Varicella-zoster virus infections of the central nervous system

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ABSTRACT

Both varicella (chickenpox), and the reactivated form of herpes zoster (shingles), may cause neurological complications with various central nervous system (CNS) manifestations. Following introduction of PCR as a diagnostic method, the possibilities to detect the virus in cerebrospinal fluid (CSF) and to explore this disease, have dramatically improved.

With the quantifiable properties of real-time PCR the question arose whether VZV viral load was correlated to the severity of neurological disease. In 97 patients, the medical records were retrospectively studied and the spectrum of clinical entities discerned. CSF VZV DNA was quantified in 66 of these cases. Baseline viral loads were higher in patients with meningitis and encephalitis as compared with those suffering from Ramsay Hunt syndrome. However, these differences did not reflect the severity of disease why this parameter was not a reliable predictor of outcome. Additionally, based on our data, VZV seems to be a more common aetiological agent of CNS infections than previously thought.

Despite the usefulness of PCR, this technique has its diagnostic limitations. In patients with late diagnosis, the VZV DNA may be absent at time of PCR analysis. Serological analysis for detection of intrathecal antibody production is then required. Using a crude VZV antigen does not properly discriminate between antibodies to VZV and HSV-1. We produced and evaluated a purified glycoprotein antigen, VZVgE. When 854 serum samples were analysed, VZVgE-ag showed equal sensitivity and at least as high specificity compared with VZVwhole-ag.

VZVgE was also evaluated as a serological antigen in CSF. Paired samples of CSF and serum from 29 patients with clinical diagnosis of VZV CNS infection (n=15) or herpes simplex encephalitis (HSE) (n=14), all confirmed by PCR were analysed. In ELISA, 11/14 HSE patients showed intrathecal antibody production with VZVwhole-ag compared with 4/14 using VZVgE-ag. In the patients with VZV CNS infection, the two antigens showed comparable results. When the CSF/serum samples pairs were diluted to identical IgG concentrations, higher CSF/serum optical density (OD) ratios were found in VZV patients using VZVgE-ag compared with VZVwhole-ag. These results show that VZVgE is a sensitive antigen for serological diagnosis of VZV CNS infection without cross-reactivity to HSV-1 IgG.

To evaluate the potential degree of brain damage in patients with VZV CNS infections, we prospectively studied the CSF concentrations of neuron-specific light chain neurofilament protein (NFL), glial fibrillary acidic protein (GFAp) and S-100β protein in 24 patients with VZV DNA positivity and acute neurological symptoms. Concentrations of CSF NFL and GFAp were moderately increased, while the S-100β levels were reduced. These results indicate that VZV might induce neuronal damage and astrogliosis, and this finding was most pronounced in the patients with VZV encephalitis.

The cognitive impairment in patients with VZV CNS infections is largely unknown. We investigated the cognitive impairment in 14 patients with predominant CNS infections caused by VZV in a 3-year follow-up. The VZV patients performed worse than controls (n=28) on 4 tests covering the domains of speed and attention, memory and learning and executive function. The VZV patients were also classified into the concept of mild cognitive impairment (MCI), which is associated with development of dementia. A greater proportion of VZV patients was classified with MCI compared with controls. These findings suggest that patients with previous VZV CNS infection might carry a risk of long-term cognitive impairment.

Key words: Varicella-zoster virus infection, central nervous system, neurological sequelae, cerebrospinal fluid, viral load, intrathecal antibody production, glycoprotein E, biomarkers, cognitive impairment

SAMMANFATTNING PÅ SVENSKA

Varicella-zoster virus (VZV) orsakar vattkoppor, oftast i barndomen. Därefter lagras virus i nervknutor längs ryggraden och i huvudet. Virus kan sedan reaktivera senare i livet och ger då bältros. Både vattkoppor och bältros kan orsaka olika neurologiska komplikationer i hjärnan såsom hjärninflammation, hjärnhinneinflammation, ansiktsförlamning och även stroke. Sedan en ny mätmetod introducerades (PCR) har möjligheterna att upptäcka virus i ryggvätskan (vilket är ett tecken på infektion i hjärnan) förbättrats betydligt.

Med hjälp av realtids-PCR kan man också mäta mängden virus. Vi undersökte om mängden virus i ryggvätskan som man mäter vid ankomst till sjukhuset kunde relateras till allvarlighetsgraden av de olika neurologiska komplikationer man kan få av vattkoppsvirus. 97 patienters journaler studerades och hos 66 av dessa patienter mätte vi mängden vattkoppsvirus i ryggvätskan. Vi kunde dock inte påvisa något sådant samband. Däremot visade det sig att vattkoppsvirus var ett av de vanligare virus som orsakade komplikationer i hjärnan, i åtminstone Västra Götalandsregionen.

Även om PCR är en bra mätmetod behöver man ibland andra tekniker. När det dröjer ett tag från sjukdomsstart tills man tar ryggprovet kan nämligen virus vara svårt att upptäcka. Då behöver man istället mäta antikropparna mot virus i ryggvätska och blod för att se om det finns tecken på vattkoppsinfektion i hjärnan. Vid sådana ”serologiska” metoder behöver man ett antigen (oftast ett protein) som fäster till antikropparna, så att man kan upptäcka dem. Antigenet måste vara specifikt, i det här fallet för vattkoppsvirus, eftersom man annars kan få ett falskt positivt svar. Därför tog vi fram ett nytt antigen, VZV glykoprotein E (VZVgE), som skulle vara renare än det antigen man använt tidigare. VZVgE visade sig vara ett både känsligt och specifikt antigen, både för analys i blod och i ryggvätskan.

Vi undersökte också om det fanns tecken på hjärnskada hos 24 patienter som fått vattkoppsvirusinfektion i hjärnan. Det gjordes med hjälp av olika markörer för sönderfall av nerv- och stödjeceller i hjärnan som mättes i ryggvätskan. Vi fann att patienter med säkerställd vattkoppsinfektion i ryggvätskan samtidigt som de hade neurologiska symptom, hade tecken på hjärnskada, i form av skadade neuron och stödjeceller. Det var mest uttalat hos de patienter som hade vattkoppsorsakad hjärninflammation.

Vi undersökte även 14 patienter tre år efter att de hade fått neurologiska komplikationer orsakade av vattkoppsvirus, med hjälp av olika neuropsykologiska tester. Vi utförde också en klassificering för att bestämma om de led av ”mild kognitiv störning”, eftersom det tidigare har associerats med ökad risk att utveckla demens. Vi fann att fler patienter med tidigare vattkoppsorsakade neurologiska komplikationer i hjärnan hade kognitiv nedsättning jämfört med friska kontrollpersoner.

LIST OF PAPERS

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

I. Persson A, Bergstrom T, Lindh M, Namvar L, Studahl M: **Varicella-zoster virus CNS disease--viral load, clinical manifestations and sequels**. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2009, **46**(3):249-253

II. Thomsson E, Persson L, Grahn A, Snall J, Ekblad M, Brunhage E, Svensson F, Jern C, Hansson GC, Backstrom M *et al*: **Recombinant glycoprotein E produced in mammalian cells in large-scale as an antigen for varicella-zoster-virus serology**. *Journal of virological methods* 2011, **175**(1):53-59.

III. Grahn A, Studahl M, Nilsson S, Thomsson E, Backstrom M, Bergstrom T: **Varicella-zoster virus (VZV) glycoprotein E is a serological antigen for detection of intrathecal antibodies to VZV in central nervous system infections, without cross-reaction to herpes simplex virus 1**. *Clinical and vaccine immunology : CVI* 2011, 18(8):1336-1342.

IV. Grahn A, Hagberg L, Nilsson S, Blennow K, Zetterberg H, Studahl M: **Cerebrospinal fluid biomarkers in patients with varicella-zoster virus CNS infections**. *Journal of neurology (Epub ahead of print)* 2013.

V. Grahn A, Nilsson S, Nordlund A, Lindén T, Studahl, M: **Cognitive impairment after neurological Varicella-zoster virus infection - a 3-year follow-up.** *Submitted*

CONTENTS

ABBREVIATIONS

CAB	Cognitive assessment battery
CD4	Cluster of differentiation 4
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computer tomography
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
GFAp	Glial fibrillary acidic protein
GMK	Green monkey kidney
GOS	Glasgow outcome scale
HAD	Hospital Anxiety and Depression scale
HIV	Human immunodeficeny virus
HRP	Horseradish peroxidase
HSE	Herpes simplex encephalitis
HSV	Herpes simplex virus
IC_{50}	Drug concentration needed to inhibit 50% of viral replication
IF	Immunofluorescence
Ig	Immunoglobulin
IQR	Interquartile range

MCI	Mild cognitive impairment
MMSE	Mini-mental state examination
MOCA	Montreal cognitive assessment scale
MRI	Magnetic resonance imaging
NFL	Neuron-specific light chain neurofilament
NIHS	National Institutes of Health stroke scale
OD	Optical density
ORF	Open reading frame
PaSMO	Parallel Serial Mental Operations
PCR	Polymerase chain reaction
PHN	Postherpetic neuralgia
PVDF	Polyvinylidene difluoride
SDMT	Symbol Digit Modalities Test
SLE	Systemic lupus erythematosus
TBE	Tick-borne encephalitis
TGN	Trans-Golgi network
TIA	Transient ischemic attack
U_L	Unique long section
U_S	Unique short section
V_{max}	Velocity max
VZV	Varicella-zoster virus
WBC	White blood cells

1 INTRODUCTION

Varicella-zoster virus (VZV) is a ubiquitous human pathogen that causes varicella, commonly called chickenpox, and in its reactivated form herpes zoster, referred to as shingles.

The earliest reports of vesicular rashes of the type we now recognise as being caused by herpes simplex and zoster date back to the ancient civilisations. It was not until 1888, however, that a relationship between herpes zoster and chickenpox was suggested [1]. The link was proven when the virus was isolated from both chickenpox and zoster and compared in the early 1950s [2]. Neurological complications presented as encephalitis and cerebellitis during primary and reactivated disease were recognised earlier [3, 4], but it was not until 1966, that VZV was isolated from the cerebrospinal fluid (CSF) [5].

1.1 Epidemiology

Varicella

Varicella occurs with a worldwide geographical distribution. As the only α-herpesvirus that is transmitted via the airborne route, it displays a typical seasonal pattern with annual epidemics occurring most frequently during late winter and spring [6][7]. This phenomenon is more common in temperate than in tropical climates. At least five genotypes of VZV exist, clade 1-5, and the different VZV strains correlate with geographical variations in prevalence [8]. Genotypes 1 and 3 are found mainly in Europe and North America, while viruses of genotypes 4 and 5 are mostly found in Africa and Asia, and genotype 2 is mostly found in Japan. The risk of being infected with the virus in susceptible household contacts exposed to varicella is approximately 90% [9], while less prolonged or intensive exposure results in transmission rates of 10-35%. In temperate climates, children usually acquire varicella during their first five to 10 years of life. In Sweden, approximately 98% of children at 12 years of age have antibodies against varicella [10]. In contrast, in many tropical countries, the incidence of varicella during childhood is low and the primary infection frequently occurs in late adolescence or early adulthood. In developed countries, average crude varicella mortality rates range from 0.3 to 0.5 per million population, and overall case fatality rates are reported to about 2-4 per 100 000 cases [11]. In countries with varicella vaccination, such as the USA, the incidence of varicella has been reduced by 76-87% [12].

Herpes zoster

Herpes zoster is described to lack seasonal pattern because the disease results from the reactivation of endogenous, latent virus and is related to host factors [13-15]. Interestingly, however, others have shown a seasonality, which mirrors the one of varicella [16, 17]. The incidence of herpes zoster increases with age (Figure 1) and the incidence is approximately three per 1000 persons per year at the age of 50, but it reaches about 10 per 1000 persons per year at the age of 80 [18, 19]. Herpes zoster is more common among patients who are immunocompromised as a result of medication or disease.

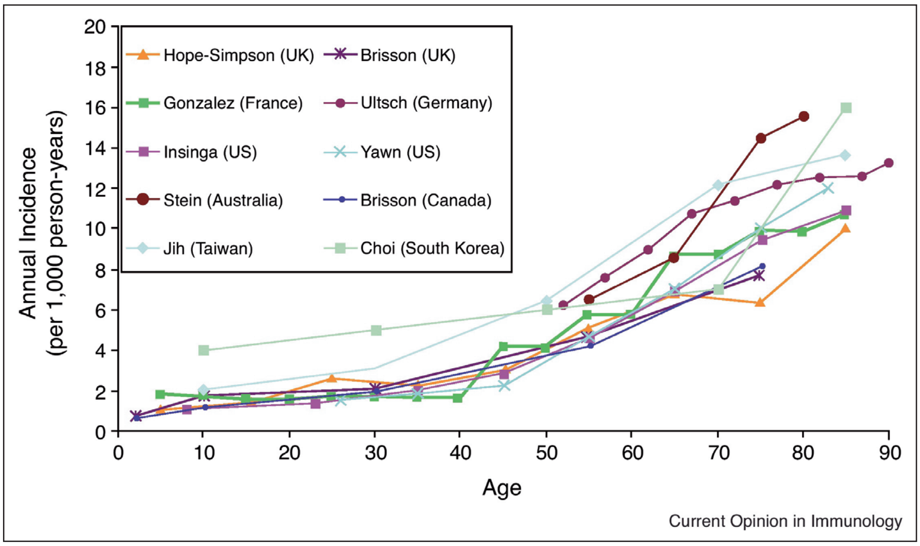

Figure 1. Effect of age on the incidence of herpes zoster [15, 20-27] (Reprinted by permission of Elsevier, Current Opinion in Immunology, Copyright 2012, reference[28]. The figure was provided by Eddy Bresnitz, MD, Merck & Co., Inc.)

1.2 The virus

VZV belongs to the herpesviridae family, which consists of more than 100 known viruses infecting non-human and human organisms and has evolved over at least 400 million years. The herpesviruses are classified into three subfamilies - α, β and γ herpesviridae based on their biological characteristics; α-herpesviruses are neurotropic and β and γ viruses are lymphotropic. In humans, all three subfamilies are represented. VZV belongs to the α-herpes viruses that diverged from the herpes viruses 180–210 million years ago and, is related most closely to herpes simplex virus (HSV) types 1 and 2, simian varicella virus and pseudorabies virus. All herpesviruses are large, double-stranded DNA viruses and a common denominator is their ability to establish latent/persistent infections. Their genomes are stable compared with those of RNA viruses.

The linear, double-stranded DNA genome of VZV consists of approximately 125 000 base pairs and codes for at least 71 viral gene products. The VZV genome is the smallest of the human herpesviruses and consists of two main coding regions covalently joined together – one unique long (U_L) section and one unique short (U_S) section. VZV DNA yields infectious virus when transfected into permissive cells. The DNA virion consists of DNA packaged in an icosahedral nucleocapsid that is surrounded by tegument proteins, which are critical for the initiation of infection. The virion is enclosed in a lipid membrane envelope in which glycoproteins form protruding spikes. The structure of VZV is shown in Figure 2.

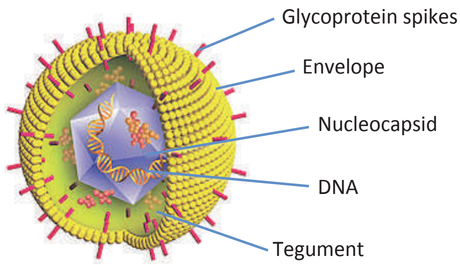

Figure 2. Varicella-zoster virus (downloaded from open domain https://en.wikipedia.org/wiki/Commons)

Viral replication

The virus is first attached to the host cell surface with the assistance of the viral glycoproteins. Following attachment the viral glycoproteins bind to the host cell surface and viral penetration occurs. This is followed by fusion with release of tegument proteins and nucleocapsid into the cytosol. The viral nucleocapsid then fuses with the outer nuclear membrane and the viral DNA genome is released into the nucleus of the host cell. In the nucleus, the expression of genes for VZV protein synthesis occurs in three stages. The first stage involves expression of the immediate early genes, whose products are transcriptional regulator genes. Once these are produced, they initiate the second stage with synthesis of early proteins. These proteins form the machinery that enters the nucleus and replicates viral DNA. The third stage is synthesis of late proteins, which are the ones that encode structural components such as the glycoproteins. These viral particles that lack tegument, envelope and the glycoproteins, bud through the inner membrane of the nucleus. Primary enveloped virions are formed in the perinuclear space, which fuse with the outer leaflet of the nuclear membrane and nucleocapsids are released into the cytoplasm. The glycoproteins are synthesised separately from the nucleocapsid. After protein synthesis, the glycoproteins are processed in the rough endoplasmic reticulum with glycosylation. The glycoproteins are then transported to the trans-Golgi network (TGN). Here, the final envelopment and synthesis of VZV virions occurs. This process is initiated by the glycoproteins. The VZV virions are then transported in vesicles in the cytoplasm and following fusion of the vesicle membrane with the plasma membrane of the cell, the virions are released onto the cell surface in elongated, densely packaged viral high-ways [29].

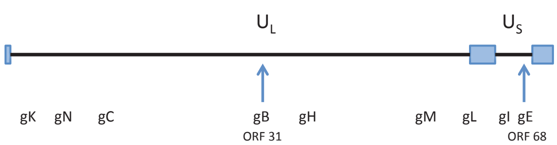

Figure 3. The VZV genome consisting of a uniqe longue (U_L) section and a uniqe short (U_S) section covalently joined together. The genes that code for the VZV glycoproteins are mostly situated in the U_L section, except the genes coding for gI and gE.

Table 1. Characterisation of the VZV glycoproteins

Glycoproteins	Gene	Main function
gB	ORF31	Critical for attachment and entry of virus into cells. Involved in intracellular trafficking.
gC	ORF14	Attaching to cells. Binding to C3b and stopping the complement cascade from destroying the infected cell. Essential for replication.
gE	ORF68	Forms a complex with gI. Essential for replication and intracellular trafficking. Behaves as an IgG Fc receptor on infected cells. Involved in attachment and entry of virus into cells.
gH	ORF37	Important for cell-to-cell spread by attachment and induction of membrane fusion.
gI	ORF67	Forms a complex with gE and is important for attachment and involved in intracellular trafficking. Essential for replication.
gK	ORF5	May be important for syncytia formation.
gL	ORF60	Facilitates maturation of gH and forms complex with gH
gM	ORF50	Important for cell-to-cell spread.
gN	ORF9A	Forms complex with gM.

VZV glycoproteins

VZV encodes nine viral glycoproteins (gB, gC, gE, gH, gI, gK, gL, gM and gN) (Table 1) which, among many functions, mediate viral attachment and cell entry, and their expression on cell membranes promotes cell fusion, permitting cell-to-cell spread of the virus. They also act as targets for the host immune response. They act either alone or in heterodimeric complexes with other glycoproteins. They are all envelope glycoproteins, except for gK, which acts only partly as an envelope glycoprotein [30]. The genes that encode for the glycoproteins are situated within the U_L region, except for gE and gI, which are encoded for in the U_S region (Figure 3).

Glycoprotein E (ORF 68) is the most abundant protein expressed in VZV-infected cells and is the major component of the virion envelope and probably the most immunogenic of the VZV glycoproteins [31]. In contrast, HSV gE, the homologue of VZV gE, is only a minor component of the HSV envelope and VZV gE and HSV gE have only 27% identity in amino acid sequence. The role of VZV gE is multifunctional. It complexes noncovalently with gI and behaves as an IgG Fc receptor on infected cells [32]. Moreover, gE and also gB, gI and gH are the targets for cytotoxic T lymphocytes and the host cellular immunity [33, 34]. The gE is also involved in cell-cell fusion in co-expression with gB [35], in addition to gH and gL, which are also important fusogens. This fusion often involves several uninfected neighbouring cells and results in giant multinucleated polykaryotes, also called syncytia. The exact mechanism for the glycoproteins mediation of syncytia formation and the movement of virus between cells is unclear. However, gE has been associated with the formation of tight junctions and additionally, it has been suggested that gE functions as a Ca^{2+} -independent adhesion protein to enhance cell-cell contact [36]. The role of gE in viral replication is essential, in contrast to its homologues in other α-herpesviruses. In virion formation, gE provides signal sequences to localise viral proteins for assembly in the TGN [37]. To achieve this, gE uses TGN specific amino acid targeting patches in their cytoplasmic tails [38]. Thereafter, gE (in complex with gI) is then involved in secondary enveloping of the virions. Many of the VZV gE functions appear to rely on a unique N-terminal region (aa1-188) [39]. Mutations in this region followed by disruption of the gE/gI complex formation, have been shown to alter cell-cell spread and secondary envelopment [40]. In addition, this region is required for VZV tropism for T-cells and skin infection [39]. Furthermore, VZV gE has been shown to bind to the cellular protein insulin-degrading enzyme which is thereby proposed to function as a cell surface receptor for VZV entry [41]. This interaction has also been attributed to the unique N-terminal region of VZV gE.

After gE, VZV gB (ORF 31) is the second most abundant VZV glycoprotein in VZV-infected cells. Its homology to HSV-1 gB (49%) is sufficient to permit the binding of cross-reactive antibodies [42]. VZV gB is the target of neutralising antibodies and is probably essential for infectivity. Like gE, it is also involved in attachment to the cell surface, cell-cell fusion [35] and intracellular trafficking.

1.3 Infectious cycle, latency and the immune response

Primary infection

VZV is predominantly spread via the airborne route, but virus may also be transmitted by fomites from skin lesions. The virus enters the body via the respiratory tract and spreads rapidly from the mucous membrane to regional lymphnodes where it undergoes the first phase of replication.

This is followed by spread of the virus to circulating T-lymphocytes during the first phase of subclinical viraemia after about four to six days. The virus is subsequently spread to reticuloendothelial tissues where it further multiplies. A second phase of viraemia after 14 days (10-21 days) has been proposed, following exit of the virus from reticulendothelial cells with subsequent viral spread to the nasopharyngeal surfaces and the skin. However, it has been shown that memory tonsillar T-cells that express skin homing markers become infected with VZV and transport the virus to cutaneous sites of replication [43]. One way or another, after 10-21 days, the virus reaches the skin, causing the typical vesicular rash of varicella. The rash is accompanied by flu-like symptoms including fever.

There are two essential components of the host response to varicella, in addition to the innate immune response − humoral and cell-mediated immunity, but their precise roles are not entirely understood. Humoral immunity is of major importance for neutralising cell-free virus mainly at sites of inoculation on reexposure to the virus. However, antibody response is suggested to be of less importance for recovery from varicella as shown in children with congenital agammaglobulinaemia who experience uncomplicated varicella [13]. IgM and IgA against VZV appear often appear within only one to two days after the rash from primary or reactivated VZV. The IgG response appears shortly after IgA and IgM. The time-course of IgM has not been well described and even though IgG in most patients seems to persist throughout the lifetime, the exact time course of VZV IgG is not well understood. Cell-mediated immunity is an even more important component of the immune response than humoral immunity, as VZV is a cell-associated virus and T-cell-mediated immunity is needed to eliminate intracellular pathogens. Both varicella and herpes zoster have been shown to be both more frequent and more severe in T-cell immunocompromised patients [44-46]. However, humoral immunity appear to supplement protection by cell-mediated immunity, as demonstrated by the success of passive immunisation with specific immunoglobulin [47].

Latency

All herpesviruses have the ability, after primary infection, to establish latency, which persists throughout the lifetime of the host. VZV establishes latency in neurons in cranial-, dorsal root- and autonomic ganglia [48-50]. One suggested pathway to the ganglia is by axonal retrograde transport. As afferent fibres of the sensory nervous system terminate in the skin, cell-free VZV that presents in varicella vesicles, have direct access to different ganglia [51]. The other suggested way is haematogenously by T-cell-mediated transport, followed by fusion of the neurons [52, 53].

Reactivation

Reactivation is associated with a decline in cell-mediated immunity, either as a natural consequence of aging or as result of immunosuppression. In lymphoma patients [54] and in bone marrow transplant recepients [55], the incidence of herpes zoster correlate with depressed VZV-specific cell-mediated immunity but not with levels of VZV antibodies [56]. Other factors that are associated with an increased risk of herpes zoster and which might influence the immunesystem are diabetes mellitus [57], genetic susceptibility [58], mechanical trauma [59] recent psychological stress [60] and white race [61]. In addition, female gender is reported as a risk factor [17, 59].

How does the decline of cell-mediated immunity induce reactivation? The presence of mediators of inflammation may influence the switch from latent to lytic infection but exactly what regulates this switch is not known. However, it has been shown that the immediate early protein 63 suppresses apoptosis of neurons [62]. In addition, this protein and immediate early protein 62 are transcriptional regulators that are only located in the cytoplasm during latent phase (as opposed lytic phase, when they are located in the nucleus). This exclusion from the nucleus probably prevents the cascade of protein synthesis that leads to lytic infection. In latent infection only six genes (including ORF 62 and ORF 63) are regularly expressed, whereas, in lytic phase, 71 genes are expressed [63]. The proteins from these six genes are only found in the cytoplasm during latent phase. The down-regulation of protein expression should be an effective way to escape the host immune system.

After reactivation, the virus multiplies and spreads within the ganglion causing neuronal necrosis and intense inflammation, which often results in severe neuralgia. The virus is then transported along microtubules within sensory axons to infect epithelial cells. In herpes zoster, skin blisters develop, accompanied by pain along the dermatome innervated by the sensory nerve (Figure 4). Normally, the pain vanishes after four to six weeks. Trigeminal (cervical) and thoracic sensory nerves are most commonly involved in VZV reactivation. Reactivation may also occur without any rash developing, ”zoster sine herpete” [64, 65].

Figure 4. Herpes zoster with thoracal distribution (downloaded from the open domain https://en.wikipedia.org/wiki/Commons)

In addition, VZV may reactivate subclinically, manifested by a rise in antibody titre [66]. Subclinical reactivation is reported in bone marrow transplant recipients [67] and also in astronauts [68], in the latter case most likely due to stress-induced depression of cell-mediated immunity.

Reinfection

In addition to subclinical reactivation of the virus, subclinical reinfection that boosts the immune response might occur. This is most common in adults who have had varicella but are then exposed to close household contact with varicella. Despite the lack of symptoms, a four-fold titre rise or greater in VZV antibodies and enhanced cellular immunity specific for VZV, has been shown after exposure to varicella [69, 70]. Furthermore, clinical reinfection with VZV has been reported in both immunocompetent and immunocompromised individuals. In a study of 1472 mostly healthy children who presented with chickenpox, 13% had a previous history of varicella [71]. Possible risk factors for clinical varicella reinfections might be primary infection at young ages (less than 12 months or in utero), mild initial first infection and genetic factors [38]. Clinical reinfection by another genotype is also possible and might not only cause varicella but also establish latency and reactivate to cause zoster [72].

1.4 VZV induced neurological disease

First, it should be mentioned that VZV causes other complications than the neurological ones. In primary VZV infection, the most frequent complication is secondary bacterial infection. Others are transient hepatitis that occurs in about 50% of children, varicella-associated pneumonia and thrombocytopenia. Reactivated VZV may involve complications such as, cutaneous with bacterial superinfection, visceral, including pneumonia and hepatitis and ocular complications.

Neurological complications (Figure 5)

Most neurological complications caused by VZV can occur in both primary and reactivated VZV, although they seem to appear more frequently in herpes zoster than in varicella. They affect both the central and the peripheral nervous systems. The incidence of CNS complications in children with chickenpox is reported to be 0.5-1.5 per 1000 [73, 74], with cerebellitis and encephalitis as the most common neurological manifestations [75, 76]. In adults with VZV-induced neurological complications, exact figures relating to the overall incidence are difficult to establish, but approximately 15% of herpes zoster patients suffer from post-herpetic neuralgia (PHN), which is defined as remaining pain along the dermatome, 90 days after onset of rash. The mean age of patients with neurological complications in varicella is reported to be four to seven years [74, 77] and, in herpes zoster 50-60 years of age [78, 79]. In patients with suspected viral CNS infections, 0.3-9% of the CSF samples are VZV DNA positive by PCR [80-84].

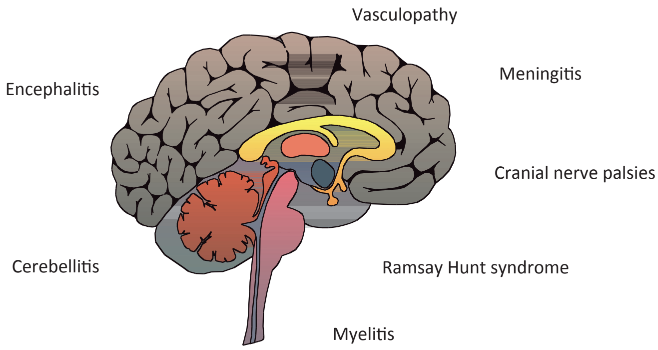

Figure 5. Neurological complications of the CNS that are associated with VZV

A vesicular rash may be absent in more than one third of the patients with reactivated VZV-induced neurological complications [64, 79, 85] and any of the neurological complications caused by reactivated VZV might develop in the absence of a rash. Since PCR was introduced and the opportunity to detect VZV DNA in the CSF has increased, VZV now appears to be a more common cause of CNS infection than was previously thought [79, 83].

Both immunocompetent and immunocompromised patients may suffer from these neurological complications but they appear to be more frequent and more severe in the latter group [86-90]. Yet, in varicella, the data are ambiguous and some studies report fewer neurological complications in immunocompromised children than in immunocompetent ones [74, 91]. This might be due to preemptive antiviral treatment in children with immunodeficiencies exposed to varicella, who are then more effectively protected.

Encephalitis is one of the most severe and common neurological complications in VZV infection. In overall terms, VZV is reported to be the most frequent viral cause of encephalitis after HSV [89, 92-94], and in children, VZV is an even more frequently identified agent than HSV [95, 96]. However, Sweden is an exception, as Tick-borne encephalitis (TBE) is reported to be the most common causal agent these recent years [97]. The overall incidence of VZV induced encephalitis in children is estimated at 0.2 per 100 000 children [75] and the average age is 5.4-6.4 years [74, 76].

Except for encephalitis, **meningitis** is a common manifestation, especially in reactivated disease. Of patients with suspected viral meningitis 4.4 to 11% are VZV DNA positive by PCR [82, 98, 99], and VZV is reported to be the second most frequent infective agent that causes meningitis, next after enteroviruses [94]. In general, patients with meningitis tend to be younger than patients with encephalitis [78, 95].

Cranial nerve palsies may involve most of the cranial nerves and the symptoms depend on which one is affected. The trigeminal nerve is the cranial nerve most commonly involved in VZV reactivation and this may be followed by symptoms from the three branches of this nerve: the optic nerve, the maxillary nerve and the mandibular nerve. If the optic nerve is involved there is a risk of serious ocular disorders with retinal necrosis. Ramsay Hunt syndrome is defined as peripheral facial palsy accompanied by rash on the ear (zoster oticus) and it is caused by VZV. In Ramsay Hunt syndrome, the vestibulocochlear nerve is often involved, together with the facial nerve, with subsequent hearing disorders.

Myelitis may occur both as an acute and as a chronic complication of VZV infection, but is generally uncommon. This complication is characterised by spinal cord involvement with paresis of the extremities, bladder or bowel incontinence and sensibility deficits. The clinical features reflect the distribution of VZV. The symptoms may appear from days to weeks after the appearance of the rash. Myelitis is more common in immunocompromised patients than in immunocompetent ones and is sometimes combined with encephalitis and other neurological complications [100, 101].

Cerebellitis is a well-known complication of childhood varicella, occurring in one per 4000 children with VZV infection [102]. The onset is acute, typically within one week of developing a rash. The disease usually lasts for two to four weeks. This neurological complication was primarily considered to be immune mediated, but the finding of VZV-DNA in the cerebrospinal fluid (CSF) in several patients indicates ongoing viral infection in the CNS [103].

Reye´s syndrome is a disease including encephalopathy and liver damage that is associated with varicella and aspirin intake. Since this association was identified [104], Reye´s syndrome is less frequently reported.

Vasculopathy

Vasculopathy caused by VZV occurs after both primary and reactivated VZV and in both immunocompetent and immunocompromised patients. In children VZV is reported to be the most common cause of acute ischaemic stroke [105]. On the other hand, the overall risk of varicella-associated stroke is estimated to be only one per 15000 children with chickenpox [106, 107]. The prevalence of VZV vasculopathy after reactivated VZV is unknown, as stroke in the elderly is often attributed to atherosclerotic disease and the CSF is not examined in those patients. Even in immunocompromised patients with stroke, CSF is not routinely examined for anti-VZV IgG antibodies. However, recent studies have revealed an increased risk of stroke after herpes zoster [108, 109].

One or several large or small cerebral arteries may be involved. Unifocal vasculopathy is most common following opthalmic distribution of herpes zoster in elderly adults. In these patients, the internal carotid artery is usually affected, followed by contralateral hemiplegia [110]. Other large arteries that are commonly involved besides the internal carotid artery, are the branches of this artery: the anterior and middle cerebral arteries and the external carotid artery [85, 110, 111]. Multifocal small vessel vasculopathy has previously been reported mostly in immunocompromised [86, 112, 113], but recent studies have shown that multifocal vasculopathy appears in both immunocompetent and immunocompromised patients [85]. Visual loss has been reported in patients with VZV infection together with small artery involvement, such as the central retinal artery and the posterior ciliary artery [114, 115]. Apart from more obvious vascular disorders such as stroke, where focal deficits depend on the location of infarction, and transient ischaemic attacks (TIA), encephalitis caused by VZV is also reported to be primarily a vasculopathy [116]. Less common manifestations of VZV vasculopathy are spinal-cord infarction, aneurysm and subarachnoid and intracerebral haemorrhage [117-119].

Neurological sequelae

Most children with neurological complications of varicella present a complete recovery without residual neurological disturbances [76, 120, 121]. In cerebellitis, complete recovery is normal although persistent cerebellar deficits may develop [122] and, in one long-term follow-up study, six of 11 children were reported with neurological sequelae including cognitive deficits [123]. Otherwise, there are few long-term follow-ups.

In adults, where neurological symptoms are mostly caused by reactivated VZV, the outcome seems to be less favourable, according to the scarce number of follow-up studies in these patients. In patients with herpes zoster-induced encephalitis, residual neurological sequelae range from mild to severe [124-127] and the mortality rate is reported to be 15-35% [93, 124, 125] the higher figure occurring in patients without treatment. Neuropsychological deficits in patients with herpes zoster encephalitis have been seen in up to 10 years after acute infection in patients not receiving antiviral treatment [124]. The neuropsychological deficits have been categorised as subcortical with slowing of cognitive processes, memory impairment and emotional and behavioural changes [126]. Yet, in another study of eight patients the neuropsychological sequelae were only very minor [127]. The risk of remaining facial palsy in patients with

Ramsay Hunt syndrome is reported to be 10-90% depending on the degree of palsy, treatment regimen and time interval between onset of disease and initiation of therapy [128-131]. The outcome in meningitis is still largely unknown, as follow-up studies are lacking. In myelitis, the outcome is shown to be good in immunocompetent patients and poorer in immunocompromised ones and the outcome appears to be less dependent on therapy regimen than on immune status [101].

Neuropathology

The neuropathogenesis of VZV infections is not well understood. One reason is that there has been no useful animal model for VZV as no small animals recapitulates disease in the human, which is in contrast to other α-herpes viruses. It is suggested that the spread of the virus to CNS in reactivated disease takes place primarily from afferent fibres from trigeminal and other ganglia via transaxonal transport [86, 116, 132, 133]. It should also be pointed out that the sensoric ganglia, which are part of the PNS, are situated very closely to the CNS, which do not only include the brain, but also the spinal cord (Figure 6). Another possibility is haematogenous spread by T-cell-mediated transport following by fusion of the neurons [52, 53] and then further transaxonal transport. Intracranial (and extracranial) blood vessels innervated by the afferent fibres then may then be infected. It has been shown that the middle cerebral artery, which is often involved in VZV CNS infections, receives its sensory innervation from the ipsilateral trigeminal ganglia [134]. In primary disease, the pathways of spread to CNS are not well described. One suggested scenario involves retrograde trafficking of virus from vesicles on the face to the trigeminal ganglion and then via the ophthalmic branch to cerebral arteries [135]. In CNS, haematogenous spread of the virus has been proposed, based on the presence of multifocal lesions at the grey-white matter junction in VZV CNS infections [86, 136].

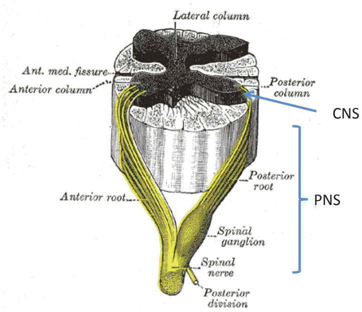

Figure 6. Illustration of that the spinal ganglia which are a part of PNS, is situated in close proximity to the spinal cord, which is a part of CNS (downloaded from open domain https://en.wikipedia.org/wiki/Commons)

As mentioned earlier, it is suggested that VZV encephalitis is primarily a vasculopathy [116, 137, 138] and, that symptoms of brain involvement are not a directly viral effect but develop secondary to productive virus within large and small cerebral arteries. Signs of vessel wall infection in the brain, such as VZV DNA and antigen in affected vessels [139, 140], Cowdry A inclusion bodies (specific for herpesvirus) and multinucleated giant cells [111, 141, 142], have been reported. In addition, in a study of virus-infected arteries, the presence of VZV primarily in early infection in the adventitia and later in the media and intima, supports the suggestion of transaxonal spread after reactivation [143]. Accordingly, in only a few cases, virus has been found in brain parenchyma [144, 145]. How the brain parenchyma with neurons and supporting cells is affected by VZV remains poorly defined. A wide range of different findings is described, including demyelination and necrosis of neurons [100, 145, 146]. In overall terms, damage to the neurons and supporting cells at spinal level appears to be more extensive than that to the brain [100, 138] and the immunocompromised host is more severely affected than the immunocompetent host [88, 136, 145, 147].

1.5 Diagnostic methods of VZV infections in the CNS

Patients with VZV CNS infection present with a wide spectrum of different neurological symptoms and vesicular rash is often absent. It is therefore important to rule out VZV in patients presenting with neurological complications indicating CNS involvement, where no other obvious causal agent is suspected. In cerebrovascular disease such as stroke and TIA, it is important to have VZV in mind if no other risk factors for cerebrovascular disease are present.

Neuroimaging and angiographic features

Brain imaging by MRI or CT reveals abnormalities in many cases of vasculopathy [85]. Abnormalities are cortical and deep, and occur in both the grey and white matter and at grey-white matter junctions [86, 112]. Most lesions are ischemic, but they may also be of haemorrhagic nature. Some lesions are enhanced on MRI with contrast, indicating blood-brain barrier damage. Both large and small arteries may be involved. As mentioned, the large arteries that are most commonly involved are the anterior and middle cerebral arteries and the internal and external carotid arteries. Typical angiographic changes include segmental constriction and occlusion, often with poststenotic dilatation [148]. On the other hand, a negative angiogram does not exclude this diagnosis, because small vessel disease is probably not detected as readily as in large arteries and VZV may manifest as exclusively small vessel disease. Although encephalitis is considered to be a vasculopathy by some important researchers in the field, the brain imaging is often negative in these patients [89, 125]. One reason might be that the CT or MRI is performed too early after onset of disease, while another reason could be that the infection of exclusively small vessels is difficult to detect, as with angiogram. In patients with zoster opthalmicus, pontine lesions are reported in several cases [149]. Ramsay Hunt syndrome might manifest as abnormalities in the 7th and 8th cranial nerves [150, 151]. Moreover, myelitis is associated with lesions on neuroimaging in the majority of patients [101]. Meningitis and radiculitis may coexist with vasculopathy [138], but neuroimaging changes are not reported.

Cerebrospinal fluid analyses

In most patients with VZV CNS infection, a mononuclear pleocytosis (white blood cells (WBC) > 4 x 10^6) is revealed in the CSF, but it might be absent, especially in vasculopathy [85]. The pleocytosis ranges from only few WBC up to several thousands [78], and tends to be less pronounced in children [74]. An elevated CSF/ serum albumin ratio indicating blood-brain barrier damage is a frequent finding in VZV vasculopathy [116] and is also reported in encephalitis, myelitis and facial palsies caused by VZV [152, 153]. On the other hand, both pleocytosis and elevated protein concentrations in the CSF are detected in uncomplicated herpes zoster [149].

Nowadays, virological diagnosis in the acute stage of disease is made by PCR for detection of VZV DNA in the CSF or by detection of intrathecal antibody production against VZV. The quantitative PCR [154], that has replaced the previously used qualitative PCR [103] has made it possible to measure the amount of viral load. Correlations between high viral load and severe manifestations, such as encephalitis have been shown [78]. Other areas in which quantitative PCR might be useful include monitoring viral response during antiviral therapy. In addition to VZV DNA findings in the CSF, VZV DNA has also been detected in the saliva of patients with cranial nerve palsies [155, 156] and this finding might be a helpful diagnostic tool in the future.

Serological analyses

Another way to diagnose VZV CNS infection is to determine intrathecal antibody production against VZV. The serum/CSF ratio of VZV IgG titres by enzyme-linked immunosorbent assay (ELISA) in CSF and serum is calculated. At some laboratories, this ratio is compared with the ratio of corresponding IgG titres against a reference virus; in Sweden, morbilli is a common reference virus, as most people carry antibodies to this virus. If the serum/CSF ratio of VZV IgG is low enough compared with the reference virus ratio, an intrathecal antibody production is assumed [157].

Serological analyses of the intrathecal antibodies are needed when PCR is negative, which is not uncommon [79, 158] especially in VZV vasculopathies [85]. One explanation of negative PCR is that the neurological symptoms associated with VZV vasculopathy often appear weeks and sometimes months after the acute VZV infection. The PCR might be negative just seven days after the outbreak of blisters preceding neurological complications, although it is sometimes positive after up to 26 days [158]. Furthermore, the same study showed that it takes at least seven days for IgG antibodies to be present in the CSF following onset of disease, which would create a ”diagnostic window” in which PCR and/or VZV IgG might be positive or negative in CSF.

Virus isolation from the CSF is nowadays rarely used and antigen detection with immunofluorescence (IF) is also becoming less fashionable and is being replaced increasingly by PCR. Both virus isolation and IF are labour intensive techniques, they are not amenable to automation and the interpretation of the results is quite subjective. In addition, the sensitivity of virus isolation is poor.

1.6 Biomarkers and cognitive dysfunction

Biomarkers in the CSF

Measuring protein biomarkers is an attractive tool for assessing neuronal death and glial pathology. Following neuronal and glial damage, proteins are released and can be quantified from the CSF, providing a source for estimating the severity of a neurological disease affecting the CNS.

Neurofilament proteins are the main component of the cytoskeleton in large myelinated axons. These proteins determine axonal radial growth and thereby conduction velocity and are composed of three subunits named according to their molecular weight [159]: light-NFL (68 kDa), medium-NFM (150 kDa) and heavy-NFH (190-211 kDa). The light chain, NFL is the most abundant of these three proteins. NFL is a sensitive biomarker of neuronal cell damage in a variety of neurological diseases. Markedly elevated levels of NFL have been demonstrated after acute ischemic events in the CNS, such as cerebral infarction, neonatal asphyxia and cardiac arrest, and these levels also correlate with severity and outcome [160-162]. In herpes simplex encephalitis, NFL increases to very high concentrations, with a peak two to three weeks after onset of neurological symptoms [163]. Other infectious neurodegenerative diseases with moderately increased NFL levels are tick-borne encephalitis (TBE) and neuroborreliosis [163, 164].

Glial fibrillary acidic protein (GFAp) is the major structural protein of astrocytes. GFAP is thought to help to maintain astrocyte mechanical strength as well as the shape of cells, but its exact function remains poorly understood [165]. The S-100 protein consists of a group of soluble dimeric proteins with different functions, where S-100β is glial-specific and is expressed primarily by astrocytes in the CNS. S-100β is distributed diffusely in the cytoplasm of astrocytes. Both GFAp and S-100β are markers of astroglial cell leakage and increase after structural damage to the CNS in various neurodegenerative diseases, such as stroke, traumatic head injury, intracranial tumour and encephalitis [166-170].

Cognitive dysfunction and mild cognitive impairment

Except for elevated biomarkers in CSF, cognitive dysfunction is reported in CNS infections as a sign of brain dysfunction. This condition sometimes lasts for several years after acute CNS infection [171]. As mentioned earlier, cognitive dysfunction has also been reported in VZV patients, long time after acute disease. One kind of cognitive dysfunction is mild cognitive impairment (MCI) which has become the most common diagnosis at Swedish memory clinics [172] and has been used to describe the transitional stage between normal cognitive function and mild dementia, before dementia is manifest [173]. MCI is connected with a higher risk of developing dementia [174-176]. However, some MCI subjects have more benign forms of cognitive impairment and do not progress to dementia and may even improve [177]. The cognitive impairment in patients with HIV has been associated with MCI, even in those on antiviral treatment and with highly surpressed viral levels [178]. In other CNS infections, this area is very sparsely investigated, but MCI has been reported in infectious brain diseases such as viral meningitis, meningoencephalitis and tick-borne encephalitis [179, 180].

1.7 Antiviral treatment

Since neurological complications of VZV infection seem to be caused by replication of VZV in the CNS, inhibition of replication is an obvious treatment. VZV is susceptible to several antiviral drugs. However, there have been no controlled studies probably because neurological complications have been regarded as a small problem and the number of patients with VZV CNS disease has been underestimated. Intravenously administered acyclovir is the therapy most frequently used for the treatment of VZV CNS infections. Another antiviral drug with therapeutic potential for oral administration is valacyclovir. In Sweden, the current recommendations in case of serious manifestations, such as encephalitis, vasculitis, myelitis and severe cerebellitis, are intravenously given acyclovir 10-15 mg/kg three times daily for seven to 14 days in adults. In vasculitis and cranial nerve palsies, additional steroid therapy may be considered to reduce the inflammation in CNS [116, 128, 181]. Valacyclovir is often used in clinical practice with a dosage of 1 g three times daily for seven days to patients with meningitis and cranial nerve palsies, although no clear recommendations exist.

Acyclovir is a synthetic acyclic purine nucleoside analogue. After administration it is phosphorylated first to acyclo-guanosine monophosphate by viral thymidine kinases and then into the active triphosphate form, acyclo-guanosine triphosphate, by cellular kinases. The active triphosphate form is incorporated into viral DNA, resulting in premature chain termination and in addition the activity of viral DNA polymerase is inhibited. Acyclovir triphosphate has greater affinity to viral than cellular polymerase, resulting in only small amounts of acyclovir being incorporated into cellular DNA. Subsequently, the toxicity of acyclovir is very low, but renal toxicity may occur after intravenous administration, especially in elderly people treated with higher doses. In addition, the accumulation of metabolites from acyclovir in the CNS, in patients with renal toxicity, is associated with neuropsychiatric side-effects [182]. CSF levels of acyclovir reach approximately 50% of the corresponding serum levels after i.v. administration [183].

Valacyclovir is a prodrug in the form of a valine ester of acyclovir that has greater oral bioavailability (about 55%) than acyclovir (10–20%) giving significantly higher serum acyclovir levels [184]. After oral administration, valacyclovir is converted by esterases to the active drug acyclovir, via hepatic first-pass metabolism. The toxicity and side-effects are similar to those of acyclovir.

1.8 VZV vaccine

The live, attenuated Oka varicella vaccine was first developed about 40 years ago [185]. The wild-type strain was isolated in Japan in 1971 from the vesicle fluid of a boy called Oka who had chickenpox. Originally, the vaccine was used to prevent primary VZV infection. But, it was soon shown that the immunocompromised vaccinated patients were also protected against zoster to some degree [186]. As a result, the Oka strain vaccine was further developed for prevention of herpes zoster. Both vaccines generate VZV-specific humoral and cell-mediated immune responses. The only difference between the vaccines is that the dosage of the zoster vaccine is about 14 times higher than the one of the varicella vaccine. Routine universal immunisation of infants is now administered in the USA, Canada, Uruguay, Sicily, Germany, Greece, South Korea, Taiwan, Israel, Australia and, recently, in our neighbouring country, Finland.

Varicella vaccine

Following the licensing of varicella vaccine in the USA in 1995, the incidence of chickenpox has fallen by > 80% in both vaccine recipients and also in the unvaccinated population, indicating herd immunity. In addition, hospitalisations and mortality due to chickenpox have markedly decreased. At the start, only one dose was administered. However, around 15% developed breakthrough disease, so a second dose of varicella vaccine was recommended in 2007. The varicella vaccine seems very safe, with very few serious complications reported. The rate of serious adverse events in the USA from 1995 to 2005 was reported as 2.6/100 000 given doses [187] and only about ten children with immunodeficiency have been described with severe Oka infections since 1995 [187-189]. In the latter group, the immunodeficiency was not known before vaccination or developed just following vaccination. Additionally, the rate of zoster in healthy vaccinated children has decreased by a factor of between four and 12 compared with children who have experienced natural infection [190]. Moreover, CNS complications after vaccination with the Oka strain appear to be very rare, and only a few cases of meningitis have been reported [191], all of which were associated with the occurrence of zoster. It is not really known if and when the immunity wanes after varicella vaccination, and so a booster dose should perhaps be given later in life.

Herpes zoster vaccine

The zoster vaccine is currently recommended in the USA, as one dose for persons over 60 years of age who are relatively healthy and, it will shortly be introduced in Sweden. The vaccine has been reported to reduce herpes zoster incidence by 51 % after a mean follow-up time of three years in a study comprising more than 38 000 adults over 60 years of age [51]. The vaccine recipients who developed zoster experienced less pain and post herpetic neuralgia was less frequent (an overall 61% lower burden of disease). The T-cell mediated immunity peaked two weeks after immunisation and then fell during the first year to remain at a level about 50% higher than pre-immunisation levels for the three-year study period. The efficacy of the vaccine in preventing zoster was markedly higher in subjects aged between 60 and 69 (64%) than in subjects ≥ 70 years of age (38%). These results were consistent with the magnitude of the boost in cell-mediated immunity, which was clearly age dependent. However, the duration of the immunity to prevent zoster after vaccination with this live zoster vaccine still needs to be determined. A follow-up study showed that the efficacy had declined to 40 %, up to 7.8 years after immunisation [192]. Furthermore, the safety of live zoster vaccine administration in immunocompromised has not yet been proven, although the vaccine has been given to VZV-seropositive HIV patients with $CD4^+$ T cells > 200 cells/ml with promising results [51].

2 AIMS

The overall aim of this thesis was to characterise and explore the clinical features of VZV CNS infections, and more specifically:

- To investigate the distribution of clinical manifestations and neurological symptoms and sequelae of VZV CNS infections.

- To analyse the viral load in the CSF and levels and kinetics of CSF biomarkers in patients with VZV CNS infection and, to correlate these findings with severity of neurological symptoms and outcome.

- To evaluate VZV glycoprotein E as a serological antigen for detection of specific intrathecal antibodies to VZV in serological analysis.

3 PATIENTS AND METHODS

3.1 Patients

All patients in this research project (Figure 7) are included from the population of Västra Götaland in Sweden, a region with 1.5 million inhabitants. Participants were included after they had given their informed consent, and the studies were approved by the Research Ethics Committee of Gothenburg University.

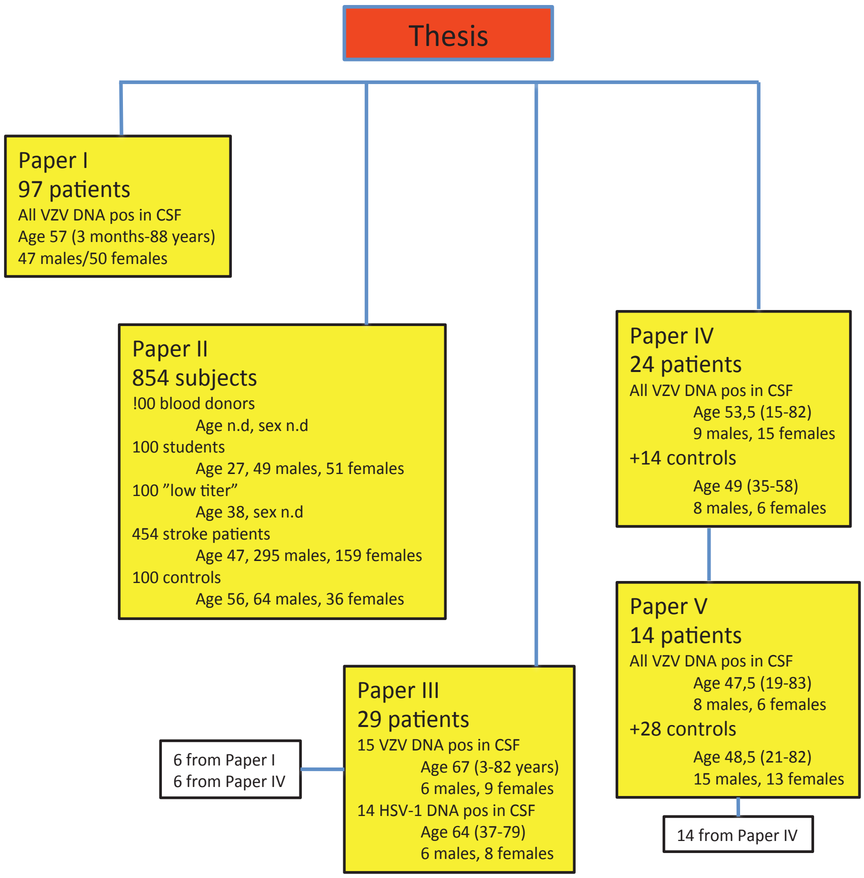

Figure 7. The number and distribution of participants in Papers I-V. Age is given as median and range, except for Paper II where age is given as the mean value

Paper I

One hundred patients from 10 different hospitals had detectable VZV DNA in their CSF at the clinical virology laboratory of Västra Götaland from 1995 to 2006. Medical records were obtained for 97 of these patients and they were included in this retrospective study. Sixty-five patients had their CSF analysed by real-time PCR and the remaining 32 patients were only analysed with qualitiative PCR due to small sample sizes. All patients had suspected neurological complications. Based on their medical records, the patients were categorised into five different clinical syndromes (encephalitis, meningitis, cranial nerve affection, encephalopathy and cerebrovascular diasease). If they did not fulfil the criteria for any of these syndromes, they were categorised under ”other symptoms”. Four patients in this last group had no neurological complications but they were lumbar punctured because of headaches and suspicion of meningitis. Ninety-two patients were assumed to have reactivated disease and five patients primary disease, based on clinical symptoms and serological testing with IgG positivity at high or moderate titres. Twelve patients were immunocompromised.

Paper II

Serum samples were collected from five groups comprising a total of 854 patients in this study. The five groups consisted of 100 blood donors, 100 medical students, 100 patients with sera with low IgG titres in VZV whole-ag ELISA, 454 patients with ischaemic stroke who had been recruited from the four stroke units in western Sweden, and 100 healthy population-based controls (age- and gender-matched with respect to the ischemic stroke patients).

Paper III

Twenty-nine patients with a clinical picture of CNS infection, consecutively sampled, and all PCR positive in CSF samples against VZV (n=15) or HSV-1 (n=14) were included. From all 29 patients, paired serum and CSF samples showed presence of intrathecal antibody production 0-4 months (1 year for 1 patient) after PCR positivity, against either VZV (n=15) or HSV-1 (n=14). All patients with HSV-1 CNS infection were diagnosed as encephalitis. The patients with VZV CNS infection were diagnosed as encephalitis (n=8), meningitis (n=4), Ramsay Hunt syndrome (n=2) or vasculitis (n=1).

Paper IV

Twenty-four patients had detectable VZV DNA in their CSF by real-time PCR and contemporary neurological symptoms and were consecutively enrolled in this study. These patients were collected from four different hospitals during the years 2007-2011. The 24 patients with VZV CNS infection were examined neurologically and sampled from CSF and serum consecutively. They were categorised as encephalitis (n=10), meningitis (n=9) or peripheral nervous disease (n=5). Four patients were immunocompromised. In addition, a control group of 14 non-infectious subjects with normal CSF findings were included. They had sought medical care because of headache or psychoneurotic symptoms.

Paper V

In this 3-year follow-up study, we included patients from the prospective study in paper IV (n=24). All patients who were still alive were asked to participate. Of these 20 patients, 15 wanted to join the study but one had to be excluded because of visual problems. Finally, 14 patients were included and performed the tests median 39.5 months (range 31-52) after acute disease. Two of these patients were immunocompromised. The control group (n=28) consisted of age- and gender-matched healthy individuals. Twenty of them were selected randomly from the Swedish National Population Register and eight individuals came from a control group initially recruited to the “Göteborg MCI study” [193] and from a student assay [194].

3.2 Methods

CSF and blood sampling

The CSF from Paper I and nine CSF samples from Paper III were collected from the routine diagnostics at the Virological Laboratory at Sahlgrenska University Hospital and the CSF from the patients with HSV-1 CNS infection in Paper III was from two previous studies [169, 195]. The CSF samples in Paper IV were collected consecutively during one year after acute disease. The CSF from the first lumbar puncture was analysed for VZV DNA, cells and albumin before storage. All CSF samples as well as the blood samples in Papers I to IV had been stored at −70°C before further analysis.

PCR

Quantification of DNA from VZV, HSV-1 and HSV-2, CMV, EBV and HHV-6 was carried out by first extracting the viral DNA from the CSF in a Magnapure LC. Second, amplification was performed in an ABI Prism 7900 real-time PCR instrument. For detection of VZV DNA, a 70 nucleotide segment of the gB region was amplified and detected by the use of primers VZVgB F, TGCAGGGCATGGCTCAGT and VZVgB R, CCCAAGAACCACATGTCCAAC, and the probe VZVgB P, CGCGGTCCCAAGTCCCTGGA. The real-time PCR for detection of VZV DNA has a lower detection limit of 100 GE/ml and a quantification rate spanning up to 10 million GE/mL.

Thirty-two CSF samples in Paper I and CSF from patients with HSV type-1 CNS infection (n=14) in Paper III were analysed by qualitative PCR. The reason was that the real-time PCR method was not available at the time of sampling and the sample sizes were to small for further analysis with real-time PCR. Real-time PCR for detecting VZV DNA was introduced 2003 in Västra Götaland. Qualitative PCR assays were performed in the Gene Amp PCR system 9600. The estimated sensitivity was between 1-10 femtogram (around 150 GE/ml). The results were scored as positive or negative.

Production and preparation of VZVgE antigen (Papers II and III)

In Papers II and III, VZVgE was used as an antigen. Initially, we used VZVgE produced by *Escherichia Coli* cells. However, the results were not satisfactory and it appeared that this antigen was not pure enough. As mammalian produced VZVgE was not available on the market, we decided to try to produce it ourselves.

The first step in the process was to generate a VZVgE mammalian expression plasmid. A coding sequence of the extracellular domain of gE from VZV was amplified by PCR. The sequences of the forward and reverse primers were 5′-AGGCAGAAGCTTACCATGGGGACAGTTAATAAACCTGT-3′, and 5′- AATAATACCGGTGGCATATCGTAGAAGTGGTGACG-3′. The amplified PCR fragment was then cut and cloned into the corresponding sites of a vector and transfected into CHO-K1 cells. These cells were cultured and cloned in several cycles in order to generate an effective and viable production of VZVgE. Western blot was used to screen for VZVgE expression. After about 25 days a pure clone of VZVgE was ensured. This clone was further adapted to serum-free suspension growth, as a serum-free VZVgE solution facilitates the subsequent purification process. This adaptation process took about nine weeks in total.

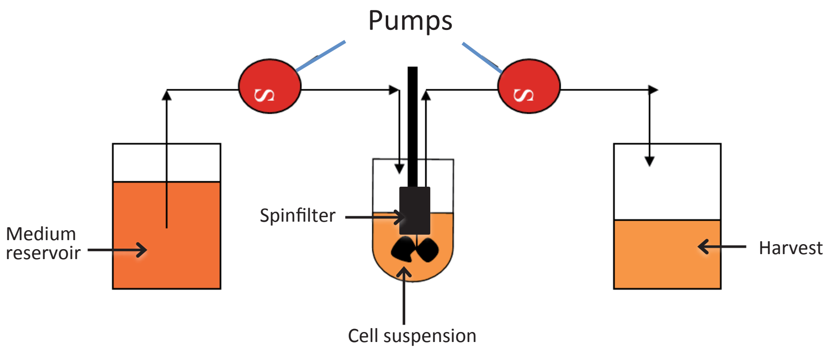

Figure 8. Bioreactor perfusion culture. During the process, continuous measures of pH, pO2, lactate and temperature of the cell suspension are performed (the figure was provided by Elisabeth Thomsson, Sahlgrenska Academy)

The following step was to produce larger quantities of VZV gE in a bioreactor perfusion culture. The advantage of this type of culture is that large volumes can be produced and, at the same time, controlled nutrient feeding is possible, thereby avoiding nutrient limitations and growth-inhibiting metabolites. A perfusion culture was set up in a 3 l Biobundle bioreactor (Figure 8). By help of a spinfilter, used medium was continuously exchanged for new to grow the culture. In all, 12.5 l of cell-free harvest was collected and centrifuged and concentrated down to a volume of 0.5 l. Partial buffer exchanges were performed five times to a final volume of 0.6 l.

Next procedure was purification of VZV gE. The bioreactor product was first centrifuged and pre-filtered. The filtrate was than applied to a 1 ml HiTrap chelating column that had been loaded with Co^{2+}. Bound protein was eluted with imidazole and then analysed by Western blot and silver staining for the presence of VZVgE (Figure 9). After quantitation of VZVgE, the antigen was ready to use.

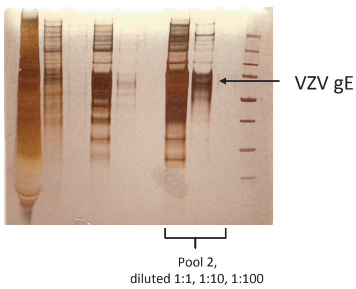

Figure 9. Presence of VZV gE by silverstaining

Gel electrophoresis and Western blot

In Paper II Western blot was used to screen for VZVgE expression from supernatants during the preparation process and also to confirm the presence of VZVgE after purification. In addition, discordant serum samples in Paper II were analysed using this technique. First, gel electrophoresis is performed. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through an agarose matrix (gel). Shorter molecules move more rapidly and migrate further than longer ones because shorter molecules migrate more easily through the pores of the gel. In our assays, the purified

VZVgE was mixed with sample buffer and heated before loading onto the gel. Gel electrophoresis was then performed, following staining with silver or colloidal blue. For Western blot, the proteins were not stained, but were transferred to nitrocellulose or PVDF membranes (Millipore) and then cut into separate strips. After washing, the strips were incubated with a block buffer solution. To analyse discordant serum samples, these were added at a dilution of 1:100 to the same solution and incubated over night. HRP-labelled polyclonal rabbit-anti-human IgG antibody was used as a conjugate for the serum samples and 4-chloro-1-naphtol was used as a substrate. To identify gE during production, a mouse monoclonal antibody VZV g Esc-56994 was used as a primary antibody and goat-anti-mouse-immunoglobulins-AP as a secondary antibody. Western blot is often referred to as a golden standard, but it is a subjective method, even if the reliability is increased by proper negative and positive control sera as well as monoclonal antibodies (in this study VZVgE). Additionally, this method is also time-consuming and labour-intensive.

Immunofluorescence

The presence of HSV IgM antibodies in Paper III and VZV IgG antibodies in Paper IV was determined by immunofluorescence. In Paper II this test was also used to evaluate discordant samples with ELISA. Briefly, infected cells present the antigen on the cell surface and if specific antibodies are available in the serum/CSF sample, they bind to the antigen. A target molecule in this antibody-antigen complex then binds to a secondary antibody which carries a fluorophore detected by an immunofluorescence microscope. HSV-1 and VZV-infected green monkey kidney (GMK) cells respectively were used for antigen presentation. As a conjugate, fluorescein-labeled goat anti-human IgM liquid globulin was used. Serum samples were titrated in two-fold steps and specific fluorescence of infected foci at a dilution of four or higher was determined as positive. Like Western blot, this is a subjective method, perhaps to an even greater degree. We used it as an additional test to ELISA and Western blot.

Enzyme-linked immunosorbent assay for detection of antibodies

In Papers II and III, ELISA was used when comparing the VZV whole antigen with VZVgE antigen and also for estimating of other antibody titres, such as IgG HSV and IgG morbilli. This test uses antibodies and colour change to identify a substance, usually antigens. Antigens from the sample are attached to a surface. The sample with possible specific antibodies is then applied and binds to the antigen. A secondary antibody which is linked to an enzyme is then applied. In the final step, a substance containing the enzyme substrate is added. The subsequent reaction produces a detectable signal, most commonly a colour change in the substrate. This colour change can be read on a spectrophotometer to determine the optical density (OD) levels at a certain wavelength, which in is turn proportional to the amount of antibodies in each well. In our assays, each well was coated with the given antigen at different dilutions depending on the antigen (1:3200 of VZVgE, 1: 1000-3000 of VZVwhole-ag and 1:1000 of HSV type-common antigen). The wells were then incubated with serum samples at a dilution of 1:100 or CSF samples at a dilution of 1:10 and diluted in two or four-fold steps. AP-conjugated affinipure F(ab′) Fragment goat anti-human IgG was used as a conjugate and Phosphatase Substrate as a substrate. The plates were read once every 10 minutes (20-80 min) on a spectrophotometer. The cut-off rate was set as a negative serum control diluted 1:200 plus 0.200 for all ELISAs. ELISA is a reliable method with high capacity as it is possible to run several samples simultaneously.

Assessment of intrathecal antibody production and blood-brain barrier damage

In most laboratories, at least in Sweden, assessment of intrathecal antibody production is performed by comparing the serum/CSF ratios of given antibody titres by ELISA to the serum/CSF ratios of the IgG titres against a reference antibody. This method was used in Paper III. In our assays, a serum/CSF sample ratio of VZV IgG four times lower than the serum/CSF sample ratio of the IgG of the reference virus was regarded as compatible with intrathecal antibody production. In addition, the CSF IgG titres had to be $\geq$ 80 using VZV whole antigen or type-common HSV antigen and $\geq$ 20 using VZVgE antigen. The problem with this method is that it is quiet rough, as the samples are always diluted in two-fold (or even four-fold) steps and when a sample is defined as negative we only know that somewhere between two dilution steps, it became negative. We therefore decided to assay the antibody in its most dynamic phase in Paper III. At first, IgG concentrations were determined in all CSF/serum pairs using a human IgG ELISA kit. Next the CSF and serum sample pairs from each patient were diluted to an identical IgG concentration of

1μg of total IgG/ml. After adding 100 μl to each well coated with either VZVgE or VZV whole-ag, ELISA was performed. The samples were all analysed in triplicates. The plates were read on a spectrophotometer and the signal was recorded every two or three minutes. The OD levels of all samples were compared at 15 minutes as most samples had reached their maximum levels at this point. In addition, velocity max (V_{max}) was estimated as the reaction time of the antigen-antibody complex and was regarded as proportional to the amount of VZV IgG in each sample. The intrathecal antibody production was then assessed by using the formula antibody index of OD_{CSF}/OD_{serum} at 15 minutes and at V_{max}. Intrathecal antibody production was assumed with an antibody index of ≥2.0 [196, 197].

Intrathecal antibody production was also assessed by determining the IgG index by calculating (CSF IgG (mg/l)/serum IgG (g/l))/albumin ratio, the reference value being < 0.63, independent of age [198]. The difference from the first described method is that the IgG index is not specific for the virus but is a general method for assessing the intrathecal antibody production of IgG in the CNS. This method was used in Paper III.

Blood-brain barrier damage was assessed in Papers III and IV by using the albumin ratio (CSF albumin (mg/l)/ serum albumin (g/l)). Reference values were < 6.8 for persons under age 45 and < 10.2 for those aged 45 and older [198]. Albumin levels in CSF and serum were measured by immunonephelometry.

CSF biomarkers

The following biomarkers were studied: GFAp, NFL and S-100β. The concentrations of GFAP and NFL were determined using previously described ELISAs [162, 199]. CSF levels of S-100β were determined using the Modular system and the S100β reagent kit (Roche Diagnostics, Basel, Switzerland).

Clinical outcome and neuropsychiatric testing

In Paper IV, clinical outcome of the patients was evaluated using Glasgow Outcome Scale. The range is from 1 to 5; death (1); persistent vegetative state - patient exhibits no obvious cortical function (2); severe disability – patient depends upon others for daily support due to mental or physical disability or both (3); moderate disability - patient is independent as far as daily life is concerned. The disabilities found include varying degrees of dysphasia, hemiparesis, or ataxia, as well as intellectual and memory deficits and personality changes (4); good recovery with resumption of normal activities even though there may be minor neurological or psychological deficits (5).

In Paper V, neuropsychiatric testing was used to estimate cognitive dysfunction. Hospital Anxiety and Depression scale (HAD) [200] was used to capture symptoms of anxiety and/or depression, which can in turn affect the cognitive capacity. NIHS [201] scale was used for screening of stroke symptoms. Other neuropsychiatric tests that were used for cognitive assessment were: Montreal Cognitive Assessment Scale (MOCA) [202] Mini-Mental State Examination (MMSE) [203] and the Cognitive Assessment Battery (CAB) by Sahlgrenska [204]. CAB is designed to capture even mild cognitive impairment (MCI) and includes the tests: Stroop test (Victoria version), Token test (6 item version), Symbol Digit Modalities Test (SDMT), Clox and cube, Naming test and Immediate and Delayed recall. Additionally, Trailmaking A, copy a complex figure and Parallel Serial Mental Operations (PaSMO) were administered [193]. Which cognitive domains these tests cover and how they are performed are described in Table 2. All tests together did not take more than one hour to perform to avoid patient fatigue. The tests were performed in a standardised sequence and no tests that could affect the performance of a memory test were administered between immediate and delayed recall.

Statistical analysis

In all papers, median and range or interquartile range (IQR) were used for group descriptives. Paired t-test was used for paired continuous data (Papers III and V). When comparing small groups or groups without normal distribution, non-parametrical statistics were used to analyse continuous variables: Mann–Whitney U test for group comparisons, Wilcoxon's signed rank test for paired data and Spearman's rank test for correlations (Papers I and IV). In Paper III, McNemar test was used for comparisons of paired categorical data. Fischer's exact test was used to test differences in proportions between patients (Papers II and V). One-sided permutation tests on the raw scores were performed in Paper V. In

Paper IV, all results of biomarkers and viral load were transformed to $\log_{10}$ before statistical analyses.

Table 2. Cognitive tests, tasks and domains

Test and task	Cognitive domain	Function	Localisation in brain
SDMT – transcribe as many symbols as possible, according to a coding key, during 90 s. **Stroop 1** – naming of coloured dots, on time **Stroop 2** – reading of colour words, coloured in that specific colour, such as green, on time **Trail making A** - connect a set of 25 numbered dots as fast as possible	Speed and attention	High order functions-planning, conseptualizing, organizing, evaluating. Difficulties include achieving insight, antisocial behaviour	Frontal lobe
Immediate and delayed recall – a text is read to the subject and repeated. After some other tasks the subject is asked to repeat the text again	Learning and memory	Memory; brief, short-term and long-term. Learning (recall, recognition)	Temporal lobes
Clox and the cube – draw a clock according to instructions and copy a complex cube **Complex figure copy**	Visuospatial functions	Ability to make sense of the visual world; shapes, angles, meaning of forms, and to reproduce what one sees.	Occipital lobe
Token test – 8 cubes in 4 different colours are to be arranged according to instructions **Naming 30 items**	Language		Temporal lobes
Stroop 3 – naming of colour words, coloured in a another colour, such as green, on time **PaSMO** - rattle of the alphabet, stating the number after each letter- that is, A-1, B-2, C-3…	Executive functions	Regulate, control, and manage other cognitive processes, such as problem solving, verbal reasoning, inhibition, mental flexibility, task switching and initiation and monitoring of actions.	Frontal lobe

4 RESULTS

Viral load of patients with VZV DNA in their CSF and concomitant neurological complications (Papers I and II)

Significant higher viral load was seen in patients with meningitis and encephalitis compared with patients with cranial nerve affection (including Ramsay Hunt syndrome) in the acute stage of disease (Figure 10). However, no difference in viral load was detected between meningitis and encephalitis. Patients were lumbar punctured median three days (interquartile range 1-5) after onset of neurological symptoms.

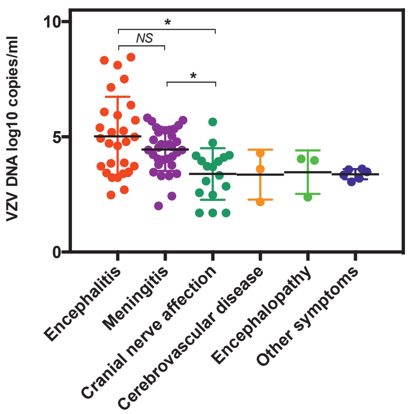

Figure 10. Viral load of 90 patients with VZV DNA in their CSF and neurological complications. The horizontal black bar in each column represents the median value. The colored horizontal bars in each column shows the interquartile range (25th to 75th). p < 0.01.*

Brain imaging, antiviral treatment and neurological sequelae of 121 patients with VZV DNA in their CSF and neurological complications (Papers I and II)

The data of brain imaging and antiviral treatment in patients in Papers I and II are summarised in Table 3. Neurological sequelae up to six months are shown in Table 4. Seventy-six of 121 patients were examined with CT or MRI of the brain. Thirty-three patients had pathological findings on brain imaging. Of these 33 patients 13 were examined by CT, 5 by MRI and 15 of them had both a CT and MRI performed. Most patients had widespread or spotted white-matter changes located subcortically and/or periventricularly (n=18). Three patients had aneurysms located in the anterior and middle cerebral artery respectively (n=2), and in the anterior communicating artery (n=1). The seven patients with cerebrovascular disease had ischemic changes in the area of the brain supported by the left middle cerebral artery, in thalamus, in right putamen and caudate nucleus, in left frontal lobe, in occipital lobe, in basal lacunar area and in right pons, while one had subarachnoid bleeding from the left anterior cerebral artery. Another patient had a suspected ischaemic event located subcortically in the left parietal lobe. The patient with myelitis had leptomeningeal changes and enlarged chiasma.

Table 3. Brain images and antiviral treatment of 121 patients with VZV DNA in the CSF and neurological symptoms

	Meningitis	Encephalitis	Cranial nerve affection	Encephalo-pathy	Cerebro vascular disease	Other symptoms[a)]
Patient No.	42	36	22	5	7	7
Patient no. with pathological CT or MRI/ patient. No. examined	0/17	17/32	3/12	2/3	7/7	1/6
Antiviral treatment ,iv, median and range (days)[b)]	6 (1-14) (No. 19)	7 (2-21) (No. 34)	6 (1-10) (No. 12)	10 (5-14) (No. 3)	10 (5-14) (No. 7)	8 (5-12) (No. 4)
Antiviral treatment, oral only, median and range (days)[c)]	7 (7-10) (No. 18)	13 (No. 1)	7 (7-7) (No. 8)	0	0	14 (No.1)
No treatment	5	1	2	2	0	4

[a)] One patient had Reyes syndrome, 2 patients had meningomyelopathy, 1 patient had chickenpox and 3 patients had varicella zoster without neurological symptoms, 1 patient had polyneuritis and 1 patient had radiculitis.
[b)] Some patients received both iv and oral treatment.
[c)] 3 patients with cranial nerve affection, 1 patient with meningitis, 1 patient with chickenpox and 1 patient with varicella zoster without neurological symptoms received acyclovir. The other patients received valacyclovir 1 g x 3, 7 to 10 days.

Table 4. Neurological sequels in 121 patients with VZV DNA in the CSF

Diagnoses (No.) and symptoms	Neurological sequelae 1 month after treatment/ patient No. followed-up	Neurological sequelae after 2-3 months[a)]	Neurological sequelae after 6 months[a)]
AAM (42)	**12/21**	**9**	**3**
-Headache	8	6	2
-Nausea	3	1	1
-Photophobia	2	1	1
-Vertigo	3	2	
-Concentration disabilities	5	3	2
-Dysphasia	1		
-Fatigue	3	1	
-Sound sensitivity	2	2	1
Encephalitis (36)	**19[b)]/22**	**11**	**6**
-Memory disturbance	5	2	
-Balance disorder/Vertigo	2	4	3
-Dysarthria	1	1	
-Other motordeficit	5	4	2
-Headache	3	3	1
-Postherpetic neuralgia	2	1	
-Bladder dysfunction	2		
-Sound sensitivity	1	1	
-Fatigue	4	1	2
-Visual disturbance	1		2
Cranial nerve affection (22)	**10/14**	**8**	**6**
-Incomplete facial paralysis	7	5	2
-Complete facial paralysis	1	1	1
-Closure defects of the eye	5	4	3
-Hearing disorders	4	3	2
-Balance disorder	1	4	3
-Fatigue	1		
Encephalopathy (5)	**2/2**	**1**	**1**
-Sensory dysfunction	2	1	
-Motor deficit	1	1	
-Concentration difficulties	1	1	1
Cerebrovascular disease (7)	**5/5**	**4**	**3**
-Headache	2	1	1
-Hyperactivity	1	1	1
-Hemiparesis	1		1
-Deafness/hearing disorder	2		
-Other motor deficit	3		
-Memory disturbance	1	1	
-Dysphasia	1		
-Visual disturbance	1	1	1
-Balance disorder			1
Other symptoms (9)	**6/8**	**5**	**4**
-PHN	3	2	2
-Headache	2	2	2
-Behavioral disorder	1	1	1
-Fatigue	1	1	1
-Motor deficit	1	1	1
-Dysphoria	1		
121 patients	54/72	38	23

[a)] Information in the charts is missing. Some patients may have been followed-up without our knowledge
[b)] Three patients had died

Antiviral treatment was administered to 107 of the 121 patients. Seventy-nine patients received i.v. acyclovir and 28 patients received oral acyclovir (n = 6) and/or valacyclovir (n = 22) for at least seven days instead of i.v. treatment. Twenty-four of the patients who received i.v. treatment were also given oral treatment additionally. I.v. treatment was started median 0 days (interquartile range: 0-1) after lumbar puncture was performed.

Three patients with encephalitis died during the acute onset of the disease from multiorgan failure. One of them was immunocompromised. Seventy-two patients had a follow-up at one month or more after treatment. At that time 54 patients still had neurological sequelae. After three months, 38 patients showed residual neurological sequelae, which remained after six months in 23 of them. Two of the patients were diagnosed with multiple sclerosis (MS) during the VZV infection.

Preparation process of VZVgE (Paper II)

The production of gE in the perfusion bioreactor was successful and enough gE was produced for large quantities of ELISA analyses. However, the productivity was somewhat low (4 mg/l/day) and was not preceded by optimisation of growth conditions, such as cultivation mode, pH, temperature, O_2 or medium composition, which could be done to make production more effective.

The IgG reactions to recombinant VZVgE in comparison to the formerly used VZVwhole-ag in ELISAs of serum (Paper II)

The IgG reactivity of 854 serum samples in ELISA with VZVwhole-ag and VZVgE in Paper II showed discordant results in eight samples, with VZVwhole-ag positivity and VZVgE negativity. Six of these samples were derived from the group of low titres and the other two showed ELISA titres of 1600 and 12800 respectively with VZVwhole-ag. These eight samples were further tested by Western blot and immunofluorescence. Two samples were judged as negative and one as positive. The five remaining discordant samples were classified as undetermined. The results were interpreted such that VZVgE was as sensitive as VZVwhole-ag (99.9% and 100% respectively) and, at least as specific (100% and 99.2% respectively).

The absorbance values in serum of the 854 patients divided into their different groups are shown in Table 5. The more obvious differences between the absorbance values when comparing the two antigens were seen in the groups of younger subjects; blood donors and students.

Table 5. The IgG antibody reactivity in ELISA of serum samples from different patient groups comparing VZV whole-ag with VZVgE-ag

	VZV gE-ag	VZV whole-ag
Pos control (n=70)	0.90 (0.83-0.98)	1.58 (1.38-1.70)
Neg control (n=70)	0.13 (0.11-0.15)	0.08 (0.08-0.12)
Stroke (n=454)	0.94 (0.60-1.41)	1.03 (0.70-1.36)
Elderly (n=100)	1.12 (0.71-1.54)	1.23 (0.89-1.56)
Blood donors (n=100)	0.44 (0.33-0.64)	1.03 (0.81-1.35)
Students (n=100)	0.49 (0.32-0.67)	0.89 (0.61-1.16)
Low titer (n=100)	0.36 (0.24-0.55)	0.37 (0.26-0.46)

Values are given as median (interquartile range). The positive and negative controls are from the same subject but 70 repetitions from each positive and negative control were performed.

Evaluation of VZVgE as an antigen for detection of intrathecal IgG VZV antibody production, without cross-reactivity to HSV-1 IgG antibodies (Paper III)

The initial titres for assessment of intrathecal antibody production, IgG indexes and albumin ratio in patients with VZV CNS infection (n=15) and herpes simplex encephalitis (HSE) (n=14) are shown in Table 6.

Only four of 14 HSE patients had intrathecal production of antibodies with VZVgE compared with 11 of 14 with VZVwhole-ag (p=0.021). On the other hand, the VZVgE-ag showed sensitivity comparable to that of VZVwhole-ag, with 14 of 15 patients with VZV CNS infection revealing intrathecal production with VZVgE compared with 15 of 15 with VZVwhole-ag. Using IgG index, six of 15 patients with VZV CNS infection and 0/14 patients with HSE showed no intrathecal antibody production. A total of seven of 15 VZV patients and five of 14 HSE patients showed no blood-brain barrier damage as measured by CSF/serum albumin ratio.

Table 6. ELISA antibody titres in serum and CSF, CSF IgG indexes and albumin ratio of 15 VZV patients and 14 HSV-1 patients with CNS infection

Patient	Sex	Age (years)	Diagnosis	Days from onset	VZV whole virus antigen		VZVgE antigen		Morbilli IgG Serum/CSF	HSV-1		GG1 Serum	CSF IgG[a] Index	Albumin ratio[b]
VZV					CSF	Serum	CSF	Serum		CSF	Serum			
1	M	45	Encephalitis	48	160	6400	40	3200	320	160	25600	pos	0.71	5.1
2	M	68	Encephalitis	113	320	12800	320	12800	320	320	102400	pos	0.49	4.93
3	F	82	Encephalitis	16	5120	102400	320	12800	160	5120	12800	neg	0.67	13.15
4	M	82	Encephalitis	n.d	640	6400	640	3200	40	neg	neg	neg	0.78	27.1
5	F	64	Encephalitis	n.d	5120	12800	640	3200	320	2560	51200	pos	0.97	9.7
6	F	38	Meningitis	6	20480	102400	10240	102400	40	20	100	neg	0.85	23.1
7	F	70	Meningitis	11	640	25600	160	6400	320	320	51200	pos	0.56	6.9
8	M	67	Meningitis	18	5120	25600	2560	25600	80	40	800	pos	0.56	43.5
9	F	81	Encephalitis	2	5120	102400	2560	102400	160	80	6400	pos	0.63	13.9
10	M	78	Meningitis	16	2560	12800	1280	6400	80	320	25600	pos	0.47	21.3
11	F	58	R-H	1	1280	102400	1280	102400	320	80	25600	pos	0.57	7.4
12	M	3	Vasculitis, left-sided hemiparesis	0	640	12800	160	3200	RS: 2560[c]	neg	neg	neg	1.57	3.39
13	F	81	Encephalitis	5	20480	102400	10240	51200	20	1280	25600	pos	0.53	68.5
14	F	63	R-H	8	2560	6400	2560	6400	160	160	6400	pos	0.65	15.5
15	F	36	Encephalitis	358	80	3200	10	400	160	640	204800	pos	1.37	5.1

HSV-1														
16	F	63	Encephalitis	88	1280	12800	10	100	320	81920	819200	pos	n.d	n.d
17	F	32	Encephalitis	6	320	12800	20	400	80	2560	12800	pos	0.77	36.2
18	M	60	Encephalitis	30	12800	10240	10	800	160	81920	204800	pos	n.d	12.8
19	F	65	Encephalitis	6	320	6400	10	400	40	2560	12800	pos	0.79	46.3
20	M	37	Encephalitis	46	5120	51200	10	400	160	20480	204800	pos	0.74	7.8
21	F	72	Encephalitis	51	40960	102400	10	200	160	5120	12800	neg	1.7	9.4
22	M	67	Encephalitis	37	2560	6400	20	400	20	81920	102400	pos	n.d	54.6
23	F	63	Encephalitis	16	20480	51200	10	200	80	81920	409600	pos	1.63	20.8
24	M	66	Encephalitis	37	40	800	10	400	320	5120	25600	pos	1.74	6.9
25	F	64	Encephalitis	30	640	6400	80	3200	320	40960	102400	pos	1.85	7.2
26	F	79	Encephalitis	30	2560	12800	40	400	640	40960	409600	pos	0.83	6.6
27	M	53	Encephalitis	34	2560	6400	20	800	320	81920	204800	pos	1.95	8.5
28	F	65	Encephalitis	46	5120	6400	10	400	40	81920	102400	pos	2.11	32.4
29	M	67	Encephalitis	26	20480	25600	10	800	80	81920	204800	pos	2.02	23.2

[a] Normal level <0.63 at age >14 (1.5 years- 14 years: < 0.80).

[b] Normal level <5.0 (1.5-14 years), <6.8 (15- 44 years), <10.2 (45-89 years)

[c] Morbilli IgG neg, RS virus IgG was used as reference.

Assessment of VZV IgG antibodies in CSF and serum samples by sample dilution to an identical total IgG concentration (1 µg/ml) (Paper III)

The different absorbance values in CSF and serum samples (diluted to identical concentrations of I µg/ml) of patients with VZV CNS infection (n=15) and HSE patients (n=14) comparing VZVwhole-ag with VZVgE, are illustrated in Figure 11. Antibody reactivity to VZVgE was almost absent in the HSE patients compared with VZVwhole-ag for these patients. The lack of reactivity was more pronounced for the CSF samples than the serum samples. In the VZV patients, the differences in the results were much less pronounced with the different antigens and the results of the CSF analyses were even similar with the different antigens. Only one patient showed very low absorbance values in CSF and serum with both antigens. The samples from this woman were taken one year after PCR positivity and, furthermore, she was diagnosed with systemic lupus erythematosus (SLE) with suspected CNS involvement.

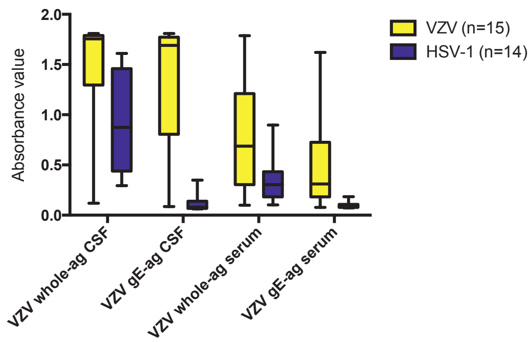

Figure 11. The absorbance values at 15 min in ELISA using either VZV whole-ag or VZV gE-ag. The horizontal bar inside each box shows the median value and interquartile range (25th to 75th percentile) within the box. Short bars outside each box show range.

In addition, we calculated the antibody index (absorbance values$_{CSF}$/absorbance values$_{serum}$) with a set ratio of ≥2.0 indicating intrathecal antibody production. At V_{max} and at 15 minutes, no intrathecal antibody production (0 of 14) was detected in the HSE patients using VZVgE, in comparison with 11 of 14 patients using the VZVwhole-ag ($p<0.001$). In the VZV patients, 12 of 15 showed intrathecal antibody production with VZVgE compared with nine of 15 patients using VZVwhole-ag ($p=0.001$).

In summary, these results showed that VZVgE was superior to VZVwhole-ag for detection of intrathecal antibody production against VZV, without signs of cross-reactivity to HSV-1.

CSF concentrations of NFL, GFAp and S-100β (Paper IV)

The individual concentrations of CSF NFL and GFAp in 24 patients with VZV DNA in their CSF and concomitant neurological complications and their controls are given in Figure 12. CSF NFL and GFAp concentrations were elevated on day 1-5 compared with the control group (p=0.002 and p=0.03). The CSF NFL levels showed a tendency to increase from day 1-5 to day 10-15, after which they decreased after three to five months. The CSF GFAp levels showed no marked differences in concentrations between day 1-5 and day 10-15 and they were normalised again after three to five months. In contrast, the CSF S-100β concentrations were decreased at all time points compared with the control group. The patients with encephalitis (n=10) had the highest CSF NFL and GFAp concentrations on day 1-5 compared with controls (p<0.001 and p=0.02). These data suggest neuronal damage and astrogliosis in the brain of patients with VZV CNS infection, which was more pronounced in patients with encephalitis in our study.

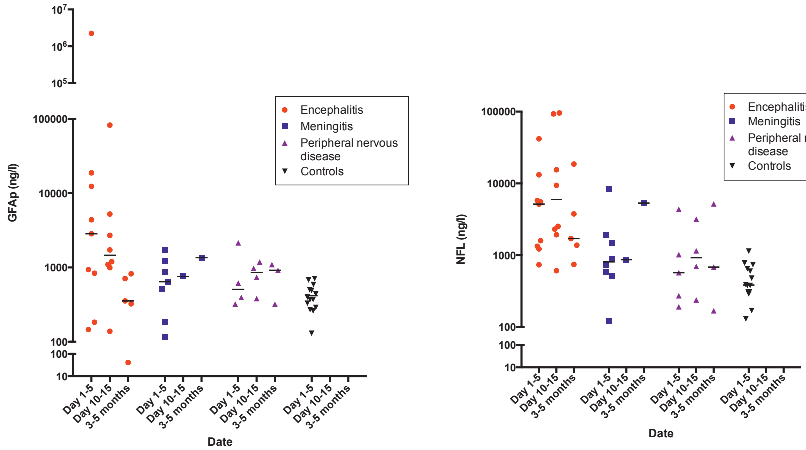

Figure 12. NFL and GFAp levels from the CSF in 24 patients with VZV CNS infection in the acute stage of disease, on day 10-15 and after 3-5 months (individual concentrations and means)

Neuropsychological test results and mild cognitive impairment in a three-year follow-up of patients with VZV infection and neurological complications (Paper V)

Fourteen patients and their age- and gender-matched controls (n=28) underwent neuropsychological testing. Fourteen tests were performed of which 12 were specific for different domains. Three tests were sub-grouped under the Stroop test (Stroop 1, 2 and 3). Seven of the tests were analysed as z-scores and seven as raw scores of which two were adjusted for age (immediate and delayed recall). A cut-off was set at -1.5 standard deviations (SD). Patients with VZV CNS infections performed significantly worse on eight tests compared with controls. However, two patients had markedly poorer results compared with both the other patients and controls and we therefore also analysed the test results with these two patients excluded. The 12 patients then, performed significantly worse on four tests compared with controls covering the domains *speed and attention*, *learning and memory* and *executive function*s. In addition, five of 12 patients were classified as MCI with cognitive impairment on at least two tests from two different domains. Our results indicate long-term cognitive impairment in patients with previously acute neurological disease caused by VZV. Moreover, these patients might run a greater risk of developing dementia based on the large proportion of patients classified as MCI.

5 DISCUSSION

The herpesviruses have evolved over the last 400 million years at the very least and have been described as existing since ancient civilisations. The link between CNS manifestations such as encephalitis and cerebellitis and VZV infection was demonstrated at the beginning of the 20th century [3, 4]. In spite of this, a large part of our knowledge on VZV CNS infections has been presented during the past few decades. One reason for this progress was the introduction of PCR for diagnostic purposes in the 1980s followed by the quantitative real-time PCR analyses. This technique, has dramatically improved the opportunity to detect the virus in CSF [103, 205]. As a result, from being under-diagnosed, VZV has emerged as one of our most common viral agents causing CNS infections including encephalitis [93, 95, 96, 125]. In addition, by the means of PCR, it has become evident that VZV CNS infections present a wide spectrum of different neurological manifestations.

Several studies of viral CNS infections and their manifestations have focused on encephalitis, but it should be emphasised that VZV CNS infections encompass various neurological complications with involvement of the brain. In our material (Papers I and IV), meningitis was the most common manifestation, followed by encephalitis and cranial nerve affection. Seven of 121 patients suffered from stroke. Few other studies have investigated the distribution of different VZV CNS manifestations, but one of them also showed meningitis to be the most common clinical entity associated with VZV CNS infections [94], while another claimed that VZV encephalitis was three times more common than VZV meningitis [95]. The diverging results might be due to different diagnostic methods, different inclusion criteria and varying clinical criteria for the CNS manifestations. Moreover, none of these studies included cranial nerve palsies or stroke as specific diagnoses. Even though it is becoming increasingly more recognised that VZV CNS infections may cause this variety of manifestations, the disease might still be under-diagnosed. A lumbar puncture is not always performed in patients with meningitis and cranial nerve palsies and, in stroke patients, VZV is probably seldom suspected as a causal agent. Furthermore, in patients with VZV CNS infection, cutaneous lesions are often lacking, all of which result in VZV being undetected, despite ameliorated diagnostic methods.

Real-time PCR has except for being a highly sensitive diagnostic method, also provided us with the opportunity to quantify the viral DNA. The question arose of whether the amount of viral DNA was correlated with clinical variables, such as severity of symptoms and neurological sequelae, and whether this parameter could be used as a predictor of outcome. In Paper I, we determined the viral load using real-time PCR in patients with different CNS manifestations. The viral load at baseline was significantly higher in the patients with meningitis and encephalitis compared with patients with cranial nerve palsies. There was no difference in viral load between the patients with meningitis or encephalitis on the other hand, which was the opposite of the findings in a previous study by Aberle et al [78]. In this last study however, patients with cranial nerve palsies were included in the meningitis group and this former group of patients showed a lower viral load in our study. Furthermore, our results for viral load were confirmed in Paper IV, even if the material was smaller. The lack of difference in viral load between the patients with encephalitis and meningitis did not correspond to the difference in neurological sequelae seen between these groups. Encephalitis was associated with more brain damage and a higher frequency of residual neurological sequelae at 12 months compared with meningitis. Thus, viral load at baseline did not correlate with the severity of neurological symptoms and subsequently, baseline viral load has hitherto not showed to be a reliable predictor of outcome in VZV CNS infections [206]. In herpes simplex encephalitis (HSE), a similar inferiority in terms of baseline viral load in the CSF as a predictor of outcome has been shown [207]. Interestingly however, in that study of HSE, the duration of HSV-1 DNA positivity in the CSF correlated with the outcome, and in PHN persistence of higher viral loads over time in peripheral blood has been associated with a longer time to recovery [208].

One might hypothesise that the here described differences in viral loads in CSF can be merely attributed to the localisation of infection in the brain and proximity to the CSF rather than the severity of neurological symptoms. If the leptomeninges are infected, there is a large contact area of the CSF with the site of infection and additionally, the leptomeninges are also close to where the CSF sample is drawn as compared with the brain. In encephalitis, large areas of the brain might be affected presumably with several blood vessels involved and the subsequent possibility for the virus to reach the CSF in large quantities through the blood-brain barrier. In VZV-induced stroke, the PCR might be negative, as vasculitis often appears several weeks to months after acute infection [85, 209]. In cerebral nerve palsies, the infection is probably more local, with less access to the CSF, which is in accordance with the levels of viral load shown in these patients in our studies (Papers I and IV) as well as in the study by Aberle et al [78].

VZV DNA might also be detected by PCR in the CSF, even in the absence of neurological symptoms. After primary infection the virus establishes latency in the dorsal root, autonomic and cranial nerve ganglia. During latent phase VZV DNA might be found in ganglia, although not detectable in the CSF [51]. Following reactivation with viral replication in the ganglia, the virus may be shed into the CSF even without neurological symptoms developing. One interesting aspect is that the CNS might actually be involved in herpes zoster without obvious symptoms of CNS involvement as indicated in an autopsy report already in the year 1900 [210] and in a later one [211]. This is not unlikely since the dorsal root ganglia located in the peripheral nervous system are situated in close proximity to the spinal cord which is a part of the CNS. A further suggestion is that the intense pain during herpes zoster might be due, at least in part, to damage of neurons in the spinal cord and not only of neurones in the ganglia [211]. In one study of 42 patients with herpes zoster but without obvious CNS manifestations, VZV DNA in the CSF was detected in 10 of them, using qualitative PCR [149]. Six patients however, had motor paresis and 10 patients had MRI or CT changes attributed to the zoster, so involvement of the brain cannot be excluded. Our study (Paper I) included four patients who had underwent lumbar punctures without any obvious signs of neurological symptoms and CNS involvement. The amounts of VZV DNA in these three patients, one of whom had chickenpox, were in the range of 1600-3200 copies/ml. Hence, the amounts of viral DNA in the CSF of patients with primary or reactivated VZV infection but without neurological symptoms are not known. As a result, even if VZV DNA findings in the CSF imply VZV CNS infection, this finding should be interpreted in conjunction with other diagnostic tools for assessment of CNS involvement, such as clinical examination and brain imaging.

Even though real-time PCR is a very useful method in many respects, this technique also has its diagnostic limitations. Viral DNA diminishes with time from the CSF and, PCR has been shown to be negative about one to three weeks after the onset of neurological symptoms [85, 158]. As a result, in a number of patients, such as those with VZV induced stroke and those with late diagnosis, serological methods with detection of intrathecal antibody production might be required to confirm the diagnosis. However, serological diagnoses have been hampered by suspected cross-reactivity between HSV-1 and VZV IgG antibodies [212-

214]. For this purpose, we evaluated VZVgE as an antigen in Papers II and III. High specificity often comes at the expense of lower sensitivity. A purified protein antigen, such as gE might lose in sensitivity. However, as previously described, VZVgE is highly immunogenic and the most abundant glycoprotein on VZV-infected cells, and there was no evident loss of sensitivity by replacement of the whole virus antigen with this single glycoprotein. A crude protein antigen as the VZVwhole-ag, on the other hand, carries a risk of cross-reactions and reduced specificity due to homologies between other envelope proteins and since such antigens also contain numerous mammalian cellular proteins. This phenomenon of low specificity was described in Paper III and was possibly involved also in the results of Paper II. In Paper II, eight serum samples showed discordant results, of which all were positive for VZVwhole-ag and negative for VZVgE. The five samples, which were judged as "undetermined" after further evaluation, were all positive by immunofluorescence (IF). The antigen of IF for VZV IgG antibodies is however also a mixture of proteins like the VZVwhole-ag used in ELISAs and thereby carries the same risk of diminished specificity. This implies that the five "undetermined samples" might have been seronegative to VZV. In one study of 634 VZV isolates collected from Sweden, two isolates showed mutations in the gE gene with absent reactions to a monoclonal antibody specific for this gE epitope [215]. Nevertheless, such findings do not change the sensitivity of gE in serological analyses, as the gE antigen probably carries several hundred of epitopes. Altogether, the sensitivity of VZVgE to specific IgG in serum appears to be as high as that for VZVwhole-ag and the specificity is probably higher for the former.

When the VZVgE antigen was evaluated for serological analysis of CSF samples, the sensitivity was confirmed and in addition VZVgE was clearly more specific than VZVwhole-ag, without cross-reactions to HSV-1 IgG antibodies. In addition to cross-reactivity, other suggestions of the co-finding of IgG antibodies to both VZV and HSV-1 have been dual infections or heterotypical responses following polyclonal B-cell activation [216]. One example of dual viral infections is Epstein Barr virus (EBV) DNA detection in concomitance with other viral DNA in the CSF, as seen in our own study (Paper I). In these cases, it is presumed that EBV is reactivated by other viral infections [217, 218]. Nonetheless, our experience is that dual infections of VZV and HSV-1 viruses are very rare, based on the lack of any concomitant presence of the DNA of these two viruses in the CSF [94]. It should also be noted that, by solving the problem of cross-reacting antibodies to VZVwhole-ag in the CSF, this particular unspecific reactivity in serum diagnostics was avoided, and the gE antigen was recently introduced as a routine antigen in the diagnostic viral laboratory of Sahlgrenska University Hospital.

Interestingly, we noticed a possible cross-reaction in the other direction, with a few patients with PCR-verified VZV CNS infection showing intrathecal antibody production to type-common HSV antigen. The possibility that the VZV infection induced a reactivation of HSV cannot be excluded. However, the possible cross-reaction in this direction appears to be much less of a diagnostic problem compared with cross-reactions of HSV IgG to the VZVwhole-ag.

It has been suggested that glycoprotein gB is the protein exposing common epitopes for VZV and HSV-1 with possible cross-reactions [213]. As mentioned, after gE, VZV gB is the second most abundant glycoprotein in VZV-infected cells and its homology to HSV-1 gB is as great as 49%. In the case of gE, the degree of similarity is 33% and, even if this percentage also might seem quite high, it appears as if VZVgE is devoid of epitopes homologues to HSV-1 protein.

Besides viral load and intrathecal antibody production, there are other markers for evaluating CNS infections. In Paper IV we measured NFL, GFAp and S-100β in the CSF over time, to investigate the neuronal damage and astroglial involvement in patients with VZV CNS infections. We found that the concentrations of NFL and GFAp were moderately higher and the S100β levels were, surprisingly, lower in patients with VZV CNS infections compared with controls. We interpreted the results as neuronal damage and reactive astrogliosis. In dementia disorders, neuroborreliosis and TBE, a similar pattern with increased GFAp but normal levels of S-100β has been shown [164, 169, 219]. This pattern is probably due to astrocyte hypertrophy with increased expression of GFAp but conserved astrocyte membrane integrity, without cell damage and with no S100β leakage [164, 165, 168, 219]. In contrast, patients with encephalitis caused by herpes simplex, another α-herpes virus, displayed markedly higher levels of NFL, GFAp and S-100β in the CSF [169].

Moreover, reactive astrogliosis is a common finding in ischemic events in the brain and is induced by different signalling molecules and probably also by direct mechanical stress [220, 221]. In an *in vitro* study, moderate mechanical stress resulted in neuronal cell death and astrogliosis and at high mechanical stress, the astrogliotic reaction was reduced and cell death, predominantly neuronal, increased [165]. Therefore, the possible brain damage in patients with VZV CNS infections, as indicated by increased concentrations of CSF biomarkers, is suggested to be of a moderate nature. Additionally, if the pathogenesis of VZV CNS infections is dominated by vasculopathy with mostly secondary effects on neurons and astroglial cells caused by ischemia, one could expect brain damage of the above profile, as opposed to a viral infection with more cytotoxic effects on brain, such as HSE [222].

With a CSF biomarker profile indicating moderate brain damage, even for the patients with encephalitis, one might assume that the clinical outcome also would be of a moderate nature in VZV CNS infections. As compared with HSE, where the neurological sequelae are often very serious, including high mortality [223], the opinion has been that patients with VZV CNS infections, including encephalitis, do not suffer from neurological sequelae to the same degree [124, 224]. Interestingly though, in two recent large studies of patients with encephalitis with various causal agents in France and England, patients with VZV encephalitis and HSE showed similar outcomes when evaluated at six months and after three years with Glasgow Outcome Scale (GOS) [89, 225]. Furthermore, in the French three-year follow-up study, all groups of infectious agents had a better outcome than VZV and HSE. Among the surviving 14 VZV patients, half of them had moderate to severe sequelae, and the outcome was favourable for the rest of them, three years after acute disease [125]. Our prospective study, which included 10 patients with encephalitis who were evaluated one year after acute disease (Paper IV), showed an outcome based on GOS in accordance with the above study. In sum, these results imply that VZV encephalitis might be more severe than previously presumed. Moreover, the wide difference in biomarker levels seen in these two viral CNS infections, do not seem to reflect the outcome, at least not in these few studies with a limited number of patients and the usage of GOS, a scale which is too rough for capturing more subtle neurological symptoms.

Among the VZV patients (Paper IV), a similar lack of correlation between clinical outcome measured by GOS and the levels of CSF biomarkers was shown. In Paper V, where more subtle methods were used, we did not analyse any correlations between the degree of cognitive impairment and levels of biomarkers, as too few patients were included in the study. Hence, the question of whether the levels of different biomarkers in VZV CNS infection might predict outcome has still not been answered.

The distribution of neurological sequelae of VZV CNS infections is previously poorly described, since there are very few follow-up studies.

In the French three-year follow-up study mentioned above [225], the most frequent neurological sequelae in the patients with VZV encephalitis were concentration problems and different motor deficits. In Papers I and IV, we were unable to distinguish any particular neurological sequelae that were more frequent than the others in the patients with encephalitis. However, few were followed-up and in addition, one of the studies was retrospective (Paper I). In the patients with meningitis, neurological sequelae were absent or only minor, which lends some support to the common notion that VZV meningitis is a fairly benign condition without sequelae. The patients with Ramsay Hunt syndrome showed typical neurological sequelae, such as remaining facial palsy and balance disorders. The recovery rate of around 75% at six months was in the lower range compared with other studies where full recovery is reported in 75-90% of patients with Ramsay Hunt syndrome [128, 130, 131]. However, the low recovery rate might be due to late and perhaps inadequate treatment without steroids [130]. In these two studies (Papers I and IV), any cognitive sequelae might have been missed.

Cognitive impairment following CNS infections has previously been reported in VZV encephalitis [126], as well as in TBE, viral meningitis and HSE [179, 180, 226]. Yet, in only one study have patients with VZV CNS infection who received antiviral treatment been followed-up including neuropsychological assessment in the long term [127]. However, that study failed to demonstrate any substantial neuropsychological deficits compared with controls, which is in contrast to our study (Paper V), where the neuropsychological assessment showed that the VZV patients performed significantly worse than controls. On the other hand, in the former study the number of participants that completed the tests was even less than in our study and, additionally, the neuropsychological tests might have differed in sensitivity. Nevertheless, the assumption that VZV CNS infections cause cognitive impairment must be taken seriously as this might lead to functional impairment in complex situations of daily living and working.

The presumptive association between VZV CNS infection and development of dementia is perhaps even more important (Paper V). Other infectious agents, including HSV-1, have been associated with development of Alzheimer's disease [227, 228]. These results have predominantly been based on the findings of the specific infectious agent in the brain, mostly detected by PCR, in patients with Alzheimer's disease [227]. The association of VZV and dementia is scarcely investigated however. The lack of VZV DNA in post-mortem human brain specimens from patients with Alzheimer's disease [229] do not necessarily exclude that an association between VZV and the development of dementia exists. Given that several manifestations of VZV CNS infections are associated with vasculopathy of the brain, one might hypothesise that damage to the vessels may lead to or contribute to vascular dementia as shown following other vascular brain injuries [230-233].

The association between VZV and dementia was based on the classification of the VZV patients regarding mild cognitive impairment (MCI). But, MCI is a heterogeneous condition which might follow various psychiatric, oncological, surgical and infectious disorders, as well as disorders caused by pharmaceutical agents, and not only typical dementia diseases. The course of the MCI syndrome and the various forms of MCI is only partially known and, most likely dependent on the underlying pathogenesis. Even though an annual rate of progression from MCI to dementia of 10% has been reported [234], some patients with clinically defined MCI have “benign” forms that remain stationary or even improve over time [177]. Taken this into account, the association between VZV CNS infections and development of dementia must be interpreted with caution.

The brain imaging abnormalities in our patients were mostly characterised as widespread and spotted white-matter changes. The development of white-matter changes is proposed to be related to ischemia of predominantly small vessels [235, 236], to which the white matter is especially sensitive [237]. These changes are seen in vascular dementia and Alzheimer’s disease but also as a consequence of normal ageing [238, 239]. VZV vasculopathies involve both large and small vessels and in one study of 30 patients as many as 37% presented with pure small-vessel involvement and white-matter changes exclusively [85]. It might be difficult to distinguish whether the brain imaging abnormalities found in our patients described as extended white matter-changes were caused by VZV, normal ageing or a combination. However, the brain images of the patients with meningitis (Papers I and IV) revealed no abnormalities, whereas more than half of the patients with encephalitis with a brain imaging performed had pathological changes, predominantly in the white matter. On the other hand, the patients with encephalitis were considerably older than the ones with meningitis. This limits the possibility to draw any far-reaching conclusions from the brain imaging studies, and an age- and perhaps gender-matched material is needed for such an evaluation.

Even though white-matter changes might be difficult to attribute to a VZV CNS infection there are other brain imaging changes where the association with this disease is more evident. Nowadays, VZV-induced vasculopathy and stroke are fairly well-known complications of the virus in both primary and reactivated VZV, but the magnitude of the problem is still clouded. In two recent reports, the prevalence of stroke among patients with previous herpes zoster was investigated [108, 109]. In one of them the risk of stroke within one year after acute disease was estimated at 1.3 and, for those with zoster opthalmicus, the risk was 4.3 compared with controls. We approached the stroke complication from another angle in Paper II, by measuring the VZV IgG levels in stroke patients and their controls. As opposed to the above study, we did not find significantly more stroke patients with a titre rise in VZV IgG (two of 454) compared with controls (unpublished material). The hypothesis was to measure the VZV IgG response before its maximum with an acute serum drawn within two weeks of symptom onset. The convalescence serum drawn three months later would then show a significant titre rise. It is however very difficult to know the time-point at which the VZV IgG titre begins to rise and when it reaches its maximum in relation to the stroke symptoms, as VZV vasculopathies might appear weeks to several months after herpes zoster [85, 209]. This might have hampered the possibility to obtain a positive result.

Despite the increased attention given to characterising and studying of VZV CNS infections, important issues remain to be investigated. Even if it is not the focus of this thesis, the issue of treatment deserves to be elucidated. As neurological complications following VZV infections appear to be more common than previously thought with sequelae ranging from mild to severe, it is of great importance to perform controlled treatment trials to assess the best therapy of choice. So far, controlled trials of this kind are lacking. The optimal dosage of neither i.v. nor oral treatment has been settled. The CSF acyclovir levels after i.v. administration are suggested to be about 50% of the levels in plasma [183], but CSF concentrations following different doses of i.v. acyclovir are inadequately described [183]. In patients with HSE, reduced mortality and morbidity have been shown when they were treated with i.v. acyclovir at a dose of 10 mg/kg three times daily for 10 days [240]. However, VZV is less sensitive to acyclovir than HSV, with an *in vitro* 50% inhibitory concentration ($IC_{50)}$ of 1.3 to 6.7 μM compared with the IC_{50} of HSV of 0.1 to 3.9 μM [241]. Compared with orally given acyclovir, the CSF acyclovir levels measured following treatment with 1 g of valacyclovir administered orally are reported to be three to five times higher [241, 242]. On the other hand, the CSF acyclovir concentrations were only partially at an inhibitory level for VZV when 1g three times daily were given to patients with multiple sclerosis [241]. Interestingly, orally given valacyclovir, as suppression therapy, to patients with HSV-2 meningitis at a dose of 0.5 g twice daily did not prevent further recurrences of meningitis [243]. Doses that are too low could also induce resistance to the drug, a phenomenon that has not been explored in VZV CNS infections. Furthermore, the role of steroids in VZV CNS disease has not been systematically investigated. Yet, considering VZV as an aetiological agent of vasculopathy with subsequent vessel wall inflammation, clinical benefits could be expected from treatment with steroids. And finally, it should be emphasised that even patients with VZV meningitis, cranial nerve palsies and other more peripheral neurological VZV manifestations might benefit from antivirals penetrating the CNS.

Then, why don't we introduce routine VZV immunisation of infants in Sweden, as has been done in the USA and several other countries? The live attenuated varicella Oka vaccine seems safe for administration to children with few complications reported. Another consideration however, is the effect of a widespread use of the varicella vaccine in a population. It is recognised that exposure to the virus boosts both the humoral and the cell-mediated immunity [59, 69, 70]. When this exposure vanishes the risk of zoster might increase, at least during a transitional period. According to modelling studies zoster might become more common for some 30 years after introduction of routine immunisation of infants [244]. However, no such increase has hitherto been described in the USA, the country with the longest history of VZV immunisation. Hence, these effects of the varicella vaccine still remain a subject of speculation. If the incidence of zoster were to increase, a zoster vaccine would become even more important, especially for immunocompromised subjects. A killed VZV vaccine would of course be of interest in this latter group. In fact, a glycoprotein E subunit vaccine has been proposed as a candidate [245]. Both $CD4^+$ T cell and humoral immunity responses were shown to be markedly higher for up to 42 months with the gE subunit vaccine compared with the Oka vaccine. The time at which this vaccine will be available has still not been finalised, however.

Future studies should involve controlled treatment trials which focus on doses of i.v. acyclovir and the opportunity to use oral alternatives, such as valacyclovir. In treatment trials, viral load and CSF biomarker concentrations should be further investigated as predictors of outcome. Neurocognitive assessment is necessary in these studies, to evaluate the neurological sequelae following the various complications of VZV CNS infections. The recognition of VZV CNS infections as vasculopathies should be further explored. The association between VZV and stroke is an important field to investigate, especially since recent data suggest that VZV might have a greater impact on stroke incidence than previously thought. Based on current knowledge on VZV CNS infections and possible future studies, the vaccination regimen to be administered in Sweden should also be discussed.

6 CONCLUSIONS

- VZV was found to be an important causative viral agent to CNS infections.
- Baseline viral load, as assayed by quantitative PCR, was higher in patients with encephalitis and meningitis as compared with those suffering from focal neurological complications. However, baseline viral load was not a reliable predictor of clinical outcome in patients with VZV CNS infections.
- VZVgE antigen was found to be a sensitive and specific antigen for serological diagnosis of VZV infections in the CNS, and this antigen appears to be devoid of cross-reactivity to HSV-1.
- Patients with VZV CNS infections, especially those with encephalitis, have signs of brain damage in form of neuronal damage and astrogliosis, as indicated by increased concentrations of CSF biomarkers.
- Patients with previous VZV CNS infections are suggested to carry a greater risk of long-term cognitive impairment compared with healthy individuals.

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