

Autopsy Findings and Inflammatory Markers in SARS-CoV-2: A Single-Center Experience

Talida Georgiana Cut¹⁻⁵, Veronica Ciocan^{4,6}, Dorin Novacescu^{3,5}, Adrian Voicu⁷,
Adelina Raluca Marinescu^{1,2}, Voichita Elena Lazureanu^{1,2}, Camelia Oana Muresan^{4,6},
Alexandra Enache^{4,6}, Raluca Dumache^{4,6}

¹Department of Infectious Diseases, Victor Babes University of Medicine and Pharmacy Timisoara, Timisoara, Romania; ²Victor Babes Clinical Hospital of Infectious Diseases and Pneumophtisiology Timisoara, Timisoara, Romania; ³Doctoral School Victor Babes University of Medicine and Pharmacy Timisoara, Timisoara, Romania; ⁴Center for Ethics in Human Genetic Identifications, Victor Babes University of Medicine and Pharmacy Timisoara, Timisoara, Romania; ⁵Academy of Romanian Scientists, Bucharest, Romania; ⁶Department of Forensic Medicine, Bioethics, Deontology and Medical Law, Victor Babes University of Medicine and Pharmacy Timisoara, Timisoara, Romania; ⁷Department of Medical Informatics and Biostatistics, Victor Babes University of Medicine and Pharmacy Timisoara, Timisoara, Romania

Correspondence: Veronica Ciocan, Department of Forensic Medicine, Bioethics, Deontology and Medical Law, Victor Babes University of Medicine and Pharmacy Timisoara, Timisoara, Romania, Tel +40722944453, Email veronica.ciocan@umft.ro

Purpose: The systemic inflammatory response related to COVID-19 can be easily investigated in living patients. Unfortunately, not every biomarker is suitable for postmortem analysis since several factors may interfere. 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 post-mortem assessment.

Patients and Methods: A total of 60 subjects divided in 2 groups were included. All subjects died outside a hospital setting and therefore did not receive specific or symptomatic therapies that could have modulated the inflammatory response. The first group included 45 subjects in which mandatory autopsy was performed in order to establish the cause of death and macroscopic examination of the lungs was highly suggestive of SARS-CoV-2 infection. As controls (Group 2), 20 subjects who died from polytrauma in high velocity car accidents and suicide were selected. Bronchial fluids collected during the autopsy procedure were used for the RT-PCR diagnosis of SARS-CoV-2 and serum samples were sent for analysis of IL-6, CRP, d-Dimers and fibrinogen.

Results: Compared with the control group, the subjects of the COVID-19 group were older (59 ± 19.5 vs 38 ± 19.15 years, $p=0.0002$) and had more underlying comorbidities such as hypertension (60% vs 35%, $p=0.06$) or were overweight (53.3% vs 30%, $p=0.08$). 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 ($p < 0.0001$). Moreover, the level of IL-6 was significantly higher in overweight patients ($r=0.52$, $P < 0.001$). In all COVID-19 subjects, the histological examination revealed features corresponding to the exudative and/or proliferative phases of diffuse alveolar damage. Large pulmonary emboli were observed in 7 cases. Gross cardiac enlargement with left ventricular hypertrophy was observed in 19 cases. The most frequent pathological finding of the central nervous system was acute/early-subacute infarction.

Conclusion: Due to the complexity of the inflammatory response, we postulate that a combination of biomarkers, rather than a single laboratory parameter, might be more effective in obtaining a reliable postmortem COVID-19 diagnosis.

Keywords: COVID-19, postmortem, diffuse alveolar damage, interleukin-6, C reactive protein

Introduction

Since the first reports from Wuhan (China) at the end of 2019, the zoonotic infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has spread rampantly, raising major public health concerns and applying continuous strain on the global medical infrastructure.¹ SARS-CoV-2 is an enveloped, positive-sense RNA single-stranded virus of β -coronavirus genus responsible for a broad spectrum of clinical manifestations ranging from asymptomatic or mild flu-like forms to acute respiratory distress syndrome (ARDS) with multiorgan failure, particularly in

elderly and severe-critically ill patients.²⁻⁵ The systemic inflammatory response to SARS-CoV-2 infection is a hallmark of 2019 coronavirus disease (COVID-19). It is initiated when the viral Spike glycoprotein (S) binds to the Angiotensin-Converting Enzyme 2 (ACE2).⁶ ACE2 is highly expressed on endothelial cells and epithelial cells of the respiratory tract.⁷ The subsequent fusion of the viral envelope with the host cell membrane leads to virus entry, replication and cell lysis which in turn activates biomolecular events such as an increase in production of C-reactive protein (CRP), interleukins 1 β , 6, 10 (IL-1 β , IL-6, IL-10), tumor necrosis factor- α (TNF- α), various glycoproteins and other acute phase reactants such as fibrinogen and procalcitonin.^{8,9}

The corresponding histological findings are represented by diffuse alveolar damage in exudative or proliferative stage, inflammatory cell infiltrates, squamous metaplasia, type 2 pneumocyte activation and fibrin microthrombi formation within pulmonary, cardiac, renal and hepatic circulation.¹⁰⁻¹³

Postmortem examinations are the gold standard for understanding the pathophysiological pathways of diseases and the prime objective of the forensic autopsy is to provide answers about the cause of death.¹⁴ In order to predict the prognosis of COVID-19 in a personalized approach, a set of time and cost-efficient biomarker signatures must be selected. Unfortunately, not every biomarker is suitable for postmortem analysis since several factors such as time elapsed from death, possible supravital reactions, leakage from cell deterioration, blood hemolysis, diffusion dependent on concentration gradients, may interfere with a correct interpretation of the results and for these reasons postmortem biochemical investigations are limited.¹⁵⁻¹⁹

COVID-19 diagnosis may be challenging in the medico-legal setting especially if clinical data or antemortem laboratory results are not available. Moreover, a reliable post-mortem diagnosis is of importance for establishing the medico-legal implications of professional liability when COVID-19 related death is ascribed to malpractice (eg, nosocomial infection, low hygienic standards, violation of the epidemiological regulations).

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 post-mortem assessment. The spectrum of our findings is presented in the context of existing literature.

Materials and Methods

Case Selection

The Romanian Ministry of Health did not recommend autopsies for Covid-19 confirmed deaths mainly because of legitimate concerns about infection risk and limited personal protective equipment (PPE) but according to Article 185 of the Code of Criminal Procedure, mandatory forensic autopsies are performed in all cases of suspicious or violent death, at the request of the prosecutor or criminal investigation bodies.²⁰ A total of 45 COVID-19-positive subjects were included (Group 1); the demographic data is presented in Table 1. All subjects died outside 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 or active pulmonary tuberculosis).

Autopsies and Tissue Processing

Complete diagnostic autopsies were performed at the Institute of Forensic Medicine, Timisoara, Romania in 24 to 200 hours following death (postmortem interval). To have a better examination of the pathological changes, we tried to prevent the process of autolysis which refers to the postmortem autodigestion of tissue by endogenous cytoplasmic enzymes of predominantly lysosomal origin by minimizing the time between autopsy and PCR sampling.²¹ Swabs of the lower respiratory tract (trachea and primary bronchi) were taken during the autopsy. Serum samples were collected and sent for analysis of IL-6, CRP, D-dimers and fibrinogen. The analysis was performed on Multiskan FC Microplate Photometer (ThermoScientific, USA) using the ELISA sandwich (enzyme-linked immunosorbent assay) principle. IL-6 ELISA Human kit (Invitrogen, USA), CRP Human ELISA kit

Table 1 Demographics and Clinical Characteristics of COVID-19 Positive Cases and Control Cases

	COVID-19 Positive Cases (n = 45)	Control Cases (n = 20)	Student's t-Test	Odds Ratio (95% CI)
Female gender	20 (44.4%)	7 (35%)		
Male gender	25 (55.6%)	13 (65%)		
Age (years)	59 ± 19.5	38 ± 19.15	p = 0.0002	
PMI (hours)	48.86 ± 32.48	28.49 ± 8.45	p = 0.007	
Comorbidities				
No comorbidities	13 (28.8%)	12 (60%)	p = 0.005	
Arterial hypertension	27 (60%)	7 (35%)	p = 0.06	2.78 (0.93–8.33)
Overweight	24 (53.3%)	6 (30%)	p = 0.08	2.66 (0.86–8.18)
Diabetes Mellitus	7 (15.55%)	2 (10%)	p = 0.55	1.65 (0.31–8.79)
Solid cancer	4 (8.88%)	1 (5%)	p = 0.59	1.85 (0.19–17.72)
Chronic kidney disease	6 (13.33%)	2 (20%)	p = 0.7	1.38 (0.25–7.54)

Note: PMI-time from death to complete diagnostic autopsy.

(Invitrogen, USA) and D-Dimer Human ELISA Kit (Invitrogen, USA) were used for the analysis. Biosafety Level 3 laboratory practices were adhered to.

Histological samples obtained after the autopsy were fixed in 10% buffered formalin for 48 h. Paraffin-embedded sections of 5- μ m thickness were stained with hematoxylin and eosin.

SARS-CoV-2 RNA Detection

The molecular analysis was performed on an ABI 7500 real-time PCR System (Thermo Fisher, USA), using the genesig[®] Real-Time PCR assay (Primer Design[™], UK). RNA extraction started with the manual pre-processing part obtaining a lysis solution composed of 200 μ L lysis buffer, 20 μ L proteinase K solution and 20 μ L of internal extraction control (IEC). Further step was to incubate the samples in a thermo block Eppendorf Max C (Eppendorf, Germany) at T=56°C for 10 minutes. During this step the cartridges for the automate RNA extraction were prepared.

In order to complete the RNA extraction, the automate system Maxwell 48 RSC System (Promega, USA) with the Maxwell RSC Viral Total Nucleic Acid Purification kit (Promega, USA) kit were used. After RNA extraction, the samples were amplified on a 7500 ABI real-time PCR (Thermo Fischer Scientific, USA) using the genesig SARS-CoV-2 RT-PCR assay (Primer Design, UK). According to the World Health Organization's (WHO) recommendations, 45 amplification cycles were used. Because the system used in this study expresses only the threshold cycle (CT) values (the limit of detection is 100 copies/reaction) and does not provide the quantitative value of the viral load, the samples were labeled as positive when specific SARS-CoV-2 genes were detected.

Statistical Analysis

Normally distributed values were presented as mean \pm standard deviation, a comparative analysis of this data was performed between group 1 and 2 using unpaired 2-sided Student's *t*-test. Categorical variables were described as percentages, and the significance was tested by the chi-square test. The data was analyzed for normality using the Shapiro–Wilk test, and the comparison of the quantitative variables of the two groups was performed using U de Mann–Whitney test. A *p* value of < 0.05 was considered statistically significant. The Odds Ratio (OR) with their confidence intervals 95% (95% CI) were calculated by Fisher's exact test. The correlations between interleukin-6 and other variables were analyzed by the Spearman correlation analysis. All these statistical calculations were performed using MedCalc 20.015 software.

Results

Using RT-PCR, all swabs from Group 1 collected from lower respiratory tract during the autopsies were positive for SARS-CoV-2. The control group was always negative on the swab result.

Among the 45 deceased COVID-19 subjects (group one), 25 (55.60%) patients were male.

In the control group, 7 (35%) subjects were female. Death occurred over almost every decade of life in COVID-19 group: 1 (0–9); 8 (30–39); 7 (40–49); 5 (50–59); 7 (60–69); 8 (70–79); 8 (80–89); 1 (90–99). The time from death to complete diagnostic autopsy, also known as postmortem interval (PMI), was longer in COVID-19 group (48.86 ± 32.48 hours vs 28.49 ± 8.45 hours, $p=0.007$).

Compared with the control group, the subjects of the COVID-19 group were older (59 ± 19.5 vs 38 ± 19.15 years, $p=0.0002$) and had more underlying comorbidities such as hypertension (60% vs 35%, $p=0.06$) or were overweight (53.3% vs 30%, $p=0.08$).

We next compared the levels of CRP, IL-6, fibrinogen and d-Dimers in postmortem plasma samples between COVID-19 group and control group. The values of the above-mentioned parameters were significantly higher in COVID-19 subjects than in control group ($p < 0.0001$) (Figure 1). The Spearman rank correlation between IL-6 and other inflammatory parameters is presented graphically in Figure 2, in the form of scatter-plots with a Local Regression Smoothing (LOESS) trend-lines for each association. 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 ($r=0.119$, $P=0.435$). Moreover, the level of IL-6 was significantly higher in overweight patients (Figure 3, $r=0.52$, $P < 0.001$).

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 (Table 2, Figure 4). Of these, 32 were in acute organizing phase and 13 showed more extensive organization. Hyaline membranes, edema and congestion were present to some extent in all cases. The interstitium contained varying amounts of lymphocytic

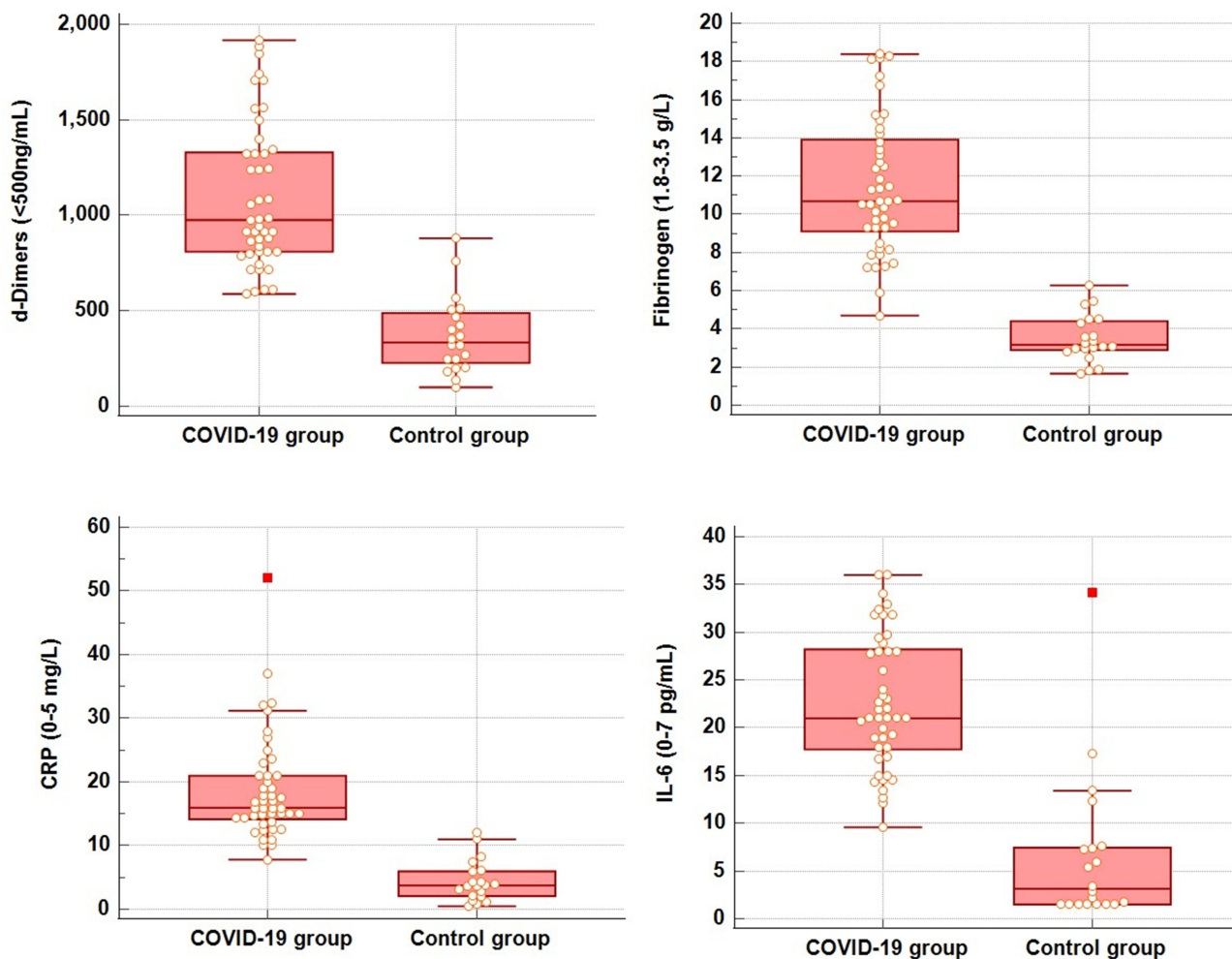


Figure 1 Statistically significant difference in the group of COVID-19 positive cases compared to the control group for the laboratory findings.

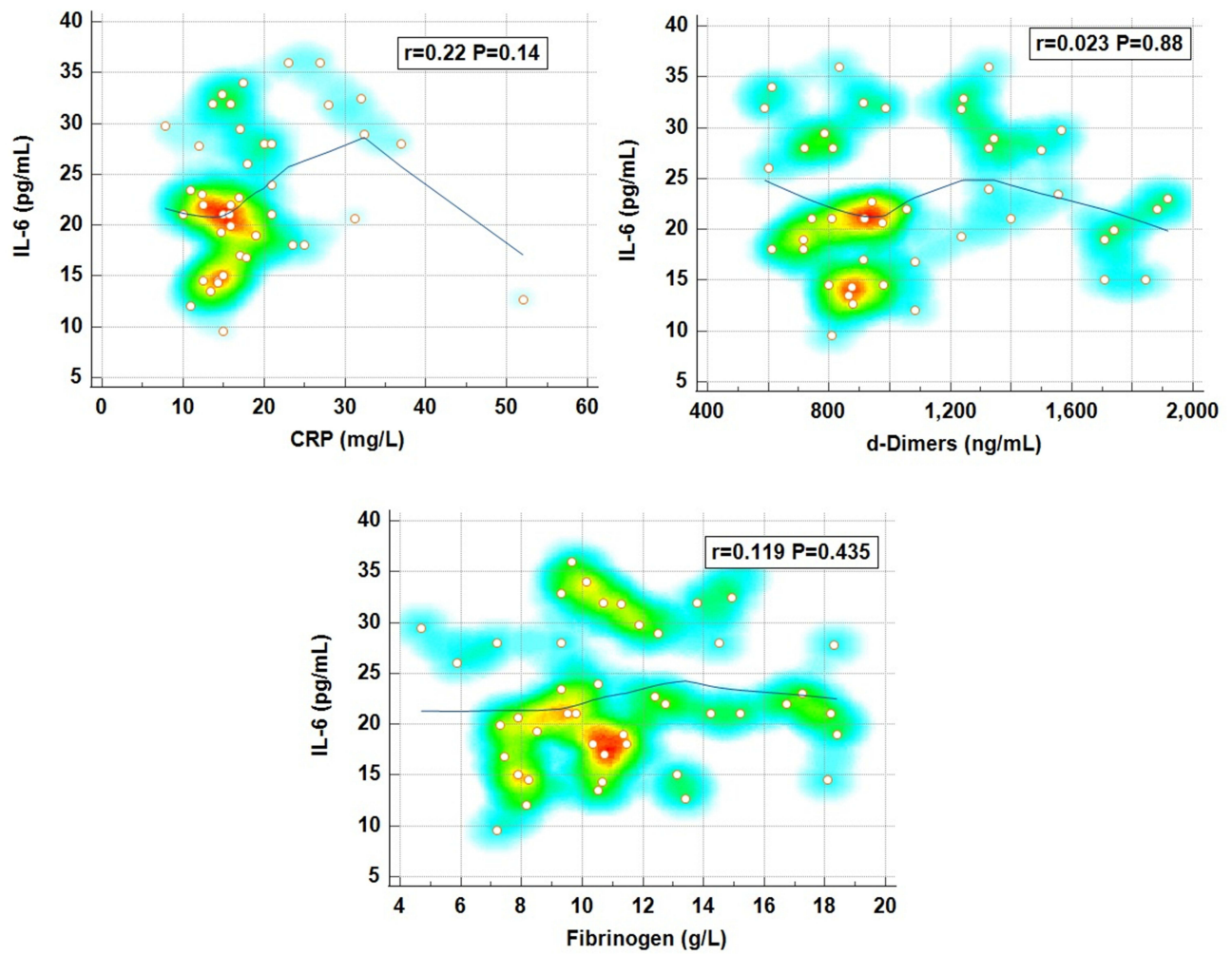


Figure 2 The Spearman rank correlation between IL-6 and other inflammatory parameters.
Note: r=Spearman's rank correlation coefficient.

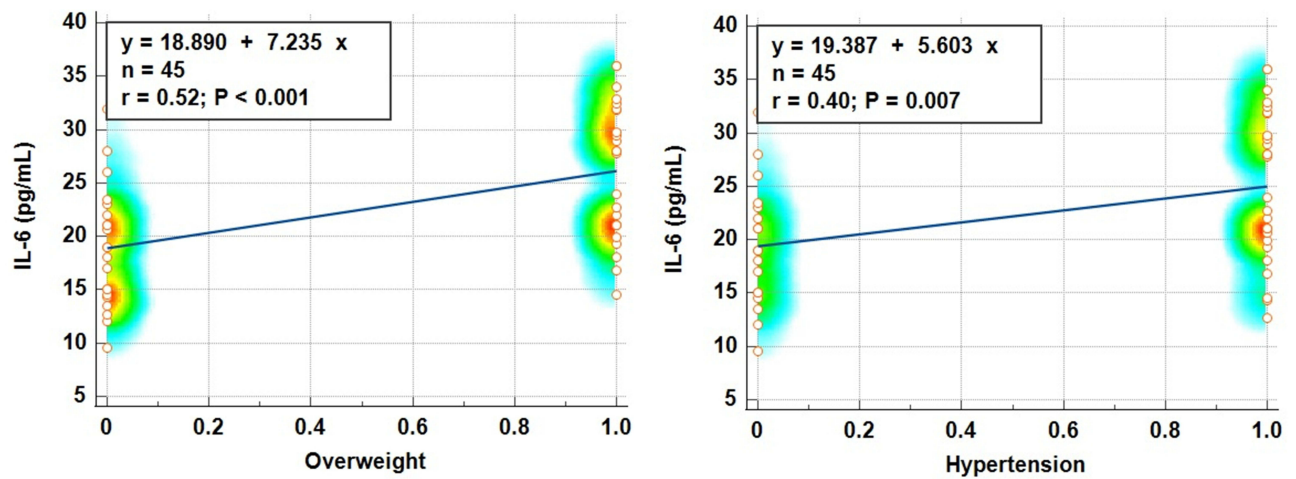


Figure 3 The Spearman rank correlation between IL-6 and comorbidities.

Table 2 Histological Data Summary

Organ	Primary Features	Cases	Precent (%)
Lungs (n=45)	Diffuse alveolar damage	45/45	100%
	Early/acute	32/45	71.1%
	Organizing	11/45	24.44%
	Fibrotic	2/45	4.44%
	Superimposed acute pneumonia	17/45	37.7%
	Septal capillary proliferation	13/45	28.8%
	Pulmonary thromboemboli	7/45	15.5%
Spleen (n=45)	Red pulp necrosis	1/45	2.22%
	Red pulp hemorrhage	4/45	8.88%
	Red pulp congestion	23/45	51.11%
	Absent germinal centers	45/45	100%
	Microthrombi	43/45	95.5%
Heart (n=45)	Cardiac enlargement	19/45	42.22%
	Myocyte hypertrophy and interstitial fibrosis	44/45	97.7%
	Interstitial chronic inflammation	4/45	8.88%
	Bacterial endocarditis	1/45	2.22%
Kidney (n=45)	Hypertensive and diabetic changes	18/45	40%
	Acute tubular injury	8/45	17.77%
	Thromboemboli	7/45	15.55%
Liver (n=45)	Early organizing thrombi	21/45	46.66%
	Venous outflow obstruction	19/45	42.22%
	Fatty liver disease	13/45	28.88%
	Cirrhosis	1/45	2.22%
Brain (n=45)	Acute-subacute infarcts	14/45	31.11%
	Microthrombi	17/45	37.77%
	Lacunar infarcts	3/45	6.66%

inflammation, tended to more diffuse in cases with extensive disease. Histological examination of the main bronchi and bronchiolar branches revealed mild non-specific alterations, focal squamous metaplasia and transmural lymphocytic infiltrates. Intravascular fibrin thrombi were observed in all cases within small arteries or arterioles. Large pulmonary emboli were observed in 7 cases. The examination of the cardiovascular system revealed that 19 hearts had gross cardiac enlargement, with the majority having left ventricular hypertrophy and moderate to marked coronary atherosclerosis (Figures 5 and 6). All but one case revealed varying degrees of interstitial fibrosis, consistent with preexisting atherosclerotic disease. Steatosis was observed in the livers of 13 subjects. This could be explained by concomitant obesity, diabetes mellitus, and/or hyperlipemia. One case showed evidence of chronic liver disease with cirrhosis of undetermined etiology. The most frequent pathological findings of the central nervous system were of acute/early-subacute infarction, observed in 14 of 45 cases, and presented as small and patchy microinfarcts located within the peripheral neocortex and the deeper gray matter structures. Widespread microthrombi were noted in 17 of 45 cases.

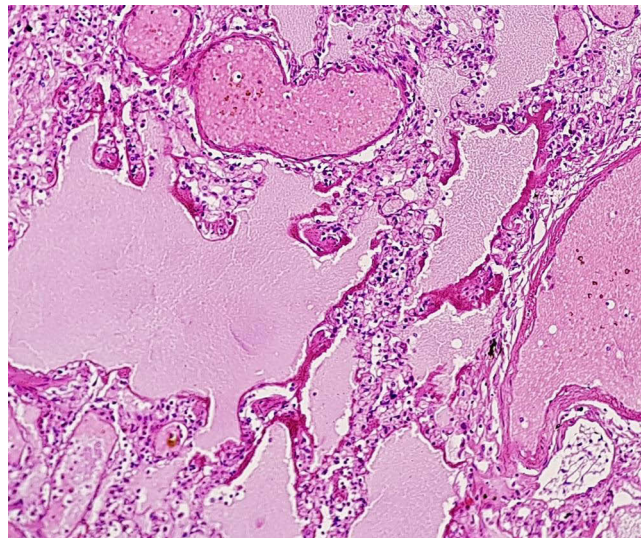


Figure 4 Lung tissue with edema, DAD with hyaline membranes and microvascular thrombi (H&E,x40).

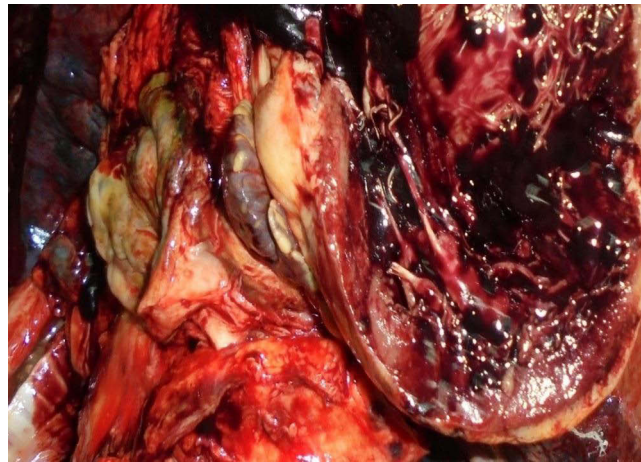


Figure 5 Macroscopic examination of heart – multiple white nodular areas highly suggestive of microinfarcts; postmortem clots intermingled with chordae tendineae.

Histological analysis of the kidneys reported acute tubular injury (ATI) in 8 cases. ATI consisted in loss of brush border, vacuolar degeneration, dilatation of the tubular lumen with cellular debris, and detachment of epithelium from the tubular basement membrane. COVID-19-related findings in the gastrointestinal and endocrine systems were less striking.

Discussion

COVID-19 primarily affects the respiratory system and early autopsy studies document respiratory failure due to ARDS as predominant cause of death, frequently accompanied by capillary microthrombosis, superimposed bronchopneumonia, pulmonary thromboembolism, and signs of multi-organ failure.²² The worldwide mortality rate for COVID-19 in 2021 was 2% with a 0.06% of severe cases.²³ Romania recorded, in October 2021, 110% more deaths than the average of Octobers in the years preceding the pandemic, namely 2017–2019.²⁴ It is the largest proportion of excess mortality among those during the entire period of the pandemic, from the entire European Union.²⁵ A study by Gebhard et al states that although the overall number of confirmed COVID-19 cases across all age groups is sex balanced in the European countries, the mortality rate is higher in men.²⁶ Similarly, in our study, the male gender is dominant in both of our groups. In a study on 80 cases published by Edler et al the average age was reported to be 79 and 76 in the study of Menter et al^{27,28} In our study, the average age for group I was 59 with the highest number of cases (n=8) reported for the third,

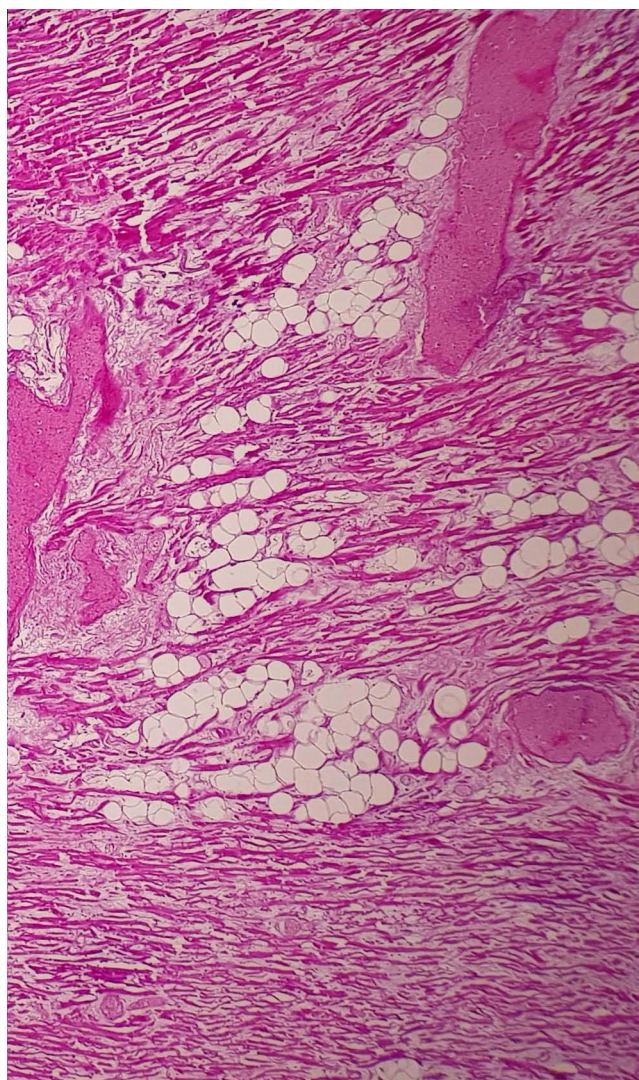


Figure 6 Inflammatory infiltration, necrosis and microvascular thrombi in myocardium (H&E,x40).

seventh and eighth decade. The fact that traumatic deaths accompanied by SARS-CoV-2 positivity were included in the study may be the reason for the lower average age compared to other studies.

The inflammatory response plays a central role in COVID-19 pathogenesis and clinical studies have reported a correlation between elevated serum levels of proinflammatory cytokines, such as $\text{INF-}\gamma$, $\text{TNF-}\alpha$, IL-6 and lung injury and poor prognosis.²⁹ A combination of three fundamental mechanisms has been proposed for the pathogenesis of pulmonary changes induced by COVID-19: severe endothelial injury associated with intracellular SARS-CoV-2 virus and disrupted endothelial cell membranes, vascular thrombosis with microangiopathy and occlusion of alveolar capillaries and new vessel growth via intussusceptive angiogenesis.³⁰ Postmortem specimens of SARS-CoV-2 infected lungs exhibited histological features of DAD with necrosis of the alveolar lining, hyperplasia of type II pneumocytes, intraalveolar fibrin deposition, infiltration of lymphocytes in the perivascular space and interstitial edema.^{1,13,31–34} Our results confirm the presence of these histological findings in all cases examined.

The immunohistochemical evaluation of proinflammatory cytokines performed by Frisoni et al on 60 cases showed a high and wide-spread lung expression of IL-6 in deaths caused by severe COVID-19 pneumonia.²⁹ 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, pro-inflammatory) as well as soluble inhibitors.^{35–37} Furthermore, Bösmüller et al noted in some patients a drop in serum IL-6 levels before death.²²

Tsokos et al engaged in a series of works aimed to assess the value of several molecules as postmortem markers of sepsis and concluded that IL-6 and CRP are good markers for the postmortem diagnosis of sepsis since the antemortem and postmortem levels were highly elevated in all individuals included in the sepsis group.³⁷

Due to its rapid response, short half-life, and high magnitude of increase, CRP is a useful indicator of infections (bacterial, viral, fungal, and mycobacterial), inflammatory diseases, necrosis such as myocardial infarction or pancreatitis, trauma, and malignancy.^{38,39} Deng et al and Shi et al found that higher levels of CRP and IL-6 in SARS-CoV-2 patients were associated with myocardial injury, ARDS development, multiorgan failure and death.^{40,41} In addition, Stassi et al observed a decrease in CRP levels with increasing PMI.¹⁸ Astrup et al found that antemortem CRP levels were higher than postmortem concentrations in septic patients, and likely suggesting molecule proteolysis due to decompositional changes along with irrelevant postmortem molecule release from hepatocytes in the early postmortem period.⁴² Similar to our findings, Maeda et al reported low postmortem CRP levels in infantile and elderly cases of pneumonia.⁴³

A common feature of severe COVID-19 is massive hypercoagulability with prominent elevation of d-Dimers, fibrin and fibrin degradation products.^{44,45} SARS-CoV-2 infection may be a trigger for venous thromboembolism with the involvement of several pathogenetic mechanisms such as endothelial dysfunction, characterized by increased levels of von Willebrand factor; systemic inflammation, and a procoagulant state induced by tissue factor pathway activation.^{46,47}

Zhou et al observed that D-dimer levels above 1000 µg/L associate a fatal outcome.⁴⁸ Our study findings support this contention since d-Dimers and fibrinogen postmortem serum levels were increased in COVID-19 group and in 7 cases pulmonary thromboembolism was present. In one of the first Romanian postmortem reports published by Dumache et al in 2021, it was presented the case of a COVID-19 patient which developed disseminated intravascular coagulation, increased d-Dimer levels and upon autopsy examination thrombi in lung vessels, findings that were also reported by Carsana et al, Edler et al and Remmelink et al^{2,13,27,32} In 30% of the subject from COVID-19 group, endotheliitis with microthrombus formation and involvement of medium-sized vessels leading to cerebral infarcts was prominent. Solomon et al reported histopathological changes in autopsies of 18 patients with COVID-19 with no suggestive changes of acute stroke on gross examination and no findings of thrombosis or vasculitis on histopathological examination, only changes of acute hypoxia in the cerebrum and cerebellum with neuronal loss.⁴⁹ In another series of postmortem analysis by von Weyhern, CNS involvement occurred in form of pan-encephalitis, meningitis and brainstem damage but the authors reported no endotheliitis in the brain.⁵⁰ Overall, the increased incidence of multifocal acute infarcts and numerous microthrombi observed in our study, could explain some of the clinical neuropsychiatric abnormalities seen in COVID-19 patients, such as mental status change or confusion. The liver damage observed in our patients is most likely a combination of metabolic, hypoxic and cytokine-induced damage, as evidence by centroacinar necrosis.

Limitations

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. This study 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-Dimers 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.

Conclusion

COVID-19 is a far more complex multiorgan and heterogenous illness than initially anticipated and further autopsy studies are needed to expand this evidence. Unfortunately, postmortem biochemical investigations are limited to few markers that are relatively stable in peripheral blood. Although CRP, IL-6, d-Dimers and fibrinogen measured from postmortem samples exhibited higher concentrations in COVID-19 group we consider that it is not advisable to reach the diagnosis of COVID-19-related death based on laboratory investigations only, especially when biochemical analyses are limited to a single parameter.

Continued autopsy studies correlated with immunostaining and electron microscopy are needed further in order to truly establish the role of these biomarkers in forensic settings.

Ethics and Consent Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Victor Babes University of Medicine and Pharmacy Timisoara, Nr. 29/15.06.2021. Written informed consent has been obtained from the next of kin to publish this paper.

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Disclosure

The authors report no conflicts of interest in this work.

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