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- Pre-analytical and analytical factors influencing the molecular diagnosis of SARS-COV-2 infection
- Prevalence of autoantibodies in ANCA-associated vasculitides and immunological associations with disease severity in the Bulgarian population
- COVID-19 induced rupioid psoriasis
- Mild course of COVID-19 infection in a patient with chronic granulomatous disease
- Erythema nodosum in SLE
- Antiphospholipid antibodies in patients with COVID-19
- Vaccine-induced humoral immunity against SARS-CoV-2

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CONTENTS

ORIGINAL ARTICLES

A. Georgieva, B. Al Hadra, N. Ivanov, E. Naumova, S. Mihailova. Pre-analytical and analytical factors influencing the molecular diagnosis of SARS-COV-2 infection 3

T. Yoneva, Y. Zdravkova, D. Kalinova, G. Kotov, E. Ivanova-Todorova, G. Vasilev, R. Rashkov, I. Sheytanov. Prevalence of autoantibodies in ANCA-associated vasculitides and immunological associations with disease severity in the Bulgarian population 11

CASE REPORT

R. Sharkova, V. Kantardjiev, V. Broshtilova. COVID-19 induced rupioid psoriasis 21

S. Lesichkova, S. Kandilarova, E. Naumova. Mild course of COVID-19 infection in a patient with chronic granulomatous disease 25

M. Nikolova, Cv. Vutova, D. Genov, N. Koleva, M. Hristova, A. Lavcheva, V. Grozeva, D. Kyurkchiev, E. Ivanova-Todorova, R. Gancheva, N. Stoilov, K. Chupetlovska, L. Dourmishev, T. Todorov, At. Kundurdjiev. Erythema nodosum in SLE 31

SCIENTIFIC REVIEWS

M. Baleva. Antiphospholipid antibodies in patients with COVID-19 36

V. Pencheva, S. Mihaylova. Vaccine-induced humoral immunity against SARS-CoV-2 44

INSTRUCTIONS TO AUTHORS 52

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ORIGINAL ARTICLE

PRE-ANALYTICAL AND ANALYTICAL FACTORS INFLUENCING THE MOLECULAR DIAGNOSIS OF SARS-COV-2 INFECTION

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Abstract. Background: The dynamics of SARS-CoV-2 replication and shedding in the respiratory tract give an idea of the clinical and epidemiological characteristics of COVID-19 infection, and of course the detection of viral RNA in human samples in itself definitively determines the diagnoses. Currently, different molecular methods are practically applied as of the latter, the final result could be influenced by a number of additional factors. **The aim** of the study was to analyze and present pre-analytical (type of sample, time of collection, severity and duration of viral persistence) and analytical factors – used reagents and methodology affecting the diagnosis of SARS-CoV-2. **Materials and methods:** Biological samples from patients (n = 1600) suspected to have COVID-19 were investigated at different stages of the disease progression. Different numbers of patients were used for the particular analytical assessments in this study. We used applied two main methods – Real Time PCR (RT-PCR) and Loop-mediated isothermal amplification (LAMP). **Results:** In the studied patients, we observed characteristic dynamics in the amount of viral load with a peak (mean Ct value of 22 ± 3) on the fifth day from the onset of symptoms and duration of persistence, which showed a certain dependence on the severity of the disease-the severe the disease was the longest the shedding was described. The use of nasopharyngeal swab showed the most reliable results compared to other biological material in the diagnosis of patients. Incorrect sampling led to inaccuracies in the results on average in 1.4% of the samples. Molecular technologies other than RT-PCR which we have compared with the gold standard for diagnosis, showed even lower sensitivity (30%) than it was previously reported and thus could be recommended as a diagnostic tool only in the first days of infection. **In conclusion**, the data presented by us reflected the experience of the team in molecular diagnostics of SARS-CoV-2 and outlined some important guidelines in everyday practice.

Key words: SARS-CoV-2, RT-PCR, LAMP, pre-analytical factors

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INTRODUCTION

Since the beginning of the pandemic of COVID-19 (corona virus disease 2019) caused by SARS-CoV-2, more than 420 million have been infected and more than 5.8 million people have died. The health, social and economic damage caused is a precedent for the 21st century [1]. As the virus spreads by both symptomatic and asymptomatic individuals, the main strategies for dealing with the pandemic remain mass testing, quarantine of positives subjects, and adherence to common anti-epidemic measures. In order for the testing to be accurate, it must be

timely and the methods used must achieve high sensitivity and specificity; to be applied to symptomatic, their close contacts and as a screening of risk contingents of the population; to be cost-effective. Pre-analytical factors (sampling technique, type of biological material, transport and storage, time from onset of symptoms) and analytical factors (type and quality of reagents used, methodology, etc.) have an impact on the sensitivity, specificity, and expediency of the different stages [2]. Furthermore they are decisive for the final result and the applicability of the method in real conditions.

The aim of the present study was to determine the influence of pre-analytical factors and more specifically – viral persistence and its relation with disease severity. Another aim was to determine the influence of analytical factors that affect the sensitivity, specificity, and promptitude of testing and to present the experience of our team to the moment.

MATERIALS AND METHODS

A thousand and six hundred patients who passed through the emergency room of University Hospital "Alexandrovska" and patients from the Infectious disease department with different course of infection at different stages were examined, both incidentally and in dynamics. Of those, 52 patients (21 women and 31 men, 34 with severe, 8 with moderate and 10 with mild course; age 23-80 years) were followed up during the course of the infection as testing data was used in order to evaluate the influence of viral persistence on PCR detection capacity and the applicability of molecular diagnostics at different stages of the disease according to severity state. The latest was evaluated according to NIH COVID-19 Treatment Guidelines [3]. Materials from the upper respiratory tract – nasopharyngeal, oropharyngeal swabs, saliva and lower respiratory tract – tracheal aspirate and bronchoalveolar lavage were examined. In selected deceased patients, biopsy materials were examined as well – lung, liver, myocardium, small intestine. We used two main methods – Real-Time PCR and Loop-mediated isothermal amplification (LAMP) and the following amplification kits: GeneFinder ™ COVID-19 Plus RealAmp Kit, Maccura 2019 n-CoV ORF1ab / N / E (RT-PCR), and Easy CoV Covid-19 salivary test (LAMP), ID Now Abbott (LAMP). The testing was performed on AB 7500 Fast device and Line Gene 9600 Fluorescent Quantitative PCR Detection System. For viral RNA yield we used automatic extraction systems based on magnetic particles: Iprep, Invitgoven, and AutoPure 32A Automatic Nucleic Acid Extraction Instrument, Maccura. Statistical analysis incuded IBM SPSS Statistics 28.0.0.0, Independent – Samples Median test, One-sample Kolmogorov – Smirnov Normal test.

RESULTS

1. Pre-analytical factors affecting the diagnosis of COVID-19

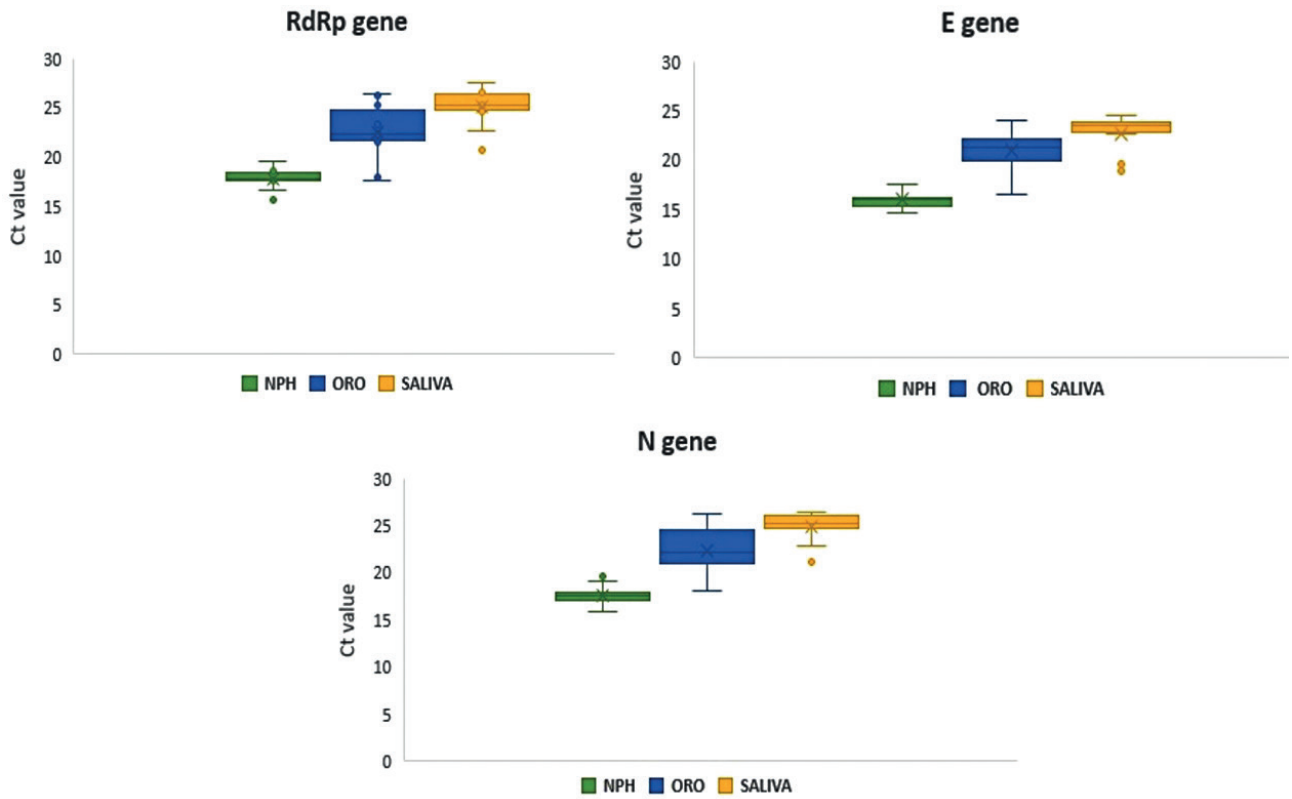
A gold standard for the diagnosis of SARS-CoV-2 infection is the highly accurate RT-PCR [4]. Although the high sensitivity and specificity of RT-PCR testing the test results might be influenced by many variables. The final results could be related to the process of sample collection, type of sample to be tested and processed, transportation, storage etc. The testing process can be divided into two phases (pre-analytical and analytical) and each step in these phases has an impact on the total testing process [5].

1.1. Type of sample (according to the anatomical place of collection)

As a respiratory infection the specimens used for diagnosis of SASR-CoV-2 are recommended to be collected from the upper respiratory tract – nasopharynx (NPH) and oropharynx (OPH). At a later stage of the viral infection and in hospitalized, acutely ill patients the virus could be detected in specimens from the lower respiratory tract – bronchoalveolar lavage (BAL) and endotracheal aspirates (ETs) [6].

We evaluated the sensitivity of the detection of SARS-Cov-2 nucleic acid in different clinical specimens from the upper respiratory tract. Nine patients had 3 specimens (nasopharyngeal, oropharyngeal swabs, and saliva) collected simultaneously at the onset of disease symptoms (Fig. 1).

The lowest Ct value for the three target genes (E, N, and RdRp) was detected in the nasopharyngeal specimens with an average Ct value of 16.0 for the E gene, 17.6 Ct for the N gene, and 17.9 Ct for RdRp. That could be explained by a high viral load in nasopharyngeal specimens. The lowest viral load indicated by the highest Ct values was observed for the saliva specimens with an average Ct value of 22.7 for the E gene, 24.9 Ct for the N gene and 25 Ct for RdRp. For all the specimens the very first gene to be detected was the E gene, followed by N and RdRp genes for which the differences in the Ct values were small.



Legend: NPH – nasopharynx; ORO – oropharynx; Ct – cycle threshold value

Fig. 1. Comparative analysis of Ct values for RdRp, E, and N genes in different clinical specimens (nasopharyngeal, oropharyngeal samples, and saliva)

We have also investigated the sensitivity of SARS-CoV-2 detection in specimens from the lower respiratory tract – BAL and ETs. Three patients with SARS-CoV-2 infection and progressive illness were tested from BAL and NPH specimens collected simultaneously. All patients were at least 21 days post the first positive PCR test and developed COVID bilateral bronchopneumonia. SARS-CoV-2 nucleic acid was detected in both specimens for the three patients with small differences in the Ct values (Figure 2). Both in NPH and BAL specimens the very first gene to be detected was the E gene with an average Ct value of 26.4 and 28.1 respectively, followed by N gene (27.1 Ct; 29.1 Ct) and ORF1ab (29.2 Ct; 30.3 Ct).

Nasopharyngeal and endotracheal specimens were taken simultaneously from four patients in severe health conditions as a result of current SARS-CoV-2 infection. Viral RNA was detected in both NPH and ETs specimens from three patients, while in one patient the viral RNA was detected in the upper respiratory tract specimen (NPH) but not in the ETs. These findings support the recommendation of the priority testing of nasopharyngeal samples.

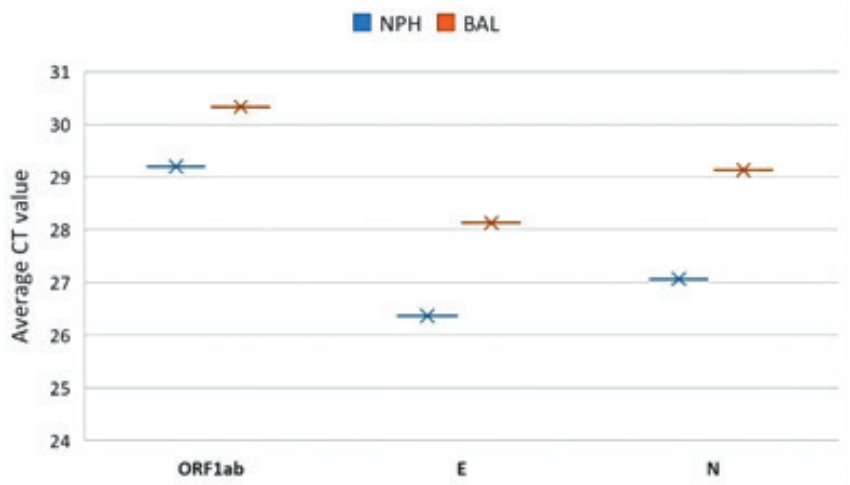


Fig. 2. Comparative analysis of the average Ct values for ORF1ab, E, and N genes in nasopharyngeal specimens (NPH) and bronchoalveolar lavage (BAL)

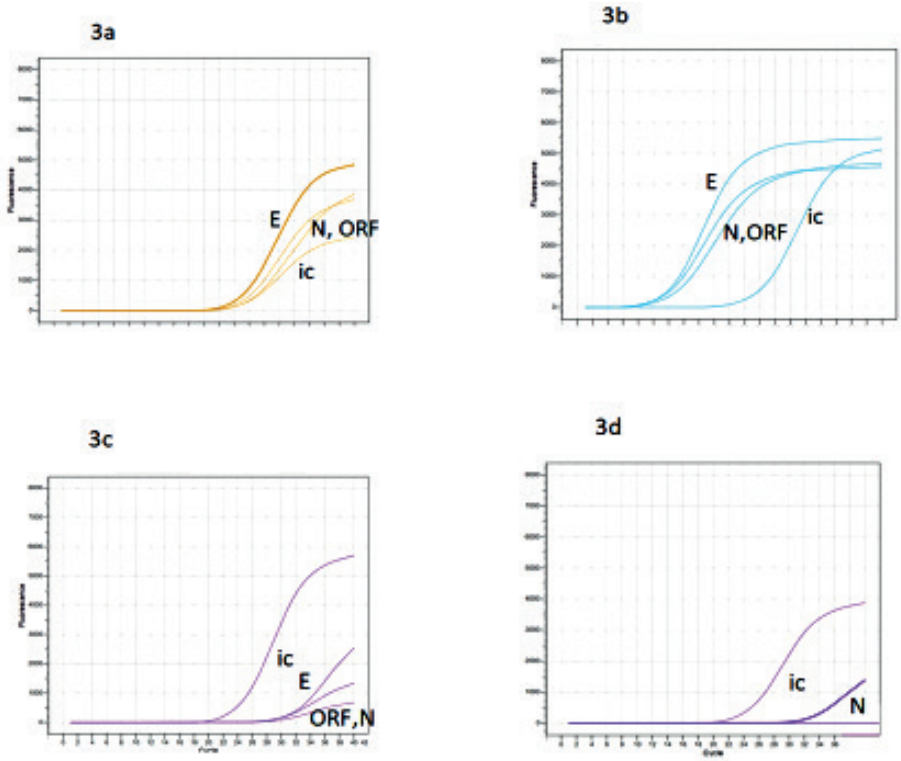
Additionally the SARS-CoV2-2 viral RNA was detected in lung, liver, myocardium and small intestine from selected deceased individuals (data not presented because of a small number of tests). We also successfully applied an adapted method that allows extraction of RNA from a paraffin block sample of the myocardium of a deceased patient. The virus was subsequently amplified and proven, which aided the postmortem diagnosis of a patient in whom no other test material was available.

1.2. Sampling technique

In our clinical practice, we have encountered samples that were suspicious of the presence of the SARS-CoV-2 virus. According to the instructions of the manifacure the definition of "uncertain" RT-PCR result is when the sample tested positive for only one of the studied genes or if both E and N genes are detected in the absence of ORF1ab or RdRp genes. In case of suspicious PCR results, the test shall be repeated with a new sample taken within 48 hours. Out of a total of 1600 tested samples at 101 it was not possible to give a definitive result. From these 101 "uncertain" for SARS-COV-2 PCR results, nineteen (or 1.4%) tested positive after a new sample collection was obtained within 24 hours. These results could be explained by an incorrect sampling technique. This observation emphasizes the importance of strict adherence to sampling guidelines and probably the need procedure to be done by trained health care providers.

1.3. Time of sample collection

If the sample collection is performed immediately right after known exposure to SARS-Cov-2 the test result most probably would be negative. The most appropriate time for testing is on the onset of symptoms where high viral loads could be detected [7]. We examined SARS-CoV-2 viral loads in 25 patients (mild and moderate course) on the first day and on the fifth day from the onset of symptoms and subsequently some of them were tested later during the course of infection – between 10th and 30th days. On the first day, a high viral load was detected (mean Ct value of 22 ± 3 for ORF1ab gene, figure 3a). This observation was followed by an increase in the viral load with a peak value around the fifth day (mean Ct value of 16 ± 3 for ORF1ab gene, fig. 3b) and its gradual decrease with a duration of 10-30 days depending on the severity of the disease and the applied treatment (Figures 3c, 3d). The other two genes, E and N, followed the same trends in time-dependent dynamics. In the late stages of the infection, low fluorescence targets of the three genes were detected, and finally only N gene is amplified with a Ct value ≥ 35) (Fig. 3d).



Legend: ic-internal control N-N gene, E-E gene, ORF-ORFab1 gene. On the X-axis are presented the PCR cycles and on the Y-axis the fluorescence level

Fig. 3. Amplification plot of E, N and ORFab1 genes during the course of infection (day 1, 5, 10 and 30)

Furthermore, in nine patients we monitored the presence of the virus in three biological specimens – NPH, ORH, and saliva. The specimens were collected simultaneously at the onset of symptoms and on the 14th day. At the onset of symptoms, the virus RNA was detected in 100% of the NPH, ORH and saliva samples. On the 14th day, the virus was still detectable in 100% of the NPH, but only in 20% of the oropharyngeal specimens, and was not detected in any of the saliva samples.

1.4. Duration of viral persistence

We studied the persistence of viral load in 52 patients with different course of the disease, who were followed up are regular intervals (patient`s data in sec. Material and methods). In the overall group the viral load persisted from 11 to 65 days. Depending on the severity of the course, we observed different viral dynamics (Fig. 4). In patients with severe course (with clinical and paraclinical data for severe respiratory insufficiency) the longest persistence of viral load was observed (20-35 days on average), in patients with moderate course – 12-32 days on average and in patients with non-severe (asymptomatic and mild) – 15-20 days on average. By applying Komogorov–Smirnov's test, we illustrated that the distribution of viral persistence, independent of disease severity, was close to normal with the highest frequency of patients with a duration of retention of viral load of 20-35 days (Fig. 5).

2. Analytical factors

2.1. Comparison of RT-PCR and LAMP technology

According to manufacturers, RT-LAMP is technology that allows the detection of less than 100 copies/ml of the SARS-CoV-2 viral genome in 30 minutes making this test one of the fastest molecular diagnostic approaches [8]. On the other hand, RT-PCR is more time consuming, but is accepted as a gold standard for diagnosis. We have simultaneously tested 30 saliva samples of patients, suspicious for COVID-19 with both methods. Nineteen of them were negative with both methods. However among the 11 RT-PCR positive results, only 3 were tested positive with RT-LAMP, while the rest 8 were false negative with the EASY COV Salivary Rapid Molecular Test (table 1). It seems that rapid technology can detect nucleic acids only at high viral loads below Ct value of 27 (ORF1ab gene).

We also used another kit based on LAMP technology – ID Now (Abbott), analyzing nasopharyngeal secretions as a testing specimen. We tested 50 patients at different stages of infection. Our results showed 100% sensitivity of the LAMP but only for patients in the initial phase of the infection with high

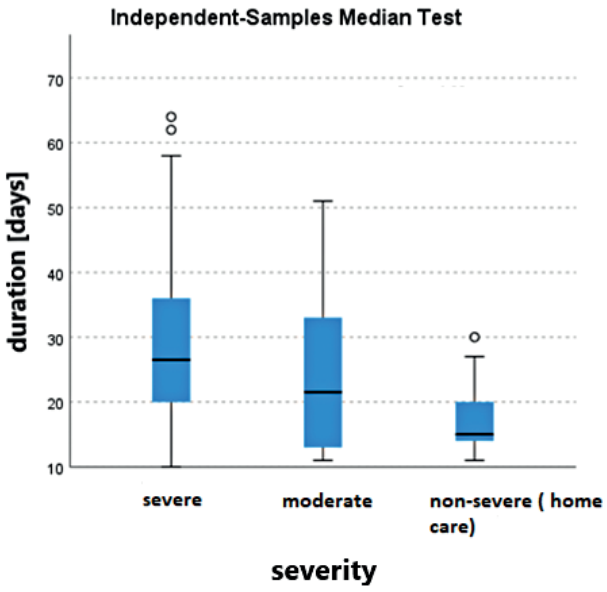


Fig. 4. Disease severity and virus persistence in 52 patients with different course of the disease-severe, moderate and non-severe (asymptomatic and mild)

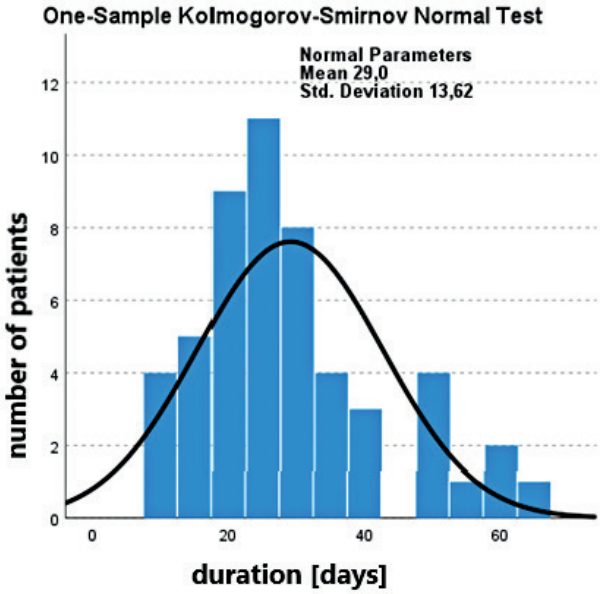


Fig. 5. Distribution of patients by duration of viral persistence

viral loads (Ct value < 30). At medium viral loads (Ct value between 30-34), the sensitivity decreases to 80-60% accordingly, and in specimens with low viral loads (Ct > 34) the sensitivity dropped dramatically – below 40% (data not presented).

Table 1. EASY COV Salivary Rapid Molecular Test (LAMP) and Maccura 2019 n-CoV ORF1ab / N / E Test (RT-PCR) comparison

Sample	LAMP test result	RT-PCR test result	ORFab gene Ct value (RT-PCR)
#1	NEGATIVE	POSITIVE	32
#2	POSITIVE	POSITIVE	16
#3	NEGATIVE	POSITIVE	30
#4	POSITIVE	POSITIVE	27
#5	POSITIVE	POSITIVE	16
#6	NEGATIVE	POSITIVE	23
#7	NEGATIVE	POSITIVE	24
#8	NEGATIVE	POSITIVE	31
#9	NEGATIVE	POSITIVE	31
#10	NEGATIVE	POSITIVE	26
#11	NEGATIVE	POSITIVE	34

DISCUSSION

In viral pandemics, including the current one, one way to deal with the pathogen is to limit its spread, which is only possible if infected people are detected and isolated as early as possible. Early and accurate diagnosis is also essential, especially in risk groups where proper and timely treatment of the disease is critical. This publication sought to reflect the experience of the molecular diagnostic team at the Department of Clinical Immunology with stem cell bank, University Hospital "Alexandrovska", outlining some key pre-analytical and analytical factors in the diagnosis of SARS-CoV-2 infection that could be of practical importance.

High levels of viral load in the upper and lower airways have been demonstrated in patients with COVID-19 within 5-6 days of onset of symptoms [9]. The use of nasopharyngeal or oropharyngeal swabs [10, 11] was recommended for early diagnosis of COVID-19, but a single nasopharyngeal swab is the method of choice. To take a suitable sample, the tampon must go deep into the nasal cavity [4]. Collected swabs should be transported immediately by transport medium to the diagnostic laboratory, ideally in refrigerated conditions [12]. A variety of studies showed that other biological samples than ORH could be used for accurate diagnosis. Patients with severe COVID-19 pneumonia showed a high viral load in bronchoalveolar lavage, but nasopharyngeal tampons were not compared in the specific study [13]. During the development of the COVID-19 pandemic, there was a need to replace the painful process of collecting a nasopharyngeal tampon, which led to the development of technologies using saliva. According to a meta-analytical study [14], having in mind that using nasopharyngeal swabs was the gold standard for diagnostics, pooled nasal and throat swabs gave the highest sensitivity of 97% (95% CI 93-100), while lower sensitivity was achieved using saliva (85%, 75-93) and nasal swabs alone (86%, 77-93) and even lower sensitivity when using throat swabs alone (68%, 35-94). A comparative analysis between samples collected by healthcare professionals and self-collection of pooled nasal and throat swabs and nasal swabs showed comparable diagnostic efficacy. The comparative analysis for virus detection in the three main biological materials used – NPH, OPH and saliva in our patients showed that the use of nasopharyngeal test was a priority over all others. The greatest amount of virus (comparable amplification of all three genes included in the kit) was detected in this biological secretion, even in seriously ill patients who have been used invasive sampling techniques such as BAL and ETs aspirates. For this reason, the diagnostic use of lavage and aspirate in Infectious ward of our hospital was applied only at the beginning of the pandemic, after which it was dropped. In the present study, we did not compare the reliability of test results from samples taken by patients themselves and those taken by healthcare professionals. However, data from the re-analysis of some samples with „uncertain results" that required re-sampling within 24 hours showed that sampling technique could be important.

Studies measuring viral load over time in COVID-19 have been somewhat controversial [15]. The viral load in the upper respiratory tract has been shown to peak at the onset of infection, usually before or within a few days after the onset of symptoms [16-18]. Some studies suggest that the viral load in the lower respiratory tract may reach its peak later, in the second week after the onset of symptoms [19]. Other studies have shown that the viral load at some point after diagnosis was similar between asymptomatic and symptomatic cases [20], but there was also

evidence that patients prone to prolonged viral shedding have more severe symptoms or these individuals are older [21, 22]. Despite the detection of viral material in samples from the upper respiratory tract in particular individuals a few weeks after the onset of symptoms, the infectious virus was usually not present after 8-14 days [23, 24]. A. Xiao et al., described the dynamic of PCR profile in 56 patients who had recovered from COVID-19. The negative RT-PCR results for SARS-CoV-2 began to dominate from week 4 after the onset of symptoms, and by the end of follow-up (6 weeks) period all tests were negative [25]. An interesting model was presented by Challenger et al. [26]. The authors observed wide variations in viral load between individuals, over 5 orders of magnitude, at any given time from the onset of symptoms. The variations were not related to age, sex, or disease severity, and additionally were not related to modelled early or late phases of immune-mediated control of viral load. Our results demonstrated specific dynamics in viral load, with a peak around the fifth day of symptoms onset. SARS-CoV-2 viral RNA detection gradually declined over time as the duration of viral shedding varied widely – from 10 days to four weeks. We also found a certain correlation between the time of viral persistence and shedding and the severity of the infection – in milder cases, the duration of viral persistence was shorter compared to moderate and severe forms of the disease. We believe that these data are important and applicable pre-analytical factors, as patients are often referred for molecular diagnosis in the later stages of the disease or spend days, even weeks after the onset of symptoms, until the condition worsens and complications occur. In these cases, when the etiology of the condition is unclear, molecular diagnostics is also required as a part of differential diagnosis. The influence of viral persistence in relation to disease severity on PCR detection capacity could generate some practical recommendations: for example, for the diagnostic detection of infection in mild disease cases, PCR is applicable on average up to the 15th day from the onset of infection; for moderate forms of the disease – up to 21st day, and for severe – up to 27 of disease onset.

The first recommended diagnostic technique for SARS-CoV-2 was RT-PCR, which still remains the gold standard and this technique is mainly used in the current COVID-19 pandemic [27]. Over time, great efforts have been made to develop rapid nucleic acid-based tests to detect COVID-19 disease, including LAMP, SHERLOCK, CRISPR and other diagnostic kits. In a study conducted by Inaba et al. [28] of 124 nasopharyngeal swab samples of 24 patients it was shown that at day 9 of the onset of symptoms, LAMP had sensitivity and specificity comparable to RT-qPCR. 92.8% of the samples were positive in both methods. However, after the 10th day after symptom onset, the positivity of RT-LAMP decreased to less than 25% and the correspondence of positivity between the two methods was below 60%. A significant decrease in the sensitivity of the method in patients with low viral load (late Cts) was reported by other studies as well [29, 30]. A comparative analysis of LAMP and RT-PCR methods performed by us confirmed the advantage of RT-PCR in the context of sensitivity in both sample types – NPH and saliva. Our data showed that LAMP technology demonstrated 100% sensitivity, however only 30% specificity. According to our results LAMP appears to be a reliable assay comparable to RT-PCR only at high to medium viral loads (Ct < 34) which makes it an appropriate method for proving the infection only in the first days of symptoms development.

In conclusion: The NPH testing is the gold standard for diagnosing and monitoring the clinical course of patients with SARS-CoV-2 infection regardless of organ involvement. The invasive methods for sampling do not bring additional information about the presence of the virus Molecular technologies other than Real-Time PCR are recommended for use as screening methods only in the first days after infection. Persistence of viral load is directly related to the severity of the infection. In individuals with severe course, the viral load persisted longer than in moderate and mild cases and based on this RT-PCR testing cold add additional value to patient`s treatment and prognosis.

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ORIGINAL ARTICLE

PREVALENCE OF AUTOANTIBODIES IN ANCA-ASSOCIATED VASCULITIDES AND IMMUNOLOGICAL ASSOCIATIONS WITH DISEASE SEVERITY IN THE BULGARIAN POPULATION

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Abstract. Antineutrophil cytoplasmic antibodies (ANCA) are autoantibodies with various specificity, which were first described by Davies et al. in 1982. They target antigens mainly found in the azurophilic and cytoplasmic granules of neutrophils and monocytes. In ANCA-associated vasculitides (AAV), cytoplasmic ANCA (cANCA) is considered a serum marker of granulomatosis with polyangiitis (GPA) (also known as Wegener's granulomatosis – WG), while perinuclear ANCA (pANCA) – a marker of microscopic polyangiitis (MPA). The aim of the present study was to analyse the immunological characteristic features of AAV patients in the Bulgarian population and their association with disease activity and severity, as well as to determine whether other known antibodies are pathogenic in the development of AAV. We conducted a prospective and retrospective analysis of patients with AAV which had been hospitalised in the Clinic of Rheumatology. Of all patients (145 in total), 127 (87.59%) had presence of ANCA. Of all ANCA-positive patients, 55.90% had been diagnosed with GPA, 36.22% with MPA and 3.94% with eosinophilic granulomatosis with polyangiitis (EGPA). The highest prevalence was reported for antibodies against proteinase-3 (anti-PR3) – in 60 patients (41.38%); in 49 patients (33.79%) we found antibodies against myeloperoxidase (anti-MPO), while in 8 patients (5.52%) antibodies had been synthetized against both antigens. Anti-PR3 was found to be the predominant type in patients with GPA, while anti-MPO was predominant in patients with MPA, the difference being statistically significant ($p = 0.0000$ for both antibodies). Patients with anti-PR3 had a higher disease activity index compared to those with anti-MPO (mean Birmingham Vasculitis Activity Score (BVAS) of 18.10) compared to those with anti-MPO (mean BVAS 16.81). An association was established between the presence of specific antibody type and disease form. In the generalized form, the presence of two antibody types was significantly more frequent ($p = 0.0253$); in the localized form, we reported the presence of anti-PR3 in significantly more cases as opposed to anti-MPO. A high number of patients with both antibodies were reported to be deceased but due to the fact that we had a relatively small number of patients with two antibody types, the analysis does not show statistically significant difference. Patients with two antibody types had a more severe disease course and the generalized form was more frequently reported (75% of cases), and a statistically significant difference was noted compared to the localized and systemic form ($p = 0.0253$). The percentage of deceased patients was higher in patients with anti-MPO (20.41% compared to 15.00%), while more patients with anti-PR3 achieved remission (26.67% compared to 20.41%), even though the differences are not statistically significant.

Various combinations of antibody overlap were reported, the most frequently observed one being between anti-MPO or anti-PR3 and anti-dsDNA; the percentage of antibody overlap was very low and was not related to the clinical presentation.

Key words: vasculitis, antineutrophil cytoplasmic antibodies (ANCA), anti-proteinase 3 (anti-PR3), anti-myeloperoxidase (anti-MPO)

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INTRODUCTION

ANCA-associated vasculitides (AAV) are classified as small vessel vasculitides with presence of antineutrophil cytoplasmic antibodies (ANCA) in the serum, as of the latest classification criteria of the Chapel Hill Consensus Conference (CHCC) of 2012. The main clinical and pathoanatomical variants of AAV include microscopic polyangiitis (MPA), granulomatosis with polyangiitis (MPA) (formerly known as Wegener's granulomatosis – WG), eosinophilic granulomatosis with polyangiitis (also known as Churg-Strauss syndrome (CSS) and single-organ vasculitis (SOV) [1, 2]. These vasculitides are associated with ANCA showing specificity towards the myeloperoxidase of human neutrophils (anti-MPOorpANCA) or proteinase-3 (anti-PR3orcANCA). So-called ANCA-negative AAV also exist. They are an analogue of seronegative rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) and are defined as such when the patient fulfils the criteria for the diagnosis of AAV but no ANCA are found in the serum. The major portion of cases of ANCA negativity are found in patients who are at an early disease stage or those with localised disease and limited involvement of the upper respiratory tract [4].

An interesting fact is that initially, one of the AAV, MPA, was not included in the classification criteria created by ACR in 1990 [3]. At that point, it was considered that MPA and polyarteritis nodosa (PAN) are different forms of one and the same disease since their clinical manifestations are fairly similar. It was subsequently discovered that they are quite different vasculitides. The difference comes from the fact that MPA is predominantly a small vessel vasculitis which explains its clinical manifestations, such as rapidly progressive glomerulonephritis, pulmonary capillaritis often with haemorrhage, which are not seen in PAN [1, 2]. After the discovery of ANCA which are only detected in patients with small vessel vasculitis and are considered an exclusion criterion for PAN, MPA was finally differentiated. Even though the contemporary classification criteria separate PAN and MPA as vasculitides of two distinct groups, MPA is still sometimes included in PAN. ANCA antibodies are a marker for clinical and pathophysiological differentiation of the small vessel vasculitides and are not present in patients with PAN [4]. Sometimes PAN and AAV may present with an indistinguishable necrotising arteritis of medium and small vessels.

ANCA are autoantibodies with various specificity, which target antigens mainly found in the azurophilic and cytoplasmic granules of neutrophils and monocytes. They were first described by Davies et al. in 1982 in patients with necrotizing glomerulonephritis through indirect immunofluorescence (IIF) [4, 5, 6]. Van der Woud et al., who described the classical pattern of staining of these antibodies, were the first to suggest that the diffuse granular cytoplasmic reaction in human neutrophils (i.e. cANCA) on immunofluorescence is a specific marker for WG [6]. The pathogenic nature of ANCA in AAV is based on in vitrofindings, in vivo animal models and clinical studies. They provide convincing evidence that ANCA are pathogenic in AAV due to the fact that the ANCA-mediated activation of neutrophils leads to their premature degranulation in the blood vessels and causes damage to the endothelial cells [7, 8, 9]. In AAV, cANCA is considered a serum marker of WG and pANCA – of MPA. In other words, the cytoplasmic pattern of staining is associated with WG and is induced by anti-PR3, while the perinuclear pattern of staining is found in MPA and is induced by anti-MPO. These dependencies, however, are not absolute. Therefore, some authors prefer to refer to them under the common denomination AAV. Nev-

ertheless, the opinion gathering increasing support is that significant differences exist between patients with anti-PR3 and anti-MPO. Establishing the diagnosis of AAV is often difficult as it is necessary to distinguish between multiple other vasculitides, as well as other connective tissue diseases with symptoms of vasculitis on the one hand, and between the different types of AAV, on the other. So far, an analysis of the immunological characteristic features of the various vasculitic syndromes among patients with AAV in the Bulgarian population has not been conducted.

The aim of the present study was to analyse the immunological characteristic features of patients with AAV in the Bulgarian population. To do so, we studies the prevalence of specific antibodies and their association with disease activity and severity. In addition, we studied the prevalence of other known antibodies and their pathogenicity in AAV.

MATERIALS AND METHODS

In the present study, we included 145 patients with AAV – 59 male and 86 female. The patients were diagnosed and treated in the Clinic of Rheumatology at UMHAT "Sv. Ivan Rilski" – Sofia, Bulgaria. We conducted a prospective and retrospective analysis which covered the patients with vasculitis who were admitted to the Clinic of Rheumatology over the period 2009-2018. Diagnosis was made based on the classification criteria developed by the American College of Rheumatology (ACR) (ACR, 1990) and the criteria for classification of the vasculitides according to CHCC [1, 2, 8, 10]. Laboratory tests were conducted in the clinical and immunological laboratory unit at UMHAT "Sv. Ivan Rilski" – Sofia, Bulgaria. The included tests served to determine disease activity (complete blood count (CBC) with differential count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), as well as disease severity and systemic involvement (creatinine, urea, liver enzymes, urine sediment, proteinuria, creatinine clearance). The presence of ANCA was evaluated through IIF on ethanol-fixed neutrophils (Inova Diagnostics VR, Inc., San Diego, CA) and confirmed for the two main types of ANCA, PR3, and MPO by using an automated Alegria VR system (ORGENTEC Diagnostika GmbH, Mainz, Germany). Furthermore, we conducted additional examinations for ANA through IIF in HEp-2 cells (ANA-Hep2, Biosystems, Barcelona, Spain), anti-dsDNA (AlegriaVR system (ORGENTEC Diagnostika GmbH, Mainz, Germany), glomerular basement membrane by immunoblot (Euroline, Anti-GBM (IgG), Euroimmune, Lubeck, Germany), anticardiolipin antibodies by Alegria VR system (ORGENTEC Diagnostika GmbH, Mainz, Germany) and rheumatoid factor (RF) (Alegria VR system (ORGENTEC Diagnostika GmbH, Mainz, Germany).

Involvement of the respiratory tract was confirmed through suitable instrumental studies: radiographs of paranasal sinuses and chest, high-resolution computed tomography (HRCT) of chest, audiogram, spirometry, lung diffusion capacity, and consultations with otorhinolaryngologists, ophthalmologists and pulmonologists.

The statistical analysis was made with SPSS 13.0. We conducted a descriptive analysis based on groups of one or several characteristic features, summarizing the indices relative share and mean.

We also performed a diagnostic analysis to test for statistically significant difference by evaluating statistical hypotheses regarding the presence of a certain association in variables measured in qualitative scales. This analysis was based on "exact: empirical indices through the Fisher's exact test in order to evaluate the difference between two relative shares.

In order to evaluate the level of significance of given empirical characteristic features, we used levels based on the assumptions of the distribution of the tested features. The cut-off value of the level of significance was set at 0.05, unless another value was explicitly stated.

RESULTS

Analysis of antibody distribution per diagnosis

Based on the criteria of ACR and CHCC, we found that a total of 145 patients with AAV had been treated in the Clinic of Rheumatology. Of them, 80 had been diagnosed with WG, 52 – with MPA, 8 – with EGPA; 5 patients had features of vasculitis and presence of ANCA but could not be classified into any of the nosological entities based on the clinical features and the conducted laboratory, immunological and instrumental examinations. These patients were excluded from the conducted comparative analysis.

Analysing our patients with AAV, we found that ANCA were present in 127 (87.59%) out of all 145 patients. Table 1 demonstrates the distribution of patients with and without ANCA based on diagnosis as a percentage of the total number of examined patients.

Table 1. Distribution of patients with and without ANCA based on diagnosis

ANCA	AAV – total (n = 145)	GPA (n = 80)	MPA (n = 52)	EGPA (n = 8)	p*	p GPA vs. MPA
ANCA (+)	127 (87.59%)	71 (88.75%)	46 (88.46%)	6 (75.00%)	0.9658-1.0000-0.6245	1.0000
ANCA (-)	15 (10.34%)	8 (10.00%)	5 (9.62%)	2 (25.00%)	1.0000-1.0000-0.4800	1.0000
Not examined	3 (2.07%)	1 (1.25%)	1 (1.92%)	1 (12.50%)	1.0000-1.0000-0.5080	1.0000
pANCA	48 (33.10%)	11 (13.75%)	31(59.62%)	2 (25.00%)	0.0027-0.0015-0.9294	0.0000
cANCA	64 (44.14%)	56 (70.00%)	7 (13.46%)	1 (12.5%)	0.0003-0.0002-0.1630	0.0000
Not examined	13 (8.94%)	4 (5.00%)	5 (9.62%)	3 (37.50%)	0.4157-1.0000-0.0484	0.4999
Anti-PR3	60 (41.38%)	48 (60.00%)	10 (19.23%)	2 (25.00%)	0.0111-0.0071-0.5832	0.0000
Anti-MPO	49 (33.79%)	12 (15.00%)	35 (67.31%)	1 (12.50%)	0.0040-0.0001-0.3882	0.0000
Anti-PR3/anti-MPO	8 (5.52%)	3 (3.75%)	4 (7.69%)	1 (12.50%)	0.7906-0.8222-0.9638	0.5551
Not examined	14 (9.66%)	5 (6.25%)	3 (5.77%)	3 (37.50)	0.5294-0.5698-0.0626	1.0000

* Fisher'sexact probability test p – used to compare each of the three groups with the total percentage in the respective row. Cases of statistically significant difference are marked in red.

Of the ANCA-positive patients, 55.90% had GPA, 36.22% – MPA and 3.94% – EGPA. ANCA were not detected in as few as 15 out of 145 patients (10.34%); in three cases antibodies were not examined since the diagnosis was established on the basis of other diagnostic criteria. We analysed and compared the three types of vasculitides separately, with particular focus on those with GPA and MPA, since the number of patients with EGPA was significantly lower. When analysing the presence of antibodies in the different vasculitic entities, we discovered that there was no statistically significant difference with regard to ANCA positivity – it was detected in 89.87% of cases of GPA, 90.19% – of MPA and 75% of cases of EGPA, which goes to show that the presence of ANCA is a particularly significant criterion for the establishment of the diagnosis in the Bulgarian population; ANCA negativity often leads to exclusion of AAV as a possible diagnosis. Due to the difference in the reference range of ANCA, we calculated the antibody titre as a numerical value indicating the times it is increased. The mean value was 11.4 in cases of GPA, 16.4 – of MPA and 4 – of EGPA. In addition, ANCA were classified according to the type of staining into cANCA and pANCA in 112 patients. Our analysis of ANCA types in ANCA-positive patients revealed that a cytoplasmic pattern was reported in 64 patients (44.14%), while the perinuclear pattern was observed in 48 cases (33.10%). We noted that cANCA occurred significantly more frequently in patients with GPA, while pANCA – in those with MPA (p = 0.0000). Furthermore, we analysed the presence of antibodies directed against specific antigens and found that the prevalence of antibodies against proteinase-3 was highest (60 patients or 41.38%). In 49 patients (33.79%) we reported antibodies directed against myeloperoxidase; in 8 patients (5.52%) antibodies were synthetized against both types of antigens. Anti-PR3 was the predominant type in patients with GPA, while anti-MPO – in those with MPA, the difference being statistically significant for both types of antibodies (p = 0.0000). In patients with EGPA, antibodies against proteinase-3 occurred twice more often. The presence of both antibodies at the same time occurred more frequently in patients with MPA; in subsequent follow-up examinations, only one type remained present.

ANCA and disease severity

When comparing disease form, severity and presence or absence of antibodies, we found that patients with AAV usually have a severe disease, with the generalised form being most often reported, which is characterised by impairments in the function of the affected organs with organ failure. We did not report statistically significant difference in the prevalence of ANCA-positive patients in the separate groups. The percentage of ANCA positivity was highest in the systemic form and lowest in the mildest form – localised disease. The severe form was in third place with regard to the presence of ANCA.

Table 2. Presence of ANCA and disease form

	AAV total (n = 145)	Localised (n = 25)	Systemic (n = 36)	Generalised (n = 49)	Severe (n = 35)	p*
ANCA (+)	127 (87.59%)	21 (84.00%)	33 (91.67%)	42 (85.71%)	31 (88.57%)	0.8644-0.6939-0.9271-1.0000
ANCA (-)	15 (10.34%)	3 (12.00%)	3 (8.33%)	5 (10.20%)	4 (11.43%)	0.8671-0.9602-1.0000-1.0000
Not examined	3 (2.07%)	1 (4.00%)		2 (4.08%)		-

* Fisher's exact probability test p – used to compare each of the three groups with the total percentage in the respective row.

When comparing ANCA-positive and ANCA-negative patients, we found that out of the former group (a total of 127 patients), 21 patients (16.53%) had localised disease form, 33 (25.98%) had systemic involvement, 42 (33.07%) had generalised disease form and 31 (24.41%) had severe disease. In ANCA-negative patients (15 in total), the most frequently reported disease form was once again the generalised one (33.33%), followed by the severe form (26.67%); the localised and systemic form were least frequently reported. These findings suggest that the presence or absence of ANCA is not a factor that determines disease severity.

Analysis of antigen specificity of ANCA

We conducted antigen specific examination for anti-PR3 and anti-MPO in ANCA-positive patients. Antibodies directed against PR3 were reported in 60 out of the examined patients; against MPO – in 49, while 8 patients had both types of antibodies. Antigen specific examination was not conducted in 15 cases of ANCA-positive patients.

Of all patients tested for presence of antibodies directed against specific antigens, a total of 109 were positive, 60 of which had antibodies against PR3, 49 – against MPO, 8 – against both antigens simultaneously. Females were the predominant sex in all three groups, the ratio male : female being 28:32 in the group of anti-PR3 positive cases and 18:31 in the group of anti-MPO positive; the mean age was 50.03 in the first group and 55.00 in the second, respectively. Statistically significant difference was reported in antibody prevalence between the separate diagnostic entities. Of patients with antibodies against PR3, 48 (80.00%) were diagnosed with GPA, 10 (16.67%) – with MPA and 2 – with EGPA (3.33%). In the group with antibodies against MPO, the highest number of patients were diagnosed with MPA – 35 (71.43%), 12 (24.49%) had GPA, 1 (2.04%) had EGPA.

We aimed at establishing a correlation between the different types of ANCA and disease severity and outcome by comparing the mean value of the Birmingham Vasculitis Activity Score (BVAS) in the two groups. In addition, we compared the different disease forms, the mildest form being the lo-

Table 3. Percentage distribution of different types of ANCA

	Anti-PR3 (n = 60)	Anti-MPO (n = 49)	Anti-PR3, anti-MPO (n = 8)	p*
Male : female	28:32	18:31	3:5	0.6914
Age	50.03	53.59	55.00	0.5084
BVAS	18.10	16.81	18.13	0.1064
Localised	14 (23.33%)	1 (2.04%)	0 (-)	0.0253
Systemic	13 (21.67%)	14 (28.57%)	1 (12.50%)	
Generalised	19 (31.67%)	20(40.82%)	6 (75.00%)	
Severe	14 (23.33%)	14 (28.57%)	1 (12.50%)	
Deceased	9 (15.00%)	10 (20.41%)	4 (50.00%)	0.1086
Remission	16 (26.67%)	10 (20.41%)	1 (12.50%)	

* Chi-square p-value (Sex, disease type, result); ANOVA Brown-Forsythe p-value (Age, BVAS). Cases of statistically significant difference are marked in red.

calised one followed by the systemic one, where no impairment in the function of the involved organs was reported, in turn followed by the generalised and severe form, characterised by organ failure with creatinine values exceeding 500 μmol/l. We found that patients with anti-PR3 had a higher activity score (mean BVAS 18.10) compared to those with anti-MPO (mean BVAS 16.81). In patients with anti-PR3 we most frequently observed the generalised form (in 31.67% of cases), while the systemic form was reported in the least number of cases (21.67%). Next, in patients with anti-MPO, the generalised form was again the predominant one (40.82% of cases), while the localised form was the least often diagnosed (in only one patient – 2.04%). Patients that had both types of antibodies simultaneously had more severe prognosis, the generalised form being the predominant one (in 75% of cases), with a statistically significant difference with the cases of localised and systemic form ($p = 0.0253$); in fact, localised disease was not diagnosed in any patient. Furthermore, we compared the groups of anti-PR3 and anti-MPO with regard to the number of deceased patients and those who achieved remission. The percentage of deceased patients was higher in the group with anti-MPO (20.41% against 15.00%), while that of patients in remission was higher in the group with anti-PR3 (26.67% against 20.41%), however neither difference proved to be statistically significant.

The conducted analysis confirmed the link between antibody type and disease form. The simultaneous presence of two antibodies was associated significantly more often with generalised disease ($p = 0.0253$). Conversely, in the localised form, we noted that anti-PR3 was found significantly more often than anti-MPO. The percentage of deceased patients who had two antibody types was high, however due to the fact that we had a small number of patients with two antibody types, the analysis did not show a statistically significant difference (even though the p-value was not much higher than 0.05).

Presence and prevalence of other antibody types typical for connective tissue diseases (CTD) and ANCA

When analysing the immunological profile of our patients, we looked for other antibody types typical for different CTD which overlapped with positive ANCA, such as antinuclear antibodies (ANA), antibodies against double-strand DNA (anti-dsDNA), against the glomerular basement membrane (anti-GBMA), anticardiolipin antibodies (ACLA) and rheumatoid factor (RF) (Table 4).

In 50.51% of cases, apart from ANCA, we found ANA, which are typical for other CTD such as systemic lupus erythematosus (SLE) and systemic sclerosis (SSc) but can be non-specifically positive in other autoimmune diseases. Surprisingly, RF, which is not characteristic of CTD and vasculitides was positive in 40.54% of patients and was reported a bit more frequently in those with GPA. Anti-dsDNA antibodies were found in 13.23% of patients (more often in cases of MPA), while ACLA – in 16.67% of patients (again, more often in cases of MPA). Antibodies against ribonucleoproteins – anti-RNP and anti-RNP/Sm were reported in only two cases. As mentioned above, patients with MPA had anti-dsDNA and ACLA more often than those with GPA but the difference was not statistically significant. In 21 patients whose main disease manifestations included glomerulonephritis and pulmonary haemorrhage, we examined the presence of anti-GBMA, which were negative in all cases, thus proving beyond doubt that these antibodies are not involved in the pathogenesis

Table 4. Prevalence of other antibodies in patients with AAV

	AAV (n = 145)	GPA (n = 80)	MPA (n = 52)	EGPA (n = 8)	p*
ANCA	127/142 (89.44)	71/79 (89.87)	46/51 (90.20)	5	1.0000
ANA	49/97 (50.51)	24/50 (48.00)	19/40 (47.50)	3	1.0000
Anti-dsDNA	9/68 (13.23)	4/35 (11.43)	5/32 (15.63)	0	0.8851
ACLA	6/36 (16.67)	2/17 (11.76)	4/18 (22.22)	0	0.7100
Anti-GBMA	0/21	0	0	0	
RF	15/37 (40.54)	10/22 (45.45)	5/14 (35.71)	0	0.8172

* Fisher's exact probability test p – used to compare between GPAand MPA. Cases of statistically significant difference are marked in red.

of AAV and their presence should be considered a marker for another disease. ANCA and anti-GBMA involve the lungs and kidneys in a similar mechanism, by causing damage to small vessels and have an identical clinical presentation. Different variants of antibody overlap were observed – the most often reported one being that of anti-dsDNA and anti-MPO (in 7 patients), anti-dsDNA and anti-PR3 (in 5 patients); in 3 patients, we noted an overlap between anti-MPO, anti-dsDNA and ACLA. In these patients, the disease presentation usually involved vasculitic manifestations – glomerulonephritis with rapid progression to renal failure in two patients; pulmonary vasculitis with pneumonitis and alveolar haemorrhage in another two patients. As mentioned above, we observed various immunological overlaps. Patients with AAV and positive ACLA most often overlapped with anti-MPO (4 cases), anti-dsDNA (3 cases), anti-PR3 (2 cases) and RF (2 cases). RF most often overlaps with anti-PR3 (in 7 patients). We reported an association between RF and joint involvement; all RF-positive patients had arthralgias or arthritis over the course of their disease.

Antibodies in PAN

In addition to our study, we analysed patients with PAN, which had been treated in the clinic (a total of 13 patients). Of the tested immunological markers, ANA were detected in two patients with PAN and RF was detected in further two patients. Anti-dsDNA antibodies were reported in one patient. A borderline titre of ANCA was found in three patients (23.08%), which did not demonstrate the typical staining pattern of AAV. Antibodies against PR3 and MPO were not found in any case. Low complement was reported in two patients with PAN.

DISCUSSION

ANCA are autoantibodies with various specificity, which were first described by Davies et al. in 1982 in patients with necrotising glomerulonephritis through IIF [4, 5, 6]. Subsequently, target antigens for ANCA such as MPO and PR3 were also discovered. The association between ANCA and vasculitides is key for the understanding of their pathogenesis and is an important diagnostic tool. In recent years, convincing evidence has been gathered regarding ANCA's pathogenicity in AAV [7, 8, 9]. This is further supported by the fact that while anti-PR3 are found only in 50% of patients with localised GPA, they are positive in 90% of patients with generalised disease. Furthermore, the levels of ANCA are associated with disease activity and relapse [2, 8, 24]. A number of studies have focused on examining the prevalence of ANCA and their relationship with various clinical manifestations in patients with AAV. According to previously published data ANCA are found in 65-75% of patients with MPA [4, 5, 6, 13]. In addition, ANCA-positivity is reported in 82-88% of patients with GPA, while the prevalence in EGPA varies between 40 and 75% [4, 13, 14, 15, 16, 17]. The results reported in our patients show, that 88.75% of cases of GPA are positive for ANCA; this number is 88.46% for cases of MPA and 75.00% for cases of EGPA. Only in 15 out of 145 patients (10.34%) we did not find antibodies, while in three cases such examination was not conducted and the diagnosis was established based on other diagnostic criteria. These data are similar to the ones mentioned above and are a little higher for MPA. This shows that the presence of ANCA is a fairly significant factor for the establishment of the diagnosis of AAV in the Bulgarian population and that negative antibodies often lead to rejection of that particular diagnosis, i.e. antibodies often have the 'upper hand' as opposed to the clinical presentation.

Anti-PR3 antibodies are considered a sensitive marker for active WG. This sensitivity depends on the difference in the testing methodology, as well as the various definitions used in the diagnosis of different conditions. If using more liberal criteria which do not require a histological verification, such as the criteria of ACR, cANCA sensitivity is only 28% [18, 19, 20]. Hagen et al. report 64% sensitivity of cANCA and 66% sensitivity of anti-PR3 as a marker for the diagnosis of WG [19, 20]. Patients with active GPA with limited involvement of the upper respiratory tract have anti-PR3 in 2/4 of cases [18 ,21]. Furthermore, anti-PR3 is also found in 50% of cases of MPA and a lower percentage of cases of necrotising pauci-immune glomerulonephritis [22, 23]. When combining all these findings, the specificity of anti-PR3 for small vessel vasculitis is calculated to be as high as 98% [25].

Anti-MPO antibodies are found in approximately 80% of cases of active idiopathic pauci-immune glomerulonephritis (necrotising and crescenting glomerulonephritis – NCGN) [22], in 50% of cases of active MPA [25], 60-70% of patients with EGPA and 20% of patients with WG [23, 26]. In addition,

anti-MPO is found in patients with other autoimmune diseases which are not part of the group of idiopathic small vessels vasculitides, such as anti-GBM disease, SLE, drug-induced glomerulonephritis. Therefore, the specificity of anti-MPO for the diagnosis of small vessel vasculitis is lower than that of anti-PR3 and is approximately 80% [4]. Between 60% and 95% of ANCA in GPA are cANCA and anti-PR3 [19]. When combining the results of IIF and those of ELISA (cANCA/anti-PR3for GPA and pANCA/anti-MPO for MPA) their diagnostic specificity rises to 99% [4, 19, 20]. When analysing antibodies directed against specific antigens in our study, we found that prevalence was highest for anti-PR3 antibodies – in 60 patients (41.38%); in 49 patients (33.79%) we found anti-MPO antibodies and in 8 patients (5.52%) antibodies were detected against both antigens. Our results further indicate that 60% of patients with GPA are anti-PR3 positive and 67.31% of patients with MPA are anti-MPO positive. In patients with EGPA, anti-PR3 were the predominant type, which is consistent with the results of other studies. Both antibodies are more often found in females; in addition, patients with anti-PR3 are younger than those with anti-MPO. Anti-PR3 are the predominant type in WG, anti-MPO – in MPA; patients with MPA and positive anti-PR3 were only 19.23%, while patients with WG and anti-MPO – 15%. These findings establish a statistically significant association between GPA and anti-PR3 and between MPA and anti-MPO ($p = 0.0000$), which confirms beyond doubt the correlation between the antibodies and the respective types of vasculitis. Our study did not find a statistically significant difference in the percentage of ANCA-positive patients when classified according to disease severity, however the systemic form had the highest percentage of ANCA positivity, while the lowest percentage was reported in the mildest form – the localised one. The severe form was in third place with regard to the presence of ANCA. These results are similar for ANCA-negative patients which shows that the presence or absence of antibodies is not a defining factor for disease severity, however it is defining with regard to the degree of systemic involvement. Our study also revealed that the localised form is associated significantly more often with anti-PR3 rather than anti-MPO ($p = 0.0253$). We also confirmed the link between the generalised form and the presence of both antibodies simultaneously.

Previous studies have demonstrated a strong association between anti-PR3 and AAV activity according to BVAS [4, 24, 27]. Such trend was also noted in our patients – those positive for anti-PR3 had a higher BVAS but the difference was not statistically significant. We did not find a correlation between disease outcome and the different types of antibodies. The percentage of deceased patients was higher in the group of anti-MPO (20.41% as opposed to 15.00%); the percentage of patients achieving remission was higher in the group of anti-PR3 (26.67% as opposed to 20.41%), however the difference was not significant. According to some studies the presence of ANCA at the point of diagnosis leads to increased mortality risk [28, 29, 30]. On the other hand, multiple studies have not found differences in survival between patients with AAV associated with anti-PR3 and that associated with anti-MPO [34, 35, 37]. According to Franssen et al., patients with anti-MPO have a poorer survival rate compared to those with anti-PR3 [31]. Such significant difference was not reported in the present study. It's worth noting the high percentage of deceased patients who had a combination of both antibodies (anti-PR3 and anti-MPO), which was not observed in those achieving remission; this attributes a poor prognosis for this combination.

ANA, which are typical for certain CTD, are not considered to participate in the pathogenesis of systemic vasculities, although they can be non-specifically positive, along with ACLA, in certain types of vasculitis, such as giant cell arteritis [32, 33]. There are only scarce data in the literature regarding the prevalence of ANA and ACLA in AAV, as well as RF. According to the study of Goitybell et al., our of 13 examined patients, ANA were not found to be positive in any case [27]. In our study, ANCA-positive patients also had high prevalence of ANA (which were reported in 50.51% of cases); RF was positive in 40.54% of patients, and was noted a bit more often in cases of WG. All patients with positive RF had clinically manifested arthritis. These results confirm the role of RF in the pathogenesis of joint inflammation. Furthermore, in the present study, antibodies specific for SLE – anti-dsDNA – were positive in 13.23%, while antibodies directed against various RNP – RNP and RNP/Sm were detected in two patients. ACLA were identified in 16.67% of patients. Patients with MPA had positive anti-dsDNA and ACLA more often than those with GPA, even

though the difference was not statistically significant (p = 1.000). In addition, in the present study, anti-GBM antibodies were not positive in any patient with AAV and renal involvement, which suggests that these antibodies likely do not play a role in the pathogenesis of glomerulonephritis in AAV. Various combinations of antibody overlap were reported, the most frequent one being the overlap of anti-dsDNA with anti-MPO and anti-PR3. The low percentage of antibody overlap confirms the key role of ANCA in AAV and suggests that the other types of antibodies are a "witness of the disease" and an immune phenomenon in a given period of the disease evolution. Over the course of continuous patient follow-up for a period of 5 years, we did not report the future development of features of overlap syndromes. It is considered that AAV as a whole do not overlap with other autoimmune rheumatic diseases and literature data on this subject are fairly scarce. Most often, such overlap occurs with rheumatoid polyarthritis followed by SLE, SSc and Sjögren's syndrome [34, 35, 36]. In our patients with AAV, we reported clinical and immunological overlap in only four patients – three with rheumatoid arthritis and one with SLE. As for PAN, the other type of vasculitis which may clinically resemble AAV, we found ANCA in three cases (23.08%), although they did not have the typical staining pattern and the antibodies directed against PR3 and MPO were negative in all cases. This confirmed the statement of CHCC from 1994 that ANCA are not related to PAN and their presence is more of an exclusion criterion for the diagnosis of PAN [3, 4].

CONCLUSION

In the current rheumatology practice, the detection of ANCA is considered a significant criterion for the diagnosis of AAV. In total, they were found in 87.59% of AAV patients in the Bulgarian population. An association exists between anti-PR3 and GPA and between anti-MPO and MPA. Our study did not report statistically significant difference in the percentage of ANCA-positive patients with regard to disease severity. Similar findings were reported in ANCA-negative patients, which confirmed that the presence or absence of antibodies is not a defining factor for disease severity. We confirmed the association between the generalised form and the simultaneous presence of both antibody types, as well as between the localised form and anti-PR3. RF can often be positive and is associated with arthritic changes. In addition, in the present study, anti-GBM antibodies were not positive in any patient with AAV. The combination between ANCA and anti-dsDNA and ACLA is associated with severe vasculitic features. ANCA were not reported in PAN and their presence should be considered an exclusion criterion for that diagnosis.

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CASE REPORT

COVID-19 INDUCED RUPIOID PSORIASIS

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Abstract. The novel coronavirus disease of 2019 (COVID-19) is associated with significant morbidity and mortality. Patients commonly present with respiratory symptoms, however, concomitant skin lesions can also evolve. Herein, an anecdotal case of rupioid psoriasis induced by the SARS-CoV-2 infection is described. A comprehensive review of the reported skin manifestations in the highlights of suggestive pathogenetic hypotheses is presented.

Key words: rupioid, ostraceous, psoriasis, SARS-CoV-2

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INTRODUCTION

In December 2019, several patients have been hospitalized for acute atypical pneumonia with unknown etiology in Wuhan, China [1]. The World Health Organization (WHO) named the disease Coronavirus Disease 2019 (COVID-19) on January 12, 2020 and on March 11, 2020 announced a world pandemic [2]. The most common symptoms of COVID-19 are fever, fatigue, dry cough, dyspnoea, headache, nasal congestion, sore throat, myalgia, and arthralgia [1, 3, 4].

Concomitant and re-convalescent skin manifestations in patients with SARS-CoV-2 infection have been reported from 0.2% to 20.5% of all cases. To date, the dermatological specialists have differentiated five lesional categories [5]: acral areas of erythema with vesicles or pustules (pseudo-chilblain) (19%), disseminated vesicular eruptions (9%), urticarial lesions (19%), maculo-papular efflorescence (47%), and livedo or necrosis (6%) [6].

Herein, psoriasiform lesions, which to date, were not included in the skin manifestations of COVID-19, are described. We report a very rare case of rupioid psoriasis, induced by the SARS-CoV-2 infection.

CASE REPORT

A 59-year-old Caucasian female presented to the Dermatology Department with diffused cone-shaped, oyster shell–like skin lesions, disseminated on the scalp, trunk, arms, and legs with three-month duration. At the time of their onset, the patient had sore throat, dry cough, anosmia, overall fatigue, muscle weakness, and gradually increasing malaise and headache. She was tested positive for SARS-CoV-2, confirming COVID-19 infection. A combined therapy with orthomolecular, anti-pyretic, and symptomatic medications was introduced. The patient recovered by the infection in a 2-week period, however, the skin efflorescence continues to spread and disseminated. No family history of any inflammatory dermatological condition was reported. The patient was in optimal general health, with no concomitant systemic disease.

On physical examination, multiple well-demarcated erythematous papules and plaques with central, thick, coin-shaped crusts presented on the trunk, upper and lower limbs (Figure 1, 2, 3) to the whole extend of 25% of the body surface area. Confluent erythematous plaques with silvery scaling diffusely covered the scalp zone. There were no nail changes and symptoms of joint involvement.

Fig. 1. Erythematous papules and plaques diffusely spread on the trunk of the patient

Fig. 2. Skin lesions involving the lower extremities

Fig. 3. Erythematous miliar, guttate and nummular papules and plaques covered with sliver-white rupioid scales

Laboratory testing was normal except lymphocytes 1.08 x 10^9/L (normal range 1.18-3.74 x 10^9/L), granulocytes of 72,6% (normal range 34-71,1%) and a C-reactive protein of 5.20 mg/L (normal range 0-5.00 mg/L). The Antistreptolysin O titer was negative. The microbiological analysis ruled out any underlying focal infection.

A 4-mm punch biopsy of a representative lesion on the trunk demonstrated psoriasiform epidermal hyperplasia (Fig. 4), thick parakeratosis, subcorneal neutrophilic microabscesses, reduced granular layer, elongation of the rete ridges, and superficial vascular ectasia (Fig. 5). The diagnosis of psoriasis vulgaris was concluded.

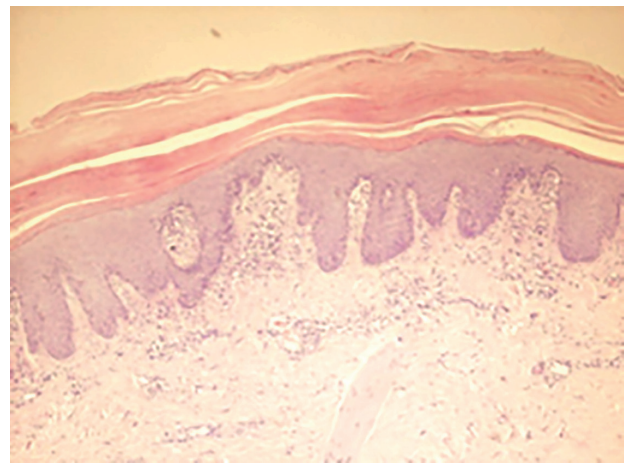

Fig. 4. Vertically alternating orhto- and parakeratosis, psoriasiform acanthosis, mild interstitial round cell inflammatory infiltrate in the papillary dermis (H&E, x 40)

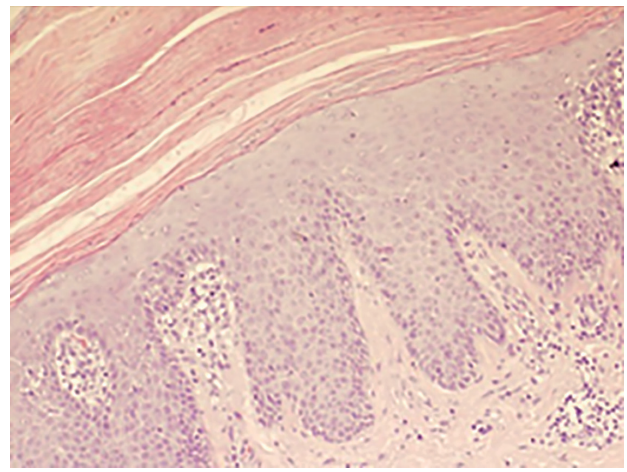

Fig. 5. Typical histological constellation of psoriasis (H&E, x 100)

Pathogenetic therapy with methotrexate 15 mg weekly in combination with narrow band UV B phototherapy, topical keratolytics, and emollients was introduced. At 3-week follow-up the patient had

marked improvement of psoriatic plaques with remarkably reduced induration and hyperkeratosis.

DISCUSSION

Psoriasis is a chronic inflammatory T-cell mediated disease with a worldwide frequency of approximately 2% to 3%. It is critically driven by IL-17 and TNF alpha inflammatory mediators. Controversial reports support the exacerbating role of superimposed or concomitant bacterial or viral infections. It is not yet clarified if these microbiological agents cause or are the result of the chronic skin inflammation [7].

The direct effect of COVID-19 on T-cell immunity may lead to the stimulation of keratinocyte proliferation and modulate various immune functions [8]. On the other hand, the increase of pro-inflammatory cytokines could reach the skin and stimulate dermal dendritic cells, macrophages, mast cells and lymphocytes in addition to polymorphonuclear cells and promote eruptions such as erythema, urticarial lesions, vesicles, etc.

The term rupioid (also known: „ostaceous") has been used to describe well-demarcated, cone-shaped plaques with thick, hard-adherent crusts on the skin resembling oyster or limpet shells [9]. Rupioid crusts were reported for the first time in 1859 by Bazin in secondary syphilis. In the last decade of the 20th century Neisser and Haslund described fully clinical appearance of rupioid crusts on erythematous infiltrative plaques, which in 1948 Grzybowski classified as clinical form of psoriasis placata [10].

Rupioid psoriasisis is an extremely rare clinical manifestation. To our knowledge, there are only 6 cases reported worldwide. Rupioid scabs have been described in the secondary syphilis, Histoplasma capsulatum infection in immunocompromised patient, Norwegian scabies, HIV, reactive arthritis and photosensitivity reactions associated with aminoaciduria [9-17]. Skin lesions progressed and disseminated about 1 to 3 months upon the incriminated trigger. All have the classic rupioid appearance of concentric circular layers of dirty, yellow, oyster-like scale. The diagnosis of rupioid psoriasis can be confirmed with a skin biopsy, which demonstrates characteristic histopathologic findings [10].

In reported cases the disease is characterized with pro-longed onset (1-3 months), nail changes and severe arthropathy [10, 11]. Some authors revealed positive family history [9]. Rupioid psoriatic flares have also been associated with drug treatments, such as intravenous corticosteroids and pembrolizumab [18, 19]. Streptococcal superantigens activate the immune system and increase of anti-streptolysine-O (ASO). It is suggested that they can induct the formation of rupioid lesions [9].

Thick, oyster-like scales in psoriasis may present during HIV infection. It is hypothesized that HIV can be a stimulatory factor to disregulated CD8+ T-cells, causing cutaneous immune system response and enhanced epidermal hyperplasia. HIV may also act as an antigen to activate host CD8+ T-cells [16]. It is important to prove HIV infection by a screening test before the initiation of biologic therapy in patient with psoriasis [17].

The solitary use of topical keratolytic and emollient therapy is conserved inadequate in rupioid psoriasis due to the poor penetration of active ingredients applied. A combined regimen of UV B phototherapy and systemic pathogenetic medications is preferred. Methotrexate, cyclosporine, adalimumab, and ustekinumab have been used with favourable therapeutic response [10, 11, 20].

Remarkably, our case features a very abrupt onset and a persistent duration over 3 months. There was no pre-existing personal or family history of psoriasis. No nail changes and joint involvement were observed. Other infectious and drug-related incriminations were also ruled out. The SARS-CoV19 is suggested to be the only possible trigger.

COVID-19 has totally changed modern medicine perspectives. Modulating the immune response, it exacerbates and triggers a variety of chronic inflammatory processes affecting different internal organs as well as the skin. Rupioid psoriasis is an extremely rare form of psoriasis with only anecdotal cases reported to date. Herein, we describe the first COVID-19-induced ostraceous psoriasis, which features a sudden onset, diffuse distribution and very refractive disease course.

CLINICAL PEARLS

- Rupioid psoriasis is a rare form of psoriasis placata
- Rupioid psoriasis has specifically well-demarcated, erythematous plaques with hyperkeratotic conical, oyster shell-like crusts
- Rupioid psoriasis features typical histological constellation

- Rupioid psoriasis is treated with combination of topical and systemic therapy

No conflict of interest.

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CASE REPORT

MILD COURSE OF COVID-19 INFECTION IN A PATIENT WITH CHRONIC GRANULOMATOUS DISEASE

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Abstract. Chronic granulomatous disease (CGD) is a rare primary immune deficiency (PID) that is classified to the Congenital defects of phagocytes. PID patients are among the risk groups for more severe SARS-Cov2 infection. However, isolated reports of patients with CGD indicate that most patients have recovered well with COVID-19 disease. **The purpose** of this report is to present a clinical case of a patient with a primary immune deficiency in which the neutrophil defect and reduced production of oxidative radicals are likely to have affected the course of the disease. We present twenty-four years old man with X-linked CGD, whose clinical manifestation started from the neonatal period. In the course of the disease, typical inflammatory and granulomatous complications occurred. The patient underwent immunomodulatory therapy with Interferon gamma 1b (Immukin), long-term alternating antibiotic prophylaxis with Trimethoprim/sulfamethoxazole and periodic courses with Itraconazole with good therapeutic response. A mild form of COVID-19 infection detected by a positive PCR test was registered in the patient. Clinical symptoms include fever up to 38.5° C, sore throat, and dry cough for 7 days. Laboratory and imaging tests were normal. Outpatient treatment with good clinical effect was performed. **Conclusion:** The patient presented by us has a primary immune deficiency associated with impaired neutrophil activity. We assume that regular treatment with interferon gamma and/or congenital error in neutrophilic inflammation had contributed to the mild course of COVID-19 infection. Whether the hypothesis shared by other authors as well that impaired neutrophil function protects against the so-called cytokine storm and subsequent tissue damage observed in severe COVID-19 is true remains to be investigated.

Key words: COVID-19 infection, Chronic granulomatous disease (CGD), neutrophil function

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INTRODUCTION

Chronic granulomatous disease (CGD) belongs to the group of congenital defects of phagocyte count, function or both according to the Phenotypic Classification for Primary Immunodeficiencies 2019.

CGD is a genetically heterogeneous disease characterized by recurrent life-threatening bacterial and fungal infections and the formation of granulomas. The etiology of CGD is related to defects in the NADPH oxidase complex of phagocytes, responsible for the generation of superoxide and other reactive oxygen species needed for intracellular killing of pathogenic microorganisms.

SARS-CoV2 disease has a broad spectrum of clinical phenotypes ranging from mild symptomatic

cases characterized by influenza-like manifestations, which occur in the majority of subjects (about 80%), to severe pneumonia requiring admission to intensive care units in 5% or invasive mechanical ventilation, or even death [1]. Although known risk factors for more severe COVID-19 infection include older age, hypertension (HTN), diabetes mellitus, obesity and cardiovascular disease [2], the association of immunocompromised patients and severity of COVID-19 infection is not well established.

We present clinical case of patient with X-linked CGD and mild course of infection with SARS-CoV2 (confirmed either with positive RT-PCR). By the age of maturity the patient was treated at the Clinic of Pediatrics and Genetic Diseases of Hospital "Sv. Georgi"; Plovdiv Since March 2017, follow-up and treatment continues at the Department of Clinical Immunology University Hospital Alexandrovska Sofia and the Expert Center for Rare Diseases – PID.

CLINICAL CASE

23-year-old man in whom X-linked chronic granulomatous disease was suspected because of family history of two deceased siblings at 2 and 5 years of age, respectively who developed post-vaccine disseminated BCG infection and fatal bronchopulmonary infection. The greatly reduced NBT test (2%) performed in the neonatal period allowed avoidance of routine BCG immunization. Clinical manifestations dated from infancy include pyoderma, perianal ragadas, chronic diarrhea and physical retardation.

At the age of three, osteomyelitis of foot was registered, with the isolated causative agent *Stenotrophomonas maltophilia*. At the age of four – purulent epididymitis with histological data for microabscesses and granulation tissue was detected. At the age of twelve, severe destructive pneumonia caused by *Enterobacter aerogenes* ESBL was observed.

Since 2011 the patient had been without clinically manifested infections requiring hospitalization and the need of parenteral antibiotic and antifungal therapy.

Immunological studies evaluating phagocyte function, cellular and humoral immune response were performed. The results showed:

- Severely reduced NBT test in the neonatal period – 2% (range 10-20%).
- DHR123 with impaired oxidative burst in the patient's neutrophils after stimulation with PMA (phorbol 12-myristate 13-acetate) (Fig. 1a).

Additionally two populations of neutrophils have been observed in his mother. One comprised of 68% cells which lacked an oxidative explosion, and the other of 32% cells which retained the ability to generate reactive oxygen species (Fig. 1b) – a typical image of female carriers of CYBB gene defect. For comparison, 100% of granulocytes of control sample showed the presence of an oxidative burst (Fig. 1c).

- Humoral immunity follow up (2017-2021) revealed progressive polyclonal IgG hypergammaglobulinemia (Fig. 2).

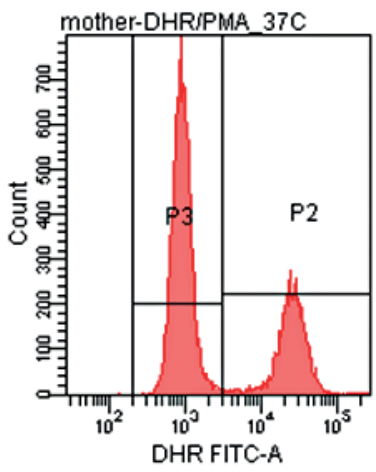

Fig. 1a. Neutrophil gate histogram showing no FITC channel fluorescence

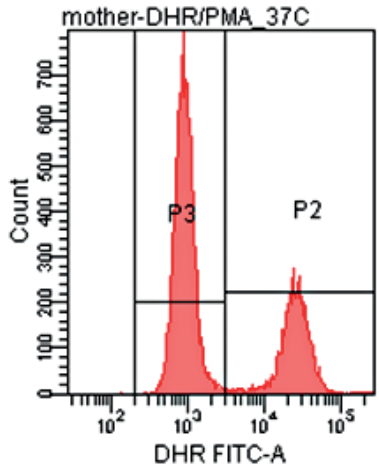

Fig. 1b. Histogram in the neutrophil gate showing two cell populations. One with no fluorescence of the FITC channel (68%) and the other (32%) with available

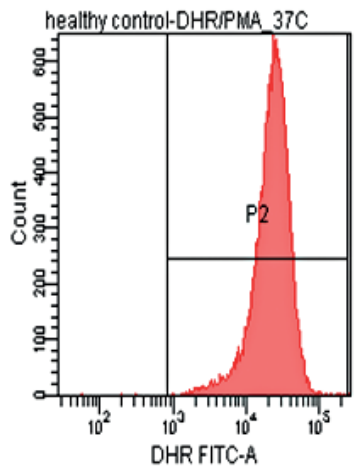

Fig. 1c. Neutrophil gate histogram showing available FITC channel fluorescence (100%)

Fig. 1. Flow cytometric assessment of DHR assay

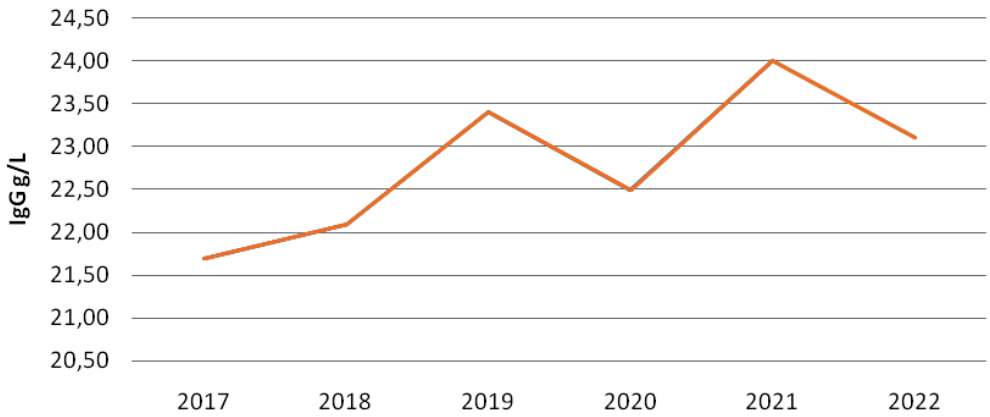

Fig. 2. Dynamic of IgG levels

- Flow cytometric analysis of leukocytes showed normal distribution of the main cell populations (total T lymphocytes and their main subsets, B-lymphocytes and NK cells). Mild relative and absolute lymphopenia and an increased percentage of B lymphocytes were observed after reinfection with COVID-19 (Table 1).
- The cytokine profile showed prevalence of Th1 immune response with elevated levels of INF-gamma, Il-12p70, Il-18, IL-2 and TNF-alpha.

The definitive diagnosis of X-linked CGD was confirmed by genetic testing, which identified new hemizygous CYBB c.252+5G > C intronic gene mutation.

Theraputic protocol for CGD consisted of prophylactic treatment with Trimethoprim/sulfamethoxazole 960 mg orally every other day, Itraconazole 100 mg/day and Interferon gamma 1b (Immukin 0.1 mg/0.5 ml) from 2012 until now – 30 mcg 3 times weekly subcutaneously.

The good clinical response achieved in this patient at this stage postpones the hematopoietic stem cell transplantation as a treatment option.

COVID-19 INFECTION

A mild form of COVID-19 infection detected by a positive PCR test was registered in the patient on March 2021. Clinical symptoms included fever up to 38.5° C, sore throat, and dry cough for 7 days. Laboratory and imaging tests were normal. Therapy with antibiotic, vitamins and antiplatelet drugs was administred.

Three months later (June 2021), a cellular and humoral immune response to COVID-19 was examined. Specific effector T lymphocytes in peripheral blood were determined by ELISpot (TSPOT. COVID-19) technology based on INF-γ secretion after stimulation with peptide mixes of SARS-CoV-2 virus. 23 spots to S antigen (panel A) and 17 spots to N antigen (panel B) were reported (positive result over 8 spots for both antigens) (Fig. 3). Virus neutralizing antibodies were determined using the cPassTM SARS-CoV-2 Neutralization Antibody Detection Kit and the percentage of neutralizing antibodies (against delta variant) was 38 (cut-off > 30%).

Based on these data, we could conclude that both humoral and cellular immunity generated during infection are insufficiently protective, which could be due to the milder course of the disease.

Eleven months later (February 2022) re-infection with SARS-CoV-2 was registered, again with a mild course. The patient had a fever of 38° C degrees in the first two days after the onset of the infection. Three weeks later, laboratory and imaging studies were per-

Table 1. Distribution of lymphocyte populations and CD4+/CD8+ ratio of the patient

Patient	TLC %	TLC mm³	T Ly %	T Ly mm³	B Ly %	B Ly mm³	NK %	NK mm³	CD4/CD8 Ratio
2017	25	1.5	72	1080	12	180	11	165	1.4
June 2021	22	1.9	72	1368	13	247	15	285	1.2
February 2022	19	1.4	66	924	19	266	14	196	1.5

formed. Mild lymphopenia – 19% (20-40%) from the blood count and increased CRP-18 mg/ml (< 5 mg/ml) are found. The other markers of inflammation and indicators of the coagulation system are within the reference range. X-ray of the lung is without infiltrative changes.

The specific T cell immune response to SARS-CoV-2 showed 9 spots to S protein and 15 spots to N protein; the percentage of neutralizing antibodies (VNT) against Omicron variant was 87 (cut-off > 30%) – Fig. 4.

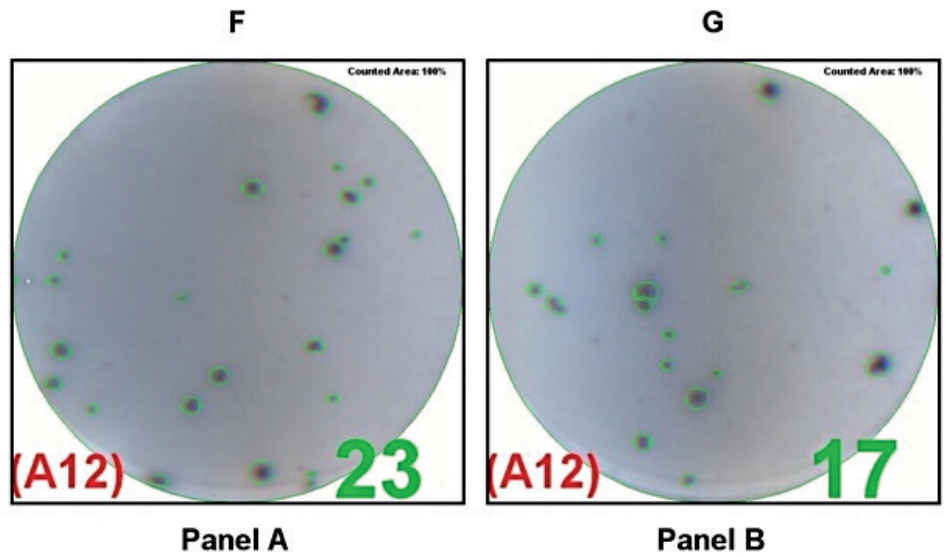

Fig. 3. ELISpot assay for a specific cellular immune response to SARS-CoV2 of the patient

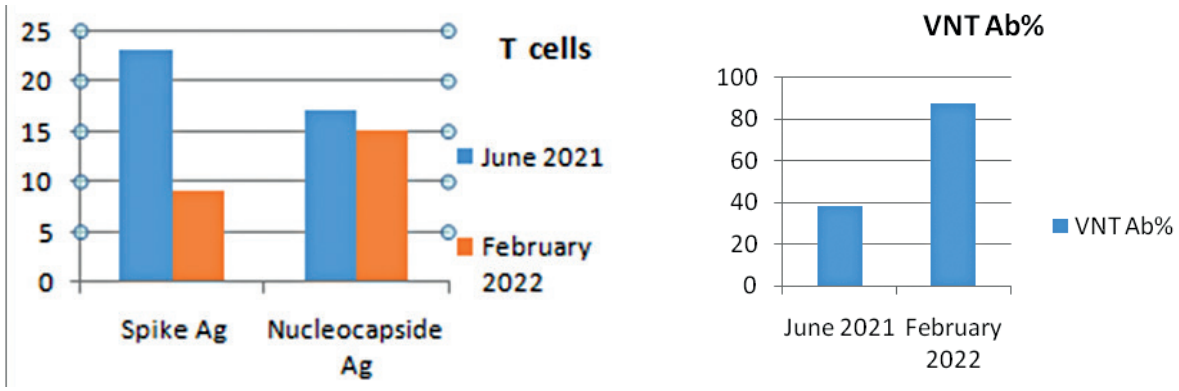

Fig. 4. Specific cellular and humoral immune response to SARS-CoV-2 of the patient after infections with delta and omicron variants

DISCUSSION

Chronic granulomatous disease is primary immunodeficiency disorder, leading to defective innate immunity. There are a few reports of patients with congenital defects in phagocytic function and COVID-19 infection in the literature. A role of the innate immune system in determining disease severity and outcome is supported by series of studies [3, 4]. An impaired immune or inflammatory response may be a predisposing factor for some PID patiens and a protective factor for others [5].

The patients with PID are among the risk groups for severe infection because the suppression of innate immune mechanisms in infected epithelial cells and, to some extent, infected monocytes/macrophages allow novel coronaviruses to proliferate without triggering the innate anti-viral response machinery of these cells [6]. SARS-CoV2 virus evade immune system recognition through suppression of these mechanisms, a phenomenon associated with more severe disease and poorer prognosis [7].

On the other hand, the neutrophils employ several anti-pathogen defense mechanisms, including

NADPH-mediated reactive oxygen species production and neutrophil extracellular trap formation, which have been implicated in lung tissue injury and hyperinflammation seen in severe COVID-19 [8]. Based on this, one might speculate that impaired neutrophil activity in CGD might protect against the exaggerated inflammatory response and consequent tissue damage observed in severe COVID-19, consistent with the mild disease course observed in our patient.

The patient presented by us is on regular therapy with interferon gamma and has been without serious infections since 2011. This is in line with the proven role of immunomodulatory therapy with interferon gamma 1b in patients with CGD. The incidence of severe infections is 0.30 (patient-years and mortality is 1.5%) patient-years [9,10].

Several authors investigated the role of different cytokines in COVID-19 patients. The interplay between the innate and adaptive immune response seems to be crucial in determining the patient's evolution, characterized by an imbalance of pro- and anti-inflammatory cytokines and the subsequent dysregulation of patient's immune response [11]. Interferons (IFNs) act as a key link between the innate and the adaptive immune response. Type I IFNs (IFN-α/β) are secreted by plasmacytoid dendritic cells (pDCs), while type II IFNs (IFN-γ) are predominantly produced by natural killer cells and in minor proportion by T cells and macrophages [12]. Both type I and type II IFNs have a plethora of antiviral effects such as inducing apoptosis of infected cells and activating macrophages, natural killer (NK) cells and T lymphocytes. In COVID-19 patients, several studies have shown a dysregulation of IFNs production [13]. Recent evidence has demonstrated that both type I and type II IFNs have immunoregulatory functions during infection and type II immune responses. IFN-γ, like type I IFN, promotes antiviral immunity through its regulatory effects on the innate immune response and acts as a key link between the innate immune response and activation of the adaptive immune response [14]. As well, IFN-γ can induce intracellular antiviral programs, including RNA-dependent protein kinase PKR as a resultant overlap in their gene expression with type I IFNs [15]. Beyond that, however, IFN-γ itself has been demonstrated to impact the function of the surrounding innate immune cells. IFN-γ induced nitric oxide production from macrophages. Further, IFN-γ has been shown to "prime" macrophages to release reactive oxygen species, through the upregulation of cellular components required for this function [16]. IFN-γ promotes polarization of macrophages to a M1 phenotype and primes these cells to produce pro-inflammatory cytokines IL-12, TNF-α, and IL-1β, which could explain the Th1 cytokine profile in our patient.

A low IFNγ level was also confirmed as an independent factor of complications in COVID patients [17]. In this context, it can be speculated that exogenous Interferon gamma therapy with its antiviral and immunomodulatory action could to facilitate further antiviral responses in our patient. Additional it is appropriate to note the long-term antibiotic prophylaxis with TPM/SMZ in the patient, which may also affect the clinical course of the infection.

Type II IFNs have different functions, eliciting T helper 1 (Th1)-driven immune responses, and also enabling induced regulatory T (Treg) cells to control and regulate immune responses [18].

Our patient had an anti-SARS-CoV2 specific effector T-cell immune response, although not strong enough, probably due to the mild course of the disease, but it undoubtedly contributes to a good outcome of the disease. The obtained results support the conclusions of other research teams about a role of T cells in viral clearance and recovery from SARS-CoV-2, suggest that cell-mediated immunity may be an important aspect of the immune response to SARS-CoV-2 infection [19].

NAbs are crucial for clearance of SARS-CoV-2 virus [20]. Almost all patients develop NAbs by week 4 of infection, and severely ill patients show higher peak, faster, and stronger NAb titers than in mild cases [21]. A similar constellation with a low percentage of virus-neutralizing antibodies is observed in our patient after the first infection with COVID-19.

Three months after the primary infection with the SARS-Cov2 delta variant, the level of neutralizing antibodies was very low in our patient. Three weeks after reinfection with the Omicron variant, there is a good antibody response. How long it will last remains to be folow up. To what extent neutralizing antibodies and their persistence are important for the clinical course and affect the frequency of re-infection in recovered individuals needs further study.

CONCLUSION

This is the first case of CGD and a mild course of COVID-19 infection described in our country. We

hypothesize that regular interferon gamma treatment contributed to the mild course of COVID-19 infection. It can be speculated that impaired neutrophil function protects against the so-called cytokine storm and subsequent tissue damage observed in severe COVID-19. Further studies are needed to prove it.

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CASE REPORT

ERYTHEMA NODOSUM IN SLE

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Abstract. Erythema nodosum (EN) is a delayed hypersensitivity reaction manifesting with painful erythematous livid nodules or plaques on the skin, usually on the shanks and above the knees or other joints. EN frequently develops at the background of or after an infection (leprosy, tuberculosis, streptococcal or viral throat infections, etc.), the intake of certain medications, pregnancy, neoplastic or inflammatory diseases and conditions (sarcoidosis, inflammatory bowel disease, etc.). EN can appear without other underlying or accompanying diseases (idiopathic EN) and subside spontaneously without treatment or necessitate immunosuppression. The histological examination reveals septal panniculitis. The authors present a female patient with EN developing at the background of systemic lupus erythematosus (SLE).

Key words: erythema nodosum, systemic lupus erythematosus, diagnosis, treatment

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INTRODUCTION

Erythema nodosum (EN) is a delayed hypersensitivity reaction manifesting with painful erythematous livid nodules or plaques on the skin, usually on the shanks and above the knees or other joints [1]. EN frequently develops at the background of or after an infection (leprosy, tuberculosis, streptococcal or viral throat infections, etc.), the intake of certain medications, pregnancy, neoplastic or inflammatory diseases and conditions (sarcoidosis, inflammatory bowel disease, etc.). EN can appear without other underlying or accompanying diseases (idiopathic EN) and subside spontaneously without treatment or necessitate immunosuppression. The histological examination reveals septal panniculitis.

EN develops both in females and in males (female : male ratio of 3-5 : 1) of all age groups, more frequently in the young, and in all racial and ethnic groups.

The majority of EN cases develop after or at the background of: infection, intake of medications, (auto)immune diseases, neoplasms, pregnancy, and approximately one third of the cases are idiopathic [1-4].

The pathogenesis of EN is unclear. It is considered a delayed type hypersensitivity reaction against

different antigens (e.g., bacterial, fungal, viral, autoantigens).

The histopathological findings in EN correspond with septal panniculitis – septal inflammation of the hypoderma, without preceding hypodermitis.

EN manifests with painful, non-ulcerative, erythematous, immobile noduli and/or plaques on the anterior shanks. The disease shows recurrent-remittent course with exacerbations during spring an autumn months. The diagnosis is based on the patient's history and the peculiar clinical findings – painful, non-ulcerative, erythematous or livid nodules and plaques on the shanks. No typical laboratory findings have been described so far. The diagnosis is supported by the typical histological picture.

EN is usually self-limiting and the skin lesions subside within 8 weeks. General measures are recommended – bed rest, lifting the affected extremity, compression, potassium jodide as a suppressor of granulomatous inflammation, treatment of the underlying disease or condition. In pregnancy, EN is treated with general measures (bed rest, elevation of the affected extremity, compression) and cautious administration of glucocorticosteroids.

The prognosis of EN is favorable, and recurrences can be observed.

CLINICAL CASE PRESENTATION

A 54-years-old female patient with history of systemic lupus erythematosus (SLE) with skin, renal and articular involvement and positive antinuclear antibodies (ANA) presented in 2020 with painful erythema-livid lesions on the shanks (Figure 1).

The patient had 3-years history of SLE with articular, skin and renal involvement, including biopsy-proven lupus nephritis (class IV lupus nephritis with 4+ mesangial deposition of IgG, IgA and C3, 1+ mesangial deposition of C1q and C4 complement fractions). After treatment with corticosteroids plus intravenous cyclophosphamide pulses (cumulative dose 7000 mg) and low-molecular weight hepatin the patient reached complete clinical and laboratory remission with persistence of low titer ANA (1:160 to 1:320). At the background of corticosteroid treatment steroid-induced diabetes manifested, with good glycemic control at the background of diet, glimepiride, empagliflozin and metformin. In August 2020 the patient noted painful red-purple nodules on the shanks that tended to merge and form painful plaques. She underwent Doppler ultrasound investigation of the lower extremities, showing normal blood flow in both arterial and venous vessels.

The clinical-laboratory investigations revealed normal erythrocyte sedimentation rate of 4 mm/I h., normal biochemical investigations (calcium, phosphate, transaminases, blood glucose profile, ionogram, and uric acid); serum creatinine of 77 mcmol/l, estimated glomerular filtration rate (Cockcroft-Gault formula) of 119 ml/min., low-grade proteinuria of 0.22 g/l, low positive ANA 1:160, negative dsDNA and ASO, negative HBs antigen and anti-HCV antibodies.

The abdominal ultrasound revealed mild liver steatosis and normal-sized kidneys with normal parenchymal size and mild increase of parenchymal echogenicity with visible renal pyramids (Figure 2). The chest X-ray revealed no hilar lymphadenopathy and no changes in lung parenchyma (Figure 3). No focal infections (ORL, dental, gynecological, urinary tract) were detected.

Fig. 1. Merging nodules and plaques on both shanks (arrows) – the typical changes in erythema nodosum

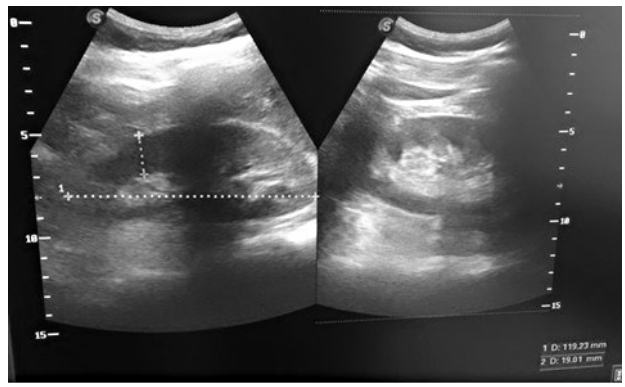

Fig. 2. Abdominal ultrasound revealing mild increase in renal parenchymal echogenicity with visible renal pyramids

Fig. 3. Chest X-ray investigation revealing no pathological findings

The diagnosis of EN was made and the patient consulted rheumatologist and dermatologist who confirmed the diagnosis. Treatment with oral corticosteroids 2+1 tablets, oral hydroxyl chloroquine 1 + 1 tablets a day and an intravenous cyclophosphamide pulse (to cumulative dose of 7 500 mg) was started and marked improvement in skin lesions was noted.

DISCUSSION AND CONCLUSION

Erythema nodosum is one of the most common forms of septal panniculitis. It develops in both males and females, with a female : male ratio of 3-5 : 1, in all racial and ethnic groups, more frequently in young age. It is most prevalent in females in the second, third and fourth decade of life and shows marked increase in incidence in spring and autumn with recurrent-remittent clinical course.

EN is usually associated with other immune-mediated diseases or conditions, such as systemic infection, autoimmune disease, etc. [1-4]:

– infections (28-48% of the cases): leprosy, tuberculosis, intestinal and throat infections, mycoplasma, Chlamydia, lymphogranuloma venereum, deep skin mycoses – blastomycosis, histoplasmosis, coccidioidomycosis; viral infections (hepatitis E, EBV), etc.

– intake of medications (3-10%): salicylates, non-steroid anti-inflammatory drugs (NSAIDs), sulphonamides, iodide drugs, bromides, oral contraceptives, etc.

– autoimmune and immune-mediated diseases (up to 30% of the cases): inflammatory bowel disease (1-4%), sarcoidosis (11-25%), Behcet's disease, SLE.

– neoplasms (paraneoplastic syndrome) – lymphoma, leukosis, solid neoplasms.

– pregnancy (2-5%).

– approximately one third of the EN cases are idiopathic.

The pathogenesis of EN is unclear. It is considered a delayed type hypersensitivity reaction against different antigens (e.g., bacterial, fungal, viral, autoantigens). The presumed factor, triggering this reaction, is the deposition of immune complexes in the hypodermal septal venules with subsequent activation of non-specific inflammatory process and generation of reactive oxygen species and inflammatory mediators (e.g., tumor-necrosis factor alpha, TNFa) and granuloma formation [1, 5-6].

The typical localization on the shanks is probably associated with the low venous and lymphatic blood flow in the lower extremities, the lack of muscle "pump" on the anterior surface of the shanks and the colonization of the skin on the lower legs with different microorganisms. The seasonal exacerbations in spring and of EN (in spring and autumn months) are probably due to the increased prevalence of streptococcal infections in spring and autumn. A link with the HLA system-II allels (HLA genes, including the TNFα gene) [6] has been suggested [7].

The estrogen/gestagen imbalance and/or the estrogens probably have a role for the development of EN. The estrogens are known to affect the T-cell and macrophage activity and the secretion of pro-inflammatory cytokines (e.g., interleukins 10 and 6 – IL-10 and IL-6), and the antiestrogens increase the levels of interleukin 2 (IL-2) and interferone-gamma (IFNg) and decrease IL-10, IL-1 and TNFa [1, 8].

The histopathological findings in EN are comparable with septal panniculitis. The early lesions are characterized by edema and lymphocytic and neutrophilic infiltration with the formation of radial granulomas (Miescher's granulomas) with radial distribution of histiocytes around the septal venules [1, 8], frequently accompanied by small perivascular hemorrhages. In patients with underlying Behcet's disease thrombophlebitic changes can be observed [9, 10], with or without eosinophilic infiltration [11]. With the evolution of the disease, the radial Miescher's granulomas evolve and epitheloid and/or multinucleated giant cells appear [12], the septae show marked thickening and

fibrosis develops, with or without propagation of the inflammatory infiltrate towards the adjacent fat tissue lobuli.

The clinical manifestations are presented by painful, non-ulcerative, erythematous, immobile noduli and/or plaques on the anterior shanks. The noduli are above the skin level 20-50 mm in size, and could merge into plaques. Such noduli have been also described above the ankles, on the thighs, shoulders, in the gluteal area, on the posterior shanks and on the face [1, 13, 14]. The appearance of the exanthema is usually preceded by prodromal signs: fever, chills, fatigue, malaise, arthralgiae, upper respiratory tract infections, dyspepsia, etc. Arthralgia and/or arthritis of the adjacent joints may develop. The skin changes usually subside within 8 weeks and residual hemorrhages and/or post-hemorrhagic hyperpigmentations can be observed (erythema contusiforme). Chronic clinical course is rare.

The diagnosis of EN is based on the patient's history and the typical clinical findings – painful, non-ulcerative, erythematous or erythema-livid nodules and plaques on the shanks. The diagnosis is supported by the histological picture, especially in the atypical clinical forms with hemorrhages or ulcerations and when the eruption is outside the shank region.

The laboratory changes depend on the underlying condition – usually representing acute-phase response with high ESR, CRP and leukocyte count, but typical laboratory findings have been described so far. In the presence of underlying disease or condition (sarcoidosis, autoimmune diseases, infections, neoplasms), the laboratory findings correspond with this condition. Of special interest is the investigation for tuberculosis and sarcoidosis, using imaging and laboratory studies, and for underlying neoplasm, or infection.

Differential diagnosis should be made with [1, 2]:

- nodular vasculitis (Bazin's erythema induratum),
- skin infections (infectious panniculitis, cellulitis),
- panniculitis in pancreatitis patients, especially in alpha-1-antitrypsin deficiency,
- panarteritis nodosa and panniculitis in autoimmune diseases,
- scleroderma,
- necrobiosis lipoidica,
- neoplastic infiltration of the hypoderma,
- lipodystrophy,
- sclerosing panniculitis, etc.

As mentioned above, EN is usually self-limiting and the skin lesions subside within about 8 weeks. General measures are of crucial importance – bed rest, lifting the affected extremity, compression, as is the proper treatment of the probable underlying condition (infection, immune-mediated disease, neoplasm), the cessation of the provoking medication(s), pain-killers, potassium jodide as a suppressor of granulomatous inflammation, antiallergic agents, glucocorticosteroids (systemic, local and intralesional), colchicine, dapsone, antimalarial agents, anti-TNFa agents (especially in patients with inflammatory bowel disease). All immunosuppressive and immunomodulating agents should be administered after underlying infection is ruled out, especially tuberculosis. In patients with Behcet's disease, immunosuppressive agents, such as cyclosporin, methotrexate and thalidomide have been used. In pregnancy, EN is treated with general measures (bed rest, elevation of the affected extremity, compression) and cautious administration of glucocorticosteroids.

The prognosis of EN quo ad vitam is favourable, as in the majority of cases spontaneous resolution is observed or the lesions respond to general measures and glucocorticosteroids. Recurrences can be observed, especially when underlying disease is present (infection, immune-mediated diseases or neoplasms).

Our patient developed EN three years after the presentation of SLE. EN had beneficial clinical evolution with treatment. Such cases of EN at the background of SLE have been described in the literature [15], showing response to the pathogenic treatment of SLE.

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SCIENTIFIC REVIEW

ANTIPHOSPHOLIPID ANTIBODIES IN PATIENTS WITH COVID-19

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Abstract. COVID-19 is a new disease caused by a SARS-CoV-2 group virus. In a short time, the disease has spread around the world and become pandemic and the medical community established a number of patterns in its development. In addition to severe lung damage, as well as damage to a number of organs and systems, it was found that a large percentage of patients have thrombotic incidents, often fatal. The question of the involvement of antiphospholipid antibodies (aPL) and antiphospholipid syndrome (APS) in these cases was raised. At present, the data on this issue are contradictory. This review presents the current knowledge on the relationship between aPL with thrombosis and the severity of COVID-19.

Key words: SARS-CoV-2 virus, COVID-19, antiphospholipid syndrome

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INTRODUCTION

At the end of 2019, the world learned about a new virus SARS-CoV-2, which causes a disease in humans, later called COVID-19. The epidemic, which started in the Chinese city of Wuhan, grew rapidly and took on the character of a pandemic. Along with the initial publications describing the picture of a respiratory disease of varying severity and with manifestations of the upper and lower respiratory tract, there were data on loss of taste and smell, and later on various other complications, incl. arterial and venous thrombosis, which in many cases are severe and can be fatal [1, 2]. Discussions on the causes of thrombosis have also begun, with some publications showing evidence of elevated antiphospholipid antibodies (aPL) in COVID-19 patients with or without thrombosis. To the group of aPL belong so call "classic" antibodies included in the laboratory criteria for diagnosis of Antiphospholipid syndrome (APS): antibodies to cardiolipin (aCL), antibodies to beta-2-glycoprotein I (aB2GPI) and lupus anticoagulant (LA). Some other antibodies – to prothrombin (aPT), to phosphathidylserine (aPS), to phosphatidylethanolamine (aPE), etc, are not included in these criteria till now. They form the group of "non criteria" antibodies, and their significance for the diagnosis of APS is discussed.

Antiphospholipid antibodies in COVID-19 – case reports

Perhaps the first report on aPL in patients with COVID-19 was made by Y. Zhang in collaboration with 35 authors [3] in April 2020 in the New England Journal of Medicine (Table 1). Peculiarities of all three patients (2 men and one woman) are: they are over 65 years old, have evidence of hypertension, 2 of them – for diabetes, 2 – for a stroke, 1 – with coronary artery disease, and one man has nasopharyngeal carcinoma. All three patients were tested for aCL and aB2GPI but not for LA and only once. The two patients of S. Hossri et al. [4] were also tested once and were positive for IgG/IgM aCL. Both have thrombotic complications – infarction of the spleen and brain in women, and occlusion of the arteries

of the left leg in men. In contrast to Y. Zhang et al., described by Ch. R. Showers et al. female patient [5] (Table 1), has several studies of aPL within 50 days, incl. and LA, according to the criteria for the diagnosis of Antiphospholipid Syndrome (APS). This woman was admitted to the clinic with advanced diabetes, hypertension and asthma and the disease was complicated by multiple thrombosis and positive IgG/IgM aCL and LA (table 1). In contrast to these patients, the patient of J. Sung and S. Anjum [6] was a healthy obese woman who developed thrombotic complications affecting the upper and lower extremities during COVID-19 and positive aCL and LA (Table 1). The 18-year-old patient of E.M. Cardoso [7] et al. (Table 1) was admitted to hospital in a severe condition: hemodynamic collapse, respiratory failure, cardiac arrest, pericardial tamponade, which progressed to acute respiratory distress syndrome (ARDS), ventricular dysfunction, pleural effusions, proteinuria and hematuria. During the hospital stay, anti-nuclear antibodies (ANA), antibodies to double stranded DNA (dsDNA), aCL and LA were positive and the patient was diagnosed with systemic lupus erythematosus (SLE). Unfortunately, aPL had not been tested after the discharge from the clinic.

N. Balanchivadze et al. [8] describe 2 men with COVID-19 (Table 1) developing pulmonary thromboembolism, in whom transient positive aPLs were detected – positive at baseline and negative after 12 weeks. R. Beyrouti et al. [9] reported 6 patients, 5 of whom developed an ischemic stroke after COVID-19, and in the sixth, the symptoms of the stroke that appeared 2 days before the infection worsened (table 1). 5/6 were positive for LA, one was positive for IgM aCL and IgG/IgM aB2GPI. In the patient of R. Escher et al. [10] despite high levels of IgM aCL/aB2GPI, no thrombotic complications were observed (Table 1). The characteristics of the male patient described by A. Maria et al. [11] were different (Table 1): this was a patient with proven and treated since 2013 primary APS (PAPS) with manifestations of venous thromboembolism, in which the condition worsened significantly during COVID-19 disease with manifestations on bilateral adrenal glands hemorrhage and limb arterial ischemia, dorsalis pedis artery occlusion. Very soon after the publication of Y. Zhang [3], the American Society of Hematology (ASH) found that only 4/27 patients with COVID-19 had LA, but none had aCL and aB2GPI, so routine aPL testing is not recommended except in patients with evidence of thrombosis or as part of a research protocol. Other authors have linked IgG aCL to the severity of COVID-19 [13]. The need for aPL studies is also emphasized, although their role in the pathogenesis of thrombosis in COVID 19 remains unclear [14, 15].

Antiphospholipid antibodies in COVID-19 – case series studies

In addition to clinical cases, studies on larger series of COVID-19 patients with thrombosis have been published (Table 2). B. Popovic et al. [16] report 11 patients with ST-elevation myocardial infarction (STEMI) and COVID-19: 4 of them (36%) had positive antibodies – 3 – aCL, 1 – aB2GPI, and all 11 patients had arterial thromboses. U. Trahtemberg et al. [17] also found no data on the development of APS in patients with COVID-19. They examined aPL in subjects with COVID-19 (COVID-19+) and in patients with a similar clinical picture without COVID-19 (COVD-19-) and found that both cases have a high frequency of aCL, and although in COVID-19+ patients these antibodies are more common (IgG aCL – 59%, IgM aCL – 32%) (Table 2), with no difference between the two groups at the admission to hospital. Antibodies against B2GPI were not detected, and those against prothrombin/phosphatidylserine (aPT/PS) were found in only 1/22 patients (5%) with COVID-19+. The long-term study (after 10 days and after discharge) showed that IgG/IgM aCL were positive in both patients. According to the authors, there is no statistically significant relationship between thrombosis and antibodies, but there is a correlation between IgG aCL, ANA and the severity of the disease.

According to M. R. Gil et al. [18], 44% of the investigated COVID-19 patients were positive for LA. This percentage was twice as high as in patients who did not test positive for SARS-CoV-2 (22%). Only 2 patients found positive IgM antibodies – 1 – aCL and 1 – aB2GPI. No statistically significant difference was found between LA positive and LA negative in terms of gender, race, ventilation capacity, mortality and therapy, but 63% of LA positives had evidence of thrombosis (Table 2), which was significantly higher than the percentage in LA negative – 34%. S. Karahan et al. [19] (Table 2), found that only LA is detected more often in critically ill patients with COVID-19 pneumonia, while the frequency of aPL and aB2GPI does not differ from that in patients with other diseases with a similar COVID-19 conditions.

Table 1. Antiphospholipid antibodies in patients with COVID 19-clinical cases ^

Author	Sex	Year	Symptoms-COVID-19	Thrombosis	Other diseases	IgG aCL	IgA aCL	IgM aCL	IgG aB2GPI	IgAaB2GPI	IgM aB2GPI	LA
Zhang Y, et al. [3]	♂	69	fever, cough, dyspnea,diarrhea, headache	multiple cerebral infarctions, ischemia in the lower limbs and in the 2 digits of the left hand	hypertension, diabetes, stroke		+		+	+		ND*
Zhang Y, et al. [3]	♀	65	fever,cough, dyspnea	multiple cerebral infarctions	hypertension, diabetes, CAD**		+		+	+		ND
Y. Zhang et al. [3]	♂	70	fever, fatigue, dyspnea, headacke	multiple cerebral infarctions	hypertension, emphysema, naso-pharyngeal Ca***, stroke		+		+	+		ND
Ch R. Showers et al. [5]	♀	63	mild asthma, progressive short-ness of breath and lethargy	multiple thrombi in the suprarenal abdominal aorta, in the splenic artery and vein, and infarcts of the spleen, stomach, and liver	diabetes with peripheral neuropa-thy and Charcot foot, hyperten-sion, asthma	+		+				+
A. Maria et al. [11]###	♂	48	fever, cough, myalgia	bilateral adrenal glands hemor-rhage and limb arterial ischemia, dorsalis pedis artery occlusion	DVT# (PAPS)##	+		+	+		+	+
J. Sung, S. Anjum [6]	♀	49	fever, cough, myalgia, tachypnea, tachycardia, oxygen saturation on room air – 87%	thrombosis in the brachial and cephalic veins bilaterally, popliteal veins, bilateral lower femoral and right peroneal veins	obesitas	+		+				+
E. M. Cardoso [7]	♀	18	respiratory failure, cardiac arrest, pericardial tamponade, ARDS§, ventricular dysfunction, pleural ef-fusions, proteinuria and hematuria	DVT	SLE	+						+
N. Balanchivadze et al. [8]	♂	59	shortness of breath, pleuritic chest pain, fever, tachycardia, hypoxemia	PE§§	thalassemia			+	+			ND
N. Balanchivadze et al. [8]	♂	41	fever, tachycardia, hypoxemia	PE	healthy before admission to the hospital	+		+				ND
R. Beyrouti et al. [9]	♂	64	fever, cough, shortness of breath, myalgia, loss of appetite	Stroke, DVT, PE	healthy before admission to the hospital			+	+		+	+
R. Beyrouti et al. [9]	♀	53	fever, malaise, cough, shortness of breath,	stroke	hypertension, diabetes, mitral valve replacement, permanent pacemaker							+

Continuation of Table 1

R. Beyrouti et al. [9]	♂∨	85	cough	stroke	hypertension, hypercholesterolaemia, atrial fibrillation, ischaemic heart disease, prostate Ca							
R. Beyrouti et al. [9]	♂	61	fever, cough, shortness of breath, tachypnoea	symptoms of stroke before hospitalisation	hypertension, stroke, chronic leg ulcers							+
R. Beyrouti et al. [9]	♂	83	fever, cough, shortness of breath, fatigue	stroke	hypertension, diabetes, ischaemic heart disease							+
R. Beyrouti et al. [9]	♂	73	shortness of breath, tachypnoea	stroke	gastric Ca							+
S. Hossri et al. [4]	♀	29	fever, vomiting, abdominal pain, cough, myalgia	splenic infarct, cerebral infarcts	sickle cell trait	+		+				
S. Hossri et al. [4]	♂	58	cough, shortness of breath	occlusions in peroneal and tibial artery of the left leg	dislipidemia	+		+				
R. Escher et al. [10]	♂	72	fever, respiratory symptoms	no	healthy			+			+	

^All patients are positive for SARS-CoV-2 on RT-PCR test; ND* – not done, CAD**– coronary artery disease, Ca*** – carcinoma; DVT# – deep venous thromboembolism, PAPS## – primary antiphospholipid syndrome, ### Data for aPL are before the onset of COVID-19, ARDS§ – acute respiratory distress syndrome, PE§§ – pulmonary embolism, ∨ LA, aCL, aB2GPI – negative

Table 2 Antiphospholipid antibodies in patients with COVID 19-case series studies

Author	N	Antibody	Thrombosis
B. Popovic et al. [16]	11	36% – aCL, aB2GPI	11 – arterial thrombosis
M.R. Gil et al. [18]	68	44% LA	63% – arterial or venous
C.I.S. Santos [20] – with thrombosis	18	10/18 (56%) – positive for at least one test – LA, aCL, B2GPI	7 – PE, 1 – aortic thrombosis, 1 – brachial vein thrombosis, 1 – stroke, 1 – heart attack, 7 – DVT
C.I.S. Santos [20] – without thrombosis	16	1/16 – LA and aCL (+), 1/16 – LA (+) - 19%	0
S. Karahan et al. [19]	31	LA – 23%; IgG aCL – 0%; IgM aCL – 6.45%; IgA aB2GPI – 6.45%	0
S. Nachate et al. [21]	9	2/9-1 – IgG aCL and IgG aB2GPI; 1 – IgG aB2GPI	0
M. Serrano et al. [25]	474	IgG/IgM – aCL/aB2GPI – 5.8%; aPS/PT– 4.6%; IgA-aβ2GPI – 15%; anyaPL – 21%.	7.4 %
U. Trahtemberg et al. [17]	22	IgG aCL – 59%, IgM aCL – 32%; aPS/PT – 5%	0

C.I.S. Santos [20] compared the presence of aPL data in two groups of COVID-19 patients: patients with thrombosis and those without thrombosis. Only the higher frequency of IgG aB2GPI in patients with thrombosis was statistically significant. It is important to note that this is one of the few studies where aPL was studied 3 months after discharge in all patients except 3 of them (2 died of thrombosis due to COVID-19 and the third dropped out of the study). It was found that 6/13 patients who were positive for LA during hospital stay, 3 months later were negative, and 1/10 with positive aPL in the first study and with evidence of thrombosis became "triple positive" – positive for aCL, aB2GPI and LA, and another – "double positive" – positive for LA and aCL. In patients without thrombosis in the second study – 1 was positive for LA and aCL, and another – only for LA.

S. Nachate et al. [21] (Table 2), also followed aCL and aB2GPI levels after 15 days and found that on admission to the hospital, only 2/9 of patients with COVID-19 had antibodies that disappeared on day 15 in one patient and aB2GPI in the other greatly decreased. In both cases there is no thrombosis and the course of the disease is not severe. On day 15, two other patients became positive for antibodies – the first for IgG aB2GPI and the second – for aCL. In both cases, the disease is severe and fatal, but without evidence of thrombosis. V. Sigureta et al. [22] – (Table 2), also followed the aPL after a different length of time (minimum 9 days) and found that LA in most cases tended to decrease. The authors did not find a difference in the frequency of aPL between patients with and without thrombosis.

In March 2021, D. Castillo-Martínez et al. published a review of 18 articles by 18 groups from Europe, Mexico, the United States and China on aPL in patients with COVID-19 [12]. Of overall 1233 patients, 60% were men, 54% had positive aPL – most frequently LA (52.3%), followed by IgM aCL-10.4%, IgG aCL – 10%, IgG aB2GPI – 7.8%, IgM aB2GPI – 3.3%.

The meta-analysis of M. Taha and L. Samavati [23], on 1159 patients from 21 studies (many of them being included in the analysis of D. Castillo-Martínez et al. [12]), published in April 2021, also confirmed the high percentage of LA – 50.7% in patients with COVID-19, emphasizing that 28,8 % of critically ill patients have IgG/IgM aCL, and 12% – IgG/IgM aB2GPI. In both cases, these percentages were significantly higher than in patients with milder disease. In a third study [24], the authors analyzed data from 23 publications involving 250 patients and reported that LA was most commonly detected in COVID-19 64%, aB2GPI in 13% of cases, and aCL in 9%, mainly of IgM %, with the IgM isotype. It is noted that no confirmatory tests were performed.

In some publications focus on the so-called "non criteria aPL" M. Serrano [25] did not find significant differences in the prevalence of IgG/IgM aCL, IgG/IgM aB2GPI and aPS/PT (antibodies to phosphatidylserine/prothrombin) among patients with COVID-19 and healthy blood donors, while IgA aB2GPI were more common in patients. Overall 35/474 patients (7.4%) in this study had various thrombotic complications, in which case "classic aPL" was more common than antibodies in patients without thrombosis.

DISCUSSION

For the relatively short period (18 months) during which COVID-19 was studied, thromboembolic complications were found to be a serious problem in the course of the disease. According to some authors, 1/3 of patients are at risk of pulmonary embolism (PE), deep venous thrombosis (DVT), ischemic stroke, myocardial infarction, as well as the development of disseminated intravasal coabulation (DIC syndrome) and ARDS [26]. One of the issues discussed in this regard is the involvement of the aPL and the possible development of APS in these cases. APS is an acquired thrombophilia characterized by manifestations of thrombosis of arterial, venous, and small vessels associated with antibodies to phospholipids or phospholipid-associated proteins. These antibodies activate vascular endothelial cells, platelets and neutrophils and create the conditions for thrombus formation. The data accumulated so far do not provide a definite answer to the question of whether aPL found in patients with COVID19 is associated with common thrombotic complications or is epiphenomenon for the following reasons:

1. The data from the research are contradictory:

– According to some authors, aPL, and especially LA, are found in a high proportion of COVID-19 patients [3, 4, 5, 6, 8, 9, 15,16, 18, 20, 25] and are associated with thrombosis. It is important to note that the risk of thrombosis in APS increases with the so-called double or triple positivity aPL (positive aCL, aB2GPI, LA, aPS / PT) [27, 28]. In COVID-19 patients, this is rare [29, 30]. In addition, according to K.M.J. Devreese et al [29], in some patients with

COVID-19, despite high aPL levels, no thrombotic complications were observed. The data of M. Gatto et al. [31] of 122 patients showed that the incidence of IgG/IgM aCL, IgG/IgM aB2GPI and LA in patients with COVID-19 was significantly lower than in patients with PAPS, but the incidence of IgG aCL and IgM aB2GPI was comparable to group of patients with autoimmune diseases (SLE, Sjogren syndrome, systemic sclerosis, rheumatoid arthritis, idiopathic inflammatory myopathies). 39% of COVID-19 patients have venous or arterial thrombosis, but this is not associated with positive aPL in a single study and these antibodies are not an appropriate screening test in the acute phase of the disease.

– According to other authors aPL are rather related to the severe course of the inflammatory process [13, 17, 19, 21, 22, 23], but according to F. Galeano-Valle et al. [32] only a small percentage of patients with COVI-19 pneumonia and thromboembolism have IgM aCL and IgM aB2GPI in low titers.

– Some authors emphasize that the determination of LA in patients with COVID-19 is inappropriate, especially in cases where they are determined by aPTT [17] due to the interference of the result with high fibrinogen, levels of factor VIII, von Willebrand factor (VWF), increased C reactive protein, binding of aPL to C4 complement and Factor H, as well as administration of anticoagulants [24, 29, 33, 34]. It is therefore advisable to examine "non criteria" aPS/PT [17] instead of LA, which have not yet been included in the consensus criteria for APS [35]. According to K.M.J. Devreese et al. [29] however, the parallel between aPS/PT and LA observed in APS was not present in patients with COVID-19.

– Of particular interest are the patients with a history of PAPS [11], as well as those with SLE and other autoimmune diseases [7, 30, 31].

– In some publications aPL have been studied once and mostly at the onset of the disease [3, 4, 6, 7, 12, 24] and have not been followed up in dynamics as recommended by Sidney recommendations [35]. Re-examination after 3 months is particularly important, as it differentiates transient aPLs during infection, after some medications, or for some other reason [35, 36].

– Serial investigations have been performed in a few studies – within 50 days [5], 10 days after the initial examination and after dehospitalization [17], 3 months after dehospitalization [8, 20], after 15 days [21], after 9 days [22], on admission, on day 10, after discharge [18]. The dynamics of antibodies in the acute phase and 30 days later is interesting [29]: in the acute phase LA is most often positive, and the concentration of aCL and aB2GPI is increased in a small proportion of patients. Positive LA levels do not correlate with aPS/PT as observed with APS. In addition, the correlation with thrombosis is unclear. One month later, the antibodies were found to be negative in most cases. A similar transient nature of aPL has also been reported by C.I.S. Santos [20], S. Nachate et al. [21] and V. Sigureta et al. [22].

– Part of the publications did not mention the cut off value for positive result [3, 7, 9, 16, 18, 20]. In addition, in most cases, the positive result is determined by the manufacturer's cut-off data. In cases where only the values recommended in the consensus are considered positive [35], the frequency of aPL decreases [37]. aPL in COVID-19 patients may be low [15, 32, 38], in medium/high titer or in high titer [37].

2. It is well-known that aPL can be elevated in various infections. They are thought to be formed by the mechanism of "molecular mimicry" and "neoipitope formation". aPL have been described in hepatitis C [39], hepatitis B [40, 41], HIV [42], cytomegalovirus [43], syphilis [42, 44], streptococcal infections [41, 45, 46, 47] and other infectious diseases. Induced by aPL infection are usually transient and non-pathogenic, but in some cases may be an important trigger for the development of APS, incl. its most severe and often fatal form – catastrophic APS (CAPS). In patients with COVID-19, an association between high levels of D-dimers, thrombosis and LA has been demonstrated at the onset of the disease [1].

3. Usually, aPL inAPS are in high titers and are directed against domain I on B2GP I, whereas in COVID-19 aB2GPI are in low titers and are directed against domain 4 and 5 [38].

4. There are many similarities and differences between APS and COVID-19 [15, 27, 48]. H. C. Suri and D. J. Bening [48] considered thrombosis in COVID-19 to be a form of CAPS, whereas according to G. Previtali et al. [15] although some patients with COVID-19 meet the clinical criteria for CAPS, a few have positive aPL. Perhaps one of the major differences between CAPS and thrombosis in COVID-19 is the normal level of fibrinogen in CAPS, as opposed to those in COVID-19 associated with thrombosis [49]. Common to both diseases are: multiorgan involvement (nervous system, cardiovascular system, kidneys, gastrointestinal tract, microthrombosis), complement activation, mild thrombocytope-

nia, high LDH, prolonged coagulation time, elevated D dimer, no bleeding, appearance after a proven infection. In CAPS, high aPLs are mandatory, in COVID-19 thrombosis is associated with aPL, but it is not clear in a large number of patients. It is not clear whether these antibodies are associated with thrombosis or are merely an epiphenomenon.

CONCLUSION

It is clear that in patients with COVID-19 the hemostatic balance is severely disturbed and there are many factors leading to prothrombotic state. It is too early to draw a definitive conclusion as to whether high aPL levels cause an increased risk of thrombosis in a viral disease such as COVID-19. Inflammation is associated with thrombosis and the presence of inflammatory mediators – CRP, cytokines, fibrinogen, etc. in COVID-19 is in favor of this. At the same time, in most studies, the conclusions on this issue are hypothetical rather than definitive. This is largely due to the use of different test methods, especially for LA, the lack of uniform criteria for determining the cut off of aPL, as well as the different phases of COVID-19 in which the studies have been performed and the lack of re-testing after 12 weeks. Therefore, the data on aPL and APS in patients with COVID-19 must be interpreted very carefully. When evaluating the COVID-19-thrombosis-aPL relationship, it is good to clarify the pre-analytical factors in advance: standardization of test methods, significance of different aPL, cut-off values, influence of therapy on results, etc.

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SCIENTIFIC REVIEW

VACCINE-INDUCED HUMORAL IMMUNITY AGAINST SARS-COV-2

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Abstract. In late 2019 in province Wuhan, China, a new species from the family of corona viruses named SARS-CoV-2 was identified. Due to its high transmission rate, infectivity, and ability to mutate, it has spread quickly and caused the current pandemic which has registered cases in more than 225 countries and by January 2022 had been accounted for the death of more than five million people. The crisis necessitated the implementation of an efficient strategy, whose core issue was to develop, manufacture and distribute effective vaccines, and inoculate a significant percent of the population as fast as possible. Due to certain technologies, which had had only limited application until then (mainly in laboratory and clinical trials), vaccines were created. Information collected for the vaccines against SARS-CoV-2 centred on their safety, efficiency and the durability of the immune response they induce. This review includes some currently conducted studies on the mechanism of action of the approved vaccines, the amplitude and durability of vaccine-induced humoral immunity, factors that influence it and their effectiveness for the variants of concern. This information is necessary for tracking and forecasting the course of the pandemic and estimate both the population risk and the individual risk.

Key words: COVID-19, SARS-CoV-2, vaccine, humoral immunity, antibodies

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INTRODUCTION

Nine species from the family of coronaviruses are known to be pathogenic to humans. Seven of them have been identified since the beginning of XX century and for years have been suspected as a probable cause of pandemic. Most of them are pathogenically weak and lead to infections of the upper respiratory tract. However, two of them were found to cause infections in the lower parts of respiratory system and other parts of human body – SARS-CoV that appeared in 2002 in Guangdong, China and which had a mortality rate of about 9,6% and MERS-CoV identified in 2012 in Saudi Arabia with mortality of 36% [1, 2]. Scientists developed more than 500 agents for immunoprophylaxis for SARS-CoV and 50 for MERS-CoV but none of them were approved for use [3]. After the outbreak of the SARS-CoV-2 pandemic in 2020, many researchers have focused on the job to produce a vaccine against the COVID-19 disease quickly. Several different technologies have been studied in the process: one using a whole attenuated virus, second by preparing protein derivates with immunogenic activity, third using enzymic derivates, fourth based on mRNA, fifth using encoding antigens of the virus or DNA recombination of other viruses to be used as viral vectors [4]. By the beginning of 2022 in Republic

of Bulgaria had been used four – two using mRNA that codes parts of the Spike-protein: the Pfizer/BioNTech vaccine (BNT162b2) and that of Moderna (mRNA1273) and two using viral vectors – the Oxford/AstraZeneka substance (ChAdOx1) and the one produced by pharmaceutical company Janssen of Johnson & Johnson (Ad26.COV2. S). Both technologies have been extensively researched but never have they had such a wide application.

mRNA VACCINES AGAINST COVID-19

After the discovery of ribonucleic acid (RNA) in 1960 many scientists have started to examine its ability to be used for treatment and prophylaxis of different diseases. In 1990 the first major research projects into using the packed in lipids RNA as a tool for developing vaccines against influenza or cancer started. However, it was only in 2012 when Guell managed to induce the immune response in mice against RSV. After him this technology has been used develop vaccines against HIV and ZIKA viruses [5, 6, 7].

There are two main factors that make it difficult to use messenger RNA (mRNA). Firstly, mRNA is an anionic molecule and due to the electrostatic interactions with the negatively charged membrane of the cell, it cannot enter the cell. Also, there are many ribonucleases in the extracellular space which quickly destroy the mRNA i.e., the molecule is unstable. Packing of the RNA in lipid nanoparticles assists its entrance into the cell and protects it from the effect of the ribonucleases. When it enters the cytosol, the mRNA undergoes a translation process, and the antigen expression begins. Once read, RNA undergoes a degradation and exits the cell via exocytosis.

Ribonucleic acid can be introduced into the muscular or epidermal cells in the place of application, in the cells of the immune system located in the tissues or in lymphoid organs. Vaccines against SARS-CoV-2 are administered intramuscular and therefore the molecule enters mainly non-immune cells around the place of injection. In them, after the antigen is synthesized, it undergoes a process of degradation in the proteosome and is expressed on the surface in combination with molecules from the major histocompatibility complex (MHC) class I and presented to CD8+ T-cells. A local inflammatory process is triggered and other cells of the immune system – dendritic cells and macrophages enter the site. The antigen enters them via endocytosis, binds to class II MHC molecules and is expressed on the surface where activates CD4 + T cells. After that the antigen-presenting cells reach lymphoid tissues via lymphatic system and through CD4+ T-cells stimulate B-cells to produce antibodies. More effective mechanism for antigen presentation is the direct interaction, which happens when the antigen reaches lymph nodes passing through the periphery. In this way it connects directly with the receptor on the surface of the B-lymphocytes, enters the plasmatic cell via endocytosis and is presented to MHC class II molecules. From this point on the B-lymphocytes may either start producing antibodies with low affinity or enter the germinal centres, where they turn into cells which produce immunoglobulins with high affinity via a process called somatic hypermutation. Part of the B-lymphocytes become memory cells and when they are stimulated again by the same antigen, they trigger faster and stronger immune response.

There are many advantages of the technology using mRNA as a vaccine. First, the possibility of unwanted side effects is minimal because there is no risk of developing infection or toxicity and unlike the viral vector vaccines, mRNA does not reach the nucleus of the cell and there is no chance of insertions and mutations of the genome. The expression of proteins is controlled due to the quickly degrading of the mRNA molecule. The other important condition when developing a vaccine is its efficacy. This requirement is met as the cell expresses only the proteins necessary for triggering specific immune response and both humoral and cellular immunity are induced. Furthermore, the manufacturing of these vaccines is not complicated because it requires only in-vitro transcription and no cell cultures are needed, and the gene sequential can be changed easily. This allows for greater productivity which is very important in the course of the pandemic [8, 9, 10, 11].

VIRAL VECTOR VACCINES

Developing of recombinant viral vector vaccines began in 1970s [12] but because of the lack of success and the limited application, it is still regarded as a new technology for vaccine production. The basic concept is the introduction of a virus that is harmless to the body but contains in its genome the DNA of the antigen against which it is necessary to create an immune response. Once inside the cell, this part of the viral DNA is transcribed to form mRNA, which is translated and expressed by the same mech-

anism as the mRNA vaccines. It has been found that this stimulates both humoral immunity through the production of antibodies, including virus-neutralizing, and cellular immunity, mainly through the production of Th1 cells [13]. The requirements for the virus to be used as a vector are that it be attenuated, have a high transduction rate and gene expression, not be integrated into the genome of the cell, i.e. no genetic toxicity. Also, it is important the population immunity to that virus to be low. Following these conditions adenoviruses have been selected as one of the best options. However, to some of them there is a high percentage of individuals with already developed immunity [14]. The main task for the scientists engaged in developing this type of vaccine is to find an adenovirus to which there are low levels of immune protection.

Adenoviruses are non-enveloped viruses that consist of nucleocapsid in which contains double-stranded DNA. Genes may be classified in two groups – those responsible for the replication (E1-E4) and the other which encode structural proteins (L1-L5) [15]. To generate a vector which is replication inactive, it is necessary genes E1 (E1A and E1B) and E3 to be removed through genetic engineering and replaced by the genes encoding the target antigen. In vaccines against SARS-CoV-2, the target antigen is spike protein [14, 16]. In addition, the proteins in the nucleocapsid can also be modified to exhibit tissue tropism, cell specificity, and immunology [17].

The vaccine of AstraZeneka and Oxford uses adenovirus, isolated from chimpanzees – ChAd, which is being reproduced in cell culture from the same species. It is administrated intramuscular and although a good immune response is observed after one dose, in order to have a higher level of protection, it is recommended to administer two doses, at intervals of about 12 weeks. In the phases of clinical trial, more than 75% efficacy against the symptomatic infection was registered 14-21 days after the second dose with patients aged 18 to more than 70. Significant difference in immunity in the different age groups was not observed [18, 19].

The Janssen vaccine uses human adenovirus as a vector – Ad26, against which it is observed higher immunity in some populations. However, in one phase of a clinical trial including 44,325 people from different continents, it showed 66% efficacy against moderate COVID-19 and 85% efficiency against severe disease 28 days after vaccination [20]. In a smaller study conducted on 25 individuals, the production of antibodies against RBD of the Spike protein and the induction of a cellular immune response were found 8 days after administration of the Johnson & Johnson vaccine [21]. The vaccine is applied intramuscular, and the indicated efficiency is observed after one dose, which makes it logistically easier to use when larger groups of people are concerned.

DURABILITY OF HUMORAL IMMUNITY AFTER VACCINATION AGAINST COVID-19

There are several studies regarding the durability of humoral immunity after application of the two mRNA vaccines – Pfizer/BioNTech and Moderna. The largest is one national Israeli research, which included 4,700,000 individuals and data provided by the health ministry about the number of vaccinated people and the number of positive PCR tests. Among them, it was found that the incidence of infections in the population over 60 years of age is higher in those vaccinated in January and decreases in those vaccinated in February, March and April [22]. In a study of 42,094 participants over 12 years of age, with no history of disease included in Phase I, II, III of the BNT162b2 clinical trials, more than 90% efficacy against severe disease was observed over the 7-day to 6-monthafter the second dose [23]. Another Israeli study compared the dynamics and durability of immune response in previously infected (n = 4,361) and the recipients of two doses of Pfizer/BioNTech vaccine (n = 2,653). One month after completion of the vaccination course or after disease, the titers of virus-neutralizing antibodies were higher in the group of vaccinated individuals. However, the decline was up to 40% in a month for vaccinated and about 5% for the recovered patients, and after six months 16,1% of vaccinated were seronegative while 10,8% of people who had been ill were seronegative after nine months [24].

When tested 90 days after the second dose of mRNA1273, 100% out of 34 healthy individuals were seropositive, but in this period a decline in the antibody titers was also observed [25]. Another study found antibodies 6 months after the end of the vaccination course, although lower than in the peak period which was 2 weeks after the second dose. Virus neutralizing activity was present in 54% of them against the Beta variant (B.1.351) and 96% against the Delta pseudovirus (B.1.617.2) after 7 months

[26]. Similar observation was made in a Bulgarian study, which includes 71 health care workers vaccinated with Pfizer/BioNTech. All the participants had virus-neutralizing antibodies on day 31 post first application while on day 111, 91.8% of individuals were positive for antibodies [27]. High values of immunoglobulins were detected in serums from 33 healthy individuals 209 days after vaccination [28].

EFFICACY OF VACCINES IN IMMUNOCOMPROMISED PATIENTS

The following studies on the effectiveness of vaccines have been found in patients with rheumatic diseases. One included 123 patients who received a single dose of Pfizer/BioNTech or Moderna vaccines in approximately equal numbers and found that 18-26 days after vaccination, 74% of them were seropositive for SARS-CoV-2 RBD. Although the study included a small number of patients without sufficient data on the type and course of immunosuppressive therapy, the lack of immune response was observed more in patients receiving mycophenolate mophetil and rituximab. In patients who were on therapy with anti-TNF, there was 94% seropositivity [29]. In another research that consisted of 264 persons who received two doses of the vaccine, without changes in the main course of therapy, 86% of them were seropositive for IgG [30]. In a study which compared two groups of people – 84 patients with immune mediated inflammatory disease and a control group of 182 healthy individuals, it was noticed 100% positive response and 99,5% virus-neutralizing activity in the control group while in the other group five persons did not manage to synthesize antibodies and the virusneutralizing activity was 90,5% [31].

Data on the vaccination of individuals on hemodyalisis are encouraging and show high percentage of seroconversion which suggest their inclusion as a priority group in vaccination campaigns [32]. When examine 50 patients who had two doses of Pfizer vaccine for a period of 90 days, 97,9% had positive values of IgG after the second application [33]. Another study of 83 persons on hemodyalisis, seronegative before vaccination, showed 26,9% seroconversion after the first dose and 89,9% after the second or third one of BNT162b2. Third injection was applied on 12 individuals who had no antibodies when completed the whole course of vaccination as it is recommended [34].

In solid organ transplantation, the necessity of strong immunosupressive therapy leads less often to positive humoral immune response after vaccination. In a study of 120 renal transplant recipients, 2-4 weeks after the second dose of Pfizer/BioNTech vaccine seropositivity was found in 43,4% compared to 98,5% in the control group of healthy individuals [35]. Positive rates of immunoglobulins were established in 65% of the immunized with mRNA1273 patients who were also immunosupressed due to the renal transplantation, which is lower than the immune response in the rest of the population [36]. A similar relationwas observed in 80 liver transplant recipients of whom 47,5% had positive results for IgG and lower titers of antibodies compared to healthy persons [37]. These studies again reported the lowest levels of seroconversion with mycophenolate mofetil therapy, while the negative effects of treatment with cyclosporine and corticosteroid therapy were less significant.

Seroconversion of 29% after the first injection and 86% after the second one with BNT162b2, was found in 263 persons with malignant neoplasms. The lack of immune response is more often associated with severe leukopenia [38]. A positive immune response in most cancer patients has been observed in other studies as well. They do not indicate a relationship between the type of neoplasm, the conducted therapy, and the degree of seropositivity. Patients with haematological malignancies had lower positive rate of immunoglobulins and lower titers [39, 40].

Two main factors must be considered when vaccinating patients undergoing immunosuppression/ immunomodulation therapy. First, it is preferable for patients to be in remission of the underlying disease to eliminate the risk of its exacerbation. Secondly the therapy and vaccination must be pursuant in time, depending on the type of medicine, the condition of the patient and the necessity of treatment for the specific disease [41].

EFFECTS OF SARS-COV-2 VACCINES IN PREVIOUSLY INFECTED

There are several studies about the immune response in seropositive persons before the first injection of mRNA vaccine. Relatively similar dynamics of antibodies was established in all of them (Figure 1). After the first application on convalescent people, IgG and virus-neutralizing antibodies were detected in serums inthe eighth day and there was a positive

correlation between the values and the severity of the disease. The main symptoms which influence the immunity are gastrointestinal disorders, fever, myalgia, dysosmia/anosmia and dysgeusia/aguesia. In naïve patients, seropositivity was observed after 11-22 days. At the first dose, previously infected showed a stronger immune response than those previously not being ill. In addition, higher values before immunization correlated with higher values of antibodies afterwards.

After the second dose in non-convalescent, the increase of the immunoglobulins continued and reached a peak two weeks after application. In seropositive before vaccination, titers entered a plateau and then no further increase was found; furthermore, in two of the studies, in the same group, there were individuals in whom decreasing IgG were detected. After the 50th day, in the convalescent group the values remained in the plateau, while in the group with only vaccinated, the ystart to decrease. The titers on day 50 in only immunized were same as the ones measured in only convalescent 60 days post the onset of the infection [42, 43, 44, 45, 46].

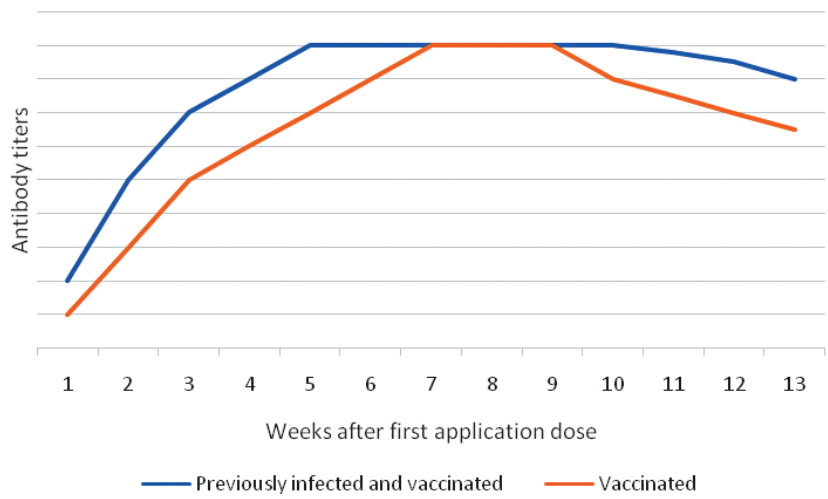

Fig. 1. Dynamics of antibody titers in previously infected and vaccinated individuals and vaccinated, seronegative before the application

An analysis of data from 215 vaccinated individuals and 130 unvaccinated but previously infected who suffered symptomatically or asymptomatically, established the following. 24 days post the first dose, higher titers were measured in those who received mRNA-1273 compared to recipients of BNT162b2, but after the second dose such a difference was not observed. After the second injection with both types of vaccines, measured titers of immunoglobulins were higher than those in convalescent people. Six (27,3%) out of the 22 individuals who received Ad26.CoV2.S did not seroconvert 24 days after the application, and in the serums of the rest of the group, the positive values the titers were lower than those measured in the serums of immunized with mRNA vaccine. However, with all three types of vaccines, stronger immune response was observed in people who were previously infected and then vaccinated (even with one dose of a vaccine), again and the increase was more significant in recipients of Pfizer/BioNTech or Moderna [47].

Both ways of inducing immune response – infection and immunization could lead to stronger and prolonged immunity, but more studies need to be conducted to reach such a conclusion. Antibodies, formed by vaccination specifically target RBD of S-protein and recognise its different parts, including more conservative core regions. This makes immunity homogeneous and more stable on mutations in RBD; therefore, more efficient against new changes in the virus. During infection the produced immunoglobulins target different proteins but those necessary for effective neutralization are less. In seropositive patients the immunity after vaccination is stronger and is preferred for the course of the pandemic [45, 48].

VARIANTS OF CONCERN

By January 2022 CDC put two variants in the category of concern – Delta (B.1.617) and Omicron (B.1.1.529). In the end of 2020 in India was detected a new genetic line – B.1.617 which includes three sublineages: B.1.617.1, B.1.617.2 (Delta) and B.617.3. Due to its high contagiousness and the ability to escape immune system, Delta pseudovirus spread quickly and by the end of 2021 was the most frequently isolated in genome sequencing in different parts of the earth. Its characteristics are a result of many mutations, eight in S-protein, two in RBD and one in the furin-cleavage site [49, 50]. Omicron variant (B.1.1.529) was reported first in South Africa on 24.11.2021. It has 32 mutations of Spike protein, 15 of which in RBD, which makes it disturbing because of its ability to escape adaptive immune response. Some of these mutations are positive and enhance the connection

between the virus and the receptor of virus-neutralizing antibodies, while other mutations are negative. One research has shown more negative than positive mutations and bigger possibility for decreased efficiency of immunity acquired after infection or vaccination [51].

SIGNIFICANCE OF HUMORAL IMMUNITY AGAINST VARIANTS OF CONCERN

The spread of the variants of concern and their ability to overcome adapted immunity in convalescent or immunized individuals is important for prediction the course of the pandemic, and for developing a strategy supported by medical professionals and governments to limit the medical, economic, and social damages they cause.

A large study has been conducted in Israel examining humoral immunity in BNT162b2 vaccinated, previously infected and convalescent, people who are immunized, against Delta variant, and differentiates three main models. The first model compares vaccinated individuals and individuals who recovered from the infection, as both groups' encounter with the antigen was at same time (there were 16,215 individuals in each group). During the follow-up period 238 infections (breakthrough infections) were detected in the group of vaccinated, of which 191 symptomatic cases, 8 hospitalization, and 19 infections in recovered people (reinfections) of which 8 symptomatic cases and 1 hospitalization. This model demonstrated a higher risk of breakthrough infections, which are more frequently symptomatic. The second model included 46,035 people in each group but the periods of vaccination and infection were different. During the follow-up, 640 infections in vaccinated and 101 in convalescent identified confirming the conclusion of the first model. The third model followed 14,025 persons with previous infection and the same number who were seropositive and vaccinated. It was noticed that infection occurs rarely with individuals who have immunity induced both ways – only 20 of them. This study identified a higher risk of breakthrough infections compared to reinfections but better immune response in recovered and immunized people [52].

When tested serums from patients who received ChAdOx1 or BNT162b2, the following percentage of efficacy was revealed. Those vaccinated with AstraZeneka/Oxford more than 15 weeks ago had no efficacy against Omicron and 41,8% against Delta pseudovirus, while those who completed the Pfizer/BioNTech vaccination course in the same period, had protection of 34-37% for Omicron and 63,2% for Delta variant. If the same vaccine was administrated 2-9 weeks ago, its effectiveness may reach 88% for both pseudoviruses. When a booster dose of Pfizer had been applied, 2 weeks later the protection was 75% in recipients of the same vaccine and 71,1% in those who had AstraZeneka previously, for B.1.1.529 and more than 90% for B.1.617.2 [53]. Similar dependencies were found in another research which measured virus-neutralizing potential in serums of patients who received either of the two mentioned vaccines as well as those with heterologous vaccination. This included a group of people with first dose of ChAdOx1 and second one of Pfizer/BioNTech; they had lower virus-neutralizing potential against Delta-variant and none for Omicron [54]. One more study was conducted comparing virus-neutralizing antibodies after booster dose against both variants of concern and the wild type in 88 recipients of mRNA 1273, 111 of BNT162b2 and 40 of Ad26.COV2. S. It concluded very low or missing immunity against the two variants in people without third dose compared to original virus. After a booster with any of the mRNA vaccines, the protection enhances significantly against all types of SARS-CoV-2 and is higher in those who were initially immunized with Pfizer or Moderna and lower in those who were injected with Ad26.COV2. S. Since almost no efficacy was established against Omicron for those with Janssen vaccine, the study demonstrated the importance of applying a mRNA booster to the population initially inoculated with this vaccine [55].

CONCLUSION

Reviewed studies proved the great significance of inoculating people who have been previously infected or who have had vector vaccine, as well as booster dose administration in recipients of mRNA vaccines. This necessitates a revision of the strategy for vaccinating a larger number of individuals in a shorter period in view of the spread of Omicron variant and the subsequent mutations in SARS-CoV-2. Inducing virus-neutralizing humoral immune response with higher potential is a key tool in dealing with the pandemic and protecting the population

from severe disease which can lead to serious complications and death.

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