

# EFFECT OF HUMAN HERPESVIRUSES 6 AND 7 INFECTION ON THE CLINICAL COURSE OF RHEUMATOID ARTHRITIS

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*Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disease affecting joints and causing symmetrical chronic progressive aseptic synovitis and erosive-destructive changes. Viruses and viral infections are considered to be the main risk factors for autoimmune disease development (especially for individuals with genetic predisposition). The goal of this study was to evaluate the frequency of HHV-6 and HHV-7 persistent infection and its activity phase in RA and osteoarthritis (OA) patients, and healthy persons. We examined also the influence of HHV-6 and -7 infections on RA activity, aggressiveness, radiographical stage, and frequency of complications as well as the presence of HHV-6 infection markers in synovial fluid and synovial tissues of RA joints of affected patients. Despite the lack of significant correlation between frequency of persistent single HHV-6, single HHV-7, and concurrent HHV-6 and HHV-7 infection and RA clinical course, we found that both active and latent HHV-6 and/or HHV-7 infection increased RA activity and progression in several clinical and laboratory parameters. Regarding the severity of the course of RA, we observed also a high prevalence of RA complications in the patient group with active single HHV-6 infection and also a more severe radiographical stage in RA patients with active concurrent HHV-6 and HHV-7 infection. Moreover, viral infection markers were found in synovial fluid and synovial tissues of affected joints of RA patients. This suggests that HHV-6 and/or HHV-7 infection has effect on the disease clinical course, but virus reactivation may be a consequence of immunosuppressive treatment.*

**Key words:** rheumatoid arthritis, HHV-6, HHV-7, viral reactivation.

## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic autoimmune inflammatory disease displaying joint lesions with symmetrical chronic progressive aseptic synovitis and erosive-destructive changes in late stage, and extra-articular lesions, which are systemic manifestations. RA is a multifactorial disease. Despite prolonged studies the role of particular aetiological factors is still unknown. For a long time it has been suggested that development of RA might cause viral infection in genetically predisposed people. Infectious agents have the potential to initiate auto-reactivity through polyclonal activation, release of previously sequestered antigens or molecular mimicry. Some studies suggest that molecular mimicry of host proteins by a pathogen can induce

autoimmune diseases (Lunardi *et al.*, 2005). However, more commonly viruses induce autoimmunity by cell death, predominantly by increased apoptosis, resulting in the release of self-antigens; increased apoptosis indeed has been suggested as a major pathogenic mechanism in autoimmune connective tissue diseases (Mackay *et al.*, 2008). As RA is a chronic, progressive disease, it is suspected that it could be caused by a worldwide-distributed infectious agent.

Severe synovial lining layer hyperplasia, neovascularisation, and inflammatory infiltration of the sublining with lymphocytes and macrophages develop in the case of RA (Beaten *et al.*, 2000; Firestein, 2003; Trembleau *et al.*, 2010; Costenbader *et al.*, 2011). Impaired T lymphocyte activation is one of the first changes observed in RA patients.

Viral antigens activate development of *naive* CD4+ T cells in T cells subclasses, which stimulates macrophages and monocytes to produce many different inflammatory cytokines, including IL-1, IL-6, and TNF- $\alpha$ , and proteolytic enzymes. Therefore, destruction of synovium, cartilage and subchondral bone occurs (Costenbader *et al.*, 2011). There is an increased level of many different cytokines in serum and synovial fluid in the case of active RA. TNF- $\alpha$  promotes production of osteoclasts and stimulates many cells and inflammatory cytokines that cause further development of inflammation (Choy and Panayi, 2001). Several studies have shown correlation of IL-6 activity and a more active and more aggressive RA course (Gottenberg *et al.*, 2012).

Several reports based on epidemiological evidence support the hypothesis that certain viruses, including human herpesviruses (HHVs), might be potential triggers of RA development. HHV-6 and HHV-7 are classified in the *Roseolovirus* genus in the *Betaherpesvirinae* subfamily. HHV-6 and HHV-7 are ubiquitous in the general population, with up to 90% of adults being seropositive. Both these viruses are the causative agents of *exanthema subitum*. After primary infection, which commonly occurs in early childhood, infection remains latent/persistent lifelong. HHV-6 and -7 may become reactivated under conditions of stress, trauma, hormonal disbalance and immunosuppression, thus contributing further toward immunosuppression (Caselli and Luca, 2007; Isegawa *et al.*, 2010). Both viruses infect cells of the immune system and therefore may modulate their function. The primary target for HHV-6 and HHV-7 replication is CD4+ T lymphocytes, pivotal cells in generation of humoral and cell-mediated adaptive immune response. Both viruses have been shown to up-regulate TNF- $\alpha$  secretion. HHV-7 might also act as a trigger factor for HHV-6 reactivation (Lusso, 2006; Wang and Pellet, 2007, Prober, 2011). IL-17 acts synergistically with TNF- $\alpha$  and induces the production of several inflammatory mediators in the affected synovium, which are further synergistically enhanced via combinations of IL-17 with other cytokines (Gonzalez-Alvaro *et al.*, 2009). IL-17 also promotes the survival of both the synoviocytes and inflammatory cells and promotes the maturation of immune cells. This leads to an increased number of synoviocytes and inflammatory cells in the synovial fluid and in the synovium, leading to the hyperplasia and exacerbated inflammation observed in joints of RA patients (Benedetti and Miossec, 2014). To assess the role of viral infection in RA we evaluated the frequency of HHV-6 and HHV-7 reactivation in RA patients, osteoarthritis (OA) patients and healthy persons. Also we examined the effect of HHV-6 and -7 infection on RA activity, aggressiveness, radiological stage and frequency of complications.

## MATERIALS AND METHODS

**Patients and controls.** RA and OA patients were recruited from the Riga East University Hospital Clinic “Linezers” and Clinic “Gaiļezers” and from the Hospital of Traumatology and Orthopaedics. The inclusion criteria were previ-

ously established diagnosis of RA or OA, or primary diagnosis of RA or OA established in the given hospitals. All RA patients fulfilled 1987 American College of Rheumatology (ACR) revised classification criteria for RA or the 2010 ACR/EULAR classification criteria for RA. Patients fulfilled relevant ACR criteria for OA affected joints: wrists (Altman *et al.*, 1990), hip (Altman *et al.*, 1991) and knee (Altman *et al.*, 1986). Patients who underwent replacement surgery fulfilled hip ACR criteria for OA. Pregnant women, alcoholic patients and patients having any other inflammatory disease were excluded from the study.

The study was performed during 2010–2013 and included 80 RA patients (67 females and 11 males) with mean age 55.8 years (range 19–80) and 78 OA patients (52 females and 26 males) with mean age 64.6 years (range 35–86) as well as a cohort of 19 healthy control persons (16 females and 3 males) with mean age 59.6 years (range 38–89). Significantly more males were in the OA patients group than in the RA group ( $p = 0.0046$ ; OR 0.3, 95% CI: 0.1 to 0.7). OA patients were significantly older ( $p < 0.0001$ ; 95% CI: 4.9 to 12.7) than RA patients; but control individual age did not significantly differ from that of RA patients ( $p = 0.3$ ; 95% CI: -3.0 to 10.7) and OA patients ( $p = 0.12$ ; 95% CI: -1.8 to 11.7). Early RA with symptoms less than 24 months (symptoms developed up to 24 months before enrolment in the study) (Emery, 1994) was detected in 33 RA patients. Fifty-five RA patients received synthetic and/or biologic DMARDs (disease modifying antirheumatic drugs). Eight of them received methotrexate alone, 35 of them — methotrexate plus one or more DMARD (hydroxychloroquine, sulfasalazine, leflunomide, tocilizumab). Seventeen RA patients received synthetic DMARDs without methotrexate and five received biological DMARD tocilizumab with or without methotrexate.

The clinical features, treatment and laboratory parameters [haemoglobin, C reactive protein (CPR), rheumatoid factor (RF) and anti-cyclic citrullinated peptide (anti-CCP)], and disease activity score DAS28 (only for RA patients) were examined for their potential association with HHV-6 and HHV-7 infections.

**Physical examination.** Clinical examination of RA patients included assessment of morning stiffness along with swelling and tenderness of joints, and development of RA complications. Full physical examination was performed, and swollen and painful joints were recorded; of the 68 joints examined, 28 were used for DAS28 calculation (Arnett *et al.*, 1988). DAS28 is a composite index that consists of the number of swollen and painful joints of 28 joints examined by a doctor, patient's general health assessment in visual analogue scale and CRP level in the last blood sample. The disease activity score for RA patients was used as a disease activity indicator. The following RA complications were recorded: polyneuropathy, myopathy, sicca syndrome, and rheumatoid nodules, damage of cervical vertebrae, osteoporosis and scleritis.

**Radiological examination of bone structure.** Plain radiography was used for RA diagnosis (van der Heijde, 1996). However, only the late signs of preceding disease activity can be visualised by radiography. Magnetic resonance imaging (MRI) can detect RA erosive change with greater sensitivity than conventional radiography (Sommer *et al.*, 2005). Therefore, MRI was used to precisely determine early changes and erosions in wrists. MRI protocols FSE T2 „fat sat” coronal plane, GR axial plane, SE T1 „fat sat” coronal plane post gadolinium, 3D SPGR coronal plane post gadolinium were used. MRI test read-out included assessment of synovitis, bone oedema, and bone erosion. Staging was based on Venables and Wheelless recommendations (Wheelless, 2012; Venables and Maini, 2013).

**Sample collection and preparation.** EDTA anti-coagulated peripheral blood, synovial tissues and synovial fluid samples from RA and OA patients and EDTA anti-coagulated peripheral blood samples from apparently healthy persons were collected. Plasma samples were separated from peripheral blood by centrifugation.

DNA was isolated from whole blood, cell-free blood plasma, synovial tissues and synovial fluid by standard phenol-chloroform extraction. Before DNA extraction, plasma and synovial fluid samples were treated with DNase I. To assure the quality of the whole blood and synovial tissue DNA, as well as to exclude contamination of plasma and synovial fluid DNA by cellular DNA debris, a beta-globin PCR was carried out.

**Laboratory analysis of disease activity.** The level of CRP and erythrocyte sedimentation rate (ESR) served as markers of RA and OA activity. ESR higher than 30 mm/h and CRP level above 8 mg/L were considered as elevated.

CRP or ESR can be used for DAS28 calculation. In our case CRP was used for calculation of DAS28, because it more corresponds to ongoing inflammation. RA patients were classified as in remission (DAS28 score  $\leq 2.6$ ), low disease activity (DAS28 score  $2.6 \leq 3.2$ ), moderate disease activity (DAS28 score  $3.2-5.1$ ) or severe disease activity (DAS28 score  $\geq 5.1$ ).

**Laboratory analysis of disease aggressiveness.** RF and anti-CCP were analysed as markers of RA aggressiveness. For RA diagnosis we used RF with or without anti-CCP. RF has sensitivity 31–54% and specificity 92–93% for RA diagnosis, and anti-CCP has sensitivity 39–50% and specificity 93–97% (Niewold *et al.*, 2007). Previous studies have shown that the anti-CCP test is positive in about 1% of the healthy population (Zendman *et al.*, 2006; Nam *et al.*, 2015) and up to 8% of OA patients (Sauerland *et al.*, 2005). RF and anti-CCP antibodies were detected also in OA patients. An RF level higher than 14 U/ml and anti-CCP antibody level higher than 17 U/ml were considered as positive. Clinical course of RA was classified by RF as non-aggressive if  $RF \leq 14$  U/ml, low aggressive if  $RF 14 \leq 42$  U/ml, highly aggressive if  $RF > 42$  U/ml and very aggressive if  $RF > 1000$  U/ml. The clinical course of RA was classified also

by anti-CCP antibody level in four aggressiveness groups: non-aggressive (anti-CCP  $\leq 17$  U/ml), low aggressive (anti-CCP  $17 \leq 51$  U/ml), highly aggressive (anti-CCP  $> 51$  U/ml) and very aggressive RA (anti-CCP  $> 1000$  U/ml).

**Detection of TNF- $\alpha$ , IL-6 and IL-17 levels.** The levels of TNF- $\alpha$ , IL-6 and IL-17 were measured by enzyme-linked immunosorbent assay (ELISA) (TNF- $\alpha$ , IL-6 by Nordic Biosite, Denmark and IL-17 by R&D systems, USA) and analysed in comparison with the presence or absence of HHV-6 and HHV-7 infection markers.

**Determination of HHV-6 and HHV-7 genomic sequences.** One microgram of whole blood and synovial tissue DNA as well as 10  $\mu$ l of plasma and synovial fluid DNA was subjected to nested PCR (nPCR) with HHV-6 and HHV-7 specific primers as described previously (Berneman *et al.*, 1992; Secchiero *et al.*, 1995). HHV-6 and HHV-7 positive and negative DNAs as well as water controls were included in each experiment. Detection of viral DNA in cell-associated material (whole blood and synovial tissues) has been used as a marker of persistent infection, while detection of viral DNA in cell-free blood plasma and synovial fluid as a marker of active infection (virus reactivation). The number of persons with latent infection was calculated as the difference between numbers with persistent and active infection.

**Synovial histopathology and immunohistochemistry.** Synovial tissues were obtained by open biopsy during elective replacement of hip without USG guide. Biopsies were performed more often in OA patients because they more often underwent joint replacement surgery. Synovial tissues were obtained from 7 RA and 54 OA patients. Of them, tissue samples from six RA and 43 OA patients were fixed in formalin and embedded in paraffin. Thereafter, paraffin-embedded tissue sections were stained with haematoxylin and eosin for routine histopathological analysis, which included evaluation of synovial lining layer thickness, vascularity of the sublining layer, inflammatory infiltration of the sublining layer, and presence of lymphoid aggregates and plasma cells, as recommended by Baeten *et al.* (2003) and Kruithof *et al.* (2005). Consecutive synovial tissue sections obtained routinely were utilised for immunohistochemistry. Immunohistochemical reactions detecting A and B strains were performed using anti-HHV-6 (2001) sc-65463 monoclonal antibody raised against viral lysate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, 1:200 dilution). Synovial CD68+ macrophages were detected by monoclonal mouse anti-human CD68 (DakoCytomation, Glostrup, Denmark, 1:100 dilution, clone PG-M1), which labels monocytes and macrophages through recognition of lysosomal glycoprotein/plasma membrane proteins. For visualisation of reaction results, a HiDef Detection™ HRP Polymer system (CellMarque, Rocklin, CA, USA) and DAB+Chromogen (Cell Marque, Rocklin, CA, USA) was used. Immunohistochemical controls included omission of the primary antibody and sections from tonsil were used as a positive control for CD68. Tissue sections were analysed using an appropriate magnification (from  $\times 100$  up to  $\times 400$ ), and im-

ages were produced with a digital camera DFC 450C on a Leitz DMRB bright-field microscope.

**Statistical analysis.** Data were analysed using SPSS and GraphPad Prism 6.0 software. Statistical differences in the detection of frequency of HHV-6 and/or HHV-7 genomic sequences in the extracted DNA samples of the individuals with RA and OA as well as the control group persons were assessed with the Fisher's exact test and T-test. The mean values of all clinical parameters and plasma cytokines levels were expressed as median with variance characterised by the interquartile region (IQR). Significance of differences between medians was assessed using the nonparametric Mann Whitney U test. Statistical significance was set at  $p < 0.05$ .

The local Ethics Committee approved the study and signed informed consent was obtained from participating patients.

## RESULTS

### Frequency of persistent HHV-6 and HHV-7 infection.

Persistent HHV-6 and/or HHV-7 infection was observed in 67 of 80 (83.8%) RA patients, 63/78 (80.8%) OA patients and in 15/19 (78.9%) control individuals. Among RA patients with persistent HHV-6 and/or -7 infection, 6/67 (9.0%) had single HHV-6 infection, 42/67 (62.7%) — single HHV-7 infection, and 19/67 (28.4%) — concurrent HHV-6 and HHV-7 infection. Among OA patients with persistent HHV-6 and/or -7 infection, single HHV-6 infection was detected in one of 63 patients (1.6%), single HHV-7 infection in 41/63 (65.1%) and concurrent HHV-6 and HHV-7 infection in 21/63 (33.3%) patients. Prevalence of single HHV-6, single HHV-7 and concurrent HHV-6 and HHV-7 persistent infection among healthy control persons was 0/15 (0%), 10/15 (66.7%) and 5/15 (33.3%), respectively (Table 1).

**Frequency of HHV-6 and HHV-7 reactivation.** All samples of patients and healthy persons with HHV-6 and/or HHV-7 genomic sequences in whole blood DNA (marker of persistent viral infection) were analysed for viral infection reactivation (viral genomic sequences in cell-free blood plasma DNA). HHV-6 reactivation was detected in 10/67

RA (14.9%), in 16/63 (25.4%) OA, and in 4/15 (26.7%) healthy persons with persistent HHV-6 and/or HHV-7 infection. The overall frequency of viral infection reactivation was 10/80 RA (12.5%), 16/78 (20.5%) OA patients, and 4/19 (21.1%) healthy control persons. Single HHV-6 reactivation was observed in 8/25 (32.0%) RA patients with HHV-6 persistent infection [2/6 (66.6%) with single HHV-6, and 6/19 (31.6%) with concurrent HHV-6 and HHV-7 infection] and in 9/22 (40.9%) OA patients [1/1 with single HHV-6 infection and 8/21 (38.1%) with concurrent HHV-6 and HHV-7 infection] as well as in 3/5 (60.0%) control group individuals with concurrent HHV-6 and HHV-7 infection. HHV-7 reactivation was detected in 13/67 RA (19.4%), in 14/63 (22.2%) OA, and in 3/15 (20%) healthy persons with persistent HHV-6 and/or HHV-7 infection. Calculated for the whole group, HHV-7 reactivation occurred in 13/80 (16.3%) RA patients, 14/78 (17.9%) OA patients as well as in 3/19 (15.8%) healthy control persons. Single HHV-7 reactivation was found in 11/61 (18.0%) RA patients with HHV-7 persistent infection [11/42 (26.2%) with single HHV-7 infection and in none from the 19 in concurrent infection group], in 7/62 (11.3%) OA patients [7/41 (17.1%) with single HHV-7 infection, in none of the 21 in the concurrent infection group] as well as in 2/15 (13.3%) control group individuals [2/10 (20%) with single HHV-7 infection, none from the 5 patients in the concurrent infection group]. Simultaneous HHV-6 and HHV-7 reactivation was observed in 2/19 (10.5%) RA, in 7/21 (33.3%) OA patients and in 1/5 (20%) healthy persons with concurrent HHV-6 and HHV-7 persistent infection (Table 1).

**Frequency of HHV-6 and HHV-7 latent infection.** There were no OA patients and healthy persons with single latent HHV-6 infection, but latent HHV-6 infection was detected in 4/6 (66.7%) RA patients. Latent HHV-7 infection was observed in 31/42 (73.8%) RA, in 34/41 (82.9%) OA patients and in 8/10 (80.0%) control group individuals with persistent single HHV-7 infection. Latent concurrent HHV-6 and HHV-7 infection was detected in 11/19 (57.9%) RA, in 6/21 (28.6%) OA patients, and 1/5 (20%) healthy person with persistent concurrent HHV-6 and HHV-7 infection.

**Presence of HHV-6 and HHV-7 DNA in synovial fluid.** HHV-6 and/or HHV-7 genomic sequences were also found

Table 1

FREQUENCY OF PERSISTENT, ACTIVE AND LATENT HHV-6 AND HHV-7 INFECTION IN PATIENTS WITH RA AND OA AS WELL AS IN HEALTHY INDIVIDUALS

Group	Type of infection							
	without infection	persistent viral infection			active viral infection			with latent infection
		HHV-6 total	HHV-7 total	from these HHV-6 + HHV-7	HHV-6 total	HHV-7 total	from these HHV-6 + HHV-7	
Patients with RA n = 80	13/80	25/80	61/80	19/80	10/25	13/61	2/19	46/67
Patients with OA n = 78	15/78	1/78	41/78	21/78	1/1	7/41	7/21	48/63
Apparently healthy persons n = 19	4/19	0/19	10/19	5/19	0/15	2/10	1/5	12/15

Table 2

FREQUENCY OF VIRUS-SPECIFIC DNA IN SYNOVIAL TISSUES' AND SYNOVIAL FLUID DNA SAMPLES OF RA AND OA PATIENTS

Patients	Viral sequence in DNA samples					
	synovial fluid DNA			synovial tissues' DNA		
	HHV-6 single	HHV-7 single	HHV-6 + HHV7	HHV-6 single	HHV-7 single	HHV6 + HHV-7
RA patients	2/6	2/6	1/6	0/7	1/7	3/7
OA patients	0/34	10/34	3/34	0/54	17/54	3/54

in synovial fluid DNA of 5/6 (83.3%) RA and 13/34 (38.2%) OA patients. A single HHV-6 genomic sequence was detected in synovial fluid DNA samples of 2/6 (33.3%) RA patients, and in none of the OA patients ( $p = 0.0192$ ; OR 38.3, 95% CI: 1.6 to 932.3), while a single HHV-7 genomic sequence was present in synovial fluid DNA samples of 2/6 (33.3%) RA and 10/34 (29.4%) OA patients. Genomic sequences of both viruses were detected in synovial fluid DNA samples of 1/6 (16.7%) RA and 3/34 (8.8%) OA patients (Table 2).

#### Presence of HHV-6 and HHV-7 DNA in synovial tissues.

HHV-6 and/or HHV-7 genomic sequences were detected in synovial tissue DNA samples of 4/7 RA (57.1%) and 20/54 (37.0%) OA patients. A single HHV-6 sequence was not detected in any of the synovial tissue DNA samples from RA and OA patients. A single HHV-7 genomic sequence was found in 1/7 (14.3%) RA and in 17/54 (31.5%) OA patient DNA samples. Significantly more often genomic sequences of both viruses were detected in synovial tissue DNA samples of RA patients (3/7, 42.9%) than in OA patients (3/54, 5.6%;  $p = 0.0166$ ; OR 12.8, 95% CI: 1.9 to 84.9). Three of seven (42.9%) synovial tissue DNA samples of RA and 34/54 (63.0%) of OA patients lacked detectable HHV-6 and HHV-7 genomic sequences (Table 2).

#### Presence of HHV-6 immunoreactivity in synovial tissues.

Cells labeled by the anti-HHV-6 antibody for HHV-6 A and B strains displayed a brown cytoplasmic staining pattern. Expression of HHV-6 in synovial tissues was demonstrated in HHV-6-positive RA and OA patients enrolled for the study and estimated by histopathology (Fig. 1A). This HHV-6-immunoexpression was moderate and/or strong. Examination of the occurrence and localisation of HHV-6-positive cells by immunohistochemistry showed their presence in the synovial lining, which often was stratified into multiple layers, but still intermixed with synoviocytes displaying immunonegativity (Fig. 1B). Some RA and OA cases demonstrated HHV-6 immunopositivity along an intimal endothelial aspect of small blood vessels. Strong

HHV-6 expression was found in large foam-like vascular wall cells (in one case of OA).

#### RA clinical parameters, treatment and frequency of complications in RA patients with different activity phase or without HHV-6 and HHV-7 infection.

The counts of painful joints and duration of morning stiffness, as well as frequency of different complications — polyneuropathy, rheumatoid nodules, osteoporosis, cervical spine damage, sicca syndrome and scleritis, in RA patients with different activity phases of HHV-6 and HHV-7 infection or without activity was very similar (Tables 3, 4). However, morning stiffness was significantly lower in RA patients with active HHV-6 infection than in patients with latent HHV-6 infection ( $p = 0.02$ ; 95% CI: 16.0 to 178.0).

Myopathic syndrome was detected significantly less often in RA patients with active HHV-7 infection, than in patients with latent HHV-6 infection ( $p = 0.0117$ ; 95% CI: 0.1 to 0.7) and patients without HHV-6 and HHV-7 infection ( $p = 0.0158$ ; 95% CI: 0.1 to 0.8). More serious complication as scleritis was observed in RA patients with only latent HHV-7, and cervical spine damage was observed in RA patients with only latent HHV-6 and HHV-7 infection (Table 3). The detection frequency of HHV-6 and/or HHV-7 active and latent infection didn't differ in RA patients receiving or not receiving DMARDs. HHV-6 and HHV-7 infection fre-

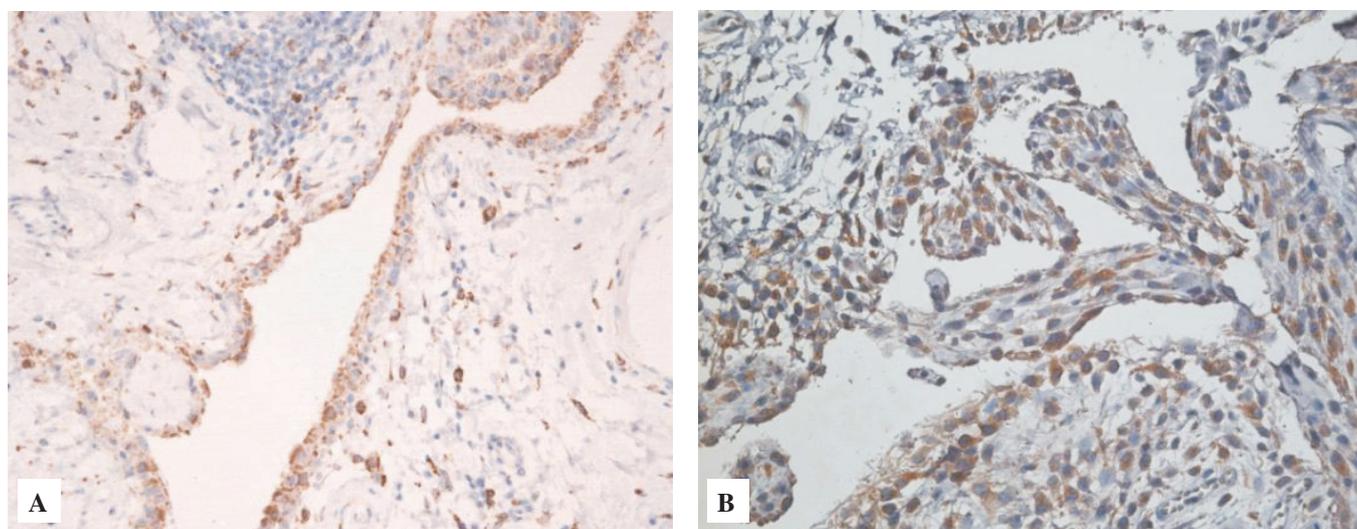


Fig. 1. **A.** Rheumatoid synovitis revealing lymphocytic infiltration and diffuse macrophage infiltration demonstrated with the anti-CD68 antibody. Original magnification  $\times 250$ ; **B.** Multiple layers of hyperplastic synoviocytes displaying HHV-6 immunoreactivity. Original magnification  $\times 250$ .

Table 3

## FREQUENCY OF COMPLICATIONS IN RA PATIENTS

Infection	Complication				
	single HHV-6 infection		single HHV-7 infection		without infection
	active	latent	active	latent	
Polyneuropathy	5/10	3/15	4/13	7/42	2/13
Myopathic syndrome	3/10	8/15	1/13	15/42	6/13
Rheumatoid nodules	3/10	1/15	1/13	3/42	1/13
Osteoporosis	4/10	6/15	0/13	8/42	2/13
Cervical spine damage	0/10	2/15	0/13	1/42	0/13
Sicca syndrome	2/10	0/15	2/13	2/42	0/13
Scleritis	0/10	0/15	0/13	1/42	0/13

quency was lower in single MTX users in comparison with "DMARDs on" group ( $p = 0.0555$ ; OR 5.3, 95% CI: 0.9 to 30.2) and "DMARDs off" group ( $p = 0.0737$ ; OR 0.2, 95% CI: 0.03 to 1.1) without statistical significance. Interestingly, HHV-6 and also HHV-7 infection reactivation were not observed in single methotrexate and in biologic treatment groups. No increase in appearance of complications such as polyneuropathy, myopathic syndrome, rheumatoid nodules, osteoporosis and sicca syndrome was observed in RA patients with active HHV-6 and/or HHV-7 infection (Table 3).

Clinical parameters did not diverge in OA patients with different activity phases of HHV-6 and HHV-7 infection.

**Disease activity and aggressiveness laboratory parameters and cytokine levels of RA and OA patients as well as radiological stage of RA patients with and without different activity phase of HHV-6 and HHV-7 infections.** There was no significant correlation between HHV-6 and/or HHV-7 infection activity stage, and DAS28 and ESR indi-

ces in RA patients. RA patients had moderate disease activity (DAS28 score 3.2–5.1) in all HHV-6 and/or HHV-7 infection groups. CRP and ESR mean levels were higher than the normal range in all RA patients groups. Of the disease activity indexes, the mean CRP level was significantly higher in the patient group with latent HHV-7 infection than in the patient group with active HHV-7 infection ( $p = 0.0031$ , 95% CI: 5.3 to 24.4).

In OA patients, the mean CRP level was higher in the patients group with latent HHV-7 infection than in patients group without HHV-6 and HHV-7 infection ( $p = 0.0488$ , 95% CI: 0.04 to 14.6), but no differences occurred in the presence of the ESR medium level in OA patients with various activity phases of HHV-6 and HHV-7 infection.

Both RA aggressiveness indexes were higher than normal range, but without statistical significance among groups with HHV-6 and/or HHV-7 infection, and the indexes were not related to the infection activity phase (Table 4), also if RA patients without HHV-6 and HHV-7 infection were used as control group (Fig. 2).

We did not find significant difference of radiographical stage in groups of RA patients with various HHV-6 and HHV-7 infection activity phases.

We observed elevated levels of RF and anti-CCP in OA patients, but the OA patients with positive anti-CCP level do not comply with the RA diagnostic criteria.

IL-6 mean level was significantly lower in RA patients with active HHV-6 than in patients with latent HHV-6 infection ( $p = 0.0478$ , 95% CI: 0.9 to 153.7). No significant differences in the mean level of TNF- $\alpha$  and IL-17 were observed in RA patients, nor in the mean level of all analysed cytokines in OA patients with different activity phases of HHV-6 and HHV-7 infection (Table 5, Fig. 2). The level of

Table 4

## MEAN RESULTS OF CLINICAL AND LABORATORY PARAMETERS IN RA PATIENTS

Infection	Parameter				
	latent HHV-6 infection, n = 15	latent HHV-7 infection, n = 42	active single HHV-6 infection, n = 10	active single HHV-7 infection, n = 13	without infection, n = 13
Painful joints count	11 (7–20)	10 (6–14)	10 (6–13)	7 (4–17)	11 (3–16)
Swollen joints count	5 (0–7)	5 (2–8)	6 (2–7)	4 (0–14)	4 (1–8)
Duration of morning stiffness (minutes)	120 (60–360)	90 (60–180)	60 (30–120)	60 (30–180)	180 (53–210)
ESR (mm/h)	17.0 (9.0–44.0)	23.0 (9.3–39.8)	17.5 (10.8–33.0)	20.0 (11.0–35.0)	28.0 (10.0–40.0)
CRP (mg/L)	9.2 (3.1–25.2)	7.5 (2.2–25.3)	6.1 (3.1–9.4)	3.2 (2.1–9.4)	4.3 (1.4–29)
DAS28	5.1 (4.4–6.4)	5.0 (3.9–5.6)	4.6 (3.9–5.3)	4.1 (3.3–4.9)	5.1 (3.8–5.9)
RF (U/ml)	56.1 (14.0–196.0)	39.8 (10.0–142.9)	77.1 (17.4–320.0)	64.0 (14.5–213.5)	27.9 (13.1–134.6)
Anti-CCP (U/ml)	43.4 (15.9–528.6)	35.9 (7.0–200.0)	55.8 (1.0–176.3)	22.6 (1.5–295.8)	149.3 (8.9–406.4)

## MEAN CONCENTRATIONS OF SERUM CYTOKINES IN RA PATIENTS

Infection	Parameter				
	latent single HHV-6 infection, n = 15	latent single HHV-7 infection, n = 42	active single HHV-6 infection, n = 10	active single HHV-7 infection, n = 13	without infection, n = 13
IL-17 (pg/ml)	130.0 (102.3–245.0)	245.0 (120.0–420.0)	180.0 (180.0–250.0)	225.0 (47.5–375.0)	150.0 (79.3–227.5)
TNF-a (pg/ml)	0,1 (0.1–52.5)	1,1 (0.1–59)	7,5 (0.1–110.0)	17,8 (2.0–75.2)	0,1 (0.1–57.5)
IL-6 (pg/ml)	31,2 (11.2–229.1)	51,5 (10.5–192.8)	8,6 (0.1–33.3)	12,5 (0.1–60.1)	49,2 (6.1–316.7)

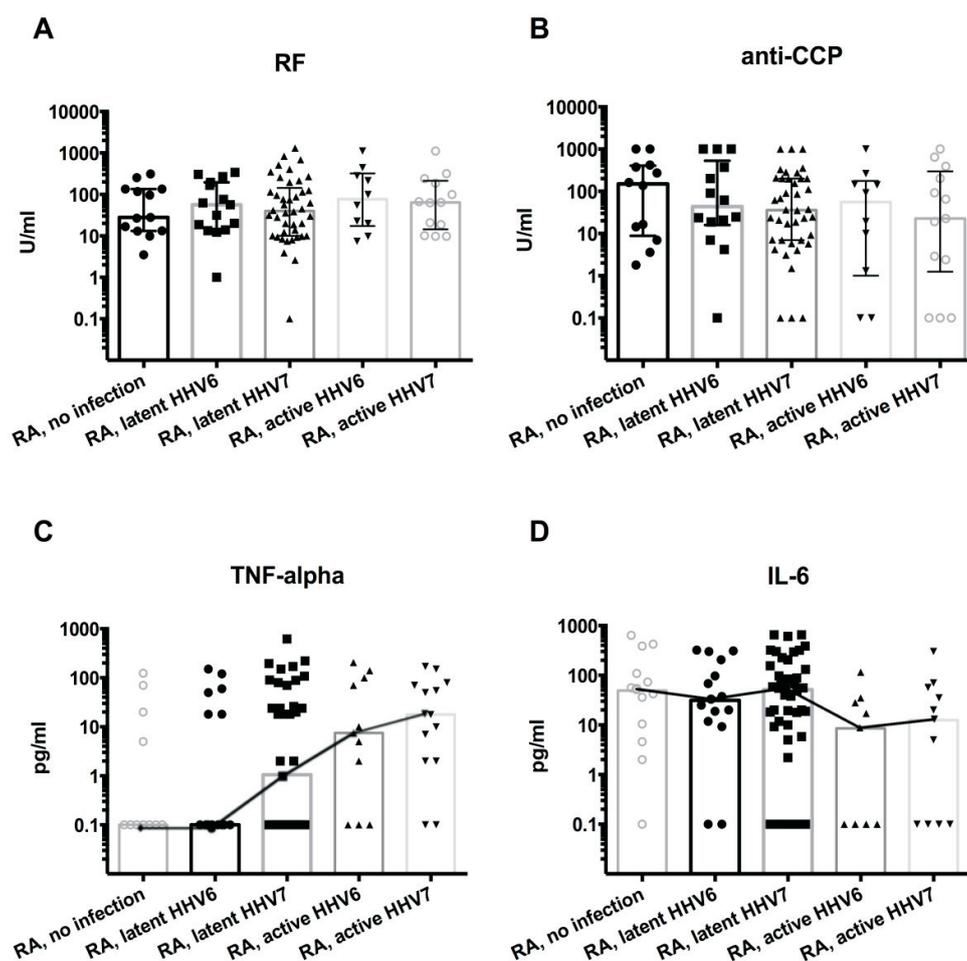


Fig. 2. Levels of RF (A), anti-CCP (B), TNF- $\alpha$  (C) and IL-6 (D) in RA patients with various persistent HHV-6 and HHV-7 infection activity phases. RA no infection group represents healthy control.

TNF- $\alpha$  tended to be higher in RA patients with active HHV-6 and/or HHV-7 infection than in the other groups.

## DISCUSSION

Viruses and viral infections are considered to be the main risk factors for autoimmune disease development (especially for individuals with genetic predisposition). The development of several autoimmune pathologies, including multiple sclerosis, autoimmune connective tissue diseases,

and Hashimoto thyroiditis, are linked to HHV-6A/B infection (Chapenko *et al.*, 2003; Nora-Krukke *et al.*, 2011; Caselli *et al.*, 2012; Broccolo *et al.*, 2013). It is also believed that human parvovirus B19, as an aetiological or co-efficient factor, is involved in the development of diseases such as RA, systemic lupus erythematosus, anti-phospholipid syndrome, systemic sclerosis and vasculitis. Our previous research has demonstrated linkage of parvovirus B19 to RA and association of the clinical development of the disease to infectious activity of the virus (Kozireva *et al.*, 2008; Kakurina *et al.*, 2015).

In this study we assess the possible association of HHV-6 and HHV-7 infection with RA and OA aetiopathogenesis.

Significantly higher prevalence of HHV-6 DNA has been observed in RA patient sera, compared with healthy control individuals (Alvarez-Lafuente *et al.*, 2009) and OA patients may have risk of EBV transmission or reactivation (Rollin, 2009). We did not find significant difference in prevalence of persistent HHV-6 and HHV-7 as well as in reactivation of HHV-6 and HHV-7 infection in RA and OA patients and healthy control groups. Despite similar frequency of persistent HHV-6 and/or HHV-7 infection in RA and OA patient groups by nPCR, HHV-6 DNA was detected significantly more frequently in synovial fluid of the RA patients group than in the OA patient group ( $p = 0.02$ , OR 38.3, 95% CI: 1.6 to 932.3). In synovial tissues of RA and OA patients, no single HHV-6 infection was found and there was no difference in the frequency of HHV-7 DNA in synovial tissues of both patient groups, but the frequency of concurrent HHV-6 and HHV-7 infection was significantly higher in synovial tissues of RA patients than in that of OA patients ( $p = 0.02$ , OR 12.8, 95% CI: 1.9 to 84.9). HHV-6 reactivity was confirmed in all PCR-positive RA cases by immunohistochemistry, but this does not exclude the presence of concurrent HHV-7 infection and possible detection of viral antigens. Demonstration of HHV-6 and/or HHV-7 DNA within the synovial joint cavity might suggest viral replication associated either with the primary immune disturbances of RA or with the immune depression produced by immunosuppressive drugs.

Effect of HHV-6 and/or HHV-7 on RA clinical parameters was different. There was no significant difference in the number of painful and swollen joints as well as pain severity in RA patients with HHV-6 and HHV-7 infection, depending on the activity phase of infection. Occurrence of morning stiffness was significantly lower in RA patients with active HHV-6 infection than in patients with latent HHV-6 infection. This can be explained by the treatment regimen — six RA patients with active HHV-6 received the severe immunosuppressive drug glucocorticoid with or without sDMARDs and only two patients received nonspecific treatment with NSAIDs. No significant difference was observed in disease clinical parameters in OA patients with various phases of HHV-6 and HHV-7 infection activity.

It is known that treatment with immunosuppressive drugs can influence reactivation of chronic viral infection (Vassilopoulos and Calabrese, 2007; Nard *et al.*, 2015), but in our study there was no effect of treatment regimen on prevalence of active HHV-6 and HHV-7 infection. No infection reactivation in RA patients groups with single methotrexate and biologic treatment was observed. These drugs are severe immunosuppressants that are prescribed in the case of severe active RA and possibly they can block reactivation of HHV-6 and HHV-7 infection. Previous studies did not find correlation between RA treatment and prevalence of HHV-6 DNA in sera (Alvarez-Lafuente *et al.*, 2005), nor significant association of the presence of cytomegalovirus and Epstein-Barr virus specific antibodies with the age, sex,

disease duration (from symptom onset), RF status, ACPA status, pain, fatigue, DAS28, HAQ disability, the SF-36 physical or mental component summary scores, or treatment with methotrexate, biologic response modifiers, or prednisone (Davis *et al.*, 2012). Other authors have accentuated the role of viral infection as a “second hit” acting in the presence of circulating ACPA and determining the transition from the pre-RA phase to chronic synovitis (van de Sande *et al.*, 2011).

There was no significant correlation between HHV-6 and HHV-7 infection and frequency of complications, such as polyneuropathy, rheumatoid nodules, osteoporosis, cervical spine damage, sicca syndrome and scleritis. However, serious RA complications like cervical spine damage and scleritis were observed only in patients with latent HHV-6 and/or HHV-7 infection. Also, the late RA sign myopathic syndrome was observed significantly often in RA patients with latent HHV-7 infection. This suggests that HHV-6/-7 infection can promote the disease progression. Analysis of the RA course severity suggests also high prevalence of RA complications in the patients group with active single HHV-6 infection as well as a more severe radiographic stage in RA patients with active concurrent HHV-6 and HHV-7 infection.

Several studies reported that anti-CMV-seropositive RA patients showed radiographic evidence of more advanced joint destruction (Davis *et al.*, 2012; Pierer *et al.*, 2012). We did not observe significant differences in radiographical stage among RA patients with different HHV-6 and HHV-7 infection stage.

The mean level of CRP was significantly higher in RA patients with latent HHV-7 infection, compared to that in patients with active HHV-7 infection. Almost all RA patients with active HHV-7 infection had received glucocorticoids and/or sDMARDs, which could reduce disease activity. Previous work also suggested lack of correlation of HHV-6 infection reactivation and RA activity (Broccolo *et al.*, 2013).

The mean levels of RA aggressiveness parameters RF and anti-CCP did not differ in RA patients with different HHV-6 and/or HHV-7 infection activity phases.

In OA patients, only the CRP mean level was significantly higher in patients group with latent HHV-7 infection, compared with that in the patients group without HHV-6 and HHV-7 infection; no significant differences in presence of ESR medium level was observed between OA patients with various activity phases of HHV-6 and HHV-7 infection. This shows that effect of HHV-6 and HHV-7 infection on OA disease activity is low.

Despite the lack of significant correlation between frequency of persistent single HHV-6, single HHV-7, and concurrent HHV-6 and HHV-7 infection, as well as its reactivation and RA clinical course, we found that both active and latent HHV-6 and/or HHV-7 infection increased RA activity and progression in several clinical and laboratory pa-

rameters. This suggests that HHV-6 and/or HHV-7 infection has effect on disease activity and aggressiveness, and that reactivation of these viruses may be a consequence of immunosuppressive treatment. 80.9% RA patients with HHV-6 and/or HHV-7 reactivation received glucocorticoids, methotrexate and leflunomide in different combinations or separately, which are strong immunosuppressants. Analysis of the severity of the course of RA suggests also high prevalence of RA complications in the patients group with active single HHV-6 infection and also a more severe radiographical stage in RA patients with active concurrent HHV-6 and HHV-7 infection. HHV-6 and HHV-7 infection markers also were found in synovial fluid and synovial tissues of affected joints of RA patients, suggesting their involvement in RA aetiopathogenesis.

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## CILVĒKA HERPESVĪRUSA 6 UN 7 INFEKCIJAS IETEKME UZ REIMATOĪDĀ ARTRĪTA KLĪNISKO GAITU

Reimatoīdais artrīts (RA) ir hroniska sistēmiska autoimūna iekaisuma slimība, kas skar galvenokārt locītavas, izraisot simetrisku hronisku progresējošu aseptisku sinovītu un vēlīni erozīvi destruktīvas izmaiņas. Vīrusi un vīrusu infekcijas tiek uzskatīti par vienu no galvenajiem riska faktoriem autoimūno slimību attīstībā (īpaši personām ar ģenētisku predispozīciju). Šī pētījuma mērķis bija noskaidrot persistētas HHV-6 un HHV-7 infekcijas sastopamību un aktivitātes fāzi slimniekiem ar RA, osteoartritu (OA) un veselām personām. Mēs noteicām arī HHV-6 un HHV-7 infekcijas ietekmi uz RA aktivitāti, agresivitāti, radioloģisko stadiju un slimības komplikāciju biežumu, kā arī vīrusu infekcijas marķieru klātbūtni RA pacientu skarto locītavu sinoviālajā šķidrumā un sinoviālajos audos. Neraugoties uz to, ka netika konstatēta ticama sakarība starp persistētu HHV-6, persistētu HHV-7, persistētu abu vīrusu dubultinfekciju un RA klīnisko gaitu, mēs noteicām, ka gan aktīva, gan latentā HHV-6 un/vai HHV-7 infekcija pēc vairākiem klīniskiem un laboratoriskiem parametriem paaugstina RA aktivitāti un progresiju. Turklāt vīrusu infekcijas marķieri tika atrasti RA pacientu skarto locītavu sinoviālajā šķidrumā un sinovija audos. Tas liek domāt, ka HHV-6 un/vai HHV-7 infekcija ietekmē slimības aktivitāti un agresiju, bet šo vīrusu reaktivāciju var veicināt imunosupresīvo medikamentu lietošana.