4. Comparison of gut microbiome profiles of CMV infected and CMV uninfected pregnant women and evaluation of any association between microbiome profiles and CMV-susceptibility host genetic variants.

2 Chapter 2: General Methodology

Synopsis

This chapter gives an overview of the methodology and experimental processes followed in meeting objectives of the overall thesis. Specific and detailed methodologies will be given in each manuscript and or publication.

2.1 Study design and setting

This was a cross-sectional study nested in the University of Zimbabwe College of Health Sciences Birth Cohort (UZ-CHS BC). The UZ-CHS BC aims to examine the impact on pregnancy outcomes, infant growth, immunity, and neuro-development of a range of factors, including co-infection with persistent viruses, maternal nutritional status and breastfeeding, together with maternal HIV status, levels of immune suppression and immune activation, and ART regimens in the HIV+ women. Participant recruitment was done at three of Harare city council high density suburb clinics namely Kuwadzana, Dzivarasekwa and Glenview. The Harare polyclinics are public health facilities which provide routine antenatal services through to post-natal care for mothers and their infants residing in and around the suburbs. The set-up of these polyclinics is the same as all the other public polyclinics throughout Zimbabwe.

Harare is the capital city of Zimbabwe with an estimated population of 1.6 million. Harare is broken down into low, medium and high-density suburbs mainly according to population density. Each of all the suburbs in Harare has one city council clinic which provides primary health care, including antenatal care to residents. Sampling for this study was done in high density suburbs.

2.2 Ethics statement

The UZ-CHS BC and the present study both received ethical clearance from the Medical Research Council of Zimbabwe with file numbers MRCZ/A/2177 and MRCZ/A/1968, respectively. Ethical clearance was also granted by the Parirenyatwa-University of Zimbabwe Joint Ethics Research Committee (JREC/111/17) and the University of Cape Town Ethics Review Board (628/2017). Study procedures and objectives were explained to potential participants by trained nurses and or study staff prior to enrolment. Only participants who provided written informed consent for both their participation and that of their to-be-born infants were recruited. To protect integrity and privacy of personal and clinical information, participants were assigned study numbers which were used throughout the study. All human and health research procedures were in accordance with the ethical standards of the committees responsible for human research (Institutional and National), as well as with the Helsinki Declaration of 1975, as revised in 2008.

2.3 Study participants and samples

HIV-infected and HIV-uninfected pregnant women in late gestation ≥20 weeks, were successfully enrolled under the UZ-CHS BC. HIV-infected women were consecutively enrolled while for the HIV uninfected women were systematically enrolled to include every 10^{th} person presenting based on the approximated HIV prevalence of 10% in Zimbabwe. The HIV status of the participants was obtained from clinic records but additional confirmatory HIV testing based on the national rapid test algorithm, at the time of sample collection. The current standard care for HIV-infected women, regardless of CD4+ count or HIV viral load is ART (Option B+) to prevent HIV MTCT, which usually consists of TENOLAM-E (Tenofovir, Lamivudine and Efavirenz). A pretested questionnaire was administered to collect demographic data and medical history. Women in late gestation (>20 weeks), aged 18-50,

with a known HIV status or who are willing to be tested for HIV were recruited into the study. The HIV participants were only enrolled into the study if they had been on antiretroviral therapy for at least 6 weeks. Furthermore, participants were unrelated, of African (Bantu) origin who gave written conset for their participation and that of their to-be-born infants. Exclusion criteria included being non-ambulatory, mentally ill as well as history of and laboratory test results suggestive of underlying liver disease.

Demographic characteristics such as HIV status, age, gestational age, parity, body mass index (BMI) and monthly income were collected. Recruitment of participants and collection of samples was carried out by trained study staff and midwives. On recruitment, the following samples were collected; 5ml whole blood in EDTA tube, 5ml blood in plain tube and stool. Plasma and serum were immediately separated and all sample stored at -80^{o}C until analysis.

The recruited pregnant women were followed up at delivery where the infants were also enrolled into the study and the mother-infant pairs followed up for two years post-delivery. Samples including amniotic fluid, breast milk, maternal and infant whole blood and infant stool were collected at each of the follow-up visits. The UZ-CHS BC recruited a total of 527 pregnant, three of whom did not have sufficient data and were excluded from all study aspects. Of the 524 enrolled participants, 280 were HIV infected and 247 were HIV uninfected. Results on CMV DNA status of participants, determined by real time PCR were also available from the cohort database. Due to loss to follow up and other factors, the numbers of participants and samples available for the various objectives were not consistent. Figure 2 is a summary of the participant and sample distribution for each of the objectives.

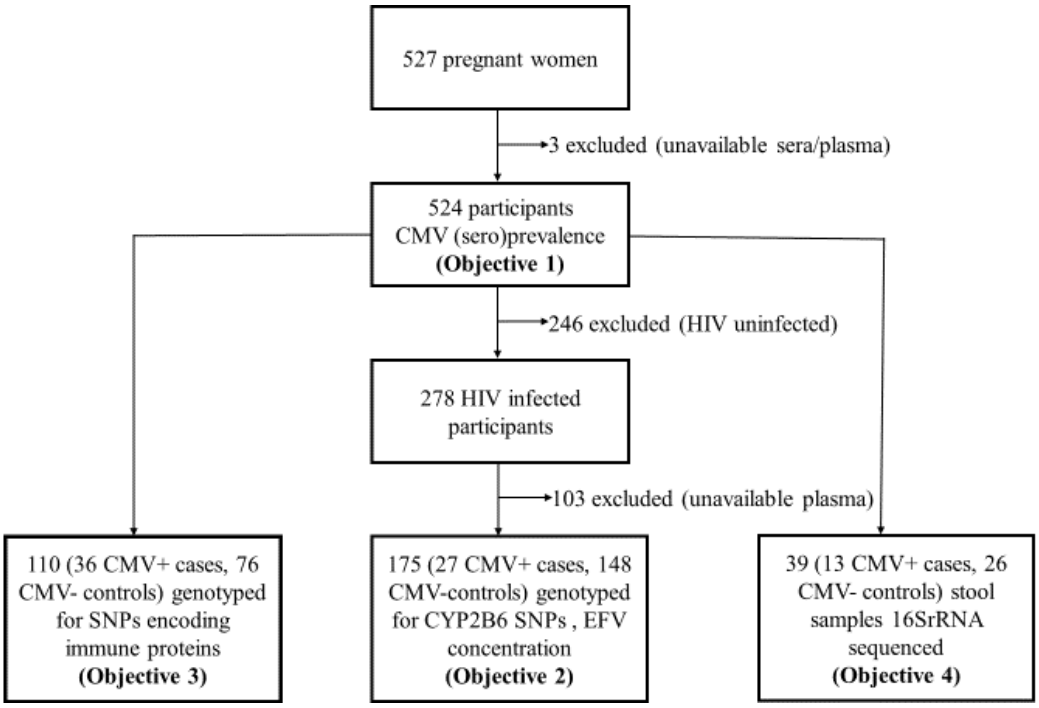

Figure 2.1: Participant and sample distribution for specific thesis objectives

2.4 Laboratory measurements

2.4.1 Determination of CMV infection status

Detection of anti-CMV IgG and IgM antibodies as well as determination of IgG antibody titre and IgG avidity in serum was done using enzyme linked immunosorbent assays (ELISA) ***as described in the method section of Chapter 3.1.***

2.4.2 Determination of plasma efavirenz concentration

Efavirenz in plasma was separated, identified and quantified using high performance liquid chromatography (HPLC). HPLC was used to separate EFV from other substances in plasma by passing through a column which acts as an adsorbent at high pressure, depending on how the different substances interact with the adsorbent (***method explained in detail in Chapter 3.2***)

2.4.3 DNA extraction, amplification, genotyping and sequencing

Extraction of host DNA was done on stored whole blood using a commercial kit as ***indicated in Chapters 3.2 and 3.3***. Following mechanical lysis of bacterial cells by bead beading, extraction of gut bacterial DNA was extracted from stool samples using a commercial kit ***as indicated in detail in Chapter 4.4***. Restriction fragment length polymorphism and Taqman were used to genotype for CYP2B6 c.516G>T and c.983T>C respectively as ***described in chapter 3.2.*** IPLEX MassArray platform, using the Agena protocol was used to genotype for the host genes that encodes proteins involved in immunity against CMV (***characterized genes are listed in methods section of chapter 3.3***). PCR amplification of the v4 region of 16SrRNA gene was carried out as instructed by manufacturer of reagents, and is ***described in the methods section of chapter 3.4***. Subsequent sequencing of the amplicons was done on the Illumina Miseq platform, also described in the same section.

2.5 Statistical analysis and bioinformatics

STATA 15, Graphpad Prism (v6) and R were used for descriptive and comparative statistics. Statistical methods employed for various objectives are described in respective chapters. Bioinformatics analysis as well as diversity comparison for bacteria profiles were done using QIIME 2 and LefSe, respectively (***as described in Chapter 3.4***). For all statistical tests, significance was determined at a p-value cut off of <0.05.

3 Chapter 3: Results

Chapter three is divided into four main sections, each responding to a particular objective and consisting of relevant publications or manuscripts. Each main section will be indicated with a synopsis and summary of findings.

3.1 Determination of the seroprevalence of CMV infection in HIV-infected and HIV-uninfected pregnant women

Synopsis

This section presents results on the objective that seek to determine seroprevalence of CMV infection among HIV -infected and -uninfected pregnant. This section consists of a paper which was published in the Viral Immunology journal (available on this link: https://www.liebertpub.com/doi/10.1089/vim.2019.0024).

3.1.1 Seroprevalence of cytomegalovirus infection among HIV-infected and HIV-uninfected pregnant women attending antenatal clinics in Harare, Zimbabwe

Nature of publication: Original research article

Journal/Publisher: Viral Immunology/Mary Ann Liebert Inc.

Candidate's contribution: conception of the project, data and sample collection, laboratory analysis of samples, data analysis, drafting of manuscript, incorporation of co-authors' comments and execution of reviewers' comments.

On investigating the seroprevalence of CMV infection and risk factors associated with CMV acquisition among pregnant women in Zimbabwe, the following main **findings are summarised:**

- 524 women; 278 HIV-infected and 246 HIV-uninfected were recruited.
- Current or active CMV infection defined as IgM positive + low avidity was detected in 4.6%, 95%CI; 3–6.9 in all women, 5.8 (16/278) in the HIV-infected and 3.3% (8/246), 95%; 1.4–6.3 in the HIV-uninfected.
- IgG seroprevalence was 99.6% (522/524), 95%CI; 98.6–99.9 in all women.
- No significant difference in the prevalence of active CMV infection between the HIV-infected and HIV-uninfected women
- The study shows a low prevalence of primary or active CMV infection among the pregnant women, but the IgG seroprevalence suggests high previous CMV exposure.
- CMV seroprevalence was not associated with the HIV status of the women, perhaps due to the ubiquitous exposure of the population to CMV.

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Seroprevalence of Cytomegalovirus Infection Among HIV-Infected and HIV-Uninfected Pregnant Women Attending Antenatal Clinic in Harare, Zimbabwe

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Abstract

This study aimed to investigate the seroprevalence of cytomegalovirus (CMV) infection and risk factors associated with CMV acquisition among pregnant women in Zimbabwe. In a cross-sectional study, pregnant women were recruited in late gestation, seeking antenatal care at council clinics in three high-density suburbs in Harare, Zimbabwe. Anti-CMV IgM and IgG antibodies were quantified in serum using an enzyme-linked immunosorbent assay. Antibody avidity tests were used to distinguish active infection from viral reactivation in anti-CMV IgM-positive cases. Five hundred and twenty four women were recruited: 278 HIV infected and 246 HIV uninfected. Current or active CMV infection defined as IgM positive+low avidity was detected in 4.6% (24/524), 95% confidence interval (CI): 3–6.9 in all women, 5.8% (16/278) in the HIV infected and 3.3% (8/246), 95% CI: 1.4–6.3 in the HIV uninfected. IgG seroprevalence was 99.6% (522/524), 95% CI: 98.6–99.9 in all women. Notably, the difference in the prevalence of active CMV infection between the HIV-infected and HIV-uninfected women was not statistically significant ($p = 0.173$). The study shows a low prevalence of primary or active CMV infection among the pregnant women, but the IgG seroprevalence suggests high previous CMV exposure. Importantly, CMV seroprevalence was not associated with the HIV status of the women, perhaps due to the ubiquitous exposure of the population to CMV.

Keywords: cytomegalovirus, seroprevalence, active infection, infection reactivation, reinfection, vertical transmission

Introduction

CYTOMEGALOVIRUS (CMV) infection is endemic worldwide, with a 30–61% seroprevalence in the developed countries (1,24) and 60–100% seroprevalence in the developing countries (26,31,38). CMV infection is usually acquired early in life resulting in an asymptomatic, subclinical, and mostly latent infection in immune-competent persons. In the context of immune dysregulation or immune compromise such as pregnancy and HIV infection, latent CMV virus can be reactivated to cause symptomatic infection (21).

In pregnancy, reactivation of CMV predisposes to transmission of the virus from the mother to the developing fetus, leading to congenital CMV (cCMV) infection (35). Unlike other antenatal viral infections such as rubella and herpes simplex virus, prior maternal immunity to CMV fails to confer full protection from acquiring CMV infection to *in utero*, peripartum, and postpartum exposed infants (4). The consequences of cCMV can be severe and include cerebral disability, psychomotor delay, speech and language disabilities, interactive disorders, visual damage, cerebral palsy, and sensorineural hearing loss for which CMV is the leading nongenetic cause (9,25).

Previous studies have informed that the risk of vertical transmission of CMV is greater in primary CMV infection (30–50% of cases) than in latent CMV reactivation or

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reinfection (0.2–3% of cases) (5,17,31). However, prevalence rates of cCMV at birth are higher (~3%) in populations with higher (nearly 100%) anti-CMV IgG seroprevalence (which indicates previous exposure to CMV) than in populations with low anti-CMV IgG seroprevalence (~0.3%) (23,34). This discrepancy suggests that both reactivated CMV and primary CMV infection are active agents of cCMV infection. The discrepancy further elaborates on the risk of reactivation or reinfection outweighing the protective effect of maternal immunity on transplacental transmission (10).

Despite the potentially disabling consequences of CMV infection during pregnancy and the unclear role of reactivated versus primary CMV infection in cCMV, there is limited information on the prevalence of CMV infection and its associated risk factors particularly among African populations. This is despite the high burden of CMV reported in the isolated studies performed in African settings. Determining CMV infection prevalence, especially among women of childbearing age, is important in estimating the risk of cCMV infection, magnitude of burden of maternal infection, as well as identifying risk groups that could be targeted for intervention (5). The current study reports the seroprevalence of CMV in pregnant Zimbabwean women. We also investigate factors associated with CMV serostatus in HIV-infected and HIV-uninfected women recruited during late gestation from clinics in Harare, Zimbabwe.

Methods

Study participants

In a cross-sectional study design, pregnant women in third trimester, presenting for routine antenatal care at three council polyclinics in the high-density suburbs of Harare, were recruited from February 2016 to August 2016. Only participants who provided written informed consent for both their participation and that of their to-be-born infants were recruited. The study was granted ethical clearance by the Medical Research Council of Zimbabwe (MRCZ/A/2177) and the University of Cape Town Ethics Review Board (628/2017). All human and health research procedures were in accordance with the ethical standards of the committees responsible for human research (Institutional and National), as well as with the Helsinki Declaration of 1975, as revised in 2008. Assuming a difference of 13% in the prevalence of CMV (29) between the HIV-infected and HIV-uninfected mothers, the minimum sample size required at 5% level of significance, where $r = 1$ and 80% power is 499 mother–infant pairs.

This study was nested into the University of Zimbabwe-College of Health Sciences Birth Cohort, which aims to recruit at least 1000 well-characterized mother–infant pairs from Harare polyclinics. Participants of the birth cohort were followed up for 2 years. The Birth Cohort study aims to examine the impact on pregnancy outcomes, infant growth, immunity, and neurodevelopment of a range of factors, including coinfection with persistent viruses, maternal nutritional status, and breastfeeding, together with maternal HIV status, levels of immune suppression and immune activation, and antiretroviral therapy (ART) regimens in the HIV-infected women. This sub-study from the Birth Cohort recruited accessed a total of 527 pregnant women, 280 were HIV infected and 247 were HIV uninfected.

The current standard care for all HIV-infected pregnant women is Option B+ (ART initiation regardless of $CD4^+$ T lymphocyte count or HIV viral load) to prevent mother-to-child transmission of HIV, which usually consists of TENOLAM-E (tenofovir, lamivudine, and efavirenz). The Harare polyclinics provide routine antenatal through to postnatal care for mothers and their infants residing in and around the suburbs. The setup of these polyclinics is the same as all the other public polyclinics throughout Zimbabwe.HIV-infected and HIV-uninfected pregnant women ≥28 weeks of gestational age were successfully enrolled under the University of Zimbabwe-College of Health Sciences Birth Cohort exploring HIV exposure, disease acquisition, and progression among children.

HIV-infected women were consecutively enrolled, whereas the HIV-uninfected women were systematically enrolled to include every 10th person presenting based on the approximated HIV prevalence of 10% in Zimbabwe. A pretested questionnaire was administered to collect demographic data and medical history. The HIV status of the participants was obtained from clinic records but additional confirmatory HIV testing based on the national rapid test algorithm, at the time of sample collection (39). Five milliliters of whole blood was collected in plain tubes and transported to the University of Zimbabwe, Department of Immunology laboratory where the serum was separated within 6 h of venipuncture. The serum specimens were subsequently stored at –80°C until anti-CMV analysis.

Determination of anti-CMV serostatus

Anti-CMV IgM and IgG antibodies were detected in serum using a commercial indirect enzyme-linked immunosorbent assay (ELISA) kit (CE 0197) according to the manufacturer's instructions (EUROIMMUN, Luebeck, Germany). Participant specimens, standards, and procedural controls, provided by the manufacturer, were run simultaneously in duplicate. Extinction coefficients were determined at 450 nm. Specimens with an extinction ratio <0.8 were considered negative, extinction ratios of ≥0.8 to <1.1 were considered equivocal while extinction ratios ≥1.1 reflected positive tests for both anti-CMV IgG and IgM antibodies. Specimens with indeterminate test results were repeated and reassigned to a positive or negative group. IgG antibody titer was concurrently determined to estimate the strength or magnitude of an immune response to the CMV antigen.

Participant specimens testing positive for anti-CMV IgM were subsequently tested for IgG avidity by ELISA according to the manufacturer's instructions (EUROIMMUN). The EUROIMMUN CMV IgG avidity assay comprises two parallel assays, one with and the other without 6 M urea added to detach low-avid antibodies (37). This enables low-avid antibodies produced at the early stage of a primary infection to be distinguished from high-avid antibodies, which are characteristics of a historical infection. The avidity index generated is a ratio of the absorbance obtained from the specimen reaction to which urea buffer was added to the absorbance from the reaction without urea buffer. An avidity index ≥0.6 was considered to be a strong indicator of a primary infection dating back >3 months, whereas an index ≤0.4 indicated primary infection dating back <3 months,

according to the manufacturer's protocol. An avidity index between 0.4 and 0.6 was considered equivocal (37).

Data analysis

Study data were collected and managed using Research Electronic Data Capture (REDCap) (20). Data were analyzed using STATA version 13.1 (StataCorp, College Station, TX). Reproductive health markers as well as demographic and clinical characteristics were compared between HIV-infected and HIV-uninfected groups using the Mann–Whitney rank-sum for nonparametric variables, and chi-squared test or Fisher's exact test for categorical data. Seroprevalences were reported with their 95% confidence intervals (CIs) and compared between HIV-infected and HIV-uninfected women using chi-squared test or Fisher's exact test. A nominal p-value ($p < 0.05$) was considered statistically significant. Univariate logistic regression analysis was performed to investigate the association of CMV anti-IgM seropositivity with demographic and clinical factors.

Results

Demographic characteristics of participants

Of the 527 participants enrolled for the main birth cohort, 524 participants' (278 HIV infected and 246 HIV uninfected) samples were accessed for this study. The demographic and clinical characteristics of the participants are shown in Table 1. The median age of HIV-infected women was significantly higher ($p < 0.001$) (30 years, 25th–75th percentile: 25–34) compared with that of HIV-uninfected women (26 years, 25th–75th percentile: 21–32). The median age of gestation among the pregnant women was 33 weeks (25th–75th percentile: 29–36) and was significantly higher ($p < 0.001$) in the HIV-uninfected group (33 weeks, 25th–75th percentile: 30–36) compared with the HIV infected (32 weeks, 25th–75th percentile: 28–35). The earlier presentation of HIV-infected women to clinic (measured by lower gestation age) could be due to improved health-seeking behavior induced by HIV treatment programs.

The median parity in the study population was one child (25th–75th percentile: 0–2), but the HIV-infected women had significantly higher parity (median = 2 children, 25th–75th percentile: 1–3) compared with the HIV-uninfected women (median = 1 child, 25th–75th percentile: 0–2) ($p < 0.001$). The median gravidity in the study population was 2.5 (25th–75th percentile: 2–4). In line with the higher parity, the HIV-infected women also had significantly higher gravidity (median = 3, 25th–75th percentile: 2–4) compared with the HIV-uninfected women (median = 2, 25th–75th percentile: 1–3) ($p < 0.001$). The HIV-infected women were significantly more likely to be divorced or cohabiting than the HIV-uninfected women. Body mass index, level of education, and monthly income were comparable between the HIV-infected and HIV-uninfected women.

Prevalence of anti-CMV antibodies

A summary of the anti-CMV antibody frequencies for this study is presented in Table 2. Of the 524 pregnant women, 522 (99.8%, 95% CI: 0.99–1) were seropositive for anti-CMV IgG. One participant from the HIV-infected group and

TABLE 1. DEMOGRAPHIC CHARACTERISTICS OF STUDY PARTICIPANTS

Characteristic	*Combined,* N = *524*	*HIV uninfected,* n = *246*	*HIV infected,* n = *278*	p *(infected vs. uninfected)*
Median age in years (25th–75th percentile)	28 (23–33)	26 (21–32)	30 (25–34)	<0.001[a]
Median gestational age in weeks (25th–75th percentile)	33 (29–36)	33 (30–36)	32 (28–35)	<0.001[a]
Median partner's age in years (25th–75th percentile)	34 (29–39)	32 (27–36)	36 (31–40)	<0.001[a]
Median BMI (25th–75th percentile)	26 (24–29)	26 (23–30)	26 (24–28)	0.093[a]
Median parity (25th–75th percentile)	1 (0–2)	1 (0–2)	2 (1–3)	<0.001[a]
Median gravidity (25th–75th percentile)	2.5 (2–4)	2 (1–3)	3 (2–4)	<0.001[a]
Income (25th–75th percentile), USD/month	237.7 (150–343)	250 (150–350)	234 (150–330)	0.6329[a]
Education, *n* (%)				
Secondary	468 (89.3)	218 (88.6)	250 (89.9)	1
Primary	25 (4.8)	12 (4.88)	13 (4.7)	0.8898[b]
Tertiary	31 (5.9)	16 (6.6)	15 (5.4)	0.5867[b]
Marital status, *n* (%)				
Married	417 (79.6)	215 (87.4)	202 (72.7)	Ref.
Single	20 (3.8)	6 (2.4)	14 (5.0)	0.0596[b]
Cohabiting	83 (15.8)	25 (10.2)	58 (20.9)	0.0004[b]
Divorced	4 (0.8)	0 (0)	4 (1.4)	0.0401[b]

[a]Mann–Whitney rank-sum test.
[b]Chi-squared test.
BMI, body mass index.

one participant from the HIV-uninfected group tested negative for anti-CMV IgG antibodies, and both were also negative for anti-CMV IgM antibodies. In the CMV IgM test, 39 individuals (7.4%, 95% CI: 4–10) of the 524 women tested positive for CMV IgM antibodies, and there was no significant difference in the prevalence of either anti-CMV IgG or IgM seropositivity between HIV-infected and HIV-uninfected participants.

We performed CMV IgG avidity test on the 39 participants who had positive results for both anti-CMV IgG and anti-CMV IgM, and 62% ($n = 24$) showed low avidity for anti-CMV IgG antibodies while the rest showed high avidity. Interestingly, anti-CMV IgG titers were significantly higher in the HIV-infected women (median = 162.2 U/mL, interquartile range [IQR]: 120.8–200) than in the HIV-uninfected women (median = 100 U/mL, IQR: 70.4–132.1) ($p < 0.001$).

Risk factors for CMV seropositivity

Using univariate and multivariate logistic regression analyses, none of the demographic or health characteristics (HIV status, age, parity, gestational age, level of education, and income) was significantly associated with the risk of being seropositive for either anti-CMV IgG or IgM antibodies (Table 3).

Table 3. Logistic Regression of Factors Associated with Anti-Cytomegalovirus Immunoglobulin M Serostatus

Characteristic	*Odds ratio (95% CI)*	p
HIV status	0.86 (0.42–1.75)	0.679
Age	0.97 (0.88–1.07)	0.517
Parity	0.66 (0.38–1.17)	0.154
Gravidity	1.38 (0.87–2.19)	0.172
Gestational age	1.00 (0.93–1.09)	0.906
Income	0.79 (0.47–1.33)	0.375
Education	1.23 (0.57–2.64)	0.601
Partner age	1.02 (0.94–1.09)	0.673
Marital status	0.67 (0.33–1.32)	0.252

Each of the variables was tested using univariate analysis, and no significance was observed when HIV-infected patients were compared with HIV uninfected.
CI, confidence interval.

Discussion

We report a seroprevalence of 99.6% for anti-CMV IgG and 7.4% for IgM antibodies, in pregnant Zimbabwean women, with no significant differences in seroprevalence observed between the HIV-infected and HIV-uninfected groups. Thus, anti-CMV seropositivity was not significantly associated with HIV status in the study population. However, high anti-CMV Ig antibody titer was significantly associated with HIV positivity. There were no significant associations between anti-CMV seropositivity and demographic characteristics, such as age, parity, gravidity, level of education, and socioeconomic status. Our findings conflict with previous reports where demographic characteristics were significantly associated with either higher or lower risk of CMV infection (2,30). However, our findings are also comparable to other reports where no significant association was found between demographic characteristics and risk of CMV infection (6,18).

Understanding the epidemiology of CMV infection during pregnancy is essential for exploring control measures since cCMV infection is associated with potentially fatal and disabling effects. The high prevalence of anti-CMV IgG antibodies (99.6%) confirms reports in other studies on Egyptian, Ghanaian, Kenyan, Malawian populations and other non-African low-income countries (3,19,22,30). In contrast, lower anti-CMV IgG prevalence has been reported in the developed countries, such as the United States of America, France, and Australia (24,36). The higher prevalence of CMV infection in the developing world compared with the developed world could be explained by lower socioeconomic class characterized by overcrowded living conditions and lower income.

Markers of lower socioeconomic class have been previously reported as risk factors for CMV infection (2). Ethnicity that narrows down to genetic variation could also contribute to the differences in CMV acquisition between the developed and developing world (14,24) where the developed world mainly consists of individuals of white ethnicity while the developed world mainly consists of individuals of black ethnicity. With a 99.6% anti-CMV IgG seroprevalence, our study demonstrates an almost ubiquitous previous contact with CMV in the study population. It is possible that the participants may have acquired the infection as they were growing up since CMV acquisition usually happens during the early years of life, especially in high CMV prevalence settings (26,35).

In previous studies carried out among infants in Zimbabwe, the prevalence of CMV infection at 6 weeks of age was greater than 70%, regardless of HIV exposure, emphasizing the early acquisition of CMV infection (11,16). However, there was no report on the prevalence of maternal CMV in both studies. The high prevalence of CMV exposure in our study suggests that most of the cCMV cases in the high CMV seroprevalence populations may be due to CMV reactivation or reinfection (nonprimary CMV infection) (4). As a result, nonprimary infection would result in a

Table 2. Prevalence of Anti-Cytomegalovirus Antibodies in the Study Population

Serostatus	*All,* N = *524*	*HIV uninfected,* n = *246*	*HIV infected,* n = *278*	p
IgG negative, *n* (%)	2 (0.4)	1 (0.4)	1 (0.4)	0.930[a]
IgG positive, *n* (%)	522 (99.6)	245 (99.6)	277 (99.6)	
IgM negative, *n* (%)	485 (92.6)	226 (91.9)	259 (93.2)	0.473[a]
IgM positive, *n* (%)	39 (7.4)	20 (8.1)	19 (6.8)	
IgM positive+LA, *n* (%)	24 (4.6)	8 (3.25)	16 (5.8)	0.173[a]

[a]Chi-squared/Fisher's exact test.
LA, low avidity.

higher number of cCMV cases than maternal primary infection. This also further explains the higher cases of cCMV in Africa despite the women being immune to CMV before pregnancy. The high seroprevalence of anti-CMV IgG antibodies in the study population nullified our effort to investigate the possible role of HIV in CMV acquisition.

In contrast to the nearly 100% anti-CMV IgG seroprevalence, we found a seroprevalence of 7.4% for anti-CMV IgM antibodies. Presence of anti-CMV IgM antibodies is indicative of primary infection, reactivation of previous infection, or reinfection with a different viral strain (12), but without clear demarcation. Overall, anti-CMV IgM seropositivity is confirmed with an anti-CMV IgG antibody avidity test to make a diagnosis of current CMV infection. In our case, 24/524 (4.6%) women were positive for anti-CMV IgM antibodies and had low avidity anti-CMV IgG antibodies as well. The rest of the anti-CMV IgM positives, 15/524 (2.9%) were a case of either possible reactivation of latent virus or reinfection with a different CMV viral strain.

Contrary to previous reports and the established coactivation between CMV infection and HIV infection (8,13), anti-CMV IgM seroprevalence was not significantly different between the HIV-infected and HIV-uninfected participants. The comparable seroprevalence of anti-CMV IgM antibodies between the HIV infected and HIV uninfected suggests that CMV infection is independent of HIV infection in our study population. The immune downregulation, which occurs during pregnancy, could sufficiently predispose the women to CMV infection, hence overshadow HIV infection. More importantly, the HIV-infected study participants were on ART, which in successful cases is sufficient for immune restoration, hence protection against CMV infection. However, the occurrence of CMV in some of the HIV-infected women who are also on ART may suggest differences in response to ART among the HIV-infected participants (33).

Anti-CMV IgG antibody avidity was performed on all anti-CMV IgM positive participants to confirm current infection. Antibody avidity test is the most accessible test of choice to differentiate between current and past infection especially in the resource-limited settings where polymerase chain reaction (PCR) for CMV DNA detection may not be available (28). In our study, 64% of the participants who had a positive anti-CMV IgM test had low avidity antibodies while the rest had high IgG avidity antibodies.

Our findings deviate from the study previously carried out among Egyptian pregnant women where all the participants who were positive for anti-CMV IgM antibodies had either high or intermediate avidity IgG antibodies (22). The discordance between anti-CMV IgM positivity and low avidity anti-CMV IgG antibodies results in our study could be due to prolonged circulation of anti-CMV IgM antibodies past period of active infection (7), hence an overestimation of active CMV infection cases when considering anti-CMV IgM results only. However, a study carried out among Kenyan pregnant women also reported discordance between anti-CMV IgM results and anti-CMV IgG antibodies avidity results where 80% of the individuals who were positive for anti-CMV IgM antibodies had low avidity antibodies (30).

Interestingly, anti-CMV IgG antibody titer was significantly associated with HIV status, with the HIV-infected individuals more likely to have higher anti-CMV IgG antibody titer. We suspect that since there is a massive production of IgG antibodies during a secondary immune response to a pathogen, there could be reactivation of CMV resulting in the surge of anti-CMV IgG production (27). However, due to compromised immunity especially in the HIV-infected individuals and immune senescence associated with HIV and CMV itself, there may be failure to mount an IgM immune response, sufficient to be detected by the ELISA (15). This results in underestimation of active CMV infection. In such cases, PCR to detect CMV DNA would be more useful, as it is the gold standard.

With such alarming levels of CMV exposure in pregnancy, understanding the factors associated with CMV infection will go a long way in ameliorating the burden of cCMV. However, our study did not find any significant association between the risk of being either anti-CMV IgG or anti-CMV IgM positive with either income or education. Education and income as markers of socioeconomic status have been linked to CMV infection, with lower income and lower level of education being associated with a higher risk of being anti-CMV seropositive (30,32). Zimbabwe has a literacy rate of $\sim$87%, ranked position 99 in the world (40), suggesting a fairly uniform and high access of written public health information. Also considering the location of the clinics where sampling was performed, the participants were most likely in the same socioeconomic group. Hence, we could not find any significant association between income and the risk of being positive for anti-CMV antibodies.

Conclusion

In conclusion, we report a high prevalence of previous CMV exposure among women of childbearing age. Given that secondary CMV infection has been equally associated with higher cCMV to primary CMV infection in the developing countries, the finding calls for interventions to prevent vertical transmission of CMV to their offspring. CMV infection is independent of HIV status in the study population. The limitation of the study could have been the sample size where we had more HIV-infected participants than HIV-uninfected participants (278 HIV infected and 246 HIV uninfected).

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Author Disclosure Statement

No competing financial interests exist.

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3.2 Investigation of possible effects of efavirenz (as a proxy for antiretroviral therapy) exposure on acquisition of CMV during pregnancy

Synopsis

From the 524 recruited participants, 278 were HIV infected who were then divided into CMV -infected and CMV -uninfected groups, who were then assigned as cases and controls, respectively (***see Figure 2***)**.** ART has resulted in restoration of longevity and normalcy of lives of people living with HIV and AIDS to approximate that of their HIV -uninfected counterparts, if well managed. Notably, 75% of the participants who were positive for CMV DNA were HIV infected. It is known that regardless of the same dosage, not all patients achieve the same concentration of drug in their plasma yet, disease or infection control is dependent on optimum therapeutic drug exposure. We therefore set out to determine the role of antiretroviral drug exposure (using EFV as a proxy) in predisposing some HIV infected pregnant women to CMV infection. This chapter comprises a manuscript accepted for publication in the South African Medical Journal. Supplementary Table 1 is in the Annex section.

3.2.1 Plasma efavirenz concentration inversely correlates with increased risk of Cytomegalovirus infection in HIV infected pregnant women

Nature of manuscript: Original research article

Candidate's contribution: conception of the project, data and sample collection, genotyping of SNPs, data analysis, drafting of manuscript, incorporation of co-authors' comments and execution of reviewers' comments.

On determining the role of EFV exposure on susceptibility to CMV infection, the **findings are summarised** as follows:

- There was an inverse association between plasma EFV concentration and CMV DNA, Participants with lower plasma EFV concentrations were significantly (p<0.001) more likely to be CMV DNA positive compared to participants with higher plasma concentrations.
- Carriers of CYP2B6 poor metaboliser genotypes (*CYP2B6 c.516T/T* and c.983T/C) were less likely to be positive for CMV DNA.
- Furthermore, poor metabolism as denoted by *CYP2B6 c.516T/T* and *c.983T/C* genotypes was significantly associated with lower CMV viral load.
- Overall, the findings suggest failure of low or subtherapeutic EFV concentration to fully recuperate immunity and protect against HIV co-infections such as HIV

ISSUES IN PUBLIC HEALTH

Plasma efavirenz concentration inversely correlates with increased risk of cytomegalovirus infection in HIV-infected pregnant women

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Background. Effective combination antiretroviral therapy (cART) has tremendously reduced HIV-associated morbidity, mortality and mother-to-child transmission. However, the benefits of cART are threatened by comorbidities, adverse drug reactions and virus resistance to existing treatment regimens. One of the most occurring comorbidities is cytomegalovirus (CMV) infection.
Objectives. To investigate the effects of cART on the occurrence of CMV infection among pregnant women.
Methods. Using a cross-sectional study design, 175 HIV-infected pregnant women were recruited, and data were obtained from their clinical records. Blood samples were collected for host DNA, CMV DNA and plasma efavirenz (EFV) measurement. CMV DNA was measured using real-time polymerase chain reaction (PCR). *CYP2B6 c.516G>T* and *CYP2B6 c.983T>C* single nucleotide polymorphisms were characterised using PCR/restriction fragment length polymorphism and TaqMan (Thermo Fisher Scientific, USA) assays, respectively. Plasma EFV concentrations were determined using high-performance liquid chromatography.
Results. There was an inverse association between plasma EFV concentration and CMV DNA. Participants with lower plasma EFV concentrations were significantly ($p<0.001$) more likely to be CMV DNA positive than those with higher plasma concentrations. This result is also supported by the observation that carriers of *CYP2B6* poor-metaboliser genotypes (*CYP2B6 c.516T/T* and *CYP2B6 c.983T/C*) were less likely to be positive for CMV DNA. Furthermore, poor metabolism as denoted by *CYP2B6 c.516T/T* and *CYP2B6 c.983T/C* genotypes was significantly associated with lower CMV viral load.
Conclusions. HIV treatment disrupts the balance between host and co-infecting microbes. Reduced or subtherapeutic levels of antiretroviral drugs, which could be exacerbated by genetic polymorphisms in drug metabolism genes and non-adherence, predispose infected individuals to an increased risk of CMV infection in pregnancy.

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Combination antiretroviral therapy (cART) has tremendously reduced HIV-associated morbidity, mortality and mother-to-child transmission (MTCT).[1] The Option B+ (lifelong cART for all HIV-infected pregnant and breastfeeding women) introduced by the World Health Organization proved to be effective, resulting in a reduction of MTCT from 33% (before ART) to <2% in sub-Saharan Africa.[2] Despite the massive success in reduction of MTCT, HIV-exposed uninfected (HEU) children experience delayed developmental milestones, disabilities and more frequent morbidities than their HIV-unexposed counterparts.[3,4] Developmental and health problems in some HEU infants occur despite maternal cART during pregnancy. Differential HIV outcomes have previously been associated with interindividual variation in response to cART.[5]

The differential outcomes of cART during pregnancy are critical in the care of HEU infants who have to endure effects of *in utero* and postpartum exposure to antiretroviral drugs.[6] Furthermore, the increasing number of HIV-infected adolescents who grow into adulthood with a strong will to procreate, further emphasises the need to understand the factors associated with different HIV treatment outcomes in pregnancy, which also determine infant outcomes.[7] Of the utmost importance during pregnancy are antenatal diseases that may result in adverse outcomes if transmitted to the developing fetus or neonate. Antenatal infections (such as cytomegalovirus (CMV), toxoplasmosis, syphilis and rubella) are more prevalent among HIV-infected than HIV-uninfected women.[8,9]

CMV is a latent beta herpesvirus that is often reactivated in immune-compromised populations such as HIV patients and pregnant women. When reactivation of CMV occurs during pregnancy, the virus can be transmitted to the fetus and/or neonate, resulting in congenital CMV (cCMV) infection.[10] Effects of cCMV may be potentially fatal and include paediatric pneumonia, neurocognitive developmental delay and sensorineural hearing loss, for which CMV is the leading non-genetic cause.[11,12] In a review by Filteau and Rowland-Jones,[13] vertical transmission of CMV *in utero* or in early infancy was hypothesised to be responsible for the health and developmental deficits in HIV-exposed children. cCMV is sustained by antenatal CMV infection or reactivation; hence, controlling maternal CMV infection is key in the prevention of cCMV. cART is protective against the emergence of opportunistic infections;[14] therefore, the occurrence of CMV in some but not all HIV-infected

pregnant women could suggest differences in protectivity of cART against co-infections.

In resource-limited settings, the choice of cART Option B+ is a combination of two nucleoside reverse transcriptase inhibitors (NRTIs) (tenofovir (TDF) and lamivudine (3TC)/emtricitabine (FTC)) and one non-nucleoside reverse transcriptase inhibitor (NNRTI) (efavirenz (EFV)). The use of EFV-based regimens among pregnant women has been recommended, as EFV has fewer drug interactions and fewer adverse drug reactions than other antiretroviral drugs.[15] Therefore, EFV is the NNRTI of choice for first-line treatment of HIV infection in pregnancy.

EFV is administered as a once-daily oral dose of 600 mg and is mainly metabolised by cytochrome P450 2B6 (CYP2B6), a hepatic enzyme encoded by the *CYP2B6* gene.[16] However, genetic polymorphisms in *CYP2B6* affect the pharmacokinetics of EFV, leading to variability in EFV steady-state concentrations.[17,18] Participants in this study were administered EFV-based cART regimens; therefore, EFV will be used as a proxy for cART in this report on susceptibility to CMV infection during pregnancy. The therapeutic effect of a drug is influenced by its rate of metabolism and excretion, which can be approximated by the plasma concentration of the drug.[4,18] Two single nucleotide polymorphisms *CYP2B6 c.516G>T* (rs3745274) and *CYP2B6 c.983T>C* (rs28399499) in the *CYP2B6* affect EFV metabolism.[19] Moreover, we also investigated associations between plasma EFV concentration and CMV infection. *CYP2B6* genotypes were also characterised to confirm their effects on variable plasma EFV concentrations.

Methods

Study participants

In this cross-sectional study, HIV-infected pregnant women were enrolled through the University of Zimbabwe College of Health Sciences birth cohort study. These women were receiving TDF, 3TC and EFV 600 mg, prescribed as part of their routine clinical care and for prevention of MTCT (PMTCT). Inclusion criteria were: gestational age ≥20 weeks, maternal age >18 years, HIV infected on cART, and presenting for routine antenatal care at any one of three council polyclinics in the high-density suburbs of Kuwadzana, Dzivarasekwa and Glen View in Harare, Zimbabwe. The HIV status of the participants was obtained from clinic records, but additional confirmatory HIV testing was done at the time of sample collection.[20] CMV DNA status and CMV viral loads were determined by real-time polymerase chain reaction (RT-PCR) using a RealStar CMV PCR Kit 1.0 (Altona Diagnostics, Germany), according to the manufacturer's instructions. Results for CMV status and CMV viral loads were available from the main cohort records.

Ethical approval

This study received ethical clearance from the Medical Research Council of Zimbabwe (ref. no. MRCZ/A/2177) and the University of Cape Town Institutional Review Board (ref. no. HREC628/2017). The study was carried out in accordance with the guidelines of the Helsinki Declaration of 2008 ethics clearance. Written informed consent was obtained from each participant prior to data and sample collection. A questionnaire was used to collect participant demographical information, and clinical parameters were obtained from the participants' medical records. A 5 mL ethylenediaminetetraacetic acid (EDTA) anticoagulated blood sample was collected from each participant at least 8 hours after administering a dose. Plasma was isolated from 3 mL of the whole blood sample (centrifugation at 3 000 g for 10 minutes) within 12 hours of venepuncture and stored at −80°C for EFV concentration measurement, while the remainder of the sample was used for DNA isolation.

Determination of plasma efavirenz concentration and *CYP2B6* genotypes

Plasma EFV concentrations were quantified at the University of Zimbabwe-International Pharmacology Specialty Laboratory (UZ-IPSL) using high-performance liquid chromatography (HPLC). A photodiode array detector scanning at 247 nm wavelength was used for quantification of EFV. The assay had a lower limit of quantitation of 500 ng/mL with an interday imprecision coefficient of variance (CV) ranging from 1.0% to 5.6%, as determined using the quality-control samples. The method used for EFV quantification was validated and approved by Clinical Pharmacology Quality Assurance (CPQA), an external quality assurance body. UZ-IPSL participates in the CPQA annual proficiency testing.

One of the factors that could affect plasma EFV is genetic variation in the *CYP2B6* gene. The *CYP2B6* gene encodes for the enzyme principally involved in the metabolism of EFV, and two polymorphisms (*CYP2B6 c.516G>T* and *CYP2B6* c.*983T>C)* have been shown to affect steady-state EFV exposure. Therefore, the contribution of *CYP2B6* genetic variation in EFV therapeutic effects and risk of CMV acquisition needed to be determined. Host genomic DNA was extracted from whole blood using the Quick-DNA Miniprep Plus Kit (Zymo Research, USA) according to manufacturer's instructions. We genotyped for *CYP2B6 c.516G>T* using restriction fragment length polymorphism.[21] *CYP2B6 c.983T>C* was genotyped using real-time allelic discrimination PCR on the Bio-Rad CFX96 (Bio-Rad Laboratories, USA). TaqMan SNP Genotyping Assay and TaqMan Universal Master Mix were used to genotype *CYP2B6 c.983T>C* (rs28399499; assay ID C_4362691_10). All components were purchased from Thermo Fisher Scientific, USA, and genotyping was done according to manufacturer's instructions. Genotyping of single nucleotide polymorphisms (SNPs) was done at the Division of Human Genetics, Faculty of Health Sciences, University of Cape Town, South Africa.

Statistical analysis

Data were compiled and managed in Research Electronic Data Capture (REDCap).[22] Statistical analyses were performed using Stata SE, version 15 (StataCorp, USA). Numerical variables were described as either median with interquartile range (IQR) for non-parametric variables or mean with standard deviation (SD) for parametric variables. Categorical variables were described as frequencies. Demographic and clinical characteristics were compared between CMV-infected and CMV-uninfected participants using *t*-tests for parametric data, Mann-Whitney tests for non-parametric data and χ^2 tests for categorical data. Plasma EFV concentrations and HIV viral load were compared among participants based on CMV DNA status using the Mann-Whitney test. The χ^2 test was used to determine the association between CMV infection sta tus and EFV therapeutic groups. The relationship between CMV DNA load and HIV viral load was determined using Spearman's correlation coefficient test. Multivariate logistic regression was then performed to control for confounders such as maternal age, gestational age, gravidity, body mass index (BMI) and CD4+ count. Plasma EFV concentrations were compared among *CYP2B6 c.516G>T* genotypes using the Kruskal-Wallis rank sum test and between *CYP2B6 c.983T>C* genotypes using the Mann-Whitney test. The correlation between EFV concentration and plasma HIV viral load was determined using Spearman's correlation coefficient. Statistical significance was set at $p<0.05$.

Results

Demographic and clinical characteristics of participants

The demographic characteristics of the study participants are presented in Table 1. A total of 175 HIV-infected pregnant women who were receiving EFV-containing first-line cART were enrolled into the study. EFV concentration values were present in all 175 participants. *CYP2B6 c.516G>T* and *CYP2B6 c.983T>C* SNP genotyping was also successfully done. All participants were in the reproductive age group (mean (range) 30 (18 - 44) years) and had been receiving EFV-based cART from a minimum of 2 months to a maximum of 140 months. The mean (SD) CD4+ cell count was 358 (215) µL, while the median (25th - 75th percentile) HIV viral load was 97 (10 - 400) copies/mL. Results for CMV DNA status of the participants were available from the main study records. Of the 175 study participants, 27 (15.4%) had CMV DNA detected and quantified in their plasma. The median (range) CMV viral load was 366 (108 - 103 355) copies/mL.

Plasma efavirenz concentration distribution

Plasma EFV concentrations were determined for all 175 participants from blood drawn at least 8 hours post dose. Participants self-reported good adherence (did not miss >1 dose since pregnancy). The median (25th - 75th percentile) plasma EFV concentration for this cohort of pregnant women was 1 850 (990 - 3 963) ng/mL. The plasma EFV concentrations ranged from 250 to15 931 ng/mL, exhibiting interindividual variability among patients despite them receiving the same 600 mg daily EFV dose. Based on previously described therapeutic ranges,[23] plasma EFV concentrations were assigned to three groups: subtherapeutic (<1 000 ng/mL), therapeutic (1 000 - 4 000 ng/mL) and supratherapeutic (>4 000 ng/mL) range. The distribution of plasma EFV concentration among participants according to categories was as follows: 44 (25%) in the subtherapeutic range, 90 (51%) in the therapeutic range, and 41 (24%) in the supratherapeutic range.

Association between CMV DNA positivity and plasma efavirenz concentration

CMV DNA-positive women may be at risk of transmitting CMV to their offspring with potentially debilitating effects – with a greater risk of transmission in HIV-infected women. We investigated the possible role of plasma EFV concentration and consequently HIV disease progression (determined by HIV viral load) on maternal plasma CMV DNA status in participants who were HIV-CMV co-infected. CMV DNA was detected in the plasma of 27 participants (15.4%).

The association between plasma EFV concentration and plasma CMV DNA positivity was determined using the Mann-Whitney test. Fig. 1 shows the effects of plasma EFV concentration on CMV DNA positivity. Reduced plasma EFV concentration (median (25th - 75th percentile) 847 (250 - 3 307) ng/mL) was significantly associated with CMV DNA positivity ($p<0.001$) compared with the higher plasma EFV concentration (median (25th - 75th percentile) 2 024 (250 - 14 039) ng/mL). Using the χ^2 test, we found a significant decrease ($p<0.001$) in the proportion of CMV positivity and plasma EFV concentration groups. The distribution of CMV DNA positivity in the three plasma EFV concentration groups was as follows: subtherapeutic (76%), therapeutic (16%) and supratherapeutic (8%). Furthermore, using Spearman's correlation test, plasma concentrations inversely correlated with CMV viral load. Log plasma EFV concentration significantly decreased with increasing CMV viral load ($r=-0.4$; $p=0.03$).

Using the Mann-Whitney test, CMV DNA positivity was significantly associated ($p<0.001$) with higher HIV viral load (median

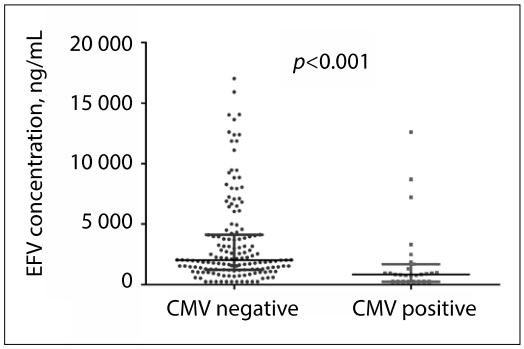

Fig. 1. Comparison of plasma EFV concentration between CMV-infected and CMV-uninfected participants. Data are presented with median and interquartile ranges. (EFV = efavirenz; CMV = cytomegalovirus.)

Table 1. Participants' demographic and clinical characteristics

Characteristic	Participants, *N*=175	CMV negative, *n*=148	CMV positive, *n*=27	*p*-value
Demographics				
Mean (SD) maternal age, years	30 (6)	30 (6)	29 (6)	0.182*
Mean (SD) gestational age, weeks	32 (5)	32 (5)	32 (4)	0.945*
Mean (SD) gravidity	3 (1)	3 (1)	3 (2)	0.285*
Mean (SD) parity	1.7 (1)	2 (1)	1 (1)	0.052*
Median (25th - 75th) BMI, kg/m^2	29 (23 - 28)	26 (24 - 28)	25 (23 - 28)	0.232†
Median time on cART (25th - 75th), months	18 (2 - 55)	18 (2 - 54)	16 (2 - 75)	0.862†
Marital status, *n* (%)				
Married	128 (73)	108 (73)	20 (74)	Reference
Single	47 (27)	40 (27)	7 (26)	0.913‡
Laboratory and clinical parameters				
Median CD4+ count (25th - 75th), cells/µL	377 (198 - 511)	405 (228 - 514)	209 (50 - 405)	0.012†§
Median VL (25th - 75th), copies/mL	97 (10 - 1 400)	49 (10 - 686)	3 033 (76 - 87 604)	<0.001†§

CMV = cytomegalovirus; SD = standard deviation; BMI = body mass index; cART = combination antiretroviral therapy; VL = HIV viral load; 25th - 75th = interquartile range.
*t-test.
†Mann-Whitney rank sum test.
‡χ^2 test.
§Statistically significant.

(25th - 75th percentile) 3 033 (76 - 87 604) copies/mL) compared with CMV-uninfected women (median (25th - 75th percentile) 49 (10 - 686) copies/mL) (Fig. 2). A stepwise multivariate logistic regression analysis was employed to account for the possible confounding factors, including maternal age, gestational age, BMI, gravidity, HIV viral load, CD4+ T-lymphocyte and plasma EFV concentration on plasma CMV DNA status (Table 2). Plasma EFV concentration and HIV viral load were the significant determinants of plasma CMV DNA positivity in the final multivariate model.

Association of efavirenz with drug response genotypes

The possible role of genetic variation in *CYP2B6* regarding the variability in plasma EFV concentration was determined by genotyping two polymorphisms, *CYP2B6 c.516G>T* and *CYP2B6 c.983C>T*. In agreement with previous findings, plasma EFV concentrations were correlated with *CYP2B6* genotypes. *CYP2B6* poor-metaboliser genotypes had significantly higher plasma EFV concentrations compared with *CYP2B6* extensive metabolisers. Comparisons across the genotype groups for both *CYP2B6 c.516G>T* and *CYP2B6 c.983C>T* were significantly different ($p<0.001$). For example, median plasma EFV concentrations for *CYP2B6 c.516G/G, CYP2B6 c.516G/T, CYP2B6 c.516T/T, CYP2B6 c.983T/T* and *CYP2B6 c.983T/C* were as follows: 1 027 ng/mL, 2 108 ng/mL, 7 074 ng/mL, 1 695 ng/mL and 7 228 ng/mL, respectively.

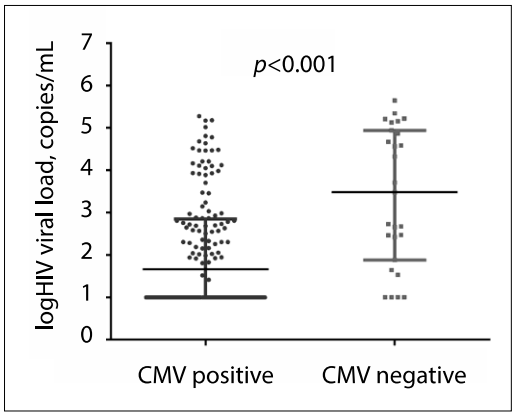

Fig. 2. Comparison of HIV viral load between CMV-infected and CMV-uninfected participants. Data are presented with median and interquartile ranges. (CMV = cytomegalovirus.)

Discussion

There is an increasing number of people living with HIV, who receive chronic cART. The biggest challenge in patient outcomes is the effect of long-term cART on susceptibility to and pathological dynamics of HIV co-infections.[24] Even in the presence of cART, which has dramatically reduced the vertical transmission of HIV, cCMV remains a public health concern owing to late and underdiagnosis.[25] In this study, we report a significant association between detection of plasma CMV DNA and subtherapeutic plasma EFV concentrations in pregnant women administered an EFV-based first-line cART regimen. The study also confirms the known significant association between plasma EFV concentrations and genetic variation in *CYP2B6*, as previously reported by Swart *et al.*[19] and Lamorde *et al.*[26]

All participants were enrolled >8 hours after the EFV dose to ensure a time-point beyond the EFV absorption phase.[27] Furthermore, all enrolled participants had been on an EFV-based regimen for >4 weeks, ensuring steady-state phase. However, drug adherence was reported by participants, which may have introduced bias. Assessment of adherence to ART is a major conundrum in the care of HIV patients due to potential patient misrepresentation. More effective methods of adherence assessment, such as the medication event monitoring systems (MEMS) cap,[28] remain largely inaccessible owing to cost. The wide range of the plasma EFV concentration (250 - 15 931 ng/mL) observed is indicative of the interindividual variability in exposure to therapy. Of note, 25% and 24% of participants were in the subtherapeutic and supratherapeutic groups, respectively. Regardless of patients receiving the same drug dosage, a great deal of variability in pharmacokinetics and treatment outcomes has also been documented for various drugs such as warfarin, digoxin and tacrolimus.

Several environmental, demographic and clinical factors have been implicated in the interindividual variability in response to therapy.[29-31] However, in this study, no association between plasma EFV concentrations and demographic markers, such as maternal

Table 2. Stepwise logistic regression of clinical and demographic variables associated with cytomegalovirus DNA status

Characteristic	Odds ratio	Standard error	*p*-value	95% CI
Gestational age	1.0	0.56	0.843	0.93 - 1.15
Gravidity	0.8	0.22	0.496	0.48 - 1.37
Maternal age	1.0	0.06	0.767	0.91 - 1.14
BMI	0.9	0.06	0.256	0.83 - 1.05
CD4+ count*	0.9	0.03	0.092	0.99 - 1.00
HIV viral load†	1.4	0.27	0.062	1.01 - 2.13
EFV concentration†	0.4	0.12	0.002‡	0.22 - 0.72‡
Remove maternal age, BMI, gestational age, count and gravidity as covariates				
CD4+ count*	0.9	0.03	0.073	0.99 - 1.00
EFV concentration†	1.5	0.12	0.004‡	0.23 - 0.71‡
HIV viral load†	0.4	0.26	0.013‡	0.99 - 2.02‡
Remove CD4+ count				
Plasma EFV concentration†	0.6	0.14	0.024‡	0.38 - 0.93‡
HIV viral load†	1.6	0.26	0.005‡	1.15 - 2.18‡

CI = confidence interval; BMI = body mass index; EFV = efavirenz.
*Square root transformed.
†Log_{10} transformed.
‡Statistically significant.

age, BMI, duration on cART and gestational age (Suppl. Table 1) was observed. Considering the distribution of plasma EFV concentration among the participants, with 25% in the subtherapeutic group and at risk of viral replication, the 400 mg once-daily dose currently recommended partly because of data from the the ENCORE1 (efficacy of 400 mg efavirenz v. the standard 600 mg dose in HIV-infected antiretroviral-naive adults) study, may not be ideal. This study concluded that an EFV dose reduction from 600 mg to 400 mg once daily would result in optimal therapy.[32] However, considering findings from the current study, further reduction of the standard dose would further predispose patients in the subtherapeutic group to viral replication and risk of developing co-infections. Perhaps, genotype-assisted dosing adjustments are ideal. It is worth noting that the ENCORE1 study was not carried out in pregnant women, and therefore may not be appropriately extrapolated to such women. Metabolic aberrations that occur during pregnancy could potentially impact drug metabolism. For example, in pregnancy, there is a general decrease in albumin content (~30%) in the third trimester, resulting in a reduced albumin availability.[33] Albumin binds EFV; therefore, increased unbound EFV may increase penetration of HIV-infected tissue cells. Furthermore, most of the results from the ENCORE1 study are premised on a different population grouping compared with participants in our study, who were predominantly of African ancestry.[34]

Understanding factors that influence exposure to drugs is important, especially during pregnancy, when there is a risk of transmission of antenatal co-infections to the developing fetus. The proportion of decreased plasma EFV concentrations and CMV viral load in the current study is a cause for concern. It is known that subtherapeutic cART concentrations sustain HIV replication. CMV and HIV have previously been shown to coactivate each other.[35] The current study further supports previous reports, which hypothesise that HIV flourishes in patients with low cART concentrations,which in turn supports CMV replication, resulting in higher CMV viral loads. We also hypothesise the contribution of CMV to poor health and hearing impairment experienced by HIV-exposed children born to mothers with high HIV viral loads. To further support the relationship between HIV replication and the likelihood of CMV infection, we found a significant association between increased HIV viral load and CMV positivity.

The observed CMV DNA positivity in this study is most likely due to CMV reactivation during pregnancy, considering the ubiquitous nature of CMV in resource-poor settings. Taylor *et al.*[36] reported that cART acts by disrupting the HIV life cycle, thereby arresting viral replication. When there is insufficient cART in circulation, HIV replicates, thereby further weakening the immune system. Latent viruses such as CMV are reactivated in the setting of compromised immunity and chronic immune activation. Hence, subtherapeutic EFV concentrations predispose to CMV reactivation, which, if occurring in pregnancy, may have adverse health outcomes on the growing fetus and/or neonate. CMV and HIV have previously been found to co-activate each other *in vitro* and have been described as 'partners in crime', with CMV viral shedding thereby activating the immune system, which then stimulates HIV replication.[13] We therefore hypothesise that the subtherapeutic EFV concentrations predisposed the pregnant women to CMV infection and perhaps reactivation. In view of these findings, dose reduction should only be implemented in view of an individual's genotype to avoid subtherapeutic drug concentrations that may predispose to co-infections.

Data in this cohort of pregnant women show that *CYP2B6 c.516T* and *CYP2B6 c.983C* variants are good correlates of plasma EFV concentrations. These findings argue for pharmacogenetic-guided EFV dosing. Previous studies have reported similar findings, such as a reduced activity of the CYP2B6 enzyme due to the *CYP2B6 c.516G>T* and *CYP2B6 c.983T>C* SNPs.[5,18,26] Consequently, carriers of the *CYP2B6 c.516T* and *CYP2B6 c.983C* variants have been associated with high CYP2B6 substrate drug concentrations, which increase with copy number (i.e. *CYP2B6 c.516T/T>>>CYP2B6 c.516G/T>>CYP2B6 c.516G/G*, *CYP2B6 c.983C/C>>>CYP2B6 c.983C/T>>CYP2B6 c.983T/T*). The *CYP2B6 c.516T* and *CYP2B6 c.983C* alleles both result in reduced enzyme function, leading to ineffective metabolism of EFV and high drug concentrations.

Conclusions

We conclude that subtherapeutic cART concentrations may predispose to viral replication and co-infections such as CMV. We also confirm that polymorphisms in the *CYP2B6* gene are potential pharmacokinetic determinants of EFV and as such should be considered for dose adjustment.

Declaration. None.

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Author contributions. DM conceived the ideas, carried out laboratory experiments, analysed data and drafted the manuscript; GM and CM co-conceived the ideas, co-supervised the pharmacology components and reviewed the manuscript draft; KM reviewed the manuscript draft, and assisted with laboratory experiments and data analysis; CD conceived the ideas, supervised all components as principal investigator and reviewed the manuscript draft.

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Conflicts of interest. None.

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Supplementary Table 1. Stepwise multivariate linear regression of clinical and demographic variables that predict plasma evavirenz concentration

Characteristic	Coefficient	Standard error	*p*-value	95% CI
CYP2B6 c.516 G/T	0.67	0.13	<0.001‡	0.42 - 0.93
CYP2B6 c.516 T/T	1.58	0.15	<0.001‡	1.28 - 1.90
CYP2B6 c.983 T/C	0.88	0.17	<0.001‡	0.55 - 1.21
Gestational age	–0.01	0.012	0.307	–0.04 - 0.01
Gravidity	–0.01	0.06	0.821	–0.12 - 0.11
Maternal age	0.01	0.013	0.577	–0.02 - 0.032
BMI	–0.004	0.014	0.767	–0.03 - 0.03
CD4+ count*	–0.01	0.01	0.133	–0.001 - 0.0000
HIV viral load	–0.26	0.04	<0.001‡	–0.35 - 0.18
Remove gravidity and gestational age as covariates				
CYP2B6 c.516 G/T	0.68	0.13	<0.001‡	0.42 - 0.94
CYP2B6 c.516 T/T	1.59	0.15	<0.001‡	1.29 - 1.89
CYP2B6 c.983 T/C	0.91	0.17	<0.001‡	0.58 - 1.23
Maternal age	0.01	0.01	0.612	–0.02 - 0.03
BMI	–0.01	0.14	0.654	–0.03 - 0.02
CD4+ count*	–0.01	0.01	0.128	–0.001- 0.00001
HIV viral load	–0.25	0.04	<0.001‡	–0.35 - 0.17
Remove BMI, maternal age and CD4+ count				
CYP2B6 c.516 G/T	0.68	0.13	<0.001‡	0.43 - 0 .94
CYP2B6 c.516 T/T	1.59	0.15	<0.001‡	1.30 - 1.90
CYP2B6 c.983 T/C	0.91	0.16	<0.001‡	0.60 - 1.23
HIV viral load†	–0.23	0.04	<0.001‡	–0.35 - 0.18

CI = confidence interval; BMI = body mass index.
*Square root transformed.
†Log_{10} transformed.
‡Statistically significant.

3.3 Determination of the role of polymorphisms in genes which encode pattern recognition proteins (TLRs 2,4,7 and 9) and immune cascade proteins (IL-1A, IL-6, IL-6R, IL-10, IL-28B, IFNAR1) in CMV reactivation during pregnancy

Synopsis

Susceptibility to CMV infection has been mainly attributed to imbalanced immunity. Functions of the immune system are regulated at genetic level. It is therefore important to determine the role of genetic variation in genes that encode proteins involved in immunity against CMV, as markers of susceptibility to CMV infection. This chapter addresses objective 3 which sets out to determine the role of host genetic variation in susceptibility to CMV infection in pregnancy. This Chapter consists of a manuscript that has been submitted to BMC Genomics. Supplementary Table 1 is in the Annex section.

3.3.1 Genetic variation in toll like Receptor 2, 7, 9 and interleukin-6 influences risk of Cytomegalovirus infection in pregnancy

Nature of manuscript: Original research article

Submitted to: BMC Medical Genetics

Candidate's contribution: conception of the project, data and sample collection, laboratory analysis of samples, initial data analysis, drafting of manuscript, incorporation of co-authors' comments and execution of reviewers' comments.

The **findings from this study can be summarised** as follows:

- This study is the first to report on genotype and allele frequencies of SNPs in TLR2, TLR4, TLR9, TLR7, IL-6, IL-10, IL-28B, IL-1A and interferon AR1 (IFNAR1) genes in a Zimbabwean population.

- The TLR2 rs1816702C/C ($p=0.002$), TLR7 rs179008C/C ($p<0.001$) and TLR9 rs352139C/C ($p=0.003$) genotypes were associated with a CMV+ status.

- In contrast, the interleukin (IL)-6 rs10499563T/C genotype was associated ($p<0.001$) with CMV- status.

- Despite previous reports on association of the SNPs in *TLR4, IL-10, IL-28B, IL-1A* and *IFNAR1* genes with CMV and other viral infections, in our study we did not observe and significant association between antenatal CMV and these SNPs.

- The lack of significant association between CMV infection status and the other SNPs could be due to: (i) low frequency in the study population, (ii) linkage disequilibrium with more significant SNPs in other studied populations which is not present in our study population.

Genetic variation in toll like Receptor 2, 7, 9 and interleukin-6 influences risk of Cytomegalovirus infection in pregnancy

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Keywords: Cytomegalovirus, Toll like receptors, interleukins, CMV DNA, Zimbabwe

Running title: Host genetics and Cytomegalovirus infection

Abstract

Background: Maternal cytomegalovirus (CMV) infection and/or reactivation in pregnancy is associated with a myriad of adverse infant outcomes. However, the role of host genetic polymorphisms in modulating maternal CMV status is inconclusive. This study investigated the possible association of single nucleotide polymorphisms in toll like receptor (TLR) and cytokine genes with maternal plasma CMV DNA status in black Zimbabweans.

Results: The *TLR2* rs1816702C/C (p=0.002), *TLR7* rs179008C/C (p<0.001) and *TLR9* rs352139C/C (p=0.003) genotypes were associated with a CMV+ status. In contrast, the interleukin (*IL)-6* rs10499563T/C *TLR2* rs1816702C/C (p=0.002) genotype was associated (p<0.001) with CMV- status. Furthermore, genotype and allele frequencies of SNPs in *TLR2, TLR4, TLR9, TLR7, IL-6, IL-10, IL-28B, IL-1A* and interferon AR1 (*IFNAR1*) genes are being reported here in a Zimbabwean population.

Conclusions: Toll like receptor and interleukin genetic polymorphisms influence CMV status in late gestation among black Zimbabweans. This is attributable to possible modulation of immune responses to CMV reactivation in a population that was previously exposed to CMV infection.

Background

Seroprevalence of Cytomegalovirus (CMV) amongst women of reproductive age ranges from 40-65% in the developed world and can reach 100% in developing countries [1, 2]. CMV infection in pregnancy, in the setting of both primary infection and reinfection, can be potentially transmitted to the foetus and or neonate, resulting in congenital CMV (cCMV). The consequences of CMV range from asymptomatic viraemia to potentially life changing conditions which include mental retardation and congenital sensorineural hearing loss. Studies have implicated maternal demographics, socioeconomics and HIV status among the strongest determinants of the biased occurrence and vertical transmission of CMV [3–7]. Furthermore, maternal immune responses to CMV infection and/or reactivation actively modulate CMV related disease outcomes [8]. Thus, variation in genes that encode components of the immune system that are directly or indirectly involved in the pathogenesis of CMV have been implicated in CMV infection outcomes [9]. However, the genetic variants, like seroprevalences and the factors influencing CMV epidemiology are heterogenous among populations hence research findings are equivocal.

Toll-like receptors (TLR) are crucial in the detection of viruses in circulation and the subsequent elicitation of an antiviral response [10, 11]. TLRs act as pattern recognition receptors of non-methylated viral CpG-containing DNA which signals the presence of CMV infection [12]. TLR2

and TLR4 are cell surface receptors while TLR3, -7 and -9 are endosomal receptors [13, 14]. TLRs facilitate viral attachment and entry resulting in CMV-elicited signalling antiviral responses such as type 1 interferon activation of nuclear factor kappa β (NF-k β) and pro-inflammatory cytokine gene expression [12, 15]. Activation of the type 1 interferon producing cascade and production of cytokines form the major cellular antiviral mechanisms against CMV [16–18]. Single nucleotide polymorphisms (SNPs) in the *TLR2, TLR4, TLR7* and *TLR9* genes were inconclusively reported to be associated with CMV infection [19–23].

In response to TLR activation, chemokine (interleukin and interferon) genes signal immediate secretion of ILs from cells such as macrophages and T-helper cells. Chemokines that trigger an immune cascade by signalling direct growth, development, maturation, activation and increased life-span of immune cells. In the case of CMV infection, chemokines signal: maturation of B-lymphocytes into plasma cells which produce anti-CMV antibodies, and activation of cytotoxic T cells for destruction of CMV infected cells [24, 25]. The differential response to CMV exposure with some but not all exposed individuals developing CMV-related diseases suggests a possible role of host genetic variation in immune response. A study by Sezgin et al [26] showed that human interleukin-10 receptor variants potentially interfere with IL-10 binding and signal transduction influence susceptibility to CMV retinitis. In a large Swiss HIV Cohort Study, the effect of *IFNL3* TT/-G substitution, the variant allele was associated with occurrence of CMV retinitis [27]. The same allele was also associated with susceptibility to CMV replication in transplant patients [28].

Detection of host genetic variants which may confer resistance to CMV infection and reactivation could reveal potential therapeutic targets against pregnancy related CMV disease. Furthermore, host genetic determinants of CMV disease outcomes could be used as predictors of adverse outcomes of maternal CMV. While the host genetics of CMV have been studied in other populations, a glaring gap in knowledge exists among Africans. The differences in genomic variation between Africans and other populations cannot be over-emphasised, hence findings from other populations may not be an accurate reflection in Africans. The aim of the present study was to determine if single nucleotide polymorphisms in genes that encode components of the immune system influence acquisition or reactivation of CMV in pregnancy.

Results

Study participants' demographic and clinical characteristics

The demographic and clinical characteristics of the 110 participants are summarised in Table 2. All participants were in child bearing age (median 28 years, 25^{th}–75^{th} percentile: 23–34). The group of

women with a positive CMV DNA (CMV+) status (median 24 kg/m^2, 25th–75th percentile: 22–27) had a significantly (p=0.006) lower body max index (BMI) than the group who tested negative for CMV DNA (CMV-) (median 26 kg/m^2, 25th–75th percentile: 24–29. CMV+ participants also had significantly lower systolic blood pressure when compared with the CMV- participants. Age, gestational age, parity, gravidity, diastolic blood pressure, pulse rate, income, level of education and HIV status were comparable between CMV+ cases and CMV- controls.

Table 2 Participants' demographic and clinical characteristics

Characteristic	**Combined (n=110)**	**CMV- (n=74)**	**CMV+ (n=36)**	**P-value**
Median age in years (25th-75th percentiles)	28 (23–34)	29 (23–34)	28 (23–33)	**0.85**
Mean gestational age, weeks ± sd	32.3 ± 4.4	32.4 ± 4.8	32.1 ± 3.5	**0.73**
Mean SBP, mmHg ± sd	112 ± 13	113 ± 14	109 ± 9	**0.037**
Mean DBP, mmHg ± sd	69 ± 10	70 ± 10	67 ± 9	**0.13**
Mean pulse rate, bpm ± sd	82 ± 10	82 ± 10	80 ± 12	**0.33**
Median BMI (25th-75th percentiles)	25.7 (23.4–28.4)	26.3 (24.3–28.8)	24.2 (21.7–27.3)	**0.006**
Median parity (25th-75th percentiles)	1 (0–2)	1 (0–2)	1 (0–2)	**0.31**
Median gravidity (25th-75th percentiles)	2 (1–3)	3 (2–4)	2 (1–3)	**0.62**
HIV infected n (%)	73 (66)	45 (61)	28 (78)	**0.08**
Median income in USD/month (25th-75th percentiles)	235 (168–300)	235 (171–300)	225 (153–332)	**0.97**
Education n (%)				**0.30**
Secondary	97 (88)	67 (91)	30 (83)	
Primary	9 (8)	4 (5)	5 (14)	
Tertiary	4 (4)	3 (4)	1 (3)	

Key: CMV=Cytomegalovirus, CMV+=CMV infected, CMV-=CMV uninfected, BMI=Body mass index

Association between SNPs and CMV infection

Genotype data for the 20 SNPs genotyped was available for all 110 participants and the SNP rs113181057 on the *IFNAR1* gene was monomorphic in the study population. There was a departure from Hardy-Weinberg equilibrium (HWE) for four of the 20 SNPs: *TLR7* rs179008 in cases, *TLR2* rs1816702 and *IL-6* rs10499563 in the controls and *IFNAR1* rs2843710 in both groups. Supplementary Table 1 is a summary of genotype frequencies in the CMV+ and CMV- negative groups and the univariable logistic regression analyses of SNPs and CMV status. Using the univariate logistic regression analysis of codominant and log additive inheritance models, 4 SNPs (rs10499563 ($p<0.001$), rs179008 ($p<0.001$), rs1816702 ($p=0.002$) and rs352139 ($p=0.003$) were significantly associated with CMV DNA status (Supplementary Table1). The *IL-6* rs10499563T>C polymorphism was significantly associated with lower risk of CMV infection. When compared to the *IL-6* rs10499563T/T genotype, the rs10499563T/C was associated with a lower risk of CMV infection as the genotype was significantly ($p<0.001$) less frequent in the CMV+ group (14%) than the CMV- group (70%). Likewise, the *TLR2* rs1816702C>T SNP was significantly associated with lower risk of CMV infection. Genotype rs1816702C/C genotype was significantly ($p=0.002$) higher in the CMV+ (47%) than the CMV- women (11%).

In contrast, *TLR7* (rs179008A>T) and *TLR9* (rs352139T>C) polymorphisms were associated with an increased risk of CMV infection. The *TLR7* rs179008C/C genotype was significantly higher in the CMV+ group than the CMV- group (31% vs. 3%; $p<0.001$. With reference to the *TLR9* rs352139T/T genotype, both the rs352139T/C and rs352139C/C genotypes were significantly ($p=0.005$) higher in the CMV+ women (28% and 58% respectively) than in the CMV- women (11% and 47% respectively). These associations remained significant after correction for multiple comparisons (Figure 1). When other models of genetic inheritance were considered, the association of *IL-6* rs10499563 maintained significant association with CMV status after Bonferonni correction (BC) in dominant, and overdominant models. SNPs rs1816702 and rs179008 also maintained significance with CMV status after BC in the dominant and recessive models (Figure 1).

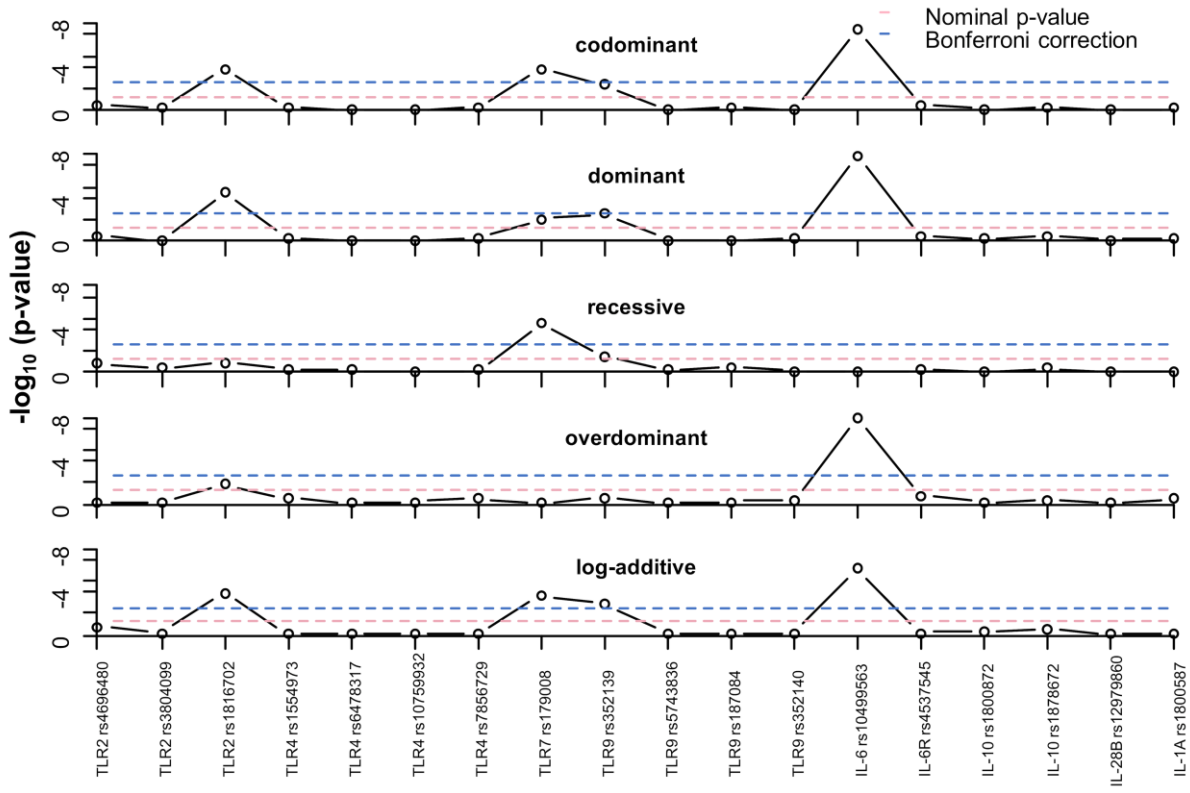

Fig. 1 Plot of $\log_{10}$ p-values for the association of gene with CMV DNA across models of genetic associations. For each figure panel, the lower dotted horizontal line is for the nominal p-value threshold for significance (0.05), while the upper dotted blue line for the Bonferroni corrected threshold p-value for significance.

Table 3 shows multivariable logistic regression analysis of SNPs (rs10499563, rs179008, rs1816702 and rs352139) that were associated with CMV status in the univariable analyses. BMI was also included in the model. All SNPs maintained significant association with CMV infection status in at least one of the models. However significant association of rs352139 with CMV status was borderline (p=0.049) in the log additive model while it was not significant in the other models. BMI's association with CMV status also substantially attenuated in the multivariate logistic regression model (p=0.068).

The *IL-6* rs10499563T>C SNP was associated with low likelihood of CMV positivity in codominant (OR=0.05; 95%CI=0.01–0.25, p=0.001) as well as in the log additive, dominant and overdominant models. The result shows the association of the C allele with lower odds of CMV infection even in heterozygous state (rs10499563 T/C). For the *TLR7* rs179008A>T SNP, the T allele was significantly associated with higher odds of CMV infection in the codominant model (OR=3.67; 95%CI=0.79–116.99; p<0.011) which, was maintained in the log additive, recessive and dominant models. Hence T allele will likely be associated with CMV+ status in both homozygous and

heterozygous states (rs179008T/T and rs179008A/T). The *TLR2* rs1816702T>C was significantly associated with decreased risk of CMV positivity both in the codominant (OR=0.05; 95%CI=0.01–0.25, p=0.001) as well as in the log additive, dominant and overdominant models. Hence, risk of CMV infection will be decreased in the homozygous state, rs1816702C/C. The TLR9 rs352139 was significantly associated with likelihood of CMV infection, only in the log additive model (OR=2.85: 95%CI=0.95-8.58; p=0.049).

Table 3 Multivariable adjusted models containing BMI and significant SNPs in univariable analysis

SNP	**Model**	**Genotype**	**OR (95%CI)**	**p-value**
TLR2 rs1816702	Codominant	C/T	0.09 (0.02-0.43)	0.001
		T/T	0.06 (0.01-0.48	
	Dominant	C/T-T/T	0.08 (0.02-0.37)	0.0003
	Recessive	T/T	0.32 90.07-1.50	0.133
	Overdominant	C/T	0.29 (0.08-1.01)	0.044
	Log additive	0,1,2	0.22 (0.08-0.62)	0.001
TLR7 rs179008	Codominant	A/T	3.67 (0.79-116.99)	0.011
		T/T	18.69 (1.59-220.04)	
	Dominant	A/T-T/T	6.05 (1.53-23.94)	0.006
	Recessive	T/T	13.15 (1.15-149.74)	0.013
	Overdominant	A/T	2.27 (0.53-9.68)	0.262
	Log additive	0,1,2	4.08 (1.46-11.39)	0.003
TLR9 rs352139	Codominant	T/C	2.87 (0.50-16.58)	0.144
		C/C	8.13 (0.90-73.63)	
	Dominant	T/C-C/C	3.58 (0.65-19.66)	0.121
	Recessive	C/C	3.65 (0.67-19.77)	0.124
	Overdominant	T/C	1.05 (0.30-3.68)	0.938
	Log additive	0,1,2	2.85 (0.95-8.58)	0.049
Il-6 rs10499563	Codominant	T/C	0.05 (0.01-0.25)	<0.001
		C/C	0.42 (0.05-3.77)	
	Dominant	T/C-C/C	0.08 (0.02-0.31)	<0.001
	Recessive	C/C	1.53 (0.21-11.39)	0.682
	Overdominant	T/C	0.06 (0.01-0.27)	<0.001
	Log additive	0,1,2	0.19 (0.06-0.58)	0.001

Comparison of variant allele frequencies from this study with other populations

The variant allele frequencies of the genotyped SNPs were compared with data from two other populations: Asians and Europeans. Table 4 gives variant allele frequencies for the genotyped SNPs in this study as well as for Asians and Europeans as reported on dbSNP.

Table 4 Comparison of variant allele frequencies of genotyped SNPs with other populations

Gene	SNP	Variant allele	Zimbabwean (This study)	Other Africans	Europeans	Asians
TLR2	rs4696480	A	0.31	0.37	0.52	0.57
TLR2	rs3804099	T	0.45	0.36	0.56	0.72
TLR2	rs1816702	T	0.47	0.43	0.12	0.00
TLR4	rs1554973	T	0.20	0.21	0.77	0.86
TLR4	rs2737190	A	0.14	0.16	0.33	0.37
TLR4	rs10759932	C	0.19	0.25	0.85	0.76
TLR4	rs7856729	T	0.38	0.33	0.13	0.10
TLR7	rs179008	T	0.23	0.12	0.23	0.00
TLR9	rs352139	C	0.42	0.61	055	0.40
TLR9	rs5743836	G	0.36	0.42	0.13	0.00
TLR9	rs187084	G	0.30	0.29	0.43	0.40
TLR9	rs352140	T	0.32	0.29	0.55	0.39
IL-6	rs10499563	C	0.31	0.27	0.23	0.16
IL-6R	rs4537545	C	0.26	0.34	0.37	0.32
IL-10	rs1800872	T	0.40	0.44	0.24	0.68
IL-10	rs1878672	C	0.25	0.26	0.45	0.05
IL-28B	rs12979860	G	0.70	0.82	0.86	0.97
IFNAR1	rs2843710	G	0.30	0.31	0.41	0.36
IFNAR1	rs113181057	C	0.00	N/A	N/A	N/A
IL-1A	rs1800587	C	0.65	0.60	0.71	0.93

Key: SNP=Single nucleotide polymorphism, TLR=Toll-like receptor, IL=Interleukin, IFNAR=Interferon α

Discussion

The outcome of an infection is determined, in part, by the intensity of the inflammatory response [32], which varies between individuals and can be regulated at the genetic level [33]. In this study, we hypothesised the possible contribution of genetic variation to the biased occurrence of CMV infection among pregnant women. SNPs may influence the rate and regulatory dynamics of gene transcription, stability of mRNA as well as production and biological activity of resultant protein. We therefore investigated possible association between CMV infection and SNPs in 19 genes which encode proteins that are or may be involved in the immune reaction cascade against CMV. The departure from HWE in polymorphic SNPs is due to their association with CMV infection mainly because the departure is being observed when cases and controls are separated but HWE is maintained when the two groups are combined. We report a significant association between each of; rs10499563, rs179008, rs1816702 and rs352139 SNPs and CMV DNA status. To our knowledge, this is the first report on SNPs and CMV infection in an African setting.

To minimise the confounding effects of age and HIV status, which are directly related to immune function, enrolled participants were age and HIV status matched. The observation that overweight women were less likely to be CMV+ contradicts findings from previous studies where CMV infection was associated with metabolic syndrome, higher BMI and or obesity [34, 35]. Our findings could be due to none of the participants having any form or history of metabolic syndrome. Hence, we were unlikely to observe any significant associations. The observation that CMV positivity is significantly associated with low systolic blood pressure contrasts with previous findings which have shown increasing systolic blood pressure with CMV positivity [36, 37]. It is worth noting that the previous studies were carried out in non-pregnant adults, hence discrepancy in findings could be due to the well documented effects of pregnancy on fluctuations in blood pressure [38, 39] masking the effects of CMV infection.

We found an association between SNP rs10499563 (-6331T>C), located within the promoter region of *IL6* gene which regulates the rate of IL6 gene transcription [40] and CMV DNA status. Individuals carrying the C allele were less likely to be CMV infected, hence likelihood of being CMV DNA positive decreased with genotypes T/T>>>T/C>>C/C. Individuals heterozygous (T/C) and homozygous (C/C) for the variant allele were significantly less likely to be CMV infected than individuals homozygous for the T allele (T/T). The *IL6* gene codes for IL6, a versatile inflammatory cytokine whose function is related to its expression in the tissue. Smith et al previously reported higher level of serum IL6, in individuals with wildtype T/T genotype compared to individuals with C/C genotype, among coronary artery bypass patients (Smith et al., 2008).

Our findings could at least in part, be explained by results from the Smith et al study. Being a pro-inflammatory cytokine, abundance of IL6 in circulation could promote CMV activation. In contrast, the low levels of IL6 associated with the rs10499563C allele would disfavour the occurrence of CMV infection. Serum IL6 levels were reported to be significantly higher among the CMV infected pregnant women compared to the CMV uninfected in a Chinese cohort [42].

We also report an association between CMV DNA status and rs179008, a non-synonymous A>T (Gln11Leu) polymorphism within exon 3 of the *TLR7* gene [43]. The resulting glycine to leucine change has been suggested to code for a functionally impaired TLR7 protein [44, 45]. In the present study, the T allele was associated with significantly lower odds of CMV positivity. Individuals homozygous for the variant allele T/T were significantly less likely to be CMV infected compared to individuals homozygous for the wildtype allele A/A.

Upon recognising pathogen associated molecular patterns (PAMP), TLR7 activate a signalling cascade which activates type I IFN, dendritic cells (DCs) and B lymphocytes [46]. Activated type 1 IFN, DCs and B cells are responsible for pathogen clearance, antigen recognition and antibody production. The induced immune cascade is critical in CMV clearance. In the presence of the T allele which results in a less potent protein, an insufficient signal is mounted by TLR7, hence carriers of the rs179008 T allele are at a greater risk of CMV infection. The rs179008 T allele has been linked with unfavourable outcomes in HIV and other viral infections. The variant was associated with increased susceptibility to HIV-1 and decreased IFNα production in HIV uninfected women [47]. The T allele has also been previously associated with a higher risk of hepatitis C infection and cCMV. Our findings are therefore contrasting with previous reports suggesting that the rs179008A>T SNP could be in linkage disequilibrium with another functional SNP or epistatic gene which masks the effects of rs179008A>T.

CMV DNA status was also associated with rs1816702C>T, a SNP located in intron 2 of the *TLR2* gene. The C variant was significantly more prevalent in cases than in controls which means that participants with the rs1816702 C/C genotype were at a higher risk of being CMV+ than those with rs1816702 T/T genotype. TLR2 recognise CMV glycoproteins B (gB) and gH in a process which facilitates entry of CMV into immune cells [15, 48]. The rs1816702 T allele is associated with significantly elevated levels of inflammatory monocytes expressing CD14+/TLR2+ receptors than rs1816702 C allele [49]. This could explain our findings of a higher risk of CMV among rs1816702C/C carriers because their immune response against CMV is impaired due to lower TLR2 expression compared to the T/T. Homozygosity for the rs1816702 C allele has also been associated with increased odds of *Mycobacteria leprae* infection and inflammatory bowel disease which were attributable to altered NFκB-mediated inflammatory response [50, 51].

The intronic SNP rs352139T>C in the *TLR9* gene was also associated with CMV DNA status. Homozygous rs352139C/C individuals were at a significantly higher risk of being CMV+ compared to homozygous T/T carriers. The effect of the C allele on risk of CMV infection was also observed in the dominant and recessive models where the significance of the compound heterozygous (T/C) and homozygous (C/C) genotypes had a greater risk than the homozygous (CC) alone, relative to the T/T genotype in both cases. The higher risk of CMV positivity in homozygous carriers of the C allele suggest that the polymorphism results in a less potent protein compared to the T allele. Since the polymorphism is intronic, it likely creates an alternative splicing site thus, affecting mRNA transcription and the final protein product. A less potent protein would have decreased ability to form dimers that are required to illicit an immune reaction. Individuals who are homozygous T/T have impaired immune responses against CMV infection, hence are more likely to experience CMV infection or reactivation. The HIV rapid progressor phenotype has been linked to homozygosity for rs352139T allele also due to reduced TRL9 potency [52].

Conflicting findings were reported reduced risk of cCMV associated with the rs352139T/T genotype among infants in Poland [53]. The conflicting effect of rs352139T variant have also been reported in bacterial infection studies in Indonesia and Mexico, perhaps due to ethnic differences [54, 55]. We suggest that rs352139 could be in linkage disequilibrium (LD) with a polymorphic regulatory region that controls *TLR9* expression or serves as a functional region SNP. LD patterns differ with level of genetic diversity among different ethnic groups; hence the effects of one SNP may vary from one population to another. To determine the possible role of genetic variability in the varying frequencies of CMV infection among populations, the minor allele frequencies for significant SNPs in our study population were compared to other populations. Whether this genetic heterogeneity among populations plays an active role in the differential prevalence of CMV is unclear and subject to further research due to the strong influence of environmental factors in CMV exposure.

Conclusions

We conclude that *TLR2, -7, -9* and *IL-6* genetic polymorphisms influence CMV status in late gestation among the black Zimbabweans. TLRs and ILs modulate immune responses to CMV, hence polymorphisms in genes encoding the receptors and cytokines could interfere with the immune mechanisms, hence their association with CMV status. We recommend that future studies consider increasing the sample size and including proteomic and transcriptomic profiles of TLRs and interleukins to fully understand the dynamics of these immunogenetic variants in CMV infection. We also recommend a mother-infant longitudinal approach that will seek to factor in the effect of these immunogenetic profiles in congenital CMV and its possible sequelae.

Materials and Methods

Study participants

This study was carried out among pregnant women seeking antenatal care at three polyclinics in Harare's Kuwadzana, Dzivarasekwa and Glenview high density suburbs who were recruited in the University of Zimbabwe College of Health Sciences Birth Cohort (MRCZ/A/1968). The general study design, setting and participants characteristics for the main cohort are described elsewhere [29]. In summary, this nested sub-study enrolled 110 women aged between 18 and 42 years who included 36 CMV infected cases and 74 CMV uninfected, age and HIV status matched controls. Whole blood and plasma specimens archived at enrolment were retrieved for host genotyping and CMV DNA detection respectively. CMV status of participants was determined by detection of CMV DNA in plasma using the real time polymerase chain reaction (PCR) kit (RealStar CMV Kit v1.0, Altona Diagnostics, Hamburg, Germany), following manufacturer's instructions.

Genotyping of candidate genes

Using candidate gene approach, 20 SNPs in 10 genes were selected for genotyping (Table 1). Selection of SNPS was based on the following criteria: previously reported association or plausible association with CMV infection and/or other viral infection, a minor allele frequency (MAF) ≥10% in African populations reported in the dbSNP database (Available from: http://www.ncbi.nlm.nih.gov/SNP/), except for the rs113181057 SNP whose MAF in African populations was not previously reported. Host genomic DNA was extracted from 200µl of whole blood using the Quick-DNA™ MiniPrep Plus Kit (Zymo Research, Irvine, CA, USA), according to manufacturer's instructions. All DNA samples were diluted to a concentration of approximately 50ng/ul in preparation of genotyping. SNPs were genotyped using Iplex GOLD SNP genotyping protocol on the Agena MassARRAY® system (Agena BioscienceTM, San Diego, CA, USA).

Table 1 Single nucleotide polymorphisms included in this study

Gene	SNP	Chrom	Genomic region	Functional effect
TLR2	rs4696480T>A	4	Intron	↓transcriptional activity
TLR2	rs3804099C>T	4	Exon	↓protein activity
TLR2	rs1816702C>T	4	Intron	↑protein levels
TLR4	rs1554973C>T	9	3'UTR	↓transcriptional activity
TLR4	rs2737190G>A	9	5'UTR	↑transcriptional activity
TLR4	rs10759932T>C	9	Promoter	↑transcriptional activity
TLR4	rs7856729G>T	9	3'UTR	Not known
TLR7	rs179008A>T	X	Exon	↓protein activity
TLR9	rs352139T>C	3	Intron	↑transcriptional activity
TLR9	rs5743836A>G	3	Promoter	↓transcriptional activity
TLR9	rs187084A>G	3	Promoter	↓transcriptional activity
TLR9	rs352140C>T	3	Exon	Not known
IL-6	rs10499563T>C	7	Promoter	↑transcriptional activity
IL-6R	rs4537545T>C	1	Intron	Not known
IL-10	rs1800872G>T	1	Promoter	↑transcriptional activity
IL-10	rs1878672G>C	1	Intron	↑susceptibility to infection
IL-28B	rs12979860T>C	19	Intron	↓protein activity
IFNAR1	rs2843710C>G	21	5'UTR	↓transcriptional activity
IFNAR1	rs113181057T>C	21	Exon	↓protein activity
IL-1A	rs1800587T>C	2	5'UTR	↑transcriptional activity

Key: SNP=Single nucleotide polymorphism, Chrom=Chromosome number, TLR=Toll-like receptor, IL=Interleukin, IFNAR=Interferon α, UTR=Untranslated region, ↑= increased, ↓= decreased, N/A=not reported

Statistical Analysis

Data were compiled and managed in Research Electronic Data Capture (REDCap) [30]. Statistical analyses were performed using Stata SE, version 15 (StataCorp, College Station, Texas, USA) and the 'Genetics' and 'SNPassoc' packages of the statistical package R (version 3.4.3 [2017-11-30], The R Foundation for Statistical Computing, Vienna, Austria). Numerical variables are described as either median and 25^{th} to 75^{th} percentiles for skewed variables or mean and standard deviation for normally distributed variables, with groups comparisons via Mann-Witney U-test and Student's t-test respectively. Categorical variables are described as frequencies and compared across groups

using Chi squared test. p-value <0.05 was considered statistically significant. Genotype and allele frequencies were calculated using ShesisPlus [31]. SNPs were tested for departure from Hardy-Weinberg Equilibrium (HWE) expectation using a Chi square goodness of fit test. Association between SNPs and CMV status was determined using univariable logistic regression analysis. Bonferroni correction was used to account for simultaneous comparison of multiple SNPs. Dominant, log-additive, codominant, recessive and overdominant inheritance models were interrogated for association of SNPs with CMV infection. Furthermore, multivariable logistic regression analysis of SNPs that were associated with CMV infection in the univariable analysis was carried out to adjust for their effect on each other in a model that also contained BMI as covariate.

Declarations

Ethics approval and consent to participate

The current study received ethical clearance from the Medical Research Council of Zimbabwe (MRCZ/A/2177) as well as the University of Cape Town Institutional Review Board (HREC628/2017). The study was carried out in accordance with the guidelines of the Helsinki Declaration of 2008 ethics clearance. All participants signed an informed consent form prior to enrolment into the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DM conceived the ideas, carried out some laboratory experiments, analysed data and drafted the manuscript; KM reviewed the manuscript draft, assisted with laboratory experiments; AK analysed data and reviewed the manuscript draft; MM read the manuscript draft; CD conceived the ideas, supervised all components as principal investigator and reviewed the manuscript draft. All authors contributed to the final version of the article.

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3.4 Comparative diversity of gut microbiota in pregnant women: effects of Cytomegalovirus infection

Synopsis to Chapter 3.4

The wide host cell tropism of CMV includes gut epithelial cells in which case it results in disruptions of the gut epithelial barrier. The disruption could result in leakage of substances from the gut which includes bacteria and their metabolites. In addition, the effects of the presence of CMV in the gut on other microbial species such as bacteria is not known. Consequences of a dysbiotic gut microbiome during pregnancy could have adverse effects on both the mother and developing foetus and or neonate. This chapter answers the fourth and last objective of the thesis, which is to determine the possible role of CMV on the gut bacterial profiles. It comprises a manuscript in preparation for submission to a relevant journal.

3.4.1 Comparative diversity of gut microbiota in pregnant women: effects of Cytomegalovirus infection

Nature of manuscript: Original research article

Candidate's contribution: conception of the project, data and sample collection, laboratory analysis of samples, data analysis, drafting of manuscript, incorporation of co-authors' comments and execution of reviewers' comments.

Main **findings from the study are summarized** as follows:

- To our knowledge, this study is the first to describe the gut bacterial profiles of pregnant women with respect to CMV infection
- Most abundant phylum for both CMV-infected and -uninfected women was Firmicutes.
- The gut microbiota of CMV-infected women had a differentially higher abundance of *P.copri* when compared to CMV uninfected women ($p<0.05$, $q<0.05$ LDA score>2.0)
- The gut bacterial profiles of CMV-uninfected women had a differentially higher abundance of *L.reuteri* and genus *Roseburia* compared to CMV -infected women ($p<0.05$, $q<0.05$ LDA score>2.0)
- These findings suggest diminished overall gut health in CMV infected participants due to diminished short chain fatty acids producing genera such as *Lactobacillus* and *Roseburia*.
- Furthermore, the differential abundance of *P.copri* in CMV infected cases confirm the previously described chronic inflammatory nature of CMV infection.
- Estimation of beta diversity of genotypes (dominant model) of the rs10499563, a SNP in the *IL-6* gene showed a trend towards statistical significance ($p=0.059$). This potentially suggests the role of genetics in shaping gut bacterial profiles.
- We previously reported the association of the rs10499563 SNP with susceptibility to CMV infection. Therefore, further, bigger studies could ascertain the contribution of each of the factors (rs10499563 SNP or CMV infection) in shaping gut bacterial profiles.

Gut microbiota profiles in pregnant women and its association with cytomegalovirus (CMV) infection

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Introduction: The human body is home to a diverse range of microorganisms collectively referred to as microbiota. Emerging data suggests that diverse microbial community is regulated by host genetic variation, environmental and dietary factors. Normal regulation of gut microbiota is disturbed by invading viruses causing dysbiosis which further influences virus infectivity. In the present article, we explore the possible effect of CMV infection on microbiota profiles of pregnant women. CMV infects gut epithelial cells, resulting in gut epithelial disjunctions, potentially causing leaky gut, gut dysbiosis and microbial translocation. In this study, we compared the gut bacterial profiles of CVM-infected and CVM-uninfected pregnant women. Comparisons of gut microbiota were also done taking into account host genetic variation in *TLR 2, 7* and *9* and *IL-6* that we have shown to affect susceptibility to CMV infection.

Methods: Using a case-control study, stools were collected from 13 CMV-infected (CMV+) and 26 CMV-uninfected (CMV-) age and HIV infection status-matched pregnant women. Microbiota were characterised using Illumina sequencing of the V4 hypervariable region of the bacterial16S rRNA gene and analysed using QIIME2. Differentially abundant bacterial taxa in women with and without CMV were identified using Linear Discriminant Analysis (LDA) effect size (LefSe).

Results: We reported on significant beta diversity ($p=0.001$, $q=0.01$) between CMV-infected and CMV-uninfected pregnant women. The gut bacterial profiles of cases had a differentially higher abundance of *P.copri* than controls ($p<0.05$, LDA score>2.0), while that of controls had a differentially higher abundance of genera *Lactobacillus* and *Roseburia* ($p<0.05$, LDA score>2.0). These differences remained significant after correcting for multiple comparisons ($q<0.05$). Genetic variants in genes known to affect susceptibility to CMV infection did not show any significant association with gut bacterial profiles. However, rs10499563 a SNP in the *IL-6* gene showed a trend towards significance on Beta diversity estimation but, did not reach statistical significance.

Discussion and Conclusion: Microbiota profiles of individuals signify their health status. *Lactobacillus and Roseburia,* genera are associated with improved gut epithelial integrity and reduced inflammation, respectively. The observed low abundance of these two genera in the CMV-infected group could explain the gut epithelia dysjunction and chronic inflammation associated with CMV infection. The chronic inflammatory nature of CMV is further explained by a differentially higher abundance of *Prevotella* in cases than in controls. In as much as CMV has been described to take advantage of compromised immunity, it may also play a role in immunomodulation through gut dysbiosis. However, the trend towards association between an immune-regulating SNP and beta diversity of gut bacterial profiles suggests that gut bacterial profiles may be determined or regulated at molecular level.

Introduction

The intestine is considered the largest immune organ in the human body and accounts for 80% of all cells that produce antibodies and consequently produces the most antibodies (40 mg/kg body weight/day). The composition of the human intestinal microbiota plays a critical role in determining the immunological, physiological and metabolic functions of the intestines (1). Alterations in gut microbiota profiles, particularly bacteria have been shown to play a role health and disease (2). Furthermore, certain disease conditions have been shown to result in gut bacterial dysbiosis. High throughput sequencing has made it possible to accurately describe structure and diversity of intestinal microbiota and its alteration in pathology.

Cytomegalovirus (CMV) is a herpesvirus that causes antenatal infection associated with disability, morbidity and mortality in CMV-exposed foetuses and neonates. Cytomegalovirus has been shown to replicate and persist in gut epithelial cells, resulting in disruption of tight junctions of intestinal cells (3). The disruption reduces transepithelial monolayer integrity thereby enhancing transepithelial permeability. The resulting permeability is characterised by translocation of gut microbial components and products into the systemic blood circulation, resulting in a deleterious inflammatory condition known as metabolic endotoxemia (4). However, it should also be noted that there is an equally strong argument supporting the thesis that increased susceptibility to infection, could be a result or a response to an already disturbed microbiome profile (5).

The role of HIV infection in microbial translocation is well documented. Support for an independent role of CMV on the gut comes from a cohort of CMV co-infected HIV patients where CMV was shown to independently result in disruption of the intestinal tight junctions (3). These findings were concluded upon observing that letermovir, an anti-CMV drug, dampened the effects of CMV on the gut epithelia. In addition, a paediatric longitudinal study reported that gut colonisation by *Staphylococcus* aureus was protective against early acquisition of CMV infection (6). These findings suggest interactions of CMV with gut microbiota.

Maternal gestational microbiota profiles have been shown to play a crucial role in shaping the infant's microbiota exposure. Microbiota exposure is believed to determine health and immune and developmental outcomes of the infant (7,8). To our knowledge, no studies have investigated the possible role of antenatal CMV infection on gut microbiota profiles. Therefore, the purpose of this study was to characterise gut microbiota profiles of CMV-infected and CMV-uninfected pregnant women. We also aimed to evaluate if CMV infection status and host genetics (*IL-6* - rs10499563, *TLR9* - rs352139, *TLR7* - rs179008, *TLR2* - rs1816702, which we previously reported to be associated with CMV infection status) play a role in shaping gut bacterial profiles.

Methods

Ethics approval

The present study received ethical approval from the Medical Research Council of Zimbabwe (MRCZ/A/2177) is a sub study of the University of Zimbabwe Birth Cohort. The study was also cleared by the Human Research Ethics Committee at the Faculty of Health Sciences, University of Cape Town (628/2017). All participants gave written informed consent for enrolment and participation in the study.

Study participants and specimen collection

This cross-sectional study was nested in University of Zimbabwe College of Health Sciences Birth Cohort that recruited pregnant women who were followed up till delivery. At delivery, infants of consenting mothers were also enrolled into the study and mother-baby pairs were followed up for two years. The general study design, setting and participants characteristics for the main cohort are described elsewhere (9). In summary, for this sub study, 39 women were enrolled and included 13 CMV-infected (CMV+) cases and 26 CMV-uninfected (CMV-), age and HIV status matched controls. Information on CMV and HIV status was available from the main cohort. Cytomegalovirus infection status of participants was determined using real time quantitative PCR while HIV status was available from patients' clinical records and confirmed at the time of sample collection, using the national rapid test algorithm. Participants enrolled for this study were between 22- and 40-weeks gestational age. Faecal specimens were collected from participants at enrolment using sterile spatulas and screw cap containers. Transportation of faecal specimens to the laboratory was done on ice. Specimens were stored at -80 °C until processing time.

Nucleic acid extraction and 16S rRNA gene amplicon library preparation

The first step towards DNA extraction was beat-beating mechanical lysis of the stool (50 mg) on the TissueLyser LT (Qiagen, FRITSCH GmbH, Idar-Oberstein, Germany). Subsequent total bacterial genomic DNA isolation from the lysed sample was done using the Zymobiomics DNA extraction kit (Zymo Research, Irvine, CA, USA), following manufacturer's instructions. Quantification of extracted

DNA was performed by spectrophotometry using Nanodrop ND 1000 (Thermo Fisher Scientific Inc., MA, USA). Empty manufacturer provided DNA extraction tubes and a Zymo mock community with eight known bacteria communities, and were included blanks and extraction controls, respectively, to check for possible contaminants. The Zymo standard was analysed for consistence in the whole pipeline from extraction to sequencing (see Figure S1). Extracted blanks spiked with a known concentration of *Mycobacterium smegmatis* were also included to control for sequencing as well as optimise the microbiota analyses. Zymo standard comprising of a synthetic mixture of genomic DNA from seven known bacteria, were used as positive controls to assess library preparation and sequencing artefacts.

Two polymerase chain reactions (PCRs) using primers targeting the V4 hypervariable region of the 16S rRNA gene were performed as previously described by Caporaso et al (10), with minor modifications. Two PCRs, rather than one, reduce the risk of non-specific binding when using adapters/sequencing primers of more than 100 bp. In the first PCR reaction, the V4 hypervariable region of the bacterial 16S rRNA gene was amplified using modified-F515 (5′ - GTGCCAGCHGCYGCGGT - 3′) and R806 (5′ - GGACTACNNGGGTWTCTAAT - 3′) primers. The reaction consisted of 12.5 µl of 2X MyTaq™ HS Mix (Bioline, MA, USA), forward and reverse primer (each at a10 µM initial concentration), 0.75 µl of dimethyl sulfoxide (catalogue no D2650, Sigma-Aldrich®, MO, USA) and 4 µl template, made up to a final volume of 25.25 µl using PCR-grade water (Thermo Fisher Scientific Inc., MA, USA). Cycling conditions included a denaturation step at 95 °C for 3 min, an amplification step at 95 °C for 30 sec, 50 °C for 30 sec and 72 °C for 1 sec (proceeding for 10 cycles); and a final extension step at 72 °C for 5 min. The expected fragment from the first PCR was approximately 375bp long. The second PCR used 4 µl of product from the first PCR as template. Composite primers F515-composite (5′ - AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNNNNNNNNGTGCCAGCHGCYGCGGT - 3′) and R806-composite (5′ - CAAGCAGAAGACGGCATACGAGATACGAGACTGATTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNNNNNNNNGGACTACNNGGGTWTCTAAT - 3′) containing adapters, barcodes and 12–15 staggered nucleotides (NNNNNNNNNNNN) were used in the second PCR to improve cluster identity and imaging in more diverse sample. Addition of barcodes was done to enable multiplexing of samples. The cycling conditions for the second PCR conditions were similar to those of the first, but with 30 cycles. The amplicon from the second PCR was approximately 400bp long.

Product amplicons from the two PCRs were cleaned using the Agencourt® AMPure® XP PCR Purification kit (Beckman Coulter, CA, USA). For both PCRs, the size of PCR products was verified and annotated on 1.5% ethidium bromide stained agarose gels, using 100 bp DNA ladder as the molecular weight marker. Amplicons were quantified using the Quanti-iT™ PicoGreen® dsDNA Reagent (Life Technologies, CA, USA) using the Infinite M1000 Pro® microplate reader (Tecan Group Ltd., Grödig, Austria) equipped with Tecan i-Control™ 1.7 software. Pooling of amplicons was done at

75 ng followed by quantification and purification of the library using the Nanodrop ND 1000 and Agencourt AMPure XP solution, respectively. The pooled 16S rRNA library was excised following agarose gel electrophoresis, and purified using the QIAquick Gel Extraction kit (QIAgen, MA, USA).

16S rRNA gene amplicon sequencing

The KAPA qPCR quantification kit (KAPA Biosystems, MA, USA), as well as the Agilent DNA 1000 kit (Agilent Technologies, CA, USA) were used to quantify and size the library. Using these metrics, the library was diluted to 4 nM using Buffer EB (Qiagen, Hilden, Germany), and denatured and neutralized using 0.2 N NaOH and hybridization buffer. A final library was prepared, diluted and spiked with 4pM of internal control (20% PhiX). Prepared library was paired-end sequenced using the MiSeq Reagent Kit v3, 600 cycles on the Illumina Miseq platform (Illumina, CA, USA) and run following manufacturer's instructions. In addition to *M.smegmatis* spiked controls, consistency of the whole procedure was determined by including duplicates and triplicates from randomly picked samples. Library preparation was carried out at the Division of Medical Microbiology, University of Cape Town and sequencing was done at Centrefor Proteomic and Genomic Research, Cape Town.

16S rRNA gene amplicon sequence data analyses

Raw sequence reads were analysed using quantitative insights into microbial ecology (QIIME2 v2019.4.0) (11). The demultiplexed raw reads were first subjected to quality-filtering, dereplication, denoising, chimera checking, and merging using QIIME2's built-in divisive amplicon denoising algorithm (DADA2) v1.10.0 (12) commands. In summary, the forward and reverse reads were both trimmed at position 5. In this order, the respective reads were then truncated at positions 235 and 190. Reads with maximum expected error (--p-max-ee) of 2.0 were discarded. Amplicon sequence variants (ASVs) were then inferred and assigned taxonomy using a pre-trained RDP Naïve Bayes Classifier (13) against the Greengenes (version 13.8) 16S rRNA database (14). The confidence for limiting taxonomy was set as 0.7. Further filtering was done to remove low-abundant ASVs (singletons and doubletons) and non-bacterial taxa, such as mitochondria, chloroplasts, archaea, and unassigned reads. Finally, an ASV table was constructed. Phylogeny of the ASVs was inferred from multiple sequence alignment of the ASVs using MAFFT v7.310 (15).

Dominance, Shannon, Simpson, and PD whole tree and Shannon equitability indices were computed for alpha diversity using an in-house R script run in the RStudio (16). Alpha diversity is the diversity within each sample in the two study groups, while beta diversity is comparison of diversity between samples. Beta diversity of the gut microbiota according to participants' metadata was computed using weighted and unweighted UniFrac distances, and Bray-Curtis dissimilarity metrics and similarities and dissimilarities of the data visualized using principal coordinate analysis (PCoA). All beta diversity analyses were computed at a subsampling depth of 5,768. Differences in beta diversity measures between participant metadata groups were tested using permutation multivariate analysis of variance

(PERMANOVA), with 999 permutations. The threshold for significance for both unadjusted and adjusted analyses were $p<0.05$ and $q<0.05$, respectively.

Statistical analysis

Comparison of relative abundance of microbial taxa and genera between CMV-infected and CMV-uninfected participants and identification of differentially abundant bacterial taxa between cases and controls was done using linear discriminant analysis (LDA) effect size (LefSe) v1.0 (17). Comparison was also done for HIV status and for the four immune-related SNPs that we have previously shown to be associated with susceptibility to CMV infection, using the dominant genetic model (i.e, for each SNP, two genotype groups were compared) using genetic data already reported (unpublished data). The p-value for LEfSe's non-parametric factorial Kruskal–Wallis sum-rank test was set at 0·05 while the threshold for differentially abundant bacterial taxa was set at a logarithmic LDA score threshold of 2.0.

STATA v15 (StataCorp, College Station, Texas, USA) was used to compare demographic characteristics between CMV-infected and CMV-uninfected participants. Mann-Whitney ranksum and t-test were used for non-parametric and parametric variables, respectively. Chi-squared test or Fisher's exact tests were used for categorical data. For all statistical tests, a value of $p<0.05$ was considered statistically significant.

Results

Study participants demographic characteristics

We characterized 16S rRNA gene sequences of faecal microbiota in 26 CMV+ cases and 13 CMV- controls. CMV+ and CMV- participants were HIV status and age matched, hence there was no significant difference in age and HIV status between the two groups. The demographic characteristics of the 39 study participants are summarised in Table 1. The age of participants ranged from 18 to 39 years. Gestational age, body mass index (BMI), gravidity and income were comparable between cases and controls since participants in the two groups were matched. Concentration of extracted gut bacterial DNA ranged from 3.6 - 36µg/ul, with a mean of 14.3µg/ul.

Table 1. Study participants demographic characteristics

Characteristic	**Combined (n=39)**	**CMV+ (n=13)**	**CMV- (n=26)**	**P-value (+ vs -)**
Mean age, years ± sd (range)	28 ± 6 (18–39)	27± 6 (18–39)	28± 6 (18–38)	0.60
Mean gestational age, weeks ± sd (range)	32 ± 4.2 (22–38)	33 ± 4.9 (20–40)	33±4.6 (22–40)	0.48
Median BMI (25^{th}–75^{th} percentile)	25 (24–29)	24 (24–26	27 (24–27)	0.07
Mean gravidity (25^{th}–75^{th} percentile)	2.6 ± 1.1 (1–5)	2.5 ± 1 (1–4)	2.8 ± 1.3 (1–5)	0.45
HIV infected n (%)	27 (69)	11 (85)	16 (62)	0.14
Mean income, USD/month (25^{th}–75^{th} percentile)	269 ± 124 (50–665)	267 ± 124 (50–665)	271±124 (72–500)	0.93

CMV+: CMV infected, CMV-: CMV uninfected, BMI: body mass index, HIV: human immunodeficiency virus.

Gut bacterial communities of participants

A total of 725,575 high-quality filtered reads were obtained from the 39 samples with an mean reads of 18,605 (range: 5,768-46,444). Using 3% dissimilarity as an indicator of an ASV (amplicon sequence variant) 1,789 ASVs were obtained which reduced to 159 after collapsing identical ASVs and removal of all spurious ASVs. Taxonomic classification revealed a typical human diversity profile in both groups (CMV+ and CMV-) at the phylum level with a total of 18 phyla which was dominated by Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes, in descending order. Figure 1 is a heatmap of the distribution of the 27 most abundant taxa according to HIV and CMV statuses of participants. The heatmap is based on average linkage hierarchical clustering of the Bray-Curtis dissimilarity matrix.

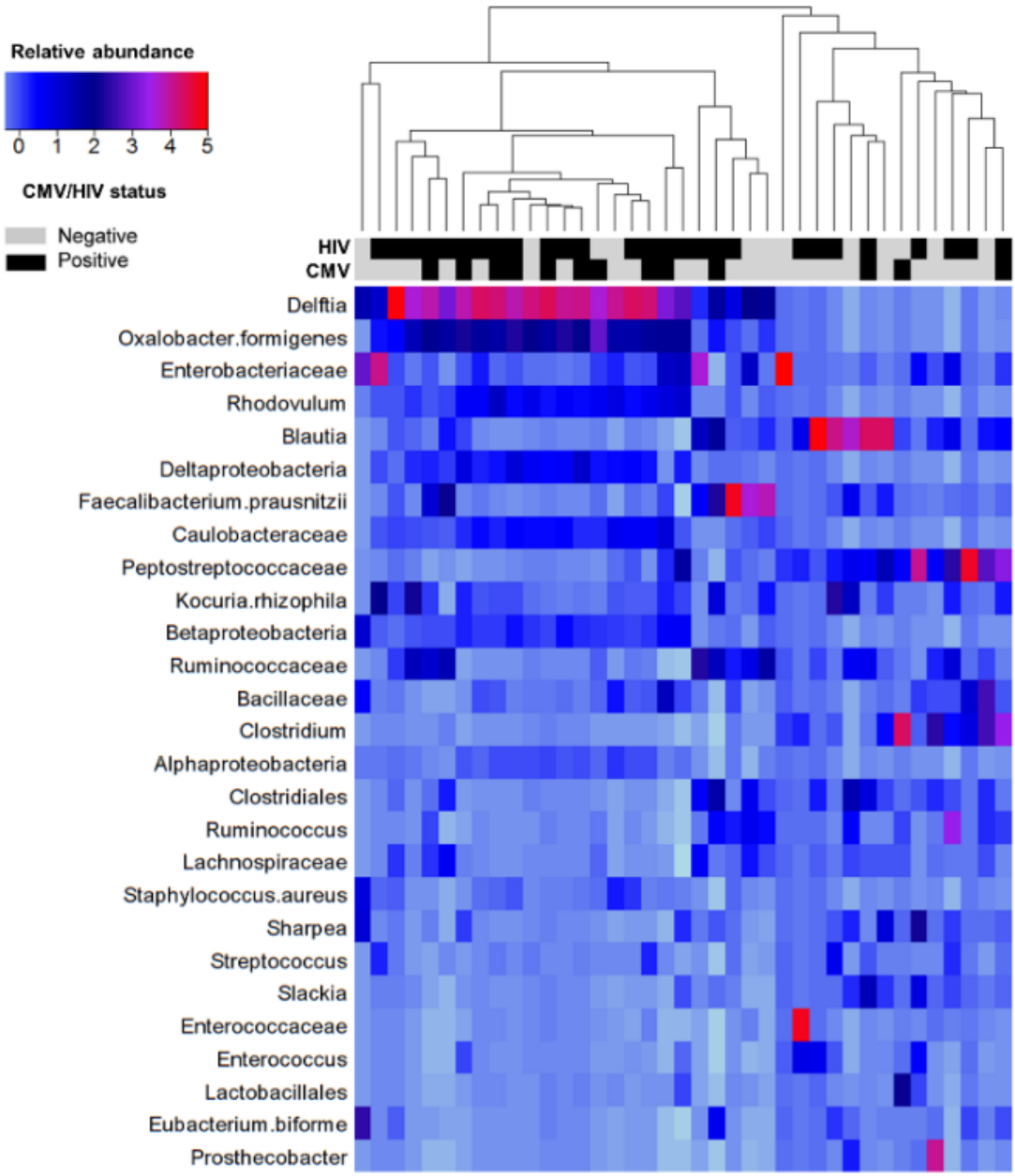

Fig. 1. Heatmap of the relative abundances of bacterial taxa in 39 gut microbiotas. Rows represent the bacterial taxa and columns the samples.

Richness and diversity analysis

The alpha diversity indices, including Simpson's, Dominance, Shannon equitability and Shannon index were calculated for each data set. The alpha diversity of stool samples of CMV-infected and -uninfected participants did not differ significantly using all four indices (Figure 2). Likewise, alpha diversity of participants' gut bacterial profiles did not differ according to any of the considered SNPs. All samples were included in determination of beta diversity and this was based on a 99.8% Good's coverage (Figure S2), this sampling depth was sufficient to estimate microbial diversity (18). To

evaluate the extent of similarity between the microbiota communities, beta diversity values were analysed based on UniFrac and Bray-Curtis distances. Principal coordinates analysis (PCoA) of the resultant beta diversity estimates were then visualized using EMPeror v3.1 (19) The plots of community structures of each faecal sample in each of the two study groups are shown in Figure 3. The PCoA results revealed that the overall microbial composition of CMV infected participants significantly deviated (p=0.001) from that of CMV uninfected participants. Hence, there was distinct clustering of samples from cases or controls. There was no significant difference on beta diversity estimation when considering all four SNPs: (rs10499563 - p=0.059, rs352139 - p=0.432, rs179008 - p= 0.097, rs1816702 - p=0.858). There was a trend towards significant beta diversity of gut bacterial profiles and the rs10499563 SNP on the *IL-6* gene. The PCoA for beta diversity estimation of gut bacterial profiles and rs10499563 is shown in Figure 4.

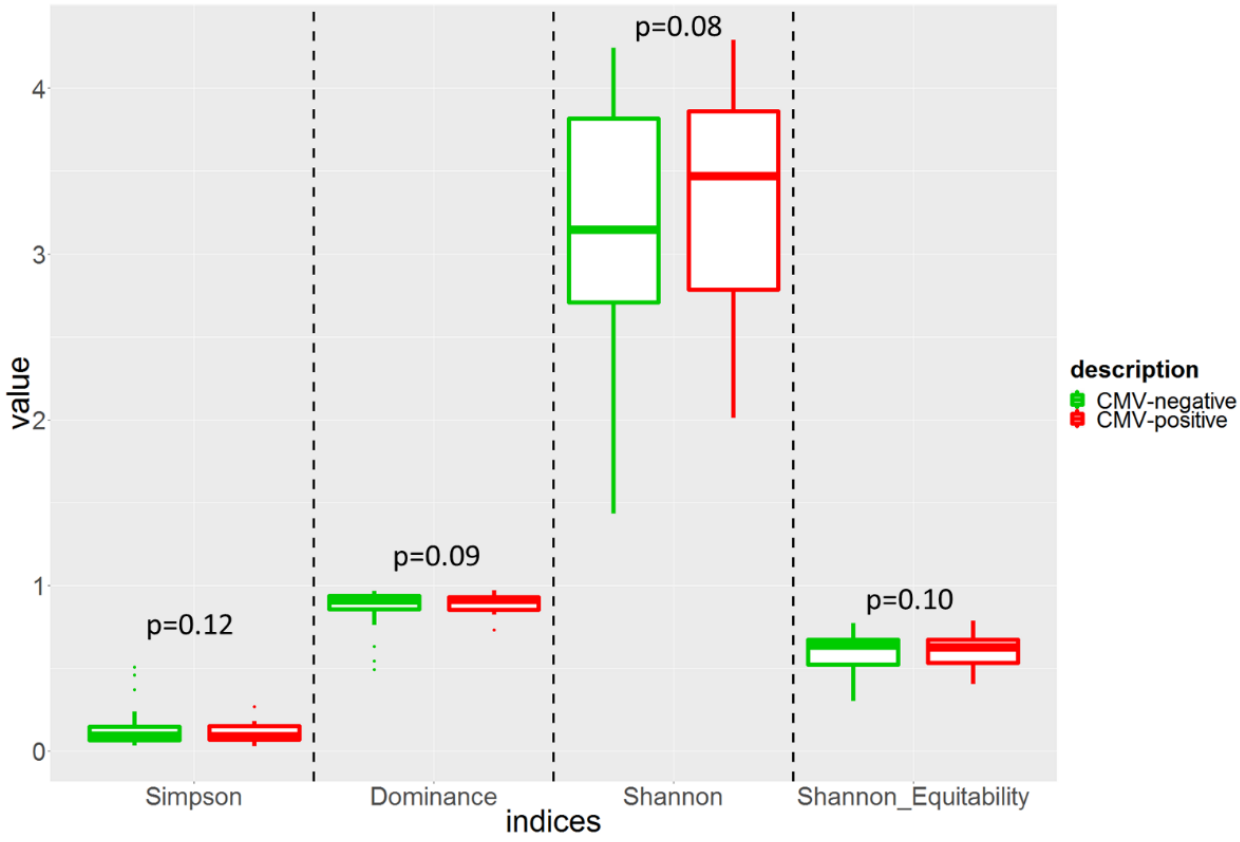

Fig. 2. Alpha diversity measures of gut microbiota grouped by CMV infection status. In each plot, the box ranges from the first to the third quartile, with the median represented by the horizontal line.

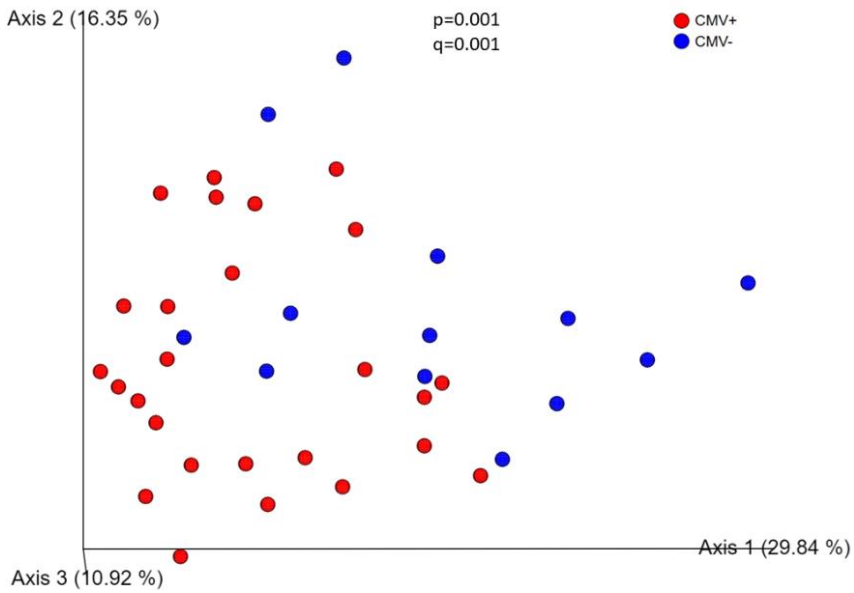

Fig. 3. Beta diversity measures of gut microbiota grouped by CMV infection status, based on weighted Unifrac distance. Key: CMV+=CMV infected, CMV-=CMV uninfected

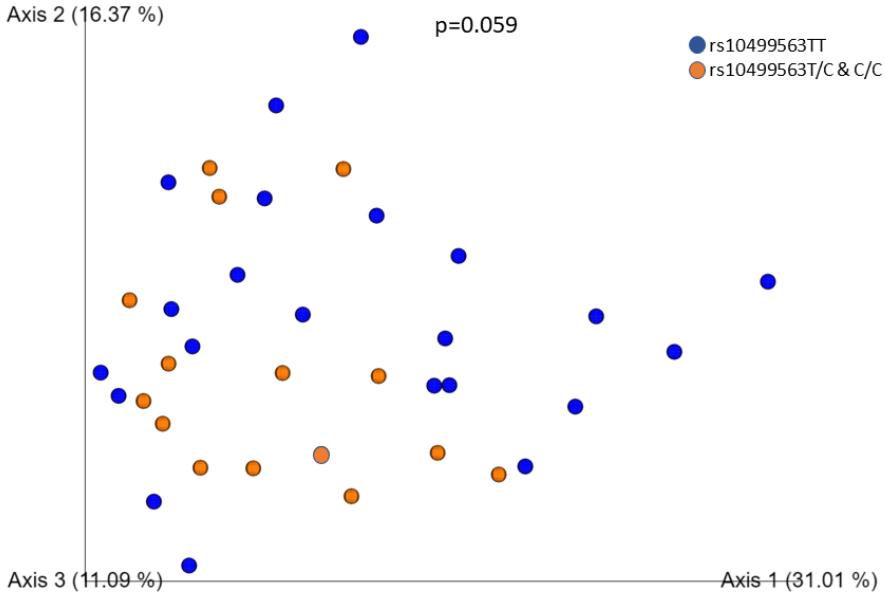

Fig. 3. Beta diversity measures of gut microbiota grouped by rs10499563 genotype (dominant model), based on weighted Unifrac distance.

Comparison of taxonomic composition according to CMV infection status

We searched for differentially abundant features (bacterial biomarkers) between CMV+ and CMV- participants using LefSe. LEfSe identified, eleven bacterial taxa were differentially abundant between CMV+ and CMV- participants (LDA score >2.0, p <0.05), Figure 5. There were no significant differences in relative abundances between the CMV+ and CMV- groups at phylum, order and class taxonomic levels. At family level, *Lactobacillaceae* was significantly less abundant in cases than controls (p<0.001, q=0.148, LDA score=6.6). *Prevotellaceae* and *Exiguobacteraceae* (p<0.001, q<0.05, LDA score=6.4 and p<0.001, q=0.174, LDA score=5.8, respectively) were significantly more abundant in CMV+ compared to CMV- participants. Genera *Prevotella, Exiguobacterium* and *Lactococcus* were identified as biomarkers for CMV infection. *Lactobacillus* and *Roseburia* were differentially less abundant in CMV+ participants.

The cladogram shown in Figure 4(a) represents the connection between the significantly different taxa at different taxonomic levels up to genus level. The significantly different taxa are shown in a tree like structure. Moreover, LDA score demonstrated that these differentially abundant taxa can be considered as potential biomarkers (LDA score > 4.0, ρ < 0.05). The histogram in Figure 4(b) show the individual differentially abundant taxa as well as their respective LDA scores. On correcting for multiple comparisons using a cut-off of q<0.05, genus *Roseburia* and *L.reuteri* remained differentially less abundant in cases than in controls. Using the same q-value cut off, *P.copri* remained differentially more abundant in cases than in controls.

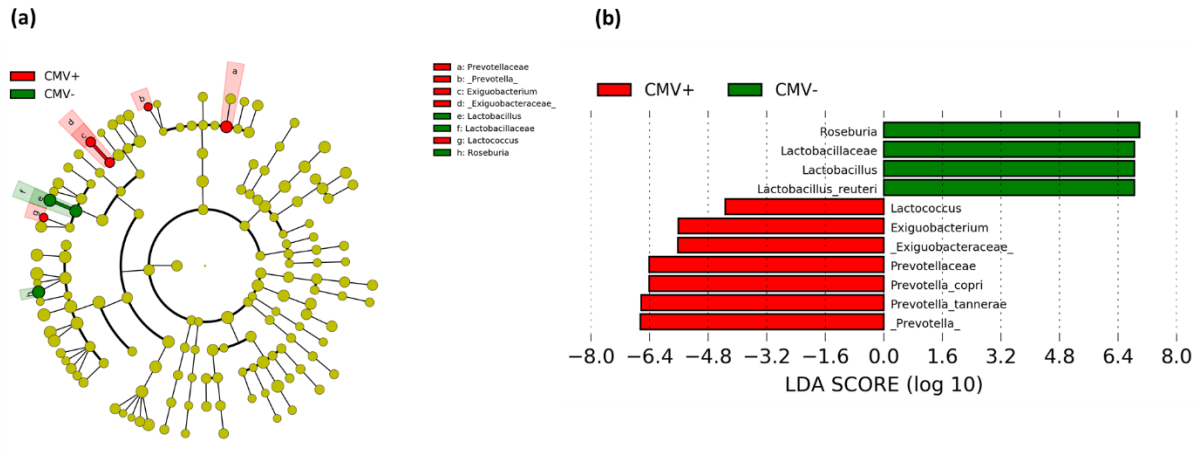

Fig. 5. (a) Cladogram showing differentially abundant taxonomic clades with an LDA score> 2.0 (p<0.05) between CMV+ and CMV- participants. **(b)** Histogram of linear discriminative analysis (LDA) effect size (LEfSe) analysis showing differentially abundant taxa between cases and controls.

Discussion

Gut microbiome plays a key role in health and disease, metabolism and permeability of the gut mucosa to metabolically active molecules. In addition, gut microbiome profiles shape the immunological environment of the gut, hence indirectly protects against pathogenic infections and influences immune responses (20,21). The aim of the present study was to determine the possible effects of CMV infection on gut bacterial profiles by comparing gut microbiome profiles of CMV-infected with -uninfected pregnant women. Determination of gut microbiome profiles in pregnancy is crucial as it has been found to play a crucial role in determining birth outcomes (22). A secondary objective was to evaluate the association between host genetic variation that affects immune response genes and microbiome profiles.

Pregnancy, particularly the differential hormone production and metabolism alters gut bacterial profiles with effects being observed in late gestation (7,8). An increase of gut bacteria associated with inflammatory and obese states, denoted by higher levels of Firmicutes, is usually observed in late gestation (23). As all participants studied here were in late gestation, differences observed with respect to bacteria profiles are highly likely not due to gestational age. Furthermore, our two groups were matched for demographic characteristics such as HIV status, age, gestational age, and income. Therefore, these factors previously identified as shaping gut bacterial profiles are not confounders in the present study.

The two major phyla in our study participants (Firmicutes and Proteobacteria) were representative of what has been previously reported in healthy populations. Proteobacteria have been reported to be more abundant in developing world populations, such as the cohort in the present study, when compared with populations from the developed world (24). There was a trend towards higher alpha diversity in gut bacterial profiles of CMV+ compared with CMV- participants. Although a greater gut microbial diversity has been widely reported to be beneficial, it has also been associated with disease states such as depression and autism (25). Greater gut microbiome diversity was reported in formula-fed babies versus breast-fed babies (26,27). Breast-fed babies were however reported to have superior neurodevelopment and higher intelligence test scores when compared with their formula-fed counterparts (28). These studies emphasise on the segregational outcomes of higher microbial diversity, depending on which bacteria are present and their possible metagenomic function. On beta diversity estimation, cases and controls significantly differentially clustered, suggesting that CMV infection potentially results in different gut bacterial profiles. The trend towards significance between beta diversity and rs10499563 SNP suggests a role of the immune-related gene in shaping gut bacterial profiles.

The presence of any possible biomarkers between cases and controls was subsequently investigated. From LEFSe analysis, it was observed that there were distinct bacterial taxa which were differentially

abundant between cases and controls. Genera *Lactobacillus* and *Roseburia* were significantly less abundant in CMV-infected than -uninfected participants. *Lactobacillus* regulates tight junction proteins for maintenance and protection against chemical-induced disruption of the epithelial barrier (29). It is for this reason the *Lactobacillus* forms a major component of commercial probiotics (30). In addition, *Lactobacillus* species produce short chain fatty acids (SCFAs), which play a role in maintaining host homeostasis and disease progression. Lactobacilli produce acetate and butyrate, which together with propionate, constitute ≥95% of all SCFAs (31). SCFA also participate in gluconeogenesis and lipid biosynthesis, thereby modulating various biological responses of host that include inflammation and oxidative stress.

Low relative abundance of *Lactobacillus* has also been associated with inflammatory conditions such as ulcerative colitis and Crohn's disease (known as inflammatory bowel disease, IBD). IBD is a classic example of gut microbiome dysbiosis condition (32). *Roseburia*, a genus which was also significantly less abundant in the CMV+ compared to CMV- in the present study produces butyrate, maintains immune function of the gut and regulates inflammation (33,34). A recent case control study reported a lower abundance of *Roseburia* in ulcerative colitis cases when compared with non- ulcerative colitis controls (35). Furthermore, higher abundance of *Roseburia* in the gut was associated with increased and T-cell differentiation and reduced inflammation by inhibiting excretion of interleukin-17 (36).

Interestingly, CMV is known as a chronic inflammation condition. We hypothesise that CMV infection may be characterised by the low abundance of *Lactobacillus* and *Roseburia*, further weakening the gut epithelia tight junctions and promoting inflammation, respectively. It is also important to note that CMV has been implicated in recurrent spontaneous abortion. In addition to bacterial and toxin leakage induced inflammation, the uncontrolled inflammation at the gut interface may lead to endometrial inflammation, resulting in spontaneous abortion. The haematogenous spread of microbes from a leaky gut to the placenta or uterus suggested by Nuriel-Ohayon et al (37), could result in endometrial inflammation, hence CMV-associated adverse effects on the pregnancy and developing foetus. CMV-induced gut leakage and dysbiosis during pregnancy could potentially have detrimental effects on the developing foetus mainly via the gut-brain axis (22).

Previous studies have also suggested and indicated the contribution of low abundance of SFCA-producing gut bacterial profiles with low vaccine success (38–40). The population-wide low success rate can be attributed to insufficient bacterial products to induce an adequate antibody response to the vaccine. Control of CMV has been greatly hampered by the low success rate of the developed vaccine. Addition of probiotics containing organisms such as *Lactobacillus* and *Roseburia* could ameliorate outcomes of CMV vaccination trials.

The differentially higher relative abundance of *P. copri* in cases than controls is in keep with previous studies that associated *Prevotella* abundance with boosted cytokine production and chronic

inflammatory conditions (41). A study carried out in HIV-infected participants reported an association between higher abundance of *P. copri* with faster disease progression and higher markers of inflammation (42). CMV is one such condition. However, *Prevotella* can be either beneficial or detrimental, depending on circumstances as abundance of *Prevotella* has also been associated with intake of a healthier, plant-based diet (43). Our findings suggest that CMV infection could potentially result in gut bacterial dybiosis.

Our findings also suggest a role of genetics in shaping gut bacterial profiles. *IL-6* gene codes for the pro-inflammatory cytokine which is associated with increased gut epithelial disjunction (44). Presence of the SNP results in reduced production of IL-6, hence the potential clustering of participants gut bacterial profiles of participants carrying different genotypes (45). We recommend that bigger prospective and functional studies be carried out to elaborate on these relationships and to ascertain the role of each factor in shaping gut bacterial profiles.

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Supplementary figures

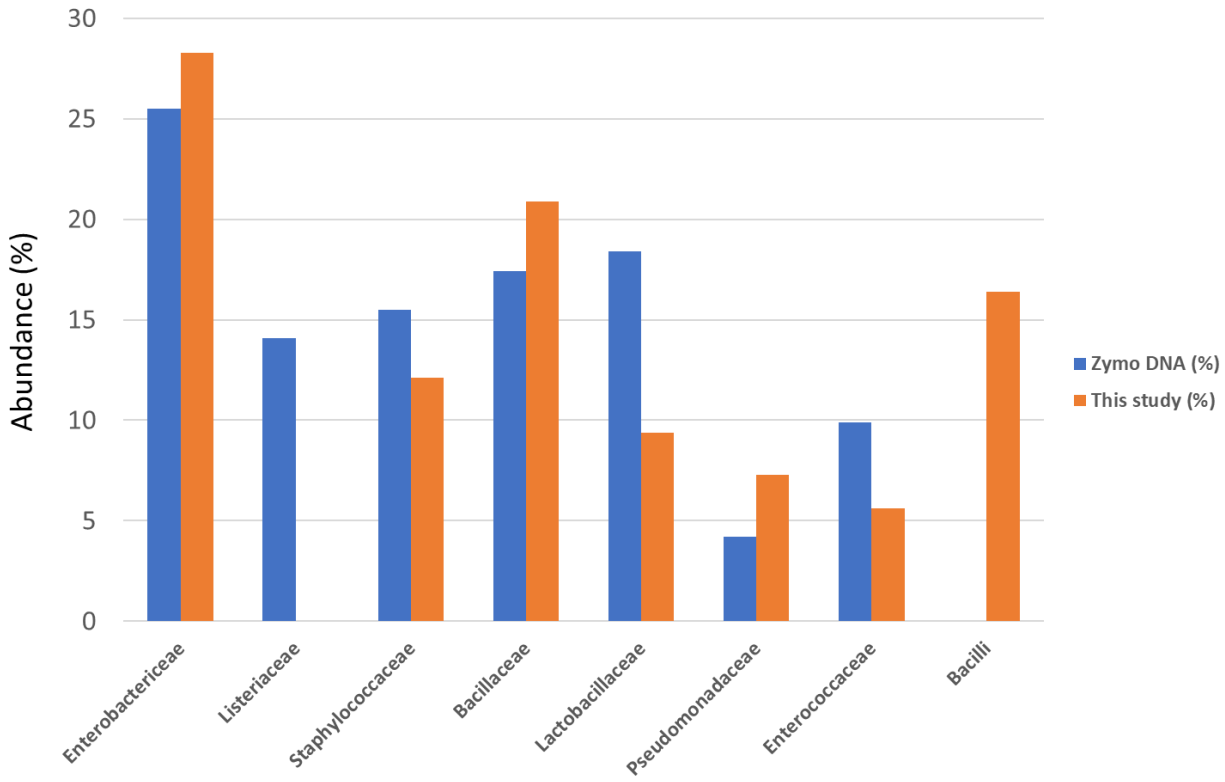

Fig. S1. Bar chart illustrating expected bacterial percentage in the Zymo commercial standard alongside the yield from this study's workflow

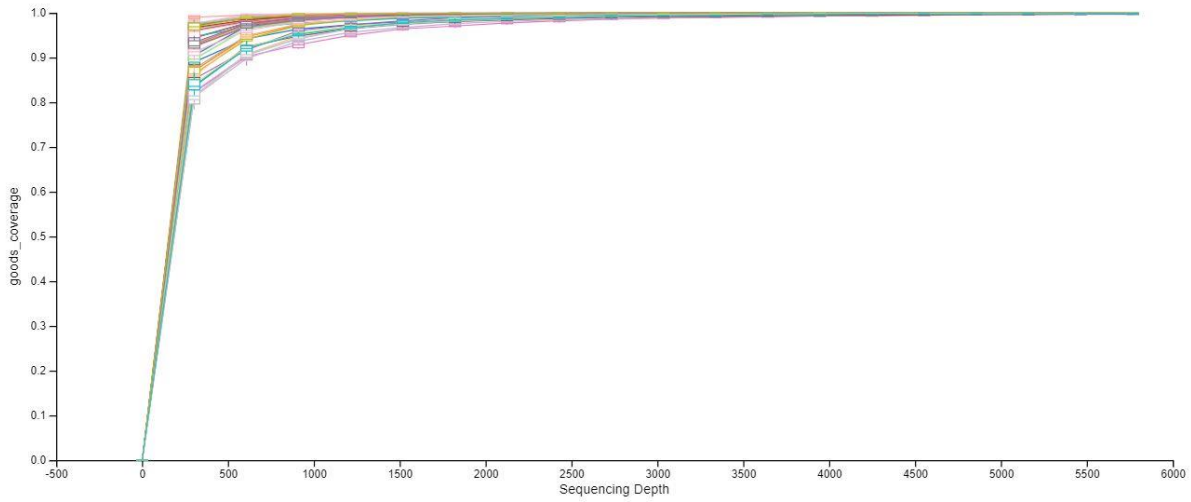

Fig. S2. Rarefaction curves depicting Goods' coverage gut bacterial sequences for all study participants

4 Chapter 4: Summary Discussion and Conclusion

4.1 Summary discussion

Antenatal CMV infection has resulted in a global burden of cCMV. The prevalence of CMV infection is higher and almost ubiquitous in the developing world (60-100%) than the developed world (0-60%), respectively (Adland, Klenerman, Goulder, & Matthews, 2015; Antona et al., 2017; Lantos, Permar, Hoffman, & Swamy, 2015). It has been suggested that the likelihood of vertical transmission of CMV is greater in the setting of primary infection than reinfection or reactivation (Mussi-Pinhata et al., 2018; Wang, Zhang, Bialek, & Cannon, 2011). However, due to cumulative effect, the prevalence of cCMV is ten times greater in the developing (3%) world when compared to the developed world (0.3%), respectively (Kenneson & Cannon, 2007; Manicklal, Emery, Lazzarotto, Boppana, & Gupta, 2013; Mussi-Pinhata et al., 2009).

Factors such as host genetic variation, socio-economic status and immune response profiles have been suggested and shown to influence susceptibility to maternal CMV infection. The factors that predispose to either antenatal CMV primary infection or infection seem to differ inter-individually and at population level (Lanzieri, Dollard, Bialek, & Grosse, 2014; Lawrence et al., 2017). Despite a higher burden of both maternal and congenital CMV infection in the developing world, particularly Africa, there is a knowledge gap on factors that contribute to the differential outcomes of CMV exposure ie. whether exposure results in CMV infection or not. This thesis aimed to determine the prevalence of CMV in a cohort of pregnant women and to investigate the role or contribution of ART exposure and host genetics to susceptibility of CMV infection.

The present study is the first to report on the prevalence of CMV infection among pregnant women in Zimbabwe and the high seroprevalence of anti-CMV IgG antibodies is further confirmation of the ubiquitous nature of CMV exposure in a low-income setting. However, despite the high CMV exposure, not all participants had active CMV infection during pregnancy or at the time of sample collection. Although immune status has been suggested to play a role in susceptibility to CMV infection (Hanley & Bollard, 2014), HIV infection status was not associated with CMV serostatus in this study. Presence of CMV DNA in plasma was also determined as a marker of active CMV infection. There was a discrepancy in the results of CMV infection status of participants using the two methods. Considering CMV DNA, participants who were HIV infected were more likely to be CMV infected compared to HIV uninfected participants. These findings confirm the inferiority of detection of anti-CMV antibodies as a diagnostic method for active CMV infection, as previously reported (S. A. Ross, Novak, Pati, & Boppana, 2011; Shannon A. Ross et al., 2014). The underestimation of HIV infected individuals who were CMV seropositive could the due to these participants being so immune compromised that they are unable to mount a sufficient anti-IgM response to be picked by the immunoassay. However, the risk of detecting latent CMD DNA by PCR, resulting in false positives is also possible (Abedi et al., 2017). It would therefore be ideal to use both methods and rigorously rule out any potential CMV infected cases. PCR technology is however not readily accessible for routine diagnosis use especially in the developing world. As a result, the bottleneck in identifying antenatal CMV infection and curb potential vertical transmission of CMV will unlikely be resolved.

Antiretroviral therapy has resulted in immune restoration, thereby significantly reducing the occurrence and episodes of opportunistic infections in people living with HIV and AIDS (Wilson & Sereti, 2013). The success of ART is highly dependent on maintenance of

optimum therapy to suppress viral replication. The optimum plasma concentrations of ART which are determined by rate of drug metabolism and excretion and can be approximated by measuring plasma concentration of the drug. Drug metabolism and excretion outcomes are also largely determined at molecular level, in the genes which encode the drug metabolising enzymes (Prakash & Agrawal, 2016). The therapeutic range of EFV has been established, aiding to therapeutic drug monitoring of ART. Previous studies have shown that ART resulted in a decrease in the occurrence of CMV retinitis among HIV infected patients. Moreover, patients on CMV retinitis maintenance dose could safely discontinue anti-CMV therapy without any rebound (Jouan et al., 2001). Antiretroviral therapy results in immune reconstitution in people living with HIV and AIDS, thereby protecting them from opportunistic infections. Having observed that some but not all HIV infected participants were CMV infected, we set out to determine the role of ART exposure in protecting pregnant women against CMV as an opportunistic infection. Individuals carrying the CYP2B6 c.516G/G and c.983T/T expectedly had low exposure of plasma EFV and were at a greater risk of antenatal CMV infection. Conversely, slow EFV metabolisers with respect to CYP2B6 genotypes (c.516T/T and c.983T/C) had higher plasma EFV exposure and significantly low likelihood of being CMV infected.

All participants were on the standard 600mg/once daily EFV dose. Findings from previous studies have inspired policy change on universal reduction of EFV dosing from 600mg to 400mg/once daily dose on all participants regardless of genotype. However, from our study findings we have shown that some women still failed to achieve therapeutic EFV concentration despite being on the 600mg/once daily dose. This could be due to pregnancy-induced metabolic aberrations which also impact on drug disposition (Perucca, Ruprah, & Richens, 1981). As a result, caution has to be taken when prescribing ART for special populations such as pregnant women. Emergence of opportunistic infections such as CMV

during pregnancy can have detrimental effects on the developing foetus and overall birth outcomes. Overall, genotype-based could be an additional tool in dosing, so that we move away from the current practice of "one size does not fit all".

In addition to HIV status which is a determinant of immune functions and plays a significant role in susceptibility to CMV, immunity is also regulated at the molecular level. Variation in genes that encode proteins involved in immune pathways against CMV may partly explain the differential outcomes of CMV exposure regardless HIV infection status. Single nucleotide polymorphisms in toll-like receptors, TLR2 (rs1816702), TLR7 (rs179008) and TLR9 (rs352139) and IL-6 (rs10499563T) genes were associated with differential risks of acquiring CMV infection during pregnancy. The presence of a SNP in the coding region of a gene may result in the coding of a protein that is structurally different from the normally produced protein, which could be the case with observations for TLR7 in this study. When a SNP occurs in the non-coding region of a gene the effect is usually on the rate or quantity of protein produced, which could be the case with observations for IL6, TLR2 and TLR9, in this study. In either case, the ultimate function of the protein will be affected as either heightened or diminished. In the case of CMV associated immune proteins, if the SNP results in a higher protein production or a protein with greater activity, immunity against CMV will also be heightened. Conversely, decreased protein production and coding of less potent proteins will result in reduced immunity against CMV and a greater risk of CMV reactivation and reinfection.

Due to the wide host cell tropism of CMV which includes gut epithelial cells, the CMV-induced disruption of gut epithelial tight junctions is likely to result in gut dysbiosis (Maidji, Somsouk, Rivera, Hunt, & Stoddart, 2017). In this study we report significantly lower differential abundance of *Lactobacillus reuteri* and *Roseburia* in CMV infected cases when compared with CMV uninfected controls. *Lactobacillus* species have been associated

with maintaining integrity of the gut epithelial cells and overall health of the gut (Yu, Yuan, Deng, & Yang, 2015). For this reason, most of the commercial probiotics contain Lactobacilli species. The low differential abundance of *Lactobacillus* in CMV infected participants suggests deranged gut health in CMV infection. Low abundance of genus *Roseburia* has been associated with chronic inflammation which is also characteristic of CMV infection.

The observed higher differential abundance of genera *Prevotella*, *Lactococcus* and *Exiguobacterium* in cases than in controls further confirms *Prevotella* association with increased cytokine release which leads to increased inflammation (Dillon et al., 2016). This observation further emphasises the role of gut bacterial profiles in the characteristic nature of CMV infection. High relative abundance of *Exiguobacterium* in the gut has been associated with community acquired pneumonia (Chen et al., 2017). Interestingly, CMV has also been implicated in severe viral community acquired pneumonia (Gonçalves et al., 2018). This suggests the possible interaction of gut bacterial profiles with disease outcomes and associated symptoms. Gut bacterial profile also been described to play a role in vaccination outcomes, with low relative abundance of short chain fatty acids producing bacteria such as *Lactobacillus* and *Roseburia* being associated with low vaccine success rates (Jamieson, 2015; Zimmermann & Curtis, 2018).

The observation of a trend towards significance ($p=0.059$) on estimating beta diversity of different genotypes of the rs10499563, a SNP in the *IL-6* gene suggests the role of genetics in shaping gut bacterial profiles. Presence of the SNP results in lower production of the pro-inflammatory cytokine IL-6 (Smith et al., 2008). It is important to note that inflammation through cytokine production plays an important role in maintaining immune balance (Zhang et al., 2008). At the same time, gut microbiota also plays a role in maintaining immunity. It

is possible that there is an interplay between host immuno-genetic variants and gut microbiota profiles.

4.2 Conclusions and Future Perspectives

Routine diagnosis mainly performed by ELISA, particularly in the developing world where there is a high HIV burden may not be reliable and results in misdiagnosis and underestimation of CMV infection cases. Hence, control of antenatal CMV and prevention of cCMV will remain a challenge. Findings from this study suggests that the differential outcomes of CMV exposure are multifactorial and hence factors such as host genetics and concentration of ART have to be taken into account to identify individuals at a higher risk of CMV infection. The multifactorial nature of CMV acquisition in addition to wide host cell tropism, presents a huge challenge to both the clinician and the drug developer in curbing CMV infection. Vaccination against CMV has been largely unsuccessful. Considering the potential role of gut bacterial profiles in CMV vaccination and disease outcomes, boosting gut bacterial profiles by SCFA producing bacteria may ameliorate vaccination outcomes.

In as much as CMV has been described to take advantage of compromised immunity, data from our study findings, particularly CMV-induced gut dysbiosis suggest that CMV may actually contribute to immune dysfunction through the gut associated lymphoid tissue. The likelihood of immune associated host genetics in shaping gut bacterial profiles warrants further investigation.

In conclusion, there are various factors associated with susceptibility to CMV infection which include host genetics and antiretroviral therapy exposure among HIV infected pregnant women. Furthermore, CMV infection has an effect on gut bacterial profiles which may result in characteristic CMV infection symptoms such as chronic inflammation.

The future direction of the current study is to perform CMV testing as well as growth and developmental tests on the infants so as to determine the rate of vertical CMV transmission and associated birth outcomes in our cohort. Moreover, there is need to assess the possible effects of maternal CMV-induced gut dysbiosis on birth outcomes.

Limitations of study

We were only able to determine the CMV infection status during pregnancy hence were not able to tell status before onset of pregnancy. This information would have allowed us to better explain the relationship between CMV reactivation and the physiological changes which occur during pregnancy. However, we were still able to associate CMV status during pregnancy with factors such as host genetics and gut bacterial profiles. Africans being the cradle of mankind, there could have been a possibility to find novel SNPs associated with CMV infection if we had used methods such as whole exome sequencing. However, we did not have sufficient funds in our project. Availability of stool samples at different time points would have allowed conclusions on the causative effects of CMV infection on gut bacterial profiles. In this case, findings can only allow us to associate CMV infection status with gut bacterial profiles. Furthermore, stool samples at different time points including a point before CMV reactivation would have given a clearer relationship between gut bacterial profiles and CMV infection.

4.3 References (Introduction and Summary Discussion chapters)

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Appendix A: Permission to include full publications in thesis

Permission to Include Publications in PhD Thesis: Doreen Zvipo Mhandire MHNDOR001

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Appendix B: Ethical clearance from the University of Cape Town Human Research Ethics Committee

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492
Email: sumayah.ariefdien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

04 September 2017

HREC REF: 628/2017

Prof C Dandara
Division of Human Genetics
Wernor Belt North N3.14.4
IDM-FHS

Dear Prof Dandara

PROJECT TITLE: INVESTIGATING THE ROLE OF HIV/AIDS STATUS AND HOST GENETIC SUSCEPTIBILITY ON THE VERTICAL TRANSMISSION OF CYTOMEGALOVIRUS IN MOTHER-INFANT PAIRS- LINKED TO 103/2009 (PHD CANDIDATE- MS D MHANDIRE)

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

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

Approval is granted for one year until the 30 September 2018.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the student: Doreen Mhandire will also be involved in this study.

Please quote the HREC REF in all your correspondence.

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

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

Yours sincerely

Signature removed avoid exposure online

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

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

HREC 628/2017

Appendix C: Ethical clearance from the Medical Research Council of Zimbabwe

Telephone: 791792/791193
Telefax: (263) - 4 – 790715
E-mail: mrcz@mrcz.org.zw
Website: http://www.mrcz.org.zw

Medical Research Council of Zimbabwe
Josiah Tongogara / Mazoe Street
P. O. Box CY 573
Causeway
Harare

APPROVAL

RESEARCH COUNCIL OF ZIMBABWE
CABINET OFFICE
15 NOV 2017
RECORDS AND INFORMATION OFFICE
P.O. BOX CY 294
CAUSEWAY HARARE

REF: MRCZ/A/2177

6 June, 2017

Doreen Mhandire
University of Zimbabwe - CHS
P.O Box A178
Avondale
Harare

RE:-Role of HIV/AIDs Status and Host Genetic Susceptibility In Vertical Transmission Of Cytomegalovirus in Mother-Infant Pairs

Thank you for the application for review of Research Activity that you submitted to the Medical Research Council of Zimbabwe (MRCZ). Please be advised that the Medical Research Council of Zimbabwe has **reviewed** and **approved** your application to conduct the above titled study.

This approval is based on the review and approval of the following documents that were submitted to MRCZ for review:-

1. Study Protocol

- **APPROVAL NUMBER** : **MRCZ/A/2177**

This number should be used on all correspondence, consent forms and documents as appropriate.

- **TYPE OF MEETING** : **Expedited review**
- **EFFECTIVE APPROVAL DATE** : **6 June, 2017**
- **EXPIRATION DATE:-** : **5 June, 2018**

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After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the MRCZ Offices should be submitted three months before the expiration date for continuing review.

- **SERIOUS ADVERSE EVENT REPORTING**: All serious problems having to do with subject safety must be reported to the Institutional Ethical Review Committee (IERC) as well as the MRCZ within 3 working days using standard forms obtainable from the MRCZ Offices or website.
- **MODIFICATIONS**: Prior MRCZ and IERC approval using standard forms obtainable from the MRCZ Offices is required before implementing any changes in the Protocol (including changes in the consent documents).
- **TERMINATION OF STUDY**: On termination of a study, a report has to be submitted to the MRCZ using standard forms obtainable from the MRCZ Offices or website.
- **QUESTIONS**: Please contact the MRCZ on Telephone No. (04) 791792, 791193 or by e-mail on mrcz@mrcz.org.zw

Other

- Please be reminded to send in copies of your research results for our records as well as for Health Research Database.
- You're also encouraged to submit electronic copies of your publications in peer-reviewed journals that may emanate from this study.

Yours Faithfully Signature removed

MEDICAL RESEARCH COUNCIL OF ZIMBABWE
2017 -06- 06
APPROVED
P.O. BOX CY 573 CAUSEWAY HARARE

.....
MRCZ SECRETARIAT
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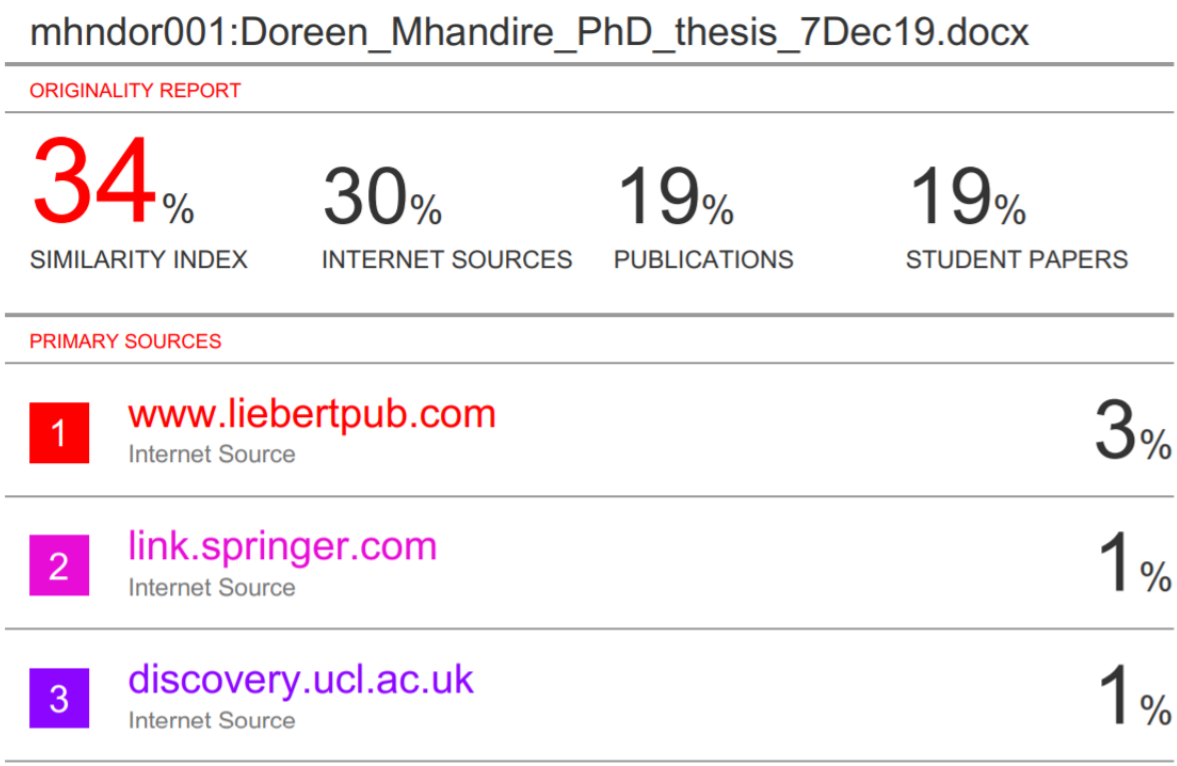
Appendix D: Turnitin plagiarism report



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All PhD candidate's publications were included in the thesis without alterations

Appendix E: Additional table for section 3.3.1

Additional Table 1 Genotype frequencies and univariate logistic regression of SNP with CMV infection status

SNP	Genotype	overall	CMV+ n (%)	CMV- n (%)	p-value	Codominant OR (95%CI)	p-value	Log additive OR	p-value
TLR2 rs4696480	T/T	50 (45.4)	14 (38.9)	36 (48.6)	0.295	1.00	0.315		
	T/A	53 (48.2)	18 (50.0)	35 (47.3)		1.32 (0.57-3.06)			
	A/A	7 (6.4)	4 (11.1)	3 (4.0)		3.43 (0.68-17.31)		1.58 (0.82-3.06)	0.172
TLR2 rs3804099	C/C	54 (49.1)	18 (50.0)	36 (48.6)	0.688	1.00	0.658		
	C/T	50 (45.4)	17 (47.2)	33 (44.6)		1.03 (0.46-2.33)			
	T/T	6 (5.4)	1 (2.8)	5 (6.8)		0.40 (0.04-3.68)		0.86 (0.44-1.69)	0.659
TLR2 rs1816702	C/C	25 (22.7)	17 (47.2)	8 (10.8)	<0.001	1.00	<0.001		
	C/T	64 (58.2)	15 (41.7)	49 (66.2)		0.14 (0.05-0.40)			
	T/T	21 (19.1)	4 (11.1)	17 (23.0)		0.11 (0.03-0.44)		0.27 (0.13-0.57)	<0.001
TLR4 rs1554973	C/C	72 (65.4)	25 (69.4)	47 (63.5)	0.568	1.00	0.564		
	C/T	31 (28.2)	8 (22.2)	23 (31.1)		0.65 (0.26-1.67)			
	T/T	7 (6.4)	3 (8.3)	4 (5.4)		1.41 (0.29-6.80)		0.92 (0.47-1.79)	0.807
TLR4 rs6478317	G/G	82 (74.5)	27 (75.0)	55 (74.3)	0.853	1.00	0.860		
	G/A	26 (23.6)	8 (22.2)	18 (24.3)		0.91 (0.35-2.34)			
	A/A	2 (1.8)	1 (2.8)	1 (1.3)		2.04 (0.12-33.83)		1.03 (0.46-2.34)	0.939
TLR4 rs10759932	T/T	69 (62.7)	22 (61.1)	47 (63.5)	0.740	1.00	0.888		
	T/C	40 (36.4)	14 (38.9)	26 (35.1)		1.15 (0.50-2.62)			
	C/C	1 (0.9)	0	1 (1.3)		-		1.04 (0.47-2.29)	0.888
TLR4 rs7856729	G/G	41 (37.3)	15 (41.7)	26 (35.1)	0.633	1.00	0.632		
	G/T	53 (48.2)	15 (41.7)	38 (51.3)		0.68 (0.29-1.64)			
	T/T	16 (14.5)	6 (16.7)	10 (13.5)		1.04 (0.31-3.44)		0.93 (0.52-1.67)	0.807
TLR7 rs179008	A/A	71 (64.5)	17 (47.2)	54 (73.0)	<0.001	1.00	<0.001		
	A/T	26 (23.6)	8 (22.2)	18 (24.3)		1.41 (0.52-3.82)			
	T/T	13 (11.8)	11 (30.6)	2 (2.7)		17.47 (3.52-86.72)		2.97 (1.63-5.43)	<0.001

	HWE (p-value)	0.0009	0.001	0.652					
TLR9 rs352139	T/T	34 (32.1)	5 (13.9)	29 (41.4)	0.007	1.00	0.005		
	T/C	54 (50.9)	21 (58.3)	33 (47.1)		3.69 (1.23-11.04)			
	C/C	18 (17.0)	10 (27.8)	8 (11.4)		7.25 (1.92-27.37)		2.70 (1.41-5.17)	0.001
TLR9 rs5743836	A/A	43 (40.6)	13 (39.4)	30 (41.1)	0.830	1.00	0.835		
	A/G	50 (47.2)	15 (45.5)	35 (47.9)		0.99 (0.41-2.40)			
	G/G	13 (12.3)	5 (15.1)	8 (11.0)		1.44 (0.40-5.26)		1.14 (0.62-2.10)	0.675
TLR9 rs187084	A/A	54 (49.5)	18 (50.0)	36 (49.3)	0.638	1.00	0.616		
	A/G	45 (41.3)	16 (44.4)	29 (39.7)		1.10 (0.48-2.54)			
	G/G	10 (9.2)	2 (5.6)	8 (11.0)		0.50 (0.10-2.60)		0.86 (0.46-1.61)	0.645
TLR9 rs352140	C/C	48 (43.6)	17 (47.2)	31 (41.9)	0.788	1.00	0.788		
	C/T	54 (49.1)	16 (44.4)	38 (51.3)		0.77 (0.33-1.76)			
	T/T	8 (7.3)	3 (8.3)	5 (6.8)		1.09 (0.23-5.15)		0.90 (0.47-1.74)	0.763
IL-6 rs10499563	T/T	47 (43.1)	29 (80.6)	18 (24.7)	<0.001	1.00	<0.001		
	T/C	56 (51.4)	5 (13.9)	51 (69.9)		0.06 (0.02-0.18)			
	C/C	6 (5.5)	2 (5.6)	4 (5.5)		0.31 (0.05-1.87)		0.13 (0.06-0.33)	<0.001
IL-6R rs4537545	T/T	63 (57.3)	23 (63.9)	40 (54.0)	0.399	1.00	0.390		
	T/C	37 (33.6)	9 (25.0)	28 (37.8)		0.56 (0.23-1.39)			
	C/C	10 (9.1)	4 (11.1)	6 (8.1)		1.16 (0.30-4.54)		0.85 (0.46-1.58)	0.607
IL-10 rs1800872	G/G	38 (34.5)	11 (30.6)	27 (36.5)	0.825	1.00	0.823		
	G/T	58 (52.7)	20 (55.6)	38 (51.3)		1.29 (0.53-3.13)			
	T/T	14 (12.7)	5 (13.9)	9 (12.2)		1.36 (0.37-5.00)		1.20 (0.65-2.20)	0.564
IL-10 rs1878672	G/G	60 (54.5)	22 (61.1)	38 (51.3)	0.515	1.00	0.494		
	G/C	44 (40.0)	13 (36.1)	31 (41.9)		0.72 (0.31-1.67)			
	C/C	6 (5.4)	1 (2.8)	5 (6.8)		0.35 (0.04-3.15)		0.67 (0.33-1.35)	0.253
IL-28B rs12979860	T/T	46 (47.9)	16 (50.0)	30 (46.9)	0.948	1.00	0.948		
	T/C	32 (33.3)	10 (31.2)	22 (34.4)		0.85 (0.33-2.23)			
	C/C	18 (18.7)	6 (18.7)	12 (18.7)		0.94 (0.30-2.97)		0.95 (0.54-1.66)	0.850
IFNAR1 rs2843710	C/C	8 (15.1)	1 (6.7)	7 (18.4)	0.282	1.00	0.250		

	C/G	45 (84.9)	14 (93.3)	31 (81.6)		3.16 (0.35-28.20)		3.16 (0.35-28.20)	0.250
	G/G	0	0	0		-			
IL-1A rs1800587	T/T	46 (45.1)	17 (50.0)	29 (42.6)	0.692	1.00	0.690		
	T/C	42 (41.2)	12 (35.3)	30 (44.1)		0.68 (0.28-1.68)			
	C/C	14 (13.7)	5 (14.7)	9 (13.2)		0.95 (0.27-3.30)		0.89 (0.49-1.60)	0.688