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Associations of Rheumatoid Arthritis Etiopathogenesis and Clinical Course with Genetic and Epigenetic Factors

SUMMARY OF DOCTORAL DISSERTATION

Medicine and Health Sciences
Medicine (M 001)

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VILNIUS 2022

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Reumatoidinio artrito etiopatogenezės ir klinikinės eigos sąsajos su genetiniais ir epigenetiniais veiksniais

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Medicina (M 001)

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To my Father

ABBREVIATIONS

ACPA – anti-citrullinated protein antibodies
ACR – American College of Rheumatology
BL – alveolar bone loss
BOP – bleeding on probing
CAL – clinical attachment loss
CpG – Cytosine-phosphate-Guanine
CRP – C-reactive protein
CYP24A1 – Cytochrome P450 Family 24 Subfamily A Member 1
CYP27B1 – Cytochrome P450 Family 27 Subfamily B Member 1
CYP2R1 – Cytochrome P450 Family 2 Subfamily R Member 1
DMARDs – disease modifying anti-rheumatic drugs
DNA – deoxyribonucleic acid
EULAR – European League Against Rheumatism
HAQ – health assessment questionnaire
HLA – human leukocyte antigen
IL – interleukin
PBMC – peripheral blood mononuclear cells
qPCR – Quantitative Polymerase Chain Reaction
PD – periodontitis
PPD – probing pocket depth
RA – rheumatoid arthritis
RAID – rheumatoid arthritis impact of disease
RF – rheumatoid factor
SNP – single nucleotide polymorphisms
TNF-α – tumor necrosis factor α
VDR – vitamin D receptor
VUHSK – Vilnius University Hospital Santaros Klinikos

1. INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory arthritis that mostly affects synovial tissue, less commonly occurs as extra-articular manifestations. Progression of the disease is associated with degraded quality of life, higher disability and mortality rates [1]. The prevalence of RA in Europe and worldwide is about 0.3 to 1.0% of the population. It affects at least twice as many women as men, and the peak incidence is at the age of 50 years [2, 3]. According to the data of the statistical data website of the Lithuanian Institute of Hygiene, the number of RA cases in Lithuania in 2020 was 15249 or 5.46 cases per 1000 of population (https://stat.hi.lt). Although RA is still a disease of unknown etiology, the combination of environmental, genetic, and epigenetic factors are thought to be potential risk factors of disease that cause gradual changes in innate and adaptive immune system leading to autoimmunogenicity, inflammatory response, and development of RA [1, 4].

Special attention was given to vitamin D in autoimmune diseases including RA during past decades. The prevalence of vitamin D deficiency is found worldwide, and in RA is higher than in the general population. Depending on the geographical region, vitamin D deficiency in RA ranges from 30 to 65 % of patients [5]. It is also recognised, that vitamin D deficiency is associated with increased disease activity rates and deteriorating quality of life [5–7]. Therefore, a suboptimal vitamin D level is often named as one of the potential risk factors of RA.

Beyond classical vitamin D effects on calcium homeostasis and bone metabolism, the active form of vitamin D, 1,25 dihydroxyvitamin D (calcitriol), leads to potential anti-inflammatory, immuno-modulatory, anti-proliferative, and even gene expression-modulating properties [8, 9]. Calcitriol contributes to the regulation and differentiation of the immune system cells and indirectly decreases the synthesis of the

inflammatory mediators (i.e. tumor necrosis factor-α (TNF-α), interleukin (IL) class cytokines, etc.), that are the key players in pathogenesis of RA [10]. Vitamin D mediates its function by binding to the vitamin D receptor (VDR) which is detected in various immune cell types, and is also present on chondrocytes and synovial cells in RA affected joints [11]. Therefore, the function of vitamin D has become the subject of research in RA. However, whether vitamin D deficiency is a consequence or the cause of the disease remains to be elucidated and the data of related studies on vitamin D and RA are inconsistent.

It is known that *VDR* gene single nucleotide polymorphisms (SNP) may influence the function as well as serum levels of vitamin D [6]. One of the most commonly analysed *VDR* SNPs in RA are *BsmI*, *ApaI*, *FokI* and *TaqI*. RA susceptibility to *VDR* gene polymorphisms differs by geographical latitude and ethnic subgroup revealing that *FokI* variant potentially increases disease risk among Europeans, while *BsmI* – Africans, whereas *BsmI* and *TaqI* is linked to RA-associated bone loss in Caucasians [12–14]. *VDR* gene polymorphisms association with RA have not been studied yet in the Lithuanian population.

Another mechanism of vitamin D action is related to its epigenetic activity – processes that affect gene expression without altering the deoxyribonucleic acid (DNA) sequence [15]. Vitamin D response can be silenced by DNA methylation of critical genes in the vitamin D signaling system (*CYP2R1*, *CYP24A1*, *CYP27B1*), including the *VDR* gene, and may lead to changes in vitamin D homeostasis [15]. Recently, epigenetic alterations were also named as potential key players in pathogenesis of RA, mostly observed as decreased DNA methylation level in RA affected joints, as well as various immune cell types [16]. The analysis of methylation pattern of vitamin D pathway genes in RA patients have not been performed yet. New data could provide more information about vitamin D deficiency cause in RA and potentially new insights in pathogenesis of the disease.

Recently, special attention has been raised to another risk factor for RA – chronic infection in the oral cavity, which potentially influences the development of RA and the course of the disease through different mechanisms of pathogenesis [17]. Periodontitis (PD) and RA are known to be associated with hyperproduction of TNF-α and IL-6 cytokines, common genetic (e.g. expression of the human leukocyte antigen (HLA)-DRB1 gene allele) and environmental risk factors, like vitamin D deficiency. Vitamin D deficiency is associated with severe PD, gingivitis, alveolar bone loss and increased RA disease activity [18–20]. Current evidence suggests that the incidence of PD is higher in patients with active RA compared to healthy population, furthermore, individuals having PD are more likely to be diagnosed with RA than those without chronic periodontitis [21, 22]. To date, there are few scientific articles that have assessed the effect of vitamin D in RA and PD together, moreover the prevalence of PD amongst Lithuanian RA patients and its association with clinical RA parameters is yet unknown.

Associations between reviewed environmental, genetic, and epigenetic factors with RA disease and their importance to its clinical course were evaluated in this doctoral dissertation.

2. AIM AND OBJECTIVES OF THE STUDY

The aim of the study was to evaluate the associations of environmental, genetic and epigenetic factors (vitamin D level, periodontitis, vitamin D receptor gene polymorphisms, vitamin D pathway genes DNA methylation level) with RA disease activity, clinical course and determine the possible association of *VDR* gene polymorphisms and RA susceptibility.

Objectives:

1. To evaluate vitamin D (25(OH)D) deficiency prevalence and its association with RA disease activity and other clinical parameters**.**
2. To compare the allele and genotype frequency of *VDR* gene polymorphisms (*BsmI*, *FokI*, *ApaI* and *TaqI*) between RA and control groups, to assess the possible association of *VDR* gene polymorphisms and RA susceptibility in studied group of the Lithuanian population and vitamin D level.
3. To analyse the epigenetic changes (DNA methylation level) of the vitamin D pathway genes (*VDR*, *CYP2R1* and *CYP24A1*), to compare the differences between RA and control subjects, and to evaluate the relationship between selected genes methylation status and vitamin D level.
4. To evaluate the prevalence of PD amongst RA patients, to assess PD severity association with RA disease activity and other clinical parameters.

3. RELEVANCE AND NOVELTY OF THE STUDY

The ability to influence the course of RA is important in both socioeconomic and psychological aspects. It is recognised that vitamin D can affect the function of innate and adaptive immune system, whereas its deficiency has been associated with a myriad of illnesses including autoimmune disorders, cardiovascular and infectious diseases [23]. Vitamin D status in health and illness depends on many factors, such as age, body mass, seasonal factors, geographic latitude, ethnic group, sunlight exposure, and use of supplements. A higher incidence of autoimmune diseases, as well as RA, is observed in people living in high latitudes, where higher vitamin D deficiency is also detected [24]. Lower than optimal vitamin D concentrations are associated with worse control of RA clinical manifestations, increased disease activity and impaired quality of life. Notwithstanding the already known and adjustable factors for vitamin D levels, RA patients

still have lower vitamin D concentrations than the general population. This could be due to a complex of innate or acquired changes occurring at the molecular level. Targeted studies of genetic and epigenetic factors in RA are necessary to expand the knowledge currently available in the scientific literature in order to assess the causes of vitamin D deficiency, its role in the course of the disease, and etiopathogenesis of RA.

In this study, for the first time in Lithuania, the distribution of *VDR* gene allele and genotype variants was evaluated in RA cases and healthy controls. The study data revealed differences of genetic diversity, contributed to the available knowledge on the prevalence of genetic polymorphisms in Lithuania and the possible influence on multifactorial vitamin D metabolism. Moreover, none of the previously published scientific articles has evaluated the methylation level of vitamin D signaling pathway genes in RA patients yet, which was also studied for the first time in this doctoral dissertation. Differentially methylated *VDR*, *CYP24A1*, and *CYP2R1* gene promoter regions were identified and correlation analysis of variables was performed. This study supported the connection of the associations between genetic and epigenetic factors with RA clinical parameters, as well as vitamin D level. This doctoral dissertation evaluated the prevalence of potential risk factors – vitamin D deficiency and chronic periodontitis – in RA patients, thus establishing the reciprocity of these factors for the course of the disease, and providing practical clinical benefits for modulating rheumatoid arthritis.

4. MATERIAL AND METHODS

A case-control (for serum vitamin D level, genetic and epigenetic factors assessment) and cross-sectional (for chronic periodontitis evaluation) study design was applied in this research. The study was carried out at Vilnius University Hospital Santaros Klinikos (VUHSK) Rheumatology Center. In cooperation with Vilnius University Life

Sciences Center *VDR* gene polymorphisms and DNA methylation analysis was performed. Vitamin D level was assessed at VUHSK Center for Laboratory Medicine, and oral health examination of RA subjects evaluated in Vilnius University Hospital Žalgiris Clinics. In cooperation with the family doctors of the VUHSK Family Medicine Center the control group subjects were enrolled in the study. This interdisciplinary research was implemented by rheumatologists, geneticists, periodontists, family doctors, and laboratory medicine physicians.

4.1. Study subjects

Totally, 206 RA patients (184 females and 22 males) and 180 age- and sex-matched healthy controls (163 females and 17 males) were enrolled at VUHSK Rheumatology Center after informed consent was obtained according to the permission of Vilnius Regional Biomedical Research Ethics Committee (Approval No. 158200-16-864-379 and 158200-18/5-1037-533). For autoimmune arthritis subjects, RA diagnosis was established by the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2010 rheumatoid arthritis classification criteria at least 1 year prior to inclusion in the study, or by the 1987 ACR classification criteria if diagnosed before the year 2010 [25, 26]. Demographic, anthropometric, and clinical/medical data from all RA patients were collected. All RA patients filled out two provided questionnaires: the health assessment questionnaire (HAQ) and rheumatoid arthritis impact of disease (RAID) score [27, 28]. The disease activity score 28 C-reactive protein (DAS28 CRP) was measured by counting 28 tender and swollen joints, the visual analogue scale (VAS 100 mm), and the C-reactive protein concentration (CRP, mg/L), and then classified as follows: high disease activity (>5.1), moderate disease activity (>3.2 to 5.1), low disease activity (2.6 to 3.2) and remission (<2.6) [29]. The control group was invited to participate in the VUHSK Family Medicine

Center by a family doctor, and if eligible, further referred to the Rheumatology Center for the informed consent form signing and data (demographic, anthropometric) and blood samples collection. All recruited subjects were >18 years old. Subjects with diagnosed cancer (<5 years, except basal cell carcinoma, *Ca in situ*), other autoimmune comorbidities (e.g., diabetes mellitus, etc.), chronic decompensated diseases (cirrhosis of the liver, etc.) and pregnancy were excluded. For the biochemical vitamin D test, genetic (*VDR* gene polymorphisms) and epigenetic (*VDR*, *CYP24A1*, *CYP2R1* DNA methylation) analysis, blood samples from all subjects were collected, coded, and labeled as required.

Ninety-three RA subjects (84 females and 9 males) underwent the periodontal clinical examination and completed an oral-health-related behaviour questionnaire at the Vilnius university Hospital Žalgiris Clinics. The rest part of RA subjects refused to participate in this stage of the research due to personal or social reasons (periodontist visit during their working hours, transportation issues, etc.).

4.2. Vitamin D evaluation

All subjects (RA patients and healthy controls) were enrolled during 2017 to 2020, from late October until the middle of May, according to known seasonal 25(OH)D serum level variations and their possible influence on disease activity and severity scores. Blood samples were collected using BD Vacutainer Serum Separator Tubes (5 mL) (BD Biosciences, NJ, USA) and prepared by standard procedures. The vitamin D serum level evaluation was performed in VUHSK, Center of Laboratory Medicine using chemiluminescent microplate immunoassay (Architect ci8200, Abbott Laboratories, IL, USA), with the ability to detect $25(OH)D_3$ from 98.6 % to 101.1 % and $25(OH)D_2$ from 80.5 % to 84.4 %. Vitamin D concentration was classified as normal (≥75 nmol/L), insufficient (>50–75 nmol/L), or deficient (<50 nmol/L) according to the Endocrine Society clinical practice guideline [30].

4.3. *VDR* gene genotyping analysis

In order to compare the allele and genotype frequency of the *VDR* gene polymorphisms (*BsmI*, *FokI*, *ApaI* and *TaqI*) between RA group and controls genotyping analysis was performed. All blood samples of enrolled participants (206 RA patients and 180 control subjects) were analysed during this research stage.

4.3.1. Cell preparation and DNA isolation

Blood samples for DNA analysis from all subjects were collected using BD Vacutainer® CPT™ mononuclear cell preparation tube sodium citrate vacutainers (8 mL) (BD Biosciences). Peripheral blood mononuclear cells (PBMC) were prepared using the manufacturer's recommendations and standardized procedures: tubes with blood samples were centrifuged (Centrifuge, Heraeus Megafuge 8 Centrifuge, Thermo Scientific (TS) part of Thermo Fisher Scientific (TFS), Wilmington, DE, USA) by stages, and cell washing steps using phosphate-buffered saline (PBS) (Biochrom, Berlin, Germany) were performed as recommended. Samples with prepared PBMC were stored at –70°C. For further genetic analysis, DNA isolation was performed using commercial GeneJET Genomic DNA Purification Kit (TFS) according to the manufacturer‘s recommendations. Isolated DNA concentration and purity were measured by a 260/280 and 260/230 ratio using a NanoDrop® 2000 spectrophotometer (TS, TFS). Each subject DNA sample was placed into two equal LoBind microcentrifuge 1.5 mL tubes (Eppendorf, Hamburg, Germany) for genotyping and DNA methylation analysis, labeled, and stored at –80°C.

4.3.2. Quantitative polymerase chain reaction (qPCR)

Four target *VDR* gene polymorphisms (*TaqI* (rs731236), *BsmI* (rs1544410), *ApaI* (rs7975232) and *FokI* (rs2228570)) were studied

using TaqMan PCR mixture Universal MasterMix II, with Uracil-N-glycosylase (UNG) (Applied Biosystems (AB) part of TFS, Foster, CA, USA) and TaqMan SNP Genotyping Assay (AB, TFS). The extracted DNA samples were normalized by diluting them in to equal concentrations. qPCR was performed according to the manufacturer's recommendations using QuantStudio 5 Real-Time PCR System (AB, TFS, USA). PCR thermal conditions were adjusted according to used TaqMan PCR mixture and were as follows: initial denaturing at 95°C for 10 min; 40 cycles of 95°C for 15 sec (denaturing) and 60°C for 1 min (annealing/extension). To assess test validity three positive controls per assay were used: samples that represent three genotype classes (homozygous major, minor and heterozygous). Genotyping data was visualized using QuantStudio 5 Real-Time PCR System program (version 1.3, AB, TFS, USA).

4.4. *VDR*, *CYP2R1*, *CYP24A1* DNA methylation analysis

Vitamin D signaling pathway genes *VDR*, *CYP2R1* and *CYP24A1* have large Cytosine-phosphate-Guanine (CpG) islands in their promoter regions and therefore can be methylated and epigenetically regulated. Considering that, selected genes were chosen for further DNA methylation analysis. In total, DNA samples of 76 subjects (35 RA patients and 41 age- and vitamin D-matched controls) were analysed.

4.4.1. DNA bisulphate conversion

Each subject DNA sample was bisulphate converted using EZ DNA Methylation™ Kit (Zymo Research, CA,USA) by manufacturer‘s recommendations. During this conversion, unmethylated cytosine is replaced by uracil and methylated cytosine remains unchanged. Converted DNA samples were labelled and stored at –20°C. Primers for selected genes polymerase chain reaction and pyrosequencing were designed using Ensembl data base (https://www.ensembl.org/index.html) and PyroMark Assay Design

Software (version 2.0.1.15, Qiagen, 2008, Germany). PCR and pyrosequencing primers are listed in Table 1. PCR method was used to amplify modified DNA. PCR cycling conditions are shown in Table 2. The success of DNA amplification was assessed by 3 % agarose gel electrophoresis.

Table 1. *VDR*, *CYP24A1* and *CYP2R1* genes PCR and pyrosequencing primers

	Primers sequences	Length, nt	%GC	Amplicon length, bp
***VDR-s*[1]**	GTTGGGTTGTTTT TGTTTGTTAAAAG	26	30.8	131
***VDR-as*[2]**	Biotin-CCTATCCTAAAAC CCCCTTTC	21	47.6	
***VDR*-seq[3]**	TTGTTTTTGTTTGT TAAAAGG	21	23.8	–
***CYP24A1-s*[1]**	AGTGTAAGGAGGT ATTAATGTTTTGA	26	30.8	130
***CYP24A1-as*[2]**	Biotin-AAAAAAACAAAA AAAACCAACTAAT ATAAT	30	13.3	
***CYP24A1*-seq[3]**	GGAGGTATTAATG TTTTGAG	20	35.0	–
***CYP2R1-s*[1]**	GGAAGTTTTGGAG AGTTGAAGAG	23	43.5	222
***CYP2R1-as*[2]**	Biotin-CCTCTCCCTACAC CTAACTCTACTTT CT	28	46.4	
***CYP2R1*-seq[3]**	AGTTGTTGAAGTA GAGG	17	41.2	–

PCR – polymerase chain reaction; nt – nucleotides; bp – base pairs; s[1] – sense primer; as[2] – antisense primer; seq[3] – pyrosequencing primer.

Table 2. PCR cycling conditions

Temperature, °C	Duration	Cycles	Stage
95 °C	15 min	1	Enzyme activation
95 °C	30 s	45	DNA strand separation
According to gene[1]	30 s		Primer annealing
72 °C	30 s		DNA strand extension
72 °C	10 min	1	Synthesis completion
4 °C	∞	1	Stopping/storing the reaction

PCR – polymerase chain reaction;[1] *VDR* – 58 °C; *CYP24A1* – 50 °C; *CYP2R1*– 55 °C.

4.4.2. *VDR*, *CYP24A1* and *CYP2R1* promoter pyrosequencing

Methylation was evaluated in 10 *VDR* gene CpG positions, 7 *CYP24A1* and 6 *CYP2R1* CpG positions by means of pyrosequencing of PCR products and performed using PyroMark Q24 platform (Qiagen, Berlin, Germany) by manufacturer's recommendations. Methylation intensity of each CpG site was generated using PyroMark Q24 Software (version 2.0.6, Qiagen, 2009, Germany) and assessed as percentage.

4.5. Oral examination

A full mouth periodontal examination (probing at six-points and X-ray examination) was performed by a periodontist at the Vilnius University Hospital Žalgiris Clinic in order to assess the presence and severity of periodontal disease in 93 RA subjects. The periodontal status of the RA patient was evaluated using the following variables: bleeding on probing (BOP, %), probing pocket depth (PPD, mm), alveolar bone loss (BL, %), clinical attachment loss (CAL, mm), and

missing teeth. PD diagnosis was approved according to the 2018 American Academy of Periodontology and the European Federation of Periodontology classification of periodontal diseases guidelines [31], and categorized as follows: stage I, initial; stage II, moderate; stage III, severe; stage IV, advanced severe PD. Patients who were not diagnosed PD acted as a periodontally healthy group (RA+PD–) and recorded when the criteria for severe, moderate, or initial disease were not fulfilled, while all others grouped as RA+PD+. Data regarding behavioral habits (e.g., smoking, alcohol consumption, etc.), as well as oral health-related behavior was collected using periodontist questionnaire. Patients, having less than 8 teeth remaining or taking medication that may affect periodontal tissue (calcium channel blockers, etc.) were excluded from the study.

4.6. Statistical analysis

Statistical analysis for the demographic, clinical, and biochemical variables was first performed using descriptive statistics (mean, median, standard deviation, etc.). Parametric (t-test, ANOVA) tests for normally distributed variables and non-parametric (Wilcoxon, Kruskal–Wallis, Mann–Whitney U test) tests for not normally distributed data were applicable for the subjects clinical and biochemical parameters and the association with genotype/allele frequency was applied after the Shapiro–Wilk normality test. For the association of two normally or not normally distributed variables, the Pearson or Spearman rank correlation coefficients were calculated, respectively. *VDR* SNP genotype and allele frequency distribution was determined by the Chi-square test. RA risk and genotype/allele association strength was assessed by computing the odds ratio (OR).
Baseline methylation levels of *VDR*, *CYP24A1* and *CYP2R1* genes were calculated. After that, qualitative and quantitative methylation analysis of listed genes' promoters CpG sites was performed. After Shapiro–Wilk normality test was applied, a parametric paired t-test or

non-parametric Mann–Whitney rank-sum test was performed for quantitative DNA methylation level (methylation intensity) analysis. To assess associations for qualitative DNA methylation level (methylation frequency) the Fisher's exact or Chi-square test was used.

Statistical analysis and data visualization were performed using Microsoft Office Excel 2016 (Microsoft Corporation, Redmond, WA, USA), R (Version 1.1.383, RStudio Inc, Boston, MA, USA), GraphPad Prism (version 7.03, GraphPad Software, San Diego, CA, USA), MedCalc (Version 14.8.1, MedCalc Software Ltd., Ostend, Belgium), Stata/MP 13.0 (StataCorp LP, College Station, TX, USA) programs and ClustVis web tool [32]. Statistically significant results were set at $p < 0.05$.

5. RESULTS

5.1. Study group characteristics

A total of 386 subjects participated in the study from 2017 until 2020 with seasonal intervals, as recorded before. Altogether, researchers enrolled 206 RA patients, comprising 184 females and 22 males, of which 76 participants were using vitamin D supplements and 130 were not, 109 were treated with biological disease modifying anti-rheumatic drugs (bDMARDs), (e.g., TNF-α inhibitors, IL-6 receptor antagonist, anti-CD20 monoclonal antibody), either alone or in combination with conventional synthetic DMARDs (e.g., methotrexate, sulfasalazine, ect.) and 97 were treated with conventional synthetic DMARDs. The mean age of the RA subjects at enrolment was 55.01 ± 11.08 years, and the mean disease duration was 11.71 ± 9.22 years. For comparison analysis, 180 age- and sex-matched healthy controls, comprising 163 females and 17 males with a mean age of 53.15 ± 10.68, were enrolled. Full descriptive subject data analysis is presented in Table 3.

Table 3. Demographical, clinical, and biochemical characteristics of RA and control group

Parameter	RA (n=206)	Controls (n=180)	*p* significance
Gender (Female/Male)	184/22	163/17	0.6879
Age (years)	55.01 ± 11.08	53.15 ± 10.68	0.0975
BMI (kg/m^2)	25.62 ± 4.64	26.46 ± 4.74	0.1005
25(OH)D (nmol/l): Deficiency (n, %) Insufficiency (n, %) Normal range (n, %)	44.96 ± 21.92 127 (62.62) 57 (27.67) 20 (9.71)	54.90 ± 22.82 79 (43.89) 73 (40.55) 28 (15.56)	p<0.0001*
Smoking (n): Yes No	 34 172	 18 162	0.0619
Disease onset (years)	43.30 ± 12.80	–	–
RA duration (years)	11.71 ± 9.22	–	–
DAS28 CRP: High activity Moderate activity Low Remission	4.16 ± 1.46 56 (27.18) 98 (47.57) 19 (9.22) 33 (16.01)	–	–
HAQ	0.95 ± 0.61	–	–
RAID	4.74 ± 2.23	–	–
CRP (mg/l)	12.55 ± 21.7	–	–
VAS (mm)	45.57 ± 20.88	–	–

Values represent the mean ± standard deviation or n,%; RA – rheumatoid arthritis; BMI – body mass index; DAS 28 – disease activity score; CRP – c reactive protein, HAQ – health assessment uestionnaire; RAID – rheumatoid arthritis impact of disease score; VAS – visual analogue scale; * –statistically significant values.

5.2. Vitamin D level and RA clinical associations

We registered 25(OH)D deficiency (<50 nmol/L) in 62.62% of RA patients (n = 127), insufficiency (>50–75 nmol/L) in 27.67% of RA patients (n = 57), and normal concentrations (≥75–125 nmol/L) in only 9.71% (n = 20) of RA patients. The mean serum concentration of 25(OH)D in RA patients was 44.96 ± 21.92 (nmol/L), being significantly lower than in the healthy controls, who had a mean concentration of 54.90 ± 22.82 (nmol/L), with $p<0.0001$ (Figure 1A). Groups subdivided by vitamin D supplementation also revealed significant differences between RA patients and controls, with $p < 0.001$ (Figure 1B). Our data confirmed a statistically significant inverse correlation between the vitamin D level, DAS28 CRP, and HAQ scores in RA patients, respectively (r-0.2614, $p=0.0017$; r-0.1893, $p=0.0065$) (Figure 1C, D). On the contrary, vitamin D levels had no significant correlation with the RAID score. However, patients using vitamin D supplementation had higher vitamin D levels and a lower RA DAS28 CRP score ($p<0.05$), suggesting that higher vitamin D concentration is associated with lower disease activity.

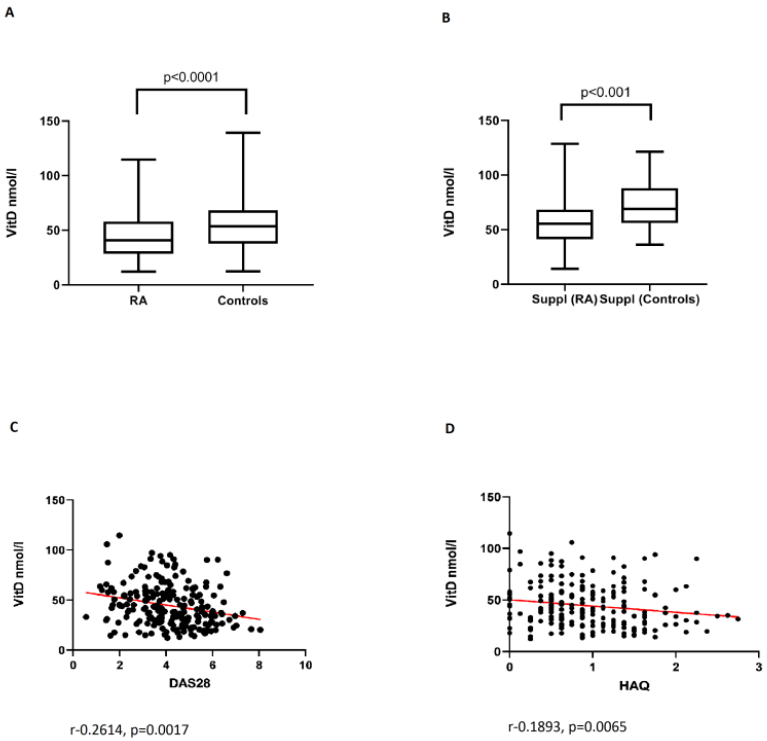


Figure 1. Vitamin D (vitD) level and correlation with clinical parameters of rheumatoid arthritis (RA). (**A**) VitD level in RA and controls; (**B**) VitD level in vitamin D supplemented (Suppl) RA and controls; (**C**) VitD correlation with disease activity score 28 C-reactive protein (DAS28 CRP); (**D**) VitD correlation with health assessment questionnaire (HAQ).

5.3. *VDR* gene polymorphisms analysis

In this study, for the first time in Lithuania, the distribution of *VDR* gene allele and genotype variants was assessed in RA patients and healthy controls. Genotype frequencies of RA patients and healthy controls were tested for compatibility with the Hardy–Weinberg equilibrium, confirming *FokI*, *ApaI*, and *BsmI VDR* loci compatibility, except for *BsmI* in RA subjects (p=0.045). No significant association

between the overall risk of RA disease for any allele or genotype of four *VDR* loci tested was found (Table 4). There was also no significant difference between RA patients and healthy controls regarding the distribution of *BsmI*, *ApaI*, *FokI*, and *TaqI* genotypes and their allele frequencies (p>0.05).

Table 4. *VDR* gene genotype and allele frequency distribution in RA and controls

	Genotype frequency, n (%)				**Allele frequency, n (%)**			**OR (95 % CI)**	**HWE**
ApaI	*AA*	*Aa*	*aa*	$X^2(p)$	*A*	*a*	$X^2(p)$		$X^2(p)$
RA (206)	56 (27)	99 (48)	51 (25)	1.172 (0.557)	211 (51)	201 (49)	0.322 (0.570)	0.983 (0.618–1.564)	0.302 (0.583)
Controls (180)	41 (23)	95 (53)	44 (24)		177 (49)	183 (51)		1.085 (0.818–1.440)	0.561 (0.454)
TaqI	*TT*	*Tt*	*tt*		*T*	*t*			
RA (206)	95 (46)	92 (45)	19 (9)	0.014 (0.993)	282 (68)	130 (32)	0.002 (0.961)	1.041 (0.519–2.092)	0.237 (0.626)
Controls (180)	83 (46)	81 (45)	16 (9)		247 (69)	113 (31)		1.008 (0.743–1.366)	0.360 (0.548)
BsmI	*BB*	*Bb*	*bb*		*B*	*b*			
RA (206)	91 (44)	101 (49)	14 (7)	0.036 (0.982)	283 (69)	129 (31)	0.001 (0.981)	0.937 (0.428–2.049)	4.028 (0.045)
Controls (180)	80 (45)	87 (48)	13 (7)		247 (69)	113 (31)		0.996 (0.735–1.351)	2.685 (0.101)

Table 4 continue

FokI	*FF*	*Ff*	*ff*		*F*	*f*			
RA (206)	43 (21)	110 (53)	53 (26)	0.085 (0.958)	196 (48)	216 (52)	0.045 (0.832)	1.071 (0.675–1.699)	1.023 (0.312)
Controls (180)	38 (21)	98 (55)	44 (24)		174 (48)	186 (52)		1.031 (0.777–1.368)	1.461 (0.227)

HWE – Hardy–Weinberg equilibrium; 95% CI – confidence interval; X^2 – Chi-square; OR – odds ratio; *p* – *p* value; RA – rheumatoid arthritis; VDR – vitamin D receptor.

Vitamin D levels comparing RA patients and healthy controls in reference to four *VDR* polymorphisms were significantly different in both groups ($p<0.005$), except for the *TaqI tt*, *BsmI bb*, and *FokI ff* genotypes (Table 5). Vitamin D concentration in RA patients and controls carrying minor alleles of *TaqI*, *BsmI*, and *FokI* variants did not differ significantly and showed similar concentrations. However, our data showed a strong tendency that the *TaqI tt* genotype had higher vitamin D levels *vs.* the *Tt* genotype ($p=0.0597$) as well as the *BsmI bb vs. Bb* genotype in RA patients ($p=0.1145$).

Table 5. Vitamin D level grouped by *VDR* polymorphism genotypes in RA and controls

Genotype	**RA**	**Controls**	***p* value**
	Vitamin D level (nmol/l, M±SD)		
ApaI			
AA	43.46 ± 23.52	54.89 ± 21.7	0.0034*
Aa	45.3 ± 22.37	53.71 ± 23.05	0.009*
aa	45.87 ± 19.44	57.49 ± 23.64	0.0087*

Table 5 continue

TaqI			
TT	46.2 ± 20.65	56.47 ± 24.3	0.0014*
Tt	42.86 ± 24.29	53.11 ± 21.33	0.0003*
tt	48.88 ± 14.8	55.86 ± 23.02	0.2869
BsmI			
BB	46.62 ± 20.71	56.78 ± 23.66	0.0012*
Bb	43.15 ± 23.97	53.62 ± 22.16	0.0002*
bb	47.16 ± 11.9	51.89 ± 22.83	0.8952
FokI			
FF	42.54 ± 18.62	54.99 ± 24.1	0.0123*
Ff	44.97 ± 23.75	56.12 ± 22.08	<0.0001*
ff	43.4 ± 20.59	46.0 ± 23.62	0.2143

RA – rheumatoid arthritis; VDR – vitamin D receptor; M±SD – mean±standard deviation; * – statistically significant values.

The CRP level (mg/l) in disease-affected subjects was significantly higher in the *ApaI aa* genotype *vs. Aa* (p=0.0049), in *FokI Ff* genotype *vs. ff* (p=0.0162), and in *aa vs. ff* (p=0.0061), suggesting that the *ApaI aa* variant is associated with a higher CRP concentration in RA subjects (Figure 2). However, there was no significant association between the DAS28 CRP, HAQ, or RAID score and any *VDR* gene polymorphism variant.

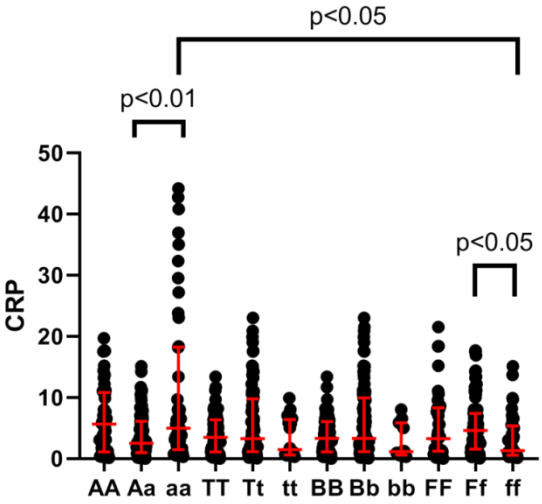


Figure 2. C-reactive protein (CRP) level and vitamin D receptor (*VDR*) gene polymorphisms in rheumatoid arthritis (RA) patients.

5.4. *VDR*, *CYP2R1*, *CYP24A1* gene DNA methylation analysis

The profile of DNA methylation changes in vitamin D pathway genes (*VDR*, *CYP2R1*, *CYP24A1*) in leukocytes of RA and healthy individuals was evaluated in this study. To our knowledge, none of previously published studies has analysed selected genes DNA methylation pattern in this group of patients. Therefore, a group of 35 RA patients and 41 healthy controls were selected for further DNA methylation analysis of vitamin D metabolism pathway genes. Average age of RA subjects' at enrolment into the study was 51.63 ± 10.45 years, mean disease duration 12.40 ± 8.22 years. For comparison analysis, age, sex and vitamin D matched healthy controls with mean age of 50.78 ± 11.85 years were enrolled. In order to precisely assess epigenetic differences in both groups, measured serum concentration of 25(OH)D in RA patients did not differ significantly in comparison with controls (50.89 ± 29.54 *vs.* 56.05 ± 28.67, $p>0.05$).

The mean methylation intensity of analysed genes in RA *vs.* control group was as follows: *VDR* gene 2.39% *vs.* 2.48%, *CYP24A1* gene 16.02% *vs.* 15.17% and *CYP2R1* 2.53% *vs.*2.41%. According to the average methylation intensity of each gene promoter regions, the threshold of methylation level was specified, and methylated vs. unmethylated samples detected to assess the methylation frequency of all analyzed CpG positions (Figure 3A and 3B). Although the differences between the cases and controls were statistically insignificant ($p>0.05$), *CYP24A1* methylation intensity was significantly higher compared to methylation intensity of *VDR* and *CYP2R1* genes promoters in both groups ($p<0.0001$) (Figure 3C and 3D).

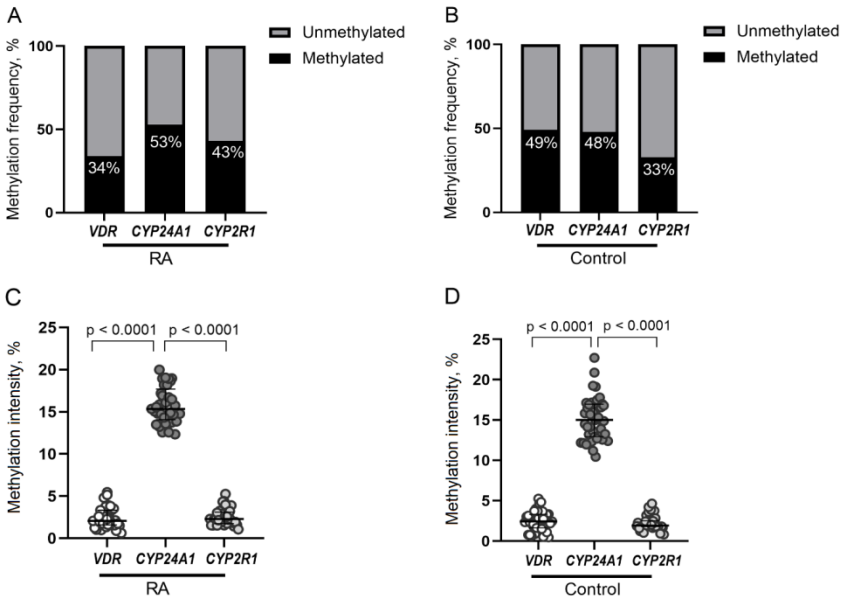
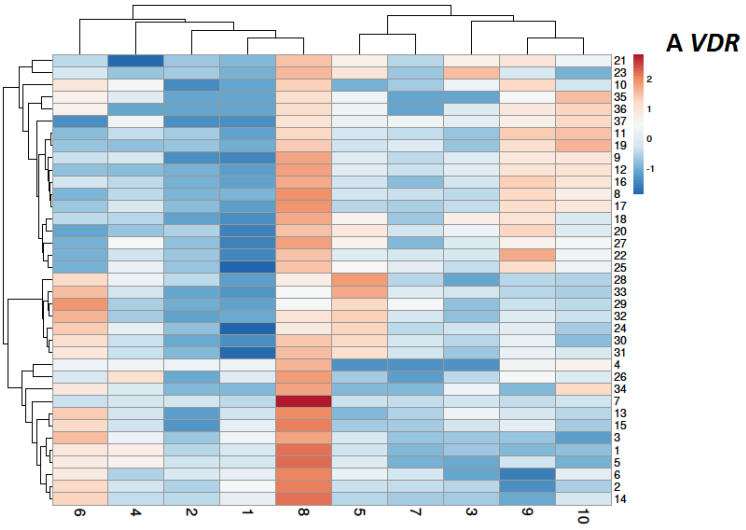


Figure 3. *VDR*, *CYP24A1* and *CYP2R1* promoter methylation frequencies (percentages) in RA (**A**) and healthy controls (**B**). Methylation intensity comparison of *VDR*, *CYPR24A1* and *CYP2R1* genes promoters in RA patients (**C**) and healthy controls (**D**). RA – rheumatoid arthritis.

The study also applied hierarchical clustering heatmap method to evaluate the methylation intensity of selected individual CpG positions located in promoter regions of *VDR*, *CYP24A1* and *CYP2R1* genes (Figure 4A, 4B and 4C). No significant differences between RA and control group were detected ($p>0.05$). However, the comparison of separate CpG positions in RA revealed several significant differences. Significantly higher methylation intensity of *VDR* 8 CpG position compared to 1–4 and 7th positions was detected (1 *vs.* 8 $p<0.0001$; 2 *vs.* 8 $p<0.0001$; 3 *vs.* 8 $p=0.0060$; 4 *vs.* 8 $p=0.0014$; 7 *vs.* 8 $p=0.0032$). Also, the 1st CpG position of *CYP24A1* gene showed higher methylation intensity level in compare to 2–7 positions ($p<0.0001$) and 3rd position in compare to 4–7 CpG positions (3 *vs.* 4 $p<0.0001$; 3 *vs.* 5 $p=0.0005$; 3 *vs.* 6 $p=0.0079$; 3 *vs.* 7 $p=0.0020$). Furthermore, 3rd CpG position of the *CYP2R1*gene was more intensively methylated than 1–5 positions (1 *vs.* 3 $p=0.0019$; 2 *vs.* 3 $p=0.0286$; 3 *vs.* 4 $p=0.0003$; 3 *vs.* 5 $p=0.0035$). Similar results were obtained from the control group analysis as well.



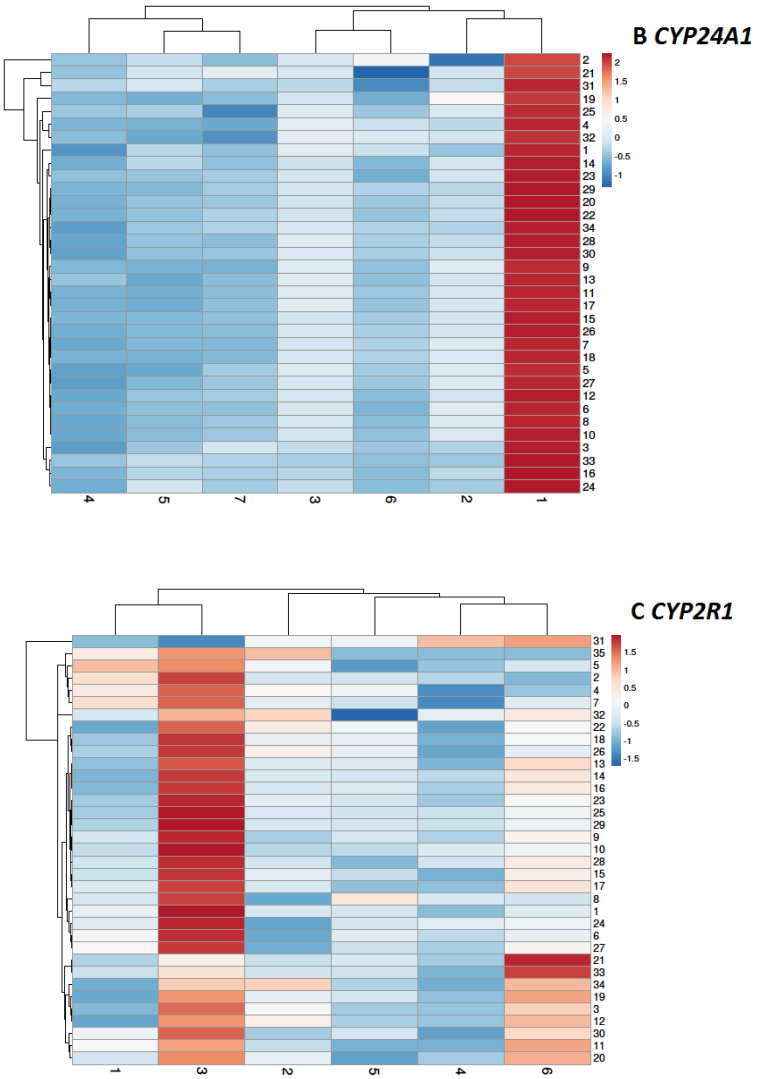


Figure 4. Hierarchical clustering heatmap. CpG methylation intensity comparison of *VDR* **(A)**, *CYP24A1* **(B)**, *CYP2R1* **(C)** promoters separate positions (X-axis) in RA group samples (Y-axis). RA – rheumatoid arthritis. The blue-to-red colour scale corresponds to the lowest and highest methylation intensity values.

Hereafter, associations between selected genes promoters' methylation pattern and demographic features in RA group have been analysed. *CYP24A1* methylation level positively correlated with age in RA patients (r=0.519, p=0.0017). Also, after comparing methylation frequencies, *CYP24A1* gene methylation was found to be significantly more frequent in older RA *vs.* younger participants (p<0.01). However, similar methylation changes were also seen in control group, possibly indicating universal age-related changes (p=0.01). No other significant associations between demographic parameters (i.e. sex, smoking status, etc.) and methylation pattern were discovered.

5.4.1. DNA methylation and RA clinical parameters

The association of DNA methylation and RA disease clinical parameters was evaluated in the study. A tendency of higher RA DAS28 CRP disease activity score in *CYP24A1* methylated *vs.* unmethylated gene promoter RA cases (3.71 ± 0.891 *vs.* 4.60 ± 0.48, p=0.0774) was found (Figure 5A). However, the study found no significant associations between selected genes DNA methylation intensity and DAS28 CRP score in RA group. Nevertheless, a higher RAID score was significantly associated with *VDR* gene promoter methylation status (4.58 ± 1.64 *vs.* 6.22 ± 0.66, p=0.018) (Figure 5B). Also, *VDR*, as well as *CYP2R1* methylated *vs.* unmethylated promoter cases had a higher HAQ score (0.82 ± 0.20 *vs.* 1.05 ± 0.23 and 0.85 ± 0.19 *vs.* 0.97 ± 0.12, respectively), however it was not statistically significant (Figure 5C, 5D). Other RA clinical variables, such as CRP, did not show any significant differences in accordance to gene's methylation level.

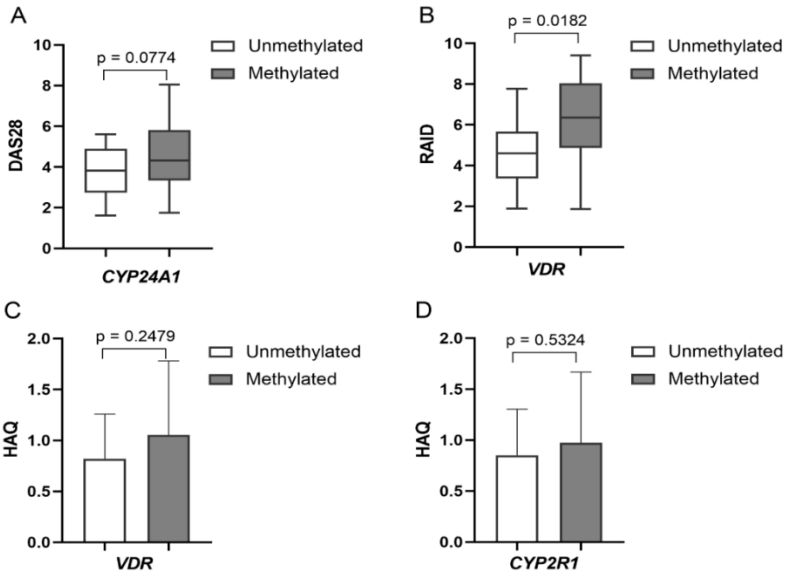


Figure 5. Comparison of cases with methylated *vs.* unmethylated *CYP24A1*, *VDR* and *CYP2R1* genes promoters and rheumatoid arthritis (RA) clinical parameters: disease activity score (DAS28) (**A**), rheumatoid arthritis impact of disease (RAID) score (**B**) and health assessment questionnaire (HAQ) score (**C** and **D**).

5.4.2. DNA methylation and vitamin D level

The study data showed higher vitamin D concentration in RA cases with methylated *vs.* unmethylated *VDR* (57.57 ± 28.93 *vs.* 47.40 ± 29.88 nmol/l), *CYP24A1* (53.23 ± 26.22 *vs.* 48.23 ± 34.41 nmol/l) and *CYP2R1* (60.41 ± 30.73 *vs.* 44.54 ± 27.63 nmol/l) gene promoters, indicating that DNA methylation pattern of aforementioned genes tend to be associated with higher vitamin D level. The differences were not statistically significant (Figure 6A–C), however, methylation intensity analysis of separate CpG positions revealed a significant positive correlation of *VDR* 8th position and vitamin D level (r=0.3485, *p*=0.0345) in RA. Furthermore, methylation intensity of *VDR* and *CYP2R1* genes promoters positively correlated with vitamin D

concentration in RA patients, though not significantly (p>0.05). Vitamin D deficient (<50 nmol/l) RA subjects revealed a significantly higher *CYP24A1* methylation intensity *vs.* vitamin D deficient controls (p=0.0104), thus indicating the disturbed vitamin D metabolism in RA (Figure 6D). Additionally, a higher *CYP2R1* methylation intensity was also revealed in vitamin D deficient, as well as normal vitamin D level RA *vs.* control group, respectively, however not significant.

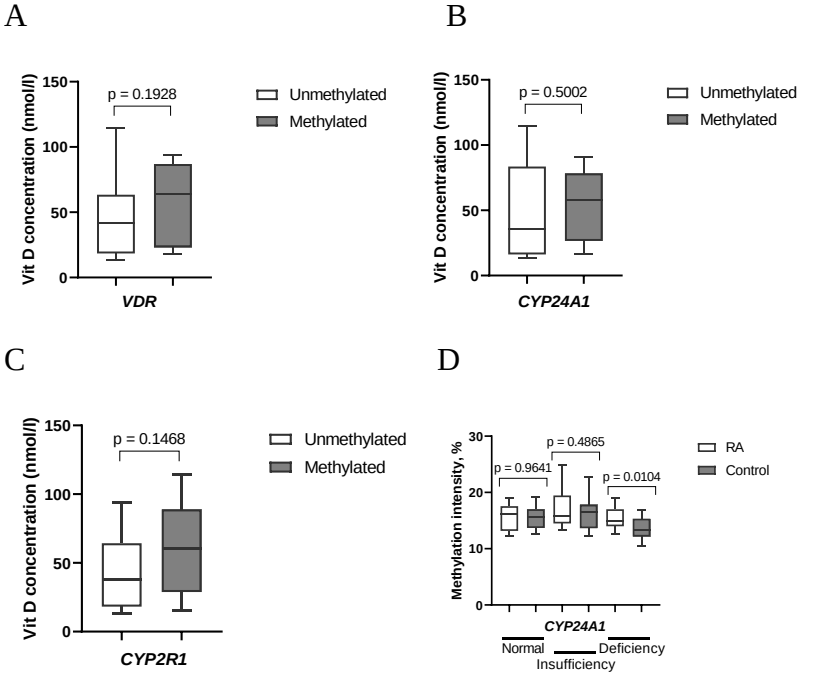


Figure 6. Vitamin D (vit D) level (nmol/l) comparison by *VDR* (**A**), *CYP24A1* (**B**) and *CYP2R1* (**C**) genes promoter methylation frequency in rheumatoid arthritis (RA) subjects; Comparison of methylation intensity of *CYP24A1* (**D**) according to vitamin D level (nmol/l) in RA subjects and healthy controls.

The methylation level of selected genes promoters did not differ significantly according to vitamin D status (normal range (≥75 nmol/l) *vs.* insufficiency (≥50–75 nmol/l) or deficiency (<50 nmol/l) and vice versa) in RA group. The use of vitamin D supplementation also did not show any statistically significant differences between the groups.

5.5. Periodontitis and rheumatoid arthritis association analysis

Periodontal examination was performed in Vilnius University Hospital Žalgiris Clinics for ninety-three RA patients (84 females and 9 males with a mean age of 54.01 ± 11.01 ranging from 29 to 75 years). The average RA disease duration was 12.65 ± 10.38 years. To distinguish differences between RA patients with diagnosed periodontitis and periodontally healthy subjects, RA+PD+ (N=63, 67.8%) and RA+PD– (N=30, 32.2%) groups were analysed. RA+PD+ diagnosis was significantly associated with sex, males were showing higher PD rates than females (p=0.029). All periodontally healthy RA subjects were non-smokers and used dental floss more frequently compared to the RA+PD+ group (p<0.05). Detailed sociodemographic data analysis of RA+PD+ and RA+PD– groups is provided in Table 6.

Table 6. Demographic and behavioural characteristics of RA+PD+ and RA+PD– group

	Total	**RA+PD+ group**	**RA+PD– group**	***p* value**
	N=93	N=63(67.8%)	N=30(32.2%)	
Age (years)	54.01±11.01	55.33±9.5	51.23±13.37	0.168
BMI (kg/m^2)	24.96±4.34	25.24±4.39	24.38±4.26	0.539
Sex (n, %)				0.029*
Female	84(90.32%)	54(85.71%)	30(100%)	
Male	9 (9.68%)	9(14.29%)	0	

Table 6 continue

Smoking status (n, %)				0.005*
Never smokers	54(58%)	36 (57.1%)	18 (60%)	
Current smokers	15(16.2%)	15 (23.8%)	0	
Former smokers	24(25.8%)	12 (16.3%)	12 (40%)	
Alcohol consumption (n, %)				0.505
Never	49(52.7%)	31 (49.2%)	18 (60%)	
Once per week	5(5.3%)	3 (4.8%)	2 (6.7%)	
Once per month	39(42)%	29(46%)	10 (33.3%)	
Annual dental prophylaxis/year				0.333
< Once	16(17.2%)	12(10.8%)	4(13.3%)	
Once	54(58%)	38(60.3%)	16(53.3%)	
Two and more times	23(24.8%)	13(20.6%)	10(33.4%)	
Dental floss				0.016*
Yes	69(74.2%)	42(66.7%)	27(90%)	
No	24(25.8%)	21(33.3%)	3(10%)	
Tooth brushing/day				0.155
Once	24(25.8%)	20(31.7%)	4(13.3%)	
Two and more times	69(74.2%)	43(68.3%)	26(86.7%)	

Values represent the mean ± SD, standard deviation or N, %; BMI – body mass index, RA – rheumatoid arthritis; PD – periodontitis; * – $p < 0.05$.

Almost one third of RA patients (N=27, 29.0%) had severe and advanced severe periodontitis (stage III+IV). The prevalence of different PD stages in RA patients is shown in Figure 7.

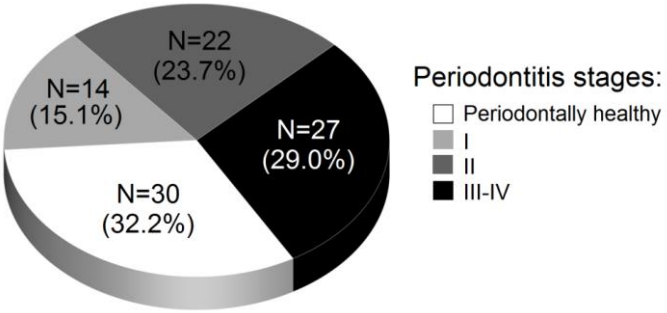


Figure 7. Periodontitis stages in rheumatoid arthritis patients.

5.5.1. Periodontitis and RA clinical associations

RA+PD+ group subjects presented a higher mean DAS28 score (4.37 ± 1.28 *vs.* 3.82 ± 1.65) and CRP level (13.82 ± 24.44 *vs.* 8.44 ± 12.99 mg/l), however this was not statistically significant ($p>0.05$). However, a significantly higher RA DAS28 CRP score was revealed in moderate-severe PD compared to the periodontally healthy-initial stage PD group (4.49 ± 1.22 *vs.* 3.86 ± 1.58, $p=0.033$) (Figure 8), indicating that PD severity could be associated with elevated RA activity and its consequences. Moreover, RA+PD+ diagnosed subjects showed higher VAS (45.81 ± 20.11 *vs.* 40.83 ± 21.75 mm) and RAID scores (4.68 ± 2.17 *vs.* 3.94 ± 2.44), although these differences were not statistically significant.

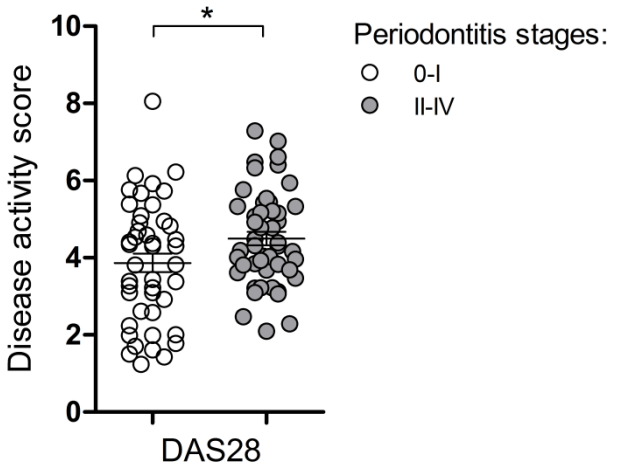


Figure 8. Disease activity score (DAS28) comparison in rheumatoid arthritis (RA)+ periodontitis (PD)+ group by PD stages; * – $p<0.05$.

5.5.2. Periodontitis analysis in RA+PD+ group

The RA+PD+ group revealed significantly higher BOP, BL, CAL, PPD periodontal parameters ($p<0.001$) and a number of missing teeth ($p=0.004$) compared with the RA+PD– group. There was no association between seropositive RA (positive rheumatoid factor (RF) and/or anti-citrullinated protein antibodies (ACPA)) and PD ($p>0.05$). Evaluating the effect of smoking on PD, ever smokers (both current and former smokers) had higher mean CAL, PPD, BOP and BL parameters compared with those who had never smoked in RA+PD+ group. Similar PD outcome shifts were also observed in elderly RA patients ($p<0.05$), older age was associated with moderate and severe PD ($p=0.006$). Interestingly, patients treated with bDMARDs or combination of bDMARDs with conventional synthetic DMARDs were less likely to be diagnosed with PD ($p=0.022$) (Figure 9A) and signaled significantly lower DAS28 score, CRP (mg/l) levels, VAS (mm) and lower PD outcome parameters – BL (%) and BOP (%)

(p<0.05) (Figure 9B) compared with conventional synthetic DMARDs-only treated RA subjects.

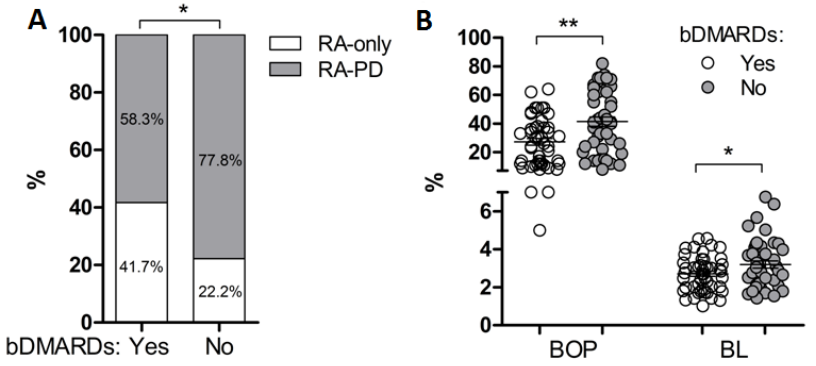


Figure 9. Biologic disease modifying anti-rheumatic drugs (bDMARDs) treatment association with periodontitis (PD) in rheumatoid arthritis (RA) subjects (A) and PD outcome parameters (B). BL – bone loss; BOP – bleeding on probing; ** – p<0.001, * – p<0.05.

5.5.3. Vitamin D association with RA and PD status

A high prevalence of vitamin D deficiency and insufficiency (<75nmol/l) was observed in RA subjects (N=80, 90.4%) in this study. The mean vitamin D concentration in the total group was 45.65 ± 21.33 (nmol/l). RA+PD– revealed a tendency of higher vitamin D concentration in compare with RA+PD+ group (48.13 ± 27.47 *vs.* 44.48 ± 17.82nmol/l, p>0.05, respectively). A significantly lower vitamin D concentration was observed in RA+PD+ group with diagnosed advanced severe (IV stage) PD compared to moderate PD (II stage) (39.61 ± 17.12 *vs.* 52.07 ± 18.23 nmol/l, p=0.031). Regarding RA characteristics, a significant inverse correlation between vitamin D level and HAQ score was confirmed (r-0.23, p=0.026), however no significant correlations between vitamin D

status and PD outcome parameters were detected, only some weak inverse correlations with PPD, CAL and BL (p>0.05).

6. CONCLUSIONS

1. Rheumatoid arthritis patients have significantly lower vitamin D level compared with healthy subjects (44.96 ± 21.92 *vs.* 54.90 ± 22.82 nmol/l, p <0.0001). Suboptimal vitamin D concentration (<75 nmol/l) is observed in 90.29% of RA subjects and is associated with higher disease activity (DAS28 CRB) score (p=0.0017) and worse dissability index (HAQ) (p=0.0065).
2. *VDR* gene polymorphisms do not increase the risk of rheumatoid arthritis in studied Lithuanian population, however certain gene genotypes could be related with vitamin D level.
3. The mean methylation intensity of analysed vitamin D pathway genes (*VDR*, *CYP24A1*, *CYP2R1)* is low and positively correlates with vitamin D concentration. Higher than control group *CYP24A1* and *CYP2R1* methylation intensity in vitamin D deficient and vitamin D sufficient RA patients is signaling of the disturbed vitamin D metabolism in RA.
4. Rheumatoid arthritis patients have high prevalence of periodontitis (67.8%), of which almost one third (29%) are diagnosed with severe periodontitis stage (III+IV). Moderate and severe periodontitis is associated with significantly higher rheumatoid arthritis disease activity measured by the DAS28 CRB score (p=0.033).

7. LIST OF PUBLICATIONS

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

1. **Punceviciene E**, Gaizevska J, Sabaliauskaite R, Venceviciene L, Puriene A, Vitkus D, Jarmalaite S, Butrimiene I. Vitamin D and VDR gene polymorphisms' association with rheumatoid arthritis in Lithuanian population. *Medicina (Kaunas)*. 2021 Apr 3;57(4):346. doi: 10.3390/medicina57040346.
2. **Punceviciene E**, Rovas A, Puriene A, Stuopelyte K, Vitkus D, Jarmalaite S, Butrimiene I. Investigating the relationship between the severity of periodontitis and rheumatoid arthritis: a cross-sectional study. *Clin Rheumatol*. 2021 Aug;40(8):3153-3160. doi: 10.1007/s10067-021-05661-3.

Scientific paper published in peer-reviewed journal of other databases:

3. **Punceviciene E**, Gaizevska J, Sabaliauskaite R, Snipaitiene K, Venceviciene L, Vitkus D, Jarmalaite S, Butrimiene I. Analysis of epigenetic changes in vitamin D pathway genes in rheumatoid arthritis patients. *Acta medica Lituanica*. 2022; 29(1), p. 7. doi: 10.15388/Amed.2021.29.1.7.

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

1. Vojinovic J, Tincani A, Sulli A, Soldano S, Andreoli L, Dall'Ara F, Ionescu R, Pasalic KS, Balcune I, Ferraz-Amaro I, Tlustochowicz M, Butrimiene I, **Punceviciene E**, Toroptsova N, Grazio S, Morovic-Vergles J, Masaryk P, Otsa K, Bernardes M, Boyadzhieva V, Salaffi F, Cutolo M. European multicentre pilot survey to assess vitamin D status in rheumatoid arthritis patients and early development of a new Patient Reported

Outcome questionnaire (D-PRO). *Autoimmun Rev*. 2017 May;16(5):548–554. doi: 10.1016/j.autrev.2017.03.002.

2. Salaffi F, Di Carlo M, Vojinovic J, Tincani A, Sulli A, Soldano S, Andreoli L, Dall'Ara F, Ionescu R, Simić Pašalić K, Balčune I, Ferraz-Amaro I, Tlustochowicz M, Butrimienė I, **Puncheviciene E**, Toroptsova N, Grazio S, Morović-Vergles J, Masaryk P, Otsa K, Bernardes M, Boyadzhieva V, Cutolo M. Validity of the rheumatoid arthritis impact of disease (RAID) score and definition of cut-off points for disease activity states in a population-based European cohort of patients with rheumatoid arthritis. *Joint Bone Spine*. 2018 May;85(3):317–322. doi: 10.1016/j.jbspin.2017.05.020.
3. Rovas A, Puriene A, Snipaitiene K, **Puncheviciene E**, Buragaite-Staponkiene B, Matuleviciute R, Butrimiene I, Jarmalaite S. Analysis of periodontitis-associated miRNAs in gingival tissue, gingival crevicular fluid, saliva and blood plasma. *Arch Oral Biol*. 2021 Jun;126:105125. doi: 10.1016/j.archoralbio.2021.105125.
4. Rovas A, Puriene A, **Puncheviciene E**, Butrimiene I, Stuopelyte K, Jarmalaite S. Associations of periodontal status in periodontitis and rheumatoid arthritis patients. J Periodontal Implant Sci. 2021 Apr;51(2):124–134. doi: 10.5051/jpis.2006060303.
5. Rovas A, Puriene A, Snipaitiene K, **Puncheviciene E**, Buragaite-Staponkiene B, Matuleviciute R, Butrimiene I, Jarmalaite S. „Gingival crevicular fluid microRNA associations with periodontitis“. *J. Oral Sci*. 2021 Oct. doi.org/10.2334/josnusd.21-0282

8. PRESENTATIONS AT CONFERENCES

1. **Puncevičienė, Eglė**; Butrimienė, Irena; Vitkus, Dalius. Vitamin D status and disease activity in rheumatoid arthitis patients. Baltic Rheumatology Conference; Vilnius (Lithuania), 2017 Sep 21-22.
2. **Puncevičienė, Eglė**; Gaiževska, Justina; Sabaliauskaitė, Rasa; Vencevičienė, Lina; Vitkus, Dalius; Jarmalaitė, Sonata; Butrimienė, Irena. Methylation analysis of vitamin D signaling pathway genes in rheumatoid arthritis patients. EULAR Congress (E-congress); 2020 June 3-6.
3. Gaiževska, Justina; **Puncevičienė, Eglė**; Vencevičienė, Lina; Sabaliauskaitė, Rasa; Jarmalaitė, Sonata; Butrimienė, Irena. Vitamin D receptor gene polymorphism distribution and methylation analysis of vitamin D metabolic pathway genes in Lithuanian rheumatoid arthritis patients. COINS 2019 - 14th international conference of life sciences; Vilnius (Lithuania), 2019 Feb 26-28.
4. **Puncevičienė Eglė**. „Vitamino D įtaka klinikinei reumatoidinio artrito eigai“. Oral presentation at the conference: „Jaunųjų reumatologų mokykla“, Kėdainiai (Lithuania), 2019 May 18-19.

9. CURRICULUM VITAE

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Education:

2015-2021 PhD student, Vilnius University, Faculty of Medicine

2008-2015 Rheumatology residency training, Vilnius University, Faculty of Medicine

2007-2008 Doctor of Medicine (MD), Vilnius University, Faculty of Medicine

2001-2007 Medical qualification, Vilnius University, Faculty of Medicine

Professional experience:

From 2015 Rheumatologist, Širvintos hospital, Širvintos

From 2016 Rheumatologist, Vilnius University Hospital Santaros Klinikos, Vilnius

From 2019 Rheumatologist, Private hospital “Kardiolitos klinikos”, Vilnius

2015-2019 Rheumatologist, Lazdynai Outpatient Clinic

Scientific publications: Published 7 scientific articles in journals having an impact factor in the Clarivate Analytics Web of Science database.

Conferences: first author and co-author of posters in multiple national and international conferences.

Projects:

2012-2015 Participant of international project „Development and validation of a new patient reported outcome (pro) questionnaire to estimate vitamin D status (D-PRO) and its correlation with quality of life, disease activity and disability in rheumatoid arthritis patients in Europe“. Study mentor prof. Maurizio Cutolo.

2017-2021 Junior researcher in The Research Council of Lithuania Scientists group project „Analysis of immunological, genetic, and epigenetic factors in pathogenesis of autoimmune arthritis“. Project leader: prof. Irena Butrimienė.

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Memberships:

From 2010 member of Lithuanian Rheumatologists Association

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Languages: Lithuanian (native), English, Russian

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