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Abstract

of the dissertation work for the academic degree of Doctor of Philosophy PhD
in the specialty 8D10102 – "MEDICINE"

**"Clinical peculiarities of the course and state of microbiocenosis in patients
with rheumatoid arthritis"**

Scientific consultant:	MD, professor Ainabekova B.A.
Scientific consultant:	MD, professor Kushugulova A.R.
Foreign consultant:	MD Zueva Y.E. (Ashkelon: Ariel University, medical faculty, Israel)
Performer:	Issilbayeva A.A.

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The research relevance

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology, characterized by joint damage, as well as the development of extra-articular manifestations. The frequency of RA is 1% in the global population [Smolen et al., 2016]. The disease develops 3-5 times more often among females, mainly at the age of 25-60 years [Sokka et al., 2009; Favalli et al., 2018], the ratio of women to men is 3:1 [Sokka et al., 2009]. In the absence of timely diagnosis and treatment, this disease in 40-70% of cases leads to disability in working age [Cross et al., 2010; Dadoun et al., 2013].

Currently, the role of endogenous and exogenous factors in the development of RA has been proven. Genetic predisposition is an important endogenous factor that interacts most with exogenous triggers [Firestein et al., 2017; Viatte et al., 2017]. In recent decades, genome-wide association studies (GWAS) have provided an opportunity to deeply study the genetic predisposition to RA. Most of the pathogenetically important HLA and non-HLA genes in the development of RA have been identified [Stahl et al., 2010; Plant et al., 2010, Okada et al., 2014...]. HLA-DRB genes provide 1/3 of the genetic predisposition to RA [Tobon et al., 2010]. In Kazakhstan Kuranov et al. Alleles of HLA-DRB1, DQB1 and DQA1 genes were studied among Astana residents and RA patients [Kuranov et al., 2014]. The role of more than 100 non-HLA gene loci was presented in a large-scale study by Okada et al., proving their key role in the RA development [Okada et al., 2014]. The strongest associations were found with the PTPN22 gene and RA in the European population [Perricone et al., 2011], PADI4 in the Asian population [Bax et al., 2011]. Currently available studies aimed at confirming previously identified single nucleotide polymorphisms of RA genes in different populations have demonstrated significant differences in genetic predisposition to this disease in different ethnic groups [Yamamoto et al., 2015; Banos-hernandez et al., 2017; Allam et al., 2020...], which in turn the queue is a prerequisite for further research in this direction.

Studies aimed at studying the triggers that lead to the activation of a particular RA gene are also relevant. Microbiocenosis is one of the leading factors occupying a significant niche in the pathogenesis of autoimmune diseases, including RA [Gevers et al., 2012]. The data obtained based on the analysis of DNA sequences of microbial communities (16S RNA sequencing) showed that the microbiocenosis of the oral cavity and gut is the most important factor affecting the manifestation of autoimmune diseases [Lynch et al., 2016; Nikitakis et al., 2017; Maeda et al., 2017]. It is known, that the gastrointestinal tract harbors the largest human microbiocenosis in general [Costello et al., 2012]. One of the other areas with microbial populations affecting the human immune system is considered to be the oral cavity, which contains more than 700 species of bacteria, the species diversity of which practically does not differ from the intestinal tract [Nikitakis et al., 2017]. Due to the available data that antibodies to cyclic citrullinated peptide (ACPA) and rheumatoid factor (RF) appear in the oral mucosa long before the debut of RA [Demoruelle et al., 2014], together with data from a number of studies that *Porphyromonas gingivalis* expressing the PADI4 gene and thereby secreting the protein peptidylarginine deiminase (PAD), responsible for the synthesis of ACPA, is considered the main

pathogenic bacterium of the oral cavity, correlating with the development of RA [Eduardo et al., 2019], and the gut plays one of the key roles in the regulation of both immunity, as well as autoimmunity [Follicular et al., 2016], the study of the microbiocenosis of these areas is of great scientific interest.

The data of the available studies devoted to the study of microbiome markers of RA are very contradictory. Thus, a number of studies have presented *Prevotella copri* as the most prominent representative of intestinal microbial communities associated with the development of RA [Scher et al., 2013]. While studies in mice have shown that *Prevotella histicola* reduces the risk of developing RA [Marietta et al., 2016]. This in turn shows that different types of bacteria belonging to the same genus may have different effects on autoimmunity in RA. In addition, numerous studies have shown differences in microbiocenosis in patients with RA in different populations. To date, there are numerous studies of the oral and gut microbiome in RA in the European and Asian populations [Wolff et al., 2014; Kishikawa et al., 2019; Jeong et al., 2019...], it should be noted that the Asian population is represented mainly by studies conducted in Japan and China [Jeong et al. al., 2019; Kishikawa et al., 2019]. The number of joint works devoted to the microbiocenosis of the oral cavity and gut is limited, mainly these works are presented in the form of literary reviews [Lorenzo et al., 2019; Prodan et al., 2019]. In Russia Gulneva M. Yu. et al. conducted the study of intestinal microbiocenosis in patients with SLE, scleroderma and systemic vasculitis [Gulneva et al., 2007]. Thus, there are no published studies of oral and gut microbiocenosis in patients with RA in Central Asian countries, including Kazakhstan.

The aim of the study: To evaluate the clinical peculiarities, genetic spectrum, condition and prognostic significance of microbiocenosis in patients with rheumatoid arthritis.

Research objectives:

1. To study the clinical peculiarities of the disease course in patients with rheumatoid arthritis.
2. To evaluate the nutritional characteristics of patients with rheumatoid arthritis.
3. To study the spectrum of genetic markers in patients with rheumatoid arthritis.
4. To investigate the state of microbiocenosis of the oral cavity and gut in women with rheumatoid arthritis.
5. To analyze the relationship of the severity of clinical manifestations with changes in microbiocenosis in patients with rheumatoid arthritis.
6. To investigate the state of microbiocenosis of the oral cavity and gut against the background of rheumatoid arthritis therapy.

7. To study the prognosis of the clinical course in the studied patients with rheumatoid arthritis.

The object of the study:

The study included 82 female patients diagnosed with rheumatoid arthritis and a control group of female persons in the number of 114 people.

Study design: Cross-sectional single-stage study

Research methods:

• Clinical methods: collection of complaints, anamnesis of the disease and life, objective examination of organs and systems;

• Questionnaire based on the HAQ, FFQ questionnaires;

• Laboratory research methods :

• general blood test;

• general urine analysis;

• biochemical blood analysis;

• protein electrophoresis;

• ACPA;

• RF;

• Ro/SSA antibodies;

• La/SSB antibodies;

• Collection of biomaterial in the form of saliva, feces and blood;

• Isolation and amplification of DNA, preparation of DNA libraries;

• Real-time PCR;

• 16S rRNA sequencing;

• Statistical method of processing (IBM SPSS Statistics 26 версия and RStudio 1.4.1717. version) and bioinformatic analysis of the data obtained.

Scientific novelty: For the first time, a comprehensive study of the microbiocenosis of the oral cavity and gut in patients with rheumatoid arthritis in the Kazakh population was conducted.

Genetic markers, HLA-DRB9 and non-HLA genes, were studied for the first time in patients with rheumatoid arthritis in the Kazakh population, while the SNP HLA-DRB9 rs9268839 was studied for the first time in the Asian population.

For the first time in the Central Asian population, the association of indicators of the gut microbiome and the effectiveness of therapy in RA has been demonstrated.

Practical significance:

The results of the study of genetic markers in patients with rheumatoid arthritis in the Kazakh population, especially SNP HLA DRB9 rs9268839, will complement and expand knowledge about the RA etiopathogenesis.

In patients with RA, significant differences of taxa in the microbiome of the oral cavity and gut were revealed depending on the RF and ACPA status, X-ray stage, FC and disease activity, which makes it possible to predict disease activity, relapses and the development of new therapeutic directions aimed at the microbiome.

The obtained data of this study on genetic and microbiomic biomarkers will be deposited and will replenish the world databases on rheumatoid arthritis.

This study will allow us to study the structure of the microbiome of patients with RA, with the possibility of correcting the identified disorders.

According to the results of the study, the developed prognostic model allows us to calculate the probability of developing deformities of small and large joints depending on the age of onset and symmetry of the articular syndrome and calculate the formation of the radiological stage from the level of CRP and the duration of the disease.

The main provisions submitted for protection:

1. The studied patients with RF-positive and ACPA-positive forms of rheumatoid arthritis had a more severe course of the disease, according to clinical and laboratory data, while the study of the nutrition of RA patients revealed significantly insufficient consumption of products containing lactose, niacin, alcohol, polyunsaturated fatty acids and vitamin E.
2. Studying genetic markers, it was revealed that a single nucleotide polymorphism HLA-DRB9 rs9268839 increases the chances of developing RA, the genotypes of RASGRP1 rs8032939 nucleotides prevailed both in the RF-positive and in the RF-negative form of the disease, SYNGR1 rs909685 prevailed in the RF negative form of RA. The genotypes of the PADI4 rs2240340a and STAT4 rs11889341 nucleotides dominated both the ACPA-positive and ACPA-negative forms of RA, the FCRL3 rs2317230 nucleotide dominated the ACPA-positive RA form.
3. Taxa in the microbiome of the oral cavity and gut significantly differed in various forms of RA depending on the RF and ACPA status, radiological stage, FC and activity of the disease, also the diversity of the gut microbiome had significant differences depending on the presence and type of RA therapy, belonging to the Prevotella enterotype increases the chances of developing rheumatoid arthritis.
4. The age of the debut and the symmetry of the articular syndrome have a prognostic effect on the likelihood of developing deformities of small and large joints, and CRP and the duration of the disease have a prognostic effect on the formation of the radiological stage of RA.

Conclusions:

1. In the studied patients with the RF-positive form of rheumatoid arthritis, the X-ray stage ($\chi^2=9,928$, $df=3$, $p=0,0019$) and the incidence of deformities

($\chi^2=4,995$, $df=1$, $p=0,025$) were significantly higher, also this form of the disease is characterized by direct correlations of CRP, albumin, gamma and beta 2 globulins of different strengths with disease activity according to DAS 28. In patients with the ACPA-positive form of the disease, the frequency of deformities was also significantly higher ($\chi^2=6,017$, $df=1$, $p=0.014$).

2. The studied women with RA significantly consumed less products containing lactose ($p= 0,003$), niacin ($p= 0,006$), alcohol ($p= 0,006$), polyunsaturated fatty acids ($p= 0,004$), vitamin E ($p= 0,001$).

3. The studied patients with RA had significantly higher carrier of the HLA-DRB9 rs9268839 gene (OR = 3,67 [95% CI: 1,58-8,54], $p = 0,001$), while in patients with RF-positive form of RA, the S/T genotype of the RASGRP1 rs8032939 nucleotide dominated, in patients with RF-negative form T/T genotype RASGRP1 rs8032939, and A/T-T/T genotypes SYNGR1 rs909685. The T/T genotypes of the PADI4 rs2240340a and STAT4 rs11889341 nucleotides dominated in the ACPA-positive form, the G/T genotype of FCRL3 rs2317230, and the C/T genotypes of the PADI4 rs2240340a and STAT4 rs11889341 nucleotides dominated in the ACPA-negative form of RA.

4. Significant differences in the microbiome of patients with RA in comparison with a healthy microbiome have been established. Significant differences of taxa in the microbiome were revealed in patients with RA depending on RF and ACPA status, radiological stage, FC and disease activity. It was determined that individuals belonging to the Prevotella enterotype have a high chance of RA developing (OR = 2,056 [95% CI: 1,038- 5,919], $p = 0,05$). In the gut microbiome at the family level, Bacteroidaceae ($p\leq 0,003$), Lachnospiraceae (g. Dorea) ($p\leq 0,00001$), Ruminococcaceae ($p\leq 0,00002$) were prevailed, and bacteria of the Coriobacteriaceae family ($p\leq 0,01$) were reduced.

5. Statistically significant differences in the α -diversity of the oral microbiome according to the Shannon ($p\leq 0,021$) and Simpson indices ($p\leq 0,002$) were revealed. The oral microbiome of the RA group was dominated by taxa at the level of the families Prevotellaceae ($p\leq 0,0001$) and Neisseriaceae ($p\leq 0,00001$), at the level of the genera Porphyromonas ($p\leq 0,001$).

6. Indicators of the gut microbiome, in contrast to the oral microbiome, are an indicator of the effectiveness of therapy: the Shannon index, $p\leq 0,05$; the Simpson index, $p\leq 0,01$ and the Bray-Curtis index, $p\leq 0,04$. At the same time, the composition of the gut microbiome of the subgroup receiving methotrexate is reliably comparable with the microbiome of a healthy control, the Bray-Curtis index $p\leq 0,01$.

7. Calculating the prognostic model, it was revealed that the probability of developing deformities of small and large joints is affected by the age of onset and the symmetry of the articular syndrome (test sensitivity 71,7%, specificity 70%, diagnostic accuracy 70,8%), while the formation of the radiological stage is influenced by the level of CRP and the duration of the disease (sensitivity of the model when predicting I X-ray stage – 71,6%, stage II – 29,4%, stage III - 37,5% and stage IV -63,6%).

Practical recommendations

1. Data on genetic and microbiome markers will be deposited in the international database of scientific and technical information, and thus complement the world data on genetic predisposition to RA and the microbiome in RA, particularly, in Central Asia.

2. Patients with RA are recommended a full diet with sufficient levels of vitamin E, niacin, polyunsaturated fatty acids, dairy products.

3. The presence of HLA-DRB9 rs9268839 polymorphism in Asian patients can be used in predicting the development of rheumatoid arthritis in the presence of triggers.

4. The age of the RA onset and the symmetry of the articular syndrome should be taken into account when using the proposed calculation of the probability of the development of deformities of small and large joints, and in the formation of the radiological stage - the level of CRP and the duration of the disease, which will prevent disability in working age

5. According to the results of the study, basic methotrexate therapy has confirmed its effectiveness concerning the microbiome in RA.

6. To increase the therapeutic effectiveness, patients with RA should be prescribed probiotic therapy with drugs containing bifidobacteria.

Approbation of the work

The dissertation work was approved at a meeting of the Department of Internal Medicine with courses in gastroenterology, endocrinology and pulmonology (Protocol No. 9 of April 20, 2022) and at an expanded meeting (Protocol No. 10A of May 30, 2022).

The main results of the research and the provisions of the dissertation are reported in the speeches:

-at the international congress "23rd Asia-Pacific League of Associations for Rheumatology Congress" (APLAR-2021), (Kyoto, Japan, August 28-31, 2021).

-at the international conference "Current Problems of Biological Safety in the Modern Condition" (Astana (Nur-Sultan), September 22-23, 2021)

-at the XXIX International multidisciplinary conference "Recent Scientific Investigation", (Shawnee, USA, 2022)

Publications.

According to the materials of the dissertation, 12 printed works were published: 2 of them articles in domestic publications recommended by Committee for Control in the Field of education and Science of the Ministry of Education and Science of the Republic of Kazakhstan at the time of publication, 1 article in the publication having 52 percentile (Q2) on CiteScore in the Scopus database and 2 articles in the publication having 48 percentile (Q3) on CiteScore in the Scopus database, 3 theses in collections international conferences, including 1 thesis in the collection of the

international conference APLAR (23rd Asia-Pacific League Associations for Rheumatology Congress) with an impact factor of 49% (Q3). 4 copyright certificates were obtained (No. 14601, No. 14602B, No. 14603, No. 14604 dated January 22, 2021). The results of the microbiome study are in print in the publication having the 97th percentile (Q1) on CiteScore in the Scopus database.

The results of the work were tested and implemented in the department of narrow specialists, at the state municipal enterprise on the right of economic management city polyclinic No. 12 Astana (Nur-Sultan).

Personal contribution of the dissertator

The work was carried out in accordance with the direction of development of science in the field of "Life and Health Sciences" approved by the Higher Scientific and Technical Commission under the Government of the Republic of Kazakhstan. The dissertator independently conducted recruiting of study participants, collecting material, carried out clinical examination of patients with rheumatoid arthritis, questioning of all study participants, independently carried out DNA isolation from saliva, blood and feces samples, preparation of DNA libraries for 16SpRNA sequencing, real-time PCR. The dissertator independently analyzed and summarized the results of the study, carried out statistical data processing, carried out writing articles under the guidance of a supervisor and consultants, independently translated articles into English.

Volume structure and dissertations. The dissertation work consists of an introduction, 3 chapters, general conclusions, a list of references, and an appendix. The work is presented on 172 pages of typewritten text, includes 53 tables, 66 figures. The list of references contains 306 titles.