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CYTOMEGALOVIRUS INFECTION IN VASCULAR
DISEASES AND TRANSPLANT REJECTION

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Till min familj
ABSTRACT

Human cytomegalovirus (HCMV) infects 60-100% of the adult population. It belongs to the Herpesviridae family and establishes latency within its host post primary infection. HCMV reactivation is dependent on inflammation and the virus has evolved numerous mechanisms to sustain an inflammatory process and thus prolong its replication period. This has led to the hypothesis that HCMV may contribute to the pathogenesis of various inflammatory diseases. Our studies herein focus on further elucidating the role of HCMV in vascular diseases and transplant rejection.

We found that: I) HCMV is present in a vast majority of abdominal aortic aneurysms where it predominantly infects the intimal smooth muscle cells. The infected cells showed an increased migratory capacity that was in part dependent on basic fibroblast growth factor. Furthermore, in the vascular lesions where HCMV was present there was an induced expression of 5-lipoxygenase, a key enzyme in the synthesis of the potent pro-inflammatory leukotriene B₄. Thus, our study supports previous findings that HCMV is found in abdominal aortic aneurysm, and that it has the potential to contribute to the pathogenesis of abdominal aortic aneurysm.

II) Native arteriovenous fistula (AVF) is the preferred form of haemodialysis vascular access however dysfunction due to neointimal hyperplasia, vascular stenosis and thrombosis is a major cause of morbidity in this patient population. In a prospective cohort study we sought to identify novel prognostic factors for failure of native AVF. Numerous biochemical and patient characteristics were analysed just prior to construction of the AVF. We identified white blood cell count, monocyte count and red blood cell distribution width as independent prognostic factors for AVF failure. Red blood cell distribution width is a novel prognostic factor and was also the most optimal. The mechanistic link between high red blood cell distribution width and AVF failure is yet to be elucidated.

III) Using the patient cohort described in (II) we investigated the potential role of HCMV in the failure of AVF. In contrary to previous reports, high anti-HCMV IgG levels were not associated with reduced AVF patency in our study. HCMV proteins could be located in the vessels of 46% of patients at the time of AVF construction but presence of HCMV proteins was not associated with reduced AVF patency. Our study does not support the hypothesis that HCMV has a role in the failure of AVF.

IV) HCMV has been associated with chronic transplant rejection however the virus has been difficult to detect in the affected organs and thus the effects of HCMV have been defined as indirect. In this retrospective cohort study we evaluated the presence of HCMV in chronic renal allograft dysfunction, in early as well as end-stage biopsies. We found that HCMV is present in a majority of grafts early post transplantation and that the HCMV levels increase as the graft deteriorates. Furthermore, high HCMV intragraft protein expression in the early biopsy is associated with reduced allograft survival. Our study provides further support for a role of HCMV in allograft rejection and suggests that direct effects of the virus are involved in chronic allograft dysfunction.
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*These authors contributed equally.
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<tr>
<td>5-LO</td>
<td>5-Lipoxygenase</td>
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<tr>
<td>AAA</td>
<td>Abdominal aortic aneurysm</td>
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<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>ApoE</td>
<td>Apolipoprotein E</td>
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<td>AVF</td>
<td>Arteriovenous fistula</td>
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<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<td>BOS</td>
<td>Bronchiolitis obliterans syndrome</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CI</td>
<td>Confidence interval</td>
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<td>C-reactive protein</td>
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<td>Dense bodies</td>
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<td>Dendritic cell</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>ds</td>
<td>Double stranded</td>
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<tr>
<td>E</td>
<td>Early</td>
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<td>EGFR</td>
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<td>Endoplasmic reticulum</td>
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<td>Human cytomegalovirus</td>
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<td>Hazard ratio</td>
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<td>Heat shock protein</td>
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<td>HSV</td>
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<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
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<td>IEA</td>
<td>Immediate early antigen</td>
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<td>Interleukin</td>
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<td>ISH</td>
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<tr>
<td>kbp</td>
<td>Kilo base pair</td>
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<td>LA</td>
<td>Late antigen</td>
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<td>LDL</td>
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<td>LTB$_4$</td>
<td>Leukotriene B$_4$</td>
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<tr>
<td>MCP</td>
<td>Monocyte chemotactic protein</td>
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<td>MCV</td>
<td>Mean corpuscular volume</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>Macrophage inflammatory protein</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NIEP</td>
<td>Non-infectious enveloped particle</td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
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<tr>
<td>nm</td>
<td>Nano meter</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>oxLDL</td>
<td>Oxidized low-density lipoprotein</td>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>PDGFR</td>
<td>Platelet derived growth factor receptor</td>
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<tr>
<td>pp</td>
<td>Phospho protein</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T cell Expressed, and Secreted</td>
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<td>RBC</td>
<td>Red blood cell count</td>
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<td>RDW</td>
<td>Red blood cell distribution width</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>ROC</td>
<td>Receiver operating characteristic</td>
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<td>SMC</td>
<td>Smooth muscle cell</td>
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<td>T cell</td>
<td>Thymus cell</td>
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<td>T&lt;sub&gt;C&lt;/sub&gt; cell</td>
<td>Cytotoxic T cell</td>
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<td>T&lt;sub&gt;H&lt;/sub&gt; cell</td>
<td>Helper T cell</td>
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<tr>
<td>T&lt;sub&gt;REG&lt;/sub&gt; cell</td>
<td>Regulatory T cell</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TIMP</td>
<td>Tissue inhibitor of matrix metalloproteinases</td>
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<tr>
<td>TLR</td>
<td>Toll like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<tr>
<td>TVS</td>
<td>Transplant vascular sclerosis</td>
</tr>
<tr>
<td>UL</td>
<td>Unique long</td>
</tr>
<tr>
<td>US</td>
<td>Unique short</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VZV</td>
<td>Varicella Zoster virus</td>
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<tr>
<td>WBC</td>
<td>White blood cell count</td>
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1 HERPESVIRIDAE

Classification into the Herpesviridae family is based on the virion architecture - characterized by linear double stranded DNA (dsDNA) contained in a core surrounded by an icosahedral capsid, a tegument and the glycoprotein containing viral envelope (as reviewed in (Pellet and Roizman, 2007; Roizman and Baines, 1991; Roizmann et al., 1992)). The viral DNA is arranged in the shape of a torus inside the core. The characteristic herpesvirus capsid consists of 162 capsomeres and is approximately 100nm in diameter. The tegument denotes the area between the virion capsid and envelope. This layer contains fully synthesized proteins that, by affecting host cell function, aid the viral infection in its earliest stage post cell entry. The envelope consists of lipids and glycoproteins of viral and host origin.

Although all herpesviruses contain dsDNA there are large variations in the length and base composition among the different herpesviruses. The length varies from 120kbp up to 250kbp and the total combined guanine and cystine content can vary from 30% to 80%. There is also considerable variation in the genome size between herpesviruses ranging between approximately 70 genes for varicella-zoster virus (VZV) up to approximately 250 for cytomegalovirus (CMV). Certain similarities in genetic architecture exist for the herpesviruses, with the majority of the herpesvirus genes having a single major open reading frame (ORF), gene overlaps are common while splicing of genes is uncommon. Gene expression during a productive herpesvirus infection is sequential and initiated by expression of immediate early (IE) genes that require no prior viral protein synthesis. These are followed by expression of early genes (E) that do not require viral DNA synthesis, leaky-late genes whose expression is augmented by viral DNA synthesis and expression of late genes (L) that requires viral DNA synthesis.

Common biological properties for all herpesviruses include:
1. Enzymes involved in nucleic acid metabolism, DNA synthesis and protein processing.
2. Viral DNA is synthesized, and capsid assembled, in the nucleus of infected cells.
3. Production of infectious virus is associated with lysis of the infected cells.
4. The ability to establish latency in their natural hosts.

Members of the Herpesviridae family are highly prevalent and most animal species can be infected. Of over 200 herpesviruses identified only eight are known to cause diseases in humans. These are: herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2) and varicella-zoster virus in the Alphaherpesvirinae subfamily; human cytomegalovirus (HCMV) and human herpesvirus 8 (HHV-8) in the Betaherpesvirinae subfamily and Epstein-Barr virus (EBV), human herpesvirus 6 and 7 (HHV-6 and HHV-7) in the Gammaherpesvirinae subfamily (Pellet and Roizman, 2007).
The subfamily classification is also based on biological properties. Alphaherpesvirinae members are characterized by a short reproductive cycle, they spread rapidly in culture and infected cells are efficiently destroyed. Sensory ganglia are the primary targets for latent infection for members of the Alphaherpesvirinae subfamily. Viruses belonging to the Betaherpesvirinae subfamily have on the contrary a long reproductive cycle and the infection does not spread rapidly in culture. The infected cells are often enlarged. Latency is primarily established in secretory glands but latent virus can be found in many other tissues as well. Viruses in the Gammaherpesvirinae group generally have specificity for either B or T lymphocytes, with the latent virus commonly found in lymphoid tissue (Pellet and Roizman, 2007).

Members of the Herpesviridae family are well adapted to the host, and are often able to deceive the immune system and replicate in certain cell types, but generally do not cause a disease that would kill the host. To achieve this herpesviruses encode numerous proteins that interfere with the host immune system in order to allow for viral replication. These abilities to alter the cellular environment can potentially contribute to common diseases such as atherosclerosis, certain cancers and other inflammatory and autoimmune diseases.
2 HUMAN CYTOMEGALOVIRUS

2.1 HISTORY OF HUMAN CYTOMEGALOVIRUS

In 1881 Ribbert, a German scientist, observed enlarged cells in kidney specimens from a stillborn infant with syphils like symptoms. The second description of these peculiar enlarged cells with an eccentrically placed nuclei and surrounded by a clear halo was made in 1904 in a report by Jesionek and Kiolemenoglou describing their presence in several organs examined from a luetic fetus. A couple of years later Löwenstein, working in Ribbert’s laboratory, found these cells in parotid glands of several infants examined. These first descriptions of cytomegalic cells with intranuclear inclusions (Figure 1) were at that time believed to be related to protozoa. In the 1920s intranuclear inclusion cells were discovered in patients infected with herpes zoster and herpes genitalis and it was hypothesized that these cells were related to viral infection rather than protozoa (as reviewed in (Ho, 2008; Riley, 1997; Weller, 2000)). In 1926 Cole and Kuttner provided further experimental evidence by inducing formation of inclusion bodies in salivary glands of guinea pigs using virus.

Figure 1. Histopathology of HCMV infected kidney tubule epithelial cells displaying the characteristic intranuclear inclusions. Obtained from CDC/Dr. Haraszti.

During the following decades many reports appeared in the literature describing cells with intranuclear inclusions in salivary glands and/or visceral organs. Clinical symptoms of a hemorrhagic disease were described in many of the cases. Many authors regarded the cytomegalic cells to be pathognomonic for this condition and in 1950 Wyatt et al suggested the name generalized cytomegalic inclusion disease (CID) (Wyatt et al., 1950);
the viral etiology was still unknown. Renal tubular cells were very frequently involved, and it was suggested that disease diagnosis could be made by identification of cells with inclusions in urinary sediments. During the 1950s the possibility to routinely grow human cells in culture enabled propagation of HCMV and it was isolated independently by three research groups; Smith and Rowe et al in 1956 (Rowe et al., 1956; Smith, 1956) and by Weller et al in 1957 (Weller et al., 1957). Weller et al proposed the term “cytomegalovirus” in 1960 as the previous term “salivary gland virus” was misleading as the virus is found in many tissues (Weller et al., 1960).

The isolation and propagation of HCMV enabled subsequent development of serological tests for identification of viral antigens as well as further in vitro studies of the biology of HCMV. This has led to many important clinical and epidemiological observations demonstrating a role of HCMV in a variety of HCMV related syndromes. Today we know that HCMV is widely prevalent and that, in addition to congenital infection and the mononucleosis syndrome, this virus is a major cause of several other clinical entities, in particular in immunocompromised individuals.

2.2 HUMAN CYTOMEGALOVIRUS STRUCTURE

Cytomegaloviruses are species specific and while these viruses share many genes they also contain genes specific to the virus infecting specific species. Thus the HCMV genome has unique genes restricting its host range to humans. The HCMV genome is around 235kbp and contains 252 ORFs likely to encode 170 proteins. More than 40 ORFs produce HCMV proteins that have a high similarity to alpha- and gammaherpesviruses’ proteins. This provides further support of a common origin for these viral subfamilies as well as conserved functions among them. In addition, only around 50 ORFs are essential for production of infectious HCMV progeny (Murphy et al., 2003). Functional characterization of the remaining ORFs remains to be performed, but it is likely that many of these protein products are of importance for optimization of the viral infectious cycle by, for example, affecting cell functions to promote virus production and counteracting the host immune system.

![Figure 2. Schematic map of the HCMV genome consisting of the unique long and unique short regions flanked by two sets of inverted repeats: TR_L/IR_L and TR_S/IR_S. Adapted from (Kotenko et al., 2000).](image)

The HCMV has an E class genome characterized by unique long regions and unique short regions flanked by terminal and internal repeats (Figure 2) (as reviewed in (Mockarski et al., 2007)). The viral genome is transcribed post infection in a highly organized and sequential order with immediate early, early and late genes. These genes are, as in other herpesviruses, interspersed across the HCMV genome. The HCMV genome also contains three types of cis-acting elements. Found at the genome termini, pac-1 and pac-2 initiate
genome packaging, determine cleavage site of the concatemeric viral DNA and terminate the encapsidation process. The oriLyt region localized in the middle of the unique long region supports CMV-dependent DNA replication.

The HCMV virion is somewhat larger than other members of the Herpesviridae family but structurally it consists of the three components common between all herpesviruses: the capsid, the tegument and the envelope (Figure 3). At least five proteins (major capsid protein, minor capsid protein, minor capsid binding protein, smallest capsid protein and portal protein) build up the icosahedral capsid (as reviewed in (Britt and Boppana, 2004; Mockarski et al., 2007)). UL85 and UL86 encode for the major and minor capsid proteins respectively. The smallest capsid protein is encoded by UL48-49 while the minor capsid binding protein is encoded by UL46. Upon self-assembly of these proteins into an icosahedral structure the viral DNA is incorporated. The mechanisms of DNA packaging still have to be elucidated, but the portal proteins seem to play a key role. It is believed that the DNA enters through a single portal as described for HSV (Britt and Boppana, 2004; Newcomb et al., 2001). The complete capsid will receive a primary envelope at the inner nuclear membrane while the tegumentation and secondary envelopment will occur in the cytoplasm. In support of this presence of the viral transmembrane glycoproteins B and H (gB and gH) in the inner nuclear membrane in infected cells has been demonstrated (Bogner et al., 1992; Radsak et al., 1990). The significance of these proteins for the primary envelopment remains to be elucidated. The primary envelope is only temporary and will be lost at the outer nuclear membrane as the naked nucleocapsid is released into the cytoplasm (as reviewed in (Eickmann et al., 2006)).

![Figure 3. The HCMV virion structure. The image is adapted from (Tomtishen, 2012).](image)

Proteins making up the tegument are sequentially added as the viral morphogenesis proceeds through its cytoplasmic phase. The tegument surrounding the nucleocapsid is made up of more than 30 viral proteins, most of which are phosphorylated. Although the function of all tegument proteins is not known some of them have been shown to regulate
viral gene expression and modulate host cell responses to the viral infection. The most abundant viral proteins in the tegument are pp65 (ppUL83), pp28 (UL99) and pp71 (UL82). The tegument protein pp28 is regarded as essential for secondary envelopment - functional deletion of it results in non-infectious progeny with accumulation of unenveloped nucleocapsids in the cytoplasm (Silva et al., 2003). Viral tegument proteins are also of importance during the early stages of infection (see section 2.3). The tegument has also recently been implicated as a site containing viral mRNA coding for several proteins. These are translated prior to viral DNA transcription and while their function still has to be fully clarified it has been suggested that they provide a framework for the packaging of tegument proteins in newly synthesized virions (Bresnahan and Shenk, 2000; Roizman, 2000).

The outer envelope consists of lipid bilayer derived from both host cell and viral glycoproteins. This secondary envelopment occurs likely in early/recycling endosomes and trans-Golgi network. The host cell proteins CD55 and CD59 (Spear et al., 1995), CD13 (Giugni et al., 1996), β2-microglobulin (Grundy et al., 1987), annexin II (Raynor et al., 1999) and topoisomerase II (Benson and Huang, 1990) have all been identified in the viral envelope. The incorporation of host proteins in the viral envelope may be of importance for the masking of viral antigenic determinants and the enhancement of viral binding. In addition, it may also provide a mechanistic link to the autoimmune phenomena observed in connection with HCMV (Varani and Landini, 2011). As the HCMV virion is transported in the exocytic pathway from the rough endoplasmic reticulum via Golgi apparatus to the plasma membrane, three major glycoprotein complexes are formed and processed. Glycoprotein complex I represents a homodimer of gB, glycoprotein complex II consists of gM (gpUL100) associated with gN (gpUL73) while glycoprotein complex III is made up of gH (gpUL75), gL (gpUL115) and gO (gpUL74). In transfection studies, insertions within the genes coding for gB, gM, gH and gL inhibited viral production in infected fibroblasts (Hobom et al., 2000). The double wrapped HCMV nucleocapsids are subsequently transported to the plasma membrane where the mature enveloped virion is released upon fusion of the transport vesicle and plasma membranes (as reviewed in (Gibson, 2006)).

HCMV infected cells also produce two types of particles that do not contain viral DNA - non-infectious enveloped particles (NIEPs) and dense bodies (DBs) (Gibson, 2006; Irmiere and Gibson, 1983). NIEPs are structurally similar to infectious virions but they lack viral DNA and have an immature capsid. DBs are even more premature enveloped particles and are predominantly composed of pp65 protein (Eickmann et al., 2006). Although both of these types of aberrant particles do not contain viral DNA they do contain viral RNA that is non-specifically incorporated into the particles (Terhune et al., 2004).
2.3 HUMAN CYTOMEGALOVIRUS ENTRY

HCMV can productively infect numerous cell types such as epithelial, endothelial and smooth muscle cells (SMCs). In some cells, such as peripheral monocytes and macrophage-granulocyte progenitors, it can enter latency. The cell attachment and entry pathway is relatively conserved among herpesviruses. It consists of distinct phases initiated by binding to specific cell surface receptors. This initiating event is followed by the viral envelope fusing with the cell membrane to release the nucleocapsid, the nucleocapsid translocation to the nucleus and finally release of the viral genome into the nucleus via nucleocapsid interactions with the nuclear pores.

HCMV binds to numerous cell types although the infection is not productive in many cases. The initial viral attachment employs cell surface heparan sulfate starting a cascade of events ultimately leading to fusion. Although several HCMV heparan sulfate binding envelope proteins have been identified gB is regarded as being crucial for the entry process (Isaacson and Compton, 2009). In support of this, soluble gB has also been shown to inhibit infection (Taylor-Wiedeman et al., 1991).

Numerous cell receptors of importance for HCMV entry have been described, which are likely acting downstream of the initial binding to cell surface heparin sulphate proteoglycans that concentrates virions at the cell surface (Compton et al., 1993). Annexin II, a protein with membrane bridging capabilities, has been shown to interact with HCMV virions potentially facilitating the entry process (Pietropaolo and Compton, 1997). However, it is not essential for viral entry and infection (Pietropaolo and Compton, 1999). Aminopeptidase N (CD13) has also been implicated as a HCMV receptor as CD13 neutralizing antibodies and compounds interacting with CD13 inhibit HCMV infection in fibroblasts (Soderberg et al., 1993a; Soderberg et al., 1993b). Later it was shown that anti-CD13 antibodies bound and neutralized virus particles prior to cell contact. Furthermore, entry of HCMV was normal in CD13 depleted cells (Giugni et al., 1996). Epidermal growth factor receptor (EGFR) is an additional receptor candidate for HCMV cell entry and its association with gB has been shown to be vital for HCMV triggered downstream signalling and entry (Wang et al., 2003). Blocking studies utilizing anti-EGFGR antibodies have not been able to confirm the vital role of EGFR for HCMV entry and in addition there are several cell types, such as hematopoietic cells, that are susceptible for HCMV but do not express EGFR on their cell surface (Isaacson et al., 2007). Platelet derived growth factor receptor alpha (PDGFR-α) is via direct interaction with HCMV gB phosphorylated and the subsequent signalling pathway activation is critical for HCMV entry and gene expression. Thus, PDGFR-α seems to be critical for HCMV infection (Soroceanu et al., 2008). The cellular integrins α2β1, α6β1, and αVβ3 have also been identified as HCMV entry receptors (Feire et al., 2004).
When the virus nucleocapsid, after the virus fusion with the cell membranes, has entered the host cell cytoplasm it is translocated into the nucleus. The fusion of HCMV virions with the cell membrane requires gH/gL complexes (Milne et al., 1998).

### 2.4 HUMAN CYTOMEGALOVIRUS REPLICATION

Initial cell gene expression alterations induced by HCMV, likely due to gB binding to the host receptor, are important for the initial phases of infection. In productively infected cells, the HCMV genome will be expressed in a highly organized and sequential order with IE, E and late LA genes (Figure 4). The HCMV genome is transcribed, utilizing the host cell machinery, with RNA polymerase II, and occurs in parallel with the synthesis and processing of host cellular mRNAs. Expression of IE gene products can be observed immediately after entry while a complete replication of HCMV requires 48 to 72 hours. The most abundantly expressed proteins in the initial phase are IE1 and IE2. They activate transcription of both viral and cellular genes in the HCMV infected cells. IE1 does this by acting via NF-κB while IE2 binds and activates transcription via TATA-binding protein, transcription factor IIB and Specificity Protein 1 (Landolfo et al., 2003; Mockarski et al., 2007). They have also been shown to block apoptosis (Zhu et al., 1995).

![HCMV gene expression and protein function](image)

The knowledge about the cellular processes enabling and initiating viral DNA synthesis are not well understood. HCMV DNA synthesis initiation depends although on activation of a core region of oriLyt that is subsequently actively transcribed. IE2 interaction with the viral UL84 protein is necessary for virus DNA replication. The HCMV genome contains six conserved ORFs that encode the core replication proteins for HCMV DNA replication - the helicase primase complex (encoded by UL70, UL102 and UL105) responsible for unwindig of the DNA strands, the single-stranded DNA-binding protein (encoded by...
UL57) preventing reannealing of DNA strands, the DNA polymerase (encoded by UL54), and the DNA polymerase processivity factor (encoded by UL44) preventing DNA polymerase dissociation form the template (Landolfo et al., 2003).

Of the currently available antiviral drugs the nucleotide analogues ganciclovir and cidofovir are both competitive inhibitors of the UL54 encoded HCMV DNA polymerase. Foscarnet, also an antiviral agent, is a pyrophosphate analogue and a non-competitive inhibitor of the pyrophosphate-binding site of HCMV DNA polymerase (Landolfo et al., 2003).

When the replication cycle is completed the viral DNA is packaged into the synthesized capsids and exported through various cellular compartments acquiring its remaining structural components (the tegument and envelope) as described in section 2.2. As mentioned previously pac-1 and pac-2 initiate genome packaging and determine cleavage site of the concatemeric viral DNA that has been synthesized. The release of virions occurs through cell lysis or cell-to-cell contacts.

2.5 HUMAN CYTOMEGALOVIRUS LATENCY AND REACTIVATION

Initially, HCMV-DNA was thought to be localized to peripheral blood lymphocytes (Schrier et al., 1985), but it was later clarified that monocytes rather than lymphocytes were harbouring latent HCMV (Taylor-Wiedeman et al., 1991). In support of this statement, reactivation of HCMV was observed in monocytes in vivo but the reactivation process was dependent on the differentiation of the monocytes into macrophages (Soderberg-Naucler et al., 1997b). The same mechanism has been shown for dendritic cell precursors harbouring latent HCMV (Reeves et al., 2005). An inflammatory milieu, where interferon (IFN)-γ and tumor necrosis factor (TNF)-α seem to be of particular importance (Soderberg-Naucler et al., 1997a), drives the differentiation of monocytes and dendritic cell precursors and thus reactivation of latent HCMV. Recently, granulocyte-colony stimulating factor stimulation of humanized mice has been shown to result in mobilization of latently HCMV infected hematopoietic cells from the bone marrow to the peripheral blood, and subsequent HCMV reactivation in macrophages spreading the virus to various organ tissues (Smith et al., 2010).

2.6 HUMAN CYTOMEGALOVIRUS AND THE IMMUNE SYSTEM

The immune system, with its innate and adaptive branches, is crucial for combating viral infections. In the initial stages of infection HCMV triggers, via binding of gB and gH to Toll like receptor 2 (TLR2) and subsequent TLR2 dependent activation of NFκB, the innate immune responses with induction of interferons, inflammatory cytokines and recruitment and activation of natural killer (NK) cells (Boehme et al., 2006; Compton et al., 2003). While the significance of NK cells in control of CMV infection is established in mice, in humans direct evidence for a role of these cells in HCMV immunity is scarce. Recurrent severe HCMV disease has been described in an adolescent without NK cells,
providing indirect evidence for their importance (Biron et al., 1989). Furthermore, HCMV has numerous mechanisms to evade NK cell-mediated cytolysis (Figure 5) providing further indirect evidence of the importance of NK cells for HCMV immunity in vivo. The viral protein UL40 promotes cell surface expression of MHC class I antigen E that is recognized by the NK cells via the inhibitory receptor CD94/NKG2A and consequently inhibits the cytotoxic activity of NK cells (Tomasec et al., 2000; Ulbrecht et al., 2000). UL18, the viral homologue of MHC class I, is also expressed on the cell surface in HCMV infected cells inhibiting NK cell cytotoxicity via the receptor Leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) (Chapman et al., 1999). In addition, five HCMV genes are known to prevent activating NK cell receptor signalling. UL16, UL142 and microRNA-UL112-1 interfere with NKG2D mediated NK cell activation while UL83 interferes with activation of NKp30 and UL141 with activation of CD226 and CD96 (as reviewed in (Jackson et al., 2011)). HCMV UL16 is also important for the resistance of infected cells to the NK cell cytolytic proteins (Odeberg et al., 2003). As part of the adaptive immune system the humoral response against HCMV includes production of antibodies against numerous HCMV structural and non-structural proteins. These antibodies are important for protection of the host from disease and inhibition of viral spread. The disease preventing capabilities of anti-HCMV antibodies has been demonstrated in renal transplant patients and in congenital infection of the foetus during pregnancy (Fowler et al., 1992; Snydman et al., 1987). The HCMV tegument protein pp150 is one of the most immunogenic, and antibodies against it are present in nearly all seropositive individuals. HCMV pp65 also elicits a strong antibody response during the acute phases of infection but the response subsides after the initial convalescence period (Landini and La Placa, 1991). Although it was initially thought that antibodies against the glycoproteins gB and gH were most important for viral neutralization (Britt et al., 1988; Urban et al., 1996) it has recently been shown that antibodies against the complex of gH/gL/UL128-131A, which mediates viral entry into cells, are of greater significance (Macagno et al., 2010).

Upon activation CD8+ T cells can develop into cytotoxic T lymphocytes and combat viral infections by killing infected cells. The significance of these CD8+ T-cells for combating HCMV infection has become evident in the hematopoietic stem cell transplant population where strong correlations are observed between recovery of the CD8+ T-cell population and protection from HCMV disease (Lilleri et al., 2006). Furthermore, transfer of ex vivo expanded CD8+ T cells with specificity against HCMV protected these patients from both primary and reactivating infection (Peggs et al., 2003). In most HCMV seropositive individuals a high CD8+ T cell response is observed against pp65 and the 72-kD immediate early protein (Jackson et al., 2011; Khan et al., 2002). During primary HCMV infection a large expansion of pp65 specific CD8+ T cells is observed and the CD8+ memory T cells maintain certain clones from the original response. HCMV is a remarkably immunodominant antigen and around 10% of the peripheral CD8+ memory
T cells are directed against HCMV (Jackson et al., 2011). The proportion of CD8+ T cells directed against HCMV proteins increases with age and these cells may constitute up to 45% of the whole CD8+ T cell repertoire (Hadrup et al., 2006). The interference with the normal MHC class I processing caused by the viral genes US2, US3, US6 and US11 (Jackson et al., 2011; Jones et al., 1996; Wiertz et al., 1996) may protect HCMV infected cells from CD8+ T cell recognition.

Figure 5. HCMV immune evasion strategies.

CD4+ helper T cells can interact with CD8+ T cells (T_{H1}) or B cells (T_{H2}). T_{H1} type cytokines are IL-2, TNF and IFN-γ while production of IL-4, IL-5, IL-6 and IL-10 is associated with a T_{H2} response. There are also various other specialist subsets of CD4+ T cells. MHC class II molecules present exogenous protein derived peptides to CD4+ T cells which induces them to secrete cytokines that promote CD8+ T cell and B cell responses as well as acting directly against HCMV. As with CD8+ T cells, HCMV specific CD4+ T cells are often recognizing pp65 and IE proteins and about 10% of the total peripheral CD4+ T cell pool is HCMV specific (Jackson et al., 2011; Sylwester et al., 2005). Indeed, data from transplant patients indicates that HCMV specific CD4+ T cells producing IFN-γ are important for the CD8+ T cell response and thus HCMV control. Most HCMV specific memory CD4+ T cells maintain this T_{H1} cytokine profile and in vitro their antiviral activity is mediated through secretion of IFN-γ and TNF-α (Davignon et al., 1996). To counteract this antiviral response HCMV infection reduces cell surface expression of the receptor for TNF-α (Baillie et al., 2003). HCMV infection appears to trigger the formation of a HCMV specific CD4+ T cell type that lacks the costimulatory
receptor CD28 as these cells are very frequently present in HCMV seropositive individuals, but not seronegative ones. The HCMV specific CD4+ CD28- T cells secrete IFN-γ and their cytotoxicity is dependent on MHC class II interaction (van Leeuwen et al., 2006; van Leeuwen et al., 2004). HCMV US2 and US3 have been shown to decrease surface expression of MHC class II thereby evading recognition by CD4+ T cells. Furthermore, HCMV infection inhibits IFN-γ stimulated MHC class II expression by interfering with the Jak/STAT signal transduction pathway (as reviewed in (Miller et al., 2001)). By encoding an IL-10 homologue HCMV is able to further decrease MHC class I and II expression as well as the expression of various proinflammatory cytokines including IFN-γ and TNF-α (Kotenko et al., 2000; Spencer et al., 2002).

In transplant recipients a subset of HCMV specific γδ T cells are expanded upon infection, which are cytotoxic to HCMV infected cells through a mechanism independent of antigen presentation via MHC class I or II. The factors on HCMV infected cells resulting in activation of γδ T cells, as well as their significance in the transplant population, remains to be elucidated (as reviewed in (Jackson et al., 2011)).

Although HCMV elicits a very strong immune response the immune system cannot prevent it from establishing latency or clear latently persistent virus. This shows the enormous potential of the immune evasion strategies utilized by HCMV, some of which were described above. In addition, HCMV UL119-118 and TRL11/IRL11 encode two Fc receptor homologues providing a possibility for the infected cells to hide viral antigens by coating the surface with IgG antibodies and thus avoiding complement binding to the Fc receptor part of IgG (Atalay et al., 2002). HCMV also induces the expression of the cell surface complement inhibitors CD46 and CD55 thereby increasing the resistance of infected cells to complement-mediated lysis (Spiller et al., 1996).

HCMV infection induces the production of numerous cytokines and chemokines including interleukin 6 (IL-6), transforming growth factor (TGF)-β and monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1β in order to create a good milieu for viral persistence. HCMV also encodes four chemokine receptor homologues: UL33, UL78, US27 and US28. These potentially enable the virus to reduce extracellular levels of chemokines and thereby the recruitment of inflammatory cells to the site of infection (as reviewed in (Soderberg-Naucler, 2006)).

2.7 CLINICAL FEATURES OF HUMAN CYTOMEGALOVIRUS INFECTION

HCMV is highly prevalent and the frequency of HCMV seropositive individuals ranges between 50% and 100% depending on factors such as socioeconomic status, age and geographical location (Mockarski et al., 2007). HCMV may be acquired at any time during life and the seroprevalence increases with age. It is transmitted via body fluids and may be acquired by children via maternal breast milk, or saliva, in day care as well as family settings. The HCMV infection rates range between 20-50% in children attending day-care
centres (1990; Pass et al., 1982; Syggelou et al., 2010). In the adult population the transfer occurs primarily through sexual contact, and via saliva and more rarely via blood transfusions or organ transplantations.

In immunocompetent individuals a primary HCMV infection is generally subclinical, however, it can cause serious illness in patients with a significant immune dysfunction such as acquired immunodeficiency syndrome (AIDS) patients or iatrogenic immunosuppressed transplant recipients. If HCMV infection is clinically evident in immunocompetent patients it is mononucleosis-like with headache, malaise, fever, sore throat, myalgias and enlarged spleen. HCMV is although the most frequent congenital infection, occurring in 0.2% to 2.5% of all births. Permanent sequelae are observed in up to 20% of infants infected (1990; Syggelou et al., 2010), the most common being hearing loss, but mental retardation and visual impairment are also observed. Primary HCMV infection of mothers during pregnancy increases the risk of transmitting the virus and affecting the infant.

Antiviral treatment of infants with congenital HCMV disease affecting the central nervous system has been beneficial for the neurodevelopment after birth with lower risk of sequelae (Oliver et al., 2009).

HCMV infection is a significant cause of morbidity and mortality in patients with AIDS, hematopoietic stem cell transplant recipients and solid organ transplant recipients. In human immunodeficiency virus (HIV) infected patients HCMV causes end-organ disease when the immunosuppression is advanced, with the most frequent clinical manifestations being retinitis, gastrointestinal disease and encephalitis, accounting for 85%, 15% and 1%
of all HCMV diseases observed in HIV-infected patients respectively (Figure 6). HCMV disease is generally defined as HCMV infection (isolation of virus or detection of viral proteins or nucleic acid in any body fluid or tissue specimen) accompanied by clinical manifestations (Ljungman et al., 2002). Highly active antiretroviral therapy is now frequently used in HIV infected patients, which has reduced the frequency of HCMV disease (as reviewed in (Steininger et al., 2006)).

In hematopoietic stem cell transplant recipients HCMV gastrointestinal disease is the most frequent complication while HCMV pneumonia remains one of the most feared with mortality rates of more than 50% (as reviewed in (Ljungman et al., 2010)). Risk factors for HCMV disease in the allogenic hematopoietic stem cell transplant recipients include HCMV serostatus, usage of high-dose corticosteroids, T-cell depletion, acute and chronic graft-versus-host disease and the use of mismatched or unrelated donors. Antiviral therapy targeting HCMV is today frequently used in hematopoietic stem cell transplant recipients and this strategy has decreased the rates of HCMV disease.

In solid organ transplant patients HCMV can cause a HCMV syndrome characterized by fever and leukopenia as well as organ specific disease. The transplanted organ is more frequently affected potentially due to it being an immunoprivileged site due to MHC mismatch that decreases the ability of MHC-restricted, virus-specific, cytotoxic T cells to clear infected cells. The highest risk for HCMV disease is observed in HCMV seronegative recipients receiving organs from seropositive donors. Today pre-emptive or prophylactic treatment with antivirals is used on a regular basis, resulting in decreased incidence of HCMV disease episodes. Nevertheless, HCMV is known to have indirect effects including allograft rejection, predisposition to other infections and potentially malignancies (as reviewed in (Dzabic and Soderberg-Naucler, 2011)). These are discussed in more detail in chapter 4.
3 HUMAN CYTOMEGALOVIRUS AND VASCULAR DISEASES

As described previously, HCMV has numerous mechanisms to interfere with cellular machinery and the immune system. Furthermore, activation of the immune system seems to be a crucial component in the viral reactivation process. Allogeneic stimulation of peripheral blood mononuclear cells from HCMV-positive donors results in reactivation of the virus (Soderberg-Naucler et al., 1997b). Taken together, inflammatory diseases or those associated with inflammation, likely provide a very suitable environment for viral reactivation and replication. The viral proteins produced in this environment may aggravate the pathological processes. This concept provides the basis for the frequent occurrence of HCMV in diseased tissues and its potential contribution to these pathological processes. Importantly, this also provides a potential treatment strategy, as efficient antiviral therapy exists today.

3.1 INFLAMMATION IN ATHEROSCLEROSIS

Several studies have identified inflammation as a key factor in the development and progress of atherosclerosis. Inflammatory and immune cells are an important component of the atherosclerotic lesion (as reviewed in (Hansson, 2005; Hansson and Hermansson, 2011; Hansson and Libby, 2006)). The fatty streak that precedes atherosclerotic lesion formation consists of macrophages and T cells. As the fatty streak progresses to form an atheroma, its’ core is made up of foam cells derived from both macrophages and SMCs and is surrounded by SMCs and a collagen rich matrix. At the growing end of the atherosclerotic lesion activated immune cells, such as T cells, macrophages and mast cells, are plentiful. Mice models of atherogenesis have enabled a more detailed characterization of the mechanisms involved in initiation and progression of atherosclerosis. Most commonly used models include mice lacking apolipoprotein E (ApoE), a key component in cholesterol metabolism, and low-density-lipoprotein (LDL) receptor-deficient mice.

The inflammatory response in the arterial wall may be initiated by infiltrating LDL and its modification, resulting in endothelial cell activation and subsequent increased expression of adhesion molecules and inflammatory genes. Expression of, in particular, vascular cell-adhesion molecule 1 (VCAM-1) is important for immune-cell recruitment and initiation of the atherosclerotic plaque formation. VCAM-1 interaction allows monocytes and T cells to enter the arterial intima, and subsequent exposure to macrophage colony-stimulating factor in the inflamed intima results in further monocyte recruitment to the plaque and the differentiation of monocytes into macrophages. In atherosclerosis mice models, expression of truncated VCAM-1 is associated with reduced severity of atherosclerosis. Furthermore, expression of chemokines, such as MCP-1, RANTES, CXCL10, CXCL11, by vascular cells are also important for the initial recruitment of inflammatory cells.
Expression of scavenger receptors, including CD36, CD68 and scavenger receptor A and B1, by intimal macrophages enables uptake and accumulation of cholesteryl esters in their cytosol, resulting in foam cell formation. Binding to scavenger receptors does not directly result in an inflammatory response but the material taken up may be presented via MHC class II resulting in an adaptive immune response. Macrophages and endothelial cells in the plaque also express Toll-like receptors that can elicit an inflammatory response directly. Endogenous and microbial molecules, such as oxidized LDL (oxLDL) and heat-shock proteins (HSP), can bind to the toll-like receptors expressed by these macrophages and activate them to release pro-inflammatory cytokines and chemokines, fuelling the inflammatory process and tissue damage in the vessel.

T-cell infiltrates are always present in atherosclerotic lesions and are mainly composed of CD4+ T-cells (as reviewed in (Hansson and Hermansson, 2011; Hansson and Libby, 2006)). They have been shown to react to several proteins, such as oxLDL and HSP60, but identifying the key antigen or antigens that the CD4+ T-lymphocytes are targeting in the atherosclerotic plaques has although been more difficult. Numerous antigens have been suggested as well as several infectious antigens, amongst those also HCMV. Activated T-cells in the atherosclerotic lesion differentiate predominantly into Th1 effector cells producing interferon-γ, TNF and CD40 ligand. The Th1 production of IFN-γ activates macrophages resulting in increased production of pro-inflammatory cytokines and pro-thrombotic mediators. Furthermore, IFN-γ inhibits endothelial and smooth muscle cell proliferation and collagen production, which may negatively affect the plaque stability. TNF is pro-inflammatory acting via the NF-κB pathway and inducing production of reactive oxygen species, proteolytic enzymes and pro-thrombotic factors, amongst others. In addition, CD40 ligation elicits an inflammatory response similar to the one triggered by TNF. All these effects form the basis for the pro-atherosclerotic action of Th1 cells.

CD4+ CD28- T cell levels increase prior to clinical myocardial infarction and they have been found in atherosclerotic plaque (Dumitriu et al., 2009). As these cells are mainly directed against HCMV this provides indirect evidence for a role of HCMV in atherosclerosis. The pathological process of atherosclerosis thus involves cells from both the innate and adaptive immune system and the activity of these inflammatory infiltrates also activates a cytokine cascade resulting in production of substantial amounts of interleukin-6 and subsequently C-reactive protein (CRP) (Hansson, 2005).

Regulatory T cells (T\textsubscript{REG}) are important in limiting immunological responses to foreign antigens and to maintain immunological self-tolerance. T\textsubscript{REG} can suppress other T cells by contact-dependent mechanisms and/or via secretion of the anti-inflammatory cytokines IL-10 and TGF-β. T\textsubscript{REG} are present in the atherosclerotic plaque and T\textsubscript{REG} deficiency enhances atherosclerotic lesion development in LDL receptor deficient mice and in mice lacking ApoE. This atheroprotective effect of T\textsubscript{REG} seems to be dependent on TGF-β (Gotsman et al., 2007; Hansson and Libby, 2006).
Humoral immunity may protect against atherosclerosis, potentially by eliminating antigens before they reach the plaque (as reviewed in (Hansson and Libby, 2006)). Antibodies specific for oxLDL have been detected in both atherosclerosis-affected humans and in animal models of atherosclerosis. These antibodies, which are mainly of IgM isotype, are targeting oxidized phospholipids in oxLDL and apoptotic bodies but recognize also phosphorylcholine in the cell wall of Streptococcus pneumoniae (Shaw et al., 2000). Clinical and experimental data in animal models have provided further support for the atheroprotective effects of anti-phosphorylcholine antibodies, potentially acting via inhibition of oxLDL uptake by macrophages (de Faire and Frostegard, 2009).

### 3.2 HCMV IN ATHEROSCLEROSIS

HCMV is able to infect various cell types in the vessel wall. HCMV antigens and nucleic acids have been detected in atherosclerotic lesions but not in atherosclerotic plaques (Pampou et al., 2000), indicating that the virus may be of importance in the early atherogenic events. In several clinical studies positive associations have been found between HCMV seropositivity and coronary artery disease (CAD) (Georges et al., 2003; Zhu et al., 2000). Furthermore, HCMV seropositivity was an independent risk factor for mortality in patients with CAD (Zhu et al., 2001). Recently a large epidemiological study showed that HCMV seropositive patients with high CRP levels are at higher risk for all-cause mortality as well as cardiovascular disease (CVD) related mortality (Simanek et al., 2011). High anti-HCMV IgG levels alone are also associated with all-cause and CVD mortality, a relation largely mediated by IL-6 and TNF (Roberts et al., 2010). In the setting of acute myocardial infarction patients tend to develop HCMV antigenemia and the prevalence of an active HCMV infection, as evident by HCMV RNA expression, is more frequent in patients with unstable coronary artery disease (Gredmark et al., 2007). Thus, these patients are likely more prone to HCMV reactivation potentially contributing to their atherosclerotic disease.

The exact molecular mechanisms linking HCMV infection to atherosclerosis and other vascular diseases remain to be elucidated (Figure 7). Endothelial cell damage is an early event in various vascular diseases, and is followed by production of inflammatory molecules by the neighbouring cells. As mentioned previously, HCMV can target many cell types in the vessel wall, including endothelial cells. The endothelial cell damage and subsequent inflammatory response may result in HCMV reactivation. In vitro HCMV infection of endothelial cells up regulates the expression of several important adhesion molecules, such as intracellular adhesion molecule (ICAM)-1 and VCAM-1, enhancing leukocyte extravasation and thus further aggravating the inflammatory response. SMC proliferation and migration is characteristic for the active inflammatory site in the vessel wall. HCMV can directly induce SMC migration via US28, a chemokine receptor homologue, binding RANTES or MCP-1 (Streblow et al., 1999). Furthermore, the increased expression of PDGFR-β observed upon HCMV infection promotes SMC migration and proliferation (Zhou et al., 1999b). In SMCs, HCMV infection results in
accumulation of the tumor suppressor protein p53 via HCMV IE86 mediated inhibition resulting in increased SMC proliferation (Speir et al., 1994).

Atherosclerotic lesions express 5-lipoxygenase (5-LO), a key enzyme in the production of pro-inflammatory leukotriene B4 (LTB₄), as well as the LTB₄ receptor. LTB₄ can induce recruitment of inflammatory cells to the lesions, as well as proliferation and migration of SMCs (Back, 2009). In advanced atherosclerotic lesions the number of 5-LO expressing cells is increased (Spanbroek et al., 2003), and expression of 5-LO correlates with atherosclerotic plaque instability (Qiu et al., 2006). Previous studies from our group have shown that HCMV infection of SMCs induces expression of 5-lipoxygenase and production of LTB₄ (Qiu et al., 2008).

Figure 7. Illustration of potential HCMV mechanisms involved in the pathogenesis of atherosclerosis.

The endothelial cell damage and the subsequent inflammatory response results in oxidization of lipoproteins and is followed by accumulation of macrophages and SMCs that by uptake of oxidized LDL are turned into foam cells. HCMV infection of SMCs upregulates scavenger receptor genes expression and results in increased uptake of modified LDL. Experimental studies have shown increased oxLDL uptake as a result of HCMV infection of cells (Zhou et al., 1996). In monocytes CMV infection in vitro induces expression of the scavenger receptor CD36 and results in increased uptake of oxLDL (Carlquist et al., 2004).

HCMV can also potentially affect the plaque destabilization process and the thrombous formation, events associated with the acute vascular pathology. Infection of endothelial cells in vitro results in increased platelet adherence and aggregation via increased expression of von Willebrand factor, ICAM-1 and VCAM-1 (Rahbar and Soderberg-Naucler, 2005). This provides a potential mechanistic explanation to the increased thrombogenicity observed in several cases of acute HCMV infection (Abgueguen et al., 2003).
Animal models have provided further support for a role of HCMV in atherosclerosis. In both rats and mice CMV has been shown to promote atherogenesis (Berencsi et al., 1998; Soderberg-Naucler, 2006; Zhou et al., 1999a). Indeed CMV infection of wild-type, as well as apo E knock-out mice, resulted in the increased expression of the atherogenic genes coding for CXCL9, CXCL10 and monocyte chemoattractant protein 1 (MCP-1) (Burnett et al., 2004). Furthermore, in apo E knock-out mice CMV infection was shown to aggravate the disease process resulting in larger atherosclerotic plaque formation. Our group has shown that CMV infection of rats accelerates intimal hyperplasia by recruitment of macrophages and increased production of MCP-1 (Grudzinska et al., 2010).

In summary, these observations provide evidence that HCMV infection may affect important functions of key cells in the atherosclerotic process (Figure 7) and potentially contribute to the pathological processes. However, further studies are needed to clarify the role of HCMV in this process.

3.3 ABDOMINAL AORTIC ANEURYSM

Aortic aneurysms are most commonly affecting the abdominal part of the aorta. Advanced atherosclerosis has for long been implicated as causative for abdominal aortic aneurysm (AAA) formation. Several studies have highlighted that although these diseases often co-exist, it is rather the AAA resulting in disturbed blood hemodynamics that aggravates the atherosclerotic process (as reviewed in (Nordon et al., 2011)). Severity of atherosclerosis has not been correlated to AAA frequency. Furthermore, several of the key pathological changes observed in AAA formation, such as alterations in the matrix composition, SMC apoptosis, and chronic inflammatory infiltrates, have also been observed in other parts of the vasculature in the diseased individuals. This evidence indicates that AAA may be a manifestation of a systemic disease, rather than being a dominant feature of atherosclerosis. In analogy with this popliteal aneurysms, which are very infrequently found in healthy individuals, are found in around 70% of the patients with AAA. Inflammatory AAA represents up to 10% of all AAA and is characterized by thickened aneurysmal wall with severe atheromatous changes, extensive perianeurysmal and retroperitoneal fibrosis, and dense adhesions of adjacent abdominal organs. It remains questionable if it is a distinct clinical and pathological entity (Rasmussen and Hallett, 1997). Histological evaluation of consecutively repaired AAAs has shown that all specimens, to a varying extent, had signs of an inflammatory process in the aneurysm wall suggesting that inflammatory AAA is the extreme end of an inflammatory process driving the pathological changes observed in atherosclerotic AAA (Rasmussen and Hallett, 1997; Rose and Dent, 1981).

Inflammation and extracellular matrix degradation are key components in the pathogenesis of AAA (as reviewed in (Hellenthal et al., 2009a, b)). The extracellular matrix is mainly composed of elastin and collagen, and the degradation of them leads to vessel wall weakening, dilatation and rupture. Evidence from animal models indicates that loss of
elastin is predominantly responsible for dilatation while collagen degradation is required for vessel rupture. Elastase is mainly responsible for degradation of elastin and its activity is inhibited by trypsin. Members of the matrix metalloproteinases (MMPs) and cysteine protease families can degrade the two main collagen types in vessels, type I and III. In addition MMPs can control and regulate the inflammatory response. MMP activity is regulated by tissue inhibitors of metalloproteinases (TIMPs). Although MMP-1, -2, -3, -8, -9, -10, -12 and -13 have all been ascribed importance in the pathogenesis of AAA, MMP-2 and MMP-9 seem to have a key role in AAA formation. The absence of MMP-2 and -9 results in lower AAA incidence in knockout mice models of AAA (as reviewed in (Pearce and Shively, 2006)). Interestingly HCMV has been shown to up regulate the expression and activity of MMP-2 (Reinhardt et al., 2006). Usage of serological levels of MMP-2 and MMP-9 to follow aneurysm progression has yielded conflicting results (Pearce and Shively, 2006).

MMPs are secreted by inflammatory cell infiltrates, which are observed mainly in the outer part of the aorta. These infiltrates consist of monocytes, lymphocytes and plasma cells that secrete proinflammatory cytokines, ultimately affecting the aortal structure (as reviewed in (Lindholt and Shi, 2006)). Extracellular matrix degradation results in release of cryptic fragments and neoepitopes that potentially further drive the inflammatory response, in addition to degrading the structural integrity of the aorta. The inflammatory cell infiltrate of the AAA wall is predominantly made up of T lymphocytes. TNF and IFN-γ are elevated in patients with AAA, and both these cytokines inhibit collagen production potentially weakening the aortic wall. TGF-1β is, on the contrary, lower in patients with AAA. As TGF-1β is a potent inducer of the cystatine proteases inhibitor cystatin C, the low systemic activity of TGF-1β potentially promotes extracellular matrix degradation. Furthermore, IL-1β, IL-2 and IL-6 are all elevated in AAA patients (as reviewed in (Hellenthal et al., 2009a, b)).

Figure 8. Implicated risk factors for AAA development.

AAA is more frequently found in males with 4-8% of men aged 65 years or more being affected. Rupture of AAA is associated with high mortality rates and in USA it accounts for around 1% of all deaths. The AAA rupture incidence in Sweden has almost doubled during the last decades from 5.6 per 100 000 in 1971-1986 to 10.6 per 100 000 in 2000-
2004 (Acosta et al., 2006). This has occurred despite twice the amount of elective repair surgeries performed. It could potentially reflect the improved treatment of patients with cardiac diseases allowing more time for the AAA to expand silently until rupture. The incidence numbers for deaths due to AAA rupture are although uncertain and are likely underestimated as fewer autopsies are performed.

Numerous risk factors for AAA development, expansion and rupture have been identified (Figure 8). Identification of risk factors is important, not only as a mean of guidance to further research into the pathogenesis of AAA but also to optimize screening programs. The mechanisms driving AAA progression are likely multifactorial. Male gender and age above 65 are clearly associated with increased incident rates of AAA (Vardulaki et al., 2000). In the late 1970s it was shown that AAA cases tend to accumulate in certain families. Further studies have shown that the risk of AAA is doubled if there is a family history of AAA (Blanchard et al., 2000). While smoking and central obesity are unquestionable risk factors for AAA (Golledge et al., 2007; Lederle et al., 2003), other classical risk factors for atherosclerosis, such as hyperlipidemia and hypertension have not shown strong and/or clear associations (as reviewed in (Nordon et al., 2011)).

3.4 **HCMV IN ABDOMINAL AORTIC ANEURYSM**

As in atherosclerosis, microorganisms such as Treponema pallidum, Chlamydia pneumoniae, and HCMV have been linked to AAA pathogenesis (Lindholt and Shi, 2006). One suggested mechanism has been via molecular mimicry meaning that certain pathogen epitopes, that are immunologically similar to host antigens, would direct a pathogen-specific response to self (Pearce and Shively, 2006).

In a study by Hendrix et al (Hendrix et al., 1989) the human abdominal aortic wall was suggested to be a site of latency for HCMV as HCMV DNA was found in 55% of arteries with, as well as without, gross changes of atherosclerosis. In other studies HCMV DNA was detected in 86% of inflammatory AAA and in 65% of atherosclerotic AAA. Importantly, HCMV RNA, as a sign of an active infection, was detected in 71% of the inflammatory AAA (Tanaka et al., 1994). Macrophages, fibroblasts and endothelial cells were most frequently HCMV infected cell types in AAA (Yonemitsu et al., 1996). It is important to note that several studies have also failed to detect HCMV in AAA specimens (Falkensammer et al., 2007; Meijer et al., 1999; Satta et al., 1998), which may be due to lower sensitivity of the detection methods used. The potential role of HCMV in the pathological process of AAA still remains to be elucidated.

3.5 **NATIVE ARTERIOVENOUS FISTULA FAILURE**

There are several haemodialysis vascular access forms available and used today but native arteriovenous fistula (AVF) is preferred (2001). Generally, native AVFs are created by a surgical anastomosis between the cephalic vein and the radial or brachial artery (Figure 9). Although AVFs are superior to many other forms of haemodialysis access, there remain
significant problems with dysfunction - an important cause of morbidity in this patient population (Feldman et al., 1996). Initial failure of the AVF to mature adequately to support haemodialysis and/or later venous stenosis followed by thrombosis are the main causes of dysfunction. The incidence rates vary between different centres, especially in regards to failure to mature, as different policies are used for selection of patients eligible for AVF. Primary non-function, that is failure to mature, can occur in up to 50% of the patients operated. The pathology of the early failure is multifactorial and it is still unclear if the stenosis formed in primary non-function AVFs is caused by venous constriction or venous neointimal hyperplasia or a combination (Allon and Robbin, 2002; Roy-Chaudhury et al., 2006). The degree of luminal stenosis is dependent on both the magnitude of neointimal hyperplasia and the degree of vasodilatation/vasoconstriction. An abnormal hemodynamic shear stress may result in endothelial activation, low levels of nitric oxide and release of proinflammatory molecules, promoting stenosis and vasoconstriction.

As AVF dysfunctions are still frequent, substantial research has focused on identifying prognostic factors influencing the AVF patency, which could provide an insight into the pathology as well as provide improved treatment. Factors contributing to primary failure include small artery and/or vein, the surgical technique used, hemodynamic stress as well as genetic susceptibility to vasoconstriction and neointimal hyperplasia (as reviewed in (Lin and Yang, 2009; Smith et al., 2012)). In addition, uremia also contributes to endothelial dysfunction and thus predisposes to venous neointimal hyperplasia. For late AVF dysfunction the frequent puncture of dialysis fistula and the potential subsequent platelet thrombi and cytokine release could also contribute to the pathology. Decreased access blood flow, due to hypotension and hypoalbuminemia, can result in higher rates of AVF thrombosis and thus dysfunction. Several hypercoagulable states are also negative predictive factors as they are associated with access thrombosis. These include elevated circulating antiphospholipid antibodies, hyperhomocysteinemia and factor V gene mutations. In a large study, no drug improved the primary patency of AVFs and only

Figure 9. An illustration of a radio-cephalic arteriovenous fistula. Attribution: Kbik at en.wikipedia
angiotensin converting enzyme inhibitors improved secondary patency. Smoking and diabetes, well known risk factors for atherosclerosis in general, have been associated with AVF failure as well. Persistent inflammation has also been identified as a risk factor for AVF thrombosis. Consistent with this the inflammatory markers CRP, IL-6 and plasminogen activator inhibitor-1 have all been associated with AVF-failure (Chou et al., 2006; De Marchi et al., 1996).

The initiating events mentioned and the subsequent SMC and endothelial cell injury are likely to result in migration of SMCs and myofibroblasts into the intima, forming the venous neointimal hyperplasia lesion. The venous neointimal hyperplasia is indeed characterized by the presence of these cells as well as by microvessel formation in the adventitia and neointima. Presence of activated macrophages and strong expression of cytokines such as basic fibroblast growth factor (bFGF), vascular vascular endothelial growth factor (VEGF), TGF-β, endothelin-1 (ET-1) and platelet derived growth factor (PDGF) as well as markers of oxidative stress have also been reported (Roy-Chaudhury et al., 2001; Weiss et al., 2001). PDGF and bFGF are important for SMC proliferation while bFGF and VEGF are potent angiogenic factors. Furthermore, there is an accumulation of extracellular matrix components that also could be aided by the increased expression of bFGF and PDGF (Rutherford et al., 1997).

3.6 HCMV IN ARTERIOVENOUS FISTULA FAILURE

CMV infection has been shown to contribute to intimal hyperplasia in several animal studies (Grudzinska et al., 2010; Kloppenburg et al., 2005; Lemstrom et al., 1995). Furthermore, HCMV infection has been associated with atherosclerosis and restenosis after angioplasty, as reviewed in chapter 3.2. HCMV US28 can promote cytokine-dependent cell growth via secretion of VEGF and IL-6 (Slinger et al., 2010) that potentially could contribute to neointimal hyperplasia and microvessel formation observed in AVF failure. The potential significance of HCMV in AVF failure has been investigated in two studies. Gagliardi et al (Gagliardi et al., 2011) analysed levels of anti-HCMV IgG in a population of 91 patients and could observe that patients with high antibody levels had higher AVF failure rates than patients with lower anti-HCMV antibody levels. Only anti-HCMV IgG levels were evaluated and the cut-off was set to approximately the top 13% of population levels of anti-HCMV IgG observed.

Grandaliano et al (Grandaliano et al., 2003) also evaluated the significance of anti-HCMV IgG levels for AVF failure in a case-control study. Anti-HCMV IgG levels of more than >250 U/ml independently increased the risk for AVF thrombosis almost 5-fold. The cut-off chosen for anti-HCMV IgG in this study is high and present in only 17% of the normal subjects. It was used as it can indicate a recent and/or active infection according to the authors. There are although more optimal analysis for detecting recent and/or active HCMV infection, such as measurement of anti-HCMV IgM levels or pp65 antigenemia. Furthermore, in neither of the studies the vessels were examined for presence of HCMV. Thus, the potential significance of HCMV in AVF failure warrants further investigation.
4 HUMAN CYTOMEGALOVIRUS IN TRANSPLANT RECIPIENTS

4.1 HCMV INFECTION IN TRANSPLANT PATIENTS

As the number of immunosuppressed patients increased during the 1970s and 1980s, due to increased numbers of AIDS patients and organ transplantations, HCMV infection emerged as a significant pathogen. When antiviral drugs targeting HCMV replication became available acute disease progression could be halted, and this became a life saving treatment for many AIDS and organ transplant patients. Despite efficient treatment for the acute phases of HCMV infection it was noted already during the 1980s that HCMV infection increased the risk of chronic graft-versus-host disease in bone marrow transplant recipients (Lonnqvist et al., 1984), as well as the risk of chronic cardiac allograft failure in heart transplant recipients (Grattan et al., 1989). With time it has become apparent that HCMV infection increases the risk for a number of long-term complications in organ transplant recipients, including acute and chronic graft rejection, bacterial and fungal infections, malignancies and post-transplant diabetes (Rubin, 1989, 2007) (Figure 10). Interestingly, active viral infection has been difficult to detect in many cases and thus the long-term negative effects of HCMV are regarded as indirect. The direct effects of HCMV infection in solid organ transplant recipients are characterized by highly active viral replication resulting in lysis of the infected cells. The direct effects can target individual organs and are often accompanied by HCMV disease characterized by prolonged fever, leukopenia as well as concurrent presence of virus in blood. The organ-invasive disease can affect the liver, gastrointestinal tract, lungs, pancreas, heart and/or retina.

4.2 HCMV AND ACUTE REJECTION

Although HCMV infection has been coupled to acute rejection episodes in solid organ transplant recipients it has been difficult to define if the virus precedes the acute rejection, or if it is simply reactivated during the inflammatory process. In renal transplant patients without evidence of HCMV disease, presence of HCMV in the kidney has been shown to be associated with increased creatinine levels, suggesting that active replication is present and affects the organ function (Helantera et al., 2006a). Studies evaluating antiviral drugs have provided further support for a role for HCMV in acute rejection. Aggressive prophylaxis in heart transplant patients reduces acute and chronic rejection (Potena et al., 2006), while pre-emptive treatment strategies only prevent HCMV disease but not acute rejection episodes (Strippoli et al., 2006). Furthermore, the potent ability of HCMV to induce and maintain inflammation could also contribute to acute rejection. In rat liver allografts acute rejection induces cyclooxygenase (COX)-2 expression, and concomitant CMV infection furthermore increased the COX-2 expression (Martelius et al., 2002). HCMV US28 induces COX-2 expression and treatment of infected cells with specific COX-2 inhibitors blocks viral DNA synthesis (Maussang et al., 2009; Zhu et al., 2002).
Furthermore, previous studies from our group have shown that HCMV infection of SMCs induces expression of 5-lipoxygenase and production of the very potent pro-inflammatory LTB₄ (Qiu et al., 2008).

Figure 10. Overview of the direct and indirect effects of HCMV in transplant recipients. Reused from (Dzabic and Soderberg-Naucler, 2011) with permission from the publisher.
4.3 HCMV AND CHRONIC REJECTION

Chronic allograft rejection is a significant problem in solid organ transplant recipients and numerous risk factors, such as donor’s age, HLA mismatches, number of acute rejection episodes, hypertension and hypercholesterolemia, have been identified. During the late 1980s the first report of HCMV infection mediating an increased risk for transplant coronary artery disease was published. It was in a cohort of 301 cardiac transplant recipients that Grattan et al observed significantly higher rates of graft rejection, as well as more severe graft atherosclerosis, in the patients experiencing a HCMV infection (Grattan et al., 1989). Since then many studies in humans and animals have evaluated and confirmed that HCMV contributes to the chronic deterioration of transplanted organs. HCMV serostatus has been important in determining which patients are at highest risk for HCMV disease, and consequently negative effects on the allograft. The highest risk is observed when the donor is HCMV seropositive and the recipient HCMV seronegative. The lowest risk for disease is observed when both the donor and recipient are seronegative. It is although known that a substantial proportion of HCMV seronegative donors are positive for HCMV DNA in their peripheral blood mononuclear cells (Larsson et al., 1998). HCMV DNA has been detected in coronary arteries of transplanted hearts and more frequently in allografts from patients with accelerated atherosclerosis (Wu et al., 1992). The transplanted organ may thus be the source for transmission of HCMV to the donor.

HCMV has been associated with cardiac allograft transplant vascular sclerosis, chronic renal allograft dysfunction, bronchiolitis obliterans and vanishing bile duct syndrome. The best evidence in support of a role of HCMV in the chronic rejection process comes from the studies utilizing antiviral therapy. In cardiac allograft recipients treatment with the potent viral replication inhibitor ganciclovir delayed allograft rejection (Merigan et al., 1992; Valantine et al., 1999). Furthermore, aggressive HCMV prophylaxis post heart-transplantation reduced acute rejection and transplant vascular sclerosis indicating that presence of HCMV, even if asymptomatic, has negative effects on the graft (Tu et al., 2006). Renal allograft survival is likewise reduced in patients with asymptomatic HCMV-infection (Sagedal et al., 2004). Persistent HCMV infection of the renal allograft, defined by presence of HCMV antigens or DNA in the allograft, but without concomitant viremia or viruria, is associated with reduced creatinine clearance and allograft survival (Helantera et al., 2006a). In lung transplant recipients, prolonged ganciclovir/valganciclovir prophylaxis reduced the incidence of bronchiolitis obliterans syndrome (BOS), graft loss due to BOS and improved the overall survival (Chmiel et al., 2008). Subclinical HCMV replication in lung allografts is also associated with BOS (Paraskeva et al., 2011).

HCMV specific T cell responses seem to be very important in controlling the HCMV subclinical infection and preventing its subsequent negative effects on the allograft (Tu et al., 2006). As described previously, a large portion of the human T cell repertoire is directed against HCMV, supporting the notion that viral reactivation occurs periodically
even in immunocompetent individuals without signs of clinical disease. Thus, low-grade replication of HCMV could potentially account for the indirect effects observed in transplant recipients. However, the techniques generally used in clinical practice are not sufficiently sensitive to detect this type of HCMV infection. Furthermore, antiviral prophylaxis prevents some of the long-term negative effects observed indicating an involvement of active viral replication in the pathogenesis. Clinically two major strategies are used for preventing HCMV: universal prophylaxis and pre-emptive therapy. In the pre-emptive therapy approach laboratory monitoring is performed regularly and antiviral therapy is initiated once viral replication reaches a certain threshold. According to the latest guidelines both of these approaches are viable, although prophylaxis is favoured over pre-emptive therapy in high-risk patients (Kotton, 2010). In a randomized controlled trial universal prophylaxis, compared with pre-emptive therapy, significantly increased long-term renal allograft survival (Kliem et al., 2008).

Studies utilizing transplant animal models have also shown that CMV infection induces earlier and more advanced lesions, implicating it as a cofactor in the development of chronic rejection. A hallmark of chronic rejection is the migration of SMCs into the neointimal space and subsequent proliferation. As mentioned previously, the CMV US28 is a chemokine receptor analogue and able to induce SMC migration. Indeed, its deletion in rat CMV resulted in a 60% reduction in intimal lesions in a rat transplant model (Streblow et al., 2005). HCMV can also inhibit apoptosis and increase the proliferation of SMCs potentially contributing to the vascular lesion. Several HCMV IE proteins interfere with cell cycle regulation and prevent infected cells from undergoing apoptosis (as reviewed in (Castillo and Kowalik, 2002)). For example, IE86 reduces the transcriptional activity of p53 potentially explaining the increased SMC proliferation contributing to restenosis (Speir et al., 1995). Furthermore, HCMV can induce expression of bFGF and PDGF-A, potent stimuli of SMC proliferation (Srivastava et al., 1999).

Figure 11. HCMV mechanisms potentially contributing to chronic allograft dysfunction.
HCMV can also potentially promote fibrosis. CMV infection in a rat transplant model resulted in increased expression of collagens I and III and myofibroblasts, enhancing the development of interstitial fibrosis in chronic renal allograft rejection (Inkinen et al., 2002). Furthermore, TGF-β1 has been suggested as a key factor in fibrosis development in chronic renal allograft dysfunction (Sharma et al., 1996). Urinary excretion of TGF-β1 is increased in renal transplant recipients diagnosed with HCMV (Helantera et al., 2006b) and HCMV induces the expression of the integrin αvβ6 resulting in activation of TGF-β1 (Tabata et al., 2008). As mentioned previously HCMV also up regulates MMP-2, potentially affecting the composition of the extracellular matrix (Reinhardt et al., 2006).

As described previously, HCMV reactivation and replication is dependent on inflammation, furthermore the virus has evolved mechanisms to sustain the inflammatory response (as reviewed (Soderberg-Naucler, 2006)). HCMV induces recruitment of inflammatory cells and induction of inflammatory cytokines including IFN-γ, TNF-α, IL-4, IL-18, RANTES, MCP-1, MIP-1α and IL-8. Furthermore, the virus also induces cellular factors involved in angiogenesis and wound healing such as ICAM-1, VCAM-1, vascular adhesion protein 1, E-selectin and TGF-β, VEGF, PDGF-A and PDGFR-β (Helantera et al., 2005; Helantera et al., 2006b; Inkinen et al., 2005; Streblow et al., 2007). This strong inflammatory and pro-fibrotic response is likely an important component in the acceleration of allograft damage observed upon HCMV infection, promoting vessel neointimal hyperplasia and leading to vessel narrowing and subsequent graft failure (Figure 11).
5 AIMS OF THE THESIS

I. Evaluate the presence and role of HCMV in AAA

II. Identify novel prognostic markers for AVF failure

III. Evaluate the presence and significance of HCMV in native AVF failure

IV. Evaluate the presence and significance of HCMV in chronic renal allograft failure
6 RESULTS AND BRIEF DISCUSSION

6.1 PAPER I – HCMV INFECTION OF SMOOTH MUSCLE CELLS IN AAA

We were able to detect HCMV proteins and/or DNA in 21 of the 22 (95%) AAA specimens included in our study utilizing PCR, *in situ* hybridization (ISH) or immunohistochemistry (IHC). Serum was unfortunately only available for 10 of the patients included in the study. In a vast majority of the cases (90%) the patients were HCMV IgG positive. Our findings related to the presence of HCMV in AAA are in analogy with earlier studies (Tanaka et al., 1994; Yonemitsu et al., 1996) described in chapter 3.4. As mentioned previously, it remains questionable if inflammatory AAA is a distinct clinical and pathological entity, and thus if the subdivision in inflammatory versus atherosclerotic AAA is relevant. Contrary to some of the previous studies the subdivision in inflammatory versus atherosclerotic AAAs was not used in our study. While high HCMV prevalence was found in some studies, others have failed to detect HCMV in AAA specimens (Falkensammer et al., 2007; Meijer et al., 1999; Satta et al., 1998). This may in part be explained by the sensitivities of the techniques used and that HCMV infection likely is a periodically-activated latent infection (Adam et al., 1997).

SMCs in atherosclerosis have been shown to harbour latent HCMV (Melnick et al., 1983) and are likely important in the formation of AAA (Henderson et al., 1999; Lopez-Candales et al., 1997). We investigated isolated medial as well as intimal SMCs from seven of the patients included in our study and could show that they all had HCMV DNA. The levels were although extremely low in the medial SMCs and were undetectable by ISH. On an average 42.0 ± 10.2% of the intimal SMCs were HCMV positive by ISH. The intimal SMCs migrated 6.6 ± 1.5 times more efficiently (*p* < 0.05) than medial SMCs, an effect potentially due to their increased HCMV infection load, as a similar migration increase was observed when the isolated medial SMCs were HCMV infected *in vitro*. Previous results from our group show that the SMC increased migration observed upon HCMV *in vitro* infection is dependent on US28, a viral chemokine receptor homologue (Streblow et al., 1999).

Using neutralizing antibodies against bFGF, PDGF-AB, IGF-1, VEGF, RANTES, MCP-1, MIP-1α, or IL-1β we were able to show that the increased migration observed in isolated intimal SMCs was in part dependent on bFGF, as anti-bFGF reduced the migration by around 60% in a dose dependent fashion. No statistically significant changes in migration were seen when the other neutralizing antibodies were added. Due to this we did not proceed with further analysis regarding the US28 pathway in the isolated SMCs as it is dependent on endogenous MCP-1 and RANTES.

Although HCMV infection has been shown to alter the cytokine and chemokine production we could not observe any significant changes in the production of MCP-1,
MIP-1α, RANTES, PDGF-AB, PDGF-BB, bFGF, EGF or VEGF by intimal or medial SMCs. Significant individual differences were observed in MCP-1 production, but they were not retained when the whole population was analysed. Furthermore, we were unable to see any statistically significant changes in the secretion of MCP-1 or bFGF upon in vitro HCMV infection of the isolated SMCs. As HCMV was previously shown to increase bFGF production in SMCs in vitro, it can be speculated that an autocrine signalling loop resulting in immediate uptake of secreted bFGF by SMCs makes it difficult to detect statistically significant differences in secreted levels. This would be in analogy with the reduced migration observed when neutralizing bFGF antibodies were added.

It is plausible that the intimal SMCs studied here had undergone a phenotypic change, as SMCs generally do not migrate without a specific stimulus. This phenotypic change may be induced or aided by the higher HCMV load that was observed in these cells as compared to the medial SMCs. It should although be noted that we used SMCs from five AAA patients and observed high inter-individual variability in migration. This makes it very difficult to predict the relevance of the effects of HCMV on SMC migration in the pathogenesis of AAA. In the complex in vivo environment, factors other than HCMV infection of SMCs may explain, in total or in part, the migration differences observed between intimal and medial SMCs. A key control would have been intimal and medial SMCs from fully HCMV negative AAA patients; unfortunately this was not available in our study.

As mentioned in chapter 3.2, 5-LO and LTB₄ have been identified as potentially important factors in atherogenesis and are induced by HCMV. We used IHC to analyse the presence of HCMV proteins as well as 5-LO, PDGFR-β, MCP-1 and RANTES. A high expression of 5-LO was found in areas with a high HCMV immunoreactivity, while only a few scattered cells expressed PDGFR-β, MCP-1 or RANTES. This supports the theoretical ability of HCMV to induce and maintain inflammation in AAA. Our findings of a high expression of 5-LO in the wall of human AAA were subsequently confirmed (Di Gennaro et al., 2010). While no significant differences in the production of MCP-1 were observed in SMCs, our IHC studies showed that inflammatory cells mainly expressed MCP-1, and although these were not maintained in the isolated cultures they may still have an important role in HCMV-induced inflammation in vivo, by producing ligand for SMC US28. AAA tissue inflammation is characterized by an abundance of activated macrophages producing proinflammatory factors driving aneurysm formation. This microenvironment is suitable for efficient replication of HCMV in tissue macrophages and replicating HCMV could potentially, via its ability to induce 5-LO expression, further exacerbate the inflammatory process. Furthermore, the presence of microbial antigens in the AAA lesions will drive the immune response.

In summary, our study confirmed previous observations of the presence of HCMV in a vast majority of AAA specimens. We furthermore showed that presence of HCMV antigens was accompanied by 5-LO expression and that HCMV induced migration in
SMCs isolated from the lesions. Although presence of HCMV in AAA does not provide evidence for the virus being a causative agent, considering how HCMV can modulate the cellular and immunological functions its presence in the vessel wall makes it a candidate in the pathogenesis of AAA.
6.2 PAPER II – RED BLOOD CELL DISTRIBUTION WIDTH IS A NOVEL PREDICTIVE FACTOR FOR ARTERIOVENOUS FISTULA FAILURE

In this prospective clinical study 68 patients receiving a native arteriovenous fistula were included and followed during 24 months. The main aim was to identify novel prognostic factors for AVF failure that could be potential new targets for improved treatment and follow up. The clinical part of the study was conducted at the Central Clinical Hospital Ministry of Internal Affairs, Warsaw, Poland.

The AVF failure rate in the included patient population was 50% at 24 months post surgery, 16% of the patients suffered an early AVF failure, which we defined as failure to mature or thrombosis within 2 months post construction, while 34% had late dysfunction. Blood samples taken prior to AVF construction were analysed for numerous biochemical parameters. As stated previously, although numerous prognostic factors are known for AVF failure they do not explain all the failures observed. When subdivided in groups according to AVF survival rates significantly higher levels of CRP, white blood cell count (WBC), red blood cell distribution width (RDW) (calculated as the standard deviation of the red blood cell volume divided by the mean corpuscular volume (Evans and Jehle, 1991)) and platelet count were detected in patients with early AVF failure. These patients had significantly lower red blood cell count (RBC) and serum albumin. There were no other significant differences in patient characteristics, medications or biochemical parameters analysed. Using FACS we also analysed serum levels of the important inflammatory cytokines IL12p70, IFN-γ, IL-17A, IL-2, MCP-1, IL-10, IL-8, IL-6, IFN-α, IL-1β and TNF-α and could not observe any differences between the groups.

By univariate analysis using Cox regression models we assessed all individual factors. All biochemical factors with a p value less than 0.2 in univariate analysis were subsequently included in the multivariable model. Correlation analysis were also performed, and for variables with a correlation coefficient >0.8, only one of the variables was kept in the final model. After adjustment for RBC, albumin, albumin-corrected calcium and CRP, the WBC (hazard ratio (HR) 1.67; 95% confidence interval (CI) 1.24 to 2.25; p<0.001), number of monocytes (HR 0.02; 95% CI 0.00 to 0.21; p=0.001), and RDW (HR 1.44; 95% CI 1.17 to 1.78; p<0.001) were significant independent predictors of AVF failure.

Receiver operating characteristic (ROC) curve analysis can be used to determine if a variable under study can distinguish between two groups, in our case the AVF failure and non-failure group. The area under the ROC curve is equal to 0.5 if there is no difference between the populations, while it equals 1 if there is perfect separation of the values of the two groups. If the ROC curve is significantly different from 0.5 there is evidence that the laboratory test does have an ability to distinguish between the two groups. Furthermore, a value corresponding to the highest average sensitivity and specificity can be generated. In ROC curve analysis, significant differences in the distribution were only found for RDW (area under the curve 0.644; CI 0.51 to 0.76; p=0.046). On the basis of ROC curve
analysis the upper quartile of RDW values (>16.2% versus ≤16.2%) was found to be the best cut-off point (sensitivity 37%, specificity 90%). Using Kaplan-Meier plots we could demonstrate that the patient population with RDW values in the highest quartile, had an increased frequency of AVF failure ($p=0.036$, log-rank test).

In respect to statistically significant correlations between WBC, RDW, monocyte numbers and the other evaluated factors, both positive and negative correlations could be found. All of them were however weak ($r^2<0.35$), and therefore their significance is questionable.

As previous studies have shown that inflammatory cells are present in the vessel wall of the vein and artery used for creating the AVF we evaluated tissue specimens taken at the time of surgery. These biopsies were stained for CD68 and CD45 in order to identify inflammatory cells. Although these cells were found in just less than 40% of all patients there were no significant differences in the number of antigen-positive cells in the arteries or veins among the three different patient populations. In order to evaluate if presence of inflammatory cells was the mechanism behind the higher failure rates observed in patients with high serum WBC and RDW we investigated if there were any associations between inflammatory cell accumulation and failure frequency. Patients with CD68 positive cells in their vessels had significantly higher serum WBC, while no significant differences were found in patients with vessels positive for CD45.

RDW, a measure of anisocytosis initially designed to aid in the evaluation of anemias, was identified as a novel prognostic marker for AVF failure. The advantage with RDW is that it is readily available, as it is routinely reported by automated blood cell count equipment used in hospital laboratories (Evans and Jehle, 1991), and very affordable. During the last years it has gained further attention as it has been identified as a strong prognostic factor for mortality in patients with acute coronary syndromes (Cavusoglu et al., 2010), pulmonary hypertension (Hampole et al., 2009), acute pulmonary embolism (Zorlu et al., 2012) and chronic and acute heart failure (Felker et al., 2007; Forhecz et al., 2009; van Kimmenade et al., 2010). Thrombosis is an important cause of AVF dysfunction but in this context RDW has been sparsely evaluated, although it has been reported to be associated with an increased risk for stroke (Ani and Ovbiagele, 2009). The risk increase associated with elevated RDW still lacks a clear mechanistic explanation. It has been suggested that it reflects inflammation, impaired renal function, malnutrition as well as ineffective erythropoiesis (Forhecz et al., 2009). Ineffective erythropoiesis is associated with inflammatory states. The proinflammatory cytokines TNF-α, IFN-γ and IL-1 are of particular importance. They cause an increased uptake and retention of iron and thereby limit the availability of iron for erythroid progenitor cells. Furthermore, they can induce apoptosis and reduce the expression of erythropoietin and the erythropoietin receptor on progenitor cells (Montagnana et al., 2012; Weiss and Goodnough, 2005). All these effects on erythropoiesis promote anisocytosis, providing a potential mechanistic link between high RDW and AVF failure.
In the case of AVF failure it is less likely that the risk increase associated with RDW was due to anemia because neither hematocrit, hemoglobin, iron, transferrin nor ferritin levels affected AVF failure risk in univariate analysis and RDW remained a significant risk factor in multivariable analysis after adjustment for these factors. Unfortunately, we did not have information on folate or B₁₂ levels and although mean corpuscular volume (MCV) levels did not differ between the groups (MCV is usually affected in these deficiencies) we cannot exclude the possibility that these deficiencies could have affected RDW in our study.

Nevertheless, it is more likely that RDW reflects an underlying inflammatory state that consequently leads to AVF failure. In analogy with this, high WBC was also an independent risk factor for AVF failure. Furthermore, there were positive correlations between CRP and RDW, although weak, as has been observed in related studies (Forhecz et al., 2009; Lippi et al., 2009). Interestingly, we did not observe an increased frequency of inflammatory cell infiltration in vessels from patients with high RDW. This could potentially be explained if RDW is a very early marker of inflammation. Importantly, RDW seems to be a superior marker of AVF failure than WBC or CRP. Although increases in WBC was an independent predictor of AVF, it was not significant in ROC analysis indicating that it would be an inferior laboratory test to RDW for predicting AVF failure. As RDW is a direct measure of anisocytosis it is plausible that it reflects the effects of erythroid or non-erythroid pathologies on red blood cell properties associated with increased thrombosis and thus AVF failure.

Malnutrition has been identified as a risk factor for AVF failure (Gagliardi et al., 2011), and also suggested as a link between RDW and mortality in heart failure (Forhecz et al., 2009). We did not observe any significant differences in body mass index, cholesterol or transferrin indicating that malnutrition is not likely the main link between high RDW and AVF failure. The samples included in our study were not analysed for prealbumin, a potentially more sensitive marker of malnutrition (Kalantar-Zadeh et al., 2003), thus we cannot conclusively state that malnutrition did not affect RDW, and so potentially AVF failure, in our study.

Although our study utilized a heterogeneous, relatively small population, recruited from one center it was strengthened by its prospective design and the comprehensive biochemical data available. We identified, for the first time, RDW as a risk factor for AVF failure. Although further studies, with larger patient populations are needed to elucidate the mechanistic link between RDW and AVF failure, it can be speculated that RDW is an integrative measure of multiple pathologic processes, such as inflammation, nutritional defects and red blood cell aggregation, explaining its strong association with AVF dysfunction.
6.3 PAPER III – HCMV INFECTION IN ARTERIOVENOUS FISTULA FAILURE

As mentioned previously the role of HCMV in the pathogenesis of arteriovenous fistula failure has been sparsely evaluated and is so far limited to the two studies (Gagliardi et al., 2011; Grandaliano et al., 2003) reviewed previously focusing on HCMV serology. We sought to further evaluate the potential role of HCMV in the cohort of patients described in paper II. The advantage of our design was the availability of arterial as well as vein biopsies in addition to serum samples. HCMV serology if measured at one time point can, due to reoccurring reactivation, render substantially weaker associations than HCMV antibody titre levels measured at several time points over a longer period of time. This is a weakness in the previous studies but also in ours, although we had the advantage of being able to examine the anatomically relevant site for virus.

Interestingly, we were unable to confirm the previous data. A vast majority of the studied patient population was HCMV IgG positive (96%) but all patients were HCMV IgM negative. Variations in titre levels were large and for subsequent analysis the patient serology data was divided in tertiles. Using Kaplan-Meier plots we did not observe any difference in AVF patency between the groups. In analogy with this, three patients that were HCMV IgG seronegative had AVF that functioned between 3 and 6 months post surgery. The cut-offs used in the previous studies, corresponding to the top 17% and the top 13% of HCMV IgG population levels, were higher than the cut-off point used by us. By using the top tertile we wanted to have an unbiased cut-off point. Nevertheless, in explorative analysis we also used higher cut-off points, but still did not see any association with AVF failure. It should be noted that neither of the previous studies was prospective in its design, neither did they analyse the HCMV serostatus prior to AVF construction.

Looking at the arterial and vein specimens with immunohistochemistry we could detect HCMV proteins in around 40% of the radial arteries examined but in only around 20% of the cephalic veins evaluated. Histologically, it was primarily the inflammatory cells that were HCMV protein positive. This provides further support for the previous reports that HCMV can be found in the vessel wall together with, as well as without, gross pathology (Hendrix et al., 1989). We were unable to detect any predictive value in the presence of HCMV proteins in the vessels for subsequent AVF failure.

Although our study does not support previous evidence for a potential role of HCMV in the failure of AVF we cannot fully exclude this idea. Frequent reactivation of latent infection complicate the investigations of the role of HCMV, and it is possible that its activity could have been triggered at a later time point than the one evaluated in our study. Further studies with serial sampling of serum, as well as vessel biopsies for evaluation of HCMV, are warranted to further clarify the potential role of HCMV in AVF failure.
PAPER IV – HCMV INTRAGRAFT PROTEIN EXPRESSION IS ASSOCIATED WITH REDUCED RENAL ALLOGRAFT SURVIVAL

As reviewed in previous sections, HCMV has been associated with acute as well as chronic allograft rejection. Although several studies have demonstrated presence of CMV DNA in renal allografts it has been difficult to detect viral protein expression. Thus, the observed negative effects of HCMV on the allograft have been described as an indirect immune phenomenon to HCMV viremia. In this study, we sought to evaluate the prevalence of HCMV proteins in renal allografts removed due to chronic dysfunction, utilizing immunohistochemistry optimized for HCMV detection in tissues, used previously for detection of HCMV in various cancers and by us for detection of HCMV in vascular tissues (Cobbs et al., 2002; Harkins et al., 2002; Harkins et al., 2010; Lucas et al., 2011; Samanta et al., 2003).

The patient population consisted of 29 patients where end-stage chronic renal dysfunction biopsies were available and for 25 of these patients the earliest available renal biopsy was also evaluated. Very few patients (7%) in our cohort received any HCMV prophylaxis. The majority (90%) were HCMV seropositive or received an allograft from a seropositive donor and in approximately 25% of the patients a verified clinical HCMV infection was observed. Surprisingly, we could detect varying levels of HCMV proteins in 93% of the examined end-stage chronic dysfunction renal allografts. In 57% of the patients the HCMV levels in the allografts were classified as high (>50% positive cells). The findings were further supported by ISH and real-time PCR, which showed extensive presence of HCMV DNA in the examined allografts. Although we made an attempt to isolate RNA from the paraffin blocks, to provide further support for on-going HCMV protein production, we were not able to isolate RNA of acceptable quality for further analysis. The high frequency of protein expression in our study is contradictory to previous studies, but more sensitive technics used in this study could potentially explain this discrepancy. Frequently automated immunohistochemistry staining is used for detection of HCMV in the clinical setting although the sensitivity of such techniques is only moderate (Lu et al., 2009; Rimsza et al., 1996). Here an optimized, manual protocol was used. Furthermore, the absence of HCMV specific histological changes does not exclude the presence of HCMV proteins (Eyzaguirre and Haque, 2008). Our results suggest that the recognized indirect effects of HCMV are likely direct, fuelled by a low-grade active virus replication in the graft.

In the early renal biopsies, all of which were obtained for clinical indications, from these patients HCMV was present in 64%. Notable is that 56% of the early biopsies showed histopathological signs of rejection. Importantly, HCMV protein levels were significantly lower in the early biopsies, and in all but two cases the HCMV graft protein levels increased or remained unchanged between the early and corresponding end-stage biopsy. In a control population of 26 patients with long-term well functioning allografts (creatinine <150µM 5 years post transplantation) the early biopsies had significantly lower HCMV protein levels than the early biopsies of the chronic allograft dysfunction cohort,
although viral proteins could be found in 42% of the patients examined. In this case 38% of the early biopsies had histopathological changes associated with rejection. These findings support the hypothesis that a low grade HCMV protein expression in the graft may fuel the inflammatory process, ultimately resulting in chronic allograft dysfunction.

Further strengthening this hypothesis is our observation that the HCMV protein expression levels in the early post-transplant biopsies are associated with a reduced renal allograft survival. Patients with high HCMV protein levels in their early biopsies lost their grafts after a mean of 18 months, which is significantly earlier than patients with no or low HCMV protein levels in their grafts (mean allograft survival of 81 and 83 months respectively). Interestingly, there were no differences in serum creatinine at the biopsy time point indicating that HCMV allograft protein level may be a superior prognostic marker to creatinine in predicting the risk of long-term graft failure.

Although our findings are potentially very significant they should be interpreted cautiously as the studied population is very heterogeneous and small. This precluded further statistical analysis that potentially could have clarified if HCMV is an independent prognostic factor. Furthermore, as this was a hypothesis generating retrospective study the patients were not optimally monitored in respect to HCMV. Thus, HCMV blood virus levels and viral shedding in urine were not assessed regularly as would be preferred in an optimal study design. Important questions for future projects are the mechanistic links between HCMV protein expression and chronic allograft dysfunction, as well as to assess the impact of antiviral therapies on the viral protein expression.
7 CONCLUDING REMARKS

My work herein has focused on further evaluating the potential significance of HCMV infection in vascular diseases and transplant rejection.

In the first article, we have shown that HCMV is present in SMCs in AAA, that it affects the SMC migratory potential, and is also associated with an expression of 5-LO. Additional studies are needed to further evaluate the role of HCMV in AAA. Evaluation of the known effects of HCMV on MMP and TIMP regulation in the context of AAA would be very valuable. Furthermore, it would be of great interest, in a prospective or retrospective study design, to investigate the potential clinical significance of the presence of HCMV in AAA by relating it to for example aneurysm size, expansion rate and/or rupture frequency. In addition, animal models of AAA provide a platform for investigating the potential effects of HCMV infection on AAA progression.

In the second article, in a prospective study design, we focused on identifying novel prognostic factors for native AVF failure. We identified RDW, WBC and monocyte count as independent prognostic factors. RDW has received a lot of attention during the last years and has been identified as a predictive variable, primarily for mortality, in various CVD populations. This is the first time RDW is identified as a prognostic factor for AVF failure. The mechanistic explanation behind the prognostic value of RDW is although lacking. As numerous processes affect RDW there may not be a simple mechanistic explanation, rather RDW is potentially a good marker because it represents an integrative measure of several pathological processes. As our study was small it will be important to validate RDW as a prognostic marker for AVF failure and preferably also investigate its stability as a marker after dialysis is initiated.

In the third paper, we used the same AVF patient cohort, to evaluate the significance of HCMV in AVF failure. We examined anti-HCMV serum antibody levels and presence of HCMV antigens in the vessels. Although we could detect HCMV proteins in the vessels, neither presence of proteins nor serum anti-HCMV IgG levels were of prognostic value for AVF failure. The lack of prognostic value of anti-HCMV IgG for AVF failure is contrary to previous findings. Our study was although very well powered to detect the hazard ratios reported to be associated with high anti-HCMV IgG levels. The shortcoming of our study design is that we only evaluated presence of HCMV at one time point and as antibody titre levels can fluctuate, larger prospective studies evaluating HCMV at multiple, fixed time points are needed to clarify its potential significance. Furthermore, in the context of AVF, rat animal models are present and evaluating the effects of CMV infection in these could provide further information.

In the fourth paper, we demonstrate that HCMV proteins are present in a majority of renal allografts, that HCMV protein expression increases as graft function deteriorates and that high HCMV protein expression early post transplantation is associated with reduced renal allograft survival. These are novel, and very interesting data indicating that the negative effects of HCMV on the graft are due to an active low-grade replication within the graft rather then due to indirect effects. The limitations of our study are its retrospective design, evaluating a small and heterogeneous patient population. Our results
need to be validated in a prospective study where blood and urine samples for evaluation of viral presence can be acquired at the same time point as the renal biopsy. Furthermore, mechanistic links between HCMV protein expression and chronic allograft dysfunction needs to be sought after. Additionally, it would be of great interest to see if anti-viral treatment of patients with high HCMV intragraft protein levels is beneficiary for allograft survival.

In summary, the current work has provided further insight into the potential significance of HCMV in the pathogenesis of abdominal aortic aneurysm, arteriovenous fistula failure and chronic renal allograft dysfunction.
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