Alphaherpesvirus infections of the central nervous system

Biomarkers, diagnostics and antiviral therapy

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"I have yet to see any problem, however complicated, which, when you looked at it in the right way, did not become still more complicated."

Poul Anderson

ABSTRACT

Herpesviruses predate the evolution of humans and are globally ubiquitous. Herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), and varicella-zoster virus (VZV) establish latency in neuronal tissue and may cause infections in the central nervous system (CNS). Despite advances in diagnostics and treatment, the disease burden remains high. The overall aims of this thesis were to explore and evaluate several aspects of HSV and VZV CNS infection, with the ultimate goal of improving clinical management, from diagnosis to treatment and prognostication.

Paper I explores cerebrospinal fluid (CSF) biomarkers in 28 patients with facial palsy caused by VZV. Biomarker expression was consistent with neurological damage and astrogliosis. This pattern was more pronounced in patients with concurrent mucocutaneous zoster rash than in those without rash, *zoster sine herpete*. Associations between biomarker concentrations and neurological outcomes could not be demonstrated.

Paper II evaluates the CXCL13 CSF biomarker as a means of discriminating between VZV and Lyme Neuroborreliosis (LNB) in cases of facial palsy. CXCL13 concentrations were significantly higher in patients with LNB facial palsy ($n = 21$), though there was some overlap with cases of VZV facial palsy ($n = 26$). Despite good performance measures, especially if analyzed early after onset of symptoms, careful interpretation is advised when concentrations are moderately increased.

Paper III assesses the performance of the FilmArray Meningitis/Encephalitis (ME) panel. The ME panel is a multiplex PCR panel for syndromic testing in CNS infections, able to detect 14 pathogens, including herpesviruses and bacteria. ME panel results were compared with routine diagnostic procedures in 4199 CSF samples from patients with suspected viral CNS infection. Discrepant results were thoroughly investigated to determine whether PCR detection was correct. A high performance level was demonstrated in calculations on individual pathogens, but 21 false negative and 20 false positive results were identified. If herpes simplex encephalitis is suspected, additional testing is warranted despite negative HSV-1 results from the ME panel. Interpretation concerning positive enterovirus, HHV-6, and *S. pneumoniae* results may also be complicated due to false positive or clinically insignificant results.

Paper IV is a pharmacokinetic study of acyclovir and its metabolite CMMG in 21 patients with acute CNS infection. Renal function, damage to the blood-brain barrier, dosage, and body weight all influenced CSF concentrations of both molecules. Acyclovir-induced neuropsychiatric symptoms (AINS) were unexpectedly identified in four patients, together with high CSF concentrations of CMMG, previously implicated in neurotoxicity. These results justify increased attention to suspected neuropsychiatric symptoms and careful consideration of dosages in acutely ill patients.

Keywords: herpesviruses, central nervous system, facial paralysis, cerebrospinal fluid, biomarkers, syndromic testing, acyclovir, neurotoxicity

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SAMMANFATTNING PÅ SVENSKA

Herpesvirusinfektioner är vanliga i hela världen. Typiska manifestationer är munsår orsakade av herpes simplexvirus typ 1 (HSV-1), genitala lesioner orsakade av herpes simplexvirus typ 2 (HSV-2) och vattkoppor samt bältros orsakade av varicella-zostervirus (VZV). Dessa virus persisterar i nervceller, och kan reaktiveras senare i livet. I ovanliga fall orsakar de infektion i centrala nervsystemet (CNS), bland annat hjärninflammation, hjärnhinneinflammation och ansiktsförlamning. I denna avhandling undersöks flera kliniska aspekter av herpesvirusinfektion i CNS, från diagnostik till behandling och prognos.

I **delarbete I** studerades utvalda biologiska markörer i ryggmärgsvätska hos 28 patienter med ansiktsförlamning orsakad av VZV. Målet var att undersöka vilka mönster dessa biomarkörer uttrycktes i och om det gick att koppla nivåer av biomarkörer till prognos i form av kvarvarande ansiktsförlamning. Vi fann tecken till nervskada och aktiverade stödjeceller, men vi kunde inte påvisa någon koppling mellan biomarkörer och restsymtom.

Ansiktsförlamning kan också orsakas av den fästingburna bakterien borrelia, som ibland är svår att diagnosticera. I **delarbete II** undersökte vi om biomarkören CXCL13 är användbar för att skilja mellan patienter med ansiktsförlamning orsakad av borrelia och VZV, då denna markör tidigare visats kraftigt förhöjd i ryggmärgsvätska vid just borrelia i CNS. I vår studie var CXCL13 på gruppnivå klart högre hos patienter med borrelia, men med överlapp i koncentrationer hos patienter med VZV-infektion. Detta kan innebära tolkningssvårigheter, särskilt om nivåerna är lågt till måttligt förhöjda, och metoden bör användas med försiktighet.

I **delarbete III** utvärderades en ny metod för att upptäcka virus och bakterier i ryggmärgsvätska, en kommersiell snabb-PCR (FilmArray) som ger svar på 14 olika analyser inom en timme. Denna jämfördes med etablerade metoder och om resultaten inte stämde överens studerades patientjournaler för att fastställa vilken metod som mest sannolikt gav rätt svar. Analyserna gjordes på 4199 ryggmärgsvätskeprover från patienter med misstänkt CNS-infektion, och snabb-PCR-metoden presterade över lag bra, även om den inte lyckades detektera 21 fall av virus och rapporterade 20 falskt positiva svar. Mest oroande var tre missade fall av HSV-1, som kan vara orsak till dödlig hjärninflammation, och vid hög misstanke om sådan infektion bör de etablerade metoderna användas även om snabb-PCR är negativ.

I **delarbete IV** undersöktes hur olika faktorer påverkar fördelningen av det antivirala läkemedlet aciklovir och dess nedbrytningsprodukt CMMG i blod och ryggmärgsvätska. Hos 21 patienter med akut CNS-infektion påverkades koncentrationerna i ryggmärgsvätska av flera faktorer: njurfunktion, läkemedelsdos, vikt, och skada på blodhjärnbarriären. Fyra patienter utvecklade neuropsykiatriska symptom, en biverkan som tidigare beskrivits tillsammans med höga koncentrationer av CMMG, vilket också dessa patienter hade. Våra fynd indikerar ett behov av ökad uppmärksamhet på neuropsykiatriska biverkningar och individuella överväganden vid val av behandlingsdos.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by Roman numerals.

I. Lindstrom J, Grahn A, Zetterberg H, Studahl M.

Cerebrospinal fluid viral load and biomarkers of neuronal and glial cells in Ramsay Hunt syndrome.

European Journal of Neuroscience. 2016 Dec;44(11):2944-9.

II. Lindstrom J, Bremell D, Grahn A, Blennow K, Zetterberg H, Studahl M.

CXCL13 in patients with facial palsy caused by varicella zoster virus and Borrelia burgdorferi: a comparative study.

Diagnostic Microbiology and Infectious Disease. 2020 Sep;98(1):115095.

III. Lindstrom J, Elfving K, Lindh M, Westin J, Studahl M.

Assessment of the FilmArray ME panel in 4199 consecutively tested cerebrospinal fluid samples.

Submitted manuscript

IV. Lindstrom J, Hellden A, Lycke J, Grahn A, Studahl M.

An unexpectedly high occurrence of aciclovir-induced neuropsychiatric symptoms in patients treated for herpesvirus CNS infection: a prospective observational study.

Journal of Antimicrobial Chemotherapy. 2019 Dec;74(12):3565-72.

CONTENT

ABBREVIATIONS IV
1 INTRODUCTION 1
1.1 A brief history of herpes 1
1.2 The herpesvirus family 2
1.3 Epidemiology 3
1.4 Infection, latency and reactivation 4
1.5 Viral infections of the CNS 8
1.6 Herpes simplex virus in the CNS 9
1.7 Varicella-zoster virus in the CNS 13
1.8 Diagnosis of herpesvirus CNS infections 16
1.9 Biomarkers 23
1.10 Treatment 25
1.11 Vaccination 33
2 AIMS 37
3 PATIENTS AND METHODS 39
3.1 Patients and CSF samples 39
3.2 Methods 41
3.3 Ethics 45
4 RESULTS AND DISCUSSION 47
4.1 Paper I 47
4.2 Discussion paper I 50
4.3 Paper II 52
4.4 Discussion paper II 54
4.5 Paper III 56
4.6 Discussion paper III 59
4.7 Paper IV 63
4.8 Discussion paper IV 66

5 CONCLUSIONS 69
6 FUTURE CONSIDERATIONS 71
ACKNOWLEDGEMENT 75
REFERENCES 79

ABBREVIATIONS

8-OH-ACV	8-hydroxy-acyclovir
AI	CSF:serum antibody index
AINS	Acyclovir-induced neuropsychiatric symptoms
AUC	Area under curve
BBB	Blood-brain barrier
CD8	Cluster of differentiation 8
CI	Confidence interval
CL_{CR}	Creatinine clearance
CMMG	9-carboxymethoxymethylguanine
CMV	Cytomegalovirus
CNS	Central nervous system
COVID-19	Coronavirus disease 2019
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9
CSF	Cerebrospinal fluid
CT	Computed tomography
Ct value	Cycle threshold
CXCL13	Chemokine [C-X-C motif] ligand 13 = B-cell chemoattractant
EEG	Electroencephalography
EFNS	European Federation of the Neurological Societies
ELISA	Enzyme-linked immunosorbent assay
GBS	Group B Streptococcus = *Streptococcus agalactiae*
GFAP	Glial fibrillary acidic protein
HHV-6	Human herpesvirus 6
HPeV	Human parechovirus
HSE	Herpes simplex encephalitis

HSV	Herpes simplex virus
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
IFN	Interferon
IL	Interleukin
LATs	Latency-associated transcripts
LNB	Lyme neuroborreliosis
ME panel	FilmArray Meningitis/Encephalitis panel
mNGS	Metagenomic next-generation sequencing
MRI	Magnetic resonance imaging
NFL	Neurofilament light chain protein
NMDAR	N-methyl-D-aspartate receptor
NPV	Negative predictive value
PCR	Polymerase chain reaction
PPV	Positive predictive value
q8h	Every eight hours
real-time PCR	Real-time quantitative PCR
RHS	Ramsay Hunt syndrome
ROC	Receiver-operating characteristic
TBE	Tick-borne encephalitis
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
VZV	Varicella-zoster virus
WBC	White blood cell

1 INTRODUCTION

Research concerning herpesvirus infections of the central nervous system (CNS) has been conducted for more than a century, which has added to the collective knowledge on which this thesis is based. The investigations in this thesis focus on the clinical perspectives of CNS infections caused by the neurotropic alphaherpesviruses – herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), and varicella-zoster virus (VZV) – mainly as they relate to immunocompetent adults.

1.1 A BRIEF HISTORY OF HERPES

Herpesviruses have been present for hundreds of millions of years [1] and symptoms attributable to herpesvirus infections have even been recorded in ancient texts. The name "herpes" originates from the Greek word "to creep," used by Hippocrates to describe spreading skin lesions, whereas zoster derives from the Greek word meaning girdle [2].

From a historical perspective, the etiology of herpesvirus infection was discovered fairly recently. What we today know as viruses were first identified by Iwanowski in 1892 when he transmitted tobacco plant virus from one plant to another after running liquified plant material through a filter, previously thought to prevent passage of germs. This discovery initially resulted in the concept of filterable viruses, since the term "virus" was used for all kinds of microbes at the time.

As research on viruses advanced, in 1919 successful inoculation of herpes simplex virus (HSV) into rabbit cornea effectively established rabbits as an animal model to study HSV [3]. By 1929, a connection was observed between HSV and neurological symptoms in the rabbits [4], and by the 1940s, the first verified human cases of herpes simplex encephalitis (HSE) were reported [5, 6].

The discovery of VZV is even more recent. The connection between primary VZV infection and herpes zoster was established in the late nineteenth century. Predating this discovery, the relationship between herpes zoster and sensory ganglia was suggested in the mid-nineteenth century. CNS manifestations of VZV were repeatedly reported in the early- to mid-twentieth century, describing meningoencephalitis in conjunction with herpes zoster [7]. However, the viral etiology was not confirmed until the development of electron microscopy in the 1940s, and VZV was ultimately isolated by Nobel Prize winner Thomas Weller in 1952 (Reviewed in [8]).

1.2 THE HERPESVIRUS FAMILY

The herpesvirus family, or Herpesviridae, includes a large number of distinctive viruses. They have in common a double-stranded DNA core encased by an icosahedral nucleocapsid, surrounded by an amorphous mass known as tegument that contains pre-synthesized proteins, as well as an envelope with glycoprotein spikes on the surface (Figure 1). Herpesviruses also share biological properties, including the ability to establish latency in their hosts. As DNA viruses, they are considered genetically stable, and although variations occur, mutation frequency is considerably lower than among RNA viruses.

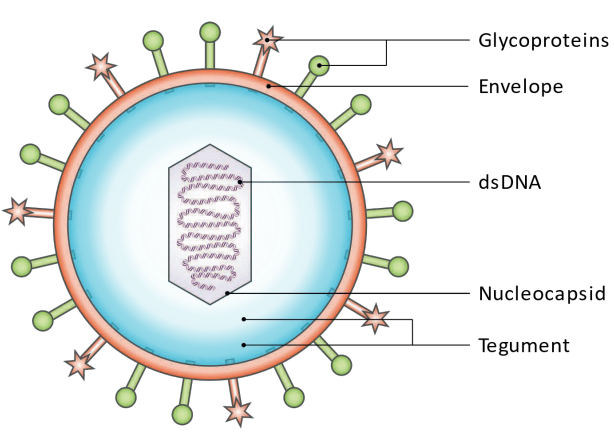

Figure 1. Herpes virion. dsDNA = double-stranded DNA

This diverse group of viruses is highly prevalent in nature, capable of infecting many different species, and nine herpesviruses are known to have humans as their primary host. The herpesviruses are placed into the subfamilies alpha-, beta-, and gammaherpesvirinae, historically classified according to biological properties, which with the discovery of genomic sequencing have remained consistent based on shared nucleotide sequences.

HSV-1, HSV-2, and VZV are alphaherpesvirinae, all of which show tropism for neurological tissue. They establish latency in neurons, and are associated with infections of the CNS. Beta- and gammaherpesvirinae establish latency in lymphoreticular tissue, and although they may cause CNS infection, this is mainly of concern in immunocompromised hosts [9].

Despite belonging to the same subfamily and infecting neuronal tissue, there are significant genetic differences between HSV-1, HSV-2 and especially VZV. The VZV genome is shorter than both HSV-1 and HSV-2, and includes a much lower proportion of G + C nucleobases (Figure 2). While HSV-1 and HSV-2 have a very high proportion of homologous genes, there is nevertheless considerable variation in sequences encoding for, e.g., glycoproteins important for tissue tropism. VZV is phylogenetically removed from HSV, and although they share a number of homologous genes, several are unique to either VZV or HSV. The resulting differences in expressed proteins likely explain the considerable differences in clinical manifestations [10].

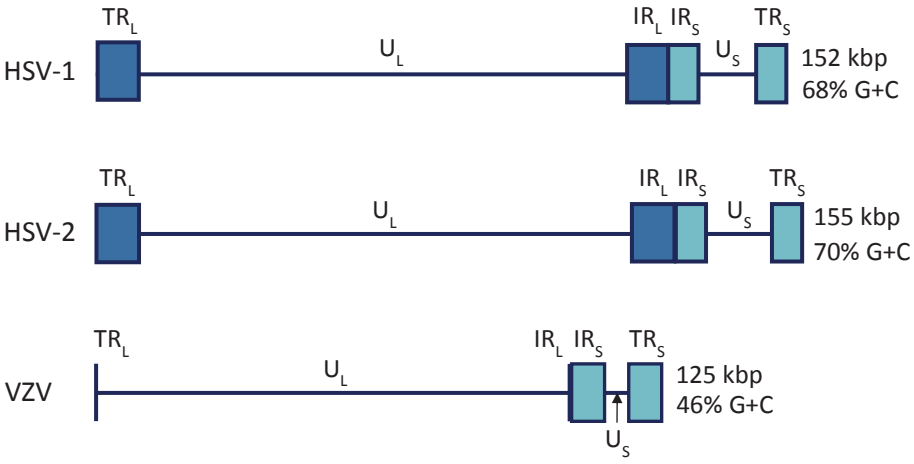

Figure 2. Genomic architecture of HSV-1, HSV-2 and VZV. U_L = Unique long region, U_S = Unique short region, TR_L = Terminal repeat long region, TR_S = Terminal repeat short region, IR_L = Internal repeat long region, IR_S = Internal repeat short region

1.3 EPIDEMIOLOGY

The herpesviruses are globally ubiquitous, albeit with variation in disease burden. HSV-1 has been estimated to affect two-thirds of all people age 0-49 years, but with geographic variation [11]. In Sweden, HSV-1 seroprevalence has been reported to be 31% among children age 0-19 years and increases with age [12]. HSV-1 typically produces oral or perioral lesions (cold sores). Other manifestations include genital herpes, ocular infections (herpes keratitis), cutaneous infections (e.g., herpes gladiatorum, herpes whitlow), and infections of the CNS.

The estimated worldwide prevalence of HSV-2 is about 11%, with the highest burden in Africa [13]. HSV-2 is associated with genital infection and may also cause cutaneous manifestations and CNS infection, but not typically oral or perioral lesions [14]. Genital infection among mothers when giving birth is of particular importance, since it may lead to life-threatening neonatal herpes infection.

The highest prevalence of herpes infections is seen with VZV. In Europe, seroprevalence ranges from as high as 97% by age five years in the Netherlands to 78% at age 15 in Italy [15]. In countries where varicella vaccine is included in the childhood immunization schedule, incidence has fallen by over 70% [16]. VZV is the cause of varicella (chickenpox) in primary infection and herpes zoster (shingles) when reactivated, but can manifest in many different ways, including in the CNS.

1.4 INFECTION, LATENCY AND REACTIVATION

1.4.1 Infection and establishment of latency

HSV infection is typically transmitted through close contact; onset of viral replication is rapid in the mucoepithelial cells of the oral region in HSV-1, and in the genital region in HSV-2. HSV gains access to the host through interactions between viral envelope glycoproteins and host cell receptors. Following fusion of the viral envelope with the cell membrane, virion contents, including proteins from the tegument and the viral capsid, are released into the cytoplasm. The capsid attaches to the cell nucleus, whereupon the viral genome is released into the nucleus for transcription. In the mucoepithelium, replication results in lysis of infected cells. After a replicative cycle at the primary infection site, new virions enter the nerve endings of sensory neurons. The capsid undergoes retrograde transport via axons to the dorsal root ganglion or cranial nerve ganglion, where it results in persistent infection [17]. Viral DNA may then undergo a replicative cycle in the nucleus of the neuron, but replication in nerve cells is not typically associated with neuronal cell death. Viral DNA may also enter into a latent state without production of new virions, thereby protecting the virus from host defenses [18] (Figure 3).

HSV genetic expression is governed by epigenetics and is vastly different in symptomatic lytic infection than in latency. In lytic replicative infection, a plethora of genes are expressed to regulate cell activity required for the production of new viruses. In latent infection, very few genes are expressed; the only viral products occurring in abundance are latency-associated transcripts (LATs), which do not appear to encode proteins. LATs instead

protect neurons from death by reducing viral lytic gene expression and protecting against apoptosis [19], possibly due to actions on heterochromatin assembly [20].

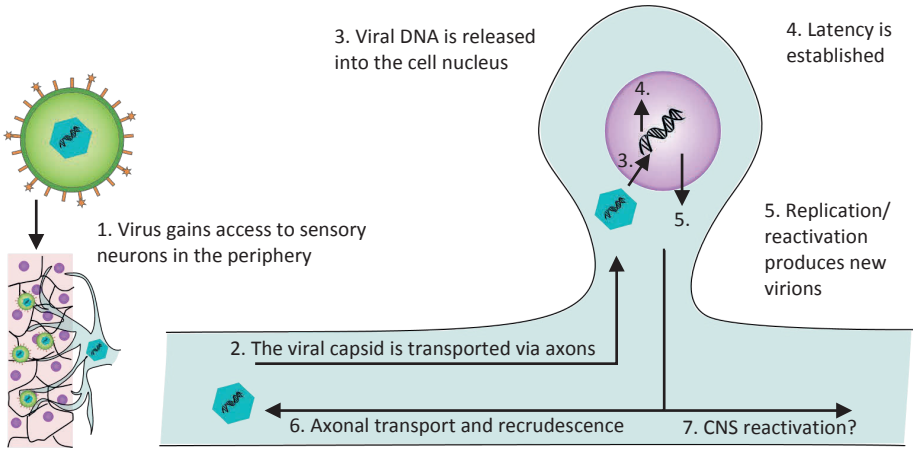

Figure 3. Alphaherpesvirus infection and establishment of latency in sensory neurons.

There are similarities in infection and establishment of VZV, but also important differences responsible for the distinctive panorama of clinical manifestations. VZV gains access to the mucosal epithelium of the respiratory tract primarily by inhalation, and shows tropism to many cell types, including T lymphocytes abundant in tonsillar crypts. VZV travels with T lymphocytes and dendritic cells to regional lymph nodes where the first phase of replication is carried out, resulting in subclinical viremia. As circulating T lymphocytes are infected and traffic through tissues, virus reaches the skin, and a typical wide-spread vesicular rash of varicella appears. The virus replicates in epithelial cells during varicella, but other cells such as endothelium of small blood vessels are also infected, shown in histological studies already in 1906 [21].

As with HSV, VZV infections start with attachment and entry of the virus into host cells, albeit involving different glycoprotein-host cell receptor interactions, which possibly explains the broader tropism of VZV [22]. Latency is established after VZV gains access to the sensory ganglia, either hematogenously or through afferent nerve fibers terminating in the skin. In contrast to HSV, VZV becomes latent in cranial nerve, dorsal root, and autonomic ganglia throughout the nervous system. In latency, VZV gene expression is highly restricted, but where only LATs are expressed in HSV latency, transcripts of genes corresponding to all stages of the VZV cycle are present. The presence of early protein 62 and early protein 63 in the

cytoplasm, as opposed to in the nucleus where they are found in the lytic phase, has been proposed as being responsible for maintaining latency. However, findings are inconsistent, and the overall mechanism is poorly understood [23].

1.4.2 Reactivation

Reactivation of latent HSV has been studied extensively, particularly in animal models, but the exact mechanisms have yet to be ascertained [24]. Certain stimuli are known to predispose to recrudescence (symptomatic reactivation), such as local injury to tissues innervated by neurons with latent virus, UV light exposure, physical and emotional stress, menstruation, and hormonal imbalances. Immune surveillance likely plays an integral part in the early containment of reactivation, especially $CD8^+$ T-cells [25], but the extent to which the immune system influences viral gene expression remains uncertain [26, 27]. Reactivation of VZV is even less well understood, in part due to lack of animal models, but factors associated with a decline in cell-mediated immunity seem to contribute [28, 29]. With reactivation, viral gene expression is deregulated from the restricted transcription in latency and replication is initiated. Virions, or perhaps capsids, then undergo anterograde transport via axons to the sensory nerve terminal, where they are released and infect the cells of surrounding tissues, possibly producing new symptoms [19] (Figure 3). In VZV reactivation, the entire ganglion is involved, resulting in recrudescence that affects an entire dermatome, while HSV recrudescence is limited to areas innervated by specific neurons [18].

The reactivation rate differs significantly between HSV and VZV. Recurrent symptomatic HSV infection is common, while episodes of VZV reactivation tend to be isolated events. Additionally, asymptomatic viral shedding frequently occurs in latent HSV infection and remains an important mode of transmission. The most probable reason is likely to be repeated reactivation in small numbers of neurons at different anatomical sites [30].

Symptoms in recurrent infection depend on the site of primary infection and are usually milder. In HSV-1, typically involving an oral primary infection, latent infection is established in the trigeminal nerve ganglion, and reactivation results in oral or perioral recurrent infection, manifesting as cold sores. However, not all primary infections give rise to symptomatic recurrences; in fact, only about one-third do so [19]. In primary genital infection, latency is established in the dorsal root ganglia of sacral nerves, and reactivation is associated with genital herpes. In contrast to oral HSV-1 infections, most patients have recurrent symptomatic genital infection [31], although to a lesser extent when caused by HSV-1 than HSV-2 [32].

The most common manifestation of VZV reactivation is herpes zoster, a vesicular rash localized to a single dermatome, often accompanied by a burning sensation due to neuritis of the infected nerve. Pain may persist after the rash resolves, and postherpetic neuralgia may be severe, as reflected in the old Scandinavian name for shingles: *Helveteseld* (Hellfire). About one-third of the population in high-endemic countries develop herpes zoster over their lifetime [33], with a higher disease burden among females and with older age [34]. Moreover, immunocompromised patients are at increased risk [35].

Depending on the site of reactivation, herpes zoster may manifest with a variety of specific complications, such as herpes zoster ophthalmicus (reactivation in the ophthalmic nerve with a risk of vision loss) and secondary bacterial infection regardless of anatomical site. Reactivation in the CNS, the subject of this thesis, is discussed below.

1.5 VIRAL INFECTIONS OF THE CNS

Viral infections of the CNS are characterized by great diversity, both in manifestation and etiology. Distinctions between the various syndromes are not always clear cut, and terms like meningoencephalitis and meningomyelitis are sometimes used to describe overlapping symptomatology. Severity ranges from mild to life-threatening, but even mild cases of acute infection may be associated with significant sequelae. Our understanding of the involved processes remains largely inadequate, as reflected by the lack of treatment options despite a significant global disease burden associated with considerable mortality and morbidity.

1.5.1 Etiology and epidemiology

Many viruses can cause CNS infection. Although herpesviruses and enteroviruses have worldwide distribution, other important viruses have more limited geographical distribution, including the West Nile virus, Japanese encephalitis virus, Tick-borne encephalitis virus (TBEV), Rabies virus, and Zika virus. Moreover, historically common causes of CNS infection include diseases now seldom seen thanks to childhood immunization, such as polio, measles, mumps, and rubella. However, due to variations in vaccine coverage and growing vaccine skepticism, knowledge of these diseases remains relevant, since outbreaks occasionally occur all over the world.

In Sweden, viral meningoencephalitis cases are reported to the Public Health Agency of Sweden (Folkhälsomyndigheten). Although an underreporting of at least 50% is presumed with 9.8 cases/100,000 inhabitants/year in 2019, the distribution of etiologies over the past five years are presented in Figure 4. In addition to the herpesviruses, which are the focus of this thesis, important viruses in Sweden are TBEV and enteroviruses, which together account for more than 50% of reported viral CNS infections.

Aseptic meningitis is the most common manifestation of viral CNS infection, with an incidence estimated at 20 cases/100,000 inhabitants/year, or about tenfold more common than encephalitis [36, 37]. The true incidence is hard to determine, as many patients have mild symptoms and do not seek medical care. The incidence of encephalitis is lower than meningitis, with 5 cases/100,000 inhabitants/year according to a British study on an adult population [38]. Children are overrepresented, with an incidence of up to 10.5 cases/100,000 inhabitants/year [39]. The geographical distribution of certain viruses may contribute to large differences in incidence, especially during outbreaks.

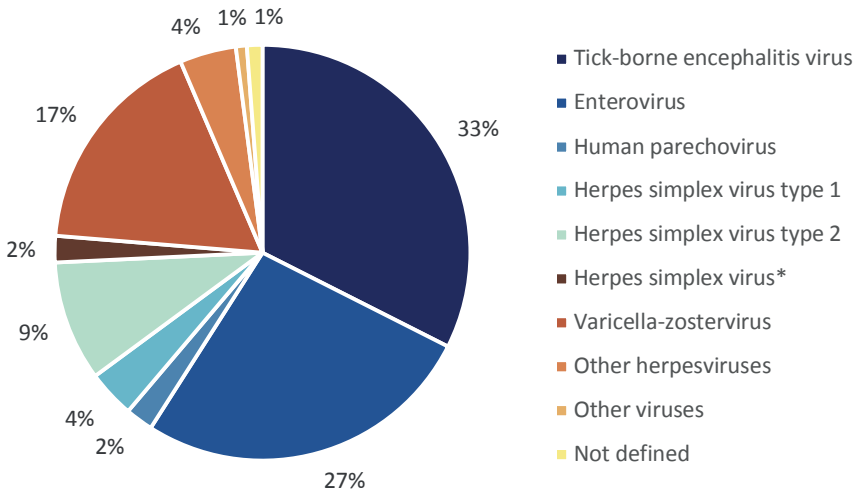

*Figure 4. Etiology of meningoencephalitis in Sweden 2015-2019. *Type not reported.*

1.6 HERPES SIMPLEX VIRUS IN THE CNS

1.6.1 Herpes simplex encephalitis

Epidemiology

Typically, HSE is caused by HSV-1, though a few percent of cases can be attributed to HSV-2 [40, 41]. Although this disease is rare, with an estimated incidence of 2-4 cases/1,000,000 inhabitants/year [42], it is the most common cause of sporadic viral encephalitis [43-45]. HSE is not regarded as an opportunistic infection, even though immunodeficiency is associated with an increase in mucocutaneous manifestations [46]. Recent case reports point to an increase in risk of HSE under certain circumstances, such as with administration of newer immunomodulatory drugs that affect lymphocyte populations [47].

Clinical symptoms and prognosis

Initially, the patient may experience influenza-like illness/prodromal gastrointestinal and respiratory symptoms with fever. As the disease progresses, typical symptoms include severe headache accompanied by fever, altered mental state with confusion and even coma, seizures which are often focal, and speech abnormalities such as dysphasia, as well as other focal neurological deficits [48]. Before modern treatment, the mortality rate was

50% or higher [49], but even with treatment, the risk of a fatal outcome is considerable at 10%-20%, and morbidity is substantial; most survivors suffer from life-long sequelae of varying degrees of severity [42, 50, 51]. Memory impairment and personality/behavioral abnormalities are well described [52, 53]. Dysphasia and anosmia (loss of smell) are frequent [42, 53], and one-fourth of patients suffer from epilepsy [54].

Pathogenesis

Although HSE may occur with primary infection, most cases are associated with reactivation of latent virus. However, it is not entirely clear how the virus gains access to the CNS. Possible pathways include retrograde axonal transport through the trigeminal or olfactory tracts to the brain [55], corresponding to the typical focal localization. A different but less supported hypothesis is local reactivation of virus; latent virus has been found in autopsy studies of the brain [56]. Hematogenous spread may also be possible, since HSV-1 can infect endothelial cells and alter permeability of the blood-brain barrier [57].

HSE is a focal, necrotizing, hemorrhagic encephalitis which usually involves the limbic system and the temporal lobe, including the hippocampus, which explains short-term memory deficits. Other areas that may be affected include the lower frontal lobe, parietal and occipital lobes [51]. Lesions may be bilateral, but in most cases more pronounced on one side of the brain. Cerebrovascular involvement is well described, often with petechial bleeding, but in some cases intracerebral hemorrhage or ischemic stroke may occur [58].

Pathologic findings associated with viral replication include ballooning of cells, fusion with multinucleated giant cells, and cell lysis. Typically, an abundance of cytotoxic T-cells are also present in the focal lesions of HSE [59]. The immunological response to infection likely contributes significantly to brain tissue damage, as supported by mild histopathological changes, but with less evidence of necrosis in immunocompromised patients [60]. In such cases, HSE may follow an atypical and more slowly deteriorating course of disease [61].

Host response

Both innate and adaptive immune components are essential in the response to HSE. In the initial innate immune response, microglia are recruited to the infection site where they produce type I interferons (IFNs) that inhibit HSV-1 replication in neurons. Recognition of viral double-stranded RNA through Toll-like receptor (TLR) 3 is crucial for initiation of the response, and genetic

defects disrupting the TLR3 pathway have repeatedly been described in HSE patients [62].

In addition, chemokines attract peripheral immune cells, including $CD4^+$ and especially $CD8^+$ T lymphocytes, which are part of the adaptive immune response. IFN-γ released from $CD8^+$ T lymphocytes is essential for inhibition of neuronal apoptosis that may be associated with massive destruction of neurons. Many additional components in the immune response, such as production of reactive oxygen species and nitric oxide, may help control the infection, but may also contribute to extensive tissue damage [47].

In cerebrospinal fluid (CSF) studies, high levels of interleukin-6 (IL-6) and IFN-γ are found during the first, acute phase of HSE. After 2-6 weeks, increases are seen in tumor necrosis factor alpha (TNF-α) and markers of T-lymphocyte activation such as soluble CD8 [63]. An increase in the ratio between proinflammatory IL-1β and IL-1 receptor antagonist has been associated with poor outcome [64]. There is also evidence of prolonged intrathecal immune activation, since markers of intrathecal inflammation such as neopterin and β2-microglobulin may be present many years after HSE [65].

Of recent interest, HSV-1 has also been shown to trigger autoimmunity toward the N-methyl-D-aspartate receptor (NMDAR) in up to one-third of patients [66]. Development of anti-NMDAR antibodies is associated with protracted recovery and, in some cases, autoimmune encephalitis [67, 68]. Whether cause or effect, a longer proinflammatory CSF response has been reported in these patients [69].

Relapse

A small subset of patients may experience clinical relapse following HSE, most cases within a few months, but sometimes after several years [70]. Although true relapses with viral replication may occur [71], immunological mechanisms, including anti-NMDAR encephalitis, may be responsible for a significant proportion of these cases [70, 72].

1.6.2 Herpes meningitis

Incidence

Herpes meningitis is almost exclusively associated with HSV-2 infection, even though HSV-1 is increasingly seen in genital herpes infection. In studies of aseptic meningitis across the board, HSV-2 is responsible for 15%-20% of total cases in the Nordic countries, with an overrepresentation of female patients [37, 73, 74]. Most cases occur among patients age 50 or younger [75].

Clinical symptoms and prognosis

Typically, symptoms of this disease are those of aseptic meningitis: increasingly severe headache over a few days, sensitivity to light and sound, nausea, and sometimes neck stiffness and fever. Acute symptoms usually resolve within days, but protracted illness has been described [76, 77]. Additionally, a subset of patients may experience lingering neurological symptoms and mental fatigue many months after an episode [78]. Mucocutaneous lesions may be associated with meningitis, but are not obligatory, and more than half of patients have never experienced genital lesions [75]. In some cases, especially in HSV-2 meningitis associated with primary genital infection, additional neurological symptoms indicative of sacral radiculitis may be present, including urinary retention, sensory disturbances, and radiating pain or weakness in the lower extremities [79].

Patients with a history of HSV-2 meningitis may experience recurrence in about 20% of cases; some patients may even have multiple recurrences in a single year [76, 78, 80]. Recurrences are usually milder than primary meningitis, often with absence of symptoms such as neck stiffness and fever [76].

Pathogenesis

The first episode of HSV-2 meningitis may be associated with primary genital infection [77], but may also result from reactivation of latent virus. In cases where genital lesions occur in association with meningitis, they usually precede CNS symptoms by a median of 1 week, supporting the theory of neuronal spread to the meninges [76].

Although it is likely that HSV-2 enters the CNS and subarachnoid space through neuronal transport, the pathogenesis of viral meningitis is poorly understood. It is evident that inflammation is present, as supported by recruitment of lymphocytes. However, since few cases of meningitis prove fatal and encephalitis has been the main focus of animal studies, there is a lack of data on viral replication sites and pathological processes.

1.6.3 Other manifestations

Rarely, HSV may cause myelitis with symptoms including sensory and motor function loss in the lower extremities and urinary bladder dysfunction, in which case prognosis is poor with sequelae such as paraplegia commonly seen, despite antiviral treatment [81].

HSV-1 has also been suggested as a potential cause of idiopathic peripheral facial palsy (Bell's palsy). The HSV-1 genome has been detected in the facial nerve of patients with Bell's palsy undergoing surgical decompression [82],

but viral replication has not been consistently proven, and antiviral treatment has not yet proven to be effective [83].

Acute retinal necrosis may be associated with herpesvirus infection, and HSV-1 or HSV-2 DNA can sometimes be detected in intraocular samples [84].

1.7 VARICELLA-ZOSTER VIRUS IN THE CNS

1.7.1 Disease panorama and incidence

The CNS manifestations of VZV infection are highly diverse, possibly explained by systemic primary infection and establishment of latency along the entire neuraxis. Additionally, a wide tropism to different cell types, with vascular endothelium possibly serving as a site of viral replication in addition to nerve cells, may contribute to the variety of syndromes. Both primary infection and reactivation with (herpes zoster) or without (zoster sin herpete) mucocutaneous manifestation may be associated with CNS symptoms [85-88]. The overall incidence of VZV-associated CNS infection is poorly investigated, but with the advent of widespread access to polymerase chain reaction (PCR) diagnostics, an increasing number of cases are attributed to VZV. The risk of severe disease may be higher among immunocompromised patients [43, 89, 90].

1.7.2 VZV encephalitis

VZV is the second most common etiology of encephalitis in Western countries [43-45]. Most patients present with an altered mental state including disorientation; associated focal neurological symptoms, such as cranial nerve palsies, are frequently observed, while seizures are uncommon [43, 45, 91]. Studies show that the mortality rate with treatment is 10%-20%, but these reflect a relatively small number of cases [43, 87, 91]. Among immunocompromised patients, most thoroughly studied in patients with AIDS, the infection tends to be severe, disseminated, and associated with poor prognosis [92]. Long-term outcome has been reported to be similar to that seen in HSE [43, 93], with cognitive and behavioral sequelae being most common [94-96].

Pathogenesis

As in HSV, VZV is likely transferred to the CNS after reactivation of latent virus in ganglia with subsequent centripetal axonal transport [92]. The pathogenesis of VZV encephalitis is debated: specifically, as to whether it is mainly a disease involving the brain parenchyma [91], or if symptoms of

encephalitis are caused by vasculopathy with secondary neuronal damage and demyelination [97]. There may be some truth in both explanations, since many cases show evidence of infection in the cerebral vascular walls [98-101], while such evidence is absent in others [91]. Spread of VZV into glial cells has been demonstrated in immunocompromised patients [92], but the extent to which this applies to more typical cases of encephalitis in immunocompetent patients remains uncertain.

1.7.3 VZV meningitis

VZV has been identified in 5%-10% of cases of aseptic meningitis [37, 44, 102]. Patients with VZV meningitis tend to be younger than those with VZV encephalitis [44]. Typical symptoms of meningitis may present with or without associated herpes zoster rash [87]. Although the prognosis of VZV meningitis is generally considered good, the outcome is not well studied, and sequelae may be underestimated, with reports of decreased quality of life [102] and mild cognitive impairment [94].

1.7.4 VZV facial palsy - Ramsay Hunt syndrome

Of particular interest to this thesis is Ramsay Hunt syndrome (RHS), palsy of the seventh cranial nerve, the facial nerve. The classic definition of RHS includes peripheral facial palsy with characteristic rash in or adjacent to the ipsilateral ear (zoster oticus), frequently with associated vestibulocochlear symptoms such as hearing impairment, tinnitus, and vertigo [103] (Figure 5). The classical definition of RHS may fail to include all VZV facial palsy cases, since PCR enables detection of VZV in the absence of rash. Studies concerning incidence are not based on uniform testing and likely underestimate the extent of peripheral facial palsy caused by VZV. A Danish study of 2,570 cases of peripheral facial palsy diagnosed RHS in 4.5%, but only patients with rash were included [104]. A German study of 509 cases found VZV in 6.6%, with rash occurring in only 62% of RHS cases, although PCR was not consistently performed [105]. Correct diagnosis of VZV is essential, since facial palsy caused by VZV is associated with a worse prognosis than idiopathic peripheral facial palsy (Bell's palsy) [104, 106]. It is also crucial to differentiate VZV facial palsy from other causes that may warrant specific treatment, such as Lyme neuroborreliosis (LNB), infection with *Borrelia Burgdorferi* sensu lato following tick-bite [107]. As symptoms at presentation may be identical regardless of etiology, appropriate diagnostic procedures must be performed.

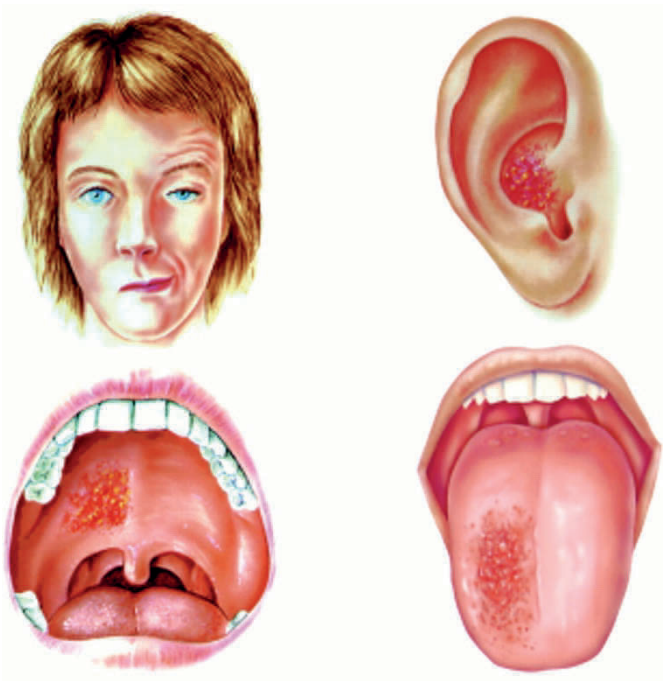

Figure 5. Ramsay hunt syndrome with peripheral facial palsy on the right side, vesicular rash in the ipsilateral ear, hard palate, and anterior two-thirds of the tongue.
[Reprinted from C J Sweeney, and D H Gilden, J Neurol Neurosurg Psychiatry, 2001;71:149-154, with permission from BMJ Publishing Group Ltd.]

1.7.5 VZV vasculopathy

Cerebrovascular disease, or vasculopathy caused by VZV, is receiving increased attention. Epidemiological studies have shown that the risk of stroke increases after an episode of herpes zoster [108, 109], especially zoster of the ophthalmic or trigeminal nerve [110, 111]. VZV is also the most frequent cause of acute ischemic stroke in children [112, 113]. Both small and large vessel involvement may occur [114], with evidence of viral replication in the arterial wall [99].

1.7.6 Other

Although VZV may manifest in other ways, including various cranial nerve manifestations, acute retinal necrosis, acute cerebellar ataxia in children, myelitis, brain stem encephalitis, and encephalopathy, the distinction between syndromes is not always clear. It is also debatable whether cranial nerve involvement truly represents infection of the CNS. In most cases, the

cranial nerves are considered to be part of the peripheral nervous system, with the exception of the olfactory and optical nerves, which are structurally similar to tracts of the CNS. Nevertheless, a similar approach to diagnosis and treatment of cranial nerve involvement is taken as in the more well-defined CNS syndromes described above. One exception is herpes zoster ophthalmicus, VZV reactivation in the first branch of the trigeminal nerve, which should be treated in the same way as peripheral herpes zoster. Also of note, in regard to herpes zoster infection without clinical symptoms of CNS involvement, diagnostic procedures including lumbar puncture may show pathological findings [115], which may raise further questions concerning the distinction between involvement of peripheral nerves and the CNS.

1.8 DIAGNOSIS OF HERPESVIRUS CNS INFECTIONS

Since there is considerable overlap between syndromes in CNS infections and their non-infectious differential diagnoses, analysis of CSF obtained through lumbar puncture is central to the diagnostic work-up.

1.8.1 CSF parameters

CSF cell count

In viral CNS infection, white blood cell (WBC) count is often moderately increased, typically with a predominance of mononuclear cells [37]. WBC count is often higher in HSV meningitis than in encephalitis, albeit with individual variations [75]. In exceptional cases, patients may present without increased cell counts, for example early in the course of HSE, where repeat CSF sampling after a few days may be necessary to demonstrate pleocytosis [51]. Other examples include VZV vasculopathy and viral CNS infections in neonates and infants.

CSF glucose and lactate concentrations

Measurements of glucose and lactate in the CSF are primarily of interest in bacterial CNS infection and are often normal or marginally abnormal in viral CNS infection [36, 116].

CSF protein

Measurements of protein or albumin reflect blood-brain barrier (BBB) integrity and are often increased in alphaherpesvirus CNS infections. The BBB shelters the brain from the rapidly changing environment of circulating blood, including any microorganisms that may be present, utilizing physical barriers and active transport mechanisms in specialized endothelial cells of

cerebral blood vessels. CNS infections may disrupt the BBB integrity, allowing increased passage of various molecules into the brain and CSF, including protein/albumin, which are often found to be increased [117]. To compensate for individual variations in serum proteins, the CSF:serum albumin ratio is often calculated [CSF albumin (mg/L)/serum albumin (g/L)] [118]. In addition to providing information valuable to the assessment of CNS infection, BBB disruption may facilitate increased transport of therapeutic agents into the CNS, previously reported with antiretrovirals [119] and antibiotics [120, 121].

1.8.2 Diagnosis of viral etiology

Since the discovery of viruses and viral culture more than 100 years ago, technological advances have dramatically changed the landscape of diagnostics. Today, cultivation of viruses is restricted to research laboratories, although special circumstances such as testing for phenotypic resistance to antivirals may still be relevant to clinical decision-making.

Detection of nucleic acids such as viral DNA or RNA using PCR-based methods is the primary tool for diagnosing viral CNS infection; however, there are situations where serological testing is crucial to the diagnostic work-up. PCR methodology relies on the presence of viral nucleic acid in the specimen, typically CSF, and generally performs very well in the acute phase of CNS infection when there is active or recent viral replication. Situations may arise where sampling is performed during late stages of disease, when PCR sensitivity is low. In such situations, serological testing may be necessary to provide an etiologic diagnosis (Figure 6).

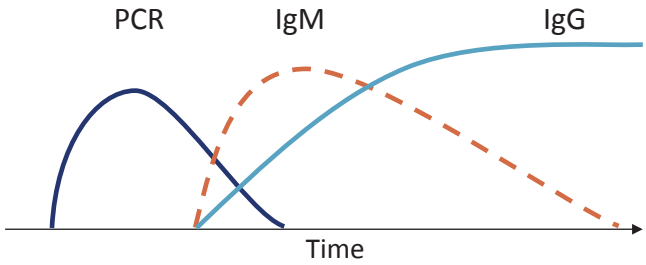

Figure 6. Choice of diagnostic methods depend on timing of sampling. Viral DNA can be detected with polymerase chain reaction (PCR) in the early stages of infection. IgM and IgG antibodies take time to develop, and can be detected in the later stages of disease.

The PCR method(s)

The PCR method was developed in the 1980s [122] and entails cyclic replication of DNA segments. Each cycle produces twice the amount of DNA, resulting in exponential replication. A primer sequence is required to initiate the reaction, tailored to a DNA sequence of the test target.

In traditional, qualitative PCR, the PCR product is analyzed after a fixed number of cycles. If the test target is present in the sample, the exponentially increased number of copies is detected, and the result is considered positive.

In real-time quantitative PCR (real-time PCR), the reaction is continuously monitored with fluorescent dyes or DNA probes. The number of cycles needed to reach a predetermined signal threshold depends on the amount of DNA in the initial sample and is referred to as the cycle threshold (Ct value). Using mathematical calculations or comparisons with control samples with known pathogen loads, the viral load (i.e., virus copies/mL CSF) in the patient sample can be estimated (Figure 7).

Multiplex PCR entails the use of several different primers and probes in the same reaction, allowing multiple pathogens to be analyzed simultaneously [123].

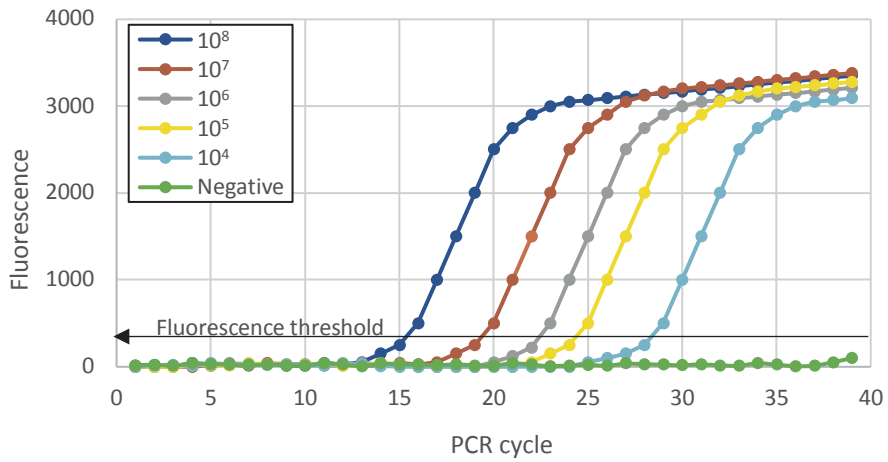

Figure 7. Real-time PCR. Amplification plots with fluorescence plotted against cycle number. Amplicons with higher initial loads break the fluorescence threshold after fewer cycles.

In recent developments, an increasing number of rapid molecular assays with turnaround times of minutes to a few hours have become available. Some utilize variations of PCR technology, while others use a relatively new technique called loop-mediated isothermal amplification [124]. These tests are available as commercial kits, tailored to testing based on patient

symptoms (e.g., diarrhea or respiratory infection), which is referred to as syndromic testing. One such multiplex panel is available for testing CSF in suspected CNS infection, the FilmArray meningitis/encephalitis (ME) panel (BioFire Diagnostics, Salt Lake City, UT). The ME panel is based on PCR technology and has a turnaround time of 1 hour [125]. Although the diagnostic accuracy of this panel has been reported as high [126], the data concerning adults is still sparse. There are also reports of false negative and positive results, the latter possibly being exacerbated by the syndromic approach, that warrant further investigation.

Interpretation of PCR results

PCR is highly sensitive and specific in most cases. There are, however, situations where interpretation can be difficult, as well as risks of false positive and false negative results.

False positive results may be generated if the sample or laboratory equipment is contaminated with PCR substrate from an unintended source (e.g., virus from skin lesions when analyzing CSF, or contamination from an adjacent sample in the preparation process). Preventive measures during handling and preparation are necessary to minimize risk of contamination. The primer sequence must also be unique to the desired organism without homologous sequences from other species.

A false positive signal may also be generated by dimerization of the PCR primers at the 3' end, with production of amplicons in small quantities typically reaching the threshold for detection after 30 cycles or more [127]. Careful selection of primers to avoid 3' complementarity and appropriate diagnostic cut-off Ct values are essential. The risk of misinterpretation is higher in qualitative PCR than in real-time PCR, since the Ct value cannot be measured.

False negative results may be generated by a poor choice of primers if the target sequence is in a variable region prone to mutations or gene polymorphism, rather than in a highly conserved region of the pathogen genome. Timing of sample retrieval may be critical, depending on disease kinetics. Additionally, many factors in sample handling and preparation must be carefully considered to optimize the method, including protocols to prevent PCR inhibitors present in many sample types that may obstruct the reaction [128].

1.8.3 HSV PCR

Qualitative PCR for HSV, introduced in the early 1990s [40, 129], has a 95% sensitivity in HSE [130]. In HSV-2 meningitis, sensitivity has been estimated

at 90% in primary infection but substantially lower in recurrent infection at 70% [73]. Real-time PCR was introduced in the early 2000s, with equal or higher sensitivity compared with qualitative PCR [131], and is now the method of choice.

HSV DNA kinetics

Despite high sensitivity, there are situations where CSF from a patient with HSE may be PCR-negative. If sampling is performed very early after the onset of symptoms (<3 days), repeat sampling may be necessary to detect HSV-1, although this is mainly demonstrated with qualitative PCR [40, 132, 133].

Although real-time PCR allows for quantification, a high initial viral load does not seem to be associated with poorer outcomes in HSE [134]. HSV-1 is generally no longer detectable in the CSF of HSE patients after 1-4 weeks of treatment [40, 134], but a longer duration before negative PCR has been associated with poorer outcome [134].

1.8.4 VZV PCR

Real-time PCR of CSF is the primary method for diagnosing VZV CNS infection [135], but sensitivity and specificity have not been thoroughly studied and may vary based on disease manifestation. In vasculopathy such as ischemic stroke, VZV DNA may be absent from the CSF [114]. Also, VZV DNA may sometimes be detectable in the CSF of patients who are asymptomatic for CNS infection, as demonstrated in 10/46 patients with herpes zoster in one study [115]. If CSF sampling cannot be performed, concurrent mucocutaneous or other specific disease manifestations may allow for VZV DNA detection in specimens from other sources, such as skin or saliva in RHS [136].

In meningitis and encephalitis, high viral load has been demonstrated, while cranial nerve manifestations, such as RHS, may be associated with low viral load [87]. Higher viral loads may be related to the severity of disease [137], but this is uncertain.

1.8.5 Serological diagnosis of HSV and VZV in the CNS

Since herpesvirus CNS infection is often the result of viral reactivation, the presence of serum antibodies alone lacks specificity. Increases in antibody titers in repeated samples, detection of IgM, or seroconversion (in primary infection) may indicate recent infection, though such findings are not specific to the CNS. However, should there be evidence of intrathecal antibody production, etiologic diagnosis can be established. Intrathecal antibody production is determined by comparing antibody titers in CSF and serum.

Corrective calculation to adjust for BBB damage is necessary, e.g., using Reibers formula [138].

In HSE, intrathecal HSV-1 antibody production has been demonstrated with high sensitivity after ten days [139]. Intrathecal antibodies may persist for many years [140], which has implications for the evaluation of future episodes of CNS symptoms. In herpes meningitis, however, the presence of intrathecal HSV-2 antibodies has not been consistently proven [76], and recurrent episodes do not appear to change serum antibody titers [80].

In VZV infection of the CNS, especially vasculopathy, where symptoms often occur in a late, PCR-negative phase, evidence of intrathecal VZV antibody production may be necessary to establish the diagnosis [114].

1.8.6 Future diagnostics

Despite significant advancements in diagnostics, in many cases the etiology of CNS infection cannot be established. The cause in half of all encephalitis cases remains unknown [43, 45], and the corresponding figure for meningitis is 40%-60% [37, 73]. Although some of these cases may be attributed to autoimmune disorders, further technological advancements may allow for identification of additional pathogens. One such technology is metagenomic next-generation sequencing (mNGS), in which all nucleic acids (DNA and RNA) from a CSF sample are amplified and sequenced. After bioinformatic processing, non-human sequences are matched to known sequences in large databases, thereby allowing for possible identification of pathogens. This technology is rarely utilized outside of research labs, but reports of successful use have been published [141], and more widespread adoption can be expected as the technology matures.

1.8.7 Neuroimaging

Computed tomography (CT) and magnetic resonance imaging (MRI) are the most frequently used neuroimaging methods. In viral CNS disease, MRI is more sensitive than CT, but CT is more readily available and may have advantages in rapidly excluding certain differential diagnoses.

In HSE, CT scan typically demonstrates uni- or bilateral focal encephalitis with edema of the temporal lobe, in some cases with hemorrhage. However, in 20%-50% of cases, findings may be unremarkable, especially if performed early after onset of symptoms [50, 51, 142]. MRI is far superior (Figure 8), and demonstrates abnormalities in almost all cases [50, 143], characteristically in the temporal lobe and adjacent structures such as the

limbic system and sometimes the thalamus. Extensive MRI abnormalities, especially bilateral, are associated with a worse prognosis [50, 144].

MRI has been shown to demonstrate leptomeningeal contrast enhancement in viral meningitis [145], though neuroimaging is mainly indicated to exclude differential diagnoses, including overlapping syndromes such as meningoencephalitis or meningomyelitis.

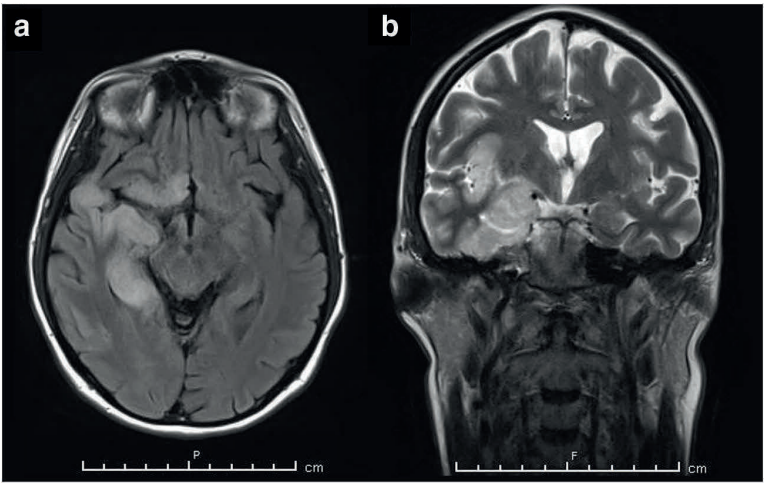

Figure 8. MRI in Herpes simplex encephalitis. (a) Axial projection with hyperintense lesions of insular, temporal, and frontal right lobes. (b) Coronal T2 projection with hyperintense lesions of temporal right lobe.
[Reprinted by permission from Springer Nature. Journal of NeuroVirology. Cerebral venous thrombosis: a rare complication of herpes simplex encephalitis, M. Vedani et al., 2019.]

Neuroimaging findings in VZV CNS infection are less uniform, much as the symptomatology, and frequently show no abnormalities [146]. However, diffuse edema and multifocal abnormalities, typically on T2-weighted images, may be present. Both cortical and deep structures, including the brainstem, may be involved, with lesions in both grey and white matter [115, 147]. Concerning vasculopathy, both small and large vessel involvement have been demonstrated in both conventional and MR angiography [114]. Involvement of the middle and anterior cerebral arteries, as well as the external carotid, are most frequently described [114, 148]. It has been proposed that varicella encephalitis is due to vasculopathy, mainly in small vessels [97], and technological limitations related to demonstration of small vessel involvement may explain the lack of neuroimaging support. In cranial nerve palsies, MRI may demonstrate inflammation, similar to that seen in the facial and vestibulocochlear nerves in RHS [149].

1.8.8 Additional diagnostic methods

Neurophysiological examination, specifically electroencephalography (EEG), may be useful in diagnosing encephalitis. EEG is often abnormal, though many changes are nonspecific. Patients with HSE may more frequently present with uni- or bilateral focal slowing, as well as periodic lateralizing epileptiform discharges (PLED) than what is seen with other types of encephalitis, but not exclusively so [150, 151]. EEG is also important in diagnosing epileptic activity that may otherwise have gone unnoticed.

1.9 BIOMARKERS

Biomarkers are biochemical substances that can be measured by analyzing samples from patients or study subjects. They are often expressed in patterns corresponding to pathological processes or immunological responses, and investigation of such patterns is a rapidly expanding research field. When measured in CSF, they may reflect biological and pathological processes in the CNS, since considerable exchange occurs between cerebral extracellular fluid and CSF. In addition to helping us understand the pathology underlying various diseases, biomarkers may aid in estimating prognosis, monitoring treatment response, and establishing differential diagnosis.

1.9.1 NFL

Neurofilament light chain protein (NFL) is a structural component of the cytoskeleton in nerve axons, both in the large myelinated axons of the CNS and in peripheral nerves [152], and is present only in insignificant amounts in other neuronal and neuroendocrine tissue [153]. Damage to these neurons results in a release of neurofilament into the surrounding extracellular fluid and CSF, where it can be measured as a sign of neuronal damage.

HSE is associated with very high NFL concentrations in the CSF, which increase 2-3 weeks after disease onset and remain increased for months [154]; and a correlation between peak NFL concentrations and impaired neurocognitive performance has recently been demonstrated [69]. In TBE and LNB, moderate elevations have been observed, though in cases of TBE with paresis, NFL concentrations are high [154, 155]. In VZV CNS infection, higher NFL concentrations have been demonstrated in CSF from patients with encephalitis than in other VZV syndromes [156], but further investigation is needed to provide more data on specific manifestations. It is possible that determination of NFL concentrations could help to identify patients with a high risk of neurological sequelae who may need more intensive treatment, for example among those with VZV facial palsy.

1.9.2 GFAP and S100B

Glial fibrillary acidic protein (GFAP) is a structural component of importance to morphological changes in astrocytes in response to stimuli and injury [157]. S100B is a protein with paracrine and autocrine effects on glial cells that is also expressed in astrocytes and present in the cytoplasm [158].

In CNS disease with extensive structural damage, GFAP and S100B concentrations become increased in response to astroglial destruction, as observed in brain infarction [159] and HSE [154]. In conditions with less pronounced structural damage, such as TBE [154], neuroborreliosis [155], and VZV CNS infections [156], a pattern of increased GFAP, but not S100B, has been observed. A suggested explanation is activation of astroglia in response to infection/inflammation but without disruption of the astroglial cell membrane.

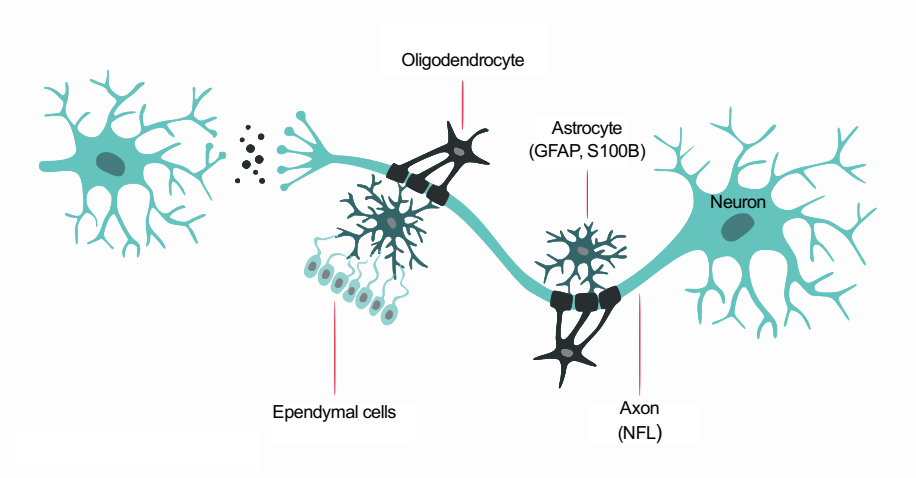

Figure 9. Origins of biomarkers. Neurofilament light chain protein (NFL) is a structural component of myelinated axons. Glial fibrillary acidic protein (GFAP) and S100B are expressed in astrocytes.
[Adapted from original artwork with permission from The Brain Tumor Charity (https://www.thebraintumourcharity.org).]

1.9.3 CXCL13

Among other biomarkers of interest in CNS infections, the Chemokine [C-X-C motif] ligand 13 (CXCL13; B-cell chemoattractant) warrants special attention. It has been proposed as a diagnostic marker in LNB, since the disease is associated with high CSF concentrations [160], possibly because it is necessary for recruitment of B-cells in the early immunological response to LNB [161]. There are, however, other CNS diseases associated with increased concentrations, such as bacterial meningitis and neurosyphilis [162, 163]. In

facial palsy, there is some overlap with suggested diagnostic cut-offs [164]; further investigation of the potential to differentiate between different etiologies (e.g., LNB and VZV) would be of interest in these patients.

1.10 TREATMENT

1.10.1 History of anti-herpesvirus therapy

The first significant step toward effective antiviral treatment for herpesviruses was taken in the late 1950s, with the synthesis of the nucleoside analogue idoxuridine [165]. During the 1960s, it was discovered to have activity against DNA viruses, both *in vitro* and *in vivo*, but toxicity prevented its use [166]. Not until the late 1970s, with the introduction of the nucleoside analogue vidarabine (adenine arabinoside), did antiviral treatment of herpesvirus infections become realistic. Vidarabine efficacy was proven for both HSE [167] and herpes zoster in immunocompromised patients [168], but it was difficult to dissolve, making administration problematic [169].

In the early 1980s, vidarabine was quickly replaced by acyclovir (9-[2-hydroxymethyl]guanine), which had the advantages of better solubility and selective phosphorylation in herpesvirus-infected cells, resulting in significantly better treatment outcomes for herpesvirus infections, including HSE [49, 170]. Although a few additional antivirals such as cidofovir and foscarnet have since been introduced, acyclovir remains the first-line treatment of HSV and VZV infections.

1.10.2 Acyclovir and valacyclovir

Mechanism of action

Acyclovir is an analogue to deoxyguanosine (Figure 10), one of the four deoxyribonucleosides that make up DNA, possessing an acyclic side chain lacking the 3'hydroxyl group required for elongation of the DNA chain during replication. Before incorporation into DNA, three phosphorylation steps are required. First, following uptake in the infected cell, acyclovir is phosphorylated into acyclovir monophosphate by viral thymidine kinase, which in HSV is a millionfold more active than host cell thymidine kinase in converting acyclovir. This explains the selective properties of acyclovir and results in concentrations of acyclovir monophosphate that are 40-100 times higher than in uninfected cells. After two additional phosphorylations by host cell enzymes, acyclovir triphosphate competes with deoxyguanosine triphosphate as a substrate for primarily viral DNA polymerase, resulting in termination of DNA elongation and inactivation of viral DNA polymerase in

infected cells [171]. VZV thymidine kinase has lower affinity for acyclovir than its HSV counterpart, and thus *in vitro,* higher concentrations are required for viral inhibition [172].

Valacyclovir is an oral prodrug to acyclovir, with significant pharmacokinetic advantages compared with oral acyclovir. It is the L-valyl ester of acyclovir, and is converted to acyclovir and the amino acid L-valine by liver hydrolases during first-pass metabolism after absorption.

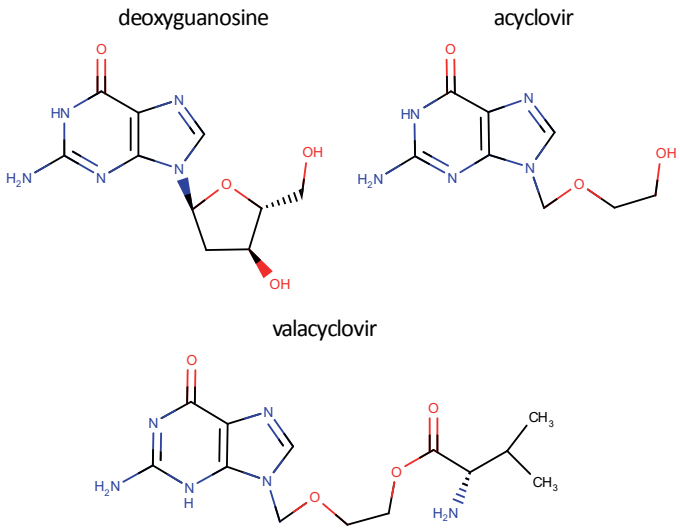

Figure 10. Chemical structures of deoxyguanosine, acyclovir and valacyclovir.

Pharmacokinetics of acyclovir and valacyclovir

Bioavailability in oral administration of acyclovir is relatively poor at 15%-30%, for which reason intravenous administration is recommended for severe disease.

The bioavailability of valacyclovir is three to five times higher than that of oral acyclovir [173]. An oral dose of 1000 mg three times daily provided acyclovir exposure (area under curve = AUC) comparable to i.v. acyclovir 5 mg/kg every eight hours (q8h) in patients with neutropenia due to chemotherapy [174], while in healthy volunteers, an oral dose of 2000 mg q6h resulted in acyclovir exposure (AUC) comparable to i.v. acyclovir 10 mg/kg q8h [175].

Acyclovir is hydrophilic, and about 15% is bound to plasma proteins [176]. It is widely distributed in the body, with high concentrations in organs such as the liver, lungs, kidneys, heart, and skin [171]. However, concentration is significantly lower in the CSF with an AUC at 20%-25% compared with

serum [177-179], suggesting that the BBB is responsible for slow diffusion into and active transport out of the CNS. Notably, the available data on CSF concentrations were obtained from patients without acute herpesvirus CNS disease and disruption of the BBB, which creates uncertainties regarding CSF concentrations during treatment. In HSE patients treated with valacyclovir 1000 mg three times daily in a very resource-limited setting [180], acyclovir concentrations in CSF were higher than those reported in patients with multiple sclerosis [179], indicating potential pharmacokinetic benefit from BBB damage.

The primary mode of elimination is via renal excretion; more than 60% of the administered dose is excreted unmetabolized, both through glomerular filtration and active tubular secretion [181]. The remainder is metabolized (Figure 11). One metabolic pathway is through alcohol dehydrogenase and aldehyde dehydrogenase, producing 9-carboxymethoxymethylguanine (CMMG), with acyclovir aldehyde as an intermediate metabolite. A second pathway is directly through aldehyde dehydrogenase to 8-hydroxy-acyclovir (8-OH-ACV). About 10% of the administered dose is excreted as CMMG and 1% as 8-OH-ACV [177, 182]. Interestingly, 8-OH-ACV more readily crosses the BBB, with similar concentrations of CMMG and 8-OH-ACV recovered in the CSF [177]. In patients with normal renal clearance, the half-life of acyclovir is less than 3 hours, increasing to almost 20 hours in patients with renal failure, and a higher proportion is excreted as metabolites in the latter group [181].

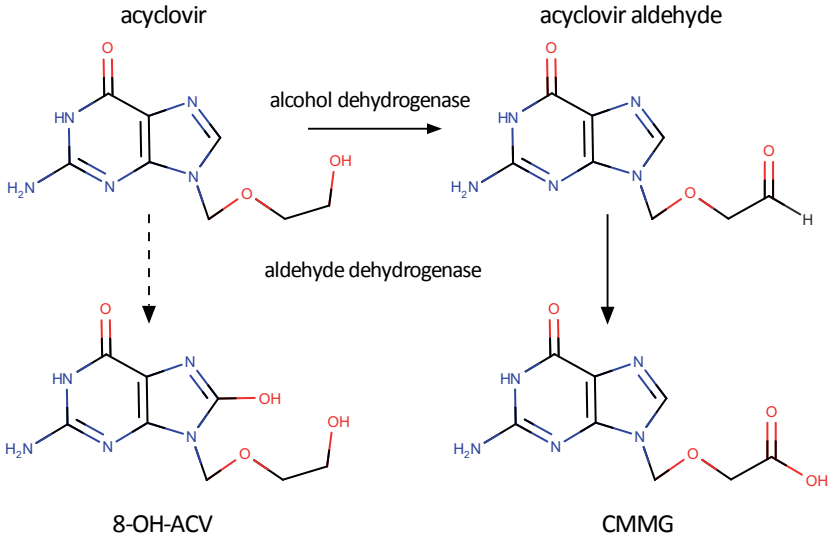

Figure 11. Metabolic pathways of acyclovir.

Acyclovir toxicity

Most patients tolerate acyclovir well in therapeutic doses. The most frequent manifestation of acyclovir toxicity is decreased renal function [183], manifesting in 10%-20% of patients treated with intravenous acyclovir [184, 185]. The most widely accepted mechanism is the formation of crystals that are insoluble in urine with precipitation in renal tubules leading to acute renal failure [186]. A second proposed mechanism is direct insult to renal tubular cells from locally produced metabolic intermediate acyclovir aldehyde, which is supported by *in vitro* experiments and reports of renal failure in the absence of crystalluria [187]. Recommendations to prevent decreased renal function include proper hydration before and during administration, dose adjustments in patients with known renal impairment, and avoidance of bolus administration [186]. Acute renal failure due to acyclovir seems to be reversible in most cases, although some patients may require temporary dialysis [184].

Less well known and likely less frequent is acyclovir-induced neuropsychiatric symptoms (AINS). First described in the 1980s [188], symptoms include but are not limited to confusion, hallucinations, ataxia, myoclonus, and even coma [189]. AINS has primarily been described in patients with acute or chronic renal failure, but the pathophysiological mechanism is unknown. High acyclovir concentrations with decreased renal clearance may contribute, but the association is inconsistent [190]. There is a stronger association between AINS and high concentrations of the metabolite CMMG, both in serum [189] and CSF [191]; CMMG or one of the other metabolites may be responsible by some yet undiscovered mechanism.

Indications and dosing regimens in CNS infections (Clinical efficacy)

HSE

In HSV CNS infection, the standard dose regimen of intravenous acyclovir is 10 mg/kg q8h, based on doses proven to be effective in 2 randomized controlled trials in a comparison with vidarabine among patients with HSE [49, 170]. The relationship between CSF concentrations and efficacy has not been studied. Many patients experience life-altering sequelae despite treatment, and higher doses of up to 15 mg/kg q8h are sometimes recommended in young patients with high creatinine clearance. However, no benefit was shown in a retrospective analysis [192], and prospective data are lacking. The duration of treatment for HSE in the randomized trials was ten days. Nevertheless, based on reports of relapses [193] and data demonstrating poorer prognosis in patients with prolonged PCR positivity [134], current recommendations are to treat for two to three weeks. Three weeks of

treatment is recommended if the patient is still PCR positive in CSF after two weeks [194].

Although valacyclovir has a better pharmacokinetic profile than oral acyclovir, it has not been sufficiently studied in acute CNS infection, including HSE. A study on high dose valacyclovir 2000 mg three times daily as suppressive follow-up treatment after HSE demonstrated no clinical benefit, but no serious adverse reactions occurred, indicating a good safety profile for further studies [195].

HSV meningitis

There are no prospective studies on antiviral treatment in HSV meningitis. Treatment may still be recommended based on experience from treatment of primary genital infection, for which randomized controlled trials have been performed. In hospitalized patients, intravenous treatment with doses of 5-10 mg/kg q8h may be appropriate, but in most cases, treatment with oral valacyclovir is probably sufficient [196]. One study on 500 mg valacyclovir two times daily for prevention of recurrent HSV2 meningitis failed to demonstrate benefit, although an increased frequency of recurrences was seen after cessation of treatment [197].

VZV CNS infection

Due to the paucity of studies in VZV CNS infections, recommendations are extrapolated from the few HSE studies that are available. As such, the recommendation for most cases of VZV encephalitis is intravenous acyclovir 10 mg/kg q8h [194]. A more liberal approach with doses up to 15 mg/kg may be taken in patients with normal renal function since acyclovir demonstrates lower *in vitro* activity against VZV. In RHS, some studies have indicated improved recovery with acyclovir treatment [198, 199], but the evidence is still weak [200].

Timing of antiviral treatment

Regardless of indication, rapid initiation of antiviral treatment is essential in herpesvirus infections. In relatively benign mucocutaneous indications such as recurrent herpes labialis [201] and herpes zoster [202], treatment is effective especially when initiated early. In HSE, a delay of treatment of more than 48 hours is associated with poor outcome [51, 53]. Thus, treatment should be initiated immediately upon suspicion of herpesvirus CNS infection.

Dose adjustments

Renal function is one determinant of dosage, since decreased renal clearance causes accumulation of acyclovir and metabolites unless doses are properly

adjusted [186]. Prolonged dosing intervals or decreased doses are recommended if the calculated creatinine clearance (CL_{CR}) is less than 50 ml/min [181]. Patients with decreased renal function should be monitored for symptoms of toxicity, including AINS, since there is considerable individual variation and risk for accumulation of metabolites. If available, it may be advisable to monitor serum and CSF acyclovir and metabolite concentrations in these patients to ensure safe and appropriate acyclovir administration.

It may also be advisable to make dose adjustments in patients with obesity, since dosage is based on body weight. Acyclovir is mainly hydrophilic and has low distribution in excess fatty tissue; moreover, many formulas used to calculate CL_{CR} may overestimate renal function in these patients [203]. Although acyclovir treatment in obese patients is as yet inadequately studied, recent reports indicate increased risk of nephrotoxicity [204]. One approach is to adjust the dose based on ideal body weight in patients with body mass index >30 [205], which results in similar or slightly lower serum concentrations of acyclovir as those found in patients with normal body weight [206].

Acyclovir resistance

Resistance to acyclovir is seldom a problem with the wild-type virus; only 0.2% of HSV-1 isolates from immunocompetent patients with herpes labialis are resistant [207]. In the growing population of immunocompromised patients, however, acyclovir resistance is of clinically significant concern. Long-term treatment or prophylaxis may select for resistant HSV strains, which may be found in up to 10% of such cases overall, with reports of over 30% in hematologic stem cell transplant recipients [208]. The prevalence of resistant VZV is undetermined but likely lower, although one study reported possible resistance in 27% of hematological patients with persistent VZV infection [209]. The primary mechanism of resistance for both HSV and VZV can be found in genetic alterations within the region coding for thymidine kinase, although other mechanisms such as alteration of viral DNA polymerase have been demonstrated [210]. Alterations in these genes may decrease viral fitness [211], and only a few HSE cases have been described [212, 213]. Moreover, resistant VZV has rarely been detected in CSF [214]. Nevertheless, testing for acyclovir resistance may be warranted in patients with viral CNS infection who do not respond to treatment as expected.

1.10.3 Foscarnet

Foscarnet is an alternative for second-line treatment of HSV and VZV CNS infection, although it is primarily used to treat cytomegalovirus (CMV) infection. It is not a nucleoside or nucleotide analogue, but rather a pyrophosphate analogue that binds directly to DNA polymerase, thereby preventing DNA chain elongation. It is relatively selective for virus-infected cells due to a 100-fold greater affinity to viral DNA polymerase compared with host cell DNA polymerase [215]. The antiviral spectrum is broad and includes herpesviruses, as well as other viruses. Importantly, it has activity against viruses with thymidine-kinase-mediated antiviral resistance [216, 217]. CNS pharmacokinetics are poorly investigated, with large variations in CSF concentrations. In rabbits, the AUC of concentrations is reported to be 20% in CSF compared with serum, but 118% in brain tissue [218]. Toxicity may be problematic, and most frequently described is nephrotoxicity, but various metabolic disturbances, including hypocalcemia, also warrant close monitoring [219]. Data concerning use of foscarnet in alphaherpesvirus CNS infection is limited, but successful treatment of acyclovir-resistant HSV-1 in HSE has been reported [213].

1.10.4 Cidofovir and brincidofovir

Cidofovir is a phosphonated nucleoside analogue and is independent of phosphorylation by viral thymidine kinase, which may be useful in acyclovir resistance. It is primarily indicated in patients with CMV retinitis, but also has activity against HSV. Adverse reactions may be severe, including nephrotoxicity and myelosuppression [171]; it has not been evaluated in CNS infection.

Brincidofovir is a lipid conjugate of cidofovir, currently under evaluation. Due to pharmacokinetic advantages, it results in 100-fold higher intracellular concentrations of cidofovir, while decreasing the risk of nephrotoxicity. Additionally, it has synergistic activity with acyclovir in treatment of HSV *in vitro* [220]. There are case reports of successful rescue treatment in infection with resistant HSV-1 [221] and VZV [222], but very little data on CNS pharmacokinetics and no prospective studies in alphaherpesvirus infection. Although the drug shows promise, development has slowed because of disappointing results in a phase III trial on CMV prophylaxis due to poor efficacy and an increase in graft-versus-host disease when compared with placebo.

1.10.5 Novel antivirals

Acyclovir remains first-line treatment for HSV and VZV infections, but increasing drug resistance and problems with toxicity and tolerability in select cases illustrate the need for additional antiviral therapies, preferably with other mechanisms of action.

Additional nucleoside analogues are under development, such as N-Methanocarbathymidine, which has been shown to be superior to acyclovir in animal models [223], though efficacy in cases of acyclovir-resistant HSV remains uncertain.

Helicase-primase inhibitors bind the helicase-primase complex and inhibit DNA replication [224]. The drug amenamevir has been demonstrated effective in treating herpes zoster [225], while pritelivir has been shown to be effective against genital herpes [226]. So far, there is very little data on distribution into the CNS [227], and there are some concerns about development of resistance, as well as safety, that warrant further investigation [220].

Although other mechanisms of action, such as targeting of viral genome injection [228] and lethal mutagenesis [229], are under investigation currently no antivirals against herpesviruses based on these strategies are close to market. Future perspectives include gene editing with CRISPR/Cas9 [230], which could target the viral genome and potentially even eradicate latent virus, thereby significantly shifting the paradigm in herpesvirus therapy.

1.10.6 Additional therapies

Immunomodulation

Corticosteroids have broad-spectrum anti-inflammatory properties through interactions of glucocorticoid receptors with genomic transcription factors, as well as through modulation of signal pathways. Use of corticosteroids in herpesvirus CNS infection has been under debate since before the availability of acyclovir. Although often used, especially in cases with suspected high intracranial pressure or cerebral edema, supporting evidence is scarce.

In HSE, a retrospective study demonstrated poorer outcomes in patients without adjunctive corticosteroid treatment [231]. However, an ambitious multinational study that was terminated early due to patient inclusion difficulties found no differences in outcomes among 41 patients [232]. Currently, one randomized controlled study is underway to evaluate early administration of dexamethasone as adjuvant therapy to acyclovir, with

results expected in 2021 (DexEnceph; ClinicalTrials.gov identifier NCT03084783).

The immunological response to infection is a contributing factor in the pathogenesis of HSE, but also necessary for combating the virus. In a retrospective study on 53 HSE patients, lower CSF concentrations of inflammatory biomarkers were associated with more extensive MRI lesions, suggesting benefit from a robust inflammatory response in early infection [69]. In an experimental HSE mouse model, initiation of corticosteroid treatment before development of symptoms increased mortality, while introduction concomitant with symptoms increased survival rate [47]. These findings suggest that it may be inappropriate to reduce the early innate immune response, and that targeting the later stages of inflammation, including prolonged immune activation, may be advantageous. Uncertainties regarding corticosteroid treatment could be explained by a lack of knowledge on optimal timing.

Evidence regarding VZV CNS infection is scarce. Corticosteroids may still be recommended in RHS to reduce edema in the facial nerve [198], even if controlled trials are lacking [233], as is also the case in vasculopathy, where inflammation is thought to highly contribute to the pathogenesis [97].

As knowledge concerning the inflammatory response increases, especially in HSE, more targeted immunomodulatory therapies are coming under consideration. So far, there are only animal model studies, with treatments targeting different TLRs involved in recognition of HSV, anti-TNF-α antibodies, inducible nitric oxide synthase inhibitors, and substances targeting matrix metalloproteinases. Although many of these studies have shown promising results, it is unknown how these strategies translate to human infection [47].

1.11 VACCINATION

1.11.1 Prevention of varicella

Live attenuated VZV vaccine

The live attenuated Oka vaccine was developed in Japan already in the 1970s, initially for primary vaccination and prevention of chickenpox in hospital outbreaks [234]. Since then, it has been commercialized and included in the routine childhood immunization schedules of many countries, including the US, Germany, and Finland. It has yet to be introduced in Sweden but is available and may be prescribed.

A meta-analysis shows an efficacy in healthy children of 81% after one dose and 92% after two doses, with 98% protection against moderate/severe varicella already after the first dose [235]. Additionally, herd immunity has resulted in a marked decrease of cases among patients ineligible for vaccination, such as infants [236]. In vaccinated children, there is a 4 to 12-fold decrease in herpes zoster [237], with a decline in CNS manifestations and only a few published cases of meningitis in association with the vaccine-strain [146]. Although it remains to be proven as vaccinated individuals reach higher ages, an overall decline in VZV reactivation, including CNS manifestations, is expected in vaccinated populations [16].

1.11.2 Prevention of herpes zoster

Live attenuated VZV vaccine

The first vaccine for the prevention of herpes zoster, marketed under the name Zostavax, was introduced in 2006 and based on the live attenuated Oka vaccine used for primary vaccination, but in a 14-fold greater dose. Initial studies demonstrated a 51% reduction in herpes zoster with a 67% reduction in post-herpetic neuralgia, but the effect was smaller among patients >70 years with only a 38% reduction of herpes zoster [238]. A retrospective study following the introduction of the vaccine, however, showed a risk reduction of >50% in all age groups, including a 2/3 reduction in herpes zoster ophthalmicus [239]. The duration of protection is uncertain, but clearly declines over time, with less protection after more than five years [240].

Recombinant glycoprotein E vaccine

In 2015, the first report was published on a recombinant glycoprotein E vaccine together with an adjuvant, marketed as Shingrix. Including 15,411 participants >50 years, the risk of developing herpes zoster was reduced by 97% [241]. In a second evaluation in 13,900 participants >70 years, the risk reduction was 90%, reducing post-herpetic neuralgia by 89% [242]. Additionally, long-term data in 70 vaccinated adults indicate lasting immunity after ten years [243]. Although the vaccines have not been compared head-to-head, Zostavax is no longer sold in the US. In the EU, Shingrix was approved in 2018, but due to low product availability, introduction in Sweden has been slow.

The impact of zoster vaccination on VZV reactivation in the CNS has not been specifically studied, but a protective effect similar to that seen for typical herpes zoster is reasonable to expect with both vaccines.

1.11.3 HSV vaccines

There are no vaccines available for HSV, although attempts have been made. Several properties of HSV impose significant problems in vaccine development, such as complex life cycles, interactions between virus and immune response, and the ability to establish latency with frequent reactivations despite presence of antibodies.

Most attempts at HSV vaccines have primarily targeted HSV-2, with the aim of cross-reactive immunity to HSV-1. In more recent attempts at live vaccine development, trials have been conducted on recombinant attenuated vaccines. Various approaches to selective attenuation have been used to make safe vaccines that elicit both humoral and cell-mediated immune responses. Promising results in animal models have either not been translated to humans due to poor immunogenicity, or have encountered problems with genetic stability, with a potential to genetically revert in experimental models [171]. Perhaps more promising are subunit vaccines, with significant safety advantages. However, a recombinant gB2 and gD2 subunit vaccine did not prevent HSV-2 infection in a clinical trial despite robust neutralizing antibody response in humans, and a gD2 vaccine with alum adjuvant and TLR4 agonist only demonstrated efficacy in seronegative women, but not in men or HSV-1 seropositive women [244].

Nevertheless, with many vaccine candidates under development, increased understanding of herpesvirus immunity, and new approaches to vaccine development, there is hope of an effective HSV vaccine in the not-too-distant future.

2 AIMS

The overall aims of this thesis were to explore and evaluate several aspects of HSV and VZV CNS infection, with the ultimate goal of improving clinical management, from diagnosis to treatment and prognostication.

The specific aims were:

- to characterize biomarker expression in patients with facial palsy caused by VZV and relate the results to neurological outcome.
- to assess the performance of new diagnostic methods pertaining to herpesvirus CNS infection: rapid multiplex PCR with the FilmArray ME panel, and the CSF biomarker CXCL13 as a tool for diagnostic differentiation in facial palsy.
- to investigate the pharmacokinetics of acyclovir in acute CNS infection, with particular consideration to acyclovir-induced neuropsychiatric symptoms.

3 PATIENTS AND METHODS

The patients included in this thesis (Figure 12) were all from Sahlgrenska University Hospital, Gothenburg, Sweden. In the retrospective studies, patients were included from the Department of Microbiology, based on results from CSF analysis (Papers I, II, III), or in the case of patients with LNB from previous study cohorts (Paper II). In the prospective study (Paper IV), patients were included from the Department of Infectious Diseases, where all sampling specific to the study was performed.

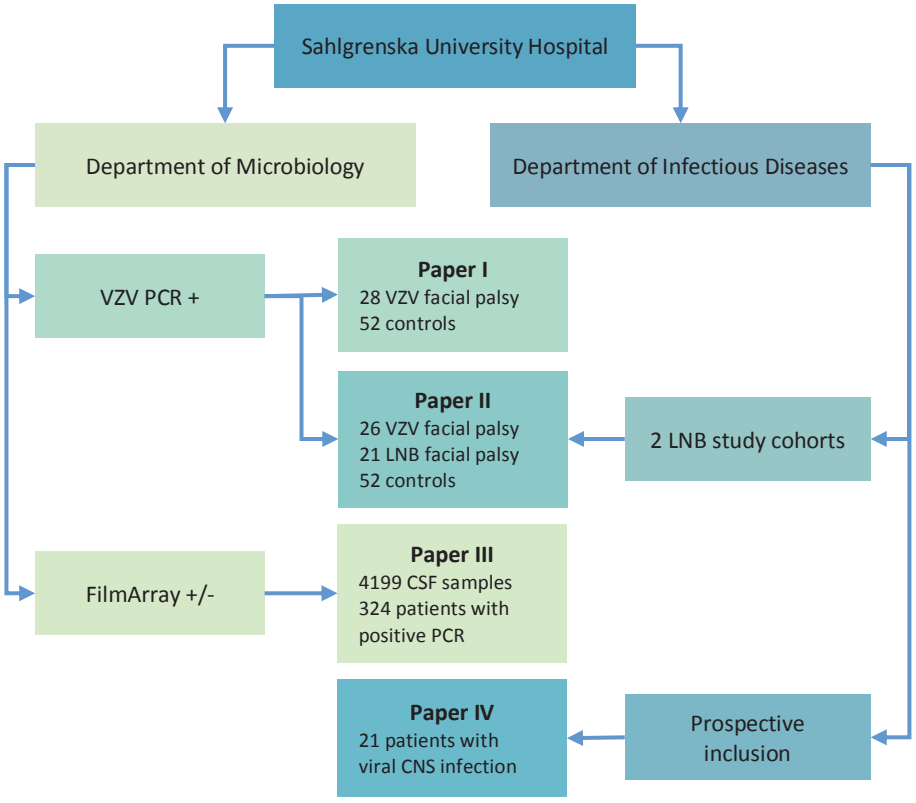

Figure 12. Flowchart of patient inclusion.

3.1 PATIENTS AND CSF SAMPLES

3.1.1 Paper I

We retrospectively identified 28 patients with facial palsy caused by VZV from collected samples that were sent to the Department of Microbiology between 2002 and 2013. Patients with other CNS symptoms were not

included, since we wanted to investigate biomarker expression specifically in VZV facial palsy. Medical records were reviewed to obtain information on demographics, symptoms and clinical course, as well as CSF sampling time points, laboratory results, and treatment.

Viral load had already been determined, since inclusion relied on positive VZV PCR in CSF, but additional CSF analyses were performed to determine biomarker concentrations.

For comparison of biomarker expression, 52 age- and sex-matched controls were included. CSF samples had been obtained from the controls at the Department of Infectious Diseases based on non-specific symptoms such as headache and general malaise, with normal CSF cell count, normal c-reactive protein in serum, and with no objective neurological symptoms or signs on neurological examination.

3.1.2 Paper II

This study included patients with similar clinical presentations, i.e., facial palsy, to investigate the use of CXCL13 in CSF as a diagnostic marker from the physician perspective. Only 26 of the patients with VZV facial palsy from paper I could be included due to an insufficient quantity of CSF for analysis in two cases.

CSF samples from 21 patients with LNB-associated facial palsy were obtained from two patient cohorts who participated in prior unrelated studies on LNB for which patients were prospectively included between 2000 and 2013. Samples from this group were collected before initiation of antibiotic treatment. All but one patient met the criteria for definite LNB as per European Federation of the Neurological Societies (EFNS) guidelines [107]. The sole exception was a patient with recent erythema migrans who did not develop Borrelia-specific antibodies and was therefore classified as possible LNB.

In addition, CSF samples from the 52 controls in paper I were included for analysis of CXCL13.

3.1.3 Paper III

In April 2017, the FilmArray Meningitis/Encephalitis (ME) panel was introduced at the Department of Microbiology primarily for diagnosis of viral CNS infection. In addition to analysis with the ME panel, CSF was analyzed using previously established methods for detection and quantification of HSV-1, HSV-2, VZV, and enterovirus, as well as other physician-ordered tests, such as bacterial cultures.

For our assessment of ME panel performance, we retrospectively included all 4199 samples from patients in Region Västra Götaland that had been analyzed during the study period, from April 2017 to February 2020. Information such as demographics, clinical data, laboratory results, and treatment was gathered from the medical records of 324 patients who demonstrated positive findings on either the ME panel or using established PCR methodology. Special attention was given to patients for whom the results from different analyses were discrepant in order to determine whether the results from either method could be considered as true or false. In these cases, three of the authors reviewed the medical records to reach agreement on whether positive PCR results were credible, despite not fully corresponding with clinical CNS infection.

3.1.4 Paper IV

This prospective study on acyclovir and CMMG in suspected or confirmed cases of herpesvirus CNS infection included adult patients admitted to the Department of Infectious Diseases. Informed consent was obtained from 21 eligible patients between September 2013 and January 2016, who were subsequently sampled on one or multiple occasions in addition to receiving standard care.

CSF and serum samples were obtained according to a schedule for the duration of acyclovir treatment, on days 1-2, 3-5, 6-9, 10-14, 15-21, and 22-30, since various factors influencing the pharmacokinetic profile of acyclovir were presumed to vary over the clinical course, including BBB damage. Time of sampling in relation to acyclovir administration was not specified.

This study entailed no interventions other than sampling, and additional data were retrospectively obtained from medical records.

3.2 METHODS

3.2.1 CSF analysis

In regard to papers I, II, and III, CSF analyses were performed on stored CSF samples. Concerning papers I and II, CSF from patients with VZV facial palsy had been stored at either –70˚C or –20˚C, and samples from patients with LNB were all stored at –70˚C, divided into small aliquots after sampling to eliminate the need for repeated freeze-thawing associated with multiple analyses. In paper III, analyses were consecutively performed as samples reached the laboratory, while samples for paper IV were stored at –70˚C until they were sent for analysis of acyclovir and CMMG concentrations.

PCR methods for detection of viruses

PCR detection of viruses in all four papers was performed using established in-house PCR methodology, including real-time PCR for HSV-1, HSV-2 [131], VZV [87, 245], enterovirus [246], CMV [247], and HHV-6 [248]. Results were reported in relation to viral load as the number of genome equivalents or copies/mL, or simply as negative. All results were retrospectively reviewed, without any study-specific deviations from established procedure.

Paper III data relied on the newly introduced FilmArray ME panel at our Department of Microbiology. This commercial test for CSF analysis includes an ME panel pouch containing freeze-dried reagents. The system purifies nucleic acid and performs a nested, multiplex PCR with identification through DNA melting point analysis [249]. The panel includes 14 different targets: *Escherichia coli* K1, *Haemophilus influenzae*, *Listeria monocytogenes*, *Neisseria meningitidis*, *Streptococcus agalactiae* (GBS), *Streptococcus pneumoniae*, CMV, enterovirus, VZV, HSV-1, HSV-2, human herpesvirus 6 (HHV-6), human parechovirus (HPeV), and *Cryptococcus neoformans/Cryptococcus gattii*. The system reports results as positive or negative.

Additional microbiological analyses

The patients with LNB facial palsy addressed by paper II were diagnosed according to the EFNS criteria, which, in addition to neurological symptoms (facial palsy) and CSF mononuclear pleocytosis, include a positive CSF/serum antibody index (AI) for *Borrelia burgdorferi* (*Bb*) sensu lato. The laboratory employed two different methods over the timespan during which LNB patients were sampled. Initially, IgM and IgG *Bb*-antibodies were measured by the Dako Lyme Borreliosis enzyme-linked immunosorbent assay (ELISA) kit (Dako Cytomation A/S, Glostrup, Denmark), from which the AI was calculated according to Hansen and Lebech [250]. Beginning in 2006, the Liaison CLIA (Diasorin, Saluggia, Italy) method replaced the earlier methodology, using AI calculation as described by Reiber and Lange [138].

In paper III, detection of pathogens, other than viruses, in CSF was performed with routine diagnostic procedures according to incoming orders to the laboratory. For identification of bacteria, in-house PCR and sequencing of 16s rRNA are available, in addition to more traditional methods such as CSF cultures. The 16s rRNA gene is highly conserved in a vast range of bacteria, and amplification of bacterial DNA can be undertaken without primers specific to a predetermined species. Nevertheless, there are segments of targeted sequences subject to interspecies variation, and

comparison to databases after sequencing allows for species identification [251].

CSF biomarkers

The CSF biomarkers investigated in paper I were selected based on experiences from previous studies on CNS infections.

NFL concentrations were measured by sandwich ELISA (Uman Diagnostics, Umeå, Sweden). S100B was measured on the Modular system using the S100B reagent kit (Roche Diagnostics, Basel, Switzerland). GFAP was measured using a previously described in-house ELISA [252].

CXCL13 concentrations (Paper II) were measured by ELISA (R&D systems), with a 7.8 pg/mL limit of detection.

Blood-brain barrier damage

Loss of BBB integrity results in increased albumin passage from serum to CSF, resulting in increased CSF concentrations. In paper III, albumin was measured by immunonephelometry on a Beckman image immunochemistry system (Beckman Instruments, Beckman Coulter, Brea, CA, USA). Since serum albumin concentrations may vary, calculation of the CSF:serum albumin ratio [CSF albumin (mg/L)/serum albumin (g/L)] is a more reliable measurement. Upper normal limits are age-dependent: 6.8×10^{-3} in patients ≤45 years of age and 10.2×10^{-3} in patients >45 years of age.

Acyclovir and CMMG concentrations

Concentrations of acyclovir and CMMG in serum and CSF were determined at the Department of Clinical Pharmacology, Karolinska University Hospital, Huddinge, Stockholm, Sweden, using liquid chromatography [253].

3.2.2 Estimation of renal function

In clinical practice, renal function is most frequently estimated by measuring serum creatinine concentration, often along with calculation of CL_{CR} as a measurement of glomerular filtration rate. In paper III, the Cockcroft-Gault formula was used for this purpose [254], and renal injury was further classified based on the RIFLE (risk, injury, failure, loss of function, end-stage renal disease) criteria [255].

3.2.3 House-Brackmann score

The severity of facial palsy (Paper I) was determined using the House-Brackmann facial nerve grading system [256], where a score of 1 corresponds to normal function and subsequent incremental degrees of facial palsy

culminate in total paralysis, which earns a score of 6. Outcome was measured as the score at the latest follow-up within three months, in which a score of 1-2 was considered good and 3-6 poor. Retrospective grading was carried out through interpretation of neurological examination records.

3.2.4 Evaluation of neuropsychiatric symptoms

Assessment of symptoms indicating a diagnosis of AINS (Paper IV) was independently performed by three of the authors, with the requirement that at least two authors reach agreement. The assessment was blinded to acyclovir administration and CMMG concentrations, although data concerning renal function and acyclovir dosage were available through retrospective investigation of medical records. Several criteria indicating AINS needed to be met. Symptoms were to be consistent with those previously described, including confusion, hallucinations, and ataxia. Fever, headache, and focal CNS symptoms that were more typical of CNS infection were considered unlikely to indicate AINS. Absolute requirements were onset of symptoms after initiation of treatment, and resolution of symptoms after dose reduction or cessation of treatment.

3.2.5 Statistical analysis

Non-parametric statistical methods were used: Mann-Whitney *U* test for comparisons between two groups (Paper I, II, IV) or Kruskal-Wallis test for three groups (Paper II), and Spearman's rank test for correlations (Paper I).

In paper II, receiver-operating characteristic (ROC) curve analysis was performed, with optimal cut-offs determined using the sensitivity = specificity approach.

In paper III, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated, with 95% confidence intervals (CI) using the Wilson/Brown method.

In paper IV, with the help of a consultant statistician, multivariable regression models were constructed to assess the impact of relevant independent variables on the studied dependent variables (acyclovir and CMMG CSF concentrations). The SAS MIXED procedure was used, considering that some of the patients were sampled at more than one time point, using the autoregressive covariance pattern found to be optimal based on the lowest Akaike information criterion. The residual plots were reviewed for assumption of normal distribution. Standard errors and 95% CIs were estimated based on robust sandwich estimators to adjust for possible deviation of residuals from a normal distribution.

The mixed models were performed using SAS software version 9.4 (SAS Institute Inc., Cary, NC, USA). GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for the other analyses.

3.3 ETHICS

Ethical approval was obtained for all studies from the Medical Ethics Committee at Gothenburg University; ref. no. 664-13 (paper I, II), ref. no 358-95 (paper II), ref. no. 407-13 (paper IV); or the Swedish Ethical Review Authority; ref. no. 2019-02073 (Paper III).

4 RESULTS AND DISCUSSION

4.1 PAPER I

Of the 28 patients with facial palsy and VZV detected in CSF, only 15 had the typical rash associated with herpes zoster and required for diagnosis of RHS. Figure 13 presents demographics and associated symptoms such as rash, fever, headache, vertigo, and hearing impairment.

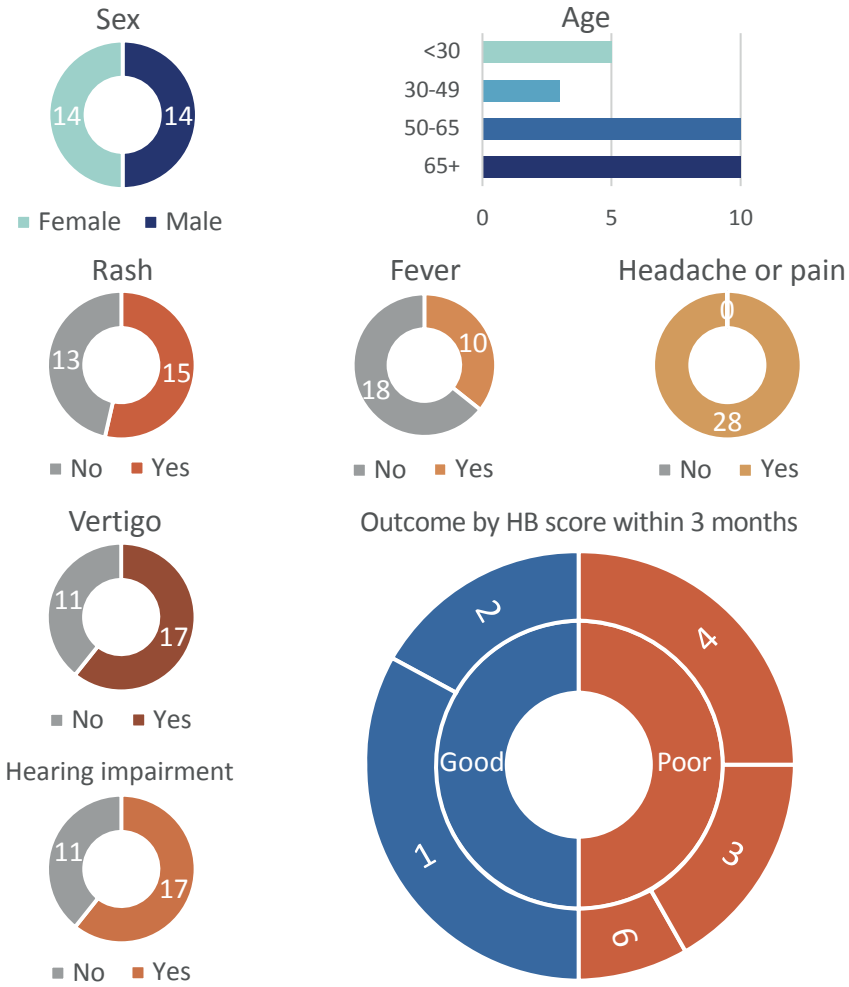

Figure 13. Demographics of 28 patients with VZV facial palsy. HB score = House-Brackmann facial nerve grading system score; scale from 1 (normal function) to 6 (total paralysis).

Acyclovir was administered to 27/28 patients and corticosteroids to 17/28 patients. MRI was performed in 10 cases; two showed signs of inflammation in the facial nerve. None of the studies indicated recent ischemic injury.

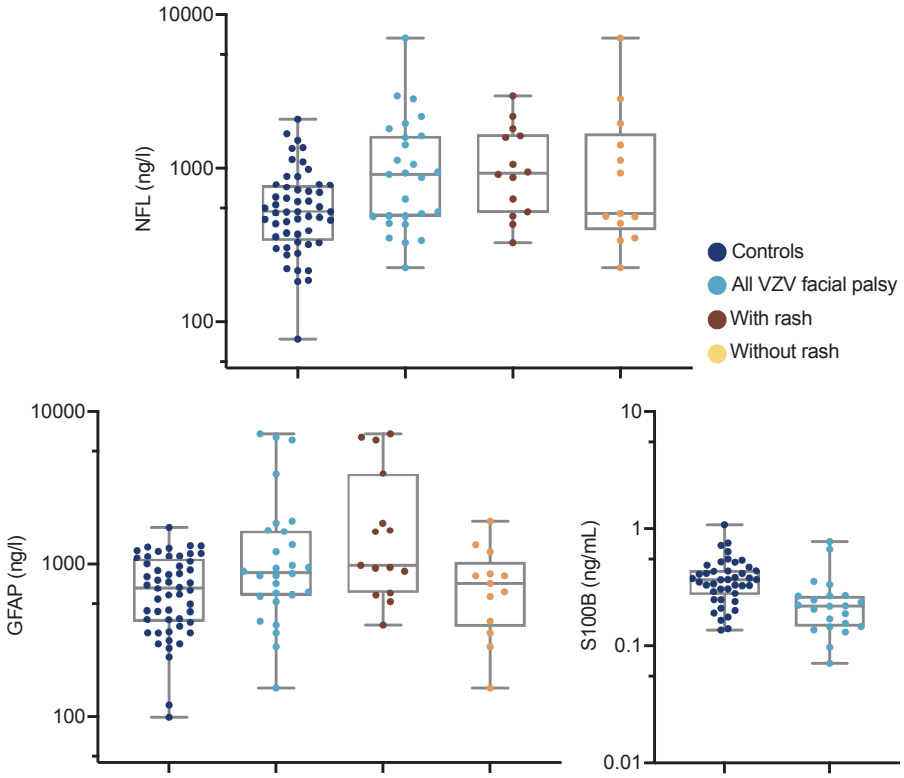

Figure 14. Concentrations of biomarkers in CSF from patients with VZV facial palsy, subgroups based on presence of rash, and controls.

Biomarkers were expressed in a pattern (Figure 14) similar to that previously described for VZV CNS infection when compared with controls [156]. NFL concentrations were significantly higher in CSF from patients with VZV facial palsy compared with controls ($P = 0.008$). Interestingly, NFL concentrations were only higher in the subgroup of patients with rash ($P = 0.005$), while patients without rash showed median concentrations (508 ng/L), actually lower than found in the control group (521 ng/L). A similar pattern was seen when GFAP was studied. GFAP concentrations were significantly higher among patients with VZV facial palsy compared with controls ($P = 0.04$) and patients in the rash subgroup ($P = 0.003$), but not in comparison with the subgroup of patients without rash. S100B concentrations were decreased compared with controls ($P = 0.001$), but with no differences between subgroups.

Viral load, as measured by real-time PCR, did not differ significantly between the subgroups with or without rash. Viral load did, however, correlate with concentrations of NFL (Spearman $r = 0.51$), GFAP (Spearman $r = 0.54$), and S100B (Spearman $r = 0.59$) (Figure 15).

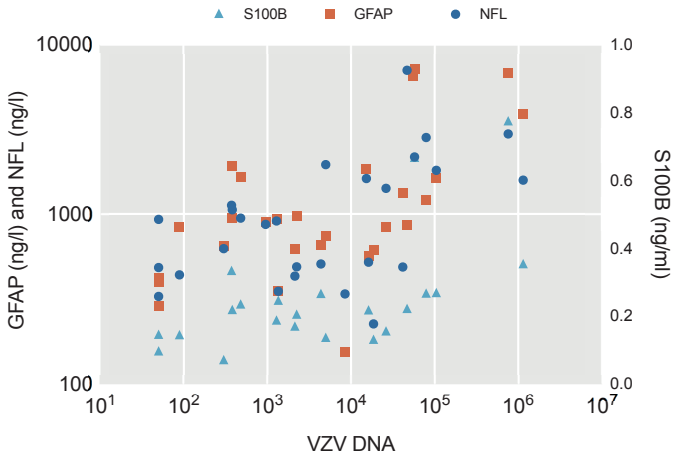

Figure 15. Correlations between CSF viral load and the CSF concentrations of NFL (individual concentrations).
[Reprinted from paper I [257] with permission from John Wiley and Sons. © 2016 Federation of European Neuroscience Societies and John Wiley & Sons Ltd.]

When looking at the outcome, no association was found between biomarker concentrations or viral load and House-Brackmann score at follow-up within three months. Patients with rash had a higher score at follow-up (median 3) than patients without rash (median 1.5), but the difference was not significant ($P = 0.16$) (Figure 16).

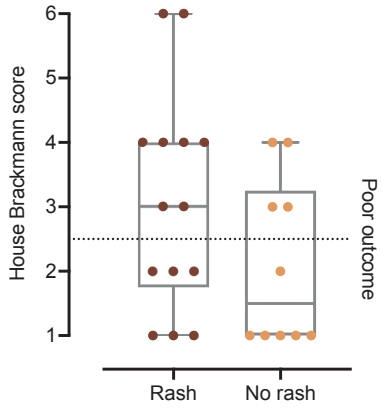

Figure 16. Outcomes measured by House-Brackmann score in VZV facial palsy patients with and without rash.

4.2 DISCUSSION PAPER I

The biomarker pattern described in this study is interpreted as a disruption of neurons and activation of astroglia, rather than the extensive necrosis associated with necrotizing encephalitis, as seen in HSE [154]. Although this pattern of biomarkers is interpreted as evidence for CNS involvement [155], it is notable that it has not yet been demonstrated that biomarker concentrations consistent with this pattern are associated with neurological or clinical outcomes. One possible explanation may be lack of sensitivity in outcome measures. There are apparent limitations to retrospective assessment of facial palsy in our study, but since similar observations have been made with different outcome measures [156], other explanations must be considered.

First, sequelae after CNS injury or infection are highly influenced by what CNS location is involved and not just by the extent of tissue damage; for example, the poorer prognosis associated with bilateral HSE [50] or the debilitating sequelae often seen in patients with small ischemic injuries to vital CNS structures. Second, in studies where an association between NFL and outcome is demonstrated, the NFL concentrations are tenfold higher than those shown in our study [69, 258]. In chronic CNS conditions such as multiple sclerosis [259], small increases in NFL concentration may be associated with poor outcome, but this does not necessarily apply to acute CNS infection, where significant fluctuations in NFL concentrations may occur. Third, individual variations due to factors such as the timing of CSF sampling may be sufficient to conceal genuine associations with outcome, given the relatively limited number of samples that have been studied to date.

Perhaps the most interesting findings in our study are the differences in patients presenting with and without rash. VZV reactivation without rash (*zoster sine herpete*) has been theorized since the early 1900s, but virologic evidence for peripheral *zoster sine herpete* was first presented in 1994 [260]. In CNS disease, serological evidence of VZV infection without rash was presented during the 1980s, and reports of VZV facial palsy without rash were published during the 1990s [88]. Biomarker concentrations in our patients without rash were similar to those of controls, which raises questions concerning differences in pathophysiology. How come rash is associated with biomarker patterns thought to be indicative of CNS involvement, while the absence of rash is not? In herpes zoster patients without CNS involvement, increased serum NFL concentrations have been demonstrated, while increased CSF NFL concentrations could potentially be explained by neuronal damage engaging nerve roots of the spinal canal [261]. Discrepancies in biomarker concentrations may represent degree of neuronal

damage rather than location.

Our results emphasize the need for a better understanding of the differences between herpes zoster with and without rash, as well as for the extent of CNS involvement in VZV facial palsy. Furthermore, there was a non-significant association between rash and outcome, which may be of importance concerning decisions on treatment strategies. Although patients with VZV facial palsy generally do not recover as well as those with Bell's palsy [262], patients with a rash may warrant more aggressive treatment targeting CNS disease. It is possible that the lack of evidence for antiviral treatment in RHS could be partly explained by the use of oral acyclovir, since intravenous or at least high-dose oral treatment may be necessary to reach adequate concentrations in the affected nerves.

4.3 PAPER II

In this study, the patients with LNB facial palsy ($n = 21$) and VZV facial palsy ($n = 26$) did not differ significantly in regard to age and sex, though some differences were noted concerning CSF sampling and storage. Patients with LNB were sampled a median of 4 days after onset of facial palsy, while those with VZV were sampled at a median of 2 days after onset ($P = 0.034$). CSF samples from the LNB group were stored in freezers for a longer duration ($P = 0.011$) (Figure 17).

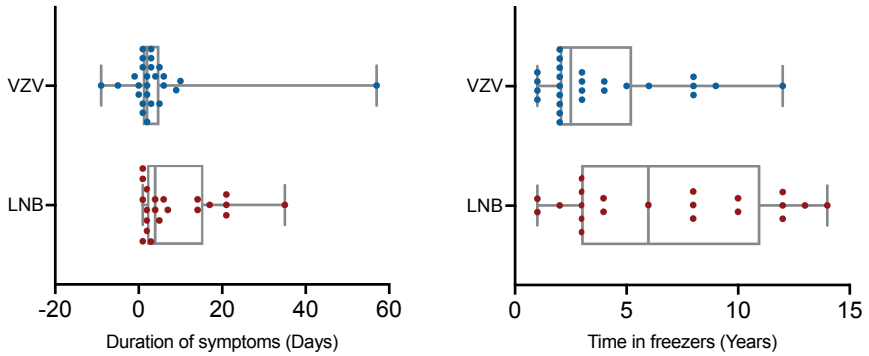

Figure 17. Duration of symptoms prior to sampling and CSF sample storage duration in patients with facial palsy caused by Lyme neuroborreliosis (LNB) and varicella-zoster virus (VZV).

CSF concentrations of CXCL13 were significantly higher in the LNB facial palsy group compared with the VZV facial palsy group ($P < 0.0001$). Six samples from the LNB facial palsy group showed overlap in concentration and fell below the cut-off previously proposed by Rupprecht et al. [263] (Figure 19). Four samples with low concentrations had undergone lengthy storage, but not the remaining two; a significant correlation between LNB CSF storage time and CXCL13 concentrations was not found. In contrast, a significant correlation was found between timing of CSF sampling and CXCL13 concentrations ($r = -0.44$, $P = 0.049$), as three of the samples with low concentrations were obtained more than 7 days after onset of symptoms.

ROC analysis found an 85.7% sensitivity and 84.6% specificity at the optimal cut-off concentration of 34.5 pg/mL when all patients were included. Considering the appropriate timing of sampling and analysis of CXCL13, we also performed ROC analysis only including patients who were sampled within one week after onset of facial palsy, which resulted in higher sensitivity and specificity at 92.3% and 90.0%, respectively, at the optimal cut-off concentration of 86 pg/mL (Figure 18).

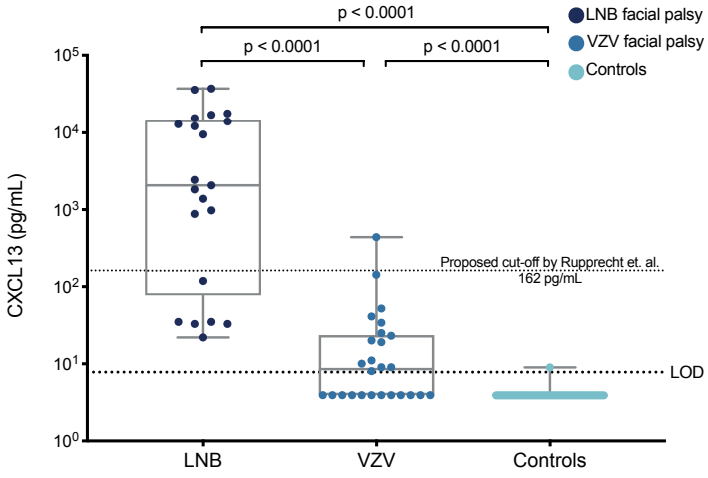

Figure 19. CSF concentrations of CXCL13 in patients with LNB facial palsy, VZV facial palsy, and controls. LOD = limit of detection.

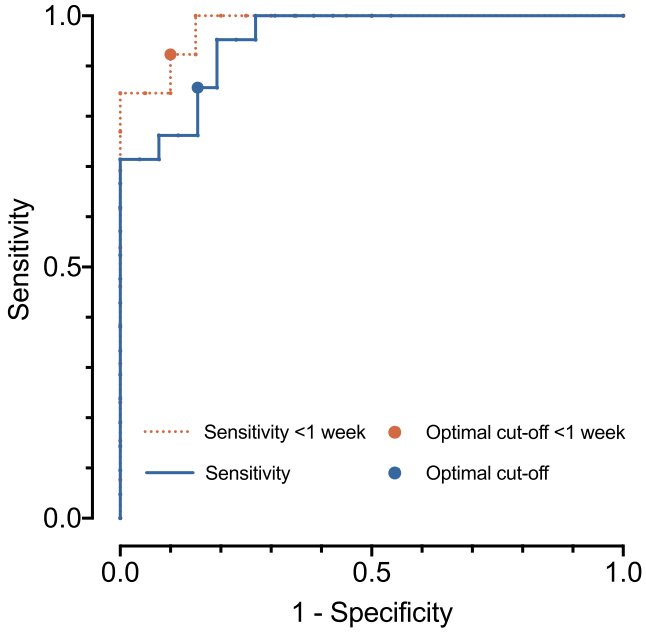

Figure 18. Receiver operating characteristic (ROC) curves of CSF CXCL13 concentrations from patients with LNB facial palsy versus VZV facial palsy. The blue ROC curve and optimal cut-off includes all patients. The orange ROC curve and optimal cut-off includes patients sampled within one week from onset of symptoms.

4.4 DISCUSSION PAPER II

We found significantly higher CXCL13 concentrations associated with LNB facial palsy compared with VZV facial palsy, and our study reaffirmed the relatively high sensitivity and specificity of CXCL13 as a diagnostic biomarker for LNB in this context.

There was, however, overlap in CXCL13 concentrations that may make interpretation of results difficult when attempting to differentiate between these two groups of patients, who show similar presentations, but require different treatments. Our ROC analyses showed lower optimal cut-offs than those previously proposed in a meta-analysis of all LNB by Rupprecht et al. at 162 pg/mL [263], implying that low CXCL13 concentrations in the LNB group should be the primary focus of concern.

One potential source of low CXCL13 concentrations is ill-timed CSF sampling. CXCL13 is part of the early immunological response in LNB [161], and we did find a significant correlation between CXCL13 and sampling time point in line with previous reports [264]. In patients sampled within one week, we calculated better performance measures at a higher cut-off point. The diagnostic utility of CXCL13 is primarily in early suspected LNB, before intrathecal *Bb* antibodies develop. Although our LNB patients were prospectively included in other studies, all but one had a definite LNB diagnosis with a positive AI, implying a longer duration of infection. Low CXCL13 concentrations could, in this context, potentially be explained by late sampling following onset of (subclinical) CNS infection, even if typical symptoms were of relatively recent presentation. In clinical practice, restricting the use of CXCL13 analysis to patients with possible LNB may mitigate this limitation.

Prior studies that included a larger number of patients with possible LNB have often failed to provide confirmation of the LNB diagnosis through repeated sampling and seroconversion [263]. Consequently, there is a paucity of evidence for the diagnostic utility of CXCL13 in the subset of patients where the analysis may be indicated. From a practical standpoint, this may be a difficult problem to study, since treatment may inhibit production of antibodies [265], and no other methods to confirm the diagnosis are available. It cannot be excluded that some patients in this group may have low concentrations similar to those found in our study.

Moreover, low CXCL13 concentrations in select patients are not unique to our study and have been described in several patients with definite LNB [266-268]. The distinction between CNS infection and isolated peripheral nerve infection remains unclear, as discussed in paper I. Low CXCL13

concentrations in some cases of LNB facial palsy could possibly be explained by more localized infection with limited biomarker expression in the CSF.

Our retrospective study design may also be the potential source of low CXCL13 concentrations. Chemokines are generally susceptible to degradation in storage, and there are indications that CXCL13 may degrade over time [163]. Nevertheless, we failed to uncover a significant correlation between CXCL13 concentration and duration of freezer storage; several of our CSF samples that had been stored for ten or more years demonstrated high concentrations similar to those found in CSF samples stored for a much shorter duration.

Despite good performance measures, CXCL13 analysis can currently only be advised in cases of possible LNB with short duration of symptoms and negative AI. Regardless of cut-off point, interpretation of low concentrations must be made with caution.

4.5 PAPER III

From assessment of the FilmArray ME panel, a total of 336 pathogens were detected in the 4199 samples. The ME panel detected 315 of these, while the remaining 21 viral pathogens were detected by real-time PCR. Figure 20 and Figure 21 present demographics, distribution of pathogens, and methods of detection.

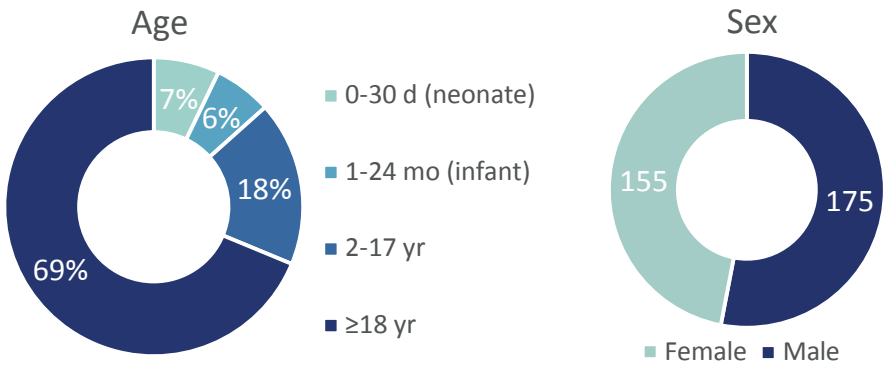

Figure 20. Age and sex distribution of patients with positive results from the FilmArray ME panel or real-time PCR

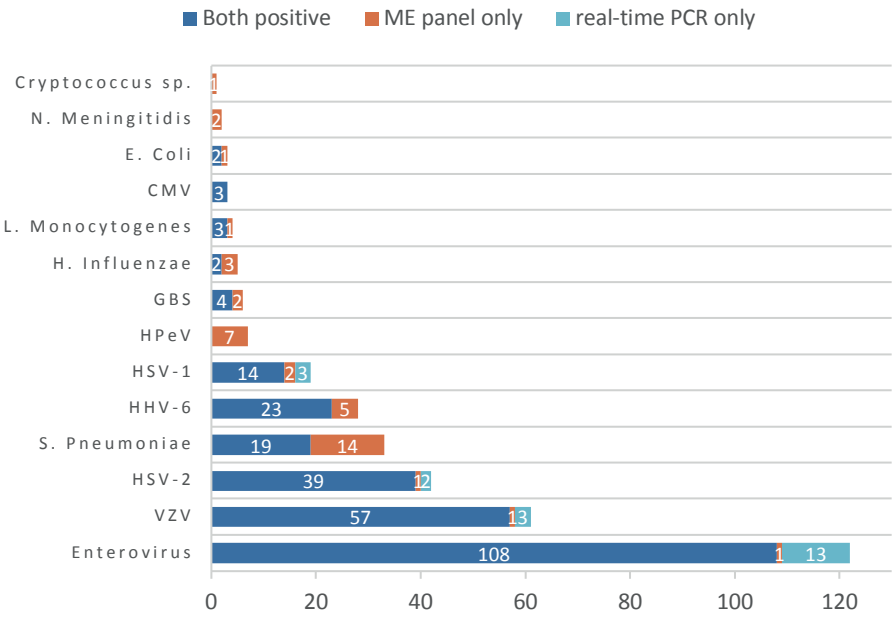

Figure 21. Pathogens detected by the FilmArray ME panel and real-time PCR.

As seen in Figure 21, a total of 41 samples were detected with the ME panel but not with routine diagnostic procedures. Only 34 results were considered discrepant, since the 7 positive HPeV results detected by the ME panel were not confirmed with real-time PCR. An additional 21 discrepant results were detected with real-time PCR, but not the ME panel.

After discrepancy analysis, it was determined that true positive PCR could not be rejected in any of the 21 cases detected by real-time PCR, but that failed detection by the ME panel. The three instances where HSV-1 was detected were of particular interest and included one case of typical HSV-1 encephalitis and one adult case of primary HSV infection with high viral load in serum. The third case was more uncertain and concerned a patient who presented with confusion and seizures. Initially, HSE was suspected, but the patient was also diagnosed with empty sella syndrome on follow-up, which could have explained the symptoms. However, true positive detection by PCR could ultimately not be ruled out. Figure 22 presents data demonstrating low viral load in false negative results.

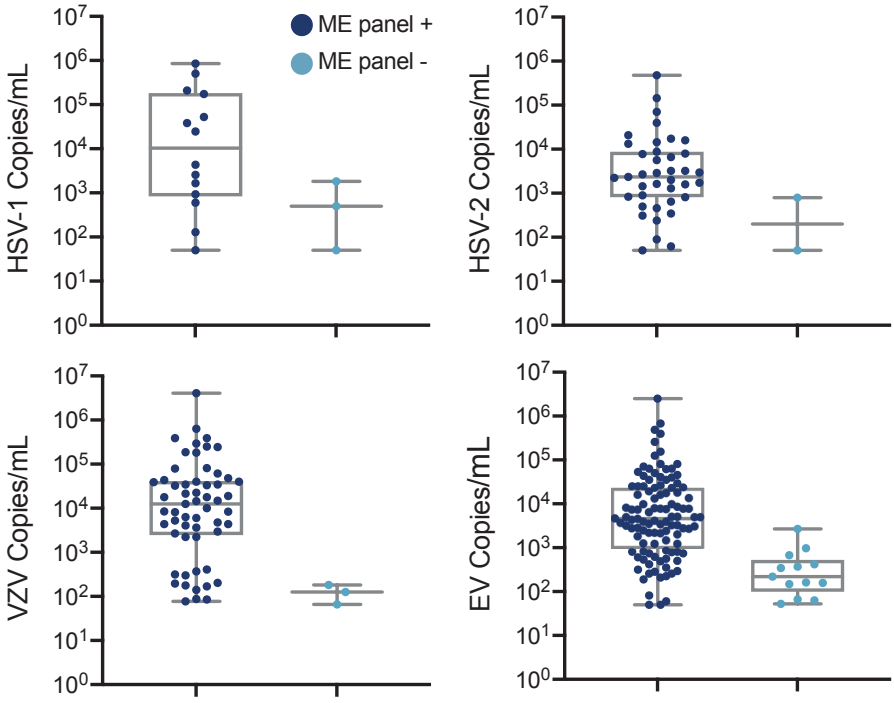

Figure 22. Viral load in CSF samples positive by the FilmArray ME panel or real-time PCR.

Of the 34 cases detected by the ME panel that were not supported by routine diagnostic procedures, 14 were true positives as determined by the discrepancy analysis. In 8 instances, bacterial detection were confirmed by positive blood culture, and the remaining 6 cases detected by ME panel were among patients with symptoms consistent with meningitis or encephalitis.

Performance measures were calculated following discrepancy analysis according to Table 1. Sensitivity was calculated for HSV-1, HSV-2, VZV, and enterovirus, for which all 4199 samples were tested with real-time PCR in addition to the ME panel. PPV and specificity were calculated for all pathogens.

Table 1. Performance of the FilmArray ME panel compared with routine diagnostic procedures after discrepancy analysis.

Target	Sensitivity[a] (95% CI)	PPV (95% CI)	Specificity (95% CI)	NPV (95% CI)
EV	89.3 (82.6–93.7)	100 (96.6–100)	100 (99.9–100)	99.7 (99.5–99.8)
HSV-1	82.4 (59.0–93.8)	87.5 (64.0–97.8)	100 (99.8–100)	99.9 (99.8–100)
HSV-2	95.1 (83.9–99.1)	97.5 (87.1–99.9)	100 (99.9–100)	100 (99.8–100)
VZV	95.1 (86.5–98.7)	100 (93.8–100)	100 (99.9–100)	99.9 (99.8–100)
CMV		100 (43.9–100)	100 (99.9–100)	
HHV-6		82.1 (64.4–92.1)	99.9 (99.7–99.9)	
HPeV		100 (64.6–100)	100 (99.9–100)	
E. coli		66.7 (11.8–98.3)	100 (99.9–100)	
H. influenzae		80.0 (37.6–99.0)	100 (99.9–100)	
L. monocytogenes		100 (51.0–100)	100 (99.9–100)	
N. meningitidis		100 (17.8–100)	100 (99.9–100)	
GBS		83.3 (43.6–99.1)	100 (99.9–100)	
S. pneumoniae		75.8 (59.0–87.2)	99.8 (99.6–99.9)	
Cryptococcus sp.		0.00 (0.00–94.9)	100 (99.9–100)	

[a]Sensitivity and NPV was only calculated for targets where all 4199 samples were tested with real-time PCR. Confidence intervals (CI) were calculated according to Wilson/Brown.

4.6 DISCUSSION PAPER III

Our assessment of the FilmArray ME panel includes a larger material than any previous study of this panel, from an unselected patient population of both adults and children, where samples were submitted for diagnosis of viral CNS infection. Our results demonstrate high performance of the ME panel, and data on individual pathogens are presented.

Although we do present data on all pathogens, our study mainly focuses on assessment of HSV-1, HSV-2, VZV, and enterovirus, where all 4199 CSF samples were subjected to state-of-the-art diagnostics using real-time PCR, allowing for complete calculations of performance measures (Table 2).

Table 2. Calculation of performance measures through cross-tabulation.

		Target condition (reference standard)		
	Total population (Pop.)	Present	Absent	Prevalence (TP+FN)/Pop.
Index test	Detected	True positive (TP)	False positive (FP)	PPV TP/(TP+FP)
	Not detected	False negative (FN)	True negative (TN)	NPV TN/(FN+TN)
		Sensitivity TP/(TP+FN)	Specificity TN/(FP+TN)	

Interpretation of these performance measures warrants further discussion, since the various performance measures differ in importance depending on the pathogen under investigation. Sensitivity and specificity are intrinsic to ME panel test. In contrast, predictive values depend on disease prevalence within the study population, and must be recalculated according to Bayes Theorem when applied to individuals or populations with a different pre-test probability or prevalence. As prevalence, or pre-test probability, increases, so too does PPV, while NPV decreases (Figure 23).

When assessing performance for HSV-1, HSV-2, VZV, and enterovirus, the primary concern is to ensure that as few cases as possible are missed by the FilmArray method if real-time PCR is not routinely utilized, for which reason sensitivity and NPV are of high priority. In our study population, NPV for HSV-1 was very high at 99.9%. However, the prevalence of HSV-1 was very low at only (14+3)/4199 = 0.4%, and we did identify three cases of false negative ME panel results. If we recalculate NPV according to Bayes theorem with a pre-test probability of 20%, corresponding to the prevalence of HSV-1 in patients with encephalitis [43, 45], NPV still remains high at 95.8%. In addition to prevalence within the tested population, individual factors may affect pre-test probability, such as results from imaging studies. With a pre-test probability of 50%, NPV would be 85%, corresponding to a 15% risk of failure to detect HSV-1. Consequently, it may be advisable to perform real-time PCR in addition to the ME panel in patients where suspicion of HSV-1 infection is high.

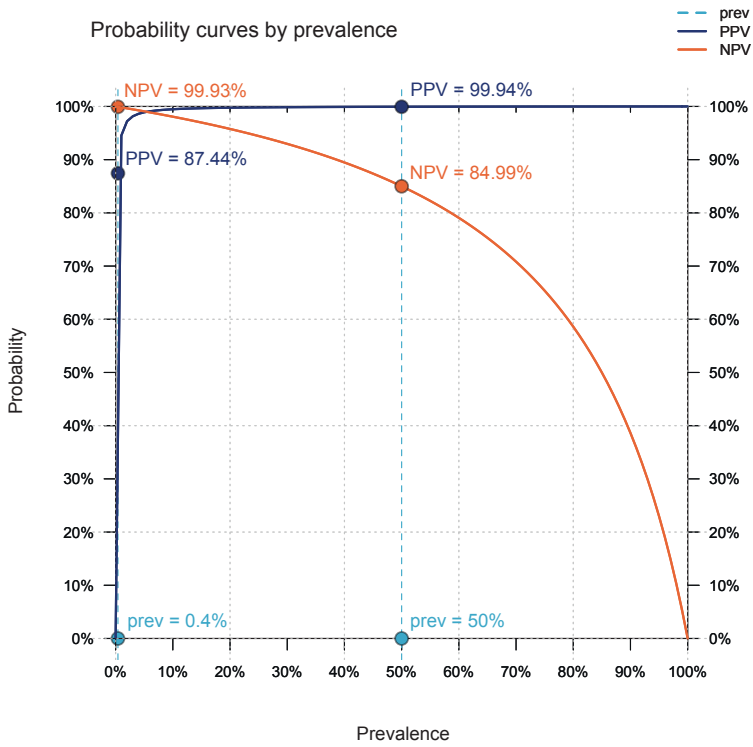

Figure 23. Probability curve of HSV-1 in the FilmArray ME panel. Vertical lines and dots depict predictive values at prevalence rates of 0.4% and 50%.

There are several potential causes for false negative results when using the ME panel, such as inappropriate primers or misinterpretation of melting curves by the FilmArray instrument [269]. In our material, false negative results were associated with lower viral loads, implicating the limit of detection as the main culprit. Although PCR is generally considered highly sensitive in the diagnosis of CNS infection, it is already relatively well known that both WBC count and HSV-1 PCR may be negative in CSF samples obtained very early after onset of symptoms in HSE [51, 130, 270]. Repeated sampling is recommended if clinical suspicion remains. In the context of ME panel performance, this knowledge may mitigate the consequences of false negative results. Similarly, detection of enteroviruses in CSF may be transient, and testing of throat and stool specimens in addition to CSF may be of value when enterovirus infection in the CNS is suspected [271].

When assessing performance concerning bacterial targets, PPV is arguably more clinically relevant than NPV, since testing does not rely on PCR alone. Additional methods such as direct microscopy, CSF cultures, and if available, PCR and sequencing of 16s-rRNA should be utilized, both for antimicrobial susceptibility testing and for identification of bacteria not included in the panel. In our material, PPV ranged from 66.7% for *E.coli* to 100% for *L. monocytogenes* and *N. meningitidis*, although CIs were large due to few positive results. Considering the low prevalence of bacterial targets in our population, where most cases were sampled for suspicion of viral infection, PPV will be considerably higher among patients in whom bacterial meningitis is suspected. *S. pneumoniae* was identified as true positives by ME panel detection in 0.6% of cases. If recalculated using an estimated prevalence of 50% for community-acquired bacterial meningitis, PPV for *S. pneumoniae* increases from 75.8% to 99.8%.

Additionally, although we did not have data on false negative ME panel results for bacteria, the ME panel detected 6 cases of *S. pneumoniae* that were adjudicated as true positives by PCR detection after discrepancy analysis despite negative routine diagnostic tests, thereby indicating the high sensitivity of this analysis. Nevertheless, it is important to note that almost 25% of detected *S. pneumoniae* cases were false positives, which should be considered when interpreting ME panel results in patients with low pre-test probability.

Syndromic multiplex panel testing is associated with complexities in interpretation depending on prevalence, and even among true positive detections of some pathogens such as HHV-6, which may be innocuously present in CSF due to factors such as chromosomal integration [272], interpretation must take into account the context of clinical significance. Still,

supported by the strong performance in our data, considerable advantages in turn-around time, and potential availability in smaller laboratories, the ME panel is a welcome addition to the diagnostic methodology arsenal for CNS infections.

4.7 PAPER IV

A total of 21 patients with suspected or confirmed herpesvirus CNS infection were prospectively enrolled and sampled on one to three occasions, depending on time of inclusion and treatment duration. In all, 34 CSF samples and 32 serum samples were obtained for further analysis. Figure 24 presents demographics and indications for treatment.

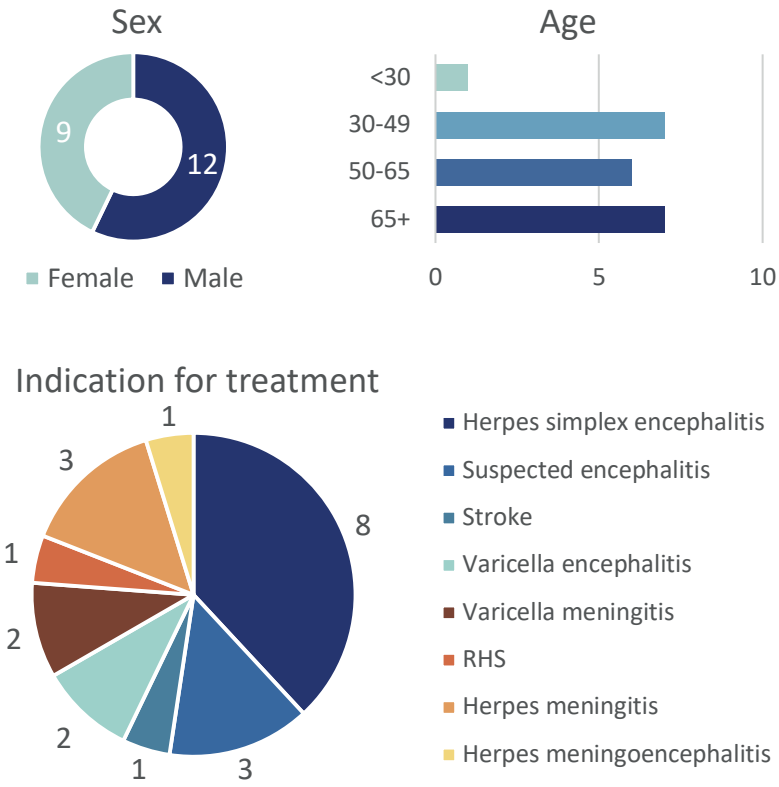

Figure 24. Demographics of 21 patients treated with acyclovir for acute CNS infection.

In our multiple linear regression model, several independent variables were associated with changes in CSF concentrations of acyclovir and CMMG (Table 3).

Table 3. Multiple linear regression models for acyclovir and CMMG CSF concentrations in 21 patients treated with acyclovir for CNS infection (samples = 34).

		Acyclovir CSF concentration		CMMG CSF concentration	
Independent variable	Comparison	β (95% CI)	P-value	β (95% CI)	P-value
Albumin ratio ($\times 10^{-3}$)	per 10 increase	1.499 (1.290 – 1.709)	<0.0001	0.388 (0.356 – 0.421)	<0.0001
Lag time (h)	per 1 increase	-0.779 (-1.350 – -0.208)	0.015	-0.046 (-0.072 – -0.020)	0.0038
CL_{CR} (mL/min)	per 10 increase	-1.428 (-2.099 – -0.756)	0.0015	-0.161 (-0.245 – -0.077)	0.0027
Dose (mg/kg)	per 1 increase	1.703 (1.215 – 2.190)	<0.0001	0.093 (0.022 – 0.163)	0.017
Weight (kg)	per 10 increase	1.996 (1.000 – 2.991)	0.0005	0.223 (0.052 – 0.394)	0.013
Age (years)	per 10 increase	0.042 (-1.402 – 1.487)	0.95	-0.026 (-0.198 – 0.147)	0.74
Gender	Female	0.774 (-2.276 – 3.824)	0.60	0.292 (-0.179 – 0.762)	0.21

Lag time = time from last dose to sampling. CL_{CR} = creatinine clearance

[Reprinted from paper IV [273] by permission of Oxford University Press on behalf of the British Society for Antimicrobial Chemotherapy.]

Decreased renal function was associated with increases in acyclovir and CMMG CSF concentrations, as were higher doses/kg of acyclovir and patient weight. An increase in lag time between acyclovir administration and CSF sampling was associated with lower concentrations. Finally, loss of BBB integrity as measured by increased CSF:serum albumin ratio was associated with increased concentrations of both acyclovir and CMMG. CSF concentrations of acyclovir and CMMG are presented in Figure 25 to assist in interpreting magnitudes of the associations between independent variables and concentrations.

Five patients developed neuropsychiatric symptoms after initiation of acyclovir treatment; all improved following decreased dose or cessation of treatment. One patient developed anxiety and decreased muscle tonus, which has not previously been described in association with AINS. Ultimately, it was concluded that it was unlikely that the symptoms were caused by acyclovir. The four remaining patients became increasingly confused under treatment, rather than showing improvement as would have been expected, but AINS was not suspected by the treating clinicians. All four patients had increased concentrations of CMMG in CSF, and only one other patient outside this group who suffered from herpes meningitis had a CMMG concentration (0.77 μmol/L) in excess of the 0.5 μmol/L that has previously been associated with AINS [191] (Figure 25). Looking at risk factors for increased CMMG concentrations, 4/4 patients had increased CSF:serum albumin ratio >10.2, indicative of BBB damage, 4/4 had decreased renal function, and 3/4 patients were treated with acyclovir doses greater than 10 mg/kg.

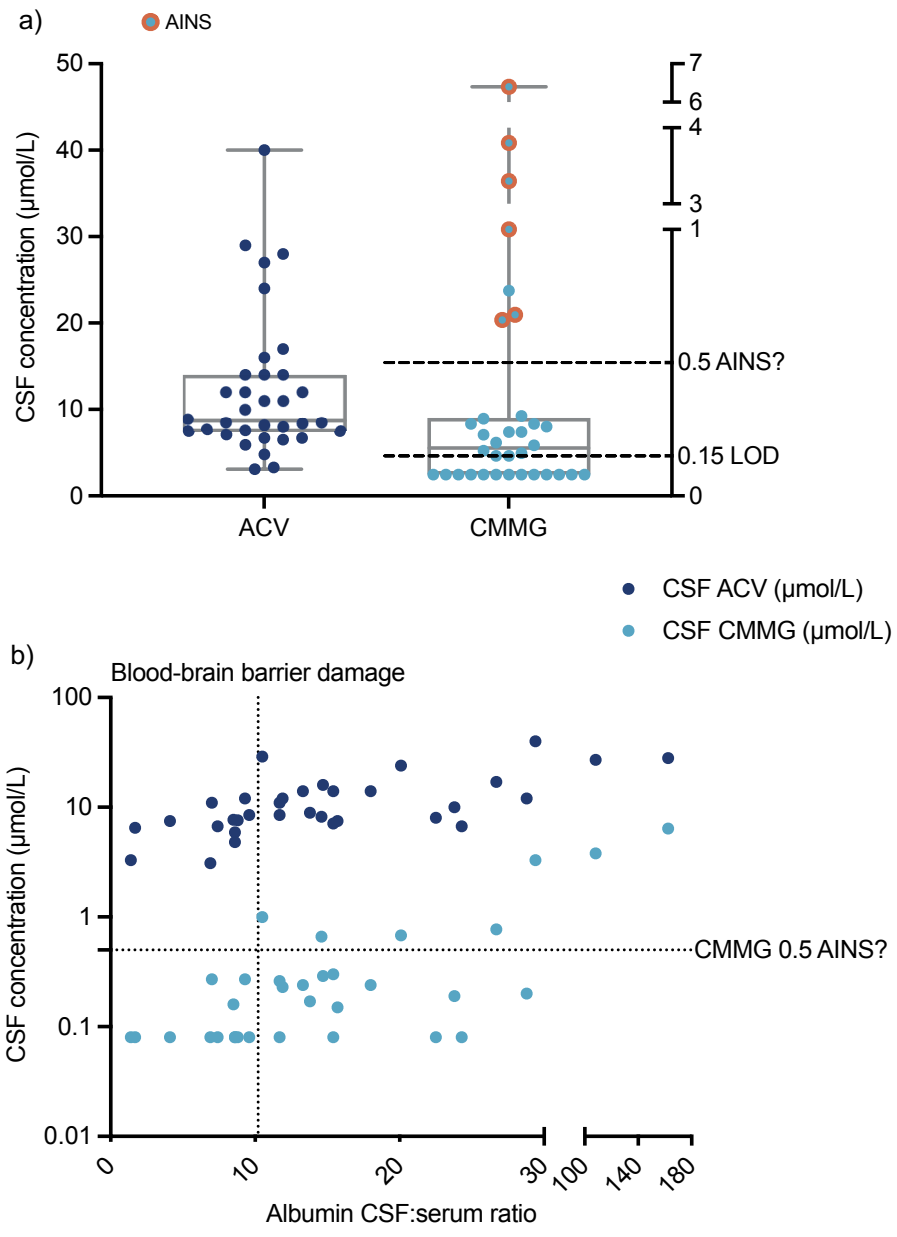

Figure 25. a) Acyclovir and CMMG concentrations in the CSF of the 21 included patients. Samples from patients with AINS are highlighted. b) Acyclovir and CMMG concentrations in relation to CSF:serum albumin ratio.

4.8 DISCUSSION PAPER IV

Prior to this study, very little was known about the pharmacokinetics of acyclovir in patients with CNS infection. Although variables such as doses and renal function are likely transferrable from patients in previous pharmacokinetic studies, data on BBB integrity were lacking.

We demonstrated an association between BBB integrity in acute CNS infection and CSF concentrations of both acyclovir and CMMG. Although increased acyclovir concentrations may be advantageous, an increase in CSF:serum albumin ratio had a greater relative impact on CMMG CSF concentrations than acyclovir CSF concentrations. Smith et al. [177] demonstrated that the CSF:plasma AUC ratio for acyclovir is considerably higher than that for CMMG, indicating substantial differences in the ability of the respective molecules to penetrate the BBB. In this context, the greater impact on CMMG CSF concentrations is reasonable, but may also be detrimental considering the association between CMMG and AINS.

Of note, we also observed that increased patient weight was associated with increased acyclovir and CMMG concentrations, possibly due to higher total doses of acyclovir when dosed according to weight. As previously mentioned, dosing according to ideal body weight may be more appropriate in obese patients because of lower acyclovir distribution to fatty tissue, but even when obesity and attendant changes in distribution are disregarded, higher body weight is not necessarily reflected by increased renal elimination. The relationship between weight and acyclovir concentrations is poorly investigated; based on our data, increased attention to symptoms of toxicity may be needed in patients with higher body weight.

Unexpectedly, we identified four patients with symptoms consistent with AINS. All of them had increased concentrations of CMMG in CSF. One other patient had a concentration higher than 0.5 μmol/L, the cut-off previously associated with AINS [191]. Although no indications of neuropsychiatric symptoms were noted in the patient medical records, the retrospective assessment in our study is a limitation, since potential symptoms of AINS may have been present but not identified. The treating clinicians did not suspect AINS in any of the four patients identified in our assessment, highlighting the difficulties in interpreting symptoms associated with AINS against the background of viral encephalitis.

Acyclovir dosing in CNS infection is not straightforward. The only dose studied in randomized controlled trials is 10 mg/kg q8h for HSE. Other dose recommendations are based on assumptions from *in vitro* studies on acyclovir susceptibility in VZV compared with HSV-1, pharmacokinetic data,

and in some cases on the hope that higher doses may further improve outcomes in selected patients. The last reason arguably applies in cases of neonatal HSV infection, where 20 mg/kg q8h for 21 days has been demonstrated to be superior to 10 mg/kg for 10 days [274]. Our results indicate that doses higher than 10 mg/kg may be unnecessary in the setting of reduced BBB integrity as seen in acute CNS infection and that the risk of AINS in this situation may offset the potential gain in many cases. There may still be patients who could benefit from higher doses. But, rather than initiating treatment with a higher dose, it may be advisable to delay increased dosing until after viral etiology has been established and due consideration given to rehydration, stable renal function, body weight, and data regarding BBB integrity. Also, if available, determination of acyclovir and CMMG concentrations in patients with unexpected neuropsychiatric symptoms or persistent cognitive impairment may provide valuable information.

5 CONCLUSIONS

In VZV facial palsy, CSF biomarker expression is consistent with a pattern of neuronal damage and astrogliosis. Unexpectedly, this pattern was only seen in patients with associated zoster rash, which raises the question of possible differences in the pathophysiology between typical Ramsay Hunt Syndrome and *zoster sine herpete*.

Analysis of the chemokine CXCL13 in CSF demonstrated good performance in discriminating between VZV facial palsy and LNB facial palsy, especially in samples obtained early after onset of symptoms, although low or moderately increased concentrations must be interpreted with caution.

Evaluation of the FilmArray ME panel indicated high performance with excellent potential to be a first-line diagnostic tool for the included pathogens. Nevertheless, caution demands additional testing should the ME panel results be negative despite strong suspicion of HSV-1 encephalitis. Moreover, interpretation may be difficult when *S. pneumoniae* and HHV-6 results are unexpectedly positive.

CSF concentrations of acyclovir and its metabolite CMMG are affected by renal function, dose, patient weight, and BBB integrity in acute CNS infection. An unexpected number of patients with unrecognized AINS were identified, indicating a need for increased attention to suspected symptoms, careful consideration of acyclovir dosages, and a liberal threshold for obtaining acyclovir and CMMG concentrations.

6 FUTURE CONSIDERATIONS

Significant advances have been made in the diagnosis and management of herpesvirus CNS infection, but considerable mortality and morbidity are still associated with these rare, but globally important, infections. Morbidity has likely been underestimated, with reports of long-lasting sequelae associated with manifestations that were previously considered benign, including viral meningitis [94, 102]. Many knowledge gaps remain where additional research may benefit patients in the future.

Our ability to diagnose herpesviruses in the CNS has dramatically improved thanks to advances in PCR diagnostics. Improved access to both diagnostics and epidemiological data has implicated herpesviruses, especially VZV, in a growing number of diseases such as ischemic stroke. Still, many cases of encephalitis and aseptic meningitis remain unexplained, and new methods are being researched to improve both turnaround time and diagnostic yield. Although technology such as mNGS will allow us to better identify pathogens, it is essential that new methods be validated and compared to established reliable diagnostic procedures, since new does not always translate to better.

Despite high diagnostic accuracy, we are still limited in our ability to prognosticate outcome in patients with herpesvirus CNS infection. An area of great promise is research into CSF biomarkers, and there are examples where CSF biomarker concentrations have been associated with prognosis. Further unraveling of pathological processes and immune response may allow us to better tailor treatment and rehabilitation to the individual patient, thereby reducing morbidity. One example is in patients with infection caused by VZV, where the distinction between peripheral reactivation and CNS involvement may be unclear and different approaches to management may be appropriate.

Perhaps the most tempting approach to combating herpesvirus CNS infection is to prevent infection in the first place, or at least to reduce the risk of reactivation. Vaccinations against varicella and herpes zoster are effective, but further evaluation is needed regarding the prevention of CNS infection. The varicella vaccine has not yet been introduced into the Swedish childhood immunization schedule, but data from several other countries support nationwide implementation. HSV vaccine development has proven difficult. On the bright side, the surge in vaccine development related to COVID-19 may also have beneficial ramifications for an HSV vaccine, and new vaccine types such as mRNA vaccines need to be explored.

In patients with manifest CNS infection, antiviral treatment with acyclovir is available, but dosing recommendations are thus far based on scanty evidence. The relationship between acyclovir concentrations and treatment effect is unknown. More data on the therapeutic window is needed for better individual dose adjustment, possibly even based on monitoring of concentrations. Moreover, although rarely observed in CNS infection, acyclovir resistance is a problem, especially among the growing number of immunocompromised patients. Therapeutic alternatives are needed, and evaluation of new antivirals for treatment of CNS infection is vital.

Finally, in addition to antiviral actions, the immune system contributes significantly to pathogenesis in herpesvirus CNS infections, but there are still large knowledge gaps concerning the involved inflammatory and immunological processes. Biomarker analyses could prove beneficial, both for identifying potential targets for treatment and for tailoring treatment to the individual. Recently, experiences from the COVID-19 pandemic have illustrated the importance of appropriate timing when administering immunomodulating drugs, including corticosteroids, for viral disease. As of now, use of corticosteroids to treat herpesvirus CNS infections has not been adequately investigated. Indeed, more specific immunomodulatory therapies are of interest considering their great potential to improve outcome in various CNS syndromes.

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