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Human Cytomegalovirus: From Novel Strain, miRNAS to Interplay with Breast Cancer

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Human Cytomegalovirus: From Novel Strain, miRNAs to Interplay with Breast Cancer

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By

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Dedicated to my Family

ABSTRACT

The prevalence of human cytomegalovirus (HCMV) ranges from 40% to 100% worldwide. A primary HCMV infection results in a lifelong latent or persistent infection. In healthy people, HCMV infection is often asymptomatic, but in immunosuppressed individuals it can cause life-threatening disease, and HCMV congenital infection may result in fetal malformation. Increasing evidence implies that HCMV infection is associated with different malignancies. Several studies have suggested different mechanisms by which HCMV can modulate the tumour and the tumour environment, which could be of relevance in tumour development and progression. However, the role of HCMV in cancer has remained highly controversial. The studies in this thesis investigated the possible role of HCMV in cancer, with a special focus on HCMV encoded-miRNAs. HCMV encodes at least 26 miRNAs, and are expressed during the lytic as well as latent phase of infection. Viral miRNAs are identified to target both host and viral mRNA transcripts and thereby regulate protein expression. They modulate various biological functions of the host cell and the virus life cycle by mediating latency, viral replication, cell cycle control, vesicle trafficking, virus assembly and immune evasion strategies.

In study I (Manuscript), we discovered a novel genetic variant of HCMV lacking intron 2 of the major immediate early (MIE) gene in 48% tumour samples obtained from breast, colon, neuroblastoma, medulloblastoma and glioblastoma patients. In contrast, this variant was less frequently detected in healthy donors, and in patients with HCMV viremia or myocardial infarction. We found, three clinical isolate that contained both the wild type HCMV and the variant HCMV strains; the variant was successfully plaque purified from endothelial cells (HUVEC). Electron microscopy analysis found dense body like particles but no HCMV particles in variant positive HUVEC cells. The variant expressed cytoplasmic IE proteins, which were of multiple sizes. Our results demonstrate a high prevalence of a novel genetic variant of HCMV in cancer patients, which may represent a new virus strain with potential oncogenic properties that warrants further investigation.

In study II (Published in PlosONE 2014), the HCMV microRNA miR-UL112-3p suggested to mediate the latency and immune evasion strategies, also highly expressed in hypertension patients and associated with increased risk of hypertension. In our study, we detected miR-UL112-3p in plasma/serum of 52% (14/27) of Diabetic Mellitus, 25% (5/20) of Glioblastoma patients, in 5% (1/20) of Rheumatoid Arthritis patients and in 10% (2/20) of Healthy Controls. Anti-HCMV IgG was detected in 85%, 65%, 75% of patients and 70% of healthy controls, respectively. Anti-HCMV IgM was found only in one Glioblastoma patient of the 87 examined patients and controls. We didn't found significant association between serology and miR-Ul112-3p.

In study III (Accepted for publication), HCMV infected cells produce mature virions and defective particles called dense bodies (DBs). In this study, we purified these particles, and

found that virions and DBs incorporate viral as well as host encoded RNAs and miRNAs within the particles. Furthermore, we demonstrated that the particle associated miRNAs can be delivered to host cells, are biologically functional, which may affect cellular processes.

In study IV (Published in PLOS One 2013), we found that HCMV proteins IE and LA were abundantly expressed in all breast cancer tissue specimens examined (n=73) and 94% of their paired sentinel lymph node specimens (n=32/34) with metastases. Moreover, we also found HCMV IE and LA proteins in 60% (20/35) sentinel lymph node specimens without metastases. HCMV infections were mostly confined to the neoplastic cells, while some inflammatory cells were also HCMV positive in 79% of lymph nodes with metastases and 60% in metastatic free lymph nodes. We didn't had enough sample to perform survival analysis, as only six patients died of breast cancer in this cohort. These six patients had high grade HCMV infection. High grade HCMV IE staining are more prevalent, due to the less patients in each group no further statistical correlation analyses with clinical prognostic factors (ER-α, PR and Elston grade) are reported. We conclude that, higher prevalence of HCMV proteins found in lymph nodes with metastasis than lymph nodes with metastasis, and bigger cohort is required to perform the correlation analysis.

Another project by Dr. Afsar Rahbar in our lab, using more number of patients found the high expression of HCMV IE proteins in breast cancer tumours tissues inversely correlation with ER, PR and HER2 levels (manuscript in revision).

In study V (Manuscript) we further studied this phenomenon *in vitro*; HCMV infection resulted in significant downregulation of mRNA and protein levels of ER-α, PR and Her-2 in breast cancer cell lines. This effect was dependent on viral gene expression. We found potential viral miRNAs which can targets of ER-α (miR-UL22A-3p, miRUL36-3p and miR-US25-2-3p) and PR (miR-UL22A-3p). Overexpression of each viral miRNAs in a breast cancer cell line resulted in downregulation of mRNA and protein levels of ER-α and PR. Thus, HCMV may promote the establishment of triple negative breast cancer, through viral miRNA induced downregulation of ER-α, PR (and Her-2 by a yet unidentified mechanism). Antiviral therapy could hence be a potential alternative treatment strategy for selected breast cancer patients.

LIST OF SCIENTIFIC PAPERS

I. Wilhelmi V*, Chato Taher*, **Mohammad AA**, Assinger A, Khan Z, Pettersson P, Odeberg J, Dzabic M, Xu X, Stragliotto G, Hartman J, Frisell J, Martling A, Varani S, Örvell C, Siesjö P, Kogner P, Peredo I, Landazuri N, Rahbar A, Yaiw KC and Söderberg-Nauclér1 C* ***Isolation and characterization of novel genetic variant of CMV highly associated with tumors of different origin*** (Manuscript)

II. **Mohammad AA**, Rahbar A, Lui WO, Davoudi B, Catrina A, Stragliotto G, Mellbin L, Hamsten A, Ryden L, Yaiw KC, Soderberg-Naucler C. ***Detection of circulating hcmv-miR-UL112-3p in patients with glioblastoma, rheumatoid arthritis, diabetes mellitus and healthy controls.*** PloS one. 2014;9(12):e113740. Pubmed Central PMCID: 4252052.

III. **Mohammad AA**, Costa H, Landázuri N, Lui WO, Hultenby K, Rahbar A, Yaiw KC, and Söderberg-Nauclér C ***Human Cytomegalovirus MicroRNAs are carried by Virions and Dense Bodies and are delivered to Target Cells*** (Accepted for publication)

IV. Taher C, de Boniface J, **Mohammad AA**, Religa P, Hartman J, Yaiw KC, Frisell J, Rahbar A, Soderberg-Naucler C***. High prevalence of human cytomegalovirus proteins and nucleic acids in primary breast cancer and metastatic sentinel lymph nodes.*** PloS one. 2013;8(2):e56795. Pubmed Central PMCID: 3579924.

V. Costa H*, **Mohammad AA***, Lui WO, Joel T, Davoudi B, Bukholm IR, Sauer T, Vetvik K, Jürgen G, Rahbar A, Söderberg-Nauclér C ***A potential role for human cytomegalovirus in triple negative breast cancer through its specific mechanisms to downregulate ER-α, PR and HER-2*** (Manuscript)

* Authors contributed equally

RELATED PUBLICATIONS

I. Costa H, Xu X, Overbeek G, Vasaikar S, Patro CP, Kostopoulou ON, Jung M, Shafi G, Ananthaseshan S, Tsipras G, Davoudi B, Mohammad AA, Lam H, Straat K, Wilhelmi V, Shang M, Tegner J, Tong JC, Wong KT, Soderberg-Naucler C, Yaiw KC. Human cytomegalovirus may promote tumour progression by upregulating arginase-2. Oncotarget. 2016 Jul 26;7(30):47221-31. Pubmed Central PMCID: 5216936.

II. Yaiw KC, Mohammad AA, Costa H, Taher C, Badrnya S, Assinger A, Wilhelmi V, Ananthaseshan S, Estekizadeh A, Davoudi B, Ovchinnikova O, Shlyakhto E, Rafnsson A, Khan Z, Butler L, Rahbar A, Pernow J, Soderberg-Naucler C. Human Cytomegalovirus Up-Regulates Endothelin Receptor Type B: Implication for Vasculopathies? Open forum infectious diseases. 2015 Dec;2(4):ofv155. Pubmed Central PMCID: 4690546.

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V. Yaiw KC, Ovchinnikova O, Taher C, Mohammad AA, Davoudi B, Shlyakhto E, Rotar O, Konradi A, Wilhelmi V, Rahbar A, Butler L, Assinger A, Soderberg-Naucler C. High prevalence of human cytomegalovirus in carotid atherosclerotic plaques obtained from Russian patients undergoing carotid endarterectomy. Herpesviridae. 2013 Nov 14;4(1):3. Pubmed Central PMCID: 4177206.

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CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
Bcl-2	B-cell lymphoma 2
BL	Burkitt's lymphoma
CDV	Cidofovir
COX-2	Cycloxygenase-2
CSC	Cancer stem cell
CTL	Cytotoxic T-cell
DC	Dendritic cells
DCs	Dendritic cells
E	Early
EBV	Epstein Barr-virus
EGFR	Epidermal growth factor receptor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked ImmunoSorbent Assay
EMT	Epithelial–mesenchymal transition
ER- α	Estrogen receptor alpha
FACS	Fluorescence-activated cell sorting
GBM	Glioblastoma Multiforme
GCV	Ganciclovir
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HER-2	Receptor tyrosine-protein kinase erbB-2
HHV	Human herpesvirus
HIV	Human Immunodeficiency Virus
HPV	Human papilloma virus
HPV	Human Papiloma Virus
HSPGs	Heparan sulfate proteoglycans
HSV	Herpes simplex virus
HTLV-1	Human T-cell lymphotropic virus type 1
IE	Immediate early
IE	Immediate Early
IHC	Immunohistochemistry
IHC	Immunohistochemistry
IL-10	Interleukin-10
IRS	Internal repeat sequences
ISH	In situ hybridization
JAK	Janus-activated kinase
kbp	Kilo Base Pair

KIR	Killer inhibitory receptor
KS	Kaposi sarcoma
L	Late
LA	Late
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
MICA	MHC Class I-related chain A
MICB	MHC Class I-related chain B
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
NF-kF	Nuclear factor kappa B
NKG2D	Natural killer group 2D
ORF	Open Reading Frame
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase chain reaction
PDGFR-α	Platelet derived growth factor receptor-α
PGE_2	Prostaglandin E2
PI3K	Phosphatidylinositol-3-kinase
PMA	Phorbol Myristate Acetage
PR	Progestrone
pRb	Retinoblastoma protein
PTEN	Phosphatase and tensin homolog
RTKs	Receptor tyrosine kinases
SLN	Sentinel lymph node
STAT	Signal transducer and activator of transcription
TERT	Telomerase reverse transcriptase
TERT	Telomerase Reverse Transcriptase
TGF-β	Transforming growth factor beta
TLR	Toll Like Receptor
TMZ	Temozolamide
TNF	Tumour necrosis factor
UL	Unique long
US	Unique short
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VGV	Valganciclovir
WHO	World Health Organization
VZV	Varicella zoster virus

1 HUMAN CYTOMEGALOVIRUS (HCMV)

1.1 The discovery of HCMV

The first observation of what is believed to be cytomegalovirus (CMV) was made by a German pathologist, Ribbert, in the year of 1881. He described stillborn babies who had died of what he thought was syphilis like symptoms. He found cells of kidney enlarged in all these babies. However, he was unable to explain the cause of this condition. In the year 1904, Jesionek and Kiolemenoglou described similar enlarged cells and described them as 'protozoan-like'. These were also found in the kidney of a still-born infant. Jesionek, in the same year, reported similar 'protozoan like' cells in the lungs, kidneys and liver of the prematurely born fetuses. In 1907 Löwenstein described cytoplasmic and nuclear inclusions in these protozoal-like cells, followed by a clear zone (Ho, 2008; Riley, 1997; Weller, 2000). In 1907, Goodpasture and Talbot used the term 'cytomegalia' to describe these abnormal cells, and they were in disagreement that the inclusion bodies were caused by a protozoan, but were unclear over what caused that cytopathology (Goodpasture E. W., 1921). In 1925, Von Glahn and Pappenheimer observed that cells infected with herpesviruses contained inclusion bodies, they suggested that cytomegalic cells were more likely caused by a virus rather than protozoa (von Glahn & Pappenheimer, 1925). Later, finding from Cole also supported that the formation of inclusion bodies are likely due to infection with viruses (Wyatt *et al.*, 1950). Later other researchers agreed to a virus as the likely cause of the pathology of these cells, and this condition of unknown viral aetiology was termed 'generalised cytomegalic inclusion disease' (Wyatt *et al.*, 1950).

In the mid of the 1950s, three different laboratories reported that they isolated a virus from tissue cultures of human adenoid and salivary gland, and named these viruses AD169, Smith and Davis (Rowe *et al.*, 1956; Smith, 1956). It was also called the 'salivary gland virus' and shortly thereafter Weller et. al. named it 'cytomegalovirus'(CMV) (Craig *et al.*, 1957). The isolation and propagation of the virus in cultures enabled further development of understanding of the nature of this virus, such as detection of virus protein, its life cycle and later its molecular pathogenesis. Recently, I together with colleagues cherished the talks held in a symposium in Germany with renowned international experts in celebrating the 60th anniversary of CMV isolation. At this meeting, they high-lighted the important work by Smith, who was a pioneer in this field and described the discovery of murine CMV, which allowed for later development of animal models to study the pathogenesis of CMV (Reddehase, 2015).

1.2 *Herpesviridae* and its subfamilies

Herpesviridae is one of the largest family of viruses. It consists of hundreds of different viruses, and they are extensively prevalent in nature. Most animal species have experienced at least one herpesvirus infection during their lifetime. These viruses are classified into the herpesviridae family, as they share similar features in virions architecture, conserved genomic segments, and upon infection they have similar biological properties (Roizmann *et al.*, 1992). A recent

classification of new viruses is mostly based on the data obtained from nucleotide and amino acid sequence similarities (McGeoch *et al.*, 2000).

The general characteristics of a *herpesviridae* family are their linear double-stranded DNA (dsDNA) genetic code with a size ranging from 120 – 240 kbp that is enclosed by an icosahedral capsid (100 -130 nm external diameter). The capsid is made of 162 hollow centred capsomeres (12 pentons and 150 hexons) and is surrounded by an asymmetric and amorphous tegument protein layer, which is further surrounded by a host-derived lipid bilayer membrane called the envelope. The envelope is studded with viral and host transmembrane proteins on its surface, which will mediate binding and entry to the next target cell.

The four most common biological properties shared among all *herpesviridae* include:

1. Herpesviruses have their own enzymes involved in nucleic acid metabolism, DNA synthesis and protein processing such as helicases, DNA polymerases, primases and tymidine kinases – the number of enzymes may vary among deferent species.

2. DNA synthesis and capsid assembly of all herpesviruses take place in the nucleus, and maturation of the virion occurs in the cytoplasm of the infected cells.

3. The spread of new viral progeny occurs by lysis of the infected host cell, or by the release of intracellular vacuoles containing mature virions.

4. Their unique ability to establish latency in their natural host, from which the virus can be reactivated. The host cell types in which they establish latency vary among the different viruses. For example, HSV-1 and -2 and VZV remain latent in neuronal cells of dorsal root ganglia (Hyman *et al.*, 1983; Kramer *et al.*, 2003). EBV latency is found in B-lymphocytes (Hurley & Thorley-Lawson, 1988) and HCMV establishes latency in myeloid lineage cells (Soderberg-Naucler *et al.*, 1997; Söderberg-Nauclér *et al.*, 2001).

Although all herpesviruses exhibit similar features, the number of open reading frames (ORF) in the genome varies considerably; from 70 – 250 (VZV - CMV).

Based on their primary variations in biological properties such as host cell tropism, latency features, replication cycle and the differences in clinical manifestations, *herpesviridae* family members are further classified into three major subfamilies. i) *Alphaherpesvirinae* ii) *Betaherpesvirinae* and iii) *Gammaherpesvirinae*. The viruses of each sub-family have variable hosts including humans. Among the all known herpesviruses, there are nine viruses that are known to primarily infect humans. These nine viruses are also classified to the three different sub-families as mentioned above. According to the ICTV website, there are also some ‘unassigned’ genera in all the three sub-families of herpesviruses (contains non-human), which consists of the viruses which needs to be classified / does not currently fit into any of the available sub-families. Hereafter, I will only discuss human herpesviruses and the biological characteristics of their sub-families.

i) *Alphaherpesvirinae*: There are three alpha herpesviruses that infect humans; HSV-1 and HSV-2, and (VZV). The replication cycle of these viruses is very short, about 12-18 h and the host cells lyses for the production of new viral progeny. They all establish a latent infection in sensory ganglia.

ii) *Betaherpesvirinae*: This sub-family includes four human viruses; Human herpesvirus 5/ HHV5/ HCMV (Genera: Cytomegalovirus), HHV6A, HHV6B and HHV7 (Genera: Roseolovirus). Infected cells frequently become enlarged in size (cytomegalic cells) and can establish latent infection in lymphoreticular cells, secretory glands and bone marrow cells. In general, members of this sub-family are more complex than the alpha herpesviruses and have a long replication cycle (> 24 h). The infection progress slowly and may or may not result in cell lysis.

iii) *Gammaherpesvirinae*: Members of this subfamily infect and replicate in lymphoblastoid cells such as T- and B- lymphocytes and also establish latency in these cells. They also infect and can cause a lytic infection in epithelioid/fibroblast cells. There are two human viruses in this subfamily; they are both considered to be oncogenic viruses; the EBV/HHV4, Genera: Lymphocryptovirus and KSHV/HHV8, Genera: Rhinovirus. EBV is associated with different types of lymphomas and KSHV with Kaposis sarcoma in AIDS patients.

1.3 Genome and its organisation

The HCMV genome consists of a linear dsDNA, the size of the genome ranges from 220 – 240 kbp among the various strains that have been sequenced. The first complete HCMV genome was published in 1990 (Bankier *et al.*, 1991; Chee *et al.*, 1990), which is almost 14 years before the complete Human genome sequence was published (Schmutz *et al.*, 2004). Since then, many researchers have sequenced various strains of HCMV; their different genomes are discussed in a recent review (Sijmons *et al.*, 2014). To compare the complexity and variations of HCMV genome sequences, a low passage clinical strain, Merlin, is today considered as the reference genome (NCBI GenBank accession NC_006273.2).

The HCMV genome is the largest of all human herpesvirus genomes, and seems to be the most complex of them. Analysis of genomes of different HCMV strains revealed about 165-252 ORFs potentially encoding about 170 proteins (Dolan *et al.*, 2004; Murphy *et al.*, 2003). However, in a recent study, researchers used ribosomal profiling and transcripts analysis and found 751 unique translated ORFs in HCMV infected cells. The investigators suggested that HCMV has a much more complex coding capability than previously believed (Stern-Ginossar *et al.*, 2012). Furthermore, HCMV also encodes for at least four polyadenylated long non-coding RNAs and 26 microRNAs that carry out important functions during virus-host interactions and virus replication (www.mirbase.org Version-21) (for detailed miRNA description, please see chapter-III).

In 2009, after the development of the next generation sequencing (NGS) technology, two variants of Towne and AD169 strains were identified (Bradley *et al.*, 2009). For the analysis of the NGS data, a known genome sequence was used as a reference genome but for the investigation of the unknown/variations of the genomes this strategy was not useful. For

unknown or variants of viruses or organisms, a reference sequence independent assembly method (de novo assembly) has been developed. Since the last five years, the NGS technology has become user-friendly and affordable. Currently, the virus-pathogen resource genome database has 243 complete HCMV genome sequences including *in vitro* genetically modified strains (www.viprbrc.org).

Organisation of the HCMV genome

The sequencing analysis of the HCMV genome has revealed that it has a very complex organisation. Overall, the HCMV genome has two major segments called the Unique Long (UL) and Unique Short (US) segments. These two segments are joined with Internal Repeated Long segments (IRL) and Internal Repeated Short segments (IRS). These are flanked with Terminal Repeated Long segments named as TRL and Terminal Repeated Short segments (TRS). The IRL and IRS segment sequences are inverse to the sequences of TRL and TRS, respectively (Mocarski *et al.*, 2007). Based on the organisation of the genome segments, the herpesvirus genomes are classified into A-F classes. The HCMV genome is classified as an E genome (Roizman & Pellett, 2001) Fig. 1.

The HCMV gene annotations are based on their location in the respective genome segments; UL, US, IRL, IRS, TRL and TRS and their positioning in the genome. For example; UL112, US28, etc.

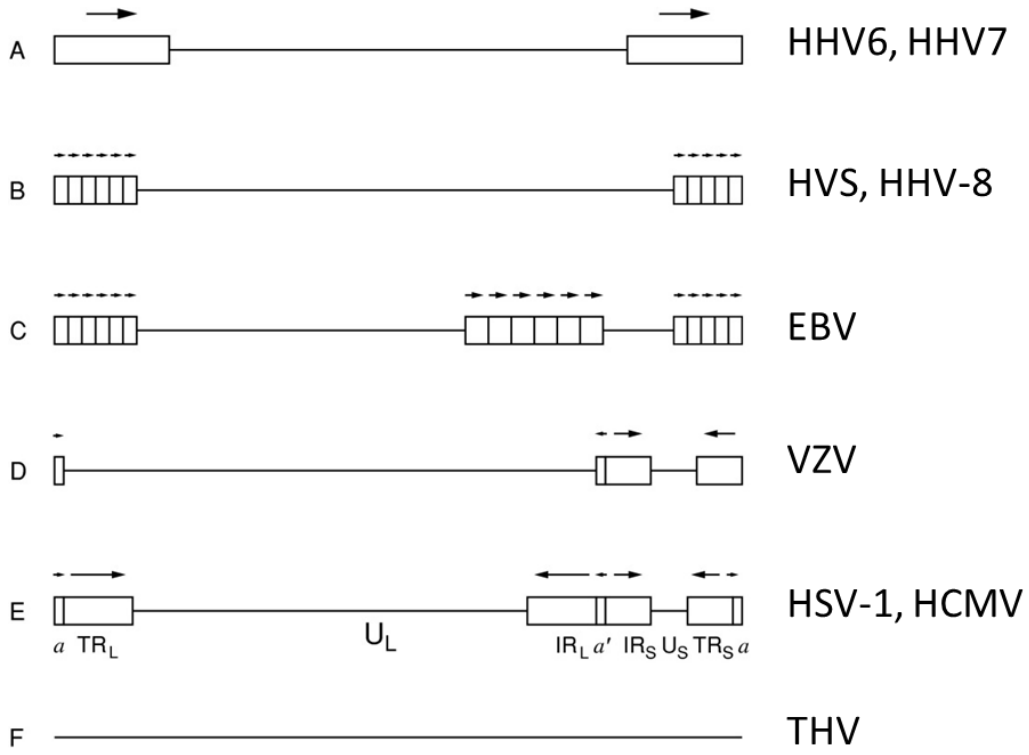

Figure. 1 Classes of herpesvirus genome structures (not to scale) defined by Roizman and Pellett (2001). Arrows = Orientations of repeats; Horizontal lines = Unique regions; Rectangles = Repeat regions.

1.4 Infected Cells Produce Virions and Defective Particles

Definition of a virion

A complete virus particle that consists of an RNA or DNA core with a protein coat or sometimes with external envelopes representing the extracellular infective form of a virus.

As described in the *Herpesviridae* section above, the HCMV virion architecture is similar to that of other herpesvirus members. The particle is spherical, about 230 nm in diameter, and contains a linear dsDNA enclosed in an icosahedral nucleocapsid of about 100 nm in diameter. A thick tegument layer further surrounds the nucleocapsid; a host-derived lipid bilayer called the envelope covers the tegument layer. The membrane of the envelope is studded with viral and host encoded transmembrane proteins; these proteins are exposed on the surface of the envelope, reviewed in (Kalejta, 2008) with the main function to target and gain entry to new cells.

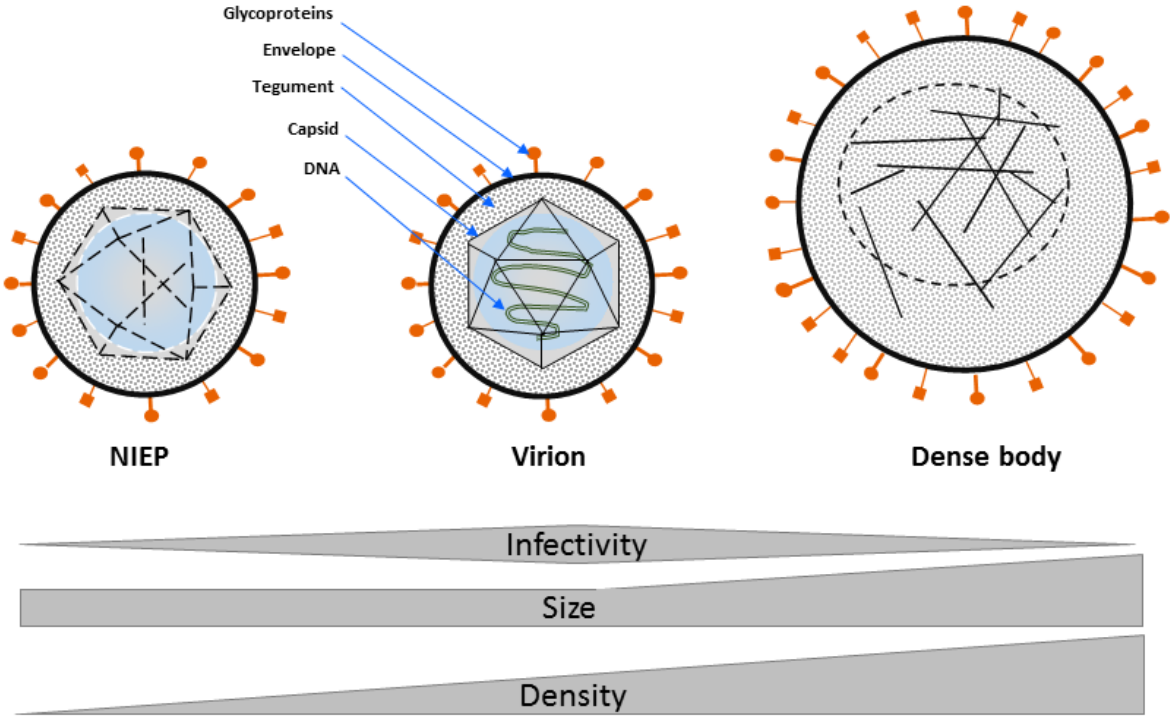

Figure. 2 HCMV infection produces virions and dense bodies (DBs) and Non-infectious enveloped particles (NIEPs). Virions and NIEPs are similar in size, about 250 nm, whereas the DB are bigger in size, about 250-600 nm. Virions are the fully mature infectious particles, NIEPs and DBs are considered non-infectious. DBs are denser than virions and NIEPs.

Capsid

The capsid is innermost core layer of a virion particle (also called nucleo-capsid), which contains and protects the genome. The shape of the capsid is icosahedral and made of 162 hollow centered capsomeres (12 pentons and 150 hexons). The major function of the capsid during the infection is to protect the DNA and to deliver the genomic code to the nucleus of

the host cell. There are three types of HCMV capsids: A-capsid (only capsid shell), -B capsid (capsid shell and assembled proteins) and –C capsid (a mature capsid containing the viral genome). These three capsids represent three different stages of capsid maturation that takes place in the nucleus of infected cells (Gibson, 1996). Capsid-A and Capsid-B can also proceed to envelopment; they then form immature virions that are defective (Irmiere & Gibson, 1985). The capsid is made of at least five proteins, i) Major Capsid Protein (UL86), ii) Minor Capsid Protein (UL85), iii) Smallest Capsid Protein (SCP, UL48- 49), iv) Assembly protein (Fragments of UL80) and v) Minor Capsid Binding Protein (MCP, UL46), as reviewed in (Gibson, 2008).

Tegument

The widest layer within the virion structure is the tegument layer, which closely surrounds the capsid; it is believed to be important to anchor the envelope to the tegumented capsid. Electron microscopy studies show that the tegument layer is amorphous and likely not well structured. The tegument layer contains the largest amount of proteins that are constituting the entire virion. Most of these tegument proteins are phosphorylated, thus, often designated with a prefix 'pp'. The functions of most of them are still unknown. The most well studied tegument proteins so far are pp65/ppUL83, pp71/ppUL82, pp150/pUL32 and pp28/pUL99 (Varnum *et al.*, 2004). These proteins play major roles during virus entry (un-coating), intracellular capsid transportation and assembly. Studies of pp65 deleted viruses show that lack of pp65 led to a growth modified viral phenotype that can replicate *in vitro* but as pp65 is essential for the formation of dense bodies (Schmolke *et al.*, 1995). Pp28 is a necessary protein for the assembly and it's known to be highly immunogenic (Meyer *et al.*, 1988). The Pp71 protein trans-activates the IE gene, which is essential for replication. In the tegument, many host encoded proteins were also found (Varnum *et al.*, 2004). The formation of the tegument layer and incorporation of these proteins are not fully elucidated yet. Along with viral and host proteins, there are also viral and host encoded RNAs present in the tegument layer, which suggest that they may play a role in HCMV biology; but this topic needs further in-depth investigations (see study-II, for more detailed description), reviewed in (Kalejta, 2008; Tomtishen, 2012).

Envelope

The envelope is a lipid bilayer, which surrounds the tegument layer and keeps the entire virion intact. It interacts with the host cell membrane on target cells and thereby plays a major role during virus attachment and entry. HCMV and host encoded transmembrane glycoproteins are studded into the membrane and decorate the particle. The viral glycoproteins gpUL55 (gB), gpUL73 (gN), gpUL74 (gO), gpUL75 (gH), UL100 (gM), gpUL115 (gL) and the pentameric complex consisting of gL, gH and UL128-131, are known to play important roles in virus entry (see section below), cell-to-cell spread and virion maturation (Ryckman *et al.*, 2008).

Defective HCMV particles

During a lytic infection, HCMV along with mature virions also produces the two types of defective particles, the dense bodies (DBs) and non-infectious enveloped particles (NIEPs) (Fig. 2). Several methods have been developed to isolate these particles from one another and also from cell culture medium (Irmiere & Gibson, 1983; Stinski, 1976; Talbot & Almeida, 1977b). Different densities of different HCMV particle types allows for their separation with ultracentrifugation techniques. NIEPs are of same size as virions but does not contain viral genome, and are this less dense than virions (Roby & Gibson, 1986). The DBs are larger in size compared to with virions and NIEPs, and ranges from approx. 250-600 nm in diameter; they are also denser than virions and NIEPs. DBs contain most of the structural proteins as are present in mature virions. However, although they contain capsid proteins, these are not organized into a capsid shell and hence no viral genome can be packed into a protective capsid. Further in depth analysis of protein and phosphoprotein contents of virions, demonstrates that DBs and NIEPs have many viral as well as host proteins packaged into the particles (Stinski, 1976; Varnum *et al.*, 2004) into the particles.

Several investigators also reported that RNA transcripts are also packaged within different particles of HCMV and can also deliver these RNA into target cells (Greijer *et al.*, 2000; Prichard *et al.*, 1998a; Sarcinella *et al.*, 2004; Terhune *et al.*, 2004). The presence of the RNA transcripts in the particles and their delivery to target cells have also been observed by other members of the herpesvirus family, as reviewed in (Amen & Griffiths, 2011b). However, still no high throughput methods have been applied for screening of all RNA molecules present in those particles. It has been suggested that particles package RNA non-specifically and the proportion to the RNAs present in the infected cells, will be reflected in the viral particles (Terhune *et al.*, 2004).

DBs can induce both humoral and cellular immune responses and can produce neutralizing antibodies against the virus (Cayatte *et al.*, 2013; Pepperl *et al.*, 2000). Several animal experiments suggested that DBs could be used as vaccines but yet the impact of the RNAs delivered by these particles warrants further investigations. Therefore, another promising strategy is being evaluated: DBs can be produced with recombinant-antigens and these were shown to induce T-cell responses to the recombined antigens without an adjuvant. This strategy is currently under development as a promising HCMV vaccine (Becke *et al.*, 2010; Mersseman *et al.*, 2008; Pepperl-Klindworth *et al.*, 2003).

1.5 Entry, Replication and Virus Assembly

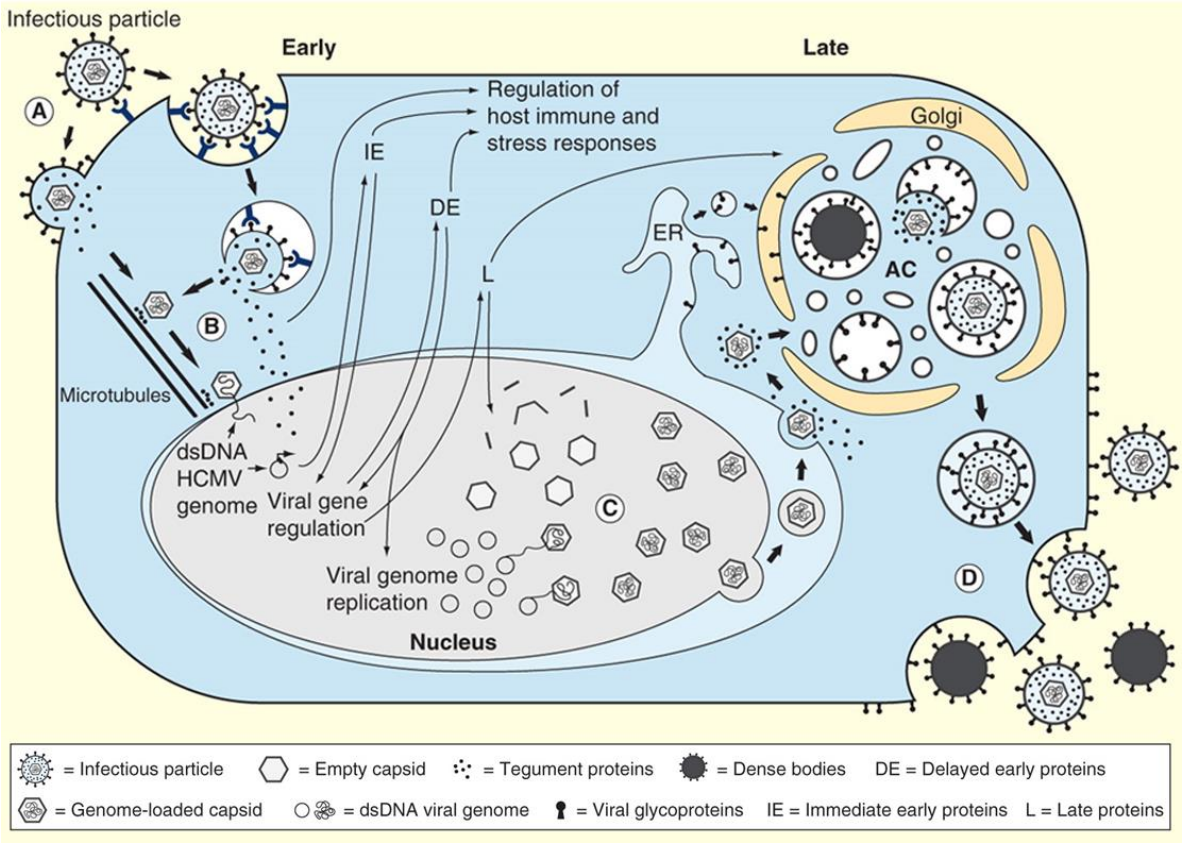

Figure 3 Overview of the human cytomegalovirus life cycle. (A) Virions enter the cell through interaction with cellular receptors. Tegument and capsid proteins are delivered to the cytoplasm. (B) The capsid travels to the nucleus, then the genome is delivered and circularized. Tegument proteins regulate host cell responses and initiate the temporal cascade of the expression of viral immediate early (IE) genes, followed by delayed early (DE) genes, which initiate viral genome replication, and late (L) genes. (C) Late gene expression initiates capsid assembly in the nucleus, followed by nuclear egress to the cytosol. Capsids associate with tegument proteins in the cytoplasm and are trafficked to the viral assembly complex (AC) that contains components of the endoplasmic reticulum (ER), Golgi apparatus, and endosomal machinery. The capsids further acquire tegument and viral envelope by budding into intracellular vesicles at the AC. (D) Enveloped infectious particles are released along with non-infectious dense bodies. Figure and figure legends are adapted from (Jean Beltran & Cristea, 2014) and modified.

Entry

Viruses have to deliver the viral genome to its host cell to begin the infection. First the viral surface proteins and the host cell membrane have to interact and facilitate entry to occur. HCMV enters almost all cell types, results in productive infection in most of them such as epithelial, endothelial and smooth muscle cells but in monocytes the infection will remain latent/non-productive. Monocytes have to differentiate into macrophages or dendritic cells before the virus can replicate and produce new virus particles (Ibanez *et al.*, 1991). This

indicates that HCMV enters the cells using various cellular receptors or that a common receptor is present on all cell types.

The entry process involves three major steps i) Attachment to the surface of the host cell ii) Interaction with entry receptors at the host cell membrane iii) Internalization of the particle or fusion of the viral envelope with the cell membrane (this depends on cell type for HCMV).

HCMV encodes for at least 57 glycoproteins among which 14 have been biochemically proven to be the structural components of the virion. Only eight of these have been found to be present in the HCMV envelope, and their functions are not yet fully elucidated (Britt *et al.*, 2004). HCMV-gB mediates attachment to cells via binding to cellular receptors including heparan sulphate proteoglycan (Jones *et al.*, 2004), integrins (Feire *et al.*, 2004) and EGFR (Wang *et al.*, 2003) to promote the entry process. HCMV gH interacts with integrin αVβ3 and act as a co-receptor (Krummenacher *et al.*, 2013). The heparan sulphate proteoglycan molecule allows the HCMV particle to attach closer to the cell membrane where viral glycoproteins bind to more specific receptors. This is mediated by gB and gH or its complexes (Compton *et al.*, 1993; Luganini *et al.*, 2010).

Several other viral protein complexes can also mediate the entry process; a homodimer of gB called gC-I, and a hetero dimer composed of gM and gN that form gC-II and a heterotrimer of gH, gL and gO that forms the gC-III complex (Boyle & Compton, 1998; Huber & Compton, 1997; Li *et al.*, 1997). HCMV has two different entry routes in different cell types. In fibroblasts, virus entry is mediated by viral envelope glycoprotein complexes gH/gL-gB and gH/gL/gO via direct fusion at the plasma membrane (Kabanova *et al.*, 2016; Wille *et al.*, 2010). Annexin-II (Pietropaolo & Compton, 1997) and Aminopeptidase N (CD13) (Söderberg *et al.*, 1993) serves as receptors to promote entry at the plasma membrane, however none of these receptors are essential for the entry and replication (Giugni *et al.*, 1996; Pietropaolo & Compton, 1997), as several other receptors can also act to mediate entry.

In endothelial and epithelial cells, monocyte/macrophages and dendritic cells, the viral pentameric complex gH/gL/UL128/UL130/UL131A is essential to mediate receptor-mediated endocytosis (Vanarsdall & Johnson, 2012). The virus then fuses with the endosomal membrane to release the capsid to the cytoplasm. Post entry, the dissociation of the tegument will take place and the capsid will be directed from the cytoplasm to the nuclear pore to deliver the DNA to the nucleus, this process also mediated by many proteins of the tegument. The viral receptor that mediates entry through receptor mediated endocytosis has been searched for during decades. Virus binding to EGFR, PDGF, and integrins result in activation of intracellular signalling processes involving activation of src and paxillin that appears to be essential for this process (Nogalski *et al.*, 2013). Our group recently discovered that the pentameric complex specifically interacts with endothelin receptor B (ETBR) and this results in similar activation cascade resulting in a downstream activation of cavelolin I that mediates the formation of the virus containing endosome. One ETBR blocker that are already approved for treatment of an unrelated disease, pulmonary hypertension (Macitentan), blocks HCMV infection in endothelial cells, epithelial cells and macrophages by more than 90% *in vitro*. ETBR thus seems to be a long searched for HCMV receptor on these cell types, and this new knowledge may open for new strategies for antiviral therapy.

Lytic replication

In general, HCMV infection of cells leads to an active replication of the viral genome and production of viral progeny that are released by lysis of the infected cells, called the lytic phase. The replication of viral DNA is key to produce more viruses during the course of infection. HCMV DNA replication is highly regulated and dependent on the expression of certain viral proteins. Once viral DNA is delivered into the host cell nucleus, gene expression takes place in three stages; first immediate early genes (IE) are expressed, followed by early genes (E) and late genes (L). The IE gene products act as transcription factors and will regulate expression of the E and L genes. The host cell transcription and translation machineries will be utilized for producing IE proteins, which are produced within 1-4 hours following initiation of the infection. IE proteins acts as trans-activators for gene expression (of both viral and cellular genes) and are therefore essential for production of E and L proteins (Fortunato & Spector, 1999). The E proteins mostly interfere with the host proteins, and also affect the transcriptional and replication machinery for viral protein production.

HCMV encodes its own proteins to regulate the replication machinery such as a DNA-polymerase (UL54) and DNA primase (UL70) to sustain an efficient production of new virus progeny (Van Damme & Van Loock, 2014). The L proteins are mainly structural components of the virions and also include proteins essential for virion assembly and egress. The HCMV genome contains a cis-acting lytic origin of DNA replication (OriLyt) element that initiates the bidirectional DNA replication model followed by a rolling circle mechanism to generate viral DNA molecules to be incorporated into new virus particles (Lehman & Boehmer, 1999; Pari, 2008). The entire replication cycle for HCMV is rather long and it takes approximately 72 h before new virions are mature and ready to infect the next cells either by release from the infected cells or by spread via cell-to-cell mechanisms (Gibson, 2006). During the latent phase of infection no replication will take place and only selected gene transcription and translation of viral proteins take place. Once in a while when the conditions are favourable, HCMV may get reactivated and a usual lytic replication will develop and spread new viral progeny (for latency, see section below).

Virus assembly

After the pre-capsid assembly step, the newly made DNA is inserted into an immature B capsid and become a fully matured C capsid. This DNA packed capsid, egresses from the nucleus through the nuclear membrane, by an envelopment and de-envelopment process (Muranyi *et al.*, 2002). It is unclear how tegumentation takes place, but as all capsids in the cytoplasm are tegumented, while none are in the nucleus, this process must take place through or rapidly after nuclear egress. The tegumented particle then egresses to cytoplasmic vacuoles for final envelopment. Mature particles are released by transport of Rab3 secretory vesicles to be released by fusion of the vacuole with the plasma membrane, or through cell lysis (Homman-Loudiyi *et al.*, 2003).

1.6 Latency and Reactivation

The latency phase can be defined as an ability of the virus genome to persist and remain dormant in infected cells from where the virus can get reactivated and produce virus when the host cell environment favours such process. Latency allows the virus to establish a persistent infection in the host, without destroying the host cells. The ability to establish latency is one of the key biological properties of herpesviruses. In healthy individuals (an immune competent host) primary HCMV infection is mostly asymptomatic and results in a latent and persistent infection.

In 1985, for the first time HCMV DNA was detected in PBMCs by the in situ hybridization technique (Schrier *et al.*, 1985) and later several others groups confirmed the presence of HCMV in blood using PCR methods (Bevan *et al.*, 1991; Stanier *et al.*, 1989). Further studies confirmed that the adherent monocytes/macrophages are major cell types which harbours the HCMV latent infection, rather than B-cells and T-cells. (Taylor-Wiedeman *et al.*, 1991). It is now established that the bone marrow derived CD34+ myeloid progenitor cells (Mendelson *et al.*, 1996; Sindre *et al.*, 1996) and their derivative CD14+ monocytes harbour the latent infection (Soderberg-Naucler *et al.*, 1997), and during the latency phase, the virus genome was localized as an episome (Bolovan-Fritts *et al.*, 1999). However T-cells and B cells also both derived from the CD34+ myeloid lineage cannot maintain the latent HCMV infection. The cellular transcription factor GATA-2 is a key player in differentiation of myeloid cells to monocytes, and is proposed to be important in maintaining latency of the viral genome in myeloid lineage cells (see below). Whether endothelial progenitor cells or mature endothelial cells also carry the latent infection is not clear. The virus is found in endothelial cells in healthy subjects, but so far no studies demonstrate that it can be reactivated from latency in this cell type. In *in vitro* studies, latently infected monocytes have to differentiate into macrophages (Soderberg-Naucler *et al.*, 1997) or dendritic cells (Reeves *et al.*, 2005) in order to induce reactivation of HCMV; the virus then enters a lytic phase. This phenomenon can cause severe disease in immunocompromised patients such as stem cell or organ transplant patients.

Mechanisms of latency and reactivation

In general, the interplay between the host and virus encoded proteins are the key to optimize host cells to maintain HCMV in a latent state of infection or allow the virus to enter the lytic phase. The mechanisms behind the latency state of a natural infection are beginning to be understood.

MIE act as a trans-activator for the transcription of most HCMV encoded genes, and is essential for replication of the virus and to proceed to the lytic phase of infection. During latency, the MIE promoter is transcriptionally repressed by cellular factors (Ets2 Repressor Factor, HP1) (Bain *et al.*, 2003; Ghazal *et al.*, 1990; Murphy *et al.*, 2002; Wright *et al.*, 2005). The chromatin around the MIE gene is also repressed by these factors and results in inhibition of the lytic cycle phase and plays a role in maintenance of the latent phase of infection. Early studies suggested that no transcription of the HCMV genome takes place during latency. Later studies noted that

a limited set of genes were transcribed also during latency. Cell culture models studying experimental infection of CD34+ positive cells, showed expression of latency associated transcripts and production of some proteins. The latency associated gene product UL138 is required for establishment of viral latency and maintenance. The HCMV-LUNA and UL138 are produced during the HCMV latency and lytic infection (Bego *et al.*, 2005), and activates CD4+ T cells and this leads to production of IL-10 and IFN-γ; one with immunosuppressive and one with immuno-activating effects that favours replication and reactivation of latent HCMV. The produced IFN-γ can promote macrophage differentiation, which could lead to reactivation and enhanced replication of HCMV (Jenkins *et al.*, 2008). UL138 also down regulates the multidrug resistance protein-1, MRP1 (Weekes *et al.*, 2013), which can lead to reduced cellular leukotriene C4 export, which may prevent DCs to reach lymph nodes, and impair an HCMV specific immune response (Robbiani *et al.*, 2000). Another strategy employed by HCMV to suppress the immune system during viral latency is provided by expression of the UL111a gene; it encodes a functional IL-10 homologue with powerful immunosuppressive effects. During the latent phase of infection UL111a undergoes alternative splicing, which leads to expression of a latency associated cmvIL-10 transcript, which produces a protein that mimics the function of the human immunosuppressive cytokine IL-10. This favours the infected cells not to be recognized by the immune system and to avoid clearance (Jenkins *et al.*, 2008). Furthermore, US28 and UL144 are also expressed during latency and manipulate the cellular environment and redirect the immune response or block immune recognition (Beisser *et al.*, 2001; Goodrum *et al.*, 2007b; Poole *et al.*, 2013).

When HCMV infects differentiated cells, it permits viral replication while in some undifferentiated cells it remains in a non-permissive state (Sissons *et al.*, 2002). For example, macrophages allow for an active infection, while monocytes do not. Latently infected HCMV is only reactivated when monocytes are differentiated into macrophages or dendritic cells, in which the virus can replicate. Therefore, the expression of latent genes is most probably directed by the cellular transcriptional milieu. Recent *in vitro* studies in infected CD34+ progenitor cells found that HCMV down regulates cellular miRNA miR-92a, which leads to upregulation of the cellular transcription factor GATA-2 (Poole *et al.*, 2011), and increased expression of cellular IL-10 (Mason *et al.*, 2012). GATA-2 is known to play an important role in myeloid cell differentiation, suggesting that GATA-2 might be a key regulator for the establishment of the HCMV latency.

1.7 Epidemiology and Clinical Features

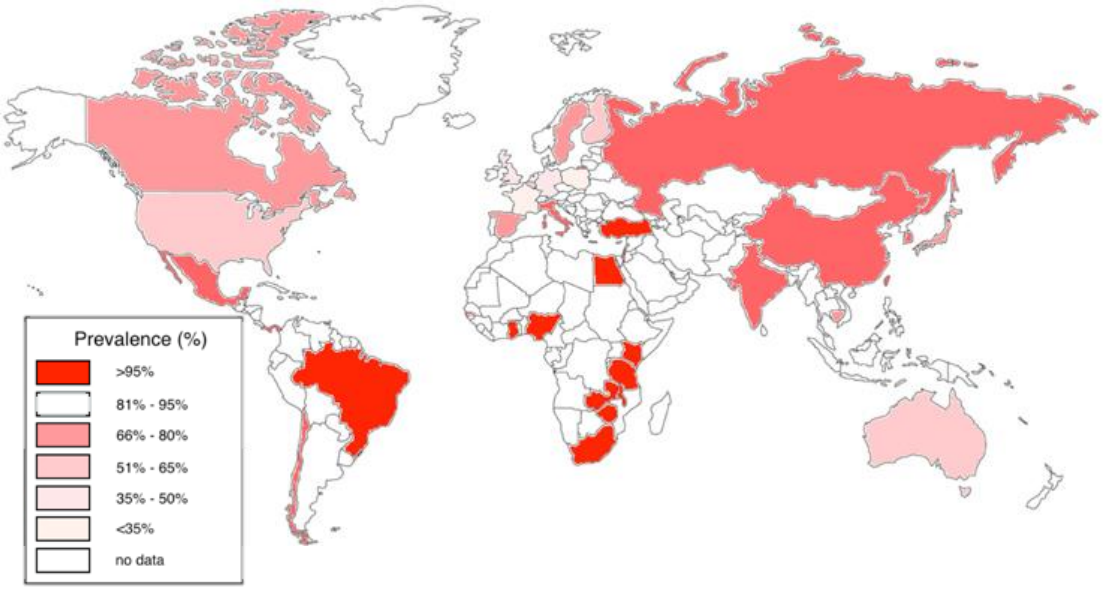

Figure 1. Worldwide HCMV seroprevalence rates in adults. Studies of adults aged 16–50 years published between 2005 and 2015 from Australia, Belgium, Brazil, Canada, Cambodia, Chile, China, Finland, France, Gambia, Germany, Ghana, India, Israel, Italy, Japan, Kenya, Mexico, Nigeria, Panama, South Africa, Spain, Sweden, Taiwan, Tanzania, Turkey, UK, USA, Zambia, and Zimbabwe. Figure and Figure legend is adapted from (Adland *et al.*, 2015)

The HCMV seroprevalence ranges from 45- 100% worldwide, and depends on socioeconmial status and/or geography location (Cannon *et al.*, 2010). HCMV can be transmitted via various routes, which includes horizontal close personal contact, vertical transmission from mother to child, also through other bodily fluids via breast feeding, urine, blood transfusion, stem cell and organ transplantation, and sexual contact. HCMV can infect humans at any age. Approximately 30-40% of children are infected with this virus by one year of age (Asanuma *et al.*, 1996). HCMV primary infection of pregnant women are at increased risk of transmitting the virus to the fetus and causing infection with a substantial risk of birth defects (Syggelou *et al.*, 2010).

In most children, the congenital infection is asymptomatic at birth. The prevalence of infection in new-borns, ranges from 0.2% - 2.5 % of all births. Only about 10-15% of these demonstrate clinical symptoms and only few present with congenital abnormalities including hearing loss, mental retardation and visual impairment. The most common clinical signs in the congenitally infected children are anaemia, thrombocytopenia, petechia, jaundice and hepatosplenomegaly. Further clinical manifestations include growth retardation, lethargy, seizures, and microcephaly (Syggelou *et al.*, 2010).

HCMV infection in healthy, immunocompetent hosts is generally asymptomatic or present with symptoms of upper respiratory tract infection or more severe mononucleosis. Common symptoms may also include headache, a sore throat, fever, malaise, lethargy, splenomegaly and

lymphadenopathy (Eddleston *et al.*, 1997). In contrast to the relative mild infection it causes in healthy people, this virus can cause life-threatening disease in immunocompromised patients.

In organ transplant patients, HCMV infection is also associated with acute and chronic rejection and other post-transplant related complications (Linares *et al.*, 2011). Significant morbidity and mortality has been reported in organ or stem cell transplant patients as well as AIDS patients. In immunocompromised patients HCMV infection can be found in various organs in the body; it can cause retinitis, pneumonitis, gastrointestinal disease and rarely also encephalitis (Ljungman, 1996). Increasing evidence also demonstrates a significant correlation between HCMV sero-positivity and atherosclerosis and increased cardiovascular mortality (Sorlie *et al.*, 2000). HCMV has also been associated with rheumatoid arthritis (Rothe *et al.*, 2016), inflammatory bowel diseases (Dimitroulia *et al.*, 2006), Sjögren´s syndrome (Shillitoe *et al.*, 1982) and Systemic Lupus Erythematosus (Perez-Mercado & Vila-Perez, 2010). These studies imply that there is a link between HCMV infection and autoimmune diseases. As stated above, HCMV can be reactivated by inflammation, and inflammation thrives viral replication. Inflammation and production of for example IL-1, IL-6 and TNF alpha produced from M1 type of macrophages may promote viral replication (Chan *et al.*, 2008). Whether the presence of HCMV is an epiphenomenon or driver of inflammatory diseases warrant further investigation. Association of HCMV with various cancer types has been extensively discussed in the Chapter-II.

1.8 Immune Responses to HCMV Infection

HCMV infection induces an innate immune response, which is followed by adaptive and cell mediated responses. Regardless of a robust host immune response, a primary infection will lead to a persistent and latent infection, from which it can reactivate whenever the environment favours (Jackson *et al.*, 2011). The immune response to HCMV may protect the infected individual from symptomatic recurrent infections, but does not protect the host from being re infected, as several strains of HCMV have been found in the same individual (Chou, 1989). HCMV has also developed multiple sophisticated strategies to evade the host immune system, and remain dormant.

HCMV and NK cell response

Rolf Kiessling and colleagues discovered NK cells at the Karolinska Institutet in 1970. They play a major role in the innate immune system, especially in viral and parasitic infections. NK cells also help driving the adaptive immunity. In 1989, recurrent HCMV associated disease was noticed in a few patients who had NK cell defects, which led to the understanding of the importance of NK cells in immune responses against viruses (Biron *et al.*, 1989). NK cells patrol tissues and can become activated by a balance of numerous activating and inhibiting cell surface ligands. The first described activation pathway was “the missing self-hypothesis”, described by Kärre et. al. at the Karolinska Institutet in 1981. They described that NK cells became activated by the loss of MHC class I molecules on the target cells. Other ligands such as MHC class I related chain A or B (MICA, MICB) that are expressed on stressed cells as well as infected and transformed tumour cells activate NK cells (Arase *et al.*, 2002). Recent

investigations found that MICA*008 and MICB are downregulated by the HCMV UL9 and miRNA miR-UL112 in infected cells, which results in escape of recognition by the NK cells (Seidel *et al.*, 2015; Stern-Ginossar *et al.*, 2008). HCMV gB and gH receptors are recognized by the TLR2 receptors that initiate production of pro-inflammatory cytokines and interferons, which subsequently results in activation of NK cells via NFkB (Boehme *et al.*, 2006). The HCMV-UL40 protein induces HLA-E expression and inhibits the functions of NK cell via CD9/NKG2 inhibitory receptor (Tomasec *et al.*, 2000; Ulbrecht *et al.*, 2000). HCMV infection induces the expression of ligands ULBP1 and 2, these ligands activate the NK-cells via the NKG2D receptor. HCMV UL16 protein binds to ULBPs and prevent the recognition by NK cells, also limiting the expression of these ULBPs on the cell surface, which results in decreased susceptibility to NK cells (Rölle *et al.*, 2003).

Cell mediated immunity to HCMV

In cell mediated immunity, $CD8^+$ T cells are the major key players involved in the control of HCMV infections. The $CD8^+$ T cells can recognize and kill the infected cells via recognizing the viral proteins presented on their surface by MHC-I molecules. Both pp65 and IE72 peptides were shown to be recognized by $CD8^+$ T cells and induced an immune response able to kill infected cells (Kern *et al.*, 2002; Kern *et al.*, 1999). The diversity of HCMV peptides that the immune system can recognize is very broad (Portland data Picker). The immune response against HCMV is remarkably strong and increases with the age; in elderly persons it may constitute approximately 50% of the CD8+ T-cell repertoire (Moss & Khan, 2004). In murine bone marrow transplant models, the removal of reconstituted $CD8^+$ T cells leads to lethal infection whereas reconstituting the bone marrow in immunocompromised mice prevents CMV disease (Polic *et al.*, 1998). Similarly, in humans, a strong correlation was found between the recovery of the $CD8^+$ T cell population and protection from HCMV disease (Cwynarski *et al.*, 2001). Patients who received ex vivo expanded CD8+ T cells were protected from HCMV primary and recurrent infections. The function of infused HCMV specific $CD8^+$ T cells was reliant on the presence of HCMV-specific $CD4^+$ cells. These observations suggest that $CD4^+$ helper T cells might be vital for effective $CD8^+$ T cell responses (Einsele *et al.*, 2002). Indeed, CD4 +T cells also play an essential role in the control of HCMV infections. CD4+ T cells recognise viral peptides presented via MHC-II molecules on the antigen presenting cells (Le Roy *et al.*, 2002). HCMV specific $CD4^+$ T cells can also act as effectors directly to virus infected cells (Gamadia *et al.*, 2004; Rentenaar *et al.*, 2000). The gamma delta T-cells represent a minor subset of T cells that are also expanded during HCMV infections, and these cells have the ability to mediate cytotoxicity of infected cells by yet unknown ligand/receptor interactions (Halary *et al.*, 2005).

Humoral immunity /Antibody responses against HCMV

The humoral immune response controls HCMV infection via specific antibodies, this helps the host to eradicate the virus via phagocytic cells or antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells. The antibodies can also be neutralizing and prevent infection of new cells as they bind to viral glycoproteins that need to interact with cellular receptors for entry. The humoral immune response helps in reducing the infection burden and limits viral

spread (Landini *et al.*, 1988). The antibody response is directed towards both structural and non-structural proteins.

1.9 HCMV Diagnosis and Virological Methods

For the diagnosis of the HCMV infections in humans, several laboratory investigations are used. These include:

Antigenemia assay

For a long time this was one of the most widely used diagnostic assays for detection of HCMV infected cells in blood samples. Isolated leukocytes in cytospots or smears were subjected to immunofluorescence staining using antibodies against HCMVs tegument protein pp65 and followed by fluorophore conjugated secondary antibodies. The method detects pp65 in granulocytes, but this does not reflect virus producing cells. Pp65 will be delivered by the virus particle to granulocytes, while HCMV does not replicate in in these cells. This method is very simple and relatively cheap to perform. The pp65-positive leukocytes correlates with risk of disease, but the threshold number that predicts disease varies with the clinical setting (Boeckh & Boivin, 1998). In research laboratories, this immunostaining method is also is used for detection of HCMV in various *in vitro* infected cells using various HCMV specific antibodies (see study-IV & V). In study-III, a similar technique was used; instead of fluorophore secondary antibodies HRP-conjugated secondary antibodies were used and later developed with DAB to visualize the staining, this method is called Immunocytochemistry.

Serology/ ELISA

Investigation of the presence of HCMV specific IgG or IgM antibodies in the serum/plasma of patients is performed with ELISA tests. The HCMV IgG antibodies determine if the infection occurred or not; to know the status of the active infection or a very recent infection, HCMV IgM antibodies has to be investigated. Furthermore, for the acute HCMV infections an avidity test for HCMV IgG antibody can also give guidance of a recent or old infection. A recent infection gives less IgG avidity and can improve the accuracy of the ELISA test, as of time the infection progresses, the avidity of antibodies produced will increase (Grangeot-Keros *et al.*, 1997). It has been reported that some IgM antibodies lack HCMV specificity, and may give false positive results (Edward S. Mocarski *et al.*, 2007). A neutralization assay can also be a reliable method for differentiation of active or non-primary infections (Eggers *et al.*, 2000). In study-I we determined the prevalence of HCMV IgG and IgM antibodies in plasma of patients and healthy blood donors using kits from the Karolinska University virology laboratory or from Hoffmann La Roche Ltd.

Virus culture

Isolation of HCMV from clinical specimens is still a gold standard method but can take 3-6 days or even a few weeks, dependent on the number of copies present in the specimen. The collected clinical specimen is co-cultured with fibroblast and followed by identification of the formation of a cytopathic effect / or a plaque, with a typical HCMV characteristic. The time for detection of HCMV can be shortened by combining the method for ocular detection of viral

plaques with immunostaining for the HCMV IE protein (Gleaves *et al.*, 1984). As this method takes a lot of time and is laborious for routine clinical laboratories, most labs prefer the PCR method to detect HCMV DNA in blood or plasma (see below). In our study-I we isolated HCMV from clinical specimens. Sometime it can be challenging to isolate one particular virus from multiple viruses in cultures, in those cases each plaque isolated will be grown in a separate tissue culture flask. Once the desired virus isolate is obtained, later it can be used for propagation of more virus.

PCR

Today, most clinical laboratories used different PCR based methods to detect viral DNA copies in blood or plasma. As this method is sensitive, highly specific and most importantly rapid and quantitative, is has recently has been automated in many laboratories to process multiple samples (Gimeno *et al.*, 2008). We have used different PCR methods in all the studies reported in this thesis.

1.1 Treatment of HCMV Infection

HCMV diseases can be treated with antiviral drugs, there are only five licensed drugs that are available today; ganciclovir, valganciclovir, foscarnet, cidofovir and fomivirsen. Except formivirsen, all other drugs are directly or indirectly targets the viral DNA polymerase UL54. Furthermore, all of these drugs have side-effects and may result in drug resistance (Mercorelli *et al.*, 2011b).

Ganciclovir (GCV) and Valganclovir (VGV)

GCV was the first antiviral drug available for the treatment of HCMV infection, GCV is still the drug of choice. VGV is a pro-drug of GCV and available as enteral formulation, which rapidly metabolize to the active form in the liver and intestinal wall (Biron, 2006). GCV is available in intravenous formulation and also as a sustained-release intraocular implant. GCV is an acyclic nucleoside analogue of 2′-deoxyguanosine, it gets incorporated instead of the nucleotides and inhibits viral DNA synthesis. The GCV has to be phosphorylated by the UL 97 viral protein kinase to become biologically active in its triphosphate form (Littler *et al.*, 1992). GCV resistance have been found in patients infected with HCMV strains containing mutations in the UL97 gene or the DNA polymerase gene (UL54) (Gilbert & Boivin, 2005). Haematological abnormalities (primarily neutropenia, anemia, and thrombocytopenia) as we as toxicity effects on the liver and kidney are the major side-effects (Markham & Faulds, 1994).

Foscarnet

This drug is manufactured as intravenous formulation. Foscarnet is used for those who cannot be treated with GCV due to dose-limiting neutropenia or leucopenia, or due to resistance to GCV (Razonable *et al.*, 2004). Foscarnet is a pyrophosphate analogue, which also inhibits viral DNA polymerase and does not require enzyme activation after intake (Mercorelli *et al.*, 2011a). Administration requires slow infusion and extensive pre-hydration, and monitoring of serum creatinine levels due to its potential nephrotoxicity as a side-effect (Naesens & De Clercq, 2001).

Cidofovir

Cidofovir is used only as a second line therapy, due to its side-effects. Cidofovir has low oral bioavailability and is only produced as intravenous formulation. Cidofovir is an acyclic nucleoside phosphonate analogue. It becomes activated by the by cellular kinases, causes premature termination in viral DNA synthesis and inhibits the viral DNA polymerase (De Clercq & Holy, 2005). It has longer intracellular half time, which allows effective treatment even by irregular dosage (Aduma *et al.*, 1995). The major side effects are renal toxicity, neutropenia, and electrolyte imbalance. Furthermore, in preclinical studies it has been shown to be teratogenic and carcinogenic (Lea & Bryson, 1996).

Fomivirsen

This drug is available as an intravitreal formulation, used for treating HCMV retinitis in immunocompromised patients, mostly HIV patients. It acts as an anti-sense RNA inhibitor (5'-GCG TTT GCT CTT CTT CTT GCG-3') with phosphorothioate linkage to protect it from degradation; it binds complementary to HCMV IE86 RNA and inhibits translation. IE86 proteins are essential for the production of early and late proteins and also essential for replication of the virus. It has a half-life of approximately 55 hours, which allows infrequent dosage, and due to its intraocular administration, it has no systemic effects during treatment (Geary *et al.*, 2002).

Novel drugs against HCMV infection

As described earlier, most anti-HCMV drugs have the side-effects or due to its teratogenic effects they can't be used for pregnant women or congenitally infected patients. In some cases however patients have been treated with acyclovir for congentical infection in utero and GCV post birth. Importantly, patients with GCV resistant infections need new therapy options. Discovery of new and less toxic anti HCMV drugs with alternative mechanisms of action may be helpful for present or for the future generations to come (Mercorelli *et al.*, 2011b).

Currently there are three new drugs are in preclinical studies.

Maribavir is an inhibitor of HCMV UL97 kinase, an important molecule that digest the nuclear lamins. Inhibiting the UL97, prevent the maturation of capsids to egress from the nucleus to cytoplasm (Prichard, 2009). It has undergone phase-II trial and in phase-III it failed. Further, it can't be used with GCV due to its antagonizing effect on the drug (Emery *et al.*, 2000; Marty *et al.*, 2011).

Brincidofovir is a lipid prodrug of Cidofovir. It has no renal toxicity compared cidofovir, instead it has certain gastrointestinal toxicity, manifested as diarrhea (Marty *et al.*, 2013). This drug also failed in its phase-III (Marty *et al.*, 2016).

Letermovir has a unique mechanism of action among other anti-CMV drugs, it inhibits the viral terminase. When viral DNA packaged into the capsid, terminase cuts the DNA (Bogner, 2002).

Our group recently identified the ETBR blocker Macitentan to be a potential future treatment option for HCMV. This drug is presently in use for treatment of patients with pulmonary hypertension and blocks HCMV infection *in vitro* by interfering with receptor binding, signalling and transcription of CMV IE proteins (Bruderer *et al.*, 2012).

Table 1. Anti HCMV drugs and treatment parameters.

Generic (Trade Name)	Route	Usual Adult Dose for Induction Treatment	Maintenance Dosage	Major Toxicity
Ganciclovir (Cytovene)	Intravenous	5 mg/kg, Every 12 h, 14–21 d	5 mg/kg Every24 h	Hematologic
Valganciclovir (Valcyte)	Oral	900 mg Two times per/d, 21 d	900 mg once/d	Hematologic
Foscarnet (Foscavir)	Intravenous	90 mg/kg, Every 12 h, 14–21 d	90–120 mg/kg once/d	Renal
Cidofovir (Vistide)	Intravenous	5 mg/kg once/wk Two time/ day, for 2 wks	5 mg/kg once every 2 wks	Renal Neutropenia
Fomivirsen	Intravitreal	330 µg day 1, day 15	330 µg once monthly	Ocular

d, day; **h**, hour; wks, weeks

2 HCMV AND CANCER

2.1 Cancer Associated Viruses

Several DNA viruses are associated with neoplasms. These are referred to as oncoviruses, and today include eight human viruses; human papillomaviruses are associated with cervical carcinoma, hepatitis B and hepatitis C viruses with hepatocellular carcinoma, Human T-cell Leukemia virus-1 with T-cell leukemia, EBV with B-cell lymphoproliferative diseases and nasopharyngeal carcinoma, Kaposi's Sarcoma associated herpesvirus with Kaposi's Sarcoma and primary effusion lymphomas (Carrillo-Infante *et al.*, 2007). Although all these viruses does not belong to the same family of viruses, or even do not have a clear pathogenicity yet they all can cause cancer. The most common features of these viruses are the abilities to infect and retain in the host cells without completely destroying the host cell, in another term they establish persistent and long term infections, and importantly they evade the host immune system, reviewed in (Pagano *et al.*, 2004). In the last few decades, observed associations of viruses with cancers suggest that about 15-20% of all known cancers may be caused by virus infections. This has been an intriguing issue for researchers to study, to understand in depth how viruses can cause cancer (Parkin, 2006). Furthermore, apart from the direct effects of these viruses on cells, chronic inflammation also plays a very important role in the cancer biology and may be caused by pathogens (Colotta *et al.*, 2009).

2.2 Koch's Postulates

Koch's Postulates, is a set of criteria that was established in 1884 by Robert Koch and Friedrich Loeffler to identify the causative agent of a particular disease. (reviewed in (Fredericks & Relman, 1996)). These criteria applies to almost all microorganisms but some of the criteria are not applicable to some viruses, such as oncogenic viruses (Herpesviruses) and parasites. These viral infections can take years to induce or promote the malignancy and can be a non-productive infection, and not able to eliminate it from the host. Due to these reason another set of criteria was developed by Frederick and Relman in 1996, especially for a potential infectious agent to be considered as aetiological agent in human cancers. The criteria for Koch's Postulates for 21st century are as follows:

i. The putative pathogen is present in most cases of the disease.
ii. Normal tissue should harbour no, or significantly less, putative pathogen.
iii. Disease resolution should be accompanied by decreased genome of putative pathogen.
iv. Microbial sequences should be present before the disease is established or correlate with disease severity.
v. The nature of the microbial organism detected should be consistent with known biological characteristics of that group of organisms.
vi. Microbe-associated sequences are detected in the diseased tissue and should be corroborated at the cellular level.
vii. Molecular evidence should be reproducible.

2.3 Prevalence of HCMV in Cancer

HCMV nucleic acids and proteins have been found in malignant tumours of the breast (Harkins *et al.*, 2010) and its metastatic tumours (Study IV), colon (Harkins *et al.*, 2002), prostate (Samanta *et al.*, 2003a), rhabdomyosarcoma (Price *et al.*, 2012), medulloblastoma (Baryawno *et al.*, 2011a), nucoepidermoid salivary gland tumours (Melnick *et al.*, 2012), neuroblastoma (Wolmer-Solberg *et al.*, 2013) and glioblastoma (Cobbs *et al.*, 2002c; Rahbar *et al.*, 2012a). HCMV proteins were mainly detected in tumour cells, and sometime it may be found in inflammatory or endothelial cells in the tumour tissue. Furthermore, no or little HCMV IE proteins were found in the healthy tissues surrounding the tumour (Harkins *et al.*, 2002). After extensive efforts, infectious HCMV has not been isolated from the primary tumours (my own observations in our lab). The prevalence of HCMV in all investigated cancers is close to 100%. However, the subject is controversial as some investigators could not find any evidence of HCMV in cancers (Forslund *et al.*, 2014; Huang *et al.*, 2014; Yamashita *et al.*, 2014). Thus, the role of HCMV in cancer is still controversial and a matter under debate. Whether HCMV infection contributes to cancers pathology, or whether it represents an epiphenomenon of cancer warrants further investigation. However, to study various aspects of HCMV on cancer cells *in vitro*, most researchers use cancer cell lines and infect them with HCMV. This may not represent the *in vivo* situation (see below).

2.4 Hallmarks of Cancer

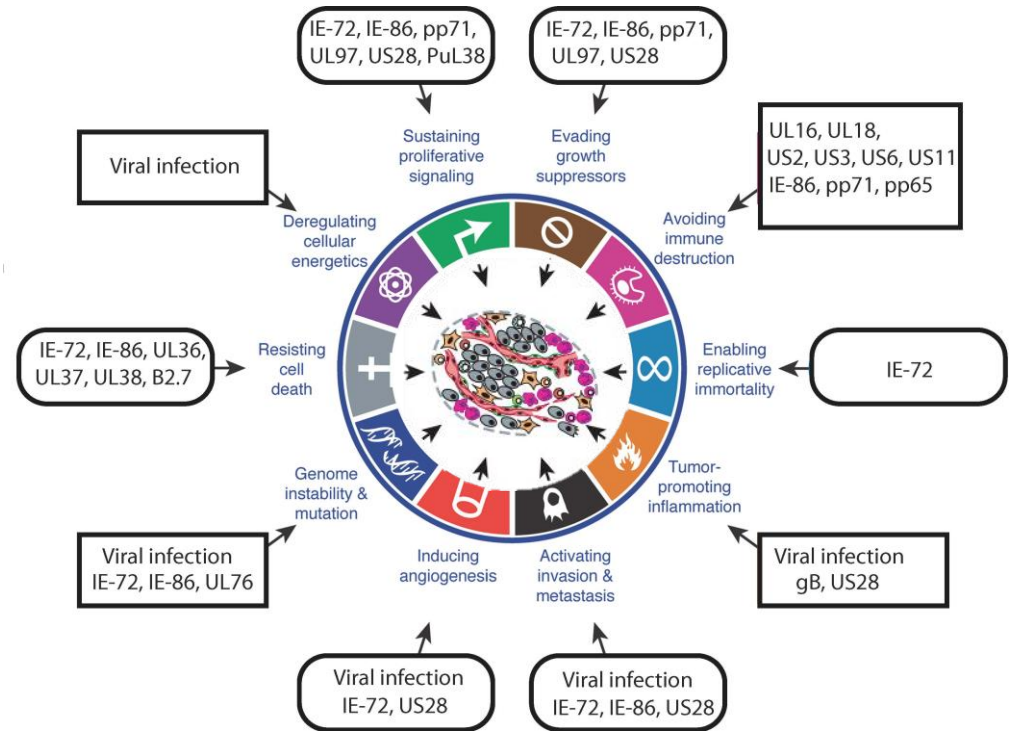

Figure 4. Overview of HCMV genes that interfere with hallmarks of cancer. Adapted and Modified from (Hanahan & Weinberg, 2011).

What differentiates malignant cells from normal cells? This question has occupied scientists for decades and a simple answer remains elusive.

Researcher all around the world have been working for many decades to identify differences between malignant and normal cells. Cancer is a disease in which the cells abnormally undergo uncontrolled proliferation and growth and are able to invade other organs. When these abnormal cells disseminate to the other part of the body via the circulation, they may give to metastatic disease, which is almost incurable. Till date, knowledge in cancer biology suggest that tumorigenesis is a multistep process and to simplify this, Hannahan and Weinberg have come up with a description of the hallmarks of cancer. These hallmarks are features shared between cancers of different origin; like uncontrolled and sustained cell growth, insensitivity to negative growth regulation, resistance to induced cell death, lack of senescence, genomic instability, angiogenesis and invasion and metastasis, reviewed by (Hanahan & Weinberg, 2011). As described in earlier sections, a recent study suggested that HCMV encodes for about 750 proteins (earlier estimated to be about 180 proteins), indicating that HCMV is a complex virus, and the functions of most of these proteins are unknown (Stern-Ginossar *et al.*, 2012). HCMV is not considered as an oncovirus, although its proteins have been found in several cancer types, and its 'oncomodulatory' roles have been studied in depth in cancers. In the following sections, I will discuss HCMV´s role in cancer by its ability to mediate the hallmark

of cancers.

2.4.1 Sustained Proliferation

In normal cells, proliferation involves a coordinated series of events through the cell cycle and activation of signalling pathways, which are often directed by growth factors and cytokines etc. In cancer cells these signals are disrupted and acquire alternative signals by which cancer cells gain capability to proliferate endlessly. Many studies have demonstrated that several signalling pathways have alterations such as disruption of autocrine signals of their own growth factors, or disruption of paracrine signals from the microenvironment (Bhowmick *et al.*, 2004; Cheng *et al.*, 2008). Cancer cells can also increase their expression of growth factor receptors that may further sustain chronic proliferation (Bhowmick *et al.*, 2004). For example, in EGF, PDGF, PTEN or P13K pathways, one or several genes are mutated, which can lead to consistent activation of the pathway, to stimulate growth, survival and proliferation (Jiang & Liu, 2009; O'Reilly *et al.*, 2006).

The Rb family of proteins are inactivated by the HCMV proteins IE72, IE86 (Castillo & Kowalik, 2002b), pp71 (Kalejta *et al.*, 2003a) and UL97 (Hume *et al.*, 2008a) and they promote the cell cycle to enter the S phase. Furthermore, both HCMV IE72 and IE86 can control the cell cycle at several checkpoints through interactions with the tumour suppressor protein p53 (Castillo & Kowalik, 2002b). Furthermore, HCMV IE proteins activate cell survival pathways in both in normal and tumour cells trough induced expression of the transcription factor NF-kB (Yurochko *et al.*, 1995). HCMV IE proteins also induce the expression of proto-oncogenes, cyclins and kinases involved in cell cycle regulation and cellular division (Jault *et al.*, 1995). IE72 can also induce proliferation in quiescent cells via trans-activating the E2F-responsive promoter by an alleviation of transcriptional repression mediated by protein p107, this plays a key role in cell cycle progression (Poma, Kowalik et al. 1996). HCMV infected cell has also acquired multiple mechanisms to activate MAPK p38, a kinase that is known to be critical for viral infection (Johnson *et al.*, 2000). Furthermore, the HCMV chemokine receptor homologue US28 induces production of IL-6, VEGF, and cyclooxygenase-2 (COX-2), and also activates STAT-3; these factors play important roles in angiogenesis, tumour cell migration, and inflammation and tumour progression (Bongers *et al.*, 2010; Maussang *et al.*, 2006). In a recent study from our lab, we demonstrate that HCMV was detected in 92% medulloblastoma. Engrafted cells containing HCMV nucleic acids in nude mice exhibited induced HCMV protein expression, which was correlated with COX-2 expression in primary tumour cells, cell lines and medulloblastoma xenografts. Importantly, the anti-viral drug valgancicovir and the COX-2 inhibitor celecoxib prevented HCMV replication *in vitro* leading to reduced production of PGE2 and decreased tumour growth. This study suggest HCMV as a potential target for modulloblastoma treatment (Baryawno *et al.*, 2011b).

2.4.2 Evades Apoptosis

Apoptosis is defined as programmed cell death and is essential for maintenance of tissue homeostasis. During infections or in pathologically alerted cells apoptosis plays as an important

role and act as an innate defence mechanism. There are two separate pathways that induce apoptosis; the extrinsic and the intrinsic pathways; both lead to caspase activation that induces cell death. The extrinsic pathway is triggered from the outside of the cell, and is activated by death receptors such as the FAS ligand receptor and the TNF-α receptor. The intrinsic pathways is regulated by mitochondrial stress, the intrinsic pathways signal may begin form DNA damage signals, loss of cell survival factors or other stress signals. The Bcl-2 family of proteins play a major role in regulating the intrinsic apoptotic pathway. Furthermore, tumour suppressor proteins such as p53 and Rb proteins are also involved (reviewed in (Danial & Korsmeyer, 2004)).

HCMV can regulate or even block apoptosis by several mechanisms. HCMV gene products are acting as anti-apoptotic proteins or interfere with apoptotic pathways in one or multiple ways. For example, IE86 binds to p53 and blocks activation of apoptosis (Tanaka *et al.*, 1999), IE72 and IE86 blocks apoptosis mediated by TNF-α receptor signalling (Zhu *et al.*, 1995). Furthermore, UL36/vICA inhibits FAS mediated caspase activation (Skaletskaya *et al.*, 2001) and UL37/vMIA inhibits the activity of the pro-apoptotic proteins Bax and Bak (Goldmacher *et al.*, 1999). Interestingly, the most abundantly expressed HCMV RNA, the non-coding RNA β2.7 inhibits apoptosis in infected U373 glioma cells via stabilizing the mitochondrial respiratory chain complex I, by preventing the relocation of an essential sub unit, GRIM-19 (Reeves *et al.*, 2007). Moreover, HCMV infection induces expression of the Bcl-2 protein in infected colon and neuroblastoma cancer cells, which led to resistance to cytotoxic drugs. When the cells were treated with antivirals, the resistance was reversed (Cinatl *et al.*, 1998; Harkins *et al.*, 2002).

2.4.3 Limitless Replicative Potential

Normal cells divide under normal circumstances for a certain number of times, and they then reach a non-dividing state called senescence. This is a natural process and is controlled by several molecular mechanisms. Senescence can be avoided by blocking the tumour suppressor proteins pRb and p53, which allow the cells to continuously divide and multiply; this process is called immortalization (Wright *et al.*, 1989). Immortalization is a very typical characteristic of tumour cells in culture, which they acquire during the transformation process (Hayflick 1997). The HCMV proteins pp71 and UL97 mediate phosphorylation and inactivation of the tumour suppressor Rb family of proteins, thereby modulating the proliferation and survival of the cells as typical oncogenic viruses do (Hume *et al.*, 2008b; Kalejta *et al.*, 2003b). Cancer cells express telomerase, which is an enzyme that protects the telomeres from shortening during each cell division and prevents cellular senescence and sustain cellular division (Deng and Chang 2007). High telomerase activity is commonly induced by oncogenic viruses, and this is vital for their long lasting survival. Telomerase activity is often induced to multiply infected cells as well as for tumour cell growth (Bellon & Nicot, 2008). Our lab has found that the HCMV IE72 protein promotes the replicative potential of tumour cells by inducing telomerase activity and telomere lengthening via a specific interaction with the hTERT promoter. Stråat et al. also found hTERT and IE1 proteins to be co-expressed in GBM tissue samples (Stråat *et al.*, 2009).

2.4.4 Genomic Instability

Cancer, and most hallmarks of cancer are considered to be the results of genomic instability, and this is one of the very important characteristics of cancer. Genetic instability and mutations occur due to deregulated DNA repair pathways. Due to genomic instability, amplifications, deletions, and re-arrangements of chromosomal segments, a point mutation or even gain or loss of entire chromosomes occur. In DNA repair pathways, p53 is the 'guardian of genome', and mutations in p53 could lead to loss of control over the DNA repair mechanism. Karyotypic instability and associated amplifications and deletions of chromosomal segments occur in most cancers due to loss of telomeric DNA, wherefore telomerase activity plays a crucial role in maintaining genome integrity (Artandi & DePinho, 2010).

In infected baby rat kidney cells, the HCMV proteins IE-72 and IE-86 together with adenovirus E1A protein induced mutations in the p53 gene as a part of a cellular transformation process. Although transformed cells had a mutated p53 gene, HCMV was not found in the transformed cells, implying a "hit and run" mechanism for virus induced cellular transformation (Shen *et al.*, 1997b). The HCMV UL76 protein can induce micronuclei, misalignment of chromosomes, lagging and bridging thereby inducing DNA damage (Siew *et al.*, 2009). Infection with HCMV induces DNA breakage in chromosome-1, specifically at positions 1q42, 1q21 and 1q23.3. Interestingly, deletion of the 1q42 chromosome segment is associated with glioblastoma (Li *et al.*, 1995), which is a tumour that is almost always positive for HCMV. Additionally, certain tumour suppressor genes in patients with breast cancer are proposed to be located at 1q21-31 (Bieche *et al.*, 1995), thus representing a potential target of 1q21 strand break consequences that can arise in HCMV infected cells.

2.4.5 Angiogenesis

Angiogenesis is a process of formation of new blood vessels, which is a very important component during solid tumour development. The growth of the tumour mass require blood vessels for its maintenance and progression. The initiation of this process is controlled by the relative balance of pro-angiogenic (e.g basic fibroblast growth factor bFGF) and anti-angiogenic factors (e.g thrombospondin-1 (TSP-1)) and the responding vascular endothelial cells. Several pro-angiogenic and anti-angiogenic factors are present in different tumour types (Hanahan & Folkman, 1996). The pro-angiogenic factors stimulate new blood vessel formation with great impact on tumour proliferation and metastasis formation (as reviewed in (Hanahan & Weinberg, 2000)). TSP-1 levels were downregulated in tumours with loss of p53 function (Dameron *et al.*, 1994). In cancers, VEGF expression could also be induced by the indirect activation of oncogenes or deregulated tumour suppressor genes (Maxwell *et al.*, 1999; Rak *et al.*, 1995).

HCMV has modulated multiple ways to promote angiogenesis and it acts as a pro-angiogenic factor. Cell-free supernatants obtained of HCMV-infected cells contain higher levels of pro-angiogenic molecules and promote angiogenesis *in vitro* (Dumortier *et al.*, 2008). In infected glioma cells, HCMV IE is also found to induce angiogenesis either via suppression of TSP-1 expression or via induced production of IL-8 (Murayama *et al.*, 1997) in glioma cells (Brat *et al.*, 2005). In infected glioblastoma cells US28 was shown to be involved in the viral induced angiogenic phenotype via induced production of VEGF (Maussang *et al.*, 2006) and also by

promoting growth and invasion via phosphorylation of STAT3 and eNOS (Soroceanu *et al.*, 2011). It has been proposed that HCMV gB proteins bind to PDGFR-alpha and this leads to intracellular phosphorylation of the receptor which leads to enhanced migration and angiogenesis in GBM cells (Soroceanu *et al.*, 2008), Furthermore, HCMV-mediated activation of COX-2 may also promote angiogenesis in GBM cells; this is also mediated by US28. COX-2 further induces the expression of VEGF, bFGF, PDGF, iNOS, and TGF-α in tumour cells, which may result in further angiogenesis and endothelial cell migration (Tsujii *et al.*, 1998).

2.4.6 Immune Evasion

Cells of the immune system are constantly patrolling tissues to identify pathologically alerted cells. Most cancer cells have developed immune evasion strategies to escape recognition by the immune system by various molecular and cellular mechanisms. Briefly, the host immune system is not effectively recognizing the cancer cells as they are disabled in presenting the antigens on their surface, they have reduced HLA class I molecules on their surface. The ability to avoid immune recognition is essential for the existence of cancer (Drake *et al.*, 2006). Furthermore, tumour-related elements such as secretion of immunosuppressive cytokines, and deficient expression of immunomodulatory molecules and resistance to apoptosis orchestrate many important roles in controlling the immune response to cancer, reviewed in (Seliger, 2005). In the tumour microenvironment, PGE_2 and the immunosuppressive cytokines TGF-β and IL-10 orchestrate the anti-tumour immune response (Gomez & Kruse, 2006). In cancer patients who have tumours heavily infiltrated with NK cells and Cytotoxic T Lymphocytes (CTL) have a better prognosis in terms of disease free overall survival time at all stages of disease than patients with low levels those cells in their tumours (reviewed in (Pages *et al.*, 2010)).

Under normal circumstances, HCMV maintains a balance with its host's immune system, it stimulates the immune response and induces the inflammation, and at the same time it has developed numerous immune evasion strategies. It has established multiple sophisticated strategies to affect immunological, molecular and cellular pathways to avoid detection by all arms of the immune system. This will allow for a persistent infection and virus spread without harming the infected cancer cells/normal cells.

HCMV infection interferes with antigen presentation

HCMV encoded gene products interfere with antigen presentation, suppression of antigen presentation and prevent the T- cell response in infected cells. HCMV US2 and US3 proteins downregulate the surface expression of MHC class-II, which avoids the recognition by the CD4+ T cells (Miller *et al.*, 2001). Furthermore, the HCMV protein US6 inhibits TAP-mediated peptide translocation in the endoplasmic reticulum (ER), US3 causes retention of MHC-I molecules into the ER, US11 causes displacement of the MHC-I heavy chain into the cytoplasm, and US2 helps to export the MHC-I heavy chain from the ER. Thus, HCMV encoded gene products interfere with antigen presentation, suppress antigen presentation and prevent the T cell response in infected cells (Soderberg-Naucler, 2006).

HCMV infection interferes with the NK cell response

NK cells provide a very important line of defence important for killing of infected cells and tumour cells. HCMV has evolved unique strategies to evade its own virally mediated downregulation of MHC class I antigen to overcome NK cell recognition, reviewed in (Soderberg-Naucler, 2006). Here, I mention some of the mechanisms: NK cell inhibitory (LIR-1) and activating (NKG2D) receptors are expressed on NK cells and regulate NK cell functions (Arase *et al.*, 2002). HCMV UL18 is an MHC class I homologue protein, which mimics MHC class-I molecule and inhibits the NK cell response by triggering the inhibitory receptor LIR-1 (Wilkinson *et al.*, 2008). This also prevents activation of NK cells via the "missing self-hypothesis".

In summary, HCMV's different roles in *in vitro* cancer studies are eminent, and in animal studies anti-viral treatment suppressed tumour growth (Baryawno *et al.*, 2011a). In patients, the time to metastasis development and the survival was shorter in breast and colon cancer patients with high grade HCMV infection (Taher *et al.*, 2014). Patients diagnosed with high grade of HCMV infection in GBM tumours had shorter survival (Rahbar *et al.*, 2012c), and importantly treatment with valganciclovir in GBM patients indicate increased survival rates (Söderberg-Nauclér *et al.*, 2013). Thus, as a pathogen HCMV also follows the 21st century Koch's postulate criteria, especially its association in cancers. Furthermore, as extensively described, HCMV directs the hallmarks of cancer via its oncomodulatory mechanisms. Although, HCMV studies so far failed to transform normal cells into cancer cells. Treatment with antivirals to CMV as add on to standard therapy give hope for improved survival of GBM patients. Thus, I believe HCMVs role in cancer needs to be further evaluated to improve the patient outcome.

3 MicroRNAS

3.1 Background

In 1958, the central dogma of molecular biology describing ribonucleic acid (RNA) as a mediator between deoxyribonucleic acids (DNA) and proteins was proposed, while proteins play the significant role in all natural molecular pathways (Crick, 1970). In the last few decades, the knowledge of RNA biology has expanded significantly. This includes the discovery of different types of RNA molecules such as transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs), small interfering RNAs (siRNAs), microRNAs (miRNAs) and long non-coding RNAs (lncRNA). Furthermore, their respective functions in biology have been elucidated by extensive research (Eddy, 2001; Fire *et al.*, 1998; Kruger *et al.*, 1982).

In the human genome, about 90% of the sequence is transcriptionally active, though only less than 2% of the genome encodes protein-coding genes (Stein, 2004; Taft *et al.*, 2010). These observations suggest that non-coding RNA plays important roles in cellular development, translation and mRNA biogenesis (Brosnan & Voinnet, 2009). In general, based on their length, non-coding RNA can be classified into two broad categories, i.e. long non-coding RNA (>200 nucleotides – 100 kb) and small non-coding RNA (<200 nucleotides). To date, more than 1500 long non-coding RNA genes had been annotated (Gencode 25) and more than 250 of them had been characterized functionally (http://www.lncrnadb.org/). Viruses, including several members of the herpesviruses, are also known to encode both lncRNAs and miRNAs. For example, human cytomegalovirus (HCMV) encodes four lncRNAs (RNA1.2, RNA2.7, RNA4.9 and RNA5.0) and 26 miRNAs. In this thesis, I will describe the basics of miRNAs biology with in-depth focus on HCMV encoded miRNAs.

3.2 Discovery of miRNA

In 1993, for the first time a small RNA molecule, lin-4, was discovered in *Caenorhabditis elegans* (*C. elegans*). Lin-4 represses lin-14 protein expression, by which it regulates the timing of larval development (Lee *et al.*, 1993; Wightman *et al.*, 1993). It was thought to be specific for *C. elegans*, and almost seven years later another study found a similar function with another small RNA named let-7 (Reinhart *et al.*, 2000). As let-7 is conserved among other species, most researchers were intrigued and investigated if these tiny RNA molecules were functional (Pasquinelli *et al.*, 2000). In 2001, using cloning methods, about 100 small RNAs with 18-22 nucleotides were identified in worms, flies and human cells by three independent laboratories and the term “miRNA” was coined (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee & Ambros, 2001).

After these studies, miRNAs were identified in several different species, and the development of the knowledge of the miRNA has expanded significantly. To date, 30,424 miRNAs have been identified from 206 species, and 2,578 human encoded miRNAs have been annotated in the miRNA database (www.mirbase.org version 20). It has been estimated that 60% of protein expression occurring in cells is controlled by miRNAs (Friedman *et al.*, 2009).

3.3 Genomic location and biogenesis

The biogenesis of miRNAs begins within the nucleus by transcription. The miRNA-encoding transcripts are located in various regions of the genome such as in introns of protein-coding genes or within the intergenic regions, and less frequently within exons or as antisense to mRNA transcripts. Furthermore, many miRNA encoding transcripts exist in clusters (Lagos-Quintana *et al.*, 2003; Rodriguez *et al.*, 2004).

Intergenic miRNAs have their own transcriptional regulatory region/promoter and these are independent units of transcription. Those miRNAs that are located within introns of genes are usually co-transcribed with their host gene transcription and their expression levels are mostly similar to their host mRNA transcripts (Aravin *et al.*, 2003; Lai *et al.*, 2003; Lim *et al.*, 2003).

The biogenesis of the miRNAs has been described using two major pathways:

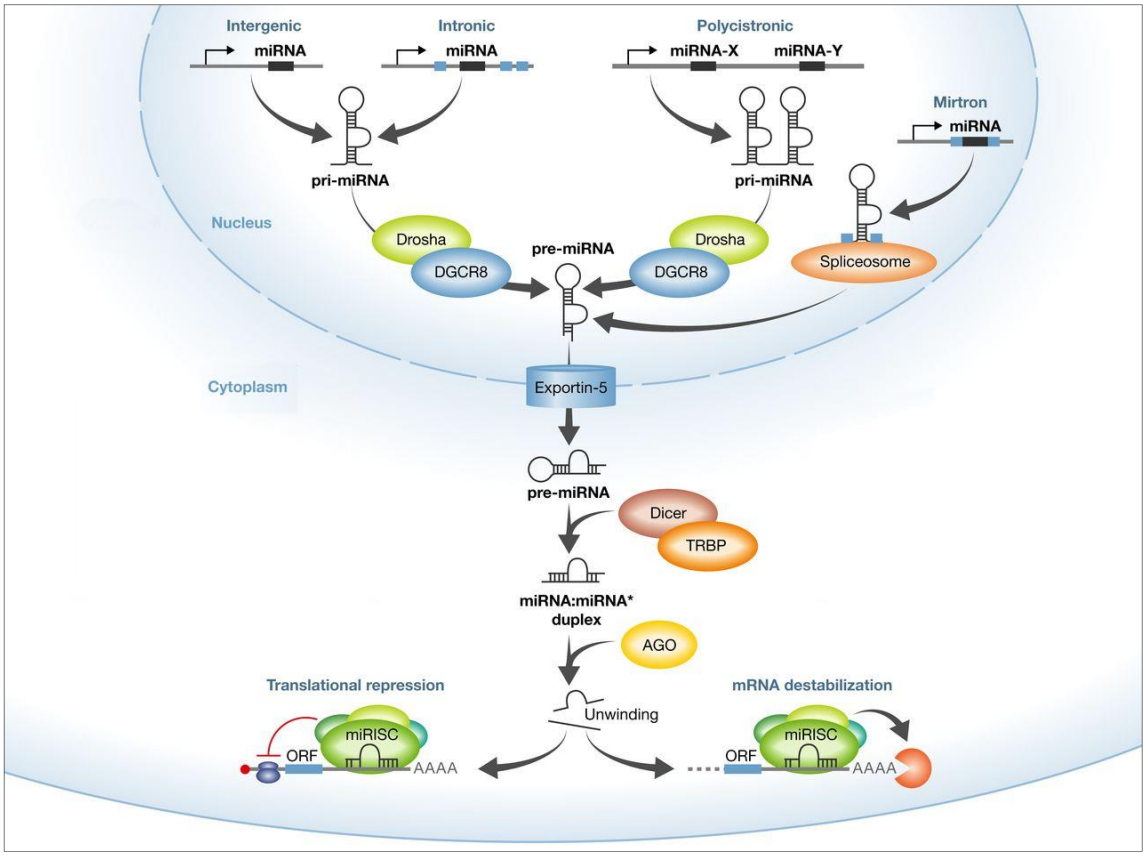

Figure 5. Overview of the miRNA biogenesis. Adapted and modified from (van Rooij & Kauppinen, 2014)

Canonical biogenesis pathway

Primary-miRNAs are produced as RNA transcripts by RNA Pol II or Pol III, and partly fold into the stem-loop structure (Borchert *et al.*, 2006; Han *et al.*, 2006; Lee *et al.*, 2004). Each stem-loop structure is cleaved at stem and become a short 60-100 bases stem-loop structure called precursor miRNA (pre-miRNA). The cleavage is mediated by two proteins: RNase III endonuclease Drosha and a double-stranded RNA-binding protein DiGeorge syndrome critical region gene 8 (DGCR8) (collectively called microprocessor complex) (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004; Lee *et al.*, 2003; Lee *et al.*, 2002). These pre-miRNAs are translocated from the nucleus to the cytoplasm of the cell by Ran-GTP and Exportin-5 protein complex (Bohnsack *et al.*, 2004; Okada *et al.*, 2009; Yi *et al.*, 2003; Zeng & Cullen, 2004). The loop structures of the pre-miRNA are then cleaved by the Dicer and TARBP2 complex and become a duplex miRNA (only stem) consisting of two strands. The passenger strand is mostly degraded and the other leading strand is incorporated into the Argonaute-containing protein complex (RNA-induced silencing complex, RISC) and targets the mRNA transcripts (Chendrimada *et al.*, 2005).

Non-canonical biogenesis pathway

Some miRNAs can be processed independently of Drosha or Dicer processing. Those miRNAs that bypass Drosha processing are termed mirtron and there are hundreds already identified (e.g. miR-877 and miR-1224). The pre-miRNA is generated directly from splicing, as it is directly adjacent to the splice sites (Babiarz *et al.*, 2008; Berezikov *et al.*, 2007; Okamura *et al.*, 2007; Ruby *et al.*, 2007). Furthermore, apart from mitrons, there are several exceptional miRNAs which are also processed via non-canonical pathways (e.g. has-miR451).

3.4 Mechanism of Function

MiRNA expression pattern is temporal and tissue-specific, suggesting their important roles in almost all biological processes (Ambros, 2004; Bartel, 2004). To date, it has been well established that miRNAs suppress gene expression at post-transcriptional level and most reports suggest that they downregulate protein expression. One miRNA can target one or multiple mRNA transcripts, and one single mRNA can also be targeted by multiple miRNAs, which make them act in very complex regulatory networks (Bartel, 2004; Tang *et al.*, 2012; Zhou *et al.*, 2013; Zisoulis *et al.*, 2012). Furthermore, the complexity of these networks increases when the host or pathogen miRNA targets each other's mRNAs and drives disease pathogenesis through their effects on biological processes. However, there are also accumulating evidence suggesting that miRNA can also induce protein expression (Mortensen *et al.*, 2011; Truesdell *et al.*, 2012; Vasudevan *et al.*, 2007). Interestingly, in HCV infection the host liver-specific miR-122 binds to 5'UTR of HCV RNA stimulating its translation (Henke *et al.*, 2008) and also protecting it from host exonuclease Xrn1 degradation (Li *et al.*, 2013). Importantly, anti-miR treatment against miR-122 alleviate from HCV infection (Janssen *et al.*, 2013).

The functional mechanisms of miRNAs have been described in two major steps: i) Target recognition, and ii) Translational inhibition or mRNA degradation.

Target Recognition

Several mature miRNA characteristics play a crucial role in targeting mRNAs. Among these, the seed region of the miRNA is a very important feature. The miRNA seed is the nucleotides located at 2 – 8 position on the 5' end of the mature miRNA. This seed sequence binds to its complementary sequence on the mRNA and regulates gene expression (Lewis *et al.*, 2003). There are several sub-types of seed regions that have been experimentally found and validated, such as 8 mer, 7 mer and 6 mer (Bartel, 2009). Apart from the seed region, additional complementary sequences throughout the miRNA gives higher. Several other determinants can also contribute to the efficacy of miRNA target recognition. These include AU-rich nucleotide compositions, the proximity of several miRNA targets, that the target site is closer to the stop codon in the 3'UTR region (at least 15 nt), or its position away from the centre of the long UTRs (Grimson *et al.*, 2007). There are several miRNA target prediction tools/ databases available online such as PicTar (http://pictar.mdc-berlin.de/) (Krek *et al.*, 2005), TargetScan (www.targetscan.org/vert_71/) (Lewis *et al.*, 2005) and Microcosm (www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/#) (Griffiths-Jones *et al.*, 2008). These tools/databases, however, are not useful to predict viral miRNA targets. In fact, to our knowledge, reptar (http://reptar.ekmd.huji.ac.il/) (Elefant *et al.*, 2010) and RNA hybrid (Krüger & Rehmsmeier, 2006) are the only tools that can be used for target predictions of viral miRNAs. As databases of published human and viral miRNA targets/ miRNAs, miRTarBase, and VIRmiRNA tools, respectively, can be very useful.

Translational Repression

In general, the imperfect base pairing of miRNA and mRNA leads to translation repression. The mechanisms by which mRNA translation is inhibited is still not fully elucidated. However, few reports suggest that miRNAs interfere with translational initiation and/or elongation

(Petersen *et al.*, 2006; Pillai, 2005). Disruption of the translational initiation complex formation can occur via dissociation of the poly-A binding proteins (PABP) from the poly-A region of the mRNA by the miRNA-RISC complex, that recruits CCR-NOT to this region (Zekri *et al.*, 2013). On the other hand, it has been suggested that during the translational elongation process, the miRNA-RISC complex promotes ribosomes drop-off (Petersen *et al.*, 2006). The translational repression is suspected when the mRNA levels are almost stable, but the protein levels are decreased, however, this might not apply for stable proteins or it depends on the miRNA turnaround time.

mRNA Degradation

In plants, miRNA-mediated target degradation of mRNAs is a common mechanism which requires a perfect base-pairing of the entire miRNA sequence with the mRNA and it is mediated by the Argonaut proteins (Yekta *et al.*, 2004). In animal cells, miRNA-mediated degradation directs the target mRNAs to the cellular mRNA decay pathway. The miRNA-RISC complex recruits the CCR4-NOT deadenylation complex to the site of the poly-A region and removes the poly-A tail of the mRNA (Behm-Ansmant *et al.*, 2006). The loss of the poly-A tail also results in mRNA degradation by the major cytoplasmic exonuclease Xrn1 (Ambros, 2004; Meister & Tuschl, 2004). The mRNA degradation is suspected when both mRNA and protein levels decrease and it can occur in the case of stable proteins.

3.5 Herpesvirus miRNAs

Viruses can encode miRNAs from their genome, despite not having their own miRNA biogenesis machinery. In 2004, for the first time, EBV was shown to encode miRNAs (organised in two clusters in the genome). These were identified in latently EBV-infected Burkitt's lymphoma cell line by small RNA cloning and validated by northern blot (Pfeffer et al., 2004). Later, several studies also found miRNAs in other viruses, including members of the herpesvirus family, retroviruses, adenoviruses and polyomaviruses. To date, 502 mature viral-encoded miRNAs have been identified in 29 different viruses, among which 277 (55%) miRNAs are encoded by 13 different herpesviruses (April-2017, www.mirbase.org). In contrast to the human miRNA annotations, viral miRNAs are annotated according to their nearby gene names. miRNA loci are arranged in clusters in the genome of most of the herpesviruses, whereas most miRNAs in HCMV are distributed across the genome. Accumulating evidence suggest that viral miRNAs and host miRNAs play a vital role in virus – host interactions and biology.

DNA viruses that replicate in the nucleus of the infected cells likely utilise the host miRNA biogenesis machinery to process the viral miRNAs. Several studies have also investigated the presence of miRNAs in RNA viruses, and they reported the presence of miRNA in bovine leukaemia virus (BLV) (Kincaid *et al.*, 2012) and HIV (Omoto & Fujii, 2006). These findings remain under debate and need more validation and functional studies. However, importantly, how miRNA biogenesis takes place for RNA viruses remains a big question to resolve.

3.6 HCMV Encoded miRNAS

In 2004, Pfeffer *et al* cloned nine miRNAs from HCMV infected human foreskin fibroblasts (Pfeffer *et al.*, 2004). The following year, more evidence was accumulated by two independent

laboratories that used in silico methods. They predicted several HCMV miRNAs and their existence was further confirmed by northern blots and cDNA cloning (Dunn *et al.,* 2005; Grey *et al.*, 2005). To date, HCMV encoded 26 miRNAs are identified and sequences are available in miRBase database (www.mirbase.org), HCMV encoded miRNAs can target 3'UTR (Kim *et al.*, 2015) and 5'UTR of both viral and host transcripts (Grey *et al.*, 2010b). In a study using a rat model, cytomegalovirus miRNAs expression is tissue specific and varies among the organs (Meyer *et al.*, 2011). A recent study found that a 15 kb segment in the UL/b0 region can act as 'miRNA decay element' (miRDE), which regulates the turn-over of particular cellular miRNAs (Lee *et al.*, 2013). To our knowledge, this is a novel mechanism of action found among the herpesviruses.

3.6.1 HCMV miRNAs Affect Several Host Cell Functions

Similar to the cellular miRNAs, the viral miRNAs encoded by HCMV regulate several biological processes such as cell cycle or apoptosis. For example, an *in vitro* screening study using co-immunoprecipitation of myc-tagged Ago2 / biotin conjugated synthetic miRNA mimic found that the HCMV miRNA miR-US25-1 targets several cell cycle controlling or tumour proliferation genes (such as cyclin E2, BRCC3, EID1, MAPRE2, and CD147) in HEK-293 cells (Grey *et al.*, 2010b). Most miRNAs are known to target the 3'UTRs, however, Grey et al was the first to show in herpesviruses that 5'UTRs is the major target site for the miR-US-25-1. By regulating cyclin E2 levels, miR-US25-1 promotes the cell cycle arrest in G1 phase, which provides favourable conditions for HCMV infection (Grey *et al.*, 2010b). In another study miR-US25-1 expression, in the presence of the oxidised low-density lipoprotein, but not alone, induced apoptosis in endothelial cells. Furthermore, miR-US25-1 expression was significantly higher in the blood of HCMV serology positive atherosclerosis patients and healthy donors (Fan *et al.*, 2014). On the other hand, miR-UL148D targets the human immediate early gene X-1 and exerts anti-apoptotic effects on cells (Wang *et al.*, 2013). Using silico methods, miR-UL148D and miR-UL70-3p were found to target several pro-apoptotic genes, but these observations need validation in *in vitro* experiments (Babu *et al.*, 2014). The over-expression of miR-US25-2-3p results in downregulation of Eukaryotic translation initiation factor 4A1 (eIF4A1) and this leads to inhibition of translation, suppression of host cell proliferation and also inhibits virus replication (Jiang *et al.*, 2015; Qi *et al.*, 2013). Another study found that miR-UL112-1 targets the BclAF1, which is an anti-viral protein that is known to be downregulated by virus delivered pp71 and UL35 proteins and essential for viral gene expression and replication (Lee *et al.*, 2012). Furthermore, miR-US25-1 also targets several cellular genes that inhibit viral replication, such as YWHAE, UBB, NPM1, and HSP90AA1 (Jiang *et al.*, 2015).

On the other hand, the HCMV infection is also studied by manipulating the miRNA biogenesis machinery. Two animal studies have been conducted using infection with mouse cytomegalovirus (MCMV) in Dicer mutant mice. These mice have increased susceptibility to infection and this is correlated with the deregulation of type-I IFNs (Ostermann *et al.*, 2012). Furthermore, when Dicer-deficient neonate mice were infected with MCMV, the virus was highly disseminated to the brain, and the infected mice exhibited increased lethality (Ostermann *et al.*, 2015).

3.6.2 HCMV miRNA -mediated Immune Evasion

Immune evasion is an important part of virus pathogenicity and not surprisingly, HCMV miRNAs target several proteins involved in the anti-viral immune response from the host. An important example is the HCMV miR-UL112 which can interfere with the cell-mediated immune response by downregulating expression of several different proteins. Bioinformatical predictions revealed that HCMV miR-UL112 is located antisense to the UL144 transcript (DNA glycosylase), and it can target the gene expression of UL144 (Stern-Ginossar *et al.*, 2009). HCMV- miR-UL112 targets and downregulates HCMV IE72 and inhibits viral replication (Grey *et al.*, 2007). IE72 is one of the major IE proteins that directs the infected cells toward the lytic phase of infection by acting as a trans-activator for the production of early and late proteins, and it is also essential for replication of the virus. It was proposed that targeting of IE72 with miR-UL112 may favour the establishment of a latent infection, thus these experiments were repeated in latency models using monocytes and THP-1 cells. Using a mutant virus that lacks the miR-UL112 binding site of the IE72 gene, it was confirmed that downregulation of IE72 also occurs in these cells. Furthermore, infected cells were not recognised by IE specific cytotoxic T-cells, suggesting that miR-UL112 plays a major role in latent infection and mediate immune evasion mechanisms (Lau *et al.*, 2016b). Another study showed that HCMV- miR-UL112 inhibits secretion of Interferon type-I and attenuates T cell activity (Huang *et al.*, 2015). The hcmv-miR-UL112-3p also targets toll-like receptor 2 (TLR2), which inhibits NFκB signalling and leads to avoidance of the innate immune response (Landais *et al.*, 2015). HCMV-miR-UL112 can also directly or together with host miRNAs (Stern-Ginossar *et al.*, 2009). HCMV- miR-UL112 also targets and downregulates HCMV IE72 and inhibits viral replication (Grey *et al.*, 2007). Also target the stress ligand MICB (3'UTRs). Downregulation of MICB expression will prevent the infected cells to be recognised by the NK cells. In addition, hcmv-miR-US25-2-3p targets tissue inhibitors of metalloproteinase-3 (TIMP3), which leads to increased shedding of MICA proteins also contributing to a decreased recognition by NK cells (Esteso *et al.*, 2014). Finally, another strategy for escape of NK cell responses involves the induction of the expression of the short form of the RNA editing enzyme ADAR1 (ADAR1-p110). This enzyme is responsible for the editing of the cellular miR-376a, which then will target HLA-E gene expression (Nachmani *et al.*, 2014). One of the mechanisms of evasion from CD8+ T cell response during HCMV infection is through the HCMV miRUS4-1 that has been shown to target ERAP1 and limit the viral peptide loading on MHC-I (Kim *et al.*, 2011). However, a later study using next generation sequencing suggested that the miR-US4-1 sequence was inaccurate, which questions the validity of the study (Stark *et al.*, 2012).

Viral miRNAs can also inhibit secretion of pro-inflammatory cytokines, which would result in reduced recruitment of immune cells at the sites of infection. HCMV infection induces the production of the pro-inflammatory cytokine IL-32 and hcmv-miR-UL112 downregulates the production of IL-32 (Huang *et al.*, 2013b), which suggest that this miRNA prevents the secretion of IL-32 to evade the immune response.

Several HCMV encoded miRNAs (UL112-1, US5-1, and US5-2) were also identified (Ago2 immunoprecipitation) to target secretory pathway genes such as VAMP3 (vesicle-associated membrane protein 3), RAB5C (RAS-related protein 5C), RAB11A (RAS-related protein 11A), SNAP23 (synaptosomal-associated protein, 23 kDa), and CDC42 (cell division control protein

42). Infection of cells with viruses lacking these miRNAs resulted in an increase in defective particle production and failed to form the virus assembly compartment and increase the secretion of TNF-α and IL-6 (Hook *et al.*, 2014). A recent study of *in vitro* infected primary myeloid cells also demonstrated that miR-UL148D prevented upregulation of ACVR1B expression, which led to the limited release of pro-inflammatory cytokines from infected primary myeloid cells. This protein is a cellular receptor of the activin signalling axis known to promote differentiation of monocytes into dendritic cells (Lau *et al.*, 2016a). hcmv-miR-UL148D also regulates production and release of the chemokine RANTES (Kim *et al.*, 2012). hcmv-miR-UL148D is accumulated in CD34+ infected cells and targets the IER5 gene which results in upregulation of CDC25B, which plays a major role in inhibition of IE-72 gene by activating the CDK-1 (Pan *et al.*, 2016). Thus, these results suggest that miR-UL148D plays a major role in modulating the immune response or controlling re-activation of latent infection.

3.6.3 *Expression of HCMV miRNAs during* Latency

Host miRNAs regulated by the HCMV infection and HCMV encoded miRNAs are known to play key roles in the establishment and maintenance of latency. As of today, it has been established that HCMV remains latent in CD34+ myeloid lineage cells. To study latency *in vitro*, several models have been established, such as infection of the monocytic leukaemia cell line THP-1, infection of CD34+ cells from donors or studies of HCMV positive CD34+ cells obtained from HCMV IgG positive individuals.

There are three *in vitro* infection studies published so far, in which the viral miRNAs are investigated during the latency phase of infection using THP-1 infected cells. One study studied the permissive (lytic), semi-permissive and non-permissive (latent) infection, and found a differential expression of viral miRNAs during the latent and lytic infection phases (Shen *et al.*, 2014). Next generation sequencing of small RNAs in HCMV infected THP-1 cells confirmed that they were differently expressed; the eight HCMV encoded miRNAs (highest to lowest in order: US25-1-5p, US25-2-5p, UL112-3p, US5-2-3p, US4-5p, US5-1-3p, US25-2-3p, UL36-5p), and these also altered the expression of the host miRNAs (39 were upregulated, and 14 were downregulated). These viral miRNAs target various biological pathways such as melanogenesis, cancer, endocytosis and WNT signalling (Fu *et al.*, 2014). Later, another study found eight out of twenty HCMV miRNAs (US22-5p, UL112-3p, UL36-5p, US29-3p, UL36-3p, US22A-5p, UL112-5p and US5-1-3p**)** in THP-1 cell line at 10 day post infection. Among these, six miRNAs (underlined) were new and were not detected by Fu et al.(Meshesha *et al.*, 2016). Finally, in a third study, 16 HCMV encoded miRNAs were examined in THP-1 infected cells and reported that only 13 were detected, with two new miRNAs which are not found in both previous studies (Fu et al and Meshesha et al). However, they only showed data from four representative miRNAs (UL112, UL22A-5p, UL70-3p and US33-3p) (Shen *et al.*, 2014). In summary, a total of 16 HCMV miRNAs were shown to be expressed in the HCMV infected THP-1 cells, though the discrepancy among the studies needs more in-depth investigation, before studying functional relevance of these miRNAs in latency.

Another approach used monocytes obtained from three healthy donors and eight HCMV encoded miRNAs (US22-5p, UL112-3p, UL36-5p, US29-3p, UL36-3p, US22A-5p, UL112-5p and US5-1-3p) were found. Interestingly, these miRNAs are the same as the miRNAs present in the *in vitro* infected THP-1 cells. Furthermore, reactivation of latent HCMV in

monocytes with dexamethasone resulted in differentiation into macrophages, and restoration of expression of 17 out of 19 HCMV encoded miRNAs, including those which were absent in monocytes. These observations suggest that viral miRNAs may play a key role in maintenance and reactivation of latent HCMV infection (Meshesha *et al.*, 2016).

3.6.4 Roles of Host miRNAs in Latent HCMV Infection

HCMV infection alters expression of host miRNAs (Lee *et al.*, 2013; Wang *et al.*, 2008), and accumulating evidence suggest that host miRNAs play crucial roles in establishing and maintaining latency. A recent *in vitro* study of HCMV infected CD34+ progenitor cells found that HCMV down-regulates the cellular miRNA miR-92a, which leads to upregulation of the cellular transcription factor GATA-2 (Poole *et al.*, 2011) and increased expression of cellular IL-10 (Mason *et al.*, 2012). GATA-2 play an important role in myeloid cell differentiation, suggesting that the GATA-2 might be a key regulator for the establishment of the latency. Furthermore, the latency associated IL10 (LAcmvIL-10) is expressed during the latent phase of infection and has been suggested to modulate the production of CCL8 via suppression of the expression of host miR-92a (Poole *et al.*, 2014).

Another interesting study described how the miR-200 family of miRNAs can target the 3'UTR of UL122 transcript (IE2) in HCMV infected CD34+ hematopoietic progenitor cells and reducing the production of the IE2 protein. Infection with a virus containing a mutated miRNA-binding site in the 3'UTR of IE2 results in increased expression of IE2 and higher production of extracellular virus. Moreover, members of the miR-200 family are highly expressed in the CD34+ hematopoietic progenitor cells and monocytes but not in macrophages, suggesting that these miRNAs may play a crucial role in suppressing lytic infection and promoting the maintenance of latent infection (O'Connor *et al.*, 2014).

On the other hand, UL138 protein is known to be required for the establishment and maintenance of latent HCMV infection (Goodrum *et al.*, 2007a; Petrucelli *et al.*, 2009). Overexpression of the miR-UL36 can downregulate UL138 during the first 24h in the lytic cell models, however, this study needs confirmation in the latent infection model (Huang *et al.*, 2013a).

Apart from the latency, HCMV can also alter host miRNA expression involved in other cellular processes. For example, two host miRNAs are upregulated in the HCMV infected endothelial cells, in which miR-217 targets SIRT1 and FOXO3A for promoting angiogenesis (Zhang *et al.*, 2013a) and miR-199a-5p targets SIRT1 and eNOS that enhances cell migration and tube formation (Zhang *et al.*, 2013b). HCMV proteins IE1, pp71, and UL26, downregulates host miR-21, which leads to decrease in expression of Cdc25a a cell cycle regulator gene, which facilitates the HCMV replication. Suggesting miR-21 inhibits the replication of the virus in neural cells (Fu *et al.*, 2015), and miR-21 is known to be one of highly expressed miRNA in almost all solid tumours.

3.6.5 HCMV miRNAs as Biomarkers of Disease

In human subjects, hcmv-miR-UL112 was found highly expressed in plasma samples from hypertension patients and associated with increased risk of high blood pressure. Furthermore, *in vitro* experiments suggested that hcmv-miR-UL112 can target IRF-1, and proposed that this

could upregulate angiotensin II type 2 receptor expression, which exerts anti-proliferative and pro-apoptotic actions and regulates blood pressure (Li *et al.*, 2011). However, it could be a consequence rather that cause because cytomegalovirus is known to be associated with cardiovascular diseases (Marques & Morris, 2012). Also, 91% of the patients with solid organ transplantation had at least one HCMV miRNA, in which miR-UL112-3p and miR-UL22A-5p were detected in over 70% of the patients (Lisboa *et al.*, 2015).

Another study assessed the expression of HCMV miRNAs in blood of solid organ transplant recipients and found that several HCMV miRNAs are expressed. In fact, about 92% of patients had at least one HCMV miRNA expressed. More specifically, hcmv-miR-UL112-3p and hcmv-miR-UL22A-5p were detected in over 70% of the patient's blood. The levels of miR-UL22A-5p were significant at baseline independently predicted the recurrence of HCMV viremia upon cessation of treatment (Lisboa *et al.*, 2015). In addition, our group detected miR-UL112 in plasma samples from patients with several HCMV-associated diseases (Study-II), and three other HCMV encoded miRNAs (miR-UL22A-3p, miR-UL36-3p and miR-US25-2-3p) (Study-V) in breast cancer tissues.

4 RESULTS AND DISCUSSION

4.1 STUDY-I

Isolation and characterization of novel genetic variant of HCMV highly associated with tumours of different origin

Background

HCMV encoded proteins or viral nucleic acids have been found in several cancer types, but not in surrounding healthy tissues, which indicates a possible role of HCMV in those tumours rather than representing a mere epiphenomenon. However, an association of HCMV with cancers has remained a controversial topic since several studies could not find evidence of HCMV in tumours. Thus HCMV´s association with cancers has continued to be a controversial topic. While investigating the wild type HCMV genome in colon cancer samples using nested PCR primers against the MIE gene (from exon-2 to exon-3), we virtually, by accident, found that the size of the PCR product in an agarose gel was smaller than expected. Surprisingly, the PCR product aligned to the HCMV genome with a frame deletion, the intron 2 of the immediate early gene, suggesting a new variant virus/strain. Next, we screened different cancer tissues and controls for the presence of the same genetic variant, and investigated the prevalence of this unique HCMV variant in cancer specimens, in clinical HCMV isolates and in healthy carriers. We named this variant virus CMVIE∆int2. Furthermore, we isolated this virus from one clinical sample, and we were able to plaque purify it from the wild type virus that was also present in the same clinical isolate.

Results and discussion

In this study, frozen tissues of glioblastoma, neuroblastoma, medulloblastoma, colon and breast cancer samples were used to isolate the DNA and RNA with Trizol Reagent (life technologies). Formalin-fixed paraffin-embedded or fresh tumour samples are used for immunohistochemistry (IHC) staining for HCMV proteins. We also examined 50 primary glioblastoma cells for HCMV antigens and nucleic acids by flow cytometry, Western blot, fluorescence in situ hybridization or nested-PCR and/or Taqman PCR.

The presence of nucleic acids of the HCMV wild type and CMVIE∆int2 variant were examined by nested-PCR and/or Taqman PCR in 322 tumor samples, 24 plasma samples of myocardial infarction patients, 11 viremic patients (9 mononucleosis and 2 transplant patients), 140 macrophage preparations from healthy blood donors, and clinical HCMV isolates (n=110). The PCR products were validated by Sanger sequencing using appropriate forward and reverse primers to the HCMV specific sequences.

Detection of a HCMV MIE gene variant lacking intron 2 was found in high prevalence in tumour tissues

A nested PCR assay using primer pairs spanning from the end of exons 2 to the beginning of exon 3 of the MIE gene were applied to investigate the HCMV IE DNA sequence. Furthermore, we developed a highly specific TaqMan PCR assay that uses DNA-specific probes to detect the CMVIE∆int2 variant. We determined that 154 of 322 (48%) examined cancer specimens

were positive for the CMVIE⊿int2 variant; 95 of 322 (47%) cancer specimens were positive for HCMV DNA wild-type virus; 68 of 322 (20%) patients were positive for both the wildtype and the CMVIE⊿int2 variant. Furthermore, 27 of 50 (54%) primary glioma cultures were positive for HCMV wildtype DNA, and 41 (82%) of 50 had the CMVIE⊿int2 variant. The CMVIE⊿int2 variant was also detected in 20 (14%) of 140 blood cell samples from healthy donors, in the plasma of 1 (9%) of 11 viremic patients, and in 2 (8%) of 24 patients with myocardial infarction. All the examined 110 clinical isolates were positive for HCMV wild type, three (3%) of the 110 clinical isolates were also positive for the CMVIE⊿int2 variant. In a few samples that were positive for the CMVIE⊿int2 variant, we found the presence of the promoter, as well as intron 1 and intron 3 regions. These observations suggest that the CMVIE⊿int2 genotype is a variant virus rather than a cDNA made from an mRNA template with the aid of a reverse transcriptase.

To our knowledge, this is the first report that found the intron-2 deleted strain of HCMV. These findings imply that a novel genetic HCMV variant, lacking intron-2 of the MIE region is associated with cancers of different origin. It often co-exists with the wild type HCMV virus, which is not surprising as multiple HCMV strains are known to exist in the same individual (Chou, 1989).

There are several studies that could not find the HCMV genome in tumour tissue using PCR assays (Baumgarten *et al.*, 2014; Holdhoff *et al.*, 2016) or NGS (Tang *et al.*, 2015). One possible explanation is that there could be more deletions or mutated segments in this CMVIE⊿int2 variant genome. As expected, we also observed that the nested PCR is more sensitive than standard PCR or Taqman PCR for the detection of the CMVIE⊿int2 variant. Our results suggest that very few copies of the CMVIE⊿int2 variant DNA are present in cancer tissue samples, and thus, it would be difficult, or even impossible, to detect using the non-target specific next-generation sequencing (NGS) methods, as this method is not sensitive enough for detection of the rare copies of the DNA or RNA among all cellular DNA copies (Depledge *et al.*, 2011). We propose that a high sensitive / target specific NGS of cancer samples is required (ongoing work using the Sure Select strategy) to identify the entire genome of this variant virus.

To further verify that the CMVIE⊿int2 variant is not a contaminant of our lab, we examined a cohort of GBM tissues in Prof Bartek's laboratory in Copenhagen. The variant was found in 6 of 70 (7%) examined glioblastoma tissue samples using new reagents and primers. Surprisingly, these results were much lower than that of observed in our laboratory. We are in the process of the finding out why the prevalence varies among the two labs, the collection and handling of the samples is a very critical step to be considered and furthermore, the prevalence could be different in a different population and variations of the CMV variant may also exist in different populations; these investigations are currently ongoing.

The localisation of the HCMV DNA in these cells has been studied using FISH. A perinuclear localisation of the HCMV nucleic acids was observed in primary glioblastoma cells, in contrast to the nucleus of *in vitro* HCMV infected normal cells, cancer cell lines or blood cells from healthy individuals.

CMVIE⊿int2 variant is associated with detection of small IE reactive proteins

We were interested to investigate further which MIE proteins could be detected in these tumour tissues and cells infected with the CMVIE⊿int2 variant. HCMV MIE proteins were detected in 79/80 (99%) and 30/33 (91%) of tumour samples by immunohistochemistry and flow cytometry, respectively. Furthermore, flow cytometry revealed that 27 of 30 (90%) glioblastoma tissue samples and 6 of 7 (86%) primary glioblastoma cell cultures expressed HCMV MIE proteins. These results were consistent with previously published observations (Cobbs *et al.*, 2002b; Harkins *et al.*, 2002; Harkins *et al.*, 2010; Samanta *et al.*, 2003b). Furthermore, we performed Western blot analysis of 59 tumour samples (15 primary glioblastomas, 10 primary glioblastoma cell cultures, 18 neuroblastomas, 12 breast cancers and 3 colon cancers). Although in few cases the expected predominant IE proteins were detected (55, 72, and 86 kDa), smaller IE-reactive proteins (40, 36, 32, 26, 19, 12 kDa) at various levels were the most frequently observed (55/59 samples, 93%) in tumour specimens.

During HCMV infection *in vitro*, multiple MIE mRNA species are made by differential splicing and polyadenylation (Awasthi *et al.*, 2004) from five exons in the MIE genes UL122 and UL123 (Castillo & Kowalik, 2002a; Stenberg *et al.*, 1989; Stenberg, 1996). The IE1-p72 protein is encoded by exons 2, 3, and 4 and the IE2-p86 by exons 2, 3, and 5 (Castillo & Kowalik, 2002a). The two major gene products, IE1-p72 protein and IE2-p86, regulate the expression of the majority of the HCMV genes and act as trans-activators for early and late gene expression. Although IE-86 protein is essential for replication of the virus, IE-72 is not (Marchini *et al.*, 2001). Five additional transcripts are initiated from exon 2 (IE2-p55, IE28, IE19, IE17.5 and IE9), and two splice variants of IE86 (IE40 and IE60) have been described (White *et al.*, 2007). These transcripts are expected to produce proteins of 60, 55, 40, 38, 31, 19, and 9 kDa, however, their functions are unknown (importantly, how they affect the replication of the virus is also unknown), and they have not been studied *in vivo*. We propose that the IE reactive proteins we detected in tumour specimens represent HCMV IE splice variant proteins. However, this has so far not been possible to confirm with immunoprecipitation and Mass spectrometry analyses. Additional studies are therefore highly warranted to investigate the identity of these proteins further and to explore the functional role of these proteins in tumorigenesis. Importantly, deletion of intron-2 might be affecting the splicing process of the IE pre-mRNA and might lead to the expression of the different IE proteins observed.

Isolation of the CMVIE⊿int2 variant strain

To isolate the intron-2 deleted HCMV strain, we investigated 110 clinical isolates, three of these were positive for both CMVIE⊿int2 variant and wildtype virus. From one of these isolates, C26, *CMVIE⊿int2* was successfully recovered in fibroblasts (MRC-5) and endothelial (HUVEC) cells. Both viruses initially replicated in this two cell types as confirmed by the expression of HCMV MIE, pp65 and gB proteins. Some IE proteins seemed to be localized in the cytoplasm of infected cells instead of the typical nuclear localisation, and western blot analyses detected IE proteins smaller as well as heavier than 70 kDa. We successfully purified the HCMV variant in HUVEC but not in fibroblasts. HUVEC cells infected with the HCMV variant showed aberrant looking plaques and small round cell structures, or cell nests, which were very typical for this virus. In the HCMV variant positive HUVEC cells, only dense bodies

were observed by electron microscopy but not the typical HCMV virus particles. In MRC-5 cells infected with the C26 isolate (containing both the variant and the wild-type virus), all types of HCMV particles were visible: virus particles, non-infectious virus particles and dense bodies. These results indicate that the HCMV variant that was plaque purified was replication defective, expressed IE splice variant proteins, it was cell associated and did not cause a lytic infection. It was hypothesised that cells expressing IE proteins in the absence of lytic virus replication might undergo transformation since expression of IE-72 and IE-86 is mutagenic and resulted in tumour transformation in the presence of the adenovirus E1A protein (Shen *et al.*, 1997a).

In primary cell cultures from glioblastoma patients, we found the variant in three well-characterized cell cultures; GBM30, GBM42 and GBM48. HCMV MIE proteins were detected both in the cytoplasm and the nucleus in GBM30 and GBM42 cells, while GBM48 cells exhibited nuclear localisation of IE proteins, as is expected in wild-type HCMV infection. Nested PCR analysis confirmed that GBM30 and GBM42 are positive for the HCMV variant and GBM48 is positive for wild-type HCMV and the variant. By western blot analysis, approx. a 70 kDa HCMV IE protein, as well as several IE reactive proteins of smaller sizes, were detected; similar in all three cell lines. Immunohistochemistry, as well as immunofluorescence showed the characteristic cytoplasmic IE protein staining pattern observed previously in HCMV variant positive cells. By Electron microscopy analysis, dense bodies were visible and virus-like structures in some cells, none, however, resembling typical HCMV particles.

The high prevalence of HCMV in different types of cancer and the detection of a HCMV variant is clinically relevant and may introduce anti-viral drugs as a new approach for treatment of cancer patients. Our group was the first to treat glioblastoma patients with anti-HCMV therapy as an adjuvant to their standard therapy. Follow-up assessments showed a significantly longer survival among patients who received valganciclovir treatment ((Söderberg-Nauclér *et al.*, 2013) and unpublished results). The fact that most of these patients may be positive for the HCMV variant, which appears to be replication defective, raised the question of how anti-viral drugs affect this virus. To assess whether anti-viral treatment could affect tumour growth in the presence of the HCMV variant, primary glioblastoma cell lines previously described (GBM30, GBM42 and GBM48) were treated with 1 µM or 2 µM Ganciclovir or left untreated. We observed that treatment with the anti-HCMV drug ganciclovir inhibited proliferation of GBM30 and GBM 42 cells by about 60% at 7 days and reduced IE protein expression.

In summary, we report for the first time the existence of the CMVIE∆int2 variant, which is highly associated with tumours. This discovery may provide new insights into the potential role of HCMV in cancer and may explain the conflicting data that has been obtained by different research groups. Thus, our methods described herein offer diagnostic tools to identify individuals who are carriers of the CMVIE∆int2 variant and allows for studies to evaluate whether this virus can be transferred through blood, breast milk and organ transplants. The clinical relevance of this new variant virus and its biological impact in different types of cancer warrants further investigation.

4.2 STUDY-II

Detection of circulating hcmv-miR-UL112-3p in patients with glioblastoma, rheumatoid arthritis, diabetes mellitus and healthy controls

Background

Inflammation plays a very critical role in reactivation of latent HCMV infection (Soderberg-Naucler *et al.*, 2001; Söderberg-Nauclér *et al.*, 1997). Thus, it is not surprising that HCMV infection is associated with several inflammation associated diseases such as, cardiovascular diseases (CVD), Rheumatoid Arthritis (RA), Glioblastoma multiforme (GBM) and Diabetes Mellitus (DM) (Cobbs *et al.*, 2002a; Lucas *et al.*, 2011; Mitchell *et al.*, 2008; Rahbar *et al.*, 2012b; Tamm *et al.*, 1993). The miR-UL-112 is a HCMV encoded miRNA that targets host and viral RNA transcripts and is linked to latency, and circulating miR-UL112 is associated with hypertension. In this study, serum/ plasma of patients with DM, GBM and RA, and Healthy Controls (HC) were analysed by ELISA for quantification of HCMV IgG and IgM levels and standard miRNA Taqman assay for the quantification of miR-UL112 levels.

Results and discussion

Samples from patients diagnosed with GBM, DM or RA and HC (87 plasma/serum) were collected and examined for the prevalence of HCMV IgG, IgM and miR-UL112-3p. The prevalence of the HCMV IgG is 85% (23/27) in DM; and not significant when compared with HC. In contrast to our results, Roberts et al. found a higher prevalence and significantly different levels of HCMV IgG compared with HC in San Antonio population (Roberts & Cech, 2005). Another study found that HCMV IgG is associated with various indicators of glucose regulation and found high prevalence of HCMV IgG to be a risk factor for the development diabetes in the elderly (Chen *et al.*, 2012). In this study, the HCMV-IgG prevalence was 65% (13/20) in GBM patients, and not significantly different as compared with the HC. In contrast to our results, a higher prevalence and significance results were obtained in Iraqi populations (Shamran *et al.*, 2015). The prevalence of HCMV IgG is not associated with risk of developing GBM (Susan Amirian *et al.*, 2013). However, our recent study demonstrated that the IgG negative patients with T-cell reactivity against HCMV peptides were positive for HCMV in their tumours and blood cells. Suggesting HCMV IgG may not be a good indicator of the HCMV activity in GBM patients (Rahbar *et al.*, 2015). We found HCMV-IgG in (14/20) 70% RNA patients, and significantly higher IgG OD values were observed in RA patients compared with HC ($p=0.009$), Similar results were obtained in another study (65.3%), and was shown to be associated severe joint disease (Pierer *et al.*, 2012).

Furthermore, the prevalence of HCMV IgM was found only in one of 20 (5%) GBM patients, all other patients and HC were negative. However, HCMV IgM OD values (background signal) were significantly higher in DM patients as compared with HC ($p=0.03$).

We found that 52% (14/27) of DM patients, 25% (5/20) of GBM patients, 5% (1/20) of RA patients and 10% of HC were positive for miR-Ul112-3p with 8, 4, 1 and 1 mean copies/10 ng of RNA, respectively. Overall DM patients had significantly higher levels of miR-UL112-3p than HCs. Moreover, no significant correlation was found between miR-UL112-3p levels and HCMV IgG or IgM OD values. To our knowledge this is the first study to examine the HCMV

miRNA miR-UL112 in these diseases and linked to DM patients. Elucidation of miR-UL112-3ps functions in DM pathogenies needs further investigations.

The miR-UL112-3p is expressed during active (Shen *et al.*, 2014), latent infection and natural infection (Meshesha *et al.*, 2016). The miR-UL112-3p acts as a translational repressor of HCMV IE-72 and prevents the production of early and late proteins and is proposed to favour latent infection by suppressing the viral replication (Grey *et al.*, 2007; Murphy *et al.*, 2008). Furthermore, several studies have found that miR-UL112-3p mediates immune evasion strategies so that the virus can be maintained in its host and avoid elimination by the host immune response. For example, miR-UL112-3p downregulates IL-32 and MICB, which prevents NK cell recognition and facilitates the establishment and maintenance of latency (Huang *et al.*, 2013b; Stern-Ginossar *et al.*, 2007).

Accumulating evidence suggests that the miRNAs are packed into microvesicles that are released from infected cells (Lasser *et al.*, 2011; Valadi *et al.*, 2007). Thus, we speculate that miR-UL112-3p released into the blood circulation from several cell types such as macrophages, monocytes, endothelial cells or even tumour cells. So far, however, there are no studies that have characterised the miRNA content of the microvesicles derived from HCMV infected cells. Importantly, based on the described functions of miR-UL112-3p, the presence of this viral miRNA in the screened patients might have an impact on disease progression.

Li et al. showed that miR-UL-112 was highly expressed in the Chinese hypertension patients. Using luciferase assays, the miR-UL112 can target IRF-1, and proposed that this could upregulate angiotensin II type 2 receptor expression, which exerts antiproliferative and proapoptotic actions and regulates blood pressure. Another recent study found that miR-UL112-3p is highly expressed in IE-86 positive GBM tissues (Liang *et al.*, 2017). Patients with higher expression of miR-UL112-3p had lower tumour free survival and overall survival rates. Furthermore, overexpression of miR-UL112-3p in GBM cell lines promoted tumour clone formation, cell proliferation, migration and invasion. miR-UL112-3p was shown to target the tumour suppressor candidate 3 (TUSC3) protein. Thus, this study suggests that miR-UL1122-3p might promote tumour progression by downregulation of the Tumour suppressor candidate-3 gene (TUSC3) (Liang *et al.*, 2017).

In summary, increased levels of miR-UL112-3p and higher prevalence and elevated levels of HCMV IgG were observed in DM patients as compared with HC. Higher prevalence of miR-UL112-3p was found in DM and GBM patients as compared with RA patients and HC. Whether miR-UL112-3p levels in the blood can serve a mediator of biological effects or representing a biomarker of the disease and/or HCMV latency warrants further investigations.

4.3 STUDY-III

Human Cytomegalovirus MicroRNAs are carried by Virions and Dense Bodies and are delivered to Target Cells.

Background

A lytic HCMV infection results in the production of mature virions, dense bodies (DBs) and Non-infectious enveloped particles (NIEPS) (Craighead *et al.*, 1972; Stinski, 1976; Talbot & Almeida, 1977a). All these particles consists of host and viral encoded proteins (Baldick & Shenk, 1996; Stinski, 1976; Varnum *et al.*, 2004) and long RNA molecules (Greijer *et al.*, 2000; Huang & Johnson, 2000; Lin *et al.*, 2012; Sarcinella *et al.*, 2004; Terhune *et al.*, 2004). In this study, we investigated whether these particles also contain miRNAs, and further examined if these miRNAs are functional when delivered to the host cells.

Characterization of virions and DBs

We isolated the virions and DBs from supernatants obtained from the HCMV infected MRC5 cells using ultracentrifugation with potassium-tartrate glycerol gradients. The isolated virions and DBs were visualized with electron microscopy-negative staining, the presence of HCMV genome, and pp65 proteins, and absence of host genome was determined by different assays. Surprisingly, we found almost similar amounts of DNA content in both virions and DBs. DBs, however, have less viral genome copies and less infectivity rate than virions (68.4 % in virions vs 3.8% in DBs). Furthermore, the infectivity observed with DBs could be caused by the contamination of few virions in the DBs preparations, as previously described by several reports (Pepperl *et al.*, 2000; Sarcinella *et al.*, 2004; Talbot & Almeida, 1977a), and thus would be expected to be infectious (Sarcinella *et al.*, 2004). Consistent with our study, the pp65 protein was found in DB preparations, but these studies did not search for viral DNA (Baldick & Shenk, 1996; Varnum *et al.*, 2004). Interestingly, one early study contradicts these results. However, Irmiere et al, did not find HCMV DNA or the amino acids in the DB sample, thus indicating that there was no DBs present in the examined sample preparation (Irmiere & Gibson, 1983).

miRNA, mRNA and lncRNAs are incorporated into virions and DBs

Before the isolation of the virions and DBs, the pellets (virus stock) were treated with RNase-ONE and, later, with the micrococcal nuclease to remove all nucleic acids. We found RNA molecules in both virions, and DBs samples, which we initially determined with NanoDrop then characterised with Agilent bioanalyzer analysis and further investigated with TaqMan PCR assays. Bioanalyzer analysis of RNA obtained from virions and DBs using two different kits found various RNA species in size ranges from 6 - 4000 nucleotides, which will include miRNAs, small RNAs and long RNA molecules. There was very little amount or no 18S and 28S rRNAs detected in virions and DBs. In contrast, cellular RNA samples had the highest content of these rRNAs. As the 18S and 28S are long RNAs and associated with ribosomes, they might not be incorporated into the particles. To our knowledge, our study is the first to perform the bioanalyzer analysis of the RNA molecules prepared from virions and DBs.

The viral transcripts IE and lncRNA2.7, and cellular transcript B2M were detected in both virions and DBs at different levels by TaqMan PCR assays. The presence of these viral

transcripts has been previously examined only in virions (Greijer *et al.*, 2000; Terhune *et al.*, 2004) (Bresnahan & Shenk, 2000). The purpose of their presence in the viral particles, however, has not been fully elucidated. The incorporation of the RNA molecules into the particle was suggested to be random, and concentration dependent (Terhune *et al.*, 2004), but another study reported that only a subpopulation of RNA molecules get incorporated into the virions (Bresnahan & Shenk, 2000) indicating there may be some selection involved. In this study, we found that IE and lncRNA2.7 levels in cells and particles had similar patterns, suggesting a concentration depence in incorporation. The translation of the delivered RNA has been determined but functional studies have not been performed (Bresnahan & Shenk, 2000). There are RNA transcripts which are found to hybridise with the viral DNA and proposed to mediate the replication of this virus by acting as an RNA primer (Prichard *et al.*, 1998b).

Among herpesvirus family members such as herpes B virus (Amen & Griffiths, 2011a), KSHV (Lin *et al.*, 2012) and EBV (Jochum *et al.*, 2012), it has been demonstrated that miRNAs are present in virions. Furthermore, delivery and function of the miRNAs from KSHV particles was studied using a luciferase reporter assays and were shown to be functional (Lin *et al.*, 2012). One previous study reported that HCMV small RNAs are abundantly expressed in the infected cells (Stern-Ginossar *et al.*, 2012). However, this is the first report determining the presence of HCMV miRNAs in different particles, virions and DBs. Here, we show the presence of 14 HCMV miRNAs (UL22A-5p, US25-1-5p, UL22A-3p, US5-2-3p, UL112-3p, US25-2-3p, US25-2-5p, US33-3p, US5-1, UL36-5p, US4-5p, UL36-3p, UL70-5p, and US25-1-3p) and 2 host miRNAs (hsa-miR-218-5p and hsa-miR-21-5p) in virions and DBs at different levels. Interestingly, the two miRNAs (UL70-5p and US25-1-3p) expressed in lowest abundance were found in both virions and DBs, but not in infected MRC-5 cells. We speculate that virions and DBs protected these two miRNAs from the cellular nuclease activity, whereas in cells these miRNAs were degraded. The overall pattern of these 14 viral miRNAs levels in these particles, are similar to that of infected MRC-5 cells, suggesting that it may be a concentration dependent incorporation. However, we do not know which other miRNAs are present in the particles and their functions, a next generation sequencing study may be required to fully elucidate whether all the miRNAs (HCMV and host encoded) are present in virions and DBs.

Virions and DBs can transfer viral miRNA to the host cell and be functional

To investigate if these miRNAs from virions and DBs are delivered to host cells and can be functional, both particles were subjected to UV-irradiation to inactivate the virus ability to replicate, and thereafter incubated with MRC-5 cells. The efficiency of inactivation of the virus with UV-treatment was determined using immunostaining against IE proteins 3 hours later. A cytoplasmic staining of IE proteins was observed, but this staining pattern disappeared 1 day later, indicating that IE proteins / RNA molecules are indeed delivered to the target cells. We did not find the IE or lncRNA2.7 transcripts in cells at 1d post treatment with UV-treated particles. Furthermore, we also examined three miRNAs and found two miRNAs; miR-US25-1-5p and miR-UL112-3p in cells incubated with UV-treated virions or DBs.

Among the detected 14 miRNAs, miR-US25-1-5p was the most abundantly expressed. To investigate if the delivered miR-US25-1 was functional, we used a luciferase reporter plasmid containing the 5'UTR of the CCNE2 gene (CCNE2) and a plasmid containing a mutation at

the miRNA binding site (MUT-CCNE2) previously described (Grey *et al.*, 2010a). We found that both UV-inactivated virions and DBs significantly decreased the luciferase levels in cells transfected with CCNE2 luciferase plasmid (12% with virions p=0.02 and 8% with DBs p=0.03), but only a 5% decrease in the MUT-CCNE2 luciferase levels. However, the observation that virions and DB still have an effect on luciferase levels when the miRNA-binding site is mutated suggest an off-target effect of delivered miRNAs or that signalling event may interfere with the luciferase levels. To address this questions further and strengthen the conclusions from this assay, we used virions and DBs isolated from a miR-US25-1 knockout virus and wild-type HCMV virus and increased the number of particles used for the experiment. Consistent with earlier experiments, luciferase levels in cells transfected with the CCNE2 plasmid and infected with WT-virions were significantly lower than in cells infected with miR-US25KO-virions (21% vs. 4% reduction). Similar results were obtained using the HCMV WT-DBs and miR-US25KO-DBs. There was no significant effect on luciferase levels in MUT-CCNE2 transfected cells when incubated with particles from WT or KO samples.

HCMV encoded miRNAs have been found to be key regulators of immune functions by targeting receptors to avoid recognition by immune cells or by inhibiting production of pro-inflammatory cytokines and molecular mechanisms of latency and reactivation (see chapter-III). More specifically, in *in vitro* studies using wild type HCMV and knock out virus infection or miRNA overexpression, it has been demonstrated that the miRNA-US25-1 targets the 5´UTRs of multiple cell-cycle-regulating genes such as CCNE2, H3F3B, and TRIM 28 (Grey *et al.*, 2010a) and several cellular genes (YWHAE, UBB, NPM1, and HSP90AA1), which can affect viral replication (Jiang *et al.*, 2015). Thus, our results suggest that when HCMV virions or DBs deliver miRNAs to the host cells, it affects various pathways by regulating several proteins.

In conclusion, this study shows that HCMV-encoded miRNAs are present in virions and DBs and can be delivered to cells. We provide proof of principle data that viral miRNAs can be delivered from virions and DBs to host cells and be functional, altering the expression of viral and cellular proteins and, thus, perhaps playing important roles in HCMV biology.

4.4 STUDY-IV

High prevalence of human cytomegalovirus proteins and nucleic acids in primary breast cancer and metastatic sentinel lymph nodes

Background *(for study-IV and -V)*

Worldwide Breast cancer is the most common cancer and a leading cause of death in women (reviewed in (Key *et al.*, 2001)). Breast cancer comprises 22% of all cancers in women (Dumitrescu & Cotarla, 2005). Several risk factors such as age, family history, diet, alcohol, etc have been associated with the breast cancer. The causes of breast cancer initiation are not known (Dumitrescu & Cotarla, 2005). In-depth studies have been performed to identify additional risk factors which may contribute to the disease. Apart from the environmental and epidemiological factors, some recent studies have focused on viral infections. It has been proposed that viral infections are responsible for about 20% of all malignancies worldwide (Kuper *et al.*, 2000).

Several studies have found the presence of the oncogenic viruses EBV and HPV in breast cancer (Heng *et al.*, 2009; Labrecque *et al.*, 1995). However, other studies could not confirm the presence of these viruses in breast cancer (Herrmann & Niedobitek, 2003; Silva & da Silva, 2011). This has created an increased interest to find other viral agents in breast cancers, but this also created enhanced controversial discussions in this field (Lawson *et al.*, 2006). As discussed elsewhere in this thesis, HCMV proteins confer pro-oncogenic properties. This virus has been extensively searched for in several types of cancer, and experimental studies are performed to elucidate whether this infection plays a major role in cancer development or progression.

To our knowledge, an association between HCMV and breast cancer was first found by Richardson et al. in 1997. They found that HCMV IgG antibody titers were higher in breast cancer patients than in healthy controls, and these data are consistent with data from more recent studies (El-Shinawi *et al.*, 2013; Richardson, 1997; Richardson *et al.*, 2004). Furthermore, several studies found the presence of the HCMV infection in the patients with breast cancer (El-Shinawi *et al.*, 2013; Harkins *et al.*, 2010; Tsai *et al.*, 2005), and others have reported a lack of evidence of HCMV nucleic acids in breast cancer specimens (Utrera-Barillas *et al.*, 2013). Several studies have reported a high prevalence of HCMV protein expression in the normal glandular epithelium (Harkins *et al.*, 2010; Tsai *et al.*, 2005), in contrast, El-Shinawi found less prevalence of the HCMV in normal glandular breast tissue specimens (El-Shinawi *et al.*, 2013).

Whether HCMV is present in breast cancer or not is under debate today. Accumulating evidence suggest a high prevalence of HCMV infection in various types of malignancies. However, it was not clear whether HCMV is also present in the metastatic tumours. In this study, we investigated the prevalence of HCMV infection in breast cancer primary tissues and its paired sentinel lymph node specimens (SLN). We used a TaqMan assay for viral DNA detection and IHC for the detection of viral proteins. To assess the association between HCMV infection and known clinical parameters, a statistical analysis was performed.

Results and discussion

High prevalence of HCMV infection in breast cancer and paired sentinel lymph node specimens

We collected breast cancer and paired sentinel lymph node (SLN) tissue specimens fixed in formalin-fixed-paraffin-embedded from breast cancer patients with (n=35) and without SLN metastasis (n=38). To detect the viral proteins, we used a high sensitive immunohistochemistry (IHC) method adapted from Cobbs et al and optimised (Cobbs *et al.*, 2002c; Taher *et al.*, 2013) and developed a TaqMan PCR assay for detection of HCMV nucleic acids.

We identified HCMV IE and LA proteins using IHC in all breast cancer specimens (n=73). Our findings were consistent with the results reported by Harkins et al (Harkins *et al.*, 2010), suggesting a high prevalence of HCMV proteins in breast cancer tissues. Both HCMV IE and LA proteins were detected in 94% of SLN positive samples and 60% of the SLN-negative group. HCMV proteins were present in the majority of metastatic tumours in SLN samples. Few HCMV-positive inflammatory cells were also found in 79% of SLN samples with metastasis, and very few HCMV-positive inflammatory cells were also detected in 60% of metastasis-free SLNs. The higher prevalence of HCMV in SLN samples might be due to the HCMV activity, infection of neuroblastoma cells increase the tumor cell adhesion and disrupts the endothelial cell monolayer integrity and favours the transendothelial migration, suggesting that HCMV infection increase the invasiveness (Scholz *et al.*, 1999; Streblow *et al.*, 1999). Furthermore, HCMV infection induces production of TGF-beta1 via inducing MMP-2 (Shimamura *et al.*, 2010). In theory, MMP-2 can mediate the degradation of extracellular matrix (Reinhardt *et al.*, 2006). HCMV induced TGF-beta production and facilitated an EMT process (Michelson *et al.*, 1994). Furthermore, infected epithelial cells treated with TGFB *in vitro* study shows a similar EMT process (Shimamura *et al.*, 2010).

We graded the tumour tissues based on the percentage of HCMV IE and LA positive cells. Sections were graded as negative (0), grade I (< 25% positive cells), grade II (25-49%), grade III (50-75%) or grade IV (> 75%). In both in the SLN-positive and -negative groups, we found that the majority of breast samples were of high HCMV grade (grade III and IV). These results were consistent with our previous findings of a high prevalence of HCMV protein detection in other tumours as well (study-I; (Rahbar *et al.*, 2012a; Taher *et al.*, 2013; Wolmer-Solberg *et al.*, 2013)). Most common grades for HCMV LA proteins were grade II and III, HCMV LA protein expression was less abundant than IE. These results indicate that there was a non-productive viral infection in these tissues. HCMV IE grade III and IV accounted for 76 % of the SLN positive samples, suggesting that most metastatic cells were IE positive. We also noted that IE protein expression was higher in the primary tumours than in SLN tissues. The HCMV IE protein expression grade was also higher in primary breast cancer specimens as compared with the SLN tissue specimens. Importantly, we noted that HCMV protein expression was mostly restricted to tumour cells, although some inflammatory cells were also stained positive for IE proteins.

High HCMV infection grade in breast cancer tissue specimens was observed in patients who died of breast cancer

In glioblastoma patients, we found a strong association between low-grade HCMV infection in the glioblastoma tumour at the time of diagnosis and prolonged survival, which implied that HCMV might be involved in tumour progression (Michaelis *et al.*, 2009; Rahbar *et al.*, 2012a). Furthermore, HCMV infection grade was associated with shorter time to tumour metastasis and shorter survival after diagnosis of distal metastasis in colon and breasts cancer patients (Taher *et al.*, 2014). In this study cohort, only seven patients died of breast cancer; this did not allow for further statistical analyses of a viral impact on survival. However, we noted that six of seven patients who died of breast cancer had high-grade HCMV infection (grade IV), while patients with low IE expression were all among the survivors. These observations imply that HCMV may influence survival and tumour progression, and HCMV targeted therapies may improve the outcome of patients.

HCMV infection level did not correlate with clinical prognostic factors

Estrogen receptor alpha (ER-α) and progesterone receptor (PR) expression status and Elston grade are well established prognostic markers for breast cancer patients, which also influence selection of patient treatment. Loss of ER-α and PR expression are predictors of poor survival (Slobedman & Mocarski, 2012). The Elston-Ellis histopathological grading provides significant prognostic information for breast cancer patients by assessing tubular formation, mitotic activity and nuclear pleomorphism. Furthermore, Elston grade I tumours have a significantly better survival than those with grade II and III tumours (Dalton *et al.*, 1994).

In breast cancer patients, we recently found an inverse correlation of high-grade HCMV positivity with the loss of expression of ER-α and PR, and a trend for reduced HER-2 expression (under review). Furthermore, in our study-V, *in vitro* experiments showed that ER-α and PR are downregulated by the HCMV encoded miRNAs. In this study, we did not find any significant association between HCMV infection grades and these prognostic indicators. This could be due to a selection of earlier cases of breast cancer, or to a low power of the study. Although the presence of an infectious agent in tumour tissues does not provide evidence for its cause of carcinogenesis, the presence of HCMV in both primary tumour and in most SLN metastases tissues, supports the hypothesis that HCMV plays an active role in tumorigenesis and metastasis development in breast cancer patients. Therefore, further studies are highly warranted to evaluate the possible mechanisms contributed to breast cancer tumorigenesis and metastatic diseases.

In an *in vitro* study, HCMV infection of neuroblastoma cells increased the tumor cell adhesion and disrupted the endothelial cell monolayer integrity and favoured trans endothelial migration, suggesting that HCMV infection increase the invasiveness and also trans-endothelial migration (Scholz *et al.*, 1999). This might be mediated by HCMV chemokine receptor US28 (Streblow *et al.*, 1999).

4.5 STUDY-V

A potential role for human cytomegalovirus in triple negative breast cancer through its specific mechanisms to downregulate ER-α, PR and HER-2

Background

Breast cancer incidence is still increasing, although the mortality is decreasing (Ferlay *et al.*, 2015). As described earlier, the expression of estrogen receptor alpha (ER-α), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER-2) are three very important prognostic markers to guide therapy (Hayes, 2005; Hortobagyi *et al.*, 1983). Breast cancer subtypes that lacks the expression of ER-α, PR, and HER-2 are known as a triple-negative breast cancer (TNBC), and these patients have a significantly worse outcome (P Kourea *et al.*, 2014; Perou *et al.*, 2000; Sørlie *et al.*, 2001). Breast cancer patients who have been treated with ER- modulators such as Tamoxifen and the ER-α antagonist fulvestrant, have reduced mortality and recurrences (Dalmau *et al.*, 2014). Similarly, HER-2 overexpressing breast cancer patients were treated with Trastuzumab (Herceptin) (Ahmed *et al.*, 2015), which increased overall survival (Slamon *et al.*, 2001). However, none of these treatments can be offered to TNBC patients, and a significant number of patients do not respond these therapies (Ahmed *et al.*, 2015; Fan *et al.*, 2015). Although, breast cancer treatment has been advanced and improved the survival during the last few decades, still for the TNBC patients are in need new therapy strategies. Several factors are linked to malignant transformation and tumour progression of the breast cancers, including many viruses. A general background of breast cancer and associations with HCMV has been described in the beginning of study-IV.

In breast cancer patients, we recently found an inverse correlation of high-grade HCMV positivity with the loss of expression of ER-α and PR, and a trend for reduced HER-2 expression (under review). These findings suggest that regulating the activity of HCMV in tumours could represent a new therapeutic option for HCMV positive cancer patients. We hypothesised that this virus might affect the expression of these receptors in breast cancer. In this study, we found that three HCMV encoded miRNA (hcmv-miR-US25-2-3p, hcmv-miR-UL36-3p and hcmv-miR-UL22A-3p) can downregulate ER-α and PR receptors. To date, 26 HCMV miRNAs have been identified in HCMVs genome (www.mirbase.org), and these miRNAs regulate several biological processes (described in chapter-III of this thesis).

Results and discussion

HCMV infection downregulates ER-α, PR and HER-2 in breast cancer cell lines

In this study, we demonstrate that HCMV *in vitro* infection downregulates the expression of ER-α, PR and HER-2 in breast cancer cell lines MCF-7 (ER+/PR+) and SKBR3 (HER-2+). The effect of HCMV was observed at mRNA and protein levels as shown by qPCR and western blot/immunofluorescence, respectively. Furthermore, downregulation of these three receptors was not affected by UV-irradiated HCMV, which suggest that viral gene expression is required to regulate the expression of these receptors. Similar results were obtained in an ovarian cancer

cells, SKOV-3. HCMV infection of the triple negative breast cancer cell line, MDA-MB-231 had no effect on the undetectable or low expression levels of ER-α, PR and HER-2.

HCMV IE and Late proteins does not affect ER-α or HER-2 levels in breast cancer cell lines

In our *in vitro* infection experiments, UV treated virus did not affect the expression of ER-α, PR and HER-2 levels suggesting that translation of viral gene products is essential, and signalling events caused by the virus entry is not involved in downregulation of these receptors. As discussed earlier in chapter-II elsewhere in this thesis, HCMV gene products have been shown to be involved in almost all biological processes such as cell cycle dysfunction, immortalization, genomic instability, etc. We hypothesised that the downregulation of ER-α, PR and HER-2 is also mediated by the viral proteins. To block the viral replication and late proteins production, we treated the breast cancer cells with ganciclovir (GCV) during infection with HCMV. Treatment of HCMV infected MCF-7 or SKBR3 cells with GCV, did not revert the downregulation of ER-α, PR or HER-2 expression, respectively, which indicate that late viral gene products are likely not involved in the regulation of ER-α, PR or HER-2 expression, but could be mediated by the IE and E proteins.

We next assessed if the IE-72 proteins (acts as trans-activator protein for the expression cascade of HCMV genes) could regulate these three receptors, and found that knock-down of IE-72 with siRNA had no effect on any of these three receptors. These results suggest that IE-72 has no role in downregulation of ER-α, PR and HER-2. However, it could be mediated by the other IE proteins.

HCMV miRNAs target ER-α and PR expression in breast cancer cell lines

Cellular and viral miRNAs are well known regulatory molecules to downregulate gene expression. We identified three HCMV encoded miRNAs (hcmv-miR-US25-2-3p, hcmv-miR-UL36-3p and hcmv-miR-UL22A-3p), which are predicted to target the ER-α; moreover, the hcmv-miR-UL22A-3p was also predicted to target PR. However, none of the HCMV encoded miRNAs are predicted to target HER-2 directly. It is possible, however, that HCMV regulates HER-2 indirectly through a cellular miRNA (ongoing work). We found that all these putatively predicted HCMV miRNAs are expressed at different levels in MCF-7 as well in MRC-5 infected cells. The levels of all the three miRNAs were higher in MRC-5 cells than in MCF-7 cells, this could be due to higher permissiveness in MRC-5 cells. The accumulation of the expressed miRNAs in MCF-7 cells with time was inversely associated with the downregulation of ER-α and PR. Similar to our results, these miRNAs were also found in infected Human Lung Fibroblast and suggested to be expressed at either 'immediate early or early' times (Shen *et al.*, 2014). miR-UL22A-3p is the first miRNA among others (initially called miR-UL23) found in infected fibroblast, endothelial, epithelial and astrocyte cells (Dunn *et al.*, 2005). Furthermore, miR-US25-2-3p (Fu *et al.*, 2014) and miR-US36-3p (Meshesha *et al.*, 2016) are expressed in the *in vitro* latent infection models, and miR-UL36-3p is found in monocytes of healthy blood donors (Meshesha *et al.*, 2016).

The bioinformatics predictions were confirmed experimentally by overexpression of these three miRNAs in MCF-7 cells. All the miRNAs were able to downregulate ER-α and PR at transcript and protein level (only ER-α). Although hcmv-miR-US25-2-3p and hcmv-miR-UL36-3p are not predicted miRNAs to target PR, still PR expression was decreased by these miRNAs, which might be expected since it has been described that ER-α regulates PR expression.

TNBC tumours have a specific molecular signature which is mediated by several pathways (Ossovskaya *et al.*, 2011). The mechanism of loss of ER-α, PR and HER2 in TNBC tumours is mostly unknown, germline mutation of BRCA1 gene is linked to the loss of these receptors (Chacon & Costanzo, 2010; Foulkes *et al.*, 2003; Lakhani *et al.*, 2005). Our results suggest that HCMV infection may contribute to the development of a triple negative phenotype in breast cancer (TNBC), which is in part mediated by these viral miRNA. TNBC patients account for about 10-15% of the all breast cancers and have limited treatment options as they do not respond to endocrine therapy or HER-2-targeted treatment (Gordon & Banerji, 2013).

There a few *in vitro* studies that have investigated the role of these miRNAs; miR-US25-2-3p can target tissue inhibitors of metalloproteinase-3 (TIMP3), which leads to increased shedding of MICA proteins leads decrease recognition of infected cells by the NK cells (Esteso *et al.*, 2014). Over-expression of miR-US25-2-3p results in downregulation of Eukaryotic translation initiation factor 4A1 (eIF4A1), and this leads to inhibition of translation, inhibits the replication of the virus (Jiang *et al.*, 2015; Qi *et al.*, 2013). UL138 protein is known to be required the establishment and maintenance of latent HCMV infection (Goodrum *et al.*, 2007a; Petrucelli *et al.*, 2009), and overexpression of the miR-UL36 can downregulate UL138 during the first 24h in the lytic cell models (Huang *et al.*, 2013a). Although, these results needs to be validated in a latent infection model to show its significance on latency. In this thesis we have extensively discussed the HCMV miRNAs in chapter-III.

Breast cancer tissues expresses HCMV miRNAs

Our results indicate that HCMV regulates the expression of ER and PR in breast cancer through viral miRNAs. Thus, we investigated if these viral miRNAs can be detected in clinical samples and how it correlates with the subtype of breast cancer. So far, 10 HCMV DNA PCR positive breast cancer tissue samples were examined for the three HCMV miRNAs by qPCR. The hcmv-miR-UL22A-3p was detected in 8/10 samples, hcmv-miR-UL36-3p was detected in 7/10 samples, while hcmv-miR-US25-2-3p was only detected in 3/10 samples. The expression of the each miRNA was highly variable among the examined miRNAs but there was only one sample negative for the three examined HCMV miRNAs.

Our *in vitro* results suggested the downregulation of ER-α and PR is mediated by the HCMV encoded miRNAs. Thus, HCMV targeted therapy may be a relevant alternative in breast cancer patients, although treatment with GCV did not restore the expression ER-α, PR and HER-2. Therefore, we highlight the possibility that different antiviral drugs targeting HCMV should be tested. In glioblastoma patients, we earlier demonstrated that anti-viral therapy may improve

survival (Rahbar *et al.*, 2012c). The presence of these miRNA in breast cancer tissues argues that they could lower the expression of ER-α and PR, and may thereby contribute to the development of TNBC. To be more specific, instead of anti-viral, treatment with anti-miRs or miRNA inhibitors against three viral miRNAs may restore the ER-α, PR and HER-2, warrants further investigation.

5 CONCLUDING REMARKS

We discovered a high prevalence of a novel genetic variant of HCMV in cancer specimens, which seems to represent a unique virus with pro-oncogenic capabilities. The altered behaviour of HCMV in tumours appears to be the explanations to controversies in the field, and that some investigators have failed to detect HCMV in cancer specimens. It is now critical to understand this variant virus role in more cancer types, and it is also essential to determine whether it spreads via blood products and organ transplants. At this moment, it is difficult to establish the association between cancer and this variant virus that exist in some healthy individuals, but in higher prevalence in cancer patients.

HCMV serology was determined and the prevalence of the HCMV encoded miRNA, miR-UL112 -3p was screened for in the blood of DM, RA, GBM patients and in HC's. No association was found between serology and miRNA levels, but we observed that the miR-UL-112 levels were significantly higher in DM patients than HC. On the basis of our results, others have found the oncomodulatory role of miR-UL112 in GBM.

Novel virus-host interaction with miRNAs has been well established. We found that host and HCMV-encoded miRNAs in virions and DBs, demonstrated that these miRNAs can be can be delivered to the host cells, where they may alter cellular protein expression and may play biologically important roles especially during the early phase of the virus life cycle.

HCMV´s role in breast cancer was elucidated. We detected HCMV proteins in significantly higher proportion in metastasis positive SLNs. Furthermore, we found that 99% of metastatic tumour tissues express HCMV proteins and nucleic acids. Despite improvements in the treatment of breast cancer, most deaths are due to metastasis. Therefore, a further understanding of the role of HCMV in the metastatic process of breast cancer is highly wanted.

The ER-α, PR and HER-2 are the most important prognostic markers for breast cancer patients. We found that HCMV encoded miRNAs downregulate the expression of ER-α and PR, and HCMV infection downregulates the HER-2 in breast cancer cells, and this may contribute to triple negative breast cancer. These observations reveal that HCMV may play an important role in the pathogenesis of TNBC, and may give hope for a new therapy option to include anti-viral therapy in the treatment of these patients, who are in desperate need of new therapy options.

In this thesis work, I together with my colleagues have worked on the several projects to understand the role of HCMV in its associated diseases in many different directions from finding a novel HCMV variant to its miRNAs and their potential links to several diseases. I believe that my thesis work covers the basic virology projects to advanced translation research projects, and by utilising a range of methods from basic methods to molecular biology methods. In several projects, we have used patient samples and liked their pathogenesis with HCMV infection, and I see a future in which the understanding of the relevance of this virus in different diseases is likely to increase, and in particular for cancer, such knowledge could lead to new treatment options for patients in a near future.

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