Rīgas Stradiņa Universitāte

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# SIGNIFICANCE OF BETA-HERPESVIRUSES (HHV-6, HHV-7) INFECTION UNDER THE CONDITIONS OF IMMUNE DISORDERS

Doctoral Thesis
for obtaining the degree of a Doctor of Medicine

Speciality – Virology

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ESF EIROPAS SOCIĀLAIS FONDS IEGULDĪJUMS TAVĀ NĀKOTNĒ EIROPAS SAVIENĪBA

Doctoral Thesis was supported by ESF project
"Support for doctoral study programs and research degrees RSU"
2009/0147/1DP/1.1.2.1.2/09/IPIA/VIAA/009

Riga, 2014

# ANNOTATION

Human herpesvirus-6 (HHV-6) and human herpesvirus-7 (HHV-7) are ubiquitous beta-herpesviruses widely distributed in the general population. They primary infection usually occurs in the early years of life and remains latent in the host for the lifelong period.

At present there are numbers of studies trying to find and evaluate the role of beta-herpesviruses infection in the development of various chronic diseases, but still there is no final answer to this question. It could be due to ther ubiquitous nature and different mechanisms of interference with the host organism that these viruses are using.

The aim of the present study was to ascertain the involvement of beta-herpesviruses infection in the pathogenesis and clinical course of chronic diseases and development of post-transplant complications due to their ability to change host-pathogen interaction.

Three groups of patients were enrolled in this study: renal transplant recipients with immunosuppressive therapy (after renal transplantation), patients with underlying disease (gastrointestinal cancer) and patients with autoimmune process (autoimmune thyroiditis). Practically healthy blood donors and thyroid tissue autopsy specimens were included as a control.

Qualitative and quantitative polymerase chain reactions (PCR) were used to detect presence of viral genomic sequences, infection activity stage, HHV-6 strain and viral load. Viral specific antibodies were detected by enzyme-linked immunosorbent assay (ELISA) and chemiluminiscence test, cytokines' expression level – by ELISA, immunocompetent cells' subpopulations – by Becton Dickinson (USA) laser flow cyto-fluorimeter, sorted and further analysed.

The results showed various frequency rates and activity stages of HHV-6 and HHV-7 infection in these three patient groups with different immune system dysfunctions, as well as differences in immune system responses and changes in immunocompetent cells' populations.

Comparison of three mentioned patients' groups revealed that patients with anti-rejection medical treatment had the most severe immunosuppression and higher beta-herpesvirus activation risk leading to the development of different complications. The lowest beta-herpesviruses infection frequency and activation rate was detected in

patients with gastrointestinal cancer, at the same time strong association of HHV-6 and HHV-7 active infection with lymphopenia was demonstrated and higher mortality rate was observed between the patients with lymphopenia than without it. In patients with autoimmune thyroiditis high HHV-6 and HHV-7 infection frequency was found, indicating that viral infection could be implicated in the disease development.

Moreover, higher viral load in thyroid tissue as in whole blood of autoimmune thyroiditis patients' was an additional evidence of HHV-6 involvement in the disease development and was as indicator that thyroid gland is one of the places of HHV-6 latency creating a precondition for autoimmune process development.

# DARBA ANOTĀCIJA

Cilvēka herpesvīruss-6 (HHV-6) un herpesvīruss-7 (HHV-7) ir plaši izplatīti limfotropi herpesvīrusi, ar kuriem inficējas agrā bērnībā, un kas saglabājas persistenti visā dzīves periodā.

Pēdējā laikā tiek pievērsta lielāka uzmanība beta-herpevīrusu infekcijas lomai vairāku hronisku slimību attīstībā, taču vienotas atbildes aizvien nav. Tas varētu būt saistīts ar šo vīrusu izplatību un dažādiem darbības mehānismiem.

Darba mērķis bija noskaidrot beta-herpesvīrusu infekcijas iesaisti hronisku slimību patoģenēzē, slimības klīniskajā gaitā un pēctransplantācijas komplikāciju attīstībā. Šajā pētījumā tika iekļautas trīs pacientu grupas: pacienti ar imūnsupresīvo terapiju (pēc nieru transplantācijas), pacienti ar pamatslimības izsauktu imūnsupresiju (kuņģa-zarnu trakta vēzis) un pacienti ar imūnsistēmas izmaiņām autoimūna procesa rezultātā (autoimūnais tireoidīts). Praktiski veselu asins donoru grupa un vairogdziedzera audu autopsijas paraugi bez patoloģiskām makro vai mikro izmaiņam tika iekļauti pētījumā kā kontroles.

Lai detektēt vīrusu genomu secību klātbūtni, infekcijas latento vai aktīvo fāzi un vīrusu slodzi, tika izmantota kvalitatīvā un kvantitatīvā polimerāzes ķēdes reakcija. Vīrusu specifisko antivielu klātbūtne plazmā tika noteikta lietojot ELISA un IFA. Citokīnu ekspresijas līmeni plazmā noteica ar ELISA. Lietojot *Becton-Dickinson* plūsmas citofluorometru, tika atdalītas un analizētas imūnkompetento šūnu subpopulācijas.

Rezultāti parādīja, ka HHV-6 un HHV-7 infekcijas biežums un aktivitātes fāze trīs pacientu grupās ar dažādu iemeslu radītiem imūnsistēmas traucējumiem ir atšķirīgi, konstatētas arī atšķirības imūnsistēmas atbildēs un izmaiņas imūnkompentento šūnu populācijās.

Salīdzinot trīs pētījumā iekļautās pacientu grupas, parādīts, ka nieres transplantāta recipientiem, kas saņēmuši pretatgrūšanas medikamentozo terapiju, bija smagāka imūnsupresija un lielāks beta-herpesvīrusu aktivācijas risks, novedot pie dažādu komplikāciju attīstības.

Lai gan pacientiem ar kuņģa-zarnu trakta vēzi vīrusu infekcijas sastopamība un aktivitāte bija zemāka, taču konstatēta izteikta asociācija starp HHV-6 un HHV-7 aktīvu

infekciju un limfopēniju, pie tam pacientiem ar limfopēniju tika novērota lielāka mirstība salīdzinot ar pacientiem bez limfopēnijas.

Pacientiem ar autoimūno tireoidītu bieži atrasta HHV-6 un HHV-7 genoma secību klātbūtne gan perifēro asiņu, gan vairogdziedzera audu DNS paraugos. Turklāt vairogdziedzera audu DNS paraugos konstatēta lielāka HHV-6 slodze nekā asins DNS paraugos, tādējādi norādot uz vairogdziedzeri kā vienu no iespējamām HHV-6 latences vietām, kas savukārt var veicināt autoimūnā procesa attīstību.

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# ABBREVIATIONS

AIDS – acquired immunodeficiency syndrome

AIT – autoimmune thyroiditis

AITD – autoimmune thyroid disease

CI – confidence interval

CMV – cytomegalovirus

CPE – cytopathic effect

CSF – cerebrospinal fluid

CTL – cytotoxic T-lymphocytes

DC – dendritic cells

DNA – deoxyribonucleic acid

ELISA – enzyme-linked immunosorbent assay

FACS – fluorescence-activated cell sorting

GIC – gastrointestinal cancer

GVHD – graft versus host disease

HHV-6 – human herpesvirus 6

HHV-7 – human herpesvirus 7

HD – Hodgkin's disease

IFA – indirect fluorescent antibody test

IgG – immunoglobulin G

IgM – immunoglobulin M

IL-1β – interleukin-1 beta

IL-2 – interleukin-2

IL-6 – interleukin-6

ICTV – International Committee on the Taxonomy of Viruses

Leu – leukocytes

Ly – lymphocytes

Mo – monocytes

NK – natural killers

nPCR – nested polymerase chain reaction

NSHL – nodular sclerosis subtype of Hodgkin's lymphoma

OR – odds ratio

p – p value

PBMC – peripheral blood mononuclear cells

PCR – polymerase chain reaction

PHA – phytohemagglutinin

TG – thyroglobulin

TNF-α – tumour necrosis factor

TPO – thyroid peroxidase

TSH – thyroid-stimulating hormone

# INTRODUCTION

Today the majority of chronic diseases already quite strongly associated with infectious agents, including viruses. In most of cases these are viruses that cause persistent infection and who themselves possess the ability to affect the body's immune system. Recently, these immunomodulating viruses have been receiving increasing attention, which is determined by several factors:

1. these viruses are widely distributed after primary infection and they exist in a persistent form throughout life;
2. they can be activated by immunosuppressant factors;
3. they by themselves possess immunosuppressant properties and they can change the body's immune status;
4. they often cause serious complications in cases of an immunosuppression caused by medication after solid organ and stem cell transplantation;
5. they cause a variety of complications in patients with immunosuppressive background caused by underlying disease, for an example in oncology patients;
6. they can initiate chronic inflammatory processes that can lead to autoimmune pathology, and neoplastic changes;
7. in the case of co-infection they can activate each other (Sprengers and Janssen, 2005; Mocarski Jr, 2002; Lucas and McFadden, 2004).

The abovementioned immunomodulating viruses mentioned above include lymphotropic herpesviruses – human herpesvirus -6 and -7 (HHV-6 and HHV-7), that belong to *Betaherpesvirinae* subfamily *Roseolovirus* genus.

HHV-6 was isolated in 1986 from the interleukin-2 (IL-2)-stimulated peripheral blood mononuclear cells obtained from the AIDS patients and patients with lymphoproliferative diseases. HHV-6 was described in two variants and later as a two species – HHV-6A and HHV-6B.

Since the moment of discovery, HHV-6 has been associated with a wide range of clinical conditions and chronical diseases, due to high ubiquitous nature of this virus. Separation of HHV-6 into two distinguished species (HHV-6A and HHV-6B) only added questions concerning involvement of this virus infection in etiopathogenesis of various diseases. HHV-6A is frequently found in multiple sclerosis (MS), chronic fatigue syndrome (CFS), acquired immunodeficiency syndrome (AIDS) and cancer

patients. HHV-6B causes *Roseola infantum*, febrile illness and encephalitis in infants and it reactivates in transplant patients, causing complications such as encephalitis, pneumonia and liver damage. In renal transplant patients, HHV-6 has been associated with the development of chronic allograft nephropathy (Chapenko et al., 2009) and graft versus host disease (GVHD) (Caiola et al., 2012). For a long time only HHV-6B was associated with the development of complications in renal transplant patients, due to it's detection particularly in the mononuclear cells of renal transplants (Helantera et al., 2008), and HHV-6B is also frequently found in the gastrointestinal tract of these patients (Lempinen et al., 2012). However, in recent study the predominance of HHV-6A viremia is reported in the plasma/serum among cohort of renal transplant patients (Csoma et al., 2011).

Data about HHV-6 implication in different cancer diseases is controversial. While a lot of researches associate HHV-6 (especially HHV-6B) with Hodgkin's lymphoma (Lacroix et al., 2010; Siddon et al., 2012) and leukaemia (Ogata et al., 2011), there are lack of studies on HHV-6 implication in solid tumour development. However, several investigators have suggested that HHV-6 possess an oncogenic potential. Cells transfected with HHV-6 can cause tumours in nude mice (Puri et al., 1991).

Recent studies have proposed a certain role for HHV-6 in several autoimmune disorders, including autoimmune acute hepatitis (Grima et al., 2008) and autoimmune hemolytic anemia/neutropenia (Yagasaki et al., 2011). The study of 2012$^{th}$ has linked HHV-6A to Hashimoto's thyroiditis (Caselli et al., 2012). However, additional evidence is required, especially taking into account the distribution of HHV-6A and HHV-6B.

HHV-7 clinical role is poorly documented, but it is possibly associated with the development of *Pityriasis rosea* (Black et al., 1999; Rebora et al., 2010) and it could act as HHV-6 activator and CMV co-factor in complication development in post-transplant patients (Chapenko et al., 2001; Chan et al., 2004; Holden and Vas, 2007; Zawilinska et al., 2011).

In this work a relation of HHV-6 and HHV-7 infection with different chronic diseases and complications development was investigated, to acquire lacking data on involvement of theses viruses in the development of immune system dysfunction. Secondary, the distribution of HHV-6A and HHV-6B infection among different patient groups in Latvia was ascertained.

## Scientific novelty of the study

1. It is the first time when HHV-6 and HHV-7 infection reactivation frequency is compared in three groups of patients with different causes of immune system dysfunction (immunosuppressive therapy, underlying disease and autoimmune process);
2. The higher risk of lymphopenia development in patients with gastrointestinal cancer during active HHV-6 and HHV-7 infection is proved and statistically confirmed.
3. Strong association between beta-herpesviruses (HHV-6 and HHV-7) infection and autoimmune thyroiditis is discovered.
4. Comparative analysis of changes in immunocompetent cell populations and severity of immunosuppression among three different patients' groups are carried out.

## Aim of the study

The aim of the present study was to ascertain the involvement of beta-herpesviruses infection in the pathogenesis and clinical course of chronic diseases and development of post-transplant complications due the ability to change host-pathogen interaction.

## Objectives of the study

1. Create the groups of patients with immune system dysfunctions caused by different factors:
    a. Renal transplant recipients with immunosuppression caused by immuno-suppressive therapy;
    b. Gastrointestinal cancer patients with immunosuppression caused by underlying disease;
    c. Patients with autoimmune thyroiditis.

2. Explore the presence and activity phase of beta-herpesviruses (HHV-6 and HHV-7) infection in patients with immune system dysfunction caused by different factors.
3. Examine the cellular immunity in renal transplant recipients, patients with underlying disease (gastrointestinal cancer) and patients with autoimmune process (autoimmune thyroiditis).
4. Clarify the association between beta-herpesviruses (HHV-6 and HHV-7) infection and post-transplant complications development, chronic disease clinical course and autoimmune process.
5. Build the database of patients, including clinical and laboratory data.

## Hypothesis of the study

1. Reactivation of HHV-6 and HHV-7 infection is occurring more often in patients with immune system dysfunctions than in control group individuals.
2. HHV-6 and HHV-7 reactivation leads to more severe imbalance of the immune system.
3. Higher viral load in thyroid tissue than in peripheral blood indicates that thyroid gland is a possible place of HHV-6 latency which could be a cause of autoimmune process development.

# 1. LITERATURE

## 1.1. Discovery and classification of human beta-herpesviruses

Intranuclear inclusions typical to cytomegalovirus infection were first noticed in 1881 by German scientists who thought they represented protozoa. After viruses were grown in cell cultures, Weller, Smith and Rowe independently isolated and grew cytomegalovirus (CMV) from man and mice in 1956 –1957. Antibodies in 30–100% of normal adults indicate not only a past infection, but the presence of a present latent infection. The presence of CMV DNA in tissues and most organs surveyed indicates the ubiquity of latent infection such as occurs in the immature fetus, in AIDS, and in transplant patients on immunosuppressive drugs. Antiviral agents can inhibit CMV replication but they cannot prevent or cure latent infection (Chen and Ho, 2008).

Human herpesvirus-6 (HHV-6) was first isolated in 1986 from AIDS patients and patients with other lymphoproliferative disorders. At the beginning this virus was called as human B-lymphotropic virus or HBLV (Ablashi et al., 1988). Human herpesvirus 7 (HHV-7) was isolated in 1990 from a healthy individual whose cells were stimulated with antibodies against CD3 and then incubated with interleukin-2 (Frenkel et al., 1990). Both of these viruses are one of the causative agents of *Exanthem subitum* and they have extensive homology (Tanaka et al., 1994; Pinheiro Rdos et al., 2013).

International Committee on Taxonomy of Viruses (ICTV) endorsed nomenclature consists of the designation of herpesviruses by serial Arabic number and the family or sub-family of the natural host of the virus (e.g., HHV-6, HHV-7, etc.). The ICTV classified human herpesviruses into three sub-families: *Alphaherpesvirinae*, containing herpes simplex 1, herpes simplex 2 and Varicella-Zoster virus (HSV-1, HSV-2 and VZV, respectively), *Betaherpesvirinae*, containing CMV, HHV-6 and HHV-7 and *Gammaherpesvirinae*, containing Epstein-Barr virus (EBV) and human herpesvirus-8 (HHV-8). Therefore, HHV-6 and HHV-7 are also called *Roseolovirus* because they are belonging to Roseolovirus genus (Yamanishi et al., 1988).

## 1.2. Structure and replication cycle of human beta-herpesviruses

### 1.2.1. Ultrastructure of beta-herpesviruses

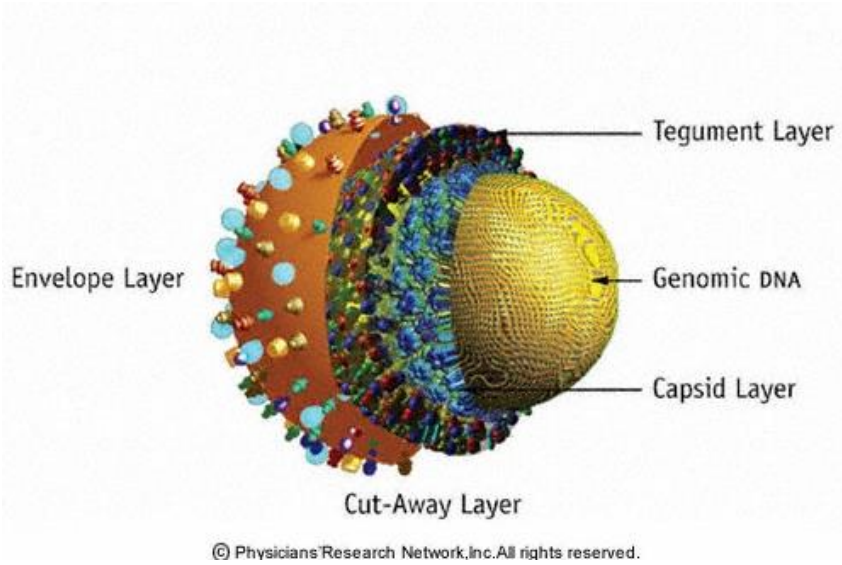



Figure 1.1. **Basic architecture of the herpesvirus virion (created by Louis E, Henderson J)**

HHV-6 is ultrastructurally similar to members of the herpesvirus family. It is an enveloped virus with an icosahedral nucleocapsid made up of 162 capsomeres.

The HHV-6 genome is arranged colineary and codes for approximately 67% of proteins in common with human cytomegalovirus (CMV), and 21% with all other herpesviruses. Sequence comparison shows that it is closely related to HHV-7 and CMV. HHV-6 shares common virion structures with other herpesviruses (Kramarsky and Sander, 1992) showing four basic elements: the core, the capsid, the tegument, and the envelope (Figure 1.1).

The core of mature virion consists of double- stranded DNA (dsDNA) closely paced in a spherical capsid. The capsid is a rigid icosahedral protein shell, 1200–1300 Å in diameter that encloses and protects the dsDNA core. The tegument is a poorly defined, asymmetric layer of host and viral proteins between the capsid and the envelope. It varies in thickness and distribution around the capsid with some of its proteins in close proximity and anchored to the capsid. The envelope is a hosts-derived lipid bilayer containing spikes of viral glycoproteins (Zhou et al., 1999).

### 1.2.2. Genomic structure of human beta-herpesviruses

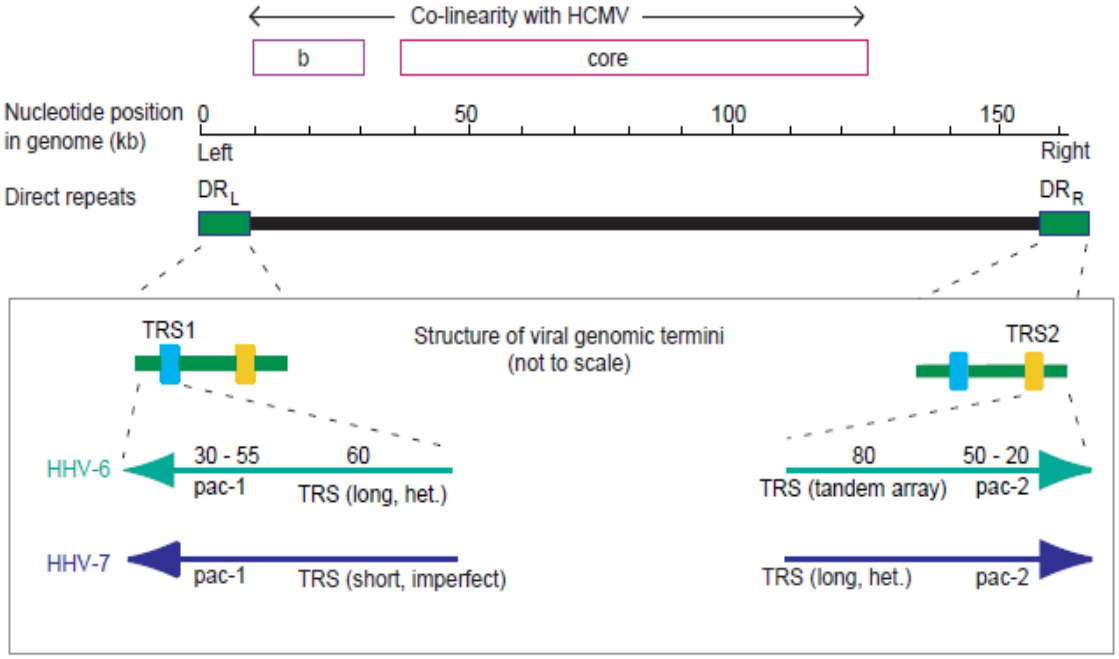



Figure 1.2. **Genome organization of beta-herpesviruses (Dewhurst et al., 1997)**

HHV-6 is genetically is very closely related to HHV-7, with only a few substantial differences in protein-coding content. Each herpesvirus contains a homolog of the EBV BMLF1-encoded transactivator, Mta (M); all members of each subgroup possess at least one IE/regulatory gene that is specific to the subgroup (e.g., gammaherpesvirus homologs of EBV BRLF1-encoded transactivator [R]), and each herpesvirus, appropriately analyzed, encodes at least one "unique" IE gene. Within the betaherpesvirus subgroup, there are both virus-common (CMV UL23, UL24, UL28, UL29, UL36, UL43, and equivalents in HHV-6 and HHV-7) and nonconserved (e.g., CMV IRS1/TRS1, and HHV-6/HHV-7 DR1, DR2, DR6, and DR7) US22 genes. The AAV-2 rep gene homolog is present only in HHV-6. It is conceivable that such event, leading to acquisition of a novel regulatory function, could be at least partially responsible for the biological and evolutionary divergence of HHV-6 and HHV-7. Other genetic differences giving rise to a novel gene products and biological properties are also candidate effectors of "evolutionary drive" (biological divergence leading to herpesvirus speciation), as would be any intragenic or intergenic sequence variations

leading to substantial alteration of gene function or expression. However, it is worth emphasizing that there is a correlation of herpesvirus IE/regulatory gene complements with herpesvirus subgroups and that each characterized herpesvirus contains at least one unique transregulatory gene. Furthermore, such unique genes are located within or adjacent to unique gene clusters that may encode well-conserved, virus-specific cellular gene homologs. This is suggestive of recent acquisition of the unique IE genes at loci susceptible to and/or tolerant of genetic variation. Thus, it seems possible that acquisition of novel functions affecting the control and tropism of herpesvirus gene expression is an important determinant of biological properties effecting segregation and evolutionary divergence. Analysis of HHV-6 and HHV-7 genomes has showed a close genetic relationship between them, and has delineated specific, localized regions of divergence between these two genomes. These divergent regions include sequences specifying potential transregulatory and replication functions, in addition to collinear repetitive elements associated with the IE-A loci. The availability of the complete nucleotide sequences of HHV-6 and HHV-7 should allow growth of rapid progress in studies of gene function and determinations of the common and unique properties of these human T-lymphotropic herpesviruses (Krueger and Ablashi, 2006).

### 1.2.3. Replication cycle of human beta-herpesviruses

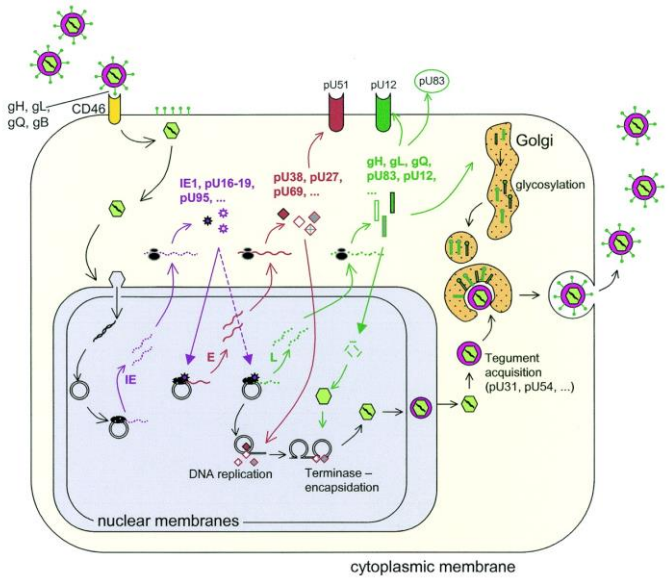



Figure 1.3. **Scheme of HHV-6 replication in cell (Bolle et al., 2005)**

Beta-herpesviruses replication cycle is similar; the difference between them is choice of receptor. If for HHV-6 main receptor is CD46, which is located almost on all cells, for HHV-7 it is CD4 (Lusso et al., 1994). However, CMV broad pathogenic tropism correlates with the ability of the virus to infect all tested vertebrate cell types *in vitro*, a characteristic that has made receptor identification extremely difficult. During virus entry, CMV induces cellular morphological changes and signaling cascades consistent with engagement of cellular integrins.

To initiate viral replication, HHV-6 glycoproteins gH, gL, gQ, and gB attach to the cell surface receptor CD46 of the host. All nucleated cells in the human body contain CD46, and thus, the range of host cells for HHV-6 is expansive. The nucleocapsid of HHV-6 is then transported to the host cell's nucleus via the cellular microtubule network. Viral DNA is then released into the nucleus (Bolle et al., 2005).

Expression of the viral genome requires utilization of host's transcriptional and translational machinery. Like other herpesviruses, both HHV-6 A and HHV-6 B express initial early (IE), early (E), and late (L) stage proteins. IE proteins, encoded by viral genes U86, U89, U95, U16-U-19, and U3, are expressed immediately after infection and are involved in regulation of subsequent viral gene expression. Early proteins, encoded by viral genes such as U27, U41, U43/U74/U77, U73, and U94 are required for replication of the viral genome and virion assembly. Late stage proteins often become incorporated into mature virions. An example of a late HHV-6 protein is that encoded by viral gene U83, which helps to establish latent infection (Dockrell, 2003; Bolle et al., 2005).

Viral DNA replication takes places in the host cell nucleus, by means of the rolling circle mechanism characteristic for herpesviruses. Virions are packaged and assembled within the host nucleus. Virus particles bud out of the nucleus into the cytoplasm, where acquire tegument proteins encoded by viral genes U31 and U54. After tegument acquisition, the virions travel to the Golgi, where they are enveloped with glycoproteins gH, gL, gQ, and gB. Mature HHV-6 particles are then released from the cell via exocytosis (Bolle et al., 2005).

## 1.3. Characteristics of HHV-6

### 1.3.1. Cell tropism

For HHV-6, main target cells appear to be $CD4^+$ lymphocytes, but natural killer (NK) cells, $CD8^+$ T cells, macrophages, epithelial, endothelial, neural cells and fibroblasts may also be infected (Bolle et al., 2005). HHV-6 uses human CD46 as a cellular receptor. Down-regulation of surface CD46 was documented during the course of HHV-6 infection. Both, acute infection and cell fusion mediated by HHV-6 were specifically inhibited with monoclonal antibodies to CD46; fusion was also blocked by soluble CD46. Nonhuman cells that were resistant to HHV-6 fusion and its entry became susceptible upon expression of recombinant human CD46. The use of a ubiquitous immunoregulatory receptor opens novel perspective for understanding the tropism and pathogenicity of HHV-6 (Santoro et al., 1999).

### 1.3.2. Two species: HHV-6A and HHV-6B

In the beginning HHV-6 was divided into two groups as variants A (HHV-6A) and variant B (HHV-6B) (Schirmer et al., 1991). The two variants are closely related but show consistent differences in biological, immunological, epidemiological, and molecular properties. HHV-6B is the major causative agent of *Exanthem subitum* (Yamanishi et al., 1988), but no clear disease has yet been associated with HHV-6A. Later, in year 2011 by ICTV it was decided to recognize both variants as a different species.

The genome of HHV-6A is 159 321 bp in size, has a base composition of 43% G + C, and it contains 119 open reading frames. The overall structure is 143 kb bounded by 8 kb of direct repeats, DRL (left) and DRR (right), containing 0.35 kb of terminal and junctional arrays of human telomere-like simple repeats (Gompels et al., 1995). Some differences have two strains of HHV-6B – HST and Z29. A total of 115 potential open reading frames (ORFs) were identified within the 161 573-bp contiguous sequence of the entire HHV-6B (HST) genome (Isegawa et al., 1999). The HHV-6B (Z29) genome is 162 114 bp long and is composed of a 144 528-bp unique segment (U) bracketed by 8793-bp direct repeats (DR). The genomic sequence allows prediction of a total of 119 unique open reading frames (ORFs), 9 of which are present only in

HHV-6B. The overall nucleotide sequence identity between HHV-6A and HHV-6B is 90%. The most divergent regions are DR and the right end of the unique region, spanning ORFs U86 to U100. These regions have 85% and 72% nucleotide sequence identity, respectively (Dominguez et al., 1999).

HHV-6A and HHV-6B replicates most efficiently *in vitro* in peripheral blood mononuclear cells (PBMCs) or cord blood lymphocytes (CBL), and several isolates have been adapted to grow efficiently in continuous T-cell lines. HHV-6A and HHV-6B differ in their capacities to replicate in specific transformed T-lymphocyte cell lines. Of the two most widely used strains of HHV-6A, strain GS is the most commonly propagated in the T-cell line HSB-2 and strain U1102 is usually propagated in J JHAN cells. HHV-6B (Z29 or HST) is grown most often in primary lymphocytes and has been adapted for growth in the Molt-3 or MT-4 –T cell lines. While T cells are most widely used for propagation of HHV-6A and HHV-6B, cell lines of neural, epithelial, and fibroblastic origin have different levels of permissiveness for HHV-6 growth *in vitro*. However, none of these cell lines are in general use for routine propagation of the virus. In patients with dual infection, only HHV-6A persisted in cerebrospinal fluid (CSF), which suggests that HHV-6A has greater neurotropism (Hall et al., 1998; Saddawi-Konefka and Crawford, 2010). A recent study has showed that HHV-6A and HHV-6B are using different cell receptors to infect T lymphocytes *in vitro*. The cellular receptor for HHV-6A entry was identified as CD46, but the receptor for HHV-6B human CD134 (Tang et al., 2013).

### 1.3.3. Transmission

Asymptomatic nature of viral transfer with body fluids is considered to be the main route of transmission. Frequent HHV-6 findings in saliva and salivary gland tissue (Di Luca et al., 1995; Zhao et al., 1997; Tanaka et al., 2012) suggest that the salivary glands are one of the HHV-6 persistence sites, and that saliva is a way of virus transmission, either to the child from the mother, or within children (Chen and Hudnall, 2006). It is important to note that all HHV-6 isolates from saliva are HHV-6B (Hall et al., 2010).

HHV-6 DNA findings in umbilical cord blood in healthy newborns and lack of IgM, as well as in abortion fetus, are indicating to a possible hereditary transmission

(Adams et al., 1998; Aubin et al., 1992). Vertical transmission frequency of HHV-6 is approximately 1-2%. A single case report show several neurological complications after intrauterine HHV-6 infection (Lanari et al., 2003). Although HHV-6B DNA was found in 20% of pregnant women cervixes, perinatal transmission does not seem characteristic (Maeda et al., 1997). Faecal-oral spread, which is very common among children, for HHV-6 is not documented, and infection by breast milk is denied. Apparently, at least in HHV-6B case, the most common transmission is through saliva. HHV-6 transmission occurs frequently in a horizontal way, usually the mother - child. It is shown that HHV-6 may be integrated into the cell genome. Chromasomaly integrated HHV-6 was first mentioned by Luppi et al (Luppi et al., 1993), the work of three unrelated cases of patients with Hodgkin's disease, non-Hodgkin's B-cell lymphoma lines and multiple sclerosis. Daibata and co-authors (Daibata et al., 1999) described a family case showing that chromasomaly integrated HHV-6 genomic DNA is stably transferred in the gene level. Integration into chromosomes can occure with both HHV-6 virus species, and some authors have found that virus DNA in serum/plasma is associated with white blood cell lysis, in which the genome of HHV-6 is integrated (Ward et al., 2005). *In vitro* experiments reveal that only one: either HHV-6A or HHV-6B can integrate, but not both at the same time.

Ward and co-authors (Ward et al., 2006) for the first time showed the presence of HHV-6 genomic sequence in hair follicle cells of the people with immune response disabilities. It is implied that the virus can be inherited and can be found in many cells of the body (Daibata et al., 1998; Daibata et al., 1999; Tanaka-Taya et al., 2004).

### 1.3.4. Epidemiology

Serologic surveys showed that virtually all full-term infants have passively acquired maternal antibodies to both HHV-6 and HHV-7 at birth. The prevalence of antibodies then falls, reaching to a lowest level by 6 months of age. By one year of age, nearly 90% of children have detectable antibody to HHV-6. Prevalence surveys of adults from various countries around the world showed HHV-6 antibodies detection rates of 88% to 90% (Ward, 2005).

Although there is no serologic test to distinguish HHV-6A and HHV-6B, it is believed that HHV-6B is more seroprevalent, causing the majority of clinically

observed infections. HHV-6A seropositivity is usually observed in immuno-compromised individuals or in adult patients who display clinical signs of *Exanthema subitum* (roseola) (Wang et al., 1999; Dockrell, 2003; Bolle et al., 2005).

HHV-6B causes 97–100% of primary HHV-6 infections in the USA and Japan and is responsible for a 97% of reactivation in transplant patients. HHV-6B primary infections can cause febrile seizures, and a large NIH funded study found HHV-6B to be responsible for over a third of status epilepticus cases in infants (Epstein et al., 2012).

HHV-6A has been found more frequently in patients with neuroinflammatory diseases such as multiple sclerosis (MS) and rhomboencephalitis (Alvarez Lafuente et al., 2006; Crawford et al., 2007). It has also been suggested as co-factor in the progression of HIV to AIDS (Lusso et al., 2007). HHV-6A is acquired later in life, usually without clinical symptoms, except in Africa where HHV-6A is more prevalent than HHV-6B in children. HHV-6B antibodies are presumed to offer partial protection against HHV-6A and vice versa (Bates et al., 2009).

## 1.4. Characteristics of HHV-7

### 1.4.1. Cell tropism

HHV-7 primary is tropic to human T cells, but appears that it has a considerably narrower *in vitro* host-cell range than HHV-6. Thus, HHV-7 infection is limited strictly to cultured primary $CD4^+$ T cells and the SupT1 cell line, whereas HHV-6 infects a wide array of cultured cells and cell lines. It is particularly striking that HHV-7 fails to productively infect many $CD4^+$ cells or cell lines, including primary, monocyte derived macrophages and commonly used $CD4^+$ T cell lines, such as Jurkat and H9 (Lusso et al., 1994).

HHV-7 productively infects $CD4^+$ T cells, inducing a cytopathic effect (CPE) that is similar to the CPE induced by HIV-1 and by HHV-6. This CPE is characterised by membrane blebbing and the presence of multinucleated giant cells (syncytia). However, HHV-7 differs from HHV-6 in its effects on the expression of immunoregulatory T-cell surface proteins (Lusso et al., 1994).

HHV-7 has no effect on the cellular expression of the CD3 cell-surface molecule, but causes a dramatic down-regulation of CD4 expression, within 6–9 days after infection. This down-modulation of CD4 antigen appears to occur via several complementary pathways, much like the inhibition of cell-surface CD4 expression that occurs in HIV-1-infected cells. Thus, HHV-7 infection has been shown to result in a decline in the total amount of CD4 protein and mRNA in SupT1 cells and infection also induces posttranslational effects on CD4 expression in primary T cells and, to a lesser extent, in SupT1 cells (Lusso et al., 1994; Takemoto et al., 2007).

### 1.4.2. Transmission

The HHV-7 transmission is showing conflicting data. Halls and co-authors in their study show that congenital HHV-6, as well as congenital CMV infection is in 1% of newborns, while congenital HHV-7 infection was not found in any cases, that suggests the transmission and pathogenesis differences between such closely related beta-herpesviruses (Hall et al., 2004). HHV-7 DNA is found in healthy individuals' saliva and salivary gland epithelium (Chen and Hudnall, 2006). Chen and Hudnall by using immunohistochemistry method, found HHV-7 antigens in healthy individuals' lymph nodes, tonsils, kidney and liver. HHV-7 is detected in pharyngeal tissue, haematological tissue (bone marrow, blood and spleen), gastrointestinal tissue (stomach, small intestine) and vagina. In above mentioned study is said that, like in the case of HHV-6, findings suggest a possible sexual and perinatal transmission of HHV-7 (Chen and Hudnall, 2006).

### 1.4.3. Epidemiology

Over 95% of adults have been infected and are immune to HHV-7, and over three quarters of them were infected before the age of six. Primary infection of HHV-7 among children generally occurs between the ages 2 and 5, which means it occurs after primary infection of HHV-6 (Yoshikawa, 2003; Epstein et al., 2012).

The prevalence of antibodies to HHV-7 increases with age; 60% of young adolescents have detectable titers (Ward, 2005).

## 1.5. Types of HHV-6 and HHV-7 infection

### 1.5.1. Primary infection

HHV-6 primary infection occurs mostly between the 6$^{th}$ and 15$^{th}$ month after birth (Enders et al., 1990; Meyding-Lamadé and Strank, 2012), and incubation period is from one to two weeks. Transplacental infection is rare, but it can be attributed directly to HHV-6 seropositivity in newborns. Twenty percent of cases of primary HHV-6 infection in children aged six to twelve months are met with acute fever (Hall et al., 1994), and are almost always caused by HHV-6B, not HHV-6A (Schirmer et al., 1991; Dewhurst et al., 1993). Many patients develop a rash on the body, neck and face, for a newborn diagnosed as erythema (*Exanthem subitum, Roseola infantum*). For unknown reasons, the majority of patients with the ES are Japanese, rather than citizens of other countries (Asano et al., 1994; Hall et al., 1994). The association between the *Exanthem subitum* and primary HHV-6B infection was revealed by Yamanishi et al in 1988. Since then, HHV-6B is considered ES disease-initiating factor in young children (Dewhurst et al., 1993). It is not clear when HHV-6A seropositivity occurs, but there is a suggestion that it is happening without significant clinical symptoms after infection with HHV-6B in adulthood. It is interesting that in several study cases more than one HHV-6 strain (or both variants) were isolated from the same patient not depending on whether is it child or an adult (Cone et al., 1996), showing that HHV-6 re-infection can happen.

The most common HHV-6 infection complications are weakness, inflamed eardrum membrane, gastrointestinal tract and respiratory system disorders (Hall et al., 1994). Fever occurs in about 10% of children with primary HHV-6 infection, 10–20% of these cases are children under the age of two (Kondo et al., 1993; Asano et al, 1994; Barone et al., 1995). Also different severe CNS complications such as meningo-encephalitis and encephalopathy were shown, but these are rare cases (Ishiguro et al., 1990; Jones et al., 1994; Yoshikawa and Asano, 2000). In one case study of infant with HHV-6B primary infection, convulsions without fever were observed, showing that HHV-6 infection may have a direct effect on the central nervous system, causing convulsions, even in the absence of fever (Zerr et al., 2002). HHV-6 DNA was found in cerebrospinal fluid of children with convulsions at primary HHV-6 infection or during the re-infection (Kondo et al., 1993). These neurological complications are resulting from direct CNS infection with HHV-6.

Mostly HHV-6 infection, although it is characterized by high fever, is calm and self-locking process, however, a multilateral HHV-6 clinical relevance, such as progressive hepatitis (Asano et al., 1990; Ishikawa et al., 2002), hepatic dysfunction (Tajiri et al., 1997), thrombocytopenia (Hashimoto et al., 2002) and hemophagocytic syndrome (Portolani et al., 1997) are described and highlighted.

Primary infection with HHV-7 usually occurs approximately in age of three. Like HHV-6, HHV-7 primary infection is able to cause *Exanthem subitum* (Tanaka et al., 1994; Asano et al., 1995) and cause damage to the CNS (van den Berg et al., 1999), but it is possible to completely imperceptible process of infection. The study, published in the 1994 by Tanaka et al., reported that five of the seven children who had typical ES signs immediately after the disease revealed HHV-7 infection (Tanaka et al., 1994). Primary HHV-7 infection may be associated with HHV-6 reactivation, as evidenced by the anti-HHV-6 antibodies titer increase in serum of sick infants and/or HHV-6 release in saliva (Hidaka et al., 1994; Asano et al., 1995). It has been showed that HHV-7 and HHV-6 can sometimes co-infect in a single cell (Kempf et al., 1997).

### 1.5.2. Persistent infection

Persistent infections are characterized as those in which the virus is not cleared by the adaptive immune response but remains in target cells of infected individuals. Persistent infections may involve phases of both silent (latent) and productive (active) infection without rapidly killing or even producing excessive damage of the host cells. Both HHV-6 and HHV-7 after primary infection in persistent form in the human body can last the entire life time. Reactivation of the virus can occur in cases of impaired immunity, for example, use of immunosuppressive agents and stress. (Sampaio et al., 2011).

#### 1.5.2.1. Latent infection

After primary infection, HHV-6 remains latent unless the immune system is compromised, at which time the virus may reactivate. HHV-6 remains latent in lymphocytes and monocytes (Mo) and persists at low levels in cells and tissues. In

immunocompetent hosts, this persistent infection is generally of no consequence. Isolated cases of pulmonary failure in immunocompetent patients have been attributed to HHV-6 when no other pathogens have been isolated; however, such cases are not common, and no causal relation has been established (Merk et al., 2005).

HHV-7 can be reactivated from latently infected peripheral blood mononuclear cells by T-cell activation, and it was first isolated from the purified CD4 (+) T-cells of a healthy individual; however, the range of cell types in which HHV-7 can establish true latency is not clear (Frenkel et al., 1990; Katsafanas et al., 1996). A variety of tissues containinfected cells at a late stage of HHV-7 infection, suggesting that HHV-7 might cause a persistent infection rather than a true latent infection (Kempf et al., 1997a).

#### 1.5.2.2. Active infection

After primary infection beta-herpesviruses remains latent, however, any immune system disbalance may cause activation (or reactivation) of viruses. Activation (or reactivation) of herpesviruses begins with the expression of "immediate early" genes. These gene products are believed to be transcription activators (Flamand et al., 2010) and may be regulated by the expression of viral micro ribonucleic acid (RNA) (Tuddenham and Pfeffer, 2011). Subsequent expression of "early genes" then occurs and activates, for instance, viral DNA polymerases. Early genes are also involved in the rolling circle replication that follows (Arbuckle et al., 2011). Viral replication results in the formation of concatemers, which are long molecules that contain several repeats of DNA (Morissette and Flamand, 2010). In the case of HHV-6, its entire genome is made over and over on a single strand. These long concatemers are subsequently cleaved between the pac-1 and pac-2 regions by ribozymes when the genome is packaged into individual virions (Borenstein and Frenkel, 2009).

Active viral infection could flow as acute or chronic infection. Acute HHV-6B infection or reactivation can cause severe encephalitis in both immunocompromised and immunocompetent individuals. HHV-6B primary infection in infant is a common cause of seizures and status epilepticus. Chronic or low level HHV-6 infection has been suggested as a trigger for subsets of patients with multiple sclerosis (HHV-6A) refractory temporal lobe epilepsy (HHV-6B) and cognitive dysfunction in transplant

patients (HHV-6B) and some patients with chronic fatigue syndrome (HHV-6A) (Caserta et al., 2001; Maeki and Mori, 2012).

## 1.6. Effects of HHV-6 and HHV-7 on immune system

### 1.6.1. Immune response after primary infection

Innate immune response to HHV-6 and HHV-7 infections induce production of immune-regulatory chemokines and cytokines. Among them, regulated upon activation, normal T cell expressed and secreted (RANTES), a proinflammatory β-chemokine, is known for inducing local responses by selectively attracting monocytes (the target for HHV-6 latency) and lymphocytes. Interleukin-1β (IL-1β) enhances inflammatory and interferon-γ (IFN-γ) responses, and is needed for IL-6-dependent, B-lymphocyte immune responses. Both IFN-α and IFN-β inhibit HHV-6 replication *in vitro* (Takahashi et al., 1992; Jaworska et al., 2010). However, tumour necrosis factor-α (TNF-α), normally an antiviral cytokine, may enhance release of extracellular HHV-6 by stimulating monocyte differentiation (Arena et al., 1997; Niiya et al., 2006).

### 1.6.2. Humoral immunity

#### 1.6.2.1. Antibody response

Beta-herpesviruses have high seroprevalence in adults; almost all infants have maternal antibodies against these viruses at birth. Antibody titers decline sharply from birth to a lowest level at 3 to 6 months of age. HHV-6 seroprevalence increases sharply from 6 months after birth till the second year of life, when the seroprevalence begins to approximate to that as in healthy adults; this is the period in which almost all HHV-6B primary infections occur (Braun et al., 1997; Magalhaes Ide et al., 2011). HHV-7 primary infection most often occurs after HHV-6, with seroprevalence not accumulating to adult levels until the early teens (Magalhaes Ide et al., 2011).

IgM antibodies develop 5 to 7 days after the onset of clinical symptoms, reach their highest titers in 2–3 weeks and disappear by 2 months post infection. At least one target of the IgM response is the 101K antigen encoded by HHV-6 *U11* (LaCroix et al.,

2000). An HHV-6-specific neutralizing IgM response develops after HHV-6 primary infection, regardless of whether it happens before or after HHV-7 infection. In contrast, while HHV-7 primary infection of HHV-6-native individuals induces IgM antibodies that can neutralize both HHV-6 and HHV-7, there is no IgM response to either virus when HHV-7 infection follows HHV-6 (Yoshida et al., 2002; Deborska-Materkowska et al., 2006).

HHV-6 IgG antibodies usually appear within 10 days to two weeks after the onset of clinical symptoms, increases in avidity over time, and remain at measurable levels for many years (Braun et al., 1997). The initial IgG response is of low avidity. Antibody avidity increases with the time, making it a useful marker for identifying recent infections and for discriminating primary infections from reactivations (Ward et al., 2001). HHV-6-specific IgG4 was detected in all bone marrow transplant recipients whose HHV-6 IgG antibody titers increased by at least 8-fold, whereas in pregnant women and in children less than three years of age, HHV-6-specific IgG4 was never detected (Carricart et al., 2004). The possibility that IgG4 antibodies may be a marker for HHV-6 reactivation warrants in the future study.

#### 1.6.2.2. Neutralizing antibodies

HHV-6 neutralizing antibodies are present at the time of birth and during and after the rash period of primary infection, but not during the febrile stage (Yoshida et al., 2002; Kawabata et al., 2011), suggesting that maternal antibodies have the capacity to block viral infection.

Despite the close genetic and antigenic relationship between HHV-6B and HHV-7, their complement-independent IgG neutralizing antibodies, as detected *in vitro*, do not cross react with each other; this is a reflection of the differences in the viral ligands that interact with the receptors (CD46 and CD4, respectively) used by these viruses to infect target cells (Lusso et al., 1994; Santoro et al., 1999; Yoshida et al., 2002). This does not exclude the possibility that antibodies against one virus provide some protection against the others *in vivo*. In contrast to neutralizing IgG, HHV-7 specific IgM antibodies neutralize HHV-6 infection (Yoshida et al., 2002), but the targets have not been identified.

### 1.6.3. Involvement of HHV-6 and HHV-7 in cellular immunity

#### 1.6.3.1. Cytokine production

An important issue in the organismal response to infectious agents is the balance between the Th1 and Th2 arms of the immune system. There have been many studies of the effect of HHV-6 infection on the ability of target cells to produce cytokines that affect this balance, with sometimes diametrically opposed results. As discussed in detail by Smith and colleagues (Smith et al., 2003), this is probably due, at least in part, to the use of different cell types or cell populations in *ex vivo* conditions that do not fully represent *in vivo* regulatory circuits. IL-12 plays a pivotal role in inducing Th1 responses, and IL-10 is one of the key effectors in shifting the balance toward Th2 responses. Under some conditions, HHV-6 infection of PBMC induces IL-10 expression, which inhibits IL-12 production (Arena et al., 1999; Li et al., 1997). Both HHV-6A and HHV-6B infections of monocytes transiently induce low levels of IL-12 production; simultaneously, these infections substantially restrict the level of IL-12 induction in response to IFN-γ and lipopolysaccharide; the sum is a net reduction in IL-12 production (Li et al., 1997; Smith et al., 2003). From these results, it has been argued that HHV-6 infection might lead to inhibition of Th1-polarized immune responses. This is supported by microarray experiments involving HHV-6B infection of a T cell line that harbors HTLV-1 (Takaku et al., 2005). In contrast, five days after HHV-6 infection, SupT1 cells expressed higher levels of IL-18 and other proinflammatory cytokines, while the level of IL-12 was unchanged and IL-10 was down-regulated (Mayne et al., 2001). In addition, HHV-6 infection induces proinflammatory chemokines, including RANTES, despite encoding a beta chemokine receptor that down-regulates RANTES transcription (Caruso et al. 2003; Grivel et al., 2003; Milne et al., 2000). From these data, it has been argued that HHV-6 infection leads to induction of a Th1 response (Mayne et al., 2001).

Interaction of HHV-6A virions with $CD4^+$ T-lymphocytes inhibits IL-2 mRNA synthesis and IL-2 production induced by PHA or OKT3 (Flamand et al., 1995). IL-2 plays a major role in regulating T-lymphocyte and NK cell functions. HHV-6A, HHV-6B, and HHV-7 each down-regulates the CXCR4 chemokine receptor leading to loss of response to the CXCR4 specific ligand, stromal cell-derived factor-1 (Hasegawa et al., 2001; Yasukawa et al., 1999).

Infection of primary astrocytes had only modest effects on expression of inflammatory genes. Interestingly, when HHV-6A-infected astrocytes were treated with mixture of pro-inflammatory cytokines TNF-α, IL-1β, and IFN-γ, anti-inflammatory genes were strongly induced (Meeuwsen et al., 2005). There was very little overlap between the genes induced by infection of astrocytes vs. the HSB-2 T cell line.

### 1.6.3.2. Involvement of T lymphocytes

Neither HHV-6A nor HHV-6B membrane glycoproteins induce T-lymphocyte proliferation in healthy adults; instead, these antigens inhibit T-lymphocyte proliferation to mitogens or antigens (Horvat et al., 1993). Antigen preparations are mainly composed of nucleocapsid proteins and tegument proteins of HHV-6A and HHV-6B and induce T-lymphocyte proliferation responses of healthy seropositive children and adults (Soldan et al., 2000); the response peaks brake out later after primary HHV-6 infection (4 weeks) in comparison with other herpesvirus infections (1 to 2 weeks) (Kumagai et al., 2006). More HHV-6 seropositive, healthy adults responded to HHV-6B antigen than to HHV-6A antigen.

As with VZV, both HLA dependent and independent cytotoxicity have been observed for HHV-6-specific $CD4^+$ T-lymphocyte clones, suggesting heterogeneous functions of $CD4^+$ T-lymphocytes (Arvin et al., 1991; Wang et al., 2006).

Cytotoxic T-lymphocytes (CTLs) are products of the Th1 pathway, which is regulated in part by IL-12. HHV-6 virions are sufficient to inhibit the ability of macrophages to produce IL-12 in response to IFN-γ or lipopolysaccharides. Although CTLs response eventually develops, it is easy to imagine that a short deferral in the development of the response helps the infection to get established (Smith et al., 2003).

HHV-7 has a selective tropism for $CD4^+$ T lymphocytes. *In vitro* the interference between HHV-7 and HIV-1 was shown. In particular, pre-exposure to HHV-7 rendered $CD4^+$ T cells totally resistant to infection by different HIV-1 isolates, including field isolates that are usually poorly sensitive to inhibition by sCD4. Thus, HHV-7 may act as effective natural inhibitor for HIV infection (Lusso et al., 1994).

HHV-6A can induce cell fusion via its cellular receptor (CD46) in the absence of viral protein synthesis (Mori et al., 2002; Santoro et al., 1999). HHV-7 infection also induces formation of giant, multinucleated $CD4^+$ T-cells, possibly due to

polyploidization of infected cells due to interrupted cell cycles (Secchiero et al., 1997). HHV-6B infection shuts off the host's DNA synthesis of the cell, but stimulates the host's protein synthesis of the cell, which possibly also interferes with the cell cycle and creates a proper intracellular milieu for viral replication (Black et al., 1992; Øster et al., 2005). In SCID-Hu thymus/liver mice, HHV-6A and HHV-6B infection affects almost all of the major lymphoid cellular subsets including $CD4^+$ and/or $CD8^+$ T-cells, but most severely depletes $CD4^-$ and $CD8^-$ intrathymic T-progenitor cells (Gobbi et al., 1999). HHV-6A replicates in and kills $CD4^+$ and $CD8^+$ T-cells with almost equal efficiency, while HHV-6B predominantly replicates in and depletes $CD4^+$ T-cells (Grivel et al., 2003).

HHV-6A and HHV-6B induce a dramatic and generalized down modulation of the CD46 molecule in both infected and non-infected cells, while CD3 was down-modulated only in infected lymphocytes, with some differences in this activity between HHV-6A and HHV-6B (Furukawa et al., 1994; Grivel et al., 2003). In contrast, CD4 is up-regulated after HHV-6A and HHV-6B infection, possibly via direct activation of the CD4 promoter in infected cells (Flamand et al., 1998; Grivel et al., 2003). HHV-6A showed a stronger effect on CD3 expression in $CD4^+$ lymphocytes in comparison with HHV-6B, but both viruses had similar effect on lymphoid tissue cultured *ex vitro* (Furukawa et al., 1994; Grivel et al., 2003). HHV-7 uses CD4 as its cellular receptor; CD4 but not CD3 expression is reduced after HHV-7 infection (Furukawa et al., 1994; Lusso et al., 1994). Down modulation of CD46 may lead to spontaneous complement activation and cytotoxicity of the affected cells. CD3 is an important component of the T-cell antigen receptor and is critical for transduction of the T-cell activation signal upon interaction with the antigen peptide-HLA complex. HHV-6 or HHV-7 infected lymphocytes lose their ability to proliferate in response to anti-CD3 antibodies and to kill virus infected cells (Furukawa et al., 1994; Smith et al., 2005).

HHV-6 viral envelope proteins inhibit T lymphocyte proliferation induced by phytohemagglutinin (PHA), IL-2, or antigens (Horvat et al., 1993; Kawabata et al., 2009). Interaction of inactivated HHV-6A viral particles with PBMC inhibits proliferation of both $CD4^+$ and $CD8^+$ T lymphocytes and their responses to IL-2. This defect is apparently due to induction of defective IL-2 receptors or defects in IL-2 induced signalling pathway in these cells, as exogenous IL-2 does not correct the HHV-6 induced proliferation defect (Flamand et al., 1995).

### 1.6.3.4. Involvement of NK cells

HHV-6 infects and kills NK cell clones cultured *in vitro*. In addition, HHV-6 also induces NK cells to express CD4 receptor, the helper T lymphocyte surface marker and the major cellular receptor for HIV-1, which can render NK cells permissive to HIV infection; this has led to speculation that HHV-6 not only causes immune suppression but also contributes to AIDS progression (Emery et al., 1999; Kumagai et al., 2006). However, HHV-6 infection of PBMC, which include a variety of cell types, results in enhanced NK activity and NK-mediated killing of HHV-6 infected cells (Flamand et al., 1996; Kumagai et al., 2006). Thus, virus-mediated killing of NK cells and NK-mediated killing of virus infected cells is regulated in part by the other cells in the system.

### 1.6.3.5. Involvement of stem cells

Although none of the *Roseoloviruses* kill blood stem cells, they do affect their growth. *In vitro*, HHV-7 inhibits the growth of granulocytic/ erythroid/ monocyte/ megakaryocytic progenitors, but not their more differentiated progeny. In contrast, HHV-6 has less effect on the progenitors, but inhibits the growth of the more differentiated progeny (Isomura et al., 2003; Knox and Carrigan, 1992; Mirandola et al., 2000). Exposure of bone marrow precursors to HHV-6 inhibited their ability to respond to growth factors such as granulocyte-macrophage colony-stimulating factor and interleukin-3, and also reduced the outgrowth of macrophages from bone marrow (Burd and Carrigan, 1993; Andre-Garnier et al., 2004). This may be due to induction of IFN-α by HHV-6 (Knox and Carrigan, 1992; Jaworska et al., 2010). HHV-6 also infects monocytes/macrophages, although these cells do not develop the typical CPE seen in $CD4^+$ T-cells after HHV-6 infection. Nonetheless, the virus does cause dysfunction of blood monocytes and blocks their differentiation to macrophages (Burd and Carrigan, 1993; Smith et al., 2005). There is disagreement as to whether HHV-6A or HHV-6B is the more potent inhibitor of hematopoietic stem cell growth (Isomura et al., 2003). The mechanism for HHV-7 inhibition of blood stem cells is unknown.

#### 1.6.3.6. Involvement of dendritic cells

Dendritic cells are important antigen presenting cells for CD4 and CD8 T lymphocytes. Immature dendritic cells support HHV-6A or HHV-6B replication (Hirata et al., 2001; Kakimoto et al., 2002; Nordström and Eriksson, 2012). Most of the infected cells are not killed, and differentiate into the mature forms, but these cells are functionally deficient and incapable of supporting lymphocyte proliferation (Kakimoto et al., 2002). This is in contrast to CMV, which selectively infects mature dendritic cells and down-regulates their cellular markers, thus impairing their function.

#### 1.6.3.7. Involvement of histocytes

Langerhans cell histiocytosis and hemophagocytic histiocytosis are characterized by dysregulated proliferation and migration of histiocytes (tissue macrophages). These diseases can be triggered by other diseases, use of certain drugs, or infections including HSV, CMV and EBV. Cases of hemophagocytic histiocytosis have been associated with HHV-6 activity following organ transplantation (Rossi et al., 2001; Jeziorski et al., 2008).

### 1.6.4. Cell apoptosis

Like other herpesviruses, HHV-6 and HHV-7 lytic infections lead to host cell death via necrotic lysis (Inoue et al., 1997; Secchiero et al., 1997; Yasukawa et al., 1998). In addition, $CD4^+$ T-lymphocytes die from virus-triggered apoptosis. This induction is dependent on viral replication of HHV-7 and is independent of viral replication of HHV-6 (Inoue et al., 1997; Secchiero et al., 1997). HHV-6-mediated apoptosis is regulated by the Fas–Fas ligand system and induced apoptosis induced by HHV-7 seems to be controlled by bcl-2 (Inoue et al., 1997; Secchiero et al., 1997). In support of this, inhibition of the apoptotic pathway by enhanced expression of bcl-2 enhances HHV-7 infection, as observed for other viruses including EBV (Razvi and Welsh, 1995). Both HHV-6A and HHV-6B incorporate the important cell regulatory protein, p53, into virion particles; p53 regulates the cell cycle and protects a portion of

infected cells from apoptosis (Øster et al., 2005). The basis for the choice between the two distinct fates (necrosis or apoptosis) of infected cells is unknown.

HHV-7 infection also induces the production of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) production, which provides a trans-acting signal that triggers apoptosis of nearby uninfected cells (Secchiero et al., 2001). Simultaneously, TRAIL receptor 1 (TRAIL-R1) is down modulated in HHV-7 infected T lymphocytes. Thus, the virus triggers the death of nearby cells, which may protect it from the activity of immune effector cells such as NK cells and CTL, while protecting infected cells from similarly triggered death.

## 1.7. Clinical manifestations

### 1.7.1. Post-transplantation complications

HHV-6 infection occurs in nearly 50% of all bone marrow and in 20–30% of solid-organ transplant recipients, 2-3 weeks following the procedure. It has been suggested that the viral infection and activation results in clinical symptoms, including fever, skin rash, pneumonia, bone marrow suppression, encephalitis, and rejection. In order to understand the viral infection in greater detail, several studies have investigat the route of viral transmission and diagnostic procedures have been carried out. HHV-6 reactivation has been increasingly associated with acute graft-versus-host disease (GVHD) and allograft rejections in the transplant settings. A recent survey of 235 allogeneic stem cell transplant patients indicated that post-transplant HHV-6 reactivation is strongly associated with delayed platelet engraftment, early post-transplantation mortality, and the development of acute graft versus host disease (GVHD) (Dulery et al., 2012).

In contrast to studies of HHV-6 infection in organ-transplant recipients, the number of studies examining HHV-7 infection in these patients is limited. According to several recent studies, HHV-7 may act as a cofactor for cytomegalovirus disease in organ-transplant recipients (Yoshikawa et al., 2003).

HHV-6 activity is very common after blood stem cell transplantation (SCT), with HHV-6B being more commonly detectable than HHV-6A. HHV-7 activity is not detected as often in PBMC, possibly due to the low $CD4^+$ cell counts after

transplantation (Boutolleau et al., 2003). Because of their high prevalence, most of the post-transplant activity of these viruses is due to either reactivation or re-infection; primary infections are rare. It is difficult to interpret serological data in these patients because they are often severely immune suppressed and exposed to many blood products. HHV-6 antibody titers do increase (including neutralizing antibodies), but often this is not linked to the presence of viral DNA in blood or infectious virus in bodily fluids (Wilborn et al., 1994). Geometric mean antibody titers were significantly higher in recipients without HHV-6 viremia than in those with viremia (Yoshikawa et al., 2002), consistent with a stronger immune response being linked to reduced virus activity. Anti-HHV-7 antibody titer increased in an allogeneic SCT patient with HHV-7 associated meningitis (Yoshikawa et al., 2003).

In renal transplant patients, HHV-6 has been associated with the development of chronic allograft nephropathy (Chapenko et al., 2009). Consequences of HHV-6 reactivation in liver transplant patients include bone marrow suppression, central nervous system dysfunction, pneumonitis, hepatitis, and increased severity of graft versus host disease, increased incidence of fungal infections and higher incidence of allograft rejection (Abdel Massih, 2009). A recent publication indicates high intrahepatic HHV-6 load, and neither CMV or EBV, and is associated with decreased graft survival following diagnosis of graft hepatitis (Pischke et al., 2012).

Acute liver failure has been associated with HHV-6 infection in both immunocompetent (Cacheux et al., 2005) and immunocompromised persons (Kuntzen et al., 2005). A recent article has demonstrated that high intrahepatic HHV-6 DNA levels were significantly associated with decreased graft survival following diagnosis of graft hepatitis, while elevated levels of CMV and EBV were not associated with decreased graft survival (Pischke et al., 2012). HHV-6 has been shown to directly infect the liver (Gallegos-Orozco and Rakela-Brödner, 2010).

It is reported that HHV-6 is a cause of many hepatobilliary diseases including fulminant hepatic failure and acute decompensation of chronic liver disease in children (Chevret et al., 2008), post-infantile giant cell hepatitis (Kuntzen et al., 2005), acute liver failure in adults (Cacheux et al., 2005), and hepatitis (Schenke et al., 2010). Liver dysfunction due to HHV-6 mononucleosis has also been well documented. An indirect role in the development of cirrhosis and hepatocellular carcinoma has also been proposed (Nakayama-Ichiyama et al., 2011). A typical liver biopsy can be very helpful in the diagnosis of HHV-6-induced liver failure (Gallegos-Orozco et al., 2010), and

HHV-6 liver infection has been successfully treated with ganciclovir antiviral therapy (Cacheux et al., 2005).

HHV-6 infection has been reported to cause cardiac complications in both immunocompromised and immunocompetent patients, including myocarditis (Leveque et al., 2011, Mahrholdt et al., 2006), dilated cardiomyopathy (Tatrai et al., 2011), sinus tachycardia (Nishimoto et al., 2012), and "idiopathic" left ventricle dysfunction (Kühl et al., 2005). Despite the recent increase in publications reporting HHV-6 induced myocarditis in immunocompromised patients, the role of the virus in the pathology of acute chronic myocarditis remains poorly defined.

A group of physicians from the University Hospital in Reims, France, recently has reported a case of fatal HHV-6 myocarditis in an immunocompetent patient (Leveque et al., 2011). Although the initial blood work and heart biopsies tested came back negative for the presence of HHV-6 infection, post-mortem frozen tissues showed evidence of chronic HHV-6 infection in regions of the heart that had not been biopsied in the initial screening process. This report demonstrates that HHV-6 can establish a nearly undetectable chronic active myocarditis in the immunocompetent individual which may eventually result in heart failure and even death. The authors emphasized the importance of endomyocardial biopsy and molecular analysis on frozen tissue – as opposed to fixed tissues – as HHV-6 was not detected in either serum or in paraffin fixed tissues of this patient. The antibody titers for HHV-6 were not elevated. Endomyocardial biopsies are not performed for most myocarditis cases in the United States, but are done routinely in Germany and in other European centers.

### 1.7.2. Cancer

HHV-6 and HHV-7 share a high degree of genomic homology and have some similar biological properties. Thus, these herpesviruses might share a similar oncogenic potential (Chan et al., 2001). HHV-6 has immunomodulating properties and is a powerful inducer of cytokines. One important mechanism for HHV-6 pathogenesis is the engagement of the primary viral receptor, CD46, a complement-regulatory cell surface molecule that provides a key link between innate and adaptive immune responses (Santoro et al., 1999).

Recently, it was shown that exposure to HHV-6 results in a dramatic inhibition of IL-12 p70 production by differentiated human macrophages in the absence of a productive viral infection, a phenomenon that is likely mediated by CD46 engagement (Smith et al., 2003). Other mechanisms of immune dysregulation by HHV-6 include defective antigen presentation by dendritic cells and aberrant cytokine production by peripheral blood mononuclear cells, such as increased secretion of IL-1β, tumor necrosis factor alpha (TNF-α), and IL-10 and decreased secretion of IL-2 associated with diminished cellular proliferation (Kakimoto et al., 2002; Arena et al., 1999; Flamand et al., 1991). Persistent IL-2 regulated HHV-6 infection of adult T-cell leukemia cells causes T cell leukemia to progress more rapidly, but *in vivo* studies have not yet confirmed a pathogenetic role of HHV-6 in this disease (Ojima et al., 2005). Virus-induced changes in cytokines secretion can lead to changes in tumor microenvironment and deviation of anti-tumour immune response. HHV-6 may also contribute to cancer circuitously through immune suppression. HHV-6 can directly infect $CD4^+$ T-cells and induce apoptosis, as an effective $CD4^+$ T cells response is believed to prevent tolerance induction by tumor antigen (Krueger et al., 1990; Schonnebeck et al., 1991; Kennedy and Celis, 2006).

There have been numerous studies aimed at finding an association between HHV-6 and Hodgkin's disease (HD), and it has proven to be very difficult to conclude definitively on this subject due to the difference in assays and patients tested. However, a link between HHV-6 and nodular sclerosis subtype of Hodgkin's lymphoma (NSHL) has recently been reinforced by several groups. A study from Yale has identified HHV-6 in 86% of NSHL cases (Siddon et al., 2012). Researchers utilized several laboratory methods - including IHC, PCR, and FISH - to identify HHV-6 DNA in tissue samples from 31 lymph node cases of Hodgkin's lymphoma. In addition, the group also sought to localize the presence of HHV-6 DNA in particular HL cell types, and identified HHV-6 DNA via IHC in malignant Reed-Sternberg cells in nearly half of the cases. This result, which is supported by previously published work by Lacroix et al and others, suggests that HHV-6 may play a direct role in the pathogenesis of NSHL (Strenger et al., 2013; Siddon et al., 2012, Lacroix et al., 2010). The study is the most comprehensive and thorough investigation conducted on the association between HHV-6 and NHSL to date, and helps to clarify previously conflicting results published in the field.

Luppi et al., (Luppi et al., 1993), reported researce findings of a higher frequency of HHV-6 DNA in a well characterized series of patients with angioimmunoblastic T cell lymphoma (AITL), a subtype of T-cell non-Hodgkin's lymphoma, compared with other lymphoma subtypes and controls. These findings have been confirmed by Zhou et al., (Zhou et al., 2007) showing a clear association between histological progression of AITL and detectable copy number of both EBV and HHV-6B in the AITL lesional tissue. While this increased viral load could reflect a role of HHV-6 in pathogenesis and progression of AITL, it also could be the consequence of increasing dysfunction of the immune system during lymphoma progression. Immunohistochemical studies so far have failed to demonstrate HHV-6 antigens in the CD4 positive T cells (the likely proliferating elements) within AITL lesions.

It is well established that human papillomaviruses (HPVs) are necessary, yet not sufficient, for the development of cervical cancer; and many essential cofactors have been implicated in the progression of this disease. Some authors have suggested a role of several human herpesviruses – namely EBV and CMV – in the development of cervical cancer, particularly through co-infection with HPV and subsequent interaction with the HPV-16 genome (Szostek et al., 2009). Multiple studies have detected HHV-6/HPV co-infection among samples from women with cervical cancer (Chen et al., 1994, Tran-Thanh et al., 2002). In one study that primarily focused on new assay development, HHV-6B was detected in a patient who later developed cervical cancer (Li 2009). Chen et al. have demonstrated that HHV-6 has HPV-transactivating capability, and HHV-6 co-infection leads to more rapid tumorgenesis (Chen et al., 2004).

Investigators from Nanjing Medical University led by Dr. Kun Yao have found HHV-6 latent infection in glioma tissues, and have isolated a strain of HHV-6A from the glioma cyst, supporting earlier studies that suggested the involvement of HHV-6 in the pathogenesis of adult and pediatric gliomas (Chi et al., 2012). Using nested PCR and immunohistochemistry methods, Dr. Yao's team identified HHV-6 DNA and its proteins in tissue from 42.5% of gliomas compared to 7.7% of normal brain tissue. In addition, elevated levels of several cytokines that were specifically promoted by HHV-6 infection in astrocyte cultures were also observed in HHV-6-positive cyst fluid samples from glioma tissues.

An earlier paper by NINDS/NIH investigators (Crawford et al., 2009) found that both early (p41) and late (gp116/64/54) HHV-6 viral antigens were three times more

frequently in adult glial tumors compared to control brain tissues. An additional study by this group utilized nested PCR, *in situ* hybridization (ISH), and IHC to detect HHV-6 in pediatric brain tumors, and demonstrated that HHV-6 viral antigens were significantly correlated with low grade-glioma tissue compared to controls (Crawford et al., 2009).

### 1.7.3. Autoimmune diseases

A role for HHV-6 has been proposed in several autoimmune disorders, including autoimmune hemolytic anemia/neutropenia (Yagasaki et al., 2011), autoimmune acute hepatitis (Grima et al., 2008), and multiple sclerosis (Tejada-Simon et al., 2003). A 2012 study linked HHV-6A to Hashimoto's thyroiditis (HT), a common autoimmune thyroid disease (AITD) (Caselli et al., 2012). A group from the Ferrara University, Italy, has published a study linking HHV-6A to Hashimoto's thyroiditis (HT), an autoimmune disorder what is the most common of all thyroid diseases. The study found that HHV-6 was detected significantly more frequently among thyroid fine needle aspirates (FNA) from HT individuals in comparison with controls (82% vs. 10%, respectively), and low-grade acute infection was identified in all HHV-6 positive HT samples compared to none (0%) of controls. In addition, the group demonstrated that thyroid cells infected with both HHV-6A and HHV-6B became susceptible to NK-mediated killing, providing evidence of a potential mechanism for HHV-6-induced autoimmunity.

It should be pointed out that the pattern of infection established by HHV-6 in HT patients was substantially different than that observed in healthy individuals. HT biopsies were more frequently positive for HHV-6 and exhibited increased viral loads compared to controls, and active HHV-6 transcription was observed in HT thyrocytes compared to latent infection in HHV-6-infected control thyroids. Furthermore, the presence of HHV-6 infection was found localized mainly in thyrocytes, rather than in lymphocytes infiltrating the lesion, and increased prevalence of latent HHV-6 infection was seen in PBMCs overall (Caselli et al., 2012).

Several studies have examined the presence of herpes virus DNA in rheumatoid arthritis (RA) synovium, but the results were inconclusive (Takei et al., 1997; Balandraud et al., 2003); other reports have supported the presence of certain viruses (HHV-6, EBV, CMV, parvovirus B19, rubella virus, human T cell leukaemia virus, and

hepatitis B) virus as potential triggers of RA, on the basis of epidemiological evidence and abnormal immune responses to these viruses (Alvarez-Lafuente et al., 2005). However, the role of viral infections in RA remains unresolved.

## 1.8. Diagnosis and treatment of HHV-6 and HHV-7 infection

### 1.8.1. Laboratory diagnostics

Nearly 100% of us have been infected with the HHV-6 virus by early childhood and have antibodies to it, and at least 30% of us have small but detectable levels of latent virus in our blood (Alvarez-Lafuente et al., 2002; Clark et al., 1996).

PCR DNA tests can detect HHV-6 in the serum during primary roseola infections and in acute post-transplant infection reactivation. However, they cannot determine reliably if a patient has a chronic central nervous system (CNS) infection that has reactivated because there is so little virus circulating outside of the tissue. HHV-6A and HHV-6B (especially HHV-6A) can migrate to the central nervous system and other organs away from the bloodstream. HHV-6A has been found to persist in the spinal fluid long after it has disappeared from the plasma (Caserta et al., 1994).

Researchers at the NINDS using autopsy material have determined that bone marrow transplant patients with active or reactivated infections in the CNS tissue have very little HHV-6 in the spinal fluid or serum (Fotheringham et al., 2007). This means that if the HHV-6 is chronically active in the brain tissue, it may be impossible to find any evidence of it in the peripheral blood or even the spinal fluid.

As is the case with pathogens with low viral copy numbers such as HHV-8 and West Nile virus, indirect evidence of the HHV-6 antibodies is easier to find than the HHV-6 virus itself. Therefore elevated IgG antibody levels (above a threshold) may be the only indication of a reactivated chronic HHV-6 CNS infection.

Another reason difficult detection of HHV-6 directly by PCR is that there is latent virus in many of our circulating white blood cells. Tests must be done in serum to differentiate active from latent infection. Furthermore, in many viral infections large numbers of virions spill into the plasma when the virus is replicating but this is not the case with HHV-6 since it is spread largely from cell-to-cell or directly through the cell

walls, therefore PCR tests must be extremely sensitive to detect persistent HHV-6 infections once the acute phase is over.

Elevated IgG antibody levels can suggest, but not prove active, chronic infection. In a 1996 study of HHV-6 in CFS patients, 89% of the patients with IgG titers of 1:320 and above were found to have active infection by culture. Stanford University infectious disease specialist Jose Montoya believes that the best evidence of smoldering CNS infection is the IgG antibody to the virus, and not the virus itself. In a pilot study, he found that when patients with high titers of HHV-6 IgG (1:320, 1:640 or higher) and EBV antibodies are treated with a potent antiviral drugs their titers fall substantially along with a significant improvement in symptoms. Elevated IgG antibody titer to HHV-6 cannot indicate with certainty that the infection is active, but high titers support a clinical diagnosis. Similarly, elevated EBV VCA (late antibody) titers also cannot indicate with certainty that an infection is active. However, antibodies to EBV early antigen (EA) disappear rapidly after an infection is over, so elevated EBV EA antibodies do predict active infection.

Individuals vary in the way they respond to the virus: some may not be able to generate antibodies due to a weak immune response. Others may generate large numbers of antibodies to many pathogens, and some healthy individuals have high titers of HHV-6 IgG antibodies. So looking at elevated antibodies to determine active infection is far from a perfect measure. Montoya is currently conducting a placebo controlled trial of Valcyte in symptomatic patients with elevated antibodies to HHV-6 and EBV. If these patients are going to show a dramatic drop in antibody titers and improvement in symptoms in response to Valcyte treatment, he will demonstrate conclusively that elevated IgG titers can be a sign of active infection and could be a reliable biomarker for monitoring patients.

Nowadays new methods of viral infection diagnosis and research have been developed. The most interesting would be the use of flow cytometry. By this method it is possible to determine immunological parameters of the patient and to sort specific cell subpopulations for the further investigation. Also it is possible to use flow cytometry to investigate viral infection properties. Recently, Caselli et al. 2012, using flow cytometry have shown HLA class II antigen expression on thyrocytes infected with HHV-6. These results became as another evidence of HHV-6 involvement in Hshimoto’s disease.

### 1.8.2. Approaches to the treatment

Virus reactivation can cause life-threatening complications in individuals with impaired immune response. Since an effective vaccine against HHV-6 is not available, a safe and effective treatment for this infection is required. There are still no clinically approved drugs or controlled trials to treat HHV-6, so most utilized therapeutic drugs are the same as for CMV-nucleoside analogues of ganciclovir and valganciclovir, in lesser extent: acyclovir, valaciclovir, cidofovir and foscarnet like pyrophosphate. Most of these drugs have side effects, therefore, researches for the new HHV-6 and all of the herpes virus group treatment strategies are carried out (Coen and Schaffer, 2003).

There are several studies that both – confirm and deny the ganciclovir exposure to HHV-6. Prophylactic treatment with ganciclovir, but not with acyclovir (Zovirax), shows the effective results in preventing reactivation of HHV-6 in bone marrow or stem cell transplant recipients (Rapaport et al., 2002). There is the lack of exposure and lack of efficacy by ganciclovir administrated orally. Another use of ganciclovir limiting feature is its side effects, most commonly neutropenia and thrombocytopenia, which is dependent from the dose (McGavin and Goa, 2001). To avoid side effects caused from ganciclovir therapy, the drug is recommended to give in small doses for prophylaxis, but it may contribute to the development of resistance (Drew et al., 2001).

Foscarnet is more effective and less cytotoxic than ganciclovir; it is usually given to patients who are resistant to ganciclovir. The foscarnet is associated with nephrotoxicity, which is the main reason why preference is given to ganciclovir (Coen and Schaffer, 2003). *In vitro*, HHV-7 is less sensitive to ganciclovir and acyclovir than HHV-6. HHV-7 is more sensitive to cidofovir than to ganciclovir or acyclovir, but the toxicity associated with this drug is not as scrupulously researched to be used in the treatment of HHV-7. At the moment one of the most effective drugs against HHV-7 is foscarnet (Bolle et al., 2005).

# 2. MATERIALS AND METHODS

## 2.1. Patient groups

Three patient groups – patients with immunosuppressive drug treatment (renal transplant recipients); patients with immune system disorders related to the underlying disease (patients with gastrointestinal cancer) and patients with autoimmune disorders (autoimmune thyroiditis) were included in the study. Practically healthy blood donors were included as control group. Post-mortem thyroid gland samples without any pathological macro or micro changes were used as the tissue controls in testing of operated thyroid glands of patients with autoimmune thyroiditis.

The research was established with the approval of the Ethics Committee of the Rīga Stradiņš University and all participants gave their informed consent prior to the examination.

### 2.1.1. Patients with immunosuppressive drug treatment

In this group 47 patients 3 months after renal transplantation were enrolled – 27 men and 20 women – mean age 49 (from 28 to 78).

Patients had received immunosuppression: induction immunosuppression therapy with monoclonal or polyclonal antibodies and triple supportive immuno-suppression therapy consisting of glucocorticoids, antiproliferative drugs and calcineurin inhibitors.

The induction immunosuppression was initiated during the renal transplantation, and there was used one of the medications containing monoclonal antibodies against interleukin-2 receptor, i.e., basiliximab (Simulect, Novartis) 20 mg intravenous (i/v) in the day of surgery and in the fourth post-operative day or daclizumab (Zenapax F. Hoffmann-La Roche) 1 mg/kg i/v in the day of surgery and 15th postoperative day, or polyclonal antibodies – anti-T lymphocyte globulin (ATG, Fresenius Biotech) 1.5–3 mg/kg i/v in the first 3–5 days after surgery.

Supportive immunosuppression was started with 500 mg i/v methylprednisolone (Solu-Medrol, Pfizer) in the day of surgery and five days after 0.5 mg/kg per oral (p/o)

prednisolone (Prednisolon, Gedeon Richter) with declining dosage (till 20 mg after month).

Anti-proliferative drugs and calcineurin inhibitors were given in the day of surgery. On top of anti-proliferative drugs, patients received mycophenolate mofetil (Cell-cept, F. Hoffmann-La Roche or Myfortic, Novartis), an initial dose was in average 2 g per day p/o or azathioprine (Imuran, GlaxoSmithKline) 100–150 mg per day p/o. Of calcineurin inhibitors cyclosporine A (Sandimmun Neoral, Novartis or Ciclosporin Sandoz, Sandoz) was used with an initial dose of 3–4 mg/kg daily p/o.

All peripheral blood samples were received from Latvian Transplantation Center. From peripheral blood samples aliquots of 200 μl blood plasma were collected and the rest of whole blood separated in aliquots of volume 500 μl. All blood samples were stored at temperature of –20 °C.

### 2.1.2. Patients with immunosuppression caused by underlying disease

In the group were included 65 patients [42 women and 23 men – mean age 54 (from 39 to 85)] with histologically confirmed various stages of gastrointestinal cancer (GIC) were enroled: I stage – 7; II stage – 28; III stage – 19 and IV stage – 11 patients. All patients were enrolled before surgery and they were without any antitumor treatment. Patients' lifespan was monitored during five years.

All peripheral blood samples were received from Latvian Oncology Center. From peripheral blood samples aliquots of 200 μl blood plasma were collected and the rest of whole blood separated in aliquots of volume 500 μl. All blood samples were stored at temperature of –20 °C.

### 2.1.3. Patients with autoimmune disorders

In this group were enrolled 44 patients [43 women and one man – mean age 45 (from 25 to 78)] with histologically confirmed autoimmune thyroiditis. Additionally patients' samples were tested for the presence of auto-antibodies to thyroid peroxidase (TPO), against to thyroglobulin (TG) and thyroid stimulant hormone receptor (TSH).

Patients were with following diagnosis: *Struma nodosa III thyreotoxicum* (n=16) and *Struma nodosa III euthyreocum* (n=28). Surgical treatment – *Thyreoidectomia totalis* was applied to all patients.

All peripheral blood samples and thyroid tissue were received from Riga East Clinical University Hospital. From peripheral blood samples aliquots of 200 µl blood plasma were collected and the rest of whole blood samples separated in aliquots of volume 500 µl. All blood samples were stored at temperature of –20 °C. Thyroid tissues were separated in 80 mg pieces and at stored at temperature of –70°C.

For FACS analysis and sorting PBMC was collected using gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, Germany), and was used at the same day for further investigation.

### 2.1.4. Practically health blood donors

In this group were enrolled 150 practically healthy persons – 77 females and 73 males – mean age 37 (from 18 to 65). All peripheral blood samples were received from State Blood Donor centre. From peripheral blood samples aliquots of 200 µl blood plasma were collected and the rest of whole blood samples separated in aliquots of volume 500 µl. All blood samples were stored at temperature of –20 °C.

### 2.1.5. Thyroid tissue autopsies

41 post-mortem thyroid tissue samples without any pathological macro or micro histological changes (with anatomical-pathological diagnosis cardiomyopathy) were enroled as a control. These controls were compared with surgically obtained thyroid gland tissue samples from patients with thyroiditis, because it is important to know the frequency of the viral genomic sequences in the whole blood of these patients and healthy thyroid tissue.

Autopsy samples have been obtained from 11 women and 30 men – mean age 52 (from 41 to 78). Thyroid autopsy specimens and blood samples from deceased individuals were taken in Riga 1st Hospital Pathology Department. Blood samples were

in aliquots of volume 500 µl and stored for subsequent DNA extraction. Tissue samples were separated into 80 mg pieces and stored at –70 ° C.

## 2.2. Molecular methods

### 2.2.1. Isolation of DNA from peripheral whole blood and tissue samples

To the 0.5 ml of blood 1 ml of lyses buffer [100mM Tris HCl (pH 7.6); 5mM $MgCl_2$; 100mM NaCl] was added, mixed until complete blood mixe with lyses buffer. Sample was centrifuged to precipitate the cells (10 000 rpm/min., for 3 min.). Supernatant was removed and 1 ml of 3× of distillated $H_2O$ was added to the residue to wash the cells with following centrifugation to precipitate the cells (10 000 rpm/min, for 3 min.).

Thyroid tissue and blood cell lysis was performed by adding 80 µl of proteinase K buffer [100mM Tris HCl (pH 8.0); 50mM EDTA (pH 8.0)], 20 µl of 20% SDS (for splitting white blood cell membranes), 30 µl of proteinase K (10 mg / ml) and 240 µl of deionized water (total volume 500 µl) added, followed by incubation at 55 °C until complete residue dissolution.

DNA was extracted using phenol-chloroform extraction method. 0.5 ml of phenol was added to the sample followed by mixing for 10 minutes then samples were centrifuged (10 000 rpm/min., for 10 min.). Supernatant was transferred to the new eppendorf tube. Then phenol and chloroform (250µl + 250µl) was added to the each sample, followed by mixing for 10 minutes and centrifugation (10 000 rpm/min., for 10 min.). Supernatant was transferred to a new eppendorf tube and 500 µl of chloroform was added followed by mixing for 10 minutes and subsequent centrifugation (10 000 rpm/min., for 10 min.). Supernatant was carefully transferred into a new tube, and DNA precipitation was done by adding 1 ml of ice-cold 96% ethanol followed by centrifugation (14 000 rpm/min., for 15 min. at 4 °C). DNA washing was performed by adding 1ml ice-cold 70% ethanol followed by centrifugation (14 000 rpm/min., for 15 min. at 4 °C). DNA samples were dried on air and dissolved in 3× distillated $H_2O$ distillate water (depending on the DNA amount).

### 2.2.2. Isolation of DNA from plasma samples

The QIAamp Blood Kit (QIAGEN, Hilden, Germany) was used to purify DNA from 200 µl of cell-free blood plasma following manufacturer's protocol. Before DNA purification the plasma samples were treated with DNase I (Fermentas, Vilnius, Lithuania).

### 2.2.3. DNA quality and quantity control

According to the manufacturer's instructions 2µl of DNA solution was taken and concentration measured using spectrophotometer NanoDrop 1000 at a wavelength of 260, resulting in the concentration of each sample, which is expressed in the unit of measurement – ng/µl. The sample purity control was done by using the 260/280 ratio diapason, which must be no less than 1.8.

Beta (β)-globin PCR with appropriate primers was used (Vandame et al., 1995) to determine the quality of DNA isolated from blood and tissue. The negative β-globin PCR results in examination of plasma-derived DNA quality indicating that DNA sample is free of cell DNA impurities what is very important to make conclusions on the virus reactivation (viremia).

Table 2.1.

**PCR master mix**

| Reagents | Volume 25µl | Concentration |
|---|---|---|
| Taq PCR buf. +KCl-$MgCl_2$ | 2.5 | 10× |
| $MgCl_2$ | 2.0 | 25mM |
| dNTP | 0.5 | 10mM |
| praimer GS268 | 0.1 | 25nmol |
| praimer GS269 | 0.1 | 25nmol |
| Taq DNA polymerase (Tag) | 0.1 | 5U/µl |
| 3x $dH_2O$ | 17.7 | |

Oligonucleotide sequences to β-globin gene were following:
GS268: 5'-ACACAACTGTGTTCACTAGC-3'
GS269: 5'-TGGTCTCCTTAAACCTGTCTTG-3'
Reaction conditions:

To the prepared mixture of master mix 2 μl of dissolved DNA (containing 200 ng of DNA) was added and amplification started. Conditions for amplification cycles were following: 95°C for one minute, 55°C for 30 seconds, 72°C for 45 seconds, the final stage after 40 cycles – synthesis – lasting seven minutes at 72°C.

Expected PCR product length was – 200 bp [(after the marker C19 PU DNA / MspI (Hpa2) Marker23 (MBI Fermantes Lithuania)], the results were visualized and processed with BioSpectrum 610 MultiSpectral Imaging System, USA.

### 2.2.4. Nested Polymerase Chain Reaction (nPCR)

The technique of nPCR was used to detect viral genomic sequences in DNA isolated from whole blood and cell free plasma (markers of persistent and active phase of persistent infection, respectively). PCR amplification of viral DNA was carried out in the presence of 1 μg of whole blood DNA or thyroid gland tissue DNA, or 10 μl of plasma DNA (which corresponded to 200 μl of plasma). HHV-6 and HHV- 7 were detected in accordance with Secchiero et al., 1995 and Berneman et al., 1992, respectively. Positive controls (HHV-6 and HHV-7 genomic DNA; Advanced Biotechnologies Inc, Columbia, MD, USA and negative controls (DNA obtained from practically healthy HHV-6 and HHV-7 negative blood donor and no template DNA) were included in each experiment.

### 2.2.4.1. Detection of HHV-6 by nPCR

Amplication of specific HHV-6 genomic sequence was performed in two cycles.

Table 2.2.

**nPCR master mix**

| Reagents (I cycle) | Volume 50µl | Reagents (II cycle) | Volume 25µl | Concentration |
|---|---|---|---|---|
| Taq PCR buf. +KCl-$MgCl_2$ | 5.0 | Taq PCR buf. +KCl-$MgCl_2$ | 2.5 | 10× |
| $MgCl_2$ | 3.0 | $MgCl_2$ | 1.5 | 25mM |
| dNTP | 1.0 | dNTP | 0.5 | 10mM |
| Praimer HV61 | 0.2 | Praimer HV63 | 0.1 | 25nmol |
| Praimer HV62 | 0.2 | Praimer HV64 | 0.1 | 25nmol |
| Taq | 0.2 | Taq | 0.1 | 5U/µl |
| 3x $dH_2O$ | 30.4 | 3x $dH_2O$ | 15.2 | |

Oligonucleotide sequences to HHV-6 U3 gene were following:

HV61: 5'- GCGTTTTCAGTGTGTAGTTCGGCAG -3'

HV62: 5'- TGGCCGCATTCGTACAGATACGGAGG -3'

HV63: 5'- GCTAGAACGTATTTGCTGCAGAACG -3'

HV64; 5'- ATCCGAAACAACTGTCTGACTGGCA -3'

Reaction conditions:

To the prepared mixture of maste mix 10µl of dissolved DNA (containing 1000 ng of DNA) was added and amplification started. Conditions for both amplification cycles were following: 95 °C for one minute, 57 °C for 30 seconds, 72 °C for 45 seconds, the final stage after 30 cycles – synthesis – lasting seven minutes at 72 °C. 5 µl of amplicone from the I cycle was used in the II cycle of amplification.

Expected PCR product length was – 258 bp [(after the marker C19 PU DNA / MspI (Hpa2) Marker23 (MBI Fermantes Lithuania)], the results were visualized and processed with BioSpectrum 610 MultiSpectral Imaging System, USA.

### 2.2.4.2. Determination of HHV-6 A and HHV-6B by nPCR and Hind III restriction

Determination of HHV-6A and HHV-6B was made in correspondance to Lyall and Cubie, 1995. Expected PCR product length was: 163bp after the marker pUC 19 DNA / MspI (Hpa2) Marker23 (MBI Fermantes, Lithuania), the results were visualized and processed with BioSpectrum 610 MultiSpectral Imaging System, USA.

Table 2.3.

**nPCR Master mix**

| Reagents (I cycle) | Volume 50µl | Reagents (II cycle) | Volume 25µl | Concentration |
|---|---|---|---|---|
| Taq PCR buf. +KCl-$MgCl_2$ | 5.0 | Taq PCR buf. +KCl-$MgCl_2$ | 2,5 | 10× |
| $MgCl_2$ | 3.0 | $MgCl_2$ | 1.5 | 25mM |
| dNTP | 1.0 | dNTP | 0.5 | 10mM |
| Praimer O1 | 0.2 | Praimer I3 | 0.1 | 25nmol |
| Praimer O2 | 0.2 | Praimer I4 | 0.1 | 25nmol |
| Taq | 0.2 | Taq | 0.1 | 5U/µl |
| 3x $dH_2O$ | 30.4 | 3x $dH_2O$ | 15.2 | |

Oligonucleotides sequences to HHV-6 LTP gene were following:

O1: 5'- AGTCATCACGATCGGCGTGCTATC- 3'

O2: 5'-TATCTAGCGCAATCGCTATGTCG-3'

I3: 5'-TCGACTCTCACCCTACTGAACGAG- 3'

I4: 5'-TGACTAGAGAGCGACAAATTGGAG- 3'

Reaction conditions:

Amplification: 95 °C – 5 min; 30 cycles: (94 °C – 1 min; 60 °C – 1min; 72 °C – 1 min); 72 °C – 10 min.

After nPCR, amplicons were used in restriction analysis with Hind III endonuclease (MBI Fermantes, Lithuania), which digests HHV-6B positive sample into two fragments: 66 bp and 97 bp, but does not digest HHV-6A positive sample.

The results were visualized and processed with BioSpectrum 610 MultiSpectral Imaging System, USA.

### 2.2.4.3. Detection of HHV-7 by nPCR

HHV-7 detection was made in correspondance to Berneman et al., 1992, with primers complementary to U10 gene.

Table 2.4.

**nPCR Master mix**

| Reagents (I cycle) | Volume 50µl | Reagents (II cycle) | Volume 25µl | Concentration |
|---|---|---|---|---|
| Taq PCR buf. +KCl-$MgCl_2$ | 5.0 | Taq PCR buf. +KCl-$MgCl_2$ | 2.5 | 10× |
| $MgCl_2$ | 3.0 | $MgCl_2$ | 1.5 | 25mM |
| dNTP | 1.0 | dNTP | 0.5 | 10mM |
| Praimer HV7 | 0.2 | Praimer HV10 | 0.1 | 25nmol |
| Praimer HV8 | 0.2 | Praimer HV11 | 0.1 | 25nmol |
| Taq | 0.2 | Taq | 0.1 | 5U/µl |
| 3x $dH_2O$ | 30.4 | 3x $dH_2O$ | 15.2 | |

Oligonucleotide sequences to HHV-7 U10 gene were following:

HV7: 5'- TATCCCAGCTGTTTTCATATAGTAAC-3'

HV8: 5'- GCCTTGCGGTAGCACTAGATTTTTTG-3'

HV10: 5'- CAGAAATGATAGACAGATGTTGG-3'

HV11: 5'- AGATTTTTTGAAAAAGATTTAATAAC-3'

Reaction conditions:

To the prepared mixture of master mix 10 µl of dissolved DNA (containing 1000 ng of DNA) was added and amplification started. The conditions for 30 amplification cycles were following: 94 °C for one minute, 60 °C for 2 minutes, 72 °C for 2 minutes, the final stage - synthesis - lasting seven minutes at 72°C.

5 µl of amplification product from the I cycle was used in the II cycle of amplification with the second set of primers and cycle conditions were: 94 °C for one minute, 55 °C for 2 minutes, 72 °C for 2 minutes in the repitition for 30 cycles, the final stage – synthesis – lasting seven minutes at 72 °C.

Expected PCR product length was – 124 bp [(after the marker pUC19 DNA / MspI (Hpa2) Marker23 (MBI Fermantes Lithuania)], the results were visualized and processed with BioSpectrum 610 MultiSpectral Imaging System, USA.

### 2.2.5. Quantitative Real-time PCR

The viral load of HHV-6 in whole blood and tissue samples from patients with HHV-6 persistent infection was determined using the HHV-6 Real-Time Alert Q-PCR kit (Nanogen Advanced Diagnostics, Buttigliera Alta, Italy) and an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA), in accordance with the manufacturer's recommendations.

Collected data were processed and analyzed with specialized software ABI 7500 system. The data are calculated to get the viral copies on $10^6$ cells.

Table 2.5.

**Reaction conditions**

| Phase | Temperature | Time |
|---|---|---|
| Decontamination | 50 °C | 2 min |
| Starting denaturation | 95 °C | 10 min |
| 45 cycles | 95 °C | 15 sek |
| | 60 °C | 1 min |

## 2.3. Immunological methods

### 2.3.1. Viral specific antibody detection

For the approval of obtained PCR results, determination of HHV-6-specific IgG class antibodies in plasma was carried out using commercial enzyme-linked immunosorbent assay (ELISA) kit (Panbio, Sinnamon Park, QLD, Australia). The reaction results were calculated and analysis carried out in accordance with the manufacturer's protocol.

To determine the presence of HHV-7-specific IgG class antibodies in plasma samples indirect fluorescent antibody test (IFA) work set (EUROIMMUN, Germany) was used following the manufacturer's developed protocol. Preparations were analyzed immunofluorescence microscope "Nikon Eclipse 80i" at 400× magnification, using the visualization program "Lucia Image Analysis Systems" Lucia 5.0 version.

### 2.3.2. Auto-antibody detection

Commercial ELISA kits (EUROIMMUN, Germany) were used to determine the presence of auto-antibodies against thyroid peroxidase (TPO), against thyroglobulin (TG) and thyroid-stimulating hormone (TSH) receptor.

### 2.3.3. Assay for cytokine determination

ELISA kits (Pierce Biotechnology, Rockford, IL, USA, and AviBion, Helsinki, Finland) were used to detect the levels of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 in plasma. The sensitivity of the ELISAs were < 3 pg/ml for IL-1β and < 2 pg/ml for TNF-α and IL-6. In addition, the expression level of soluble IL-2 receptor (sIL-2R) was measured using a solid-phase competitive chemiluminescent enzyme immunoassay (CLIA; Siemens, Los Angeles, CA, USA) in accordance with the manufacturer's recommendations. The assay was able to detect 5 U/ml. All samples were tested in duplicate.

### 2.3.4. FACS analysis and sorting

BD FACSAria II flow cytometer (USA) and BD FACSDiva software were used for sorting and analysing of peripheral blood mononuclear cells (PBMC). Commercial monoclonal antibodies conjugated with different flourochromes (anti- CD3-FITC, CD4-PerCP-Cy7, CD8-PE, CD16-V450, CD19-APC, CD45-V500, CD56-V450 and CD95-PerCP-Cy 5.5) were used to distinguish and sort lymphocytes populations shown below.

All procedures were performed with standard BD protocols for staining and sorting PBMC's.

## 2.4. Statistical analysis

All calculations were performed with the MedCalc Software version 12.3 (Ostend, Belgium). Statistical differences in the prevalence of latent and active HHV-6

and HHV-7 infection were assessed by Fisher's exact test. Serum levels of cytokines were expressed as the mean ± SD. Immunological parameters were analyzed using t-test. Regression analysis was used to assess the continuous variable values; a value of $p < 0.05$ was considered to be significant, CI 95% deviation and odds ratio.

# 3. RESULTS

## 3.1. Involvement of HHV-6 and HHV-7 infection in the development of post-transplant complications

### 3.1.1. Cellular immune parameters in renal transplant recipients

Analysis of cellular immune parameters in renal transplant recipients after 3 month of immunosuppressive drug treatment in average showed decreased numbers of lymphocytes below 1400 cells in 1 $mm^3$ of peripheral blood, which indicates to lymphopenia (in accordance with Pauls Stradins clinical university hospital, Clinical immunological center laboratory references of year 2008). Also, percentage ratio of $CD4^+$ and $CD19^+$ subsets had tendency to decrease, however, $CD95^+$ was elevated (Table 3.1.).

Table 3.1.

**Absolute count of immunocompetent cells in renal transplant recipients (n = 47)**

| Parameters | (n = 47) | | |
|---|---|---|---|
| | Mean absolute count ± SD | Count (%) | Reference interval |
| Leu | 7490 ± 2500 | | |
| Ly | 1230 ± 650 | 17.04 | 18–42 % |
| $CD3^+$ | 940 ± 590 | 72.02 | 66–80 % |
| $CD4^+$ | 370 ± 300 | 34.72 | 35–50 % |
| $CD8^+$ | 350 ± 260 | 34.32 | 23–38 % |
| $CD38^+$ | 360 ± 280 | 29.74 | 20–35 % |
| $CD16^+$ | 110 ± 70 | 14.62 | 8–17 % |
| $CD19^+$ | 90 ± 60 | 8.60 | 9–18 % |
| $CD95^+$ | 560 ± 400 | 44.43 | 10–30 % |
| $CD25^+$ | 40 ± 40 | 3.53 | 2–5 % |
| $CD4^+/CD8^+$ | 1.20 ± 0.70 | | 1.3–2.3 |

### 3.1.2. Presence of HHV-6 and HHV-7 infection markers in renal transplant recipients

Persistent beta-herpesviruses infection was detected in 42 out of 47 (89%) renal transplant recipients. HHV-6 persistent infection was found in 15 out of 47 (32%)

recipients, HHV-7 persistent infection – in 40 out of 47 (85%) recipients, that is statistically higher (p = 0.02) than in healthy blood donors – 101 out of 150 (67%).

Persistent single HHV-6 infection was found in two out of 47 recipients, however, single HHV-7 infection was detected in 27 out of 47 recipients and persistent HHV-6 + HHV-7 infection – in 13 out of 47 individuals (Table 3.2.).

Latent single HHV-6 infection was detected in one out of two, HHV-7 latent infection in 16 out of 27 (59%) and HHV-6 + HHV-7 latent infection in 5 out of 13 (39%) renal transplant recipients. Activation rate was higher in patients with double HHV-6 + HHV-7 infection – 8 out of 13 (62%) than in recipients with single HHV-6 infection – one out of two or HHV-7 infection – 11 out of 27 (41%) (Table 3.2.). In all HHV-6 positive samples HHV-6B was defined.

Table 3.2.

**Presence of beta-herpesviruses infection in renal transplant recipients and healthy blood donors**

| | HHV-6 persistent (latent/active) infection | HHV-7 persistent (latent/active) infection | HHV-6 + HHV-7 persistent (latent/active) infection | Without HHV-6 or HHV-7 infection |
|---|---|---|---|---|
| Renal transplant recipients (n = 47) | 2 (4.3%) (1/1) | 27 (57%) (16/11) | 13 (27.6%) (5/8) | 5 (11%) |
| Blood donors (n = 150) | 13 (8.6%) (13/0) | 83 (55%) (71/12) | 30 (20%) (30/0) | 24 (16%) |

Presence of beta-herpesvirus infection was slightly higher in renal transplant recipients in comparison with healthy blood donors (89% and 84%, respectively). However, HHV-6 + HHV-7 infection was higher in renal transplant recipients (28%) than in healthy blood donors (20%), but statistical analysis showed no significance. There was no difference between renal transplant recipients and blood donors (57% and 55%, respectively) in the presence of HHV-7 persistent infection. However, presence of HHV-6 persistent infection was higher in blood donors than in renal transplant recipients (9% and 4%, respectively) (Figure 3.1.).

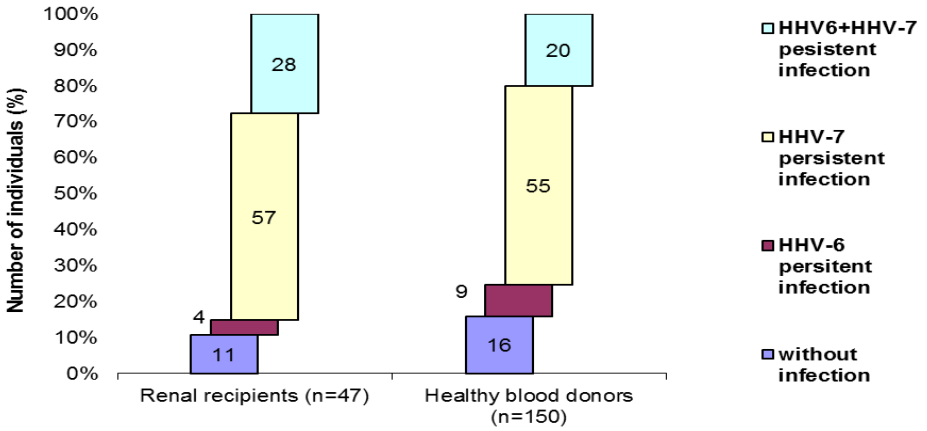



Figure 3.1. **Comparison of beta-herpesvirus infection in renal transplant recipients and healthy blood donors**

There was no significant difference in the presence of latent HHV-6 infection between renal recipients and healthy individuals. However, HHV-7 latent infection was significantly higher ($p = 0.006$) in healthy blood donors (85%) than in renal transplant recipients (59%). Also, presence of double HHV-6 + HHV-7 latent infection was significantly higher ($p < 0.0001$) in healthily individuals (100%) than in renal transplant recipients (59%).

HHV-6 and double HHV-6+HHV-7 active infection was found only in renal transplant recipients (one out of two and 8 out of 13, respectively). Rate of active HHV-7 infection was significantly higher ($p = 0.006$) in renal transplant recipients 11/27 (41%) than healthy blood donors 12/83 (14%) (Table 3.2.).

Although, HHV-6 genomic sequence was detected in 15/47 (32%) of renal transplant recipients, presence of anti- HHV-6 specific IgG class antibodies was detected in 36/47 (77%) of patients.

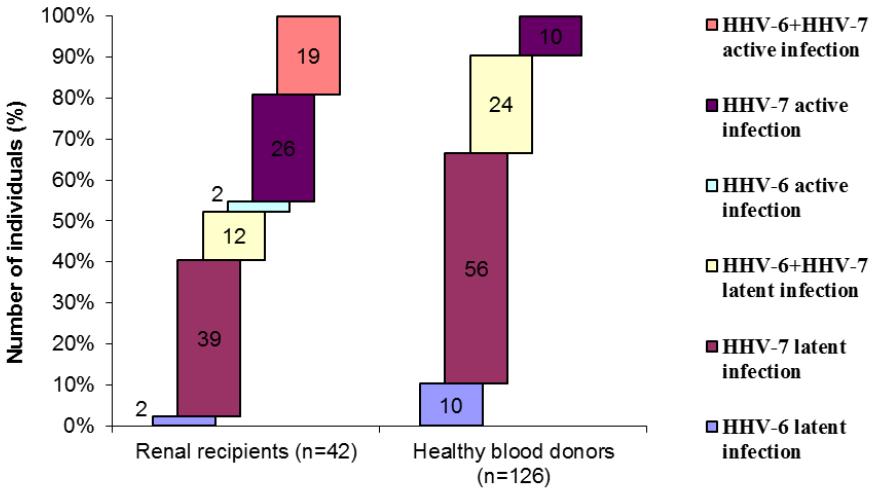



Figure 3.2. **Comparison of latent and active beta-herpesviruses infection presence in renal transplant recipients and healthy blood donors**

### 3.1.3. Analysis of immunocompetent cell populations in renal transplant recipients dependently on lymphocytes' count

It was possible to divide renal transplant recipients into two groups according to the lymphocyte count: group I (n = 17) – patients without lymphopenia (lymphocytes > 1400 cells in $1mm^3$ of peripheral blood) and group II (n = 30) – patients with lymphopenia (lymphocytes < 1400 cells in $1mm^3$ of peripheral blood), in accordance with Latvian oncological clinical laboratory references of year 2008.

Comparative analysis of immunocompetent cell populations in both renal transplant recipient groups showed significant differences in all rates. The mean absolute number of lymphocytes in the group I was two times higher than in the group II (1890 ± 440 $\times 10^6$/L and 850 ± 410 $\times 10^6$/L, respectively) (Table 3.3.). Comparative analyses of the lymphocyte subsets between the group I and II ($CD3^+$, $CD4^+$, $CD8^+$, $CD38^+$, $CD16^+$, $CD19^+$, $CD25^+$ and $CD95^+$) showed significant decrease of Ly subsets in the group II – approximately two fold decrease in comparison with the group I (Table 3.3.).

Table 3.3.

**Absolute counts of immunocompetent cells populations in the I and II renal transplant recipients' groups**

| Parameters | Group I Ly > 1400 (n = 17) | | Group II Ly < 1400 (n = 30) | | $p \leq 0.05$ |
|---|---|---|---|---|---|
| | Absolute count ± SD | Count % | Absolute count ± SD | Count % | |
| Leu | 8450 ± 2490 | | 6950 ± 2370 | | 0.04 |
| Ly | 1890 ± 440 | 24.92 | 850 ± 410 | 12.57 | < 0.0001 |
| $CD3^+$ | 1510 ± 390 | 80.12 | 620 ± 400 | 67.43 | < 0.0001 |
| $CD4^+$ | 630 ± 300 | 40.53 | 230 ± 190 | 31.43 | < 0.0001 |
| $CD8^+$ | 580 ± 210 | 38.35 | 220 ± 200 | 32.03 | < 0.0001 |
| $CD38^+$ | 520 ± 350 | 26.18 | 270 ± 170 | 31.77 | 0.002 |
| $CD16^+$ | 150 ± 70 | 10.00 | 90 ± 50 | 17.23 | 0.001 |
| $CD19^+$ | 130 ± 60 | 7.00 | 70 ± 50 | 9.50 | 0.0006 |
| $CD95^+$ | 850 ± 440 | 43.71 | 400 ± 270 | 44.83 | 0.0001 |
| $CD25^+$ | 80 ± 40 | 4.29 | 30 ± 30 | 3.10 | < 0.0001 |
| $CD4^+/CD8^+$ | 1.24 ± 0.70 | | 1.18 ± 0.70 | | |

### 3.1.4. Presence of HHV-6 and HHV-7 infection in renal transplant recipients with and without lymphopenia

HHV-6 persistent infection was found in 8 out of 17 (47%) group I patients and in 7 out of 30 (23%) patients of the group II. However, HHV-7 persistent infection was presented in 15 out of 17 (88%) group I patients and in 25 out of 30 (83%) patients of the group II. Presence of single HHV-6 persistent infection was detected only in 2 out of 30 (6.6%) patients with lymphopenia. Higher presence of single HHV-7 persistent infection was found in the group II of patients in comparison to the group I (66% and 41%, respectively). However, higher presence of double HHV-6 + HHV-7 persistent infection was detected in 8 out of 17 (47%) renal transplant recipients without lymphopenia comparing to the group II patients with lymphopenia (5/30, 17%).

Single HHV-6 latent infection was revealed only in one patient from the group II (with lymphopenia); however, frequency of single HHV-7 latent infection between both groups showed no significant difference (57% in the group I and 60% in the group II, respectively).

Double HHV-6 + HHV-7 latent infection was more prevalent in the group I patients (4/8) comparing with the group II patients (1/5) (Table 3.4.).

Presence of single HHV-6 active infection was found only in one renal transplant recipient with lymphopenia, however, activation rate of single HHV-7 active infection showed no significant difference between both groups (3/7 in the group I and 8/20 in the group II, respectively). Activation rate of double HHV-6 + HHV-7 active infection was more frequent in patients from the group II (4/5) than from the group I (4/8) (Figure 3.3.).

Table 3.4.

**Presence of beta-herpesviruses (HHV-6 and HHV-7) infection in the group I and II**

| Patients | HHV-6 persistent (latent/active) infection | HHV-7 persistent (latent/active) infection | HHV-6 + HHV-7 persistent (latent/active) infection | Without HHV-6 or HHV-7 infection |
|---|---|---|---|---|
| group I (n = 17) | 0 (0%) | 7 (41%) (4/3) | 8 (47%) (4/4) | 2 (12%) |
| group II (n = 30) | 2 (6.6%) (1/1) | 20 (66%) (12/8) | 5 (17%) (1/4) | 3 (10%) |

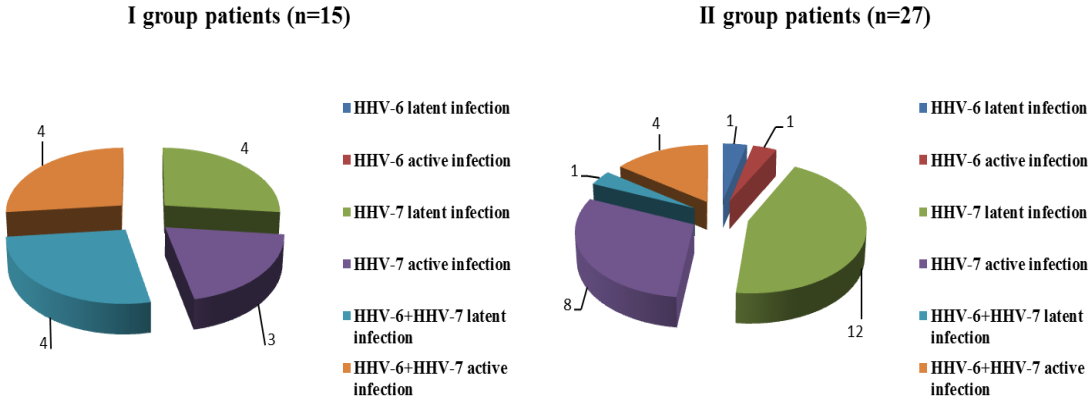



Figure 3.3. **Presence of latent and active beta-herpesviruses (HHV-6 and HHV-7) infection in the patients of group I and II**

### 3.1.5. Comparative analysis of immunocompetent cell populations in renal transplant recipients without and with lymphopenia dependently on beta-herpesviruses infection

Comparative analysis of lymphocytes (Ly) populations in the group I and II was performed dependently on beta-herpesviruses infection (Table 3.5.). Each group was subdivided into three subgroups: renal transplant recipients without infection, with latent and with active HHV-6 and/or HHV-7 infection. Both groups' patients with latent viral infection had tendency to increase in all cell populations (lymphocytes. $CD3^+$, $CD4^+$, $CD8^+$, $CD38^+$, $CD16^+$, $CD19^+$, $CD25^+$ and CD4/CD8 ratio) comparing with the subgroups without HHV-6 and HHV-7 infection and with active viral infection.

The most severe decrease of immunocompetent cells populations was detected in the group II patients with active beta-herpesviruses infection, especially in lymphocytes absolute count and $CD38^+$ cells (to 21% and to 56%, respectively), comparing to the group with latent viral infection (Table 3.5.). Although, the group II patients with active HHV-6 or HHV-7 had significantly higher decrease ($p = 0.001$) in $CD38^+$ subpopulation (to 56%) comparing to group with latent viral infection, the group I patients with active viral infection had significant ($p = 0.0001$) downfall in $CD8^+$ subpopulation (to 80%). Also, big shift in $CD95^+$ subpopulation was detected between both groups with latent and active viral infection. While in the group I patients $CD95^+$ population increases to 18% during latent infection, in the group II $CD95^+$ significantly ($p < 0.05$) decreases to 28%. Also, higher decrease of $CD95^+$ was detected in group II patients with active beta-herpesviruses infection (to 25%) than in the group I (to 9%).

Table 3.5.

**Comparative analysis of immunocompetent cells populations dependently on beta-herpesviruses infection in the group I and II**

| Parametrs | Without HHV-6 and/or HHV-7 | | p | Latent HHV-6 and/or HHV-7 | | p | Active HHV-6 and/or HHV-7 | | p |
|---|---|---|---|---|---|---|---|---|---|
| | Group I (n = 2) | Group II (n = 3) | | Group I (n = 8) | Group II (n = 14) | | Group I (n = 7) | Group II (n = 13) | |
| Leu | 8900 ± 850 | 6770 ± 2520 | 0.35 | 8090 ± 3100 | 7700 ± 2500 | 0.75 | 8740 ± 2210 | 6180 ± 2120 | 0.02 |
| Ly | 1750 ± 70 | 830 ± 380 | 0.045 | 2050 ± 520 | 940 ± 400 | 0.0001 | 1740 ± 360 | 750 ± 430 | 0.0043 |
| $CD3^+$ | 1270 ± 170 | 630 ± 370 | 0.11 | 1700 ± 410 | 660 ± 430 | 0.0001 | 1370 ± 350 | 570 ± 400 | 0.0003 |
| $CD4^+$ | 340 ± 220 | 250 ± 190 | 0.65 | 760 ± 320 | 210 ± 190 | 0.0001 | 560 ± 240 | 230 ± 210 | 0.005 |
| $CD8^+$ | 570 ± 40 | 200 ± 130 | 0.033 | 650 ± 230 | 240 ± 240 | 0.0009 | 130 ± 60 | 210 ± 170 | 0.24 |
| $CD38^+$ | 420 ± 200 | 390 ± 210 | 0.88 | 540 ± 410 | 430 ± 290 | 0.46 | 530 ± 340 | 190 ± 110 | 0.0035 |
| $CD16^+$ | 160 ± 50 | 100 ± 30 | 0.18 | 150 ± 90 | 110 ± 70 | 0.27 | 140 ± 50 | 80 ± 60 | 0.037 |
| $CD19^+$ | 140 ± 10 | 60 ± 20 | 0.015 | 130 ± 70 | 70 ± 60 | 0.046 | 130 ± 60 | 60 ± 50 | 0.012 |
| $CD95^+$ | 760 ± 40 | 600 ± 340 | 0.57 | 900 ± 610 | 430 ± 290 | 0.022 | 820 ± 250 | 320 ± 240 | 0.0004 |
| $CD25^+$ | 70 ± 30 | 20 ± 20 | 0.1 | 80 ± 30 | 30 ± 30 | 0.0012 | 70 ± 50 | 20 ± 20 | 0.0048 |
| $CD4^+/CD8^+$ | 0.60 ± 0.42 | 1.10 ± 0.26 | 0.18 | 1.36 ± 0.74 | 1.31 ± 0.94 | 0.89 | 1.27 ± 0.77 | 1.06 ± 0.43 | 0.44 |

### 3.1.6. Complications development in renal transplant recipients

Development of complications was found in 14 out of 47 (30%) renal transplant recipients. Five patients had acute rejection (AR), four patients were with microbial infections and others had melanoma, sepsis, CMV disease, chronic allograft nephropathy (CAN) and acute hepatitis of non-identified etiology.

Analysis of patients with complications and beta-herpesviruses infection revealed that 10 out of 14 (71%) renal transplant recipients with complication were with active HHV-6 or/and HHV-7 infection and only 4 out of 14 (29%) with latent infection.

The logistic regression analysis also showed that patients with active viral infection have higher risk of complication development (OR 4.5; 95% CI 1.12–18.13; $p = 0.03$) than the patients with latent viral infection (OR 0.22; 95% CI 0.06–0.89; $p = 0.03$).

## 3.2. Involvement of HHV-6 and HHV-7 infection in the clinical course of gastrointestinal cancer (GIC)

### 3.2.1. Cellular immune parameters in patients with GIC

Immunocompetent cell population analysis in 65 patients with gastrointestinal cancer showed slight decrease of $CD19^+$ lymphocytes subpopulation (8.29%), however, slight increase was identified in $CD16^+$ and $CD25^+$, according to the reference interval. Furthermore, higher increase in $CD95^+$ subpopulation was detected (Table 3.6.).

Table 3.6.

**Absolute count of immunocompetent cells populations in the patients with gastrointestinal cancer**

| Parameters | (n = 65) | | |
|---|---|---|---|
| | Mean absolute count ± SD | Count (%) | Reference interval |
| Leu | 6970 ± 2310 | | |
| Ly | 1770 ± 780 | 25.39 | 18–42 % |
| $CD3^+$ | 1240 ± 610 | 75.46 | 66–80 % |
| $CD4^+$ | 690 ± 340 | 39.15 | 35–50 % |
| $CD8^+$ | 520 ± 330 | 30.35 | 23–38 % |
| $CD38^+$ | 510 ± 280 | 28.74 | 20–35 % |
| $CD16^+$ | 350 ± 270 | 19.55 | 8–17 % |
| $CD19^+$ | 150 ± 100 | 8.29 | 9–18 % |
| $CD95^+$ | 860 ± 420 | 49.92 | 10–30 % |
| $CD25^+$ | 140 ± 130 | 8.14 | 2–5 % |
| $CD4^+/CD8^+$ | 1.43 ± 0.75 | | 1.3–2.3 |

### 3.2.2. Presence of HHV-6 and HHV-7 infection in patients with GIC

HHV-6 and HHV-7 persistent infection was detected in 44 out of 65 (68%) patients with GIC. In contrast, presence of HHV-6 and HHV-7 persistent infection was higher in healthy donors, where 126 out of 150 (84%) individuals had persistent viral infection ($p = 0.01$).

In total HHV-6 persistent infection was detected in 16 out of 65 (25%) patients and HHV-7 infection in 41 out of 65 (63%) patients with GIC, which coincides with frequency in healthy blood donors (43 out of 150; 29% and 101 out of 150; 67%, respectively).

Presence of single HHV-6 and HHV-7 infection was more frequent in healthy donors than in GIC patients (9%; 55% and 4.6%; 43%, respectively). However, patients with GIC and healthy donors had the same level of persistent double HHV-6 + HHV-7 infection frequency (Figure 3.4.).

Presence rate of single HHV-6 and HHV-7 latent infection was higher in healthy blood donors (13/13; 100% and 71/83; 85%, respectively) than in patients with GIC (2/3 and 21/28, respectively). Frequency of double HHV-6 + HHV-7 latent infection was also higher in healthy blood donors (30/30; 100% versus 7/13; 54% in patients with GIC).

However, single HHV-6 active infection was found only in patients with GIC (one of two). Activation rate of single HHV-7 infection was 15% in healthy blood donors, in contrast to 25% in patients with GIC. Presence of double active viral infection was found only in 6 out of 7 patients with GIC (Table 3.7.).

Table 3.7.

**Presence of beta-herpesviruses infection in patients with GIC**

| | HHV-6 persistent (latent/ active) infection | HHV-7 persistent (latent/active) infection | HHV-6 + HHV-7 persistent (latent/active) infection | Without HHV-6 or HHV-7 infection |
|---|---|---|---|---|
| Patients with gastrointestinal cancer (n = 65) | 3 (4.6%) (2/1) | 28 (43%) (21/7) | 13 (20%) (7/6) | 21 (32%) |
| Blood donors (n = 150) | 13 (8.6%) (13/0) | 83 (55%) (71/12) | 30 (20%) (30/0) | 24 (16%) |

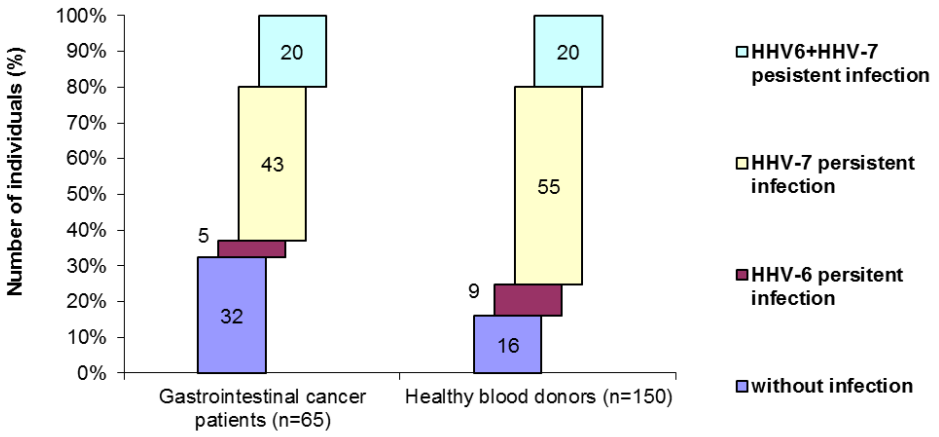



Figure 3.4. **Comparison of beta-herpesvirus infection presence in patients with GIC and healthy blood donors**

Comparison of beta-herpesviruses active and latent infection presence in two groups with persistent infection showed following results. Presence of a single HHV-6 and HHV-7 latent infection was higher in healthy blood donors (13/126, 11.4% and 71/126, 56.3%) than in patients with GIC (2/44, 4.5% and 21/44, 47.7%). Also,

presence of double HHV-6 + HHV-7 latent infection was more frequent in healthy blood donors (26% versus 15.9% in patients with GIC). However, reactivation of beta-herpesviruses (HHV-6 and HHV-7) was higher in patients with GIC than in donors (Figure 3.5.). Single HHV-6 active infection was found only in patients with GIC (1/44, 2.3%). Although, single HHV-7 active infection was found in 12 out of 126 (9.5%) of healthy blood donors, in patients with GIC it was found more frequently– in 7out of 44 (15.9%). Presence of double active viral infection was found only in patients with GIC (6/44, 13.6%).

Although, HHV-6 genomic sequence was detected in 16 out of 65 (25%) of patients of GIC, presence of anti- HHV-6 specific IgG class antibodies was detected in 33 out of 65 (51%) patients. HHV-7 genomic sequence was detected in 63% of patients by nPCR, however, presence of HHV-7 specific IgG class antibodies was detected in 75%.

In all HHV-6 positive GIC patients HHV-6B was detected.

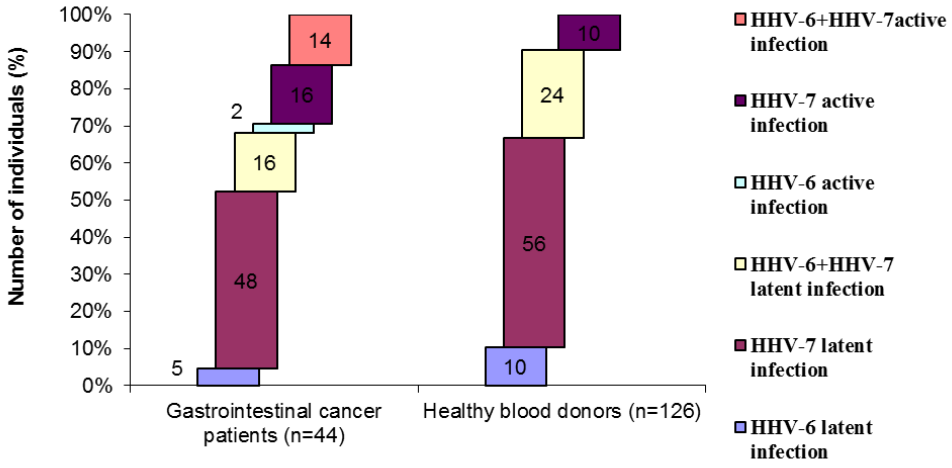



Figure 3.5. **Comparison of latent and active beta-herpesviruses infection in patients with GIC and healthy blood donors**

### 3.2.3. Analysis of immunocompetent cell populations in patients with GIC dependently on lymphocytes' count

It was possible to divide patients with GIC into two groups according to the lymphocytes count: group I – patients without lymphopenia (lymphocytes > 1400 cells

in 1mm$^3$ of peripheral blood) and group II with lymphopenia (lymphocytes < 1400 cells in 1mm$^3$ of peripheral blood), in accordance with Latvian oncological clinical laboratory references of year 2008.

Comparative analysis of immunocompetent cells' populations in both immunocompetent (lymphocytes > 1400) and immunocompromesed (lymphocytes < 1400) GIC patients groups showed significant differences in almost all rates. The mean number of leukocytes (Leu) in the group I (7910 ± 1960 $\times 10^6$/L) was significantly ($p = 0.002$) higher (27%) than in the group II (5830 ± 2210 $\times 10^6$/L). The mean absolute number of lymphocytes in the group I was two times (50%) higher than in the group II (2270 ± 700 $\times 10^6$/L; 1140 ± 210 $\times 10^6$/L, respectively; $p = 0.0001$). Comparative analyses of lymphocytes' subsets between the groups I and II ($CD3^+$, $CD4^+$, $CD8^+$, $CD38^+$, $CD16^+$, $CD19^+$, $CD25^+$ and $CD95^+$) showed significant decrease of immunological parameters in the group II - approximately in two times in comparison with the group I (Table 3.8.).

Table 3.8.

**Absolute counts of immunocompetent cells populations in the GIC patients' groups I and II**

| Parameters | Group I Ly > 1400 (n = 35) | | Group II Ly < 1400 (n = 30) | | p ≤ 0.05 |
|---|---|---|---|---|---|
| | Absolute count ± SD | Count % | Absolute count ± SD | Count % | |
| Leu | 7910 ± 1960 | | 5830 ± 2210 | | 0.002 |
| Ly | 2270 ± 700 | 28.83 | 1140 ± 210 | 19.88 | 0.0001 |
| $CD3^+$ | 1600 ± 580 | 70.77 | 780 ± 180 | 70.77 | 0.0001 |
| $CD4^+$ | 880 ± 330 | 39.29 | 450 ± 130 | 39.0 | 0.0001 |
| $CD8^+$ | 680 ± 370 | 29.11 | 330 ± 120 | 31.08 | 0.0001 |
| $CD38^+$ | 660 ± 300 | 29.03 | 320 ± 90 | 28.4 | 0.0001 |
| $CD16^+$ | 460 ± 310 | 19.69 | 220 ± 130 | 19.4 | 0.0015 |
| $CD19^+$ | 190 ± 110 | 8.03 | 110 ± 60 | 7.0 | 0.0018 |
| $CD95^+$ | 1130 ± 370 | 50.89 | 520 ± 130 | 48.8 | 0.0001 |
| $CD25^+$ | 180 ± 150 | 8.63 | 80 ± 50 | 7.5 | 0.047 |
| $CD4^+/CD8^+$ | 1.58 ± 0.85 | | 1.18 ± 0.57 | | |

### 3.2.4. Presence of HHV-6 and HHV-7 infection in GIC patients with and without lymphopenia

Frequency of single HHV-6 persistent infection was significantly ($p < 0.05$) higher in patients with lymphopenia (2/35; 6.6%) than in patients without it (1/30;

2.6%), however, presence of single HHV-7 persistent infection was higher in patients from the group I (20/35; 57%) than from the group II (8/30; 27%). Double persistent infection was more frequently detected in patients with (9/30; 30%) than in patients without (4/35; 11%) lymphopenia.

There was significant difference in the presence of single HHV-6 latent infection between two analysed groups, however, higher rate of single HHV-7 latent infection was found in the group I patients (17/20; 85%) comparing with the group II patients (4/8). Frequency of double latent infection was found more frequent in patients without lymphopenia (4/4) than in patients with lymphopenia (3/9) (Table 3.9.).

In the group of patients without lymphopenia significantly ($p = 0.002$) lower incidence of active beta-herpesviruses infection was detected (3/25, 12%) than in patients with lymphopenia (11/19, 58%). Active single HHV-6 infection was detected only in one out of two patients with GIC from the group II. Presence of single HHV-7 active infection was higher in (4/8) patients from the group II comparing with patients from the group I (3/20; 15%). Double HHV-6 + HHV-7 active infection was found only in patients with lymphopenia (6/9) (Figure 3.6.).

Table 3.9.

**Presence of latent and active beta-herpesviruses (HHV-6 and HHV-7) infection in GIC patients group I and II**

| Patients | HHV-6 persistent (latent/active) infection | HHV-7 persistent (latent/active) infection | HHV-6 + HHV-7 persistent (latent/active) infection | Without HHV-6 or HHV-7 infection |
|---|---|---|---|---|
| Group I (n = 35) | 1 (2.8%) (1/0) | 20 (57%) (17/3) | 4 (11%) (4/0) | 10 (29%) |
| Group II (n = 30) | 2 (6.6%) (1/1) | 8 (27%) (4/4) | 9 (30%) (3/6) | 11 (37%) |

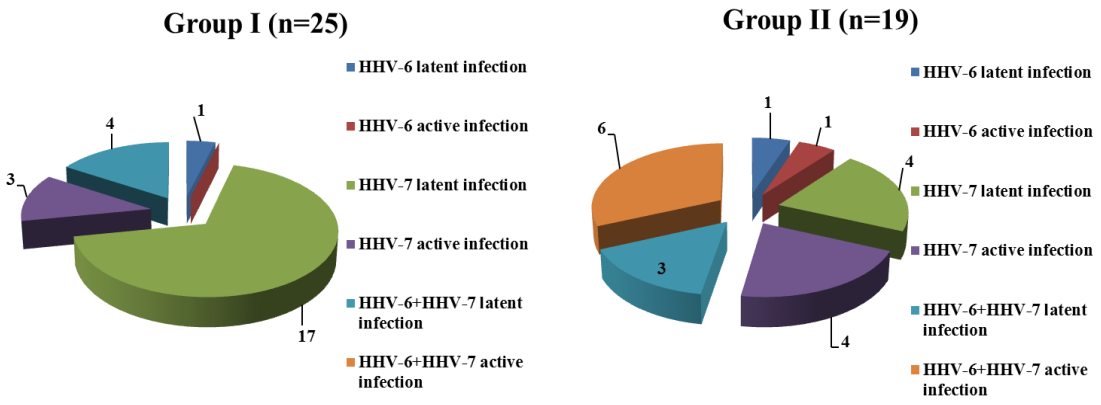



Figure 3.6. **Presence of latent and active beta-herpesviruses (HHV-6 and HHV-7) infection in GIC patients group I and II**

### 3.2.5. Comparative analysis of immunocompetent cell populations in GIC patients without and with lymphopenia dependently on beta-herpesviruses infection

Comparative analysis of immunocompetent cell populations in the group I and II was performed dependently on beta-herpeviruses infection. Each group was subdivided into three subgroups: GIC patients without, with latent and with active HHV-6 and/or HHV-7 infection.

The group I patients with active viral infection had tendency to increase all cellular immunological parameters (leukocytes, monocytes, lymphocytes and $CD3^+$, $CD4^+$, $CD8^+$, $CD38^+$, $CD16^+$, $CD19^+$, $CD25^+$, $CD95^+$) comparing with subgroups with and without latent HHV-6 and HHV-7 infection. The most dramatic decrease was found in $CD3^+$ and $CD8^+$ subpopulations (to 16% and to 21%, respectively) in the group II patients with active beta-herpesviruses infection. Tendency to increase was detected in both patient groups' B lymphocytes ($CD19^+$) during active viral infection. However, patients without lymphopenia had increase to 105% of $CD19^+$ subpopulation, but in patients with lymphopenia only to 18% (Table 3.10.).

The logistic regression analysis showed that patients with active viral infection have higher risk of lymphopenia (OR 3.33; 95% CI 0.72–15.51, p = 0.0035) than patients with latent viral infection (OR 0.33; 95% CI 0.10–1.07; p = 0.0035).

Table 3.10.

**Comparative analysis of immunocompetent cell subpopulations dependently on beta-herpesviruses infection in GIC patients without and with lymphopenia**

| Parametrs | Without HHV-6 and/or HHV-7 | | p | Latent HHV-6 and/or HHV-7 | | p | Active HHV-6 and/or HHV-7 | | p |
|---|---|---|---|---|---|---|---|---|---|
| | Group I (n = 10) | Group II (n = 11) | | Group I (n = 22) | Group II (n = 8) | | Group I (n = 3) | Group II (n = 11) | |
| Leu | 7670 ± 1610 | 5420 ± 2480 | 0.02 | 7720 ± 2050 | 5800 ± 2090 | 0.03 | 10030 ± 1420 | 6310 ± 2110 | 0.01 |
| Ly | 2260 ± 870 | 1130 ± 230 | 0.0005 | 2110 ± 490 | 1230 ± 60 | < 0.0001 | 3400 ± 420 | 1070 ± 250 | < 0.0001 |
| $CD3^+$ | 1580 ± 790 | 770 ± 140 | 0.003 | 1490 ± 360 | 870 ± 110 | 0.0001 | 2500 ± 270 | 730 ± 240 | < 0.0001 |
| $CD4^+$ | 890 ± 450 | 420 ± 120 | 0.003 | 850 ± 280 | 500 ± 50 | 0.002 | 1110 ± 240 | 440 ± 180 | 0.0002 |
| $CD8^+$ | 670 ± 520 | 340 ± 90 | 0.052 | 600 ± 180 | 370 ± 120 | 0.002 | 1190 ± 470 | 290 ± 130 | 0.0001 |
| $CD38^+$ | 680 ± 320 | 330 ± 140 | 0.004 | 630 ± 300 | 310 ± 40 | 0.006 | 780 ± 160 | 300 ± 50 | < 0.0001 |
| $CD16^+$ | 490 ± 330 | 250 ± 180 | 0.04 | 410 ± 320 | 210 ± 70 | 0.1 | 650 ± 190 | 210 ± 90 | 0.0001 |
| $CD19^+$ | 180 ± 90 | 80 ± 50 | 0.004 | 170 ± 70 | 110 ± 50 | 0.04 | 350 ± 280 | 130 ± 80 | 0.03 |
| $CD95^+$ | 1170 ± 460 | 500 ± 100 | 0.0001 | 1040 ± 230 | 570 ± 160 | 0.0002 | 1620 ± 230 | 510 ± 150 | < 0.0001 |
| $CD25^+$ | 170 ± 100 | 110 ± 60 | 0.11 | 180 ± 180 | 50 ± 30 | 0.05 | 220 ± 90 | 80 ± 40 | 0.001 |
| $CD4^+/CD8^+$ | 1.68 ± 0.85 | 1.24 ± 0.49 | 0.16 | 1.55 ± 0.9 | 1.31 ± 0.49 | 0.5 | 1.37 ± 0.56 | 1.29 ± 0.65 | 0.8 |

### 3.2.6. Pro-inflammatory cytokines levels in GIC patients with and without lymphopenia

There were no significant changes in the serum levels of IL-6, IL-1β, sIL-2R and TNF-alpha between GIC patients of the group I and II. However, in the group I and II of patients with active viral infection the levels of IL-6 and sIL-2R had tendency to increase, nonetheless, level of TNF-alpha in the group II patients with active viral infection was lower than in patients with latent and without viral infection, and also lower in comparison with the group I patients with active viral infection (Table 3.11.).

Table 3.11.

**Levels of IL-1beta, IL-6, sIL2R and TNF-alpha in the group I and II patients with GIC**

| Patients groups | IL-1beta (pg/ml) N = < 5.0 | IL-6 (U/ml) N = 3.4 | sIL2R (U/ml) N = 223-710 | TNF-alpha (pg/ml) N = 8.1 |
|---|---|---|---|---|
| Group I (n = 10) Without HHV-6 and/or HHV-7 | < 5.0 | 4.08 ± 1.23 | 527 ± 159.58 | 15.13 ± 5.15 |
| Group II (n = 11) Without HHV-6 and/or HHV-7 | < 5.0 | 5.15 ± 2.76 | 559.80 ± 379.10 | 15.58 ± 5.66 |
| Group I (n = 22) HHV-6 and/or HHV-7 latent | < 5.0 | 5.44 ± 2.99 | 604.78 ± 242.33 | 11.62 ± 1.08 |
| Group II (n = 8) HHV-6 and/or HHV-7 latent | < 5.0 | 5.55 ± 1.91 | 617.00 ± 173.20 | 13.83 ± 3.06 |
| Group I (n = 3) HHV-6 and/or HHV-7 active infection | < 5.0 | 7.20 ± 11.88 | 851 ± 528.55 | 14.40 ± 5.31 |
| Group II (n = 11) HHV-6 and/or HHV-7 active infection | < 5.0 | 6.18 ± 2.91 | 733.67 ± 388.76 | 9.78 ± 4.23 |

### 3.2.7. Gastrointestinal cancer clinical outcomes dependently on beta-herpesviruses infection

Stages of gastrointestinal cancer were defined in accordance to TNM evaluation (Evaluation of the (primary) tumor 'T'; evaluation of the regional lymph nodes 'N'; evaluation of distant metastasis 'M'). First stage was defined only in 9% of patients. The majority of patients (43%) were with second stage of cancer. Third stage was defined in 30% patient and the most severe – fourth stage was defined in 18% patients with GIC.

There was no association between beta-herpesviruses infection and stages of cancer; however, fourth stage of cancer was more prevalent in patients with HHV-6 and/or HHV-7 persistent infection (23%) than in patients without it (6%) (Table 3.12.).

Table 3.12.

**Stages of GIC frequency dependently on beta-herpesviruses infection**

| Stage of cancer | Without HHV-6 and/or HHV-7 (n=21) | Persistent HHV-6 and/or HHV-7 infection (n=44) |
|---|---|---|
| I stage | 4 (19%) | 3 (7%) |
| II stage | 9 (44%) | 19 (43%) |
| III stage | 7 (31%) | 12 (27%) |
| IV stage | 1 (6%) | 10 (23%) |

| Stage of cancer | Latent HHV-6 and/or HHV-7 infection (n=30) | Active HHV-6 and/or HHV-7 infection (n=14) |
|---|---|---|
| I stage | 1 (4%) | 2 (15%) |
| II stage | 13 (44%) | 6 (39%) |
| III stage | 8 (26%) | 4 (31%) |
| IV stage | 8 (26%) | 2 (15%) |

Also, no association between lymphopenia and stages of cancer was found (Figure 3.7.). However, mortality rate was higher in patients with lymphopenia (53%) than in patients without it (34%), but there was no statistical significance.

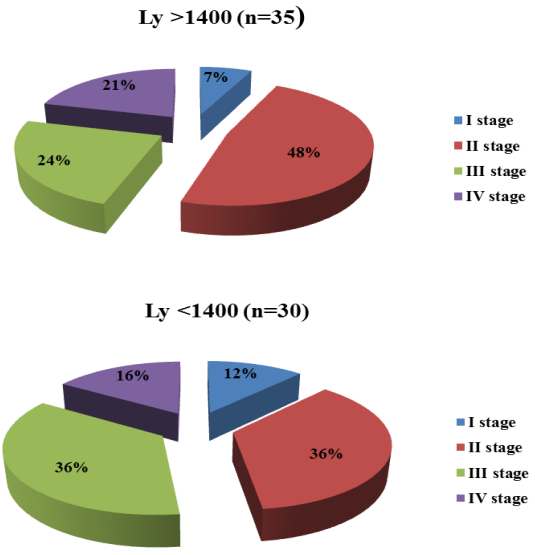



Figure 3.7. **Stages of GIC in patients without and with lymphopenia**

### 3.3. Involvement of HHV-6 and HHV-7 infection in the development of autoimmune thyroiditis (AIT)

To ensure autoimmune thyroid disorders in 44 patients, they were examined for auto-antibodies against thyroid peroxidase (TPO), against thyroglobulin (TG) and thyroid stimulating hormone (TSH) receptor by ELISA. In 15 patients elevated titers of auto-antibodies against the TSH receptor, in four patients against TG, auto-antibodies against TPO in seven patients were detected, two patients had elevated plasma levels of all three auto-antibodies. Eight patients were positive for auto-antibodies against both TSH receptor and TPO, and seven patients for TG and TPO (Figure 3.8.).

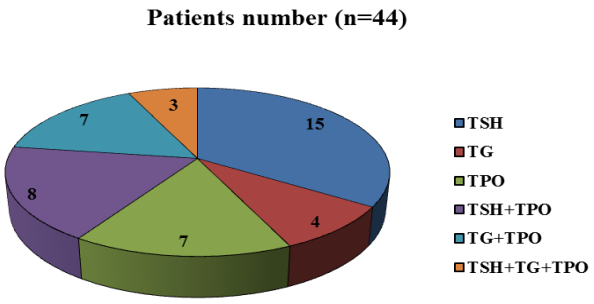



Figure 3.8. **Presence of auto-antibodies in AIT patients**

After surgery the histological examination of thyroid gland tissue was made. Histological conclusions showed that in 11/44 (25%) of tissue samples follicular adenoma was found. In majority of samples (30/44, 68%) micro- macrofollicular colloid struma was diagnosed and in 3/44 (7%) samples carcinoma was found (Figure 3.9.).

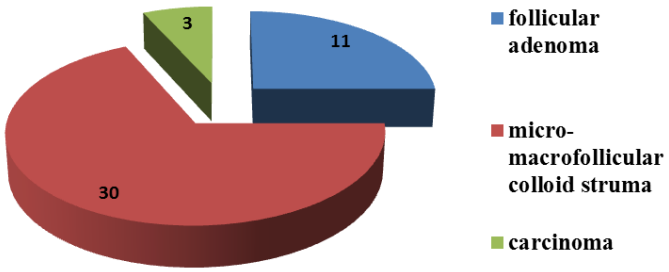



Figure 3.9. **Frequency of macro and micro changes in thyroid gland tissues of AIT patients**

### 3.3.1. Cellular immune parameters in patients with AIT

Analysis of cellular immune parameters in 44 patients with AIT showed slight decrease of $CD8^+$ and $CD19^+$ lymphocyte subsets, however, mean count of natural killers ($CD16^+$) was found elevated. Also, big increase in $CD95^+$ population was detected, according to reference interval (Table 3.13.).

Table 3.13.

**Absolute count of immunocompetent cells populations in patients with AIT**

| Parameters | (n = 44) | | |
|---|---|---|---|
| | Mean absolute count ± SD | Count (%) | Reference interval |
| Leu | 6138 ± 1419 | | |
| Ly | 2031 ± 579 | 33.8 | 18–42 % |
| $CD3^+$ | 1415 ± 403 | 69.7 | 66–80 % |
| $CD4^+$ | 536 ± 153 | 37.9 | 35–50 % |
| $CD8^+$ | 305 ± 87 | 21.6 | 23–38 % |
| $CD16^+$ | 406 ± 115 | 20.00 | 8–17 % |
| $CD19^+$ | 176 ± 50 | 8.7 | 9–18 % |
| $CD95^+$ | 1251 ± 355 | 61.6 | 10–30 % |
| $CD4^+/CD8^+$ | 2.6 ± 0.90 | | 1.3–2.3 |

### 3.3.2. Presence of HHV-6 and HHV-7 infection markers in patients with AIT

Presence of beta-herpesviruses infection markers in blood samples were found more frequently in patients with AIT than in healthy blood donors (93% and 84%, respectively). HHV-6 persistent infection was detected in 12 out of 44 (27%) patients with AIT. The same infection frequency was detected in healthy blood donors (43/150, 29%). HHV-7 persistent infection was revealed in 40 out of 44 (91%) patients with AIDT. Presence of HHV-7 was found to be significantly lower ($p = 0.03$) in healthy individuals (only 75%).

Single HHV-6 and HHV-7 persistent infection was more frequently observed in blood donors (8.6% and 55%, respectively) comparing to AIT patients (2.3% and 36%, respectively). Frequency of double HHV-6 + HHV-7 persistent infection was much higher in patients with AIT than in blood donors (55% against 20%, respectively) (Table 3.14., Figure 3.10.).

Table 3.14.

**Presence of HHV-6 and HHV-7 infection in patients with AIT**

| | HHV-6 persistent (latent/active) infection | HHV-7 persistent (latent/active) infection | HHV-6 + HHV-7 persistent (latent/active) infection | Without HHV-6 or HHV-7 infection |
|---|---|---|---|---|
| Patients with AIT (n = 44) | 1 (2.3%) (1/0) | 16 (36%) (10/6) | 24 (55%) (11/13) | 3 (7%) |
| Blood donors (n = 150) | 13 (8.6%) (13/0) | 83 (55%) (71/12) | 30 (20%) (30/0) | 24 (16%) |

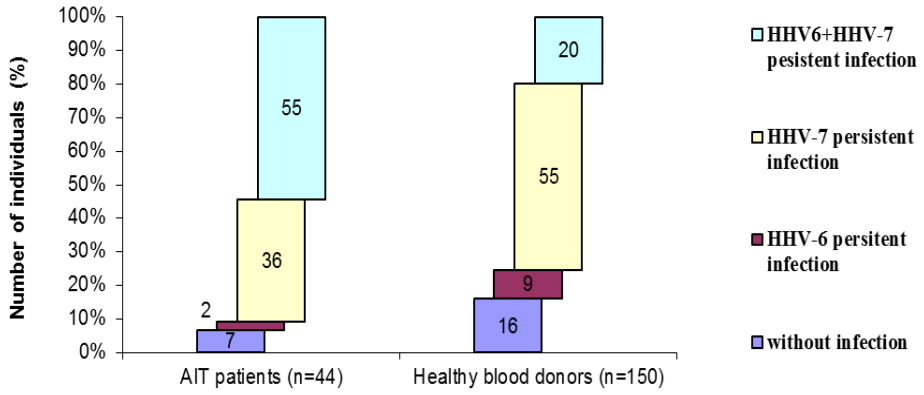



Figure 3.10. **Presence of HHV-6 and HHV-7 persistent infection in patients with AIT and healthy blood donors**

Presence of single HHV-6 latent infection was detected only in one patient with AIT and in 13 healthy blood donors. Single HHV-7 latent infection was more frequently detected in healthy blood donors than in patients with AIT (56% and 23%, respectively) (Figure 3.11). However, activation rate of single HHV-7 infection was significantly higher ($p<0.0001$) in patients with AIT comparing with blood donors (38% and 15%, respectively) (Table 3.14.). There was no significant difference in frequency of double HHV-6 + HHV-7 latent infection between patients and donors (25% and 23%, respectively). However, double HHV-6+HHV-7 active infection was found only in patients with AIT (13/44, 30%).

Single HHV-6 active infection was not found neither in patients with AIT and in healthy blood donors (Figure 3.11.).

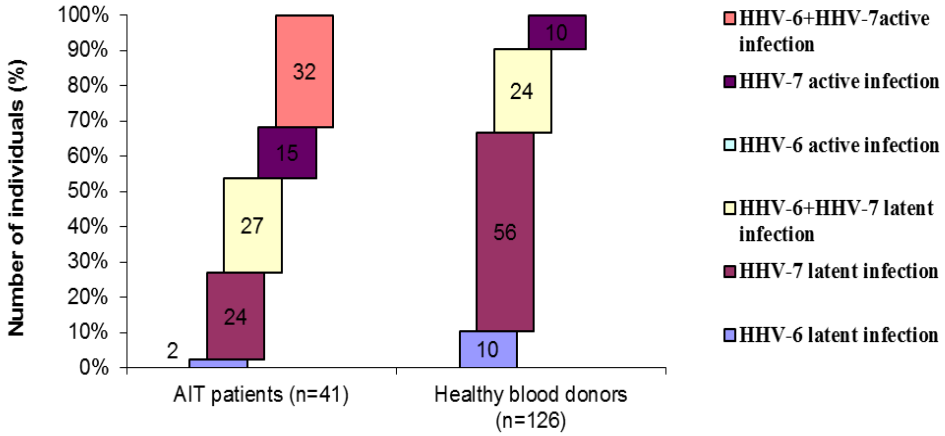



Figure 3.11. **Presence of HHV-6 and HHV-7 active infection in patients with AIT and blood donors**

### 3.3.3. Analysis of immunocompetent cell populations dependently on viral infection in patients with AIT

Comparative analysis of immunocompetent cell populations in the patients with AIT was performed dependently on beta-herpesviruses infection. Patients were subdivided into three subgroups: AIT patients without, with latent and with active HHV-6 and/or HHV-7 infection.

As in previous groups, patients with latent HHV-6 and/or HHV-7 infection had increase almost in all immunological parameters (Ly, $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$, $CD19^+$ and $CD95^+$) comparing to patients without viral infection and patients with active viral infection. In patients with active HHV-6 and/or HHV-7 infection decrease in all immunocompetent cell populations was observed in comparison with the patients with latent infection however statistical analysis showed no significance (Table 3.15.).

Table 3.15.

**Analysis of cellular immune parameters in AIT patients dependently on beta-herpesvirus infection**

| Parameters | Without HHV-6 and/or HHV-7 (n = 3) | Latent HHV-6 and/or HHV-7 (n = 21) | Active HHV-6 and/or HHV-7 (n = 20) |
|---|---|---|---|
| Leu | 5833 ± 1401 | 6073 ± 1297 | 6252 ± 1597 |
| Ly | 1883 ± 188 | 2087 ± 732 | 1994 ± 427 |
| $CD3^+$ | 1312 ± 131 | 1454 ± 510 | 1390 ± 297 |
| $CD4^+$ | 497 ± 49 | 551 ± 193 | 527 ± 112 |
| $CD8^+$ | 283 ± 28 | 314 ± 110 | 300 ± 64 |
| $CD16^+$ | 376 ± 37 | 417 ± 146 | 399 ± 86 |
| $CD19^+$ | 163 ± 16 | 181 ± 63 | 173 ± 37 |
| $CD95^+$ | 1160 ± 115 | 1285 ± 450 | 1228 ± 263 |
| $CD4^+/CD8^+$ | 0.9 ± 0.3 | 1.33 ± 0.85 | 1.14 ± 0.56 |

### 3.3.4. Presence of HHV-6 and HHV-7 infection in thyroid gland tissue

In this study thyroid tissue DNA taken from patients with autoimmune thyroiditis and from control group autopsy materials (thyroid tissue without macro or micro histological changes) were enrolled.

Beta-herpesviruses HHV-6 and HHV-7 persistent infection was detected in 43 out of 44 (98%) of AIT patients' thyroid tissue DNA samples and in 31 out of 41 (76%) control group autopsies' DNA samples ($p = 0.003$). In 42 (95%) of the 44 patients with AIT in tissue DNA samples HHV-6 genomic sequence was found, whereas in the control group HHV-6 genome sequence was found in 25 out of 41 (61%) autopsy tissue DNA samples ($p < 0.0001$).

HHV-7-specific genomic sequence was presented in 33 (75%) out of 44 patients with AIT and in 16 (39%) out of 41 thyroid tissue DNA samples extracted from control group autopsies ($p = 0.001$). There was no significant difference in the presence of single HHV-6 and HHV-7 genomic sequences in patients' and control group thyroid tissue DNA, however, simultaneous presence of HHV-6 and HHV-7 genomic sequences in tissue DNA samples was significantly higher ($p < 0.0001$) in patients with AIT (Table 3.16.).

Presence of HHV-6 genomic sequence in thyroid tissue DNA samples was significantly more prevalent ($p = 0.01$) than the presence of HHV-7 genomic sequence (95% and 75%, respectively).

Table 3.16.

**Presence of beta-herpesviruses (HHV-6 and HHV-7) infection in AIT patients' thyroid tissue DNA samples and autopsy materials**

| | HHV6 | HHV-7 | HHV-6 + HHV-7 | Without |
|---|---|---|---|---|
| AIDT (n = 44) | 10 (23%) | 1 (2.3%) | 32 (73%) | 1 (2.3%) |
| Control group (n = 41) | 15 (36%) | 6 (15%) | 10 (24%) | 10 (24%) |

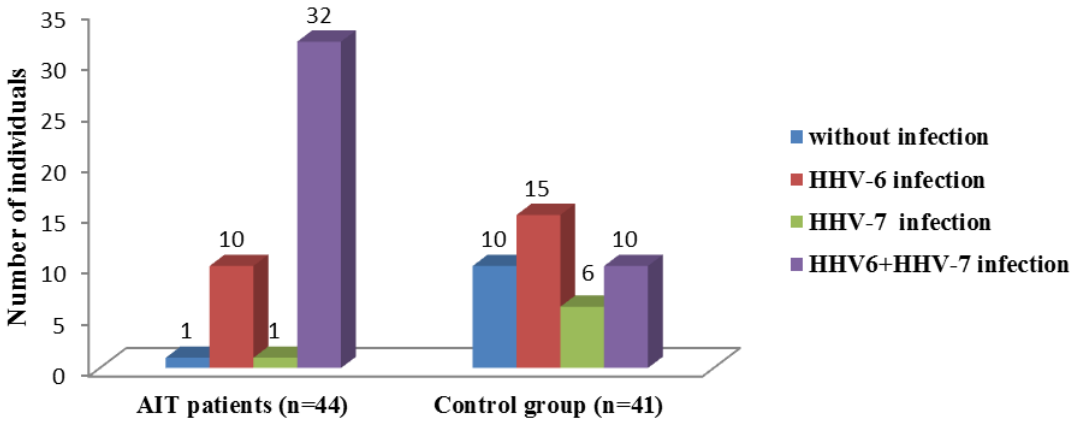



Figure 3.12. **Presence of beta-herpesviruses infection genomic sequences in tissue DNA samples of patients with AIT and control group autopsies**

### 3.3.5. Comparison of HHV-6 infection presence in thyroid gland tissue and blood samples

In 43 (98%) of the 44 patients with AIT HHV-6 genomic sequence in blood and/or thyroid tissue DNA samples was found: in 17 (39%) patients, HHV-6 genomic sequence was detected in both tissue and blood DNA samples, while in 25 (58%) patients viral genomic sequence was detected only in tissue DNA samples. In contrast, HHV-6 genome sequence was found in 25 (61%) DNA samples isolated from control groups' autopsy materials: in 14 (34%) cases in both blood and tissue DNA samples,

and in 11 (27%) cases only in tissue DNA samples (Table 3.17.). Presence of HHV-6 genomic sequence in thyroid gland tissue only was significantly higher in patients with AIT comparing to the control group ($p = 0.008$).

Table 3.17.

**Presence of HHV-6 sequence in peripheral blood and thyroid tissue DNA of patients with AIT and control group autopsy material**

| Distribution of HHV-6 genomic sequence | Number of patients with AIT (n = 44) | Number of control group samples (n = 41) |
|---|---|---|
| Only blood DNA | 1 | 0 |
| Blood DNA + tissue DNA | 17 | 14 |
| Only tissue DNA | 25 | 11 |
| Blood DNA + plasma DNA | 0 | 0 |
| Tissue DNA + blood DNA + plasma DNA | 0 | 0 |
| Without | 1 | 16 |

### 3.3.6. FACS sorting of lymphocytes subpopulations

To explain differences in the presence of HHV-6 genomic sequence in thyroid tissue and peripheral blood DNA samples it was decided to sort main lymphocyte subpopulations.

For the following DNA extraction and real-time polymerase chain reaction, the main lymphocyte subpopulations were sorted ($CD4^+$, $CD8^+$, $CD16^+$, $CD19^+$ and $CD95^+$) from the peripheral blood mononuclear cells (PBMC) of 7 patients with AIT (Figure 3.13.).

In average $8\times10^6$ peripheral blood mononuclear cells were taken for the FACS sorting. An average efficiency of cell sorting was 80%.

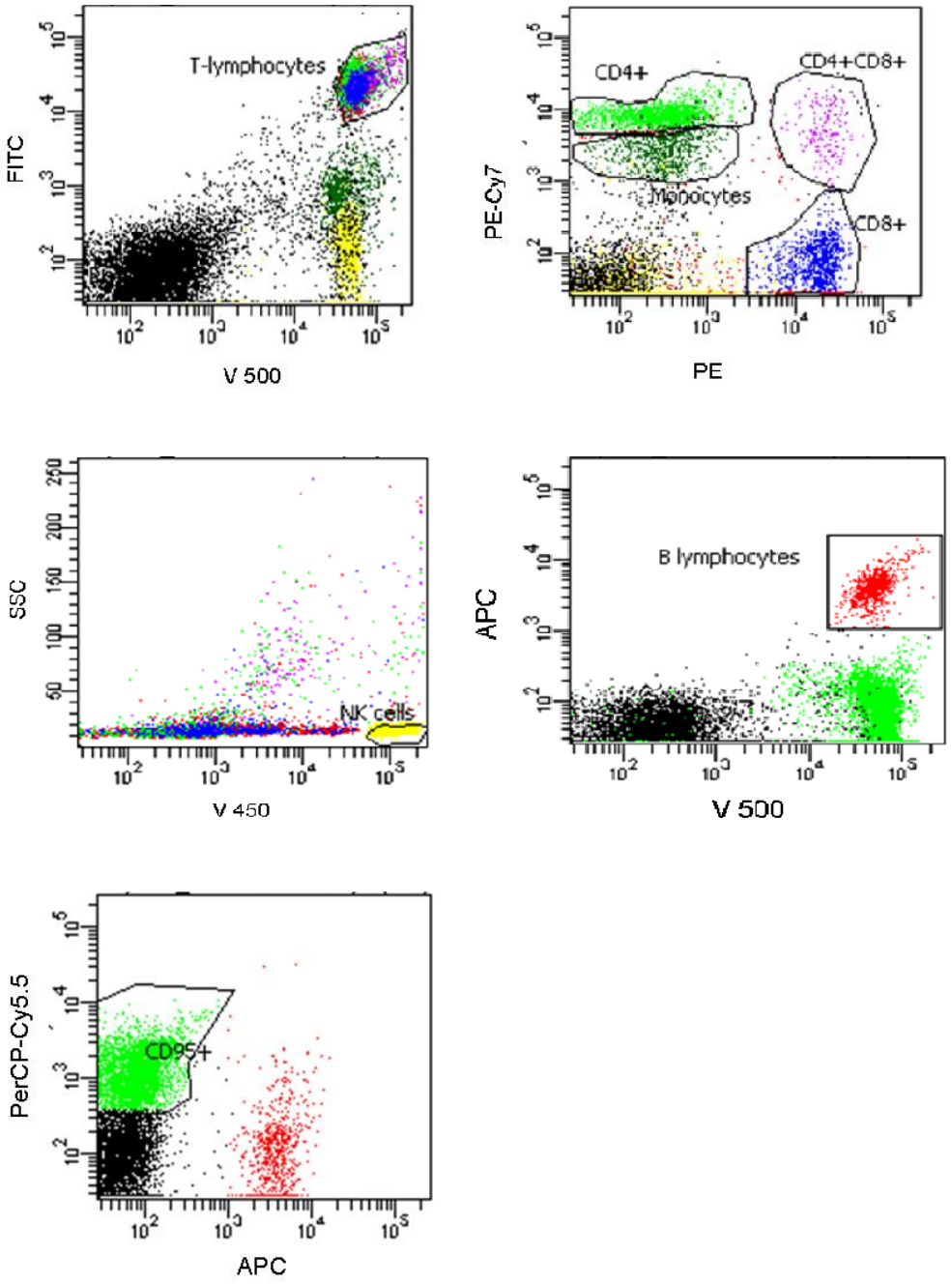
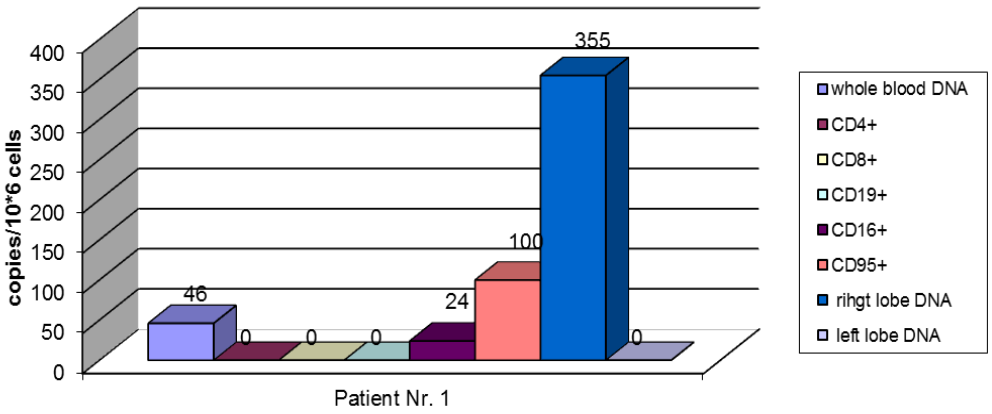



Figure 3.13. **Cell gating of main lymphocyte subpopulations for the FACS sorting**

### 3.3.7. HHV-6 load in blood and tissue DNA

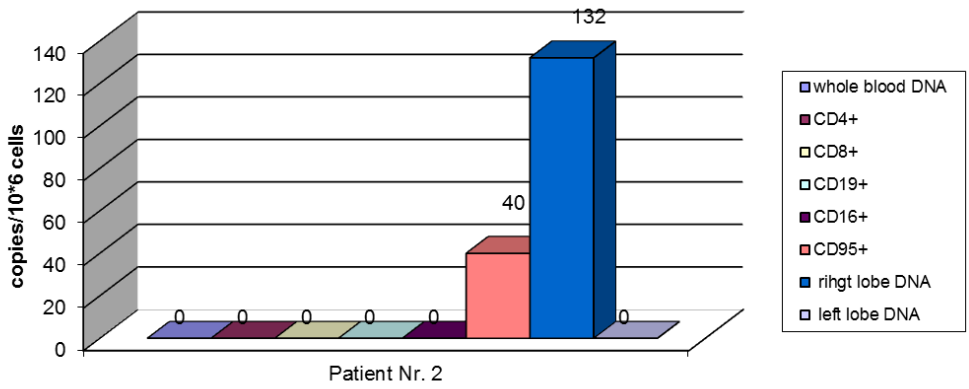
Using real-time polymerase chain reaction HHV-6 load was determined in DNA samples from patients' whole blood, tissue and main lymphocyte subpopulations ($CD4^+$, $CD8^+$, $CD16^+$, $CD19^+$ and $CD95^+$) DNA samples. In patients' blood samples the average viral load was 98 ± 44 copies/1 × $10^6$cells while in the tissue samples viral load in average was 2552 ± 2015 copies/1 × $10^6$cells.

To ensure that detected HHV-6 sequence in DNA samples is due to the involvement of thyroid gland tissue but not due to lymphocyte infiltration, the main lymphocyte subpopulations DNA was also examined by real-time polymerase chain reaction. Substantial HHV-6 viral load in lymphocyte subpopulation was detected in two out of seven patients. In first patient, who was positive for the presence of HHV-6 genomic sequence by nPCR in whole blood and tissue DNA, HHV-6 load was detected in NK and $CD95^+$ cells (24 and 100 copies/1 × $10^6$cells, respectively). In this patient also lower HHV-6 load was detected in whole blood DNA (46 copies/1×$10^6$cells) comparing to tissue DNA, where HHV-6 load was 355 copies/1×$10^6$cells (Figure 3.14.). In second patient, who was positive for the presence of HHV-6 genomic sequence by nPCR only in tissue DNA, HHV-6 load was detected only in $CD95^+$ cells. HHV-6 load in $CD95^+$ cells was 40 copies/1x$10^6$cells (Figure 3.15.). In other five patients' lymphocyte subpopulations ($CD8^+$, $CD16^{+,}$ and $CD95^+$) DNA insignificant (according to the manufacturer's protocol) HHV-6 load was detected (range 14–20 copies/1 × $10^6$cells).




| | Whole blood DNA | Plasma | Thyroid gland right lobe | Thyroid gland left lobe |
|---|---|---|---|---|
| HHV-6 viral sequence by nested PCR | + | – | + | – |

Figure 3.14. **HHV-6 load in DNA samples of whole blood, tissue and main lymphocyte subpopulations DNA samples in Patient Nr. 1; nested PCR results**




| | Whole blood DNA | Plasma | Thyroid gland right lobe | Thyroid gland left lobe |
|---|---|---|---|---|
| HHV-6 viral sequence by nested PCR | – | – | + | – |

Figure 3.15. **HHV-6 loads in DNA samples of whole blood, tissue and main lymphocyte subpopulations DNA samples in Patient Nr. 2; nested PCR results**

## 3.4. Comparative analysis between patients with different immune system dysfunctions

### 3.4.1. Comparison of beta-herpesviruses infection frequency in all patient groups and healthy blood donors

The highest frequency of HHV-6 and HHV-7 persistent infection was detected in patients with AIT (93%) in comparison with other two groups. The significantly lowest rate of beta-herpesviruses presence (68%) was detected in patients with GIC ($p < 0.05$) comparing to the renal transplant recipients and patients with AIT (Figure 3.16.).

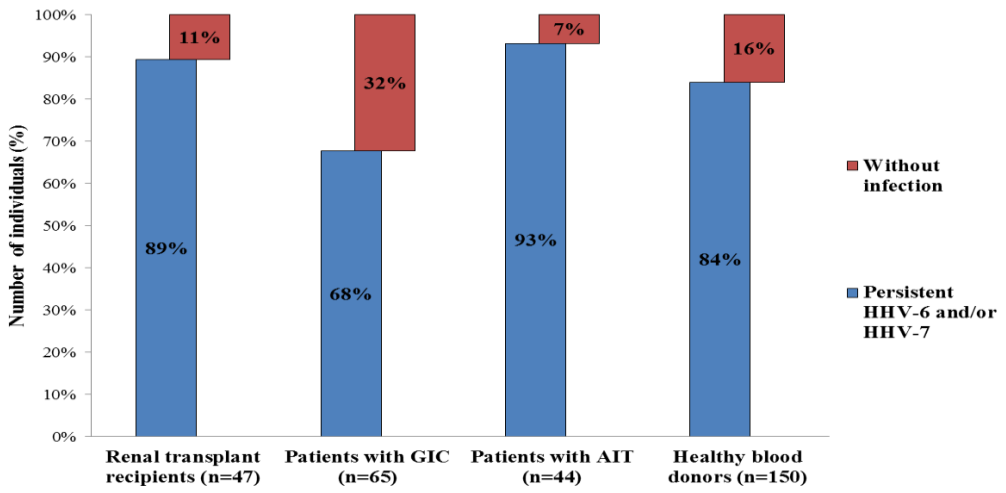



Figure 3.16. **Presence of beta-herpesviruses persistent infection in three groups of patients**

There was no significant difference in frequency of single HHV-6 latent infection between the groups, however, in patients with GIC higher frequency of single HHV-7 latent infection was detected in comparison with AIT patients ($p = 0.04$) and with renal transplant recipients (without statistical significance). Double (HHV-6 + HHV-7) latent infection was found higher in patients with AIT (27%) than in renal transplant recipients (12%) and in patients with GIC (16%), however, statistical analysis showed no significance.

Single HHV6 active infection was found only in renal transplant recipients and in patients with GIC without significant difference in frequency. Higher frequency of single HHV-7 active infection was found in renal transplant recipients (26%) than in patients with GIC (16%) and patients with AIT (15%). Presence of double HHV-6 + HHV-7 active infection was more frequently detected in patients with AIT (32%) comparing to renal transplant recipients (19%) and patients with GIC (14%) (Figure 3.17.).

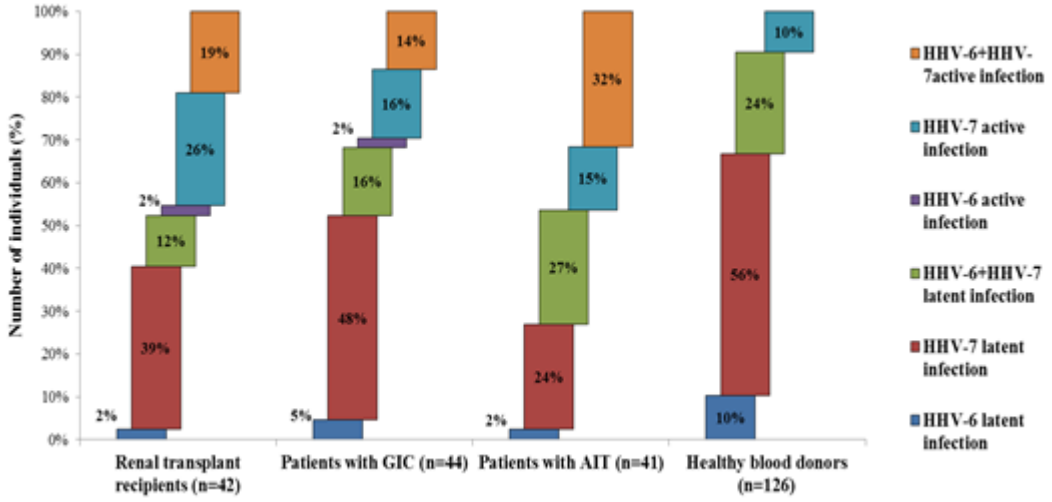



Figure 3.17. **Presence of latent and active beta-herpesviruses infection in three groups of patients**

### 3.4.2. Comparison of immunocompetent cell populations dependently on beta-herpesviruses infection

Despite of more severe immunosuppression in renal transplant recipients, in this group of patients' level of leukocytes (Leu) was the highest between all patients group. If in renal transplant recipients and patients with GIC mean absolute count of Leu had increased during beta-herpesviruses latent infection and had decreased during active infection, in patients with AIT increase of Leu absolute count was observed during latent as well as active infection.

Comparative analysis of immunocompetent cell populations dependently on beta-herpesviruses infection in patients with AIT and two other groups of patients with immunosuppression of different origin showed remarkable differences in immunological parameters' changes. During HHV-6 and/or HHV-7 latent infection in patients with AIT constant increase (to 11%) in all immunocompetent cell populations (Ly, $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$, $CD19^+$ and $CD95^+$) was observed and constant decrease (to 4%) during active infection.

Increase of Ly count was detected in renal transplant recipients (to 13%) and in patients with GIC (to 14%) in case of beta-herpesviruses latent infection. However, during HHV-6 and/or HHV-7 active infection decrease in Ly number was found in both groups (to 19% in renal transplant recipients and to 12% in patients with GIC). Almost

the same tendency was observed in level of $CD3^+$ subpopulation (increase during latent infection to 18% in transplant recipients and to 15% in patients with GIC, decrease during active infection – to 18% in renal transplant recipients and to 12% in patients with GIC). Remarkable increase of $CD4^+$ number was detected in renal transplant recipients (to 41%) during beta-herpesviruses latent infection comparing to GIC patients (to 19%) and patients with AIT (to 11%). In all three groups of patients also increase in $CD8^+$ subpopulation (to 11% in all groups) was observed. However, increase in $CD16^+$ subpopulation was observed during latent infection only in patients with AIT (to 11%).

Number of leukocytes and levels of another immunocompetent cell subpopulations ($CD3^+$, $CD4^+$, $CD8^+$, $CD38^+$, $CD19^+$, $CD25^+$ and $CD95^+$) were increased in renal transplant recipients and patients with GIC during latent beta-herpesvirus infection comparing to the subgroups without viral infection. Comparison of both groups showed significant differences in numbers of $CD4^+$, $CD16^+$, $CD19^+$ and $CD25^+$ subpopulations ($p < 0.05$) between patients with latent and active beta-herpesvirus infection. In renal transplant recipients with active viral infection mean absolute number of $CD4^+$ lymphocytes was decreased to 14% and in GIC patients to 19% in comparison with patients with latent infection. The most dramatic decrease was detected in number of $CD16^+$, where in renal transplant recipients with active viral infection this subpopulation was decreased to 24% and in the patients with GIC only to 11% comparing both patients groups with latent viral infection. Another interesting finding was revealed in patients' groups with active beta-herpesvirus infection (Figure 3.18; Table 3.18.). Number of B lymphocytes ($CD19^+$) had tendency to decrease in renal transplant recipients (to 10%) and in patients with AIT (to 4%), however in GIC patients it had tendency to increase (to 26%).

By comparative analysis of immunocompetent cell subpopulations in all three patients groups greater differences in cell counts were detected between renal transplant recipients and patients with AIT. Mean absolute count of Ly was significantly higher ($p < 0.0001$) in patients with AIT during latent ($2087 \pm 732$) and active ($1994 \pm 427$) HHV-6/7 infection comparing with transplant recipients ($1350 \pm 700$ and $1100 \pm 630$, respectively). However, no significance was detected between patients with AIT and patients with GIC. Also, in patients with AIT significantly higher ($p < 0.0001$) mean absolute count of $CD16^+$ and $CD19^+$ was found in comparison with renal transplant recipients. Significantly ($p < 0.005$) higher amount of $CD95^+$ was detected in AIT patients comparing to renal transplant recipients and patients with GIC. In its turn mean

absolute number of $CD8^+$ was significantly ($p < 0.0001$) higher in GIC patients with latent and active beta-herpesviruses infection comparing to patients with AIT (Table 3.18.).

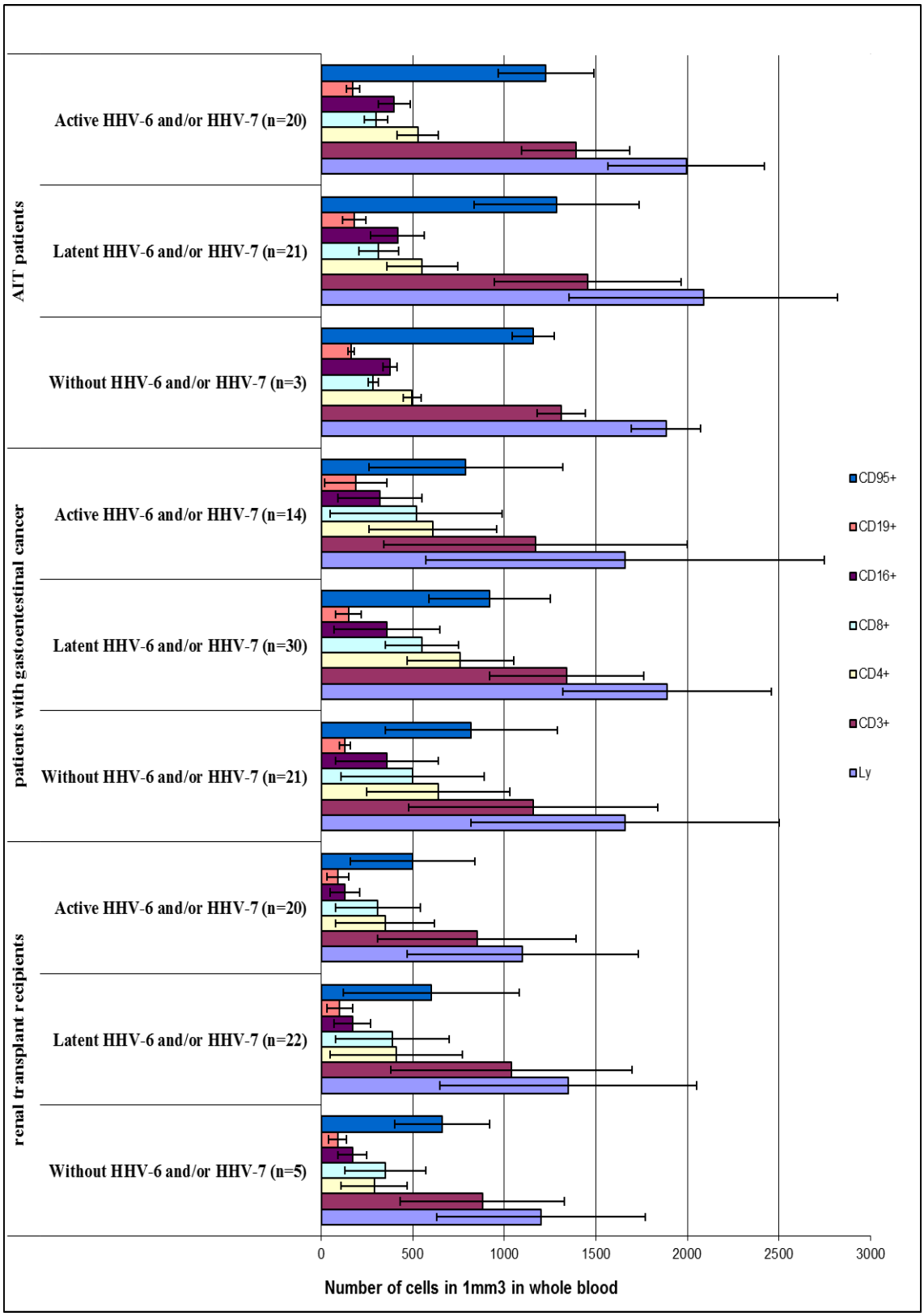



Figure 3.18. **Comparison of immunocompetent cell populations dependently on beta-herpeviruses infection in three different patients groups**

Table 3.18.

**Immunocompetent cell populations in three different patients groups dependently on beta-herpeviruses infection**

| | Renal transplant patients (n = 47) | | | Patients with gastrointestinal cancer (n = 65) | | | Patients with AIT (n = 44) | | |
|---|---|---|---|---|---|---|---|---|---|
| Parametrs | Without HHV-6 and/or HHV-7 (n = 5) | Latent HHV-6 and/or HHV-7 (n = 22) | Active HHV-6 and/or HHV-7 (n = 20) | Without HHV-6 and/or HHV-7 (n=21) | Latent HHV-6 and/or HHV-7 (n = 30) | Active HHV-6 and/or HHV-7 (n = 14) | Without HHV-6 and/or HHV-7 (n = 3) | Latent HHV-6 and/or HHV-7 (n = 21) | Active HHV-6 and/or HHV-7 (n = 20) |
| Leu | 7620 ± 2170 | 7840 ± 2660 | 7080 ± 2440 | 6490 ± 2360 | 7240 ± 2190 | 7170 ± 2520 | 5833 ± 1401 | 6073 ± 1297 | 6252 ± 1597 |
| Ly | 1200 ± 570 | 1350 ± 700 | 1100 ± 630 | 1660 ± 840 | 1890 ± 570 | 1660 ± 1090 | 1883 ± 188 | 2087 ± 732 | 1994 ± 427 |
| $CD3^+$ | 880 ± 450 | 1040 ± 660 | 850 ± 540 | 1160 ± 680 | 1340 ± 420 | 1170 ± 830 | 1312 ± 131 | 1454 ± 510 | 1390 ± 297 |
| $CD4^+$ | 290 ± 180 | 410 ± 360 | 350 ± 270 | 640 ± 390 | 760 ± 290 | 610 ± 350 | 497 ± 49 | 551 ± 193 | 527 ± 112 |
| $CD8^+$ | 350 ± 220 | 390 ± 310 | 310 ± 230 | 500 ± 390 | 550 ± 200 | 520 ± 470 | 283 ± 28 | 314 ± 110 | 300 ± 64 |
| $CD38^+$ | 400 ± 180 | 400 ± 300 | 310 ± 270 | 500 ± 300 | 550 ± 300 | 420 ± 230 | | | |
| $CD16^+$ | 170 ± 80 | 170 ± 100 | 130 ± 80 | 360 ± 280 | 360 ± 290 | 320 ± 230 | 376 ± 37 | 417 ± 146 | 399 ± 86 |
| $CD19^+$ | 90 ± 50 | 100 ± 70 | 90 ± 60 | 130 ± 90 | 150 ± 70 | 190 ± 170 | 163 ± 16 | 181 ± 63 | 173 ± 37 |
| $CD95^+$ | 660 ± 260 | 600 ± 480 | 500 ± 340 | 820 ± 470 | 920 ± 330 | 790 ± 530 | 1160 ± 115 | 1285 ± 450 | 1228 ± 263 |
| $CD25^+$ | 40 ± 30 | 50 ± 40 | 40 ± 40 | 140 ± 90 | 150 ± 120 | 120 ± 80 | | | |
| $CD4^+/CD8^+$ | 0.9 ± 0.3 | 1.33 ± 0.85 | 1.14 ± 0.56 | 1.46 ± 0.69 | 1.48 ± 0.83 | 1.24 ± 0.62 | 0.9 ± 0.3 | 1.33 ± 0.85 | 1.14 ± 0.56 |

# 4. DISCUSSION

Today there are a lot of studies trying to find and evaluate a role of beta-herpesviruses infection in etiopathogenesis of different kinds of chronic diseases, but the final answer to this question is still not found. It could be due their ubiquitous nature and different mechanisms of interference that these viruses are using. But one common feature can unite all viruses – it is disturbance in the immune system, especially immunosuppression, caused by different origin that is a base for beta-herpesviruses reactivation. Beta-herpesviruses in the host can function both – directly and indirectly, that means the viruses infect cells involved in the cellular and humoral immune response formation, and at the same time these viruses alter cell surface receptor expression, as well as proinflammatory cytokine (IL-12, IL -1ß, IL-6, TNF-α, IFN-γ) and chemokine expression levels, thereby contributing to a local inflammation (Vossen et al., 2002).

This work shows results obtained by comparison of beta-herpesviruses infection and disease or complications development in three different groups of patients with different types of immune system dysfunctions. Renal transplant recipients have immunosuppression caused by medical treatment (immunosuppressants), GIC patients – immunosuppression caused by underlying disease and patients with autoimmune thyroiditis (AIT) – immunosuppression caused by immune system dysfunction.

Comparison of immunocompetent cell populations shows more severe immunosuppression in renal transplant recipients (almost two fold decrease in all parameters comparing with other two patient groups). This fact is crucial for the patients with beta-herpesviruses active infection because this group has showed more pronounced downfall of all Ly subsets than in other two groups of patients and reactivation of these viruses can cause more severe complications.

In this group also higher incidence of beta-herpesviruses infection comparing with GIC patients (89% versus 68%) is found. Single HHV-7 active infection is more prevalent in renal transplant recipients (26%) in comparison to patients with GIC (16%) and patient with AIT (15%), however without statistical significance. This finding indicates that HHV-7 important role in immunosuppression is worsening the clinical outcome in these patients.

Comparative analysis of cellular immune parameters dependently on beta-herpeviruses infection shows significantly lower level (14%, $p < 0.0001$) of natural killer cells ($CD16^+$) in renal transplant recipients with active viral infection than in GIC patients (19.5%) and patients with AIT (20%). Decrease of $CD16^+$ number in this group could be caused by immunosuppressive therapy and higher rate of HHV-7 activation.

Significant difference ($p < 0.0001$) in number of $CD19^+$ cells is found between recipients and GIC patients with active beta-herpesvirus infection. In renal transplant recipients number of B lymphocytes ($CD19^+$) is decreased by 10%, however, in GIC patients number of $CD19^+$ cell have tendency to increase (in average by 26%). In spite of immunosuppression in GIC patients, their immune system is able to fight with active viral infection comparing with the renal transplant recipients. Renal transplant recipients' group is the only one where $CD95^+$ population of cells is decreasing in the cases of beta-herpesviruses latent infection (to 9%) and with active infection (16%). However, in patients with GIC and patients with AIT increase of $CD95^+$ during latent viruses infection (to 12% and to 11%, respectively) is defined and decrease during active infection (to 14% and to 4%). Such difference may be caused by more sever immunosuppression in renal transplant recipients, where immunosuppressant drugs were used.

Interesting finding is associated with lymphocyte count – not all renal transplant recipients had lymphopenia. Despite of severe immunosuppression caused by medication after transplantation, in 17/47 (36%) of recipients absolute lymphocytes count is > 1400 cells in 1 $mm^3$ of peripheral blood. This group of recipients has significantly higher count of all immunocompetent cell populations, especially of $CD3^+$, $CD4^+$, $CD8^+$ and $CD25^+$ populations ($p < 0.0001$) than in patients group with lymphopenia 30/47 (64%). Although, there is no difference in frequency of beta-herpesviruses persistent infection in renal transplant recipients without lymphopenia (88%) and with lymphopenia (90%), single HHV-6 active infection is detected only in recipient with Ly < 1400 cells in 1 $mm^3$ of peripheral blood. Also, double HHV-6 + HHV-7 active infection is more prevalent in renal transplant recipients with (80%) than in recipients without (50%) lymphopenia. These results may be explained by important role of immunosuppression in herpesviruses reactivation or vice versa.

Development of complications is detected in 30% of recipients and significantly higher rate ($p = 0.048$) is found in renal transplant recipients with active beta-herpesviruses infection (10/20, 50%) than in recipients with latent infection

(4/22, 18%). Also, logistic regression analysis shows higher risk of complication development in patients with active viral infection than with latent infection (OR 4.5; 95% CI 1.12–18.13; p = 0.03 and OR 0.22; 95% CI 0.06–0.89; p = 0.03, respectively). This is an evidence of the HHV-6 and HHV-7 implication in complications development.

Concerning the patients with GIC, at present there is too little information on the influence of beta-herpesviruses infection on the clinical course of the disease. Gastrointestinal malignancies are associated with a compromised immune system and viruses, such as immunotropic and immunomodulating HHV-6 and HHV-7 may be able to utilize cellular mechanisms responsible for the immune response inhibition. Modulation of functional properties of host immune factors is an important mechanism of evading the immune response or creating an environment in which the virus can survive.

Analysis for immunocompetent cell populations shows that only 46% of patients have lymphopenia (Ly < 1400 cells in $1mm^3$ peripheral blood), so it is interesting to compare these patients with GIC patients without lymphopenia (Ly > 1400 cells in 1 $mm^3$ peripheral blood).

These results show that in GIC patients group with lymphopenia the activation of HHV-6 and HHV-7 infection is significantly more frequent (p = 0.003). Moreover, comparative analysis of immunocompetent cell populations shows decrease of preferable beta-herpesviruses cell target populations (absolute count of lymphocytes and $CD3^+$, $CD4^+$, $CD8^+$ and $CD38^+$ populations). Such difference in cell populations could be another evidence of HHV-6 and HHV-7 involvement in this disease progression. It should be pointed out that only in GIC patients without lymphopenia during active beta-herpesviruses infection increase in absolute count of lymphocytes (to 9%), $CD3^+$ (to 13%), $CD4^+$ (to 19%) and $CD8^+$ (to 9%) subpopulations is identified; however in all patients with lymphopenia these populations are decreased. This fact can be explained by the effect of immunosuppression on more frequent beta-herpesviruses activation which in turn subsequently affects the count of immunocompetent cells (decreasing their count). In conformation of this statement logistic regression analysis was done and it shows that patients with active viral infection have higher risk of lymphopenia (OR 3.33; 95% CI 0.72–15.51; p = 0.0035) than patients with latent viral infection (OR 0.33; 95% CI 0.10–1.07; p = 0.0035).

Another interesting observation is noticed in natural killer cell population ($CD16^+$), which do not increase even in GIC patients with lymphopenia during active viral infection. Decrease in this subpopulation could cause abnormal anti-viral response that leads to worse progression of co-infections and worst outcomes in GIC patients.

There are no significant changes observed in serum levels of IL-6, IL-1beta, sIL-2R and TNF-alpha between immunecompetent and immunecompromised GIC patients with latent and without HHV-6 and HHV-7 infection, but in the GIC patients with lymphopenia during active viral infection level of TNF-alpha is decreased comparing to the other GIC patients groups. However, it is hard to compare GIC patients with active viral infection because of higher HHV-6 and HHV-7 distribution in the GIC patients group with lymphopenia and low presence of active infection in the another group without lymphopenia. Decreased level of TNF-alpha and increased levels of IL-6 and sIL2R in patients with active and persistent virus infection confirms that HHV-6 and HHV-7 possesses immunomodulating properties. Both facts, decrease in lymphocytes' subpopulations and changes in levels of interleukins, could show possible mechanisms of how HHV-6 and HHV-7 influence the clinical course of the disease. First mechanism is direct influence on lymphocytes by infecting them and inducing cell lyses, and second – influence through changes in interleukins expression. Combining studies of both virus and cancer mediated immune suppressive mechanisms will help us to understand the complicated host-tumour interactions. The further investigation is important to evaluate not only changes in pro-inflammatory cytokines but also in anti-inflammatory cytokines expression in patients with GIC. It could explain ways of virus influence on the course of disease and would help to choose the most efficient tactic of the treatment.

Although, development of the most sever (fourth) stage of GIC is more prevalent in patients with persistent beta-herpesviruses infection (23%) than without viral infection (6%), there is no significant association between cancer stages development and beta-herpesviruses infection. However, higher mortality rate (53%) is found between the patients with than without (34%) lymphopenia. This fact indicates that lymphopenia is an important factor that impacts the course of the disease, in its turn strong association of lymphopenia development and active beta-herpesviruses infection is shown.

All these results show importance of HHV-6 and also HHV-7 involvement in immunosuppression in both mentioned patients groups.

It has been shown that the herpesviruses may be involved in a variety of chronic inflammatory autoimmune processes, including autoimmune thyroid disease pathophysiology (Posnett, 2008; Leite et al., 2010; Caselli et al., 2012). In Latvia this is the first study of HHV-6 and HHV-7 infection involvement in autoimmune thyroid etiopathogenesis. Autoimmune thyroid diseases are widespread endocrine disorders (Huber et al., 2008) and are more common in women (Saravanan and Dayan, 2001). This research also shows that 43 out of 44 patients with AIT are women, which coincides with other published data mentioned before.

Viral infections have been frequently cited as important environmental factors implicated in AIT (Prummel et al., 2004; Desailloud and Hober, 2009), but no specific virus has yet been conclusively associated to the disease. In particular, herpesviruses have been implicated in this disease, with conflicting evidence. Case reports suggested a potential association between herpesvirus infection and AIT (Thomas et al., 2008; Shimon et al., 2003; Caselli et al., 2012), but in other report, when thyroid specimens were analyzed no EBV, CMV or HSV-1 DNA is detected (Mori et al., 1998). A recent study analyzed the presence of herpesvirus DNA in post-operative thyroid specimens from tissue blocks, and HHV-6 was detected by single round PCR in 2 out of 15 (13.3%) Hashimoto thyroiditis tissue specimens, whereas in Grave's disease or multi nodular goitre tissues no HHV-6 DNA is detected (Thomas et al., 2008).

In this work significantly ($p = 0.003$) higher rate of HHV-6 and HHV-7 genomic sequence presence in DNA samples extracted from AIT patients thyroid tissue (43/44, 98%) is shown in comparison to the control group (31/41, 76%). In 43/44 of AIT patients' thyroid tissue DNA HHV-6 genomic sequence is found. These results show a possible influence of HHV-6 on AIT development. Interesting, that 25/44 (57%) of patients have HHV-6 genomic presence only in thyroid tissue DNA samples, which evidences on HHV-6 latency in thyroid glands. However, presence of HHV-6 DNA in lymphocytes infiltrates in tissue could not be excluded. Although, real-time PCR results show that average HHV-6 viral load is higher in tissue DNA samples, rather than in whole blood DNA, which could strengthen the evidence of HHV-6 latency in thyroid gland. These results are advocating to the involvement of HHV-6 infection in AIT development, but further investigation is needed to make general conclusion.

HHV-7 genomic presence is detected in 40 AIT patients' DNA samples (91%), however, presence in thyroid tissue DNA samples is detected in 33/44 (75%) of patients.

While there is a lack in a direct evidence of HHV-7 infection in association with thyroid autoimmune diseases, an interesting fact remains that in other autoimmune diseases, more frequently active HHV-6 infection is found rather than HHV-7 active infection. For example, Italian researchers study in patients with autoimmune connective tissue diseases HHV-7 active infection has been found in none of the patients, but active HHV-6 infection – 26 out of 58 patients (Broccolo et al., 2009).

Although, role of HHV-7 in the AIT development is less known, it could interact with HHV-6 infection, which could be explained by the fact that HHV-7 infection increases CD46 and CD59 molecule expression levels of target cells (Takemoto et al., 2007). Both viruses could influence the AIT development by several mechanisms. Indirect mechanism is to attract lymphocytes by persistent antigen production in thyroid tissue or directly worsen situation by thyrocyte lysis by infecting them.

Presence of HHV-6 and HHV-7 genomic sequences in patients' with thyroid disease tissue DNA samples are detected more frequently than in control group ($p < 0.0001$, $p = 0.0001$). Results of this study are in contrary to the Syrian group of researchers carrying out a study in which the 50 investigated patients with autoimmune thyroid disease and 50 patients without autoimmune component are negative for the presence of HHV-6 genomic sequence in thyroid gland tissue DNA (AL-Zarzour and Monem, 2011). In contrast, a similar study in Greece, where researchers report that in 72.2% of individuals with thyroid disease one of the herpesviruses is found in thyroid tissue (Thomas et al., 2008), however the possibility that there are differences in terms of the incidence of the virus due to geographic regions, the quality of the material analyzed, and study methods used could not be excluded. In addition, in the same study, the authors report that HHV-7 genomic DNA sequence in tissue is more frequently found than HHV-6 genomic sequences which do not match well with the results of our study, because the statistical analysis shows the opposite – that HHV-6 genomic sequence in tissue DNA is found more frequently than HHV-7 (95% and 75%, respectively; $p = 0.01$).

Comparative analysis of immunocompetent cell populations in patients with AIT and other two groups of patients shows two significant differences. First of all, in patients with AIT was not found any signs of immunosuppression; second, significant higher rate of lymphocytes with CD95 receptor ($p < 0.0001$, mean abs. count $1251 \pm 355$) comparing with other groups is detected. In literature such huge increase of $CD95^+$ cells indicates an abnormal apoptosis signalling pathway, which is common in

patients with autoimmune diseases. Another evidence of involvement of HHV-6 in AIT development could be the presence of this virus genomic sequence in $CD95^+$ cells, but not in $CD4^+$ and $CD8^+$ after cell sorting. Immunomodulating abilities of HHV-6 could be one of the reasons of disorders in apoptotic signalling pathway.

Also, results in patients with AIT shows that not in all patients' whole blood DNA (25/44, 57%) samples HHV-6 genomic sequence is detected, however, almost all patients (43/44, 98%) have HHV-6 genomic sequences in thyroid tissue DNA samples. This can be explained by a very small load of HHV-6 genomic sequence in blood and insufficient sensitivity of PCR. The same problem is showed in renal transplant recipients and patients with GIC, where PCR results do not match with ELISA results. Presence of HHV-6 persistent infection by PCR is detected in 36% of recipients and in 25% of patients with GIC, however, anti-HHV-6 IgG class antibodies are detected in 77% and in 51% of patients, respectively.

The HHV-6B is detected in all DNA samples from patients with HHV-6 infection. This might suggest that either the HHV-6A is infrequent in these patients or it might be limited to sites other than the peripheral blood. Also, one of the causes of dominant HHV-6B presence in patients could be connected with geographical distribution.

This work shows different ways of beta-herpesviruses interaction with the host organism and their involvement in etiopathogenesis of chronic diseases, complication development and clinical course worsening. In renal transplant recipients beta-herpesviruses activity is associated with worsening of immunosuppression during treatment. Also, substantial role in immunosuppression more likely plays also active HHV-7 infection in this group. On the other hand, in patents with GIC lower beta-herpesviruses (HHV-6 and HHV-7) distribution is found than in other two groups of patients – only in 68% of patients. However, development of lymphopenia in immunocompromised patients with GIC is strongly associated with the active beta-herpesviruses infection. In case of AIT, especially HHV-6 is associated with this disease development (because of its high frequency and higher viral load in thyroid gland tissue in comparison with peripheral blood). HHV-6 and HHV-7 infection, possibly, could be a primary cause, by expressing antigens in thyroid glands during the latent stage of infection or it could play secondary role by infecting and destroying thyrocytes.

# 5. CONCLUSIONS

1. Renal transplant recipients have higher risk of immunosuppression strengthening and complications development associated with HHV-6 and HHV-7 active infection.
2. Active beta-herpesviruses infection in patients with gastrointestinal cancer is associated with decrease of lymphocytes' total count, increase of IL-6, sIL-2R and decrease of TNF-alpha expression levels leading to the deepening of immunosuppression.
3. HHV-6 and HHV-7 persistent infection is not associated with gastrointestinal cancer stage, at the same time activation of HHV-6 and HHV-7 leads to the worsening of clinical course and outcome of underlying disease.
4. HHV-6 and HHV-7 genomic sequences are frequently detected in thyroid tissue as well as in whole blood DNA samples of patients with autoimmune thyroiditis therefore the association of viral infection with the disease development could not be excluded.
5. Higher viral load in thyroid tissue of autoimmune thyroiditis patients than in whole blood is additional evidence of HHV-6 involvement in the disease development and indicates that thyroid gland is one of the places of HHV-6 latency.
6. HHV-6B is revealed in renal transplant recipients, patients with gastrointestinal cancer and autoimmune thyroiditis, which shows dominant distribution of this species in Latvia.

# 6. RECOMMENDATIONS

1. Highly sensitive molecular and serological methods are required to detect HHV-6 and HHV-7 infection, which allows distinguishing latent and active phase of persistent infection.
2. HHV-6 and HHV-7 active infection in renal transplant recipients is associated with immunosuppression caused by medication. Therefore, patients with beta-herpesviruses persistent infection should be monitored for the active phase to prevent development of complications in time.
3. In 46% of patients with GIC association between active beta-herpesviruses (HHV-6 and HHV-7) infection and lymphopenia is found. Therefore, patients with beta-herpesviruses persistent infection should be monitored for the active phase to avoid the deepening of lymphopenia in time.
4. Taking into account that thyroid gland is one of the HHV-6 latency places it is recommended to monitor the activity of beta-herpesviruses infection activity in patients with the first signs of thyroid gland diseases to prevent deepening of the autoimmune process.

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# 8. THE LIST OF PUBLICATIONS

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# 10.Acknowledgments

I would like to express my sincere of gratitude to my supervisors – Assoc. Prof. Modra Murovska and Senior Researcher Svetlana Chapenko for invaluable assistance, support and guidance. Also, I would like to express gratitude to Prof. Sandra Lejniece for support and valuable advices.

I would like to express gratitude to Vice-Rector for Science, Prof. Iveta Ozolanta and Scientific Secretary, Ingrida Kreile for support and consultations during doctoral studies.

Finally, I would like to thank all my colleges for moral support.