Histopathological findings in the advanced natural evolution of the SARS-CoV-2 infection

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Abstract
We are reporting a case of natural evolution and pathological data from a young person that was diagnosed with coronavirus disease 2019 (COVID-19). All data has been collected from the autopsy of a 30-year-old female, which was performed by the Department of Forensic Medicine from Emergency County Hospital, Drobeta Turnu Severin, Mehedinţi County, Romania. The infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was confirmed by reverse transcription polymerase chain reaction (RT–PCR) on the lung tissue which was obtained during autopsy. This case provides the opportunity to study the natural evolution of COVID-19 pneumonia in a young person with clinical signs of pneumonia but without associated comorbidities. The patient had not received any treatment. The histopathological examination of the lung revealed a process of productive proliferation, proteinaceous and fibrin-macrophagic interalveolar spaces exudate, and lesions consistent with vasculitis. In the heart, we identified a cardiac thrombus. These changes are likely to suggest an advanced natural evolution of SARS-CoV-2 virus infection.

Keywords: COVID-19 pneumonia, histopathology, advanced natural evolution, SARS-CoV-2 infection.

Introduction
At the end of 2019, in China, Wuhan City, Hubei Province, a new outbreak of respiratory infection is supposedly to have started, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which was named coronavirus disease 2019 or COVID-19 [1].

At the beginning of April 2020, World Health Organization (WHO) reported approximately one million confirmed cases of COVID-19 worldwide, with more than 50 000 deaths [2], and at the end of June, more than 10 million cases were confirmed, with more than half a million deaths worldwide. The rapid spread of the virus, its great contagion and the production of a large number of diseases and deaths, has created a serious situation worldwide, which has never been encountered before and which has worried the whole world [3–6].

The Coronaviridae family is a family of viruses with a linear single-stranded ribonucleic acid (RNA) genome that causes infections in humans and various domestic and wild animals. In humans, it can cause serious illness including Middle East respiratory syndrome (MERS) and SARS. SARS-CoV-2 has as its main mode of transmission, the airway, by inhaling respiratory droplets. Another route of transmission are the hands that come in contact with contaminated surfaces and transmit infection through mucosal surface, such as mouth, nose, and eyes [7].

Most of the SARS-CoV-2 infected patients develop a mild form of disease, while severe forms of disease are being found in elderly patients with associated comorbidities, immunocompromised cases, etc. The group of patients who develop severe forms of disease have a high mortality rate, which requires assisted ventilation. Depending on the degree of testing performed in different countries, the reported mortality is in the range of 0.3–10% [8].

Currently, the histopathological (HP) data collected from autopsies or biopsies is scarce [9]. In Romania, the first patient confirmed with COVID-19 was on February 26, 2020. At the end of April 2020, Romania registered 13 163 positive cases with 780 deaths, and at the end of
June, about 27 000 cases and 1600 deaths were registered.

To the best of our knowledge, this is the first report of complete autopsy with HP data on a Romanian COVID-19 patient and adds to very scant literature on COVID-19 autopsied HP worldwide data in general.

**Case presentation**

We present here the case of a 30-year-old woman (S.V.C.) from the rural area of Mehedinți County, Romania, who (as it results from the police investigation), at the beginning of April (around 06.04.2020) showed mild respiratory symptoms (dry cough, sore throat and retro-sternal pain, loss of appetite). The patient did not consult the family doctor nor did she seek hospital admission as she considered it to be common cold. The symptoms intensified progressively, and starting with 17.04.2020 the patient presented difficulties in breathing, had high grade fever, chest pain, loss of appetite, but again, no medical doctor was consulted. The death occurred at home, on 18.04.2020. In order to establish the diagnosis of death, a forensic expertise was requested, the patient not being known with any previous condition. It is important to mention that the deceased woman was not in isolation at home or institutionalized quarantine, had no history of travel abroad or contact with a confirmed case of SARS-CoV-2. The forensic autopsy was performed after the end of the working schedule in an isolated room, both the coroner and the forensic technician wearing personal protective equipment consisting of: hazmat protective suits, gloves, boots, protective eyewear, face shields.

Following the autopsy, blood was collected to determine blood alcohol levels, biological samples for the HP examination, but also three lung fragments were collected preferentially from the basal portions of the lungs for the diagnosis of SARS-CoV-2. Lung fragments collected postmortem for the diagnosis of SARS-CoV-2 were sent to the Cantacuzino National Military-Medical Institute for Research & Development, Bucharest, Romania. The technique used was reverse transcription polymerase chain reaction (RT–PCR) method, the result being positive for SARS-CoV-2.

This autopsy provided the opportunity to study the natural evolution and histopathology of COVID-19. The organs were examined macroscopically and then the usual pathological techniques were performed. Biological samples were collected from organs for HP examination: lung, heart, liver, kidneys, pancreas. The biological specimens were fixed in 10% neutral buffered formalin for 24 hours at room temperature. After processing and paraffin embedding, tissue blocks were processed for routine sectioning and staining for Hematoxylin–Eosin (HE), Periodic Acid–Schiff (PAS), Masson’s trichrome, Orcein, and Silver impregnation according to producer’s protocols (Bio-Optica, Italy). Moreover, serial sections were also processed for immunohistochemistry. Briefly, deparaffinized and rehydrated sections were processed first for antigen retrieval by microwaving in citrate buffer, pH 6, for 20 minutes, incubated in 1% hydrogen peroxide in distilled water for 30 minutes to block the endogenous peroxidase activity, kept for another 30 minutes in 3% skimmed milk in phosphate-buffered saline (PBS), then incubated with the primary mouse anti-human antibodies, at 4°C, for 18 hours. Next day, the signal was amplified for 30 minutes utilizing an anti-mouse peroxidase polymer-based secondary system (Vector Laboratories, Burlingame, CA, USA), then detected with 3,3’-Diaminobenzidine (DAB) (Vector Laboratories), and the slides were coveredslipped in DPX (Sigma-Aldrich, St. Louis, MO, USA) after a HE staining. Negative controls were obtained by omitting the primary antibodies. The following primary antibodies have been utilized (Table 1):

| Name          | Clone         | Target                  | Dilution |
|---------------|---------------|-------------------------|----------|
| CK AE1/AE3    | AE1/AE3       | Pan-cytokeratin         | 1:100    |
| CK7           | OV-TL 12/30   | CK7                     | 1:50     |
| CD3           | F7.2.38       | T-lymphocytes           | 1:25     |
| CD20          | L26           | B-lymphocytes           | 1:50     |
| CD68          | KP1           | Macrophages             | 1:100    |
| α-SMA         | 1A4           | Smooth muscle actin     | 1:100    |
| CD31          | JC70A         | Endothelial cells       | 1:50     |
| CD34          | QBEnd 10      | Endothelial cells       | 1:50     |
| Collagen IV   | CIV22         | Collagen IV             | 1:50     |

Slides were evaluated and imaged utilizing a Nikon 90i microscope equipped with a Nikon DS-Ri2 16 MP complementary metal oxide semiconductor (CMOS) camera and the Nikon NIS Elements AR software. In order to evaluate and quantify the reticulin versus mature collagen stroma on silver stained slides, we have utilized a Nuance FX multispectral camera and the Nuance analysis software (Perkin Elmer, Hopkinton, MA, USA). After building a spectral library from areas with individual reticulin or collagen stroma, on a control lung tissue, we were able to efficiently unmix and characterize the individual contribution of each stromal component as signal areas. A total of 10 random images (with 20× objective) were utilized to this extent from the pathological lung specimen, and from the control lung slide. An analysis of variance (ANOVA) test was utilized to compare the mean areas of collagen and reticulin stained areas between the pathological specimen and the control case, and $p<0.05$ was deemed significant.

At the external examination of the body, no traumatic skin lesions were found on the head, trunk and limbs. We noticed a discrete facial cyanosis that extended to the upper thorax. No other traumatic or medical comorbidity was identified.

Macroscopically, we noticed a bilateral pneumonia, more accentuated in the right middle and lower lobe. On the surface of both lungs, we observed multiple pale purple areas alternating with whitish areas. On palpation, crackles were absent in the right lower lobe and diminished in the right middle lobe and left lower lobe. On the section surface, the right lower lobe appeared dry, red-brown, and consolidated (Figure 1). Under mechanical pressure, the lungs expressed white-yellowish secretions. Other autopsy findings were cardiomegaly with dilation of the right ventricle and blood clots in the heart (Figure 2), moderate hepatomegaly, and small hemorrhagic foci in the kidneys.
diffuse alveolar damage in different phases of evolution, around them with narrowing of the lamina. Despite this, diameter vessels presented with fibroblastic proliferation mononuclear cells in their walls, and overall small fibroblastic proliferation, arterioles showed inflammatory alveolar septae accumulated lymphocytes, plasma cells and cytoplasmic viral inclusions. Besides stasis, engorged by the presence of “mosaic” lesions, power, the histology of the lungs was profoundly affected by the presence of “mosaic” lesions, i.e., the almost total disappearance of airspace. There was a massive interstitial stasis, and the alveolar spaces were replaced by edema and hemorrhage (Figure 3A), with a glycoproteinaceous exudate forming isolated PAS-positive globular condensations (Figure 3B), and a productive-proliferative accumulation of admixed mononuclear, epithelial and fibroblastic cells in most of the alveolar spaces (Figure 3, C–F). Overall, areas with plasma cells, macrophages and fibroblasts predominated (Figure 3C), and seemed to alternate with less dense areas filled mostly with denudated and hypertrophic epithelial-looking cells (Figure 3D) and foamy macrophages (Figure 3E). Even more dense regions were also present with a more homogenous population of whorled fibroblasts–fibrocytes forming round/ovoid structures (Masson-like bodies) (Figure 3F). We did note only on occasion multinucleated syncytial desquamated epithelial-like cells, and there were no visible intranuclear or intracytoplasmic viral inclusions. Besides stasis, engorged alveolar septae accumulated lymphocytes, plasma cells and fibroblastic proliferation, arterioles showed inflammatory mononuclear cells in their walls, and overall small diameter vessels presented with fibroblastic proliferation around them with narrowing of the lamina. Despite this diffuse alveolar damage in different phases of evolution, we did not identify hyaline membrane formation; there was a complete collapse and denudation of the respiratory epithelium in bronchioles and bronchi. Although there was abundant stasis and hemorrhage, there were only rare thromboses, and only in larger caliber vessels (Figure 4A).

We further explored the stromal component of the lung, considering this massive intra-alveolar cellular proliferation. While it was not surprising that elastic fibers were almost completely missing from the remnant interstitial stroma (Figure 4B), a trichrome staining identified almost no collagen fibers in the remaining septae or in the proliferative areas (Figure 4C). A dual collagen–reticulin silver staining was next performed and, surprisingly, revealed a very dense meshwork of reticulin fibers in these areas. Slides were subjected to multispectral unmixing in order to separate and quantify reticulin–collagen ratios, and if we compared the pattern of this lung with a control tissue, this revealed that while in control lungs collagen and reticulin participate almost in the same ratios (non-significant statistical difference), there was a significant disproportionate massive increase in reticulin (p<0.0001) with a small decrease in collagen for the COVID-19 case (Figure 4, D–I).

In the heart, there was recent intracardiac thrombosis (Figure 5A), vascular leukostasis with thrombi formation mainly in the small sub-epicardium vessels (Figure 5B), and a massive interstitial edema that obliterated the intercalated disks in between the myocardial cells (Figure 5C). On occasion, scant mononuclear inflammatory cells were present, as well as petechial hemorrhages (Figure 5D). In the liver, there was a moderate lymph cell infiltrate in portal spaces (Figure 5E), passive hyperemia with an unusual enlargement of the sub-endothelial Disse spaces (Figure 5F). In the pancreas, there was interstitial fibrosis and lipomatosis separating the acinar lobules. Kidneys present dystrophic lesions and focal tubular necrosis at the level of proximal convolute tubules, with an apparent preservation of the distal tubules (Figure 6A), few hematic intratubular cylinders, vascular stasis and widespread focal microthrombosis (Figure 6B). In the renal medulla, stasis and petechial hemorrhages were frequent (Figure 6, C and D).

In the lung, we intended to further characterize the epithelial, stromal and inflammatory changes with the aid of immunohistochemistry. Despite the dense cellular proliferations, alpha-smooth muscle actin (α-SMA)-positive myofibroblasts were present mainly in the thickened alveolar spaces (Figure 7A), and most of the cells proliferating in the alveolar spaces were cluster of differentiation 68 (CD68)-positive macrophages (Figure 7B) and T-lymphocytes (Figure 7C), with only very rare B-lymphocytes (Figure 7D). As already suggested by HE-stained slides, a staining for pan-keratin (CK) AE1/AE3 or the CK7 revealed extensive proliferated, thickened and/or detached epithelial cells through the alveolar septae, even there where alveolar spaces were completely filled with the proliferative cellular areas (Figure 8, A and B). While the overall architecture of blood vessels seems also intact even in these proliferative regions, staining for endothelial cells (CD31, Figure 8C), and basement membranes (collagen IV, Figure 8D) revealed discontinuous and fragmented vascular profiles.
Figure 3 – Histopathology of the lungs showed (A) massive interstitial stasis, edema and hemorrhage, (B) PAS-positive depositions, and (C–F) a productive-proliferative accumulation of mononuclear, epithelial (arrows) and fibroblastic cells (arrow heads). HE staining: (A and F) ×200; (C–E) ×400. PAS staining: (B) ×400. HE: Hematoxylin–Eosin; PAS: Periodic Acid–Schiff.

Figure 4 – Histopathology of the lungs (continued) revealed (A) only rare thromboses, (B) loss of elastic and collagen fibers (C). PAS staining: (A) ×200. Orcein staining: (B) ×200. Masson’s trichrome staining: (C) ×200. PAS: Periodic Acid–Schiff.
Figure 4 – Histopathology of the lungs (continued). However, silver staining for reticulin fibers revealed a massive proliferation of these stromal elements in the parenchyma (D and E), and spectral separation and quantification revealed (F–I) a massive increase in reticulin fibers compared to control tissue and collagen fibers. Silver staining: (D, E, G and H) ×400. In (I), error bars represent standard deviation. ***p<0.001 on ANOVA testing. ANOVA: Analysis of variance; RGB: Red, green, blue; COVID-19: Coronavirus disease 2019; ctrl: Control.

Figure 5 – Histopathology of the heart revealed (A) recent intracardiac thrombosis, (B) thrombi in the small sub-epicardium vessels (C), and (D) a massive interstitial edema that obliterated the intercalated disks between the myocardial cells. The liver showed (E) moderate lymph cell infiltrate in portal spaces, and (F) enlargement (arrows) of the sub-endothelial Disse spaces. HE staining: (A, B, D and E) ×200; (C and F) ×400. HE: Hematoxylin–Eosin.
Figure 6 – Histopathology of the kidney showed (A) dystrophic epithelial lesions and tubular necrosis in proximal convolute tubules with remnant distal tubules (arrows), (B) widespread focal microthrombosis, (C and D) stasis and petechial hemorrhages. HE staining: (A and C) ×200; (B and D) ×400. HE: Hematoxylin–Eosin.

Figure 7 – Immunohistochemistry in the lungs showed (A) α-SMA-positive myofibroblasts were present mainly in the thickened alveolar spaces, with most of the cells proliferating in the alveolar spaces being (B) CD68-positive macrophages, and (C) CD3-positive T-cells, with a only a few (D) CD20-positive B-cells. (A–D) images: ×400. α-SMA: Alpha-smooth muscle actin; CD: Cluster of differentiation.
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**Figures**

8 – Immunohistochemistry in the lungs (continuation) also showed (A and B) extensive proliferated and thickened pneumocytes immunopositive for pan-CK AE1/AE3 and CK7, and apparent intact (C) CD31-positive endothelial cells, and (D) collagen IV-positive basement membranes. (A, B and D) images: ×400; (C) image: ×200. CK: Cytokeratin; CD: Cluster of differentiation.

## Discussions

This study describes the case of a 30-year-old woman who died under suspicious conditions at home, for which a forensic autopsy was performed. Correlating the data presented in the police investigation (young woman, without comorbidities and who did not take medication for the symptoms she presented before death), together with the HP examination, we consider that this case represents an illustrative example of fatal evolution in COVID-19 without interventions, thus presenting a natural course of the disease.

The symptomatology was not specific, family interview revealing both respiratory and non-respiratory features (cardiac and digestive). Other studies recently published pointed out to unspecific symptoms, especially in the first phases of the disease, which can lead to a late correct diagnosis [10, 11].

The main HP features observed in our COVID-19 patient were of diffuse alveolar lesions in the stage of proliferative/fibroblastic organization, but without important stromal proliferation. For instance, we show gross destruction of the lung parenchyma and disappearance of aerated spaces, along with fibrinous protein and macrophage exudate, and fibroblastic proliferation but not in stroma. Similar HP features consistent of severe pneumonia and acute respiratory distress syndrome (ARDS) have also been described in other studies [9, 12]. Notably, in this patient, formation of hyaline membranes was not observed.

Fibroblast proliferation also plays a role in COVID-19 disease progression as in other pneumonia/ARDS. Hsiao *et al.* (2005) conducted a case study and found that lung samples collected from a patient whose disease began 17 days ago showed reactive fibroblast proliferation [13]. In response to lung injury, epidermal growth factor receptor (EGFR) activation occurs as a key signal for excessive proliferation of fibroblasts with a role in wound healing [14].

While we could not examine blood of this patient, patients infected with SARS-CoV-2 show suppression of immunity with a sustained decrease in the number of peripheral lymphocytes, mainly CD4-positive and CD8-positive T-cells [15]. The mechanism is not fully understood, but it appears that SARS-CoV-2 can directly infect T-cells [16]. Secondary bacterial infections, characteristic of patients infected with SARS-CoV-2, could explain lymphopenia. Regardless of the cause, degree of lymphopenia is correlated with the severity of disease and is shown to be a critical factor associated with mortality [17]. Xu *et al.* (2020), in a case report, pointed out that the cytotoxicity of T-cells can explain the severe decrease in immunity [18].

SARS-CoV-2 shares clinical and biological characteristics, but also genome sequences with SARS-CoV [19–22], and the configuration of the structural protein S (spike) is shown to be the same in SARS-CoV and SARS-CoV-2 [23]. Li *et al.* (2020) suggested that SARS-CoV-2 may lead to abnormal coagulation by directly attacking
vascular endothelial cells with the expression of high levels of angiotensin-converting enzyme 2 (ACE2) receptors [16]. Carsana et al. (2020) conducted an extensive study on the lung fragments of 38 deaths with COVID-19 and observed fibrin thrombi in small arterial vessels in 33 deaths; the conclusion of this finding was that COVID-19 may be directly related to thrombosis or may explain severe hypoxemia in ARDS [24].

While the precise cause of death in this patient is unknown, cytokine storm has been linked with mortality in a subgroup of patients of COVID-19. This phenomenon describes an uncontrolled cascade activation of pro-inflammatory cytokines that culminates in multiple organ dysfunction and ultimately death. Lung epithelial cells, macrophages, and dendritic cells express cytokines [25]. In response to SARS-CoV-2 infection, alveolar macrophages and epithelial cells produce proinflammatory cytokines and chemokines, resulting in uncontrolled inflammation. This process is also enhanced by the dramatic decrease in lymphocytes, resulting in a dysfunctional adaptive immune response, which leads to an uncontrolled viral infection with a role in the progression of lung damage [16].

One of the important features observed in this patient was also evidence of failure of coagulation cascade. Interestingly, coagulation cascade may be activated by the cytokine storm [26]. SARS-CoV-2 can lead to venous thromboembolism involving several pathological mechanisms, such as endothelial dysfunction, systemic inflammation and a procoagulant state [27]. A study of 12 confirmed autopsies of SARS-CoV-2 found that seven of 12 patients had deep vein thrombosis (this was found after autopsies because patients were not suspected before death) [28]. In a study of 191 patients, half of those who died had coagulopathy with very high levels of D-dimers [16]. Thrombus formation is reported under hypoxic conditions, and it is known that many patients with SARS-CoV-2 suffer from severe hypoxemia [29]. Zhang et al. (2020) suggested that thrombi can also be formed by indirect causes, respectively by antiphospholipid antibodies which can modulate the immunity in such a way [30]. On the one hand, it is suggested that antiphospholipid antibodies act under several conditions to promote thrombus formation, such as antibodies having various roles, such as in promoting platelet aggregation, binding to platelet membrane proteins, inhibiting fibrinolysis and anticoagulant activity, promoting inflammation; binding to beta 2 glycoprotein I (β2GPI) expressed on the surface of endothelial cells, and causing the cells to turn into a procoagulant and pro-inflammatory phenotype. However, all these conditions are not sufficient to promote thrombosis, and a second condition is required, respectively the endothelial lesion that may be the direct result of the SARS-CoV-2 infection [31].

After analyzing published articles about COVID-19 pneumonia and considering the HP images mentioned above, we believe that SARS-CoV-2 could act as a possible trigger to fulminant systemic autoimmune-like disease with pulmonary predominance. The lesions initiated on the vascular wall by viral particles makes the individual person to elicit a hyperergic response with autoantibody formation (possible antiphospholipidic) and aberrant fibroblastic proliferation with narrowing vascular lumens, thrombosis and leading to a fatal outcome.

In the present study, we have also identified microscopic changes in organs other than lung. Thus, at the level of the myocardium, we have shown thrombosis, microhemorrhages, interstitial edema, and discontinuities at the level of the intercalated disks (Eberth’s lines). Lesions of the intercellular junctions appeared areal, of variate intensity, and might explain the cardiac failure symptoms that can appear in COVID-19 patients – as it is the case here with the enhanced symptomatology that occurred before the death. Other studies have also shown that patients infected with SARS-CoV-2 develop heart pathology that exteriorizes as different clinical signs and symptoms [32–36].

Another severely affected organ in the present case was the kidney, which showed extensive tubular necrosis mainly in the proximal convoluted tubules, along with vascular thrombosis, stasis and petechial hemorrhages. Numerous clinical studies have shown that hospitalized patients infected with SARS-CoV-2 have elevated plasma urea, uric acid or creatinine, proteinuria or hematuria, features of renal impairment [37–39]. About 7–20% of these patients develop acute renal failure [38, 40]. To date, the pathogenic mechanism by which kidney damage occurs in cases of SARS-CoV-2 infection is unknown. According to some authors, the virus can enter the bloodstream from the lungs and from there into the kidneys. In addition, it appears that the virus penetrates easily into kidney cells due to the presence of large amounts of ACE2 receptors in these cells that facilitates SARS-CoV-2 binding to cell surface and is known to be important in causing infection [41]. Other studies state that kidney damage is multifactorial, and hypovolemia, renal hypoperfusion, heart failure, sepsis, massive release of cytokines, and variate nephrotoxins being the main factors involved in kidney failure [42, 43].

The liver lesions in our case were less extensive than those in the lung, heart and kidney. The presence of a moderate inflammatory infiltrate was predominantly composed of lymphocytes in the Kierman spaces, moderate collagen fibrosis in the porto-biliary spaces, and enlargement of the sinusoidal capillaries and of Dise spaces. Some studies have shown that about 2–11% of patients infected with SARS-CoV-2 have liver damage, but elevated serum transaminases occur in about 14–53% of cases [44]. With a more severe SARS-CoV-2 infection, more extensive liver lesions and higher serum transaminase levels have been described [45, 46].

We consider that SARS-CoV-2 infection simultaneously affects many organs, though pathophysiological mechanisms are not yet specified, and depending on the reactivity of each organism, age, presence or absence of comorbidities, lead to milder or more serious forms of disease.

**Conclusions**

SARS-CoV-2 infection is a systemic disease with multiple clinical forms, ranging from an asymptomatic evolution to sever deadly evolution. We emphasize the importance of the autopsy, as HP findings could play an important role in understanding the pathophysiology of the SARS-CoV-2 infection. So far, by extracting the knowledge and data from medical reports, articles and prior research, there has been no pathology data about the
macroscopic and microscopic reports about the natural advanced evolution of COVID-19 disease. The HP analysis, mainly in lung and heart tissues of this patient, may help to understand pathogenesis of COVID-19 illness and maybe to direct future research to study. Lastly, based on these data we support an autoimmune mechanism of the disease with SARS-CoV-2 as a trigger.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper. All authors read and approved the final manuscript.

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