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HUMAN CYTOMEGALOVIRUS IN CONGENITAL INFECTIONS AND EMBRYONAL MALIGNANCIES OF THE NERVOUS SYSTEM

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To you, the wind beneath my wings

ABSTRACT

Cytomegalovirus (CMV) is a herpesvirus that may cause severe or even fatal disease in immunocompromised patients as well as severe developmental disturbances in foetuses infected in utero. In contrast, only mild symptoms are generally observed in immunocompetent individuals. Virus infections are very common in and the global prevalence is 70-90%. After a primary infection, the virus establishes latency, from which it can be reactivated by stress, inflammation or concomitant diseases. Most likely, a reactivated infection is controlled by a functioning immune response. However, due to the virus' sophisticated strategies, the infection will never be cleared but remain in the host throughout life.

In the western world, 0.15-2.0% of all live births are congenitally infected by CMV. The virus can transfer across placenta at any time during pregnancy and cause a wide range of symptoms from foetal death to mild concentration disorders. Clinical observations and data from studies *in vitro* and in animals suggest that neural stem and progenitor cells (NPCs) are vulnerable targets for CMV infection and that it is the damage in these cells that cause the developmental disorders of the brain. In paper I and II, we demonstrate that NPCs were readily infected by CMV and expressed both immediate early (IE) and late (L) CMV proteins. Interestingly, infected NPCs were unable to differentiate into neuronal or astrocytic lineage cells. Moreover, CMV decreased proliferation and induced apoptosis in infected cells. Our data points to that the clinical findings of microcephaly and disturbed migration in a CMV infected brain may be caused by cell loss due to infection of NPCs.

CMV has ways of manipulating its' host cell into the perfect viral residence. Many of these strategies are also causing the host cell to increase proliferation, change migration patterns, counteract apoptotic signals and more. This makes CMV a good candidate for oncogenicity and in the 70's it was proposed to be an oncogenic virus. In recent years new evidence of CMV as a player in tumour development has come forth. CMV proteins and nucleic acids are found in tumour tissue and CMV infected cancer cells are reported to grow more aggressively than uninfected cells. In paper III and IV we show that CMV proteins and DNA are found in 97% and 87% of neuroblastoma and medulloblastoma tumours, respectively. These tumours are reported to express high levels of cyclooxygenase-2 (COX-2) and treatment by COX-2 inhibitors have resulted in significantly reduced tumour growth *in vivo* and *in vitro*. CMV also causes up-regulation of COX-2 expression with subsequent high levels of the end product, prostaglandin E_2 (PGE_2). We confirmed that both neuroblastoma and medulloblastoma cells increase COX-2 levels *in vitro* in response to infection, most likely due to IE or early gene expression. Therefore, we investigated whether treatment with antiviral drug ganciclovir and COX-2 inhibitor celecoxib inhibited tumour growth *in vitro* and *in vivo*. We found that when combining the two drugs, tumour growth was decreased significantly both *in vivo* and *in vitro* in both tumour types. Our observations may lead to more effective treatments against severe paediatric malignancies.

POPULÄRVETENSKAPLIG SAMMANFATTNING

CMV är ett herpesvirus som infekterar större delen av jordens befolkning, 70-90% beräknas bära på viruset. Har man en gång smittats av CMV, stannar viruset kvar i kroppens celler där det etablerar latens och kan reaktiveras då och då på grund av stress eller sjukdom. Hos en individ med ett normalt immunförsvar ger en CMV-infektion vanligen mycket milda symptom, men en del upplever symptom som påminner om körtelfeber. För en person med rubbat immunförsvar, såsom en AIDS-patient eller en patient som genomgått en transplantation, kan infektionen bli dödlig. Dessa personer kan drabbas av lunginflammation, ögoninfektion och inflammation i hjärnan. Om en kvinna smittas eller reaktiverar sin CMV-infektion under graviditeten kan viruset ta sig genom moderkakan och infektera barnet. Detta kan i sin tur leda till att barnet drabbas av allvarliga utvecklingsstörningar, nedsatt hörsel, synproblem, koncentrationssvårigheter och kan i värsta fall leda till döden. De här problemen uppstår till följd av att CMV orsakar stora skador på flera organ, framförallt hjärnan. Fostrets hjärna utvecklas inte ordentligt efter infektion, den blir mindre än normalt och inte korrekt uppbyggd. Vi tror att hjärnans dåliga utveckling beror på att cellerna inte mognar och utvecklas som de ska. Vi undersökte hur de omogna cellerna, hjärnans stamceller, påverkas efter en infektion *in vitro* och vi observerade att hjärnstamcellerna är mycket mottagliga för CMV-infektion. Viruset gör att stamcellerna inte kan mogna ut till olika celltyper i hjärnan, som nervceller och supportceller (astrocyter). Vi upptäckte också att både hjärnstamcellerna och de mer mogna celltyperna inte kan dela sig korrekt och många av dem dör i inducerad celldöd, apoptos. Dessa fynd gjorde vi i humana celler odlade i laboratoriet. Detta betyder att, även om vi kan tänka oss att mekanismen är densamma i själva hjärnan, så krävs fler studier för att säkerställa att så är fallet. Mina experiment grundar sig på en isolerad celltyp under artificiella förhållanden och det är möjligt att när cellerna sitter i sin naturliga miljö, med andra celler runt omkring som påverkar dem, så blir händelseförloppet inte detsamma. Vad vi är säkra på är att CMV har förmågan att hämma cellernas mognad, hämma deras celldelning och inducera celldöd.

Jag har också undersökt CMVs roll i uppkomst och utveckling av tumörer. Det finns virus som kan ge upphov till tumörer, Epstein Barr-virus kan ge lymfom och Pappilloma-virus kan ge livmoderhalscancer, men CMV räknas (än så länge) inte till den gruppen. Det finns en forskningsrapport från 70-talet där en forskargrupp lyckades göra tumörceller av vanliga celler genom att infektera dem med CMV, men detta har inte kunnat upprepas. De senaste tio åren har forskare hittat proteiner och gener från CMV i tumörceller och det har lyft frågan om CMVs roll i cancer upp på dagordningen igen. Vi har undersökt om CMV förekommer i två typer av nervsystemstumörer som drabbar barn, medulloblastom och neuroblastom. Medulloblastom är en hjärntumör och neurblastom sitter i det perifera nervsystemet. Gemensamt för dem båda är att de kan uppkomma mycket tidigt, neuroblastoma till om med innan födseln, och att de i sina mest aggressiva former är mycket svårbehandlade. Vi undersökte vävnad från tumörer och hittade CMV i över 85% av

fallen. Vi undersökte vidare hur en aktiv CMV-infektion påverkar cellernas tillväxt och fann att infekterade celler oftast växer snabbare och mer aggressivt än oinfekterade. Vi kunde också observera att de infekterade cellerna har ett högre uttryck av ett inflammatoriskt enzym, cyklooxygenas-2 (COX-2) som bildar prostaglandin E_2 (PGE_2). PGE_2 stimulerar celler till att växa och bidrar också till nybildningen av blodkärl, vilket är en mycket viktigt del i utvecklingen av en tumör. Det fanns också kliniska observationer och djurstudier som pekade på att vanliga antiinflammatoriska droger som hämmar COX-2 kunde bidra till en bättre utveckling för patienter med neuroblastoma och medulloblastom. Vår hypotes är att CMV i tumörcellerna gör dem mer aggressiva och att vi skulle kunna förbättra behandlingen av dessa sjukdomar genom att lägga till en antiviral drog mot CMV. Vi testade detta *in vitro* och såg att den antivirala drogen ganciklovir tillsammans med COX-2-hämmaren celecoxib verkligen kunde minska tumörcellernas tillväxt, särskilt i infekterade celler. Genom djurstudier slog vi fast att dessa droger i kombination ger en god tumörhämmande effekt. Våra resultat kan vara början till ett stort genombrott i kampen mot medulloblastom och neuroblastom och med vidare forskning kan vi kanske förbättra nuvarande behandlingstekniker och öka överlevnad såväl som livskvalitet för barn med cancer i nervsystemet.

LIST OF PUBLICATIONS

I. **Late human cytomegalovirus (HCMV) proteins inhibit differentiation of human neural precursor cells into astrocytes.**

Jenny Odeberg*, Nina Wolmer*, Scott Falci, Magnus Westgren, Erik Sundström, Åke Seiger och Cecilia Söderberg-Naucler

(*Authors contributed equally to the manuscript)

II. **Human cytomegalovirus inhibits neuronal differentiation and induces apoptosis in human neural precursor cells.**

Jenny Odeberg, Nina Wolmer, Scott Falci, Magnus Westgren, Åke Seiger och Cecilia Söderberg-Naucler.

III. **Human Cytomegalovirus infection in Neuroblastoma patients opens up for new treatment options using targeted therapies.**

Nina Wolmer-Solberg*, Ninib Baryawno*, Dieter Fuchs, Lonneke Verboon, Lova Segerström, John-Inge Jonssen, Baldur Sveinbjörnsson, Afsar Rahbar, Cecilia Söderberg-Nauclér* and Per Kogner*

(*Authors contributed equally to the manuscript and share primary and senior authorship, respectively.)

IV. **High prevalence of HCMV in medulloblastoma; reduced tumor growth using valganciclovir and celecoxib.**

Baryawno, N.*, Wolmer-Solberg, N.* Rahbar, A., Sveinbjörnssen B., Fuskevåg, O-M, Kogner, P., Johnsen, JI.*, and Söderberg-Nauclér, C.*

(*Authors contributed equally to the manuscript and share primary and senior authorship, respectively.)

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LIST OF ABBREVIATIONS

APC	Antigen Presenting Cell
APC	Adenomatosis polyposis coli gene
BDNF	Brain Derived Neurotrophic Factor
CDK	Cyclin dependent kinase
CMV	Cytomegalovirus
CNS	Central Nervous System
COX-2	Cyclooxygenase 2
cpe	cytopathic effect
CT	Computed tomography
DC	Dendritic cell
dpi	days post infection
E	Early (CMV genes)
EBV	Epstein Barr-virus
EGF(R)	Epidermal Growth Factor (Receptor)
FGF	Fibroblast growth factor
GCV	Ganciclovir
HBV	Hepatitis B virus
HCMV	Human Cytomegalovirus
HCV	Hepatitis C virus
Hh	Hedgehog
HHV	Human Herpesvirus
hpi	hours post infection
HPV	Human papilloma virus
HSV	Herpes Simplex Virus
IE	Immediate Early (CMV genes)
L	Late (CMV genes)
MAPK	Mitogen activated protein kinase
MCMV	Murine Cytomegalovirus
MHC	Major histocompatibility complex
MIEP	Major Immediate Early Promoter
MOI	Multiplicity of infection
MRI	Magnetic Resonance Imaging

NGF	Nerve growth factor
NK cell	Natural Killer cell
NPC	Neural Progenitor Cells
PDGF(R)	Platelet Derived Growth Factor (Receptor)
PI3kinase	Phosphoinositide 3-kinase
PNET	Primitive Neuroectodermal Tumour
RhCMV	Rhesus Cytomegalovirus
STD	Sexually transmitted disease
TNF	Tumor necrosis factor
Trk	Tropomyosin receptor kinase
UL	Unique Long (CMV gene nomenclature)
US	Unique short (CMV gene nomenclature)
VEGF(R)	Vascular Endothelial Growth Factor (VEGFR)
VZV	Varicella Zoster Virus

AIMS

CMV can cause severe disturbances in the developing nervous system. For every 1000 live births in Sweden, at least 2 will be born with congenital CMV infection, and it may cause anencephaly, microcephaly and migration disorders. It has been suggested that the virus targets the stem cell rich areas of the brain and that clinical findings are due to infections of the cells residing there. These immature cells of the brain and nervous system are also the source of tumour cells in embryonal malignancies neuroblastoma and medulloblastoma. The overall goal of my thesis work has been to investigate the role of CMV infection in the foetal nervous system during congenital infection and in childhood neural malignancies. To achieve this overall goal, I set up the following specific aims:

- To investigate the possible cellular mechanisms behind clinical findings of congenital infection, such as cell loss and migration disturbances. I used an *in vitro* system based on human foetal neural progenitor cells. I differentiated the cells into neurons or astrocytes and investigated the impact of CMV infection on these processes (**paper I and II**).

- To investigate whether CMV proteins and/or nucleic acids can be found in embryonal malignancies medulloblastoma and neuroblastoma. If present, to further investigate if antiviral treatment is affective to prevent tumour growth *in vivo* and *in vitro* (**paper III and IV**).

INTRODUCTION

During a meeting in Bonn in 1881 Dr Hugo Ribbert, a Swiss pathologist of the Zürich UniversitätsSpital, reported on findings in a still born child with syphilis like symptoms. He had observed abnormal enlarged cells displaying intranuclear inclusions in the kidney, what we now recognize as cytopathological observations relating to CMV infection. Later on, in 1904 Jesionek reported on similar observations when he, in a premature foetus also with syphilis like symptoms, found these abnormal cells in the liver, kidneys and lung. He was searching for the agent behind complex multisystem failures involving several different organs affecting infants. At this time, these findings were believed to be the result of a protozoan infection. Ribbert, convinced that Jesionek had the accurate explanation, reinterpreted his own findings from 1881 and agreed on the hypothesis of protozoan infection. After Jesionek, others reported on enlarged cells and intra nuclear inclusions especially in the salivary glands but also in lungs, adenoids, kidneys and liver in infants and older children. The reports suggested similarities between these observations and the signs of herpesvirus infections. Goodpasture and Talbot initiated the term cytomegalia regarding these observations in 1921 and the hypothesis that the symptoms must arise from a prenatal event was posted by Mueller in 1922. Close to 1950, the term "generalized cytomegalic inclusion disease" was used and in 1953, Minder was able to detect virus-like particles in the inclusion part of the cytomegalic cells by electron microscopy. In the mid 50's cell culturing *in vitro* enabled isolation of CMV in three different laboratories, almost simultaneously, Margaret Smith isolated the strain named Smith, Weller the Davis strain and Rowe perhaps the most used strain in laboratories today, AD169. In the laboratory of Weller, the discovery was entirely by chance as they set out to extract another infectious agent, *Toxoplasma Gondii* from liver cells from an infant with hepatosplenomegaly, cerebral calcifications and chorioretinitis. However, they observed enlarged, rounded cells and when retaining these in culture, it soon became clear that what they had isolated was not related to *Toxoplasma* but instead the answer to the question first raised by Ribbert in 1881, the agent causing cytomegaly.

Once CMV was isolated and cultured in the laboratory, the path was set for diagnostics, producing antigens for serology, and *in vitro* studies of CMV (as reviewed in [1-3]).

CYTOMEGALOVIRUS

Cytomegalovirus (CMV) belongs to the family of herpesviruses, which to date also includes Epstein Barr Virus (EBV), Human Herpesvirus (HHV) 6, 7 and 8, Herpes Simplex (HSV) 1 and 2 and Varicella Zoster Virus (VZV). This family is divided further into the α, β, γ subfamily depending on their biological properties such as replication cycle, site of latency and growth ability in culture. CMV belongs to the β-herpesvirus subfamily, grouped together with HHV 6 and 7 because of similar biological properties such as the cytomegalic effect, host cell preferences and slow growth in *in vitro* cultures. Herpesviruses are large DNA viruses and share the ability to establish latency after a primary infection, virion assembly procedure and certain genes. Although they all establish latency, they favor different host cells. EBV resides in B cells, HSV and VZV in neural cells (as reviewed in [4] and CMV has been reported to establish latency in cells of myeloid lineage [5].

They are old viruses, very well adapted to the human host after co development during the course of evolution. For CMV, there is a species specific variant for each mammal. This phenomenon creates a problem when trying to establishing animal models for CMV, since the different species variants differ and the most difference lies in the virus-host cell interaction [6]. Since CMV works in close relation with the immune response, the interspecies differences in immune response are not trivial. Common animal models used today include mice, rats, guinea-pigs and monkeys. Mice are desirable as a model system being small, genetically well characterized, widely used with many transgenic set ups readily available and easily bred. Moreover, murine CMV (MCMV) has been widely used in *in vivo* latency and reactivation studies [7]. However mouse MCMV does not cross placenta which makes it unfavourable as a model for viral transmission during pregnancy, and promoting the guinea pig model as a better choice. For the role of CMV in vascular disease, the rat model has proved efficient. Monkeys and the rhesus CMV (RhCMV) is closely linked to HCMV infection in humans, but of cost and ethical reasons, monkeys are not an optimal model for large scale, basic research but rather a necessity used mainly in drug trials. Species variants do not readily infect other species although it can be done in the laboratory setting, using direct means of administration and high viral inoculum titers, however the relevance of results obtained by this method can be debated.

PATHOGENESIS, EPIDEMIOLOGY AND CELLULAR TROPISM

HOST ENTRY

CMV has co-evolved with humans as hosts during thousands of years and generally it manages to sustain a balance with the immune system and remaining in a normal, immunocompetent host for the duration of host life without causing acute damage. It is in its' very interest to keep its' host well enough to live on and spread the infection to new hosts. A classic hypothesis is that viral infections are either a) highly infectious,

easily spread and cause severe symptoms, or b) less infectious, require close contact to infect, but in return symptoms are mild or non-existent, which makes it near impossible to avoid contagious individuals.

CMV is transmitted by all body fluids and typically enters the body via mucosal surfaces, where virus has been detected in epithelial cells of the upper airways and genitourinary tract, via blood or transplanted organs or regarding congenital infection, via cells of the placenta (as reviewed in [8]). From the primary site in infection, the virus is carried to other organs primarily by leukocytes and vascular endothelial cells [9]. Even during active infection, the presence of free viral particles in plasma is rare and blood products can be cleared of CMV infection by simply removing the leukocyte fraction [10]. The spread of infection is aided by CMV recruiting inflammatory cells such as monocytes and neutrophils which can in turn be infected and used as viral vehicles [11].

Infection can occur anytime in life, but incidence has three clear peaks (Fig1):

1. The first years of life including congenital and intra partum infections as well as transmission of virus via breast milk and close contact with other children.
2. Puberty and sexual debut
3. Old age

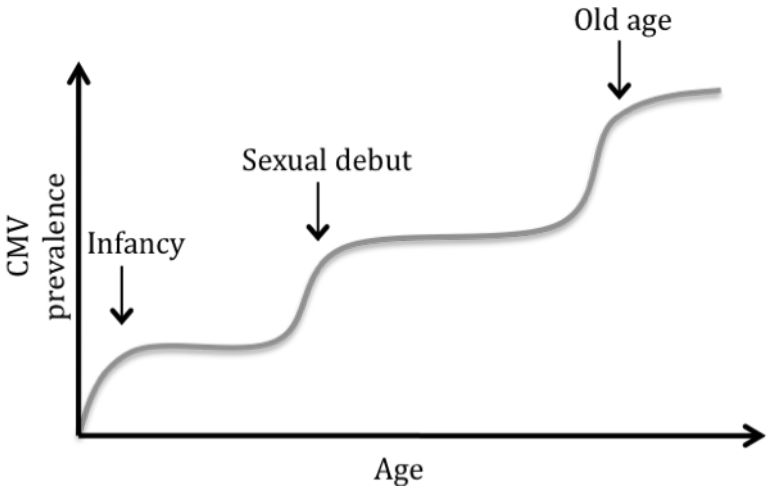

Figure 1. CMV prevalence

Herpesvirus infections are very common and cytomegalovirus is no exception, with a prevalence of 70-90% world-wide. The distribution is highly dependent on socio economic factors with higher prevalence and earlier occurrence in the developing world and disfavoured populations of the developed countries.

EPIDEMIOLOGY OF CMV

There are no epidemics of CMV reported and no seasonal variation in incidence. CMV is not highly infectious, unlike viruses such as the noroviruses, where a few viral particles are enough to infect a human. To be infected it is probably necessary to come in direct contact with a large amount of viral particles, such as when in direct contact

with infected body fluids. However, since CMV positive individuals shed virus in saliva, urine and other body fluids during long periods of time, sometimes without any symptoms at all, contact with CMV is abundant and difficult to avoid. Toddlers are an important reservoir of virus, distributing it to the rest of the population via care takers, other children and their parents. Some studies show that among CMV negative parents to CMV shedding toddlers, 45% seroconvert compared to 0% for parents of non shedding children. Day care workers have a heavily increased incidence, up to ten fold higher (2% vs. 20%) than the average population (represented by a group of blood donors) as reported in an American study and this risk is further increased to 30% among care takers working with children below one year of age [12, 13]. Young children can actively shed virus for years, while shedding in adults is limited to shorter period of time (weeks or months). A study of day care children in US showed that up to 80% of children in the age of 13-24 months shed virus, in comparison to only 7-8% in children cared for at home. After the age of 3 years, shedding is dramatically decreased [14, 15]. Interestingly, reports from Swedish day care centres show no such high viral shedding. This may be due to Swedish children being older when admitted to day care [16].

Another important route of transmission is sexual contacts. A report based on female patients of an American STD clinic showed an incidence of 37% of active CMV infection, which represents a twenty fold increase compared to blood donors (as reviewed in [17]). In the elderly population CMV prevalence is almost 100%. It does not come as a surprise that individuals having lived the longest have also been the most exposed to contamination.

SYMPTOMS OF CMV INFECTION

In the immunocompetent host, a primary CMV infection normally results in subclinical symptoms similar to those of a common influenza infection, such as mild fever, fatigue and moderate pain. In some individuals, a more severe pathology occurs, including adenopathy, hepatitis and splenomegaly. These differences in displayed symptoms are probably due to viral load, viral phenotype, site of viral entry or host immune system composition. Lockridge et al. showed that monkeys infected by RhCMV intravenously or intraperitoneally presented with more severe symptoms regarding liver dysfunction and haematological disturbances than administration via mucosal surfaces. This was also confirmed in the mouse model by Jonjic [3]. Others have through animal models advocated the hypothesis of increased viral load as the main denominator for more severe symptoms and convincing evidence is given in [18-20]. It is also an appealingly simple explanation that it is only certain extra virulent CMV strains that cause these symptoms. Animal studies show that when deleting small parts in the CMV genome related to virulence, although the virus is fully replicative, large variations in efficiency of infection are observed. Last but not least, several investigators have pointed to anomalies within the host's immune system as being the critical factor. Individuals with insufficient NK cell function is more likely to develop CMV disease [21, 22]. The ability for antigen presentation by MHC is also important [23]. As with many

questions within the field of host defences, the answer most probably involves a little of all and maybe other, still unknown, explanations.

Although CMV is considered to be rather particular in its choice of host cell and do not readily infect just any cell type *in vitro*, post mortem examinations of patients suffering from severe CMV disease reveals virus in almost every organ in the body. Most common are infections of blood cells, endothelial, epithelial, neuronal, stromal, smooth muscle cells and fibroblasts. Apart from the acute effects of CMV, the last decades many reports have been published on the role of persistent infection in inflammatory diseases such as atherosclerosis, colitis and colonic diverticulitis [24-28]. It has been proposed that CMV residing in mononuclear cells is recruited to the site of inflammation where activation of monocytes also reactivates the virus. CMV replication and the local inflammation stimulate one another, favouring the viral persistence but simultaneously damaging the tissue. According to the literature, CMV is most probably a late arrival worsening the development of chronic inflammatory disease, rather than its cause. Once CMV replication has been initiated by the milieu at the site of inflammation, it begins to influence the pathologic process. Through further stimulation of immune responses in haematological cells as well as endothelial and epithelial cells where it resides, the local inflammation is further augmented. By these mechanisms, it is the host immune response and not the viral infection per se that causes tissue damage (as reviewed in [29-32]).

CMV infects several types of blood cells including both mononuclear and polymorphonuclear cells. Patients receiving blood transfusions are at risk for CMV mainly through contaminated leukocytes. Infected blood cells can be found in the peripheral circulation but they are extremely scarce. Instead, they are found in abundance at sites of inflammation suggesting that CMV is residing in blood cells awaiting recruitment to inflammatory sites where its host cell is activated and thereby also activating the resting CMV into an active infection. This has been demonstrated in macrophages, where a latent infection is reactivated in response to allostimulation [5].

CMV IN THE IMMUNOCOMPROMISED HOST

During the last forty years, a new patient group has emerged for whom the threat of CMV is ever greater; an increasing number of AIDS patients and individuals with induced immunosuppression- the transplant patients and other patients undergoing immunosuppressive therapy like chemotherapy against cancer. Other immunosuppressed at risk for CMV infection are individuals with congenital immune deficiencies. The severity of symptoms in immunocompromised individuals has for a long time been considered to depend on viral load, but there are contradictory reports on this [33-37]. When measuring viral load, investigators tend to use blood as a sample source but Brune et al. reported that there is not always a direct correlation between viral load in the organ in question and viral load in the blood [38]. In fact, it seems it is predominantly the viral load in the specific target organ that is a determinant for organ damage, not just blood viral load. This is interesting in the light of findings of CMV in

cancer tissue where many tumour cells are infected. However, it is not always possible to detect traces of active infection in the systemic circulation, neither by serology nor by tracing CMV DNA in blood cells. One might speculate that this is not only due to a localized infection but also a sign of CMV induced immunosuppression, inhibiting a proper antibody response.

In bone marrow and solid organ transplant patients, CMV poses a lethal threat and although the drugs currently on the market, such as ganciclovir, cidofovir and foscarnet have made a substantial difference for the short term graft survival numbers and thereby overall patient survival, the long term risks of graft rejection remains. Matching CMV negative recipients with CMV negative donors for solid organ and bone marrow transplant as well as for blood transfusions, substantially decrease the risk of CMV disease. In CMV positive organ transplant recipients, pre-emptive strategies where antiviral drugs are administered prior to transplantation, are used to prevent reactivation. It is also standard care to administer ganciclovir for long periods of time after transplantation. For AIDS patients, CMV is the most prominent opportunistic infection causing severe and sometimes fatal disease. A previous report shows that CMV is even linked to clinical progression of HIV infection [39]. CMV causes retinitis, esophagitis, colitis and less frequently encephalitis, pneumonitis, gastritis and hepatitis in AIDS patients and severity of CMV disease is heavily linked to immune status where especially individuals with low and very low CD4 counts are at high risk. Previously, the incidence of these diseases were 40%, but since highly active antiretroviral treatment regimes was introduced the incidence of these diseases has decreased substantially [40-42].

CMV PARTICLE

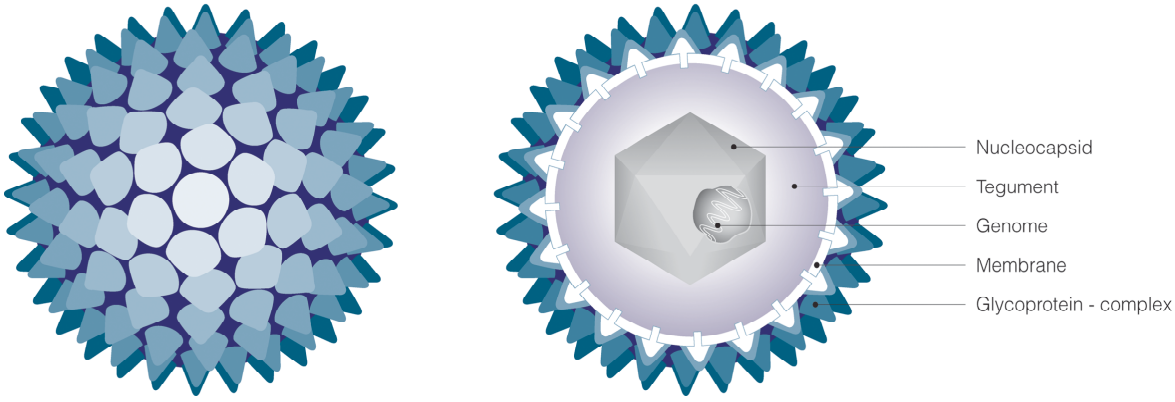

Figure 2. The CMV particle

The CMV particle consists of an outer envelope and an inner capsid harbouring the double stranded, linear DNA genome (Fig 2). Between the capsid and the envelope is the tegument layer, similar to the cytoplasm of a cell, with a mixture of molecules important during the first steps in infection. The virus contains 30-40 polypeptides of

various sizes, enabling CMV to rapidly influence its host cell even before its own genes are transcribed.

The CMV genome is large over 200kb, depending on strain, and human CMV is the largest with its 235kb. It includes 250 open reading frames, for which we do not know all functions. The genome of human CMV is arranged in a class E genome structure; two separate components of unique sequences (unique long, UL and unique short, US) with sequence repeats at both ends.

The capsid is icosahedral (T=16) and composed of seven proteins arranged in multimeres. The most prominent protein is the Major Capsid Protein (MCP), which is one of the most highly conserved proteins common to all herpesviruses. In simian and human CMV there are three different forms of capsids, representing different stages of maturation. The A capsid contains no DNA and is accumulated due to packing difficulties, the B capsid is a precursor of a mature capsid but also without DNA and the C capsid, which is the fully mature form. There are also three different forms of whole viral particles; the virions, the non infectious enveloped particles and the dense bodies. The virion is the released, fully infectious particle. The non infectious enveloped particles consist of an enveloped B particle. The dense bodies are small, defectively enveloped particles. Neither of the latter are able to infect new host cells.

The layer between capsid and the outer envelope, the tegument, consists of around 25 proteins and other molecules that are released in the cell cytoplasm upon viral entry. They are factors essential to start the infection and manipulate the cell into replicating the viral genome and start producing virally encoded proteins. Among these proteins is the pp65, a common antigen for antigenemia tests. It is more abundant in virions from laboratory strains than in those from clinical isolates, which is interesting since it does not seem to be essential for *in vitro* viral replication [43]. The UL97 kinase is another member of the tegument proteins, it is a DNA polymerase and the phosphorylator activating ganciclovir (described more in antiviral treatment chapter). Interestingly, in the tegument is also RNA. CMV is rather unique as a DNA virus in that it also harbours RNA. The tegument RNA is released and expressed directly upon viral entry [44]. It has also been reported that the tegument harbours RNAi for regulatory purposes.

In the outer envelope covering the whole viral particle, viral proteins are interspersed in a lipid bi-layer acquired from host cell nuclear and intra cellular membranes. In this bi-layer are two large glycoprotein complexes. One of them contains multimeres of gB, the most common and a highly conserved glycoprotein and the other complex consists of gH, gL and gO. Both complexes are essential for viral entry. gB is the major heparan sulphate proteoglycan (HSPG) binding molecule, essential for viral binding, entry, cell-cell transmission and release. It also binds a 30-36kDa cellular protein suggested to be the CMV cellular receptor. Due to its high abundance, relatively low sequence variation and the high amount of neutralizing antibodies against it found in human sera, gB has been a popular target in attempts to establish a CMV vaccine. In addition, certain

transmembrane viral receptors are present in the envelope; UL33, UL78, US27 and US28 [45-47].

Apart from the cellular proteins and lipids in the virion itself, CMV particles are also known to bind to $\beta_2$microglobulin, actin and various cellular receptors and enzymes [48-50]. The role of actin and $\beta_2$microglobulin is most probably related to viral binding and intracellular transport [48, 49].

CMV AND THE HOST CELL

From binding of the host cell to release of new viral particles, CMV needs 48-72 hours, depending on viral strain and host cell type. The course of infection on a cellular level can be divided into four steps, 1) viral binding of and entry into the host cell, 2) replication of viral genes, 3) assembly of viral particles and 4) egress.

1. Viral binding to the host cell.

Glycoproteins on the viral envelope surface, most importantly gB, binds to cell surface heparan sulphate proteoglycans [51] before binding between elements of the viral envelope and cell surface receptors. It is believed that the binding to HSPGs is weak and unspecific and that viral entry requires a stronger, more specific binding to a cellular receptor. The attempts to elucidate this receptor have been many, but although several have been suggested, the discussion on which is the right one or even if the right one has yet been found is still ongoing.
A 30kDa cellular protein was isolated as the receptor, but not identified [52-54]. Annexin II was suggested as being the mystery 30kDa protein after studies on its association to CMV particles [55], but later experiments from the same laboratory revealed that blocking of annexin II had no effect on viral entry [56]. Binding of MHC class I molecules by viral MHC class I homologue pUL18 was also suggested, but virus with deleted UL18 proved to replicate well. CD13, aminopeptidase N, was shown to be indispensible for viral entry [57] and neutralizing antibodies against CD13 potently inhibited CMV infection [58]. Epidermal growth factor receptor (EGFR) was reported to be a possible receptor in 2003 when Wang et al. showed interaction between EGFR and gB as necessary of viral entry [59]. However, Isaacson et al. did not agree and published four years later that EGFR is indeed not needed for CMV entry into host cells [60] but rather that it is the EGFR related integrins that are the true CMV receptors, as earlier posted by both laboratories [61, 62]. The question remains open, but one speculation is that since CMV is the master of diversity, it is not a question of one single receptor, but rather a group of receptors that can be targeted for viral binding and entry. After binding, the viral envelope fuses with the plasma membrane, releasing the capsid with its tegument into the cytoplasm.
The proteins and RNA of the tegument immediately interacts with cellular processes to prepare for viral replication. The capsid goes to the nucleus along the microtubulii, with the help of tegument proteins UL47 and UL48, and empties the viral DNA through the nuclear pores (as reviewed in [63]).

2. Replication

The CMV genome is expressed in phases called the immediate early (IE), early (E) and late (L) phase. The products of the immediate early and early genes include manipulative proteins, used to gain access to the cellular replication and translational machinery, avoid immune recognition and stimulate the cellular proliferation and metabolism. The L genes include structural proteins of the capsid, tegument and envelope, essential for building new viral progeny, but also additional manipulative proteins. Genes from the different phases interact to regulate each other in a cascade manner; once the IE genes are expressed, the IE proteins activate the transcription of the E and L genes as well as regulate the continuous expression of IE genes (Fig 3). Expression of the IE and E genes is carried out entirely with the help of cellular transcription factors [64] and tegument proteins [64-66], which means it is not dependent on transcription of viral genes [67]. However, for the L genes the viral polymerases are needed. With the expression of the L genes CMV has all the proteins necessary to start replicating its DNA and produce new viral particles. Not dependent on any additional viral gene expression, IE can be expressed even in cells that cannot support a full, productive infection. This fact combined with the knowledge of the manipulative function of the IE proteins has made them prime suspects for mediating pathogenic effects in inflammatory diseases and cancer.

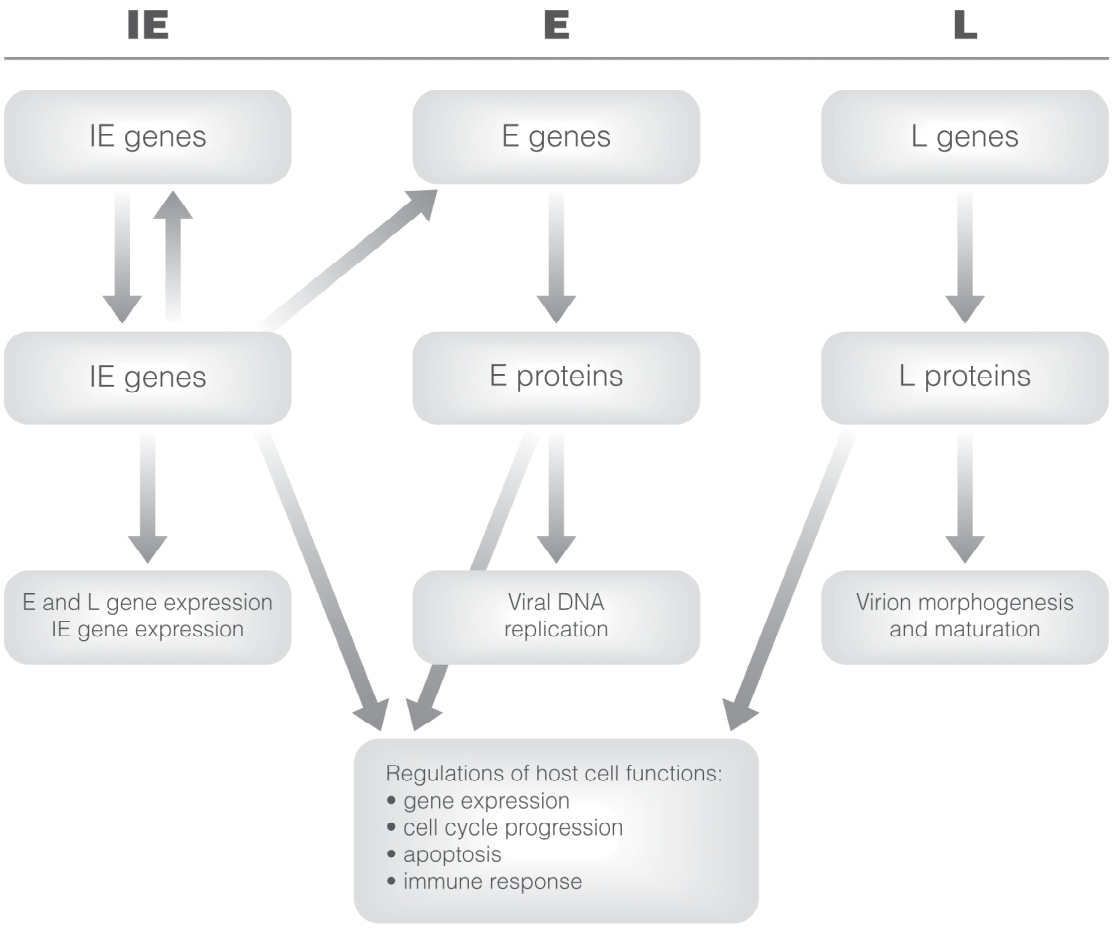

Figure 3. The expression pattern of CMV genes

3. Assembly

In the nucleus, the CMV genome is packaged into newly formed capsids. It is the accumulation of capsids in nuclear inclusions that gives the trade mark “owls eye” appearance of infected cells [8]. The route of the capsid from the nucleus and further on out of the host cell is not clear, but it is suggested to be a two step scenario with a built in maturation process. The capsid attaches to the inner leaflet of the nuclear membrane, thereby receiving a primary tegument and envelope which is lost again upon exiting the perinuclear space [68]. Through further maturation and processing through cytoplasmatic compartments, the final tegument and envelope will form (as reviewed in [69]). The cytoplasmatic envelopment of herpesvirus particles was first suggested for by Siminoff et al. in 1966 in a study regarding HSV1 [70]. This two step model is supported by electron microscopy findings showing distinct differences between primary, perinuclear virions and the fully matured. However, Sanchez et al. found that two prominent tegument proteins, pp150 and pp28 were consistently and exclusively found in the cytoplasm, leading to the conclusion that CMV assembly may take place in the cytoplasm in association with microtubulii and unidentified vesicular structures [71]. Data from our group shows that glycoprotein gB as well as CMV capsids colocalize with markers of the Golgi network and secretory, but not lysosomal vesicles. This implies that CMV buds into the Golgi compartments and vacuoles with the plasma membrane as a final destination [72] and that it is parts of these membranes that form the envelope. This hypothesis is further strengthened by data from experiments where blocking of the Golgi process causes accumulation of non enveloped CMV capsids [73].

4. Egress

The mechanisms of viral release are not entirely clear, but it probably involves fusing of the vacuole membrane and release of viral particles from the plasma membrane. The spread of new viral particles from one host cell starts 48-72 hours after infection and can continue for days until cell lysis. Interestingly, as none of the proteins essential for viral entry is indispensible during egress, these two processes are apparently mechanistically completely different [69]. pUL131 has been implicated to be involved in the viral egress from fibroblasts since over expression of this protein causes delayed viral release. However, this was not observed in other cell types [74]. CMV glycoprotein gO has also been suggested as important in virion maturation and viral egress since deletion mutants show severely reduced release of infectious viral particles [75].

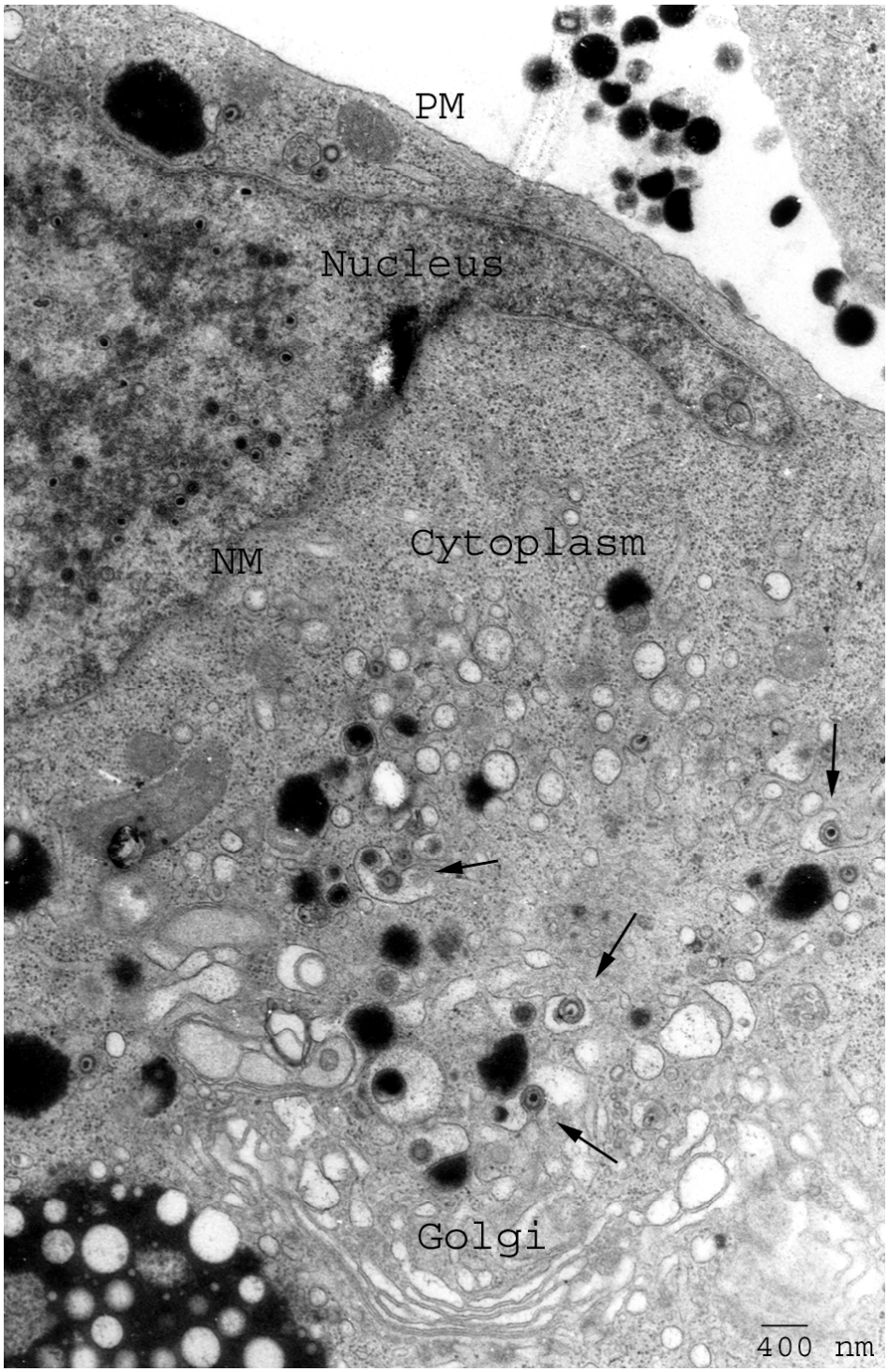

Figure 4. Production of viral particles in cellular compartments. (Photo: Mohammed Homman-Loudiyi, printed with permission)

LATENCY AND REACTIVATION

Upon primary infection, CMV like other herpesviruses establishes latency and remains in the body for as long as the host lives. It will remain undetected by the immune system as long as it is latent, but during reactivation periods, the immune system will try to limit the infection and so maintain the continuous balance between virus and the host immune response. Reactivation can occur in response to stress, inflammatory stimuli or other cellular events. Reactivations rarely give any severe symptoms in an immunocompetent host as the immune system most likely keeps reactivating virus under close surveillance. As for primary infection, the clinical picture is very different for the immunocompromised host, as previously mentioned. During latency, none or only genes required for keeping CMV in status quo in the host cell, will be actively transcribed. No viral progeny is produced (as reviewed in [76]). It has been suggested that CMV genome remains in the nucleus arranged in an episomal manner [77]. Although CMV infects a variety of organs during acute infection, it is very restrictive in where it establishes latency. CMV DNA has been reported in cells of the myeloid lineage, primarily myeloid progenitor cells in the bone marrow and peripheral blood monocytes (as reviewed in [76]). Less than 1 in 10 000 mononuclear cells harbour CMV DNA during latency [78], but still it is believed to be the major site of latency [79]. No other progenies of the bone marrow progenitors, such as PMNL, B or T cells have been reported to carry virus during latency. Endothelial cells, smooth muscle cells and neural progenitor cells [80] have also been suggested as sites of latency but no CMV DNA has been isolated from these cells in healthy donors [81].

Reactivation of CMV from myeloid cells requires differentiation. Söderberg et al. reported reactivation of CMV in peripheral mononuclear cells from healthy individuals in response to allogenic stimulation [5], a potent differentiation signal. Reactivation of CMV in bone marrow progenitors was reported by Reeves et al., when he subjected cells to *in vitro* differentiation to dendritic cells. However, not blood from all donors responded by reactivation of CMV and the authors hypothesize that this could be due to latent viral load [82].

CONGENITAL INFECTION

EPIDEMIOLOGY AND TRANSMISSION OF CONGENITAL CMV

Although antiviral treatment has improved the prognosis for many patient groups previously at great risk of severe CMV infection, the risk for and pathological development resulting from congenital infections have not changed a lot. The incidence in the developed countries range from 0.15 to 2% (as reviewed in [83]), much depending on population seroprevalence. The incidence in the developing world is not clear but studies on small populations suggest an incidence at least twice the incidence in developed countries [84, 85]. Previous reports state that the risk of attracting a primary infection during pregnancy is 1-4% [86-88], while the risk of reactivation of a latent infection is as high as 10-30% [89]. However, the risk of transmission is 30-40% during primary infection, compared to 1-3% for reactivated infection [89]. Fig 5.

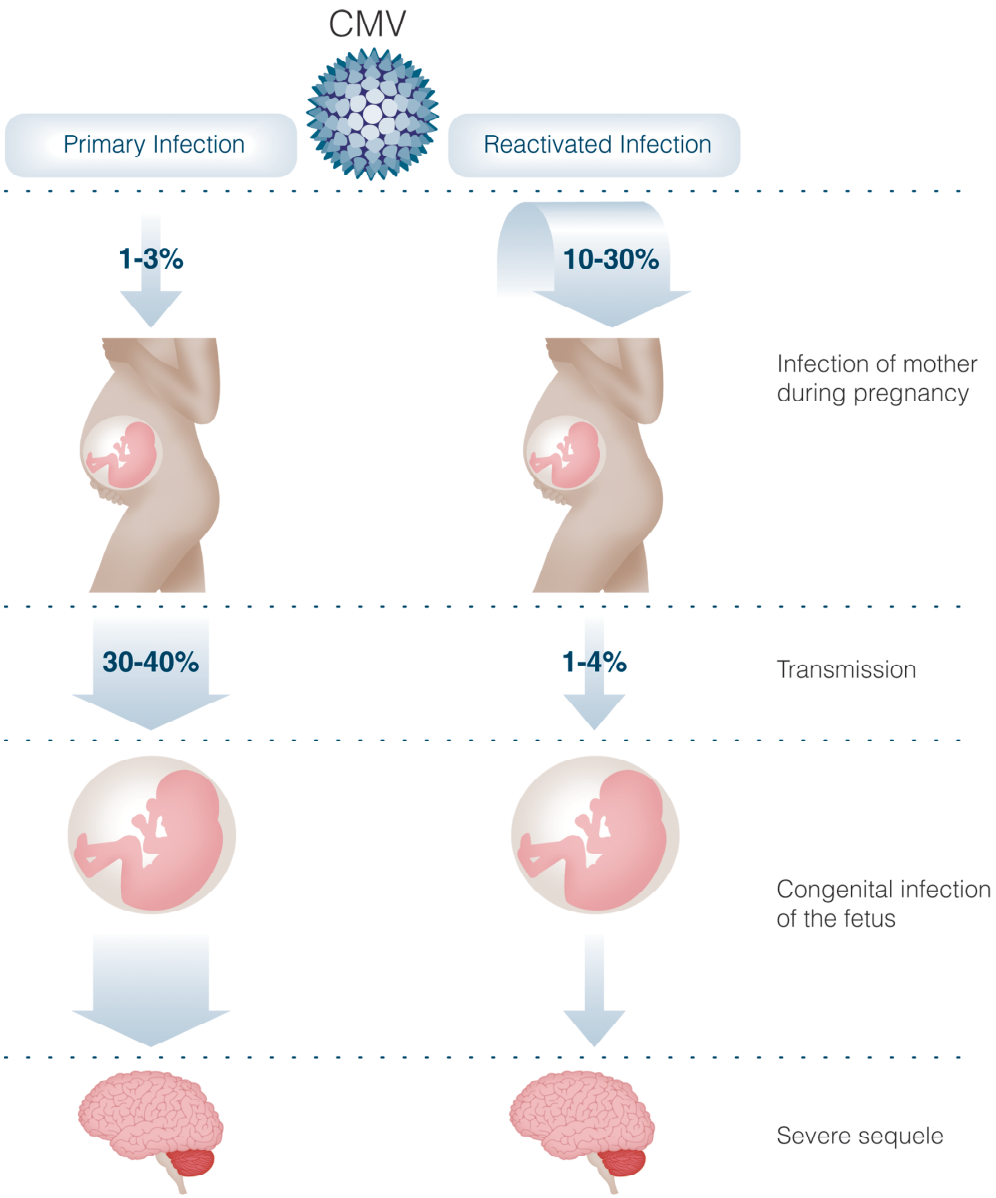

Figure 5. Transmission risk

A study of more than 6000 women from high and low income background in United States showed that when derived from a primary infection, the congenital infection is more likely to result in clinical disease [87]. In a study of high seroprevalence pregnant women, the number of cases with CMV related hearing loss was similar to those of groups of lower seroprevalence [90]. The risk of severe sequelae of congenital infection could therefore be higher in upper socio economic groups, since they have a lower seroprevalence and subsequently a higher risk of getting a primary infection during pregnancy [87].

CLINICAL SYMPTOMS

For children born with CMV, 10-15% display symptoms during the neonatal period. These symptoms include growth reduction, petechiae, hepatosplenomegaly, jaundice, purpura, thrombocytopenia, anemia and neurologic symptoms such as seizures, lethargy and microcephaly. It is not uncommon that these children are born prematurely (as reviewed in [17]). Sensory neural hearing loss is one of the most common sequelae, and it ranges from mild hearing impairment to total deafness. It may be a progressive event, deteriorating as the child gets older. According to a study by Engman et al., the risk for hearing deficits is 20 times higher in children with congenital CMV than in non infected infants [91] and Ogawa et al. showed that 15% of sensorineural hearing loss cases are caused by congenital CMV [92]. Visual impairment is also a rather common result of CMV infection, this is diagnosed early and there are no reports on this being a progressive process.

The effects on CNS are often irreversible, but sequelae involving other organs can be transient. However, there are cases where infants with CNS damage at birth have been treated early with good results (Cecilia Söderberg-Nauclér, personal communication). Even if the infants appear asymptomatic during their neonatal period, they may present with symptoms at a later stage. Psychomotor function, learning disabilities, concentration disorders, mental retardation, autism [93], schizophrenia [94], and additional hearing deficits (as reviewed in [95] and [96]). The severity of sequelae is dependent on time of infection and to some extent also on viral load. Blood viral load has been reported to predict hearing deficits in infants born asymptomatic but not in those symptomatic at birth [97]. Other studies have found correlations between higher viral load and symptomatic congenital CMV as compared to asymptomatic infection [98] and as a predictor for sequelae severity [99]. To date, the most important predictor of symptomatic infection is viral load in the amniotic fluid [100]. As for the time factor, it has been reported that an early infection, occurring during the first trimester when the progenitor cells of the brain migrate and differentiate, can cause migration disturbances and devastating brain injuries. Later on, the infection may result in white matter lesions as it targets the time of myelination (as reviewed in [101]).

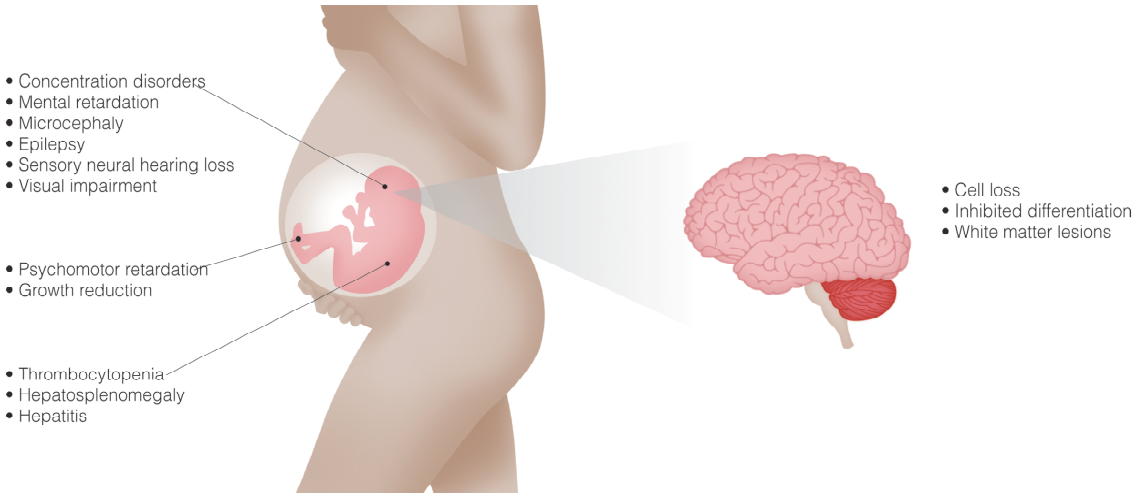

Figure 6. Sequelae of congenital CMV infection

DIAGNOSIS

In utero diagnosis of CMV is normally done by PCR for viral DNA in the amniotic fluid or the cord blood. Due to higher sensitivity and less risk of complications, such as spontaneous abortion, the use of amniotic fluid is preferable over cord blood. Diagnosis in the neonatal period is done by virus isolation from urine, CMV DNA PCR of urine or blood, or serology. When looking at the serological response, it is important to focus on IgM antibodies since IgG is transferred from the mother via placenta and is not an indication of an endogenous response in the infant (as reviewed in [95]). Diagnosis retroactively is possible by CMV DNA PCR of Guthrie cards, the dried blood spots (DBS) collected during the neonatal period originally to detect metabolic diseases. However, since the sample size is very limited, the risk of false negative results is evident. The brain abnormalities seen in children with congenital CMV include calcifications, ventricular dilatation, atrophy, white matter gliosis, chronic lesions, parenchymal and ependymal cysts and cortical malformations such as polymicrogyria. Ultra sound examinations in utero can help predict outcome and neurological sequel, but only by computed tomography (CT) is it possible to get a complete picture of the total encephalopaty (as reviewed in [102]). There are strong correlations between the damages seen on CT and clinical outcome. However, for white matter gliosis the correlation, if any, is not clear (as reviewed in [96]).

TO TREAT OR NOT TO TREAT?

Some countries use large screening regimes, testing for CMV during the course of pregnancy by amniocentesis and ultrasound to detect any signs of CMV infection. In Sweden, there is no such routine based screening. One reason often used in the debate on routine screening in Sweden, is the question of possible intervention. Apart from terminating the pregnancy, treatment options are limited since the drugs currently on the market have been observed to be teratogenic in animal models [103, 104]. No human has been reported, in fact a case report of a pregnant transplant patient who was

treated with ganciclovir, rendering no teratogenic effects, suggests there are none [105]. Still, there is no routine use of these drugs against congenital CMV infection during pregnancy. Treatment of symptomatic children during the neonatal period has proven somewhat efficacious, but the effect on viral replications seems transient and the long term benefits are unclear [106-108]. However, diagnosis at birth may still be beneficial since it enables early interventions to counteract any milder cognitive disturbances. There is a strong belief that such disturbances if caught early can be significantly reduced (as reviewed in [95]). It has been suggested that treating the mother with anti CMV IgG can protect the foetus, but there are no large studies on this [109, 110]. The best future option may be to avoid infection by vaccinations. There are several vaccine attempts being tried out to limit the overall spread of CMV and above all prevent primary infection in the pregnant woman, but the results are so far disappointing (as reviewed in [111]). The most efficient method to avoid CMV infection to date is decreased direct contact and increased hygiene. Forceful information on avoiding contact with contagious bodily fluids from children and others combined with frequent hand washing with soap was suggested to decrease incidence of CMV infection in pregnant women [112, 113] (and reviewed in [114, 115]).

FETAL TRANSMISSION – THE ROLE OF PLACENTA

How the virus travels from mother to foetus has been debated over the years, but the consensus hypothesis today illustrates virus from maternal circulation infecting the cells of the placenta, mainly the cytotrophoblasts. These cells are situated right at the interface between mother and foetal circulation. Infection could occur either through the syncytiotrophoblasts that cover the cytotrophoblasts or by infection of the invasive cytotrophoblasts in the uterine wall. These cells have been reported as permissive *in vitro* and to get impaired function in response to infection. Migration and differentiation is inhibited and this interference results in poor perfusion of the placenta, a most crucial function for foetal development. This decreased blood flow may play an important role in the developmental disturbances that CMV causes in the growing foetus; without proper blood flow, the organs will develop poorly. The invasiveness and the pseudovasculogenesis process, where invading cytotrophoblasts imitate and ultimately replace maternal vascular endothelial cells, is highly important and failure during this process may have devastating consequences and can result in miscarriage.

Cytotrophoblast invasiveness into the uterine wall is dependent on MMP-9 function, a matrix metalloproteinase which has been shown to be down regulated by CMV in macrophages [116]. Furthermore, interleukin 10 (IL-10) has been shown to down regulate MMP-9 in cytotrophoblasts [117] and interestingly, CMV induces IL-10 expression [118] as well as code for an IL-10 homologue [118, 119]. This data implies that CMV infection in the placenta can cause severe disturbances in foetal development, even without ever reaching the foetus itself. It is worth mentioning that CMV in the foetus is not a certain consequence of infected placental cells. In the guinea pig model, only 27% of foetuses where infected, even though CMV was present in the

trophoblasts of placenta [120]. This observation has also been confirmed in humans by McDonagh et al., where CMV was found in 69% of term placentas from uncomplicated births [121]. No additional analysis of the infants was made in this study to determine whether the children themselves were CMV positive or whether any of these children presented symptoms later on. Authors conclude that asymptomatic congenital CMV infection may be much more common than previously stated.

TARGET CELLS IN THE STEM CELL AREAS

The encephalopaties seen on ultrasound and CT are the results of viral influence on several cell types. Reports show that most cells of the brain are possible to infect with CMV *in vitro*. However, not all cells are fully permissive, meaning they may not support a full viral replication and production of new viral particles. Brain vascular endothelial cells, astrocytes, microglia/macrophages, neurons, neuronal stem and progenitor cells, and oligodendroglial cells, can all be infected *in vitro* (as reviewed in [96]) (**paper I and II**). Infection of different cell types have also been shown *in vivo* in adult AIDS patients suffering from CMV encephalitis [122]. Several reports show that it is the early, immature cells of the brain that are the primary target for CMV during congenital infection in animals.

The Tsutsui group in Japan has worked extensively with CMV mouse models, and they showed that mouse embryos infected via placenta frequently showed MCMV infected cells in the brain, mainly in the ventricular walls. Infected cells also travelled to the hippocampus and the cortical plate [123]. This was further shown in brain slice cultures from neonatal mice infected by whole immersion of the tissue *in vitro* [124]. In the brain slice culture method, the brain is thinly sliced and kept in culture dishes in growth medium. This method allows *in vitro* observations of the different cell types of the brain, in their natural contact with each other. It was evident that the subventricular zones, derived from ventricular walls, was the primary site of CMV infection.

The ventricular wall and subsequently the subventricular area forms, together with parts of the hippocampus, the neuronal stem and progenitor cell reservoir of the brain. From this location, immature cells migrate and differentiate on their way to designated areas. Precursor cells differentiating into glial lineage will become the support cells of the brain and guide neurons on their way to cortex as well as dividing into new neurons by asymmetrical division. The neurons themselves migrate along the glial fibres, maturing as they go. These features are seen mostly during the brain development, but during the last decade revolutionary reports have been published on the plasticity of the adult brain and the ability to regenerate. Thinking of the brain plasticity concept, taken together with the knowledge of CMV infecting NPCs and possibly establishing latency there [80], it would not be unreasonable to think of CMV in adult diseases of the brain. It is a thrilling thought that CMV could reactivate from this stem cell pool at any time, travelling to new sites in the brain and contribute to disease development and progression, as in the case of brain tumours or inflammatory diseases. Through the

homing of NPCs to sites of injury, inflammation sites or to what has been termed as the vascular niche, CMV has by infecting these immature brain cells found itself perfect means of regeneration and transport.

NEURAL STEM CELLS AS TARGET CELLS

NPCs of the foetal brain are readily infected by CMV *in vitro* as well as *in vivo*. Our group and others have shown that CMV can infect human NPCs [125-127] even at rather low multiplicity of infection (MOI). Infection using MOI 0.1 of a clinical isolate like strain resulted in >50% IE positive cells 7 days post infection (**paper I**). It was a remarkable difference between the infection efficiency of clinical isolate like strains such as TB40 or VR1814 and laboratory strain Towne. Towne consistently resulted in infection levels lower than that of clinical isolate like strains. However, this effect was more pronounced in low MOI infections than high, indicating a viral saturation (**paper I**).

Cheeran et al. reported that infected NPCs cannot withhold normal proliferation rates, but they are decreased to 50% of uninfected cells and this is supported by data from the mouse model, where clonogenic capacity of the neural stem cells is impaired by MCMV infection [128]. In the human NPCs, neither apoptosis nor other cell death was seemingly affected [125, 126]. In our laboratory, we have observed heavily reduced cell numbers in infected NPC cultures, but no significant effect on proliferation (unpublished data). In accordance with Cheeran's data, we do not detect any large induction of apoptosis, therefore drawing the conclusion that our NPCs die of apoptosis unrelated death in response to CMV infection, 7 dpi (unpublished data). However, Cheeran et al. showed no such increase. We all concur that NPCs support a productive infection through the release of new, infectious viral particles (**paper I** [125, 126]). The lack of proliferation and increased cell death may play an important role in the cerebral atrophy often observed in congenital CMV.

We further argue that NPCs do not function properly in neither differentiation into neural or glial lineage, nor in migration. We have observed that infected NPCs do not migrate as readily *in vitro* as uninfected cells when stimulated with astrocyte conditioned medium, in some cases the migration ability is completely abolished. Interestingly, we have detected discrepancies between NPCs from different donors. A minority of cultures did not show any effect on migration ability in response to infection despite repeated attempts. They all originate from fore brain tissue isolated from human foetuses aborted at 5-12 weeks of gestation. We have not yet been able to isolate the factor determining whether a culture will be responsive to infection or not regarding infection effect on migration. The CMV effect on migration has also been reported *in vivo* in mice [129]. In this experiment, MCMV was injected into the ventricle of mice foetuses and the brains examined postnatally.

An interesting hypothesis on neural stem cells of the ventricular area being possible sites of latency has been posed and backed up by data from the mouse model. MCMV

was injected into the ventricles of young or neonatal mice and after 4 weeks the viral production was below detection limits by plaque assay. The mice were kept for 180-210 days and the infection was considered latent. Brains were collected and put into brain slice cultures where CMV replication was detected after one week, peaking at 2-3 weeks as determined by reporter gene expression and viral production by plaque assay. Reactivated cells were detected mainly in the ventricular areas, and a smaller amount in the cerebral cortex. Reactivation occurred in 75% of neonatally infected animals but also in some of the mice infected during adulthood [80]. These data imply that CMV can remain latent in immature cells of the ventricular area or brain endothelial cells and reactivate in response to unknown stimuli. This is intriguing in the context of brain diseases such as inflammatory conditions or cancer, where an active CMV infection could influence the course of disease, worsening disease progression.

When examining data from our laboratory and others and pondering over the differences in results in some contexts, a couple things are worth pointing out. There are two major types of *in vitro* cultured NPCs used here. We have used human foetal forebrain tissue, prepared according to Carpenter et al. [130] and further cultured as neurospheres. McCarthy et al. also used neurosphere cultures, but derived from a larger part of the brain (telencephalon, diencephalon and rostral brain stem). It is also possible that discrepancies are due to specific cellular location and viral load, as suggested in the review of Tsutsui [131].

Neurosphere cultures are considered more heterogeneous than adherent cultures but also less differentiated (as reviewed in [131, 132]). Cheeran et al., however have used adherent cultures derived from foetal brain tissue in a similar manner [133] but cultivated in different media, on different surfaces and passaged using different dissociated techniques. Although all methods results in highly proliferative and for many passages stable cultures expressing similar levels of stem cell markers nestin and CD133, these basic differences most probably influence the neural progenitor cells and their properties. Even so, general findings such as permissiveness to infection, manipulated differentiation and reduction in cell number are consistent between us.

DIFFERENTIATING CELLS; ASTROCYTES

Astrocytes make up 70% of the adult brain cell pool and are important structural and maintenance cells that also contribute to immune responses by releasing cytokines and chemokines attracting immune cells such as microglia and immune cells from the circulation. They are fully permissive to infection, with expression of IE proteins, up regulation of IE promoter and produce large amounts of virus a few days post infection [134]. This makes them a prime possible source of virus, disseminating infection to other parts of the brain. Yet, what if the very production of astrocytes and other cells of the glial lineage was inhibited? Some of the neurological sequelae from congenital CMV infection are due to cell loss and we set out to test whether this cell loss included astrocytes. Human foetal NPCs were stimulated to differentiate towards glial lineage by removing single growth factors and adding serum and at the same time infected with

CMV. As with NPCs in the previous text, CMV readily infected the cells, yielding >70% IE and >50% gB positive cells in response to MOI 0.1 7 d.p.i. with clinical isolate strain TB40. We observed a significantly reduced ability for the NPCs to differentiate into astrocytes, at MOI 1 the presence of glial marker Glial Fibrillar Acidic Protein (GFAP) was reduced to 10% of the amount GFAP positive cells in uninfected cultures.

In addition to reduction in differentiation, we observed an increased apoptosis and reduced proliferation (**paper I**). This is in accordance with data from the mouse model, where differentiation into glial lineage cells is also impaired upon infection [128]. All three effects were dependent on viral replication, inhibition of differentiation and induction of apoptosis on L genes and the decrease in proliferation on IE or E genes (**paper I**). Furthermore, we found that once the differentiation process has started and the cells are committed to glial lineage, although note yet fully differentiated, CMV cannot influence further differentiation. However, CMV can still cause cell loss since the proliferative capacity is still reduced and cells are still pushed towards apoptosis. In conjunction with the fact that even fully differentiated astrocytes are permissive, this means that, even if progenitor cells escape infection, astrocytes further on in the differentiation process can still be infected and the infection result in severe cell loss.

DIFFERENTIATING CELLS; NEURONS

To determine the effect of CMV infection on the brains supply of neurons, we infected NPCs and then stimulated neuronal differentiation by withdrawing growth factors and switching to a neuron promoting medium. It was clear that neuronal differentiation was blocked in a virus concentration dependent manner. We also observed reduced proliferation and increased apoptosis indicating that CMV not only inhibits the production of neurons but also reduces the over all cell number by limiting cell production and induce cell death (**paper II**). As for astrocytes, the block of differentiation requires expression of the L genes. However, both the increased apoptosis and the decreased proliferation seems to depend on IE or E genes. Fully differentiated neurons do not seem to be fully permissive, even though receiving CMV and expressing some of its genes. Shinmura et al. showed that MCMV has the ability to enter these cells in vivo [135]. However, Cheeran et al. observed a reduction in CMV permissiveness *in vitro* upon differentiation into neurons and stated that viral infection in human neuronal cultures are supported only by remaining astrocytes and not by neurons, since neurons appeared to be unable to express L proteins or produce virus particles. They furthermore observed that this abortive infection was blocked, not at viral binding or entry, but at the activation of the major IE promoter which was activated to a lower degree in neuronal cells [126].

Here it is interesting to point out that our observations diverge. We observed that 72 hours after initiation of differentiation cells were still permissive to CMV infection in that they expressed both IE and L proteins gB and pp65 upon infection with the clinical isolate strain TB40 to similar levels as that of cells infected earlier during

differentiation (**paper II**). The discrepancies in data may be explained by the fact that Cheeran et al. differentiated neural progenitor cells into neural cells for a longer period of time (>7 days compared to 3 days) and by use of different stimuli (BDNF and PDGF compared to Neurobasal medium supplemented with B27 supplement, Gibco) before infection. Thereby their neurons are further differentiated than ours which were infected at 72 hours after initiation of differentiation. It is also possible that in our cultures it is the remaining nestin positive, immature, cells that express the L genes and not the β(III) tubulin positive neuronal cells. However, since no inhibition of differentiation is detected in the cells infected at 72 hours, the expression of stem cell and neuronal markers are equal to uninfected cultures. That means that there are, at 7 d.p.i. only 40% nestin positive cells remaining and 60% are β(III) tubulin positive. In the cells infected at 72 hours, 60% express L genes. These numbers indicate that at least some of the L expressing cells are also expressing neuronal marker β(III) tubulin (**paper II**).

Luo et al. also showed permissiveness in neuronal cells, although to a lesser extent than in astrocytes [127]. The production of viral progeny is remarkably slow in neuronal cultures in comparison. Although their neuronal cultures are astrocyte contaminated, which means it cannot be ruled out that the viral production stem from astrocytes and not neurons at all. However, they do show that β(III) tubulin positive cells also express UL44, an E-L gene [127]. If the statement from Cheeran et al. was true also in this case, there would not have been any expression of UL44, which is downstream of the MIE promoter. Luo et al. interestingly also observed that a small subpopulation of cells expressing viral antigens had a morphology resembling uninfected cells and stayed alive for up to 12 days. By this observation, they suggest that CMV is able to persistently infect neurons of the developing brain [127]. This correlates very well to the findings in mouse neuronal cells which were shown to evade the innate immune system as persistently infected [136].

IMMUNE RESPONSE TO CMV INFECTION

Both innate and adaptive immune functions are important in fighting a CMV infection. The infection is controlled by induction of a strong immune response upon a primary infection or reactivation, and NK cells, T cells and antibodies are believed to be important.

Natural killer (NK) cells are important anti-viral cells killing virally infected cells. NK cell are recruited to the site of infection early after infection and are thus central in the initial viral control. NK cells recognize virally infected cells and tumour cells mainly by the "missing-self hypothesis" which describe a down regulation of MHC class I molecules commonly employed by viruses in order to avoid T cell recognition. When activated, NK cells rapidly kill target cells, and also secrete anti-viral cytokines such as IFN-γ and TNF-α. The importance of NK cells in the immune response against CMV has been described both in humans and in animal models. Patients with a deficient NK cells response often suffer from CMV associated pneumonia [22], and mice with a depleted NK cell repertoire have been shown to have an increased susceptibility to MCMV [137].

Both CD4+ and CD8+ T cells are crucial in controlling CMV infection and a loss of T-cell reactivity is correlated to increased viral replication and disease development [138-140]. CD8+ T cells recognize CMV infected cells by binding of the T-cell receptor to CMV fragments presented by MHC class I molecules expressed on the surface of the infected cell. CMV specific T cells recognize a wide range of CMV epitopes, but pp65 and IE1 are believed to be immune-dominant [141, 142]. Upon recognition and in combination with cytokine stimulation, T cells are activated and large CMV-specific T cell pools are formed. These cells are highly cytotoxic and kill infected cells, as well as secrete anti-viral cytokines that induce cell apoptosis. A strong T cell response mediates protective immunity against CMV and is vital in the recovery after CMV infections [143, 144]. In fact, CMV is believed to be among the most immuno-dominant antigens and the CMV specific immune response is remarkably strong. Also, the CMV specific T-cell response increases with age and has in elderly persons been described to constitute up to 50 % of the CD8+ T cell repertoire [140].

Although the CMV specific CD8+ T-cell response is very strong, it is not alone sufficient to control CMV replication. Data from mice studies points to the importance of an effective CD4+ T cell response in recovery from infection [145]. CD4+ T cells recognize pathogen peptides displayed on MHC class II molecules expressed on professional antigen-presenting cells (APCs), and secrete cytokines important in the anti-viral defence. In humans, APCs presenting CMV IE1-particles on MHC class II molecules have been shown to activate CMV specific CD4+ T cells *in vitro* leading to decreased virus titres [146].

The humoral immunity comprises of secretion of antibodies from B cells differentiated into effector plasma cells. Specific antibodies bind to and neutralize pathogens by steric hindrance, and also mediate recognition and elimination by phagocytocing cells. In addition, antibody coated particles induce complement activation as well as antibody-

dependent cell cytotoxicity (ADCC) and elimination by NK cells. CMV specific antibodies are not able to neutralize the virus and do not mediate a full protection against infection nor protect against re-infections. Rather, antibodies directed against CMV are believed to reduce the severity of CMV related diseases and limit viral spread within the host [147]. The importance of CMV specific neutralizing antibodies have also been reported in mice, where antibodies were shown to limit viral activity [148].

IMMUNE EVASION

The immune system is an important player in controlling CMV infection although clearing the infection is not possible, an infection always leads to life-long latency. CMV will establish a balance between the host and the pathogen, where the pathogen remains in the system of the host, but in levels controlled by the immune system. This balance poses a controversy in argument where CMV on the one hand induces and/or sustains immune responses. On the other hand, CMV is suppressing immune responses to avoid detection by down regulation of recognition molecules, manipulation immune cell cross talk, inhibiting cellular migration and inducing senescence in T cell populations. This tells us that CMV is well adapted to the human immune system and has a great many strategies to manipulate it in any direction it desires.

In order to avoid the immune system, CMV targets both innate and the adaptive immune responses. To evade elimination by CD8+ T cells, CMV has developed several mechanisms to interfere with the expression of MHC class I and class II molecules on infected cells. The CMV encoded proteins gpUS2, gpUS11, gpUS3, gpUS10 and gpUS6 hamper the MHC class I surface expression [149, 150]. gpUS2 and gpUS3 have also been shown to decrease MHC class II surface expression, as have CMV protein pp65 [151, 152]. Both MHC class I and class II expression is inhibited by the CMV encoded IL-10 homologue [153], and although the mechanism is still to be described, CMV is also believed to inhibit the IFN-γ induced MHC class II surface expression by disrupting the Jak/Stat pathway [154]. In addition to regulating the expression of MHC molecules on the surface of infected cells, CMV induce the release of soluble molecules from infected dendritic cells that hamper T cell proliferation [155, 156].

In concordance with the missing self-hypothesis, a down-regulation of MHC class I molecules would activate NK cells to eliminate the virally infected cell. To counteract this, CMV has developed several mechanisms to avoid the cytotoxic actions of NK cells. Two CMV encoded MHC class I homologues have been described; UL18 and UL142 [157, 158] although their importance in NK cell evasion is debated. NK cell activation is mainly regulated by the expression of activating and inhibitory receptors on the NK cell surface. CMV uses several proteins to prevent binding to NKG2D, one of the most prominent NK cell activating receptors. CMV protein UL16 binds to NKG2D activating ligands often expressed on infected cells, thus preventing NKG2D mediated NK cell activation [159]. UL16 is also believed to protect infected cells from cytolytic proteins released from activated NK cells [160].

To counteract the action of CMV specific antibodies, the virus expresses two Fc receptor homologues; UL119-118 and TRL11/IRL11. These molecules may bind in an unspecific fashion to virally infected cells, thereby masking viral antigens displayed on the cell surface [161, 162]. Also, activation of complement is hampered by viral induction of cellular proteins CD35, CD46 and CD55, that inhibit opsonization by phagocytosing cells and complement-mediated cell lysis [163, 164].

DCs and macrophages are vital cells of the immune system. DCs are the most potent APCs, inducing the innate immunity and regulating the subsequent adaptive immunity, while macrophages are potent phagocytosing cells. CMV has been described to interfere with their functions in multiple ways; differentiation of monocytes into both DCs and macrophages *in vitro* is hampered resulting in cells with a reduced endocytocis and migration capacity [165, 166]. CMV also decreases the expression of MHC class I and class II molecules and costimulatory molecules on DCs, as well as hamper their cytokine release [167, 168]. As mentioned above, CMV encodes an IL-10 homologue with strong inhibitory effects on dendritic cells [169]. Also, CMV encodes chemokine homologues which may contribute to viral production and viral spread by attracting CMV susceptible neutrophils [170]. In addition, CMV induce secretion of several host cell encoded pro- and anti-inflammatory cytokines and chemokines important in regulating the immune response [28], possibly contributing to the high cytokine serum levels often seen in patients with an active CMV infection.

In addition to counteract effector cell activation and recognition, CMV has developed multiple mechanisms to inhibit apoptosis of the infected cell; CMV genes IE1, IE2, UL36, UL37 and UL144 have been shown to block apoptosis [171-174]. Induction of apoptosis is a central mechanism in the host immune system, being an efficient method to eliminate both virally infected cells and tumour cells.

CANCER; WHAT, WHY AND WHEN?

How does a tumour develop? It is caused by instability of the genome, first and foremost mutations in genes regulating cellular proliferation. Having stated that, it is notable that this is not primarily a fully hereditary disease (with a few exceptions), environmental factor play a very important role. However, predisposing genetic set ups are found in many cancer forms. The influence of environmental factors is illustrated by the global pattern of cancer incidence. Every corner of the world has its own high risk patterns for different cancers and when migrating, people tend to acquire the risk pattern of their place of residence rather than their place of origin, which would be expected if this was this predominantly a hereditary form of disease. No, to get cancer several incidents are required, as well as a mix of genetic and environmental occurrences like a chain of events.

ONCOGENES

There are two major classes of tumour inducing genes, the oncogenes that unnaturally forcefully promote cell division and the tumour suppressor genes that loose their ability to control cell division. The making of an oncogene from the original, normal allele, the proto oncogene, can occur in several different ways; a) deletion or point mutation changing the function, b) gene amplification rendering too many copies of the gene, c) chromosome rearrangement when the gene segment moves to another position and may be hyperactive by proximity to a strong enhancer or fusion to an actively transcribed gene or d) insertion of a pathogen derived oncogene, pushing cell division forward. A selection of oncogenes is listed in table 1. Further cancer promoting damages of the cellular genome is facilitated by the genomic instability of tumours cells. Tumour cells are constitutively transcriptionally active and since most check points of correct DNA replication has been inactivated or circumvented, the cell is vulnerable to further mutations.

TABLE 1

Oncogene	Oncogene origin	Proto-oncogene function
N-MYC	DNA amplification	Transcription factor
v-fos	Viral homologue	Transcription factor
Erb A	Viral homologue	Transcription factor
BCL2	Chromosomal translocation	Anti apoptotic protein
MDM2	DNA amplification	Forms complex with p53
EGFR	DNA amplification	EGF receptor
TRK	DNA transfection	NGF receptor
SRC	Viral homologue	Protein tyrosine kinase
RAS (H-, K-, N-)	Viral homologue	GTPase

Selected oncogenes, from [175]

INFECTIONS AND CANCER

Approximately 20% of all cancers today are believed to be caused or promoted by infectious agents. Several viruses have been reported to cause cancer and other still promoting tumours initiated by other causes.

EBV

Epstein Barr virus (EBV) was discovered as a tumour virus in Burkitts lymphoma. It has been reported in Hodgkin- and non Hodgkin lymphoma and nasopharyngeal carcinoma as well. EBV is a γ-herpesvirus and establishes latency in infected resting B cells and epithelial cells. The genes responsible for immortalization, primarily the EBNAs (Epstein-Barr virus Nuclear Antigen) and LMPs are episomally arranged and expressed during latency. They are believed to be essential both for immune evasion and cellular transformation (as reviewed in [176]).

HHV8

Human Herpesvirus 8 (HHV8) is also called Kaposis sarcoma herpesvirus because of its relation to the aetiology of Kaposis sarcoma, a cancer type mainly affecting immunocompromised individuals, heavily associated with HIV infection. Like EBV, it belongs to the γ-herpesvirus family. Both genes expressed during latent and active infection are involved in oncogenesis, where the latent genes are believed to be in charge of the actual oncogenic properties, while the active infection genes promote tumour development and angiogenesis (as reviewed in [177]).

HPV

Human Papilloma virus (HPV), are DNA viruses and there are many types of HPV, of which only a few are heavily linked to cancer. It is a sexually transmitted virus, infecting the immature cells of the epidermis of the genital tract and oral mucosa and can give rise to cancer in these areas. After infection, parts of the HPV genome can be incorporated into the host genome and it is then that the oncogenic proteins E6 and E7 can exert its oncogenic potential (as reviewed in [178]).

HBV/HCV

Hepatitis B and C virus (HBV, HCV) are the major risk factors for hepatocellular carconima. HBV is a DNA virus of the hepadna family and it is able to incorporate into the host cell genome. However, there are no universal incorporation sites identified and it does not appear essential for oncogenesis since 20% of HBV positive cancers do not show any signs of incorporation. Chronic HBV infection is the main causal factor for hepatocellular carcinoma. HBV exerts oncogenesis via the HB x gene which in turn interacts with oncogenes *c-myc* and *c-myb* as well as tumour suppressor genes such as *p53*. HCV on the other hand is an RNA virus that does not incorporate into the host genome. The mechanism of oncogenesis is not fully understood although several HCV

proteins have been found to have oncogenic properties *in vitro* and in animal models. It is also known that chronic HCV infection of the liver is correlated to extensive angiogenesis which may in turn be linked to high COX-2 expression and multidrug resistant patterns of the tumour (as reviewed in [179, 180]).

HTLV-1

Human T cell lymphotropic virus, type 1 (HTLV-1) is a retrovirus able to transform and immortalize T cells. It is associated to T-cell leukemia and lymphoma and its' oncogenic properties are exerted by the Tx protein which can regulate the cell cycle and inhibit tumour suppressors (as reviewed in [181]).

CMV

The role of CMV as a tumorigenic virus has been debated for decades. At present there is no evidence of tumorigenicity but instead, the term oncomodulation has been used for CMV. This is discussed below.

True for all above mentioned viruses is that only a minority of individuals that attract the infection get the tumour, but the vast majority of those that get the tumour have attracted the infection.

Bacterial as well as parasitic infections have also been associated to tumour development (e.g. *Helicobacter pylorii* and gastric cancer, *Shistosoma haematobium* and bladder, gastric and liver cancer) however they will not be further discussed here.

TUMOR CELL GROWTH

The primary requirement for a tumour to form is unrestricted cell division. This can be achieved either by actively support growth signalling independently of the environment, overriding proliferation regulatory pathways or losing the ability for apoptosis. But it takes more to make a malignant tumour; solid tumours need the ability to stimulate angiogenesis for supply of oxygen and nutrients and the tumour cells need the ability to pass lamina to grow invasively and metastasize (as reviewed in [182, 183]).

It is in this case important to remember the function of a normal cell. The cell will proliferate only in response to growth stimuli and proceed through the cell cycle only when check points have been passed. Otherwise the cell will go quiescent or undergo apoptosis either by the intrinsic pathway mediated by p53 and resulting in mitochondrial release of cytochrome c, or the extrinsic pathway in response to binding of death receptors such as the Fas/Fas ligand interaction or the binding of TNFα to TNF receptor 1. However activated, the order of apoptosis will be carried further by the caspase cascade, a highly regulated chain of protein interactions executing the death program. Every time the cell divides, the end of the chromosomes, the telomeres, are

shortened just a little bit. After a certain number of cell divisions, the telomeres are used up and the cell will detect chromosomal damage and undergo apoptosis.

But in a cancer cell, no external growth factors from the surrounding cells are necessary as the cell can trigger itself by autocrine stimulation (glioblastomas producing PDGF and TGFα), receptors can be over expressed or constitutively activated (EGFR up regulation in brain and breast tumours) or the intracellular signalling pathways may be defective (the oncogene Ras activating the MAPK mitogenic cascade). In addition, as soon as a tumour cell has divided enough to be surrounded by neighbouring tumour cells, they stimulate each other by cell-to-cell growth signalling (as reviewed in [182]). It has even been suggested that tumour cells persuade surrounding, non tumour cells such as fibroblasts, to provide extra proliferative signals [184]. The cancer cells also evade antigrowth signals. These signals are mainly mediated by non phosphorylated pRb which bind to E2F transcription factors and thereby inhibit replication. When phosphorylated, pRb releases the E2Fs and replication proceeds. The pRb function is lost in many types of tumours, further promoting perpetual cell growth. Loss of pRb function is could be mediated by mutation of the Rb gene, by viral oncogenic influence, like that of human papilloma virus protein E7 or by TGF-β related mechanisms as TGF-β is the regulator of pRb phosphorylation. Immature cells generally divide more than differentiated cells, and therefore evading terminal differentiation is another route of the tumour cell to keep up proliferation. The oncogene *cmyc* disrupts the balance within the Myc-Mad-Max complex interplay blocking differentiation and in colon cancer, the APC-β-catenin pathway blocks differentiation in the colonic crypts (as reviewed in [182]).

INHIBITION OF APOPTOSIS

One of the most studied phenomenon of tumour cells is their capacity to avoid apoptosis, which in turn has taught us a lot about the apoptotic mechanisms in the process. The apoptosis evading mechanisms are many; p53 could be mutated (occurs in approximately 50% of all cancers) [185] or inactivated by protein binding, the PI3 kinase-AKT/PKB anti apoptotic pathway can be hyper activated by Ras activation or loss of repressors or inhibiting Fas death receptor activation by expressing a decoy receptor quenching the signalling [186]. In order to completely "breach the mortality barrier" [182], tumour cells bypass the telomere shortening system mentioned above either by the enzyme telomerase or the ALT (Alternative Lengthening of Telomeres) mechanism, both resulting in telomerase perseverance. It is estimated that telomere maintenance can be found in all cancer forms [187, 188].

ANGIOGENESIS

As the tumour cells keep growing, unrestricted by contact inhibition or other stop signals, the supply of oxygen and nutrients will begin to cease. The tumour needs to stimulate angiogenesis in order to get new blood vessels into the tumour area. Solid tumours that cannot master this will not grow to any large extent and may remain

benign. Tumour cells are more resistant to hypoxia than most normal cells, but the rapid growth requires a continuous supply of nutrients, as reviewed by [182]. Tumours cells frequently over express VEGF and FGF or increase their release by recruiting endothelial cells and promoting vessel formation and by down regulating expression of inhibitors such as thrombospondin-1 [189]. Thrombospondin-1 may also be regulated by p53, which means the loss of p53 function is related to decreased levels of this inhibitor [190].

INVASION AND METASTASIS

Last but not least, aggressive tumours grow invasively and they metastasize and it is estimated that 90% of all cancer deaths are due to metastases [191]. Invasion and metastasis are aided by several mechanisms, like the loss of E-cadherin expression on tumour cell surface due to mutations or proteolysis [192], changes in N-CAM appearance from a highly adhesive to a low adhesive form [193] and changes in cell surface integrin profiles [194, 195]. In addition, in order to protrude extracellular matrix, matrix degrading proteases are of the essence. The expression of proteases is increased, protease inhibitors are down regulated and inactive forms of proteases are activated (as reviewed in [182]).

CANCER STEM CELLS

Is there a single cellular source of cancer and if so, can it be isolated? The tough nut to crack in many cancer treatments is how to get rid of the whole tumour and nothing but the tumour to ascertain minimal risk of the cancer returning and minimal risk of damage to surrounding tissue. Radical surgery, whenever possible, is the obvious reply to this statement, but that is rarely practically possible. In most cases, some cells remains. By subsequent treatment like radiation and chemotherapy we may still cure the cancer, but every day there are many proofs of the contrary. Would treatment success rise if we could eradicate the root, the basis, and the foundations of the tumour? It is safe to say that it is most probably so.

The theory of the cancer stem cell is the theory of a small subpopulation of tumour cells being long-lived, highly resistant to standard cancer treatment and a possible source of recurrent disease. The cancer stem cell is the foundation, dividing into new cancer stem cells as well as cancer supporting tumour cells and without it, the tumour would not be as aggressive. Their progeny, also capable of division themselves, is rather the source of tumour bulk. Cancer stem cells have been isolated from many but not all solid tumours including glioblastoma, medulloblastoma, astrocytomas, prostate cancer, breast cancer etc. It has been suggested that cancer stem cells are somatic stem or progenitor cells transformed by mutations or other alterations of their proliferative pattern. It is believed that some tumours simply do not have cancer stem cells, but constitutes a more homogenous tumour mass where all cells contribute equally to tumour growth. Compelling pieces of evidence for cancer stem cells are increased

growth and chemotherapy resistance *in vitro* in isolated cancer stem cell population as well as unsurpassed tumorigenicity in animal models [196] observed when establishing tumours in animals by injecting tumour cells. Cancer stem cells are not easily susceptible to conventional therapies. This property may be exactly what will form the basis for future cancer treatment, targeting specifically the cancer stem cells. An evident obstacle is how to detect and target them *in vivo*, taking care not to harm healthy stem cell pools. At present, cancer stem cells are isolated based on expression of regular stem cell markers like CD133, nestin, integrin patterns, CD34 etc. (as reviewed in [197-199]).

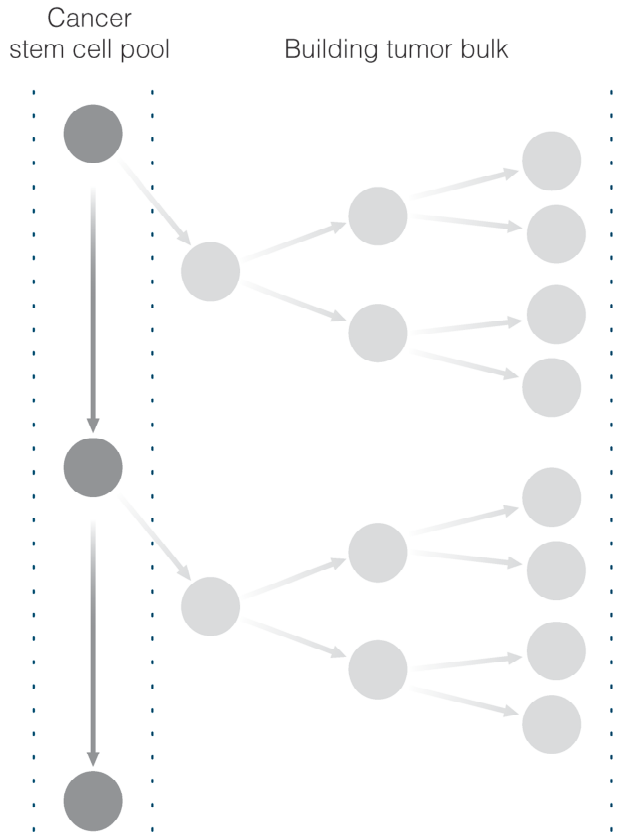

Figure 7. Schematic picture of cancer stem cell growth

IMMUNE SURVEILLANCE?

Cancer happens all the time. At least it should considering that during a human life time, there will be 10^{16} cell divisions. During these 10^{16} cell divisions every single gene will experience 10^{10} mutations and although just a small percentage of these may be tumour promoting, the actual number of oncogenic mutations would logically be high. However "only" one in five individuals will at some point in life get cancer [183, 191]. And it may be benign. It may not even be noticed. But it may also be fatal within a dramatically short period of time.

It is possible that our immune system is able to protect us from tumours to a certain extent and that tumour cells are being kept under close observation. This has been

termed “immune surveillance” and has been a much debated hypothesis. However, it has been shown that cancer patients a) do generate an immune response against tumour antigens, although this response is rather weak, b) immune cells infiltrate tumour tissue and c) immunosuppressed individuals suffer a higher risk of developing tumours. However, the role of the immune system as an adversary in cancer development is not clear. In fact, it may be the other way around. In sites of inflammation, where the immune response is evidently active, the risk of tumour development is higher, e.g. inflammatory bowel disease as well as colon cancer and inflammation caused by irritants of the lower airways predispose for lung cancer. Animal studies also suggest a role for macrophages as accomplices in tumour development through the NFκB pathway. The environment created by infiltrating immune cells contribute to tumour cell mutations and result in a process of “immunosculpting” or “immunoediting”, meaning a situation where the tumour is not only evading the immune system, but manipulating it. Especially macrophages have been reported to aid in tumour progression, where by a paracrine loop macrophages stimulated the growth of breast cancer cells. The macrophages produce and release EGF, with promoted cell growth among the breast cancer cells and the tumour cells in response stimulated the macrophages further by releasing colony stimulating factor 1 (CSF-1) (as reviewed in [200]).

Facts remain, that under some circumstances, the immune system can recognize and kill tumour cells. NK cells, dendritic cells and T cells recognize tumour antigens, tumour associated antigens (TAA) or tumour specific antigens (TSA) displayed by MHC class I, and possibly MHC class II. Tumour antigens include mutation specific antigens (e.g. p53 and RAS), differentiation antigens (mucin), over expressed antigens (WT, MMD2) or tumour type specific antigens such as cancer testis antigen or the rearranged immunoglobulins in B cell neoplasms. However, most of these antigens are also present to some extent on non tumour cells, which poses a problem since immune cells responsive to these targets may get eliminated during maturation (as reviewed in [201]).

CMV AND CANCER

CMV AS AN ONCOGEN, A HISTORICAL PERSPECTIVE

CMV was first implied as an oncovirus in 1970s by Rapp et al. who observed CMV in prostate tumours [202] and isolated a virus strain from tumours that was oncogenic in animal models [203]. In 1980 Giraldo et al. suggested a link to Kaposis sarcoma [204]. Considering the biological properties of CMV, it was the perfect candidate for oncogenesis. It can persist in its host for a long time, modulate immune responses, manipulate the whole cellular machinery and clinical findings suggested a link. However, in order to be a proper oncovirus, a virus should fulfil the classical Koch's postulate of correlation between pathogen and the disease. Sanford and Geder et al. isolated CMV from the prostate tumour where they found it and managed to show that it could transform fibroblasts *in vitro* and was oncogenic in an animal model [205]. However, these results were never repeated elsewhere and the idea of CMV as an oncogenic virus was abandoned.

CMV IN TUMOR TISSUE

In 2002, Cobbs et al. reported a remarkably high prevalence of CMV proteins and nucleic acids in glioblastoma tissue samples. The pattern was not the same as seen in CMV inclusion disease, the hallmark "owls eye" staining and plenty of cytomegalic cells. Instead, a rather a weak expression of IE and L proteins was observed in almost every cancer cell in 100% of examined patients. Importantly, the surrounding healthy brain tissue was negative [206]. This was in accordance with a case report from 1991 where an AIDS patient with astrocytoma also was shown to have CMV positive tumour cells and negative areas surrounding it [207].

Several additional reports where to come from the laboratory of Dr Cobbs as well as others involving both glioblastoma and other types of cancer but presenting the same findings; a majority of cells positive for CMV proteins and nucleic acids, but at low levels. Tumours investigated included brain [206, 208-210], colon [211] and prostate [212]. In our laboratory, we have found CMV proteins and DNA in a majority of glioblastoma, cancers of the colon, prostate, breast, ovaries and cervix as well as melanoma and sarcoma (manuscripts in preparation). Connections to T-cell lymphoma have been made without evidence of CMV in actual tumour cells but based on serological findings [213]. In a case study of a patient suffering from severe neuroblastoma, Nigro et al. have suggested a connection between neuroblastoma and CMV. They observed active CMV infection by serology, viremia tests and virus culture both at time of neuroblastoma diagnosis and at the time of recurrence [214]. Since neuroblastoma is a tumour of immature neural cells and occurs early in life, and CMV is known to targets immature neural cells during foetal development it is possible that CMV could be present and play a role in tumour aetiology and/or progression. Furthermore, Fortunato et al. observed that CMV causes specific chromosomal breaks

at 1q21 and 1q42 [215], just the site where copy number variations have been linked to neuroblastoma [216]. We decided to test whether CMV is indeed detected in neuroblastoma tumours. In **paper III**, we examined 36 neuroblastoma tumour tissues and found that CMV proteins could be detected in 97% of investigated samples. IE proteins were more often detected than L proteins (97% vs. 87%) and when grading the tissues based on estimated number of positive cells, we observed that a majority of tissues showed high grade expression of IE proteins, but low grade expression of L. The same pattern was observed in medulloblastoma tumour tissues, where 87% where positive for CMV IE proteins and 77% for L. These tumours also showed a similar pattern in infection grade with a majority of tissues being high grade IE and low grade L (**paper IV**).

It should be mentioned that the findings of CMV in tumour tissue is still a controversial issue and there are contradictory findings by [217, 218]. The differences in results can be due to differing experimental protocols. However, since all laboratories examined tissues from different origins, this cannot be confirmed at present. It is difficult to imagine that the dramatic differences in results should mirror an actual difference in prevalence between examined patient groups.

If CMV is contributing to tumour formation or development, there ought to be a correlation between severity of infection and severity of disease. For glioblastoma, this seems indeed to be the case. Scheurer et al. reported on higher levels of CMV proteins in more malignant tumours. In the group of grade IV glioblastoma multiforme, the highest grade of malignancy in gliomas, 79% of investigated tissues were positive for CMV by immunohistochemistry and in situ hybridization compared to 48% for lower grade tumours [210]. This is in accordance with results from our laboratory showing that glioblastoma patients with low levels of CMV antigens in the tumour survives longer after glioblastoma diagnosis than those with high level of infection. Patients with low grade CMV infection have a mean survival of 42 months compared to 14 months in patients with high grade CMV infection in the tumour at diagnosis, p=0.008 (Rahbar et al, submitted manuscript). Although a clear tendency of differences in infection grade, in neither neuroblastoma nor medulloblastoma was it possible to evaluate the prognostive influence of CMV due to small sample sizes and a highly heterogeneous patient groups (**paper III and IV**).

CMV AS AN ONCOMODULATOR

So CMV has not been shown to transform viruses since the work from Geder, Sanford et al. back in the 1970s [205]. The current hypothesis on CMV and cancer is instead evolving around the term oncomodulation, suggested by Cinatl in a review article from 1996 [219]. This means that CMV is not causative, but in predisposed cells, CMV can influence tumour progression and promote a more aggressive tumour phenotype.

Bentz et al. reported that CMV stimulates endothelial cell proliferation and motility, resulting in angiogenesis simply by binding to host cell surface. This effect was dependent on binding to host cell EGFR and β_1 and β_3 integrin, which in turn activated PI3 kinase and MAPK pathway. Authors state that this was not dependent on VEGFR, neither viral binding thereof nor autocrine activation upon stimulated VEGF release, since VEGFR neutralizing antibodies did not influence viral effect [220]. This is in contrast to the data from the laboratory of Martine Smit mentioned below, where CMV infection caused subsequent up regulation of VEGF [221]. However, Bentz et al. have used another target cell, the human endothelial cell itself while Smits lab used mouse fibroblasts. Moreover, Bentz et al. used neutralizing antibodies toward VEGFR1 and 2, but not VEGFR3 [222], which has been shown to play an important role in tumour progression [223], and KSHV has been reported to up regulate this receptor on endothelial cells during infection [224].

CMV ONCOMODULATORY PROTEINS

CMV present an impressive array of biological properties related to tumour development. These include increased proliferation, inhibition of apoptosis, increased migration, release of stimulatory factors, increased angiogenesis, chemotherapy resistance and telomerase activity. The most prominent effector proteins are listen here:

IE1 AND IE2

IE1 and IE2 inhibit apoptosis when transfected into HeLa cells [171]. CMV infection has been reported to elevate p53 levels in infected cells [225] and IE2 has been reported to associate with p53 [226], suggesting that IE2 binding of p53 is one of the mechanisms for apoptosis blockage reported by Zhu et al. IE2 can also inhibit p53 functions by binding to histone acetyltransferases of p53 co activators and block p53 dependent gene activation [227]. However, IE1 seems to be working in a p53 independent manner. IE1 has been reported to deregulate PI3 kinase pathway, and interfere with Rb apoptotic functions in transfected glioblastoma cell lines. It is however interesting to note that in some glioblastoma cell lines IE1 transfection resulted in growth arrest rather than increased proliferation [228]. In addition, CMV infection induces telomerase activation in fibroblasts, thereby possibly inhibiting growth arrest and apoptosis. This effect is most probably mediated by IE1 activation of the telomerase associated promoter, hTERT [229].

PPUL97

ppUL97, a virus encoded kinase, hyperphosphorylates pRb [230] and may prevent pRb from blocking G1 to S-phase continuation of the cell cycle. Moreover, ppUL97 was recently reported to mimic the enzymatic properties of cyclin dependent kinase-1 (CDK-1), showing preferences for CDK-1 favourite binding sites [231]. CDK-1 is an important mitogenic mediator, regulating the G2 to M-phase transition and maintaining

the cell in a mitotic state [232]. Indeed, Hume et al. reported that ppUL97 does exert CDK functions and that ppUL97 in addition is unresponsive to the factors regulating normal CDK effect. Therefore, ppUL97 may stimulate unguarded proliferation [233].

PPUL144

ppUL144 is a TNF receptor homologue, although it does not appear to bind any ligands from the TNF family. Instead, it has been suggested to inhibit apoptosis, however, the mechanism for this is yet unknown (as reviewed in [234]).

VICA AND VMIA

(viral Inhibitor of Caspase-8-induced Apoptosis and Mitochondria-localized Inhibitor of Apoptosis)
vICA (*UL36*) and vMIA (*UL37*) blocks apoptosis. vICA does so by inhibiting caspase 8 activation. vMIA is situated in the mitochondrial membrane, inhibiting cytochrome c release. vMIA also neutralizes the activity of Bax (as reviewed in [234]).

PUL38

pUL38 blocks apoptosis by inhibiting the proteolytic cleaving of caspase 3 [235].

PP71

The primary function of pp71 is to stimulate the expression of IE proteins via MIEP. Since pp71 is a phospho-protein of the tegument layer, it is released fully functional immediately upon viral entry. In addition, pp71 can stimulate quiescent cells to enter the cell cycle by binding and degrading tumour suppressor pRb [236].

US28

US28 is a G-protein coupled receptor (GPCR) homologue expressed on the surface of infected cells, that is constitutively active and signals independent of ligand binding. It does however bind to β chemokines with strong affinity. US28 has been reported to be involved in key cancer cell functions such as cell migration [237] and angiogenesis [238]. Lately it was reported that US28 could induce a transformed phenotype in fibroblasts *in vitro* and *in vivo* and that these effects are due to induction of vascular endothelial growth factor (VEGF) expression via up regulation of cyclooxygenase-2 (COX-2) in infected cells [221].

All of the above mentioned viral proteins belong to the IE and E classes. As already stated, the IE/E proteins are responsible for the majority of host cell manipulation effects. It is therefore not surprising that we find less L expression in both neuroblastoma and medulloblastoma (**paper III and IV**). We hypothesize, that this expression patterns mirrors a situation characterized by priority of tumour progression rather than viral production. It is most likely not a question of high viral progeny production in these tumours, if any such production even goes on at all. Cobbs et al.

observed CMV like particles by electron microscopy in glioblastoma, although rare, indicating that new CMV particles really are formed, at least in the glioblastoma tumours [206]. Are these viral particles still infectious? This could of course be tested by trying to isolate infectious virus particles from fresh tumour tissue or primary tissue cultures, a very interesting experiment indeed. If no such virus particles were to be discovered, one might argue that a) the infection is on a very low level, close to latency, and therefore almost non productive. Still, with the right stimuli this infection could be reactivated and the virus isolated or b) the role of viral host cell manipulation is no longer driven by the virus but in a way hijacked by the tumour, favouring tumour progression rather than ultimate virus production. However, this has proved very difficult to achieve in practice.

On the other hand, there also reports on that viral proteins mediate decreased proliferation (**paper I and II,** [228]), promote differentiation [239], increase apoptosis (**paper I and II**) and induce cell cycle arrest [228, 240]. This is most probably a question of host cell type, viral strain and other environmental influences. Some cells support a lytic infection and die from infection whilst other, like many immortalized cell lines do not even display cytopathic effect. However in the case of the findings from Cobbs et al., it is interesting to note that even tumour cells can be inhibited in their proliferative capacity by CMV [228].

CHILDHOOD CANCERS AND EMBRYONAL NEURAL TUMORS

Cancer in children is less common than cancers in adults, and with distinct different biological features. Children with cancer have today a much improved chance of cure with more than 75% being long-term survivors [241]. Each year approximately 16 children per 100 000 children below 15 years of age are diagnosed with a malignancy, i.e. almost 300 per year in Sweden [241]. The overall childhood cancer incidence, and survival, in the Nordic countries is the highest among all international reports, but in contrast to the rest of Europe the incidence is not increasing significantly [242, 243]. Leukemias, brain tumours and other solid tumours are the largest diagnostic groups with 31%, 28% and 41% each, with the embryonal tumours medulloblastoma and neuroblastoma as two of the most common solid tumours, constituting 5.7% and 4.8% respectively, of all childhood malignancies. However, these tumours are still having a relatively poor survival with 9.2% and 9.6% respectively, of all deaths due to cancer in children.

The aetiology of childhood cancers remains mainly obscure, although convincing evidence from studies in both biology and epidemiology indicates that these tumours, in particular embryonal tumours, may be considered as developmental disorders [244-246].

NEUROBLASTOMA

Neuroblastoma is the second most common cause of death in infants in the western world, only accidents take more victims. 90% of the cases are diagnosed before 5 years of age. Approximately half of cases are categorized as high risk and the survival for this group is less than 40%, despite heavy treatment (as reviewed in [247]). Recent Swedish population based data however, indicate a significant improvement in prognosis for children with high-risk neuroblastoma during the last decade [248].

CLINICAL FEATURES OF NEUROBLASTOMA

The neuroblastoma tumours can arise anywhere along the sympathetic nervous system, but in most cases the primary tumour is formed in the abdomen, by the adrenal medullas or in the neck, chest or pelvis. Tumours form from immature neuro ectodermal cells of the neural crest. This is the cell population that during foetal development will become adrenal medulla and sympathetic ganglia. Metastases are common and typically form along the routes of the nervous system, through the lymphatic system or in distant sites such as cortical bone, bone marrow or the liver. Already at time of diagnosis, 40% of all cases present with metastases.

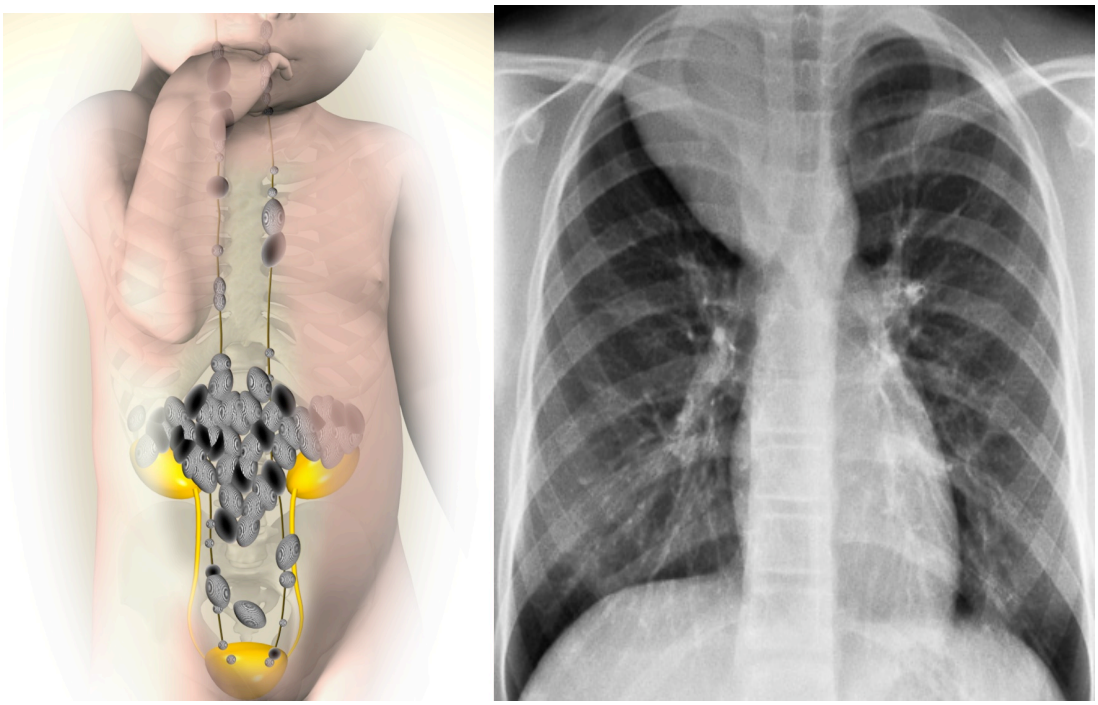

Figure 8. Schematic image of neuroblastoma in the abdomen, and X-ray showing neuroblastoma in the mediastinum. (Pictures from Per Kogner, printed with permission)

Neuroblastoma is a tumour disease with very heterogeneous clinical features and the symptoms depend a) on where the primary tumour is situated, b) if and in that case where the metastases have developed and c) if the patient develops paraneoplastic syndrome. Paraneoplastic syndrome includes symptoms derived from excretion of hormones or cytokines from the tumour cells or host immune response to the tumour. The most common symptoms are diffuse, the patient often present with pain (34%), fever (28%) and weight loss (21%). More specific and highly indicative symptoms are lower limb paresis, severe diarrhoea that is unresponsive to standard treatment, acute cerebellar encephalopathy or hypertension. However, these symptoms are rare, they each occur in less than 5% of patients. Some completely asymptomatic tumours are diagnosed by coincidence (as reviewed in [249]).

A fascinating trait of neuroblastoma tumours is their ability to spontaneously regress. This happens occasionally in most cancer types, but for neuroblastoma it is 10-100 times more common than with other cancers. In addition, some tumours mature into benign ganglioneuromas (as reviewed in [250]). To date, there are no clear explanations to why this occurs or any reliable marker for predicting which tumours will regress and which will remain.

Screening programs have been attempted to detect tumours early and thereby increasing treatment success. However, although the number of detected cases doubled, the number of severe cases detected was the same and no differences in survival were observed as a consequence of the screening program [251, 252]. Instead of being beneficial, it increases the risk of unnecessary treatment.

Neuroblastomas are divided into stages, depending on severity of disease (Described shortly in table 2).

TABLE 2

Stage	Clinical features	Risk group
1	Localized tumour with complete resection by surgery, no spread to non tumour-associated lymph nodes.	Low
2A	Localized tumour, incomplete surgical resection, no spread to non-adherent lymph nodes. Possible MYCN amplifications.	Low or intermediate risk depending on level of surgical resection. High risk if tumours show MYCN amplifications.
2B	Localized tumour, resection may be complete or incomplete. Spread to lymph nodes. MYCN amplifications	Low or intermediate risk depending on level of surgical resection. High risk if tumours show MYCN amplifications.
3	Unresectable tumour with tumour cells spread to lymph nodes. Tumour and/or lymphatic spread can be unilateral or bilateral. MYCN amplifications can occur.	Intermediate risk. High risk for children above 18 months at diagnosis, especially for those with MYCN amplifications.
4	Any primary tumour with heavy metastases to lymph nodes, bone marrow, one, liver, skin or other organs. MYCN amplifications occur.	Mostly high risk, but children below one year at diagnosis or without MYCN amplifications have intermediate risk.
4S	Localized tumour as in stage 1 and 2, but with metastases to skin, liver or bone marrow. Diagnosed in infants below one year of age.	Low to high risk depending on age at diagnosis, MYCN amplifications and if disease is symptomatic

(reviewed in [247])

Up until recently, the only tools in predicting neuroblastoma prognosis was by looking at disease stage and age at diagnosis. These blunt tools clearly results in over estimation of treatment need. Prognostic value increases when also evaluating occurrence of MYCN amplifications, DNA content, differentiation marker expression, NTF receptor expression and chromosomal changes (as reviewed in [253]) displayed in table 3.

TABLE 3

Good prognosis	Poor prognosis
Diagnosis early in life	Diagnosis > one year of age
Near-triploidy	Metastasis at diagnosis
TrkA expression	MYCN amplification
Differentiation markers expressed by tumour cells	Allelic loss at 1p
	Combined MYCN amplification and TrkB expression
	Chromosomal alterations detected by multivariate analysis [254, 255].

NEUROBLASTOMA GENETICS

Several studies describe a sub population of neuroblastomas caused by hereditary factors. In these studies, it was suggested that approximately 20% of neuroblastomas arise from autosomally dominant germinal mutations and that this sub population has a lower mean age at diagnosis (9 months vs 18 months) (as reviewed in [256]). A twin study showed that for very young children, there is a concordance for neuroblastoma development, but for older twin pairs no such concordance was observed [257]. This is cause for speculation that early neuroblastomas may be more related to inherited predisposition whilst neuroblastoma in older children is the result of random mutations and other environmental influences.

CELLULAR FEATURES OF NEUROBLASTOMA

MYCN AMPLIFICATIONS

MYCN amplifications are detected in 20% of neuroblastoma tumours and are correlated to poor prognosis both in high and low stages. Therefore it has been added to determinants for prognosis. Yet, it underestimates number of severe cases since they only occur in 30% of high stage neuroblastomas. In normal cells, MYCN proteins associate with the molecule Max to form transcription activator ultimately pushing cell cycle progression through G_1. In absence of MYCN, Max instead turns into a suppressor (as reviewed in [256]). The number of MYCN copies correlates to the amount of MYCN protein and despite a short half life, a high protein production can still reach MYCN levels 100 times that of a normal cell. It does not seem very farfetched to conclude that this would promote continued cell growth. MYCN amplifications and high MYCN protein levels in cell lines are correlated to high malignancy [258]. Interestingly, some neuroblastoma cell lines display elevated MYCN protein levels even without gene amplifications [259], implying other regulations of MYCN protein levels than based on transcription. In the study of Nakagawara et al.,

MYCN protein levels was only correlated to a degree of malignancy when associated with MYCN amplifications [258].

CHROMOSOMAL CHANGES, 1P DELETIONS AND OTHERS

1p deletions can be detected in 30% of neuroblastoma tumours with an over representation in high stage tumours [260, 261]. It is highly associated with MYCN amplifications and there is no clear evidence of 1p deletions being an isolated prognostic factor or only observed in association with MYCN amplifications [260]. When isolating the chromosomal deletion of 1p as correlated to neuroblastoma, it was hypothesized that affected sites on this chromosome harbour the neuroblastoma suppressor gene and that loss of this gene inheritably gave rise to neuroblastoma. However, no such gene was identified and more recent theories imply that it is a mixture of several genes affected by the allelic loss at 1p [262]. Other chromosomal abnormalities reported in neuroblastoma include allelic loss of 11q, 17q gain (as reviewed in [253]), and deletions in 2q, 3p, 4p, 9p, 14q, 16p and 19q (as reviewed in [249]).

NEUROTROPHINS AND THEIR RECEPTORS

When the early neuroblasts differentiate into normal, functional cells, they do so by responding to the stimuli from the neurotrophin signalling pathway. The receptors, termed Tropomyosin receptor kinase (Trk) where first discovered as an the product of an oncogene [263]. The receptors TrkA, B and C bind their respective ligands nerve growth factor (NGF) (A), brain-derived neurotrpohic factor (BDNF) and neurotrophin-4 (NT-4) (B) and neurotrophin-3 (NT3) (C). When binding to NGF, TrkA induces differentiation, but in the absence of NGF, TrkA causes induction of apoptosis (as reviewed in [264]).

Trk A expression has been associated to young patient age and positive outcome [265]. Since TrkAs primary function is differentiation, it is believed to aid in maturation of tumour cells into less aggressive types as perhaps in tumour regress as well. Whatever the mechanism is, it may be related to its negative correlation to MYCN amplifications [258]. Trk C seems to share the benign properties of TrkA [266]. Trk B on the other hand, is expressed in tumours of unfavourable outcome and is associated with MYCN amplifications. In this case the tumour cells also express the ligand, BDNF enabling a paracrine or autocrine stimulation. The activation of TrkB results not only in tumour cell proliferation but also enhanced angiogenesis, invasiveness and drug resistance [267-269]. Trk signalling has been suggested as a possible chemotherapy target and there are substances, most of them TrkB inhibitors, currently being tested (as reviewed in [264]).

OTHER RELEVANT PROTEINS

Drug resistance in tumour cells are often connected to the expression of multidrug resistance (MDR) gene family. Expression of *MDR-1* has been linked to survival in neuroblastoma patients, where it was also found to be associated with observations of MYCN amplification [270].

The expression of telomerase, the enzyme maintaining the telomeres and working to sustain continued cell division, can be detected in many tumor types and is one of the hall marks of malignancy. Hiyama et al. investigated the telomerase expression in 100 neuroblastoma tumours and found expressed in 94% of them but in none of the investigated healthy adrenals or benign ganglioneuromas. High telomerase expression was linked to poor prognosis and associated with MYCN amplifications [271].

NEUROBLASTOMA TREATMENT

Treatment of neuroblastoma tumours is a multimodal approach including surgery, chemotherapy, radiation and drugs based on individual patient needs. Chemotherapy may or may not be combined with bone marrow rescue. Although hospitalization and treatment is a discomfort to anyone, heavy chemotherapy regimens as well as steroid treatments are harmful for a developing child and the risks of treatment sequelae are significant. Therefore, identifying markers that predict which tumors to treat and which will regress spontaneously is of the outmost importance. Clinical observations combined with data from animal studies indicate that non steroidal anti inflammatory drugs, (NSAIDs) improves disease outcome. The anti-angiogenic drug avastin has also been implicated as a possible choice of treatment after good results in treatment of adult tumours of the nervous system [272] and neuroblastoma animal models [273].

Two major challenges in neuroblastoma treatment remain; to improve treatment regimes to increase progression free survival without treatment sequelae and to improve prognostic markers to avoid over treatment.

CMV IN NEUROBLASTOMA

CMV as a mediator in the aetiology of neuroblastoma development is a compelling thought. Neuroblastoma originates in the early neural cells, cells that are permissive to infection *in vivo* [274] and *in vitro* [126]. The virus favours immature cells and effectively inhibits differentiation in several cell types **(paper I and II)** [165, 166, 275]. CMV has, through congenital infection, the opportunity to reach the foetus very early on in development. If CMV plays a role in neuroblastoma tumour cells, it must be able to exist in the tumour cells without causing an acute lytic infection and indeed, Cinatl et al. showed that neuroblastoma cells infected *in vitro* can remain persistently infected for more than a year in culture [276, 277]. Furthermore, Cinatl moved on to show that these cells displayed a more aggressive phenotype with resistance to cytotoxic agent, increased survival and increased invasiveness compared to non infected neuroblastoma cells. In addition, CMV infected cells expressed more MYCN protein than uninfected cells, suggesting a role for CMV in promoting cellular proliferation mediated by increased MYCN in neuroblastoma cells [277, 278].

The hypothesis of CMV in neuroblastoma is supported by clinical observations in the form of the case study from Nigro et al., describing a child suffering from 4S neuroblastoma. At first diagnosis, she presented with symptoms typically associated with congenital CMV in addition to her neuroblastoma and tested positive for CMV serology, CMV DNA-emia and virus culture. During early childhood she was also diagnosed with mental retardation. At five years of age she had a neuroblastoma relapse which was preceded by a mononucleosis like state and active CMV infection was determined by serology, DNA-emia and virus culture. In addition, she had decreased number of immune cells and here NK cell activity was low [214]. This case shows that neuroblastoma and CMV infection could be linked, however no proof was admitted of virus in the tumour cells.

Now we can show that 97% of examined neuroblastoma tissues are positive for CMV proteins. To our knowledge, we are the first to show CMV in the neuroblastoma tumour cells, both in tumour tissue samples and commonly used cell lines (**paper III**). Although we rarely found any CMV RNA or protein in neuroblastoma cell lines, when injected into an animal to establish xenograft tumours, the cells appeared to start expressing both early and late proteins (unpublished data). Is this a reactivation of latent CMV or simply a forceful up regulation of viral expression? This is difficult to say, but what we can hypothesize is that CMV protein expression could be stimulated by the complex environment with cell-cell contact, soluble factors and other influences from the surroundings available inside living tissue in contrast to a cell culture. In the neuroblastoma tissues examined we find both early and late proteins, indicating that it is an active infection going on. In order to mimic this active infection situation, we used *in vitro*-infected neuroblastoma cells to investigate the effect of active CMV on neuroblastoma cells. As Cinatl et al. stated already in 1996, CMV infection of neuroblastoma cells causes changes in gene expression, in particular those related to tumour progression properties [219, 276, 279]. In the light of our finding of CMV in tumour tissue, we propose a new strategy for cancer treatment based on combinations of chemotherapy and antiviral drugs.

MEDULLOBLASTOMA

Medulloblastoma is the most common malignant brain tumour in children, affecting approximately 6 children per one million children and year in Sweden [280]. It primarily affects young children, with a peak around 3 to 9 years of age, even though adult cases do occur. It is estimated that 1-2% of all adult brain tumours are medulloblastomas (as reviewed in [281]). For children, the five year survival is between 55% and 75% in Europe and United states with the highest survival rates reported in northern Europe [282]. The term “medulloblastoma” was introduced by Baily and Cushing in 1925 when they characterized this tumour as being formed from a CNS precursor cell, the medulloblast. However, no medulloblasts have ever been identified and their existence is questionable (reviewed in [283]). Later on,

medulloblastomas was instead incorporated into the group of primitive neuroectodermal tumours (PNETs) [284].

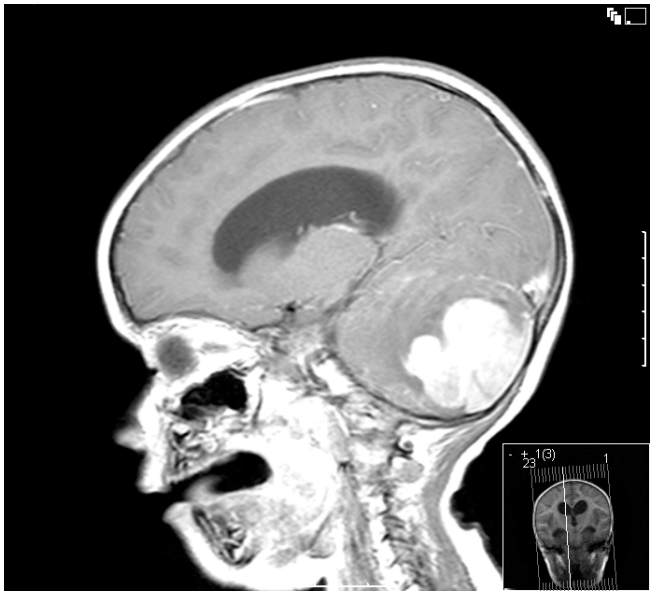

Figure 9. Medulloblastoma tumour. (Picture from Per Kogner, printed with permission)

CLINICAL FEATURES OF MEDULLOBLASTOMA

Medulloblastoma tumours arise from the posterior fossa or closely related areas and can readily spread along the ventricular surfaces, via the subarachnoidean space or along nerve roots. Metastases outside the brain are rare, but can occur and in such cases the bone marrow is the most common target. Patients with ventriculoperitoneal shunts also risk spread to the peritoneal cavity (as reviewed in [285]). Primary symptoms of medulloblastoma are often related to ventricular obstruction of cerebrospinal fluids and/or cerebellar dysfunction. About 75% of patients present with headache, vomiting and lethargy which are rather unspecific symptoms. If the tumour has invaded the brainstem, symptoms related to cranial nerve dysfunction develop or stiff neck and possible head tilt. In infants macrocephaly can be observed or "sun-setting sign", when the iris of the eye seems set below the rim of the lower eye lid. Symptoms are often progressive as the tumour grows and diagnosis is generally determined a couple of months after onset of symptoms. 30% of patients have disseminated disease at diagnosis, however this is rarely symptomatic (as reviewed in [285]).

CELLULAR FEATURES OF MEDULLOBLASTOMA

CELLULAR ORIGIN OF MEDULLOBLASTOMA

The tumour stems from immature cells in the germinal zones, the pool of stem and progenitor cells of the brain. It has been suggested that there are two different subpopulations of medulloblastoma, one arising from the ventricular zone and the other from the external granular layer, where the cells are generally considered slightly more mature and neural restricted. The tumours from the external granular layer form mostly

desmoplastic medulloblastomas and have a favourable outcome. The tumours form the ventricular zone cells, which are less lineage restricted, are termed classic midline medulloblastomas, and are related to a poorer prognosis. Furthermore, according to the review from Pietsch [286], tumours from these two sites of origin have different genetic markers [287] and therefore may be the result of different oncogenic insults. Classic midline medulloblastomas has been linked to loss of heterozygocity in 17p and MYC amplifications while the desmoplastic type is linked to loss of heterozygocity in 9q and the inactivation of *PTCH [288]*.

TABLE 4

Good prognosis	Poor prognosis
>3 years of age at diagnosis	<3 years of age at diagnosis
Local tumour, no metastasis	Disseminated disease at diagnosis
Total surgical resection	Minimal surgical resection
Increased expression of NT3 receptor (TrkC)	Tumour located outside the posterior fossa
Expression of γ- and β-catenine	Increased expression ERBB2
Increased expression of survivin	Increased expression of PDGFRα
	Increased levels of the RAS/MAPK pathway
	MYCN or *MYCC* amplifications
	p53 immunopositivity

(reviewed in [281])

PROGNOSTIC MARKERS

Medulloblastomas are divided into two groups, the average risk-group and the high risk-group. Risk assessment is primarily based on basic observations during surgery and by MRI. The average risk-group is characterized by localized tumour mass with non disseminated disease at time of diagnosis, extensive surgical resection and no tumour invasion of the brainstem. Tumours of the high risk-group show disseminated disease at diagnosis, minimal or at least subtotal surgical resection and possibly also invasion of the brainstem. It is debated whether brainstem invasion is really of predictive value or not. It has also been reported that individuals below three years of age at diagnosis have a poorer prognosis than older patients. This may be due to treatment bias of milder radiotherapy in young patients as they are more vulnerable to treatment sequelae. Another hypothesis is that there are biological differences between the medulloblastoma tumour cells in younger as compared to older children (as reviewed in [289]). A selection of the most significant prognostic markers identified for medulloblastoma are listed in table 4.

PTCH AND THE HEDGEHOG PATHWAY

PTCH is a part of the hedgehog signalling (Hh) pathway, coding for an Hh receptor expressed on the surface of developing cells. *PTCH* has a suppressive role in the

pathway so when it is silenced, there is an over activation of the Hh signalling. The interest in Hh signalling from a medulloblastoma point of view is easy to see; it regulates the proliferation of external granule cells, one of the possible cell sources for medulloblastoma. Activation of this signalling pathway inhibits differentiation and drives proliferation of the immature cells [290]. In addition to the *PTCH* mutation, other mutations within the Hh pathway have been identified in association to medulloblastoma, further strengthening the evidence that this pathway has crucial importance in medulloblastoma development, both for desmoplastic and classical medulloblastoma [291-293]. One report indicated that *Hh* too could be mutated in medulloblastomas [294] while other investigators found no such association [295, 296].

APC AND WNT

The *Wnt* (human homologue to the *Drosophila* gene *wingless*) pathway, similarly to the *Hh* pathway mentioned above, regulates proliferation and is implicated in several types of sporadic colon cancers (see Turcots syndrome, mentioned below). Wnt signals through a membrane receptor, Frizzled and its effector Dishevelled, and activates a complex of APC, β-catenine and other factors. When the signalling pathway is active, β-catenine translocates to the nucleus and stimulates transcription of oncogenes *c-myc* and *cyclin D*. Defects in this pathway in tumours is characterized by an over activation causing uncontrolled mitogenic stimulus. Several types of oncogenic disturbances are reported in this pathway and in medulloblastoma, missense mutations of AXIN1 [297], mutations rendering defective *APC* [298] and activating mutations of *β-catenine* are reported [299] all causing an increase of nuclear β-catenine resulting in increased proliferation.

ERBB2

The ERBB family is cell surface receptor tyrosine kinases that mediates growth signals by activating the PI3kinase and the MAPK pathways. Gilbertson et al. reported that ERBB2 was prominently expressed in 86% of investigated medulloblastoma tissue samples and that this expression, when co localized with another family member, ERBB4 and the ligand NRG1, was significantly correlated to disseminated disease at diagnosis [300]. In patients with over expression of ERBB2, survival was down to 30% [301].

PDGFRα

There is a study by MacDonald et al. which points to PDGFRα as predictor of metastasis formation and poor clinical outcome. Indeed, they found that increased expression of this receptor in metastasizing medulloblastomas, but not in those from localized tumours. PDGFRα readily activates the MAPK pathway in medulloblastoma cells, mediating several tumour promoting signals and this pathway is over activated in medulloblastoma cells. MacDonald et al. also showed that neutralizing antibodies against PDGFRα blocks cellular migration, suggesting PDGFRα as a therapeutic target to reduce formation of metastases [302]. Gilbertson et al. provided a possible

mechanism for this receptor up regulation by identifying an activating mutation in the PDGFRα gene, however this mutation was only found in 2 of 28 (7%) tumours tested [303].

MYCN AND MYCC

MYCN and MYCC have important roles in medulloblastoma tumour growth as prime targets of the *Wnt* [304] and *Hh* [305, 306] pathways, responding to mitogenic signals. Several studies suggest that medulloblastomas have a high expression of both these factors [307, 308]. Amplification of these genes are common in other tumours, however in medulloblastoma it appears as though they are not amplified on a DNA level [309, 310], but rather that their abundance is due to elevated expression [311].

TRKC

The expression of the receptor tyrosine kinase TrkC is strongly linked to a favourable clinical outcome. For cases with low or no TrkC expression, the five year survival was reported to be 49%, whilst it was 89% for cases with high expression in the tumour cells. Based on this, TrkC expression is suggested as an addition to routinely used prognostic markers (as reviewed in [312]).

SURVIVIN

Survivin, a potent inhibitor of apoptosis and strongly expressed during neurogenesis, has been linked to tumour formation and is currently investigated in the context of new drugs against cancer [313]. In a study of 82 medulloblastoma cases, high expression of survivin was reported to be predictive of poor survival [314-316].

P53

p53 immunopositivity in tumour tissue has been reported as a predictor of poor survival [317], but data from Adesina et al. suggests that p53 mutations, although common in many types on cancers, are very rare in medulloblastomas [318]. However, many other studies have failed to determine any correlation at al between p53 and medulloblastoma (as reviewed in [319, 320]).

NOTCH

Medulloblastomas have been reported to show chromosomal gain in the *Notch2* locus. Notch2 is a receptor within the Notch family promoting proliferation and survival of neural stem and progenitor cells and inhibiting differentiating. Fan et al. reported on 15% of medulloblastomas having gained *Notch2* copy numbers and activation of the pathway was correlated to poorer outcome. In addition, Notch inhibiting drugs was efficient in suppressing tumour growth *in vitro* [321].

CHROMOSOMAL CHANGES AND SOMATIC MUTATIONS

Several reports point out loss of 17p, sometimes associated with an isochromosome 17q, as the most common chromosomal abnormality in medulloblastoma and it is found in up to 50% of all cases [322]. Interestingly, this is only found in classic medulloblastoma, not in the desmoplastic form (as reviewed in [286]). The tumour suppressor gene *HIC-1* is located at 17p13.3 and it has been reported to be silenced by epigenetic mechanisms, implying a role in tumour development of medulloblastomas [323].

GENETIC PREDISPOSITION

Reports from sibling- and twin studies claim that there is an inherited genetic factor at play in development of some, though not all medulloblastomas (as reviewed in [319]). Most of the hereditability can be assigned to two familial syndromes that have been correlated to medulloblastoma development; Turcots syndrome and Gorlins syndrome. Individuals born with Turcots syndrome have a predisposition for colonic polyps and colon cancer in addition to CNS tumours including medulloblastoma. The syndrome is coupled to mutations in *adenomatosis polyposis coli* (*APC*) on chromosome 5, which has been suggested as a tumour suppressor gene in this case. Interestingly, even though familial APC mutations predispose for medulloblastoma (the relative risk is 92 compared to normal population), somatic mutations of APC seem very rare in sporadic medulloblastoma (as reviewed in [324]). Recent reports however show that occurrences may be as high as 10%, although this has yet to be confirmed [298]. Gorlins syndrome, or nevoid basal cell carcinoma syndrome (NBCCS), is a congenital syndrome involving the *PTCH* gene at chromosome 9q22.3. Patients have an increased risk of developing basal cell carcinoma, skeletal abnormalities and 3% of them develop desmoplastic medulloblastoma. There are reports on loss of heterozygocity at 9q in medulloblastoma patients [325, 326] and inactivation of the *PTCH* gene [327].

TREATMENT

For patients of all ages, medulloblastoma is notoriously difficult to treat although it is highly responsive to both radiation and chemotherapy. The difficulties rather lie in the position of tumour and the risk of severe negative treatment effects in the central nervous system. Treatment follows a individual based multimodal regime including surgery, radiotherapy and chemotherapy. Whenever possible, radical surgery is the first step, followed by radiotherapy and chemotherapy to establish and maintain over tumour growth. Highly resective surgery is one of the prognostic factors, less residual tumour mass generally results in a better outcome for the patient. However, aggressive surgery in the brain is not without risk. In a study from 2006, Robertson et al. report that a staggering 24% of patients that underwent surgery against medulloblastoma developed cerebellar mutism syndrome (CMS) within 1-2 days after surgery. CMS symptoms include ataxia, mutism, personality changes, obtundation and cranial nerve

deficiencies. In 50% of the cases, some or all symptoms remained one year after surgery [328]. Radiation also results in negative effects and taken together the list of possible treatment sequelae is long; e.g. growth reduction, progressive intellectual deterioration including disturbed organization and learning abilities, decreased fine motor skills, visual deficiencies, memory deficits, endocrinologically related sequelae from disturbed levels of thyroid, sexual and adrenocortical hormones. As a result from all this, patients can experience psychosocial impairment resulting in difficulties fitting into normal social contexts, finding employment or sustaining personal relationships. Despite disabilitating physiological sequelae, the social dysfunctions have been rated by medulloblastoma survivors as the factor most decreasing the quality of life [329, 330].

MEDULLOBLASTOMA AND CMV

There are no case reports suggesting that CMV has a role in medulloblastoma, not as a risk factor for development nor for tumour progression. However, several studies strongly support an infectious agent influencing the risk of developing medulloblastoma. Most recently, Altieri et al., investigated the role of many siblings and risk for CNS tumour development and found that the relative risk was elevated for children with three or more younger siblings compared to children who had none (RR=2.3). Authors suggest that this is indicative for an infectious agent involved in malignancy development. The relative risk was also elevated for astrocytoma (1.34), ependymoma (2.61), meningioma (3.71) and neuroblastoma (2.13) [331]. The idea of viral aetiology of medulloblastoma has long been debated and mainly it is the polyoma viruses that have been investigated as candidate pathogens. There is evidence of viral presence in tumour tissue presented during the last decade, showing viral proteins and nucleic acids with immunohistochemistry and PCR respectively. It is simian virus 40 (SV40) and JC virus (JCV) that has been identified in tumours, while a third polyoma virus, BK virus (BKV) although found in other brain malignancies, was not associated with medulloblastoma. In addition, medulloblastoma-like tumours have been created by intracranial injections of polyoma viruses in animals (as reviewed in [332]). However, there are contradictory reports from studies where no or very low amounts of polyoma virus was found in medulloblastoma tumour cells [333-335].

We have shown that CMV proteins can be detected by immunohistochemistry in 87% of tested tissues. In addition, we found CMV DNA in conventionally used cell medulloblastoma cell cultures (**paper IV**). We hypothesize that the presence of virus, although in low levels but in many cells, is influencing the tumour progression by its unique abilities for manipulation. HCMVs preference for immature cells based on clinical observations as well as animal and *in vitro*-data heavily argues that CMV targets the stem cell rich areas of the brain during infection during brain development (as reviewed in [96]). These stem cell areas are the sites of origin for medulloblastoma and the stem and progenitor cells are the sources of tumour cells. We therefore claim it likely that CMV would be able to infect these cells before or at the time of tumour

initiation and subsequently influence the tumour growth. We do know that medulloblastoma cells are possible to infect *in vitro* and although it is possible to get reach high infection efficiencies with about 75% IE positive cells 3 dpi, no cytopathic effect is observed and the cells are not seemingly negatively affected. Instead, we observed an increase in cellular viability in response to infection (unpublished data). As mentioned in the neuroblastoma chapter, we infect the cells *in vitro* to mimic a natural, active infection in the tumour. As previously stated, it is not certain that CMV has any true oncogenic properties, but it is oncomodulatory in its effects on angiogenesis, proliferation, apoptosis, migration etc (as reviewed in [336]). The role of cancer stem cells in medulloblastoma is currently being investigated, but it is safe to state that the immature cells do exist in the medulloblastoma tumour, that the tumour cell composition is somewhat heterogeneous and that the tumour cells express stem cell like properties. Calabrese et al. reported on a sub population of medulloblastoma cells that expressed the stem cell marker CD133 and that is repeatedly found in the vicinity of small blood vessels or in association with endothelial cells [337]. Interestingly, we observed that although CMV proteins are not readily detected in cell lines in culture, we could detect CMV IE and L in xenografts of the same cell line. When the xenograft was harvested after about two weeks on site in the animal, IE and L proteins were detected throughout the tumour. IE was rather evenly distributed, but L was primarily localized around small vessels (**paper IV**). We do not have any immunohistochemistry data on whether these cells are $CD133^+$ or not, these experiments are ongoing, but it is a fine hypothesis that CMV should be most activated in immature cells where it can easily replicate.

However, the virus would not enter the brain by injection in the natural setting. In the case of medulloblastoma, congenital infection could be a source for CMV in the brain. The HCMV preference for immature cells in addition to clinical observation as well as animal data heavily argues that CMV targets the stem cell rich areas from where medulloblastoma arises. In 1958, Stewart proposed that childhood malignancies could be caused by maternal infection during pregnancy and he showed a correlation between leukemia and maternal chickenpox during pregnancy [338]. Later on, Bithel published a paper where he suggested that maternal chickenpox was also associated with medulloblastoma [339]. There are, to our knowledge, to date no clinical findings suggesting CMV as a factor in medulloblastoma development

ANTIVIRAL TREATMENT AGAINST CMV

STANDARD OF CARE DRUGS

Currently there are four drugs against CMV available in the market, ganciclovir, cidofovir, foscarnet and fomivirsen. Furthermore, the anti herpes drug aciclovir could possibly be added to the list. However, the effect against CMV for aciclovir is limited and therefore it is not routinely used. Ganciclovir, cidofovir and foscarnet target the viral DNA polymerase, UL54. That implies that early viral genes that are transcribed by cellular polymerases are not affected, but without the late genes no new viral particles can be formed and virus cannot spread in the body. Fomivirsen on the other hand is an antisense inhibitor of IE mRNA and thereby blocks all classes of CMV gene expression. It is registered as a drug against CMV retinitis, requiring intraocular administration.

Ganciclovir requires phosphorylation by the CMV kinase UL97 into a monophosphate form, and further by cellular kinases into the active triphosphate form. Cidofovir requires only cellular kinases for activation and foscarnet none at all. Foscarnet is a pyrophosphate analogy that inhibits the viral polymerase by binding to its pyrophosphate site. Ganciclovir and Cidofovir are nucleoside analogs that are incorporated into the newly formed viral DNA strand, thereby slowing down polymerase activity. These drugs are considered non toxic to uninfected cells since they target the viral polymerase and in the case of ganciclovir, needs viral phosphorylation for activation. As with all components, toxicity is only a matter of concentration and the doses needed for antiviral effects are generally nontoxic to uninfected cells although all three components can act on cellular targets as well although with significantly lower affinity (as reviewed in [3, 340]).

Ganciclovir is the first treatment choice for pre-emptive treatment, prophylaxis as well as CMV disease therapy and has considerably improved the prognosis primarily for immunosuppressed patients. But as for many drugs, resistant strains are appearing and, the first was reported by Biron in 1986 [341] and since then many more have been isolated, most of which have UL97 mutations. Luckily, cidofovir and foscarnet are still effective against most ganciclovir resistant strains.

The bioavailability of ganciclovir is poor and therefore a valylester derivative of the drug was established- valgangiclovir. It is hydrolyzed into ganciclovir upon passing the gut mucosa and bioavailability after oral administration is considerably higher than ganciclovir. It is therefore especially suitable for long term treatments since it can be self administered without difficulty. However, long-term treatment patients should be monitored for appearance of ganciclovir resistance as it has been reported that resistance occurrence increases with increased treatment duration [342].
All mentioned drugs have possible side effects. Ganciclovir can result in negative effects on the blood cells, including thrombocytopenia, leukopenia, anaemia, eosinophilia and also gastointestinal disturbances and nephrotoxicity. As previously

mentioned, ganciclovir is not routinely administered to pregnant women due to risks of teratogenic effects. Side effects of cidofovir and foscarnet are generally related to nephrotoxicity.

NEW DRUGS AGAINST CMV ARE ON THE WAY

New drugs against CMV are constantly being tested and at present, maribavir has come far, reaching clinical trials. It belongs to a group of phenylenediaminesulphonamides that inhibits viral replication by still unclear mechanisms. It is effective against strains resistant to ganciclovir and cidofovir. Clinical trials have been challenging since maribavir is only effective against HCMV, rendering all animal models useless. In 2007 it was approved in United States as an orphan drug against CMV disease and viremia and phase II and III studies in transplant patients are ongoing. It is not associated with the haematological toxicities or nephrotoxicity of other CMV drugs, but it has been reported to cause taste disturbances. As trials go on, further insight into possible side effects will be provided (as reviwed in [343]). Recent reports state that one phase III trial failed and another trial was discontinued when maribavir effect was reported as indistinguishable from placebo [344].

In addition there are other drugs in pipeline, *in vitro* studies predict possible success for small interfering (si)RNA against UL 54 [345] and the UL97 inhibitors indolocarbazoles [346].

CMV VACCINES

Although studied for decades, to date no effective CMV vaccine has reached the market. Even the need for a vaccine has been debated, but there seems to be a consensus around that a CMV vaccine would be highly beneficial to fight congenital infections since these are almost impossible to treat once they have occurred and therefore prevention would be the way to go. Transplant patients would also be a patient group with evident benefits. Different attempts have been made using both live virus and single subunits as immunogens. The live vaccine, constructed from the Towne or Toledo strains elicited an immune response and were considered safe with few exceptions, but failed to prevent infection in several groups such as young women and transplant patients (as reviewed in [347]). The gB based vaccine has been reported to be well tolerated and indeed be protective, although no cell mediated response was mediated. Just out of phase II trials, it is the best attempt this far, however further studies are needed (as reviewed in [348]).

IMMUNE THERAPY- THE FUTURE OF ANTIVIRAL TREATMENT?

Antiviral drugs have had a most beneficial impact on patients at risk for CMV disease, but their success is somewhat hampered by side effects and resistance development. If

we instead could modify the immune system into an active antiviral response and counteract CMVs immune evasion strategies, we may be able to avoid the risk of CMV disease without the long term use of antiviral drugs. It is evident from studies in transplant patients that individuals who are able to mount a specific T-cell response against CMV antigens are less likely to get severe CMV disease [349]. By adoptive T-cell transfer, the endogenous T-cell response can be boosted up to a level where it can gain control of the virus or, in the setting of prophylaxis, prevent reactivation. T cells can be presented to the CMV antigen in several ways. Riddell generated CMV specific $CD8^+$ T cells by exposing them to in vitro infected donor fibroblasts and then administering them to stem cell transplanted patients. He managed to induce a clonal expansion producing enough T cells for prophylactic administration with good and long term T-cell response as a result [144]. When Einsele et al. repeated the protocol but added both $CD4^+$ and $CD8^+$ T cells, results improved; viral reactivation was prevented in 6 out of 7 patients [350]. In order to find a protocol fast and safe enough for routine practice, alternative ways to generate the CMV specific T cells have developed. One is by presenting the antigen to the T cells by the help of APCs, another is the use of HLA peptide tetramers. The APCs are pulsed with CMV lysates or single peptides, generally pp65, and then present this to the T cells. Tetramers can be used with or without the presence of APCs. By subsequent isolation and expansions, adequate numbers of CMV specific T cells can be generated from peripheral blood. The tetramer technology is not yet developed enough for full scale clinical practice and it has the evident drawback of being HLA type specific [351, 352]. This line of therapy, though very promising, needs further testing and evaluation before substituting standard antiviral drugs of today. A remaining problem is also how to develop this technique to include seronegative donors, since they do not possess any CMV specific T-cell pool to expand [353].

TREATING CANCER BY TARGETING CMV; A FUTURE OPTION FOR IMPROVED PATIENT OUTCOME?

The evidence for a role of CMV in cancer is rapidly growing. However, whether CMV plays a true causative role in tumorigenicity or in modulating the tumour to a more malignant phenotype remains to be shown. Regardless, the presence of CMV in the tumour cells but not in surrounding tissues opens for new targets for cancer treatment. Such strategies may target the virus at different levels. The most obvious to test is the current anti-viral therapies specific against CMV. Currently, ganciclovir is the most frequently used and the most efficient anti-viral drug used in humans [340]. Ganciclovir was in fact initially developed as an anti-cancer drug.

However, also other drugs may inhibit CMV replication. CMV induces COX-2 in infected cells [354, 355] and COX-2 inhibitors are efficient in preventing CMV replication [355, 356]. Elevated COX-2 expression is detected in tumour tissue and is correlated to a bad prognosis [357-361]. Clinical observations as well as studies in animals and *in vitro* have shown that inhibiting COX-2 activity reduces tumour growth (personal communication, Per Kogner) and [359, 362, 363]. In addition, Lau and colleagues showed that inhibiting COX-2 increased the effect of chemotherapy by stabilizing p53 and so increased the induction of apoptosis [364].

Current evidence suggests that the effect of COX-2 inhibitors on CMV infection is mediated by lack of PGE2, which appears to be important for efficient CMV replication [354, 355]. The fact that the two drugs ganciclovir and COX-2 inhibitors may interfere with CMV replication through different mechanisms [355, 356] was the rationale behind our efforts to try to prevent tumour growth by using these drugs.

We tested whether ganciclovir and COX-2 inhibitor celecoxib used separately or in combination could prevent tumour growth *in vitro* and *in vivo*. We found that both ganciclovir and celecoxib prevented the colony forming capacity of medulloblastoma and neuroblastoma cell lines *in vitro* by approximately 20-50%. When used in combination, a synergistic effect was observed that resulted in a 75-97% inhibition of colony formation(**paper III and IV**). We proceeded with animal studies; xenografts of medulloblastoma or neuroblastoma cell lines were established in nude mice and the animals were treated with the oral form of ganciclovir (valganciclovir, Valcyte) and per oral treatment of celecoxib twice daily. At 12 days, the growth of the medulloblastoma tumours were inhibited by 40% by either valganciclovir or celecoxib treatment. When used in combination, we observed a synergistic effect, and the tumour growth was decreased by 70% (**paper IV**). These observations suggest that interfering with virus replication and/or inflammatory processes in these tumours may be a new and efficient strategy to be used in combination with conventional therapy for treatment of patients.

In animal experiments of neuroblastoma xenografts, we observed a trend, but not statistically significant reduced tumour growth when valganciclovir or celecoxib were used separately. However, in combination, they significantly reduced tumour growth; we observed a 47% reduction of tumour growth (**paper III**). The reasons for

differences in the efficacy of valganciclovir and celecoxib in preventing tumour growth of two very different tumour forms is not surprising, and may be explained by several mechanisms. The neuroblastoma tumours appeared to be more aggressively growing than the medulloblastomas tumours. They were hemorrhagic and rapidly growing, wherefore the animals had to be euthanized two days earlier than the medulloblastomas animals.

The xenografts were established by cell lines that have been adapted for growth *in vitro* over several decades, which may have altered their phenotype significantly. We therefore do not know how efficient this therapy would be in primary tumours. In the worlds first clinical study (VIGAS), we are currently evaluating the safety and efficacy of valganciclovir treatment as add on therapy in patients with the brain tumour malignant glioblastoma. 42 patients were recruited into this double blind study. All patients were over 18 years old and had a successful removal of 90% of their tumour mass by surgery, and had histology proven CMV infection in their tumours. Patients received 900mg valganciclovir or placebo twice daily for 3 weeks and thereafter 450 mg twice daily as maintenance dose for an additional 21 weeks. After the study duration of 6 months, we allowed patients who demanded valganciclovir on prescription, to receive the drug. This decision now complicates the statistical evaluation of the two cohorts, which is currently ongoing. Among patients who have received the drug, we currently observe a better outcome, but this observation may also be influenced by other factors.

In addition to valganciclovir and COX-2 inhibitors, angiogenesis blockers interfering with VEGF, may also be interesting to further evaluation in the context of CMV and cancer. As CMV protein US28 induces COX-2 expression and thereby VEGF production [221, 238], interfering with VEGF in tumours may in fact also interfere with the effects by CMV in cancer. Avastin (bevacizumab), an antibody blocking the activity of VEGF, was recently approved for treatment of glioblastoma patients. There are no current survival data available for Avastin in this patient group, but approval was based on prolonged progression free survival [365].

The efficacy of this drug has however been debated, as patients receiving Avastin may decrease their uptake of contrast enhancing fluid during MRI/ CT scans which may result in a false picture of the size of the patients tumour [366]. A global phase III trial is about to be initiated to further evaluate the efficiency of Avastin in glioblastoma patients. 900 patients are planned to be recruited world wide [367]. There is data from xenograft animal models of neuroblastoma showing that Avastin reduces tumour growth by 30-63%. [273]. However, the long term effects of anti-angiogenic treatment in children remains to be determined.

As for CMV, this drug would be excellent to combine with valganciclovir and celecoxib. To my knowledge, one glioblastoma patient has received a combination of avastin and valganciclovir. This patient was treated by Dr Cobbs in San Francisco after consultation with my supervisor, Cecilia Söderberg-Nauclér. The patient had a very large “butterfly” glioblastoma occupying the majority of the patients frontal brain. After successful surgery, he had a massive relapse within 3 weeks. He was treated with valganciclovir in combination with Avastin, and recovered remarkably well. The

patient died approximately 18 months later of a pneumonia, but was at that time tumour free (personal communication, Cecilia Söderberg-Nauclér, Charles Cobbs). In future studies, we therefore aim to further evaluate these drugs in combination as well as in combination with conventional chemotherapeutic agents to prevent tumour growth of brain tumours.

Will this cure brain tumours? If our hypothesis is correct, it is likely that these drugs will improve patient outcome, but unlikely that they will cure these diseases. If CMV is present in stem cells of the tumour, it will be of outmost importance to eliminate these cells from the patient to obtain a curative treatment. Surgery will therefore always be the most important step in removal of these cells from the tumour, which will be followed by combined drug and radiation therapy. I believe that CMV specific therapy has its place in such therapy in the future. One option to further improve along this strategy is to use drugs in combination together with CMV specific immunotherapy. One currently ongoing study by Sampson and colleagues at Duke University is evaluating whether dendritic cell vaccinations utilizing pp65 mRNA can improve the outcome for patients with malignant glioblastomas. 13 patients were 100% disease free at 6 months and disease progression was delayed (personal communication, Charles Cobbs, Cecilia Söderberg-Nauclér).

Yet another study has demonstrated a strong CMV specific immune response in a DC vaccination protocol [368]. Prins and colleagues used tumour lysates to stimulate DC with before the autologous primed DC were transferred back to the patient. Several patients, one of whom was described in the case report in NEJM, had a robust response against CMV. This particular patient is healthy and is coming back for re-stimulation of his immune response regularly five years after diagnosis of malignant glioblastoma (personal communication Cecilia Söderberg-Nauclér, Robert M. Prins).

In summary, many anecdotal observations in combination with animal data and human studies are pointing to the fact that interfering with CMV in glioblastomas, medulloblastomas and neuroblastomas may be an efficient strategy for treatment of patients. The biological evidence for this connection is compelling, and as these strategies appear to be safe and very well may specifically target tumour cells, It is my opinion that their efficacy in patients urgently should be further evaluated. This could possibly also help to elucidate key steps in tumour biology.

How is it possible be that CMV could be involved in so many different cancer forms, and if so, how can the virus play specific but different roles in different cancer forms originating from so diverse tissues?
Several studies have demonstrated that the expression profiles of different CMV mRNA/proteins are different in different tissues. For example, microarrays of mRNA from the salivary glands of RCMV infected animals, is quite different from the profiles expressed by other tissues, such as the liver and lungs [369]. Likewise, the expression of CMV proteins may also be cell and tissue specific. Furthermore, Dr Cobbs has recently found that the expression of IE as well as gB proteins appear to be higher in cells expressing the stem cell marker CD133 (personal communication; Charles Cobbs), compared to other cells in the tumour. Thereby, distinct mechanisms may be diversely prominent in different cell types.

Currently, we do know that CMV DNA, RNA and proteins are present in the patients' tumour tissues, but we do not know if the whole viral DNA is intact in cancer cells. Despite attempts to grow out infectious virus from numerous malignant glioblastoma tumour cells, we have not been able to retrieve infectious virus. Viral proteins are readily detected in the tumour, as well as in early established primary cultures from glioblastomas patients. However, after a few passages, the cultures become protein negative, but remain DNA positive, with occasional expression of IE and or pp150 RNA (Afsar Rahbar, unpublished observations). In commercially available cell lines, we also detect CMV DNA, but not in every PCR sample. By using the FISH technique, we detect viral DNA only in 1-5 out of 100 cells in these cell lines (Afsar Rahbar, unpublished observations). In both neuroblastoma and medulloblastoma tumours, we observe the same pattern; CMV protein positivity is detected in the vast majority of samples from the patients´ tissues. We have no access to primary tumour samples from these patients, but when we analyze well established cell lines, no CMV protein reactivity is found. DNA is present in the cell lines, but only in very few cells, and only occasionally do we detect CMV RNA. However, when these cells are transferred to animals to establish xenografts, viral protein expression is readily induced. Interestingly, in xenografts in nude mice, the expression of IE is wide spread over the whole tumour; cells that may represent the bulk of the tumour. In sharp contrast, L protein expression is predominantly detected around vessels in the tumour (**paper IV**). We hypothesize that perhaps L protein expression is restricted to cells in close proximity to the vessel; and thereby provide evidence for a cell type specific expression of CMV proteins. We hypothesize that these cells may be represented by stem cells, as these are known to home to vascular niches. Singh et al demonstrated that only cells with the stem cell marker CD133 obtained by positive selection from both malignant glioblastomas and medulloblastomas tumours were able to form new tumours when injected into the brain of NOD-SCID (non-obese diabetic, severe combined immunodeficient) mice. While injection of 100 $CD133^+$ cells resulted in established tumour, 10^5 $CD133^-$ cells did not result in any tumour formation [196]. Although debated, it implies a compelling evidence of tumour initiating cells.

$CD133^+$ cells infected by CMV could be capable of conferring tumorigenicity; $CD133^+$ cells, different from more mature daughter cells could represent the tumour initiating cell. When the stemness is lost and the cells differentiate, the expression pattern may change, as exemplified by loss of expression of proteins in cell cultures and the wide spread IE expression in tumours cells that lack expression of late proteins. Investigations of mRNA expression of $CD133^+$ compared to more differentiated cells reveal that US28, an extremely important protein in oncomodulation, is expressed in $CD133^+$ cells (personal communication, Charles Cobbs, Dan Streblow).

Regardless of what the expression pattern may be, it will be of outmost importance to reveal the CMV DNA sequence in the tumours. In a collaborative project with Dr Timothy Kowalik, University of Massachusetts, we are currently trying to sequence the whole viral genome from cancer cells, and compare it with viral DNA from the patients´ blood cells. It is possible that only parts of the CMV genome is maintained in cancer cells, and thereby infectious virus is not produced. It is also possible that a variant CMV strain is present in tumours; a close relative to the CMV we detect in normal cells, but distinct in its phenotype, and able to replicate its genome

preferentially in tumour cells. Therefore, the results of these experiments are highly wanted.
It is also a possibility that CMV interacts with other pathogens and thereby give rise to tumorigenic effects. Although one might consider interactions with bacteria or parasites, I believe interactions with other viruses are more likely due to their abundance in the global population and the higher possibility for genetic interplay. Worth mentioning here is the human endogenous retroviruses, viral sequences remaining in our genome after germ cell infections many generations ago. They are implied to be influential factors during tumour development (as reviewed in [370]) and it is plausible that CMV could interact with expression of such viral genes. Another exciting hypothesis is the possibility of completely new, yet unknown viruses that like CMV could master host cell manipulation and thereby contribute to CMV oncomodulation.

Whatever the agent or factor may be, accumulating results from the scientific community on CMV in a wide range of diseases strongly supports an hypothesis of CMV infection effects as context- and organ dependent.

CONCLUSIONS

The work included in this thesis spans over three different clinical disorders and include methods from molecular biology to studies of patient samples and animal studies. Although divergent areas, the theme of CMV and its impact on nervous system progenitor cells is consistent. In my thesis projects, we found that:

- CMV readily infects human foetal neural precursor cells with high efficiency. Infected cells express both IE and L proteins, but viral excretion appears to be relatively low (**paper I**).
- CMV infection in neural progenitor cells inhibit their differentiation into astrocytes and neurons (**paper I and II**). This effect is dependent on expression on L genes since foscarnet treatment abolished the effect of CMV infection. Once the cells are committed to neural or astrocytic lineage, CMV has, as far as we could determine, no further effect on differentiation.
- In addition to inhibiting differentiation, CMV decreases proliferation and induces apoptosis in infected cells. We observed that this is at least in part dependent on the expression of IE and/or E genes (**paper I and II**).
- In my projects aiming at further understanding the role of CMV in the paediatric malignancies medulloblastoma and neuroblastoma, we found that CMV proteins are present in 97% of neuroblastoma and in 87% of medulloblastoma tumours. Generally, more IE proteins than L were detected in both tumours. In addition, we found CMV DNA in six different commercially available cell lines, which were used for further *in vitro* and *in vivo* studies (**paper III and IV**).
- In both neuroblastoma and medulloblastoma cells, CMV infection results in up regulation of the inflammatory enzyme COX-2 and its product PGE_2 (**paper III and IV**).
- Suppression of COX-2 activity by celecoxib in combination with the anti-viral drug ganciclovir, reduced tumour growth *in vitro* by up to 75% in neuroblastoma cells and by up to 97% in medulloblastoma cells. This effect was more prominent in actively infected cells than in control cells carrying dormant CMV DNA (**paper III and IV**).
- Celecoxib and ganciclovir in combination reduced tumour growth of xenografts of medulloblastoma in nude mice by 70-75%. In mice carrying more aggressively growing neuroblastoma xenografts, tumour growth was reduced by 47% by combinatory treatment of both the drugs (**paper III and IV**). These observations suggest that anti-viral treatment and COX-2 inhibitors significantly reduce tumour growth of both neuroblastoma and medulloblastoma in vivo and thereby imply their potential future valuable role in clinical therapy for children.

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