Herpes Virus Retinitis-

Clinical and Virological Characteristics

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Cover illustration: Frosted branch angiitis. Joanna von Hofsten, watercolour

Herpes Virus Retinitis-Clinical and Virological considerations

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In the memory of my beloved mother

ABSTRACT

In this thesis, we first aimed to determine whether there may be herpes virus deoxyribonucleic acid (DNA) in the aqueous humour of asymptomatic individuals (*paper II*). Presence of herpes simplex virus (HSV), varicella zoster virus (VZV), cytomegalovirus (CMV) and Epstein Barr virus (EBV) in aqueous humour was measured by polymerase chain reaction (PCR) in patients eligible for cataract surgery. None of the samples were positive for herpes virus suggesting that shedding, at least frequent shedding, in aqueous humour is unlikely.
Acute retinal necrosis (ARN) is a diagnosis based on criteria describing clinical signs. In *paper III*, we investigated all intraocular samples positive for HSV, VZV, CMV or EBV in south-western Sweden over almost ten years. Thirteen patients were identified with clinical signs that met the criteria for ARN. All cases were caused by alpha herpes viruses, the subgroup of herpes viruses including HSV1, HSV2 and VZV with similar tropism for neuronal tissues. Viral load in intraocular samples, measured by PCR, did not correlate with visual prognosis. However, a trend towards higher viral load in samples taken earlier, compared with later, in the disease process was observed. This thesis includes two of the first reported cases of ARN with deep sequencing of the viral genome from aqueous humour *(paper IV).* Varicella zoster virus in aqueous humour in ARN exhibited a comparatively low genetic heterogeneity similar to vesicle fluid in shingles.
We performed a retrospective national study including all patients with *Cytomegalovirus* diagnosis over a period of 11 years, *(paper V).* Sixty-three patients with CMV retinitis were identified. The most common predisposing factors were haematopoietic stem cell transplantation (27%), and haematological malignancies (24%). We also found two patients with no other immunosuppression than that related to diabetes mellitus (DM) (3.2%). Clinical characteristics of patients with delayed diagnosis at >30 days from symptom onset were compared with those of patients with early diagnosis. Presence of intraocular inflammation (IOI) (p=0.003) and increased intraocular pressure (p=0.023) as well as old age (p=0.01) were risk factors. One reason for late diagnosis was the misconception that the patient was not immunocompromised, because of DM or as described in our case report of a patient treated with ruxolinitib for myelofibrosis *(paper 1).*

Keywords: Cytomegalovirus, acute retinal necrosis, sequencing, retinitis

SAMMANFATTNING PÅ SVENSKA

Herpesvirus finns överallt omkring oss och har följt oss och våra förfäder under miljontals år. Virusets evolution är sammanflätad med vår. Herpesvirus har lärt sig att stanna kvar i våra kroppar efter infektion och till stor del undvika och hämma det immunologiska svar som vi använder oss av för att bekämpa dem. De allra flesta har blivit smittade av herpesvirus men alla uppvisar inte sjukdom. Den här avhandlingen handlar om två typer av herpesinfektion i näthinnan som är mycket ovanliga.
I *delarbete II* har vi även utrett om man kan hitta spår av herpesvirus i ögats inre vätska i främre segmentet, kammarvattnet, hos personer som inte har några symptom. Det har nämligen visat sig att utsöndring i herpesvirus i olika kroppsvätskor som till exempel tårvätska kan ske utan att individen har symptom, i synnerhet för herpes simplex virus (HSV). Provtagning av kammarvatten kräver sterila förhållanden och innebär en viss risk liksom andra invasiva provtagningar och kirurgi. Därför bedömdes i övrigt friska patienter som planerades att opereras för gråstarr vara lämpliga kandidater. Vi exkluderade patienter som uppvisade tecken till inflammation men inkluderade dem som hade andra ögonsjukdomar. Kammarvattnet analyserades med Polymerase Chain Reaction (PCR) för herpes simplex virus 1 och 2 (HSV1, HSV2), varicella zoster virus (VZV), cytomegalovirus (CMV) och Epstein Barr virus (EBV). Ingen av de 30 individerna hade spår av virus vid provtagningstillfället. Vi bedömer förekomst av virus i kammarvatten hos personer utan symptom vara osannolik. Följaktligen kan man dra slutsatsen att om virus DNA finns i ett prov från en sjuk patient har fyndet klinisk signifikans.

Akut retinal nekros (ARN) har sedan 90-talet varit en diagnos baserad på kriterier som beskriver det kliniska uttrycket. ARN är ett ovanligt tillstånd med smältning av näthinnan och inflammation i näthinnans kärl. Man ser cirka ett fall på två miljoner invånare per år. Nyligen har det framkommit förslag att specificera denna diagnos med avseende på vilka virus som kan orsaka tillståndet. Man har tidigare menat att HSV1, HSV2, VZV men även EBV och CMV kan utgöra relevanta patogener. Vi utförde en studie (*delarbete III*) där vi granskade journaler på patienter som av olika anledningar hade genomgått provtagning av kammarvatten eller glaskropp (gelformad vätska i ögats inre bakre del) och som varit PCR-positiva för HSV1, HSV2, VZV, CMV eller EBV under nästan 10 år i Sydvästra Sverige, en region med en befolkning av 2 miljoner. De 13 patienter som uppfyllde kriterierna för ARN var uteslutande de som hade diagnosticerats med HSV1, HSV2 eller VZV. Dessa virus tillhör en undergrupp av de humana herpesvirus som kallas alfaherpesvirus. Typiskt för dessa är deras benägenhet för att infektera celler i slemhinna och hud, insidan av kärl samt att de befäster sin persisterande infektion i sensoriska nervceller. Ingen av de 13 patienter som varit CMV-positiva med PCR eller de två som var EBV-positiva i materialet uppfyllde kriterierna för ARN.
PCR-metoden som utfördes kunde både visa vilket virus som fanns i provet samt mängden av virus DNA. Kammarvatten- eller glaskroppsproverna som togs vid diagnos hade olika mängder DNA av undersökta virus. Vi jämförde mängderna med synutfallet samt om det fanns en koppling mellan tidpunkten för provtagning. Det fanns inget direkt samband mellan sämre syn efter sjukdom och höga virusmängder initialt, något som har föreslagits tidigare. Däremot fanns en tendens till att virusmängderna minskade om provet togs sent i sjukdomsförloppet, dvs långt tid efter symptomdebut.
Två patienter från *delarbete III* deltog i *delarbete IV* som utgör analys av VZV-virusets DNA där vi med djupsekvensering kunnat täcka hela arvsmassan i ett fall och nästan hela i det

andra fallet. Detta är bland de första rapporterna med djupsekvensering som publicerats av herpesvirus som påvisats i kammarvatten. Man kunde se att det fanns en mycket liten variation i gensekvenserna i VZV vilket talar för att det inte rör sig om flera olika populationer av samma virus i provet samt att viruset inte avvek från virus som beskrivits från andra delar av kroppen.

CMV är ett herpesvirus som mer än hälften av alla i Sverige har antikroppar emot. Trots att denna infektion är så vanlig är det mycket få som under sitt liv kommer att utveckla symptom på CMV infektion. Detta virus är en så kallad opportunist som ger klinisk sjukdom först när värden har ett försvagat immunförsvar. Cytomegalovirusretinit (CMVr) är en sådan manifestation som utgör en infektion i näthinnan och dess blodkärl. Tillståndet är mycket ovanligt och därför valde vi att inkludera alla patienter som fått diagnosen *Cytomegalovirus* på alla ögonkliniker i Sverige under 11 år i *delarbete V*. Efter att ha granskat journalerna från dessa patienter kvarstod 63 personer av 110 som hade haft CMVr. Orsak till försämrat immunförsvar var stamcellstransplantation (27%), hematologisk cancer (24%), HIV (16%), autoimmun/reumatisk sjukdom (16%), organtransplanterade (14%) samt diabetes som enda orsak (3.2%). Att diabetes i sig skulle kunna orsaka CMVr var oväntat. Diagnosen CMVr var i vissa fall fördröjd med mer än 30 dagar från att patienten fått besvär. I denna grupp var intraokulär inflammation i form av förekomst av vita blodkroppar i kammarvatten eller glaskropp mycket vanligare samt att det intraokulära trycket oftare var förhöjt. Patienterna var äldre och fler hade diabetes. En del av dem med försenad diagnos bedömdes inte tillräckligt försämrade i sitt immunförsvar för att misstänkas kunna utveckla CMVr. En av dessa presenterade vi i en fallrapport (*delarbete I*) då patienten medicinerades med ett relativt nytt läkemedel, ruxolitinib, som vid denna tidpunkt inte bedömdes påverka immunförsvaret nämnvärt. Ett intressant fynd var att patienten vid provtagning uppvisade ett mycket lågt antal av en speciell vit blodkropp, benämnd natural killer cells, något som har visat sig kunna utlösas av medicinering med ruxolitinib.

LIST OF PAPERS

I von Hofsten J, Johnsson Forsberg M, Zetterberg M.
Cytomegalovirus Retinitis in a Patient Who Received Ruxolitinib. *N Eng J Med.* 2016 Jan 21;374(3):296-7

II von Hofsten J, Bergström T, Zetterberg M.
Absence of Herpesvirus DNA in Aqueous Humor from Asymptomatic Subjects. *Clin Ophthalmol.* 2022 Mar 30;16:959-962

III von Hofsten J, Bergström T, Zetterberg M.
Alpha herpes virus type and viral load in intraocular fluids in patients with acute retinal necrosis. BMJ Open Ophthalmol. 2019 Apr 9;4(1):e000247

IV von Hofsten J, Ringlander J, Norberg P, Zetterberg M, Andersson M, Lindh M, Bergström T.
Deep Sequencing of Varicella-Zoster Virus in Aqueous Humor From a Patient With Acute Retinal Necrosis Presenting With Acute Glaucoma. *Open Forum Infect. Dis.* 2020 May 26;7(6):ofaa198

V von Hofsten J, Zetterberg M.
Risk factors for Cytomegalovirus Retinitis in a National Survey in Sweden *Ocul Immunol Inflamm.* 2023 Jan 10;1-8. doi:10.1080/09273948.2022.215479

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ABBREVIATIONS

AAO American Academy of Ophthalmology

AIDS aquired immune deficiency syndrome

AMD age-related macular degeneration

ARN acute retinal necrosis

ART anti retroviral therapy

BARN bilateral acute retinal necrosis

BCVA best corrected visual acuity

BL Burkitt´s lymphoma

bp base pair

BRB blood-retina barrier

CD cluster of differentiation (defines type of lymphocyte)

CMV cytomegalovirus

CMVr cytomegalovirus retinitis

CSF cerebrospinal fluid

CT cycle threshold

CTL cytotoxic T-cell

DM diabetes mellitus

DNA deoxyribonucleic acid

EBV Epstein barr virus

EHV-1 equid herpes virus 1

ELISA enzyme-linked immunosorbent assay

GCA giant cell arteritis

GWC Goldmann-Witmer coefficient

HHV human herpes virus

HIV human immunodeficiency virus

HPC haematopoietic progenitor cells

HSCT haematopoietic stem cell transplantation

HSV herpes simplex virus

HZ herpes zoster

HZO herpes zoster ophthalmicus

IFN interferon

Ig immunoglobulin

IL interleukin

IM infectious mononucleosis

IOI intraocular inflammation

IOP intraocular pressure

IRL internal repeat long (region)

IRS internal repeat short (region)

IRU immune recovery uveitis

JAK janus kinase

JAK-STAT janus kinase-signal transducer and activator of transcription

logMAR log of the minimum angle of resolution

MDV Marek's disease virus

MHC major histocompatibility complex

ML maximum likelihood

MRI magnetic resonance imaging

mRNA messenger ribonucleic acid

NK cell natural killer cell

OCT optical coherence tomography

ORF open reading frame

PAMP pathogen associated molecular pattern

PSS Posner-Schlossman syndrome

qPCR quantitative polymerase chain reaction

RD retinal detachment

RNA ribonucleic acid

RPE retinal pigment epithelium

SCID severe combined immunodeficiency

SD standard deviation

SNP single-nucleotide polymorphism

SUN Standardization of Uveitis Nomenclature

TRL terminal repeat long (region)

TRS terminal repeat short (region)

UL unique long (region)

US unique short (region)

VZV varicella zoster virus

INTRODUCTION

HERPES VIRUS

Herpes viruses are a group of large, enveloped DNA viruses. The virions (virus particles) are between 120 and 260 nm in diameter, (Figure 1). The innermost nucleocapsid is the carrier of DNA consisting of between approximately 125,000 to 240,000 base pairs (bps). The surface of the envelope is covered with a large number of glycoproteins that enable the virus to infect and fuse with host cell membranes. [1,2] The characteristics of these glycoproteins, which are often used as serological antigens, determine which cell receptors the virus can bind to, thus defining tropism.

Figure 1. Herpes simplex virus 2 (HSV2) virion as seen with electron microscopy. Illustration by J von Hofsten. White chalk on black paper.

Millions of years of evolution have resulted in a ubiquitous spread of herpes viruses over our planet. Numerous animal species are infected, despite their apparent discrepancies in morphology. An oyster and a human have this group of viruses in common. Each virus has developed species specificity but many core genes are conserved between species. Obviously, fossils cannot enlighten us about the course of the herpes virus evolution. However, phylogenetic analyses of virus genome take us back to a common ancestor in molluscs, estimated to have evolved 400 million years ago. [3] The phylogenetical networks can show us the similarities between genomes of herpes viruses infecting different species (Figure 2).

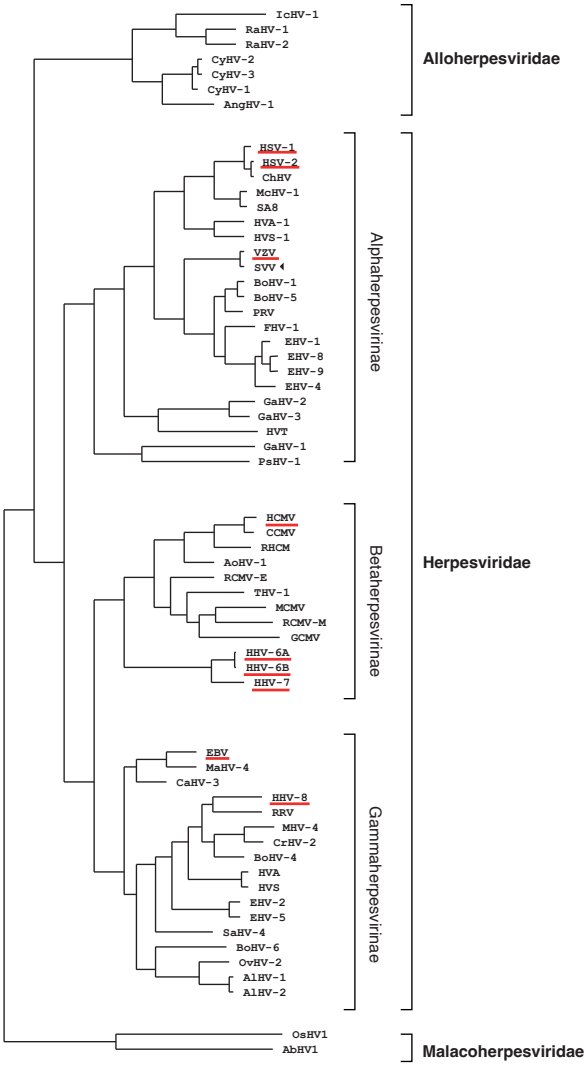

Figure 2. Phylogenetic tree of herpesvirus of different species. The human herpes viruses (HHVs) are underlined. Created by Peter Norberg.

Today, there are nine known human herpesviruses (HHVs) (Table 1). They are divided into groups named alpha, beta and gamma according to the viruses' preferred site of latency and infection. [4] The genetic sequences are in keeping with this classification and as presented in this thesis,so are the clinical characteristics. Five of these HHVs are being studied in the context of ocular disease: herpes simplex virus 1 (HSV1), herpes simplex virus 2 (HSV2), varicella zoster virus (VZV), cytomegalovirus (CMV) and Epstein Barr virus (EBV).

Name	HHV number	Group	Cell tropism/latency
Herpes simplex virus 1	HHV-1	Alpha	muco-epithelial cells, endothelial cells/neurons
Herpes simplex virus 2	HHV-2	Alpha	muco-epithelial cells, endothelial cells/neurons
Varicella zoster virus	HHV-3	Alpha	muco-epithelial cells, endothelial cells/neurons
Epstein Barr virus	HHV-4	Gamma	epithelial cells/B-lymphocytes
Cytomegalovirus	HHV-5	Beta	endothelial cells, epithelial cells, fibroblasts/HPCs
Human herpes virus 6A	HHV-6A	Beta	CD4+ T-lymphocytes, muco-epithelial cells, endothelial cells, neurons/monocytes
Human herpes virus 6B	HHV-6B	Beta	
Human herpes virus 7	HHV-7	Beta	
Kaposi´s sarcoma herpes virus	HHV-8	Gamma	endothelial cells, epithelial cells, monocytes/B-lymphocytes

Table 1. Human herpes viruses (HHVs)
HPC=haematopoietic progenitor cell.

GENETICS

Herpesvirus virions contain double stranded DNA organized in a linear form. At entry into the nucleus of a host cell during infection, DNA is released from the capsid and forms a circle. Knowledge on the complexity of viral genes has increased over the last years. Features previously associated with eukaryotic genes have been observed. [5,6] For example, with the help of deep sequencing it has been shown that ribonucleic acid (RNA) splicing of transcripts of viral genes is not uncommon. [7,8] This enables the relatively small genome to provide a larger number of functional proteins. To infect different cell types, herpes virus must encode proteins necessary for virus entry, metabolism, regulation of gene expression and synthesis of new viral DNA with structural proteins. Mechanisms for immune evasion and regulation of latency are crucial as well.

In this thesis work, the sequence of the VZV genome was studied. Varicella zoster virus has approximately 125,000 bps.[9] About 105,000 bp consist of the unique long region (UL) flanked by sections with nucleotide repeats named the terminal repeat long (TRL) and internal repeat long (IRL). Adjacent to these is the unique short region (US), flanked by terminal repeat short (TRS) and internal repeat short (IRS). Forty core genes are conserved among alpha, beta, and gamma herpes viruses, all localized in the UL region. Among the type-specific genes for VZV are for example those coding for envelope glycoproteins. Varicella zoster virus has more than 71 open reading frames (ORFs). Open reading frames are spans of DNA sequence between start and stop codons. In other words, they constitute the parts of the genome that are transcribed to RNA.
In the host, RNA polymerase II transcribes DNA into messenger RNA (mRNA) for translation and production of proteins. RNA polymerase I and III produce RNA that is non-coding. This non-coding RNA, from originally being regarded as useless, has subsequently been recognized to have several important functions for the host as well as for the virus. Open reading frame 36 is the gene coding for thymidine kinase and ORF28 codes for DNA

polymerase. Both are used for the synthesis of DNA although their function is also required for targeted treatment with acyclovir, as described later.

One of the most effective tools of survival that evolution has given to herpes viruses is the ability to infect and establish latency in their host. As a lytic infection provides a very short period of replication and survival within the same host, there are many advantages for a virus in entering a different type of cycle where the host cell can survive and still harbour the virus. In herpes virus, several investigations have pointed out the importance of the production of viral non-coding RNA for maintaining latency and evading host immune reponse.[10-14]

Herpes viruses, in particular VZV, are considered genetically stable especially compared with many RNA-viruses. The latter are prone to massive, uncontrolled replication with a high mutation rate. This can have advantages, such as rapid adaptation to environmental changes, antiviral medication or immune pressure. [15,16] The numerous mutations give rise to a swarm of variants called quasispecies.[17-19] Herpes virus, by contrast, has well-controlled replication with a 3′-5′exonuclease proof-reading ability. Among other functions, the 3′-5′exonuclease removes erroneously incorporated bases.[20] This results in a low mutation rate. Given the high prevalence of VZV-infection globally, it seems to be a successful strategy. However, investigations of laboratory manipulated herpes viruses with partially impaired 3′-5′exonuclease, in this case Marek's disease virus (MDV), have suggested a possible increase of virulence with diverse subpopulations of virus. [21] Marek's disease virus shares common features with VZV and likewise belongs to the alpha herpes group. In keeping with this, genetic variability of human VZV is shown to be increased in cerebrospinal fluid (CSF) in patients with encephalitis compared with meningitis, independent of viral load. Some of the encephalitis patients in the same investigation had evidence of simultaneous infection from different clades. [22]

Cytomegalovirus exhibits remarkable levels of genetic instability in cell culture. In clinical samples, signs of frequent recombination events can be observed. [23-25] Recombination can occur in the event of two viruses infecting the same cell and sharing genetic material. Variability has typically been seen in certain regions, therefore named "hypervariable loci". [26] For example, these can be regions coding for glycoproteins necessary for immune evasion and tropism. Evolution of these sites may favour infection. There are reasons to believe that co-infection and, therefore possible recombination in this manner may be a strategy employed by CMV.

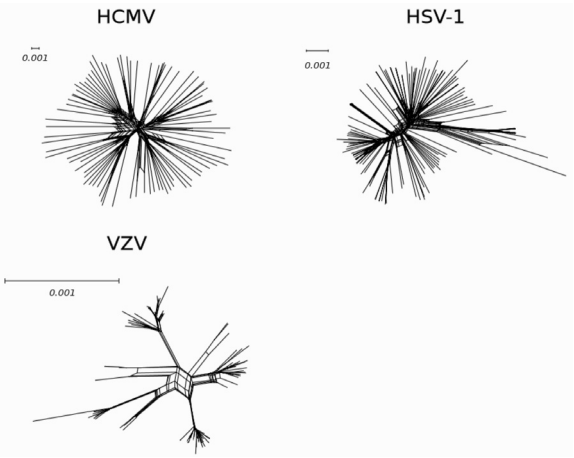

Figure 3. Genetic diversity of herpes viruses. Source: Cagliani et al 2020. Microorganisms. Evolution and genetic diversity in primate cytomegaloviruses. https://www.mdpi.com/2076-2607/8/5/624. Reprinted with permission from Creative Commons Attribution license.

With sequencing of the virus genome, single-nucleotide polymorphisms (SNPs) can be detected, that is, positions in the nucleotide sequence that vary between isolates. Similar sequences are classified into the same clade. A clade in a phylogenetic tree can be described as a branch with its leaves, a group of genomes with a common ancestor. As illustrated in Figure 3 above, VZV has more defined clades than CMV and HSV-1. Furthermore, there is some geographic diversity, where certain clades are more frequently found in a certain geographic region, for example in Europe (Figure 20 in the Results section) than in others. Clade 2 is seen in Asian subjects and includes the strain of the live vaccine. Recombination within and between clades was previously considered rare, given the belief that reinfection does not occur in VZV. Reactivation of zoster is frequently caused by the same virus that is responsible for the primary infection/varicella.[27] However, both reinfection and co-infection in the same individual have been observed. [28,29] Recombination events are not as rare as was previously believed.[30] Increased human migration has led and will further lead to less separated clades over time. Because of this, the live virus vaccine, consisting of a mixture of genomes based on the Oka strain, may recombine with other clades. The clinical impact of this is unknown. However, viral sequences obtained from patients with acute retinal necrosis (ARN) and from meningitis cases have been found to derive from vaccine strains. [31,32] In the case of ARN, no signs of recombination were observed.
Equine herpes virus 1 (EHV-1) is an alpha herpes virus closely related to VZV. It causes upper respiratory tract infection in horses, although outbreaks of abortion and/or myeloencephalopathy are seen in clusters. Genomic analysis of EHV-1 in outbreaks has revealed shared point-mutations and similar genotypes, suggesting that the genetic characteristics may partly determine clinical manifestation. [33,34]

ALPHA HERPES VIRUS

Herpes simplex virus 1, HSV2 and VZV share common features. Target cells for primary infection include muco-epithelial cells and latency is established mostly in sensory ganglia. The reproductive cycle of alpha herpes viruses is short. Genomic analysis reveals a high conservation among genes in HSV1 and HSV2 with up to 80 % of identity in DNA sequence. [35,36] Recombination between HSV1 and HSV2 has been reported, mostly with HSV2 containing novel elements of HSV1. [37,38]

HERPES SIMPLEX VIRUS 1 AND 2

On a global level, about two-thirds of all humans carry antibodies against HSV1 in serum; in Sweden the number was 79.4% in individuals with a mean age of 63 years. [39-41]
HSV2 is probably a younger virus, assumed to have infected our human ancestors from other primates.[42,43] An HSV2 seropositivity of 4-24% has been reported in a European population, and 13% in Sweden.[40,41] Given their resemblance, some cross-protection exists for HSV2 infection in individuals seropositive for HSV1.[44,45]

In HSV1, primary infection is usually asymptomatic but can present as a gingivostomatitis, which is more common in children. [46] Conjunctivitis (bilateral or unilateral) is another manifestation of primary infection. The most common site for HSV2 is the genital mucosa and primary infection usually occurs in adolescence through sexual contact. Both HSV1 and 2 are transmitted by direct contact with skin or mucous membranes.

After infection, latency is established preferentially in the closest sensory ganglion. The trigeminal ganglion is the preferred site for HSV1 and the sacral ganglia for HSV2. The virus can periodically reactivate and travel through anterograde axonal transport back to the skin, mucosa or eye.[47] There are reasons to believe that there is reactivation from the autonomic ganglia as well as sensory ganglia. In genital herpes reactivation, autonomic dysregulation such as urinary retention has been observed. Polymerase chain reaction analyses of autonomic ganglia from human cadavers have shown that they can harbour HSV1 and VZV DNA. [48]
Latency was previously considered a passive state of the virus. Newer insights in epigenetics have revealed substantial viral activity controlling latency. [49,50] Therefore, one can argue that the boundaries between latency and a low-level chronic infection are very subtle.

Periodically the virus needs to replicate and spread to other hosts. Reactivation can be initiated, usually following a change in host cell metabolism in response to external signals. Individuals carrying herpesviruses regularly spread infectious virions, a process known as “shedding”, during disease or asymptomatically. Asymptomatic shedding is another example of active virus replication during a stage we consider as latency. In both asymptomatic and symptomatic individuals, HSV1 exhibit regular shedding from saliva and tears. [51-53]
The most common manifestation of HSV1 reactivation is mucocutaneous vesicles presenting as cold sores. Among all viruses, HSV1 causes the most ocular complaints. It has been estimated that the incidence of ocular herpes simplex disease in the USA and Europe is 24 per 100,000 person-years. [54] Reactivation of HSV1 is most often seen in the form of epithelial keratitis lesions with a characteristic dendritic shape (Figure 4). Other clinical signs include stromal keratitis, uveitis, neurotrophic keratitis. These latter manifestations are often seen after many recurrences. Neurotrophic keratitis is a hallmark of decreased sensibility of the

cornea. Typically, initially appearing dendritic lesions appear broader and corneal wounds are more leaf-shaped (Figure 5). Prolonged viral activity has affected the corneal nerves and important cell-to-cell communication is interrupted. Both HSV1 and HSV2 can cause neonatal conjunctivitis after vaginal delivery. Herpes simplex conjunctivitis in adults is uncommon. It is estimated to be the causative virus in around 4% of follicular conjunctivitis cases. [55-57] Rarely, HSV1, HSV2 and VZV can lead to acute retinal necrosis (ARN), as described below. Apart from cases with ARN and conjunctivitis, HSV2 is rarely diagnosed in ocular disease.

The most dreaded reactivation of HSV is encephalitis. In around one in 250,000 individuals per year, reactivation occurs with focal encephalitis. Symptoms include fever and altered consciousness and behaviour. Encephalitis is caused by HSV-1, but only rarely by HSV-2.[58,59] Before the introduction of antiviral treatment, the mortality rate was >70%; this has now decreased to a 10-20% in treated patients. Neurological sequelae such as epilepsy are not uncommon.[60-62]

Meningitis is most often a reactivation of HSV2 and has a milder course. Nausea, neck stiffness, headache and photophobia are main symptoms. As inflammation is localized to the meninges and does not involve the brain parenchyma itself, most patients have a favourable outcome. However, a recent study reported 11% of patients experienced a substantial effect on daily living even after 6 months. [63] Between 20% and 30% of patients with HSV meningitis will develop recurrent disease.

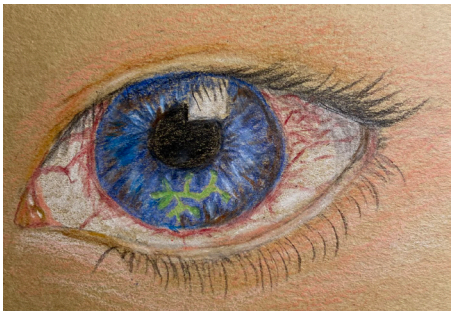

Figure 4. Dendritic keratitis caused by herpes simplex virus 1 (HSV1). Illustration by J von Hofsten, colour pencil.

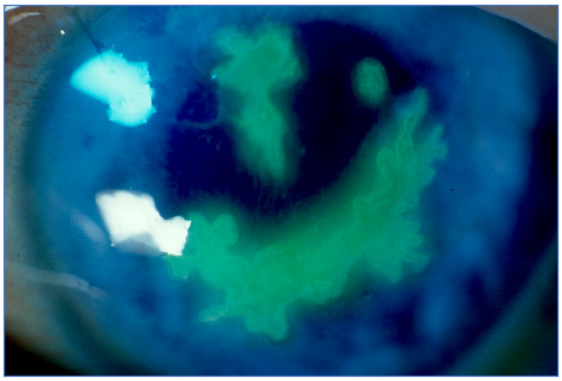

Figure 5. Geographic keratitis in herpes simplex virus 1 infection. Photo by Maud Leindahl, St. Erik Eye Hospital, Stockholm.

VARICELLA ZOSTER VIRUS

In Sweden, close to all individuals (97.9%) have been exposed and have developed antibodies against VZV by the age of 60.[40] Varicella zoster virus is considered the most contagious of all HHVs with transmission through aerosol inhalation in contrast to CMV, EBV and HSV, where transmission is by direct contact with, for example saliva or genital secretions. Primary infection gives rise to varicella, also known as chickenpox, an endemic disease globally, with regular outbreaks among small children. In young individuals, the clinical symptoms are relatively mild but tend to become more serious in adults. A rash with blisters over the entire skin is typically combined with fever. Complications that may occur are encephalitis, hepatitis and haemorrhagic states, bacterial pneumonia or sepsis.[64] After infection, VZV establishes latency in a sensory/autonomic ganglion or in the dorsal roots of the spine. This occurs either by retrograde axonal transport or by haematogenous spread. Asymptomatic reactivation with shedding of virus through saliva is very rare. In investigations, quantitative PCR (qPCR) tends to be negative in nearly all healthy individuals.[65-67] Under extreme conditions, such as space flights, low levels of VZV DNA have been found even in healthy subjects.[68] This is in contrast to 88-100% positivity of VZV in saliva samples from herpes zoster (HZ) patients.[66,69]

The most common symptomatic reactivation from latency manifests as HZ/shingles. This is characterized by vesicular eruptions in a dermatome of the skin. Pain and a burning sensation are often experienced in this area days to weeks before the blisters form. Lesions last for up to 2 weeks while the pain can last much longer. If the pain prevails for > 3 months, it is defined as post-herpetic neuralgia. Despite a small area of engagement, HZ seems to have a systemic impact. As early as 1998, immunocompetent individuals with HZ without signs of meningitis, encephalitis or myelitis were studied and found to have abnormal CSF, some with detectable VZV DNA, and magnetic resonance imaging (MRI) lesions. [70]

Even in the absence of zoster skin lesions there are central nervous system (CNS) manifestations such as meningitis and encephalitis. [32] The risk of stroke is increased after HZ, especially within 3 months after onset. [71-73] In particular, HZ ophthalmicus (HZO), where the ophthalmic branch of the trigeminal nerve is engaged, is associated with an increased risk of stroke, which is even higher in untreated individuals.[71,74] Varicella zoster virus-induced

vasculopathy is considered to be the mechanism behind stroke and probably behind encephalitis, meningitis and cranial nerve engagement. Histology of arteries shows neointimal proliferation, necrosis and inflammation; at times multi-nucleated giant cells are seen. Affected arteries exhibit stenosis, aneurysms, or haemorrhage. Because of its resemblance to giant cell arteritis (GCA), samples from GCA patients have been analysed and found to test positive for VZV DNA, suggesting VZV as a potential trigger for GCA. [75,76] Later investigations, however, have shown conflicting results because of the absence of expressed VZV proteins in affected arteries. [77,78]

Herpes zoster is a reactivation of VZV. So why are there so many anecdotes of grandparents diagnosed with shingles after having physical contact with grandchildren having chickenpox? In temperate zones of the world, shingles seems to have a slightly higher incidence during the summer and/or when UV-index is high. [79-82] As HSV reactivation is triggered by light, this could explain reactivation of VZV as well. However, chickenpox has its own more distinct cycle with recurrent outbursts.[83] When the numbers of chickenpox and shingles are compared over time, they exhibit mirror images of each other. Moreover, after a peak in chickenpox, there is a significant rise in shingles within 3 months. [79,84] Although controversial, these data may suggest reinfection with VZV to be responsible for some cases of shingles.

CYTOMEGALOVIRUS

Cytomegalovirus (CMV) belongs to the beta herpes virus group and comes from the Greek *cyto* (cell) and *megalo* (large) which refers to the enlarged cells seen in CMV-infection. The seroprevalence globally is between 45% and 100%, where developing countries generally exhibit higher numbers. [85] Sweden has a seroprevalence of between 50 and 83%, the latter observed in older individuals.[40,86]

Primary infection is asymptomatic although CMV may manifest as fever, lymph node enlargement and, occasionally, hepatitis. Congenital CMV infection is the most common infectious cause of intrauterine infection. Transplacental transmission of CMV occurs in 1 % of all pregnancies worldwide .[87] In about 10-15 % of these cases, it results in disability, from sensorineural hearing loss to advanced malformation and death of the foetus.

Shedding of virus after primary infection is extensive and can continue for weeks to months. This is especially true for congenital CMV. Shedding is more common in children than in elderly individuals despite the higher prevalence of seropositivity with age. Transmission is by contact with most body fluids. The highest frequency of shedding and viral load is found in urine. [88]

Latency is primarily located in CD34+ haematopoietic progenitor cells (HPCs) found in blood and bone marrow. About one in 10,000 to 100,000 CD34+ HPCs is infected in an individual. [89] Recent investigations have reported expression of viral microRNAs inducing the myelosuppressive cytokine transforming growth factor beta (TGFβ), indicating a state of activity even during latency.[90] On reactivation, preferred target cells are epithelial cells, endothelial cells and fibroblasts. Symptomatic reactivation is rare in an immunocompetent host. In the immunocompromised, manifestations of end organ disease include oesophagitis, colitis, hepatitis, encephalitis and retinitis. The latter is discussed in sections below.

Despite the opportunistic characteristics of CMV, corneal endotheliitis and anterior uveitis have been shown to be caused by CMV in immunocompetent individuals. Surprisingly, CMV endotheliitis of the cornea has been observed in predominantly Asian subjects. [91,92] Moreover, corneal graft failure has been linked to CMV infection in many reports from East Asia[93,94] but not in the UK.[95]
In Caucasian individuals, anterior uveitis without corneal involvement is uncommon but most probably it is underdiagnosed. Uveitis is limited to the anterior chamber and is often associated with high intraocular pressure (IOP). Posner Schlossman syndrome (PSS), also called "glaucomatocyclitis crisis", is defined as a state of recurrent attacks of high IOP combined with discrete non-granulomatous anterior uveitis.[96] From being considered an idiopathic condition, many reports now support the hypothesis of CMV as the leading cause of PSS. [91,97-99]

EPSTEIN BARR VIRUS

Epstein Barr virus (EBV) is a member of the gamma herpes virus family. Members of this family share characteristics, which include oncogenic associations. Another HHV in this group is Kaposi's sarcoma virus. Epstein Barr virus infects and establishes latency in B-lymphocytes. A recent review describes the ability of EBV to interact with several important cellular pathways involved in oncogenesis. [100] One of these is the Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway.

Seroprevalence of EBV was previously estimated to 90% globally.[101] More recent publications report a prevalence of 85.3%, 98% and 99.8% in populations in the UK, Germany and China, respectively. [102-104] Primary infection with EBV is most often asymptomatic but can manifest as infectious mononucleosis (IM). It typically occurs in teenagers and presents with low-grade fever, lymphadenopathy and tonsillitis. In some cases, the disease progresses with splenomegaly, hepatomegaly and a longstanding fatigue. Serious complications include bone marrow depression, hepatitis, myocarditis and Guillain-Barré syndrome. Epstein Barr virus encephalitis is rare and occurs in conjunction with IM. The virus is mainly transmitted orally and asymptomatic shedding is common.[105] Other means of acquiring EBV is by blood or transplantation of organs.
As mentioned above, EBV has oncogenic features. In fact, the virus was first discovered in 1964, by Epstein and Barr in a Burkitt's lymphoma (BL) from an African child. [106] In endemic areas in Africa and New Guinea, EBV positive BL is the most common childhood tumour. Surprisingly, the percentage of EBV-positive BL is much lower in Europe and the USA, 10-20% compared with 100% in Africa. Other tumours with EBV-association are for example Hodgkin's lymphoma, nasopharyngeal carcinoma, gastric carcinoma and diffuse large B-cell lymphoma. Patients with immunosuppression, caused by human immunodeficiency virus (HIV) for example, have a higher EBV-positivity in various types of lymphomas. Virally induced oncogenesis occurs early in infection. In samples of lymph nodes from patients with IM, Reed-Sternberg cells, usually pathognomonic for Hodgkin´s lymphoma, have been found. [107] Epstein Barr virus has been linked to chronic inflammatory diseases as well, most recently to multiple sclerosis.[108,109]

IMMUNITY AND INFLAMMATION

The human body has a wide range of defence mechanisms to protect itself from intruding organisms and viruses. The immune system is divided into the innate and the adaptive part, each playing a pivotal role in protection against viral infection.

Parts of the innate immunity, i.e. the unspecialized part of the immune response, detects virion DNA, classified as pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors for example toll-like receptors on the cell surface, or by cytoplasmic sensors.[110,111] These initiate a cascade of events with expression of inflammatory genes, such as those coding for cytokines, chemokines and interferons (IFNs).[112] These substances activate the immune system and attract its cells to the affected compartment. Immune cells that are part of the innate immune system include: neutrophiles, monocytes, macrophages, dendritic cells, eosinophiles, basophils, mast cells and natural killer cells (NK-cells).

NK-cells are crucial for the body's defence against virus. As the name implies, their main function is to kill infected cells and thereby eliminate reservoirs of infection. Natural killer cells have also been claimed to be the utmost important defence against herpes virus.[113,114] Deficiency or lack of function of NK-cells has been shown to result in serious herpetic disease.[115,116] Other parts of the innate immune system include the complement system and autophagy, both of which have effects against herpes virus.

Adaptive immunity, on the other hand, consists of lymphocytes specialized in defeating a specific antigen. The thymus, the spleen and all lymph nodes are immunological organs where the "education" of these lymphocytes take place. Antigen presenting cells from the innate immune system present antigen for naïve T-lymphocytes. They also produce interleukin (IL), to guide the proliferation and differentiation of the T-cell into CD4+ T-cells or CD8+ T-cells. Some CD8+ T-cells evolve to become cytotoxic T-cells (CTLs), capable of killing infected cells. The CD4+ T-cells, also called "helper T-cells", have numerous functions. They are crucial for activation of CD8+ T-cells as well as for refining antibody production in B-lymphocytes. CD4+ T-cells produce large amounts of cytokines to recruit and attract phagocytes to the area of infection.
After antigen recognition, B-cells differentiate to become antibody-producing plasma cells and memory cells. As stated above, the process of creating high-affinity antibodies, for example immunoglobulin G (IgG) requires help from CD4+ T-cells. T-independent antibody response is limited to low-affinity antibodies, such as IgM. Immunoglobulins (Ig) are relatively large, Y-shaped molecules. In the upper arms of the Y, the fragment antigen-binding (Fab) region, there are two antigen binding sites. At the bottom of the Y, the fragment crystallizable (Fc) region determines the biological function. There are several isoforms of immunoglobulin (Ig). Immunoglobulin M (IgM) is expressed early in infection and typically produced by immature B-cells. The specificity for antigen is low. Immunoglobulin M is composed of 5-6 Y-shaped Igs connected at the Fc region in a pentamer/hexamer. Immunoglobulin G, on the other hand, is monomeric and the most abundant and specialized Ig. As stated above, only mature B-cells can secret IgG and they have the highest specificity of all Ig-types. Both IgM and IgG can combat viruses either by direct neutralization of extra-cellular virus or by activating the complement system. Immunoglobulin G provides antibody-dependent cell-mediated cytotoxicity as well. However, antibodies do not seem to prevent reactivation or cell to cell spread of herpesvirus.[117]

A cellular pathway involved in control of inflammatory response as well as in haematopoiesis is JAK-STAT (Figure 6). It is essential for both innate and adaptive immunity. Cytokines such as IL-6 and IFN can bind to the JAKreceptor on the cell surface and via this pathway promote transcription of pro-inflammatory genes. Loss-of-function of these genes can result in severe combined immunodeficiency (SCID).[118] Gain-of-function can result in myeloproliferative disease. For example, mutations in the Janus kinase 2 (JAK2) gene are present in 50% of patients with myelofibrosis.[119] Activation of this pathway has also been observed in EBV-positive diffuse large B-cell lymphoma tumours in contrast to EBV-negative lymphoma.[120] The JAK-STAT pathway is important for the defense against herpes viruses and will be discussed in more detail later in this thesis (see "Results and Discussion, Papers I and IV").
An interesting feature of herpes viruses is that an ongoing inflammation in the host is a driver of reactivation. For example, herpes simplex reactivation is particularly common after intraocular surgery which induces postoperative inflammation as well as in bacterial keratitis. Moreover, the treatment of many manifestations of herpes virus includes substantial use of steroids to inhibit inflammation. In some cases, such as HSV- or VZV-induced iritis and stromal/endothelial keratitis, steroids could be used exclusively without antiviral treatment, with adequate clinical response.

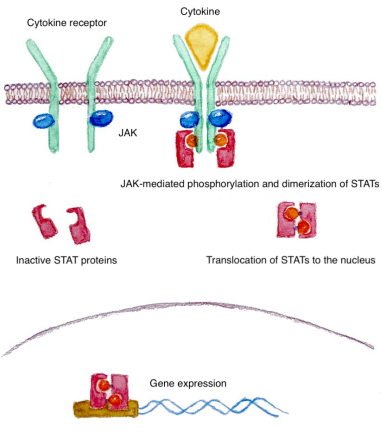

Figure 6. The Janus kinase signal transducer of transcription (JAK-STAT) pathway. Illustration by J von Hofsten. Watercolour pencil

Immune evasion

For many of the restrictions used by the host, countermeasures have evolved in the virus. More than half of a herpes virus´s genome is devoted to immune evasion. By studying what viral genes remain conserved over an extensive span of time, we can draw conclusions on what parts of the immune system that are crucial to evade from an evolutionary perspective. It has been shown that all HHVs encode at least one chemokine homologue with antagonistic or agonist effect. [112] As mentioned above, NK-cell activity is important in the defence against herpes viruses which have several mechanisms to inhibit NK-cell activity. For example, NK-cells target cells lacking normal major histocompatibility complex (MHC) on the surface as this is a sign of infection. (Figure 7) This downregulation of MHC is caused by the virus to prevent discovery by the adaptive immune system. Therefore, for example CMV has been shown to produce decoy ligands to simulate an uninfected state of the host cell.[116,121,122] The JAK/STAT pathway has been shown to be inhibited by alpha herpes viruses to promote infection.[120,123]

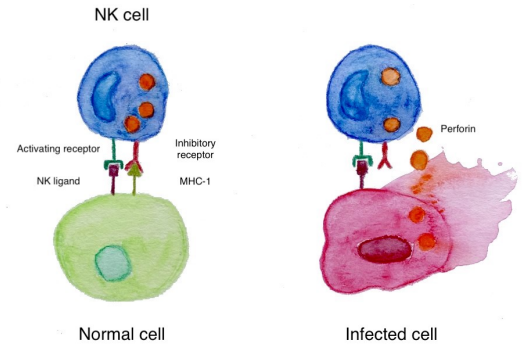

Figure 7. Lysis of infected cell by natural killer (NK) cell activity due to downregulation of major histocompatibility complex (MHC) receptors on the cell surface. Illustration by J von Hofsten. Watercolour.

ANATOMY OF THE EYE

The eye is a tissue mainly derived from the ectoderm during foetal development. The cornea and lens have similarities with skin and the retina and optic nerve are basically part of the CNS. The uvea, including iris, ciliary body and choroid, derive from the neural crest and mesoderm. This middle layer is a highly pigmented vascular bed (Figure 8). Aqueous humour fills the anterior compartment of the eye, between the lens and the cornea, while a major part of the eye contains a gel called the vitreous. Although only separated by the zonule fibres, these two liquids are very different. Aqueous humour is similar to plasma and CSF and has a formation rate of about 2.5 µl per minute. [124] As described in other mammalian species, 1.0 to 1.5 % of the volume of the anterior chamber is exchanged per minute.[125] In the vitreous, which is more like a gel, the turnover, by diffusion or bulk flow of particles, is much slower.[126]

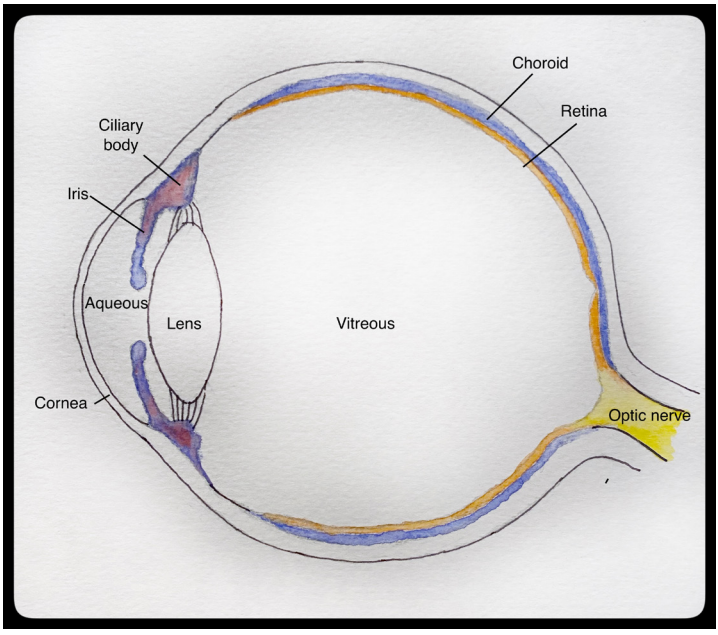

Figure 8. Anatomy of the eye. Illustration by J von Hofsten. Watercolour pencil and ink marker.

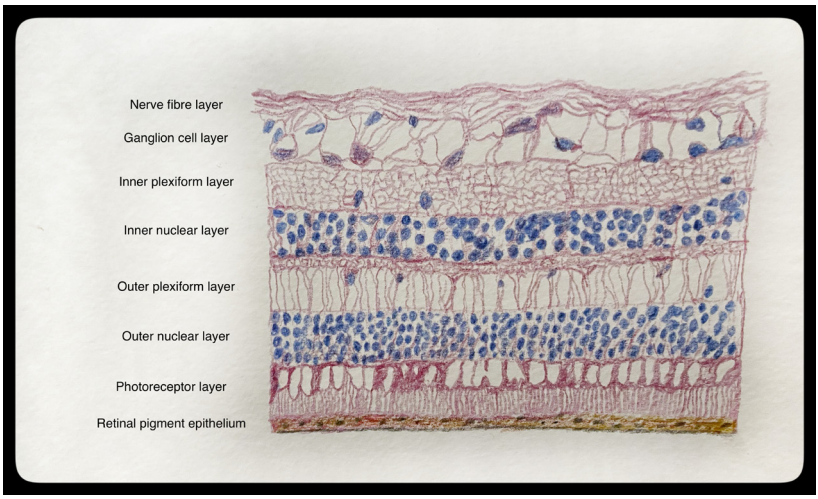

Figure 9. Layers of the retina. Illustration by J von Hofsten. Colour pencil.

Figure 9 shows the layers of the retina overlying the retinal pigment epithelium (RPE) and choroid. Light beams are refracted by the cornea and lens and pass through these layers to be perceived by sensitive light receptors (rods and cones) in the deepest layer above the RPE. The visual information is then transferred through horizontal cells and bipolar cells in the outer plexiform layer, which synapse with amacrine cells and ganglion cells in the inner plexiform layer. The axons of the ganglion cells form the nerve fibre layer and gather to form the optic nerve into the CNS. In the fovea, which is the part of the retina where the image of the central visual field centre is focused, the retina consists of fewer layers to obtain a shorter path for the light beams and a clear image. Moreover, the fovea has a greater proportion of the photoreceptors called “cones” that provide higher sensitivity to colour and contrast. Rods are more abundant in the peripheral retina and are more sensitive to light, thus playing an important role in scotopic visual processes (dark).

The RPE is a densely pigmented layer below the retina and above the sclera. It has several important functions. This dark wall is responsible for absorbing scattered light, not only to optimize visual quality but also to reduce oxidative stress. Phagocytosis of photoreceptor outer segment membranes is another means of protection against oxidative stress provided for the photoreceptors by the RPE. Constant interaction between the RPE and, on one side, the photoreceptors and, on the other side the choroid vessels include RPE secretion of growth factors. Disturbed functions of the RPE can result in many disorders, including age-related macular degeneration (AMD).
Because of the importance of sustained clarity of vision for survival, there is immune privilege intraocularly. For example, an antigen presented intraocularly can induce tolerance if this antigen is presented later elsewhere in the body. There are several mechanisms, many unknown, that regulate tolerance [127,128] Resembling the blood-brain barrier, the blood-retina barrier (BRB) is a mechanism by which the eye protects the retina. [129] It protects against infectious agents, as well as immune cells. The outer BRB is maintained by the RPE. The RPE acts as a physical barrier between the outer blood stream in the choroid and the intraocular environment. Moreover, it communicates with the immune system by secretion of fas-ligand for example, which suppresses inflammation. The inner BRB is an immunological

barrier of adjacent microglia, pericytes, perivascular macrophages and the endothelium itself in the retinal vessels. [130] These endothelial cells are connected to each other by tight junctions, which also is the case for RPE. Disruption of the BRB can result in serious disease; for example, it enables the development of CMV retinitis as described below.

RETINITIS

CYTOMEGALOVIRUS RETINITIS

Clinical signs and diagnosis

Cytomegalovirus has a predilection for infecting endothelial cells. It has been shown that CMV viraemia can spread via the vascular endothelium and infect surrounding tissues in the retina, resulting in cytomegalovirus retinitis (CMVr).[131,132] In autopsy eyes with clinically diagnosed CMVr, capillaries have been shown to be partly devoid of endothelial cells. Adjacent neuronal cells, glial cells and RPE have been shown to stain positive for CMV antigens in analyses using immunofluorescence and in situ hybridization. [132] More recent investigations with optical coherence tomography (OCT) have enabled us to visualize layers of the retina non-invasively. Examinations by OCT of active CMVr show a full thickness disruption of all retinal layers including the RPE.[133-135] In healed lesions, thinning of the underlying choriocapillaries has been observed. [134] Typical clinical signs of CMVr include wedge-shaped necrosis along vessels accompanied by haemorrhages and vasculitis affecting both venules and arterioles (Figure 10, 11). Depending on the level of immunosuppression in the host, there are inflammatory cells in the anterior chamber and vitreous.

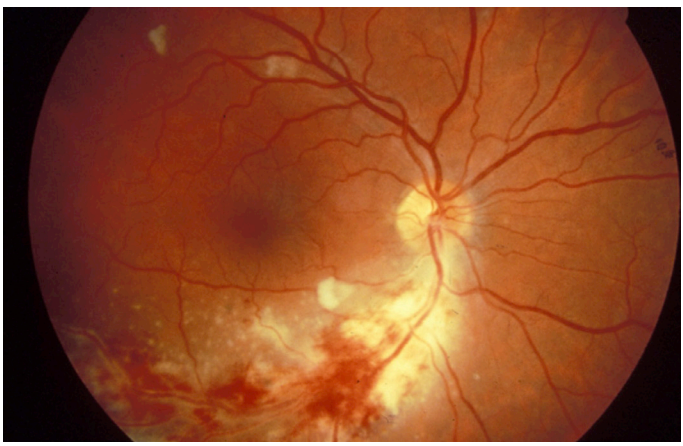

Figure 10. Typical clinical findings in cytomegalovirus retinitis (CMVr). Reprinted from Ophthalmology, vol 228. Standardization of Uveitis Nomenclature (SUN) Working Group. Classification Criteria for Cytomegalovirus Retinitis. pp 245-254, 2021. With permission from Elsevier.[136]

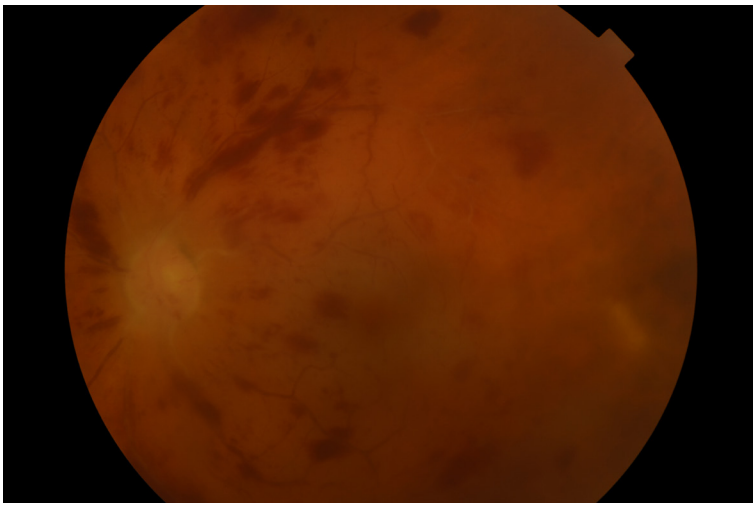

Figure 11. Cytomegalovirus retinitis (CMVr) with numerous haemorrhages and arteritis. Photograph used with permission from the patient.

A granular, indolent form of retinitis is an alternative presentation. Here, lesions are located more peripherally and there is less haemorrhage. Granular patches could also be signs of inactive retinitis resembling atrophy after previous necrosis. In 11-14% of cases, a different clinical picture is seen with frost-branch angiitis where arterioles and/or venules resemble frost-covered tree branches (Figure 12 and cover illustration).[137-139] The exact pathology of the perivascular exudates is not known but they seem to consist of thickening of the vessel wall, with considerable leakage seen on fluorescein angiography.

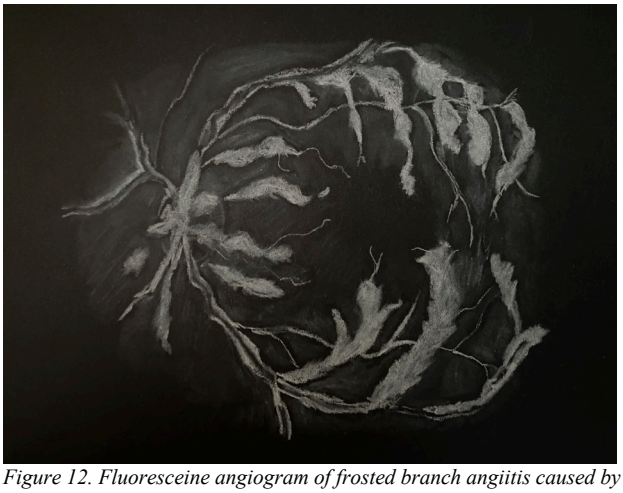

Figure 12. Fluoresceine angiogram of frosted branch angiitis caused by cytomegalovirus (CMV). Illustration by J von Hofsten. White chalk on black paper.

In rare cases, Kyrieleis plaques can be observed at arterioles, resembling glistening cuffs. These were first described in tuberculosis by Kyrieleis in 1933. [140] There is, to date, no exact explanation of the mechanism behind the plaques. Initially described as segmental periarteritis, later investigations with fluorescein angiography and OCT have rather displayed inflammation in the vessel wall, resembling endotheliitis. [141,142] There is no evidence of occlusion of the lumen or leakage of contrast.

Classification criteria for CMVr, suggested by the Standardization of Uveitis Nomenclature Working Group (SUN)[136] are as follows (Table 2):

Criteria (requires #1 and #2 and either #3 or #4)

1. Necrotizing retinitis with indistinct borders due to numerous small (<50μm) satellites

AND

2. Immune compromise, either
   a. Systemic (e.g. AIDS, organ transplant, chemotherapy) OR
   b. Ocular (e.g. intraocular corticosteroids or chemotherapy)

AND (#3 or #4)

3. Characteristic clinical picture ([a or b or c] And d)
   a. Wedge-shaped area of retinitis OR
   b. Hemorrhagic appearance of the retinitis OR
   c. Granular appearance of the retinitis AND
   d. Absent to mild vitritis

OR

4. Evidence of intraocular infection with cytomegalovirus
   a. Positive PCR for cytomegalovirus from either the aqueous or vitreous specimen

Exclusions

1. Positive serology for syphilis using a treponemal test
2. Intraocular specimen PCR-positive for infection by herpes simplex virus, varicella zoster virus, or *Toxoplasma gondii* (unless there is immune compromise, morphologic evidence for >1 infection, the characteristic picture of cytomegalovirus retinitis, and the intraocular specimen also has a positive PCR result for cytomegalovirus)

Table 2. Classification criteria for cytomegalovirus retinitis (CMVr), suggested by the Standardization of Uveitis Nomenclature (SUN) Working Group, August 2021. AIDS=aquired immune deficiency syndrome; CMV=cytomegalovirus; HSV=herpes simplex virus; PCR=polymerase chain reaction:VZV=varicella zoster virus.

Mandatory for diagnosis are signs of necrotizing retinitis in an immunocompromised patient together with either laboratory evidence of intraocular CMV infection or a characteristic clinical picture including absent or mild vitritis. If these criteria are met, the correct diagnosis is presumably found in 93.1-93.7% of cases. Viraemia and especially the level of DNA in serum is a risk factor for developing CMVr.[143] However, only 32.9-56.1% of patients with CMVr have measurable CMV DNA in serum at diagnosis.[144,145] Polymerase chain reaction analysis of samples of aqueous or vitreous humor have proven to be positive for CMV DNA in 93-100% in active CMVr, which makes it very useful for diagnosis. [146,147] The viral load is typically high, significantly higher in aqueous humour compared with other CMV-related ocular disease such as endotheliitis of the cornea or anterior uveitis.[147]

Immunosuppression:

Cytomegalovirus retinitis is only seen in immunosuppressed individuals. Before the emergence of the HIV pandemic, CMVr was a rare event. In the early phase of HIV infection, CD4 + T-lymphocytes are infected; later they become gradually depleted, which is the main mechanism of immunosuppression in aquired immune deficiency syndrome (AIDS). The level of CD4+ T-cells in serum is shown to be correlated to the risk of developing CMVr where <50 cells/µl comes with a high risk of retinitis.[148-150]
Prior to the introduction of anti-retroviral therapy (ART), around 30% of HIV-infected individuals succumbed to this potentially blinding retinitis.[151] With appropriate ART against HIV, the incidence of CMVr has decreased by about 80% [152], although developing countries still struggle with high numbers due to a high prevalence of HIV-positive individuals and lack of access to medication. For example, the percentage of adults with HIV-infection in Malawi is estimated to be 10.6%. [153] In low-middle income countries, screening of HIV positive patients with low levels of CD4+ T-cells or visual complaints has revealed a high prevalence of CMVr. In South East Asia, up to 30% of screened HIV-positive individuals have been reported to have CMVr. [154] By contrast, South Africa has low levels of CMVr, 1.3%. The discrepancy between South Africa and Asia has been studied and confounding factors, such as CD4+ T-cell levels taken into account, could not explain the differences in prevalence. Among others, one explanation may be genetic differences in virus or host. [155,156]

Medically induced immunosuppression is rising in developed countries. The number of solid organ transplantations and, even more so, haematopoietic stem cell transplantation (HSCTs) has expanded and become more available for patients. The field of research on medically induced immunosuppression is growing, providing potent treatment for an expanding number of patients with autoimmune disease, transplants, cancer, etc. Around 3% of the American population are prescribed drugs than induce immunosuppression. [157] Consequently, the majority of cases of CMVr in Western countries are likely to be caused by medically induced immunosuppression. Multiple substances may be needed for adequate effect in some patients. New drugs are introduced with potent effect on the immune system. One of these is ruxolitinib described in *paper I*, where the immunosuppressive effect was underestimated, thereby delaying diagnosis of CMVr.

Cytomegalovirs retinitis is an end-organ disease of CMV reactivation. Other manifestations are colitis, oesophagitis, encephalitis and pneumonitis. Retinitis is considered more common in HIV-positive individuals compared with HSCT or solid organ recipients, in whom gastrointestinal CMV disease is seen more frequently. [158,159]An explanation could be that, with the increased level of immunosuppression and higher viral loads the virus could pass the BRB. Gastrointestinal disease may be underdiagnosed as well. [160] Co-existing HIV retinopathy/microvasculopathy could potentially increase the risk of viral replication in the retina.

Treatment:

At present, mainly three substances are used for treating CMVr: ganciclovir, foscarnet and cidofovir. All inhibit replication of the virus. There are no virucidal treatments, nor is there a way of eradicating a virus in latency.

Ganciclovir is a nucleotide analogue of deoxyguanosine. Triphosphorylation is required for effect. The first step is catalyzed by the viral enzyme phosphotransferase coded from the

UL97 gene. Ganciclovir monophosphate is further phosphorylated by cellular enzymes. With this mechanism, essentially only CMV infected cells are targeted for treatment. Ganciclovir triphosphate is a competitive inhibitor of the incorporation of guanosine trisphosphates in replication of the DNA and can be described as a chain terminator. Ganciclovir can be administered intravenously or intravitreally. Valganciclovir, a prodrug, is given orally. Sustained-release implants with ganciclovir were available previously but were later discontinued on the market as CMVr cases were decreasing after the introduction of ART and the introduction of valganciclovir. Unfortunately, side effects associated with systemic ganciclovir include bone marrow depression. Surveillance with regular blood counts is necessary.

Foscarnet is a pyrophosphate analogue that binds directly to the viral DNA polymerase and inhibits DNA chain elongation. It does not require activation by viral enzymes. Therapeutic concentrations do not affect human DNA polymerase. Metabolic toxicity and nephrotoxicity are severe side effects of foscarnet when given systemically. Intravitreal treatment with foscarnet is, however a preferred choice, especially in viral resistance of ganciclovir. High doses of foscarnet (2.4 mg) intravitreally have proven to be safe and effective in CMVr.[161]

Cidofovir is a nucleotide analogue of cytosine with one phosphate group already present. It does not need phosphorylation by viral enzymes but by cellular enzymes. Cidofovir is a competitive inhibitor of the deoxycytosine triphosphate. Treatment comes with significant risk of neutropaenia and nephrotoxicity. Moreover, cidofovir can induce uveitis. [162]

Intravitreal therapy with ganciclovir or foscarnet was shown to have an increased effect on preventing progression of retinitis compared to only systemic treatment, probably because of higher levels of ganciclovir in the vitreous. Systemic treatment, however, decreases the risk of other end-organ diseases.[163] Moreover, systemic therapy as opposed to only intraocular therapy, has been shown to reduce the mortality in AIDS-patients by 28%.[164] Consequently, many ophthalmology clinics use a combination of intravitreal injection and systemic treatment. Combined intravitreal and systemic treatment was recommended in all patients except naïve patients with peripheral lesions (where monotherapy with oral valganciclovir was considered enough) by Jabs in 2008. [165]
However, no definitive guidelines have been established for CMVr treatment.

Resistance to antiviral drugs is rarely seen in treatment-naïve individuals.[166] Unfortunately, even after 3 months of treatment in immunosuppressed patients, resistance tends to occur. [167] Mutations in the *CMV UL97* gene, which codes for phosphotransferase (which phosphorylates ganciclovir) is most commonly the cause of ganciclovir resistance. Several mutations in the *UL54* gene, which codes for DNA polymerase and affects CMV replication affects ganciclovir as well as cidofovir. Mutations in other loci of the *UL54* gene can lead to foscarnet resistance. Development of ganciclovir resistance has become a worrying issue with resistance rates of 14.1% according to a recent meta-analysis. [168]
Drug resistance can be measured by two methods. Phenotypic resistance is determined by measuring the concentration of the drug necessary to inhibit virus-induced cytopathic effect by 50% (IC_{50}). Genotype resistance can be measured by sequencing part of the viral genome for mutations known to confer phenotypic resistance. In a study with samples from 94 clinically resistant CMV patients, only 26.6% were found to be genetically explained. [169] The need for extending the mutation databases is therefore crucial.
Investigations with sequencing of the *UL97* gene have shown 93.5% agreement between vitreous and blood samples.[170] This may suggest that isolates of virus in different body

compartments are genetically similar. However, more recent reports have found discrepancies between body compartments in the same individual, suggesting heterogeneity in virus strains. [169] Moreover, it has been proposed that individuals carry several CMV types in blood and only certain sequences of CMV can gain access to the retina by immune evasion. [171] As mentioned previously, CMV seems to promote reinfection and recombination; therefore, a heterogeneous population of virus variants can be evolutionarily beneficial.

Due to emerging treatment refractory strains of CMV, a new drug is available on the market to be considered in cases of resistance. Maribavir is a benzimidazole riboside taken orally. It has effects on viral DNA replication, encapsidation and nuclear egress of viral capsids by inhibition of the CMV UL97 protein kinase. Phase 3 studies of treatment refractory CMV infections in post-transplant patients have exhibited superior viral clearance and symptom control compared to valganciclovir/ganciclovir, foscarnet or cidofovir (55.7% vs 23.9%) with less systemic side effects.[172] Satisfactory results of using maribavir on treatment refractory CMVr have been reported.[173] However, there is a substantial risk of recurrence at treatment cessation and treatment-induced UL97 mutations resistance is not uncommon (25%). The latter unfortunately including some conferring cross-resistance to ganciclovir. [174]

Immune recovery uveitis

In the rise of ART, immune systems of HIV-positive patients recovered with retained strength to combat opportunistic infections. Ironically, in some of the cases, this has also led to an immense immune reaction known as “immune recovery uveitis (IRU)”. In the 2000s, IRU was believed to be the leading cause of severe vision loss in patients with HIV and CMVr.[175] Signs of prominent anterior and posterior inflammation, optic disc oedema, macular oedema, epiretinal membrane and occasionally, neovascularization can be seen. The definition of IRU is not precise. Karavellas et al defined it as vitritis of 1+ or greater severity with visually important floaters, a decrease in vision by one or more lines, or both, with or without associated papillitis and macular changes. [176] In some investigations, a mere increase in inflammatory cells from baseline was considered to constitute possible IRU. [177] Immune recovery uveitis is mostly discussed in HIV-positive patients. [177-179] However, IRU is most certainly an issue even in other predisposing states with CMVr such as inflammatory disease when immunosuppressive medication is withdrawn. [180-182] As in many manifestations of herpes virus disease, the balance act between targeting the virus and suppressing harmful inflammation remains difficult.

Other complications

Besides IRU including macular oedema and formation of epiretinal membranes, the risk of retinal detachment in CMVr was previously 24-50%, [183-186] with a reduction to 10-25% in more recent studies. [138,187,188] Treated or untreated, eyes with CMVr can progress to blindness and phthisis.

Mortality

The mortality of HIV-positive individuals with CMVr in the ART era is estimated to 22-26% per person-years. [164,189] By comparison, a 23-36% mortality rate has been reported for non-HIV patients. [180,189] In the latter group, those with HSCT have the highest mortality rate. [190] The survival rate in one study was reported to be dramatically different for HIV-positive

patients with IRU compared to those without IRU, with a 5-year survival of 63.7% and 1.4%, respectively. [164] In a cohort of HIV-positive patients, high plasma viral load of CMV at the diagnosis of CMVr was correlated with a higher mortality.[145]

ACUTE RETINAL NECROSIS

Clinical signs and diagnosis

Acute retinal necrosis (ARN) is a disease first described by Uruyama in 1971. [191] Initially, there was no suspicion of viral aetiology, there was a mere description of clinical signs. Figure 13 and 14 show the typical fundus appearance of ARN.
Subsequently, Culbertson et al identified herpes-like virions by electron microscopy in a retina from an enucleated eye. [192] The first established criteria for ARN were as follows:

(1) one or more foci of retinal necrosis with discrete borders located in the peripheral retina
(2) rapid progression in the absence of antiviral therapy
(3) circumferential spread
(4) evidence of occlusive arteriolar vasculopathy
(5) prominent inflammatory reaction in the vitreous and anterior chamber.

These criteria were formulated by the Executive Committee of the American Uveitis Society in 1994. [193] The disease is thought to be caused by the viruses of the herpes group; HSV, VZV, EBV or CMV. New diagnostic criteria were suggested in 2015 by the Japanese ARN group. Based in their clinical experience, one criterium was added to the description of clinical signs: the presence of HSV1, HSV2 or VZV in intraocular fluids by PCR, or indications of local antibody production by antibody index or Goldmann Witmer coefficient (GWC) (described in "Methods"). [194] In their investigation, none of the CMV-positive patients had a clinical picture that met these criteria for ARN. Despite this, some still consider both EBV and CMV to cause ARN. [195-197] In keeping with the results of the Japanese group, the SUN Working Group recently presented new criteria of ARN in 2021 (Table 3). [198]

Criteria
1. Necrotizing retinitis involving the peripheral retina

AND (either #2 OR #3)

2. Evidence of infection with either HSV or VZV
   a. Positive PCR for either HSV or VZV from either an aqueous or vitreous specimen

OR

3. Characteristic clinical picture
   a. Circumferential or confluent retinitis AND
   b. Retinal vascular sheathing and/or occlusion AND
   c. More than minimal vitritis[a]

Exclusions
1. Positive serology for syphilis using a treponemal test
2. Intraocular specimen PCR-positive for cytomegalovirus or *Toxoplasma gondii* (unless there is immunocompromise, morphologic evidence for >1 infection, the characteristic clinical picture of acute retinal necrosis, and the intraocular fluid specimen has a positive PCR for either HSV or VZV)

Table 3. Classification criteria for acute retinal necrosis (ARN) by the Standardization of Uveitis Nomenclature (SUN) Working Group, 2021. [a]Vitritis criterion not required in immunocompromised patients. HSV=herpes simplex virus, VZV=varicella zoster virus, PCR=polymerase chain reaction, VZV=varicella zoster virus.

These criteria were based on 252 cases of ARN and were created to be used for machine learning in uveitis. Positive PCR for either VZV or HSV in intraocular fluids was a relative criterion and most importantly, presence of CMV or toxoplasma was an exclusion criterion. Epstein Barr virus is not mentioned. Polymerase chain reaction for herpes virus DNA in intraocular fluids has been shown to have a high sensitivity in ARN, 89-100%.[196,199-203]

Acute retinal necrosis is very rare; approximately one case per 2 million is seen annually. Symptoms include vision loss and/or floaters, sometimes with periorbital pain and photophobia. As described in the criteria, necrosis and arteritis are main features of ARN. Other common clinical signs include mutton fat precipitates and increased IOP. Kyrieleis plaques have been observed, as described in CMVr.[141] Unilateral disease is most common although bilateral disease may develop and is sometimes referred to as "bilateral ARN (BARN)". Surprisingly, ARN is affecting immunocompetent individuals. There have been reports of human leukocyte antigen (HLA) types that may predispose for ARN, such as HLA-DQw7. [204]Also, in more fulminant types of ARN, HLA-DR9 has been shown to be more prevalent.[205]

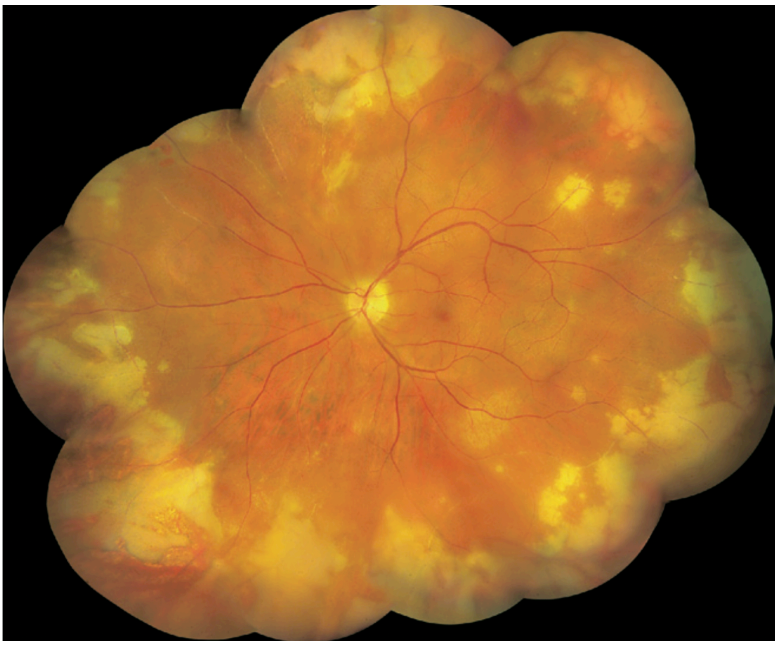

Figure 13. Wide-field fundus photo of a typical presentation of acute retinal necrosis (ARN).
Source: Aizman et al. 2007. Treatment of acute retinal necrosis syndrome with oral antiviral medications, Ophthalmology, vol 114, pp 307-312. Reprinted with permission from Elsevier.

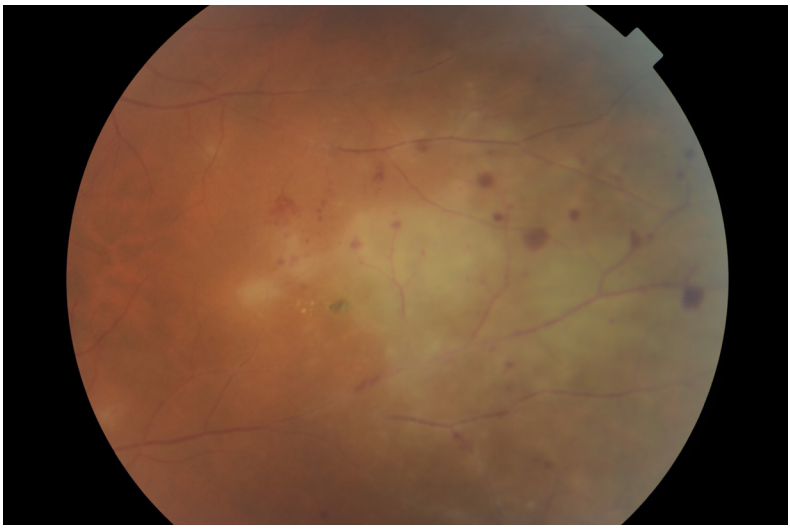

Figure 14. Photograph of peripheral necrosis, haemorrhages and arteritis in acute retinal necrosis (ARN), in this case caused by herpes simplex virus 2 (HSV2). Photograph used with permission from the patient.

Individuals with immunosuppression can develop ARN or another manifestation classified as progressive outer retinal necrosis (PORN), a subtype of ARN. The main aetiology is VZV and clinical features are similar to ARN, however, there is minimal or absent inflammatory reaction in the anterior chamber or vitreous humor. The level of CD4+ T-cells in these patients is very low. [206,207]
Histology of the retina from patients with ARN shows full layer retinal necrosis with eosinophilic intranuclear inclusions and arteritis. [192] On OCT, a disorganization of all retinal structures is seen with thinning in the resolution phase. [208]

Complications

Complications are common in ARN, even with adequate treatment. Rates of retinal detachment (RD) are between 16% and 80%, usually around 50%. [199,200,209-211] Blindness, secondary glaucoma, retinal vascular occlusion, epiretinal membrane and finally phthisis are not uncommon. [212]

Treatment

Given the rarity of the disease, no prospective randomized controlled trials have been performed to give precise recommendations on treatment. However, a high dose of antivirals is recommended systemically as soon as ARN is suspected. The mechanism of action for acyclovir is similar to that previously described for ganciclovir. It requires a series of phosphorylation steps intracellularly to provide antiviral effect. The first step is taken by the viral thymidine kinase and the remaining steps are catalysed by cellular enzymes. Acyclovir is an analogue to deoxyguanosine. When incorporated into the DNA chain in replication, it induces chain termination. Valacyclovir is a prodrug with better bioavailability and can be given orally. It has a clinically proved effect equal to intravenous treatment.[213] Plasma and

concentrations are comparable as well.[214] Adequate vitreous concentrations of acyclovir has been detected in patients with noninflamed eyes after oral treatment with Valacyclovir 1 gram, three times daily. [215]
Famciclovir is another oral guanosine analogue used in some countries and has proved to be equivalent to acyclovir.[216,217]
Foscarnet and ganciclovir are mainly used for treating CMVr, as previously described. Two case series have reported combination treatment with intravitreal injections in ARN with decreased risk of RD and visual loss. [203,218] Consequently, such adjunctive treatment was recommended in a thorough review by the American Academy of Ophthalmology (AAO) in 2017.[219] There are reasons to believe that adequate concentrations are more easily achieved with combined treatment.

The thymidine kinase of VZV is two to eight times less effective than that of HSV. [220] Despite this, ARN caused by VZV is seldomly treated with higher concentrations of acyclovir. Resistance to acyclovir is uncommon in immunocompetent individuals. As in CMV, mutations in genes for thymidine kinase or DNA polymerase, (*UL23* and *UL30,* respectively for HSV and *UL36* and *UL28* for VZV) determine resistance to acyclovir.

Given the intensity of ocular inflammation in ARN, anti-inflammatory treatment is required in most patients. Oral steroids are often prescribed 3 days after the initiation of antiviral therapy and are thereafter slowly tapered. High doses of steroids are recommended in case of engagement of the optic nerve.

Prophylactic laser photocoagulation is sometimes advocated in ARN patients to reduce the risk of RD. [200,221,222] Other investigations consider it ineffective or even potentially harmful. [223,224] Treatment with laser is performed at the edge of the peripheral necrosis with the aim of decreasing the risk of RD. The rationale behind the treatment is to provide a stronger adherence of the retina to protect against traction of the vitreous and thus preventing RD. On the other hand, laser energy could potentially induce further inflammation in an infected tissue. Moreover, patients with considerable inflammation cannot receive this treatment because of a dense vitreous. This may result in an inclusion bias as severe disease is not included. It may explain why reviews and meta-analyses have come to different conclusions on the matter whether prophylactic laser is protective against RD or not. [225,226]

Previously, prophylactic vitrectomy was frequently chosen by physicians. Early removal of the vitreous may reduce the viral load and by removing traction may also decrease the risk of RD. Lower incidence of RD has been reported, [199,223] but also higher [210] and unchanged incidence rates have also been reported. [209,227] More importantly, final best corrected visual acuity (BCVA) is not favoured by prophylactic vitrectomy; it may even further deteriorate vision.[199,210,228] The mechanism behind this may be the stress of surgery on a fragile tissue.

Prognosis

Visual prognosis depends mostly on baseline best corrected visual acuity (BCVA)[212,213,229,230] and presence of RD. [213,230,231] Most eyes deteriorate in BCVA over time.
The causal virus appears to influence visual prognosis. In investigations with a larger number of cases, VZV has been correlated with a low visual outcome and higher risk of RD, compared to HSV. [203,212,230,232] Unsurprisingly, the risk of RD depends on the extent of the

retinitis, i.e. the number of quadrants of the retina involved. [231,233] High viral load in intraocular fluids at diagnosis may correlate with prognosis as well. [234-236]

Vaccine and prophylaxis

The risk of fellow eye involvement in ARN is substantial and is one of the reasons for prolonged antiviral treatment, usually for at least 3 months after onset. Some patients require lifelong treatment with low doses of acyclovir. As in HZ, reactivation in ARN does not seem to boost immunity and prevent future reactivations. There is to date no vaccine against HSV. Two vaccine strategies are used to prevent shingles. Zostavax is a live vaccine based on the Japanese Oka-strain (clade 2). The efficacy is modest (61.1%) in individuals aged 60-69,[237] and declines significantly in older age and over time. [238] Moreover, the vaccine cannot be administered to immunocompromised individuals. Shingrix is a younger subunit vaccine based on VZV glycoprotein E. Surprisingly, this vaccine has exhibited a superior clinical response with increased humoural as well as T-cell response. Protection against shingles has been reported to be up to 98%[239] with >85% protection in individuals >80 years of age and over time (>4 years of follow-up). [240] As antibody levels have not been shown to prevent reactivation,[117] T-cell response is believed to be crucial in controlling disease. Glycoprotein E specific CD4+ and CD8+ responses have been shown to be tenfold or higher in individuals vaccinated with Shingrix compared with those vaccinated with live vaccine. [241]
Most likely, this targeted immune response explains the increased efficacy. Moreover, vaccination with a live virus may alter the immune response due to immune evasion of the virus and therefore be less effective despite providing multiple epitopes.
There are no investigations on the protection of vaccine against ocular disease caused by VZV. The risks of administering live vaccine have been discussed in a case report with a patient with ARN where deep sequencing of the vitreous revealed the sequence of the vaccine. [31] The patient had been vaccinated with live vaccine 6 weeks prior to presentation despite being immunocompromised (rheumatoid arthritis and diabetes mellitus (DM)). Surprisingly, a case of ARN, 2 months after receiving the first dose of the subunit vaccine has been reported as well in a severely immunocompromised patient. The reason for decreased protection was probably absent, or low, immune reaction to the vaccine or simply that the vaccine does not protect against ARN. In this case, wild-type virus strain was detected in aqueous humour.[242]

ROLE OF EPSTEIN BARR VIRUS IN UVEITIS

There are several unanswered questions on the role of EBV in uveitis. There is an uncertainty regarding its pathogenesis and relevance in uveitis and what type of treatment is preferred. Samples from healthy individuals are very seldom positive in analyses with modern methods (qPCR, RNA-sequencing, antibody index).[243-245] In eyes with uveitis, EBV has been detected by PCR in 1-10% of intraocular samples from uveitis patients that required investigation.[244-247] Not rarely, EBV was found in conjunction with other viruses/pathogens or another plausible reason for uveitis was established. Immunosuppressed states have been shown to predispose for the presence of EBV. [243,244]

Intravenous acyclovir or ganciclovir has been suggested as treatment of EBV in uveitis. There have been reports of absence of EBV DNA in intraocular fluids in 73% of cases treated systemically with acyclovir or ganciclovir. [245]

The significance of testing for EBV in uveitis patients has been debated and some have considered it unnecessary as the pathogenesis is unclear. [246] However, recent investigations have found an increased risk of lymphoma or leukaemia in EBV-positive eyes. [247,248] The significance of viral load of EBV in intraocular samples and pathogenesis of uveitis has not been studied extensively.

AIMS

The aim of this thesis was to shed light on two rare but serious types of herpes virus retinitis and to investigate their viral and clinical characteristics.

Specific aims of this thesis are:

Paper I – to report a case of CMVr in a patient with myelofibrosis treated with a new drug and to enlighten physicians about the risk of serious CMV-related disease.

Paper II – to investigate whether any herpes virus DNA is present in the aqueous humour of asymptomatic individuals, measured by PCR.

Paper III – to describe a cohort of ARN-patients and correlate viral loads in samples of aqueous humour to visual prognosis. Further, to investigate whether any of the EBV- or CMV-positive patients met the criteria of ARN and to compare clinical parameters in relation to virus type.

Paper IV – to report an ARN patient with unusual presenting signs. In addition, to perform deep sequencing on aqueous humour from two patients with VZV ARN and to characterize viral heterogeneity as well as phylogenetically define their viral strains.

Paper V – To describe a large cohort of CMVr patients and characterize immunosuppressive conditions promoting retinitis. Moreover, to investigate which factors correlate with delayed diagnosis of this condition.

METHODS

PATIENTS AND CLINICAL DATA

The patients described in *paper I and paper IV* were identified at the Department of Ophthalmology at Halland Hospital in Halmstad, which is the largest hospital in Halland region with a catchment population of 333,000.

In *paper II*, consecutive patients were recruited at the Department of Ophthalmology at Halland Hospital in Halmstad between March 2016 and March 2017. Patients who were planned for elective cataract surgery were invited to participate in the study at the preoperative visit. Thirty individuals were included. Active intraocular inflammation and/or treatment with systemic or topical steroids in the last 3 months were considered as exclusion criteria. We chose to include patients with cornea guttata who stated that none of their relatives had known corneal dystrophy.

In *paper III*, patients were included from Halland as well as its neighbouring region; Västra Götaland with a total population of 1.7 million. Most retinitis cases in Västra Götaland were referred to the tertiary referral clinic of the region; Sahlgrenska University Hospital, Mölndal for diagnosis and treatment. Patients were identified retrospectively by searching for aqueous or vitreous samples positive for herpes viruses in the laboratory database of the Clinical Virology unit of the Microbiology laboratory at Sahlgrenska University Hospital. Only patients who met the criteria for ARN as stated by the American Uveitis Society in 1994[193] and who exhibited positive PCR results for any herpes virus on aqueous or vitreous tap were included. Between May 2007 and December 2016, 13 patients (15 eyes) met the criteria for ARN.

The cohort studied in *paper V* consisted of patients with CMVr diagnosed in Sweden (population 10 million) between January 2008 and December 2018. Identification of these patients was possible by searching the National Patient Register [249] for the diagnosis code of *cytomegalovirus* in all ophthalmology departments in Sweden. Medical records from clinics (including private practices) treating these patients were reviewed. The following background characteristics were extracted from the medical charts: age, sex, comorbidity, laterality, time of follow-up. The clinical signs at diagnosis and treatment regimen were noted for each patient. Inclusion criteria were set to signs of retinitis with positive PCR for CMV DNA in intraocular fluids or serum. Patients with previously diagnosed CMVr, and therefore recurrent disease, were excluded. Congenital CMV or patients with exclusively anterior uveitis were excluded as well. Out of 110 individuals with a diagnosis of *Cytomegalovirus,* 63 remained for further analysis, (Figure 15).

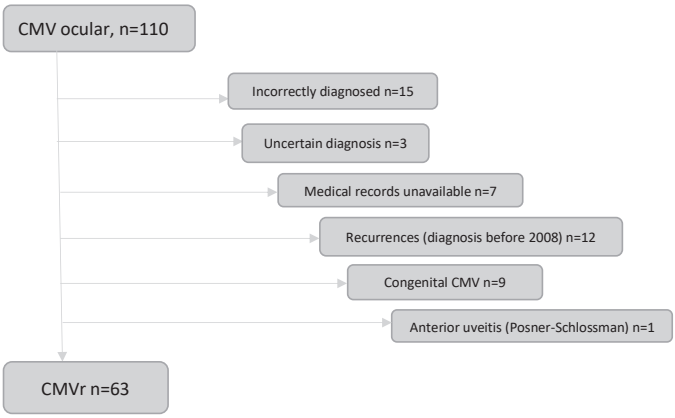

Figure 15. Flow chart of inclusion of patients in the population study on cytomegalovirus retinitis (CMVr).

METHODS OF SAMPLING

In *papers I-IV* aqueous or vitreous samples were collected under sterile conditions in surgery units at the Department of Ophthalmology at Halland Hospital in Halmstad or at Sahlgrenska University Hospital in Mölndal. An amount of 100-200 µL of aqueous humor was collected with a 30-gauge needle. In *paper III*, the vitreous samples were collected at Sahlgrenska Hospital by a vitreoretinal surgeon in conjunction with vitrectomy. These vitreous biopsies of 3 x 0.3 mL were undiluted and performed with closed infusion line.

LABORATORY METHODS

DNA EXTRACTION

Aqueous and vitreous samples were analysed in the Clinical Virology unit at Sahlgrenska University Hospital in Gothenburg. DNA was extracted from 100 µL of samples by MagNa Pure LC Robot (Roche Diagnostics, Mannheim, Germany) using the MagNa Pure DNA isolation kit according to the manufacturer´s instructions. Extraction of DNA was performed in samples subsequently analysed with qPCR or deep sequencing, as described below.

QUANTITATIVE POLYMERASE CHAIN REACTION

The first available methods of PCR were time-consuming and required multiple manual steps, which increased the risk of contamination. Sensitivity and specificity were low and the amount of DNA in a sample could not be assessed.
Quantitative PCR (qPCR) is an established method for detecting nucleic acids in a sample, with the advantage of following the amplification in real time. Moreover, the amount of nucleic acids can be determined. Reaction mixture of template DNA, oligonucleotide primers, nucleotides, buffers, a fluorescence dye and a thermo-stable polymerase enzyme (Taq polymerase) are added to the sample. The first step of the process consists of a rapid raise in temperature to 95°C to make the DNA-strands dissociate into single strand DNA. The temperature is then lowered to 58°C enabling the primers to attach to the DNA. As the temperature is increased to 72°C, the Taq polymerase has its peak activity with amplification of the primer bound DNA strands. The fluorescence of these strands is measured by a fluorometer. This cycle is repeated around 40 times and amplification can be followed in real time, thus enabling us to see the exponential curve of amplificated strands. The steeper the curve, the higher the amount of genome in the sample. To describe the amount of viral DNA, the cycle threshold (CT) value is used for the cycle where fluorescence becomes detectable and is in the exponential phase of amplification, (Figure 16). A low CT therefore means higher viral load and can be calculated to number of copies per mL. [250] Although qPCR measures the presence of DNA in a sample, the method cannot define whether there is a live virus present or not. Previously used methods, such as isolation and plaque assays (virus culture in cells) are less sensitive and required more laboratory work but have the advantage of detecting viability of the virus.
Quantitative PCR was used in all papers included in the thesis. In *papers I-IV*, all samples were analysed at the Clinical Virology unit at Sahlgrenska University Hospital in Gothenburg. An amount of 10 µL of prepared samples was used for each reaction.

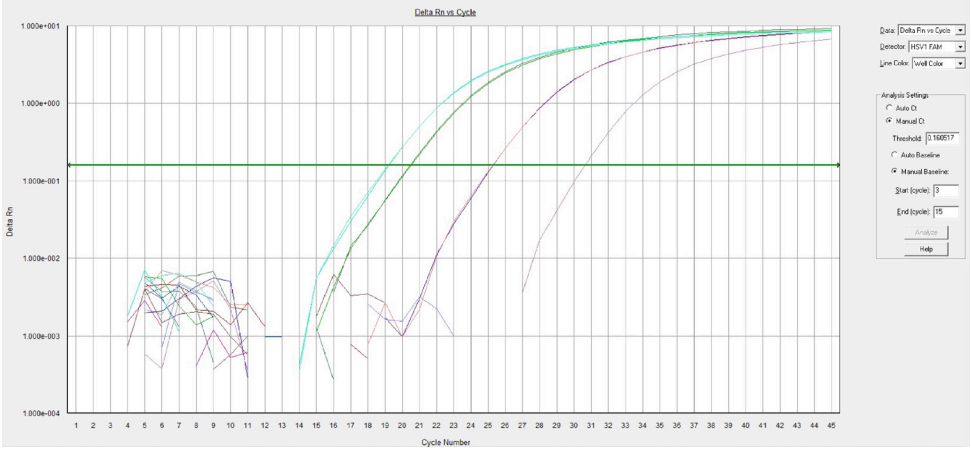

Figure 16. Cycle threshold (CT) curve of real time quantitative polymerase chain reaction (qPCR) with multiple samples, each passing the threshold of detection (green horizontal line) at different cycles (x-axis). Diagram provided by Maria Johansson at the Virology Department at Sahlgrenska University Hospital.

FLOW CYTOMETRY

Flow-cytometric immunophenotyping of lymphocytes and NK-cells was performed in *paper I.* This is an important analysis exploring the subpopulations of immune cells in peripheral blood.[251] It is regularly used in the follow-up of leukaemia and lymphoma to measure residual disease. In flow cytometry, the sample cells are separated in a small channel where each cell is enlightened by an argon-ion laser beam. The cells will refract the light commensurate to size and morphology. If monoclonal antibodies against highly specific cell surface antigens (CD markers) tagged with fluorescent dyes have been added to the samples and subsequently bound to cells, these will excite and emit fluorescent light. Back-scattered light is then measured at different angles by sensors. These data are compiled and integrated into a comprehensive picture of the cell population. In this case, AQUIOS CL flow cytometer (Beckman Coulter® Miami, FL, USA) was used with Tetra Reagents panel 1 and 2. The distribution of T-cells and NK-cells was of interest regarding immune response. CD 4+ T-cells were identified based on presence of antigen CD3 and CD4. Natural killer cells were defined by presence of CD16 and CD56. The analysis was performed at the Department of Immunology at Skåne University Hospital in Lund.

SEROLOGY

In *paper III*, serology (IgG and IgM) in patients with ARN was analysed by enzyme-linked immunosorbent assay (ELISA). This is a classic method of detecting antibodies by presenting a certain antigen and allowing binding of the corresponding antibody from a patient sample. Thereafter a new antibody against the host species, and marked with fluorescent dye, is added. This antibody binds to the sample antibody and the amount of fluorescein is measured by a sensor. In *paper III*, routine ELISA was used with antigen glycoprotein G1 for HSV1, glycoprotein G2 for HSV2 and glycoprotein E for VZV. The use of the latter is preferred as there has been cross-reactivity between HSV and VZV with previous antigens and glycoprotein E has been shown to be specific for VZV. [252]
As described in the Introduction section, the sensitivity and specificity of IgM is low.

GOLDMANN-WITMER COEFFICIENT

The method of measuring local antibody production in intraocular fluids and comparing their concentration with serum concentration is used in some centres. This method can be used instead of, or complementary to, PCR in intraocular samples. The formula for the calculation of GWC is as follows:

$$\frac{titre\ of\ aqueous\ virus\ IgG}{titre\ of\ serum\ virus\ IgG} \ x \ \frac{total\ IgG\ in\ serum}{total\ IgG\ in\ aqueous}$$

A value of >3 or 4 is considered to indicate local antibody production.
In the early 1990s, the GWC method appeared in the literature as a reliable tool to search for viral or parasite infection, especially as PCR methods had less sensitivity and specificity at the time. [253] GWC is mostly used in the Netherlands and is not available in Sweden or Denmark. The analysis requires substantial manual laboratory work compared with the

modern automated workflows in antibody detection. Another limitation is the small sample size of aqueous humour, sometimes forcing the clinician to choose only one analytic method. A recent review estimated the role of GWC to be small compared to PCR in posterior uveitis and retinitis. However, in anterior uveitis, Goldmann-Witmer coefficient is still a valuable diagnostic tool compared with PCR. [254] GWC was not used in our investigations but is presented here for completeness.

DEEP SEQUENCING AND BIOINFORMATIC ANALYSIS

Deep sequencing, next generation sequencing or massive parallel sequencing, has revolutionized the field of investigating genomes in a precise and affordable way. Sanger sequencing, which once was the only sequencing method, was time-consuming and expensive and therefore not practical in clinical work. Not only genomes of humans as well as viruses can be sequenced, moreover, deep sequencing can be used in epigenetics such as measuring gene expression by mRNA, histone modification and DNA-methylation.

In *paper IV*, aqueous humour samples from two patients with ARN were analysed by using the IonTorrent/Ion S5 system (Life Technologies, Carlsbad, CA, USA).[255,256] This platform was commercially released in 2010 and offers deep sequencing, which means that large number of reads are made for each nucleotide position. We used a protocol that can be classified as shotgun sequencing, (Figure 17). No amplification by PCR or other target enrichment was done before sequencing. The process of sequencing is initiated by building libraries where fragmentation of DNA occurs. In this case, enzymatic fragmentation was used to create fragments of a size of 200-300 bps. Adaptors bind to the DNA pieces and each of these in turn bind to a bead. With the use of Ion Chef (Thermo Fisher Scientific, Waltham, MA, USA), emulsion PCR is used to amplify the DNA on the bead to identical templates. This augments the signal in the following step, the sequencing. The beads are placed on a chip with millions of wells. Each well containing a bead is now flooded with one nucleotide at a time. If the nucleotide is incorporated to the single strand DNA, a hydrogen ion is released in the well, changing the pH. An ion-sensitive layer in the bottom of each well detects this change and records and thus a base is called. In this way, the nucleotide positions of each fragment can be read. The word “deep” in “deep sequencing” refers to how many times a specific nucleotide position is detected in this process, each time is defined as a “read”. The higher the number of reads on one nucleotide position, the deeper the sequencing and the more reliable are the results.

The enormous amount of data from a sequencing requires massive bioinformatical processing. This includes a quality check of the fragments with among others, removal of short reads and adaptor trimming. Further analyses include aligning the reads to a reference genome which in this case was done using GLC Genomic Workbench aligner (QIAGEN, Hilden, Germany). As described in the Introduction, SNPs are variations in the nucleotide sequence that vary between isolates. Single-nucleotide polymorphisms are used to classify the isolates into different clades. If the viral genome in the sample is homogenous, the SNPs are distributed with 100% identical nucleotide sequence at each position. However, if there are SNPs in one position exhibiting a different nucleotide, there is a heterogeneity in the sample. To distinguish these so-called “minority SNPs”, a thorough quality check removing sequencing errors is required to rule out sequencing errors.

Determining SNPs when data have been aligned to a reference strain is a step towards phylogenetic analysis. Phylogeny can be described as the relationship between species or as the branching pattern of ancestor-descendant relationships among species. Since the 1960´s, this has been studied by analysis of the genome. Phylogenetic trees are presentations of relationships and are bifurcating graphs with branches and nodes. The tree can have a root, which is the common ancestor of all other genetic sequences above. If the tree is unrooted, the path of evolution is not as clear and the order of different evolutionary steps is not illustrated. Phylogenetic trees can be built using several algorithms. When using maximum likelihood (ML), the probability of observing the nucleotide sequences in a given tree is calculated. The length of the branches represents the genetical distance between nodes. Bootstrapping, a statistical method for calculating a reliability value is often required. This is a way of rebuilding the tree through many iterations and the bootstrap value can be presented at the internal nodes and presents the credibility. Bayesian inference is another method, which is faster than ML and can be used with shorter algorithm runs and no bootstrapping; however, results may not be as reliable.

Changes in genes can appear by spontaneous mutations and can result in harmful, neutral or beneficial effects for the organism or virus. Natural selection will lead to maintaining genetic changes that favour fitness. Recombination, compared to spontaneous mutations, is a rapid way for a virus to change larger amounts of genetic material. At best, the virus may receive beneficial mutations and rid itself of the harmful ones. In this way, a large stride in evolution can be achieved in one step. Illustrating recombination in phylogenetic trees is more complicated. As genetic sequences from different clades are interchanged, phylogenetical trees become inadequate in presenting the genetic landscape. A phylogenetical network created by split decomposition is a better option. In contrast to ML that construct trees by optimizing parameters, split decomposition takes different or conflicting signals into account. A network created in this way will include the recombination by connecting the strains with parallel bold lines. In this material, SplitsTree (University of Tübingen, Tübingen, Germany) was used for presentation of the VZV strains.[257]

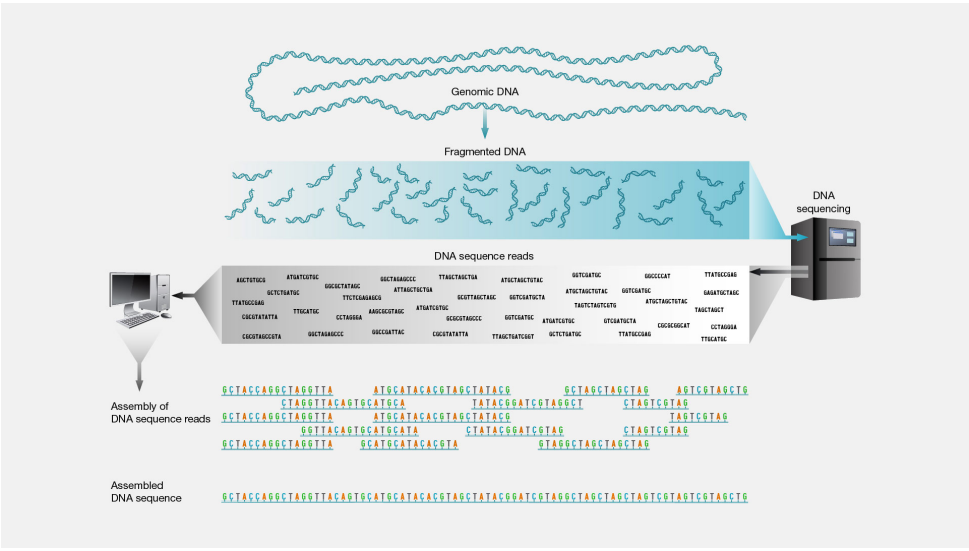

Figure 17. Shotgun sequencing. Courtesy: National Human Genome Research Institute, Bethesda, MD, USA; genome.gov.

LogMAR

Best corrected visual acuity was measured using Snellen charts and described in decimal in all papers. To enable statistical calculation, it was converted to log of the minimum angle of resolution (logMAR) using the formula LogMAR= - Log (decimal acuity).[258] For low vision, the following logMAR values were assigned; counting fingers, 2.6 logMAR; hand motion, 2.9 logMAR; light perception, 3.1 logMAR and no light perception, 3.4 logMAR, in accordance with Johnson et al. [259]

STATISTICAL ANALYSIS

For statistical analysis in *paper III* and *V*, IBM SPSS statistics (SPSS Inc., Chicago, IL, USA), version 24 (*paper III*) and 27 (*paper V*), was used. In *paper III*, parametric tests were used for continuous parameters presented with mean and standard deviation (SD) or range and significance was analyzed with Student's *t*-test. To determine differences in categorical parameters between groups, Chi-square test was used for significance testing. Viral load was presented as copies/mL as this is standard in publications in ophthalmology journals. When investigating correlations between viral load and visual prognosis or days to sampling, copies/mL was converted to log 10 for graphical presentation and calculation of Pearson´s coefficient/R. In *paper V*, parameters in early or late diagnosis were compared using Chi-square test or Fisher´s exact test. Continuous parameters were presented with mean and SD or median and range or interquartile range according to the distribution of the data. Mann-Whitney U-test was used to compare BCVA at presentation with BCVA at last follow-up, as data were skewed. However, presentation of BCVA was presented as mean and SD as well since previous studies have used parametric arithmetic procedures, despite small cohorts.

ETHICAL CONSIDERATIONS

Papers II-V were preceded by ethical application and approval. *Paper I* is a case report and ethical permission was not required in the publication process. However, the patient has approved by written informed consent. *Paper II*, with a prospective study design, required written consent from all participating patients. In the retrospective study of ARN patients in *paper III*, consent was only required if the patients had to undergo further sampling, including sequencing of host genome from serum. Three of these 13 patients were asked for consent and agreed to additional analyses being conducted. Results of these are not yet published. We obtained written consent from both patients in *paper IV*. In *paper V*, the ethical board did not require individual consent due to retrospective study design and because it was conducted on a national level.

Ethical considerations for *paper I*, *III* and *IV* include the risk that individual patients can be identified. Only 13 patients were included in *paper III* and presented by case number in tables due to the rarity of ARN. However, no sensitive personal information is revealed in the article.

In *paper II*, all participants were subject for cataract surgery by the author. As the patient is in some way in a dependency state in relation to their surgeon, this situation may put pressure on the patient not feeling comfortable to decline participation. Written information about the study was sent to the patients in advance to minimize this risk. Participation was only discussed if the patients themselves asked for it at the subsequent visit.

In *paper II*, aqueous sampling was performed in conjunction with cataract surgery. The additional risk of this procedure is very small. None of the patients had complications caused by the sampling. Complications are exceedingly rare when sampling is performed simultaneously with other surgery. [260,261]

RESULTS AND DISCUSSION

Paper II

In *paper II,* we wanted to investigate the presence, or absence of viral or parasitic DNA in the anterior chamber in patients eligible for cataract surgery and without intraocular inflammatory disease. Thirty aqueous samples were collected and in total, 180 analyses were performed. No DNA from HSV1, HSV2, CMV, EBV, VZV or *toxoplasma gondii* was found in any of the samples. Toxoplasma was included in the analysis because of its known pathogenesis and latency in intraocular inflammatory disease. Patients' mean age was 75.3 (±6.60) years. We chose to include patients with ocular comorbidities (53%) such as glaucoma (20%), AMD (10%) and diabetic retinopathy (3.3%). One patient with previously recurrent anterior uveitis and scleritis and presently quiescent was included. Five patients (17%) with cornea guttata were included, none of these patients were aware of any hereditary pattern of corneal dystrophy. We included these patients because of previous reports of CMV endotheliitis causing decompensation of the cornea. [92-94,262] Cytomegalovirus shedding is prolonged after infection, as has been seen in urine months after primary infection.[88] Therefore, we hypothesized that DNA may be present in aqueous humour even after signs of inflammation had vanished.
Asymptomatic shedding of viral particles is seen in HSV1, HSV2, CMV and EBV infections. Shedding of VZV is considered rare between clinical reactivations. Many bodily fluids seem to be involved in the shedding of virus. Herpes viruses have been detected in saliva, urine, tears and vaginal secretions. The most likely reason for shedding is the need of the virus to spread to other hosts. In a previous investigation, 50 individuals exhibited shedding of HSV1 in tears at least once in 30 days, even in those with seronegativity (26%). [51] Despite the proximity of tears and aqueous humour, intraocular fluids seem to be free from viral DNA. Most previous investigations agree on the absence of herpes DNA in the anterior chamber in patients without coincident inflammation.[243,245,263-267]

However, with intraocular inflammation there are more factors to consider. The aqueous humour is normally cell-free in healthy subjects. Inflammatory cells invade the intraocular fluids in uveitis. The type of cells and cytokines depend on the aetiology of uveitis. [268,269] As mentioned previously, 1-10% of uveitis patients investigated with PCR of intraocular samples were positive for EBV DNA. [244-247] As EBV remains latent in B-lymphocytes, an explanation may be that the higher presence of these cells in the aqueous humour may be sufficient for detection. However, EBV is seldomly diagnosed in blood samples. Another explanation may be that EBV reactivates in inflammatory or infectious disease without being the driver of inflammation. A positive test may therefore give the physician the false impression of having found the infectious cause of uveitis and thus potentially missing another treatable disease. Cases of vitreoretinal lymphoma have been diagnosed in patients with EBV positive uveitis. [244,247]

An interesting case report of an immunocompromised individual with uveitis exhibiting high levels of EBV (6.2×10^6) in vitreous humour was presented by Mashima et al. [270] This case resembled CMVr although herpes viruses other than EBV were negative by PCR. Ganciclovir and later acyclovir were given intravenously without any effect on viral load or clinical signs. Investigation of lymphoma was negative. The patient was subsequently treated with methotrexate and the viral load surprisingly decreased to zero.

Cerebrospinal fluid is very similar to aqueous humour, produced in an immune-privileged environment by filtration of plasma. For ethical reasons, it is not fair to sample healthy individuals for CSF and most investigations include patients with other neurological disease. In an investigation of 115 patients diagnosed with multiple sclerosis and optic neuritis, no virus was detected in CSF by nested PCR. [271] Later observations have revealed 5 % EBV positivity in a cohort of 167 patients with suspected bacterial CNS infection. Epstein Barr virus was detected together with other herpes viruses that were more consistent with clinical disease in two cases.[272] Apparently, there is a resemblance between these compartments in the body and investigations of either area may be of importance in understanding EBV in inflammatory disease.

Papers I and V

In *paper I*, a case of CMVr is reported in a patient with myelofibrosis treated with ruxolitinib, a drug with the aim to reduce spleen size and provide symptom relief. Characterization of lymphocytes by CD-markers in blood revealed a very low number of NK-cells; 20 cells/mm^3 (normal range 80-570 cells/mm^3). CD4+ T-cells were normal at 870 cells/mm^3 (normal range 400-1,200 cells/mm^3).

In *paper V*, 63 patients were included in the study. The patient in *paper I* is one of the participants. We used the classification of immunosuppression by Bonten et al [273] and Rothova et al[274] to identify immunocompromised states (Table 4):

Presence of one or more of the following conditions:

- Congenital immunodeficiency syndromes
- Human Immunodeficiency Virus infection with CD4+cells <300/mm^3
- Sepsis and short recovery period immediately after major surgery
- Leukemia*
- Lymphoma*
- Hodgkin's disease*
- Multiple myeloma*
- Generalized malignancy*
- Receipt of an organ or bone marrow transplant
- Concurrent use of immunosuppressive therapy, including steroids in the daily dose of above 7.5 mg for at least 4 weeks (inhaled, intraarticular and topical steroids were not considered immunosuppressive.)
- Chronic renal failure (defined as receipt of renal dialysis or transplant) or nephrotic syndrome.

Table 4. Criteria for immune deficiency or suppression as characterized by Rothova et al 2018,[274] adapted from Bonten et al 2015.[273]
**=presence defined as having been treated by or been eligible for treatment by radiotherapy and/or chemotherapy within the last 5 years.*

Predisposing factors for CMVr were having previously received HSCT (27%), haematological malignancy (24%), autoimmune disease (16%), HIV (16%) and solid transplant recipients (14%), (Table 5). Two patients did not suffer from immunosuppressive states according to the above criteria by Bonten/Rothova, but both had DM as a potential risk factor.

Subjects were divided into CMVr diagnosed within 30 days from onset of symptoms: early diagnosis (n=35) to those with >30 days of delay: late diagnosis (n=18). Ten patients had missing data of symptom onset.

Main findings in this paper include the abundance of intraocular inflammation (IOI) that was frequently observed in all patients (70%) and particularly in those with delayed diagnosis (89%) compared with early diagnosis (60%) (p=0.030). Increased IOP was also significantly more common in the late diagnosis group (p=0.023). In this group, the main reason for delay was either misinterpreting retinal signs as diabetic retinopathy or considering the diabetic patient immunocompetent (5/18). Intraocular inflammation (IOI) was considered too pronounced to be consistent with CMVr in 4 out of 18 with late diagnosis.

Patient characteristics	Early diagnosis n=35 (%)	Late diagnosis n=18 (%)	p-value
Gender: female	21 (60)	6 (33)	0.086†
Age, yrs: median (range)	47 (16-77)	69.5 (48-88)	0.001‡
Comorbidity			
HIV	7 (20)	1 (5.6)	0.240†
Haematological malignancy	8 (23)	5 (28)	0.743†
Recipient of HSCT	13 (37)	2 (11)	0.059†
Autoimmune disease	4 (11)	4 (22)	0.421†
Solid organ transplant	3 (9.6)	4 (22)	0.345†
Diabetes	0	2 (11)	0.111†
Diabetes (total)δ	6 (17)	8 (44)	0.033†
Clinical signs (either eye)			
Retinal haemorrhages	28 (80)	15 (83)	0.769†
White retinal lesions	34 (97)	17 (94)	1.000π
Intraocular inflammation*	21 (60)	16 (89)	0.030†
Intraocular pressure>21 mmHg	3 (8.6)	6 (33)	0.023†

Table 5. Comparison of patient characteristics and clinical signs in patients with cytomegalovirus retinitis diagnosed early or late after symptom onset.

**signs of vitritis, anterior chamber cells and/or keratic precipitate; δ + other type of immunosuppression*

† Chi-square test, ‡ Mann-Whitney U-test. π Fisher's exact test. HIV = human immunodeficiency virus; HSCT = haematopoietic stem cell transplantation

Medically induced immunosuppression is increasing. Novel therapies are being introduced to target numerous inflammatory diseases and, in transplanted individuals to prevent rejection. Moreover, HIV is a global burden with 38.4 millions of people living with the disease, many without access to ART.[275] In this investigation in Sweden, HSCT recipients were the most common immunosuppressed group developing CMVr. The risk of aquiring CMVr has been estimated to be between 0.12% and 2.5 % of patients with HSCT, rising to 5.6% if viraemia is detected. [143,180,276] The discrepancy in incidence may depend on choice of preemptive therapy against CMV, i.e. antiviral prophylaxis in vulnerable patients.

None of the patients with CMVr in the present research had received local immunosuppressive treatment, such as intravitreal or periocular steroids. There have been reports on immunocompetent patients developing CMVr with mere local immunosuppression.[277] In one investigation, the risk of developing CMVr after intravitreal triamcinolone injection was estimated to be 0.41%. [278]

In our material, there were two patients with no immunosuppression, both with DM. In previous case series, CMVr was observed in DM with no other immunosuppressive factors. [139,279,280] The effect of hyperglycaemia on the immune system, such as impairing phagocytosis and reducing cytokine production and NK-cell cytotoxicity is a plausible explanation for the pathogenesis. [281-283] Diabetes mellitus, alone or in combination with intravitreal steroids, as a risk factor for CMVr has been discussed in a review of CMVr in HIV-negative patients. [284] However, in Sweden's population of 10 million, there still are very few cases of CMVr in patients with DM and no other immunosuppressive diagnosis. Rothova et al found no CMVr in a cohort of 93 DM patients with uveitis.[274] Regardless, suffering from delayed diagnosis and having permanently reduced vision because retinal findings have been misinterpreted is unfortunate. Still, DM in combination with other immunosuppression may increase the risk of CMVr.

In 2016, when *paper I* was published, ruxolitinib (Jakafi/Jakavi®) was a relatively new treatment for myelofibrosis. Ruxolitinib was the first approved JAK-STAT inhibitor and inhibits both janus kinase 1 (JAK1) and 2 (JAK2). It affects haematopoiesis and inflammation. Myelofibrosis is a myeloproliferative neoplasm with ineffective clonal haematopoiesis where mutations in the JAT2 gene are present in 50% of all cases. [119] Treatment with ruxolitinib has been documented to reduce spleen size and decrease the inflammatory response. However, molecular remission and/or reduced bone marrow fibrosis is very rare. [285-287] Despite the effect on the JAK-STAT pathway with decreased viral defence, as described in the Introduction, the risks of treatment were not widely discussed at the time. However, as early as 2013, Heine et al presented data on ruxolitinib impairing dendritic cell function in vivo and in vitro, with differentiation of dendritic cells leading to impaired function of T-cell activation .[288] A later investigation showed decreased NK-cell count and function induced by ruxolitinib both in vitro and in vivo, which was correlated to increased rates of infections.[289] In *paper I*, we used flow cytometry to characterize lymphocytes and NK-cells in the patient's peripheral blood. The CD4+ T-cell count was assumed to be low, as seen in AIDS patients with CMV reactivation. However, CD4+ T-cell levels were normal. Natural killer cell levels, on the other hand, were very low. It is plausible that NK-cell levels may have been suppressed by ruxolitinib.
Subsequent review articles discuss the risk of infection when treating patients with ruxolitinib.[290,291] For example, an investigation presented odds ratio for HZ was >5 in treated individuals. [290] Janus kinase 1 and JAK2 have separate mechanisms of action. Effects on selective inhibitors of JAK2 have been investigated. Treatment with a JAK2-specific inhibitor has been shown to induce far less functional compromise of NK-cells. [292]

Papers published before 2010 were almost exclusively on CMVr in HIV-positive individuals. After 2010, papers on CMVr in non-HIV-patients started appearing. [138,143,159,180,188] Differences in clinical signs were analysed and conclusions sometimes drawn on very small cohorts.[138]
In particular, the level of IOI was presumed to be higher in non-HIV CMVr. There seems to have been a conception of absence of inflammation in CMVr in AIDS patients. However, most cases series could not confirm a more pronounced inflammation in non-HIV CMVr.[138,159,180] Moreover, an early investigation from 1997 including 337 patients with AIDS and CMVr reported IOI in 75%.[293] A recent report from the SUN Working Group, giving detailed descriptions of vitreous haze, vitreous cells, anterior chamber cells and anterior chamber flare, did not find a significant difference in any of these except that less vitreous haze was seen in AIDS than in non-HIV CMVr. That investigation was the basis for suggestion of classification criteria for CMVr. [136] These criteria included the absence of

vitritis/presence of mild vitritis. In our investigation, one reason for late diagnosis was the presence of IOI, as defined previously as presence of any keratic precipitates, aqueous cells or flare, or vitreous cells, visible on slit-lamp examination.[293] The diagnosis of CMVr was dismissed in the medical records of individual patients because of IOI. The amount of IOI depends on the immune response of the host at the time of examination. Immune recovery may occur during retinitis. All patients with CMVr have an impaired immune response, a precondition for viral entry into the retina. Therefore, we as physicians must be aware of different stages of IOI in CMVr.

Treatment with intravitreal injections for CMVr has been discussed in the literature. As the pathogenesis of CMVr includes a systemic infection, systemic treatment is a reasonable option. Treating one eye with only intravitreal injections has been associated with higher risk of fellow eye involvement and other end-organ disease. [163]
Systemic treatment with antiviral medication has also been shown to decrease mortality rates in CMVr [164,294] However, as anaemia or other bone marrow deficiencies are contraindicated in ganciclovir treatment, there may be a selection bias making systemic treatment an option for the less severely ill.
Intravitreal treatment may be the only option for CMVr patients in low-income countries. The cost of treating a patient with oral valganciclovir/intravenous ganciclovir is tenfold that of treating them with intravitreal ganciclovir (induction dose 8 injections the first month, maintenance 4/month).[295] Changes in BCVA at follow-up seem to be equal between intravitreal and systemic treatment.[182,295]

In our cohort, 21% of patients received intravitreal injections as adjuvant treatment. Eighty percent of these patients had intravitreal injections added to the treatment regimen to enhance the antiviral effect when systemic treatment had proved to be unsatisfactory. The remaining patients were converted to intravitreal injections because of bone marrow depression. Therefore, a selected subgroup with severe disease had adjuvant therapy. Despite inconclusive research results, a combination of intravitreal and systemic antiviral treatment should be considered, as suggested by Jabs in 2008. [165]

The visual outcome was presented in different ways in *paper V*. The BCVA data were not normally distributed; hence, non-parametric statistical procedures were chosen to provide a fair description of visual change. However, both mean and median values are presented in the paper as previous publications have used this method in even smaller cohorts. Median BCVA at diagnosis was worse in the late diagnosis group (p=0.012). In both groups, median BCVA was unchanged between diagnosis and last follow-up. On an individual level, 50% of patients improved after treatment. Our results are in keeping with other investigations.[182,188,295-297]
Comparing visual outcomes is complicated as the cohorts are small and participants have serious comorbidities. Different follow-up times may also influence results as BCVA tend to worsen over time, even after 12 months. [188,298]

Papers III and IV

Both patients in *paper IV* were included in paper III. Between 2007 and 2016, 13 patients (15 eyes) in the regions of Halland and Västra Götaland met the criteria for ARN and had herpes virus DNA in intraocular samples, six with VZV, four with HSV1 and three with HSV2. The medical records of patients with positive PCR for EBV (n=1) and CMV (n=12) and combined EBV+CMV (n=1) were reviewed as well and none of these met the criteria for ARN.

Acute retinal necrosis was diagnosed according to certain defined criteria of clinical signs based on the knowledge that was available in the 1990´s. [193] Attempts to update the criteria were made by Takase et al in 2015. [194] These authors emphasize that HSV and VZV are the pathogenic viruses responsible for the clinical signs described as ARN. Also, cases with negative test results on PCR or local antibody production against HSV or VZV were considered to be "virus-unconfirmed ARN". Among the 409 controls with other uveitis types, 32 cases were CMVr and none of them resembled the clinical picture of ARN, just as in our investigation. Recently, the SUN Working Group has further updated the classification criteria, likewise emphasizing HSV and VZV as pathogens. [198] In my opinion, retinal necrosis caused by another pathogen, such as EBV, CMV or even toxoplasma, can resemble ARN but should not be defined as such. As acyclovir is the mainstay treatment for ARN, this treatment of CMV in a clinical manifestation resembling ARN would be insufficient, and acyclovir treatment of toxoplasma would have no effect. As the laboratory methods for detecting viral DNA are steadily improving, the need for criteria for diagnosing viral disease is decreasing. The rate of positive PCR on intraocular fluids in patients with ARN is approaching 100%.[196,200,210] I suggest an alternative name for ARN such as "alpha herpes virus retinitis". This name would then refer to the causative viruses. Moreover, the "acute" part of "acute retinal necrosis" is very relative. In some patients, the clinical course is rapid; in others, a very slow progression is observed.

When comparing patient characteristics between HSV and VZV, patients with VZV ARN were older than patients with HSV ARN. In the published article, statistical calculations with Student's t-test reached significance (p=0.049), while a more correctly chosen nonparametric method of Mann-Whitney U-test did not (p=0.101). The median age of patients with HSV and VZV was 43 and 57 years, respectively. These results are in keeping with previously reported average age of acquiring VZV ARN, between 50 and 60 years of age. [196,203,213,230] The age difference may be explained by VZV reactivation in immunosenescence.

Visual prognosis was not significantly different between HSV and VZV ARN. Borderline significance (p=0.074) was reached for BCVA at diagnosis with a lower BCVA in VZV. The prognosis of ARN relative to virus type has been discussed previously. In three larger case series, VZV has been shown to have a worse visual outcome and increased risk of RD. [203,212,230] This difference may be due to virulence of the virus, or to the treatment regime. As mentioned in the Introduction, in VZV the thymidine kinase that activates acyclovir is not as efficient as it is in HSV and therefore VZV infection is often treated with higher doses. As ARN is mostly treated with equally high doses regardless of pathogen, insufficient dosing of medicine may occur depending on virus type. Intravenous acyclovir in a dose of 10 mg/kg is equivalent to approximately 2 grams of valaciclovir, [214] which is recommended three times daily in the initial treatment of ARN. Fifteen mg/kg has been proposed as a short induction therapy in cases suspected to be caused by VZV. Even higher doses have been suggested recently, of up to 10 grams of oral valacyclovir a day.[299] However, the risk of such high doses must be taken into account, especially in longer treatment durations. Clinical efficiency is not

necessarily correlated with higher doses. [300] Unfortunately, higher doses of acyclovir increase the risk of nephrotoxicity and also the risk of acyclovir-induced neuropsychiatric symptoms. [301]

Fifty percent of the HSV1 ARN patients in this investigation previously had had herpes simplex encephalitis in childhood. Cases of ARN during encephalitis or decades later have been reported.[196,302,303] Developmentally delayed patients with previous neonatal HSV1 encephalitis are at risk of having their diagnosis delayed because of difficulties in verbalization of their ocular symptoms or difficulties in cooperating at slit-lamp examination.[304] This was the case for one patient in this investigation who advanced to bilateral ARN before a diagnosis was found.

In 23% (3/13) of patients, DNAemia was present within 5-12 days after symptom onset. The viral loads in serum were close to detection level and simultaneously sampled intraocular fluids were very high (10^7-10^8), suggesting spill over from the small compartment of the eye.

Viral loads of vitreous and aqueous humour samples were considered equal in this investigation. There was no statistical difference in viral load between the fluids (p=0.683) in this material, despite the kinetic differences of production and clearance as described in the Introduction. Previous papers report around a tenfold higher viral load in vitreous compared with aqueous humour taken simultaneously. [234,305] Figure 18 shows no correlation between low BCVA at follow-up and viral load in the intraocular fluid.

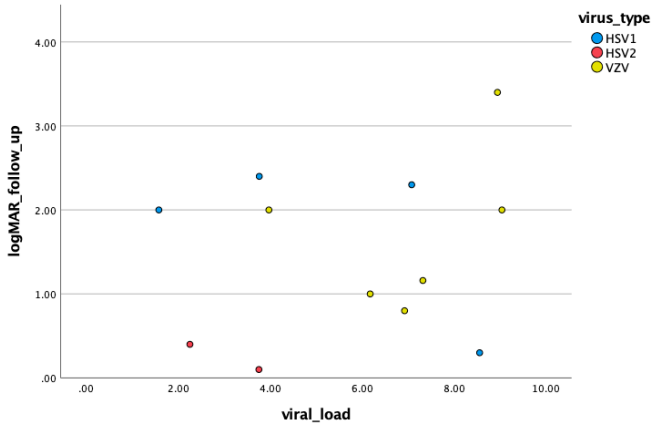

Figure 18. No correlation was found between viral load and final visual acuity. Pearson´s R=0.062, R^2=0.004; p=0.857.

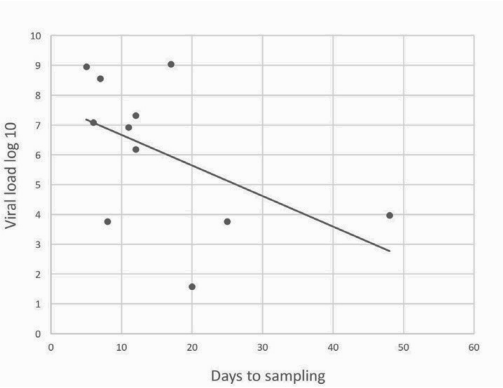

Figure 19. Scatter plot of time to sampling (days) by viral load (log 10). Pearson´s R=-0.512, R^2=0.262; p=0.107. Used with permission from BMJ Open Ophthalmology.

However, there was a tendency of higher viral loads in samples taken earlier in the disease process as shown by regression analysis (Pearson´s R=-0.512, R^2=0.262) (Figure 19). This finding has recently been confirmed in ARN (in an investigation including CMVr).[306] The timing of sampling is of importance as viral loads typically are high at the beginning of an infection and decline after immune response. Not many investigations consider when in the infectious process the sample is taken. In a paper with the same number of patients as in our investigation, all samples were taken within 5 days after onset of symptoms. Its authors found a correlation between high viral load and RD, as well as worse visual prognosis and extent of retinitis. [235] Perhaps viral load in intraocular fluids can correlate to extent of disease, as in VZV anterior uveitis where high viral load was associated with pronounced iris atrophy. [307] However, this information may not be of great interest in the clinical setting if it merely mirrors the clinical signs that are obvious to the examining physician. Viral loads have also been suggested to determine the length of treatment. Repeated measurements of aqueous and vitreous humour during treatment have shown a very slow decline in viral DNA. [308,309] Some have drawn the conclusion that this implies active infection and motivates prolonged treatment. [308] However, the disadvantage of PCR is that it does not measure live virus; it measures DNA fragments and therefore is not a valuable tool to monitor infection.

In *paper IV*, an ARN case exhibiting atypical presenting signs is presented and the genomes of two VZV isolates in ARN patients are sequenced. The patient exhibited no inflammatory signs but had a marked increase in IOP at the first visit. At follow-up, the patient developed an intense inflammatory state and subsequently met the criteria for ARN. An aqueous sample was taken 17 days from symptom onset and a very high viral load of VZV was detected by PCR: 1.1 x 10^9 copies/mL. Reports of deep sequencing of VZV in aqueous humour are very rare with one previous report published. [31] Shotgun sequencing by the Ion Torrent/Ion S5 system was performed on raw material in aqueous humour without previous enrichment or amplification. Libraries of 10,000,000 reads were mapped to a reference genome from GenBank (Dumas strain; GenBank accession No. NC_0011348.1). In total, 68.576 reads aligned to VZV. The complete genome of VZV was covered with an average depth of 98.91 reads and a maximum of 235 reads. A second aqueous sample from a patient with VZV ARN with classical signs was analysed as well. This sample had a lower, albeit still high viral load of 8.4 x 10^6 copies/mL. Altogether 4,815 reads were aligned to VZV and 98% of the total viral genome was covered. Average coverage depth was 6.28 and maximum depth, 244 reads.

Phylogenetical analysis revealed that both isolates were of commonly observed clades in Europe and had low genetical heterogeneity, with fewer than ten SNPs (Figure 20). Further analysis determining whether the heterogeneous within-host population could be explained either by spontaneous mutations occurring after infection or by multiple infections with different strains was performed. We concluded that SNPs were the results of spontaneous mutations after primary infection and recombination events were unlikely.

In this investigation, sequenced VZV genome from the aqueous humour of two patients with ARN did not exhibit genomic heterogeneity. Low variability may be due to the presumed reactivation from a single adjacent ganglion and a bottle-neck effect in the passage to the retina. Moreover, low variability in combination with absent or very low levels of viral DNA in serum makes an alternative haematogenous spread of virus prior to ARN unlikely. Isolates clustered in clades that are commonly seen in Europe. This suggests that virus characteristics may not influence type or severity of disease.

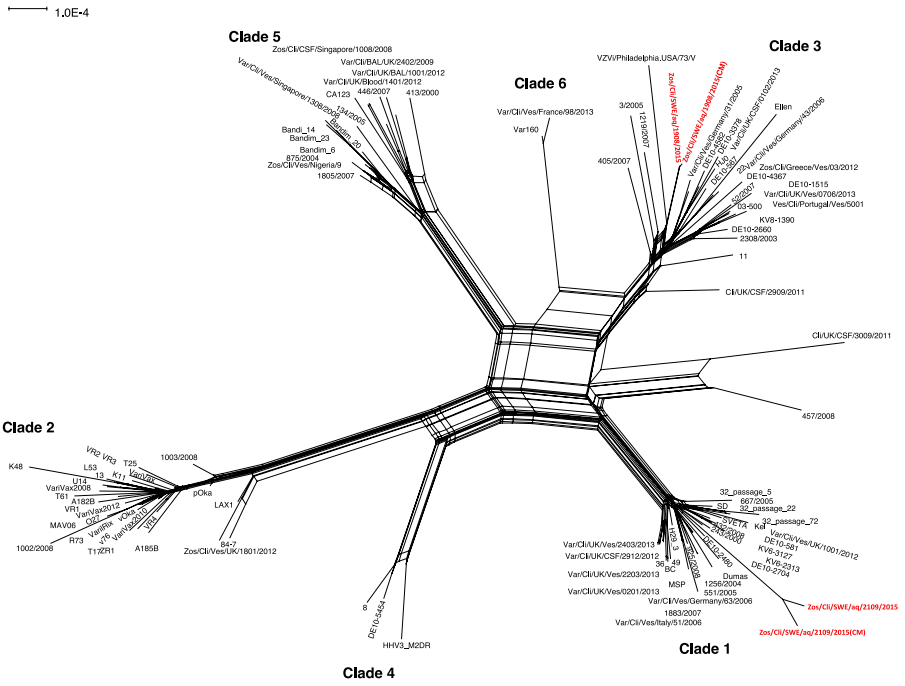

Figure 20. Phylogenetic network of varicella zoster virus (VZV). The two individual sequences from aqueous humour with adjacent minority consensus strains are marked in red. By Peter Norberg. Used with permission from Open Forum Infectious Diseases.

STRENGTHS AND LIMITATIONS

Studies of rare conditions will inevitabily be flawed by insufficient data which is an overall limitation of this presented research. In *paper III*, only 13 ARN patients were identified in the regions of Halland and Västra Götaland over a span of almost 10 years. Needless to say, most case series with ARN patients are small and conclusions are sometimes drawn that are not backed by statistical significance. In *paper V*, an attempt was made to gather a larger cohort for the study of CMVr by including patients from a population of 10 million over an one-year period. There were 63 patients in total; still, subgroups were not large enough to generate reliable statistical results. In many publications on ARN and CMVr, parametric calculations are being made despite small cohorts. *Paper III* is unfortunately no exception and nonparametric calculations would have been more appropriate. Significance is found in age differences between patients with ARN depending on viral etiology. Using nonparametric calculations with Mann-Whitney U-test, the p-value increases to non-significance (i.e., $p<0.05$). However, previous investigations have reached statistical significance in age difference using correct statistical calculations and some with larger cohorts.
In *paper V*, nonparametric calculations are being used more generally. Unfortunately, visual acuity at diagnosis and at last follow-up is compared using Mann-Whitney U-test, instead of the correct Wilcoxon signed ranks test suitable for nonparametric calculations of paired samples. When Wilcoxon is used, the *p*-value remains nonsignificant, but closer to significance when comparing all subjects.

The retrospective nature of *papers III* and *V* is a limiting factor as well, which obviously is also a consequence of low incidence of the studied conditions. The SUN classification of uveitis, which is a tool based on objective grading of clinical findings, was not used in any of the medical records. Moreover, the zones of the retina, proposed by Holland et al [310] were not used to describe the extent of retinitis and therefore the extent could not be specified.

In *paper IV*, Ion Torrent sequencing was performed on aqueous humour. No previous amplification by PCR or other enrichment was done which is an advantage as these manipulations of a sample can influence the sequencing results. This unbiased routine is not generally employed in ocular samples. As different types of sequencing and bioinformatics are used in investigations, this may be a limitation when comparing results. For example, the depth of sequencing in each position varies and SNPs may be easier to find in sequences with greater depths.

Nevertheless, despite limitations, *paper V* includes a unique cohort of patients on a national basis. Because of the possibility to search for codes of diagnosis in the National Patient Registry in Sweden, most patients with CMVr could be identified. Analyses performed at the Virology Department in Gothenburg were of high quality and state of the art in all papers.

CONCLUSIONS

Herpes virus DNA is shed in body fluids secreted externally to enable transmission. In the presented research, intraocular fluids, in this case aqueous humour sampled from asymptomatic individuals were free from herpes virus DNA. This makes frequent shedding from aqueous humour unlikely.

Acute retinal necrosis is caused by HSV1, HSV2 or VZV, according to our investigation. Patients with EBV or CMV in intraocular fluids are not likely to meet the criteria for ARN. The explanation may lie in the differences in viral tropism. Further definitions of ARN are needed with respect to causative virus. I agree with the latest criteria proposed by the SUN Working Group. Viral load in aqueous humour is not correlated to visual prognosis. Time from symptom onset to sampling may influence the viral load.

Deep sequencing can successfully be performed on aqueous humour even without previous enrichment or amplification by PCR. Genetic heterogeneity in ocular fluids in ARN is similar to what has been seen in vesicle fluid in shingles in contrast to CSF in encephalitis. In other words, subpopulations of virus seem to be rare in aqueous humour samples from VZV ARN.

There are several immunosuppressive states predisposing for CMVr in Sweden, and of these, HSCT is the most common. In patients with a delayed diagnosis of > 30 days, old age, presence of IOI and increased IOP are more common. Diabetes mellitus may be a risk factor for CMVr and diabetic retinopathy may also be mistaken for CMVr.

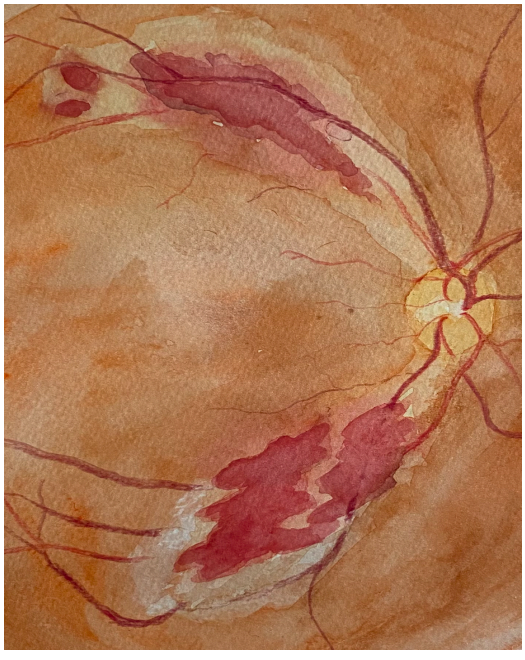

Figure 21. Cytomegalovirus retinitis (CMVr) with necrosis and haemorrhages adjacent to the retinal vessels in the fundus. Watercolour illustration by J von Hofsten.

FUTURE PERSPECTIVES

In this thesis, I investigated the presence of herpes virus DNA in aqueous humour in healthy subjects, performed in 2016. With this and similar studies before and after this investigation, the absence of herpes virus DNA in healthy subjects can be confirmed. However, presence of herpes virus DNA in patients with uveitis is not clear regarding EBV. In the last couple of years, the pathogenic role of EBV in uveitis has been discussed. Does EBV cause uveitis? Is EBV a bystander in IOI caused by other pathogens? Can EBV be detected in noninfectious uveitis? And, most importantly, does EBV indicate malignant disease, such as lymphoma or leukaemia? Future investigations may give answers to these questions. Multicentre studies would be preferable to include many participants. Viral load is mentioned in a few case series and may not be correlated with pathogenesis, but larger investigations are needed. Epstein Barr virus positivity is often seen in samples with simultaneous infection with other viruses or organisms. It is seen in idiopathic uveitis, where it is assumed to be caused by autoinflammatory or autoimmune states. Still, are there pathogens that we do not search for in our analyses? Deep sequencing is a valuable tool in these cases. Metagenomic protocols may help us to discover other aetiologies in uveitis. In a paper from 2021, Doan et al reported that RNA sequencing could be equally sensitive to PCR. Moreover, infectious agents in addition to those suspected by the physician could be detected, for example fungal endophthalmitis in a patient with idiopathic uveitis. [311] Enterovirus is a pathogen in CNS disease and has been suggested to cause uveitis. [312] Not only may sequencing reveal presence of virus but may also detect mutations in host genes characteristic of vitreoretinal lymphoma. [313] Routine implementation of deep sequencing will improve the diagnostic workup in many ophthalmologic conditions. Lower reagent and sequencing cost in combination with standardizations of bioinformatic workflows may enable clinicians to use this method in daily practice.

There are still no answers to why some individuals develop ARN. There is an ongoing project with Professor Trine Mogensen at the Department of Infectious Diseases Aarhus University Hospital, in Aarhus, Denmark, investigating host genetics (using exome sequencing) and studying fibroblast gene expression in VZV infection based on two of the VZV ARN patients in *paper III* and ARN patients from Copenhagen. These results have not yet been published. Their research group have previously reported on serious VZV infection of the CNS in three children with rare missense mutations in genes encoding RNA polymerase III, a DNA sensor in the innate immune response. Reduced IFN induction was observed in vitro in peripheral blood mononuclear cells on infection with VZV. [314]

We agree on the latest update on the definition of ARN by the SUN group and recommend further focus on alpha herpes virus as pathogens for ARN. As I previously suggested, an alternative term of “alpha herpes virus retinitis” would be more descriptive of the causative virus.

In ARN and CMVr, there is a need for developing guidelines for treatment with recommendation of intravitreal and intravenous/oral therapy. At least in CMVr, international randomized studies may be possible to convey, comparing combined intravitreal and systemic antivirals with systemic treatment only.

There are several aspects of CMVr that are not entirely clear. According to the existing literature and the classification criteria by the SUN Working Group, indolent or granular retinitis is a manifestation of CMVr. Haemotogenic spread transmitting the virus to retinal

vessels, and further to the retina by breached BRB is a plausible explanation for the haemorrhagic or fulminant form of retinitis adjacent to these vessels. By contrast, the granular form is located mostly in the periphery away from the retinal vessles and retinal haemorrhages are sparse. An alternative pathogenesis for the characteristic appearance of granular retinitis may involve spread from choroidal vessles. By studying the structural differences between these manifestations, we may come closer to understand the mechanisms of viral spread in CMVr.

Furthermore, the effect of Shingrix in preventing ocular disease caused by VZV is not known. Beyond ARN, there are other, more frequently observed manifestations of VZV, such as recurrent stromal keratitis and uveitis, which may be affected by a vaccine that may alter the host's immune response to VZV. A multi-centre investigation of Shingrix in patients with recurrent ocular VZV disease would be valuable.

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