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i. Title page

St George's, University of London

Investigating human cytomegalovirus shedding in
seropositive pregnant women by studying the
literature, clinical samples, and experiences of
participating women

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Thesis for the degree of Doctor of Philosophy

30th June 2024

I declare that this thesis is an original report of my research,
has been written by me and has not been submitted for any
previous degree. All collaborative contributions have been
indicated clearly and acknowledged. References have been
provided for all supporting literature and resources.

Signed  _____
Date 30th June 2024

ii. Abstract

Congenital cytomegalovirus (cCMV) infection causes significant morbidity and can be a result of primary or non-primary maternal HCMV infection. Diagnosing non-primary maternal infection in human cytomegalovirus (HCMV)-seropositive pregnant women is challenging. It is possible that detection of HCMV shedding in the bodily fluids of HCMV-seropositive pregnant women may aid in identifying those with non-primary infection. This hypothesis was examined using two, complementary, methodologies: a systematic review to understand the prevalence of HCMV shedding in HCMV-seropositive pregnant women, and an observational cohort study. The observational cohort study investigated the prevalence and risk factors of HCMV shedding, the HCMV-specific cellular mediated immunity (CMI), the hygiene-related contact with children's bodily fluids, and the experiences of participating in a study, in HCMV-seropositive pregnant women. To achieve this, serial sampling of saliva, urine, vaginal secretions, and blood, alongside completion of questionnaires were performed, followed by an interview. The prevalence of HCMV shedding in any bodily fluids of HCMV-seropositive women at any point in pregnancy was approximately 20%, consistent with the estimate calculated from the systematic review. Risk of HCMV shedding during pregnancy was associated with lower number of pregnancies. An HCMV-enzyme-linked immunosorbent spot (ELISPOT) assay, specifically the responses towards the phosphoprotein(pp) 65 antigen, was more likely to detect HCMV-specific CMI during pregnancy and identified that a lower response to pp65 was associated with a higher likelihood of HCMV shedding. Most hygiene-related contact with children's bodily fluids was

not found to be associated with HCMV shedding. Study participation during pregnancy in women with children was shown to be feasible, although reducing involvement around the time of birth would improve study compliance and experience. In conclusion, HCMV shedding in HCMV-seropositive women during pregnancy, may be used as an endpoint for the evaluation of preventative and therapeutic strategies against maternal and cCMV infection.

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vi. Abbreviations

ANOVA	Analysis of variance
aOR	adjusted odds ratio
B	blood
BLASTN	Nucleotide Basic Local Alignment Search Tool
Bp	base pair
C	cervical secretions
cCMV	congenital cytomegalovirus
CD	cluster of differentiation
CE	Conformité Européene
CI	confidence interval
CMI	cellular-mediated immunity
CMV	cytomegalovirus
CNS	central nervous system
COVID-19	coronavirus disease 2019
CO ₂	carbon dioxide
D	detected
DC	dendritic cell
df	degree of freedom
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
D-PBS	Dulbecco's phosphate-buffered saline
E	early
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot
f	frequency
FBS	foetal bovine serum
FDA	Food and Drug Administration
g	glycoprotein
GCSE	general certificate of secondary education
ge/ml	genome equivalent per millilitre
GP	general practitioner
GW	gestational age in in weeks
HCMV	human cytomegalovirus
HLA	human leukocyte antigen
HRA	Health Research Authority
HSC	haematopoietic stem cell
IE	immediate-early
IFN- γ	interferon-gamma
IgG	immunoglobulin G
IgM	immunoglobulin M
IGRA	interferon gamma release assays
IQR	interquartile range
IU/ml	international units/millilitre

I ²	Higgins I ² score
Kb	kilobase
L	late
lncRNA	long non-coding RNA
LR	logistic regression
LUNA	latency unique natural antigen
MHC	major histocompatibility complex
MI	multiple imputation
MICE	multivariate imputation by chained equations
miRNA	micro ribonucleic acid
ml	millilitres
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	multiple sites
MT	multiple time-points
N	total observations
NCT	National Clinical Trial
ND	not detected
ng/μL	nanogram per microlitre
NGS	next-generation sequencing
NHS	National Health Service
NIEP	non-infectious enveloped particle
NK	natural killer
OR	odds ratio
ORF	open reading frames
ONS	Office for National Statistics
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PhD	Doctor of Philosophy
pp	phosphoprotein
PRISMA	Preferred Reporting Items for Systematic reviews and Meta-analyses
QFN	QuantiFERON
RCF	relative centrifugal force
REC	Research Ethics Committee
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RR	risk ratio
S	saliva
SD	standard deviation
SNHL	sensorineural hearing loss
SS	single site
ST	single time-point
SWLP	South West London Pathology
T	trimester
TP	time-point
U	urine

UK	United Kingdom
UL	unique long
US	unique short
USA	United States of America
V	vaginal secretions
vIL-10	viral interleukin-10
WHO	World Health Organization
°C	degree Celsius
μL	microlitre

vii. Attribution of work

All work was carried out by the author (S.S), unless otherwise stated as declared here.

Chapter	Declaration
Chapter <u>1</u>	Nil
Chapter <u>2</u>	- The text in section <u>2.1</u> was adapted from Sapuan <i>et al.</i> (2022) which was written by S.S and edited by co-authors. - The text in section <u>2.2.5.11</u> was adapted from Sapuan <i>et al.</i> (2023) which was written by S.S and edited by co-authors.
Chapter <u>3</u>	- Anastasia Theodosiou (A.T) was the second reviewer for the systematic review. Her roles included screening all the titles and abstracts identified in the literature searches, reviewing all potential full texts for inclusion, reviewing extracted data from the selected full-text articles, and critically appraising the selected full-text articles. - Chrissie Jones (C.J) had a role as the third reviewer for the systematic review should there be any disagreements between the first and second reviewers, however this did not occur. - The text was adapted from Sapuan <i>et al.</i> (2022) which was written by S.S and edited by co-authors.
Chapter <u>4</u>	- Study screening, recruitment, and follow-up of participants for the observational cohort study was assisted by the clinical research team at St George's Hospital National Health Services (NHS) Trust and St George's, University of London. - Human cytomegalovirus (HCMV) serology analysis for the observational cohort study screening was performed by laboratory staff at South West London Pathology (SWLP) at St George's Hospital NHS Trust. - HCMV deoxyribonucleic acid (DNA) polymerase chain reaction (PCR) analysis of saliva, urine, and vaginal secretions, and HCMV-QuantIFERON analysis of blood, of study participants, was performed by NgeeKeong Tan (N.K.T) at SWLP. - The genomic sequencing analysis of HCMV DNA isolates of study participants were performed at the laboratories in University College London under the supervision of Judith Breuer (J.B) and Paul Griffiths (P.G). Claire Atkinson (C.A) performed the extraction of HCMV DNA from the study samples, Charlotte Williams (C.W) performed genomic library preparation of extracted HCMV DNA, and Cristina Venturini (C.V) analysed suitable hybridised and sequenced genome for within-host HCMV diversity and to generate a HCMV sequencing phylogenetic tree. - Tushna Vandrevale (T.V.), a psychologist at Kingston University, London provided guidance on the writing of the end-of-study interview guide script. - The text in sections <u>5.5</u> was adapted from Sapuan <i>et al.</i> (2023) which was written by S.S and edited by co-authors.
Chapter <u>5</u>	
Chapter <u>6</u>	
Chapter <u>7</u>	Nil

Table describing the attribution of work related to this thesis.

Table 1. Attribution of work

viii. COVID-19 statement

The coronavirus disease 2019 (COVID-19) pandemic had a significant impact on the running of the observational cohort study of human cytomegalovirus (HCMV) shedding in HCMV seropositive pregnant women (chapters 2, 4-7). Identification, recruitment, and follow-up of participants were halted due to the government lockdowns and restrictions, reducing the total number of participants recruited into and the number of participants completing the study follow-ups. Sample collections were also ceased during these periods. Certain aspects of the methods, in particular sample storage, were modified to optimise the number of samples collected and to minimise impact on the study compliance and power of the findings. However, some sample collection that required real-time sample analysis, in particular blood for analysis with HCMV-enzyme-linked immunosorbent spot (ELISPOT) assay, was not possible. Additionally, I was also pregnant and gave birth to my child during the COVID-19 pandemic, requiring me to shield over many months, followed by maternity leave. Therefore, I was not personally able to perform some of the study procedures. Please see the attributions of work under section [vii](#).

ix. Acknowledgements

I am extremely grateful to my supervisors, Prof. Paul Heath, Dr Blair Strang, and Dr Chrissie Jones, for their guidance, patience, support, advice, and wisdom in my journey of completing this thesis.

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I am very grateful to Merck, Sharpe & Dohme for providing funding for some of the vital work in the observational cohort study.

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Finally, many thanks are owed to my family, especially to my husband William for his patience and encouragement, and to my daughter Sofia to whom I dedicate this thesis.

Thank you.

1. Introduction

1.1. Human cytomegalovirus infection: The implications and challenges in pregnant women and their unborn infants

1.1.1. Modes of human cytomegalovirus infection

HCMV can infect a broad range of cell types *in vivo* that includes epithelial cells of glandular and mucous tissues, smooth muscle cells, fibroblasts, macrophages, hepatocytes, dendritic cells, and vascular endothelial cells (Gugliesi *et al.*, 2020). Upon entry of HCMV into a host for the first time (primary HCMV infection), epithelial cells and internal mucosa are usually the first site of HCMV infection with subsequent viral replication, and from there HCMV may infect monocytic cells that traffic around the body expelling many viral particles which can lead to infection of multiple tissues such as lungs, liver, and spleen (Gugliesi *et al.*, 2020). Subsequently HCMV can infect secretory organs such as salivary glands and breast, which shed the virus into the environment for months, even years (Gugliesi *et al.*, 2020).

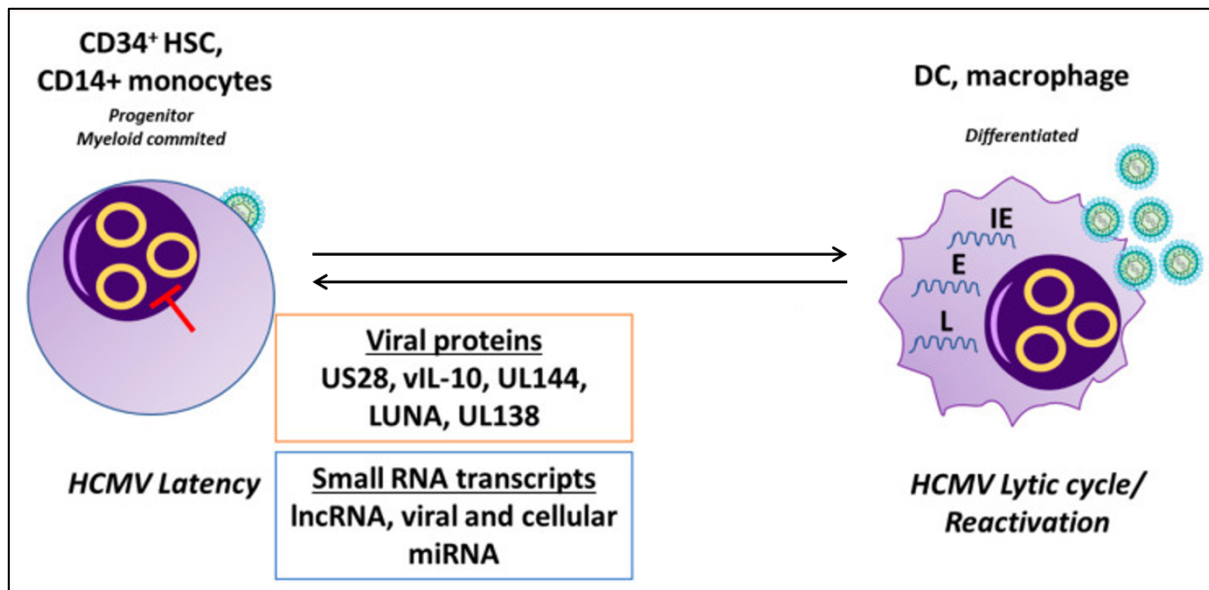
Similar to other members of the herpesviruses, after a primary HCMV infection, HCMV can establish latency in the host (Gugliesi *et al.*, 2020). The main site where HCMV is known to establish latency is in cells of the myeloid lineage and their progenitors, cluster of differentiation (CD) 34+ cells (Gugliesi *et al.*, 2020). The ability to establish a lifelong latent infection by maintaining the HCMV genome without any production of infectious progeny, is an important biological characteristic of HCMV, making it a pathogen able to cause opportunistic infections, where the dormant genome can be reversed and initiate a

productive infection in response to any number of stimuli even after many years, a phenomenon known as reactivation (Griffiths, Baraniak and Reeves, 2015; Gugliesi *et al.*, 2020). Latency was historically considered to be a silent state of the infection, characterised by overall suppression of viral gene expression aimed at preventing the detection and activation of the immune system (Gugliesi *et al.*, 2020). However, recent studies have shown latency to be a dynamic phase of the infection, where viral gene expression is responsible for subverting host cell functions, such as cell survival, genome carriage, and immune evasion (Gugliesi *et al.*, 2020). Viral gene expression in latently infected cells is restricted and markedly different from the gene expression during lytic infection (La Rosa and Diamond, 2012). To establish latency, HCMV must stop the production of infectious viral particles through suppression of lytic gene expression (Griffiths, Baraniak and Reeves, 2015; Gugliesi *et al.*, 2020). As described, the expression of major IE genes is required for HCMV replication (Isomura and Stinski, 2003). A key event required to initiate a state of latency involves the repression of major IE genes, which is sufficient to prevent the expression of E and L genes as well. The mechanism of repression is argued to be a concert of effects. Concurrently, differentiation of latent CD34+ cells or monocytes (CD14+) to macrophages or dendritic cells induces reactivation of the major IE genes with subsequent expression of IE genes and re-entry into the lytic cycle (Gugliesi *et al.*, 2020).

The long-term maintenance of latency and subsequent reactivation appear to be controlled by inherent cellular functions important for controlling normal gene expression and dynamic regulation of the MIE genes (Griffiths, Baraniak and Reeves, 2015; Gugliesi *et al.*, 2020). As latency appears to be a very complex phenomenon, reactivation is often the result of a closely intertwined crosstalk between cellular and viral signals triggering multiple pathways (Figure 1) (Gugliesi *et al.*, 2020). IE gene expression alone does not seem to be

sufficient to induce the production of infectious particles, where the observation that differentiation of experimentally latently infected monocytes into monocyte-derived macrophages can lead to a fully permissive phenotype implies that the differentiation status is a critical determinant of reactivation (Gugliesi *et al.*, 2020). In addition, mounting evidence indicates that inflammation may also play a role in HCMV reactivation (Gugliesi *et al.*, 2020). A better understanding of latency and subsequent reactivation may be crucial to elucidate HCMV pathogenesis and develop therapeutics targeting latent virus reservoirs. This is a particularly important aspect given that all commercially available drugs for the treatment of HCMV diseases target only lytic but not latent infections (Gugliesi *et al.*, 2020).

The reactivation of a latent HCMV infection is what is known as a non-primary HCMV infection (Gatherer *et al.*, 2011; Schleiss M.R., 2017; Gugliesi *et al.*, 2020). In addition, as already described, HCMV is a genus of viruses made of different strains, and multiple-strain HCMV infection or HCMV mutation giving rise to other HCMV strain(s) can also occur (Suárez *et al.*, 2019). The event by which a host is infected with different HCMV strain(s) to the strain(s) from the primary HCMV infection, known as reinfection, is also a non-primary infection (Griffiths, Baraniak and Reeves, 2015). Thus, combinations of primary and non-primary infections can be attributed to HCMV disease, including HCMV disease observed during pregnancy.



Following primary infection, HCMV can establish latency in CD34⁺ myeloid progenitor cells and is carried down the myeloid lineage. In latently infected CD34⁺ cells and monocytes, there is a targeted suppression of lytic viral gene expression. HCMV utilises several viral proteins and small RNA transcripts, including viral and cellular miRNAs, during latent infection to alter the signalling environment within the cell to maintain the status of latency. Differentiation of these cells to macrophages and dendritic cells causes the derepression of the major IE genes and allows initiation of the lytic transcription program, which involves a temporal cascade of viral gene transcription, allowing reactivation of de novo virus production. (Gugliesi *et al.*, 2020) (section 1.1.1).

Abbreviations: CD; cluster of differentiation; DC, dendritic cell; E, early; HCMV, human cytomegalovirus; HSC, hematopoietic stem cell; IE, immediate-early; L, late, lncRNA, long non-coding RNA; LUNA, latency unique natural antigen, miRNA, micro ribonucleic acid; RNA, ribonucleic acid; UL, unique long; US, unique short; vIL-10; viral interleukin-10.

Figure 1. Key cellular, immune and viral components involved in the intertwined relationship between lytic, latent and reactivation of HCMV infection (Gugliesi *et al.*, 2020)

1.1.2. Dissemination of human cytomegalovirus infection

HCMV exploits both vertical and horizontal transmission (Gugliesi *et al.*, 2020).

Horizontally, HCMV efficiently spreads through direct contact with infectious bodily fluids of an infected hosts, such as urine, saliva, tears, breast milk, cervicovaginal secretions, semen, and blood. HCMV can be transmitted sexually and through blood transfusions (Mocarski, Shenk and Pass, 2007; Saldan *et al.*, 2017). Latently infected myeloid cells can also persist in bone marrow, thereby contributing to the risk of transferring HCMV along with organs and tissues following transplantation (Gugliesi *et al.*, 2020).

Vertical transmission can occur from the mother to the foetus in-utero through the placenta when the placental barrier is breached allowing HCMV to enter foetal circulation, or through the vaginal canal (cervicovaginal secretions) during birth, causing cCMV infection in the foetus (Mocarski, Shenk and Pass, 2007; Saldan *et al.*, 2017; Gugliesi *et al.*, 2020). The major source of HCMV infection for pregnant women from young children, whose saliva and urine may contain high levels of HCMV and direct contact with these infectious bodily fluids can cause horizontal transmission of HCMV (Mocarski, Shenk and Pass, 2007; Griffiths, Baraniak and Reeves, 2015; Saldan *et al.*, 2017).

1.1.3. Clinical presentation of human cytomegalovirus infection and at risk groups

In healthy, immunocompetent children and adults, HCMV infection is mostly asymptomatic or causes flu-like symptoms, including in pregnant women (Gugliesi *et al.*, 2020).

However, HCMV is a serious health risk for immunocompromised individuals such as patients suffering from human immunodeficiency virus (HIV) or undergoing bone or organ transplants (Gugliesi *et al.*, 2020). Transplant patients may display a spectrum of clinical

manifestations, such as gastrointestinal and respiratory symptoms in less severe disease, and meningoencephalitis, hepatitis, myocarditis, and pancreatitis in more severe disease (Gugliesi *et al.*, 2020).

It is also dangerous in a foetus or an infant when transmitted vertically from an infected pregnant woman to result in cCMV infection (Gugliesi *et al.*, 2020). Up to 25% of children with cCMV infection will have life-long problems, the most common being SNHL (Korndewal *et al.*, 2017; Luck *et al.*, 2017). Visual impairment, developmental delay, learning disability, behavioural difficulties, cerebral palsy, epilepsy, and autism spectrum disorder have also been associated with cCMV infection (Korndewal *et al.*, 2017; Luck *et al.*, 2017; Retzler *et al.*, 2019). Children with cCMV infection may require significant input from health and social care services, which can have a large impact on families and is associated with a significant cost to society (Dollard, Grosse and Ross, 2007; Retzler *et al.*, 2019).

Clinical features of a newborn with cCMV infection could include microcephaly, petechiae, purpura, icterus, hepatosplenomegaly, and/or a small-for-gestational-age habitus (Schleiss M.R., 2017). However, around 90% of newborn infants with cCMV infection are asymptomatic, showing no obvious and visible symptoms of the virus (Boppana *et al.*, 2001). When cCMV infection is identified, diagnostic neuroimaging is warranted (Schleiss M.R., 2017). Cranial ultrasonography, head computed tomography (CT) scans, or brain magnetic resonance imaging (MRI) can be used to detect CNS pathologies (Schleiss M.R., 2017). Cranial ultrasound can readily detect periventricular calcifications, structural anomalies, and mild-to-moderate ventricular dilatation (Schleiss M.R., 2017). MRI provides more detailed information, including identification of neuronal migration defects (polymicrogyria, lissencephaly), hippocampal dysplasia, white matter abnormalities, and cerebellar hypoplasia (Schleiss M.R., 2017). Careful initial audiological evaluation for sensorineural

hearing loss (SNHL) is required in infants with cCMV infection (Schleiss M.R., 2017).

Moreover, serial longitudinal audiological assessments are warranted since SNHL may be late in onset and/or progressive in nature, even if it is not evident at birth (Schleiss M.R., 2017). Ophthalmologic evaluation is also warranted in infants with cCMV infection to evaluate for HCMV retinitis, particularly in those children with symptomatic disease, abnormal CNS imaging studies, or both (Schleiss M.R., 2017).

1.1.4. Relevant incidence and prevalence rates of human cytomegalovirus infection

Antibodies of immunoglobulin (Ig) G class to HCMV reflect past infection, known as being HCMV-seropositive (Griffiths, Baraniak and Reeves, 2015). The worldwide HCMV seroprevalence in adults ranges from 50% to 100% (Griffiths, Baraniak and Reeves, 2015; Gugliesi *et al.*, 2020). Infection can be acquired at any age (Griffiths, Baraniak and Reeves, 2015). People born into poor socio-economic circumstances may acquire HCMV earlier than do others (Griffiths, Baraniak and Reeves, 2015). HCMV spread in the global population is wide-ranging, likely due to the asymptomatic mode of infection, and the ability for the virus to shed in bodily fluids for a prolonged period (Gugliesi *et al.*, 2020). While seroprevalence rates have been reported in the literature, the incidence and prevalence of HCMV infection in the global population has not been reported.

Approximately 40% of HCMV-seropositive solid organ transplant recipients reactivate latent HCMV when given immunosuppressive drugs, and those with seropositive donors can also be reinfected with new strains of HCMV whereby approximately 78% of HCMV-seropositive donors transmit HCMV to seronegative recipients (Griffiths, Baraniak and Reeves, 2015).

Approximately 2% of HCMV-seronegative women who enter pregnancy have seroconverted by the time of delivery (Griffiths, Baraniak and Reeves, 2015). Among the women with primary infection during pregnancy, around 30-40% of them transmit virus across the placenta to produce intrauterine infection (Varnum *et al.*, 2004). This is not usually recognised until delivery, because the women's symptoms are not sufficient for them to seek medical attention, although retrospective enquiry can identify symptoms of fever and malaise (Griffiths, Baraniak and Reeves, 2015). The foetus can also be infected as a result of non-primary maternal infection, i.e., by reactivation of latent maternal infection or by maternal reinfection with a new strain of HCMV (Griffiths, Baraniak and Reeves, 2015). These women usually have no symptoms to report and as described there are no diagnostic tests to detect these infections, making it even more challenging to diagnose them (Griffiths, Baraniak and Reeves, 2015; Saldan *et al.*, 2017). Approximately 0.2 to 1% of women who are HCMV-seropositive prior to pregnancy deliver infants with cCMV infection (Raynor, 1993; Griffiths, Baraniak and Reeves, 2015). However, the abundance of HCMV-seropositive women in the antenatal population means that, even in developed countries, more infants with cCMV infections are born to HCMV-seropositive women than to those experiencing primary infection during pregnancy (Pass and Anderson, 2014; Griffiths, Baraniak and Reeves, 2015; Scaramuzzino *et al.*, 2022).

cCMV infection is the most common congenital infection in the United Kingdom (UK) and worldwide (Peckham *et al.*, 1983; Griffiths *et al.*, 1991; Luck *et al.*, 2017). With an estimated incidence of 3 per 1000 live births, it is more common than many better-known congenital conditions, such as Down's syndrome and cystic fibrosis (Peckham *et al.*, 1983; Griffiths *et al.*, 1991; Luck *et al.*, 2017).

1.1.5. Diagnosis of human cytomegalovirus infection

Serologic assays are the primary tools for diagnosing a primary HCMV infection and the gold standard for determining HCMV seroconversion, which is the detection of HCMV IgG, with or without IgM detection, in a previously known non-immune (HCMV IgG and IgM undetected, i.e., HCMV-seronegative) individual (Saldan *et al.*, 2017). The diagnosis of non-primary HCMV infection cannot rely on conventional serological findings (pre-existing HCMV-seropositivity), thus making its diagnosis challenging (Saldan *et al.*, 2017).

For cCMV infection, laboratory confirmation is best identified by detection of HCMV DNA by polymerase chain reaction (PCR) in samples collected within the first three weeks of life (Schleiss M.R., 2017). The finding of HCMV shedding in an infant beyond three weeks of age, which is the shortest incubation period for HCMV infection, may reflect acquisition of infection in the postnatal period, commonly after ingestion of breast milk and cannot be assumed to be diagnostic of congenital infection (Schleiss M.R., 2017). It may be possible to clarify when the infection occurred by testing a bloodspot stored by the neonatal screening laboratory (Schleiss M.R., 2017). Urine and saliva are both acceptable for PCR assay to diagnose cCMV infection (Schleiss M.R., 2017). Blood (or plasma) PCR may be of interest for prognostic purposes in infants with symptomatic cCMV infection, but it is not sufficiently sensitive to stand alone as a diagnostic test for cCMV, since congenitally infected infants may not have DNAemia (Schleiss M.R., 2017).

Serodiagnosis of cCMV is challenging and serological tests for HCMV antibodies are generally not useful in this setting (Schleiss M.R., 2017). Maternal HCMV IgG antibodies that cross the placenta may yield a positive result when infant serum is analysed, therefore a positive HCMV IgG serology is non-informative. HCMV IgM serology has a low sensitivity for

diagnosis of cCMV, although its presence does suggest a greater likelihood of symptomatic disease in infants with congenital infection (Schleiss M.R., 2017).

Pregnant women who are found to have HCMV infection may have a diagnosis of intrauterine infection by amniocentesis and testing of amniotic fluid for HCMV DNA by PCR (Griffiths, Baraniak and Reeves, 2015). This test is reliable if performed after 21 weeks of gestation to reduce the risk of a false negative result, and at least 6 weeks after the presumed time of maternal infection to consider time for HCMV to move from the maternal sites of infection to cross the placenta and for foetal kidney function to become sufficiently established for viruria to be detectable by sampling amniotic fluid (Griffiths, Baraniak and Reeves, 2015).

1.1.6. Immune responses to human cytomegalovirus infection

During primary HCMV infection, antibodies specific for HCMV proteins, including tegument (e.g., pp65 and pp150), envelope glycoproteins (e.g., gB and gH), and non-structural proteins (e.g., IE1-encoded protein), are produced in the host (La Rosa and Diamond, 2012). The role of antibodies for protection against and control of HCMV has been debated, however, studies indicate that humoral immunity is crucial in restricting viral dissemination and may contribute to minimising the clinical manifestations of the disease (La Rosa and Diamond, 2012).

The magnitude of the HCMV-specific T-cell immune responses, referred to here as cellular mediated immunity (CMI), is the most striking aspect of the dynamic, life-long interaction between the host and HCMV (La Rosa and Diamond, 2012). The frequencies of HCMV-specific CMI in healthy subjects have been observed to be higher than those observed for other human viruses (e.g., influenza, adenovirus, measles, mumps, other

herpes viruses) (La Rosa and Diamond, 2012). The properties of HCMV that determine such large and long-lasting CMI in the absence of detectable HCMV viremia have not been elucidated (La Rosa and Diamond, 2012). However, the relatively prolonged, but reduced viral replication during primary infection may induce recruitment of HCMV-specific T-cells into the memory compartment. T-cells are essential to restrain HCMV viral replication and prevent disease (La Rosa and Diamond, 2012). HCMV-specific CMI dominate the memory compartments of infected individuals, comprising on average approximately 10% of both the CD4+ and CD8+ memory compartments in peripheral blood (La Rosa and Diamond, 2012). Levels of HCMV-specific CMI continue to rise throughout life, and very large responses may be observed *ex vivo* against single epitopes contributing to a substantial fraction of CD8+ cells in healthy HCMV-seropositive individuals (La Rosa and Diamond, 2012). HCMV-seropositive immunocompetent individuals can maintain protection from symptomatic disease even with minimal HCMV-specific CMI, however, the level of HCMV-specific CMI required for viral control remains unclear (La Rosa and Diamond, 2012).

In-depth *ex vivo* analysis indicate that pp65 (UL83) and IE1 (UL123) are recognised by more than 50% of individuals, with several other ORFs including UL48, UL55, UL122, UL48, UL32, UL123, UL99 and UL82 also recognised by both CD4+ and CD8+ HCMV-specific cells (Sylwester *et al.*, 2005). Studies investigating HCMV-specific CMI as an essential host factor in the control of viral replication to prevent HCMV disease have been mainly performed on individuals in transplantation setting (La Rosa and Diamond, 2012). A variety of assays to measure HCMV-specific CMI have also been developed. These provide a useful technique to target those in the transplantation settings who are susceptible to HCMV infection and end-organ disease. This information is incorporated into guidelines on the management of HCMV in solid organ or stem cell transplantation (Valle-Arroyo *et al.*, 2020). Commercially

available assays to perform this include the HCMV-QuantiFERON and HCMV-enzyme-linked immunosorbent spot (ELISPOT) assays, which measure interferon gamma (IFN- γ) release from memory T-cells following exposure or stimulation with HCMV-specific antigens, reflecting the ability of the HCMV-specific CMI to control HCMV infection (Lee *et al.*, 2017). Although both HCMV-QuantiFERON and HCMV-ELISPOT assays are interferon gamma release assays (IGRAs) and used as proxies to measure HCMV-specific CMI, their characteristics are different, as described in Table 2 (Saldan *et al.*, 2016; Lee *et al.*, 2017; Oxford Immunotec Ltd, 2017; QIAGEN, 2018).

Category	HCMV-QuantiFERON	HCMV-ELISPOT
HCMV antigenic peptides used for HCMV-specific CMI <i>ex vivo</i> stimulation	HLA Class I restricted and combined pool of peptides: pp65, pp28, pp50, IE1, IE-2, and gB	Complete protein-spanning mixtures of overlapping entire protein sequence regardless of HLA type, of separate pool of peptides: pp65 and IE1
Method	ELISA of whole blood, which measures the level of secreted IFN- γ	ELISPOT assay of PBMCs, which measures the number of IFN- γ -secreting cells.
Principle	HCMV-specific CD8+ cell responses	HCMV-specific CD4+ and CD8+ cell responses
Result value	IU/ml	Spot count
HCMV-specific CMI result interpretation	- 'Detected' if the [HCMV peptide minus negative control (Nil)] value is ≥ 0.200 IU/ml and $\geq 25\%$ of Nil value - 'Undetected' if the [HCMV peptide minus Nil] value is < 0.200 IU/ml or ≥ 0.200 IU/ml but $< 25\%$ of Nil value - Indeterminate if the positive control (Mitogen) value is < 0.500 IU/ml or Nil value is > 8.000 IU/ml	- 'Detected' if the [IE1 minus Nil] or [pp65 minus Nil] spot count is ≥ 1, where higher number of spots indicates higher level of response - 'Undetected' if the [IE1 minus Nil] and [pp65 minus Nil] spot count is 0, or a negative value - Indeterminate if the Mitogen spot count is ≤ 20

Table comparing the HCMV-specific CMI assays HCMV-QuantiFERON and HCMV-ELISPOT (Saldan *et al.*, 2016; Lee *et al.*, 2017; Oxford Immunotec Ltd, 2017; QIAGEN, 2018).

Abbreviations: Enzyme-linked immunosorbent assay, ELISA; enzyme-linked immunosorbent spot, ELISPOT; glycoprotein, g; HCMV, human cytomegalovirus; HLA, human leukocyte antigen; interferon gamma, IFN- γ ; IE, immediate-early; IU/ml, international units per millilitre; PBMC, peripheral blood mononuclear cells; pp, phosphoprotein (section 1.2.3).

Table 2. Comparison between HCMV-QuantiFERON and HCMV-ELISPOT

When immunocompetent individuals are infected with HCMV, they will likely develop protective HCMV-specific CMI that control viral replication sufficiently to prevent the development of end-organ HCMV disease and will likely either be ‘asymptomatic’ or only have a mild HCMV disease (Sinclair *et al.*, 2004; Valle-Arroyo *et al.*, 2020). The time course of the appearance of HCMV-specific adaptive immune responses is difficult to define in asymptomatic healthy subjects since the time of onset of primary infection typically goes unnoticed (Sinclair *et al.*, 2004; Valle-Arroyo *et al.*, 2020). Thus, the laboratory correlates of this immune protection in immunocompetent HCMV-seropositive individuals have been poorly investigated and are not fully understood (Sinclair *et al.*, 2004; Valle-Arroyo *et al.*, 2020). Correlates of immune protection have also been poorly investigated in HCMV infection in pregnant women, especially where HCMV-seropositive pregnant women with latent infection are concerned. In a study to compare HCMV-QuantiFERON and HCMV-ELISPOT assays in pregnant women, HCMV-ELISPOT was found to be more effective than HCMV-QuantiFERON in differentiating primary from non-primary HCMV infections (Saldan *et al.*, 2016). HCMV-ELISPOT also displayed significantly higher values for pregnant women with primary HCMV infection than those who were HCMV-seropositive with or without non-primary infection, whereas in comparison the values of that for HCMV-QuantiFERON were not significantly different (Saldan *et al.*, 2016). The ability of either assay to differentiate between HCMV-seropositive pregnant women with and without non-primary HCMV infection was not described (Saldan *et al.*, 2016). Pregnancy may pose an immunological challenge in an immunocompetent woman when faced with HCMV infection; while continuing to accommodate a foetus during HCMV infection, immunomodulation could occur in pregnancy, and may vary throughout pregnancy (Lissauer *et al.*, 2011). The

longitudinal measurements of HCMV-specific CMI could provide valuable insight into the changes in immune function as pregnancy progresses.

1.1.7. Antiviral treatment of human cytomegalovirus infection

For over two decades, the mainstay of treating HCMV infection has been through ganciclovir or valganciclovir, an oral prodrug that is rapidly converted to ganciclovir, together with the off-label use of foscarnet in some cases (Griffiths, Baraniak and Reeves, 2015). Cidofovir has activity against HCMV, but its renal toxicity precludes use in routine clinical practice (Griffiths, Baraniak and Reeves, 2015). Ganciclovir (or valganciclovir) is used for pre-emptive therapy or prophylaxis in transplant patients, and to treat certain HCMV end-organ disease in certain immunodeficient patients (Griffiths, Baraniak and Reeves, 2015).

Currently, there is no licensed antiviral drug for the treatment of maternal HCMV infection, however valaciclovir has been used off-label especially in research setting, with some evidence in favour of pharmacological interventions during pregnancy to prevent and improve prognosis of cCMV infection (Scarpini *et al.*, 2021).

Intravenous ganciclovir and oral valganciclovir is also used to treat symptomatic infants with cCMV infection involving the central nervous system (CNS) (Griffiths, Baraniak and Reeves, 2015), which has been shown to have modest short term-benefits on hearing and neurodevelopmental outcomes (Kimberlin *et al.*, 2003, 2015; Griffiths, Baraniak and Reeves, 2015; Leung *et al.*, 2022). Treatment with ganciclovir for more than 6 weeks is typically not recommended because of potential toxicity. Neonates who are unable to commence antiviral treatment with valganciclovir, which is bioavailable in neonates with less potential for toxicity, can begin treatment on ganciclovir and be transitioned to

valganciclovir when clinically suitable (Griffiths, Baraniak and Reeves, 2015; Leung *et al.*, 2022). Antiviral treatment with valganciclovir within the first month of life and for up to 6 months has been recommended since 2017 for infants with moderately to severely symptomatic cCMV involving the CNS (Griffiths, Baraniak and Reeves, 2015; Leung *et al.*, 2022). However, the use of valganciclovir in infants also comes with significant side-effects (Leung *et al.*, 2022). Neutropenia occurs in as many as two-thirds of neonates treated with ganciclovir and one-fifth of those treated with valganciclovir (Leung *et al.*, 2022). Other short-term side effects include raised aminotransferases and thrombocytopenia; data on long-term side effects such as impaired fertility and carcinogenicity are still lacking (Leung *et al.*, 2022). It is also important to highlight that these antiviral drugs improve outcomes in symptoms of those cCMV-infected infants more significantly affected, it does not cure the disease. Therefore, prevention of cCMV infection is still the favourable outcome and measures to prevent cCMV infections through the prevention or treatment of maternal HCMV infection are imperative.

Three new antiviral drugs have entered clinical trials fairly recently: Maribavir, brincidofovir, and letermovir (Griffiths, Baraniak and Reeves, 2015).

1.1.8. Human cytomegalovirus vaccine

To date, there is no licensed HCMV vaccine to treat HCMV infection, including cCMV infection. Major difficulties in the development of HCMV vaccine include the capacity of HCMV to evade the immune response, unclear immune correlates for protection, and a need to determine which may be the best target populations for vaccine administration (Das *et al.*, 2023). There are however several HCMV vaccines under clinical development. A recombinant, soluble gB vaccine with MF59 adjuvant (manufactured by Sanofi Pasteur) has

been studied in several phase II clinical trials and reported some degree of control of HCMV replication and seroconversion (Griffiths, Baraniak and Reeves, 2015). One studied the vaccine on patients undergoing stem cell transplantation, which showed the vaccine reduced the need for preventive therapy, based on the HCMV-ELISPOT assay results as the correlate of protective immunity, providing evidence in its capacity to provide protection against primary infection and to boost natural immunity (Griffiths, Baraniak and Reeves, 2015). Another trial studied the same vaccine on HCMV-seronegative women within 1 year of giving birth, which found 50% protection against HCMV seroconversion at follow-up, providing evidence in its capacity to potentially reduce the incidence rate of primary and cCMV infection (Pass *et al.*, 2009).

A live-attenuated, AD169 genetically engineered vaccine (manufactured by Merck Sharp & Dohme) has also been recently studied in a phase II clinical trial in healthy HCMV-seronegative non-pregnant females of childbearing age with regular exposure to young children, which found that although it was well tolerated and immunogenic, it did not reduce the incidence HCMV-seroconversion at follow-up (Scarpini *et al.*, 2021).

Both the vaccines above have not been studied in healthy HCMV-seropositive women. A more recent messenger ribonucleic acid (mRNA)-1647 vaccine (manufactured by Moderna) is being studied in a phase II clinical trial in healthy HCMV-seronegative and seropositive adults (National Clinical Trial (NCT) number NCT04232280) (Das *et al.*, 2023). The findings from this study may provide valuable insight into the vaccine development to prevent non-primary HCMV infection. However, no HCMV vaccine has been clinically trialled on pregnant women, where concerning cCMV infection, the vaccine should be able to protect both seronegative women from primary infection and seropositive women from non-primary infection (Das *et al.*, 2023). Although data indicate that the HCMV vaccine should ideally

stimulate both the cellular and humoral components, the exact details of immune mechanisms against HCMV with regards to cCMV infection is still unclear, (Das *et al.*, 2023).

1.2. Human cytomegalovirus: Understanding the virus and its structure

1.2.1. Historical perspective of human cytomegalovirus

Human cytomegalovirus (HCMV) was thought to have been first observed in 1881 by Ribbert, noting the characteristic appearance of HCMV-infected cells that were later known as the “owl’s eye” inclusions (Ho, 2008). In 1910, these features came to medical attention when the characteristic “owl’s eye” inclusions were seen in cells from the kidneys, lungs, and livers of stillbirths, giving rise to recognising as an infection, that could infect the foetus (Ho, 2008). After human cells and viruses were grown in cell culture, Weller, Smith, and Rowe isolated HCMV strains independently in 1956 to 1957 from humans, where it was thought that Weller proposed the term "cytomegalovirus", due to the enlarged cytoplasm observed upon infection, and subsequently isolated a HCMV strain from an infant with congenital CMV (cCMV) infection (Ho, 2008). In 1964, the aforementioned features came to medical attention again when the characteristic “owl’s eye” inclusions were seen in cells from the lungs of patients undergoing organ transplantation, giving rise to the recognition of its ability to transmit through transplanted organs (Griffiths, Baraniak and Reeves, 2015). In 1984, the complete HCMV genome was sequenced (the AD169 strain; GenBank AC146999), and subsequently, using high-throughput genomic sequencing technology, more HCMV genomes and strains were discovered, giving rise to our ability to recognise the genomic complexity of HCMV (Griffiths, Baraniak and Reeves, 2015; Gugliesi *et al.*, 2020).

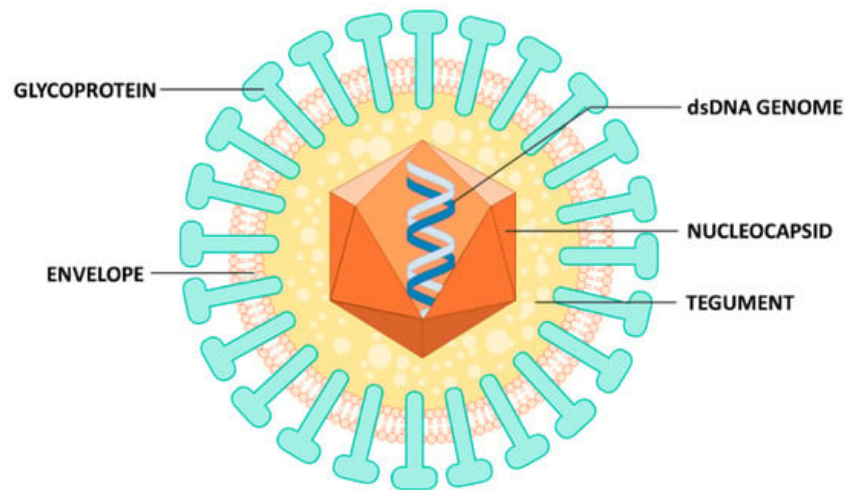
The complexity of HCMV infection and the wide spectrum of disease manifestations sparked a major interest in determining the origin and mechanisms of HCMV infection (Ho, 2008; Griffiths, Baraniak and Reeves, 2015; Gugliesi *et al.*, 2020).

1.2.2. Classification of human cytomegalovirus

Cytomegalovirus (CMV) is a genus of viruses and a member of the viral order known as *Herpesvirales* (commonly referred to as herpesviruses), a large group of deoxyribonucleic acid (DNA) viruses that cause infections and diseases in animals, including humans (Gugliesi *et al.*, 2020). CMV belongs to the *Betaherpesvirinae* subfamily (Gugliesi *et al.*, 2020). Human CMV (HCMV) is one of nine human herpesviruses (HHV 1 to 9), known to infect humans (Gugliesi *et al.*, 2020). HCMV is also known as human herpesvirus-5 (HHV-5) or human betaherpesvirus 5 (Gugliesi *et al.*, 2020). HCMV is also commonly known just as CMV (Schleiss M.R., 2017).

1.2.3. Structure of human cytomegalovirus

Like other herpesviruses, the HCMV virion is composed of an icosahedral nucleocapsid that contains a double-stranded DNA genome, with an outer layer of proteins called tegument, surrounded by a cellular lipid coating known as envelope, which contains the viral glycoproteins (Varnum *et al.*, 2004; Gugliesi *et al.*, 2020). The virion is a quasi-spherical shape (Figure 2) (Varnum *et al.*, 2004; Gugliesi *et al.*, 2020).



Structure of HCMV virion. It contains a double-stranded DNA genome enclosed within an icosahedral nucleocapsid, surrounded by tegument and coated by an envelope with protruding glycoproteins (Gugliesi *et al.*, 2020) (section [1.1.3](#)). Abbreviations: DNA, deoxyribonucleic acid; HCMV, human cytomegalovirus.

Figure 2. Structure of HCMV virion (Gugliesi *et al.*, 2020)

1.2.4. Analysis of human cytomegalovirus infection by microscopy

The morphologic alteration of an HCMV infected cell is well-described; the cytoplasm and nucleus become enlarged with voluminous inclusion bodies made up of clusters of newly formed viruses and lysosomes, leading to the clear demarcation of the nuclear membrane with a surrounding halo, giving rise to the characteristic “owl’s eye” inclusion when viewed under a light microscope (Ho, 2008; Gugliesi *et al.*, 2020) (Figure 3 (Ho, 2008), Figure 4 (Griffiths, Baraniak and Reeves, 2015)). The formation of such inclusion bodies generally causes increased cellular volume, a phenomenon defined as cytomegaly, which granted HCMV its name (Gugliesi *et al.*, 2020).



Image under microscopy of non-infected cells and an enlarged HCMV-infected cell with intranuclear inclusion with surrounding halo, an "owl's eye" nucleus, and cytoplasmic inclusion (Ho, 2008) (section [1.2.1](#)).
Abbreviations: HCMV, human cytomegalovirus.

Figure 3. Owl's eye inclusion of HCMV-infected cell (Ho, 2008)

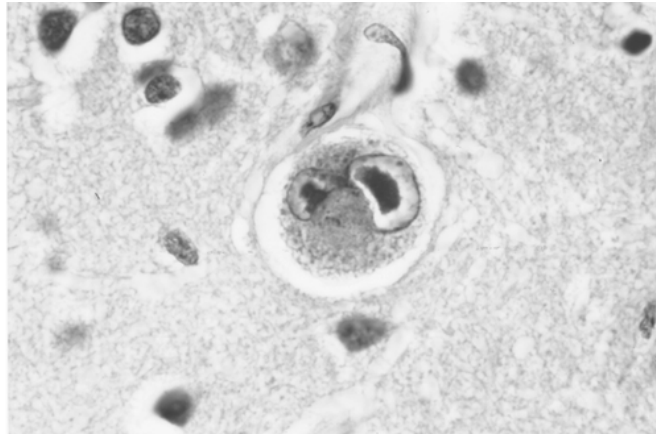


Figure 3. Appearance under microscopy of a HCMV-infected epithelial cell bearing intranuclear inclusion, an “owl’s eye” inclusion (Griffiths, Baraniak and Reeves, 2015) (section [1.2.1](#)).
Abbreviations: HCMV, human cytomegalovirus.

Figure 4. Owl’s eye inclusion of HCMV-infected cell (Griffiths, Baraniak and Reeves, 2015)

1.2.5. Human cytomegalovirus genome

The HCMV genome is approximately 230 kilobase in length, the largest among known human viruses, and encodes over 200 unique open reading frames (ORFs) (Varnum *et al.*, 2004; Schleiss M.R., 2017). The first complete HCMV genome sequenced was the AD169 strain (GenBank AC146999) in 1984 from a plasmid library when little was known about the sequence or location of genetic information in the viral genome (Suárez *et al.*, 2019). Over a decade later more HCMV genomes were sequenced giving rise to the discovery of other HCMV strains, mostly derived from laboratory strains isolated in cell culture, and characterised using mainly Sanger sequencing of polymerase chain reaction (PCR) amplicons, complemented subsequently by other, increasing high-throughput sequencing methods (Suárez *et al.*, 2019). Advances in high-throughput sequencing technology made it possible to generate a wealth of HCMV genome information directly from clinical material to define HCMV natural populations (Suárez *et al.*, 2019). Current approaches to whole genomic sequencing involve the next-generation sequencing (NGS) technology, such as that used in the Illumina technology (Venturini *et al.*, 2022). To date, the complete genomes of 351 full-length HCMV strains have been published and analysed (Gugliesi *et al.*, 2020). It is now understood that early HCMV genomes sequenced, such as AD169, do not faithfully represent HCMV genomes from clinical material, due to excessive passage of HCMV in cell culture (Wilkinson *et al.*, 2015). Laboratory studies of HCMV are now shifting to so-called “low passage” strains of HCMV, such as the Merlin strain (GenBank AY446894) (Wilkinson *et al.*, 2015).

The HCMV genome has been discovered to exhibit several evolutionary phenomena, including mutation, multiple-strain infection, recombination, and gene loss (Suárez *et al.*, 2019). HCMV can be highly polymorphic among and within hosts, with a high level of intra-

host variability (Suárez *et al.*, 2019; Gugliesi *et al.*, 2020). Many of these genetic alterations may in turn influence HCMV cell tropism, immune evasion, and disease outcomes (Suárez *et al.*, 2019; Gugliesi *et al.*, 2020).

1.2.6. Transcription from the human cytomegalovirus genome and the function of human cytomegalovirus proteins

The expression of HCMV genes, like that of all other herpesviruses, occurs in a temporal cascade consisting of immediate-early (IE), early (E), and late (L) genes (Gugliesi *et al.*, 2020). The HCMV IE genes include the major IE genes IE1 (unique long (UL) 123) and IE2 (UL122), which play a critical role in the efficiency of replication (Isomura and Stinski, 2003). The IE1 protein is necessary for efficient replication after a low multiplicity of infection (the ratio of the numbers of viral particles to the numbers of host cells) by antagonising the intrinsic and innate response to infection, and the IE2 protein is essential for early viral gene expression and viral replication (Isomura and Stinski, 2003).

Studies have yielded information regarding the proteins that compose the capsid, tegument, and envelope (glycoproteins) (Varnum *et al.*, 2004; Schleiss M.R., 2017). The HCMV virion is composed of many viral and cellular proteins, however the complete protein composition of the HCMV virion is unclear and may depend upon which strain is being studied and how that strain was prepared for analysis (Varnum *et al.*, 2004; Schleiss M.R., 2017). HCMV infection of cells in culture generates three different types of particles: infectious virions, non-infectious enveloped particles (NIEPs), and dense bodies. Infectious virions are composed of the same viral proteins as NIEPs, but NIEPs lack viral DNA (Varnum *et al.*, 2004). Dense bodies are uniquely characteristic of HCMV infection and are non-replicating enveloped particles composed primarily of the tegument protein

phosphoprotein(pp)65 (produced from the UL83 gene) (Varnum *et al.*, 2004). The quantities of these different HCMV particles are dependent on the viral strain and the multiplicity of infection (Varnum *et al.*, 2004). Mass spectrometry, used to identify and determine the relative abundances of viral and cellular proteins in the purified HCMV AD169 strain, revealed its overall predominant viral protein to be pp65 (Varnum *et al.*, 2004). The capsid of both NIEPs and infectious virions is assembled with five virally encoded proteins: UL86, UL85, UL80, UL48-49, and UL46 (Varnum *et al.*, 2004). The capsid is surrounded by a protein structure known as the tegument (Varnum *et al.*, 2004). Approximately 20 to 25 known HCMV virion-associated tegument proteins have been identified in virion preparations, many are phosphorylated and most have unknown functions (Varnum *et al.*, 2004). Cytoplasmic viral capsids containing tegument are enveloped with nine virally encoded glycoproteins: glycoprotein(g) B (UL55), gM (UL100), gH (UL75), gL (UL115), gO (UL74), gN (UL73), gp48 (UL4), gpTRL10, and UL33 (Varnum *et al.*, 2004). Previous studies have identified that there are approximately 30 to 60 viral proteins that compose a HCMV virion (Varnum *et al.*, 2004). In addition to viral proteins, a small number of cellular proteins, including aminopeptidase N, β 2-microglobulin, protein phosphatase I, annexin II, and actin-related protein 2/3, have been shown to be associated with the HCMV virion (Varnum *et al.*, 2004).

1.3. The potential of studying human cytomegalovirus shedding in pregnant women

As outlined above, HCMV infection is usually asymptomatic or associated with non-specific flu-like symptoms in immune-competent individuals, including pregnant women. The delay that commonly occurs in diagnosing a woman with maternal CMV infection due to its 'silent' nature, especially non-primary maternal infection in a HCMV-seropositive pregnant woman. Thus, HCMV infection is often diagnosed retrospectively following the detection of cCMV infection in her foetus or infant, such as on routine antenatal ultrasound scan, on newborn clinical examination, on routine newborn hearing screening, or when older children present with hearing loss or unexplained developmental delay. This means that the opportunities to diagnose, treat and potentially improve the outcome of maternal HCMV infection, especially in its manifestation of cCMV infection, are currently limited.

Both primary and non-primary HCMV infection, including in women during pregnancy, can result in excretion of HCMV particles in the individual's bodily fluids, such as (but not limited to) saliva and urine (Mocarski, Shenk and Pass, 2007; Cannon, Hyde and Schmid, 2011). This presence of HCMV in a bodily fluid, which may be detected by culture techniques or through HCMV DNA detection by PCR, can be referred to as HCMV shedding (Mocarski, Shenk and Pass, 2007; Cannon, Hyde and Schmid, 2011). HCMV shedding has the potential to transmit HCMV infection, where an individual can become infected with HCMV through contact with bodily fluids of another individual who is shedding HCMV (Boppana *et al.*, 2001; Mocarski, Shenk and Pass, 2007; Saldan *et al.*, 2017). Furthermore, even when the primary infection is resolved by an effective cellular immune response and has entered a

latent phase, HCMV may still be present in the bodily fluids with a potential to transmit HCMV infection (Mocarski, Shenk and Pass, 2007; Saldan *et al.*, 2017).

Young children may shed HCMV for a prolonged period, making this age group an important source of HCMV transmission to pregnant women (Mocarski, Shenk and Pass, 2007; Cannon, Hyde and Schmid, 2011; Griffiths, Baraniak and Reeves, 2015). Despite the burden of adverse health outcomes associated with cCMV infection, most pregnant women are not aware of HCMV and cCMV infection, and advice about it is not routinely provided as part of antenatal care in the United Kingdom (Cannon *et al.*, 2012; Wizman *et al.*, 2016; Montague *et al.*, 2022). A pregnant woman may reduce her risk of acquiring HCMV infection by the avoidance of direct mucosal contact with a young child's saliva and urine that may shed HCMV, such as not kissing directly on the lips, not sharing food and drink with them and hand washing after contact with saliva or urine (Adler *et al.*, 2004; Manicklal *et al.*, 2013; Barber *et al.*, 2020). There is evidence to suggest that this might be achieved through educational interventions (Adler *et al.*, 1996; Vauloup-Fellous *et al.*, 2009; Revello *et al.*, 2015).

If detection of HCMV shedding was shown to be a suitable method to diagnose maternal HCMV infection in a timely and non-invasive way, especially in challenging situations when the diagnosis is difficult such as with non-primary maternal HCMV infection in HCMV-seropositive pregnant women, it could be a very useful tool or a convenient endpoint for the evaluation of preventative and therapeutic strategies against maternal infection, and potentially against cCMV infection. First and foremost, a better understanding of the natural history of HCMV shedding in HCMV-seropositive women during pregnancy is paramount.

1.4. Summary and aims

1.4.1. Summary

cCMV infection continues to cause significant morbidity to the affected infants with far-reaching consequences to their families. Diagnosing non-primary maternal HCMV infection in HCMV-seropositive pregnant women is challenging. HCMV shedding in bodily fluids of HCMV-seropositive pregnant women may suggest that she has developed a non-primary maternal HCMV infection. Non-primary maternal HCMV infection in a HCMV-seropositive pregnant woman has the potential to transmit cCMV infection to her unborn infant. In fact, most cases of cCMV infection globally are due to non-primary maternal infections. Despite this there are significant gaps in knowledge about the diagnostic, preventative, and therapeutic measures of vertical transmission in HCMV-seropositive pregnant women. HCMV shedding may aid in identifying HCMV-seropositive pregnant women at higher risk of having an infant with cCMV infection, however the epidemiology and assessment of HCMV shedding in HCMV-seropositive pregnant women has also not been well studied.

1.4.2. Aims

To this end, I investigated HCMV shedding in HCMV-seropositive pregnant women through the study of literature, clinical samples, and experiences of participating women, with the aims outlined below:

1. To systematically review the prevalence of HCMV shedding in HCMV-seropositive pregnant women (Chapter three)
2. To investigate the prevalence of HCMV shedding in saliva, urine, and vaginal secretions of HCMV-seropositive women during pregnancy and postpartum period in a prospective study (Chapter four)
3. To investigate the HCMV-specific CMI in HCMV-seropositive women during pregnancy and postpartum period (Chapter four)
4. To investigate the feasibility of genomic sequencing of HCMV shedding isolates obtained from a study of HCMV-seropositive pregnant women (Chapter four)
5. To investigate the hygiene-related contact with children's bodily fluids of HCMV-seropositive women during pregnancy and postpartum period (Chapter five)
6. To investigate the experiences of HCMV-seropositive women participating in a study investigating HCMV shedding (Chapter five)
7. To investigate the feasibility of performing future large-scale studies to evaluate the relationship between HCMV shedding in HCMV-seropositive women during pregnancy with cCMV infection (Chapter five)
8. To investigate the risk factors for HCMV shedding in HCMV-seropositive women during pregnancy (Chapter six)

2. Methods

This chapter describes the methods used to address the specific research questions of the thesis. The methods were broadly divided into two categories: a systematic review (section [2.1](#), which covers Results chapter [3](#)) and an observational cohort study (section [2.2](#), which covers Results chapters [4](#), [5](#), and [6](#)).

2.1. Systematic review: The prevalence of human cytomegalovirus shedding during pregnancy up to delivery in seropositive women

2.1.1. Systemic review protocol and registration

The systematic review protocol was written in accordance with the criteria set out by the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (Shamseer *et al.*, 2015; Page *et al.*, 2021). The protocol was registered (registration number: CRD42021275205) with the International prospective register of systematic reviews (PROSPERO) (University of York, 2016) on 25th August 2021 ([Appendix A](#)).

2.1.2. Literature search strategy

The search strategy was developed with guidance from the academic liaison librarian and the Cochrane information specialist at St George's, University of London. The literature search was conducted on three electronic bibliographic databases; Medline, Embase, and Web of Science Core Collection. There were no publication period restrictions. The following

search terms were used in different combinations: cytomegalovirus, CMV, shedding, excretion, replication, DNA, PCR, culture, seropositive, IgG, pregnant, maternal. These search terms were chosen as they were related to or described the systematic review condition, context, and population as described under Inclusion Criteria. The search terms were adapted for use with the bibliographic database. The searches were re-run before the final analyses to identify further studies for inclusion.

2.1.3. Data inclusion criteria from literature search

As this was a systematic review of observational epidemiological studies reporting prevalence data, the *CoCoPop* mnemonic (condition, context, and population) was used to determine the inclusion criteria (Munn *et al.*, 2015) for the studies:

Condition: HCMV shedding, defined as the detection of HCMV by culture techniques or the detection of HCMV DNA by PCR, in any bodily fluid of pregnant or postpartum women. HCMV shedding could also be described as HCMV excretion or HCMV replication. Only studies with any of the possible definitions and descriptions of HCMV shedding in any bodily fluids of pregnant or postpartum women were included. Studies where only HCMV shedding in foetal bodily fluids were looked at were excluded.

Context: HCMV-seropositive status (defined as the detection of HCMV IgG in the serum), established prior to or at the beginning of HCMV shedding assessment. Those with suspicion of or confirmed primary maternal HCMV infection (detected HCMV IgM in the serum, seroconversion from non-detected to detected HCMV IgG in pregnancy with or without detected HCMV IgM, or low HCMV IgG avidity in the serum) were excluded.

Population: Pregnant women of any age and ethnicity, at any trimester including at delivery, and in any country. Women with suspicion of or confirmed immunosuppressive,

immunocompromised or immunodeficient condition or status, such as HIV-positive, transplant recipient, on immunosuppressive therapy, were excluded.

Observational studies including cohort, cross-sectional and case-control studies were included. Experimental studies, case reports, case series, review articles, full articles not in English, conference abstract and unpublished studies were excluded.

2.1.4. Systemic review objectives

Primary: To systematically review the prevalence of HCMV shedding in any bodily fluid at any point during pregnancy up to delivery in HCMV-seropositive women

Secondary: Meta-analyses of the prevalence estimates of HCMV shedding at any point during pregnancy up to delivery in HCMV-seropositive women, in any bodily fluid and according to bodily fluid site

2.1.5. Data extraction for systematic review

Step 1: The first reviewer (S.S) performed the search according to the *Search Strategy* to identify the studies. Identified studies were screened, deduplicated and catalogued on Mendeley Reference Manager.

Step 2: Abstracts of the studies retrieved from Step 1 were screened independently by two blind reviewers (S.S and A.T, see section [vii](#)) according to the *Search Strategy* using Rayyan Qatar Computing Research Institute to identify studies that potentially meet the *Inclusion Criteria*.

Step 3: Any disagreement between the two reviewers over the eligibility of abstracts from Step 2 would be resolved through discussion with a third reviewer (C.J, see section [vii](#)).

Step 4: Full-text articles of abstracts selected from Step 3 following consensus from all reviewers, were reviewed independently by two blind reviewers (S.S. and A.T) to identify studies that met the *Inclusion Criteria*.

Step 5: Any disagreement between the two reviewers over the eligibility of full articles from Step 4 would be resolved through discussion with the third reviewer (C.J).

Step 6: Data were extracted from the selected full-text articles from Step 5 following consensus from all reviewers, by the first reviewer (S.S). A standardised data extraction template was used to extract data from the included studies for data synthesis. Data extraction on studies included study design, country, setting and sample size. Data extraction on demographic included women's age, ethnicity, any details on their children. Data extraction on HCMV shedding included bodily fluid site(s), time-point(s), detection method(s), prevalence in any bodily fluid, and prevalence according to specific bodily fluid site. Data extraction was performed once.

Step 7: Extracted data from Step 6 were reviewed by the second reviewer (A.T).

Step 8: Any disagreements between the two reviewers over the extracted data from Step 7 would be resolved through discussion with the third reviewer (C.J).

2.1.6. Critical appraisal of selected studies for systematic review

Full-text articles selected from Step 5 of the *Data Extraction* were critically appraised independently by two blind reviewers (A.T and S.S) using the Joanna Briggs Institute Critical Appraisal Checklist for Prevalence Studies 2013 (Munn *et al.*, 2015), to assess the methodological quality and to determine the extent to which the studies have addressed the possibility of bias in its design, conduct and analysis. The results of this appraisal did not

preclude the studies from being included in the review but were used to inform data synthesis and interpretation of the study results.

2.1.7. Statistical analysis of the systematic review

A flow diagram of the identification of studies as derived from the *Data Extraction* was produced, adapted from the PRISMA 2020 flow diagram for systematic review (Page *et al.*, 2021). A narrative synthesis of the included studies, structured around the condition, context and population specified for this review, and the quality assessment based on the critical appraisal, was produced. Individual study characteristics structured around the data extraction categories were summarised in a descriptive table. Meta-analyses of the prevalence of HCMV shedding in HCMV-seropositive pregnant women, overall, and separately by bodily fluid site (where at least two studies reported prevalence data by the respected bodily fluid site that could be extracted), were performed to provide pooled estimates of HCMV shedding prevalence in HCMV-seropositive pregnant women, displayed as forest plots. The meta-analysis was performed using Microsoft Excel (Neyeloff, Fuchs and Moreira, 2012), grouped by random-effects model with 95% CI to allow for between-study variation, and to adjust the weight of each study based on the assumption that observed variability is due to both sampling error and true variability in the population (Neyeloff, Fuchs and Moreira, 2012; Munn *et al.*, 2015). Analysis of heterogeneity across the studies was performed through the calculation of Higgins I^2 (I^2) to assess the extent of inconsistency of findings across the studies included in the meta-analysis (Neyeloff, Fuchs and Moreira, 2012; Munn *et al.*, 2015). The minimum sample size for studies to include in the meta-analysis was 94, calculated based on the adequate sample size in prevalence study formula (Pourhoseingholi, Vahedi and Rahimzadeh, 2013); $n = [Z^2P(1-P)]/d^2$ ($Z=1.96$, $p=0.425$,

d=0.10). The prevalence estimated from the study with the highest prevalence was used in the prevalence study formula as the expected prevalence was not known. A forest plot with a pooled estimate of the effect sizes (Neyeloff, Fuchs and Moreira, 2012) on the overall prevalence of HCMV shedding in HCMV-seropositive pregnant women was created for each meta-analysis. Each study contributed with only one estimate for each meta-analysis to determine the pooled estimate.

2.2. Observational cohort study of human cytomegalovirus shedding in seropositive pregnant women

2.2.1. Ethical approval of observational cohort study

Screening of participants was ethically approved by the National Health Service (NHS) Health Research Authority (HRA), South-Central Oxford Research Ethics Committee (REC) (16/SC/0683), and London-Brent REC (19/LO/0161). Recruitment of participants, collection of completed questionnaires, saliva (including that of the participants' newborn infants), urine, vaginal and blood samples, and analysis of questionnaires and samples besides genomic sequencing, were ethically approved by the NHS HRA and London-Brent REC (19/LO/0161). Genomic sequencing analysis of HCMV DNA isolated from saliva, urine and vaginal secretions was ethically approved by the NHS HRA and London-Queen Square REC (17/LO/0916). All participants gave written informed consent, including towards testing their samples for further ethically approved research to help improve the understanding of infections.

2.2.2. Identification of study participants

Between 21st March 2019 to 3rd December 2020, identification of study participants ('study screening') for the observational cohort study took place in St George's Hospital, London, UK. Note that no participants were recruited between 13th March 2020 to 29th September 2020 due to the COVID-19 pandemic disruption. Pregnant women attending their antenatal combined screening and blood analysis appointment (occurring between 10 to 14 gestation weeks) were approached to seek their written informed consent to screen their blood for HCMV serostatus (in addition to the routine antenatal screening blood tests they were having done during this appointment), provided they met all the eligibility criteria:

- aged 18 years or more
- living with at least one child aged less than 4 years old
- sufficient understanding of verbal and written English

Their contact details and ethnicity were sought as part of this written consent. When a consented woman proceeded to have her routine antenatal screening blood tests (occurring between 10 to 14 gestation weeks), the clinical staff performing the blood tests were asked to collect additional blood for the screening blood test (5ml of venous blood placed into a serum separating blood tube) to test for HCMV IgG and HCMV IgM. The blood test form was completed with the woman's details and the screening blood test request label attached ([Appendix B](#)), and the screening participation notification label were attached to the women's antenatal clinical notes ([Appendix C](#)). Along with routine clinical blood samples, the screening blood samples were collected by the St George's Hospital NHS Trust sample collection service and delivered to the microbiology laboratory at St George's

Hospital NHS Trust, which is part of the NHS South West London Pathology (SWLP), to be tested for HCMV serology (see section [vii](#)). HCMV IgG and IgM titres were elucidated using their routine clinical HCMV enzyme immunoassay. The results of HCMV serology were reported as part of the routine clinical investigation results system (iCLIP) at St George's Hospital NHS Trust. The women were informed that if their HCMV serology results revealed that they were HCMV-seropositive (HCMV IgG detected, HCMV IgM not detected), they and their general practitioners (GPs) will be contacted with information about the observational cohort study. If the result revealed that they were HCMV-seronegative (HCMV IgG and IgM not detected), they would have been invited into a different study known as the *RACE-FIT* (*Reducing Acquisition of CMV through antenatal Education: A feasibility study to assess an educational intervention to prevent cytomegalovirus infection in pregnancy*; NCT04021628), which is not part of this thesis. For the observational cohort study, which is as part of this thesis, a copy of the signed written consent form is provided to the consented women ([Appendix D](#)). A poster about the study was also on display within the antenatal departments at St George's Hospital NHS Trust ([Appendix E](#)).

2.2.3. Recruitment of study participants for the observational cohort study

As outlined above, women who were found to be HCMV-seropositive with no serological evidence of acute infection (HCMV IgG detected, HCMV IgM not detected) were contacted via post ([Appendix F](#)) to notify them of their HCMV-seropositive results and contacted via telephone to recruit into the study. Additionally, their GPs were also informed ([Appendix G](#)). A face-to-face study appointment was arranged to seek informed written consent again ([Appendix H](#)) to recruit them into the observational cohort study, provided they met all the following eligibility criteria:

- living with at least one child aged less than 4 years old
- willing to have saliva, urine, and vaginal secretion sampling to be tested for HCMV DNA
- willing to be followed up until the postpartum period (6 weeks postpartum)
- no known or suspected immunodeficiency of any aetiology, including the use of oral steroid therapy equivalent to more than one milligram per kilogram of prednisone per day for more than one week

More information about the observational study was provided using a study information sheet ([Appendix I](#)). A copy of the signed written consent form was provided to the consented women, as well as to their GPs including a study participation letter to their GPs ([Appendix J](#)).

2.2.4. Study time-points for the observational cohort study

For all recruited women, four study time-points were planned:

- 1st time-point: As soon as possible in pregnancy following screening, aimed at up to 16 weeks and 6 days in gestational age ('early in pregnancy')
- 2nd time-point: From 17 weeks and 0 days to 26 weeks and 6 days in gestational age ('middle of pregnancy')
- 3rd time-point: From 27 weeks and 0 days in gestational age to the day before birth ('late in pregnancy')
- 4th time-point: Day 0 to day 42 post-birth ('postpartum period')

If a participant delivered prematurely, provided the participant was willing to continue with the study and the investigator had no concern with the participant remaining in the study, the subsequent time point would be the 4th time-point, even if a 3rd time-point was not conducted. The timing for each study time-point was chosen following a discussion

with the study management team and the South London Maternal and Paediatric Infection and Immunity Patient and Public Involvement group, to strike a balance of coinciding with routine clinical appointments to promote convenience of their participation and study compliance, while capturing study samples throughout pregnancy at varying stages of pregnancy. Where a visit was not possible to coincide with routine clinical appointments, standalone study appointments were scheduled. Text message reminders for each study time-points were sent to the contact numbers provided by the participating women on the day before the scheduled time-points, as well as text message reminders at 37 weeks of gestational age to remind them to inform the study team when they have given birth to conduct the 4th time-point.

After the 4th time-point, women who participated were also invited to take part in an end-of-study telephone interview.

2.2.5. Study parameters for the observational cohort study

The methods for each study parameter are described in detail, including each respective statistical analysis, in separate headings:

- HCMV serostatus distribution of women screened for the study
- Demographics of participants recruited into the study
- HCMV shedding of participants recruited into the study
- HCMV-specific cellular-mediated immunity of participants recruited into the study
- Genomic sequencing of HCMV of participants recruited into the study
- Screening for cCMV in newborn infants of participants recruited into the study
- Recruitment and retention of participants in the study
- Information seeking and familiarity with HCMV of participants recruited into the study

- Hygiene-related contact with children's bodily fluids of participants recruited into the study
- Study participation feedback and experience of participants recruited into the study

Some parameter findings were integrated together to investigate for risk factors of HCMV shedding in HCMV-seropositive pregnant women, with their respective methods also described in separate headings below. For some of these parameters*, multiple imputation using the multivariate imputation chained equation (MICE) technique were also performed as part of the logistic regression models, to account for the missing data to the HCMV shedding detection variables which were assumed to be missing at random and compare their findings with that in complete case analysis to take into consideration possible biased estimates (Graham, 2009). To this end, the probability of the missing value depended only on observed values and not on unobserved values where after controlling all the variables included in the imputation models, any remaining missingness was completely random (Graham, 2009):

- *The relationship between demographics and human cytomegalovirus shedding in seropositive pregnant women
- *The relationship between hygiene-related contact with children's bodily fluids and human cytomegalovirus shedding in seropositive pregnant women
- The relationship between human cytomegalovirus-specific cellular mediated immunity and human cytomegalovirus shedding in seropositive pregnant women
- Correlating hygiene-related contact with children's bodily fluids and human cytomegalovirus-specific cellular mediated immunity, with sequenced HCMV shedding in seropositive pregnant women

2.2.5.1. Investigation of human cytomegalovirus serostatus distribution in pregnant women screened for the observational cohort study

Women who consented to the study had their blood tested to assess for their HCMV serostatus as described in section 2.2.2.

Study data were collected and managed using REDCap electronic data capture tools hosted at St George's, University of London. Data was organised on Microsoft Excel 2019. Data analysis was conducted using STATA/SE 15.1. All missing data were assumed missing at random. Descriptive statistics with graphical display (pie chart) were generated on the women screened found to be HCMV-seropositive and HCMV-seronegative, with 2-sided 95% confidence interval (CI).

2.2.5.2. Investigation of demographics in pregnant women screened and recruited into the observational cohort study

Through the self-completion of a questionnaire at the 1st study time-point, information was collected on age, ethnicity, country of birth, duration of living in the UK, any other residing countries, highest education qualification, if they work regularly with children, living arrangements, amount and type of households, number of occupants per bedroom, and number and age of children.

Study data were collected and managed using REDCap electronic data capture tools hosted at St George's, University of London. Data was organised on Microsoft Excel 2019. Data analysis was conducted using STATA/SE 15.1. All missing data were assumed missing at random. Descriptive statistics with graphical display (bar and pie charts) were generated on the ethnicity distribution and HCMV seroprevalence rate according to ethnicity, of screened

and recruited women, according to the following categories: White British, White Other, Black, South Asian, Asian Other, and Mixed. Descriptive statistics on other demographic information collected were generated in tabular form.

2.2.5.3. Investigation of human cytomegalovirus shedding in participants of the observational cohort study

At each time-point, with the help of a written guide ([Appendix K](#)), samples of saliva, urine, and vaginal secretions were self-collected from participants to detect and quantify HCMV DNA using polymerase chain reaction. Self-sampling of saliva was performed using a swab called eNAT® (manufactured by Copan), a flocked swab in a tube packaged with 1.0 ml of Guanidine-thiocyanate based medium to stabilise the ribonucleic acid (RNA) and DNA of viruses (Copan Italia S.p.A., 2020). Self-sampling of urine was performed using a collection kit called cobas® PCR Media Urine Collection Kit (manufactured by Roche), a kit that contains a disposable transfer pipette to transfer the urine into a tube containing 4.3 ml of nucleic acid transport and storage stabilising medium (Roche Molecular Systems, 2015). Self-sampling of vaginal secretions was performed using cobas® PCR Media Dual Swab Sample Kit (manufactured by Roche), a kit that contains one woven, polyester bud swab (to be used), one flocked polyester swab (not to be used), and a tube containing 4.3 ml of nucleic acid transport and storage stabilising medium (Roche Molecular Systems, 2017). Following self-sampling of saliva, urine and vaginal secretions, the samples were transported at room temperature and stored in a freezer at -70 to -80 degree Celsius (°C) within eight hours of collection. During the UK government COVID-19 pandemic lockdown restrictions, the samples were stored in the participant's home freezer immediately following self-sampling using a written guide ([Appendix L](#)). Once COVID-19 lockdown

restrictions were lifted, the study team collected the frozen samples at the participants' homes and transported them at 0 to 4°C to the laboratory to be stored at -70 to -80°C. All stored samples were retrospectively tested for HCMV DNA in batches by N.K.T, with the assistance of S.S (see section [vii](#)). HCMV DNA detection and quantification were performed using the Roche cobas® 6800 real-time PCR system. Using the Conformité Européene (CE) marked and Food and Drug Administration (FDA) approved cobas® HCMV plasma assay as reference, the HCMV DNA PCR assays for saliva, urine and vaginal secretions were developed and validated with technical support from Roche Diagnostics and calibrated to the 1st World Health Organization (WHO) International Standard for HCMV (Tan, Pope and Carrington, 2022; Tan, Carrington and Pope, 2023). Detected HCMV DNA were quantified in international units/millilitres (IU/ml). The lower limit of detection of HCMV DNA for saliva, urine, and vaginal secretions were 200.0, 30.9, and 80.6 IU/ml respectively. The lower limit of quantification for saliva, urine, and vaginal secretions were 200.0, 100.0, and 100.0 IU/ml respectively. The upper limit of quantification for saliva, urine, and vaginal secretions was 250,000.0 IU/ml.

Study data were collected and managed using REDCap electronic data capture tools hosted at St George's, University of London. Data was organised on Microsoft Excel 2019. Data analysis was conducted using STATA/SE 15.1. A descriptive flow chart of collected and analysed samples were generated. All analysed samples with HCMV DNA PCR results in IU/ml were displayed in tabular form. Results with HCMV DNA detected but below the assay limits for quantification were assigned a value of 1.0 IU/ml for the purpose of statistical analysis. Descriptive statistics of the proportion of samples with HCMV shedding (i.e., HCMV DNA detected), and the median quantity of detection, were displayed in tabular and graphical forms where suitable with 2-sided 95% CI and interquartile range (IQR)

respectively, cumulatively and at each time-point. All missing data were assumed missing at random. Analysis on the prevalence of HCMV shedding were done with 2-sided 95% CI during pregnancy (1st to 3rd time-points) and including postpartum (1st to 4th time-points) separately. The distribution and quantity of HCMV shedding in women found to shed on at least one occasion were illustrated in graphical forms.

2.2.5.4. Investigation of human cytomegalovirus-specific cellular-mediated immunity in participants of the observational cohort study

At any study time-points when agreed, blood was collected to assess for HCMV-specific CMI as measured by two interferon gamma release assays, 1) HCMV-QuantiFERON and 2) HCMV-ELISPOT.

1. HCMV-QuantiFERON: For each blood sample, 1 ml of venous whole blood was collected by venepuncture into every assay blood collection tube: a negative control tube, a HCMV Antigen tube and a Mitogen (positive control) tube. Blood was then transported at room temperature to SWLP at St George's Hospital, to be tested by N.K.T, with the assistance of S.S (see section vii). The HCMV-QuantiFERON assay was used according to the manufacture's instruction (QIAGEN, 2018). Collected blood tubes were incubated at 37°C within 16 hours of collection for 16–24 hours, before being centrifuged for 15 minutes at 2000-3000 relative centrifugal force (RCF). The plasma was then harvested using a pipette and loaded directly into the QuantiFERON®-CMV ELISA plate to perform immediate testing or retained in the blood collection tubes and stored for up to 28 days at 2-8°C before being tested. Harvested plasma were examined with the HCMV-QuantiFERON assay (QIAGEN, 2018) using the QuantiFERON®-CMV ELISA kit included:

- microtitre plate of strips of individual wells coated with a mouse anti-human IFN- γ monoclonal antibody
- human IFN- γ *Standard* solution (recombinant human IFN- γ , bovine casein, 0.01% w/v Thimerosal)
- *Green Diluent* (bovine casein, normal mouse serum, 0.01% w/v Thimerosal)
- *Conjugate* reagent (mouse anti-human IFN- γ , horseradish peroxidase, 0.01% w/v Thimerosal)
- *Wash Buffer* (0.05% v/v ProClin® 300)
- *Substrate* solution (H₂O₂, 3,3',5,5' Tetramethylbenzidine)
- *Stop* solution (0.5 M H₂SO₄)

The *Standard* solution was reconstituted with deionised or distilled water and prepared in a dilution series of four IFN- γ concentrations (*Standards* 1-4) in *Green Diluent*. To perform ELISA for each blood sample, 50 microlitre (μ l) of *Conjugate* was added to the required wells. 50 μ l of test plasma samples and *Standards* 1-4 were added separately to the appropriate wells and incubated for 120 minutes at room temperature. During the incubation, *Wash Buffer* was prepared by diluting one part with 19 parts deionised or distilled water and mix thoroughly. The incubated plate was washed with prepared *Wash Buffer* at least 6 times and 100 μ l of *Substrate* was added. After a further incubation for 30 minutes at room temperature, 50 μ l of *Stop* was added and results were calculated using the QuantiFERON-CMV Analysis Software. HCMV-specific CMI were measured based on the level of secreted IFN- γ measured in IU/ml by the assay, when presented with a combined pool of HCMV antigens. With suitable quality control (negative control $\leq$ 8.0 IU/ml, positive control any result), a result was considered reactive (HCMV-specific CMI detected) for a measured IFN- γ level (HCMV Antigen minus

negative control results) of equal to or above 0.2 IU/ml and equal to and above 25% of the negative control value (QIAGEN, 2018). With suitable quality control (negative control ≤ 8.0 IU/ml, positive control ≥ 0.5 IU/ml), a result was considered non-reactive (HCMV-specific CMI not detected) for a measured IFN- γ level (HCMV Antigen minus negative control results) of less than 0.2 IU/ml or equal to or above 0.2 IU/ml but less than 25% of the negative control value (QIAGEN, 2018). A test was considered indeterminate when the positive control was less than 0.5 IU/ml with measured IFN- γ level (HCMV Antigen minus negative control results) of less than 0.2 IU/ml or equal to or above 0.2 IU/ml, or any result with a negative control value more than 8.0 IU/ml (QIAGEN, 2018).

2. HCMV-ELISPOT: For each blood sample, venous whole blood was collected into a lithium heparin blood collection tube. Blood was then transported at room temperature to a containment level 2 laboratory at St George's University of London (Institute for Infection & Immunity) for the preparation of peripheral blood mononuclear cells (PBMCs) (within 8 hours of blood draw). To isolate PBMCs, the heparin-treated blood was mixed with a balanced salt solution, Dulbecco's phosphate-buffered saline (D-PBS), at a 1:1 ratio. Without mixing, the diluted blood solution was then layered over a Ficoll solution (Histopaque-1077) within a conical tube at a 2:1 ratio (Figure 5A). The tube(s) containing diluted blood and Ficoll solution was centrifuged at 500 RCF, maximum acceleration, 20°C, and for 20 minutes. After centrifugation, several layers of different cell types at different density are formed, from bottom to top: erythrocytes, granulocytes, Ficoll, PBMCs and plasma (Figure 5B).

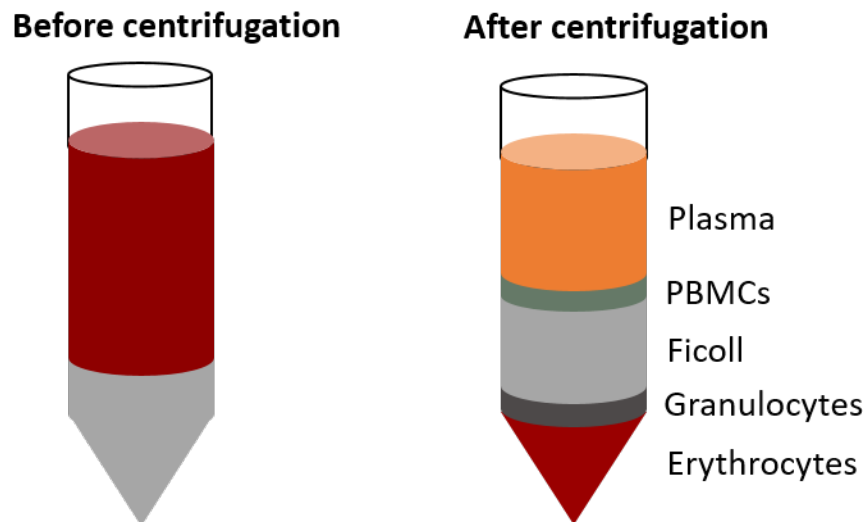


Figure illustrating the layers of different media and cells within a conical tube before and after centrifugation, as part of the PBMC preparation to perform the HCMV-ELISPOT assay.
 Abbreviations: ELISPOT, enzyme-linked immunosorbent spot; HCMV, human cytomegalovirus; PBMC, peripheral blood mononuclear cell (section [2.2.5.4](#)).

Figure 5. A. Layers of different media and cells within conical tube of blood and Ficoll solution before centrifugation. B. after centrifugation

The top layer of plasma was discarded (Figure 5, orange), revealing the layer of PBMCs (Figure 5, green) which were transferred into a new conical tube to be washed. PBMC washing was performed twice by mixing PBMCs with 37°C serum-free medium (Roswell Park Memorial Institute (RPMI) 1640), centrifuged at 400 RCF at 20°C (10 and 5 minutes for the 1st and 2nd wash, respectively, with brake and acceleration on the maximum setting), and removal of supernatant to leave only a cell pellet of PBMCs in the tube. The washed PBMC pellet resuspended in serum-free RPMI 1640 was examined. Isolated PBMCs were examined with the HCMV-ELISPOT assay according to the manufacture's instruction (Oxford Immunotec Ltd, 2017) using the T-SPOT.CMV test kit included:

- microtitre plate of strips of individual wells coated with a mouse monoclonal antibody to IFN- γ
- *IE1 solution* (CMV-A)
- *pp65 solution* (CMV-B)
- *Positive Control* (a mitogen solution containing phytohaemagglutinin)
- *Conjugate Reagent* (concentrated solution of mouse monoclonal antibody to IFN- γ conjugated to alkaline phosphatase)
- *Substrate Solution* (Novex™ alkaline phosphatase chromogenic substrate, made of 5-bromo-4-chloro-3-indolyl-1-phosphate and nitroblue tetrazolium)

Suspended PBMC pellet with serum-free AIM V™ medium was mixed with Trypan Blue at a ratio of 1:1, to determine the number of viable cells (cells that did not absorb the blue dye) present in the cell suspension using an automated haematology analyser, Bio-Rad TC20 Automated cell counter. The cell suspension was diluted to 2.5×10^6 PBMCs/ml in AIM V™ medium with guidance using the T-SPOT® handheld measurement guide. Fifty μ l of the following four solutions were added to the four-well strip plate (one solution

per well): AIM V™ medium (*Nil Control*), *IE1 Solution*, *pp65 Solution*, *Positive Control*.

One hundred µl of cell suspension (containing 250,000 PBMCs) were added to each well containing the solutions, and the plate are then incubated for spot development in a humidified incubator at 37°C with 5% carbon dioxide (CO₂) for 16-20 hours. After incubation, the cell culture medium was discarded from the plate. Cell washing was done by adding 200 µl of D-PBS to each well and discarded, four times. Fifty µL *Conjugate Reagent* solution (diluted 200-fold in D-PBS) was added to each well and incubated at 2-8°C for 1 hour. After incubation, the conjugate solution was discarded, and four cycles of D-PBS cell washing was performed again. Fifty µL of *Substrate Solution* was added to each well and incubated at room temperature for 7 minutes before being washed with distilled water and dried. Spot-forming cells were counted by the HCMV-ELISPOT manufacturer using an automated ELISPOT reader (*CTL CORE* model). HCMV-specific CMI were measured based on the count of spot-forming cells which represents IFN-γ secreting cells, when presented with the HCMV antigens IE1 and pp65 separately. The T-SPOT.CMV test results were interpreted by subtracting the spot count in the *Nil Control* well from the spot counts in CMV-A (IE1) and CMV-B (pp65) wells separately. A spot count of equal to or less than 20 in the *Positive Control* well would consider the result as 'indeterminate' (Oxford Immunotec Ltd, 2017). Following quality control interpretation, detected HCMV-specific CMI was a positive IE1 or pp65 spot count, where the number of spot count was also indicative of the strength of the cellular immune response to HCMV (Oxford Immunotec Ltd, 2017).

Study data were collected and managed using REDCap electronic data capture tools hosted at St George's, University of London. Data was organised on Microsoft Excel 2019. Data analysis was conducted using STATA/SE 15.1. All missing data were assumed missing at

random. All analysed samples with HCMV-QuantiFERON results in IU/ml and their respective HCMV-specific CMI interpretation were displayed in tabular form. Descriptive statistics of the proportion of samples with detected HCMV-specific CMI as measured by HCMV-QuantiFERON were described and displayed in graphical forms with 2-sided 95% CI. All analysed samples with HCMV-ELISPOT results in number of spot count and their respective HCMV-specific CMI interpretation were displayed in tabular form. Descriptive statistics of the proportion of samples with detected HCMV-specific CMI as measured by HCMV-ELISPOT were described and displayed in graphical forms with 2-sided 95% CI. Comparison of the descriptive statistics of the proportion of samples with detected CMI and the median spot count with 2-sided 95% CI and IQR respectively, between that towards IE1 and pp65 antigens, were displayed in tabular and graphical forms. Paired results of HCMV-ELISPOT towards IE1 and pp65 antigens were compared using McNemar test for the proportion of detected CMI and using Wilcoxon signed rank test for the number of spot count, using significance at 5%, cumulatively and at each time-point. Paired results of HCMV-QuantiFERON and HCMV-ELISPOT were displayed in tabular and graphical forms. Paired results of the spread of ELISPOT spot count towards IE1 and pp65 antigens when HCMV-specific CMI was detected or not detected by HCMV-QuantiFERON were compared using Mann-Whitney U test with significance at 5%, cumulatively and at each time-point. Cohen's kappa statistic test was performed to assess the level of agreement between HCMV-QuantiFERON and HCMV-ELISPOT on detecting and not detecting HCMV-specific CMI, cumulatively and at each time-point.

2.2.5.5. Investigation of genomic sequencing of human cytomegalovirus in participants of the observational cohort study

Genomic sequencing using the Illumina genomic sequencing technology was performed at the laboratories in University College London. Saliva, urine, and vaginal secretions samples with detected HCMV DNA at a quantity of more than 390.0 IU/ml on more than one occasion within a participant, were re-tested and extracted for HCMV DNA to be analysed again with real-time PCR, using the QIAamp® UltraSens® Virus kit (manufactured by Qiagen) according to the manufacture's instruction (QIAGEN, 2015), by C.A, with the assistance of S.S (see section [vii](#)). The kit included:

- QIAamp spin columns
- *Buffer AC* solution
- *Buffer AR* solution
- *Buffer AB* solution
- *Buffer AW1* solution
- *Buffer AW2* solution
- *Buffer AVE* solution
- *Carrier RNA* solution
- *Proteinase K* solution

At room temperature, 0.8ml of *Buffer AC* and 5.6 µl of *Carrier RNA* were added to 1 ml of sample and incubated for 10 minutes. The sample was then centrifuged at 1200 RCF for 3 minutes. After removal of supernatant, 300 µl of warmed (60°C) *Buffer AR* and 20 µl of proteinase K were added, and vortexed until the pellet was resuspended, and incubated for 10 minutes at 40°C. Three hundred µl of *Buffer AB* was added to the sample and vortexed to adjust binding conditions, and 700 µl of the lysate was applied to a QIAamp spin column and

centrifuged at 3000-5000 RCF for 1 minute. Remaining contaminants and enzyme inhibitors were removed by centrifugation in two wash steps with 500 µl Buffer AW1 at 6000 RCF for 1 minute and 500 µl Buffer AW2 at 20,000 RCF for 3 minutes respectively. The viral nucleic acids were eluted in *Buffer AVE* and centrifuged at 6000 RCF for 1 minute twice to ensure maximum recovery of viral nucleic acids (QIAGEN, 2015). Those samples with extracted HCMV DNA were subjected to genomic library preparation by C.W, where samples that produced genomic library pre-hybridisation were hybridised and sequenced. Genomic sequencing was performed according to the SureSelect^{XT} Target Enrichment System for the Illumina Platform protocol for 200 nanograms (ng) genomic DNA samples (Agilent Technologies, 2021) in steps described below:

1st: Sample preparation

- Step 1. Shear the DNA to prepare it for analysis and cleave it into appropriate fragment size
- Step 2. Assess the quality of DNA
- Step 3. Repair the ends of DNA
- Step 4. Purify the sample using *AMPure XP* beads
- Step 5. Deoxyadenosine-tail the 3' end of the DNA fragments
- Step 6. Purify the sample using *AMPure XP* beads
- Step 7. Ligate the paired-end adaptor
- Step 8. Purify the sample using *AMPure XP* beads
- Step 9. Amplify the adaptor-ligated library
- Step 10. Purify the amplified library with *AMPure XP* beads
- Step 11. Assess quality and quantity

2nd: Hybridisation and capture

- Step 1. Hybridise DNA samples to the probe
- Step 2. Prepare streptavidin-coated magnetic beads
- Step 3. Capture the hybridised DNA using streptavidin-coated beads

3rd: Indexing and sample Processing for multiplexed sequencing

- Step 1. Amplify the captured libraries with indexing primers
- Step 2. Purify the amplified captured libraries using *AMPure XP* beads
- Step 3. Assess indexed library DNA quantity and quality
- Step 4. Pool samples for multiplexed sequencing
- Step 5. Prepare sequencing samples

Samples that were hybridised and sequenced were subjected to quality control (more than 90% genome coverage) (Cudini *et al.*, 2019; Venturini *et al.*, 2022), where samples that passed the quality control were analysed by C.V, with the assistance of S.S. Analysis included assessing for within-host HCMV diversity (diversity of equal to or more than 0.005 raises the possibility of a mixed HCMV infection) (Cudini *et al.*, 2019; Venturini *et al.*, 2022) and the generation of a HCMV sequencing phylogenetic tree using SplitsTree software to compare with existing HCMV genomes sourced from GenBank sequence database, that are of similar and dissimilar ancestry based on Nucleotide Basic Local Alignment Search Tool (BLASTN) programme (Cudini *et al.*, 2019).

2.2.5.6. Investigation of congenital cytomegalovirus screening in newborn infants of participants in the observational cohort study

If written consent was provided by the participating women and agreed in real time, at the 4th time-point saliva was collected from their newborn infants to test for HCMV DNA using PCR (described in section 2.2.8).

The proportion of newborn infants who were tested for HCMV DNA and who were found to have HCMV DNA detected were calculated with 2-sided 95% CI, as well as the proportion of those with their mothers found to have HCMV shedding in their bodily fluids on at least one occasion during pregnancy, also calculated with 2-sided 95% CI.

2.2.5.7. Investigation of recruitment and retention in participants of the observational cohort study

Information was collected on the numbers of women who were approached for HCMV serostatus screening, who did or did not meet the eligibility criteria, and who were successfully recruited into the HCMV serostatus screening for the observational cohort study. Information was also collected on the numbers of women who were recruited into the observational cohort study, who completed each time-point, and who completed the study. The definition of completing a time-point is the collection of at least one sample of saliva, urine, or vaginal secretions, at said time-point. The definition of completing a study is completing the 4th time-point.

Study data were collected and managed using REDCap electronic data capture tools hosted at St George's, University of London. Data was organised on Microsoft Excel 2019. Data analysis was conducted using STATA/SE 15.1. All missing data were assumed missing at random. Descriptive flow charts were generated to illustrate the number and proportion of women who were approached and eligible for study screening, recruited into the observational cohort study, and completed each time-point and the study. The margin of error of recruited participants were compared descriptively to that of the target sample size (see section [2.2.21](#)).

2.2.5.8. Investigation of information seeking and familiarity with human cytomegalovirus in participants of the observational cohort study

Through the self-completion of a questionnaire at the 1st and 4th time-points (at the beginning and at the end of the study respectively), information was collected on the sources of information about HCMV if sought, and on their level of agreement, using a five-point Likert scale with six statements, about their perceptions on how HCMV infection is spread (Price *et al.*, 2014).

Study data were collected and managed using REDCap electronic data capture tools hosted at St George's, University of London. Data was organised on Microsoft Excel 2019. Data analysis was conducted using STATA/SE 15.1. All missing data was assumed missing at random. The perceptions on how HCMV infection is spread at the beginning of the study were analysed descriptively using mean scores (out of 5, where higher mean scores indicated higher level of agreement) with 2-sided 95% CI and displayed in tabular form. Paired t-test was performed comparing the perception means scores on how HCMV infection is spread at the beginning and at the end of the study, with 2-sided 95% CI and significance at 5%.

2.2.5.9. Investigation of hygiene-related contact with children's bodily fluids in participants of the observational cohort study

At each time-point, through the self-completion of questionnaires, information was collected on the frequency (using a five-point Likert scale) of specific hygiene behaviours (based on fourteen statements) in relation to participants' contact with their children's

bodily fluids (such as saliva, mucus, urine, and faeces) over the preceding two weeks, defined as “hygiene-related contact with children’s bodily fluids”.

Study data were collected and managed using REDCap electronic data capture tools hosted at St George’s, University of London. Data was organised on Microsoft Excel 2019. Data analysis was conducted using STATA/SE 15.1. All missing data was assumed missing at random. The reported frequencies of hygiene-related contact with children’s bodily fluids by HCMV-seropositive women at each time-point were analysed descriptively using mean scores (out of 5, where higher mean scores indicated higher level of frequency) with 2-sided 95% CI and displayed in tabular form. Using repeated measures within-subjects analysis of variance (ANOVA) with significance at 5%, the frequency mean scores of hygiene-related contact with their children’s bodily fluids between different time-points were analysed.

2.2.5.10. Investigation of study feedback in participants of the observational cohort study

Through the self-completion of a questionnaire at the 4th time-point, information was collected on their level of agreement, using a five-point Likert scale with 32 statements, about their feedback on screening, recruitment, study procedures and structure.

Study data were collected and managed using REDCap electronic data capture tools hosted at St George’s, University of London. Data was organised on Microsoft Excel 2019. Data analysis was conducted using STATA/SE 15.1. All missing data was assumed missing at random. Feedback was analysed descriptively using mean scores (out of 5, where higher mean scores indicated higher level of agreement) with 2-sided 95% CI and displayed in tabular form.

2.2.5.11. Investigation of study participation experiences in participants of the observational cohort study

At the end of the observational cohort study, women who participated were invited to take part in an end-of-study, individual, semi-structured, interview to evaluate their experiences of study participation. The interview guide consisted of open and closed-ended questions with prompts (Figure 6), which were written by S.S with guidance from T.V (see section vii), designed around each study procedure to identify as much study participation experiences as possible. The series of questions were divided into two categories: the first was to explore the participants' experiences on their participation focusing on the specific study procedures (described as 'direct' experiences, see Figure 6, black font), and the second was to evaluate any unintended consequences of study participation (described as 'indirect' experiences, see Figure 6, blue font). The interviews were conducted by phone, which were audio-recorded and transcribed verbatim.

We will ask you some questions about your experience of being in the study and how you think it could be improved.

First, we would like to ask you about your 'direct' experience of participating in the study:

- *Why did you decide to participate in the study?*
- *What did you like about the study?*
- *What did you learn by participating in the study?*
- *What (if anything) did you find inconvenient or difficult?*
- *Did you feel comfortable being approached for the first time about the study in the combined screening clinic? Did you feel you had enough information about the study before agreeing to have your blood screened for CMV? Is there any other information you would have liked at this stage?*
- *Did you discuss your participation with someone else before you decided to participate?*
- *What was your experience of having to complete the Background Questionnaire at the beginning?*
- *What was your experience of having to complete the Contact Questionnaire at each study visit? Was having it emailed to you most convenient? Do you remember if you complete it at every stage?*
- *What was your experience of having to complete the Feedback Questionnaire at the end? Was having it emailed to you most convenient?*
- *What was your experience of having to do the self-sampling of saliva, urine and vaginal secretions?*
- *Was self-sampling instead of sampling by a study team most convenient?*
- *Did you give bloods as part of the study? What was your experience of this?*
- *How did you feel about having four study visits?*
- *How did you feel about the study timings?*
- *Is receiving a text reminder for the visits most convenient?*
- *How easy or difficult was it to contact the study team (if you did) at the time of delivery?*
- *How did you feel about your baby being offered testing for congenital CMV infection? Did your baby have the test? If so, how did you feel before and after the test? What about how your partner felt?*
- *As a result of participating in the study did you access more information about CMV? If so, where from? Did you find the information you access useful?*
- *What changes would you make in the way the study was conducted?*

Now, we would like to ask you about any 'indirect experience' of participating in the study:

- *Has your participation in the study led you to make any changes to your behaviours? To guide you with your answers, we will prompt each behaviour according to the study contact questionnaire:*
 - *I wash my hands with soap after changing a dirty (poo) nappy*
 - *I clean my hands with alcohol gel after changing a dirty (poo) nappy*
 - *I wash my hands with soap after changing a wet nappy (urine only)*
 - *I clean my hands with alcohol gel after changing a wet nappy (urine only)*
 - *I wash my hands with soap after wiping my child(ren)'s nose*
 - *I clean my hands with alcohol gel after wiping my child(ren)'s nose*
 - *I put my child(ren)'s dummy in my mouth (for example, if fallen on floor)*
 - *I eat left-overs on my child(ren)'s plate*
 - *I share cutlery with my child(ren) to eat with after they have used it*
 - *I drink from my child(ren)'s cup or bottle after they have had a drink from it*
 - *I kiss my child(ren) on the lips*
 - *Any other behaviour*
- *If you did, why do you think you changed your behaviour? How easy was it to change these behaviours? What helped you? What did you find particularly difficult to change? What made it difficult? Are there ways in which this could be made less difficult?*
- *Did you discuss your behaviour change with your partner? If so, what changes did he/she make? How supportive was your partner/family? Did he/she find it easy/difficult to incorporate these changes?*
- *Did you discuss this with your family members or friends? How supportive were they?*
- *If your behaviours changed, have they become normal in your household? Which ones? If no, why?*

Figure illustrating the Interview guide. It consisted of open and closed-ended questions, divided into two categories: the first (black font) was to explore the participants' experiences on their participation in the observational cohort study focusing on the study procedures, and the second (blue font) was to evaluate any subsequent non-specifically directed consequences of study participation (section 2.2.5.11). Abbreviations: CMV, cytomegalovirus.

Figure 6. Post-study interview guide

Descriptive statistics of the demographics of interviewed women were displayed in tabular form. The transcribed interview data was analysed following the six phases of Thematic Analysis described by Braun and Clarke: 1) familiarisation; 2) coding; 3) generating themes; 4) reviewing themes; 5) defining and naming themes; and 6) reporting (Braun and Clarke, 2006). The codes, themes and subthemes generated were summarised in tabular forms. Where extracted quotes by the participants were included in the report, these are written in italic font and signified by the double quotation marks, and any omitted material are signified by the symbol '...'.

2.2.5.12. Investigation of the relationship between demographics and human cytomegalovirus shedding in participants of the observational cohort study

A full description of the investigation of demographics and of HCMV shedding, in participants of the observational cohort study, can be found in sections [2.2.7](#) and [2.2.8](#) respectively.

Study data were collected and managed using REDCap electronic data capture tools hosted at St George's, University of London. Data was organised on Microsoft Excel 2019. Data analysis was conducted using STATA/SE 15.1. All missing data was assumed missing at random. Simple and multiple logistic regression (LR) to calculate odds ratio (OR) using significance at 5% and 2-sided 95% CI, were analysed using the detection of HCMV shedding as the outcome variable and the demographic variables as the predictor variables, with and without multiple imputation (MI). The MI model used was multivariate imputation by chained equations (MICE), to impute missing values in the detection of HCMV shedding, repeated 20 times. The LR model was performed separately with HCMV shedding detected

at different sites (saliva, urine, vaginal secretions, and in any bodily fluid) and time-points (1st, 2nd, 3rd, and at any time-point during pregnancy) as the outcome variable.

2.2.5.13. Investigation of the relationship between hygiene-related contact with children's bodily fluids and human cytomegalovirus shedding in participants of the observational cohort study

A full description of the investigation of hygiene-related contact with children's bodily fluids and of HCMV shedding, in participants of the observational cohort study, can be found in sections [2.2.8](#) and [2.2.14](#) respectively.

Study data were collected and managed using REDCap electronic data capture tools hosted at St George's, University of London. Data was organised on Microsoft Excel 2019. Data analysis was conducted using STATA/SE 15.1. All missing data was assumed missing at random. At each time point during pregnancy, simple and multiple LR to calculate OR using significance at 5% and 2-sided 95% CI, were analysed using the detection of HCMV shedding as the outcome variable and the hygiene-related contact variables as the predictor variables, with and without multiple imputation (MI). The MI model used was multivariate imputation by chained equations (MICE), to impute missing values in the detection of HCMV shedding, repeated 20 times. The LR model was performed separately with HCMV shedding detected at different sites (saliva, urine, vaginal secretions, and in any bodily fluid) as the outcome variable.

2.2.5.14. Investigation of the relationship between human cytomegalovirus-specific cellular mediated immunity and human cytomegalovirus shedding in participants of the observational cohort study

A full description of the investigation of HCMV-specific CMI and of HCMV shedding, in participants of the observational cohort study, can be found in sections [2.2.8](#) and [2.2.9](#) respectively.

Study data were collected and managed using REDCap electronic data capture tools hosted at St George's, University of London. Data was organised on Microsoft Excel 2019. Data analysis was conducted using STATA/SE 15.1. All missing data was assumed missing at random. At each time point during pregnancy, simple and adjusted (to age and ethnicity) multiple LR to calculate OR using significance at 5% and 2-sided 95% CI, were analysed using the detection of HCMV shedding as the outcome variable and the HCMV-specific variables as the predictor variables. MI was not suitable as there was a large number of missing data in both the outcome and predictor variables to be imputed. The LR model was performed separately with HCMV shedding detected at different sites (saliva, urine, vaginal secretions, and in any bodily fluid) as the outcome variable.

2.2.5.15. Investigation of the relationship between hygiene-related contact with children's bodily fluids, human cytomegalovirus-specific cellular mediated immunity, and sequenced HCMV shedding, in participants of the observational cohort study

A full description of the investigation of hygiene-related contact with children's bodily fluids, of HCMV-specific CMI, and of genomic sequencing of HCMV shedding, in

participants of the observational cohort study, can be found in sections [2.2.9](#), [2.2.10](#) and [2.2.14](#) respectively.

Results of hygiene-related contact with children's bodily fluids and HCMV-specific CMI of HCMV-seropositive women at the same time-point (2nd) when their HCMV shedding in vaginal secretions were sequenced, were summarised in tabular form, and described. The HCMV-ELISPOT spot counts towards IE1 and pp65 were compared to the median counts of other HCMV-seropositive pregnant women at the same time-point.

2.2.6. Sample size for the observational cohort study

Target sample sizes were calculated for i) the number of HCMV-seropositive women to be recruited into the observational cohort study, and ii) the number of participants recruited into the observational cohort study to do the end of study interview. For the number of HCMV-seropositive women to be recruited into the observational cohort study, the sample size calculation was to achieve an acceptably precise estimate of the percentage of HCMV-seropositive women who shed HCMV during pregnancy. Using an estimated proportion (prevalence of HCMV shedding at least once in any bodily fluid in HCMV-seropositive women throughout pregnancy) of 30% based on a previous study (Barbosa *et al.*, 2018), a target sample size of 200 participants would provide a 95% confidence levels for this estimate of 6% precision (i.e., 24-36%). For the number of participants to do the end-of-study interview, the sample size calculation was to achieve data saturation, that is until additional interviews will not provide new information over and above that already obtained. Based on a previous study, a target sample size of 20 interviews would likely reach data saturation (Hagaman and Wutich, 2017).

2.3. Statistical analysis

2.3.1. Statistical analysis of the systematic review

A flow diagram of the identification of studies as derived from the *Data Extraction* was produced, adapted from the PRISMA 2020 flow diagram for systematic review (Page *et al.*, 2021). A narrative synthesis of the included studies, structured around the condition, context and population specified for this review, and the quality assessment based on the critical appraisal, was produced. Individual study characteristics structured around the data extraction categories were summarised in a descriptive table. Meta-analyses of the prevalence of HCMV shedding in HCMV-seropositive pregnant women, overall, and separately by bodily fluid site (where at least two studies reported prevalence data by the respected bodily fluid site that could be extracted), were performed to provide pooled estimates of HCMV shedding prevalence in HCMV-seropositive pregnant women, displayed as forest plots. The meta-analysis was performed using Microsoft Excel (Neyeloff, Fuchs and Moreira, 2012), grouped by random-effects model with 95% CI to allow for between-study variation, and to adjust the weight of each study based on the assumption that observed variability is due to both sampling error and true variability in the population (Neyeloff, Fuchs and Moreira, 2012; Munn *et al.*, 2015). Analysis of heterogeneity across the studies was performed through the calculation of Higgins I^2 (I^2) to assess the extent of inconsistency of findings across the studies included in the meta-analysis (Neyeloff, Fuchs and Moreira, 2012; Munn *et al.*, 2015). The minimum sample size for studies to include in the meta-analysis was 94, calculated based on the adequate sample size in prevalence study formula (Pourhoseingholi, Vahedi and Rahimzadeh, 2013); $n = [Z^2P(1-P)]/d^2$ ($Z=1.96$, $p=0.425$,

d=0.10). The prevalence estimated from the study with the highest prevalence was used in the prevalence study formula as the expected prevalence was not known. A forest plot with a pooled estimate of the effect sizes (Neyeloff, Fuchs and Moreira, 2012) on the overall prevalence of HCMV shedding in HCMV-seropositive pregnant women was created for each meta-analysis. Each study contributed with only one estimate for each meta-analysis to determine the pooled estimate.

2.3.2. Statistical analysis of the observational cohort study

Study data were collected and managed using REDCap electronic data capture tools hosted at St George's, University of London. Data was organised on Microsoft Excel 2019. Data analysis was conducted using STATA/SE 15.1. All missing data were assumed missing at random.

Descriptive statistics with graphical display (pie chart) were generated on the women screened found to be HCMV-seropositive and HCMV-seronegative, with 2-sided 95% confidence interval (CI).

Descriptive statistics with graphical display (bar and pie charts) were generated on the ethnicity distribution and HCMV seroprevalence rate according to ethnicity, of screened and recruited women, according to the following categories: White British, White Other, Black, South Asian, Asian Other, and Mixed. Descriptive statistics on other demographic information collected were generated in tabular form.

A descriptive flow chart of collected and analysed saliva, urine, and vaginal secretion samples were generated. All analysed samples with HCMV DNA PCR results in IU/ml were displayed in tabular form. Results with HCMV DNA detected but below the assay limits for quantification were assigned a value of 1.0 IU/ml for the purpose of statistical analysis.

Descriptive statistics of the proportion of samples with HCMV shedding (i.e., HCMV DNA detected), and the median quantity of detection, were displayed in tabular and graphical forms where suitable with 2-sided 95% CI and interquartile range (IQR) respectively, cumulatively and at each time-point. All missing data were assumed missing at random. Analyses on the prevalence of HCMV shedding were done separately on data that included missing data and data that excluded missing data and described in the results, with 2-sided 95% CI. Results during pregnancy (1st to 3rd time-points) and including postpartum (1st to 4th time-points) were also analysed and described separately. The distribution and quantity of HCMV shedding in women found to shed on at least one occasion were illustrated in graphical forms.

All analysed blood samples with HCMV-QuantiFERON results in IU/ml and their respective HCMV-specific CMI interpretation were displayed in tabular form. Descriptive statistics of the proportion of samples with detected HCMV-specific CMI as measured by HCMV-QuantiFERON were described and displayed in graphical forms with 2-sided 95% CI.

All analysed blood samples with HCMV-ELISPOT results in number of spot count and their respective HCMV-specific CMI interpretation were displayed in tabular form. Descriptive statistics of the proportion of samples with detected HCMV-specific CMI as measured by HCMV-ELISPOT were described and displayed in graphical forms with 2-sided 95% CI.

Comparison of the descriptive statistics of the proportion of samples with detected CMI and the median spot count with 2-sided 95% CI and IQR respectively, between that towards IE1 and pp65 antigens, were displayed in tabular and graphical forms. Paired results of HCMV-ELISPOT towards IE1 and pp65 antigens were compared using McNemar test for the proportion of detected CMI and using Wilcoxon signed rank test for the number of spot count, using significance at 5%, cumulatively and at each time-point. Paired results of

HCMV-QuantiFERON and HCMV-ELISPOT were displayed in tabular and graphical forms. Paired results of the spread of ELISPOT spot count towards IE1 and pp65 antigens when HCMV-specific CMI was detected or not detected by HCMV-QuantiFERON were compared using Mann-Whitney U test with significance at 5%, cumulatively and at each time-point. Cohen's kappa statistic test was performed to assess the level of agreement between HCMV-QuantiFERON and HCMV-ELISPOT on detecting and not detecting HCMV-specific CMI, cumulatively and at each time-point.

All saliva, urine and vaginal secretion samples that were retested for HCMV DNA extraction in preparation for genomic library sequencing, that were later hybridised and sequenced, were displayed in tabular forms. The quality control analysis of hybridised and sequenced samples were also displayed in tabular forms. Analysis of samples that passed the quality control included assessing for within-host HCMV diversity (diversity of equal to or more than 0.005 raises the possibility of a mixed HCMV infection) (Cudini *et al.*, 2019; Venturini *et al.*, 2022) and the generation of a HCMV sequencing phylogenetic tree using SplitsTree software to compare with existing HCMV genomes sourced from GenBank sequence database, that are of similar and dissimilar ancestry based on Nucleotide Basic Local Alignment Search Tool (BLASTN) programme (Cudini *et al.*, 2019).

The proportion of newborn infants who were tested for HCMV DNA and who were found to have HCMV DNA detected were calculated with 2-sided 95% CI, as well as the proportion of those with their mothers found to have HCMV shedding in their bodily fluids on at least one occasion during pregnancy, also calculated with 2-sided 95% CI.

Descriptive flow charts were generated to illustrate the number and proportion of women who were approached and eligible for study screening, recruited into the observational cohort study, and completed each time-point and the study. The margin of

error of recruited participants were compared descriptively to that of the target sample size (see section [2.2.6](#)).

The perceptions on how HCMV infection is spread at the beginning of the study were analysed descriptively using mean scores (out of 5, where higher mean scores indicated higher level of agreement) with 2-sided 95% CI and displayed in tabular form. Paired t-test was performed comparing the perception means scores on how HCMV infection is spread at the beginning and at the end of the study, with 2-sided 95% CI and significance at 5%.

The reported frequencies of hygiene-related contact with children's bodily fluids by HCMV-seropositive women from the self-completed questionnaire at each time-point were analysed descriptively using mean scores (out of 5, where higher mean scores indicated higher level of frequency) with 2-sided 95% CI and displayed in tabular form. Using repeated measures within-subjects analysis of variance (ANOVA) with significance at 5%, the frequency mean scores of hygiene-related contact with their children's bodily fluids between different time-points were analysed.

Feedback from the self-completed questionnaire was analysed descriptively using mean scores (out of 5, where higher mean scores indicated higher level of agreement) with 2-sided 95% CI and displayed in tabular form.

Descriptive statistics of the demographics of women that participated in the end-of-study interview were displayed in tabular form. The transcribed interview data was analysed following the six phases of Thematic Analysis described by Braun and Clarke: 1) familiarisation; 2) coding; 3) generating themes; 4) reviewing themes; 5) defining and naming themes; and 6) reporting (Braun and Clarke, 2006). The codes, themes and subthemes generated were summarised in tabular forms. Where extracted quotes by the

participants were included in the report, these are written in italic font and signified by the double quotation marks, and any omitted material are signified by the symbol '...'.

Some parameter findings were integrated together to investigate for risk factors of HCMV shedding in HCMV-seropositive pregnant women. For some of these parameters, multiple imputation using the multivariate imputation chained equation (MICE) technique were also performed as part of the logistic regression models, to account for the missing data to the HCMV shedding detection variables which were assumed to be missing at random and compare their findings with that in complete case analysis to take into consideration possible biased estimates (Graham, 2009). To this end, the probability of the missing value depended only on observed values and not on unobserved values where after controlling all the variables included in the imputation models, any remaining missingness was completely random (Graham, 2009).

Simple and multiple logistic regression (LR) to calculate odds ratio (OR) using significance at 5% and 2-sided 95% CI, were analysed using the detection of HCMV shedding as the outcome variable and the demographic variables as the predictor variables, with and without MICE, to impute missing values in the detection of HCMV shedding, repeated 20 times. The LR model was performed separately with HCMV shedding detected at different sites (saliva, urine, vaginal secretions, and in any bodily fluid) and time-points (1st, 2nd, 3rd, and at any time-point during pregnancy) as the outcome variable.

At each time point during pregnancy, simple and multiple LR to calculate OR using significance at 5% and 2-sided 95% CI, were analysed using the detection of HCMV shedding as the outcome variable and the hygiene-related contact variables as the predictor variables, with and without MICE, to impute missing values in the detection of HCMV shedding, repeated 20 times. The LR model was performed separately with HCMV shedding

detected at different sites (saliva, urine, vaginal secretions, and in any bodily fluid) as the outcome variable.

At each time point during pregnancy, simple and adjusted (to age and ethnicity) multiple LR to calculate OR using significance at 5% and 2-sided 95% CI, were analysed using the detection of HCMV shedding as the outcome variable and the HCMV-specific variables as the predictor variables. MICE was not suitable as there was a large number of missing data in both the outcome and predictor variables to be imputed. The LR model was performed separately with HCMV shedding detected at different sites (saliva, urine, vaginal secretions, and in any bodily fluid) as the outcome variable.

Results of hygiene-related contact with children's bodily fluids and HCMV-specific CMI of HCMV-seropositive women at the same time-points when their HCMV shedding in vaginal secretions were sequenced, were summarised in tabular form, and described. The HCMV-ELISPOT spot counts towards IE1 and pp65 were compared to the median counts of other HCMV-seropositive pregnant women at the same time-point.

3. Systematic review: The prevalence of human cytomegalovirus shedding during pregnancy up to delivery in seropositive women

3.1. Background

I sought to systematically review the prevalence of HCMV shedding in HCMV-seropositive pregnant women. A full description of the methods can be found in section [2.1](#).

3.2. Results

3.2.1. Search strategy and study selection

The search strategy and number of records identified (total underlined) used for the database during the final search before the final analyses accessed on 7th December 2021, are described below:

MEDLINE:	Search strategy	Number of records
	1 cmv.ti.	3338
	2 cytomegalovirus.ti.	23897
	3 shed*.tw.	100233
	4 excret*.tw.	177052
	5 replicat*.tw.	307142
	6 dna.tw.	1058718
	7 pcr.tw.	538521

8	culture.tw.	607491
9	seropositiv*.tw.	40287
10	igg.tw.	142743
11	pregnan*.tw.	525404
12	matern*.tw.	290291
13	exp Cytomegalovirus/	21607
14	exp virus shedding/	3869
15	exp pregnant woman/	10143
16	1 or 2 or 13	32682
17	3 or 4 or 5 or 6 or 7 or 8 or 14	2447886
18	9 or 10	177111
19	11 or 12 or 15	692267
20	16 and 17 and 18 and 19	<u>185</u>

Embase:	Search strategy	Number of records
1	cmv.ti.	6044
2	cytomegalovirus.ti.	28079
3	shed*.tw.	119671
4	excret*.tw.	199564
5	replicat*.tw.	367776
6	dna.tw.	1260290
7	pcr.tw.	778640
8	culture.tw.	760810
9	seropositiv*.tw.	50883

10	igg.tw.	194568
11	pregnan*.tw.	675147
12	matern*.tw.	375166
13	exp Cytomegalovirus/	42232
14	exp virus shedding/	8021
15	exp pregnant woman/	90110
16	1 or 2 or 13	55181
17	3 or 4 or 5 or 6 or 7 or 8 or 14	3040571
18	9 or 10	237359
19	11 or 12 or 15	878644
20	16 and 17 and 18 and 19	<u>337</u>

Web of Science Core Collection

Search Strategy (TI=(cmv) OR TI=(cytomegalovirus)) AND (TS=(shed*) OR TS=(excret*) OR TS=(DNA) OR TS=(PCR) OR TS=(culture)) AND (TS=(seropositive) OR TS=(IgG)) AND (TS=(pregnan*) OR TS=(maternal))

Number of records 187

Out of 709 articles generated, 395 articles were identified for abstract evaluation following deduplication. Only one article (Zelini *et al.*, 2022) from the final search was not identified during the initial search on 25th August 2021, which was selected for both abstract and full article inclusion in the systematic review by the first and second reviewers independently, in addition to the eight studies making it nine studies in total for inclusion in

the systematic review. Three studies initially appeared to have met the inclusion criteria but were excluded: the seropositive status of the pregnant women with HCMV shedding was unclear in two studies (Fujikawa *et al.*, 2003; Al-Mousawi and AL-Hajjar, 2021), and the source of shedding was of a foetal bodily fluid for one study (Wenig *et al.*, 2008). A simplified flow diagram describing the study selection and search strategy is displayed in Figure 7.

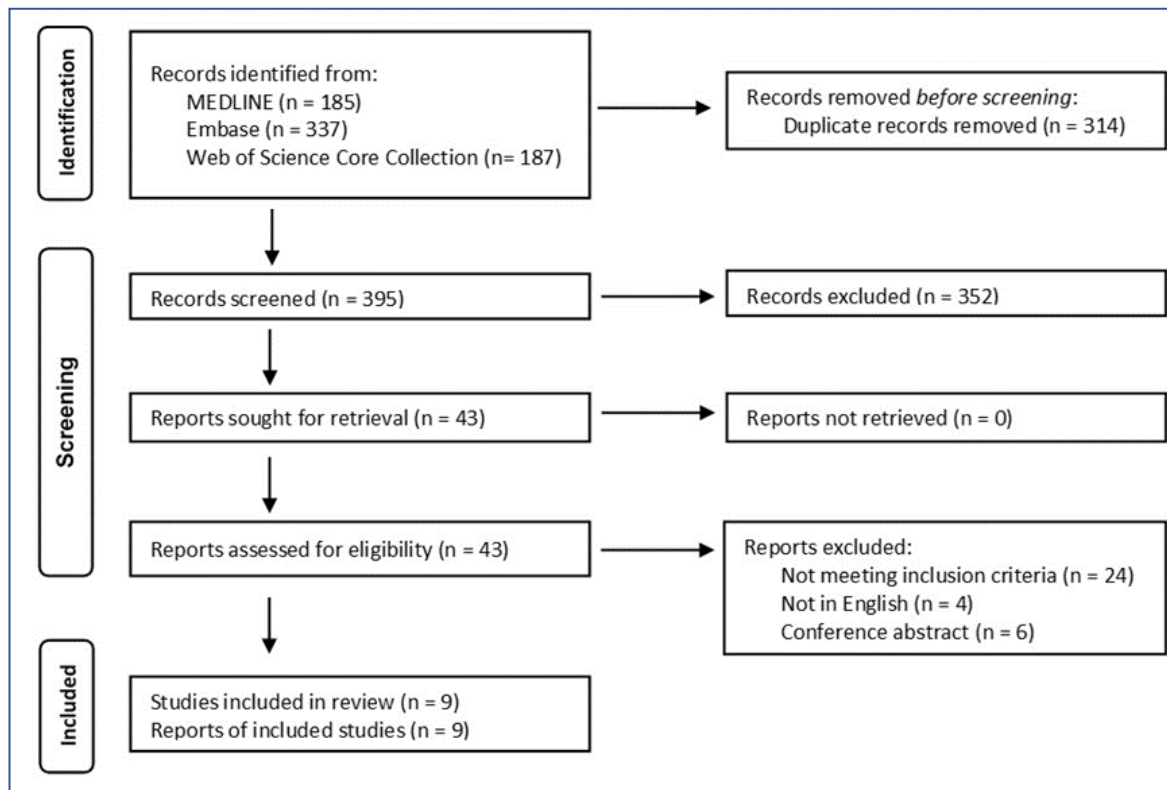


Figure illustrating the study selection and search strategy used for the identification of studies in the systematic review (see section 2.1 for the methods used, and section 3.2 for the description of results).
Abbreviations: n, total studies.

Figure 7. Flow diagram of the identification of studies for the systematic review

A description of the nine studies included in the systematic review derived from the data extraction is shown in Table 3. Only data that met the inclusion criteria from included studies are stated, data not meeting the inclusion criteria from these same studies are not stated. Data extraction on studies included study design, country, setting and sample size. Data extraction on demographic included women's age, ethnicity, and any details on their children in relation to the HCMV shedding data. Data extraction on HCMV shedding included bodily fluid site(s), time-point(s), detection method(s), prevalence in any bodily fluid, and prevalence according to specific bodily fluid site. Data extraction was performed once.

No	Study	Study Design	Country	Setting	Demographic a. Age (years) b. Ethnicity c. Data on children in relation to the HCMV shedding data	No. of participants	HCMV shedding site(s)	HCMV shedding time-point(s)	HCMV shedding design	HCMV shedding method	HCMV shedding prevalence overall (% [95% CI] (x/n))	HCMV shedding prevalence by site (Site: % [95% CI] (y/n))
1	Gehrz (1981)	Cohort study	USA	Unknown	a. Unknown b. Unknown c. No	8	U, C	1 st T, 2 nd T, 3 rd T, at delivery	MS, MT	Culture	0% [0%,36.9%] (0/8)	U: 0% [0%,36.9%] (0/8) C: 0% [0%,36.9%] (0/8)
2	Faix (1983)	Single-centre cohort study	USA	Pregnant women attending one obstetric clinic for adolescents	a. Range 13-19 b. White, Black c. No	45	U, C, S, B	1 st prenatal visit, <20 GW, 20-30 GW, at delivery	MS, MT	Culture	35.6% [21.9%,51.2%] (16/45)	U: 13.3% [5.1%,26.8%] (6/45) C: 22.2% [11.2%,37.1%] (10/45) S: 8.9% [2.5%,21.2%] (4/45) B: 2.2% [0.1%,11.8%] (1/45)
3	Chandler (1985)	Cohort study	USA	Pregnant women attending their first prenatal clinic	a. Mean 24 b. White, Black, Asian, Mexican American, native American, Mixed c. No	564	C	1 st prenatal clinic visit (mean 14.3 GW)	SS, ST	Culture	14.0% [11.3%,17.2%] (79/564)	C: 14.0% [11.3%,17.2%] (79/564)
4	Shen (1993)	Multi-centre cohort study	Taiwan	Pregnant women attending two obstetric clinics	a. <26, 26-35, >36 b. Unknown c. Yes	350	U, C	14-16 GW, 24-26 GW	MS, MT	CMV DNA PCR	27.1% [22.6%,32.1%] (95/350)	U: 11.1% [8.0%,14.9%] (39/350) C: 20.6% [24.0%,36.5%] (66/220)
5	Kyriazopoulou (1996)	Cohort study	Greece	Unknown	a. Unknown b. Unknown c. No	32	B	17-20 GW	SS, ST	CMV DNA PCR	0% [0%,10.9%] (0/32)	B: 0% [0%,10.9%] (0/32)
6	Sahiner (2015)	Cohort study	Turkey	Pregnant women planned for elective C-section	a. Mean 30 b. Unknown c. No	101	U, B	Just before delivery	MS, ST	CMV DNA PCR	5.5% [2.2%,12.5%] (6/101)	U: 5.0% [1.6%,11.2%] (5/101) B: 2.0% [0.2%,7.0%] (2/101)
7	Mujtaba (2016)	Multi-centre cohort study	Pakistan	Pregnant women seeking antenatal care at two hospitals	a. Mean 27 b. Unknown c. No	347	B	1 st - 3 rd T	SS, ST	CMV DNA PCR	9.2% [6.4%,12.8%] (32/347)	B: 9.2% [6.4%,12.8%] (32/347)
8	Barbosa (2018)	Multi-centre cohort study	Brazil	Pregnant women attending first antenatal visit at six centres	a. Range 14-41(mean 24) b. White, Black, Mixed Black c. Yes	120	U, S, V, B	1 st T, 2 nd T, 3 rd T	MS, MT	CMV DNA PCR	30.8% [23.3%,39.6%] (37/120)	S: 18.3% [11.9%,26.4%] (22/120) U: 10.8% [5.9%,17.8%] (13/120) V: 5.8% [2.4%,11.7%] (7/120) B: 0.8% [0%,4.6%] (1/120)
9	Zelini (2022)	Single-centre cohort study	Italy	Pregnant women attending first antenatal visit at one centre	a. Range 19-49 b. Unknown c. Yes	228	U, S, V, B	9-16 GW, 17-25 GW, 26-37 GW, at delivery	MS, MT	CMV DNA PCR	42.5% [36.0%,49.2%] (97/228)	Unknown

Table detailing the characteristic of studies included in the systematic review, ordered by year of publication in ascending order (see section 2.1 for the methods used, and section 3.2 for the description of results).
Abbreviations: B, blood; C, cervical secretions; CI: confidence interval; DNA: deoxyribonucleic acid; GW, gestation in weeks; MS, multiple sites; MT, multiple time-points; n, included population; PCR: polymerase chain reaction; S, saliva; SS, single site; ST, single time-point; T, trimester; U, urine; USA, United States of America; V, vaginal secretions; x, no. of women with HCMV shedding in at least one site during at least one time-point; y, no. of women with HCMV shedding in the specific site during at least one time-point;
*Average score of the critical appraisal performed by two authors using the Joanna Briggs Institute Critical Appraisal Checklist for Prevalence Studies 2013 (Munn *et al.*, 2015)

Table 3. Characteristics of studies included in the systematic review derived from the data extraction

3.2.2. Methodologies of selected studies

All studies used a cohort study design, with a sample size ranging between 8 to 564 women (Table 3). Across the studies, the bodily fluid sites for HCMV shedding detection were saliva, urine, vaginal secretions, cervical secretions, and blood. Six studies (Table 3; study number 1, 2, 4, 6, 8, 9) implemented sample collection from at least two (range 2-4) bodily fluid sites, and three studies (Table 3; study numbers 3, 5, 7) from a single bodily fluid site. Five studies (Table 3; study numbers 1, 2, 4, 8, 9) applied sample collection at multiple (range 2-4) time-points, and four studies (Table 3; study numbers 3, 5, 6, 7) at a single time-point. Six studies (Table 3; study numbers 3, 5, 6, 7, 8) included sample collection from women only during pregnancy, and three studies (Table 3; study numbers 1, 2, 9) as well as at delivery. Timing of sampling varied between the studies, with some reporting it according to range of gestational weeks, trimester of pregnancy, or proximity to (just before or just after) delivery. Five studies (Table 3; study numbers 1, 2, 4, 8, 9) reported at least two (range 2-4) sampling time-points, and four studies (Table 3; study numbers 3, 5, 6, 7) reported a single time-point. Six studies (Table 3; study numbers 4, 5, 6, 7, 8, 9) utilised PCR to detect HCMV DNA as the method to assess HCMV shedding, and three studies (Table 3; study numbers 1, 2, 3) used viral culture techniques.

3.2.3. Demographics of selected studies

The studies represented four continents: three from North America, three from Asia, two from Europe, and one from South America. Reporting of population age varied across studies, with mean age reported in four studies (Table 3; study numbers 3, 6-8), age range in three studies (Table 3; study numbers 2, 8, 9), and age category in one study (Table 3; study number 4). In two studies (Table 3; study numbers 1, 5), age was either not reported or it

was not possible to extract the relevant data. Data on the women's living children in relation to the HCMV shedding assessment was only reported in three studies (Table 3; study numbers 4, 8, 9) which were also varied. Shen *et al.* (Table 3; study number 4) found no difference in HCMV shedding between groups of women who had children attending nursery or kindergarten and those who did not in urine (11.8% vs. 10.8%, p-value 0.83) and in cervical secretions (28.0% vs. 30.6%, p-value 0.73). Barbosa *et al.* (Table 3; study number 8) reported that pregnant women living with or providing daily care to children aged 3 to 6 years old were twice as likely to shed HCMV in any site as those not living with or providing daily care to children of this age (58% vs 26%: adjusted RR 2.21 [95% CI 1.37,3.56]). Zelini *et al.* (Table 3; study number 9) reported on whether participants did or did not have children aged less than 36 months and found no difference in HCMV shedding between these groups (28.6% vs. 20.7%, p-value 0.18).

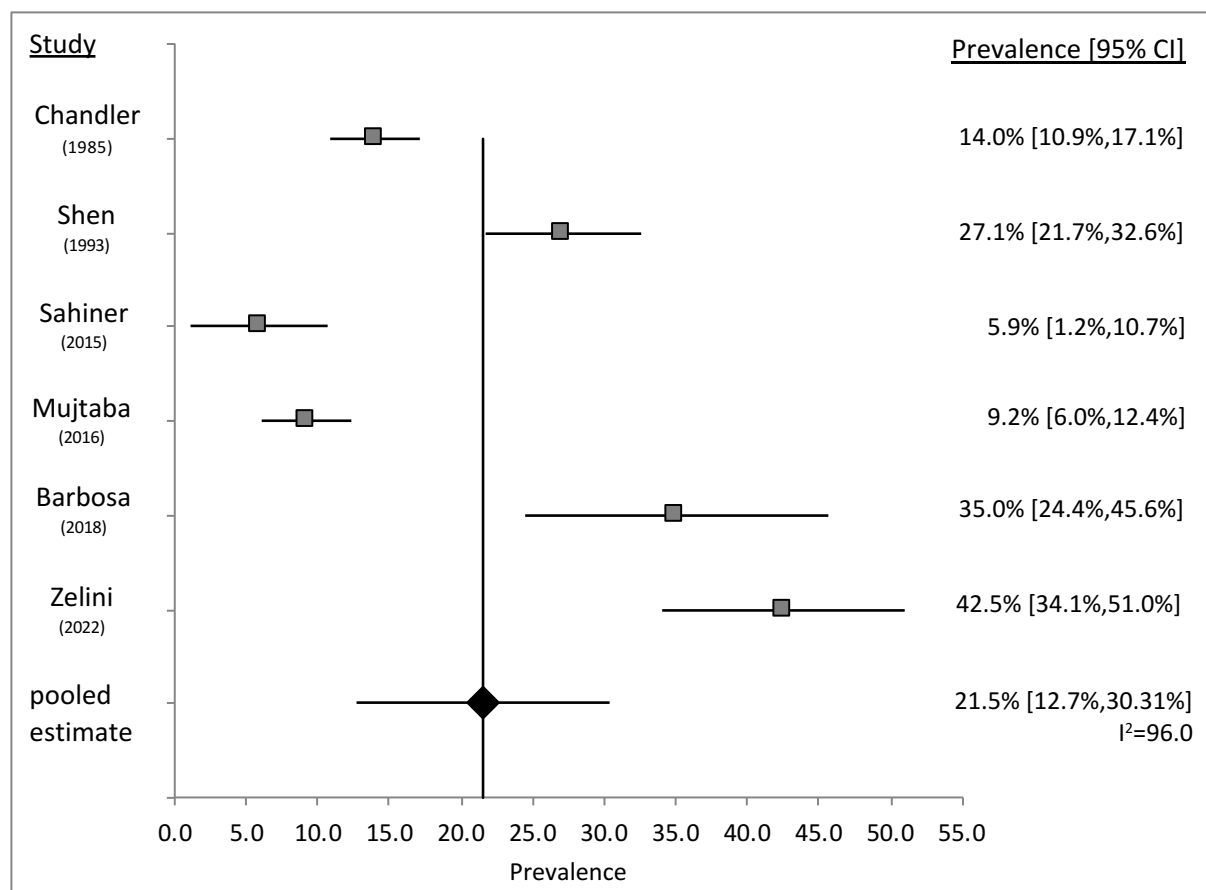
3.2.4. Prevalence of HCMV shedding of selected studies

The prevalence of HCMV shedding in pregnant women, at least once in any bodily fluid and at any point during pregnancy up to delivery, ranged from 0% to 42.5%. The highest prevalence was reported by Zelini *et al.* (Table 3; study number 9), and the lowest by Gehrz *et al.* (Table 3; study number 1) and Kyriazopoulou *et al.* (Table 3; study number 5), where both were of a sample size below the minimum sample size deemed suitable for a meta-analysis for a pooled prevalence estimate. We were able to extract data on prevalence according to specific bodily fluid site from all studies except one (Table 3; study number 9). The highest site-specific shedding prevalence was reported by Faix *et al.* (Table 3; study number 2) in cervical secretions, and the lowest by Gehrz *et al.* (Table 3; study number 1) in urine and cervical secretions.

Only three out of nine included studies (Table 3; study numbers 2, 5, 9) have data that were extractable on the prevalence of cCMV infection in HCMV-seropositive pregnant women meeting the inclusion criteria. Faix *et al.* (Table 3; study number 2) reported 6.3% (1/16) of cCMV infection in infants born to women with HCMV shedding detection, compared to 0% (0/29) in infants born to women with no HCMV shedding detection. Kyriazopoulou *et al.* (Table 3; study number 5), which found no HCMV shedding detection in the pregnant women tested, reported 12.5% (4/32) of cCMV infection in the foetuses (tested via amniotic fluid). Zelini *et al.* (Table 3; study number 9) reported 0% of cCMV infection in infants born to women with and without HCMV shedding detection.

3.2.5. Pooled prevalence and meta-analyses of selected studies

The pooled prevalence estimate of HCMV shedding in any bodily fluids in HCMV-seropositive pregnant women in the meta-analysis was 21.5% [95% CI 12.7%,30.3%], as shown in Figure 8. Six studies met the sample size criteria (Table 3; study numbers 3, 4, 6, 7, 8, 9). The pooled prevalence had a high level of heterogeneity among the study results ($I^2=96.0\%$). The prevalence from individual studies included in the meta-analysis ranged from 5.9% to 42.5%, such that the highest prevalence was seven times greater than the lowest prevalence.



Forest plot displaying a random effects meta-analysis of the prevalence of HCMV shedding in any bodily fluid of HCMV-seropositive pregnant women on selected studies in the systematic review. Each square represents the prevalence for each study included in the meta-analysis, placed in position to the corresponding study on the y-axis and the corresponding prevalence estimate on the x-axis. The pooled estimate prevalence is marked by a diamond. The length of the line on either side of each square and diamond represents the extent of the prevalence 95% CI. The prevalence estimate and 95% CI for each study and for the pooled estimate are also displayed on the far right, including Higgins I^2 score for the pooled estimate displayed as I^2 to analyse heterogeneity across the studies (see section 2.1 for the methods used, and section 3.2 for the description of results).

Abbreviations: CI, confidence interval; I^2 , Higgins I^2 score.

Figure 8. Forest plot of the prevalence of HCMV shedding in any bodily fluid of HCMV-seropositive pregnant women on selected studies of the systematic review

Subgroup meta-analyses of site-specific prevalence of HCMV shedding in pregnant women were possible for cervicovaginal secretions (cervical and vaginal secretion estimates were grouped together), urine, and blood, with an order of highest to lowest pooled prevalence estimated in cervicovaginal secretions (18.5% [9.3%,27.7%]), urine (9.7% [4.8%,14.3]), and blood (3.9% [0%,8.5%]). All subgroup meta-analyses had high levels of heterogeneity across the studies as measured by Higgins I^2 (Table 4).

Site of HCMV shedding	Studies included	Studies	Prevalence range	Pooled prevalence estimate (% [95% CI])	Heterogeneity (Higgins I ² %)
Blood	Three	Sahiner (2015), Mujtaba (2016), Barbosa (2018)	0.8%-9.2%	3.9% [0%*,8.5%]	90.5
Cervicovaginal secretions	Three	Chandler (1985), Shen (1993), Barbosa (2018)	12.5%-30.0%	18.5% [9.3%,27.7%]	88.3
Urine	Three	Shen (1993), Sahiner (2015), Barbosa (2018)	5.0%-13.3%	9.7% [4.8%,14.3]	68.7

Table detailing the systematic review subgroup meta-analyses of the prevalence of HCMV shedding in HCMV-seropositive pregnant women according to site of detection, performed on selected studies with suitable sample size, ordered alphabetically by the bodily fluid site.

*Substituted from a negative lower CI of -0.8% (see section 2.1 for the methods used, and section 3.2 for the description of results).

Abbreviations: HCMV, human cytomegalovirus; CI, confidence interval; I², Higgins I² score.

Table 4. Systematic review subgroup meta-analyses of the prevalence of HCMV shedding in HCMV-seropositive pregnant women according to bodily fluid site

3.2.6. Critical appraisal of selected studies

The critical appraisal of included studies is described in Table 5. Using the Joanna Briggs Institute Critical Appraisal Checklist for Prevalence Studies 2013 (Munn *et al.*, 2015), the two reviewers agreed on most (range 7-9) of the nine-criteria checklist of the critical appraisal tool. Gehrz *et al.* (Table 3; study number 1) investigated mainly on HCMV-specific immunity instead of on HCMV shedding in pregnant women, where the study design and results did not directly address the question of estimating HCMV shedding prevalence, and limited information was provided on both population and methods used to test the condition intended for the systematic review. Moreover, the sample size was small. Faix *et al.* (Table 3; study number 2) also investigated mainly on HCMV-specific immunity, where they did not exhaustively address HCMV shedding prevalence in their methods and results, and the study population was also limited to adolescents. While investigating HCMV shedding in HCMV-seropositive pregnant women was a main objective for Chandler *et al.* (Table 3; study number 3), it was assessed in a single bodily fluid site (cervical secretions) at a single time-point (during their first prenatal clinic). Investigating HCMV shedding in HCMV-seropositive pregnant women was also a main objective for Shen *et al.* (Table 3; study number 4), tested twice in pregnancy in both urine and cervical secretions. Kyriazopoulou *et al.* (Table 3; study number 5) reported limited details on the study population, methods and statistical analysis, and the sample size was also small. For Sahiner *et al.* (Table 3; study number 6), the study population was limited to pregnant women undergoing elective caesarean section, with both urine and blood collected to investigate for HCMV shedding once just before delivery. Mujtaba *et al.* (Table 3; study number 7) described their assessment of HCMV shedding in blood at a single time point at various stages of pregnancy in HCMV-seropositive women either with or without detected IgM, where only data on

179 those without IgM detection were extracted, but limited details on how the study
180 population was sampled was reported. Investigating HCMV shedding in HCMV-seropositive
181 pregnant women was a main objective for Barbosa *et al.* (Table 3; study number 8), tested
182 three times in pregnancy in multiple bodily fluid sites, as well as at one month postnatally
183 which was not included in the data extraction. Investigating HCMV shedding in HCMV-
184 seropositive pregnant women was also a main objective for Zelini *et al.* (Table 3; study
185 number 9), tested three times in pregnancy and once at delivery in multiple bodily fluid
186 sites. However, as the study population included women with immunodeficiency and
187 immunosuppression, only suitable data that were extractable were included in the
188 systematic review. The average quality assessment score of the critical appraisal performed
189 on the studies is also described in Table 5; from the six studies included in the meta-
190 analyses, three (Table 3; study numbers 3, 4, 9) had a high score, two (Table 3; study
191 numbers 7, 8) a medium score and one (Table 3; study number 6) a low score.

Study	Reviewer	1. Sample frame appropriate to address the target population	2. Study participants sampled appropriately	3. Sample size adequate	4. Study participants & setting described in detail	5. Data analysis conducted with sufficient coverage of the sample	6. Valid methods for condition identification	7. Condition measured in a standard and reliable way for all participants	8. Appropriate statistical analysis	9. Adequate response rate or low response rate managed appropriately	Average score	Quality assessment score
1 Gehrz (1981)	S.S. A.T.	? x	? ?	x x	x x	? ?	✓ ✓	? ?	? ?	✓ ✓	2	22% (low)
2 Faix (1983)	S.S. A.T.	x x	? ?	? ?	✓ ✓	x x	✓ ✓	? ?	✓ ✓	✓ ✓	4	44% (moderate)
3 Chandler (1985)	S.S. A.T.	✓ ✓	✓ ✓	✓ ✓	✓ ✓	✓ ✓	✓ ✓	? ?	✓ ✓	✓ ✓	8	89% (high)
4 Shen (1993)	S.S. A.T.	✓ ✓	✓ ✓	✓ ?	✓ ✓	✓ ✓	✓ ✓	? ?	✓ ✓	x x	6.5	72% (high)
5 Kyriazopoulou (1996)	S.S. A.T.	? ?	? ?	x x	x x	✓ ?	✓ ✓	? ?	? ?	? ?	1.5	17% (low)
6 Sahiner (2015)	S.S. A.T.	x x	? ?	? ?	x x	✓ ?	✓ ✓	✓ ✓	x x	✓ ✓	3.5	39% (low)
7 Mujtaba (2016)	S.S. A.T.	✓ ?	? ?	✓ ?	✓ ✓	? ?	✓ ✓	✓ ✓	✓ ✓	✓ ✓	6	67% (moderate)
8 Barbosa (2018)	S.S. A.T.	✓ ✓	✓ x	✓ ✓	✓ ✓	x x	✓ ✓	? ?	✓ ✓	x x	5.5	61% (moderate)
9 Zelini (2022)	S.S. A.T.	x x	✓ ✓	✓ ✓	✓ ✓	✓ ✓	✓ ✓	? ?	✓ ✓	✓ ✓	7	78% (high)

Critical appraisal of studies included in the systematic review using the Joanna Briggs Institute Critical Appraisal Checklist for Prevalence Studies 2013 (University of York, 2016) on the studies ordered by year of publication in ascending order (see section 2.1 for the methods used, and section 3.2 for the description of results).
Abbreviations: ✓, yes; x, no; ?, unclear.

Table 5. Critical appraisal of studies included in the systematic review

3.3. Discussions

Firstly, there is evidence that HCMV shedding is detected in HCMV-seropositive women during pregnancy, up to delivery. Furthermore, a pooled prevalence estimate of 21.5% of HCMV-seropositive pregnant women was found to shed HCMV on at least one occasion during pregnancy from at least one bodily fluid. As HCMV shedding may suggest that an individual is acutely infected with HCMV, HCMV shedding in a HCMV seropositive pregnant woman may suggest that she has developed a non-primary maternal HCMV infection (Saldan *et al.*, 2017; Mussi-Pinhata *et al.*, 2018). It is known that there is an association between cCMV infection and non- primary maternal HCMV infection (Boppana *et al.*, 2001; Yamamoto *et al.*, 2010; De Vries *et al.*, 2013; Mussi-Pinhata *et al.*, 2018). Therefore, HCMV shedding may aid in identifying HCMV-seropositive pregnant women at higher risk of having an infant with cCMV infection. According to bodily fluid site, the pooled prevalence estimate in the analysis was highest for cervicovaginal secretions, which may suggest that cervicovaginal secretions are the most suitable site for assessing HCMV shedding in HCMV-seropositive pregnant women. As the vertical transmission of HCMV from a pregnant mother to her foetus occurs either transplacentally or during vaginal delivery (Luck *et al.*, 2017; Saldan *et al.*, 2017), contact with HCMV-infected cervicovaginal secretions may provide a mechanism for this (Mocarski, Shenk and Pass, 2007; Saldan *et al.*, 2017; Adnane, Meade and O'Farrelly, 2018). Thus, assessment of cervicovaginal secretions may be a practical way of identifying HCMV-seropositive pregnant women with non-primary HCMV infection.

The association between HCMV shedding in pregnant women and the vertical transmission of cCMV has been poorly investigated. Evidence has been gathered here to suggest that this important gap in our knowledge needs to be addressed, and it may

contribute towards the development of disease burden models and therapeutic or preventative strategies against cCMV infection.

The potential risk factors for HCMV shedding in HCMV-seropositive pregnant women also needs to be investigated further. Exposure to young children was identified as a risk factor for HCMV shedding in one of the three included studies in the systematic review, that reported varying data on this. Young children commonly shed HCMV for prolonged periods of time and have been shown to be a common source of HCMV transmission to pregnant women (Cannon, Hyde and Schmid, 2011; Saldan *et al.*, 2017). Exposure to young children is likely to be a risk factor for HCMV shedding in HCMV seropositive pregnant women too, however this was not shown conclusively in all studies.

While this review aims to understand the prevalence of HCMV shedding in HCMV seropositive pregnant women in order to facilitate the estimation of disease burden, an understanding of the distribution of HCMV shedding during pregnancy up to delivery would also contribute to this. Five of the nine studies included in the systematic review adopted a longitudinal study design, which could add important information on the disease burden to contribute towards the development of preventative and therapeutic strategies for cCMV infection.

There is a paucity of published literature on the prevalence of HCMV shedding in HCMV-seropositive pregnant women and this systematic review identified only nine relevant studies. As addressed, this may be due to the value of this epidemiological information being underestimated. The appraised quality of the studies included in this systematic review and the meta-analyses also varied, few with low scores where the prevalence and pooled prevalence estimates should be interpreted with caution.

Although the studies represented a considerable range of countries and continents, only studies published in English were included, which may limit the applicability of these results internationally. Indeed, a third of the studies were from the United States of America (USA), and no studies represented Africa or Australia continents. Therefore, it may be difficult to generalise the findings for populations that were not studied.

The high level of statistical heterogeneity (Jonathan J Deeks *et al.*, 2021) identified across the studies included in the meta-analyses means that the pooled prevalence estimates from the meta-analyses should be interpreted with caution. The population demographics such as ethnicity, socio-economic status, age, and status of exposure to children also varied amongst all studies included in this systematic review. Due to this variability, it was not possible to determine the association of these factors with HCMV shedding, but it may have had an impact on the prevalence of HCMV shedding found.

To my knowledge, this review is the first to systematically search the literature to summarise the available evidence on the prevalence of HCMV shedding in HCMV seropositive pregnant women. HCMV shedding could aid in identifying HCMV-seropositive pregnant women at an increased risk of having an infant with cCMV, and the findings from this review may contribute towards the development of disease burden models and therapeutic or preventative strategies against cCMV infection. There is insufficient evidence at present about the global prevalence of HCMV shedding in HCMV-seropositive pregnant women, where more research is needed to assess this important epidemiological question in different populations.

4. Investigating clinical samples of human cytomegalovirus-seropositive pregnant women: seroprevalence, shedding, cellular mediated immunity, and genomic sequencing

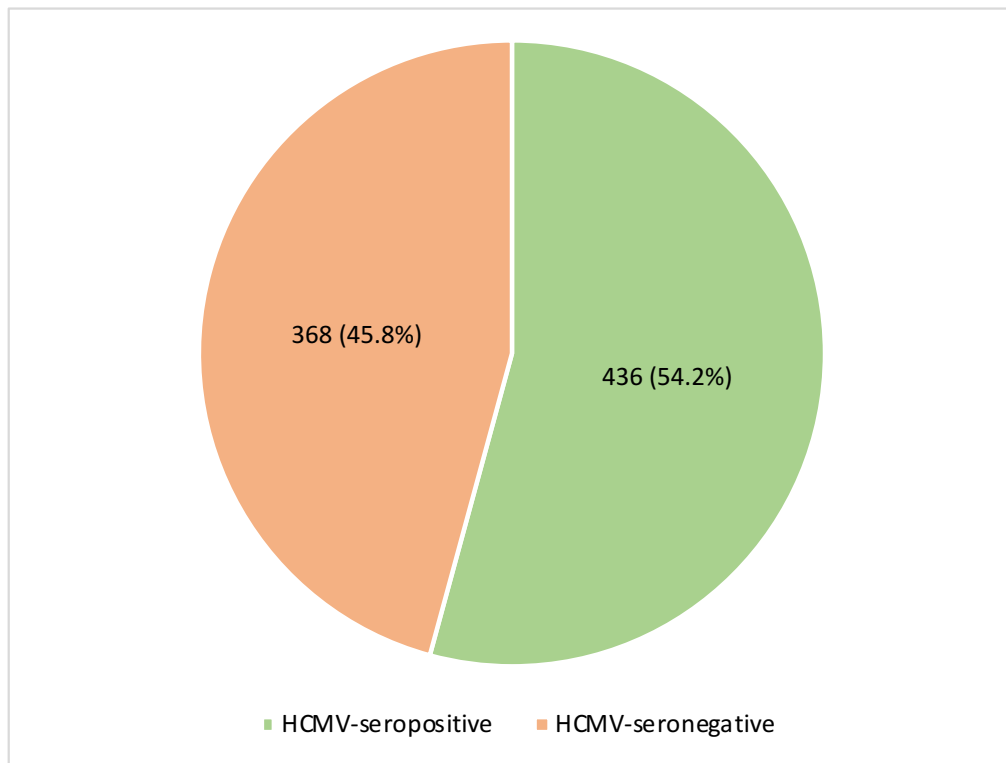
4.1. Human cytomegalovirus serostatus distribution in pregnant women screened for the observational cohort study

4.1.1. Background

I sought to investigate the HCMV serostatus distribution in pregnant women screened for the observational cohort study, to understand the HCMV seroprevalence in the study cohort. A full description of the methods can be found in section [2.2.5.1](#).

4.1.2. Results

From the 804 women screened for the observational study, 436 [54.2%; 95% CI: 50.7-57.7%] were found to be HCMV-seropositive and 368 [45.8%; 95% CI: 42.3-49.3%] HCMV-seronegative (Figure 9), giving a HCMV seroprevalence rate of 54.2% in the study cohort.



Pie chart showing the HCMV serostatus distribution (orange for seronegative, green for seropositive) in pregnant women screened for the observational cohort study (see section [2.2.5.1](#) for the methods used, and section [4.1.2](#) for the description of results).
Abbreviations: HCMV, human cytomegalovirus.

Figure 9. HCMV serostatus distribution of pregnant women screened for the observational cohort study

4.1.3. Discussions

The HCMV seroprevalence rate found in this ethnically diverse cohort in London (see section [4.2.3](#)), is less than the seroprevalence rate of 60-70% in female of reproductive age in England found in a literature review published in 2010 which reviewed 8 studies in England over the period of 1970-2004 (Cannon, Schmid and Hyde, 2010). More recently in 2020, a Health Survey for England cross-sectional study found a seroprevalence rate of 28.3% at age 22–24 years across England, however considerable variation in the seroprevalence rate was observed by region, ethnicity, and sex, where it was highest in London (47.5%), non-white (61.6%, versus 19.2% in white) and women (26.9%, versus 20.4% in men) (Winter *et al.*, 2020). Thus, the observational cohort study which is participated only by women living in London may likely have a higher HCMV seroprevalence rate than the average population living across England. It is possible that the HCMV seroprevalence rate in England has dropped over the last decade.

An up-to-date HCMV seroprevalence rate in female of reproductive age across the whole of the UK, which represents a diverse population, is needed to contribute towards the development of disease burden models and therapeutic or preventative strategies.

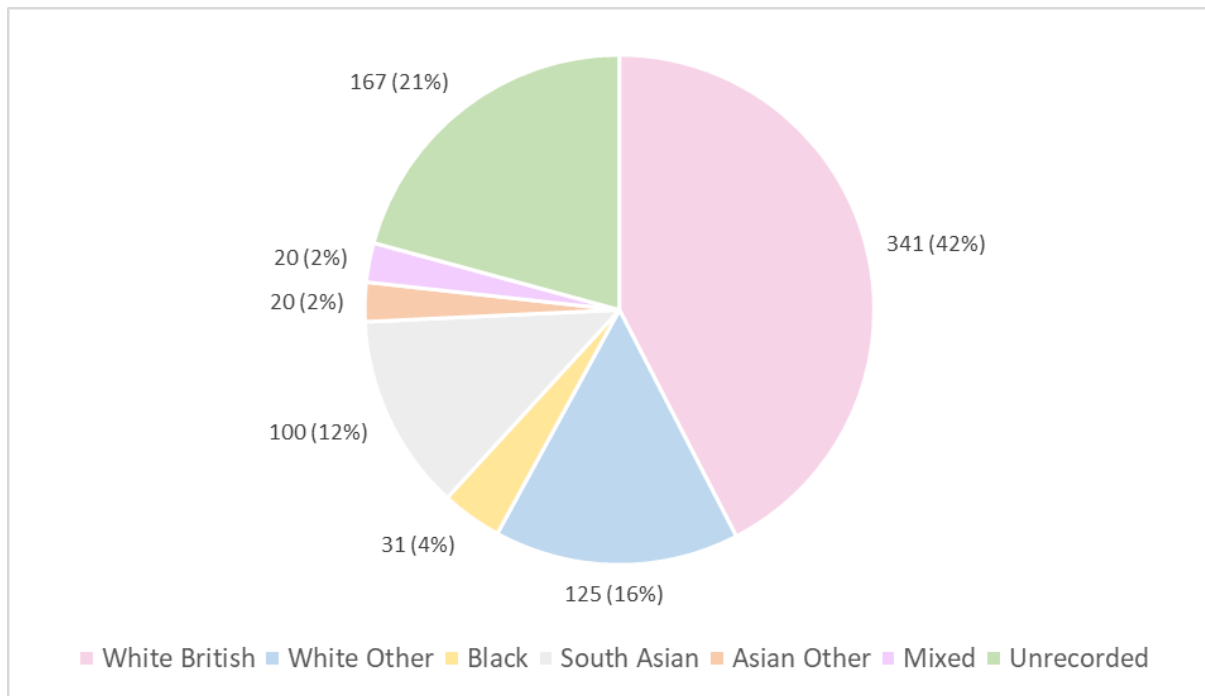
4.2. Demographics of pregnant women screened and recruited into the observational cohort study

4.2.1. Background

We sought to investigate the ethnicity distribution of pregnant women screened and recruited into the observational cohort study, as well as investigating other demographic information in those recruited. A full description of the methods can be found in section [2.2.5.2.](#)

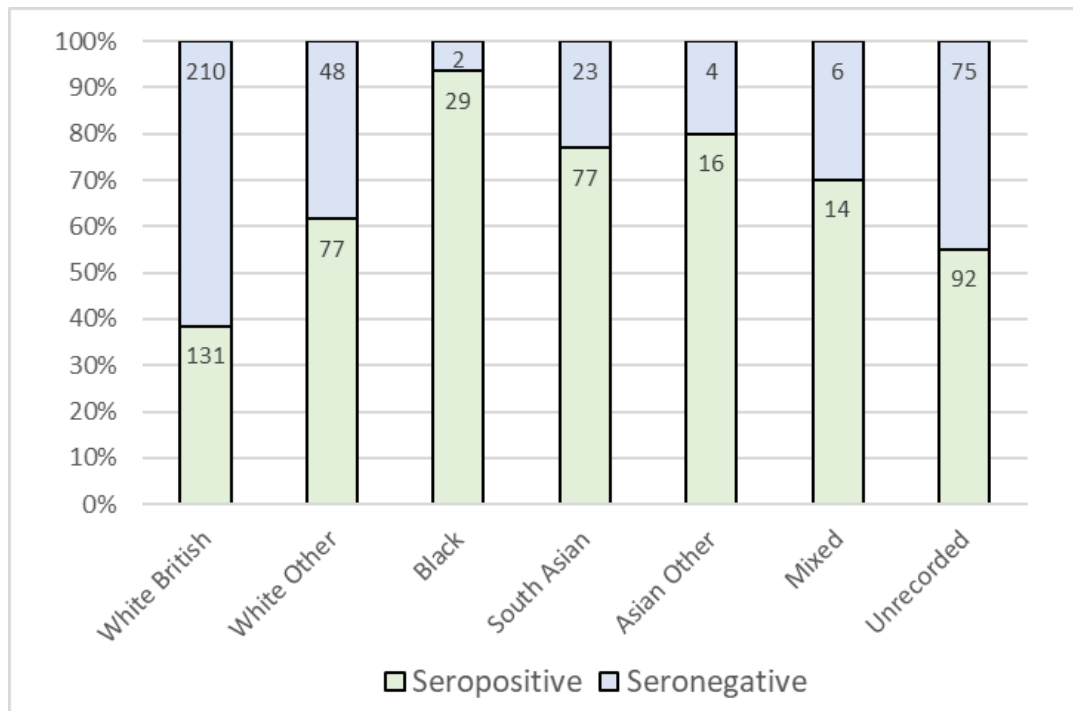
4.2.2. Results

Ethnicity was recorded for 637 of the 804 women screened for their HCMV serostatus (Figure 10). The HCMV seroprevalence rate varied by ethnicity as described: 38.4% in White British, 61.6% in White Other, 93.5% in Black, 77.0% in South Asian, 80.0% in Asian Other, and 70.0% in Mixed (Figure 11). From the 436 women found to be HCMV-seropositive, 160 (36.7%) were recruited into the observational study. Figure 12 showed the ethnicity distribution of recruited women, and Table 6 described the rest of the demographic sought in recruited women. There was a symmetrical distribution of age for the recruited women where both the median and mean for age was 34 years.



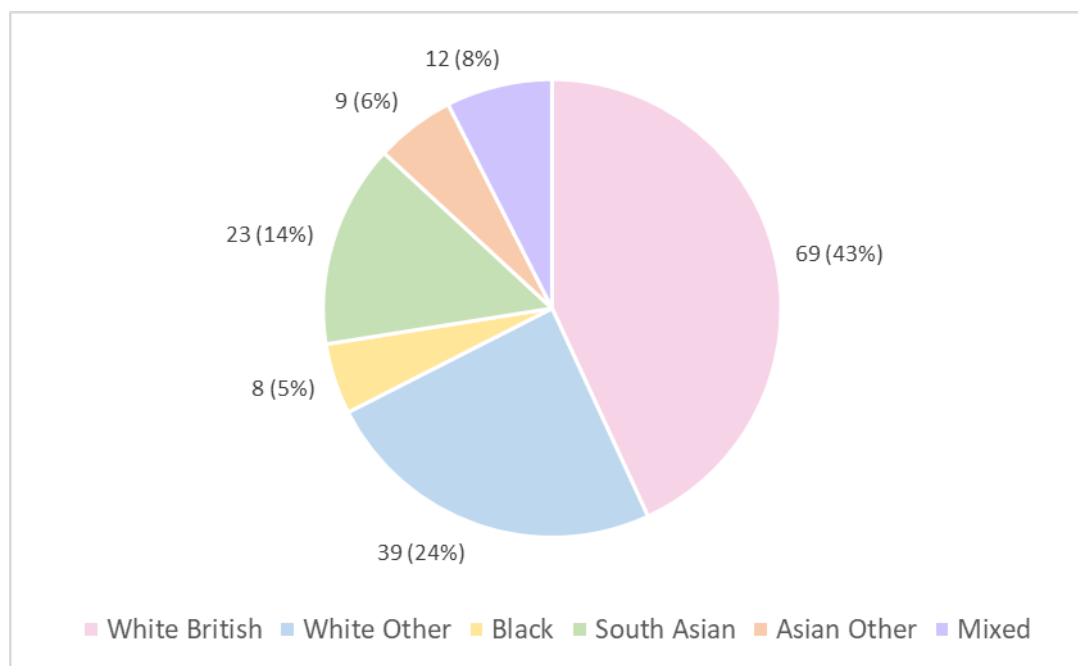
Pie chart showing the ethnicity distribution of pregnant women screened for the observational cohort study (see section [2.2.5.2](#) for the methods used, and section [4.2.2](#) for the description of results).

Figure 10. Ethnicity distribution of women screened for the observational cohort study



Bar chart showing the HCMV serostatus distribution, by ethnicity, of pregnant women screened for the observational cohort study (see section 2.2.5.2 for the methods used, and section 4.2.2 for the description of results).
Abbreviations: HCMV, human cytomegalovirus

Figure 11. HCMV serostatus by ethnicity in women screened for the observational cohort study



Pie chart showing the ethnicity distribution of HCMV-seropositive pregnant women recruited into the observational cohort study (see section 2.2.5.2 for the methods used, and section 4.2.2 for the description of results).
Abbreviations: HCMV, human cytomegalovirus.

Figure 12. Ethnicity distribution of HCMV-seropositive women recruited into the observational study

Demographic		frequency (%)
Age (years) N=160	<30	20 (12)
	30-34	65 (41)
	35-39	65 (41)
	>39	10 (6)
Ethnicity N=160	White British	69 (43)
	White Other	39 (24)
	South Asian	8 (5)
	Asian Other	23 (14)
	Black	9 (6)
Born in UK N=160	Mixed	12 (8)
	Yes	90 (56)
Duration in UK (years) N=160	No	70 (44)
	<5	14 (9)
	5-15	44 (27)
Highest education N=160	>15	102 (64)
	PhD/Masters	50 (31)
	Undergraduate	59 (37)
	Diploma	28 (18)
Gravidity N=160	A-levels/GCSE	23 (14)
	2	88 (55)
	3	35 (22)
No. of children N=160	>3	37 (23)
	1	125 (78)
	2	26 (16)
Age of children (years) N=205	>2	9 (6)
	<1	4 (2)
	1	56 (27)
	2	73 (36)
	3	32 (16)
	4	7 (3)
	5	8 (4)
	6	5 (3)
	7	4 (2)
	8	7 (3)
Work regularly with children N=160	>8	9 (4)
	Yes	27 (17)
No. of households N=160	No	133 (83)
	<=3	116 (73)
No. of people per bedroom N=160	>3	44 (27)
	1	5 (3)
	2	130 (81)
	3	25 (16)

Table summarising the demographics of HCMV-seropositive pregnant women recruited into the observational cohort study (see section 2.2.5.2 for the methods used, and section 4.2.2 for the description of results).
Abbreviations: A-levels, advanced levels; GCSE, general certificate of secondary education; HCMV, human cytomegalovirus; N, total observations; PhD, Doctor of Philosophy.

Table 6. Demographics of HCMV-seropositive women recruited in the observational cohort study

4.2.3. Discussions

This cohort represented an ethnically diverse group of women, and all the categories of ethnicity groups in accordance with the Office for National Statistics (ONS). It is established here that the HCMV seroprevalence rate found varied by ethnicity, consistent with the literature (Winter *et al.*, 2020) as described in section 4.1.3, with higher prevalence rates in non-white ethnicities. There was a symmetrical distribution of age of recruited women. The age range in this cohort is wide up to above 40 years old, which may have an impact on the HCMV seroprevalence rate (Winter *et al.*, 2020). A reasonable proportion of women were also born outside the UK or have not always lived in the UK, which could also impact on the HCMV seroprevalence rate (Cannon, Schmid and Hyde, 2010; Winter *et al.*, 2020). There is a reasonable proportion of women with a higher degree qualification, which may not represent the true population although this cannot be confirmed as there was no comparable data available through the ONS. Majority of them live in a house with few households occupying the house and per bedroom; this non-crowding environment may have an impact on the HCMV shedding found in the women, where living in crowded households has been found to be associated with HCMV shedding (Barbosa *et al.*, 2018).

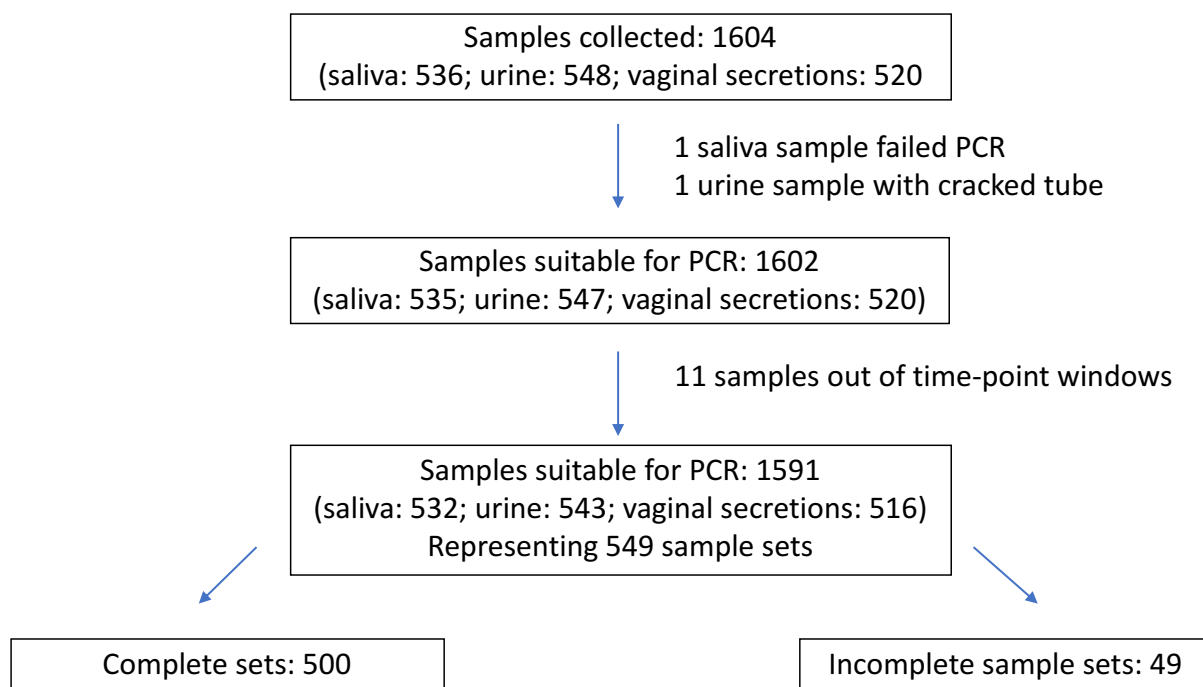
4.3 Human cytomegalovirus shedding in participants of the observational cohort study

4.3.1. Background

I sought to investigate the prevalence and distribution of HCMV shedding in saliva, urine, and vaginal secretions of HCMV-seropositive women during pregnancy and the postpartum period who were recruited into the observational cohort study. A full description of the methods can be found in section [2.2.5.3](#).

4.3.2. Results

Altogether, 1604 samples (saliva: 536; urine; 548, vaginal secretions; 520) were collected from 160 women (Figure 13). Two samples were not suitable for HCMV DNA PCR analysis (one saliva sample failed the PCR testing, one urine sample had a cracked tube), and 11 samples (saliva: 3; urine: 4; vaginal secretions: 4) were not suitable for study analysis due to being collected beyond the time-point windows. The 1591 samples (532 saliva, 543 urine, 516 vaginal secretions) suitable for study analysis represented 549 sets, of which 500 sets were complete (all three samples of saliva, urine and vaginal secretions at a study time-point were collected and suitable), and 49 were incomplete (not all three samples of saliva, urine and vaginal secretions at a study time-point were collected and suitable) and considered as missing data (Figure 13).



Flowchart illustrating the number of urine, saliva, and vaginal secretion samples of HCMV-seropositive women in the observational cohort study that were collected and analysed with PCR to detect HCMV DNA. (see section 2.2.5.3 for the methods used, and section 4.3.2 for the description of results).

Abbreviations: DNA, deoxynucleic acid; HCMV, human cytomegalovirus; polymerase chain reaction, PCR.

Figure 13. Flowchart of saliva, urine and vaginal secretion samples of HCMV-seropositive women recruited into the observational cohort study available for HCMV DNA PCR analysis

Of the 1591 samples analysed, 466, 424, 405 and 296 represented the 1st, 2nd, 3rd, and 4th time-points, respectively (Table 7). Twelve samples that were collected during the 1st time-point were analysed as part of the 2nd time-point analysis as they were collected during gestation weeks that represented the 2nd time-point time range. Table 7 shows the results of HCMV DNA detection via PCR in saliva, urine, and vaginal secretion samples, at different time-points, of HCMV-seropositive women during pregnancy (1st to 3rd time-points) or postpartum period (4th time-point). Table 8 describes the spread of the quantity of HCMV shedding detected and their quartiles, while Table 9 and Figure 14 show the proportion of samples with HCMV shedding by bodily fluid site at each time-point and cumulatively.

Woman	TP1: S	TP1: U	TP1: V	TP2: S	TP2: U	TP2: V	TP3: S	TP3: U	TP3: V	TP4: S	TP4: U	TP4: V
1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
4	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
8	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
11	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	ND
12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
13	D: 521	D: 10900	D: 32000	D: 402	D: 4930	D: 3660	D: 137	D: 373	D: 6480	ND	D: 2490	D: 282
14	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-
16	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
17	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
18	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
19	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
20	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
21	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
22	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
23	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
24	ND	ND	ND	ND	ND	ND	ND	ND	D: 106	ND	ND	ND
25	ND	ND	ND	ND	ND	ND	ND	ND	D: 398	ND	D: 1	D: 639
26	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	D: 210
27	ND	ND	ND	ND	ND	D: 170	ND	ND	D: 353	-	-	-
28	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
29	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
30	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
31	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
32	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
33	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
34	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
35	ND	ND	ND	-	-	-	ND	ND	ND	ND	-	-
36	ND	ND	ND	ND	ND	ND	-	-	-	-	-	-
37	ND	ND	D: 439	ND	ND	ND	ND	D: 1	ND	ND	ND	ND
38	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
39	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
40	ND	ND	ND	-	ND	ND	ND	ND	ND	-	-	-
41	ND	ND	ND	ND	ND	D: 1	ND	ND	ND	ND	ND	ND
42	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
43	ND	ND	ND	-	-	-	-	-	-	-	-	-
44	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
45	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-
46	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
47	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
48	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
49	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
50	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
51	ND	ND	ND	ND	ND	ND	ND	ND	D: 1010	ND	ND	-
52	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
53	ND	ND	ND	ND	ND	D: 1	ND	ND	ND	ND	ND	ND
54	ND	ND	D: 132	ND	ND	ND	ND	ND	ND	ND	ND	ND
55	ND	ND	ND	ND	ND	ND	ND	D: 1	ND	ND	ND	-
56	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
57	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
58	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
59	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
60	ND	ND	ND	ND	ND	ND	-	-	-	-	-	-
61	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
62	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
63	ND	ND	ND	ND	D: 1	D: 209	ND	D: 1	D: 883	-	-	-
64	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
65	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
66	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

67	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
68	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
69	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-
70	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
71	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
72	ND	ND	ND	-	-	-	ND	ND	ND	ND	ND	-
73	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	ND	-
74	ND	ND	ND	ND	ND	ND	-	-	-	-	-	-
75	ND	D: 1	ND	ND	D: 208	ND	ND	D: 1	ND	ND	D: 1	ND
76	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
77	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
78	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
79	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
80	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
81	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
82	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	D: 1	ND
83	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
84	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
85	ND	D: 1	ND	ND	ND	ND	ND	ND	ND	-	-	-
86	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
87	ND	ND	D: 133	D: 768	D: 601	ND	D: 634	D: 1	ND	-	-	-
88	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
89	-	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND
90	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
91	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
92	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
93	ND	ND	ND	ND	D: 1	ND	ND	ND	ND	-	-	-
94	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
95	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
96	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
97	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
98	ND	D: 1	D: 9630	ND	D: 1130	ND	ND	D: 232	D: 947	-	-	-
99	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
100	-	-	-	ND	ND	ND	ND	ND	ND	-	-	-
101	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
102	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
103	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
104	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
105	ND	ND	ND	ND	ND	ND	ND	ND	D: 163	ND	ND	ND
106	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
107	ND	ND	ND	ND	ND	D: 103	ND	ND	ND	-	-	-
108	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
109	ND	ND	ND	ND	ND	ND	-	ND	ND	-	-	-
110	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
111	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
112	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
113	ND	-	ND	ND	ND	ND	-	-	-	-	-	-
114	ND	D: 1	D: 265	D: 693	ND	ND	D: 539	D: 1	ND	-	-	-
115	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
116	ND	ND	ND	ND	ND	ND	-	-	-	-	-	-
117	ND	ND	ND	ND	ND	ND	-	-	-	-	-	-
118	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
119	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
120	ND	ND	ND	ND	ND	ND	ND	ND	D: 216	ND	ND	ND
121	D: 227	ND	D: 1270	ND	D: 339	D: 7500	ND	D: 1	D: 3170	ND	D: 439	D: 166
122	D: 689	D: 1	ND	ND	ND	ND	-	-	-	-	-	-
123	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
124	ND	ND	ND	ND	ND	ND	-	-	-	-	-	-
125	ND	ND	ND	ND	-	ND	-	-	-	-	D: 241	ND
126	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
127	ND	ND	ND	ND	ND	ND	-	-	-	-	-	-
128	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
129	ND	D: 135	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
130	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
131	ND	ND	ND	-	-	-	-	-	-	-	-	-
132	ND	ND	ND	ND	D: 1	ND	ND	ND	ND	ND	ND	ND
133	ND	ND	ND	-	-	-	-	-	-	-	-	-

134	ND	ND	ND	-	-	-	-	-	-	-	-	-
135	ND	ND	ND	-	-	-	-	-	-	ND	ND	ND
136	ND	ND	ND	-	-	-	-	-	-	-	-	-
137	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	ND
138	ND	ND	ND	-	-	-	-	-	-	-	-	-
139	ND	ND	D: 800	-	-	-	-	-	-	ND	ND	ND
140	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	ND
141	ND	ND	ND	-	-	-	-	-	-	-	-	-
142	ND	ND	ND	ND	ND	D: 302	ND	ND	ND	ND	ND	ND
143	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	-
144	ND	ND	ND	-	-	-	-	-	-	-	ND	ND
145	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	ND
146	ND	ND	ND	ND	ND	ND	-	ND	ND	-	ND	ND
147	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	ND	D: 260
148	ND	D: 1	ND	ND	D: 1	ND	ND	ND	ND	-	ND	ND
149	ND	ND	ND	-	-	-	-	-	-	-	-	-
150	ND	ND	ND	-	-	-	-	-	-	-	ND	ND
151	ND	ND	ND	-	-	-	-	-	-	-	ND	ND
152	ND	ND	ND	ND	ND	ND	ND	ND	ND	-	-	-
153	ND	ND	ND	ND	D: 1	D: 657	ND	ND	ND	ND	ND	-
154	ND	ND	ND	-	-	-	ND	ND	ND	ND	ND	ND
155	ND	ND	ND	-	-	-	ND	ND	ND	ND	ND	ND
156	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	-
157	ND	ND	ND	-	-	-	ND	ND	ND	-	ND	-
158	ND	D: 399	D: 1270	ND	ND	ND	ND	ND	ND	ND	D: 1	ND
159	-	-	-	ND	ND	D: 101	ND	ND	ND	ND	ND	-
160	-	-	-	ND	ND	ND	-	-	-	ND	ND	ND

Table detailing the results of HCMV DNA detection via PCR (quantified in IU/ml) in saliva, urine, and vaginal secretion samples at different time-points, of HCMV-seropositive women in the observational cohort study. Where HCMV DNA was detected but below the PCR assay limit for quantification, a value of 1.0 IU/ml was assigned for the purpose of statistical analysis. Women with at least one result of detected HCMV DNA at any point during pregnancy or postpartum period is in green font (see section 2.2.5.3 for the methods used, and section 4.3.2 for the description of results).

Abbreviations: D, detected; DNA; deoxyribonucleic acid; HCMV, human cytomegalovirus; IU/ml; international unit per millilitre; ND, not detected; PCR, polymerase chain reaction; S, saliva, TP1, 1st time-point, TP2, 2nd time-point; TP3, 3rd time-point; TP4, 4th time-point; U, urine; V, vaginal secretions; -, not available. Where HCMV DNA was detected, in green font, the quantity displayed is in IU/ml.

Table 7. HCMV DNA detection in HCMV-seropositive women during pregnancy and the postpartum period

Site	1 st time-point			2 nd time-point			3 rd time-point			4 th time-point			All time-points		
	Samples with HCMV shedding	Shedding quantity (IU/ml)		Samples with HCMV shedding	Shedding quantity (IU/ml)		Samples with HCMV shedding	Shedding quantity (IU/ml)		Samples with HCMV shedding	Shedding quantity (IU/ml)		Samples with HCMV shedding	Shedding quantity (IU/ml)	
		Median	IQR		Median	IQR		Median	IQR		Median	IQR		Median	IQR
S	3	521	231	3	693	183	3	539	249	0	-	-	9	539	287
U	9	1	134	10	105	535	9	1	0	7	1	339	35	1	289
V	9	134	1005	10	190	467	10	641	744	5	260	72	34	328	827.2

Table summarising the quantity (median and IQR) of HCMV DNA PCR detected in saliva, urine, and vaginal secretion samples at each time-point and cumulatively, of HCMV-seropositive women in the observational cohort study. Where HCMV DNA was detected but below the PCR assay limit for quantification, a value of 1.0 IU/ml was assigned for the purpose of statistical analysis (see section 2.2.5.3 for the methods used, and section 4.3.2 for the description of results).

Abbreviations: DNA, deoxyribonucleic acid; HCMV, human cytomegalovirus; IQR, interquartile range; IU/ml, international unit/millilitre; PCR, polymerase chain reaction; S, saliva; U, urine; V, vaginal secretions.

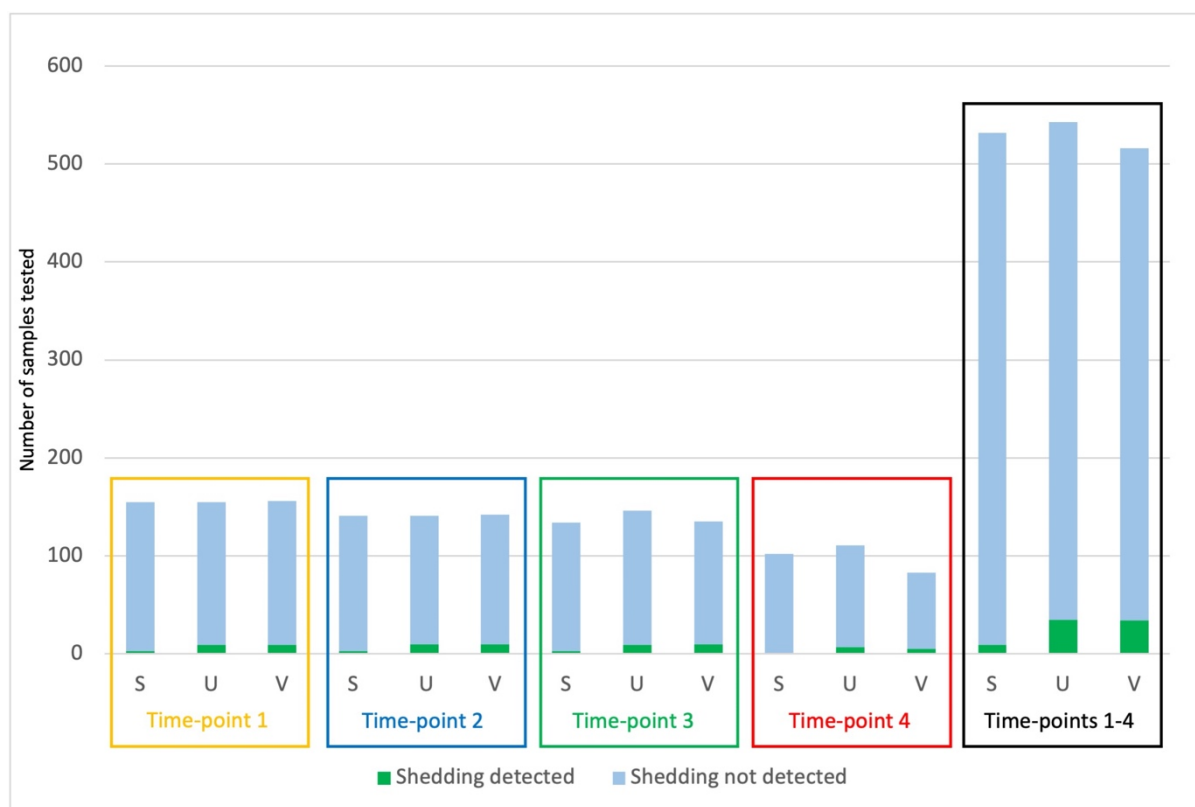
Table 8. Spread of quantity of HCMV shedding in HCMV-seropositive women during pregnancy and the postpartum period

Site	1 st time-point			2 nd time-point			3 rd time-point			4 th time-point			All time-points		
	No. tested	HCMV shedding	% shedding [95% CI]	No. tested	HCMV shedding	% shedding [95% CI]	No. tested	HCMV shedding	% shedding [95% CI]	No. tested	HCMV shedding	% shedding [95% CI]	No. tested	HCMV shedding	% shedding [95% CI]
S	155	3	1.9 [0.4,5.6]	141	3	2.1 [0.4,6.1]	134	3	2.2 [0.5,6.4]	102	0	0 [0,3.6]	532	9	1.7 [0.8,3.2]
U	155	9	5.8 [2.7,10.7]	141	10	7.1 [3.5,12.7]	136	9	6.6 [3.1,12.2]	111	7	6.3 [2.6,12.6]	543	35	6.5 [4.5,8.9]
V	156	9	5.8 [2.7,10.7]	142	10	7.0 [3.4,12.6]	135	10	7.4 [3.6,13.2]	83	5	6.0 [2.0,13.5]	516	34	6.6 [4.6,9.1]

Table summarising the proportion of saliva, urine, and vaginal secretion samples with HCMV DNA detected via PCR, at each time-point and cumulatively, of HCMV-seropositive women in the observational cohort study (see section 2.2.5.3 for the methods used, and section 4.3.2 for the description of results).

Abbreviations: CI; confidence intervals; DNA, deoxyribonucleic acid; HCMV; human cytomegalovirus; PCR, polymerase chain reaction; S, saliva; U, urine; V, vaginal secretions; %, percentage.

Table 9. Proportion of samples with HCMV shedding by bodily fluid site in HCMV-seropositive women during pregnancy and the postpartum period



Bar chart detailing the proportion of saliva, urine, and vaginal secretion samples with HCMV DNA detected via PCR, at each time-point and cumulatively, of HCMV-seropositive women in the observational cohort study (see section 2.2.5.3 for the methods used, and section 4.3.2 for the description of results).

Abbreviations: DNA, deoxyribonucleic acid; HCMV, human cytomegalovirus; PCR, polymerase chain reaction; S, saliva; U, urine; V, vaginal secretions.

Figure 14. Proportion of samples with HCMV shedding in HCMV-seropositive women during pregnancy and the postpartum period

The prevalence of HCMV shedding detection at any time-point during pregnancy was 22.0% [27/123; 95% CI: 15.0-30.3%] in any bodily fluid, 3.3% [4/123; 95% CI: 0.9-8.1%] in saliva, 13.0% [16/123; 95% CI: 7.6-20.3] in urine, and 16.3% [20/123; 95% CI: 10.2-24.0%] in vaginal secretions. The distribution and quantity of HCMV shedding detected during pregnancy in these 27 women found to shed on at least one occasion is shown in Figure 15.

The prevalence rate is slightly higher when postpartum period is included. The prevalence of HCMV shedding detection at any time-point during pregnancy or postpartum period was 25.8% [17/66; 95% CI: 15.8-38.0%] in any bodily fluid, 3.0% [2/66; 95% CI: 0.4-10.5%] in saliva, 13.6% [9/66; 95% CI: 6.4-24.3] in urine, and 19.7% [13/66; 95% CI: 10.9-31.3%] in vaginal secretions. The distribution and quantity of HCMV shedding detected during pregnancy in these 17 women found to shed on at least one occasion is shown in Figure 16.

Woman	1st time-point: 12-16 gestation weeks	2nd time-point: 17-26 gestation weeks	3rd time-point: 27 gestation weeks - prebirth
1	▲ 521, ●10900, ◆32000	▲ 402, ●4930, ◆3660	▲ 137, ●373, ◆6480
2			◆106
3			◆398
4		◆170	◆353
5	◆439		●1
6		◆1	
7			◆1010
8		◆1	
9	◆132		
10			●1
11		●1, ◆209	●1, ◆883
12	●1	●208	●1
13	●1		
14	◆133	▲ 768, ●601	▲ 634 ●1
15		●1	
16	●1, ◆9630	●1130	●232, ◆947
17			◆163
18		◆103	
19	●1, ◆265	▲ 693	▲ 539, ●1
20			◆216
21	▲ 227, ◆1270	●339, ◆7500	●1, ◆3170
22	●135		
23		●1	
24		◆302	
25	●1	●1	
26		●1, ◆657	
27	●399, ◆1270		

▲	Saliva
●	Urine
◆	Vaginal secretions

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A tabular and graphical display of the distribution of HCMV shedding detection (including the quantity in IU/ml) in the samples, according to time-point and bodily fluid type, of the 30 HCMV-seropositive women with HCMV shedding detected on at least one time-point during pregnancy (see section 2.2.5.3 for the methods used, and section 4.3.2 for the description of results).
Abbreviations: HCMV, human cytomegalovirus; IU/ml, international unit/millilitre.

Figure 15. HCMV-seropositive women with HCMV shedding detected in their samples at any point during pregnancy

Woman	1st time-point: 12-16 gestation weeks	2nd time-point: 17-26 gestation weeks	3rd time-point: 27 gestation weeks - prebirth	4th time-point: Birth - 6 weeks postpartum	
1	▲521, ●10900, ◆32000	▲402, ●4930, ◆3660	▲137, ●373, ◆6480	●2490, ◆282	▲ Saliva
2			◆106		● Urine
3			◆398	●1, ◆639	◆ Vaginal secretions
4				◆210	
5		◆170	◆353		
6	◆439		●1		
7		◆1			
8		◆1			
9	◆132				
10	●1	●208	●1	●1	
11			◆163		
12			◆216		
13	▲227, ◆1270	●339, ◆7500	●1, ◆3170	●439, ◆166	
14	●135				
15		●1			
16		◆302			
17	●399, ◆1270			●1	

A tabular and graphical display of the distribution of HCMV shedding detection (including the quantity in IU/ml) in the samples, according to time-point and bodily fluid type, of the 34 HCMV-seropositive women with HCMV shedding detected on at least one time-point during pregnancy or the postpartum period (see section 2.2.5.3 for the methods used, and section 4.3.2 for the description of results).
Abbreviations: HCMV, human cytomegalovirus; IU/ml, international unit/millilitre.

Figure 16. HCMV-seropositive women with HCMV shedding detected in their samples at any point during pregnancy or the postpartum period

4.3.3. Discussions

The prevalence of HCMV shedding in any bodily fluid at any point during pregnancy in HCMV-seropositive women was 22%. This finding, is in keeping with the literature (Sapuan *et al.*, 2022) and the meta-analysis finding in the systematic review which estimated a pooled prevalence of 21.5% (95% CI: 12.7-30.3%) for HCMV shedding in any bodily fluid at any point in pregnancy up to delivery (see section 3.2.5) . The prevalence of HCMV shedding in vaginal secretions and urine of HCMV-seropositive pregnant women in the observational cohort study were also similar to the pooled prevalence estimates in the systematic review, and similarly the highest HCMV shedding by bodily fluid to be in vaginal secretions (Sapuan *et al.*, 2022). This may suggest that vaginal secretion sampling is a suitable source of bodily fluid for the assessment of HCMV shedding in HCMV-seropositive pregnant women. The prevalence of HCMV shedding in any bodily fluid at any point during pregnancy including postpartum up to 6 weeks postnatal age in HCMV-seropositive women was 26%, slightly higher than when postpartum period was excluded. Another study also found the prevalence rate of HCMV shedding to be higher when including postpartum period compared to just during pregnancy (35.0% versus 30.8%) (Barbosa *et al.*, 2018). Other factors may be at play during the postpartum period to impact on the HCMV shedding of HCMV-seropositive women, such as shedding of HCMV into other bodily fluid sites such as breast milk (Barbosa *et al.*, 2018), and immune system alterations (Dye, Lenz and Leuner, 2022).

Overall, HCMV shedding detection during pregnancy was least common in saliva, and most common in vaginal secretions. The proportion of HCMV shedding for observed by bodily fluid site was similar at any time-point during pregnancy. It is important to consider that the lower limit of detection of HCMV DNA with the PCR assay for saliva was 200.0

IU/ml, much lower than that for urine (30.9 IU/ml) and vaginal secretions (80.6 IU/ml), which may suggest that there may be lower quantity of HCMV shedding in saliva that was undetected by the assay. The collection of saliva samples were also done as a swab stored in 1 ml of viral transport media, compared to vaginal secretion samples which was also a swab but stored in a higher volume of transport media at 4.3 ml (see section [2.2.5.3](#)), where the HCMV DNA recovery may have been affected when tested (Tan, Pope and Carrington, 2022). Nonetheless, saliva sampled with flocked swab and stored in eNAT® media has been shown to recover the highest amount of HCMV DNA compared to a foam swab in transport media or dry swab (Tan, Pope and Carrington, 2022). Retrospective testing of HCMV DNA in the saliva, urine and vaginal secretion samples meant that the samples were stored frozen for a period of time before being tested. Some samples that were impacted by the COVID-19 disruptions were also stored for a period of time in -20°C, before being stored in -80°C. However, it has been observed that HCMV DNA recovery for urine and vaginal swab in cobas® PCR Media and saliva swab in eNAT® media stored for 3 months in -20°C or -80°C did not differ (Tan, Carrington and Pope, 2023). No difference was also observed in HCMV DNA recovery for urine and vaginal swab in cobas® PCR Media when stored for 2 years in -80°C (Tan, Carrington and Pope, 2023).

The quantity of HCMV shedding detected was also limited by the lower limit of quantification of the PCR assay (for saliva, urine, and vaginal secretions were 200.0, 100.0, and 100.0 IU/ml for saliva, urine, and vaginal secretions respectively; see section [2.2.5.3](#)). As a result, a considerable number of samples with HCMV shedding detected that were below the assay limits, especially for urine, was quantified as 1.0 IU/ml and therefore statistical analysis for this should be interpreted with caution.

4.4. Human cytomegalovirus-specific cellular mediated immunity in participants of the observational cohort study

4.4.1. Background

I sought to investigate the HCMV-specific cellular mediated immunity, as measured by the HCMV-QuantiFERON and HCMV-ELISPOT assays, of HCMV-seropositive women during pregnancy and the postpartum period who were recruited into the observational cohort study. A full description of the methods can be found in section [2.2.5.4](#).

4.4.2. Results

Altogether, 185 blood samples were collected from 88 women to perform the HCMV-QuantiFERON assay. Three samples had indeterminate results, and therefore 182 samples were included in the analysis: 72 from the 1st time-point, 52 from the 2nd time-point, 42 at the 3rd time-point and 16 at the 4th time-point. Table 10 illustrates all the results of HCMV-specific CMI as measured by HCMV-QuantiFERON and Figure 17 illustrates the proportion of detected and undetected HCMV-specific CMI at each time-point and cumulatively. Of the 182 samples, HCMV-specific CMI were detected in 71.4% [130/182; 95% CI: 64.3-77.9%] of samples and undetected in 28.6% [52/182; 95% CI: 22.1-35.7%] of samples. Of the 88 women, 95.5% [84/88, 95% CI: 88.8-98.8%] either had all detected HCMV-specific CMI [58/88, 65.9%, 95% CI: 55.0-75.7%] or all undetected HCMV-specific CMI [26/88, 29.5%, 95% CI: 20.3-40.2%], where only 4.5% (4/88, 95% CI 1.3%,11.2%) of women had a combination of both detected and undetected HCMV-specific CMI. Of the 88 women, 70.5% [62/88, 95% CI: 59.8-79.7%] had at least one result with detected HCMV-specific CMI.

Women	1 st time-point		2 nd time-point		3 rd time-point		4 th time-point	
	HCMV QFN level (IU/ml)	HCMV-specific CMI interpretation	HCMV QFN level (IU/ml)	HCMV-specific CMI interpretation	HCMV QFN level (IU/ml)	HCMV-specific CMI interpretation	HCMV QFN level (IU/ml)	HCMV-specific CMI interpretation
1	7.565	Detected	8.551	Detected	8.407	Detected	-	-
2	14.432	Detected	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-
4	0.413	Detected	0.201	Detected	0.339	Detected	0.505	Detected
5	1.708	Detected	0.648	Detected	0.754	Detected	-	-
6	11.304	Detected	-	-	9.404	Detected	-	-
7	5.050	Detected	2.608	Detected	1.201	Detected	-	-
8	0.025	Undetected	0.000	Undetected	0.016	Undetected	0.015	Undetected
9	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-
11	0.128	Undetected	0.196	Undetected	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	-	-	-	0.144	Undetected	-	-
14	0.068	Undetected	0.002	Undetected	-	-	-	-
15	4.684	Detected	4.188	Detected	7.257	Detected	-	-
16	20.233	Detected	-	-	8.300	Detected	-	-
17	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-
19	0.000	Undetected	0.002	Undetected	-	-	-	-
20	0.000	Undetected	0.000	Undetected	0.000	Undetected	-	-
21	2.186	Detected	0.443	Detected	-	-	-	-
22	13.888	Detected	9.810	Detected	-	-	-	-
23	0.634	Detected	2.686	Detected	0.375	Detected	-	-
24	0.670	Detected	0.644	Detected	0.412	Detected	1.711	Detected
25	5.907	Detected	3.331	Detected	2.713	Detected	-	-
26	0.512	Detected	1.574	Detected	6.589	Detected	-	-
27	0.049	Undetected	0.185	Undetected	-	-	-	-
28	0.103	Undetected	0.260	Detected	0.556	Detected	1.108	Detected
29	0.102	Undetected	0.032	Undetected	-	-	-	-
30	10.399	Detected	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-
32	0.011	Undetected	0.019	Undetected	-	-	-	-
33	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-
35	2.131	Detected	-	-	5.253	Detected	-	-
36	0.974	Detected	-	-	-	-	-	-
37	3.669	Detected	6.998	Detected	-	-	8.322	Detected
38	3.264	Detected	6.421	Detected	2.396	Detected	-	-
39	2.038	Detected	3.378	Detected	3.574	Detected	-	-
40	0.201	Detected	-	-	-	-	-	-
41	1.039	Detected	1.676	Detected	2.959	Detected	-	-
42	-	-	-	-	0.073	Undetected	-	-
43	-	-	-	-	-	-	-	-
44	3.780	Detected	4.607	Detected	-	-	-	-
45	0.231	Detected	-	-	0.114	Undetected	-	-
46	0.000	Undetected	-	-	-	-	0.000	Undetected
47	0.507	Detected	0.683	Detected	-	-	-	-
48	-	-	-	-	-	-	-	-
49	-	-	-	-	-	-	-	-
50	7.797	Detected	8.049	Detected	4.457	Detected	-	-
51	-	-	-	-	1.734	Detected	3.311	Detected
52	2.653	Detected	-	-	1.615	Detected	-	-
53	0.000	Undetected	0.074	Undetected	-	-	0.003	Undetected
54	0.231	Detected	0.306	Detected	-	-	-	-
55	-	-	-	-	-	-	-	-
56	1.495	Detected	1.408	Detected	0.971	Detected	-	-
57	-	-	0.640	Detected	-	-	-	-
58	-	-	0.108	Undetected	-	-	-	-
59	0.596	Detected	0.607	Detected	0.457	Detected	-	-
60	-	-	-	-	-	-	-	-
61	5.277	Detected	4.367	Detected	-	-	-	-
62	0.000	Undetected	-	-	-	-	-	-
63	-	-	-	-	-	-	-	-
64	43.582	Detected	-	-	44.680	Detected	-	-
65	-	-	-	-	9.325	Detected	-	-
66	-	-	-	-	-	-	-	-
67	0.525	Detected	-	-	-	-	-	-
68	1.753	Detected	1.557	Detected	1.481	Detected	0.593	Detected

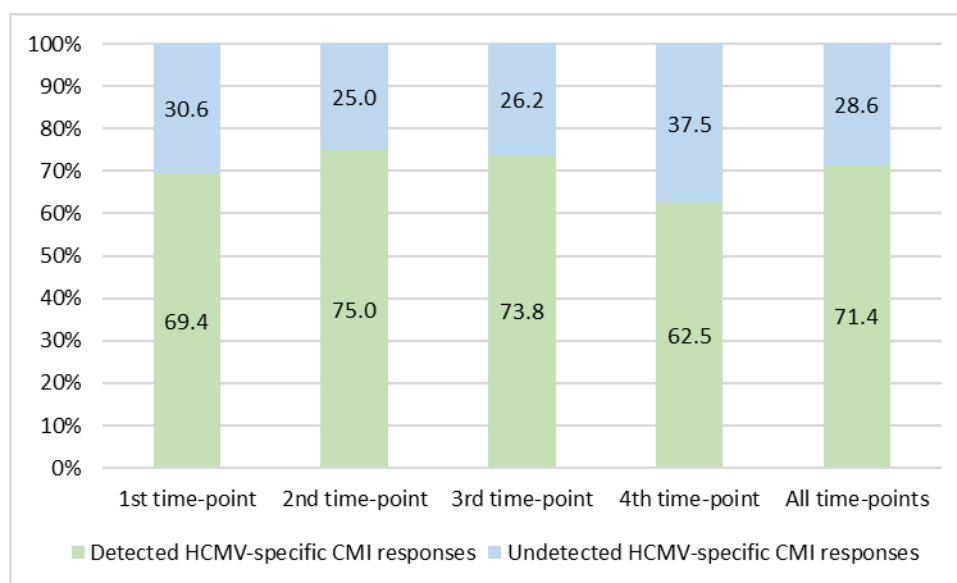
69	8.359	Detected	-	-	-	-	-	-
70	0.115	Undetected	-	-	0.008	Undetected	0.010	Undetected
71	41.966	Detected	44.570	Detected	-	-	-	-
72	-	-	0.257	Detected	-	-	-	-
73	-	-	-	-	-	-	-	-
74	1.116	Detected	-	-	-	-	-	-
75	0.007	Undetected	-	-	-	-	-	-
76	0.484	Detected	-	-	0.061	Undetected	-	-
77	-	-	-	-	-	-	-	-
78	-	-	-	-	-	-	-	-
79	8.049	Detected	58.509	Detected	-	-	-	-
80	-	-	-	-	-	-	-	-
81	-	-	-	-	-	-	-	-
82	-	-	-	-	-	-	-	-
83	-	-	-	-	-	-	-	-
84	-	-	-	-	-	-	-	-
85	44.662	Detected	-	-	-	-	-	-
86	1.546	Detected	3.829	Detected	-	-	-	-
87	44.664	Detected	41.744	Detected	39.385	Detected	-	-
88	-	-	-	-	-	-	-	-
89	-	-	6.117	Detected	7.258	Detected	-	-
90	-	-	-	-	0.058	Undetected	-	-
91	0.499	Detected	-	-	0.135	Undetected	-	-
92	-	-	-	-	-	-	-	-
93	-	-	8.856	Detected	-	-	-	-
94	-	-	-	-	-	-	-	-
95	-	-	-	-	-	-	-	-
96	-	-	-	-	-	-	-	-
97	-	-	-	-	-	-	-	-
98	0.278	Detected	1.083	Detected	0.324	Detected	-	-
99	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-
101	-	-	-	-	-	-	-	-
102	-	-	-	-	-	-	-	-
103	-	-	-	-	-	-	-	-
104	0.000	Undetected	-	-	-	-	-	-
105	-	-	0.684	Detected	-	-	-	-
106	-	-	-	-	-	-	-	-
107	-	-	-	-	-	-	-	-
108	-	-	-	-	-	-	-	-
109	0.002	Undetected	-	-	-	-	-	-
110	-	-	-	-	-	-	-	-
111	-	-	-	-	-	-	-	-
112	-	-	-	-	-	-	-	-
113	-	-	-	-	-	-	-	-
114	-	-	-	-	-	-	-	-
115	-	-	-	-	-	-	-	-
116	-	-	-	-	-	-	-	-
117	-	-	-	-	-	-	-	-
118	-	-	-	-	-	-	-	-
119	0.007	Undetected	0.028	Undetected	-	-	-	-
120	-	-	-	-	-	-	-	-
121	-	-	4.922	Detected	-	-	-	-
122	-	-	-	-	-	-	-	-
123	2.330	Detected	-	-	-	-	-	-
124	0.049	Undetected	-	-	-	-	-	-
125	-	-	-	-	-	-	-	-
126	-	-	-	-	-	-	-	-
127	-	-	-	-	-	-	-	-
128	-	-	-	-	-	-	-	-
129	0.126	Undetected	-	-	-	-	-	-
130	0.061	Undetected	-	-	-	-	-	-
131	4.977	Detected	-	-	-	-	-	-
132	5.305	Detected	-	-	-	-	-	-
133	-	-	-	-	-	-	-	-
134	-	-	-	-	-	-	-	-
135	-	-	-	-	-	-	-	-
136	-	-	-	-	-	-	-	-
137	-	-	-	-	-	-	-	-
138	-	-	-	-	-	-	-	-
139	-	-	-	-	-	-	-	-
140	-	-	-	-	-	-	-	-

141	-	-	-	-	-	-	-	-
142	-	-	-	-	-	-	-	-
143	-	-	-	-	-	-	-	-
144	-	-	-	-	-	-	-	-
145	-	-	-	-	-	-	-	-
146	33.183	Detected	-	-	-	-	-	-
147	-	-	-	-	-	-	-	-
148	-	-	-	-	-	-	-	-
149	-	-	-	-	-	-	-	-
150	-	-	-	-	-	-	-	-
151	-	-	-	-	-	-	-	-
152	-	-	2.802	Detected	6.080	Detected	-	-
153	-	-	2.567	Detected	-	-	1.347	Detected
154	9.206	Detected	-	-	-	-	41.945	Detected
155	-	-	-	-	0.802	Detected	2.531	Detected
156	6.136	Detected	5.879	Detected	3.802	Detected	4.390	Detected
157	0.140	Undetected	-	-	0.115	Undetected	-	-
158	0.043	Undetected	0.024	Undetected	0.01	Undetected	0.044	Undetected
159	-	-	3.311	Detected	2.675	Detected	-	-
160	-	-	0.031	Undetected	-	-	0.016	Undetected

Table detailing the results of HCMV-specific CMI that passed the quality control as measured by the HCMV-QuantiFERON (QFN) assay in blood samples, at each time-point and cumulatively, of HCMV-seropositive women in the observational cohort study. Results showing undetected CMI are in red font. Where results were not available these are stated as ‘-’ (see section 2.2.5.4 for the methods used, and section 4.4.2 for the description of results).

Abbreviations: CMI, cellular mediated immunity; HCMV, human cytomegalovirus; IU/ml, international unit/millilitre; QFN, QuantiFERON.

Table 10. HCMV-specific CMI as measured by HCMV-QuantiFERON in HCMV-seropositive women during pregnancy and the postpartum period



Bar chart detailing the proportion of detected and undetected HCMV-specific CMI, as measured by HCMV-QuantiferON, at each time-point and cumulatively, in HCMV-seropositive women in the observational cohort study (see section 2.2.5.4 for the methods used, and section 4.4.2 for the description of results).

Abbreviations: CMI, cellular mediated immunity; HCMV, human cytomegalovirus.

Figure 17. Proportion of detected and undetected HCMV-specific CMI as measured by HCMV-QuantiferON in HCMV-seropositive women during pregnancy and the postpartum period

Altogether, 115 blood samples were collected from 88 women to perform the HCMV-ELISPOT assay. Seven samples had indeterminate results, and therefore 108 samples were included in the analysis: 49 from the 1st time-point, 25 from the 2nd time-point, 26 at the 3rd time-point and 8 at the 4th time-point. Table 11 illustrates all the results of HCMV-specific CMI as measured by HCMV-ELISPOT and Figure 18 illustrates the proportion of detected and undetected HCMV-specific CMI at each time-point and cumulatively. At the 1st time-point, HCMV-specific CMI were detected in 89.8% [44/49, 95% CI: 77.8-96.6%] of samples towards IE1 antigen and in 100% [49/49, 95% CI: 92.8-100%] of samples towards pp65 antigen, which means HCMV-specific CMI were detected in 100% [49/49, 95% CI: 92.8-100%] of samples (following the definition of a HCMV-specific CMI to be detected towards at least one antigen). At the 2nd time-point, HCMV-specific CMI were detected in 88.0% [22/25, 95% CI: 68.8-97.5%] of samples towards IE1 antigen and in 96.0% [24/25, 95% CI: 79.7-99.9%] of samples towards pp65 antigen, which means HCMV-specific CMI were detected in 96.0% [24/25, 95% CI: 79.7-99.9%] of samples. At the 3rd time-point, HCMV-specific CMI were detected in 84.6% [22/26, 95% CI: 65.1-95.6%] of samples towards IE1 antigen and in 96.2% [25/26, 95% CI: 80.4-99.9%] of samples towards pp65 antigen, which means HCMV-specific were detected 96.2% [25/26, 95% CI: 80.4-99.9%] of samples. At the 4th time-point, HCMV-specific CMI were detected in 87.5% [7/8, 95% CI: 47.4-99.7%] of samples towards IE1 antigen and in 100% [8/8, 95% CI: 63.1-100%] of samples towards pp65 antigen, which means HCMV-specific CMI were detected in 100% [8/8, 95% CI: 63.1-100%] of samples. Cumulatively, HCMV-specific CMI were detected in 98.1% [106/108, 95% CI: 93.5-99.8%] of samples and undetected in 1.9% [2/108, 95% CI: 0.2-6.5%] of samples. All [67/67, 95% CI: 94.6-100%] women had at least one result with detected HCMV-specific CMI.

Women	1 st time-point					2 nd time-point					3 rd time-point					4 th time-point				
	Spot count to IE1	IE1 CMI interpretation	Spot count to pp65	pp65 CMI interpretation	Overall HCMV-specific CMI	Spot count to IE1	IE1 CMI interpretation	Spot count to pp65	pp65 CMI interpretation	Overall HCMV-specific CMI	Spot count to IE1	IE1 CMI interpretation	Spot count to pp65	pp65 CMI interpretation	Overall HCMV-specific CMI	Spot count to IE1	IE1 CMI interpretation	Spot count to pp65	pp65 CMI interpretation	Overall HCMV-specific CMI
1	392	Detected	418	Detected	Detected	396	Detected	302	Detected	Detected	246	Detected	302	Detected	Detected	-	-	-	-	-
2	51	Detected	541	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	415	Detected	550	Detected	Detected	-	-	-	-	-	229	Detected	392	Detected	Detected	150	Detected	206	Detected	Detected
5	158	Detected	193	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	327	Detected	290	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	28	Detected	138	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	59	Detected	116	Detected	Detected	-	-	-	-	-	104	Detected	53	Detected	Detected	93	Detected	169	Detected	Detected
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	17	Detected	131	Detected	Detected	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	8	Detected	69	Detected	Detected	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	31	Detected	173	Detected	Detected	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	-	8	Detected	146	Detected	Detected	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	27	Detected	22	Detected	Detected	7	Detected	76	Detected	Detected	-	-	-	-	-	-	-	-	-	-
20	11	Detected	62	Detected	Detected	11	Detected	87	Detected	Detected	-	-	-	-	-	-	-	-	-	-
21	194	Detected	214	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	602	Detected	290	Detected	Detected	722	Detected	651	Detected	Detected	-	-	-	-	-	-	-	-	-	-
23	414	Detected	405	Detected	Detected	238	Detected	184	Detected	Detected	222	Detected	337	Detected	Detected	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-	-	79	Detected	250	Detected	Detected	42	Detected	126	Detected	Detected
25	185	Detected	267	Detected	Detected	-	-	-	-	-	47	Detected	102	Detected	Detected	-	-	-	-	-
26	72	Detected	346	Detected	Detected	123	Detected	422	Detected	Detected	-	-	-	-	-	-	-	-	-	-
27	22	Detected	2	Detected	Detected	47	Detected	122	Detected	Detected	-	-	-	-	-	-	-	-	-	-
28	8	Detected	196	Detected	Detected	0	Undetected	150	Detected	Detected	4	Detected	89	Detected	Detected	1	Detected	119	Detected	Detected
29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	-	-	-	-	-	45	Detected	110	Detected	Detected	-	-	-	-	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	21	Detected	359	Detected	Detected	-	-	-	-	-	60	Detected	473	Detected	Detected	-	-	-	-	-
36	7	Detected	143	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37	0	Undetected	149	Detected	Detected	79	Detected	103	Detected	Detected	-	-	-	-	-	-	-	-	-	-
38	123	Detected	83	Detected	Detected	-	-	-	-	-	59	Detected	177	Detected	Detected	39	Detected	68	Detected	Detected
39	304	Detected	317	Detected	Detected	-	-	-	-	-	14	Detected	38	Detected	Detected	-	-	-	-	-
40	24	Detected	166	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
41	1	Detected	260	Detected	Detected	0	Undetected	0	Undetected	Undetected	5	Detected	366	Detected	Detected	-	-	-	-	-
42	-	-	-	-	-	-	-	-	-	-	73	Detected	243	Detected	Detected	-	-	-	-	-
43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
44	50	Detected	241	Detected	Detected	139	Detected	281	Detected	Detected	-	-	-	-	-	-	-	-	-	-
45	4	Detected	73	Detected	Detected	-	-	-	-	-	1	Detected	51	Detected	Detected	-	-	-	-	-
46	3	Detected	187	Detected	Detected	-	-	-	-	-	-	-	-	-	-	0	Undetected	64	Detected	Detected
47	29	Detected	268	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

49	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50	21	Detected	154	Detected	Detected	-	-	-	-	-	0	Undetected	198	Detected	Detected	-	-	-	-
51	-	-	-	-	-	-	-	-	-	-	81	Detected	8	Detected	Detected	33	Detected	5	Detected
52	29	Detected	135	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-
53	-	-	-	-	-	169	Detected	13	Detected	Detected	-	-	-	-	-	62	Detected	1	Detected
54	-	-	-	-	-	6	Detected	243	Detected	Detected	-	-	-	-	-	-	-	-	-
55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
56	-	-	-	-	-	-	-	-	-	-	34	Detected	81	Detected	Detected	-	-	-	-
57	-	-	-	-	-	29	Detected	274	Detected	Detected	-	-	-	-	-	-	-	-	-
58	-	-	-	-	-	218	Detected	138	Detected	Detected	110	Detected	92	Detected	Detected	-	-	-	-
59	0	Undetected	116	Detected	Detected	0	Undetected	148	Detected	Detected	0	Undetected	157	Detected	Detected	-	-	-	-
60	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
61	321	Detected	317	Detected	Detected	180	Detected	140	Detected	Detected	-	-	-	-	-	-	-	-	-
62	17	Detected	43	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-
63	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
64	3	Detected	36	Detected	Detected	-	-	-	-	-	48	Detected	149	Detected	Detected	-	-	-	-
65	0	Undetected	13	Detected	Detected	-	-	-	-	-	4	Detected	86	Detected	Detected	-	-	-	-
66	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
67	5	Detected	103	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-
68	161	Detected	182	Detected	Detected	-	-	-	-	-	23	Detected	25	Detected	Detected	-	-	-	-
69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
70	0	Undetected	36	Detected	Detected	-	-	-	-	-	0	Undetected	0	Undetected	Undetected	-	-	-	-
71	73	Detected	207	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-
72	-	-	-	-	-	147	Detected	127	Detected	Detected	-	-	-	-	-	-	-	-	-
73	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
74	10	Detected	124	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-
75	208	Detected	9	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-
76	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
77	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
78	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
79	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
81	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
82	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
83	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
84	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
85	55	Detected	132	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-
86	5	Detected	83	Detected	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-
87	234	Detected	171	Detected	Detected	108	Detected	99	Detected	Detected	0	Undetected	4	Detected	Detected	-	-	-	-
88	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
89	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
90	-	-	-	-	-	-	-	-	-	-	145	Detected	32	Detected	Detected	-	-	-	-
91	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
92	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
93	-	-	-	-	-	62	Detected	100	Detected	Detected	-	-	-	-	-	-	-	-	-
94	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
96	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
97	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
98	-	-	-	-	-	247	Detected	139	Detected	Detected	-	-	-	-	-	-	-	-	-
99	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
101	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

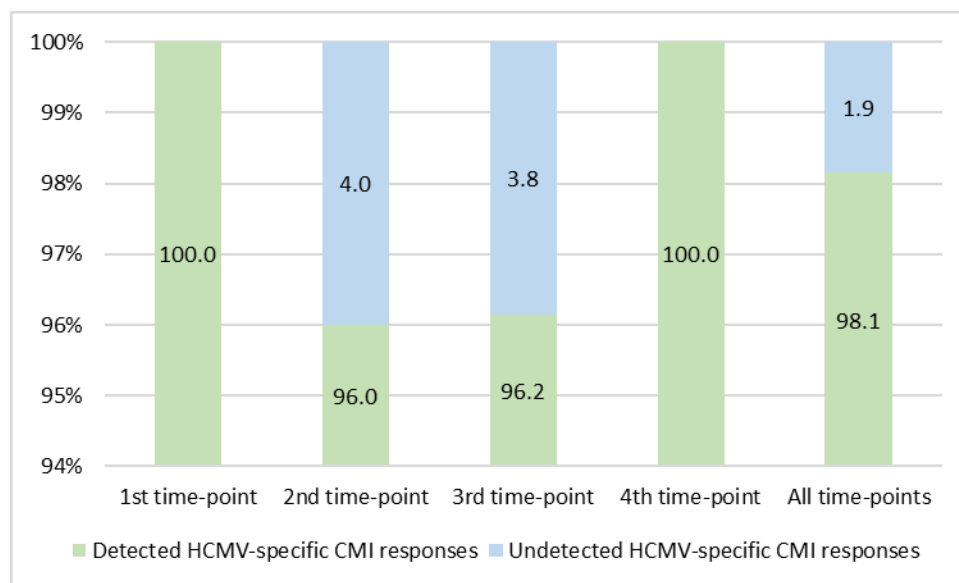
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155	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
156	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
157	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
158	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
159	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
160	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table detailing the results of HCMV-specific cellular mediated immunity (CMI) that passed the quality control as measured by the HCMV-ELISPOT assay in blood samples, at each time-point and cumulatively, of HCMV-seropositive women in the observational cohort study. Spot count above 0 to either IE1 or pp65 antigen were interpreted as an overall detected HCMV-specific CMI. Results showing either IE1 or pp65 undetected CMI are in blue font. Results showing overall undetected CMI are in red font. Where results were not available these are stated as '-' (see section 2.2.5.4 for the methods used, and section 4.4.2 for the description of results).

Abbreviations: CMI, cellular mediated immunity; ELISPOT, enzyme-linked immunosorbent spot; HCMV, human cytomegalovirus, IE1, immediate-early 1; pp65, phosphoprotein 65.

Table 11. HCMV-specific CMI as measured by HCMV-ELISPOT in HCMV-seropositive women during pregnancy and the postpartum period



Bar chart detailing the proportion of detected and undetected HCMV-specific CMI, as measured by HCMV-ELISPOT, at each time-point and cumulatively, in HCMV-seropositive women in the observational cohort study (see section 2.2.5.4 for the methods used, and section 4.4.2 for the description of results).

Abbreviations: ELISPOT, enzyme-linked immunosorbent spot; CMI, cellular mediated immunity; HCMV, human cytomegalovirus.

Figure 18. Proportion of detected and undetected HCMV-specific CMI as measured by HCMV-ELISPOT in HCMV-seropositive women during pregnancy and the postpartum period

Table 12 and Figure 19 describe the spread of the level of HCMV-specific CMI as measured by HCMV-ELISPOT towards IE1 and pp65 antigen separately. A Wilcoxon signed rank test revealed a significant difference in the HCMV-ELISPOT spot count between the IE1 and pp65 antigens at the 1st time point [$Z=-4.37$, $p<0.05$], the 3rd time-point [$Z=-3.46$, $p<0.05$] and across all time-points cumulatively [$Z=-5.85$, $p<0.05$]. A Wilcoxon signed rank test revealed no difference at the 2nd time-point [$Z=-1.58$, $p=0.11$]. The sample size was not large enough to calculate with Wilcoxon signed rank test at the 4th time-point.

Table 13 shows the distribution of detected and undetected HCMV-specific CMI as measured by HCMV-ELISPOT towards IE1 and pp65 antigen separately. A McNemar test showed that the proportions of detected CMI towards IE1 and pp65 antigen were not significantly different at individual time-points (1st time point, 2-sided $p=0.07$; 2nd time-point, 2-sided $p=0.48$; 3rd time-point, 2-sided $p=0.25$; 4th time-point, 2-sided $p=1.00$) but were significantly different cumulatively [2-sided $p<0.05$].

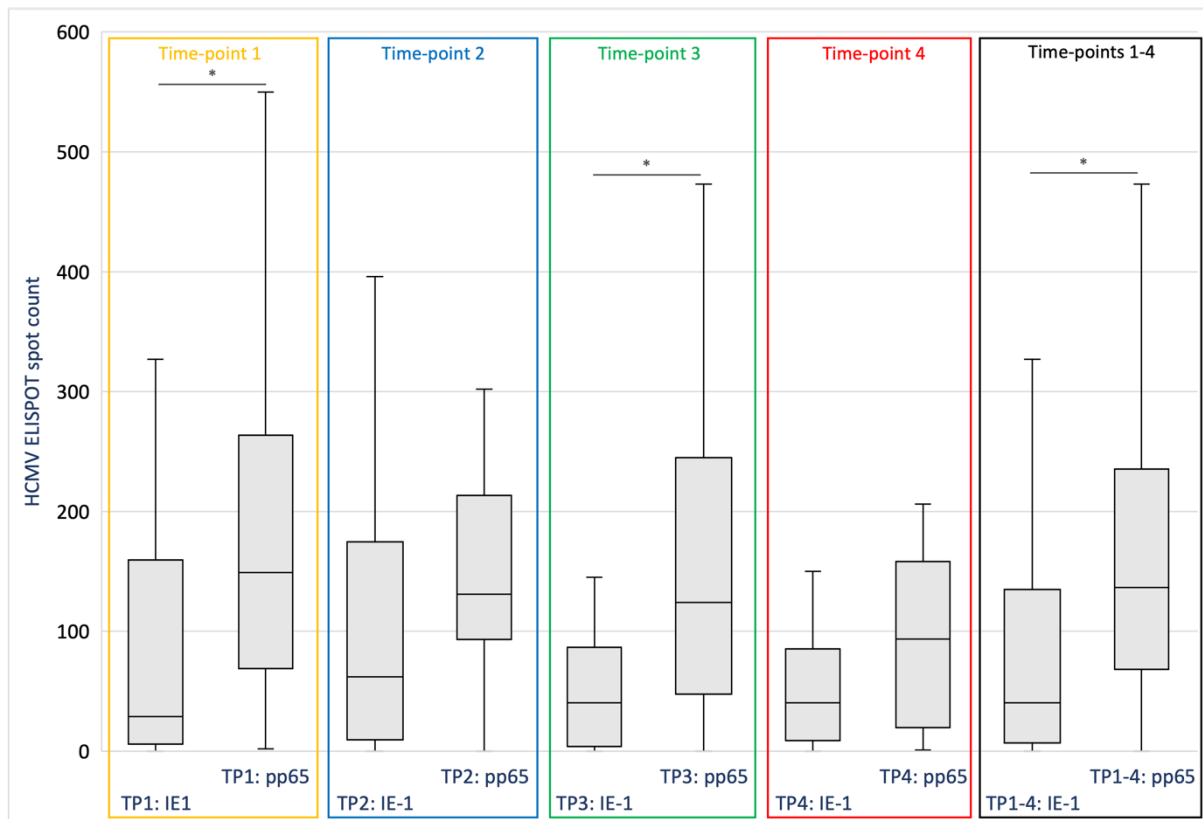
HCMV antigen	1 st time-point (N=49)		2 nd time-point (N=25)		3 rd time-point (N=36)		4 th time-point (N=8)		All time-points (N=108)	
	HCMV-ELISPOT spot count		HCMV-ELISPOT spot count		HCMV-ELISPOT spot count		HCMV-ELISPOT spot count		HCMV-ELISPOT spot count	
	Median	IQR	Median	IQR	Median	IQR	Median	IQR	Median	IQR
IE1	29	151	62	158	41	76	40	45	40	120
pp65	149	187	131	85	124	180	93	88	137	156

Table summarising the quantity (median and IQR) of HCMV-specific CMI, as measured by HCMV-ELISPOT (towards IE1 and pp65 antigens separately), at each time-point and cumulatively, of HCMV-seropositive women in the observational cohort study.

IQR, interquartile range; IU/ml, international unit/millilitre; S, saliva; U, urine; V, vaginal secretions (see section 2.2.5.4 for the methods used, and section 4.4.2 for the description of results).

Abbreviations: ELISPOT, enzyme-linked immunosorbent spot; HCMV, human cytomegalovirus; IE1, immediate-early 1; IQR, interquartile range; N; total observations, pp65, phosphoprotein 65.

Table 12. Spread of HCMV-ELISPOT spot count towards IE1 and pp65 antigens in HCMV-seropositive women during pregnancy and the postpartum period



Boxplot detailing the HCMV-ELISPOT spot count towards IE1 and pp65 antigens at each time-point and cumulatively, of HCMV-seropositive women in the observational cohort study. A significant result (p-value <0.05) is marked with an asterisk symbol (see section 2.2.5.4 for the methods used, and section 4.4.2 for the description of results).
Abbreviations: ELISPOT, enzyme-linked immunosorbent spot; HCMV, human cytomegalovirus; IE1, immediate-early 1; pp65, phosphoprotein 65; TP1, 1st time-point; TP2, 2nd time-point; TP3, 3rd time-point; TP4, 4th time-point; TP1-4, all time-points.

Figure 19. Spread of HCMV-ELISPOT spot count towards IE1 and pp65 antigens by time-points in HCMV-seropositive women during pregnancy and at postpartum

HCMV antigen	1 st time-point (N=49)		2 nd time-point (N=25)		3 rd time-point (N=26)		4 th time-point (N=8)		All time-points (N=108)	
	HCMV-ELISPOT CMI		HCMV-ELISPOT CMI		HCMV-ELISPOT CMI		HCMV-ELISPOT CMI		HCMV-ELISPOT CMI	
	Detected	Undetected	Detected	Undetected	Detected	Undetected	Detected	Undetected	Detected	Undetected
IE1	44	5	23	2	23	3	7	1	97	11
pp65	49	0	24	1	25	1	8	0	106	2

Table comparing the observations of detected and undetected HCMV-specific CMI, as measured by HCMV-ELISPOT, between those towards IE1 and pp65 antigens, at each time-point and cumulatively, of HCMV-seropositive women in the observational cohort study (see section 2.2.5.4 for the methods used, and section 4.4.2 for the description of results).

Abbreviations: CMI, cellular mediated immunity; ELISPOT, enzyme-linked immunosorbent spot; HCMV, human cytomegalovirus; IE1, immediate-early 1; N; total observations; pp65; phosphoprotein 65.

Table 13. Level of HCMV-ELISPOT CMI detection towards IE1 and pp65 antigens in HCMV-seropositive women during pregnancy and the postpartum period

Altogether, 105 blood samples from 67 women were tested with both HCMV-QuantiferON and ELISPOT assays as illustrated in Table 14: 48 from the 1st time-point, 25 from the 2nd time-point, 25 at the 3rd time-point and 7 at the 4th time-point. For HCMV-QuantiferON, HCMV-specific CMI were detected in 73.3% (77/105, 95% CI: 63.8-81.5%) of samples and undetected in 26.7% [28/105, 95% CI: 18.5-36.2%] of samples. For HCMV-ELISPOT, HCMV-specific CMI were detected in 98.1% [103/105, 95% CI: 93.3%-99.8%] and undetected in 1.9% [2/105, 95% CI: 0.2-6.7%] of samples. HCMV-specific CMI detection matched in 73.3% (77/105; 95% CI: 63.8-81.5%) of the samples (either detected by both assays or undetected by both assays). The interrater reliability with Kappa statistic showed no agreement at the 1st time-point [Kappa=0.000, 95% CI: 0.000 to 0.000] and 2nd time-point [Kappa=-0.077, 95% CI: -0.217-0.063], a fair agreement at the 3rd time-point [Kappa=0.286, 95% CI: -0.163-0.735], and a slight agreement across all time-points cumulatively [Kappa=0.032, 95% CI: -0.071-0.136]. The sample size was not large enough to calculate with Kappa statistic at the 4th time-point.

Table 15 and Figure 20 compare the spread of HCMV-ELISPOT spot count towards IE1 and pp65 antigens when HCMV-specific CMI was detected or not detected by HCMV-QuantiferON. The distribution in the two groups deferred significantly towards pp65 at the 1st [Mann-Whitney U= 510, p<0.05], 2nd [Mann-Whitney U= 29, p< 0.05] and cumulative [Mann-Whitney U= 423, p<0.05] time-points. They did not defer significantly towards IE1 at any time-point or cumulatively.

Women	1st time-point					2nd time-point					3rd time-point					4th time-point				
	HCMV-ELISPOT			HCMV-QuantIFERON		HCMV-ELISPOT			HCMV-QuantIFERON		HCMV-ELISPOT			HCMV-QuantIFERON		HCMV-ELISPOT			HCMV-QuantIFERON	
	Spot count to IE1	Spot count to pp65	CMI	Level (IU/ml)	CMI	Spot count to IE1	Spot count to pp65	CMI	Level (IU/ml)	CMI	Spot count to IE1	Spot count to pp65	CMI	Level (IU/ml)	CMI	Spot count to IE1	Spot count to pp65	CMI	Level (IU/ml)	CMI
1	392	418	Detected	7.565	Detected	396	302	Detected	8.551	Detected	246	302	Detected	8.407	Detected	-	-	-	-	-
2	51	541	Detected	14.432	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	415	550	Detected	0.413	Detected	-	-	-	-	-	229	392	Detected	0.339	Detected	150	206	Detected	0.505	Detected
4	158	193	Detected	1.708	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	327	290	Detected	11.304	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	28	138	Detected	5.050	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	59	116	Detected	0.025	Undetected	-	-	-	-	-	104	53	Detected	0.016	Undetected	93	169	Detected	0.015	Undetected
8	-	-	-	-	-	17	131	Detected	0.196	Undetected	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	8	69	Detected	0.002	Undetected	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	31	173	Detected	7.257	Detected	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	8	146	Detected	8.300	Detected	-	-	-	-	-
12	27	22	Detected	0.000	Undetected	7	76	Detected	0.002	Undetected	-	-	-	-	-	-	-	-	-	-
13	11	62	Detected	0	Undetected	11	87	Detected	0.000	Undetected	-	-	-	-	-	-	-	-	-	-
14	194	214	Detected	2.186	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	602	290	Detected	13.888	Detected	722	651	Detected	9.810	Detected	-	-	-	-	-	-	-	-	-	-
16	414	405	Detected	0.634	Detected	238	184	Detected	2.686	Detected	222	337	Detected	0.375	Detected	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-	-	79	250	Detected	0.412	Detected	42	126	Detected	1.711	Detected
18	185	267	Detected	5.907	Detected	-	-	-	-	-	47	102	Detected	2.713	Detected	-	-	-	-	-
19	72	346	Detected	0.512	Detected	123	422	Detected	1.574	Detected	-	-	-	-	-	-	-	-	-	-
20	22	2	Detected	0.049	Undetected	47	122	Detected	0.185	Undetected	-	-	-	-	-	-	-	-	-	-
21	8	196	Detected	0.103	Undetected	0	150	Detected	0.260	Detected	4	89	Detected	0.556	Detected	1	119	Detected	1.108	Detected
22	-	-	-	-	-	45	110	Detected	0.019	Undetected	-	-	-	-	-	-	-	-	-	-
23	21	359	Detected	2.131	Detected	-	-	-	-	-	60	473	Detected	5.253	Detected	-	-	-	-	-
24	7	143	Detected	0.974	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	0	149	Detected	3.669	Detected	79	103	Detected	6.998	Detected	-	-	-	-	-	-	-	-	-	-
26	123	83	Detected	3.264	Detected	-	-	-	-	-	59	177	Detected	2.396	Detected	-	-	-	-	-
27	304	317	Detected	2.038	Detected	-	-	-	-	-	14	38	Detected	3.574	Detected	-	-	-	-	-
28	24	166	Detected	0.201	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	1	260	Detected	1.039	Detected	0	0	Undetected	1.676	Detected	5	366	Detected	2.959	Detected	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	73	243	Detected	0.073	Undetected	-	-	-	-	-
31	50	241	Detected	3.780	Detected	139	281	Detected	4.607	Detected	-	-	-	-	-	-	-	-	-	-
32	4	73	Detected	0.231	Detected	-	-	-	-	-	1	51	Detected	0.114	Undetected	-	-	-	-	-
33	3	187	Detected	0.000	Undetected	-	-	-	-	-	-	-	-	-	-	0	64	Detected	0.000	Undetected
34	29	268	Detected	0.507	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	21	154	Detected	7.797	Detected	-	-	-	-	-	0	198	Detected	4.457	Detected	-	-	-	-	-
36	-	-	-	-	-	-	-	-	-	-	81	8	Detected	1.734	Detected	33	5	Detected	3.311	Detected
37	29	135	Detected	2.653	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	-	-	-	-	-	169	13	Detected	0.074	Undetected	-	-	-	-	-	62	1	Detected	0.003	Undetected
39	-	-	-	-	-	6	243	Detected	0.306	Detected	-	-	-	-	-	-	-	-	-	-
40	-	-	-	-	-	-	-	-	-	-	34	81	Detected	0.971	Detected	-	-	-	-	-
41	-	-	-	-	-	29	274	Detected	0.640	Detected	-	-	-	-	-	-	-	-	-	-
42	-	-	-	-	-	218	138	Detected	0.108	Undetected	-	-	-	-	-	-	-	-	-	-
43	0	116	Detected	0.596	Detected	0	148	Detected	0.607	Detected	0	157	Detected	0.457	Detected	-	-	-	-	-
44	321	317	Detected	5.277	Detected	180	140	Detected	4.367	Detected	-	-	-	-	-	-	-	-	-	-
45	17	43	Detected	0.000	Undetected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46	3	36	Detected	43.582	Detected	-	-	-	-	-	48	149	Detected	44.680	Detected	-	-	-	-	-

47	-	-	-	-	-	-	-	-	-	-	4	86	Detected	9.325	Detected	-	-	-	-	-
48	5	103	Detected	0.525	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
49	161	182	Detected	1.753	Detected	-	-	-	-	-	23	25	Detected	1.481	Detected	-	-	-	-	-
50	0	36	Detected	0.115	Undetected	-	-	-	-	-	0	0	Undetected	0.008	Undetected	-	-	-	-	-
51	73	207	Detected	41.966	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
52	-	-	-	-	-	147	127	Detected	0.257	Detected	-	-	-	-	-	-	-	-	-	-
53	10	124	Detected	1.116	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
54	208	9	Detected	0.007	Undetected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
55	55	132	Detected	44.662	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
56	5	83	Detected	1.546	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
57	234	171	Detected	44.664	Detected	108	99	Detected	41.744	Detected	0	4	Detected	39.385	Detected	-	-	-	-	-
58	-	-	-	-	-	-	-	-	-	-	145	32	Detected	0.058	Undetected	-	-	-	-	-
59	-	-	-	-	-	62	100	Detected	8.856	Detected	-	-	-	-	-	-	-	-	-	-
60	-	-	-	-	-	247	139	Detected	1.083	Detected	-	-	-	-	-	-	-	-	-	-
61	0	37	Detected	0.000	Undetected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
62	-	-	-	-	-	28	28	Detected	4.922	Detected	-	-	-	-	-	-	-	-	-	-
63	38	81	Detected	2.330	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
64	56	49	Detected	0.126	Undetected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
65	8	7	Detected	0.061	Undetected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
66	54	65	Detected	4.977	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
67	2	219	Detected	5.305	Detected	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table detailing the results of HCMV-specific CMI interpretations as measured by both the HCMV-ELISPOT and QuantiFERON assays on the same blood samples at different time-points, of HCMV-seropositive women in the observation cohort study. Undetected CMI is in red font. Where results were not available for both assays these are stated as ‘-’ (see section 2.2.5.4 for the methods used, and section 4.4.2 for the description of results).

Abbreviations: CMI, cellular mediated immunity; ELISPOT, enzyme-linked immunosorbent spot; HCMV, human cytomegalovirus; IE1, immediate-early 1; IU/ml, international units per millilitre; pp65, phosphoprotein 65.

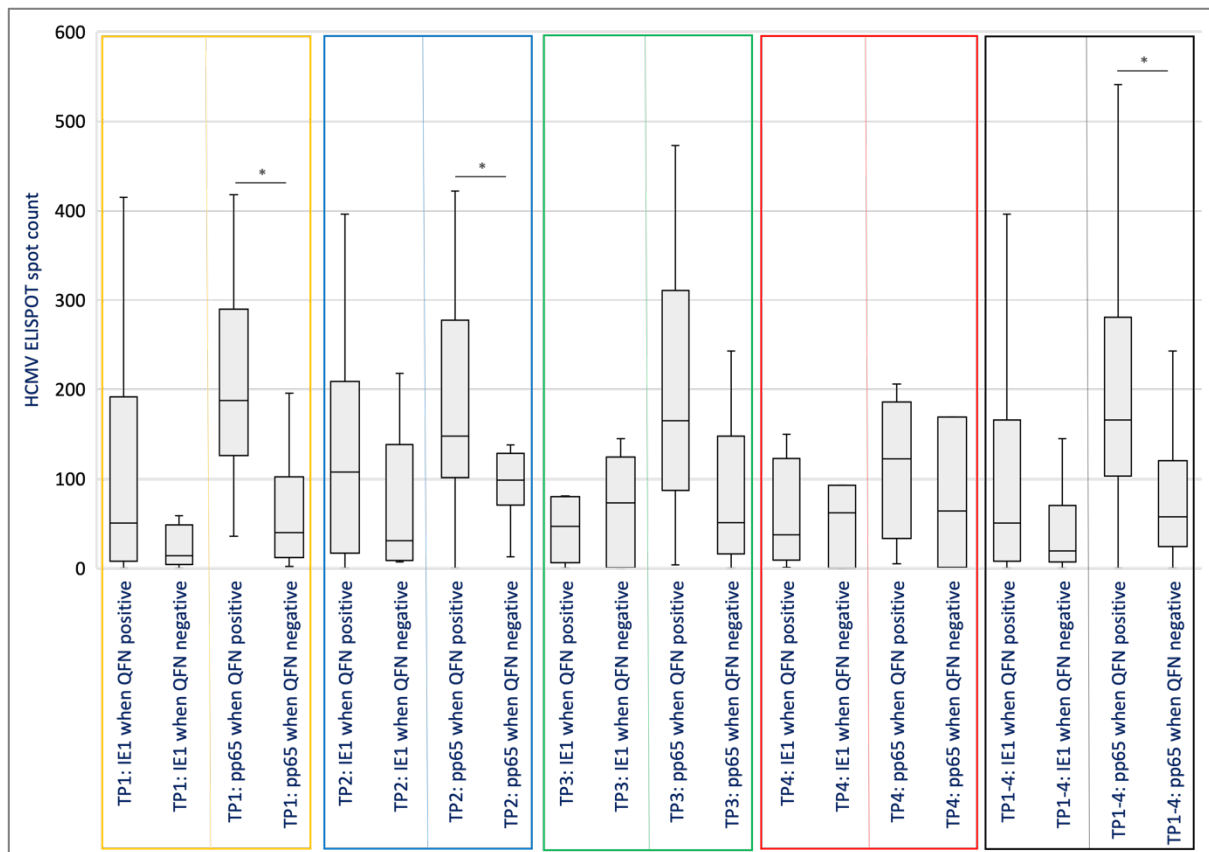
Table 14. Results of HCMV-specific CMI as measured by both HCMV-ELISPOT and HCMV-QuantiFERON in HCMV-seropositive women during pregnancy and the postpartum period

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HCMV QFN CMI detected	1 st time-point				2 nd time-point				3 rd time-point				4 th time-point				All time-points			
	IE1		pp65		IE1		pp65		IE1		pp65		IE1		pp65		IE1		pp65	
	Median [IQR]	Mann- Whitney U [p]	Median [IQR]	Mann- Whitney U [p]	Median [IQR]	Mann- Whitney U [p]	Median [IQR]	Mann- Whitney U [p]	Median [IQR]	Mann- Whitney U [p]	Median [IQR]	Mann- Whitney U [p]	Median [IQR]	Mann- Whitney U [p]	Median [IQR]	Mann- Whitney U [p]	Median [IQR]	Mann- Whitney U [p]	Median [IQR]	Mann- Whitney U [p]
Yes	51 [181]	140 [0.07]	188[162]	49 [<0.05]	108 [192]	53 [0.40]	148 [176]	29 [<0.05]	33 [65]	47 [0.84]	153 [193]	25 [0.10]	38 [79]	-*	123 [104]	-*	48 [153]	510 [0.22]	157 [175]	423 [<0.05]
No	14 [36]		40 [74]		31 [99]		99 [54]		73 [124]		51 [132]		-*		-*		20 [60]		58 [92]	

Table comparing the spread (median and IQR) of HCMV-ELISPOT spot count towards IE1 and pp65 antigens when HCMV-specific CMI was detected or not detected by HCMV-QuantiFERON, on the same blood samples, at each time-point and cumulatively, of HCMV-seropositive women in the observational cohort study. Mann-Whitney U test was performed to compare the findings seen, any p-value of less than 0.05 is described as p<0.05, representing a statistically significant result (see section 2.2.5.4 for the methods used, and section 4.4.2 for the description of results).
Abbreviations: CMI, cellular mediated immune; ELISPOT, enzyme-linked immunosorbent spot; HCMV, human cytomegalovirus; IE1, immediate-early 1; IQR, interquartile range; p, p-value; pp65, phosphoprotein 65; QFN, QuantiFERON.
*Sample size not large enough to calculate for the relevant test.

Table 15. Spread of HCMV-ELISPOT spot count towards IE1 and pp65 antigens when HCMV-specific CMI was detected or not detected by HCMV-QuantiFERON, in HCMV-seropositive women during pregnancy and the postpartum period



Boxplot comparing the spread of HCMV-ELISPOT spot count towards IE1 and pp65 antigens when HCMV-specific CMI were detected ('positive') or were not detected ('negative') with HCMV-QuantiFERON, at each time-point and cumulatively, in HCMV-seropositive women in the observational cohort study. A significant result (p-value <0.05) is marked with an asterisk symbol (see section 2.2.5.4 for the methods used, and section 4.4.2 for the description of results).

Abbreviations: CMI, cellular mediated immunity; ELISPOT, enzyme-linked immunosorbent spot; HCMV, human cytomegalovirus; IE1, immediate-early 1; QFN, QuantiFERON; pp65, phosphoprotein 65; TP1, 1st time-point; TP2, 2nd time-point; TP3, 3rd time-point; TP4, 4th time-point; TP1-4, all time-points.

Figure 20. Spread of HCMV-ELISPOT spot count towards IE1 and pp65 antigens when HCMV-specific CMI was detected or not detected by HCMV-QuantiFERON, in HCMV-seropositive women during pregnancy and the postpartum period

4.4.3. Discussions

At every time-point and cumulatively during pregnancy in HCMV-seropositive women, HCMV-specific CMI was detected in around 70% of blood as measured by HCMV-QuantiFERON. Around 70% of HCMV-seropositive women also had at least one event of HCMV-specific CMI detected in their blood during pregnancy as measured by HCMV-QuantiFERON. There was no comparable study that has looked at the proportion of HCMV-specific CMI detection as measured with HCMV-QuantiFERON in immunocompetent HCMV-seropositive pregnant women in the literature to compare the findings with.

When HCMV-specific CMI was measured with HCMV-ELISPOT, it was detected in almost all blood of HCMV-seropositive pregnant women during pregnancy, where all of them had the detection found in at least one event during pregnancy. Similar to HCMV-QuantiFERON, there were no comparable study found with similar conditions in the literature.

As a reminder, although both are IGRAs and used as proxies to measure HCMV-specific CMI, there are some differences between HCMV-QuantiFERON and HCMV-ELISPOT in their characteristics. HCMV-QuantiFERON measures HCMV-specific CD8+ cell responses against a pool of HCMV-specific antigens based on the level of secreted IFN- γ , whereas HCMV-ELISPOT measures HCMV-specific CD4+ and CD8+ cell responses against IE1 and pp65 antigen separately based on the number of IFN- γ -secreting cells (Saldan *et al.*, 2016; Lee *et al.*, 2017; Oxford Immunotec Ltd, 2017; QIAGEN, 2018).

HCMV-specific CMI detection was higher when measured by HCMV-ELISPOT than that of HCMV-QuantiFERON. This could reflect the inclusion of HCMV-specific CD4+ cells as part of the HCMV-ELISPOT assay which are not present in the HCMV-QuantiFERON assay (Saldan *et al.*, 2015). It has been found that while the level of CD8+ cell IFN- γ response was

higher than that of CD4+ in healthy HCMV-seropositive individuals, the CD4+ cell responses were still at higher values in HCMV-seropositive individuals than that in HCMV-seronegative individuals (Sinclair *et al.*, 2004), and therefore the additional CD4+ cell component in the HCMV-ELISPOT could have contributed to the higher rate of HCMV-specific CMI seen.

The higher detection and level of HCMV-specific CMI as measured by HCMV-ELISPOT towards pp65 antigen than that towards IE1 antigen could also be of important significance. Unique to herpesviruses, tegument is a structure that contains virus encoded proteins, where pp65 is the most abundant tegument in HCMV (Chevillotte *et al.*, 2009) and is responsible for the modulation and evasion of a host's CMI during HCMV infection (McLaughlin-Taylor *et al.*, 1994). Pp65 targets both humoral and cellular immunity and is a dominant target antigen of HCMV-specific CD8+ cells (McLaughlin-Taylor *et al.*, 1994). Previous studies have found that while HCMV-specific CD8+ cell responses to pp65 and IE1 are comparatively strong, HCMV-specific CD4+ responses to pp65 are greater than those to IE1 in HCMV-seropositive immunocompetent individuals (Sylwester *et al.*, 2005; Slezak *et al.*, 2007), where the importance of CD4+ cell component in HCMV-specific CMI has already been discussed. Pp65 may therefore be a dominant antigen in shaping HCMV-specific CMI for both CD4+ and CD8+ cells in immunocompetent HCMV-seropositive individuals, including pregnant women.

A consideration to be made with the considerable amount (34.1%) of HCMV-seropositive women that had undetectable HCMV-specific CMI during pregnancy when measured with the HCMV-QuantiFERON, so called to be discordant (i.e., HCMV-seropositive and undetectable HCMV-specific CMI) (Valle-Arroyo *et al.*, 2020), is as to whether it was due to an artifact to the assay and/or it identified pregnant women with immunological characteristics that were different from the non-discordant (i.e., HCMV-seropositive and

detectable HCMV-specific CMI) (Valle-Arroyo *et al.*, 2020) pregnant women. Comparing findings with a different assay may help ascertain this, which was done with the HCMV-ELISPOT where the HCMV-ELISPOT spot count towards pp65 antigen was higher (i.e., higher level of HCMV-CMI detection) in samples with detectable HCMV-specific CMI by HCMV-QuantiFERON than that in samples with undetectable HCMC-specific CMI by HCMV-QuantiFERON [157 versus 58, Mann-Whitney U= 423, $p<0.05$] time-points. which may suggest it was related to specific immunological characteristics that were different from the non-discordant pregnant women. Moreover, majority of the women had either all detected or all undetected HCMV-QuantiFERON HCMV-specific CMI instead of the combination of both, where most of the discordant findings of results were persistent in these women. This may also suggest that it was related to specific immunological characteristics in these pregnant women, which may be as discussed related to the absence of HCMV-specific CD4+ cell detection and only inclusion of CD8+ cell detection in the HCMV-QuantiFERON assay (Saldan *et al.*, 2015). Other variables that may suggest an impact to the HCMV-specific CMI should also be looked at, such as HCMV shedding which was explored in a later chapter in this thesis (see section [6.3](#)).

It is important to remember that not only are the methods, principles, and antigens involved for both IGRAs different, but also the results interpretation (Saldan *et al.*, 2015). For HCMV-ELISPOT, a detection would include any number of spots above zero (following the deduction of the value in the negative control), and for the HCMV-QuantiFERON, not any value above zero (following the deduction of the value in the negative control) meet the requirement for a detection and therefore this could suggest that the HCMV-QuantiFERON assay has a narrower range of result interpretation to be detected than that of HCMV-

ELISPOT. However, as the methodology of both IGRAs are different from each other, a direct comparison of the results interpretation may not be suitable.

Optimal monitoring of HCMV-specific CMI detection, and potentially therefore risk of cCMV transmission in HCMV-seropositive women during pregnancy, may be better achieved with HCMV-ELISPOT. Although both assays poorly agreed on their HCMV-CMI detection rating, higher level of HCMV-specific CMI in HCMV-ELISPOT were more likely when HCMV-CMI was detected in HCMV-QuantiFERON than when it was not detected. Pp65 may be a dominant antigen in shaping HCMV-specific CMI in HCMV-seropositive pregnant women.

4.5. Genomic sequencing of human cytomegalovirus shedding isolates obtained from participants of the observational cohort study

4.5.1. Background

I sought to investigate the feasibility of genomic sequencing of HCMV shedding isolates obtained from HCMV-seropositive pregnant women in the observational cohort study. A full description of the methods can be found in section [2.2.5.5](#).

4.5.2. Results

Table 16 describes the saliva, urine, and vaginal secretion samples of the HCMV-seropositive women in the observational cohort study that were retested for extraction of HCMV DNA, to produce to genomic libraries for whole genome sequencing. From 20 samples, 17 were able to have HCMV DNA extracted and therefore subjected to genomic

library preparation. Nine out of 17 HCMV DNA samples that did not produce genomic library did not shear well and produced sample input amount below the threshold (2.9 ng) required for production of genomic libraries. Eight out of 17 samples achieved sample input amount above the threshold (2.9ng) to shear DNA suitable to produce genomic library, i.e., produce PCR cycles post-capture (Table 17). These 8 samples were subjected to hybridisation and sequencing (Table 17), before being subjected to quality control (Table 18). From 8 samples that were hybridised and sequenced, one sample passed the quality control (more than 90% genomic coverage) as described in Table 18.

Women	Time-point	Sample site	Quantity of detected HCMV DNA (IU/ml)	Quantity of extracted HCMV DNA (ge/ml)	Subjected to genomic library preparation
1	1	Urine	10900	25781	Yes
	1	Vaginal secretions	32000	73875	Yes
	2	Urine	4930	7945	Yes
	2	Vaginal secretions	3660	2752	Yes
	3	Vaginal secretions	6480	5314	Yes
	4	Urine	2490	4151	Yes
2	3	Vaginal secretions	398	0	No
	4	Vaginal secretions	639	1775	Yes
3	2	Saliva	768	0	No
	2	Urine	601	2467	Yes
	3	Saliva	634	605	No
4	1	Vaginal secretions	9630	22895	Yes
	2	Urine	1130	1544	Yes
	3	Vaginal secretions	947	9878	Yes
5	1	Vaginal secretions	1270	16505	Yes
	2	Vaginal secretions	7500	7144	Yes
	3	Vaginal secretions	3170	4116	Yes
	4	Urine	439	0	No
6	1	Urine	399	2210	Yes
	1	Vaginal secretions	1270	4151	Yes

Table describing the samples retested for extraction of HCMV DNA, of HCMV-seropositive women in the observational cohort study. Those with HCMV DNA extracted were subjected to genomic library preparation (see section 2.2.5.5 for the methods used, and section 4.5.2 for the description of results).

Abbreviations: DNA, deoxyribonucleic acid; HCMV, human cytomegalovirus; ge/ml, genome equivalents per millilitre; IU/ml, international units per millilitre

Table 16. Retesting of HCMV DNA detected samples for extraction in preparation for genomic library sequencing, of HCMV-seropositive women during pregnancy and at the postpartum period

Women	TP	Site	Quantity of extracted HCMV DNA (ge/ml)	Qubit concentration (ng/μL)	Sample input amount (ng)	Estimated Genome Copies/ge extract	Estimated ge input	Number of PCR cycles (pre-capture)	Number of PCR cycles (post-capture)	Bait design	Final library concentration (Qubit) (ng/μL)	Mean Peak Size (bp)	Index sequence	Proportion of run achieved (%)
1	1	U	25781	<0.05	<2.9	0.32	18.7	13	19	612971	12.8	680	AACGCTTA	4.4335
	1	V	73875	0.4	25	0.92	53.6	13	0	-	-	-	-	-
	2	U	7945	<0.05	<2.9	0.10	5.8	13	0	-	-	-	-	-
	2	V	2752	0.6	34	0.03	2.0	13	19	612971	13.1	720	GGAGAACA	4.7624
	3	V	5314	2.1	121	0.07	3.9	13	19	612971	11.9	509	CATCAAGT	4.7813
	4	U	4151	<0.05	<2.9	0.05	3.0	13	0	-	-	-	-	-
2	4	V	1775	<0.05	<2.9	0.02	1.3	13	0	-	-	-	-	-
3	2	U	2467	<0.05	<2.9	0.03	1.8	13	0	-	-	-	-	-
	3	S	605	<0.05	<2.9	0.00	0.2	13	0	-	-	-	-	-
4	1	V	22895	0.3	19	0.29	16.6	13	19	612971	1.88	328	AAGGTACA	1.9915
	2	U	1544	<0.05	<2.9	0.02	1.1	13	0	-	-	-	-	-
	3	V	9878	1.4	80	0.12	7.2	13	19	612971	0.782	328	CGCTGATC	1.4408
5	1	V	16505	<0.05	<2.9	0.21	12.0	13	0	-	-	-	-	-
	2	V	7144	7.8	454	0.09	5.2	11	19	612971	12.5	436	GGTGCGAA	4.1811
	3	V	4116	2.1	124	0.05	3.0	13	19	612971	4.9	348	CCTAATCC	2.6315
6	1	U	2210	<0.05	<2.9	0.03	1.6	13	0	-	-	-	-	-
	1	V	4151	0.1	3	0.05	3.0	13	19	612971	13.3	533	CTGAGCCA	4.4159

Table describing the extracted HCMV DNA that were subjected to genomic library preparation, from samples of HCMV-seropositive women in the observational cohort study. The sample input amount requires to be >2.9ng to shear DNA suitable to produce genomic library. Where genomic library was produced, i.e. PCR cycles were produced post-capture, these samples were hybridised and sequenced (see section 2.2.5.5 for the methods used, and section 4.5.2 for the description of results).

Abbreviations: Bp, base pair; DNA, deoxyribonucleic acid; HCMV, human cytomegalovirus; ge/ml, genome equivalents per millilitre; IU/ml, international units per millilitre; ng/μL, nanogram per microlitre; PCR, polymerase chain reaction; S, saliva; TP, time-point; U, urine; V, vaginal secretions; -, no results available.

Table 17. Genomic library hybridisation and sequencing of samples with extracted HCMV DNA in HCMV-seropositive women during pregnancy and the postpartum period

Women	Time-point	Sample Site	Reference Length	Mapped reads	Breadth	Coverage (%)	Minimum Depth	Maximum Depth	Average Depth
1	1	Urine	235646	312	8854	3.8	0	19	0.07
	2	Vaginal secretions	235646	658	28244	12.0	0	53	0.24
	3	Vaginal secretions	235646	2590	156500	66.4	0	55	1.44
4	1	Vaginal secretions	235646	468	27328	11.6	0	63	0.21
	3	Vaginal secretions	235646	166	3413	1.5	0	46	0.03
5	2	Vaginal secretions	235646	15299	229572	97.4	0	109	9.36
	3	Vaginal secretions	235646	446	15901	6.8	0	83	0.15
6	1	Vaginal secretions	235646	1747	89613	38.0	0	56	0.92

Table describing the quality control assessment of hybridised and sequenced HCMV DNA, from samples of HCMV-seropositive women in the observational cohort study. Sample that passed the quality control, i.e., achieving more than 90% genomic coverage is in green font (see section 2.2.5.5 for the methods used, and section 4.5.2 for the description of results).
Abbreviations: DNA, deoxyribonucleic acid; HCMV, human cytomegalovirus.

Table 18. Quality control assessment of hybridised and sequenced HCMV DNA from samples of HCMV-seropositive women during pregnancy

The hybridised and sequenced HCMV DNA that passed the quality control was from a vaginal secretion sample of a 37-year-old White British pregnant woman during the 2nd time-point. The within host diversity was 0.002, below the threshold that raises the possibility of a mixed HCMV infection (≥ 0.005 (Cudini *et al.*, 2019; Venturini *et al.*, 2022)) and therefore there was no evidence found of mixed infection. Figure 21 illustrates the HCMV sequencing phylogenetic tree generated to compare the sequenced HCMV genome with previously described genomes. One hundred six HCMV sequences were used to make this tree: the genome sequence and 105 sequences (91 sequences from populations similar to the genome sequence, and 14 sequences from geographically diverse population (African and Israeli sequences) to the genome sequence). The genome sequence was found to cluster closely with European and North American sequences, in particular with three sequences from Glasgow, Hannover and Ohio.

4.5.3. Discussions

More than half of the HCMV DNA samples did not shear well, which means that the samples could not be cleaved into appropriate fragment sizes to prepare them for genomic sequencing analysis (Agilent Technologies, 2021); this may be due to the quantity and quality of the HCMV DNA samples. Most of the HCMV DNA samples that did not shear well were of lower viral loads. Although few of these samples were actually of the higher viral loads, overall, the viral loads of these samples were still lower (in the hundreds to the lower end of thousands) when compared to the higher thousands to millions of viral loads that may be seen in blood of an individual with primary HCMV infection. The HCMV DNA may also not have sheared well due to the sample type (saliva, urine, vaginal secretions) where there may high levels of contaminating DNA from the host and other commensal microorganisms (Bachmann *et al.*, 2018). There may also be presence of DNA amplification inhibitors in the clinical sample matrix (Bachmann *et al.*, 2018). The success of genomic sequencing is dependent on the quality and quantity of DNA extracted from a specimen, including in HCMV, where several methods have been introduced to prepare and optimise a sample to improve the sequencing ability, such as methods to reduce the presence of host DNA and to separate out microbial DNA (Bachmann *et al.*, 2018).

Only one sample of a pregnant woman with HCMV-seropositive produced sufficient genomic coverage to be fully sequenced and analysed. This sample also sheared well and had the highest sample input amount. Interpretation can only be made on this individual. Evidence of more than one HCMV strain in this sample was not found, i.e., a singular HCMV strain was seen which may suggest that the HCMV shedding seen was a result of reactivation. It would be valuable to relate this hypothesis to the individual's hygiene-related contact with her children's bodily fluids and to that of other HCMV seropositive

pregnant women with shedding detected (see section [6.2.3](#)), as well as her HCMV-specific CMI status found at the time, where in a later chapter, this will be explored (see section [6.4](#)). The sequenced HCMV genome, of a European woman, was also consistent with the European HCMV sequences sourced from GenBank sequence database.

As emphasised, the interpretation here can only be made on an individual, with only one HCMV DNA sample sequenced. Nonetheless, the interpretation provided with some possible answers towards the mode of non-primary HCMV infection responsible, which in turn provided with some possible answers towards the contributing factors to look into. Therefore, investigating genomic sequencing of HCMV shedding isolates obtained from HCMV-seropositive pregnant women may be valuable, and methods to optimise its ability to be sequenced are worth investigating.

4.6. Screening for congenital cytomegalovirus infection in newborn infants of participants in the observational cohort study

4.6.1. Background

I sought to investigate the feasibility of screening for congenital cytomegalovirus infection in newborn infants of the HCMV-seropositive pregnant women in the observational cohort study. A full description of the methods can be found in section [2.2.5.6](#).

4.6.2. Results

Sixty-nine newborn infants of HCMV-seropositive mothers had saliva samples taken to test for HCMV DNA, of which none [0%, one-sided 97.5% CI: 0-0.05%] had detectable HCMV DNA. Of these 69 newborn infants tested for cCMV infection, 59 had HCMV-seropositive mothers tested for HCMV DNA on their saliva, urine, and vaginal secretions samples at all of the study time-points during pregnancy, of which 15.3% [9/59; 95% CI: 7.2-27.0%] had HCMV shedding detected in their bodily fluids on at least one occasion during pregnancy.

4.6.3. Discussions

No infants screened were found to have cCMV infection, which is consistent with the literature, of which the vertical transmission following a non-primary maternal infection is reported to be 0.2-1% (Raynor, 1993). Of course, in this cohort none of the HCMV-seropositive women were confirmed to have non-primary maternal infection, and with only around 15% of the women (with their newborn infants tested for cCMV infection) who were found to have HCMV shedding during pregnancy, which may suggest non-primary infection, the vertical transmission rate may be expected to be lower. The experiences of participating women with having their newborn infants screened for cCMV infection were discussed in section 5.5. Studying a larger scale of HCMV-seropositive pregnant women and following up until birth, and therefore a larger scale of newborn infants to be screened for cCMV infection, would provide clearer epidemiological information on the vertical transmission rate in HCMV-seropositive pregnant women as well as associations with other risk factors, such as HCMV shedding.

5. Feasibility of the observational cohort study and experiences of participants

5.1. Recruitment and retention of participants in the observational cohort study

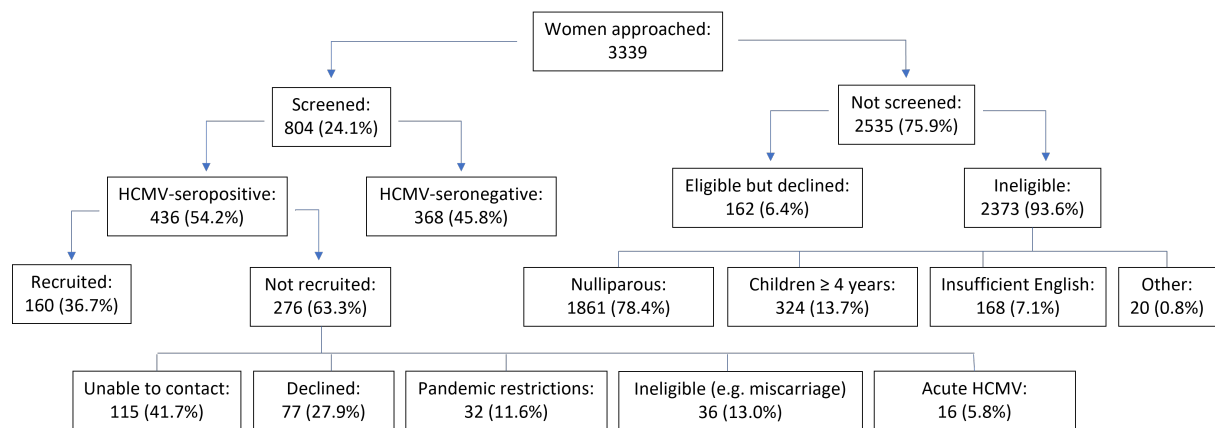
5.1.1. Background

I sought to investigate the recruitment and retention of participants in the observational cohort study. A full description of the methods can be found in section 2.2.5.7.

5.1.2. Results

Figure 22 illustrates the flow of the women approached, those who consented to testing for HCMV serology (study screening) and those recruited into the observational study. A total of 3339 women were approached for screening, of whom 804 (24.1%) were tested for HCMV serology. There were 2535 women who were not tested for HCMV serology, the main reason of which was ineligibility (2373; 93.6%) however 162 women (6.4%) were eligible for the study but declined to participate. Reasons for ineligibility were predominantly due to being nulliparous (1861; 78.4%), followed by living with children equal to or older than 4 years of age (324; 13.7%), and insufficient command of English (168; 7.1%). Of the 804 women screened, 436 (54.2%) were found to be HCMV-seropositive and

368 (45.8%) HCMV-seronegative. From the 436 women found to be HCMV-seropositive, 160 (36.7%) were recruited. Using an estimated proportion of HCMV-seropositive women who shed HCMV at least one during pregnancy of 30% (see section [2.2.6](#); 6% precision with target sample size of 200) to calculate the study sample size, the precision was 7% for 160 recruited women. The 276 (63.3%) HCMV-seropositive women not recruited were due to, in order of commonest to least common reason, inability to be contacted (115; 41.7%), declined by the women (77; 27.9%), meeting ineligibility criteria such as miscarriage (36; 13.0%), the COVID-19 pandemic restrictions (32; 11.6%), and suspicion of or confirmed acute (lytic) HCMV infection (16; 5.8%) (Figure 22).



Flow diagram illustrating the number and proportion of pregnant women approached, screened (tested for HCMV serology), and recruited into the observational cohort study (see section 2.2.5.7 for the methods used, and section 5.1.2 for the description of results). Abbreviations: HCMV, human cytomegalovirus

Figure 22. Flow diagram of women screened and recruited into the observational cohort study

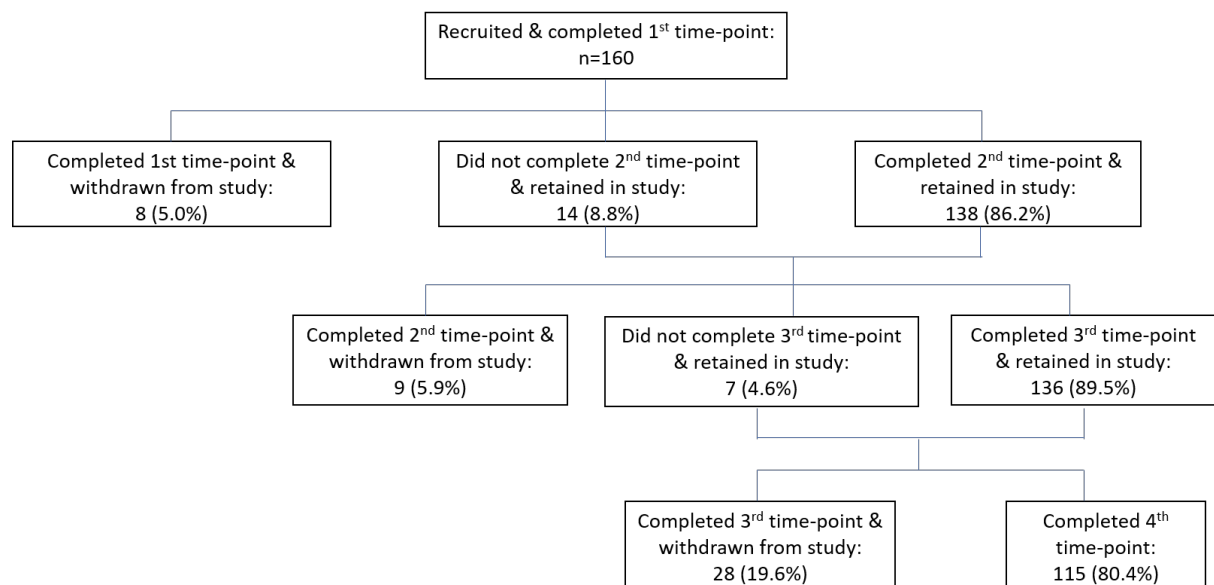
From 160 women who completed the 1st study time point, 8 (5.0%) were withdrawn thereafter. Of these, 7 (87.5%) were lost to follow up (1 [14.3%] prior to the COVID-19 pandemic restrictions and 6 [85.7%] during to the COVID-19 pandemic restrictions). One of the 8 (12.5%) was withdrawn during the COVID-19 pandemic disruption due to the 1st time-point being completed out of window.

Fourteen (8.8%) did not complete the 2nd time-point due to 1 (7.1%) missing the appointment, 4 (28.6%) having their 1st time-point late during the 2nd time-point gestation window and therefore missed the 2nd time-point, and 9 (64.3%) due to the COVID-19 pandemic restrictions and declining to freeze their samples at home. One hundred thirty-eight (86.2%) completed the 2nd time-point.

From 152 women remaining in the study after the 2nd time-point, 9 (5.9%) were withdrawn because of loss to follow up in 7 (77.8%), of which 4 (57.1%) were before the COVID-19 pandemic restrictions and 3 (42.9%) were during the COVID-19 pandemic restrictions. Two of the 9 (22.2%) were withdrawn during the COVID-19 pandemic disruption due to the 3rd time-point being completed out of window. Seven (4.6%) did not complete the 3rd time-point, out of which 6 (85.7%) were due to the COVID-19 pandemic restrictions and declining to freeze their samples at home, and 1 (14.3%) were due to having given birth during the 3rd time-point gestation window and therefore completed a 4th time-point instead of a 3rd time-point. One hundred thirty-six (89.5%) completed the 3rd time-point.

From 143 women remaining in the study after the 3rd time-point, 28 (19.6%) were withdrawn because of loss to follow up in 26 (92.9%), of which 6 (23.1%) were before the COVID-19 pandemic restrictions and 20 (76.9%) were during the COVID-19 pandemic restrictions. Two of the 28 (7.1%) were withdrawn during the COVID-19 pandemic

disruption due to the 4th time-point being completed out of window. One hundred fifteen (80.4%) women completed the study. Figure 23 illustrates the flow of retention and withdrawal at each time-point for the recruited women.



Flow diagram illustrating the number and proportion of participants who were withdrawn and completed the observational cohort study at each time-point (see section 2.2.5.7 for the methods used, and section 5.1.2 for the description of results).

Figure 23. Flow diagram of completion of time-points and study by women recruited into the observational study

5.1.3. Discussions

The proportion of women eligible for the study screening (testing for HCMV serostatus) was much lower than anticipated; more than 90% of the women approached were ineligible. This is due to majority of them being nulliparous, followed by not living with/having a child aged younger than 4 years. It is unknown if this would be seen in different parts of London or indeed other parts of the UK, or whether this is a product of local factors, but is something to be considered in the design of a larger scale trial. Although this made identification of participants for the observational cohort study more challenging, moreover with the study screening period halted twice due to the COVID-19 pandemic disruption, overall, of those who were eligible more than 90% agreed to take part in the study screening, which is encouraging for future work. In this cohort, 5.8% of HCMV-seropositive women screened were found to be suspicious of having an acute HCMV infection. Although this proportion is slightly higher than that in the literature where primary maternal HCMV infection occurs in around 1-4% of women (Nigro, 2009), it is not expected that all of them had confirmed primary maternal HCMV, but the suspicion of it made them ineligible to be recruited.

Although the total number of participants was slightly less than the target sample size of 200, the percentage of precision (based on the estimated proportion used for the sample size calculation) for the recruited participants remained narrow and similar to that of the target sample size. The COVID-19 pandemic did impact on recruitment for the observational study, as well as the completion of all visits for the participants, where some participants had to withdraw early, or missed some visits in between due to inability to see participants. Nevertheless, majority completed the study. As described, a substantial amount of saliva, urine and vaginal secretion samples were not able to be collected during

the COVID-19 pandemic disruption due to study visits that could not take place during this period. To allow sample collection to continue during this period, participants were asked to self-sample as usual and store the samples in their freezer until collection by the study team is possible, which improved on the sample number, however only some participants agreed to do so. Moreover, a good proportion of participants declined vaginal sampling during the post-partum period at the 4th time-point, likely due to the circumstances of it being soon after delivery in an effort to coincide with a routine appointment (in this case, on labour ward or postnatal ward) like with other time-points; the timeline and setting for this visit window would need to be strongly considered in the design of a larger scale trial to ensure sample collection are not compromised, whilst also striking a balance to ensure ease of visit attendance for participants (e.g., home visits could be emphasised for a visit during the postpartum period).

5.2. Information seeking and familiarity with human cytomegalovirus in participants of the observational cohort study

5.2.1. Background

I sought to investigate the information seeking of and familiarity of HCMV in participants of the observational cohort study. A full description of the methods can be found in section 2.2.5.8.

5.2.2. Results

Before recruitment into the study, 36.9% (59/160) of women had sought additional information regarding HCMV, since being approached for study screening. The source of information in order of most to least common: NHS website (29/59; 49.2%), search engine (22/59; 37.3%), CMV Action (11/59; 18.6%), midwife/obstetrician/GP (9/59; 15.3%). From 58 recorded responses in the study questionnaires, 34.5% (20/58) of women sought additional information at some point during their study participation. The source of information in order of most to least common: NHS website (13/20; 65.0%), search engine (7/20; 35.0%), midwife/obstetrician/GP (4/20; 20.0%), CMV Action (3/20; 15.0%).

The perceptions on how HCMV infection is spread by the 160 participating women at the beginning of the study are summarised in Table 19.

Beginning of study: Perception on how HCMV infection is spread	Mean score [95% CI] (N=160)
HCMV can spread via saliva	3.88 [3.73-4.04]
HCMV can spread via urine	3.51 [3.33-3.68]
HCMV can spread via faeces	3.41 [3.25-3.57]
HCMV can spread via hugging	2.43 [2.26-2.61]
HCMV can spread via kissing	3.59 [3.43-3.76]
HCMV can spread via casual contact	2.53 [2.35-2.70]

Table summarising the perceptions on how HCMV infection is spread by the HCMV-seropositive women at the beginning of the study. Mean score out of 5, where higher mean scores indicated higher level of agreement to the statement on how HCMV is spread (see section 2.2.5.8 for the methods used, and section 5.2.2 for the description of results).

Abbreviations: CI, confidence interval; HCMV, human cytomegalovirus; N, total observations.

Table 19. Participants' perceptions on how HCMV infection is spread at the beginning of the observational cohort study

From 58 recorded responses in the study questionnaires, there were significant differences to the participants' perceptions on how HCMV is spread for all type of bodily fluids at the end of the study compared to at the beginning, as summarised in Table 20.

Perception on how HCMV infection is spread	Mean score [95% CI] (N=58)		Paired t-test value (df)	p-value
	Beginning of study	End of study		
HCMV can spread via saliva	4.07 [3.80-4.33]	4.57 [4.41-4.73]	-3.76 (57)	<0.05
HCMV can spread via urine	3.64 [3.32-3.95]	4.31 [4.08-4.54]	-4.01 (57)	<0.05
HCMV can spread via faeces	3.57 [3.30-3.84]	4.34 [4.13-4.56]	-4.92 (27)	<0.05
HCMV can spread via hugging	2.28 [1.97-2.58]	3.67 [3.33-4.07]	-6.43 (27)	<0.05
HCMV can spread via kissing	3.64 [3.36-3.92]	4.28 [4.04-4.51]	-4.22 (27)	<0.05
HCMV can spread via casual contact	2.26 [1.98-2.54]	3.28 [2.93-3.62]	-5.11 (27)	<0.05

Table summarising the perceptions on how HCMV infection is spread at the end of the study compared to the beginning by the same HCMV-seropositive women, available in 58 women. Mean score out of 5, were higher mean scores indicated higher level of agreement to the statement on how HCMV is spread (see section 2.2.5.8 for the methods used, and section 5.2.2 for the description of results). Abbreviations: CI, confidence interval; df, degree of freedom; HCMV, human cytomegalovirus; N, total observations.

Table 20. Participants' perceptions on how HCMV infection is spread at the end of in comparison to the beginning of the observational cohort study

5.2.3. Discussions

Just over a third of participating women sought additional information regarding HCMV both at the beginning during the time of study screening and later during their participation in the observational cohort study, the most common source being the NHS. It is reassuring that most pregnant women refer to a reputable source for additional information on their health and wellbeing, but this also highlights the importance of up-to-date and robust information is available and accessible to them through their routine antenatal care.

At the beginning of participation, the participating women agreed highly on the ability of HCMV infection to spread via saliva, urine, faeces, and kissing, and less so on hugging and casual contact. Interestingly, at the end of participation their agreement on all mode of spread increased. A previous study has found that a change in knowledge occur following an educational intervention in research (Price *et al.*, 2014; Calvert *et al.*, 2021). Although educational intervention was not part of this study, it has been shown here that the women sought additional information at the beginning and later during their study participation on their own accord. However, the change in knowledge or perceptions of how HCMV infection is spread here may not necessarily reflect increased knowledge and may be influenced by general increased awareness of hygiene; acquisition of information through the study participation was also discussed in-depth in section 5.5.

5.3. Hygiene-related contact with children's bodily fluids

5.3.1. Background

I sought to investigate the frequency of specific hygiene behaviours in relation to contact with children's bodily fluids, defined as "hygiene-related contact with children's bodily fluids", in participants of the observational cohort study. A full description of the methods can be found in section [2.2.5.9](#).

5.3.2. Results

Table 21 summarises the average frequencies of hygiene-related contact with children's bodily fluids by participating women, collected through the completion of questionnaires at different time-points, with mean scores out of 5 where higher mean scores indicated higher level of frequency of the contact.

From 160 women, 93 (58.1%) completed these questionnaires at all three time-points during pregnancy; for the different time-points during pregnancy, there were statistically significant differences among the mean frequency scores for washing hands with soap after handling their children's faeces [$F(2, 184) = 223.30, p < 0.05$] and washing hands with soap after wiping their children's noses [$F(2, 184) = 3.14, p < 0.05$] (Table 22)

From 160 women, 59 (36.9%) completed these questionnaires at all four time-points of the study; for the different time-points during pregnancy and at postpartum, there were statistically significant differences among the mean frequency scores also for washing hands with soap after handling their children's faeces [$F(3, 174) = 177.03, p < 0.05$] and washing

1573 hands with soap after wiping their children's noses [$F(3, 174) = 6.12, p < 0.05$], as well as
1574 putting their children's used dummy in their own mouth [$F(3, 174) = 2.88, p < 0.05$] (Table 23).

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Contact	1 st time-point: Mean score (SD) N= 154	2 nd time-point: Mean score (SD) N= 121	3 rd time-point: Mean score (SD) N= 113	4 th time-point: Mean score (SD) N= 75
Wash hands with soap after handling child's faeces	2.02 (1.51)	4.60 (0.83)	4.65 (0.71)	4.63 (0.63)
Wash hands with just water after handling child's faeces	1.83 (1.18)	1.79 (1.18)	1.83 (1.18)	1.83 (1.04)
Clean hands with gel after handling child's faeces	2.11 (1.08)	2.11 (1.15)	1.99 (1.12)	2.12 (1.06)
Wash hands with soap after handling child's urine	4.03 (1.04)	3.90 (1.06)	3.88 (1.03)	3.93 (0.91)
Wash hands with just water after handling child's urine	2.14 (1.22)	2.31 (1.26)	2.28 (1.19)	2.27 (1.08)
Clean hands with gel after handling child's urine	2.09 (1.13)	1.99 (1.06)	2.00 (1.05)	2.08 (1.04)
Wash hands with soap after wiping child's nose	2.75 (1.10)	2.71 (1.09)	2.84 (1.03)	2.91 (1.02)
Wash hands with just water after wiping child's nose	2.34 (1.12)	2.40 (1.02)	2.39 (0.99)	2.36 (1.01)
Clean hands with gel after wiping child's nose	1.82 (0.94)	1.88 (0.93)	1.79 (0.92)	1.81 (0.89)
Put child's used dummy in own mouth	1.79 (1.23)	1.65 (1.09)	1.71 (1.09)	1.48 (0.92)
Eat child's leftover food	3.25 (1.12)	3.13 (1.14)	3.00 (1.13)	3.03 (1.11)
Eat with child's used cutlery	2.86 (1.24)	2.86 (1.29)	2.78 (1.28)	2.59 (1.09)
Drink from child's used cup	2.48 (1.25)	2.40 (1.29)	2.37 (1.27)	2.04 (1.16)
Kiss child on lips	3.41 (1.36)	3.42 (1.42)	3.51 (1.33)	3.25 (1.40)

Table summarising the reported frequencies of hygiene-related contact with children's bodily fluids, by HCMV-seropositive women in the observational cohort study at different time-points. Mean score out of 5, where higher mean scores indicated higher level of frequency of the contact (see section 2.2.5.9 for the methods used, and section 5.3.2 for the description of results).

Abbreviations: HCMV, human cytomegalovirus; N, total observations; SD, standard deviation.

Table 21. Frequency of hygiene-related contact with children's bodily fluids by HCMV-seropositive pregnant women at different time-points in the observational cohort study

Contact	1 st time-point: Mean score (SD) N=93	2 nd time-point: Mean score (SD) N=93	3 rd time-point: Mean score (SD) N=93	Repeated measures ANOVA (df _{contact} = 2, df _{error} = 184) N=279	
				F-value	P-value
Wash hands with soap after handling child's faeces	1.87 (1.46)	4.61 (0.87)	4.67 (0.73)	223.30	0.000
Wash hands with water after handling child's faeces	1.75 (1.12)	1.78 (1.21)	1.87 (1.24)	0.36	0.698
Clean hands with gel after handling child's faeces	2.13 (1.04)	2.09 (1.08)	2.03 (1.11)	0.50	0.607
Wash hands with soap after handling child's urine	4.10 (1.02)	3.90 (1.06)	3.92 (0.96)	2.70	0.070
Wash hands with water after handling child's urine	2.16 (1.20)	2.30 (1.21)	2.33 (1.22)	0.81	0.445
Clean hands with gel after handling child's urine	2.11 (1.11)	1.97 (0.98)	2.02 (1.05)	1.13	0.324
Wash hands with soap after wiping child's nose	2.70 (1.03)	2.68 (1.10)	2.87 (0.97)	3.14	0.045
Wash hands with water after wiping child's nose	2.41 (1.18)	2.39 (1.03)	2.46 (1.02)	0.20	0.821
Clean hands with gel after wiping child's nose	1.82 (0.94)	1.82 (0.90)	1.84 (0.94)	0.05	0.162
Put child's used dummy in own mouth	1.71 (1.15)	1.56 (1.03)	1.73 (1.09)	1.84	0.162
Eat child's leftover food	3.28 (1.06)	3.13 (1.17)	2.99 (1.14)	4.40	0.134
Eat with child's used cutlery	2.81 (1.25)	2.82 (1.33)	2.78 (1.29)	0.05	0.950
Drink from child's used cup	2.48 (1.30)	2.41 (1.34)	2.47 (1.29)	0.39	0.676
Kiss child on lips	3.42 (1.43)	3.37 (1.47)	3.51 (1.40)	2.43	0.091

Table comparing the frequency, between different time-points during pregnancy, of hygiene-related contact with children's bodily fluids by HCMV-seropositive women. Mean score out of 5, where higher mean scores indicated higher level of frequency of the contact. Blue font indicates where there is a statistically significant difference among the mean scores for the different time-points during pregnancy. Abbreviations: ANOVA, analysis of variance; Df, degree of freedom; HCMV, human cytomegalovirus; N, total observations; SD, standard deviation (see section 2.2.5.9 for the methods used, and section 5.3.2 for the description of results).

Table 22. Comparing the frequency, between different time-points in pregnancy, of hygiene-related contact with children's bodily fluids of HCMV-seropositive women in the observational cohort study

Contact	1 st time-point: Mean score (SD) N=59	2 nd time-point: Mean score (SD) N=59	3 rd time-point: Mean score (SD) N=59	4 th time-point: Mean score (SD) N=59	Repeated measures ANOVA (df _{contact} = 3, df _{error} = 174) N=236	
					F-value	P-value
Wash hands with soap after handling child's faeces	1.80 (1.46)	4.71 (0.70)	4.81 (0.43)	4.71 (0.56)	177.03	0.000
Wash hands with water after handling child's faeces	1.71 (1.13)	1.56 (1.05)	1.69 (1.10)	1.73 (1.00)	0.42	0.735
Clean hands with gel after handling child's faeces	2.14 (0.97)	2.03 (1.03)	1.93 (1.08)	2.10 (1.06)	1.20	0.313
Wash hands with soap after handling child's urine	3.97 (0.98)	3.97 (0.98)	3.98 (0.92)	4.00 (0.87)	2.16	0.094
Wash hands with water after handling child's urine	2.07 (1.03)	2.07 (1.03)	2.14 (1.12)	2.24 (1.07)	0.39	0.758
Clean hands with gel after handling child's urine	1.97 (0.95)	1.97 (0.95)	1.93 (1.01)	2.08 (1.04)	1.06	3.660
Wash hands with soap after wiping child's nose	2.49 (0.97)	2.49 (0.97)	2.75 (0.86)	2.93 (1.01)	6.12	0.001
Wash hands with water after wiping child's nose	2.24 (0.95)	2.24 (0.95)	2.36 (0.89)	2.43 (1.04)	0.33	0.807
Clean hands with gel after wiping child's nose	1.71 (0.77)	1.71 (0.77)	1.73 (0.94)	1.85 (0.89)	0.67	0.569
Put child's used dummy in own mouth	1.46 (0.90)	1.46 (0.90)	1.64 (1.09)	1.37 (0.83)	2.88	0.038
Eat child's leftover food	2.95 (1.17)	2.95 (1.17)	2.88 (1.13)	2.92 (1.16)	2.01	0.115
Eat with child's used cutlery	2.56 (1.29)	2.56 (1.29)	2.59 (1.25)	2.51 (1.10)	0.66	0.580
Drink from child's used cup	2.17 (1.26)	2.17 (1.26)	2.25 (1.21)	1.98 (1.17)	2.49	0.062
Kiss child on lips	3.12 (1.45)	3.12 (1.45)	3.29 (1.40)	3.15 (1.46)	1.51	0.215

Table comparing the frequency, between different time-points during pregnancy and at postpartum, of hygiene-related contact with children's bodily fluids by HCMV-seropositive women. Mean score out of 5, where higher mean scores indicated higher level of frequency of the contact. Blue font indicates where there is a statistically significant difference among the mean scores for the different time-points during pregnancy (see section 2.2.5.9 for the methods used, and section 5.3.2 for the description of results). Abbreviations: ANOVA, analysis of variance; Df, degree of freedom; HCMV, human cytomegalovirus; N, total observations; SD, standard deviation.

Table 23. Comparing the frequency, between different time-points in pregnancy and the postpartum period, of hygiene-related contact with children's bodily fluids of HCMV-seropositive women in the observational cohort study

5.3.3. Discussions

Compared to other types of hygiene-related contact with children's bodily fluids, there appeared to be a stark difference to the average level of frequency of hand washing with soap when handling children's faeces at the start of the study, when compared to the rest of pregnancy and at postpartum. It is unclear why this was the case. Whether the increased level of hygiene with handling children's faeces was related to study participation, such as a change in knowledge which could include their increased perception that HCMV infection can spread via faeces (see section [5.5.2](#)), or a general increased awareness of hygiene as found in previous research where trial participation can have an impact on the participants' thinking about their own behaviour with a possibility to influence change (Torkan *et al.*, 2018). Or whether it was related to the timing in pregnancy, such as being preoccupied with antenatal appointments during the 1st trimester.

Women were also found to have a higher frequency of hand washing with soap when wiping children's noses with increasing trimesters and further into the study. It would be valuable to explore if there was a difference seen between those with and without HCMV shedding, which is discussed later in this thesis (see section [6.2](#)). These findings were also correlated with the findings in genomic sequencing later in this thesis (see section [6.4](#)).

5.4. Study feedback in participants of the observational cohort study

5.4.1. Background

I sought to investigate the study feedback in participants of the observational cohort study. A full description of the methods can be found in section [2.2.5.10](#).

5.4.2. Results

Out of 115 women who completed the 4th time-point, 58 (50.4%) completed the feedback questionnaire, as summarised in Table 24 in mean scores out of 5 where a higher mean score indicated a higher level of agreement to the feedback statement. Positive feedback was found for most of the statements related to study screening, recruitment, procedures, and structures. However, lower agreement mean scores were reported on their willingness to attend study visits not coinciding with routine antenatal appointments [mean score= 3.31, 95% CI: 2.99-3.63], their preference for home visits [mean score= 3.22, 95% CI: 2.98-3.47], and on their willingness to attend the last study visit at a later date by travelling with their newborn infants instead of during their postnatal admission in hospital [mean score= 3.14; 95% CI: 2.85-3.42].

Category	Feedback Statement	Mean score [95% CI] N= 58
Study screening	The language and information on the study poster were just right	4.03 [3.80-4.27]
	Happy to be approached by the study team during antenatal appointment	4.57 [4.32-4.82]
	The language and information on the screening invitation letter were just right	4.38 [4.11-4.65]
	Happy to ask the study team questions at screening which were appropriately addressed	4.48 [4.23-4.74]
	Satisfied with the information received at screening and understood the next steps	4.50 [4.29-4.76]
Study recruitment	The language, information on the letter about the HCMV-seropositive results and study invitation were just right	3.98 [3.72-4.24]
	Happy to contact the study team after receiving the letter about the seropositive results and study invitation	4.43 [4.18-4.68]
	Satisfied with the information on the phone and happy to ask questions which were appropriately addressed	4.52 [4.26-4.77]
	The language and information on the Participant Information Sheet were just right	4.45 [4.22-4.68]
	Happy to complete all study procedures at recruitment visit	4.55 [4.30-4.80]
	Happy to ask the study team questions at recruitment visit which were appropriately addressed	4.59 [4.33-4.84]
Study procedures	Satisfied with the information received at recruitment visit and understood the next steps	4.57 [4.34-4.80]
	Willing to complete the questionnaire on demographics	4.64 [4.39-4.88]
	Willing to complete the questionnaires on contact activities with children's bodily fluids at all study visits	4.62 [4.38-4.87]
	The self-sampling written instructions were simple and clear	4.53 [4.31-4.76]
	Willing to perform the self-sampling of saliva at all study visits	4.71 [4.47-4.95]
	Willing to perform the self-sampling of urine at all study visits	4.64 [4.39-4.89]
	Willing to perform the self-sampling of vaginal secretions at all study visits	4.26 [3.94-4.58]
	Willing to perform all self-sampling at all study visits	4.40 [4.13-4.67]
	Willing to have blood samples taken at all study visits	4.36 [4.10-4.62]
Study structure	Willing to have additional blood samples taken on top of antenatal blood samples	4.16 [3.87-4.44]
	Happy to have the option to have newborn tested for cCMV infection	4.52 [4.27-4.76]
	Willing to be part of all four study visits	4.71 [4.48-4.93]
	Study visit timelines were not troublesome	4.59 [4.37-4.80]
	Happy to receive study visit text reminders	4.74 [4.52-4.96]
	Willing to attend study visits that do not coincide with antenatal appointments	3.31 [2.99-3.63]
	Prefer to have study visits at the same time as antenatal appointment	4.62 [4.36-4.88]
	Prefer to have study visits at home	3.22 [2.98-3.47]
	Willing to inform the study team to contact when in labour/soon after birth	4.09 [3.82-4.36]
	Willing to have study visit soon after birth whilst still in hospital	4.38 [4.15-4.60]
	Willing to bring newborn to hospital to have study visit after being discharged from hospital	3.14 [2.85-3.42]

Table summarising the feedback on the study by participants of the observational cohort study. Mean score out of 5, where higher mean scores indicated higher level of agreement to the feedback statements (see section 2.2.5.10 for the methods used, and section 5.4.2 for the description of results).

Abbreviations: cCMV, congenital cytomegalovirus infection; CI, confidence interval; N, total observations.

Table 24. Feedback on the study by HCMV-seropositive women in the observational cohort study

5.4.3. Discussions

From the feedback received, there was a high agreement mean scores on screening, recruitment, study procedures and structure. However, as feedback was only received from half of the women who completed the study and just over a third of the total recruited women, interpretation of this should be done with caution. The timing of when feedback was sought via the feedback questionnaire, i.e., for most participants soon after delivery (4th time-point), was likely to be a main reason for poor feedback completion. The timing for this will need to be evaluated in the design of a larger scale trial to ensure robust experience and feedback from participants will be captured. Based on the feedback sought, most participants were happy with self-sampling technique including for vaginal secretions, which is reassuring for future work. As discussed in section 5.1.3, it is emphasised here that promoting flexibility and ease of completion of the 4th time-point would be beneficial, such as a home visit instead of an additional visit to the hospital if a visit on the postnatal ward after delivery was not possible, to promote study compliance and experience of participating women.

5.5. Learning from experiences of participating women

5.5.1. Background

I sought to investigate the study participation experiences in participants of the observational cohort study. A full description of the methods can be found in section 2.2.5.11.

5.5.2. Results

Seventy-eight participating women were invited to take part in an interview via email in chronological order of recruitment, of which 20 responded and agreed to take part in the interview. Each interview lasted between 18 and 40 minutes, and each of the 20 participating women were interviewed once. Table 25 describes the demographics of the women interviewed.

Demographic Category	Demographic Details	Frequency (%; N=20)
Age	30-34	7 (35%)
	35-39	11 (55%)
	40-44	2 (10%)
Ethnicity	White	17 (85%)
	Black/ African/ Caribbean/ Black British	0 (0%)
	Asian/Asian British	2 (10%)
	Mixed/multiple ethnic groups	1 (5%)
Birth country in relation to UK	Born in UK	14 (70%)
	Not born in UK	6 (30%)
Length living in UK	5-15 Years	6 (30%)
	>15 years	14 (70%)
Education	PhD or equivalent	1 (5%)
	Masters degree or equivalent	8 (40%)
	Undergraduate degree	8 (40%)
	Postgraduate certificate, diploma or equivalent	3 (15%)
Number of pregnancies mean (SD)		3 (1.07)
Number of children aged <4 years	1	20 (100%)
	>1	0 (0%)
Living arrangement	Living in a couple (irrespective of marital status)	20 (100%)
	Not living in a couple	0 (0%)
Type of household members	A cohabiting couple or single parent family (parents and children)	19 (95%)
	Multi-generational families (grandparents, parents and children)	1 (5%)

Table summarising the demographics of HCMV-seropositive women participating in the post-study interview (see section 2.2.5.11 for the methods used, and section 5.5.2 for the description of results).
Abbreviations: HCMV, human cytomegalovirus; N, total observations; PhD, Doctor of Philosophy; SD, standard deviations; UK, United Kingdom; %, percentage.

Table 25. Demographics of HCMV-seropositive women in the post-study interview

The codes, subthemes and themes that emerged from the interviews are outlined in Table 26. The following two main themes were identified: 1) research participation is contingent on a balance of convenience and inconvenience, and benefits and risks, and 2) research participation in pregnancy is associated with subsequent non-specifically directed changes to usual behaviour influenced by awareness, knowledge, and support.

Themes	Subthemes	Codes	Ref
1. Research participation in pregnancy is contingent on a balance of convenience and inconvenience, and benefits and risks	1.1. Research involvement should be convenient	Study participation was easy	37
		Study participation or procedure was convenient	168
		Study participation or procedure was flexible	6
		Study participation or procedure was accommodating	16
		Study procedures were fine to do	101
	1.2. Additional health screening is a benefit	Study procedures were easy to do	47
		Being informed of maternal HCMV status was valuable	4
		Being informed of newborn HCMV status was appropriate	19
		The study was informative	21
		The awaiting newborn HCMV screening did not concern the participant	33
	1.3. The benefits of research procedures around the time of birth needs to be balanced against the risks of poor research compliance	The awaiting newborn HCMV screening did not concern the partner	12
		Inability to remember the completion of or to complete the feedback questionnaire	8
		Difficulty to perform the study procedures around birth	5
		Difficulty to coordinate the study visit around birth	7
		Communication with participant around birth and newborn period is better over text than email or phone	2
	1.4. Collaboration between clinical and research teams needed to streamline research activities alongside routine care	Inconvenience found with the study due to uncertainty on who, how and when to provide sample to study team during a routine appointment	5
		Participant could benefit from better coordination and streamline between study and clinical team to help with running the study alongside routine appointments	4
	1.5. Research involvement should have the right balance of low intensity and high monitoring	Participant could sometimes feel disengaged from the study journey	2
		Participant would benefit from a more robust system of regular contact points to keep participant informed of the study participation progress	3
		Participant would benefit from a more robust system of regular contact points to keep participant informed of the study outcome progress	2
		Participant would benefit from a more robust system of regular contact points to remind participant of the study aims	5
2. Research participation in pregnancy is associated with subsequent non-specifically directed behavioural modification influenced by awareness and support	2.6. A reflection on behaviour can result in behavioural modification	Study participation induced participant's reflection on her hygiene and behaviour	3
		Study participation induced participant's reflection on how her behaviour could impact on HCMV transmission	4
		Study participation induced participant's reflection on how her behaviour could have an impact	4
		Study participation induced participant's reflection on how her behaviour could impact on germ spreading	1
		Study participation induced participant's reflection to her behaviour, enough to result in her changing her behaviour	13
	1. Behaviour is modified to reduce the risks of infection	Induced participant's own reflection to her hygiene and how her behaviour could impact on germ spreading, enough to result in her changing her behaviour	5
		Induced participant's own reflection to her hygiene and how her behaviour could impact on germ spreading not limited to HCMV, enough to result in her changing their behaviour	3
		Induced participant's own reflection to her hygiene and how her behaviour could impact on HCMV spreading, enough to result in her changing her behaviour	6
		Behavioural change decided and made by participant was made easy by being able to be organised preplanning through being informed	5
	Feeling informed empowers behavioural modification	Behavioural change decided and made by participant was made easy by feeling informed	6
	Behavioural modification is a time-limited risk-reduction measure	Participant recognised the behavioural change they decided to make was to reduce the risk to her unborn child and balanced this with her usual behaviour in the household	4
	Behavioural modification is aided by prompts and partnership	Participant discussed with partner to participate in the study	9
		Participant discussed with partner to change behaviour during the study	8
		Partner supportive of participant's behavioural change during the study	7
		Participant did not discuss with partner to change behaviour during the study	4
		Partner also changed behaviour alongside the participant during the study	3
		Partner did not change behaviour like participant did during the study	2
		Participant forgot to perform the behavioural change they decided to make whenever it was not at the forefront of her mind	5
		Behavioural change made by participant did not continue post-study due to the lack of prompt	1
		Behavioural change made by participant could be difficult due to the impracticality	4
		Behavioural change made by participant was helped by changing routine and preplanning	4
		Behavioural change made by participant could be difficult due to changing routine	1
		Behavioural change made by partner could be difficult due to changing routine	1

Table summarising the emerged codes, subthemes, and themes from the post-study interview of HCMV-seropositive women in the observational cohort study. The references represented the number of observations the relevant code emerged from the transcription of the interviews (see section 2.2.5.11 for the methods used, and section 5.5.2 for the description of results).
Abbreviations: cCMV, congenital cytomegalovirus; HCMV, human cytomegalovirus; ref; references.

Table 26. Codes, subthemes and themes emerged from the post-study interviews of HCMV-seropositive women in the observational cohort study

Theme 1: Research participation in pregnancy is contingent on a balance of convenience and inconvenience, and benefits and risks

Five subthemes were identified that relate to this overarching theme:

Subtheme 1.1: Research involvement in pregnancy should be convenient

The study procedures and study visits, as well as how the study was conducted (for example, the ability to self-sample, the ability to complete questionnaires via email in participants' own time, study visits coinciding with routine antenatal appointments), were frequently described as "easy", "convenient", "flexible", and "accommodating". These positive experiences facilitated their participation.

"I guess it was kind of something that happened whilst I was pregnant, and because I was at the hospital so much anyway, it just kind of happened alongside me being there, it wasn't an inconvenience for me to do it, because I was there anyway doing the things I needed to do, and I was doing it in addition to that."

"It definitely, it made it easier to keep on top of the appointments, that made it easier for everyone to keep track of...it couldn't have been, I honestly don't think it could have been any easier, everything, all my appointment took place when I would be waiting anyway..."

"It was good because they were booked ahead of, I mean when I knew my appointment times, I'd let you guys know, and then we'd arrange to meet where I was, so either in the antenatal clinic, or um, I remember I saw her the day before the C section, she came to meet me then as well."

"The main thing really is that you came and met me at the house or at the appointments, for someone like me I had lots of appointments and it then meant that I didn't have any additional appointments on top of that, it didn't make it a no no, it made it really simple and there's no excuse not to do it, if you know what I mean."

Subtheme 1.2: Additional health screening is a benefit

Most participants felt that being informed about HCMV infection, and the ability to find out their own HCMV serostatus, were valuable and informative. Most participants and their partners also felt that the opportunity to have their newborn infants tested for cCMV infection was a benefit of participation. Most participants described that they had not put much thought into the outcome of the HCMV test of their newborn during the study.

"...the CMV (HCMV) thing, it's not tested in women that are pregnant, that they're not tested for it, and I think that they should be...once you've found out that you've got it, it might be too late, and there might be, like, bad effects to the baby."

"So I was a bit worried that I had the virus early in my pregnancy, so, again I'm a bit vague on the timeline of events and things like that. But it was helpful to be able to have the test and find out whether I had it before."

"...it's fine because even if he has got something then it would be identified and he would be referred appropriately, as opposed to if I hadn't taken part in the study, then not that it would have happened..."

"I was happy for him to have it done because it was something that he wouldn't have had done if I had not known about it, I think it would have been tricky if you had said to me "there's all this stuff, you know, we're trying to find out about it, um and your baby might have it, sorry we're not going to be able to do anything about that", but the fact that he had the test at the end, it was kind of a nice conclusion to it all"

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1832 **Subtheme 1.3: The benefits of research procedures around the time of birth should be**

1833 **balanced against the risks of poor research compliance**

1834 Some participants felt that study procedures, such as self-sampling of vaginal secretions or

1835 completing questionnaires, were challenging to perform around the time of birth. Some

1836 found the practicalities difficult, whilst others had trouble in remembering to perform the

1837 procedures.

"I think again, just the last one, doing the vaginal swab at the last one, when I had the baby, probably not wise really, you don't know what's happened down there, and doing that one was really, I think I shouldn't have done it really. I think you are in a bit of a daze, you don't know what kind of damage and it's uncomfortable, but I felt like I should do it, probably should have said no for that last one..."

"So the test the swabs that you wanted once he was born, that was quite tricky to negotiate, because he wasn't really feeding, I had constant people coming, and I think when one of your colleagues came, somebody else was asking me about a research study, there were like a fair few coming that day, and I kind of get it because it's important and everybody kind of has got their research thing that they're doing, but that was may be the trickiest."

"...actually, when my baby was born, that time, it's really hard to give you the sample and everything, because you're bleeding...most likely you're in pain, and you forget as well, so I think it was like, how is it that I can remember, and give you everything, when you're in so much pain."

"Honestly if it was the end of the study it would have been around when I gave birth, so I couldn't even tell you I did it or not."

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Subtheme 1.4: Collaboration between clinical and research teams needed to streamline research activities alongside routine care

Some participants felt that even though having the study visits running alongside routine antenatal appointments was convenient, the lack of involvement by the clinical team with the study caused problems. This included the inability to provide their study samples to the clinical team, and the clinical team being unaware of the study. The participants felt that they were unable to discuss the study with the clinical team, which was challenging.

"Sometimes it would be difficult to know when somebody would come to collect my samples, so it was always tied in as I said with my appointments, but you know no time was made with me a lot of the time, either it's you know your time for the scan is on this day at this time, we will come and collect it at some point while you are at the hospital...I was always waiting for a phone call or I had to phone to try and find someone, so I'd say that was probably the only thing, I was never 100% sure if someone was actually going to come and collect it."

"...for me as I said it was fine but the midwives, it all seemed like they didn't know that it was going to happen, and it was all really quick; "oh is it ok if you take some extra blood for this"...the other midwife was just probably slightly taken aback, and wasn't entirely clear with what she should be doing, and I would just say if that coordination could happen better."

"I guess when I was in hospital, the midwife said if they had been aware they would have taken the blood for you, you know label it, and etc, I mean it must be absolutely impossible to tie in with all the deliveries especially you know as I was two weeks late, but maybe, I don't how you would go about informing the midwives but that might make it easier for you, you know we did all the kind of swabs and bloods and things, it might be easier for you, they said to me there's so many research programmes going on, if they were aware they could have done it"

"It was quite difficult because after she (newborn) arrived, my husband said to the midwife, to different midwives a couple of times that she is participating in this study and they were like, 'oh yeah we'll let them know' and I don't what came of it."

Subtheme 1.5: Research involvement should have the right balance of low intensity and high monitoring

Although the study was designed to keep participation convenient, easy, and non-time consuming, some participants felt that to have a regular contact, reminder and refresher throughout the study would be beneficial. This is because they were pregnant as well as

1853 caring for a young child and could lose track of their place in the study journey, or even
1854 forget about the study altogether.

"Um, sometimes I forgot um when you know what um how many visits were left and things like that, um and what I need to take, again I don't think that could have been made much easier um and maybe I'm forgetting that um, I know I had a folder that um, I don't know if on the folder there was like, how many visits, um, how many samples were needed and , um, but, I know they were all broken up inside, but I don't know if there were like a summary of what were, and a timeline maybe on the front of the folder might be handy but maybe that it is there and I forgot."

"I think, to be frank with you it probably stretched over quite a long period of time, you know, I suppose to be honest I probably forgot about it at points at times, so I think there are a couple of times, or maybe just once I was supposed to drop in samples or something at the hospital coinciding with a visit, but I completely forgotten"

"I think perhaps more regular sort of contact points would have been useful...sort of at the full front of your mind a bit more, as it's over a long period of time, and you know if you're pregnant and you're working you're just busy, and you've got another child, just to make it as easy as possible for the participants, I think the whole nudge theory, it's never harmful to give people a gentle reminder before they have to do it, I think would be helpful."

"...or like a quick sheet for the mother, as it did make me, "oh, you're taking part in a survey, you don't even know what it's for?" and I just felt a little bit like, "oh yeah maybe I should", I did know at one point, but you know what your memory is like when you're pregnant...it would be like, as well as giving more information, a little bit more empowering to have it as an easy reference for."

1855

1856 **Theme 2: Research participation is associated with subsequent non-specifically directed**
1857 **behavioural modification influenced by awareness and support**

1858 Five subthemes were identified that relate to this overarching theme:

1859

1860 **Subtheme 2.1: A reflection on behaviour can result in behavioural modification**

1861 Completion of the serial questionnaires to assess for hygiene-related behaviour with their
1862 children encouraged most of the participants to reflect upon their hygiene practices. In
1863 some, this resulted in a change to their behaviours during study participation.

"I think you obviously sometimes feel like it's a test don't you, especially when it's to do with hygiene and stuff, but they were absolutely fine, it kind of made me feel like, 'oh, should I be doing these things', am I right or am I wrong."

"I guess I was conscious when I was doing the form that because I was part of the study, I was being more careful about my behaviour and things. So I just felt like I was, I wasn't doing any of the behaviours that you were asking about. And so then I wondered how, I don't know, how that was affecting the results."

"I learned, all the questions about hand hygiene and all you are doing with your existing child, I never even thought about them in my daily life until I was reading them, so it certainly made me think about hygiene a bit more I suppose, and do I actually share cutlery with my child etc so that was quite interesting doing that going through, it certainly made me think I suppose than just do as I think you do with a toddler so that was good"

"It made me quite sort of be careful with what I do when I was changing the nappy, it really made me think, because normally when I change her nappy I would just change with a hand gel and whether that was sufficient, so it really made me think if I should use water and soap every time when I change the nappy."

1864

1865 **Subtheme 2.2: Behaviour is modified to reduce the risks of infection**

1866 Most participants' decision to change their behaviour, was motivated by a desire to reduce
1867 the risks of spreading infection in general, not just of HCMV infection specifically.

"I think because I wanted to be more hygienic anyway, I carry tissue around to wipe her nose, the dummy thing I think that's just a bad habit, I think it's not very clean, so I just sort of clean it instead of sucking on it, I think because I was asked those questions it made me think I should do those definitely."

"Generally thinking more about hygiene and thinking actually things can pass, thinking doing things like that you can spread it around, so instead of just rinsing with water use soap, I don't know I guess that you live amongst germs so much and if you are part of a study, it makes you think about it more and you know, this may help in other area as well."

"I think it's knowing a bit more about it, before I was always quite vigilant about, like, with poo, washing my hands with soap and stuff, and I wasn't with wet nappy and wiping their noses, I think I was probably like you know do it half-heartedly and not with soap, so I was more vigilant and washing with soap with that kind of stuff, from the reading and understanding a bit more about you know why it's important to do that for CMV and other germs."

"Just because it was almost a reaction to you know reading about it, it made me think there probably is an infection transmission risk here both ways, and why aren't I doing something to minimise that"

1868

1869 **Subtheme 2.3: Feeling informed empowers behavioural modification**

1870 Most participants who made changes to their behaviour during their study participation,
1871 described them to be an easy adjustment because of the sense of feeling informed,
1872 empowering them to act on their reflections. Some participants even felt empowered to

1873 relax their hygiene practices during pregnancy because of their understanding that being
1874 HCMV-seropositive puts them at a lower risk of transmitting HCMV infection to their unborn
1875 child, compared to primary HCMV infection in pregnancy.

"It was fine really, you know it was just something I wasn't really consciously doing, and just being a bit more, thinking more about it, so yeah very easy."

"Before it was something that I was conscious of, and that I was trying to follow guidelines to avoid contact but I did find it quite difficult, because when you have a toddler you do have contact all the time and they're not the most hygienic creatures so either you just stop contact altogether or you kind of have to put up with that higher level of risk, but it's quite anxiety-inducing if you do feel like you're potentially risking your unborn child's health, but you also don't want to, you know, affect your existing child emotionally by seeming to reject them...I think guidelines aren't very easy to follow in practice, which is why I sort of found it quite a relief to know that I wasn't sort of at risk, to be slightly less cautious without feeling too guilty about it."

"Because I learned a bit more about the virus, um, what it do to the baby, um, and what kind of behaviour potentially more risky, um, so yeah, I tried to avoid them, because I learned a bit more about it"

"Because I learned about the risk of contracting the virus and that then that could cause harm to my child."

1876

1877 **Subtheme 2.4: Behavioural modification is a time-limited risk-reduction measure**

1878 Some participants who made behavioural changes to their hygiene practices during
1879 pregnancy recognised that the behavioural modifications were to reduce the risks of HCMV
1880 infection to their child before birth, which allowed them to revert to normal practices for
1881 them following delivery of the infant.

"...because it's easier, because I don't have to be careful anymore because baby is born, so not a concern anymore for me on what you have to be careful with, you know all of this it was part of these, kind of, safety instructions while you're pregnant."

"But the other ones, I guess, because I'm probably unlikely to get pregnant again. So I guess I started like sharing a bottle and things like that. So we haven't really kept them up because I feel like the risk is low."

"I mean I eat his food, I'm not pregnant so I do"

"but um, things like sharing the cutlery or eating from the plate or wiping the nose, um, yea, has gone back to what it was before"

1882

1883 **Subtheme 2.5: Behavioural modification is aided by prompts and partnership**

Some participants found it difficult to maintain the behavioural modifications, after they were no longer being prompted by the study questionnaire to assess for hygiene-related behaviour with their children. Some participants also found the behaviour changes they made during the study were difficult due to the impracticality and having to change their routine. These difficulties were eased through the support and reminders they received from their partners. Most participants who made behavioural changes involved their partners in their decision.

"...my partner helped reminding me when he saw me doing it."

"Yes. I think he (partner) would explain it to my son as well. He would tell him that he couldn't drink from my drink or tell him that he couldn't share my food...we would explain why as well."

"Because my husband and I did it (behavioural change), it wasn't hard."

"Never easy to change a behaviour. I mean it comes and goes, it's not necessarily conscious behaviour change, it's just all of a sudden you sort of doing it again and then you don't and then you think this is what I've got to do and you don't think twice about it."

5.5.3. Discussions

Convenience of participation was a key reason for pregnant mothers, especially those with young children, to take part and remain in the study. This reaffirms that convenience should be at the core of study design. Paradoxically, low-intensity study involvement reduced engagement between the participant and the study team, which could impact on study compliance and retention. In future studies, ensuring that the design involves regular contacts, monitoring, and reminders, whilst still maintaining convenience of study participation, would be beneficial to both the participants and research team.

A collaboration between the research and clinical teams was essential to ensure that research procedures and clinical appointments can run in synchrony, as also shown in

previous research (MacNeill *et al.*, 2016). The timing of research involvement was another key consideration when designing a maternal study. It is essential to weigh up the benefits for a pregnant woman to complete a study procedure around the time of birth against the risks of poor study compliance.

Antenatal screening for HCMV infection and neonatal screening for cCMV infection are not part of routine maternity care in the UK. The fact that pregnant women valued the ability to know their own and their newborn infants' HCMV status without inducing unintended anxiety or concerns, was reassuring not only for future studies, but also for the potential implementation of routine antenatal and neonatal HCMV screening. The concern of inducing anxiety in pregnant women through the acquisition of information on HCMV without being able to provide solutions to treat it, has been recognised to be a factor in the reluctance of health care professionals to include HCMV in routine antenatal education (Montague *et al.*, 2022). However, in line with findings here, the same research also found that pregnant women were keen to acquire knowledge about HCMV and were motivated to reduce risks of HCMV to their unborn child (Montague *et al.*, 2022).

This was a non-interventional study, where no recommendations to perform hygiene-based behaviours were made, nor was information provided of any hygiene-based behaviours that could promote the risk of HCMV infection to them or their unborn child. However, the serial questionnaires to assess the pregnant women's hygiene-based behaviours used induced the reflection of and heightened awareness into their own behaviour, some enough to have resulted in a change to their behaviours during study participation. These findings are in line with previous research that had highlighted that trial participation had an impact on the participants' thinking about their own behaviour, with a possibility to influence change (Torkan *et al.*, 2018). The potential for subsequent non-

specifically directed changes of behaviour through study participation should be evaluated when designing a trial to consider its influence on the study results.

Knowledge of HCMV serostatus empowered women participating in the study to make informed decisions on their behaviour. Most pregnant women modified their behaviour to reduce contact with saliva and urine of their young child, with the aim of reducing the risk of a new HCMV infection which could be passed on to their unborn child. However, some did not adapt their behaviours because of the perception that the risk of vertical transmission was significantly reduced, as they already had HCMV immunity. The ability of the knowledge of maternal HCMV serostatus to have an impact on the perceptions to risk that may result in a behavioural change, may be an important consideration in the evaluation of antenatal HCMV screening.

The findings have highlighted that behavioural change messages about HCMV in pregnancy should be framed as a short-term measure instead of a long-term measure to make it more attainable, and, in line with previous research (Montague *et al.*, 2022), as a risk-reduction measure. It was also shown that behavioural change messages about HCMV should be framed in the context of advice about other infections, to make behavioural change measures during pregnancy more achievable, relatable, and practical for women. The findings also identified the value of involving partners in antenatal education on HCMV, especially on the continual support they were able to provide to the pregnant women in implementing behavioural measures to reduce the risks of HCMV. Moreover, it was shown that continual prompts was a key factor to sustain behavioural change measures throughout pregnancy, which can be provided by their partners. Research also supports the inclusion of partners for behavioural change (Appleton and Pharoah, 1998; Montague *et al.*, 2022).

Although the study was limited to 20 participating pregnant women, thematic saturation was achieved. It provided rich data highlighting the experiences of participating women and reveals the potential facilitators and barriers towards engaging pregnant women in HCMV antenatal education and risk-reduction measures. The lack of ethnic diversity may have had an impact on the findings and therefore warrants further investigation.

Finally, while the behavioural modification identified was specific to pregnant women with existing children, most of the themes and subthemes identified from the findings can be applied in most context of maternal research. Research involving pregnant women, especially in those with existing children, should be centred around convenience of participation, and it should also be informative, empowering, holistic, and supportive.

6. Investigating for risk factors of human cytomegalovirus shedding

6.1. The relationship between demographics and human cytomegalovirus shedding in participants of the observational cohort study

6.1.1. Background

I sought to investigate the relationship between demographics and HCMV shedding in HCMV-seropositive pregnant women in the observational cohort study. A full description of the methods can be found in section [2.2.5.12](#).

6.1.2. Results

The associations between HCMV shedding during pregnancy in HCMV-seropositive pregnant women and demographics were assessed separately by site of shedding (saliva, urine, vaginal secretions, or any bodily fluids) at any time-point; and by time-point of shedding (early, middle, or late in pregnancy) in any site. They were also analysed separately as single and multiple logistic regression (LR) models, as well as with and without multivariate imputation by chained equations (MICE).

An increasing number of previous children was significantly associated with HCMV shedding in saliva with simple LR [OR 3.40 (95% CI: 1.08-10.71), $p=0.036$] but not multiple LR [OR 5.47 (95% CI: 0.86-34.53), $p=0.071$] or with MICE (total observations imputed= 36) for both simple [OR 1.72 (95% CI: 0.96-3.09), $p=0.069$] and multiple [OR 2.80 (95% CI: 0.86-9.09), $p=0.087$] LR. Three other factors (younger age, born in the UK, and longer duration of

1997 living in the UK) were found to be significantly more likely when HCMV shedding was
 1998 detected in saliva during pregnancy with MICE (total observations imputed= 36) using
 1999 simple LR, but not with multiple LR and not without MICE with both simple and multiple LR.

2000 Ethnicity was significantly different between women with and without HCMV
 2001 shedding detected in urine with both simple [OR 0.34 (95% CI: 0.15-0.80), p=0.013] and
 2002 multiple [OR 0.29 (95% CI: 0.09-0.98), p=0.046] LR without MICE. However, this was not of
 2003 statistical significance with MICE (total observations imputed= 31) for both simple [OR 0.91
 2004 (95% CI: 0.72-1.14), p=0.422] and multiple [OR 1.01 (95% CI:0.78-1.31), p=0.936] LR.

2005 Working regularly with children was more likely when HCMV shedding was detected in urine
 2006 only with simple LR without MICE [OR 3.27 (95% CI: 1.06-10.10), p=0.039], not with multiple
 2007 LR [OR 2.24 (95% CI: 0.52-9.58), p=0.277] and with MICE (total observations imputed= 31)
 2008 for both simple [OR 1.78 (95% CI: 0.76-4.20), p=0.185] and multiple [OR 1.49 (95% CI: 0.58-
 2009 3.83), p=0.402] LR. Three other factors (born in the UK, longer duration of living in the UK,
 2010 lower level of highest education qualification) were found to be significantly more likely
 2011 when HCMV shedding was detected in urine only with MICE (total observations imputed=
 2012 31), but not without MICE.

2013 Longer duration of living in the UK was significantly more likely when HCMV
 2014 shedding was detected in vaginal secretions with simple LR with MICE (total observations
 2015 imputed= 31) [OR 1.79 (95% CI: 1.01-3.17), p=0.046], but not with multiple LR [OR 1.60 (95%
 2016 CI: 0.70-3.65), p=0.261] and not without MICE for both simple [OR 1.17 (95% CI: 0.58-2.34),
 2017 p=0.667] and multiple [OR 1.79 (95% CI: 0.55-4.19), p= 0.418] LR.

2018 Decreasing gravidity was significantly more likely with HCMV shedding in any bodily
 2019 fluids with multiple LR in both with [OR 0.52 (95% CI: 0.31-0.89), p=0.017] and without [OR
 2020 0.36 (95% CI: 0.16-0.81), p=0.014] MICE (total observations imputed= 37) (Table 27).

2021 Increasing number of children was significantly more likely when HCMV shedding
2022 was detected at the 1st time point with multiple LR without MICE [OR 21.00 (95% CI: 1.48-
2023 297.87), p=0.024]. However, this was no longer significant with simple LR without MICE [OR
2024 1.54 (95% CI: 0.67-3.53), p=0.306] and with MICE (total observations imputed= 6) for both
2025 simple [OR 1.28 (95% CI: 0.60-2.76), p=0.523] and multiple [OR 7.15 (95% CI:0.97-52.47),
2026 p=0.053] LR. Ethnicity was significantly different between women with and without HCMV
2027 shedding detected at the 1st time-point only with simple LR with [OR 0.51 (95% CI: 0.29-
2028 0.90), p=0.019] and without [OR 0.34 (95% CI: 0.14-0.86), p=0.022] MICE (total observations
2029 imputed= 6), but not with multiple LR with and without MICE.

2030 Increasing number of children was significantly more likely when HCMV shedding
2031 was detected at the 2nd time point only with multiple LR [OR 0.45 (95% CI: 0.23-0.86),
2032 p=0.017] with MICE (total observations imputed= 20), but not with simple LR with MICE [OR
2033 0.63 (95% CI: 0.38-1.03), p=0.066] and not without MICE for both simple [OR 0.64 (95% CI:
2034 0.32-1.28), p=0.204] and multiple [OR 0.50 (95% CI:0.20-1.22), p= 1.28] LR.

2035 Working regularly with children was found to be significantly more likely when
2036 HCMV shedding was detected at the 3rd time-point only in simple LR without MICE [OR 3.96
2037 (95% CI 1.25-12.56), p= 0.019] but not with multiple LR [OR 3.79 (95% CI: 0.99-14.54), p=
2038 0.052] and not with MICE (total observations imputed= 27) for both simple and multiple LR.

2039
2040
2041
2042
2043
2044
2045
2046
2047
2048

		HCMV shedding detected at least once in any bodily fluids during pregnancy		Logistic regression							
		Yes (N=27)	No (N=96)	Without MICE (N= 123)				With MICE (N= 160, imputed N=37)			
				Simple		Multiple		Simple		Multiple	
Demographics		x (%)	x (%)	OR [95% CI]	p	OR [95% CI]	p	OR [95% CI]	p	OR [95% CI]	p
Age (years)	<30	2 (7.4)	10 (10.4)	2.15 [0.73-6.34]	0.164	3.17 [0.89-11.34]	0.076	0.87 [0.41-1.82]	0.707	1.32 [0.54-3.21]	0.541
	30-39	21 (77.8)	81 (84.4)								
	>39	4 (14.8)	5 (5.2)								
Ethnicity	White British	15 (55.6)	36 (37.5)	0.84 [0.60-1.16]	0.288	0.95 [0.62-1.47]	0.829	0.97 [0.79-1.19]	0.785	1.07 [0.84-1.36]	0.580
	White Other	5 (18.5)	30 (31.2)								
	South Asian	2 (7.4)	13 (13.5)								
	Asian Other	3 (11.1)	5 (5.2)								
	Black	2 (7.4)	6 (6.3)								
	Mixed/Other	0 (0)	6 (6.3)								
Born in UK	Yes	12 (44.4)	50 (52.1)	1.36 [0.56-3.21]	0.484	1.02 [2.67-3.93]	0.972	2.39 [1.23-4.64]	0.010	2.33 [0.87-6.24]	0.094
	No	15 (55.6)	46 (47.9)								
Duration in UK (years)	<5	2 (7.4)	12 (12.5)	1.23 [0.65-2.36]	0.519	1.41 [0.52-3.80]	0.502	1.80 [1.05-3.07]	0.032	1.25 [0.57-2.75]	0.577
	5-15	8 (29.6)	28 (29.1)								
	>15	17 (63.0)	56 (58.3)								
Highest education	PhD/Masters	3 (11.1)	14 (14.6)	1.02 [0.67-1.56]	0.929	0.99 [0.60-1.63]	0.965	0.87 [0.64-1.19]	0.394	0.84 [0.59-1.21]	0.359
	1 st degree	4 (14.8)	14 (14.6)								
	Diploma	12 (44.4)	35 (36.5)								
	A-level/GCSE	8 (29.6)	33 (34.4)								
No. of pregnancies	2	16 (59.3)	51 (53.1)	0.61 [0.34-1.10]	0.101	0.36 [0.16-0.81]	0.014	0.80 [0.54-1.19]	0.274	0.52 [0.31-0.89]	0.017
	3	10 (37.0)	19 (19.8)								
	>3	1 (3.7)	26 (27.1)								
No. of children	1	21 (77.8)	78 (81.2)	1.12 [0.50-2.51]	0.787	2.73 [0.63-11.82]	0.178	1.43 [0.82-2.51]	0.208	2.70 [0.97-7.53]	0.057
	2	5 (18.5)	14 (14.6)								
	>2	1 (3.7)	4 (4.2)								
Children aged 1 year	Yes	7 (25.9)	33 (34.4)	0.67 [0.26-1.74]	0.410	1.39 [0.03-71.94]	0.872	1.07 [0.55-2.08]	0.839	2.05 [0.29-14.45]	0.471
	No	20 (74.1)	63 (65.6)								
Children aged 2 years	Yes	17 (63.0)	42 (43.8)	2.19 [0.91-5.26]	0.081	3.61 [0.08-170.82]	0.514	1.13 [0.60-2.14]	0.697	2.78 [0.40-19.43]	0.302
	No	10 (37.0)	54 (56.2)								
Children aged 3 years	Yes	3 (11.1)	20 (20.8)	0.48 [0.13-1.74]	0.261	1.10 [0.02-52.22]	0.962	0.88 [0.39-1.95]	0.747	2.38 [0.34-16.80]	0.384
	No	24 (88.9)	76 (79.2)								
Work with children	Yes	20 (74.1)	81 (84.4)	1.89 [0.68-5.25]	0.222	1.60 [0.47-5.46]	0.452	1.25 [0.54-2.87]	0.606	1.11 [0.44-2.76]	0.828
	No	7 (25.9)	15 (15.6)								
No. of households	<=3	20 (74.1)	70 (72.9)	0.94 [0.36-2.49]	0.905	0.91 [0.20-4.16]	0.899	1.95 [0.52-2.14]	0.885	0.63 [0.20-2.05]	0.445
	>3	7 (25.9)	26 (27.1)								
No. of people per bedroom	1	1 (3.7)	3 (3.1)	0.92 [0.33-2.60]	0.878	0.73 [0.21-2.56]	0.625	1.00 [0.46-2.14]	1.000	0.84 [0.35-2.05]	0.703
	2	22 (81.5)	78 (81.3)								
	3	4 (14.8)	15 (15.6)								

Table summarising the association of HCMV shedding in saliva, urine and/or vaginal secretions at least once in pregnancy with demographics. Simple and multiple logistic regression performed with detection of HCMV shedding in saliva at 1st, 2nd, or 3rd time-point as the outcome variable, with and without MICE. Statistically significant results (p-value <0.05) are in blue font (see section 2.2.5.12 for the methods used, and section 6.1.2 for the description of results).

Abbreviations: A-level, Advanced level; CI, confidence interval; GCSE, General Certificate of Secondary Education; HCMV, human cytomegalovirus; MICE, multivariate imputation by chained equations; N, total observations; OR, odds ratio; p, p-value; PhD, doctor of philosophy, United Kingdom; x, frequency; %, percentage.

Table 27. Association of HCMV shedding in any bodily fluids during pregnancy with demographics in HCMV-seropositive women

6.1.3. Discussions

The addition of multiple LR here in the assessment of demographics as potential risk factors for HCMV shedding in HCMV-seropositive women during pregnancy is important, as some of the demographics may have an interaction effect with each other. For example, the number of pregnancies, the number of children, the number of households, and the number of people per bedroom are likely to correlate positively with each other. By including multiple independent demographic variables, more factors that influence HCMV shedding can be accounted for while reducing the error and bias in the estimates and controlling for confounding variables. The analysis also included multiple imputation using the MICE method to account for the missing HCMV shedding data, to compare their findings with that in complete case analysis to take into consideration possible biased estimates.

The only demographic factor significantly associated with HCMV shedding during pregnancy in HCMV-seropositive women with multiple LR, with and without MICE, is the number of pregnancies, where there was an inverse relationship between the number of pregnancies an individual had experienced and HCMV shedding in any bodily fluids at any point during pregnancy. This was primarily seen in pregnant women with at least three previous pregnancies. “Trained immunity” of the innate immune system (natural killer (NK) cells) in subsequent pregnancies of HCMV-seropositive women has been hypothesised previously based on observed NK cells in maternal uterine tissue (decidua) of multigravida pregnant women, which increases with the number of pregnancies, suggesting that the observed pregnancy-trained decidual NK cells could be induced by decidual HCMV exposure and aid in the protection against HCMV disease in subsequent pregnancies (Feyaerts, Joosten and van der Molen, 2020). This trained immunity in subsequent pregnancies of HCMV-seropositive pregnant women has also been hypothesised to have the potential to

provide improved protection against intrauterine cCMV infection, where previous epidemiological studies have reported that HCMV-seropositivity during previous pregnancies reduces the risk of cCMV infections in future pregnancies, especially during the first trimester of pregnancy when the NK cells are abundantly present and the rate of cCMV infection is lowest compared to later trimesters (Hamilton *et al.*, 2014; Feyaerts, Joosten and van der Molen, 2020). The risk of vertical transmission is 30–40% in cases of primary maternal infection, while the transmission risk is 0.2–1% in case of a non-primary maternal infection (Yamamoto *et al.*, 2010; Griffiths, Baraniak and Reeves, 2015). Therefore, a lower number of pregnancies in HCMV-seropositive women, which had been associated with a lower level of trained innate immunity and a lower level of protection against HCMV disease and intrauterine cCMV infection, may also be a risk factor for HCMV shedding. Thus, HCMV shedding may be a proxy of HCMV non-primary maternal infection and vertical transmission.

HCMV-seropositive pregnant women living with a greater number of children was found to be associated with HCMV shedding early in pregnancy with multiple LR at a high OR of 21, although this may be a biased estimate as the result was not of statistical significance with MICE. Nevertheless, it is a biologically plausible association and consistent with other known risk factors, such as living in crowded households (Barbosa *et al.*, 2018). This may be of concern as maternal primary HCMV infection in the 1st trimester is known to be associated with a higher risk of symptomatic cCMV infection (20-30% versus 9% in the 2nd trimester) (Picone *et al.*, 2013), and young children may shed HCMV for a prolonged period and therefore is an important source of HCMV transmission to pregnant women (Mocarski, Shenk and Pass, 2007).

Although working regularly with children was found to be significantly more likely when HCMV shedding was detected late in pregnancy only with simple LR, the p-value of that in multiple LR was close to a significance level. While this may be a biased estimate as the result was not of statistical significance with MICE, this finding may be important. The risk of maternal-fetal transmission in primary HCMV infection increases with gestation (Picone *et al.*, 2013); while the same is not known for non-primary infection, it may be sensible for HCMV-seropositive women working regularly with children to take extra caution during the 3rd trimester to reduce the risk of vertical transmission.

Finally, ethnicity was found to be significantly associated with HCMV shedding in urine during pregnancy with multiple LR, contributed primarily by White British where the number of White British women who shed in urine was around twice more than that in those who did not shed. While this may be a biased estimate as the result was not of statistical significance with MICE, it warrants further exploration in future larger scale studies.

6.2. The relationship between hygiene-related contact with children's bodily fluids and human cytomegalovirus shedding in participants of the observational cohort study

6.2.1. Background

I sought to investigate the relationship between the frequency of specific hygiene behaviours in relation to contact with children's bodily fluids, defined as "hygiene-related

contact with children's bodily fluids", and HCMV shedding in HCMV-seropositive pregnant women in the observational cohort study. A full description of the methods can be found in section 2.2.5.13.

6.2.2. Results

The associations between HCMV shedding in HCMV-seropositive pregnant women and hygiene-related contact with children's bodily fluids were looked at separately by site of shedding (saliva, urine, vaginal secretions, or any bodily fluids) and at each time-point during pregnancy, summarised in different tables. They were also done separately as single and adjusted LR models, as well as with and without MICE when there were observations to be imputed.

For HCMV shedding detection in saliva, at the 1st time-point, LR without MICE for assessing hand washing with soap when handling children's faeces was not possible because of perfect collinearity. However, LR was possible with MICE (total observations imputed= 1), where higher frequency of this contact-type was significantly more likely when shedding was detected for both simple [OR 2.42 (95% CI: 1.03-5.64), p=0.042]] and adjusted (aOR 2.29 (95% CI: 1.02-5.16), p= 0.045] LR to age, highest level of education, number, and age of children. No significant differences were seen for this contact at the 2nd and 3rd time-point.

For HCMV shedding detection in urine, at the 1st time-point, hand washing with soap when handling's children's faeces was significantly done more frequently by the women with shedding detected, as analysed without MICE for both simple [OR 93.48 (95% CI: 8.58-1017.92), p= 0.000] and adjusted [aOR 198.89 (95% CI: 6.03-6555.75), p=0.003] LR to highest level of education and number of children, and also without MICE (total

observations imputed= 2) for both simple [OR 6.18 (95% CI: 1.78-21.41), $p= 0.004$] and adjusted [aOR 6.26 (95% CI: 1.94-20.22), $p= 0.002$] LR.

For HCMV shedding detection in vaginal secretions, at the 1st time-point, hand washing with soap when handling's children's faeces was significantly done more frequently by the women with shedding detected as analysed without MICE for both simple [OR 13.50 (95% CI: 2.64-69.38), $p= 0.002$] and adjusted [aOR 70.80 (95% CI: 3.12-1604.03), $p=0.007$] LR to age, ethnicity, highest level of education, number, and age of children. At the 1st time-point, eating children's leftover food was significantly done less frequently by the women with shedding detected, as analysed without MICE for both simple [OR 0.51 (95% CI: 0.28-0.95), $p= 0.033$] and adjusted [aOR 0.43 (95% CI: 0.21-0.88), $p=0.021$] LR to age, ethnicity, highest level of education, number, and age of children. Also, at the 1st time-point, drinking from children's used cups was significantly done less frequently by the women with shedding detected, as analysed with adjusted LR [aOR 0.40 (95% CI: 0.17-0.97), $p= 0.042$] but not simple LR [OR 0.49 (95% CI: 0.24-1.03), $p=0.058$]. There were no observations to be imputed therefore MICE was not performed.

For HCMV shedding detection in any bodily fluid, at the 1st time-point, LR without MICE for assessing hand washing with soap when handling children's faeces was not possible because of perfect collinearity. However, LR was possible with MICE (total observations imputed= 2) where higher frequency of this contact-type was significantly more likely when shedding was detected for both simple [OR 4.31 (95% CI: 2.00-9.26), $p=0.000$] and adjusted (aOR 3.90 (95% CI: 1.83-8.30), $p= 0.000$] LR to age, ethnicity, highest level of education, number, and age of children. At the 1st time-point, eating children's leftover food was significantly done less frequently by the women with shedding detected, as analysed without MICE for both simple [OR 0.59 (95% CI: 0.36-0.97), $p= 0.038$] and

2186 adjusted [aOR 0.59 (95% CI: 0.31-0.99), p=0.049] LR, and with MICE (total observations
 2187 imputed= 2) for simple LR [OR 0.61 (95% CI 0.39-0.98), p=0.039]. Also, at the 1st time-point,
 2188 drinking from children's used cups was done significantly less frequently by the women with
 2189 shedding detected, as analysed without MICE for adjusted LR [aOR 0.54 (95% CI: 0.29-0.99),
 2190 p= 0.045] and with MICE (total observations imputed= 2) for simple [OR 0.60 (95% CI 0.37-
 2191 0.99), p=0.047] and adjusted [aOR 0.53 (95% CI 0.29-0.96), p= 0.037] LR. At the 3rd time-
 2192 point, hand washing with soap when handling children's urine was significantly done less
 2193 frequently by the women with shedding detected, as analysed without MICE for both simple
 2194 [OR 0.50 (95% CI: 0.29-0.87), p= 0.013] and adjusted [aOR 0.45 (95% CI: 0.24-0.83), p=0.012]
 2195 LR, and also without MICE (total observations imputed= 2) for both simple [OR 0.59 (95% CI:
 2196 0.36-0.98), p= 0.040] and adjusted [aOR 0.54 (95% CI: 0.31-0.96), p= 0.037] LR. Finally at the
 2197 2nd time-point, hand washing with soap when wiping children's noses was significantly done
 2198 more frequently by the women with shedding detected, as analysed with MICE for simple
 2199 and adjusted LR but not without MICE. However, at the 3rd time-point, the opposite was
 2200 found where hand washing with soap when wiping children's noses was significantly done
 2201 less frequently by the women with shedding detected, as analysed with MICE for simple and
 2202 adjusted LR but not without MICE (Table 28).

Contact with children's bodily fluids	1 st time-point										2 nd time-point										3 rd time-point									
	Shedding (N=14)	No shedding (N=138)	Logistic regression								Shedding (N=13)	No shedding (N=106)	Logistic regression								Shedding (N=11)	No shedding (N=100)	Logistic regression							
			Without MICE (N=152)				With MICE (N=154, imputed N=2)						Without MICE (N=119)				With MICE (N=121, imputed N=2)						Without MICE (N=111)				With MICE (N=113, imputed N=2)			
			Simple		Multiple		Simple		Multiple				Simple		Multiple		Simple		Multiple				Simple		Multiple					
	Mean (SD)	Mean (SD)	OR [95% CI]	p	aOR [95% CI]	p	OR [95% CI]	p	aOR [95% CI]	p	Mean (SD)	Mean (SD)	OR [95% CI]	p	aOR [95% CI]	p	OR [95% CI]	p	aOR [95% CI]	p	Mean (SD)	Mean (SD)	OR [95% CI]	p	aOR [95% CI]	p	OR [95% CI]	p		
Wash hands with soap after handling child's faeces	4.8 (0.4)	1.8 (1.3)	*	-	*	-	4.31 [2.00-9.26]	.000	3.90 [1.83-8.30]	.000	4.5 (0.8)	4.6 (0.8)	0.82 [0.45-1.49]	.514	0.76 [0.39-1.48]	.415	0.82 [0.47-1.45]	.499	0.76 [0.41-1.41]	.380	4.5 (0.7)	4.7 (0.7)	0.73 [0.36-1.47]	.372	0.73 [0.35-1.52]	.399	0.81 [0.40-1.64]	.561	0.82 [0.40-1.70]	.594
Wash hands with water after handling child's faeces	1.6 (0.9)	1.9 (1.2)	.84 [0.49-1.42]	.509	0.86 [0.47-1.59]	.634	0.77 [0.45-1.31]	.339	0.82 [0.44-1.51]	.516	1.9 (0.9)	1.8 (1.2)	1.10 [0.69-1.74]	.688	1.23 [0.73-2.06]	.436	1.06 [0.68-1.65]	.797	1.19 [0.73-1.95]	.479	2.2 (1.0)	1.8 (1.2)	1.26 [0.79-2.00]	.327	1.23 [0.77-1.98]	.381	1.14 [0.72-1.79]	.585	1.13 [0.71-1.80]	.607
Clean hands with gel after handling child's faeces	2.4 (1.2)	2.1 (1.1)	1.25 [0.77-2.02]	.361	1.24 [0.69-2.21]	.468	1.27 [0.81-1.20]	.300	1.29 [0.731-2.29]	.377	2.1 (1.1)	2.1 (1.1)	0.99 [0.59-1.65]	.958	0.86 [0.46-1.60]	.627	1.08 [0.86-1.71]	.739	1.04 [0.62-1.75]	.881	2.5 (1.6)	1.9 (1.1)	1.46 [0.88-2.41]	.142	1.55 [0.91-2.64]	.108	1.55 [0.97-2.49]	.069	1.69 [1.01-2.81]	.045
Wash hands with soap after handling child's urine	4.2 (1.1)	4.0 (1.0)	1.25 [0.69-2.24]	.463	1.27 [0.61-2.64]	.521	1.40 [0.78-2.53]	.258	1.48 [0.72-3.04]	.291	3.8 (1.2)	3.9 (1.1)	0.95 [0.56-1.61]	.849	1.04 [0.57-1.88]	.907	0.97 [0.58-1.60]	.893	1.01 [0.58-1.76]	.971	3.1 (1.4)	4.0 (0.9)	0.50 [0.29-0.87]	.013	0.45 [0.24-0.83]	.012	0.59 [0.36-0.98]	.040	0.54 [0.31-0.96]	.037
Wash hands with water after handling child's urine	1.9 (1.0)	2.2 (1.2)	0.78 [0.47-1.30]	.345	0.64 [0.35-1.16]	.143	0.71 [0.43-1.18]	.185	0.59 [0.33-1.06]	.079	2.1 (0.9)	2.3 (1.3)	0.83 [0.51-1.36]	.463	1.01 [0.58-1.75]	.984	0.78 [0.49-1.26]	.316	0.93 [0.56-1.55]	.783	2.5 (1.4)	2.3 (1.2)	1.13 [0.68-1.88]	.626	1.16 [0.68-2.01]	.584	1.08 [0.67-1.74]	.743	1.10 [0.65-1.84]	.725
Clean hands with gel after handling child's urine	2.4 (1.2)	2.1 (1.1)	1.31 [0.84-2.06]	.238	1.25 [0.73-2.14]	.412	1.32 [0.86-2.03]	.198	1.27 [0.75-2.15]	.374	2.2 (0.8)	2.0 (1.1)	1.19 [0.71-2.02]	.506	1.22 [0.67-2.24]	.510	1.29 [0.81-2.06]	.286	1.39 [0.82-2.35]	.223	2.3 (1.1)	2.0 (1.0)	1.31 [0.76-2.27]	.337	1.37 [0.77-2.44]	.280	1.43 [0.86-2.39]	.166	1.52 [0.89-2.62]	.122

Wash hands with soap after wiping child's nose	3.0 (1.3)	2.7 (1.1)	1.23 [0.76-2.03]	.397	1.43 [0.76-2.66]	.264	1.06 [0.66-1.70]	.800	1.21 [0.66-2.21]	.531	3.2 (1.1)	2.6 (1.1)	1.55 [0.91-2.63]	.107	1.87 [0.98-3.59]	.059	1.71 [1.03-2.84]	.039	1.90 [1.03-3.51]	.039	2.3 (1.3)	2.9 (1.0)	0.50 [0.25-1.02]	.055	0.53 [0.25-1.08]	.082	0.47 [0.24-0.92]	.029	0.50 [0.25-0.99]	.049
Wash hands with water after wiping child's nose	2.3 (1.1)	2.4 (1.1)	0.94 [0.57-1.55]	.807	1.08 [0.60-1.96]	.794	0.87 [0.54-1.40]	.553	0.99 [0.55-1.78]	.977	2.2 (0.9)	2.4 (1.0)	0.76 [0.42-1.36]	.351	0.75 [0.39-1.46]	.402	0.74 [0.42-1.29]	.287	0.72 [0.39-1.33]	.292	2.0 (1.1)	2.4 (1.0)	0.60 [0.29-1.26]	.180	0.61 [0.28-1.30]	.197	0.74 [0.39-1.40]	.360	0.73 [0.38-1.42]	.358
Clean hands with gel after wiping child's nose	2.1 (1.1)	1.8 (0.9)	1.42 [0.84-2.39]	.195	1.45 [0.74-2.84]	.282	1.32 [0.79-2.19]	.286	1.27 [0.66-2.49]	.472	1.8 (0.7)	1.9 (1.0)	0.95 [0.51-1.79]	.881	0.92 [0.46-1.85]	.824	0.98 [0.54-1.75]	.937	0.92 [0.49-1.74]	.808	1.6 (0.8)	1.8 (0.9)	0.81 [0.39-1.69]	.579	0.84 [0.39-1.80]	.646	0.87 [0.45-1.69]	.691	0.90 [0.45-1.82]	.778
Put child's used dummy in own mouth	1.9 (1.2)	1.8 (1.2)	1.11 [0.72-1.69]	.643	1.03 [0.52-1.71]	.909	1.15 [0.78-1.71]	.476	1.06 [0.65-1.72]	.818	1.7 (1.4)	1.7 (1.1)	1.03 [0.62-1.74]	.897	0.92 [0.52-1.64]	.788	1.01 [0.62-1.66]	.958	0.92 [0.54-1.58]	.770	1.8 (1.3)	1.7 (1.1)	1.11 [0.64-1.91]	.711	1.21 [0.67-2.20]	.523	1.13 [0.68-1.88]	.627	1.23 [0.70-2.17]	.460
Eat child's leftover food	2.6 (1.4)	3.3 (1.1)	0.59 [0.36-0.97]	.038	0.59 [0.31-0.99]	.049	0.61 [0.39-0.98]	.039	0.58 [0.33-1.02]	.059	3.3 (0.9)	3.1 (1.2)	1.18 [0.70-1.99]	.544	1.24 [0.70-2.21]	.454	1.20 [0.73-1.98]	.465	1.25 [0.74-2.12]	.410	2.8 (1.3)	3.0 (1.1)	0.87 [0.50-1.50]	.611	0.73 [0.38-1.39]	.336	1.00 [0.60-1.67]	1.00	0.85 [0.46-1.57]	.604
Eat with child's used cutlery	2.4 (1.1)	2.9 (1.3)	0.68 [0.43-1.09]	.112	0.63 [0.35-1.13]	.121	0.66 [0.42-1.03]	.068	0.59 [0.33-1.06]	.076	3.0 (1.2)	2.8 (1.3)	1.11 [0.71-1.75]	.637	1.13 [0.68-1.88]	.644	1.21 [0.79-1.86]	.381	1.29 [0.79-2.08]	.307	2.8 (1.5)	2.8 (1.3)	1.04 [0.64-1.70]	.866	1.01 [0.60-1.71]	.969	1.17 [0.74-1.85]	.507	1.15 [0.69-1.90]	.593
Drink from child's used cup	1.9 (1.1)	2.6 (1.3)	0.60 [0.35-1.02]	.057	0.54 [0.29-.99]	.045	0.60 [0.37-0.99]	.047	0.53 [0.29-0.96]	.037	2.1 (1.1)	2.4 (1.3)	0.81 [0.50-1.31]	.384	0.88 [0.51-1.51]	.644	0.96 [0.63-1.46]	.838	1.09 [0.67-1.76]	.730	2.1 (1.3)	2.4 (1.3)	0.88 [0.53-1.46]	.625	0.83 [0.48-1.43]	.496	0.96 [0.60-1.52]	.846	0.89 [0.54-1.48]	.656
Kiss child on lips	3.4 (1.4)	3.4 (1.4)	0.97 [0.65-1.45]	.883	0.86 [0.53-1.40]	.551	0.98 [0.67-1.43]	.915	0.91 [0.57-1.46]	.690	3.8 (1.2)	3.4 (1.4)	1.23 [0.79-1.92]	.349	1.29 [0.79-2.11]	.307	1.21 [0.80-1.82]	.364	1.23 [0.79-1.89]	.361	3.8 (1.4)	3.5 (1.3)	1.24 [0.75-2.08]	.400	1.27 [0.75-2.17]	.377	1.35 [0.82-2.21]	.243	1.40 [0.83-2.34]	.207

Table summarising the association of the frequency of hygiene-related contact with children's saliva and urine, according to whether shedding in any bodily fluids was detected at least once or not detected, in HCMV-seropositive women at different time-points of pregnancy. Simple and multiple (adjusted to age, ethnicity, education, number, and age of children) logistic regression performed with detection of HCMV shedding in saliva at 1st, 2nd, or 3rd time-point as the outcome variable, with and without MICE. Statistically significant results (p-value <0.05) are in blue font (see section 2.2.5.13 for the methods used, and section 6.2.2 for the description of results).
Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; HCMV, human cytomegalovirus; MICE, multivariate imputation by chained equations; N, total observations; OR, odds ratio; p, p-value; SD, standard deviation; -, results not available; *, excluded from regression model because perfect collinearity.

Table 28. Association of HCMV shedding in any bodily fluids in HCMV-seropositive pregnant women with the frequency of hygiene-related contact with children's bodily fluids

6.2.3. Discussions

It was hypothesised that if shedding reflected recent acquisition of a new HCMV strain then a decreased level of hygiene would be shown to be associated with this. In fact, only less washing of hands with soap after handling child's urine late in pregnancy was associated with a risk of HCMV shedding. A potential explanation is that reduced soap washing will increase the risk of exposure to new HCMV strains through contact with young children as their urine is known to be an important source of exposure, allowing reinfection to occur (Manicklal *et al.*, 2013; Hamilton *et al.*, 2014; Saldan *et al.*, 2017; Barber *et al.*, 2020). However, this was only seen at one time-point during pregnancy, and not with several other activities involving handling of children's bodily fluids. Paradoxically, an increased level of hand hygiene when handling children's faeces early in pregnancy was more likely when shedding was detected in saliva, urine, and vaginal secretions. Moreover, a decreased level of hand hygiene when wiping children's noses was associated with risk of HCMV shedding late in pregnancy but the complete opposite was seen during the mid-point of pregnancy.

Most of the HCMV shedding detected in HCMV-seropositive women during pregnancy may therefore be due to reactivation of existing HCMV strains, not due to acquisition of new strains. Alternatively, it may be that acquisition of new strains in pregnancy can occur despite optimal hygiene. It is also possible that reporting of hygiene behaviour is not accurate as it is subjective and reliant on memory; although this would likely impact on those who shed and those who did not shed equally.

As discussed in section [4.5.3](#), evidence of more than one HCMV strain in the sample sequenced was not found which may suggest that the HCMV shedding seen was more likely to be a result of reactivation. This hypothesis is supported by the findings here (see section

6.4), although more genomic sequencing of strains would facilitate a better understanding of this issue.

6.3. The relationship between human cytomegalovirus-specific cellular mediated immunity and human cytomegalovirus shedding in participants of the observational cohort study

6.3.1. Background

I sought to investigate the relationship between HCMV-specific CMI and HCMV shedding in HCMV-seropositive pregnant women in the observational cohort study. A full description of the methods can be found in section 2.2.5.14.

6.3.2. Results

The association between HCMV shedding in HCMV-seropositive pregnant women and HCMV-specific CMI as measured by HCMV-ELISPOT and HCMV-QuantiFERON via LR were looked at separately by site of shedding (saliva, urine, vaginal secretions, or any bodily fluids) and at each time-point during pregnancy, summarised in Table 29. They were also done separately as single and multiple LR adjusted to age and ethnicity.

There was no significant difference in the HCMV-QuantiFERON assessment between women who shed on at least one occasion and women who did not shed at all for all time-points and for any bodily fluids (Table 29).

The HCMV-ELISPOT spot counts to both IE1 and pp65 antigens were lower at all time-points when HCMV shedding was detected in the women, significantly so at the 2nd

2262 time-point for the pp65 antigen in any bodily fluids ([OR: 0.9; 95% CI: 0.9-0.9, p=0.044] and
2263 [aOR 0.9 (95% CI 0.9-0.9), p= 0.046]), as well as in vaginal secretions specifically ([OR 0.9
2264 (95% CI 0.9-0.9), p=0.034] and [aOR 0.9 (95% CI: 0.9-0.9), p= 0.039]) (Table 29).

Bodily fluid site	HCMV-specific CMI detection by assay	1 st time-point (N=72)							2 nd time-point (N=51)							3 rd time-point (N=42)						
		x + y	Shedding	No shedding	Logistic regression				x + y	Shedding	No shedding	Logistic regression				x + y	Shedding	No shedding	Logistic regression			
					Simple		Multiple					Simple		Multiple					Simple		Multiple	
					OR [95% CI]	p	aOR [95% CI]	p				OR [95% CI]	p	aOR [95% CI]	p				OR [95% CI]	p	aOR [95% CI]	p
Saliva	Detection by QuantiFERON: f (%)	0 + 72	0 (0)	50 (69.4)	-	-	-	-	1 + 50	1 (100)	37 (74.0)	*	-	*	-	2 + 40	1 (50.0)	30 (75.0)	0.3 [0.0-5.8]	.452	0.3 [0.0-5.8]	.452
	QuantiFERON IU/ml: median (IQR)		-	1.0 (5.2)	-	-	-	-		41.7 (0)	1.5 (4.2)	1.1 [1.0-1.2]	.071	1.1 [0.9-1.2]	.071		19.8 (39.2)	1.3 (4.6)	1.1 [0.9-1.2]	.062	1.1 [0.9-1.2]	.062
	ELISPOT IE1 count: median (IQR)		-	29 (151)	-	-	-	-		108 (0)	47 (172)	1.00 [0.9-1.0]	.940	1.0 [0.9-1.0]	.940		0 (0)	47 (76)	*	-	*	-
	ELISPOT pp65 count: median (IQR)		-	149 (187)	-	-	-	-		99 (0)	138(156)	1.0 [0.9-1.0]	.611	1.0 [0.9-1.0]	.611		4 (0)	146 (190)	0.9 [0.6-1/2]	.440	0.9 p0.6-1.2]	.440
Urine	Detection by QuantiFERON: N (%)	5 + 67	2 (40.0)	48 (71.6)	0.2 [0.0-1.7]	.162	0.1 [0.0-1.2]	.076	5 + 46	5 (100)	33 (71.7)	*	-	*	-	3 + 39	2 (66.7)	29 (74.4)	0.7 [0.1-8.5]	.771	0.7 [0.1-8.5]	.771
	QuantiFERON IU/ml: median (IQR)		0.1 (0.2)	1.1 (5.2)	1.0 [0.9-1.1]	.457	1.0 [0.9-1.1]	.430		4.9 (6.3)	1.0 (4.0)	1.0 [0.9-1.1]	.204	1.0 [0.9-1.1]	.341		0.3 (39.2)	1.5 (5.1)	1.1 [0.9-1.2]	.133	1.1 [0.9-1.2]	.133
	ELISPOT IE1 count: median (IQR)		56 (153)	28 (153)	1.0 [0.9-1.0]	.921	1.0 [0.9-1.0]	.793		85 (133)	46 (167)	1.0 [0.9-1.0]	.906	1.0 [0.9-1.0]	.906		0 (0)	47 (76)	*	-	*	-
	ELISPOT pp65 count: median (IQR)		49 (123)	160 (186)	1.0 [0.9-1.0]	.157	1.0 [0.9-1.0]	.071		100 (56)	139 (164)	1.0 [0.9-1.0]	.234	1.0 [0.9-1.0]	.234		4 (0)	146 (190)	0.9 [0.6-1.2]	.440	0.9 [0.6-1.2]	.440
Vaginal secretions	Detection by QuantiFERON: N (%)	5 + 67	4 (80.0)	46 (68.7)	1.8 [0.2-17.3]	.600	1.7 [0.2-16.4]	.649	6 + 45	4 (66.7)	34 (75.6)	0.65 [0.1-4.0]	.641	0.7 [0.1-4.3]	.672	5 + 37	4 (80.0)	27 (73.0)	1.5 [0.1-14.9]	.739	1.9 [0.2-20.6]	.591
	QuantiFERON IU/ml: median (IQR)		0.3 (3.4)	1.0 (5.2)	1.0 [0.9-1.1]	.371	1.0 [0.9-1.1]	.387		2.1 (3.1)	1.4 (4.4)	0.9 [0.7-1.2]	.561	0.9 [0.7-1.2]	.563		0.4 (1.4)	1.5 (5.9)	0.7 [0.4-1.3]	.276	0.8 [0.5-1.3]	.313
	ELISPOT IE1 count: median (IQR)		117 (234)	29 (151)	1.0 [0.9-1.0]	.850	1.0 [0.9-1.0]	.842		38 (94)	71 (190)	1.00 [0.9-1.0]	.440	1.0 [0.9-1.0]	.488		79 (34)	31 (100)	1.0 [0.9-1.0]	.871	1.0 [0.9-1.0]	.866
	ELISPOT pp65 count: median (IQR)		160 (22)	143 (202)	1.0 [0.9-1.0]	.863	1.0 [0.9-1.0]	.858		21 (69)	140 (157)	0.9 [0.9-0.9]	.034	0.9 [0.9-0.9]	.039		102 (242)	146 (192)	1.0 [0.-1.0]	.624	1.0 [0.9-1.0]	.821
Any bodily fluid (saliva, urine, and/or	Detection by QuantiFERON: N (%)	8 + 64	5 (62.5)	45 (70.3)	0.7 [0.2-3.2]	.652	0.6 [0.1-2.8]	.487	9 + 42	7 (77.8)	31 (73.8)	1.2 [0.2-6.9]	.804	1.2 [0.2-6.7]	.838	6 + 36	5 (83.3)	26 (72.2)	1.9 [0.2-18.6]	.572	2.7 [0.3-28.0]	.411
	QuantiFERON IU/ml: median (IQR)		0.3 (24.1)	1.1 (5.2)	1.0 [0.9-1.1]	.106	1.0 [0.9-1.1]	.104		2.6 (3.8)	1.0 (4.2)	1.0 [0.9-1.1]	.558	1.0 [0.9-1.1]	.671		1.1 (2.4)	1.3 (5.5)	1.0 [0.9-1.1]	.395	1.0 [0.9-1.1]	.300

vaginal secretions)	ELISPOT IE1 count: median (IQR)		56 (153)	28 (135)	1.0 [0.9-1.0]	.840	1.00 [0.9-1.0]	.889		62 (141)	45 (172)	1.0 [0.9-1.0]	.624	1.0 [0.9-1.0]	.579		63 (57)	33 (100)	1.0 [0.9-1.0]	.747	1.0 [0.9-1.0]	.739
	ELISPOT pp65 count: median (IQR)		132 (100)	160 (191)	1.0 [0.9-1.0]	.203	1.00 [0.9-1.0]	.149		99 (109)	148 (164)	0.9 [0.9-0.9]	.044	0.9 [0.9-0.9]	.046		55 (170)	148 (190)	1.00 [0.9-1.0]	.306	1.0 [0.9-1.0]	.443

Table summarising the association of HCMV-specific cellular mediated immunity, according to whether shedding in specific bodily fluid sites were detected at least once or not detected, in HCMV-seropositive women at different time-points of pregnancy. Simple and multiple (adjusted to age and ethnicity) logistic regression performed with detection of HCMV shedding at 1st, 2nd or 3rd time-point as the outcome variable. Statistically significant results (p-value <0.05) are in blue font. Detected HCMV-specific CMI by HCMV-QuantiFERON is defined as a quantity ≥ 0.2 IU/ml and meeting the quality control. Detected HCMV-specific CMI by HCMV-ELISPOT is defined as a positive IE1 or pp65 spot count and meeting the quality control, where the number of spots is indicative of the strength of the cellular immune response to HCMV (see section 2.2.5.14 for the methods used, and section 6.3.2 for the description of results).

Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; CMI, cellular-mediated immunity; ELISPOT, enzyme-linked immunosorbent spot; f, frequency; HCMV, human cytomegalovirus; IE1, immediate-early 1; IU/ml, international units/millilitres; N, total observations; OR, odds ratio; pp65, phosphoprotein 65; p-value; SD, standard deviation; x, total frequency with HCMV shedding detected; y, total frequency with HCMV shedding not detected; *, excluded from regression model because of perfect collinearity.

Table 29. Association of HCMV shedding in any bodily fluids in HCMV-seropositive pregnant women with HCMV-specific cellular mediated immunity

6.3.3. Discussions

A lower HCMV-ELISPOT spot count, i.e., a lower level of HCMV-specific CMI towards pp65 antigen, was associated with HCMV shedding during pregnancy in HCMV-seropositive women. As discussed in section [4.4.3](#), the HCMV-QuantiFERON assay only includes HCMV-specific CD8+ cells whereas the HCMV-ELISPOT assay also includes HCMV-specific CD4+ cells (Saldan *et al.*, 2015). It has been found that both CD4+ and CD8+ cell IFN- γ response is high in healthy HCMV-seropositive individuals (Sinclair *et al.*, 2004), and therefore both types of T-cells are important components of HCMV-specific CMI. The pp65 antigen is the most abundant antigen in HCMV (Chevillotte *et al.*, 2009) and is responsible for the modulation and evasion of a host's CMI during HCMV infection (McLaughlin-Taylor *et al.*, 1994). pp65 are targeted by both humoral and cellular immunity and is a dominant target antigen of HCMV-specific CD8+ cells (McLaughlin-Taylor *et al.*, 1994). Previous studies have found that while HCMV-specific CD8+ cell responses to both pp65 and IE1 are comparatively strong, HCMV-specific CD4+ cell responses to pp65 are greater than CD4+ responses to IE1 in HCMV-seropositive immunocompetent individuals (Sylwester *et al.*, 2005; Slezak *et al.*, 2007). pp65 may therefore be a dominant antigen in shaping HCMV-specific CMI, for both CD4+ and CD8+ cells, in immunocompetent HCMV-seropositive individuals, including pregnant women.

In support of this, lower HCMV-specific CMI to pp65 antigen as measured by HCMV-ELISPOT was associated with a higher likelihood of HCMV shedding in HCMV-seropositive pregnant women. This suggests that pp65 may be a correlate of protection against shedding and potentially therefore a suitable endpoint for vaccine studies. Further work is required to explore this hypothesis. There are currently HCMV vaccines in clinical and pre-clinical developmental phases that include the pp65 antigen, including but not limited to vaccines

manufactured by Merck Sharpe & Dohme, City of Hope, Astellas, Moderna and Pfizer (Plotkin *et al.*, 2020).

As the HCMV-QuantiFERON assay does not measure CD4+ cell responses towards the pp65 antigen, this may explain the lack of association between HCMV shedding detection and HCMV-specific CMI as measured by HCMV-QuantiFERON.

The HCMV-specific CMI to pp65 antigen, as assessed with HCMV-ELISPOT, may be important in the prediction and control of HCMV shedding in HCMV-seropositive women during pregnancy. The HCMV-specific pp65 antigen may prove to be a suitable target for the evaluation of preventative and therapeutic strategies against cCMV infection in the context of non-primary maternal HCMV infection.

6.4. The relationship between hygiene-related contact with children's bodily fluids, human cytomegalovirus-specific cellular mediated immunity and sequenced HCMV shedding, in participants of the observational cohort study

6.4.1. Background

I sought to investigate the relationship between hygiene-related contact with children's bodily fluids, human cytomegalovirus-specific cellular mediated immunity and sequenced HCMV shedding in a HCMV-seropositive pregnant woman in the observational cohort study. A full description of the methods can be found in section [2.2.5.15](#).

6.4.2. Results

The reported frequencies of hygiene-related contact with children's bodily fluids and the HCMV-specific CMI as measured by HCMV-QuantIFERON and HCMV-ELISPOT at the 2nd time-point, of the HCMV-seropositive pregnant woman who had her HCMV shedding sequenced (obtained from her vaginal secretion sample also at the 2nd time-point, see section [4.5.2](#)), is summarised in Table 30. No decreased levels of any hygiene-related contact with children's bodily fluids during the 2nd time-point were found to be associated with HCMV shedding detection in HCMV-seropositive pregnant women (see section [6.2](#)), therefore no comparison could be made with the hygiene-related contact findings of the women in question here. The median ELISPOT spot counts towards IE1 and pp65 antigens at the 2nd time-point for all HCMV-seropositive women were 62 and 131 respectively (see section [4.4.2](#)). The median ELISPOT spot counts towards IE1 and pp65 antigens at the 2nd time-point for those with HCMV shedding detected were 62 and 99 respectively (see section [6.3.2](#)). The HCMV ELISPOT spot counts towards IE1 and pp65 antigens for the woman in question here were 28 and 28 respectively (Table 30), lower than that of all HCMV-seropositive women including those with HCMV shedding detected.

Frequency of hygiene-related contact with children's bodily fluids (mean score)	Wash hands with soap after handling child's faeces	5
	Wash hands with just water after handling child's faeces	2
	Clean hands with gel after handling child's faeces	2
	Wash hands with soap after handling child's urine	4
	Wash hands with just water after handling child's urine	2
	Clean hands with gel after handling child's urine	2
	Wash hands with soap after wiping child's nose	2
	Wash hands with just water after wiping child's nose	1
	Clean hands with gel after wiping child's nose	2
	Put child's used dummy in own mouth	1
	Eat child's leftover food	3
	Eat with child's used cutlery	3
	Drink from child's used cup	2
	Kiss child on lips	3
HCMV-specific CMI	HCMV-QuantiFERON	Detected
	HCMV-ELISPOT towards IE1 antigen (spot count)	Detected (28)
	HCMV ELISPOT towards pp65 antigen (spot count)	Detected (28)

Table summarising the reported frequencies of hygiene-related contact with children's bodily fluids (mean score out of 5, where higher mean scores indicated higher level of frequency of the contact), and HCMV-specific CMI as measured by HCMV-QuantiFERON and HCMV-ELISPOT at the 2nd time-point, of a HCMV-seropositive pregnant individual with sequenced HCMV DNA from a vaginal secretion sample also at the 2nd time-point (see section 2.2.5.15 for the methods used, and section 6.4.2 for the description of results). Abbreviations: CMI, cellular mediated immunity; ELISPOT, enzyme-linked immunosorbent spot; HCMV, human cytomegalovirus; IE1, immediate early 1; pp65, phosphoprotein 65.

Table 30. Hygiene-related contact with children's bodily fluids and HCMV-specific CMI of a HCMV-seropositive woman with sequenced HCMV DNA during the second trimester of pregnancy

6.4.3. Discussions

As discussed in section 4.5.3, evidence of more than one HCMV strain in the subject's sequenced HCMV DNA sample from the 2nd trimester was not found, which may suggest that the HCMV shedding seen was a result of reactivation. This hypothesis is supported by the findings in section 6.2.3 where most of the HCMV shedding detected in the HCMV-seropositive pregnant women may be due to reactivation of those strains that caused the original infection in the women. Certainly, during the 2nd time-point there was no decreased level of hygiene-related contact with children's bodily fluids found to be associated with HCMV shedding detection in HCMV-seropositive women.

As discussed in section 6.3.3, lower HCMV-ELISPOT spot counts towards pp65 antigen was associated with HCMV shedding. For the subject, her lower-than-average HCMV ELISPOT spot counts towards pp65 antigen when compared to all HCMV-seropositive women, irrespective of shedding status, appeared to have put her at increased risk of shedding. Moreover, her lower and therefore suppressed HCMV-specific CMI which is important to restrain HCMV viral replication and prevent disease (La Rosa and Diamond, 2012), may potentially put her at risk of reactivation, supporting the hypothesis made on her sequenced sample to suggest that the HCMV shedding seen may be a result of reactivation. Genomic sequencing of more HCMV-seropositive pregnant women would facilitate a better understanding of this issue.

Genomic sequencing assessment in HCMV-seropositive pregnant women, together with other clinical and non-clinical information, can provide valuable insights into the pathogenesis of HCMV shedding and non-primary HCMV maternal infection.

7. Conclusions and future work

7.1. Conclusions

In this thesis, HCMV shedding in bodily fluids of HCMV-seropositive pregnant women was investigated, an important issue not only in maternal health, but also in child health. The outputs of my thesis included a systematic review of published data on HCMV shedding in HCMV-seropositive pregnant women, which highlighted important gaps in our understanding of HCMV shedding in HCMV-seropositive pregnant women; an observational cohort study was conducted to improve our understanding of this. The investigations in the observational cohort included the investigation of risk factors associated with HCMV shedding in HCMV-seropositive pregnant women, as this could have direct clinical relevance and help in our understanding of the mechanisms of HCMV infection in HCMV-seropositive pregnant women. The observational cohort study provided us with novel information and highlighted the complexity not only of HCMV shedding but also of the immune responses to HCMV infection, which may impact many future studies of virus-host interaction, most importantly in combatting cCMV infection.

In the systematic review, a pooled prevalence estimate of approximately 20% of HCMV-seropositive pregnant women shedding HCMV on at least one occasion during pregnancy was found. According to bodily fluid site, the estimate was highest for cervico-vaginal secretions. This suggests that cervico-vaginal secretions may be the most suitable bodily fluid for assessing HCMV shedding in HCMV-seropositive pregnant women. Moreover, as cervico-vaginal secretions represent a mixture of vaginal, cervical, and uterine secretions (Adnane, Meade and O'Farrelly, 2018) and, as vertical transmission of HCMV can

2419 occur either transplacentally or during vaginal delivery (Luck *et al.*, 2017; Saldan *et al.*, 2017)
2420 via contact with such secretions (Mocarski, Shenk and Pass, 2007; Saldan *et al.*, 2017),
2421 analysis of these samples may provide insight into the risk of vertical transmission in HCMV-
2422 seropositive pregnant women.

2423 A cohort of HCMV-seropositive pregnant women at a large tertiary hospital in South
2424 London was identified, representing a multinational and ethnically diverse group of
2425 pregnant women. The seroprevalence rate of approximately 55%, varied by ethnicity, was
2426 found for this cohort and was consistent with data presented elsewhere (Winter *et al.*,
2427 2020).

2428 The prevalence of HCMV shedding on at least one occasion during pregnancy in
2429 HCMV-seropositive women in the study cohort was approximately 20%, consistent with the
2430 systematic review. Similarly, the highest HCMV shedding prevalence by bodily fluid site was
2431 seen in vaginal secretions. This supports the conclusions made from the systematic review.

2432 Although many pregnant women had to be approached at their routine early
2433 pregnancy appointment to identify those already living with children of young age to be
2434 eligible to participate in the study, the majority who were eligible agreed to take part, and
2435 agreed to have their HCMV serostatus established. The post-study interview of the
2436 participating women identified that HCMV screening for themselves and their babies (to test
2437 for cCMV infection), were perceived benefits from participating in the study, without any
2438 additional concerns.

2439 In the observational cohort study, there was an inverse relationship between the
2440 number of pregnancies an individual had experienced and HCMV shedding. At first sight,
2441 this result seems implausible, as exposure to more children seems likely to lead to more
2442 exposure to HCMV and therefore a higher likelihood of reinfection. One possible

explanation is that “protection” may be acquired via “trained immunity” of the innate immune system in subsequent pregnancies of HCMV-seropositive women, which has been hypothesised previously based on observed NK cells in maternal uterine tissue, known as decidua, of multigravida pregnant women, which increases with the number of pregnancies, suggesting that the observed pregnancy-trained decidual NK cells may be induced by decidual HCMV exposure and aid in the protection against HCMV disease in subsequent pregnancies (Feyaerts, Joosten and van der Molen, 2020). This trained immunity in subsequent pregnancies of HCMV-seropositive pregnant women had also been hypothesised to have the potential to provide improved protection against intrauterine cCMV infection, where previous epidemiological studies have reported that HCMV-seropositivity during previous pregnancies reduces the risk of cCMV infections in future pregnancies, especially during the first trimester of pregnancy when the NK cells are abundantly present and the rate of cCMV infection is lowest compared to later trimesters (Hamilton *et al.*, 2014; Feyaerts, Joosten and van der Molen, 2020). As we know, the risk of vertical transmission is 30–40% in cases of primary maternal infection, while the transmission risk is 0.2–1% in case of a non-primary maternal infection (Yamamoto *et al.*, 2010; Griffiths, Baraniak and Reeves, 2015). Therefore, a lower number of pregnancies in HCMV-seropositive women, which has been associated with a lower level of trained innate immunity and a lower level of protection against HCMV disease and intrauterine cCMV infection, may also be a risk factor for HCMV shedding as seen here. Thus, HCMV shedding may be a proxy of HCMV non-primary maternal infection and vertical transmission.

It was also found that HCMV-seropositive women with higher number of children may be more at risk of HCMV shedding during pregnancy, in particular early in pregnancy. This is of concern as maternal HCMV infection early in pregnancy is most associated with a

higher risk of symptomatic cCMV infection (Picone *et al.*, 2013). It was also found that working regularly with children may be a risk factor for HCMV shedding late in pregnancy, which is when there may be a higher risk of vertical transmission (Picone *et al.*, 2013). These observations may be explained by a high number of individuals in the households studied (Barbosa *et al.*, 2018) and an increased exposure to children, who are a well-known source for HCMV shedding (Mocarski, Shenk and Pass, 2007; Cannon, Hyde and Schmid, 2011).

The assessment of HCMV-specific CMI as measured by HCMV-QuantiFERON and HCMV-ELISPOT assays in the observational study cohort found that HCMV-ELISPOT (specifically responses towards the pp65 antigen) was more likely than HCMV-QuantiFERON to detect HCMV-specific CMI in HCMV-seropositive women during pregnancy. Additionally, greater HCMV-specific CMI in HCMV-ELISPOT towards pp65 was found when HCMV-CMI was also detected in HCMV-QuantiFERON. Importantly, lower HCMV-specific CMI to pp65 as assessed with HCMV-ELISPOT was found to be associated with a higher likelihood of HCMV shedding in HCMV-seropositive pregnant women. Thus, HCMV-specific CMI may have a role in controlling HCMV shedding in HCMV-seropositive women during pregnancy.

Pp65 is the most abundant antigen in HCMV virions (Chevillotte *et al.*, 2009) and is a dominant antigen in shaping HCMV-specific CD4+ and CD8+ CMI in immunocompetent adults (McLaughlin-Taylor *et al.*, 1994; Sylwester *et al.*, 2005; Slezak *et al.*, 2007). HCMV-ELISPOT measures HCMV-specific CMI based on CD4+ and CD8+ cell responses, whereas for HCMV-QuantiFERON the measurement of HCMV-specific CMI is based only on CD8+ cell responses (Saldan *et al.*, 2016; Lee *et al.*, 2017; Oxford Immunotec Ltd, 2017; QIAGEN, 2018). Therefore, optimal monitoring of HCMV-specific CMI in HCMV-seropositive women during pregnancy may be better achieved with HCMV-ELISPOT than with HCMV-QuantiFERON. Furthermore, as pp65 may be a correlate of protection against HCMV

shedding it may be useful in the prediction and control of HCMV shedding in HCMV-seropositive women during pregnancy. Pp65 also has the potential to be a suitable endpoint for vaccine studies and for the evaluation of other preventative and therapeutic strategies against cCMV infection in the context of non-primary maternal HCMV infection.

In the observational cohort study, although decreased hand hygiene was associated with risk of HCMV shedding, this was only found during the 3rd trimester. For most other hygiene-related contact with children's bodily fluids, decreased level of hand hygiene was not found to be associated with HCMV shedding during pregnancy. It is possible that the acquisition of new strains in HCMV-seropositive women during pregnancy may therefore occur despite optimal hygiene or possibly, that the reporting of hygiene behaviour was not accurate. Another possibility is that most of the HCMV shedding detected in HCMV-seropositive women is in fact due to reactivation of existing HCMV strains. This latter hypothesis is supported by the virus genomic sequencing result. Unfortunately, HCMV genomic sequencing analysis was possible on one HCMV shedding isolate in vaginal secretions obtained from a HCMV-seropositive participant during pregnancy. There was no evidence of mixed infection in the HCMV genome sequenced, suggesting that the HCMV shedding may be due to reactivation, rather than reinfection. For this specific participant, the HCMV-specific CMI at the time of shedding showed that the HCMV-ELISPOT spot count to pp65 antigen was lower than the average of that of other HCMV-seropositive participants with HCMV shedding. This evidence of suppressed HCMV-specific CMI may therefore explain her risk of shedding and of reactivation. Genomic sequencing of HCMV in HCMV-seropositive women found to be shedding during pregnancy, together with the assessment of hygiene-related contact with children's bodily fluids and of HCMV-specific CMI as

2514 measured by HCMV-ELISPOT, may provide valuable information regarding non-primary
2515 maternal infection.

2516 Most women recruited completed the observational cohort study. Reassuringly,
2517 most of the feedback received by participating women on the study screening, recruitment,
2518 procedures, and structure was positive. The findings from the post-study interview of
2519 HCMV-seropositive women who participated in the observational cohort study provided a
2520 richer understanding of their experiences in the study and perspectives on participation in
2521 research. Importantly, these data also identified the potential facilitators and barriers
2522 towards engaging pregnant women with HCMV risk-reduction measures. As previously
2523 discussed, antenatal screening for HCMV infection and neonatal screening for cCMV
2524 infection are not part of routine maternity care in the UK. The fact that pregnant women
2525 valued the ability to know their own and their newborn infants' HCMV status without
2526 inducing unintended anxiety or concerns, was reassuring for the potential implementation
2527 of routine antenatal and neonatal HCMV screening. It was found that study participation
2528 during pregnancy in women with young children is feasible. They identified that their
2529 participation was contingent on a balance of convenience and inconvenience, and benefits
2530 and risks. For instance, it was identified that the benefits of research procedures around the
2531 time of birth needs to be balanced against the risks of poor research compliance. For
2532 example, issues with study compliance were mainly found with self-sampling of vaginal
2533 secretions and completion of the feedback questionnaire during the 4th time-point, which
2534 mainly took place soon after the birth of their newborn infants. The early postpartum period
2535 can be challenging for the mother, and therefore for future studies research involvement
2536 around this time should be as minimal as possible to improve on study compliance and
2537 experience of participation. As vaginal secretions were identified to be the most suitable

bodily fluid for the assessment of HCMV shedding, it was reassuring that most participants were happy with self-sampling of these secretions. Participation increased their awareness of hygiene-based practices, leading to behavioural modifications that put them in contact with urine and saliva of their children without instructions to do so, in line with previous findings that trial participation can impact on participants' thinking about their behaviour with a possibility to influence change (Torkan *et al.*, 2018). Indeed, a change in their knowledge of HCMV infection during the study was found, where their perceptions of how HCMV infection can transmit increased for all bodily fluid sites. Evidently, it was found that the level of some hygiene-related contact with children's bodily fluids such as hand hygiene when handling children's faeces and noses increased during study participation, which may be related to a change in knowledge during study participation. A previous study has also found that a change in knowledge can occur following an educational intervention in research (Price *et al.*, 2014; Calvert *et al.*, 2021). While educational intervention was not part of the observational cohort study, it was found that some of the women sought additional information on HCMV during study participation on their own accord, mostly on NHS-based sources, highlighting the importance of resourceful, reliable and adequate information to be available and accessible to pregnant women as part of their routine antenatal care. While these behavioural modifications may interfere with their household routines, they recognised it to be a time-limited risk-reduction measure and felt empowered by the knowledge they had gained through study participation and the support they had received from their partners. Maternal research and indeed risk-reduction measures should be centred around being convenient, informative, empowering, holistic, and supportive.

7.2. Future work

Although the work in this thesis has addressed the questions outlined in the aims of the investigation, it has also revealed some areas for future work.

Firstly, more work is required to improve the epidemiological knowledge relating to the HCMV-seroprevalence rate in pregnant women in different populations worldwide. Up to date HCMV seroprevalence rates need to be investigated in many communities and there needs to be an understanding of how they may be affected by specific determinants such as ethnicity, country, and age. These data will contribute towards the development of disease burden models and therapeutic or preventative strategies of maternal HCMV and cCMV infection.

Additionally, there is work to be done to improve our knowledge of HCMV shedding prevalence in the bodily fluids of HCMV-seropositive women during pregnancy in different populations worldwide. The systematic review performed here included studies from North America, South America, Europe, and Asia continents, with no studies from Africa and Australia continents. More observational longitudinal studies worldwide to assess HCMV shedding in bodily fluids of HCMV-seropositive women during pregnancy to assess its prevalence, distribution, and determinants, are needed to assess this important epidemiological question which can contribute towards the development of preventative and therapeutic strategies for cCMV infection in the context of non-primary maternal infection.

Additionally, the ability to detect HCMV shedding is limited to the limitations of the PCR assays used to detect HCMV DNA. These limitations include sensitivity of the assay and the inability to understand if more than one HCMV strain is detected in the assay. Improvements in HCMV DNA PCR sensitivity and viral genomic sequencing will be necessary

for further studies to assess the epidemiology of HCMV shedding, and the presence of different HCMV strains in HCMV shedding in different bodily fluids.

As stated above, the inclusion of HCMV-specific CD4+ cells as part of the HCMV-ELISPOT assay, which are not present in HCMV-QuantIFERON, is likely responsible for the higher HCMV-specific CMI seen in HCMV-ELISPOT. Plus, it was likely that pp65 is a dominant antigen in shaping HCMV-specific CMI for both CD4+ and CD8+ cells in HCMV-seropositive pregnant women. This could be further evaluated by separating the HCMV-specific CD4 or CD8+ cells from the PBMCs generated to test with HCMV-ELISPOT, using anti-CD4 or anti-CD8+ magnetic cell separation beads and retesting with the HCMV-ELISPOT (Jackson *et al.*, 2017). The findings in HCMV-ELISPOT with and without separation of CD4 or CD8+ cells could then be compared, especially with respect to pp65 antigen. Moreover, lower HCMV-specific CMI to pp65 antigen as measured by HCMV-ELISPOT were associated with a higher likelihood of HCMV shedding in HCMV-seropositive pregnant women, suggesting that pp65 may be a correlate of protection against shedding and potentially a suitable endpoint for vaccine studies. There are currently HCMV vaccines in the pre-clinical and clinical developmental stages that induce CMI responses to pp65. These include, but are not limited to, vaccines manufactured by Merck Sharpe & Dohme, City of Hope, Astellas, Moderna and Pfizer (Plotkin *et al.*, 2020). As highlighted here, it is imperative that the investigation of these vaccines using CMI responses to pp65 as a biomarker are studied in HCMV-seropositive pregnant and non-pregnant women.

One of the main objectives of this work was to evaluate the feasibility of performing larger scale studies to evaluate the relationship between HCMV shedding in HCMV-seropositive women during pregnancy with cCMV infection. Here, it was shown that HCMV shedding may have the potential to be a proxy not only to non-primary maternal infection,

2610 but potentially to vertical transmission. If confirmed by further studies, the assessment of
2611 HCMV shedding in HCMV seropositive women during pregnancy may be a valuable endpoint
2612 for the evaluation of preventative and therapeutic strategies against maternal and cCMV
2613 infection. At a vertical HCMV transmission rate of 0.2 to 1% following non-primary HCMV
2614 maternal infection (Raynor, 1993), a large sample size of several hundreds to thousands
2615 would likely be required to provide sufficient power to the findings to investigate this. While
2616 a larger sample size may mean a larger undertaking, a redesign of the study through what
2617 has been learned in this work could accommodate this, such as: 1) sample collection of just
2618 urine and vaginal secretions, not saliva, for the study of HCMV shedding, 2) removal of
2619 sampling in the postpartum period for the study of HCMV shedding, 3) Using only HCMV-
2620 ELISPOT assay, not HCMV-QuantiFERON, for the study of HCMV-specific CMI.

2621 Finally, viral genomic sequencing analysis of one HCMV shedding isolate from a HCMV-
2622 seropositive woman was able to indicate if infection of multiple HCMV strains was present,
2623 thus allowing assessment of reactivation or reinfection. The suggestion that infection was
2624 due to HCMV reactivation was guided by the findings in the woman's HCMV-specific CMI
2625 analysis and self-reported hygiene-related contact with children's bodily fluids. Thus, further
2626 work on genomic sequencing analysis of HCMV shedding isolates in HCMV-seropositive
2627 women during pregnancy is recommended. This will provide valuable information on HCMV
2628 strains associated with shedding, which could contribute towards therapeutic or
2629 preventative strategies of maternal HCMV and cCMV infection. Development of viral
2630 genomic sequencing has many challenges. For example, methods to reduce the presence of
2631 host DNA and to remove microbial DNA are required and methods to optimise the quality
2632 and quantity of extracted HCMV DNA should be explored (Bachmann *et al.*, 2018). This will

allow us to sequence many more HCMV genomes from diverse samples and not encounter the technical limitations faced in this study.

7.3. Final comments

Non-primary maternal HCMV infection in a HCMV-seropositive woman, although a relatively mild disease, has the potential to transmit CMV infection to her unborn infant, a potentially severe disease with far reaching consequences to the infant and the family. HCMV shedding in bodily fluids of HCMV-seropositive pregnant women may suggest that she has developed non-primary maternal infection, and aid in identifying those at higher risk of having an infant with cCMV infection. HCMV shedding has the potential to be a proxy of vertical transmission, which could impact clinical management. Assessment of HCMV shedding alongside HCMV-specific CMI in HCMV seropositive women during pregnancy may be a valuable endpoint for the evaluation of preventative and therapeutic strategies against maternal HCMV and cCMV infection.

8. References

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3010 **Appendix A**

The prevalence of cytomegalovirus (CMV) shedding in CMV-seropositive pregnant women: a systematic review

Shari Sapuan, Anastasia Theodosiou, Christine Jones

Citation

Shari Sapuan, Anastasia Theodosiou, Christine Jones. *The prevalence of cytomegalovirus (CMV) shedding in CMV-seropositive pregnant women: a systematic review*. PROSPERO 2021 CRD42021275205 Available from:

https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42021275205

Review question

To systematically review the prevalence of cytomegalovirus (CMV) shedding in pregnant women who are CMV-seropositive.

Searches

The following electronic bibliographic databases will be searched: MEDLINE, EMBASE and Web of Science Core Collection. The following search terms will be used in different combinations: cytomegalovirus, CMV, shedding, excretion, replication, DNA, PCR, culture, seropositive, IgG, pregnant, maternal. These search terms only include terms relating to or describing the review condition, context, and population. The search terms will be adapted for use with other bibliographic database. There will be no language and publication period restrictions. The searches will be re-run just before the final analyses and further studies retrieved for inclusion.

Types of study to be included

Included: observational studies including cohort, cross-sectional and case-control studies.

Excluded: experimental studies, case reports, case series, review articles, conference abstract and unpublished studies.

Condition or domain being studied

Following an acute infection with cytomegalovirus (CMV) known as primary CMV infection, CMV can establish a life-long latency in an infected individual and may reactivate known as non-primary CMV infection, which could also occur from a reinfection with a different CMV strain. At any stage of the infection, it could result in excretion of CMV in the individual's bodily fluids, known as 'shedding', where another individual can become infected with CMV through contact with these infected bodily fluids shedding CMV. CMV can infect any individuals, but the extent of the disease depends on the status of the individual's immune response, where most immune-competent individuals, including pregnant women, will have mild or no symptoms, but it can cause severe symptoms in immune-incompetent individuals, including infants when contracted through the vertical transmission of CMV from women during pregnancy or delivery, known as congenital CMV infection.

Participants/population

Include studies involving pregnant women of any age and ethnicity, at any trimester, in any country. Include studies involving pregnant women up to 6 months post-birth if data were collected from within pregnancy. Exclude studies of women where data were only collected after birth. Exclude studies where only pregnant women with any known or suspected immunosuppressive, immunocompromised or immunodeficient condition or status (e.g., HIV-positive, transplant recipient, on immunosuppressive therapy) were included.

Intervention(s), exposure(s)

As this is a systematic review of observational epidemiological studies reporting prevalence data, the 'exposure' for this review refers to the 'condition' or variable of interest as part of the mnemonic 'CoCoPop' (Condition, Context, Population), which is CMV shedding. CMV shedding is defined as the detection of CMV in any bodily fluids (e.g., saliva, urine, vaginal secretions, cervical secretion, blood, breast milk) by culture or CMV DNA detected by polymerase chain reaction (PCR). CMV shedding could also be described as CMV excretion or CMV replication. Only include studies with any of the possible definitions and descriptions of CMV shedding in any maternal bodily fluids. Exclude studies where only CMV shedding in foetal bodily fluids are looked at (e.g., only amniotic fluid was looked at due to suspicion or confirmation of congenital CMV infection in the foetus or infant).

Comparator(s)/control

Not applicable

Context

Include studies where the pregnant women are CMV-seropositive (defined as the detection of CMV-specific immunoglobulin G (CMV IgG) in the serum from performing a CMV serology) and was established prior to or at the beginning of data collection for CMV shedding. Exclude studies where only CMV-specific serological blood test suggestive of primary maternal CMV infection were included (i.e., women who seroconvert from non-detected to detected CMV IgG in pregnancy with or without detected CMV-specific immunoglobulin M (CMV IgM), or low CMV IgG avidity and detected CMV-IgM).

Main outcome(s)

Prevalence estimates of CMV shedding in CMV-seropositive pregnant women.

Additional outcome(s)

Epidemiological distributions of CMV shedding in CMV-seropositive pregnant women.

Data extraction (selection and coding)

Step 1. One reviewer will perform the search according to the search strategy outlined in this protocol to identify the studies.

Step 2. Titles and abstracts of the studies retrieved from the search will be screened independently by two reviewers to identify studies that potentially meet the inclusion and exclusion criteria outlined in the protocol.

Step 3. Any disagreement between the two reviewers over the eligibility of abstracts will be resolved through discussion with a third reviewer.

Step 4. Full-text articles of abstracts selected following consensus from all reviewers, will be reviewed independently by two reviewers to identify studies that meet the inclusion and exclusion criteria outlined in the protocol.

Step 5. Any disagreement between the two reviewers over the eligibility of full articles will be resolved through discussion with a third reviewer.

Step 6. Data will be extracted from the selected full-text articles by the first reviewer.

Step 7. Extracted data will be reviewed by the second reviewer.

Step 8. Any disagreements between the two reviewers over the extracted data will be discussed with a third reviewer.

A standardised, pre-piloted form will be used to extract data from the included studies for data synthesis. Extracted information will include: study design; study setting; study population and participant demographics and baseline characteristics; details of the condition; details of the context; study methodology; outcomes and times of measurement; information for assessment of the risk of bias. Data extraction will be performed once.

To determine pooled estimates, each study contributed with only one estimate. Whenever a study generated multiple estimates according to different diagnostic or methodological definitions, one estimate was selected according to a defined protocol to ensure that data on each subject were extracted only once.

Risk of bias (quality) assessment

The Joanna Briggs Institute (https://jbi.global/sites/default/files/2019-05/JBI_Critical_Appraisal-Checklist_for_Prevalence_Studies2017_0.pdf) will be used by all reviewers to select the studies for Steps 4-6 of the data extraction process, to assess the methodological quality of the full articles included in the review and to determine the extent to which the studies have addressed the possibility of bias in its design, conduct and analysis. The results of this appraisal will be used to inform synthesis and interpretation of the results of the study.

Strategy for data synthesis

A narrative synthesis of the included studies, structured around the characteristics of the condition, population and context specified for this review will be provided. Cumulative graphs or forest plots (with or without a pooled estimate) will be provided to display the epidemiological distribution of the prevalence data found from the included studies. If suitable, meta-analysis to determine a single 'true' estimate of the prevalence (of the condition specified for this review) and its variance across studies will be conducted, by performing a standardised recalculation of rates from the studies to generate a distribution allowing the estimation with some probability the extent to which the populations differ. A summary of the study design will also be provided.

Analysis of subgroups or subsets

None planned.

Contact details for further information

Shari Sapuan
[REDACTED]

Organisational affiliation of the review

St George's University of London

Review team members and their organisational affiliations

Dr Shari Sapuan. St George's University of London

Dr Anastasia Theodosiou. University of Southampton

Dr Christine Jones. University of Southampton

Collaborators

Professor Paul Heath. St George's University of London

Type and method of review

Epidemiologic, Meta-analysis, Narrative synthesis, Systematic review

Anticipated or actual start date

01 September 2021

Anticipated completion date

01 February 2022

Funding sources/sponsors

None.

Conflicts of interest

None.

Language

English

Country

England

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3015 **Appendix B**

This patient is pregnant and participating in the RACE-FIT study.

Please test for CMV IgG and if positive for IgG avidity and IgM.

If there are any problems with the sample please contact
the research team on 07920158212 or 02087253887.

Many thanks.

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3030 **Appendix C**

This person has consented to have their CMV serology tested as part of CMV research (RACE-FIT study, REC ref:18/SC/0360 and cCHIPS study, REC ref: 19/LO/0161)

Date of blood sampling:.....

The test results will be communicated directly to the patient.

Signed..... Name..... Date/...../.....

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Study Title:	RACE FIT/cCHIPS
Protocol number:	RACE FIT: Version XX, XX.XX.XX cCHIPS: Version XX, XX.XX.XX
Study Sponsor:	RACE FIT: St George's University Hospitals NHS Foundation Trust cCHIPS: St George's, University of London
REC reference	RACE FIT: 18/SC/0360 cCHIPS: 19/LO/0161
IRAS number	RACE FIT: 239977 cCHIPS: 257710



St George's University Hospitals NHS Foundation Trust and St George's University of London, would like to invite you to have a blood test for a virus called cytomegalovirus (CMV) at the same time as your routine 'combined screening' blood tests. This is being done to allow us to identify whether you have had the CMV virus before so that we can contact you about relevant research investigating CMV in pregnancy.

CMV is a common virus. In healthy adults, CMV causes mild illness and most people don't know if they have had CMV before. However, if a woman catches CMV for the first time when she is pregnant or if the CMV virus reactivates or she is infected with another strain of the virus, there is a chance that the infection can be passed on to her baby. In a small number of cases, this can cause problems in the baby, such as problems with hearing or development. The chance of passing CMV infection to the baby is lower if a woman has had CMV infection before pregnancy.

CMV is the most common infection passed from mother to baby and there is currently no vaccine available to protect women and their infants. We are conducting two research studies investigating CMV in pregnancy. The first of these involves women who have never had the virus before. These women will be invited to take part in a study exploring if an educational film in early pregnancy leads to changes in the day to day activities of women and whether this reduces the risk of getting CMV for the first time in pregnancy. The second study will involve women who have had CMV infection before and will explore the characteristics of the CMV virus to better understand the effects it can have in pregnancy.

If you have a child less than four years of age, we would like to invite you to have your blood checked for evidence of previous CMV infection or active (current) infection. This will be done by testing a sample of blood taken with other routine blood tests in early pregnancy. We will collect an extra 5 mls (about a teaspoon). You do not have to take part in this study and have your blood tested. If you choose not to have this extra test done, it will not affect the care you receive and you will still have the routine blood tests performed.

If you have active infection, the risk to the baby is still low, but we will refer you to a doctor with expertise in this area so that the best care can be provided for you and your baby. If you do not have active infection you will be informed of your results and may be contacted about involvement in further research. **At this stage we are asking for your consent to test for active or past CMV infection by taking a small extra amount of blood - we are not asking for your consent to take part in further research.**

St George's University Hospitals NHS Foundation Trust is the sponsor for this study based in the United Kingdom. We will be using information from you in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. St George's University Hospitals NHS Foundation Trust will keep identifiable information about you until we have reviewed the results from your blood test and contacted you about this, including giving you information about further research if that is appropriate. Your rights to access, change or move your

information are limited, as we need to manage your information in specific ways in order for the research to be reliable and accurate. If you withdraw from the study, we will keep the information about you that we have already obtained. To safeguard your rights, we will use the minimum personally-identifiable information possible. St George's University Hospitals NHS Foundation Trust will use your name and contact details to contact you about your blood results including, if appropriate, information about research you could be involved in, and make sure that relevant information about the study is recorded for your care, and to oversee the quality of the study. Individuals from St George's University Hospitals NHS Foundation Trust and regulatory organisations may look at your medical and research records to check the accuracy of the research study. The only people in St George's University Hospitals NHS Foundation Trust who will have access to information that identifies you will be people who need to contact you to communicate your blood results or inform you about further research or audit the data collection process.

St George's University Hospitals NHS Foundation Trust will keep a copy of the consent form which will include your name, for 10 years after the study has finished, but will not retain your contact details after you have been informed of the blood test results.

You can find out more about how we use your information here:

<https://www.stgeorges.nhs.uk/about/privacy-notice/>

For general information on how the NHS uses research data please visit:

<https://www.hra.nhs.uk/information-about-patients/>

If you would like to read more about CMV there is information available on the NHS choices website

(<https://www.nhs.uk/conditions/cytomegalovirus-cmv/>)

Chief Investigator for RACE FIT: Dr Chrissie Jones (Associate Professor Paediatric Infectious Diseases)

Principal Investigator for RACE FIT: Dr Anna Calvert (Research Fellow)

Chief Investigator for cCHIPS: Prof Paul Heath (Professor in Paediatric Infectious Diseases)

Principal Investigator for cCHIPS: Dr Shari Sapuan (Research Fellow)



Contact details

Patient addressograph label

EDD: _____

Planned date of 12/40 scan: _____

Phone number: _____

Email address: _____

Name of GP practice: _____

Address of GP practice (if known): _____

Consent	Please initial
I _____ have read and considered the information above and I am happy to have my blood tested for active or past CMV infection.	
I understand that this blood sample will be stored for two years for my clinical care.	
I understand that my contact details will be kept by the study team to allow them to contact me with the results of my blood test and provide information about further research studies and that my consent form will be kept for ten years.	
I understand that if I am found to have active infection I will be referred to a doctor with expertise in this area and my GP will be informed.	
I consent to providing details of my ethnicity to the study team which will be stored in an anonymous database with my serology results.	
OPTIONAL: I consent for my sample to be used for other ethically approved research.	
OPTIONAL: I consent to being sent a questionnaire by the study team to ask for my feedback about the study process.	

Signed: Date: Name (block capitals):

ARE YOU PREGNANT?

Would you like to be tested for cytomegalovirus (CMV)?

CMV can cause problems for a developing baby if a baby catches the infection in pregnancy



We are carrying out two studies to investigate CMV in pregnancy.

We are inviting women to have their blood tested for CMV at the same time as routine bloods in early pregnancy.

Women who have never had CMV infection before will be asked if they would like to take part in a study to investigate if an educational film can reduce the chance of pregnant women catching CMV.

Women who have had CMV infection before will be asked if they would like to take part in a study to explore the characteristics of the virus to better understand the effects it can have in pregnancy.

PLEASE CONTACT US TO HEAR MORE:
<INSERT CONTACT NAME(S) AND PHONE NUMBER(S)>
VACCINESFORCHILDREN@SGUL.AC.UK





The RACE-FIT study was funded by the NIHR Research for Patient Benefit Programme and the cCHIPS study was funded by the MRC Investigator Studies Programme. The views expressed are those of the author(s) and not of the NHS, the Department of Health, the NIHR or the MRC Investigator.

Combined RACE-FIT and cCHIPS_poster_V3.0_5/11/18

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Study Title:	cCHIPS
Protocol number:	Version 3.0, 25/03/2020
Study Sponsor:	St George's, University of London
REC reference	19/LQ/0161
IRAS number	257710

Thank you for allowing us to test a sample of your blood when you had your early pregnancy 'combined screening' blood tests.

The results of this blood test show that **you have previously been infected with the CMV virus** - this is sometimes referred to as being 'seropositive'. About half of the general population is 'seropositive' and this is very unlikely to cause any problems during your pregnancy. Although you have immunity against one strain of the virus, it is possible for this infection to reactivate or for you to be infected with a different strain of the virus.

St George's University of London and St George's University Hospitals NHS Foundation Trust are inviting pregnant women who are CMV seropositive to take part in a study, called the cCHIPS study. This study will explore the characteristics of the CMV virus during pregnancy in women who have had CMV before in order to better understand the effects it can have in pregnancy and identify strategies that might help prevent CMV infection in babies in the future.

If you are interested in participating or would like to find out more about the cCHIPS study or your blood test results, please contact a member of the study team using the contact information provided below. A member of the study team will also contact you to provide you with further information and can answer any questions about the study or your blood test results.

If you would like to read more about CMV there is information available on the NHS choices website:
<https://www.nhs.uk/conditions/cytomegalovirus-cmv/>

Thank you for having participated in the first step of this important study, which we hope will provide information to help us prevent CMV infection in babies.

Yours sincerely,

Chief Investigator for cCHIPS: Prof Paul Heath (Professor in Paediatric Infectious Diseases)
Principal Investigator for cCHIPS: Dr Shari Sapuan (Research Fellow)
Ms Asma Khalil, Consultant Obstetrician specialising in Maternal and Fetal Medicine
Robin Parsons, Research Midwife

Telephone number: 0208725 3887 / 0208725 4851
Email: vaccinesforchildren@sgul.ac.uk

cCHIPS_seropositive letter to women
V1.0: 05/12/18

St George's University Hospitals

NHS

NHS Foundation Trust





cytomegalovirus shedding characteristics
in pregnant women

Study Title:	cCHIPS
Protocol number:	Version 3.0, 25/03/2020
Study Sponsor:	St George's, University of London
REC reference:	19/LQ/0161
IRAS reference:	257710

Dear Doctor,

Re: Name: _____ Date of Birth: ____ / ____ / ____

 NHS number: _____

This patient, registered at your practice, consented to have her CMV serostatus checked as part of screening for participation in clinical research being conducted by St George's University Hospitals NHS Foundation Trust and St George's University of London.

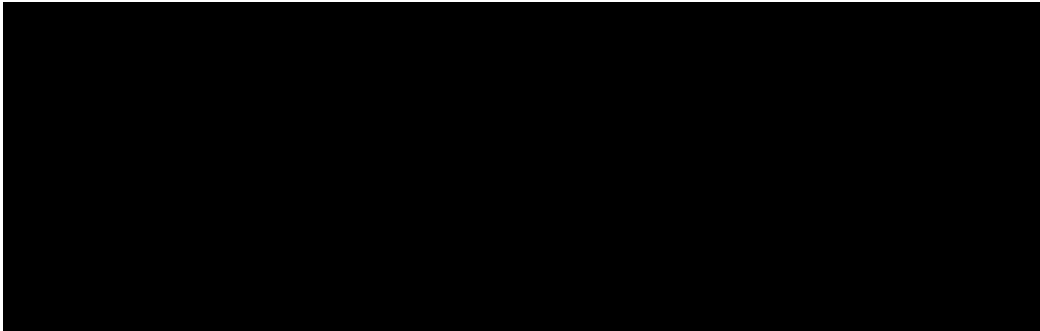
We are inviting women in early pregnancy, who have children less than 4 years old, to have their serology checked for CMV. Based on the serology results we are asking women if they would like to take part in one of our research studies investigating CMV. If women have evidence of past CMV infection (IgG positive with high avidity [suggesting infection more than 3 months ago] and IgM negative), we will invite them to participate in the cCHIPS study, which aims to recruit 200 CMV seropositive women to explore the characteristics of the CMV virus during pregnancy in seropositive women, in order to better understand the effects it can have in pregnancy and identify ways that it might be possible to prevent vertical transmission.

Your patient was found to have evidence of past CMV infection (IgG positive with high avidity [suggesting infection more than 3 months ago] and IgM negative). We have provided information to her about her CMV test results and informed her that whilst re-infection with a different strain or reactivation are unlikely, this can happen.

This means that she is eligible to take part in the cCHIPS study. We have made her aware about the cCHIPS study and a member of the study will make contact with her to provide her with further information and invite her to participate. We are writing to you to ensure that you have the necessary information about your patient's serostatus if she approaches you with any questions or concerns about this result.

If you would like any further information about the study or this result, please do not hesitate to contact a member of the study team.

Yours sincerely,



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Informed Consent Form

Study Title:	cCHIPS
Protocol number:	Version 3.0, 25.03.20
Study Sponsor:	St George's, University of London
REC reference:	19/L0/0161
IRAS number:	257710

Participant number: _____

Participant name: _____

DOB: __/__/____

	Please initial
I have read and considered the Participant Information Sheet (version 2.0 dated 08/02/2019) about the cCHIPS study. It provided enough information so that I understand what is involved and any questions I had about the study have been answered to my satisfaction.	
I consent to my GP being informed about my participation in this study. I agree that if the study team is unable to contact me during this study they may ask my GP for my contact details and if I change my GP they may use my NHS number to find details of my new GP.	
I understand that the researchers, the study sponsor, regulatory authorities or my NHS Trust may look at relevant sections of medical notes and data collected during the study. I give permission for these individuals to have access to these notes where it is relevant to my participation in this research.	
I agree to the processing of my personal data as explained in the section on "How will my personal data be handled?" of the Participant Information Sheet (version 2.0, dated 08/02/2019), which I have read and understood.	
I agree to be contacted by the study team to remind me of the study visits.	
I agree to perform the self-sampling of saliva, urine and vaginal secretions as instructed on the Self-sampling Instructions (version 1.0, dated 19/12/18) for each visit, and for my samples to be tested as explained in the section on "What would happen to any samples?" of the Participant Information Sheet (version 2.0, dated 08/02/2019).	
I agree to complete the Contact Questionnaire for each visit. I also agree to complete the Background Questionnaire provided to me at the initial visit and the Feedback Questionnaire at the last visit.	
I agree to contact the study team to let them know that I have come to the hospital to have my baby or soon after delivering my baby.	
I understand that at the end of the study I might be invited to take part in an interview with the study team that will be audio recorded and that I can decline this invitation.	
I understand that participation is voluntary and that I may withdraw from the study at any time without having to give a reason and that this will not affect my routine care.	
I agree to take part in this study.	
OPTIONAL: I agree to the collection of my blood sample at each study visit to be tested as explained in the section on "What would happen to any samples?" of the Participant Information Sheet (version 2.0, dated 08/02/2019). [You can still take part in the study if you do not give consent for this. If you do not consent to this, please strike through and do NOT initial this section]	
OPTIONAL: I agree that any of my remaining samples may be used for further ethically approved research to help improve the understanding of infections. I understand that the sample will be used anonymously and it will not be possible to link the results of any tests back to me. [You can still take part in the study if you do not give consent for this. If you do not consent to this, please strike through and do NOT initial this section]	
OPTIONAL: I agree to the collection of a saliva swab from my baby to test him/her for congenital CMV infection and be contacted by the study team to inform me of the results. If my baby's saliva swab test was suggestive of congenital CMV infection, I agree for the study team to refer my baby to a children's specialist and that my GP will be informed of this referral. [You can still take part in the study if you do not give consent for this. If you do not consent to this, please strike through and do NOT initial this section]	
OPTIONAL: I agree that remaining sample from my baby may be used for further ethically approved research to help improve understanding of infections. I understand that the sample will be used anonymously and it will not be possible to link the results of any tests back to my baby. [You can still take part in the study if you do not give consent for this. If you do not consent to this, please strike through and do NOT initial this section]	
Signed: _____ Date: _____	
Name (block capitals): _____	
I have explained and discussed the study with the above participant. I have answered all her questions regarding the study and I am satisfied that the above signature denotes her informed consent to take part in the trial.	
Signature of Study Doctor/Nurse/Midwife: _____ Date: _____	
Name (block capitals): _____	

When completed, 1 copy for participant's family, 1 copy for researcher site file, 1 for GP.

cCHIPS_Informed Consent Form_V2.0, dated 08/02/19



Study Title:	cCHIPS
Protocol number	Version 3.0, 25.03.20
Study Sponsor	St George's, University of London
REC reference	19/LO/0161
IRAS number	257710

Participant Information Sheet

St George's, University of London, in collaboration with St George's University Hospitals NHS Foundation Trust, is conducting a study called *cytomegalovirus shedding characteristics in pregnant women*, better known as the cCHIPS study, to explore the characteristics of cytomegalovirus (CMV) shedding during pregnancy in women who have previously been infected with CMV, known as being 'seropositive', in order to understand better the effects it has in pregnancy and identify ways that might be able to help prevent CMV infection in babies in the future.

Before you decide if you would like to join this study, you need to understand the reasons why this study is being done and what it would involve. Please take the time to read the following information carefully and talk to others about the study if you wish.

In summary:

- The study involves four study visits; early in pregnancy, middle of pregnancy, late in pregnancy and after birth. As much as possible these visits will be coincided with your routine antenatal appointments.
- At each visit, you will be taking your own samples of urine, saliva and vaginal secretions to check how much CMV (if any) is excreted in these samples. We will provide you with simple instructions on how to do this.
- If you agree to it, a blood sample will also be collected at each visit to look at your immune response; as much as possible this will be performed at the same time as your other routine antenatal blood tests.
- At each visit, you will also complete a short questionnaire on the contact you've had with your child(ren)'s saliva and urine.

Why is this study being performed?

Cytomegalovirus, better known as CMV, is a common virus, which in most healthy adults and children causes only a mild illness, like getting a "cold". After being infected with CMV, the person can sometimes excrete or 'shed' the CMV virus into his or her body fluids, such as urine, saliva and vaginal secretions, for a variable amount of time without any obvious signs and without causing any visible symptoms.

If a woman acquires CMV infection when she is pregnant there is a chance that the infection can be passed on to her baby, known as congenital CMV infection. In a small number of cases, this can cause problems in the baby, such as deafness or developmental delay. Overall, CMV

is the most common infection passed from mothers to babies (called a congenital infection), and there is currently no vaccine available to protect women and their babies against CMV.

Congenital CMV infection is much more likely to happen if a woman acquires CMV infection for the first time when she is pregnant. However, there is also a small chance that congenital CMV infection can happen in a woman who has previously been infected with CMV i.e. those who are 'CMV seropositive'. This can happen if she gets the infection again, but with a different CMV strain (type), or if the CMV strain she had previously becomes active again.

We know relatively little about CMV shedding during pregnancy in seropositive women, such as how much, how often, which bodily fluid and when in pregnancy CMV shedding occurs, and what its association is with congenital CMV. We also do not know if there is any association between CMV shedding and the level of immunity (protection) against CMV in the pregnant woman. The more we understand about CMV shedding and how it affects seropositive women in pregnancy, the more likely we are to identify ways that might help prevent congenital CMV infection in the future.

Why have I been chosen to take part and do I have to take part?

We are approaching you because you are pregnant, have a child aged less than four years of age and your blood test result shows that you have had CMV infection before (sometimes called CMV seropositive). We are approaching women with children less than four years because these children frequently become infected with CMV; this does not cause harm to them, but this means that you are more likely to be exposed to CMV compared to women who do not have young children.

You do not have to take part in the study and if you do choose to take part you would be able to withdraw from the study at any time and without giving a reason. If you do not wish to take part in this study or withdraw from the study before the end, your routine antenatal or other medical care will not be affected in any way. Your routine antenatal care team will still be responsible for your antenatal care throughout your pregnancy if you decide to take part.

What happens if I take part?

If you agree to participate we will arrange a convenient time for you to come to St George's Hospital for the first study visit ('Visit 1'). If this is not possible, we will arrange a suitable time to see you at your home.

At this visit we will discuss the study with you and will ask you to sign a consent form.

After this, we will ask you to complete a questionnaire called 'Background Questionnaire' on an electronic hand-held device or on paper form which should take about 5 to 10 minutes to collect information on your background including your sociodemographic details, your awareness of CMV, your health and your pregnancy. This is so that we can identify if there might be an association between CMV shedding and any of this information.

You will also be asked to complete a questionnaire called 'Contact Questionnaire' on the contact you have had with your child(ren) in the preceding two weeks which should take about 5 minutes on an electronic hand-held device or on paper form, to identify if the type of contact you have had with your child(ren)'s saliva and urine have any association with CMV shedding.

We will then go through the simple written instructions on how to perform self-sampling of saliva, urine and vaginal secretions which you will need to do for each study visit. We will collect the samples from you to check how much CMV (if any) is excreted in these samples. We will not need to supervise you in collecting these samples.

If you have agreed to blood sampling, we will collect around 12mls (about two teaspoons) of your blood at the same time. The blood sample is to look at the level of your immunity against CMV, called CMV-specific T cell immune responses. You will be provided with information about how to contact the study team.

At the second and third study visits ('Visit 2' and 'Visit 3'), the self-sampling of saliva, urine and vaginal secretions and blood sampling will be as for the first study visit. You will also complete the 'Contact Questionnaire' which will be emailed to you a day in advance. If email is not convenient for you, we can post you the questionnaire in paper form and you can return it to us via the enclosed return envelope. A text reminder will be sent to you the day before the pre-arranged second and third visits to remind you of the visit date, to complete the questionnaire emailed to you, and to perform the self-sampling of saliva, urine and vaginal secretions on the visit date.

The second study visit will be aimed to coincide with your routine 20 week antenatal appointment to make it most convenient for you. You can either perform the self-sampling at home before you come to this appointment, or at the appointment venue itself, and leave them with your routine antenatal team; or if it had been pre-arranged for the study team to see you we will take the samples directly from you. You will need to complete the questionnaire in your own time on the date of the second study visit, as this will be emailed to you (or posted) directly.

Similarly for the third study visit, we will aim to coincide this with your routine 28 week antenatal appointment. You will have a routine NHS blood test performed at this appointment and we hope to collect the study blood sample at the same time. The procedures and processes will be the same as has been described for your second study visit.

If the second and third study visits cannot be coincided with your routine antenatal appointments, we will arrange a convenient time for you to come to St George's Hospital. If this is not possible, we will arrange a suitable time to see you at your home.

For the fourth study visit ('Visit 4'), the study team will aim to see you on the labour or postnatal wards, if this is convenient for you. Again, we will try to arrange for your study blood sample to be collected at the same time as your routine blood sample. The self-sampling of saliva, urine and vaginal secretions, and the questionnaire on the contacts you have had with your child(ren), will be the same as for the other study visits. We will also ask you to complete a study feedback questionnaire called 'Feedback Questionnaire' which should take about 10 minutes on an electronic hand-held device or on paper form.

As part of the fourth study visit, we will also offer to test your baby for congenital CMV infection by taking a saliva swab from your baby. As you have previously been infected with CMV, there is a low likelihood that your baby will have congenital CMV and this is not part of routine NHS care. Your baby's routine care will not be affected in any way whether you choose for your baby to be tested for congenital CMV infection or not. If your baby is found to have congenital CMV infection, we will ensure that your baby is referred to a children's doctor specialising in infections so that your baby can be checked and the doctor will be able to discuss with you what management is appropriate and whether your baby needs treatment.

If the fourth study visit cannot be performed while you are still in hospital, we will arrange a convenient time for you and your baby to come to St George's Hospital within the first seven days of life. If this is not possible, we will arrange a suitable time to see you and your baby at your home.

You may also be invited to take part in a process evaluation at the end of the study in the form of an interview by phone, skype or face to face at your convenience. This is so that we can explore in depth your experiences of participation in the study.

Visit No.	Visit 1	Visit 2	Visit 3	Visit 4
Visit Schedules	As soon as possible up to 16 weeks and 6 days gestational age (early in pregnancy)	20 gestational weeks antenatal appointment or in 2 nd trimester from 17 weeks and 0 days to 27 weeks and 6 days gestational age (middle of pregnancy)	28 gestational weeks antenatal appointment or in 3 rd trimester from 28 weeks and 0 days onwards (late in pregnancy)	Labour/postnatal ward after delivery or from the time of delivery to 7 th day after delivery
Visit Procedures	•Background questionnaire •Contact questionnaire •Self-sampling of saliva, urine & vaginal secretions •+/- blood sample	•Contact questionnaire •Self-sampling of saliva, urine & vaginal secretions •+/- blood sample	•Contact questionnaire •Self-sampling of saliva, urine & vaginal secretions •+/- blood sample	•Contact questionnaire •Feedback questionnaire •Self-sampling of saliva, urine & vaginal secretions •+/- blood sample •+/- saliva sample of newborn

The diagram above summarises what will happen in the study.

What are the possible disadvantages of taking part?

This study involves taking samples of saliva, urine and vaginal secretions and if possible blood tests, if you agree to them. Blood tests can be uncomfortable, but they will be done by a member of the study or clinical team with experience in taking blood tests to minimise any discomfort, and efforts will be made to coincide them with your routine antenatal blood tests. The self-sampling of saliva, urine and vaginal secretions will also be quick and minimally invasive and the instructions provided will be easy to follow. You will be required to attend four study visits and to complete one to two questionnaires for each visit. We will always try to arrange study visits to coincide with your routine antenatal appointments or at a time convenient for you.

What are the possible benefits of taking part?

All newborn babies of women participating in the study will be offered testing to see if they have congenital CMV infection. In the unlikely event that your baby has congenital CMV infection, we will be able to refer your baby in a timely manner to an appropriate children's doctor with expertise in infection, to ensure that he or she receives the appropriate management as soon as possible. Currently, babies are not routinely screened after birth for CMV infection in the NHS and they would only be tested if any problems were picked up later on. If babies are found to be infected and need treatment, this should usually be started before they are one month old. This study allows any babies with infection to be tested and if needed, to be given treatment as early as possible.

There may not be any direct benefit to you, however you will contribute to research that will give us a better understanding of CMV infection in mothers and their babies and may point to ways that might help to prevent congenital CMV infection in babies in the future.

What are the side effects or harm when taking part?

We do not anticipate any side effects or harm from your participation in this study. If you have concerns regarding CMV infection during participation in this study, please discuss this with the study team. You will also be able to discuss with your routine antenatal care team who will still be responsible for your antenatal care throughout your pregnancy where your routine medical care will not be affected in any way.

What happens when the research stops or if new information becomes available?

If new or relevant information becomes available during the course of the study, we will inform you as soon as possible.

What if there is a problem?

St George's, University of London is the study sponsor and holds insurance to cover participants for injury caused by their participation in this study. If something does go wrong and you are harmed during the study because of someone's negligence, then you may have grounds for legal action for compensation, but you may have to pay your legal costs. The National Health Service complaints mechanism will still be available to you.

What if you wish to complain about the trial?

If you have any concerns about the way you have been treated during this study or wish to make a complaint, then you can talk to the cCHIPS study team who will do their best to answer your questions or concerns (contact details at the end). Alternatively, you may contact the Patient Advice and Liaison Service:

Phone number: 02087252453

E-mail: pals@stgeorges.nhs.uk

The National Health Service complaints mechanisms are also available to you.

How will my personal data be handled?

St George's, University of London is the sponsor for this study and is based in the United Kingdom. We will be using information from your medical records in order to undertake this study and will act as the data controller for this study. This means that we are responsible for looking after your information and using it properly. Personal data collected will include your name, date of birth and address as well as your contact details, medical history and details of your pregnancy and results of your blood tests. This information will be stored on secure password protected NHS or University computers and will be kept until the study is over to allow us to make contact with you to explain the results of the study. After this point it will be destroyed. If you were invited and took part in the process evaluation interview at the end of the study, this interview will be audio recorded. The recording will start after introductions have been done and will only contain your first name. These recordings will be stored securely on NHS or University computers before being transcribed. After transcription the audio files will be destroyed. St George's University Hospitals NHS Foundation Trust collaborates closely with St George's, University of London and often shares research information. The only people with access to this research information will be members of the research team across both the university and hospital, your GP, the sponsors and or regulatory authorities who may wish to check the study is being carried out within the appropriate guidelines. This will be after you consent to be included in the study. St George's University Hospitals NHS Foundation Trust may keep information collected for the purpose of the study for up to 5 years after the study has finished. This is to ensure integrity of the results. All data will be stored in a secure manner.

The data will only be used for the purposes of this study and any data released outside the above group will not contain any information that could be traced back to you.

Your rights to access, change or move your information are limited, as we need to manage your research information in specific ways in order for the research to be reliable and accurate. If you withdraw your consent to participate in a research project, this will not mean we will have to remove all data as well. We will keep the information about you that we have already obtained to ensure research integrity is maintained in the public's interest. In the event that you lose capacity to consent during the study, you would be withdrawn from the study. The research team would retain tissue and personal data collected and continue to use it confidentially in connection with the purposes for which consent was sought prior to the loss of capacity. To safeguard your rights, we will use the minimum personally-identifiable information possible.

You can find out more about how we use your information here:
<https://www.stgeorges.nhs.uk/about/privacy-notice/>

For general information on how the NHS uses research data please visit:
<https://www.hra.nhs.uk/information-about-patients/>

What would happen to any samples?

We will send all the study samples to the laboratory at St George's Hospital where they will be tested; the saliva, urine and vaginal secretion samples will be tested for presence of CMV virus and the amount present (CMV DNA), the blood samples will be tested for immunity level against CMV (CMV-specific T cell immune responses). Once the study tests are complete, there may be small amounts of samples remaining from you. We would like your permission to use any of your samples remaining after these tests for future ethically approved research to help improve our understanding of infections. You will be asked to consent for this separately in the consent form. If you are not happy for your left over samples to be used for any other tests, please cross out the relevant section in the consent form and the samples will be destroyed according to routine practice after all study tests are completed.

If you agree for your baby to have a saliva sample performed to test for congenital CMV, this will be tested for the presence of CMV virus and the amount present (CMV DNA).

What would happen to the results of the research study?

We will write to you at the end of the study to inform you of our findings. This will be around 12 months after the end of the study. This may be some time after your participation in the study has ended. We will retain your contact details in a secure database at the site to enable us to contact you. We plan to publish the results (which will not contain any of your identifiable details and this cannot be traced back to you) in medical journals so that other doctors can learn about the findings of the study. If you wish, you can also be sent a copy of the published research.

The intellectual property that we anticipate to generate through this study is all information based, which might identify ways to help prevent congenital CMV infection in the future. All Intellectual Property Rights and Know How (IP) related to this study are and shall remain the property of the Study Sponsor, St George's University of London, excluding pre-existing IP related to clinical procedures of any hospital and pre-existing IP related to analytical procedures of any external laboratory.

Who is organising and funding the research?

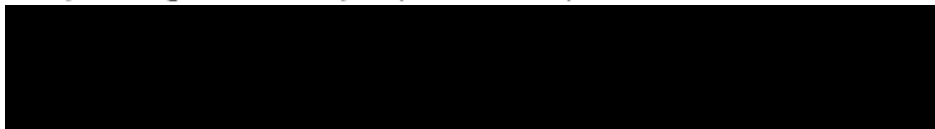
The study is funded by Merck Sharp & Dohme (MSD) as part of the Merck Investigator Studies Program (MISP). MSD has no role in the design, collection, management, analysis and interpretation of the study results, writing of report or the decision to submit for publication. MSD will not have access to your identifiable information and any study information shared with MSD such as the study report will not contain any identifiable information. All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect the interests of donors/participants. This study has been reviewed and given favourable opinion by London – Brent Research Ethics Committee, as well as approval by the Health Research Authority and St George's Joint Research and Enterprise Service.

Further information

If you have any questions, or would like further information please contact:

Chief Investigator: Prof Paul Heath (Professor in Paediatric Infectious Diseases)

Principal Investigator: Dr Shari Sapuan (Research Fellow)



If you would like to read more about CMV there is information available on the NHS choices website: <https://www.nhs.uk/conditions/cytomegalovirus-cmv/>

We do hope that you will take part in this study. Your contribution would allow us to have a better understanding of CMV infection and how it affects seropositive women in pregnancy, and may allow us to identify ways that can prevent congenital CMV infection in babies in the future.

Thank you for reading this leaflet and thinking about taking part in the study.

St George's University Hospitals 
NHS Foundation Trust





Study Title:	cCHIPS
Protocol number	cCHIPS: Version 3.0, 25.03.20
Study Sponsor	St George's, University of London
REC reference	19/L0/0161
IRAS number	257710

Notification of Study Participation

Date: __/__/____

Dear Doctor,

Re:

Name: _____

Date of Birth: __/__/____

NHS number: _____

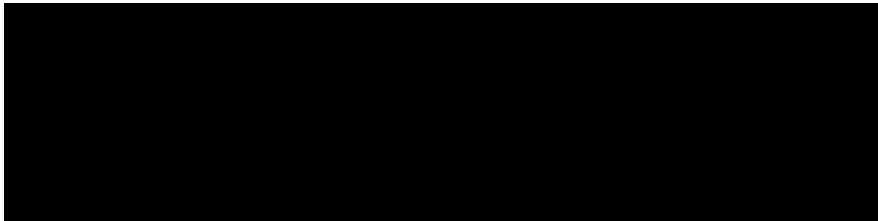
This patient, registered at your practice, has agreed to participate in the cCHIPS study. This study, conducted by St George's, University of London, in collaboration with St George's University Hospitals NHS Foundation Trust, is an observational, longitudinal, natural history, feasibility cohort study which aims to recruit 200 CMV seropositive women with children aged less than four years of age to explore the characteristics of the CMV virus during pregnancy in seropositive women, in order to understand better the effects it can have on pregnancy and identify ways that might be able to help prevent vertical transmission. CMV serology was tested along with the combined screening bloods at around 11 gestational weeks. The participant was found to be seropositive (IgG positive with high avidity [suggesting infection more than 3 months ago] and IgM negative).

The first study visit has taken place and I have enclosed a copy of the informed consent form for your records. There are four study visits in total (early in pregnancy, midway through pregnancy, late in pregnancy and postpartum) whereby at each visit the patient will complete a contact questionnaire and perform self-sampling of saliva, urine and vaginal secretions to be tested for CMV DNA, as well as a blood sample to be tested for CMV-specific cellular mediated immune responses if consent was obtained. At the last study visit, her newborn baby will also be offered to be screened for congenital CMV infection by performing a saliva swab. If there is suggestion of congenital CMV in her baby we will arrange the baby for appropriate follow up.

If you would like any further information about the study, or have any questions or concerns, please do not hesitate to contact us.

Many thanks.

Yours sincerely,



cCHIPS_letter to GP_study participation notification, V1.0, dated 19/12/18



Study Title:	cCHIPS
Protocol number	Version 2.0, dated 08/08/2019
Study Sponsor	St George's, University of London
REC reference	19/L0/0161
IRAS number	257710

Participant Self-Sampling Instructions

For each study visit, you will be required to take your own sample of saliva, urine and vaginal secretions. Please ensure you wash your hands before and after taking the samples. If you have any questions please contact the study team at 0208725 3887 / 0208725 4851 or email vaccinesforchildren@sgul.ac.uk.

Saliva	Urine
1. In the swab pack labelled 'Saliva', you will see a collector tube and a swab. Peel open the pouch. 2. Remove the swab from the pack holding onto the stick end above the score line. Do not touch the cotton end. 3. Insert dry swab into mouth on the inner cheek. Twist the swab a full circle against the inner cheek 2-3 times to collect enough saliva. 4. Carefully uncap the tube collector and avoid spillage of the fluid in it. Insert sampled swab into the collector tube, when the score line on the swab is at the opening of the tube, snap the swab on the side of the tube. Discard the remaining stick. Replace the cap onto the tube and screw it firmly closed. 5. Write your initials and the date of the sample. 	1. You will see a specimen pot labelled 'Urine'. 2. Remove the silver cap from the pot. Do not touch inside the pot. 3. Pass some urine into the toilet, then without stopping the flow of urine, pass urine directly into the pot (mid stream) half full. 4. Replace the silver cap onto the pot and screw it firmly closed. 5. Write your initials and the date of the sample.
Vagina 1. In the swab pack labelled 'Vagina', you will see a collector tube and two swabs; one thicker with cotton end, one thinner with brush end. Peel open the pouch. Discard the thinner swab with brush end. You will use the thicker swab with cotton end to collect your vaginal secretions sample. 2. Hold the swab on the stick end above the score line. Do not touch the cotton end. 3. Insert dry swab around 5cm into vagina (like using a tampon but not as far in). Twist the swab gently for about 30 seconds while rubbing the swab against the vagina walls. Remove swab from the vagina carefully. 4. Carefully uncap the collector tube and avoid spillage of the fluid in it. Insert sampled swab into the collector tube, when the score line on the swab is at the opening of the tube, snap the swab on the side of the tube. Discard the remaining stick. Replace the cap onto the tube and screw it firmly closed. 5. Write your initials and the date of the sample.  	
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6. Insert the collector tube containing your saliva swab, the specimen pot containing your urine and the collector tube containing your vaginal swab into the bag provided. Store the samples in ambient temperature; do not put the samples in the fridge.
7. Give the bag (containing your saliva swab, urine and vaginal swab) to the study team, or bring this with you to your study visit/routine antenatal appointment to leave it with the study team or your routine midwife, depending on what was arranged with the study team.

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St George's University Hospitals NHS Foundation Trust







Study Title:	cCHIPS
Protocol number	Version 1.0, dated 25/03/2020
Study Sponsor	St George's, University of London
REC reference	19/L0/0161
IRAS number	257710

Participant Self-Sampling and Sample Storing Instructions during COVID-19 Pandemic

Thank you for agreeing to store your saliva, urine and vaginal secretion samples at home for your remaining cCHIPS study visits over the COVID-19 outbreak, until we are safe to collect them to avoid face-to-face contact with study participants for your safety and wellbeing. In the envelope you have received you will find this written instructions, swab pack(s) labelled 'Urine' to store your urine sample(s), spare sampling kit labelled 'Visit 4' if you are due for your Visit 4, red bag(s) and clear airtight container to store and freeze your samples securely. If you have any questions please contact the study team at 07920158212 or email vaccinesforchildren@sgul.ac.uk.

1) Self-sampling of Saliva, Urine and Vaginal Secretion Samples

The instructions on how to collect the samples are the same as previously provided to you, *with additional step no.4 to the urine sampling in red and italic font*. As usual please wash your hands before and after taking the samples.

Saliva	Urine
1. In the swab pack labelled 'Saliva', you will see a collector tube and a swab. Peel open the pouch. 2. Remove the swab from the pack holding onto the stick end above the score line. Do not touch the cotton end. 3. Insert dry swab into mouth on the inner cheek. Twist the swab a full circle against the inner cheek 2-3 times to collect enough saliva. 4. Carefully uncap the tube collector and avoid spillage of the fluid in it. Insert sampled swab into the collector tube, when the score line on the swab is at the opening of the tube, snap the swab on the side of the tube. Discard the remaining stick. Replace the cap onto the tube and screw it firmly closed. 5. Write your initials and the date of the sample. 	1. You will see a specimen pot labelled 'Urine'. 2. Remove the silver cap from the pot. Do not touch inside the pot. 3. Pass some urine into the toilet, then without stopping the flow of urine, pass urine directly into the pot (mid stream) half full. 4. <i>In the swab pack labelled 'Urine' in the envelope received, you will see a collector tube and a pipette. Peel open the pouch. Carefully uncap the collector tube and avoid spillage of the fluid in it. Holding onto the pipette bulb, pinch the pipette bulb and insert the pipette tip into the urine collected. With the pipette tip still in the urine, release your pinch on the pipette bulb that will draw urine into the pipette. Whilst still holding onto the bulb without pinching, transfer the pipette with urine into the collector tube. Then pinch the bulb again to release the urine into the collector tube. Repeat the full process of no.4 until the amount of urine in the collector tube is anywhere between the two marked lines on the collector tube. Replace the cap onto the collector tube and screw it firmly closed. The full instructions are also available on the back of the 'Urine' swab pack. Discard the silver cap specimen pot containing the remaining urine sample.</i> 5. Write your initials and the date of the sample.  Pipette bulb Pipette tip Continue overleaf on next page

Vagina

1. In the swab pack labelled 'Vagina', you will see a collector tube and two swabs; one thicker with cotton end, one thinner with brush end. Peel open the pouch. Discard the thinner swab with brush end. You will use the thicker swab with cotton end to collect your vaginal secretions sample.
2. Hold the swab on the stick end above the score line. Do not touch the cotton end.
3. Insert dry swab around 5cm into vagina (like using a tampon but not as far in). Twist the swab gently for about 30 seconds while rubbing the swab against the vagina walls. Remove swab from the vagina carefully.
4. Carefully uncap the collector tube and avoid spillage of the fluid in it. Insert sampled swab into the collector tube, when the score line on the swab is at the opening of the tube, snap the swab on the side of the tube. Discard the remaining stick. Replace the cap onto the tube and screw it firmly closed.
5. Write your initials and the date of the sample.



2) Storing of Saliva, Urine and Vaginal Secretion Samples

The instructions on how to store the samples are different as previously provided to you, as detailed here.

1. As usual, insert the collector tube containing your saliva swab, the collector tube containing your urine and the collector tube containing your vaginal swab back into the green bag labelled with the appropriate visit number (or if you are performing samples for your post delivery visit i.e. Visit 4, the spare sampling kit labelled 'Visit 4' enclosed in the envelope received) within the zip lock compartment.
2. Now, insert this green bag containing your samples into the red bag enclosed in the envelope received, remove the seal strip from the red bag and seal the red bag securely to ensure samples will not leak out of the red bag. One red bag is for one set of samples (i.e. one red bag per one set of visit samples), we will enclose enough red bags to last for your remaining cCHIPS study visits.
3. Finally, open the lid of the clear airtight container enclosed in the envelope received, insert the sealed red bag containing your samples into the clear airtight container and replace the container lid firmly closed.
4. Place the clear airtight container containing the sealed red bag with your samples into your freezer at home upright so that the collector tubes containing the samples within the sealed red bag are also held upright in the freezer; this will ensure that your samples and the fluid within each collector tubes are at the bottom end and able to mix together when just being frozen to maintain the integrity of your samples. After one hour, the samples would have frozen well and the clear airtight container can be placed flat in the freezer if necessary.
5. For any new set of visit samples in a new sealed red bag, please store this in the existing clear airtight container by removing it from the freezer to insert the additional sealed red bag containing the new set of samples, ensuring the clear airtight container is firmly closed and returning it to the freezer again; as there is a new set of samples just being frozen, remember to place the clear airtight container upright again in the freezer for at least one hour before being placed flat if necessary.
6. Remember; only store your samples in the freezer once they have been sealed securely in the red bag and enclosed in the clear airtight container provided. This avoids cross contamination between your samples and anything that may be in the freezer.
7. Do not store the unused sample kits in the freezer. The unused sampling kit should remain at room temperature and only be stored in the freezer (using the instructions provided) once you have performed the sampling at the appropriate time, depending on when your next sample visit is due.
8. The frozen samples should remain in the freezer until the study team can collect the samples from you at your home once it is safe to do so.
9. The study team will guide you on when to do your samples.