

**The impact of advances in molecular virology on the clinical
epidemiology and management of central nervous system viral
infections in infants**

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Declaration

I, Seilesh Kadambari, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed: _____

Date: _____

Abstract

Molecular virological techniques have rapidly replaced cell culture as the diagnostic tool of choice across NHS laboratories. This thesis studies two of the commonest causes of CNS viral infections in infants less than 90 days of age to determine whether the introduction of Polymerase Chain Reaction (PCR) based techniques have impacted significantly on their management and epidemiology.

Congenital CMV (cCMV) is the commonest congenital infection in the UK and a leading cause of sensorineural hearing loss (SNHL). In the absence of a screening programme the great majority are detected in early childhood outside the time when antiviral therapy has been shown to be effective. This thesis describes a series of coordinated pilot studies that suggest that it is potentially feasible to integrate testing for cCMV using salivary PCR into the Newborn Hearing Screening Programme (NHSP) and start appropriate antiviral management within the first month of life. A programme of work that needs to be conducted before potential national implementation is also outlined. Conventional molecular techniques were used to characterise the variation of five genetic loci of CMV in congenitally infected infants to better understand the current and future medical interventions available.

Enterovirus (EV) infections most commonly infect young infants. PCR has been shown

to detect viruses with greater accuracy than cell culture. National data over a 10 year surveillance period were analysed to demonstrate a seven fold increase in viral meningo-encephalitis diagnosis rates associated with increased use of PCR. Enteroviruses accounted for 92% of all cases of viral meningo-encephalitis in those aged less than 90 days. The year on year increase in EV infections coincided with increasing PCR-based laboratory diagnosis, which accounted for 36% of reported cases in 2000 and 92% in 2011. These data provided the rationale for the design and implementation of a British Paediatric Surveillance Unit (BPSU) study characterising the burden of EV and human parechovirus meningitis in children aged less than 90 days.

This thesis supports the notion that advances in molecular virology can significantly inform the investigation and management of CNS viral infections (as exemplified by cCMV) and alter the apparent epidemiology (as exemplified by EV infections in infants less than 90 days old).

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Abbreviations

BPAIIG	British Paediatric Allergy, Immunology and Infection Group
BPSU	British Paediatric Surveillance Unit
DBS	Dried Blood Spot
CASG	Collaborative Antiviral Study Group
cCMV	Congenital Cytomegalovirus
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CT	Computerised Tomography
CTU	Clinical Trials Unit
DEAFF	Detection of early antigen fluorescent foci
DH	Department of Health
ECCI	European Congenital Cytomegalovirus Initiative
EV	Enterovirus
ESPID	European Society for Pediatric Infectious Diseases
GCV	Ganciclovir
HCMV	Human Cytomegalovirus
HIG	Human Immoglobulin
HFMD	Hand Foot and Mouth Disease
HPeV	Human Parechovirus
IQR	Interquartile range
IUGR	Intrauterine Growth Restriction
IV	Intravenous
MIC	Minimum inhibitory concentration
MRC	Medical Research Council
MRI	Magnetic Resonance Imaging
NDCS	National Deaf Children's Society
NPV	Negative Predictive Value
ORF	Open Reading Frames
PBMC	Peripheral Blood Monocyte
PCR	Polymerase Chain Reaction
PD	Pharmacodynamic
PHE	Public Health England
PK	Pharmacokinetic
PO	Oral
PPV	Positive Predictive Value
R&D	Research and Development
RCT	Randomised Controlled Trial
RT-PCR	Real-time polymerase chain reaction
SD	Standard Deviation
SNHL	Sensorineural Hearing Loss

Chapter 1

Introduction

1.1 Cell Culture v Polymerase Chain Reaction

Growing viruses in cell culture started in the 1950's gaining a Nobel prize for the discoverers and enabling production of live attenuated vaccines including against polio (1).

The use of cell culture to determine viral of specimens relies on growing live viruses to enable detection. Shell vial culture, where viral growth is measured by antigen detection, was shown to reduce the time to detect slowly growing viruses such as cytomegalovirus (CMV) to 24 hours and was adopted into clinical practice from the early 1980's (2,3). The gold standard in the diagnosis of congenital CMV infection has been viral culture of urine (4,5).

Enteroviruses (EV) are shed in the oropharynx and faeces and can be detected in blood, cerebrospinal fluid (CSF), urine, or tissues in cases of severe disease. Viral culture has been the "gold standard" for the diagnosis of EV infection in different clinical specimens such as faeces, throat swabs and CSF. Enteroviruses may be shed for weeks after the acute infection so isolation of the virus from stool needs to be interpreted with caution. Viral culture enables identification of the serotype causing disease and remains

an important epidemiological surveillance tool. Obtaining positive EV or CMV culture results often comes too late to influence clinical decision making.

CMV DNA detection using PCR is automated, rapid and reproducible (6). Real time PCR can be used to provide greater accuracy to detect CMV. CMV PCR, with the capacity for real time quantification of viral genomes, has been increasingly used in clinical practice since the early 2000's (2). A number of studies have shown that CMV PCR is of greater sensitivity compared to urine culture in immunosuppressed patients and solid organ transplant (SOT) recipients (2,7–10).

The sensitivity of cell culture for enteroviruses ranges between 53%-75% and they usually take 4-8 days to grow (11–14). A prospective national surveillance study conducted by de Louvois and colleagues between 1985-7 assessed the incidence of meningitis in infants (15). Viral infection was confirmed in only 4% (16/423) of meningitis cases in infants less than one month. However, 92/423 (22%) of CSF samples showed evidence of pleiocytosis (CSF white cell count $>20 \times 10^6/l$) but no pathogen was grown when using cell culture. A study conducted between 2011-2012 in three UK paediatric referral centres aimed to assess the aetiology of childhood meningitis (16). This study, conducted almost 30 years later, identified no aetiology in 35 out of 84 (42%) of children with pleiocytosis.

Real time PCR is replacing viral culture across laboratories as the diagnostic tool of choice to identify viruses (17–20). PCR methods only require small amounts of biological samples, can be used to test different samples using batched testing and deliver results within 24 hours of samples arriving in the laboratory. Real time PCR also allows quantitative analysis with, for example, comparison of fluorescence threshold

(C_T) values or determination of copy numbers relative to a standard curve of known concentrations.

PCR methodology was applied to detect EV soon after it was first developed (21,22). PCR based assays use a reverse transcription step to convert RNA to cDNA (RT-PCR) and are able to detect almost all EV serotypes because they share common genomic sequences. Several studies have shown that PCR is able to detect EVs with greater sensitivity rates ranging between 92 - 96% and specificity rates of 93 – 96% compared to viral culture (23–28). One study has shown that at least two thirds of all cerebrospinal fluid (CSF) specimens from meningitis patients who had negative results by cell culture had positive results by EV-PCR (16). RT-PCR is at least as sensitive and certainly more rapid than virus recovery in cell culture (29).

Congenital CMV and enteroviruses are both important viral causes of CNS infection in infants less than 90 days. This thesis will discuss how molecular techniques including PCR can be used to inform the management of cCMV and epidemiology of enterovirus infections in this age group.

1.2 Cytomegalovirus (CMV)

1.2.1 Epidemiology

Vertical transmission of CMV infection can occur through three main routes: (i) intrauterine; (ii) intrapartum and (iii) post-natal. Intrauterine transmission is the most important route as it may result in major neurological sequelae. Primary maternal infection, maternal reinfection with a different viral strain or reactivation of latent

maternal infection can all cause in utero transmission. Maternal CMV infection in seronegative women with primary infection during pregnancy is transplacentally transmitted to the fetus in 32% of cases (30). However, maternal to fetal transmission occurs in only 1% - 2% of seropositive cases which is either due to reactivation or reinfection with a new strain (30,31).

Congenital CMV (cCMV) is the most common congenital infection in the U.K. Data from studies conducted over 20 years ago show that approximately 0.3% of live births in the U.K are infected (32,33). Congenital CMV is the leading non genetic cause of sensorineural hearing loss (SNHL) (34,35). Approximately 12.7% of infected newborns are symptomatic at birth (34). Around 13.5% of infants who are asymptomatic then develop sequelae including SNHL in childhood (34). The most recent national prospective surveillance study in the U.K was conducted by the British Paediatric Surveillance Unit between 2001 – 2002 (36). This study only recorded 86 confirmed cases which gives a prevalence of 0.06 per 1000 live births. The authors of the BPSU study noted that challenges in case ascertainment arose due to difficulty in confirming congenital infection (obtaining samples within three weeks of life), diagnosing cases (the majority are asymptomatic and not reported) and differences in local unit protocols (some neonatal units screen all newborns on admission but most do not). Since 2001, there have been no further attempts over to define the incidence of cCMV in the U.K.

Antiviral treatment has been shown in a Randomised Control Trial to prevent further hearing loss and improve developmental outcomes when started in the first month of life in babies born with central nervous system (CNS) disease (37). At present in the UK testing for cCMV only occurs after a newborn is confirmed with hearing loss, and this typically occurs too late for treatment to be offered or effective. Testing saliva is

essentially 100% sensitive for detection of the virus but there is no NHS framework to take saliva samples from every baby at birth or for laboratories to efficiently process them. Integrating saliva testing for cCMV into the routine newborn hearing test would ensure that affected newborns could be treated within the time during which treatment is known to work. This will reduce the overall burden of childhood deafness (and developmental disability), and overall costs to the NHS.

1.2.2 Clinical disease

1.2.2.1 Symptomatic infection

The typical physical signs of symptomatic disease include blueberry muffin rash (due to extramedullary haematopoiesis), petechiae (associated with underlying thrombocytopenia), Intrauterine Growth Restriction (IUGR), microcephaly, hepatosplenomegaly and jaundice. Laboratory results are consistent with hepatic and reticuloendothelial involvement. Biochemical abnormalities can include conjugated hyperbilirubinaemia, thrombocytopenia and elevated hepatic transaminases in the majority of symptomatic newborns (38). CNS features include seizures, focal neurological findings, chorioretinitis, optic atrophy, pigmentary retinopathy and strabismus (39). Mortality is reported in 4-12% of those symptomatic at birth (38,40,41).

1.2.2.2 Sensorineural hearing loss

CMV related hearing loss can be either unilateral or bilateral and may be profound. SNHL most frequently affects young children coinciding with the critical period for speech and language development. SNHL has a lifelong impact on the individual, significantly impairs quality of life and adversely affects future life outcomes. The

SNHL caused by cCMV is often progressive, worsening through early childhood (42).

A retrospective Dutch study showed that CMV DNA was detected on Dried Blood Spot (DBS) of 8% (14/179) children aged between 3 – 5 years of age with permanent childhood hearing impairment and in 23% (9/39) who had profound hearing loss (43). Other retrospective cohort studies have reported rates between 15-40% (44–46). A systematic review of studies of children with cCMV showed that approximately 14% of children infected with cCMV will develop SNHL of some type and 3-5% will develop bilateral moderate to profound SNHL (47). The review found that 15-20% of all cases of bilateral to profound SNHL are due to cCMV.

The risk of SNHL is highest in newborns with symptomatic disease. However, approximately two thirds of the overall burden of CMV associated SNHL lies in the asymptomatic group due to their higher abundance in the population (48). A retrospective cohort study found rates of SNHL in 22-65% of babies with symptomatic CMV compared to 6-23% of those asymptomatic at birth (49). A meta-analysis of global rates of CMV related SNHL found overall hearing loss in around 35% compared to 11-12% of those born with symptomatic and asymptomatic cCMV at birth respectively (34).

1.2.2.3 Asymptomatic infection

Congenital CMV is most commonly asymptomatic. Approximately 10% of asymptomatic children will develop SNHL over the first 5–7 years of life (35). Hearing loss can be bilateral and is often progressive or with delayed onset therefore requiring prolonged audiological follow up (35). Because hearing is often normal at birth, only

50% of cases of SNHL caused by CMV are expected to be detected by neonatal hearing screening programmes (35). Asymptomatic newborns should therefore be regularly followed up till the age of 6 years to identify late onset hearing loss (50).

The NHSP is part of routine neonatal care in the U.K. and successfully screens over 95% of newborns in the UK. Integrating a targeted screening programme for cCMV into an already successful screening programme would be feasible, enable quality evaluation, rigorous follow up of infected newborns and enhance the diagnostic pathway along with potential cost savings. Integrating detection of congenital CMV through the NHSP would consolidate the link between screening, identification and management of the virus within standard NHS processes. Chapters 2 of this thesis proposes that no other viable screening options exist. Chapter 3 demonstrates that integrating testing for c CMV into the NHSP is effective.

1.2.3 Treatment

A recent survey of clinicians revealed significant variation amongst clinicians in the management of newborns with cCMV (Personal communication). I led a multidisciplinary team of audiologists, paediatricians and virologist to publish the first evidence based management guidelines to treat cCMV in 2011 (50). A RCT demonstrating 6 months of treatment using valganciclovir if started in the first month of life in symptomatic newborns was published in 2015 (37). Chapter 7 describes updated management guidelines that include data from this recently published RCT. The guidelines recommend that only symptomatic newborns are treated with valganciclovir if started in the first month of life. Guidance is also provided regarding safety monitoring and follow up of both symptomatic and asymptomatic newborns. It is

anticipated that these guidelines will be widely adopted across neonatal units in the UK and provide a useful template for clinicians to treat infants with symptomatic disease.

1.2.3.1 Antivirals in clinical use

Data from a recently completed placebo-controlled, double blind, randomized study comparing 6 weeks versus 6 months of valganciclovir conducted by the Collaborative Antiviral Study Group (CASG 112) were recently published (37). The primary objectives of the study were to compare hearing outcomes, safety profiles, assess neurological outcomes and monitor CMV viral loads in symptomatic neonates up to one month of life who received 6 weeks versus 6 months of VGCV. In total, 109 subjects were recruited between 2008 and 2011. The results showed superior language ($p=0.0046$) and receptive communication ($p=0.0031$) scores at 24 months in the 6-month treatment group compared with the 6-week group. During the first 6 weeks of treatment 19.3% of subjects experienced severe neutropenia ($<0.75 \times 10^9/L$). 21.3% of subjects between week 6 and month 6 who were treated with valganciclovir had severe neutropaenia compared to 26.5% who were on placebo ($p=0.6353$). The authors concluded that 6 months of oral valganciclovir treatment in symptomatic newborns, when started less than a month of age, improved audiological and neurodevelopmental outcomes to at least 2 years of age. Moreover, fewer participants had neutropaenia in the first 6 weeks of treatment with oral valganciclovir (19.3%) than compared to the previous RCT which evaluated ganciclovir efficacy (63%). No other phase III treatment trials are being conducted to assess treating cCMV.

1.2.3.2 Vaccine candidates

Development of a vaccine against cCMV infection is a major public health priority (51). Three phase II vaccine trials have been conducted in the last six years with promising results (52–54). The most recent vaccine trials have used glycoprotein B (gB) as a

vaccine target. A phase II RCT recruited 70 seronegative and 70 seropositive patients awaiting kidney or liver transplant (53). Patients received either gB vaccine with MF59 adjuvant or placebo, each given at baseline, 1 month and 6 months later. Glycoprotein B antibody titres were raised in both seronegative (geometric mean titre 12 537 (95% CI 6593–23 840) versus 86 (63–118) in recipients of placebo recipients; $p < 0.0001$) and seropositive (118 395; 64 503–217 272) versus 24 682 (17 909–34 017); $p < 0.0001$) recipients of vaccine. Seronegative recipients with seropositive donors had a shorter duration of viremia ($p = 0.0480$) and the number of days of treatment with ganciclovir or valganciclovir was reduced compared to placebo.

1.2.3.3 Polyclonal and monoclonal antibody studies

A recent randomised double blind placebo controlled trial enrolled 124 pregnant women with primary CMV infection between 5 – 26 weeks gestation (55). Women were randomised to receive either 100U/kg HIG or placebo every 4 weeks till 36 weeks or till CMV detection in the amniotic fluid. Treatment started within 6 weeks after the onset of infection. The rate of congenital infection was 30% in the treatment group compared to 44% in those who received placebo ($p = 0.13$). Moreover, obstetric adverse events (preterm delivery, IUGR, hepatitis cholestasis and postpartum eclampsia) occurred in 13% of the HIG group compared to only 2% in those receiving placebo. The authors concluded that HIG did not show any statistical difference in prevention of cCMV in 123 women who received either HIG or placebo (55).

1.2.3.4 Genetic variation and implications for medical intervention

Gaps in understanding the molecular epidemiology of cCMV act as one of the barriers to successfully implementing a vaccination programme to protect infants from congenital disease (56). A successful vaccine to protect against cCMV would need to consider the genotypic variation of CMV including in glycoprotein B gene. It is also unclear whether one strain of virus has a predilection for one body compartment over another strain. Distinct differences in CMV strains might occur between different compartments and could help explain variation in disease outcome or viral transmission. No data exist detailing whether the virus segregates with the patient or the body compartment.

Chapter 6 provides novel data which contributes to our understanding of the molecular epidemiology of therapeutic targets for cCMV including antiviral treatment, vaccine targets and regions for monoclonal antibody binding.

1.2.3.4.1 UL97

The Virology Immunity Congenital CMV (VICC) study started recruitment in 2008 across 9 tertiary paediatric centres in the UK (57). The primary objectives were to study the cellular immunity and viral loads in blood, saliva and urine of infants with cCMV. To date, this is the only known study to prospectively collect blood, saliva and urine samples in infected infants treated with 42 days ganciclovir therapy. The study showed that CMV viral loads in blood, saliva and urine fell at the point ganciclovir was started in 17 cCMV infected infants (57). However, a rebound in viral load was seen within 1 week of stopping treatment in 4 out of 8, 6 out of 9 and 1 out of 5 patients with blood, urine and saliva samples available respectively with corresponding median increase in

VL being 0.52, 1.03 and 2.05 log₁₀.

There had previously been no studies have yet evaluated whether the rebound in viral load after 42 days of therapy is due to mutations in the UL97 gene leading to resistance to ganciclovir. Results from the Virology Immunity Congenital CMV (VICC) study demonstrated a decline in CMV viral loads in blood, saliva and urine following 42 days treatment with ganciclovir which had been administered in 17 infected newborns. A rebound in viral load can be seen, particularly in the urine and saliva compartments, when treatment stops at day 42 (Figure 1).

Data presented in chapter 6 are the first to demonstrate that six weeks of treatment with ganciclovir does not frequently select for resistance in the UL97 gene. These results, in part, have helped to inform evidence based management guidelines described in chapter 7.

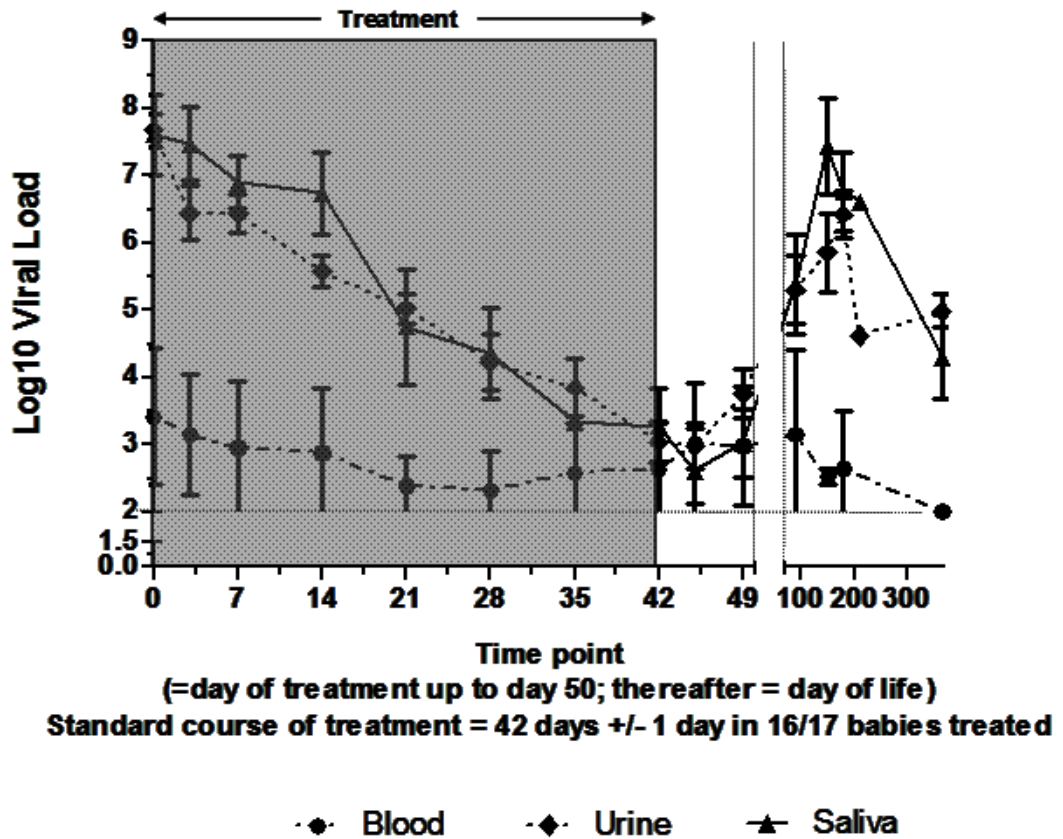


Figure 1: Mean viral load over time in different body fluids in 17 babies treated for congenital CMV demonstrating decline in viral loads upon starting treatment with ganciclovir and rebound recorded in each body compartment upon treatment stopping at day 42. Data obtained from Viral load and Immunity on Congenital CMV study with permission from Dr S. Luck.

(Error bars represent standard deviation).

1.2.3.4.2 *Glycoprotein B*

Glycoprotein B has been used in recent phase II trials in women of childbearing age and solid organ transplant recipients as a target for vaccines (53,54).

Glycoprotein B is a major envelope glycoprotein encoded by the UL55 gene, which is implicated in virus entry, cell to cell transmission and fusion of infected cells. It is a major target for both humoral and cellular immune responses. Four distinct genotypes (gB 1 – 4) have been identified in cCMV infected infants (58). Studies have shown co-infection with multiple strains occur (59). Studies in transplant patients have suggested that mixed gB genotypes may be associated with more severe clinical disease (60).

Chapter 6 shows that significant genetic variation exists in gB. These are important findings which would need to be considered in future vaccine development. Moreover, these results indicate infants may be infected with more than one strain of virus through transplacental passage.

1.2.3.4.3 *UL 128 – UL 131a*

The UL128-131a gene loci form a pentameric complex with gL and gH. The UL128-131a region is a major determinant of virus entry and replication into epithelial cells. The gH/gL/UL28-131a complex induces early, highly potent and long lasting neutralising antibody responses to control virus dissemination *in vivo* (61). Studies have shown a correlation between neutralising antibody that is targeted at the gH/gL/UL128-UL131a complex (62). The neutralising antibody response during primary infection has been directed against two or three gene products of the UL128-131a region (63). Sequence variation amongst different strains of CMV is explained due to the unusually

high variation of a subset of genes and may be a significant problem for developing antibody based programmes (64). Characterising the UL128-131a region is important in order to understand whether significant sequence variability exists which may make resistance to HIG therapy more likely.

Results outlined in chapter 6 show that there were no changes in the binding site for monoclonal antibody. This would suggest that there should not be any reduction in binding affinity between monoclonal antibody against the virus.

1.2.4 Diagnosis

One of the initial studies conducted by Demmler and colleagues showed that PCR using primers to immediate early and late genes was 93% sensitive and 100% specific when testing samples from congenitally infected newborns (65). Another of the earlier studies, by Warren and colleagues, found that PCR was 89.2% sensitive and 95.8% specific when compared to standard tissue culture of saliva samples (66). Higher sensitivities have been reported when regions with low genetic variability between strains have been targeted (67). Nested PCR is time intensive and carries a risk of contamination but has higher sensitivity rates. Urine can inhibit the enzymes used in PCR assays and consequently deliver false negative results.

1.2.4.1 Screening for cCMV

There are no ongoing studies or systematic reviews that have assessed the effectiveness of integrating cCMV testing into the Newborn Hearing Screening Programme (NHSP).

Identification of newborns with hearing loss but no other features of CMV disease would allow for enhanced audiological monitoring to detect hearing deficits early.

Language outcomes in infancy have demonstrably improved with hearing screening programmes (68). Prompt intervention of late onset hearing loss in early childhood, including early fitting of cochlear implants has been shown to improve language development (69). In the absence of routine testing at birth, the majority of asymptomatic newborns are missed so infection is only detected in early childhood when SNHL may be profound. Detecting cCMV in the newborn period could result in significant cost savings, avoid unnecessary diagnostic tests, minimise therapeutic interventions and provide parents with a cause for their newborns hearing loss (70).

There is currently no national screening programme to detect cCMV. At present in the UK testing for cCMV only occurs after a newborn is confirmed with hearing loss, and this typically occurs too late for treatment to be offered or effective.

Testing for cCMV using saliva by real time PCR is accurate, cheap, rapid and ensures prompt identification in the newborn thereby enabling antiviral treatment to be started in the first month. This will reduce the overall burden of childhood deafness (and developmental disability), and overall costs to the NHS. Integrating testing into the NHSP would enhance the diagnostic care pathway. It is estimated that an additional 113 newborns will be identified and treated with CMV related SNHL through integrating salivary testing into the NHSP (71).

Chapter 2 provides a comprehensive review of which strategies are available to test for cCMV and which future research areas need to be addressed before national implementation could be introduced (72). Chapters 3 and 4 attempt to provide data to answer some of the research questions proposed in chapter 2.

Chapter 3 describes the BEST 2 study assessed the feasibility of integrating testing for

cCMV using salivary swabs into the NHSP in south west London over 6 months (73). Parents of newborns <22 days old who were referred after their initial newborn hearing for further audiological testing, were approached by hearing screeners to obtain a saliva sample for CMV DNA PCR. Hearing screeners completed a five point Likert psychometric questionnaire to assess their opinions on the feasibility of cCMV testing through the NHSP. In total, 80% (203/255) newborns who were eligible had a saliva swab taken by the hearing screener. >99% results were delivered within the first month of life. Two newborns were identified with cCMV and both seen on day 10 of life by the paediatric specialist. All saliva samples tested delivered a result using real time PCR. All hearing screeners (20/20) stated that integrating testing for cCMV into the NHSP was a good idea and felt confident to perform this. This pilot study showed that it is feasible for hearing screeners to obtain saliva swabs to test for CMV DNA using real time PCR in newborns referred after their initial hearing screen.

Chapter 4 describes results from a national survey of members of the British Association Paediatricians of Audiology (BAPA) and British Association of Audiovestibular Physicians (BAAP) to determine the service hurdles in managing newborns with cCMV related SNHL. Audiovestibular physicians stated that the time taken to manage the extra newborns identified through testing would place undue pressure on their service. These results are important in understanding how to develop any potential national screening programme.

Chapter 5 reviews a novel approach to identify pCMV early in extremely preterm infants and using pre-emptive therapy to minimise adverse clinical outcomes. To my knowledge, this is the first published review to systematically describe how to identify and manage pCMV. Importantly, it also offers a unique approach to better detecting

pCMV on neonatal units and outlines where future research should be prioritised.

1.3 Enteroviruses

Conjugate vaccines against *Neisseria meningitidis* group C, *Streptococcus pneumoniae* and *Haemophilus influenzae type b* (Hib) have ensured that serious bacterial infections and bacterial meningitis are now a rare cause of disease in young infants and children (74–77).

Most cases of infant meningitis are now due to viral causes (78,79). Population based surveillance data published from the U.K show that mumps meningitis occurred in only 0.35% of all cases during 2002 – 2006 with the virus less commonly identified since two doses of vaccine was introduced to the universal immunisation schedule in 1996 (80). Approximately 85% of all cases of viral meningitis are now therefore caused by enteroviruses (EVs) (78).

There are few recent data in the UK that attempt to define the incidence, characterise the clinical burden or describe the epidemiology of EV infections in infants. The introduction of advanced molecular techniques to detect EV infections may have altered the clinical epidemiology of EV infections.

Chapter 8 of this thesis will describe longitudinal national data over a 10-year period obtained through the national communicable diseases database highlighting a seven-fold increase in laboratory confirmed reports of viral meningo-encephalitis. Chapter 9 provides a detailed analysis of epidemiological trends in EV infections in England and

Wales over a similar 12-year period and highlights the impact of increasing use of PCR EV disease trends. Chapter 10 outlines a British Paediatric Surveillance Unit (BPSU) study which aims to evaluate the clinical burden of EV meningitis in infants less than 90 days old.

1.3.1 Serotype diversity in the U.K

The last surveillance study conducted in the UK to characterise the molecular epidemiology of circulating EV serotypes was conducted over 20 years ago (81). This study analysed culture positive laboratory reports between 1975 and 1994 and demonstrated that Coxsackie A9, E7, E9, E11, E19 and E30 accounted for 70% all culture positive results. Chapter 9 will provide contemporary national data describing trends in EV reporting and circulating serotypes. These data show no particular predominance of any strain which may reflect the increase in PCR use to identify EV. Knowledge of current circulating serotypes is important in identifying any association between serotype and phenotype, designing future treatment trials and monitoring disease outbreaks.

1.3.2 Clinical disease

The spread of EV occurs predominantly through faecal-oral spread. Poliovirus infections and poliomyelitis have become exceedingly rare in most developed countries as a result of routine immunisation programmes (82,83). The World Health Organization (WHO) regions of the Americas and the Western Pacific were certified polio-free in 1994 and 2000, respectively. Europe has been declared free of polio since 2002. No cases of Polio virus 2 have been recorded since 1999 (84). No cases of Polio 3 have been reported since November 2012 (85). A recent report stated that global

eradication will be achieved if safety is guaranteed to healthcare workers delivering vaccine programmes, maintaining government commitment and continued surveillance (86).

Non Polio EV's are a major cause of infection in children, with up to 10 to 30 million infections annually in neonates and young infants (87–89).

EVs replicate in the respiratory tract, small intestine and several other secondary organs. Most EVs cause only mild disease including low grade fever, self-limiting rash or general malaise. In some instances, the disease manifestations can be more severe and include acute flaccid paralysis, myocarditis, meningitis, encephalitis and severe generalised infection in the newborn (90).

1.3.2.1 EV meningitis

Diagnosis on clinical grounds is difficult to establish in neonates. EV meningitis in infants is often accompanied by involvement of other organ systems (91). Fever and irritability are the most common features and nuchal rigidity rare (92).

CSF pleocytosis is an uncommon finding in infants with EV meningitis. A study in South Korea showed that 68%-77% of neonates did not have any evidence of CSF pleocytosis (93). Other studies have shown pleocytosis to be absent in 22.3% and 50% of all cases of EV meningitis in infants less than a month old (94,95). CSF pleocytosis is therefore an unreliable marker of EV meningitis in infants. The lack of pleocytosis may be due to immunological immaturity in neonates or the short duration between obtaining CSF and disease onset.

Chapter 8 provides the first national longitudinal surveillance study of viral meningitis trends for over 20 years. The study highlights that EV causes over 90% of all viral meningo-encephalitis cases in infants less than 90 days old in England and Wales. These data outline which areas of research should be prioritised in order to better understand this condition.

1.3.2.2 Neonatal sepsis syndrome

A prospective, population-based survey in Finland of neonates (up to day 29 of life) presenting with suspected systemic infection found that at least 3% of these episodes had been caused by EV's which was the same proportion diagnosed with bacterial sepsis during the same period (96). Risk factors for serious disease include onset in the first few days of life, maternal illness before the or at delivery, absence of

transplacentally acquired neutralising antibody, prematurity, severe hepatic dysfunction and infection with echovirus 11 or coxsackie B viruses (97,98). Factors associated with infection may also include lower socioeconomic status, lack of breastfeeding and low haemoglobin (99). The most common presenting features associated with neonatal EV infection are fever, irritability, poor feeding and lethargy (100,101). Approximately half of the infants with neonatal EV infection have evidence of hepatitis or jaundice during the course of the illness, while hepatomegaly is detected in around 20% (102). Hepatitis is more often associated with Echoviruses (11, 6, 7, 9, 14, 17, 19, 21) which can lead to persistent hepatic dysfunction and intrahepatic calcification (97).

1.3.2.3 Long term clinical outcomes

Studies conducted over 20 years ago have reported overall mortality rates ranging between 0% and 42% (100,102–104). Neurological, cognitive and developmental delay have all been evaluated in follow up studies of infants with EV meningitis (105–108). The outcomes in each of these studies show conflicting results. Sells and colleagues showed that infants with viral meningitis less than 12 months of age had smaller head circumferences, lower IQ's and delayed language at 1 and 6 years after the infection than compared to controls (105). However, studies by Farmer of infants affected by coxsackie B5 infection, Bergman of infants less than 12 months with viral meningitis and Rorabaugh of infants with aseptic meningitis all showed no neurodevelopmental or language delays at one year of follow up (106–108). Each of these studies were conducted over 20 years ago and used crude developmental tools, tested at inconsistent times after the acute illness and evaluated small numbers of infants with EV meningitis caused by different serotypes.

The most recent prospective study to assess developmental outcomes in infants less than 90 days with EV meningitis was conducted in 1995 in the USA (109). Sixteen infants were enrolled in a case controlled study that included cognitive, developmental, speech and language assessments conducted by paediatricians and speech and language therapists. The study showed that affected infants only had subtle delays in receptive language at 3 years of age.

Chapter 10 will describe the first prospective study in the UK to evaluate the burden and outcomes in infants with EV meningitis. This will provide the largest dataset in Europe and aims to evaluate the incidence, clinical features, laboratory markers and clinical

outcomes in infants less than 90 days.

1.3.3 Treatment

The mainstay of management remains supportive treatment, which may include mechanical ventilation, inotropic support, extracorporeal membrane oxygenation, cardiac pacemakers and organ transplants in the most severe cases.

1.3.3.1 Antivirals

Developing antivirals against EV has not been a priority for the pharmaceutical industry. The biggest EV disease burden in South East Asia is caused by EV 71. However, potential revenue from marketing EV71 antivirals may be limited in these resource-deprived settings. Manufacturing antivirals against RNA viruses needs to take into account their high mutation rate (110). The need for multiple drug agents to act on different viral targets to minimise resistance and limited cross genotypic activity further increases the challenge to successfully trial new antivirals.

Pleconaril had been shown to inhibit the replication of some enterovirus serotypes and rhinoviruses (111). The drug inhibits the uncoating of viral RNA and progeny virions during EV replication. Pleconaril has also been shown to have good oral bioavailability. A double blind placebo controlled randomised controlled trial of pleconaril in infants up to 12 months with suspected EV meningitis showed no clear evidence of efficacy and led to early termination of the trial by the pharmaceutical sponsor (112). Infants were randomised to receive pleconaril (5mg/kg/dose) orally three times/day for 7 days or placebo. Twelve subject received pleconaril and 8 placebo. There were no differences in symptoms or length of hospital stay in either group. Overall, 10/12 (83%) in the pleconaril group and 6/9 (75%) in the placebo group had adverse events which most

commonly included diarrhoea rash and fever.

1.3.3.2 Immunoglobulin (IVIG)

Intravenous immunoglobulin preparations contain neutralising antibodies to EV's. One study showed that administering IVIG prophylactically in all neonates on a neonatal unit prevented the spread of echovirus 11 beyond the index case (113) and prevention of echovirus 6 in another neonatal outbreak (114).

There have been case reports of IVIG improving clinical outcomes in neonates with severe EV meningitis (99,115). However, the only blinded randomised study conducted in neonates with enterovirus meningitis infection showed no statistical difference in clinical outcome with IVIG (116). Sixteen neonates less than 2 weeks of age and their mothers were enrolled. Randomised administration of IVIG (750mg/kg) to 9 neonates did not increase neutralisation titers significantly or reduce the daily incidence of viraemia or viruria compared to controls (116).

1.3.4 Diagnosis

1.3.4.1 PCR

Several studies have shown a high sensitivity ranging between 67% - 100% of EV specific PCR assays in comparison to cell culture (116–120) and specifically a statistically significant improvement in detection of EV in CSF samples to diagnose CNS disease using PCR compared to culture (102,121,122). One step RT-PCR assays allow for reverse transcription and DNA amplification in a single, closed reaction tube with real time detection of amplicons. One step assays have the added advantage of requiring minimal handling of genetic content so reducing the risk of any contamination. Several studies have shown high sensitivity and specificity rates using a one-step assay (13,123–126).

1.3.4.2 Comparing Non Polio EV RT-PCR and cell culture

The first prospective multicentre study comparing the detection rates of EV and Human Parechovirus (previously known as Echovirus 22 and Echovirus 23) using PCR and cell culture in different specimens (CSF, stool, blood, NPA and urine) of paediatric patients was published recently (127). A total of 296 nasopharynx, 281 stool, 250 urine, 189 blood and 141 CSF specimens were available for PCR analysis. 146 nasopharynx, 159 stool and 80 CSF specimens were available for cell culture. EV was detected in 140 (49%) and Human Parechovirus (HPeV) in 44 (15%) children. Both EV and HPeV real time PCR had a higher sensitivity and negative predictive value than EV and HPeV viral culture respectively. EV and HPeV PCR in stool specimens had the highest sensitivity (99.2% and 95.1%) of all specimens. An EV was detected in all positive patients if RT-PCR was performed on both faeces and CSF samples or in both faeces

and urine samples.

EV replication in the gut can persist for several weeks. Indeed, a number of studies have shown that the EV load is higher in faecal samples than CSF during EV meningitis (128–130) suggesting that faecal samples should be submitted for EV testing in symptomatic patients to increase the diagnostic yield as they are a good target for RNA detection. This study also showed that HPeV has a lower detection rate in culture compared to EV, which could be due to poor cultivability of certain HPeV serotypes, sample quality and low number of viral particles in the specimen at the time of sampling.

One of the few national surveillance studies looking at long term trends in enterovirus infection was conducted in South Korea (131). The study team evaluated clinical and epidemiological disease during 1999 – 2011 which covered a period where three different detection methods were used (1999-2004 phase I cell culture; 2005 – 2008 phase II RT-PCR was introduced; 2008 – 2011 phase III real time RT-PCR). During 1999-2005 genotyping was detected using neutralisation assays and since 2005 sequencing of the VP1 region was used. Cell culture (phase I) detected 20.5% of all EV infections, the introduction of RT-PCR (phase II) detected 26.4% and real time RT-PCR (phase III) 39.2% of EV was detected. During the 13 year period, 44 different genotypes were detected among 3,128 EV positive samples. The 5 main genotypes were EV 71, E 30, CB5, E6 and CB2 accounting for 14.9%, 12.5%, 9.3%, 8.4% and 6% of the total EV infections respectively. The authors note that the increase in number of reports over the 13 year study period is due to more sensitive molecular assays being used to replace cell culture and also due to a number of outbreaks.

Data from chapter 9 was obtained by interrogating the national communicable diseases

database. These data show that EV was identified using PCR in 36% in 2001 and rose to 92% in 2011. This is the first study to highlight that changing diagnostic practices have altered the apparent epidemiology of EV infections nationally. Notably, the increased use of sensitive molecular assays have led to a year on year increase in EV infections. The greatest burden of disease was in infants less than 90 days who accounted for almost one quarter of all reported cases across. These data have been used to inform a BPSU study which will aim to define the clinical burden of EV meningitis in the UK and is outlined in chapter 10.

1.3.4.3 Impact of PCR on clinical practice

Diagnosing EV early can have a clinical and economic impact as this potentially can lead to stopping unnecessary antimicrobial drugs, minimising extensive investigations and reducing the length of hospital stay.

A recent study in France compared the impact of PCR in managing patients during two separate outbreaks of E30 in 2000 and 2005 in a single tertiary unit (132). Molecular analysis of the genetic strains and the patient demographics (age and gender) during both outbreaks were similar thus implying that management of patients should not have varied. However, PCR was used during the 2005 outbreak and was shown to reduce hospital length of stay from 5.4 days to 2.2 days at a cost saving of 322 000 Euros. Results of PCR assays were made available within 1.9 days of receipt of the sample. In 2000, cell cultures inoculated with CSF samples delivered a positive result in 10 days and negative result in 14 days at the earliest.

The largest study evaluating the effect of an EV CSF PCR result on length of stay in infants under 90 days of life was conducted over 6 enteroviral seasons in a single tertiary US centre (133). The authors showed that the length of stay was shorter where results were known before discharge. Moreover, a positive PCR result led to a 1.5 day decrease in length of stay and 33.7% shorter duration of antibiotic use but a delay in delivering the result by 24 hours increased the length of stay by 13.6%.

1.4 Summary

Section 1 of this thesis considers how PCR can be used to better diagnose and manage infants with cCMV. This section describes a prospective clinical cohort study evaluating the feasibility of targeted testing through the Newborn Hearing Screening Programme (NHSP) and outlines strategies to implement national screening. This section also describes a series of experiments which use PCR to define the genetic intervention of therapeutic interventions available to manage cCMV. Finally, evidence based guidelines to better diagnose and manage infants with cCMV will be outlined. Data presented in section 1 will test the hypothesis that advances in molecular virology can significantly inform the investigation and management of CNS viral infections as exemplified by congenital CMV.

Understanding how the introduction of molecular diagnostic assays may have influenced the epidemiology of EV infection is important in order to prioritise future research. In part, the lack of any phase II or III treatment trials is due to our poor understanding of the incidence, clinical burden and circulating serotypes of EV in the UK. Section 2 of this thesis evaluates how molecular testing has influenced the epidemiology of viral meningitis and enterovirus disease in infants less than 90 days old in England and Wales over 10 years. Data from these studies will outline the gaps in our understanding of the clinical and epidemiological burden of EV meningitis in infants order to inform future treatment trials.

1.5 Aims

The aims of this thesis are to address the following hypotheses:

Section 1: Advances in molecular virology can significantly inform the investigation and management of CNS viral infections as exemplified by congenital CMV

- Evaluate the feasibility of diagnosing cCMV through clinical integration within the Newborn Hearing Screening Programme using salivary PCR **(chapters 2, 3 and 4).**
- Describe a novel strategy to identify pCMV disease using salivary PCR **(chapter 5)**
- Conduct a series of molecular sequencing and genotyping studies to better understand the medical interventions available to manage cCMV **(chapters 6)**
- Outline evidence based management guidelines to identify and treat cCMV **(chapter 7).**

Section 2: Advances in molecular virology are altering the apparent epidemiology of CNS viral infections as exemplified by enteroviral infections in infants under 90 days

- Determine the current epidemiology of enterovirus meningitis in infants less than 90 days in England and Wales (**chapter 8**).
- Determine the current epidemiology of enterovirus infections in infants less than 90 days in England and Wales (**chapter 9**)

Section 1

Changing management of cCMV due to PCR

Chapter 2

Clinically targeted screening for congenital CMV - potential for integration into the National Hearing Screening Programme.

Acta Paediatr. 2013 Oct;102(10):928-33

<https://www.ncbi.nlm.nih.gov/pubmed/23927434>

Chapter 3

Evaluating the feasibility of integrating salivary testing for congenital CMV into the Newborn Hearing Screening Programme in the UK

Eur J Paediatr 2015

<https://www.ncbi.nlm.nih.gov/pubmed/2574311>

Chapter 4

Integrating rapid diagnostic testing for congenital CMV into the Newborn Hearing Screening Programme: the audiovestibular physician's perspective

Arch Dis Child Fetal Neonatal Ed. 2015 Sep;100(5):F466-7

<https://www.ncbi.nlm.nih.gov/pubmed/26126843>

Chapter 5

Pre-emptive screening strategies to identify postnatal CMV diseases on the neonatal unit

Pediatr Infect 2016

<https://www.ncbi.nlm.nih.gov/pubmed/27455438>

Chapter 6

Characterising variation in five genetic loci of cytomegalovirus during treatment for congenital infection

J Med Virol 2016

<https://www.ncbi.nlm.nih.gov/pubmed/27486960>

Chapter 7

Fifteen Minute Consultation: Diagnosis and management of Congenital CMV

Arch Dis Child Educ Pract Ed 2016

<https://www.ncbi.nlm.nih.gov/pubmed/27307463>

Section 2

Changing epidemiology of enterovirus infections due to PCR

Chapter 8

Seven-fold increase in viral meningo encephalitis reports in England and Wales during 2004-2013

J Infect 2014

<https://www.ncbi.nlm.nih.gov/pubmed/24887614>

Chapter 9

Enterovirus infections in England and Wales, 2000-2011:

The impact of increased molecular diagnostics

Clin Microbiol Infect 2014

<https://www.ncbi.nlm.nih.gov/pubmed/25039903>

Chapter 10

Discussion

Data from this thesis supports both hypotheses tested. Molecular virology can inform the investigation and management of CNS viral infections as exemplified by congenital CMV. Advances in molecular virology are altering the apparent epidemiology of CNS viral infections as exemplified by EV infections in infants under 90 days.

The key findings and contributions to the research for each of the publications in this thesis are discussed below:

10.1 Section 1

Chapter 2 outlines the criteria that need to be met to screen for cCMV and highlights which future research areas need to be prioritised. A novel strategy to test for cCMV through integration into the NHSP by obtaining salivary swabs to test for CMV DNA PCR in newborns who do not pass their initial hearing screen is detailed. This would ensure that CMV related SNHL is detected early in the newborn period and treatment can be started in the first month of life in symptomatic infants as per evidence based guidelines. Targeted screening would only identify approximately half of all CMV related SNHL cases. The CHIMES study team showed that 44% of infants would be missed as they developed SNHL after the newborn period (134). Furthermore, Williams and colleagues have shown that parental anxiety is not heightened in infants screened and identified with CMV related SNHL but no studies have evaluated this in parents of asymptomatic infants (135). However, the

review argues that most of the Wilson-Junger criteria to screen for cCMV would be met. The review states that it is important to conduct prospective cohort studies to determine the feasibility of integrating salivary testing for cCMV into the NHSP and evaluate whether audiology services could conduct the further burden of testing that would arise from implementing screening. These areas have not been investigated previously and are essential before considering national implementation of any screening programme. Chapters 3 and 4 examined these areas further.

Chapter 3 describes the first prospective clinical cohort study to evaluate the feasibility of integrating testing for cCMV using salivary CMV DNA PCR into the NHSP. The study showed that 80% (203/255) of newborns who did not pass their hearing screen had a saliva swab taken to test for cCMV. Over 99% of swabs taken delivered a result in the first month of life enabling treatment to be started in the appropriate timeframe. Moreover, newborn hearing screeners felt confident in obtaining saliva samples after receiving appropriate training. Kimberlin and colleagues have recently showed that 6 months of valganciclovir therapy causes severe neutopaenia in a fifth of cases and results in modest benefits to auditory outcomes (37). Kimberlin highlights that it is essential that are cases are selected for treatment carefully and closely monitored. This study had three major limitations. Firstly, 20% of referrals were made after 21 days of life and thus ineligible. Of the eligible cases, 20% of parents refused to take part in the study due to most commonly not wanting their newborn to be part of a research study. Finally, the initial hearing screen is only performed in infants after 34 weeks gestation which means extremely premature babies could not participate. These are the first published data showing it is feasible to integrate testing for cCMV into the NHSP and thus removes delay in the clinical care pathway by enabling early diagnosis and timely treatment. This study already provides the template for local and

regional audiology networks in England to screen for cCMV and has been adopted in Germany, Ireland, southern Italy and certain states in the USA.

Chapter 4 describes results from a national survey of audiovestibular physicians which highlight 69% (n=42/61) of respondents strongly agreed that targeted testing of cCMV through integration into the NHSP was a good idea. Furthermore, 80% (n=49/61) thought it was feasible to integrate testing. However, 79% (n=41/52) of respondents stated that the biggest hurdle to service development would be the increased time taken for physicians to manage cases identified through any future screening programme. Chapter 11 will outline a multi regional study which aims to address this concern and investigate other areas of research, as detailed in chapter 2, that should be prioritised.

Chapter 5 proposes a novel strategy to identify pCMV early using serially collected saliva samples in extremely preterm infants. This approach builds on work described in previous chapters and aims to provide a coherent methodology using salivary CMV PCR assays to identify congenital and postnatally acquired CMV early in at risk populations through serial saliva collection. This chapter also provides a concise summary of the risk factors, clinical features and investigations that need to be conducted in managing infants with pCMV disease. Hamprecht and colleagues have advocated that heat pasteurization of breast milk will prevent pCMV transmission to preterm infants (136,137). However, the American Academy of Pediatrics recently published guidance that the benefits of giving fresh breast milk in seropositive mothers outweighed the risks (138). Only once small placebo controlled trial conducted over 20 years ago shows that CMV immunoglobulin reduced the risk of transmission compared to placebo (139). A meta analysis by Strippoli and colleagues show that preemptive screening reduces the risk of CMV disease in adult solid organ transplant patients (140). Postnatal CMV is a poorly defined illness which affects an extremely

vulnerable cohort. This is the first review to offer a strategic mechanism to identify disease early using preemptive screening and provides the rationale for a pilot study which is discussed in chapter 11.

Chapter 6 used population based sequencing to characterise the UL97 gene (target for ganciclovir), Glycoprotein B (vaccine target) and UL128, UL130 and UL131a (target for HIG) genes in infants with symptomatic cCMV who were treated with ganciclovir. This is the first study to use serial blood, saliva and urine samples from infected infants treated to characterise the gene loci that underpin these medical interventions. This study showed that 6 weeks of treatment with ganciclovir did not confer resistance and are used to help inform treatment guidelines discussed in chapter 7. The region sequenced covered codons 439 – 645 of the UL97 gene. Chou and colleagues have shown that this area includes all clinically relevant ganciclovir mutations (141). It is possible, however, that as yet undefined mutations exist outside this region. These results suggest that the rebound in viral load seen at the end of treatment is more likely due to the natural history of CMV dynamic replication rather than antiviral resistance (142). Genotyping gB in infected infants reveals multiple CMV genotypic variety and the emergence of new strains at 3 months. In contrast to Ross's study of babies not reported to be symptomatic, this study found no evidence of body site compartmentalisation, because genetic variants segregated with individual patients rather than by body site (143). No maternal samples were available for sequencing and so it was not possible to demonstrate the presence of the same genotypes present in a mother and her infected newborn as has been shown by Yamamoto et al (144). Future studies could consider prospectively collecting samples from other family members to determine if they are the source of CMV reinfections during the first year of life. No mutations were identified in the UL128, UL130 and UL131a regions of any of the samples sequenced which suggest there should be no reduction in binding affinity between monoclonal antibody and virus. These

findings have not been published previously and will support the development of vaccine design and trials of HIG in susceptible populations.

Chapter 7 consolidates themes discussed in previous chapters and recent treatment trials to outline evidence based management guidelines for cCMV which aim to identify disease early and treat appropriately. The guidelines recommend commencing 6 months of treatment using oral valganciclovir in symptomatic infants if started in the first month of life. Evidence from a recently published randomised controlled trial are used to inform the guidelines and advocate close monitoring for marrow suppression and renal impairment throughout treatment (37). It is anticipated that these guidelines will be widely adopted across units in the UK and minimise variation in treatment amongst clinicians which currently occurs.

Data presented in this section show that molecular tools, such as PCR, can improve diagnosis through identifying CMV related SNHL using salivary swabs in newborns who do not pass their initial hearing screen and inform management by characterising the molecular epidemiology of therapeutic targets and thus inform evidence base guidance. However, implementing routine testing for cCMV using salivary PCR may increase diagnostic uncertainty amongst clinicians treating mildly symptomatic cases and could heighten parental anxiety in parents of asymptomatic newborns. There are no published data evaluating the incidence of treatment resistance in infants who receive 6 months of valganciclovir treatment. Analysis of large international datasets through registries is required to improve understanding of clinical outcomes of mildly symptomatic infants and better inform which newborns to treat.

10.2 Section 2

Chapter 8 describes data obtained through interrogation of a national communicable diseases database. The study shows a seven-fold increase in viral meningo-encephalitis reported across England and Wales. The burden of disease was highest in infants less than 90 days old who account for over a quarter of all cases. Specifically, 92% of all cases of viral meningo-encephalitis in infants less than 90 days old was due to EV's. In 2013, the incidence of EV meningo-encephalitis was 313/100,000 in infants less than 90 days old compared to the overall incidence of viral meningo-encephalitis across all age group of 3.9/100,000. In 2011, a study from New York state reported that 340 of 2,357 specimens from patients with suspected meningo-encephalitis were positive for at least one virus, including enteroviruses (129; 5.5%), EBV (85; 3.6%), HSV (67; 2.8%), and VZV (44; 1.9%) (145). A prospective US cohort study conducted over 20 years ago showed that enteroviruses were grown by cell culture to be responsible for 89% of 169 aseptic meningitis cases in <2 year-olds (146). In Brazil, enteroviruses were responsible for around 40% of aseptic meningitis cases, followed by HSV-2 (17%), VZV (17%), EBV (12%), HSV-1 (7%) and mumps (7%) (147), while a national prospective Spanish study involving 17 hospitals identified a virus in 250 of 581 meningo-encephalitis cases, including enteroviruses (n=161,66.4%), HSV-1 (n=31, 12.4%), VZV (n=28, 11.2%) and HSV-2 (n=3, 1.2%) (148). A major disadvantage of such studies is that, while many collected detailed clinical and microbiological data on the confirmed cases, most were restricted to specific age-groups and/or did not provide longitudinal data to assess trends over time, thus making it difficult to compare with our study. This is the first population based longitudinal surveillance study of viral meningo-encephalitis for over two decades and demonstrates the significant burden of EV meningo-encephalitis in infants less than 90 days old. This study highlights that further research should focus on evaluating the clinical burden and long term outcomes of infants who have been affected by EV meningo-encephalitis in order to inform any future treatment trials. Studies attempting to address this

will be discussed in chapter 11.

Chapter 9 investigates the epidemiology of EV infection in more detail. Two large national datasets were interrogated. This is one of the largest published studies to describe the impact of molecular detection techniques on viral pathogens since being introduced into routine clinical practice. The study showed that PCR has significantly altered the apparent epidemiology of EV infections. A year on year increase in EV confirmed laboratory reports was noted between 2006 – 2011 which coincided with use of PCR. PCR was used to detect EV infections in 36% of cases in 2006 and rose to 92% in 2011. Infants less than 3 months accounted for almost a quarter of all reported cases during the 12-year surveillance period. Compared to overall rates, the incidence of EV infection was almost 100-fold higher in <3 month-olds, far above rates for bacterial infections in this age-group (149). In Denmark, the incidence of hospital admissions for non-polio EV during 1977-2001 was estimated to be 10.7 per 100,000 person-years, with the highest incidence in <6 month-olds (38.7 per 100,000 years) (150). Comparison is complicated by differences in country-specific clinical practice (e.g. threshold for investigating and hospitalising patients with suspected viral infections), investigation (e.g. threshold for performing lumbar puncture in patients with fever) and diagnosis (e.g. availability of viral cultures or PCR-testing for routine clinical samples). The proportion of EV samples undergoing molecular typing remained very low through the study and showed no predominance in strain. This study reveals an apparent increase in EV disease which is most significant in infants less than 90 days old. Importantly, this study highlights in which areas future research should focus. Primarily, knowledge of the clinical burden of EV disease in infants less than 90 days old is essential in order to inform evidence based management and direct healthcare resources. Prospective data collection through clinical and laboratory cohort studies should focus on determining the association between circulating serotypes and clinical manifestations in order to inform future treatment

trials. A prospective national surveillance study which aims to collect data to fill the gaps in our understanding in these areas will be outlined in chapter 11.

Section 2 shows that PCR has altered the apparent epidemiology of EV infections in infants less than 90 months. National surveillance data collected over a decade highlight a seven-fold increase in EV meningo-encephalitis as a consequence of increasing PCR use in NHS and hospital laboratories. A quarter of all cases occur in infants less than 90 days old. These studies are the largest published datasets in Europe to describe longitudinal trends in EV disease and evaluate the impact of PCR in disease trends and clearly highlight which future areas of research should be prioritised. Management of this condition varies considerably amongst paediatricians due to the lack of any robust clinical studies to determine the burden or long term outcomes of disease. There are currently no phase 2/3 treatment trials in Europe in part due to the lack of any current epidemiological data. Large robust prospective surveillance studies are necessary to gather these data on order to inform evidence based guidance and support future treatment trials.

Chapter 11

Future steps

This chapter will detail research I intend to conduct over the next 3 years of my career and build on work described earlier in this thesis. I will outline a series of research questions that have arisen through this thesis and detail studies that attempt to address these.

11.1 Multiregional Implementation of integration of rapid diagnosis for cCMV within the Newborn Hearing Screening Programme. The Benefits of Evaluating Salivary Testing for congenital CMV (BEST 3 Study)

11.1.1 Research question

How effective is integrating salivary testing for cCMV into the Newborn Hearing Screening Programme (NHSP) to prevent audiological deterioration in infants with CMV related sensorineural hearing loss (SNHL)?

This proposed study builds on work outlined in chapters 2, 3 and 4.

11.1.2 Background

The BEST 3 study will determine the proportion of newborns with CMV related SNHL that will be detected through hearing screeners taking a saliva swab to test for CMV DNA PCR after not passing the initial hearing screen. Subsequently, it will be possible to determine how many newborns will not develop any further audiological deterioration as a consequence of being identified and treated early. BEST 3 will be the first study to conduct a cost outcome analysis of integrating testing for cCMV into the NHSP.

The study team will collaborate with the NHSP and CMV Action to develop an interactive educational package for hearing screeners that can be integrated into the NHSP training curriculum. The study team will engage with local clinical commissioning groups and other stakeholders to develop the infrastructure to test for cCMV by integration within the NHSP and build the case for national implementation. Public awareness of CMV remains minimal in the UK. The study team will work in collaboration with CMV Action to promote CMV awareness to healthcare professionals and pregnant women alike during the antenatal period.

11.1.3 Research Plan

Study design

Prospective observational cohort study

Primary outcome measures

- Proportion of newborns with CMV related SNHL who will be identified through integrating salivary PCR testing for cCMV into the NHSP.
- Proportion of newborns with CMV related SNHL identified through integrating salivary PCR testing for cCMV into the NHSP that are treated and do not develop audiological deterioration

Inclusion criteria:

- Infants “referred” for any further audiology testing after completing newborn hearing screening

Methodology

This study comprises of five work packages:

1. Multi region screening study

The Newborn Hearing screen

Parents will be given a Participant Information Sheet (PIS) by the newborn hearing screeners at the start of their newborns AABR. The AABR typically lasts 45 minutes. Newborns referred for further audiological testing after having “no clear response” on the AABR will then be given the opportunity to enrol in the study. Newborn hearing screeners will provide more detailed information about the study at this point.

Assent processes

In this study, assent for the swab would be taken by the NHSP screener. The screener would take a sample of saliva using a flocked swab, placing it in the inner surface of the cheek for one minute and then into a sterile tube before posting to the CMV Virology Laboratory at the Royal Free Hospital. Documentation of assent will be made electronically on the NHSP website and an email confirming that the saliva swab has been taken with a confidential study number will be sent to the study coordinator.

Virology

Newborns diagnosed with cCMV will be seen by the relevant regional paediatric infectious diseases specialist. No drug treatment will be offered as part of the study protocol. Antiviral therapy will be offered to families with a baby with symptomatic cCMV by regional Paediatric Infectious Disease services following national guidelines (50).

Negative result processing

Negative results will be sent from the virology department at the Royal Free Hospital to the study coordinator who will record the result on eSP and post results to the parents and G.P.

Positive result processing

Laboratory personnel will call and email both the study coordinator and local PID specialist with the results of any positive swabs on the same day as processing the sample. In the event of obtaining a positive CMV result, the PID specialist will liaise with the local audiology

team to expedite the newborns definitive audiological assessment. This is already current practice and part of routine clinical care.

2. Education programme

The educational programme will be conducted in conjunction with the NHSP Training team and CMV Action. CMV Action will provide “funding in kind” to help develop the programme.

The study team in collaboration with the NHSP and CMV Action will develop a comprehensive educational programme for hearing screeners taking part in the study. All hearing screeners are required to pass an Objective Structured Clinical Examination (OSCE) and online assessment prior to being certified to screen. This educational package will pilot material that may be used in the formal hearing screener training as part of any future national implementation. A two hour study session was delivered to screeners during BEST 2. The session has since been tailored using written and verbal feedback from screeners. A study specific training session will be given at each site. This will include didactic teaching (overview of importance of testing for cCMV), case based discussions (2-3 case based sessions with role play of “real-life scenarios” and feedback) and study specific handouts.

3. Health economic analysis

This programme of work will include a health economic assessment which will estimate the cost-effectiveness of the enhanced diagnostic pathway compared to current standard practice in a large cohort. The cost-effectiveness analysis will take into account the audiological, neurocognitive and associated health benefits expected in patients treated through the proposed pathway. These benefits will be compared to the direct health costs of the actual expenditure of testing, confirmation of diagnosis and subsequent treatment of diagnosed cases. Moreover, the model will take into account recent data from a large randomised controlled trial which showed that 6 months of oral valganciclovir provided improved

audiological and cognitive outcomes at 12 and 24 months of life (37). The cost-effectiveness analysis will follow the NICE guidelines for health technology assessment.

4. Commissioning consultation

The study team will engage with local clinical commissioning groups within the five regions using a structured questionnaire to understand and develop the framework to implement integration of cCMV testing into the NHSP established clinical care pathway.

5. Attitudinal work

All newborn hearing screeners in England will be invited to complete a brief 5 point Likert confidence questionnaire at the start and end of the study in order to assess attitudes and confidence towards testing for cCMV.

11.2 Pre-emptive screening of preterm babies using salivary PCR to identify and manage postnatal CMV on the neonatal unit (POPS CMV study)

11.2.1 Research question

Is it feasible to collect saliva swabs routinely on neonatal units to detect early, asymptomatic, postnatal CMV infection?

This proposed study builds on work outlined in chapter 5.

11.2.2 Background

This single centre study will provide preliminary pilot data that will be used to scrutinise any association between viral kinetics and onset of disease which might assist in defining a group of babies that could benefit from early antiviral therapy to prevent severe CMV-related morbidities. Data will also be collected to better understand the clinical burden of pCMV and outcomes at discharge.

It is hoped that results from this pilot study will be used to gain external funding to facilitate collaboration with other centres in the U.K and USA on perinatal CMV. The Collaborative Antiviral Study Group (CASG) are currently enrolling into a treatment study to assess the pharmacokinetics and pharmacodynamics of ganciclovir in preterm infants (<https://clinicaltrials.gov/ct2/show/NCT01602614?term=david+kimberlin&rank=1>). It has not however been possible to collaborate with the group as there have been no studies in the U.K that have demonstrated the burden of CMV disease in preterm infants. It is hoped that new data might allow for collaboration with the CASG or provide information sufficient to power a study in other U.K sites.

11.2.3 Research plan

Study design

Prospective observational cohort study

Primary outcome measures

- Assess the feasibility of pre-emptive screening for pCMV on NICU
- Gather pilot data to assess any association between viral load, gestational age and clinical features in infants with pCMV

Inclusion criteria

- Infant <32 weeks or <1500gm who has CMV DNA isolated on saliva swab taken during weekly screening after 21 days of life

Methodology

Identification of infants: The neonatal team will inform the study nurse/research fellow that an infant <32 weeks gestation/<1500g has been admitted to the neonatal unit

Consent: Patient Information Sheet will be given to parents for all eligible participants as part of admission and the neonatal team will ask parents if they are happy to be approached to discuss the study further. Informed written consent for weekly swabs and collection of data

will be taken by the study team. Neonatal nursing staff will take a sample of saliva using a flocked swab, placing it in the inner surface of the cheek for one minute and then into a sterile tube before posting to the Royal Free Hospital, London on a set day each week. Samples will be taken within the first 14 days of life and then on a weekly basis from 22 days of life until discharge from NICU.

Laboratory processes: Swabs will be posted for immediate testing for CMV DNA by real time quantitative PCR.

Result processing: Positive results will be telephoned within 24 hours and negative results emailed weekly to the study coordinator from the laboratory. The study nurse/research fellow will inform the neonatal team and parents immediately in the event of a positive result.

Data collection: All clinical and demographic data will be anonymised, collected and stored onto a password protected Excel spreadsheet. Data will be backed up onto the St George's, University of London shared drive which is backed up centrally every 24 hours by the university IT.

Clinical samples: Parents will be consented to collect serial blood, saliva and urine samples from cases where saliva PCR is positive. This will enable to staging and monitoring if CMV disease progression.

11.3 Characterising the burden of enterovirus and parechovirus meningitis in infants less than 90 days old in the UK and Republic of Ireland (BPSU EV/HPeV meningitis Surveillance Study)

11.3.1. Research question

What is the clinical burden (rate of hospitalisation including NICU/PICU admission) of enterovirus (EV) and parechovirus (HPeV) meningitis in the United Kingdom and Republic of Ireland?

This proposed study builds on work outlined in chapters 8 and 9

11.3.2 Background

This will be the first UK study to link the molecular types of EV/ HPeV circulating in the UK and Ireland with clinical disease, such as severity, laboratory markers or outcomes. This provides a unique opportunity to prospectively collect clinical information and understand whether specific strains might be associated with more severe infection in young infants. The results of the BPSU study may also provide an evidence-base for national guidance on the prevention, investigation and management of EV/HPeV infections in young infants. The study will allow comparison between disease severity and source of positive specimens. It will also be able to assess whether the disease severity is associated with the amount of virus present in the blood and/or CSF by measuring the viral load. In the absence of any licenced medication it is unclear what proportion of clinicians use blood products such as immunoglobulins to affect the course of disease and what the outcomes at discharge are. The BPSU study may be able to identify possible impact of such therapies on the outcome of disease. Finally, this BPSU study will serve to promote awareness of EV/ HPeV meningitis amongst paediatricians and, thus, aim to improve case detection, recognition and management.

11.3.3 Research plan

Study design

Prospective enhanced national surveillance study

Primary outcome measure

- To estimate age-group specific hospitalisation rates per 100,000 childhood population for enterovirus (EV) and parechovirus (HPeV) meningitis

Inclusion criteria

Any infant aged less than 90 days old with clinical symptoms of meningitis* AND laboratory confirmation of enterovirus or parechovirus from any site**

* fever (≥ 38 degrees Celsius), coma, seizures, neck stiffness, apnoea, bulging fontanelle, irritability, lethargy, poor feeding

**CSF, blood, stool, throat, peri-anal swab

Exclusion criteria

Isolation of enterovirus or parechovirus with no clinical symptoms of meningitis*.

* fever (≥ 38 degrees Celsius), coma, seizures, neck stiffness, apnoea, bulging fontanelle, irritability, lethargy, poor feeding

Methodology

Paediatricians will be asked to notify any cases in the previous month through the BPSU orange card system. At the same time, potential cases will also be identified using LabBase2, a voluntary electronic database used by almost all hospital microbiologists in the UK to report clinically significant infections to the PHE, as well as the PHE Virus Reference Department (VRD) electronic database, MOLIS. LabBase2 and MOLIS notification will be compared with the BPSU cases using a number of identifiers including NHS number, date of birth, gender, admitting hospital, date of diagnosis and source of sample. Paediatricians and microbiologists will also be reminded to submit the EV/HPeV specimen to the PHE virus reference laboratory for molecular typing if this has not already been done. No additional samples will be taken from patients. Meningitis charities, including Meningitis Research Foundation and Meningitis UK/Meningitis Trust, have been informed of the study and agreed

to promote awareness on their website. The study team will not be contacting any parent directly.

In keeping with existing national guidance, NHS hospital microbiology labs are advised to submit all positive EV/HPeV strains to PHE Virus Reference Department (VRD) for molecular typing. Before starting the study, the study team will write to all microbiologists through the national microbiology network to promote the study and remind them to submit all positive EV/HPeV clinical samples to PHE VRD for molecular typing, especially in infants less than 90 day-olds. For all cases reported through the BPSU, the study team will also remind the microbiologist of the reporting hospital in writing to ensure all positive clinical samples are submitted to PHE VRD.

11.4 Neurodevelopmental follow up of enterovirus and parechovirus meningitis in infants less than 90 days old

11.4.1 Research question

What are the neurodevelopmental outcome of enterovirus (EV) and human parechovirus (HPeV) meningitis in infants less than 90 days old?

This proposed study builds on work outlined in chapters 8 and 9

11.4.2 Background

This study will be the first in Europe to comprehensively evaluate neurodevelopmental outcomes in infants with EV and HPeV meningitis. It will also provide a unique opportunity to link molecular subtype with phenotype. Data from this study will be used to inform the evidence base to manage infants with EV and HPeV meningitis beyond the inpatient setting and define which healthcare needs are required for this vulnerable population. Furthermore,

an accurate data of the natural history of the condition is crucial to evaluate potential future developments of anti-viral and immunotherapeutic strategies.

11.4.3 Research plan

Study design

Prospective observational cohort study

Primary outcome measures

- Determine if babies who had meningitis caused by viruses (caused by enterovirus or parechovirus) in the first three months of life have developmental delay in early childhood
- Define any link between developmental outcomes of infants with enterovirus or parechovirus meningitis and specific genetic type of virus
- Assess the effects of enterovirus and parechovirus meningitis in young infants on the quality of life of both the survivors and the adult family members of survivors

Methodology

Eligible infants will be identified using Public Health England (PHE) LabBase2 or Molis database (PHE's national database). UK National Health Service hospital microbiology laboratories in England and Wales routinely report enterovirus and parechovirus cases pathogens electronically on a voluntary basis to PHE. Both datasets do not contain clinical data.

The study team will contact the family's GP to inform them that we are proposing to approach the family regarding this study. Unless the GP indicates otherwise, families will

then be contacted by post and provided with a patient information leaflet on the study and invited to participate. If parents agree, the study team will discuss the study procedures in more detail, provide the opportunity for parents/carers to ask questions and arrange a time and date for the study visit. If the parents have not replied to the original invitation one further invitation letter will be sent after approximately two weeks.

Parents will be asked to provide written consent for themselves and their child. No study procedures will be performed until written informed consent is given. All individuals and families will be free to withdraw from participation at any time without giving a reason and without this affecting the ongoing care, and this will be explained in the consenting process.

Identifying cases through in this manner will ensure that this population based study minimises any risk of selection bias. This study will provide the largest and most comprehensive dataset in Europe of infants with neurodevelopmental outcomes following enterovirus/parechovirus meningitis as an infant. It will therefore be possible to address the study aims as outlined above. This will be the first study to link which enterovirus/parechovirus types are associated with poorest outcomes and evaluate the effects of immune modulating therapies on long term outcomes.

Neurodevelopmental assessments

The children will have an assessment of their cognitive, language and motor development using the third edition of the Bayley Scales of Infant Development (BSID III). This assessment is done on one occasion and takes approximately 60 – 90 minutes. A member of the study team with training in developmental paediatrics and use of BSID III will perform the assessment in the child's home. If any of the children are aged over 36 months their current progress will be evaluated using information from their parents, general practitioner and any educational or paediatric assessments which have taken place previously.

Basic information on neurological status, functional disabilities (hearing, vision and speech and language), general health problems (respiratory, renal and gastrointestinal function) and growth will be collected at the same time as the Bayley Scales is performed. This information will be collected using questions from the 2008 Health Status Questionnaire (HSQ).

Quality of life assessments

Parents and other family members (over the age of 16 years) of survivors of bacterial infections who have been recruited to the follow-up study will be invited to complete a PEDsQL to assess the health status of their child by proxy. The questionnaire will be given to the adult with parental responsibility for the child by the study team at the start of the assessment visit. The same assessment will also be independently completed by a member of the study team during the visit. The same adult will be asked to complete an EQ-5D-5L questionnaire to assess their own health status. Additional forms will be provided for other family members who are eligible to participate (i.e. over 16 years of age). The study team will collect any questionnaires that have been completed by the end of their visit and provide a stamped addressed envelope for the return of any additional questionnaires.

Results

The GP (with parental permission) and parents of each child will receive a copy of the results of the child's assessment. A paediatric doctor from the study team will be available to answer questions relating to the assessment. If any areas of concern, or need for specialist referral, are identified this will be included in the assessment report sent to the child's GP so that the appropriate referral can be made locally. Information about relevant support groups (e.g. Meningitis Research Foundation) will also be provided to them.

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