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ORIGINAL ARTICLE

CHANGES IN THE CYTOKINE PROFILE IN PATIENTS DURING COVID-19 INFECTION

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Abstract. *COVID-19 has proven to be a disease that affects not only the respiratory tract but also leads to a state of generalized systemic hyperinflammation and overall immune dysregulation. An important role in its pathogenesis is the disturbance of many cytokines – a condition which, in its most pronounced form, is also called a „cytokine storm".* ***Objective:*** *To evaluate the serum cytokine levels during COVID-19 infection as potential biomarkers for the severity and course of infection.* ***Materials and methods:*** *By design, the study is a retrospective cross-sectional, in which the serum concentrations of 10 pro- and anti-inflammatory cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF-α, and GM-CSF) were investigated in 36 individuals (divided into 3 groups by severity – with a mild form of the infection/presymptomatic, moderately severe and severe/critical) within two periods – before and after the second week from the onset of symptoms of the disease.* ***Results:*** *In the period up to the 2nd week, the serum concentrations of IFN-γ ($p = 0.029$), IL-1β ($p = 0.017$), and IL-5 ($p = 0.014$) showed a statistically significant correlation with the disease severity, however in the later stage of the disease the cytokine levels did not show any clinical value.* ***Conclusion:*** *Cytokine testing could be used to predict the severity of COVID-19 infection which could support individual therapeutic decisions. Analysis of a larger group of patients is needed to unfold the full potential of such testing.*

Key words: *COVID-19, cytokines, hyperinflammation, dysregulation, biomarkers, severity*

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INTRODUCTION

To date, coronavirus disease 2019 (COVID-19), which originated in Wuhan, China, has spread to over 200 countries, including Bulgaria [1] infecting nearly 700,000,000 people of all ages, of whom approximately 1% have succumbed to the infection. Studying the immune response both during natural infection and after vaccination provides important information about the pathogenesis of the disease as well as about the immune response to the virus [2, 3]. To date, there is a body of evidence supporting the

important role of various cytokines in the pathogenesis of COVID-19. Studies have shown pathologically altered levels of several cytokines and chemokines, some of which are: interleukin (IL)-1b, IL-2, IL-6, IL-7, IL-8, IL-10, IL-12, IL-17A, IL-18, IL-23, TNFα, IFNg, G-CSF, GM-CSF, MCP-1, etc. [4, 5]. For this reason, the expression "cytokine storm" is often used to describe the immunopathogenesis of the disease. The term was first used in the medical literature for graft-versus-host reaction in 1993 [6] and describes a state of uncontrolled over-release of multiple, mostly pro-inflammatory cytokines, which in turn trigger the release of other cytokines. A vicious cycle of positive feedback is generated, resulting in dysregulation and dysfunction of the immune mechanisms [4]. The term "cytokine storm" partially overlaps with the concepts of "hyperinflammation" and "cytokine release syndrome". However, more specific criteria – which are the cytokines involved and what should be their levels in order to observe this phenomenon are not standardized currently. The term „cytokine storm" is also mentioned in the literature when describing the pathogenesis of many infectious diseases, including cytomegalovirus (CMV), Epstein-Barr virus (EBV)-associated hemophagocytic lymphohistiocytosis (HLH), group A streptococcal infections, influenza, SARS-CoV-1 (severe acute respiratory syndrome – coronavirus 1), MERS-CoV (Middle East respiratory syndrome – coronavirus) and others. The term gained serious popularity in 2005 with the spread of bird flu. Apart from infectious diseases, however, a „cytokine storm" is also observed in diseases proceeding with a process of intense inflammation ("hyperinflammation") and massive cytolysis such as acute pancreatitis, acute respiratory distress syndrome (ARDS), macrophage activation syndrome (MAS), as an adverse reaction in CAR-T-cell (chimeric antigen receptor T-cell) therapy and others [6].

Due to the complex interactions, pleiotropicity, and cascading cytokine interactions, the search for causal relationships has been extremely complex and has so far not led to an unequivocal categorical model of the exact sequence of events. Often, a parallel study of a large set of biological molecules is applied to discover different clinically relevant and prognostic markers for the course and severity of the infection in patients, including disease-related complications and post-infectious effects on the body.

OBJECTIVE

We hypothesized that serum cytokine levels monitored during the COVID-19 infection have the potential to prove to be useful biomarkers bearing information on the immunological phenotype, severity, and long-term consequences of the disease. Therefore, in the present study, we aimed to evaluate the serum concentrations of selected key pro- and anti-inflammatory cytokines in patients with different severity of COVID-19 infection in order to highlight those that would have prognostic significance for the course of the disease.

MATERIALS AND METHODS

Clinical material

Patients with COVID-19 infection diagnosed and treated at the University Hospital "Alexandrovska" and University Hospital "Sv. Anna" for the period April – November 2020 were included.

In the testing period of 2 weeks after the onset of the first symptoms of COVID-19 infection (or 8 days after a positive RT-PCR test), we evaluated 36 individuals (9 with a mild form of infection/asymptomatic; 16 with the moderately severe course; 11 – with the severe/critical course; men – 20; women – 16). Of them 24 patients were followed up for 2 weeks after initial disease presentation, (7 mild/asymptomatic, 10 moderate; 7 severe/critical). The severity of infection was categorized based on the CDC COVID-19 treatment guidelines [8], as follows:

- *mild/asymptomatic infection*: Individuals who test positive for SARS-CoV-2 using a virologic test (i.e., a nucleic acid amplification test (NAAT) or an antigen test) but who have no symptoms that are consistent with COVID-19 and individuals who have any of the various signs and symptoms of COVID-19 (e.g., fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhea, loss of taste and smell) but who do not have shortness of breath, dyspnea, or abnormal chest imaging.
- *moderate illness*: Individuals who show evidence of lower respiratory disease during clinical assessment or imaging and who have an oxygen saturation measured by pulse oximetry (SpO_2) ≥ 94% on room air at sea level.
- *severe/critical illness*: Individuals who have SpO_2 < 94% on room air at sea level, a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2) < 300 mm Hg, a respiratory rate > 30 breaths/min, or lung infiltrates > 50% and individuals who have respiratory failure, septic shock, and/or multiple organ dysfunction.

Test procedure

To measure the serum levels of cytokines, we used a multiplex method based on magnetic-sphere fluo-

rimetric technology (Invitrogen ProcartaPlexTM Human Cytokine Magnetic10-plex Panel) and a detection system Luminex®100™. Individual serum concentrations of 10 pro- and anti-inflammatory cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF-α and GM-CSF) was analysed. For initial results interpretation and calculation, we applied programs xPONENT® for detection and ProcartaPlex Analyst 1.0 for software analysis.

Statistical methods

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM Corp., Armonk, NY, USA). Categorical variables are presented as numbers and percentages, continuous variables with median, first, and third quartile (Q1 and Q3). The distribution of continuous variables was tested using the Kolmogorov-Smirnov test. The relationship between disease severity and categorical variables was assessed by Chi-square test or Fisher's exact test. For continuous variables, the association with disease severity was examined with a Kruskal-Wallis test. Comparisons between groups were performed with the Mann-Whitney test. Binary logistic regression (univariate and multivariate) was applied to estimate the dependence of the severity of the disease on the various factors. For statistically significant we accepted p values < 0.05.

RESULTS

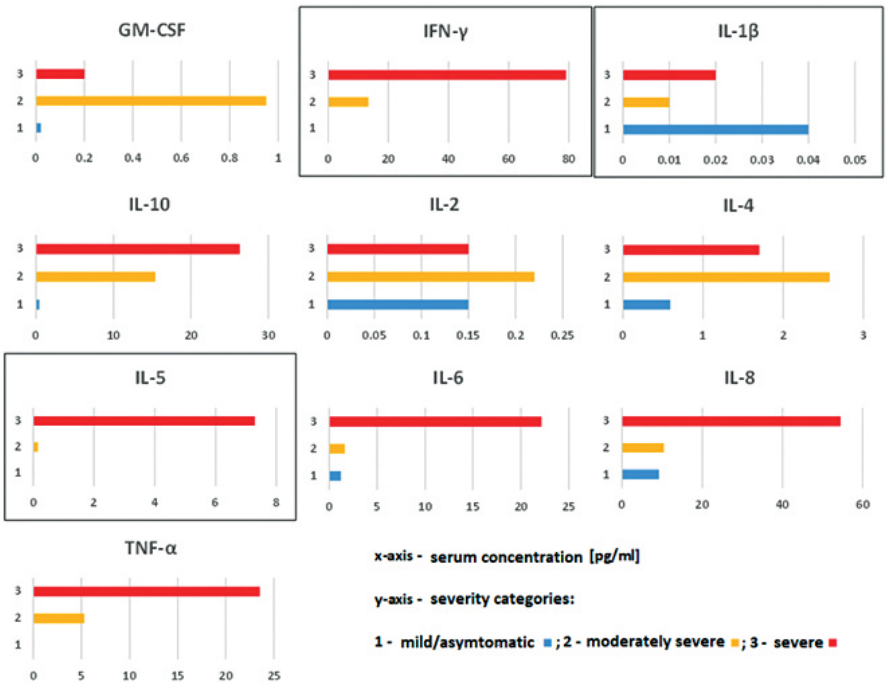
Determination of cytokine levels in the early infectious period (up to the 2nd week from the onset of symptoms)

The results for the individual cytokines depending on the severity of the infection are presented in Table 1 and Figure 1.

Table 1. Serum concentratons of cytokines up to the 2nd week from the onset of symptoms with regards to the disease severity

Indicator (up to 2 weeks)	Severity	N	Mean	Median	SD	Min	Max	Q1	Q3	p-value
GM-CSF	mild/asymptomatic	9	0,02	0,00	0,05	0,00	0,16	0,00	0,01	0,520
	moderately severe	16	0,95	0,00	3,49	0,00	14,01	0,00	0,04	
	severe/critical	11	0,20	0,00	0,45	0,00	1,47	0,00	0,16	
IFN-γ	mild/asymptomatic	9	0,01	0,00	0,03	0,00	0,06	0,00	0,03	**0,029**
	moderately severe	16	13,36	0,10	52,61	0,00	210,65	0,00	0,44	
	severe/critical	11	78,97	0,00	261,77	0,00	868,24	0,00	0,16	
IL-1β	mild/asymptomatic	9	0,04	0,04	0,04	0,00	0,10	0,01	0,07	**0,017**
	moderately severe	16	0,01	0,00	0,02	0,00	0,07	0,00	0,01	
	severe/critical	11	0,02	0,01	0,02	0,00	0,07	0,00	0,04	
IL-10	mild/asymptomatic	9	0,41	0,07	0,81	0,00	2,48	0,00	0,50	0,176
	moderately severe	16	15,39	0,45	31,38	0,00	105,47	0,02	14,56	
	severe/critical	10	26,27	2,77	54,39	0,00	174,68	0,02	38,44	
IL-2	mild/asymptomatic	9	0,15	0,05	0,30	0,00	0,94	0,00	0,14	0,542
	moderately severe	16	0,22	0,00	0,36	0,00	0,94	0,00	0,33	
	severe/critical	11	0,15	0,00	0,45	0,00	1,49	0,00	0,09	
IL-4	mild/asymptomatic	9	0,59	0,00	1,19	0,00	3,66	0,00	0,65	0,439
	moderately severe	16	2,58	0,26	6,53	0,00	24,85	0,00	1,18	
	severe/critical	11	1,70	0,00	5,33	0,00	17,75	0,00	0,26	
IL-5	mild/asymptomatic	9	0,01	0,00	0,02	0,00	0,07	0,00	0,01	**0,014**
	moderately severe	16	0,15	0,12	0,22	0,00	0,89	0,02	0,12	
	severe/critical	11	7,29	0,02	16,04	0,00	49,90	0,00	4,45	
IL-6	mild/asymptomatic	9	1,21	0,04	2,85	0,00	8,72	0,00	0,96	0,163
	moderately severe	16	1,63	0,59	3,23	0,00	13,24	0,21	1,45	
	severe/critical	10	22,13	1,93	55,21	0,00	178,39	0,07	13,12	
IL-8 (CXCL8)	mild/asymptomatic	9	9,19	3,35	15,57	1,01	48,90	1,45	9,97	0,075
	moderately severe	16	10,38	4,61	10,89	0,49	34,71	1,72	17,65	
	severe/critical	11	54,44	23,27	76,49	1,88	259,18	3,15	77,70	
TNF-α	mild/asymptomatic	9	0,02	0,00	0,03	0,00	0,08	0,00	0,03	0,109
	moderately severe	16	5,29	2,06	12,75	0,00	51,86	0,00	3,78	
	severe/critical	11	23,51	0,20	59,64	0,00	197,32	0,00	4,12	

N – number of individuals tested, Mean – the average concentration of particular cytokine, SD – standard deviation, Min – the lowest value, Max – the highest value, Q1 – first quartile, Q3 – third quartile.



N – number of participants, Mean – average concentration, SD – standard deviation, Min – lowest value, Max – the highest value, Q1 – first quartile, Q3 – third quartile. *Note: "severe" – to be read as severe/critical*

Fig. 1. Mean serum concentrations of tested cytokines in the early infectious period (up to week 2 of symptom onset) with regards to the severity of the disease

Based on the statistical analysis we observed an association of serum concentrations of IL-1β, IL-5, IL-8, and IFN-γ with the severity of the disease. However, data for IFN-γ, IL-1β, and IL-5 reached statistical significance. IL-8 values remained close but outside the accepted limit for statistical significance. It is important to note here that a single sample with values of IL-6 1686.85 pg/ml and IL-10 1508.12 pg/ml, was excluded from the analysis due to its potential to generate serious statistical bias. Further elaboration of associations with established statistical significance ($p < 0.05$) by the Kruskal-Wallis test with the non-parametric Mann-Whitney test showed levels of significance between individual groups (mild/asymptomatic: moderate; mild/asymptomatic: severe/critical; moderate-severe: severe) in Table 2:

Table 2. Intergroup comparison of statistical significance levels with the Mann-Whitney test

Indicator	Compared groups		
	mild/asymptomatic	mild/asymptomatic	moderately severe
	moderately severe	severe/critical	severe/critical
	P	P	P
IFN-γ	**0,015**	0,583	0,079
IL-1β	**0,006**	0,060	0,316
IL-5	**0,002**	**0,041**	0,900

From the presented data it is clear that the serum concentrations of IFN-γ and IL-1β differ significantly only between the groups of patients with a mild/asymptomatic and moderately severe form of course, and IL-5 levels – both between mild/asymptomatic and moderate, and between mild/asymptomatic and severe.

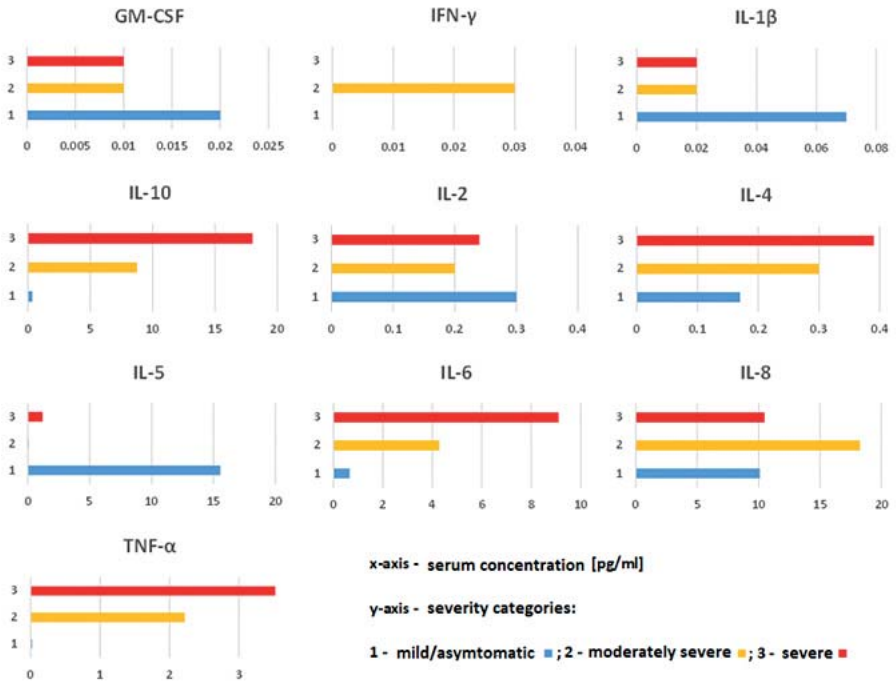
Determination of cytokine levels in the period after the 2nd week from the onset of symptoms

The results we obtained in the study for the patients with mild infection/asymptomatic, moderately severe, and severe course, during this later period are presented in Table 3 and Figure 3. No statistically significant associations were found. Close to statistical significance ($p = 0.07$) was only the level of TNF-α between studied groups. When comparing the results from the two testing periods (up to and after the 2nd week), there was a tendency for higher levels of IL-6, and IL-10 in the group with the severe course and increased concentration of IL-5 in the group of mild/asymptomatic subjects during the period after two weeks.

DISCUSSION

In this study, we tested the concentrations of 10 cytokines in 36 patients with different severity of COVID-19 infection and found associations of some cytokines with disease severity in the early stage of the illness (up to week 2 of symptom onset).

It is now well known that the delayed response to type I interferons in coronavirus infections is one of the main factors associated with a more severe course of the disease [9, 10]. Due to the early manifestation of



N – number of participants, Mean – average concentration, SD – standard deviation, Min – lowest value, Max – the highest value, Q1 – first quartile, Q3 – third quartile. Note: "severe" – to be read as severe/critical

Fig. 3. Serum concentrations in the period after the 2nd week from the onset of symptoms in the different groups depending on the severity of the disease

Table 3. Serum concentrations of cytokines after 2nd week from the onset of symptoms with regards to the disease severity

Indicator (after 2 weeks)	Severity	N	Mean	Median	SD	Min	Max	Q1	Q3	p-value
GM-CSF	mild/asymptomatic	7	0,02	0,00	0,04	0,00	0,11	0,00	0,02	0,900
	moderately severe	10	0,01	0,00	0,02	0,00	0,07	0,00	0,01	
	severe/critical	7	0,01	0,00	0,01	0,00	0,02	0,00	0,02	
IFN-γ	mild/asymptomatic	7	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,232
	moderately severe	10	0,03	0,00	0,08	0,00	0,25	0,00	0,01	
	severe/critical	7	0,00	0,00	0,00	0,00	0,00	0,00	0,00	
IL-1β	mild/asymptomatic	7	0,07	0,04	0,08	0,00	0,24	0,01	0,10	0,194
	moderately severe	10	0,02	0,00	0,03	0,00	0,07	0,00	0,06	
	severe/critical	7	0,02	0,00	0,04	0,00	0,10	0,00	0,04	
IL-10	mild/asymptomatic	7	0,36	0,00	0,80	0,00	2,14	0,00	0,35	0,376
	moderately severe	10	8,75	0,49	19,16	0,00	61,74	0,00	8,58	
	severe/critical	7	17,99	0,02	28,32	0,00	63,18	0,00	55,01	
IL-2	mild/asymptomatic	7	0,30	0,09	0,55	0,00	1,51	0,00	0,28	0,374
	moderately severe	10	0,20	0,00	0,58	0,00	1,84	0,00	0,05	
	severe/critical	7	0,24	0,00	0,55	0,00	1,49	0,00	0,09	
IL-4	mild/asymptomatic	7	0,17	0,00	0,31	0,00	0,78	0,00	0,39	0,613
	moderately severe	10	0,30	0,00	0,46	0,00	1,31	0,00	0,59	
	severe/critical	7	0,39	0,00	1,04	0,00	2,76	0,00	0,00	

Continuation of Table 3

IL-5	mild/asymptomatic	7	15,55	0,00	41,11	0,00	108,78	0,00	0,07	0,958
	moderately severe	10	0,06	0,00	0,12	0,00	0,37	0,00	0,12	
	severe/critical	7	1,18	0,00	3,01	0,00	8,01	0,00	0,25	
IL-6	mild/asymptomatic	7	0,66	0,00	1,41	0,00	3,80	0,00	0,81	0,204
	moderately severe	10	4,28	0,76	7,86	0,00	24,62	0,32	5,18	
	severe/critical	7	9,09	1,23	20,42	0,00	55,16	0,00	5,88	
IL-8 (CXCL8)	mild/asymptomatic	7	10,09	3,22	12,51	1,28	28,53	2,83	28,21	0,918
	moderately severe	10	18,25	5,08	38,50	1,92	127,12	2,67	12,40	
	severe/critical	7	10,47	8,01	9,20	1,01	24,33	1,85	20,79	
TNF-α	mild/asymptomatic	7	0,02	0,03	0,02	0,00	0,03	0,00	0,03	0,070
	moderately severe	10	2,22	2,74	2,30	0,00	7,07	0,03	3,09	
	severe/critical	7	3,60	0,03	8,01	0,00	21,61	0,00	2,74	

this phenomenon – even before the development of a pronounced clinical picture, before the patient seeks medical care, and because enough data has already accumulated in the world literature, we chose not to include type I IFN in the investigated cytokine set.

The overall picture shows a simultaneous increase of cytokines with extremely divergent functions: IFN-γ as a classical Th1 mediator associated with enhanced cell-mediated immunity; IL-5 – representative of Th2 immune response and with a key role in the development and mobilization of eosinophils and the generation of humoral immunity; IL-1β, representative of the classic pro-inflammatory triad – IL-1β, IL-6, and TNFα, obtained as a result of activation of the NF-κB-pathway associated with the acute inflammatory phase of innate immunity.

The differences in the rest of the investigated cytokines did not show statistical significance, but some showed trends. The other two pro-inflammatory cytokines – IL-6 and TNFα, and IL-10, mainly recognized for its anti-inflammatory properties, were also elevated. Usually, the mobilization of a Th1 or Th2 immune response is associated with the activation of counter-regulatory mechanisms that lead to an inhibition in the production and effect of cytokines of the "opposite" class. The fact that high levels of cytokines were simultaneously detected in both groups suggests the presence of an over-stimulus that overcomes the threshold of this inhibition. Ultimately, the multidirectional "unfocused" cytokine background leads to an overall dysregulation in the immunological response and an increased "braking distance" of the overactivated immune system.

IFN-γ levels showed a clear statistically significant relationship with disease severity. But what is the immunological logic behind this? Contrary to expectations that in the first phase of the disease, elevated Th1 cytokines would have a beneficial effect on viral clearance and overall survival of patients with COVID-19, the exact opposite trend was observed. Luo et al. found that despite increased total plasma concentration of IFN-γ, the proportion of IFN-γ-producing CD4+ T-lymphocytes decreased and that of CD8+ increased accordingly in the severe course [11]. Coppock et al. described decreased T-cell production of IFN-γ in response to mitogen stimulation, which they suggested was due to exhaustion as a consequence of the initial overactivation [12]. A study by Gadottie et al. [13] also found a relationship between serum IFN-γ concentrations and the severity of the course of COVID-19.

IL-1β as one of the major pro-inflammatory cytokines already mentioned shows surprisingly low concentrations (0.00-0.24 pg/mL) and a little albeit statistically significant difference between groups. However, we could speculate that this could be due to its short half-life of 21 minutes. Data in support of this finding are also provided by Del Valle et al. [14].

Increased levels of IL-5 in severe/critical and moderate forms are supported by the publications of Zhang et al. [15] and Liu et al. [16], while the team of Ghazvai et al. [17] did not find a statistically significant difference in serum IL-5 concentrations in patients with COVID-19 and healthy controls. IL-5 is one of the main type 2 cytokines. Its increase in the early phases of infection may cause impaired viral clear-

ance due to clonal/immune deviation. The phenomenon has been described in several infectious diseases such as leprosy, tuberculosis, leishmaniasis, HIV/AIDS, etc. [18] and represents a kind of "delusion" of the immune system, in which it deviates in the direction of developing an immune response that is suboptimal or generally unsuitable for fighting the specific pathogen.

Although the association with IL-6 was not among those found to be statistically significant in our study, we could speculate that it would have reached significance in a larger number of patients. The major role of IL-6 was presented in a meta-analysis by Mulchandani et al. [4]. The publication included 23 studies with a total of 2477 subjects (mild to moderate = 993 and severe/critical 1484). The data indicated the overall statistical significance of the association between serum IL-6 concentration and severity ($p = 0.00001$). Chang et al. [19] found that the IL-6 levels in non-severe forms of COVID-19 were not significantly different from the levels in healthy controls, in contrast to severe cases where there was a statistically significant difference. Other cytokines with marked differences in the three studied patient' groups but without statistical significance were IL-8, TNFα, and IL-10. IL-8 (CXCL8) is a cardinal chemokine in the chemotaxis of neutrophils and other immune cells to the site of inflammation. Several publications have shown its correlation with leukocytosis, neutrophilia, severity, and mortality in COVID-19 [20, 21, 22]. It was demonstrated that together, TNF-α and IL-8 trigger neutrophil NETosis [23, 24] and directly correlate with the risk of developing ARDS [22]. IL-10 is one of the main anti-inflammatory and immunoregulatory cytokines, and its increased levels are usually associated with a state of homeostatic immune tolerance or with a counterregulatory response, being negative feedback of increased pro-inflammatory cytokines. In cases with severe COVID-19, increased IL-6 induces the expression of IL-10, which, however, is unable to limit the unfolding systemic inflammatory process, and even impairs its resolution [25].

Of note, limitation of our study is the small study sample. The studied patients were a small group, therefore it is difficult to draw cardinal conclusions.

In conclusion, the cytokine correlation observed in the present study could be used to stratify patients into subgroups by severity/risk of complications, and type of disease course. Thus cytokine testing in selected patients could have prognostic and therapeutic value. However, a larger group of patients needs to be studied to support these observations.

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Disclosure Summary: *The authors have nothing to disclose.*

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ORIGINAL ARTICLE

ROLE OF BACTERIAL AND VIRAL INFECTIONS AND CO-INFECTIONS IN MISCARRIAGES

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Abstract. Aim: *To investigate the potential role of the following bacterial/viral panel (Chlamydia trachomatis, Ureaplasma urealyticum/parvum, Mycomplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV6, HHV7, HHV8) as causative factors for miscarriages in women by testing endometrial biopsies. Anaerobic and aerobic microorganisms causing dysbiosis and endometrial bacterial colonization by unbalanced growth were additionally tested.* ***Materials and methods:*** *In total, 65 patients with a history of early and late miscarriages were analyzed. DNA extractions, real-time qPCR, agarose gel-electrophoresis were applied. Comparative analysis of the current with previously obtained data on the described panel in menstrual tissue samples was performed.* ***Results:*** *In 64,6% of all tested endometrial biopsies bacterial and/or viral pathogens were detected. In 49,23% of all tested samples we found bacterial, while in 15,3% – viral pathogens. These results are similar to our previous data on menstrual tissue samples of infertile women – 61,1% infected, as 48,8% had bacterial and 22,2% had viral pathogens. Gardnerella vaginalis and Ureaplasma parvum were detected in 31,25% and 3,12% of all bacterial infected endometrial biopsies, significantly lower in comparison to the estimated rate of 69,31% and 61,36% on menstrual tissue. Anaerobic and aerobic dysbiosis were detected in 53,33% and 27% of the bacterial infected endometrial samples. In 13,33% a dysbiosis with a mixed etiology was found, while in 7% a dysbiotic condition with a totally absent findings of targeted bacteria and Lactobacillus was observed. EBV, CMV, HHV6 and HHV7 were detected in 30%, 30%, 20% and 20% of the positive for viral factors endometrial biopsies and in 40%, 7,5%, 10% and 42,5% in menstrual tissue samples. In the current study 62,5% bacterial co-infection and 12,5% bacterial/viral co-infection variants were found. Infections with the rest of the target pathogens were not detected in the endometrial biopsies. In contrast to the endometrial biopsy results, Mycomplasma hominis, Ureaplasma urealyticum and HSV2 were detected in our previous research on menstrual tissue samples.* ***Conclusions:*** *Our research suggests a possible dysbiosis as a consequence of bacterial/viral endometrial colonization, associated with miscarriages. We prove that menstrual tissue, containing parts of the functional endometrial layer, is a reliable and accurate noninvasive sample for infectious screening of the upper genital tract.*

Key words: *endometrium; inflammation; HHVs; infertility; menstrual tissue*

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INTRODUCTION

Miscarriage represents one of the most common and still unclear adverse pregnancy outcomes. Miscarriage is the spontaneous loss of a pregnancy before 12th gestational week (early miscarriage) or between 12th and 24th gestational week (late miscarriage). Unfortunately, a miscarriage occurs in one of five pregnancies and could cause serious physiological and psychological implications for the suffering patient and couples [1]. The reasons for a miscarriage are often unknown but in 50% of early miscarriages the fetus exhibits chromosomal aberrations, as morphological and structural alteration or abnormal chromosomal numbers [2]. Besides genetic causes, other factors are associated with increased risk for miscarriage as well: advanced age of both parents, especially for woman, ethnic origin, mother's psychological condition, very low or very high pre-pregnancy body mass index (BMI), stress, non-steroidal anti-inflammatory drugs, smoking, alcohol consumption [3-4].

It is known, that a number of infections are associated with miscarriage and other unfavorable outcomes such as stillbirth, preterm delivery [5-6]. Different infections are the cause of approximately 15% of early and 66% of late miscarriages [7-8].

The diagnostics of pathogenic microorganisms in the upper genital tract resulting in endometrial colonization, dysbiosis and inflammation is of a great significance. Some bacterial communities are able to multiply and colonize the female endometrium through mechanisms of virulence, such as mucin degradation, biofilm formation and antimicrobial resistance, leading to a state of dysbiosis [9]. The population of commensal microorganisms also plays an essential role in maintaining an eubiotic equilibrium, including Lactobacillus. Unbalanced bacterial fractions may also affect the intrauterine metabolic composition and thus, the growth of pathogenic bacteria in that imbalanced and unregulated, even toxic microenvironment is stimulated.

AIM

To investigate the potential role of the following bacterial/viral panel (*Chlamydia trachomatis, Ureaplasma urealyticum/parvum, Mycomplasma hominis/genitalium, Gardnerella vaginalis, HSV1/2, EBV, CMV, VZV, HHV6, HHV7, HHV8*) as causative factors for miscarriages in women by testing endometrial biopsies. Anaerobic and aerobic microorganisms causing dysbiosis and endometrial bacterial colonization by unbalanced growth were also tested.

MATERIALS AND METHODS

Patients and samples: In total, 65 females at the average age of 31,2 years were selected, mainly on the basis of their history of early (5th-12th gestation week) – 85% and/or late recurrent miscarriages (13th-27th gestation week) – 15%. No autoimmune disorders or other acquired diseases were present. The clean status in regard to the target bacterial/viral panel in cervical-vaginal swabs was the leading criteria for the patient selection. Anaerobic and aerobic microorganisms causing inflammation, dysbiosis and endometrial bacterial colonization by unbalanced growth were also tested. All selected women were tested for the described bacterial/viral pathogens in the upper genital tract by analyzing endometrial biopsies. The examination was performed at least 2 months after the miscarriage. Comparative analysis of the current with previously obtained data on the same pathogens in menstrual tissue samples was made.

Endometrial biopsies were performed using endometrial suction catheter following the established standards in the gynecological practice. As a transport medium for DNA preservation 2 ml 0.5M EDTA with pH 8.0 was used. Protein digestion with proteinase K (15 µl) for 24 hours at 65oC was performed and sediment was collected after centrifugation for 15 min at 8000 rpm. Total DNA was extracted from the 120 µl sediment using AmpliSens DNA isolation kit (Ecoli s.r.o, Slovak Republic). As a DNA carrier, 2 µl (15 mg/µl) glycogen was added after the preparation with a lysis buffer, simultaneously with the addition of the DNA-sorbent component. An additional washing step with 75% ethanol was included, resulting in a higher yield and purity of the obtained genomic viral/bacterial DNA. The amplification of the target viral and bacterial DNA fragments was performed, using AmpliSens commercial amplification kits (Ecoli s.r.o, Slovak Republic) and DNA Technology commercial amplification kits (Moscow, Russia) based on Real-Time qPCR.

RESULTS

The current research does not include healthy control group, because the procedure for obtaining endometrial biopsy is invasive.

In 49,23% of all tested endometrial biopsies we found bacterial, while in 15,3% viral pathogens. These results are similar to our previous data on menstrual tissue samples of infertile women – 48, 8% had bacterial and 22,2% had viral pathogens.

Gardnerella vaginalis and *Ureaplasma parvum* were detected in 31,25% and 3,12% of all bacterial infect-

ed endometrial biopsies, which are significantly lower percentages in comparison to the estimated rates on menstrual tissue – 69,31% and 61,36%, respectively. Anaerobic and aerobic dysbiosis were detected in 53,33% and 27% in all endometrial samples with bacterial invasion. Dysbiosis with a mixed etiology was reported in 13,33%, while in 7% a dysbiotic condition without the target bacteria or *Lactobacillus* was found (Fig. 1).

Active infection of *EBV, CMV, HHV6* and *HHV7* was detected in 30%, 30%, 20% and 20% of all infected with viral factors endometrial biopsies (Table 1) and in 40%, 7,5%, 10% and 42,5% of the menstrual tissue samples, respectively.

In the current study 62,5% bacterial and 12,5% bacterial/viral co-infection variants were found. Infections with the rest of the target pathogens were not detected in the endometrial biopsies, namely *Chlamydia trachomatis, Ureaplasma urealyticum, Mycomplasma hominis/genitalium, HSV1/2, VZV*, and *HHV8*. In contrast to the endometrial biopsies results, *Mycomplasma hominis, Ureaplasma urealyticum* and HSV2 were detected in our previous research on menstrual tissue samples with frequencies of 2,27%, 2,27% and 2,5%, respectively. All patients included in the current study had molecular-genetic testing for thrombophilia and in a high proportion (67,69%, 44/65) a genetically risk profile was detected.

DISCUSSION

Our study revealed positive active infection for the targeted bacterial and/or viral pathogens in 64,6% of the endometrial biopsies of patients with a history of recurrent early and/or late miscarriages. In 49,23% of all tested samples we found bacterial, while in 15,3% viral pathogens, which is consistent with our previously unpublished data on menstrual tissue of infertile women, where 48,8% had bacterial and 22,2% had viral pathogens [10].

Active infection with *Gardnerella vaginalis* was detected in 31,25% of all endometrial biopsies with bacterial invasion, which is two times lower in comparison to our previously reported results (in 69,31% of the menstrual tissue samples). Approximately one half of the cases with *Gardnerella vaginalis* dominant active infection were presented in co-infection variant with other anaerobic bacteria predominantly (*Peptostreptococcus spp., Eubacterium spp., Atopodium vaginae, Megasphaera spp., Veilonella spp., Dialister spp.*). The cases of Gardnerella vaginalis dominant active co-infection with mixed etiology, including anaerobic/aerobic bacteria (*Staphylococcus spp.*) were much rarer.

The presence of an abundant reservoir of *Gardnerella vaginalis*, combined with other potentially pathogenic bacteria configured in a polymicrobial biofilm, attached to the endometrium closely to the myometrium and amniotic compartment was reported by other researchers, which is sustained by our work [11-12]. Our data confirms the hypothesis that the endometrial bacterial colonization, resulting in dysbiosis and inflammation has a key role in the pathogenesis of non-viable pregnancy and adverse outcome as miscarriage. The implication of bacterial endotoxins, macrophages, *IL-1, TNF*, etc. in pro-inflammatory response against infection was already proved. We sustain the hypothesis that endometrial infection leads to pathogenic intrauterine inflammatory-immunological changes in the host, which disrupt the endometrial functions by decreasing its receptivity to embryo implantation and development [13-14].

The low frequency of *Gardnerella vaginalis* in the endometrial biopsies in comparison to menstrual tissue (31,25%) is considered as a normal finding, because all tested women were negative for the targeted pathogens in the cervical-vaginal swabs. The number of the currently tested endometrial biopsies (65) is relatively small, compared with the number of the previously tested menstrual tissue samples (180). It is so, because the endometrial biopsies were selected only on the basis of a positive history for recurrent miscarriages, while the menstrual tissue samples selection included a great variety of reproductive and healthy problems. The current study included testing of about 50% of the sexual partners of the selected women for the targeted bacterial pathogens by microbial culture (data not provided). Their negative status further explains lower frequency of *Gardnerella vaginalis* and *Ureaplasma parvum* in the present study and is interpreted as a limited possibility for sexual pathogens transmission. It can explain also partially the lower *Gardnerella vaginalis* frequency in endometrial biopsies, compared with examined menstrual tissue. We do not exclude also a variant of bacterial re-activation and uncontrolled growth in the upper genital tract due to unbalanced and negatively changed environment particularly in the cases of opportunistic pathogens.

Ureaplasma parvum was found in only 3,12% of all bacterial infected endometrial biopsies, which is extremely low percentage compared to our data from menstrual tissue samples – 61,36%. Our results do not comply with data in the literature, according to which *Ureaplasma parvum* infection is associated with recurrent miscarriages [15]. Our explanation of the data regarding the low frequency of *Ureaplasma parvum* is similar with the interpretation of *Gardnerel-*

la vaginalis results – negative results in the cervical-vaginal swabs, negative status among up to 50 % of the tested sexual partners and very strict criteria for inclusion in the study.

Anaerobic and aerobic dysbiosis were detected in 53,33% and in 27% from all endometrial samples with bacterial invasion. Dysbiosis with a mixed etiology was reported in 13,33%, while in 7% a dysbiosis without the targeted bacteria or Lactobacillus was found. Our data on the detected anaerobic and aerobic bacteria in the endometrial biopsies (Fig. 1) correlates with the results from high-tech studies on endometrial microbiome [16-17]. The most prevalent variant of *Gardnerella vaginalis* dominant anaerobic dysbiosis was in combination with *Prevotella bivia, Porphyromonas spp.* and the second one, *Mobiluncus spp., Corynebacterium spp., Eubacterium* and *Atopobium vaginae. Staphylococcus spp.* was detected predominantly in the cases of aerobic dysbiosis.

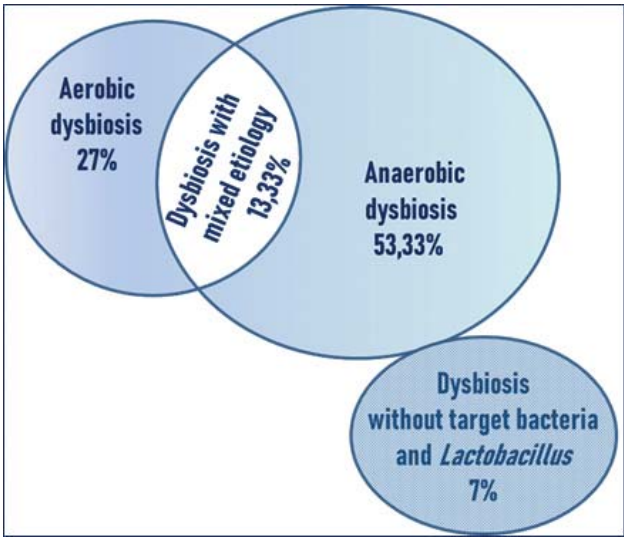

Fig. 1. Types of dysbiosis, as a consequence of unbalanced bacterial growth, endometrial colonization and inflammation. **Anaerobic dysbiosis** /positive bacterial findings/: *Mobiluncus spp., Corynebacterium spp. Eubacterium spp., Megasphaera spp., Veillonella spp., Dialister spp. Eubacterium spp., Gardnerella vaginalis, Prevotella bivia, Porphyromonas spp., Peptostreptococcus spp., Atopobium vaginae, Lachnobacterium spp., Clostridium spp.*; **Aerobic dysbiosis** (positive bacterial findings): *Streptococcus spp., Enterobacteriaceae spp., Staphylococcus spp.*

As a common feature of dysbiotic conditions, we accept the decreasing proportion of *Lactobacillus* to different degree, as was also reported in the literature [18-19]. Some scientific groups establish bacterial interactions (networks) and prove that *Lactobacillus* is negatively associated with *Gardnerella, Bifidobacterium and Atopobium* [17], as we also observed regarding *Gardnerella* and *Atopobium*.

It is worth mentioning that in the lower genital tract (cervical-vaginal swabs) all patients studied represented normocenosis and conventional normocenosis, but in the endometrium we found moderate and strong dysbiosis and co-infection. These differences are not a surprising finding, because the physiological nucleus of vaginal and endometrial microbiome was not clarified and determined. This ambitious task requires numerous future investigations. Some scientists report coinciding results concerning the vaginal and endometrial microbiome [20-21], but others describe significant differences, as it was established in our work [22]. The endometrial microbiome is significantly affected by hormonal changes and evidence for that is the exogenous progestin, which significantly alters the endometrial microbiome by reducing the phylotype diversity of *Lactobacillus spp.* [23]. Probably that could explain the differences which we found in our study.

According to our results *Chlamydia trachomatis*, *Mycoplasma hominis/genitalium* and *Ureaplasma urealyticum* were not detected, which does not comply completely with data in the published literature [24]. Only *Mycoplasma hominis* and *Ureaplasma urealyticum* were detected with a very low frequency (2,27%) in noninvasive menstrual tissue samples. *Mycoplasma hominis/genitalium* were reported with negligibly low frequency (2,9%) in the published data [25].

Active infection with *EBV, CMV, HHV6* and *HHV7* was detected in 30%, 30%, 20% and 20% of the positive for viral factors endometrial (Table 1), and in 40%, 7,5%, 10% and 42,5% of the menstrual tissue samples [10]. We suppose that these differences are due to the smaller number of the tested endometrial biopsies (65) compared to the menstrual tissue samples (180). If the endometrial biopsies are increased, then we expect to obtain more similar to the menstrual tissue samples results. Infections with *Herpesviridae* family have a direct negative, even cytotoxic effect on male spermatozoa. By vertical viral transmission during fertilization *HHVs* affects negatively the tissues of the female upper genital tract and the implanted newly formed embryo [26]. A common cause of failure in vitro fertilization attempts or miscarriages is the reactivation of *HHVs* as a result of hormonal stimulation by pregnancy hormones. Most probably a complex cascade of immunological rejection of the embryo was triggered in our cases with positive HHVs findings and recurrent miscarriages. Generally the unfavorable scenario includes increased NK cells level, disturbed Th1/Th2 balance, increased cytotoxicity, increased fibrosis and reduced levels of Tregs resulting from viral active infections [27-28].

Table.1. Positive results in the group of viral infected probands and co-infection variants.

Viral factor	Viral frequency	Description of viral-bacterial co-infection variant
*EBV**	30%	–
*CMV**	30%	● *CMV* + moderate aerobic dysbiosis with dominant *Streptococcus spp*.
		● *CMV* + dysbiosis with a mixed etiology: *Lachnobacterium spp*. + *Clostridium spp., Enterobacteriaceae* and *Streptococcus spp*.
*HHV6**	20%	● *HHV6* + strong anaerobic dysbiosis with a dominant *Eubacterium spp*.
*HHV7**	20%	–

*After applied individualized therapy (1,5-3 months), an entirely bacterial and/or viral cleaning of the upper genital tract was observed. Re-examination was performed on endometrial biopsies again.

The viral pathogens *HSV1/2, VZV* and *HHV8* were not detected in the tested endometrial biopsies, which is in agreement with the published data for negligibly low frequencies of these viral factors [29]. In the menstrual tissue samples we detected only **HSV2** with very low frequency (2,5%), which again supports the literature data. There is a controversial data for the impact of *HHV7* in contrast to the categorically proven association of *EBV* and *CMV* to the reproductive failure, including miscarriages, primary unexplained infertility, and even pathogenesis of preeclampsia in association to *HHV6* [30]. Our data assumes a potential association between the active asymptomatic infection with *HHV7* in the female endometrium with recurrent miscarriages and points *HHV7* as a risk co-factor implicated in the fertility loss. Further investigations are needed and they would be of a great importance, because *HHV7* turned out to be morphologically similar to *HHV6* and even more it participates simultaneously with *HHV6* in other diseases [31-32].

In 67,69% of all patients included in the current study a genetic risk profile for thrombophilia was detected. The high proportion of a risk molecular profile to thrombophilia in the current study determines its role as an independent risk factor or co-factor for the complex nature of miscarriages. Genetic risk profile to thrombophilia, combined with positive bacterial/viral status in endometrium could increase significantly the risk of a pregnancy loss.

In 15% of the patients included in the study both menstrual tissue and endometrial biopsy samples were tested. The observed negative or positive bacterial/viral findings were absolutely identical in both samples. The reported data confirms once again that the noninvasive menstrual tissue sample, containing parts of the functional endometrium is representative for assessment of the infectious status in the upper genital tract.

Finally, an individualized therapy (1,5-3 months) was applied to all bacterial/viral infected patients. A total bacterial and viral cleaning of the upper genital tract was observed by retesting new endometrial biopsies. Simultaneously a maintaining therapy for minimizing the risk of thrombophilia (during future pregnancy) was applied where necessary. It is worth mentioning that in 97% of the patients where an adequate therapy was applied, a natural conception was achieved. In the remaining 3% of the cases an endometrial polyp of hyperplastic type was found and in these patients the treatment still goes on.

CONCLUSIONS

Our pilot data shows that an individualized approach for treatment of fertility loss can gain positive impact in the fight against recurrent miscarriages.

Powerful tools of the modern medicine and molecular biology contribute to clarify easily reproductive problems and to perform adequate and successful therapy in the affected couples.

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ORIGINAL ARTICLE

CHANGES OF SERUM ANGIOTENSIN PEPTIDES, PRO-ENDOTHELIN-1 LEVELS IN WOMEN ONE YEAR AFTER PREECLAMPSIA AND THEIR ASSOCIATION WITH CARDIOVASCULAR RISK FACTORS

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Abstract. Introduction: *Women who suffered preeclampsia (PE) have two to four times higher risk for development of cardiovascular disease (CVD) compared with women with a history of normotensive pregnancy. Microvascular and endothelial dysfunction, mediated by different vasoactive factors have been suggested as attainable pathophysiological pathways. The study aimed to: (1) determine changes in circulating levels of key vasoactive peptides in sera of women with history of PE and in women who had a normal pregnancy 1 year after delivery and (2) investigate whether an association exists between these molecules and cardiovascular risk factors.* ***Materials and methods:*** *The current research examined 32 women one year after preeclampsia (mean age 25.3 ± 6.3 years) and control group of 20 women one year after normal pregnancy (mean age 25.6 ± 5.6 years). The enzyme-linked immunosorbent assay (ELISA) was used to determine serum levels of angiotensin II (AngII), angiotensin-(1-7) [Ang-(1-7)] and pro-endothelin-1 (Pro-ET-1).* ***Results:*** *We found that at 1 year after delivery 38.46% of women who suffered PE have developed hypertension and 5.77% have developed diabetes mellitus. Women who had normal pregnancy developed neither hypertension, nor diabetes mellitus 1 year after delivery. Serum AngII levels in women one year after PE were statistically significantly lower than in women one year after normotensive pregnancy 0.9 (0.55÷1.7) vs. 2.3 pg/ml (2.0 ÷ 2.9) (KW = 20.849; p = 0.0001). Ang-(1-7) concentrations in women one year after PE were lower than in women one year after normal pregnancy, but not significantly 1.7 (0.3÷4.5) vs. 3.2 ng/ml (0.2÷8.0) (p > 0.05). Levels of serum Pro-ET-1 in women one year after PE were statistically significantly higher than in women one year after normal pregnancy 322.65 (261.75÷391.85) vs. 248.7 pmol/L (231.05÷282.15) (KW = 6.639; p = 0.009). AngII showed correlation with AH grade (r = -0.33; 0.02), Ang-(1-7) (r = 0.27; 0.05), DBP (r = -0.28; p = 0.04), mean arterial pressure (r = -0.43; p = 0.002), pulse pressure (r = -0.28; p = 0.04), BMI (r = -0.30; p = 0.03), TC (r = -0.31; p = 0.03) and LDL (r = -0.30; p = 0.03). Pro-ET-1 correlated with age (r = 0.30; p = 0.03), mean arterial pressure (r = -0.25; p = 0.05) and pulse pressure (r = 0.41; p = 0.003).* ***Conclusion:*** *Our data showed an association between key vasoactive peptides and major CVD risk factors in women one year after PE. We suggest that imbalance between AngII, Ang-(1-7) and Pro-ET-1 could have a potential imply on the vascular wall after PE, reflecting persistent microvascular and endothelial*

injury/dysfunction postpartum. Further studies are warranted to clarify these vasoactive peptides' role in the ongoing vascular endothelial function after delivery and the pathogenic mechanisms determining development of CVD in previously PE women.

Key words: *angiotensin-II, angiotensin-(1-7), pro-endothelin-1, history of preeclampsia, cardiovascular risk factors*

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INTRODUCTION

Preeclampsia (PE) is a hypertensive disorder of pregnancy, which globally affects about 8 million pregnancies [1]. It is a major cause of maternal and perinatal morbidity and mortality [2]. PE is defined by the occurrence of new-onset hypertension (140/90 mm Hg) and either proteinuria (0.3 g in a 24h urine sample) or end-organ dysfunction developing after 20 weeks of gestation [3]. According to the current understandings preeclampsia is a systemic disease with generalized endothelial cells injury/dysfunction and multi-organ involvement [4].

Evidence exists that women with a history of preeclampsia are at a 2-4 times greater risk for the development of cardiovascular disease (CVD) postpartum than women with a history of normotensive pregnancy. Moreover, previously PE women are significantly more likely to die of CVD compared with women who have had normal pregnancy [5]. It have been estimated that women with early-onset or severe preeclampsia have highest risk of CVD later in life [6-10]. The exact pathophysiological mechanisms leading to this increased CV risk still remains unclear and might in fact be multifactorial. It has been hypothesized that the initial vascular damage and dysfunction sustained during a pregnancy complicated by preeclampsia may persist chronically together with the compromised vascular endothelial function. Vasoactive peptides angiotensin II, angiotensin-(1-7) and pro-endothelin-1 have been suggested to play a key role in these processes.

Normal pregnancy is characterized with hemodynamic changes which activate the renin angiotensin system (RAS), resulting in water and sodium retention and increased BMI. Angiotensin II (AngII) and angiotensin-(1-7) [Ang-(1-7)] are major components of RAS. They have counter-regulatory impact in the systemic circulation and in tissues important in cardiovascular regulation [11]. AngII is a potent vasoconstrictor, while Ang-(1-7) has antihypertensive actions via vasodilatory effects on vascular smooth muscle cells [12, 13]. Therefore, Ang-(1-7) is accepted as an endogenous counter-factor of AngII and balance of the two peptides may be required to regulate normal vascular activity. Dysregulation of AngII and Ang-(1-7) might be one of the possible reasons for development of cardiovascular and renal diseases. Large number of studies has demonstrated that circulating levels of AngII are lower in clinically manifested PE, compared with uncomplicated pregnancies [14-19]. It has also been revealed that concentrations of AngII are markedly diminished at diagnosis of PE and are closely associated with the severity of disease. Evidence exist that, AngII sensitivity of the vascular system is elevated in pregnancies at high risk for preeclampsia, long before PE is overt. Moreover, sensitivity of AngII remains higher in former-preeclamptic patients compared to normal pregnancies [20] and microvascular damage sustained during a preeclamptic pregnancy may persist postpartum in women with a history of PE [21]. It can be pointed-out that most researchers have evaluated concentrations of AngII and Ang-(1-7) pre-pregnancy or intrapartally. Interestingly, the balance between circulating AngII and Ang-(1-7) in PE women post-partum (where the residual effects of the preeclamptic pregnancy on vascular wall structure and function have been suggested to persist) has not been fully determined.

There is growing evidence for impaired endothelial function, generalized vascular constriction and increased resistance in PE pathophysiology. Hence, the reduced placental perfusion might be related with widespread dysfunction of the maternal vascular endothelium [22]. Considering that, Verhaar et al. (2001) demonstrated that women who have had PE have persistent microvascular dysfunction postpartum, mediated in part, by increased sensitivity to AngII [23]. Thus, maternal vascular endothelial cells are believed to take a central part in the pathogenesis of PE [23-28]. In this context, endothelins have been suggested to play key role in vascular function and

dysfunction in PE. They are a group of endogenous peptides that express a strong and long-lasting vasoconstrictor effect [29, 30]. Endothelin-1 (ET-1) is the most potent vasoconstrictor agent, originally isolated from the endothelial cells of aortic media. The biological effects of ET-1 are vasoconstriction, bronchoconstriction and aldosterone secretion. ET-1 is known as the mature peptide and it has a very short half-life (about a few minutes). Pro-endothelin-1 (pro-ET-1) is endothelin-1 precucursor's fragment. Contrary to ET-1, pro-ET-1 is able to be detected for hours in the circulation [31, 32]. Studies have suggested that it can be used to indirectly assess the release of mature peptides in pathological conditions such as preeclampsia [33, 34]. However, there are no data in the literature on evaluation of serum pro–ET-1, AngII and Ang-(1-7) concentrations in women one year after preeclampsia, where pathways of endothelial activation and vascular injury have not been fully understood.

AIM

This study aimed to: (1) determine circulating AngII, Ang-(1-7) and Pro-ET-1 levels in sera of women who suffered preeclampsia and women who had normotensive pregnancy at 1 year after delivery and (2) investigate for an association between these vasoactive peptides and cardiovascular risk factors.

MATERIALS AND METHODS

Subjects

The following inclusion criteria applied: women who have had 1 year ago clinical symptoms and laboratory criteria for preeclampsia; maintaining a regular diet and exercise routine throughout the research; signed informed consent form to take part in the investigation. The diagnostic criteria of preeclampsia used in this study were concordant with the definition given in the European Society of Cardiology 2018 Guideline for the management of cardiovascular diseases during pregnancy: gestational hypertension with significant proteinuria > 300 mg/24 h urine collection or the extrapolated amount from a timed collection [35]. The following criteria applied for patients exclusion: presence of diabetes mellitus, renal and heart disease, signs of chorioamnionitis, presence of a fetus with a chromosomal abnormality.

The study was part of a university scientific project under the National Program "Young Scientists and Postdoctoral Students-2", approved by the Ethics Committee of Medical University – Pleven (Protocol N70/2023). All participants signed informed consent. Study procedures followed all guidelines for ethical standards of the responsible committee on human experimentation as well as the Helsinki Declaration of 1975, as revised in 2000. All patients were residing in the Clinic of Obstetrics and Gynecology, University Hospital "Georgi Stranski", Pleven, an year ago (in 2022). Sera of subjects were taken from February to March 2023 for the aims of the present investigation. The study group consisted of 32 women one year after preeclampsia (mean age 25.3 ± 6.3 years) and a control group of 20 women one year after normotensive pregnancy (mean age 25.6 ± 5.6 years).

ELISA

Enzyme-linked immunosorbent assay (ELISA) was used for determination of AngII, Ang-(1-7) and Pro-ET-1 levels in serum samples. The following ELISA kits were used (Human Angiotensin II ELISA Kit, AssayGenie), [Human Angiotensin 1-7 ELISA Kit (Colorimetric), Novus Biologicals (Bio-techne)] and [(Human pro-ET1(Pro-endothelin 1) ELISA Kit, AssayGenie] according to the manufacturer's instructions.

Blood pressure

Arterial blood pressure was measured using a standard aneroid sphygmomanometer, to the nearest 2 mm Hg, in the dominant arm after at least 10-min rest in the supine position. Blood pressure measuring was performed by the Riester blood pressure measuring tool – Type-Precisa® N; Ø 64 mm aluminium, single-tube, cotton hook cuff, adult, No.1362-104.

Statistical analyses

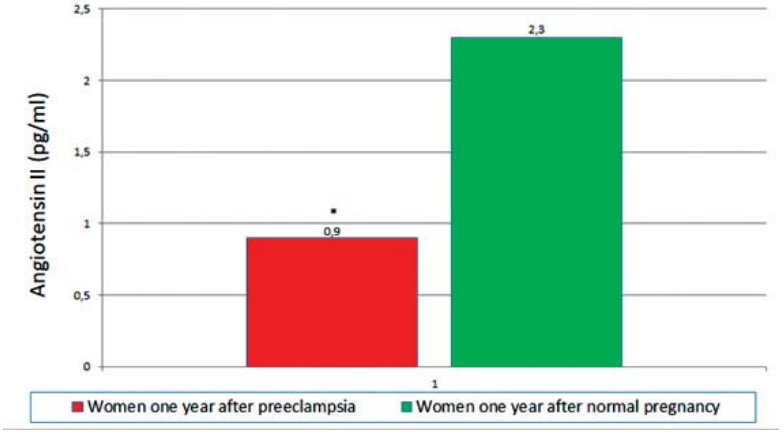
In order to analyze the research data the following computer programs were used: Excel (Microsoft Corporation, Redmond, WA), SPSS and Statgraphics Plus (Manugistics, Rockville, MD) for Windows. The level of significance was determined as ($p < 0.05$). Stnd. Skewness and Stnd. Kurtosis tests were used for checking the normality of distribution and equality of variances. To discover significant differences between groups, Student's t-test and ANOVA with mean ± SD was used in cases with normal distribution (LSD, Tukey HSD, Scheffe, Bonferroni, Newman-Keuls, Duncan). χ^2 and Kruskal–Wallis H test with median (M) value was used in cases with different from normal distribution, together with first and third quartile Q1 and Q3; (twenty-fifth and seventy-fifth percentile P25 and 75P). Pearson type of correlation was used.

RESULTS

Clinical data of women who had preeclampsia and women who had normotensive pregnancy at 1 year after delivery are presented in Table 1.

We found that at 1 year after delivery 38.46% of women who suffered PE have developed hyperten-

sion and 5.77% have developed diabetes mellitus. Women who had normotensive pregnancy developed neither hypertension (AH), nor diabetes mellitus (DM) 1 year after delivery. Serum AngII levels in women one year after PE were statistically significantly lower than in women one year after normal pregnancy 0.9 (0.55 ÷ 1.7) vs. 2.3 pg/ml (2.0 ÷ 2.9) (KW = 20.849; p = 0.0001) (Table 2) (Figure 1) Ang-(1-7) concentrations in women one year after PE were lower than in women one year after normal pregnancy, but not significantly 1.7 (0.3 ÷ 4.5) vs. 3.2 ng/ml (0.2 ÷ 8.0) (p > 0.05) (Table 2) (Figure 2). Levels of serum Pro-ET-1 in women one year after PE were statistically significantly higher than in women one year after normal pregnancy 322.65 (261.75 ÷ 391.85) vs. 248.7 pmol/L (231.05 ÷ 282.15) (KW = 6.639; p = 0.009) (Table 2) (Figure 3). AngII showed correlation with AH grade (r = -0.33; 0.02), Ang-(1-7) (r = 0.27; 0.05), DBP (r = -0.28; p = 0.04), mean arterial pressure (r = -0.43; p = 0.002), pulse pressure (r = -0.28; p = 0.04), BMI (r = -0.30; p = 0.03), TC (r = -0.31; p = 0.03) and LDL (r = -0.30; p = 0.03). Pro-ET-1 correlated with age (r = 0.30; p = 0.03), mean arterial pressure (r = -0.25; p = 0.05) and pulse pressure (r = 0.41; p = 0.003).

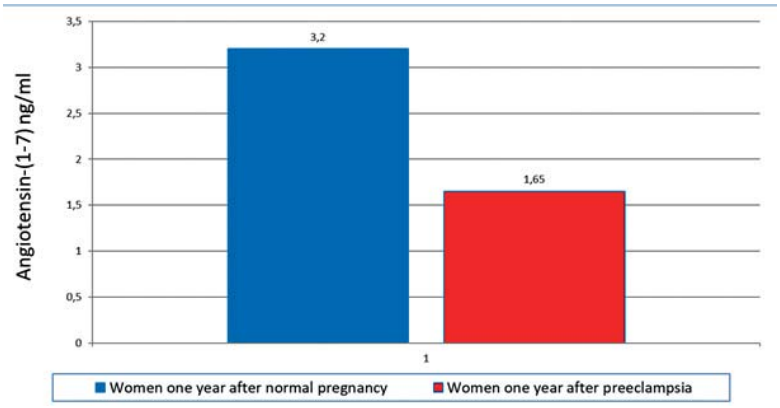


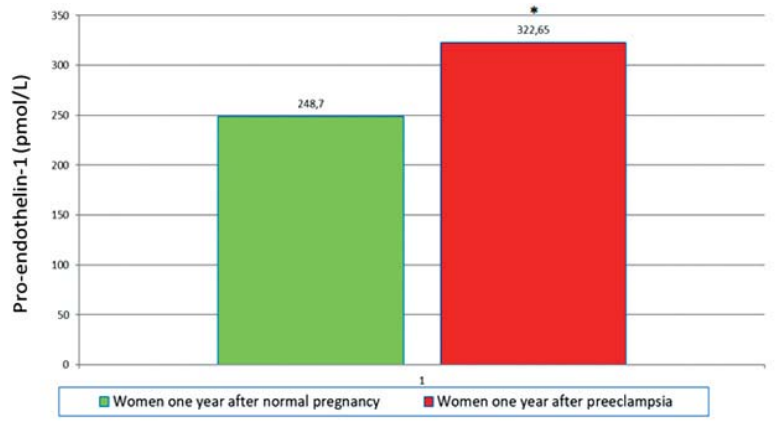
Serum AngII levels in women one year after preeclampsia were statistically significantly lower than in women one year after normal pregnancy 0.9 (0.55 ÷ 1.7) vs. 2.3 pg/ml (2.0 ÷ 2.9) (p = 0.0001). Values were presented as mean ± SD; *P < 0.05 compared with women one year after normal pregnancy.

Fig. 1. Serum Angiotensin II levels in in women one year after preeclampsia and one year after normal pregnancy determined by ELISA

Ang-(1-7) concentrations in women one year after preeclampsia were lower than in women one year after normal pregnancy, but not significantly 1.7 (0.3 ÷ 4.5) vs. 3.2 ng/ml (p > 0.05). Values were presented as mean ± SD; P > 0.05 compared with women one year after normal pregnancy.

Fig. 2. Serum Angiotensin-(1-7) levels in women one year after preeclampsia and one year after normal pregnancy determined by ELISA





Levels of Pro-ET-1 in women one year after preeclampsia were statistically significantly higher than in women one year after normal pregnancy 322.65 (261.75 ÷ 391.85) vs. 248.7 pmol/L (231.05 ÷ 282.15) (p = 0.009). Values were presented as mean ± SD; *P < 0.05 compared with women one year after normal pregnancy.

Fig. 3. Serum Pro-endothelin-1 levels in women one year after preeclampsia and one year after normal pregnancy determined by ELISA

DISCUSSION

Preeclampsia is one of the most common pregnancy disorders. PE occurs as a complication in approximately 2–8% of all pregnancies throughout the world and has been identified as one of the leading causes of maternal and perinatal morbidity and mortality. It is a well-known fact that hypertensive disorders in pregnant women are associated with an increased risk of developing future cardiovascular diseases after delivery [36, 37, 38]. Furthermore, women who develop preeclampsia are twice as likely to die from heart failure or stroke [39, 40]. A large proportion of women who experienced early-onset preeclampsia had major cardiovascular risk factors in the fifth decade of life, compared with healthy controls [41]. However, the exact pathways of development of CVD after preeclamptic pregnancy remain unclear.

Current approaches towards preeclampsia provide data that it is a systemic disease characterized by new-onset hypertension and signs of damage to another organ system, most often involving alteration of liver or kidneys function with or without proteinuria. Generalized endothelial involvement is believed to be one of the main pathogenic pathways in the development of preeclampsia. In addition, studies have shown that some groups of vasoactive molecules associated with vessel remodelling as angiotensin peptides and endothelins are released into the circulation of hypertensive disorders of pregnancy [14-21, 29-34]. There is not enough information for their role in vascular endothelial function postpartum.

Given the increased risk of CVD development in women who have suffered PE and the effect of major vasomodulatatory factors on the vascular function and endothelial activation, we examined women with a history of PE. Our data confirmed that there is a link between PE and CVD: 38.46% of women who suffered PE have developed AH at 1 year after delivery and 5.77% have developed DM. Women who had normal pregnancy developed neither AH, nor DM one year after delivery. Present results demonstrated that serum AngII were statistically significantly lower in women one year after preeclampsia than in women one year after normal pregnancy. Current data is analogic with AngII concentrations in previous studies involving patients with clinically manifested PE [43, 44, 54, 56, 57]. Of note, results in our research were measured in women who had PE, 1 year after delivery. This finding assumes that the initial vascular damage and dysfunction sustained during the preeclamptic pregnancy persists chronically, probably mediated by increased sensitivity to AngII. This processes can favor persistent microvascular constriction and increased resistance. The insignificantly lower Ang-(1-7) concentrations in women one year after preeclampsia compared with these in women one year after normal pregnancy show failure of RAS' regulatory mechanisms. Moreover, the elevated levels of Pro-ET-1 in women one year after preeclampsia than in women one year after normotensive pregnancy are similar to previous studies with clinically manifested preeclampsia [71, 72]. This is also evidence for persisting endothelial damage postpartum. We suggest that Pro-ET-1 plays an important role in the vascular endothelial dysfunction after PE.

AngII and Ang-(1-7) are key components in the two renin angiotensin system arms. They exhibit counter-regulatory effects in the systemic circulation, as well as in tissues important in cardiovascular regulation [42]. Considering the altered regulation of maternal RAS system during preeclampsia, Wallukat et al. [43] and Siddiqui et al. [44] found that levels of renin, AngII and aldosterone are lower in PE, compared to uncomplicated pregnancies. Authors also showed increased presence of AngII type 1 receptor agonistic autoantibodies (AT1-AA). Therefore, Irani et al. (2011) concluded that this process mimics the effect of AngII, in the plasma of preeclamptic women during pregnancy [45]. Although, the levels of renin, AngII and aldosterone seem to normalize within 3–4 months after delivery [25], sensitivity of AngII remains higher in former-preeclamptic patients compared to normal pregnancies [46,47]. It seems that increased sensitivity to angiotensin II during and after a hypertensive pregnancy increases the risk of persistence or development of hypertension after delivery [48–52].

Zhang et al. (2017) analysed serum AngII in relation with the clinical manifestation of PE. ELISA was used to measure serum AngII, urinary angiotensinogen and urinary TGFb1 in preeclampsia, pregnancy-induced hypertension and normotensive pregnancy patients. They found that following algorithms (measuring urinary angiotensinogen or a combination of urinary angiotensinogen, serum AngII and urinary TGFb1) can help in early diagnosis of PE [53]. In their investigation, Chase et al. (2017) compared AngII concentrations prior to pregnancy, and changes during pregnancy, between women who developed preterm PE compared with other pregnancy outcomes. Researchers investigated 55 subjects longitudinally and divided on groups-gestational hypertension, term PE and preterm PE. Researchers found that pre-pregnancy AngII levels were significantly higher in women who went on to develop preterm PE, term PE. Third trimester AngII levels were similar between all groups. The change of AngII level during pregnan-

cy was significantly lower in preterm PE pregnancies compared to all other outcomes. Authors concluded that AngII levels are reduced intrapartum in preterm PE [54].

Merril et al. (2002) demonstrated for the first time, increased plasma Ang-(1-7) in normal pregnant subjects compared with nonpregnant subjects and decreased Ang-(1-7) in preeclamptic subjects compared with normal pregnant subjects. Investigators concluded that in preeclampsia the decreased plasma Ang-(1-7) in the presence of elevated AngII is consistent with the development of hypertension [55]. Recently, Brosnihan KB et al., 2020 demonstrated results from longitudinal study of angiotensin peptides in normal and pre-eclamptic pregnancy. The researchers reported that plasma AngII levels were elevated in normal pregnant subjects as early as 16 weeks of gestation and maintained throughout gestation. In PE subjects plasma Ang II concentations were elevated at 16-33 weeks as compared with postpartum levels. PE subjects showed reduced plasma AngII (at 35-37 weeks of gestation) compared with normal pregnant subjects. Plasma Ang-(1-7) was unchanged in both groups [56].

Leanos-Miranda et al. (2018) examined AngII concentrations in preeclampsia using a cross-sectional study. Authors found that concentrations of AngII are markedly diminished at diagnosis of PE and are closely associated with the severity of disease. Besides that, researchers also reported that "women who subsequently developed preeclampsia had lower AngII levels than women with normal pregnancies, and these changes became significant at 24 weeks onward" [57]. The study of Baev et al. (2019) compared changes of AngII and Ang-(1-7) levels in early and late-onset preeclampsia. They found increase in the production of AngII and absence of compensatory increase of Ang-(1–7) in early-onset preeclampsia group. The investigators concluded that this may be an important pathogenetic mechanism of early-onset preeclampsia features [58].

The researches of Van der Graaf [20] and Stanhewicz et al. showed that increased angiotensin II sensitivity contributes to microvascular dysfunction in women who have had preeclampsia. More concretely, Stanhewicz's study evaluated endothelium-dependent dilatation, AngII sensitivity, and the therapeutic effect of AngII receptor blockade on endothelium-dependent dilatation in vivo in the microvasculature of 12 women with a history of PE and control group of 12 women who had a healthy pregnancy. The research team found that women with preeclampsia had significantly reduced endothelium-dependent dilatation and NO-dependent dilation and that PE is associated with augmented vasoconstrictor sensitivity to AngII. Stanhewicz et al. made important conclusions: "Microvascular damage sustained during a preeclamptic pregnancy may persist postpartum. The mechanisms mediating this dysfunction include a reduction in NO-dependent dilatation and an increased sensitivity to angiotensin II" [21].

Khlestova G et al. studied dynamics of renin, AngII, and Ang-(1-7) during pregnancy and predisposition to hypertension-associated complications [59]. Authors reported that a change exists between proportions of AngII and Ang-(1-7). This shift attests to high predisposition of pregnant women to hypertension-related complications. Velloso et al. investigated plasma levels of Ang-(1-7) in preeclamptic women and found reduced Ang-(1-7) concentrations in patients [60]. However, no one of these investigations determined Ang-(1-7) concentrations postpartum.

Endothelins are a family of endogenous peptides that express long-lasting vasoconstrictor effect [61, 62]. ET-1 is predominantly released by vascular endothelium. This peptide has strong vasoconstrictor characteristics [63, 64]. ET-1 has very short half-life (less than 5 minutes) and rapidly cleared from the circulation, and therefore it is difficult to measure. Pro-endothelin-1 has been reported as endothelin-1 precucursor's fragment which is able to be detected for several hours in the circulation [65]. This allows the possibility pro-ET-1 to be used to indirectly assess the release of mature ET-1 in sera. A large majority of researches describe endothelins as key mediators in vascular tone regulation in pathological conditions as sepsis and cardiovascular diseases [66-70]. Additionally, data indicates that endothelins have also shown to predict prognosis in heart failure [66].

To the current moment, there are only two studies in the literature which describe pro-ET1 circulating levels in hypertensive disorders of pregnancy. In 2014, Velman et al. prospectively investigated the plasma concentrations of this peptide in 147 pregnant women in the obstetric triage units of the University Hospital of Zurich, Switzerland. Of the 147 pregnant women included in the study, 27 (18.4%) of them were diagnosed with PE. Pro-ET1 levels were significantly higher in PE patients compared to controls. Moreover, the authors found that pro-ET1 was an independent predictor for the development of PE [71].

In another study, Malte et al. (2018) investigated pro-ET1 levels in 215 third-trimester pregnant women admitted to the Department of Obstetrics, Aarhus University Hospital, Denmark with suspected PE and 94 women with normal pregnancy. The following patient groups were formed: women with subclinical PE, es-

sential hypertension, gestational hypertension, moderate PE and severe PE/HELLP. The researchers found that pro-ET1 levels could be used to predict the progression of mild to moderate PE to severe PE/HELLP in the 1-2 week arm from this biomarker's AUC score of 0.82 and 0.78, respectively. Malte et al. concluded that pro-ET1 is a promising indicator for predicting the development of PE/HELLP syndrome in the third trimester of pregnancy [72].

To this date, there have been no investigations to measure serum angiotensin peptides and pro-ET-1 levels in patients with history of preeclampsia. Studies have involved women with clinically manifested hypertensive disorders of pregnancy, but none of the above commented researches have focused on changes of vasoactive peptides' levels after preeclampsia. The major findings of the present study were: disrupted balance between AngII, Ang-(1–7), pro-ET-1 and relationship between them and major cardiovascular risk factors after preeclamptic pregnancy. Hereby, we suggest that altered steadiness between these vasomodulating factors might play an important role in the pathogenic mechanisms of chronically persistent vascular damage and dysfunction determining development of CVD after PE. To the best of our knowledge, our study has provided for the first time the equilibrium between the two main RAS pathways and pro-ET-1 at one year after delivery in women who suffered PE.

The current study reveals that in previously preclamptic women, an altered balance between the vasoconstrictor and vasodepressor arms [Ang II/Ang (1-7)] of the RAS is observed 1 year after delivery. Based on the similar observations of the above-mentioned investigations of decreased AngII/Ang (1-7) levels in clinically manifested PE, we suggest that factors like increased angiotensin II sensitivity might contribute to vascular function and dysfunction in women with a history of preeclampsia. Thus favors microvascular constriction, increased resistence and endothelial dysfunction. Further longitudinal studies with serial measurements and more specific methods like Western blots, gelatine zymography and immunohistochemical analysis would allow more precise assessment of AngII, Ang-(1-7), Pro-ET-1 impact on vascular wall and elucidate their role in the pathogenesis of CVD after PE.

CONCLUSION

Current evidence demonstrated for the first time that in women who had PE, serum levels of AngII and Ang-(1-7) are decreased at 1 year after delivery. Contrary to that, Pro-ET-1 levels are elevated one year after PE. Our study gives arguments that altered balance between crucial vasoactive peptides might be involved in the pathogenic pathways of chronically persistent microvascular and endothelial injury/dysfunction after preeclamptic pregnancy. Consequently, changes of serum AngII, Ang-(1-7) and Pro-ET-1 levels postpartum might contribute to the mechanisms determining development of CVD after PE.

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ORIGINAL ARTICLE

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IN VITRO EVALUATION OF CORN SILK EXTRACT AS A POTENTIAL ALTERNATIVE THERAPY FOR VULVOVAGINAL CANDIDIASIS

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Abstract. Aim: *To determine the effectivity of corn silk extract in inhibiting the growth of C. albicans.* ***Materials and Methods:*** *Corn silk used in this study was Zea mays L. var. indurata and was collected from Desa Suka Makmur, Deli Serdang, Sumatera Utara Province, Indonesia. Tested C. albicans is C. albicans from VVC patients, obtained from the Microbiology Laboratory of Faculty of Medicine, Universitas Sumatera Utara. Preparation of this study included corn silk extraction by maceration method with 96% ethanol, followed by dilution with 10% DMSO (Dimethyl Sulfoxide) into concentrations of 75%, 50%, 25%, and 12.5%. Well diffusion method was used to detect growth inhibition of C. albicans and agar dilution was used to determine MIC (Minimum Inhibitory Concentration) and MFC (Minimum Fungicidal Concentration) of corn silk extract. Data were statistically analyzed with Kruskal-Wallis analysis.* ***Results:*** *From this study, corn silk extract of all concentrations showed an inhibitory effect against C. albicans. The mean diameter of inhibition zones for each concentration respectively from highest to lowest concentration were 27.4875 ± 0.3838 mm, 26.7250 ± 0.2533 mm, 25.7250 ± 0.2598 mm, and 24.9375 ± 0.2462 mm. Kruskal-Wallis analysis showed significant results (p-value 0.001). MIC and MFC were 1.5625% and 6.25%, respectively.* ***Conclusions:*** *Corn silk extract of concentrations 75%, 50%, 25%, and 12,5% have antifungal activity against C. albicans. The higher the concentration of corn silk extract, the higher the efficacy in inhibiting the growth of C. albicans.*

Key words: *Candida albicans; corn silk extract; vulvovaginal candidiasis*

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INTRODUCTION

Vulvovaginal candidiasis (VVC) is an infection caused by Candida species, especially *C. albicans* [1]. The incidence of VVC is high, approximately 78% of women have one episode of VVC and 34% have two or more episodes in a lifetime [2].

Over the past few years, treatment of VVC is challenging due to the increasing resistance of *C. albicans* to azoles [3]. According to previous study, 97.4% *C. albicans* was resistant to fluconazole and 94.9% was resistant to ketoconazole [4]. A study in Bulgaria showed *C. albicans* isolates were resistant to all azoles, even to echinocandins [5]. For this reason, proven alternative therapy for VVC is needed to treat azole-resistant VVC. Natural products especially plants are believed to be potential alternative therapies as they contain biologically active molecules,

one of those plants is corn [6]. Corn has silk which is widely believed to be inutile, but unexpectedly has been proven to be useful as sunblock [7]. Corn silk also has antidiabetic activity [8]. Corn silk contains alkaloids, flavonoids, terpenoids, and tannins [9]. Those phytochemical compounds are known for their antifungal effects [10, 11, 12, 13]. Thus, corn silk may be a potential alternative therapy for VVC.

This study aims to determine the effectivity of corn silk extract in inhibiting the growth of *C. albicans* with the expectation that corn silk may be an alternative therapy for VVC.

MATERIALS AND METHOD

This study was conducted at the Microbiology Laboratory of Faculty of Medicine at Universitas Sumatera Utara. The protocol of this study has obtained approval from the Research Ethics Commission of Universitas Sumatera Utara /H. Adam Malik General Hospital, Medan, Indonesia with ethical clearance number 98/KEP/USU/2021.

Materials

Corn silk used in this study was Zea mays L. var. indurata and was collected from Desa Suka Makmur, Deli Serdang, Sumatera Utara Province, Indonesia. The corn silk used in this study had previously been identified by Herbarium Medanese with identification result number 6539/MEDA/2021. Moldy and dried-out corn were excluded from this study. Tested *C. albicans* is *C. albicans* from VVC patients, obtained from the Microbiology Laboratory of Faculty of Medicine Universitas Sumatera Utara. Contaminated culture was excluded from this study.

Preparation of Corn Silk Ethanol Extract

Corn silk collected was air-dried and ground into 400 g powder, then extracted by maceration method with 96% ethanol. Extraction was more effective if the ground particle was finer [14]. After 24 hours of maceration process, the maceration solution was filtrated and evaporated to obtain thick corn silk extract. Dilution using 10% DMSO was done to attain corn silk extract of concentrations 75%, 50%, 25%, and 12,5% [15].

Inhibitory Assay of Corn Silk Extract on Growth of C. albicans

Inhibitory assay of corn silk extract on growth of *C. albicans* was done with well diffusion method. The assay was preceded by culture identification and confirmation using conventional tests. *C. albicans* from the culture was suspended in sterile distilled water and adjusted to 1×106 colony forming units (CFU/mL) (0.5 McFarland standard) using a nephelometer, then the suspension was streaked on Sabouraud Dextrose Agar (SDA) plates using sterile swabs. The number of samples (SDA plates) was determined using Federer's formula. Federer's formula is (t-1) (n-1) ≥ 15, where the t value was the number of test groups and the n value was the number of samples required. A total of 4 samples (SDA plates) were required. On every SDA plate, 6 wells of diameter 6 mm were made. Each well was introduced to different test groups which were divided into 6 test groups as followed, group I (positive control using fluconazole), group II (negative control using DMSO solution), group III (75% corn silk extract), group IV (50% corn silk extract), group V (25% corn silk extract) and group VI (12,5% corn silk extract). The SDA plates were then incubated at 37 oC for 24 hours. Inhibition zones formed after the incubation period were measured using a Vernier caliper in millimeters [16].

MIC Determination

The MIC of corn silk extract was determined by agar dilution method using SDA. Dilution of corn silk extract was needed beforehand by using two-fold serial dilution with starting concentration determined by the lowest concentration that still showed inhibitory activity in well diffusion method. SDA plates were prepared by incorporating the diluted corn silk extract into molten test agars, with each plate containing a different concentration of corn silk extract. A total of 32 inocula were added to each plate. The inoculated plates were left to dry at room temperature for no more than 30 minutes, then incubated at 37oC for 24 hours. Growth of the inocula was observed to determine the MIC. This procedure was conducted in quadruplicate [17].

MFC Determination

The MFC of corn silk extract was determined by streak plate method. The streak plate method was conducted by transferring part of the agar which concentration showed no growth of *C. albicans* in the agar dilution method into sterile SDA. Incubation was conducted at 37ºC for 24 hours. Growth of inocula was observed to determine the MFC. This procedure was conducted in quadruplicate [18].

Methods: Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences (IBM SPSS Statistics 25.0). Statistical analysis was performed by Kruskal-Wallis analysis followed by Post Hoc Mann-Whitney test. The result is considered as statistically significant if p-value < 0.05.

RESULTS

Inhibitory assay of corn silk extract on growth of C. albicans

The inhibitory effect was determined by the diameter of inhibition zones formed. The results of the diameter measurement are presented in Table 1. The diameter of the inhibition zones formed can be seen in Figure 1.

The diameters of inhibition zones formed by all concentrations of corn silk extract were larger than the ones formed by positive control (fluconazole). The diameter of inhibition zones was directly proportional to the concentration of corn silk extract. The smallest diameter of inhibition zones (24.9375 ± 0.2462 mm) was formed by the smallest concentration of corn silk extract (12.5%), followed by ascending concentration to the largest concentration of corn silk extract (75%) where the largest diameter of inhibition zones (27.4875 ± 0.3838 mm) was formed.

MIC determination

The MIC determination was performed using a two-fold serial dilution of corn silk extract to attain concentrations of 12.5%, 6.25%, 3.125%, and 1.5625%. After an incubation period, growth of inocula was observed and MIC was read as the lowest concentration of corn silk extract that showed an 80% or greater reduction in growth as compared to the control [17]. Results of growth observation are presented in Table 2 and can be seen in Figure 2. The MIC is corn silk extract of concentration 1.5625%.

Table 1. Results of inhibition zones measurement

Interventions		Diameter of Inhibition Zone (mm)				Mean (mm)	Mean ± SD
		Replication					
		I	II	III	IV		
Concentration of corn silk extract (%)	75	27.7	27.05	27.9	27.3	27.4875	27.4875 ± 0.3838
	50	26.65	26.6	27.1	26.55	26.7250	26.7250 ± 0.2533
	25	25.65	25.65	26.1	25.5	25.7250	25.7250 ± 0.2598
	12.5	24.9	25.25	24.95	24.65	24.9375	24.9375 ± 0.2462
Fluconazole (positive control)		17.5	16.7	20.55	24.4	19.7875	19.7875 ± 3.4939
DMSO (negative control)		0	0	0	0	0	0

Fig. 1. Inhibition zones formed in well diffusion method: C+ refers to positive control (Fluconazole), C – refers to negative control (DMSO), 75 refers to concentration 75%, 50 refers to concentration 50%, 25 refers to concentration 25% and 12.5 refers to concentration 12.5%

Table 2. Results of growth observation in agar dilution method

Concentration of corn silk extract (%)	Growth observation			
	Replication			
	I	II	III	IV
12.5	-	-	-	-
6.25	-	-	-	-
3.125	-	-	-	-
1.5625	-	-	-	-
Positive control	+	+	+	+
Negative control	-	-	-	-

Note: (-): no growth of *C. albicans* was observed; (+): growth of *C. albicans* was observed; Positive control: plate containing SDA inoculated with *C. albicans*; Negative control: plate containing SDA and corn silk extract not inoculated with *C. albicans*

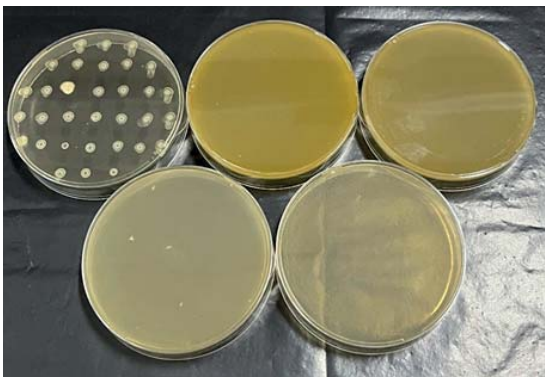

Fig. 2. Results of agar dilution method with corn silk extract concentration of 12.5% (upper middle), 6.25% (upper right), 3.125% (lower left), and 1.5625% (lower right), positive control (upper left)

MFC determination

Table 3. Results of growth observation in streak plate method

Concentration of corn silk extract (%)	Growth observation			
	Replication			
	I	II	III	IV
12.5	-	-	-	-
6.25	-	-	-	-
3.125	+	+	+	+
1.5625	+	+	+	+
Positive control	+	+	+	+
Negative control	-	-	-	-

Note: (-): no growth of *C. albicans* was observed; (+): growth of *C. albicans* was observed; Positive control: plate containing SDA inoculated with *C. albicans*; Negative control: plate containing SDA and corn silk extract not inoculated with *C. albicans*

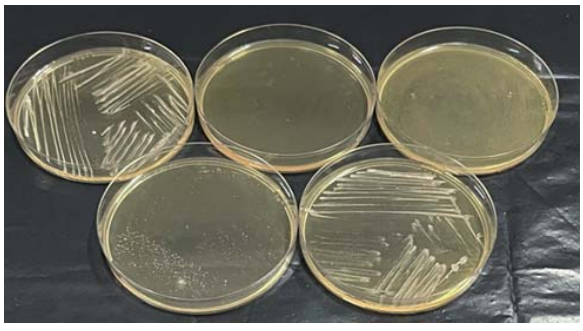

Fig. 3. Results of streak plate method with the upper ones are positive control, concentration of 12.5%, 6.25%, and the lower ones are concentration of 3.125% and 1.5625% respectively from left to right

Statistical analysis

Kruskal-Wallis analysis showed a significant result (p-value < 0.05), interpreted as a significant effect of corn silk extract in inhibiting the growth of *C. albicans* was found. Mann Whitney test showed a significant result (p-value< 0.05), which suggests that each concentration showed significant differences in inhibiting growth of *C. albicans* compared to positive control (fluconazole).

Table 4. Result of Kruskal Wallis analysis

Null Hypothesis	Test	Sig.	Decision
There is no effect of corn silk extract in inhibiting the growth of Candida albicans	Kruskal Wallis analysis	0.001	Reject the null hypothesis

Table 5. Result of Mann Whitney test

	Sig.
Comparison between corn silk extract of concentration 75% and positive control	0.021
Comparison between corn silk extract of concentration 50% and positive control	0.021
Comparison between corn silk extract of concentration 25% and positive control	0.020
Comparison between corn silk extract of concentration 12.5% and positive control	0.021

DISCUSSION

Based on the results of this study, all four concentrations of corn silk extract showed an inhibitory effect against *C. albicans*. The higher the concentration of corn silk extract used, the larger the diameter of inhibition zones formed. This result is in agreement with a previous study which showed that the higher the concentration, the higher the efficacy in the inhibition of growth [19]. The diameter of inhibition zones formed by positive control fluconazole was smaller than those formed by corn silk extract of concentration 75%, 50%, 25%, and 12.5%. This result indicates corn silk extract concentrations of 75%, 50%, 25%, and 12.5% are superior in inhibiting the growth of *C. albicans* compared to fluconazole as standard therapy for VVC [20]. This is suspected to be caused by the resistance of *C. albicans* to fluconazole [21].

The inhibitory effect of corn silk extract (Zea mays L. var. indurata) on the growth of *C. albicans* is known to originate from secondary metabolites contained in corn silk extract such as alkaloids, flavonoids, tannins, and terpenoids which existence has been confirmed in a previous report [9]. Alkaloids cause damage to cell membrane integrity and impair mitochondrial function thus inhibiting the growth of *C. albicans* [10]. Flavonoids interrupt the formation of cell wall, inhibit RNA and protein synthesis, and disrupt plasma membrane integrity in Candida species [11].

Terpenoids inhibit DNA replication in Candida species [12]. Tannins inhibit the growth of mycelium and disrupt the integrity of cell walls and membranes in Candida species [13].

CONCLUSION

In conclusion, corn silk extract of concentrations 75%, 50%, 25%, and 12.5% have an antifungal effect against *C. albicans*. The higher the concentration of corn silk extract, the higher the efficacy in inhibiting the growth of *C. albicans*. The MIC and MFC of corn silk extract obtained from this study are 1.5625% and 6.25%, respectively.

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ORIGINAL ARTICLE

LINGUISTIC VALIDATION, ADAPTATION, AND RELIABILITY OF THE LIVERPOOL ELBOW SCORE'S PATIENT-ANSWERED QUESTIONNAIRE IN BULGARIAN

THE BULGARIAN LES-PAQ QUESTIONNAIRE

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Abstract. Background: *There is a lack of upper-limb organ specific patient-reported questionnaires that are linguistically validated and culturally adapted for the Bulgarian population. The Liverpool Elbow Score (LES) is a mixed elbow-specific score with excellent psychometric properties of the patient-answered questionnaire (PAQ). There is no Bulgarian version. The aim of this study was to perform a linguistic validation and cultural adaptation of the LES−PAQ in Bulgarian, and to test the validity and reliability of the Bulgarian version.* ***Materials and Methods:*** *The study was conducted in a single institution. Participant selection criteria were defined – included were mentally healthy adult patients with a diagnosis of osteochondritis dissecans of the radiocapitellar joint, elbow arthritis, lateral epicondylitis and elbow contracture. Excluded were patients with coexisting upper limb conditions, illiterate and non-native Bulgarian speakers. The Bulgarian LES−PAQ was created. The process consisted of forward translation, reconciliation and backward translation. An expert committee agreed upon a pre-final Bulgarian version. A final version was decided after cultural adaptation. The psychometric properties and reliability of the Bulgarian LES−PAQ was tested.* ***Results:*** *101 patients were included in this study. We did not observe a ceiling or floor effect. Cronbach's α coefficient was 0.858. Intraclass correlation coefficient was 0.864 (95% CI 0.77 to 0.92; $p < 0.05$). A moderate negative correlation was revealed between the LES-PAQ and the DASH ($r = -0.591$, $p < 0.05$), and a high positive correlation with SF-12 ($r = 0.867$, $p < 0.001$).* ***Conclusion:*** *The Bulgarian LES−PAQ is a reliable and valid instrument for assessing elbow conditions such as osteochondritis dissecans of the radiocapitellar joint, elbow arthritis, lateral epicondylitis and elbow contracture. It may assist Bulgarian healthcare professionals in both research and daily work.*

Key words: *linguistic validation, elbow, patient-reported outcomes, questionnaire*

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INTRODUCTION

Clinical outcome measures are clinician-reported, patient-reported or mixed. Self-reported questionnaires reflect the patients' perception of their health and are ever more often being used in addition to clinical assessment – they are easy to use and convenient [1, 2]. Elbow pathologies are an important share of patients who present in orthopedic/rheumatologic practices [3]. It is essential to properly diagnose and treat such conditions, as they affect the working-age group and impair quality of life. There is a variety of elbow-specific scores available in the literature [2, 4]. They are used to assess function, evaluate the effectiveness of treatment, and compare different methods [5]. However, patient-reported outcomes require language fluency and to our knowledge, there is only one formally validated Bulgarian version of a scale that assesses the upper limb – the Disability of Arm, Shoulder, and Hand (DASH) questionnaire [6]. Bulgarian elbow-specific scores that are linguistically validated and culturally adapted for the Bulgarian population currently do not exist.

The Liverpool Elbow Score (LES) is a mixed elbow-specific outcome measure that was developed by Sathyamoorty et al. in 2004 [4]. LES consists of two parts: the first one is a 6-item clinical assessment score (CAS, C1-6) with three subscales, evaluating the range of motion, strength, and ulnar nerve involvement; the second part is a 9-item patient questionnaire (PAQ, P1-9) assessing function and the ability to perform activities of daily living (P1-P7), levels of pain (P8), and participation in sports and leisure activities (P9). Items are scored from 0 to a variable maximum of 3 or 4 points. The interpretation consists of a final score combining PAQ and CAS. The score is normalized to 10 for ease of interpretation, via a formula, with a maximum score of 10 points and a minimum of 0 points. The lower the score, the greater the disability [13]. LES was originally intended to assess patients after elbow replacement surgery, but it was also found as a valid outcome measure in assessing soft-tissue elbow conditions (tendinopathy, contractures).

The purpose of this study was to perform a linguistic validation and cultural adaptation of the LES−PAQ in Bulgarian, and to test the validity and reliability of the Bulgarian version in patients with osteochondritis dissecans of the radiocapitellar joint, elbow arthritis, lateral epicondylitis and elbow contracture.

MATERIALS AND METHODS

We obtained permission from the original author for the linguistic validation of LES−PAQ. The study was conducted in a single institution. Verbal informed consent was obtained from all the participants. Institutional Review Board approval was granted before conducting this research.

Participant selection criteria were defined. Included were mentally healthy adult patients (above 18 years of age) with a diagnosis of osteochondritis dissecans of the radiocapitellar joint, elbow arthritis, lateral epicondylitis and elbow contracture. Excluded were patients with coexisting upper limb conditions, illiterate and non-native Bulgarian speakers. A total of 101 participants were selected for the study (Table 1).

Table 1. Demographic data

Age	47.6 ± 17.2 years	
Sex	71 male/30 female	
BMI	25.2 ± 4.7 kg/m^2	
Dominant hand involvement	74/101	
Diagnosis	Osteochondritis dissecans of the radiocapitellar joint	16/101
	Elbow arthritis	19/101
	Lateral epicondylitis	40/101
	Elbow contracture	26/101

The Bulgarian LES−PAQ was created. We followed Beaton's guidelines [8]. The process consisted of forward translation, reconciliation and backward translation. This was done blindly by two independent researchers – one English native (fluent in Bulgarian), and one Bulgarian native (fluent in English). An expert committee that consisted of two orthopedic elbow surgeons and a rheumatologist agreed upon a pre-final Bulgarian version. It was given to the participants for cultural adaptation. Additional comments from the patients regarding understandability and clarity were taken into notice. A final version of the questionnaire was agreed upon.

The Bulgarian LES−PAQ was self-administered by the participants twice within a one week interval for reliability testing. The participants were clinically

stable during the test-retesting. No treatment was given in this period to ensure a minimum change in clinical condition.

The sample distribution was discovered with the Kolmogorov-Smirnov test. A p-value ≤ 0.05 was considered statistically significant. Floor or ceiling effect was also assessed. The threshold was accepted to be 15% of respondents obtaining the minimum or maximum score respectively [9]. Intraclass correlation coefficient (ICC) and Chronbach's alpha were calculated [10]. Satisfactory internal consistency was accepted for values above 0.7 [11]. Construct validity was assessed by comparing the Bulgarian LES-PAQ, SF-12 version 1, and DASH through Pearson's correlation coefficient.

The obtained data were summarized and analyzed through Microsoft Excel 2019 and the Real Statistics Resource Pack for Excel 2019, Release 7.7.1 [12].

RESULTS

Normal distribution was found for the outcome measure scores. No floor or ceiling effects were seen. None of the participants scored the minimum – 0 points. 4/101 of the patients reached the maximum 6 points score, and in the second study, 6/101 of the patients scored maximum values.

ICC was 0.864 (95% CI 0.771 to 0.921; p = 0.000). Cronbach's α was 0.858 (p < 0.05). Item-total correlations were between 0.641 and 0.795.

Test-retest reliability of the Bulgarian LES-PAQ was established. We found highly positive correlations for all questions and the total score (r = 0.862, p = 0.000) (Table 2).

The LES-PAQ showed a highly negative correlation with the Bulgarian DASH (r = -0.591, p < 0.05) and a highly positive correlation with the SF-12 version 1 physical health subscale (r = 0.867, p < 0.001).

DISCUSSION

The most important finding of this study, the Bulgarian LES-PAQ demonstrated good reliability, satisfactory internal consistency, and validity to assess function in Bulgarian-speaking patients with osteochondritis dissecans of the radiocapitellar joint, elbow arthritis, lateral epicondylitis and elbow contracture. No floor or ceiling effects were discovered. Vishwanathan et al. measured the internal consistency of LES to be 0.87 [13]. In the original developmental and validation study of LES, the authors found good internal consistency (Cronbach's α = 0.997) [4]. The internal consistency of the scale was 0.858 (p < 0.05), and the test-retest reliability of the Bulgarian LES-PAQ was 0.862 (p < 0.05).

Sun and Fan observed high correlations with DASH (r = 0.88 preoperatively and 0.87 postoperatively, p < 0.001), while the Bulgarian LES-PAQ showed a highly negative correlation (r = -0.591, p < 0.05).

Sathyamoorthy et al. also measured validity with DASH and SF-12 correlation testing. High correlation was found with DASH (r = -0.76, p = 0.000) and a low correlation with SF-12 physical health subscale (r = -0.39, p = 0.000) [14].

The Bulgarian LES−PAQ can be routinely used in clinical practice by orthopedic surgeons, rheumatologists, physiotherapists and other health professionals as well. Also, the score has the properties to evaluate the patients remotely [14]. This can be done via email

Table 2. Internal consistency and test–retest reliability of the Bulgarian LES-PAQ (n = 101)

PAQ	Mean (SD)	Re-test Mean (SD)	r (p-value) *	Chronbach's alpha if item deleted
Other arm use (Question 1)	2.48 (1.00)	2.48 (0.97)	0.788 (0.000)	0.829
Combing hair (Question 2)	2.67 (0.83)	2.58 (0.86)	0.629 (0.000)	0.841
Washing self (Question 3)	2.67 (0.83)	2.66 (0.94)	0.676 (0.000)	0.838
Feeding (Question 4)	2.88 (0.88)	2.88 (0.80)	0.529 (0.000)	0.846
Dressing (Question 5)	2.73 (0.89)	2.90 (0.89)	0.709 (0.000)	0.846
Household activities (Question 6)	2.83 (0.98)	2.70 (0.89)	0.644 (0.000)	0.848
Lifting (Question 7)	2.96 (0.79)	2.94 (0.87)	0.699 (0.000)	0.839
Pain (Question 8)	2.71 (1.07)	2.90 (0.97)	0.493 (0.000)	0.854
Sport and leisure activities (Question 9)	3.33 (0.79)	3.34 (0.85)	0.561 (0.000)	0.846

* Pearson's correlation coefficient

or telephone call because the questionnaire is short, easy to use, and understandable.

The LES consists of both clinician and patient-answered sections. In addition to clinical findings, using an elbow score with good psychometric properties in the native language could improve the process of assessment. This would help to better understand the patient's condition and measure the effect of treatment.

LES was originally intended to evaluate patients after elbow replacement surgery, but has since been validated in cases of elbow stiffness, tendinopathy and elbow fractures [12, 19]. In our study, we investigated the properties of LES−PAQ in patients with osteochondritis dissecans of the radiocapitellar joint, elbow arthritis, lateral epicondylitis and elbow contracture. The questionnaire may be used in a wide range of elbow conditions with validity and reliability.

A disadvantage of the LES is the need for a somewhat complex formula that normalizes the score from 0 to 10 for ease of interpretation. Additionally, the mentioned scoring system did not include data that provided information about the patient's quality of life [13]. Quality of life may be evaluated using a different questionnaire.

CONCLUSION

The Bulgarian LES−PAQ is a reliable and valid instrument for assessing elbow conditions such as osteochondritis dissecans of the radiocapitellar joint, elbow arthritis, lateral epicondylitis and elbow contracture. It may assist Bulgarian healthcare professionals in both research and daily work.

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ORIGINAL ARTICLE

ATTITUDE OF ATHLETES TOWARDS DIETARY SUPPLEMENTS

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Abstract. *At the beginning of 2023 we carried out a survey among active athletes asking about their attitude towards dietary supplements.* ***Aim:*** *The aim of the study was to evaluate the attitude of athletes towards dietary supplements using a survey method. The objective of the study was to determine whether active athletes use dietary supplements.* ***Materials and methods:*** *The participants in the study were asked to anonymously complete a survey consisting of 16 questions. In January 2023, we surveyed 50 active athletes from the „Vasil Levski" National Sports Academy (35 men and 15 women). Forty-six of the participants were between 18 and 22 years old and 4 were between 23 and 27 years old.* ***Results:*** *The majority (94%) of the surveyed athletes trusted the advertisement of a particular dietary supplement. Ninety per cent had complete trust in the pharmacists' advice. Pharmacists played a significant role in the choice of dietary supplements. A high percentage (60%) of the respondents who used dietary supplements had not sought medical advice prior to their use. Ninety per cent of the respondents used dietary supplements for weight loss in order to fit in a particular category; 78% indicated that they used dietary supplements for improvement of their sports achievements; 72% used supplements to increase their stamina and 46% – to shorten their time for recovery. A campaign should be initiated to encourage the use of dietary supplements only when they are prescribed by physicians.*

Key words: *dietary supplements, sports, athletes, survey*

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INTRODUCTION

Dietary supplements are an integral part of each athlete's diet. The information on their use in athletes' regimens changes daily. We conducted this survey to gain an insight into the attitude of 50 active athletes towards dietary supplements.

The United States of America's Food and Drug Administration (FDA) uses the following definition of supplements: A dietary supplement is a product for ingestion that contains a "dietary ingredient" intended to supplement the diet. The "dietary ingredient" can include one or more components. Dietary supplements can be taken in different forms: tablets, capsules, gels, liquids or powders [1]. The types of dietary supplements include herbs or other botanicals, minerals, vitamins, amino acids, concentrates, metabolites, constituents, extracts, etc. [1].

One of the main roles of proteins is to facilitate muscle recovery remodelling through muscle protein syn-

thesis (MPS) [2]. It has been proven that increased dietary intake of proteins improves immunity during intensive bouts of training and in cyclists [3, 4].

During intensive training a carbohydrate-rich diet is recommended (5-7 g/kg per day) [5, 6, 7].

Creatine is synthesized mainly by the liver, also by the pancreas and the kidneys. The main sources of creatine are meat (from 3 to 5 g creatine per 1 kg of raw meat) and fish. Creatine can support recovery and increases the MPS after exercise [8, 9, 10].

MATERIALS AND METHODS

The participants were asked to complete an anonymous survey consisting of 16 questions. In January 2023, we surveyed 50 active athletes from the Vasil Levski National Sports Academy (35 men and 15 women). Forty-six of the respondents were between 18 and 22 years old and 4 were between 23 and 27 years old. The role of internet advertisements is best illustrated by the answer to the following question: "Where do you prefer to buy your supplements from?" Eighty-four per cent of the respondents indicated that they were buying dietary supplements online, while 68% preferred to visit pharmacies.

When asked: "What advertisements influence you the most?" 98% answered that they trusted product advertisement articles the most.

The majority (94%) of the surveyed athletes trusted the advertisement of a particular dietary supplement.

Pharmacists played a significant role in the choice of dietary supplements: 90% of the surveyed athletes had complete trust in the pharmacists' advice.

A high percentage (60%) of the surveyed athletes using dietary supplements had not consulted a physician prior to starting their use.

One of the most important questions in the survey is: "What is your source of information on the different types of supplements?" Our study established that the respondents were obtaining their information from friends (98%) and the internet (88%) (Table 1).

When asked what amount of money they could spend monthly on dietary supplements, approximately half of the participants replied they could spend more than BGN 50.

Ninety-eight per cent of the respondents indicated that they are buying dietary supplements for personal use. The answers to the question: "During what period of the year are you buying dietary supplements the most?" showed that 60% of the participants buy the most in spring and a similar percentage (54%) – in summer.

Table 1. Answers to the question: "What is your source of information on the different types of supplements?"

What is your source of information on the different types of supplements?	N	%
Friends	48	96.0
The internet	44	88.0
Radio and television	4	8.0
Advertisements	4	8.0
Other	2	4.0

Each athlete has individual needs and so each uses dietary supplements for different purposes in the beginning of summer. Ninety per cent of the respondents were using the supplements for weight loss and 68% – for detoxication (Table 2).

Table 2. Answers to the question "What supplements are you taking in early summer?"

What supplements are you taking in early summer?	N	%
For detoxication	34	68.0
For weight loss	45	90.0
Vitamins	23	46.0
Probiotics to increase immunity	3	6.0
Digestive enzymes	4	8.0
I do not take any supplements	1	2.0
Other	6	12.0

Seventy-eight per cent of the athletes indicated that they used dietary supplements in order to improve their sports achievements, 72% used them for increased stamina and 46% – for faster recovery (Table 3).

Table 3. Answers to the question: "What is your purpose for using dietary supplements?"

What is your purpose for using dietary supplements?	N	%
improvement in sports achievements	39	78.0
increased stamina	36	72.0
improvement of circulation and nutrient intake	8	16.0
decreased sense of effort	12	24.0
decreased time for recovery	23	46.0
decreased muscle pain	15	30.0
decreased risk of injury	6	12.0
better muscle strength and health	35	70.0
support for the immune system	4	8.0
improvement in general health	12	24.0

The dietary supplements used most frequently by active athletes for appropriate recovery after training are: creatine – 84%, carbohydrates – 54%, multivitamins – 54%, L-carnitine – 42% (Table 4).

Table 4. Answers to the question: "What supplements are you using to ensure appropriate recovery after training?"

What supplements are you using to ensure appropriate recovery after training? Please list them:	N	%
Branched-chain amino acids (BCAA)	13	26.0
L-glutamine	6	12.0
Whey protein	4	8.0
Creatine	42	84.0
Carbohydrate supplements	27	54.0
L-carnitine	21	42.0
Leucine	17	34.0
Multivitamins	27	54.0
Beta-alanine	15	30.0
Magnesium	26	52.0

Active athletes reported that they most often take: creatine – 84%, protein powder – 60%, magnesium 54%, multivitamins – 54% (Table 5).

Table 5. Answers to the question about what dietary supplements are taken by the surveyed athletes

You are taking:	N	%
Protein powder	30	60.0
Multivitamins	27	54.0
Creatine	42	84.0
Protein bars	12	24.0
BCAAs	9	18.0
Fish oil	6	12.0
Magnesium	27	54.0
Linseed oil	19	38.0
Dextrose	7	14.0
Beta-alanine	14	28.0

Sixty per cent of the athletes had not encountered any adverse effects related to their intake of dietary supplements, while 26% thought that supplements may lead to adverse effects.

In a study conducted by Jovanov et al., they found that most respondents used protein supplements (54.5%), and 41.4% indicated their trainers as the main source of information about supplements. The main motivation for taking supplements was to increase sports performance in 35.4%. The majority of athletes (72.1%) were aware of the associated health risks of using dietary supplements [11].

In every textbook on human physiology, it is written how important it is to take in micro and macro elements, and this can also be done through nutritional supplements, but only under medical supervision [12].

DISCUSSION

Eighty-eight per cent of the respondents are obtaining their information on dietary supplements from the internet. In a study conducted by Jovanov et al., they found that 41.4% cited their trainers as their primary source of information about supplements.

The role of team coaches and physicians is to assist in the choice of safe and beneficial supplements. Eighty-four per cent of the respondents indicated that they are buying dietary supplements online. This behaviour entails health risks, as not all products sold on the internet are certified. The majority (94%) of the surveyed athletes trust the advertisements of dietary supplements. This may not always be the best decision and consulting a physician is mandatory. The most common supplements taken by active athletes are creatine (84%) and protein powder (60%). In a study by Jovanov et al., they found that most respondents used protein supplements (54.5%).

This result is consistent with the worldwide tendencies among athletes. The majority (78%) of the respondents indicated that they use dietary supplements for improvement of their athletic achievements, 73% use supplements to increase their stamina and 46% – to shorten their recovery period. In a study by Jovanov et al., they found that the main motivation for taking supplements was to increase athletic performance at 35.4%.

Taking supplements is a part of the athletes' everyday life, but only 26% of the respondents think that this may lead to adverse effects. In a study by Jovanov et al., athletes (72.1%) were aware of the health risks associated with using dietary supplements.

All risks and benefits connected to taking dietary supplements need to be explained to them.

CONCLUSIONS

The lack of sufficient opportunities to recover between training and competitions coupled with international trips is a significant problem faced by all athletes. They need to plan effective strategies for recovery under the guidance of their coaches. One of the most

important strategies is taking dietary supplements during the recovery process. Unfortunately, our team did not find such a large-scale study in Bulgaria, and this motivated us to do this study.

Only a small proportion of athletes consults a physician before they use dietary supplements. A campaign should be initiated to encourage the use of dietary supplements only when they are prescribed by physicians.

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ORIGINAL ARTICLE

COMPARISON OF ULTRASONOGRAPHY AND CONE-BEAM COMPUTED TOMOGRAPHY ACCURACY IN MEASURING THE SOFT TISSUE THICKNESS OF MAXILLARY AND MANDIBULAR GINGIVA IN A SHEEP MODEL

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Abstrtact. Background: *To date, few studies have compared the accuracy of cone-beam computed tomography (CBCT) and ultrasonography in measuring the soft tissue thickness of the maxillary and mandibular gingiva.* ***Aims:*** *To compare the accuracy of ultrasonography and CBCT in measuring the soft tissue thickness of the maxillary and mandibular gingiva in a sheep model.* ***Materials and Methods:*** *In this study, 38 different landmarks (26 points from the upper jaw and 12 points from the lower jaw) were evaluated. The gingival soft tissue thickness was measured using a digital caliper, ultrasonography, and standard and high-resolution CBCTs. The measurements were finally compared with each other.* ***Results:*** *Regarding the thicknesses < 2 mm, no significant difference was seen between the measurements of the digital caliper and ultrasonography (mean difference < 0.1 mm, p = 0.140). Conversely, data analysis indicated significant differences between CBCTs measurements and digital caliper and ultrasonography measurements. Regarding thicknesses > 2 mm, digital caliper measurement was not significantly different from ultrasonography and high-resolution CBCT measurements (mean differences < 0.1 mm) but differed from the standard CBCT measurement. Also, a significant difference was observed between ultrasonography and standard CBCT measurements but not between ultrasonography and high-resolution CBCT (mean differences < 0.1 mm). Finally, mean differences between standard and high-resolution CBCT measurements were statistically significant.* ***Conclusion:*** *According to the results, ultrasonography can be a reliable option for measuring gingival soft tissues regardless of their thickness, while CBCT may be more suitable for thicker gingival tissues. Clinicians should carefully consider the measurement accuracy of different imaging methods when planning dental procedures.*

Key words: *ultrasonography, cone-beam computed tomography, soft tissue, measurement accuracy*

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INTRODUCTION

Periodontal disease is a chronic inflammatory condition affecting the supporting tissues of teeth, including the gingiva. Accurate assessment of gingival tissues, including gingival thickness, is essential for the diagnosis and management of periodontal disease. Various imaging modalities have been used to measure the thickness of gingival tissues, including cone-beam computed tomography (CBCT) and ultrasonography [1-3]. However, the accuracy of these techniques in measuring the soft tissue thickness of the maxillary and mandibular gingiva has not been well-established.

CBCT is a non-invasive imaging modality that has been used in dentistry for many years. It provides high-resolution images of both hard and soft tissues, making it an excellent tool for the assessment of periodontal tissues [4, 5]. A number of studies have investigated the accuracy of CBCT in measuring gingival thickness, with conflicting results [6-8].

Ultrasonography is another non-invasive imaging technique used in dentistry to measure the gingival thickness. It emits high-frequency sound waves that are reflected back from the tissues, producing an image [9]. Ultrasonography has been shown to be highly accurate in measuring the thickness of soft tissues in other parts of the body, but its accuracy in measuring gingival thickness has yet to be well-established [9, 10].

To date, few studies have compared the accuracy of CBCT and ultrasonography in measuring the soft tissue thickness of the maxillary and mandibular gingiva. Therefore, the purpose of this paper is to compare the accuracy of CBCT and ultrasonography in measuring these soft tissues in a sheep model. The use of a sheep model is significant because sheep have periodontal tissues similar to those of humans, making them a valuable animal model for periodontal research. Moreover, the results of this study could have significant implications for the diagnosis and management of the periodontal disease. Accurate imaging modalities for measuring gingival thickness can aid in the early detection and treatment of periodontal disease. Additionally, using a sheep model can provide valuable insight into the accuracy and reliability of these imaging techniques.

MATERIALS AND METHODS

In the present study, 38 different landmarks were assessed. Specifically, 26 points were allocated to the upper jaw and 12 points to the lower jaw, where the average gum thickness was less than two millimeters in the buccal and lingual regions. In addition, the posterior dental region was selected for the study due to the similarity of gum tissue thickness in the back of the upper and lower jaw with that of human gum tissue. To ensure that the points covered the gums in the longitudinal axis of the tooth and the buccal, palatal, and lingual interdental gums, we selected the location of the points accordingly. We also considered some points in the mid-palatal suture of the upper jaw. Finally, the approximate location of the buccal, lingual, and palatal points in the upper third of the cortical plate of the posterior alveolar ridge of the maxilla and mandible was considered. In total, 26 maxillary sites were selected for the study, with five points selected in the buccal and five points in the palatal ridge of the right and left posterior alveoli of the maxilla. Furthermore, six points were selected in the midpalatal suture. In the mandible, 12 points were considered, with three points in the buccal cortical plane and three points in the lingual cortical plane on both the left and right sides.

All procedures conducted in our study complied with the ethical guidelines outlined in the Helsinki Declaration.

Direct measurement of soft tissue thickness

To directly measure the thickness of soft tissue, an endodontic spreader with a silicone stop was inserted perpendicularly into the mucosa inside the mouth until it reached the alveolar bone at designated locations. The silicone stop was fixed against the gum in this position. Subsequently, the endodontic spreader was removed, and the distance between the silicone stop and the tip of the spreader was measured using a digital caliper with a sensitivity of 0.01 mm. To ensure accuracy in CBCT scans, orthodontic wire with a diameter of 5 mm was placed in the same predetermined locations as the spreader measurements (Fig. 1). Two expert dental radiologists measured the landmarks to ensure reliability with excellent interobserver agreement (kappa = 0.96).

Fig. 1. Imaging the sheep's maxilla after placing the orthodontic wire at the designated points

CBCT imaging

We obtained CBCT images of all teeth using the Giano (Newtom, Verrona, Italy) CBCT system in a private dental clinic in Babol, northern Iran. The radiation settings were set to a Field of View (FOV) of 8×11, radiation time of 9 seconds, current of 3 mA, and voltage of 90 kVp. To display the images, we used a 19-inch LCD-19 monitor with a distance of approximately 60 cm from the observer to the monitor in a dimly lit room. The outline of the soft tissue on the outer border of the cortical bone was visible as a white line in the images. We measured the soft tissue thickness perpendicular to the cortical bone in the CBCT images, examining a total of 35 points, with each point analyzed separately. To ensure accuracy, we used multiplanar reconstruction (MIPR) to select coronal images with a slice thickness of 1 mm (Figs 2 and 3). We repeated these steps for each region and point of interest.

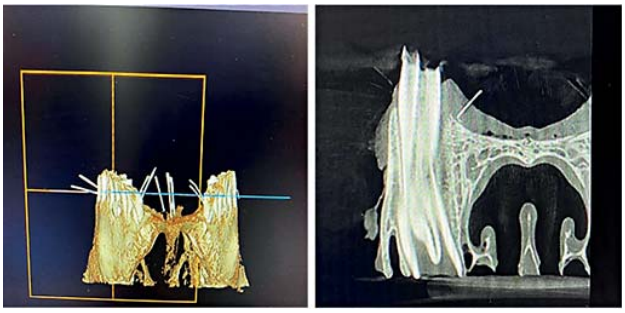

Fig. 2. Cone-beam computed tomography image of the sheep maxilla after placing the orthodontic wire at the designated points

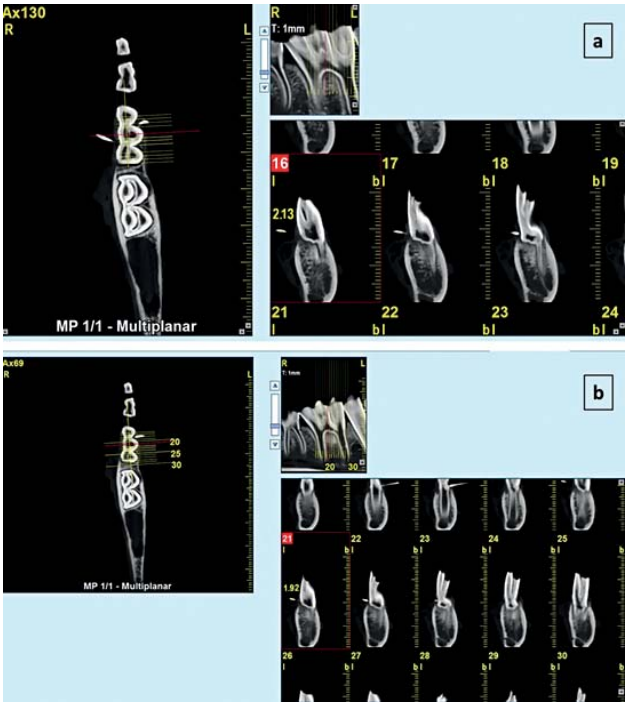

Fig. 3. Measurement of gingival thickness using high-resolution (a, 2.13 mm) and standard (b, 1.92 mm) cone-beam computed tomography

Ultrasonography imaging

An ultrasonography machine with a linear probe operating at a frequency of 8 MHz (L3-12A) was utilized. This method boasted a good signal-to-noise ratio, which allowed for a selectable 80 dB dynamic range and improved soft tissue contrast. Both ultrasonography gel and specialized gel-based pads were utilized to guide sound waves. Initially, the wire was placed with the tangential surface probe, and then the wire insertion point was identified. Next, the wire was removed, and the software's embedded electronic caliper was utilized to obtain high-resolution grayscale images at the desired levels for measuring gum thickness.

Statistical analysis

SPSS V22 was used to analyze the data. The Kolmogorov-Smirnov test was used to assess the normality of the data. In order to compare the measurements made by CBCT (both standard and high-resolution modes) and ultrasonography with digital caliper, sample T-test (for parametric data) and Mann-Whitney or Wilcoxon tests (for non-parametric data) were used. A p-value less than 0.05 was statistically considered significant.

RESULTS

Table 1 summarizes the comparison of the measurement accuracy of the digital caliper, ultrasonography, and CBCTs (standard and high-resolution) regardless of jaw thickness. Data analysis showed no statistically significant difference between digital caliper and ultrasonography measurements (mean difference < 0.1 mm, p = 0.578). On the other hand, standard and high-resolution CBCT measurements were significantly different from the digital caliper and ultrasonography measurements.

Regarding the thicknesses < 2 mm, no significant difference was seen between the measurements of the digital caliper and ultrasonography (mean difference < 0.1 mm, p = 0.140). Conversely, data analysis indicated significant differences between CBCTs (standard and high-resolution images) measurements and digital caliper and ultrasonography measurements. Table 2 represents these results.

Comparison of the measurement accuracy of the digital caliper, ultrasonography, and CBCTs concerning thicknesses greater than 2 mm are summarized in Tables 3 and 4. Regarding thicknesses 2-4 mm and > 4 mm, we found that digital caliper measurement was not significantly different from ultrasonography and high-resolution CBCT measurements (mean differences < 0.1 mm) but

differed from the standard CBCT measurement. Also, a significant difference was observed between ultrasonography and standard CBCT measurements but not between ultrasonography and high-resolution CBCT (mean differences < 0.1 mm). Finally, mean differences between standard and high-resolution CBCT measurements were statistically significant.

Table 1. Comparison of the measurement accuracy of the digital caliper, ultrasonography, and cone-beam computed tomography regardless of the gingival thickness

Imaging (Mean ± SD, mm)		Mean difference ± SD (mm)	P-value
Digital caliper (3.24 ± 1.57)	Ultrasonography (3.24 ± 1.56)	0.001 ± 0.01	0.578
	s-CBCT (3.18 ± 1.56)	0.06 ± 0.03	< 0.001
	hr-CBCT (3.23 ± 1.56)	0.01 ± 0.01	0.001
Ultrasonography (3.24 ± 1.56)	s-CBCT (3.18 ± 1.56)	0.06 ± 0.03	< 0.001
	hr-CBCT (3.23 ± 1.56)	0.005 ± 0.01	0.030
s-CBCT (3.18 ± 1.56)	hr-CBCT (3.23 ± 1.56)	-0.05 ± 0.03	< 0.001

s-CBCT, standard cone-beam computed tomography images; hr-CBCT, high-resolution cone-beam computed tomography images

Table 2. Comparison of the measurement accuracy of the digital caliper, ultrasonography, and cone-beam computed tomography for the gingival thicknesses < 2 mm

Imaging (Mean ± SD, mm)		Mean difference ± SD (mm)	P-value
Digital caliper (1.52 ± 0.26)	Ultrasonography (1.51 ± 0.26)	0.006 ± 0.01	0.140
	s-CBCT (1.45 ± 0.27)	0.07 ± 0.04	< 0.001
	hr-CBCT (1.50 ± 0.26)	0.02 ± 0.01	< 0.001
Ultrasonography (1.51 ± 0.26)	s-CBCT (1.45 ± 0.27)	0.06 ± 0.03	< 0.001
	hr-CBCT (1.50 ± 0.26)	0.008 ± 0.01	0.042
s-CBCT (1.45 ± 0.27)	hr-CBCT (1.50 ± 0.26)	-0.05 ± 0.04	0.001

s-CBCT, standard cone-beam computed tomography images; hr-CBCT, high-resolution cone-beam computed tomography images

Table 3. Comparison of the measurement accuracy of the digital caliper, ultrasonography, and cone-beam computed tomography for the gingival thicknesses 2-4 mm

Imaging (Mean ± SD, mm)		Mean difference ± SD (mm)	P-value
Digital caliper (3.17 ± 0.69)	Ultrasonography (3.17 ± 0.68)	-0.004 ± 0.01	0.138
	s-CBCT (3.13 ± 0.68)	0.04 ± 0.03	< 0.001
	hr-CBCT (3.17 ± 0.69)	-0.001 ± 0.01	0.668
Ultrasonography (3.17 ± 0.68)	s-CBCT (3.13 ± 0.68)	0.04 ± 0.03	< 0.001
	hr-CBCT (3.17 ± 0.69)	0.003 ± 0.002	0.136
s-CBCT (3.13 ± 0.68)	hr-CBCT (3.17 ± 0.69)	-0.04 ± 0.03	0.001

s-CBCT, standard cone-beam computed tomography images; hr-CBCT, high-resolution cone-beam computed tomography images

Table 4. Comparison of the measurement accuracy of the digital caliper, ultrasonography, and cone-beam computed tomography for the gingival thicknesses > 4 mm

Imaging (Mean ± SD, mm)		Mean difference ± SD (mm)	P-value
Digital caliper (5.25 ± 1.00)	Ultrasonography (5.25 ± 1.00)	0.004 ± 0.02	0.373
	s-CBCT (5.19 ± 1.00)	0.06 ± 0.02	< 0.001
	hr-CBCT (5.25 ± 1.00)	0.005 ± 0.01	0.052
Ultrasonography (5.25 ± 1.00)	s-CBCT (5.19 ± 1.00)	0.06 ± 0.02	< 0.001
	hr-CBCT (5.25 ± 1.00)	0.001 ± 0.01	0.823
s-CBCT (5.19 ± 1.00)	hr-CBCT (5.25 ± 1.00)	-0.06 ± 0.03	< 0.001

s-CBCT, standard cone-beam computed tomography images; hr-CBCT, high-resolution cone-beam computed tomography images

DISCUSSION

The objective of the study was to compare the accuracy of different measurement tools for assessing the soft tissue thickness of the maxillary and mandibular gingiva of sheep. Three measurement tools were compared: digital caliper, ultrasonography, and CBCT at standard and high resolutions. The results of the study showed that the accuracy of the measurements varied depending on the thickness of the gingiva and the type of measurement tool used. Overall, the results of this study showed that ultrasonography and digital caliper measurements were comparable in accuracy, with no significant differences observed between them. In contrast, standard and high-resolution CBCT measurements showed significant differences from conventional measurements.

Regarding the thickness of the soft tissue < 2 mm, the difference between ultrasonography and digital caliper data was not statistically significant. In contrast, CBCT (standard and high-resolution images) measurements differed significantly from conventional methods. This finding is in agreement with previous studies that have demonstrated the superior diagnostic performance of ultrasonography in detecting periodontal disease compared to other imaging modalities [11-14].

Concerning thicknesses greater than 2 mm, digital caliper, ultrasonography, and high-resolution CBCT measurements showed no significant differences; however, the standard CBCT measurement showed significant differences from the given techniques. The mean difference between digital caliper and CBCT methods was < 0.1 mm, but this difference is not clinically important. Additionally, no significant difference was observed between high-resolution CBCT and ultrasonography measurements. These findings support the use of ultrasonography and high-resolution CBCT as reliable and accurate methods for measuring soft tissue thickness and may be considered a potential substitute for conventional methods.

In their study, Moudi et al. [7] reported that there was no significant difference between digital caliper and CBCT measurements for the jaws soft tissue thicknesses less than 2 mm, which was inconsistent with our findings. The authors also stated that the accuracy of CBCT was 0.1 mm, which was in agreement with our results. Fourie et al. [15] investigated the accuracy of CBCT in measuring the soft tissue thickness of 11 facial landmarks of seven cadavers and finally concluded that CBCT was a reliable tool for representing facial soft tissue. In a pig jaw model, Lau et al. [8] evaluated the CBCT reliability in the measurement of the thickness of four mandibles and reported that there was no significant difference between the direct physical and CBCT measurements, which was consistent with our findings obtained for thicknesses > 2 mm.

Our findings are supported by previous studies that showed the high accuracy of CBCT in diagnosis and treatment planning in dentistry, and its superior resolution and diagnostic capability compared to other imaging modalities such as CT and MRI [2, 16-18]. However, the significant difference observed between measurements obtained by standard CBCT, digital caliper, and ultrasonography measurements may be attributed to the low spatial resolution of standard CBCT and its poor ability to distinguish between soft tissues with similar radiographic densities. This finding highlights the importance of using high-resolution CBCT to avoid errors and confirm the accuracy of the measurements obtained; however, it is important to note that CBCT involves exposure to ionizing radiation, which may limit its use in specific patient populations such as pregnant women and children. Finally, the superiority of ultrasonography in measuring soft tissue thickness could be attributed to its non-invasive nature, short time, and user-friendliness.

The results of this study have important implications for dentists and periodontists in the planning and execution of dental procedures such as implant placement, crown and bridge restorations, and periodontal surgery. The use of digital caliper and ultrasonography measurements may be more accurate than CBCT imaging for areas of thinner tissue, while the use of high-resolution CBCT imaging may be more appropriate for areas of thicker tissue. These findings can help clinicians make informed decisions about the imaging methods used to measure gingival tissue thickness in their patients.

It should be mentioned that the current study was conducted on sheep models, and the results may not be fully generalizable to human subjects. Therefore, further studies are needed to validate the findings of this study in humans with sufficient sample sizes.

CONCLUSION

This study provides valuable information about the accuracy of different imaging methods and emphasizes the importance of choosing the appropriate method depending on the thickness of the gingival tissue. According to the results, ultrasonography can be a reliable option for measuring gingival soft tissues regardless of their thickness, while CBCT may be more suitable for thicker gingival tissues. Therefore, clinicians should carefully consider the measurement accuracy of different imaging methods

when planning dental procedures. Future studies can be conducted on these findings by investigating the accuracy of different imaging methods in measuring tissue thickness in other animal models and human subjects.

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CASE SERIES

ROLE OF ELECTROPHYSIOLOGAL STUDIES FOR DETECTION OF SIMULATION AND AGGRAVATION IN OPHTHALMOLOGY

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Abstract. Objective: *To present the importance of the electrophysiological studies for detection of malingering and aggravation in ophthalmology.* ***Materials and methods:*** *Six eyes of three patients underwent a complete clinical examination, fundus-autofluorescence (FAF), fluorescein angiography (FA), optical coherence tomography (OCT), visual field testing, electrophysiological (EF) studies – full-field, multifocal and pattern electroretinography (ffERG, mfERG and PERG) and visual evoked potentials (VEPs), for detection of simulation or aggravation.* ***Results:*** *After the electrophysiological studies' results, which are objective and non-manipulable, we purposefully reviewed and repeated some of the tests already done, which allowed a comprehensive interpretation of the results. It turned out that discrete changes in targeted search can be detected in several of the studies performed, which greatly facilitates the correct diagnosis.* ***Conclusion:*** *EF studies are objective methods for studying the visual analyzer's function, that can not be manipulated, which makes them indispensable for detecting simulation and aggravation in ophthalmology. A detailed extensive study of the degree of simulation and aggravation among the ophthalmological patients is needed, which will enrich our knowledge and make us more precise in our expertise.*

Key words: *electrophysiology, electroretinography, visual evoked potentials, aggravation, simulation*

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INTRODUCTION

The Electrophysiological (EF) studies are objective methods for evaluating the visual analizer's function. These include different types of electroretinography (ERG) – fullfield ERG (ffERG), focal ERG (FERG), multifocal ERG (mfERG), pattern ERG (PERG), electrooculography (EOG) and visual evoked potentials (VEPs) [1-6].

ERG and EOG are used for diagnosis and monitoring of many retinal diseases, and VEPs depend on the functional integrity of the entire visual pathway from the retina, through the optic nerve, optic tract, optic radiation to the visual cortex [1, 7].

EF methods are also used for objective measurement of visual acuity and visual field in uncooperative patients, in young children and for detection of simulation [1, 8, 9].

The problem of simulation and aggravation in medicine is still relevant today, despite the development of technologies and the availability of increasingly better diagnostic equipment. The dilemma of whether to declare a patient a simulant has always troubled the conscientious physician. On the other hand, there are still patients who try to fake an illness, for psychological, economic, social or other reasons [10-12].

OBJECTIVE

To present the importance of the electrophysiological studies for detection of malingering and aggravation in ophthalmology.

MATERIALS AND METHODS

Six eyes of three patients aged between 20 and 36 years underwent a complete clinical examination, fundus-autofluorescence (FAF), fluorescein angiography (FA), optical coherence tomography (OCT), visual field testing, electrophysiological (EF) studies – full-field, multifocal and pattern electroretinography (ffERG, mfERG and PERG) and visual evoked potentials (VEPs), for detection of simulation or aggravation.

The study followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all subjects after the nature and possible consequences of the study had been explained.

RESULTS

All three patients were refered for expertise for a possibility of simulation.

After the electrophysiological studies' results, which are objective and non-manipulable, we purposefully reviewed and repeated some of the tests already done, which allowed a comprehensive interpretation of all results. It turned out that discrete changes in targeted search can be detected in several of the studies performed, which greatly facilitates the correct diagnosis.

Patient 1

A 36-year-old woman of Roma origin was presented to our clinic with complaints of reduced distance and near vision for 5-6 years, pain and heaviness in the eyes and difficulty seeing in the dark. According to the patient, her father and her brother had the same complaints, but we do not have any evidence of this.

Observation: She moved on her own in the unfamiliar environment of the doctor's office, did not bump into objects, did not turn her head to look away. She oriented herself correctly and reached to pick up an object, on which was written precisely a certain small font.

Subjectively: Her right eye best corrected visual acuity (BCVA) was 0.08, the left eye BCVA was 0.1, with color vision impaired, but inconclusive. The visual field testing showed completely black perimetry in both eyes (Fig. 1).

Objectively: The anterior and posterior eye segments were clinically healthy. FAF, FA, OCT were normal (Fig. 2, 3). All EF tests were done – insignificant changes in the photopic ERG of the right eye was found. The other EF tests – scotopic ERG, mfERG and PERG of both eyes were normal. VEPs were also normal and corresponding to visual acuity at least 0.8 (Fig. 4). She refused to be tested for genetic analysis.

Conclusion: High simulation probability.

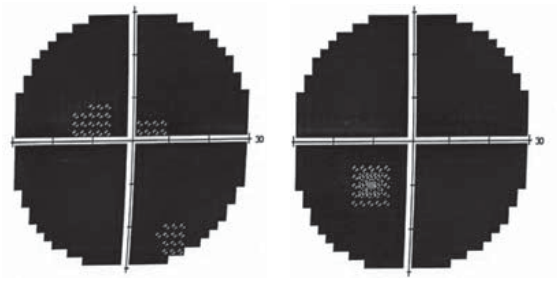

Fig. 1. Computer automated perimetry of the first patient – completely dark perimetry both eyes

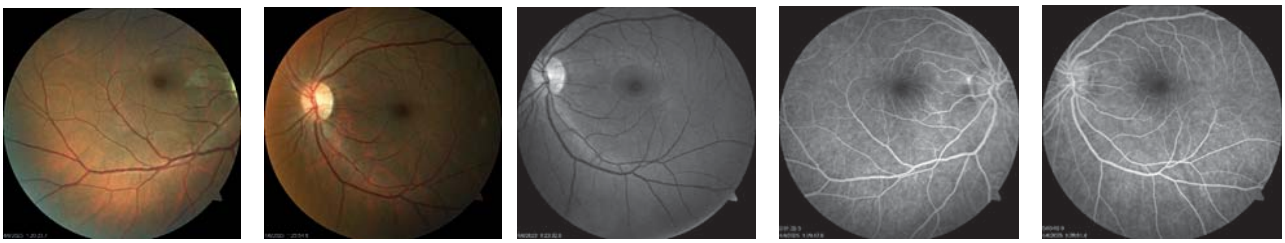

Fig. 2. Fundus-photography, FAF and FA of the first patient – normal pictures, as you can see the patient did not cooperate during the examination

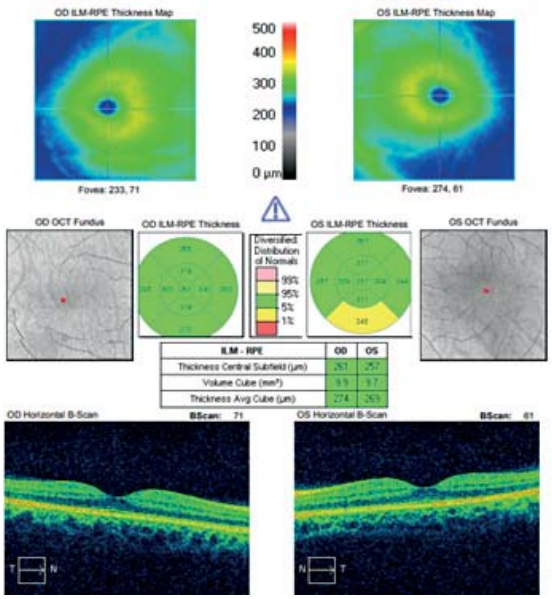

Fig. 3. OCT of the the first patient – insignificant thickening of the inferior part of the left macula

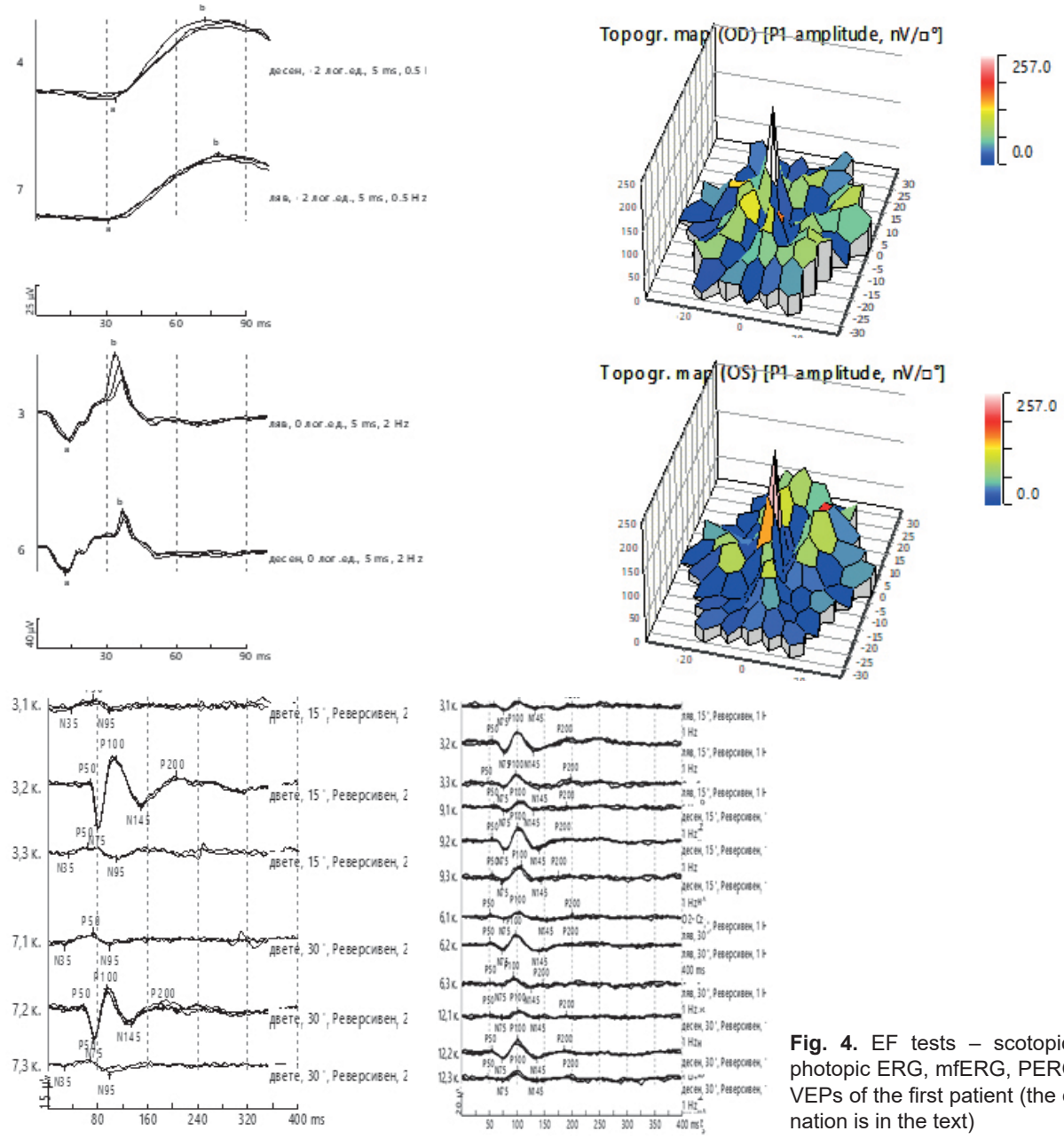

Fig. 4. EF tests – scotopic and photopic ERG, mfERG, PERG and VEPs of the first patient (the explanation is in the text)

Patient 2

A 20-year-old woman with complaints of reduced vision and photophobia for about a year was presented to our office.

Subjectively: BCVA OD = 0.3, BCVA OS = 0.5, the color vision was impaired. The visual field testing demonstrated concentric peripheral narrowing in both eyes, the central vision was also affected in the right eye (Fig. 5).

Objectively: The anterior and posterior eye segments were clinically healthy. Localized spots of hypofluorescence peripherally was detected on FAF. Abnormal speckled hypofluorescence was detected on FA at the choroidal phase, and speckled hyperfluorescence was observed peripherally around the vessels in some sections at the late phases (Fig. 6). On OCT – preserved foveolar contour with areas of thinned retina parafoveolarly bilaterally were found (Fig. 7). In scotopic ERG – slightly reduced amplitude with a changed wave configuration were detected. The diffuse photopic ERG was normal. In mfERG – reduced local central photopic activity in left eye was found (Fig. 8).

Conclusion: Clinically and electrophysiologically, it is probably a rod-cone dystrophy. Peripheral blood was taken for genetic testing, still not ready.

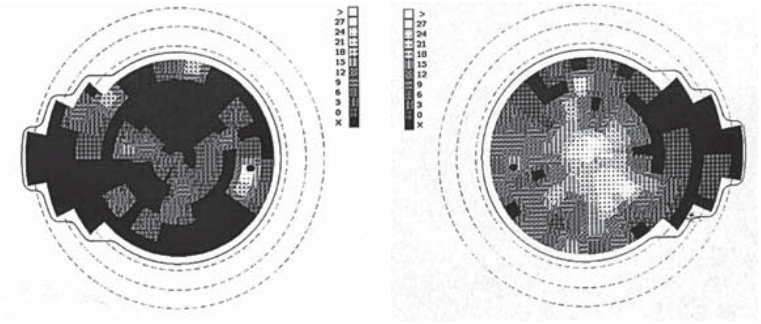

Fig. 5. Computer automated perimetry of the second patient (the explanation is in the text)

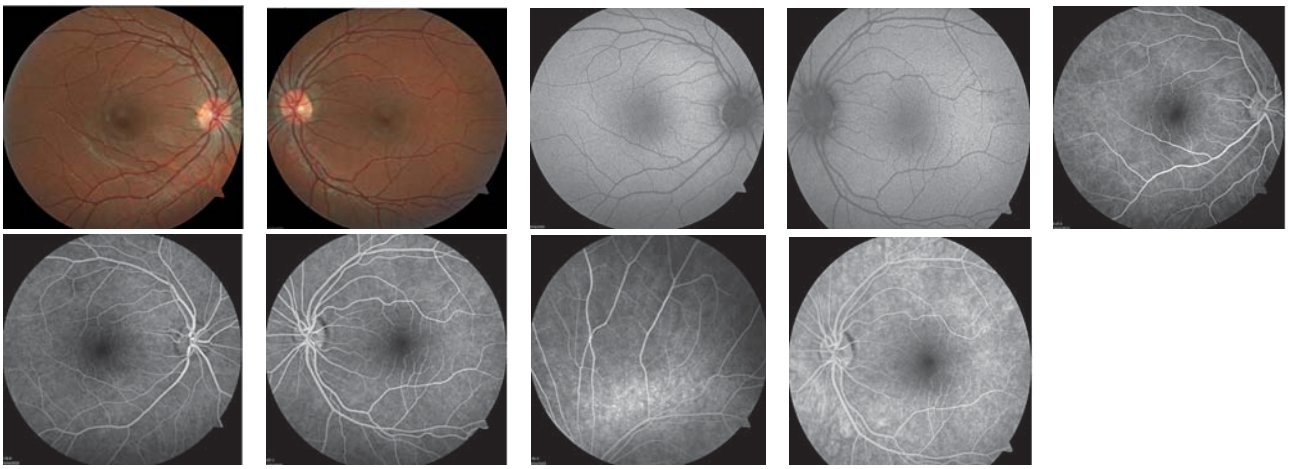

Fig. 6. Fundus-photography, FAF and FA of the second patient (the explanation is in the text)

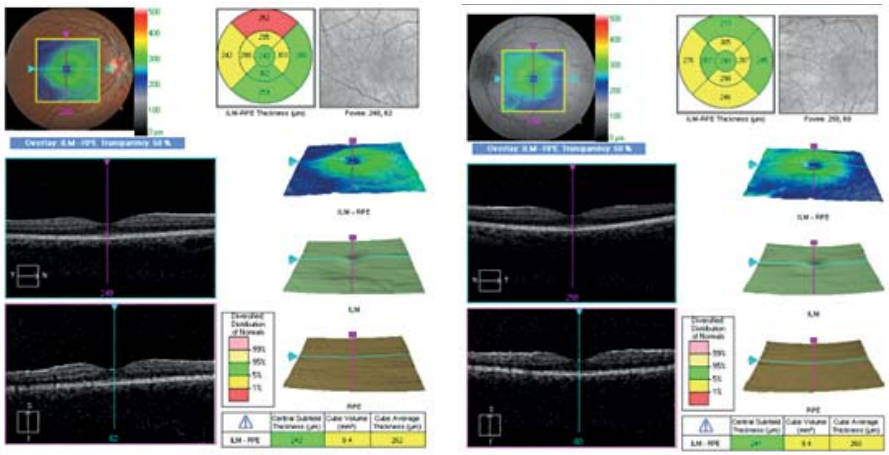

Fig. 7. OCT of the the second patient right and left eye (the explanation is in the text)

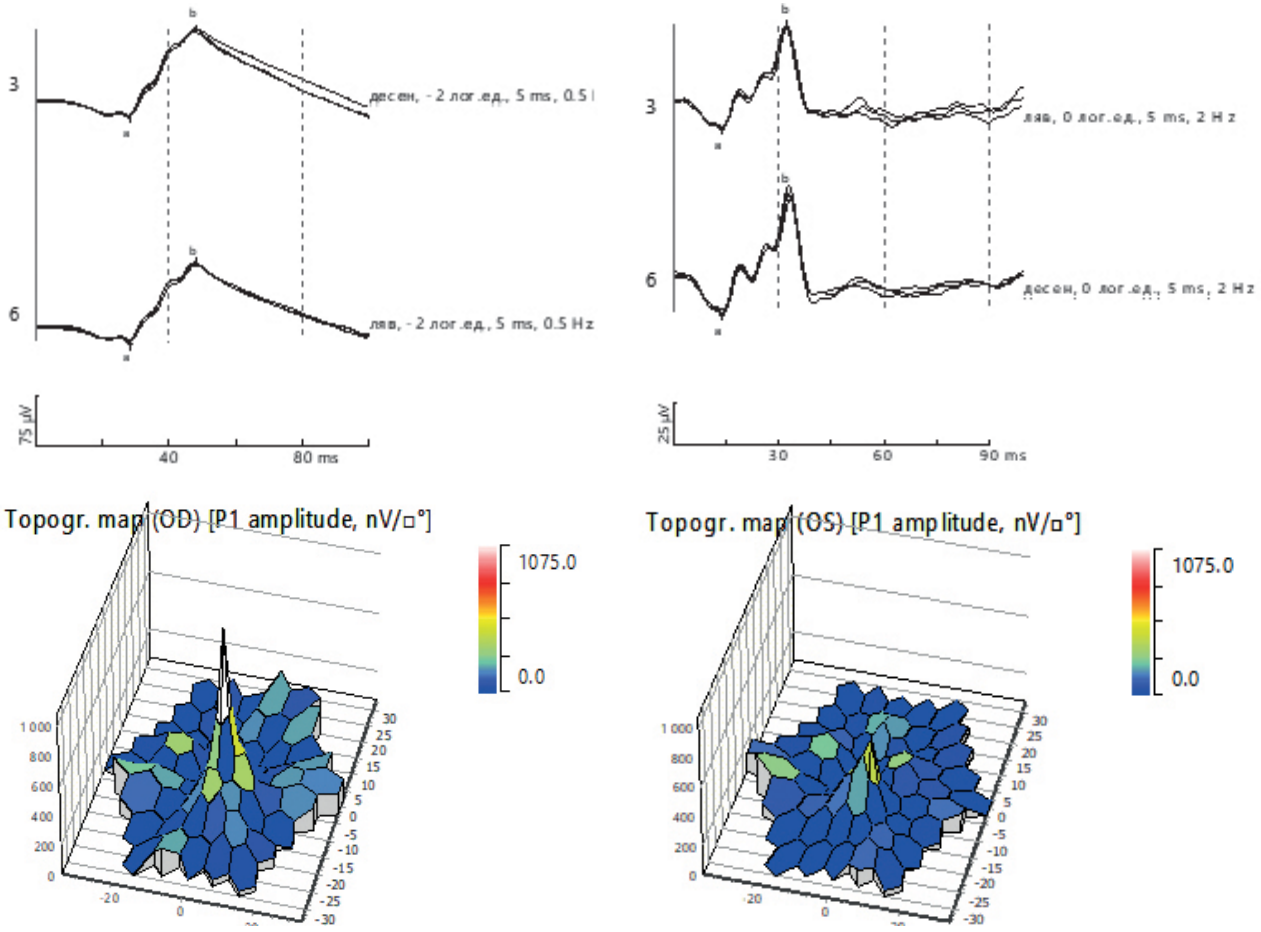

Fig. 8. EF tests – scotopic and photopic ERG and mfERG of the second patient (the explanation is in the text)

Patient 3

A 22-year-old woman with complaints of reduced vision and photophobia for about a year was presented to our clinic.

Subjectively: BCVA OD = 0.3, BCVA OS = 0.3, the color perception was impaired. The visual field testing was normal (Fig. 9).

Objectively: The anterior and posterior eye segments were clinically healthy. No pathology on FAF was found. On FA at the late phase, abnormal spots of hyperfluorescence was found peripherally around the vessels and nasally of the head of the optic disk in some sections (Fig. 10). On OCT – a slightly smoothed foveolar contour with areas of thinned retina in the macula bilaterally were detected (Fig. 11). Normal scotopic ERG bilaterally was found. In the diffuse photopic ERG – slightly reduced amplitude in left eye was demonstated. In mfERG reduced local central photopic activity in left eye was detected. PERG was normal, due to the very early changes in photoreceptors that were compensated by the healthy bipolar and ganglion cells. In VEPs changed configuration and slightly reduced amplitude, more in the left eye in central stimulation was found (since the macula has a large representation in the visual cortex) (Fig. 12). The changes demonstrated macular damage and visual acuity at least 0.3.

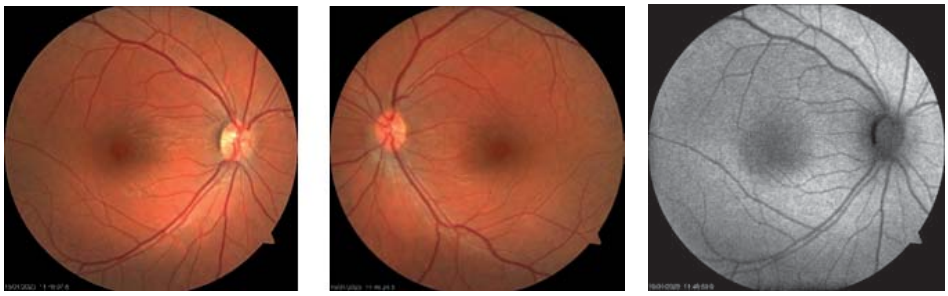

Fig. 9. Computer automated perimetry of the third patient (the explanation is in the text)

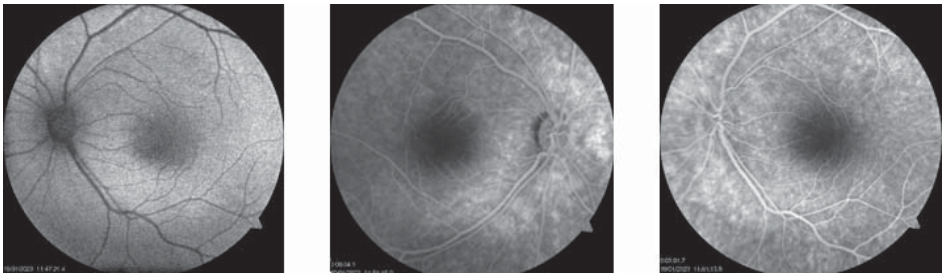

Fig. 10. Fundus-photography, FAF and FA of the third patient (the explanation is in the text)

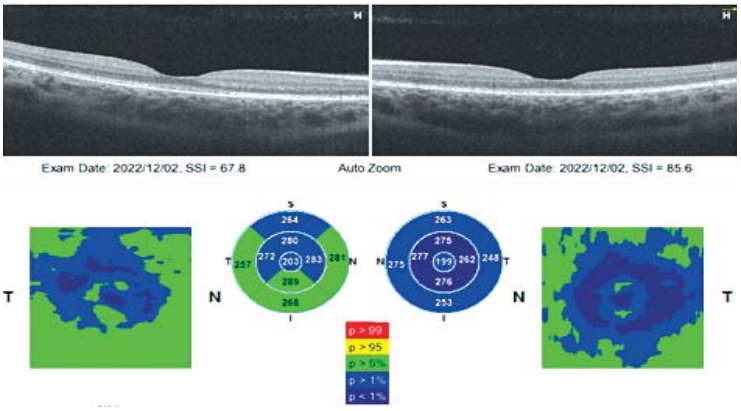

Fig. 11. OCT of the thethied patient right and left eye (the explanation is in the text)

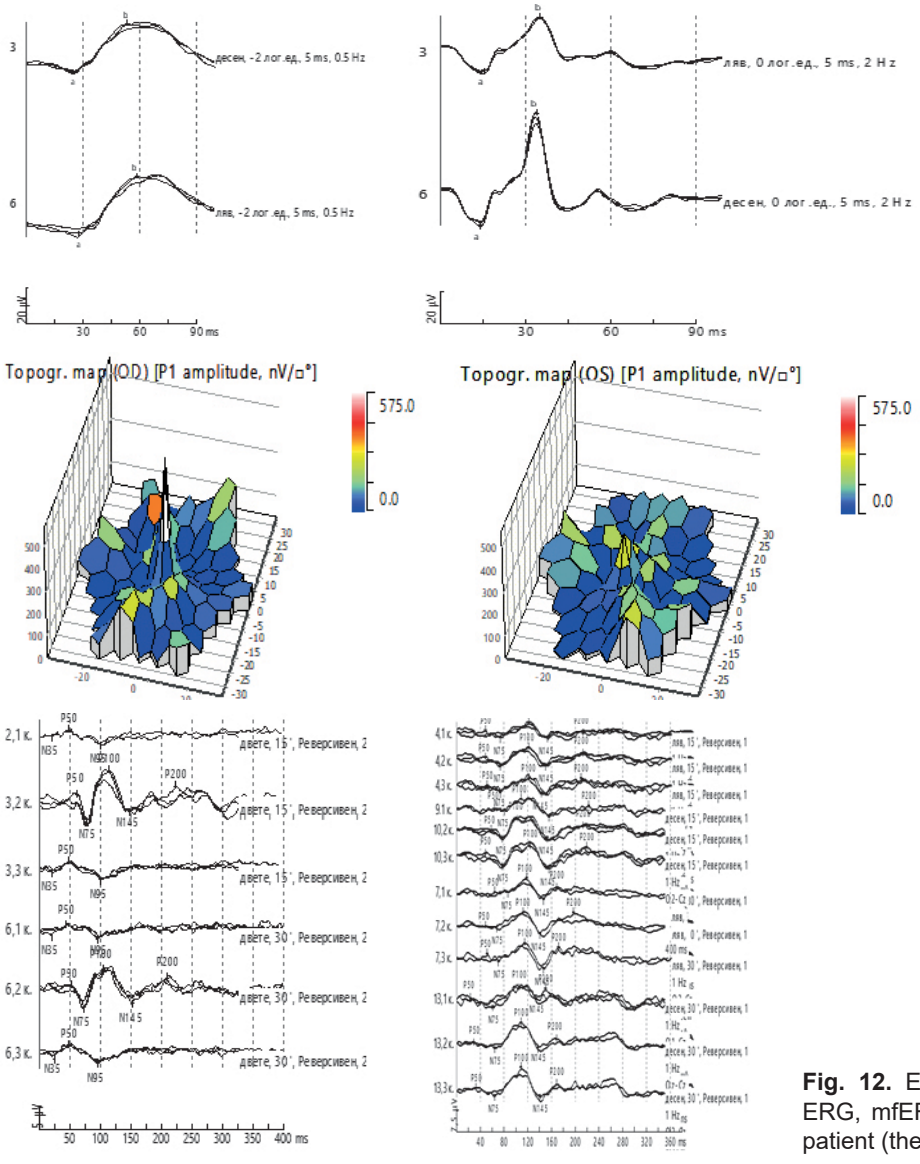

Fig. 12. EF tests – scotopic and photopic ERG, mfERG, PERG and VEPs of the third patient (the explanation is in the text)

Conclusion: Clinically and electrophysiologically, it is probably an initial stage of maculopathy. Peripheral blood was taken for genetic testing, a mutation causing macular dystrophy was found.

DISCUSSION

The problem of expertise in detecting simulation and aggravation is very complex. The doctor's mission is to cure and empathize with the patient's pain and suffering and to do everything according to the medicine achievements to relieve the patient suffering. However, although rare, this is used by unscrupulous patients who, for some psychological, social or financial reason, try to simulate or aggravate their condition.

On the other hand, by acting suspiciously, the doctor may miss some very initial or non-specific symptom and thus deprive the suffering patient of his right to be diagnosed, treated and benefit from some social benefits that could alleviate suffering and improve his life [11].

Of the three presented patients, only in the first one the suspicions of simulation were confirmed. In our country, there are no published studies on the degree of simulation in eye diseases. Worldwide, there are single such publications that confirm the role of EF studies to objectively study the visual analyzer's function, since simulation can only be performed in subjective functional studies, such as computer perimetry or subjective visual acuity or color perception study [1, 12-18].

The essence as well as the exact description of all modern electrophysiological methods is described by Fishman GA. and co-authors in 2001. They illustrate in great detail their importance for an objective study of the visual analyzer function, which makes them indispensable in the diagnosis of simulation and aggravation [1]. This, at the stage the modern ophthalmology is, cannot be achieved with the rest of the available imaging and functional studies we know. In support of this are several articles published this year that fully support the claims of Fishman GA. and co-authors on the relevance and irreplaceability of the EF methods for this type of expertise [19-24].

The role of EF methods for detection of simulation was also described by Gundogan F. et al. in a young woman who claimed to have no visual perception in the left eye for 2 years. All standard ophthalmological examinations performed, did not identify an organic cause of blindness. Absence of an afferent pupillary defect was also found. VEPs demonstrated that the visual acuity of the left eye was at least 0.3 [25].

A large-scale study of 155 scientific reports on the role of EF studies, and in particular of VEPs for objective measurement of the visual function, was published by Hamilton R. et al., in 2021. Their analysis clearly proves the objectivity of EF studies for simulation expertise, as well as for objective examination of visual acuity in children and uncooperative patients [12].

In the available literature, a comprehensive study on the degree of simulation and aggravation in ophthalmological patients was not found. The most similar is the study of Streppel M. and Brusis, who studied another similar analyzer – the auditory system and presented a prospective study on 61 patients who underwent a subjective hearing examination – audiogram, followed by an objective examination – auditory evoked potentials and found, that only 42% of those surveyed had no simulation. 10% of the remaining patients showed a severe degree of simulation, and the remaining 48% were found to have a mild or moderate degree of simulation [11]. The published results are quite alarming considering that the studies were not conducted among suspected for simulation patients.

In the eye, there is a greater opportunity for an objective study of its structure, especially with the modern diagnostic methods, which eliminates some of the hesitation. But nevertheless, there remains a significant number of patients, such as the ones presented by us, in whom the suspicion of simulation must be ruled out. Another concern of the ophthalmologist with intact and well-functioning eye structures is the presence of some ophthalmo-neurological problem that would lead to reduced vision, but performing VEPs testing eliminates these doubts. A similar conclusion was reached by Gruber H. [10].

CONCLUSION

EF studies are objective methods for study the visual analyzer's function, that can not be manipulated, which makes them indispensable for detecting simulation and aggravation in ophthalmology. Unfortunately, they are very little distributed in the clinical practice, they are more often used for scientific purposes. A detailed extensive study of the degree of simulation and aggravation among the ophthalmological patients is needed, which will enrich our knowledge and make us more precise in our expertise.

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CASE REPORT

A RARE CASE OF ADULT PRECURSOR B-CELL LYMPHOBLASTIC LYMPHOMA/LEUKEMIA PRESENTING WITH MULTIPLE OSTEOLYTIC BONE LESIONS AS SOLE MANIFESTATION

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Abstract. *Primary precursor B-lymphoblastic lymphoma/leukemia (B-LBLL) of bone is uncommon neoplasm that accounts for less than 1% of primary bone tumors. It has been commonly reported in pediatric population but is rare in adults. We present a case of a 20-year-old man with multiple osteolytic lesions as sole presentation of acute lymphoblastic leukemia, whose diagnosis was established by bone biopsy. A peripheral blood smear and the bone marrow aspirate showed no blast cells. The patient received treatment according to BFM 99 protocol and achieved complete response confirmed by F-FDG-PET/CT.*

Key words: *acute lymphoblastic lymphoma/leukemia, bone lesions, PET/CT*

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common leukemia in childhood and is a relatively rare neoplasm in adults [1]. Patients with ALL usually present with fever, bone and joint pains, lymphadenopathy, hepatosplenomegaly, bleeding manifestations. Primary precursor B-lymphoblastic lymphoma/leukemia (B-LBLL) of bone is uncommon presentation that accounts for less than 1% of primary bone tumors. It has been commonly reported in pediatric population but osteolytic bone lesions as sole presentation of B-LBLL in adults is extremely rare [2].

Only few cases of B-LBLL in adult patients with osteolytic lesions and hypercalcemia have been reported worldwide [3, 4], whereas multifocal skeletal B-LBLL as sole extra medullary disease is casuistic.

CASE PRESENTATION

We describe a case of a 20-year-old man who developed severe lower back pain after sport activity. Analgesics were prescribed without pain relief and the patient was sent for lumbar computer tomography (CT) scan and magnetic resonance imaging (MRI). The CT scan showed multiple osteolytic bone lesions of lumbar spine, whereas the MRI was negative. The osteolytic lesions were interpreted as metastatic and 18F-fluorodeoxyglucose – positron emission computer tomography (18F-FDG-PET/CT) was requested in order to determine the primary tumor location. 18F-FDG-PET/CT revealed numerous lytic lesions with high metabolic activity of skull, femur, humerus, thoracic and lumbar vertebrae, ribs, sacrum, and pubis (Fig. 1). No lymphadenomegaly or organomegaly was detected. The patient was sent to the Hematol-

ogy Clinic for diagnostic workup with a suspected diagnosis of multiple myeloma (MM).

At the time of presentation in the hematology department there were no other physical complaints. Medical and family history was irrelevant. Physical examination revealed no peripheral lymphadenomegaly or hepato- and splenomegaly.

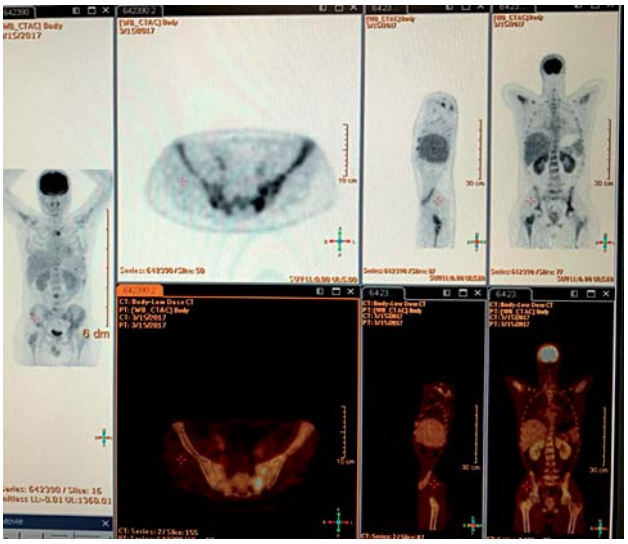

Fig. 1. F-FDG-PET/CT imaging revealed numerous lytic lesions with high metabolic activity of skull, femur, humerus, thoracic and lumbar vertebrae, ribs, sacrum, and pubis

The laboratory results revealed grade 1 anemia, serum creatinine level – 116 mcmol/l (range 44-97 mcmol/l), c-reactive protein – 164 mg/l (normal < 2.9 mg/l), lactate dehydrogenase – 337 U/l (range 84-246 U/l), calcium – 3.2 mmol/l (range 2.1-2.5 mmol/l). Values for platelets, leucocytes, leucocyte differentiation, liver enzymes, total protein and albumin were normal. Electrophoresis of protein in serum and urine was normal. No free light chains (FLC) in serum and urine were detected as well. The parathormone (PTH) level was within the normal reference ranges. The bone marrow aspirate revealed normocellular bone marrow; normal maturation of the three lineages of hematopoiesis; no plasma cell or blast cell infiltration. Cytogenetical and molecular analysis did not show abnormalities. Ultrasound of parathyroid glands was normal. Biopsy of a skull lesion was performed, and it revealed the diagnosis of precursor B-LBLL. Morphology and immunohistochemistry revealed diffuse proliferation of lymphoblasts with telangiectasia in the tumor parenchyma, soft tissue septa with hyalinization. Blast cells express CD45, CD99, TdT, CD10, CD43; Ki67 – 50% (Fig. 2).

Treatment according to BFM 99 protocol was initiated. He received remission-induction chemotherapy consisting of prednisolone (40 mg/m^2), daunorubicin (40 mg/m^2 on day 15 and 22), vincristine (2 mg on day 8, 15, 22 and 29) and PEG-l-asparaginase (1000/m^2 on day 8 and 21). There were no signs of cerebral invasion on cerebrospinal fluid examination and prophylactic intrathecal administration of methotrexate (15 mg) and dexamethasone (4 mg) was given according to the protocol. After the induction phase a complete remission, proved by F-FDG-PET/CT, was achieved. Due to the absence of a sibling donor he eventually proceeded to consolidation chemotherapy.

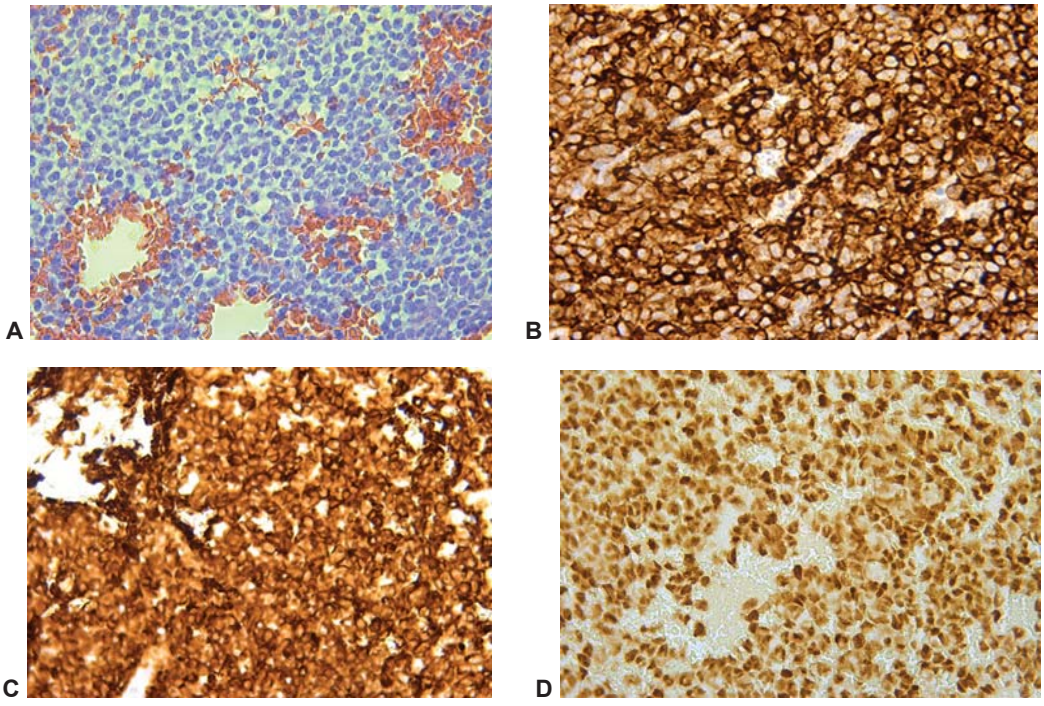

Fig. 2. Morphology and immunohistochemistry of scull lesion (**A**) H&E, 400x, (**B**) CD10, 400x, (**C**) CD99, 400x, (**D**) TdT, 400x

DISCUSSION

Various myeloid and lymphoid malignancies can present with bone involvement and osteolytic lesions such as acute megakaryocytic leukemia [5], chronic myeloid leukemia [6], non-Hodgkin' s lymphomas, adult T-cell leukemia, hairy cell leukemia, chronic lymphocytic leukemia, MM, Waldenstrom's macroglobulinemia, eosinophilic granuloma [7].

Osteolytic bone lesions are a rare presentation of adult B-LBLL and only a few cases are reported worldwide [4, 8, 9, 10, 11]. Most information regarding B-ALL associated hypercalcemia and osteolysis comes from reports in pediatric patients [12]. A retrospective analysis of 83 children with ALL suggests that children with few (one to four) bone lesions have an indolent form of leukemia with short duration and good prognosis. On the other hand, more than four bone lesions suggest longer duration of disease and poor prognosis [13]. The association with hypercalcemia is observed between 0.6% and 4.8% of children with this ALL/L [12, 14, 15, 16]. The differential diagnosis is wide ranging. Several malignancies can be associated with hypercalcemia such as rhabdomyosarcoma, hepatoblastoma, lymphoma, multiple myeloma, brain tumors, neuroblastoma, angiosarcoma, and less commonly ALL and acute myeloid leukemia [13]. The most common cause of malignancy-related hypercalcemia is ectopic production of PTH-related protein (PTHrP). The cause of hypercalcemia in ALL is not well defined and may be related to the production of an osteoclast activating factor by leukemic blasts. Other factors such as transforming growth factor – α (TGF-α), tumor necrosis factor-α (TNF), interleukin-1, prostaglandin E2 (PGE2) have also been implicated [17, 18, 19]. In one series of 21 patients 11 had PTHrP – mediated hypercalcaemia, while two patients had raised TNF-α and interleukin-6 [18]. ALL presenting with hypercalcemia has a distinctive clinical profile, biology, pathological features and poor response to treatment.

B-LBLL presenting as a solitary bone tumor without bone marrow or other sites involvement is extremely rare and less than 10 cases have been reported in the English language literature [20]. Clinically it may be confused with other primary bone tumors or metastatic neoplastic diseases which confirms the importance of histological verification especially in these cases with no blood or bone marrow presentation. 18F-FDG-PET/CT appears a method of choice combining the sensitivity of metabolic imaging with the specificity of anatomic imaging and may be useful for the initial diagnosis, determination of optimal biopsy site and for monitoring therapeutic response.

Despite the distinctive clinical profile and poor response to conventional chemotherapeutic agents, the curability of B-LBLL in contrast to other metastatic malignancies makes early and accurate diagnosis of paramount importance.

Disclosure Summary: *The authors have nothing to disclose.*

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REVIEW

CURRENT THERAPEUTIC OPTIONS IN ACTIVE MODERATE-TO-SEVERE THYROID-ASSOCIATED OPHTHALMOPATHY

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Abstract. *Active moderate-to-severe TAO is a disease, the treatment of which requires a multidisciplinary team, an individualized approach and strict follow-up, yet it is often suboptimal. Recent knowledge about the pathogenesis of the disease and the randomized controlled trials conducted in recent years made it possible to use new therapeutic combinations and biological agents. The currently recommended first-line therapy for active moderate-to-severe TAO is the combination of intravenous glucocorticoids (GCs) in moderate doses and mycophenolate or, in more severe cases, a high-dose GC treatment alone. There are several options for second-choice therapy, if needed: a new course with GCs, combination of orbital radiotherapy and GCs (preferably intravenous), cyclosporine and oral GCs, azathioprine and oral GCs, rituximab, tocilizumab or teprotumumab. The clinical manifestations of TAO should also be considered when choosing second-line treatment. Thus, for example, teprotumumab best affects diplopia, orbital radiotherapy – visual disturbances and diplopia, while intravenous GCs, mycophenolate, cyclosporine, rituximab and tocilizumab – the inflammatory manifestations of TAO. However, the question of the availability of the new drugs in routine clinical practice remains unsolved.*

Key words: *thyroid-associated opthalmopathy, treatment, glucocorticoids, therapeutic response*

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INTRODUCTION

Thyroid-associated ophthalmopathy (TAO) is a disease affecting the retroorbital soft tissues, that is etiopathogenetically associated with an autoimmune thyroid disease, most commonly Graves' disease [1]. The frequency of TAO is relatively low, especially its more severe forms, which occur in only 5-6% of cases [2]. The etiology and pathogenesis of the disease are not fully understood. It is believed that a number of risk factors (thyroid dysfunction, smoking, stress, radioiodine therapy) can trigger the disease in genetically predisposed individuals [3, 4]. In the pathogenesis of TAO, the interaction between TSH-receptor antibodies (TRAb) and the corresponding receptor expressed on the surface of retroorbital fibroblasts, as well as the post-receptor interaction with the IGF-1 receptor [5, 6], play a major role. A number of cytokines and immune cells also take part in this process [7]. Ultimately, the chronic local autoimmune inflammatory process in TAO leads to transformation of the retroorbital tissues with accumulation of fatty tissue, hydrophilic substances, edema and fibrosis [8, 9].

The clinical picture of TAO is varied and depends on the severity and type of ocular involvement. Manifestations of TAO are: proptosis, motility defiicits, retraction of the eyelids, increased intraocu-

lar pressure, double vision, edematous manifestations, variety of subjective symptoms, corneal defects, and in the most severe cases even reduction or loss of vision [10]. Depending on the severity of the changes, the form of TAO is classified as mild, moderate-to-severe and sight-threatening [11] (Table 1). Moderate-to-severe forms have some of the following manifestations: proptosis ≥ 3 mm, lid retraction ≥ 2 mm, moderate or severe soft-tissue involvement, persistent or intermittent diplopia. The activity of the inflammatory process is evaluated by calculating the so-called clinical activity score (CAS), based on which TAO is classified as active (if CAS ≥ 3) or inactive (if CAS < 3) [11] (Table 2). In patients with TAO, the quality of life is greatly impaired [12]. Ocular changes negatively impact both visual function and appearance, the latter being associated with significant psychosocial problems.

The diagnosis of TAO is based on a detailed evaluation of thyroid hormone and immunologic status, followed by meticulous assessment of ocular involvement by a team of endocrinologists and ophthalmologists [13]. Correct determination of the form and activity of the disease is a key factor when choosing the optimal therapeutic strategy. In the treatment of moderate-to-severe forms, various immunomodulatory agents are used, and the therapeutic strategy requires a close multidisciplinary interaction and an individualized approach taking into account the needs of the individual patient.

Table 1. Classification of TAO severity according the consensus of the European Thyroid Association and European Group on Graves' Orbitopathy

Severity of TAO	Signs and symptoms
Mild	Mildly affected quality of life One or more of the following: – eyelid retraction < 2 mm – mild soft tissue involvement – proptosis < 3 mm – no or intermittent diplopia – mild corneal involvement
Moderate-to-severe	Moderately or severely affected quality of life or one or more of the following: – eyelid retraction ≥ 2 mm – moderate or severe soft tissue involve – ment proptosis ≥ 3 mm – constant or inconstant diplopia
Sight-threatening	Dysthyroid optic neuropathy Corneal breakdown

THERAPEUTIC AGENTS FOR TREATMENT OF ACTIVE MODERATE-TO-SEVERE TAO

Control of risk factors. A primary measure in all patients with TAO is the control of risk factors – cessation of smoking, control of hyperthyroidism, avoidance of iatrogenic hypothyroidism, prophylaxis with oral glucocorticoids (GCs), if radioiodine therapy is necessary [2]. This favors the natural evolution of the disease regardless of its form, and ensures an optimal response to immunosuppressive therapy in cases indicated for such treatment [3].

Statins. Literature data indicate a protective effect of statins regarding the occurrence of TAO in patients with Graves' disease [14]. Until recently, it was thought to be due to the well-known anti-inflammatory properties of this class of drugs. In a recent cross-sectional study by Sabini et al. including 250 patients with Graves' disease, 133 of whom with TAO, a positive correlation was found between the presence of TAO and the levels of total and LDL-cholesterol, suggesting a possible role of cholesterol in the occurrence of TAO [15]. In addition, the authors observed a relationship between lipid levels and TAO activity, which in turn indicates that cholesterol could be related to the clinical manifestations of TAO. Based on the obtained results, it can be speculated that in addition to the anti-inflammatory properties of statins, their antilipemic effect also protects against the development of TAO. According to the latest consensus of the European Thyroid Assosiation (ETA) and the Eu-

Table 2. Evaluation of TAO activity

	Initial CAS evaluation
1.	Spontaneous retrobulbar pain
2.	Pain when moving the bulb
3.	Eyelid hyperemia
4.	Injection
5.	Eyelid edema
6.	Chemosis
7.	Edema of caruncle/plica
	Follow-up:
8.	Increase in proptosis by ≥ 2 mm for a period of one to three months
9.	Decrease in eye movement by > 8° in any direction for a period of one to three months
10.	Decrease in visual acuity by ≥1 line of the Snellen chart for a period of one to three months

ropean Group on Graves' Orbitopathy (EUGOGO), in patients with TAO and hypercholesterolemia, statin treatment should be considered [11].

Local treatment. Enlarged palpebral aperture, exophthalmos, blink rate, eyelid retraction, lagophthalmos, impaired bulbar elevation and absent Bell phenomenon in patients with TAO can lead to dry eyes (the so-called dry eye syndrome) with subsequent risk of corneal damage [16]. In addition, researchers studying tear film composition in patients with TAO reported higher tear fluid osmolarity [17]. The involvement of the lacrimal glands in the autoimmune inflammatory process inevitably affects the amount and composition of their secretion. In the proteomics of tear fluid in TAO, increased levels of inflammatory proteins were found, while those of protective proteins were reduced. In long-standing disease, fibrosis of the lacrimal glands occurs with the development of secondary Sjogren's syndrome.

Therefore, patients with TAO should apply artificial tears containing hyaluronic acid several times a day to protect the ocular surface, relieve dry eye symptoms, and prevent corneal damage [11]. In case of severe lagophthalmos, it is recommended to apply bandages to the eyes at night and use viscous gels and ointments with prolonged action.

GCs. For decades, GCs have been well known for their anti-inflammatory, immunomodulatory and immunosuppressive actions. This makes them widely used to treat certain inflammatory and autoimmune diseases. Since the 1950s, they have also been used to treat severe forms of TAO [18], and are currently the well-established therapy of first choice in patients with active moderate-to-severe TAO [19].

Nagayama et al. were the first investigators to use intravenous GCs to treat TAO [20]. Subsequently, various authors studied the short-term and long-term effectiveness and safety of intravenous GC administration in TAO [21-24]. The regimens used significantly differed in terms of dose, duration, GC administration scheme, observation period, etc. However, what these studies have in common is the observed good results regarding the ocular manifestations of TAO. Geest et al. conducted the first prospective, randomized, double-blind, placebo-controlled trial to evaluate the effectiveness of methylprednisolone (MP) pulse therapy, including 15 patients with TAO. Despite the small number of patients, the results undoubtedly showed that intravenous GCs had good efficacy in the absence of serious side effects in patients with active moderate-to-severe TAO [25]. Macchia et al. were the first research team to directly compare the effectiveness of intravenously and orally administered GCs [21]. The authors observed a significantly better effect of venous GCs (84% vs. 57%, $p < 0.01$), as well as a lower incidence of side effects. Later studies confirmed the better effectiveness and safety profile of intravenous administration compared to oral administration [26-28].

The effectiveness and safety of three different cumulative doses of intravenous GC treatment – 7.47, 4.98 and 2.25 g., were evaluated in a large multicenter randomized clinical trial by Bartalena et al. [29]. The authors found that short-term efficacy was significantly better using the highest cumulative dose compared to the two lower doses ($p = 0.03$ and $p = 0.01$ when compared with the moderate and low doses, respectively). At the 6th month of follow-up after discontinuation of GC treatment, 33% of patients in the high-dose regimen group, 21% of the moderate-dose group, and 40% of the low-dose group had a recurrence of TAO. Severe side effects were seen slightly more often in the high-dose regimen group.

The 2016 ETA and EUGOGO consensus recommends that GC treatment in moderate-to-severe forms of TAO should be administered as intravenous infusions – 500 mg MP weekly for 6 weeks, followed by 250 mg MP weekly for 6 weeks with a total cumulative dose 4.5 g with a proven good benefit/risk ratio [19]. The effectiveness of the this regimen, evaluated in various studies, varies between 35-80%, and in terms of the effect on CAS – 64-83% [26, 29, 30].

Cases of serious cardiovascular and liver damage after intravenous GC treatment of TAO have been described in the literature [31-38]. A meta-analysis of 14 studies (over 1000 patients with TAO) found morbidity and mortality due to GC treatment to be 6.5% and 0.6%, respectively [39]. Lendorf et al. described 5 cases (out of a total of 67 treated patients) having serious cardiovascular side effects after administration of high-dose intravenous GC treatment (1 g MP daily for 5 consecutive days), 2 deaths due to cardiovascular and cerebrovascular complications, 3 cases of coronary thrombosis with elevated cardiac enzymes [38].

Serious liver damage has also been reported in patients with TAO treated with intravenous GCs, including acute liver failure at a total cumulative dose of 10-24 g [35-37]. Marinól et al. reported 7 cases of acute liver injury during or after administration of intravenous GCs [36], and the authors suggested several possible pathogenetic mechanisms: 1) direct hepatotoxic effect of GCs; 2) activation of viral hepatitis (hepatitis B, cytomegalovirus) in the settings of the immunosuppression induced by GCs; 3) reactivation of the immune system after the abrupt ces-

sation of venous GCs with the development of autoimmune hepatitis. Cases of hepatitis and acute liver failure have not been reported when using oral GC therapy, possibly due to the lower dose and gradual tapering off [39]. In order to minimize the risk of side effects during the GC course, it is accepted that the single daily dose should not exceed 750 mg of MP, the cumulative dose of MP should be less than 8 g, and the administration of GCs should not be done on consecutive days [11]. Otherwise, the risk increases two-fold, including for severe cardiovascular and cerebrovascular events and liver failure [39]. In order to prevent severe complications, a preliminary assessment of liver function is necessary – examination of liver enzymes, viral markers, liver autoantibodies and exclusion of patients with active viral hepatitis, severe liver damage, severe cardiovascular disease, uncontrolled arterial hypertension, psychosis, active infection and decompensated diabetes mellitus [40].

Orbital irradiation. Orbital irradiation has been used for years to treat the severe forms of TAO. Conventional external beam radiation and cobalt therapy (telegammatherapy) are no longer used at the expense of supervoltage linear accelerators (orbital radiotherapy, OR) [41]. OR has nonspecific anti-inflammatory properties, which are exerted on the highly radiosensitive lymphocytes infiltrating the retrobulbar spaces [42]. Under the action of OR, their secretory activity becomes suppressed. In addition, the T-helper/T-suppressor ratio changes under the influence of low-dose irradiation. It has also been established that OR suppresses the synthetic and secretory activity of retroorbital fibroblasts.

Commonly applied regimens for the treatment of patients with TAO are with a total dose of 16-20 Gy, divided into 10 sessions of 1.6-2.0 Gy [41]. However, at present, there is still no definitive consensus regarding the optimal OR regimens and doses for the treatment of TAO. While, according to some researchers, the effectiveness of OR is better at higher doses – 20-24 Gy [43], according to others the effect is identical at doses from 10 to 24 Gy, but with a higher risk of side effects for the higher doses [44]. Pitz et al. proposed a more prolonged administration of OR in order to ensure an optimal safety profile with preserved therapeutic efficacy (1 Gy per week for 20 weeks) [44].

Literature data regarding the effectiveness of OR in the treatment of TAO are conflicting. Donaldson et al. were the first to use a 4-6 MeV linear accelerator to treat active severe TAO in a group of 23 patients, with a therapeutic response observed in 65% of cases, even in those resistant to GC treatment [45]. The effect was weaker when long-standing disease was present. On the other hand, Gorman et al. observed comparable effectiveness of OR and sham-irradiation [46]. In a double-blind randomized trial by Mourits et al. the effectiveness of OR was compared with sham-radiation. The authors found a therapeutic response in 60% of OR patients versus 31% of those on sham-radiation ($p = 0.04$). Diplopia and eye muscle disturbances were best affected, while soft-tissue manifestations and proptosis were barely affected [47]. In other studies, in addition to eye muscle disorders OR was also found to have a good effect on soft tissue manifestations and visual acuity [41]. Similar results were observed in the study by Gabrovski et al. from 2013 – good therapeutic response in about 60% of Bulgarian patients with the best effect on visual acuity, soft tissue manifestations, eye muscle disturbances and corneal involvement [48]. Prummel et al. found that the therapeutic effect of OR was similar to that of oral GCs with less side effects [49]. The prospective randomized controlled trials of Bartalena et al. and Marcocci et al. demonstrate a higher effectiveness of the combined use of OR and oral GCs than when they are used as monotherapies [50, 51]. The administration of oral GCs alongside OR is also benefitial in terms of prevention of the exacerbation of ocular manifestations, which may occur as a result of OR-induced destruction of immune cells retroorbitally with leakage of cytokines [52].

In a retrospective study including 210 patients with TAO treated between 2000 and 2010 with fractionated OR (total dose 20 Gy), if necessary, combined with GCs/surgery with a mean follow-up of 11 months, it was found that 84% of patients had symptomatic improvement, and a complete therapeutic response was observed in 44%, with 1/3 of them treated with OR alone [53]. In a pilot prospective double-blind controlled study, Marcocci's research team compared the effectiveness and safety of OR combined with oral and intravenous GCs [27]. In the intravenous GCs group, a therapeutic response was observed in 83% (vs. 63% for the oral GCs and OR group) with better tolerability and fewer side effects. To date, there are no randomized controlled trials comparing the effectiveness of combined OR and intravenous GCs with that of monotherapy with intravenous GCs [52]. In two retrospective studies from 2016 and 2017, the teams of Kim and Oeverhaus found better efficacy of combined treatment with intravenous GCs and OR compared to monotherapy with GCs [54, 55].

In the course of OR, there may be a mild and transient exacerbation of the ocular manifestations, mainly soft tissue edema, injection and chemosis, for the prevention of which low doses of oral GCs are successfully used [52]. The development or

worsening of a pre-existing cataract is a well-known dose-dependent side effect. The risk of developing cataracts is reduced by using a fractionated regimen, well-collimated irradiation techniques, and radiation fields to protect the lens from irradiation. The median latency period for cataract development is 2 years, ranging from 6 months to 25 years [41]. Radiation-induced retinopathy has been reported in patients using much higher cumulative doses than those used for TAO, and in some cases this side effect could be explained by errors in dosing or radiation technique. Preexisting diabetic retinopathy increases the risk of further retinal damage. In a study investigating the long-term safety of OR, it was found that within 17 years after radiotherapy with a daily dose of 1 or 2 Gy, radiation-induced retinopathy occurred in 5% of patients with TAO and diabetes mellitus or arterial hypertension [56]. After OR, there is a theoretical risk of carcinogenesis in patients < 35 years of age. Absolute contraindications for OR are hypertensive and diabetic retinopathy, and diabetes mellitus is a relative contraindication [57, 58].

Mycophenolate. Mycophenolate exerts a potent reversible inhibition of the enzyme inosine monophosphate dehydrogenase, which is of key importance in the synthesis of purines in immune cells and also selectively depletes the available guanosine triphosphate in them [59]. The drug is widely used in clinical practice as an immunosuppressant after organ transplants and in some autoimmune diseases. It has antiproliferative effects on B and T-lymphocytes, induces T-cell apoptosis, inhibits antibody production, reduces the expression of adhesion molecules and chemoattractants, and suppresses fibroblast proliferation. In one Chinese randomized trial, mycophenolate monotherapy was found to be more effective than combined intravenous and oral GC treatment in terms of overall therapeutic response (79% vs. 51% at 12th week and 91% vs. 68% at 24th week), individual eye parameters (CAS, proptosis, diplopia) and recurrence rate (0% vs. 6%) [60]. The EUGOGO multicenter randomized trial from 2018 found that combining an intravenous GC regimen (total dose 4.5 g) with a moderate dose of oral mycophenolate resulted in a significantly better therapeutic response at 6th month compared to intravenous GC monotherapy (71% vs. 53%, p = 0.026) with a comparable safety profile and recurrence rate [61, 62]. Furthermore, the results of these studies showed that approximately 70% of patients on GC monotherapy versus 90% of those treated with mycophenolate achieved a significant improvement in CAS.

Cyclosporine. Cyclosporine is an immunosuppressant, the effect of which is due to inhibition of calcineurin and suppression of T-cell proliferation and the production of certain interleukins [63]. The effectiveness of cyclosporine was initially evaluated in small uncontrolled studies reporting good results [64, 65]. In their prospective randomized controlled trial involving 40 patients with severe TAO, Kahaly et al. compared the effect of oral prednisolone monotherapy with that of the combination of oral prednisolone and cyclosporine [66]. The authors found a significantly better efficacy of the combined treatment, especially regarding diplopia and proptosis. In addition, the recurrence rate in the prednisolone and cyclosporine group was significantly lower. The combination treatment was relatively well tolerated by the patients, with the most frequently observed adverse effects being gingivitis, hypertrichosis, paresthesias, elevation of liver enzymes and worsening of arterial hypertension. Prummel et al. conducted a single-blind, randomized trial in which they compared the effectiveness of cyclosporine and oral prednisone used as monotherapies, then examined the effect of the combination of the two drugs in non-responders to monotherapy [67]. Oral prednisolone produced a therapeutic response in significantly more patients (61% vs. 22%, p = 0.018), but with more frequent occurrence of severe and moderate side effects. Non-responders to monotherapy responded to combination therapy in 56-62% of cases and it was better tolerated than prednisolone monotherapy. The cited studies used low doses of cyclosporine – 5-7.5 mg/kg/day in order to prevent serious side effects – hepatotoxicity, nephrotoxicity, etc. To date, there are no controlled studies comparing the effect of cyclosporine and intravenous GCs.

Rituximab. Rituximab is a chimeric monoclonal antibody directed against the CD20 antigen on the surface of B-lymphocytes in peripheral blood and lymphoid organs, which exerts immunosuppression through B-cell depletion [68].

Fassi et al. and Salvi et al. were the first research groups to report the effectiveness of rituximab administered in single cases of severe TAO resistant to conventional GC therapy [69, 70]. Subsequently, in a double-blind randomized study by Salvi et al., including 32 patients with moderate-to-severe TAO, the effect of rituximab was compared with that of intravenous GCs. The authors found a significantly greater decline in CAS at each follow-up point in the rituximab group. At 24th week, 100% of patients in the rituximab group improved compared to 69% in the intravenous GC group (p < 0.001) [71]. During follow-up, patients treated with rituximab had no recurrences and the number of corrective surgical interventions was significantly lower (p = 0.049). In a

prospective double-blind placebo-controlled study by Stan et al. the effectiveness of rituximab appeared to be similar to that of placebo with a more frequent occurrence of side effects, some of which were moderate and severe [72]. However, in this study, the duration of TAO was greater compared to that of Salvi et al. In the cited studies, the total dose of administered rituximab was 500 mg (once) or 2000 mg (2 x 1000 mg). Later, similar effectiveness was found using a lower single dose (100 mg) [73]. The most frequently reported side effects were mild and transient: myalgias, arthralgias, nausea, diarrhea, headache, fever, rashes, etc. Two cases of acute deterioration of the eye status were also reported, including development of dysthyroid optic neuropathy in two patients, which was associated with the abrupt release of cytokines following B-cell depletion [71]. An increased risk of infections and progressive multifocal encephalopathy are possible serious side effects reported with the use of rituximab for the treatment of oncohematological and rheumatological diseases [74, 75].

Teprotumumab. Based on the current knowledge about the pathogenesis of TAO, and more precisely the demonstrated TSH- and IGF-1-postreceptor interaction, some researchers suggest effectiveness of IGF-1 receptor blockers for the treatment of TAO [76]. Teprotumumab is a human monoclonal antibody that extracellularly binds to and blocks the IGF-1 receptor, also leading to receptor internalization and enhanced degradation [77]. Smith et al. conducted a multicenter, double-blind, randomized, placebo-controlled clinical trial to evaluate the efficacy and safety of teprotumumab in patients with active moderate-to-severe TAO [78]. At 6th month, a therapeutic response was observed in 69% of patients in the teprotumumab group versus 20% for the placebo group ($p < 0.001$). The only side effect of the drug was worsening of glycemic control in patients with diabetes. In a similar study by Douglas et al. teprotumumab was found to be significantly more effective than placebo in terms of proptosis, CAS, diplopia, and quality of life in the absence of serious side effects [79]. In 2021, a systematic review was published by Kahaly et al., which evaluated the long-term effectiveness of teprotumumab on the patients from the two randomized trials [80]. One year after treatment, significant improvement in proptosis and diplopia was found in 67% and 69% of cases, respectively, and long-lasting improvement in the overall therapeutic response – in 83% of patients. The most common side effects reported were muscle cramps, nausea, vomiting, hair loss, etc. A case of severe teprotumumab-induced amyloid encephalopathy in a patient with TAO refractory to high-dose GC therapy has also been reported [80]. To date, there are no comparative studies evaluating the effectiveness of teprotumumab and that of intravenous GCs. Although the drug is approved by the United States Food and Drug Administration for the treatment of TAO, its use in real clinical practice is currently limited.

Tocilizumab. In vitro, interleukin-6 (IL-6) has been shown to stimulate the expression of TSH-receptors on the surface of fibroblasts and preadipocytes from the retroorbital spaces of patients with TAO and thus plays a key role in the pathogenesis of TAO [81, 82]. Tocilizumab is a human monoclonal antibody that binds to the IL-6 receptor and thus prevents the interaction of IL-6 with its receptor [83]. Perez-Moreiras et al. were the first research team to use Tocilizumab for the treatment of active moderate-to-severe TAO resistant to GC therapy [84]. Their study was uncontrolled and included 18 patients with TAO. The results showed a very good efficacy of tocilizumab in terms of CAS, proptosis, visual disturbances, diplopia and reduction of TRAb. The reported side effects were mild and transient – fatigue, arthralgias, nausea, etc., but two cases of neutropenia requiring adjustment of the dosage regimen were also recorded. In recent years, new data on the effectiveness of the drug in TAO have been accumulated from several randomized trials. A placebo-controlled study by Perez-Moreiras et al., including 32 patients with GC-resistant TAO, showed that tocilizumab was more effective than placebo in significantly reducing CAS (93.3% vs. 58.8% at 16th week), as well as in terms of reducing the severity of ocular manifestations and especially proptosis [83]. In 2021, the results of a retrospective longitudinal study were published, in which the effect of the medication administered in patients with GC-resistant TAO in real clinical practice was evaluated [85]. The authors found very good efficiency in terms of CAS – 90.9% of patients achieved a reduction in CAS by ≥ 2 points after the first infusion of the drug, and after the forth infusion (at 16th week) 74% of patients had CAS 0-1. Side effects were observed in almost half of the patients, the most common of which were: an increase in cholesterol, development of neutropenia, leukopenia and thrombocytopenia, an increase in liver enzymes, allergic reactions, including one case of anaphylactic shock [85]. In an uncontrolled observational study of 48 patients resistant to existing therapies, tocilizumab was well tolerated and 92% of patients showed clinical improvement [86]. To date, there are no large randomized clinical trials investigating the effect of the drug in untreated TAO.

Azathioprine. Azathioprine is an immunosuppressant with a mycophenolic-like mechanism of action that is often used to treat certain autoimmune and

inflammatory diseases. In a small prospective controlled study by Perros et al. azathioprine monotherapy was found to be ineffective for the treatment of TAO [87]. In a retrospective study by Chalvatzis et al. in 88 patients with TAO, a satisfactory therapeutic response was observed in 61.4%, 64.8%, 69.3% and 73.9% of the patient at the 3rd, 6th, 12th and 18th months, respectively, of combined treatment with GCs, azathioprine and OR [88]. The results of a randomized trial showed that azathioprine was particularly effective in preventing recurrence of ocular manifestations after GC treatment [89]. The most common side effects of the drug were nausea, vomiting, hepato- and hemotoxicity.

CURRENT RECOMMENDATIONS FOR TREATMENT OF ACTIVE MODERATE-SEVERE AND SEVERE TAO

The most recent EUGOGO and ETA consensus for the treatment of TAO is from 2021 and contains clear recommendations for first- and second-line therapies for the treatment of active moderate-to-severe TAO [11].

First-line treatment. In view of the new data on the better efficacy of the combination of mycophenolate and intravenous GCs versus monotherapy with intravenous GCs [60, 61], this combination treatment is recommended as first-line therapy for most patients with moderate-to-severe active TAO [11]. The recommended scheme for administration of the GCs is: 500 mg per week for 6 weeks, then – 250 mg per week for another 6 weeks, and for mycophenolate – 0.72 mg/day for 24 weeks. For more serious symptoms (constant/inconstant diplopia, severe soft tissue manifestations) as an alternative first-choice treatment a higher-dose intravenous GC therapy is recommended – 750 mg weekly for 6 weeks, then – 500 mg weekly for another 6 weeks, total cumulative dose 7.5 g. It is recommended to evaluate the therapeutic response at the 6th week of treatment. In case of registered worsening or lack of effect on the ocular manifestations, the treatment should be discontinued and the application of an appropriate second-line therapy should be discussed. When TAO becomes inactive, patients may be referred for surgical treatment to correct residual ocular manifestations [11].

Second-line treatment. In case of insufficient or absent response after first-line therapy, the authors of the 2021 consensus suggest the following therapeutic options:

Repeat course with GCs. A repeated course with intravenous GCs can be administered if additional treatment is needed and after a careful assessment of liver function at least 3-4 weeks after the previous one. The recommended total dose is 7.5 g MP [11].

OR and GCs. The combination of OR and oral GCs is by far the most widely used second-line therapy for moderate-to-severe active TAO [50, 51]. In view of the data on the better effectiveness of intravenous than oral GCs, and of the combination of OR and intravenous GCs over OR and oral GCs [27], the expert opinion is that if second-line treatment is needed, combined administration of OR and pulse therapy with GCs should be applied [11].

Other possible therapies: cyclosporine and oral GCs, azathioprine and oral GCs, rituximab, tocilizumab, teprotumumab.

According to the current recommendations, the clinical manifestations of TAO should also be considered when choosing second-line treatment [11]. Thus, for example, teprotumumab best affects diplopia, OR – visual disturbances and diplopia, while intravenous GCs, mycophenolate, cyclosporine, rituximab and tocilizumab – the inflammatory manifestations of TAO. Rituximab should be avoided when there is a risk of dysthyroid optic neuropathy.

ASSESSMENT OF THE THERAPEUTIC RESPONSE

Currently, the most common method to evaluate the effect of treatment is using the so-called Bartalena's criteria. Different authors use different modifications both of the criteria and of the definition of the therapeutic response through them [22, 23, 25]. Other authors use the inactivation of TAO or a significant decrease in CAS as a primary endpoint [55]. Another ways to evaluate the effectiveness are: through the change in the severity of TAO, using the so-called ophthalmological index based on the NOSPECS classification or by evaluating the need for additional therapy, including corrective surgical treatment [42, 55]. The need of unified criteria in determining the effectiveness of a given therapy is discussed in the most recent EUGOGO and ETA consensus [11]. The authors suggest that the assessment of the therapeutic response should be performed at well-defined time intervals after the end of treatment, using subjective (patient-reported outcome, PRO) and objective assessment (clinician-reported outcome, CRO) of the treatment effect. It is preferable that the subjective evaluation is based on the quality of life questionnaire, while for the objective evaluation of the response to the treatment, the recently proposed composite index including only objective parameters is recommended: ≥ 2 mm reduction in eyelid retraction, ≥ 1 point reduction in 5-point CAS (subjective symptoms – pain on movement and spontaneous pain are excluded), ≥ 2 mm reduction in proptosis, improvement in bulbar movement by ≥ 8°. Improve-

ment of 2 or more of these criteria in one eye in the absence of deterioration in the other should be considered a positive response to treatment. Other ocular parameters, serological and imaging features are recommended to be included as secondary endpoints of treatment. Optimally, the effect of the treatment should be reported 3 months after its completion and possibly followed up at the 6th month.

CONCLUSION

Active moderate-to-severe TAO is a disease requiring a multidisciplinary team approach, an individualized approach and strict follow-up, yet its treatment is often suboptimal. According to the current recommendations, GCs remain the first-line therapy for moderate-to-severe TAO, combined or not with mycophenolate. Recent knowledge about the pathogenesis of the disease and the randomized controlled trials conducted in recent years made it possible to use new therapeutic combinations and biological agents. The question of the availability of the new drugs in routine clinical practice remains unsolved.

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REVIEW

THE DEATH OF SPERM CELLS

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Abstract. *A major factor affecting male fertility is excessive death of germ cells, both immature germ cells and mature spermatozoa. It can be due to various factors causing testicular and/or post-testicular damage, such as infections, obstructive conditions, toxins, oxidative stress, hormonal imbalance, hyperthermia, and anti-sperm antibodies. Massive death of spermatozoa leads to a high proportion of dead sperm cells in the ejaculate (necrozoospermia or necrospermia) while death of immature germ cells can lead to low sperm count (oligozoospermia or oligospermia). Cell death can occur both by necrosis and by apoptosis; in recent decades, it has been found that apoptosis of mature spermatozoa is not only possible but quite common, and can contribute to infertility. Treatment approaches are primarily directed to the underlying condition, i.e. removing the cause(s) of sperm cell death whenever possible, but include also attempts to bypass the cell death event by intracytoplasmic sperm injection with testicular spermatozoa.*

Key words: *spermatozoa, male germ cells, necrozoospermia, male infertility, sperm apoptosis*

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Male infertility is a condition having a severe impact on individuals and families, and affecting more and more people: its age-standardized prevalence rate has increased by 0.291% since 1990 [1]. Studying its mechanisms is important because it can help identify the underlying causative factors and eventually find the best course of treatment. The first prerequisite for sperm cells to be functional is, of course, being alive. If there is excessive death of germ cells, either immature germ cells or mature spermatozoa, fertility will be reduced or abolished altogether. The aim of this review is to summarize the current state of knowledge about male germ cell death.

FACTORS CAUSING DEATH OF SPERM CELLS AND THEIR PRECURSORS

The presence of a high proportion of dead spermatozoa in the ejaculate, sometimes referred to as necrospermia or necrozoospermia, greatly reduces fertility. It can be due to various factors causing testicular and/or post-testicular damage, and their accurate assessment is needed to develop an adequate approach to every case [2, 3]. In addition, excessive death of immature germ cells in the testis can lead to too few of them completing their differentiation and ever reaching the ejaculate, leading to low sperm count – oligozoospermia (oligospermia) [4].

Examples of factors able to cause testicular damage leading to male germ cell death are varicocele, local hyperthermia, and hormonal imbalance. Examples of factors causing post-testicular damage include inflammatory conditions of the male genital tract such as epididymitis and prostatitis, and obstructive conditions, such as structural changes in autosomal dominant polycystic kidney disease, or a history of vasectomy even if it has been reversed. Systemic factors such as generalized infection or intoxication, spinal cord injury, aging, and anti-sperm antibodies can cause both testicular and post-testicular damage [5].

Infections of the male genital tract, while having only a minor impact on sperm viability in most cases, are thought to cause about 40% of cases of necrozoospermia. The mechanisms involved seem to differ between individual pathogens. They may involve substances directly produced by the pathogen, such as bacterial lipopolysaccharide, or mediators of inflammation caused by the infection, such as cytokines secreted in chronic bacterial prostatitis [3]. In this respect, sperm cells are similar to their partners, the oocytes, in which prostaglandin exposure can induce accelerated degeneration [6]. Viruses can also have an impact. In addition to the well-known correlation between mumps orchitis and future infertility risk, new data have shown acute decrease in sperm count and chronic reduction in testicular size and weight after SARS-CoV-2 infection of an experimental model. These effects seem to occur through Sertoli cell damage [7].

Toxic damage can be due to environmental, occupational or iatrogenic exposure to toxic substances, as well as substance abuse. Its negative effects on male germ cells, though difficult to evaluate especially with common pollutants, are pervasive and long-term [4]. When prescribing drugs to male patients, the potential impact on semen quality should be taken into account. For example, anti-parasitic drugs, being toxic to eukaryotic cells, can have detrimental effects on male germ cells. In particular, niridazole which is used to treat schistosomiasis has been reported to cause reversible spermatogenic arrest [8]. Oxidative stress is so important that it deserves a category of its own: some studies have found increased concentrations of reactive oxygen species in the seminal plasma of about a third of infertile men [9]. Oxidative stress can affect male germ cells by multiple mechanisms: it can directly damage mature spermatozoa, which are very susceptible to it due to the high content of polyunsaturated fatty acid in their membranes, and at the same time unable to repair the damage because of the loss of most of the cytoplasm; it can make late spermatids retain excess residual cytoplasm; and it might also disturb earlier stages of spermatogenesis. Chlorine disinfectants, which act by causing oxidative damage, have been shown to interfere with chromosome segregation in hematopoietic cells of a primate model [10], and data from patients with Robertsonian translocations suggest that problems with chromosome mis-segregation can lead to apoptosis [11]. The impact of varicocele on male fertility is thought to be partly based on generating oxidative stress in addition to heat stress [9].

Hormonal imbalance is another factor associated with male germ cell death. Hyperthyroidism has been shown by experiments in rats to cause prophase I arrest of spermatocytes, and in humans, it is associated with a decrease in the vitality, number and motility of spermatozoa [12]. Gonadotropins and testosterone regulate the survival of immature germ cells in the testis, with their effect being at least partly mediated by Sertoli cells creating the microenvironment for differentiating spermatogenic cells. Deprivation or excessive levels of these hormones can cause germ cell death. It is noteworthy that, while estradiol can support germ cell survival when appropriately balanced with follicle-stimulating hormone and testosterone, on its own it has a pro-apoptotic effect [13]. In recent decades, there is a widespread concern that environmental pollution with chemical mimicking the action of estrogens can be a contributing factor to the deteriorating sperm cell counts and male fertility worldwide [14].

Anti-sperm antibodies, both autoantibodies produced by the male and isoantibodies produced by his female partner, are especially important when directed against surface antigens. In addition to hindering sperm motility by agglutination, they could mediate their destruction by turning them into a target for defense mechanisms such as phagocytosis and complement. In fact, the sperm immobilization test, which is routinely used for detection of anti-sperm antibodies [15], detects immobilization resulting from lysis by complement. It is hypothesized that formation of anti-sperm antibodies is the basis of lingering effects after vasectomy reversal [3], and that such antibodies against epitopes cross-reacting with antigens of pathogens contributes to the impact of infections on male fertility [15].

NECROSIS VERSUS APOPTOSIS

In addition to the above described situations of spermatogenic cell death caused directly by an overwhelming external factor, i.e. necrosis, their death by apoptosis is the subject of a growing number of studies. Immature male germ cells, similarly to other dividing and differentiating cells, are well known to undergo apoptosis. During the first spermatogenic wave, apoptosis of large numbers of spermatogonia is used to optimize their ratio to Sertoli cells; in later life, testicular germ cells respond by apoptosis to moderately damaging factors [16]. Hormonal imbalance, especially

testosterone deficiency or excess, increases the level of male germ cell apoptosis [13, 17]. Certain toxins, such as estrogen-mimicking chemicals and plasticizers which are widespread environmental pollutants, have also been shown to exercise testicular damage by inducing germ cell apoptosis [4, 14]. This pro-apoptotic action can be based both on the extrinsic pathway using signaling though death receptors (Fas), and the intrinsic pathway using mitochondria-associated proteins of the Bcl-Bax family [13]. The well-known detrimental effect of hyperthermia on spermatogenesis is also due to inducing apoptosis, though it is still unclear why spermatogenic cells of humans and most other mammals are uniquely susceptible to heat-induced apoptosis and require a lower temperature than is maintained in the rest of the body [18]. Experiments on rats have shown that only prolonged and intensive heat can change the mode of cell death from apoptosis to necrosis, which is supposed to be caused by severe oxidative stress resulting from the treatment [19].

Apoptosis of mature spermatozoa was regarded as more controversial. It was initially thought that they cannot undergo this type of cell death due to their terminal differentiation including cessation of transcription and protein synthesis. This assumption was supported by the methodological difficulties in assessing apoptosis in sperm cells with their already highly condensed nuclei, leading to difficult detection of DNA fragmentation by TUNEL protocols standardized for somatic cells, and making it impossible to use as a criterion the morphological changes of the nucleus which are a hallmark of somatic cell apoptosis [20]. In recent years, however, evidence accumulated that spermatozoa possess the molecular apparatus of apoptosis, particularly caspases, and are able to perform it. TUNEL test has been optimized for sperm cells, and other tests to detect DNA damage have been introduced, such as the Comet assay quantifying the DNA fragmentation level. Both double- and single-strand breaks can be detected [20]. Moreover, the proportion of sperm cells displaying apoptotic markers has been shown to be higher in infertile patients than in healthy donors, and increases after subjecting the cells to potentially damaging treatment such as freezing [22, 23]. These data show that sperm cell apoptosis not only exists but also is of practical importance with regard to male infertility and assisted reproduction. In fact, spermatozoa may be better “suited” for apoptosis than mature oocytes which are arrested in metaphase II and need to exit meiosis in order to develop the full apoptotic sequence [24].

Numerous teams have studied the influence of factors able to trigger apoptosis in spermatozoa, as well as the possible mechanisms of the process. Mitochondria known to be important for apoptosis of somatic cells can have the same role in sperm cells, as shown by experiments using the betulinic acid, a plant terpenoid inducing release of cytochrome C from the mitochondria into the cytosol [25, 26]. Bacterial lipopolysaccharide, which is present in the seminal plasma in male genital tract infections, causes apoptosis of spermatozoa by binding their surface Toll-like receptor 4 (TLR4) [27]. Once inside the female genital tract, sperm cells undergo capacitation which, while a necessary prerequisite for their fertilizing ability, increases their susceptibility to apoptosis through oxidative stress [28]. There are yet no data whether anti-sperm antibodies against surface antigens can cause apoptosis but this possibility should be considered in future research, because such pathways have been described in other cell types [e.g. 29].

ASSESSMENT AND TREATMENT OF NECROZOOSPERMIA

During semen analysis, sperm motility is a proof of vitality. Dead spermatozoa are of course immotile, but live spermatozoa can also be immotile for various reasons, i.e. a molecular defect in their motility apparatus [30]. To distinguish between the two situations, if the observed motility of ejaculated spermatozoa is poor (< 40%), a vitality test is performed by assessing their membrane integrity. The recommended methods are staining by eosin (alone or in combination with nigrosin) or hypo-osmotic swelling [31]. If the result proves an excessive proportion of dead spermatozoa, more examinations and tests are performed to reveal the cause(s). An important parameter in this respect is sperm DNA fragmentation, which may be involved in the causation of cell death and invariably follows it [3]. Electron microscopic observation, though not obligatory, can also be very helpful and show interesting sequences of structural degeneration [32, 33]. The role of epididymal factors in some cases of necrozoospermia was first revealed by this method [34].

Treatment of necrozoospermia depends on its presumed cause, and there is yet no standardized protocol [35]. Depending on the revealed causative factors, there are two approaches to help the patient, which are not mutually exclusive. The first one is to address the supposed cause(s) of germ cell death: treating the infection, minimizing the exposure to heat, removing the toxins, surgically correcting the varicocele, correcting the hormonal profile etc. The second approach is to try to bypass the stage and place where cell death is induced. When epididymal factors are suspected, the exposure of sperm cells to them can be shortened by repeated ejaculations. In severe necrozoospermia, expecially when it is thought to be caused by post-testicular damage, intracytoplasmic sperm injection (ICSI) with testicular spermatozoa is recommended [3]. When perform-

ing ICSI with immotile spermatozoa, application of a modified hypo-osmotic swelling test is recommended in order to select a viable sperm cell [35]. In all cases, treatment should be personalized based on extensive examinations and tests to reveal the etiology of sperm damage, and the aim should be not only giving the patient an opportunity to reproduce but also restoration of his general health and well-being.

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REVIEW

DECENTRALIZED CLINICAL TRIALS – CURRENT ENVIRONMENT, POTENTIAL BARRIERS AND FACILITATORS FOR IMPLEMENTATION AND RISK MITIGATION: A REVIEW OF THE LITERATURE

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Abstract. Introduction: *Decentralized clinical trials are a new approach in health technology research and development that take advantage of innovative digital technologies in data collection for clinical trial purposes. Decentralized clinical trials achieve shorter participant recruitment periods, better adherence to assigned therapy, lower drop-out rates and shorter trial duration overall. Participants reported greater convenience compared to traditional clinical trials due to the studies being conducted at home and the removal of transport and time barriers.* ***Materials and methods:*** *A literature review was conducted using the Scoping Review methodology defined by Arskey and O'Malley in 2005 and further updated in 2010 and 2014. PubMed, Scopus, and Google Scholar scientific databases were searched using predefined inclusion and exclusion criteria and keywords: virtual clinical trials, and/or decentralized clinical trials and barriers and challenges.* ***Results:*** *The literature review found 40 articles that met the specified inclusion and exclusion criteria. The results of the different studies in this area show that decentralized clinical trials achieve shorter recruitment periods, better adherence to assigned therapy, lower dropout rates from trials, and shorter trial duration overall. Participants reported greater convenience compared to traditional clinical trials due to the studies being conducted at home and the removal of transport and time barriers. Major challenges with this type of trial is the difficulty in conducting physical examinations, invasive therapies by the investigators, and privacy protection.* ***Conclusion:*** *The adoption of a regulatory framework for digital healthcare, education of medical professionals and patients about innovative technologies are necessary, especially in countries from Central and Eastern Europe.*

Key words: *decentralized clinical trials, digital transformation, patient access, challenges*

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INTRODUCTION

Decentralized clinical trials (DCT) are a novel approach in the research and development (R'n'D) of innovative health technologies. They incorporate the utilization of different digital technologies in the clinical research practice (apps, electronically monitoring devices, telemedicine, etc.) and online social engagement programs [1].

The difference with the traditional clinical trials is in the data collection approach. While traditional clinical trials use hybrid approach, using protocol predefined semi-virtual collection data (e-mail, telephone, etc.), DCTs can distribute data collection leveraging innovative digital methods as telemedicine, sensor-based technologies in order to accommodate the process to where participants live in real-world settings [1]. This approach also reduces the participants` burden in terms of travel costs, time loss, etc. and facilitates the recruitment process as it allows trial participants to take part in the clinical research from anywhere [2].

Nevertheless, the benefits of DCTs, their implementation in the practice is still a challenge and may vary in the different countries. There are some potential barriers that might impact their implementation. The success of DCTs is dependent of the extend of digitalization of the certain health care systems in the different countries [3, 4].

Most of the Western European countries have already adopted legislation rules for digital health which facilitates the implementation of DCTs. The majority of the Eastern European health care systems, however, still experience difficulties in setting appropriate framework for the digitalization of the health sector and all related activities not only in terms of regulatory changes, but also to cultural transformation and the need for tailored training for health care professionals, investigators and patients [5].

As in the recent years scoping review of the literature is becoming popular [6] this prompted our interest to apply this methodology to analyze the benefits and challenges of the implementation of DCTs, especially in Central Eastern European countries.

METHODOLOGY

A scoping review of the literature based on the methodology defined by Arskey and O,Malley in 2005 and further refined in 2010 and 2014 [6, 7, 8] was conducted. We searched the PubMed database on predefined key words: virtual clinical trials, and/or decentralized clinical trials. The search was limited to articles published after 2013 and with abstracts or free full texts. We also considered articles published on official biomedical information centres and articles addressing the COVID-19 pandemic and its effect on CTs. As exclusion criteria we considered: 1) articles published before 2013, 2) not focusing on DCTs, 3) articles addressing the implementation of virtual reality technology in CTs that are not linked to DCTs.

RESULTS

The scoping review identified 40 articles on total meeting the inclusion and exclusion criteria for the predefined observed period – Figure 1. The objectives of the selected articles are summarized in Table 1.

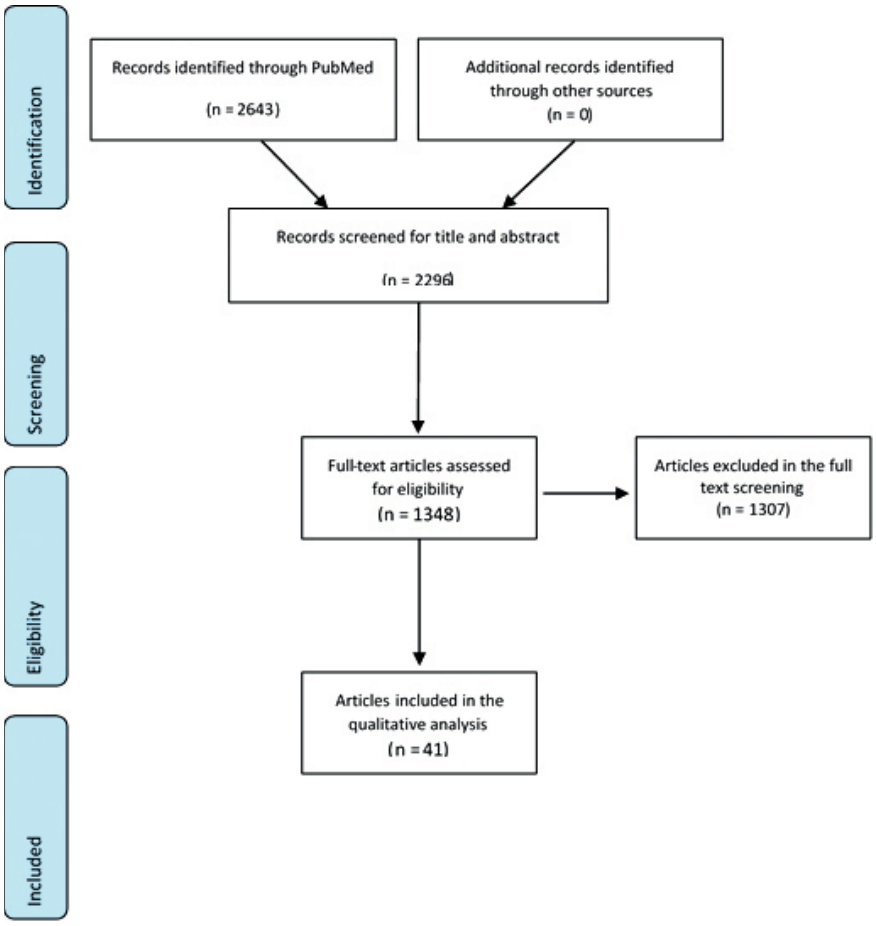

Fig. 1. Flow-diagram of articles screened in the scoping literature review

The common objective of the selected articles aims to identify the possibilities of implementing digital technologies during the clinical trials and to facilitate the decentralization of patient monitoring and data collection.

Based on the conducted literature review we summarized the main advantages and challenges that might be considered in terms of digital transformation of clinical trials – Table 2.

Table 1

Article	Objectives
Zarka A et al., 2020 [11]	Taking advantage of technology and web platforms allow patients to be home-based at every stage of the clinical trial
Apostolaros M et al., 2019 [12]	To promote a decentralized approach during clinical trials using new technologies and methodologies
Van Norman GA, 2021 [13]	To outline major advantages of DCTs and possible obstacles considering cyber security
Krishna S et al., 2020 [14]	To discuss the role of social media and online communities in boosting trial accrual
Yu Zhuang et al., 2018 [15]	To explore potential use of "block chain" technology in DCTs
De Brouwer W et al., 2021 [16]	To describe technologies modernizing CTs
Sundquist S et al., 2021 [17]	To review existing models for remote access to trials; assess national health system readiness; identify needs and enabling mechanisms; and to develop recommendations that would serve as a framework for a Canadian approach
Tan AC et al., 2020 [18]	To provide evidence of feasible and cost-effective future cancer CTs
Haak D et al., 2015 [19]	To analyze requirements for a DICOM-based system interconnection of EDCS and research PACS and to propose an entirely web-based solution.
Lehrach H, 2015 [20]	To analyze the impact of VCTs on drug development process
Sommer C et al., 2018 [21]	To compare the feasibility and acceptance by patients of DCTs compared to conventional clinical trials
Dorsey ER et al., 2021 [22]	To analyse approaches to design clinical trials around the needs of participants rather than sites, embracing digital measures of health, and advancing decentralized studies
Spertus JA et al., 2021 [23]	To design a patient-centered DCT aiming to test the efficacy of canagliflozin for heart failure treatment based on clinical trial leveraging mobile technologies
Lee EQ et al., 2021 [24]	To leverage the COVID-19 experience for a future in which hybrid/DCTs for brain tumors become the new normal
Gouda P et al., 2021 [25]	Voice technology represents a novel and promising tool for cardiovascular CTs
Persky S, 2020 [26]	To explore potential benefits of virtual reality to provide a "virtual site" for VCTs (virtual clinical trials)
Hashem H et al., 2020 [27]	To discover moves towards conducting DCTs across satellite sites
McGoohan K et al., 2020 [28]	To explore the views of Parkinson's disease (PD) patients and care givers on CTs adaptations during COVID-19
Ali Z et al., 2020 [29]	To examine whether reward-based VCTs would enable nationwide recruitment, high adherence, and reduce dropouts
Yaakov RA et al., 2021 [30]	To address regulatory compliance, data security, privacy, and ownership in relation with the conduct of VCTs
Wang H et al., 2020 [31].	To present a modular model to conduct in silico cancer immunotherapy VCTs on patient cohorts of interest
Kadakia KT et al., 2021 [32]	To highlight an oncology-specific paradigm for VCTs
Zhuang Y et al., 2021 [33]	To develop a comprehensive block chain framework for VCTs
Ieronimakis KM et al., 2020 [34]	To describe video-telecommunication adoption for research consent
Schneider RB et al., 2020 [35]	To establish virtual follow-up of CT participants, compare changes in online and smartphone-based assessments, and explore novel digital markers of PD

Continuation of Table 1

Marra C et al., 2021 [36]	To compare the use of connected digital products in CTs before and after the onset of COVID-19
Getz K et al., 2020 [37]	To use a virtual AI-based platform to identify the unreported intentional non-adherence in CTs
Singh V et al., 2021 [38]	To assess the status and challenges of ongoing CTs during the COVID-19 pandemic
Coert R et al., 2021 [39]	To better understand the barriers and facilitators to VCT adoption by determining the factors that influence individuals' considerations regarding VCTs from the perspective of various stakeholders
Samei E et al., 2020 [40]	To assess the VCTs as an efficient means to conduct clinical research and to ensure that the VCT closely models that reality
Gouda P et al., 2021 [25]	To determine whether voice-based technologies increase cost-effectiveness in cardiovascular CTs
Chiamulera C et al., 2021 [41]	To address limitations for the implementation of telemedicine technologies for virtual clinical trials
Petrini C et al., 2022 [42]	To review on some the ethical implications and requirements of DCTs in order to encourage further ethical reflection
Goodson N et al., 2022 [43]	To present a range of challenges and opportunities for researches to adopt and adapt DCT approaches to obtain reliable evidence
Coyle J et al., 2022 [44]	To learn from representatives of academic institutions, pharmaceutical companies, small-medium enterprises, and patient representatives about their experiences developing and implementing DCT methods and to provide conceptual focus on participant engagement in DCTs
Adams D et al., 2022 [45]	To assess the association of remote technology and other decentralization tools used to reduce participation-related time and travel with the likelihood to enroll in cancer clinical trials
Moore J et al. 2002 [46]	To assess the barriers and benefits in conducting DCTs in rare diseases
Rogers A et al., 2022 [47]	To evaluate, using quantitative and qualitative approaches, published data on the design and conduct of decentralized clinical trials (DCTs)
De Las Heras B et al., 2022 [48]	To analyses the role of DCTs in providing environment for remote collection and assessment of data in clinical research
DiMasi JA et al., 2023 [49]	To assess the financial net benefits of DCTs
De Jong A et al., 2022 [50]	To identify regulatory challenges and opportunities for the implementation of DCTs in EU

Table 2. Advantages and disadvantages for digital transformation in clinical trials

Advantages	Challenges
– DCTs have the potential to bring many advantages to the medical field. DCTs could reduce physical barriers for participating in clinical trials – Since the start of the COVID-19 pandemic, many clinical trials were halted or completely terminated due to the high risk of infection through physical contact. DCTs offer the option to conduct clinical trials in a safe, ensuring and secluded environment, at the patient's home. Implementation of communication software might improve patient feedback as well as patient compliance – Furthermore, by shifting the CTs' location directly to the patient's home, recruitment times would be drastically improved and transport-related costs would be minimized – DCTs have positive impact on the financial aspects of drug development in the clinical research phase – DCTs are an important aspect of the digital health	– Need for implementing telecommunication software for the purpose of connecting to participants in DCTs – Need for implementing software ensuring patient data confidentiality, cryptency of data sharing, and data integration for the needs of health information exchange and clinical trials – Costs of conducting educational programs for performing and participating in DCTs – Difficulty in complete evaluation of patient condition due to lack of physical attendance

DISCUSSION

The COVID-19 pandemic accelerated the adoption of decentralized clinical trials, in terms of remote monitoring. Studies show that due to the increased consumption of COVID-19 related healthcare resources, epidemiologic measures and lockdowns, patients' access to trial sites was reduced by 80% [50].

A report by McKinsey and Company, published in 2021, shows that decentralization of clinical trials could improve the access to trials and would provide opportunities to reach a larger number and potentially a more diverse pool of patients [51].

In the recent years we are witnessing the important and inevitable process of digitalization of healthcare.

WHO defines this process as supportive transition to equitable and universal access to quality health services. Digital health can help make health systems more efficient and sustainable.

The results from the scoping review show that most of the challenges reported are could be also valid for Central and Eastern European countries for which there is identified need for regulatory framework adoption for digital health, education of healthcare specialists and patients for innovative technologies [49].

CONCLUSIONS

Most of the reported challenges of conducting decentralized clinical trials are also valid for Bulgaria. The adoption of a regulatory framework for digital healthcare, education of medical professionals (physicians and pharmacists) and patients about innovative technologies are necessary.

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INSTRUCTIONS TO AUTHORS OF ARTICLES
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A scientific paper represents a newly produced or adapted knowledge. The scientific text is meant to be a result of careful consideration and evaluation of all details of the report. Presentation of scientific information implies strict logic and consistency, concise and accurate expression, objective statement of facts; convincing argumentation. Clarity of expression is a priority. Scientific presentation is characterised by specific requirements for the structural and compositional form of the various genre forms (article, review, case description, abstract).

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Keywords are used to thematically categorise the article in databases and to search for references accordingly. It is the author's responsibility to choose these accurately according to the most significant concepts of their work. The number of keywords for each type of publication is usually between 3 and 8, and may be single words or short phrases generally accepted in the particular field of knowledge.

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ORIGINAL ARTICLE

Original research articles must entail proprietary work presented via a thoroughly described methodology. The following format is to be followed: Introduction & Purpose – Materials and methods – Results – Discussion – Findings/ Conclusion(s).

Introduction: Its main function is to answer the questions: what are the motives for conducting the study and what is its immediate purpose. Additional functions: to provide background information needed to understand the article; a brief review of similar studies by other authors; a link to similar problems; a purpose statement; an assessment of the importance of the problem.

Materials and methods. Outlines the characteristics of the groups studied, including: does the material meet the stated aim of the study; analyses the method of selection – inclusion/exclusion criteria, randomisation technique; discusses the comparability of the groups compared; explains the method used as appropriate for the stated aim, states the statistical methods, declares compliance with ethical rules.

Results. Should contain all the results and only the results. The text shall be supplemented by illustrative material - photographs, diagrams, tables. The author is expected to judge the objectivity or subjectivity of the results and whether they answer the questions posed. Avoid duplication of information (same data presented as table and figure; in text and in illustration).

Discussion. Interpret the data and its significance, discuss problems with the methods/techniques used, compare similarities and differences with other studies in the literature review, and highlight the contribution of the results to illuminating the problem posed. Controversies, unresolved issues, unwanted phenomena, unexpected results, doubts, alternative interpretations and hypotheses, statistical differences, limitations of the study are discussed. The data is summarized. The following should be avoided: unsubstantiated claims, exaggeration of the significance of data, digressive/peripheral issues, attacks on other studies and authors or their uncritical retelling, emotional appeals to the reader.

Conclusion. Summarizes the main results and draws conclusions from the study.

SCIENTIFIC REVIEW

It summarizes the contents of a number of sources dedicated to a single subject during a defined period of time. Discussion of authors and texts should be consistent with the thematic and issue relevance to the purpose of the study. It is intended to consider the experience, current status and trends of a given issue, to evaluate the material in a reasoned way, and to offer concrete, practically usable conclusions and recommendations. A logically connected, coherent exposition is expected, without mechanically retelling the sources. Subheadings are thematic.

CLINICAL CASE REPORT (CASUISTRY) / CLINICAL SERIES

Clinical Case Reports consist of Introduction, Clinical Case Description, Discussion and Conclusions. An extended review section is used to demonstrate the significance of the presented case. The telegraphic style with duplication of a case history, is unacceptable.

The LIST OF REFERENCES at the end of the paper should cover only those publications that have actually been cited and are necessary to outline the foundation on which the research is built. Do not offer an abundance of literature at the expense of its relevance. Minimize self-citation. Citation of Bulgarian sources is strongly recommended.

Citations of bibliographic references within the text are indicated by numbers in square brackets in the order of their appearance. The bibliography list at the end is arranged in the order of appearance of the sources within the text. Each source is listed on a new line, with an Arabic number. Sources are structured in the following manner:

- Articles*:* Author(s). The article title. Journal title (abbreviated), year, volume, number (issue) in round brackets, papers (from-to). *Example: Yakub YN, Freedman RB, Pabico RC. Renal transplantation in systemic lupus erythematosus. Nephron, 2019, 27(1):197-201*.
- Papers from a compendium / digest / collective book: Author(s). Title. In: The book title. Edition number, editors. Place of publication (city), publishing house, year of publication, pages (from-to). *Example: Wilkinson AH. Evaluation of the transplant recipient. In: Handbook of Kidney Transplantation. 6th ed. G. M. Danovitch (Ed.). Boston, Little, Brown and Co., 2019, 109-122.*
- Books. Author(s). Title. Place of publication (city), publishing house, year of publication, pages (from-to). *Example: Sheytanov Y. Systemic Vasculitis. Sofia, Medicina i Fizkultura, 2019, 8-11.*
- When citing electronic publications which are available online, a link is added to the source description along with the date it was accessed on, or a DOI number.

In cases of up to three authors, their surnames followed by their initials (without periods) are written. In cases of more than three authors, "et al." is written after the name of the third author.
When citing Bulgarian sources, the source should be presented in Latin, with the title translated in English, while the names of the authors and the journals or publishers should be transliterated (e.g., *Bulg. sp. psihiatr.* or *Med i fizk.*).

ETHICAL NORMS IN PUBLISHING

Authors must warrant that they submit for publication their own studies and in case different author's data and/or text are used, these are specified by citations. Strict adherence to copyright issues is maintained – texts with more than 10% verbatim repetition of another publication are returned for revision..
A secondary publishing of the same science paper in a periodical is permissible only in the form of a secondary publication reflecting the data from an original publication, which is explicitly quoted. The corresponding author declares that the material has not been published previously, except in the form of an abstract for a scientific event, and has not been submitted to another journal/publisher.
All researchers contributing to the concept and fulfillment of the scientific study should be listed as co-authors. The corresponding author must ensure that they have approved the final version of the paper as submitted for publication, and have been informed on the critical notes and recommendations for correction following peer review.
Authors assume the responsibility for the contents of their publications. Presented papers and the studies described in them should comply with the established ethical standards on performance of the clinical and/or experimental studies on human subjects (the Helsinki Declaration) and animals used for experiments. Patients must not be referred to by their names or initials, and any images through which they can be identified, must not be presented.
Declaring conflict of interests, or lack of such conflict as well as absence or presence of financial association of study and the institutions performing it, is obligatory.

STATISTICAL PROCESSING

Statistical methods must be described sufficiently so readers with access to original data can check the presented results. The results must be presented quantitatively (if possible) by appropriate indicators for the measurement of error or uncertainty (e.g., confidence intervals). Avoid using only p-values in the verification of a hypothesis since this approach does not generate sufficient quantitative information. Quoting the correct p-values in addition to the appropriate confidence intervals is desirable.
The number of measurements (sample size) must be stated and the method of calculation justified. Describe the randomisation procedures, if any. The statistical programs / applications used must be described. The used statistical terms, abbreviations and symbols should be defined unambiguously.

LAYOUT AND FORMATTING

The article must start with its title (without abbreviations), the names of the authors (without academic or other titles), their workplaces designated by numeric indices, abstract and keywords.
The corresponding author must provide their contact details (e-mail & optionally: postal address, telephone number).
Approximate word count expected of submitted papers:

Type of publication	*Word count in the main text*	*Word count in the abstract*	*Number of references*
Original article	2500-4000	200-300	30
Review	3000-5000	100-200	50
Clinical case report	1000-3000	100-200	20

MS Word files are acceptable. There are no specific requirements on the font size and type, spacing, margins and other formatting.
Illustrative tables, figures, images etcare positioned at their corresponding places within the text with captions and notes. Captions of figures must not be shown within the image. Images with good quality (at least 300 dpi) and appropriate file format (.jpg, .tif, .png) are required. Tables must be presented in an editable format rather than as images.
Specific abbreviations used in the text are to be entered in brackets the first time the full name appears in the text. Measurement units should follow the SI system.

The materials have to be sent by e-mail to the organizational secretary of the journal.