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THE VALIDITY OF THE SPECIFIC BIOMARKER OF RHEUMATOID ARTHRITIS FROM THE ASPECT OF SMALL SAMPLE SIZES

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Abstract:

In our study, we processed the selected laboratory parameters of patients with rheumatoid arthritis and other rheumatic diseases. The objective of the study was to compare the differences of anti-CCP examinations between the observed groups of patients. The most specific and in practice the most used laboratory parameters to support the diagnosis of rheumatoid arthritis are anti-CCP. The importance of this parameter was confirmed by the results of our work (p = 0.01). An unexpected information bonus of our study was the use of statistical tests for small groups (n = 10 and n = 11 respectively), the low number of which was the result of a pandemic situation in connection with the Covid-19 disease and restrictive anti-epidemiological measures. It is the example of the often-required statistical analysis which must always be supplemented by a suitable interpretation of the results, even from the rational level of the benefit of the parameter for the physician's decision algorithm.

1 Background

To obtain reliable results of examinations of biomedical laboratory parameters, we need sufficiently numerous sample groups. Only in that case, we can verify the real informative value of the tested parameter from the aspect of a specific disease. Therefore, many biomedical parameters are only statistically related to the diseases. This means that examination of selected samples with a large number will show a higher relative risk of disease in an individual with specific characteristics. However, it is a selected risk that is given by statistical probability and is not deterministic. The typical examples are polymorphisms of several genes. Their carriers have a statistically increased probability of the disease, but they have no confidence that the disease will really manifest in them. The opposite example are parameters, the presence of which also means the presence of the disease in almost all cases. This group also includes antibodies against cyclic citrulline peptide (anti-CCP), which are important laboratory parameters for monitoring rheumatoid arthritis.

1.1 Rheumatoid arthritis

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Rheumatoid arthritis (RA) is a chronic inflammatory disease of an autoimmune nature that can occur in all age groups of patients. Most frequently begins between the ages of 25 – 50. The maximum incidence was observed in women before menopause. RA mainly affects the joints, their synovial lining, so it is called arthritis (from the Latin arthritis = inflammation of the joints), but the disease process often also affects non-joint components of the musculoskeletal system, for example muscles or tendons, and internal organs. It usually begins as symmetric polyarthritis, often with non-specific systemic symptoms. Its course is very variable. Acute exacerbations are often alternated with periods of remission [1, 2].

1.2 Anti - CCP

Already in the early stages of RA, antibodies to cyclic citrulline peptides appear in most patients during the inflammatory process. Compared to another laboratory parameters (such as rheumatoid factor - RF), they are much more specific for RA, up to 96 %, with the same sensitivity (approx. 80 %) [1].

These antibodies are formed by converting the amino acid arginine to citrulline. Citrulline is a non-standard amino acid that is formed by post-translational modification of arginine in proteins using the enzyme peptidyl arginine deaminase (PAD). PAD is found intracellularly inactive. It needs a much higher concentration of calcium ions than is commonly found in a living cell to activate. This increases only when the integrity of the cell is violated, when calcium ions can penetrate inside the cell and activate PAD. Protein citrullinations often occur in cell differentiation, inflammatory responses, cell apoptosis, gene regulation, and the aging process. Citrullination of the protein loses its basic charge, which can affect the entire structure of the protein and create a new epithelium that can be recognized by the immune system as an antigen. In RA, specific antibodies are formed against it, mainly in the IgG isotype [3].

2 Aim

The goal of our study was to verify the validity of parameter anti-CCP in the conditions of examination of patients from the local Caucasus population, which was significantly limited by another external factor (pandemic Covid-19). It is in accordance with good laboratory practice that the laboratory verifies the validity and informative value of the determined biomedical parameter in the catchment population.

3 Material and methods

3.1 Sample

A total of 21 individuals were evaluated, which were divided into two groups. Group A consisted of only 10 patients diagnosed with seropositive RA. From the point of view of gender representation, there were 8 women and two men in the sample. Group B consisted of patients with other diseases of the musculoskeletal system, and connective tissue (for example systemic lupus erythematosus, polymyalgia rheumatica, and others) and served as a comparative/control group. The total number of patients in this group was also only 11, of which the number of men was 3 and the number of women was the same as in the first case, i.e., 8. In individuals, we observed differences in the incidence of autoantibodies between the two groups. Specifically, these were the parameters of anti-CCP and anti-nuclear antibodies (ANA). In patients who were indicated for further typing of ANA, we tested differences in antibodies to histones and SS-A. Data were obtained in cooperation with specialist clinics in the Košice self-governing region during the period 10/2020 – 01/2021 i.e., during the second serious wave of the Covid-19 pandemic, which significantly reduced the number of individuals examined for other diseases. The processed data of all patients were deidentified and reduced in the database to numerical data of basic laboratory and physiological parameters in terms of protection of personal data of patients.

3.2 Methods

After a basic examination of ANA by the immunofluorescence method, an immune specific test was subsequently indicated, which was performed by immunoblotting (IB). The immune specification consisted of testing the serum for the presence of antibodies against DNA, histones and against so-called Extractable Nuclear Antigen (ENA), including SS-A, Jo-1 and many more. Immunoblot assays are used to detect several autoantibodies simultaneously.

3.3 Data processing

We used a Microsoft Excel platform to process the data. First, we focused on the basic properties of the monitored files and obtained data on the number, arithmetic mean, standard deviation, median, as well as the minimum and maximum value of parameters in the files. To verify the proportionality of the gender representation in the files, we applied a 2x2 contingency table using a chi-square test. A nonparametric Mann-Whitney test was used to evaluate the difference between the groups due to the low number of sets, the independence of both sets and the nature of the numerical values. The results were also compared with Student's t-test. We set $p < 0.05$ as the level of significance.

4 Results and discussion

4.1 Basic characteristics of groups

First, we verified the balance of both genders in groups A and B. The results are shown in Table 1. The data show that overall women (n = 16) predominated over men (n = 5), but their representation in both groups was equal and did not show statistically significant differences (p = 0.70).

To compare the age characteristics of both groups A and B, we used the nonparametric Mann-Whitney test as the main comparison test due to the smaller number of individuals in the sets. The differences in the medians of the age of both groups were not statistically significant (p = 0.94) and this result was also confirmed by a parametric two-tailed t-test (p = 0.99; Table 2).

By comparing the mean age between men and women, we did not find statistically significant differences (Table 3, p = 0.54, t-test). The result of the verification of the equality of variances by the F-test was on the border of statistical significance (p = 0.05), therefore we performed the Welch approximation of the t-test, which considers the situation of inequality of variances. However, even after this calculation, the results did not show significant differences (p = 0.34; t-test, Welch approximation).

Based on the above findings, we state the homogeneity of the files in terms of age and gender representation.

4.2 Comparison of differences in anti-CCP between groups

Analysis of anti-CCP showed significant differences between the two sets (Table 4). For anti-CCP, of course, the intensity class was not

Table 1 Verification of the proportionality of gender in both files

Parameter		*Gender*		*Total*	χ^2	*p*	*d.f.*
		Man	Woman				
Group	A	2	8	10	0.15	0.70	1
	B	3	8	11			
Total		5	16	21			

Legend: χ^2 – test characteristic, *p* – probability value of the test criterion of the chi-square test, *d.f.* – degrees of freedom

Table 2 Basic statistical parameters of age in files

Parameter	*n*	$\bar{x}$	*sd*	x_m	*min.*	*max.*	*p (t-test)*	*p (M-W)*
Group A	10	51.9	16.2	56.5	20	71	0.99	0.94
Group B	11	51.8	16.3	55.0	20	75		

Legend: *n* – number of individuals, $\bar{x}$ – arithmetic mean, *sd* – standard deviation, x_m – median, *min.* – minimum value, *max.* – maximum value, *p* – probability value of the test criterion of the tests used (Mann-Whitney test and t-test)

Table 3 Comparison of average age between genders

Gender	*n*	$\bar{x}$	*sd*	x_m	*min.*	*max.*	*p(t-test)*
Men	5	55.8	6.1	55.0	51	66	0.54
Women	16	50.6	17.8	56.5	20	75	

Legend: *n* – number of individuals, $\bar{x}$ – arithmetic mean, *sd* – standard deviation, x_m – median, *min.* – minimum value, *max.* – maximum value, *p* – value of the t-test test criterion

Table 4 Statistical comparison of anti-CCP (kIU/l) between patients with seropositive RA and patients with other rheumatic diseases.

Parameter	*n*	$\bar{x}$	*sd*	x_m	*min.*	*max.*	*p (t-test)*	*p (M-W)*
Group A	7	276.6	246.8	271.5	6.3	600	0.02	0.01
Group B	3	0.6	0.5	0.5	0.2	1.2		

Legend: *n* – number of individuals, $\bar{x}$ – arithmetic mean, *sd* – standard deviation, x_m – median, *min.* – minimum value, *max.* – maximum value, *p* – value of the test criterion of the tests used (Mann-Whitney test and t-test)

evaluated, but its concentration is given in kIU/l directly. We tested this parameter with the aid of the t-test, and due to character of the parameter we chose a two-sample t-test with unequal variance. The t-test revealed a significant statistical significance of the differences between the two sets at the level of p = 0.02. Due to the very small number in group B, we used a more suitable Mann-Whiteny test, the value of which did not differ significantly from the student's t-test (p = 0.01). Therefore, we can conclude that there are statistically significant differences in studied groups.

The most useful markers to support the diagnosis of rheumatoid arthritis are antibodies against cyclic citrulline peptide [4]. According to the data available, we observed differences in the parameter between patients with RA and patients with other connective tissue diseases. In group A, anti-CCP antibodies were determined in 7 patients, but in group B we tested anti-CCP in only 3 patients (Table 4). However, this is only a consequence of the effectiveness of the indication of examinations in our health system, because if RA is not suspected, the doctor does not unnecessarily indicate the examination of the parameter. However, despite this disproportion, statistically significant differences with a high level of significance were verified by both applied tests (t-test, p = 0.02, Mann-Whitney test, p = 0.01). Interpretation of the findings given in table no. 4 must again consider the low numbers of individuals in the groups, especially in group B of patients with other rheumatic diseases. Therefore, we must evaluate the results on two levels: statistical and rational. In the case of low numbers of individuals, the authors (for example de Winter [5]) admit acceptance of the results, provided that a high rate of false positive results is considered in the case of different number of compared files and different variability of the observed parameter in them [5]. Even the possible use of modifications of tests that work with rank values (as we use the Mann-Whitney test) in the case of low numbers does not provide an interpretatively "safe" solution [6]. Moreover, these literature sources are not related to laboratory examination methods, and in particular the work of Zimmerman and Zumbo [6] is more than a quarter of a century old. Therefore, from the point of view of statistical data processing, we can only carefully state that our results suggest agreement with the assumption of a high association of the parameter

with rheumatoid arthritis and thus support the usefulness of anti-CCP as part of the classification criterion for RA diagnosis. At this point, we would move in the evaluation of this parameter from the statistical level to the rational level. If the range of group A was 593.7 kIU/l (minimum 6.3 kIU/l; maximum 600 kIU/l) and the range of group B was 1.0 kIU/l (minimum 0.2 kIU/l; maximum 1.2 kIU/l) thus, assuming the representativeness of the sample and the homogeneity of the target population, we can assume that in the case of patients with rheumatoid arthritis, the numerical values of anti-CCP will be in a much wider range with significantly higher concentrations. This fact makes anti-CCP a very beneficial laboratory parameter.

5 Conclusion

Our results show that sufficient file count cannot be understood as a fixed mantra, but any data processing results of small sample groups must be interpreted in the broader context of the biomedical significance of a particular laboratory parameter. Such an approach can be useful in cases of extremely low sample numbers due to the unexpected influence of an external factor.

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