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The Significance of Sociodemographic, Clinical, Radiological and Laboratory Findings in the Outcomes of Early Undifferentiated Arthritis

SUMMARY OF DOCTORAL DISSERTATION

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To my Family

ABBREVIATIONS

ACR – American College of Rheumatology
ANA - anti-nuclear antibodies
Anti-CCP - anti-cyclic citrullinated peptides
bDMARDs – biologic disease modifying drugs
BMI – body mass index
CASP-1 - caspase-1
CASPAR - ClASsification criteria for Psoriatic Arthritis
CHORI - Children‘s Hospital Oakland Research Institute
CI – confidential interval
CN – control group
CRP – C- reactive protein
DAMPs - damage-associated molecular patterns
DAS 28 – disease activity score 28 calculated based on assessment of 28 tender and swollen joints
DMARDs - disease modifying antirheumatic drugs
ESR – erythrocyte sedimentation rate
EULAR - European League Against Rheumatism
HAQ – health assessment questionnaire
HLA B27 – human leucocyte antigen B 27
IgG4 RD – immunoglobulin G4 related disease
IL – interleukin
MAF – minor allele frequency
MMP – matrix metalloproteinase
NLRP - Nucleotide-binding and oligomerization domain NOD-like receptor
NSAID - non-steroidal anti-inflammatory drugs
OA – osteoarthritis
OR – odds ratio
PAMPs - pathogen-associated molecular patterns
PD - Power Doppler
PsA – psoriatic arthritis
RA – rheumatoid arthritis

RF – rheumatoid factor
SF – synovial fibroblasts
SJC – swollen joint count
Sjogren's syndrome – SjS
SLE – systemic lupus erythematosus
SLICC'12 - 2012 Systemic Lupus International Collaborating Clinics criteria
SNP – single nucleotide polymorphism
SpA – spondyloarthropathy
TJC – tender joint count
TLR – Toll-like receptor
TNF – α – tumor necrosis factor α
UA – early undifferentiated inflammatory arthritis
US – ultrasound
VAS – visual analogue scale
VDR – vitamin D receptor
VEGF - vascular endothelial growth factor
VitD – vitamin D
VitD3 - 1α,25-Dihydroxy vitamin D3
VUHSK – Vilnius University Hospital Santaros Klinikos

1. INTRODUCTION

Early undifferentiated inflammatory arthritis (UA) is an autoimmune joint disease of inflammatory origin (1, 2). The early differential diagnosis of undifferentiated inflammatory arthritis and the establishment of a defined diagnosis of the disease (hereinafter referred to as 'disease outcome') is essential. The early treatment of arthritis applying "window of opportunity" and "treat-to-target" strategies leads to better treatment outcomes, helps to avoid destructive joint changes, and reduces the risk of impaired quality of life and mobility (3, 4).

The prevalence of inflammatory arthritis ranges from 115 to 271 per 100,000 adults (5). Still, there is no general consensus on the exact duration of the disease as defined by the term "early arthritis" (6). In literature data, this term ranges from <12-16 weeks to 2-3 years (6-9). The prevalence of early undifferentiated arthritis ranges from 23% to 81% in early arthritis cohorts (on average, UA is diagnosed in about 30% of patients consulting their doctors due to inflammatory arthritis) (10). Throughout the disease, UA outcomes can differ greatly. Data from UA patients' cohort studies vary widely and from 20% to 60% of patients are known to make a full recovery (6), while in about one-third of patients the disease progresses to RA or other chronic inflammatory rheumatic diseases (psoriatic arthritis (PsA), ankylosing spondylarthritis (AS), etc.), or even to multiple overlapping rheumatic diseases (11). Despite the fact that recently developed diagnostic and classification criteria for inflammatory rheumatic diseases are noted for great sensitivity and specificity (12-15), there is still a large number of patients who fail to meet these criteria even while suffering from chronic inflammatory rheumatic disease for a long time (16) It is also important to mention that the sensitivity and specificity of classification criteria for inflammatory rheumatic diseases decline in early arthritis cohorts (16, 17). Therefore, a proportion of UA patients fail to have an early diagnosis.

Laboratory tests that are used to diagnose inflammatory rheumatic diseases and assess their activity include C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP), anti-nucleolar antibodies (ANA), and human leucocyte antigen B27 (HLA-B27). These tests are also included in the diagnostic and classification criteria for inflammatory rheumatic diseases (12, 15, 18). The discovery of anti-CCP has revolutionized the early diagnosis of rheumatoid arthritis (RA) (12). Although the sensitivity of this test in RA accounts for 92.70% and the specificity equals 79.93% (19), in early RA diagnosis sensitivity drops to 41-77% (20). Laboratory markers that are applied diagnosing rheumatic diseases (RF, anti-CCP, ANA, HLA-B27) are also frequently detected in healthy individuals, as well as in individuals with other diseases (viral hepatitis C, oncological diseases, etc.) (21-26). Meanwhile, an increase in disease activity indices (ESR and CRP) may also be due to infection or other inflammatory diseases (27, 28). Therefore, the search for new laboratory markers to differentiate inflammatory joint diseases in patients with UA remains crucial to increasing the sensitivity and specificity of the current criteria.

In arthritis, inflammation of synovial layer is observed (synovitis). The processes that occur during synovitis lead to destructive processes in the cartilage and bone. Angiogenesis is particularly characteristic of inflammatory rheumatic diseases and is one of the key components of synovitis etiopathogenesis (29). Vascular endothelial growth factor (VEGF) is involved in almost all stages of the angiogenesis process and promotes both physiological and pathological angiogenesis (30, 31), and is also a key factor in the initiation and maintenance of synovial pannus (32, 33). Elevated VEGF levels are detected in early inflammatory arthritis (34), and VEGF levels were also associated with inflammatory rheumatic disease activity and radiological progression (35). For all these reasons, VEGF alone or in combination with other laboratory markers that are already applied in the diagnosis

of inflammatory rheumatic diseases could be a useful marker in UA differential diagnosis.

The role of genetic factors in the etiopathogenesis of inflammatory arthritis is widely discussed (36, 37). Single nucleotide polymorphism (SNP) analyses are among the most common genetic studies in the search for inherited risk factors in inflammatory arthritis and other systemic inflammatory diseases (38-40). Rheumatoid arthritis is one of the most common forms of inflammatory arthritis, and it is known to have an overall heritability of 66% (36, 41). rs2476601, rs833070, rs6920220 polymorphisms have been linked to the risk of developing RA and other systemic connective tissue diseases (systemic lupus erythematosus (SLE), Sjogren's syndrome (SjS)) (35, 42, 43). The meta-analysis showed that individuals with rs2476601 polymorphism were statistically more likely to have positive RF and anti-CCP values (44). The analysis of rs6920220 polymorphism demonstrated an association with the increased risk of developing RA (45). A study of *VEGF* gene polymorphism rs833070 confirmed tested SNP association with elevated plasma VEGF levels and synovial changes observed on ultrasound (US) examination of the joints (46). After a systematic literature analysis, only one study evaluating the significance of rs2476601 polymorphism for UA outcomes was detected (47), while no literature could be found analyzing the other two polymorphisms in UA cohorts that were tested in the present dissertation.

Radiological tests are very important in the diagnosis and the follow-up of inflammatory arthritis. One of them is the US examination of the joints, which is very important for the differential diagnosis of UA. In the early stages of inflammatory arthritis, in patients with less distinguished symptoms, US testing can help identify different types of inflammatory arthritis and distinguish autoimmune arthritis from osteoarthritis (48). It is worth mentioning that a strong correlation was observed between VEGF levels and the abnormalities of joints detected by ultrasound in patients with RA (49). It was also found that

UA patients who later were diagnosed with RA, PsA or other chronic inflammatory rheumatic disease had higher synovitis and power Doppler (PD) scores at the onset of disease than those whose arthritis remained undifferentiated (50).

Synovial fibroblasts (SF) are known to play an important role in the etiopathogenesis of inflammatory arthritis, activation of these cells leads to the onset of the inflammatory process in the joint (51). They are also able to act as innate immune system cells by attracting neutrophils and responding to Toll-like receptor (TLR) stimuli by producing pro-inflammatory cytokines (52). Tumor necrosis factor α (TNFα) and interleukin (IL)1β, produced by B lymphocytes, are also key factors in the transformation of SF into aggressive matrix metalloproteinase (MMP) secreting cells that cause destruction of the surrounding tissues (53). TLRs are characterized by their ability to recognize molecules found in most bacteria and viruses (54). TLRs play a key role in regulating SF function during RA (55, 56). TLR4 has been implicated to be the most important in the etiopathogenesis of RA (57, 58). Studies suggest that TLR4 plays an important role in SF proliferation, migration, and inflammatory response during RA (58, 59). Recently, the role of inflammasomes (nucleotide-binding oligomerization domain receptor or NOD-like receptor 1 (NLRP1) and NLRP3) in the etiopathogenesis of inflammatory rheumatic diseases has been investigated (60). These inflammasomes are supramolecular complexes that assemble in the cytosol of cells and respond to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (60). NLRP1 and NLRP3 increase the levels of caspase-1 (CASP-1) and IL-1β, which are involved in the immune response and cell pyroptosis (60, 61). Meanwhile, the vitamin D receptor (VDR) is known to suppress proinflammatory cells and stimulate tolerogenic (obtaining immuno-suppressive properties) regulatory T cells (62). There are a number of studies investigating the role of TLRs and only a few studies examining the role of NLRP1 and NLRP3 in the etiopathogenesis of

RA and OA have been published (58, 59, 63-67). After a systematic literature review had been carried out, we could not find a single study analyzing *TLR1*, *TLR2*, *TLR4*, inflammasome (*NLRP1*, *NLRP3*), and *VDR* gene expression and measuring MMPs secretion levels all together. Although all of the above-mentioned markers have been implicated in the etiopathogenesis of arthritis, there are still many unanswered questions about their importance both for UA and for other inflammatory arthritis.

The significance of sociodemographic, clinical, radiological, and laboratory (cell count, biochemical, immunology, and genetic) variables (and their interrelationships) for the outcomes of UA was assessed in this dissertation. Processes occurring in synovial fibroblasts of the inflamed joint (SF of the knee joint) were also investigated in an attempt to understand better the factors contributing to the chronicity of inflammation.

2. AIM AND OBJECTIVES OF THE STUDY

The aim of the study was to assess the significance of sociodemographic, clinical, radiological, and laboratory findings in the outcomes of early inflammatory undifferentiated arthritis.

Objectives:

1. To determine the clinical outcome of patients who were diagnosed with early undifferentiated inflammatory arthritis, at this study entry, after a 12-month follow-up period. To assess the factors which may have contributed to the disease outcomes.
2. To assess the association between vascular endothelial growth factor levels and clinical, radiological, and laboratory disease parameters, as well as early undifferentiated inflammatory arthritis outcomes.
3. To investigate single nucleotide polymorphisms (rs2476601, rs833070, rs6920220) and assess their association with clinical, laboratory, and ultrasound parameters in early undifferentiated

inflammatory arthritis and to determine their significance for the probability of progression to RA.

4. To determine levels of *VDR*, *TLR1*, *TLR2*, *TLR4*, *NLRP1*, and *NLRP3* genes expression and matrix metalloproteinases (MMP-1, MMMP-7, MMP-8, MMP-12, MMP-13) secretion in synovial fibroblasts of patients with early undifferentiated inflammatory arthritis and to compare the results with the data from long-standing RA, OA, and noninflammatory synovial fibroblasts analysis, to determine changes characteristic of early inflammatory arthritis.
5. To assess the role of vitamin D and TNFα in the expression of the genes studied (*VDR, TLR1, TLR2, TLR4, NLRP1*, and *NLRP3)* and the secretions levels of matrix metalloproteinases (MMP-1, MMP-7, MMP-8, MMP-12, MMP-13) in early undifferentiated arthritis synovial fibroblasts and compare the results with the data obtained from the analysis of longstanding RA, OA, and noninflammatory synovial fibroblast samples.

3. STATEMENT DEFENDED

6. In routine clinical practice, various sociodemographic, clinical, laboratory, and ultrasound tests are assessed during the first visit of patients with early undifferentiated arthritis, but only some of them are important for the prediction of a chronic inflammatory rheumatic disease, as the outcome of UA.
7. The vascular endothelial growth factor is an important laboratory marker of early inflammatory arthritis, and its value correlates with findings that are applied in everyday practice to the differential diagnosis of inflammatory arthritis and confirms inflammatory processes in the joint: clinical evaluation, laboratory, and ultrasound test results. By defining the range of VEGF values, the test could be used in the early differential diagnosis of inflammatory arthritis.

3. In the early stages of inflammatory arthritis, single nucleotide polymorphism rs6920220 is associated with the number of affected joints.
4. *VDR, TLR1, TLR2, TLR4, NLRP1,* and *NLRP3* gene expression and MMP-1, MMP-7, MMP-8, MMP-12, and MMP-13 secretion are detected in fibroblasts from inflammatory and non-inflammatory synovial fibroblasts. In early undifferentiated inflammatory arthritis synovial fibroblasts *TLR4* and *NLRP3* gene expression was higher than that in rheumatoid arthritis.

4. RELEVANCE AND NOVELTY OF THE STUDY

The opportunity to assess the course of UA at the very onset of the disease is very important for many reasons. Knowing which patients will recover spontaneously during the disease can help avoid unnecessary treatment, which undoubtedly might have its side effects. Meanwhile, for patients with chronic inflammatory rheumatic disease, treatment initiated during the window of opportunity period leads to faster and sustained disease remission, besides, lower doses of disease modifying drugs (DMARDs) are needed to achieve full remission, also it delays treatment with biological disease modifying drugs (bDMARDs), prevents structural damage of the joints, preserves patients' functional capacity and ability to work (1), and reduces the socioeconomic burden of inflammatory rheumatic diseases (68). The sensitivity and specificity of diagnostic and classification criteria for inflammatory rheumatic diseases that are applied in daily practice are not sufficient in early arthritis cohorts, which results in a significant proportion of patients being diagnosed too late when structural damage to the joints is already observed. Also, the laboratory parameters that are currently used in daily rheumatology practice may be negative or at the normal range values disease in patients with chronic inflammatory rheumatic diseases. For example, 15-45% of patients are diagnosed with seronegative RA (69), which means that they have neither elevated RF nor anti-CCP values). Moreover, there

are some diseases, such as PsA, that do not have any specific laboratory parameters that could assist in the differential diagnosis (70). Therefore, there is a great need for a search for new prognostic markers, or their combinations, to improve the early diagnosis of inflammatory rheumatic diseases and to ameliorate UA outcomes.

The present study is the first one in Lithuania to investigate the cohort of patients with early undifferentiated arthritis. The study revealed the sociodemographic, clinical, laboratory, and ultrasound characteristics of UA patients. It also helped realize how quickly UA patients have their first visit with a rheumatologist, given that the duration from the disease onset to the first rheumatology consultation is crucial for UA outcomes. Based on literature analysis, this research was also the first in Lithuania and the second in the world to assess the link between VEGF serum levels and the abnormalities observed during joint ultrasound in UA patients, and it improved scientific knowledge of the relationship between these two tests and the potential new applications of the laboratory marker in the early diagnosis of UA. The association between VEGF levels and the tests that are applied in the inflammatory arthritis classification criteria was also assessed. During the study, for the first time in Lithuania, SNPs (rs2476601, rs833070, rs6920220) that are associated with the etiopathogenesis of RA were investigated in the UA population, and their significance for disease outcomes was assessed. In addition, for the first time, based on the published literature analysis, the expression of *TLR1*, *TLR2*, *TLR4*, inflammasomes (*NLRP1*, *NLRP3*), and *VDR* genes, and secretion of MMPs and IL-1β were investigated in the knee joint synovial fibroblasts of the subjects with early undifferentiated inflammatory arthritis, and data were compared with the results obtained from the analysis of RA, OA, and noninflammatory (control group (CN)) synovial fibroblasts. The data obtained improved the existing knowledge of the etiopathogenesis of inflammatory rheumatic diseases.

5. MATERIAL AND METHODS

The study consists of two parts:

1. A prospective study, in which patients with early undifferentiated inflammatory arthritis (referred as early undifferentiated arthritis (UA)) were followed for 12 months to measure outcomes of UA. Patient selection, inclusion and clinical examination were carried out in Vilnius University Hospital Santaros Klinikos (VUHSK) Rheumatology Centre. Patients' enrolment and investigation based on the study protocol was carried out by the author of the dissertation. Routine laboratory tests that are usually applied in inflammatory arthritis diagnostics and an additional test for vascular endothelial growth factor (VEGF-A) were performed in cooperation with VUHSK Laboratory Medical Centre. The analysis of genetic factors (SNPs) was performed in cooperation with the Children's Hospital Oakland Research Institute (CHORI).
2. A comparative study of synovial fibroblasts in patients with different types of knee arthritis (AA, RA, OA, and CN), assessing differences in the gene expression levels of inflammasomes *NLRP1* and *NLRP3*, *TLR1*, *TLR2*, *TLR4,* and *VDR*, and in secretion levels of MMPs (MMP-1,MMP-7,MMP-8,MMP-12,MMP-13) and IL-1β, looking for markers that would ameliorate the differential diagnosis of inflammatory arthritis at a very early stages of the disease. The recruitment, enrolment, and clinical examination of the participants in this part of the study were performed by a rheumatologist (the author of the dissertation) and traumatologists from the VUHSK. Post-operative residual tissue (knee synovial samples) from the patients who agreed to take part in this study was forwarded to the State Research Institute Centre for Innovative Medicine, Department of Regenerative Medicine where a synovial fibroblast analysis was carried out.

This study was approved by Vilnius Regional Biomedical Research Ethics Committee (Approval No. 158200-15-800-310, 158200-16-859-368, and 158200-18/5- 43 1037-533). All patients were enrolled in this study after they had signed informed consent.

5.1. Prospective study of patient's cohort with early inflammatory undifferentiated arthritis

Patients were recruited for this study after a rheumatology consultation at VUHSK, Rheumatology Centre to clarify the diagnosis of inflammatory arthritis. A total of 155 UA patients were enrolled (99 females and 56 males). Patients were observed for 12 months in total (the first visit at study entry, after 6 months, and after 12 months).

Inclusion criteria: patients signed informed consent; adults (>18 years old) with inflammatory arthritis at least in one joint; as assessed by the treating rheumatologist; patient-reported duration of arthritis less than 12 months; at the study entry, patients did not meet any inflammatory rheumatic diseases diagnostic and/or classification criteria (2010 American College of Rheumatology (ACR)/ European League Against Rheumatism (EULAR) rheumatoid arthritis classification criteria (12), ClASsification criteria for Psoriatic Arthritis (CASPAR) (13), Assessment of SpondyloArthritis International Society (ASAS) classification criteria for axial and peripheral spondylarthritis (SpA) (15), Systemic Lupus International Collaborating Clinics (SLICC'12) criteria for systemic lupus erythematosus (14), or other autoimmune rheumatic diseases (71-73)). Exclusion criteria: patients disagree to participate in the study; under the age of 18; pregnancy; arthritis due to trauma; septic arthritis; paraneoplastic arthritis; microcrystal arthropathies; osteoarthritis; at the time of enrollment patient fulfilled diagnostic and/or classification criteria for inflammatory rheumatic diseases.

All patients who met the inclusion criteria, had no exclusion criteria, agreed to participate in the study, read, understood, and signed the

informed consent form were invited to participate in this study. The study sample size was decided based on the data from epidemiological studies on early arthritis (74, 75). The present author consulted part of the patients enrolled in this study at the 'Early Arthritis Rheumatology Office' of the VUHSK outpatient clinic. The patient came to a rheumatologist for a consultation when the condition of registration stated: 'suspected to have the onset of inflammatory arthritis lasting <12 months for the first time in life'. During the enrolment period, 421 patients were registered at the 'Early Arthritis Rheumatology Office' and arrived for a consultation, including 77 who met the inclusion criteria and 41 who agreed to participate in the study. Other patients in this study cohort were enrolled when they came for a consultation with the rheumatologist without applying the case assignment to said < 12-month disease registration. Patients were followed up for a total of 12 months (visit 0, after 6 months, and 12 months later) to assess the rheumatological state and UA outcomes.

During the study baseline visit, sociodemographic data of each patient (age, gender, smoking history, education level, presence of rheumatic disease in blood relatives, body mass index (BMI) were calculated), and clinical disease characteristics (presence of comorbidities and infection, patient-reported duration of arthralgia and joint swelling; 68 tender joint count (68 TJC) and 66 swollen joint count (66 SJC); patient's global assessment of disease activity on a 100 mm visual analog scale (patient's global VAS), physician's global assessment of disease activity on a 100 mm visual analog scale (physician's global VAS), patient's joint pain assessment on a 100 mm visual analog scale (patient's joint pain VAS); disease activity score 28 (DAS 28) calculated based on an assessment of 28 tender and swollen joints and ESR (76), Health Assessment Questionnaire (HAQ) score (77) were recorded.

Laboratory tests that are applied to evaluate disease activity (ESR, CRP) were performed on all early UA patients. RF, Anti-CCP, ANA, and HLA B27 (tests that are applied in inflammatory rheumatic

diseases classification criteria) analysis was carried out at the discretion of the treating rheumatologist as clinically indicated. Blood samples were collected during the baseline visit for the detection of VEGF levels and SNPs rs2476601, rs833070, rs6920220 expression.

If allowed, US examination of the affected (tender and/or swollen) joints was performed during each visit (at the beginning of the study, after 6 and after 12 months) by the present author. All affected joints (tender and swollen) were evaluated. The methodology for US is given in Section 4.1.3.

During follow-up visits this sociodemographic, clinical, and laboratory data were recorded: the patient's age, BMI, number of tender and swollen joints (68 TJC, 66 SJC), the patient's global VAS, the physician's global VAS, the patient's joint pain VAS, DAS 28, ESR, CRP values, HAQ, as well as US changes in tender and/or swollen joints. The final diagnosis of 17 patients who did not attend a follow-up visit after 12 months was verified by phone call (phone number was provided in patients' questionnaires).

During a follow-up after 6 and/or 12 months, UA outcomes were evaluated. UA outcomes: 1) the inflammatory rheumatic disease diagnosis was made based on rheumatic diseases diagnostic or/and classification criteria (12-15, 71-73); 2) the patient's arthritis resolved completely (74); 3) UA diagnosis remained. If the patient failed to show up for the final (after 12 months) follow-up, and his/her UA outcome was already known after a 6- month follow-up, this diagnosis was used for a final data analysis.

Based on UA outcome during the follow-up, patients were finally divided into groups:

I. Patients who were diagnosed with rheumatic inflammatory disease or UA diagnosis remained:
    1) RA patient group – diagnosis based on ACR/EULAR 2010 RA classification criteria (12);

2) SpA patient group: a) patients who were diagnosed with reactive arthritis (ReA) (78); b) axial or peripheral SpA (15); c) patients who were diagnosed with PsA (13);
3) Patients who were diagnosed with other autoimmune inflammatory diseases: a) patients who were diagnosed with SLE (14); b) patients who were diagnosed with mixed connective tissue disease (79); c) patients who were diagnosed with immunoglobulin G4 related disease (IgG4 RD) (80), dermatomyositis (72) or other inflammatory rheumatic disease (81, 82);
4) Patients with UA during follow-up visits.

II. Patients whose arthritis resolved completely (remission was observed for a 6-month follow-up (no swollen joints) without any need for non-steroidal anti-inflammatory drugs (NSAIDs), steroids or DMARDs).

III. Patients who failed to come to other visits in 6 and/or 12 months or decided to discontinue participation in this study.

In this part of the study, the association between conventional clinical, laboratory, and radiological parameters that are applied in everyday rheumatology practice and VEGF levels, as well as SNPs, were analyzed, and the importance for UA outcomes was calculated.

5.1.1. Vascular endothelial growth factor analysis

VEGF analysis was performed for the first 76 UA patients that were enrolled in this study and who agreed to undergo US examination of tender and swollen joints. Blood samples were collected during the baseline visit for the detection of VEGF levels. Patients' venous blood was drawn on an empty stomach, avoiding lipemia. Serum samples were tested for human VEGF-A. Blood samples were collected using BD Vacutainer Serum Separator Tubes (5 mL) (BD Biosciences, NJ, USA). Before the investigation, serum samples were stored frozen at −20 ◦C. Prior to an assay, the frozen samples were brought to room temperature gradually and mixed gently. The enzyme-linked

immunosorbent assay (ELISA) was performed to measure VEGF-A levels in sera, following the manufacturer's instructions. The ELISA kit for human VEGF-A was from IBL International, Germany (catalog No. BE55101). Samples were analyzed in duplicate. Four subjects had exceptionally high VEGF-A levels and were excluded from a further statistical analysis.

5.1.2. Single nucleotide polymorphism analysis

A pilot study for rs2476601, rs833070, rs6920220 polymorphism detection was performed for 92 patients with UA and for those who agreed to undergo US examination of tender and swollen joints. Genomic DNA was extracted from frozen whole blood using the Gentra Puregene Blood Kit (Qiagen). Each patient sample was genotyped on an Illumina BeadLab1000 platform using the ImmunoChip V2 and the Infinium HD assay. Genotypes were called using a score threshold of 0.15 in the Illumina BeadStudio software. Data cleaning was performed using the PLINK software (version 1.9) (http://pngu.mgh.harvard.edu/purcell/plink/). When analyzing the data, on the basis of UA outcomes, patients were divided into two groups: patients who were diagnosed with RA during the follow-up period (RA group) and patients who were diagnosed with other inflammatory rheumatic diseases or whose arthritis resolved completely (non-RA group). To analyze the significance of tested SNP on sociodemographic, clinical, laboratory, and instrumental variables, patients were divided into two groups based on genotype: rs2476601 *AA*+*AG* and *GG*; rs833070 *GG*+*AG* and *AA*; rs6920220 *AA*+*AG* and *GG*.

5.1.3. Ultrasound analysis

At the baseline, and after 6 and 12 months, ultrasound of all tender and swollen joints was performed using 12 MHz linear transducer (General Electric LOGIQ E Portable Ultrasound). Synovitis, PD, and

erosion findings were scored using a scale of 0 to 3. Scores from each joint were added up to calculate synovitis, PD, and erosions (Table 1) (83, 84). If joints that were tender or swollen during baseline visit were no longer tender or swollen during the follow-up visits, a US analysis was performed for these joints all the same.

Table 1. Ultrasound imaging scoring

Pathologic change	Sonographic scoring system
Synovitis	None=0, Mild=1, Moderate=2, Severe=3.
Power Doppler (PD)	No flow in the synovium=0, Single vessel signals=1, Confluent vessel signals in less than half of the area of the synovium=2, Vessel signals in more than half of the area of the synovium=3.
Erosions	None=0, Surface irregularity where no defect detected in 2 perpendicular planes=1, Surface defect seen in 2 perpendicular planes=2, Defect forming severe bone destruction=3.

Adapted from (83, 84).

5.2. Differences between *TLR1*, *TLR2*, *TLR4*, *VDR*, *NLRP1*, *NLRP3* gene expression and MMP-1, MMP-7, MMP-8, MMP-12, MMP-13 and IL-β secretion levels in synovial fibroblast of patients with early inflammatory undifferentiated arthritis, rheumatoid arthritis, osteoarthritis, and control group

A total of 19 patients were enrolled in this part of the study (7 patients diagnosed with RA, 4 with OA, 4 with UA and 4 controls (CN)). Patients with RA and OA underwent total knee joint replacement operation. Meanwhile patients from AA and CN groups had arthroscopic knee surgeries. Operations were performed by three senior surgeons who reaffirmed the need and decided on the type of surgery. In this research, only residual postoperative tissues (synovial tissues) were used, which, otherwise would have been destroyed as medical waste.

Inclusion criteria: patients signed informed consent, adults (>18 years old); patients who, according to the medical indications determined by orthopedic surgeons - traumatologists who treated them, needed to undergo planned knee joint operations; RA patients met ACR/EULAR 2010 classification criteria (12), OA patients met ACR classification criteria for knee OA (85); UA patients - patients whose arthritis duration was <12 months and at the time of enrollment did not meet any diagnostic or classification criteria of inflammatory rheumatic diseases (12-15, 79); CN group - patients who were operated due to meniscus or cruciate ligament tear and were not diagnosed with inflammatory rheumatic disease (12-15, 79) or osteoarthritis (85) were determined as the CN group. Exclusion criteria: patients refused to participate in the study; under the age 18; pregnancy; arthritis due to causes other than mentioned above in the inclusion criteria; comorbidities, such as diabetes, autoimmune thyroiditis, malignancies, and other autoimmune inflammatory conditions were not enrolled in this study.

Sociodemographic (age, gender) data, comorbidities, and medications used were assessed. CRP, RF, vitamin D (VitD) and anti-CCP tests were performed on all patients at the Centre for Laboratory Medicine of Vilnius University Hospital.

5.2.1. Synovial tissue and cell culture preparation

Cells were isolated from synovial tissues as described in (86). Briefly, mechanically minced synovial tissues were incubated overnight in Dulbecco's modified Eagle's medium (DMEM) (with 1 g/L D-glucose, sodium pyruvate, L-glutamine, phenol red, Invitrogen) in a humidified 5% CO2 incubator at 37°C. After incubation, synovial tissues were digested with 0.1% collagenase (Type I, Biochrom, Cambridge, UK) at 37°C in a shaking mode overnight. Isolated cells were centrifuged at 400×g for 10 min and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (Biochrom), 1% stock solution of penicillin (10,000 units/ ml), streptomycin (10

mg/ml), and amphotericin B (0.025 mg/ml, Biological Industries, Haemek, Israel). Passages 2–4 SFs were plated into 25–cm2 culture flasks in DMEM containing 10% FBS and stimulated or not for 72 h with or without 100 ng/ml TNF-a (Thermo Fisher Scientific, Waltham, MA, USA) (with additional stimulation after 36 h) and 1 or 0.01 nM of vitD3 (Sigma, St. Louis, MO, USA). At the end of experimental stimulation, cell culture supernatants were collected under sterile conditions and stored at −80°C until a further analysis; cells were lysed in RLT buffer (RNeasy kit, Qiagen, Hilden, Germany) and used for a gene expression analysis.

5.2.2. RNA extraction, cDNA synthesis, and Quantitative Real-Time PCR

RNA was extracted with RNeasy Mini Spin columns (Qiagen) according to the manufacturer's instructions, and RNA concentration and purity were measured with the SpectraMax® i3 (Molecular Devices, San Jose, CA, USA) spectrophotometer. Before synthesizing the first complementary DNA strand, RNA samples were treated with DNase I (Thermo Fisher Scientific) and cDNA synthesis was performed with the Maxima®First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer protocols. qPCRs were performed using the Maxima® Probe qPCR Master Mix (2×) (Thermo Fisher Scientific) and AriaMx real-time PCR system (Agilent Technologies, Santa Clara, CA, USA). The TaqMan® Gene Expression Assays (Applied Biosystems, Waltham, MA, USA) for 8 genes were used for the gene expression analysis, using primers as indicated in Table 2. The qPCR reaction volume was 25 ml with 0.5 ml of 20× Taqman® Gene Expression Assay mix. All reactions were run in triplicates. Cycle conditions were as follows: the initial denaturation step for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C for denaturation and 60 s for annealing and extension. Each RNA sample was controlled for genomic DNA contamination by reactions without reverse transcriptase (RT), and reagent contamination was

checked by reactions without template (NTC). Relative gene expression quantification was calculated using the 2−DCT×1,000 method. The geometric mean of two reference genes—RPS9 and B2M—was used to normalize gene expression. qPCR data were analyzed with the help of AriaMx (Agilent Technologies) software.

Table 2. The TaqMan Gene Expression Assays used for gene expression analysis

Gene, assay ID	Encoded protein
RPS9 Hs02339424_m1	40S ribosomal protein S9
B2M Hs00984230_m1	Beta-2 microglobulin
TLR-1 Hs00413978_m1	Toll-like receptor 1
TLR-2 Hs02621280_s1	Toll-like receptor 2
TLR-4 Hs00152939_m1	Toll-like receptor 4
VDR Hs01045843_m1	Vitamin D receptor
NLRP1 s00248187_m1	PYD domains-containing protein 1
*NLRP3*Hs00918082_m1	PYD domains-containing protein 3

4.2.3. Detection of secreted proteins by Luminex and ELISA assays

The analysis of IL-1β concentration in non-diluted supernatants was performed using commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA); levels of MMP-1, MMP-7, MMP-8, MMP-12, and MMP-13 were measured using Luminex Technology and ProcartaPlex Human MMP-Panel 5 plex panel (Affymetrix, eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. Cell culture medium was used for background normalization.

5.3. Statistical analysis

The mean and standard deviation was used to describe the quantitative characteristics of the research. Frequencies (n) and percentages (%) were used for qualitative characteristics. Data distribution normality was assessed using the Shapiro-Wilk normality test. Depending on

applicable assumptions, Student's t-test for independent samples was used to compare means of a particular qualitative characteristic of different samples. The analysis of variance ANOVA was conducted to compare quantitative variables of more than two samples. Differences in the qualitative characteristics of experimental groups were assessed using the Chi-square test. To compare nonparametric data sample means the Mann-Whitney U and the Kruskal–Wallis tests were applied. The related samples were compared using a nonparametric Wilcoxon signed-rank test. Correlations between all cohort data were calculated using the Spearman's correlation test. The prognostic value of the analyzed parameters for UA outcomes were assessed using logistic regression. The association strength between the risk for UA to progress into chronic inflammatory arthritis and both VEGF levels and analyzed SNP (rs2476601, rs833070, and rs6920220), were assessed by computing odds ratio (OR) with 95% confidence intervals (95%CI). All SNPs were tested for Hardy Weinberg Equilibrium and all had minor allele frequencies (MAF) ≥10%. A statistical analysis and visualization were performed using Microsoft Office (Microsoft Corporation, Redmond, W.A., USA), GraphPad Prism (version 9, GraphPad Software, San Diego, CA, USA), SPSS (version 26.0 IBM Corp, Armonk, NY, USA), and PLINK (version 1.9). The selected level of significance $p<0,05$.

6. RESULTS

6.1. Prospective 12-month-long study of patient cohort with early inflammatory undifferentiated arthritis

6.1.1. Sociodemographic, clinical, radiological and laboratory finding characteristics of the study group

A total of 155 patients diagnosed with UA, with a disease duration of less than 12 months, were enrolled in this part of the study (from Jul 2016 until 2018). Part of the UA patients were enrolled in the 'Early

Arthritis Rheumatology Office'. During the study enrollment period, a total of 421 patients were consulted in this office, however, after the rheumatologist's evaluation, it was determined that only 77 (18.25 %) patients met the inclusion criteria and only 41 (9.7%) of them agreed to participate in this study and signed informed consent. The remaining study population (114 patients) were enrolled during a routine visit to a rheumatologist's office at VUHSK outpatient clinic. After 6 months 123 patients were admitted during a follow-up visit and after 12 months – this figure stood at 83 patients. Out of 155 study cohort, 99 (63.9%) were females and 56 (36.1%) were males, the mean age at enrollment was 44.32±16.59 years. Before study entry, UA patients were seen by family doctors mainly twice (mode 1, median 3 [0-5]) and by a rheumatologist in the nearest outpatient clinic – once (mode 0, median 1 [0-3]). The mean duration before the first visit to the rheumatologist was 5.01±3.14 months, and in VUHSK 5.72±3.36 months, 67 (43.2%) UA patients were seen by a rheumatologist for the first time within the "Window of opportunity" timeframe. A full description of sociodemographic, clinical, instrumental, and laboratory findings parameters in UA patient's cohort is presented in Table 3.

Table 3. Sociodemographic, clinical, instrumental and laboratory finding parameters in early undifferentiated arthritis patient's cohort at the study entry and during follow-ups after 6 and 12 months

Variables	At the study entry n=155	After 6 month n=123	After 12 months n=83
Sociodemographic data			
Age, years	44.32±16.59	44.64±16.81	44.46±17.25
Females	99 (63.9)	80 (65.0)	65 (65.0)
Males	56 (36.1)	43 (35.0)	35 (35.0)
Daily smokers	27 (17.4)	-	-
Education, years	13.36±2.34	-	-
BMI, kg/m^2	24.12±3.25	-	-
Presence of rheumatic diseases in family	55 (35.5)	-	-
Clinical data			
Presence of comorbidities	115 (74.2)	-	-
Tested for infections	103 (66.5)		
Active infection detected	54 (34.8)	-	-
Patient's global VAS, mm	48.12±16.44	35.77±18.36	32.77±17.02
Patient's joint pain VAS, mm	46.55±17.25	33.75±20.52	29.22±18.18
Physician's global VAS, mm	46.10±13.93	31.52±16.68	28.47±16.11
Duration of joint swelling, months	572±3.36	-	-
Duration of joint pain, months	7.22±5.17	-	-

Duration of morning stiffness, min	45 [0-300]	30 [0-300]	30 [0-180]
66 SJC	4.08±3.45; 3 [1-20]*	2.10±2.60; 1 [0-16]*	1.66±2.25; 1 [0-16]*
28 SJC	3.29±2.83; 3 [0-18]*	1.78±1.87; 1 [0-8]*	1.41±1.59; 1 [0-10]*
68 TJC	7.01±5.68; 7[0-28]*	3.82±3.30; 3 [0-18]*	3.63±3.46; 3 [0-15]*
28 TJC	5.19±4.21; 4[0-18]*	2.90±2.53; 3 [0-10]*	2.61±2.47; 2 [0-10]*
DAS 28 (ESR)	4.72 ±0.91	3.67±1.24	3.15±1.25
HAQ score	0.74 [0.00-2.75]	0.49 [0.00-2.38]	0.43 [0.00-2.13]
Laboratory findings data			
ESR, mm/h	25 [2-144]	19 [1-72]	14 [1-65]
CRP, mg/l	6.7 [0.16 – 144.30]	4.6 [0.14-63.27]	3.9 [0.20-39.87]
VEGF[1] pg/mL	365.27 [25.75 – 3438.23]	-	-
RF[3], kU/l	72.21 [9.59-814.20]	-	-
RF positive (>30 kU/l)	62 (40.8)	-	-
Anti-CCP[4], U/ml	81.57 [2-300]	-	-
Anti-CCP positive (≥5U/ml)	58 (43.6)	-	-
RF and anti-CCP positive	54 (40.6)	-	-
HLA-B27[5] positive	35 (29.7)	-	-
ANA[6] positive (titre >1:40)	28 (43.1)	-	-
Ultrasound findings data			
Number of examined patients	n=115	n=81	n=55
Number of examined joints	815	613	429

Synovitis score	6.54±5.20; 5 [1-30]*	3.84±4.55; 2[0-18]*	3.65±3.96; 2 [0-16]*
PD score	3.42±3.41; 2 [0-15]*	1.89±2.87; 0 [0-10]*	1.71±2.31; 0 [0-10]*
Erosions (grade)	1.42±2.23; 0 [0-10]*	1.84±2.59; 0 [1-10]*	1.98±2.69; 0 [0-10]*
Presence of erosions	46 (40.0)	40 (49.4)	27 (49.1)

Continuous data are presented and median [minimum and maximum] values; or mean ± standard deviation, counts as numbers and valid percentages; n - number of patients; * the limits are presented to the parametric and nonparametric indices to provide the minimum and maximum values of the variable, providing more detailed information on the cohort of the study. BMI, body mass index; VAS, visual analogue scale; SJC, swollen joints count; TDJ, tender joints count; HAQ, Health Assessment Questionnaire; DAS 28 (ESR), disease activity score 28 using on erythrocyte sedimentation rate; ESR, erythrocyte sedimentation rate; CRP, C - reactive protein; RF, rheumatoid factor; Anti-CCP, anticitrullinated protein antibodies; HLA B27, human leukocyte antigen B27; ANA, anti-nuclear antibodies; VEGF, vascular endothelial growth factor; PD, Power Doppler; totally tested [1]- 76 [3]- 152, [4]-133, [5]- 118, [6]- 65.

6.1.2. Early undifferentiated arthritis outcomes

After 6 months, a total of 123 (79.4%) patients came for the first follow-up visit after rheumatologists' evaluation: 44 (35.8%) were diagnosed with RA, 65 (52.8%) – with SpA, 7 (5.7%) with other autoimmune inflammatory diseases, and 7 (5.7%) had UA diagnoses. During the final study visit in 12 months, 83 patients were admitted to the rheumatology office. 17 patients' diagnosis was verified by a survey on a phone or/and by reviewing the medical electronic records. After 12 months outcomes of 100 UA patients were evaluated: 35 (35.0%) were diagnosed with RA, 27 (27.0%) with SpA, 10 (10.0%) with other autoimmune inflammatory diseases, and 28 (28.0%) patients' arthritis resolved completely. All diagnoses that were known after a 12-month follow-up were used in this study for a final UA outcomes analysis. In case the disease outcome became known only after 6 months and the patient did not come to the final visit, the patient's diagnosis after 6 months was considered as the final outcome of the disease. In all study cohort (155 patients) these UA outcomes were confirmed: 50 (32.3%) were diagnosed with RA, 33 (21.3%) with SpA (8 (5.2%) with reactive arthritis, 16 (10.3%) with axial or peripheral SpA, 9 (5.8%) with PsA)), 12 (7.7%) with other autoimmune inflammatory diseases (2 (1.3%) with SLE, 1 (0.6%) with dermatomyositis, 8 (5.2%) with an undifferentiated connective tissue disease, 1 (0.6%) with IgG4 RD)), arthritis of 28 (18.1%) patients resolved completely, and outcomes were unknown for 32 (20.6%) UA patients (Table 4).

Table 4. Early undifferentiated arthritis patient cohort outcomes after 6- and/or 12-month follow-up

Outcome (diagnosis/ arthritis resolved/ patient failed to come to follow-up visits)	0 week	After 6 months	After 12 months			All enrolled patients UA outcome n=155 (%)
	Attended visit n=155 (%)	Attended visit n=123 (%)	Attended visit n=83 (%)	Survey on phone n=17 (%)	General data n=100 (%)	
UA	155 (100)	7 (5.7)	0 (0)	0 (0)	0 (0)	0 (0)
RA	0	44 (35.8)	30 (36.1)	5 (29.4)	35 (35.0)	50 (32.3)
SpA	0	65 (52.8)	24 (28.9)	3 (17.6)	27 (27.0)	33 (21.3)
Other autoimmune inflammatory diseases *	0	7 (5.7)	10 (12.0)	0 (0)	10 (10.0)	12 (7.7)
Arthritis resolved**	0	-	19 (22.8)	9 (53.0)	28 (28.0)	28 (18.1)
Follow-up data is missing	-	-	-	-	-	32 (20.6)

*Diseases: SLE, dermatomyositis, undifferentiated connective tissue disease, IgG4 related disease. **During 6 months follow-up, patients with no clinical signs of synovitis, and without using medication (nonsteroidal anti-inflammatory drugs, glucocorticoids, disease modifying drugs) were still assigned to patients group with inflammatory arthritis, as in this study, arthritis was assessed as resolves, if no clinical symptoms of synovitis ≥6 months. n(%) – number of patients; UA, early undifferentiated arthritis; RA, rheumatoid arthritis; SpA, spondyloarthropathies (reactive arthritis, axial or peripheral spondylarthritis, psoriatic arthritis).

6.1.3. Analysis of sociodemographic, clinical, laboratory and ultrasound findings between patient groups whose arthritis resolved or who were diagnosed with chronic inflammatory rheumatic disease during the study follow-up

Patients whose arthritis resolved completely during the study follow-up had significantly higher education (p=0.008).

Clinical data analysis revealed that patients who were later diagnosed with chronic inflammatory rheumatic disease, had a greater number of tender and swollen joints (p<0.001), a longer duration of morning stiffness (p<0.001), higher DAS 28 scores (p<0.001) at the onset of the disease. Patients whose arthritis resolved, at study entry significantly more often suffered from active infection (p<0.05).

The analysis of laboratory tests demonstrated that patients whose arthritis resolved at the onset of the disease had significantly lower ESR and CRP values (p<0.05 and p<0.001, respectively). RF and/or anti-CCP values were significantly more often positive (p<0.001 and p<0.001, respectively) in the patient group that was later diagnosed with chronic inflammatory rheumatic disease. The analysis of HLA-B27 and ANA tests showed no statistically significant difference between the analyzed groups.

Ultrasound examinations of swollen and/or tender joints were performed on 115 subjects during the first visit, 81 subjects after 6 months, and 55 subjects after 12 months. The analysis of US findings revealed that the patient group that later was diagnosed with chronic inflammatory rheumatic disease, at the study entry had significantly higher scores of synovitis (p<0.001), PD (p<0.001), grades of erosions (p<0.001) and erosions (p=0.001) were more often frequent. All descriptive data is presented in Table 5.

Table 5. Comparison of sociodemographic, clinical, laboratory and ultrasound findings between patient groups whose arthritis resolved and who were diagnosed with chronic inflammatory rheumatic disease during a 12-month follow-up

Variables	Chronic inflammatory rheumatic disease* n=95	Arthritis resolved n=28	*p* values**	UA outcome unknown n=32
Sociodemographic data				
Age, years	44.43±17.44	42.18±15.12	0.653	45.78±15.55
Females	67 (70.5)	15 (53.6)	0.094	18 (56.3)
Males	28 (29.5)	13 (46.4)		14 (43.8)
Daily smokers	15 (15.8)	6 (21.4)	0.486	5 (15.6)
Education, years	12.97±2.24	14.36±2.63	**<0.001**	13.53±2.09
BMI, kg/m^2	23.74±3.22	24.51±3.32	0.279	24.68±3.26
Presence of rheumatic diseases in family	35 (36.8)	9 (32.1)	0.648	10 (31.3)
Clinical data				
Presence of comorbidities	74 (77.9)	19 (67.9)	0.277	23 (71.9)
Presence of active infection [1]	28 (29.5)	13 (46.4)	**0.015**	13 (40.6)
Patient's global VAS, mm	48.63±16.98	46.96±14.63	0.925	46.37±15.83

Patient's joint pain VAS, mm	47.43±17.54	45.68±15.94	0.584	43.72±16.95
Physician's global VAS, mm	46.73±14.23	45.18±12.44	0.569	44.19±13.87
Duration of joint swelling, months	5.84±3.49	5.84±3.35	0.935	4.44±3.06
Duration of joint pain, months	7.17±4.93	6.46±3.21	0.728	7.12±6.90
Duration of morning stiffness, min	60 [0-300]	25 [0-120]	**<0.001**	60 [0-300]
66 SJC	4.73±3.87; 4 [1-20]↓	2.54±2.50; 2 [1-13] ↓	**<0.001**	3.53±2.17; 3 [1-11] ↓
28 SJC	3.85±3.07; 3 [0-18] ↓	1.88±2.05; 1 [0-10]↓	**<0.001**	2.86±2.17; 2 [0-10] ↓
68 TJC	7.93±5.85; 6 [1-28] ↓	3.93±3.89; 3 [1-19] ↓	**<0.001**	7.37±5.69; 6 [0-24] ↓
28 TJC	5.9±4.31; 4 [0-18] ↓	2.71±2.98; 2 [1-13]↓	**<0.001**	5.21±4.14; 4 [0-16] ↓
DAS 28 (ESR)	4.88±0.89	4.09±0.70	**<0.001**	4.78±0.91
HAQ score	0.77±0.53	0.67±0.40	0.565	0.73±0.67
Laboratory findings data				
ESR, mm/h	29 [2-144]	16 [2-97]	**0.013**	35 [6-110]
CRP, mg/l	7.8 [0.39-111.50]	3.42 [0.16-18.00]	**<0.001**	6.27 [0.29-144.30]
VEGF[3], pg/mL	372.04 [25.75-3438.23]	350.04 [53.97-1437.3]	0.759	-
RF positive[4], >30 kU/L	48 (50.5)	4 (16.0)	**0.002**	10 (31.3)
RF negative[4], ≤30 kU/L	47 (49.5)	21 (84.0)		22 (68.8)
Anti-CCP positive[5], 5>UmL	47 (56.6)	3 (13.0)	**<0.001**	8 (29.6)
Anti-CCP negative[5], 5≤UmL	36 (43.4)	20 (87.0)		19 (70.4)
RF and anti-CCP positive	44 (53.0)	3 (13.0)	**0.001**	7 (21.9)
RF and anti-CCP negative	39 (17.0)	20 (87.0)		20 (74.1)

HLA-B27[6] positive	22 (31.0)	6 (25.0)	0.578	7 (30.4)
HLA-B27[6] negative	49 (69.0)	18 (75.0)		16 (69.6)
ANA[7] positive, titre >1:40	20 (46.5)	4 (28.6)	0.238	4(50.0)
ANA[7] negative, titre ≤1:40	23 (53.5)	10 (71.4)		4 (50.0)
Ultrasound findings data				
Number of examined patients	n=68	n=18		n=29
Number of examined joints	452	127	-	216
Synovitis score	7.34±5.73	2.94±1.11	**<0.001**	6.69±4.56
PD score	3.00 [0-12]	1.00 [0-3]	**<0.001**	3.97 [0-15]
Erosions (grade)	1.00 [0-8]	0.00 [0-3]	**<0.001**	1.28 [0-10]
Presence of erosions	36 (52.9)	1 (5.6)	**0.001**	9 (31.0)

Continuous data are presented and median [minimum and maximum] values; or mean ± standard deviation, counts as numbers and valid percentages; n- number of patients; ǂ the limits are presented to the parametric and nonparametric indices to provide the minimum and maximum values of the variable, providing more detailed information on the cohort of the study. BMI, body mass index; VAS, visual analogue scale; SJC, swollen joints count; TDJ, tender joints count; HAQ, Health Assessment Questionnaire; DAS 28 (ESR), disease activity score 28 using on erythrocyte sedimentation rate; ESR, erythrocyte sedimentation rate; CRP, C - reactive protein; RF, rheumatoid factor; Anti-CCP, anticitrullinated protein antibodies; HLA B27, human leukocyte antigen B27; ANA, antinuclear antibodies; VEGF, vascular endothelial growth factor; PD, Power Doppler; totally tested: [1] –103, [3] – 76, [4] –152, [5]- 133, [6] – 118, [7] – 65; *- patients group that after 6 and/or 12-month follow-up were diagnosed with rheumatoid arthritis, spondyloarthropathy or other autoimmune inflammatory diseases. ***p*- significant if <0.05.

6.1.4. The prognostic value of sociodemographic, clinical, laboratory and ultrasound findings in early undifferentiated arthritis outcomes

In the present study it was determined that a greater number of tender and swollen joints, higher DAS 28 and HAQ scores were associated with an increased risk of developing a chronic inflammatory rheumatic disease (RA, PsA, SpA, etc.). Higher ESR, CRP, and RF values, as well as higher synovitis and PD scores seen in the US of affected joints, were also associated with poor UA prognosis and a higher risk of developing a chronic inflammatory rheumatic disease (Table 6).

Table 6. The importance of sociodemographic, clinical, laboratory and ultrasound findings in early undifferentiated arthritis outcomes

Variables	OR [95% CI]	*p* value
Sociodemographic data		
Age, years	1.008 [0.983-1.034]	0.534
Education, years	0.779 [0.646-0.980]	**0.009**
BMI, kg/m^2	0.928 [0.815-1.058]	0.267
Clinical data		
Patient's global VAS, mm	1.006 [0.980-1.033]	0.636
Patient's joint pain VAS, mm	1.,006 [0.981-1.032]	0.633
Physician's global VAS, mm	1.008 [0.977-1.040]	0.601
Duration of joint swelling, months	0.985 [0.808-1.129]	0.944
Duration of joint pain, months	1.039 [0.936-1.153]	0.476
Duration of morning stiffness, min	0.938 [0.850-1.035]	0.203
66 SJC	1.403 [1.081-1.822]	**0.011**
28 SJC	1.667 [1.209-2.299]	**0.022**
68 TJC	1.222 [1.072-1.392]	**0.003**
28 TJC	1.374 [1.129-1.672]	**0,002**
DAS 28 (ESR)	3.503 [1.792-6.848]	**<0.001**
HAQ score	1.492 [0.599-3,715]	**0.039**
Laboratory findings data		
ESR, mm/h	1.028 [1.002-1.055]	**0.037**
CRP, mg/l	1.155 [1.046-1.275]	**0.04**
VEGF, pg/ml	1.000 [0.999-1.002]	0.513

RF, kU/l	1.038 [1.001-1.076]	**0.045**
Anti-CCP, U/ml	1.031[0.997-1.067]	0.075
Ultrasound findings data		
Synovitis score	1.794 [1.234-2.609]	**0.002**
PD score	1.963 [1.264-3.050]	**0.003**
Erosions (grade)	5.808 [0.943-3.769]	0.058

OR, odds ratio; 95% CI, confidence interval; BMI, body mass index; VAS, visual analogue scale; SJC, swollen joints count; TDJ, tender joints count; HAQ, Health Assessment Questionnaire; DAS 28 (ESR), disease activity score 28 using on erythrocyte sedimentation rate; ESR, erythrocyte sedimentation rate; CRP, C - reactive protein; RF, rheumatoid factor; Anti-CCP, anticitrullinated protein antibodies; HLA B27, human leukocyte antigen B27; ANA, antinuclear antibodies; VEGF, vascular endothelial growth factor; PD, Power Doppler; *p*- significant if <0.05.

6.1.5. Relationship between VEGF levels and sociodemographic, clinical, laboratory and ultrasound findings in early undifferentiated arthritis patients, as well as the significance to disease outcomes

A total of 76 patients were enrolled in this part of the study, 51 (6.71%) were females, mean age 43±15.81 years, 13 (17.1%) were daily smokers, mean education – 13.49±2.05 years. BMI of patients was 24.02±3.18 kg/m². As many as 27 subjects (35.5%) reported the history of rheumatic diseases in blood relatives (RA, PsA, SpA, SLE, etc.). At the time of enrolment, median VEGF level was 365.27 [25.75-3438.23] pg/ml.

Correlation of VEGF values with the analysis of tested parameters. The correlation of VEGF with all tested parameters was evaluated. The results of the statistically significant correlation are presented in Table 7.

Table 7. Correlation of VEGF levels with sociodemographic, clinical, laboratory, and ultrasound data in an early undifferentiated arthritis patient cohort

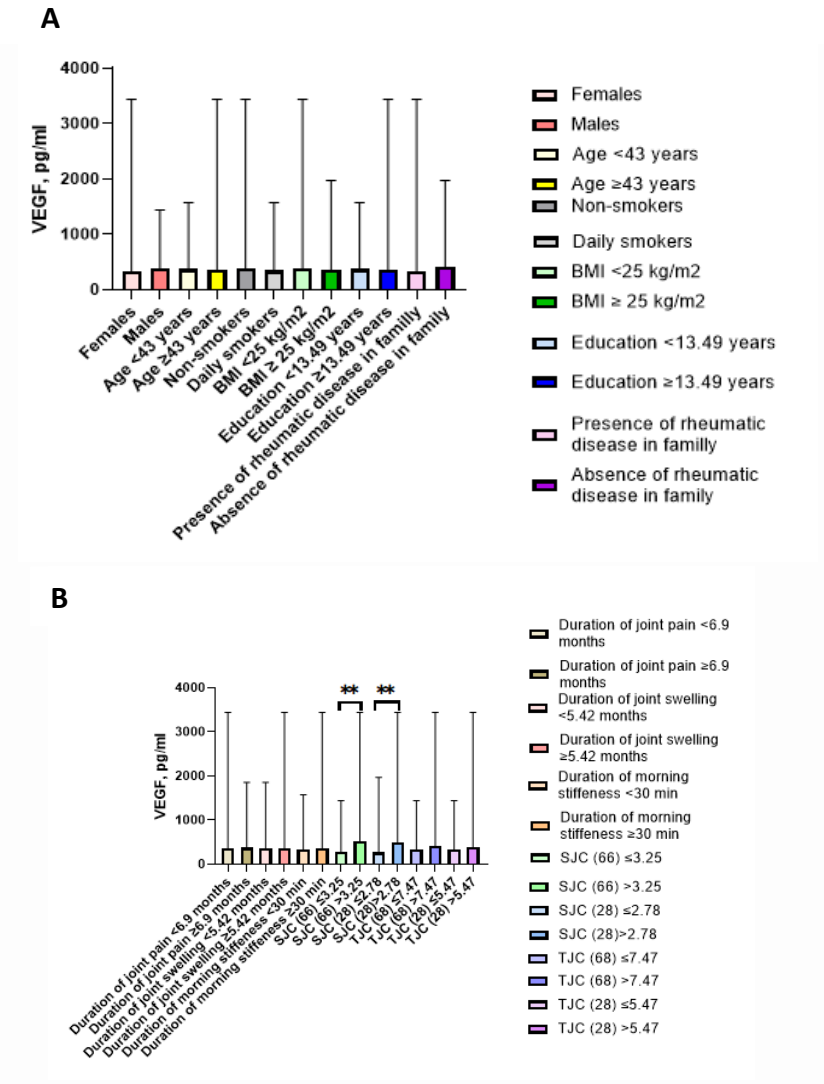
Variables	Correlation coefficient, *r*	*p* value
Clinical data		
66 SJC	**0.428**	**0.006**
28 SJC	**0.375**	**0.001**

Laboratory findings data		
ESR, mm/h	**0.256**	**0.029**
CRP, mg/l	**0.375**	**0.001**
RF, kU/l	**0.263**	**0.022**
Ultrasound findings data		
Synovitis score	**0.332**	**0.003**
PD score	**0.370**	**0.018**
Erosions (grade)	**0.256**	**0.026**

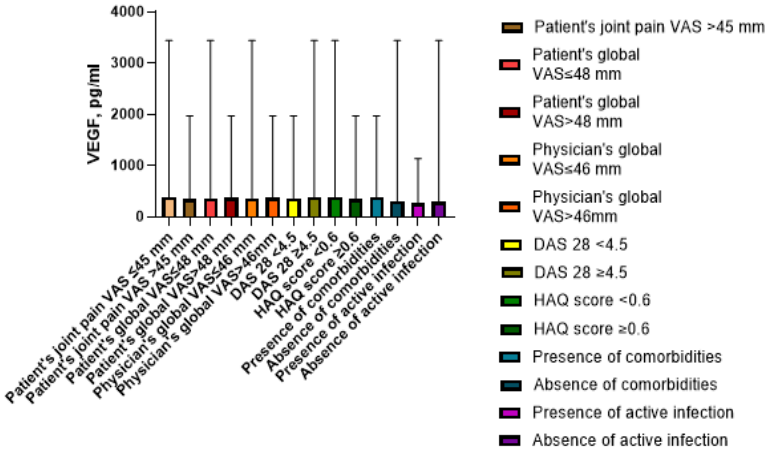
SJC, swollen joints count; ESR, erythrocyte sedimentation rate; CRP, C - reactive protein; RF, rheumatoid factor; PD, Power Doppler; *p*- significant if <0.05.

Distribution of VEGF values among tested groups divided by the median or the average of quantitative parameters and positive/negative values or identified/unidentified features of qualitative parameters. No statistically significant difference was detected between VEGF level distribution and analyzed sociodemographic parameters (Figure 1A). In the patient groups divided by mean values of affected joints, VEGF values were statistically higher in the groups above mean value of 66 SJC (788.75 and 390.34 pg/mL, respectively; $p = 0.005$) and 28 SJC (665.95 and 381.35 pg/mL, respectively; $p = 0.004$). Although no statistically significant differences were confirmed between VEGF values in patient groups divided by mean values in 68 TJC, 28 TJC (Figure 1B), as well as DAS 28 and HAQ (Figure 1C). VEGF value was statistically significantly higher in the RF-positive (708.97 pg/mL) as compared with that in the RF-negative (427.17 pg/mL) patient group ($p = 0.024$) and in patients whose CRP values were ≥ 5mg/l (664.03 pg/mL), compared with those whose CRP values were <5mg/l (375.86 pg/mL) ($p = 0.008$). VEGF value was higher, however, not significantly in anti-CCP-positive patient groups as compared with anti-CCP negative, RF and anti-CCP positive as compared with RF and anti-CCP negative (Figure 1D). Patients with synovitis grade above the mean value had statistically higher levels of VEGF than those that were below (782.16 and 385.34 pg/mL, respectively; $p = 0.007$). Patient groups where PD score was above the mean value also had

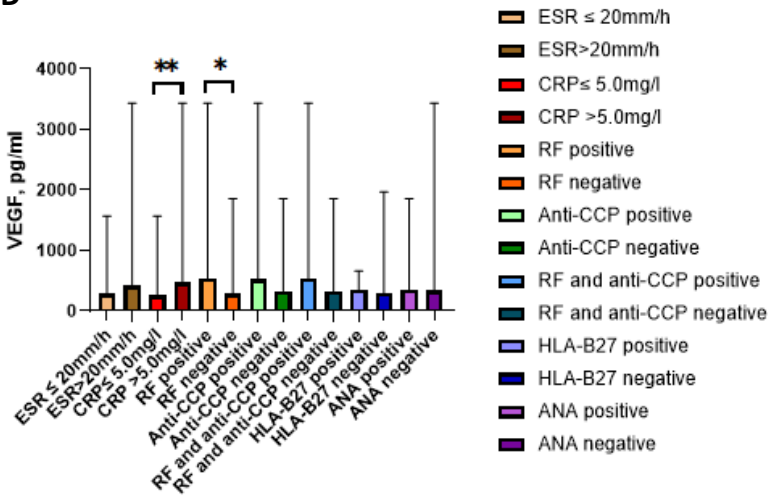
statistically higher VEGF values than those that had a lower PD score (718.21 and 398.29, respectively; $p = 0.042$) (Figure 1E).



C


VEGF, pg/ml
4000
3000
2000
1000
0
Patient's joint pain VAS ≤45 mm
Patient's joint pain VAS >45 mm
Patient's global VAS≤48 mm
Patient's global VAS>48 mm
Physician's global VAS≤46 mm
Physician's global VAS>46mm
DAS 28 <4.5
DAS 28 ≥4.5
HAQ score <0.6
HAQ score ≥0.6
Presence of comorbidities
Absence of comorbidities
Presence of active infection
Absence of active infection

D


VEGF, pg/ml
4000
3000
2000
1000
0
**
*
ESR ≤ 20mm/h
ESR>20mm/h
CRP≤ 5.0mg/l
CRP >5.0mg/l
RF positive
RF negative
Anti-CCP positive
Anti-CCP negative
RF and anti-CCP positive
RF and anti-CCP negative
HLA-B27 positive
HLA-B27 negative
ANA positive
ANA negative

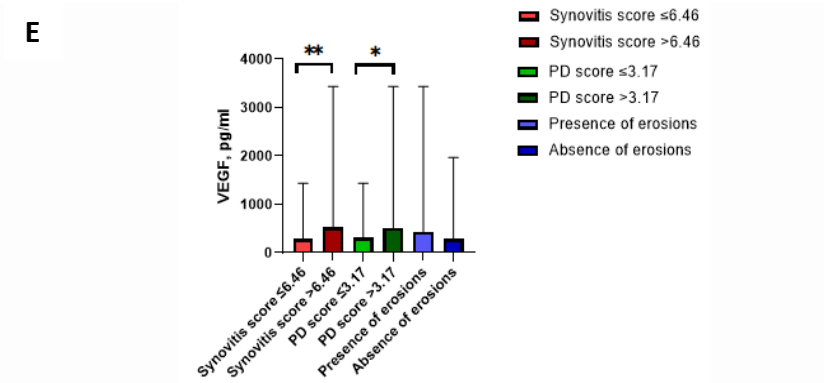


Figure 1. Distribution of VEGF values between sociodemographic parameters (**A**), clinical (**B** and **C**), laboratory (**D**) data, and ultrasound findings (**E**).

BMI, body mass index; VAS, visual analogue scale; SJC, swollen joints count; TDJ, tender joints count; HAQ, Health Assessment Questionnaire; DAS 28 (ESR), disease activity score 28 using on erythrocyte sedimentation rate; ESR, erythrocyte sedimentation rate; CRP, C - reactive protein; RF, rheumatoid factor; Anti-CCP, anticitrullinated protein antibodies; HLA B27, human leukocyte antigen B27; ANA, antinuclear antibodies; VEGF, vascular endothelial growth factor; PD, Power Doppler; * $p<0.05$, ** $p<0.01$, p- significant if <0.05.

Distribution of tested findings based on VEGF median values. Between patient groups divided by VEGF median value, no statistical difference was identified between the analyzed sociodemographic parameters. Clinical data analysis showed that the number of patients with infection was statistically higher in the patient group with VEGF levels lower than the median value (p = 0.046). This study also revealed that patients with higher than median VEGF values had a significantly higher number of swollen joints than the patients in the group with lower than median values: 66 SJC (4.0 ± 2.7 and 2.5 ± 1.37, respectively; p = 0.019), and 28 SJC (3.37 ± 1.85 and 2.18 ± 1.16, respectively; p = 0.016), however, there were no statistical differences between 68 and 28 TJC. Higher than median VEGF values were also in the groups of patients with higher CRP and positive RF and anti-CCP values (p = 0.039, p = 0.014, and p = 0.041, respectively). As to the patient groups divided by the VEGF median value, the grade of

synovitis and the grade of erosions seen in the US were statistically higher in the group with higher than the median VEGF values (p = 0.049 and p=0.018, respectively).

VEGF prognostic value in early undifferentiated arthritis outcomes. After a 12 -month follow-up in this part of study, 23 out of 76 analyzed patients developed RA, 23 - SpA (peripheral or axial SpA, reactive arthritis, PsA), 10 had other autoimmune inflammatory diseases (SLE, undifferentiated connective tissue disease, IgG 4 DR), and arthritis resolved completely in 20 patients. In patients who during the follow-up period developed SpA, VEGF levels were significantly lower as compared with VEGF levels of the patients who later were diagnosed with other inflammatory rheumatic diseases or whose arthritis resolved completely. (p = 0.046) (Table 8) and RA group separately (p = 0.028) (Figure 2). In the RA outcome group as compared with other patients in this study, VEGF levels were higher, although not statistically significant. In this study, no significant difference was confirmed between the patients whose arthritis resolved completely and the remaining study population (Table 8).

Table 8. Distribution of VEGF levels between undifferentiated arthritis patient cohort outcomes in 12 -month follow-up

Diagnosis	Patients with confirmed diagnosis (N)	***p* value	VEGF (pg/ml) in confirmed diagnosis patient's group*	VEGF (pg/ml) in other study population*	*p* value
Rheumatoid arthritis	23	0.108	525.65 [25.75-3438.23]	297.43 [53.97-1854.91]	0.134
Spondyloarthropathies[1]	23		272.3 [58.47-1020.74]	412.77 [25.75-3438.23]	**0.046**
Arthritis resolved	20		350.03 [53.97-1437.35]	372.04 [25.75-3438,23]	0.794

Other autoimmune inflammatory disease[2]	10		481.59 [87.82-1854.91]	353.27 [25.75-3438.23]	0.734

Continuous data are presented in median [min-max]; n - number of patients; *other early undifferentiated arthritis (UA) outcome groups taken all together; [1]spondyloarthropathies: reactive arthritis, axial or peripheral spondylarthritis, psoriatic arthritis; [2]other autoimmune inflammatory diseases: systemic lupus erythematosus, undifferentiated connective tissue disease, IgG 4 related disease; VEGF; vascular endothelial growth factor; ***p* value calculated among all UA outcome groups using the Kurskal-Wallis test; p significant if <0.05.

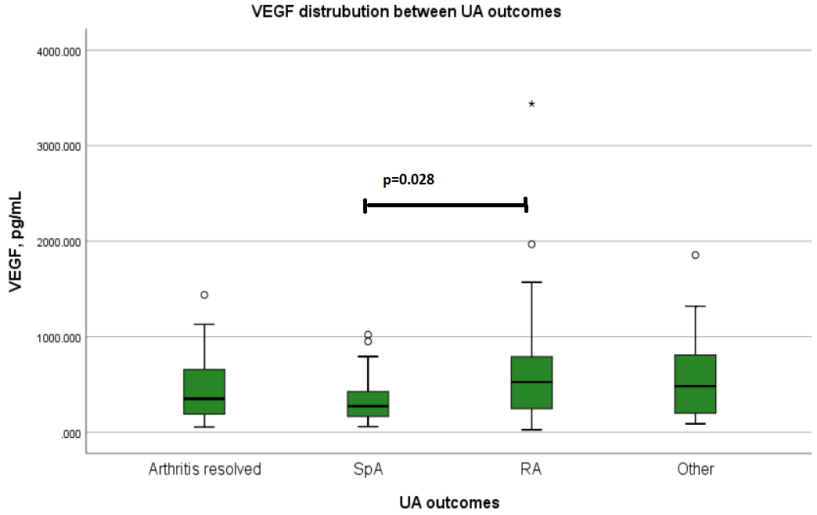


Figure 2. Distribution of VEGF levels detected at the disease onset among early undifferentiated arthritis outcome groups.
SpA, spondyloarthropathies (reactive arthritis, axial or peripheral spondylarthritis, psoriatic arthritis); RA, rheumatoid arthritis; other; other autoimmune inflammatory diseases (systemic lupus erythematosus, undifferentiated connective tissue disease, IgG 4 related disease); UA; undifferentiated arthritis; VEGF; vascular endothelial growth factor.

Between the patient groups divided by VEGF median value at the study entry, there was a significantly greater number of patients who were confirmed with SpA diagnosis after a 12 - month follow-up in the group with VEGF values below the median (Table 9).

Table 9. Association between undifferentiated arthritis study cohort outcomes based on VEGF median levels

Diagnosis	VEGF< 365.27 pg/ml, N=38 (%)	VEGF≥ 365.27 pg/ml, N=38 (%)	*p* value
Rheumatoid arthritis	8 (21.1)	15 (39.5)	0.080
Spondyloarthropathies[1]	16 (42.1)	7 (18.4)	**0.025**
Of them: reactive arthritis	9 (23.7)	3 (7.9)	0.059
Other autoimmune inflammatory diseases[2]	4 (10.5)	6 (15.8)	0.497
Arthritis resolved	10 (26.3)	10 (26.3)	1.000

[1] -spondyloarthropathies: reactive arthritis, axial or peripheral spondylarthritis, psoriatic arthritis; [2] - other autoimmune inflammatory diseases: systemic lupus erythematosus, undifferentiated connective tissue disease, IgG 4 related disease; p significant if <0.05.

Logistic regression showed no statistically significant associations between UA outcomes and VEGF levels (Table 10).

Table 10. Association between early undifferentiated arthritis study cohort outcomes and VEGF level

UA Outcome	OR [95% CI]	*p* value
Rheumatoid arthritis	0.999 [0.998 – 1.000]	0.075
Spondyloarthropathies*	1.001 [0.998 – 1.004]	0.376
Other autoimmune inflammatory diseases	1.000 [0.998 – 1.001]	0.732
Arthritis resolved	1.000 [0.999 – 1.002]	0.513

OR, odds ratio; 95% CI, Confidential interval; UA, undifferentiated arthritis; *spondyloarthropathies: reactive arthritis, axial or peripheral spondylarthritis, psoriatic arthritis; other autoimmune inflammatory diseases: systemic lupus erythematosus, undifferentiated connective tissue disease, IgG 4 related disease, p significant if <0.05.

6.1.6. Analysis of single nucleotide polymorphisms (rs2476601, rs833070, rs6920220) and their importance in early undifferentiated arthritis patients' outcomes

A total of 92 patients with UA were enrolled in this part of the study. After a 12-month follow-up, 27 (29.4%) patients were diagnosed with

RA (RA-group). As many as 65 (70.6%) patients were classified in the non-RA group (patients who were either diagnosed with other chronic rheumatic inflammatory diseases (44 (47.8%)) (SpA, other autoimmune rheumatic diseases) or fully recovered from arthritis (21 (22.8%) after a 12 -month follow-up).

This study did not confirm any significant differences in the genotype distribution of tested polymorphisms between RA and non-RA groups (Table 11).

Table 11. Association between rs2476601, rs833070, rs6920220 polymorphisms and the risk of developing rheumatoid arthritis in early undifferentiated arthritis cohort

SNP	Chr	Position base pair	Minor allele	MAF (%)	n	Odds ratio (OR)	*p* value
rs2476601	1	113834946	A	0.1	92	0.99	0.98
rs833070	6	43774889	G	0.5	92	1.0	0.97
rs6920220	6	137685367	A	0.8	92	0.48	0.13

OR, odds ratio; SNP, single nucleotide polymorphism; Chr; chromosome; MAF, minor allele frequency, n, number of patients; p significant if <0.05.

The analysis of the distribution of the tested SNPs recessive allele (homozygous and heterozygous) and a comparison of its association with clinical, laboratory parameters, and US data, showed that subjects with the rs6920220 *AA+AG* genotype had a statistically significantly lower number of tender joints at the baseline as compared with those with the *GG* genotype (68 and 28 TJC) (Table 12).

Table 12. SNPs (rs2476601, rs833070, rs6920220) genotype association with clinical, laboratory and ultrasound findings in early undifferentiated arthritis cohort

Single nucleotide polymorphism	**rs2476601**		**rs833070**		**rs6920220**	
Variable		***p* value**		***p* value**		***p* value**
Patients' groups divided by genotype, n	*AA+AG/GG* 28/64	-	*GG+GA/AA* 27/65	-	*AA+AG/GG* 30/62	-
66 SJC	2.96±1.88; 2 [1-8]*	0.619	3.00±1.90; 3 [1-8]*	0.485	3.1±2.68; 2 [1-14]*	0.171
	3.27±2.26; 3 [1-14]*		3.25±2.24; 2 [1-14]*		3.21±1.84; 3 [1-12]*	
28 SJC	2.46±1.32; 2 [1-5]*	0.718	2.48±1.34; 2 [1-5]*	0.385	2.53±1.59; 2 [1-8]*	0.262
	2.84±1.72; 2 [0-8]*		2.83±1.71; 2 [0-8]*		2.82±1.62; 2 [0-8]*	
68 TJC	6.61±4.62; 6 [1-17]*	0.368	6.78±4.62; 6 [1-17]*	0.901	5.87±6.25; 4 [1-28]*	**0.019**
	7.64±6.21; 6 [0-28]*		7.55±6.21; 6 [0-28]*		8.03±5.44; 7 [0-24]*	
28 TJC	4.96±3.05; 4 [1-14]*	0.654	5.07±3.05; 5 [1-14]*	0.836	4.37±3.84; 3 [0-18]*	**0.025**
	5.54±4.02; 4 [0-18]*		5.49±4.02; 4 [0-18]*		5.85±3.63; 5 [0-14]*	
ESR, mm/h	35.08 [2-144]	0.754	74.44 [2-144]	0.590	32.07 [2-110]	0.853
	31.51 [2-88]		31.34 [2-88]		32.77 [2-144]	
CRP, mg/l	20.04 [0.20 – 111.50]	0.732	20.62 [0.20-111.50]	0.646	16.90 [0.16-111.50]	0.500
	16.60 [0.16-144.30]		16.42 [0.16-144.30]		18.01 [0.20-144.30]	
RF, kU/l	72.49 [20.00-814.20]	0.637	74.44 [20.00-814.20]	0.417	62.47 [9.59-814.20]	0.674

	50.45 [9.59-306.00]		49.97 [9.59-306.00]		47.13 [20.00-240.30]	
Anti-CCP, U/l	83.10 [2-300]	0.136	84.51 [2-300]	0.387	62.47 [2-300]	0.871
	57.62 [2-300]		57.49 [2-300]		66.24 [2-300]	
	12 (44.4)		13 (46.4)		15 (55.6)	
VEGF, pg/ml	537.69 [25.75-3438.23]	0.563	672.50 [68.67-3438.23]	0.773	540.49 [115.88-3438.23]	0.323
	455.86 [68.67-1854.91]		464.04 [25.75-1854.91]		504.92 [25.75-1967.50]	
Synovitis score	6.29± 4.22; 5 [2-16]*	0.806	6.37±4.28; 5 [2-16]*	0.938	5.87±5.31; 4 [2-25]*	0.215
	6.37±4.77; 5 [2-25]*		6.34±4.74; 4 [2-25]*		6.5±4.22; 5 [2-21]*	
PD score	2.79±2.84; 2 [0-10]*	0.627	2.89±2.85; 2 [0-10]*	0.798	2.70±3.26; 2 [0-12]*	0.164
	3.17±3.13; 3 [0-14]*		3.12±3.14; 2 [0-14]*		3.23±2.94; 3 [0-14]*	
Erosions (grade)	1.18±1.89; 0 [0-6]*	0.722	1.22±1.91; 0 [0-6]*	0.988	1.03±1.90; 0 [0-8]*	0.468
	1.31±2.23; 0 [0-10]*		1.29±2.22; 0 [0-10]*		1.39±2.23; 0 [0-10]*	
Presence of erosions (%)	9 (32.1)	0.803	9 (33.3)	0.643	9 (30.0)	0.336
	25 (39.1)		25 (38.5)		25 (40.3)	

Continuous data are presented and median [minimum and maximum] values; or mean ± standard deviation, counts as numbers and valid percentages; n- number of patients; * the limits are presented to the parametric and nonparametric indices to provide the minimum and maximum and median values of the variable, providing more detailed information on the cohort of the study. SJC, swollen joints count; TDJ, tender joints count; ESR, erythrocyte sedimentation rate; CRP, C - reactive protein; RF, rheumatoid factor; Anti-CCP, anticitrullinated protein antibodies; HLA B27, human leukocyte antigen B27; ANA, anti-nuclear antibodies; VEGF, vascular endothelial growth factor; PD, Power Doppler; *p*- significant if <0.05.

6.2. Differences between TLR*1, TLR2, TLR4, VDR, NLRP1* and *NLRP3* gene expression, as well as MMP-1, MMP-7, MMP-8, MMP-12, MMP-13 and IL-β secretion levels in synovial fibroblast of patients with early inflammatory undifferentiated arthritis, rheumatoid arthritis, osteoarthritis, and control group

6.2.1. Baseline characteristics of the knee synovial fibroblast study patient cohort

A total of 19 patients (7 in RA, 4 in OA, 4 in UA, 4 in CN group) were included in this part of the study. Mean age was 53.1 years (± 11.9), and 14 (73.7%) were females. No differences in age or sex were detected between different pathology patient groups. Ten (52.6%) patients had positive RF values and nine (47%) patients had positive anti-CCP values. All RA patients were RF and anti-CCP positive, UA patients had positive RF and/or anti-CCP values, OA and CN patients were RF and anti-CCP negative. The highest value of CRP ($p < 0.05$) was detected in RA group. In all study cohort (n = 19) patients, RF showed correlation with anti-CCP (r −0.869, $p < 0.001$), CRP (r −0.628, p −0.004), as well as anti-CCP and CRP (r −0.539, $p < 0.017$). There were no differences in VitD levels between the tested patient groups. All RA patients and one UA patient were treated with DMARDs. Basic sociodemographic and clinical data of patient cohort is presented in Table 13.

Table 13. Baseline demographic, laboratory tests and treatment history characteristics

Characteristic	**All patients** N-19	**EA** N-4	**RA** N-7	**OA** N-4	**CN** N-4
Females N (%)	14 (73.7%)	2 (50%)	6 (85.7%)	4(100%)	2 (50%)
Age, years: mean (SD)	53.1 (11.9)	40.5 (7.4)	62.29 (7.3)	60.75(2.87)	42.0(5.1)

Anti – CCP, positive N (%)	9 (47%)	2 (50%)	7(100%)	0	0
Anti- CCP, (CU), median	12.6	70.7*	1610.5*	-	-
Anti - CCP, min-max	4.1 - 2776.8	4.6-199.6	12.6-2776.8	-	-
RF, positive N (%)	10 (52.6%)	3(75%)	7(100%)	0	0
RF, (IU/ml) median	23.2	102.8	110.1	-	-
RF, (IU/ml) min-max	20.0 - 1221.5	20.0 - 597.6	23.2 - 1221.5	-	-
CRB (mg/l), mean (SD)	7.1(7.5)	3.5(3.9)	13.9(7.9)	2.2(3.1)	3.6(3.3)
CRB (mg/l) median	4.8	2.6**	17.1**	0.9**	2.97**
CRB (mg/l), min-max	0.1-22.3	0.63-9.12	1.5-22.3	0.1-6.7	0.98-7.8
VitD (nmol/l):					
Normal result (%)	4 (21.05%)	1 (25.0%)	2 (28.6%)	1 (25%)	0 (0%)
mean (SD)	51.14(29.2)	54.4(30.8)	49.99(37.1)	61.01(29.6)	39.97(15.2)
Ever DMARD treatment N (%))	7(36.8)	1(25)	7(100)	0	0
Ever used TNF-α (N (%))	3(15.8)	0	3(42.9)	0	0
Ever used RTX (N (%))	2(10.5)	0	2(28.6)	0	0
Ever used MTX (N (%))	4(21.1)	1(25)	3(42.9)	0	0
GK	7(36.8)	0	7(100)	0	0

UA, early arthritis; RA, rheumatoid arthritis; OA, osteoarthritis; CN, control group. SD standard deviation. Anti-CCP, anti-cyclic citrullinated peptide; CU, chemiluminescent units; CRP, C-reactive protein; RF, rheumatoid factor; DMARD, disease modifying antirheumatic drugs; TNF-α, tumor necrosis factor alpha; RTX, Rituximab; MTX, Methotrexat; GK, glucocorticosteroid. *$p < 0.05$ (RA compare UA group), ** $p < 0.05$ (RA compared to UA, OA, CN groups). CRP (elevated if >5mg/l), general blood test, anti-CCP (positive if >10 U/ml), RF (positive if >30 U/mL), vitD (range 75-100 nmol/l).

6.2.2. Expression of *NLRP1* and *NLRP3* inflammasomes and *VDR* gene in synovial fibroblasts

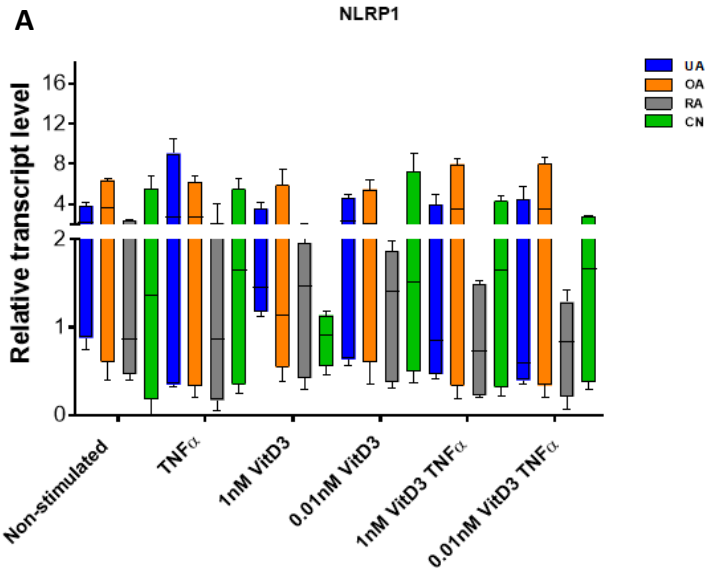
In this study, expression of *NLRP1*, *NLRP3* and *VDR* genes was detected in SFs of all the patients enrolled (Table 14), and expression levels in non-stimulated cells were similar in all tested pathology groups (Figure 3). Stimulation with TNF-α 100ng/ml and/or 0,01 or 1 nM of vitD3 had no effect on the expression of *NLRP1* and *VDR* in either the whole cohort or in either group separately. After stimulation with TNF-α or TNF-α and 0,01 nM vitD3, the expression of the *NLRP3* increased in the whole study cohort.

Table 14. Effects of stimulation on the expression of *NLRP1*, *NLRP3* inflammasomes, *VDR* genes in synovial fibroblast

Analyzed gene	**Stimulation**	**Relative transcript level** Median [range]
NLRP1	non - stimulated	1.23 [0.01-6.76]
	TNFα 100 ng/ml	1.04 [0.05-10.43]
	1 nM vitD3	1.17 [0.29-7.47]
	0.01 nM vitD3	1.55 [0.31-9.03]
	1 nM vitD3 TNFα 100 ng/ml	0.87 [0.18-8.57]
	0.01 nM vitD3 TNFα 100 ng/ml	0.7 [0.06-8.7]
NLRP3	non - stimulated	0.08 [0.01-0.96]*
	TNFα 100 ng/ml	0.13 [0.01-5.01]*
	1 nM vitD3	0.08 [0.01-0.76]
	0.01 nM vitD3	0.09 [0.01-0.66]
	1 nM vitD3 TNFα 100 ng/ml	0.09 [0.01-5.95]
	0.01 nM vitD3 TNFα 100ng/ml	0.11 [0.02-3.42]*
VDR	non - stimulated	21.86 [1.77-58.76]
	TNFα 100 ng/ml	26.17 [0.74-107.66]
	1 nM vitD3	24.32 [3.61-56.33]
	0.01 nM vitD3	25.9 [3.46-60.66]
	1 nM vitD3 TNFα 100 ng/ml	29.4 [3.3-89.67]
	0.01 nM vitD3 TNFα 100 ng/ml	29.47 [0.82-72.63]

NLRP, NOD-like receptor family, pyrin domain containing; VDR, vitamin D receptor; TNF α, tumor necrosis factor alpha; vitD3, 1α,25-Dihydroxy vitamin D3. Nonparametric related samples Wilcoxon signed-rank test: * p value <0.05 (compare non-stimulated and stimulated related samples).

Under stimulation with TNF-α alone, expression of the *NLRP3* gene tended to increase in all patient groups, with the exception of RA. Consequently, stimulation with TNF-a resulted in a significantly higher expression of *NLRP3* in CN, OA, and UA patient groups, as compared with that in the RA group. In the presence of vitD3, stimulation with TNF-a resulted in similar although somewhat less-expressed differences between the patient groups. VDR was similarly expressed in all the groups in this study.



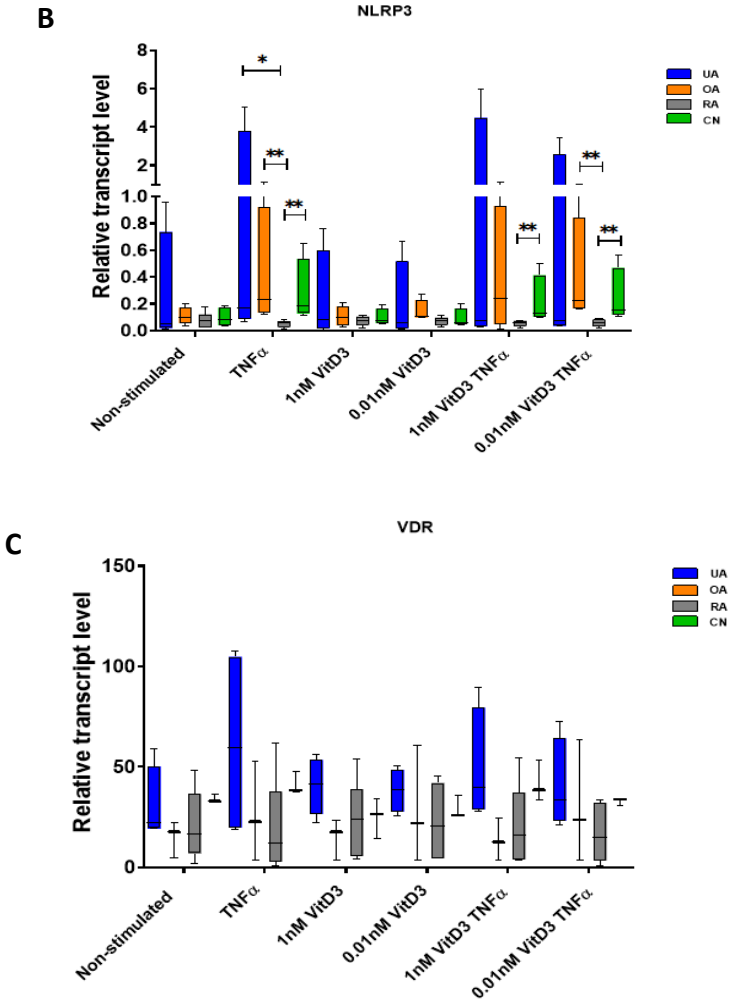


Figure 3. Relative NOD-like receptor family, pyrin domain containing (NLRP)1 (**A**) and NLRP3 (**B**) inflammasome and vitamin D receptor (VDR) (**C**) gene expression levels.

UA - early undifferentiated arthritis, OA - osteoarthritis, RA - rheumatoid arthritis, CN – control group; TNF-α tumor necrosis factor α, VitD3 - 1a,25-dihydroxy vitamin D3. The box length represents the interquartile range with a median. The whiskers represent the minimum and maximum data values. *$p < 0.05$; **$p < 0.01$.

6.2.3. Expression of *TLR1*, *TLR2*, and *TLR4* genes in synovial fibroblasts

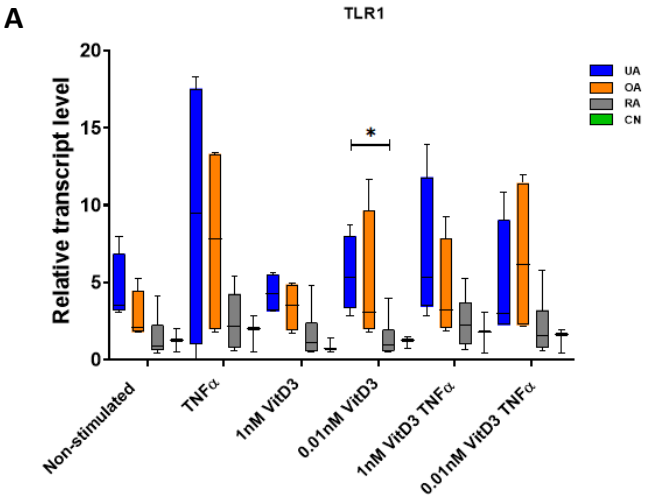
Expression of *TLR1*, *TLR2* and *TLR4* genes was detected in SFs of all enrolled patients. After stimulation with TNF-a alone or in combination with vitD3 (at doses 0.01 or 1 nM) an increase in *TLR1* and *TLR2* and a decrease in *TLR4* gene expression in related samples in all study cohort was detected ($p < 0.05$). Stimulation with 0.01 or 1 nM vitD3 alone had no significant effect on *TLR1*, *TLR2*, and *TLR4* gene expression, however, the related sample analysis revealed that 0.01 nM vitD3 decreased the effect of TNF- α on *TLR1* and *TLR4* gene expression ($p < 0.05$) (Table 15).

Table 15. Effects of stimulation on the expression of *TLR1*, *TLR2*, *TLR4* genes in synovial fibroblast cultures

Analyzed gene	**Stimulation**	**Relative transcript level** Median [range]
TLR1	non-stimulated	1.87 [0.41-7.97]*
	TNFα 100 ng/ml	2.85 [0.08-18.32]*‡
	1 nM vitD3	1.73 [0.49-5.60]
	0.01 nM vitD3	1.79 [0.5-11.65]
	1 nM vitD3 TNFα 100 ng/ml	2.86 [0.46-13.91]*
	0.01 nM vitD3 TNFα 100 ng/ml	2.23 [0.43-11.99]*‡
TLR2	non-stimulated	0.03 [0.003 -3.00]*
	TNFα 100 ng/ml	1.64 [0.32-13.54]*
	1 nM vitD3	0.03 [0.01-4.99]
	0.01 nM vitD3	0.01 [0.01-6.49]
	1 nM vitD3 TNFα 100 ng/ml	1.47 [0.02-13.30]*
	0.01 nM vitD3 TNFα 100 ng/ml	1.55 [0.3-8.81]*
TLR4	non-stimulated	11.52 [2.67-272.82]*
	TNFα 100 ng/ml	5.84 [0.43-156.4]*‡
	1 nM vitD3	11.7 [1.68-130.88]
	0.01 nM vitD3	15.45 [1.69-110.73]
	1 nM vitD3 TNFα 100 ng/ml	5.46 [1.5-155.41]*
	0.01 nM vitD3 TNFα 100 ng/ml	5.12 [0.47-142.23]*‡

TLR, Toll-like receptor; TNF α, tumor necrosis factor alpha; vitD3, 1α,25-Dihydroxy vitamin D3; Nonparametric Wilcoxon signed-rank test: * - p value <0.05 (compare non-stimulated and stimulated related samples); ‡ - p value<0.05 (compare stimulated TNFα 100 ng/ml and stimulated TNFα 100 ng/ml with 0.001 nMvitD3 related samples).

Without any stimulation, the expression of *TLR2* gene was significantly higher in the RA, as compared with that in UA patient group, while the expression of *TLR4* was lower in RA group, as compared with that in UA and OA groups (3.9- and 3.5-fold respectively). Differences between OA and RA groups were also observed in the presence of 1 nM vitD3 stimulation. Similar results were obtained for *TLR1* gene expression; however, differences between the groups did not reach the level of statistical significance, except for UA and RA groups under stimulation with vitD3. Cell stimulation with TNF-α resulted in significant upregulation of *TLR2* gene expression in UA, OA, and RA groups. Similar effects were observed in both, in the absence or presence of TNF-α with vitD3 stimulation (Figure 4).



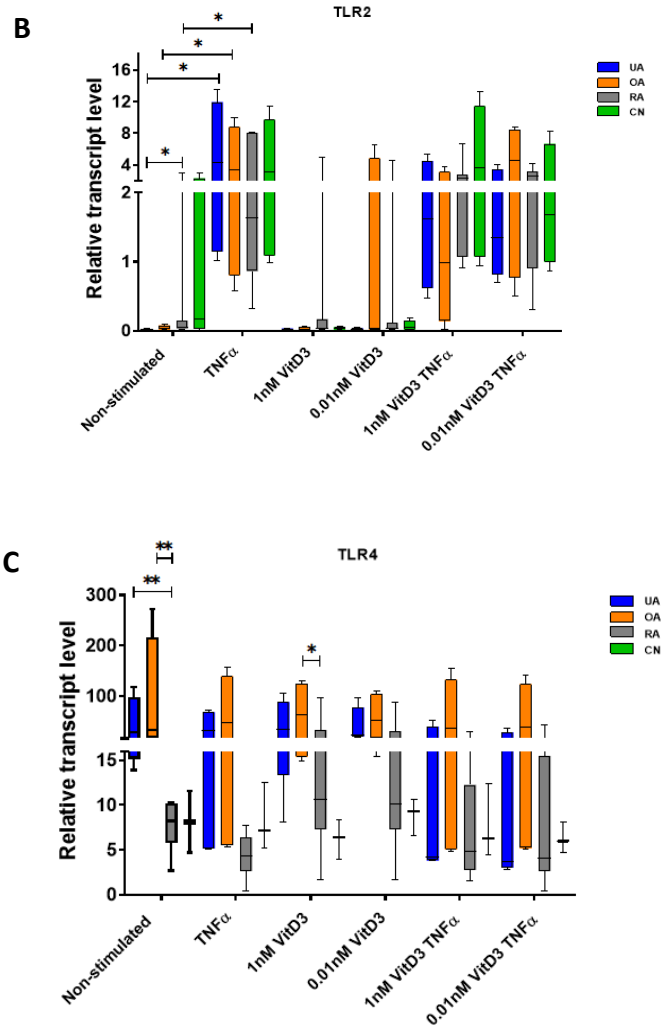


Figure 4. Relative gene expression levels of Toll-like receptor (TLR)1 (**A**), TLR2 (**B**), and TLR4 (**C**).

UA - early undifferentiated arthritis, OA - osteoarthritis, RA - rheumatoid arthritis, CN – control group; TNF-α tumor necrosis factor α, VitD3 - 1a,25-dihydroxy vitamin D3. The box length represents the interquartile range with a median. The whiskers represent the minimum and maximum data values. *p < 0.05; **p < 0.01.

6.2.4. Secretion of MMPs and IL-1b by synovial fibroblasts

Secretion of MMP-1, MMP-7, MMP-8, MMP-12, and MMP-13 was analyzed in SFs without and after stimulation and related sample analysis was carried out. After stimulation with TNF-α, secretion of all MMPs increased significantly. A similar effect was observed after stimulation with TNF-α and 1 nM vitD3, while stimulation with 1 nM vitD3 alone had no effect on the level of MMPs. Difference between stimulation with TNF-α alone or TNF-α in combination with 1 nM vitD3 MMP secretion was not significant in the related sample analysis (Table 16).

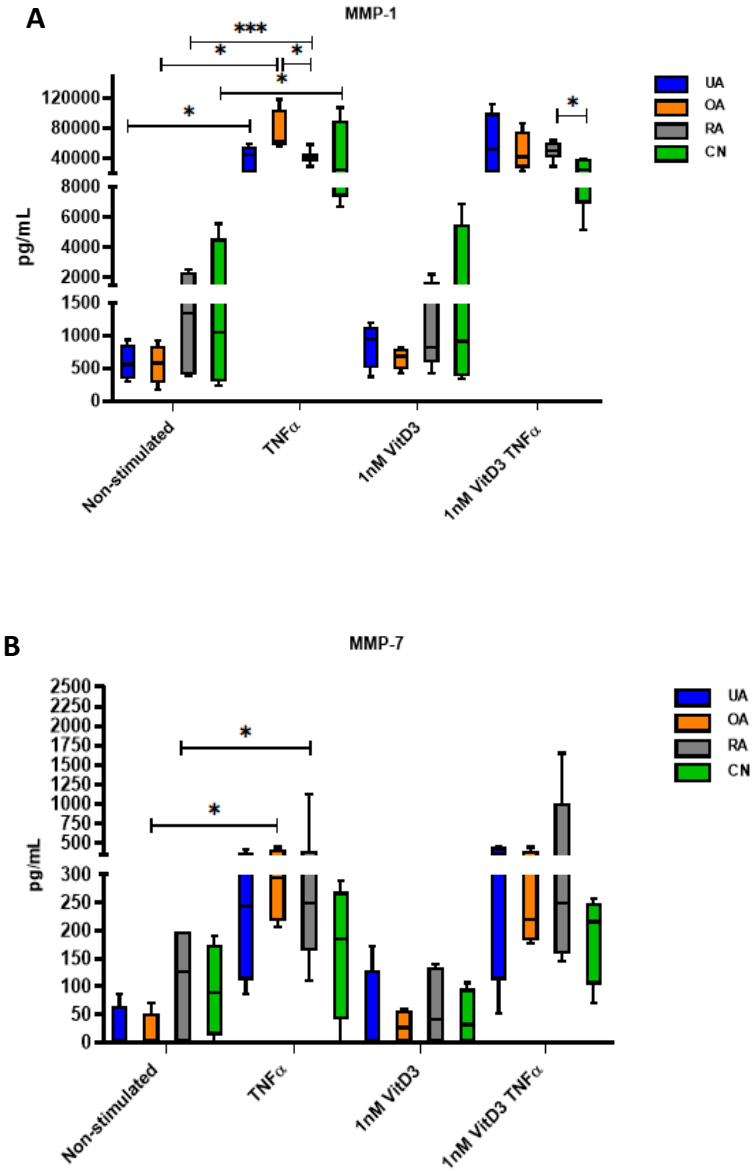
Table 16. Levels of MMP-1, MMP-7, MMP-8, MMP-12, MMP-13 secretion in supernatants of synovial fibroblast

MMP	Stimulation	Median [range]; pg/ml
MMP-1	non-stimulated	586.18 [177.32-5567.35]***
	TNFα 100 ng/ml	43632.13 [6666.88-118020.06]***
	1 nM vitD3	815.26 [347.74-6876.54]
	1 nM vitD3 TNFα 100 ng/ml	41103.14 [5184.93-112105.89]***
MMP-7	non-stimulated	0.00 [0.00 – 198.92]***
	TNFα 100 ng/ml	245.55 [0.00 – 1126.93]***
	1 nM vitD3	0.00 [0.00 – 172.45]
	1 nM vitD3 TNFα 100 ng/ml	241.4 [52.87 – 1649.88]***
MMP-8	non-stimulated	58.68 [0.00 – 303.18]***
	TNFα 100 ng/ml	906.86 [275.33 – 1605.19]***
	1 nM vitD3	58.68 [0.00 – 343.53]
	1 nM vitD3 TNFα 100 ng/ml	821.58 [296.30 – 2082.79]***
MMP-12	non-stimulated	162.73 [0.00-1842.93]**
	TNFα 100 ng/ml	743.13 [302.63 – 2078.4]**
	1 nM vitD3	0.00 [0.00 – 675.1]
	1 nM vitD3 TNFα 100 ng/ml	847.49 [302.63 – 2689.64]**
MMP-13	non-stimulated	33.08 [0.00-993.27]***
	TNFα 100 ng/ml	328.7 [54.31-2173.26]***
	1 nM vitD3	28.1 [0.00-722.72]

	1 nM vitD3 TNFα 100 ng/ml	292.51 [22.64-3217.84]***

MMP, matrix metalloproteinase; TNF-a, tumor necrosis factor alpha; vitD3, 1a,25-dihydroxy vitamin D3. Nonparametric Wilcoxon signed-rank test: *p-value < 0.05; **p-values < 0.01; ***p < 0.001 (comparison of MMP secretion levels (Luminex technology) in 72 h non-stimulated and stimulated related samples)

After analyzing secretion of MMPs without and under stimulation with TNF- α, 1 nM vitD3, and TNF-α with 1 nM vitD3 (Figure 5), no differences in patient groups in all MMPs secretions in unstimulated SFs, were confirmed. Stimulation with 1 nM vitD3 alone had no impact on the expression of all tested MMPs in different pathology SFs. Under stimulation with TNF-α, the levels of MMP-1 were significantly increased in all tested groups (UA, OA, RA, CN). MMP-1 secretion was higher in SFs of the OA group, as compared with that in RA ($p < 0.05$). Levels of MMP-7 were significantly upregulated in OA ($p < 0.05$) and RA ($p < 0.05$) groups, and levels of MMP-8 and MMP-12 were upregulated in UA, OA, and RA groups ($p < 0.05$). Under stimulation with TNF- α, the secretion of MMP-13 increased in all groups, although the increase was statistically significant only in UA and OA groups. Furthermore, under stimulation with TNF- α, the levels of MMP-13 were statistically higher in RA group, as compared with those in CN group. Stimulation with 1 nM vitD3 alone had no effect on the levels of MMPs in SFs of different pathology patient groups. Combined stimulation with 1 nM vitD3 and TNF- α had relatively similar effects on the expression of MMPs to those of stimulation with TNF-α alone.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1nM VitD3 TNFα
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MMP-7
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1750
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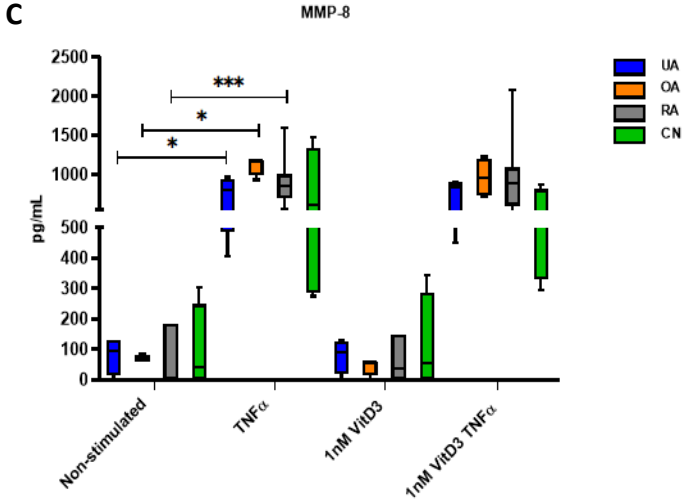
MMP-8
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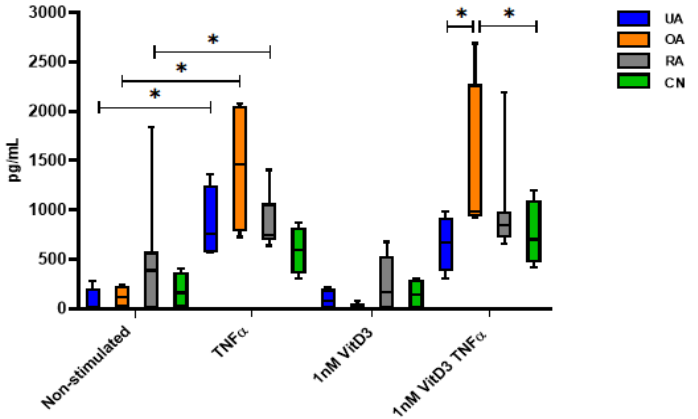
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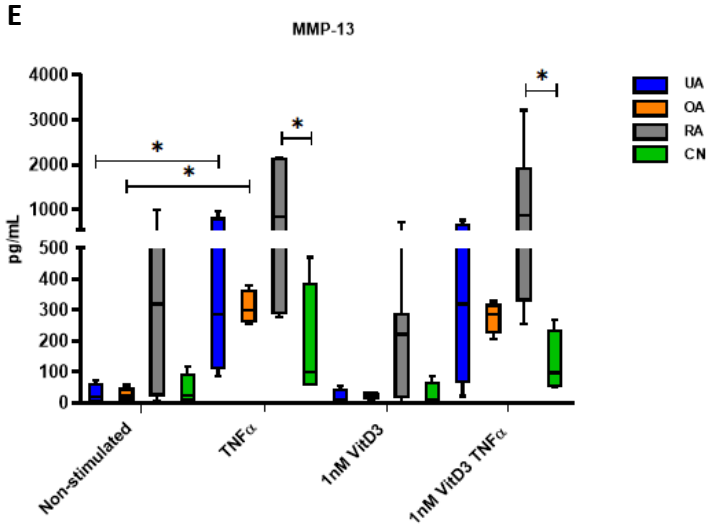

Figure 5. Secretion of matrix metalloproteinases (MMP)-1 (**A**), MMP-7 (**B**), MMP-8 (**C**), MMP-12 (**D**), and MMP-13 (**E**), determined by Luminex technology in culture supernatants of synovial fibroblasts. UA - early undifferentiated arthritis, OA - osteoarthritis, RA - rheumatoid arthritis, CN – control group; TNF-α tumor necrosis factor α, VitD3 - 1a,25-dihydroxy vitamin D3. The box length represents the interquartile range with a median. The whiskers represent the minimum and maximum data values. *$p < 0.05$; ***$p < 0.001$.

Secretion of IL-1β was also analyzed, although no traces of IL-1β were detected in supernatants of either tested group even after stimulation with TNF- α, even if the ELISA test was chosen with sensitivity as low as 1 pg/ml.

6.2.5. Correlation analysis between characteristics of synovial fibroblasts and patient age, serum levels of CRP, RF, anti-CCP, vitD

Correlations between *NLRP1*, *NLRP3*, *TLR1*, *TLR3*, *TLR4* and *VDR* gene expression levels, MMP-1, MMP-7, MMP-8, and MMP-12 secretion levels in non-stimulated and stimulated with TNF-α SF

samples, patient age, serum levels of CRP, RF, anti-CCP, vitD were analyzed in the whole study cohort. Confirmed significant correlations are presented in Table 17. Correlations between serum levels of CRP, anti-CCP, RF, and vitD are described in Section 5.2.1. In statistical analysis, patient age had negative correlations with *VDR* and *NLRP3* gene expression levels in TNF-α stimulated samples (Table 17).

NLRP1 gene expression in the non-stimulated and stimulated with TNF-α SF samples correlate with the expression of *TLR1*, *TLR2*, *TLR4*, and *VDR* in TNF-α in stimulated and TLR4 non-stimulated with TNF-α SF samples (Table 17).

NLRP3 gene expression levels correlate with serum anti-CCP and RF levels in non-stimulated samples, whereas TNF-α stimulated SF samples correlate with serum anti-CCP and CRP levels. After stimulation with TNF-α, *NLRP3* gene expression levels correlate with gene expression levels in *TLR4* in non-stimulated and stimulated with TNF-α and *VDR* in stimulated with TNF-a SF samples (Table 17).

VDR and *TLR4* gene expression levels correlate in non-stimulated SF samples. *VDR* gene expression levels in stimulated with TNF-α SF samples correlate with the *TLR1*, *TLR2*, *TLR 4*, *NLRP1*, and *NLRP3* gene expression levels (Table 17).

In analysis between *TLR* gene expression levels and serum laboratory tests, only *TLR4* gene expression in non-stimulated samples correlates with serum anti-CCP levels. In the correlation analysis between *TLR* gene expression and MMP secretion levels, only one correlation was confirmed between *TLR4* gene expression in non-stimulated and MMP-12 stimulated with TNF-α samples. There were also multiple correlations between expressions of different *TLRs* (Table 17).
MMP-13 stimulated with TNF-α had a weak correlation with *TLR4* in non-stimulated SFs. In the correlation analysis between MMPs secretion levels and serum laboratory tests, only MMP-13 secretion in non-stimulated SF samples correlated with serum CRP levels (Table 17).

Table 17. Analysis of correlation between *TLR1, TLR2, TLR4, NLRP1, NLRP3, VDR* gene expression and MMP1, MMP7, MMP8, MMP12, MMP13 secretion levels in synovial fibroblasts and patient age, serum levels of CRP, RF, anti-CCP, and vitD in whole patient cohort

	MMP-12 TNFα	MMP-13 NS	MMP-13 TNFα	*** Other MMPs	*VDR* TNFα	*VDR* NS	*TLR4* TNFα	*TLR4* NS	*TLR2* TNFα	*TLR2* NS	*TLR1* TNFα	*TLR1* NS	*NLRP3* TNFα	*NLRP3* NS	*NLRP1* TNFα	*NLRP1* NS
Age					-0,594*								-0,516*			
Anti-CCP								-0,583*					-0,746**	-0,498*		
RF														0,518*		
VitD																
CRP		0,472*	0,644*										-0,606**			
***NLRP1* NS**					0,593*		0,579*	0,615*	0,647**		0,594*				0,911**	
***NLRP1* TNFα**					0,689**		0,703**	0,571*	0,723**		0,618*					
***NLRP3* NS**													0,482*			
***NLRP3* TNFα**					0,561*		0,703**	0,609*								
***TLR1* NS**								0,752**								
***TLR1* TNFα**					0,807**		0,600*	0,733**	0,662**							
***TLR2* NS**								0,525*								
***TLR2* TNFα**					0,774**		0,532*									
***TLR4* NS**	0,487*				0,632*	0,647**	0,637**									
***TLR4* TNFα**					0,739**											
***VDR* NS**					0,644**											
***VDR* TNFα**																

[a] Only statistically significant results are presented after whole study cohort (19 patients) data correlation analysis. Anti-CCP, anti-cyclic citrullinated peptides; RF, rheumatoid factor; VitD, vitamin D; CRP, C-reactive protein; NS, non-stimulated; TNF-a, after stimulation with 100 ng/ml TNF-α; NLRP, NOD-like receptor family pyrin domain containing; TLR, Toll-like receptor; VDR, vitamin D receptor; MMP, metalloproteinases. *$p \leq 0.05$; **$p \leq 0.01$; ***Other MMP (MMP-1, MMP-7, MMP-8) did not correlate with TLRs and NLRPs.

7. CONCLUSIONS

1. After a 12-month follow-up period, as many as 50 (32.3%) out of 155 enrolled UA patients, were diagnosed with RA, 33 (21.3%) of them - with SpA (reactive arthritis, axial or peripheral SpA, PsA), 12 (7.7%) - with another inflammatory rheumatic disorder (SLE, undifferentiated connective tissue disease, IgG4 RD), 28 (18.1%) had made a complete recovery, and the outcome was unknown in 32 (20.6%) UA patients due their withdrawal from the present study.
2. After a 12-month follow-up, patients with longer morning stiffness at baseline, a greater number of swollen and tender joints, and higher RF, ESR, CRP, DAS 28, US, and power Doppler values were at a higher risk of developing a chronic inflammatory rheumatic disease. Patients with a lower education were also classified as a higher risk group to develop chronic inflammatory conditions.
3. VEGF levels, measured at the study baseline, were noted for two UA outcomes - RA and SpA. Statistically significantly lower VEGF concentrations were detected in the subjects with SpA as compared to those in RA group or the remaining study population.
4. Higher VEGF sera levers were closely associated with poor prognosis markers for RA (positive RF, anti-CCP, increased ESR, CRP values, and a greater number of swollen joints) as well as changes detected in the US (higher grades of synovitis, PD, and erosions).
5. Patients with infection had lower VEGF levels than those without infection. This suggests that this test could be used to differentiate between infectious and non-infectious origins of elevated ESR and CRP values.
6. In this pilot study of patients with early undifferentiated arthritis, the analyzed SNPs were not associated with a higher risk of developing RA. Patients with the SNP rs6920220 *GG*

and *GA* genotypes had a significantly greater number of tender joints at baseline.

7. A significantly higher *TLR4* gene expression in unstimulated SF cells and *NLRP3* gene expression in TNFα-stimulated SF cells detected in UA group as compared to RA group suggests a possible impaired activation pathway of these systems due to a long-lasting inflammation, or, as a consequence of a long-lasting treatment, and it also demonstrates the important role of these receptors at the early stages of etiopathogenesis of inflammatory arthritis. No differences in *NPRPs* and *TLRs* genes expression were detected between UA and OA and CN groups.
8. After SF stimulation with TNFα in combination with VitD3, MMP-12 secretion was statistically lower in UA group as compared with that in OA group suggesting that vitamin D might play a greater protective role in acute short-term inflammation than in chronic low-activity inflammation.

8. LIST OF PUBLICATIONS

Scientific papers published in journals having impact factor (IF) in the Clarivate Analytics Web of Science databases:

1. **Sakalyte R**, Denkovskij J, Bernotiene E, Stropuviene S, Mikulenaite SO, Kvederas G, Porvaneckas N, Tutkus V, Venalis A, Butrimiene. The Expression of Inflammasomes NLRP1 and NLRP3, Toll-Like Receptors, and Vitamin D Receptor in Synovial Fibroblasts From Patients With Different Types of Knee Arthritis. I.Front Immunol. 2022 Jan 19;12:767512. doi: 10.3389/fimmu.2021.767512. eCollection 2021.
2. **Sakalyte R**, Bagdonaite L, Stropuviene S, Naktinyte S, Venalis A. VEGF Profile in Early Undifferentiated Arthritis Cohort. Medicina (Kaunas). 2022 Jun 20;58(6):833. doi: 10.3390/medicina58060833.

Other scientific paper related to doctoral dissertation, but indirectly to the objective if the dissertation and to the objectives of the dissertation and published in the journals having impact factor (IF) in the Clarivate Analytics Web of Science databases:

1. Sillat T, Barreto G, Clarijs P, Soininen A, Ainola M, Pajarinen J, Korhonen M, Konttinen YT, **Sakalyte R**, Hukkanen M, Ylinen P, Nordström DC. Toll-like receptors in human chondrocytes and osteoarthritic cartilage. Acta Orthop. 2013 Dec;84(6):585-92. doi: 10.3109/17453674.2013.854666. Epub 2013 Nov 18.

9. PRESENTATIONS AT CONFERENCES

1. **Šakalytė, Regina**; Sigita Stropuviene, Algirdas Venalis. Management of Early Arthritis in Vilnius University Santaros Klinikos Rheumatology Center (Comparing EULAR 2016 Recommendations). Baltic Rheumatology Conference; Vilnius (Lithuania), 2017 Sep 21-22.
2. **Šakalytė, Regina**; Sigita Stropuviene, Algirdas Venalis. Some Rare Connective Tissue Diseases May Start as Arthritis (Data from Vilnius University Hospital Santaros Klinikos Rheumatology Center). Innovations in the treatment of rare diseases (liet. *Retų ligų gydymo inovacijos*); Vilnius (Lithuania) 2018 Mar 16.
3. **Šakalytė, Regina**; Sigita Stropuviene, Algirdas Venalis, Irena Butrimiene. Delay of Rheumatologist Consultation Leads to Joint Dammage Confirmed by Ultrasound (Data from Vilnius University Hospital Santaros Klinikos Rheumatology Center Early Arthritis Study). Life Science Baltics; Vilnius (Lithuania) 2018 Sep 27-28.
4. **Šakalytė, Regina**; Jaroslav Denkovskij, Eiva Bernotiene, Gabriele Mourad, Giedrius Kvederas, Narunas Porvaneckas, Sigita Stropuviene, Vytautas Tutkus, Algirdas Venalis, Irena Butrimiene. Comparative analysis of TLRs and inflammasomes genes expression in different ethiology arthritis after stimulation with inflammatory stimuli and vitamin D. European League Against Rheumatism, (EULAR); Madrid (Spain) 2019 Jun 12-15.
5. **Šakalytė, Regina**. „Ankstyvo artrito diagnostikos ir gydymo gairės“. Oral presentation at the conference: „ Jaunųjų reumatologų mokyla“; Kėdainiai (Lithuania), 2019 May 18-19.

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2. Novella-Navarro M, Plasencia-Rodriguez C, Nuno L, Balsa A. Risk Factors for Developing Rheumatoid Arthritis in Patients With Undifferentiated Arthritis and Inflammatory Arthralgia. Front Med (Lausanne). 2021;8:668898.
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NOTES

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