Rīgas Stradiņa universitāte

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INVOLVEMENT OF LATENT/PERSISTENT PARVOVIRUS B19, HHV-6 AND HHV-7 INFECTIONS IN ETHIOPATHOGENESIS OF RHEUMATOID ARTHRITIS AND RELATIONSHIP WITH CLINICAL AND RADIOLOGICAL FINDINGS

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

Speciality – Rheumatology

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TABLE OF CONTENTS

ABBREVIATIONS AND KEY WORDS

Anti-CCP antibodies	anti-cyclic citrullinated peptide antibodies
B19V	parvovirus B19
CD	designation of lymphocyte receptors
CMV	cytomegalovirus
CRP	C-reactive protein
DIP	distal interphalangeal
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
ESR	erythrocyte sedimentation rate
GC	glucocorticoids
Hb	haemoglobin
HHV-6	human herpes virus 6
HHV-7	human herpes virus 7
HLA	human leukocyte antigens
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukine
MCP	metacarpophalangeal
MMP-9	matrix metalloproteinase-9
MTP	metatarsophalangeal
MTX	methotrexate
NSAID	non-steroidal anti-inflammatory drugs
OA	osteoarthritis
PBL	peripheral blood leukocytes
PG	prostaglandin
PIP	proximal interphalangeal
PCR	polymerase chain reaction
SJC	swollen joint count
PNS	peripheral nervous system
RA	rheumatoid arthritis
RNA	ribonucleic acid
RF	rheumatoid factor
TJC	tender joint count
DMARD	disease-modifying antirheumatic drugs
TNF-α	tumour necrosis factor alpha

INTRODUCTION

Topicality of the research paper

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by progressing aseptic, symmetric polyarthritis, which with time causes erosive changes in joints and their ankylosis. Prolonged and aggressive progress of the disease may also cause internal injuries. RA is the most common inflammatory arthritis in adults. About 0.5 – 1% of people of the world have it. There are no accurate data about RA patients in Latvia, but based on disease incidence in nearby countries, these may be about 10,000 residents of the country. Due to different factors, RA is frequently diagnosed too late, when it has already made irrevocable changes to joints and extraarticular changes, complicating the use of effective therapy. As the disease progresses, gross joint deformations develop, which significantly restrict patient's work and self-care. As inflammatory cytokines spread throughout the body as blood circulates, an extraarticular damage may develop – an internal injury, which may reduce survival. Typically, RA starts in small joints of palms and feet, their fingers and toes. With time inflammation may affect almost every joint. Internal injuries most frequently affect the lungs, the cardiovascular system, the peripheral nervous system and the kidneys. New diagnostic methods have been developed recently, which allow to establish the diagnosis at an early stage, as well as a new group of drugs was created – biological disease-modifying anti-rheumatic drugs (bDMARD). The new group of drugs, acting on different stages of pathogenesis of the disease, provide for more effective control of progress of the disease and prevention its progression. Costs of bDMARD limit their availability. The risk of development of potential side effects also creates a known danger. Although RA is not completely curable and its treatment is life-long, it is possible to achieve disease remission. Every patient may have different progress of the disease – from mild, slowly progressing arthritis with

periodic remissions to aggressive, quickly progressing and very active arthritis with early development of deformations. Different progress of the disease requires the development of a personalised treatment strategy for each patient, because treatment efficiency and tolerability vary. Treatment with DMARD started at an early stage is more effective. Unfortunately, no treatment strategy excludes exacerbation of the disease and the development of progression.

RA is an autoimmune disease, which is based on immune system disorders. It was ascertained that RA develops in genetically predisposed individuals affecting a range of different external and internal factors. Despite many long-term studies have been carried out, the precise disease agent is unknown, therefore, it is necessary to continue to study possible factors, which may promote the development of the disease. The awareness of these factors would facilitate treatment of the disease. Smoking, large consumption of caffeine, cold injury and psychoemotional or physical stress, as well as different infectious agents – both bacteria and viruses, can be mentioned as external factors promoting the disease. The most well studied viral agents are parvovirus B19 (B19V), rubella virus, human herpes virus and other. B19V is a non-enveloped single-stranded DNA virus found throughout the world. B19V causes the disease only in humans targeting predecessors of their red blood cells. To be noted, its clinical presentation in patients may vary. The most common clinical syndromes of B19V are erythema infectiosum, polyarthritis, transient aplastic crisis and pure red cell aplasia, as well as *hydrops fetalis*, while skin lesion, hepatitis, neurological diseases, changes in blood cell composition, as well as rheumatological diseases, including RA, are less common. Human herpes virus 6 and 7 (HHV-6 and -7) are double-stranded DNA viruses. They are frequently found in persons with neuroinflammatory diseases. The first infection usually happens in childhood. As the virus does not get fully eliminated, it creates a persistent life-long infection. An injury, physical and emotional stress, hormonal disbalance or immune suppression

may contribute to reactivation of the infection. The correlation between B19V, HHV-6 and -7 and the development of RA has been studied for a long time, however, published data are ambiguous and often contradictory.

Available data on the effect of B19V and herpes virus infection on clinical and laboratory activity and aggressiveness parameters, as well as the radiological stage of RA are incomplete. The doctoral thesis summarises analyses of biological materials of patients treated in the Linezers Clinic, the Gaiļezers Clinic of the Riga East Clinical University Hospital, the Hospital of Traumatology and Orthopaedics, and laboratory biological material analysis and research conducted in the Oncovirology Laboratory of RSU A. Kirhenšteins Institute of Microbiology and Virology and the Histology Laboratory of RSU Institute of Anatomy and Anthropology from 2008 to - 2016. RA is the most common autoimmune inflammatory arthritis, which justifies the need for such studies. The obtained data may help to understand the development of the disease and to discover and eliminate factors promoting RA in a timely manner.

Hypotheses of the research paper

1. B19V, HHV-6 and -7 infections are one of those, which affect the development and progress of RA.
2. B19V, HHV-6 and -7 infections affect the clinical and laboratory activity, aggressiveness and radiological stage of RA.

As a null hypothesis it is assumed that B19V, HHV-6 and -7 infections do not affect ethiopathogenesis of RA, activity and aggressiveness of the disease based on clinical and laboratory parameters, and radiological stage.

Goal of the research paper

By comparing RA patients with OA patients and healthy control group individuals, to confirm or reject the role of B19V, HHV-6 and -7 infections in development of RA, as well as to evaluate the impact of different stages of activity of B19V, HHV-6 and -7 infections the clinical and laboratory activity, aggressiveness and radiological stage of RA.

Objectives of the research paper

1. To obtain data on presence and activity stage of B19V, HHV-6 and -7 infections in each individual included in the study, using molecular biology and serology methods:
   a. to determine the presence of B19V, HHV-6 and -7 genomic sequences in DNA samples isolated from PBL, in cells free of plasma, synovial fluid and synovial tissue in RA and OA patients and to determine the presence of B19V-specific IgG and IgM class antibodies in plasma/serum in all subjects included in the study;
   b. to determine the presence of B19V, HHV-6 and -7 genomic sequences in DNA samples isolated from PBL and cell free plasma in healthy individuals;
2. To determine the effect of therapy on the B19V, HHV-6 and -7 infectious activity stage in RA patients.
3. To determine the frequency and rate of T lymphocyte proliferative response to B19V antigens in RA patients compared to healthy subjects. Determine the rate and frequency of T lymphocyte proliferative response in RA patients, depending on the treatment received.
4. To determine changes in levels of MMP-9 and cytokines IL-2, IL-6, IL-10, IL-12, IL-17, and TNF-α depending on B19V, HHV-6 and -7 the infection

activity stage and on the B19V infection duration before the patient was included in the study in all study groups.

5. To determine the relationship between the RA and OA disease course by clinical and laboratory parameters and the radiological stage of RA with the B19V, HHV-6 and -7 infection activity stage and the B19V infection duration before the patient was included in the study.
6. In statistical data processing, to confirm or reject the null hypothesis.
7. Based on the obtained results, to draft recommendations for clinical practice.

1. MATERIAL AND METHODS

1.1. Included persons and inclusion criteria

The prospective study includes patients from the Linezers Clinic and the Gaiļezers Clinic of the Riga East Clinical University Hospital, and the Hospital of Traumatology and Orthopaedics, who were treated there from 2010 to 2016. Inclusion criteria included primary or remotely diagnosed RA or OA in these clinics. All RA patients met the RA classification criteria set by the American College of Rheumatology in 1987 (Arnett et al., 1988) or early RA classification criteria set by the American College of Rheumatology and the European League Against Rheumatism in 2010 (Aletaha et al., 2010). The exclusion criteria included pregnant women, alcoholics and patients with other inflammatory diseases. OA patients corresponded to the OA classification criteria set by the American College of Rheumatology (Altman et al., 1986., Altman et al., 1990., Altman et al., 1991). Potentially healthy individuals without known chronic inflammatory diseases were included in the study as a control group.

1.1.1. Demographic characteristics of patients with rheumatoid arthritis

103 Europeoid RA patients were included in the study. Those were 13 men (12.9%) and 90 women (87.1%), with average age 56.3 ± 12.8 (ranging from 19 to 82). The number of men among RA patients was statistically credibly smaller than in the OA patient group ($p = 0.001$) and in the group of healthy control individuals ($p = 0.0047$). RA patients were considerably older compared to healthy individuals ($p = 0.0047$). The average duration of the disease in this group was 93.7 (0–576) months.

The RA patient group included: 37 patients with early RA, whose duration of symptoms was 24 months or less (Emery, 1994) and 66 remotely diagnosed patients.

1.1.2. Demographic characteristics of patients with osteoarthritis

Since has been reported that inflammation play an role in the course of OA in recent years, OA patients have been used as a control arm for RA patients.

78 OA patients were examined in the study: 26 were Europeoid men (33%) and 52 were Europeoid women (67%), with average age 64.6 ± 12.0 (ranging from 35 to 86). OA patients were statistically credibly older than RA patients ($p < 0.0001$) and healthy control individuals ($p < 0.0001$). The average duration of the disease in the group was 78.6 (2–300) months.

1.1.3. Demographic characteristics of control group subjects

43 potentially healthy Europeoid control group individuals without known chronic inflammatory diseases were included in the study. This group included 15 men (34.9%) and 28 women (65.1%), with average age 51.7 ± 11.9 (ranging from 38 to 89).

The study was approved by the Ethics Committee of the Rīga Stradiņš University and all individuals signed an informed consent form before their inclusion in the study.

At the beginning of the study, every patient in both disease groups had their RA and OA clinically evaluated and the treatment used, the duration of its administration was re-confirmed, as well as the disease history was collected.

1.1.4. Materials used in the study (peripheral blood, synovial fluid, synovial tissues)

Samples of peripheral blood, synovial tissues and fluid of RA and OA patients (when possible based on indications), surgical material of cartilage tissues and bone tissues were obtained simultaneously. Peripheral blood samples were taken from healthy control group individuals (see Table 1.1). Blood for biochemical analysis was collected into vacutainers (Vacutest Clotactivator; 6 ml), blood for molecular biological analysis and T lymphocytes were collected into a vacutainers with EDTA (Vacutest K_2EDTA 5.4 mg; 3 ml × 6). Plasma was separated from peripheral blood using centrifugation 1400g × 15 minutes. Peripheral blood leukocytes (PBLs), which were not used for the proliferation test, were aliquoted (10^6 cells per tube) and frozen to −70 °C for further use. Superficial layer of the supernatant, plasma, was aliquoted into 200 µL per tube and frozen to −20 °C. Using a standard procedure, on the same day during an endoprosthetic joint surgery a traumatologist obtained synovial tissues biopsy material and synovial fluid of the hip joint for further histological and electron microscopic examination (see morphology section).

1.2. Clinical examination methods used

1.2.1. Tender and swollen joint count

In RA patients, the duration of morning stiffness, the general evaluation of health condition, which was marked at the visual analogue scale (VAS) (Anderson, 2012) from 0 to 100 mm was re-confirmed during a clinical examination. A full physical examination was conducted, during which the tender and swollen joint count was determined by evaluating 68 joints. The

tender and swollen joint count was analysed in connection with presence or lack of markers of B19V, HHV-6 and HHV-7 infections.

Table 1.1

Characteristic of samples taken from groups of RA and OA patients and healthy control group individuals

Patient group / Collected materials	RA patients	OA patients	Healthy individuals
Serum (2 × 5 ml)	103	78	43
Plasma (2 ml)	103	78	43
PBL (10^6 cells/ml)	103	78	43
Synovial fluid (3–5 ml)	6	33	–
Synovial tissues (1 cm^3 on average)	7	54	–
Surgical cartilage tissue material (1 cm^3 on average)	5	57	–
Surgical bone tissue material (1 cm^3 on average)	5	60	–

1.2.2. Clinical laboratory indicators

All clinical laboratory indicators were determined in respective clinics in a local laboratory.

Laboratory analysis of haemoglobin, lymphocytes and platelets: The reference interval of haemoglobin level was determined as 12.0–17.5g/dL, the interval of absolute lymphocyte count – 2.5–4.0 × 10^9/L and the interval of platelet count – 150–400 × 10^9/L. The results beyond these reference intervals were interpreted as reduced or increased level, respectively. The results of haemoglobin, lymphocyte and platelet levels were analysed in connection with presence or lack of markers of B19V, HHV-6 and HHV-7 infections.

Laboratory analysis of the disease activity: C-reactive protein (CRP) and the results of erythrocyte sedimentation rate (ESR) were evaluated as RA and OA activity indicators. ESR higher than 30 mm/h and CRP level higher

than 8 mg/L were evaluated as increased and corresponding to an active disease.

Laboratory analysis of the disease aggressiveness: Rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (anti-CCP) were analysed as RA aggressiveness indicators. These parameters were also determined in OA patients. The RF level above 14 IU/ml and anti-CCP antibody level above 17 IU/ml were evaluated as increased, taking into account local laboratory reference intervals. Based on the RF result, the clinical presentation of RA was classified as non-aggressive, if RF ≤ 14 IU/ml, weakly aggressive – if RF 14 ≤ 42 IU/ml, highly aggressive – if RF > 42 IU/ml and very highly aggressive – if RF > 1000 IU/ml. Based on the level of anti-CCP antibodies, the clinical presentation of RA was classified as non-aggressive, if anti-CCP ≤ 17 IU/ml, weakly aggressive – if anti-CCP 17 ≤ 51 IU/ml, highly aggressive – if anti-CCP > 51 IU/ml and very highly aggressive – if anti-CCP > 1000 IU/ml (*Aletaha et al., 2010*). The results of disease aggressiveness indicators were analysed in connection with presence or lack of markers of B19V, HHV-6 and HHV-7 infections.

1.2.3. DAS28

The CRP result, using a standard formula, was used for the calculation of DAS28 in the group of RA patients, which is an evidence of existing inflammation pattern. According to DAS28 indicators, RA patients were classified into four groups:

- remission (DAS28 ≤ 2.6),
- low disease activity (DAS28 2.6 ≤ 3.2),
- medium disease activity (DAS28 3.2 ≤ 5.1),
- high disease activity (DAS28 ≥ 5.1).

The results of disease activity indicators were analysed in connection with activity stage or lack of B19V, HHV-6 and HHV-7 infections.

1.2.4. Radiologic examinations

In the Gaiļezers Clinic of the RECUH and in the Latvian Oncology Centre, RA patients had an X-ray and in case of doubt also a magnetic resonance imaging (MRI) for both palms to re-confirm presence of early changes and erosions. A two independent radiologists with long work experience interpreted examination results. The MRI protocol was as follows: FSE T2 "fat sat" in coronary plane, GR in axial plane, SE T1 "fat sat" in coronary plane after gadolinium injection, 3D SPGR in coronary plane after gadolinium injection. Presence of synovitis, bone marrow oedema and bone erosion was evaluated on MR images. After the interpretation of X-ray and MRI results, RA patients were broken down into four groups, based on Venables and Wheelss recommendations (Venables, Maini, 2013, Wheeless, 2012) (see Table 1.2). The radiological stage of RA was analysed in connection with activity or lack of B19V, HHV-6 and HHV-7 infections.

Table 1.2

Characteristics of radiological stages of RA

Stage	Characteristics
I	Early RA stage. Characterised by synovitis or inflammation of the synovial membrane. Typically, the X-ray does not show destruction of joints, but only swelling of soft tissues.
II	Moderate RA stage. The inflammation spreads into synovial tissues, affecting the joint cavity, also the cartilage. Destruction of cartilage tissues and joint space narrowing are forming gradually.
III	Marked RA stage. Pannus forms in the synovium, the cartilage covering the joint surface gradually disappears and the bone becomes exposed. The X-ray shows marginal erosions. Developed deformations (subluxations).
IV	Final or terminal stage of RA. The active inflammation process reduces, fibrous tissues are forming, the bones forming the joint merge – ankylosis develops.

1.2.5. Complications and their frequency

Existing complications of the disease were re-confirmed in RA patients. Complications included PNS impairment, which includes polyneuropathy and carpal tunnel syndrome, myopathy, Sjogren's syndrome and rheumatoid nodules, as well as specific damage of cervical vertebrae, osteoporosis and scleritis.

1.3. Methods for determination of markers of B19, HHV-6 and HHV-7 infections

1.3.1. Determination of persistent B19 infection and its stage of activity (nPCR)

DNA was isolated from full blood, cell-free plasma, synovial tissues and synovial fluid, using standard phenol-chloroform extraction. Before isolating DNA, samples of plasma and synovial fluid were treated with DNase I. To guarantee the quality of DNA of PBLs and synovial tissues, as well as to exclude contamination of the plasma and synovial fluid with DNA of cells, beta-globin polymerase chain reaction (PCR) was carried out.

The presence of B19V genomic sequences was determined using nested PCR. DNA was isolated from PBLs and plasma, using cleavage with proteinase K i followed by a standard phenol-chloroform extraction technique. The quality of the isolated DNA was determined using PCR amplification of the 200-bp fragment of the beta-globin gene. Nested PCR of B19V VP-1 and B19V NS-1 was carried out as described above (*Barah et al., 2001*). Presence of B19V genomic sequences in the DNA, which was isolated from cell-free plasma (viremia), was used as a marker of active infection, while presence of B19V genomic sequences in the DNA, which was isolated from peripheral blood leukocytes, was used as a marker of latent persistent infection. The viral load

was determined using real-time PCR technology with a TaqMan probe for VP-1 gene, according to the manufacturer's protocol (*Roboscreen*, Germany).

1.3.2. Determination of B19 specific antibodies (*recomWell, recomLine*)

B19 specific IgM class and IgG class antibodies were determined using ELISA *recomWell* kit (Microgen, Germany), based on the manufacturer's recommendations. Presence in plasma of antibodies of the virus against certain B19V was re-confirmed using the *recomLine* Parvovirus IgM and IgG test (Microgen, Germany) and guided by the manufacturer's recommendations.

1.3.3. Determination of proliferative response of T lymphocytes to B19V and its proteins

Proliferative activity of PBMCs was determined on the third and sixth day after the addition of ^{3}H-thymidine to the cells, which were cultured in presence of B19V (final concentration – 10^6 viral genomes/ml) or VP1/VP2 B19 peptide LASEESAFYVLEHSSFQLLG [(Caslo Laboratory ApS, Denmark) final concentration 10 µg/ml] (Kasprowicz et al., 2006). The final concentration of B19V was calculated based on the viral load in the serum material, which was determined using real-time PCR (serum with high viral load was received from prof. K. Haedman, Haartman Institute, Helsinki University). PBMCs were isolated from heparinised blood with Ficoll-Hypaque density gradient centrifugation (400 × g, 30 min), washed with RPMI-1640 (GIBCO), then centrifugated and resuspended by RPMI-1640 with 10% foetal bovine serum supplement, supplemented with penicillin (10^5 U/L) and streptomycin (100 mg/L) (GIBCO). The obtained PBMCs were cultured in 9–12 wells in two 24-well plates in concentration 1×10^6 cells per well. B19V was added to 3–4 wells and VP1/VP2 B19V peptide to 3–4 wells of each

sample. On day three or six of the culturing ^{3}H-thymidine (25 Ci/mmol, Amersham, England) 2 μCi/well was added and the cells were cultured for four hours, then the cells were collected and transferred to Millipore filters (pore size 1.5 μm). The filters were twice rinsed with PBS and three times with 5 ml of 5% trichloroacetic acid to deposit DNA. The DNA was fixed with 1 ml of 96% ethanol and air dried at 37 °C. The inclusion of ^{3}H-thymidine was measured using the Packard liquid scintillation analyser. The proliferative response of PBMCs was considered positive, if the inclusion of ^{3}H-thymidine in the cells stimulated by the virus or peptide at least doubled compared to the negative control. The data were expressed as count per minute (Δcpm): mean cpm (test antigen), mean cpm (*media*) and as simulation indices (SI). SI = mean cpm (test antigen) / mean cpm (*media*). PBMC simulation with phytohemagglutinin M (GIBCO) (2μg/ml) followed by the addition of identical ^{3}H-thymidine and culturing was used as a positive control.

1.3.4. Determination of persistent HHV-6 and HHV-7 infection and its stage of activity (nPCR)

One microgram of blood and synovial tissue DNA, as well as 10 μl of plasma and synovial fluid DNA were used for nested PCR with specific HHV-6 and HHV-7 primers as described above (Secchiero et al., 1995; Berneman et al., 1992). Each experiment included HHV-6 and HHV-7 positive and negative control, as well as water control. The presence of HHV-6 and HHV-7 sequences in DNA, which was isolated from peripheral blood and synovial tissues, was used as a marker of persistent infection, but identification of DNA in cell-free plasma and synovial fluid – as a marker of active infection (reactivation). The number of persons with persistent infection at latent stage was obtained by calculating the difference between the total number of patients with persistent infection and persistent infection at active stage.

1.3.5. Determination of HHV-6 and HHV-7 specific antibodies (ELISA)

As incidence of HHV-6 specific IgG class antibodies in the adult population is almost 100% (Braun et al., 1997), they cannot be used to differentiate between remote and persistent infection. Furthermore, kits for determination of HHV-6 IgM class antibodies, which would allow determining presence of an active infection, were not commercially available, when the doctoral thesis was written, therefore, the antibodies were not determined.

According to literary data, HHV-7 specific IgG class antibodies in the adult population are as common as HHV-6 specific IgG class antibodies (Clark et al., 1993). As kits for determination of HHV-7 IgM and IgM class antibodies were not commercially available, when the doctoral thesis was written, they were not determined.

1.3.6. Immunohistochemical determination of B19V, HHV-6 and HHV-7 antigens

Synovial tissues from 6 RA and 43 OA patients were fixed in 10% neutral formalin, washed, dehydrated and contaminated with paraffin. 4–5 microns thick cuts made from 4 to 5 microns thick, dewaxed, rehydrated and dried haematoxylin for routine histopathological analysis and diagnosis detection, which include determination of the thickness of the upper lining of the synovial membrane and the lower layer (sublining), vascularization and inflammatory infiltration, the presence of lymphoid follicles according to previous recommendations (Baeten et al., 2003; Kruithof et al., 2005). The resulting sequential paraffin cuttings of synovial tissue were used for immunohistochemical analysis. Immunohistochemical reactions were performed using the anti-HHV-6 (2001) sc-65463 monoclonal antibody to detect both HHV-6A and HHV-6B type (*Santa Cruz Biotechnology, Inc., Santa*

Cruz, California, USA, 1: 200 dilution) and anti-parvovirus B19 NCL-PARVO monoclonal antibody, which allows the determination of VP1 / VP2 proteins (Leica Biosystems, Novocastra, 1:20 dilution).

Synovial macrophages were determined using a monoclonal anti-CD68 antibody (*DakoCytomation, Glostrup, Denmark*, 1: 100 dilution, PG-M1 clone) that recognizes lysosomes.

HiDef Detection ™ HRP Polymer System (*CellMarque, Rocklin, California, USA*) and DAB + Chromogen (*Cell Marque, Rocklin, California, USA*) were used to visualize the reaction results. Immunohistochemical controls include the replacement of primary antibodies with a buffer. The tissue cuttings were analyzed using an increase (×100 to ×400) and the preparations were analyzed by the *Leitz DMRB* light field microscope using the DFC 450C digital camera.

1.3.7. Determination of the level of proinflammatory cytokines

Levels of MMP-9 and cytokines TNF-α, IL-6, IL-10, IL-12 and IL-17 were determined using ferment immune sorption test (ELISA) (TNF-α, IL-6 with *Nordic Biosite, Denmark* and MMP-9 and IL-17 with *R&D system, USA*, IL-10, IL-12 with *Affymetrix eBioscience, USA*) and the results were analysed for the presence of lack of markers of B19V, HHV-6 and HHV-7 infection.

1.4. Therapy used for treatment of rheumatoid arthritis

20 (19.6%) of RA patients used only NSAID without base therapy or other immunosuppressants. RA patients used the following drugs as monotherapy or in different combinations: 49 (47.6%) – GC, 41 (39.8%) – MTX, 19 (18.4%) – leflunomide, 10 (9.7%) – hydroxychloroquine, 31 (30.1%)

sulfasalazine. 24 (23.3%) RA patients used a combination of different sDMARDs without MTX. Overall, 64 (62.1%) RA patients received sDMARD. 35 (54.7%) of them receive one sDMARD and 29 (45.3%) two or more sDMARD. 8 (7.8%) RA patients received sDMARD as monotherapy or in combination with other immunosuppressants.

RA patients were evaluated for the total impact of immunosuppressive therapy on the stage of activity of B19V, HHV-6 and HHV-7 persistent infection (B19V based on *recomLine* and *recomWell*, based on proliferative response of T lymphocytes to B19V antigens and incidence of B19V DNA sequences in cell-free plasma, HHV-6 and HHV-7 – based on incidence of virus-specific sequences in cell-free plasma).

1.5. Statistical processing methods

The data were analysed using SPSS Version 23 (SPSS Inc., Statistical Package for the Social Sciences, US product) and GraphPad Prism 6.0 software. Generally used descriptive and conclusive statistical methods, which are described in medical, biological, mathematical and general statistical literature, were used in the study. Mean levels of all clinical, laboratory parameters and plasma cytokines and MMP are expressed as medians with dispersion, which is characterised by interquartile region (IQR).

The following statistical methods were used for the verification of hypotheses according to the set objectives and type of data used:

1. ANOVA is a one-way analysis of variance used for comparison of mean values of a quantified variable in more than two independent samples;
2. The Fisher's exact test evaluates the credibility of differences between proportions of two samples;

3. The Chi-square test is a non-parametric test, which allows evaluating matching between two qualitative variables;
4. The Mann-Whitney test is a non-parametric criterion, which is used to determine the quantitative value of a value of interest in a small sample;
5. Student's t-test is a non-parametric criterion, which is used for the comparison of mean values of quantified variables of two independent samples.

The differences in characteristics for testing of hypotheses were evaluated with 95% confidence, which matches the significance level $p = 0.050$. The null hypothesis was rejected, because p value was smaller than 0.050, but if p value was bigger than 0.050 and smaller than 0.100, then the value was considered a trend of statistical difference.

2. RESULTS

2.1. Clinical and laboratory characteristics of patients included in the study

2.1.1. Patients with rheumatoid arthritis

According to the mean indicators of the disease activity, the disease of RA patients was with high activity. The most common RA complications were polyneuropathy 21.3%, rheumatoid nodules 16.3%, myopathic syndrome 26.6% and osteoporosis 20%, but less common specific damage of cervical vertebrae 3.8%, Sjogren's syndrome 6.3% and scleritis 1.3%. The total number of complications in the group of RA patients was 80. 52.5% of RA patients did not have any complications.

In the group of RA patients, the mean platelet count and haemoglobin corresponded to the reference range, while the mean number of lymphocytes was 1.76 ± 0.68 x 109 / L, which was consistent with lymphopenia. Among RA patients, mean laboratory disease activity indicators were low. In the group of RA patients, remission according to DAS28 was found in 7.1% of patients, low disease activity – in 6.1%, medium high RA activity – in 49.5% and high disease activity – in 37.4% of RA patients. The total RA aggressiveness by RF and by anti-CCP was evaluated as high.

Mean cytokine levels among RA patients were not evaluated as markedly increased.

Based on the results of radiological examinations, RA patients were broken down into four radiological stages: stage I was found in 8.7% of patients, stage II – in 31.1% of patients, stage III – in 46.6% of patients and stage IV – in 13.6% of RA patients.

2.1.2. OA patients

The clinical characteristics of the OA group corresponded to medium active disease.

In OA patients, the mean platelet counts and haemoglobin corresponded to the reference range, while the mean number of lymphocytes was 2.07 ± 0.41 x 10^9 / L, which was consistent with lymphopenia. Based on laboratory activity parameters, the OA patient group corresponded to mildly active OA. In the OA patient group, mean RF and anti-CCP levels corresponded to the respective reference interval – the mean RF level was 7.0 IU/ml (IQR 4.2–10.4) and the mean anti-CCP level was 7.0 IU/ml (IQR 7.0–12.3).

Mean cytokine levels among RA patients were not evaluated as markedly increased.

2.1.3. Control group individuals

Control group individuals were practically healthy persons. The level of MMP-9 and cytokines in healthy control group individuals was determined without stated changes.

2.2. Finding of markers of a viral infection in RA patients

2.2.1. Finding of markers of a B19V infection in RA patients

The determination of B19V antibodies according to *recomWell* and PCR test was carried out for 96 RA patients. Based on *recomWell* and PCR results, RA patients were broken down into five groups: (1) 37 RA patients with remote B19V infection (presence of B19 IgG class antibodies only), (2) 15 RA patients with acute B19V infection (presence of B19 IgM class antibodies and/or

viremia), (3) 14 RA patients with B19V persistent latent infection (presence of B19 IgG class antibodies and B19V DNA in blood), (4) 19 RA patients with B19V persistent active infection (presence of B19 IgG class antibodies and viremia) and (5) 11 RA without B19V infection (without B19V specific antibodies). The acute infection group also included RA patients with B19 IgM, IgG class antibodies and without viral genome in the DNA and presence of IgG NS-1 class antibodies. Full blood DNA PCR may be negative, because the test was conducted at an acute infection stage, when B19 IgG class antibodies have already appeared and the viral load is low. The persistent active infection group also included RA patients with B19 IgM and IgG class antibodies, no viral genome in the DNA, but with presence of NS-1 specific IgG class antibodies. The PCR finding may be negative due to low viral load.

SJC was considerably higher in RA patients with active persistent B19V infection than in patients with remote B19V infection ($p = 0.018$). The duration and the radiological stage of the disease in these RA groups had no statistically credible difference.

The incidence of different RA complications in RA groups with different stages of activity of B19V infection was similar. The total number of complications in these RA groups did not significantly differ. Stages of activity of B19V infection in RA patients with a different treatment strategy did not significantly differ.

The level of ESR and CRP, RA laboratory activity indicators, between RA patient groups with different stages of B19V activity did not statistically credibly differ, and the level of anti-CCP, DAS28 and disease aggressiveness indicator, between them was similar. In RA patients with acute B19V infection, the RF level was considerably higher compared to RA patients without B19V infection ($p = 0.015$), compared to RA patients with remote B19V infection ($p = 0.001$) and to RA patients with latent persistent infection ($p = 0.027$). The degree of aggressiveness separately by RF and separately by anti-CCP level in

RA patients with different stages of activity of B19V infection was similar. Furthermore, the total level of aggressiveness, when RF and anti-CCP levels were evaluated at the same time, was higher in RA patients with acute B19V infection than in patients without B19V infection ($p = 0.013$) and in patients with remote B19V infection ($p = 0.002$).

The mean haemoglobin, platelet and lymphocyte counts in these groups did not significantly differ.

The TNF-α level in the group of RA patients with remote B19V infection was lower than in groups of RA patients with active persistent B19V infection ($p = 0.001$) and with acute B19V infection ($p = 0.002$). In RA patients with acute B19V infection, the TNF-α level was higher compared to RA patients without B19V infection ($p = 0.043$). The IL-17 level was higher in RA patients without B19V infection compared to the group of RA patients with remote B19V infection ($p = 0.027$). The MMP-9 level in RA patients with active persistent B19V infection was found to be statistically credibly lower than in RA patients with remote B19V infection ($p = 0.006$) and then in patients without B19V infection ($p = 0.001$). The cytokine level among the mentioned patient groups did not significantly differ.

The determination of B19V antibodies according to *recomLine* test was carried out for all 103 RA patients. Based on *recomLine* and manufacturer's recommendations/protocols, RA patients were broken down into four groups: (1) 29 RA patients with years long B19V infection, (2) 34 RA patients with months to years long B19V infection, (3) 14 RA patients with weeks to months long B19V infection and (4) 19 RA patients without B19V infection. In seven RA patients the period, when they were infected with B19V, was unclear, therefore, their data were not used for further processing. The groups characterise the time period from the infection until the patient was included in the study.

No statistically credible differences in clinical parameters of the disease (TJC, SJC, VAS, morning stiffness) and in the radiological stage, DAS28, laboratory disease indicators (ESR, CRP) aggressiveness (RF and anti-CCP) and other clinical (Hb, platelets, lymphocytes) indicators, as well as levels of MMP-9 and cytokines were found, taking into account the period of being infected with B19V before the inclusion in the study.

The incidence of RA complications in RA patients with different periods of infection with B19V before the inclusion in the study did not significantly differ (evaluating based on *recomLine*).

The mean level of IL-17 cytokine in RA patients with months to years long B19V infection was lower than in patients without B19V infection ($p = 0.007$), while the mean level of IL-17 cytokine with weeks to months long B19V infection was higher than in RA patients with years long B19V infection ($p = 0.025$) and in patients with months to years long RA infection ($p = 0.001$). Levels of other cytokines in RA patients with different period of infection with B19V before the inclusion in the study with RA did not differ.

The difference in proliferative response of T lymphocytes was found between RA patients and healthy control individuals. Lymphocytes of 73.6% of RA patients and lymphocytes of only 25.0% of healthy individuals responded with proliferation to B19V antigens. T lymphocytes of RA patients responded to B19V antigens (viruses and/or VP1/VP2 peptides) more frequently and much faster than control group individuals. On the third day of culturing proliferation of T lymphocytes in presence of B19V antigens was stated in 25/52 (48.0%) RA patients and only in 2/25 (8.0%) healthy individuals ($p = 0.00068$). On the sixth day of culturing of T lymphocytes, 33/52 (63.5%) RA patients and only in 8/25 (32.0%) control group subjects ($p = 0.01436$) responded to B19V antigens.

Having analysed the response of T lymphocytes to B19V antigens depending on markers of B19V infection, it was determined that all RA patients

(4/4) and all control group individuals (6/6), in whom no markers of B19V infection were found, did not respond to B19V antigens. In 10/14 (74.1%) RA patients with active B19V viral infection and both control group subjects with an active viral infection, T lymphocytes responded to B19V antigens on the third day of proliferation. Also, on the third day of proliferation, T lymphocytes of 13/29 (44.8%) remotely (years long) infected RA patients, including patients with remote B19V infection, responded to viral antigens, but T lymphocytes of remotely infected persons did not respond to B19V antigens on the third day ($p=0.00071$). Furthermore, on the sixth day of proliferation, T lymphocytes of 20/29 (68.9%) RA patients, who were infected several years ago, and T lymphocytes of 6/17 (41.1%) years long infected control individuals ($p = 0.0347$) responded to viral antigens with proliferation. The proliferative response of T lymphocytes to B19V antigens was found in 2/5 (40.0%) RA patients, in whom IgG class antibodies and B19V genome sequence PBL (persistent infection) was found, the proliferative response of T lymphocytes to B19V antigens was determined in 2/5 (40.0%) patients on the third day and in 4/5 (80.0%) patients on the sixth day. No persistent infection was found in the group of healthy individuals.

On the third day, the proliferative response of T lymphocytes in RA patients with acute B19V infection to B19 VP-1/VP-2 peptide was detected in 9/14 (64.2%) and on the sixth day – in 8/14 (57.1%) patients. T lymphocytes of both control group individuals with acute B19V infection responded to B19V antigens, but only on the sixth day. The proliferative response of T lymphocytes in remotely infected RA patients on the third day was detected in 8/29 (27.5%) and on the sixth day – in 7/29 (24.1%) patients. Furthermore, T lymphocytes of control group subjects with years long B19V infection before the inclusion in the study responded to B19V antigens only on the sixth day. T lymphocytes in 2/5 (40.0%) RA patients with latent persistent B19V infection responded with proliferation on the third day and 4/5 (80.0%) patient

– on the sixth day. Thus, the proliferative response of T lymphocytes to B19V VP-1/VP-2 peptide was determined both in RA patients infected with the virus and in control group individuals, but in RA patients this response was considerably faster, because on the third day it was detected only in the RA patient group ($p = 0.009$; 0/19 vs 10/34).

Higher mean indicators of stimulation of T lymphocytes were found in groups of RA patients and healthy subjects with markers of active B19V infection and with markers of latent persistent infection (see Tab. 2.1).

Table 2.1

Mean indicators of stimulations of T lymphocytes with B19V antigens in groups of RA patients and healthy individuals

RA patients with markers of active B19V infection (n = 14)		**Remotely infected RA patients**				**RA patients without markers of B19V infection (n = 4)**	
3 days peptide/ virus	**6 days peptide/ virus**	**IgG only (remote B19V infection) (n = 29)**		**IgG with B19 DNA PBL (latent persistent B19V infection) (n = 5)**		**3 days peptide/ virus**	**6 days peptide/ virus**
		3 days peptide/ virus	**6 days peptide/ virus**	**3 days peptide/ virus**	**6 days peptide/ virus**		
2.00 ± 0.18 1.76 ± 0.15	2.17 ± 0.16 2.24 ± 0.14	1.55 ± 0.12 1.49 ± 0.12	1.63 ± 0.09 1.88 ± 0.12	1.87±0.55 1.30±0.22	2.63 ± 0.63 2.67 ± 0.64	0.99 ± 0.10 1.13 ± 0.03	1.04 ± 0.05 1.07 ± 0.07
Control subjects with markers of active B19V infection (n = 2)		**IgG only (remote B19V infection) (n = 17)**				**Control subjects without markers of B19V infection (n = 6)**	
3 days peptide/ virus	**6 days peptide/ virus**	**3 days peptide/ virus**		**6 days peptide/ virus**		**3 days peptide/ virus**	**6 days peptide/ virus**
1.78 ± 0.03 2.01 ± 0.01	2.21 ± 0.12 2.62 ± 0.10	1.06 ± 0.04 0.99 ± 0.03		1.56 ± 0.20 1.46 ± 0.16		1.11 ± 0.10 1.04 ± 0.08	0.96 ± 0.04 0.99 ± 0.06

2.2.2. Finding of markers of a B19V infection and other laboratory indicators depending on the therapy used

Patient group, which did not receive DMARD as therapy

If we compare RA patients, who received NSAID only, with RA patients, who received any of immunosuppressive drugs (sDMARD, bDMARD, GC), no significant differences were found by the B19V infection activity stage (according to the *recomWell* test) or by the period of infection with B19V before the inclusion in the study (according to the *recomLine* test). Among users of immunosuppressive drugs, proliferative response of T lymphocytes to B19V antigens on the third day was infrequent (45.2%), but on the sixth day it was credibly infrequent (66.7%) than among non-uses of these drugs (80% and 100%, $p = 0.078$ and $p = 0.046$, respectively). The incidence of B19V IgM NS-1 and B19V IgG NS-1 antibodies did not differ among groups of users and non-users of immunosuppressants. The level of cytokines and MMP-9 did not statistically credibly differ among users and non-users. No significant differences in B19V infection activity phases according to the *recomLine* test compared to RA patients, who do not use DMARD.

The incidence of B19V DNA sequences in blood, plasma, synovial fluid or synovial tissues, as well as IgG class antibodies against B19V NS-1 did not significantly differ in RA patients, who use and do not use sDMARD. No statistically credible differences by any parameters were found in the RA patients, who used only one sDMARD. Furthermore, if an RA patient used two or more sDMARD, the proliferative response of T lymphocytes to B19V antigens on the third and sixth day was credibly lower than in the group on non-users of sDMARD ($p = 0.001$ un $p = 0.001$, respectively). The incidence of different stages of B19V infection according to the *recomLine* test and the

incidence of B19V DNA sequences in blood, plasma, synovial tissues and synovial fluid in the group of RA patients, who did not use at least two sDMARD. Neither the use of sDMARD generally, nor the number of sDMARD used significantly affected the level of MMP-9 and cytokines in respective treatment groups.

Patient group, which received glucocorticoids as therapy

No difference was found in the incidence of different stages of B19V infection according to *recomWell*, compared to their incidence in groups of users and non-users of GC. The use of GC did not affect the proliferative response of T lymphocytes to B19V antigens on the third or sixth day. The incidence of IgM class antibodies against B19V NS-1 and IgG class antibodies against B19V NS-1 in both groups did not statistically differ. B19V DNA sequences in blood were found less frequently in GC users than non-users ($p = 0.044$). The incidence of B19V DNA sequences in plasma, synovial fluid and tissues did not statistically differ.

Patient group, which received methotrexate as therapy

The incidence of different stages of B19V infection determined according to the *recomWell* test and B19V infection according to the *recomLine* test, did not significantly differ among MTX users and non-users. The incidence of IgG and IgM class antibodies against B19V NS-1 did not differ among users and non-users of MTX. The incidence of B19V genome sequences in the DNA isolated from blood, cell-free plasma, synovial fluid and tissues did not credibly differ.

To evaluate the potential effect of MTX on the immune response of B19V specific cells to viral antigens, the proliferative response of T lymphocytes was compared in RA patients with and without MTX therapy on the third and sixth day. As a result, it was stated that in both treatment groups (with and without MTX) the T lymphocytes proliferative response on the sixth day was considerably higher than on the third day ($p = 0.0001$ and $p = 0.0001$). In RA patients, who did not receive MTX therapy, the detected mean T lymphocytes proliferative activity of cells was considerably higher both on the third and sixth day compared to the mean T lymphocytes proliferative activity of cells in control group individuals on the third and sixth day (see Fig. 1.1).

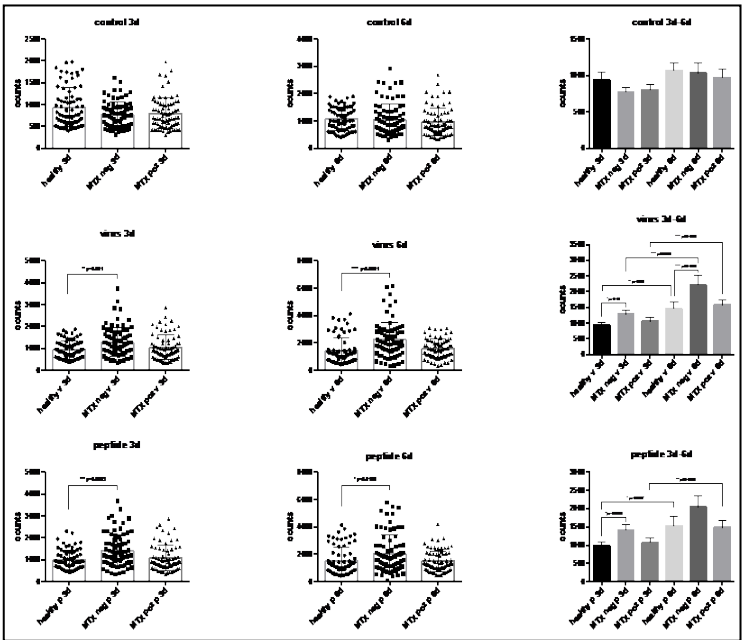

Figure 1.1. **Proliferative response of T lymphocytes in RA patients and control group individuals** (upper row images: intact lymphocyte cultivation with phytohemagglutinin M; median row images: lymphocyte cultivation with B19V genome; lower row images: lymphocyte cultivation with B19V VP1 / VP2 peptide)

No significant differences in T lymphocytes proliferative activity was found between RA patients with MTX therapy and RA patients without MTX therapy, as well as between RA patients with MTX therapy and healthy individuals. Taking into account high individual variability in RA patients with and without MTX therapy, RA patients and control group subjects were broken down into two subgroups depending on the mean T lymphocyte count. The T lymphocytes proliferative activity, which was determined without adding B19V antigens (negative control), was assumed as 100%. Thus, one subgroup included RA patients or healthy subjects, whose proliferative activity is similar to the negative control group, but the second subgroup – RA patients or healthy subjects, whose proliferative activity is higher than in the negative control group. Using this analysis, considerable differences were found on the third and sixth day of T lymphocytes proliferation between RA patients, who receive and who do not receive MTX.

The subgroup with high T lymphocytes proliferative activity on the third day of culturing included considerably less RA patients receiving MTX than RA patients, who do not receive MTX therapy ($p < 0.025$), but it was significantly higher than in the control group ($p < 0.0374$). In RA patients, who had faster proliferative response of T lymphocytes to B19V antigens and who did not receive MTX, proliferative activity was also higher than in the control group ($p = 0.0001$). The group with high T lymphocytes proliferative activity on the sixth day of culturing included less RA patients receiving MTX than patients without MTX therapy ($p < 0.012$), but it was similar to the number of healthy control individuals. Both on the third and sixth day of culturing, the number of RA patients with high T lymphocytes proliferative activity and without MTX therapy was significantly higher compared to the number of healthy control subjects ($p < 0.0001$).

Patient group, which received sDMARD without MTX as therapy

In the group of RA patients, who used sDMARD without MTX, the incidence of B19V infection activity stage according to *recomWell*, as well as B19V infection according to the *recomLine* test and proliferative response of T lymphocytes to B19V antigens on the third and sixth day of culturing, as well as IgM class antibodies against B19V NS-1 did not statistically differ from the RA patients, who did not use sDMARD without MTX. The incidence of B19V, HHV-6 and HHV-7 specific DNA in DNA samples isolated from blood, cell-free plasma, synovial fluid and tissues did not significantly differ. In the group of users of sDMARD without MTX, IgG class antibodies against B19 NS-1 were found more frequently (50.0%) than in the group of non-users (26.9%, $p = 0.046$).

Patient group, which received bDMARD as therapy

Among users and non-users of bDMARD, the incidence of B19V infection activity stage according to *recomWell*, the duration of B19V infection before the inclusion of the patient in the study according to the *recomLine* test and proliferative response of T lymphocytes to B19V antigens on the third and sixth day of culturing, as well as IgG and IgM class antibodies against B19V NS-1 did not statistically differ. The incidence of B19V genome in the DNA isolated from blood, cell-free plasma, synovial fluid and tissues did not significantly differ.

2.2.3. Finding of markers of a HHV-6 and HHV-7 infections in RA patients

Persistent HHV-6 and/or persistent HHV-7 infection was found in 67/80 (81.3%) RA patients included in the study. Among the RA patients with persistent HHV-6 and/or HHV-7 infection 6/67 (9.0%) had HHV-6 infection only, 42/67 (62.7%) – HHV-7 infection only and 19/67 (28.4%) – HHV-6 and -7 infection at the same time (see Table 2.2).

Table 2.2

Incidence of persistent, active and latent HHV-6 and HHV-7 infection in RA and OA patients, and in healthy individuals

Type of infection / Group	No infection	Persistent viral infection			Active viral infection			With latent infection
		HHV-6	HHV-7	HHV-6 + HHV-7	Only HHV-6	Only HHV-7	HHV-6 + HHV-7	
RA n=80	13/80	6/67	42/67	19/67	8/67	11/67	2/67	46/67
OA n=78	15/78	1/63	41/63	21/63	9/63	7/63	7/63	40/63
Healthy control individuals n=19	4/19	0/15	10/15	5/15	3/15	2/15	1/15	9/15

Reactivation of HHV-6 was found in 10/67 (14.9%) RA patients. Reactivation of HHV-6 infection only was found in 8/25 (32.0%) RA patients with persistent HHV-6 infection. Furthermore, reactivation of HHV-7 was found in 13/67 (19.4%) RA patients. Reactivation of HHV-7 infection was found in 11/61 (18.0%) RA patients with persistent HHV-7 infection. Two of 19 RA patients with persistent infection of both viruses (HHV-6 and HHV-7) demonstrated reactivation of both of these viruses (10.5%) (see Table 2.2).

Among RA patients, persistent HHV-6 monoinfection at latent stage was found in 4/6 (66.7%) patients. Persistent HHV-7 infection at latent stage was found in 31/42 (73.8%) RA patients. Simultaneous persistent HHV-6 and HHV-7 infection at latent stage was found in 11/19 (57.9%) RA patients.

2.2.4. Finding of markers of HHV-6 and HHV-7 infections in connection with RA clinical activity and laboratory indicators

TJC and SJC and strength of pain at VAS, as well as frequency of complications – rheumatoid nodules, specific damage of cervical vertebrae, osteoporosis, Sjogren's syndrome, scleritis and polyneuropathy in RA patients with different stages of activity of persistent HHV-6 and HHV-7 infection or also without an infection were similar. However, morning stiffness was longer in RA patients with latent HHV-6 infection than with active HHV-6 infection ($p = 0.02$). Mean TJC and SJC and the duration of morning stiffness were higher in RA patients than in OA patients without HHV-6 and HHV-7 infection. The myopathic syndrome is less common in RA patients with active HHV-7 infection compared to RA patients, who had latent HHV-6 infection ($p = 0.0117$) and patients without HHV-6 and HHV-7 infection ($p = 0.0158$). Such a serious RA complication as scleritis was found only in patients with latent HHV-7 infection, but specific damage of cervical vertebrae – in RA patients with latent HHV-6 and HHV-7 infection.

Disease activity indicators DAS28 and ESR in RA patients at different stages of activity of persistent HHV-6 and/or HHV-7 infection did not differ. In all groups, where HHV-6 and/or HHV-7 infection was found, RA activity according to the DAS28 indicators was medium high. CRP and ESR indicators in all RA patient groups were also increased.

Mean values of both disease aggressiveness indicators RF and anti-CCP in RA patients were increase, but without significant differences at different stages of activity of persistent HHV-6 and HHV-7 infection.

The breakdown of radiological stages between RA patients with different persistent HHV-6 and/or HHV-7 infection activity stages was similar.

In RA patients without HHV-6 and HHV-7 infection, the mean values of CRP and ESR were higher compered to OA patients without infection ($p < 0.05$).

Mean levels of haemoglobin, platelets and lymphocytes in RA patients with different stages of activity of persistent HHV-6 and HHV-7 infection did not differ.

The mean level of IL-6 was considerably lower in RA patients with active HHV-6 infection than in patients with latent HHV-6 infection ($p = 0.0478$). The mean levels of TNF-α and IL-17 in RA patients with different persistent HHV-6 and HHV-7 infection activity stages were similar. The mean level of IL-10 was considerably lower in RA patients with active HHV-7 infection compared to RA patients with latent HHV-7 infection and RA patients without infection ($p = 0.0488$ and $p < 0.0001$, respectively). The mean level of MMP-9 was considerably lower in RA patients with active HHV-7 infection compared to RA patients with latent HHV-7 infection and patients with active HHV-6 infection ($p = 0.0199$ and $p = 0.006$, respectively).

Significant differences were found in mean levels of IL-6 and anti-CCP between RA and OA patients and healthy individuals without HHV-6 and HHV-7 infection. In RA patients without both viral infections, the mean level of IL-6 ($p = 0.0186$), RF ($p = 0.0007$) and anti-CCP ($p = 0.0346$) was considerably higher than in OA patients without infection. RA patients with active HHV-6 infection showed higher mean TJC ($p = 0.0013$) and SJC ($p = 0.003$) and the duration of morning stiffness ($p = 0.0195$), as well as the mean level of RF ($p = 0.001$), anti-CCP ($p = 0.0063$) and MMP-9 ($p = 0.0252$)

compared to OA patients with active HHV-6 infection. Similarly to RA patients with active HHV-7 infection, TJC ($p = 0.0365$) and SJC ($p = 0.0419$), as well as the mean level of RF ($p < 0.0001$) and IL-10 ($p = 0.0064$) was higher than in OA patients with active HHV-7 infection. In RA patients with latent HHV-6 infection, the mean TJC and SJC and the mean level of RF was considerably higher compared to OA patients with latent HHV-6 infection ($p < 0.05$). Similarly, in the group of RA patients with latent HHV-7 infection the mean TJC ($p < 0.0001$) and SJC ($p = 0.007$) and the mean level of anti-CCP ($p = 0.03$), TNF-α ($p < 0.0001$) and IL-6 ($p < 0.0001$) was higher compared to OA patients with latent HHV-7 infection.

2.2.5. Finding of markers of HHV-6 and HHV-7 infections and other laboratory indicators depending on the therapy used

The incidence of active and latent HHV-6 and/or HHV-7 infection did not differ among groups of RA patients, who received and who did not receive DMARDs. No reactivation of HHV-6 or HHV-7 infection was found in groups of users of MTX monotherapy and bDMARDs. In RA patients with active HHV-6 and/or HHV-7 infection the incidence of complications like myopathic syndrome, rheumatoid nodules, osteoporosis, polyneuropathy and Sjogren's syndrome did not differ.

2.2.6. Finding of markers of B19V, HHV-6 and HHV-7 infections in RA patients synovial fluid and tissues

B19V genomic sequences were found in DNA of synovial fluid of 1/6 (16.7%) RA patients and samples of synovial tissues of 3/7 (42.9%) RA patients.

HHV-6 and/or HHV-7 genomic sequences were found in the DNA of synovial fluid of 5/6 (83.3%) RA patients. Only HHV-6 genomic sequences were found in 2/6 (33.3%) RA patients and in no OA patient ($p = 0.0192$). Furthermore, only HHV-7 genomic sequences were found in DNA samples of synovial tissues of 2/6 (33.3%) RA patients. Genomic sequences of both viruses were found simultaneously in DNA samples of synovial fluid of 1/6 (16.7%) RA patients (see Table 2.3).

HHV-6 and/or HHV-7 genomic sequences were found in DNA samples of synovial tissues of 4/7 (57.1 %) RA patients. HHV-6 genomic sequences were not found in DNA samples of any RA patient, and only HHV-7 genomic sequences were found in the DNA samples of synovial tissues of 1/7 (14.3%) RA patients. Genomic sequences of both viruses (HHV-6 and HHV-7) were found in DNA samples of synovial tissues of RA patients at the same time credibly more frequently, in 3/7 (42.9%), compared to 3/54 (5.6%) of OA patients ($p = 0.0166$). Furthermore, no viral sequences were found in DNA samples of synovial tissues of 3/7 (42.9%) RA patients (see Table 2.3).

Table 2.3

Incidence of B19V, HHV-6 and HHV-7 genomic sequences in DNA samples of synovial fluid and synovial tissues of RA and OA patients

Viral sequence in DNA samples / Patients	Synovial fluid DNA				Synovial tissue DNA			
	B19V	HHV-6	HHV-7	HHV-6 + HHV7	B19V	HHV-6	HHV-7	HHV6 + HHV-7
RA patients	1/6	2/6	2/6	1/6	3/7	0/7	1/7	3/7
OA patients	6/33	0/34	10/34	3/34	3/57	0/54	17/54	3/54

The cells marked with HHV-6 specific antibody, have brown cytoplasmic staining. Expression of HHV-6 antigens was observed in synovial tissues of HHV-6 positive RA and OA patients included in the study, where presence of viral genomic sequences was found. HHV-6 positive cells are often located in the upper layer of the synovial membrane, as well as in the vessel wall of the endothelium (see Fig. 2.2A). In expressions with anti-B19 antibody, expression was detected in the nucleus of the cells. Immunoexpression was demonstrated in synovial cells (see Fig. 2.2B), vascular endotheliocytes and myocytes and red bone marrow cells.

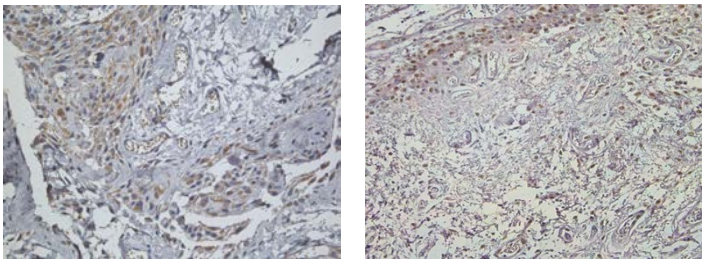

Figure 2.2 A: **Synovial expression of HHV-6.** Original magnification × 250.
B: **Expression of the B19 cell surface core of the synovial membrane.**
Original magnification × 200;
Captured by prof. V. Groma.

HHV-6 antigen was determined as cytoplasmic staining in cells of synovial membrane, cartilage tissues, bone tissues, vessel walls and inflammation infiltrates, but B19V capsid proteins VP-1/VP-2 – as staining of nuclei in synovial cells, endotheliocytes, vascular myocytes and red bone marrow cells.

2.3. Finding of markers of a viral infection in OA patients

2.3.1. Finding of markers of B19V infection in OA patients

The determination of B19V antibodies according to the *recomWell* test and *PCR* was carried out for 78 OA patients. Based on *recomWell* and PCR results, OA patients were broken down into five groups: (1) 44 OA patients with remote B19V infection (presence of B19 IgG class antibodies), (2) 9 OA patients with active persistent B19V infection, (3) 9 OA patients with latent persistent B19V infection, (4) 5 OA patients with acute B19V infection and (5) 11 OA patients without B19V infection.

Antibodies to 4–5 different B19V antigens were found in 59.8% of OA patients. IgG class antibodies against B19 NS-1 were found in 22.1% of OA patients.

No specifically credible differences were found in OA patients in the duration of the disease, clinical parameters of the disease, as well as laboratory indicators depending on the stage of activity of B19V infection, which are defined according to the *recomWell* test and PCR.

Higher MMP-9 level was determined in the group of OA patients without B19V infection compared to the group with active persistent B19V infection ($p = 0.022$). Higher IL-17 level was found in OA patients without B19V infection compared to OA patients with remote B19V infection ($p = 0.034$). Higher TNF-α level was determined in OA patients with active persistent B19V infection compared to OA patients with latent persistent B19V infection ($p = 0.022$), remote B19V infection ($p = 0.007$) and without B19V infection ($p = 0.027$). The level of other determined cytokines in groups of OA patients depending on the stage of activity of B19V infection does not credibly differ.

In groups of OA patients with different stages of activity of B19V infection B19V genome sequences were more commonly found in patients with latent persistent B19V infection ($p = 0.045$).

The determination of B19V antibodies according to *recomLine* test was carried out for 77 OA patients. Based on *recomLine* results, OA patients were broken down into four groups: (1) 19 OA patients with years long B19V infection, (2) 31 OA patient with months to years long B19V infection, (3) 9 OA patients with weeks to months long B19V infection and (4) 11 OA patients without B19V infection. In seven individuals in the OA patient group the stage of B19V infection was unclear, therefore, their data were not used for further processing.

In OA patients with different periods of infection with B19V before the inclusion in the study, which were determined according to the *recomLine* test, no significant differences in clinical parameters of the disease, duration of the disease, TJC, SJC and VAS, were found. Only the duration of morning stiffness was found to be longer in patients with months to years long B19V infection than in patients with weeks to months long B19V infection ($p = 0.037$).

No significant changes were found in OA laboratory parameters, CRP, ESR, RF, anti-CCP, haemoglobin and lymphocyte count, taking into account the duration of the period of infection with B19V before the inclusion in the study. A significantly higher mean level of platelets was found in OA patients without B19V infection, as well as in OA patients with years long B19V infection than in patients with weeks to months long B19V infection ($p = 0.027$ and $p = 0.018$, respectively).

A statistically credibly higher mean level of IL-17 was determined in the group of OA patients without B19V infection compared to OA patients with years long B19V infection ($p = 0.01$) and compared to OA patients with weeks to months long B19V infection ($p = 0.05$) before the inclusion in the study. Furthermore, a considerably lower mean MMP-9 level was found in

OA patients with weeks to months long B19V infection before the inclusion of the patients in the study compared to patients without B19V infection ($p = 0.001$). Levels of other indicators in OA patients with different periods of infection with B19V before the inclusion in the study (determined according to the *recomLine* test) did not significantly differ.

No significant differences in the incidence of IgG class antibodies against B19V NS-1 depending on the period of infection with B19V before the onset of the disease (determined according to the *recomLine* test) were stated.

2.3.2. Finding of markers of HHV-6 and HHV-7 infections in OA patients

Persistent HHV-6 and/or persistent HHV-7 infection was found in 63/78 (80.8%) OA patients. Among the OA patients with persistent HHV-6 and/or -7 infection 1/63 (1.6%) had HHV-6 infection only, 41/63 (65.1%) – HHV-7 infection only, but 21/63 (33.3%) patients – HHV-6 and -7 infection at the same time.

Reactivation of HHV-6 was found in 16/63 (25.4%) OA patients. Reactivation of HHV-6 infection only was found in 9/22 (40.9%) OA patients. Furthermore, reactivation of HHV-7 was found in 14/63 (22.2%) OA patients. Reactivation of HHV-7 infection was found in 7/62 (11.3%) OA patients. Simultaneous reactivation of both viruses was found in 7/21 (33.3%) OA patients with persistent HHV-6 and HHV-7 infection (see Table 2.2).

There were no OA patients with persistent HHV-6 monoinfection at latent stage. Persistent HHV-7 infection at latent stage was found in 34/41 (82.9%) OA patients and simultaneous persistent HHV-6 and HHV-7 infection at latent stage was found in 6/21 (28.6%) OA patients.

2.3.3. Finding of markers of B19V, HHV-6 and HHV-7 infections in synovial fluid and tissues of OA patients

B19V genomic sequences were found in DNA of synovial fluid of 6/33 (18.2%) OA patients and DNA samples of synovial tissues of 3/57 (5.3%) of OA patients (see Table 2.3).

HHV-6 and/or HHV-7 genomic sequences were found in the DNA of synovial fluid of 13/34 (8.8%) OA patients. HHV-6 only genomic sequences were not found in any OA patient. Furthermore, HHV-7 only genomic sequences were found in DNA samples of synovial tissues of 10/34 (29.4%) OA patients. Genomic sequences of both viruses were found simultaneously in DNA samples of synovial fluid of 3/34 (8.8%) OA patients (see Table 2.3).

HHV-6 and/or HHV-7 genomic sequences were found in DNA samples of synovial tissues of 20/54 (37.0%) OA patients. HHV-6 only genomic sequences were not found in DNA samples of synovial tissues of any OA patient, and HHV-7 genomic sequences were found in DNA samples of synovial tissues of 17/54 (31.5%) OA patients. HHV-6 and HHV-7 genomic sequences were found simultaneously in DNA samples of synovial tissues statistically credibly more frequently, in 3/7 (42.9%) RA compared to 3/54 (5.6%) OA patients ($p = 0.0166$). Furthermore, no viral sequences were found in DNA samples of synovial tissues of 34/54 (63.0%) OA patients (see Table 2.3).

HHV-6 immunopositivity was found in individual OA patients. One OA patient had massive HHV-6 expression in large foam cells of vascular walls (see Fig. 2.2 B).

The incidence of B19V DNA sequences in synovial fluid and synovial tissues of OA patients with different periods of infection with B19V before the inclusion in the study, which were determined according to the *recomLine* test, did not significantly differ.

2.3.4. Relationship between markers of a viral infection and OA clinical progress and laboratory indicators

Clinical parameters of OA patients at different stages of activity of persistent HHV-6 and HHV-7 infection did not differ.

The mean level of CRP was credibly higher in OA patients with latent HHV-7 infection than in patients without HHV-6 and HHV-7 infection ($p = 0.0488$), but the mean level of ESR in OA patients with different stages of activity of persistent infections did not statistically differ. Differences between OA and RA patient groups are described in section 2.2.5.

Mean level of all determined cytokines in OA patients at different stages of activity of persistent HHV-6 and HHV-7 infection did not significantly differ.

2.4. Finding of markers of a viral infection in healthy control group individuals

2.4.1. Finding of markers of B19V infection in healthy individuals

The determination of B19V antibodies according to the *recomWell* test and PCR was carried out for 19 control group individuals. Based on *recomWell* and PCR results, healthy individuals were broken down into five groups: (1) 12 control group individuals with remote B19V infection, (2) one with active persistent B19V infection, (3) one with latent persistent B19V infection, (4) one with acute B19V infection and (5) 4 healthy control group individuals without B19V infection.

Antibodies against no more than four different B19V antigens were found in serum of healthy control group individuals. No significant difference in proliferative response of T lymphocytes on the third and sixth day, or

MMP-9 and determined level of cytokines was found among control group individuals with different stages of activity of B19V infection. Also, the frequency of presence of B19V sequences in the DNA isolated from full blood, cell-free plasma and synovial tissues, as well as the incidence of IgG class antibodies against B19V NS-1 did not significantly differ.

If we compare healthy control group individuals with remote B19V infection, and RA patients with remote B19V infection, no significant differences in MMP-9 and levels of checked cytokines were found. The incidence of B19V genomes in synovial tissues and synovial fluid in these groups did not significantly differ.

No significant differences in MMP-9 and cytokine levels, as well as incidence of B19V genome in synovial tissues and synovial fluid were found, when comparing OA patient group with healthy control group individuals with remote B19V infection, in both studied groups.

MMP-9, cytokine levels and the incidence of B19V genome in synovial tissues and synovial fluid in the group of RA patients without B19V infection and in the group of healthy individuals without B19V infection did not differ.

No significant differences in MMP-9 and cytokine levels, and incidence of B19V genomes in synovial tissues and synovial fluid were found, when comparing OA patient group without B19V infection with the group of healthy individuals without B19V infection.

The determination of B19V antibodies according to *recomLine* test was carried out for 37 healthy control group individuals. Based on *recomLine* results, healthy individuals were broken down into four groups: (1) 8 control group individuals with years long B19V infection, (2) 12 healthy individuals with months to years long B19V infection, (3) four control group individuals with weeks to months long B19V infection and (4) 13 healthy individuals without B19V infection.

In the group of healthy individuals with different periods of infection with B19V before the onset of the disease determined according to the *recomLine* test, the proliferative response of T lymphocytes on the third or sixth day, as well as the incidence of IgG class antibodies against B19V NS-1 and the incidence of B19V genome sequences in the DNA isolated from full blood, cell-free plasma did not statistically credibly differ. Levels of MMP-9 and checked cytokine levels in healthy individuals with different periods of infection with B19V before the onset of the disease, which were determined according to the *recomLine* test, did not significantly differ.

2.4.2. Finding of markers of HHV-6 and HHV-7 infections in healthy control group individuals

Persistent HHV-6 and/or persistent HHV-7 infection was found in 15/19 (78.9%) healthy control group individuals. In the group of healthy control individuals, HHV-6 infection only was not found in any of 15 subjects (0%), HHV-7 infection only was found in 10/15 (66.7%) individuals and simultaneous HHV-6 and -7 infection was found in 5/15 (33.3%) subjects (see Table 4.2).

Reactivation of HHV-6 was found in 4/15 (26.7%) healthy subjects. Reactivation of HHV-6 infection only was found in 3/5 (60.0%) healthy control group individuals with simultaneous HHV-6 and HHV-7 infection. Reactivation of HHV-7 was found in 3/15 (20.0%) healthy subjects. Reactivation of HHV-7 infection was found in 2/15 (13.3%) healthy control group individuals with persistent HHV-7 infection. Simultaneous reactivation of both viruses was found in 1/5 (20.0%) healthy individuals with persistent HHV-6 and HHV-7 infection (see Table 2.2).

There were no control group subjects with persistent HHV-6 monoinfection at latent stage. Persistent HHV-7 infection at latent stage was

found in 8/10 (80.0%) healthy control group individuals. Simultaneous persistent HHV-6 and HHV-7 infection at latent stage was found in 1/5 (20.0%) healthy control group subjects.

3. DISCUSSION OF RESULTS

RA is chronic progressing systemic disease, which is characterised mainly by chronic aseptic synovitis and erosive, destructive joint damages. As the disease progresses, internal injuries may develop in part of patients (Gibofsky, 2012). Both progressing course of the disease with marked development of deformations and the development of an extraarticular damage leads to functional failure, loss of work capacity, incapacitation of patients and reduces their survival. RA is the most common inflammatory arthritis and its incidence increases with age, which creates a serious socioeconomic burden. The newly discovered group of drugs – biological disease-modifying anti-rheumatic drugs do not ensure complete recovery from RA. Despite many conducted studies, the precise ethiopathogenesis of RA is still unclear. It is important to continue studies, which might probably discover and re-confirm the factors causing the disease and affecting its progress. Viruses and viral infections are an important risk factor for the development of autoimmune diseases, especially in genetically predisposed persons. Two thirds of RA patients have been associated with HLA-DR * 4, which according to literature indicate a chronic course of disease (Colmegna, Alberts-Grill, 2009). A correlation between the development of several autoimmune diseases, including connective tissue diseases, multiple sclerosis and Hashimoto's thyroiditis and HHV-6 A/B infection was found (Chapenko et al., 2003; Nora-Krukle et al., 2011; Caselli et al., 2012; Hayem, Hayem, 2012; Broccolo et al., 2013). It was determined that several viral infections, including B19V, HHV-6 and HHV-7, may affect the development and progress of arthritis. B19V infection induced arthritis often meets RA criteria (Colmegna, Alberts-Grill, 2009; Kerr, 2000; Khoqueer, 2009) and prolonged B19V viralemia may contribute to the development of pronounced and prolonged arthritis (Ogawa et al., 2008). In

33% of patients with RA and in 45% of axial spondyloarthritis synovial fluid DNA have been found the Varicella zoster genome sequences, but they have not been detected in DNA isolated from synovial fluid in OA patients and mononuclear cells in RA patients. Herpes simplex 1 and 2 genome sequences have also been detected in 33% of RA patients isolated from both blood and synovial fluid DNA (Burgos et al. 2015). It is believed that the factor causing or promoting human B19V as a disease may affect the development of RA, systemic lupus erythematosus, scleroderma, vasculitis and antiphospholipid syndrome. B19V induced arthritis reminds of RA's progression with symmetrical small joint damage (Franssil, Hedman, 2006; Kerr, 2000; Takasawa et al., 2004).

However, several studies deny the correlation between B19V and RA development. This may be related to different progress of RA, as well as the influence of other factors in the development of RA. Data of conducted studies are sometimes difficult to interpret and compare, because it is hard to select RA patients with similar duration, progress of RA and received treatment. Several studies are being conducted to re-confirm the response of cells to B19V antigens and potential expression of B19V genome in the patient’s body (Kozireva et al., 2008; Murai et al., 1999; Stahl et al., 2000; Takahashi et al., 1998). Although the B19V-specific immune response of cells is being studied, there are quite a few studies of the development of response in case of acute and persistent B19V infection. The role of persistent B19V infection in the development of RA is evidenced by high incidence of B19V infection among RA patients, lack of anti-B19 IgM class antibodies in most of RA patients with viremia and presence of anti-B19 IgG class antibodies in these patients, frequent finding of anti-B19 NS-1 IgG class antibodies in RA patients compared to healthy individuals and relatively low viral load in RA patients with viremia (von Poblotzki et al., 1995; Hemauer et al., 2000; Kerr, Cunniffe,

2000; Lee et al., 2011). This study determined the potential correlation between B19V, HHV-6 and HHV-7 infections and ethiopathogenesis of RA and OA.

The thesis summarises the studies conducted in the Linezers Clinic and the Gaiļezers Clinic of the Riga East Clinical University Hospital, the Hospital of Traumatology and Orthopaedics, and RSU A. Kirhenšteins Institute of Microbiology and Virology and RSU Institute of Anatomy and Anthropology from 2008 to 2016. The study includes 103 RA and 78 OA patients, as well as 43 healthy control group individuals. The stage of B19V, HHV-6 and HHV-7 infection was determined in patient groups and healthy individuals and the role of these infections in the development of RA was studied. B19V antibodies were analysed and B19V, HHV-6 and HHV-7 in DNA was determined in the RSU A. Kirhenšteins Institute of Microbiology and Virology. The histological and immunohistochemical analysis of synovial tissues was conducted in the RSU Institute of Anatomy and Anthropology.

Only 20 of 103 patients included in the study did not receive immunosuppressants (GC, DMARD), because the disease has started in these patients only recently and they were included in the study before their treatment started. In the study group of RA patients, most patients received for treatment MTX as monotherapy or in combination with other base drugs (both synthetic and biological). Overall, 80.4% of the RA patients included in the study use different immunosuppressants as monotherapy or in combination for treatment. According to EULAR (European League Against Rheumatism) recommendations, synthetic and biological DMARD are the basis for treatment of RA. In case of active RA, the drug of first choice is MTX as monotherapy or in combination with other base drugs (Smolen et al., 2014).

According to mean disease activity indicators (TJC, SJC, VAS value, duration of morning stiffness, DAS28 index) most of RAs have medium high and high disease activity. In the group of RA patients, remission according to DAS28 was found in 7.1% of patients, low disease activity – in 6.1%, medium

high RA activity – in 49.5% and high disease activity – in 37.4% of RA patients. The study included patients, who were treated in the hospital mainly with exacerbation of the disease, therefore, most RA patients have increased disease activity indicators.

According to radiological examinations, most RA patients correspond to the third radiological stage, which evidence of aggressive progress of the disease with the development of erosions in the study group (Schett, Gravallese, *2012*). In the RA group, different complications were found in 46.9% of patients, which is close to the incidence of complications indicated in reference data, 40% (Cojocaru et al., 2010). RA patient group most commonly had myopathy or muscle atrophy, PNS impairment and osteoporosis. The formation of muscle atrophy is mainly promoted by pain caused by the disease, oedema, stiffness and defamations of joints, which reduces the range of movements. The development of osteoporosis is promoted by osteoclast dysfunction, systemic activity of cytokines, patient's age, use of GCs, ankylosis caused by disease activity and progression, inadequate intake of calcium and vitamin D and positive RF (Mobini et al., 2012).

In order to avoid B19V differences in circulation caused by seasons and age, blood samples were taken from RA and OA patients, and from healthy individuals simultaneously. The total RA aggressiveness by RF and by anti-CCP is evaluated as high. When evaluating separately by RF and anti-CCP level, their increase is more frequently minor (50.3% and 53.8%, respectively) or marked (34.4% and 35.6%, respectively). Higher aggressiveness of the disease provides for earlier erosive changes, faster progress of the disease with the development of deformations and extraarticular damages (Heidari et al., 2009), the need to use DMARD combinations in treatment and longer treatment time to achieve a remission.

In the group of RA patients, the mean platelet count and haemoglobin corresponds to the reference range, but the mean lymphocyte count corresponds

to lymphopenia. The development of lymphopenia is supposedly related to the use of DMARD in RA treatment (Crowson et al., 2012).

The clinical characteristics of the OA group correspond to medium active disease. As in a degenerative arthritis, lower mean TJC and the mean SJC is determined than in the RA patient group. The mean VAS indicator and the mean duration of morning stiffness also correspond to the progress of degenerative arthritis.

Similarly to the RA patient group, in the OA patients the levels of platelets and haemoglobin corresponds to the reference range, but the count of lymphocytes corresponds to lymphopenia. Lymphopenia may be caused not only by DMARD, but also by NSAID (Mikaeloff et al., 2008). The mean laboratory inflammation indicators CRP and ESR in the OA group are comparatively low. In OA patients, the mean level of RF and anti-CCP corresponds to the reference interval, although 1% of healthy control group individuals (Nam et al., 2016; Zendman et al., 2006) and up to 8% of OA patients may have positive anti-CCP antibodies (Sauerland et al., 2005).

Based on *recomWell* and PCR results, RA patients are broken down into five groups: (1) 37 RA patients with remote B19V infection (presence of B19 IgG class antibodies only), (2) 15 RA patients with acute B19V infection (presence of B19 IgM class antibodies and/or viremia), (3) 14 RA patients with latent persistent B19V infection (presence of B19 IgG class antibodies and B19V DNA in blood), (4) 19 RA patients with active persistent B19V infection (presence of B19 IgG class antibodies and viremia) and (5) 11 RA without B19V infection.

Naciute and colleagues have determined that the B19V genome sequences are more likely to be detected in RA patients serum DNA compared to healthy subjects (Naciute et al., 2016), while Akt and colleagues found anti-B19 IgG antibodies in patients with various diseases, however, the most commonly in the RA group was 72,2% (Aktas et al., 2016). It has also been

shown that the sequences of B19V genome isolated from plasma and/or PBL and synovial fluid DNA in RA patient's samples are more frequent (Kozireva et al., 2008). Only the presence of anti-B19 IgG antibodies may also indicate pre-infection with B19V, which is consistent with published epidemiological data (Chen et al., 2006; Jorgensen et al., 2008; Kerr, 2000; Tzang et al. 2009).

The acute B19V infection group includes 46.7% of RA patients with early RA – with duration of the diseased up to one year. It has also been previously established that RA patients are more likely to find B19V markers than healthy controls (Chen et al., 2006) and acute arthritis patients with positive B19V markers are more likely to develop advanced arthritis (Oğuz et al., 2002). This suggests that patients with early RA have a high incidence of B19V infection. Acute B19V infection was found in 15.3% of RA patients compared to 5.3% control group individuals, what suggests that B19V is meaningful in the development of RA. Anti-B19 IgG class antibodies were determined in all RA patients with viremia, which, in turn, evidences that B19V is meaningful in the development of RA. Prolonged B19V viremia can also contribute to the development of pronounced and prolonged arthritis (Ogawa et al., 2008).

Only SJC out of all clinical parameters was credibly higher in the group of RA patients with active persistent B19V infection than in patients with remote B19V infection, which evidences that B19V infection may affect activity of the disease. Other clinical parameters and duration of the disease do not significantly differ. References contain different data about the correlation between B19V infection and TJC and SJC (Oiwa et al., 2011). The total number of complications and the incidence of each complication separately in studied RA patient groups does not significantly differ.

The obtained data shows that B19V infection activity stage in patients with different RA treatment strategies does not differ significantly.

B19V infection can lead to erosive arthritis (Kerr, 2000; Mayer, 2003; Lungquist, 2005), although we did not find a significant difference in radiological stages in patients with different stages of B19V infection activity.

Our previous study showed that higher RA activity was detected in patients with established B19V markers (Kakurina et al., 2015). In this study he mean level of laboratory indicators CRP, ESR and anti-CCP, as well as combined diseases activity indicator DAS28 in RA patient groups with different stages of B19V activity does not significantly differ. At the same time, the mean RF level is higher in the group of RA patients with acute B19V infection compared to patients without B19V infection, as well as in RA patients with remote B19V infection, and latent persistent B19V infection. When evaluating the total aggressiveness of the disease by the degree of increase in RF and anti-CCP summarily, it is found to be higher in RA patients with acute B19V infection than in patients without B19V infection and in patients with remote B19V infection. Also, several authors have previously found that B19V promotes the production of various antibodies, including RF production (Kerr, 2000; Kerr, 2016; Meyer, 2003; Page et al. 2015) and that after acute B19V infection an increase in RF levels (Kerr, 2016; Murai et al., 1999) and erosive arthritis develops three years later (Murai et al., 1999). Naciute and colleagues have identified higher serum sickness and aggressiveness and lower hemoglobin levels in RA patients with B19V genomic sequences. DNA-derived from cell-free serum. Increased association of IL-6 with higher disease activity after DAS28 and CRO (Naciute et al., 2016) was also found. Our data also suggest that B19V can impact the progression of RA and promote its progression.

The mean level of haemoglobin, lymphocytes and platelets in groups of RA patients with different stages of activity of B19V infection does not significantly differ.

Inflammatory cytokines, especially TNF-α, play an important role in the development and progress of RA. B19V promotes the production of various hemoglobin and cytokines (Kerr et al., 2004). In patients with acute B19V infection and with active persistent B19V infection, the level of TNF-α is determined as credibly higher than in patients with remote B19V infection. In RA patients with acute B19V infection, the TNF-α level is also significantly higher than in patients without B19V infection. Both Kerr and Barash with colleagues found that a B19V infection at its acute stage and thereafter may increase the level of TNF-α (Barash et al., 2003; Kerr et al., 2001), thus promoting the development of RA after reactivation of acute B19V infection or persistent B19V infection. The highest IL-17 level is found in the group of RA patients without B19V infection. However, this is contrary to the recently published data that B19V-specific CD4+ T cells secrete IL-17 (Kumar et al., 2015). It is possible that the IL-17 level in this study was affected by the received RA treatment in respective groups. The detected MMP-9 level is credibly lower in RA patients with active persistent B19V infection than in patients with remote B19V infection, and in patients without B19V infection, although Tzang et al, revealed an increase un MMP-9 activity under the influence of B19-VP1u (Tzang et al., 2009). References contain data that a B19V infection also increases the level of IL-6 (Kerr, 2016; Naciute et al., 2016; Tzang et al., 2009). Isa et al. determined that early after acute B19V infection IL-12 increases, then IL-2, while IL-10 is low (Isa et al., 2007). In this study, the mean level of IL-2, IL-6, IL-10 and IL-12 in patients with different stages of activity of B19V infection does not significantly differ. This may be related to different times of blood sampling after acute B19V infection.

The expression of the B19V genome sequences was found in both synovial tissue and synovial fluid samples in RA patients (Takahashi et al. 1998; Kerr 2000; Mehraein et al. 2003; Sasaki 2007; Colmegna; Alberts-Grill 2009; Khoqueer 2009). However, it is more commonly found in early RA

sinovial tissues than in synovial fluid (Stahl et al., 2000). In our study, the incidence of B19V genome sequences in synovial tissue and synovial fluid samples of RA does not differ. This is most probably related to the small number of samples in study groups.

Based on *recomLine* results, RA patients are broken down into four groups: (1) 29 RA patients with years long B19V infection, (2) 34 RA patients with months to years long B19V infection, (3) 14 RA patients with weeks to months long B19V infection and (4) 19 RA patients without B19V infection. In seven RA patients the period, when they were infected with B19V, is unclear, therefore, their data were not used for further processing. The groups characterise the time period from the infection with B19V until the patient was included in the study.

Antibodies against at least 5–6 different B19V antigens are found in serum of 34.0% of RA patients and only in 21.0% of healthy control individuals in the group. Anti-B19 NS-1 IgG class antibodies, which are one of indicators of persistent B19V infection (Poblotzki et al., 1995; Kerr et al., 2000; Hemauer et al., 2000), are found in 32.0% of RA patients and only in 10.5% of healthy control individuals. Furthermore, antibodies to 4–5 different B19V antigens are found in 59.8% of OA patients, IgG class antibodies against B19 NS-1 are found in 22.1% of OA patients. The data evidence that antibodies against different B19V antigens may develop in RA patients more frequently and in larger amounts. The presence of anti-B19-NS-1 IgM class antibodies has been reported to indicate RA diagnosis (Tzang et al., 2009), and antibodies against the B19V NS-1 protein are associated with chronic but not acute arthritis (Kerr, Cunniffe, 2000).

No significant differences were found in RA patients in clinical parameters of the disease and radiological stage, laboratory – disease activity and aggressiveness and other clinical indicators, as well as MMP-9 and in cytokine levels determined during the study, taking into account the different

period of infection with B19V before the inclusion in the study, which was determined according to the *recomLine* test results. Furthermore, if aggressiveness of the disease is evaluated separately based on the RF value, then RA patients with months to years long B19V infection have higher RF level than RA patients with weeks to months long B19V infection, but the medium high RF level was more commonly found in RA patients with weeks to months long B19V infection compared to months to years long B19V infection. This is an indication of the impact of long B19V infection on promotion of aggressiveness and progression of the disease. The incidence of complications in groups of RA patients with a different period of B19V infection before the inclusion in the study does not significantly differ.

From determined cytokines, only the mean level of IL-17 cytokine in RA patients with months to years long B19V infection is determined as lower than in patients without B19V infection, but the mean level of IL-17 cytokine with weeks to months long B19V infection was determined as higher than in RA patients with years long B19V infection and in patients with months to years long infection. Different data prevent from making conclusions about the impact of the duration of B19V infection on the IL-17 level. Kumar et al. earlier showed that B19V CD4+ T cells promote production of IL-17 (*Kumar et al., 2015*).

Earlier, it was demonstrated that T lymphocytes of patients with remote B19V infection, proliferate under the influence of B19V recombinant VP-1/VP-2 protein (*von Poblotzki et al., 1996; Corcoran et al., 2000*). Von Poblotzki found out that these lymphocytes are CD4+ T cells. Further studies have confirmed that the immune response is turned against the non-structural NS-1 protein of the virus and against VP1/VP2 B19V proteins (*Corcoran et al., 2000; Mitchell, 2001; Tolfvenstam et al., 2001*). The response of most CD4+ T cells in turned against structural proteins (*Isa et al., 2006*), but the response of CD8+ cells is mainly turned against NS-1 protein. The response of CD8+

T cells develops during an acute infection and preserved for months or even intensifies after liquidation of the acute infection. This suggests that CD8+ T cells may play a significant role in the control of B19V infection (*Norbeck et al., 2005*). All the individuals with acute, recent or persistent B19V infection, but not patients with remote infection, respond to the unique region of the VP-1 protein (*Franssila et al., 2005; Lindner et al., 2005*). Individuals with persistent B19V infection showed larger number of responses to the structural protein compared to anti-B19 seropositive healthy individuals (*Isa et al., 2006*). Kasprowicz et al have found an immunodominant peptide (LASEESAFYLEHSSFQLLG) in B19V capsid proteins, which in the target of subpopulations of lymphocytes in recently and remotely infected individuals (*Kasprowicz et al., 2006*). The response of lymphocytes in acutely infected individuals may be determined *ex vivo* and in remotely infected individual – only via culturing of lymphocytes. It is sought that in case of persistent B19V infection, virus-specific antigens circulate in the human body, which is ensured by prolonged periodic expression of B19V proteins. In such cases, the proliferative response of T lymphocytes may be found much earlier than in remotely infected individuals. In this study, VP-1/VP-2 peptide is used to determine the frequency and quickness of proliferative response of T lymphocytes in RA patients and healthy control group individuals with acute and remote B19V infection in both groups. The impact of therapy on proliferative response of T lymphocytes against B19V antigens was also evaluated, which was not studied before.

Markers of B19V infection and proliferative response of T lymphocytes against B19V antigens is more frequently found in early RA patients. This suggests that the frequency of B19V infection is not related to the duration of the disease or prolonged immunosuppressive therapy.

Most RA patients (64.2%) and all healthy individuals with acute B19V infection, as well as 41.4% of RA patients and 35.2% of control persons with

longer B19V infection respond to the B19V peptide. These data are consistent with the results found by of Kasrowicz et al. (*Kasprowicz et al., 2006*). However, the quickness of T lymphocytes proliferative response between remotely infected RA patients and control group individuals differs. Part of remotely infected RA patients respond to antigens on the third day, while none of healthy control persons with remote B19V infection respond to B19V infection on the third day. The results of the study show that T lymphocytes of recently infected RA patients respond to VP-1/VP-2 faster than T lymphocytes of remotely infected control group individuals. The proliferative response of T lymphocytes evidence of their high sensitivity to viral antigens and potential involvement of persistent B19V infection in the development of RA. Having analysed the data of proliferative response of T lymphocytes in the RA patient group, significant differences were found depending on the use of MTX. Strong proliferative response of T lymphocytes against B19V antigens on the third and sixth day, was found more frequently in RA patients not receiving MTX therapy compared to patients, who used MTX as monotherapy or in combination with other drugs. It is possible that MTX delays the proliferative response of T lymphocytes of RA patients against B19V antigens. At the same time, the number of RA patients receiving MTX with a strong T lymphocytes proliferative response on the third day is markedly bigger than the number of control group persons, but on the sixth day this difference disappears. This prevents from drawing final conclusions about the inhibiting effect of MTX therapy on the response of B19V specific cells. This is the first study, where the frequency and quickness of proliferative response of T lymphocytes to B19V antigens, including VP-1/VP-2 peptide, is determined in RA patients with acute and remote B19V infection and the impact of RA therapy on it is evaluated.

The impact of RA therapy on B19V markers and laboratory tests is evaluated. If we compare RA patients, who have received NSAID only, with RA patients, who have received any of immunosuppressive drugs (sDMARD,

bDMARD, GC), no significant differences are found by the B19V infection activity stage (according to the *recomWell* test) or by the period of infection with B19V before the inclusion in the study (according to the *recomLine* test). The proliferative response of T lymphocytes on the third day tends to be lower, on the sixth day it is credibly lower in the group of users rather than non-users of DMARD. This evidences that both GC and DMARD delays the proliferative response of B19V-specific T lymphocytes. The incidence of B19V NS-1 specific IgM and B19V IgG antibodies does not differ among groups of users and non-users of immunosuppressants. In users of immunosuppressants, the incidence of B19V DNA sequences in DNA samples isolated from cell-free plasma, is lower than among non-users. The level of cytokines and MMP-9 does not differ among users and non-users of immunosuppressants.

In RA patients, who received sDMARD as therapy, IgG class antibodies against B19V NS-1 are found more frequently compared to those, who did not received this therapy. This may be related to the longer duration of the disease in the group of sDMARD users. The incidence of B19V DNA sequences in blood, plasma, synovial fluid or synovial tissues does not differ in RA patients, who use and do not use sDMARD. No statistically credible differences by any parameters are found in the RA patients, who use only one sDMARD. Furthermore, if an RA patient uses two or more sDMARD, the proliferative response of T lymphocytes to B19V antigens on the third and sixth day are credibly lower than in the group on non-users of sDMARD. The incidence of markers of B19V infection according to the *recomWell* and according to the *recomLine* test, and the incidence of B19V DNA sequences in blood, plasma, synovial tissues and synovial fluid in the group of RA patients, who use or do not use at least two sDMARD, does not differ. This allows to conclude that when combined sDMARD therapy is used, the proliferative response of T lymphocytes against B19V antigens reduced, but it does not significantly affect other B19V markers.

No difference is found in the incidence of different stages of B19V infection according to *recomWell*, different period of infection with B19V infection before the inclusion in the study according to the *recomLine*, when comparing groups of users and non-users of GC. The use of GC does not significantly affect the proliferative response of T lymphocytes to B19V antigens on the third or sixth day, the incidence of IgG and IgM class antibodies against B19V NS-1. B19V DNA sequences in blood are found less frequently in GC users, while the incidence of B19V DNA in plasma, synovial fluid and tissues does not statistically differ. Overall, GCs do not significantly affect B19V markers.

The frequency of incidence of different stages of B19V infection determined according to the *recomWell* test, different periods of infection with B19V before the inclusion of the patient in the study (according to the *recomLine* test) does not significantly differ among MTX users and non-users. The incidence of IgG and IgM class antibodies against B19V NS-1, the incidence of B19V genome sequences in the DNA isolated from blood, cell-free plasma, synovial fluid and tissues does not statistically credibly differ among users and non-users of MTX. In both treatment groups (with and without MTX) the proliferative response on the sixth day is considerably higher than on the third day. In RA patients, who have not received MTX therapy, the detected mean proliferative activity of cells is considerably higher both on the third and sixth day compared to the mean proliferative activity of cells in control group individuals on the third and sixth day. No significant differences in proliferative activity are found between RA patients with and without MTX therapy, as well as between RA patients with MTX therapy and healthy individuals. Data of the study show that the biggest number of RA patients with high proliferative response of T lymphocytes is in the group of patients, who do not receive treatment with MTX.

In the group of RA patients, who use sDMARD without MTX, as well as in the group of RA patients, who use bDMARD, the incidence of B19V infection activity stage according to *recomWell*, as well as B19V infection period according to the *recomLine* test and proliferative response of T lymphocytes to B19V antigens on the third and sixth day of culturing, as well as IgM and IgG class antibodies against B19V NS-1 does not statistically differ from the RA patients, who do not use sDMARD without MTX. The incidence of and B19V specific DNA in DNA samples isolated from blood, cell-free plasma, synovial fluid and tissues does not significantly differ. Data evidence that markers of B19V infection are most seriously affected by MTX rather than other sDMARD.

Based on *recomWell* and PCR results, OA patients are broken down into five groups: (1) 44 OA patients with remote B19V infection, (2) 9 OA patients with active persistent B19V infection, (3) 9 OA patients with latent persistent B19V infection, (4) 5 OA patients with acute B19V infection and (5) 11 OA patients without B19V infection.

No specifically credible differences are found in OA patients in the duration of the disease, clinical parameters of the disease, as well as laboratory indicators depending on the stage of activity of B19V infection, which are defined according to the *recomWell* test. This suggests that the activity of B19V infection does not significantly affect progress of OA. The only changes found in determined cytokines are in mean levels of MMP-9, IL-17 and TNF-α. To be noted, a considerably higher mean level of MMP-9 and IL-17 is found in the groups of OA patients without B19V infection. Only higher TNF-α level was determined in OA patients with active persistent B19V infection compared to OA patients with latent persistent B19V infection, remote B19V infection and without B19V infection. The level of other cytokines in groups of OA patients depending on the stage of activity of B19V infection does not credibly differ. In groups of OA patients with different stages of activity of B19V infection, the

frequency of incidence of B19V genome sequences in synovial tissues differs. They are more frequently found in patients with latent persistent B19V infection. Data suggest that different stages of activity of B19V infection do not significantly affect progress of OA.

Based on *recomLine* results, OA patients are broken down into four groups: (1) 19 OA patients with years long B19V infection, (2) 31 OA patient with months to years long B19V infection, (3) 9 OA patients with weeks to months long B19V infection and (4) 3 healthy individuals without B19V infection. In seven individuals in the OA patient group the stage of B19V infection was unclear, therefore, their data were not used for further processing.

In OA patients with different periods of infection with B19V before the inclusion in the study, which were determined according to the *recomLine* test, no significant differences in most clinical parameters and laboratory of the disease are found. Out of clinical parameters, only the duration of morning stiffness is credibly longer in patients with months to years long B19V infection than in patients with weeks to months long B19V infection. Out of laboratory parameters, only the mean level of platelets is significantly higher in OA patients without B19V infection, as well as in OA patients with years long B19V infection than in patients with weeks to months long B19V infection. This evidence of a comparatively small impact of B19V infection on the clinical and laboratory progress of OA.

A statistically credibly higher mean level of IL-17 is determined in the group of OA patients without B19V infection, compared to OA patients with years long B19V infection and compared to OA patients with weeks to months long B19V infection before the inclusion in the study. Levels of determined cytokines in OA patients with different periods of infection with B19V before the inclusion in the study (determined according to the *recomLine* test) do not significantly differ. Furthermore, a considerably lower mean MMP-9 level is found in OA patients with weeks to months long B19V infection compared to

patients without B19V infection. Data of the study suggest that the duration of B19V infection does not significantly affect production of cytokines in OA patients. Aslan et al. has determined a higher IL-6 level in OA patients, but its increase depends on the degree of OA damage in the joint (*Aslan et al., 2008*). We would probably get different data, if we have broken OA patients down into groups depending on the degree of damage in the affected joint.

Based on *recomWell* and PCR results, healthy individuals are broken down into five groups: (1) 12 control group individuals with remote B19V infection, (2) one healthy individual with active persistent B19V infection, (3) one control group individual with latent persistent B19V infection, (4) one control group individual with acute B19V infection and (5) 4 healthy individuals without B19V infection. Acute and active persistent B19V infection is found less frequently in healthy individuals than in RA and OA patients.

Antibodies against no more than four different B19V antigens are found in serum of healthy control group individuals. No significant difference in proliferative response of T lymphocytes on the third and sixth day, or MMP-9 and cytokine levels is found among healthy control group individuals with different stages of B19V infection determined according to the *recomWell* test. Also, the frequency of presence of B19V sequences in the DNA isolated from full blood, cell-free plasma and synovial tissues, as well as the incidence of IgG class antibodies against B19V NS-1 does not significantly differ.

B19V may alter plasma cytokine levels in RA patients and healthy subjects (Naciute et al., 2016; Naciute et al., 2017). In our study comparing healthy control group individuals with remote B19V infection, and RA patients with remote B19V infection, no significant differences in MMP-9, IL-2, IL-6, IL-12, IL-17 and TNF-α levels were found. Furthermore, higher IL-10 level was determined in the group of RA patients with remote B19V infection than in the group of healthy individuals with remote B19V infection. This evidence that other factors rather than B19V infection affect only the IL-10 level. The

incidence of B19V genomes in synovial tissues and synovial fluid in these groups did not significantly differ.

No significant differences in MMP-9 and cytokine levels, as well as incidence of B19V genome in synovial tissues and synovial fluid were found, when comparing OA patient group with healthy control group individuals with remote B19V infection, in both studied groups.

In RA patients, the presence of HHV-6 genome and increased viral loads can be statistically significantly higher than healthy controls (Alvarez-Lafuente et al., 2005). Broccoli with colleagues also found that HHV-6 genomic sequences from cell-free serum (viremic) and anti-HHV-6 IgG antibodies were significantly more common in connective tissue diseases, including RA, compared to healthy control subjects (Broccolo et al. 2013). We have not found significant differences in reactivation of persistent HHV-6 and HHV-7 infection, as well as reactivation of HHV-6 and HHV-7 in groups of RA and OA patients, and healthy individuals. In the chronic inflammation process, lymphocytes containing HHV-6 and HHV-7 genome sequences are found in the joint, which evidence of their potential role in the development of the disease. It is still unclear, why so much HHV-7 is found in synovial tissues of OA patients. Despite similar incidence of persistent HHV-6 and HHV-7 infection in RA and OA patient groups, HHV-6 in DNA is found in synovial fluid of RA patients with direct PCR significantly more frequently than in the OA patient group. No HHV-6 monoinfection is found in synovial tissues of RA and OA patients, as well as the incidence of HHV-7 in DNA in synovial tissues of both patient groups is similar. Furthermore, the frequency of simultaneous incidence of HHV-6 and HHV-7 infection in synovial tissues of RA patients is significantly higher than in tissues of OA patients. Reactivity of HHV-6 in all PCR positive RA cases was confirmed by immunochemistry, but it does not exclude simultaneous presence of HHV-7 infection and potential determination of viral antigens. The determination of HHV-6 and/or HHV-7 in DNA in the

joint cavity may evidence that replication of the viruses may be related to primary RA immune disorders or with immune depression caused by immunosuppressive drugs.

Different effect of HHV-6 and/or HHV-7 on clinical parameters of RA is observed. TJC and SJC, as well as VAS in RA patients with different stages of activity HHV-6 and/or HHV-7 infection do not significantly differ. The duration of morning stiffness is credibly shorter in patients with active HHV-6 infection than with latent HHV-6 infection. Such an observation is explained by the strategy of treatment being used – six RA patients with active HHV-6 infection received strong immunosuppressing glucocorticoid with or without sDMARD and only two patients of this group received non-specific therapy with NSAID. No significant differences in clinical parameters of the disease are found in groups of OA patients at different stages of activity of HHV-6 and HHV-7 infection.

In previous studies, it was found that treatment with immunosuppressive drugs may cause reactivation of a chronic viral infection (Vassilopoulos, Calabrese, 2007; Nard et al., 2015). Komar and colleagues found that the HHV-6 viral genome was not more commonly detected in healthy subjects in patients with juvenile rheumatoid arthritis, Crohn's disease and ulcerative colitis who are taking abatacept in the treatment of biological medicinal products (Comar et al., 2013). The impact of immunosuppressive therapy on the incidence of HHV-6 and HHV-7 infection was not found in this study. At the same time, reactivation of the infection was identified neither in the MTX nor in the biological drug monotherapy groups. These drugs are strong immunosuppressants and they probably inhibit reactivation of HHV-6 and HHV-7 infection. Some studies have identified high incidence of HHV-6 in DNA in RA patients compared to healthy control group persons (Alvarez-Lafuente et al., 2009) and transmission or reactivation of the Epstein-Barr virus in case of OA (Rollin et al., 2009). Furthermore, Davis et al. have no found a

significant correlation between presence of specific antibodies of cytomegalovirus and Epstein-Barr virus and the duration, activity, aggressiveness and treatment of RA (Davis et al., 2012). Other authors believe that the viral infection plays a secondary role – it develops in presence of circulating ACPA and promotes the transition of the pre-inflammatory phase of RA into chronic synovitis (van de Sande et al., 2011).

In the study, we have not stated a significant correlation between HHV-6 and HHV-7 infection and the development of RA complications. Cervical spine damage and scleritis are observed only in the group of RA patients with latent HHV-6 and/or HHV-7 infection. One of the late RA complications, myopathic syndrome, is stated significantly more frequently in RA patients with latent HHV-7 infection than with active HHV-7 infection. This suggests that not only active, but also latent infection may contribute to progression of the disease. The frequency of complications is higher in the group of RA patients with active HHV-6 monoinfection.

Several studies have determined that anti-CMV (cytomegalovirus) positive RA patients develop more marked destructive changes in joints (Davis et al., 2012; Pierer et al., 2012). Furthermore, in this paper were have stated a more severe roentgenological stage in RA patients with simultaneously active HHV-6 and HHV-7 infection.

The mean level of CPR, inflammation marker, is significantly higher in RA patients with latent HHV-7 infection than in patients with active HHV-7 infection. Almost all RA patients with active HHV-7 have received therapy with glucocorticoids and/or sDMARD, which may considerably reduce activity of the disease. Also Broccolo et al. have not found any correlation between reactivation of HHV-6 infection and RA activity (Broccolo et al., 2013).

The mean level of RA aggressiveness indicators, RF and anti-CCP, does not significantly differ in groups of RA patients with different markers of HHV-6 and/or HHV-7 infection.

In the OA patient group, the mean level of CPR, disease activity indicator, is found to be significantly higher in patients with latent HHV-7 infection than in patients without HHV-6 and HHV-7 infection. At the same time, the mean ESR level in groups of OA patients with different HHV-6 and HHV-7 activity stages does not significantly differ, which may suggest that HHV-6 and HHV-7 infections have low impact on the OA activity.

Despite the fact that no significant differences were found in the incidence of persistence HHV-6 only and HHV-7 only and simultaneous HHV-6 and HHV-7 infection and reactivation between groups of RA and OA patients and control group persons, it is determined that both active and latent HHV-6 and/or HHV-7 infection increases RA activity and progression based on several clinical and laboratory parameters. This may suggest that HHV-6 and/or HHV-7 infection influences the activity and aggressiveness of the disease, but reactivation of these viruses may develop, if induced by immunosuppressive therapy. 80.9% of patients with reactivation of HHV-6 and/or HHV-7 receive marked immunosuppressants separately or in different combinations. At the same time, Cavada and his colleagues determined that MTX and tofacitinib therapy did not affect HHV-6 viral load in juvenile idiopathic arthritis patients (*Kawada et al., 2012*). High incidence of RA complications in patients with active HHV-6 only infection, as well as more severe radiological stage of RS in patients with simultaneous active HHV-6 and HHV-7 infection may suggest impact on the severity of RA progress. The impact of HHV-6 and HHV-7 on the disease activity and aggressiveness is more marked in the group of RA patients than in the group of OA patients.

4. CONCLUSIONS

1. The presence of B19V, HHV-6 and -7 infection markers in both RA and OA patients and healthy individuals is found, but their frequency and stage of activity are different:
    a. Acute and active persistent B19V infections are more common in RA patients compared to other study groups. In addition, 46.7% of RA patients with acute B19V infection are at an early stage of the disease, which indicates the potential role of the virus in RA development. Anti-B19 NS-1 IgG antibodies, one of the B19V transient infection rates, are also more commonly detected in RA patients than in other study groups.
    b. Only in the RA patients synovial fluid DNA samples were found only genotypes of HHV-6, but compared with OA patients, the sequences of HHV-6 and -7 genomic sequences were more likely to be detected in synovial tissue DNA samples of RA patients, indicating a significant increase in HHV-6 and - 7 infection involvement in RA development.
2. Stages of infection with B19V, HHV-6 and -7 were not significantly different in RA patients with different RAs. It should be noted that no reactivation of HHV-6 or HHV-7 infection was detected in MTX monotherapy and bSMARMs in user groups, but the stages of B19V infectious activity did not differ.
3. In RA patients, the T lymphocyte proliferative response to B19V antigens is more frequent and faster compared to healthy subjects, suggesting persistent B19V infection in RA patients. Treatment with MTX suppresses the T lymphocyte proliferative response to B19V antigens.
4. RA patients with active B19V infection have the highest levels of TNF-α that may contribute to the development of RA following acute B19V

infection or after persistent B19V infection reactivation. In the case of OA, TNF-α levels are higher in patients with active persistent B19V infection, suggesting that B19V reactivation also affects OA. The levels of inflammatory cytokines for healthy individuals in groups with different stages of B19V, HHV-6 and -7 infection are similar. The duration of B19V infection prior to the individ being included in the study does not affect the cytokine production changes in any of the study groups.

5. When evaluating clinical parameters, tender joint count is a larger in RA patients group with active persistent B19V infection, suggesting that the B19V infection affects the disease activity, and B19V infection does not significantly affect the frequency of RA complications. In RA patients with latent persistent HHV-6 infection, there is longer morning stiffness duration, but in the group with latent persistent HHV-6 and HHV-7 infection there are more serious complications. When evaluating laboratory parameters RA patients with B19V viraemia are more likely to have high levels of RF as well as overall aggressiveness of the disease as measured by simultaneous rate of RF and anti-CCP elevation, suggesting a role for B19V in the progression of RA. Laboratory activity and aggressiveness of RA disease are not significantly affected by persistent HHV-6 and -7 infection. B19V, HHV-6 and -7 infection do not affect the radiological stage of the RA and the course of OA.
6. Infections of B19V, HHV-6 and -7 affect the development and progression of RA - primarily an acute and active persistent B19V infection and a latent/ persistent HHV-6 and -7 infection, thus no zero hypothesis is confirmed.

5. PRACTICAL RECOMMENDATIONS

Based on the data obtained during the study, recommendations for clinical practice are:

1. If the presentation of arthritis develops in a patient with a viral infection, virologic testing should be conducted to re-confirm the agent, because B19V, HHV-6 and HHV-7 may stay in latent form, then reactivate and contribute to the development of RA.
2. Arthritis promoted by viruses may also be early RA.
3. If acute and/or active persistent B19V infection develops in an RA patient, the drug of choice is MTX. Combined therapy can be used, when needed, but one of DMARD should be MTX.
4. When progress of RA aggravates, it may be necessary to determine markers of the viral infection to start proper arthritis treatment in a timely manner.

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