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PhD THESIS

PREDICTIVE BIOMARKERS IN SARS-CoV-2 INFECTION. CLINICAL AND FORENSIC ASPECTS

– A B S T R A C T –

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The theoretical section of the thesis is centered on exploring the pathophysiological mechanisms of COVID-19, including its complications and significant biomarkers, which are then detailed in the special part. To accomplish this, an extensive review of pertinent literature was conducted, emphasizing the immune-mediated mechanisms of COVID-19, the direct effect of SARS-CoV-2 on tissues and currently available biomarkers for viral detection, clinical work-up and post-mortem assessment.

The special part follows up on identifying potential prognostic biomarkers at different clinical spectrum stages of COVID-19 and includes information on potential post-mortem implications as well as key histopathological findings within each organ system due to SARS-CoV-2 infection. Another crucial element of this dissertation includes investigating and providing relevant data regarding emerging co-infections and superinfections in COVID-19, while also defining promising novel research directions.

This doctoral thesis aims to integrate clinical, histological and laboratory data, monitoring trends rather than a single value, thereby improving COVID-19 clinical management and patient outcomes.

The global spread of SARS-CoV-2 has plunged our modern society into an economic and healthcare crisis and more than three years into the pandemic, multiple factors regarding the disease progression and complications remain unelucidated, thus posing important questions to motivate further research.

The restricted use of biomarkers in postmortem scenarios due to discrepancies in reported data and the absence of external validation studies, coupled with the established potential and promising biological implications noted for specific molecules, has given rise to this research initiative.

The global research population of the current thesis is comprised of 507 COVID-19 cases from 2020-2022 divided in:

- 418 cases managed clinically at Victor Babes Clinical Hospital of Pneumophtisiology and Infectious Diseases Timisoara, within the Infectious Diseases II Department;
- 89 autopsy cases at the Institute of Forensic Medicine.

The global research population was evaluated through RT-PCR for SARS-CoV-2 RNA and only cases with a positive molecular test were included further in the studies. For cases managed in the Infectious Diseases Department, treatment was conducted in accordance with the Romanian Ministry of Health's COVID-19 guidelines, as published and implemented at the time of patient hospitalization. Based on disease severity, the patients were administered antiviral therapy (remdesivir or favipiravir), interleukin-1 receptor antagonists, humanized

monoclonal antibodies against the interleukin-6 receptor, steroid treatment with dexamethasone or methylprednisolone, low molecular weight heparin, broad-spectrum antibiotics, proton pump inhibitors, antitussives, or antipyretics.

According to the Romanian National Institute of Public Health, clinical criteria include the presence of at least one of the following signs or symptoms: cough, fever, shortness of breath, sudden onset of anosmia, ageusia or dysgeusia, in children – the presence of gastrointestinal symptoms (vomiting and accelerated intestinal transit), radiologic signs of COVID-19 compatible lesions, and epidemiologic criteria that include at least one of the following:

- direct contact with a COVID-19 confirmed case, within 14 days before the date of symptom onset;
- residents or staff members in institutions caring for a vulnerable population, during 14 days before the date of onset, or institutions with confirmed SARS-CoV-2 transmission [61, 121].

Upon initial evaluation of COVID-19 cases, the presence of at least one of the following risk factors was considered sufficient grounds for hospitalization: age ≥60 years, cardiovascular disease, chronic obstructive pulmonary disease, asthma, emphysema, diabetes, malignancy, obesity (body mass index ≥ 30 kg/m^2), chronic kidney disease, immunocompromised state (solid organ transplantation, recipient of immunosuppressive therapy, HIV/AIDS), pregnancy, sickle cell disease, dyspnea or increased respiratory rate (≥30 breaths per min), spontaneous oxygen saturation ≤94% (in room air) or a drop in saturation to <90% (requiring oxygen supplimentation), lymphopenia < $1000/mm^3$, D-dimer > 250 ng/mL, and CRP > 10 mg/L.

Moving forward, for all the cases which met our study inclusion criteria, complete data sets were collected, focusing on the variables considered relevant for further statistical processing.

Depending on the clinical form of the infection and following the definitions of Romanian Public Health's COVID-19 guidelines the patients were classified thus: patients with a mild form of the disease, patients with a moderate form of the disease and patients with signs of severe pneumonia.

Cases managed at the Institute of Forensic Medicine Timisoara were divided in two studies thusly:

- Study I, where a total of 45 COVID-19-positive subjects were included (Group 1). All subjects died outside of a hospital setting and therefore did not receive specific or

symptomatic therapies that could have modulated the inflammatory response. As controls (Group 2), we selected a total of 20 subjects who died from polytrauma in high velocity car accidents (n=10), and suicide (jumping from height n=5, hanging n=4, intoxication n=1). The case exclusion criteria was the presence of concomitant known infectious lung diseases (for example history of or active pulmonary tuberculosis). The aim of this study was to summarize key histopathological findings within each organ system due to COVID-19 and to assess if serological inexpensive and widely available biomarkers such as CRP, IL-6, fibrinogen and d-Dimers, associated with adverse outcomes in COVID-19, can be implemented in a postmortem assessment.

- Study II was conducted on 61 autopsy cases and aimed to analyze the expression of microRNAs from postmortem positive and negative SARS-CoV-2 cases. After the postmortem molecular analysis was performed, 44 cases tested positive for SARS-CoV-2 infection (Group 1) and the remaining 17 negative cases were considered the control group (Group 2). The study was conducted between 1st of September 2020 and 31st of March 2021 and the case exclusion criteria was normal aspect of the lungs upon macroscopic examination.

Study III is a retrospective case series, describing the evolution of 11 COVID-19 patients out of all 1648 cases hospitalized at Victor Babes Clinical Hospital of Pneumoftiziology and Infectious Diseases Timisoara, within the Infectious Diseases Department, in the second outbreak of COVID-19 (from 1st of October 2020 until 31st of January 2021).

Infections diagnosed within 48 h of hospital admission were classified as co-infections. Infections identified after 48 h of admission were classified as superinfections. Biological samples for the diagnosis of co-infection/superinfection in COVID-19 patients in Study IV, were collected after evaluating the following criteria:

- clinical criteria: purulent sputum, persistent fever (>38°C), deterioration of ventilatory parameters, or hemodynamic instability.
- laboratory criteria: worsening of leukocytosis or leucopenia, increased procalcitonin, or C-reactive protein.
- radiological criteria: progression/worsening of the chest radiological pattern, or onset of a pattern characteristic for bacterial pneumonia such as basal consolidation, nodules, cavitation, or pleural effusion.

Expectorate sputum was selected as sampling technique and routine microbiological investigations were conducted at the medical microbiology laboratory of Victor Babes Clinical Hospital of Pneumoftiziology and Infectious Diseases Timisoara, using standard bacteriology. All isolates were first identified using the VITEK® 2 GN, VITEK® 2 GP ID cards (BioMérieux,

Marcy l'Etoile, France). Antimicrobial susceptibility tests were performed using the VITEK 2 GN AST-N222 and VITEK 2 AST GP 67 cards (BioMérieux, Marcy l'Etoile, France).

For Study I, the levels of CRP, IL-6, fibrinogen and d-Dimers in postmortem plasma samples were significantly higher in COVID-19 subjects than in control group ($p< 0.0001$). The level of IL-6 was significantly higher in overweight patients (r=0.52, P<0.001). No significant positive correlations were found between the plasmatic level of IL-6 and other immune-inflammatory parameters: CRP (r=0.22, P=0.14), d-Dimers (r=0.023, P=0.88), fibrinogen (r=0.119, P=0.435). Upon microscopic examination of the COVID-19 group, the lungs of all subjects were heavy, congested, generally with abundant edema fluid with patchy involvement. In all the cases of group one, histological examination revealed features corresponding to the exudative and/or proliferative phases of diffuse alveolar damage. Of these, 32 were in acute organizing phase and 13 showed more extensive organization.

For Study II, our results show that both the COVID-19 group, and the control group were matched well for age and gender (Shapiro-Wilk test; p = 0.505). The mean age for the SARS-CoV-2 infected patients was 58.5±18.72 (raging between 29 and 91 years) and for the control group was 52±17.49 (ranging between 25 and 83 years). Among study population and controls, 23/44 (52.7%) and 9/17 (52.9%) were male, respectively. ROC analysis revealed the power of the 3 microRNAs as predictive biomarker in SARS-CoV-2 postmortem confirmed cases, with the area under the curve (AUC) as follows: microRNA- 6501-5p = 0.762; microRNA-5695 = 0.837 and microRNA-29b-3p = 0.855. The combined AUC value was 0.985, a much higher value than each single microRNA, thus proving the higher discriminatory power.

Study III, followed the evolution of 11 patients with COVID-19, nine men and two women, aged between 36 and 78 years, mean age 58.27±12.39 years, hospitalized after two to seven days, median 4.8 (2–7) days, since the onset of symptoms of SARS- CoV-2 infection, the diagnosis being confirmed by a real-time polymerase chain reaction. After 1 to 15 days of hospitalization, with a median of 4.45 (2–6) days, all subjects developed at least one of the following complications: PT, PM, PP, and SE. Eight patients died after 12 to 40 days, median 23.5 (14–33.75) days of hospitalization, with a median interval of 19.5 (9.25–25.5) days after the occurrence of the air leak. The remaining three, with reduced pulmonary injury at admission, did not require surgical drainage and were discharged in good clinical condition after a median in-hospital stay of 7 (2–7) days.

For the fourth study of this research initiative, there were 46 individuals with a positive sputum culture growing pathogenic bacteria, 67 patients with positive sputum cultures for respiratory tissue-associated commensal bacteria, 51 cases identified with fungal growth in their sputum cultures, and 243 patients with negative sputum cultures. It was observed that

the majority of patients were elderly people over the age of 65, of which more than 52% were men. However, there were no statistically significant findings between the four comparison groups regarding their background ($p > 0.05$).

It was observed that patients with fungal infections had a significantly shorter average duration from symptom onset until hospitalization (4.7 days), compared with 6.1 days in patients with pathogenic bacteria identified in the sputum samples, 5.9 days among those with commensal flora infections, and 6.8 days in the control group ($p < 0.001$). However, the time elapsed from the first positive COVID-19 PCR test until hospitalization was approximately 4 days, without significant differences between groups ($p > 0.05$). Most sputum samples were taken within 48 h from hospital admission, and more than 80% of all samples showed multidrug resistance.

The parallel pathogen identification among sputum samples was classified by pathogenic bacteria for the respiratory tract, commensal human pathogens of the respiratory tract, and fungal infections of the respiratory tract. In total, 31.5% of samples were positive for *Pseudomonas aeruginosa*, followed by 26.2% with co-infections with *Klebsiella pneumoniae*, among patients admitted with COVID-19. The third most common pathogenic bacteria identified in the sputum samples was *Escherichia coli*, followed by other Gram-negative bacilli, and *Acinetobacter baumannii* in 9.3% of samples.

Commensal human pathogens caused respiratory infections in 67 patients with COVID-19, the most prevalent being *Streptococcus penumoniae* in 34.1% of patients, followed by methicillin-sensitive (21.6%) and methicillin-resistant *Staphylococcus aureus* (17.0%). The remaining sputum samples were confirmed for *Moraxella catarrhalis* in 9.1% of the samples and *Haemophilus influenzae* in 6.5%. Lastly, fungal infections among non-critical COVID-19 patients admitted to the infectious disease department identified a majority of 53.4% of samples positive for *Candida spp.* growth, followed by 41.1% of samples with *Aspergillus spp.* growth.

Inflammatory markers were also statistically significantly different among the four study groups. High procalcitonin levels were observed among patients with pathogenic bacteria and commensal flora respiratory co-infections (60.9% and 61.2% of samples outside the normal range, respectively), while IL-6, ESR, and CRP were equally elevated in the three groups with positive samples compared with the control group ($p < 0.05$).

The three groups with positive microbial growth on sputum cultures had an equally proportional distribution of patients admitted to the ICU, with an average of 30% of them being admitted, compared with only 17.3% among hospitalized COVID-19 patients with negative

sputum cultures (p = 0.003). Consequently, the duration of hospitalization, ICU stay, and mortality was much higher in these three groups than in the patients with negative samples.

Since the pandemic began, Romania has navigated multiple COVID-19 infection waves, characterized by oscillating daily case counts with peaks and troughs in transmission rates. The healthcare system has been strained with a considerable number of hospital admissions and fatalities. Mortality trends have not remained static but have seen surges during times of heightened transmission. Vulnerable groups, such as senior citizens, and individuals with pre-existing conditions like hypertension, diabetes, and cardiovascular diseases, have been disproportionately affected.

Examining the situation in Romania, especially within the COVID-19 framework, holds importance for numerous reasons. A comprehensive understanding of Romania's COVID-19 landscape can enrich the broader public health strategy, offer valuable data on viral transmission, evaluate treatment efficacy, gauge the healthcare system's resilience, and allow an in-depth review of its impact on at-risk groups. This is particularly relevant given Romania's aging population and communities like the Roma who may be especially vulnerable.

The worldwide mortality rate for COVID-19 in 2021 was 2% with a 0.06% of severe cases and early autopsy studies document respiratory failure due to ARDS as predominant cause of death, frequently accompanied by capillary microthrombosis, superimposed bronchopneumonia, and pulmonary thromboembolism. Romania recorded, in October 2021, 110% more deaths than the average of Octobers in the years preceding the pandemic, namely 2017–2019. It is the largest proportion of excess mortality among those during the entire period of the pandemic, from the entire European Union.

The postmortem serological investigations carried out in our study revealed higher serum expression of IL-6 for the COVID-19 group however this difference does not reflect magnitude and duration of IL-6-mediated signaling, which is dependent on the complex interplay of membrane-bound (cis-signaling, classically anti-inflammatory) and soluble IL-6 receptors (trans-signaling, proinflammatory) as well as soluble inhibitors.

Postmortem studies are prone to limitations, the most important of which consists in the inability to assess illness dynamics, as the evaluation takes place at the end of the disease course. Study I is limited due to the small sample size and population distribution. Complete medical records for every patient were not available, leading to difficulties in correlating the clinical course with pathological findings. Additionally, CPR, IL-6, d-Dimes and fibrinogen measured from postmortem samples could not be compared with the values present before death because all the subjects included in the study died in out-of-hospital settings.

Since the start of the COVID-19 pandemic, only a few studies worldwide have evaluated microRNAs expression patterns in postmortem biological samples of SARS-CoV-2 infected individuals. Taking this into consideration, the second part of the thesis aimed to explore the diagnostic potential of circulating microRNAs (microRNA-6501-5p, microRNA-5695 and microRNA-29b-3p) in postmortem detection of SARS-CoV-2 infection.

Altogether, our findings demonstrate the relevance of miR-6501-5p, miR-29b-3p, and miR-5695 in endothelial dysfunction and inflammatory response in patients with SARS–CoV-2 infection and the occurrence of severe lung injury and immunothrombosis. These clinical events are the most damaging occurrences correlated with fatal outcomes in patients with severe forms of COVID-19. The results support the need for multivariable approaches when evaluating miRNAs as clinical indicators.

It is relevant to point out that the small sample size, the lack of other respiratory diseases as controls and the use of other miRNA signatures/panels are the main limitations of this study. The risks of bias includes the inability to verify the reported figures, and the limited number of studies specifically concerning postmortem biochemical blood analysis. Lastly, we did not perform immunostaining or electron microscopy on the pulmonary tissue specimens. Further large-scale studies are needed to address these limitations.

As the COVID-19 pandemic evolved and the number of cases increased worldwide, several scientific papers were published in the medical literature, starting with March 2020, reporting patients who had developed spontaneous PT, PM, or even PP, in the absence of invasive mechanical ventilation. These conditions were initially considered rare complications of the SARS-CoV-2 infection. By analyzing the database of the two Infectious Diseases Departments of the Victor Babes Clinical Hospital of Pneumophtisiology and Infectious Diseases Timisoara, among all patients hospitalized for SARS-CoV-2 infection during the first COVID-19 outbreak (28 February to 31 July 2020), when hospital admission was mandatary for all individuals infected with SARS-CoV-2, in contrast with the cited studies, we found no mention of such complications.

On the contrary, after the second outbreak of the pandemic, since 1 October 2020 until the end of January 2021, of all 1648 patients admitted in the hospital, we observed the occurrence of these complications in 11 subjects, leading to a prevalence of 0.66%, similar to around 1% reported for hospitalized patients in the medical literature.

In our study population, PM, PT, PP and SE occurred spontaneously, after several days of disease progression, often coinciding with the aggravation of pulmonary lesions, but in the absence of invasive mechanical ventilation or non-invasive positive pressure ventilation. PT was diagnosed the most frequently (in eight cases—72.72%), followed by PM, associated

with SE in all these patients (seven cases—63.63%), while PP was identified only in one subject (9.09%).

In contrast to other studies where lower mortality was reported, in our study population, PT, PM, and PP frequently led to a fatal outcome (72.72%), despite intensive care measures, including ECMO in one case. Additionally, it was observed that the survivors were hospitalized sooner after the onset of symptoms, with an earlier start of specific therapy to avoid the progression of the existing pulmonary lesions and recorded no secondary infections as expressed by low levels of serum procalcitonin. As with many early pandemic reports, Study III is limited due to the small sample size and population distribution.

Data on bacterial or fungal pathogens and their impact on the mortality rates of Western Romanian COVID-19 patients are scarce. As a result, the purpose of the fourth part of this research initiative, was to determine the prevalence of bacterial and fungal co- and superinfections in Western Romanian adults with COVID-19, hospitalized in in-ward settings during the second half of the pandemic, and its distribution according to sociodemographic and clinical conditions. Furthermore, sputum cultures are cost efficient and have allowed us to assess the regional respiratory pathogens associated with COVID-19 pneumonia.

In the framework of Study IV, we found rather high rates of bacterial co-infections and secondary infections (23.3% and 16.95%, respectively) compared to a study conducted by Timpau et al., where the reported figures were significantly lower (1.4% and 6.8%, respectively), but similar to the prevalence rates observed by Langfort et al., Novacescu et al., and Contou et al.

We hypothesize that an imbalance in the respiratory microbiota such as in a SARS-CoV-2 infection can determine commensal organisms to act as pathogenic. ACE2, the receptor for SARS-CoV-2, is an interferon-stimulated gene and thus could be modulated by the respiratory microbiome.

Among the etiologic agents responsible for fungal co-infections and superinfections, *Candida spp*. (53.4%) and *Aspergillus spp*. (41.1%) were the most frequently detected species. Opportunistic invasive fungal disease in the setting of severe respiratory viral illness is not novel, being well described in the context of severe influenza, parainfluenza, and respiratory syncytial virus infections. Although such a high rate of fungal co- and superinfections might come across as a potential overestimation, given the fact that our study population excluded critically ill patients, it can be explained in the context of poor oral hygiene, immunity dysregulation, and viral cytopathic effects on ductal epithelial cells.

Regarding the distribution of antimicrobial resistance among COVID-19 patients, 6.5% of samples and 4.5% of samples in the pathogenic bacteria and commensal human pathogens groups, respectively, were resistant to more than five antimicrobials. This can be explained by the current context in which the combination of the fear of COVID-19 and the lack of adequate knowledge of the utility of antibiotics has a direct impact on over-the-counter access to antibiotics, especially in low- and middle-income countries such as Romania, with weak antibiotic control measures.

Study IV has limitations. By applying a strict definition of bacterial co-infection based on sputum samples taken within 48h of admission, our study deliberately decreased sensitivity for bacterial co-infection overall and excluded other types of pathogens. We eliminated culture results from bacterial species likely to represent cross-contamination. The validity of sputum culture is improved by strict case definition and adequate radiographic review, but in the absence of a gold standard, sputum diagnostics is often underexposed. Finally, COVID-19 vaccination status of the patients was not included in the statistical analysis due to the heterogeneity of the data (low vaccination rate or incomplete vaccination schedule recorded in the region and three available vaccines on the Romanian market).

The impact of COVID-19 on patients in Romania encompasses multiple dimensions, from public health and epidemiology to healthcare system strain, vulnerable populations, variants, socioeconomic consequences, international collaboration, and policy implications. Additionally, emergency scenarios like this pandemic often bring about various medicolegal challenges.

Despite the initial praise of the population towards health professionals in the fight against COVID-19, there were subsequently important medico-legal repercussions also due to diseases not related to the pandemic.

The concept of causality is fundamental in legal medicine and the difficulties encountered when trying to differentiate between deaths *with* COVID-19 and deaths *due to* COVID-19, represent a substantial problem.

The dead speak for themselves, but their language, in particular with regard to the taxonomy of COVID-19, cannot be radically different from that of the living, considering both confirmed and probable cases of COVID-19.

COVID-19, a far more complex multiorgan and heterogenous illness than initially anticipated, has significantly impacted Romania. As such, the identification of high-risk patients and the proper allocation of healthcare resources during the pandemic may be carried out with

the use of laboratory data, which have the potential to be utilized as early indicators to enhance the management of COVID-19 patients.

The primary aim of the current doctoral dissertation is centered on SARS-CoV-2 infection, by focusing on several biomarkers with potential use in postmortem diagnosis of COVID-19, histological aspects and disease complications, especially due to alveolar rupture, co-infections and super-infections.

Overall, by focusing on Western Romanian COVID-19 patients along with possible forensic aspects of SARS-CoV-2 infection, this doctoral thesis provides future researchers and policymakers with a comprehensive understanding of the pandemic's effects within this country. This knowledge is essential for devising targeted interventions, informing policy decisions, and contributing to the global efforts aimed at overcoming the challenges posed by COVID-19.

In conclusion, despite populations historically having suffered a range of pandemics which have depleted them demographically, the epidemiological outbreak of COVID-19 in late 2019 has highlighted that we remain a fragile species, subject to environmental stressors in survival.