Rīgas Stradiņa universitāte

Santa Rasa

ASSOCIATION OF PERSISTENT VIRAL INFECTIONS WITH MYALGIC ENCEPHALOMYELITIS/ CHRONIC FATIGUE SYNDROME

Summary of the Doctoral Thesis
for obtaining the degree of a Doctor of Medicine

Speciality – Microbiology and Virology

Riga, 2017

The Doctoral Thesis was carried out at Rīga Stradiņš University (RSU) A. Kirchenstein Institute of Microbiology and Virology in collaboration with RSU Health Centre "Ambulance", Latvian Centre of Infectious Diseases, Pauls Stradiņš Clinical University Hospital Neurology clinic and Latvia State Blood Donor centre.

Scientific supervisor:

Dr. med., Associate Professor **Modra Murovska**,
Rīga Stradiņš University, Latvia

Official reviewers:

Dr. med., Professor **Juta Kroiča**,
Rīga Stradiņš University, Latvia

Dr. habil. med., Professor **Aija Žileviča**,
University of Latvia

Dr. biol. sci. **Mykolas Mauricas**,
State Research Institute Centre for Innovative Medicine
Department of Immunology, Lithuania

Defence of the Doctoral Thesis will take place at the public session of the Doctoral Council of Medicine on 11 December 2017 at 15.00 in Hippocrates Lecture Theatre, 16 Dzirciema Street, Rīga Stradiņš University.

The Doctoral Thesis is available in the RSU library of and at RSU webpage: www.rsu.lv.

ESF EIROPAS SOCIĀLAIS FONDS IEGULDĪJUMS TAVĀ NĀKOTNĒ EIROPAS SAVIENĪBA

The Doctoral Thesis has been carried out by the financial support of European Social Fund project "Support for doctoral study programs and the acquisition of scientific degree at Rīga Stradiņš University" (2009/0147/1DP/1.1.2.1.2/09/IPIA/VIAA/009).

Secretary of the Doctoral Council:

Dr. med., Associate Professor **Simona Doniņa**

TABLE OF CONTENTS

Abreviations 5
Introduction 6
Scientific novelty of the study 8
Aim of the study 8
Objectives of the study 8
Hypothesis of the study 9
Structure of the study 9
1. Material and Methods 10
1.1. Patients and biological material 10
1.2. Molecular methods 11
1.3. Immunological methods 13
1.4. Phylogenetic analysis 14
1.5. Statistical analysis 14
2. Results 15
2.1. Patients with ME/CFS 15
2.2. Analysis of XMRV genomic sequences in patients with ME/CFS 15
2.3. Involvement of HHV-6 in development of ME/CFS 15
2.3.1. Presence of HHV-6 specific antibodies 15
2.3.2. Frequency of HHV-6 genomic sequences 16
2.3.3. HHV-6 load 16
2.3.4. Presence HHV-6 antigens 17
2.3.5. Association of HHV-6 with ME/CFS clinical symptoms 17
2.3.6. Level of cytokines in case of HHV-6 infection 18
2.4. Involvement of HHV-7 in development of ME/CFS 19
2.4.1. Presence of HHV-7 specific antibodies and genomic sequences 19
2.4.2. HHV-7 load 19
2.4.3. Association of HHV-7 with ME/CFS clinical symptoms 20
2.4.4. Level of cytokines in case of HHV-7 infection 21
2.5. Involvement of B19V in development ME/CFS 22
2.5.1. Presence of B19V specific antibodies 22
2.5.2. Frequency of B19V genomic sequences 22
2.5.3. B19V load 22
2.5.4. B19V antibody reaction patterns 23
2.5.5. Association of B19V with ME/CFS clinical symptoms 23
2.5.6. Level of cytokines in case of B19V infection 25
2.5.7. B19V phylogenetic analysis 26
2.6. Involvement of HHV-6, HHV-7 and B19V infection/co-infection in development of ME/CFS 27

2.6.1. Frequency of virus infection/co-infection 27
2.6.2. Viral load in patients with co-infection..... 28
2.6.3. Cytokine level in ME/CFS patients with viral infection/co-infection 30
2.6.4. ME/CFS typical clinical symptoms in patients with infection/co-infection 31
2.6.5. Severity of ME/CFS in patients with infection/co-infection... 33
3. Discussion 35
4. Conclusions 53
5. Recommendations 55
6. List of publications 56
6.1. Papers in journals included in the international databases 56
6.2. Papers in other journals and collections of articles 56
6.3. Presentations at international conferences/congresses 57
6.4. Presentations at local conferences/congresses 59
7. References 60
Acknowledgements 67

ABREVIATIONS

B19V	human parvovirus B19
bp	base pairs
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CNS	central nervous system
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EBV	Epstein-Barr virus
ELISA	Enzyme-Linked Immunosorbent Assay
HHV	Human Herpesvirus
ICD-10	International statistical classification of diseases and related health problems
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
IQR	interquartile range
ME/CFS	Myalgic Encephalomyelitis/Chronic Fatigue Syndrome
$MgCl_2$	magnesium chloride
ml	millilitre
µl	microliter
nm	nanometre
ng	nanogram
pg	picogram
mRNA	messenger ribonucleic acid
nPCR	nested polymerase chain reaction
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
RT	reverse transcription
SD	standard deviation
TNF-α	tumor necrosis factor alpha
XMRV	xenotropic murine leukemia virus- related virus

INTRODUCTION

Myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is a chronic, complex disease involving central nervous system (CNS) and immune system disorders, cell energy metabolism and ion transport dysfunction, as well as cardiovascular abnormalities (Carruthers et al., 2011). This illness mainly is characterized by severe chronic fatigue, including such clinical symptoms as tender cervical or axillary lymph nodes, muscle pain, joint pain without swelling or redness, post-exertional malaise for more than 24 hours, impaired memory/concentration, headaches, sore throat and un-refreshing sleep (Fukuda et al., 1994; Carruthers et al., 2011).

Prevalence of ME/CFS is reported depending on the applied criteria for diagnostics and it is determined from 0.76% of clinically diagnosed up to 3.48% of self-reported population (Johnston et al., 2013). Still there is no consensus on a single case definition for this disease. Diagnosis is based on differential diagnostics and clinical symptoms, therefore it is necessary to identify specific biomarkers for ME/CFS. However, currently there is no effective and standardized diagnostic tests, prophylactic and treatment strategies for this disease (Albright et al., 2011; Bansal et al., 2012).

Viral infections have been considered as one of potential etiological factors for ME/CFS, which accompanied by immune disturbances can facilitate maintenance of disease symptoms (Bansal et al., 2012; Fischer et al., 2014). Many patients confirm an onset of ME/CFS with flu-like symptoms. Moreover, the observed immune abnormalities could be caused by a viral infection or a viral infection follows immune disturbances. Still, the role of viral infections in ME/CFS remains obscure (Morinet and Corruble, 2012; Underhill, 2015). Infectious agents that have been studied in association with ME/CFS are hepatitis C virus, human immunodeficiency virus, coxsackie B, Epstein-Barr virus (EBV), human herpesvirus (HHV)-6, human parvovirus B19 (B19V) and

such bacteria as borrelia, chlamydia and mycoplasma. However, an association of a single specific infectious agent and ME/CFS has not been established (Nicolson et al., 2003; Bansal et al., 2012; Chapenko et al., 2012; Halpin et al., 2017; Loebel et al., 2017).

Studies on the association of xenotropic murine leukemia virus-related virus (XMRV) with ME/CFS started after a published report on frequently detectable XMRV in patients with ME/CFS (Lombardi et al., 2009). Furthermore, ME/CFS could result from reactivation of persistent herpesvirus infection, which is found more frequently in patients with ME/CFS compared to donors (Chapenko et al., 2006; Krueger and Ablashi, 2006). Similarly, B19V infection is present at onset of ME/CFS, therefore it could be one of causal factors of this disease (Fremont et al., 2009). Some researchers report that the reactivation of these viruses could serve as an objective biomarker (Ablashi et al., 2000; Chapenko et al., 2006; Kerr et al., 2010; Aoki et al., 2016). On the contrary, others find no association of HHV-6, HHV-7 and B19V infection with ME/CFS etiopathogenesis (Koelle et al., 2002; Cameron et al., 2010). Also immune system disorders are determined in various studies by the analysis of changes in several cytokine production in patients with ME/CFS (Mensah et al., 2017; Russell et al., 2016). Therefore, it is important to conduct studies in order to clarify the role of these viruses in ME/CFS, as well as to determine etiological, progression and maintenance mechanisms, and biomarkers for this disease.

This study was conducted in RSU A.Kirchenstein Institute of Microbiology and Virology, where the first studies on ME/CFS were started in Latvia. To clarify the role of HHV-6, HHV-7, B19V and XMRV in ME/CFS, presence of viral infection markers and infection activity phase was studied using methods of molecular biology, immunology and statistics, and findings correlated with the clinical signs and course of the disease. All used materials and methods are described in details later.

Scientific novelty of the study

In this study frequency and activity of persistent viral infection and co-infection, viral load, time from infection onset, level of cytokines and association with clinical symptoms in patients with ME/CFS were estimated to clarify the involvement of HHV-6, HHV-7, B19V and XMRV infections in the development of ME/CFS.

Aim of the study

To determine the involvement of human herpesvirus-6, human herpesvirus-7, parvovirus B19 and xenotropic murine leukemia virus related virus in etiopathogenesis of myalgic encephalomyelitis/chronic fatigue syndrome.

Objectives of the study

1. To detect the presence of XMRV provirus genomic sequences in DNA extracted from peripheral blood of ME/CFS patients.
2. To estimate the frequency of HHV-6 and HHV-7 specific antibodies and genomic sequences, infection activity phase, viral load, as well as HHV-6 type and antigen expression in patients with ME/CFS.
3. To detect the frequency of B19V specific antibodies and genomic sequences, infection activity phase, viral load, genotype and period of time from B19V infection appearance in ME/CFS patients.
4. To determine the expression level of cytokines (IL-6, TNF-α, IL-12, IL-4 and IL-10) in patients with persistent infection/co-infection in latent and active phase.
5. To analyse the association of HHV-6, HHV-7 and B19V infection/co-infection with ME/CFS clinical symptoms.

6. To estimate the influence of infection activity on severity of ME/CFS clinical course.

Hypothesis of the study

1. Persistent viral infections, like beta-herpesviruses HHV-6 and HHV-7, and parvovirus B19V infections, are ME/CFS trigger factors and are associated with the development of ME/CFS.
2. The activity phase of virus infection is of the greatest importance because an active infection causes much deeper immunological disturbances and is associated with a more severe ME/CFS clinical course.
3. XMRV could be associated with ME/CFS development (confirm or deny this hypothesis).

Structure of the study

Doctoral thesis is written in English. It contains following parts: introduction, scientific novelty, aim, objectives and hypothesis of the study, as well as literature review, materials and methods, results, discussion, conclusions, recommendations, list of publications, references' section and acknowledgements. Thesis is written on 111 pages with seven tables and 19 figures, and contains 229 references to literature sources. Results of this study are published in 10 papers and reported in 20 local and international conferences/congresses as oral or poster presentation.

1. MATERIAL AND METHODS

1.1. Patients and biological material

The study was done in accordance with safety and ethical standards, as well as laws and requirements of the Republic of Latvia and the European Union. The cohort was established with the approval of the Ethics Committee of Rīga Stradiņš University issued on September 27, 2012. All enrolled patients gave their informed consent prior to the study.

Two hundred patients [130 (65%) female and 70 (35%) male, mean age 38 ± 12] with clinically diagnosed ME/CFS corresponding to 1994 Fukuda Centers for Disease Control and Prevention (CDC) criteria were included in this cross-sectional study. For clinical ME/CFS diagnosis in Latvia according to International Statistical Classification of Diseases and Related Health Problems (ICD-10), G93.3 – postviral fatigue syndrome (benign myalgic encephalomyelitis), R53 – fatigue and weakness and B94.8 – consequences of other defined infectious and parasitic diseases were used. Inclusion and exclusion criteria were applied for ME/CFS patients to be included in the study. As a control group 150 age and gender matched apparently healthy individuals (blood donors) were enrolled in this study.

Samples were collected from the Riga East University Hospital Latvian Centre of Infectious Diseases, RSU Health Centre "Ambulance", Pauls Stradiņš Clinical University Hospital Neurology clinic and Latvia State Blood Donor centre. Blood samples were collected in vacutainers with ethylenediaminetetraacetic acid – EDTA, transported to A.Kirchenstein Institute of Microbiology and virology laboratory, where aliquots of whole blood samples, blood plasma and peripheral blood mononuclear cells (PBMCs) were prepared and stored.

1.2. Molecular methods

DNA was **isolated** from peripheral blood by phenol-chloroform extraction method and from blood plasma samples – using QIAamp DNA Blood Kit, (Qiagen GmbH, Germany).

RNA was **extracted** from PBMCs with Tri Reagent (Applied Biosystems, USA). Presence of RNA was analysed electrophoretically in 1% agarose gel and visualized electrophoretically.

Concentration of the extracted DNA and RNA was measured spectrophotometrically.

Complementary DNA (cDNA) was synthesized with reverse transcription (RT) using commercially available RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA).

To assure the **quality of cDNA and DNA** from peripheral blood and to exclude possible contamination of plasma DNA by cellular DNA, polymerase chain reaction (PCR) was carried out to detect β-globin gene sequence according to Vandamme et al., 1995.

Nested PCR (nPCR) was used to detect viral or proviral genomic sequences in patients and apparently healthy individuals' DNA. Presence of XMRV provirus *env* and *gag* sequences was detected in DNA extracted from peripheral blood, according to Lombardi et al., 2009 and Lo et al., 2010. Sensitivity of the nPCR was five copies per reaction and XMRV VP62 plasmid as a positive control was used (Urisman et al., 2006; Dong et al., 2007).

nPCR was used also to amplify HHV-6, HHV-7 and B19V specific genomic sequences in DNA isolated from peripheral blood (a marker of persistent infection) and cell-free blood plasma (a marker of an active infection). The detection of HHV-6 (U3 gene) and HHV-7 (U10 gene) genomic sequences was performed in accordance with Secchiero et al., 1995 and Berneman et al., 1992, respectively. HHV-6 and HHV-7 genomic DNA

(Advanced Biotechnologies Inc, Columbia, MD, USA) was used as a positive controls. Sensitivity of HHV-6-specific primers was three copies and HHV-7 – one copy per reaction (Tomsone et al., 2001; Kozireva et al., 2008). Presence of human parvovirus B19V genomic sequence was determined according to Barah et al., 2001, using primers complementary to NS1 gene. Viremic serum DNA was used as a positive control. Sensitivity of primers was 1–10 copies per reaction (*Barah* et al., 2001).

HHV-6A and HHV-6B according to Lyall and Cubie, 1995 **were differentiated**. Following nPCR, amplification products were digested with HindIII restriction endonuclease (Thermo Scientific, USA) which cleaves HHV-6B 163 bp amplification product into 66 bp and 97 bp fragments, whereas does not cleave HHV-6A.

PCR was used to amplify virus specific DNA sequences in **cDNA** samples, which were obtained from RNA that was extracted from PBMCs. HHV-6 U89/90 immediate-early gene expression was detected according to Van den Bosch et al., 2001, though HHV-7 U57 and B19V NS1 gene expression was detected in accordance with Ito et al., 2013.

Electrophoretic analysis was done in agarose gel to separate and identify DNA fragments amplified by PCR.

HHV-6, HHV-7 and B19V load was estimated using DNA extracted from peripheral blood by **real-time PCR** according to manufacturer's instructions. HHV-6 load was determined with HHV-6 Real-TM Quant and B19V – with Parvovirus B19 Real-TM Quant kit (Sacace Biotechnologies, Italy). HHV-7 load was detected using Human Herpes Virus 7 genomes genesig kit (Primerdesign, United Kingdom) and in-house real-time PCR amplifying HHV-7 U90 and PI15 gene sequences based on previous report by Prusty et al., 2013.

1.3. Immunological methods

IgM and IgG class antibodies against HHV-6 were detected with HHV-6 IgM and HHV-6 IgG **enzyme linked immunosorbent assay (ELISA)** kits (Panbio, Australia) and HHV-6 IgG Antibody ELISA kit (Advanced Biotechnologies, Columbia MD, USA). HHV-7 specific IgG class antibodies were analysed with immunofluorescence method using HHV-7 IgG IFA Kit (Advanced Biotechnologies, Columbia MD, USA). B19V specific IgM and IgG class antibodies were estimated with Biotrin Parvovirus B19 Enzyme Immunoassay (Biotrin Ltd, Dublin, Ireland), *recomWell* Parvovirus B19 Enzyme Immunoassay and *recomLine* Parvovirus B19 IgM and IgG kits (Mikrogen Diagnostik, Germany). Antibodies against antigens of B19V (Vp-2p; VP-N; VP-1S; VP-2r; VP-C; NS-1) were identified to determine time period after B19V infection onset by various reaction patterns (Pfrepper et al., 2005).

Determination of cytokine level in blood plasma was performed. IL-4 level was detected using Endogen Human **ELISA** kit (Pierce Biotechnology, Rockford, IL, USA), IL-6 level – with eBioscience Human IL-6 Platinium ELISA (eBioscience Europe/International, Austria), IL-10 level – using BIOSOURCE IL-10 EASIA (Enzyme Amplified Sensitivity Immunoassay) from BioSource Europe S.A., Belgium and eBioscience Human IL-10 Platinium ELISA (eBioscience Europe/International, Austria), IL-12 (p70) level – with eBioscience Human IL-12p70 Platinium ELISA (eBioscience Europe/International, Austria), and TNF-α level – using Biorbyt Human TNFα ELISA kit (Biorbyt, United Kingdom).

HHV-6 antigen expression was detected by indirect immunofluorescence in PBMCs with following primary mouse antibodies: anti-p41 (clone 6A5D12), HHV6B specific anti-gH (gp100) (clone OHV-3)

and HHV6A and HHV6B specific anti-gB (gp116) (clone OHV-1) and secondary antibodies [rabbit anti-mouse FITC-conjugated (Dako) sera].

1.4. Phylogenetic analysis

To perform phylogenetic analysis part of human parvovirus B19V NS genes (396 bp) were aligned by Multiple Sequence Comparison by Log-Expectation – MUSCLE implemented in the MEGA 6 software. Trees were reconstructed with the neighbour-joining and maximum likelihood methods using the MEGA 6 and PhyML 3.0 (Guindon et al., 2010; Tamura et al., 2013).

1.5. Statistical analysis

Statistical analysis was done by GraphPad Prism 7.0 program. Discrete variables were described as numbers and percentage, and difference in frequency of gender, virus-specific antibodies, antigens, virus presence markers between groups was estimated using Chi-square and Fisher´s exact tests as appropriate. Continuous variables were expressed as average ± standard deviation (SD) or median (interquartile range – IQR). Considering data distribution, viral loads and cytokine levels were analysed with Analysis of variance – ANOVA and Mann-Whitney nonparametric tests. A value of $p \leq 0.05$ was considered to be statistically significant.

2. RESULTS

2.1. Patients with ME/CFS

Out of 200 enrolled ME/CFS patients, 79% were in age between 25–50 years. All patients had more than 6 months lasting unexplained chronic fatigue ($p < 0.0001$). Impaired memory, decreased concentration and sleep disturbances were most frequently observed symptoms in patients with ME/CFS. All included patients reported that onset of ME/CFS symptoms occurred 6–36 months before inclusion in this study, mean (± SD) 10.2 ± 4.2 months. In 85% of patients onset of ME/CFS symptoms had started 8–12 months before inclusion in the study.

2.2. Analysis of XMRV genomic sequences in patients with ME/CFS

XMRV proviral *gag* and *env* gene sequences were not detected in any of DNA samples isolated from peripheral blood of 150 patients with ME/CFS and 30 apparently healthy individuals. Only positive controls gave amplimers of 735 bp and 351 bp after the first round, and 410 bp and 218 bp after the second round amplification that corresponds to the expected XMRV *gag* and *env* gene fragments, respectively.

2.3. Involvement of HHV-6 in development of ME/CFS

2.3.1. Presence of HHV-6 specific antibodies

HHV-6 specific antibodies were detected in 92.1% (151/164) of analysed ME/CFS patients' and 76.7% (69/90) apparently healthy individuals' blood plasma samples ($p = 0.0009$). Anti-HHV-6 IgG class antibodies had 90.9% (149/164) patients and 76.7% (69/90) apparently healthy individuals ($p = 0.0026$), though IgM class antibodies had 6.1% (10/164) patients and 2.2% (2/90) apparently healthy individuals ($p = 0.2227$).

2.3.2. Frequency of HHV-6 genomic sequences

Using nPCR presence of HHV-6 genomic sequences was detected in 53% (106/200) of patients with ME/CFS and in 28.7% (43/150) of apparently healthy individuals ($p < 0.0001$). From them presence of HHV-6 genomic sequence in DNA isolated from peripheral blood leukocytes (marker of a persistent infection in latent phase) had 42% (84/200) of patients and 28.7% (43/150) of apparently healthy individuals ($p = 0.0133$), though presence of genomic sequence in cell free blood plasma (marker of a persistent infection in active phase) – 11% (22/200) of ME/CFS patients and none of apparently healthy individuals ($p < 0.0001$). HHV-6A was detected in one and HHV-6B in the rest of the analysed patients with ME/CFS ($p < 0.0001$). Using RT-PCR HHV-6 U89/90 gene expression was revealed in 78% (57/73) of the analysed ME/CFS patients PBMCs with previously detected HHV-6 genomic sequence in peripheral blood DNA by nPCR.

2.3.3. HHV-6 load

HHV-6 load was elevated (> 10 copies/10^6 cells) in 66% (66/100) of the analysed patients with ME/CFS and in 2/10 apparently healthy individuals with previously detected by nPCR presence of HHV-6 genomic sequence ($p = 0.0064$). In patients with a persistent HHV-6 infection in a latent phase median (IQR) HHV-6 load was 279 (1022–54.5) copies/10^6 cells, whereas in patients with a persistent HHV-6 infection in an active phase – 1927 (6732–348.5) copies/10^6 cells ($p = 0.0019$). In 43.6% (34/78) of patients with a persistent HHV-6 infection in a latent phase the viral load was < 10 copies/10^6 cells. Six patients' median (IQR) viral load was 1209033 (1464421–808183) copies/10^6 cells.

2.3.4. Presence HHV-6 antigens

Analysing 36 patients PBMCs by indirect immunofluorescence, in six patients with ME/CFS p41 expression was shown [samples were positive using HHV-6 specific anti-p41 (clone 6A5D12) antibody], in 15 patients gp100 expression was found [samples were positive using HHV6B specific anti-gH (gp100) (clone OHV-3) antibody], and in seven patients with ME/CFS gp116 expression was present [samples were positive using HHV6A and HHV6B specific anti-gB (gp116) (clone OHV-1) antibody].

2.3.5. Association of HHV-6 with ME/CFS clinical symptoms

Occurrence of typical ME/CFS clinical symptoms and presence of markers of a persistent HHV-6 infection in latent and active phase in patients with ME/CFS are summarized in Table 2.1.

Table 2.1

Occurrence of typical ME/CFS clinical symptoms in ME/CFS patients with persistent HHV-6 infection in latent and active phase

Symptoms	Latent HHV-6, n (%)	Active HHV-6, n (%)	P value
Chronic fatigue >6 months	84 (100%)	22 (100%)	> 0.9999
Post-exertional malaise	53 (63.1%)	14 (63.6%)	> 0.9999
Impaired memory	55 (65.5%)	17 (77.3%)	0.4418
Decreased concentration	62 (73.8%)	14 (63.6%)	0.4261
Sleep disturbances	64 (76.2%)	18 (81.8%)	0.7762
Subfebrility	49 (58.3%)	13 (59.1%)	> 0.9999
Lymphadenopathy	48 (57.1%)	10 (45.5%)	0.3469
Muscle pain	46 (54.8%)	12 (54.5%)	> 0.9999
Multi-joint pain	36 (42.9%)	10 (45.5%)	> 0.9999
Headache of new type	37 (44%)	9 (40.9%)	0.8146

2.3.6. Level of cytokines in case of HHV-6 infection

Table 2.2 shows median (IQR) concentration level (pg/ml) and percentage of patients with an elevated level of pro-inflammatory (IL-6, TNF-α and IL-12) and anti-inflammatory cytokines (IL-4 and IL-10) in ME/CFS patients with a persistent HHV-6 infection in latent and active phase.

Table 2.2

Level of cytokines in patients with ME/CFS with persistent HHV-6 infection in latent and active phase

Assessed parameters / Cytokine, sensitivity	IL- 6, 0.92 pg/ml	TNF- α, < 1 pg/ml	IL- 4, < 2 pg/ml	IL-10, < 1 pg/ml	IL-12 (p70), 2.1 pg/ml
Persistent HHV-6 infection in latent phase					
Median (IQR)	3.8 (5.2–2.0)	68.0 (126.4–39)	< 2	15.5 (36.4–8)	12.0 (16.8–4.6)
Patients with elevated level (%)	28.8	80.0	0.0	78.5	96.6
Persistent HHV-6 infection in active phase					
Median (IQR)	4.4 (26.9–1.3)	67.0 (113.6–55)	< 2	20.0 (52.4–11.9)	15.1 (18.6–14)
Patients with elevated level (%)	55.0	81.0	0.0	88.9	100.0
Mann-Whitney test [latent vs active infection (pg/ml)]	0.9493	0.919	–	0.4789	*0.0386
Fishers' exact test [elevated cytokine level latent vs active infection (number of patients)]	*0.0356	> 0.9999	> 0.9999	0.6759	> 0.9999

* statistically significant ($p < 0.05$)

– undetectable

2.4. Involvement of HHV-7 in development of ME/CFS

2.4.1. Presence of HHV-7 specific antibodies and genomic sequences

HHV-7 specific IgG class antibodies was detected in 84.6% (11/13) of patients with ME/CFS and 93.8% (30/32) of the analysed apparently healthy individuals ($p = 0.5672$).

Marker of persistent HHV-7 infection was detected by nPCR in 92% (184/200) of patients with ME/CFS and 75.3% (113/150) of apparently healthy individuals ($p < 0.0001$). From them presence of HHV-7 genomic sequence in DNA extracted from peripheral blood leukocytes was revealed in 58% (116/200) of ME/CFS patients and 67.3% (101/150) of apparently healthy individuals ($p = 0.0766$), whereas presence of genomic sequence in DNA from blood plasma – 34% (68/200) of patients and 8% (12/150) of apparently healthy individuals ($p < 0.0001$).

Using RT-PCR HHV-7 U57 gene expression was detected in 45.7% (58/127) of analysed ME/CFS patients PBMCs with previously detected HHV-7 in peripheral blood DNA by nPCR.

2.4.2. HHV-7 load

HHV-7 load was elevated (> 10 copies/10^6 cells) in 67.3% (113/168) of the analysed patients with ME/CFS and in 31.4% (16/51) of the analysed apparently healthy individuals with previously detected HHV-7 genomic sequence by nPCR ($p < 0.0001$).

In ME/CFS patients with persistent HHV-7 infection in a latent phase median (IQR) viral load was 196.7 (533–132) copies/10^6 cells and in an active phase – 238.6 (410.6–80.2) copies/10^6 cells ($p = 0.3502$). HHV-7 load < 10 copies/10^6 cells was detected in 37.1% (39/105) of the patients with a persistent HHV-7 infection in a latent phase and 25.4% (16/63) of the patients with a

persistent HHV-7 infection in an active phase, as well as 68.6% (35/51) of apparently healthy individuals.

In one patient with ME/CFS HHV-7 load in whole blood DNA was 1140127.6 copies/10^6 cells and in hair follicle DNA – 1188811.8 copies/10^6 cells. In addition, in the same patient's mother's hair follicle the HHV-7 load was 2591031.6 copies/10^6 cells.

2.4.3. Association of HHV-7 with ME/CFS clinical symptoms

Occurrence of typical ME/CFS clinical symptoms and presence of markers of a persistent HHV-7 infection in latent and active phase in patients are shown in Table 2.3.

Table 2.3

Occurrence of typical ME/CFS clinical symptoms in ME/CFS patients with persistent HHV-7 infection in latent and active phase

Symptoms	Latent HHV-7, n (%)	Active HHV-7, n (%)	P value
Chronic fatigue (>6 months)	116 (100%)	68 (100%)	> 0.9999
Post-exertional malaise	68 (58.6%)	40 (58.8%)	> 0.9999
Impaired memory	79 (68.1%)	44 (64.7%)	0.7458
Decreased concentration	79 (68.1%)	50 (73.5%)	0.5059
Sleep disturbances	78 (67.2%)	53 (77.9%)	0.1325
Subfebrility	64 (55.2%)	42 (61.8%)	0.4406
Lymphadenopathy	63 (54.3%)	31 (45.6%)	0.2864
Muscle pain	67 (57.8%)	32 (47.1%)	0.1711
Multi-joint pain	55 (47.4%)	30 (44.1%)	0.7596
Headache of new type	51 (44%)	27 (39.7%)	0.6437

2.4.4. Level of cytokines in case of HHV-7 infection

Median (IQR) concentration (pg/ml) and percentage of patients with elevated levels of pro-inflammatory and anti-inflammatory (IL-6, TNF-α, IL-12 and IL-4, IL-10) cytokines levels among ME/CFS patients with persistent HHV-7 infection in latent and in active phase is shown in Table 2.4.

Table 2.4

Level of cytokines in ME/CFS patients with persistent HHV-7 infection in latent and active phase

Assessed parameters / Cytokine, sensitivity	IL-6, 0.92 pg/ml	TNF-α, < 1 pg/ml	IL-4, < 2 pg/ml	IL-10, < 1 pg/ml	IL-12 (p70), 2.1 pg/ml
Persistent HHV-7 infection in latent phase					
Median (IQR)	2.9 (5.2–1.5)	55.7 (125.6–32.8)	< 2	12.5 (30–6.3)	14.2 (16.4–8.2)
Patients with elevated level (%)	30.7	65.2	0.0	83.5	100.0
Persistent HHV-7 infection in active phase					
Median (IQR)	4.6 (6.5–2)	57.5 (120.8–36.5)	< 2	20.0 (50–10.4)	15.3 (17.7–12.7)
Patients with elevated level (%)	37.3	83.6	0.0	73.9	95.3
Mann-Whitney test [latent vs active infection (pg/ml)]	0.131	0.4814	–	*0.0421	0.1071
Fishers' exact test [elevated cytokine level latent vs active infection (number of patients)]	0.4145	*0.0096	> 0.9999	0.1841	0.1044

* statistically significant ($p < 0.05$)

– undetectable

2.5. Involvement of B19V in development ME/CFS

2.5.1. Presence of B19V specific antibodies

B19V specific IgG class antibodies were found in 70% (140/200) of patients with ME/CFS and 67.4% (60/89) of the analysed apparently healthy individuals ($p = 0.6803$) blood plasma samples. None of apparently healthy individuals had B19V specific IgM class antibodies, though 8% (16/200) of ME/CFS patients had IgM class antibodies ($p = 0.0038$).

2.5.2. Frequency of B19V genomic sequences

Using nPCR B19V genomic sequence in DNA isolated from peripheral blood and/or blood plasma was detected in 29% (58/200) of ME/CFS patients and in 3.8% (4/104) of apparently healthy individuals ($p < 0.0001$). Presence of B19V genomic sequences in DNA from peripheral blood leukocytes was detected in 12% (24/200) of patients with ME/CFS and in 1.9% (2/104) of apparently healthy individuals ($p = 0.002$). However, presence of B19V genomic sequences in DNA from blood plasma was found in 17% (34/200) of patients with ME/CFS and 1.9% (2/104) of apparently healthy individuals ($p < 0.0001$). B19V NS1 gene expression in PBMCs was detected by RT-PCR and 25 out of 58 (nPCR positive) analysed ME/CFS patients were positive.

2.5.3. B19V load

In nine of 24 patients with a latent/persistent B19V infection the viral load was [median (IQR)] 5.6 (27.4–0.8) copies/μg DNA (37.2 copies/10^6 cells) and in 15 patients < 0.2 copies/μg DNA. In addition, in 11 out of 34 patients with an active B19V infection the viral load was 38.2 (217.5–17.7) copies/μg DNA (251.8 copies/10^6 cells) and in 23 patients < 0.2 copies/μg DNA

(p = 0.0289). All apparently healthy individuals with B19V infection had a viral load < 0.2 copies/μg DNA.

2.5.4. B19V antibody reaction patterns

By analysing B19V specific antibody reaction patterns of 75 patients with ME/CFS (39 with and 36 without the presence of B19V genomic sequence in DNA from peripheral blood and/or blood plasma) with *recomLine* kit, an acute B19V infection was revealed in one patient, a recent infection (weeks to months after infection onset) in 41% (16/39) of the patients with B19V genomic sequence and in 30.6% (11/36) without it (p = 0.4706). A sustained infection (months to years after infection onset) was observed in 56.4% (22/39) of patients with and 27.8% (10/36) – without B19V genomic sequence in peripheral blood and/or plasma DNA (p = 0.0191). 41.7% (15/36) of patients without B19V genomic sequence were without B19V specific antibodies. 51.3% (20/39) of the analysed patients with the presence of B19V genomic sequence in DNA isolated from peripheral blood and/or plasma had developed B19V specific NS1 antibodies.

2.5.5. Association of B19V with ME/CFS clinical symptoms

The occurrence of typical ME/CFS clinical symptoms and the presence of B19V infection markers in patients with ME/CFS are summarized in Table 2.5.

The onset of ME/CFS symptoms was determined six months up to three years before inclusion in the study. In 93.3% (70/75) of patients with B19V infection onset of symptoms had occurred 8.3 ± 1.7 months before inclusion in this study and in 6.7% (5/75) of the patients symptoms had started 2.4 ± 0.5 years before. In patients with a recent B19V infection symptoms had started

8.3 ± 1.6 months ago and in patients with a sustained infection – 12.1 ± 7.8 months ago (from them 25% – more than 12 months ago).

Table 2.5

Occurrence of typical ME/CFS clinical symptoms in ME/CFS patients with latent/persistent and active B19V infection

Symptoms	Latent/persistent B19V, n (%)	Active B19V, n (%)	P value
Chronic fatigue (>6 months)	24 (100%)	34 (100%)	1.0000
Post-exertional malaise	15 (62.5%)	18 (52.9%)	0.5923
Impaired memory	12 (50%)	24 (70.6%)	0.1694
Decreased concentration	13 (54.2%)	18 (52.9%)	1.0000
Sleep disturbances	15 (62.5%)	22 (64.7%)	1.0000
Subfebrility	9 (37.5%)	20 (58.8%)	0.1820
Lymphadenopathy	10 (41.7%)	17 (50%)	0.5992
Muscle pain	14 (58.3%)	16 (47.1%)	0.4351
Multi-joint pain	9 (37.5%)	15 (44.1%)	0.7872
Headache of new type	14 (58.3%)	13 (38.2%)	0.1828

Figure 2.1 shows the percentage of ME/CFS typical clinical symptoms in the analysed patients with and without detectable NS1 antibodies in the presence of B19V genomic sequence in whole blood DNA. From them 55% of patients with and 21.1% without NS1 antibodies had multi-joint pain ($p = 0.0294$). Muscle pain was observed in 65% and 42.1%, while lymphadenopathy – 65% of patients with and 31.6% without NS1 antibodies, respectively ($p = 0.1517$ and $p = 0.0369$).

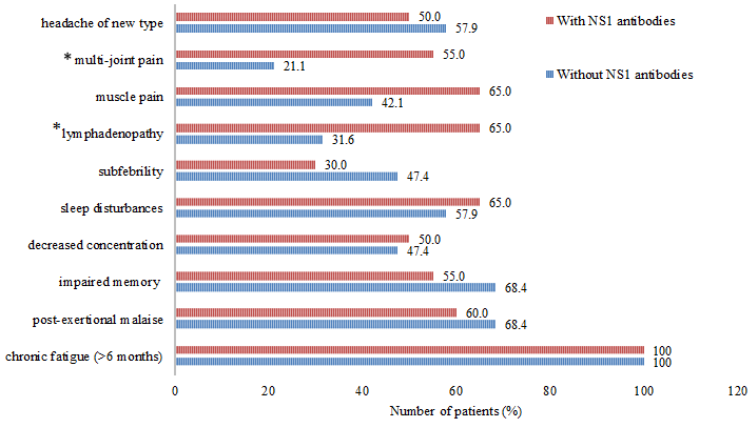

Figure 2.1 **Number of ME/CFS typical symptoms in patients with presence of B19V genomic sequence with and without B19V specific NS1 antibodies**
* statistically significant ($p < 0.05$)

2.5.6. Level of cytokines in case of B19V infection

Pro-inflammatory (IL-6, TNF-α and IL-12) and anti-inflammatory (IL-4 and IL-10) cytokines median (IQR) concentration (pg/ml) and percentage of patients with an elevated cytokine level in ME/CFS patients with a latent/persistent and active B19V infection is depicted in Table 2.6.

Table 2.6

Level of cytokines in patients with ME/CFS with latent/persistent and active B19V infection

Assessed parameter \ Cytokine, sensitivity	IL-6, 0.92 pg/ml	TNF-α, < 1 pg/ml	IL-4, < 2 pg/ml	IL-10, < 1 pg/ml	IL-12 (p70), 2.1 pg/ml
Latent/persistent B19V infection					
Median (IQR)	3.0 (3–2.1)	69.9 (133–29)	< 2	11.6 (29.6–5.9)	13.8 (15.1–8.1)
Patients with elevated level (%)	8.3	76.2	0.0	75.0	100.0
Active B19V infection					
Median (IQR)	4.5 (6–1.5)	106.4 (153–42.9)	< 2	15.8 (82.5–10)	14.9 (18.6–12.7)
Patients with elevated level (%)	45.5	72.7	0.0	92.9	92.9
Mann-Whitney test [latent vs active infection (pg/ml)]	0.625	0.3581	–	0.096	0.0612
Fishers' exact test [elevated cytokine level persistent vs active infection (number of patients)]	*0.0031	> 0.9999	>0.9999	0.1234	0.4949

* statistically significant ($p < 0.05$)

– undetectable

2.5.7. B19V phylogenetic analysis

Although two B19V genotypes (genotype 1 and 2) were revealed in Latvia, the results of phylogenetic analysis of the B19V NS1 gene showed genotype 1 circulation in patients with ME/CFS. The majority of Latvian isolates (also from patients with diagnoses other than ME/CFS) was clustered with genotype 1. Consistent tree topologies were observed with both of neighbour-joining and maximum likelihood methods. The gene diversity for genotype 1 was low – 0.3 ~ 1.1%.

2.6. Involvement of HHV-6, HHV-7 and B19V infection/co-infection in development of ME/CFS

2.6.1. Frequency of virus infection/co-infection

Using nPCR markers of persistent viral infection/co-infection was revealed in 96.5% (193/200) of patients with ME/CFS and in 85.3% (128/150) of apparently healthy individuals (p = 0.0003) (Figure 2.2).

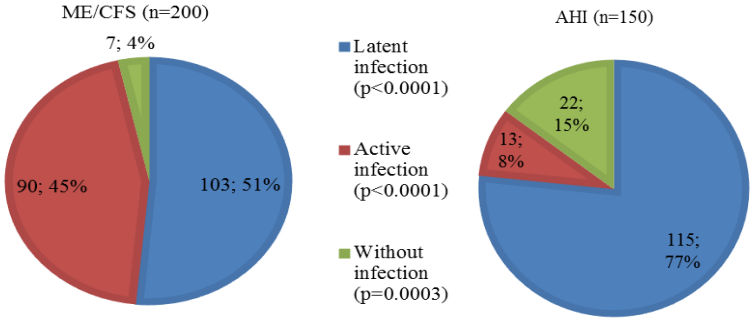

Figure 2.2 **Frequency of persistent HHV-6, HHV-7 and B19V infection in patients with ME/CFS and apparently healthy individuals**
AHI – apparently healthy individuals

Figure 2.3 shows the frequency of markers for persistent HHV-6, HHV-7 and B19V infection/co-infection in latent or active phase in groups of patients with ME/CFS compared with apparently healthy individuals.

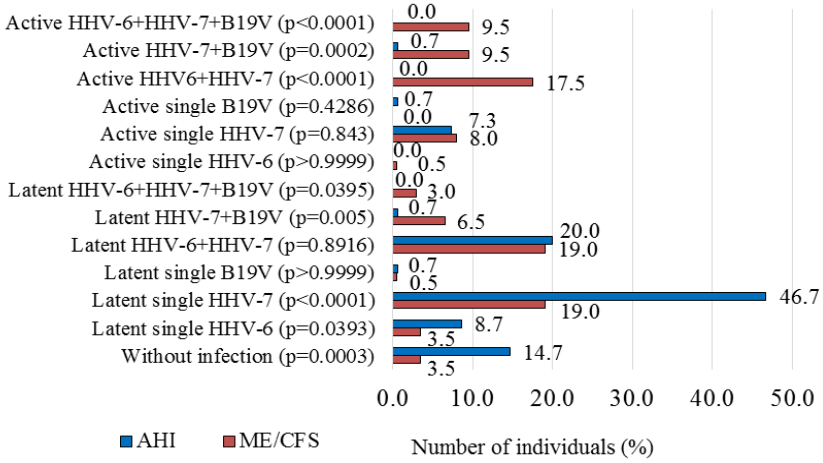

Figure 2.3 **Frequency of persistent HHV-6, HHV-7 and B19V infection/co-infection (%) in latent or active phase**
HHV-human herpesvirus, B19V – human parvovirus B19, AHI – apparently healthy individuals

2.6.2. Viral load in patients with co-infection

The median HHV-6 load in patients with a persistent infection/co-infection in a latent phase was (IQR) 262 (474–29.7) copies/10^6 cells, whereas in an active phase – 653.2 (4136–190.5) copies/10^6 cells ($p = 0.0251$) (Figure 2.4.a).

HHV-7 load was 166.5 (398.6–123.8) copies/10^6 cells in patients with a persistent infection/co-infection in a latent phase and 248.5 (422–105.6) copies/10^6 cells in ME/CFS patients with an active infection ($p = 0.55$) (Figure 2.4.b).

In patients with a persistent infection/co-infection in a latent phase B19V load was 14.7 (27.4–0.7) copies/µg DNA (96.8 copies/10^6 cells) and in patients with an infection in an active phase – 38 (217.8–18) copies/µg DNA (250.8 copies/10^6 cells) ($p = 0.0444$) (Figure 2.4.c).

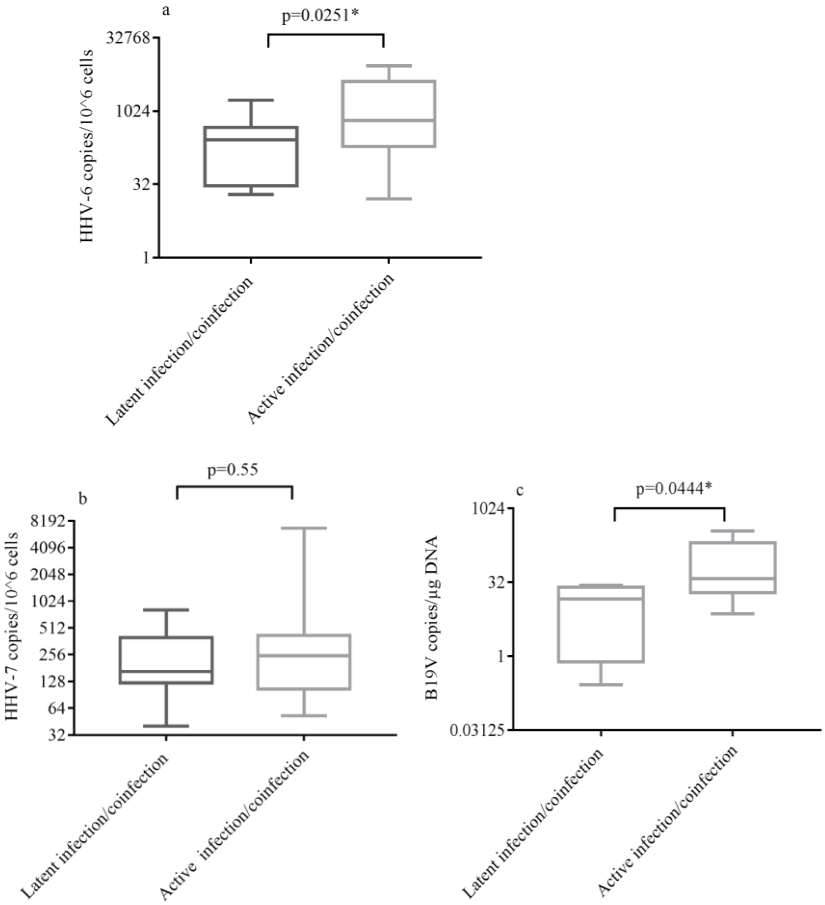

Figure 2.4 **HHV-6 (a), HHV-7 (b) and B19V (c) viral load in ME/CFS patients with persistent infection/co-infection in latent and active phase**
* statistically significant ($p < 0.05$)

2.6.3. Cytokine level in ME/CFS patients with viral infection/co-infection

According to the used cytokine detection protocols, the mean (range) of IL-6 level in apparently healthy individuals was 6.4 (< 0.92–13) pg/ml. Median (IQR) level of IL-6 in ME/CFS patients with a persistent HHV-6/HHV-7/B19V infection and/or co-infection in a latent phase was 2.5 (5.1–1.9) pg/ml and in patients with a persistent single HHV-6 or HHV-7 infection in an active phase – 4.2 (5.3–1.3) pg/ml. IL-6 level in case of a persistent double (HHV-6+HHV-7 and HHV-7+B19V) infection in an active phase was 4.7 (10.2–2) pg/ml and in case of a persistent triple (HHV-6+HHV-7+B19V) infection in an active phase – 3.3 (6–1.8) pg/ml. In all ME/CFS patients without infection IL-6 level was < 0.92 pg/ml ($p = 0.1289$).

TNF-α level in apparently healthy individuals was under the detection level (< 2.3 pg/ml). In ME/CFS patients with a persistent HHV-6/HHV-7/B19V infection and/or a co-infection in a latent phase median (IQR) TNF-α level was 59 (133–29) pg/ml and with a single HHV-6 or HHV-7 infection in an active phase – 44 (68–26.5) pg/ml. In patients with a persistent double (HHV-6+HHV-7 and HHV-7+B19V) infection in an active phase TNF-α level was 62.5 (117.2–43) pg/ml, though with a persistent triple (HHV-6+HHV-7+B19V) infection in an active phase – 123 (178–74.7) pg/ml. In ME/CFS patients without infection the TNF-α level was 59 (77.5–12) pg/ml ($p = 0.0492$).

IL-12 level in apparently healthy individuals was under the detection level (< 2.1 pg/ml). In ME/CFS patients with a persistent HHV-6/HHV-7/B19V infection and/or co-infection in a latent phase IL-12 median level was 13.4 (15.6–5.6) pg/ml. In patients with a persistent single (HHV-6 or HHV-7) infection in an active phase and with a double (HHV-6+HHV-7 and HHV-7+B19V) infection in an active phase IL-12 level was 15.5 (16.8–13.3) pg/ml and 15.5 (18.4–13.3) pg/ml, respectively. In case of a persistent triple

(HHV-6+HHV-7+B19V) infection in an active phase IL-12 level was 16 (31.3–13.9) pg/ml and patients without infection – 14.6 (15.1–3.1) pg/ml ($p = 0.0063$).

In apparently healthy individuals the mean IL-10 level was 10.3 (8.1–12.5) pg/ml. The median (IQR) IL-10 level in ME/CFS patients with a persistent HHV-6/HHV-7/B19V infection and/or co-infection in a latent phase was 12.4 (30–7) pg/ml, whereas in patients with a persistent single (HHV-6 or HHV-7) infection in an active phase – 20 (25–18.3) pg/ml. In addition, in ME/CFS patients with a persistent double (HHV-6+HHV-7 and HHV-7+B19V) and a triple (HHV-6+HHV-7+B19V) infection in an active phase IL-10 level was 21 (98.8–10.3) and 22 (130–12.2) pg/ml, respectively. Median IL-10 level was 5 (11.6–3.1) pg/ml for patients without infection ($p = 0.0023$).

2.6.4. ME/CFS typical clinical symptoms in patients with infection/co-infection

ME/CFS clinical symptoms in case of a persistent single HHV-6, HHV-7 and B19V infection and co-infection (double HHV6+HHV-7, HHV-7+B19V and triple HHV-6+HHV-7+B19V) in latent and active phase with p value (Fisher's exact test) are shown in Table 2.7.

Table 2.7

Occurrence of typical ME/CFS clinical symptoms in ME/CFS patients with persistent HHV-6, HHV-7 and B19V infection/co-infection in latent and active phase

Infection phase \ Symptoms	chronic fatigue (> 6 months)	post-exertional malaise	impaired memory	decreased concentration	sleep disturbances	subfebrility	lymphadenopathy	muscle pain	multi-joint pain	headache of new type
Latent single	100	52.2	76.1	80.4	76.1	47.8	56.5	52.2	60.9	45.7
Active single	100	52.9	58.8	82.4	76.5	70.6	41.2	52.9	29.4	35.3
p value	> 0.9999	> 0.9999	0.2158	> 0.9999	> 0.9999	0.1557	0.395	> 0.9999	*0.0452	0.5708
Latent double	100	62.7	66.7	66.7	64.7	58.8	58.8	58.8	39.2	37.3
Active double	100	50.0	70.4	72.2	70.4	64.8	46.3	48.1	48.1	46.3
p value	> 0.9999	0.2385	0.8337	0.6718	0.6768	0.5525	0.2424	0.3294	0.4323	0.4295
Latent triple	100	66.7	50	66.7	83.3	33.3	33.3	66.7	16.7	100
Active triple	100	78.9	57.9	47.4	78.9	47.4	52.6	52.6	47.4	31.6
p value	>0.9999	0.6061	> 0.9999	0.6447	> 0.9999	0.6609	0.6447	0.6609	0.3449	*0.052

* statistically significant ($p < 0.05$)

2.6.5. Severity of ME/CFS in patients with infection/co-infection

Severe course of disease was experienced by 18.7% and moderate – by 81.3% of patients with ME/CFS ($p < 0.0001$).

In patients with severe and moderate ME/CFS median (IQR) IL-6 level was 1.5 (5.2–1.1) pg/ml and 4.5 (5.7–2) pg/ml, respectively ($p = 0.0506$). TNF-α level was 103 (150.7–44.7) pg/ml in patients with severe and 58 (123–32) pg/ml with moderate ME/CFS ($p = 0.0434$). In patients with ME/CFS and a severe course of the disease IL-12 level was 19.9 (37.3–15.5) pg/ml and with a moderate course – 13.8 (16.4 –7.4) pg/ml ($p = 0.0494$). Moreover, IL-10 level was 35 (90–8.8) pg/ml in patients with severe and 12.4 (25–6.7) pg/ml with moderate severity of ME/CFS ($p = 0.025$) (Figure 2.5).

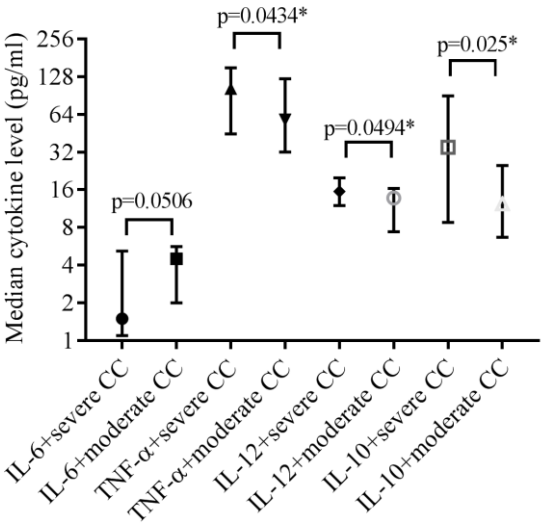

Figure 2.5 **Median (IQR) IL-6, TNF-α, IL-10 and IL-12 level in ME/CFS patients with severe and moderate course of the disease**
* statistically significant, CC – clinical course

HHV-6 load in ME/CFS patients with a severe course of the disease was median (IQR) 1134 (2962–34.5) copies/10^6 cells and with a moderate course – 391.8 (3190–162.8) copies/10^6 cells (p = 0.7656). Though HHV-7 load in patients with severe ME/CFS was 303.6 (514.8–174) copies/10^6 cells and with a moderate disease course – 175.7 (402.7–90) copies/10^6 cells (p = 0.0254), but B19V load in cases of severe and moderate ME/CFS was 8 (13.5–2.5) copies/μg DNA (53 copies/10^6 cells) and 30 (79.1–5.6) copies/μg DNA (197.8 copies/10^6 cells), respectively (p = 0.2353) (Figure 2.6).

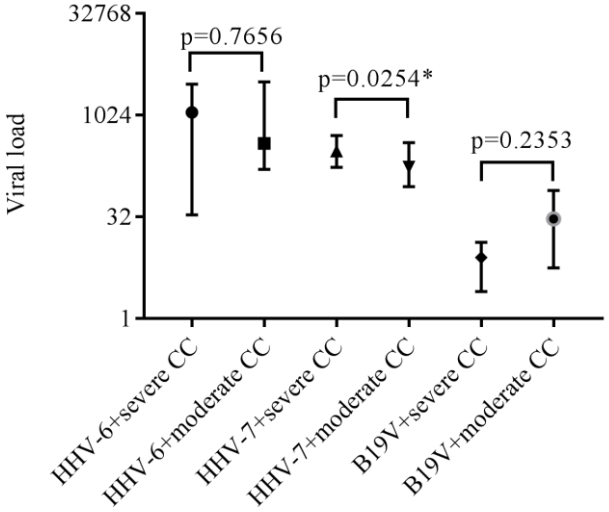

Figure 2.6 **Median (IQR) HHV-6, HHV-7 (copies/10^6 cells) and B19V (copies/μg DNA) load in ME/CFS patients with severe and moderate course of the disease**

* statistically significant, CC – clinical course

3. DISCUSSION

ME/CFS is a multifactorial disorder characterized by several symptoms, which frequently follow after a virus infection or prolonged stress. After decades of great effort in research, there is still no accordance regarding frequency and severity of immune disturbances in this condition (Bansal et al., 2012; Committee on the Diagnostic Criteria for Myalgic Encephalomyelitis/Chronic Fatigue Syndrome et al., 2015). ME/CFS is recognized as a heterogeneous disease with a lack of homeostasis in multiple organ systems accompanied by psychosocial problems that reduce the quality of life (Winger et al., 2015; Glassford, 2017).

Virus infections are believed to be one of ME/CFS potential triggers, because infectious-like symptoms are present in many ME/CFS patients during sudden onset of the disease. Fatigue can be the consequence of a post-viral infection and immunological dysfunctions may be caused or facilitated by a virus infection in patients with ME/CFS. However, there is no consensus on implication of a virus infection in ME/CFS (Morinet and Corruble, 2012). It is not clear, whether an active HHV-6A, HHV-6B, HHV-7 and B19V infection cause ME/CFS or follows the disease.

In this study the biological material of 200 patients' with clinically diagnosed ME/CFS and 150 apparently healthy individuals is analysed with molecular and serological laboratory methods to determine presence, load and activity phase of HHV-6A, HHV-6B, HHV-7, B19V and XMRV, as well as the level of cytokines in association with clinical symptoms.

ME/CFS is more prevalent in females compared to males. It is reported that 65 to 80% of adult ME/CFS cases are females (Underhill, 2015). Likewise, in this study 65% of patients with ME/CFS are females, albeit the prevalence of ME/CFS could be higher than currently assessed. Onset of ME/CFS can happen at any age, though patients are characterized by a peak at the age of 10 to 19

years and 30 to 39 years (Bakken et al., 2014). This corresponds to average age of adult patients in our study (38 ± 12 years).

XMRV as potential causative factor of ME/CFS was considered since 2009, when 67% of patients and 3.7% of healthy donors are reported to be XMRV positive (Lombardi et al., 2009). Next year the publication revealed murine leukemia virus related virus gene sequence in 86.5% of ME/CFS patients and in 6.8% of controls (Lo et al., 2010). However, these reports are in contrary to this study result, where XMRV specific *gag* and *env* gene sequences are absent in DNA isolated from peripheral blood of ME/CFS patients and apparently healthy individuals. The obtained results are in line with majority of published researches worldwide, where XMRV is not detected in patients with ME/CFS and is concluded not to be associated with human diseases (Groom and Bishop, 2012).

Trigger factor could differ from causal factor of ME/CFS aetiology and Underhill in 2015 discusses that related pathogens instead of different ones causes this disease (Underhill, 2015).

The presence of virus-specific IgG class antibodies in blood plasma or serum is observed in patients with history of an infection, whereas virus-specific IgM class antibodies and virus genomic sequence in DNA from cell-free plasma is present during an acute or primary infection and an active viral infection (Liefeldt et al., 2005). Considering that the primary infection with these viruses usually occurs in early childhood, in case of a persistent infection in the adults the presence of virus genomic sequence in DNA isolated from cell-free plasma is marker for virus reactivation. It is accompanied by detectable virus genomic sequence in DNA isolated from whole blood or blood leukocytes indicating persistent virus infection (Traylen et al., 2011).

In this study, the frequency of B19V specific IgG class antibodies is similar in patients with ME/CFS (70%) and apparently healthy individuals (67.4%) ($p = 0.6803$). Furthermore, none of apparently healthy individuals but

8% of patients with ME/CFS have IgM class antibodies ($p = 0.0038$). Other B19V seroprevalence studies likewise do not find a significant difference between the presence of B19V specific IgG class antibodies in patients and control group, reporting that B19V seroprevalence in population varies from 60% to 80%, but others show B19V specific IgG class antibodies in 74% and IgM – in one patient with ME/CFS (Cooling et al., 1995; Zhang et al., 2010). Considering that B19V seroprevalence increases from 2% in children under age of 5 years up to 85% in elderly people (Servant-Delmas et al., 2010) and the mean age of this study cohort is 38 ± 12 years, results on frequency of IgG class antibodies are consistent with worldwide population.

The presence of B19V NS1 specific antibodies accompanied by the presence of B19V DNA is found in 20/39 (51.3%) analysed ME/CFS patients, asserting the persistence of B19V infection. These results coincide with Kerr et al., publication, where IgG class antibodies against NS1 protein are detected in more patients (41.5%) than controls (7%) whereas IgM class antibodies are found in three patients and one donor. Detectable B19V specific NS1 antibodies indicate on a severe and persistent infection, therefore immune system of a part of the patients is not able to control the virus sufficiently (von Poblotzki et al., 1995; Kerr et al., 2010).

In this study B19V genomic sequence is more frequently detected in patients with ME/CFS (29%) than apparently healthy individuals (3.8%) ($p < 0.0001$). A significant difference is revealed between patients (12%) and apparently healthy individuals (1.9%) in case of a latent/persistent B19V infection (virus genomic sequence only in DNA isolated from leukocytes) ($p = 0.002$). In addition, an active B19V infection (B19V genomic sequence also in cell-free blood plasma DNA) is significantly often detected in patients with ME/CFS (17%) than apparently healthy individuals (1.9%) ($p < 0.0001$). Furthermore, B19V replication is proven in patients with a persistent B19V infection by the detection of NS1 gene expression in patients PBMCs. Other

studies have also reported the detection of B19V DNA, messenger ribonucleic acid (mRNA) and protein in macrophages, T and B cells, follicular dendritic cells and in monocytes (Takahashi et al., 1998), whereas B19V infection of monocyte cell line U937 can be abortive due to a lack of viral particle production (Munakata et al., 2006).

The results of our study are in accordance with publications by other researchers, who conclude that at least in part of patients B19V could be involved in etiopathogenesis of ME/CFS due to the detection of B19V infection markers in 40% of patients and almost 15% of controls (Fremont et al., 2009). In this study the frequency of elevated B19V load is significantly often estimated in ME/CFS patients than in apparently healthy individuals ($p = 0.0003$). Moreover, in case of an active B19V infection the viral load is higher than in patients with a latent/persistent B19V infection. Reports from other studies show the detection of B19V genomic sequence with real-time PCR only in patients with ME/CFS and not in control group (Kerr et al., 2010). However part of researchers report no association of B19V infection with ME/CFS, because markers of B19V are not detected in all ME/CFS cases (Sanders and Korf, 2008). Furthermore, reviewing neurological aspects of B19V infection, nine reports have published a correlation of ME/CFS with an acute B19V infection, however two studies deny it (Barah et al., 2014).

The analysis of B19V IgG and IgM class antibodies' patterns discloses an acute infection in only one ME/CFS patient. Recent infection is observed in more patients with the presence of B19V genomic sequence (41%) detected by nPCR than without it (30.6%), though not statistically significant ($p = 0.4706$). Furthermore, a sustained infection is significantly often found in patients with (56.4%) than without (27.8%) detectable B19V genomic sequence in DNA from whole blood ($p = 0.0191$). The data of this study show that B19V infection can persist for years. In patients with a recent B19V infection, ME/CFS symptoms started 8.3 ± 1.6 months ago and with a sustained infection

– 12.1 ± 7.8 months ago, indicating that B19V infection is a potential promoter of this disease. In addition, a severe clinical course of ME/CFS is experienced by more patients with a sustained (28.1%) than with a recent BV19 infection (18.5%), thus pointing to the gradual increase in the symptoms of the disease during an infection. Findings of B19V infection in ME/CFS tends to confirm the hypothesis of B19V as a possible trigger for this disease, however ME/CFS could be caused by various factors and some infectious agents may contribute to forming a subset of this illness (Kerr et al., 2010). No specific pathogen is detected yet and rather could be viral than bacterial due to failure of antibiotic treatment (Underhill, 2015).

In our study HHV-6 seropositivity is revealed in 92.1% of patients with ME/CFS and 76.7% of analysed apparently healthy individuals showing a difference between these groups. Whereas IgM class antibodies are found in 6.1% of patients and only 2.2% of controls ($p = 0.2227$). Published results on the prevalence of HHV-6 antibodies by other researchers are discrepant. Some report on a higher frequency of IgM class antibodies among patients with ME/CFS (50%) compared to donors (28.5%), while some do not find any difference between patients and control groups (Levine et al., 2001; Burbelo et al., 2012). Despite potential differences in geographic distribution, the prevalence of HHV-6 IgG class antibodies in apparently healthy individuals (76.7%) from this study correspond to previously published in Greece (78.8%) that does not differ from the age and gender of adults (Politou et al., 2014). However, Ablashi with colleagues detected IgM class antibodies more frequently in ME/CFS patients (57.1%) and donors (16%) than in our study (6.1% and 2.2%, respectively) (Ablashi et al., 2000).

According to PCR results a persistent HHV-6 infection in a latent phase is observed in more patients with ME/CFS than in apparently healthy individuals (42% vs 28.7%, $p = 0.0133$). Moreover, a persistent HHV-6 infection in an active phase is detected only in patients with ME/CFS (11%)

and none of donors ($p < 0.0001$). Similar to this study results, Di Luca with colleagues found HHV-6 genomic sequences in 44% of patients and 29% of donors (Di Luca et al., 1995). Analysing a larger cohort, HHV-6 is reported to be present in 70% of patients and 20% of controls (Buchwald et al., 1992). Furthermore, 30.5% of patients and 9% of donors have an active HHV-6 infection (Nicolson et al., 2003). Publications show the correlation of an active HHV-6 replication with ME/CFS, which can be emerged from reactivation of a latent virus infection (Ablashi et al., 2000; Buchwald et al., 1992; Sairenji et al., 1995). However, other studies show no difference in the frequency of HHV-6 infection between patient and control groups (Wallace et al., 1999; Reeves et al., 2000; Cameron et al., 2010). Important is a fact that some study cohorts are too small to draw general conclusions about association of a virus infection with ME/CFS. HHV-6A is detected in only one patient with ME/CFS showing that HHV-6B is prevalent among ME/CFS patients in Latvia. Similarly, in another study HHV-6B is more present (75%) than HHV-6A (9.7%) in patients with ME/CFS (Burbelo et al., 2012). Controversial, in other studies HHV-6A is more prevalent in patients with ME/CFS but HHV-6B – in controls (Di Luca et al., 1995; Ablashi et al., 2000). These differences can be explained by geographic location, because another study in Latvia also reports on the detection of HHV-6B in Latvian patients with other diseases, like autoimmune thyroiditis (Sultanova et al., 2017).

The first gens, which are transcribed after HHV-6 infection are immediate early (IE) genes. IE U89/90 α-gene is expressed in 78% of analysed patients with the presence of HHV-6 showing HHV-6 transcription, as well as optimization of a cell for virus gene expression and replication in these patients with ME/CFS (Mirandola et al., 1998; De Bolle et al., 2005). The detection of specific mRNA is not always a marker for new protein synthesis. Accumulation of transcripts can occur prior to protein synthesis (Mirandola et al., 1998). Moreover, mRNA is not detected in all cases due to potential replication site in

lymphoid tissue or other organs, instead of PBMCs (Van den Bosch et al., 2001).

HHV-6 reactivation and replication in these ME/CFS patients are proven by the detection of HHV-6 early and late proteins with virus-specific monoclonal antibodies. In our study analysing PBMCs from 36 patients, in six patients early p41 antigen expression is found. Moreover, HHV-6B reactivation is identified by detection of gp100 expression in 15 patients with ME/CFS. Furthermore, in seven patients with ME/CFS gp116 expression is revealed, which reacts with HHV-6A and HHV-6B and is associated with virus replication (De Bolle et al., 2005).

The analysis of a virus copy number by real-time PCR reveals that in patients with a persistent HHV-6 infection in an active phase the viral load is almost seven times higher than in patients with a persistent infection in a latent phase ($p = 0.0019$). The detection of elevated viral load in all patients with a persistent HHV-6 infection in an active phase as well as a significantly higher viral load in patients with an active infection suggests the usage of an elevated viral load as a marker to distinguish latent from active infection phase.

During an active infection, the virus replicates through a rolling circle mechanism and maintains the viral genome as a circular episome. Some patients with HHV-6 related diseases have very high viral loads that can show chromosomally integrated HHV-6 instead of high level of viral replication. Therefore, in patient treatment and association of the virus with this disease it is important to distinguish an active viral infection from integration (Clark, 2016). Telomeric repeats at the end of HHV-6 genome allow integration into host cell telomeres during homologous recombination (Kaufer and Flamand, 2014). Integration in germ line cells can be transmitted to progeny, therefore the virus integrates in a specific chromosome of every nucleated cell (Gravel et al., 2015). It is proved that germ line integrated HHV-6 can be activated to a transmissible infectious form in cell culture (Prusty et al., 2013 b) and can

produce infectious virions in patients with immunodeficiency (Endo et al., 2014). Literature data suggest HHV-6 integration in case of detected virus genome copy in every cell of the body (Gravel et al., 2015). In this study possible HHV-6 chromosomal integration is suspected in six ME/CFS patients, due to a high viral load – more than one HHV-6 copy per cell [median (IQR) 1209033 (1464421–808183) copies/10^6 cells].

Dr. Prusty with colleagues analysed cells from patients with detected HHV-7 sequences in DNA isolated from peripheral blood and demonstrate telomeric integration of HHV-7 into chromosomes (Prusty et al., 2017). HHV-7 chromosomal integration was estimated in one Latvian ME/CFS patient with more than one copy of HHV-7 per cell (1140127.6 copies/10^6 cells) and confirmed with HHV-7 load detection in DNA from hair follicle (1188811.8 copies/10^6 cells), as well as with fluorescence in situ hybridisation analysis by Prusty et al., 2017. Germ line integration of HHV-7 was confirmed by the detection of more than two HHV-7 copies per cell (2591031.6 HHV-7 copies/10^6 cells) in patient's mother's hair follicle DNA.

In our study, HHV-7 specific IgG class antibodies are detected in 84.6% of ME/CFS patients and 93.8% of apparently healthy individuals corresponding to HHV-7 seroprevalence of around 90% among worldwide adult population (Caselli and Di Luca, 2007). The frequency of a persistent HHV-7 infection in a latent phase is similar between patients with ME/CFS (58%) and apparently healthy individuals (67.3%) ($p = 0.0766$) while a persistent HHV-7 infection in an active phase is more often found in patients with ME/CFS (34%) than apparently healthy individuals (8%) ($p < 0.0001$). Moreover, self-assembled HHV-7 U57 gene, which forms an icosahedral capsid, is expressed in 45.7% of analysed patients with HHV-7, indicating on active replication. Worldwide studies on HHV-7 in patients with ME/CFS are very scarce. Some find HHV-7 specific antibodies in 91.4% of patients and 88% of controls, whereas some in

all ME/CFS patients and 88% of controls (Sairenji et al., 1995; Ablashi et al., 2000).

Patients with a persistent HHV-7 infection in an active phase have a higher viral load than with a latent infection, though without statistical significance ($p = 0.3502$). The data published by Oakes et al., coincides with our results – they also do not find any statistical difference between HHV-7 copy number in patients and controls DNA isolated from PBMCs and saliva (Oakes et al., 2013).

Recently published research data also support the hypothesis on herpesviruses involvement in ME/CFS development due to expression of herpesviruses encoded deoxyuridine triphosphate nucleotidohydrolases – dUTPases that activates humoral immune response (Halpin et al., 2017). The percentage of HHV-6, HHV-7 and co-infection is estimated to be similar between patients and controls, nevertheless HHV-7 is revealed twice as much as HHV-6 (Wallace et al., 1999). Elsewhere differences in detection of HHV-6, HHV-7 and B19V in twins with and without ME/CFS are not observed (Koelle et al., 2002). Furthermore, Fremont with colleagues estimate similar amount of HHV-6 and HHV-7 positive cases with high loads in gastric and intestinal mucosa tissue from patients and donors. In the same work, B19V is detected significantly more in ME/CFS than in control group (Fremont et al., 2009).

Previous studies by colleagues in our laboratory demonstrate the reactivation of HHV-6 and HHV-7 in patients with ME/CFS (Chapenko et al., 2006). The analysis on the presence of herpesviruses genomic sequences in healthy blood donors in Latvia are also conducted (Kozireva et al., 2001). Subsequently, an active HHV-6, HHV-7 and B19V infection and a simultaneous dual or triple infection of these viruses is present in patients with ME/CFS (Chapenko et al., 2012).

Analysing co-infections of HHV-6/HHV-7/B19V in this study, a persistent infection/co-infection is more frequently found in patients with ME/CFS (96.5%) than apparently healthy individuals (85.3%) ($p = 0.0003$). From them a persistent infection/co-infection in a latent phase is revealed in half of patients with ME/CFS (51.1%) and ¾ of apparently healthy individuals (76.7%) ($p < 0.0001$). However, a persistent infection/co-infection in an active phase is present significantly more often in patients (45%) than in apparently healthy individuals (8.7%) ($p < 0.0001$), showing the relevance of an active viral infection in ME/CFS.

Notably, single B19V infection is detected in only one patient and two apparently healthy individuals from this cohort. Considering that herpesviruses can be helper viruses for subfamily of parvoviruses – dependoviruses replication, hypothetically they could serve as triggers for B19V infection (Streiter et al., 2011).

The data of this study demonstrate no differences in the frequency of a persistent single infection in an active phase among patients with ME/CFS and apparently healthy individuals, whereas a persistent double HHV-7+B19V infection in an active phase is observed significantly more patients compared to apparently healthy individuals ($p = 0.0002$). Moreover, an active double HHV-6+HHV-7 and active triple co-infection is found only in patients with ME/CFS ($p < 0.0001$ and $p < 0.0001$, respectively), distinctly indicating on the involvement of an active co-infection in the development of ME/CFS. HHV-6 and B19V load is significantly higher in patients with an infection/co-infection in an active than in a latent phase ($p = 0.0251$ and $p = 0.0444$, respectively). In addition, HHV-7 load is higher in patients with a severe compared to a moderate course of ME/CFS, therefore it could be linked with symptoms severity ($p = 0.0254$). Other researchers have not analysed viral loads according to co-infections, though they find a similar tendency of higher HHV-7 prevalence and load, as well as a higher B19V frequency in patients with

ME/CFS compared to controls suggesting B19V involvement in ME/CFS. Controversial to our research, they do not determine the difference of HHV-6 frequency and load in patients and controls (Fremont et al., 2009).

An active virus may be undetectable in many patients' body fluids because of possible latency in other tissues than peripheral blood (Hüfner et al., 2007). Therefore, although the presence of a virus infection is not detected in all cases of ME/CFS, it could form a subgroup of this disease. Similarly, endocrine, immunological, psychosocial, genetic factors and factors predisposing oxidative stress are considerable in dividing patients into subgroups (Sanders and Korf, 2008).

It is hypothesized that ME/CFS could be caused by neurotropic viruses, like HHV-6 and HHV-7, which can infect neurons and immune cells to impair CNS capillaries and micro-arteries, leading to brain damage. This infection initiates immune system disturbances that in its turn can lead to a chronic infection. Immunosuppression and activated immune complexes may cause chronic inflammation, which facilitates the establishing of a persistent infection (Krueger and Ablashi, 2006; Broderick et al., 2010; Glassford, 2017). Furthermore, chronic immune system activation is accompanied by alterations in regulation of cytokine production (Sairenji and Nagata, 2007).

Cytokines are small proteins, involved in cell signalling, mostly providing a balance between humoral and cell mediated immune response. Pro-inflammatory cytokines ensure systemic inflammation and anti-inflammatory – inhibition of pro-inflammatory cytokines (Mensah et al., 2017). The analysis of cytokine levels in this study reveals that patients with ME/CFS have elevated levels of pro-inflammatory (IL-6, TNF-α and IL-12) and anti-inflammatory (IL-10) cytokines comparing to apparently healthy individuals. Moreover, ME/CFS patients with a persistent infection in an active phase have higher levels of cytokines than patients with a persistent infection in a latent phase, however not in all cases statistical difference is confirmed. In

contrary, the level of IL-4 is not elevated in patients with ME/CFS, what is supported by other studies where no difference is found in the level of IL-4 between ME/CFS patients and healthy controls (Nakamura et al., 2010; Brenu et al., 2011; Lidbury et al., 2017). Elsewhere IL-4 is found to be higher in women with this disease compared to women in control group (Fletcher et al., 2009).

The inconsistency among studies on cytokine levels in patients with ME/CFS are explained by variations in patient and control recruitment in terms of diagnostic criteria, onset, duration and phase of the disease, as well as time of sample collection and used laboratory methods (Mensah et al., 2017). Findings on increased level of several cytokines after exertion are found in patients with severe symptom flare (White et al., 2010). In addition, disease duration for more than three years is reported to impact immune signatures (Hornig et al., 2015).

It is shown that HHV-6 can infect monocytes/macrophages and inflammatory cytokines can contribute in the reactivation of this virus from a latent phase (Aoki et al., 2016). Viral infection induced prolonged state of immune disbalance accompanied by changes in cytokine level can lead to the development of ME/CFS clinical symptoms. Findings in this study correspond to Brenu et al., findings of Th1/Th2 cytokine response imbalance that is reflected by increased levels of TNF-α and IL-10, suggesting a persistent chronic infection (Couper et al., 2008; Brenu et al., 2011).

Despite frequent findings of elevated IL-6 in patients with active single HHV-6 and B19V infection, analysing the level of cytokines in case of co-infections, no difference is confirmed in the level of IL-6 among patient groups without infection, with a latent infection/co-infection, active single, double and triple co-infection ($p = 0.1289$). Though a significant difference is revealed in levels of TNF-α, IL-12 and IL-10 among five above mentioned groups ($p = 0.0492$, $p = 0.0063$ and $p = 0.0023$, respectively). Our study results

are in accordance with those in the published report on equally raised IL-6 level without any difference between patients' and donors' group (Brenu et al., 2011). Other researchers also do not find differences in the level of IL-6 amongst ME/CFS and control cases (Ter Wolbeek et al., 2007; Lidbury et al., 2017). Further analysis discloses a higher level of IL-6 in patients with an active double co-infection than in patients with a latent infection/co-infection and without infection ($p = 0.0319$ and $p = 0.0418$, respectively). Despite the fact that a level of IL-6 is elevated only slightly, the results show differences among patients with a persistent co-infection in latent and in active phase, which can be observed only analysing certain ME/CFS patients groups with co-infection. Other studies also report on a raised level of IL-6 in patients with ME/CFS (Fletcher et al., 2009). Taking into account that average onset of ME/CFS among patients included in this study is 10.2 ± 4.2 months, discrepant results can be explained by a difference in the duration of disease. Findings of high IL-6 level concern older patients with the duration of ME/CFS for more than two years but a low level of IL-6 concern younger patients with a recent occurrence of disease (early disease) (Russell et al., 2016).

Particularly higher level of TNF-α is found in patients with an active triple co-infection if compared to a latent infection/co-infection and an active double co-infection, presenting a role of an active co-infection with multiple viruses in increase in TNF-α level, which indicates on inflammation that could be caused by a viral infection ($p = 0.0045$ and $p = 0.0158$, respectively). A study by Brenu and co-authors shows a higher level of TNF-α in ME/CFS patients, however some authors do not find any difference between patients and controls (Fletcher et al., 2009; Nakamura et al., 2010; Brenu et al., 2011; Lidbury et al., 2017). It is shown that low-level inflammation and activation of cell-mediated immunity is observed in ME/CFS cases and the high level of TNF-α correlates with several clinical symptoms, therefore an increase of

inflammatory mediators might explain why these disease symptoms exist (Maes et al., 2012).

Similarly, in case of an active triple co-infection the level of IL-12 is more elevated than in a latent infection/co-infection, active single and active double infection cases ($p = 0.0003$, $p = 0.0125$, $p = 0.0195$, respectively). The same tendency in IL-12 level is observed between patients with an active triple co-infection and without infection ($p = 0.0636$). Elevated level of IL-12 is admitted to have a good biomarker potential in ME/CFS (Fletcher et al., 2009). Russell and co-workers also record increased expression of IL-12 in their study (Russell et al., 2016). In contrary, elsewhere a decreased level of IL-12 is reported (Visser et al., 2001).

Significantly higher level of IL-10 is observed in patients with an active double co-infection compared to patients without infection and with a latent infection/co-infection ($p = 0.029$ and $p = 0.0035$, respectively). In addition, ME/CFS patients with an active triple co-infection have a higher level of IL-10 in comparison to patients without infection, with a latent infection/co-infection and an active single infection ($p = 0.0107$, $p = 0.0034$ and $p = 0.0321$, respectively). The same tendency of IL-10 level increase in patients with ME/CFS is presented in several studies (Visser et al., 2001; Ter Wolbeek et al., 2007; Nakamura et al., 2010; Brenu et al., 2011). However, some researchers find a similar level of IL-10 in patients and controls, but some find a higher level of IL-10 even in healthy controls (Kavelaars et al., 2000; Patarca, 2001; Fletcher et al., 2009; Lidbury et al., 2017).

Cytokines can serve as markers for virus induced changes in cell immunity and an elevated level of certain cytokines can be associated with inflammation caused by a virus infection (Nastke et al., 2012). The data of this study show that the level of cytokines is changed significantly in case of co-infections. Moreover, patients with an active co-infection demonstrate a higher level of pro-inflammatory and anti-inflammatory cytokines. It is proved,

that the level of pro-inflammatory cytokines correlates with the severity of ME/CFS and sleep disturbances (Milrad et al., 2017). Besides, the level of TNF-α in patients with ME/CFS correlates with a degree of fatigue (Bansal et al., 2012). In our study a level of TNF-α, IL-12 and IL-10 is statistically significantly higher in patients with a severe course of ME/CFS compared to those with a moderate course ($p = 0.0434$, $p = 0.0494$ and $p = 0.025$). Inversely, the level of IL-6 tends to be higher in patients with moderate severity of the disease ($p = 0.0506$). Stress and fatigue are estimated to be greater in patients with an elevated level of IL-6 (Lattie et al., 2012). A moderate course of ME/CFS is experienced by most of the patients in this study (81.3%) that could be because of the level of IL-6, which is not significantly elevated.

It is possible that a virus infection causes a cellular immunity dysfunction, which induces virus reactivation. Subsequently, viral proteins facilitate cytokine secretion, resulting in emergence of ME/CFS typical symptoms, such as fatigue, fever, sleep and cognitive disorders (Bansal et al., 2012). Chronic pain can be caused by inflammatory signals that are spread by glial cells, whereas inflammatory cytokines and neuronal stimulation can activate glial cells (Yasui et al., 2014; Glassford, 2017).

Besides chronic fatigue for more than six months, which all patients with diagnosed ME/CFS have, impaired memory, decreased concentration and sleep disturbances are the most frequently observed symptoms in these patients. The presence of typical ME/CFS symptoms is reported more frequently among patients in the Netherlands and the United Kingdom. Besides these symptoms, cognitive dysfunction, sleep disturbances and post-exertional malaise are most frequent reported and are acknowledged to be essential symptoms of ME/CFS (Collin et al., 2016).

The analysis of single HHV-6, HHV-7 and B19V infection does not reveal any statistically significant differences in occurrence of typical ME/CFS clinical symptoms among patients with a persistent infection in a latent and an

active phase. Though, patients with B19V genomic sequence and antibodies to NS1 protein significantly more frequently have multi-joint pain (55%) than patients' with B19V genomic sequence and without NS1 antibodies (21.1%) ($p = 0.0294$). Muscle pain and lymphadenopathy are more frequently observed in patients with (65% and 65%) than without (42.1% and 31.6%) presence of NS1 antibodies ($p = 0.1517$ and $p = 0.0369$, respectively). Such B19V associated ME/CFS clinical manifestations as fatigue, lymphadenopathy, joint and muscle pain could be consequences to B19V infection. Reports are published on B19V as a cause of ME/CFS typical clinical symptoms, therefore in some studies B19V is reported to be one of the trigger factors for at least part of ME/CFS patients that corresponds to the results of our study (Matano et al., 2003; Appel et al., 2007; Fremont et al., 2009).

Further analysing the occurrence of ME/CFS typical symptoms in case of HHV-6/HHV-7/B19V co-infection, a significant difference is not found between the groups of patients with a persistent co-infection in latent and active phase. However, multi-joint pain is observed in more patients with a persistent single infection in latent than in active phase ($p = 0.0452$) and in more patients with a persistent double and triple infection in active than in latent phase, though without statistical significance. In addition, headache of a new type is present in more patients with a persistent triple co-infection in latent than in active phase ($p = 0.0052$). Subfebrility is present in more patients with a persistent single, double and triple co-infection in active than in latent phase, though predominance is not statistically significant. Taking into account that diagnosis of ME/CFS was set up at least six months after the onset of the first symptoms and samples were collected even longer period after onset of ME/CFS, a clear difference between a persistent infection in latent and active phase is difficult to identify. These results may indicate a possible role of a persistent infection in ME/CFS development or consequences of a virus infection that could be in an active phase during the onset of this disease and is

in a latent phase on sample collection time, although the clinical symptoms persist.

A lack of statistical differences comparing clinical symptoms between groups of ME/CFS patients with persistent infection in latent and active phase could also be due to the diagnostic criteria – all patients have to have chronic fatigue for at least six months and four out of eight typical ME/CFS symptoms, therefore a variation of symptom frequency is limited. Noteworthy is also a fact that various numbers of patients – from six up to 54 patients, in analysed groups with latent and active single, double and triple infection that affects statistical analysis.

The frequency of ME/CFS clinical symptoms also is different between various countries and depends on patient characteristics, comorbidities and patient-reported measures. It is still not clear whether a group of symptoms leads to a chronic illness or symptoms are developed during chronic illness (Collin et al., 2016). Such flu-like symptoms as fatigue, joint, muscle and extremities pain, tender lymph nodes and headache are present not just in a majority of patients in other studies, but also in many patients from this study. Friedberg with colleagues report immune and viral factors as the most frequent causes of ME/CFS in patients with short and long duration of this illness, whereas persistent stress as second most frequent etiological factor for ME/CFS is mentioned (Friedberg et al., 2000).

It is necessary to conduct longitudinal studies in order to assess immune functions and symptom severity variations over time, what in some studies already is showed (Hardcastle et al., 2015; Mensah et al., 2017). Moreover, results should be compared not only between ME/CFS patients and controls, but also with other comorbidities, like multiple sclerosis or depression to assess specificity of suggested biomarkers. A biomarker must be selected considering costs and a possibility to use it in clinics, because expensive and very complex methods will most likely not be incorporated in routine practice (Fischer et al.,

2014). Therefore, chosen methods in this study are cost-effective for routine analysis in laboratories. Controlled trials in future will enable assessment of antiviral therapy and resulting in clinical improvement will approve association with the disease (Clark, 2016). Considering ME/CFS heterogeneity, use of biomarkers will enable to define subtypes of the disease. In addition, longitudinal and standardized studies determining phenotype and measures of ME/CFS course and therapy effectiveness with follow-up measurements in dynamics should be accomplished. This will allow prognosis of the disease development and promote development of a specific definition for diagnostics and a treatment plan (Fischer et al., 2014).

4. CONCLUSIONS

1. No evidence of XMRV infection in patients with ME/CFS and apparently healthy individuals is found, therefore the hypothesis on XMRV association with ME/CFS development is denied.

2. Persistent HHV-6 and HHV-7 infection in an active phase is presented significantly more frequently and with a higher viral load among patients with ME/CFS than apparently healthy individuals, and HHV-6B is prevalent in Latvian ME/CFS patients.

3. A more common finding of B19V (genotype 1) active infection with a higher B19V load in ME/CFS patients than in apparently healthy individuals and the coincidence of the infection time with the onset of the disease symptoms point to B19V as a possible trigger factor in ME/CFS development.

4. HHV-6, HHV-7 and B19V persistent co-infection in an active phase is significantly more widespread among patients with ME/CFS compared to healthy donors and is characterized by a higher a viral load and level of cytokines in comparison to the latent phase of infection. Therefore, markers of HHV-6, HHV-7 and B19V infection could be used as one of biomarkers in ME/CFS diagnostics.

5. The level of cytokines is elevated in patients with ME/CFS indicating immune response to inflammation that could be caused by a viral infection. Also persistent HHV-6, HHV-7 and B19V co-infection in an active phase might significantly influence elevation of pro-inflammatory and anti-inflammatory cytokine levels, which can lead to immune disturbances and the development of ME/CFS symptoms.

6. ME/CFS patients with viral infection markers are more likely to disclose the clinical symptoms of the disease defined in the diagnostic criteria, which are also common with respect to the appropriate virus infection.

7. A higher HHV-6 and HHV-7 load and a significantly elevated level of pro-inflammatory cytokines TNF-α, IL-12 and anti-inflammatory cytokine IL-10 in patients with a more severe ME/CFS clinical course advocate on the involvement of these viral infections in ME/CFS development.

5. RECOMMENDATIONS

1. To improve knowledge of general practitioners in Latvia about ME/CFS to assure recognition and proper diagnostics of this illness.

2. To inform society about ME/CFS existence and characteristics to increase tolerance and understanding of this disease.

3. To use more than one ME/CFS case definition in diagnostics to assure sensitivity and specificity of diagnosis.

4. To analyse the presence of active HHV-6, HHV-7 and B19V infection markers in patients with ME/CFS to assess potential etiological factors and manage treatment strategies according to disease severity and infection activity phase, considering necessity of antiviral treatment application.

6. LIST OF PUBLICATIONS

6.1. Papers in journals included in the international databases

1. Chapenko, S., Krumina, A., Logina, I., **Rasa, S**., Chistjakovs, M., Sultanova, A., Viksna, L. and Murovska, M. Association of active human herpesvirus-6, -7 and parvovirus B19 infection with clinical outcomes in patients with myalgic encephalomyelitis/chronic fatigue syndrome. *Advances in Virology*. 2012, 2012, 1–7.
2. **Rasa, S**., Nora-Krukle, Z., Chapenko, S., Krumina, A., Roga, S., Murovska, M. No evidence of XMRV provirus sequences in patients with myalgic encephalomyelitis/chronic fatigue syndrome and individuals with unspecified encephalopathy. *New Microbiologica*. 2014, 37, 17–24.
3. Prusty, B. K., Gulve, N., **Rasa, S**., Murovska, M., Hernandez, P. C., Ablashi, D. V. Possible Chromosomal and Germline Integration of Human Herpesvirus 7 (HHV-7). *The Journal of General Virology*. 2017, 98 (2), 266–274.

6.2. Papers in other journals and collections of articles

1. **Rasa, S**., Chapenko, S., Krumina, A., Chistyakovs, M., Viksna, L., Logina, I., Gintere, S., Murovska, M. Role of beta-herpesviruses infection in the development of chronic fatigue syndrome/myalgic encephalomyelitis. *Immunomodulating Human Herpesviruses and their Role in Human Pathologies*. 2011, 16–20.
2. Chistyakovs, M., Čapenko, S., Sultanova, A., **Rasa, S**., Krūmiņa, A., Murovska, M. Beta-herpesviruses HHV-6 and HHV-7 infection and cytokines level changes in plasma from patients with chronic fatigue syndrome. *Collection of Scientific Papers: Research articles in medicine & pharmacy, 2010: Medical Basic Sciences*. 2011, 2, 121–125.
3. **Rasa, S**., Čapenko, S., Krūmiņa, A., Kozireva, S., Murovska, M. Association of parvovirus B19 with chronic fatigue syndrome. Collection of Scientific Papers: Research articles in medicine & pharmacy, 2011: Internal Medicine. Surgery. Medical Basic Sciences. Stomatology. Pharmacy. 2012, 1, 217–224.
4. **Rasa, S**., Čapenko, S., Krūmiņa, A., Nora-Krūkle, Z., Murovska, M. Xenotropic murine leukemia virus related virus, human herpesvirus-6, herpesvirus-7 and parvovirus B19 association with chronic fatigue syndrome/myalgic encephalomyelitis. *Collection of Scientific Papers: Research articles in medicine & pharmacy, 2012: Internal Medicine. Surgery. Medical Basic Sciences. Stomatology. Pharmacy*. 2013, 2, 242–249.
5. Krumina, A., Vasiljeva, G., Ivanovs, A., Gintere, S., Kovalchuka, L., **Rasa, S**., Chapenko, S., Murovska, M., Viksna, L, Logina, I.

Assessment of Value of Fatigue Severity and Symptoms in Patients with Chronic Fatigue Syndrome/Myalgic Encephalomyelitis and Fibromyalgia. *British Journal of Medicine & Medical Research*. 2014, 4 (36), 5866–5877.

6. **Rasa, S**., Čapenko, S., Krūmiņa, A., Nora-Krūkle, Z., Roce, A., Murovska, M. Interleukin-10 level in blood plasma in case of beta-herpesvirus co-infection in patients with myalgic encephalomyelitis/chronic fatigue syndrome. *Collection of Scientific Papers: Research articles in medicine & pharmacy, 2013: Internal Medicine. Surgery. Medical Basic Sciences. Stomatology. Pharmacy*. 2014, 204–211.
7. **Rasa, S**., Chapenko, S., Vanaga, A., Mihailova, M., Logina, I., Krumina, A., Murovska, M. Human parvovirus B19 infection status in patients with myalgic encephalomyelitis/chronic fatigue syndrome and fibromyalgia. *Collection of Scientific Papers: Research articles in medicine & pharmacy, 2014: Internal Medicine. Surgery. Medical Basic Sciences. Stomatology. Pharmacy*. 2014, 55–63.

6.3. Presentations at international conferences/congresses

1. Logina, I., Krumina, A., Chapenko, S., **Rasa, S**., Chistyakov, M., Sultanova, A., Murovska, M. Pain in chronic fatigue syndrome/myalgic encephalomyelitis patients: relation to active HHV-6, HHV-7 and parvovirus B19 infection/co-infection. *European Journal of Pain Supplements 5*, DOI:10.1016/S1754-3207(11)70434-5. Abstract of Pain in Europe VII. The Congress of the European Federation of IASP. Hamburg, Germany, September 21–24, 2011, pp.127.
2. Murovska, M., Chapenko, S., Krumina, A., Logina, I., **Rasa, S**., Chistyakov, M., Sultanova, A., Viksna, L. Presence of Active HHV-6, HHV-7 and Parvovirus B19 infection/Co-infection in patients with Chronic Fatigue Syndrome/Myalgic Encephalomyelitis. *CFS/ME Biennial International Conference Translating Evidence into Practice Syllabus*. Canada, Ontario, Ottawa, September 22–25, 2011.
3. **Rasa, S**., Chapenko, S., Nora-Krukle, Z., Krumina, A., Murovska, M. Detection of XMRV, HHV-6 and HHV-7 genomic sequences in blood of patients with chronic fatigue syndrome/myalgic encephalomyelitis. *22nd Annual Meeting of the Society for Virology programme*. Essen, Germany, March 14–17, 2012, pp. 543.
4. **Rasa, S**., Nora-Krukle, Z., Chapenko, S., Krumina, A., Murovska, M. Detection of xenotropic murine leukemia virus-related gene sequences in blood of patients with chronic fatigue syndrome/myalgic encephalomyelitis in Latvia. *International Medical Meeting Abstract Book*. Riga, Latvia, September 7–9, 2012, pp. 48.
5. **Rasa, S**., Krumina, A., Nora-Krukle, Z., Chapenko, S., Murovska, M. Association of human herpesvirus-6 and 7 and parvovirus B19 with chronic fatigue syndrome/myalgic encephalomyelitis. *School of*

Translational Immunology Abstract Book. Belgrade, Serbia, September 19–21, 2012, pp. 149.

6. **Rasa, S**., Chapenko, S., Nora-Krukle, Z., Krumina, A., Viksna, L., Murovska, M. Co-infection of HHV-6 and HHV-7 in patients with myalgic encephalomyelitis/chronic fatigue syndrome. *8th International Conference on HHV-6&7 Program Book*. Paris, France, April 8–10, 2013, pp.76.
7. **Rasa, S**., Chapenko, S., Krumina, A., Viksna, L., Murovska, M. Occurrence of typical clinical symptoms and markers of human parvovirus B19 infection in patients with myalgic encephalomyelitis/chronic fatigue syndrome. *IACFS/ME 11th Biennial International Conference Translating Science into Clinical Care Syllabus*. San Francisco, California, USA, March 20–23, 2014, pp. 24.
8. **Rasa, S**., Chapenko, S., Vanaga, A., Mihailova, M., Krumina, A., Logina, I., Murovska, M. Serologically approved parvovirus B19 infection status in viremic cases of patients with myalgic encephalomyelitis/chronic fatigue syndrome and fibromyalgia. *15th Biennial International Parvovirus Workshop Abstract Book*. Bordeaux, France, June 22–26, 2014, P-15.
9. Murovska, M., **Rasa, S**., Cistjakovs, M., Roga, S., Krumina, A., Logina, I., Chapenko, S. Possible involvement of human herpesvirus-6 infection in the development of myalgic encephalomyelitis/chronic fatigue syndrome, fibromyalgia and encephalopathy. *International Union of Microbiological Societies Congresses Abstracts Resumes*. Montreal, Canada, July 27–August 1, 2014, pp. 613.
10. **Rasa, S**., Chapenko, S., Zazerska, Z., Krumina, A., Murovska, M. Evidence of human parvovirus B19 infection in patients with myalgic encephalomyelitis/chronic fatigue syndrome. *Program & Poster Abstracts Thirty-First Annual Clinical Virology Symposium and Annual Meeting of the Pan American Society for Clinical Virology*. 31st Clinical Virology Symposium. Daytona Beach, USA, April 26–29, 2015, pp. 90–91.
11. **Rasa, S**., Chapenko, S., Zazerska, Z., Vilmane, A., Krumina, A., Murovska, M. Level of TNF-alpha in myalgic encephalomyelitis/chronic fatigue syndrome patients with beta-herpesviruses infection. *1st Baltic Conference Immunological modelling: theory and practice programme and abstracts*. Riga, Latvia, May 13–15, 2015, pp 88–89.
12. Murovska, M., Nora-Krukle, Z., Gravelsina, S., **Rasa, S**., Zazerska, Z., Chapenko, S., Lin, Y. C., Lin, J. H., Liu, H. F. Parvovirus B19 detection frequency and genotype analysis in various Latvian patients' cohorts. *XVIth International Parvovirus Workshop abstracts*. Ajaccio, Corsica, France, June19–23, 2016, pp. 77.
13. **Rasa, S**., Chapenko, S., Krumina, A., Zazerska, Z., Murovska, M. Occurrence, phase and status of human parvovirus B19 infection in patients with myalgic encephalomyelitis/chronic fatigue syndrome. *Journal of Clinical Virology*, Abstracts of the 19th Annual Meeting of

the European Society for Clinical Virology. Lisbon, Portugal, September 14–17, 2016, 82, S141.

6.4. Presentations at local conferences/congresses

1. Svilpe, S., Krūmiņa, A., Čistjakovs, M., **Rasa, S**., Čapenko, S., Murovska, M., Logina, I. Evaluation of neurological symptoms in patients with myalgic encephalomyelitis/chronic fatigue syndrome. *Rīga Stradiņš University scientific conference abstracts 2010*. Riga, Latvia, March 18–19, 2010, pp. 161.
2. Chistyakovs, M., **Rasa, S**., Chapenko, S., Krumina, A., Sultanova, A., Murovska, M. Plasma Levels of Various Cytokines and Activation of Immunomodulating Beta-herpesviruses in Patients with Chronic Fatigue Syndrome. *Rīga Stradiņš University scientific conference abstracts 2010*. Riga, Latvia, March 18–19, 2010, pp. 196.
3. **Rasa, S**., Čapenko, S., Krūmiņa, A., Kozireva, S., Murovska, M. Association of Parvovirus B19 with chronic fatigue syndrome. *Rīga Stradiņš University scientific conference abstracts 2011*. Riga, Latvia, April 14–15, 2011, pp. 220.
4. Krūmiņa, A., Logina, I., Svilpe, S., Gintere, S., Čapenko, S., **Rasa, S**., Čistjakovs, M., Sultanova, A., Vīksna, L., Murovska, M. Findings of active HHV-6, HHV-7 and parvovirus B19 co-infections in patients with myalgic encephalomyelitis/chronic fatigue syndrome. 3rd United World Latvian scientists congress and 4th Letonika congress Medicine section *"Medical science and Latvian public health in XXI century" abstracts*. Riga, Latvia, October 24–27, 2011, pp. 54–55.
5. **Rasa, S**., Čapenko, S., Krūmiņa, A., Nora-Krūkle, Z., Roce, A., Murovska, M. Interleukin-10 expression level in case of beta-herpesviruses co-infection in patients with myalgic encephalomyelitis/chronic fatigue syndrome. *Rīga Stradiņš University scientific conference abstracts 2013*. Riga, Latvia, March 21-22, 2013, pp. 189.
6. **Rasa, S**., Čapenko, S., Nora-Krūkle, Z., Mihailova, M., Logina, I., Krūmiņa, A., Murovska, M. Human parvovirus B19 infection status in patients with myalgic encephalomyelitis/chronic fatigue syndrome and fibromyalgia. *Rīga Stradiņš University scientific conference abstracts 2014*. Riga, Latvia, April 10–11, 2014, pp. 207.
7. **Rasa, S**., Čapenko, S., Zazerska, Z., Krūmiņa, A., Murovska, M. Human parvovirus B19 infection occurrence and load in patients with myalgic encephalomyelitis/chronic fatigue syndrome and apparently healthy individuals. *Rīga Stradiņš University scientific conference abstracts 2015*. Riga, Latvia, March 26–27, 2015, pp. 206.

7. REFERENCES

1. Ablashi, D. V., Eastman, H. B., Owen, C. B. et al. Frequent HHV-6 reactivation in multiple sclerosis (MS) and chronic fatigue syndrome (CFS) patients. *Journal of Clinical Virology*. 2000, 16 (3), 179–191.
2. Albright, F., Light, K., Light, A. et al. Evidence for a heritable predisposition to Chronic Fatigue Syndrome. *BMC Neurology*. 2011, 11: 62.
3. Aoki, R., Kobayashi, N., Suzuki, G. et al. Human herpesvirus 6 and 7 are biomarkers for fatigue, which distinguish between physiological fatigue and pathological fatigue. *Biochemical and Biophysical Research Communications*. 2016, 478 (1), 424–430.
4. Appel, S., Chapman, J., Shoenfeld, Y. Infection and vaccination in chronic fatigue syndrome: myth or reality? *Autoimmunity*. 2007, 40 (1), 48–53.
5. Bakken, I. J., Tveito, K., Gunnes, N. et al. Two age peaks in the incidence of chronic fatigue syndrome/myalgic encephalomyelitis: a population-based registry study from Norway 2008–2012. *BMC Medicine*. 2014, 12, 167.
6. Bansal, A. S., Bradley, A. S., Bishop, K. N. et al. Chronic fatigue syndrome, the immune system and viral infection. *Brain, Behavior and Immunity*. 2012, 26 (1), 24–31.
7. Barah, F., Vallely, P. J., Chiswick, M. L. et al. Association of human parvovirus B19 infection with acute meningoencephalitis. *Lancet*. 2001, 358, 729–730.
8. Barah, F., Whiteside, S., Batista, S., Morris, J. Neurological aspects of human parvovirus B19 infection: a systematic review. *Reviews in Medical Virology*. 2014, 24 (3), 154–168.
9. Berneman, Z.N., Gallo, R. C., Ablashi, D. V. et al. Human herpesvirus 7 (HHV-7) strain JI: independent confirmation of HHV-7. *Journal of Infectious Diseases*. 1992, 166, 690–691.
10. Brenu, E. W., van Driel, M. L., Staines, D. R. et al. Immunological abnormalities as potential biomarkers in Chronic Fatigue Syndrome/Myalgic Encephalomyelitis. *Journal of Translational Medicine*, 2011, 9, 81.
11. Broderick, G., Fuite, J., Kreitz, A. et al. A formal analysis of cytokine networks in Chronic Fatigue Syndrome. *Brain, Behavior, and Immunity*. 2010. 24 (7), 1209–1217.
12. Buchwald, D., Cheney, P. R., Peterson, D. L. et al. A chronic illness characterized by fatigue, neurologic and immunologic disorders, and active human herpesvirus type 6 infection. *Annals of Internal Medicine*. 1992, 116 (2), 103–113.
13. Burbelo, P. D., Bayat, A., Wagner, J. et al. No serological evidence for a role of HHV-6 infection in chronic fatigue syndrome. *American Journal of Translatioinal Research*. 2012, 4 (4), 443–451.
14. Cameron, B., Flamand, L., Juwana, H. et al. Serological and virological investigation of the role of the herpesviruses EBV, CMV and HHV-6 in post-infective fatigue syndrome. *Journal of Medical Virology*. 2010, 82 (10), 1684–1688.

15. Campadelli-Fiume, G., Mirandola, P., Menotti, L. Human Herpesvirus 6: An Emerging Pathogen. *Emerging Infectious Diseases*. 1999, 5, 353–366.
16. Carruthers, B. M., van de Sande, M. I., De Meirleir, K. L. et al. Myalgic Encephalomyelitis: International Consensus Criteria. *Journal of Internal Medicine*. 2011, 270 (4), 327–338.
17. Caselli, E., Di Luca, D. Molecular biology and clinical associations of Roseoloviruses human herpesvirus 6 and human herpesvirus 7. *The New Microbiologica*. 2007, 30 (3), 173–187.
18. Chapenko, S., Krumina, A., Kozireva, S. et al. Activation of human herpesviruses 6 and 7 in patients with chronic fatigue syndrome. *Journal of Clinical Virology*. 2006, 37 (1), 47–51.
19. Chapenko, S., Krumina, A., Logina, I. et al. Association of active human herpesvirus-6, -7 and parvovirus B19 infection with clinical outcomes in patients with myalgic encephalomyelitis/chronic fatigue syndrome. *Advances in Virology*. 2012, 2012, 1–7.
20. Clark, D. A. Clinical and laboratory features of human herpesvirus 6 chromosomal integration. *Clinical Microbiology and Infection*. 2016, 22 (4), 333–339.
21. Collin, S. M., Nikolaus, S., Heron, J. et al. Chronic fatigue syndrome (CFS) symptom-based phenotypes in two clinical cohorts of adult patients in the UK and The Netherlands. *Journal of Psychosomatic Research*. 2016, 81, 14–23.
22. Cooling, L. L., Koerner, T. A., Naides, S. J. Multiple glycosphingolipids determine the tissue tropism of parvovirus B19. *The Journal of Infectious Diseases*. 1995, 172 (5), 1198–1205.
23. Couper, K. N., Blount, D. G., Riley, E. M. IL-10: the master regulator of immunity to infection. *Journal of Immunology*. 2008, 180 (9), 5771–5777.
24. De Bolle, L., Naesens, L., De Clercq, E. Update on human herpesvirus 6 biology, clinical features, and therapy. *Clinical Microbiology Reviews*. 2005, 18 (1), 217–245.
25. Di Luca, D., Zorzenon, M., Mirandola, P. et al. Human herpesvirus 6 and human herpesvirus 7 in chronic fatigue syndrome. *Journal of Clinical Microbiology*. 1995, 33 (6), 1660–1661.
26. Dong , B., Kim, S., Hong, S. et al. An infectious retrovirus susceptible to an IFN antiviral pathway from human prostate tumors. *Proceedings of the National Academy of Sciences of the United States of America*. 2007, 104, 1655–1660.
27. Endo, A., Watanabe, K., Ohye, T. et al. Molecular and virological evidence of viral activation from chromosomally integrated human herpesvirus 6A in a patient with X-linked severe combined immunodeficiency. *Clinical Infectious Diseases*. 2014, 59, 545–548.
28. Fischer, D. B., William, A. H., Strauss, A. C. et al. Chronic Fatigue Syndrome: The Current Status and Future Potentials of Emerging Biomarkers. *Fatigue*. 2014, 2 (2), 93–109.
29. Fletcher, M. A., Zeng, X. R., Barnes, Z. et al. Plasma cytokines in women with chronic fatigue syndrome. *Journal of Transplantational Medicine*. 2009, 7, 96.

30. Fremont, M., Metzger, K., Hulstaert, J., De Meirleir, K. Detection of herpesviruses and parvovirus B19 in gastric and intestinal mucosa of chronic fatigue syndrome patients. *In Vivo*. 2009, 23, (2), 209–213.
31. Friedberg, F. Chronic fatigue syndrome, fibromyalgia, and related illnesses: a clinical model of assessment and intervention. *Journal of Clinical Psychology*. 2010, 66 (6), 641–665.
32. Fukuda, K., Straus, S. E., Hickie, I. et al. The chronic fatigue syndrome: a comprehensive approach to its definition and study. *Annals of Internal Medicine*. 1994, 121, 953– 959.
33. Glassford, J. A. The Neuroinflammatory Etiopathology of Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS). *Frontiers in Physiology*. 2017, 8, 88.
34. Gravel, A., Dubuc, I., Morissette, G. et al. Inherited chromosomally integrated human herpesvirus 6 as a predisposing risk factor for the development of angina pectoris. *Proceedings of the National Academy of Sciences of the United States of America*. 2015, 112 (26), 8058–8063.
35. Groom, H. C., Bishop, K. N. The tale of xenotropic murine leukemia virus-related virus. *The Journal of General Virology*. 2012, 93 (5), 915–924.
36. Guindon, S., Dufayard, J. F., Lefort, V. et al. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Systematic Biology*. 2010, 59, 307–321.
37. Halpin, P., Williams, M. V., Klimas, N. G. et al. Myalgic encephalomyelitis/chronic fatigue syndrome and gulf war illness patients exhibit increased humoral responses to the herpesviruses-encoded dUTPase: Implications in disease pathophysiology. *Journal of Medical Virology*. 2017, 89 (9), 1636–1645.
38. Hardcastle, S. L., Brenu, E. W., Johnston, S. et al. Longitudinal analysis of immune abnormalities in varying severities of Chronic Fatigue Syndrome/Myalgic Encephalomyelitis patients. *Journal of Translational Medicine*. 2015, 13, 299.
39. Hornig, M., Montoya, J. G., Klimas, N. G. et al. Distinct plasma immune signatures in ME/CFS are present early in the course of illness. *Science Advances*. 2015, 1(1), e1400121.
40. Hüfner, K., Arbusow, V., Himmelein, S. et al. The prevalence of human herpesvirus 6 in human sensory ganglia and its co-occurrence with alpha-herpesviruses. *Journal of Neurovirology*. 2007, 13 (5), 462–467.
41. Ito, K., Shimizu, N., Watanabe, K. et al. Analysis of viral infection by multiplex polymerase chain reaction assays in patients with liver dysfunction. *Internal medicine (Tokyo, Japan)*. 2013, 52 (2), 201–211.
42. Johnston, S., Brenu, E. W., Staines, D., Marshall-Gradisnik, S. The prevalence of chronic fatigue syndrome/ myalgic encephalomyelitis: a meta-analysis. *Clinical Epidemiology*. 2013, 5, 105–110.
43. Kaufer, B. B., Flamand, L. Chromosomally integrated HHV-6: impact on virus, cell and organismal biology. *Current opinion in virology*. 2014, 9, 111–118.
44. Kavelaars, A., Kuis, W., Knook, L. et al. Disturbed neuroendocrine-immune interactions in chronic fatigue syndrome. *The Journal of*

Clinical Endocrinology & Metabolism. 2000, 85, (2), 692–696.

45. Kerr, J. R., Gough, J., Richards, S. C. et al. Antibody to parvovirus B19 nonstructural protein is associated with chronic arthralgia in patients with chronic fatigue syndrome/myalgic encephalomyelitis. *The Journal of General Virology*. 2010, 91 (4), 893–897.
46. Koelle, D. M., Barcy, S., Huang, M. L. et al. Markers of viral infection in monozygotic twins discordant for chronic fatigue syndrome. *Clinical Infectious Diseases*. 2002, 35 (5), 518–525.
47. Kozireva, S. V., Zestkova, J. V., Mikazane, H. J. et al. Incidence and clinical significance of parvovirus B19 infection in patients with rheumatoid arthritis. *The Journal of Rheumatology*. 2008, 35, (7),1265–1270.
48. Kozireva, S., Nemceva, G., Danilane, I. et al. Prevalence of Blood- Borne Viral Infections (Cytomegalovirus, Human Herpesvirus-6, Human Herpesvirus-7, Human Herpesvirus-8, Human T-Cell Lymphotropic Virus- I/II, Human Retrovirus-5) Among Blood Donors in Latvia. *Annals of Hematology*. 2001, 80, 669–673.
49. Krueger, G. R., Ablashi, D. Human Herpesvirus-6, Second Edition, *General Virology, Epidemiology and Clinical Pathology. Perspectives in Medical Virology*. Netherlands: Elseviere, 2006. – Pp. 251–262.
50. Lattie, E. G., Antoni, M. H., Fletcher, M. A. et al. Stress management skills, neuroimmune processes and fatigue levels in persons with chronic fatigue syndrome. Br*ain, Behavior, and Immunity*. 2012, 26 (6), 849–858.
51. Levine, S., Eastman, H., Ablashi, D. V. Prevalence of IgM and IgG Antibody to HHV-6 and HHV-8 and Results of Plasma PCR to HHV-6 and HHV-7 in a Group of CFS Patients and Healthy Donors. *Journal of Chronic Fatigue Syndrome*. 2001, 9 (1–2), 31–40.
52. Lidbury, B. A., Kita, B., Lewis, D. P. et al. Activin B is a novel biomarker for chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) diagnosis: a cross sectional study. *Journal of Translational Medicine*. 2017, 15 (1), 60.
53. Liefeldt, L., Plentz, A., Klempa, B. et al. Recurrent high level parvovirus B19/genotype 2 viremia in a renal transplant recipient analyzed by real-time PCR for simultaneous detection of genotypes 1 to 3. *Journal of Medical Virology*. 2005, 75 (1), 161–169.
54. Lo, S. C., Pripuzova, N., Li B. et al. Detection of MLV-related virus gene sequences in blood of patients with chronic fatigue syndrome and healthy blood donors. *Proceedings of the National Academy of Sciences of the United States of America*. 2010, 107 (36), 15874–15879.
55. Loebel, M., Eckey, M., Sotzny, F. et al. Serological profiling of the EBV immune response in Chronic Fatigue Syndrome using a peptide microarray. *PloS One*. 2017, 12 (6), 1–17.
56. Lombardi, V. C., Ruscetti, F. W., Das Gupta, J. et al. Detection of Infectious Retrovirus, XMRV, in Blood Cells of Patients with Chronic Fatigue Syndrome. *Science*. 2009, 326 (5952), 585–589.

57. Lyall, E. G., Cubie, H. A. Human herpesvirus-6 DNA in the saliva of paediatric oncology patients and controls. *Journal of Medical Virology*. 1995, 47 (4), 317–322.
58. Maes, M., Twisk, F. N., Kubera, M., Ringel, K. Evidence for inflammation and activation of cell-mediated immunity in Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS): increased interleukin-1, tumor necrosis factor-α, PMN-elastase, lysozyme and neopterin. *Journal of Affective Disorders*. 2012, 136 (3), 933–939.
59. Matano, S., Kinoshita, H., Tangawa, K. et al. Acute parvovirus B19 infection mimicking chronic fatigue syndrome. *Internal Medicine*. 2003, 42 (9), 903–905.
60. Mensah, F. K. F., Bansal, A. S., Ford, B., Cambridge, G. Chronic fatigue syndrome and the immune system: Where are we now? *Neurophysiologie Clinique/Clinical Neurophysiology*. 2017, 47 (2), 131–138.
61. Milrad, S. F., Hall, D. L., Jutagir, D. R. et al. Poor sleep quality is associated with greater circulating pro-inflammatory cytokines and severity and frequency of chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) symptoms in women. *Journal of Neuroimmunology*. 2017, 303, 43–50.
62. Mirandola, P., Menegazzi, P., Merighi, S. et al. Temporal mapping of transcripts in herpesvirus 6 variants. *Journal of Virology*. 1998, 72 (5), 3837–3844.
63. Morinet, F., Corruble, E. Chronic Fatigue Syndrome and Viral Infections. An International Perspective on the Future of Research in Chronic Fatigue Syndrome. Ed. by Snell C. R. ISBN: 978-953-51-0072-0 – Croatia: InTech, 2012. – Pp. 1–12. Available from: http://www.intechopen.com/books/an-international-perspective-on-the-future-of-research-in-chronic-fatigue-syndrome/chronic-fatigue-syndrome-and-viral-infections.
64. Munakata, Y., Kato, I., Saito, T. et al. Human parvovirus B19 infection of monocytic cell line U937 and antibody-dependent enhancement. *Virology.* 2006, 345, 251–257.
65. Nakamura, T., Schwander, S.K., Donnelly, R. et al. Cytokines across the night in chronic fatigue syndrome with and without fibromyalgia. *Clinical and Vaccine Immunology*. 2010, 17 (4), 582–587.
66. Nastke, M. D., Becerra, A., Yin, L. et al. Human CD4+ T cell response to human herpesvirus 6. *Journal of Virology*. 2012, 86 (9), 4776–4792.
67. Nicolson, G. L., Gan, R., Haier, J. Multiple co-infections (Mycoplasma, Chlamydia, human herpes virus-6) in blood of chronic fatigue syndrome patients: association with signs and symptoms. *APMIS: Acta Pathologica, Microbiologica et Immunologica Scandinavica*. 2003, 111 (5), 557–566.
68. Oakes, B., Hoagland-Henefield, M., Komaroff, A. L. et al. Human endogenous retrovirus-K18 superantigen expression and human herpesvirus-6 and human herpesvirus-7 viral loads in chronic fatigue patients. *Clinical Infectious Diseases.* 2013, 56 (10), 1394–1400.
69. Patarca, R. Cytokines and chronic fatigue syndrome. *Annals of the New York Academy of Sciences*. 2001, 933, 185–200.

70. Pfrepper, K. I., Enders, M., Motz, M. Human parvovirus B19 serology and avidity using a combination of recombinant antigens enables a differentiated picture of the current state of infection. *Journal of veterinary medicine B, Infectious diseases and veterinary public health*. 2005, 52 (7–8), 362–365.
71. Politou, M., Koutras, D., Kaparos, G. et al. Seroprevalence of HHV-6 and HHV-8 among blood donors in Greece. *Virology Journal*. 2014, 11, 153.
72. Prusty, B. K., Gulve, N., Rasa, S. et al. Possible Chromosomal and Germline Integration of Human Herpesvirus 7 (HHV-7). *The Journal of General Virology*. 2017, 98 (2), 266–274.
73. Prusty, B. K., Krohne, G., Rudel, T. Reactivation of chromosomally integrated human herpesvirus-6 by telomeric circle formation. *PLoS Genetics*. 2013, 9 (12), e1004033 b.
74. Prusty, B. K., Siegl, C., Hauck, P. et al. Chlamydia trachomatis infection induces replication of latent HHV-6. *PLoS One*. 2013, 8 (4), e61400.
75. Reeves, W. C., Stamey, F. R., Black, J. B. et al. Human herpesviruses 6 and 7 in chronic fatigue syndrome: a case-control study. *Clinical Infectious Diseases*. 2000, 31 (1), 48–52.
76. Russell, L., Broderick, G., Taylor, R. et al. Illness progression in chronic fatigue syndrome: a shifting immune baseline. *BMC Immunology*. 2016, 17, 3.
77. Sairenji, T., Nagata, K. Viral infections in chronic fatigue syndrome. *Nihon Rinsho*. 2007, 65 (6), 991–996.
78. Sairenji, T., Yamanishi, K., Tachibana, Y. et al. Antibody responses to Epstein-Barr virus, human herpesvirus 6 and human herpesvirus 7 in patients with chronic fatigue syndrome. *Intervirology*. 1995, 38 (5), 269–273.
79. Sanders, P., Korf, J. Neuroaetiology of chronic fatigue syndrome: an overview. *The World Journal of Biological Psychiatry*. 2008, 9 (3), 165–171.
80. Secchiero, P., Carrigan, D. R., Asano, Y. et al. Detection of human herpesvirus 6 in plasma of children with primary infection and immunosuppressed patients by polymerase chain reaction. *The Journal of Infectious Diseases*. 1995, 171 (2), 273–280.
81. Servant-Delmas, A., Lefrere, J. J., Morinet, F., Pillet, S. Advances in human B19 erythrovirus biology. *Journal of Virology*. 2010, 84, 9658–9665.
82. Streiter, M., Malecki, M., Prokop, A. et al. Does human bocavirus infection depend on helper viruses? A challenging case report. *Virology Journal*. 2011, 8, 417.
83. Sultanova, A., Cistjakovs, M., Gravelsina, S. et al. Association of active human herpesvirus-6 (HHV-6) infection with autoimmune thyroid gland diseases. *Clinical Microbiology and Infection*. 2017, 23 (1), 50.e1–50.e5.
84. Takahashi, Y., Murai, C., Shibata, S. et al. Human parvovirus B19 as a causative agent for rheumatoid arthritis. *Proceedings of the National Academy of Sciences of the United States of America*. 1998, 95, 8227–8232.
85. Tamura, K., Stecher, G., Peterson, D. et al. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*. 2013, 30, 2725–2729.

86. Ter Wolbeek, M., van Doornen, L.J., Kavelaars, A. et al. Longitudinal analysis of pro- and anti-inflammatory cytokine production in severely fatigued adolescents. *Brain, Behavior and Immunity.* 2007, 21 (8), 1063–1074.
87. Tomsone, V., Logina, I., Millers, A. et al. Association of human herpesvirus 6 and human herpesvirus 7 with demyelinating diseases of the nervous system. *Journal of Neurovirology*. 2001, 7 (6), 564–569.
88. Traylen, C. M., Patel, H. R., Fondaw, W. et al. Virus reactivation: a panoramic view in human infections. *Future Virology*. 2011, 6 (4), 451–463.
89. Underhill, R. A. Myalgic encephalomyelitis, chronic fatigue syndrome: An infectious disease. *Medical Hypotheses*. 2015, 85 (6), 765–773.
90. Urisman, A., Molinaro, R. J., Fischer, N. et al. Identification of a Novel Gammaretrovirus in Prostate Tumors of Patients Homozygous for R462Q RNASEL Variant. *PLOS Pathogens*. 2006, 2 (3), e25.
91. Van den Bosch, G., Locatelli, G., Geerts, L. et al. Development of reverse transcriptase PCR assays for detection of active human herpesvirus 6 infection. *Journal of Clinical Microbiology*. 2001, 39 (6), 2308–2310.
92. Vandamme, A. M., Fransen, K., Debaisieux, L. et al. Standardisation of primers and an algorithm for HIV-1 diagnostic PCR evaluated in patients harbouring strains of diverse geographical origin. The Belgian AIDS Reference Laboratories. *Journal of Virology Methods*. 1995, 51 (2–3), 305–316.
93. Visser, J., Graffelman, W., Blauw, B. et al. LPS-induced IL-10 production in whole blood cultures from chronic fatigue syndrome patients is increased but supersensitive to inhibition by dexamethasone. *Journal of Neuroimmunology*. 2001, 119 (2), 343–349.
94. von Poblotzki, A., Hemauer, A., Gigler, A. et al. Antibodies to the nonstructural protein of parvovirus B19 in persistently infected patients: implications for pathogenesis. *The Journal of Infectious Diseases*. 1995, 172, 1356–1359.
95. Wallace, H. L. 2nd, Natelson, B., Gause, W., Hay J. Human herpesviruses in chronic fatigue syndrome. *Clinical and Diagnostic Laboratory Immunology*. 1999, 6 (2), 216–223.
96. White, A. T., Light, A. R., Hughen, R. W. et al. Severity of symptom flare after moderate exercise is linked to cytokine activity in chronic fatigue syndrome. *Psychophysiology*. 2010, 47 (4), 615–624.
97. Winger, A., Kvarstein, G., Wyller, V. B. et al. Health related quality of life in adolescents with chronic fatigue syndrome: a cross-sectional study. *Health and Quality of Life Outcomes*. 2015, 13, 96.
98. Yasui, M., Yoshimura, T., Takeuchi, S. et al. A chronic fatigue syndrome model demonstrates mechanical allodynia and muscular hyperalgesia via spinal microglial activation. *Glia*. 2014, 62 (9), 1407–1417.
99. Zhang, L., Gough, J., Christmas, D. et al. Microbial infections in eight genomic subtypes of chronic fatigue syndrome/myalgic encephalomyelitis. *Journal of Clinical Pathology*. 2010, 63 (2), 156–164.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor *Dr. med.*, Assoc. Professor Modra Murovska for guidance, support, motivation and contribution, as well as to Senior Researcher *Dr. habil.biol*. Svetlana Chapenko for training, consultation and collaboration at work. I would like to thank Professor Angelika Krūmiņa, Professor Ludmila Vīksna and Professor Ināra Logina for providing the clinical material and for contribution in preparation of clinical section, as well as to my colleagues in RSU A. Kirchenstein Institute of Microbiology and Virology for support during PhD studies.

I would like to express my gratitude to Vice-Rector for Science, Professor Iveta Ozolanta and Scientific Secretary Ingrīda Kreile for support during doctoral studies.

This study was funded in parts by the projects No. 6.2.-25/2013/0039; RSU ZP 13/2013; (2009/0147/1DP/1.1.2.1.2/09/IPIA/VIAA/009); 316275 and COST Action 15111.