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COVID-19 COVID-19 LOOKING BACK POHĽAD SPÄŤ

Bratislava, 23. - 24. 6. 2023

LEKÁRSKA FAKULTA
Univerzita Komenského
v Bratislave

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Foreword

The COVID-19 pandemic has significantly disrupted everyday life, but it has also brought increased workload and new research opportunities for professionals and scientists in various fields. Their primary objective is to protect and improve public health.

At the Faculty of Medicine, Comenius University in Bratislava, several pre-clinical and clinical departments have undertaken research projects to investigate the characteristics of SARS-CoV-2, the human body's response to the infection, the progression and treatment of COVID-19, the mechanism and dynamics of the transmission, and other related areas.

These research activities have not only produced valuable findings but also fostered collaboration among different faculty departments and cooperation with scientific institutions in Slovakia and abroad.

In light of recent progress, the Faculty of Medicine of Comenius University in Bratislava is organizing a conference titled "COVID-19: A Look Back" on 23-24 June 2023. The conference will provide an opportunity to present the results of joint efforts while also raising new questions. It will facilitate the exchange of experiences and participation in scientific meetings, contributing to future research endeavors.

Alexandra Bražinová

Pavol Janega

Predslov

Pandémia ochorenia COVID-19 znamenala v mnohých aspektoch ochromenie bežného života. Pre odborníkov a vedcov pôsobiacich vo viacerých oblastiach však znamenala najmä nárast objemu práce a otvorenie nových možností pre výskum s primárnym cieľom chrániť a zlepšovať zdravie populácie.

Viaceré predklinické a klinické pracoviská Lekárskej fakulty Univerzity Komenského v Bratislave realizovali výskumné projekty zamerané na objasnenie charakteristík vírusu SARS-CoV-2, reakcie ľudského organizmu na infekciu spôsobenú týmto patogénom, priebeh a liečbu ochorenia COVID-19, mechanizmus a dynamiku jeho šírenia a ďalších súvisiacich oblastí.

Tieto aktivity priniesli nielen cenné zistenia, podnietili aj spoluprácu medzi pracoviskami fakulty a spoluprácu s ďalšími vedeckými inštitúciami na Slovensku aj v zahraničí.

Vzhľadom na tento vývoj organizuje Lekárska fakulta Univerzity Komenského v Bratislave 23-24. júna 2023 konferenciu "COVID-19: Pohľad späť". Konferencia prináša príležitosť prezentovať výsledky spoločného úsilia ale aj nastoľuje nové otázky. Umožňuje vymeniť si skúsenosti a zúčastniť sa na vedeckých stretnutiach, ktoré pomôžu budúcemu výskumu.

Alexandra Bražinová

Pavol Janega

Scientific papers

1. The monitoring of immunological parameters in patients with COVID-19

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Introduction

The coronavirus disease 2019 (COVID-19) has been a global pandemic for more than 3 years and has affected more than 700 million people so far. While in some patients the course of this infection may be mild or even asymptomatic, for many others, especially older patients with chronic diseases, it may be life-threatening or even fatal (1,2).

Why some individuals develop severe disease when others are asymptomatically infected remains unclear, the immune protection being one explanation.

Several studies show that the immune response to the coronavirus SARS-CoV-2 is complex and includes both specific and non-specific, humoral and cellular immune responses. Significant differences in immunological parameters have been described between patients with mild and severe COVID-19 disease. The available results show that the most significant changes were mainly observed in the specific cellular immune response, in patients lymphopenia with a significant decrease in T lymphocytes was observed in all populations (CD3+, CD4+, CD8+) with a significant representation of naïve T lymphocytes (CD3+CD4+CD45RA+) with a simultaneous decrease in memory T lymphocytes, especially in patients with a severe course of the disease. A reduced incidence of cytotoxic T lymphocytes and regulatory T lymphocytes was also noted in these patients. On the other hand, it is interesting that changes in the expression of HLA-DR antigen on helper T lymphocytes were not recorded. The observed eosinopenia is also an interesting phenomenon, while it is known that eosinophils have a wide spectrum of antiviral mechanisms and effects. Publications show that eosinopenia and lymphopenia can represent an important diagnostic and prognostic factor for COVID-19. An interesting observation from non-specific cellular immunity is the massive secretion of cytokines, the so-called cytokine storm, increased values of a wide range of pro-inflammatory cytokines were observed in patients with a severe course of the disease: IL-2R, IL-6, IL-8, IL-10 and TNF-α, another interesting observation is the cytokine profile characteristic of Th17 lymphocytes.

In contrast to observed changes in cellular immunity, on the side of humoral immunity, significant and statistically significant changes were not observed in patients with COVID-19. On the side of humoral immunity the occurrence of specific antibodies against SARS-CoV-2 was mainly monitored.

Many publications show that the monitoring of selected immunological parameters could be beneficial in identifying patients at risk of a severe course of the disease. (3 – 7).

In our study, we aimed to analyse the changes in inflammatory parameters and selected immune parameters in relationship to various degrees of COVID-19 severity and clinical outcomes.

Patients and methods

All patients included in the study were RT-PCR confirmed positive for SARS-CoV-2 RNA retrieved from nose and throat swabs. Patients were divided into 3 groups according to the severity of their COVID-19 disease (according to the clinical manifestations of cough, shortness of breath, fever, pneumonia):

1. asymptomatic patients (none of the clinical manifestations, N = 37),
2. patients with mild/moderate symptoms (at least one of the clinical manifestations, but a condition not requiring hospitalization of the patient, N = 296),
3. patients with severe symptoms (at least one of the clinical, condition requiring patient hospitalization, N = 41).

The immune profile was analysed at the latest on the 5th day after finding out the positive result of the PCR test and consisted of:

- inflammatory parameters: CRP, procalcitonin,
- 5-parameters differential blood cell count,
- lymphocytes immunophenotyping by flow cytometry: (CD3+HLADR+, CD3+CD4+HLADR+, CD14+HLADR+) the basic panel (CD3+,CD3+CD4+,CD3+CD8+,CD19+,CD3-CD16+56+), T regulatory lymphocytes, memory/naive T lymphocytes (CD45RA+CD4+/CD45RO+CD4+) and markers of cell activation (CD3+HLADR+, CD3+CD4+HLADR+, CD14+HLADR+)
- cytokines: IL-2, IL-6, IL-8, IL-10, IL-17, TNF-α.

Results

As anticipated, the different clinical subsets according to COVID-19 severity (groups 1–3) were characterized by different changes in immune biomarkers and lymphocytes subsets. We found elevated concentrations (out of reference range) of inflammatory markers CRP and IL-6, but nor PCT in patients with severe symptoms (group 3) compared to the asymptomatic patients and patients with mild/moderate symptoms (group 1 and 2) (1 and 2), these differences were statistically significant (CRP - group 3 versus group 1: p = 7.32E-07, group 3 versus group 2: p = 2.00E-06, IL-6 - group 3 versus group 1: p = 0.016, group 3 versus group 2: p = 0.016). The total leucocytes (Leu), platelets (PLT) and both neutrophiles percentage (Neu %) and absolute count (Neu Abs) was significantly higher in patients with severe symptoms (group 3) compared to the asymptomatic patients and patients with mild/moderate symptoms (group 1 and 2) (Leu - group 3 versus group 1: p = 3.64E-07, group 3 versus group 2: p = 7.33E-08, PLT - group 3 versus group 1: p = 0.00784, group 3 versus group 2: p = 0.00153, Neu Abs- group 3 versus group 1: p = 8.89E-09, group 3 versus group 2: p = 2.80E-09). In the opposite, we found statistically significant decreased amount (in percentage and also absolute number) of

lymphocytes (Ly) and in percentage of eosinophiles (Eo) in patients with severe symptoms (group 3) in comparison with the asymptomatic patients and patients with mild/moderate symptoms (group 1 and 2) (Ly % - group 3 versus group 1: p = 2.86E-20, group 3 versus group 2: p = 3.21E-26, Ly Abs – group 3 versus group 1: p = 3.62E-06, group 3 versus group 2: p = 5.08E-08, Eo % - group 3 versus group 1: p = 3.74E-05, group 3 versus group 2: p = 2.53E-07). In the subpopulations of lymphocytes we found the statistically significantly increased percentage but not the absolute count of B lymphocyte in the group 3 compared to group 1 and 2, however, these values were within the reference range (Bly % - group 3 versus group 1: p = 3.81E-04, group 3 versus group 2: p = 1.05E-06). For the other lymphocyte subpopulations, we recorded a statistically significant decrease in the absolute numbers of T lymphocytes and NK cells, which is related to the decrease in the absolute number of total lymphocytes. Concerning to the activation markers, we noted the statistically significant decreased amount of HLA-DR antigent on monocytes (HLA-DRCD14+) in patients with severe symptoms (group 3) compared to the asymptomatic patients and patients with mild/moderate symptoms (group 1 and 2) (HLA-DR+CD14+ % - group 3 versus group 1: p = 6.57E-09, group 3 versus group 2: p = 3.20E-11).

Conclusions

In our study, we demonstrated statistically significant differences in several monitored parameters between groups of patients with severe symptoms compared to the asymptomatic patients and patients with mild/moderate symptoms. The biggest differences we noticed in inflammatory parameters (CRP, IL-6) and in almost all values of blood count parameters (e.g. absolute count of leucocytes, platelets, lymphocyte, neutrophiles and eosinophiles). Concerning lymphocytes subpopulations we noted the most significant differences between the mentioned groups in the percentage of B lymphocytes, although the values were in the reference range in all three groups. In the group with a severe symptoms the values were statistically significantly higher, which may be due to their activation during the production of specific anti SARS-CoV-2 antibodies. In the case of monitored activation markers (HLA-DR on lymphocytes and monocytes), we recorded a statistically significant decrease of HLA-DR on monocytes in patients with a severe symptoms, which may indicate a failure of the function of monocytes as antigen-presenting cells.

Immune system and the changes in its reactivity play an evident role in various aspects of the COVID-19 pathology, from the increased susceptibility to infection in general, to the modulation of the clinical course and determining the clinical outcome of the disease.

Acknowledgments

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2. Longitudinal analysis of antibody dynamics after vaccination against SARS-CoV-2

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) belongs to 7 coronaviruses that are pathogenic for humans and is responsible for COVID-19 pandemic. Coronaviruses are enveloped, single-stranded RNA viruses with positive RNA polarity. They have 4 main structural proteins: nucleocapsid (N), spike (S), small envelope protein (E) and membrane protein (M). Protein S has two subunits; S1 ensures attachment to the host cell and S2 fusion with the cell membrane when the virus enters the cell. The M protein forms an essential part of the viral membrane. N protein is attached to the virus genome. The E protein is the smallest protein of the virus. M, N and E proteins are involved in viral replication. (1). After the initial outbreak in Wuhan, China, SARS-CoV-2 quickly spreads worldwide, including Slovakia. During the first wave we succesfully managed to minimize the number of cases, but the following waves overwhelmed us. Although most people infected with SARS-CoV-2 may be asymptomatic or develop only mild to moderate disease, some have developed life-threatening and often fatal pneumonia (2,3). As the virus strains evolved, the rate of symptoms decreased and now the infection is considered as a common cold virus.

Vaccination against the SARS CoV2 virus is aimed at creating neutralizing antibodies against the surface S protein of the virus. Several types of mRNA or vector-based vaccines are available. After the introduction of the mRNA vaccine on the Slovak market, we began to monitor the development of the level of antibodies after vaccination. We monitored the level of antibodies against S1/S2 protein for 11 months after vaccination.

Aims

The primary aim was to determine the antibody response after vaccination. The second goal was to monitor the development of antibody levels in the time since vaccination and to determine which factors influence these antibody levels.

Methods

Our cohort consisted of subjects vaccinated with the Comirnaty mRNA vaccine from BioNTech Manufacturing GmbH. Vaccination was carried out during January / February 2021. The group consisted of 250 employees - medical workers of Medirex JSC laboratories in the age range from 21 to 69 years. 202 women and 48 men were represented. Quantitative determination

of IgG antibodies against the S1/S2 protein of SARS CoV2 was performed on a LIASON® XL device by chemiluminescence analysis. The measurement range of the diagnostic kit is from 3,8 to 400 AU/ml. The positivity level of antibodies is equal or more than 15 AU/ml. A total of 6 measurements of antibody levels were carried out. The observation period was set at 2 weeks after the first dose, and 2, 12, 24, 36 weeks after the second dose. After 36 weeks, our group was divided into two subgroups. The first subgroup was boosted with the 3rd dose of vaccine (159 people) and the second subgroup remained as a control group (91 people). In the boosted group the sixth measurement was performed 2 weeks after the third dose of vaccine and in the control group at 48 weeks after the second dose of vaccine, which corresponds to the 11th month.

We also monitored the influence of selected factors on antibody levels in our group. We compared the effect of age, gender, occurrence of associated diseases and BMI on the level of antibodies. From associated diseases, we focused on the presence of diseases of the cardiovascular system, oncological diseases, immunosuppressive diseases, respectively the use of drugs affecting the activity of the immune system and autoimmune diseases.

Due to the research methodology and data quality requirements, we excluded 29 probands from the set of further analyses. We excluded 14 persons from the beginning of the measurement to the 5th sampling, due to the overcome or suspected overcome SARS-CoV-2 infection. In another 15 persons, by comparing the interquartile range, we identified extreme values in samples 1 to 5, which could significantly distort the results of the statistical analysis.

We tested the statistical significance of the differences in the average level of antibodies in the R language using the ANOVA method for repeated measurements.

Results

Of the 250 employees tested, 221 participants had positive antibody levels two weeks after the first dose (88.4%); 7 participants had borderline or equivocal values (2.8%) and 22 participants did not develop antibodies (8.8%). After the application of the second dose of the vaccine, we noted the presence of antibodies in the entire test set, and in all study participants antibodies were detected up to the 36th week (5th collection) from the administration of the second dose of vaccine. In one participant, at week 36, antibodies dropped below the positivity threshold. In the sixth sampling, all boosted with 3rd dose of vaccine exceeded the upper limit of antibody level measurement (400 AU/ml) but two probands, who had levels approaching 400 AU/ml. In the control group, antibodies continued to decline, except for those infected with SARS-CoV-2. The average antibody level in the non-infected control group was 126.9 AU/ml.

As part of the comparison of the influence of selected factors, we noted a significant influence of gender and younger age on the development of the average value of antibodies over time. Antibody levels in women were statistically significantly higher during the entire measurement period [$F(3.02;661.71)=4.39$; $p=0.004$]. When analyzing the formation of antibodies in connection with age, we noted differences in the level of antibodies depending on the age category [$F(12.09;652.68)=1.81$; $p=0.043$]. During all samplings there was a statistically significant difference between the group under 39 years and the group over 60 years old. After taking into account age and gender, we did not confirm the effect of BMI on

antibody levels in our cohort. The influence of associated diseases was also not confirmed in our group. Only 25 probands had associated diseases within the set. Some had combinations of the observed diseases.

Conclusions

In our study we can clearly confirm 88.4% presence of antibody response after the first dose, 100% after the second dose and its persistence in the 36th week in 99.6% of participants. During all measurements, mean antibody levels were higher in women and younger age groups under 39 years. Many other studies have confirmed higher levels of antibodies in women and younger ages (4,5,6). We did not confirm the effect of BMI or associated diseases on antibody levels. Obesity is an important factor influencing the level of antibodies, but BMI is not an optimal indicator of obesity (7,8). Until now, a protective level has not been established on the basis of which a vaccine booster would be recommended. Previous recommendations were based on time since last dose and did not consider individual antibody status. The effort to unify the results of the antibody response into BAU/ml units (binding antibody units) could facilitate the definition of the minimum protective level. Even for potential other pandemics, the unification of measurement units would contribute to a better interpretation of results and the setting of vaccination schemes.

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3. The potential of circulating markers from plasma for COVID-19 diagnosis

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Introduction

During COVID-19 infection, significant changes in cell-free DNA (cfDNA) composition and quantity occur, primarily due to cell death mechanisms such as apoptosis, necrosis, NETosis, and active DNA release [1]. The level of cfDNA has the potential to predict the severity of the disease. Lymphocytes and leukocytes, undergoing apoptotic and necrotic cell death, are considered the primary sources of cfDNA in COVID-19 patients. However, the clearance of dead cell material by phagocytes may be overwhelmed in severe cases, leading to an accumulation of cfDNA [2].

NETosis, the formation of neutrophil extracellular traps (NETs), is another mechanism that plays a role in response to viral infections, including COVID-19. NETs are networks of decondensed neutrophil DNA, histones, and antimicrobial proteins that are released to capture and eliminate pathogens. While NETs have a beneficial role in neutralizing microorganisms, their persistence and inefficient clearance in SARS-CoV-2 infection can contribute to inflammation and tissue damage, potentially leading to increased levels of cfDNA [3].

In severe cases of COVID-19, where multiple organs are affected, excessive activation of inflammasomes and the release of pro-inflammatory cytokines can occur, known as a "cytokine storm." Inflammasomes are protein complexes of the innate immune system that regulate inflammation during viral infections. Additionally, pyroptosis, an inflammatory form of cell death, may also be involved, particularly in monocytes. These mechanisms further contribute to the inflammatory response and the release of cfDNA [4].

Overall, the release and accumulation of cfDNA in COVID-19 patients can be influenced by apoptotic and necrotic cell death, NETosis, and inflammatory processes such as the cytokine storm and pyroptosis [5]. Monitoring cfDNA levels may provide insights into the severity of the disease and its impact on different organs. The aim of our study was to determine

whether cfDNA has the potential to serve as a non-invasive biomarker in COVID-19, similar to its established role in other pathologies.

Methods

Collecting samples

The total number of samples used for the analysis of cfDNA in the presented study is 322 and consists of two groups: samples from positive-tested individuals for the SARS-CoV-2 virus within the PanClinCov project (ITMS2014: 313011ATL7), and a control group of samples. The control group comprised 104 samples from the population branch collected within two projects, PREVELynch (ITMS: 13011V578) and GENOScan (ITMS2014: 313011Q927). The positivity of patient samples was confirmed through antigenic or PCR tests. They were divided into three groups based on the virus variant: Alpha, Delta, and Omicron. The samples were collected during different COVID-19 waves, and based on this criterion, they were divided into three groups according to the dominant prevailing virus variant: Alpha - 80 samples, Delta - 80 samples, and Omicron - 58 samples. The variants in individual patients were not confirmed by sequencing nasopharyngeal swabs.

Isolation of cell free DNA

Cell free DNA from plasma samples was isolated using the QIAamp® DNA Blood Mini Kit (QIAGEN GmbH, Hilden, DE, USA) following the manufacturer's instructions with some protocol modifications. For the isolation, 680 µl of plasma was used. To maintain the ratios, the volumes of all other solutions used, such as Proteinase K, AL buffer, and absolute ethanol, were adjusted accordingly. The wash solutions were increased to 600 µl for AW1 and 700 µl for AW2. An extra purification step using 700 µl of absolute ethanol was added for column purification. The elution volume was 37 µl of millipore water. After isolation, the samples were quantified using the Qubit dsDNA HS (High Sensitivity) Assay Kit on a Qubit 3.0 fluorometer (Invitrogen Inc., Waltham, MA, USA).

Library preparation and quality control

The sequencing libraries were prepared using the TruSeq® Nano DNA Library Prep Kit (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol with some modifications. Library preparation started by end-repair step omitting DNA fragmentation. After that, the size selection step was replaced by capturing all fragments using paramagnetic beads at a ratio of four times the bead volume to the sample volume. The remaining steps followed the manufacturer's protocol. The resulting volume of the sequencing library was 25 µl.

The final sequencing libraries were quantified by the Qubit dsDNA HS Assay Kit on a Qubit 3.0 fluorometer (Invitrogen Inc., Waltham, MA, USA) following the manufacturer's instructions. The distribution of fragment lengths was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) with the Agilent High Sensitivity DNA Kit, prepared according to the manufacturer's instructions.

NGS sequencing

The prepared libraries were normalized to 4 nM, pooled, denatured, and diluted to a final loading concentration of 1.6 pM. An estimated read count of 5 million reads per sample was

used. Pair-end sequencing with 2 x 76 bp read length on NextSeq 500 instrument was performed.

Bioinformatic analysis

The sequencing quality was assessed using the FastQC tool, and subsequently, the sequencing reads were trimmed using Trimmomatic to remove adapters, low-quality ends, and short ambiguous reads. The trimmed reads were then mapped to the reference human genome GRCh38.P14 (HG38) using the BWA-MEM tool. The mapped reads were sorted, deduplicated, and converted to binary format using Samtools. Mapping quality was checked and mapping statistics were generated using Qualimap 2.

All statistic analysis and differences between sample groups were evaluated using Mann-Whitney U test at significance level of 0.05.

Results

To identify the potential use of circulating free DNA (cfDNA) as a diagnostic tool, we focused on plasma-derived cfDNA. In our analysis of a potential biomarker for COVID-19 diagnosis, we have investigated two parameters so far: the concentration of cfDNA after isolation and the length profile of this DNA in control samples and samples from COVID-19 patients obtained during different waves of the pandemic.

Based on the evaluation of the obtained data, we observed a statistically significant difference in the concentration of cfDNA after isolation between the two previously mentioned groups (control and COVID-19 patients) with $p<0.0001$. The weighted median concentration in the control group was 0, and the average concentration reached 0.076 ng/µl. The weighted median concentration in the COVID-19 patient group was 1.148, and the overall average concentration was 3.179 ng/µl (Figure 1).

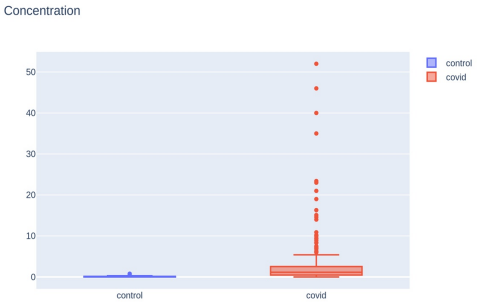

Figure 1: Box plots displaying the weighted median concentrations of isolated cfDNA in the control group (blue) and COVID-19 patient group (red). The Y-axis represents the concentration in ng/µl.

After dividing the patient group into three categories (Alpha, Delta, and Omicron) based on the timing of sample collection, all three groups were significantly different from the control group ($p<0.0001$), with no statistically significant difference in cfDNA concentrations among the individual categories of patient samples. The average concentration of cfDNA in the Alpha category was 3.28 ng/µl, in the Delta category was 3.48 ng/µl, and in the Omicron category was 2.61 ng/µl (Figure 2).

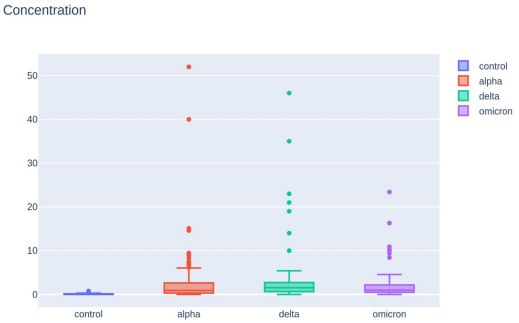

Figure 2: Box plots displaying the weighted median concentrations of isolated cfDNA in the control group (blue) and patient samples divided into three groups: Alpha (red), Delta (green), and Omicron (purple). The Y-axis represents the concentration in ng/µl.

As the second parameter for cfDNA analysis, we evaluated its length profile. After bioinformatic processing of sequencing reads and their mapping to the human reference genome GRCh38, we observed a statistically significant difference in the length of cfDNA fragments between the control and patient groups with a value of $p<0.0001$ (Figure 3), similar to the concentration of cfDNA. Control samples had, on average, a 5.27 bp longer weighted median than patient samples (133.35 bp vs. 128.08 bp).

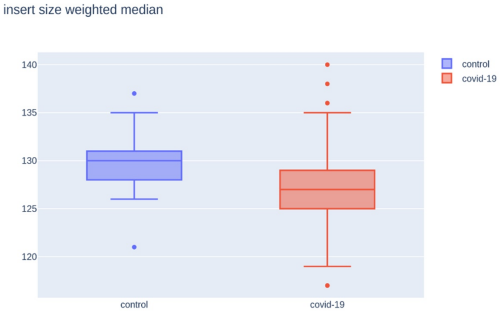

Figure 3: Box plots displaying the weighted median lengths of sequenced cfDNA fragments in the control group (blue) and COVID-19 patient group (red). The Y-axis represents the length in bp.

When COVID-19 samples were divided into separate groups, significant differences were found between the control group and the Alpha, Delta, and Omicron groups ($p<0.0001$ for Alpha and Delta, $p<0.001$ for Omicron). Fragment length differences were observed within the COVID-19 groups. The Delta group had the shortest fragments (126.01 bp), 7.34 bp shorter than the control group. Alpha and Omicron groups had weighted median lengths of 127.58 bp and 128.38 bp, respectively. Significant differences were observed between Alpha and Delta ($p<0.05$) and between Delta and Omicron ($p<0.0001$), but not between Alpha and Omicron ($p=0.149$) (Figure 4).

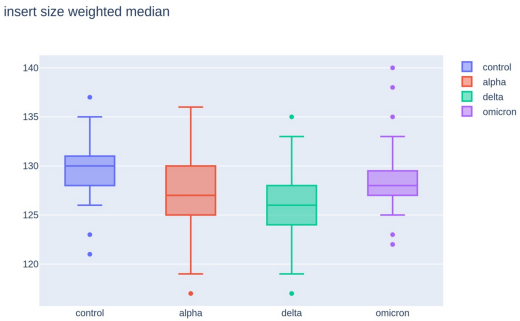

Figure 4: Box plots displaying the weighted median lengths of sequenced cfDNA fragments divided into four groups: control (blue), Alpha (red), Delta (green), and Omicron (purple). The Y-axis represents the length in bp.

Discussion

Increased cfDNA levels have been observed in various pathologies, including COVID-19, and can serve as a biomarker. Further research aims to predict disease severity based on cfDNA profiles. It is worth noting that several studie**s** focusing on free nucleic acids have reported increased concentrations of cfDNA as a potential non-invasive biomarker in various pathological conditions. These conditions include monitoring graft acceptance or rejection [6], oncological diseases [7], non-invasive prenatal diagnostics, and as a biomarker for the development of sepsis [8]. One of the most significant advantages of cfDNA as a biomarker is its potential to detect the disease state before clinical symptoms or histopathological changes become apparent. This early detection capability is exemplified in cases of transplant rejection and has the potential to be utilized in the context of COVID-19 as well. Notably, Andargie *et al.* [9] conducted a study that revealed a positive correlation between elevated cfDNA levels, the severity of COVID-19, and markers such as C-reactive protein and D-dimers. Furthermore, the amount of cfDNA detected upon admission allowed the identification of patients who would later require intensive care or face mortality during hospitalization. This critical insight implies that cfDNA analysis could be utilized in managing the hospitalization of COVID-19 patients, especially in overcrowded healthcare facilities.

Another difference was observed in the length of cfDNA fragments, which can serve as emerging biomarkers. COVID-19 patient samples showed higher proportions of short cfDNA

fragments, possibly due to tissue damage caused by the virus. Variation in fragment size was also observed among different variants, potentially reflecting inflammatory processes. Lower mortality and milder inflammation were found in patients infected with the Omicron variant compared to Alpha and Delta variants. These changes may be caused by increased necrosis, apoptosis, and metabolic burden.

Conclusion

Comprehensive studies on cfDNA fragmentomics in COVID-19 patients are lacking, but they could greatly contribute to diagnostic biomarkers and disease monitoring. We have ongoing research on cfDNA and plan to predict disease severity using cfDNA profiles. This next phase will provide valuable insights into cfDNA as a predictive tool in COVID-19 management.

Acknowledgments

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4. Seroprevalence study of SARS-CoV-2 specific antibodies in Slovakia

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Introduction

The coronavirus SARS-CoV-2 is a causative agent of Covid-19, a highly infectious disease, that has had a global impact with more than 767 million confirmed cases and 6.9 million deaths from March 2020 to June 2023(1). Several new variants of the virus have been described during the Covid-19 pandemic, including the so-called variants of concern (VOCs): Alpha; Beta; Gamma; Delta; Omicron (2). VOCs have a potential to increased transmission or virulence, the ability to reduce the effectiveness of vaccination and also to reduce neutralization by antibodies obtained through natural infection. Our understanding of the global scale of SARS-CoV-2 infection remains incomplete (3). Routine surveillance data underestimates infection, there is an occurrence of asymptomatic infections and uneven access to diagnostics. Seroprevalence studies allow to understand the true extent of infection as well as to estimate the rate of undiagnosed and unreported cases.

Here we describe the results of the seroprevalence study of SARS-CoV-2 specific antibodies in Slovakia. The aim of the study was to find out the infection-induced seroprevalence and the overall seroprevalence (from infection or vaccination) of selected regions of Slovakia.

Methods

This is a cross-sectional observational study of the state of the antibody response to the SARS-CoV-2 virus in non-vaccinated residents of selected regions of Slovakia. The study is population-based, age-stratified, cross-sectional and it took place in November 2021. Participants of the study were volunteers who met the following criteria:

- permanent/temporary residence in the given region
- age 12 years and more
- not vaccinated against Covid-19

Study participants underwent sampling of capillary blood from the finger and completing a questionnaire for the collection of personal and epidemiological data, such as age, data on vaccination status, data on overcoming and the course of the disease Covid-19 and on the contact with a person tested positive for SARS-CoV-2 using the Ag/real time PCR test.

Participants were tested in 27 sampling sites in five districts and two cities of Slovakia: Čadca (CA; 4 sampling sites), Považská Bystrica (PB; 5 sampling sites), Komárno (KN; 3 sampling sites), Kežmarok (KK; 3 sampling sites), Lučenec (LC; 2 sampling sites), Košice (KE; 4 sampling sites) and Bratislava (BA; 6 sampling sites).

Antibodies of the IgG class against the S1 subunit of the S protein of the SARS-CoV-2 were determined from the collected biological samples of capillary blood from the finger using the ELISA method. Samples were examined in the Biomedical Research Centre of the Slovak Academy of Sciences, where they were checked and arranged according to the assigned codes. Then an area of the dried blood drop pinched with pliers was defined. The dried blood sample was dissolved in an extraction buffer and analysed using the ELISA sets (EUROIMMUN, Germany) for the presence and level of antibodies against SARS-CoV-2.

In the resulting statistics, we distinguished several levels of the test result according to the measured numerical test result (S/C unit, i.e. the signal intensity ratio compared to the calibrator):

- negative result (<0.8 S/C),
- borderline result (0.8 – 1.1 S/C),
- low antibody result (1.1 – 3.5 S/C)
- a result with a high level of antibodies (>3.5 S/C)

The estimate of overall seroprevalence in population includes the percentage of fully vaccinated two weeks before the study and those unvaccinated who had a positive antibody test in the study.

Results

A total of 3785 volunteers participated in the study. Out of them 1477(39%) were males and 2308 (61%) were females. The mean age was 43,3 years. The study group was divided in four age categories: 12 – 18 years (303/8%), 19 – 39 years (1249/33%), 40 – 59 years (1476/39%) and 60 and over (757/20%). According to the questionnaire answers 2546 (67%) of the participants were not aware about overcoming Covid-19, 116 (3%) stated overcoming without any symptoms and 1123 (30%) had Covid-19 with clinical symptoms. In 1476 (39%) of the participants, the presence of antibodies against SARS-CoV-2 was detected, 2157 (57%) of the participants had a negative result, in 152 (4%) the result was borderline. In all age categories were negative results in more than 50% of participants (Fig.1). The highest proportion of detected antibodies was in category 12 – 18 year. A high antibody level had 25% participants in categories 12 -18 years and 60 and over.

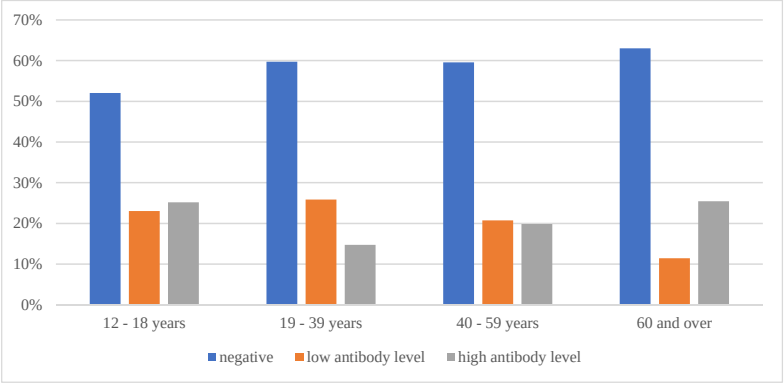

Figure1: Infection-induced SARS-CoV-2 antibody levels by age group in percentage

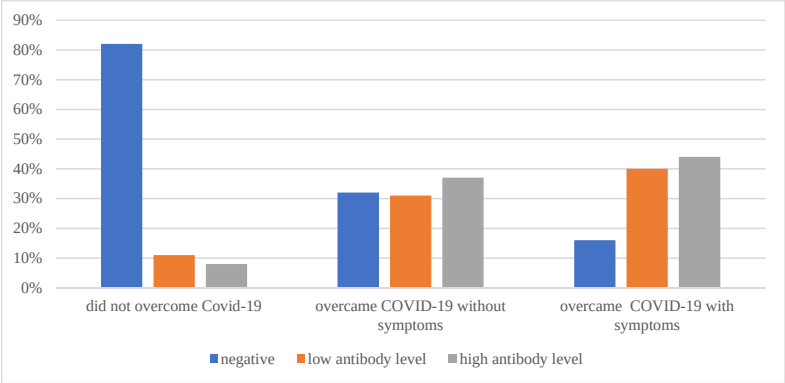

Figure 2: Infection-induced SARS-CoV-2 antibody levels according to the way of overcoming Covid-19

Up to 82% of those who did not overcome the disease of Covid-19 had a negative result of the antibody test (Fig.2). On the other hand, 18% of them had SARS-CoV-2 specific antibodies which suggests they had overcome the infection without knowing. Among those who overcame the disease of Covid-19 asymptomatically, up to 32% had a negative result of the antibody test, frequently even after it was recently overcome. Among those who overcame the symptomatic course, including mild symptoms, the antibody test was negative in only 16% of cases, mostly when the disease had been overcome earlier. In this group, 44% had a high level of antibodies, among them mainly those who had overcome the disease during the last 8 months.

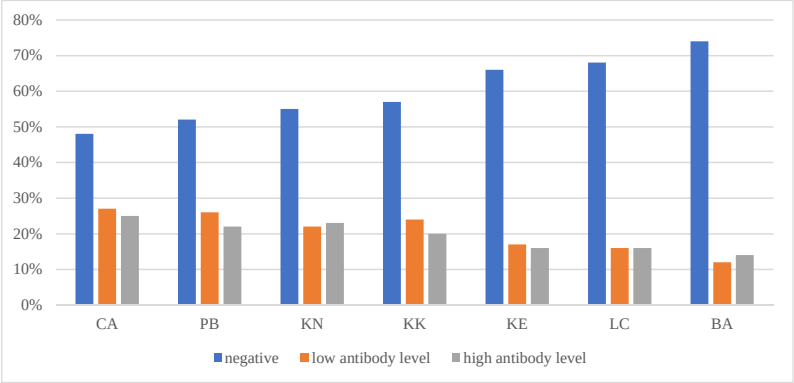

Figure 3: Infection-induced SARS-Cov-2 seroprevalence in selected districts of Slovakia

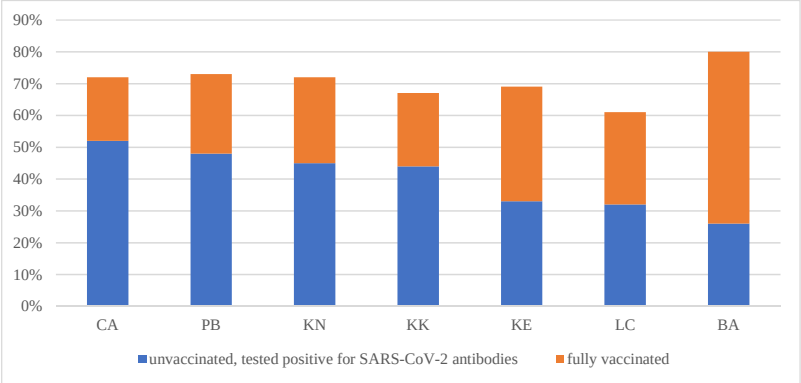

Figure 4: The overall SARS-CoV-2 seroprevalence in selected districts of Slovakia

The range of the antibody response present in the selected regions was from 26% in Bratislava to 52% in Čadca (fig.3). Čadca district was the only one where the percentage of people tested positive for antibodies exceeded the percentage of people with a negative result. The total estimated level of immunization of the population is estimated as the sum of the number of fully vaccinated in a given region and the percentage of those who were tested positive in this study (Fig. 4). The range of the population with antibody response against SARS-CoV-2 was from 67% in Kežmarok to 80% in Bratislava district. The highest vaccination coverage and at the same time the lowest percentage of those who overcame Covid-19 was in Bratislava. On the contrary, the Čadca district had the lowest vaccination coverage and at the same time the highest percentage of people who have overcame the infection.

Discussion

We conducted a population-based, cross-sectional seroprevalence study in Slovakia in November 2021. Up to now (June 2023) there are more than 4000 seroprevalence studies from 145 countries all around the world registered in a global SARS-CoV-2 seroprevalence dashboard (SeroTracker) (4). This allows to estimate the true extent of the pandemic and compare estimates between regions. Confirmed and reported Covid-19 cases undercount the true number of infections because many people have asymptomatic infections or were not diagnosed especially in the case of a mild course of the infection.

In September 2021, global seroprevalence attributable to infection was 35.9% and global seroprevalence from infection or vaccination (overall seroprevalence) was 59.2%, which means that 40% of global population was susceptible. (3). There were regional variations driven by differences in the extent of SARS-CoV-2 infection and vaccination. In European high-income countries (HIC EUR) overall seroprevalence in November 2021 was around 90% mostly because of approximately 70% vaccination coverage (3,5). Our findings in Slovakia were 39% seroprevalence attributable to infection and overall seroprevalence 71%. This follows that a third (29%) of the population of Slovakia was completely susceptible to SARS-CoV-2 infection.

To assess the true burden of disease we compared reported cases of Covid-19 in the year 2021 and infection-induced seroprevalence. The incidence of Covid-19 in Slovakia in 2021 was 17,73% (6). Infection-induced seroprevalence was 39%. Estimated seroprevalence to case ratio is 2.2:1, suggesting that there were actually twice as many cases of infection as were reported. Seroprevalence to case ratio in HIC EUR was 1.9:1 and globally 10.5:1(3). Moreover, waning immunity after infection likely underestimates the extent of past infections.

Considering infection-induced seropositivity in different age groups, in the 12 – 18 years olds it points to the possible exposure to Covid-19 in schools. Lower seroprevalence (63% seronegative persons) in adults 60+ could be explained by immunosenescence or extra careful behaviour to avoid the infection and hence a lower proportion of people with evidence of past infection.

However, the study results should be interpreted considering a few limitations of the study: First, the size of the tested sample in each of these locations is sufficient for statistical representativeness, but its composition did not fully correspond to the age composition or gender distribution in the given region. Second, people who participated in the study were volunteers, so it may be affected by selection bias.

The testing in our study took place in different districts in November 2021, somewhere before the peak of the wave of the delta variant SARS-CoV-2 at the regional level, somewhere after it. Therefore, the infection-induced seropositivity was after overcoming SARS-CoV-2 infection caused by the alfa or delta virus variant, which were circulating in Slovakia in 2021. Immediately after the retreat of the delta wave more transmissible and immune-escaping Omicron variant exceeded the previous variants in the number of cases in January – February 2022. In both vaccinated and previously infected individuals there is a high risk of reinfection with the Omicron variant.

Seroprevalence studies give an estimate of the exposure to infection, do not serve as a calculation of the size of the population that cannot be infected with the virus or does not spread the infection. They may help to identify high-risk groups in population and lead to

targeted disease control strategies. However, seroprevalence estimates remain indicative of protection against severe disease and death.

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- *Biomedical Research Centre of the Slovak Academy of Science*
- *Institute of Health Analysis, Ministry of Health of the Slovak Republic*

We also thank students of medicine 5th year who participated at sampling.

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5. Looking back at the COVID-19 pandemic from the perspective of specific antibody levels

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Introduction

When COVID-19 pandemic reached Slovakia in March 2020, the research teams of the Biomedical Research Center of the Slovak Academy of Sciences (BMC SAS) immediately adapted spaces of the BSL3 laboratory, introduced RT qPCR detection of SARS-CoV-2 viral RNA and offered their testing expertise and capacity to the Public Health Authority (PHA) of the Slovak Republic. During the first and second wave of the pandemic, when the state testing capacity was at critically low level, the BMC SAS researchers performed routine testing of more than 20,000 samples from PHA and several hospitals. Later, the testing was provided to artists, sportsmen, to policymakers to ensure safety during the GLOBSEC meetings, and to other professionals endangered by the pandemics. Testing was also performed at weekly basis to the employees of the Slovak Academy of Sciences.

The activities of the BMC SAS proved to be highly useful in several aspects of the pandemic management in Slovakia. Besides systematic testing, they included isolation, characterization and deposition of SARS-CoV-2 virus from Slovak patients, infectivity testing for the needs of clinical facilities, sequencing and genomic analyses of the SARS-CoV-2 variants. Sequencing allowed early identification of the onset of alfa, delta and Omicron variants with direct impacts on decisions of state authorities on anti-pandemic measures. All these activities contributed to the development and clinical validation of innovative diagnostic tests in collaboration with MultiplexDX. The kits were equipped with the positive control RNA obtained at the BMC SAS from the virus isolates and certified by the State Institute for Drug Control for IVD use. Our studies were the basis for the introduction and validation of gargling as an alternative sampling method, suitable for testing of school children, into the standard operational procedure for SARS-CoV-2 diagnostics in Slovakia and for the evaluation of saliva for virus diagnostics in combination with LAMP methodology. The laboratories of the BMC SAS performed the quality tests and provided expert background to the concluding statement on pharmaceutical quality of the SputnikV vaccine. In connection with the efforts to resolve pandemic situation, our renowned researchers participated in expert discussions with the authorities of the Slovak Republic - the President, the Prime Minister, the Deputy Prime

Minister, representatives of several ministries and members of the crisis staff. They communicated their expert opinions individually or as part of an initiative "Science helps - COVID-19".

Furthermore, BMC offered antibody testing for the employees of the institutes and non-research organizations of the SAS located throughout the country in two consecutive studies (summer 2021 – (1), September 2022 – (2) and a pre- and post-Omicron wave antibody testing (mid-January 2022 and end-April 2022) for the BMC employees (3).

Subjects and Methods

The study, performed under the IMPROVE project (APVV-PP COVID-20-0017), was approved by the Ethic committee of Bratislava Self- Governing Region by its decision No. 09833/2020/HF and amendment 07071/2021 from June 30, 2021. All volunteers received detailed written information about the study, signed informed consent, and provided relevant anamnestic information.

We introduced and validated a new approach based on the self-sampling of capillary blood on a card for dry blood collection from finger pricks using lancet as a suitable alternative to a venous blood serum sample. Defined area of the card was punched out, submerged into the sample buffer of the ELISA and incubated for 1 hour at 37°C. The extracted sample was used for serological analysis by an anti-SARS-CoV-2 IgG ELISA (EUROIMMUNE Medizinische Labordiagnostika AG, Germany) according to manufacturer's protocol. Levels of IgG antibodies specifically binding to the SARS-CoV-2-encoded spike protein subunit 1 (anti-S1 IgG) containing the receptor binding domain were measured, considering the signal-to-calibrator (S/C) ratio of <0.8 negative, ≥0.8 and <1.1 borderline, and ≥1.1 positive. Participants received detailed description on their personal results with a remark that the test result represents only partial information of the immune response and cannot determine an individual's risk of subsequent infection.

Results and discussion

We conducted a total of 3 serological studies among SAV employees, each in a different, specific phase of the development of the pandemic. Thanks to early introduction and good compliance of the society to strict epidemiological measures, the country has managed the first pandemic wave (3/2020 – 5/2020; ancestral Wuhan-Hu-1 strain) relatively successfully. However, the second wave between 10/2020 and 4/2021, caused by the spread of the B.1.1.7 (alpha) variant of the virus, resulted in devastating consequences in sense of several thousand deaths, exhausting of the health care system and decreased cooperation and compliance of the society. During the second wave, the public vaccination program started in Slovakia (1/2021). Our first study from August 2021 (1928 participants) gave us an insight into the state of antibody immunity after the wave of the SARS-CoV-2 B.1.1.7 variant and at the same time after about half a year of nationwide vaccination in Slovakia (1). The third wave, caused by the spread of the B.1.617.2 (delta) variant, hit the country in 9/2021 with gradual transition to the fourth wave (B.1.1.529, omicron variant) in January 2022. The fourth wave lasted until the end of 4/2022. In our second study (3), only BMC employees took part (263 participants), two blood samples were analyzed - before the start of the Omicron wave in Slovakia (January 2022)

and after it subsided (April 2022). We therefore had the opportunity to monitor the effect of this variant on the development of antibody immunity in specific individuals and the effect of initial antibody levels on subsequent infection. So far, the last serological study (2), in which 1365 SAS employees participated, was carried out in October 2022. The monitoring of antibody levels during this period gave us a comprehensive view of immunity after all the main waves of the COVID-19 pandemic had subsided, at a time when most of the participants were vaccinated with booster doses of vaccines and many have overcome the disease repeatedly. The pandemic in Slovakia resulted in over 21 thousand deaths from more than 1.8 million reported coronavirus cases (https://www.worldometers.info/coronavirus/country/slovakia/).

The works brought several significant results, however, we can consider realization on academic grounds to be a disadvantage, due to the much higher vaccination rate compared to the Slovak average, and probably the higher willingness to comply with anti-pandemic measures.

The most important findings of our studies regarding the humoral immune response to the SARS-CoV-2 virus and the vaccine against COVID-19 are the following:

mRNA vaccines appear to be more effective in inducing antibody response compared to adenovirus vector vaccines. Comparison of the relative levels of anti-S1 IgG induced by full vaccination revealed considerable differences among the vaccine types (Fig. 1a). The highest relative antibody levels were induced by mRNA vaccines Comirnaty and Spikevax, whereas Vaxzevria and SputnikV induced medium antibody levels. The lowest IgG levels were induced by the one-shot vaccine Janssen. This observation is in line with the published data showing similar vaccine type relationships not only with respect to IgG levels, but also to the levels of neutralization antibodies, which represent only a fraction of the total anti-S1 IgG that is considered a surrogate of vaccine protective effect (4,5)

Immune protection of hybrid immunity, repeated infection, bolus vaccination. As expected, data analysis revealed that antibody levels principally reflected the disease severity in the subgroup of participants who reported COVID-19 and remained without subsequent vaccination. Importantly, in persons who underwent full vaccination by any vaccine following COVID-19, the antibody levels significantly increased irrespective of the symptoms. This was particularly striking in the category of participants with no, mild and moderate symptoms, who, if not vaccinated, did not develop robust humoral response to natural infection (Fig. 1b). This finding suggested that in those cases, vaccination is very beneficial (or critical) for providing better immune protection from reinfection.

We also confirmed increasing levels of anti-S1 IgG with increasing number of vaccine doses administered, and/or with multiple overcoming of the disease.

(a) (b)

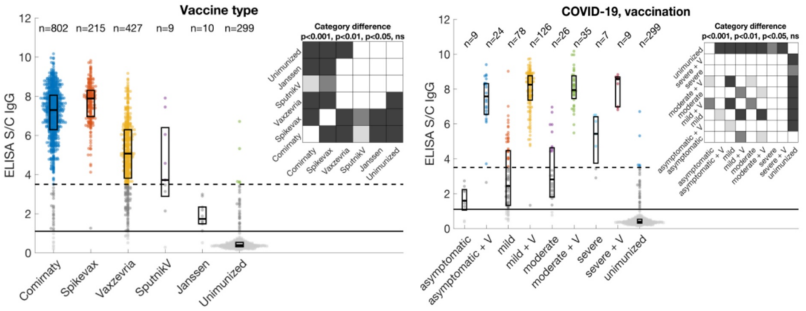

Figure 1: Relative anti-S1 IgG levels in **(a)** study participants vaccinated by indicated vaccines and **(b)** participants who reported COVID-19 (stratified according to self-reported severity of COVID-19 symptoms) and/or vaccination. (1)

Time-related decrease of anti-S1 IgG in response to infection. The levels of virus-specific IgG antibodies showed a decreasing trend of dependence on the time elapsed from the date of detection of the infection. The estimated average rates of decline were comparable for groups of participants who overcame COVID-19 with mild and moderate symptoms and slightly higher for those with severe and critical symptoms.

Higher initial anti-S1 IgG prevented Omicron infection. The key result of "Omicron serological study" is the finding of a significant difference ($p = 0.005$) in the median levels of anti-S1 IgG antibodies in the group of participants who were subsequently infected with the Omicron variant of SARS-CoV-2 ("+OMI, BEFORE") versus the group in which no breakthrough infection occurred during the monitored period ("-OMI, BEFORE"). The results also naturally showed an increase in antibody levels after overcoming the infection ($p < 0.0001$) and an expected decrease in antibody levels caused by the effect of waning immunity in the "-OMI" group ($p < 0.001$) (Fig. 2).

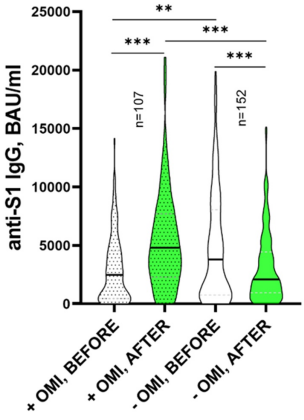

Figure 2: Levels of anti-S1 IgG (BAU/ml) before and after Omicron wave in those who got infected with the Omicron variant ("+OMI") and those who did not ("-OMI") (3)

Lower immunogenicity of Omicron variant. A very interesting finding is also the fact that the Omicron variant of SARS-CoV-2 (B.1.1.529) elicits a much lower antibody response than the previous variants of the virus (Fig.3). We observed this in a group of unvaccinated participants, by comparing those who overcame the COVID-19 disease in 2020 or 2021 with those who overcame it only in 2022 (dominance of the Omicron variant in the EU).

Seroprevalence at the end of the pandemic. In the period between the first and the last study, the administration of booster vaccine doses against COVID-19 began in Slovakia (October 2021) and new variants of SARS-CoV-2 (delta and omicron) caused 2 significant waves of the COVID-19 disease. It is therefore not surprising that in the last study (10/2022), the total seropositivity to the S1-protein of the SARS-CoV-2 virus was 96.04%, 87.11% of the participating employees were vaccinated, and 65.05% of the participants had overcome the COVID-19 disease at least once.

Since 1004 individuals participated in both studies, we observed a significant ($p<0.0001$) increase in the median of anti-S1 IgG (Fig. 4a). We divided the both studies participants into four groups: "-VAC, -C19 T"; "+VAC, -C19 T"; "-VAC, +C19 T" and "+VAC, +C19 T", according to their vaccination status (+VAC = vaccinated whenever since December 2020) and according to their COVID-19 test positivity (+C19 T = had at least one positive test for COVID-19 during the pandemic). An increase in antibody levels in all groups could be observed (Fig. 4b). In the "-

VAC, -C19 T" group, the significant increase of anti-S1 IgG ($p < 0.0001$) was probably caused by asymptomatic infections or by not confirming the suspicious infection by a test for COVID-19.

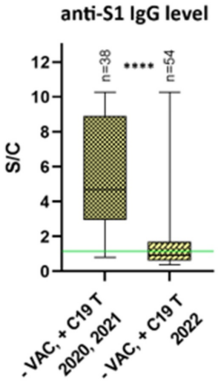

Figure 3: Levels of anti-S1 IgG (S/C) in unvaccinated participants stratified according to the date of positive testing for COVID-19. 2020, 2021 – assumption of overcoming the original strain/Alpha/Delta variant of SARS-CoV-2; 2022 – assumption of overcoming the Omicron variant of SARS-CoV-2 (2)

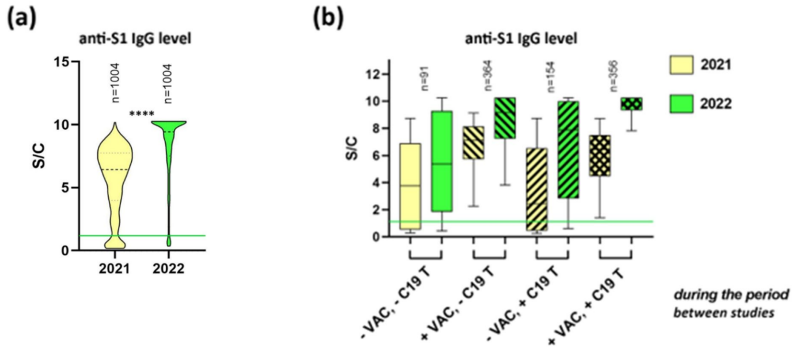

Figure 4: Comparison of relative levels of anti-S1 IgG in 2021 and 2022. **(a)** Truncated violin plot visualization of antibody levels in participants who participated in both serological studies. **(b)** Participants stratified according to the vaccination status (one or more doses

whenever since December 2020) and/or COVID-19 overcoming (one or more times) during the period between studies (September 2021 – October 2022). (2)

COVID-19 pandemic represented an unprecedented burden for public health systems worldwide including Slovakia. The goal of the studies was to reduce the negative impact of the pandemic through the improvement of our understanding of SARS-CoV-2 immune response. One of the main objectives of the studies was to improve our understanding of the immune response to SARS-CoV-2 infection and vaccination. Cross-sectional seroprevalence studies performed on more than 1900 Slovak Academy of Sciences employees in the period before the onset of the second pandemic wave revealed that mRNA vaccines produce higher antibody response than the adenovirus-based vector vaccines, antibody levels reflect the clinical course in COVID19 patients, vaccination significantly improves antibody levels in patients with asymptomatic or mild clinical course, antibody levels decrease with increasing time since vaccination or infection. Long-term analyses confirmed that antibody levels are significantly increased by vaccination also in the patients with moderate and severe disease and that the second vaccine dose is crucial for the improvement of antibody level longevity. We observed, that higher initial anti-S1 IgG antibodies prevented the infection with the Omicron variant, the one with lower immunogenicity.

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6. Possibilities of SARS-cov-2 genome assembly and transcriptomic analysis of COVID-19 clinical samples

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Introduction

Since emergence of COVID-19 disease caused by virus named SARS-CoV-2, disease became worldwide and target of investigations (Edwards et al., 2022; Koelle et al., 2022; Wu et al., 2020) Many procedures of bioinformatics, that have been used daily on various analyses were applied to research of COVID-19 disease. One of them, an assembly of a genome is necessary for many downstream analyses. There are multiple assemblers suitable for an assembly of transcripts or RNA genomes (like SARS-CoV-2 virus genome) from RNA-seq reads. Although usually combination of long and short reads is suggested, we tested performance of SPAdes and Trinity on our short-RNA reads data (Grabherr et al., 2011; Prjibelski et al., 2020). Quality of assemblies can be measured by contiguity, completeness and accuracy. These parameters are represented by various metrics – N50, L50 and their derivations, proportion of the genome assembled compared to complete genome and its fidelity.

Not only virus itself can be studied by RNA-seq method, but also changes in human transcriptome caused by the virus presence. Infection by virus results in changes in human metabolism. There are changes in signaling pathways that regulate processes of DNA repair and replication, immune response, transcription, metabolism, cell cycle, and apoptosis (Jamison et al., 2022). Significant for clinical purposes are cytokine storms (excessive and uncontrolled immune response), which cause release of a large amount of pro-inflammatory cytokines into the bloodstream, followed by widespread inflammation and damage to tissues and organs (Henderson et al., 2020; Mehta et al., 2020; Wang et al., 2020). These changes are reflected at transcriptome profile, which were studied in our research.

Methods

Study Approval

Sample collection was performed as part of the clinical study approved by the Ethical Committee of Bratislava Self-Governing District under the identifier 03228/2021/HF from January 12, 2021. All patients have filled out the questionnaires with relevant in-formation regarding their health status in relation to COVID-19 and signed informed consent.

Sample collection

Nasopharyngeal swabs from patients suspected of having COVID-19 were obtained in two primary regimens. Patients hospitalized with severe symptoms of the disease at the collaborating hospitals were enrolled in the study. Patients with mild or any symptoms of the

disease were recruited in mobile testing facilities for SARS-CoV-2 by a company providing routine laboratory diagnostics from the population during the COVID-19 pandemic. In total, 96 samples were part of this study (72 COVID-19 positive patients and 24 healthy donors) were used for analysis of transcriptome. Additional 7 COVID-19 positive patients were part of genome assembly testing.

Nucleic acid extraction

Nasopharyngeal swabs specimens were collected from COVID-19 patients and controls and stored in viRNAtrap collection medium (GeneSpector, Czech Republic) at 4°C. Total RNA was extracted using Sera-Xtracta Virus/Pathogen Kit (Cytiva, UK) according to manufacturer instructions. 400 µl of the nasopharyngeal swab medium was used for the extraction with a final elution volume of 50 µl. RNA was quantified with the Qubit™ RNA High Sensitivity Assay Kit (Invitrogen). RNA isolates were stored at -80°C.

RT-qPCR

The presence of SARS-CoV-2 was determined by RT-qPCR using the COVID-19 Real-Time Multiplex RT-PCR Kit (Labsystems Diagnostics, Finland) and RT-qPCR plat-form ABI QuantStudio 6 Real-Time PCR System (ThermoFisher, USA) utilizing the original manufacturers' protocols. Amplification cycles threshold of Ct value <40 was needed to evaluate the sample as positive.

RNA library preparation and sequencing

The metatranscriptomic libraries were prepared using KAPA RNA HyperPrep Kit with RiboErase (HMR) (Kapa Biosystems, South Africa) according to the original protocol of the manufacturer. For quantity and quality control of prepared libraries a Qubit 1X dsDNA High Sensitivity Assay Kit on Qubit 3.0 (Invitrogen) and Agilent High Sensitivity DNA Kit on Agilent 2100 Bioanalyzer (Agilent) instruments were used. Sequencing of pooled libraries was performed on NextSeq 500 and NextSeq 2000 (Illumina) platforms using 2x75 or 2x100 paired-end sequencing setup, respectively.

Data analysis

Quality control of raw reads was done by FastQC v0.11.9 (Andrews, 2010). Subsequently, reads were processed by Trimmomatic (Bolger et al., 2014) and again checked on quality by FastQC. After final affirmation of sufficient quality of reads by FastQC, reads were mapped to the human genome hg38 by BWA-MEM algorithm v0.7.17 (Li, 2013). Reads were mapped as paired set, otherwise parameters of mapping were set to default. Same way it was done on SARS-CoV-2 genome.

Genome assembly

We performed an assembly of SARS-CoV-2 genome by Spades and Trinity assemblers (Grabherr et al., 2011; Prjibelski et al., 2020). We tested multiple strategies of assembly. In 2 strategies we proceeded with reads mapping on SARS-CoV-2 genome, in other cases, we tested Spades assembler on reads that didn't map to human genome. All strategies tested are shown on the Table 1. For deeper evaluation and comparison of performance (resemblance to a reference genome, N50 value and other values), we used command line (Conda) instance of Quast software (Gurevich et al., 2013).

Table 1. Strategies of SARS-CoV-2 assembly.

Strategy	Reads filtering	Assembler	Mode
1	mapping on SARS-CoV-2 genome	coronaspades.py	
2	not mapping on human hg38 genome	coronaspades.py	
3	not mapping on human hg38 genome	Spades	--rna
4	not mapping on human hg38 genome	Spades	--rnaviral
5	mapping on SARS-CoV-2 genome	Trinity	

Differentially expressed genes analysis

In search of human genes affected by infection of SARS-CoV-2 or category of infection, we further analysed .bam file with human-mapped reads. Gene expressions were quantified by FeatureCounts v.2.0.1 (Liao et al., 2014). Statistical comparison was done by R instance of Deseq2 v.1.38.3. (Love et al., 2014). Genes under condition of adjusted p-value < 0.1 were considered significant hits, which is a default value according to DESeq2 manual.

Identification of altered pathways

To find out which pathways are altered from the set of differentially expressed genes, we used R instance of gProfiler2 v.0.2.1. (Kolberg et al., 2020). Genes, which were under p-value threshold 0.1 were used as a query for the analysis, ordered by p-values and separated to down-regulated and up-regulated set. Databases of KEGG pathways, Wiki pathways and Reactome were used, for the purpose of better visibility, just KEGG terms were chosen for visualization. Set of genes which were part of differentially expressed genes analysis were set as custom background. Domain scope were set to "known". For the visualization, barplot function in R was used.

Results

In this paper, we report results of the analysis of RNA-seq data from nasopharyngeal swabs. Multiple assembly strategies of the SARS-CoV-2 genome were performed on 79 samples by tools Spades and Trinity assemblers. We tested their performance and tried to answer the question if it is possible to assemble RNA virus genome from short RNA-seq reads either in de-novo fashion or with a help of reference genome and tools adjusted to specific virus. Results showed that although using only reads mapping on SARS-CoV-2 genome and mode specifically designed to it led to improvement of genome assembly. However, less specific SPAdes modes and Trinity assembler were able to assemble genomes as well. Our data shown, that it is possible to predict assembly success by RT-qPCR Ct value. Samples with Ct value < 25 were always successfully assembled.

Analysis of differentially expressed genes was performed on 72 COVID-19 positive patients and 24 healthy donors to characterise changes for diseased patients and changes based on disease severity. By statistical test (using DESeq2) 16,365 genes were reported with an adjusted p-value < 0.1 compared to COVID-19 negative controls while 4539 genes were reported with a p-value < 0.1 when comparing mild and severe disease. Subsequently, pathway analysis was

performed, where unsurprising pathways where reported to be overrepresented in the set of differentially expressed genes. Most of the reported pathways were related to the immune response to various diseases, other immune-related pathways, and signaling pathways mostly related to the immunity.

Discussion

This work is the report about our recent research on RNA-sequencing of COVID-19 patients. We investigated possibilities of SARS-CcoV-2 virus assembly from short read Illumina sequencing from 79 samples and performed differentially expressed genes analysis on human transcripts on 96 samples (72 COVID-19 positive and 7 COVID-19 negative controls). We proved that SARS-CoV-2 or other RNA-virus assembly is possible even from short read RNA-seq and success of assembly can be predicted by RT-qPCR Ct value. Here we tested how can be genome assembled when using advantages of already available and annotated reference genome, but also without it. Since assembly was possible even de-novo without using reference, we concluded this method would be possible for assembly of unknown new viruses that might come in the future.

We studied transcriptome of 72 COVID-19 positive patients and 24 healthy donors. By comparison of measured gene expressions between COVID-19 positive patients and healthy controls or comparison between patients with severe and mild symptoms, we observed dramatic changes in gene expressions. Because of the extent of these changes, we couldn't assign some specific genes as markers, but we were able to assign pathways to them. Comparing to similar studies and previous knowledge results were not surprising (Rhoades et al., 2021). Immunity related terms and signaling pathways were assigned, apart from the different disease terms, which observation is probably caused by similarities between processes in different diseases.

Acknowledgments

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7. Extracellular mitochondria contribute to neutrophil extracellular traps formation during SARS-CoV-2 infection

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Introduction

In the early stages of the COVID-19 pandemic, it was observed that a severe SARS-CoV-2 infection results in the destruction of lung tissue and impaired oxygenation. Initial treatment approaches utilizing mechanical ventilation were however met with high mortality, highlighting the need for a better understanding of COVID-19 pathology.(1) Subsequent studies associated thrombosis with disease severity and identified neutrophilia as a marker of poor outcome.(2, 3) This led to the hypothesis that the neutrophil extracellular traps (NETs) cause thrombi formation, thereby reducing the efficiency of artificial ventilation. NETs are web-like structures composed of DNA and antimicrobial proteins, capable of capturing and eliminating bacteria.(4) While the primary function of NETs is host defense, their excessive production is implicated in disseminated intravascular coagulation during systemic inflammation. Multiple studies have also observed an association between increased NETs production and the severity of COVID-19.(5) In order to improve the treatment not just for COVID-19 patients, but also other viral respiratory tract infections, a comprehensive understanding of the mechanisms underlying NETs induction is crucial. In this work, we have therefore aimed to analyze the potency of SARS-CoV-2 to induce NETs formation.

Methods

Neutrophil isolation

Healthy adult donors volunteered and consented to the blood collection under ethical approval given by the Ethics Committee of the University Hospital Bratislava (workplace Ruzinov under number EK 218/2020). Blood was collected by venous puncture into heparinized tubes, neutrophils isolated by 1-Step Polymorphs (Accurate Chemical & Scientific Corp, USA) and resuspended in RPMI 1640 with 10% FBS (PAN-Biotech, Germany).

SARS-CoV-2 preparation

SARS-CoV-2 was cultured on Vero E6 cell line in complete DMEM medium with 10% FBS (Gibco, UK). Virus titter was calculated by plaque forming assay. Live virus was used for

induction of NETs detected via fluorescence microscopy. All following experiments used a heat-inactivated virus.

Mitochondria isolation and quantification

Placental tissue was homogenized in STE buffer (250 mM saccharose, 2 mM EGTA, 5 mM Tris-HCl, pH=7.4) and mitochondria were isolated by performing a series of three differential centrifugations at 500 x *g* for 3 minutes at 4°C and 8000 x *g* for 10 min at 4°C.

Isolated mitochondria were quantified by qPCR (forward: 5'-CATAAAAACCCAATCCACATCA-3', reverse: 5'-GAGGGGTGGCTTTGGAGT-3') on the QTower3 (Analytik Jena GmbH, Germany) using the Advanced Universal SYBR Green Supermix (Bio-Rad, USA).

Detection of NETs by fluorescence microscopy

Neutrophils were seeded onto 0.01% poly-L-lysine coated coverslips and stimulated with DMEM medium collected from control or SARS-CoV-2 infected Vero cells and 100 nM phorbol 12-myristate 13-acetate (PMA) for 3 hours at 37°C and 5% CO_2. Neutrophils were then fixed with 2% paraformaldehyde and stained with anti-citrullinated histone H3 antibody (ab219407, Abcam, UK) followed by and 200 nM SYTOX™ Green Nucleic Acid Stain (Invitrogen, USA). Slides were mounted and images collected with an Axiolab 5 fluorescence microscope (Zeiss, Germany).

Detection of NETs by live-cell microscopy

Neutrophils were seeded on a 96-well tissue culture flat bottom plate and stained with 1.25 µg/ml Hoechst 33342 (Merck, USA) and 200 nM SYTOX™ Green Nucleic Acid Stain (Invitrogen, USA). Neutrophils were treated with isolated mitochondria (MOI20) and media from control or SARS-CoV-2 infected Vero cells and immediately imaged using a Cytation 7 Cell Imaging Multi-Mode Reader (BioTek, USA). NETs were considered to be >20 µm SYTOX™ Green positive objects and relative NETs formation was calculated from the area under the curve (AUC) of NETs formed over time normalized to the number of Hoechst positive cells.

Flow cytometry of mitochondria and DNA containing particles

Quantity and size of DNA-associated microparticles stained with 200 nM SYTOX™ Green Nucleic Acid Stain (Invitrogen, USA) and cell-free mitochondria stained with 100 nM Mitoview™ (Biotium, USA) was analysed by a DxFlex flow cytometer (Beckman Coulter, USA) on a combination of V-SSC-A and FITC-A channels. Particle size was calculated according to Flow Cytometry Sub-micron Particle Size Reference Kit (Invitrogen™, USA).

Statistical analysis

T-test and ANOVA was performed using GraphPad Prism v8.00 for Windows (GraphPad Software, La Jolla, CA, USA). Data are presented as a mean and standard deviation (SD). P values < 0.05 were considered statistically significant.

Results

SARS-CoV-2 infected and control medium induce NETs formation

The induction of NETs by live SARS-CoV-2 virus was first analysed by fluorescent microscopy. NETs were identified according to positive staining for histone H3 citrullination and a web-like morphology of released DNA. Treatment with both SARS-CoV-2 conditioned medium and uninfected medium from Vero cells induced the formation of NETs with a comparable intensity and promoted histone citrullination in majority of the neutrophils. PMA used as a positive control induced massive NETs formation while control neutrophils were negative for both histone H3 citrullination and NETs release (Fig 1).

SARS-CoV-2 infected and control cell media contain extracellular mitochondria

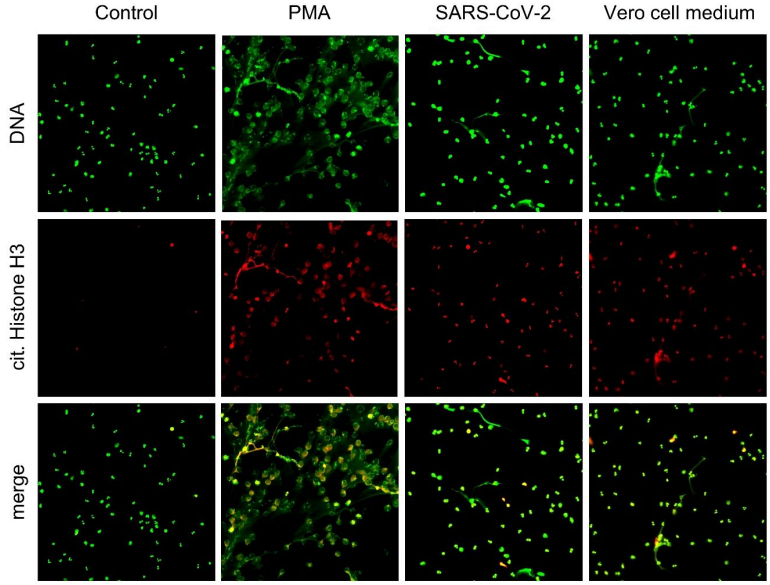

Fig 1 – Detection of NETs in response to SARS-CoV-2 conditioned and control media. Representative images of NETs formation in response to mitochondria (MOI 20), medium from SARS-CoV-2 infected cells (PFU) and control cells incubated with neutrophils for 3 h. NETs are identified according to positive staining for citrullinated histone H3 (red) and DNA (green).

Content of both cell media that induced NETs formation was then analysed by flow cytometry for the presence of DAMPs respresented by submicron particles associated with nucleic acids or mitochondrial components (Fig 2A). The concentration of nucleic acid-associated particles was ~11-times higher in media from SARS-CoV-2 infected cells when compared to the control media, with more than 90% of the particles forming a population with an estimated size up to

150 nm ($p < 0.01$) (Fig 2A, B). When analysed for the presence of mitochondrial components, both media from control and SARS-CoV-2 infected cells contained a similar amount of particles sized between 120-500 nm (Fig 2A, C). Quantity of mtDNA in both media was additionally assessed by qPCR with no difference being observed in mitochondrial DNA copy number (Fig 2D).

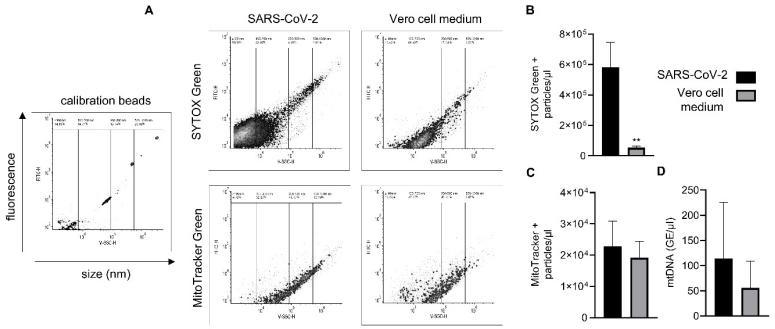

Fig 2 – Analysis of submicron particles in SARS-CoV-2 conditioned and control media. (A) Calibration of flow cytometry for the detection of submicron particles with representative images of nucleic acid-associated particles detected by SYTOX Green and MitoTracker Green. (B, C) Quantity of nucleic acid- and mitochondria-associated particles in media analysed by flow cytometry. (D) Analysis of mtDNA quantity by qPCR expressed as concentration of genomic equivalents / µl. All analyses were independently repeated 3 times. ** = $p < 0.01$

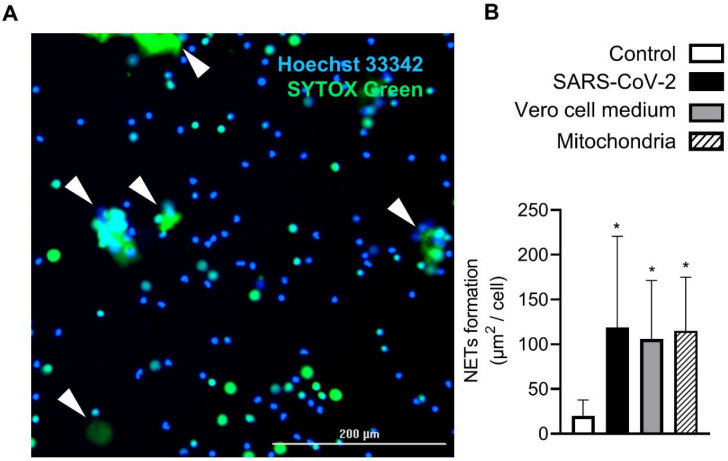

Fig 3 –Live-cell microscopy analysis of NETs formation. (A) Representative image from live-cell microscopy. NETs marked by white arrows are identified according to positivity for SYTOX Green and size > 20 µm. (B) Relative NETs formation in response to mitochondria (MOI20) and medium from SARS-CoV-2 infected (PFU/ml) and control cells. n = 11, * = $p < 0.05$.

Exogenous mitochondria induce NETs similarily to SARS-CoV-2 infected and control media

Finally, NETs formation in response to exougenously administered mitochondria and inactivated SARS-CoV-2 infected or control Vero cell media was analysed by live-cell microscopy (Fig 3A). Concentration of extracellular mitochondria was set to MOI20, which is an average concentration of mitochondria found in both types of previously tested media. All of the samples induced NETs when compared to control and the intensity of the response did not differ between the treatments ($p < 0.05$) (Fig 3B).

Discussion

Formation of NETs was since their discovery found to be induced by a plethora of stimuli including viruses, although their antiviral properties still remain questionable. At the beginning of the pandemic, several studies have shown that SARS-CoV-2 infection results in an aberrant neutrophil activation and release of NETs. Herein, we have tested the capacity of both live and inactivated SARS-CoV-2 virus harvested from media of infected Vero cells to induce NETs formation.

Back in 2020, two research teams were fast to report that both live and heat-inactivated SARS-CoV-2 induce the formation of NETs, but a formaldehyde fixation inhibits their capacity to do so.(6, 7) Veras and colleagues have even proposed a mechanism were the virus stimulates NETs release upon binding to neutrophil angiotensin converting enzyme (ACE2).(6) In both studies, it was observed that SARS-CoV-2 could induce the formation of NETs even at very low concentrations, starting from a MOI of 0.5-1. This dose was surprisingly found to be lower than what is typically required for other pathogenic stimuli, such as bacteria or fungi.

Similar to these studies, we have also cultivated SARS-CoV-2 on an immortalized Vero cell line that is standardly used as a substrate for virus isolation. Interestingly, we have observed that both media derived from SARS-CoV-2 infected Vero cells containing live or heat-inactivated virus and the control media exhibited equal potency in inducing NETs formation. This suggests that the neutrophils can be activated by other components of the media than just the virus itself. Successful spread of a virus requires lysis of the infected cell, which in the case of coronaviruses results in a massive destruction of pneumocytes.(8) We have therefore hypothesized that mitochondria released from dead cells accumulated in cultivation media and were acting as so-called DAMPs (damage-associated molecular patterns). Due to their evolutionary origin, mitochondria share several bacterial features such as formylated peptides and unmethylated DNA that were already described to induce NETs.(9, 10) Indeed, our flow cytometry analysis revealed that media derived from both SARS-CoV-2 infected and control cells contained a lot of mitochondria, which was also confirmed by qPCR.

We have therefore stimulated neutrophils with isolated mitochondria set to a dose found in the Vero cell media and observed a similar rate of NETs induction. We thus propose that

mitochondria released from dying cells during SARS-CoV-2 infection contribute to the aberrant activation of neutrophils resulting in formation of NETs. While this phenomenon needs a more extensive verification, understanding the mechanism of NETs induction during viral infection is vital not just for the COVID-19 pandemic but extends to other viral infections as well.

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8. Sumarizácia výsledkov a pandemických trendov detekcie SARS-CoV-2 v slovenskej populácii

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Úvod

Ako je dnes už známe, pandémia Covid-19 je v modernej svetovej histórii bezprecedentná a spustila príval výskumných aktivít v rôznych oblastiach verejného zdravia, pričom toto úsilie prinieslo rovnako bezprecedentnú záplavu referenčných epidemiologických údajov [1]. Od prvého prípadu detekcie vírusu SARS-CoV-2, ktorý bol zaznamenaný v decembri 2019 v čínskom Wu-chane, sa globálna priestorová dynamika nákazy týmto ochorením rýchlo menila a stále sa mení. Infekcia sa rozšírila z Ázie do Európy a USA, Južnej Ameriky a napokon do celého sveta [2].

Možným spôsobom, ako monitorovať šírenie chorôb a odhadovať vývoj nárastu/ poklesu Covid-19 prípadov, je vykonať rozsiahle laboratórne, ale aj domáce testovanie na detekciu SARS-CoV-2 v populácii – toto opatrenie bolo prijaté aj na území Slovenska. Optimálnou laboratórnou testovacou metódou na zistenie prítomnosti vírusu SARS-CoV-2 vo vzorke je RT-qPCR (Quantitative Real-Time Reverse Transscription Polymerase Chain Reaction). K 6. júnu 2023 Slovensko zaznamenalo celkovo 1 981 096 pozitívnych prípadov koronavírusu z 7 412 925 vykonaných RT-qPCR testov [3].

Monitorovanie RT-qPCR amplifikácie je založené na princípe fluorescencie za využitia fluorescenčných látok viažucich sa na amplifikovanú DNA. Pre každú vyšetrovanú vzorku je následne na základe nameraných fluorescenčných signálov vytvorená amplifikačná krivka [4]. Prahová hodnota cyklu (Ct) predstavuje počet PCR cyklov potrebných na to, aby generovaný fluorescenčný signál prekročil úroveň pozadia – detegovaný údaj Ct hodnoty je súčasťou pozitívneho výsledku RT-qPCR testu [5]. I keď ide o relatívnu veličinu, existuje konsenzus, že jej hodnota môže do určitej miery odrážať vírusovú záťaž a následne indikovať stav infekcie – nízke Ct hodnoty napovedajú o vysokej vírusovej záťaži jedinca a naopak vysoké Ct hodnoty naznačujú potenciálne nízku vírusovú záťaž a teda relatívne nízku úroveň infekčnosti jedinca [6].

Nakoľko má vírus SARS-CoV-2 schopnosť vysokej frekvencie rekombinácie genómu, hrozbou na úrovni populácie sú možné nepredvídateľné zmeny virulencie [7]. Pre dohľad nad šírením obdobných vírusových infekčných ochorení je preto dôležité nájsť taký indikátor, ktorý dokáže predpovedať epidemické/ pandemické trendy v populácii. Autori Pujadas a kol. svojimi výsledkami determinujú, že vírusová záťaž detegovaná na základe Ct hodnôt môže predpovedať úmrtnosť pacientov s Covid-19 [8]. Výsledky českých kolegov Musalkova a kol.

potvrdzujú, že zaznamenaná priemerná týždenná Ct hodnota s určitým časovým oneskorením môže predpovedať zmeny, resp. nárast počtu pozitívnych Covid-19 pacientov [9].

Na základe vyššie uvedených zistení sme sa v našej štúdii zamerali na výsledky RT-qPCR detekcie vírusu SARS-CoV-2 v laboratóriách Medirex a.s. v kontexte s parametrami vírusovej záťaže (odhadom pomocou hodnôt Ct) medzi charakterizovanými podskupinami pozitívnych jedincov detegovaných v dátumovom období marec 2020 – september 2022 s cieľom zhrnúť výsledky a trendy laboratórneho testovania prítomnosti SARS-CoV-2 vo vzorkách pochádzajúcich zo slovenskej populácie z obdobia masívnej Covid-19 PCR detekcie.

Materiál a metódy

Štúdia analyzovala údaje poskytnuté akreditovaným laboratóriom Medirex. Údaje predstavujú výsledky RT-qPCR testov hodnotených od marca 2020 do septembra 2022. RT-qPCR detekcia bola vykonaná v centrálnych laboratóriách v Bratislave, Košiciach a Nitre. Počas tohto obdobia laboratóriá zanalyzovali 1 420 572 testov.

Prítomnosť SARS-CoV-2 sa stanovovala z nazofaryngeálnych výterov a vzoriek slín, nakoľko odberové miesta na Slovensku poskytovali tieto dve možnosti odberu biologického materiálu. Automatizovaná izolácia nukleovej kyseliny (RNA) na magnetických časticiach bola uskutočnená pomocou súprav Sera-Xtracta Virus/Pathogen Kit (Cytiva) a Zybio Nucleic Acid Extraction Kit (Zybio) s využitím systému KingFisher™ Flex Purification System (Thermo Scientific), Zybio EXM 3000 Nucleic Acid Isolation System a Zybio EXM 6000 Nucleic Acid Isolation System (Zybio). Testovanie sa uskutočnilo metódou RT-qPCR s použitím súprav COVID-19 Real Time Multiplex RT-PCR Kit (Labsystems Diagnostics), SARS-CoV-2 Nucleic Acid Detection Kit (Zybio) a Real Time Multiplex RT-PCR Kit (Liferiver) pomocou qPCR platforiem ABI 7500 (Fast) Real-Time PCR System (Applied Biosystems), QuantStudio 5 a QuantStudio 6 Real-Time PCR System (ThermoFisher). Údaje získané pre každý test zahŕňali denné číslo vzorky, dátum odberu, ID pacienta, vek, pohlavie, miesto odberu vzorky, výsledok testu, a v prípade pozitívneho výsledku aj hodnotu Ct vírusového génu E - hraničná hodnota do 40 (pre kit SARS-CoV-2 Nucleic Acid Detection Kit (Zybio)) alebo 41 (pre kity Real Time Multiplex RT-PCR Kit (Labsystems Diagnostics) a Real Time Multiplex RT-PCR Kit (Liferiver)) - t.j. sekvencia nukleovej kyseliny bola identifikovaná v čase, keď PCR prešla 39 alebo 40 cyklami. Hodnota Ct bola spolu s pozitívnym výsledkom testu laboratóriom Medirex, a.s. uvádzaná a archivovaná od 3. decembra 2020, čo vysvetľuje nižšiu početnosť pozitívnych PCR testov s uvedenou Ct hodnotou v porovnaní s celkovým počtom pozitívnych PCR testov v rámci tejto štúdie.

Údaje boli kategorizované do deviatich skupín podľa veku/ úrovne navštevovanej školy: novorodenci a batoľatá (0-2 roky); deti predškolského veku (3-5 rokov); žiaci ZŠ I (6-8 rokov); žiaci ZŠ II (9-13 rokov); žiaci ZŠ III (14-15 rokov); mládež I/ žiaci SŠ (16-19 rokov); mládež II/ študenti VŠ (20-26 rokov); dospelí (27-65 rokov) a seniori (66 rokov a starší).

Výsledky

Od marca 2020 do septembra 2022 laboratóriá Medirex, a.s. vykonali celkovo 1 420 572 RT-qPCR testov na diagnostické účely detekcie SARS-CoV-2 vo vzorke, čo za dané obdobie predstavuje viac ako 19 % z celkového počtu testov RT-qPCR na Slovensku. Celková pozitivita

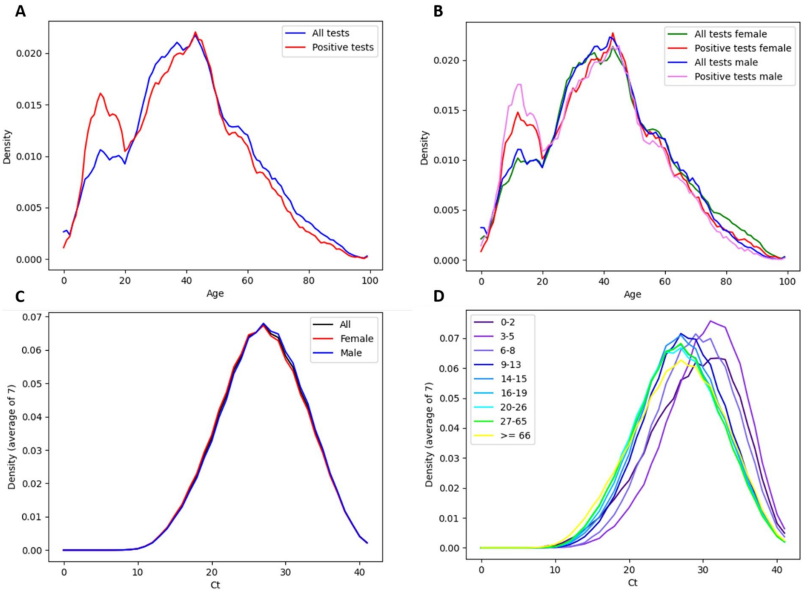
analyzovaných testov bola 24,64 % (350 067 testov), pričom najvyššiu mieru pozitivity (nad 30 %) dosiahli vekové kategórie: 6-8 r, 9-13 r, 14-15 r, 16- 19 r (Tabuľka 1).

Obrázok 1 zobrazuje vekové rozdelenie testovaných jedincov a pozitívnych jedincov (1A), respektíve vekové rozdelenie testovaných a pozitívnych jedincov v závislosti od pohlavia – muž/ žena (1B). Najviac testovaných jedincov bolo vo veku 43 rokov (30 895 jedincov). Rozdelenie hodnôt Ct u pozitívne testovaných jedincov pravdepodobne nie je unimodálne, pričom priemerný medián hodnôt Ct je rovný 27,9 (1C). Zistené hodnoty Ct sa pohybovali od 9,5 do 41. Najvyššie priemerné hodnoty Ct boli dosiahnuté vo vekovej skupine 3-5 r, rovné číslu 30,75, najnižšie priemerné hodnoty Ct boli dosiahnuté vo vekovej skupine > 65 r a dosahovali číslo 27 (1D).

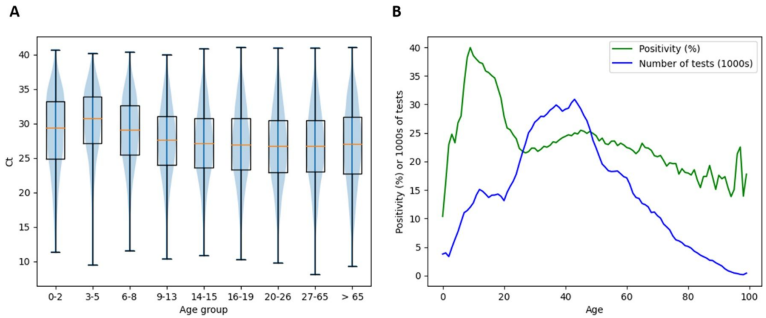
Obrázok 2 znázorňuje rozdelenie hodnôt Ct/ celkovej pozitivity v závislosti od vekovej skupiny jedincov. Husľový graf ukazuje postupné znižovanie hodnôt mediánu Ct spolu so zvyšujúcim sa vekom kategorizovaných vekových skupín. Najvyššia hodnota mediánu Ct bola zistená vo vekovej skupine 3-5 r, rovnala sa číslu 30,75. Najnižšia hodnota mediánu Ct bola zistená v skupinách 20-26 r a 27-65 r, rovnala sa číslu 26,7 (2A). Celková pozitivita nad 30 % bola zaznamenaná u jedincov vo veku 7-19 r (2B).

Tabuľka 1: Počet pozitívnych/ negatívnych RT-qPCR testov a celková pozitivita testov pre jednotlivé vekové kategórie.

Veková kategória	Počet pozit. testov	Počet negat. testov	Celkový počet testov	Pozitivita (%)
0-2 r	1 806	9 273	11 079	16,30
3-5 r	4 757	14 201	18 958	25,09
6-8 r	10 689	21 112	31 801	33,61
9-13 r	26 193	42 544	68 737	38,11
14-15 r	9 983	17 968	27 951	35,72
16-19 r	18 839	37 543	56 382	33,41
20-26 r	28 861	90 078	118 939	24,27
27-65 r	216 104	703 904	920 008	23,49
>65 r	32 835	133 882	166 717	19,70
Celkovo	350 067	1 070 505	1 420 572	24,64



Obrázok 1: **(A)** Vekové rozloženie všetkých testovaných jedincov (modrá krivka), pozitívne testovaných jedincov (červená krivka). **(B)** Vekové rozdelenie všetkých testovaných žien (zelená krivka), pozitívne testovaných žien (červená krivka), všetkých testovaných mužov (modrá krivka), pozitívne testovaných mužov (ružová krivka). **(C)** Rozdelenie Ct hodnôt všetkých diagnostických testov (čierna krivka), testov vykonaných u žien (červená krivka), testov vykonaných u mužov (modrá krivka). **(D)** Rozdelenie Ct hodnôt všetkých diagnostických testov – údaje sú rozdelené podľa vekových skupín jednotlivcov. **(A-D)** Údaje sú zobrazené ako grafy hustoty.



Obrázok 2: (A) Distribúcia Ct pozitívnych diagnostických testov v rôznych vekových skupinách. Dáta sú zobrazené ako husľové grafy a vrúbkované boxploty sú doplnené o hodnoty Ct mediánu (oranžové segmenty). **(B)** Rozdelenie miery pozitivity podľa veku jedincov (zelená krivka) a rozdelenie počtu testov podľa veku jedincov (modrá krivka).

Diskusia

Pandémia Covid-19 zasiahla mnohé oblasti medicíny a zdravotnej starostlivosti po celom svete. Jej ďalší vývoj bolo/ je veľmi ťažké predpovedať, pričom neschopnosť predvídať nárasty alebo poklesy v prípadoch infekcie SARS-CoV-2 výrazne ovplyvnila schopnosť autorít verejného zdravotníctva reagovať na krízu vyvolanú pandémiou a prijímať potrebné opatrenia vo verejnom sektore. Včasná detekcia vírusu SARS-CoV-2 pomocou diagnostických metód je preto stále kľúčová – RT-qPCR poskytuje vysoký diagnostický potenciál vďaka v súčasnosti dobre rozvinutej laboratórnej infraštruktúre. V našej štúdii sme analyzovali, či a ako korelujú priemerné Ct hodnoty pozitívnych RT-qPCR testov so zvyšujúcim sa vekom jedincov subpopulácie obyvateľov Slovenska testovaných v jednom akreditovanom laboratóriu.

Skutočná lokálna prevalencia a počet vykonaných testov ovplyvňujú hodnotu miery pozitivity. Vo všeobecnosti, čím viac testov bolo diagnostikovaných, tým sa miera pozitivity viac približuje skutočnej prevalencii [10]. Analyzované testy tejto štúdie predstavujú > 19 % všetkých diagnostických RT-qPCR testov na Slovensku (za daný dátumový interval) z > 93 % okresov SR, takže by mali byť reprezentatívne pre populačnú mierku.

Z údajov vyplynulo, že najvyššiu mieru pozitivity mali žiaci základných a stredných škôl (6-19 r) (Tabuľka 1, Obrázok 2B), tento jav pravdepodobne súvisí s nižšou frekvenciou testov v daných vekových podskupinách. Okrem toho mali žiaci počas veľkej časti pandémie zatvorené školy [11] a nemuseli sa podrobovať pravidelnému PCR testovaniu (povinné napr. pre niektoré skupiny zamestnancov na pracovisku). Preto boli maloletí zrejme podrobení PCR diagnostike prítomnosti SARS-CoV-2 až v prípade príznakov ochorenia Covid-19, čo mohlo prispieť k zistenej vyššej miere celkovej pozitivity v tejto vekovej podskupine.

Výsledky niekoľkých štúdií preukazujú, že distribúcie Ct hodnôt v prípadoch skríningu SARS-CoV-2 sú typicky bimodálne [12, 13]. Existuje predpoklad, že nižšia maximálna Ct hodnota zodpovedá pacientom s vysokou infekčnosťou a vyššia maximálna Ct hodnota bimodálneho rozdelenia zodpovedá pacientom s nízkou infekčnosťou [13]. Bimodálna distribúcia je typická

aj pre iné vírusové infekcie, pričom najnižšie Ct hodnoty označujú podiel jedincov s vysokou vírusovou záťažou v akútnej fáze infekcie a vyššie Ct hodnoty sú typické pre skorú fázu infekcie alebo fázu rekonvalescencie [6, 14, 15]. Tento predpoklad by mohol vysvetľovať, prečo rozdelenie priemerných Ct hodnôt našich údajov nie je unimodálne.

Ďalej sme sa zamerali na distribúciu Ct hodnôt v rôznych vekových skupinách, aby sme určili, ktoré skupiny môžu najviac prispieť k šíreniu infekcie. Graf na obrázku 2A determinuje, že pokles priemerných Ct hodnôt bol konzistentný so zvyšujúcim sa vekom jedincov v kategorizovaných vekových podskupinách. Toto pozorovanie je v súlade s tvrdením, že deti s infekciou SARS-CoV-2 sú často asymptomatické alebo mierne symptomatické a len zriedka sú prípadom indexu v prenosových reťazcoch v domácnostiach [16].

Existuje niekoľko faktorov, ktoré môžu potenciálne ovplyvniť zistené Ct hodnoty, jedným z nich je typ vzorky (napr. nazofaryngeálna, predná sliznica nosa, sliny, spútum) [17]. Detekcia nižších Ct hodnôt u detí môže súvisieť s kvalitou odberu, nakoľko je potrebné zaviesť odberovú paličku do nosohltana [18]. Odber slín bol zavedený do praxe práve pre komplikácie odberov z nosohltanu u určitých skupín jedincov – výraznú bolesť pri výteroch z nosohltanu popísalo 58 % mladých ľudí oproti žiadnej pri odbere slín, pričom odber slín preferuje až 90 % detí. [19]. Výsledky štúdií preukazujú, že detekcia zo vzoriek slín funguje rovnako alebo lepšie ako výtery z nosohltanu v nemocničných, pohotovostních či skríningových zariadeniach na detekciu SARS-CoV-2 pomocou RT-qPCR [20-23]. Priemerná hodnota Ct v slinách však môže byť vyššia v porovnaní s priemernou hodnotou Ct z nazofaryngeálnych výterov [24]. Preto sa ako vhodnejšia vzorka na diagnostiku RT-qPCR zvyčajne odporúča kombinovaný výter z nosa a hrdla [17]. Keďže sme do tejto štúdie chceli zahrnúť všetky vzorky od marca 2020 do septembra 2022 poskytnuté laboratóriom Medirex, a.s. s priradeným pozitívnym/negatívnym výsledkom, údaje zahŕňajú výtery z nosohltanu aj vzorky slín. Výsledky reprezentujúce detekciu SARS-CoV-2 zo vzoriek slín však predstavujú menej ako 5 % všetkých vzoriek, a preto nepredpokladáme, že by táto skutočnosť mohla ovplyvniť celkovú výpovednú hodnotu priemerných hodnôt Ct pre údaje tejto štúdie.

Poďakovanie

Táto práca vznikla vďaka podpore v rámci Operačného programu Integrovaná infraštruktúra pre projekt: Výskum progresívnych metód diagnostiky COVID-19 a biomarkerov umožňujúcich skorú detekciu jedincov so zvýšeným rizikom ťažkého priebehu ochorenia, kód ITMS: 313011ATA2, spolufinancovaný zo zdrojov Európskeho fondu regionálneho rozvoja a s podporou Agentúry na podporu výskumu a vývoja na základe zmluvy č. PP-COVID-20-0056.

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9. Detection of SARS-CoV-2 in the saliva samples using loop-mediated isothermal amplification

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Introduction

The first cases of unknown acute atypical respiratory infection emerged in the city of Wuhan (China) in December 2019. Soon, novel betacoronavirus SARS-CoV-2 [1] was identified. Due to the rapid spread worldwide, the World Health Organization (WHO) declared a pandemic in March 2020 [2]. Testing and rapid identification of infected persons continues to play a key role in limiting the spread of the virus in the population.

Reverse real-time polymerase chain reaction (qRT-PCR) became very quickly the standard and accurate diagnostic method for the detection of SARS-CoV-2 ribonucleic acid (RNA) in nasopharyngeal swab samples. However, the collection of nasopharyngeal swabs faces several challenges. Firstly, it requires specialized medical personnel to perform the sample collection. These medical personnel are exposed to the risk of transmission of the virus from the infected patients. Secondly, laboratory facilities and time to provide results (from several hours to 1 day) are important factors when comes to prompt diagnostic. Moreover, other disadvantages are possible difficulties in sampling and patient discomfort [3]. Non-invasive sampling suitable for the diagnostic of viral infection – such as saliva collection, could resolve many difficulties associated with nasopharyngeal swabs. Patients can collect saliva by themselves, not exposing medical personnel to the risk of infection [4].

Even though qRT-PCR is the diagnostic standard for the presence of viral RNA in samples, it requires technical equipment such as temperature cycler with fluorescence detection. An alternative method to qRT-PCR is loop-mediated isothermal amplification coupled to reverse transcription (RT-LAMP), which can also detect specific viral RNA sequences [5]. RT-LAMP reaction is isothermal, and it does not require a temperature cycler or other special equipment except for a thermostat maintaining the temperature at 65°C [6]. The result can be analyzed by visual inspection using a pH-sensitive dye after only 30 minutes of incubation [7]. The reaction time, simple procedure, use of saliva as a sample and visual analysis of the result are ideal and important for quick diagnosis of the patient, for example in the hospital. The aim of this study was to verify whether the diagnosis of the SARS-CoV-2 is possible using the RT-LAMP method on saliva samples.

Materials and methods

Sample collection and processing

One cohort of saliva samples was collected from volunteers (n=120) suspected of being infected with the SARS-CoV-2 virus at the University Hospital Ružinov in Bratislava. At the same time, nasopharyngeal swabs were collected by the medical staff and sent to the diagnostic laboratory for qRT-PCR. Diagnostic laboratory provided information whether samples were positive or negative.

Second cohort of saliva samples was collected from volunteers (n=58) tested positive on antigen test (STANDARD Q COVID-19 Ag, SD Biosensor, South Korea) at the University Hospital Ružinov in Bratislava. Saliva samples were divided, and one aliquot was used for RNA isolation and second aliquot was used directly in RT-LAMP. Total RNA was isolated from 400 µL of the whole saliva using RNeasy Plus Mini kit (Qiagen, Hilden, Germany) followed manufacturer protocol.

Whole saliva in both cohorts was collected by volunteers by spitting into a sterile tube and delivered to our laboratory. To avoid potential infections, but also as a part of sample processing, saliva samples were inactivated by heating at 95°C for 5 min. In the saliva samples, with a sufficient volume, the pH value of the saliva was measured. Saliva samples were used for direct testing in the RT-LAMP reaction and aliquots were stored at −20°C.

Colorimetric RT-LAMP

To assess sensitivity of RT-LAMP reaction 2-fold serial dilution from 100 copies to 1 copy of the EDX SARS-CoV-2 RNA Standard (Exact Diagnostics, Fort Worth, Texas, USA) was used as the RNA template for the RT-LAMP. Primers used in RT-LAMP are targeting spike protein S-123 and were designed by Yan et al. 2020 [9]. All primers were synthetized by Microsynth AG, Balgach, Switzerland. A 10 µl reaction mixture contained WarmStart® Colorimetric LAMP 2x Master Mix DNA and RNA (New England Biolabs, Ipswich, MA, USA) – 5 µl; 10x primer mix – 1 µl; nuclease free H_2O – 3 µl and RNA template – dilutions of SARS-CoV-2 RNA Standard – 1 µl. The results of the color reactions were evaluated with naked eye after a 30 min incubation at 65°C in an Eppendorf ThermoMixer C (Eppendorf, Hamburg, Germany).

For testing saliva samples, RT-LAMP reaction with a total volume of 10 µl was prepared by mixing WarmStart® Colorimetric LAMP 2x Master Mix DNA and RNA (New England Biolabs, Ipswich, MA, USA) – 5 µl; 10x primer mix [9] – 1 µl; nuclease free H_2O – 1 µl; examined saliva sample or positive control (EDX SARS-CoV-2 RNA Standard) or negative control (H_2O) - 3 µl. The RT-LAMP reaction was performed at 65°C for 30 min in an Eppendorf ThermoMixer C (Eppendorf, Hamburg, Germany). After incubation, we observed the reaction with the naked eye and determined the result based on color change. In the case of a positive sample or control, the reaction mixture changed color from pink to yellow, and in the case of a negative sample, the reaction mixture remained pink.

One-step qRT-PCR

qRT-PCR reaction with total volume 10 µL was prepared by mixing QIAGEN QuantiTect SYBR Green RT-PCR master mix (Qiagen, Hilden, Germany) - 5 µL; 500 mM forward and reverse primers [10] for N1 and N3 gene of SARS-CoV-2 (Microsynth AG, Balgach, Switzerland) – 0.5 µL each; QuantiTect RT mix – 0.1 µL; nuclease free H_2O – 1.4 µL and RNA template – 2.5 µL. As a positive control we used viral RNA isolated from cell culture supernatant obtained from Vero E6 cells infected with SARS-CoV-2 virus strain Slovakia/SK-BMC5/2020. One-step real-time qRT-PCR thermocycling conditions were set as described in the Table 1 using Eppendorf realplex^4 Mastercycler epgradient S (Eppendorf, Hamburg, Germany).

Table 1: Thermocycling conditions for one-step qRT-PCR detection of N1, N3 genes of SARS-CoV-2.

Step	Temperature	Time	Cycles
1. Reverse transcription	50°C	30 min	1
2. Initial denaturation	95°C	15 min	1
A. Denaturation	95°C	15 sec	45
B. Annealing	55°C	30 sec	
C. Extension	65°C	30 sec	
• melting curve			

Statistical analysis

We compared the results of saliva RT-LAMP with the results of qRT-PCR of nasopharyngeal swabs. Based on this, we determined which samples were positive, false positive, negative, and false negative. Subsequently, we mathematically determined the accuracy, sensitivity, and specificity of the RT-LAMP reaction. All graphs were done using GraphPad Prism 9.3.1 Software (GraphPad Software, San Diego, California, USA).

Results

The sensitivity of RT-LAMP reaction was assessed using 2-fold dilution of EDX SARS-CoV-2 RNA Standard from 100 copies to 1 copy per reaction. We evaluated results based on color change (yellow – positive or pink – negative) after 30-minute incubation at 65°C. The primer mix [9] we used shown the lowest limit of detection (LOD) at 6 copies of the EDX SARS-CoV-2 RNA Standard per reaction (Figure 1).

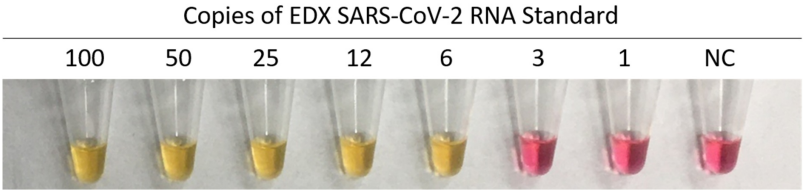

Figure 1: Sensitivity of RT-LAMP using the 2-fold dilution of EDX SARS-CoV-2 RNA Standard. Yellow color – positive result; pink color – negative result; NC - negative control.

In the first cohort, we have tested saliva samples (n=120) using RT-LAMP. We compared RT-LAMP results to the qRT-PCR results (positive/negative) from diagnostic laboratory and evaluated three samples as positive (2.5%), two samples as false positive (1.7%), one hundred three samples as negative (85.8%) and twelve samples as false negative (10%) (Figure 2). We calculated the accuracy of the RT-LAMP test as the proportion of positive and negative samples against all tested parameters. Based on our results, the accuracy of the RT-LAMP reaction is 88.3%. We calculated the sensitivity of the RT-LAMP test as the proportion of positive samples to positive and false negative samples. Based on our results, the sensitivity of the RT-LAMP reaction is 20%. As a third parameter, we calculated the specificity of the RT-LAMP reaction as the proportion of negative samples against negative and false positive samples. The specificity of the RT-LAMP reaction is 98% based on our results.

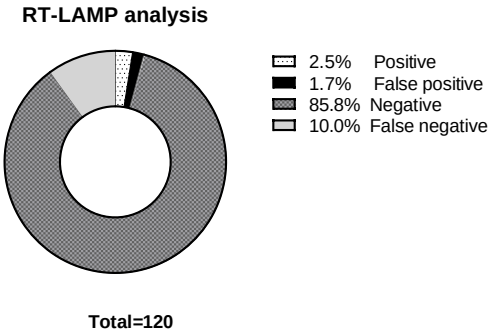

Figure 2: Percentage analysis of RT-LAMP results with direct use of saliva.

In the second cohort, we have tested in RT-LAMP saliva samples (n=58) previously tested positive on COVID-19 antigen test. We compared RT-LAMP results to the Ct values obtained from one-step qRT-PCR (Figure 3) and evaluated twenty-three samples as positive (39.7%), nine samples as false positive (15.5%), nine samples as negative (15.5%) and seventeen samples as false negative (29.3%) (Figure 4). We calculated the accuracy of the RT-LAMP test as the proportion of positive and negative samples against all tested parameters. Based on

our results, the accuracy of the RT-LAMP reaction is 55%. We calculated the sensitivity of the RT-LAMP test as the proportion of positive samples to positive and false negative samples. Based on our results, the sensitivity of the RT-LAMP reaction is 58%. As a third parameter, we calculated the specificity of the RT-LAMP reaction as the proportion of negative samples against negative and false positive samples. The specificity of the RT-LAMP reaction is 50% based on our results.

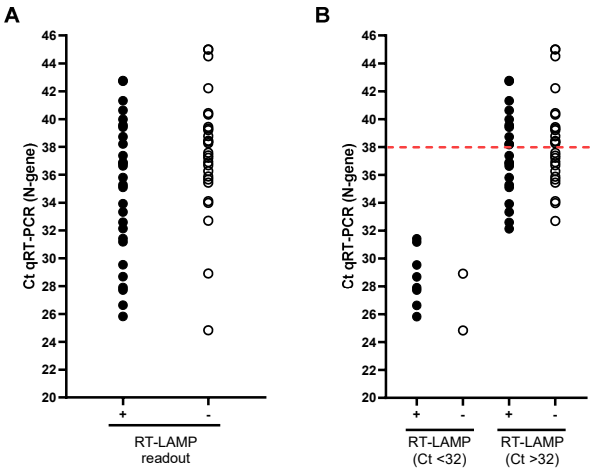

Figure 3: Detection of SARS-CoV-2 in saliva samples using RT-LAMP. A – Comparison of RT-LAMP and qRT-PCR results. Average Ct values of N1 and N3 gene (qRT-PCR) of 58 COVID-19 antigen positive samples (y-axis) were compared to the RT-LAMP readout (x-axis) after 30-minute incubation at 65℃ where yellow color presented positive (+) result and pink color presented negative (-) result. B - Comparison of RT-LAMP and range of Ct values (qRT-PCR). Average Ct values of N1 and N3 gene (qRT-PCR) of 58 COVID-19 antigen positive samples (y-axis) were divided to two range groups from Ct <32 and Ct >32 and compared to the RT-LAMP readout (x-axis) after 30-minute incubation at 65℃ where yellow color presented positive (+) result and pink color presented negative (-) result. Red dashed line indicates cut off – samples with Ct values below 38 are positive and samples with Ct value 38 and above are negative for presence of viral RNA.

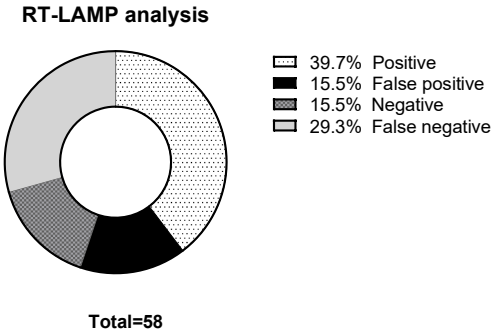

Figure 4: Percentage analysis of RT-LAMP results with direct use of saliva.

In the second cohort, we were able to measure pH value in 57 from 58 saliva samples. We divided samples to four groups: true negative, true positive, false negative and false positive samples and compared pH values (Figure 5). Analysis did not show any significant differences.

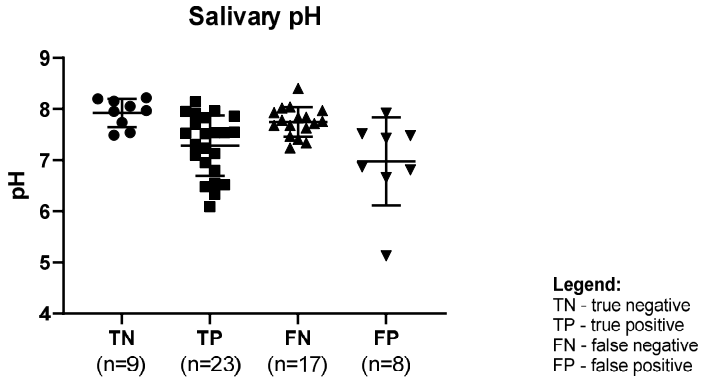

Figure 5: Effect of salivary pH on RT-LAMP.

Discussion

The aim of this study was to verify whether the diagnosis of the SARS-CoV-2 is possible using directly saliva samples in the RT-LAMP method without previous nucleic acid extraction. In our study we assessed sensitivity of RT-LAMP using EDX SARS-CoV-2 RNA Standard with LOD 6 copies per reaction. Furthermore, we tested two cohorts of saliva samples in RT-LAMP. Our results showed that in the first cohort of saliva samples (n=120) we are able to detect SARS-CoV-2 directly from saliva using the RT-LAMP with an accuracy of 88.3%, a specificity of 98%, but a very low sensitivity - in our case, only 20% compared to qRT-PCR (positive/negative) results. In the second cohort of saliva samples (n=58) we detected SARS-CoV-2 directly from saliva with the accuracy of 55%, the specificity of 50% and sensitivity of 58% compared to Ct values of SARS-CoV-2 N gene in qRT-PCR.

Saliva itself is probably one of the factors affecting the sensitivity of the RT-LAMP reaction. The main disadvantage of saliva as a direct diagnostic fluid is low salivation in some patients or high viscosity of saliva, which results in difficulties with its processing in the laboratory. Furthermore, Uribe-Alvarez et al. in their study showed that a low pH of saliva leads to a false positive RT-LAMP result, and at the same time, the stabilization of pH fluctuations due to the acidity of saliva leads to a significant improvement in the reliability of the RT-LAMP test [8]. However, in our study comparison of pH values between true positive and false positive results did not show any difference.

Based on our results, we can confirm that we can detect the SARS-CoV-2 virus directly in a saliva sample without prior RNA isolation using the RT-LAMP method. Saliva as a diagnostic fluid has many advantages – it can be simply, non-invasively collected anywhere, and at the same time it does not require trained medical personnel. However, some characteristics of saliva (e.g. low pH) can negatively affect the result of the RT-LAMP reaction [8]. Nevertheless, RT-LAMP represents a rapid method with a simple procedure and detection of the result, which are of utmost importance for testing at the point of care (bedside testing) in various emerging diseases such as COVID-19. However, for the use of saliva and the RT-LAMP method in clinical practice, further optimization to increase the sensitivity of the diagnostic test is necessary.

Acknowledgements

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10. The national wastewater surveillance program: a useful method for regular monitoring of circulating SARS-CoV-2 variants

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Introduction

The World Health Organization still considers the pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) a public health threat, even three years after the declaration of the Coronavirus disease (COVID-19) pandemic (1). The recent statement from the WHO ending the global health emergency does not mean that the system built to cope with the pandemic will be dismantled (2). Many states initially developed surveillance systems to detect and monitor the SARS-CoV-2 virus, but as the pandemic evolved, new variants of concern emerged. Clinical based surveillance tended to be outbalanced due to limitations like the availability of testing capacities and high costs of individual testing (3). Wastewater based (WWB) surveillance emerged as a viable alternative to estimate the percentages of circulating variants in the population, but there are limitations in the quality of isolated RNA and different viral loads (4). To overcome these limitations, pipeline processing and new bioinformatic tools needed to be developed to analyze wastewater based WWB samples.

This study presents data collected from more than 60 municipal wastewater collecting facilities in the Slovak Republic from January to August 2022. We compared WWB data with clinical specimens to show how these two separate epidemiology approaches worked alongside during the observed time. Collecting sludge from all environments is a feasible method to monitor SARS-CoV-2 virus abundance and variant composition circulating in the community. WWB surveillance can play an essential role in the next epidemic or pandemic of human pathogens, as data generated by WWB are very informative, just like data gained by collecting and sequencing individual clinical samples.

Methods

Wastewater sample collection and processing

This study analyzed data from wastewater samples collected over a period of nine months in 2022 (January-August) from 64 different municipal wastewater treatment plants (WWTP). The samples, collected once per week or every second week, were taken by external partners and consisted of sewage influent water prior to any treatment. Using an automatic collector, the samples were collected over 24 hours and transported in 250 mL composite samples at 4°C to Regional Public Health Authorities (RPHA) including the Public Health Authority of the Slovak Republic in Bratislava (PHA SR).

The primary sewage influent samples were processed (centrifugation, clarification of supernatant and final pellet resuspension) by PHA SR. RNA was extracted with the QIAamp Viral RNA Mini kit following the manufacturer's protocol.

SARS-CoV-2 clinical samples data

For this study, we analyzed data from positive SARS-CoV-2 cases in the Slovak Republic that were deposited in the public repository Global Initiative on Sharing All Influenza Data – GISAID (5). The data is readily available to the public. We selected individual samples and their corresponding metadata (n = 24,247) based on the date of sample collection, which ranged from January 1, 2022 to August 31, 2022. The samples were collected randomly from all districts of the SR through testing centers (6).

RNA isolation

We extracted nucleic acid from all concentrated wastewater samples. The RNA isolation process was carried out at the PHA SR. Using the QIAamp Viral RNA Mini Kit, we extracted viral RNA from the concentrated samples according to the manufacturer's protocol. After RNA isolation, we stored the samples in a 96-well plate at −80 °C. The plates with isolated RNA were then processed by Comenius University Science Park in Bratislava (CU SP) sequencing center.

NGS sequencing

The genomic laboratory at CU SP used Illumina sequencing platforms to sequence the SARS-CoV-2 virus. We prepared whole-genome sequencing libraries in 96-well plates, with 95 samples and one non-template control, following the Illumina COVIDSeq Test protocol. The libraries were amplified using the COVIDSeq V4 Primer Pool based on the ARTIC protocol and tagmented with IDT for Illumina PCR Unique Dual Indexes Set 1-4 (384 Indexes). The libraries were then purified, pooled according to the manufacturer's guidelines, quantified, and normalized to 2 nM. The Illumina sequencing systems used were NextSeq 500 and NextSeq 2000, with 2 x 74 bp paired-end sequencing parameters for NextSeq 500 and 2 x 100 bp paired-end for NextSeq 2000.

Bioinformatic pipeline

We used the Cutadapt tool (7) to remove any adapters and low-quality ends from the sequenced reads, based on quality control statistics generated by FastQC (v0.11.5) (8). Then we eliminated any extraneous RNA fragments using a decontamination process. To map the reads to the SARS-CoV-2 reference genome, we used BWA (9) and to sort and index the generated SAM/BAM files, we used SAMtools (10). We removed duplicated reads originating from the same DNA fragment and produced by PCR using the Picard tool (11). Quality statistics were summarized using the Qualimap 2 tool (12). Finally, we used the Freyja tool (13) to

determine the proportions of the selected SARS-CoV-2 variant (Delta, Omicron - BA.1/BA.2/BA.3/BA.4/BA.5) by measuring SNV frequency and sequencing depth at each position in the genome. All computational analyses were written and executed using the SnakeLines framework (14,15), and they are independent of the length of the sequencing reads.

A new system called NarCoS (short for Národné COVID-19 Sekvenovanie) was launched in February 2022 to improve data transfer during sequencing processes. It integrates sample data, metadata management, and processing information, allowing for unified analysis, verification, and batch uploading to repositories. The system's data analysis module is the previously mentioned pipeline. NarCoS is an integrated system for national COVID-19 sequencing.

Results

Wastewater sample collection

In Slovakia, 69.3% of the population is connected to public sewage systems (16). This study analyzed samples from 64 WWTPs. Based on the 2021 census, cities where participated WWTPs are located represents approximately 2,180,000 residents (17). The samples were collected from eight regions in the Slovak Republic, with coverage ranging from 31% to 59.6% of the population in each region. The WWTPs serve areas with populations ranging from 5,870 to 172,000 people (17). SARS-CoV-2 positivity in all samples was confirmed by PHA SR. A total of 1,715 virus-positive sewage samples were collected over nine months in 2022, and 1,126 high-quality samples were used for further analysis.

Wastewater SARS-CoV-2 lineages compositions

We used the bioinformatic deconvolution tool Freya (13) to analyze all wastewater samples and received results in the form of the proportion of selected SARS-CoV-2 variants and sublineages. The category - Minor sublineages, represents data of virus genomes with small proportions from entire sample set for specific time point. At the start of 2022, the most prevalent VOC Delta (B.1.617.2) began to decline and be replaced by the next VOC Omicron (B.1.1.529). In February 2022, most of the viruses detected in positive wastewater samples were new VOC Omicron. The dominant BA.1 sublineage (B.1.1.529.1) was gradually outcompeted in March and April 2022 by the BA.2 (B.1.1.529.2) sublineage. BA.2 was detected in wastewater samples until the end of May (21-22/2022). However, the new Omicron sublineage BA.5 (B.1.1.529.1.5) started to spread in the population and continued to do so until week 28 and beyond. From weeks 24-26/2022, most of the wastewater samples were marked as unknown, and we could not assign to specific sublineage. These results were the similar across all state WWTPs. At the beginning of June (22-23/2022), we started to detec Omicron sublineage BA.5 in some wastewater samples. By week 28 and beyond, almost all positive samples showed the presence of the BA.5 sublineage (Figure 1).

We also analyzed wastewater samples separately for each region to track development of the SARS-CoV-2 epidemic in different parts of Slovakia. Delta variant decreased similarly in all regions and was not detected after the fourth week of most regions. From the fifth week, Omicron outcompeted Delta in all regions and the pattern shown in figure 1 was similar on regional level.

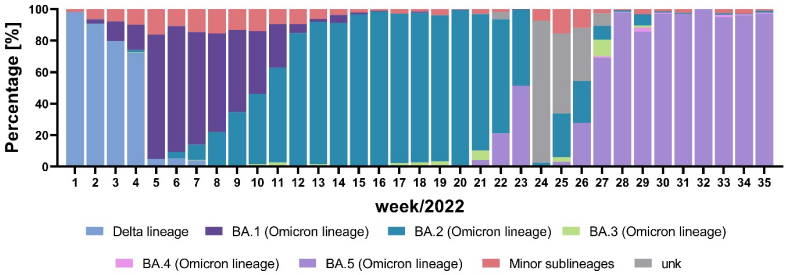

Figure 1: The weekly occurrence of SARS-CoV-2 lineages in Slovakia, as determined from samples taken from wastewater.

Wastewater and clinical surveillance comparison

We compared and verified longitude observations of wastewater based epidemiology by integrating surveillance data from SARS-CoV-2 sequenced clinical cases. Using metadata from public repositories and samples collected from January to August 2022, (n = 24,247), we divided SARS-CoV-2 samples and their corresponding variants into six groups. We expressed the total cases per week for each sublineage as a proportion and analyzed them. The result was that wastewater based epidemiology system accurately detected the onset of new sublineages within one or two weeks after detection of first clinical cases.

Discussion

In 2022, two years after the first cases of an unidentified lung disease, later linked with the SARS-CoV-2 virus (18), the Delta variant and its sublineages were still the most prevalent in Slovakia (19). However, many countries, including those in Europe, began to see a surge in a new variant called Omicron (20), which had more mutations than any previous variants. In Slovakia, the WWB epidemiology team already started a virus-sequencing program a few weeks before Omicron wave. As part of this effort, all regions of Slovakia began routinely collecting wastewater for sequencing and variant determination.

This study provides an overview of the spread of SARS-CoV-2 variants in Slovakia using a wastewater based (WWB) surveillance approach. Over 60 WWTPs were used to monitor the situation across the country. The Omicron variant was detected in wastewater samples in the second and third week of 2022, when a decline of Delta variant was noticeable in clinical samples. The Omicron variant quickly became dominant in all regions of Slovakia. The BA.1 sublineage was initially dominant but was later outcompeted by the BA.2 sublineage, which remained dominant until the emergence of the BA.5 sublineage. The quality of the samples used in WWB monitoring is crucial to the surveillance process. We have found that this

approach is sensitive and reliable, especially when compared to clinically positive cases. Additionally, WWB surveillance is an effective method for monitoring the prevalence of SARS-CoV-2 variants.

Overall, the study demonstrated the high potential, reliability, and accuracy of using the WWB surveillance approach for future epidemiology tools for public health safety policy.

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11. The impact of COVID-19 pandemic on mental health of Slovak families with typically developing children or children with autism spectrum disorder

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Introduction

Soon after the beginning of COVID-19 pandemic, first research studies from around the world started pointing out several negative consequences on children's mental health, e.g. problems with concentration, irritability, restlessness, nervousness, loneliness, time spent in front of screens increased at the expense of time spent outside with friends (1, 2). Parents were observed to experience increased stress (3) and negative affect (4), while mothers, parents with fewer social contacts, parents with more children or younger children (5) were even more affected.

In times of natural disasters or pandemics, various vulnerable groups are prone to be significantly more affected, including people with previous mental disorders (6). Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder characterized by deficits in reciprocal social interaction and communication and narrowly defined, repetitive patterns of behavior and interests (7). Other symptoms of ASD, which could cause specific challenges during the pandemic, include difficulty coping with changes, poor adaptation strategies, low tolerance of uncertainty, cognitive deficits, executive dysfunction, poor emotion recognition and self-regulation, or frequent comorbidities such as ADHD, anxiety or depression (8).

When comparing the negative consequences that the pandemic had on children with ASD versus neurotypical children, children with ASD were mainly negatively affected by disruption of their everyday routines, while neurotypical children by social isolation (9). The closure of schools and the introduction of online learning resulted in either increase or decrease of stress in different individuals with ASD (10), as it caused loss of some interventions previously offered by school facilities, but on the other hand it also reduced demands for obligatory socialization. Other research studies reported an increase in aggression, negative changes in diet, deterioration of communication skills, sleep problems or worsening of ASD symptoms such as mannerisms, movement and speech stereotypies in individuals with ASD since the beginning of the pandemic (11). Problem behavior increased in more than one third of children with ASD, and the children who struggled with problem behavior before the pandemic had a two times higher chance of experiencing it more intensively and more often (12).

Caring for children with ASD is generally more challenging, due to the disorder symptoms, a stigma associated with the condition, as well as frequent comorbidities such as gastrointestinal or sleep problems. Even before the pandemic, parents and caretakers of

children with ASD were found to experience more parental stress than parents of neurotypical children, but also more than parents of children with disorders such as Down's syndrome, cerebral palsy or intellectual disability (13). During the COVID-19 pandemic, the prevalence of depression, anxiety and stress among parents of children with special needs increased (14), as did their problems with managing daily responsibilities and free time planning (12). Both children with ASD and their parents experienced a significant increase in anxiety, and higher levels of stress were observed in parents of younger children or children with higher symptom severity (15).

Methods

Due to the uneasy pandemic situation and related safety measures, out data collection was conducted online. A robust parent survey was created (18), containing three main sections: demographic questions, a section about the mental health of parents, and a section about their children. To measure the prevalence of depression, anxiety and stress symptoms of parents and caregivers we used Depression Anxiety and Stress Scale (DASS-42). Additionally, we asked the parents about the most prominent stressors they experienced during each wave of the pandemic, choosing between lockdown duration, fears of the infection, frustration and boredom, unavailability of goods and services, insufficient or unclear information, work insecurity/finances or more demanding child care. In the section about their children, we used two subscales of Vineland Adaptive Behavior Scales–Third Edition measuring the prevalence of internalizing (such as distress, sadness, apathy or social withdrawal) and externalizing (attention deficits, problems with self-regulation, aggression or disobedience) maladaptive behavior.

The survey was firstly sent to the parents of children recently diagnosed with ASD in the Academic Research Centre for Autism, Institute of Physiology, Faculty of Medicine, Comenius University in Bratislava and subsequently shared online. In three consecutive cross-sectional surveys, we gathered answers from 506 parents and caregivers, 236 of whom have children with ASD. 179 participants were surveyed during the first wave, 153 during the second wave and 174 during the third wave of COVID-19 pandemic in Slovakia.

Data and statistical processing of the data was performed using a statistical program SPSS (Statistical Package for Social Science) version 25. From the statistical methods package we used Chi-square with Yate continuity correction, t-test for comparison of two independent samples, Pearson correlation analysis, and analysis of covariance (ANCOVA).

Results

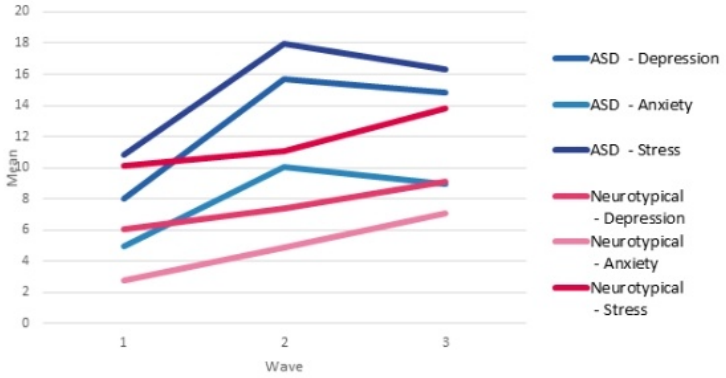
Depression, anxiety and stress of parents

To analyze the changes in prevalence of depression, anxiety and stress in parents of children with ASD and parents of neurotypical children, we performed a two-way factorial analysis of covariance. Parents of autistic children already experienced elevated anxiety during the first wave, before any negative changes were found in parents of typically developing children. We found the interaction effect of the pandemic period and the child's diagnosis with stress ($F(2,500) = 4,646$, $p = 0.010$, $\eta 2 = 0.018$) and depression ($F(2,500) = 4,233$, $p = 0.015$, $\eta 2 = 0.017$).

Interaction effect of the pandemic period with the diagnosis (ASD vs neurotypical) was not observed for anxiety (F (2,500) = 2.222, p = 0.109, η2 = 0.009). In all cases we also recorded statistically significant main effects.

During the second wave, prevalence of anxiety, depression and stress experienced by parents in both groups increased, but it increased significantly more in parents of autistic children. A more detailed analysis showed the scores in all three subscales deteriorated in parents of children with ASD between the first and the second wave (D: t = 4.393, p <0.001, d = 0.70; A: t = 3.648, p <0.001, d = 0.58; S: t = 4.373, p <0.001, d = 0.70), while the decline was confirmed exclusively in anxiety (t = 2.26, p = 0.025, d = 0.33) for parents of neurotypical children. During the second wave, we also observed statistically significant differences between the parents of children with ASD and of neurotypical children, in all three subscales (D: t = 4.70, p <0.001, d = 0.75; A: t = 3.57, p <0.001, d = 0.68; S: t = 4.26, p <0.001, d = 0.57).

There were no significant differences in stress, anxiety or depression in parents of children with ASD between the second and third wave. On the contrary, we observed a significant increase of stress in parents of neurotypical children (t = 2,018, p = 0.045, d = 0.31). Therefore, during the third wave, both groups of parents only differed in the prevalence of depression, which remained higher in parents of children with ASD (t= (177) = 3.560, p <0.001, d = 0.54), as also illustrated in Graph 1.



Picture. 1 Changes in the prevalence of parental depression, anxiety and stress between the first and third pandemic wave

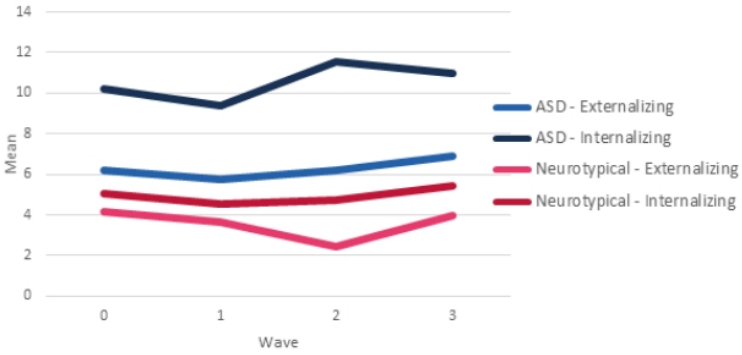
Parental stressors

In general, more demanding child care was the most prevalent pandemic stressor for both groups of parents with maximum values of up to 78.87% for parents of ASD children and up to 59.14% for parents of neurotypical children. During the second wave, the importance decreased to 57.75% for parents of children with ASD and 37.80% for parents of neurotypical children and was replaced by Insufficient or unclear information as the most prevalent

stressor for both groups, selected by 42.25% of parents of children with ASD and 54.88% of parents of neurotypical children.

Maladaptive behavior

To analyze the changes in maladaptive behavior of children with ASD and neurotypical children, we performed a two-way factorial analysis of covariance. Autistic children scored significantly higher in both internalizing and externalizing maladaptive behavior throughout the pandemic. We did not observe the interaction effect of the pandemic period and the child's diagnosis with internalizing ($F(3,677) = 1.151$, $p = 0.328$, $\eta 2 = 0.005$) or externalizing maladaptive behavior ($F(3,677) = 2.132$, $p = 0.095$, $\eta 2 = 0.009$). More detailed analysis of the main effects showed a significant effect of the diagnosis, children with ASD scored significantly higher values in internalizing ($F(3,677) = 203.022$, $p < 0.001$, $\eta 2 = 0.231$) as well as externalizing maladaptive behavior $F(3,677) = 101.215$, $p < 0.001$, $\eta 2 = 0.130$) during all data collections. The main effect of pandemic waves was lower, there were no statistically significant changes between the waves. Between the first and second wave, we found a significant increase in internalizing maladaptive behavior of children with ASD ($t = 2.324$, $p = 0.021$, $d = 0.38$). Contrastingly, in the group of neurotypical children, we recorded a statistically significant decrease of externalizing maladaptive behavior between the first and the second wave ($t = 2.594$, $p = 0.010$, $d = 0.39$) as also shown in the Graph 2. During the third wave, there were no significant changes for children with ASD, but for neurotypical children, we found another increase almost to the original maximum from the first wave ($t = 3.055$, $p = 0.003$, $d = 0.47$) (19).



Picture. 2 Changes in the prevalence of interalizing and externalizing maladaptive behavior of children, from before the pandemic to the third pandemic wave

Discussion

Our results turned out to be partially in contrast with foreign research studies, which reported a significant deterioration of the mental health in both children and parents (1-5) during the

first wave of the COVID-19 pandemic. In our research sample, the prevalence of depression, anxiety and stress was relatively low in both groups of parents during the first wave, with the exception of increased anxiety in parents of children with ASD. When interpreting the results, it is necessary to consider the specifics of the development of the pandemic situation in Slovakia during the first, second and third wave of the pandemic. During the first wave, despite relatively low numbers of infected people, strict measures were quickly implemented to reduce the spread of the virus, including closure of all services and shops except grocery shops and pharmacies, the closure of all schools and facilities providing therapies for children with ASD. However, thanks to the favorable pandemic situation, the first wave did not last long and Slovakia was considered one of the countries that managed the first wave of the pandemic well and with minimal losses.

During the second wave, Slovakia was dramatically more affected, whether in terms of the number of infected, number of deaths or the impact on the health system and the economy. According to our research, during the second wave, both groups of parents experienced an increased level of symptoms of depression, anxiety and stress, but the mental health of parents of children with ASD deteriorated much more significantly, in accordance with foreign studies from this time period (12, 14, 16). Between the first and second wave, externalizing maladaptive behavior (attention deficits, problems with self-regulation, aggression or disobedience) of children with ASD did not change significantly, but it decreased in neurotypical children, who probably adapted more flexibly to the situation. Conversely, internalizing maladaptive behavior (anxiety, fear, sadness, apathy, withdrawal) did not change in neurotypical children, but increased significantly in children with ASD.

While there were no significant changes in the psychological health of parents of children with ASD between the second and third wave, we observed a significant increase in stress in parents of neurotypical children. During the third wave, the differences between the parents of both groups almost evened out, the differences in stress and anxiety were statistically insignificant, but we found a persistently higher prevalence of depression in parents of children with ASD. In neurotypical children, we observed a significant increase in externalizing maladaptive behavior, with minimal changes on the part of children with ASD. The third wave in Slovakia was very similar to the second wave in terms of the number of infected people, but the safety measures were more relaxed and schools were being reopened. As we also monitored in our research, the availability of various interventions for children with ASD also increased significantly. We can assume that the improvement in the availability of inverventions as well as the return to schools could mean that the parents of autistic children received more necessary support, which prevented any further deterioration of their mental health. In line with another comparable research study (17), our conclusions indicate that there are significant differences in the mental health challenges that people faced during each of the COVID-19 pandemic waves.

Naturally, the results of our research must be interpreted with regard to its limitations, especially the fact that the data collection was realized online using self-report methods, and that it was a series of three cross-sectional studies. However, our results clearly show that the COVID-19 pandemic had a negative impact on the mental health of many families in Slovakia, but the impact on families with children with autism spectrum disorder was more severe. The increased availability of various interventions and the opening of schools, which can provide

more support mainly for parents of children with ASD, are one of the possible explanations why these differences have almost leveled off during the third wave.

These findings provide several practical implications, in case of future global pandemics as well as for the future of mental health research. One of our main missions is to draw the attention to individuals with autism and their families and to ensure their needs are considered, even when creating pandemic safety measures. For example, as our results indicate, keeping at least the schools for children with special needs open during the pandemic can be greatly beneficial to the children because of many necessary interventions the schools provide, as well as could significantly lighten the strain on their parents. Additionally, for psychologists and other specialists providing different interventions and therapies for children with ASD, being able to swiftly adapt them into the online form is imperative.

Furthermore, even though this research can only provide the data about the immediate impact of the pandemic, we believe that the full extent of consequences can perhaps only be assessed after the passage of some time. Our clinical observations, acquired while providing diagnostics in the Academic Research Center for Autism, provide additional information about these children, who are now in our waiting list or coming for a differential diagnosis. We observe two distinct groups that were significantly affected, 1) children born during the COVID-19 pandemic, which had limited possibilities to freely socialize with broader family, their peers or any new people and missed valuable communication learning possibilities 2) children who turned two years during the pandemic, which is the age when parents oftentimes notice the first differences, while comparing the children to their peers, which was limited because of the lockdowns and social distancing.

To sum up, even though it can be easy to underestimate long-term consequences of COVID-19 pandemic, for all the specialists working with children with or without ASD, it is absolutely crucial to take into consideration the possible impact that the pandemic could have on their development and overall mental health.

Acknowledgments

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12. Collection of clinical data and samples of patient with COVID-19 in MGA project biobank for use in basic and applied research

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Abstract

Coronavirus disease 2019 (COVID-19) has rapidly infected millions of people worldwide. Recent studies suggest that patients with comorbidities are at higher risk of Covid-19. We established a Medirex Group Academy n.p.o. (MGA) project biobank with the aim of collecting high-quality and well-annotated human biospecimens, in the effort to understand the pathogenic mechanisms underlying oncologic, civilization and infectious diseases (e.g., COVID-19) and identify potential screening, diagnostics or therapeutic targets. Here we describe our experience and briefly review the characteristics of the biobanks for COVID-19 that have been so far established. The aim of the article is to inform about the current state of studies, a set of data and results obtained thanks to the Medirex Group Academy projects primary focused on COVID-19 topic (Promedicov-19, DIACOVID) for use in current and future basic and applied research and to define future perspectives and development strategies for the established biobank of samples and clinical data. In the article, we present examples of collected anamnestic and clinical data and discuss various advantages or disadvantages of the currently used approach of collecting samples and data for a better adjustment of the results of studies in the future.

Introduction

SARS-CoV-2 infection causing COVID-19 has continued to spread across the world and represents a severe clinical, diagnostic and epidemiological challenge. It is important to react to this challenge and support disease-related research by standardized high quality biobanking and acquisition of related data by an established network and infrastructure. The pandemic showed the need and demand for information sharing and research. This includes rapid and safe collection as well as sharing of subsequent analysis of high-quality biosamples and associated data to globally support research (2).

MGA project biobank was founded as a local unit to support specific research projects of Medirex Group Academy n.p.o. (MGA). MGA is a non-profit organization settled in Nitra, Slovakia and focused primarily on the field of science and research, especially in biomedicine. The main goal of the projects implemented by MGA is the search for new biomarkers in order to increase preventive, diagnostic and therapeutic approaches with the potential to implement the knowledge of science and biomedical research into diagnostic and therapeutic practice. In connection with the implementation of many biomedical projects focused on different medical fields (oncology, infectiology, civilization diseases), there was a need for systematic and organized sample collection. Therefore, MGA biobank was established in

October 2020 as a multispecialistic academic project research biobank. The systematic collection of samples continues to these days in cooperation with many health care providers from Slovakia and Czech Republic.

Among projects focused on topic of infection diseases especially on COVID-19 are projects Promedicov-19 and DIACOVID, which are supported by VA-COVID-19 study. The main goal of Promedicov project is to create a (universal) system for early and rapid detection, identification and diagnosis of new infectious diseases with pandemic potential, implemented in the pilot phase in direct connection with the current COVID-19 pandemic (3). The goal of DIACOVID project is through research of serious civilizational diseases (diabetes mellitus, intestinal bowel diseases) and their complications caused by acute respiratory tract targeting viral diseases (the disease COVID-19 will serve as a model) obtain such knowledge that will enable the support and development of laboratory and clinically applicable innovative procedures for personalized diagnostics and therapy of such patients (4). All these projects were designed under the professional supervision of the guarantor of the entire project with regard to the fulfilment of the set scientific goals and were approved by the relevant ethical committees. The protocol and sampling schedules for patients enrolled in these studies differed depending on the branch of the study in which they were included or according to the primary diagnosis. The samples and data obtained in these studies were subsequently used for research by our project partners from various academic and scientific institutions in Slovakia. (e. g. Slovak Academy of Science, Geneton s.r.o., Comenius University Science Park).

Methods

Ethics committee and patient informed consent form

The clinical study was approved by the relevant multicentric and local ethics committee of the participating clinical sites before the actual implementation. The multicentric ethics committee was the Ethics Committee of the Bratislava Self-governing Region (EK BSK), which issued approval for VA-COVID- 19 study realization (decision number: 03228/2021/HF). The involved hospitals included University hospital Bratislava Ruzinov, University hospital Bratislava – Academician Ladislav Dérer hospital, Hospital Malacky). All patients signed an informed consent prior to participating in the study, in which all information regarding the research, the collection protocol, and the use of the provided information and samples for research purposes were stated.

Collection of anamnestic and clinical data

We obtained several types of information from the participants involved in the study. In addition to the anamnesis, which consisted of basic physiological and anthropometric data, information was also collected about the participant's state of health, clinical history, medication use, family history, various sociodemographic indicators, and the results of laboratory and experimental examinations. All anamnestic and clinical data were obtained mainly by questionnaire. Some parts of the questionnaires could be filled in by the participants themselves, mainly the parts that contained questions about the participant's lifestyle, sociodemographic characteristics of the participant. The next part consisted of questions focused on the participant's clinical history, and these parts were filled in together with the admitting doctor or nurse. Some groups of patients were also sampled for biochemical,

serological and immunological examinations. The results of these examinations were also collected and recorded in an electronic system for administration and management of studies.

Promedicov-19

Patients with a different course of COVID-19 were part of the VA-COVID-19 study and the Promedicov-19 project. In the case of COVID-19 positive patients, regardless of the course, repeated blood samplings were carried out in the biobank (1x 5 ml of blood in a serum gel tube, 1x 10 ml of blood in an EDTA tube) and naso and oropharyngeal swabs (1x tube with viRNAtrap medium) in three terms after diagnosis of infection. At the same time, a set of biochemical, serological and immunological examinations was carried out to monitor the development of the infection. For patients whose infection was not confirmed, only swab samples were taken, which remained after the diagnosis and were sent to the biobank in a tube with viRNAtrap medium. Patients with post-covid syndrome had 1x 10 ml of blood drawn into an EDTA tube, which was stored in the biobank for genomic analyses.

DIACOVID

Patients with a primary diagnosis of diabetes mellitus (DM) and/or inflammatory bowel disease (IBD) (ulcerative colitis or Crohn's disease) were included in the DIACOVID project. In addition to the listed diagnoses, another inclusion criterium for these patients was overcoming the disease of COVID-19. The study also included the recruitment of individuals into the control group, in whom the presence of DM or IBD was ruled out by anamnesis and who had overcome COVID-19. All patients underwent a venous blood sampling of 3x10ml into an EDTA Vacutainer tube and a stool collection of 1x10ml into a tube with DNA/RNA Shield solution (Zymo Research). In addition, patients with IBD underwent sampling of native tissue, which was obtained by biopsy during an endoscopic examination indicated as part of standard diagnostic and treatment procedures.

Primary processing and archiving of samples in the biobank

After the samples were delivered to the laboratory, some samples were primarily processed according to a standardized protocol. Blood samples collected in EDTA Vacutainer tubes intended for proteomic and genomic analyses were processed by one-step (2200 g, 10 min., 4°C) and two-step (2200 g, 10 min., 4°C; 16000 g, 10 min., 20 °C) centrifugation, and the blood samples collected in serum-gel tubes were processed by one-step centrifugation (3000 g, 10 min., 4 °C). After centrifugation, the samples were preserved in the form of aliquots of whole blood (2-3x 500µl), plasma (proteomics - 2x 500µl, remaining aliquots of 1800 µl; genomics - 2x 680µl, remaining aliquots of 1800 µl) or serum (2x1ml). Stool and swab samples were prepared in the form of 1 ml aliquots and stored in the biobank. Native tissues and FFPE blocks were stored without primary processing.

Results

Current status of patient recruitment in projects focused on COVID-19 topic

Promedicov -19

The recruitment of patients with a diagnosis of COVID-19 began in April 2021. Patients with asymptomatic and mild manifestations of the disease, or a control group of COVID-negative

patients, were obtained through the sampling centres Medirex a.s.. 2 hospital workplaces ensured the recruitment of patients with a severe COVID-19 (University Hospital Bratislava - University hospital Bratislava – Academician Ladislav Dérer Hospital, University Hospital Bratislava — Ruzinov). Sanatorium Dr. Guhra in Tatranska Polianka was involved in the recruitment of patients with post-covid syndrome. The recruitment of patients has ended and the current status of the number of enrolled patients in individual groups is 288 COVID-19 negative, 48 COVID-19 positive - asymptomatic, 305 COVID-19 positive with a mild course, 78 COVID-19 positive with a severe course and 258 patients with post-covid syndrome (Table 1).

DIACOVID

The recruitment of patients began in June 2022 at the IBD gastroenterology centre and at the internal clinic of the University Hospital Bratislava – Ruzinov. In this study, we are currently registering 50 patients with a diagnosis of DM, 53 patients with IBD, 11 patients with IBD and DM, and 154 patients in the control group (as of March 31, 2023).

Analysis of selected anamnestic data about the participants enrolled to DIACOVID and Promedicov-19

Part of the collection protocol for all participants was the acquisition of anamnestic and clinical information through an anamnestic questionnaire, which was filled in by the patient himself or by a doctor with the patient. During the implementation, our goal was to obtain the most consistent data about patients, so that, if necessary, we could compare these data between patients from different studies and thus obtain more information for possible use in future research. An example of the same data collected across studies was basic anthropometric and physiological information about the participant. For the purposes of this report, we have chosen for comparison some basic data about the participant, such as gender, age, height, weight, waist, BMI, blood group, education. Detailed results from the analysis of these data for participants enrolled in individual studies (DIACOVID, Promedicov-19) and groups as well as summary results are shown in Table 1. Although a total of 1,244 participants were included in the studies, 1,227 patients completed the anamnestic questionnaire as of March 31, 2023. In DIACOVID study 168 (62,45%) women and 96 (35,69%) men were enrolled. The median age of the patients in the study was 44,5 years. The height median of the patients was 173 cm, weight 81 kg, waist 91,25 cm and BMI 25,4. The most frequent blood group was A with 85 cases and 0 with 61 cases. In the study Promedicov-19, the gender ratio between men and women was more balanced. Median Age of participants was 48,6 years, hight 173,1 cm, weight 81,8, waist 72,8 cm, BMI 26,6. The most frequent blood type was A and 0. In the both studies, the common level of the highest education attained was university.

Table 1: The basic anthropometric and physiological information about the participants enrolled to DIACOVID and Promedicov-19 study. DM+ - diabetes mellitus positive; IBD+ - intestinal bowel disease positive

			Gender				Age Median	High Median *(cm)*	Weight Median *(kg)*	Waist Median *(cm)*	BMI	Blood group *(N)*				Education *(N)*		
	Patient group	Subjects *(N)*	Male *(N)*	Male (%)	Female *(N)*	Female (%)						0	A	B	AB	VŠ	SŠ	ZŠ
DIACOVID																		
	DM+	50	25	50,00 %	23	46,00 %	49,5	174	88,5	94	27,5	5	19	8	1	20	25	0
	IBD+	53	29	54,72 %	23	43,40 %	39	176	77,5	90	24	15	15	5	7	31	21	0
	DM+IBD+	12	5	41,67 %	6	50,00 %	55,5	172	90	100	28	3	3	1	0	1	9	1
	Healthy control	154	37	24,03 %	116	75,32 %	34	170	68	81	22	38	48	24	12	97	40	2
	DIACOVID total subjects	**269**	**96**	**35,69 %**	**168**	**62,45 %**	**44,5**	**173**	**81**	**91,25**	**25,4**	**61**	**85**	**38**	**20**	**149**	**95**	**3**
Promedicov -19																		
	COVID negative	288	146	50,69 %	135	46,88 %	40	175	78	80	25	81	80	41	20	214	62	3
	COVID positive																	
	Asymptomatic cases	46	26	56,52 %	20	43,48 %	42	176,5	78	89	25	15	16	6	4	26	20	0
	Mild cases	305	126	41,31 %	179	58,69 %	39,5	172	75	88	24	76	95	36	27	195	103	7
	Severe cases	78	43	55,13 %	32	41,03 %	63,5	170	88	0	29	4	7	2	2	22	38	4
	Post covid syndrome	258	138	53,49 %	118	45,74 %	58	172	90	107	30	44	51	20	13	66	172	13
	Promedicov-19 total subjects	**975**	**479**	**49,13 %**	**484**	**49,64 %**	**48,6**	**173,1**	**81,8**	**72,8**	**26,6**	**220**	**249**	**105**	**66**	**523**	**395**	**27**
Total		**1244**	**575**	46,22 %	**652**	52,41 %												

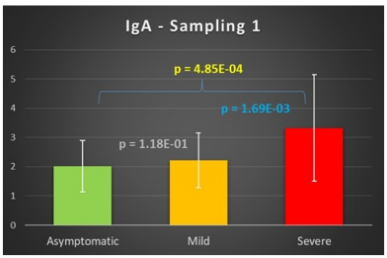
Comparison of serum total IgA and IgG levels in patients with varying COVID-19 severity enrolled to Promedicov-19 project

In addition to selected anamnestic data, information about the results of selected laboratory parameters that were examined during the course of the study was also collected from subjects included in the Promedicov -19 project. For the purposes of this article, we have chosen to evaluate the levels of total serum immunoglobulins of the IgA and IgG classes. These parameters were investigated in patients with acute COVID-19 who, based on initial symptoms, were assigned to the group with asymptomatic, mild or severe course of COVID-19. They were examined 3-5 days after confirming the positive result of the PCR test (Sampling 1).

The mean ± SD of IgA in asymptomatic subjects was 2.01 ± 0.88 (g/L), in mild cases 2,23 ± 0.94 (g/L) and in severe group 3,32 ± 1.82 (g/L). Figure 1 A shows the mean ± SD of total IgA among the three groups. According to the Welch's t-test, there were statistically significant changes in terms of IgA among the asymptomatic and severe group (P value < 0.05). Among mild and severe group, the difference was on the borderline of significance.

The mean ± SD of IgG in asymptomatic subjects was 11,34, in mild cases 11,44 ± 2.34 (g/L) and in severe cases 11,11 ± 0.54 (g/L). The mean ± SD of the total IgG among three groups of asymptomatic, mild, and severe is shown in Figure 1B. According to the Welch's t-test, there were no statistically significant changes in IgG among the three groups.

A.



B.

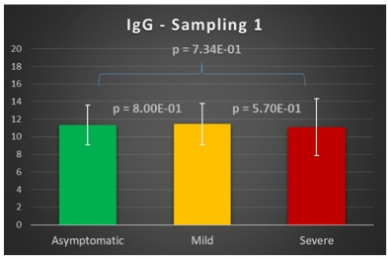


Figure 1.: Total IgA and IgG in Sampling 1 between asymptomatic, mild and severe COVID-19 patient. A: Total IgA levels B. Total IgG levels.

Current status of samples from patients with COVID-19 in the biobank

Currently (as of March 31, 2023), the biobank is a repository of 4,669 different biological samples (blood, stool, nasopharyngeal swab, native tissue) stored in the form of aliquots obtained from 1,244 patients with acute or overcome COVID-19 participating in the Promedicov-19 and DIACOVID project.

Table 2: The current status of MGA biobank for DIACOVID and Promedicov-19 study.

MGA biobanking - stage			MGA biobanking - sample distribution			
	Sampling material	Number of received samples	Number of received aliquots	Type of analysis	Input volume/amount	Number of released aliquots
DIACOVID	Blood	906	604	genomics		
	Plasma		1,510	genomics		
			1,208	proteomics		
	Stool	275	275	microbiome		
	Tissue	63	63	genomics		
Promedicov-19	Blood	2,235	3,352	genomics		
	Serum		2,235	serology	1 ml	921
	Swab	1,190	2,380	genomics	400 µl	87
Total		4,669	11,627			1008

The first samples began to be collected in April 2021 (Promedicov-19) and June 2022 (DIACOVID). 3,141 blood samples, 275 stool samples, 1,190 naso- and oropharyngeal swab samples, and 63 native tissue samples are stored in the biobank (Table 2). The collection and storage of samples in the biobank continues throughout the entire duration of individual projects. Archived biological material is gradually used for various types of analysis (genomics, proteomics, microbiome, serology, immunology, histology). So far, more than 921 aliquots have been released for serology analysis and 87 aliquots from swab samples for genomics analysis for the participating partner research organizations (Comenius University Science Park, Medirex Group Academy, Geneton s.r.o.) (Table 2). The results of the implemented experimental analyses are currently in the processing phase and will be part of professional publications that will be gradually published during the years 2023, 2024 and 2025.

Discussion

Biobanking plays a critical role in diagnostics, biomarker research and development of novel treatment approaches for various diseases. In urgent need of understanding, preventing and treating coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the importance of biobanking including data sharing and management further increased (1). In Slovakia, during COVID-19 pandemic, MGA n.p.o. established a project biobank for collecting the COVID-19 patient biospecimens of different kind (blood, swab, stool, tissue). We evolved a sustainable biobanking mechanism for the standardised collection, characterisation, and archiving of specimens, and sharing these specimens to facilitate and accelerate diagnostic test development and evaluation for diseases of epidemic potential. Our long-term goal is to continue building a biobank of biological samples and clinical data from different medical fields therefore we plan to continue with the implementation of further clinical studies and continue to work on improving and optimizing the processes of data collection, samples and biobanking.

Acknowledgements

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13. Metatranskriptómová analýza z nazofaryngeálnych výterov pacientov s ochorením COVID-19

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Úvod

Mikrobióm respiračného traktu hrá dôležitú úlohu v zachovaní respiračného zdravia. Mnoho štúdií poukazuje na vplyv mikrobiómu respiračného traktu na náchylnosť na infekcie respiračného traktu vrátane ochorenia COVID-19. Pochopenie interakcií medzi hostiteľom, vírusom a respiračným mikrobiómom môže vniesť svetlo do hľadania nových potenciálnych terapeutických cieľov pri prevencii a liečbe týchto infekcií. Vírus SARS-CoV-2 primárne vstupuje do organizmu cez respiračný trakt, ústnu dutinu a nos (1). Mikrobióm je teda jednou z prvých entít, ktorá čelí infekcii. Hrá dôležitú úlohu pri stimulácii imunitného systému a ochrane pred patogénmi (2). Keď je narušená homeostáza mikrobiómu (mikrobiálna dysbióza), patogény prerastú a kolonizujú dýchacie cesty, čo nakoniec vedie k infekcii dolných dýchacích ciest (3).

Kolonizácia horného dýchacieho traktu začína pri narodení a je ovplyvnená spôsobom pôrodu. Počas vaginálneho pôrodu je dieťa vystavené materskému vaginálnemu mikrobiómu a počas pôrodu cisárskym rezom je dieťa vystavené matkinej pokožke a mikrobiómu prostredia. Počas prvého týždňa života je nosohltan kolonizovaný *Staphylococcus aureus* bez ohľadu na spôsob pôrodu. Hoci *S. aureus* je primárne známy ako patogénna baktéria, môže byť dôležitou komenzálnou baktériou v nosohltane počas raného života. Wang a kol. ukazujú, že *S. aureus* významne zmierňuje chrípkou sprostredkované imunitné poškodenie pľúc u myší indukciou alveolárnych makrofágov (4). V neskorších týždňoch sa početnosť *S. aureus* znižuje a druhom ako *Corynebacterium*, *Dolosigranulum* a *Moraxella* sa početnosť zvyšuje. Zvýšená početnosť týchto 3 druhov je dôležitá pre zdravý vývoj nosohltanovej mikroflóry v neskorších štádiách života. V prvých mesiacoch života deti narodené cisárskym rezom vykazovali variabilný mikrobiálny profil a úbytok množstva *Corynebacterium* a *Dolosigranulum*, čo viedlo k nárastu respiračných infekcií. Početnosť *S. aureus* nie je významne znížená, zatiaľ čo sa začínajú objavovať *Prevotella*, *Veillonela* a *Porphyromonas* (5). Naproti tomu deti narodené prirodzene a dojčené vykazovali vyšší výskyt prospešných baktérií (6). Mohlo by to byť spôsobené aj prenosom prospešnej mikroflóry, ako je *Lactobacillus* a *Bifidobacterium*, do mlieka počas dojčenia (7). Dojčené deti majú tiež vyšší výskyt *Corynebacterium* a *Dolosigranulum* a nižší

výskyt respiračných ochorení v porovnaní s deťmi kŕmenými umelým mliekom. Mikroflóra detí môže byť ovplyvnená mnohými rôznymi aspektmi, ako je napríklad typ pôrodu a kŕmenia, prítomnosť súrodencov, predchádzajúce infekcie, používanie antibiotík, očkovanie, ročné obdobie, vystavenie rôznym prostrediam (domov, škôlka, park), atď. V prvom roku života je expozícia mikrobiálnym spoločenstvám rozhodujúca pre tvorbu imunitného systému (8). Mikrobióm sa počas života vyvíja a je vidieť rozdiely medzi dieťaťom, dospelým jedincom a starším človekom. Mikrobióm respiračného traktu dospelých je menej denzný zato diverzita je vyššia. U starších ľudí začína bakteriálna diverzita klesať, mikrobióm je menej stabilný a početnosť patogénnych baktérií stúpa. Tieto zmeny majú pravdepodobne za následok vyššiu náchylnosť na respiračné ochorenia (9).

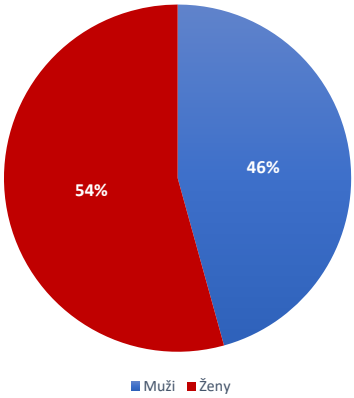
Cieľom práce bolo zistiť odlišnosti v kompozícií nazofaryngeálneho mikrobiómu medzi negatívnymi jedincami a pacientmi s ochorením COVID-19. Zároveň sme chceli zistiť či sa líšia medzi sebou aj jednotlivé skupiny s rôznou závažnosťou priebehu COVID-19.

Materiál a metódy

Do štúdie bolo zapojených 151 pacientov, ktorí boli rozdelení do skupín podľa výsledku PCR testu. Negatívni pacienti (n=72) slúžili ako kontrolná skupina. Pacienti s pozitívnym výsledkom PCR testu boli následne rozdelení do troch skupín podľa závažnosti priebehu ochorenia: bezpríznaková skupina (n=24), skupina s miernym priebehom ochorenia (n=25) a skupina s ťažkým priebehom (n=30). Pacienti so závažným priebehom ochorenia boli hospitalizovaní univerzitných nemocniciach v Bratislave: Ružinov a Kramáre. Charakteristika pacientov zameraná na vek a pohlavie je zhrnutá v tabuľke 1 a grafe 1. Odbery výterov z nosohltanu boli realizované od marca 2021 do októbra 2022 do skúmavky obsahujúcej viRNAtrap médium (GeneSpector, ČR) a uskladnené pri 4°C. Pomocou kitu Sera-XtractaTM virus/Pathogen Kit (Cytiva, UK) bola vyizolovaná celková RNA, ktorá bola uskladnená pri -80°C. Všetky vzorky boli podrobené RT-PCR analýze na potvrdenie alebo u negatívnych kontrol na vyvrátenie prítomnosti SARS-CoV-2 vírusu. Vzorka bola COVID pozitívna ak mala hodnotu Ct < 40. Na RT-PCR sme použili COVID-19 Real-Time PCR system RT-qPCR kit (Labsystems Diagnostic, Fínsko) na ABI QuantStudio 6 Real-Time PCR System RT-qPCR (ThermoFisher Scientific, USA). Transkriptómové knižnice boli vytvárané podľa protokolu KAPA RNA HyperPrep Kit with RiboErase (HMR) (Roche, USA) s použitím duálnych adaptérov TruSeq CD (Illumina, USA). Kvalita knižníc bola skontrolovaná pomocou fluorometrickej analýzy za použitia QubitTM dsDNA HS Assay (Invitrogen, USA) a fragmentovej analýzy za použitia High Sensitivity DNA (Agilent Technologies, USA). Obojstranné sekvenovanie sa uskutočnilo na platforme NextSeq 2000 (Illumina, USA). Väčšina čítaní bola namapovaná na ľudskú referenciu hg38 pomocou BWA-MEM a nemapované čítania boli analyzované pomocou Kraken 2 v2.1.2, aby sa odhalila a zmerala prítomnosť mikrobiálneho transkriptu.

Tabuľka 1. Charakteristika pacientov s jednotlivých študovaných skupín zameraná na vek a pohlavie (M-muži, Ž-ženy, SD-smerodajná odchýlka).

	Počet	M	Ž	vek (medián)	SD	M	SD	Ž	SD
negatívni pacienti	72	26	46	37 (25-75)	10.59	42 (25-58)	9.5	35 (25-75)	11.45
bezprízнakoví pacienti	24	14	10	42 (20-49)	7.86	39 (21-49)	9.35	43 (36-48)	3.72
mierni pacienti	25	13	12	37 (17-57)	11.83	38 (17-57)	12.77	32.5 (19-57)	10.54
ťažkí pacienti	30	16	14	68 (32-90)	13	69.5 (32-90)	13.6	64 (41-77)	11.7
celkom	**151**	**69**	**82**	**39.5**		**40.5**		**39**	

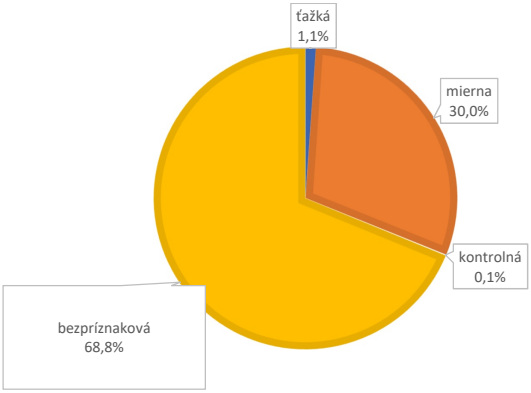


Graf 1. Percentuálne zastúpenie pohlaví v rámci všetkých študovaných skupín.

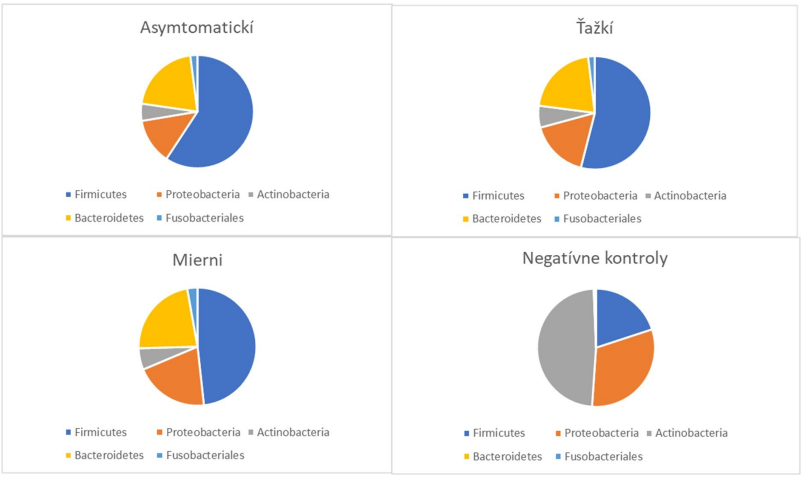
Výsledky

151 vzoriek zo 4 študovaných skupín sme podrobili metatranskriptómovému sekvenovaniu, pričom priemerný počet čítaní na vzorku bol 45,3 milióna (27,7-133 milióna). Vo všetkých 4 skupinách bolo odlišné zastúpenie bakteriálnych taxónov. Pacienti z negatívnej a ťažkej skupiny mali výrazne zníženú relatívnu početnosť v porovnaní s pacientami z asymptomatickej a miernej skupiny (graf 2.). U všetkých skupín s COVID pozitívnymi pacientmi boli najpočetnejšími kmeňmi *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* a *Fusobacteriales*. Avšak kontrolná skupina mala výrazne odlišné zastúpenie bakteriálnych kmeňov, najpočetnejšími boli kmene *Actinobacteria, Proteobacteria* a *Firmicutes*. Zvyšné dva

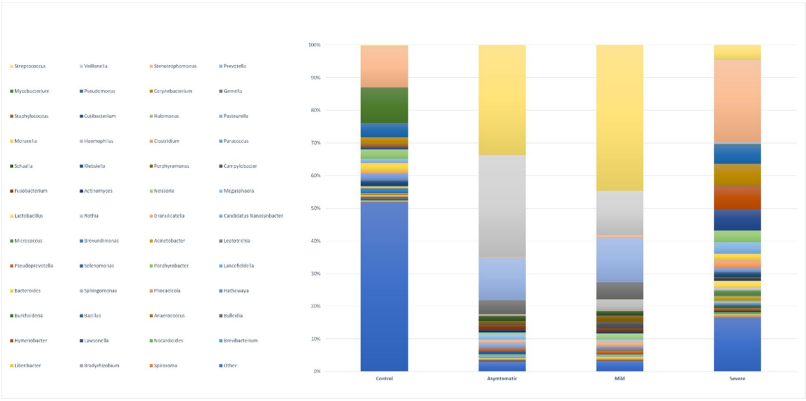
bakteriálne kmene *Bacteroidetes* a *Fusobacteriales* mali u negatívnej skupiny zanedbateľný pomer (graf 3.). Celkovo sme vo všetkých skupinách identifikovali 944 druhov a 531 rodov. Kontrolná negatívna skupina mala najnižší počet identifikovaných druhov (58). Dokonca niekoľko násobne nižší ako v COVID-19 pozitívnych skupinách (ťažká 725, bezprízнаková 692 a mierna skupina 574 druhov). Čo sa týka zastúpenia rodov v jednotlivých skupinách, v negatívnej skupine je najpočetnejším rodom *Stenotrophomonas* nasledovaný rodmi *Mycobacterium* a *Pseudomonas*. Bezprízнаkovej a miernej skupine dominoval rod *Streptococcus* nasledovaný rodmi *Prevotella* a *Veillonella*. Skupina s pacientmi s ťažkým priebehom COVID-19 bol najpočetnejším druhom rovnako ako u kontrolnej skupiny *Stenotrophomonas*, ďalej *Staphylococcus* a *Corynebacterium* (graf 4.). Na úrovni druhov boli v kontrolnej skupine najpočetnejšie baktérie *Stenotrophomonas maltophilia*, *Halomonas sp*. JS92-SW72 a *Mycobacterium canettii.* V oboch skupinách bezprízнаkových a miernych pacientov boli najviac zastúpené druhy *Veillonella atypica* a *Prevotella melaninogenica.* Tak ako aj na úrovni rodov, tak aj na úrovni druhov kontrolná skupina a skupina s ťažkým priebehom zdieľa najpočetnejšie druhy *Stenotrophomonas maltophilia*, *Halomonas sp.* JS92-SW72 nasledované *Mycobacterium canettii* (graf 5.).



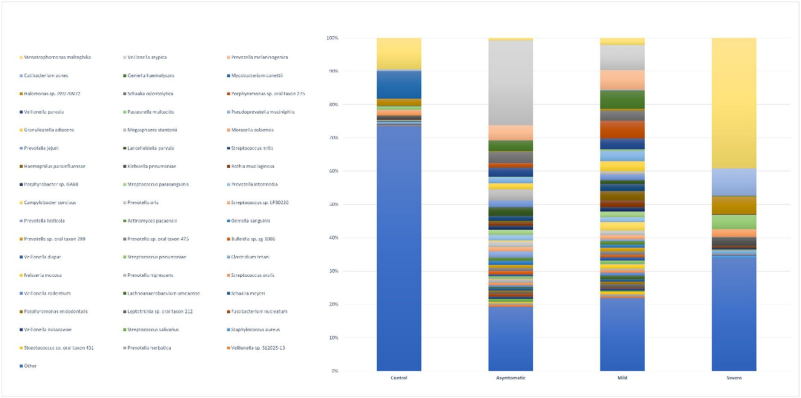
Graf 2. Relatívna početnosť baktérií vo všetkých študovaných skupinách



Graf 3. Percentuálne zastúpenie bakteriálnych kmeňov vo všetkých sledovaných skupinách



Graf 4. Relatívna početnosť rodov vo všetkých 4 skupinách



Graf 4. Relatívna početnosť druhov vo všetkých 4 skupinách

Diskusia

V našej štúdií sme zisťovali odlišnosti v kompozícií nazofaryngeálneho mikrobiómu medzi negatívnymi jedincami a pacientmi s ochorením COVID-19 s rôznou závažnosťou ochorenia od bezpríznakového priebehu, mierneho priebehu až po ťažký priebeh kedy boli pacienti hospitalizovaní v nemocnici. Početnosť baktérií je v skutočnosti početnosť transkriptov, ktoré ale predstavujú aktívnu časť mikrobiómu. Kontrolná skupina mala najnižšiu početnosť baktérií a taktiež mala aj najnižšiu početnosť bakteriálnych druhov zo všetkých 4 skupín. Výrazne vyššiu početnosť mali skupiny s bezpríznakovým a miernym priebehom. Zaujímavé je, že skupina pacientov so závažným priebehom COVID-19 mala nízku početnosť baktérií ale najvyššiu početnosť identifikovaných bakteriálnych druhov. Nízku početnosť baktérií môže vysvetliť fakt, že väčšine tejto skupiny boli v nemocnici podávané antibiotiká. Pravdepodobným vysvetlením vyššej početnosti bakteriálnych druhov v rámci COVID-19 pozitívnych skupín voči negatívnym kontrolám môže byť fakt, že vírusová infekcia zvyšuje pravdepodobnosť bakteriálnej superinfekcie. Pri pohľade na COVID-19 pozitívne skupiny, všetky 3 vykazujú rovnaké percentuálne zastúpenie profilu bakteriálnych kmeňov s najdominantnejším kmeňom *Firmicutes*, nasledovaný *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* a *Fusobacteriales*. Avšak ak sme sa pozreli na negatívnu skupinu, najpočetnejším kmeňom bol *Actinobacteria*, nasledovaný *Proteobacteria* a *Firmicutes*, pravdepodobne preto, lebo v nosovej dutine zdravých ľudí sú zvyčajne prítomné baktérie bežne vyskytujúce sa na pokožke z kmeňa *Actinobacteria* (rod *Corynebacterium*), *Proteobacteria* a *Firmicutes* (rod *Staphylococcus*) (10).

Poďakovanie

Táto publikácia vznikla vďaka podpore v rámci Operačného programu Integrovaná infraštruktúra pre projekt: Výskum progresívnych metód diagnostiky COVID-19 a biomarkerov umožňujúcich skorú detekciu jedincov so zvýšeným rizikom ťažkého priebehu ochorenia , kód ITMS: 313011ATA2, spolufinancovaný zo zdrojov Európskeho fondu regionálneho rozvoja.

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14. Severe retroperitoneal hemorrhage in covid-19 with low-molecular-weight heparin therapeutic dose – case report

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Introduction

COVID-19 has been associated with thrombosis as one of the disease's complications [1]. Once patients are hospitalized with COVID-19, they usually receive treatment accompanied by anticoagulants to prevent thrombosis [2]. The drug name, dosage, or duration are not yet strictly defined, as there is still a lack of guidelines in this matter. The thromboembolic pathologies and mortality have increased in individuals who are infected with COVID-19 [3]. Clinical studies [4, 5, 6] already in first pandemic period described that COVID-19 patients show a predisposition to thromboembolism. On the other hand, as mentioned by Tanal [2] findings of nontraumatic focused haemorrhages are unexpected. Several clinical reports [7-12] describing cases of severe spontaneous abdominal or retroperitoneal bleeding were published during the most fulminant phase of pandemic. Retroperitoneal hematoma is a radiologically diagnosed rare diagnosis, defined as the bleeding in the retroperitoneal space usually without associated trauma or iatrogenic manipulation. It is usually seen in patients receiving systemic anticoagulation, mostly including Warfarin [13]. Other than anticoagulant therapies and clotting disorders, it has been associated with hematologic diseases, malignancies, trauma, and Evans syndrome [14] . Compared to other areas of bleeding, as shown by Tanal [2], retroperitoneal hematoma diagnosis can be challenging due to asymptomatic or non-specifically symptomatic conditions. Its treatment can be even more challenging, because there are usually other comorbidities that should be taken care of [2].

Case presentation

A 43-year-old patient was admitted to the intensive care unit (ICU) of the Pneumological Department on the 28th December 2020, because of bilateral coronavirus disease 2019 (COVID-19) bronchopneumonia. According to the chest X-ray the bilateral inflammatory infiltrates were present. The blood test showed signs of dehydration, severe hypoxemia and hypocapnia and the inflammation parameters were elevated. Initial management included the high flow nasal oxygen therapy treatment and intravenous antibiotic therapy (ceftriaxone, moxifloxacin, fluconazole), systematic corticoids (dexamethasone) and low-molecular-weight heparin (LMWH) in the full anticoagulation dose (Clexane - 2x0,8ml subcutaneously). Also the regular antiviral therapy during the treatment (acidum ascorbicum, vigantol, cetirizine, famotidine) was applied. Because the patient underwent in past bilateral transplantation of lungs, standard post-transplant treatment with tacrolimus for immunosuppression at a dose of 2.5 mg / 24 h was continued. From 5th January 2021, the patient complained about experiencing stomach pain within hypogastrium, bilaterally, more on his left side. The symptoms and pain worsened with time. During the ultrasound examination of the abdomen,

the fluid formations were described in the left mesohypogastrium of the size 120x100x80mm, suspicious in retroperitoneum and in the right musculus rectus abdominis - suspicious hematomas. The patient was hemodynamically unstable, in objective findings dominated hypotension, tachycardia, anaemia (Hb 90g/l). Diagnosis was confirmed by Computed tomography (CT) of the abdomen and small pelvis - discovered active bleeding into the abdominal cavity, in left retroperitoneum (Fig. *1*, *2*) and right musculus rectus abdominis. Surgical revision was indicated and performed in biosafety level 3 (BSL-3), 150 minutes after the CT examination. The duration of the surgery was 1 hour, and revision, haemostasis, Mikulicz tamponade and drainage were done, with perioperative blood loss approx. 2500ml. After the surgery, the patient was transferred to the ICU of the anaesthesiology department, where the complex therapy (vasopressor support, supplementing the circulating volume with crystalloids and blood derivatives, 6x blood transfusions, 7x fresh frozen plasma and 6g of fibrinogen. Protamine and Tranexamic acid were given for antagonization of LMWH) continued. Unfortunately, multiple organ failure (MOF) with fatal complications developed 10 hours after the surgery.

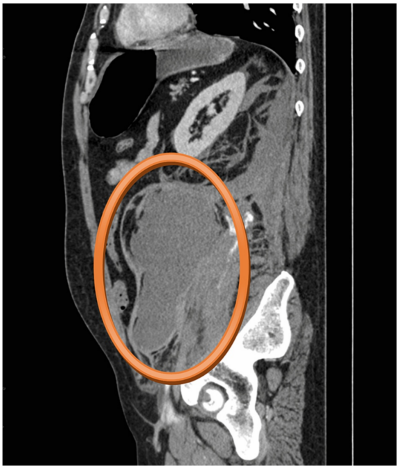
Discussion

The pandemic period of more than two years has brought a number of challenges to all parts of our society, especially to medical professionals, including surgeons. COVID-19 may appear in severe, complicated form and as shown by Al-Ani et al., Huang et al. and Chen et al. [4, 5, 6], it creates a thromboembolic situation in patients, independent of their age and comorbidities [15]. Thus, anticoagulant therapies are usually added to these patients, and this is recommended in international guidelines [16] as well. In our hospital, the therapeutic strategy is to reach the therapeutic dose of LWMH therapy, which seems beneficial to the majority of patient. However, as presented in this case, a life-threatening condition may develop. The work of Yeoh et al. [17] shows, that diagnosis of retroperitoneal hematoma requires a high degree of clinical suspicion as patients do not exhibit any clinically apparent signs and symptoms until a substantial amount of blood loss has occurred. Yeoh et al. [17] recommend to suspect it in patients who present with significant groin, flank, abdominal, back pain or hemodynamic instability after an interventional procedure or in patients who are anticoagulated. As shown by Kalayci [11,12], the ideal and simple diagnostic method is to check the haemoglobin level regularly. A contrast enhanced CT scan of the abdomen remains the imaging modality of choice for early detection and prompt intervention [17]. As showed in our case report, COVID-19 patients treated with anticoagulants were at risk of developing spontaneous retroperitoneal hematoma. Although rare, it should remain as a probable cause of bleeding, especially when patients present with flank pain, anaemia and signs of hypovolemia.

Conclusions

In COVID 19 hospitalized patients we rarely had to deal with a specific surgical complication - severe bleeding into the retroperitoneal space and abdominal cavity. There was no history of previous injury and also clinical signs were non-specific. Patient complained of abdominal pain, objective signs were hypotension, tachycardia and anaemia. The only possible life-saving method is quick diagnosis and follow-up surgery. Prevention could be the optimization of

anticoagulant therapy. Although treatment of LMWH in patients with severe COVID-19 bronchopneumonia was necessary, a high risk of spontaneous bleeding should be considered at therapeutic doses. Treating physicians must keep in mind the high risk of profuse bleeding, which is very difficult to treat, even surgically. It may be suspected in unexpected anaemia and non-specific abdominal symptoms. Indication for an immediate imaging examination is the only way to get us to the right diagnosis.



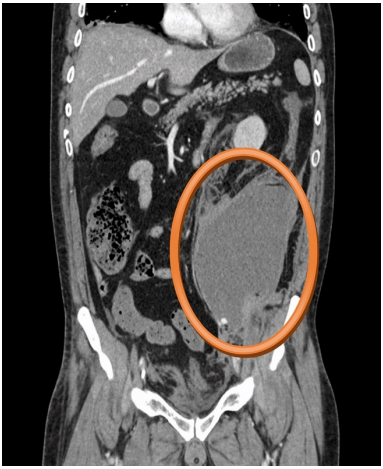


Fig. *1* and *2*: The hematoma in the left retroperitoneum was of size 170x125x120mm, attached to the left musculus psoas major and presented with active bleeding.

Acknowledgement

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15. Effect of covid-19 pandemic on surgical treatment in general surgical department

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Goals of the study

This study is focused on routine surgical praxis in hospital during the pandemic of COVID 19 disease. We observed the influence on surgical therapy, planed operations as well as on acute ones. Except of general effect of pandemics on surgery we observed the changes in management of patients with acute appendicitis and described case of massive non-traumatic abdominal bleeding caused by anticoagulant therapy.

Methods

In order to evaluate the effect of COVID 19 pandemics on surgical therapy in IV. Department of Surgery we performed a retrospective study, that compared the periods of reprofilization to COVID department with control periods of the same duration length (as showen in Tab 1.)

Table 1: Observed time periods included in the study

COVID group A	1.11.2020 - 30.4.2021	Second pandemic wave of COVID 19 in Slovakia
COVID group B	1.11.2021 - 30.4.2022	Third pandemic wave of COVID 19 in Slovakia
Control group 1	1.11.2018 - 30.4.2019	Control to COVID group A
Control group 2	1.11.2019 - 30.4.2020	Control to COVID group B

The main factors observed during higher mentioned periods were the numbers of surgeries in total, planed and acute surgeries, oncological and non-oncological cases and we focused separately on patients admitted acutely because of acute appendicitis. All the criteria summarised in Tab. 2.

Table 2.: Factors observed in our study

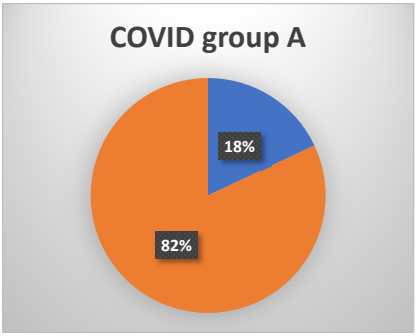
Surgery	planed oncological case		
	planed non-oncological case		
	acute case		
Acute appendicities	Age		
	Sex		
Acute appendicities	Therapy	conservative	
		surgery	laparoscopy
			laparotomy
			BSL - 3
Acute appendicities	time from onset to operation		
Acute appendicities	total hospitalisation time		
Acute appendicities	histological result	phlegmon	
		gangrene	
		chronic appendicities	
		negative	

Results

In order to compare the number of surgeries, we analysed in Covid group A 211 surgeries in total, this number consisted of 38 planed operations and 173 acute ones. Covid group B included 355 surgeries in total, 121 planed and 234 acute ones. Control group 1 included 406 planed and 298 surgeries. Control group 2 included 518 planed and 239 acute surgery. Overall findings show, that during the reprofilization planed surgeries formed only 28% of all compared to the controls, where planed surgeries were 63%. The most alarming effect was in the planed surgery cases. Meanwhile in control groups together 924 patents were operated, in both COVID groups only 159 underwent planed surgery. It means decrease of more than 82%. In acute patients the fall-off was not so massive, only 24%. (Tab. 3, Fig 1-4)

Table 3: Numbers of surgeries

	Planned surgeries	Acute surgeries	Total
COVID group A	38	173	211
COVID group B	121	234	355
Control 1	406	298	704
Control 2	518	239	757



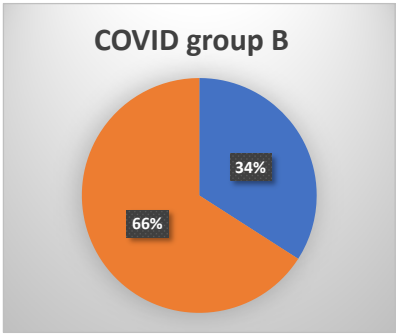
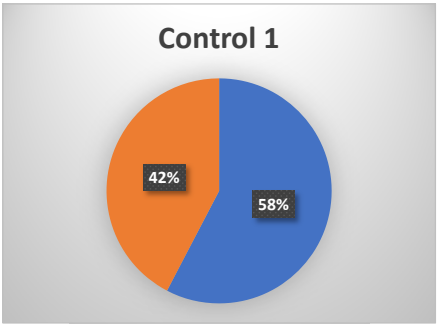


Figure 1 and 2: Number of surgeries in COVID groups. BLUE = planned, ORANGE = acute



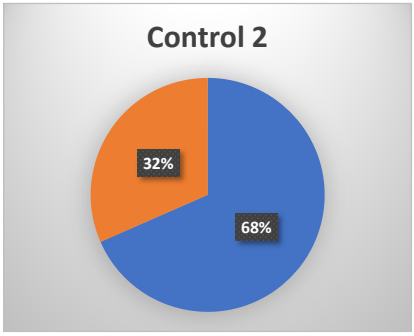


Figure 3 and 4: Number of surgeries in Control groups. BLUE = planned, ORANGE = acute

Also, in oncological surgeries we observed a significant decrease. In both control groups together 112 patients underwent surgical intervention, meanwhile in both Covid groups together it was only 52. (Tab 4)

Table 4: Numbers of surgeries

	Number of planned oncological surgeries
COVID group A	26
COVID group B	26
Control 1	60
Control 2	52

Part of our study specially focused on patients admitted to our department because of acute appendicitis. Together 314 patients were included and 237 (100 female and 137 male) underwent surgery. In Covid group A we performed 46 appendectomies, in Covid group B 66, in Control 1 we performed 52 appendectomies and in Control 2 we performed 73 appendectomies (Tab.5)

Table 5 – Patients admitted with signs of acute appendectomy

	Hospitalized	Surgical therapy	Conservative
COVID group A	59	46	13
COVID group B	84	66	18
Control 1	76	52	24
Control 2	95	73	22
Together	314	237	77

Relation between conservative and surgical therapy in all the observed groups show no important difference. Conservative therapy was 22% in Covid group A, 21% in Covid group B, 32% in Control 1 and 23% in Control 2.

Interesting results showed the observation od COVID 19 patients, admitted because of acute appendicitis. Together 10 patients (4 female and 6 male) with average age 55,2 years underwent appendectomy laparotomicly in BSL-3 conditions (Tab.6). The rate between female and male did not show any significant difference (Tab.7), but the average age in non-Covid patients was 43,5 year, but in Covid positive patients with acute appendectomy it was 12 years more. Difference was also in the rate between appendectomies and all the acute operations performed. In Covid group A appendectomies formed 21,8% of all the surgeries performed, in Covid group B 18,5%, but in Control 1 it was only 7,3% and in Control 2 in was 9,6% (Tab. 8 and 9).

Table 6: – Appendectomies

	Surgery	Surgery in BSL – 3
COVID group A	46	6
COVID group B	66	4
Control 1	52	0
Control 2	73	0

Table 7: Patients treated surgically - appendectomy

APPENDECTOMY	Female	Male	Together
COVID group A	23 (50%)	23 (50%)	46
COVID group B	25 (38%)	41 (62%)	66
Control 1	22 (42%)	30 (58%)	52
Control 2	30 (41%)	43 (59%)	73

Table 8: - Appendectomies vs. all the surgeries

	Surgeries in total	Acute	Appendectomy
COVID group A	211	173	46
COVID group B	355	234	66
Control 1	704	298	52
Control 2	757	239	73

Table 9: - Appendectomy – laparotomy vs laparoscopy

APPENDECTOMY	Surgery	Laparotomy	Laparoscopy
Covid group A	46	28	18
Covid group B	66	39	27
Covid 19 positive	10	10	0
Control 1	52	30	22
Control 2	73	36	37

Last very interesting finding was in histopathological result. We observed the rate of gangrenous / perforated appendicities. In Covid group A it was 34,8%, in Covid group B it was 44%, meanwhile in control groups the rate of gangrene was lower, 21,2% in Control 1 and

28,8% in Control 2 (Tab 10). So, in the Covid periods there was a higher rate of complicated appendicities. All the histopathological results of patients in Covid periods confirmed the diagnosis, so no surgery was incorrectly indicated (Tab. 10).

Table 10: Appendectomies – histopathological results

APPENDECTOMY	Phlegmon	Gangrene	Chronic	NEGAT
COVID group A	30	16 (34,8%)	0	0
COVID group B	34	29 (44%)	3	0
Control 1	36	11 (21,2%)	3	2
Control 2	48	21 (28,8%)	0	4

The reason of higher rate of gangrenous / perforated appendicities first seemed to be the delay between onset and operation in pandemic situation. On one hand patients were afraid to go to the emergency department, because it was the ideal place to catch COVID 19, on the other hand, the time patients had to spend in emergency in order the estimate their diagnosis could be influenced by numbers of Covid 19 patients needed diagnostics and therapy. After the statistical evaluation between the onset of symptoms and surgery surprisingly we found out, that during Covid periods the time between onset to surgery was shorter than in non-pandemic periods. This was valid also for gangrenous/perforated appendicities (Tab. 11 and 12).

Table 11: - Appendicities – average time between onset of first simptoms and surgery

APPENDECTOMY	AVERAGE TIME BETWEEN ONSET OF FIRST SIMPTOMS AND SURGERY
COVID group A	34 hours
COVID group B	44 hours
Control 1	41 hours
Control 2	40,4 hours

Table 12: Gangrenous / perforated appendicities - average time between onset of first symptoms and surgery

GANGRENOUS / PERFORATED APPENDICITIES	AVERAGE TIME BETWEEN ONSET OF FIRST SIMPTOMS AND SURGERY
COVID group A	45,8 hours
COVID group B	54,4 hours
Control 1	59,3 hours
Control 2	63 hours
COVID positive patients	33,6 hours

In spite of all challenges pandemic era brought to our society, we were able to provide the same quality of medical care to patients with acute appendicitis. I tis shown in time necessary for hospitalisation. There is no difference between Covid ang Control group. The only exception, were the patient Covid 19 positive with appendicities. They required longer hospital

Table 13: - Length of hospital stay

ACUTE APPENDICITY	Hospital stay average	Hospital stay median	Time frame (days)
Covid group A	5,3	4	2 – 18
Covid group B	5,2	5	2 – 18
Control 1	5,7	5	2 – 19
Control 2	5,2	5	2 – 13
COVID positive	8,4	8	4 – 18

Discussion

COVID 19 brought many challenges also to surgeons. A lot of stereotypes were changed, surgeons had to face a lot of new situations, some surgeries had to be performed under complicated condition in BSL-3 and as showen in Results both planned and acute surgeries were restricted. In order to evaluate the effect of COVID 19 pandemics on surgical treatment we realized a retrospective study and compared our experience with similar cases from abroad. The transformation to covid department underwent also the Department of Plastic Surgery of Elizabeth Queen Hospital in Birminghame [(1)], where 83 medical doctors were responsible for covid department with 36 beds, meanwhile in our department 22 medical doctors were responsible for covid department with 52 beds. Both departments provided also standard surgical care to non-covid patients with serious restriction of planned surgeries. Department in Birmingham was reprofilised for period of 6 weeks and doctors worked in 12

hours shift, but our department was work for 6 months in second and also 6 months in third pandemic way and work was 8 or 24 hours.

From surgical point interesting findings were observed in acute appendicities. Similar work was published by authors from Ankara, Turkey [(2)]. The number of patients included was similar, we had 237 and study from Turkey included 214 patients. Both studies showed higher rate of gangrenous / perforated appendicities, in Ankara 27,8%, in our study up to 44%.

Interesting was the change of the therapeutic strategy in Scotland, Ninewells [(3)], were surgeons limited surgical therapy of acute appendicities to 56,3% of cases, the rest was treated by antibiotics only, what is in contrast to our therapeutic management – appendectomy in both pandemic and non-pandemic period.

Acute appencitis was observed in multicentric study in Germany [(4)], in 41 departments on 1915 patients. Similar to our results, the number of appendectomies during pandemic was a little bit lower – 10,4% less in Germany, 13,5% less in our department and the number of complicated appendicities was higher from 58,2% to 64,4% in Germany and form 25,6% to 40,2% in our department.

We also observed a specific complication of COVDI 19 treatment [(5)], that required surgical therapy – sever non-traumatic intraabdominal haemorrhage, that occurred also in other hospitals, as showed in work of Kovácsová et. al. [(6)].

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16. Genomic surveillance of SARS-CoV-2 in the Slovak Republic: reconstruction of two years' experience

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Introduction

The Coronavirus disease (COVID-19) pandemic, declared by the World Health Organization (WHO) on March 11th, 2020 (1), has resulted in over 767 million confirmed infections and over 6,9 million deaths as of June 7th, 2023 (2). The recent declaration by WHO, ending COVID-19 global health emergency (3), can lead to wrong conclusion that all countermeasures created last three years are already not necessary at all. For example, next-generation sequencing (NGS) has been the primary method to track emerging changes in the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus genome, with significant mutational changes observed throughout the pandemic (4,5). The resulting sequencing data are increasingly being analyzed to understand the links between transmission dynamics, pathogenicity, vaccine efficacy, and immune escape.

The SARS-CoV-2 virus genome is approximately 29.9 Kb in size, with mutations occurring in various regions (6). Changes in structural proteins have had the most significant impact on virus properties, resulting in different genetic lineages. Nomenclature systems from GISAID (7), Nextstrain (8), and Pango (9) are currently used to track and name SARS-CoV-2 genetic lineages. WHO has introduced a uniform nomenclature using Greek letters and has divided the lineages into three categories: Variants of Concern (VOCs), Variants of Interest (VOIs), and Variants under Monitoring (VUMs). VOCs, such as the Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), and Omicron (B.1.1.529) variants, have had a significant impact on transmissibility, severity, and immune response (10).

To monitor novel or emerging variants, the European Center for Disease Prevention and Control (ECDC) recommends a sequencing rate of 5% of all COVID-19 positive samples (11). Many countries in the world have launched national viral genome surveillance programs, allowing real-time tracking of SARS-CoV-2 viruses' diversity. For the same purpose, the Slovak Republic formed an expert workgroup for genomics surveillance under the Ministry of Health, coordinated by the Public Health Authority of the Slovak Republic (PHA SR). The national SARS-CoV-2 sequencing program, launched in March 2021, has contributed to the global endeavor to monitor the COVID-19 pandemic's development (12). Additionally, an integrated central information system has been developed to facilitate the scheduling of Illumina sequencing laboratories, enabling more robust and automated data and metadata transfer, and unifying analysis through automated batch database uploading. This system, abbreviated as NarCoS (National COVID-19 Sequencing), has been operating since late February 2022 and has significantly enhanced the robustness, speed, and accuracy required to accommodate the rapidly increasing number of sequenced samples. This study presents the major results obtained by six participating laboratories using Illumina sequencing technology from the program's launch until March 2023.

Methods

Collecting SARS-CoV-2 samples

Medical subjects provided nasopharyngeal swabs for routine COVID-19 testing. Swabs were refrigerated and transported to a central laboratory PHA SR in Bratislava. Samples were selected randomly and selectively (positive travel anamnesis, atypical course of COVID-19, severe case subgroups) for genome sequencing, with a limit of RT-PCR cycle threshold ≤ 30 for successful sequencing.

RNA isolation of selected samples

The samples selected for sequencing had fresh nucleic acid extracted from the primary source, independently of the initial RT-PCR testing material. RNA isolation was done at four different workplaces (PHA SR in Bratislava and Regional PHA in Trenčín, Banská Bystrica and Košice). RNA was isolated using various kits (RNAdvance Viral RNA Extraction Kit, QIAamp Viral RNA Mini Kit and Quick-RNA™ Viral Kit) following the manufacturer's protocol. RNA was stored in a 96-well plate at -80°C and processed by the relevant sequencing center.

Massive parallel sequencing of SARS-CoV-2 genome

Six genomic laboratories performed sequencing on Illumina sequencing platforms (Comenius University Science Park in Bratislava - CU SP, PHA SR; Jessenius Faculty of Medicine of Comenius University - JFM CU; Regional Public Health Authority in Banská Bystrica - RPHA BB; Regional Public Health Authority in Trenčín - RPHA TN; Regional Public Health Authority in Košice - RPHA KE). The SARS-CoV-2 whole-genome sequencing libraries were manually prepared in 96-well plates (95 samples, 1 non-template control) based on the Illumina COVIDSeq Test protocol. After cDNA synthesis and amplification using COVIDSeq™ V3 Primer Pool Pool (replaced by the new COVIDSeq™ V4 Primer Pool from December 2021), PCR amplicons were tagmented using IDT® for Illumina PCR Unique Dual Indexes Set 1–4 and libraries were purified and pooled following the manufacturer's guidelines. All laboratories used Illumina platforms - three used MiniSeq, one used MiSeq, one used NextSeq 550, and

one used NextSeq 500 and NextSeq 2000. The sequencing parameters were initially different for each platform, but they were later unified for all platforms for 2 × 74 bp paired-end reads except NextSeq 2000 with 2 x 100 bp paired-end.

Bioinformatic analysis

Detailed information about the pipeline is available in Goga paper (13). To study the SARS-CoV-2 virus, the researchers used a pipeline process that involved trimming the reads, removing human RNA fragments, mapping the reads to the SARS-CoV-2 virus's genome, and checking the quality of the consensus sequences. The sequences that met the coverage criteria were uploaded to public repositories - European Nucleotide Archive (14) and GISAID (7). The computational analyses were independent of the sequencing reads length. In February 2022, an integrated system called NarCoS (Národné COVID-19 Sekvenovanie—National COVID-19 Sequencing) was launched to optimize data transfers and allow unified analysis, verification, and batch uploading to repositories. The previously mentioned pipeline became the data analysis module of this system.

Results

SARS-CoV-2 capture in the Slovak population

We conducted an analysis of Slovak Republic's SARS-CoV-2 pandemic data by examining a specific set of sequenced samples from a particular period (March 1, 2021 – March 31, 2023). During a span of 25 months, more than 1.8 million (n = 1,865,471) positive SARS-CoV-2 cases were confirmed by RT-PCR [32]. Of all the positive samples, COVID-19 dedicated laboratories randomly selected and sent an over 47,000 samples (n = 47,677) for sequencing and SARS-CoV-2 variant determination, representing 2.56% of the total. Needs to be mentioned, that this percentage is slightly higher as only data generated by collaborating laboratories using Illumina sequencing and described in the methods are included here. Out of all the suitable RNA samples, 94.2% (n = 44,890) were successfully sequenced and analyzed to determine the SARS-CoV-2 virus variant. Finally, 86.2% (n = 41,079) of the high-quality consensus sequences met all criteria for submission to the GISAID repository for sharing with the scientific community.

Of these, 54.8% were female with an average age of 46.1 (median = 45), and 45.1% were male with an average age of 44.5 (median = 44). Unknown sex was associated with only 0.1% of samples. Samples were collected randomly from all districts of the Slovak Republic.

The surveillance program and its sequencing volume over time

Slovakia's strategy for monitoring the SARS-CoV-2 virus focused on collecting samples from state and private laboratories, hospitals, and citizens returning from countries where new virus variants had been reported. Depending on the current epidemiological situation, genomic laboratories sequenced statistically anywhere from 12 to 1650 samples per week. In the summer of 2021, the number of positive cases decreased, resulting in a drop in sequencing volume to an average of 100 samples per week. However, in early 2022, the new Omicron variant and its sublineages caused a surge in infections, leading to increased sequencing numbers of over 1500 positive cases per week. As a result, a more comprehensive approach to handling and analyzing data became necessary.

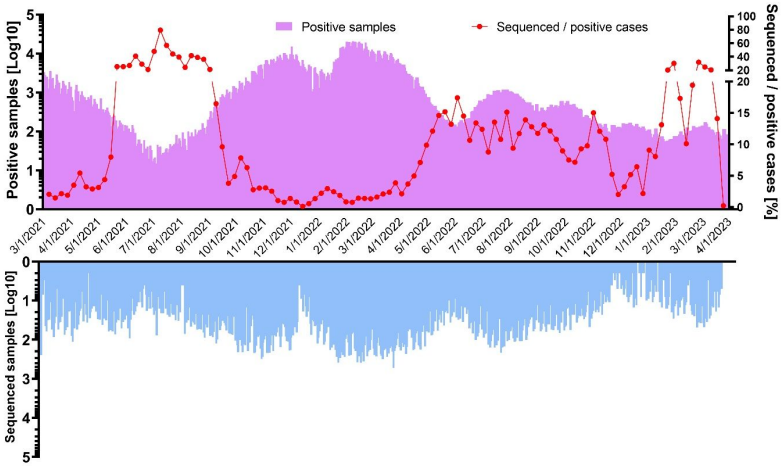

Figure 1: Number of confirmed cases of SARS-CoV-2 in Slovakia over time. The purple bars indicate the daily number of positive test results, while the red line shows the weekly coverage. The blue bars represent the number of samples that were sequenced. The data reveals that there was a peak in positive cases in late 2021 and early 2022.

In order to accurately determine the prevalence of virus variants, a sufficient number of samples need to be sequenced. Ideally, this would be 5% of all positive cases during the same period. We analyzed the proportion of weekly sequencing volumes compared to weekly positive cases and found that during June, July, and August of 2021, the proportion of sequenced cases was over 40%. However, from the beginning of October 2021 till April 2022 of the analyzed period, the VOCs Delta (B.1.617.2) and Omicron (B.1.1.529) variants were dominant. This period is characterized by a very high number of SARS-CoV-2 positive cases in the population, which was reflected in a lower percentage of sequencing coverage, averaging 2.35%. During the summer months of 2022 (June to September), the proportion of samples determined to be variants ranged from 10% to 15%. Towards the end of 2022 and the beginning of 2023, the percentage of SARS-CoV-2 samples with a determined variant fluctuated between 2% and 30% of the total positive samples obtained during that time (Figure 1).

Virus lineages proportions in Slovak population over time

We analyzed each SARS-CoV-2 consensus sequence using the GISAID Pangolin pipeline to determine the lineage in time of deposition to the GISAID repository. Our study identified 425 unique sublineages of the SARS-CoV-2 virus. We have selected a list of the 18 most prevalent

lineages, which accounted for over 1% of all analyzed samples (Table 1). The most common lineage detected during the analyzed period was BA.2, present in 17.14% of SARS-CoV-2 positive cases, followed by BA.1.1, B.1.1.7, AY.43 and BA.2.9 (Table 1). We identified four variants of concern (VOC) out of 5 WHO: Alpha, Beta, Delta, and Omicron. We also detected two variants of interest (VOI): Kappa and Mu. Alpha was prevalent in the Slovak population until the end of June 2021, followed by an increase in Delta lineages until the last weeks of 2021. The Omicron variant was detected at the beginning of 2022, with dominant sublineage BA.2 followed by BA.5. During the end of 2022 and throughout 2023, multiple SARS-CoV-2 recombinants were spreading throughout the population. The XBB recombinants have been particularly prevalent since February 2023.3.

Table 1: List of 18 sublineages with more than 1% proportion among all determined SARS-CoV-2 samples collected in Slovak Republic.

Sublineage	%	Sublineage	%	Sublineage	%
BA.2	17.14	AY.122	3.66	BA.5.2.1	2.11
BA.1.1	8.42	BA.1	3.37	BF.5	1.92
B.1.1.7	7.67	BA.5.1	3.35	AY.126	1.64
AY.43	7.63	BA.5.2	2.63	AY.43.9	1.45
BA.2.9	6.47	BA.1.1.1	2.47	BE.1.1	1.28
AY.4	3.87	AY.9.2	2.46	BA.1.17.2	1.23

Discussion

We analyzed the COVID-19 pandemic in Slovakia, focusing on genomic surveillance of the SARS-CoV-2 virus. This was done through Illumina technology-based labs coordinated by the PHA SR. We used publicly available sequences and metadata to describe the pandemic's development in the Slovak population (population 5.45×10^6) (15).

The first global wave of the virus in Slovakia was under control due to strict government measures, but the situation worsened in October 2020 with the new lineage Alpha (B.1.1.7). (16) This variant, which originated in England, caused a worldwide increase in COVID-19 cases (17). Despite facing challenges with the new lineage B.1.1.7, Slovakia implemented a national sequencing program in March 2021 to stay on top of the virus's developments (18). With the collaboration of six sequencing laboratories, the country continues to monitor and detect SARS-CoV-2 variants and sublineages. Slovakia's proactive approach serves as an inspiration for other nations in the fight against COVID-19 (19).

We looked at three significant waves of SARS-CoV-2 VOCs. The first wave, known as the Alpha variant (B.1.1.7), lasted from March 1, 2021, to mid-July 2021, and had fewer daily positive

cases compared to the subsequent waves. However, the impact on total confirmed deaths was higher than the Delta or Omicron waves, with a total of 81,098 positive tests and 5,136 confirmed deaths. This was due to the high number of hospitalized and ICU patients, with the highest daily hospital occupation being $715/1x10^6$ people and the highest daily ICU occupation being $112/1x10^6$ people (16).

The Delta (B.1.617.2) variant and its AY lineages caused the second wave of COVID-19 in Slovak Republic, with the first cases being detected and sequenced in July 2021. These variants and their sublineages led to an increase in SARS-CoV-2 positive cases among the population (n = 498,683) but resulted in fewer total deaths (n = 4,932) compared to the previous period analyzed. The highest daily statistics included $2083/1x10^6$ people for positive cases, $17.8/1x10^6$ people for confirmed deaths, $615/1x10^6$ people for hospitalizations, and $115/1x10^6$ people for ICU patients (16).

Starting in January 2022, the third wave of the COVID-19 pandemic began with the discovery of the Omicron (B.1.1.529) variant, which was followed by subsequent variants BA.1, BA.2 and BA.5 The impact of the Omicron variant was different from previous waves of the pandemic until the end of March 2023. During the 15 months, there were around 1,000,000 positive SARS-CoV-2 samples, with the highest daily positive cases being $4148/1x10^6$ people. Unfortunately, the high increase in infections resulted in 1848 confirmed deaths, with the highest daily number being $7.16/1x10^6$ people. However, hospitals had a lower burden, with the highest hospital and ICU patient occupancy at 534 and $50/1x10^6$ people, respectively (16).

The number of COVID-19 samples being sequenced varies over time and depends on the number of positive cases. During months with a high number of positive cases, the surveillance program was only able to identify variants in about 2% of the samples tested, despite original plans to sequence 500 samples per week. However, this number increased during the Delta and Omicron waves, leading to bottlenecks in processing and uploading data. To address this issue, an integrated central information system called NarCoS was developed and has been in operation since February 2022. NarCoS has helped increase the efficiency and accuracy of sample sequencing by allowing for the scheduling of Illumina sequencing laboratories, more robust and automated data transfer, unified analysis, and automated batch database uploading. This system has been instrumental in accommodating the need to increase the number of samples sequenced rapidly.

Although the COVID-19 pandemic has calmed down, it has not yet displayed a typical seasonal respiratory disease pattern. However, recent months have shown that newly detected lineages cause fewer issues compared to those that spread two to three years ago. Even the current recombinant XBB.1.16 circulating strain does not tend to produce severe COVID-19 symptoms compared to previous Omicron sublineages (20). Nonetheless, genomic surveillance remains a crucial aspect of epidemiology tools for future epidemics and pandemics, as emphasized by the WHO's global genomic surveillance strategy for pathogens over the next ten years (21).

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17. Antibiotic therapy and a risk factor of multidrug-resistant bacteria superinfection in COVID-19 patients

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Introduction

Clinical presentation of Coronavirus infection disease 2019 (COVID-19) varies from asymptomatic or mild infection of upper respiratory airways to life threatening respiratory failure requiring admission to intensive care unit (ICU)1. At the ICU setting, the bacterial or fungal superinfections are relatively common, especially during large COVID-19 outbreaks associated with significant strain to local health care system 2. One of the most important bacterial pathogens causing superinfections in COVID-19 patients at the ICU setting during outbreaks is multidrug resistant *Acinetobacter species* (MDR-Ab). It is well known that development of superinfection caused by MDR-Ab impairs the prognosis of COVID-19 patients significantly. 3,4Despite the fact that COVID-19 is primarily a viral disease, the antibiotics are abundantly prescribed in COVID-19 patients, even without any clinical proofs of bacterial superinfection.7 The inadequate use of antibiotics in COVID-19 might be associated with the risk of development of multidrug resistant bacterial superinfection, especially in ICU setting.

Methods

To investigate the association of empiric antibiotic therapy with the risk of MDR-Ab infection in COVID-19 patients admitted to ICU, we conducted a retrospective case-control study. The study included 90 patients admitted to the ICU of the Department of Infectology and Geographical Medicine, University Hospital in Bratislava, with severe COVID-19 requiring High flow nasal oxygen treatment or mechanical ventilation. The inclusion criteria were SARS-CoV-2 infection verified by PCR and presence of respiratory failure requiring high flow nasal oxygen therapy or mechanical ventilation. All patients underwent standard microbial screening on a regular basis. Bacterial superinfection was defined as clinically, laboratory and microbiological verified infection occurring ≥ 48 h after ICU admission. Acinetobacter strains were isolated and identified using blood agar cultivation followed by matrix-assisted laser desorption/ionization (MALDI-TOF). Acinetobacter isolates were considered as multidrug-resistant according to international expert proposals for standard definitions for acquired resistance.8

Results

The study included 58 male and 32 female patients. MDR-Ab was isolated in 37 patients. The isolation of MDR-Ab was regarded as superinfection in 33 patients (36.7%). Fifty-four (60%) patients were exposed to antibiotics during ICU stay. Ceftriaxone was the most utilized antimicrobial drug used in 35 (64.8%) patients. Exposure to ceftriaxone was independently associated with the risk of MDR-Ab superinfection (odds ratio [OR] 4.1, 95% confidence interval [CI] 1.4–11.9, $P < 0.05$) in multivariate binary logistic regression. Other antimicrobials were not associated with the risk of MDR-Ab superinfection.

Discussion

MDR-Ab is one of the most important pathogens causing superinfections in COVID-19 patients in ICU settings 3,4,5,6. MDR-Ab was also the most common pathogen isolated in our study population. It was also the most common pathogen causing clinically relevant superinfection. Its ability to develop resistance to various antimicrobials, to avoid standard decontamination procedures and to be easily transmitted by hands of hospital personnel are the features that facilitate development of large MDR-Ab outbreaks during pandemic. 3 The overuse of antibiotics was proposed as possible factor contributing to the risk of development of MDR-Ab infection in COVID-19 patients, however the more solid evidence are scarce. The ceftriaxone was the most utilized antimicrobial drug in our study cohort. It was found to be independently associated with the risk of development of MDR-Ab superinfection. Previous authors found association with use of other broad spectrum antibiotics like piperacillin-tazobactam and carbapenems.9, 10 The lack of association of carbapenem or other antimicrobial drugs with MDR-Ab development in our study was caused by low number of subject treated by these antibiotics. According to results of our study and also evidence from previous studies, we propose that exposure to broad-spectrum antibiotics increases the risk of Acinetobacter colonization in COVID-19 ICU patients and therefore facilitates the MDR-Ab superinfection. Therefore, antibiotics should be used in COVID-19 patients only if there is strong suspicion for bacterial superinfection.

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18. Epidemiology and characteristics of headache associated with COVID-19 in hospitalized patients in Slovakia

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Coronavirus disease is a clinical presentation of the infection of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which became global pandemic in 2020. Though respiratory symptoms are the most common clinical indicators, multiple neurological symptoms and related complications have also been reported. Among the neurological symptoms, headache has been consistently identified as a widespread and debilitating symptom, impacting anywhere from 6 to 70% of individuals infected with the virus. This broad spectrum of reported prevalence is likely due to methodological discrepancies among studies, such as how data was collected, the specific population studied, whether the study was prospective or retrospective, the severity of the disease in the studied population, and whether the primary focus of the research was headaches specifically or COVID-19 symptoms in general. Although headache associated with COVID-19 has proven to be a common, disabling, often persisting and difficult to treat symptom for neurologists worldwide, epidemiological data in Slovakia are lacking. Therefore, the goal of our study was to establish the prevalence and characteristics of headache associated with COVID-19 infection in hospitalized patients in Slovakia.

19. Koncentrácia 25-hydroxyvitamínu D v sére signifikantne klesá u pacientov s COVID-19 pneumóniou počas prvých 48 hodín po prijatí do nemocnice

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Úvod

Vitamín D je v kontexte pandémie ochorenia COVID-19 (z angl. Coronavirus Disease 2019) veľmi diskutovanou témou a viaceré práce poukázali na fakt, že hodnoty vitamínu D v sére sú nezávislým prediktorom mortality u pacientov s COVID-19 pneumóniou a ťažký deficit vitamínu D je spojený s excesívnou mortalitou u týchto pacientov (1). Ťažká forma ochorenia COVID-19 spôsobuje hyperaktiváciu systémovej imunitnej odpovede, ktorá je vyvolaná najskôr hypoxickým prostredím v dôsledku respiračného zlyhania a neskôr sa rozvíjajúcou cytokínovou búrkou (2). Počas tejto akútnej fázy systémového zápalu možno na úrovni organizmu očakávať metabolické zmeny tak makronutrientov, ako aj mikronutrientov; metabolity vitamínu D nie sú výnimkou. Koncentrácia 25-hydroxyvitamínu D (25[OH]D) v sére reflektuje zásoby vitamínu D v tele a za normálnych okolností je najlepším biomarkerom, ktorým môžeme zhodnotiť status vitamínu D u konkrétneho pacienta (3). Úloha 25(OH)D ako biomarkera počas akútneho zápalového ochorenia je však oveľa menej jasná. Niektoré práce popisujú signifikantné zmeny v koncentrácii 25(OH)D, ktoré sa môžu u akútne chorého pacienta objaviť už v priebehu hodín (4). Preto niektorí autori uvádzajú, že jednorazové stanovanie koncentrácie 25(OH)D v sére nie je dostačujúce na adekvátne zhodnotenie zásob vitamínu D počas akútnej fázy zápalu (4). Vzhľadom k diskutovej role vitamínu D v kontexte pandémie ochorenia COVID-19, naším cieľom bolo prospektívne sledovať zmeny v koncentrácii 25(OH)D počas akútnej COVID-19 infekcie u pacientov s pneumóniou a hypoxemickou respiračnou insuficienciou a identifikovať vhodné časové okno pre zhodnotenie koncentrácie 25(OH)D počas akútneho ochorenia.

Materiál a metódy

Do práce boli zaradení pacienti, ktorí boli hospitalizovaní s diagnózou akútnej COVID-19 pneumónie na V. internej klinike Lekárskej fakulty Univerzity Komenského v Bratislave a

Univerzitnej nemocnice Bratislava v časovom období od 1. novembra 2021 do 31. decembra 2021.

Exklúzne kritériá boli nasledovné: (a) pacienti bez potreby oxygenoterapie, (b) pacienti, ktori nespĺňali kritériá pre ťažkú formu ochorenia, (c) pneumónia COVID-19 nebola primárnou diagnózou v čase prijatia do nemocnice, (d) pacienti mali inú infekciu (napr. uroinfekciu) počas sledovaného obdobia.

Pacienti počas hospitalizácie nedostávali preparáty vitamínu D. Ťažká forma ochorenia bola definovaná nasledovne: počet dychov za minútu >30; závažný respiračný distres; alebo saturácia krvi kyslíkom <90% bez oxygenoterapie (5).

Prvý odber venóznej krvi sme realizovali hneď pri prijatí pacienta (deň 0), a následne sme realizovali odber venóznej krvi každých 24 hodín: 2. vzorka odobraná v 24. hodine (deň 1), 3. vzorka odobraná v 48. hodine (deň 2), 4. vzorka odobraná v 72. hodine (deň 3), a 5. vzorka odobraná v 96. hodine (deň 4).

Hodnota 25(OH)D (ng/ml) v sére bola stanovená pomocou automatického elektrochemiluminisenčného systému (Eclesys Vitamin D Total II, 2019, Roche Diagnostics GmBH, Mannheim, Germany). Detekčný limit 25(OH)D bol 3 ng/ml.

Štatistická analýza bola realizovaná poocou štatistického sowftvéru Analyse-it (Leeds, UK) v 5.40.2 alebo R (v 3.6.0). Výsledné dáta sú prezentované ako priemer ± štandardná odchýlka, ak boli normálne distribuované, alebo ako medián a kvartilové rozpätie, ak neboli normálne distribuované. Shapiro-Francia test bol použitý na testovanie normality distribúcie študovaných parametrov. Zmeny medzi jednotlivými časovými bodmi boli hodnotené párovým t-testom u normálne distribuovaných parametrov a Wilcoxonovým testom u ostatných parametrov. Vzťahy medzi sledovanými parametrami boli vypočítané pomocou Pearsonovho korelačného koeficientu alebo binominálnou logistickou regresiou. P hodnota menej ako 0.05 bola považovaná za signifikantnú.

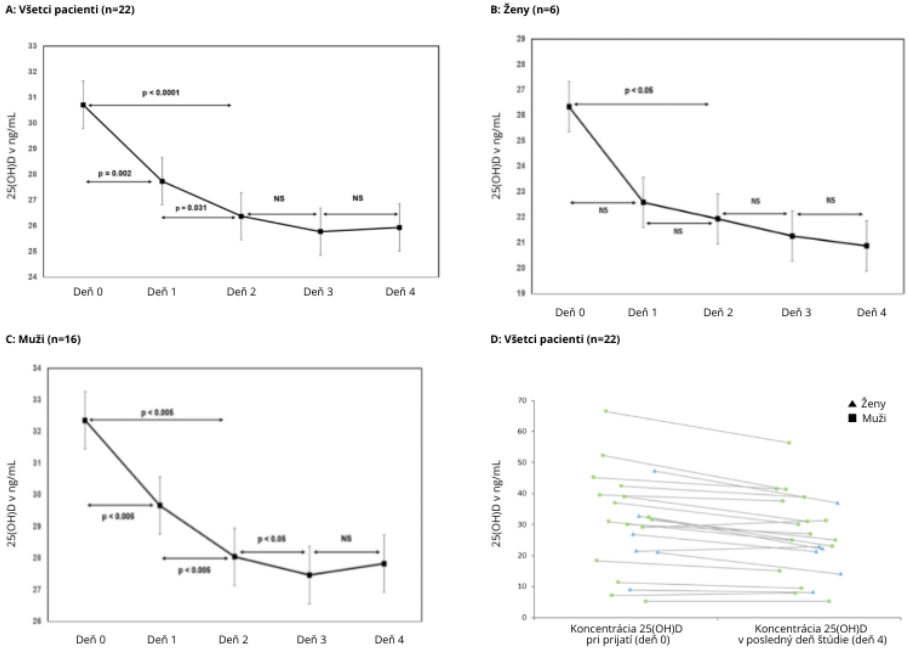
Štúdia bola realizovaná v súlade s Helsinskou deklaráciou a schválená Etickou komisiou Univerzitnej nemocnice Bratislava (rozhodnutie Etickej komisie: EK/011/2021).

Výsledky

Inklúzne kritériá splnilo 22 pacientov (6 žien, 16 mužov, priemerný vek 60,6 roka). 12 pacientov (55%) malo anamnézu artériovej hypertenzie, 7 pacientov (32%) malo diabetes mellitus, 2 pacienti (9%) mali koronárnu chorobu srdca a 5 pacientov chronické ochorenie obličiek. Priemerná hodnota BMI bola 29,74; 10 pacientov malo nadváhu (BMI > 25) a 8 pacientov bolo obéznych (BMI > 30). Všetci pacienti vyžadovali liečbu kyslíkom, 12 pacientov (55%) bolo liečených vysokoprietokovou oxygenoterapiou cez nazálnu kanylu (HFNV). 2 pacienti vyžadovali intubáciu a umelú pľúcnu ventiláciu. 4 pacienti po sledovanom období zomreli (po 4 dňoch hospitalizácie). Symptómy ochorenia COVID-19 (horúčka, triaška, kašeľ, nauzea, celková slabosť, myalgie, cefalea) boli prítomné v priemere 7,45 dňa pred prijatím do nemocnice. Dýchavica bola prítomná u 36% pacientov dlhšie než 48 hodín pred prijatím a u 64% pacientov do 48 hodín pred prijatím do nemocnice. Priemerná koncentrácie 25(OH)D pri prijatí bola 30,71 ng/mL; 59% pacientov malo suficientné hodnoty

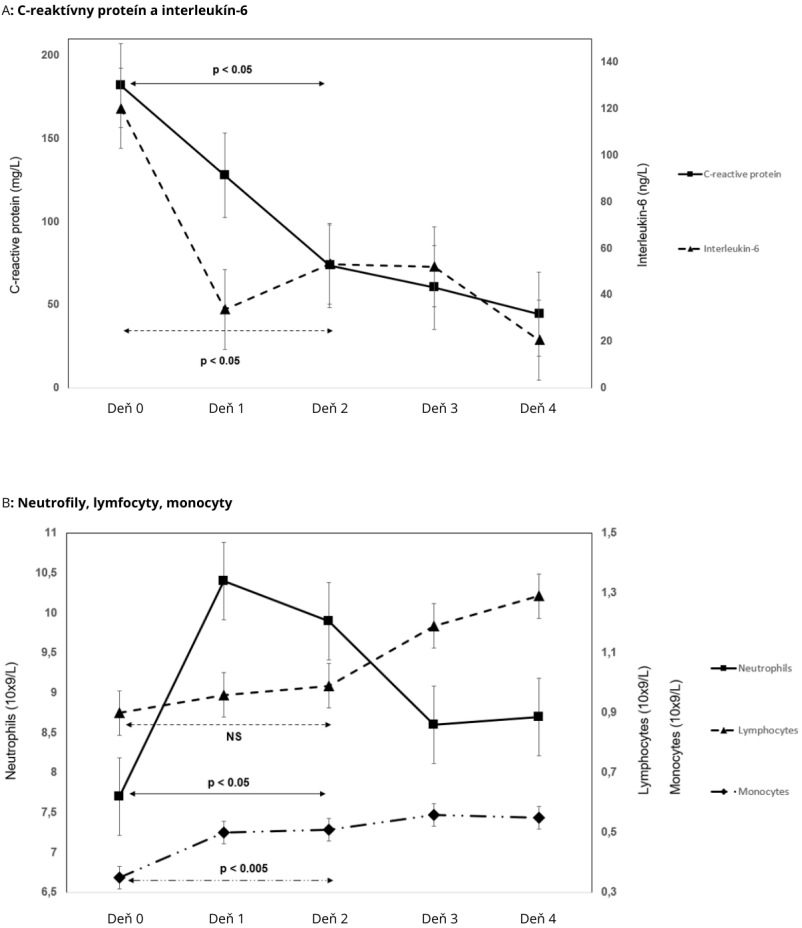
25(OH)D (>30 ng/mL), 18% pacientov bolo v pásme insuficiencie (30-20 ng/mL) a 5% pacientov bolo v pásme deficitu vitamínu D (<20 ng/mL). 45% pacientov užívalo preparáty vitamínu D pred prijatím do nemocnice.

Priemerná sérová koncentrácia 25(OH)D signifikantne klesla za prvých 48 hodín po prijatí do nemocnice (30,7 ng/mL vs. 26,4 ng/mL; p<0.0001) (Obrázok 1A). Po prvých 48 hodinách sme nepozorovali ďalší signifikantný pokles medzi 2. a 3. dňom (26,4 ng/mL vs. 25,9 ng/mL; p=0.23) a medzi 3. a 4. dňom (25.8 ng/mL vs. 25,9 ng/mL; p=0.70). Pokles v sérovej koncentrácii 25(OH)D bol prítomný u mužov aj u žien, ale pokles koncentrácie bol počas prvých 48 hodín výraznejší u mužov (p<0.005 vs p<0.05). Ďalší pokles v sérovej koncentrácii 25(OH)D po 48 hodinách bol prítomný u mužov, ale nie u žien (Obrázok 1B a 1C). U väčšiny pacientov v sledovanom období (n=17) došlo k poklesu koncentrácie 25(OH)D (Obrázok 1D).



Obrázok 1.: Kinetika koncentrácie 25(OH)D v sére počas sledovaného obdobia u všetkých pacientov (A), u žien (B), a u mužov (C). Počas sledovaného obdobia došlo k poklesu v koncentrácii 25(OH)D takmer u všetkých pacientov (D).

Absolútna zmena koncentrácie počas sledovaného obdobia (od prijatia po deň 4) bola 4,8 ng/mL (p<0.0001) a nebola asociovaná s mortalitou (p=0.21) alebo s nutnosťou HFNV (p=0.64). Na 4. sledovaný deň počet pacientov s neadekvátnou koncentráciou 25(OH)D (<30 ng/mL) vzrástol o 18% (p=0.018).



Obrázok 2.: Kinetika zápalových parametrov počas sledovaného obdobia. A: C-reaktívny proteín a interleukín-6. B: neutrofily, lymfcyty a monocyty.

Počas sledovaného obdobia sme pozorovali signifikantný pokles kreatinínu, albumínu, hemoglobínu a hematokritu (deň 1 vs deň 5, všetko $p<0.05$).

Koncentrácie C-reaktívneho proteínu (CRP) a interleukínu-6 (IL-6) počas sledovaného obdobia signifikantne poklesli (deň 0 vs deň 4, všetko $p<0.0005$) (Obrázok 2A). Prítomný bol signifikantný vzostup neutrofilov medzi prijatím a 1. dňom a následný pokles medzi 1. a 3. dňom ($p<0.0001$) (Obrázok 2B). Lymfocyty sa počas prvých 48 hodín výraznejšie nemenili; po 48 hodinách sme pozorovali vzostup v koncentrácii lymfocytov (deň 0 vs. deň 4; $p<0.05$) (Obrázok 2B). Koncentrácia monocytov počas sledovaného obdobia signifikantne vzrástla ($p<0.005$) (Obrázok 2B).

Diskusia

V našej práci sme preukázali signifikantný, 16%-ný pokles v koncentrácii 25(OH)D u pacientov s COVID-19 pneumóniou počas prvých 2 dní hospitalizácie. Výsledkom tohto poklesu je nárast počtu pacientov s neadekvátnymi hodnotami vitamínu D počas hospitalizácie (vzostup zo 41 na 59% počas sledovaného obdobia). V tejto štúdii zmeny v koncentrácii 25(OH)D neboli inverzne asociované s markermi zápalu CRP a IL-6. Pokles 25(OH)D sme pozorovali u oboch pohlaví, u mužov bol pokles výraznejší a pretrvával aj po prvých 48 hodinách.

Existujú viaceré práce hodnotiace zmeny 25(OH)D v kontexte zápalovej reakcie rôznej etiológie (najčastešie operačný výkon). Táto štúdia je však prvá, ktorá hodnotí zmeny v koncentrácii 25(OH)D prospektívne u pacientov s akútnou zápalovou reakciou infekčnej etiológie.

Koncentrácia 25(OH)D klesá už hodiny (6-48 hodín) po začiatku operačného výkonu, a zmena v koncentrácii oproti východzej hodnote môže byť až 40% (4). Vzhľadom k inverznému vzťahu medzi CRP a 25(OH)D v perioperačnom obdbí v týchto štúdách, viacerí autori uvažujú o sérovej koncentrácii 25(OH)D pri akútnych stavoch iba ako o negatívnom reaktante akútnej fázy zápalu (6). Pozorované zmeny v týchto štúdiách však môžu byť skôr v dôsledku samotného operačného výkonu alebo anestézy, než v dôsledku zápalu ako takého. Inverzný vzťah medzi hodnotami CRP a 25(OH)D bol však pozorovaný aj pri akútnej pankreatitíde (7) a po intravenóznej infúzii bisfosfonátov (8). Naproti tomu u pacientov po akútnom koronárnom syndróme (9) a u pacientov po malárii (10) neboli pozorované signifikantné zmeny v koncentrácii 25(OH)D ani významná korelácia s markermi zápalu. Treba však podotknúť, že v oboch prípadoch boli hodnoty 25(OH)D merané až po viacerých dňoch po nástupe symptómov, čo mohlo ovplyvniť schopnosť pozorovať zmeny v koncentrácii, respektíve pokles 25(OH)D.

V tomto kontexte je zaujímavé, že naši pacienti boli symptomatickí v priemere viac ako 7 dní a pociťovali dýchavicu takmer 2,5 dňa pred prijatím, a napriek tomu sme u väčšiny z nich detekovali signifikantný pokles koncentrácie 25(OH)D. Ťažká forma ochorenia COVID-19 sa však rozvíja najčastejšie 8-12 dní po začiatku symptómov a rozsah pľúcnych lézií je najvýraznejší medzi 6-11 dňom po začiatku symptómov (11). Je teda možné, že u našich pacientov bol rozsah pľúcnej manifestácie ochorenia na svojom vrchole a pozorovali sme funkčný deficit vitamínu D. Klesajúca koncentrácia vitamínu D by potom súvisela s požiadavkami periférnych tkanív, v tomto prípade pľúcneho parenchýmu. Cirkulujúci 25(OH)D predstavuje substrát pre konverziu na aktívnu formu vitamínu D – 1,25(OH)D2

bunkami respiračného epitelu a alveolárnymi makrofágmi. Metabolická dráha vitamínu D zohráva kľúčovú úlohu v homeostáze zápalovej reakcie na úrovni pľúcneho parenchýmu a bráni rozvoju syndrómu akútnej respiračnej tiesne (ARDS) viacerými mechanizmami (12). Abrishami s kolektívom autorov preukázali, že hospitalizovaní pacienti s COVID-19 a vyššími hodnotami 25(OH)D majú signifikantne menšie postihnutie pľúcneho parenchýmu na CT hrudníka (13).

Aj iné faktory, než priamy efekt zápalu môžu ovplyvniť sérové koncentrácie 25(OH)D počas akútneho ochorenia. Môže sa jednať o hemodilúciu pri podávaní väčšieho množstva infúznych roztokov, redistribúciu telesných tekutín v jednotlivých kompartmentoch počas akútneho stavu, alebo pokles viažúcich bielkovín pre 25(OH)D u kriticki chorých pacientov, či už sa jedná o vitamín D viažuci proteín (VDBP) alebo o albumín. V kontexte predkladanej štúdie je dôležité spomenúť aj možný efekt glukokortikoidov. Všetci pacienti dostávali počas sledovaného obdobia denne 6 miligramov dexametazónu intravenózne. Dexametazón zvyšuje expresiu vitamín D-24-hydroxylázy, ktorá degraduje metabolity vitamínu D, medzi inými aj 25(OH)D (14).

Naša práca má niekoľko limitácií. Po prvé, sledovali sme malú skupinu pacientov. Naším primárnym cieľom bolo pozorovať zmeny 25(OH)D a preto naša práca nemá dostatočnú silu preukázať vzťah medzi zmenami v koncentrácii 25(OH)D a mortalitou. Po druhé, nemali sme kontrolnú skupinu. Liečba glukokortikoidmi bola, a aj stále je základom liečby pacientov s hypoxemickou respiračnou insuficienciou pri ochorení COVID-19. Preto nebolo možné hodnotiť zmeny u pacientov s ťažkým priebehom COVID-19 bez podávania glukokortikoidov, a tak jednoznačne nemôžeme vylúčiť možný efekt podávanej liečby glukokortikoidmi na zmeny koncentrácie 25(OH)D. Po tretie, nemali sme k dipozícii hodnoty sérového 25(OH)D u našich pacientov pred hospitalizáciou. Tiež sme nestanovovali VDBP, takže nemôžeme zhodnotiť mieru, v akej ovplyvnili koncentráciu 25(OH)D zmeny v koncentrácii VDBP. Prínos našej práce spočíva v tom, že sme prospektívne sledovali pomerne homogénnu, jasne zadefinovanú skupinu pacientov s akútnou ťažkou hypoxemickou respiračnou insuficienciou pri COVID-19 pnuemónii. Všetky hodnoty 25(OH)D boli stanovené rovnakou metodikou u všetkých pacientov v presne definovaných intervaloch. Naša práca je prvá, v ktorej boli hodnoty 25(OH)D u pacientov s ťažkou formou COVID-19 sledované prospektívne počas dlhšieho časového obdobia. Je to tiež prvá práca s prospektívnym dizajnom, ktorá skúma zmeny 25(OH)D počas akútneho infekčného ochorenia.

Záverom možno povedať, že sérová koncentrácia 25(OH)D u akútne chorých pacientov s COVID-19 počas prvých 48 hodín po prijatí signifikantne klesá. Počet pacientov s neadekvátnou sérovou koncentráciou 25(OH)D sa počas hospitalizácie zvýšil o 18%. Po 48 hodinách sme nepozorovali ďalší signifikantný pokles v koncentrácii 25(OH)D, čo môže mať praktické implikácie pre stanovovanie hodnôt vitamínu D počas akútnych zápalových ochorení. To, či nízka hodnota 25(OH)D pri ochorení COVID-19 reflektuje funkčný deficit vitamínu D a má kauzálny vzťah k horšej prognóze ochorenia, alebo predstavuje „iba“ negatívny reaktant akútnej fázy zápalu, sa musí objasniť v prospektívnych, randomizovaných štúdiách so suplementáciou vitamínu D.

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20. Pacienti s COVID-19 pneumóniou, ktorí majú sérovú koncentráciu 25(OH)D < 12 ng/mL, majú vyššie riziko úmrtia v nemocnici

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Úvod

Nový koronavírus SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus 2) využíva ACE-2 (Angiotensin-Converting Enzyme 2) receptor na vstup do makrofágov a monocytov, čo u niektorých pacientov vedie k rozvoju hyperinflamačného syndrómu, syndrómu akútnej respiračnej tiesne a orgánovému poškodeniu (1). Imunomodulačné účinky vitamínu D by mohli pozitívne ovplyvniť excesívnu zápalovú odpoveď pri ochorení COVID-19 (Coronavirus Disease 2019) (2). Hoci je 25-hydroxy vitamín D (25[OH]D) považovaný za najlepší marker zásob vitamínu D v tele, presné definíce deficitu respektíve nedostatočnej koncentrácie vitamínu D sú stále predmetom diskusie (3). Vysoká miera zhody však panuje v tom, že hodnota 25(OH)D < 12 ng/ml predstavuje absolútny deficit vitamínu D (4). V kontexte ochorenia COVID-19 môžeme predpokladať, že pacienti s aboslútnym deficitom vitamínu D nemajú dostatočnú koncentráciu 25(OH)D v periférnych tkanivách (napr. pľúcnom parenchýme), čo v konečnom dôsledku môže viesť k nedostatočnej imunomodulačnej funkcii vitamínu D v týchto tkanivách a tým k ťažšiemu priebehu ochorenia COVID-19 (5). Naším cieľom bolo vyhodnotiť mortalitu pacientov hospitalizovaných s COVID-19 pneumóniou v kontexte absolútneho deficitu 25(OH)D.

Materiál a metódy

Štúdia bola realizovaná na V. internej klinike LF UK a UNB v Bratislave. Do štúdie boli zaradení hospitalizovaní pacienti medzi 1. novembrom 2020 a 30. aprílom 2021 , ktorí spĺňali všetky nasledovné inklúzne kritériá:

- pneumónia asociovaná s COVID-19 bola primárnou diagnózou pri prijatí,
- pacient mal pozitívny RT-PCR test na SARS-CoV-2,
- pacienti mali pri prijatí vyšetrenú sérovú koncentráciu 25(OH)D,

- pacienti počas liečby neboli preložení do iného zdravotníckeho zariadenia.

Pacienti boli monitorovaní do prepustenia z nemocnice (preživší) alebo do doby úmrtia v nemocnici (zomrelí). Terapeutický manažment bol realizovaný v súlade s vnútornými odporúčaniami pre liečbu ochorenia COVID-19 v Univerzitnej nemocnici Bratislava, Ružinov. Tieto odporúčania vychádzali z odporúčaní publikovaných Centrom pre kontrolu a prevenciu chorôb (USA) dostupných online (https://www.cdc.gov/coronavirus/2019-ncov/hcp/clinical-guidance-management-patients.html). Laboratórne paramtere boli stanovované pomocou štandardných komerčných laboratórnych setov. Odber venóznej krvi bol realizovaný u každého pacienta v raňajších hodinách medzi 7:00-8:00 hod. Odber arteriálnej krvi za účelom stanovenia hodnôt krvných plynov bol realizovaný ± 2 hodiny od odberu venóznej krvi. Hodnota 25(OH)D (ng/ml) v sére bola stanovená pomocou automatického elektrochemiluminisenčného systému (Eclesys Vitamin D Total II, 2019, Roche Diagnostics GmBH, Mannheim, Germany). Detekčný limit 25(OH)D bol 3 ng/ml. Hodnota 25(OH)D < 12 ng/ml bola považovaná za absolútny tkanivový deficit vitamínu D (4). Charlson Comorbidity Index bol použitý za účelom získania číselného údaja vyjadrujúceho mieru polymorbidity u daného pacienta. Na jeho výpočet bola použitá online aplikácia (www.mdcalc.com/charlson-comorbidity-index-cci.com). Výsledné dáta sú prezentované ako priemer ± štandardná odchýlka alebo ako čísla a percentá. Pre porovnanie skupín (preživší vs zomrelí a < 12 ng/ml vs > 12 ng/ml) sme použili analýzu rozptylu pre kontinuálne premenné a chi-square test pre kategorické premenné. Za účelom zhodnotenia prediktorov mortality bola použitá multivariantná regresná analýza. Do modelu boli zaradené nezávislé premenné na základe preštudovania publikovanej literatúry na danú problematiku.Vo finálnom modeli bola pre identifikovanie nezávislých prediktorov mortality využítá metóda backward selection. Hodnoty $p < 0.05$ boli považované za signifikantné.

Výsledky

Celkovo bolo v sledovanom období hospitalizovaných 558 pacientov, 201 pacientov nespĺňalo inklúzne kritériá. Analyzovaných bolo 357 pacientov (198 mužov a 159 žien). 168 (47%) pacientov zomrelo počas hospitalizácie. Rozdiely medzi základnými demografickými, klinickými a laboratórnymi parametrami u preživších a zomrelých sú uvedené v **tabuľke č. 1**.

Tabuľka 1: Základné demografické, klinické a laboratórne charakteristiky preživších a zomrelých.

Parameter	Preživší (n=189)	Zomrelí (n=168)	*p* hodnota
Vek (roky)	63.5±13.9	73.5 ±10.5	<0.0001
BMI (kg/m2)	29.8±7.6	30.4 ±7.1	0.45
Muži/ženy, n (%)	104(55)/85(45)	94(56) /74(44)	0.86
Artériová hypertenzia, n (%)	130 (69)	132 (78.5)	0.05

Tabuľka 1 (pokračovanie): Základné demografické, klinické a laboratórne charakteristiky preživších a zomrelých.

Chronické srdcové zlyhávanie, n (%)	17 (9)	35(21)	0.002
Diabetes mellitus bez komplikácií, n (%)	48 (26)	27(16)	0.03
Diabetes mellitus s komplikáciami, n (%)	23(12)	40(24)	0.003
Chronické ochorenie obličiek, n (%)	34 (17)	65(39)	<0.0001
Charlson comorbidity index	3.6 ±2.7	5.7±2.6	<0.0001
Preparáty vitamínu D pred hospitalizáciou, n (%)	31(16)	32 (19)	0.51
Vysokoprietoková oxygenoterapia, n (%)	44 (24)	132 (79)	<0.0001
Umelá pľúcna ventilácia, n (%)	2 (1)	22 (13)	<0.0001
Leukocyty (10x9/L)	7.4 ±3.3	9.0±5.1	0.0006
Neutrofily (10x9/L)	6.0±3.1	7.7±4.6	0.0001
Lymfocyty (10x9/L)	1.0 ±0.8	0.8 ±0.9	0.08
Trombocyty (10x9/L)	263.2 ±110.5	231.2 ±97.0	0.0042
CRP (mg/L)	107.3 ±88.1	153.6 ±90.9	<0.0001
IL-6 (ng/L)	122.7±435.5	215.5±476.5	0.07
D-dimér (mg/L)	2.7±4.6	3.6±5.1	0.09
Procalcitonín (ug/L)	1.5±10.4	2.6±9.7	0.30
25(OH)D (ng/ml)	24.6±14.6	20.8±11.1	0.007
Vírusová nálož (CT e gene)	24.2±5.0	21.0±4.6	<0.0001
Saturácia krvi kyslíkom (%)	89.6±7.47	86.1±10.3	0.0003

80% pacientov malo buď insuficientné alebo deficientné hodnoty 25(OH)D pri prijatí (<30 ng/ml). 74 (21%) pacientov malo ťažký tkanivový deficit 25(OH)D (<12 ng/ml). Pozorovali sme nižšiu koncentráciu 25(OH)D, nižší počet trombocytov, nižšiu hodnotu saturácie krvi kyslíkom

(SpO2), a vyšší vek, hodnotu C-reaktívneho proteínu (CRP), vírusovú nálož, počet leukocytov, neutrofilov, a vyššiu hodnotu Charlson Comorbidity indexu u pacientov, ktorí zomreli v porovnaní s preživšími (všetko p<0.005). Sérová koncentrácia 25(OH)D pri prijatí bola nezávisle asociovaná s mortalitou (p=0.0398). Vek, CRP, SpO2, trombocyty, BMI a Charlson Comorbidity Index boli takisto nezávisle asociované s mortalitou (všetko p < 0.05). Výsledky multivariantnej lineárnej regresnej analýzy sú uvedené v **tabuľke č. 2**.

Tabuľka 2: Výsledky lineárnej regresnej analýzy. Uvedené sú iba výsedky s $p < 0.05$.

Nezávislé premenné	Coefficient	Std. Error	t	p	$r_{partial}$	$r_{semipartial}$	VIF
	0,2628						
Vek (roky)	0,01049	0,002462	4,258	<0.0001	0,2365	0,2075	1,815
BMI (kg/m2)	0,007869	0,003524	2,233	0,0263	0,1266	0,1088	1,063
CRP (mg/L)	0,001071	0,000275	3,899	0,0001	0,2176	0,19	1,086
Charlson Comorbidity Index	0,02327	0,01136	2,048	0,0414	0,1163	0,09976	1,797
Trombocyty (10x9/L)	-0,0007356	0,000239	-3,077	0,0023	-0,1732	0,1499	1,031
Saturácia krvi kyslíkom (%)	-0,008144	0,002905	-2,803	0,0054	-0,1582	0,1366	1,09
25(OH)D (ng/mL)	-0,00395	0,001913	-2,065	0,0398	-0,1172	0,1006	1,028

Pacienti s hodnotou 25(OH)D < 12 ng/ml mali v porovnaní s pacientmi s hodnotou 25 (OH)D > 12 ng/ml vyššiu prevalenicu chronického ochorenia obličiek, vyššiu hodnotu Charlson Comorbidity Indexu a vyššiu vírusovú nálož. Nebol prítomný rozdiel v hodnotách zápalových parametrov (CRP, IL-6, prokalcitonín,neutrofily, celkové leukocyty, lymfocyty) medzi jednotlivými podskupinami. Pacienti s hodnotou 25(OH)D < 12 ng/ml mali o 11% vyššiu mortalitu oproti pacientom s hodnotou 25(OH)D > 12 ng/ml (p<0.05). Porovnanie klinických parametrov vo vzťahu k absolútnemu deficitu 25(OH)D (<12 ng/ml) je uvedené v **tabuľke 3**.

Tabuľka 3: Porovnanie klinických a laboratórnych parametrov vo vzťahu k absolútnemu deficitu 25(OH) (< 12 ng/ml vs > 12 ng/ml).

Parameter	25(OH)D ≥12 ng/ml (n=283)	25(OH)D <12 ng/ml (n=74)	*p* hodnota
Vek (roky)	67.9±13	69.4±14.7	0.39
BMI (kg/m2)	30.5±7.4	28.7±7.2	0.08
Muži/ženy, n (%)	164(58) / 119 (42)	34 (46) /40 (54)	0.07
Artériová hypertenzia, n (%)	210 (74)	52 (70)	0.60
Chronické srdcové zlyhávanie, n (%)	38 (13)	14 (19)	0.41
Diabetes mellitus bez komplikácií, n (%)	62 (22)	13 (17)	0.41
Diabetes mellitus s komplikáciami, n (%)	50 (18)	13(18)	0.8
Chronické ochorenie obličiek, n (%)	70 (25)	29 (40)	0.007
Charlson Comorbidity Index	4.4±2.8	5.3±3.09	0.022
Preparáty vitamínu D pred hospitalizáciou, n (%)	50(18)	13 (17)	0.81
Vysokoprietoková oxygenoterapia, n (%)	141 (49)	35(46)	0.56
Umelá pľúcna ventilácia, n (%)	18 (6)	4 (5)	0.61
Leukocyty (10x9/L)	8.4±4.6	8.1±3.6	0.65
Neutrofily (10x9/L)	7±4.2	6.8±3.5	0.73
Lymfocyty(10x9/L)	0.97±0.8	1.07±1.1	0.43
Trombocyty (10x9/L)	251±108	246±100	0.75
CRP (mg/l)	131.6±93	125.15±91	0.59
IL-6 (ng/l)	171.1±475	143.6±330	0.65

Tabuľka 3 (pokračovanie): Porovnanie klinických a laboratórnych parametrov vo vzťahu k absolútnemu deficitu 25(OH) (< 12 ng/ml vs > 12 ng/ml).

D-dimer (mg/L)	3.4±5.2	3.2±4.8	0.79
Prokalcitonín	1.7 ±9.3	2.8±12.1	0.41
25(OH)D (ng/ml)	26.7±12	8.2±2.6	<0.0001
Saturácia krvi kyslíkom (%)	87.7±9.3	87.6±10.5	0.9
Vírusová nálož (CT e gene)	23.2±5.2	21.5±4.9	0.04
Zomrelí/Preživší, n (%)	127(44)/166(54)	41(55)/31(46)	0.05

Diskusia

Výsledky štúdií už z obdobia pred pandémiou ochorenia COVID-19 naznačujú, že imnuologické účinky vitamínu D môžu zohrávať významnú úlohu v modulovaní excesívnej zápalovej reakcie na úrovni pľúcneho parenchýmu (6). Suplementácia vitamínu D napríklad znižuje incidenciu akútnych respiračných ochorení, pričom pacienti s najťažším deficitom 25(OH)D zo suplementácie najviac benefitujú (7). Viaceré práce naznačujú, že nízka hodnota 25(OH)D je asociovaná aj s horším priebehom a vyššou mortalitou ochorenia COVID-19 (8). Podľa niektorých autorov však nízka hodnota 25(OH)D je u pacientov s COVID-19 pneumóniou spôsobená dôsledkom tzv. reverznej kauzality. Sérová koncentrácia 25(OH)D v tomto prípade predstavuje negatívny reaktant akútnej fázy zápalu, a nízka hodnota jednoducho znamená silnejšiu zápalovú reakciu (9). V našej štúdii sme zistili, že hodnota 25(OH)D pri prijatí je nezávisle asociovaná s mortalitou. Absolútny deficit 25(OH)D (<12 ng/ml) je spojený s 11% vzostupom mortality. Napriek tomu, že pacienti s absolútnym deficitom 25(OH)D mali signifikantne vyššiu mortalitu, medzi skupinami (<12 ng/ml vs. > 12 ng/ml) sme nepozorovali významné rozdiely v hodnotách zápalových markerov. Toto zistenie by mohlo podporovať tvrdenie, že deficit 25(OH)D nie je iba vedľajší produkt zápalovej reakcie, ale skôr potenciálne modifikovateľný rizikový faktor ochorenia COVID-19. Zistenie, že absolútny deficit 25(OH)D je signifikantne asociovaný s mortalitou, môže byť relevantné aj pre plánovanie intervenčných štúdii so suplementáciou vitamínu D pri ochorení COVID-19. Vitamín D je tzv. prahový mikronutrient, t.z. pokiaľ sa dosiahne určitá prahová koncentrácia pre konkrétny fyziologický účinok, ďalším zvýšením koncentrácie mikronutrientu nad túto prahovú hodnotu nedôjde k zvýrazneniu želaného efektu (10). Teda ak sa do intervenčnej štúdie zaradia iba pacienti s koncentráiou 25(OH)D nad touto prahovou hodnotou, nemožno zo suplementácie očakávať významnejší benefit. To môže byť jedným z hlavných dôvodov, prečo výsledky intervenčných štúdií nepreukazujú pri suplementácii vitamínom D želaný efekt (10). Napríklad štúdia realizovaná v Brazílii nepreukázala signifikantný benefit suplementácie vitamínu D na redukciu dĺžky hospitalizácie u pacientov so stredne ťažkým až ťažkým ochorením COVID-19. Avšak ani do placebo skupiny, a ani do intervenčnej skupiny neboli zaradení pacient s ťažkým deficitom 25(OH)D, pričom v oboch skupinách mali pacienti koncentráciu 25(OH)D vyššiu než 20 ng/ml (11). Záverom možno konštatovať, že hodnota 25(OH)D sa javí ako potenciálny prediktor

ťažkého priebehu ochorenia COVID-19 a jeho stanovovanie môže byť relevantné z hľadiska stratifikácie rizika ale aj plánovania liečebnej stratégie (12).

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21. Prevalencia deficitu vitamínu D u hospitalizovaných pacientov s ochorením COVID-19 v Slovenskej republike signifikantne klesla počas trvania pandémie v priebehu rokov 2020-2022

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Úvod

Už v prvej polovici roka 2020, na začiatku pandémie ochorenia COVID-19 (Coronavirus Disease 2019), viaceré krajiny zaviedli rozsiahle prísne opatrenia s cieľom obmedziť šírenie vírusu SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2). Zatiaľ čo z hľadiska šírenia ochorenia boli tieto aktivity prevažne úspešné, v odbornej literatúre sa začalo diskutovať o možných negatívnych vplyvoch týchto opatrení na životný štýl, spôsoby stravovania, fyzickú aktivitu, náladu a sociálny život. Ammar a kolektív preukázali, že lockdown počas pandémie viedol k významnému zníženiu všetkých foriem fyzickej aktivity, k zvýšeniu času stráveného v sede, a k negatívnym zmenám v stravovacích návykoch (1). Bogataj Jontez a kolektív v malej štúdii poukázali na to, že po lockdowne mali aj mladí zdraví jedinci zvýšené hodnoty glykémie, celkového aj LDL cholesterolu (2).

Vitamín D a jeho vplyv na ochorenie COVID-19 bol od začiatku pandémie diskutovaný odbornou aj laickou verejnosťou. Viaceré štúdie poukazovali na negatívny prognostický význam deficitu vitamínu D z hľadiska závažného priebehu ochorenia COVID-19 (3). Vitamín D vzniká v koži po expozícii slnečnému UV-B žiareniu (hlavný zdroj vitamínu D), alebo ho získavame v jedle, či formou suplementácie (4). Viacerí autori uvažovali o tom, či počas pandémie vzhľadom k zmene stravovacích návykov a najmä k menej času stráveného na slnku počas lockdownu, nedôjde k prehĺbeniu deficitu vitamínu D na populačnej úrovni (5). Na druhej strane, rastúcie povedomie o potenciálnych extraskeletálnych účinkoch vitamínu D (najmä vo vzťahu k imunitnému a respiračnému systému) mohlo viesť k zvýšenej miere suplementácie vitamínom D a tým aj k zlepšeniu statusu vitamínu D na úrovni populácie.

Cieľom predkladanej práce bolo porovnať priemerné koncentrácie 25(OH)D v sére medzi druhou (2020/2021) a treťou (2021/2022) vlnou pandémie u hospitalizovaných pacientov s ochorením COVID-19 v Slovenskej republike.

Metódy

Analyzovali sme pacientov hospitalizovaných na V. internej klinike LF UK a UNB Ružinov počas druhej (Skupina 1) a tretej (Skupina 2) vlny pandémie ochorenia COVID-19. Pacienti z oboch vĺn pandémie boli hospitalizovaní počas zimného obdobia: analyzovaní pacienti z druhej vlny boli hospitalizovaní v období od 1. decembra 2020 do 28. februára 2021 a pacienti z tretej vlny pandémie boli hospitalizovaní v období od 1. decembra 2021 do 28. februára 2022. 101 (61 mužov/ 40 žien) pacientov z tretej vlny pandémie spĺňalo naše inklúzne kritériá. Týchto pacientov sme porovnávali so 101 (61 mužov/ 40 žien) pacientmi z druhej vlny pandémie. Pacientov sme párovali najskôr podľa pohlavia a následne podľa veku $\pm$ 1 rok. Ak bolo k výberu viac možností, vybrali sme pacienta s najbližšou hodnotou BMI. Celkovo sme analyzovali 202 pacientov (102 mužov a 100 žien), ktorí spĺňali naše inklúzne kritériá. Inklúzne kritériá boli nasledovné: a) COVID-19 primárna diagnóza pri prijatí, b) ťažký priebeh ochorenia COVID-19, c) detekcia SARS-CoV-2 pomocou RT-PCR, d) hodnota 25(OH)D vyšetrená pri prijatí do nemocnice.

Ťažký priebeh ochorenia COVID-19 bol definovaný ako klinický obraz pneumónie a aspoň jedna z nasledujúcich situácií: a) počet dychov za minútu > 30, b) ťažký respiračný distres, c) saturácia krvi kyslíkom < 90 % bez oxygenoterapie (6).

Zmeny koncentrácie 25(OH)D boli hodnotené na úrovni celej kohorty, ale aj vzhľadom na pohlavie (muži, ženy) a vek (< 65 rokov, > 65 rokov).

Hodnota 25(OH)D (ng/mL) v sére bola stanovená pomocou automatického elektrochemiluminisenčného systému (Eclesys Vitamin D Total II, 2019, Roche Diagnostics GmBH, Mannheim, Germany). Detekčný limit 25(OH)D bol 3 ng/mL.

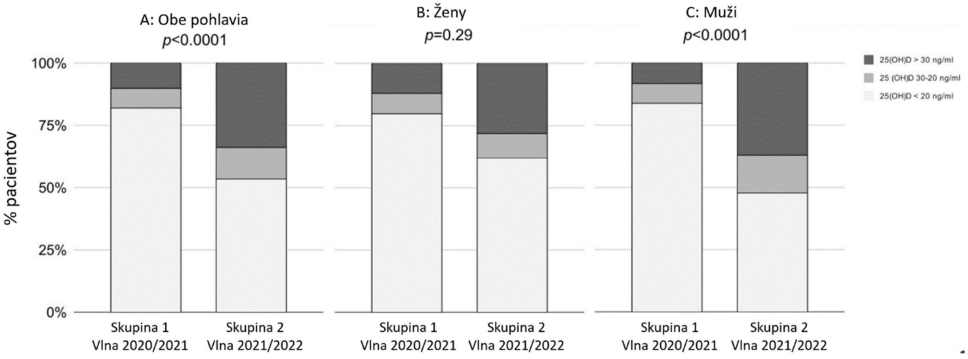
Koncentrácia 25(OH)D > 30 ng/mL bola považovaná za dostatočnú, koncentrácia 25(OH)D medzi 20-30 ng/mL za insuficientnú, a koncentrácia 25(OH)D < 20 ng/mL za deficit vitamínu D.

Na štatistickú analýzu kontinuálnych premenných sme využili nepárovy t-test priemerných hodnôt a na štatistickú analýzu kategorických premenných sme využili chí-kvadrát test nezávislosti. Za účelom analýzy kategórií vitamínu D (dostatočná koncentrácia, nedostatočná koncentrácia a deficit) sme použili chí-kvadrát test s kontingenčnými tabuľkami. Priemerné koncentrácie 25(OH)D sme na úrovni celej kohorty a v rámci pohlaví a vekových skupín porovnávali pomocou nepárového t-testu priemerných hodnôt. Na úrovni celej kohorty sme analyzovali vzťah medzi mortalitou a sérovou koncentráciou 25(OH)D po úprave súboru vzhľadom k pohlaviu a veku. Bola použitá logistická binárna regresná analýza s úmrtím na COVID-19 ako závislou premennou. Štatistickú analýzu sme realizovali pomocou programu SPSS (ver. 21.0; IBM Corp., Armonk, NY, USA). P hodnota < 0.05 bola považovaná za štatisticky významnú.

Výsledky

Z každej vlny pandémie sme analyzovali 101 pacientov (61 mužov, 40 žien, priemerný vek 69 rokov). Priemerná koncentrácia 25(OH)D pri prijatí počas druhej vlny pandémie (Skupina 1) bola 17.8 ng/mL a vzrástla na hodnotu 25.2 ng/mL počas tretej vlny pandémie (Skupina 2)

(p < 0.0001). Pri prijatí malo zo Skupiny 1 82 % pacientov deficit 25(OH)D a 10 % pacientov malo dostatočné koncentrácie 25(OH)D. V Skupine 2 malo 54 % pacientov deficit 25(OH)D a 34 % pacientov malo dostatočné koncentrácie 25(OH)D v sére (p < 0.0001) (Obrázok č. 1).



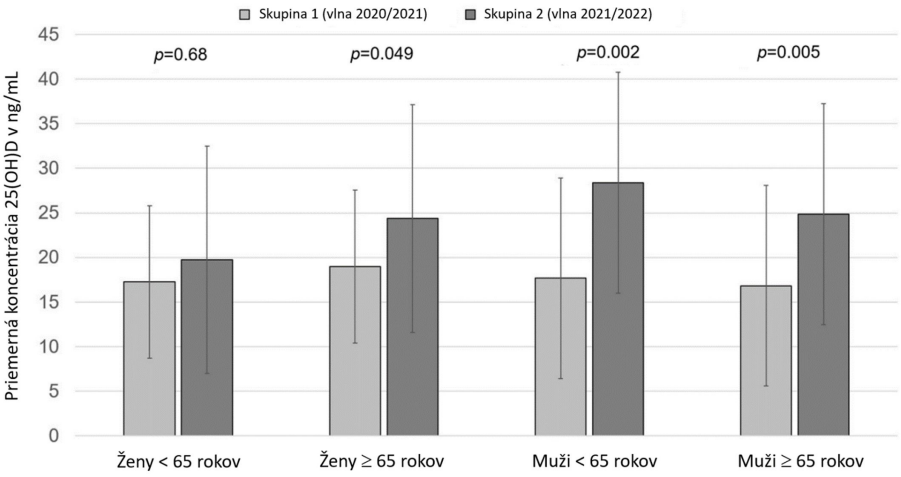
Obrázok 1: Percentuálne zastúpenie pacientov podľa jednotlivých hodnôt 25(OH)D v sére pri prijatí a zmeny medzi vlnami pandémie COVID-19 v rámci celej kohorty (A), u žien (B) a u mužov (C).

Nepozorovali sme výraznejší rozdiel v prevalencii chronických ochorení medzi skupinami s výnimkou chronického ochorenia obličiek, ktoré sme častejšie pozorovali v Skupine 1 (p < 0.0001) a demencie, ktorá bola častejšia v Skupine 2 (p = 0.02). Zastúpenie ochorení najčastejšie asociovaných s kardiopulmonálnou rezervou – chronické srdcové zlyhávanie, chronické pľúcne ochorenia, anémia a embólia do pľúcnej artérie, sa medzi skupinami signifikantne nelíšilo. Množstvo pacientov, ktorí predhospitalizačne užívali preparáty vitamínu D vzrástlo medzi vlnami z 18 % na 44 % (p < 0.0001).

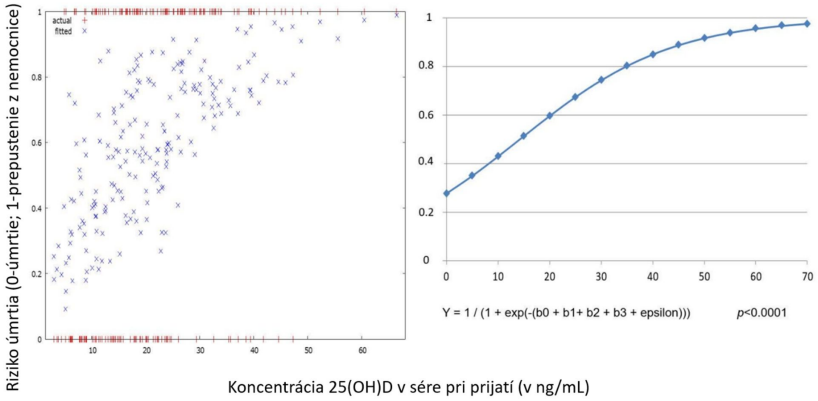
U mužov počet vitamín D deficientných pacientov klesol z 84 % na 48 % a počet pacientov s adekvátnou hodnotou 25(OH)D stúpol z 8 % na 37 % (p < 0.0001) (Obrázok č. 1). Priemerná koncentrácia 25(OH)D vzrástla o 9.1 ng/mL, z priemernej hodnoty 17.2 ng/mL na 26.3 ng/mL (p < 0.0001). U žien počet vitamín D deficientných pacientov klesol z 80% na 62 % a množstvo pacientov s adekvátnou hodnotou vzrástlo z 13 % na 28 % (p = 0.29) (Obrázok č. 1).

Prevalencia deficitu vitamínu D klesla u mladších aj starších pacientov. U pacientov < 65 rokov počet vitamín D deficientných pacientov klesol z 81 % na 44 %, a množstvo pacientov s adekvátnou koncentráciou 25(OH)D stúplo z 3 % na 34 %. U starších pacientov (>65 rokov) prevalencia deficitu vitamínu D klesla o 24 % a prevalencia adekvátnych hodnôt 25(OH)D v sére stúpla o 20 %. U mužov sme pozorovali štatisticky významný pokles vitamín D deficientných pacientov v oboch vekových skupinách; v podskupine mladších mužov (< 65 rokov) o 44 % a v podskupine starších mužov (> 65 rokov) o 29 % (p < 0.001, resp. p = 0.002). U žien prevalencia deficitu vitamínu D klesla v oboch vekových skupinách, avšak pokles nebol štatisticky významný. U starších žien (> 65 rokov) sme pozorovali pokles prevalencie deficitu

vitamínu D o 19 % a vzostup prevalencie adekvátnej sérovej koncentrácie 25(OH)D o 16 %, ktorý bol hranične štatisticky významný (p = 0.056).



Obrázok 2: Zmeny priemerných koncentrácii 25(OH)D pri prijatí u hospitalizovaných pacientov s ochorením COVID-19 medzi druhou (vlna 2020/2021) a treťou (vlna 2021/2022) vlnou pandémie.



Obrázok 3: Vzťah medzi koncentráciou 25(OH)D v sére pri prijatí a rizikom úmrtia v nemocnici pre celú kohortu pacientov hospitalizovaných pre ťažký priebeh ochorenia COVID-19. Y = závislá premenná (úmrtie počas hospitalizácie), b1 = nezávislá premenná (vek), b2 = nezávislá premenná (pohlavie), b3 = nezávislá premenná (koncentrácia 25(OH)D)

Najväčšiu zmenu priemernej koncentrácie 25(OH)D sme zaznamenali v kohorte mužov < 65 rokov (10.7 ng/mL, p = 0.002) a najmenšiu zmenu priemernej koncentrácie 25(OH)D sme pozorovali v kohorte žien < 65 rokov (2.5 ng/mL, p = 0.68) (Obrázok č. 2). Okrem podksupiny mladších žien bola zmena priemernej koncentrácie 25(OH)D pri prijatí vo všetkých ostatných sledovaných podksupinách štatisticky významná (Obrázok č. 2).

Binárna logistická regresná analýza na celej kohorte pacientov preukázala, že v sledovanom súbore existuje signifikantný inverzný vzťah medzi koncentráciou 25(OH)D a mortalitou, ktorý pretrváva aj po úprave súboru vzhľadom k veku a pohlaviu. Vzostup sérovej koncentrácie o jeden ng/mL viedol k 7 % redukcii rizika úmrtia na ochorenie COVID-19 (p < 0.0001).

Diskusia

V predkladanej štúdii sme pozorovali signifikantný pokles v prevalencii deficitu vitamínu D v kohorte hospitalizovaných pacientov pre ochorenie COVID-19 medzi druhou a treťou vlnou pandémie v Slovenskej republike. Prevalencia deficitu vitamínu D poklesla o 28 % a prevalencia adekvátnej hodnoty vitamínu D stúpla o 24 %. V sledovanom období sa viac než zdvojnásobil počet pacientov, ktorí predhospitalizačne užívali preparáty vitamínu D. Tieto výsledky sú prekvapujúce vzhľadom k známej vysokej prevalencii deficitu vitamínu D v európskych krajinách (7). Vzostup sérovej koncentrácie 25(OH)D o jeden ng/mL bol asociovaný so 7 % redukciou rizika úmrtia na ochorenie COVID-19.

Bolo publikovaných niekoľko prác skúmajúcich zmeny koncentrácií 25(OH)D v sére počas pandémie ochorenia COVID-19, pričom väčšina z nich sa sústredila na populáciu mladšiu než 18 rokov. Meta-analýza 5 štúdií zahŕňajúca 4141 ľudí < 18 rokov preukázala signifikantný pokles priemerných koncentrácií 25(OH)D počas pandémie v porovnaní s hodnotami pred pandémiou COVID-19. Tento pokles nebol pozorovaný v podskupine pacientov < 1 rok, kde nebola pozorovaná žiadna zmena, alebo dokonca vzostup priemernej koncentrácie (8).

V štúdii u pacientov > 19 rokov (sledované obdobie pandémie do novembra 2021) z Južnej Kórey sa podobne ako v našej práci preukázal signifikantný vzostup priemernej koncentrácie 25(OH)D počas pandémie (9). V kontraste s našou prácou, v tejto kohorte pacientov boli zmeny výzamnejšie u žien, pričom najväčší vzostup bol pozorovaný v podskupine starších žien.

V analýze porovnávajúcej 12 predpandemických mesiacov a prvých 12 mesiacov pandémie COVID-19 v Írsku sa preukázal signifikantný medziročný nárast priemernej koncentrácie 25(OH)D o 2 ng/mL (10).

Somagutta a kolektív analyzovali trendy vo vyhľadávaní jednotlivých mikronutrientov prostredníctvom internetových prehliadačov. Zadávanie kľúčových slov súvisiacich s vitamínom D vzrástlo od roku 2004 do roku 2021 osemnásobne a medzi rokmi 2019-2021 sa zdvojnásobilo (11). Toto bolo spôsobené zrejme rastúcim záujmom o možné imunomodulačné funkcie vitamínu D a mohlo sa pretaviť aj do väčšej miery suplementácie na populačnej úrovni.

Počas pandémie COVID-19 bolo zároveň o liečebných modalitách diskutovaných množstvo dezinformácií. Podľa štúdií až 73 % ľudí udáva, že boli počas pandémie vystavení nejakej forme dezinformácií (12). Zároveň existuje priamy vzťah medzi mierou dezinformácií a nedôverou voči vakcinácii (12). V kontexte pandémie CVID-19 treba spomenúť, že potenciálne imunomodulačné účinky vitamínu D boli často nadhodnotené a výsledky štúdií často dezinterpretované. Podľa štúdií informačné portály, ktoré označovali epidemiologické opatrenia za zbytočné, zároveň často zdôrazňovali preventívne či kuratívne účinky vitamínu D voči COVID-19 (13). Viac než 70 % hospitalizovaných pacientov na našej klinike počas tretej vlny pandémie nebolo plne zaočkovaných napriek tomu, že v tom čase bola už vakcinácia v Slovenskej republike široko dostupná. Môžeme špekulovať, že za pozorovaným vzostupom priemerných koncentrácií 25(OH)D počas pandémie môže byť sčasti tendencia týchto pacientov inklinovať k alternatívnym liečebným stratégiám (14).

Naša práca má niekoľko limitácií. Analyzovali sme realtívne malú skupinu pacientov. Jedná sa o kohortu pacientov iba z jedného centra v špecifickom geografickom regióne (nadmorská výška, počet slnečných dní, stravovacie návyky). Nepoznali sme presné dávky vitamínu D, ktoré pacienti predhospitalizačne užívali. Predkladaná práca je však prvá, ktorá porovnáva zmeny sérových koncentrácií u hospitalizovaných pacientov s chorením COVID-19 medzi jednotlivými vlnami pandémie. Faktory, ktoré môžu významnou mierou ovplyvniť koncentráciu 25(OH)D ako vek, pohlavie a hodnota BMI nehrajú v našom porovnaní významnú úlohu, vzhľadom k tomu, že pacienti boli medzi jednotlivými vlnami párovaní na základe týchto premenných. Vzorky krvi na stanovenie 25(OH)D boli zároveň počas jednotlivých vĺn odoberané v rovnakom časovom období (zimné mesiace).

Záverom možno povedať, že počas obdobia pandémie medzi rokmi 2020 až 2022 sme pozorovali u hospitalizovaných pacientov s ochorením COVID-19 singifikantný medziročný vzostup priemernej koncentrácie 25(OH)D o 7.45 ng/mL. Do budúcna je potrebná analýza tohto trendu na širšej populačnej úrovni.

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22. Automatizácia SARS-CoV-2 testovania v Slovenskej a Českej republike.

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Úvod

Pandémia koronavírusu SARS-CoV-2 spôsobujúca ochorenie COVID-19 bola spojená s kritickou situáciou týkajúcou sa nemocničnej sféry zdravotníckeho systému, ale aj kladením bezprecedentne vysokých nárokov na klinické diagnostické laboratóriá, ktoré museli v krátkom čase riešiť množstvo problémov spojených so zabezpečením potrebných testovacích kapacít pri dodržaní požadovaných princípov správnej laboratórnej praxe. Rýchla a vysokovýkonná laboratórna diagnostika sa totiž v čase celosvetovej pandémie ukázala ako jeden z hlavných nástrojov monitorovania a hodnotenia priebehu pandemických vĺn, čím bola nosnou pri nastavovaní bezpečnostných opatrení, či už na regionálnej alebo celoštátnej úrovni rovnako v Českej aj v Slovenskej republike. Bolo teda potrebné realizovať dovtedy nevídané počty diagnostických testov v čo najkratšom čase. V rámci tímov zo Slovenska a Českej republiky, boli vytvorené vysokovýkonné pracoviská, ktoré umožnili dosiahnutie počtov analyzovaných vzoriek plne porovnateľných so špecializovanými riešeniami od popredných výrobcov laboratórnych systémov.

Medzi hlavné problémy patrili absencia jasne definovaných laboratórnych postupov („zlatých štandardov“) pre vyžadovaný typ plošného testovania, nedostatok základných pomôcok pre odber biologického materiálu a odberových miest, nedostatok certifikovaných vyšetrovacích súprav a asociovaného laboratórneho prístrojového vybavenia a v neposlednom rade nedostatočné kapacity laboratórií, ktoré by mali vybudovanú infraštruktúru určenú na prácu v režime s vysokou priepustnosťou analýz. Hoci boli známe a funkčné automatizované laboratórne riešenia, problémom bola ich nedostupnosť na svetovom trhu, keďže záujem o ich využívania prudko narástol a ich výroba nedokázala pokryť všeobecné požiadavky. To bol hlavný dôvod, prečo laboratóriá na celom svete museli začať zostavovať laboratórne systémy z aktuálne dostupných zariadení a výsledkom bola ich veľká heterogenita a tiež vznik špecifických požiadaviek na optimalizáciu a nastavovanie takýchto častokrát unikátnych riešení pre čo najlepšiu ekonomickú a pracovnú efektivitu.

Cieľom práce je popísať optimalizáciu laboratórnych procesov vzniknutých kombináciou manuálneho a automatizovaného spracovania a analýzy pacientskych vzoriek vedúcu k zvyšovaniu výkonu diagnostických laboratórií, Medirex a.s. (Slovensko) a Spadia Lab a.s. (Česká republika), počas pandémie SARS-CoV-2. Okrem toho práca poukazuje na využitie konkrétneho laboratórneho pipetovacieho poloautomatu, ako nástroja pre rutinnú prácu v postpandemickom období.

Materiál a metódy

Celý postup možno zhrnúť do 6 bodov: Odber a príjem vzoriek (za „zlatý štandard" odberu vo všeobecnosti považoval nazofaryngeálny ster), primárne spracovanie vzoriek, extrakcia RNA, príprava PCR mastermixu, RT-qPCR (*real time quantitative PCR*), vyhodnotenie výsledkov.

Extrakcia RNA

V prvých fázach rutinného diagnostického testovania SARS-CoV-2, kedy v laboratóriách Medirex ani Spadia neexistovala automatizáciu umožňujúca infraštruktúra bola extrakcia nukleových kyselín realizovaná prostredníctvom zaužívaných kolónkových kitov. Ich použitie vyžaduje značnú manuálnu pracnosť a viackrokový protokol. V záujme automatizácie sa všeobecne akceptovaným priemyselným štandardom stalo využívanie kitov obsahujúcich magnetické partikuly. Práve tento spôsob extrakcie spolu s dobrou dostupnosťou relatívne konštrukčne a ovládateľnosťou jednoduchých automatických systémov pracujúcich v štandardizovanom 96-jamkovom formáte viedol k tomu, že sa takýto spôsob extrakcie stal v našich krajinách najpoužívanejší.

RT-qPCR

V čase, kedy sa pandemická vlna dostala po prvýkrát do našich krajín, boli už vo svete dostupné štandardizované a validované protokoly pre detekciu SARS-CoV-2 v klinických vzorkách na základe štúdií publikovaných všeobecne akceptovanými autoritami (napr. protokol WHO (1)), ktoré metodicky odkazovali na metódu RT-qPCR a aj preto sa stala akceptovaným „zlatým štandardom" (2). Realtime PCR cykléri sú síce relatívne často používané v molekulárno-genetických laboratóriách, preto bolo možné relatívne rýchlo prevziať takto dostupné protokoly, často už v podobe komerčne dostupných kitov, a používať ich bez ďalšieho zdržania v rutinných diagnostických laboratóriách.

Vyhodnotenie a manažment vzoriek – laboratórny informačný systém

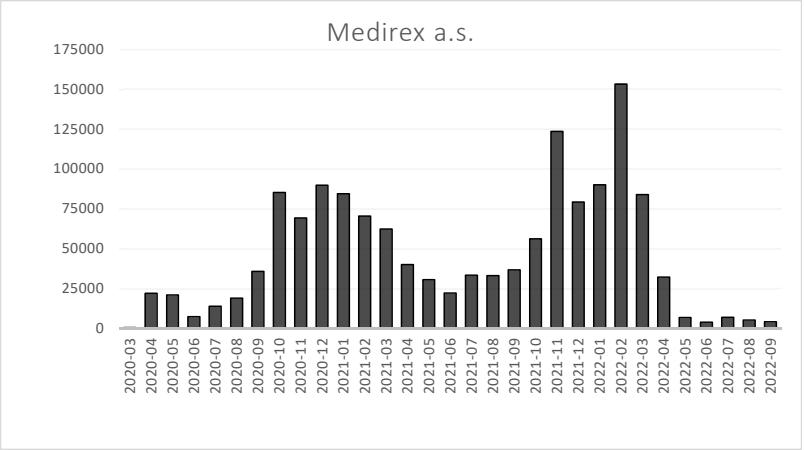
Laboratórny informačný systém (LIS) bol nedielnou súčasťou príjmu, spracovania vzoriek a vyhodnotenia ich analýz. V českých aj slovenských laboratóriách už boli zavedené LIS systémy, na ktorých pozadí sú komplexné a robustné softvérové riešenia, ktorých zmeny si často vyžadujú samotné úpravy v týchto softvéroch, ale aj ich detailné testovanie, keďže diagnostická sféra musí podliehať najvyššej úrovni ochrany z pohľadu citlivosti spracúvaných. Pre minimalizáciu rizika zámeny vzoriek boli často už na odberových miestach označené unikátnymi, pseudoanonymnými identifikátormi (v laboratóriách čiarovými kódmi), ktoré vzorku sprevádzali celým procesom po vydanie výsledku a jeho analýzu. Pre zabezpečenie posunu informácii centrálnym inštitúciám, ktoré mali úlohu v monitoringu priebehu pandémie a v manažmente protipandemických opatrení, bolo nevyhnutné upraviť funkcionality LIS systémov tak, aby výmena týchto informácii prebiehala paralelne a bez nežiaduceho časového zdržania. Pri manuálnom a automatickom spracovaní (Medirex) a aj pri plne automatizovanom riešení (Spadia Lab) čiarové kódy automatizáciu zjednodušili a minimalizovali potenciálne riziko zámeny vzoriek, ktoré sú najčastejšie zdrojom problémov pri manuálnom spracovaní a analýze vzoriek. LIS systémy boli upravené tak, aby bolo možné používať na získanie informácií o vzorkách prichádzajúcich do laboratórií na formátovanie buď manuálne čítačky čiarových kódov alebo sa čiarové kódy načítavali aj priamo v pipetovacích automatoch. Samotný výsledok bol prostredníctvom automatizovaných IT služieb odosielaný jednak klientom (pacient, lekár, klinika) ale aj do národných informačných systémov, v ktorých

sa dáta tohto typu zbierali zo všetkých laboratórií v rámci Slovenska – Národné centrum zdravotníckych informácií (NCZI) a Českej republiky - Informačního systému infekčních nemocí (ISIN).

Výsledky

Laboratóriá Medirex a.s.

Kapacita laboratórií sa s narastajúcim počtom zariadení (súvisiacou aj s optimalizáciou priestorového usporiadania) ako aj s prechodom až na trojzmenný režim práce postupne zvyšovala.

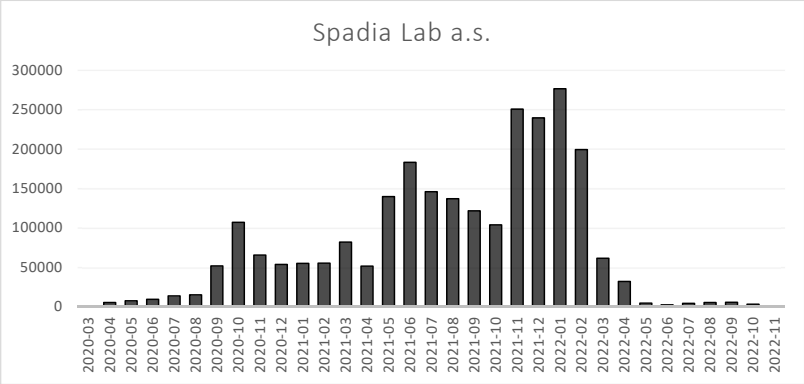


Obrázok 1: Prehľad počtov analyzovaných vzoriek v SARS-CoV-2 diagnostických laboratóriách spoločnosti Medirex a.s. v období od marca 2020 do septembra 2022.

Laboratóriá sa nachádzali na 3 miestach – v Bratislave, Košiciach a Nitre, pričom posledné menované bolo najväčšmi automatizované. Laboratórne priestory v Nitre („kovidovňa“) boli vo finálnej podobe vybavené 2 BSL2 laminárnymi boxami pre manuálne formátovanie vzoriek do 96-jamkového formátu, 3 izolačné automaty KingFisher Flex (ThermoFisher Scientific, USA) pre extrakciu nukleových kyselín, 1 pipetovací systém Opentrons OT2 (Opentrons, USA) pre prípravu PCR platní, 3 realtime PCR systémy QuantStudio 5 a 1 systém QuantStudio 6 (Life Technologies, USA) pre samotnú RT-qPCR. Bratislavské a Košické priestory mali podobne zavedené manuálne formátovanie, ale extrakcia nukleových kyselín bola realizovaná automatizovanými systémami KingFisher Flex (ThermoFisher Scientific), Zybio EXM3000 a Zybio EXM6000 (Zybio, Čína). Bratislavské laboratória navyše disponovali aj pipetovacím systémom Agilent Bravo (Agilent, USA). Na RT-qPCR v Bratislave a Košiciach slúžili 3 Roche LightCycler 480 (Roche, USA) a 3 QuantStudio 5 (Applied Biosystems). Z pohľadu počtov

vzoriek bolo v období od marca 2020 do septembra 2022 realizovaných v laboratóriách Medirex a.s. 1 420 572 testov. Za sledované obdobie bolo najviac testov realizovaných na prelome januára a februára 2022, kedy bolo za štyri týždne analyzovaných 195 589 vzoriek. V trojzmennej prevádzke len v laboratóriách v Nitre bolo možné vyšetriť > 9 000 vzoriek denne (Obr. 1).

Systém Opentrons OT2 (Opentrons, USA), hoci preň boli optimalizované pipetovacie protokoly, ktoré bolo možné využiť v nitrianskej „kovidovni“, na jeho využitie v rutine nedošlo. V súčasnej dobe prebieha testovanie a optimalizácia pre prípravu sekvenačných DNA knižníc podľa protokolu využívaného na inom diagnostickom oddelení. Testované sú protokoly na 8 a na 24 vzoriek s rôznou koncentráciou.



Obrázok 2: Prehľad počtov analyzovaných vzoriek v SARS-CoV-2 diagnostických laboratóriách spoločnosti Spadia Lab a.s. v období od marca 2020 do novembra 2022.

Laboratóriá Spadia Lab a.s.

Podobne ako na Slovensku, aj české laboratória Spadia Lab postupne navyšovali svoju kapacitu. Okrem prechodu na dvojzmenný režim došlo k navyšovanie počtu pipetorov, izolátorov a cyklerov a preusporiadaniu laboratórií. Ich centralizácia v Ostrave ale zabezpečila unifikovaný prístup a plnú automatizáciu už od formátovania vzoriek z odberových skúmaviek do 96-jamkových platní pomocou Biomek i5 (Beckman Coulter) platformy. Takéto zvýšenie kapacity aj nad 10 000 vzoriek denne predišlo spusteniu trojzmennej prevádzky. Finálna plne automatizovaná linka obsahovala 3 pipetovacie systémy na formátovanie primárnych vzoriek Biomek i5 (Beckman Coulter), 4 extraktory KingFisher Flex (Thermo Scientific), pipetovací systém Biomek i7 (Beckman Coulter) na predprípravu reakčných platničiek pre extrakciu aj PCR mastermix a pipetovací systém Agilent Bravo (Agilent) na prenos RNA do PCR reakčnej platničky. Takto pripravené platničky sa vkladali do jedného z 18 RT-qPCR cyklerov Biorad CFX96 resp. CFX Opus 96 (Biorad). Z pohľadu počtov vzoriek bolo v období od marca 2020 do novembra 2022 realizovaných v laboratóriách Spadia Lab a.s. 2 499 921 testov. Maximálnu

kapacita laboratória bola dosiahnutá v období november 2021 až január 2022, kedy bolo mesačne vyšetrených priemerne viac ako 250 000 vzoriek, čo zodpovedá dennému priemeru > 10 000 vzoriek (Obr. 2).

Diskusia

Naše vyhodnotenie poukazuje na to, že už čiastočná alebo plná automatizácia pracovných procesov umožňuje významné navýšenie laboratórnych testov, aby boli kompletne schopné pokryť požiadavky zo strany objednávateľov testov. V uvádzaných laboratóriách boli pracovné tímy schopné realizovať riešenia ktoré mali potenciál byť vo veľkom rozsahu nasadené aj počas budúcich pandémií alebo v iných prípadoch naliehavej potreby verejného zdravia. Okrem už uvádzaných výhod automatizácie stojí za zmienku aj vzniknutá odolnosť laboratórií voči výpadkom pracovnej sily, ktorá je problematická predovšetkým v čase pandémie, jednak z dôvodu pracovnej neschopnosti, či z dôvodu protipandemických opatrení. V Medirex a.s. stratifikácia laboratórií v troch mestách neumožnila väčšiu úroveň automatizácie celého procesu v takom rozsahu ako keby bolo testovanie úplne centralizované.

Ďalšie možnosti ako navýšiť laboratórne kapacity je spájať vzorky, resp. poolovať. Teda by sa pripravil pool viacerých vzoriek a ďalej by bol vyšetrovaný ako jedna vzorka a v prípade pozitivity by sa jeho vzorky vyšetrovali samostatne (3). V priebehu pandémie bolo tiež predstavených niekoľko profesionálnych jednoúčelových uzatvorených diagnostických systémov, ktoré poskytujú porovnateľné a vyššie denné kapacity aké boli dosiahnuté v našich laboratóriách. Napríklad Thermo Fisher Scientific Amplitude solution umožňuje spracovať cca 8 000 vzoriek za 24 hodín (2) alebo extrémne automatická linka LGC Biosearch Technologies' SARS-CoV-2 testing system založená na platforme Nexar, umožňujúca dosiahnuť až 150 000 testov za deň s minimalizáciou manuálnych úkonov (4). V čínskych veľkomestách došlo aj k vybudovaniu špacializovaných Huo-Yan Air Laboratory, ktorých kapacita presahovala 10 000 vzoriek denne, no zaujímavými sú možnosť ich vybudovania v expresnom čase do 24 hodín (5). Využitie takýchto systémov je ale neflexibilné a pri nárokoch v našom regióne aj ekonomicky neefektívne.

V porovnaní s uzavretými systémami boli komponenty našich riešení zaradené v rámci súčasnej infraštruktúry laboratórií a sú tak k dispozícii pre ďalšie použitie aj v prípade ďalších situácií vyžadujúcich klinické diagnostické laboratóriá. Príkladom je pipetovací automat Opentrons OT2 (Opentrons, USA), ktorý je testovaný na aktualizáciu krokov pre prípravu DNA sekvenačných knižníc.

Poďakovanie:

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23. Polygenic risk score and its utilization for estimation of risk for diabetes mellitus and COVID-19 diseases

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Introduction

Turn of the 20th and 21th century was linked in the field of the genetic research with huge expectations put in the Human Genome Project. It was estimated that such program has potential to uncover genetic background of the diseases and terminate decades-lasting effort for disease risk prediction. The project revealed fascinating discoveries however the finding that between genes and with them associated diseases don't pay simple relationships but on the contrary, there were identified whole complex of interactions across the genome which include hundreds to millions of single-nucleotide polymorphisms. Moreover, diseases associated with such polymorphisms have polygenic character with only small effect from single variants on overall phenotypic manifestation of the disease. The most passable fail-back in research of disease risk prediction seemed to be return back to previous theory based on estimation of cumulative risk of many factors influencing on disease development as possible. Continuously expanding genetic-database network altogether with modern hardware equipment for data-intensive statistical analytical approaches have enabled calculation of common genetic risk for specific diseases and their combination. One such method is polygenic risk score.

Basics of genetic alterations and their role in hereditary diseases

Differences in nucleotide sequence of DNA strand between individuals within population are assigned as genetic variations. There are five main types of genetic alterations: i) structural variants; ii) single-nucleotide variants; iii) variations assigned as insertions and deletions; iv) copy number variations and v) variations assigned as translocations and inversions.

Replacement of one base in base pair is refered as single nucleotide variation (SNV) at level of individuals and as single-nucleotide polymorphism (SNP) for whole population. It is estimated that each person carries several millions of such single-nucleotide polymorphisms while average nucleotide diversity (π), defined as average proportion of differences in nucleotides among randomly selected subjects, is somewhere between 1:1000 and 1:1500 (1-2).

Above mentioned changes in genome can be either hereditary but these can occur also spontaneously during cell division or can be shaped by environmental factors, respectively. Majority of variants has minimal or any phenotypic expression and therefore don’t have any impact on the health state of the carrier. On the other hand, there are also variants which phenotypic expressions can be manifested even as disease. In comparison with Mendelian inheritance, by which changes occur within one gene or small number of genes of large effect, gene variants represent type of polygenic inheritance which is characterised by changes of large amount of genes of weak to moderate outcome.

Although single-nucleotide polymorphisms are already used in genetic analysis, the prediction of risk for hereditary diseases based on multiple single-nucleotide polymorphisms remains big challenge for the future with respect to polygenic character of heredity in large number of diseases.

Projects for mapping of genomic variations like HapMap or 1000 Genome Project in cooperation with smaller platforms enabled realization of Genome-wide association studies (GWAS) on large cohorts (3-4). GWAS have enabled identification of thousands of genetic variations (mostly single-nucleotide polymorphisms) that are tightly linked with some phenotypic traits or directly with some disease. Newly acquired knowledge about genes as well as with them associated pathways was successfully used for construction of model for genetic risk prediction. Such an estimation for expression of genetic variation's impact across human genome in one number is polygenic risk score (PRS).

Polygenic Risk Score

In one of the simplest way could be polygenic risk score expressed as sum of n- single-nucleotide polymorphisms weighted by their effect size β:

$$\sum_{i}^{n} x_{ij} \beta_i$$

where n is count of the incorporated SNP, x_{ij} is the number of copies for the i-th SNP in the genotype of j-th subject and β_i represents effect size of appropriate polymorphism calculated from GWAS analysis.

In graphical interpretation could be polygenic risk score expressed also as genetic risk percentile (5). Individuals with a polygenic risk score close to the population mean have the estimation of genetic risk similar to population's risk. Individuals with polygenic risk score up to 10th percentile have the lowest genetic risk while a person with polygenic risk score in 91st to 100th percentile would be considered to have the highest genetic risk.

Commonly, for calculation of polygenic risk score are commonly used hundreds to thousands of single-nucleotide polymorphisms but with rising number of SNPs included in the analysis, there is rising not only statistical power but also the noise ratio. Therefore in estimation of polygenic risk score model, there is needed to find balance between lower number of incorporated SNPs with overall highest accuracy on the one hand and on highest number of included SNPs with overall lower accuracy of the analysis (6). Moreover, estimation of such equilibrium is complicated also due to factors such as genetic background of the disease, genotyping density and sample size. Therefore for the estimated model of polygenic risk score is optimalization on independent validation dataset reacquired, by which is eliminated risk of overfitting. One of the complications is also linkage disequilibrium associated with tightly closeness of identified genes which can be cause of false positive.

The aim in estimation of polygenic risk score model is applicability on the widest population selection possible.

Despite the fact that polygenic risk score is relatively widespread in praxis, there is still limited amount of guides for optimal polygenic risk score construction as well as for interpretation of outcomes from such analysis (8).

With rising interest for polygenic risk score, there are improved not only models of calculations but also data availability from already existing databases. Currently, there si also option to share data required for analysis, for example via platforms like Polygenic Score Catalog or Cancer PRS-Web (9-10).

Utilization of polygenic risk score for estimation of common risk for diabetes mellitus and COVID-19

On the one hand, the world is influenced by the pandemic of coronavirus but with the respect to the prevalence of diabetes, which is about 10 percent, we can also speak about period of diabetes pandemic. Therefore there is enormous need to estimate cumulative risk for both diseases with the respect to large number of evidence about mutual interplay of both diseases.

Already at the beginning of the COVID-19 pandemic were estimated age, sex and associated comorbidities as possible risk factors for disease risk prediction. In that time, there were not available relevant data that could be usable for estimation of their effect on the disease severity. This fact led to incorrect estimation of infection risk as well as risk of severe course of the disease. From laboratories was relatively soon reported that almost all critically ill patients suffer for severe hyperglycemia. Thus, it was used as one of the markers of disease severity. Today is still unclear if SARS-CoV-2 infection is able to induce diabetogenic state by similar mechanism as in pathogenesis of type 1 and type 2 diabetes or it is atypical form of diabetes (11). There are known multiple mechanisms common for both diseases, such as chronic inflammation state manifested by increased level of proinflammatory cytokines IL-11, IL-16 and TNF-α as well as by increased level of C-reactive protein (CRP) and by adhesion of monocytes to vascular endothelium (12-13). Increased levels of chemokines CCL1, CCL2, CCL4 and CXCL10 are also observed. Norouzi et al. described in COVID-19 patients significantly higher levels of inflammatory cytokines and chemokines in state of acute hyperglycemia with further increased risk of multiorgan failure (14).

The site of entrance for SARS-CoV-2 virus in the organism are angiotensin converting enzyme (ACE2) receptors that are widely distributed in multiple type of tissues and which play important role in diabetes. After the infection, the virus is replicated also in endocrine and exocrine secretory cells of pancreas with further impairment of pancreatic β-cells. Estimated is also relationship between systemic inflammation and immune dysfunction as well as hyperglycemia and insulin resistance resulted from impaired function of pancreatic β-cells (14-15). Reciprocally, in comparison with healthy individuals, diabetic patients have higher risk of coronavirus infection with overall worse prognosis and with higher mortality rate (16).

With increasing knowledge about SARS-CoV-2 infection, there is also growing evidence of genetic background of COVID-19 disease, which could contribute to the infection risk- and diseases severity estimation. Dite et al. identified 64 single-nucleotide polymorphisms and clinically important risk factors which were consequently used for creation of model of severe

course of SARS-CoV-2 infection in patients over 50 years (17). They also show that their model has higher discrimination ability for the severity of the disease course in comparison with other models based only on age and sex. Although there is rising number of such a models, there still aren't available any systematic analysis of common loci that are characteristic for both diseases.

Conclusion

The topic of disease course prediction has become more and more important from the point of view of patient as well as from the socio-economic impact because of constantly rising health care expenses paid from public resources. Constant technological development and implementation of modern approaches in genetic research has led to creation of several models for infection risk prediction as well as for course of disease prediction. One of such method is polygenic risk score, which has wide potential for application in biomedical research and in clinical praxis.

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24. Importance of mitochondrial energy production and endogenous Coenzyme Q_{10} for patients with post-COVID-19

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Introduction

WHO named the disease caused by SARS-CoV-2 virus as COVID-19 (**Co**rona **Vi**rus **D**iseases 20**19**) (1). The clinical symptoms of COVID-19 can be divided into two categories: *Post-acute COVID-19* (sub-acute COVID-19) including symptoms present between 4-12 weeks and *post-COVID-19 syndrome* (long, chronic COVID-19) including symptoms present more that 12 weeks up to several months (2). The main symptoms include shortness of breath, pain of muscle, and joints, cough, hair, taste and smell loss, sleep and memory disturbances, depression, impaired quality of life of patients.

Mitochondria have of key importance for the survival and replication of the SARS-CoV-2 virus. They are involved in the regulation of several metabolic processes in health and diseases, and in antiviral responses. The dynamics and bioenergetics of the host mitochondria can be manipulated by a virus and the mitochondrial antiviral signaling protein (MAVS) activates NF_k-B and the induction of interferons (3). SARS-CoV-2 virus causes dysfunction and aggregation of platelets, can increase the production of reactive oxygen species, reduce the antioxidant protection of the organism and cause damage of the host mtDNA (4). The SARS-CoV-2 virus can target intracellular and extracellular mitochondria, which play a central role in the primary host defense mechanisms against viral infections. This virus changes intracellular distribution of mitochondria. Hijacking of immune cells mitochondria by the SARS-CoV-2 virus belongs to the molecular mechanisms that could be a key factor in the pathogenesis of the virus and in the induction of the disease COVID-19 (5). The exact mechanism of the effect of the SARS-CoV-2 virus on mitochondrial functions is not fully understood. We assumed that SARS-CoV-2 virus might manipulate mitochondrial bioenergetics and endogenous coenzyme Q_{10} (CoQ_{10}) levels (6, 7).

It is believed, that the most effective strategy to prevent COVID-19 infection is vaccination (8). New strategies to prevent and reduce the negative impact of the SARS-CoV-2 virus on society include the environmental protection. Mountain spa rehabilitation in the High Tatras is beneficial for chronic pulmonary diseases, improving fatigue, joint pain, depression and quality of life of patients (9, 10, 11). CoQ_{10} in the form of ubiquinol and ubiquinone is used for

targeted supportive therapy of reduced mitochondrial energy production in patients with various diseases.

We determined the effect of the SARS-CoV-2 virus on platelet mitochondrial bioenergetics and endogenous levels of CoQ_{10} in patients with post-acute COVID-19 (vaccinated, non-vaccinated) and the effect of targeted supplementary therapy with ubiquinol and mountain spa rehabilitation on impaired of platelet mitochondrial bioenergetics and endogenous levels of CoQ_{10} in patients with post-COVID-19 syndrome.

Methods

1**.** *High-Resolution Respirometry (HRR):* In platelets isolated from whole blood, mitochondrial bioenergetics was evaluated by the HRR method with the use of an O2k-Respirometer (Orobors Instruments, Austria) (12, 13, 14) as described in detail previously (15). 2. *High-Performance Liquid Chromatography (HPLC):* Coenzyme $Q_{10\text{-TOTAL}}$ (ubiquinol + ubiquinone) in platelets and plasma was determined by HPLC method (16, 17).

Human subjects: 1. Control group (***C)*** - healthy volunteers; 2. Group of patients with post-acute COVID-19 (***PAC19***); 3. Group of vaccinated patients with post-acute COVID-19 ***(V+PAC19***); 4. Group of patients with post-COVID-19 syndrome on mountain spa rehabilitation ***(MR);*** 5. Group of patients with post-COVID-19 syndrome on simultaneous supplementary therapy with ubiquinol and mountain spa rehabilitation (***MRQ***).

Data analysis: Unpaired Student´s t-test was applied to evaluate differences between parameters of post-COVID-19 patients and control group. Paired Student´s tests were applicated to evaluate differences between parameter of MR and MRQ groups before and after therapy. The P values <0.05 were considered statistically significant. The results are expressed as the mean ± sem.

ClinicalTrials.gov, Ethics Committees: These studies were carried out according to the principles expressed in the Declaration Helsinki, study protocols were approved by the Ethics Committee, Bratislava, Slovakia, No. EK/012/2021/UNB and Ethics Committee of Medical Faculty, Comenius University in Bratislava, No. 34/2022. Studies are registered by ClinicalTrials.gov ID: NCT05178225 and ClinicalTrials.gov ID: NCT05421234. Written informed consent form was obtained from each subject before the start of the study.

Results

Study 1: Effect of SARS-CoV-2 virus on platelets mitochondrial energy production and endogenous CoQ_{10} level in patients with post-acute COVID-19

In January – February 2021 ten patients 4-7 weeks with post-acute COVID-19 were included in the study (7 women and 3 men), with the mean age 59.9±5.4 years. Control group consisted of 15 healthy volunteers (6 men, 9 women). No differences of basal values platelet mitochondrial respiration were found in comparison with control data. The CI-linked respiration associated with adenosine triphosphate (ATP) production by oxidative phosphorylation (OXPHOS) capacity in the post-acute-COVID-19 patients was significantly reduced (p=0.027), reaching 65.0% of the control values. After addition of cytochrome c

respiration was significantly decreased (p=0.040), reaching 69.0% of the control values. CI-linked electron transfer (ET) capacity, reached 68% (p=0.036) of control values. Complex CII-linked respiration was not affected by SARS-CoV-2 virus.

The concentration of coenzyme $Q_{10\text{-TOTAL}}$ ($CoQ_{10\text{-TOTAL}}$) (ubiquinone + ubiquinol) in platelets of the patients with post-acute COVID-19 was significantly reduced (p=0.002) to 58.9±3.60 pmol.10^{-6} cells vs control data 84.1±5.3 pmol.10^{-6} cells, reaching 70% of control group values. The concentration of $CoQ_{10\text{-TOTAL}}$ in blood and plasma was reduced to 69.1% (p=0.014) and 76.3% (p=0.034) of control group values (18, 19, 20).

Study 2: Effect of vaccination on platelet mitochondrial energy production and endogenous CoQ_{10} level in patients with post-acute COVID-19

In October 2021 in Bratislava, ten vaccinated human volunteers (5 men, 5 women) were included in the study, with the mean of age 41.5±3.8 years. All participants were infected with the SARS-CoV-2 virus at a party. Five patients vaccinated twice with the Astra Zeneca vaccine were infected with the SARS-CoV-2 virus two months after vaccination. Four patients vaccinated with the BionTech/Pfizer vaccine were infected with the SAS-CoV-2 virus 2-3 months after the second vaccination. One patient vaccinated with the Moderna vaccine, was infected with the SARS-CoV-2 virus one month after vaccination. The control group consisted of 10 healthy volunteers.

The parameters of platelet mitochondrial function of vaccinated patients with post-acute COVID-19 (V+PAC19) were not significantly changed in comparison with control data. The concentration of CoQ_{10} was significantly lower in platelets (p=0.046), reaching 80±6% of control values; in whole blood the CoQ_{10} concentration reached 78±10% (p=0.085); in plasma the CoQ_{10} concentration was significantly lower (p=0.010), reaching 67±8% of control values (19, 20). Our pilot result showed that vaccination prevented damage of platelet mitochondrial bioenergetics, while vaccination did not prevent the deficit of endogenous CoQ_{10} levels of patients with post-acute COVID-19.

Study 3: Effect of mountain spa rehabilitation on platelet mitochondrial energy production and endogenous CoQ_{10} level in patients with post- COVID-19 syndrome

Environmental strategies play a vital role in pandemic prevention similar to COVID-19. Mountain spa rehabilitation is beneficial for chronic pulmonary diseases, improving fatique, joint pain, psychological stress, and improving quality of life of patients with various diseases (10). In May and June 2021, fourteen patients with post-COVID-19 syndrome were included in the study (MR group). The mean age of patients was 58.97±2.64 years (8 men, 6 women). The patients were 4-6 months after COVID-19 infection. Platelets mitochondrial function was evaluated before MR and after 16-18 days of MR in Sanatorium of Dr. Guhr, Tatranská Polianka, Slovakia. The control group consisted of fifteen healthy individuals (6 men and 9 women). After MR improved lungs function (Borg Scale, 6-MWT, SpO_2), and 51.8% clinical symptoms of COVID-19 in patients disappeared.

Platelet mitochondrial bioenergetics parameters were reduced in patients with post-COVID-19 syndrome in comparison with the control group. Basal mitochondrial respiration (CI-linked

LEAK respiration) was lower by 14.2%, CI-linked respiration coupled with ATP production (OXPHOS capacity) was significantly reduced (p=0.0004) by 45.8%; Maximal mitochondrial oxidative capacity (the electron transfer capacity, ET) was significantly reduced (p=0.0002) by 45%; ET capacity with CI&CII-linked substrates was decreased by 9.7% in patients with post-COVID-19 syndrome in comparison with values of the control group. After MR mitochondrial parameters improved. Basal respiration increased by 47.8% (p=0.029) vs before MR; OXPHOS capacity associated with ATP production was improved by 12.3% vs before MR. Endogenous concentration of $CoQ_{10\text{-}TOTAL}$ in platelets, blood and plasma in patients with post-COVID-19 syndrome did not significantly differ from the control group and did not change after MR (9, 10, 11, 21).

Study 4: Effect of ubiquinol with mountain spa rehabilitation on platelet mitochondrial energy production and endogenous CoQ_{10} level in patients with post-COVID-19 syndrome

Patients with post-COVID-19 syndrome were on mountain spa rehabilitation and simultaneously supplemented with ubiquinol in daily dose 2x100 mg (MRQ group) during 16-18 days in Tatranská Polianka, Sanatorium Dr. Guhr, Slovakia. MRQ group consisted of 22 patients (14 men, 8 women) with the mean of age 57.8±2.5 years. Control group consisted of 15 healthy volunteers (6 men, 9 women), mean of age 51.3±2.3 years. After MRQ improved lungs function (Borg Scale, 6-MWT, SpO_2), and 62.8% clinical symptoms of COVID-19 in patients disappeared.

Platelet mitochondrial bioenergetics parameters were reduced in patients with post-COVID-19 syndrome in comparison with the control group. After MRQ basal mitochondrial respiration increased by 47.6% (p=0.0025) vs before MRQ. CI-linked respiration coupled with ATP production (OXPHOS capacity) increased by 25.4% vs before MRQ (p=0.043). ET capacity increased by 22.4% (p=0.042), and ET capacity with CI&CII-linked substrates increased by 9.5% (p=0.055) vs before MRQ (22, 23). The concentration of $CoQ_{10\text{-}TOTAL}$ in platelets in patients with post-COVID-19 syndrome did not initially differ from the control values. After MRQ the concentration of $CoQ_{10\text{-}TOTAL}$ significantly increased in blood and plasma, in PLT $CoQ_{10\text{-}TOTAL}$ increased by 68% compared before MRQ (p<0.0001) (21, 22).

Next our study showed platelet mitochondrial dysfunction and reduced endogenous CoQ_{10} levels in infertile men with post-COVID-19 (24).

Discussion

***Study 1*:** Our results indicate modulation of mitochondrial function by SARS-CoV-2 virus. The deficit of CI-linked OXPHOS could be caused by a decreased activity of CI or by impaired electron transfer from CI to CIII. Reduced CoQ_{10} concentration in platelets indicated that its deficit could be limiting for the electron transfer from CI to CIII (18), or SARS-CoV-2 virus reprogrammed energy production in platelets mitochondria towards the preference of glycolysis instead of OXPHOS. We suppose that the protease (PDB 6Y84) of the SARS-CoV-2 virus may form a direct bond with the CoQ_{10} structure and block the endogenous CoQ_{10} biosynthesis in patients with post-acute COVID-19 (19).

***Study 2**:* Vaccines prevented declines in platelet mitochondrial respiration and energy production in patients 2 weeks after acute infection with the SARS-CoV-2 virus. Parameters of platelet mitochondrial function of vaccinated patients with post-acute COVID-19 (V+PAC19) were not significantly changed in comparison with control data (20). We assume in agreement with other that vaccination may protect platelet mitochondrial bioenergetics by next mechanisms: by reduction of inflammatory signalling in megakaryocytes (25) and by blocking the entry of the SARS-CoV-2 virus into blood and into cells (26). An alternative mechanism of infection by the SARS-CoV-2 virus, independent on the ACE2 receptor, is the binding of the spike protein of SARS-CoV-2 to platelets via the CD42b receptor (27).

During vaccination against the SARS-CoV-2 virus, the immune system recognizes the entry of a foreign spike protein into the body, and antibodies begin to form. Vaccination prevent entry of SARS-CoV-2 into platelets, virus cannot bind to ACE2 or CD42b receptors and mitochondrial function is protected (27). A deficit of endogenous CoQ_{10} biosynthesis is one of the main causes of muscle weakness and fatique in patients with post-COVID-19 disease, reduced mitochondrial function can contribute to the COVID-19 progression. SARS-CoV-2 virus reduced endogenous CoQ_{10} antioxidant level in vaccinated patients with post-acute-COVID-19. The exact mechanisms for the depletion of CoQ_{10} during SARS-CoV-2 infection remain to be determined (28).

Study 3: European Association of Spa Rehabilitation (ESPA) recommends spa rehabilitation for the patients with post-COVID-19 syndrome (10). The rehabilitation improved pulmonary function, exercise capacity, and quality of life of patients with post-acute phase of COVID-19 (10, 11). Mountain spa rehabilitation in High Tatra, Slovakia is beneficial for chronic lung diseases, for alleviating fatigue, joint pain, mental stress, sleep disorders and the quality of life of patients with various diseases (15). In our patients with post-COVID-19 syndrome, MR improved the functional parameters of the lungs, increased the vitality and quality of life of the patients. After MR, the mean improvement of mitochondrial parameters representing OXPHOS and ET capacity was 11.4%. MR improved multiple clinical symptoms in patients with post-COVID-19 syndrome. Endogenous concentration of CoQ_{10} in patients with post-COVID-19 syndrome did not change after MR (15, 21).

Study 4: Mountain spa rehabilitation in combination with ubiquinol significantly improved several clinical symptoms of patients with post-COVID-19 syndrome. MRQ improved bioenergetics of platelet mitochondria, increased coenzyme Q_{10} concentration in platelets by 68% ($p<0.0001$) and in plasma by 232% ($p<0.0001$) compared to the values before the treatment (21, 22). Mountain spa rehabilitation and ubiquinol supplementation is recommended for patients with post-COVID-19 syndrome.

Highlight

1. In patients with post-acute COVID-19 (without vaccination) and in patients with post-COVID-19 syndrome, platelet mitochondrial ATP production was significantly reduced.
2. Vaccination prevented reduction of the platelet mitochondrial respiration and energy production in patients with post-acute COVID-19.
3. In all patients with post-acute COVID-19 endogenous coenzyme Q_{10} level was decreased.
4. After mountain spa rehabilitation of patients with post-COVID-19 syndrome, lungs function and clinical symptoms improved, mitochondrial energy production and CoQ_{10} concentration increased. Better results were achieved with simultaneous ubiquinol supplementation.
5. Mountain spa rehabilitation and ubiquinol supplementation is recommended for patients with post-COVID-19 syndrome.
6. Determination of CoQ_{10} concentration and platelet mitochondrial function could be useful for estimation of mitochondrial health and monitoring the treatment of patients with post-COVID-19.

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25. Can ACE2 blockade be a suitable rat model for monitoring the consequences of COVID-19?

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Rising deaths due to the consequences of the COVID-19 pandemic necessitated an immediate search for a relevant, most suitable animal model that would allow understanding the mechanisms of this disease, developing targeted therapies, and testing more appropriate drugs for the comorbidities that most often accompany COVID-19 (1). Indeed, 2- or 3-dimensional models of tissue cultures and organoids derived from human lungs, bronchi, alveoli, blood vessels and other tissues have strong limitations for more complex study targets (2). Especially for purposes where fully differentiated cells and tissues, complete immune functions or systemic circulation are required, animal models still remain essential.

Histological analysis, surface visual inspection, and radiological imaging were the predominant approaches used to assess the development of inflammation and lung damage after experimental SARS CoV 2 infection. However, these pathological observations have so far only been documented in some experimental animals. This is probably due to the focus of studies on early viral infection and its transmission. However, for rigorous drug efficacy studies, it is important that disease-relevant clinical signs to be measured in such a way that the effects of potential therapeutics on the consequences of COVID-19 can be statistically determined. That is why it is important to develop a relevant biomodel for monitoring the consequences of COVID-19. Previous studies for in vivo monitoring of COVID-19 infection have been described in macaques (3), cats (4), ferrets (5), hamsters (6), mink (7) and transgenic mice that express human angiotensin I converting enzyme 2 (ACE2). These models have been used successfully for transmission and immunity studies, but only partially simulate the mechanisms involved in the consequences of COVID-19 (8). The biggest limitation in these ACE2 transgenic mouse model is its lethal effects caused by neuroinvasion affecting central nervous system (9, 10).

Ongoing studies continue to confirm that cardiovascular pathologies such as hypertension, diabetes, obesity, and heart failure are the most common comorbidities in patients with Covid-19 (11). Patients with cardiovascular disease are usually treated with renin-angiotensin system (RAS) blockers, such as angiotensin-converting enzyme (ACEi) inhibitors or angiotensin II receptor (Ang II) blockers. Part of this system - angiotensin converting enzyme (ACE2) is the gateway for SARS-CoV-2 entry into the target cells. Both ACE and ACE2 belong to the ACE family of dipeptidyl carboxydipeptidases and exhibit different physiological functions (2,3). Explaining the relationship between SARS-CoV-2 and membrane ACE2 will help to better understand not only the infection itself, but also the implications of COVID-19.

Similar to other CoV, during viral, the spike proteins (S) on the envelope of SARS-CoV-2 are cleaved into S1 and S2 subunits. The S1 contains the receptor binding domain and directly binds to the peptidase domain of ACE 2 to enter host cells (12,13). ACE2 is a monocarboxypeptidase, which cleaves Ang I into Ang 1-9 and Ang II into a heptapeptide, Ang 1-7. Both peptides have vasodilatory and antiproliferative functions by activating the MAS/G receptor. The ACE2/Ang 1-7/MAS1 axis provides a counter-regulatory mechanism within the RAS that balances the deleterious effects of ACE/Ang II/AT1 receptor axis (14). Mice deficient in MAS1 or ACE2 receptors exhibit cardiac systolic dysfunction, increased blood pressure, myocardial interstitial fibrosis, endothelial dysfunction, and exhibit increased susceptibility to intravascular thrombosis, chronic kidney disease, and metabolic abnormalities (15). ACE2 activation prevents the deleterious effects of Ang, such as cell death, fibrosis, angiogenesis, and thrombosis formation (15). Recent autopsy results on SARS-CoV-2 infected humans showed diffuse alveolar damage with capillary congestion accompanied by microthrombi in vascular beds (16). However, pathological examinations have not investigated if SARS-CoV-2 infection leads to total destruction of ACE2 receptors.

Interestingly, in an animal model of SARS-CoV, Oudit et al. found a marked decreased ACE2 expression in the heart of infected mice (17). Ang-(1-7), the main product of ACE2, by binding to MAS induces many beneficial actions, such as vasodilation, inhibition of cell growth and anti-thrombotic effects. In addition, it has protective effect on the brain and prevents ischemic stroke (18).

Clinical studies indicate that in most cases the respiratory distress occurs many days after the infection, suggesting that this may not be a direct effect of the initial viral infection but rather the hosts reaction to the loss of function of ACE2 and dysregulation of Ang II/ACE2 pathways as well activation of host proteases. Binding of the spike protein to ACE2 leads to shedding of ACE2 receptors by various proteases, which in turn leads to the loss of protective function of the ACE2/MAS axis (Fig. 1). In addition, activation of ACE/RAS/Ang II and alternative pathways leads to an excessive production of Ang II at the tissue level. This process may further shift the balance of protective Ang (1-7)/MAS and ACE2 function to the detrimental effects of increased Ang II (19). Therefore, induction of the downstream pathway of ACE2, by activating the ACE2/Ang1-7/MAS axis may prove a useful strategy in preventing lung and cardiovascular damage associated with SARS-CoV-2 infections. Conversely, ACE2 blockade, e.g. via MLN-4760 (20) can simulate pulmonary and cardiovascular complications associated with a SARS-CoV-2 infection. In this sense, ACE2 blockade may be suitable for the development of an animal model of the consequences of COVID-19, including the rat.

Indeed, many laboratories prefer to use a rat model for their testing which does not require the equipment of larger animals and compared to mice is better adapted to simulate human diseases apart from transgenic mice. Although there is yet no relevant rat model for monitoring the consequences of severe COVID-19, there is a relatively good basis of biomodels including spontaneously hypertensive, obese, diabetic, or immunodeficient rats, in which appropriate intervention including ACE2 inhibition can induce COVID-19-like consequences. The development of a rat model for monitoring the consequences of COVID-19 would bring new possibilities for monitoring drugs and substances that have the potential to treat COVID-19. Future studies are needed to standardize relevant rat model of COVID-19 and protocols to allow comparisons of different drug candidate interventions.

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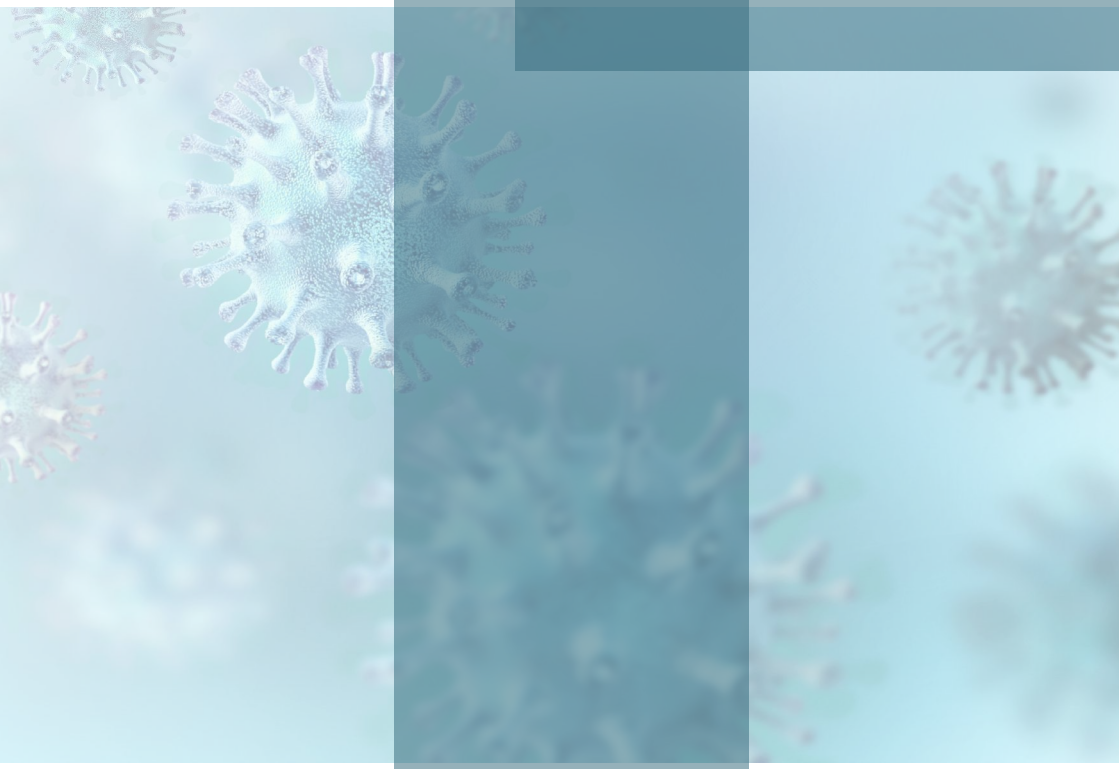

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