CONGENITAL CYTOMEGALOVIRUS: USING MODERN VIROLOGICAL METHODOLOGY TO DEFINE NATURAL HISTORY AND RATIONALISE TREATMENT

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I, Suzanne Elizabeth Luck confirm that the work presented in this thesis is my own. Part of this work has already been published in a peer reviwed journal (Luck et al. 2011) (Appendix D). Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

Congenital cytomegalovirus (CMV) infection is responsible for long-term morbidity in the form of sensorineural hearing loss and neurodevelopmental impairment in a high proportion of those with symptomatic infection at birth but only around 14% of those without symptoms. In contrast newborns acquiring infection posnatally, generally from maternal breast milk, do not seem to have any associated long-term sequelae. These differing clinical outcomes remain to be explained.

In immunocompromised adults, quantitative CMV DNA PCR has given valuable insight into viral dynamics. Such data have informed treatment, particularly in the transplant setting, where CMV disease has been shown to be directly correlated with various measures of viral load in both blood and urine. Pre-emptive therapy has been adopted in many units as a direct result. Newer immunological techniques have also led to the realisation that a large proportion of the human host's cellular immune response is directed towards maintaining CMV in a latent state lifelong following primary acquisition of this virus. No equivalent data exist for the natural history, viral response to treatment or associated immune responses in neonates.

This thesis therefore aimed to apply quantitative, virological and immunological techniques already successfully employed in adult patient groups to help define natural history and immune responses along with other measures of treatment response in this neonatal patient group through multicentre collaboration.

The results presented give the first calculations of virus dynamics in response to antiviral treatment in this age group. The possibility is raised of suboptimal drug exposure in the clinical setting and data presented on both viral load and CMV-specific immunological responses have potentially important implications for treatment in these infants and for optimal sampling times which will inform future clinical trials and the continued search for biomarkers of treatment efficacy.

Table of Contents

List of Tables

List of Figures

Abbreviations

APC	Allophycocyanin
AUC	Area under Curve
Bd	Twice daily
BPAIIG	British Paediatric Allergy, Immunology and Infection Group
BPSU	British Paediatric Surveillance Unit
CASG	Collaborative Antiviral Study Group
cCMV	Congenital Cytomegalovirus
CHIPS	Collaborative HIV Paediatric Study
C_{max}	Maximum drug concentration
CNS	Central Nervous System
CPE	Cytopathic Effect
CT	Computerised Tomography
CTU	Clinical Trials Unit
DBS	Dried Blood Spot
DEAFF	Detection of early antigen fluorescent foci
DMSO	Dimethyl sulphoxide
ECCI	European Congenital Cytomegalovirus Initiative
EEG	Electroencephalogram
EMA	European Medicines Agency
ESPID	European Society for Pediatric Infectious Diseases
FIT-C	Fluoroscein isothiocyanate
GCV	Ganciclovir
GI	Gastrointestinal
HCMV	Human Cytomegalovirus
HLA	Human Leucocyte Antigen
HPLC	High Performance Liquid Chromatography
IC_{50}	50% inhibitory concentration
IE1	Immediate early antigen 1
IQR	Interquartile range
IUGR	Intrauterine Growth Restriction
IV	Intravenous
MIC	Minimum inhibitory concentration
MRC	Medical Research Council
MRI	Magnetic Resonance Imaging
NaN_3	Sodium azide solution
NPV	Negative Predictive Value
NSHPC	National Study of HIV in Pregnancy and Childhood
ORF	Open Reading Frames
PBMC	Peripheral Blood Monocyte
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PD	Pharmacodynamic
PENTA	Paediatric European Network for Treatment of AIDS
Per-CP	Peridinin-Chlorophyll-Protein Complex
PIAG	Patient Information Advisory Group
PIC	Participant Identification Centre
PK	Pharmacokinetic
PO	Oral
PPV	Positive Predictive Value
R&D	Research and Development
RCT	Randomised Controlled Trial

RFLP	Restriction Fragment Length Polymorphisms
R-PE	R-Phycoerythrin
RT-PCR	Real-time polymerase chain reaction
SD	Standard Deviation
SEB	Staphylococcus enterotoxin B
SNHL	Sensorineural Hearing Loss
SSEP	Somatosensory Evoked Potential
SSIF	Site Specific Information Form
$T_{1/2}$	Half-life
TDM	Therapeutic drug monitoring
USS	Ultrasound Scan
VEP	Visual Evoked Potential
VGCV	Valganciclovir
VICC	Viral load and Immunology in Congenital CMV
VL	Viral load
VTM	Virus Transport Medium

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CHAPTER 1.

1. INTRODUCTION

1.1. DESCRIPTION OF THE VIRUS

Cytomegalovirus (CMV) derives its name (cyto-megalo-virus) from the typical histological appearance of enlarged cells with intranuclear (Owl's eye) inclusions first observed in post mortem specimens in the late 19th and early 20th Century. It was some years after initial histopathological changes were observed in post mortem specimens from newborns, however, that they were identified as being viral in origin and that CMV was named as the aetiological infective agent (Weller et al. 1960). CMV is now known as a member of the betaherpes virus sub-family (Human Herpes Virus 5) and is ubiquitous in humans. As with other herpesviruses, infection is characterised by a primary infection, establishment of latency and subsequent reactivation throughout the hosts' lifetime. Virus is detectable in most human body fluids during active infection. More recently it has been established as the most common congenital infection in the developed world (Cannon 2009).

What follows below is an overview of the aspects of CMV most relevant to the focus of this thesis.

1.1.1. *CMV structure*

Electron microscopy of CMV shows the characteristic herpesvirus appearance including a central icosahedral capsid encasing the DNA core, surrounded by a layer of amorphous tegument and an outer, lipid bilayer, envelope.

The genome of CMV is the largest of all herpes viruses. Its linear, double-stranded, genome was demonstrated to be 230kb on sequencing of strain AD169 in the early 90's (Chee et al. 1990). Some years later, however, it became apparent that this highly passaged strain of CMV lacked a large portion of the genome present in 'wild-type' human CMV (HCMV), containing around 22 additional genes. In 2004 Dolan *et al* proposed that Merlin, a low-passage clinical virus strain, 235 kb in size and encoding 165 genes was closer to wild-type virus (Dolan et al. 2004). Some of the mutations present in AD169, and other laboratory strains of human CMV, are likely to facilitate the high production of virus observed following infection of fibroblasts in traditional laboratory tissue culture systems. The difference between laboratory and clinical isolates therefore has implications for translational research

into the pathogenesis of CMV and has led to recent debate regarding the most relevant cell lines to be used in laboratory-based research (Gerna et al. 2008).

Proteins are produced in sequence with immediate early genes being transcribed first (alpha), followed by early (beta) and late (gamma) genes, which are mainly transcribed after DNA replication has occurred. Immediate early proteins e.g. IE1 and IE2 regulate cellular functions, early proteins generally have enzymatic functions within the cell e.g. *pUL54* is a DNA polymerase and *pUL97* is a protein kinase essential for CMV replication and involved with the first phosphorylation step necessary for activation of ganciclovir (GCV). Late proteins are generally structural e.g. surface glycoproteins.

Envelope is derived from budding from the inner nuclear membrane of the host cell, which has been modified by insertion of herpesvirus glycoproteins (gp). These glycoproteins are therefore of importance due to their early interaction with the host immune system. One of the most abundant envelope components is glycoprotein B (gB). This protein can be detected on both infected cells and virions and has been found to be one of the major targets of neutralising antibody in the host response to CMV infection (Britt et al. 1990). It is also the fragility of this envelope which leaves the virus susceptible to damage and renders CMV sensitive to drying, acids, detergents and organic solvents.

CMV is often divided into distinct genotypes based on these envelope glycoproteins with gB, for example, having four main genotypes (Pignatelli et al. 2004). The more detailed subtyping of distinct CMV strains is traditionally derived from restriction endonuclease analysis (RFLP). Whole genome sequencing is now leading to a full appreciation of the extensive genetic variation exhibited by HCMV.

1.1.2. *CMV replication (including permissive cell types)*

Following virus entry a multitude of factors regulate expression of genes necessary for CMV to complete its replicative cycle. These are likely to be dependent both on the cell type infected and the virus strain involved along with corresponding host immunological regulatory mechanisms. Although wild-type virus can be propagated in a wide range of cell lines only human fibroblasts give rise to high-titres of virus production *in vitro* (Spiller et al. 1997). Cultures of placental cytotrophoblasts have

been shown to be fully permissive to infection; enhanced replication and more virus is released in first trimester trophoblast infections when compared to term in these *in vitro* models (Halwachs-Baumann et al. 1998;Halwachs-Baumann 2006).

In vivo, CMV is found in a multitude of cells (Bissinger et al. 2002;Sinzger and Jahn 1996). CMV DNA has also been detected in placentas at post mortem (McDonagh et al. 2004). During active CMV infection the predominant cell types expressing CMV DNA are, however, endothelial cells, granulocytes, monocytes, B cells and T cells (Emery 2001a;Hassan-Walker et al. 2001).

The differences in cell types allowing for infection *in vitro* when compared to *in vivo* observations highlights the need for caution when extrapolating results from laboratory-based systems to the human host.

1.2. PATHOGENESIS

1.2.1. *Incubation period, viral dissemination and proposed mechanisms of tissue damage*

The period between infection of a new host and the onset of detectable virus excretion has been estimated to be around 4-8 weeks from data derived from transplant recipients and perinatally infected neonates (Hamprecht et al. 2001;Stagno et al. 1975). This is based on cell culture data and may be shortened in light of data emerging from studies using polymerase chain reaction (PCR).

Historical concepts of viral spread postulate that initial entry into the host leads to establishment of local viral infection followed by primary viraemia enabling subsequent virus transmission to the liver and spleen. Further virus propagation in these organs leads to a secondary viraemia followed by generalised virus dissemination. There is some experimental evidence in animal models of CMV infection to support this theory, however, recent data using novel experimental methods have challenged this mode of spread (Lockridge et al. 1999;Sacher et al. 2008). There are no corresponding data in humans and the factors involved with virus spread within the human host following initial virus entry therefore remain elusive. Although cell free virus has been proposed to have a role in the initial spread of community acquired HCMV this is supported by limited data. Spread

within the host is possibly predominantly by cell associated virus. Certainly infectivity in the blood compartment has been shown to be mediated mainly by PBMCs and endothelial cells (Emery 2001a;Percivalle et al. 1993). PBMCs, although not being fully permissive to viral replication themselves, can transmit virus from infected endothelial cells or fibroblasts to infect further susceptible fibroblasts (Gerna et al. 2000). The production of chemokine-like proteins along with activation of pathways which increase PBMC recruitment to sites of inflammation are also likely to enhance virus dissemination as summarised in a recent review (Goodrum et al. 2012). Further understanding of the key events involved with early establishment of infection and dissemination to multiple organs might give crucial insight into why disease in neonates so readily involves the central nervous system (CNS).

The delayed detection of virus in fetuses of mothers with primary infection is consistent with placental infection preceding transmission to the unborn baby. Placental infection, without fetal involvement, has also been reported (McDonagh et al. 2006). In experimental models using explants of first trimester placenta CMV replication in trophoblasts is likely followed by subsequent transmission to the stromal fibroblasts and then on to fetal endothelial capillary cells (Gabrielli et al. 2001). Stromal fibroblasts are therefore thought important in propagating placental infection.

The lack of detectable viraemia in many women transmitting CMV to their unborn child, however, raises uncertainty regarding the exact mechanism of fetal transmission. Despite this uncertainty a number of proposals have been made including the possibility that antibody-coated virus is transcytosed using placental Fc receptors or that cell-associated virus is reactivated from latency in placental monocytes with subsequent infection of different layers of the syncytiotrophoblast as reviewed by Revello and Gerna (2004). Whatever events precede it is likely that the final step in fetal infection involves infection of fetal endothelial cells and subsequent haematogenous dissemination.

Both lytic and non-lytic productive infections have been described dependent on the source of cells and infecting virus. Acute tissue damage due to direct viral cytopathogenic effect has been directly associated with viral copy number in

individual organs (Mattes et al. 2000). Indirect damage is also proposed through inflammatory responses, chemokine alterations and cell apoptosis induced by CMV infection (Britt 2007).

1.2.2. *Possible mechanisms for pathogenesis associated with congenital infection*

In keeping with the observation that intrauterine growth retardation (IUGR) occurs in both congenital infection and other maternal conditions affecting placental function recent data have implicated changes in placental vasculature induced by HCMV as contributing to placental insufficiency and subsequent fetal hypoxia (Maidji et al. 2010).

Although the exact mechanisms of CMV-induced sensorineural hearing loss (SNHL) remain unknown, CMV has been detected in non-neuroepithelial cells of both the cochlear and vestibular apparatus of the inner ear on post mortem examination of temporal bones (Strauss 1990). CMV inclusion bodies have been noted in the stria vascularis and vestibular (Reissner's) membrane but not other critical structures in post mortem studies. However, a wider distribution of viral antigen, including the organ of Corti and spiral ganglia, have been reported using immunofluorescent staining for viral antigen (Strauss 1990). Studies in animal models have raised the possibility that hearing loss is more likely a result of CMV-induced inflammation in the inner ear than of direct cytopathic effect (Darmstadt et al. 1990;Keithley et al. 1989).

CMV infection in mouse models has been shown to alter the expression of factors important in differentiating neural stem/progenitor cells and may thus disturb neuronal cell migration (Kosugi et al. 2000). These models have also suggested that acute infection is characterised by a ventriculo-encephalitis and lytic infection with subsequent chronic infection being sustained both by neuron-specific activation of the early CMV promoters along with evasion of innate host immunity and possible anti-apoptotic mechanisms as reviewed by Tsutsui et al (2005). Reactivation of infection has been observed in mouse brain slice cultures with the possibility that differentiation of neural stem cells into progenitor cells reactivates latent virus in a similar way to that reported during differentiation of haematopoietic stem cells

(Tsutsui et al. 2002). This proposed model for chronic neurotropic infection might explain the neurological sequelae and progression of clinical findings noted during the first few years of postnatal life which are characterised by rapid brain growth and neuronal differentiation.

1.2.3. *Viral persistence and latency*

Persistent virus excretion is common in HCMV and may either arise from chronic low-level virus production following primary infection or following intermittent reactivation of virus from sites of proposed latency, whereby viral genomic DNA is detectable with no associated early gene products.

In congenitally infected infants a median duration of virus excretion in urine of 3.9 years has been reported (Noyola et al. 2000). It is likely that this results from persistent infection of glandular epithelial cells although the mechanisms behind this remain elusive. Urinary excretion of up to a year has also been reported in healthy adult hosts (Klemola et al. 1969).

Latency is established primarily in cells of the myeloid lineage and in dendritic cells (Hahn et al. 1998;Soderberg-Naucler et al. 1997). Differentiation of monocytes to macrophages has been proposed as an important trigger for subsequent reactivation from this latent state, resulting in intermittent low level detection of virus. As mentioned earlier reactivation in neural stem or progenitor cells has also been proposed in mouse models of infection. It is likely that the associated cytokine milieu and host immune status play an important role in the balance between virus latency and reactivation (Soderberg-Naucler et al. 2001;Tsutsui, Kawasaki, & Kosugi 2002).

1.3. EPIDEMIOLOGY AND TRANSMISSION

1.3.1. *Determinants of population seroprevalence*

CMV is endemic with maternal seroprevalence ranging from 60–100% in different populations studied (Embil et al. 1969;Miles et al. 2007;Staras et al. 2006;Tookey et al. 1992;Vyse et al. 2009). Seroprevalence increases with age although in many populations, particularly those studied from low-income countries, a high proportion of individuals seroconvert early in life, presumably due to high rates of seropositivity

in maternal groups and concomitant early postnatal or childhood transmission (Miles et al 2007). In the UK seroprevalence is estimated at only around 15% in those aged 1-4 years of age increasing to 80% by ≥65 years of age with a relatively high proportion of children acquiring CMV infection in early life (Peckham et al. 1987;Vyse, Hesketh, & Pebody 2009).

Studies in various Western populations have also shown large variations within populations according to ethnicity and socio-economic status (Colugnati et al. 2007;Staras et al. 2006). A large US study showed a 10-15% higher seroprevalence in low- and middle-income households compared to high-income households (Staras et al. 2006). Consistent with this finding, higher seroprevalence was observed in women from lower social class in a study of women attending antenatal clinics in West London (Tookey, Ades, & Peckham 1992). Increased seropositivity was also noted in women with Asian and African/Caribbean ethnic origin compared to white women in both this and a more recent South West London cohort (Khare et al. 2004;Tookey, Ades, & Peckham 1992).

Working in daycare settings with toddlers has also been associated with an increased seroconversion rate but not work within healthcare settings (Adler 1989;Dworsky et al. 1983).

1.3.2. *Route of acquisition*

As outlined above congenital infection is thought most likely to follow placental infection following either haematogenous spread or possibly ascending infection via the maternal genital tract (Fisher et al. 2000). The delayed detection of CMV DNA in amniotic fluid, of around 6-8 weeks after the estimated date of maternal primary infection, is consistent with the incubation period for CMV and supports placental infection as the main route of acquisition in this group (Revello and Gerna 2004).

Postnatal infection either follows ingestion of infected vaginal secretions during passage through the birth canal or via breast milk soon after delivery (Hamprecht et al. 2001;Reynolds et al. 1973). CMV is detectable in breast milk at some stage during lactation in 96% of seropositive mothers, and acquisition via breast milk is likely to be the predominant mode of transmission during the first year of life (Hamprecht et al. 2001). As many mothers shed no virus from other body fluids

localised reactivation of CMV infection in breast epithelium is most likely responsible for the virus excretion observed in these women (Hamprecht et al. 2008).

Thereafter infection probably results from direct contact with infected body fluids. The high burden of virus excreted in saliva and urine in toddlers has been implicated as a significant source of infection in this age group (Adler 1985). This is supported by studies whereby saliva containing CMV has been found contaminating toys in daycare centres (Hutto et al. 1986). In teenage years, where CMV has been occasionally found to cause an infectious-mononucleosis like illness, similar to EBV, saliva has likewise been implicated as a significant mode of transmission. Although CMV has been isolated in large titres in both vaginal secretions and semen whether CMV is truly sexually transmitted remains debated. Importantly for this thesis, population analyses suggest that women of childbearing age are more likely to acquire primary CMV infection from their children than from sexual contacts (Pass et al. 1986;Staras et al. 2008). Transmission via blood transfusions was a common route of acquisition, particularly in transplant recipients and premature babies, prior to the introduction of measures to limit this mode of transmission by use of CMV-negative red cells and leucocyte depletion using white cell filters during transfusion. Likewise CMV seronegative solid organ recipients are at particular risk of acquiring infection from seropositive organ donors with virtually no risk if they receive an organ from a seronegative donor (Atabani et al. 2012). Seropositive recipients may also be reinfected following acquisition of new strains of CMV from the organ donor. It is hypothesised that infection is transmitted either via CMV-infected parenchymal cells or leucocytes infiltrating the donated organs.

1.3.3. *Factors associated with intrauterine transmission*

In mothers with primary infection around 33% have been found to transfer infection to their unborn fetus (Kenneson and Cannon 2007). Natural maternal immunity prior to conception has been shown to provide 69% protection against delivering a neonate with cCMV infection compared to seronegative women in the same population (Fowler, Stagno & Pass 2003). Transmission of infection is still reported, however, in 1% of fetuses born to women with pre-existing immunity to CMV (Fowler, Stagno, & Pass 2003). Infections in women with pre-existing immunity are proposed

to follow either reinfection with a new strain of CMV or reactivation of pre-existing infection from sites of latency. Epidemiologically it is difficult to prove the exact origin of infecting virus and to ascertain relative significance of these two routes. Mothers with pre-existing immunity nonetheless remain a significant source of transmission in many populations, with 90% of all cCMV cases being born to seropositive mothers in populations with a high underlying maternal seroprevalence (Kenneson and Cannon 2007;Mussi-Pinhata et al. 2009).

Risk of transmission also seems to be greatest in women acquiring CMV in later stages of pregnancy (Griffiths and Baboonian 1984), perhaps explained by the proposed role of Fc receptors in transcytosis of antibody-coated virus across the maternal and fetal placental interfaces (Fisher et al. 2000). Transmission to the unborn fetus is increased in the presence of maternal antibody with both low avidity and poor neutralising activity (Boppana and Britt 1995;Lazzarotto et al. 1999). Up to the 18th week of pregnancy a low avidity index is 100% sensitive in identifying women who will have an infected newborn (Lazzarotto et al. 2000).

Correspondingly, women with impaired cellular immune responses (such as in HIV infection) are more likely to transmit infection antenatally (Guibert et al. 2009;Rola-Pleszczynski et al. 1977).

1.4. DIAGNOSIS

For many years CMV was diagnosed using its cytopathic effect (CPE) on fibroblast cultures. Subsequently electron microscopy enabled the virion to be visualised. These ‘direct’ methods are labour intensive, and generally require a high virus burden or prolonged periods of cell culture to enable detection. Furthermore it can be difficult to differentiate between CMV and other, related, herpes viruses (Mattes et al. 2000).

Modern diagnostic techniques now enable direct detection of CMV viral DNA using small amounts of body fluid or tissue. Owing to its capacity for high throughput, high sensitivity and ability to quantify viral DNA, PCR has now largely superseded these older tissue-culture based methods in combination with the use of indirect detection using serological measures of detectable anti-CMV antibody.

1.4.1. *Serological (Indirect measures)*

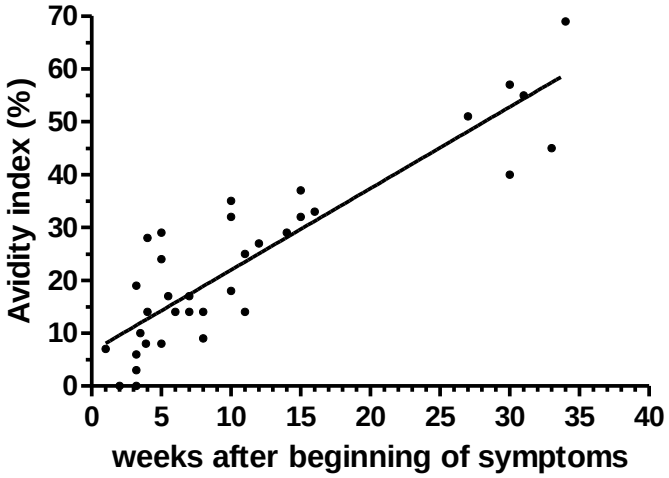
- **IgG; IgM; IgG Avidity**

IgM is, as with other infections, the first detectable antibody produced in response to CMV infection. CMV IgM is generally positive within 4 weeks of infection and persists for around 2-3 months. Sensitivity is poor, however, with at best only 70% of congenitally infected newborns having detectable IgM antibody (Donner et al. 1993;Revello et al. 1999). False positives are commonly reported, particularly during pregnancy, from cross-reactivity with rheumatoid factor IgM, and with EBV. Furthermore IgM can persist for up to one year, even in immunocompetent hosts, and IgM detection during reinfection with new CMV strains or reactivation has also been reported as reviewed by Revello and Gerna (2002). IgM assays are therefore rarely helpful on their own in confirming primary infection.

IgG sensitivity and specificity varies, as with IgM, dependent on the commercial assay being used but all assays are generally acceptable in immunocompetent patients. Unless seroconversion is observed CMV IgG measurement is of limited value in identifying primary CMV infection. Given the absence of any screening programmes for CMV in the UK CMV IgG is therefore only of value in excluding CMV infection, with a negative result 4-8 weeks after potential exposure giving reasonable confidence that a mother has not acquired infection. CMV IgG is transplacentally transferred and, as with other congenital infections, is therefore of no value in diagnosing CMV infection in the newborn.

In recent years the development of CMV IgG avidity assays has assisted in differentiating between primary and non-primary CMV infections. Data show that a low CMV IgG avidity index is associated with infection within the preceding 3 months and maturation to high avidity usually occurs over a period of 6 months (Lazzarotto et al. 1997) (Figure 1-1). The presence of low avidity CMV IgG in pregnant women has been reported to be highly sensitive for identifying women who will have an offspring with CMV infection (Lazzarotto et al. 2000).

Figure 1-1 Correlation between CMV Avidity Index and number of weeks after primary HCMV infection in pregnant women



Correlation coefficient = 0.8726

Reproduced from Lazzarotto et al. (1997)

Each data point represents the avidity index measured at different time points, in weeks, after a CMV-infected pregnant woman reported symptoms of primary HCMV infection. Correlation analysis gives a correlation coefficient of 0.8726, indicating a strong association between the two parameters.

1.4.2. *Culture vs PCR (Direct measures)*

- **Culture**

Historically the gold standard for diagnosis was tissue culture based methods such as the DEAFF (detection of early antigen fluorescent foci) test, often referred to as shell vial assay in the US. By detecting proteins produced early in the replicative cycle of CMV using fluorescent-labelled monoclonal antibody, virus is detected earlier than waiting for cytopathic effect. This method remains labour intensive, however and cultures still need to be left up to 21 days in order to be able to report a negative result.

- **PCR detection**

QUALITATIVE

Over recent years PCR of both tissues and various body fluids has been shown valid for the diagnosis of CMV disease (de Vries et al. 2012;Demmler et al. 1988). Sensitivity and specificity of this method of detection depends on the area of the CMV genome being probed with higher sensitivity reported when areas with low genetic variability between strains are targeted (Boeckh and Boivin 1998). There are no other significant differences between different PCR assays with regards to test performance. Nested PCR, while more sensitive, has associated disadvantages of both increased labour and risk of background contamination. A further disadvantage of PCR is that CMV does not need to be actively replicating in order to be detected. It is therefore important that the amount of DNA extracted from clinical samples is not sufficient to detect latent infection. Once this is done, many clinical studies have shown correlation between CMV detection by PCR and active disease leading to this being adopted as the new 'gold standard' according to updated virological texts (de Vries et al. 2012;Griffiths et al. 2008). Where urine is being assayed, inhibition of the enzymes used in the PCR mixture is a potential cause of false negative results. Virus is generally sufficiently abundant in urine of congenitally infected infants for this to have little impact on diagnostic utility and can additionally be controlled for by using an internal laboratory control.

QUANTITATIVE (REAL TIME) PCR.

Real-time PCR (RT-PCR) has led to the development of assays which give an estimate of the virus burden in a given body compartment, "viral load" (VL). These assays generally involve an oligonucleotide probe with an associated fluorescent dye and 'quencher' meaning that fluorescence is only detected if the DNA sequence of interest is recognised. The fluorescence generated during consecutive cycles of amplification directly relates to the amount of DNA present in a sample and is reported in relation to a standard curve generated during each diagnostic run from serial dilutions of a known amount of target DNA. During the log linear phase of DNA amplification this provides an accurate reflection of the DNA content of the input sample but due to the non-linearity at either end of this curve accuracy correspondingly decreases at very low or very high VL.

Inter-lab variations up to 4.3 $\log_{10}$ have also been reported and in one study only 58% of all samples came within a proposed acceptable variation of 0.5 $\log_{10}$ from the 'expected' value (Pang et al. 2009). This has historically prevented comparison of quantitative results derived from different laboratories.

1.4.3. *Differentiation of congenital vs postnatal infection*

The diagnosis of congenital infection currently relies on the availability of clinical samples taken within the first 21 days of life. This proposed cut-off is derived from historical studies of congenitally and postnatally infected babies using tissue culture (Stagno et al. 1975). The increased sensitivity of PCR detection raises the possibility of earlier virus detection, thus potentially decreasing this diagnostic cut-off to around 14 days (de Vries et al. 2012). CMV DNA is detectable in blood before viruria in a proportion of postnatally infected neonates with further implications for defining congenital infection if this body fluid is used diagnostically (Hamprecht et al. 2001).

- **Diagnostic utility of different body fluids**

CMV is not uniformly detectable in the blood and is therefore not the specimen of choice for diagnosis of cCMV (Bradford et al. 2005;Kimberlin et al. 2008). Urine has been the gold standard for diagnosis but it is not always easy to obtain such specimens in infants, particularly in the out-patient setting. Virus detection in

salivary samples has been shown to be comparable to that in urine, particularly in the setting of large scale screening studies (Warren et al. 1992;Yamamoto et al. 2006). Although there are no published data regarding false positive results, concerns exist regarding contamination of neonatal saliva with maternally derived CMV-DNA, particularly in samples obtained from breastfed neonates. Diagnosis should therefore always be subsequently confirmed using another body fluid.

CSF is not part of the routine assessment of the neonate with cCMV infection in most institutions as there are insufficient data that this adds anything to less invasive investigations and clinical examination. CMV detection in CSF has been associated with cCMV disease and correlated with poor outcome (Halwachs-Baumann et al. 2002;Troendle Atkins et al. 1994). However, CMV was also weakly detectable in 2/100 controls being evaluated for other diagnoses in one of these studies (Troendle Atkins et al. 1994). Larger studies including adequate numbers of both symptomatic and asymptomatic infants along with long-term follow-up are needed before the role of testing CSF can be fully defined.

CMV has been detected in perilymph in children undergoing cochlear implantation up to 7 years of age (Bauer et al. 2005;Di et al. 2009). Obtaining a diagnosis at this stage has limited value, however, other than for epidemiological purposes and might purely be reflecting CMV detection in the perilymph following a recent acquisition of CMV (Di et al. 2011).

Amniotic fluid is often obtained antenatally from women with suspected primary CMV infection, or with fetal findings consistent with a diagnosis of congenital infection. To maximise virus detection, samples need to be taken at least 6-8 weeks after maternal primary infection and >21 weeks gestation to ensure sufficient fetal urinary production (Donner et al. 1994;Enders et al. 2001;Guerra et al. 2000). If samples are taken in accordance with this guidance then specificity is reported to be around 100% (Guerra et al. 2000). Sensitivity of around 80% is reported if high load virus detection is taken to indicate a positive result but increases to 100% if lower quantities of virus detection are used to indicate positivity or if amniotic fluid PCR is combined with other diagnostic tests (Enders et al. 2001;Guerra et al. 2000). A

positive result indicates infection only, however, and is not diagnostic of fetal disease.

- **Retrospective diagnosis using dried blood spots (DBS)**

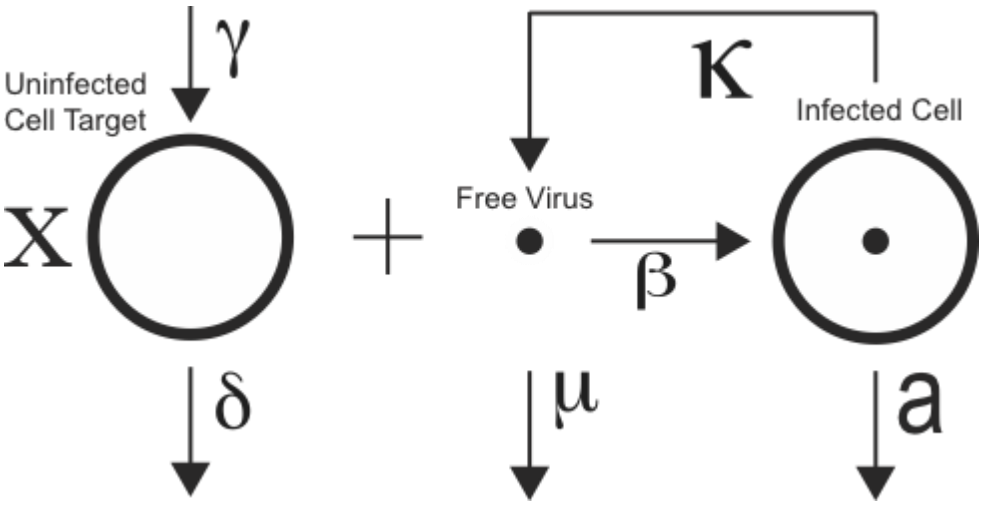
Detecting clinical symptoms and signs of cCMV and obtaining relevant samples within the narrow time frame necessary to make a definitive diagnosis of cCMV is challenging and likely contributes to underdiagnosis (Townsend et al. 2011). The availability of DBS, taken on all babies shortly after birth, has therefore raised interest in the use of these clinical specimens to aid retrospective diagnosis of congenital infection. CMV DNA was first successfully extracted and detected from DBS in the late 1990s (Barbi et al. 1996b;Shibata et al. 1994). Since that time there have been many further reports on the retrospective diagnosis of cCMV using DBS in babies exhibiting clinical features of disease and, more recently, as a possible screening tool for detecting cCMV in large cohorts of asymptomatic neonates (Barbi Binda, & Caroppo 2006;Boppana et al. 2010).

Specificity is generally close to 100% but sensitivity has been reported variably as 30-100% (Barbi et al. 2008;Boppana et al. 2010). Variations in sensitivity depend not only on the specific patient group being tested but also on the method of DNA extraction and the region of CMV being targeted as summarised in Barbi's review (2006) of this methodology. The lack of detectable CMV DNA in the blood of many congenitally infected infants further limits the sensitivity of this test and although a positive result is of benefit in confirming cCMV a negative result by no means excludes the diagnosis.

1.4.4. *Defining virus dynamics using quantitative PCR*

The basic model of virus dynamics is similar to epidemiological models of infectivity, albeit at a cellular level. A number of nonlinear differential equations can be used to mathematically explain the interactions between uninfected cells and free virus leading to infection of cells and subsequent production and release of further virion as shown in Figure 1-2. These equations in active infection are difficult to interpret and subject to many variables with many of the rates involved being time dependent (Herz et al. 1996).

Figure 1-2 Cartoon of basic model of virus dynamics



γ = rate of repletion of uninfected cells (constant rate)

δ= rate of cell death of uninfected cells

β = rate of free virus infecting uninfected cells

κ = rate of virion production by infected cells

μ = rate of death/clearance of free virus

a= rate of death of infected cells

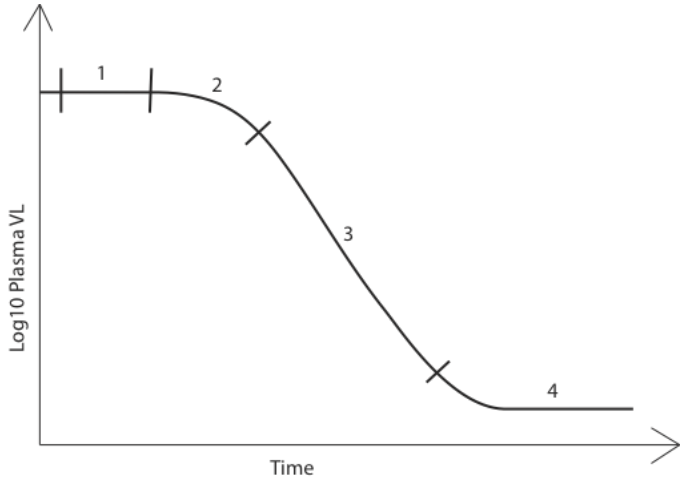
Cartoon showing the basic model of virus dynamics as described in more detail by Perelson et al. (2002). Uninfected cells (x) are infected with free virus at rate β. Infection leads to productively infected cells which produce new virions at a rate, κ. Uninfected cells, infected cells and free virus die at the rates δ, a, and μ, respectively. Uninfected cells are replenished at a constant rate (γ).
This model can be further described by a number of inter-related differential equations which have been used to estimate virus half-life ($T_{1/2}$) and other parameters.

The stable VL observed in chronic HIV infection had for many years been assumed to imply slow virus replication which was discrepant with laboratory observations of rapid virus mutation. Studies in the 1990's led to the observation that antiviral treatment with protease inhibitors effectively inhibited virion production resulting in a rapid logarithmic decline in HIV VL within the first few weeks of treatment (Ho et al. 1995;Wei et al. 1995). By preventing infection of new cells the normal cycle of virus production was disrupted allowing mathematical biologists to break down the complex equations describing virus dynamics into linear differential equations as reviewed by Perelson (2002). These differential equations were subsequently used to estimate upper limits of half-life ($T_{1/2}$) of plasma virions using the equation ln2/c, where c is the rate of clearance from plasma; c can be derived from measuring the slope of the curve of VL during the phase of exponential decline (phase 3 in Figure 1-3) (Perelson 2002). Serial VL measurements obtained during these treatment studies confirmed that HIV was, in fact, a rapidly replicating virus with a $T_{1/2}$ of around 6 hours (Ho et al. 1995;Perelson et al. 1996;Wei et al. 1995). These models have been used to further describe the phases of viral decline observed when measuring quantitative VL during antiviral treatment (Figure 1-3).

Similarly the slow appearance of CMV cytopathic effect in fibroblast cell culture of around 7-20 days had led to the assumption that CMV was a slowly replicating virus. Applying the models of virus dynamics deployed for HIV, and assuming GCV efficacy to be 100%, Emery *et al* (1999) established that the $T_{1/2}$ of CMV in HIV-infected patients was around 2.56 ± 0.36 days. Similar calculations in liver and bone marrow transplant recipients receiving IV GCV gave $T_{1/2}$ values of 2.36 ± 1.2 days and 1.52 ± 0.67 days respectively thus proving that CMV was also a more rapidly replicating virus than had been previously appreciated. By carrying out more frequent sampling in a number of patients during early treatment these estimates were further refined to give estimates of $T_{1/2}$ of around 1.0 ± 0.3 days during early stages of treatment (Emery et al. 1999).

Subsequently these authors, using similar models of exponential decay, confirmed that GCV had an efficacy of close to 100% (91.5%) in adult patients (Emery and Griffiths 2000). In contrast oral GCV, due to its poor oral bioavailability, had a corresponding efficacy of only 46.5%. Oral valganciclovir (VGCV), with its

Figure 1-3: Schematic of classical phases of plasma virus decline following antiviral treatment



1. Stationary phase reflecting pharmacological and intracellular delay
2. Transition phase reflecting decay of free virus and productively infected cells
3. Rapid decline reflecting decay of productively infected cells (exponential)
4. Levelling off due to: non-optimal drug dose, emergence of drug resistant virus, reservoirs of virus-producing cells that are not affected by drug

In the initial phase the plasma virus load stays constant. The duration of this phase is dependent on the pharmacological delay (defined as the time the drug needs to reach an effective concentration) and the intracellular delay (defined as the time between infection of a cell and production of new virus particles). Subsequently there is a smooth transition to an approximately exponential decline that primarily reflects the decay of productively infected cells. The length and smoothness of the transition phase is determined by a combination of the half-lives of free virus and productively infected cells. Finally, decline levels off due to the reasons stated in 4, above.

Adapted from Herz et al. (1996)

enhanced oral bioavailability, has been shown to have a similar efficacy to that of i.v. GCV and in a group of solid organ transplant patients treated pre-emptively with oral VGCV $T_{1/2}$ was estimated at 2.16 days compared to 1.73 days in those receiving i.v. GCV (P=0.63) (Mattes et al. 2005). Oral VGCV can thus be considered equivalent to GCV for the purposes of calculations of viral dynamics in this patient group.

1.5. CLINICAL DISEASE ATTRIBUTED TO CMV

1.5.1. *Adult disease*

Disease in immunocompetent hosts is rare and generally mild although occasionally primary infections are associated with an infectious mononucleosis-type illness (Klemola et al. 1969). In immunocompromised hosts, however, CMV is a leading cause of morbidity and mortality. Prospective studies in patients post-transplantation show that viraemia always precedes all forms of end organ disease and that a high VL is a pre-requisite (Emery et al. 2000). In these patients a 'CMV syndrome' has been described in which a spiking pyrexia accompanies viraemia and leucopenia.

Patterns of acute disease often involve specific organs dependent on the patient group affected as shown in Table 1-1. The factors determining these different manifestations, such as the predominance for significant eye involvement in HIV co-infected subjects, and pneumonitis following bone marrow transplantation, are currently unknown but are likely to involve a complex interplay between viral factors (including genetic variations between different strains) and host adaptive and innate immune responses together with the known viral immune modulatory functions.

Encephalopathy is also observed in HIV-infected patients although it usually only occurs in the most severely immunocompromised (<50 CD4 cells/mm^3) (Cinque et al. 1997). This can be either subacute with progressive dementia or an acute presentation associated with necrotising ventriculitis and high mortality, despite antiviral therapy. Polyradiculopathy and peripheral neuropathies have also been described. CNS disease is only rarely described in non-HIV immunosuppressed patients or immunocompetent individuals (Schober and Herman 1973).

CMV itself may cause immunosuppression and morbidity from associated secondary

Table 1-1 Pathology associated with CMV disease in different patient groups

Symptoms	Solid organ recipients	Bone marrow recipients	AIDS patients	Congenital infection	Premature babies/ postnatal infection
Fever	+	+	+		+
Hepatitis	++	+		++	++
Gastrointestinal	+	+	+		++
Retinitis	+	+	++	++	
Pneumonitis	+	++		+	++
Encephalopathy/ CNS			+	++	
Polyradiculopathy			+		
Myelosuppression		++		++	+
Immunosuppression	+				
Rejection	+				
Addisonian		+			
Audiological				++	

Adapted from Emery (2001a)

bacterial or fungal superinfection and graft rejection are described (Ljungman et al. 2002). The evidence supporting a causal role for CMV in these diverse conditions is their reduction in double-blind, randomised, placebo-controlled trials of anti-CMV prophylaxis in transplant patients (Lowance et al. 1999). CMV has also been associated with other conditions such as immunosenescence, extended stay in intensive care and excess mortality in the whole USA population although whether CMV is causative in these conditions remains unclear (Koch et al. 2006;Limaye and Boeckh 2010).

1.5.2. *Postnatally acquired CMV in neonates*

Pneumonitis, lymphadenopathy, hepatitis, enterocolitis and aseptic mengingitis have all been associated with postnatal acquisition of CMV infection although antiviral treatment has not shown consistent symptomatic improvement (Barampouti et al. 2002;Cheong et al. 2004;Kumar et al. 1984;Rongkavilit et al. 2004). A 'sepsis-like syndrome' has also been reported in up to 25% of premature babies closely associated with the first detection of CMV in body fluids (Hamprecht et al. 2001). Current evidence does not support any concerns regarding neurological morbidity or SNHL associated with such postnatally acquired infections as reviewed by Luck and Sharland (2009).

1.5.3. *Symptomatic congenital CMV (CMV inclusion disease)*

CMV inclusion disease historically describes the multiorgan pathology observed in the most severely affected newborns with congenital infection. Symptomatic disease at birth is detected in only 13% of all CMV-infected neonates according to a recent meta-analysis (Dollard et al. 2007). Many authors consider only those with observable manifestations of cCMV disease to be symptomatic, thus excluding those babies identified as having SNHL at birth alone. Similarly whether babies with isolated neuro-radiological abnormalities should be considered to have symptomatic disease is contentious with the role of MRI in particular remaining uncertain. Such differences in definitions are partly responsible for the variable figures of symptomatic infection at birth and may have significance when considering long-term follow-up data.

The most common manifestations of symptomatic cCMV at birth are

Table 1-2 Frequency of clinical and laboratory abnormalities in babies born with congenital CMV disease

CLINICAL FEATURES OF CONGENITAL CMV	
CLINICAL	
IUGR	26-50%
Microcephaly	20-53%
Hepato-splenomegaly	45-70%
Petechiae	45-75%
Jaundice	41-70%
Pneumonitis	7.30%
Chorioretinitis	14-17%
Seizures	7-13%
Other neurological abnormalities	19-37%
Dental enamel defects	11%
LABORATORY:	
Thrombocytopenia	50-77%
Anaemia	7%
Raised ALT/AST	48-83%
Conjugated hyperbilirubinaemia	47-81%
CSF abnormalities	46%
IMAGING:	
Abnormal Cr USS	56%
Abnormal CT	71-80%
Abnormal MRI	89%
OTHER	
Abnormal VEP*	43%
Abnormal SSEP*	71%
Abnormal EEG*	67%

* = only reported in Kylat, Kelly, & Ford-Jones (2006)

VEP = Visual Evoked Potential
SSEP = Somatosensory Evoked Potentials
EEG = Electroencephalogram

Data Taken From:
Kylat, Kelly & Ford-Jones (2006)
Boppana et al (1992)
Noyola et al (2001)

hepatosplenomegaly, jaundice, petechiae (associated with underlying thrombocytopenia), blueberry muffin rash (due to extramedullary haematopoiesis), microcephaly and IUGR with variable frequencies (Table 1-2). Pneumonitis and myocarditis are less common. Mortality is reported in 4-12% of those symptomatic at birth (Boppana et al. 1992;Fowler et al. 1999;Kylat, Kelly, & Ford-Jones 2006). Although non-specific for cCMV, a combination of the above clinical findings should certainly raise the suspicion of a congenital infection. IUGR alone, however, can arise following a multitude of antenatal challenges to placental function and whether babies should be considered to have CMV disease with this finding alone is contentious.

CNS features include seizures, focal neurological findings and abnormalities in tone and posture which may be difficult to appreciate clinically in the newborn. Ocular pathology includes chorioretinitis, optic atrophy, pigmentary retinopathy and strabismus (Stagno et al. 1977). The manifestations of cCMV-associated SNHL are described in more detail below.

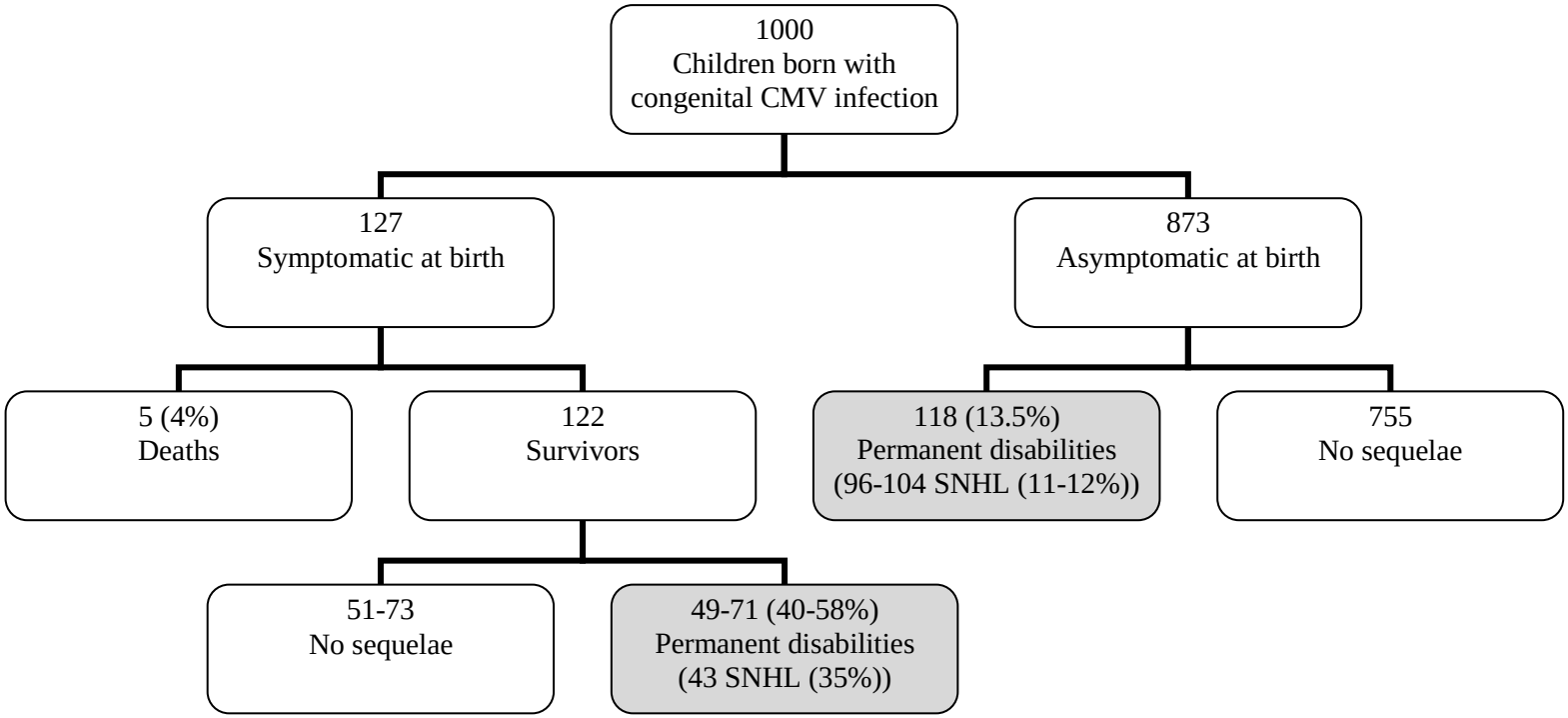
- **Long term sequelae**

Many of the non-CNS symptoms and signs of cCMV resolve over the first few months of life although liver involvement has been reported to persist for some months (Kimberlin et al. 2003). Approximately 50% of those with early signs of disease will exhibit long-term sequelae in some form (Dollard, Grosse, & Ross 2007). Most commonly these manifest as SNHL and developmental delay in one or more areas resulting in significant disability. Visual loss from early chorioretinitis has also been reported along with dental abnormalities and slow growth. Importantly large cohort studies have also reported development of sequelae, particularly SNHL, in 13% of babies considered asymptomatic at birth (Dollard, Grosse, & Ross 2007).

- **Sensorineural hearing loss**

Second only to genetic mutations, cCMV is a leading cause of SNHL at birth and accounts for around 25% of all SNHL at 4 years of age (Nance et al. 2006). Although the risk is greatest in those symptomatic at birth the ‘asymptomatic’ group account for up to 2/3 of the overall burden of CMV-associated SNHL due to the higher absolute numbers of babies in this group (Figure 1-4)(Dahle et al. 2000).

Figure 1-4 Estimated frequency of sequelae in 1000 children born with congenital CMV infecion



Figures from Dollard, Grosse, & Ross (2007)
SNHL = Sensorineural hearing loss
69-71% of babies destined to have CMV-attributable SNHL will be asymptomatic at birth

A number of large cohort studies from Birmingham Alabama provide the most comprehensive data of SNHL in congenitally infected babies to date. These studies found hearing loss in 22-65% of babies with symptomatic CMV compared to 6-23% of those asymptomatic at birth (Fowler and Boppana 2006). Although the Alabama population may not be representative of the situation worldwide, a recent meta-analysis reported overall hearing loss in around 35% compared to 11-12% of those born with symptomatic and asymptomatic cCMV at birth respectively, which would be consistent with these earlier data (Dollard, Grosse, & Ross 2007).

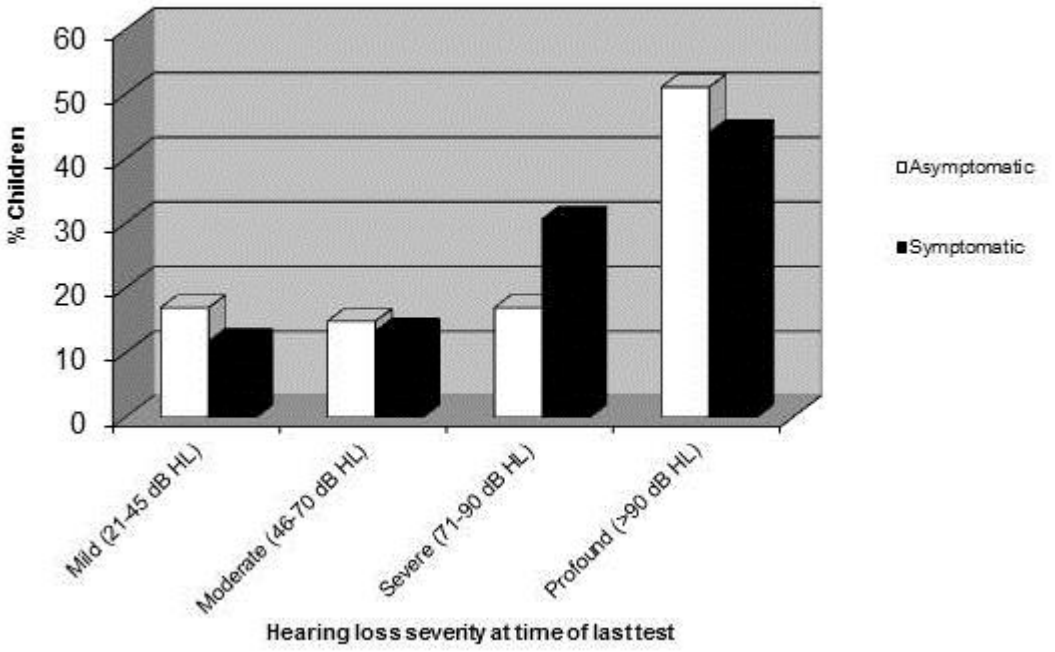
These cohort studies have demonstrated that SNHL can be of any type and severity with severe or profound hearing loss documented in around 70% of those with symptomatic or asymptomatic infection (Figure 1-5) (Dahle et al. 2000); bilateral hearing loss is reported in 67% of those with symptomatic infection, however, compared to 48% of asymptomatic babies. Late (delayed) onset hearing loss is also described in approximately 27% and 38% of those symptomatic and asymptomatic at birth respectively with cCMV-associated SNHL. Significantly this means that less than half of individuals destined to have SNHL attributable to cCMV will have deficits detectable on newborn hearing screening at birth (Fowler et al. 1999). Also characteristic of cCMV is the progressive loss observed in around 50% of affected babies (Dahle et al. 2000). The pathogenesis of this delayed and progressive loss remains unknown. Fluctuations in hearing are also reported (defined as deterioration in hearing assessment followed by subsequent improvement with no other obvious cause) with this phenomenon being more frequently described in asymptomatic babies (54% vs 29% in symptomatic) (Dahle et al. 2000).

If cumulative hearing loss is plotted, the majority of SNHL presents in the prelingual phase of development with symptomatic babies having a slightly earlier onset of hearing loss when compared to those that are asymptomatic (median onset 33 months compared to 44 months) (Dahle et al. 2000)(Figure 1-6).

- **Neurological progression**

Long-term cognitive deficit has been documented in around 42% of symptomatic babies and 6.5% of asymptomatic babies on meta-analysis (Dollard, Grosse, & Ross 2007). Seizures are common and have been reported in 13% of babies <6 months of

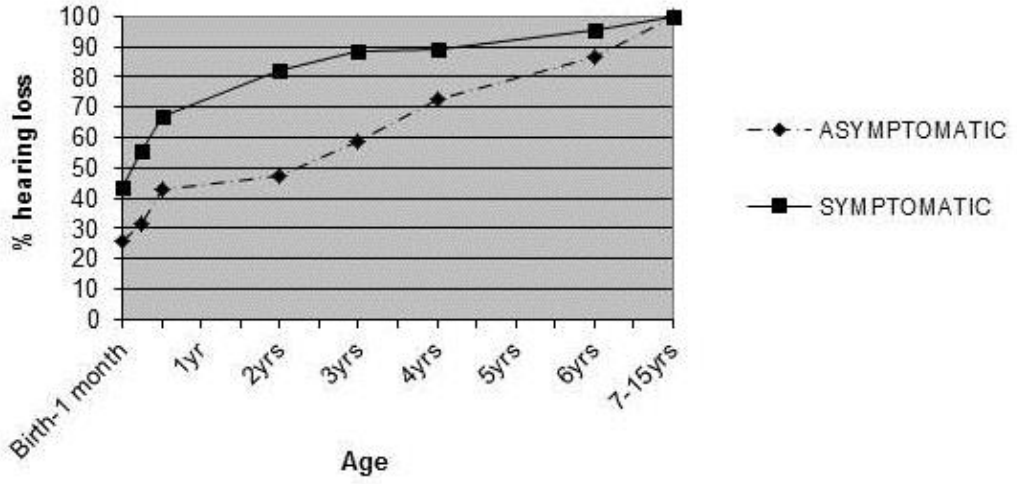
Figure 1-5: Severity of SNHL reported at last hearing test in babies with 'symptomatic' or 'asymptomatic' congenital CMV at birth



Degree of SNHL based on average pure-tone thresholds at last audiologic evaluation (percentage of ears) in babies with ('symptomatic') and without ('asymptomatic') clinically evident CMV disease at birth. If SNHL was determined by auditory brainstem response, hearing thresholds were calculated based on nHL as follows: mild (30-45 dB), moderate (50-70 dB), severe (>70 dB). Chronological age at last evaluation ranged from 1month to 19 years (median 5 years).

Data taken from Dahle et al. (2000)

Figure 1-6: Cumulative hearing loss over time in 85 babies with 'symptomatic' and 48 with 'asymptomatic' congenital CMV infection at birth



Hearing was measured by either auditory brainstem response or pure-tone audiometry depending on the child's age and developmental level. The criteria for hearing loss for pure-tone audiometry was the presence of thresholds greater than 20 dB HL at one or more frequencies. For ABR, hearing loss consisted of thresholds greater than 25 dB nHL. Chronological age at last evaluation range 1month – 19 years; Median 5 years

Data taken from Dahle et al. (2000)

age and a further 16% later in childhood (Kylat, Kelly, & Ford-Jones 2006). Some studies have reported delayed detection of neurological manifestations although it is unclear whether this is due to true clinical progression or difficulties in detecting subtle neurological impairments in newborns (Conboy et al. 1986;Pass et al. 1980;Reynolds et al. 1974;Stagno et al. 1977).

- **Visual loss**

Early studies reported chorioretinitis associated with both symptomatic and asymptomatic cCMV infection (Stagno et al. 1977). As with other sequelae, symptomatic babies are more likely to have long-term visual impairment than those asymptomatic at birth (22-50%) (Anderson et al. 1996;Coats et al. 2000). Although generally thought to be stable in immunocompetent children case studies reporting delayed onset, reactivation and progression of CMV chorioretinitis in those congenitally infected would seem to refute this (Barampouti, Rajan, & Aclimandos 2002;Boppana et al. 1994).

1.6. RISK FACTORS FOR SYMPTOMATIC DISEASE

There has been much interest in finding markers present at birth which predict long-term sequelae of CMV disease. Studies are generally weakened by the inclusion of only small numbers of asymptomatic babies that subsequently develop sequelae. It is arguably this group of babies that present the biggest management challenge and that early treatment might be hypothesised to benefit.

1.6.1. *Antenatal factors*

Severity of placental pathology has been associated with the severity of manifestations in the newborn (Mostoufi-zadeh et al. 1984).

In addition to increasing likelihood of CMV transmission, primary rather than non-primary infection during pregnancy has been shown to be associated with more frequent and more severe neonatal disease (Foulon et al. 2008;Fowler et al. 1992;Ross et al. 2006;Yamamoto et al. 2011). However, symptomatic cCMV is still reported in women with pre-existing immunity and in populations with a high seroprevalence the majority of affected babies are born to women with previous exposure to CMV (Ahlfors et al. 1983;Colugnati et al. 2007;Yamamoto et al. 2011).

In keeping with observations in other congenital infections it is now generally accepted that infection in the third trimester, although associated with increased transmission, is likely to carry a smaller risk of severe symptomatic disease (Daiminger et al. 2005;Liesnard et al. 2000;Revello and Gerna 2004). This might relate to enhanced concomitant transfer of maternal anti-CMV antibody at these later gestations or to the more advanced development of the CNS and/or immune system in these more mature fetuses.

Babies with abnormal antenatal ultrasound scan (USS) findings are more likely to be born with symptomatic infection. However, one Italian study reported symptomatic infection in 68/131 (52%) congenitally infected babies with normal USS (Guerra et al. 2008). Another, more recent, study reported enhanced sensitivity of detection of cerebral lesions with prenatal MRI with a sensitivity of 83% for predicting symptomatic infection and a corresponding negative predictive value (NPV) of 94% (Doneda et al. 2010). Other authors have proposed additional prognostic value in conducting fetal blood sampling (FBS) to determine fetal platelet counts in addition to serial USS, despite the associated 1-3% risk of fetal loss with this procedure (Benoist et al. 2008;Fabbri et al. 2011).

The utility of measuring amniotic fluid VL is discussed further below (see 1.6.4).

1.6.2. *Clinical findings at birth as predictors for long term sequelae*

Petechiae and IUGR at birth have been shown to be independent risk factors for subsequent hearing impairment and petechiae have additionally been associated with abnormal CT findings (Boppana et al. 1997;Rivera et al. 2002). Other studies have reported microcephaly (taking into account whether head circumference (OFC) was appropriate for weight) as being the most specific predictor for poor neurological outcome with the combination of normal CT and head circumference being associated with a good prognosis in those with symptoms at birth (Noyola et al. 2001).

1.6.3. *Neuroimaging and long term sequelae*

Cranial USS has a good positive predictive value (PPV) and NPV with regards to subsequent neurological abnormalities in all CMV infected neonates (Ancora et al.

2007). Conversely in symptomatic infection CT scan has been shown to have a good NPV but only 50% PPV in identifying a poor neurodevelopmental outcome (Noyola et al. 2001). Although CT has a better sensitivity for detecting intracerebral calcifications in one recent study it added nothing diagnostically to cranial USS (Ancora et al. 2007). MRI has, however, been found to identify additional information in 6/8 children in 2 unrelated studies (Ancora et al. 2007;de Vries et al. 2004).

There is currently no literature evaluating the prognostic benefit of carrying out MRI in large cohorts of asymptomatic babies in addition to cranial USS. It also remains largely unknown whether more subtle changes, such as lentriculostriatal vasculopathy, should be considered indicative of symptomatic CNS disease.

1.6.4. *Viral load (threshold concept)*

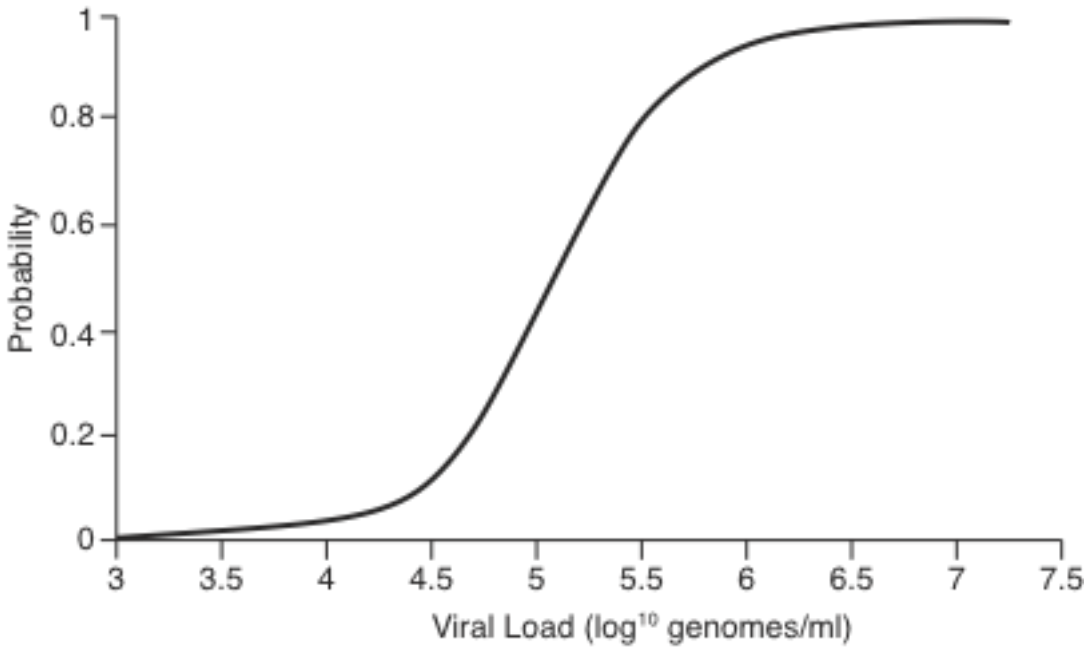
- **Adult transplant patients (threshold concept)**

A series of studies conducted in adult transplant patients have shown that risk of CMV disease is associated with blood VL independent of donor seropositivity (Cope et al. 1997a;Gor et al. 1998;Hassan-Walker et al. 1999). VL in urine was also shown to be associated with disease in renal transplant patients independent of presence of viraemia (Cope et al. 1997b). Indeed, VL seemed to fully account for the increased risk of disease in seronegative patients. Both peak VL and rate of increase are associated with increased risk of CMV disease in these transplant recipients (Cope et al. 1997a;Emery et al. 2000).

These observations led to the proposal of a 'threshold' effect with a defined level of viraemia being significantly associated with risk of disease; such was this association that many transplant centres have since adopted a 'pre-emptive' strategy to CMV prophylaxis (Figure 1-7)(Cope et al. 1997a). Such treatment strategies have proposed benefits in decreasing both subsequent 'late-onset' CMV complications and healthcare costs (Emery 2001b). Similarly VL has been shown to be of predictive value in those with HIV that develop CMV disease (Bowen et al. 1997).

These observations have led to interest in whether a similar threshold for disease exists in those congenitally infected.

Figure 1-7 Blood viral load and association with probability of CMV disease in liver transplant patients



Disease probability curves computed from univariate logistic regression analysis of blood virus load in liver transplant recipients. CMV viral load was measured using quantitative CMV DNA PCR.

Reproduced from Cope et al. (1997a)

- **Congenital CMV**

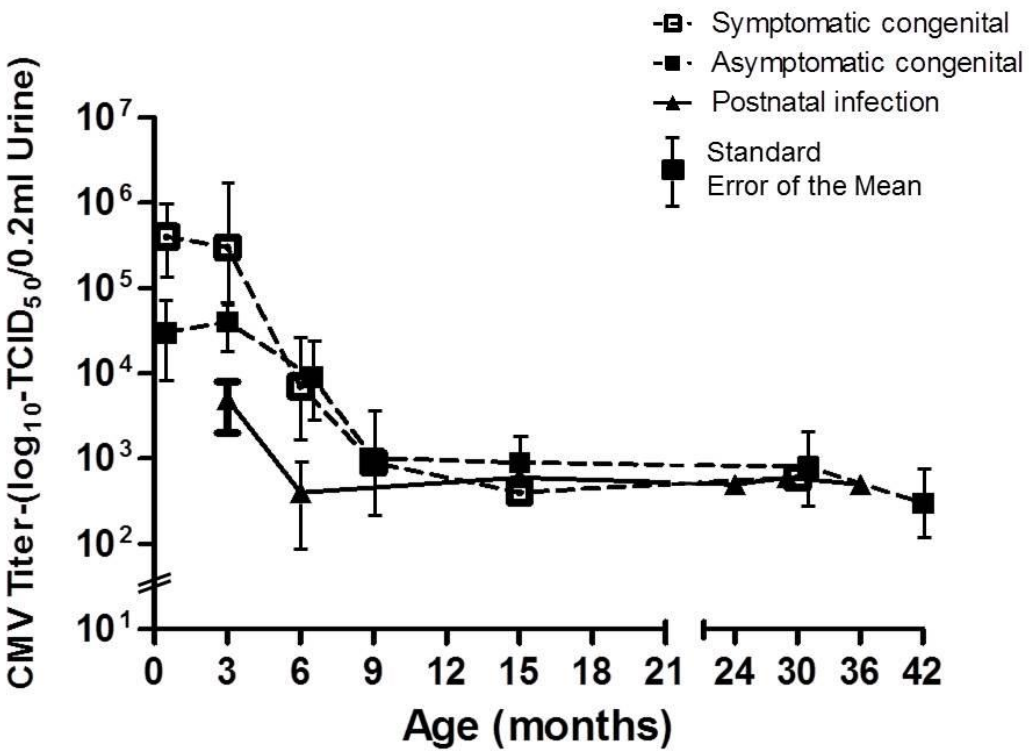
Stagno *et al* (1975) showed in their early studies of the natural history of cytomegalovirus infection that the amount of virus detectable in urine using tissue culture was significantly higher in those babies with symptomatic infection over the first 6 months of life when compared to those without clinically evident infection (Figure 1-8). Furthermore virus titer was higher in congenitally infected babies when compared to those postnatally infected up to 9 months of life.

More recent studies have not only associated the detection of CMV in the blood with symptomatic infection but have also found similar quantitative associations between CMV DNA PCR in blood, urine and DBS and clinical severity of disease (Boppana et al. 2005;Bradford et al. 2005;Lanari et al. 2006;Rivera et al. 2002;Walter et al. 2008). Amniotic fluid VL similarly correlates with symptomatic cCMV in the newborn although the overlap between groups limits utility of this finding in clinical practice (Revello and Gerna 2002). Asymptomatic babies are generally under-represented in these cohorts and many babies without symptoms have comparable VL to those with disease. Low/undetectable virus, however, is consistently reported to be associated with a good overall prognosis and a good NPV for disease; research in this area is ongoing.

There are conflicting data reporting on the duration of CMV excretion and symptoms with viruria being variably reported as decreased and increased in congenitally infected neonates developing SNHL (Noyola et al. 2000;Rosenthal et al. 2009). Noyola et al. (2000) reported no significant difference, however, in duration of viruria in symptomatic compared to asymptomatic neonates.

As discussed in 1.4.3 above the value of obtaining CSF and perilymph in CMV-infected newborns has not been fully evaluated in asymptomatic babies. Whether detection of virus or VL in these samples is predictive of subsequent neurological sequelae is therefore currently unknown.

Figure 1-8 Quantitative viruria over time in babies infected with cytomegalovirus congenitally (symptomatic and asymptomatic) and postnatally



Data shown are from 38 infants with congenital infection (8 with symptoms of disease at birth) and 17 with postnatal infection. Congenital infection was confirmed by the presence of viruria within the first week of life.
Viruia was quantified using titration of tissue cultures of human skin fibroblasts.

Redrawn from Stagno et al. (1975)

1.6.5. *Role of genetic polymorphisms*

It has been demonstrated by a number of authors that multiple strains of CMV may coexist in congenitally infected infants (Arav-Boger et al. 2002;Ross et al. 2011). There has been interest in whether specific genetic subtypes predict symptomatic infection. Studies have failed to show an association between subtypes of US28, gB or UL146 and UL147 despite demonstrating an unexpectedly diverse genetic variation in the UL146 gene locus which encodes for an α–chemokine homolog (Arav-Boger et al. 2006b). Arav-Boger *et al* described an association between UL144 subtype C and risk of symptomatic infection (Arav-Boger et al. 2002;Arav-Boger et al. 2006a;Arav-Boger et al. 2006b). This has not, however, been confirmed by other researchers (Picone et al. 2005).

1.7. IMMUNE RESPONSE

Many CMV gene products have a proposed role in evading usual host immune responses which include the down regulation of MHC Class I and II expression, production of MHC Class I-related decoy molecules, production of chemokine and chemokine receptor homologues along with the ability to alter cell signalling pathways as reviewed in detail elsewhere (Mocarski 2004;Ploegh 1998). A full understanding of these interactions is further complicated by the discrepancies often reported between data derived from animal models, *in vitro* systems and *in vivo* observations (Goodrum, Caviness, & Zagallo 2012).

Despite these diverse immune evasive strategies a large proportion of the T-cell repertoire in CMV-infected hosts has been shown to be directed towards CMV peptides and immune evasion genes themselves can be targets of CD8+ T cells (Elkington et al. 2003;Sylwester et al. 2005).

The decreased need for antiviral treatment for CMV in HIV infected patients associated with use of highly active anti-retroviral therapy signifies the importance of host immunological responses in the overall control of CMV in this situation. A complex balance therefore exists between virus, host immune responses and virus immunomodulation.

1.7.1. *Innate immunity*

CMV has been reported to employ multiple strategies to decrease NK cell receptor activity including encoding genes which interfere with NK cell receptor binding with activation ligands along with enhanced signalling through inhibitory NK receptors as reviewed by Jackson et al (2011). The process of CMV binding to and entering cells also activates production of interferon and inflammatory cytokines through TLR-2 recognition of gB and gH (Isaacson et al. 2008).

1.7.2. *Adaptive immunity - antibody mediated*

The envelope glycoprotein, gB (gpUL55), has been proposed as the predominant target for neutralising antibodies, accounting for 40-70% of the total serum neutralising response (Britt et al. 1990). Recent studies evaluating CMV hyperimmune globulin, however, have implicated pentameric gH/gL/UL128-131A complexes as the predominant target for neutralising antibodies and *in vitro* studies using endothelial cell lines (the cell line predominantly infected *in vivo*) have also challenged the role of gB as the major neutralising epitope (Macagno et al. 2010).

Clinical studies would infer that antibodies are important in preventing infection based on decreased transmission from seropositive mothers and in seropositive transplant recipients as described earlier. Passive transfer of maternal antibody has also been proposed as a contributory factor to the lack of disease associated with postnatally transmitted infection and as an explanation for the altered CMV–specific antibody responses observed in postnatally compared to congenitally infected babies (Stagno et al. 1975). Recent studies investigating the use of a recombinant gB vaccine have correspondingly shown some promising initial results (Griffiths et al. 2011;Pass et al. 2009). The contribution of antibody to overall virus control remains undefined, however, and intact cellular immune responses are also likely to be necessary to minimise symptomatic infection and maintain CMV in a latent state following primary infection.

1.7.3. *Adaptive immunity – cellular immune responses*

In adults symptomatic patients have been observed to have impaired cellular immunity with both functional and phenotypic differences in T cells. Broadly

speaking chronic CMV infection induces phenotypic changes favouring a CD28-/CD27- CD8+ "late" T-cell phenotype and cells characteristically express perforin, granzyme and other cytotoxic effectors but lack the chemokine receptor CCR7. This pattern is distinct when compared to other chronic viral infections (Appay et al. 2002). Given the asymptomatic nature of infection, responses during primary infection in immunocompetent adults are not easy to define. During primary infection in renal transplant recipients, however, CD8+ T cells are detectable shortly after CMV antibody responses and show a similar activated phenotype with high expression of Ki67, granzyme B, perforin and CD45RO+, CCR7-, CD28-; they are predominantly CD27+, however (Gamadia et al. 2003;van de Berg et al. 2008).

Immune therapy with reconstitution of CMV-specific CD8+ lymphocytes has been shown to decrease active disease and reactivation of infection in mice and humans highlighting the important role of this component of host immunity in immunocompromised individuals (Riddell et al. 1992;Steffens et al. 1998;Walter et al. 1995). The appearance of late onset CMV disease has similarly been proposed to be due to the intense viral suppression accompanying prophylactic antiviral treatment strategies and a consequent delay in reconstitution of CMV-specific immune responses (Boeckh et al. 2003;Li et al. 1994).

Studies using HLA-matched Class I tetramers incorporating overlapping peptides from 213 known or predicted HCMV open reading frames (ORFs) have shown that 10% of the overall CD8+ and CD4+ memory T-cell pool is able to recognise CMV (Sylwester et al. 2005). Although these studies demonstrated T-cell responses to a diverse range of CMV epitopes in individual hosts, responses to pp65 and IE1 are consistently present and remain relatively immunodominant between subjects. Responses to pp65 and IE1, however, only represent ~40% of all T-cell responses to CMV. Detail of other known Class I and Class II T lymphocyte epitopes are reviewed elsewhere (Gandhi and Khanna 2004). A direct correlation between viral load and virus specific CD8+ve T cells has been shown in HIV (Ogg et al. 1998). Similar correlations have also now been shown between the calculated proportion of pp65-specific CD8+ T-cells secreting IFNg and CMV VL (Mattes et al. 2008). No association was demonstrated between CD8+ T-cell responses and virus shedding, however, in healthy children (Chen et al. 2004).

Other authors have proposed a more pivotal role for IFN-g secreting CD4+ cells with CD8+ effector cells and CMV-specific antibody alone being ineffective in controlling virus in renal transplant and allogeneic stem cell transplant patients (Gamadia et al. 2003;Widmann et al. 2008). Consistent with this observation a longer duration of virus shedding has been observed in healthy children associated with deficient CD4+ T cell immunity (Tu et al. 2004).

High numbers of CD57+ T cells have been found associated with CMV seropositivity in a number of situations and are proposed to result from chronic antigenic stimulation (Brenchley et al. 2003;Wang et al. 1997;Wang and Borysiewicz 1995). CD57+ve lymphocytes have been shown to have decreased proliferative capacity and expression of CD57 is thought to be independently associated with replicative senescence and an increased susceptibility to cell apoptosis on activation along with decreased telomere length (Brenchley et al. 2003). This may have relevance when considering the "immune risk" phenotype also recently described in elderly CMV seropositive subjects (Pawelec et al. 2009).

1.7.4. *Differences in immune responses to CMV in congenitally infected babies*

Previous studies have suggested various abnormalities in the immune response to CMV in young children and between those with symptomatic and asymptomatic infection.

Activated NK cells are decreased in symptomatic compared to asymptomatic congenitally infected infants with low numbers of activated CD16+ NK cells being significantly correlated with decreased killing of CMV-infected fibroblasts (Cauda et al. 1987).

Studies in neonates suggest similar phenotypic changes in lymphocyte populations during acute CMV infection to adults with loss of CD28 and CD27 along with expression of Ki67 and RA to RO+ shift (Marchant et al. 2003;Miles et al. 2008). These changes are distinct in infected neonates compared to healthy, uninfected controls (Elbou Ould et al. 2004). However, lymphocyte proliferation and IFN-g production have been shown to be decreased in both congenitally and postnatally infected infants compared to adults (Elbou Ould et al. 2004;Pass et al. 1983b). These

deficits are more evident in younger infants (less than 1-2 years of age) and seem to be a selective defect in response to stimulation with CMV antigens as other stimuli do produce responses (Elbou Ould et al. 2004;Gehrz et al. 1982;Hayashi et al. 2003;Pass et al. 1983b). Indeed, enhanced activation has been observed following non-specific antigen stimulation in CMV-infected infants compared to healthy neonatal controls (Elbou Ould et al. 2004). Correspondingly Hayashi et al, also found decreased CMV specific CD8+ lymphocyte frequencies in HLA-A24-positive babies with symptomatic cCMV compared to postnatally infected infants or adults (Hayashi et al. 2003). Others have demonstrated T-cell responses but propose that younger infants might respond preferentially to IE1, rather than pp65 as seen in adults, particularly in those that were not HLA-A2 positive (Gibson et al. 2004).

Results comparing asymptomatic and symptomatic populations are conflicting with authors variably reporting increased and decreased markers of T cell activation and cytotoxic killing in asymptomatic compared to symptomatic babies (Cauda et al. 1987;Rola-Pleszczynski et al. 1977). Decreased numbers and activation of CD4+ T cells have been demonstrated in symptomatic compared to asymptomatic congenitally infected infants (mean age 4 years) and uninfected controls (Cauda et al. 1987;Elbou Ould et al. 2004).

In contradiction to speculation that immune tolerance might play a role in these observations are studies of fetal immune responses which have shown CMV-specific responses in fetuses as young as 28 weeks gestation (Elbou Ould et al. 2004;Marchant et al. 2003).

1.8. ANTIVIRAL TREATMENT

There are currently only four licensed drugs for the systemic treatment of CMV, all of which act on the viral DNA polymerase and have major side effects which limit their use. The two drugs most commonly used for the treatment of cCMV are GCV and its oral prodrug VGCV. These drugs and their clinical utility in the treatment of cCMV are therefore discussed in more detail below. Cidofovir and foscarnet are rarely used in the treatment of cCMV and data are sparse; these drugs are therefore not discussed further here.

1.8.1. *Ganciclovir*

2'-deoxyguanosine, GCV, has been used in children since 1989 (Fan-Havard et al. 1989). It is a synthetic guanosine analogue which undergoes phosphorylation in infected cells to its triphosphorylated form (Figure 1-9). The first phosphorylation is initiated by a protein kinase homolog encoded by HCMV pUL97, meaning that phosphorylation occurs preferentially in infected cells, thus ensuring high levels of active drug in these cells (Biron et al. 1985;Sullivan et al. 1992). Intracellular GCV-triphosphate has a half-life of around 16.5 hours making it additionally effective in inhibiting CMV replication (Crumpacker 1996). Although GCV-triphosphate is a competitive inhibitor of the viral DNA polymerase unlike acyclovir triphosphate it is not an obligate chain terminator meaning that synthesis of viral DNA continues, albeit at a slower rate, with an associated risk of side effects including mutagenicity if drug is activated in non-infected cells.

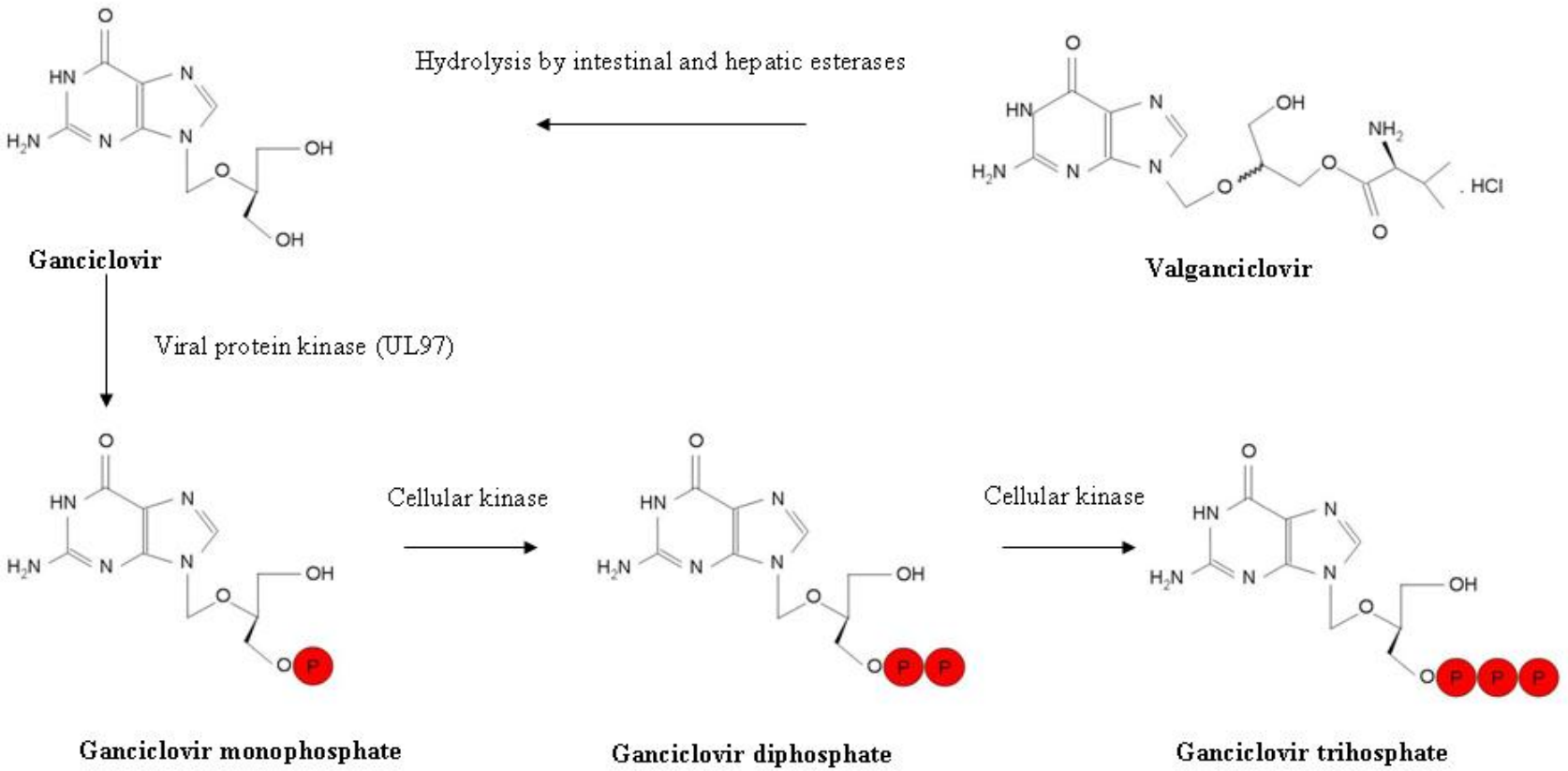
- **Formulation and dose, pharmacokinetics**

The drug is excreted by the kidneys, with almost 100% of drug eliminated unchanged in the urine (Jacobson et al. 1987); dose must therefore be adjusted in renal impairment. There is minimal protein binding.

In vitro minimum inhibitory concentration (MIC) and 50% inhibition of virus replication (IC_{50}) for GCV are reported to be 0.31-1.63 mcg/ml and 0.02-3.48 mcg/mL respectively (Fishman et al. 2000). Peak concentrations of GCV of 4.5-10mcg/mL and trough levels of 0.8mcg/mL have been reported in adults receiving doses of 7.5-10mg/kg/day i.v. GCV in two divided doses (Crumpacker 1996). Oral doses of 1000mg GCV three times daily were recommended for prophylaxis but due to the low bioavailability (~6%) and correspondingly low peak levels achieved the use of this drug has been superseded by oral VGCV and oral GCV is not currently available. Bioavailability in children has been reported to be even more problematic (Frenkel et al. 2000).

Levels of 0.7 mcg/mL have been detected in the CSF 3.5 hours after a 2.5mg/kg dose of i.v. GCV where simultaneous serum concentrations were 2.2mcg/mL (Fletcher et al. 1986). These levels only just reach the MIC for GCV and may thus limit efficacy in this body compartment.

Figure 1-9 Structure of ganciclovir and valganciclovir and steps to tri-phosphorylated, active drug



Reports of the use of GCV in children emerged in the late eighties; over the subsequent two decades there have been very few further pharmacological studies in the paediatric age group and even fewer involving neonates. Rapid changes in many parameters impacting on pharmacokinetics (PK), including renal function, make dosing in the neonatal age group particularly challenging. In neonates plasma levels comparable to those found in adults can be achieved with i.v. GCV doses of 6mg/kg twice daily although some studies report two-fold higher levels in adults compared to neonates (Table 1-3)(Pacifici 2005;Trang et al. 1993;Zhou et al. 1996). Changes in body weight and age have been correlated with changes in the volume of distribution (Vd) and creatinine clearance (Cl) of GCV respectively (Trang et al. 1993;Zhou et al. 1996). A more recent study in congenitally infected infants aged 30 days or less at onset of treatment demonstrated a 73% increase in renal excretion of GCV from 3.8mL/min/kg to 6.8mL/min/kg between days 4 and 34 of treatment, and a corresponding decrease in area under curve (AUC) of 41% over the same time period, following a dose of 6mg/kg i.v. GCV (Kimberlin et al. 2008). Furthermore in contrast to the multicompartmental PK reported in adults, neonatal studies have shown that PK is adequately described by a single-compartment model with zero-order input and first-order elimination (Trang et al. 1993).

- **Toxicity**

Toxicity limits the use of most antivirals effective against CMV with one of the commonest side effects of GCV being bone marrow suppression. Grade 3-4 neutropenia (based on NIAID DMID toxicity definitions) has been reported in around 65% of babies undergoing treatment with i.v. GCV often necessitating a decrease in dose (Kimberlin et al. 2003;Whitley et al. 1997). This is in contrast to levels of neutropenia of around 15% quoted in adult studies. Neurotoxicity has been rarely reported in adults, generally in association with decreased renal function (Ernst and Franey 1998). Although uncommon this emphasizes the need for close monitoring of both haematological and biochemical parameters during treatment.

Animal models have shown decreases in spermatogenesis causing infertility associated with treatment. Increased tumours, lymphoma cell mutagenesis and DNA damage have also been reported in rodent models with less drug exposure than

Table 1-3: Pharmacokinetics of ganciclovir in neonates and adults

Age	Single dose+	Cl (l/h/kg)	Vd (l/kg)	Cmax (µg/ml)	$T_{1/2}$ (h)	Ref	No. cases
4-49 days	4mg/kg	0.19 ± 0.03	0.67 ± 0.07	5.5 ± 0.4	2.4 ± 0.4	Trang, 1993	14
2-30 days	6mg/kg	0.21 ± 0.02	0.75 ± 0.07	7.0 ± 0.5	2.4 ± 0.4	Trang, 1993	13
n.a. (newborns)	4-6mg/kg	0.42 ± 0.08	1.8 ± 0.32	n.a.	n.a.	Zhou, 1996	27
Adult	5mg/kg	0.28 ± 0.11	1.1 ± 0.30	6.6 ± 0.8‡	4.3 ± 1.6	Pacifici, 2005	n.a.

Figures are mean ± SEM

\+ = Ganciclovir was administered by an intravenous infusion over 1 hour
‡ = Following a single 6mg/kg dose over 1 hour of infusion.

n.a. = not available
Cl = clearance
C_{max} = Maximum drug concentration
SEM = Standard error of mean
$T_{1/2}$ = drug half-life
Vd = Volume of distribution.

Table adapted from Pacifici (2005)

anticipated in humans (Genentech 2013;Tomicic et al. 2002). Data show that GCV crosses the placenta, probably by simple diffusion, resulting in both embryotoxicity and teratogenicity in animal models (Genentech 2013).

In view of the concerns relating to carcinogenesis and gonadal toxicity, long-term follow-up has been conducted of 47 children enrolled as neonates into a phase II trial (NCT 00031421). Although unpublished no adverse effects have been noted in relation to cancer incidence, sexual development or pubertal development in this small number of subjects (personal communication).

- **Resistance**

Resistance to GCV can be secondary to mutations in pUL97 or in the viral polymerase encoded by the UL54 gene. Recent studies have detected a number of mutations in pUL97 with only a small number conferring GCV resistance (Chou 2010). Resistance in adult studies is associated with low plasma levels of GCV; the use of oral GCV for prolonged periods was correspondingly proposed to select for resistance (Emery and Griffiths 2000).

1.8.2. *Valganciclovir*

VGCV is the L-valyl-ester prodrug of GCV and after oral administration it is rapidly converted to GCV by hepatic and gastrointestinal esterases (Figure 1-9) (Razonable and Paya 2004). Unlike GCV, VGCV is a substrate for peptide transporters in the intestinal wall, thus explaining its enhanced bioavailability. It was licensed for use in adult transplant recipients in 2001 in the USA and a few years later gained a European marketing authorisation for use as prophylaxis against CMV disease in solid organ transplant recipients.

- **Formulation and dose, PK**

At around 60%, oral bioavailability in adults is around 10 times greater than for oral GCV (Sugawara et al. 2000). More recently, oral bioavailability in neonates and children has been shown to be around 50% with an increased bioavailability reported over time in neonates sampled over a 6 week treatment period (Acosta et al. 2007;Burri et al. 2004).

Adult studies have shown GCV C_{max} following oral VGCV administration is comparable to that achieved with iv GCV (Razonable and Paya 2004). VGCV was originally available only as tablets with subsequent reports of extemporaneously produced VGCV solution being used in the paediatric age group (Anaizi et al. 2002;Henkin et al. 2003). Oral VGCV solution gained marketing authorisation in the UK in 2009. At the onset of this thesis uncertainty therefore existed regarding dosing of VGCV in infants.

There is no published literature regarding CSF concentrations of GCV following oral VGCV administration. Data from studies of oral valaciclovir, however, show stable levels of aciclovir in the CSF when compared to serum with data indicating slow passive diffusion into this compartment and active transport out (Lycke et al. 2003). This might imply that peak serum levels of nucleoside drugs are not as important for CSF penetration as AUC.

- **Toxicity**

Side effects are essentially those of GCV with some reported increase in gastrointestinal side effects (Martin et al. 2002). Neurotoxicity has been reported in one child treated with VGCV (Peyriere et al. 2006). The same cautions regarding possible long-term carcinogenicity and impaired fertility need to be taken into consideration although no long term data currently exist for humans.

1.8.3. *Treatment efficacy and pharmacodynamics*

- **Clinical efficacy in adults**

In adults GCV has been used to treat and prevent CMV disease in transplant patients and those with HIV with variable efficacy dependent on the underlying condition, drug used (including formulation and dose) and clinical stage at time of treatment initiation (Griffiths 2009). Once clinical manifestations of disease are present treatment is of limited efficacy in preventing long term morbidity in either of these patient groups. Where disease processes have already been initiated the host immune response to CMV is therefore likely critical in continuing to cause tissue damage even once virus burden is decreased by antiviral treatment. Treatment strategies in transplant patients therefore consist of either prophylactic therapy (antiviral agents

commenced dependent on certain risk factors irrespective of virus detection) or pre-emptive therapy (antiviral therapy commenced at an asymptomatic stage generally dependent on predetermined levels of virus detection) with advocates for both strategies (Singh 2006a;Snydman 2006). The main proposed benefit of pre-emptive strategies is a decrease in late-onset disease which has emerged as a significant problem with the prophylactic use of potent antivirals (Singh 2006b). Studies to date have generally reported comparable efficacy between oral VGCV and i.v. GCV, including small studies in children, with the exception of liver transplant recipients (Clark et al. 2004;Jain et al. 2005;Paya et al. 2004).

Antiviral treatment for CMV retinitis is generally successful in preventing sight-loss in those with AIDS and it may be of some benefit in treating CMV polyradiculitis and other CMV-related peripheral neuropathies but is rarely efficacious in treating CMV ventriculoencephalitis (Cinque, Marenzi, & Ceresa 1997). It is speculated that this might be due to the low levels of GCV detectable in the CSF which are close to the *in vitro* MIC for CMV. In support of this CMV has been detected in CSF, with no associated pp65 antigenaemia, at the end of 3 weeks treatment with GCV and at autopsy in HIV patients with diffuse encephalopathy that subsequently died (Cinque et al. 1995).

The majority of studies of clinical efficacy in adults focus on acute CMV-related morbidity and mortality post-transplantation or the clinically visible manifestations of HIV retinitis. The ongoing uncertainty regarding the best therapeutic options to prevent late-onset disease post-transplantation highlight the difficulties of obtaining longer term follow-up data of less clearly defined morbidities and the need for early biomarkers for such morbidities.

- **Virological efficacy in adults**

Virological efficacy has been described in adult patient groups with 71% of transplant patients with a median starting VL of 4.3 $\log_{10}$ (range 2.7-5.9) clearing viraemia within 14 days of starting pre-emptive i.v. GCV treatment; none of 24 failed to clear viraemia within 50 days of treatment (Mattes et al. 2004). Similarly median duration of detectable viraemia of 14 and 15 days were observed in patients treated with GCV and oral VGCV respectively in a subsequent study in transplant

recipients with similar viral dynamics reported in both treatment groups. A number of subjects failed to become CMV PCR negative, however, even after treatment courses of up to 49 days (Mattes et al. 2005). Likewise 85% of 45 patients with HIV and CMV retinitis who were CMV PCR positive at treatment onset had no viraemia 21 days after commencing i.v. GCV (Bowen et al. 1996). Those that remained viraemic had a higher starting VL and a shorter time to disease progression and death (Bowen et al. 1996).

- **Congenital CMV clinical efficacy**

The Collaborative Antiviral Study Group (CASG) has conducted a series of studies evaluating the optimal treatment for babies with cCMV. The first of these studies compared 6mg/kg i.v. GCV twice daily (bd) with a lower dose of 4mg/kg bd and showed the higher dose suppressed virus more effectively with no significant increase in side effects (Whitley et al. 1997). Improved clinical outcome was also reported compared to historical data. A subsequent randomised controlled trial (RCT) found that 6 weeks treatment with this higher dose of GCV decreased long-term sequelae in babies with cCMV and CNS disease (Kimberlin et al. 2003). Although treatment was randomized there was no placebo arm, as this would have necessitated central line insertion which was not thought ethical. Results were further confounded by the availability of follow-up data in only 42 of the 100 subjects initially enrolled. Despite these limitations a significant treatment advantage was shown with 0/25 treated babies having hearing deterioration from baseline at 6 months compared to 7/17 (41%) of controls ($p < 0.01$); this difference remained significant at one year follow-up. Short-term benefits were also reported in growth and resolution of liver function. Subsequent neurological follow-up of these babies also showed fewer neurodevelopmental delays in the treated cohort (Oliver et al. 2009).

Other, uncontrolled, case cohort studies have reported a benefit from antiviral treatment of symptomatic babies at birth (Halwachs-Baumann et al. 2002;Michaels et al. 2003;Nigro et al. 1994;Rojo and Ramos 2004;Tanaka-Kitajima et al. 2005;Whitley et al. 1997). Duration of treatment and dose of medication varies greatly in these cohorts, however, as summarised by Luck et al. (2006). Recent

studies have reported clinical benefit with higher drug doses and longer treatment courses, particularly with the availability of oral VGCV solutions (Amir et al. 2010;Baquero-Artigao and Romero Gomez 2009;Meine Jansen et al. 2005). Publication bias is obviously of concern and the need for placebo-controlled RCTs is further highlighted by the fluctuations in SNHL described earlier (Dahle et al. 2000).

- **Congenital CMV virological efficacy**

An association between clinical efficacy and suppression of viruria at treatment termination was shown in early studies but virus was seen to subsequently rebound after treatment cessation (Nigro, Scholz, & Bartmann 1994;Whitley et al. 1997). There were no large studies reporting on virological efficacy with treatment for cCMV at the start of my work.

1.9. AIMS OF THESIS

The aims of this thesis were primarily to apply modern virological and immunological techniques to further define the natural history of disease in congenitally infected babies and to inform treatment strategies in the neonatal age group.

Specific objectives were to:-

1) Evaluate PK in neonates and correlate with drug efficacy.

2) Describe pharmacodynamics (PD) of antiviral treatment in neonates.

3) Apply VL measurements and calculations of virus dynamics to infected neonates (including VL measurements in different body fluids)

4) Establish collaborations within the UK and European Union to facilitate multicentre studies and data collection.

5) Evaluate sequential immunological responses in the neonatal age group using methodology validated in adult patients.

6) Define optimal time points at which sampling should be conducted to facilitate correlation of PK with PD.

One or more of these objectives are addressed in each of the following Chapters.

CHAPTER 2.

2. GENERAL METHODS

2.1. INTRODUCTION

This chapter describes methods developed by others and used during work in a number of different chapters of this thesis.

2.2. QUANTITATIVE CMV PCR

2.2.1. *Outline*

Most DNA extractions and CMV PCR analyses were carried out by the diagnostic team in the laboratory at the Royal Free Hospital alongside samples received for routine clinical analysis. DNA was extracted according to manufacturer's instructions and standard laboratory protocols in place at the time from 110uL of blood or urine or 1mL of viral transport medium (VTM) from salivary swabs into 110uL phosphate buffered saline (PBS) using either the semi-automated easyMag™ or the fully automated QIAsymphony™ system (which work on the same technology). Quantitative VL was then measured using an in-house real-time TaqMan PCR detecting a target within the highly conserved glycoprotein B (gB) gene of HCMV (primer 1 5'-GAGGACAACGAAATCCTGTTGGGCA-3' [gB1] and primer 2 5'- TCGACGGTGGAGATACTGCTGAGG-3' [gB2]). The 150 base pair product was detected in real time by use of a 29-mer TaqMan probe (5'-CAATCATGCGTTTGAAGAGGTAGTCCACG-3' [gb-P3]), with PCR and cycling conditions as described elsewhere and using 5uL of input purified or control DNA (Mattes et al. 2005). Each TaqMan PCR run contained a dilution series of cloned HCMV gB plasmid from 200-200x10^6 copies/ml. CMV VL was calculated using the ABI TaqMan sequence detection system software. The limit of detection was 1 copy per 5uL input (200 copies/mL).

2.2.2. *Limitations*

Urine can inhibit the PCR reaction. Extraction of DNA prior to PCR analysis minimises this risk and inhibition has not been problematic during routine diagnostic analysis within our laboratory.

For samples received in the Royal Free diagnostic laboratory VL results are likely to be reasonably consistent over time despite the long study period involved due to the

internal controls and similar PCR conditions used. Results from different labs are not, however, comparable although log changes should, nonetheless, be meaningful as intra-assay variation is low.

2.3. GANCICLOVIR LEVELS

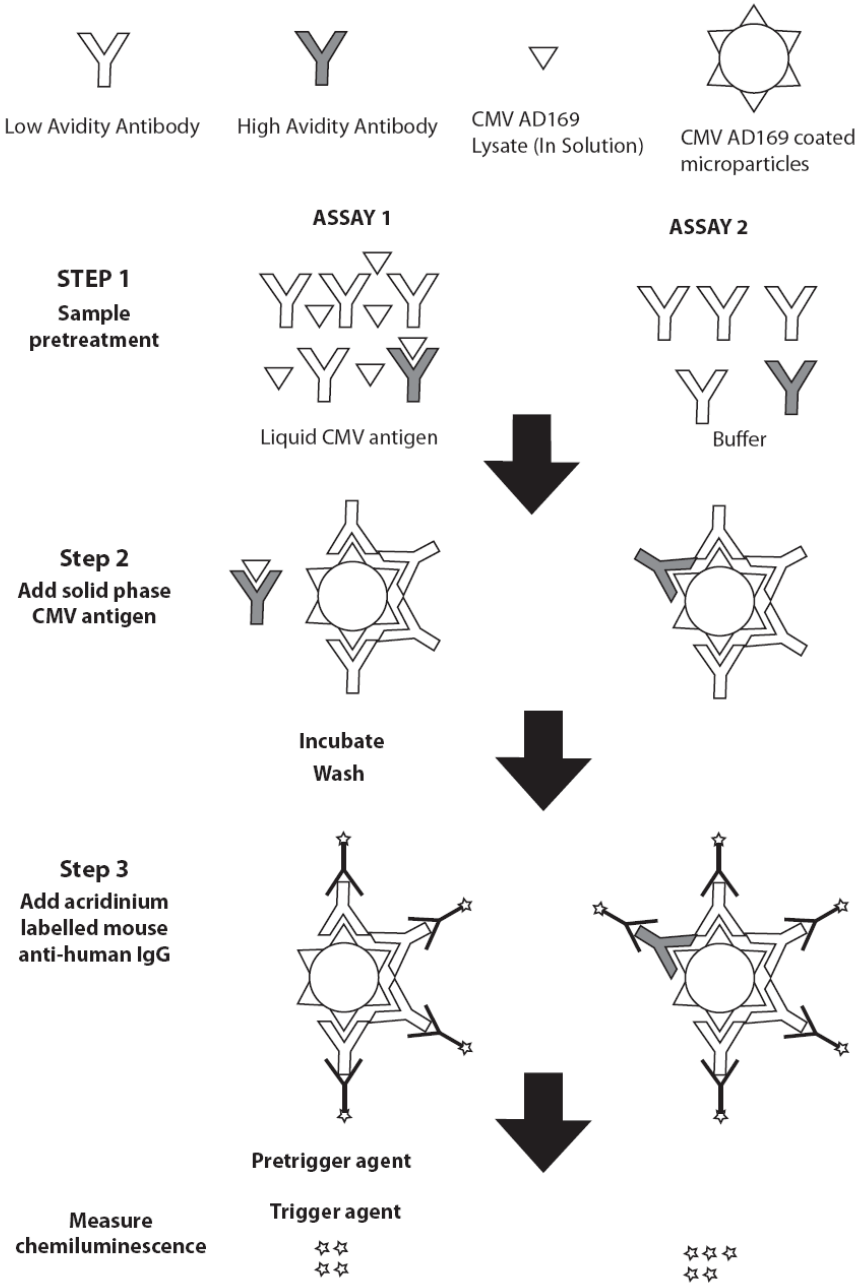
All samples were assayed by the United Kingdom Antimicrobial Reference laboratory (BCARE) using a validated high performance liquid chromatography (HPLC) assay and following the standard protocol in use at the time of analysis. In brief, chromatography was performed on a Techsphere C8 column using a mobile phase of 1% phosphoric acid in a 10g/L solution of octane sulphonic acid, with sample preparation by 1:1 dilution with perchloric acid and quantification by the external standard method. Both the intra- and inter-assay precision, coefficient of variation (CV), were below 10% and the limit of detection ranged from 0.5 down to 0.1 mg/L depending on the instrumentation used over the period of the study.

2.4. CMV IGG AVIDITY ASSAYS

IgG avidity was measured using diagnostic equipment and protocols available at the time and were conducted by laboratory staff alongside samples being processed for diagnostic purposes. Some earlier samples were analysed using the VIDAS® (Biomerieux) system which measures avidity using a denaturing agent to remove low avidity antibody. Most samples, however, were analysed using the completely automated Abbott ARCHITECT® system which in contrast uses a two-step chemiluminescent microparticle immunoassay (CMIA) utilising the finding that high, but not low, avidity antibody binds to antigen in a liquid phase to remove high avidity antibody prior to measuring remaining low avidity antibody (Curdt et al. 2009)(Figure 2-1). CMV IgG avidity index is subsequently calculated as a percentage using the relative light units (RLUs) of paired samples: 100 – [ratio (RLU with liquid phase antigen (Assay 1))/(RLU without liquid phase antigen (Assay 2))] x 100.

To enable the most appropriate dilution protocol to be selected it is necessary for a total CMV IgG to first be measured on the ARCHITECT® system. If samples have a total IgG concentration of >250IU then a 1:10 dilution of the serum is

Figure 2-1 Cartoon showing steps in Abbott ARCHITECT® IgG avidity assay



Two aliquots of sample are used. Assay 1 is pretreated with liquid CMV antigen which binds to high avidity antibody in the sample. Assay 2 is pretreated with buffer only leaving both high and low avidity antibody unbound in the sample. Solid phase CMV antigen (CMV lysate-coated paramagnetic microparticles) is then added to both samples. The samples are incubated and washed, removing high avidity antibody bound to the liquid phase antigen in Assay 1 but leaving both high and low antibody bound to the solid phase antibody in Assay 2. An acridinium-labelled mouse anti-human IgG is then added to both samples followed by a pre-trigger (hydrogen peroxide) and a trigger agent (sodium hydroxide) which results in chemiluminescence, the amount of which is measurably related to the amount of antibody bound to the solid phase antigen in both samples. IgG avidity is then calculated using the relative light units (RLU's) measured in the 2 assays.

automatically conducted in keeping with evidence that this improves avidity accuracy (Dangel et al. 2006).

Values of <50.0% and ≥60.0% are interpreted as indicating a low and high CMV IgG avidity respectively with results between 50 and 60% representing an equivocal 'grey zone' which cannot be interpreted.

2.4.1. *Limitations*

Using this commercial platform it is possible that more serum was needed than if manual assays had been conducted. Minimum serum recommended by the manufacturer is ~150uL. There were only two samples where serum was insufficient for avidity analysis so this had minimal impact on my studies.

2.5. PBMC EXTRACTION, EX-VIVO STAINING AND CYTOKINE ASSAYS

I spent some time adapting and refining adult protocols, developed previously by others in the laboratories at the Royal Free Department of Virology, to maximise buffy coat and PBMC yield from the smaller blood samples obtainable from neonates. Data from our lab demonstrated a marked decrease in viable PBMCs and cytokine production following freezing and thawing processes, it was therefore decided that fresh samples would provide the most reliable source of material in my studies particularly with additional restricted sample sizes. Most of the assays were then conducted by me, with some assistance by others in the lab towards the end of the studies.

2.5.1. *PBMC extraction*

EDTA blood (~1.0-1.5mls) was diluted with an approximately equal volume of RPMI-1640 (Gibco, UK) and carefully layered over an approximately equal volume (2-4mls) of Ficoll-Paque (GE Healthcare, UK) solution in a 15ml Falcon tube, to prevent mixing. The tube was centrifuged with no brake (778g for 20 minutes) to separate the PBMC-containing buffy coat. PBMCs were then harvested using a pipette, ensuring no Ficoll or blood contamination, and placed in a clean 15ml Falcon tube. 10mls RPMI was added and mixed gently with the harvested PBMC to wash. After further centrifugation with the brake on (15mins at 600g) supernatant was

removed and the cell pellet resuspended in a further 10mls RPMI. 10uL of this solution was removed, diluted 1:1 with trypan blue, inserted into a FastRead 102™ haemocytometer and viable PBMCs counted. Cell count/ml of blood was calculated using the formula: No. of cells per grid x 10^4 x dilution factor. At least 3 different grids were counted and the average number of PBMCs per grid calculated. Whilst cells were being counted, remaining cells were centrifuged (500g, 5min) then resuspended in warmed 10% FCS/RPMI to give ~$2x10^6$ PBMCs/ml and then stained according to either the ex-vivo or stimulation protocols below.

2.5.2. *Ex-vivo staining*

~$1x10^6$ PBMCs were placed in a FACS tube and centrifuged (500g, 5min) to pellet. Supernatant was discarded and cells resuspended in residual fluid. If subjects had been identified as being HLA-A2 positive cells were incubated for 10 minutes at room temperature, protected from light, with 10µl of R-PE labelled A*02:01/NLVPMVATV (CMV) pentamer (Proimmune); if HLA-A2 negative, cells were incubated with 10uL CD28-R-PE (BD Pharmingen) in place of pentamer. Cells were washed in 2mls 0.1% sodium azide in PBS (PBS/0.1% NaN_3) and centrifuged at (500g, 5 minutes).

Pellets were resuspended in residual fluid and incubated at room temperature for 15 minutes protected from light with 10µl CD8 PerCP Cy5.5 and 10µl CD57 FITC (BD biosciences). Cells were washed as before, pellets resuspended, and 100µl Caltag A (Fixative) (Invitrogen) added while vortexing. Cells were then incubated at room temperature for 15 minutes protected from light before washing again.

Following centrifugation, pellet was again resuspended in supernatant and 100µl Caltag B (Permeabilising solution) (Invitrogen) added while vortexing along with 10µl Granzyme B APC (APC Invitrogen). Cells were incubated at room temperature for a further 15 minutes, protected from light before washing as before and resuspending in 100µl of 4% paraformaldehyde. Samples were stored at 4°C, protected from light, until acquisition (maximum of 24 hours).

2.5.3. *PBMC overnight cultures for cytokine detection*

CD4 and CD8 positive PBMC production of the cytokines IFNγ, TNFα and IL-2 following stimulation of PBMCs was conducted by me using protocols developed by others in our laboratory as follows.

Essentially ~1x10^6 PBMC in 500µl of 10%FCS in RPMI were added to sterile polypropylene FACS compatible culture tubes. Dependent on the number of cells available stimulation was conducted with reagents as shown below.

1. Matched pp65 MHC class I peptide: 5µl of CMV pp65 NLVPMVATV, Proimmune peptide (i.e. corresponding to peptide folded with MHC Class I HLA-A2 specific pentamers used in ex-vivo assays described earlier).
2. CMV lysate: 2.5µl of HCMV AD169 purified whole-virus lysate (Autogen Bioclear cat no.10-144-000).
3. IE-1 pool/pp65 pool: 2µl of appropriate peptide pool (JPT Technologies PepMix containing a mix of 15-aa overlapping peptides spanning the HCMV-pp65 and IE-1 proteins)
4. SEB: 1ul of 1mg/ml *Staphylococcal aureus* enterotoxin B stock.
5. No antigen (background): 2µl of DMSO only.

5µl of pure anti-CD28 (BD bioscience) was added as a costimulatory antibody to CMV lysate, pp65/IE-1 pools and no antigen controls. Previous work by others had shown that adding this reagent to SEB and matched pp65 MHC class I peptides did not further increase cytokine production (data not shown).

Where cell numbers were limited stimulations were prioritised, as detailed below:-

1. If <3x10^6 PBMC and subject HLA-A2 positive ex-vivo pentamer staining and matched peptide stimulation were conducted along with positive (SEB) and no antigen background control. Although fewer than <1x10^6 cells were present in each tube this was felt to be an acceptable compromise to ensure that there were no obvious problems with the assay. If <3x10^6 PBMC and subject HLA-A2 negative then stimulation was conducted with CMV lysate, along with the positive control (SEB) and no antigen background control.

2. If >4x10^6 PBMC stimulation with IE-1 peptide pool and pp65 peptide pool were added and an ex-vivo tube (CD28, CD57 and granzyme B) was carried out for the HLA-A2 negative subjects.
3. If > 6 x10^6 PBMC all assays were included.

Tubes were vortexed to mix and placed in a 37°C CO_2 incubator for 2 hrs. 500µl of Brefeldin A (BFA)(Sigma) in 10% FCS/RPMI (concentration 20µg BFA/ml) was then added to each tube (to give a final concentration of 10µg BFA/ml), vortexed to mix and cells returned to the incubator overnight. Previous time course assays in adult subjects had indicated that optimal cytokine production was seen 12 -14 hours after addition of BFA. Cells were therefore left overnight for ~12 hours and no longer than 14 hours.

Following overnight stimulation PBMC cultures with no antigen, pp65 peptide pool, IE-1 peptide pool were divided between 2 tubes to be labelled with either CD4 (A) or CD8 (B). Cell suspensions were then centrifuged at 500g for 5 minutes, supernatant discarded and the pellet resuspended in residual fluid. For surface staining 10µl of CD4 PerCP Cy5.5 (BD biosciences) was added to CMV lysate, No Ag (A), pp65 pool (A), IE-1 pool (A) and SEB. 10µl of CD8 PerCP Cy5.5 (BD biosciences) was added to matched pp65 MHC class I peptide, No Ag (B), pp65 pool (B), IE-1 pool (B). Tubes were incubated at room temperature for 15 minutes, protected from light. Cells were then washed in 2mls PBS/0.1% NaN_3 and spun (500g, 5 minutes). Pellets were resuspended in supernatant and 100µl Caltag A added while vortexing. Cells were incubated for a further 15 minutes at room temperature, protected from light before a further wash.

Cells were resuspended in residual fluid and 100µl Caltag B added while vortexing. Simultaneously 10µl IL-2 FITC (BD biosciences), 10µl TNFα PE (BD pharmingen) and 2µl IFNγ APC (BD pharmingen) was added. Samples were mixed and incubated at room temperature for 15 minutes protected from light prior to washing, centrifuging and resuspending in 4% paraformaldehyde. Samples were stored protected from light at 4°C until acquisition (within 24 hours).

- **Acquisition**

Cells were acquired using Cellquest software and BD 4-colour FACSCalibur™. Initial instrument settings were created using single staining set-up protocols with manual compensations. Given the small number of cells available fine tuning was carried out at each analysis if required.

The lymphocyte population was defined according to forward and side scatter characteristics and subsequently the CD4+ or CD8+ cells defined as a subset of this population by gating on PerCP Cy5.5+ cells. Acquisition and storage was then set to 50,000 CD4+ or CD8+ cell events to enable small changes in CMV-specific populations to be detected (i.e. down to approximately 1:1000). Data were saved under the subject number, time point of analysis and stimulating antigen/ex vivo protocol prior to transferring to an AppleMac computer for analysis using FlowJo software as described further in Chapter 6 of this thesis.

CHAPTER 3.

3. ANALYSIS OF GANCICLOVIR LEVELS IN CHILDREN FROM THE UK NATIONAL REFERENCE LABORATORY

3.1. INTRODUCTION

GCV and its oral prodrug, VGCV, are the most commonly used antiviral drugs with activity against HCMV. Plasma GCV levels have been used to estimate drug exposure and to develop suggested doses for treatment and prophylaxis in adults (Fishman et al. 2000). As discussed in the Introduction, measurement of plasma GCV levels has also subsequently been used in small groups of children to derive equivalent paediatric dosing regimes.

Therapeutic drug monitoring (TDM) for GCV is available. The optimal GCV levels for ensuring treatment efficacy while avoiding toxicity are, however, largely unknown and lacking a firm evidence base.

3.1.1. *Optimal GCV levels and drug efficacy*

Values for MIC and IC_{50} are derived from CMV grown *in vitro* on human foreskin fibroblasts. As described in the Introduction, CMV mutates rapidly during multiple passages and CMV cultured on fibroblast cell lines may lack large portions of the genome which are required for growth on epithelial cells (Wang and Shenk 2005); moreover human foreskin fibroblasts are not representative of the cells generally associated with CMV disease *in vivo*. Caution is therefore needed when applying the results of such *in vitro* assays to the situation *in vivo* with wild-type CMV infection of different cell lines.

I*n vivo* data has been obtained from specific patient groups but data correlating these with clinical efficacy are lacking. The therapeutic ranges often quoted originate from an early study in only 6 adults. 5 bone marrow transplant patients and 1 AIDS patient with CMV retinitis or pneumonitis were studied and peak and trough plasma concentrations of 4.75-6.2 ug/mL and 0.25-0.63 ug/mL, respectively, recorded. Drug levels achieved have also been found to vary dependent on the patient group being studied (Scott, Partovi, & Ensom 2004).

Lower trough levels (<0.6 mg/L) have been associated with a higher incidence of disease progression in a study of CMV retinitis in HIV patients (Piketty et al. 2000). Oral GCV prophylaxis is also hypothesised to select for GCV-resistant virus, and possible treatment failure, due to the low serum drug levels achieved, (Emery and

Griffiths 2000;Limaye et al. 2000). Other authors have not been able to correlate drug levels with clinical or virological efficacy (Fishman et al. 2000;Kimberlin et al. 2008).

Despite the lack of data in adult populations, studies so far in children have largely strived to show equivalent drug exposure to previous adult studies. Data correlating GCV levels and efficacy in neonates are lacking. These babies have notable differences in PK to adults and may additionally have an impaired ability to recognise and control CMV due to a relatively immunological immaturity.

The relevance of drug levels for clinical outcome, therefore, currently remains undefined in all age groups. The use of TDM is correspondingly somewhat contentious and in the adult population generally reserved for those at particular risk of toxicity, such as those with renal impairment (Scott, Partovi, & Ensom 2004).

3.1.2. *GCV levels and toxicity*

Data are also lacking to identify a specific threshold at which GCV levels may be considered undesirable and toxicity tends to have a high degree of inter-patient susceptibility. Despite toxicity being relatively common, previous reports have failed to establish a correlation between GCV levels and clinical toxicity in adults (Erice et al. 1987;Fishman et al. 2000). A recent study of VGCV in neonates did, however, correlate both C_{max} and AUC_{12} with neutropenia (Kimberlin et al. 2008).

Although AUC is the parameter reported in many PK studies its measurement is not always practical in the clinical setting, particularly in the paediatric age group. AUC has been shown to correlate with both C_{max} and trough levels in cystic fibrosis patients undergoing transplantation, due to the first-order kinetics exhibited by GCV (Snell et al. 2000).

As GCV is only active once it has been tri-phosphorylated intracellularly whether measuring plasma GCV levels is a true reflection of levels of active drug is also uncertain and it is possible that phosphorylation varies between individuals and in different tissues; similarly clinical virus isolates have been shown to vary in their ability to phosphorylate GCV (Crumpacker 1996).

There are also possible differences between results reported from PK studies and those from samples acquired during routine clinical care. A rapid decrease in the GCV concentration measured in plasma has been reported in blood samples stored at room temperature as opposed to on ice, at 0^{o}C, prior to centrifugation. This is proposed to be due to increased intracellular transport of drug into erythrocytes at room temperature. If plasma is separated immediately after acquisition, however, GCV levels have been reported to remain stable for up to 2 hrs at room temperature (Figure 3-1)(Boulieu and Bleyzac 1994). Other authors have demonstrated in experimental models that, after an initial period of equilibration, serum and intracellular GCV levels are comparable and stable for 4-6 hours at room temperature with diffusion across a concentration gradient hypothesised as being responsible for some of the shift in drug between the two compartments (Perrottet et al. 2008). It is therefore conceivable that levels measured in clinical samples, where separation of plasma may be delayed, are not comparable with those reported in the tightly controlled PK studies from which therapeutic ranges are derived.

3.1.3. *Aims of this Chapter*

Clinical experience and the shortage of evidence for GCV TDM in infants using standard dosing regimens led me to hypothesise that drug levels achieved in infants may frequently be less than those quoted as optimal in adults. I therefore reviewed the current UK evidence base available by analysing the concentrations of GCV found in samples received for TDM of GCV by the UK Antimicrobial Reference Laboratory.

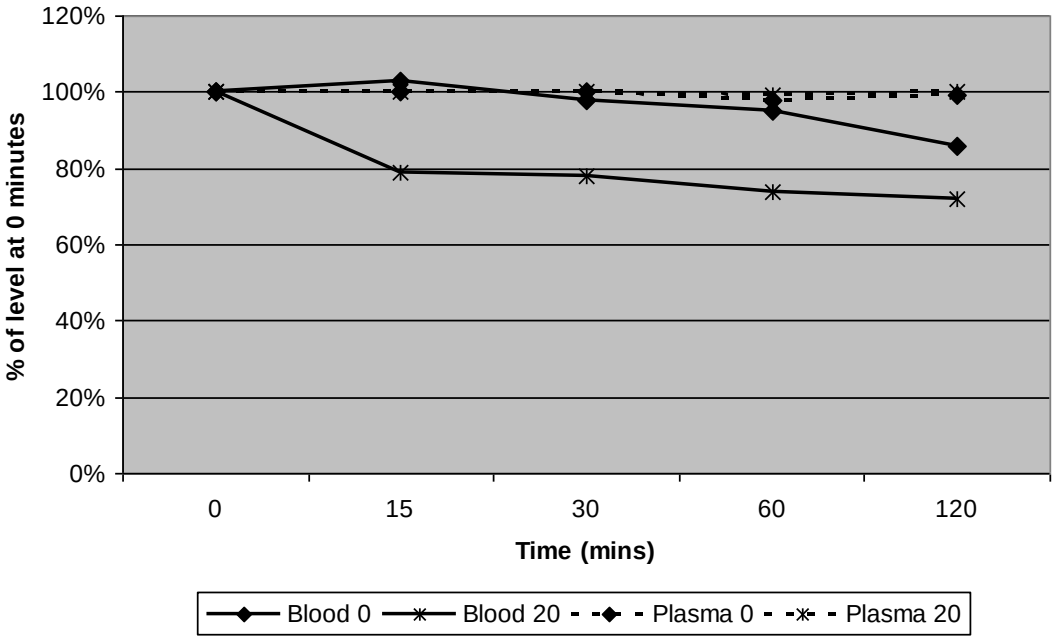
3.2. METHODS

3.2.1. *Study overview*

Serum GCV levels (reported in mg/L) received and analysed by the United Kingdom Antimicrobial Reference laboratory in Bristol from 01/11/1999 to 31/3/2007 were reviewed.

The standard advice issued to clinicians for sampling around that time (Antimicrobial Reference Laboratory, Guidelines for Users, 10^{th} Edition 2004) recommended that separated serum samples (minimum volume 100uL) should be sent. The

Figure 3-1 Ganciclovir levels in plasma and blood over time



Blood was stored at 0 and 20°C for up to 120 minutes prior to centrifugation (Blood 0 and Blood 20).
Plasma was separated immediately and stored at 0 and 20°C for up to 120 minutes prior to analysis (Plasma 0 and Plasma 20).

Compiled from data presented in Boulieu and Bleyzac (1994)

recommended timing of samples was one pre-dose and one post-dose with post-dose samples taken either 1 hour after the end of i.v. drug administration or 2 hours after oral administration. This guidance also suggested that GCV assays might be of value in patients with renal failure and to monitor oral absorption, particularly in patients receiving VGCV.

Data were anonymised and comprised of date of birth of subject, timing of sample acquisition (pre-, post- or random) and GCV level measured in the sample. No clinical data or data relating to dose or formulation of medication were available despite laboratory guidance requesting that this information should accompany clinical samples.

Results were analysed in different paediatric age groups and compared to those observed in samples from adults (those aged older than 18 years).

3.2.2. *Measurement of ganciclovir levels*

All samples were assayed as described in the Methods of this thesis.

3.2.3. *Data analysis*

In instances where there was no reported GCV detectable in the sample, a value of 50% of the detection limit for the assay at that time was used for analysis. Results were analyzed in different paediatric age groups and median values compared to those from adults using Mann Whitney U test.

Given literature reporting resistance and decreased efficacy with trough levels below 0.6mg/L, 2 x 2 contingency tables and Fisher's exact test were used to compare the percentage of individuals in each age group with levels either <0.6 mg/L or ≥ 0.6 mg/L. Similarly the proportion of peak samples above and below 3.0 mg/L (the upper limit of the IC_{50} often quoted from *in vitro* studies) and those above and below 7.0 mg/L (the lower reference value for i.v. GCV often quoted) was compared between age groups.

3.3. RESULTS

Specimens were received from 129 patients at 339 time points over the 89 month time period. Only 173 (51%) of samples received at each time point were paired pre- and post- levels.

Specimens were received from 65 patients aged <18 years (50% of all patients), at 192 different time points (57% of all specimens) 117 (61%) of which were paired pre- and post- levels. Although specimens were received on 95 occasions from children aged <6 months, only 10 of these were from 4 neonates (aged <28 days).

3.3.1. *Trough levels*

A total of 199 trough levels were received with 130 (65%) of these being from subjects aged <18 years. Table 3-1 and Figure 3-2 show the distribution of trough levels for each of the age categories.

In those patients aged either <6 months or 6-12 months, there was a significantly higher percentage of levels <0.6 mg/L than seen in adults (64.8% and 53.9% respectively vs 15.9%; P<0.001). There was also a trend towards more levels <0.6 mg/L in those patients aged 1-5 years compared to adults but, possibly due to small numbers, this did not reach statistical significance (N=16; 37.5% vs 15.9%; P = 0.079). Those aged 5-18 years had significantly more trough levels <0.6mg/L than adults although small numbers may again have influenced results (N=15; 80% vs 15.9%; P<0.001).

Correspondingly the younger age groups also had fewer samples with levels that might be associated with clinical toxicity with all paediatric age groups having ≤ 7% of trough measurements ≥ 6.0 mg/L compared to 19% in adults.

3.3.2. *Peak levels*

198 post-dose levels were received. Median levels in all age groups were 3.7-5.7 mg/L (Table 3-1). Approximately one third of patients aged <18 years had peak levels <3.0 mg/L and in infants aged 6-12 months the modal peak level was 2.0-3.0 mg/L, with nearly 35% of peak levels being <3.0 mg/L. Although a trend towards lower peak levels can be seen in the younger age groups (Figure 3-3) a statistically

Table 3-1 Peak and trough ganciclovir levels (mg/L) in different age groups

	TROUGH LEVELS				PEAK LEVELS				
AGE	**No. of samples**	**MODE**	**MEDIAN (IQ range)**	**<0.6 (%)**	**No. of samples**	**MODE**	**MEDIAN (IQ range)**	**<3.0 (%)**	**>7.0 (%)**
<28 days	8	<0.6	1.3 (0.3-1.9)	50.0	8	Insuff nos	4.7 (3.4-5.8)	25.0	12.5
<6 months[1]	71	<0.6	0.4 (0.1-1.0)	64.8	67	3-4	4.8 (3.5-6.3)	20.9	17.9
6-12 months	26	<0.6	0.5 (0.1-1.0)	53.9	29	2-3	5.2 (2.7-8.0)	34.5	34.5
1-5 years	16	<0.6	1.3 (0.2 – 2.3)	37.5	17	4-5	4.7 (2.4-7.9)	29.4	29.4
5-18 years	15	<0.6	0.3 (0.2-0.5)	80.0	14	3-4; 5-6[2]	3.7 (2.4-5.6)	35.7	14.3
>18 years	69	1-2	2.1 (1.0-4.2)	15.9	70	4-5	5.7 (3.7-8.2)	20.0	35.7

1) Includes those babies <28 days of age
2) 3 children in each group

Figure 3-2: Trough levels of ganciclovir in different age groups

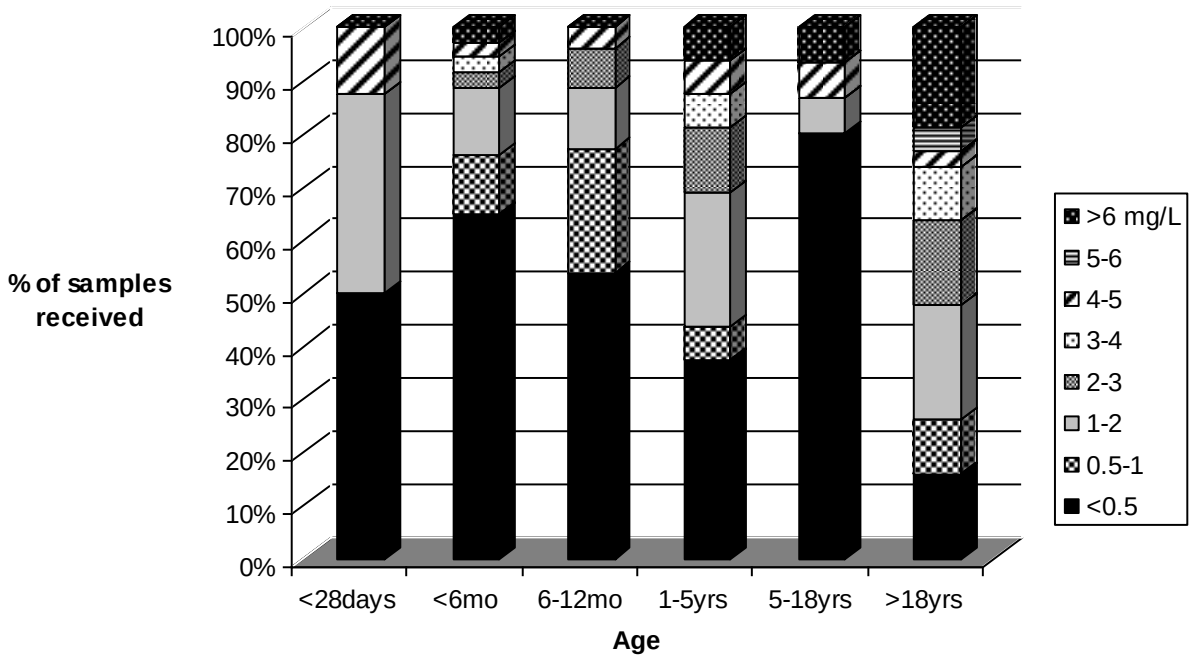
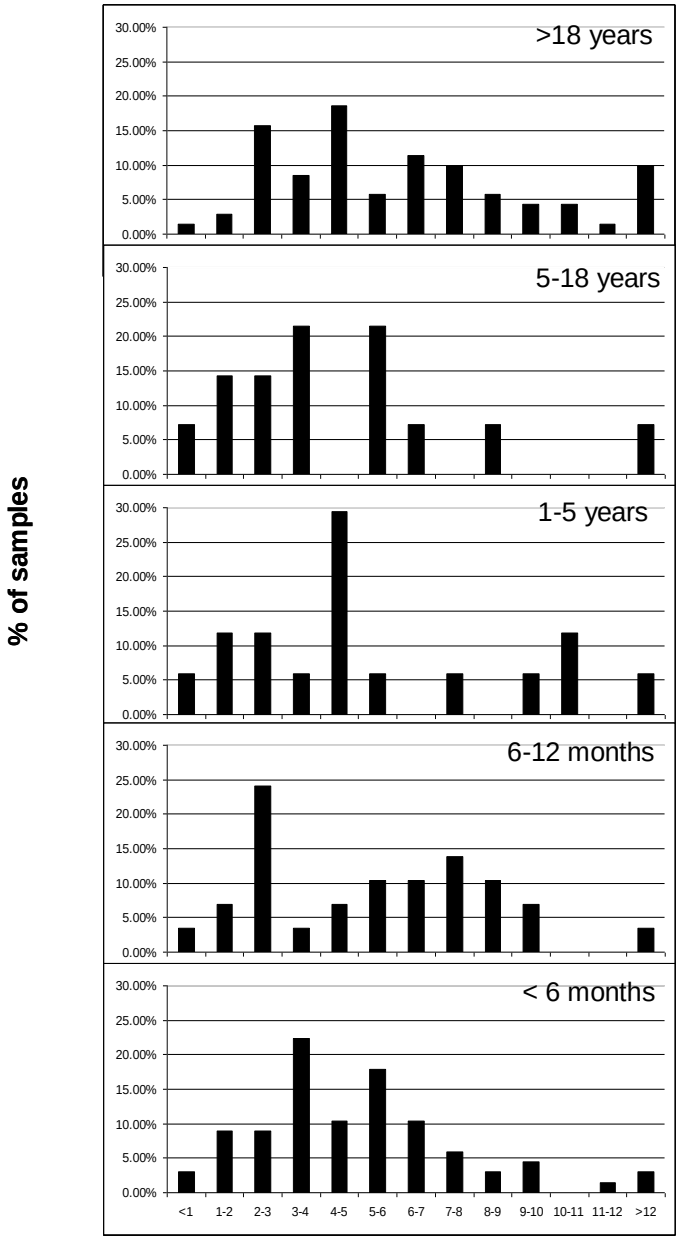


Figure 3-3: Peak (post-dose) levels of ganciclovir in different age groups



significant difference was only found when comparing the median peak levels in those aged >18 years with those aged <6 months (5.7 mg/L vs 4.8 mg/L respectively P=0.047). However, the lowest median peak levels were actually found in those aged 5-18 years (3.65 mg/L; IQR 2.35-5.63) with 35% of levels being <3.0mg/L.

3.4. DISCUSSION

In just over seven years, samples were received on 339 occasions from a total of 129 patients throughout the UK and although the reasons for TDM were not available, this represents one of the largest cohorts reported to date for GCV TDM. In earlier studies patient numbers have generally been low (<20) and the patients have either had tightly defined disease states or the studies have been conducted under clinical trial conditions. The data presented here may give a clearer picture of the concentrations found in samples taken from patients under more typical clinical conditions.

Levels from children accounted for more than half of sampling events received by this national reference laboratory, despite the lack of any validated reference values for paediatric age groups. There were significantly more trough levels <0.5 mg/L reported in young children compared to adults. Low trough levels have previously been reported in a small study of paediatric transplant patients, with levels <0.5mg/L being significantly more common in younger patients (Vethamuthu et al. 2007). My observation that fewer babies <28 days had trough levels <0.5 mg/L when compared to the <6 month age group as a whole would be in keeping with the almost exclusive renal excretion of GCV alongside known renal immaturity in newborns, leading to a corresponding decrease in GCV excretion when compared to older infants.

Peak GCV levels of >7.0 mg/L (the reference value often quoted for TDM) were rarely achieved in any of the age groups studied. Although a significant difference was only seen between median peak levels in those aged <6 months old compared to adults the lowest median levels were actually noted in the 5-18 year age group.

Peak levels were also in keeping with those found in other clinical studies and were well above published *in vitro* values for MIC and IC_{50} (Fishman et al. 2000;Fletcher et al. 1986). The relatively high frequency of peak levels <3.0mg/L in younger age groups would, however, give rise to concerns regarding treatment failure particularly if the disease-causing virus had an IC_{50} at the upper reported range of 3.48mg/L.

This may have additional relevance in the neonatal age group where immature host immunological responses may further favour viral persistence.

I cannot offer a full explanation for the lower peak levels and higher proportion of trough levels <0.5mg/L seen in 5-18 year olds from these anonymised data which lack clinical and pharmacological information. Despite the small numbers included these data do suggest that under-dosing may also affect this older paediatric age group.

Trough levels >0.6mg/L have been proposed by some authors as being optimal (Emery and Griffiths 2000;Limaye et al. 2000;Piketty et al. 2000). The lower levels reported here and elsewhere raise concerns that paediatric age groups are receiving inadequate doses of GCV and/or VGCV and might likewise be susceptible to acquisition of resistant virus and treatment failure.

Although the lower levels seen in the paediatric population may imply that toxicity is correspondingly less likely, toxicity tends to have a high degree of inter-patient variability and a specific threshold above which GCV levels may be considered undesirable has not been described. Despite the low levels of drug observed here studies in neonates have found similar levels of severe neutropenia to those observed in adults (Kimberlin et al. 2003). Interestingly Kimberlin's study of targeted drug dosing to a proposed optimal AUC of 27 mg x h/L, has been one of the only published studies to correlate GCV exposure with neutropenia (Kimberlin et al. 2008).

The limitations of such retrospectively obtained data must, however, be taken into consideration. These limitations include the possibility that these clinical samples may not have been taken within the timeframes required to make them true trough or peak values and may additionally have been left for some time prior to plasma separation with the potential for inaccurate readings as discussed above.

A further weakness of this study is the lack of clinical information, making the underlying indication for TDM sampling a potential bias. The relatively high proportion of paediatric samples may reflect the fact that almost without exception investigations of GCV levels in adult populations over the past two decades have failed to correlate levels with either measures of clinical and virological efficacy or with neutropenia (a measure of potential toxicity). These findings have led other

authors to challenge the utility of TDM in adult patients, other than in specific sub-groups (Scott, Partovi, & Ensom 2004). A possible confounding factor may therefore be that levels were only sent from patients in whom there were concerns about treatment failure, toxicity or dosing of medication, particularly in adults. Interpretation is further limited by the absence of information on the drug administered (GCV or VGCV), dose given (including adjustment for renal failure), and route of administration. Given that the oral solution of VGCV only became available for use as extemporaneously prepared solutions in the UK around 2006 and that oral GCV has also not been available for some time it can reasonably be assumed that the majority of younger children received i.v. GCV; this assumption cannot be applied to older age groups.

Although the clinical relevance of the lower levels presented here is therefore uncertain the significantly decreased exposure to GCV during standard treatment courses when compared to adults is notable. Recent reports have suggested dosing algorithms for oral VGCV based on both weight and creatinine clearance (Vaudry et al. 2009). Given that such algorithms do not currently exist for i.v. GCV and the wide inter- and intra-patient variability reported in younger children when weight alone is used for dosing, the development of such algorithms would seem beneficial. In the absence of such data TDM may offer some interim utility to guide GCV dosing, particularly in situations where renal function is uncertain or compromised, for example in prematurity (Vethamuthu et al. 2007). A role for TDM is therefore proposed in younger children particularly if longer treatment courses are being considered and during early infancy where rapid changes in renal function occur. An additional role could be proposed for those infants where virological response seems inadequate. Based on existing data and concerns regarding under-treatment a trough of 0.5-1.0 mg/L should be targeted. Although not necessarily translatable to younger children the peak GCV reference level used for adults of 7-9mg/L following GCV administration (5-7mg/L when using VGCV) are also suggested as an initial goal. In the presence of adequate trough levels and good virological response adjusting dose based purely on a mildly suboptimal peak GCV level would not, however, seem justifiable based on current evidence.

The need for dosing algorithms for i.v. GCV, such as those derived for VGCV, seem apparent from these data. Moreover detailed PK/PD studies in different paediatric

age groups (and at early gestations) would also be desirable. In all age groups the use of surrogate markers for clinical efficacy (either virological or immunological) when designing such trials may help to define the optimal dose. Until such studies are conducted the true utility of TDM with regard to clinical outcomes will remain somewhat uncertain. My observation that the receipt of paired samples in children exceeded those received for adult patients shows that obtaining such samples is achievable. I would propose that paediatric PK/PD studies should therefore be considered a priority alongside those performed in adult patient populations.

CHAPTER 4.

4. DATA FROM CASG #109 (PHASE I/II PHARMACOKINETIC TRIAL COMPARING ORAL VALGANCICLOVIR SYRUP WITH STANDARD INTRAVENOUS GANCICLOVIR)

4.1. INTRODUCTION

As described in the Introduction, work carried out in immunosuppressed adult patients led to the discovery that CMV had a more rapid half-life than previous dogma had dictated (Emery et al. 1999). There have been no previously published data relating to viral dynamics of CMV in the paediatric age group.

Using pre-existing data of blood VL obtained during a PK study of oral VGCV treatment in neonates this chapter therefore aims to explore viral dynamics in this population.

Some assumptions must be made in order to apply existing mathematical models to this age group. The equation used for analysis of $T_{1/2}$ of viral decline requires virus to be in steady state prior to treatment commencing. There have been no studies of sequential VL in blood prior to treatment in the neonatal age group, however, older data show that the amount of virus in urine (measured using $TCID_{50}$) is reasonably stable for the first 3 months of life in both symptomatic and asymptomatic babies (Figure 1-8)(Stagno et al. 1975). Although it is likely that the majority of symptomatic infants will have acquired infection some weeks prior to delivery and will therefore have reached steady state some may not have acquired infection until later in pregnancy, however, meaning that they may still be undergoing changes in viral equilibrium during the course of their primary infection.

The second assumption is that VGCV has the same efficacy with regards to treating CMV in neonates as it does in adults. Given that the dose of medication actually ingested orally in neonates may be more erratic than in adults this needs to be taken into consideration.

The aim was for the results of this analysis to inform the design of a protocol for a larger RCT assessing efficacy of longer treatment courses with VGCV.

4.2. METHODS

4.2.1. *Study overview*

Through collaboration with the US-based CASG data for blood VL (measured by CMV DNA PCR) were obtained from a Phase I/II PK study evaluating VGCV in neonates (CASG #109). The primary aim of this ethically approved study was to evaluate the dose of oral VGCV giving GCV exposure equivalent to the 6mg/kg bd

dose of i.v. GCV previously described for the treatment of cCMV (Trang et al. 1993;Whitley et al. 1997). Population PK were used to describe the PK of GCV, measured by serum GCV levels, following administration of IV GCV and oral VGCV to neonates. Additional samples were obtained during the study for safety monitoring and VL (Acosta et al. 2007;Kimberlin et al. 2008).

The first 5 subjects were recruited on a regime (Version 1.0) that involved i.v. GCV 6mg/kg bd for Days 1-4 followed by oral VGCV at a dose of 14mg/kg bd on Days 5 and 6 and a further five weeks of i.v. GCV 6mg/kg bd with an interruption on days 35-36 for oral VGCV (Figure 4-1). Due to recruitment difficulties the protocol was subsequently modified (Version 2.0) so that an initial oral dose of VGCV was followed by 14 days of i.v. GCV 6mg/kg bd and the treatment course completed with oral VGCV. This enabled more rapid recruitment of the remaining 19 infants. Version 2.0 was carried out using a targeted AUC approach with the dose of oral VGCV being adjusted according to AUC_{12} measured following the initial VGCV dose to achieve a target AUC of 20-55 mg x h/L. In this second version 9 babies therefore received 14mg/kg, 6 received 16mg/kg and 4 received 20mg/kg VGCV bd. It should be noted, therefore, that in Version 1.0 the majority of the treatment course was with i.v. GCV whereas in Version 2.0 4 weeks of the 6 week course was with a targeted dose of oral VGCV.

Blood samples for VL were scheduled in both versions of the protocol at baseline (day 1) and days 7, 14, 28, 42, 56 and 180. Samples taken on days 56 and 180 were therefore 14 days and 138 days (19.7 weeks) after treatment termination respectively.

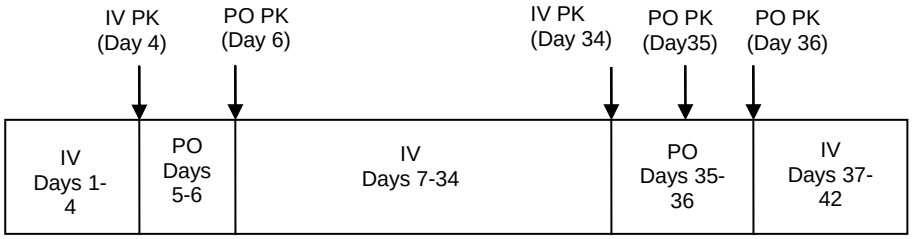
4.2.2. *Subjects*

Subjects were all neonates who received antiviral treatment for symptomatic cCMV aged 8-34 days at the time of their first blood sample for PK analysis. Further details of these patients are given by Acosta *et al* (2007) and Kimberlin *et al* (2008) in their publications of the full results of this study.

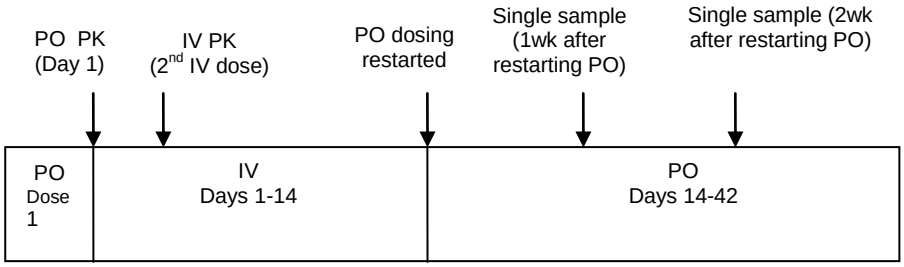
In brief, the median age of participants at study enrolment was 27 days (range 9-31), 46% were female. All participants were culture positive for CMV from a urine or throat swab specimen taken within 30 days of birth but not all had a detectable viraemia.

Figure 4-1 Schematic representation of study design for CASG Study #109

Version 1.0



Version 2.0



PO = oral medication
IV = intravenous medication
PK = Pharmacokinetic sample
GCV = ganciclovir
VGCV = valganciclovir

Treatment duration was 6 weeks in both protocol versions.
In Version 1.0 6mg/kg of GCV was given i.v. on Days 1-4 with a dose of 14mg/kg oral VGCV on Days 5 and 6 followed by a further five weeks of i.v. GCV 6mg/kg bd with an interruption on Days 35-36 for oral VGCV.
In Version 2.0 an initial oral dose of VGCV was followed by 14 days of i.v. GCV 6mg/kg bd and the treatment course completed with oral VGCV.

Reproduced from Acosta (2007)

4.2.3. *Data acquisition and Analysis*

Anonymised data were obtained for all 24 patients in the form of a list of subject numbers and dates of sample acquisition along with corresponding whole blood VL measurements (copies/ml).

7/24 (29%) participants had no detectable viraemia at entry to the study. The VL in the remaining 17 participants at each of the study visits is summarised in Table 4-1. One subject had undetectable VL at entry and throughout the study period excepting a measurement of 1486 copies/ml on day 28 (subject 68). The remaining 6 subjects with no viraemia at study entry had no virus detectable throughout the study period.

Where VL was expressed as <200 copies/ml the mid-point value of 100 copies/ml was taken for analysis to enable log transformation of data. Log_{10} VL over time, at the actual time of sample acquisition, is shown graphically for each subject in Figure 4-2. Samples were not always obtained on the exact day intended according to protocol. Except where specified, specimens were analysed according to the closest intended sampling time point. A calculation of skew indicated that data were probably not normally distributed. Analysis was therefore carried out using non-parametric methods and using GraphPad prism 5 software.

Comparison of medians in the same groups at different time points was conducted using Wilcoxon matched pairs. Comparison of medians between different groups was performed using Mann-Whitney U test.

For calculations of $T_{1/2}$ of virus decline the slope of decline of log_e (ln) VL was first estimated using segmental regression in GraphPad prism. X0 constraint for decline was set at the point where the phase of most rapid viral decline appeared to end (Figure 4-3). As described earlier (see 1.4.4), the slope derived was then used to determine $T_{1/2}$ in blood by using the formula (-ln2)/slope.

4.3. RESULTS

17/24 babies (70.8%) were viraemic at baseline. Median starting log_{10} VL in those with viraemia was 3.3 log_{10} (IQR 3.1-4.5).

Examination of the baseline VL in these babies raised the possibility of 2 distinct groups defined by a baseline VL of greater than or less than 4.0 log_{10} (Figure 4-4). These groups were therefore considered separately in some analyses.

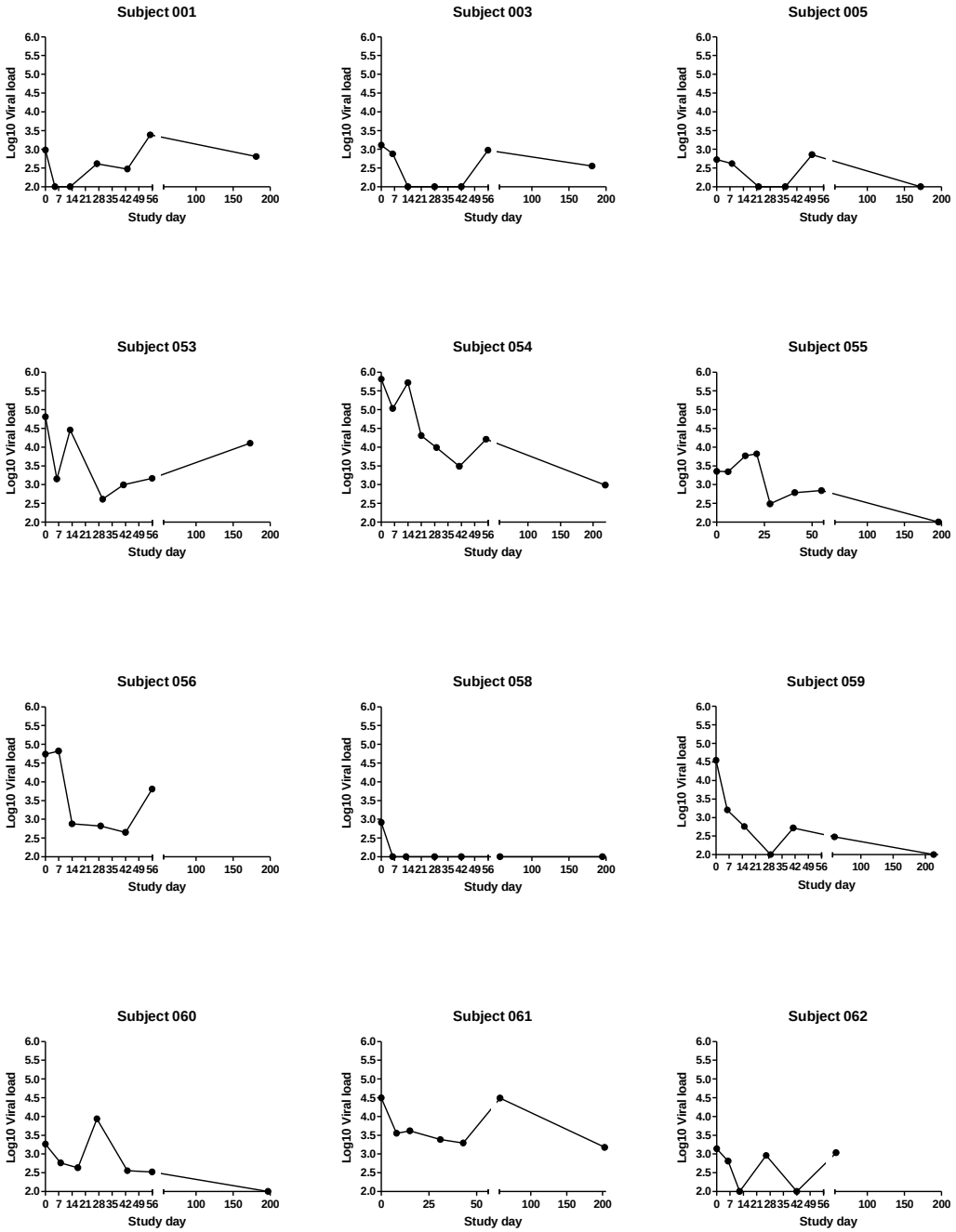
Table 4-1 Viral load in 18 neonates receiving 42 days antiviral therapy.

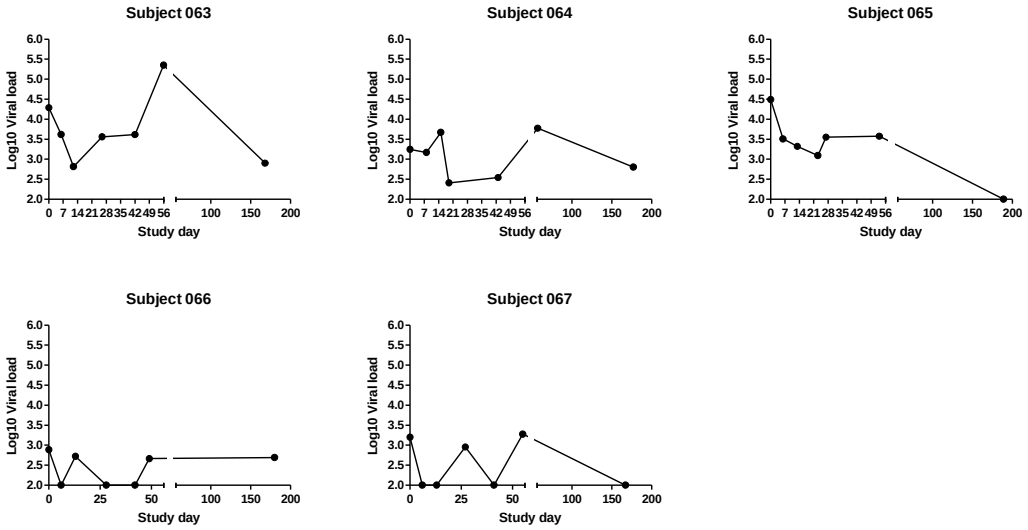
SUBJECT	VIRAL LOAD (copies/ml) taken at specified study day						
	D1	D7	D14	D28	D42	D56	D180
1	956	<200	<200	412	298	2418	644
3	1296	756	<200	<200	<200	942	358
5	530	414	NS	<200	<200	724	<200
53	64828	1414	28800	410	990	1478	12704
54	651200	108666	527768	9706	3082	16258	974
55	2264	2210	5888	308	614	692	<200
56	54692	66414	756	664	448	6452	NS
58	822	<200	<200	<200	<200	<200	<200
59	35038	1602	572	<200	528	300	<200
60	1844	576	430	8694	360	332	<200
61	31514	3602	4146	2432	1966	31008	1512
62	1384	647	<200	914	<200	1082	NS
63	19400	4152	652	3620	4116	223922	796
64	1772	1486	4682	NS	348	5950	640
65	31072	3218	2094	3552	NS	3752	<200
66	776	<200	528	<200	<200	464	492
67	1584	<200	<200	902	<200	1904	<200
68	<200	<200	<200	1486	<200	<200	<200

NS = No Sample
Pale shading = those with undetectable viral load at end of treatment.
Darker shading = excluded from analysis.

Viral load in 18 babies with viraemia detected at any time point during a study of treatment with 6 weeks antiviral therapy (Kimberlin et al, 2008).
Viral load was measured using quantitative CMV PCR and reported in copies/ml.
Treatment was with i.v. ganciclovir or p.o. valganciclovir according to two different protocols as summarised in Figure 4-1.

Figure 4-2 Log_{10} viral load over time in 17 viraemic babies treated for congenital CMV

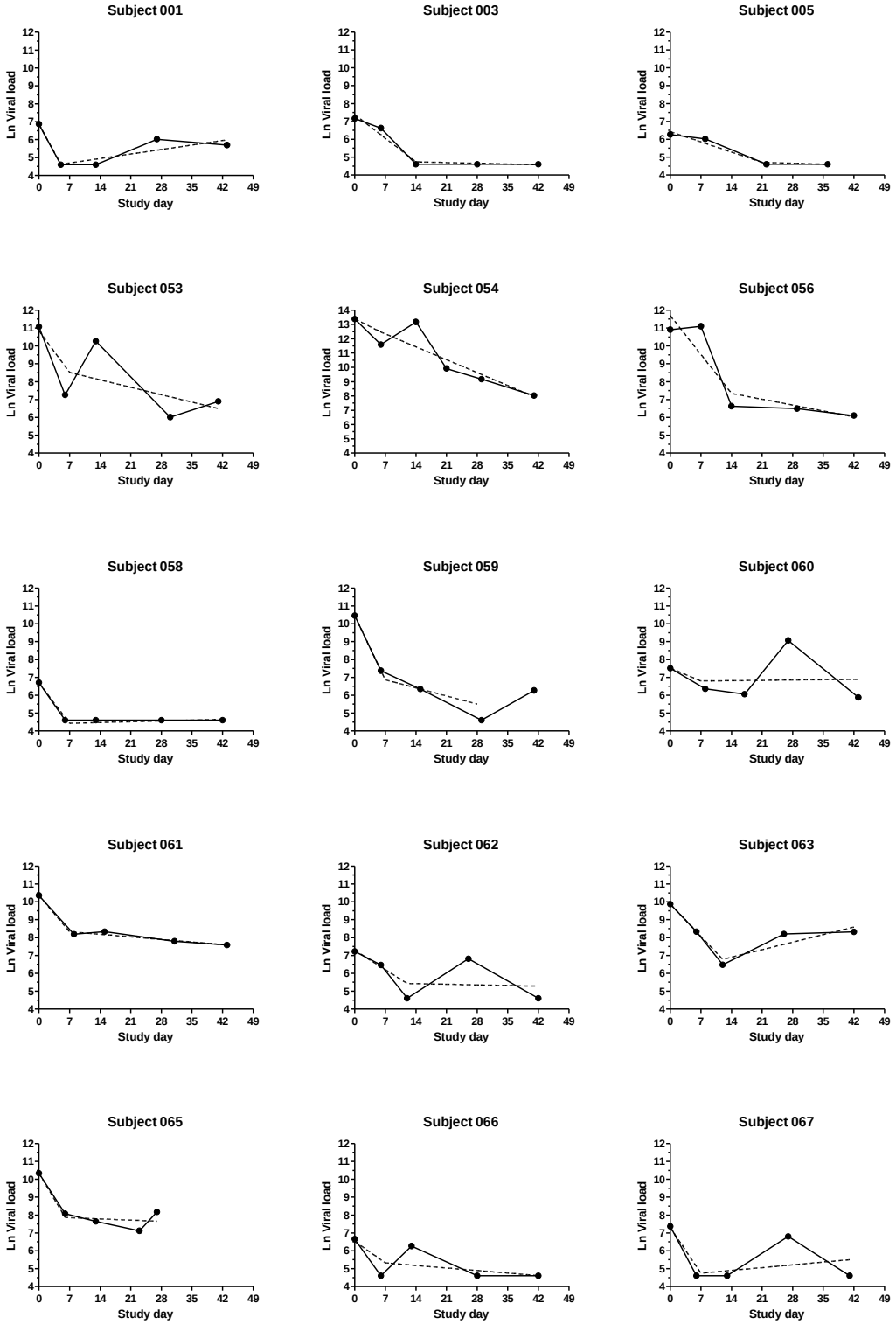




Viral load, measured using quantitative CMV PCR, is shown plotted at the actual time of sample acquisition in 17/24 babies with congenital CMV that were enrolled in a study of antiviral treatment and that were viraemic at the onset of treatment.

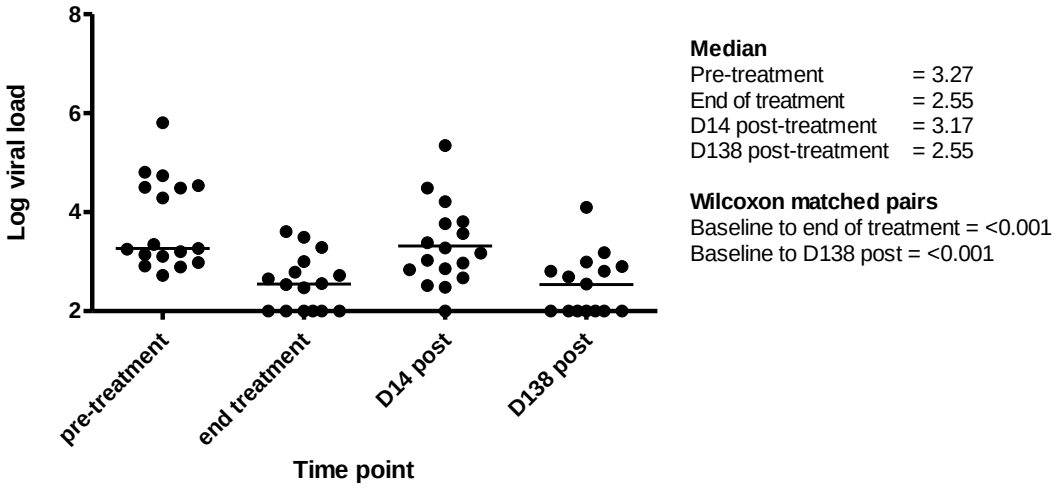
Two different treatment protocols were utilised as shown in Figure 4-1: subjects 1-5 received mainly i.v. GCV for 42 days with oral VGCV 14mg/kg on days 5 and 6, subjects 53-67 received i.v. GCV days 0-14 followed by varying doses of oral VGCV to achieve a targeted AUC of 20-55 mg x h/L to complete 42 days treatment.

Figure 4-3 Examples of segmental regression plots of Ln viral load during treatment in 15 babies treated for congenital CMV



Log_e (ln) viral load was plotted in GraphPad prism according to the intended day of sampling in the study protocol (Kimberlin 2008). The slope of virus decline was then estimated using segmental regression. X0 constraint for decline was set at the point where the phase of most rapid viral decine appeared to end. Data is only shown for 15/17 viraemic babies included in subsequent analyses of viral half-life.

Figure 4-4: Median viral load at baseline, and after treatment in 17 viraemic babies treated for congenital CMV



Samples for CMV viral load were taken according to protocol (Kimberlin 2008) and are shown for baseline (pre-treatment), study day 42 (end of treatment), day 56 (D14 post-treatment), and day 180 (D138 post-treatment).

4.3.1. *Viral suppression with treatment*

Only 6/16 subjects (37.5%) who had a sample analysed around day 42 (D42) had undetectable viraemia. However, when $\log_{10}$ VL was plotted at baseline and at the end of the treatment course all subjects were shown to have a reduction in VL (Table 4-2; Figure 4-5). Median VL at D42 was 2.5 $\log_{10}$ (IQR 2.0-2.8), significantly lower than median VL at baseline ($p<0.01$), with a median decrease in VL at D42 of 1.0 $\log_{10}$ (IQR 0.7-1.4).

In the group of babies with baseline VL >4.0 $\log_{10}$ copies/ml median reduction in VL was 1.8 $\log_{10}$ copies/ml (IQR 1.4-2.0) compared to only 0.8 $\log_{10}$ copies/ml (IQR 0.7-1.1) in the 11 subjects with a lower starting VL, a statistically significant difference ($p=0.02$). 6/11 (54.5%) of subjects with a lower starting VL had no virus detectable by the end of the treatment course, meaning that they had reached maximal decline. Indeed, all babies with undetectable VL at the end of treatment were in the group with a starting VL <4.0 $\log_{10}$ and there was a significantly lower baseline $\log_{10}$ VL in those with undetectable compared to detectable VL at D42 (3.0 $\log_{10}$; IQR 2.8-3.2) vs 4.4 $\log_{10}$ (IQ 3.3-4.8; $p<0.01$).

In all 6 subjects with no detectable viraemia at the end of treatment CMV DNA became undetectable within 2 weeks of commencing treatment (subject 5 was missing a D14 specimen but was undetectable at next sampling). However, only 3 of these 6 subjects had a consistently undetectable VL following the first undetectable level; the other 3 subjects had intermittent low level virus detection. 2 further subjects had an undetectable VL at some time during treatment (one in the first 2 weeks of treatment and one at D28).

4.3.2. *Viral load decline*

When considering change in VL during treatment in the group as a whole most of the change in VL was observed during the first two weeks treatment (Figure 4-6). In those with a higher starting VL, however, decline continued up to D28 before reaching a nadir.

The median slope of decline in all subjects over 42 days of treatment was calculated to be 0.02 $\log_{10}$ copies/day. Decline during the first 14 days was more rapid, however, with an observed decrease of 0.07 $\log_{10}$ copies/day.

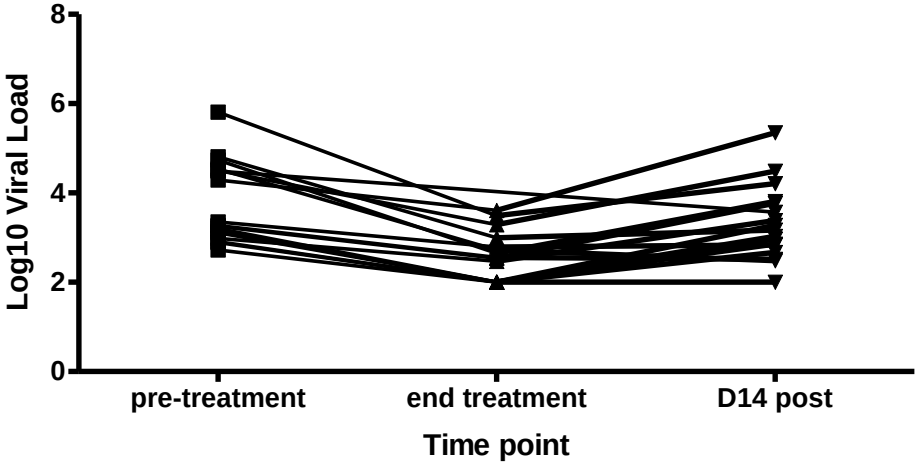
Table 4-2 Change in viral load in 17 babies with congenital cytomegalovirus at various time points during and after antiviral treatment

Subject	Log_{10} VL D1	Change in Log_{10} VL D1 to D42	Change in Log_{10} VL D42 to D56	Change in Log_{10} VL D56 to D180	Change in Log_{10} VL D42 to D180
1	2.98	-0.51	0.91	-0.57	0.33
3	3.11	-1.11	0.97	-0.42	0.55
5	2.72	-0.72	0.86	-0.86	0.00
53	4.81	-1.82	0.17	0.93	1.11
54	5.81	-2.32	0.72	-1.22	-0.50
55	3.35	-0.57	0.05	-0.84	-0.79
56	4.74	-2.09	1.16	MD	MD
58	2.91	-0.91	0.00	0.00	0.00
59	4.54	-1.82	-0.25	-0.48	-0.72
60	3.27	-0.71	-0.04	-0.52	-0.56
61	4.50	-1.20	1.20	-1.31	-0.11
62	3.14	-1.14	1.03	MD	MD
63	4.29	-0.67	1.74	-2.45	-0.71
64	3.25	-0.71	1.23	-0.97	0.26
65	4.49	MD	MD	-1.57	MD
66	2.89	-0.89	0.67	0.03	0.69
67	3.20	-1.20	1.28	-1.28	0.00
median	**3.27**	**-1.01**	**0.88**	**-0.84**	**0.00**
IQR	**(3.11-4.50)**	**(-1.36 - -0.71)**	**(0.14 - 1.17)**	**(-1.25 - -0.45)**	**(-0.54 – 0.32)**

VL = viral load
D = study day
MD = missing data
IQR = Interquartile range

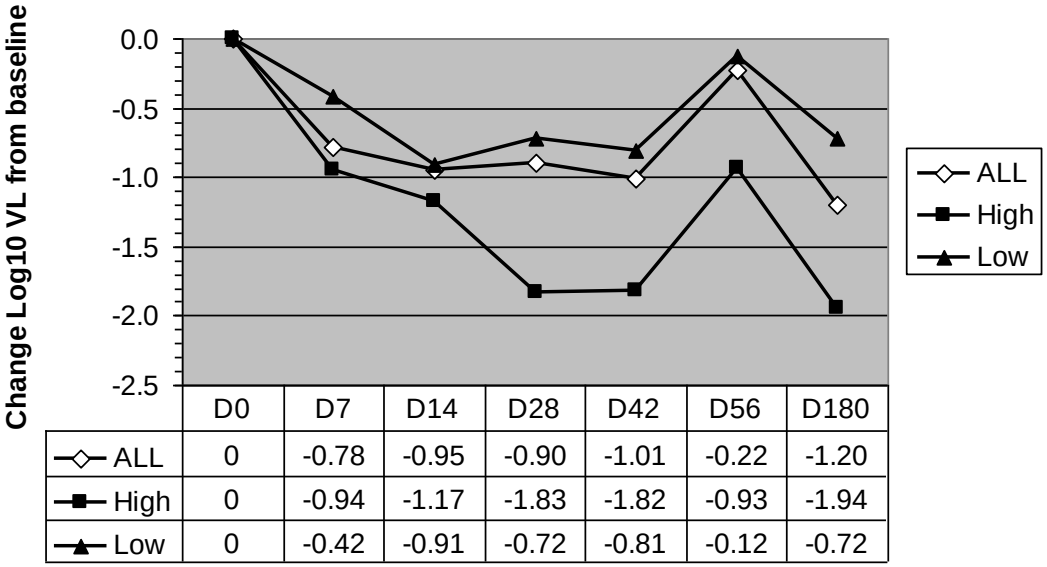
Data shown is for baseline log_{10} viral load and change from this baseline value at the end of a 42 day treatment course (D1 to D42). Change in viral load between: end of treatment (D42) and day 56 (D56), D56 and day 180 (D180) and D42 and D180 are also shown.

Figure 4-5: Viral load in 17 babies treated for congenital CMV at baseline, end of treatment and 14 days post-treatment.



Decrease in viral load during treatment and subsequent rebound following treatment discontinuation is shown graphically for 17 babies treated for congenital CMV. Viral load was measured using quantitative CMV PCR at baseline (pre-treatment), at the end of 42 days antiviral treatment (end treatment) and 14 days after treatment discontinuation (D14 post).

Figure 4-6 Median change in viral load from baseline in 17 viraemic infants during and after 42 days treatment for congenital CMV



	D0	D7	D14	D28	D42	D56	D180
ALL	0	-0.78	-0.95	-0.90	-1.01	-0.22	-1.20
High	0	-0.94	-1.17	-1.83	-1.82	-0.93	-1.94
Low	0	-0.42	-0.91	-0.72	-0.81	-0.12	-0.72

VL = viral load
High = babies with baseline VL >4.0 $\log_{10}$ copies/ml
Low = babies with baseline VL <4.0 $\log_{10}$ copies/ml
D = day of study

D7, 14, 28, 42 = treatment
D56 = 14 days post-treatment
D180 = 138 days post-treatment

Change in viral load ($\log_{10}$) from baseline was calculated for all viraemic babies with samples available at specified time points. Median change for all study participants was then plotted (all). Babies with a baseline VL >4.0 $\log_{10}$ copies/ml (high) were then analysed separately to those with baseline VL <4.0 $\log_{10}$ copies/ml (low).

In order to more accurately estimate viral $T_{1/2}$, Ln2 of the VL at the exact time point of sampling during treatment were used to plot graphs for each subject (Figure 4-3) and the phase of most rapid decline used for further calculations of $T_{1/2}$ as already described. $T_{1/2}$ during the phase of most rapid decline was thus calculated to be 2.4 days (IQR 1.9-4.5) (Table 4-3).

Taking into consideration the more rapid initial decline observed in those with a higher starting VL (and shown graphically in Figure 4-6) data from subjects with high and low starting VL were also analysed separately. There was no significant difference, however, between the median $T_{1/2}$ of decline in those with a high baseline VL compared to those with VL <4.0 log_{10} (median 3.86 vs 2.23 p=0.36) (Table 4-3). $T_{1/2}$ during the phase of maximal decline was plotted against baseline log_{10}VL for each subject. There was no obvious correlation between baseline VL and $T_{1/2}$ using standard regression models (Figure 4-7).

4.3.3. *Viral rebound after treatment discontinuation*

After treatment was discontinued an initial rebound in VL was seen in all but 3 participants who had data available at Day 56 (D56) (Table 4-2). In the group as a whole rebound was to a level similar to baseline (median at baseline 3.27 vs 3.17 log_{10} D56 (p=0.06)). Those with a high starting VL, however, had a VL at D56 that was a median of 0.9 log_{10} lower than at baseline, compared to a median of only 0.1 log_{10} decline in those with a low baseline VL (Figure 4-6). The difference between VL at baseline and D56 in those with starting VL >4.0 log_{10} was still, however, statistically non-significant (p=0.1)

4.3.4. *Long-term control of virus*

9/15 (60%) of the subjects with data available still had viraemia detectable at D180. All subjects had lower median VL at D180 compared to baseline, with a median decrease of 1.2 log_{10} (IQR 0.7-1.4); at D180 VL was lower than at D56 in all but 3 subjects (Table 4-2).

4.3.5. *Clinical outcome and association with viral dynamics*

Data for these analyses were limited. There did not appear to be a relationship, however, between starting VL, rate of viral decline or rate of virus rebound between

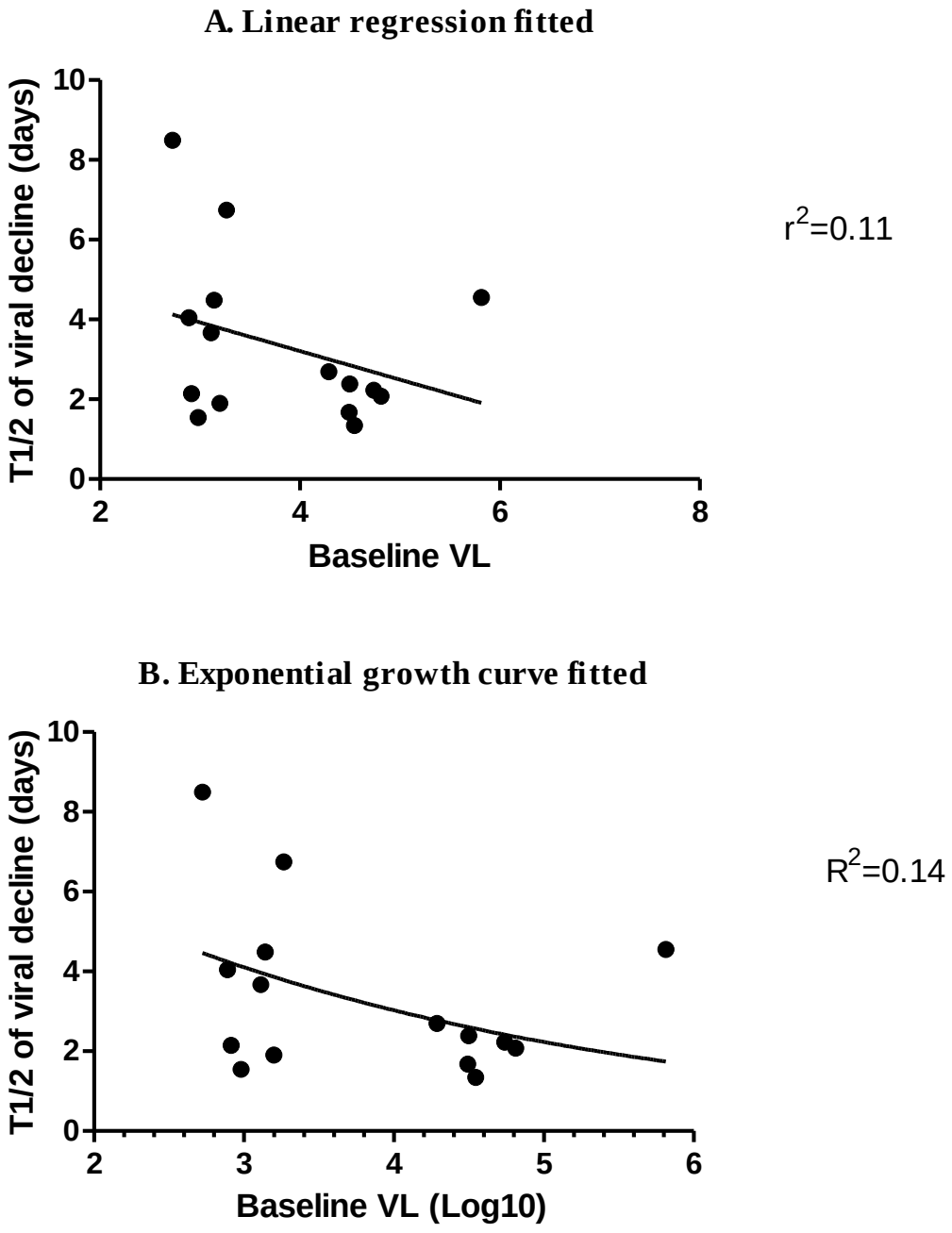
Table 4-3 Viral half life of decline and baseline viral load

	Subject	Baseline Log_{10} VL	Slope	$T_{1/2}$
BASELINE VL <4.0	**1**	2.98	-0.45	1.55
	3	3.11	-0.19	3.67
	5	2.72	-0.08	8.50
	55	3.35	0.02	-39.05
	58	2.91	-0.32	2.15
	60	3.27	-0.10	6.75
	62	3.14	-0.15	4.49
	64	3.25	-0.06	12.45
	66	2.89	-0.17	4.04
	67	3.20	-0.36	1.91
	MEDIAN	3.13	-0.16	3.86
	IQR	(2.93 - 3.24)	(-0.29 - -0.09)	(1.97 - 6.18)
BASELINE VL >4.0	**53**	4.81	-0.33	2.08
	54	5.81	-0.15	4.55
	56	4.74	-0.31	2.23
	59	4.54	-0.51	1.35
	61	4.50	-0.29	2.39
	63	4.29	-0.26	2.69
	65	4.49	-0.41	1.68
	MEDIAN	4.54	-0.31	2.23
	IQR	(2.91 - 4.77)	(-0.37 - -0.27)	(1.88 - 2.54)
ALL SUBJECTS	**MEDIAN**	3.27	-0.29	2.39
	IQR	(3.11 - 4.52)	(-0.35 - -0.16)	(1.99 - 4.49)

VL = Viral load
IQR = interquartile range
$T_{1/2}$ = half-life of virus decline

The slope of virus decline was calculcated using segmental regression in GraphPad prism as described in the methods to this chapter and shown graphically in Figure 4-3. This slope was then used to calculcate virus half-life using the formula (-ln2)/slope.

Figure 4-7 Relationship betweten starting viral load and half-lilfe of virus decline using standard regression models



VL = viral load
$T_{1/2}$ = half-life of virus decline

The relationship between starting viral load and virus half-life was scrutinised using common methods of regression and using GraphPad prism software. Two such analyses are shown above.

days 42 and 56 and either overall mental score or motor developmental score. When the IQ and motor score of those babies who had no virus detectable at D180 was compared to those with continued viraemia there was also no appreciable difference between the two groups. There was no apparent association between higher starting VL and more severe hearing loss nor did there appear to be an association between hearing deterioration and viral decline or rebound.

4.4. DISCUSSION

Consistent with other studies only 71% of infants were viraemic at birth, (Bradford et al. 2005); others have reported that viraemia is detectable in all babies albeit with 10% having only low levels of detection (<100 copies CMV DNA per 10^5 polymorphonuclear leucocytes) (Lanari et al. 2006).

63% of babies still had virus detectable in the blood at the end of a 6 week treatment course. This differs from unpublished data from our laboratory which found 66% of infants treated with a dose of 5mg/kg GCV bd had no CMV detectable in the urine at the end of a course of similar duration and with published data in which undetectable levels of virus were reported at the end of treatment in all subjects receiving higher GCV dosing regimes (Nigro, Scholz, & Bartmann 1994;Whitley et al. 1997). Given that blood VL is usually found to be a number of logs lower than that found in urine one might anticipate that it would be easier to suppress virus to undetectable levels in this body fluid after 6 weeks treatment. Indeed Whitley *et al* found that of 7 babies treated with i.v. GCV all 3 that were positive for CMV in leucocyte culture at enrolment were negative after two weeks treatment (Whitley et al. 1997). It is possible that the above discrepancy with previous literature reflects both differences in viral dynamics between these two body fluids and a higher sensitivity for detection using CMV PCR compared to older culture-based methodology.

A significant difference was observed in VL at start and end of treatment. The median decrease in VL from baseline observed in the group as a whole was 1.0 $\log_{10}$ (IQR 0.7-1.4) with maximum decrease in VL occurring during the first 2 weeks of treatment. This was the period where GCV was predominantly used for treatment. In those with a starting VL >4.0 $\log_{10}$ a greater decrease of 1.8 $\log_{10}$ (IQR 1.4-2.0) during 6 weeks treatment was observed and these babies additionally had a more rapid and more sustained initial decline in VL over the first 28 days of treatment.

Although some of this more prolonged decline may purely be a function of the higher starting point this does not fully explain the flattening of viral decline after the first 14 days of treatment in other subjects given that 6/11 of those with lower starting VL continued to have virus detectable at the end of treatment.

Starting VL and $\log_{10}$ decrease over the first 14 days of treatment in this group of infants are similar to data reported in an adult study of VGCV treatment in transplant patients (Table 4-4)(Mattes et al. 2005). However, in this study all patients receiving i.v. GCV had 2 negative CMV PCR results by day 33 of treatment and those receiving VGCV had a median time for 2 negative results of 20 days (Table 4-4).

From the data presented here $T_{1/2}$ of viral decline in neonates treated for congenital CMV is around 2.39 days (1.99-4.49). This value is comparable to that found in groups of GCV-treated adult patients with HIV (2.56 +/-0.36 days); following bone marrow transplantation (1.52 +/-0.67 days) and liver transplantation (2.36 +/-1.2 days) (Emery et al. 1999). It is also similar to the $T_{1/2}$ of 2.16 days (range 0.75 to 6.93) reported in adult solid organ transplant patients given pre-emptive VGCV treatment (Mattes et al. 2005).

Older studies have reported a rapid decline of approximately 2 $\log_{10}$ in viruria between the ages of 3 and 9 months in untreated babies after an initial period of stability (Stagno et al. 1975). There are no comparable studies of natural history of viraemia in untreated babies with cCMV. In the absence of a control group of untreated participants in this study it is therefore possible that the decrease in blood VL over time observed here is not significantly different to that in untreated babies and is purely reflecting the natural history of cCMV infection.

In support of a PD effect of treatment on VL is the subsequent rebound in VL observed in this study, as with others, following discontinuation of treatment (Nigro, Scholz, & Bartmann 1994;Tanaka-Kitajima et al. 2005;Whitley et al. 1997). Rebound in VL in the subjects presented here was not, however, to a level statistically different to baseline. The lower VL at D180 compared to D56 along with the continued low level viraemia detectable towards the end of treatment may be explained by natural oscillations in VL once steady state has been reached, as observed in other viral infections and in CMV-infected adults. To confirm this hypothesis further blood sampling would be needed in the period after treatment in

Table 4-4 Comparison of viral load decrease during antiviral treatment for CMV in babies and adults

Characteristic	Treatment		
	iv GCV[1]	VGCV[1]	VGCV - neonate
Treatment length (days)	16 (1-33)	20 (6-49)	42
Baseline viral load (log_{10})	3.55	3.81	3.27
Half-life, median days	1.73 (0.87-11.55)	2.16 (0.75-6.93)	2.34 (2.14-2.71)[2]
Log_{10} decrease, genomes/ml			
Day 7	-0.64 (-1.79 to 0.57)	-1.07 (-1.96 to -0.07)	-0.78 (-0.98 to -0.23)
Day 14	-1.17 (-3.07 to 1.07)	-0.98 (-2.12 to 0.29)	-0.95 (-1.18 to -0.31)

Data are expressed as median (range)
1 Treatment was continued until 2 consecutive negative CMV PCR results were obtained
2 These data are expressed as median (interquartile range) due to outliers.

Babies received treatment for congenital CMV disease with a combination of i.v. GCV and p.o. VGCV according to two separate protocols (Figure 4-1). Adult patients were enrolled in a study of pre-emptive treatment for CMV infection following solid organ transplantation (Mattes 2005).

order to define VL fluctuations over time. This persistent low level viraemia may also be reflecting immature neonatal immune responses and/or continued virus production from sanctuary sites as discussed further in the final chapter of this thesis.

The results presented here raise a number of areas warranting further investigation.

1) More information is needed relating to the natural history of blood VL in babies with cCMV infection. This includes data confirming steady state of viraemia at birth and trends in blood VL over time in babies not receiving treatment. Without a good knowledge of the natural history of VL it is difficult to evaluate the contribution of treatment in suppressing VL and to define target outcome measures.

2) Taking into consideration the more rapid viral decline noted during the first 7-14 days in these subjects and that observed during a more intensive sampling study of viral dynamics in HIV co-infected patients, more frequent sampling during the first 1-2 weeks of treatment may allow for more accurate measures of viral half-life.

3) Further investigation into the different patterns of viral decline is needed. This includes further consideration as to why 5/11 subjects with a low (<4.0 $\log_{10}$) baseline VL continued to have virus detectable at the end of a 42 day treatment course. In light of the data relating to potentially subtherapeutic GCV levels presented earlier in this thesis whether drug exposure is sufficient to suppress virus in this age group should be a primary concern. The first two weeks of treatment in this study was with i.v. GCV and the fact that viral decline in blood during the first two weeks of treatment was comparable between this group of neonates and adult data would suggest that treatment in the first few weeks of life was adequate (Mattes et al. 2005). PK and PD analysis of this study, however, also did not find an overall association between any PK parameters and viral suppression (Kimberlin et al. 2008). Notably the dose of oral VGCV used later in the study was individually targeted and oral bioavailability of drug was shown to increase with postnatal age; drug exposure during the latter stages of treatment should therefore have been optimal. There are little data, however, to confirm that the target AUC defined is optimal for targeting treatment in neonates. Moreover factors affecting drug availability to CNS compartments in this age group are unknown for both GCV and VGCV. It is therefore possible that differences in PK of VGCV led to the initial viral decline observed not being sustained.

Less likely is that lower levels of drug exposure during early weeks of treatment at the standard GCV dose might encourage the emergence of virus mutations conveying drug resistance. Current data do not support this theory, as discussed in the introduction to this thesis.

These persistent, low levels, of viraemia may alternatively represent low levels of continued viral replication in a subset of cells in these infants that are either exposed to lower drug levels or possibly with decreased levels of the cellular enzymes needed to complete phosphorylation of GCV/VGCV. This will be explored in more detail in the final chapter of this thesis.

4) The rapid viral rebound after treatment termination observed in neonates is also unique to this age group. If the 'threshold' concept described in adult transplant recipients applies to these neonates then the rebound back up to baseline noted here may have implications for disease progression. Clinical improvement has, however, been documented in previous studies after only 6 weeks i.v. GCV treatment despite these observations (Kimberlin et al. 2003). Maturing neonatal immune responses are also likely to be of relevance in ensuring prolonged viral control. Further studies of the immunological response to CMV in neonates may therefore enable identification of the key components determining immunological control in this age group.

5) Key to future therapeutic studies in this age group are data correlating clinical outcome with virological response in accessible body fluids. Whether the inability to fully control virus in blood during 6 weeks of treatment has any clinical implications or reflects viral replication in relevant cells in the CNS and auditory apparatus remains unknown.

CHAPTER 5.

5. THE EUROPEAN CONGENITAL CYTOMEGALOVIRUS INITIATIVE AND THE UK CMV TREATMENT REGISTRY

5.1. INTRODUCTION

5.1.1. *The European Congenital CMV Initiative*

It is widely recognised that for uncommon conditions, such as symptomatic cCMV, the small number of cases identified by any one centre limits good quality research. A group of professionals within Europe with an interest in cCMV was therefore established in 2005. The group included clinicians from the fields of virology, obstetrics, neonatology, paediatrics, immunology and epidemiology who had made significant contributions to cCMV research over the preceding decades. It was anticipated that the establishment of a collaborative network of interested parties within Europe would help address some of the barriers to research by facilitating studies involving larger cohorts of affected babies in multicentre studies conducted throughout the European Union.

5.1.2. *Rationale for the establishment of a treatment registry for congenital CMV*

Our group had experience of working within other successful neonatal infection networks, most notably the Paediatric European Network for Treatment of AIDS (PENTA, http://www.pentatrials.org/). This collaboration, established in 1991, between paediatric HIV centres in Europe is co-ordinated by the HIV Division of the Medical Research Council (MRC) Clinical Trials Unit (CTU) in London along with other international organisations. The large studies made possible through this network have led to many notable improvements in the treatment of paediatric HIV and significant findings with regards to paediatric dosing of anti-retrovirals (Bergshoeff et al. 2005;Burger et al. 2004).

Within the UK, and also co-ordinated by the MRC CTU, the CHIPS dataset, has successfully maintained treatment records that, at the end of March 2009, covered 93% of the 1560 children identified as HIV infected. This cohort study has provided valuable observational data regarding treatment commencement, drug resistance and other follow-up data for HIV-infected children treated in the UK (CHIPS 2013).

Data originating from this dataset have previously raised important concerns regarding underdosing of anti-retroviral medication during childhood which has relevance for other paediatric medications (Menson et al. 2006). Based on the

paediatric GCV levels presented earlier in this thesis we had reason for similar concerns in relation to the treatment of babies for cCMV. The use of treatment registries, particularly where small numbers of patients are treated with a drug, has become increasingly recognised as a valuable pharmacovigilance tool (EMEA 2007).

In April 2006 the members of ECCI met in Veyrier-du-Lac, France, and agreed broad recommendations for clinical management of cCMV. Given the limited evidence-base on which to guide treatment of these babies and the availability of new antiviral treatments, such as oral VGCV, it was agreed that a European Registry of babies treated for cCMV should be established, in order to monitor treatment of these infants while the results of future RCTs were awaited.

At the time this need was identified oral VGCV solution was not licensed anywhere within the European Union (EU), and no published dosing recommendations existed for the paediatric age group. Despite this, given the challenges of administering i.v. GCV for 6 weeks, case reports of the use of VGCV in neonates using extemporaneously prepared solutions were emerging with varying doses and duration of treatment reported (Anaizi, Dentinger, & Swenson 2002;Burri et al. 2004;Clark et al. 2004;Henkin, Griener, & Ten Eick 2003;Meine Jansen et al. 2005). The publication of such case studies suggesting clinical benefit raised concerns regarding emergent publication bias. Oral VGCV was already known to have been used in some neonatal units within the UK and interest in its use was anecdotally increasing, particularly in cases where i.v. access was difficult, and in less severely ill babies to enable earlier discharge home. There were therefore concerns that the availability of VGCV might lead to the treatment of babies outside the current evidence base for benefit, either in older age groups than previously studied, with only mildly symptomatic cCMV or for longer durations of treatment (Meine Jansen et al. 2005;Muller et al. 2008;Schulzke and Buhrer 2006). Furthermore, although of potential benefit through cost savings to healthcare systems, there were also concerns that earlier discharge may lead to less frequent review of treated babies, less rigorous monitoring of side effects and failure to adjust drug doses for weight-gain during treatment.

I carried out a survey evaluating the views and perception of treatment and its success amongst members of the UK CMV parent support group. Contrary to published literature many members of this group felt that, in their experience,

medication had not been particularly beneficial and was associated with worrying side effects (personal correspondence). Finally, although we were aware of one long-term follow-up study of outcomes up to puberty in the small cohort of neonates studied by Kimberlin *et al* in their RCT of GCV treatment these data have not been published to date and there was no ongoing surveillance in place to follow the large numbers of children that would be necessary to identify small increases in outcomes such as carcinogenicity.

The need for larger surveillance data relating to babies treated for cCMV, in addition to formal RCTs, was therefore evident.

5.1.3. *Aims of the CMV Registry*

Given that data on symptomatic cCMV had previously been obtained on a large scale in both the UK and the US (Demmler 1991;Lynn et al. 2004), the limited resources at our disposal and our primary concerns regarding antiviral treatment it was agreed that the focus of this registry would be on the short and long term toxicity of treatment along with some markers of clinical and virological efficacy.

The CMV Registry was therefore set up with the primary aims of:

1) Holding a central register of any babies treated for cCMV in the UK, and ultimately Europe, both within and outside of any formal treatment studies.
2) Developing a national knowledge base of patterns of antiviral usage in cCMV and changes over time.
3) Recording short-term side effects of antiviral usage in cCMV.
4) Recording possible markers of treatment efficacy, including virological and audiological data.
5) Providing the possibility for longer term surveillance of any potential toxicities associated with antiviral usage in this age group both within the database itself and by linkage to national cancer registries.

To pilot the concept and facilitate a more rapid instigation it was agreed that the Registry would initially be set-up to recruit babies from within the UK and subsequently extended to include other European partners dependent on subsequent funding and interest within these countries.

5.1.4. *Anticipated numbers within the UK*

In the U.K. epidemiological studies estimate around 350 cases of symptomatic cCMV per year (Peckham et al. 1983). A two year British Paediatric Surveillance Unit (BPSU) study carried out from February 2001 to February 2003 identified 93 confirmed cases and a further 69 suspected cases of cCMV. Of the confirmed cases 40% had neurological symptoms and 33% of those received treatment, translating to approximately 6 cases of treated cCMV per year in the UK (Lynn et al. 2004).

We anticipated that more babies than this previous estimate would be treated annually and eligible for enrolment based on a number of observations. Firstly the publication by Kimberlin *et al* (2003) gave the first evidence that GCV treatment might improve outcome in the most severely affected babies meaning that clinicians might now be more likely to treat babies with CNS involvement. Newer diagnostic methods, including rapid CMV PCR and retrospective diagnosis using DBS, were emerging and likely to aid confirmation of a diagnosis of cCMV. A multicentre, multinational study of treatment with VGCV of babies with any symptoms of cCMV (not just those with CNS involvement) was in the final stages of protocol development (NCT00466817). Selected UK centres were proposed as likely study centres and widespread publicity had already commenced, including educational talks to clinical groups throughout the UK, to increase the profile of cCMV. Anecdotally there was already an increased awareness amongst clinicians of the problems associated with cCMV, both antenatally and postnatally, enabling more prompt identification of symptomatic babies and initiation of treatment where indicated. We were aware of 4 babies receiving treatment for cCMV in South London alone in the year preceding the implementation of the Registry (January-December 2006).

UK annual births were around 700,000 in 2007 and using estimates of cCMV prevalence at birth of 3-7 per 1,000 live births, 12.7% symptomatic, around 80-350 babies would be anticipated to be born annually in the UK with symptomatic cCMV (Dollard, Grosse, & Ross 2007). If ~40% of these neonates had CNS signs or symptoms, around 32-140 babies per year would be potentially eligible for treatment in the UK. We would anticipate that maybe only 50% of these would be identified and treated and a further 50% brought to our attention meaning that the Registry would aim to capture 8-35 treated babies annually.

We had already established through the CMV Parent Support Group that parents were receptive to any research proposals that might inform treatment of cCMV (personal correspondence). Maximal recruitment was proposed by publicising the study within neonatology, paediatric infectious diseases, audiology and virology networks throughout the U.K. It was intended that the linked website would additionally provide an educational resource for both health professionals and the public.

5.2. METHODS

The Registry was set up as a web-based database, enabling access from any internet connection using a unique username and password. The use of individual login codes enabled audit of the data trail if required. Local investigators had access to data generated from their own institution; only the central study team were able to access the complete dataset.

Our group had experience of successfully using similar databases for other national cohort studies and similar collation of data from babies treated for cCMV in many centres throughout the UK was therefore felt feasible. It was anticipated that most babies would be treated in tertiary neonatal units with input from paediatric infectious diseases specialists, as with the treatment of paediatric HIV and other uncommon neonatal conditions. Capturing the majority of treated babies was therefore proposed by the involvement of around 18 key centres throughout the UK.

Inclusion criteria were defined as:-.

1) Any baby with confirmed cCMV (diagnostic sample obtained within 21 days of life) born between 1/1/2002 and 1/1/2017 AND
2) Receipt of any treatment for cCMV within the first 5 years of life.

Treatment and duration were not specified since the Registry aimed to reflect current practice and changes over time. Data were stored in a pseudo-anonymised form using a unique study reference number but with inclusion of date of birth and hospital number to prevent double entries of the same case from different sites, prevent confusion in the case of twins and to facilitate follow-up reminders. The local study team stored more detailed enrolment information enabling retrospective identification of each baby registered if required.

The initial proposal involved clinicians entering baseline data on treated babies as soon as they were identified, with reminders automatically generated from the

database at one and two years to capture defined follow-up datasets relating particularly to neurological and audiological outcomes.

A set-up grant was successfully obtained through the European Society for Pediatric Infectious Diseases (ESPID) which provided funds for development of the database through an independent database developer (Dataphiles Ltd). Hardwear was purchased using internal funds made available from St George's University London and the Royal Free Charity.

5.2.1. *Database development and piloting*

A website was initially set-up, as proposed by the ECCI group (www.ecci.ac.uk), to act as a platform for the CMV Registry, with a link to the password-protected login page.

An SQL server with 128 bit SSL encryption of the data between the user's computer and the database, giving a level of data protection equivalent to that used by internet banking at the time, was subsequently set up.

A questionnaire containing key demographic data and data relating to disease manifestations to be collected was devised. The focus was to obtain information relevant to antiviral drug treatment, including medication formulation and dose, reasons for commencing treatment, side effects experienced and any associated alterations to treatment along with drug levels and any viral load results. Proposed data collection sheets were sent to a number of neonatologists and general paediatricians for comments on acceptability of length and detail of proposed questions and any additional/unnecessary data fields. The data collected are now available in the form of an ethically approved 'Letter requesting information from clinicians' which enables data collection from other units in the case of neonates transferred to or from a study site during treatment (Appendix A).

A separate dataset relating to hearing outcomes was developed in collaboration with a trainee in audiological medicine with an interest in cCMV to provide a semi-quantifiable measure of long-term outcome. This information is also available in an ethically approved 'Letter requesting information from audiologists' that can be sent to audiological departments to obtain detailed audiolgical data (Appendix B). This form is particularly important as audiology services are often under the auspices of different NHS Trusts than acute neonatal and paediatric care.

The datasets discussed above were translated into specific questions along with drop-down menus to closely define data entry options and decrease variability in entries.
Several rounds of database testing were subsequently performed to identify errors in translation of data fields and programming and to refine drop-down menus and data entry values permitted (for example format of numbers generated and length of additional text allowed).
During the lengthy period of set-up it was evident that for any data collection to commence the proposed follow-up fields would have to be sacrificed in order for the project to start recruitment. It was decided at that point that exporting data from the database at regular time intervals (e.g. 6 monthly) would allow clinicians to update the database at the time of annual reviews while still enabling the central study team to capture changes in outcome over time. Manual notifications to clinicians would therefore be necessary in order to remind them that follow-up data input was due.

5.2.2. *Ethics*

Ethics approval was obtained in February 2007. With a recent increase in focus towards ensuring children are also informed of research that involves them, the ethics committee made a requirement that there was some mechanism in place to notify children that their data were being held. This proved challenging in that the central research team, to comply with data protection requirements, were unaware of contact details of families. In addition, children with cCMV would be anticipated to have varying degrees of disability with many potentially never having sufficient cognitive function for this to be relevant. Ethics approval was therefore granted on the basis that parents would be provided with a letter at enrolment to give to their child at an undefined time in the future when their child was felt to be able to understand the implications of their data being held in the database.
Due to a change in the regulations since the inception of the idea for such a Registry it became necessary for individual parental consent to be obtained for data entry meaning that local site research and development (R&D) approval had to be gained. As the process of site specific approval and informed consent was necessary even for basic data collection it was decided that consent would additionally be obtained for surplus clinical samples to be retrieved and stored along with testing of DBS to compliment other work in progress in our group.

Enquiries were made with both ethics committees and the UK Central Cancer Registry (UKACR) regarding processes to facilitate future linkage with cancer registries. As advice was limited and application through the UKACR involved securing additional funds it was decided to obtain consent from parents for possible future data linkage while not formalising this plan until the Registry was more established.

It was evident early on in study set-up that changes in the ethics requirements for such data collection might prove challenging for case ascertainment.

5.2.3. *Approach of sites, local approvals and consent*

An ethically approved advertisement letter was sent out to members of the British Paediatric Allergy, Infection and Immunology Group (BPAIIG) encouraging them to notify the study team of any treated babies, and asking for expressions of interest from those who had already seen cases. Further eligible babies were identified through clinical enquiries to members of the study team.

I undertook to complete all paperwork required for local site approvals, asking the local investigator only to approve drafted documents and provide signatures. Once local site approval had been obtained study files containing all relevant documents were sent out along with login details for the Registry.

Consent could either be obtained from parents at the next out-patient visit or via telephone and postal consent (as approved in a later ethics amendment). The postal consent option was particularly aimed at increasing recruitment of previously treated children that were no longer under regular follow-up with the local study team to decrease unnecessary hospital visits for parents and associated out-patient workload for local clinicians.

5.2.4. *Ongoing data management*

The Registry was backed up daily and data also manually exported into Excel on a regular basis.

Small amendments and updates were made following set-up, mainly to update the website but also to address further technical issues identified during database usage.

5.2.5. *Data analysis*

In general median values with IQR were used for summarising linear data as it was uncertain in most cases whether a normal distribution was being followed.

As with my previous analysis of GCV levels, for levels stated as being undetectable below a certain level, 50% of this value was entered for analysis. In addition, for the reasons specified in Chapter 3, pre-dose levels above and below 0.6mg/L were specifically scrutinised, with >0.6mg/L being the proposed optimal trough value; similarly post-dose GCV levels above 7.0mg/L and 5.0mg/L for GCV and VGCV treatment respectively were examined.

5.3. RESULTS

5.3.1. *Site Accrual and numbers recruited*

From February 2007 to May 2010 17 UK sites received R&D approval (14 in England; 1 in Scotland and 2 in Wales).

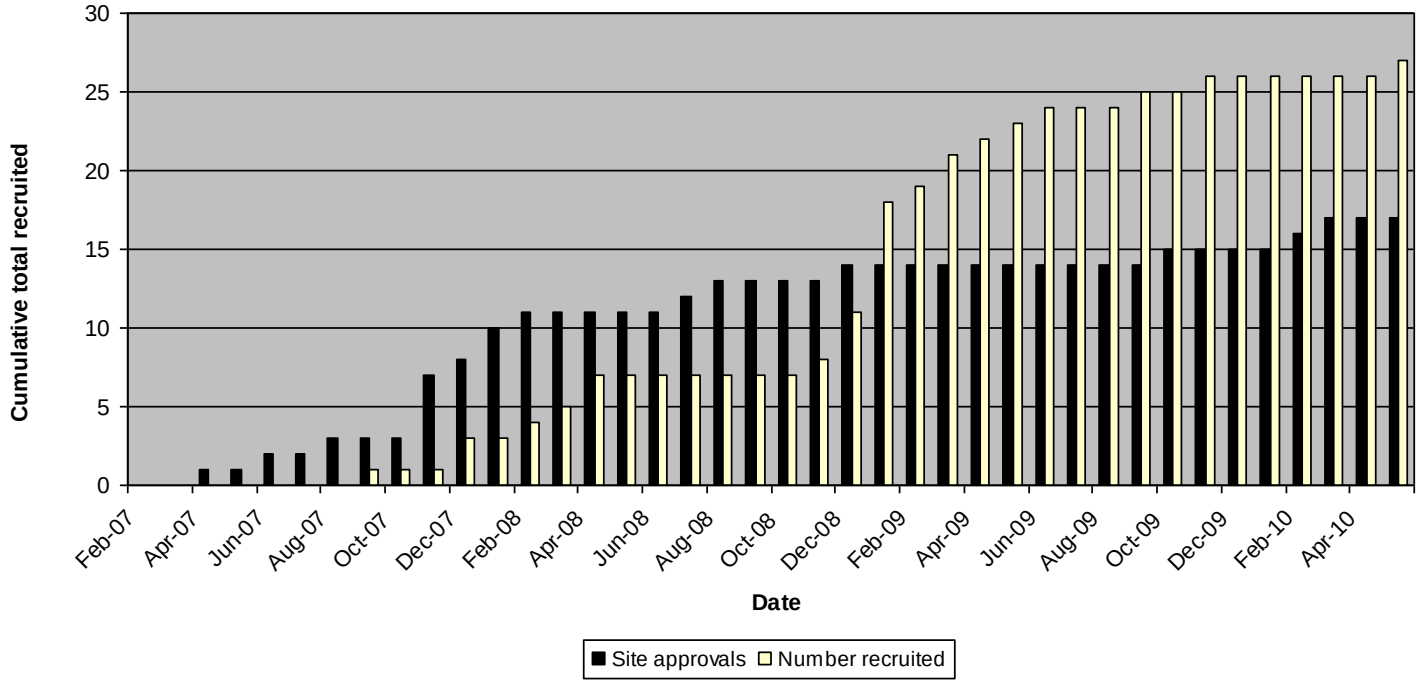
The median time between application for site approval and approval being obtained was 78 days (range 23-169) (measured from date of first submission of documents to the local R& D department to date of letter of site approval).

A further 10 sites were in various stages of approval at the time of writing with documentation required for R&D and ethics submission drafted for all of these sites.

Up to May 2010 27 babies had been enrolled from 10 centres (Figure 5-1; Figure 5-2). Although 7 sites had not yet entered data on a baby despite having relevant approvals, most of the 10 sites that had entered cases had entered data for more than one baby (Figure 5-2). I was aware of 8 babies treated in approved study sites where parental consent was not possible (1 declined due to concerns regarding access to data by regulatory agencies; 2 had difficult relationships with clinicians during treatment and were not pursued for consent; 1 mother did not formally decline but consent was not gained despite several approaches; 3 were contacted by letter and declined to get in touch with the study team and in 1 family language constraints prevented informed consent).

During the same time period (February 2007 – May 2010) I had information on a further 16 treated babies who would have been eligible for inclusion in the Registry (as summarised in Table 5-1 and considered further in the discussion of this chapter).

Figure 5-1: Recruitment over time into the UK congenital CMV Treatment Registry



Data shown as cumulative number of recruiting sites (site approvals) and number of babies recruited from February 2007 to May 2010.

Figure 5-2: Number of babies enrolled at each site

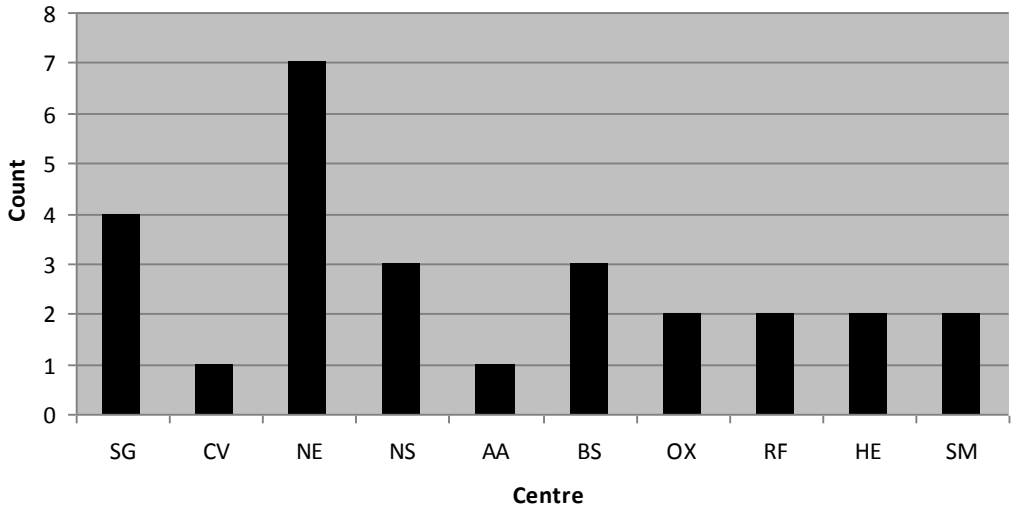


Table 5-1 Reasons for failed recruitment of babies managed by research team but not currently enrolled into Registry

No.	DoB	Centre	Symptoms	Treatment	Reason for failed recruitment
1	08/02/06	SG	CNS + multiple systemic including PPHN	GCV, Foscarnet	Retrospective – baby died, parents did not respond to invitation letter
2	01/06/06	SG	CNS	GCV	Retrospective – parents moved; no response to invitation letter
3	14/09/08	SG	CNS only	GCV, VGCV	Consent not obtained.
4	21/05/08	SM	CNS only	GCV	Parents declined – (agreed to associated study)
5	Aug 09	SM	Subtle CNS	GCV	Parents transferred care to GOSH, not further approached for study
6	05/08/09	RF	CNS only	GCV	Difficult clinical relationship with parents therefore not approached.
7		HE	Partial details – CNS.	?GCV	Parents direct correspondence with study team. Verbally happy for consent but consent not returned.
8	26/12/07	PM	Severe CNS	VGCV	SSIF prepared. Local team not submitted.
9	02/01/08	W	CNS	GCV/VGCV	SSIF prepared, Local team not submitted.
10	21/4/08	WYo	CNS	GCV/VGCV	SSIF prepared. Local team not submitted.
11	1/7/08	NH	CNS	?	SSIF prepared. Local team not submitted.
12	13/2/09	NM	CNS	GCV/ VGCV	Parents met, consent not possible due to language barrier.
13	03/09	LB	CNS	?GCV	SSIF prepared. Local team not submitted.
14	19/05/09	RL	CNS (SNHL)	GCV, VGCV	Local team expressed interest but initial paperwork not completed.
15	12/6/09	EB	CNS	VGCV	Site paperwork not commenced
16	8/7/09	BA	? details	? no details	Site paperwork not commenced
17	08/09	GW/BCH	CNS + Hepatitis	GCV	Site paperwork not commenced
18	09/09	DE	CNS, hepatitis	GCV,VGCV	Site approvals commenced
19	11/09	MC	? full details	GCV	Site to start approvals process
20	18/01/10	MA	CNS	GCV	Site paperwork not commenced
21	17/6/09	SH	CNS, IUGR	GCV, ?VGCV	Site approvals commenced
22	09/08/09	EC	CNS	GCV	SSIF prepared. Local team not submitted
23	16/04/09	BF	CNS	?GCV	Site approvals obtained. Eligible baby not yet consented.
24	19/07/07	BS	CNS	GCV, VGCV	Local clinicians not able to obtain consent. Language barriers.

CNS = Central Nervous System
PPHN= Persistent Pulmonary Hypertension of the Newborn
IUGR = Intrauterine growth restriction
SNHL = Sensorineural hearing loss
SSIF = Site Specific Information Form
GCV = Ganciclovir
VGCV = Valganciclovir

5.3.2. *Baseline demographics of enrolled babies*

The majority of babies (21/27: 78%) were term (≥37/40 gestation)(Figure 5-3); 59% were male. Symptoms and signs of disease are summarised in Table 5-2. Two babies were reported to have no abnormal clinical findings. CNS symptoms/signs were documented in 21/27 babies, with thrombocytopenia and hepatosplenomegaly being the next most common clinical findings entered. Birth weight ranged from 1000-4000g and was <0.4th Centile (<2.7 Standard Deviations below the mean) in 5/27 (19%). OFC at birth was recorded in 22/27 babies and was <0.4th Centile in 7/22 (32%); in 6/22 (27%) OFC was ≥1 Centile measurement below that of weight.

Only 8/27 babies currently enrolled were born prior to 2007 (Figure 5-4). There was no obvious over-representation of any particular month (or season) of birth for treated babies (data not shown).

Only 2 babies were reported to have co-morbidities with 1 having co-infection with HIV and a further baby having multiple congenital abnormalities (hypospadias; bilateral inguinal hernias; branchial cyst).

5.3.3. *Reasons for commencing treatment*

CNS disease was the commonest reason for treatment (22/27 = 81%) (Figure 5-5). In 11/22 additional reasons for commencing treatment were also stated including 5 for high VL, 4 for hearing, 3 for hepatitis and 1 eye problems. 3/5 babies treated without CNS disease appeared to have mild, or no clinical symptoms.

Almost 20% of babies had a high VL stated as a reason for commencing treatment, although in only one baby was treatment started purely for this indication; treatment was only given for 23 days in this case. 4 (15%) babies had treatment commenced for hearing loss and an equal number for hepatitis although these indications were always accompanied by others. A further 4 babies had treatment commenced for 'other' reasons; 2 for petechiae (in one case this was the only apparent reason for commencing treatment); one following "advice from tertiary ID team" with no other reason or symptoms specified and one for "symptomatic CMV and failure to thrive" with hepatitis and eye involvement also documented.

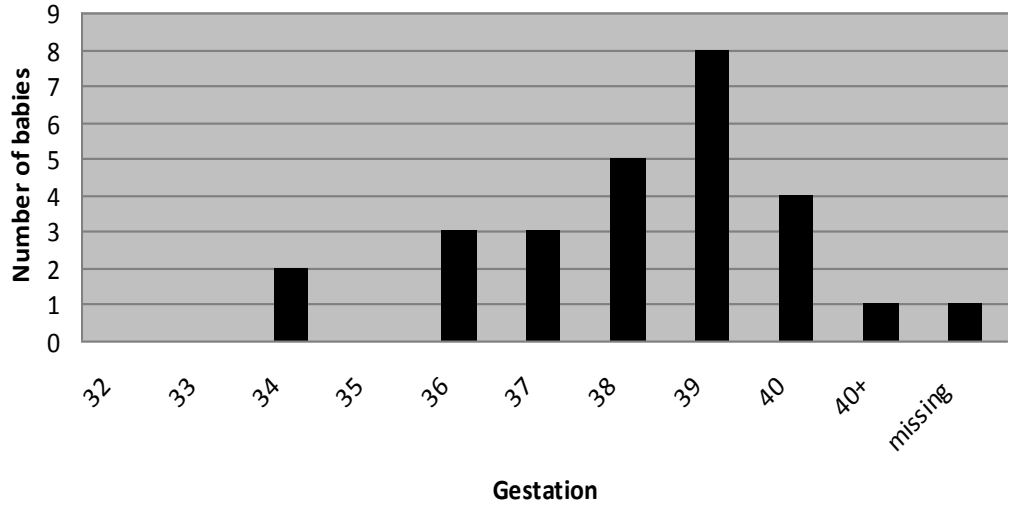
Table 5-2 Symptoms and signs at presentation in 27 babies treated for congenital CMV

Symptom/sign	Number (%)
Central Nervous System	21 (78)
Hepatitis	5 (19)
Hepatosplenomegaly	8 (30)
Pneumonitis	1 (4)
Thrombocytopenia	18 (67)
Anaemia	6 (22)
Neutropenia	4 (15)
Chorioretinitis	3 (11)
Skin	10 (37)
Bowel	1 (4)
Hearing	15 (56)

Definitions used:

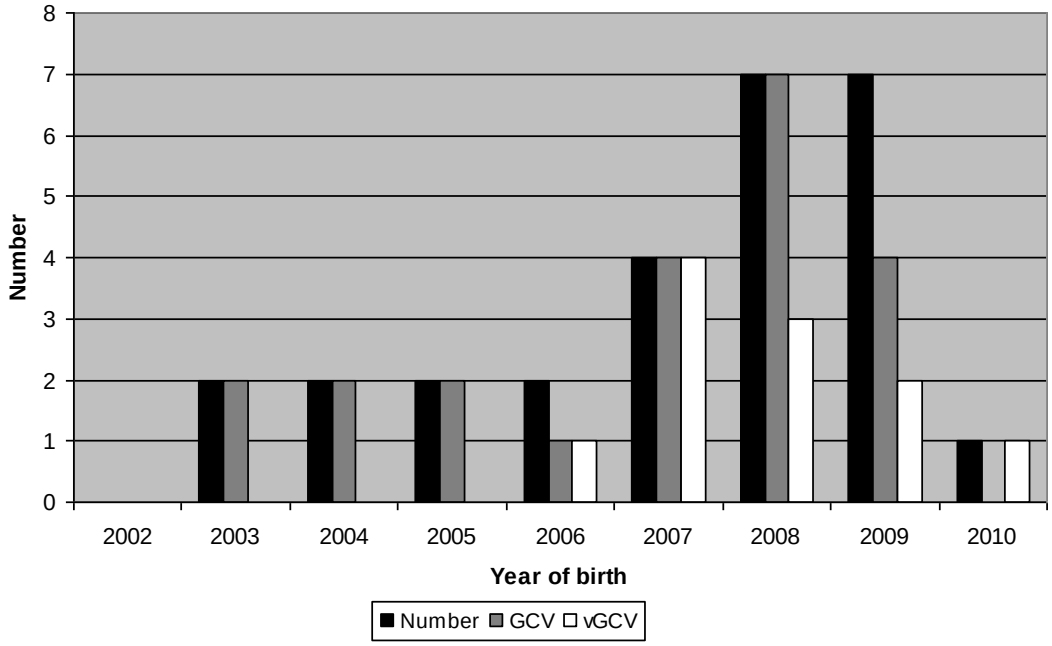
Central nervous sytem: microcephaly, seizures, abnormal tone, poor suck, abnormal neuroimaging consistent with CMV (intracranial calcification, ventriculomegaly, abnormal periventricular hyperechogenicity, hyperechogenicity of lenticulostriatal vessels).
Hepatosplenomegaly: liver or spleen >4cm below the costal margin
Hepatitis = ALT (Alanine Transferase) >100 IU/L OR jaundice/raised serum bilirubin (conjugated and without other obvious attributable cause).
Thrombocytopenia = platelet count $<100 \times 10^9$/L
Anaemia = Hb <13.5 g/dL
Neutropenia = Neutrophil count $< 0.5 \times 10^9$/L
Skin = petechiae or purpura (or other recognised manifestation of congenital CMV)

Figure 5-3: Gestation of babies enrolled



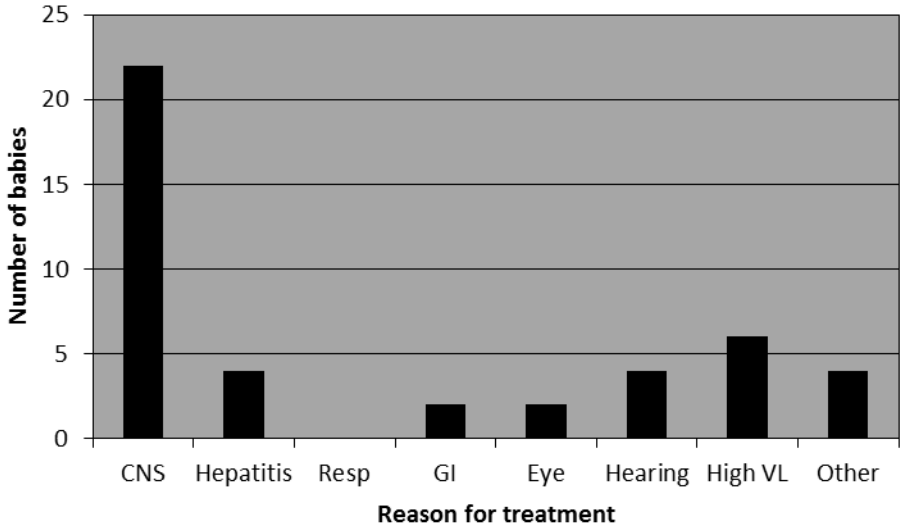
Recorded gestation of babies enrolled in the UK CMV Treatment Registry (N=27).
Missing = data not recorded.

Figure 5-4: Number of enrolled babies treated with ganciclovir or valganciclovir



GCV = ganciclovir
VGCV = valganciclovir

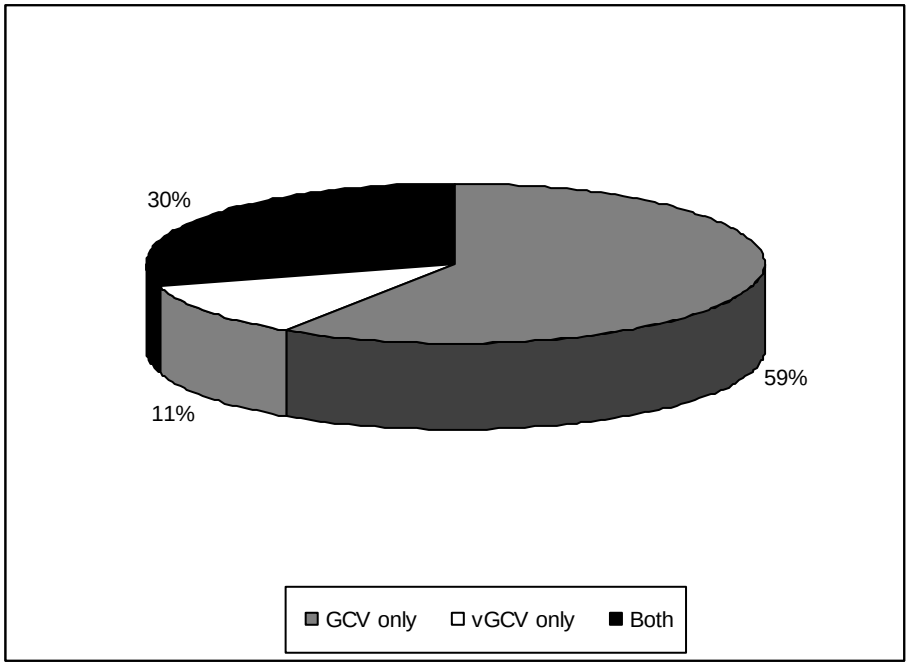
Figure 5-5 Indication for treatment in babies with congenital CMV



CNS = Central nervous system
GI = Gastrointestinal
VL = Viral load

Data showing indication for treatment in 27 babies with congenital CMV as recorded in the UK CMV Treatment Registry. In most babies more than one indication was entered.

Figure 5-6 Treatment received



GCV = Ganciclovir
vGCV = Valganciclovir

Treatment given in 27 babies with congenital CMV as recorded in the UK CMV Treatment Registry.

5.3.4. *Treatment received*

In 16/27 (59%) babies GCV was the sole drug used for treatment (Figure 5-6). In a further 8/27 (30%) of babies GCV was used sequentially with VGCV; GCV was the starting drug in all these cases. In the 8 babies in whom sequential GCV and VGCV were used the reason for switching to oral therapy was stated as problems with i.v. access in 2 cases (suspected in a 3rd case from correspondence regarding management), to enable discharge in 2 cases (and suspected as the reason in a 3rd case from the same site) and to enable prolonged therapy in one case (suspected as the reason in a further case that also received 42 days GCV followed by VGCV). VGCV treatment was first documented as used in the Registry in a baby born in 2006; there was no increase in its use over time (Figure 5-4). VGCV was used in the treatment of a total of 11 babies although it was the sole drug used in only 3 cases (Figure 5-6).

In the majority of babies (24/27) treatment was commenced in the first 30 days of life. Treatment was started at 31 days of age in one baby; the remaining two babies in whom treatment was started later were born in 2003.

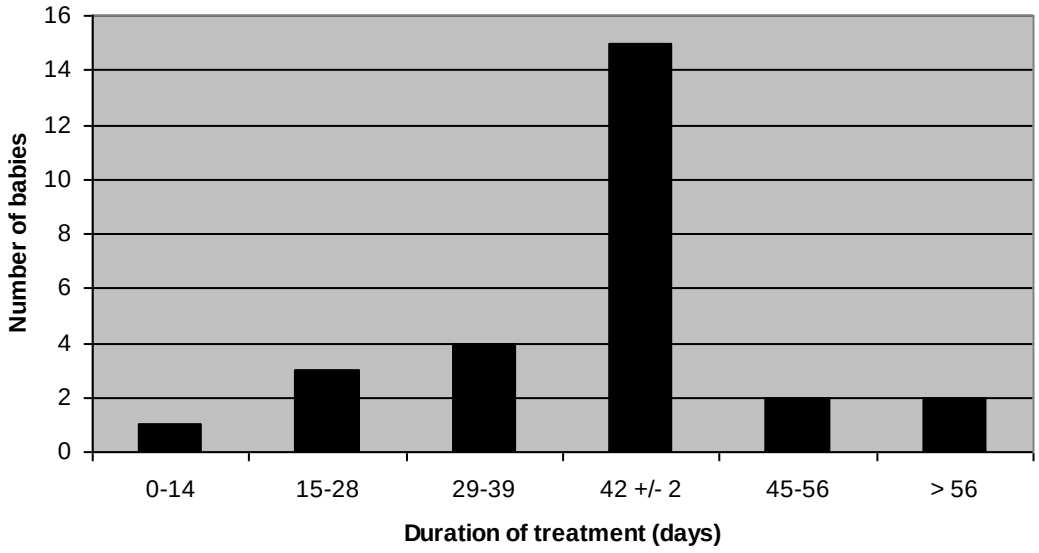
The majority of babies (15/27) received a 42 day course of treatment (+/- 2 days) (Figure 5-7). In the 8 babies who received <40 days of treatment 3 were documented to have side effects during treatment (2 neutropenia and 1 *Staphylococcus aureus* line sepsis) but in only one of these was it documented that treatment was stopped due to side effects (neutropenia). 4 babies received a longer duration of therapy, with 2 of these receiving >56 days treatment. There is no documented reason for the longer treatment course in any of these babies.

The starting dose of GCV was 6mg/kg/dose bd in 16/24 (66%) and 5mg/kg in a further 7/24. In 3/7 in whom a dose of 5mg/kg was commenced the dose was subsequently increased to 6mg/kg.

In 6/11 of those treated with VGCV, the starting dose was 15 or 16mg/kg/dose bd. In the other 5 babies the starting dose ranged from 10mg/kg to 40mg/kg bd. The dose was changed in 3 babies receiving VGCV; dose was increased in 2 of these babies (one from 15mg/kg to 15.5mg/kg and one from 16mg/kg to 18mg/kg) and decreased in one (from 25mg/kg to 15mg/kg bd).

I am unable to assess from the data currently entered into the Registry how often dose was adjusted for increases in weight.

Figure 5-7 Duration of treatment



Duration of antiviral treatment given in 27 babies treated for congenital CMV and enrolled in the UK CMV Treatment Registry

5.3.5. *Ganciclovir levels*

8 babies had 36 GCV levels recorded; 14 pre- and 16 post-dose levels and 6 untimed. No babies had reported renal impairment. For i.v. GCV median pre- and post- dose levels recorded were 0.2 mg/L (IQR 0.1-0.4) and 4.6 mg/L (IQR 2.9-5.1) respectively (Figure 5-8A); dose of GCV was 6mg/kg in all instances. Corresponding levels for those receiving VGCV were 0.5mg/L (IQR 0.1-0.8) and 4.0mg/L (IQR 3.0-4.3) pre- and post-dose respectively (Figure 5-8B). These levels were obtained, however, on varying weight based doses of VGCV. Due to these different dosing regimens linear regression was carried out of both the pre- and post-dose levels (Figure 5-8B). A strong linear association was demonstrated between dose of VGCV and post-dose levels ($r^2 = 0.73$) with a weaker association between pre-dose levels ($r^2 = 0.58$). In one subject (UKBS3), in whom multiple levels were taken, both pre- and post- levels were seen to increase gradually over a 15 day period whilst on the same dose of VGCV (12mg/kg bd).

Trough levels were <0.6mg/L in 4/4 and 6/10 of those receiving GCV and VGCV respectively. Peak levels were >7.0mg/L in 0/6 of those receiving GCV and >5.0 mg/L in 2/9 (22%) of those receiving VGCV.

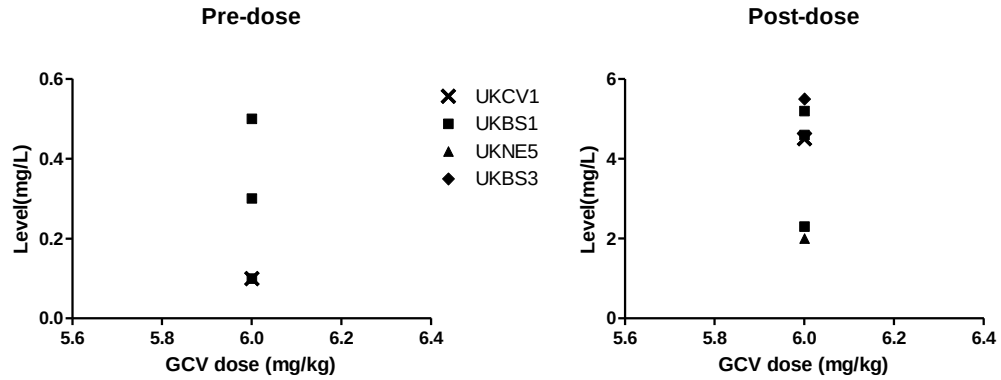
Due to insufficient data it was not possible to describe in any detail the relationship between VL and drug levels. No obvious association emerged in the few patients with serial measurements available (Figure 5-9).

5.3.6. *Side effects*

16 side effects were documented in 13 subjects (12 receiving GCV and one VGCV) (Figure 5-10). The commonest side effect documented was neutropenia, which occurred in 7/27 (26%) treated babies, followed by line infection which was reported in 5/22 (23%) babies receiving i.v. GCV. In only one baby, however, was the treatment course documented to be curtailed due to side effects (the only recipient of VGCV that had side effects documented). In the remaining 6 babies treatment was continued but either interrupted for brief periods or switched to VGCV (N=4). In the 5 babies with reported line infection 3 had a growth of *S. aureus* documented, one coagulase negative staphylococcus and in one no organism was documented. In 3 of these babies the course was completed with i.v. GCV, in one the course was

Figure 5-8 Pre- and Post- dose ganciclovir levels following administration of i.v. ganciclovir and oral valganciclovir

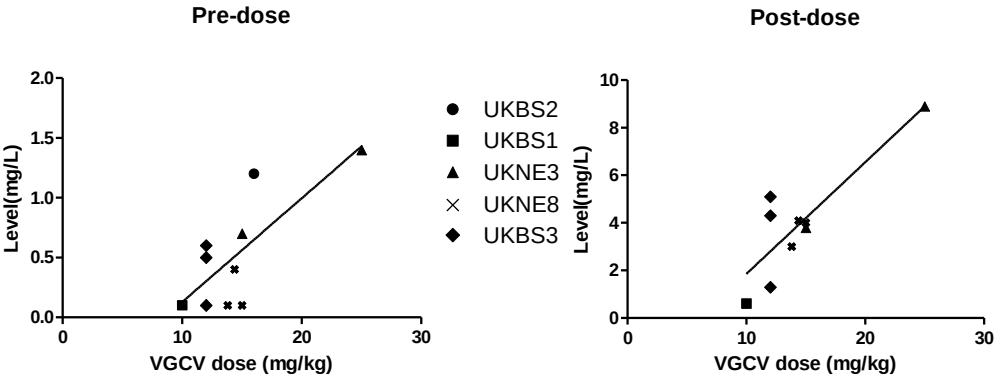
A: IV Ganciclovir



Median = 0.2 (0.1-0.35)

Median = 4.6 (2.9-5.1)

B: Oral Valganciclovir



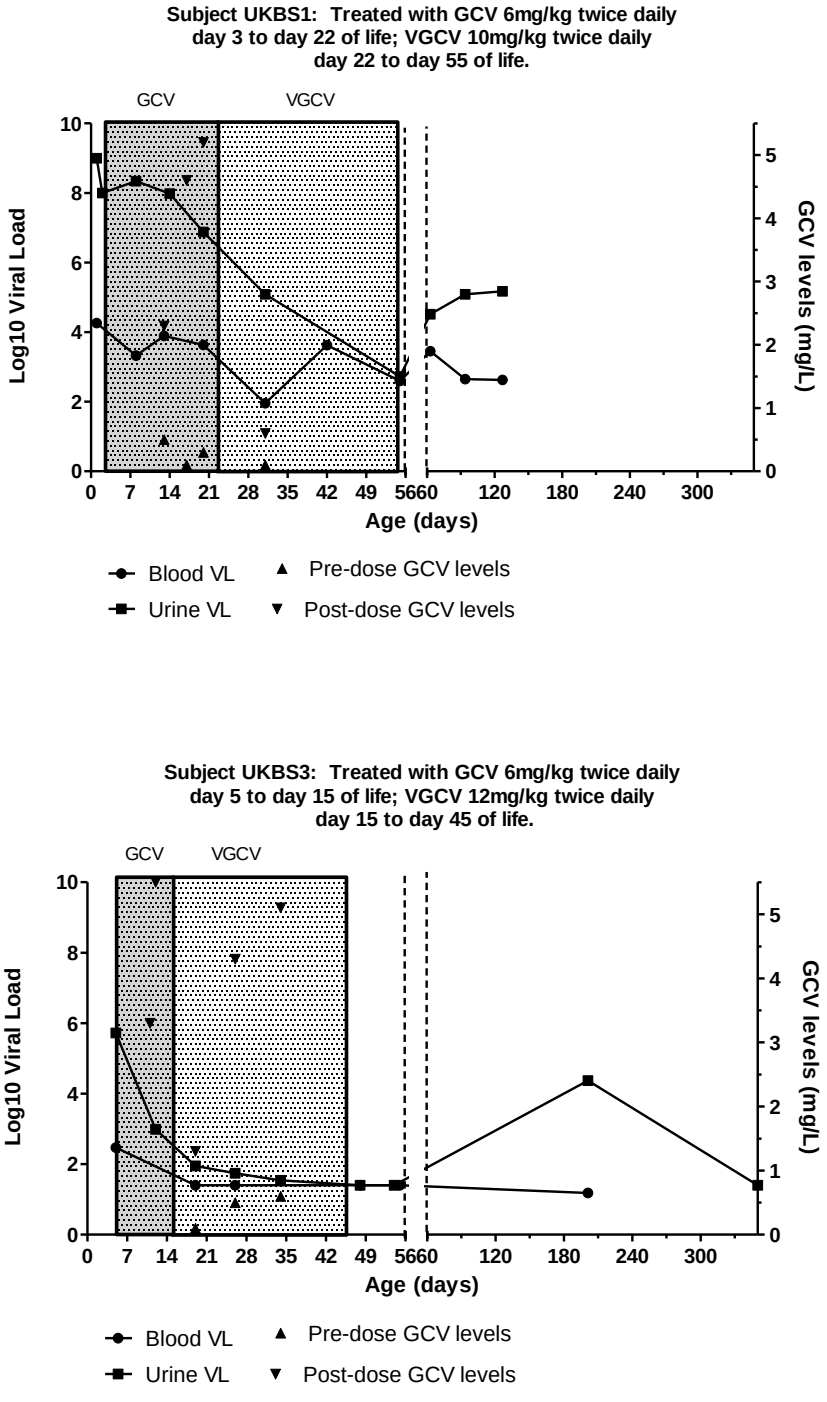
r^2= 0.58
Median = 0.45 (0.1-0.8)

r^2 = 0.73
Median = 4.0 (3.0-4.3)

GCV = ganciclovir
VGCV = valganciclovir

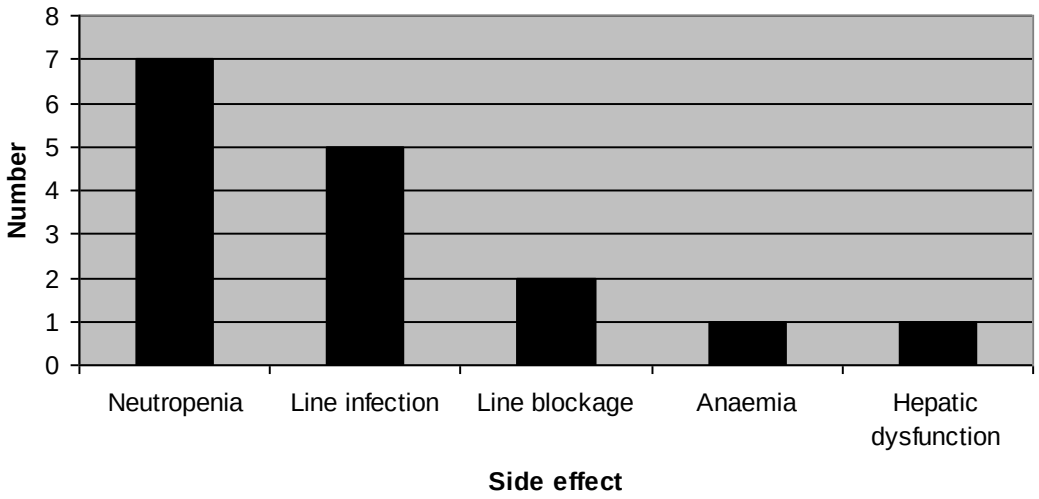
Ganciclovir levels (mg/L) recorded following GCV (A) and VGCV (B) administration in 7 babies treated for congenital CMV.

Figure 5-9 Viral load and ganciclovir levels over time in two subjects treated for congenital CMV



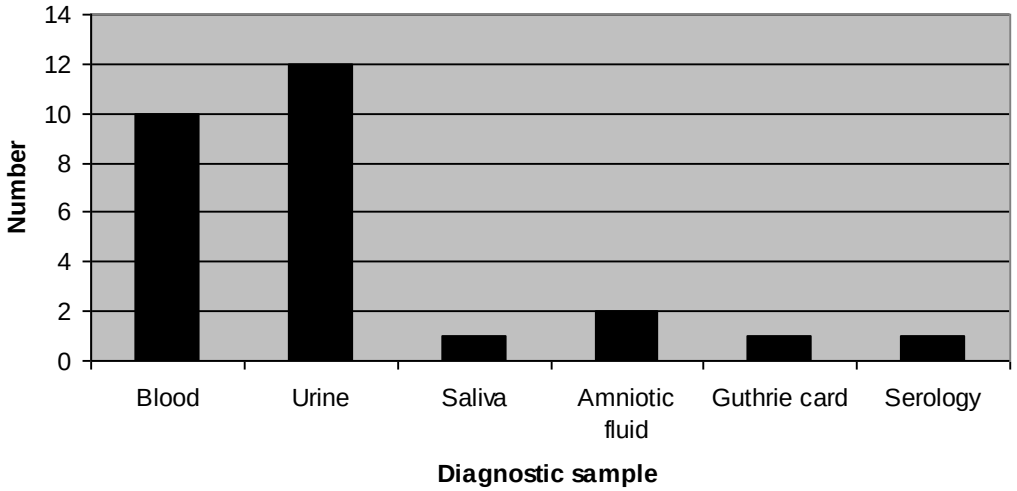
GCV = ganciclovir
VGCV = valganciclovir
VL = viral load

Figure 5-10 Side effects recorded during treatment for congenital CMV



Side effects during treatment with GCV or VGCV as recorded in 13 of 27 babies enrolled in the UK CMV Treatent Registry.

Figure 5-11 Source of sample used to diagnose congenital CMV



Sample recorded in the UK CMV Treatment Registry as the sample used to make a diagnosis of congenital CMV in 27 babies subsequently receiving treatment.

completed with VGCV and in one a 37 day course was given with no reason specified for this duration of therapy.

5.3.7. *Virology at diagnosis and during treatment*

Most babies had diagnosis confirmed on urine or blood (12/27 and 10/27 respectively) (Figure 5-11). Age at time of diagnostic sampling ranged from 1 to 17 days in 25 babies in whom this was recorded with a median value of 1 day.

In 3 babies diagnosis was documented to have been made antenatally with 2/3 specimens being amniotic fluid.

25 subjects had at least one VL measurement recorded. 13 of these babies were also recruited into the Viral Load and Immunology study (VICC) discussed in Chapter 6 of this thesis. The detailed analysis of these data, along with those obtained from a further 3 babies with detailed VL available but entered in the Registry alone (UKCV1, UKBS1, UKBS2), are therefore summarised in more detail in this later chapter.

CMV DNA was detected in blood at baseline (first sample taken after birth and only included in analysis if obtained within 7 days of treatment commencing) in 19/23 (83%) babies in whom samples were recorded; mean VL in these 19 babies was 4.3 $\log_{10}$ copies/ml (SD 0.68). Most samples were obtained within the first week of life (median day 4; range day 0-27) and there was no apparent decrease in VL with increasing age of subjects (data not shown). 8 babies, however, had 2 blood specimens analysed prior to, or within 2 days of, commencing treatment (a median of 10 days between samples; range 6-28 days). All but one of these babies had a decrease in VL over time (mean decrease of 0.82 $\log_{10}$ copies/ml; SD 0.66 copies/ml). In the majority of these babies it was thought likely that the VL was measured in the same laboratory.

Virus was detectable in urine in all 17 babies who had results recorded. 13 babies had a quantified VL recorded (mean baseline 7.32 $\log_{10}$ copies/ml; SD 1.2). 3 babies had 2 specimens analysed prior to, or within 2 days of treatment commencing (1-8 days between samples). VL was a mean 0.51 $\log_{10}$ copies/ml (SD 0.43 copies/ml) lower at start of treatment compared to baseline.

VL at the end of the course of treatment was not often reported. In those with data recorded 9/15 (60%) had virus still detectable in the blood and 5/10 (50%) in urine.

10/15 babies had a decrease in blood VL recorded during the first 14 days (+/- 4 days) treatment. Mean change in VL for all 15 babies was -0.79 $\log_{10}$ copies/ml (SD 1.14). All except 2/13 babies had a decrease in VL from baseline to day 42 treatment (+/- 4 days), with a mean change in VL of -0.94 $\log_{10}$ copies/ml (SD 0.82).

When considering urine 11/13 babies had a decrease in VL in the first 14 days (+/- 4 days) of treatment (mean change -1.66 $\log_{10}$ copies/ml; SD 1.21). Between days 14 and 42 all 8 babies with results entered showed a decreased VL with a mean decrease of 1.88 $\log_{10}$ copies/ml (SD 0.91). All 7 babies with values available from day 42 (+/- 4 days) of treatment had a decrease when compared to baseline with a mean decrease of 4.14 $\log_{10}$ copies/ml (SD 1.24).

Most babies received GCV for the majority of their treatment. There were therefore insufficient data to compare VL decline between those receiving VGCV and GCV.

5.3.8. *Clinical outcome*

Data are still being updated, particularly with regards to hearing outcomes. 9/27 (33.3%) babies with date of data entry recorded were, however, more than two years of age at the time of last data entry. 5 of these were recorded as being asymptomatic. 10/27 (37.0%) babies with data available were reported to be asymptomatic at the time of data last being entered; no babies entered in the Registry are reported to have died to date.

16 babies were recorded as having hearing loss; detailed hearing data is currently recorded for 14 of these babies. Newborn Hearing Screening Programme (NHSP) was reported to have detected hearing loss in 8/10 babies with right sided hearing loss, data was not entered for the remaining 2 babies. Similarly 9/11 babies with documented left ear involvement were detected by the NHSP, in one baby whether hearing loss had been detected at the point of screening was unknown and in the remaining baby data was unrecorded. In 8 babies hearing loss was bilateral when last recorded, 3 left sided only and 2 right sided only, in 1 laterality was not recorded. Severity of hearing loss was severe to profound in 4/6 and 4/5 babies with data available for right and left ears respectively. In 1 baby progression of hearing loss was reported and in one improvement noted. Data were, however, largely entered as ‘not recorded’ in this field. 2 babies were noted as having been referred for cochlear implantation.

Detailed neurological outcome data is not currently entered into the Registry.

5.4. DISCUSSION

5.4.1. *Number accrued and relation to previous studies*

27 babies treated for cCMV in the UK had been entered into the ECCI Registry from 10 participating sites up to 1/5/2010. Summary data available at the onset of this study reported approximately 6 babies being treated per year during the two year surveillance period (Lynn et al. 2004). The data currently entered for 2003 would imply that the Registry has a fairly low case ascertainment, certainly for earlier years. This is not surprising given the comparatively well-established nature of the BPSU and the more stringent enrolment requirements for this new treatment Registry. Although recruitment has not yet reached the numbers predicted, the enrolment of 7 babies born in 2008 and 2009 is close to the predicted estimates. The higher number of cases recruited in recent years is likely to reflect improved case ascertainment but would also be in support of my hypothesis that more babies would be treated following recent evidence particularly as these numbers exceed those identified through the more extensive and robust BPSU reporting system.

The difficulties encountered with ensuring good case enrolment and data collection are discussed further below.

5.4.2. *Is treatment in the UK evidence based?*

As one of our initial concerns was regarding the use of antiviral treatment in babies in whom there was no evidence base it is interesting to compare the data currently entered into this treatment registry with the entry criteria for the only existing RCT of treatment for cCMV (Kimberlin et al. 2003). In this study treatment with 6mg/kg bd i.v. GCV was commenced and continued for 42 days duration in neonates aged ≤1 month with clinically apparent cCMV and evidence of CNS involvement. Participants were all ≥1200g in weight and ≥ 32 weeks' gestation.

- **Basic demographics**

All treated babies currently enrolled in the Registry were ≥32 weeks' gestation and all but one baby was ≥1200g in weight (Figure 5-3). Treatment was commenced within 31 days of age in all but 2/27 babies. The 2 babies treated outside the age at which treatment has been proved effective were both born in 2003.

- **Clinical symptoms**

22/27 babies were reported to have CNS findings at birth. In the majority of cases treatment was therefore commenced according to existing evidence. However, in nearly 20% of babies treatment was commenced for indications other than CNS disease with 2/27 (7%) having no symptoms recorded at birth. There is therefore a notable proportion of babies entered being treated in whom side effects might outweigh the currently unproven benefits of such treatment.

Our comparatively low findings of hepatitis and hepatosplenomegaly (19% and 30% respectively) compared to other studies (Table 1-2) is interesting and may be due, in part, to recruitment bias as major paediatric liver units are not currently active study sites for the Registry. It is also possible that these babies are not being uniformly treated in the UK in keeping with a current lack of evidence that doing so is of any benefit.

It is not currently possible to ascertain from this database how frequently clinicians are treating babies with SNHL alone. Although often considered a CNS manifestation of cCMV disease, SNHL may be the only abnormal finding in babies with CMV infection making its presence as an indication for treatment contentious (Stehel et al. 2008). Most of the babies included in the Registry did, however, have other documented findings of symptomatic CMV.

- **Treatment formulation, dose and duration**

Treatment was commenced with GCV in 24/27 babies and in 8 babies subsequently changed to VGCV; difficulty with i.v. access was cited as the reason for this change in ~25%. In other cases the change of medication was to facilitate discharge home or to enable prolonged courses of therapy. Treatment duration was around 42 days in 16 babies. 7 babies received a shorter course of treatment with side effects reported to account for just under half of these. In 4 babies a longer course of therapy was given, with VGCV used in all cases to complete treatment; no information is recorded regarding the justification for these longer treatment courses. The dose of GCV used was 5-6mg/kg bd in the majority of babies treated with this drug (21/22). Dose of VGCV, however, varied more considerably from 10-40mg/kg bd. The evidence-based VGCV dose of 16mg/kg bd was only published in 2007, however 2

of the 6 babies treated with VGCV born in or after 2008 were still commenced on alternative doses of medication (Acosta et al. 2007).

According to my data VGCV is therefore being used frequently both to enable earlier discharge and to enable longer treatment courses. The treatment of a number of babies with prolonged courses of VGCV is of some concern given the absence of any RCTs reporting any additional benefit from such treatment strategies. This will hopefully be addressed once the results are published of forthcoming RCTs (NCT00466817).

There has not been a noticeable increase in VGCV use over time in babies enrolled into the Registry to date. However, oral VGCV solution gained a manufacturing authorisation in the UK in 2009. Due to the lag between clinical cases being identified and recruitment into the Registry an associated increase in VGCV may not be observed until late in 2010/2011. Trends in VGCV use therefore warrant continued monitoring.

It is notable that there are few premature babies included. Although these babies fall outside the dosing evidence available it would be surprising if such neonates were being denied treatment if symptomatic. The current lack of involvement of premature neonates is unfortunate as inclusion in such a Registry would allow information regarding dosing and safety to be accumulated to inform treatment of other similarly affected babies.

Although treatment in the UK seems to be largely following the evidence available there are notable exceptions with regards to the treatment of less symptomatic babies and longer courses of treatment along with variable VGCV dosing. Given that to be enrolled in the database clinicians are far more likely to have had contact with the study team and discussed treatment options the cases enrolled in the Registry have inherent selection bias. It is therefore even more likely that treatment elsewhere in the UK, and Europe, is not so closely in keeping with this published evidence. The only way for this to be addressed it to increase overall case ascertainment as discussed further below.

5.4.3. *Pharmacokinetics and pharmacodynamics*

Despite the dose of GCV given being in keeping with that used in previous studies 10/14 (71%) babies had trough GCV levels <0.6mg/L (Kimberlin et al. 2003;Whitley et al. 1997). Similarly peak drug levels rarely reached those proposed as being

therapeutic for adults. This is consistent with data presented earlier in this thesis and raises further concerns that in clinical practice recommended doses of medication are associated with underdosing.

In general the trough GCV levels reported during GCV treatment were less optimal than those whilst receiving VGCV. This may be due to the fact that VGCV dose appeared to be adjusted when low GCV levels were documented whereas GCV dose generally remained unchanged throughout treatment. It may also be due to the PK changes previously documented in neonates over time whereby increased renal excretion of GCV is counter-balanced by increased bioavailability of GCV following VGCV administration over the same time period (Kimberlin et al. 2008). Thus VGCV treatment is proposed to give a more stable AUC over 6 weeks' treatment when compared to i.v. GCV. In support of this latter proposal in one baby (UKBS3) in whom serial GCV levels were recorded from specimens taken between days 19 and 34 of life whilst receiving the same dose of 12 mg/kg bd of VGCV, an increase in both pre- and post- levels was observed (Figure 5-9).

5.4.4. *Clinical and virological response to treatment and outcome*

Urine analysis was limited due to laboratories often not reporting a quantitative CMV VL. Although ethical approval permitted retrieval of these specimens in many cases samples had been destroyed by the time full consent had been obtained or laboratories did not respond to these requests. Where samples were obtained these were often in the form of DNA extracts meaning that accurate comparisons of VL were not feasible.

Consistent with other studies virus was detectable in all urine samples tested at baseline but only 83% of blood samples (Bradford et al. 2005;Kimberlin et al. 2003). Decrease in VL between two 'baseline' measurements (two measurements taken prior to or within only a couple of days of starting treatment) in both blood and urine indicates that there is some spontaneous control of virus postnatally in both these body fluids, as already observed in early culture-based studies of urine (Stagno et al. 1975). Given the lower starting VL in blood it is notable that a larger decline prior to treatment was seen in this body fluid when compared to urine. There is currently no published literature describing natural history of VL using quantitative CMV PCR of blood or urine in untreated babies. This is important to take into consideration when

drawing any conclusions from results of viral decline in either blood or urine from uncontrolled treatment studies and attributing this decline to treatment efficacy.

Most babies in the Registry with results available had a decline in urine and blood VL between start and end of treatment but virus was still detectable at the end of treatment in 60% and 50% of blood and urine samples respectively.

Although the Registry holds insufficient data to allow correlations to be made between drug levels and viral decline the continued detection of virus at the end of treatment raises questions regarding treatment efficacy in this cohort. The low GCV levels observed both here and earlier in this thesis are possibly significant when considering that virus is still detectable in a high proportion of blood and urine samples in these babies at the end of treatment. The treatment duration of 6weeks should also be questioned particularly taking into account the continued decrease in urine VL noted in one baby with samples available during a prolonged treatment course. However, if the threshold theory for symptomatic CMV proposed in adult patients is applicable to these babies then transiently decreasing peak VL might be sufficient to allow the neonatal immune system to mature and gain subsequent virological control. With this in mind RCTs are essential in evaluating any longer treatment courses before exposing babies to unnecessary antiviral agents for prolonged periods.

5/10 (50%) babies with data entered at more than 2 years of age have remained asymptomatic; this is consistent with the 50% long term sequelae reported in a meta-analysis of untreated babies with symptoms at birth(Dollard, Grosse, & Ross 2007). This is not therefore consistent with the reported improvement in neurological and hearing outcomes with treatment published elsewhere (Kimberlin et al. 2003;Oliver et al. 2009). This may be due to the inclusion in this Registry of less symptomatic babies. It is, however, also possible that less rigorous monitoring of treatment in the clinical setting, particularly where VGCV is used, results in suboptimal treatment when compared to the constrained scenario of a RCT.

The data currently available are insufficient to enable any conclusions to be drawn with regards to VL and progression of symptoms. The lack of ability to capture follow-up data and record accurate audiological and neurological outcomes in the Registry further limits the interpretation of any follow-up data. It is interesting to note the above concerns, however, when considering design of any future treatment studies for cCMV and in developing clinical guidelines on treatment.

There are currently no deaths reported. Numbers in the Registry are, however, small and we are aware of a number of babies who had passed away and in whom consent was not obtained due to concerns about distressing parents further. This sort of recruitment bias is unavoidable in any Registry where consent is required, and limits conclusions that can be drawn from this select cohort.

5.4.5. *Safety*

In keeping with other reports, the most common side effect documented was neutropenia, observed in 26% of our treated babies, and rarely leading to termination of treatment (Kimberlin et al. 2003;Kimberlin et al. 2008). The relatively few reports of neutropenia associated with VGCV use in our cohort may reflect the fact that this was generally started later on in the treatment course, when side effects have been reported to be less common(Kimberlin et al. 2003). It is also consistent with data emerging from other studies indicating that neutropenia occurs less frequently with VGCV treatment (Kimberlin et al. 2008). Overall the frequency of neutropenia (cells <500 cells/mm^3) reported here is less than grade 3 and 4 neutropenia documented in previous RCTs but comparison is limited by the variable neutrophil values (<250 up to <899 cells/mm^3, according to age) involved with reporting toxicities in these previous trials.

Line infections were the commonest reason for switching to VGCV and were reported in 5/27 (19%) babies. *Staphylococcus aureus* was the reported infective organism in 3 out of 4 in whom an organism was recorded. Given that *S aureus* has been reported to be the infective organism in only around 10% of all episodes of neonatal central line infections this trend also warrants further monitoring (Benjamin, Jr. et al. 2001).

5.4.6. *Recruitment barriers*

Case ascertainment bias has already been mentioned as a factor limiting interpretation of data in a number of areas already discussed above. Difficulties encountered with recruitment, which have a bearing on ensuring widespread inclusion of treated babies, is therefore worthy of further discussion. During the 39 month period of data collection reported here the study team were aware of a further 24 babies, who would have been eligible for enrolment (Table 5-1). In 8 cases consent was not possible. In one of these cases consent was declined due to parental

concern regarding regulatory agencies having possible access to clinical notes and personal details when monitoring the study.

Due to many factors, including out-patient capacity and minimising parental inconvenience, the anticipated recruitment from a core number of centres with paediatric infectious diseases specialist input did not occur. Most commonly telephone advice was sought by managing clinicians regarding evidence and recommendations for treatment. Babies were therefore often treated locally with subsequent monitoring of treatment and associated side effects seeming anecdotally variable. This is of particular concern where oral VGCV is being used in the out-patient setting and where junior team members may be primarily involved with monitoring treatment.

The associated difficulties in gaining numerous site approvals has been a significant hindrance to recruitment and a rate-limiting factor in enrolling a significant number of babies as highlighted in Table 5-1. The reported median duration of time of 78 days from approach to a site's R&D department to approval is somewhat under-representative of the time actually taken for submissions as this does not take into account the time taken to obtain the Principal Investigator's approval for the drafted site documents prior to initial R&D submission. In a number of cases this took over one year of correspondence and reminders. At some sites no progress had been made with approvals in two years despite numerous contacts including meetings with apparently interested paediatricians. This likely reflects both time constraints for NHS clinicians in being able to review these documents and changes in R&D funding streams during the period of this study.

Despite the over-riding principles of multicentre study ethics approval, 3 sites were not happy to proceed with approvals until significant amendments had been made to study documents or the proposed recruitment methods. In many cases completion of further site specific R&D forms and paperwork were required and further discussion was often needed with those involved with the local approval process prior to permission being granted to enable one potential recruit to be approached and consented.

The above problems are inherent in any voluntary reporting system, particularly one where a complex set of approvals and consent are required. Indeed, a surveillance study of babies exposed pre- and post-natally to anti-retroviral medication (the

CHildren exposed to AntiRetroviral Therapy (CHART) study) conducted through the well-established NSHPC, but requiring parental consent, likewise achieved only 33.5% case enrolment. This would be in keeping with our enrolments in earlier study years compared to the currently published information from BPSU and our knowledge of clinical cases notified to us. It is also notable that even well-established datasets such as the CHIPS/NSHPC described earlier have had to alter processes in recent years and have reported difficulties with ensuring individual institution approvals are in place to enable data sharing that was previously commonplace for similarly rare conditions (personal correspondence).

The restrictive nature of recent regulatory changes led to a call for a review of the regulation and governance of medical research by the Academy of Medical Sciences commissioned by the Health Secretary in March 2010. Given my experience with this multicentre study I submitted evidence relating to how the current approvals process has limited data sharing in studying rare conditions such as cCMV which necessitate multicentre collaborations. There is ongoing review of current processes in order to ensure that there is a balance between regulatory requirements put in place to protect individuals whilst ensuring that the conduct of clinically important research is not unduly inhibited.

5.4.7. *Implications of these data*

Despite the limitations noted above data acquired in this registry can still serve a valuable role in hypothesis generation and in providing pilot data for more scientifically robust studies. The cohort of 27 treated babies described here still represents a reasonable sized cohort of treated babies when compared to previously published literature for cCMV and raises a number of points warranting future consideration as summarised below.

1) My data would suggest that there is increasing use of antiviral treatment for babies with cCMV in the UK. These babies are treated in multiple different centres, often with little experience of managing associated toxicities and further follow-up. In some areas this has been identified as a concern at a regional level and addressed by centralisation of referral and treatment. It is suggested that this should be considered throughout the UK while taking into consideration associated parental inconvenience and distress at what is already a difficult time. In the meantime, the ECCI Treatment Registry

provides both a means of reviewing treatment trends within the UK and a platform for providing further evidence based information to guide individual clinicians.

2) As originally hypothesised the main reasons for VGCV being used are documented as difficulties with i.v. access and to enable early discharge; in 2 cases it was used to enable a prolonged treatment course. There remains no clinical efficacy data for VGCV when compared to i.v. GCV and both clinicians and parents should be aware of this when making treatment decisions. The stable, and more optimal, GCV drug levels observed with VGCV are, however, encouraging and may indicate an improved drug exposure over time in babies treated with this drug. It will be of interest to monitor trends in VGCV usage in this age group following the marketing authorisation of oral VGCV syrup in 2009.
3) As with our previous data GCV levels documented in the Registry would suggest possible underdosing of these neonates based on recorded serum drug levels. The additional data presented here of continued virus detection in a significant number of both urine and blood samples at the end of a 6 week treatment course would raise further concerns regarding virological efficacy. Further work is needed to ascertain whether this is of any significance with regards to clinical outcomes.
4) The lack of a standardised unit for quantifying CMV VL limits conclusions that can be drawn from virological data from multiple sites, such as that held in the treatment Registry. Work is in progress to address this using the recently described international unit system for reporting VL (nibsc.ac.uk). The treatment guidelines mentioned above will in addition hopefully encourage clinicians to take samples at similar time points bringing further uniformity to date entered.
5) The data presented here showing decreased VL in both blood and urine of up to 1.0 $\log_{10}$ copies/ml between first measurement and treatment commencement in babies support the need for RCTs to compare both virological and clinical outcomes in these neonates in whom natural history data are lacking. In order to partially address this deficiency a more tightly controlled study of CMV VL involving prospective data acquisition and sampling of different body fluids at specified time points with centralised

analysis of samples was designed and is described further in Chapter 6 of this thesis.

5.4.8. *Further work proposed as a result of current data analysis*

The identification of improvements in the database to capture data more accurately and make the Registry more user friendly are ongoing. Translating the audiology dataset into such defined menus proved particularly challenging due to the variable nature of some of the data captured during audiology assessments. Simplification of the audiology datasets would aid interpretation of follow-up outcomes. Additional fields to enable capture of neurological outcomes will also ultimately be of importance in ensuring relevant follow-up data is recorded. The need to make it clear whether SNHL is the only indication for treatment would be beneficial in assessing outcomes in this specific, and possibly different, group of symptomatic babies. Refining the database to enhance capture of data relating to reasons for courses of treatment that are longer or shorter than the currently recommended 42 days would also be informative.

As an outcome of this data analysis further contact with lead clinicians and site approvals for the main paediatric hepatology units is considered a priority along with revisiting other means of collating such data. Eliminating the need for informed consent via approval from the Patient Information Advisory Group (PIAG), looking at methods of recruiting interested families directly via the website and involving sites as participant identification centres (PICs) are all possible means of easing the recruitment process and thus increasing case ascertainment.

In addition the study team has been approached by partners in a number of European countries who are keen to share their cohorts of treated babies. This would lead to inclusion of data on over 100 treated babies, providing a more powerful dataset and allowing for comparisons of treatment trends between countries.

CHAPTER 6.

6. VIRAL LOAD AND IMMUNOLOGY IN CONGENITAL CMV (VICC) STUDY

6.1. INTRODUCTION

On reviewing the literature available at the start of my studies it was evident that data relating to the natural history of VL in different body fluids in symptomatic and asymptomatic infants with cCMV was lacking. Moreover, studies describing immune responses in CMV-infected infants consisted, in the main, of observations from single time points, often taken some years after birth, and also often combining results from babies with both symptomatic and asymptomatic congenital infection (Cauda et al. 1987;Hayashi et al. 2003;Pass et al. 1983b;Pedron et al. 2011). Few studies have documented changes in immunological responses with time or how treatment with antiviral agents might alter these responses or evaluated the relationship between immune responses and VL in congenitally infected babies.

A prospective study evaluating VL and CMV-specific immune function at specific time points over the first two years of life was therefore designed. With the known inter-lab variations in quantitative measurements of CMV VL, centralisation of samples was felt essential in order to provide robust, comparative data in the different groups of CMV infected babies (Pang et al. 2009). Our lab had developed methodology for evaluating CMV-specific immune responses in multiple samples over prolonged time periods in adult transplant and HIV co-infected patients. In order to be able to draw direct comparisons with adult data I planned to modify these techniques, using reduced samples sizes, to define CMV-specific immune responses over time in CMV-infected infants.

CMV IgG avidity is now carried out routinely in adults with suspected primary CMV infection but there is limited information on antibody avidity in the neonatal age group. As small amounts of blood are required for antibody assays this prospective study also offered an opportunity to study CMV IgG avidity maturation in infants over time.

My investigation of GCV levels in paediatric age groups had raised concerns regarding underdosing, but was limited by the lack of associated clinical and pharmacological data. Increased use of VGCV was suspected in the UK with no published data on comparative levels of GCV achieved at the start of this study. This study therefore also aimed to provide some PK data of treatment with both GCV and

VGCV in babies undergoing treatment along with some PD assessment in the form of observed virological response.

This chapter therefore presents the results of this study which essentially aimed to address viral load and immune responses over time in congenitally and postnatally infected infants.

6.2. METHODS

6.2.1. *Study overview*

4 different patient groups were identified for study as defined below

1. any treated babies
2. babies with symptomatic cCMV at birth, not receiving treatment
3. babies with asymptomatic cCMV at birth, not receiving treatment
4. babies with postnatally acquired CMV infection, not receiving treatment.

CCMV was defined by CMV detection (by culture or PCR) in any sample acquired <21 days of age. To confirm a diagnosis of postnatal CMV a subject required both a sample of urine taken at age <21 days testing negative for CMV followed by a subsequently positive sample. In order for valid comparisons to be made with babies with cCMV, postnatally infected babies were only eligible if CMV was identified up to 3 months chronological age.

Sampling was conducted according to pre-defined protocols with a plan for 1-2 years follow-up (Appendix C). In view of the VL analysis presented earlier in this thesis and knowledge of viral dynamics in other viral infections the protocol specified frequent sampling early in treatment and following discontinuation of drug with the aim of more clearly defining viral dynamics in this population. Due to the requirement for fresh PBMCs, samples for cellular immunity were only acquired from study sites based around London. I retrieved and transported all samples obtained for cellular immunity directly to the laboratory to ensure no sample loss or wastage.

Full ethical approval was gained in May 2007 and the initial recruitment plan was for a total of 45 babies to be included over a period of two years aiming for 20 symptomatic congenitally infected babies (10 of which to have been treated), 5

asymptomatic congenitally infected and 20 postnatally infected babies. As there were no previous data in this field no formal statistical power calculations were possible and the aim was therefore for the production of descriptive data which could inform future hypothesis-generation.

6.2.2. *Saliva swabs*

At the onset of my studies use of salivary culture as a diagnostic tool in adults was described but there was little literature on saliva collection in neonates or of PCR detection of CMV in such specimens (Warren et al. 1992;Yamamoto et al. 2006). There were no studies to my knowledge evaluating VL in this body fluid. Initial literature searches and discussions with other laboratories led to the investigation of the use of cotton swabs in medium or saliva absorbed onto filter paper (Yamamoto et al. 2006;Zerr et al. 2000). The cotton swabs used in previous studies were not available in the UK so initial experiments were conducted using filter paper strips (Sno-strips®). Preliminary experiments using gB plasmids suspended in saliva, salivary samples from CMV positive healthy subjects and blood samples from subjects known to be CMV viraemic, resuspended in saliva following FACS-lysis to remove haem contamination, and using various different DNA extraction methods failed to give a good yield of CMV DNA from these strips. Use of nylon flocked salivary swabs (Sterilin™) was therefore investigated (Figure 6-1). These swabs have been shown to have better sample release than conventional nylon or polyester swabs (94% compared to 25%) and in increased virus detection from nasal and nasopharyngeal swabs (Unpublished data, Copan Innovation March 2006 and Daley et al. 2006). Using neonatal flocked swabs, in sterile containers (MicroRheologics™ Product code 516C) good DNA yield was found from a swab obtained from a baby with asymptomatic CMV and extracted with Qiagen mini-kit™ using their recommended buccal swab spin extraction protocol (QIAamp® DNA Mini Kit and QIAamp DNA Blood Mini Kit Handbook February 2003). These swabs were therefore evaluated further.

- **Assay set-up**

Salivary swabs were weighed pre- and post- saturating in samples of human saliva. Saliva was verified as being close to 1uL = 1ug solution in weight. Average salivary volume retained was thus estimated to be ~27uL per neonatal flocked swab.

Figure 6-1 Photograph of flocked salivary swabs (Sterilin™) used for saliva acquisition

DNA extraction was trialled using a number of different commercial kits available at the time with similar DNA yield. For practical reasons Qiagen DNA minikit™ was adopted for use in the study as they were in current use in the diagnostic laboratory. PBS was used for the initial step of the extraction procedure following further work showing that this gave similar DNA yield to swabs vortexed in virus transport medium (VTM) (data not shown). Swab extractions during early stages of these studies were all carried out by hand following the manufacturers' buccal swab spin protocol. During latter stages, and following further comparisons of DNA yield, swab extraction was fully integrated into the diagnostic laboratory protocols as further described below. Swabs were processed using VTM as the solution routinely in use in the diagnostic laboratory at the time.

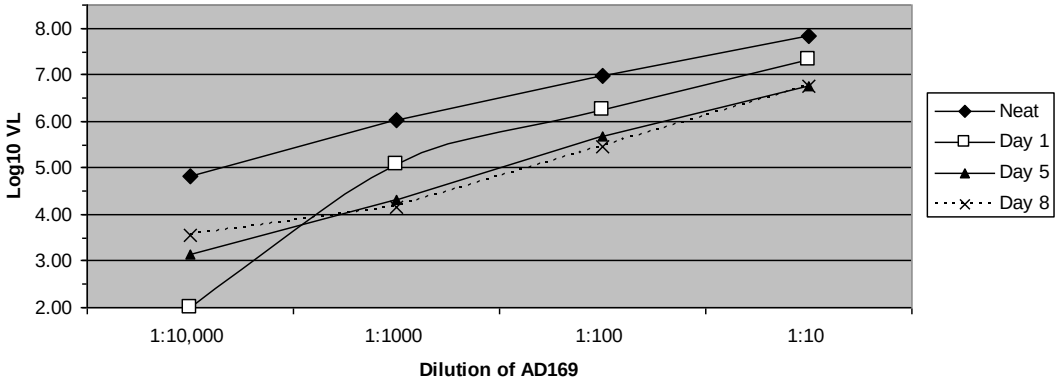
To investigate stability swabs dipped in PBS spiked with known dilutions of cell-associated AD169 were left at room temperature for up to one week before extraction. Initial DNA yield from swabs was 2.8-0.5 $\log_{10}$ lower than the starting salivary solution when extracted on Day 1 (Figure 6-2). There was a further decrease in CMV DNA yield from swabs extracted on day 1 of acquisition compared to day 5 but less difference in those extracted on day 8 compared to day 5. Differences were greatest in starting solutions with lower virus concentration (Figure 6-2).

- **Limitations**

A decrease in DNA acquisition from flocked swabs compared to starting solution was noted. It was uncertain whether the use of laboratory preparations of AD169 accurately mimicked CMV DNA detectable in clinical samples but no other means of comparing CMV DNA extraction from fresh salivary solutions compared to swabs was available to us. Variable salivary absorption is likely in clinical samples thus potentially further limiting accurate quantification of DNA copy number. The protocol for saliva acquisition therefore advised investigators to leave the swab inside the baby's mouth for around 60 seconds to ensure full saturation with saliva; this could also be assessed visually. As obtaining liquid saliva samples from neonates is impractical, obtaining swabs offered the best solution available at the time despite these limitations.

In contrast to other liquid samples where only 110uL of starting solution is taken in our laboratory for DNA extraction and quantification it is necessary to extract the

Figure 6-2 CMV viral load detectable in flocked salivary swabs following extraction at different time points



'Neat' is AD169 diluted with saliva followed by direct extraction of nucleic acid from this solution.
For other samples flocked swabs were dipped in this 'neat' salivary solution until saturated and then extracted on different days following sample preparation.

whole 1mL of salivary swab solution to accurately estimate virus quantity present. This was subsequently incorporated into standard laboratory protocols.

The salivary sample protocol also stipulated that samples should be obtained from babies at least one hour after a feed to minimise potential contamination with maternal CMV DNA in breast feeding mothers.

6.2.3. *Viral load*

CMV VL was quantified using real time PCR in the Royal Free diagnostic laboratory, by the laboratory staff, according to standard protocols in use at the time. Salivary swabs were processed according to the protocol also described earlier and VL adjusted to give an estimated quantity of virus/mL. Log_{10} VL was plotted for each baby at each defined time point with the allowances for sampling as per protocol (Appendix C). Where VL result was reported as <200 a value of 100 (as the mid-point between undetectable and 200) was entered for analysis to enable Log_{10} or Log_e transformation of data.

VL results of treated babies reported here are from 11 babies recruited into this study along with 3 babies whose data is recorded solely in the CMV Registry (as discussed in Chapter 5) and 3 babies whose samples were received at the Royal Free laboratory for clinical management and in whom basic data regarding treatment was known. Baseline was considered to be before, or within 7 days of, treatment commencing. If a number of samples were available prior to treatment commencing the sample taken closest to the date of drug initiation was used. End of treatment was considered to be date of treatment termination +/- 3 days. Where VL in different body fluids was being compared, however, samples were only considered if taken within one day of each other. It is important to note that day of treatment does not equate to day of life; at time point 'Month 3' a few babies were still receiving treatment. Month 3 data was therefore omitted from many analyses in these subjects. Medians were used for most VL comparisons, to enable comparison with data presented earlier in this thesis, although starting VL appeared more normally distributed in the babies included in this study.

6.2.4. *Ganciclovir levels*

Ideally samples were spun and serum separated in the hospital of acquisition to prevent sample degradation. Serum samples were then sent to the Royal Free

laboratories where they were frozen at -80^{o}C as soon as the study team were aware of arrival. A form was developed to capture details of timing of doses, most recent blood creatinine level, most recent weight and whether the sample had been taken from a central line (if i.v. drug being given). Samples were packaged in dry ice and sent by courier in one batch towards the end of the third year of the study to the UK reference lab where analysis was conducted by the team there according to their standard protocols (see 2.3). Only the unique participant study ID was attached to samples along with dates and times of sample acquisition.

6.2.5. *HLA-A2 Class 1 MHC typing.*

This analysis was carried out by me simultaneously to PBMC extraction in new study patients so that at the point of specific antigen stimulation HLA-A2 status had been ascertained.

- **Initial set-up**

Although pentamers were available for HLA-A2, -A24, -B7 and -B35 CMV epitopes and monoclonal antibody was available for HLA-A2, -A24 and –B7 initial set-up focussed on HLA-A2 and HLA-B7 as these were known to be predominant HLA-types in the ethnic groups anticipated to be most represented in my studies (Proimmune handbook 2007).

Initial assays were conducted using aliquots of samples of blood from subjects with known HLA types. During assay set-up the optimal amount of anti-HLA-specific antibody necessary for reliable detection was titrated and the minimum time that allowed for effective staining was evaluated by leaving samples incubating for 15, 20 and 30 minutes prior to analysis (data not shown). Further work established that gating on CD14+ monocytes gave the most accurate reflection of HLA-type. After carrying out anonymised testing of 5 subjects it was established that rapid testing was likely to be inaccurate without including additional control samples which would increase both sample sizes required and analysis time. In order to keep testing as rapid and accurate as possible the final protocol therefore only involved testing for HLA-A2, the most predominant HLA type in Caucasian populations.

In the final protocol 5uL HLA-A2-FITC conjugated mouse anti-human monoclonal antibody (BD pharmingen) was added to 50uL whole blood. In a separate FACS

tube 5uL isotype control mouse IgG_{2b}, κ, clone 27-35 (BD pharmingen) was added to 50uL whole blood as a negative control. 5uL anti-CD3 PerCP (BD pharmingen) was added to both samples. Samples were mixed well and then incubated in the dark for 20 minutes at room temperature before adding 1ml BD FACS™ Lysing solution (diluted 1:10 with distilled water) and agitating vigorously. Cells were left for a further 10 minutes prior to washing with 2mls PBS/0.1% NaN_3 and centrifuging (500g for 5 minutes) at room temperature. Supernatant was removed and cells resuspended in 2mls PBS/0.1% NaN_3 prior to a further wash cycle as above. Cells were then resuspended in 200μL 2% paraformaldehyde and immediately FACS acquired and analysed using a standard acquisition template. Examples of positive and negative results are shown in Figure 6-3.

- **Reliability**

1/5 samples tested falsely negative for HLA-A2 during assay set-up despite altering control cut-offs; there were no false positive results. As the purpose of this assay was to identify subjects eligible for further analysis with HLA-A2 specific pentamers and peptides false negatives were less concerning than false positive samples and this was therefore felt acceptable.

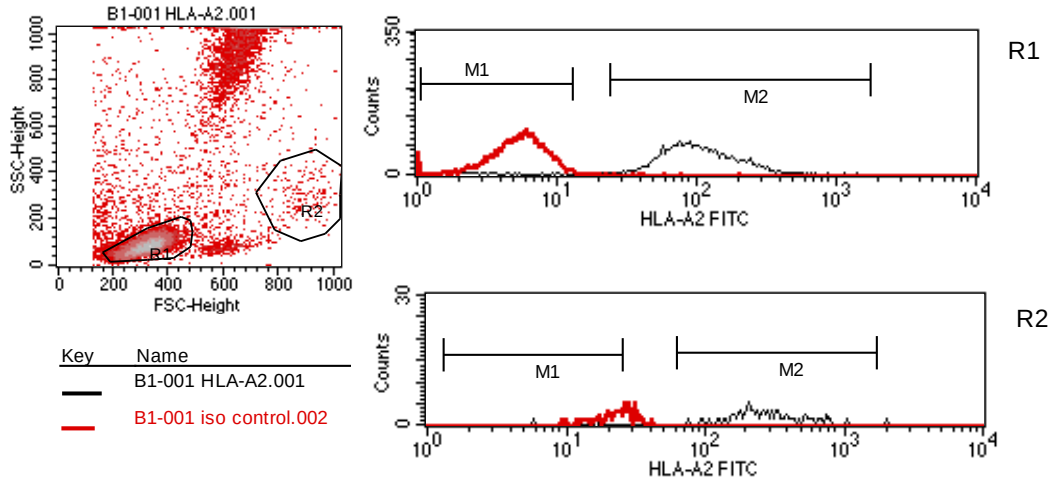
A concurrent study assessing CMV responses in 24 CMV seropositive adults found only one participant assessed as being HLA-A2 positive using the same assay had no measurable lymphocyte population staining positive with R-PE fluorochrome-labelled HLA-A*02:01 matched CMV (NLVPMVATV) pentamers (Proimmune). Median CMV-specific CD8 cells were 0.54% (IQR 0.14-1.30).

- **Limitations**

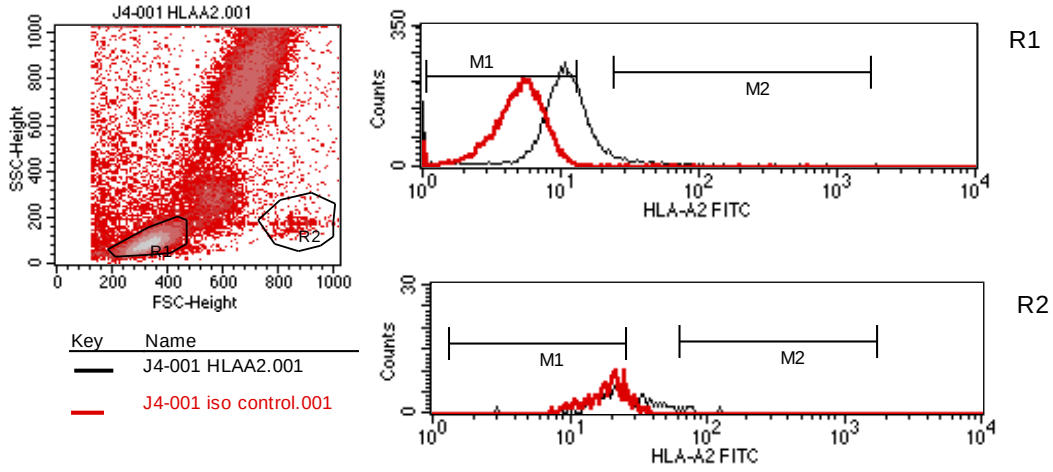
The monoclonal antibody used identifies HLA-A2 but for peptide binding specific HLA sub-groups might also be important with HLA*0201 being the HLA type associated with the immunodominant CMV NLV peptide incorporated in commercially available pentamer preparations. It would therefore be possible that a subject that was HLA*0205, for example, might bind the HLA*A2 monoclonal antibody but produce a negative result on pentamer testing thus giving a false negative immunological result. Ideally samples should subsequently undergo more detailed, genetic analysis, to determine specific HLA-A2 alleles; this was not possible in this study.

Figure 6-3 Examples of subjects considered HLA-A2 positive and negative using rapid FACS-analysis

A. Example of subject considered HLA-A2 positive



B. Example of subject considered HLA-A2 negative



HLA-A2 status was evaluated using fluorochrome-labelled monoclonal antibody to stain whole blood samples.

Samples were stained with either 5uL HLA-A2-FITC conjugated mouse anti-human monoclonal antibody (BD pharmingen™) (HLA-A2) or 5uL mouse IgG_{2b}, κ, clone 27-35 (BD pharmingen™) (iso control) as isotype control.

R1 = lymphocyte gate
R2 = monocyte gate
M1 is cut-off set for cells considered to be negative for staining for HLA-A2 antibody; M2 cut-off for HLA-A2+ve cells

6.2.6. *PBMC phenotyping and stimulation assays*

PBMCs were extracted as described in Chapter 2 of this thesis. Cells were stained according to either the ex-vivo or stimulation protocols also described in Chapter 2. Analysis was conducted using FlowJo (Version 8.7.1) software on an Applemac computer prior to exporting to Microsoft Excel spreadsheets for further analysis. FACS plots were exported to Microsoft PowerPoint and both saved and printed. All data were stored by subject number and sampling time point only. In all analyses initial lymphocyte gates were drawn according to forward and side scatter characteristics.

Ex-vivo analysis of CMV-specific HLA-matched CD8+ T cell populations

Using the lymphocyte subset the CD8+ cell population was further defined using a box plot of the CD8-PerCP Cy5.5+ cells (Figure 6-4).

HLA-A2 NLV(CMV)+ cells were subsequently identified as a subgroup of these CD8+ cells using a further box plot around this easily visible population. Quadrant analysis was applied to the CD8+ cells to quantify CD57+ and Granzyme B+ subsets. These quadrants were then copied to the A2-NLV(CMV)+/CD8+ population to ensure comparability of subsets between the two different populations.

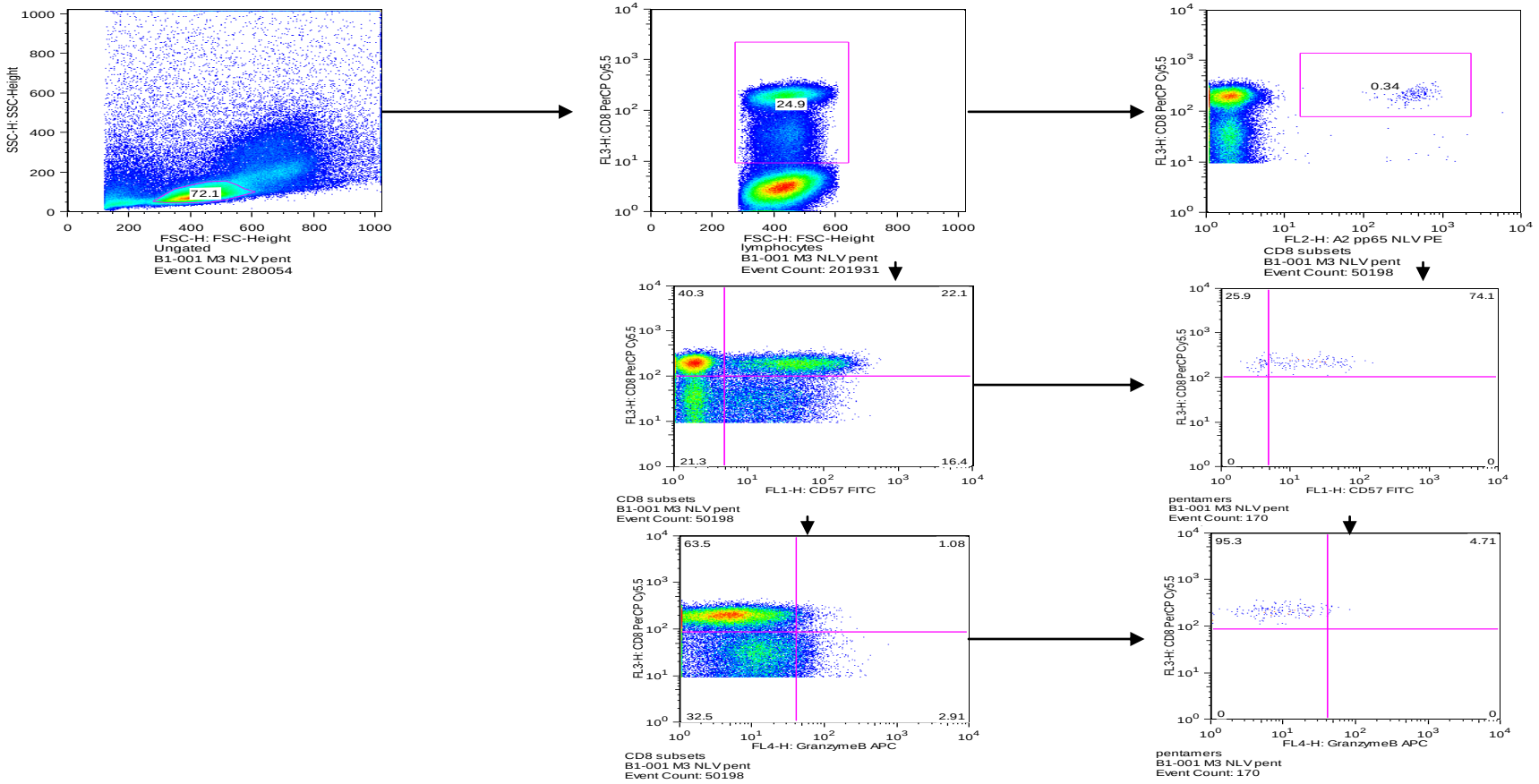
Ex-vivo analysis of CMV-specific non-HLA-matched CD8+ T cell populations

For subjects that were not identified as being HLA-A2+ lymphocyte and CD8+ populations were characterised as above. Quadrants were then drawn characterising CD28+ and CD57+ lymphocyte populations. To further aid correct definition of CD57+ lymphocytes histograms were additionally drawn.

Cytokine producing CD4+ or CD8+ T cells

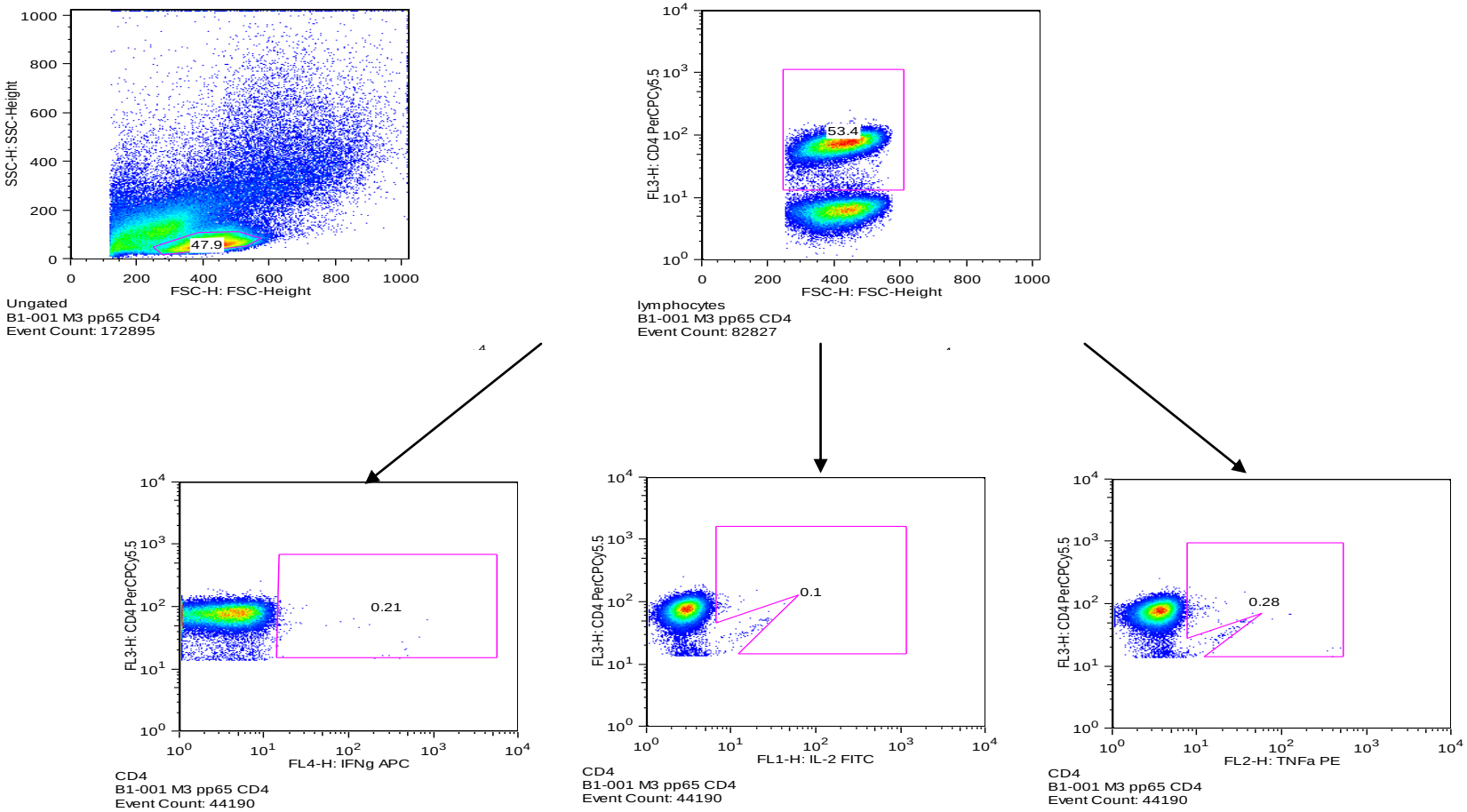
Taking the lymphocyte subset the CD4+ or CD8+ cell population was further defined (Figure 6-5). Taking either the CD4+ or CD8+ populations IFNγ producing cells were defined using a further box plot. This box was then applied to all CD4 and CD8 analyses for consistency. For IL-2 FITC and TNFα PE analyses a polygonal shape was applied to enable elimination of a non-specific staining ‘spur’ present in these channels. This was thought to be due to non-specific staining of PBMCs and was noted both in my analyses and those of adult transplant patients (Figure 6-5).

Figure 6-4 Gating analysis for ex-vivo PBMC analyses



PBMCs were extracted and stained according to the protocols detailed in Chapter 2. Lymphocyte populations were selected and then further defined by CD8+ve staining cells. CMV-specific HLA-A*0201 pentamer (A2 pp65 NLV) positive cells were selected using a further box plot. Quadrant analysis of Granzyme B and CD57 was carried out on CD8+ve cells and then copied to the CD8+/pp65NLV+ population to enable comparison.

Figure 6-5: Gating analysis for intracellular cytokine assays



PBMCs were extracted, stimulated, and stained according to the protocols in Chapter 2. During FACS analysis lymphocyte populations were first selected followed by CD4 or CD8 according to sample type. Cytokine production was then defined for each cytokine separately (IFNg, IL-2 and TNFa) and these settings ideally applied to all samples from a participant at a given time point.

In most cases it was possible to define the same cytokine 'gates' for both CD4+ and CD8+ lymphocyte populations but where these did not adequately define populations separate gating was conducted for CD4+ and CD8+ lymphocytes.

Further analysis was conducted in Excel with 'no antigen' negative controls deducted as background from any response seen. Where cytokine production in non-stimulated 'no antigen' controls was greater than that seen in antigen-stimulated samples, these 'negative' responses were represented as zero response to enable data to be presented on a positive axis.

It was decided that data from all samples would be included, even if it was not possible to acquire the intended 50,000 events, in view of the limited number of samples and sample size anticipated and the aim to provide descriptive data.

6.3. RESULTS

11 sites gained approval between June 2007 and September 2008. As with the CMV Treatment Registry the approvals process ranged considerably between sites with one site taking 14 months from initial submission to final approvals. In 4 of the 11 sites it was felt feasible to obtain samples for immunological analysis. In 2 sites it proved difficult to ensure samples for VL analysis were received by the Royal Free once babies had been recruited.

6.3.1. *Demographics and baseline clinical data*

20 babies were enrolled between May 2008 and April 2010 from 9 sites. 16 of these babies had received treatment for cCMV, 1 had symptomatic cCMV but had thrombocytopenia and normal cranial USS at birth and therefore was not treated, 2 were asymptomatic at birth and 1 had postnatal infection.

Of the 16 babies receiving treatment 13 were also enrolled in the CMV Treatment Registry. Demographics and descriptive data for these 13 babies are therefore more fully reported in Chapter 5 of this thesis.

- **Symptoms and severity at baseline**

8/19 (42%) mothers of congenitally infected babies were reported to have been diagnosed antenatally; no mothers received treatment. One of these babies was born without symptoms of CMV at birth and was not treated. The other 7 received treatment although 1 baby had no abnormal findings at birth and one had subtle

abnormalities on cranial USS only, with no clinical or biochemical/haematological abnormalities.

Of the congenitally infected babies 16/19 were born ≥37/40 with the remaining babies being born at 34/40 (2 babies) and 35/40 gestation.

Of the 17 symptomatic babies 14 had neurological involvement reported at baseline with 9 scans reported to be abnormal; 16 received treatment. Abnormal scan findings are summarised in Table 6-1. 9 babies had hearing loss already identified at baseline but only one had hearing loss as their only abnormal finding. 5 of these babies also had abnormalities noted on neuroimaging; 1 had normal neuroimaging and the remaining 3 had no neuroimaging results documented. 10/17 symptomatic babies had haematological involvement, with thrombocytopenia predominating; 5 of these babies also had skin lesions visible. Only 3/17 had hepatosplenomegaly and 3/17 hepatitis with 0/17 having pneumonitis or bowel involvement. 1 baby had ophthalmic abnormalities but it was uncertain if these were CMV-related. The symptomatic, untreated baby had IUGR (0.4th Centile) with OFC on 2nd Centile and thrombocytopenia noted on day 3 which resolved spontaneously by day 23 of life. The postnatally infected baby was born at 27/40 birth weight 740g (9th Centile) and had CMV detected on NPA on day 88 of life. Hepatitis was noted on day 37 of life which resolved by day 196 and pneumonitis was present from days 124-234 of life without treatment being given.

All except 2 treated babies were commenced on i.v. GCV, with the other 2 treated with VGCV exclusively. 3 babies were subsequently switched to oral VGCV treatment (1 after 6 weeks; 1 after 3 days following i.v. access difficulties and 1 at Day 10 with no reason documented). Treatment course was 42 days (+/- 1 day) for all except 2 babies, one of whom had a prolonged course of treatment of 81 days and the other who received only 18 days of treatment, treatment being discontinued once no evidence of CNS involvement had been confirmed.

6.3.2. *Clinical follow-up*

Of those congenitally infected with symptoms at birth, 1 of the 9 babies initially thought to have hearing loss was reported to have normal hearing at 12 months; 2/9 had received or were on the waiting list for cochlear implantation. No babies with normal hearing at birth had developed SNHL. 3 babies had neurological sequelae

Table 6-1 Abnormal neuroimaging findings in 9 babies with congenital cytomegalovirus infection and neurological abnormalities reported at birth

Subject	Neuroimaging	Abnormality
N1-001	CT scan	Intracranial calcification & diffuse white matter hypodensity
M1-001	Antenatal USS	Prenatal ventriculomegaly
	Postnatal MRI	Asymmetrical sulci, possible neuronal migration defect. Bilateral (mild) lateral ventriculomegaly.
B1-002	Antenatal MRI	Synechiae posterior horn, lateral ventricles bilaterally at 32/40
	Postnatal MRI	Abnormal septations, localised ventricular prominence.
D1-001	Cranial USS and MRI	USS: abnormal. MRI: bilateral cerebellar malformation, diffuse white matter abnormalities, small area of polymicrogyria.
D1-002	Postnatal Cranial USS and MRI	USS: bilateral caudo-thalamic subependymal cysts. Cyst left lateral ventricle, bilateral calcific lenticulostriate vasculopathy. MRI diffuse white matter changes, frontal cortex possible early polymicrogyria.
D1-003	MRI age 4 months	Widespread abnormal white matter and polymicrogyria, dysplastic cerebellum. Subependymal cysts.
H1-002	Postnatal Cranial USS	Periventricular calcification
H1-003	Modality unknown	Periventricular calcification
H1-004	CT scan	Periventricular calcification
A1-001	Cranial USS at birth	Calcification
	MRI and CT 4 months	Possible white matter changes left temporal region. No calcification noted.
A1-002	Antenatal USS	Ventriculomegaly.
	Postnatal MRI	Bilateral perisylvian polymicrogyria. Diffuse white matter signal abnormality. Small punctate lesions with signals suggestive of calcified haemorrhage in parietal lobes. Ventricular septations in occipital horns, lateral ventricles. Subependymal cysts.

documented with 2 of them having significant delay with neurodevelopmental age estimated at around 6 months on formal testing at around one year. One baby had a significant retinal scar documented which had not been recorded in earlier documentation received. No seizures were documented. Overall 9 of the 16 (56%) congenitally infected babies with follow-up data available had some kind of permanent impairment at 12 months with the majority of these involving hearing.

At two year follow-up clinical data was available for 17/19 congenitally infected children. In many cases this was through correspondence with parents only as many families had moved areas. Both asymptomatic babies in the study remained asymptomatic. 7/17 with hearing data available had continued hearing loss with 2 of these likely conductive rather than sensorineural. 1 baby was reported to have new hearing loss, but this was suspected to be conductive in nature. No further babies had received cochlear implantation. 9/14 babies with data available had neurological impairment with most of these babies having delays in more than one area. No babies had visual loss documented although no further information was available for the baby noted to have scarring present at 12 months of age.

6.3.3. *Viral Load*

The main analysis of VL presented here is of 17 babies treated for cCMV in the neonatal period. Data has been pooled from 3 main sources, as described above, with the majority (11/17 babies) being part of the VICC study. Some results were available for 4 untreated babies with these small numbers limiting the value of detailed comparisons of VL between different patient groups.

- **Viral load in untreated babies**

CMV was only detectable in blood at a low level (log_{10} 2.54 copies/ml) in 1/16 samples analysed in the 4 babies not receiving treatment for cCMV (Table 6-2); this was in the baby that was symptomatic at birth but not treated. In contrast virus was detectable in both urine and saliva at >4.0 log_{10} from all 4 untreated babies at all time points up to 12 months of age (M12).

The congenitally infected babies had first recorded urine and salivary VL ranging 4.7-6.9 log_{10} and 5.8-8.4 log_{10} respectively. There was continued viruria at high levels on sampling at age 6-12 months (M6-M12) in all 4 untreated babies but in

Table 6-2 Viral load in different body fluids at specified time points in babies with CMV infection not receiving treatment.

BLOOD				
Time point	M2-001	H3-001	M3-001	J4-001
Baseline	-	2.0	2.0	-
D14	-	-	-	-
D28	2.0	2.0	-	-
M3	2.0	2.0	-	-
M6	2.5	2.0	2.0	2.0
M12	2.0	2.0	2.0	-
M24	2.0	2.0	2.0	-

URINE				
Time point	M2-001	H3-001	M3-001	J4-001
Baseline	-	4.7	6.9	-
D14	-	-	-	-
D28	6.5	4.5	-	-
M3	5.8	5.4	-	-
M6	5.4	5.2	6.3	8.7
M12	5.3	5.8	-	6.0
M24	2.0	4.8	2.0	-

SALIVA				
Time point	M2-001	H3-001	M3-001	J4-001
Baseline	-	7.8	8.4	-
D14	-	-	-	-
D28	5.8	7.6	-	-
M3	8.8	6.4	-	-
M6	5.8	6.0	9.1	8.7
M12	4.1	4.4	6.0	6.0
M24	5.3	5.3	2.0	

M2-001 symptomatic at birth, not treated
H3-001 and M3-001 asymptomatic at birth (age at baseline H3-001 = 8 days, M3-001= 47 days)
J4-001 postnatally infected, not treated
D = day of treatment M = Month of age

only 1/3 babies with samples available at age 24 months (M24). Urinary VL decreased from baseline to M6-12 in 3/4 babies.

There was a spontaneous decrease in salivary VL observed in all 4 untreated babies from first measurement to M12 (decrease of $\log_{10}$ 1.7, 3.4, 2.4 and 2.7 respectively). Virus was still detectable in saliva, however, in 2/3 subjects with samples received at M24, and had increased from the M12 values in both babies.

- **Baseline VL in blood, urine and saliva in treated babies and suppression at end of treatment**

In babies treated for cCMV, virus was detectable in 15/17 (88%) of baseline blood samples obtained within 7 days of treatment and all of the 15 urine and 8 saliva samples. Mean VL at baseline was 3.8 (SD ± 0.8), 7.7 (± 0.9) and 7.3 (± 1.5) in blood, urine and saliva respectively with corresponding median values of 3.8 (IQR 3.3-4.2), 7.7 (IQR 7.0-8.4) and 7.2 (IQR 6.8-8.3).

In 6 babies there was more than one blood, and in 5 babies more than one urine, sample taken prior to treatment commencing. In all except one pair of urine specimens the samples were analysed in the same laboratory. In 2/6 blood samples both VL were within 0.5 $\log_{10}$ of each other. In the remaining 4 samples VL varied from 0.6-4.3 $\log_{10}$ between samples; in all cases VL decreased over time. In 2 patients with more than 2 blood samples taken prior to treatment, VL continued to decrease over 21 and 29 days measured. In 3/5 urine samples where 2 samples were obtained prior to treatment a difference of <0.5 $\log_{10}$ was observed. The remaining 2 subjects showed decreases of 1.0 and 1.6 $\log_{10}$ over 2 and 23 days respectively. No babies had more than 2 urine specimens analysed prior to treatment.

At the end of 42 days treatment (or within 3 days of this date) CMV remained detectable by PCR in 9 of 14 blood samples (65%), 9 of 12 urine samples (75%) and 4 of 7 salivary swabs (57%). Median VL in those babies with virus detectable in blood, urine and saliva was 2.8 $\log_{10}$ (IQ 2.5-3.3), 2.9 $\log_{10}$ (IQ 2.7-3.9) and 4.0 $\log_{10}$ (IQ 3.6-4.7) respectively.

- **Viral decline in blood, urine and saliva and viral half-life calculations**

VL was around 4.0 $\log_{10}$ higher at start of treatment in urine and saliva when compared with blood but this difference narrowed during the treatment course

(Figure 6-6). In keeping with this observation VL declined at a greater rate between start and end of 42 days treatment in urine and saliva when compared to blood with a median change of -1.2 log_{10} (IQ range -1.8 to -0.9) in 14 paired blood samples compared to -4.4 log_{10} (IQ range -5.5 to -3.8) in 10 paired urine samples and -4.8 log_{10} (IQ range –5.2 to -3.9) in 7 paired saliva samples (Figure 6-7). In 2/14 paired blood samples an increase in VL or no change was observed whereas decline was noted in all urine and salivary samples during treatment.

Taking into account observations earlier in this thesis that viral decline appeared to be most rapid in the first week of treatment viral decline was analysed separately for the first 3 and 7 days of treatment and compared to that over the full 42 day treatment course (Table 6-3). Samples were limited, particularly for decline between days 0 and 3. Viral decline over the first 7 days of treatment was nonetheless observed to be more rapid in both blood and urine when compared to that observed over the full 42 days of treatment. Salivary samples were even fewer but in the one pair of samples available there was no difference in the rate of decline between days 0-7 and the full 42 days treatment. A more rapid decline was, however, observed during the first 3 days of treatment compared to over the full treatment course in the 2 babies with samples available.

Virus half life of decline ($T_{1/2}$) was calculated using segmental regression of plots of Ln VL during treatment in GraphPad Prism for each body fluid where sufficient samples were available (see 4.2.3).

Median virus $T_{1/2}$ in blood of 14 babies with sufficient data available was calculated to be 2.4 days (IQR 1.9-3.3). In 8 of these babies the slope was fitted to viral decline during the first 7 days of treatment but in the other 6 subjects maximal decline was over a longer time period, in part due to limited sample availability during early time points. Median $T_{1/2}$ in those babies with viral decline analysed in the first 7 days of treatment was 2.1 (IQR 1.4-2.7) days compared to 2.9 (IQR 2.3-3.9) days in those with analysis over a longer time period (a non-statistically significant difference).

Median $T_{1/2}$ in urine in 14 babies was 2.03 days (IQR 1.29-2.64). In 7 maximal viral decline was noted in the first 7 days of treatment; in these babies median $T_{1/2}$ was observed to be 1.25 days (IQR 0.66-2.46) compared to 2.26 days in the remaining 7 babies (IQR 1.91-2.75) a difference which was again not statistically significant.

Figure 6-6 Mean viral load over time in different body fluids in 17 babies treated for congenital CMV

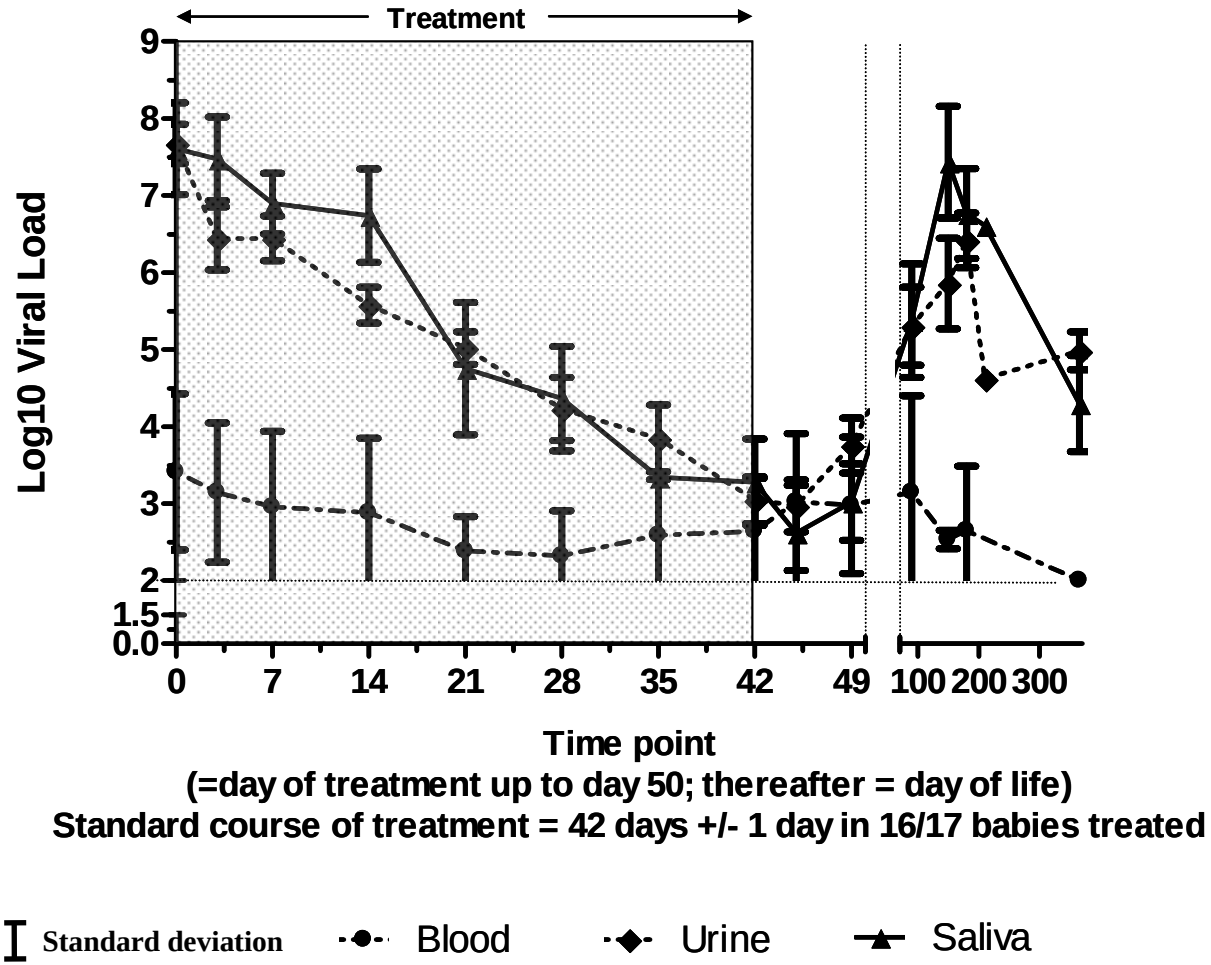
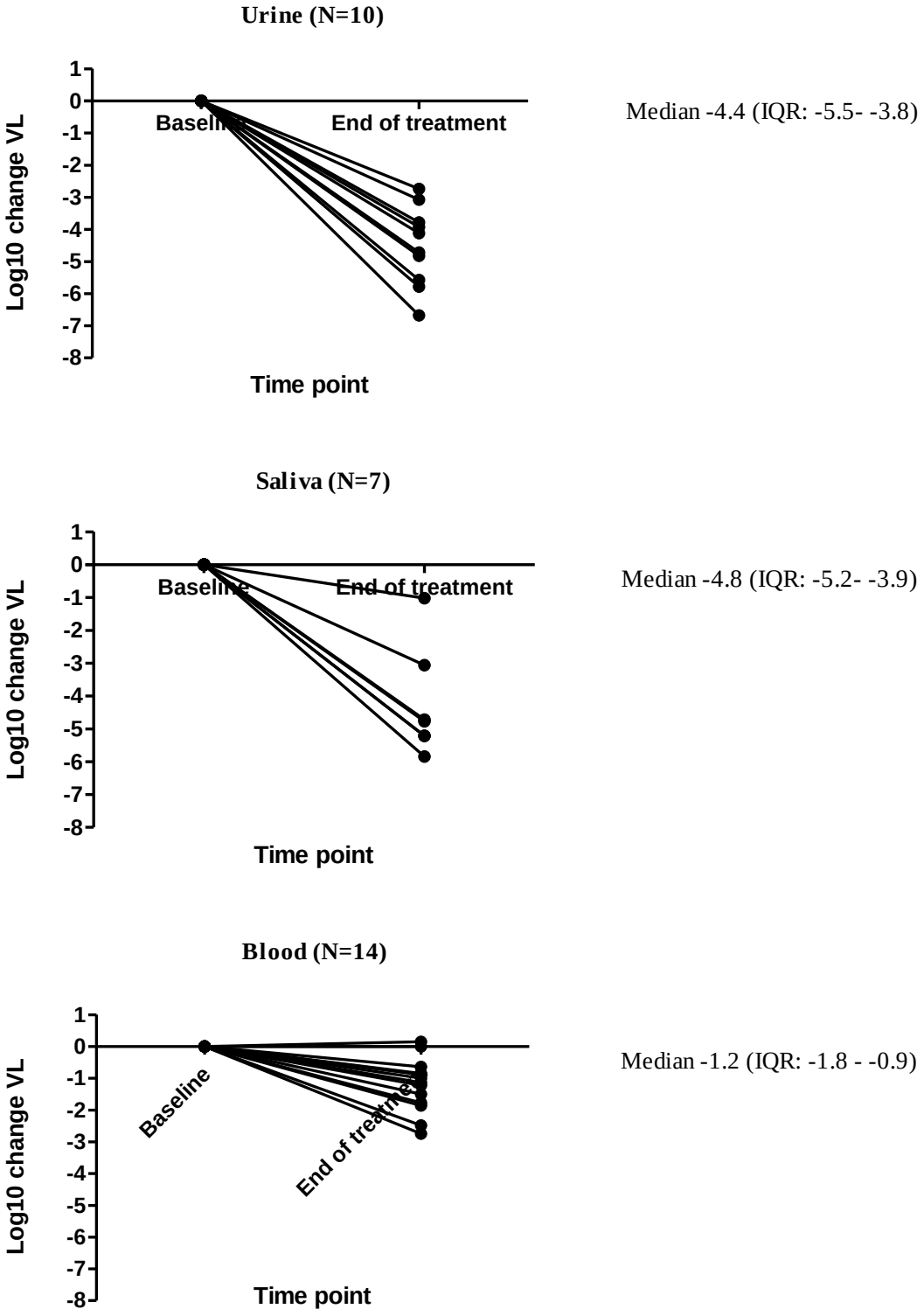


Figure 6-7: Change in Log_{10} viral load between baseline and end of 42 days antiviral treatment in babies treated for congenital CMV



VL= Viral Load
IQR = Interquartile range
Viral load measured using quantitative CMV PCR before treatment onset and at the end of 42 days antiviral treatment (+/- 3 days).

Table 6-3: Table of median viral decline in blood, urine and saliva at different time points during treatment in 17 babies with congenital CMV.

Body fluid	Time point		
	D0-D42 viral decline (log_{10} copies/day) (IQR)	**D0-3 viral decline (log_{10} copies/day) (IQR)**	**D0-7 viral decline (log_{10} copies/day) (IQR)**
Blood	0.03 (0.04-0.02) N=12	0.3 (0.5-0.1) N= 2	0.12 (0.16-0.10) N=12
Urine	0.10 (0.14-0.08) N= 13	0.63 (0.85-0.38) N= 4	0.21 (0.3-0.07) N= 12
Saliva	0.11 (0.12-0.07) N=7	0.25 (0.49-0.02) N=2	0.11 N=1

D= Day of treatment
N= Number of paired samples available for analysis.
IQR = Interquartile Range

There were only 2 babies with maximum viral decline noted in the first 3 days of life, partly due to samples available, these babies had a $T_{1/2}$ calculated of 0.78 and 0.25 days.

Saliva specimens were available during treatment at more than 1 time point for 8 babies. In two babies VL increased over the first 7 days of treatment and in a further two the first saliva sample was only acquired after day 6 of life; data from these babies were therefore excluded in this analysis. Median $T_{1/2}$ of viral decline in the remaining 4 babies was calculated to be 1.49 (IQR 1.38-2.37).

Treatment in the group of babies studied above was with i.v. GCV in 11 babies, oral VGCV only in 2 and the remaining 4 babies received a mixture of GCV and VGCV, all commencing with GCV for at least 6 days. There was no obvious difference in viral decline in those treated with GCV compared to VGCV, although numbers were too small to allow meaningful comparisons.

- **Rebound**

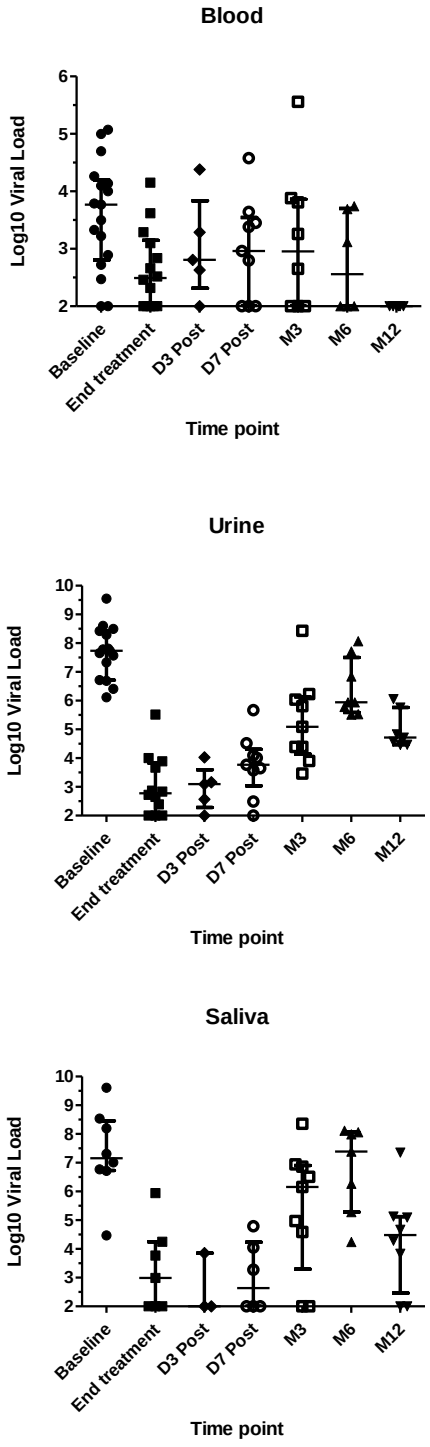
Once treatment was stopped rebound of virus was seen within 1 week in 4/8, 6/9 and 1/5 of those patients with blood, urine and saliva samples available respectively with corresponding median increase in VL being 0.52, 1.03 and 2.05 $\log_{10}$. In samples where no rebound was seen virus was undetectable at the end of treatment in 2/4 blood, 1/3 urine and 2/4 saliva; in the remaining subjects virus was still detectable but continued to decrease after treatment discontinuation.

Based on mean and median values maximal rebound was observed at M3 in blood and at M6 in urine and saliva samples (Figure 6-6 and Figure 6-8). Median VL was not significantly different from baseline for blood at M3 (3.78 $\log_{10}$ vs 2.96 $\log_{10}$ at baseline and M3 respectively; P=0.3) or saliva at M6 (VL 7.39 $\log_{10}$ and 7.16 Log_{10} respectively; p = 0.72). Urine VL was, however, significantly lower at M6 compared to baseline (median 5.94 $\log_{10}$ vs 7.74 $\log_{10}$ respectively; P= <0.01).

- **Long-term viral control**

Data presented here are from all enrolled babies (treated and untreated). Virus was still detectable in 0/10 blood, 9/9 urine and 9/11 saliva samples available at M12 and 0/6 blood, 4/6 urine and 3/6 saliva samples available at M24.

Figure 6-8 Viral load in blood, urine and saliva, during and after treatment in 17 babies treated for congenital CMV



Baseline = start of treatment
End treatment = end of 42 day treatment course
D3 and D7 Post = 3 and 7 days after treatment discontinued
M3, 6, 12 = age 3, 6 and 12 months of life
Error bars represent median and interquartile range

In urine median VL of 9 samples at M12 was 4.83 $\log_{10}$ (IQR 4.53-5.78) and of 6 samples at M24 was 2.76 $\log_{10}$ (IQR 2.00-3.89), significantly lower than the baseline of 7.66 $\log_{10}$ ($p<0.01$)(Figure 6-9).

Similarly saliva VL was significantly lower at M12 and M24, measuring 4.40 $\log_{10}$ (IQR 3.84- 5.13) and 2.76 $\log_{10}$ (IQR 2.00- 5.30) respectively, than the baseline median of 7.56 (IQR 6.76-8.44)($p <0.01$)(Figure 6-9).

At M24 there was no association apparent between those with virus detectable and whether they were symptomatic or asymptomatic at birth or had ongoing abnormalities detectable. However, none of the babies in this cohort had confirmed progressive SNHL.

- **Prolonged treatment**

Only one baby had a course of 81 days treatment (42 days i.v. GCV followed by 39 days oral VGCV) (Figure 6-10). In this baby virus continued to be intermittently detectable at low levels in blood from day 42 to end of treatment (max 2.6 $\log_{10}$); VL continued to decrease in urine from 3.67 $\log_{10}$ on day 42 of treatment to become undetectable at day 91 and in saliva became undetectable on day 49 treatment and remained undetectable. 7 days after treatment was terminated rebound was observed in urine VL up to 1.5 $\log_{10}$ above end of treatment levels with no corresponding rebound in blood or saliva VL. Virus was detectable at high levels at M6 and M12 in urine and saliva with no corresponding viraemia. This baby had no hearing loss but significant neurodevelopmental delay at two years of age.

6.3.4. *Ganciclovir Levels*

Most samples were received as whole (unseparated) clotted blood in our laboratory. There was therefore significant delay between sample acquisition and separation and freezing of serum in most cases.

31 samples were received from 8 babies, a further 8 samples were sent from 2 babies directly to the UK national reference laboratory. Results were available for 12 pre-dose, 9 post-dose and 15 random samples; timing was unspecified in 3 samples. 8 paired samples were available from 5 patients; all babies were aged < 6 months.

9/12 (75%) of pre-dose samples had GCV levels of <0.5mg/L whilst on treatment (Figure 6-11A); 2/3 babies with pre-dose levels 0.5mg/L or above were being treated with oral VGCV.

Figure 6-9 Viral load in urine and saliva at baseline and over 2 years follow-up from 20 babies with congenital CMV (both treated and untreated)

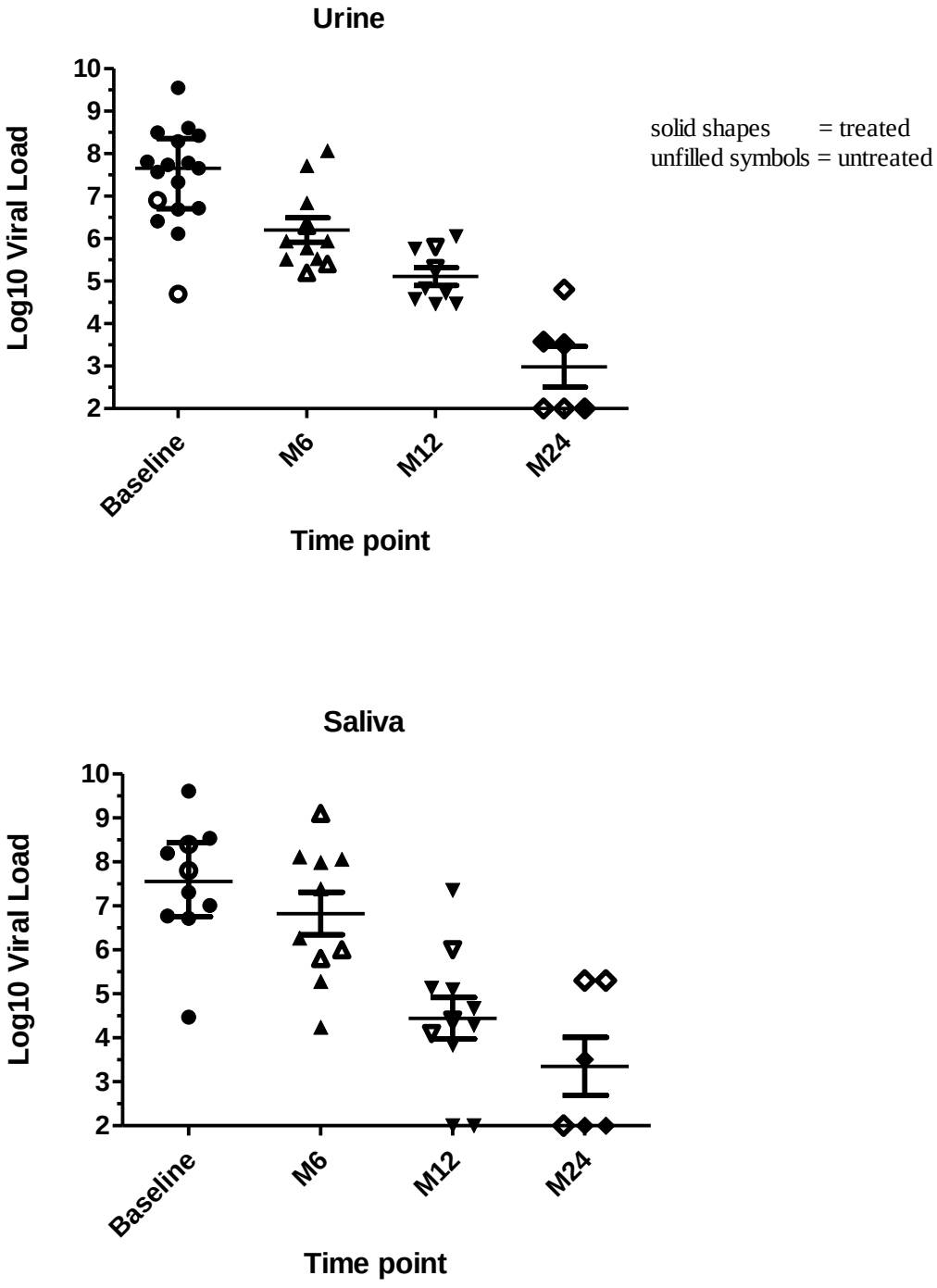
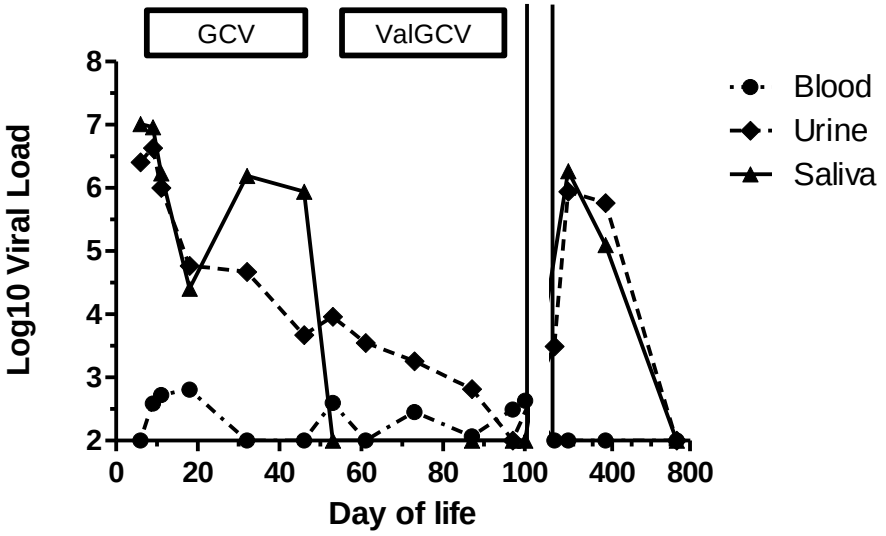


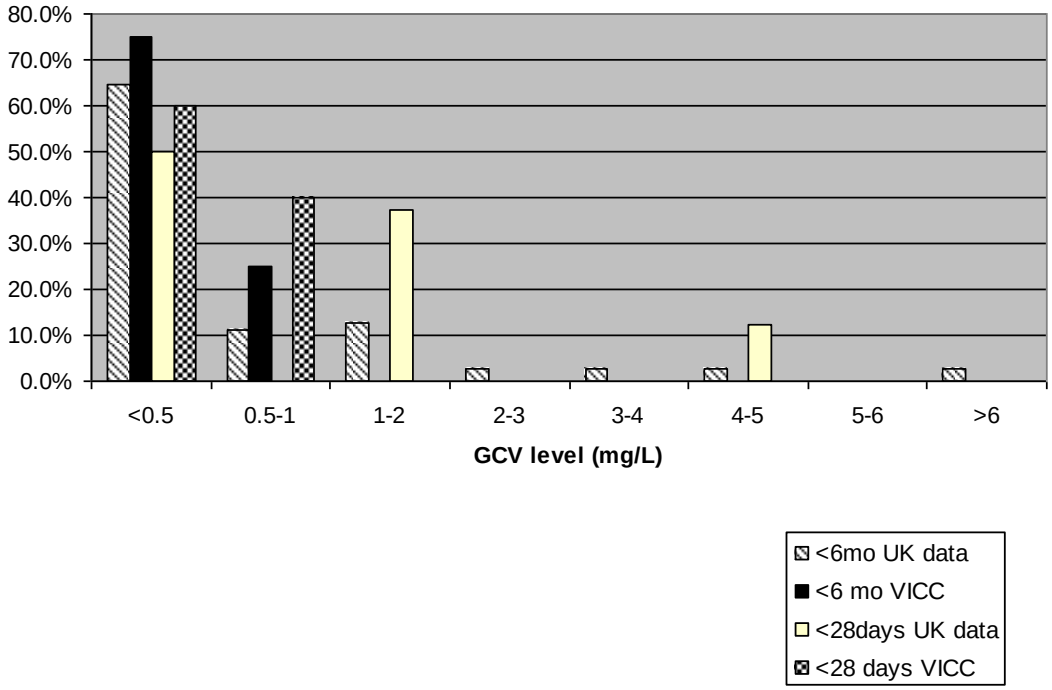
Figure 6-10 Viral load during treatment and follow-up in subject N1-001



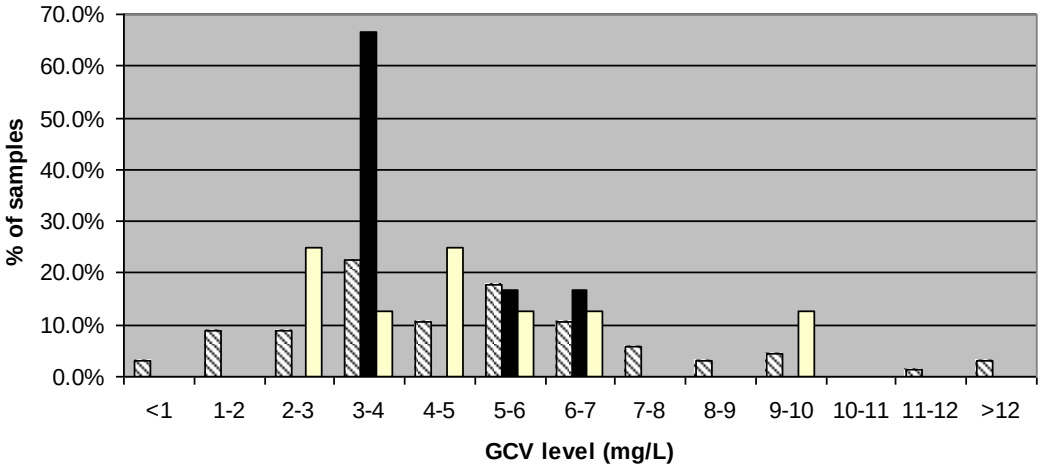
Subject was treated for 81 days with ganciclovir (GCV) followed by valganciclovir (ValGCV) and followed up for 2 years to monitor neurodevelopmental and audiological outcomes.

Figure 6-11 Pre- (A) and Post- (B) dose ganciclovir levels measured in babies in the VICC study alongside data from the UK National Reference laboratory.

A. Pre-dose



B. Post-dose



GCV = ganciclovir
6mo = 6 months of age
28 days = 28 days of age

Data shown here is from both the VICC study and the analysis of GCV levels from the UK reference laboratory described in Chapter 3 to allow for comparison.

There were no post-dose levels >7.0 mg/L in babies treated with GCV (Figure 6-11B); Mode post-dose level was 3.0-4.0 mg/L. 1/3 babies being treated with VGCV had post-dose GCV level >5.0 mg/L.

Neutropenia was noted in 2 babies with results available. Both of these babies had low pre- (0.1; 0.2 mg/L) and post-dose (3.7; 3.6mg/L) GCV levels. There were no toxic GCV levels recorded.

In 4 babies multiple samples were available over time. In one of these babies, treated with GCV, a drop was noted in levels between D7 and D42 of treatment (pre- 0.9 and 0.1 respectively; post- 6.7 and 3.7 mg/L). In the other 3 babies values were similar at D7 and D42 of treatment; one of these babies received 42 days of GCV, one 42 days oral VGCV and the other received 10 days GCV followed by VGCV. Drug dose was increased for weight in all 4 subjects.

There was detailed VL data available for 6 babies with GCV levels analysed. At the end of treatment virus was undetectable in 2/6 (33%), 3/6 (50%) and 4/6 (66%) of blood, urine and saliva samples respectively. Viral decline in the 6 babies with data available was not notably different to that of the group as a whole. There was no apparent trend between those babies achieving undetectable VL at the end of treatment and GCV levels.

6.3.5. *Cellular Immunity*

Samples were obtained from 8 babies (7 congenitally infected and receiving treatment and 1 postnatally infected) at various time points for measures of cellular immunity. More than 1 sample was obtained from 5 babies permitting observation of changes over time.

3 babies were determined to be HLA-A2 positive, 4 were negative and 1 was not tested. Samples were fully acquired (50,000 G2 events +/- 5000 cells) in 67/86 (78%) CD4 samples and 28/58 (48%) CD8 samples. As stated earlier all samples were included in this analysis.

- **CMV-specific lymphocyte populations and lymphocyte phenotype in CMV-infected infants.**

The mean CD4/CD8 ratio was 2.12 (SD 0.8). Although not statistically significant, CD4/CD8 ratios showed a trend towards decreasing over time with median (IQR) at time points D0-14, D28-M3, M6-M12 of 2.2 (1.6-3.3), 2.0 (1.7 - 3.1) and 1.9 (1.4-

2.1) respectively (Table 6-4). Most of this decrease could be accounted for by a decrease in CD4 percentage over time with median values at D0-14, D28-M3 and M6-12 of 62.7% (IQR 53.7-70.1%), 55.3% (IQR 52.8-68.8%) and 53.1% (IQR 47.7-57.0%) respectively. CD8 levels, however, remained relatively stable with only a small increase at M6-12 to 27.0% from a median of 26.8% at D0-14.

Analysis of samples from HLA-A2 negative subjects revealed that most CD57+ cells were also CD28-, with changes in CD57+ populations mirroring those of CD57+/CD28- lymphocytes. There were insufficient flow cytometer channels to examine both CD28 and CD57 populations alongside CMV-specific pentamer frequencies. Changes in CD57+ populations were therefore reviewed alone to enable comparison between all patients, with the assumption that most of these cells were not expressing CD28.

Median frequency of CD8+/CD57+ lymphocytes was 27.2% (IQR 15.7-37.8%). This is comparable to the proportion of CD8+/CD57+ cells observed in healthy, CMV seronegative adults included as controls (Figure 6-12).

In the 3 patients that were HLA-A2+, median CMV specific Class I MHC restricted pp65 (NLV) pentamer frequencies were 0.50% (range 0.02-1.65%). There was no observable increase in proportion of CMV specific lymphocytes over the first year of life (Figure 6-13). In these babies a significantly higher proportion of CMV-specific (CD8+/NLV+) lymphocytes were CD57+ compared to the CD8+ population as a whole with a median frequency of 71.1% (IQR 60.7-79.5%)($p<0.001$)(Figure 6-12). There was no difference observed in total percentage of CD8+/CD57+ lymphocytes between those subjects that were HLA-A2+ compared to those that were not and no association apparent between the frequency of CD8+/NLV+ lymphocytes and total CD8+ lymphocytes expressing CD57 (data not shown).

- **PBMC Stimulation assays**

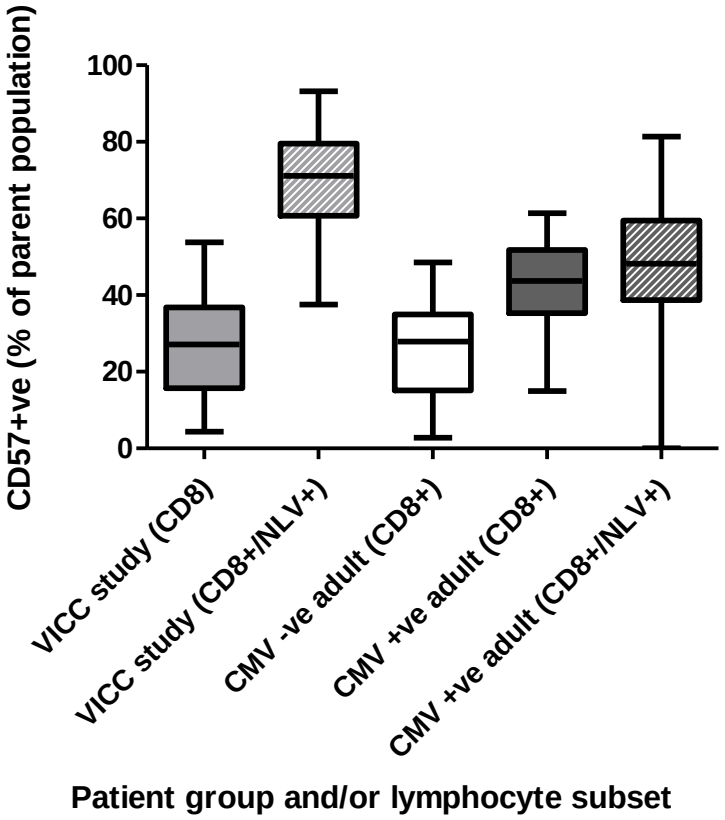
IFNg responses to stimulation with SEB, the positive control, were barely detectable or absent in all babies except B1-002 at D0-D14 (Figure 6-14A and B). This poor IFNg response persisted throughout the time frame being studied with maximum IFNg-secreting CD4+ T cell frequencies at M6-12 of 1.0%, (median 0.2%) compared to maximum IL-2 and TNFa frequencies at this same time point of 8.8% and 9.7% respectively (median 2.3% and 2.8%; $p<0.05$). Some IL-2 and TNFa production

Table 6-4: Median CD4 and CD8 T cells and CD4/CD8 ratio at different time points in 8 babies with congenital CMV

Age	Median CD4 % (IQR)	Median CD8 % (IQR)	Median CD4/CD8 ratio (IQR)
D0-14 (N=4)	62.7 (53.7-70.1)	26.8 (21.7-32.7)	2.2 (1.6-3.3)
D28-M3 (N=5)	55.3 (52.8-68.8)	26.6 (21.8-32.5)	2.0 (1.7-3.1)
M6-12 (N=9)	53.1 (47.7-57.0)	27.0 (26.2-34.8)	1.9 (1.4-2.1)

% = percentage of total lymphocyte number
IQR = interquartile range
D = day of treatment
M= month of life
N = number of samples included in analysis

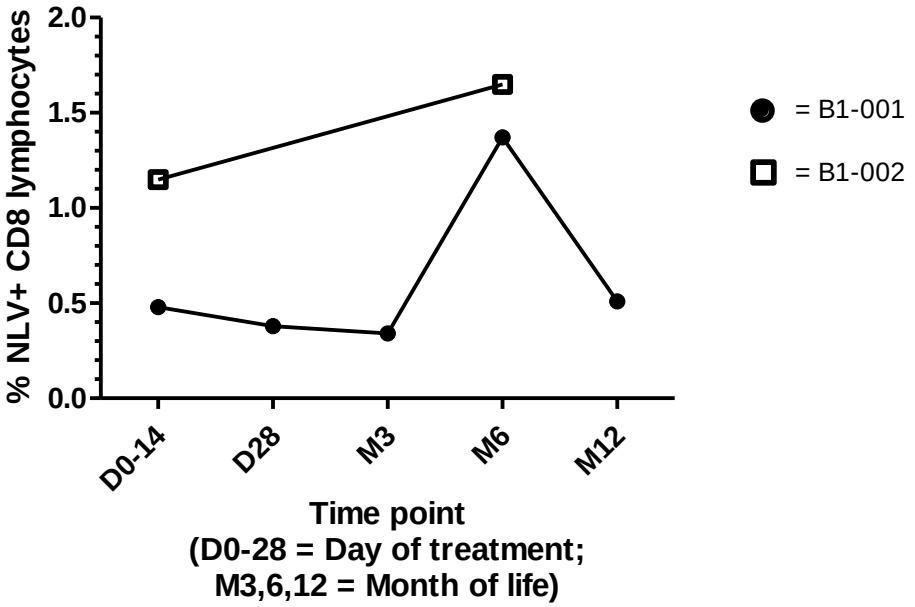
Figure 6-12 Frequency of CD57+ lymphocytes in CMV infected infants and healthy adults



Box plots show interquartile range with line at median; Whiskers show minimum and maximum values.

Infants were all CMV-infected and enrolled in the VICC study of viral load and immunity in congenital CMV.
Adult patients were aged 51-70 years (median 60.5 years). Adults were either positive or negative for CMV IgG (CMV +ve or CMV –ve respectively).
Frequencies shown are CD57+ lymphocytes as a percentage of total CD8+ lymphocytes (CD8+) or as a percentage of CMV-specific lymphocytes (CD8+/NLV+) as defined by detection of class I MHC HLA-A*0201 pp65 (NLV) restricted pentamer staining.

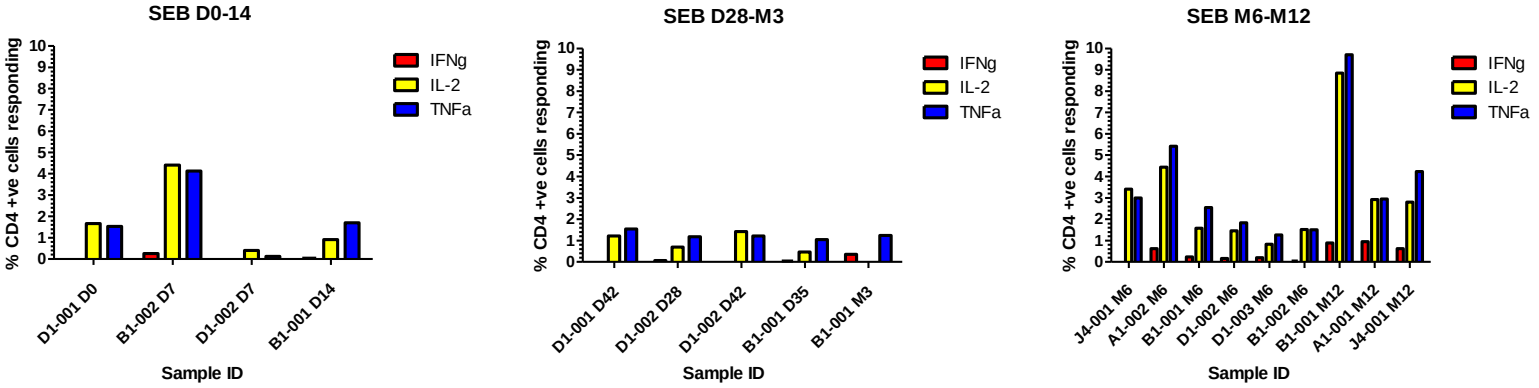
Figure 6-13: Frequency of CMV-specific lymphocytes over time in two HLA-A2 positive babies treated for congenital CMV.



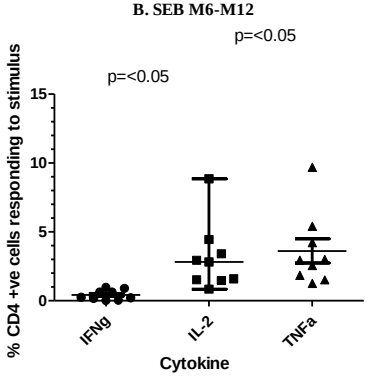
CMV specific lymphocytes as a percentage of total lymphocytes (%NLV+/CD8 lymphocytes) were quantified using FACS analysis of fluorescently labelled HLA-A*0201 Class I restricted pentamers loaded with CMV pp65 (NLV) peptide (Proimmune) and using protocols as described in Chapter 2 and Chapter 6.2.6.

Figure 6-14: CD4+ lymphocyte cytokine production in response to stimulation with *Staphylococcal aureus* enterotoxin B

A. Individual patient responses over time



B. SEB M6-M12



PBMC's were extracted, stimulated and stained according to protocols detailed in Chapter 2 of this thesis.

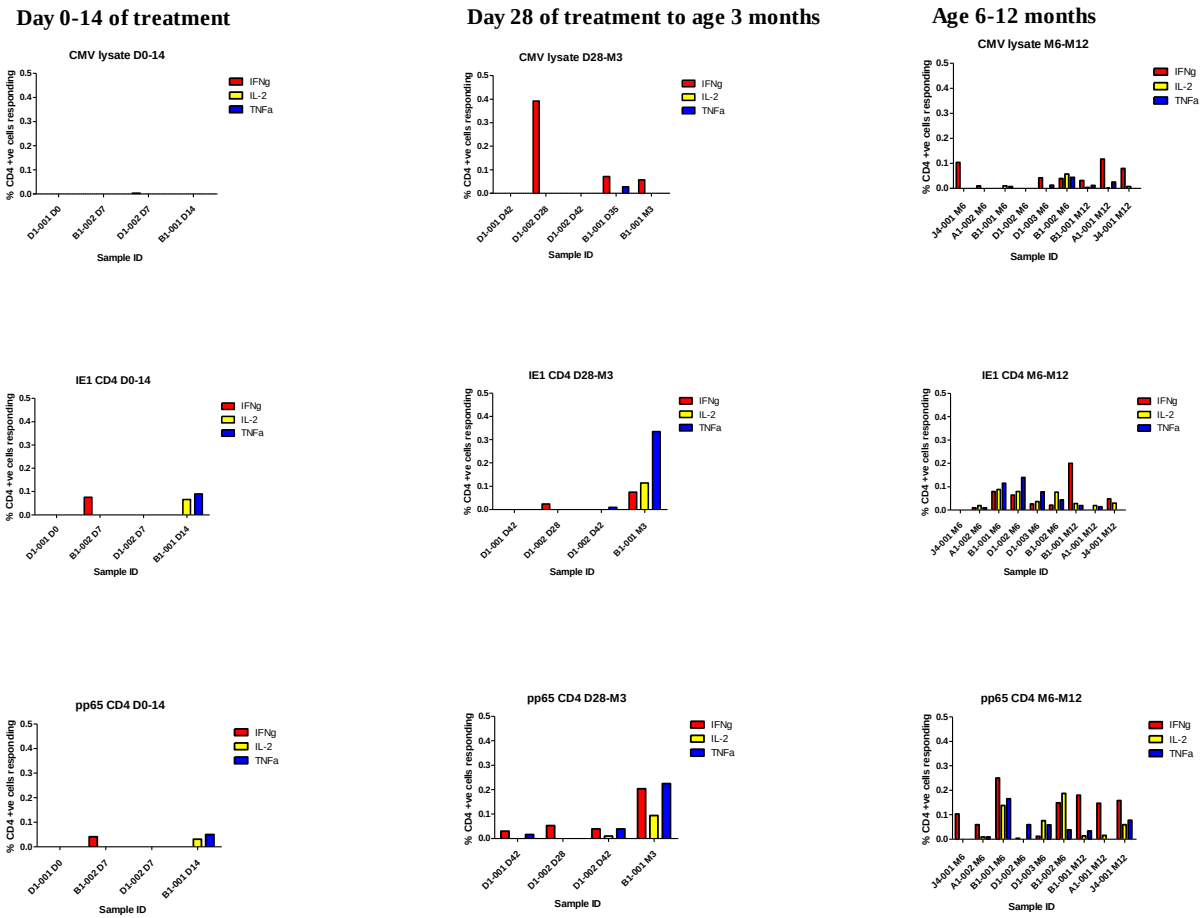
was seen by CD4+ cells following SEB stimulation at most time points from most ies, with median IL-2 and TNFa responses 1.3% (range 0.4-4.4%) and 1.6% (range 0.1-4.1%) respectively at D0-14 and 0.7% (range 0.0-1.4) and 1.2% (range 1.0-1.5%) at D28-M3.

In contrast CD4+ lymphocyte cytokine responses were <0.1% in all subjects for all CMV-specific stimuli at D0-14 (Figure 6-15). With only one exception the response to stimulation with CMV lysate was ≤ 0.1% for any cytokine production at all time points. Maximal CD4+ cytokine response was seen at M6-12 in response to stimulation with pp65 peptides with all patients showing some response to stimulation with this antigen with median (range) IFNg, IL-2 and TNFa lymphocyte frequencies being 0.13% (0.0-0.25%); 0.0% (0.0-0.19%); 0.04% (0.0-0.17%) respectively. There was a general trend towards increased production of all cytokines with increasing age. In contrast to the responses elicited with SEB IFNg was the predominant cytokine produced in response to CMV-specific stimulation in most cases. In only one infant (D1-002) was IE1 the most potent stimulator of CD4+ lymphocyte cytokine production with this baby also showing predominant TNFa responses at M6.

CD8+ T-cell cytokine responses, when elicited, were greater for both IE1 and pp65 stimulation than those seen in CD4+ lymphocytes. In keeping with the responses observed for CD4+ T-cells responses were generally greater following stimulation with pp65, involved IFNg secretion predominantly and increased over time (Figure 6-16). In 4 samples only (D1-001 at D42, B1-001 M3, D1-002 at M6 and A1-001 at M12) cytokine production was maximal following IE-1 pooled peptide stimulation. Median responses following pp65 peptide stimulation for IFNg, IL-2 and TNFa at M6-12 were 0.15 % (range 0.0-0.85%), 0.03% (range 0.0-0.25%) and 0.13% (0.0–0.48%) respectively. Corresponding results following IE1 stimulation were 0.1% (range 0.00-0.83%); 0.04% (range 0.00-0.11%) and 0.04% (range 0.00-0.35%).

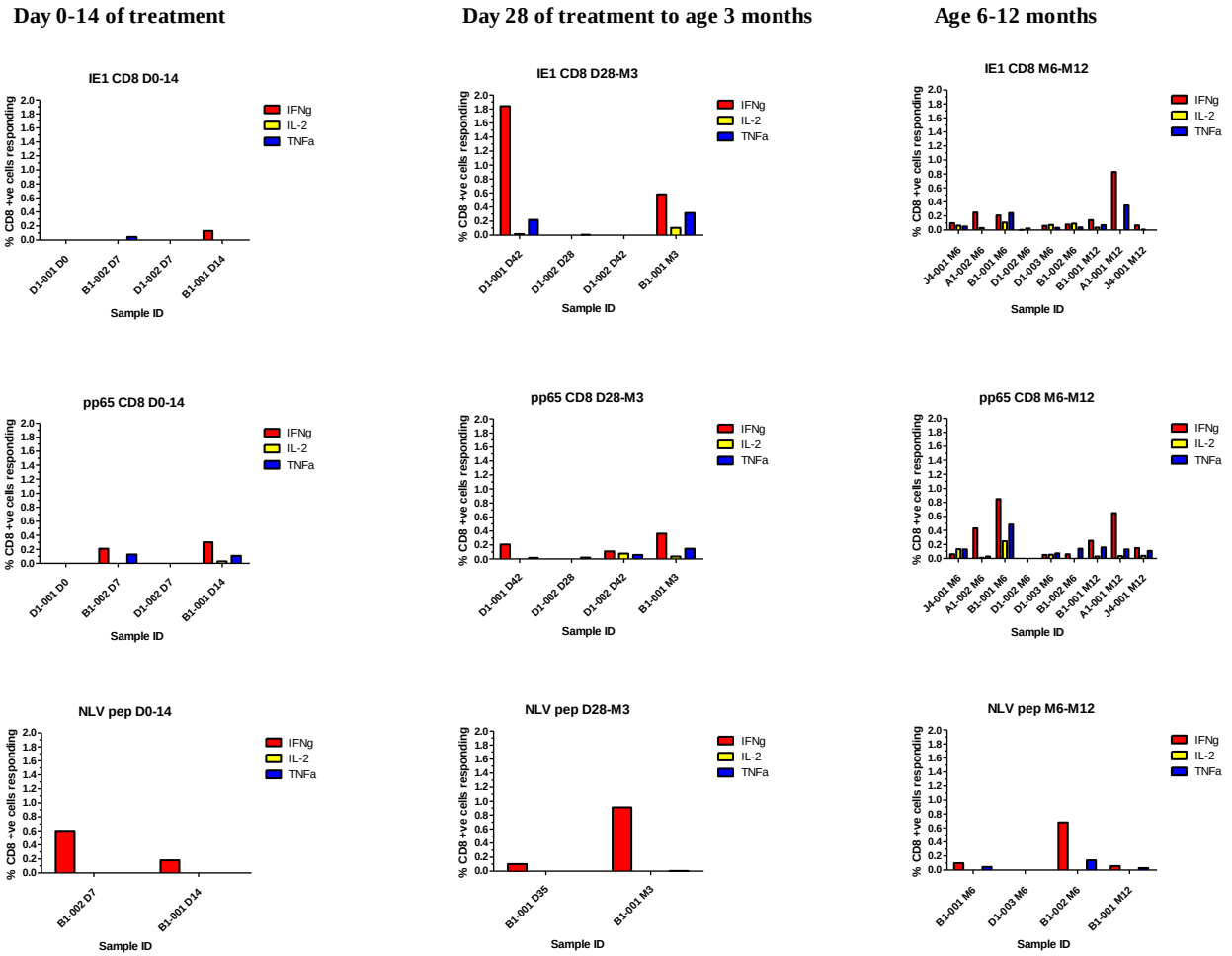
An IFNg response was seen following stimulation with NLV peptide in 7 of 8 CD8+ samples from HLA-A2 positive subjects (range 0.06-0.91%). Somewhat surprisingly NLV peptide stimulation produced higher responses than with stimulation with pooled peptide preparations. In keeping with results for stimulation with other antigens IL-2 and TNFa production was minimal.

Figure 6-15: CD4+ lymphocyte cytokine production following stimulation with CMV-specific stimuli over time



PBMCs extracted and stimulated according to protocols described in detail in Chapter 2 of this thesis.

Figure 6-16: CD8+ lymphocyte cytokine production following stimulation with CMV-specific stimuli over time



The responses seen in the only postnatally infected baby (J4-001), who had samples available from M6 and M12, were not notably different from those observed in congenitally infected babies.

There was no obvious association between the frequency of CD8+/NLV+ lymphocytes on ex-vivo FACS analysis and the frequency of IFNg production by CD8+ lymphocytes in response to matched pp65 (NLV) peptide stimulation in the small number of samples available (Figure 6-17).

- **Viral load and correlates with CMV-specific T cell responses**

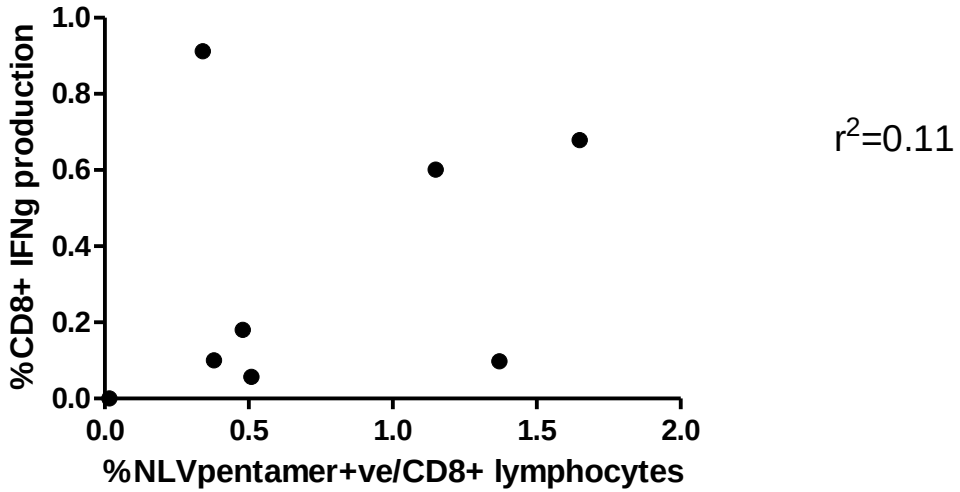
Given that minimal cytokine responses were observed at earlier time points only data for responses at M6-12 were scrutinised for any relationship between CD4+ and CD8+ lymphocyte responses and VL. Most subjects had no viraemia at these later time points. There was no obvious trend visible between cytokine responses to any stimulus between the 2 patients with viraemia at M6 compared to the 5 patients with no viraemia detectable (data not shown). For urine and saliva, VL was compared to IFNg responses observed in CD4+ and CD8+ T cells to all stimuli (Figure 6-18). There was a weak association observed between urine VL and IE1 stimulated CD8+/IFNg+ ($r^2 = 0.52$) and between salivary VL and pp65 stimulated CD4+/IFNg+ lymphocyte frequencies ($r^2 = 0.57$). There was a stronger inverse relationship observed between the frequency of CD8+/NLV pentamer+ lymphocytes and urine VL in the 4 samples evaluable ($r^2 = 0.76$).

6.3.6. *Serum IgG Avidity*

52 serum samples were received for CMV IgG analysis (38 from babies and 14 samples from 8 mothers who agreed for samples to be retrieved or taken as part of the study). Only maternal samples taken closest to their baby's birth are considered further here. Baby samples taken closest to specific time points were used for analysis.

20/40 (50%) samples had total CMV IgG levels >250 AU/mL (the upper limit given routinely by the automated system used in our laboratory); 30/40 (75%) had levels >200 AU/mL. There was no obvious trend over time in total CMV IgG levels with some babies showing increasing levels over time (H1-002, H1-003) and others decreasing (D1-002, B1-001, H3-001)(Figure 6-19A). Interestingly the 3 babies with lowest IgG levels measured were asymptomatic (K1-001, H3-001, M3-001).

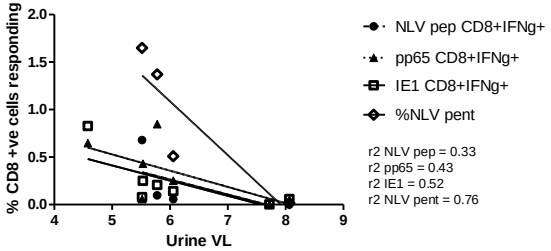
Figure 6-17: Relationship between CMV specific lymphocyte population and frequency of IFNg production following stimulation with CMV-specific peptide

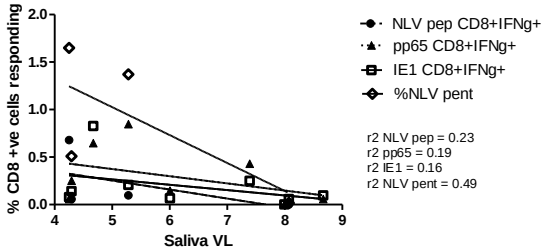


CMV specific lymphocytes were defined by percentage of HLA-A2 Class I (NLV) pentamer+/CD8+ lymphocytes in HLA-A2 positive subjects as described in Chapter 2.
IFNg production was measured using FACS analysis of fluorochrome-stained PBMCs following stimulation with NLV peptide, the immunodominant CMV peptide in HLA-A2 positive populations as also described in Chapter 2.

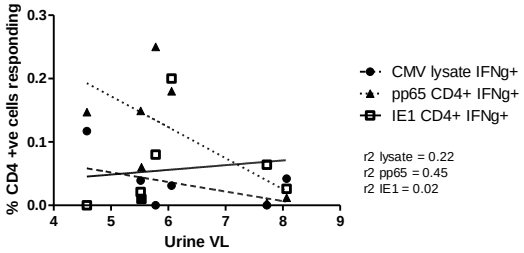
Figure 6-18 Salivary and urinary viral load and frequency of CMV-specific CD8+ (A) and CD4+ (B) lymphocytes at 6-12 months of age in 6 babies with congenital CMV infection following stimulation with CMV specific stimuli.

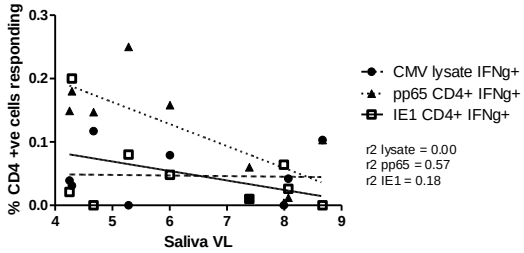
A. CD8+ lymphocyte responses





B. CD4+ lymphocyte responses

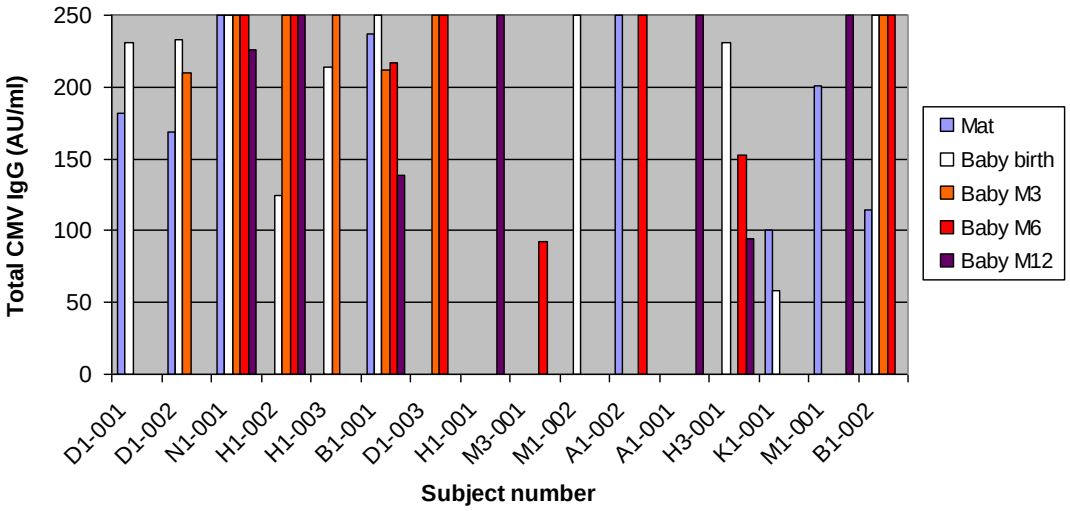




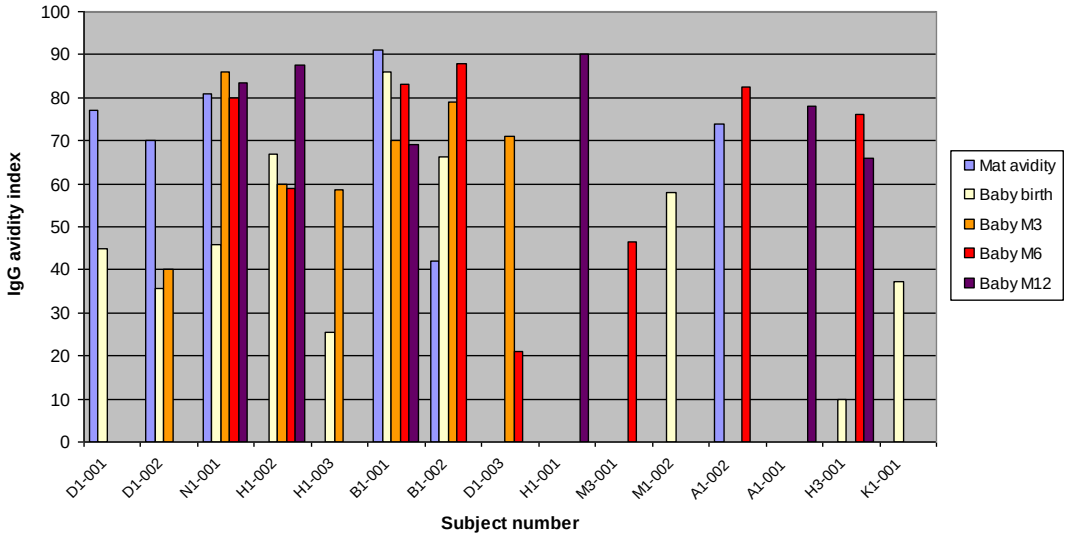
VL = viral load
Stimulation was with pooled peptides spanning the pp65 and IE1 proteins of CMV (A and B).
CD4+ lymphocytes were stimulated with whole cell CMV lysate (B)
In 3 HLA-A2 +ve subjects additional CD8+ responses were assessed following stimulation with HLA-A2 restricted pp65 (NLV) peptide (NLVpep) and frequency of MHC Class 1 HLA-A2 pp65 (NLV) restricted pentamer populations were measured (NLVpent).

Figure 6-19: Total CMV IgG (A) and CMV IgG (B) avidity measurement over time in 16 congenitally infected infants.

A. Total CMV IgG



B. CMV IgG avidity



Mat = maternal serology obtained closest to birth of baby
M3, M6 and M12 = age 3, 6 and 12 months

Measurements shown are for babies at different time points and, where available, the sample from their mother taken closest to the baby's birth.
Total CMV IgG was reported as a maximum value of >250IU and shown here as 250IU.
Avidity index is a percentage of total CMV IgG as described in more detail in Chapter 2.

Table 6-5: Table of total CMV IgG levels and avidity index in 7 mother and baby pairs.

All except one baby (J4-001) had confirmed congenital CMV infection; J4-001 had symptomatic, postnatally acquired, CMV infection.

Subject	Day of baby life	Mat IgG	Mat avidity index	Mat avidity (high/low/ equiv)	Day of baby life	Baby IgG	Baby avidity index	Baby avidity (high/low /equiv)
D1-001	-29	182	77	H	26	231	45	L
D1-002	-134	168	70	H	25	233.2	36	L
N1-001	-169	>250	81	H	6	>250	46	L
B1-001	-16	237	91	H	38	>250	86	H
M1-001	27	200	66	H	421	>250	Insuff	Insuff
A1-002	-10	>250	74	H	159	>250	83	H
K1-001	17	100	14	L	15	58.1	37	L
J4-001	196	102	94	H	196	168.5	NV	L
J4-001					405	140	42	L

H = high avidity
L = low avidity
Equiv = equivocal
Insuff = insufficient sample for analysis
NV = no value

9 congenitally infected babies had serum samples available from close to birth. 6/9 were found to have low CMV IgG avidity index, 2 high avidity and 1 equivocal measurements; by month 12 0/6 samples analysed had low avidity (Figure 6-19B). Of the 6 babies with initially low avidity around birth 2 had high avidity on subsequent measurement (M3 and M6), 1 had increased to become equivocal, 1 remained low; 2 babies had no further samples available for analysis.

Serum was available from 8 mother and baby pairs. 7 of these babies were congenitally infected. In congenitally infected cases 6/7 of mothers had CMV IgG avidity index >70 in the sample taken closest to baby's birth, consistent with infection more than 4 months previously (Table 6-5). In the related baby samples avidity index was <50 in 4; >70 in 2 and not quantifiable in 1 due to insufficient sample (Table 6-5). In all 4 samples with low avidity samples had been taken at <30 days of life; those with high avidity were acquired at day 38 and day 140. In the one baby with postnatal infection the maternal sample showed a high avidity index with a corresponding sample on the same day of life in the baby showing a low avidity. In both samples total measured CMV IgG levels were relatively low. A subsequent sample taken 7 months later in this baby, aged 405 days of life, revealed a similar total CMV IgG level and a continued low avidity index (42).

6.4. DISCUSSION

The aim of this study was to compare VL and cellular immunity between treated and untreated babies and those with and without symptoms. Understandably investigators found it easier to approach parents of babies that received treatment for recruitment into this study. There is therefore an over-representation of babies with CNS findings of CMV given that this was the main indication for antiviral treatment at the onset of this study. Only one baby had SNHL with no other findings of cCMV. This is consistent with current uncertainty regarding indications for treatment in CMV-infected infants with SNHL alone along with the difficulties in acquiring samples early enough in postnatal life to confirm congenital infection in otherwise asymptomatic neonates who 'fail' NHSP testing. The 9/16 (56%) of babies here that had some evidence of permanent neurodevelopmental or sensory impairment on follow-up at 2 years of age is also consistent with figures from other studies of babies with symptomatic infection at birth and is a reminder of the significant damage associated with this presentation.

It also became apparent during the course of the study that fewer neonatal units than anticipated were checking for CMV in the neonatal period thus decreasing the number of babies that could be enrolled in the postnatally infected group, as congenital infection could not be excluded. In spite of the small numbers of babies involved some interesting observations are still possible, particularly in those that received treatment.

6.4.1. *Viral load*

The undetectable viraemia in all but one sample obtained from babies not undergoing treatment for CMV disease is notable. Larger numbers would, however, be needed before any firm conclusions could be drawn from these data. Other authors have previously commented on the association between symptomatic disease and detectable viraemia (Barbi et al. 1996a;Boppana et al. 2005;Lanari et al. 2006). The association between VL and development of symptoms has not, however, been maintained when larger numbers of asymptomatic babies are included in analysis (Ross et al. 2009). The negative predictive value of having no CMV viraemia at birth, as in our study, does however seem to be of value in predicting good hearing outcomes in asymptomatic babies (Ross et al. 2009).

Others have reported a decrease in VL in both blood and urine with antiviral treatment for cCMV. Systematic follow-up of VL in different body fluids over prolonged periods and after treatment termination has not, however, previously been reported. The similarity in salivary and urinary VL both at baseline and in response to treatment is striking despite the potential limitations of salivary swab VL quantification in neonates when compared to sampling of other body fluids. Similarly rebound in VL measured in both urine and saliva was more marked than in blood, peaking at around 6 months of life. Both blood and saliva VL rebounded to levels not significantly different to those detected pre-treatment. In contrast, however, urine rebounded to a level significantly lower than baseline. These observations may have significant implications when considering treatment duration which will be explored in more detail, alongside results from earlier chapters, in the final chapter of this thesis.

Median $T_{1/2}$ in blood observed here of 2.4 days (IQR 1.9-3.3) is remarkably similar to that calculated in Chapter 4 (2.4 days; IQR 1.99-4.27). The $T_{1/2}$ calculated during

the first week of life of 2.1 days was not notably different from this value, although small numbers limit analysis. These values in blood are comparable to those calculated for CMV viral decline in HIV-infected and liver transplant patients described elsewhere (Emery et al. 1999). This would imply that viral dynamics, and thus ability to control CMV in the blood, in congenitally infected babies is similar to that observed in immunocompromised adult patient groups and is consistent with data relating to viral dynamics of HIV in children where likewise similar dynamics to adults have been described (Palumbo et al. 2007).

Median $T_{1/2}$ in urine was similarly 2.03 days (IQR 1.29-2.64), however, in contrast median $T_{1/2}$ calculated during days 1-7 of treatment in this body fluid was only 1.25 days (IQR 0.66-2.46). Median $T_{1/2}$ of viral decline in the 4 babies with sufficient salivary samples was also shorter at 1.49 days (IQR 1.38-2.37) although small numbers may evidently be biasing this analysis.

Despite the consistency of the results for $T_{1/2}$ obtained above it is important to remember the uncertainty regarding the validity of these calculations in this patient group. My observations that VL decreased spontaneously prior to treatment in the few cases where serial samples had been obtained alongside historical tissue culture data showing viral decline in the pre-treatment era and a lack of any other natural history studies undertaking multiple sampling in untreated infants all raise doubt as to whether virus in these babies is truly in steady state. It is difficult to justify multiple sampling in this patient group as unnecessary blood tests provide discomfort, raise further parental anxiety and offer no valid prognostic information based on our current knowledge. Possible strategies to address this will be discussed in the closing chapter of this thesis.

There are no published data to my knowledge of viral decline, measured using quantitative real-time PCR, in other body fluids. A number of observations would suggest different viral dynamics in urine and saliva compared to blood. These include the shorter $T_{1/2}$ following treatment initiation, higher starting VL and marked rebound up to 6 months of age (3 months after treatment completion) presented here along with the long periods of virus detection noted both here and by others (Noyola et al. 2000). There are no current data describing whether there is ongoing viral replication in the CNS or inner ear associated with the progressive clinical manifestations which are known to occur in these neonates. If viral replication is

ongoing and viral dynamics in these body compartments were shown to more closely reflect that of virus infecting the glandular and renal ductal epithelium than in blood then treatment focused on maintaining viral suppression in these body fluids could be proposed.

6.4.2. *Ganciclovir levels.*

Firstly, the difficulties in interpreting results of samples obtained outside formal, rigorously regulated, PK studies should be noted. The fact that many samples were received unseparated from sites and that the time between collection and processing of samples was not recorded introduces many possible biases in analysis.

Despite the low GCV levels observed for all subjects around 1/3 of babies had virus undetectable in blood at the end of treatment. This is comparable to the proportion having undetectable viraemia in the PK study described in Chapter 4 of this thesis whereby treatment was tailored to a specific AUC (Acosta et al. 2007). The low levels observed throughout this study may therefore be largely attributable to deterioration of GCV in these clinical samples as illustrated by studies of sample degradation mentioned in Chapter 3 (Figure 3-1) (Boulieu and Bleyzac 1994). It is therefore likely that levels measured in clinical samples, unless processed rapidly, do not accurately reflect "true" levels of drug exposure in these babies. Given that the doses of GCV and VGCV used in the babies studied here were generally those derived from previous PK/PD studies and that viral decline appears similar in both groups studied this is the most likely explanation. Alternatively, however, given that only 1/3 of babies in either study had undetectable VL at the end of the treatment it could be argued that the GCV dose thought to be optimal, from early dosing studies in severely affected neonates, is in reality insufficient in both these groups of babies. It is possible that these less severely ill neonates have better renal function and correspondingly higher GCV clearance than those from whom PK data were initially obtained. However, this does not fully explain the reason that few babies treated according to the targeted AUC approach in the more recent CASG study remained viraemic at the end of treatment. Further work regarding whether babies are receiving correctly targeted therapy needs further consideration whilst taking into account the known and frequent associated toxicities of antiviral agents.

6.4.3. *Cellular immunity*

- **T-lymphocyte phenotypic marker expression and CMV-specific lymphocyte populations**

The trend in decreasing CD4/CD8 ratios over time observed here was not statistically significant but is consistent with the decrease in CD4/CD8 ratios and decreasing CD4+, but not CD8+ lymphocytes, reported with age elsewhere in healthy newborns (Shearer et al. 2003;Thomas and Linch 1983). Shearer *et al* found 52% CD4+ cells at birth, decreasing to 46% over the first year of life and CD8+ cells decreasing from 18% to 17% over the same time period, comparatively lower frequencies than the 63% CD4+ cells reported here which decreased to 53% at age 6-12 months and CD8+ frequencies of 27%. An increase in total CD8+ T cell percentage in CMV infected neonates has also been noted by other authors (Pass et al. 1983a). Pass *et al* in their study of 48 cCMV infected neonates and 27 healthy controls found a significantly higher number of CD8+ T cells and a corresponding significantly lower CD4/CD8 T cell ratio in symptomatic, but not asymptomatic congenitally infected infants <1 year of age. Although we also report here a higher CD8+ T cell frequency the associated increase in CD4+ T cells led to the observation of a CD4/CD8 ratio comparable to healthy neonates. Due to the limited phenotypic markers being identified in this study I am unable to report on what lymphocyte subsets have been correspondingly decreased in the infants studied here. The lack of a control group of normal, age-matched neonates further limits drawing conclusions from these data.

The observation that CD57+/CD8+ lymphocytes were predominantly CD28- is in keeping with phenotypic analysis of CD8+ lymphocytes of healthy, CMV seropositive adults studied as controls, and by others (Weekes et al. 1999). My observation that a higher proportion of CMV-specific lymphocytes express CD57 would be in keeping with data suggesting that high levels of terminally differentiated, CD57+, lymphocytes are generated as a result of chronic antigenic stimulation such as in chronic viral infections and autoimmune disease as described in more detail in the introduction to this thesis (1.7.3) (Wang et al. 1997;Wang and Borysiewicz 1995). It is more intriguing why this correlation was not seen in our older group of CMV positive subjects. The CMV positive infants studied here had an overall frequency of CD8+ lymphocytes expressing CD57 similar to that seen in CMV negative adults. The lack of a similar association in older CMV-infected

adults may be explained by the high proportion of CD8+ lymphocytes dedicated to controlling CMV along with other factors coming into play during aging in those who have had chronic infection and immune stimulation with CMV over a period of many years (Pawelec et al. 2009).

The median frequency of 71% CD57+/NLVpentamer+/CD8+ T cells is also consistent with the elevated CD57+ cell populations observed by Marashi et al (2011) in CMV infected adult patients with common variable immune deficiency (CVID). Marashi reported higher proportions of CD57+ lymphocytes in those with active inflammation compared to those with no concurrent inflammatory disease. Although the data presented here do not allow comparison between symptomatic and asymptomatic babies the significantly increased CD57+ lymphocyte population in the symptomatic babies studied here would be consistent with the possibility that these babies also have ongoing inflammatory disease.

In the 3 HLA-A2+ subjects studied here the frequency of CD8+ lymphocytes recognising the immunodominant NLV peptide ranged from 0.02-1.7% (median 0.5%). Some response was noted at all time points in all 3 participants. This is in contrast to data presented by Hayashi *et al* reporting negligible CMV pp65-specific/CD8+ T cells in congenitally-infected HLA-A24+ infants (Hayashi et al. 2003). Although CMV-specific, NLV pentamer+ lymphocytes were detected in the neonates studied here Marashi *et al* (2011), using the same protocol and reagents, reported relatively higher mean frequencies of 4.8% in CVID patients with active inflammation; 1.4% in those without inflammation and 0.66% in healthy adults. Mattes *et al* (2008) in their study of CMV in adult renal transplant recipients similarly reported median CMV-specific CD8+ lymphocyte frequencies of 1.19% (0.27-5.2%) in those patients that developed viraemia in the post-transplant period; lower median frequencies of 0.5% (0.03-6.46%) were observed following resolution of viraemia. In contrast significantly lower CMV-specific CD8+ lymphocyte frequencies of 0.28% (0.02-3.35%) and 0.37% (0.01-6.92%) were observed in subjects that did not develop viraemia at days <50 and >50 post-transplant respectively. The frequency of CMV-specific lymphocytes reported in infants in my study is therefore most consistent with healthy adults in Marashi's CVID studies or in post-viraemic, recovering, adult transplant patients. This is in spite of the continued excretion of virus in urine and saliva and clinical progression known to

occur in infants with cCMV. The frequency of CMV-specific CD8+ lymphocytes could therefore be considered to be inappropriately low. The prolonged presence of virus in various body fluids in cCMV may thus be a function of inadequate CD8+ lymphocyte responses in infants with active infection.

In further contrast with findings in adult transplant and CVID patients there was no correlation between the frequency of CD8+ lymphocytes producing IFNg in response to stimulation with NLV-peptide and that of NLV pentamer+/CD8+ T cells (Mattes et al. 2008). Indeed although CMV-specific CD8+ lymphocytes were detected using MHC-Class I pentamers, corresponding cytokine responses on stimulation with matched (NLV) pp65 peptide were negligible. CMV infected neonates therefore not only have fewer numbers of CMV specific CD8+ T cells but these cells seem to be functionally impaired. This lack of association may be partly attributable to the small numbers of viraemic patients studied here when compared to data derived from adult patient groups. In support of this hypothesis the only subject that was viraemic at the time of sampling (D1-003 at M6) showed no IFNg response following stimulation with NLV-peptide and correspondingly only 0.016% NLV pentamer+/CD8+ lymphocytes (the lowest observed in the 8 samples analysed). More data in viraemic and non-viraemic infants is needed before this association can be fully excluded.

- **CD4 and CD8 T cell cytokine responses**

The high number of samples that were not fully acquired was partly due to the limited amount of blood available, compounded by the necessity to split samples into CD4+ and CD8+ aliquots to compensate for the limitations of 4-channel flow cytometry. The lower ratio of CD8+ lymphocytes compared to CD4+ also contributed to the higher proportion of CD8+ samples which were not completely acquired. Although interpretation of results needs to take this into account the selection of 50,000 G2 events was based on an ability to be able to detect infrequently (i.e. 1:1000) occurring events such as CMV-specific NLV pentamer+ lymphocyte populations. The acquisition of over 30,000 events in all but 2 samples (one of which was a no antigen control sample) therefore still enables valid comparison between samples.

My finding that SEB stimulation produced little CD4+ lymphocyte IFNg secretion in early postnatal life in the neonates studied here is in keeping with studies of non-specific T-cell activation in response to other antigens in young children which likewise have shown impaired IFNg production (Connell et al. 2010). As IFNg is central to the cell mediated response to chronic viral infection and the predominant cytokine secreted by CD4+ lymphocytes the absent responses witnessed in these younger CMV positive infants is of interest. Others in our lab, using similar assays in adults undergoing liver transplantation, have reported median frequencies of CMV-specific CD4+ cells producing IFNg, and IL2 in CMV-PCR positive patients of 0.4% (range 0.05-1.96%) and 0.07% (range 0.05-0.08%) respectively following stimulation with CMV-lysate (Nebbia et al. 2008). In patients who controlled CMV infection (CMV PCR-) cytokine production was significantly higher with median IFNg and IL2 responses of 0.97% (0.05-2.9%) and 0.32% (range 0.05-1.2%) respectively. The median IFNg and IL2 secretion observed following pp65 peptide stimulation at 6-12 months of age in the non-viraemic infants presented here (0.13% (0.0-0.25%) and 0.0% (0.0-0.19%) respectively) is therefore comparatively low. Other authors have also documented absent or decreased CD4+ IFNg responses in CMV-infected children aged <2 years (Hayashi et al. 2003;Pedron et al. 2011). The observation that this response increased over time and that IFNg emerged as the predominant cytokine secreted following CMV-specific antigen stimulation would be in keeping with the observation that primary CMV infections are characterised by mainly IFNg-secreting effector CD4+ T cells and that this response is diminished in young children. The lack of secretion of other cytokines in response to stimulation with CMV-specific antigens observed here is in contrast to the TNFa and IL2 secretion observed in response to SEB stimulation. In the context of prolonged virus detection in cCMV this observation would be in keeping with the emerging significance of polyfunctional T cells in enabling viral control (Nebbia et al. 2008). The high VL observed in cCMV infection along with the known immunomodulatory properties of CMV may all be contributing to the poor polyfunctional CD4+ T cell cytokine responses observed in these infants. Alternatively, the low responses may reflect immunological immaturity.

CD8+ responses were higher than those observed for CD4+ lymphocytes with median CD8+/IFNg cytokine responses of 0.16% (range 0.0-0.85%) reported in the

infants studied here. Although closer to figures reported in adults they remain comparatively low. In adult liver transplant patients CD8+/IFNg+ lymphocyte responses in patients having CMV DNA detectable by PCR at any time have been reported as 0.4% (range 0.05-2%) following pp65 peptide stimulation with no difference between these patients and those not experiencing DNAemia at any time point (Nebbia et al. 2008).

As reported in CMV-infected adults the infants studied here had maximal CD4+ and CD8+ cytokine responses in response to stimulation with pp65. Although Gibson *et al* found IE1 to be possibly more important as an antigenic determinant than pp65 in neonates it may be noteworthy that only one baby in their study population had symptomatic infection (Gibson et al. 2004). Somewhat surprisingly cytokine responses following stimulation with whole protein CMV lysate were minimal in my studies, with pooled pp65 or IE1 peptide stimulation leading to more efficient cytokine production. In further contrast to Gibson's findings, and that of others, I found no notable differences in responses following stimulation with pp65 and IE1 peptides between HLA-A2+ve and HLA-A2-ve participants. Given the small numbers of participants and the lack of asymptomatic controls it is not possible to draw too many conclusions from these data. However, in summary it would seem that congenitally infected neonates are mounting similar responses to immunosuppressed CMV positive adult groups but they have fewer CMV-specific CD8+ lymphocytes than found in these adult groups and the CD4+ and CD8+ lymphocytes are functionally impaired, with respect to cytokine secretion in response to CMV antigen.

It is interesting to note that in perinatal HIV infection, where viral set-point is also significantly higher than observed following primary infection in adulthood, immature T cell responses have similarly been observed (Sandberg et al. 2003). This may have relevance when considering further treatment studies in neonates and would justify the relevance of concomitant immunological monitoring alongside such studies as has been successfully employed for studies of perinatal HIV infection (www.pentatrials.org).

6.4.4. *CMV total IgG and IgG avidity*

There were few paired maternal and neonatal samples available for comparing CMV IgG avidity; in 3/5 of these pairs avidity was lower in the infants than in mothers. A previous study, conducted in term neonates, reported a higher mean IgG avidity in fetal cord blood of normal deliveries when compared to maternal placental samples leading the authors to conclude that high avidity IgG1 is preferentially transcytosed via the placental Fc receptor and aids suppression of viral replication in the placenta, preventing symptomatic fetal infectionIgG avidity in term neonates (Nozawa et al. 2009). If this was replicated in a larger cohort it would be intriguing to speculate why. One further study has reported on IgG avidity in premature babies, noting a correlation between high avidity anti-CMV-IgG and low CMV viral load (Fedorova et al. 2005). Only one postnatally infected, premature, baby was included in my study. A low IgG avidity was observed for a prolonged period of time in this neonate associated with hepatitis and pneumonitis. Although viraemia was not detectable by 6 months of age salivary VL continued to be detectable at 6.0 $\log_{10}$ at one year of age. As serum samples are relatively easy to obtain and process differences in IgG avidity in asymptomatic and symptomatic, congenitally infected neonates may warrant further investigation.

6.4.5. *Study limitations*

The small numbers of asymptomatic and postnatally infected babies recruited here limits meaningful comparison of data between patient groups. It is hoped that some of these deficiencies can be addressed by encouraging recruitment of asymptomatic/less symptomatic infants from other studies which are currently recruiting or in the process of being set-up. Ensuring that investigators are aware of the need for control data in order to inform treatment strategies for both symptomatic and asymptomatic infants will be key to encouraging recruitment into these groups. The need to separate samples taken for drug sampling as soon as possible after acquisition also needs reinforcing in future studies addressing this area.

The small numbers of participants and small sample sizes obtainable in neonates provided obvious limitations to the cellular immunology data presented here. My assay choice necessitated PBMC separation and stimulation of fresh blood samples further limiting eligible participants. This decision was made following observations

by others in our laboratory that thawed frozen PBMCs had diminished cytokine responses when compared to freshly extracted PBMCs (unpublished data). Such methodology is feasible when examining large numbers of patients attending one institution but may not be optimal when embarking on multicentre studies of uncommon diseases. However, the validity of freezing samples of lymphocytes in these neonates where only small samples are available in the knowledge that cytokine responses are also diminished could also be challenged. This clearly needs further thought when considering future multicentre collaborative efforts; the data presented here may correrspondingly inform future study protocols aiming to characterise the key factors in immunological control of cCMV and ultimately those associated with neurodevelopmental sequelae.

CHAPTER 7.

7. DISCUSSION

The aim of this thesis at the outset was to investigate the potential for modern virological methodology to define natural history of disease and to inform the design of trials. This chapter discusses the main conclusions of the varied studies presented here along with studies already in progress that have been informed by preliminary data from work in this thesis and suggested future work.

7.1. IMPLICATIONS OF DATA IN THIS THESIS FOR TREATMENT

When considering treatment efficacy, clinical benefit is obviously paramount. The outcomes of most interest in cCMV, namely hearing and neurological outcome, require follow-up of a number of years, however, in order to fully assess their impact.

Although the studies described in this thesis are unable to add to the current literature regarding long term clinical efficacy, the virological and PK data presented suggest that current treatment for cCMV might be inadequate with regards to dose, duration and monitoring and that VL measurements could be used as biomarkers to study this sytematically.

7.1.1. *Dose.*

Based on the PK and virological data presented here, and by others, it is proposed that standard doses of GCV (6mg/kg bd i.v.) and VGCV (16mg/kg bd) may not be sufficient in many congenitally infected babies. Although the VGCV dose now commonly used is based on a tightly controlled PK study, doses of up to 20mg/kg bd were needed in some babies to achieve the target AUC (Acosta et al. 2007). The higher amount of virus observed in congenitally infected infants along with the immune impairments documented both in my studies and elsewhere would also question whether higher target drug levels are indicated in infants. Certainly the reported variability in drug dose required to achieve similar AUC, along with possible differences in bioavailability where oral drug is being used might suggest that individualised dosing is necessary to provide maximal therapeuetic benefit in this age group (Acosta et al. 2007).

In light of the data presented here I would propose that if VL is not decreasing steadily, consideration should be made to increasing drug dose, particularly in babies with no renal impairment. Although further work is needed to evaluate validity of GCV levels taken in the clinical setting they might have a supporting role in such

circumstances to ensure that levels are not already in the high normal range particularly in the context of reports of higher C_{max} and AUC_{12} being associated with increased neutropenia (Kimberlin et al. 2008).

7.1.2. *Formulation*

Data presented here and published elsewhere indicate that GCV levels are more consistently sustained and AUC varies less over time following oral VGCV administration than with i.v. GCV (Kimberlin et al. 2008). However, in sick neonates concerns remain regarding ability to fully absorb oral drug and to ensure compliance. PK parameters of relevance for adequate drug exposure in brain parenchyma and the auditory system also remain uncertain. It remains an additional challenge to ensure that oral treatment is sufficiently closely monitored to ensure that adequate dosing is maintained throughout the treatment course. In light of the data in this thesis I would recommend initiating treatment with i.v. GCV, particularly in those with significant disease. Oral treatment could then subsequently be considered once a virological response has been observed. In less symptomatic babies, particularly those who might not historically have received treatment, oral dosing could be considered from the outset thus decreasing the risk of side effects associated with i.v. drug administration. Parents should be appropriately informed, however, of the limited evidence regarding the clinical efficacy of such an approach.

7.1.3. *Duration*

It is important to note that the 6 week treatment course, derived from the only RCT of treatment in cCMV, was not evidence-based but based on prior natural history data along with what was thought acceptable to parents when considering administration of an i.v. drug (Kimberlin et al. 2003). This study did, however, show improved outcome with treatment.

My studies and data presented elsewhere, however, clearly show that virological control is not achieved in the majority of babies with 6 weeks treatment. Regardless of underlying pathological processes longer treatment courses would potentially both decrease direct viral damage and enable normal immunological control to be established. This will be investigated further in a recently completed study comparing 6 weeks to 6 months treatment (CASG #112; NCT00466817).

7.1.4. *Drug Toxicity*

The toxicities reported in the UK CMV Registry are generally in keeping with those reported in other studies and rarely led to drug being discontinued. However, neutropenia with GCV administration was possibly less than expected from previous reports. Recent studies have correlated higher C_{max} and AUC_{12} with lower neutrophil counts which might further support concerns that the low levels and low toxicity found in my clinical studies represent true underdosing of drug in the clinical setting (Kimberlin et al. 2008).

Consistent with other studies, neutropenia was also reported less commonly with oral VGCV treatment in the clinical studies presented here which might further support its role in treating less severely affected infants (Kimberlin et al. 2008). It is possible that bone marrow toxicity correlates with the peak level of GCV while efficacy correlates with either AUC of concentration versus time, or time above a minimum value. This observation could, however, also be explained by the fact that many babies included in the studies presented here were having less than the now recommended dose of 16mg/kg bd VGCV and VGCV was often used later in the treatment course, when neutropenia has also been reported to be less frequent (Kimberlin et al. 2003).

7.1.5. *Therapeutic Drug Monitoring*

Despite the concerns regarding accuracy of samples taken in the clinical setting the study of UK GCV levels showed comparatively low levels in children compared to adults and similar sampling bias should be applicable to samples taken in both groups.

It is interesting to note the higher trough GCV levels observed following VGCV administration when compared to i.v. GCV. Although the recommended target C_{max} is lower following VGCV dosing (5.0mg/L) when compared to GCV (7.0mg/L) in these clinical studies similar C_{max} was achieved independent of drug used. The increasing GCV levels with increasing VGCV dose not only support the linear PK reported in published literature but also imply that these clinical levels are reflecting potentially clinically relevant changes in drug exposure.

Although early studies of treatment of cCMV showed an association between drug dose, virological response and clinical efficacy both my data and other recent PK/PD

studies of VGCV use have not been able to correlate measures of quantitative VL with any PK parameters (Kimberlin et al. 2008;Nigro, Scholz, & Bartmann 1994;Whitley et al. 1997).

7.2. NATURAL HISTORY OF VIRAL LOAD IN CONGENITAL CMV

In adults CMV VL is successfully used to govern decisions regarding both starting and stopping treatment (Atabani et al. 2012). This thesis provides detailed evaluation of virological response to treatment in younger children and the first calculations of viral dynamics of CMV in neonates undergoing antiviral treatment.

7.2.1. *Viral dynamics and virological response to treatment in neonates*

- **Blood**

Analysis of viral response to treatment in viraemic newborns presented here (16 from CASG #109 study (chapter 4) and 14 from the VICC study (chapter 6)) show that 63% (total 19/30; 10/16 and 9/14 respectively) still had detectable viraemia at the end of a standard 6 week treatment course. This is in contrast to studies in adult transplant recipients in whom the majority of patients, with comparable starting VL, have no virus detectable within 7 weeks of treatment (Mattes et al. 2005). Although all babies having undetectable viraemia at the end of treatment in the CASG #109 study had a lower starting VL at treatment onset this did not fully account for the high proportion remaining viraemic at the end of treatment. A nadir in viral decline was observed in both studies despite a good initial response to treatment, consistent with the 'levelling off', observed in classical models of virus decline (Figure 1-3). $T_{1/2}$ of virus decline in blood during the initial stages of treatment from both these studies are also almost identical (2.4 (IQR 1.9-4.5) and 2.4 (IQR 1.9-3.3) days respectively) and consistent with those observed in adults. This would imply similar virus dynamics in these early phases of treatment to those observed in immunocompromised adults, who exhibit more complete virological control when followed over a longer period.

Drug resistance or non-compliance are possible explanations for this observation, particularly given the low GCV levels also reported in my studies. Interestingly no published data to my knowledge have addressed compliance in adults or children. Non-compliance is unlikely in the studies presented here, however, given the

predominant use of i.v. GCV in clinical studies, the targeted dose approach used in CASG #109 and the fact that VL in the urine and saliva showed continued decline in response to treatment (see below). Data from adult studies and preliminary analysis of UL97 sequences in samples obtained during the VICC study would also suggest that drug resistance is not likely to be contributing significantly during the short courses of treatment described here (unpublished data).

The prolonged viraemia observed here might alternatively reflect continued virus production in 'sanctuary sites', with clearance of virus-infected cells into the blood stream, for example following granulocyte phagocytosis, accounting for persistent detection of CMV DNA. Given the increased neurological morbidity observed in cCMV compared to immunocompromised adults the CNS or auditory system would be the most obvious sources of such virus reservoirs, possibly explained by poor penetration of GCV into the CNS. An alternative explanation for this persistent viraemia might be virus production following reactivation from latency during cellular differentiation in these neonates, who are in a rapid phase of growth (Figure 7-1).

The data presented here also support other reports that viraemia is rarely detectable in asymptomatic babies, or those without CNS symptoms at birth.

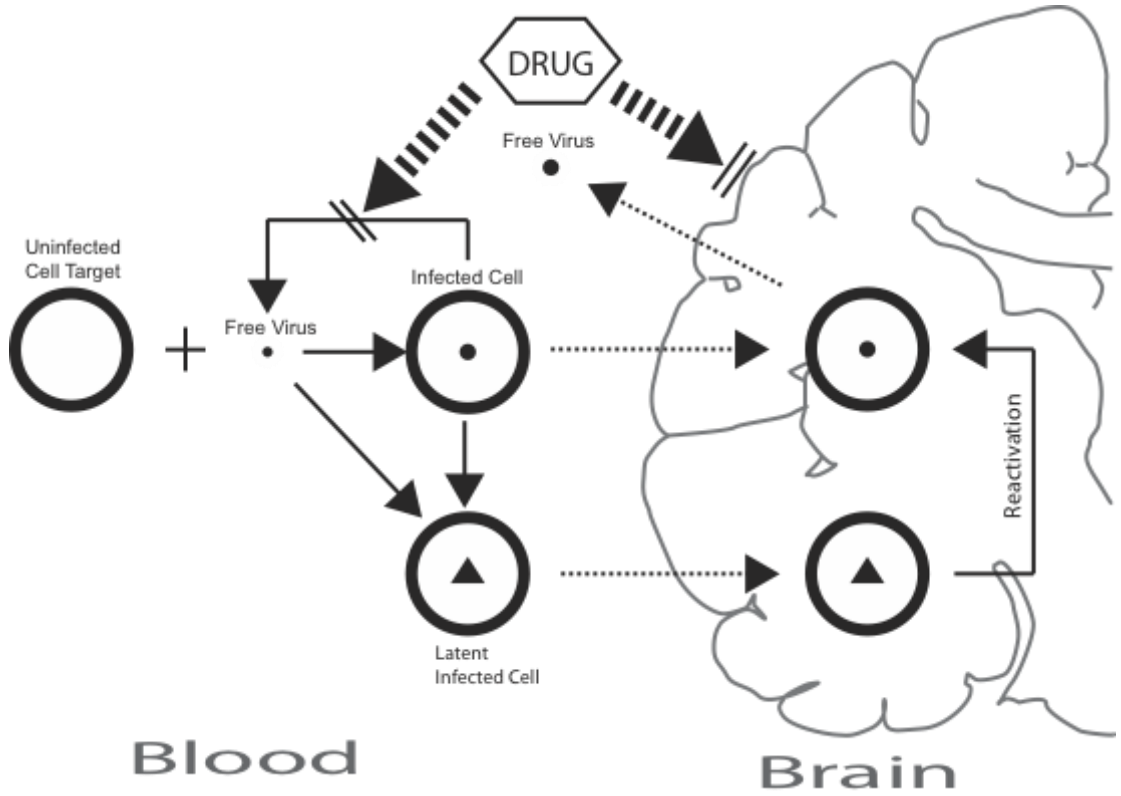
- **Urine**

Studies in adult renal transplant recipients have shown that viruria is an independent risk factor for CMV disease and prolonged virus excretion in this body fluid in infants corresponds with the ongoing pathogenesis observed over the first few years of life in cCMV (Cope et al. 1997b). A shorter $T_{1/2}$ was observed in urine in the VICC study compared to blood. No comparable $T_{1/2}$ data for urine have been published in adults.

- **Saliva**

A number of large cohort studies have reported on the benefits of using saliva for screening with benefits regarding ease of sample acquisition and decreased risk of leakage during transport compared to urine (Boppana et al. 2011;Yamamoto et al. 2006). None of these larger screening studies have commented on VL. The data presented here show that VL in saliva has similar dynamics to that in urine in a number of aspects with persistent detection over time and a shorter $T_{1/2}$ than blood.

Figure 7-1 Proposed model of ongoing viraemia in babies with congenital cytomegalovirus



The above cartoon shows a postulated mechanism for ongoing viraemia in babies with congenital cytomegalovirus.
Antiviral drug prevents infected cells from releasing free virus into the bloodstream but the same drug has poor penetration through the blood-brain-barrier leading to low efficacy of treatment of infected cells in the central nervous system (CNS). In addition to virus in the CNS not being killed it is possible that virus in latently infected cells within the CNS becomes reactivated, possibly during neuronal differentiation, to give rise to further productively infected cells. Virus from these sources is subsequently detectable in the blood following cell degradation or virus release.

There are no comparable quantitative data from adult patient cohorts.

7.2.2. *Viral rebound and subsequent control*

Rebound of virus to a level not significantly different from baseline was observed in blood in my studies, with a peak at around 3 months observed in the VICC study. Similarly saliva was also noted in the VICC study to rebound to a peak level not significantly different to baseline at 6 months of age. In contrast, rebound in urine was to a level significantly lower than baseline at around 6 months. In both the CASG #109 study and VICC study viraemia was becoming controlled without treatment by around 5-6 months of age. Virus was spontaneously decreasing in all body fluids by one year of age. It is noteworthy that this virological control corresponds with the emergence of CMV specific immune responses also described within this thesis.

7.2.3. *Virus persistence in cCMV*

Although the persistent detection of virus in urine of both congenitally and postnatally infected children is well described, the reasons for this observation are largely unknown. It may be that the ongoing detection of virus in urine and saliva reflects ongoing viral replication permitted by ineffective immunological control along with poor inhibition by antiviral drug activity specific to these sites. This is unlikely in the context of the short $T_{1/2}$ of viral decline observed in both urine and saliva compared to blood and the continued decrease in viruria observed during prolonged antiviral treatment in the VICC study.

An alternative explanation for virus persistence is that virus detectable in urine and saliva has itself adapted in some way to evade host immunological control in certain body compartments.

7.2.1. *Potential applications of salivary viral load findings*

My work in assessing the use of dry salivary swabs led to approval for funding of a targeted screening study (CMV BEST; NCT01162330). The purpose of this study was to evaluate the added benefit of detecting CMV in babies identified as having potential SNHL through the UK NHSP. This study has recently completed recruitment and showed that parents found it easier to obtain salivary swabs and that these are as sensitive and specific as urine for diagnosing cCMV (Williams et al.

2012). The acceptability of this sampling method offers potential for testing saliva as part of the NHSP in the UK to enable early confirmation of cCMV in children with hearing impairment. A larger study to assess feasibility of integrating salivary testing for CMV at the point of referral for further audiological investigation is shortly due to commence (CMV BEST 2).

- **Utility of saliva as a diagnostic/monitoring sample**

The ease of both acquisition and transport of salivary samples raises the possibility of monitoring VL in untreated symptomatic and asymptomatic babies in non-hospital settings to further define natural history in these groups particularly in those subsequently developing SNHL. Further data are needed, however, to determine the role of possible breast milk contamination in these samples and optimal sampling time following breast feeds.

7.3. RELEVANCE OF OBSERVATIONS HERE FOR CMV DISEASE

7.3.1. *Threshold concept*

Challenging the relevance of ongoing virus detection in the pathogenesis associated with cCMV is the prolonged viruria which has also been noted in postnatally acquired infection, with no convincing long-term morbidity, alongside the decreased duration of excretion noted in asymptomatic cCMV by some authors (Noyola et al. 2000;Stagno et al. 1975;Vollmer et al. 2004). Although higher VL in both urine and blood have also previously been reported to be associated with disease in cCMV it has not yet been possible to identify a critical 'threshold' associated with disease. Lack of viraemia has, however, consistently been shown to have good NPV for disease and the documented clinical response to only 6 weeks antiviral treatment might also support the benefits of lowering virus burden, albeit temporarily. The data presented here are the first to describe quantitative VL in urine over time in treated infants. It is therefore of note that only urine VL was shown to decrease significantly from baseline with treatment in my studies. Although other authors have reported decreased duration of excretion in symptomatic babies it is possible that peak viruria at 6 months or AUC might be of greater significance for development of sequelae during this critical period of neurological and audiological maturation.

This concept of a critical threshold of VL during the natural history of disease would also be consistent with the higher initial viral inoculum postulated to be received during congenital infection following direct transmission of virus into the bloodstream via the placenta. Such direct inoculation has been hypothesised to overwhelm host innate immune responses leading to increased susceptibility to organ damage. As postnatally infected babies most commonly acquire infection from breast milk there is not only time during primary amplification for immune mechanisms to be triggered but also for concomitant transmission of maternal antibodies, particularly if breast feeding is ongoing. Mucosal immunity and other innate responses might thus also have an important role in differentiating between HCMV pathogenesis following infection acquired via these two different routes.

7.4. IMMUNOLOGICAL CONTROL AND IMPLICATIONS FOR TREATMENT

Although the most important determinants of immunological control remain debated, clinically significant acute CMV disease is observed almost uniquely in those with impaired immunological function.

The data presented here give new information regarding evolving immune responses over time in babies with symptomatic cCMV. These data show that VL decreases, without antiviral treatment, at around the same time as CMV specific IFNg responses emerge. This impaired IFNg response is consistent with observations in other infections in neonates and is in keeping with the more severe disease often exhibited in this age group. The reported role of IFNg in promoting neural differentiation and neurogenesis might be of further relevance to the neurological damage observed in these chronically infected neonates (Gonzalez-Perez et al. 2012). In view of the emerging importance of polyfunctional T cell responses in gaining virological control in CMV and other chronic viral infections the relative lack of other cytokine responses is also of likely importance.

The relatively diminished CMV-specific pentamers and decreased production of TH1 cytokines observed in PBMCs here would challenge the suggestion by some authors that an overactive immunological response is responsible for the decreased duration of virus excretion observed in babies with SNHL attributable to cCMV compared to those that do not develop such sequelae (Caviness et al. 2013;Noyola et al. 2000).

The relevance of the increased CD57+ cells observed here is uncertain but would imply that there is certainly some ongoing antigenic stimulation leading to associated immunological recognition and response in these neonates. It is possible that immunological responses in the CNS and inner ear are distinct from those observed in PBMCs here. My observations suggest, however, that ongoing viral replication, reflected by prolonged virus detection in saliva and urine, are partly due to immature immunological responses to CMV in neonates. These diminished responses could help explain the ongoing audiological and cerebral pathogenesis also observed in cCMV.

7.5. OTHER WORK INFORMED BY THIS THESIS

7.5.1. *#109 analysis*

Work based on my analysis of the CASG #109 data informed the sampling protocol for the subsequent CASG study (CASG #112; NCT00466817) evaluating 6 months vs 6 weeks treatment with oral VGCV whose results are expected immenently.

7.5.2. *ECCI website and CMV Treatment Registry*

The European collaborations established during the work presented here generated interest in an application for FP-7 funding of work to define some of the unknown treatment parameters for CMV in the paediatric age group which reached the final review stages. An expansion of the Registry in its current form was incorporated into this application to further evaluate PK and PD of GCV in all children and preliminary data from work in this thesis formed a large part of the application. Furthermore the use of the Registry for ongoing pharmacovigilance, which is currently lacking for antiviral treatment in cCMV and considered increasingly important by the EMA, was a core component of this application (EMEA 2007).

A smaller application was subsequently made through other funding streams to support the role of the CMV treatment Registry and there is continued European interest in unified data sharing.

7.6. FURTHER WORK PROPOSED

One of the biggest unknowns when discussing treatment adequacy in this group of patients is what the short-term aims of treatment are. In cCMV not only is there a lack of correlation between levels of virus and adverse clinical outcomes but

therapeutic agents currently available have their own potentially significant morbidities.

Treatment studies offer a unique opportunity to simultaneously assess clinical response whilst also answering questions regarding natural history of disease and potential biomarkers. By incorporating virological and immunological measurements into study protocols our overall understanding of the pathological processes contributing to long-term damage and their potential reversibility may be further evaluated. In addition unified agreement with regards to what is considered to be 'symptomatic' disease at birth along with standardised VL measurements and recommendations for follow-up would lead to the possibility of combining data from smaller studies.

Obviously the availability of less toxic therapeutic agents would also shift the risks of treatment in favour of treating babies born with less obvious disease.

7.6.1. *New drug treatments/studies*

Newer antiviral therapies targeting different parts of the CMV life cycle are in early stages of development as summarised in a recent review (Emery and Milne 2011). Although EMEA/FDA initiatives will hopefully increase the likelihood of early data in paediatric groups the use of such treatments for cCMV are still likely decades away. When evaluating new treatments in the absence of reliable biomarkers the need for RCTs in neonates are paramount given the difficulties in assessing SNHL in this age group along with the known fluctuations in hearing thresholds which can lead to improvement in hearing independent of antiviral treatment. The difficulties in objectively assessing milder neurological involvement at birth also create potential bias when assessing treatment response in uncontrolled studies.

7.6.2. *Work to support treatment studies*

- **Standardisation of CMV VL**

Sending samples to a central laboratory for CMV PCR is increasingly untenable given current market constraints within the NHS. It is anticipated that the recent international standardisation of quantitative CMV PCR will facilitate further research relating to natural history of VL and their widespread adoption is eagerly awaited (Fryer et al. 2013). Areas where this would be of particular benefit are in evaluating

natural viral decline prior to treatment initiation and enabling comparisons of peak VL across sites in babies not entered into ethically approved trials, particularly with regards to defining a potential 'threshold' for treatment initiation. The data held within the CMV Registry would also have added value if standardisation is widely adopted across Europe.

- **Standardised definitions of symptomatic infection**

Inconsistent definition of 'symptomatic' infection at birth complicates interpretation of currently published data. It is likely that analysis of data is altered by including all babies with evidence of SNHL at birth in the 'symptomatic' group from the outset. A consistent definition of symptomatic and asymptomatic infection in neonates, similar to the consensus published for disease in infected adults would aid future collaborative studies (Ljungman, Griffiths, & Paya 2002).

The need for such definitions also has wider implications for defining which babies should be treated antenatally as obstetric practice often aims to treat *in utero* those babies anticipated to have disease warranting treatment postnatally. Furthermore The US Institute of Medicine has assigned the search for a cytomegalovirus vaccine as a level 1 priority with one of the main objectives being prevention of symptomatic congenital disease (Stratton et al. 2000). A standard definition of cCMV disease would therefore be of additional significance when moving forwards with future vaccine strategies. It is proposed that such work should be a focus of work for the ECCI group in coming years.

Whether carrying out neuroimaging such as MRI on all babies identified as having infection at birth might alter our understanding of those more likely to have future pathology is also currently uncertain. Neurological signs/symptoms are difficult to elucidate in the early postnatal period and it is possible that CNS disease is under-recognised at birth. It is therefore suggested that MRI should be incorporated into the protocol of any future studies identifying or evaluating 'asymptomatic' neonates. Large neonatal imaging studies assessing the prognostic value of MRI in babies with perinatal hypoxia (ePrime: NCT 01049594) might also have implications for rarer conditions such as cCMV and imaging techniques such as TBBS could warrant future investigation as a biomarker for disease.

- **Monitoring of different body fluids**

Data from adult studies suggests that analysis of clinically accessible samples mirrors that of target organs (Bowen et al. 1996;Cope et al. 1997b). However, which body fluid most closely reflects virus dynamics in relevant target organs in cCMV remains unknown and remains an important question to address when considering monitoring of these babies. Although monitoring viraemia has historically been considered of greatest importance the persistent virus detection in urine and saliva in cCMV and differences in virus dynamics described here might suggest that monitoring of these body fluids are of greater relevance in this patient group. Genetic sequencing and modelling to further define which body compartment detectable virus originates from, might assist in assessing the relative merits of monitoring of different body fluids and choosing drugs able to penetrate into any identified sanctuary sites.

It could also be informative to explore differences in natural history and virus dynamics between postnatally and congenitally infected infants.

7.6.3. *Maximising treatment efficacy with currently available preparations*

Until newer therapeutic options are available research should focus on maximising efficacy of agents currently available.

- **Pharmacokinetics**

The parameters affecting penetration of drug into the CSF and brain parenchyma are complex and not well described for GCV (Strazielle and Ghersi-Egea 2005). There are no studies of CSF or brain penetration of GCV in the paediatric age group and drug availability to this compartment might also differ in neonates due to differences in either the blood-brain barrier and/or transport systems involved. The most important body compartment to target for drug sampling, however, might be the inner ear and perilymph. Although being closely related to the CSF, it is likely that drug distribution in the cochlear and other clinically relevant parts of the audiovestibular system involves even more complex interactions. Modelling is currently being developed to further assist administration of other intratympanic treatments (Salt et al. 2008). It is tempting to speculate that differences in hearing outcome during treatment for cCMV result from factors relating to actual drug exposure in these sanctuary sites and experimental strategies addressing GCV delivery to the inner ear might be worth pursuing in future. Given that guinea pig

models have been used both for pioneering research into sampling of the intratympanic space and as models of cCMV hearing loss utilising these animal models could be considered as a first line of investigation. Sampling of perilymph at cochlear implantation might also be a means of obtaining such PK data in children. A single oral dose of VGCV administered prior to surgery might enable valuable *in vivo* data to be obtained although the ethics of such a proposal would need careful consideration. It is possible that advances in drug modelling will enable such data to be ultimately derived from other more easily measurable parameters in the future.

There has also been little/no research into the ability of different cell lines to phosphorylate GCV monophosphate to its di- and triphosphate and whether neonates have altered enzymatic maturity compared to adults. This might also affect drug efficacy and could be investigated further initially in *ex vivo* systems.

- **Formulation**

Some historical comparison between oral VGCV treatment and i.v. GCV will be possible from clinical outcomes in the recently completed CASG study of 6 weeks vs 6 months VGCV treatment in congenitally infected babies (NCT00466817) (Kimberlin et al. 2003). In the absence of a clinical trial directly comparing clinical outcomes in GCV compared to VGCV surrogate markers for treatment response and drug penetration into clinically relevant areas are even more urgently needed.

- **Duration**

Publication in 2014 of results of the above CASG study (NCT00466817) evaluating 6 weeks vs 6 months of treatment should inform the debate as to whether a more prolonged treatment course is of clinical benefit and whether any short term toxicities limit such treatment. If virological control in urine, rather than blood, is found to correlate with morbidity in cCMV then an even longer treatment course of up to one year would be indicated based on my data.

- **Therapeutic drug monitoring**

It would be informative to evaluate how delay in separating samples in the clinical setting affects reported levels returned. These data could be obtained by splitting adequately sized and timed samples obtained for clinical need into aliquots and separating serum at different time points after receipt prior to determining GCV

levels. If separation delay leads to the same decline in levels observed experimentally then clinicians and laboratories should be aware of the necessity for urgent processing and freezing of such samples to ensure validity of results.

As discussed earlier whether target levels for the neonatal group should be redefined warrants further consideration.

- **Pharmacovigilance and Development of the CMV Treatment Registry**

The use of ECCI as a platform for publishing guidelines to support good practice in treatment is ongoing and recent suggested management guidelines published by our group are currently accessible through this site (Kadambari et al. 2011).

The CMV Treatment Registry does not currently hold data on sufficient numbers of treated neonates or of sufficient duration of follow-up to allow for any comments regarding long-term toxicity. Collaboration with other European partners with cohorts of babies treated for cCMV is, however, planned which would expand this dataset. Data collection proformas have already been shared to facilitate uniform data collection whilst formalised funding is sought. The future amalgamation of these cohorts would further inform long-term safety in this patient group and provide a potentially powerful tool for ongoing pharmacovigilance as currently supported by EMEA guidance (EMEA 2007). There are currently no other initiatives planned, to our knowledge, to monitor these longer term toxicities of treatment making this an important longer term goal.

Preliminary data published by collaborators in Spain indicate that very different courses of treatment are being given in this European country (del Rosal et al. 2012); it will therefore be of further interest to compare and contrast data from these different cohorts.

7.6.4. *Treatment of asymptomatic babies*

The observation that $T_{1/2}$ in blood from two independent data sources in neonates are similar to values obtained in immunologically compromised adults might allow for correlates to be made with adult treatment strategies such as that of ‘pre-emptive’ therapy. It might therefore be rational to sample asymptomatic babies regularly and only initiate treatment if VL reaches a certain threshold. Although a recent study has shown a reasonable negative predictive value of a VL of 3500 CMV ge/ml, as

already discussed there is currently a lack of information regarding a VL 'threshold' for progression to disease in cCMV (Ross et al. 2009). Now that an oral drug preparation with an acceptable side effect profile is available such regular sampling in babies may be considered unacceptable compared to adopting a 'treat all' approach for those with any virus detectable at birth. The consistent finding in previous studies that having no virus detectable is associated with a decreased likelihood of CMV-associated disease would also support this strategy (Boppana et al. 2005;Lanari et al. 2006;Ross et al. 2009). It is evident that an RCT in babies without symptoms at birth is needed, particularly in light of the data presented here and of other publications which suggest that some clinicians are already opting to treat such babies, particularly if a high VL is found at presentation (Gandhi et al. 2010).

It is possible that combining parameters such as initial platelet count and neuroimaging with VL might assist in defining a group at 'lower risk' of long-term sequelae. Targeting treatment to babies not in this low risk group might be a feasible future proposal for a RCT in 'asymptomatic' babies. Costs of neuroimaging along with the costs of treating those not destined for any long-term impairment (both financial, drug-related and psychologically to families) would be needed before results of such studies could be adopted into routine clinical practice.

7.6.5. *How to address missing natural history data in untreated groups*

Although serial blood sampling would seem difficult to justify in this age group serial, daily, measurements of VL in saliva or urine in untreated babies would be feasible.

It is hoped that studies addressing other areas of cCMV research will increase the likelihood of recruiting asymptomatic babies (CMV BEST 2). Any RCT of treatment in asymptomatic babies will obviously offer valuable opportunities for contributing to natural history data in this group and would justify any possible parental anxiety associated with obtaining such data. Treatment studies which are currently in planning stages to evaluate treatment of older congenitally infected children might also contribute to natural history of VL in babies asymptomatic at birth, particularly regarding VL at the time of disease progression (NCT01649869).

7.6.6. *Parallel studies that could inform treatment strategies*

The reasons for continued virus production from sanctuary sites in the face of antiviral treatment are possibly key to maximising treatment efficacy in this age group. It is possible that this continued virus detection may be a function of altered drug exposure, for example across the blood-brain-barrier, to different levels of cellular enzymes needed to complete GCV tri-phosphorylation in different body fluids or to diminished immunological control in neonates. Further studies investigating these differences might be informative.

- **Compartmentalisation**

Coinfection with multiple different virus strains is well documented (Arav-Boger et al. 2002;Ross et al. 2011). Recent data in adults have interestingly shown differential viral decline dependent on gB genotype and with mixed compared to single genotype infection, however the role of different virus genotypes in clinical pathogenesis is as yet undefined (Emery et al. 2012).

UL144 is a TNF-a receptor homologue located in a hypervariable region of the CMV genome. Some authors have found that genotype C is more commonly found in symptomatic infection, although this has not been confirmed by others. Scrutinising UL144 genotype in different body compartments and comparing with TNF-a detectable in blood and/or CSF is of potential future interest.

The application of high-throughput sequencing to model evolution of virus populations within a particular body fluid/compartment might provide further insight into the origin of virus detectable in blood (Renzette et al. 2011). Such studies may also contribute to our understanding of the origin of virus responsible for persistence and rebound observed in cCMV.

Whether certain virus genotypes have a predilection for different body compartments is therefore of potential future interest and further work using samples obtained during my studies is in progress.

- **Immunological correlates**

It is tempting to postulate that in neonates, with their maturing immune systems, the high VL seen in primary infection pushes the balance in favour of virus causing symptomatic disease.

In light of conflicting results from my immunological studies and speculation from studies of duration of urinary CMV excretion in babies with and without SNHL further work to examine immune response in larger numbers of symptomatic and asymptomatic babies is warranted. Sampling of CSF and blood for immunological assays alongside VL at enrolment into treatment studies is suggested. Use of immunological techniques which allow for centralisation of samples is likely to be of greatest value in enabling inclusion of large enough numbers of babies for meaningful comparisons to be made despite the possible limitations of analysing frozen samples. An alternative would be shared protocols to process fresh samples in a number of large centres.

The low CMV Ig avidity noted at birth in symptomatic babies compared to their mothers is also of potential interest and warrants further investigation particularly in the context of the emerging use of antenatal immunoglobulin both within and outside of controlled trials. Monitoring such changes might also be of utility when considering future trials of CMV vaccine or therapeutic antibody in mothers.

- **Defining biomarkers**

The search for biomarkers remains a priority. Having a surrogate marker for poor clinical outcome would enable shortened follow-up periods in therapeutic studies. This would ultimately decrease funding needed for such studies and thus facilitate the conduct of RCT's in this patient group. It is hoped that results of some of the studies mentioned earlier in this discussion will add important information regarding such biomarkers, particularly in those initially defined as being asymptomatic. My observations of timing of peak VL and virological control should inform such future protocols and thus aid future evaluation of VL with clinical outcomes.

The natural history data presented here suggest that blood sampling at birth followed by 3 and 6 months to define peak VL and those remaining viraemic for a prolonged period might further inform the published literature on the relationship between VL and disease in asymptomatic babies.

The difference in virus dynamics in urine compared to blood might suggest greatest value in investigating this body fluid further as a biomarker for treatment efficacy. Additional work evaluating whether peak urinary or salivary VL, or AUC, is of relevance for likelihood of disease in these babies is also suggested.

In Hepatitis C infection in adults a better early viral response has been associated with need for shorter treatment duration to achieve a sustained virological response (Manns et al. 2006). More recently a similar correlation has been shown between first phase virus decline and $\log_{10}$ VL reduction in HCMV at day 21 of treatment in adult solid organ transplant recipients (Emery et al. 2012). Although data presented in Chapter 4 did not reveal any association between initial viral decline and neurological or audiological outcome further work involving larger numbers of babies to evaluate whether initial response to treatment correlates with subsequent clinical outcome might be of future value.

When considering the importance of VL and clinical symptoms further data to compare and contrast virus dynamics in postnatally and congenitally infected infants might also be of benefit. These data would not only assist in defining natural history in these two different groups but might also be informative for evaluating potential benefits of treatment in symptomatic postnatally infected neonates.

Given the individual differences in virus dynamics exhibited in different babies, identification of markers of immunological control may further enable treatment duration to be individually tailored to take into account differences in immune maturation. Use of a rapid IFNg capture assay (similar to that used to enhance diagnosis of TB) might thus be of benefit in monitoring immunological maturation in cCMV and in informing treatment duration. I would therefore suggest that future treatment studies could focus on levels of IFNg release following CMV stimulation and correlated with virological response to further evaluate this as a biomarker of interest.

I would propose that, in keeping with treatment of other viral infections in adults and children, it is likely that treatment will ultimately be tailored according to responses involving a number of the above biomarkers rather than follow a prescribed duration to maximise efficacy whilst minimising potential toxicity.

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APPENDICES

Appendix A. Letter requesting information from clinicians

PRINT ON TRUST HEADED PAPER

Dear Dr……..…..…. Date:……

Short study title: National Congenital CMV Registry

Study title: A National Registry of Babies, Infants and Children Under 5 Treated for Congenital Cytomegalovirus (CMV) Infection.

The parent/guardian of your patient ……..……..……has given their consent for him/her to be entered into this study.

This is a surveillance study of babies, infants and children receiving treatment for congenital CMV. Information will be entered regarding the above child's symptoms, treatment and blood test results onto a web-based database.

To ensure accurate data collection I would be grateful if you could complete the attached form relating to information that we may not have access to in our clinical notes. If you have already entered this baby's details onto the registry please can you let us know so that data is not duplicated.

Once the form is completed please return to Dr S. Luck, Dept of Virology, Royal Free Hospital, Rowland Hill Street, Hampstead, London, NW3 2PF.

A copy of the Patient Information Leaflet is enclosed for your perusal. Should you have any further questions relating to your patient's participation in this study, please contact myself or Dr Sue Luck: email sluck@doctors.org.uk or tel 0208 7253922.

Yours sincerely

Dr ……

Data from paediatricians for children with congenital CMV.

Child's name: _____ **Date of birth**:_____
Referring hospital: _____
Date of completion: _____

CLINICAL DETAILS
Gestation (completed wks)_____
Birth wt (g)_____ Wt cent _____ OFC (cm)_____ OFC cent _____

MICROBIOLOGICAL DIAGNOSIS
Specimen diagnosis made on — Blood / urine / saliva / NPA / serology / Guthrie
Post natal age taken (days) _____
CMV viral load on diagnosis? — Whole Blood / Plasma/ Urine / Not done / Other
CMV viral load value (if done) _____ copies/ml

SYMPTOMS & SIGNS
(see Appendix 1 for details)

SYMPTOMS	**YES**	**NO**	**N/K**	**Date Onset**	**Date Resolutn**	**DETAILS**
Symptoms at birth?						
Neurological						
Hepatitis						
Hepato- &/or spleno-megaly						
Pneumonitis						
Haematological						
Chorioretinitis						
IUGR (<0.4th cent)						
Skin (petechiae/purpura)						
Bowel disease						

ANTENATAL PERIOD
Was CMV diagnosed in mother antenatally? YES NO
Gestation diagnosed (completed wks) _____
Mother treated antenatally? YES NO
If yes (please circle) IVIG / HIG / ACICLOVIR / Other (specify) _____
Gestation treated _____

BABY
Indication for treatment — CNS symptoms / other symptoms / high viral load / other
(please specify) ……

Antiviral treatment received

	Ganciclovir	Valganciclovir	Foscarnet	Other (specify)
Date started				
Date finished				
Dose (mg/kg/dose)				
Dose freq.				
Route				

Dose adjustment during treatment (please note it is particularly important to fill in any changes if drug levels have been taken or serial viral loads done so that we can accurately draw conclusions from the data). Please continue on a separate page if more dose adjustments were made than space allows.

Drug	Date	Dose change	Drug	Date	Dose change

SIDE EFFECTS (See Appendix 2 for Definitions)

Date	Event	Dose of drug (mg/kg/day)	Action taken i.e. stopped, decreased,	Date resolved	Renal imp? Yes/ No

Was treatment stopped earlier than planned? Yes / No
If yes was this due to:- difficult IV access / Side effects / other factors (please state)
_____

DRUG LEVELS (if available)

Date	Level	Pre- or Post- dose (if post timing)	Renal imp at time of level? Yes/ No

Evidence of resistance? YES NO
If yes enclose details _____

VIRAL LOAD (If available)
Please continue on a separate page if more viral load results than space allows.

Date	Level Copies/ml	Specimen type (whole blood /plasma/urine)	Date	Level Copies/ml	Specimen type (whole blood /plasma/urine)

OUTCOME

Duration as in-patient _____

Follow-up planned? YES NO

If yes when _____

Thank you very much for your help in collecting this important data.
If you require any further information regarding this study please go to www.ecci.ac.uk or contact Dr S. Luck (Clinical Research Fellow): email sluck@doctors.org.uk or Tel 0208 725 3922.

Please return these forms to Dr S Luck, Dept Virology, Royal Free Hospital, Rowland Hill Street, Hampstead, London, NW3 2PF.

APPENDIX 1
Symptom definition
Neurological symptoms:-
Microcephaly,
Seizures,
Abnormal neuroimaging consistent with CMV (intracranial calcification, ventriculomegaly, abnormal periventricular hyperechogenicity, hyperechogenicity of lenticulostriatal vessels)
Abnormal CSF indices
Abnormal tone
Poor suck

Other symptoms: -
Thrombocytopenia - $\leq 100 \times 10^9$/L (100 000/mm^3)
Neutropenia $\leq 0.5 \times 10^9$ cells/L ($\leq$ 500cells/mm3)
ALT >100 IU/L
Abnormal renal function – raised creatinine above reference range (see below), decreased urine output (see below), decreased creatinine clearance.
Hepatomegaly or splenomegaly, measured 4cm below costal margin.

APPENDIX 2
Side effects
Thrombocytopenia $\leq 100 \times 10^9$ cells/L or decrease >50% from baseline
Neutropenia $\leq 0.5 \times 10^9$ cells/L ($\leq$ 500cells/mm3) or decrease >50% from baseline
Seizures
Renal impairment see below
Gastrointestinal disturbance
Hepatic impairment AST >540 IU/L (10X Upper limit normal) and increasing after treatment initiated.
ALT >150 IU/L and increasing after treatment initiated.

Renal impairment
Defined as serum creatinine

7 days old:	>2.5 mg/dL	=> 200 umol/L
7–60 days old:	>1.5 mg/dL	= > 130 umol/L
61–90 days old:	>1.2 mg/dL	= > 105 umol/L

Urine output decreased (<1ml/kg/hr for > 24hrs)
Creatinine clearance < 50% for age.

Appendix B. Letter requesting information from audiologists

PRINT ON HOSPITAL HEADED PAPER

Dear Dr……..…..… Date:…….

Short study title: National Congenital CMV Registry

Study title: A National Registry of Babies, Infants and Children Under 5 Treated for Congenital Cytomegalovirus (CMV) Infection.

The parent/guardian of your patient ……..……..……has given their consent for him/her to be entered into this study.

This is a surveillance study of babies, infants and children receiving treatment for congenital CMV. Information will be entered regarding the above child's symptoms, treatment and blood test results onto a web-based database.

To ensure accurate data collection I would be grateful if you could complete the attached form relating to hearing data that we may not have access to in our clinical notes.

Forms should then be returned to Dr S Luck, Dept of Virology, Royal Free Hospital, Rowland Hill Street, Hampstead, London, NW3 2PF.

If you have already entered this baby's details onto the registry please can you let us know so that data is not duplicated.

A copy of the Patient Information Leaflet is enclosed for your perusal. Should you have any further questions relating to your patient's participation in this study, please contact myself or Dr Sue Luck: email sluck@doctors.org.uk or tel 0208 7253922.

Yours sincerely

Dr ……

Hearing data for children with congenital CMV known to have hearing impairment.

Child's name: _____ **Date of birth**:_____
Referring hospital: _____
Date of completion: _____

Sensorineural Hearing Loss (SNHL)[1] detected? Yes / no / not recorded
(circle appropriate response)

Ear(s) affected? Right only / Left only / Both

Hearing loss detected on Newborn Hearing Screen?
Right: yes / no / not recorded
If yes, Oto-acoustic emissions present?
Yes / no

Left: yes / no / not recorded
If yes, Oto-acoustic emissions present?: yes / no

If No, age of onset of hearing loss (months)?
Right: **Left:**

Initial ABR thresholds (dBnHL)? **Date of assessment:**		
Please circle ac or bc[2]	Right (insert value)	Left (insert value)
Click *ac / bc*		
1KHz Tone pip/burst *ac / bc*		
0.5 KHz Tone pip/burst *ac / bc*		

Did the test results fit with auditory neuropathy/dysynchrony?[3]
Right: yes / no / not recorded **Left:** yes / no / not recorded

Current Severity/ audiometric pattern?[4]
Right: (circle appropriate response)
mild / mild-moderate / moderate / moderate-severe / severe / severe-profound / profound

high-frequency sloping/
low-frequency sloping/
mid-frequency/ flat

Left: (circle appropriate response)
mild / mild-moderate / moderate / moderate-severe / severe/ severe-profound / profound

high-frequency sloping/
low-frequency sloping
mid-frequency/ flat

If soundfield results only:
Mild / mild-moderate / moderate / moderate-severe / severe / severe-profound / profound
High frequency sloping / low frequency sloping/mid-frequency/flat

Has sensorineural hearing changed since birth?
If yes details e.g. has it progressed[5], improved[6], fluctuated[7] with time or treatment?
Right:
Yes / no ……
……

Left:
Yes / no……..
……

Most recent behavioural hearing thresholds? Date of assessment:			
Please circle ac / bc[2]	Right (value)	Left (value)	Soundfield (value)
0.5 KHz (dBHL) *ac / bc*			
1 KHz (dBHL) *ac / bc*			
2 KHz (dBHL) *ac / bc*			
4KHz (dBHL) *ac / bc*			
Other responses (please specify stimulus & sound intensity)			

Most recent ABR thresholds (dBnHL)?[2] **Date of assessment:**		
Please circle ac/bc[2]	Right (insert value)	Left (insert value)
Click *ac / bc*		
1KHz Tone pip/burst *ac / bc*		
0.5 KHz Tone pip/burst *ac / bc*		

Other causes of SNHL? Yes / no / not recorded
(If yes, details if known) .....

Any other information?
.....
.....

Notes
1. Defined as air conduction (ac) thresholds >20dBHL for pure tones, or >30dBnHL for clicks or tone pips on auditory brainstem response testing (ABR) when middle ear function was normal, and as bone conduction (bc) thresholds >20dBHL for pure tones, or > 30dBnHL for clicks or tone pips on ABR, when middle ear function was abnormal
2. Please provide **ac** thresholds if middle ear function was normal and **bc** thresholds if middle ear function was abnormal (using high frequency tympanometry in infants <4 months old and standard tympanometry in infants >4 months old).
3. Defined as absent or extremely abnormal ABR and normal oto-acoustic emissions.
4. Defined using British Society of Audiology criteria:
Mild: 21-40, Moderate: 41-70, Severe: 71-95, Profound: > 95dBHL
5. Progression: most recent audiometric threshold poorer than previous audiometric thresholds by > 10dBHL for at least two frequencies for that ear.
6. Improvement: most recent audiometric threshold better than previous audiometric thresholds by > 10dBHL for at least two frequencies for that ear.
7. Fluctuation: consecutive worsening and improvement in audiometric thresholds >10dBHL during follow-up period, (in the absence of otitis media with effusion).

Thank you very much for your help with collecting this important data.

Please return these forms to Dr S Luck, Dept Virology, Royal Free Hospital, Rowland Hill Street, Hampstead, London, NW3 2PF.

Appendix C. Sampling protocol

TREATED BABIES

DAY / MONTH OF STUDY	TREATMENT					POST TREATMENT			CHRONOLOGICAL AGE[d]			
	D0	D3	D7	D14	D28	D42 (STOP)	D3	D7	AGE 3 months	AGE 6 months	AGE 12 months	AGE 24 months
		+/- 1D	+/- 2D	+/- 2D	+/- 2D	-3D	+/- 1D	+/-2D	+/- 2wks	+/- 2wks	+/- 30D	+/- 30D
Viral load (urine & blood) [a]	X	X	X	X	X	X	X	X	X	X	X	X
Oral swab (saliva) viral load	X	X	X	X	X	X	X	X	X	X	X	X
Ganciclovir Level [b]		X	X (x2)	X	X	X (x2)						
HLA-Type [c]		X										
T-cell immunity	X			X	X	X			X	X	X	X
	+/-1wk			+/-1wk	+/-1wk	+/- 1wk			+/- 30D	+/- 30D	+/- 30D	+/- 30D
IgG Avidity	X								X	X	X	
Min volume of blood required	1.4ml	0.5ml	0.4ml	1.3ml	1.3ml	1.4ml	0.2ml	0.2ml	1.4ml	1.4ml	1.4ml	1.2ml
Max volume blood taken	4ml	1.2ml	0.9ml	3.7ml	3.7ml	3.9ml	0.5ml	0.5ml	4ml	6ml	6ml	5.5ml

a If babies continue treatment longer than 42 days alternate weekly viral load to be continued until 3months of age. If treated longer than 3months for monthly viral load until one year of age.
b On D7 and D42: GCV levels to be taken 1) 1hr post dose and 2) before next dose post-dose
VGCV levels take 1) 2hrs post-dose and 2) before next dose
At other time points levels to be taken at random time points with time recorded (these can deviate from times specified as long as not exceeding number of tests above).
Please ensure to fill in Ganciclovir level form and send with blood specimens.
If CSF is taken and sufficient specimen please send for GCV levels along with a corresponding blood level.
c HLA-type is only required if initial sample is HLA-A2 or HLA-B7 postitive on initial FACS analysis (researcher will contact you and request this specifically).
d Chronological age samples are separate to those obtained during treatment, and may occasionally coincide with timing of samples taken during treatment. It is not necessary to take repeat samples if a sample exists from within the time period specified. Eg treatment stopped at 10 weeks of age no 3 month needed.

Priority of samples: Viral load specimens to be taken first, followed by Ganciclovir levels, avidity and any extra blood for T-cell immunity.

UNTREATED SYMPTOMATIC AND UNSYMPTOMATIC CCMV BABIES

DAY / MONTH OF STUDY	Baseline	Age D14	Age D28	Month 3	Month 6	Month 12	Month 24
		+/-1 wk	+/- 1wk	+/- 2 wks	+/- 2 wks	+/- 30D	+/- 30D
Blood viral load	X	X	X	X	X	X	X
Urine viral load	X	X	X	X	X	X	X
Oral swab (saliva) viral load	X	X	X	X	X	X	X
HLA-Type[a]		X					
T-cell immunity	X		X	X (+/- 30D)	X (+/- 30D)	X	X
IgG Avidity	X			X	X	X	X
Min volume of blood required	1.4ml	0.4ml	1.2ml	1.4ml	1.4ml	1.4ml	1.4ml
Max volume taken	4ml	1ml	3.5ml	4ml	6ml	6ml	6ml

a HLA-type is only required if initial sample is HLA-A2 or HLA-B7 postitive on initial FACS analysis (researcher will contact you and request this specifically).

Priority of samples: Viral load specimens to be taken first, followed by Ganciclovir levels, avidity and any extra blood for T-cell immunity.

POSTNATALLY INFECTED BABIES (UNTREATED)

DAY / MONTH OF STUDY	Baseline	Day 28	Month 3	Month 6	Month 12	Month 24
		+/- 1 wk	+/- 2 wks	+/- 2 wks	+/- 30D	+/- 30D
Blood viral load [a]	X	X	X	X	X	X
Urine viral load	X	X	X	X	X	X
Oral swab (saliva) viral load	X	X	X	X	X	X
HLA-Type[b]		X				
T-cell immunity	X	X	X (+/-30D)	X (+/- 30D)	X	X
IgG Avidity	X		X	X	X	X
Min volume of blood required	1.4ml	1.4ml	1.4ml	1.4ml	1.4ml	1.4ml
Max volume taken	4ml	4ml	4ml	6ml	6ml	6ml

a Baseline should be taken and then time periods as appropriate to the babies age (i.e. if postnatal infection at age 10 weeks next sample will be Month 3).

b HLA-type is only required if initial sample is HLA-A2 or HLA-B7 postitive on initial FACS analysis
(researcher will contact clinician and request this specifically).

BLOOD VOLUMES REQUIRED & SPECIMENS

Assay	Sample	Min Amount	Amount requested
Viral load	EDTA	0.2ml	0.5ml
GCV levels	Serum (clotted blood)	0.1ml clotted (50µL serum)	0.2ml clotted (100µL serum)
PBMC	EDTA	1ml 1ml	3ml < 6 months 5ml > 6 months
HLA-typing	EDTA	0.2ml	0.5ml
IgG avidity	Serum (clotted blood)	0.2mL clotted (100µL serum)	0.5mL clotted (200µL serum)