Subtle vascular and astrocytic changes in the brain of coronavirus disease 2019 (COVID-19) patients

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Abstract
Background and purpose: In the central nervous system, a multitude of changes have been described associated with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, such as microglial activation, perivascular lymphocyte cuffing, hypoxic-ischaemic changes, microthrombosis, infarcts or haemorrhages. It was sought here to assess the vascular basement membranes (vBMs) and surrounding perivascular astrocytes for any morphological changes in acute respiratory syndrome (coronavirus disease 2019, COVID-19) patients.

Methods: The light microscopy morphology of the vBMs and perivascular astrocytes from brains of 14 patients with confirmed SARS-CoV-2 infection was analysed and compared to four control patients utilizing fluorescent immunohistochemistry for collagen IV and astrocytes (GFAP), endothelia (CD31), tight junction 1 (TJ1) adhesion protein, as well as the aquaporin 4 (AQP4) water channel. On 2D and 3D deconvoluted images from the cortex and white matter, vessel densities, diameters, degree of gliosis, collagen IV/GFAP and GFAP/AQP4 colocalizations were calculated, as well as the fractal dimension of astrocytes and vBMs viewed in tangential planes.

Results: Fractal dimension analysis of the GFAP-stained astrocytes revealed lower branching complexities and decreased GFAP/collagen IV colocalization for COVID-19 patients. Interestingly, vBMs showed significantly increased irregularities (fractal dimension values) compared to controls. Vessel diameters were increased in COVID-19 cases, especially for the white matter, TJ1 protein decreased its colocalization with the endothelia, and AQP4 reduced its co-expression in astrocytes.

Conclusions: Our data on the irregularity of the basement membranes, loss of endothelial tight junction, reduction of the astrocyte end-feet and decrease of AQP4 suggest subtle morphological changes of the blood–brain barrier in COVID-19 brains that could be linked with indirect inflammatory signalling or hypoxia/hypercapnia.

Keywords
aquaporin 4, astrocytes, brain blood vessels, COVID-19, fractal dimension, vascular basement membranes
INTRODUCTION

In the city of Wuhan in China, in mid-December 2019, a new type of coronavirus infection appeared, which spread rapidly in a large area, and then in all of China and other countries. On 12 March 2020 the World Health Organization (WHO) announced that the infection with this coronavirus had reached the threshold of a pandemic. On 10 January 2020, the genome of the virus, isolated from the lower respiratory tract of an infected patient, was sequenced and confirmed to be a new type of coronavirus, which was named 2 days later by the WHO ‘2019 novel coronavirus (2019-nCoV)’ [1].

Although initially considered a respiratory disease, subsequent data have shown that coronavirus disease 2019 (COVID-19) infection may be affecting other organs including the kidneys, skeletal muscle, heart, brain, liver or the skin. Infection with this new coronavirus may be asymptomatic in some people, but others may develop severe respiratory symptoms that require supportive ventilatory treatment. People with cardiovascular risk factors, pre-existing lung disease or old age are at risk of developing a severe form of the disease [2].

Although the main signs of the infection are respiratory symptoms, fever or muscle pain, it has been observed that almost a third of patients also suffer neurological complications [3, 4], ranging from mild ones such as nausea, vomiting, headache, dizziness or loss of smell/taste to severe ones such as encephalopathy, Guillain–Barré syndrome, stroke or seizures [4, 5]. All of this suggests an important neurotropism of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [6, 7].

The blood vessel walls and the blood–brain barrier (BBB) play a central role in maintaining the integrity of the brain parenchyma, and all the components of the BBB were shown to be affected either directly by neurotropic viruses like SARS-CoV-2 or indirectly through their responses to the systemic inflammatory cascades [8–11]. In SARS-CoV-2 infection, the vascular system is also affected as proved by injuries of the blood vessels’ basement membranes and endothelia, which favour the appearance of thrombi in both large blood vessels and capillaries [12, 13]. Although the pathophysiology, symptoms and management of large vessel thrombo-embolic phenomena are relatively well described (e.g., pulmonary embolism, stroke), the exact subsequent changes in the structure and ultrastructure of the blood vessels as well as their persistence in time and their immediate and long-time physiological effects remain unclear [14, 15].

In the present paper, the aim was to perform a detailed light microscopy morphological evaluation of the vascular endothelium and the perivascular astroglia sheath, in a comparative study on brain fragments collected at necropsy both from patients diagnosed with SARS-CoV-2 and from patients who died of non-neurological causes prior to the onset of the pandemic.

METHODS AND MATERIALS

Patients and specimens

In this study, telencephalon brain tissue specimens (superior frontal lobe) collected during autopsy from 14 patients (male, N = 9; female, N = 5) with SARS-CoV-2 infection, confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) on lung biopsy, and an average age of 64.79 ± 15.03 years (range 39–88 years) were analysed (Table 1). All COVID-19-positive specimens were collected during forensic autopsies performed on COVID-19 cases at the National Institute of Legal Medicine Mina Minovici, Bucharest, Romania, between June 2020 and November 2020. The cases were confirmed to be SARS-CoV-2 positive through a PCR test done during hospitalization and using lung tissue specimens sampled during the forensic autopsy. COVID-19 was the initial cause of death in all cases, and the average admission-to-death time was 6.64 ± 6.16 days (range 0–24 days) (Table 1). Moreover, equivalent brain tissue specimens from four control patients who died from non-central-nervous-system (non-CNS) and non-respiratory-related causes were collected from the neurodegenerative brain bank archive at the Laboratory for Microscopic Morphology and Immunology of the University of Medicine and Pharmacy of Craiova and utilized as control cases for all pathological comparative analysis (average age 67.80 ± 5.5 years, range 64–69 years). Here patients included in our database in 2014 were used, thus clearly excluding the presence of SARS-CoV-2 infection even in the absence of any genetic or immune tissue testing. The study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova (no. 209/08.12.2021), according to the Declaration of Helsinki.

Based on the initial histopathological reports, representative tissue blocks containing both cortex as well as subcortical white matter were selected. Tissue blocks with widespread haemorrhage or liquefactive necrosis were excluded.

Tissue processing and immunohistochemistry

Selected paraffin-embedded archived tissue blocks were sectioned as sections 5 μm thick for pathology reconfirmation and as sections 10 μm thick for further analysis.

In order to assess the presence of SARS-CoV-2-infection-related antigens, 5 μm thick lung and brain tissue fragments were stained with an anti-SARS-CoV-2 spike glycoprotein antibody (Table 2). After deparaffination and antigen retrieval by microwaving in 0.1 M citrate buffer pH 6.0 for 20 min, the slides were kept for a further 30 min in 3% skimmed milk (Bio-Rad Laboratories GmbH, München, Germany) in phosphate-buffered saline for blocking unspecific antigen sites and then incubated with the primary antibody for 18 h at 4°C. The next day, the sections were incubated with a goat anti-rabbit horseradish peroxidase conjugated polymer (Vector Laboratories, Burlingame, CA, USA) for 1 h, and then the signal was detected with 3,3′-diaminobenzidine (DAB) (Vector). Moreover, in order to test for the specificity of the antibody, after dilution optimization, a blocking peptide competition assay was run. Briefly, the primary antibody was preincubated with the SARS-CoV-2 spike glycoprotein complementary peptide for 18 h at 4°C at a mass ratio of 10:1 with that of the antibody, and the next day this mix was further incubated on the slides and detected in the same conditions as the unblocked primary antibody (Table 2). Also, in order to evaluate the presence of the
SARS-CoV-2 spike glycoprotein in the epithelial versus non-epithelial components of the lung parenchyma, an enzymatic double staining was performed by simultaneously incubating a batch of slides with the anti-SARS-CoV-2 antibody (rabbit) and an anti-cytokeratin 7 antibody (CK7) (mouse) (Table 2). After 18 h of incubation the signal was simultaneously amplified with a mix of goat anti-rabbit horseradish peroxidase and goat anti-mouse alkaline phosphatase labelled polymers (Vector), and detections were performed sequentially with Vector Red and DAB. All the slides were counterstained with haematotoxin, and coverslipped with either a xylene-based medium (DPX, Sigma-Aldrich, Hamburg, Germany) for single immunohistochecmistry or with a glycerol-based medium (Dako) for double staining. The same simple staining enzymatic detection protocol was utilized for an anti-glial fibrillary acidic protein (anti-GFAP) staining (rabbit) for all brain sections, and the slides were evaluated for the presence and type of gliotic reactive changes.

Furthermore, 10 μm thick seriate sections were processed for double immunofluorescence for the detection of GFAP/collagen IV, GFAP/aquaporin 4 (AQP4) and CD31/tight junctions (Table 2). The sections were processed for antigen retrieval by microwave in citrate buffer and blocked for 30 min in 3% skimmed milk in phosphate-buffered saline, and then primary pairs of antibodies were added simultaneously on the slides for 18 h at 4°C, as follows (Table 2): (i) GFAP, rabbit, Dako/collagen IV, mouse, Dako; (ii) tight junction protein 1 (TJP1), rabbit, Novus Antibodies/CD31, mouse, Dako; and (iii) GFAP, mouse, Dako/AQP4, rabbit, Santa Cruz. The next day, after thorough washing, the signals were simultaneously detected with a mix of anti-mouse biotinylated (1:300, Dako) and anti-rabbit Alexa Fluor 594 secondary antibodies (1:300, Thermo Fisher Scientific, Waltham, MA, USA), for 2 h at room temperature. The biotin was further amplified and visualized with streptavidin Alexa Fluor 488 (1:300, Thermo Fisher Scientific). In all cases, the slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) for 15 min, incubated for 20 s in the perimeter of the initial ROIs. In these areas comprising the ves-

Image acquisition and statistics

High resolution images were acquired utilizing a Nikon 90i motorized microscope (Nikon Europe BV, Amsterdam, the Netherlands) equipped with plan apochromat high numerical aperture (NA) immersion objectives (40x, NA = 0.95; 60x, NA = 1.27; and 100x, NA = 1.45), a high resolution low noise 16Mp DS-Ri Nikon CMOS camera, a high resolution motorized stage (Prior OptiScan ES111, Prior Scientific, Cambridge, UK), a Prior LED fluorescence source, and highly selective custom-made filters to eliminate the cross-talk of the fluorophores and to ensure a reliable quantification for DAPI, Alexa 488, Alexa 555 and Alexa 594 spectra (Chroma Technology Corp., Bellows Falls, VT, USA). Image grabbing and analysis were based on the Nikon NIS-Elements Advanced Research and Image ProPlus AMS (Media Cybernetics, Bethesda, MD, USA) image analysis packages running on a dedicated Dell Precision Tower 7910 graphic station (2x Intel Xeon 8-Cores E5-2630 v3 2.40GHz, 64GB DDR4, nVidia Quadro M4000 8GB GDDR5).

Moreover, in order to qualitatively evaluate the presence of the SARS-CoV-2 spike glycoprotein in the lung samples double stained with CK7, light microscopy images were acquired on our microscope through a Nuance FX multispectral camera and with the aid of the Nuance analysis software (Perkin Elmer, Hopkinton, MA, USA). After building a spectral library from individual slides stained with haematoxylin, DAB or Vector Red, it was possible to efficiently unmix, separate and colocalize SARS-CoV-2 spike glycoprotein and CK7 expressions. Also, the same methodology was utilized to separate the spectral signature of red blood cells from endothelia (CD31) on immunofluorescence detection.

Further, for GFAP/collagen IV, tight junction/CD31 and GFAP/AQP4 analysis, 10 images were randomly captured utilizing the 40x objective for the white and grey matter for each of the patients included in the study but did not consider areas with large penetrating arterioles surrounded by meninges. Images were obtained by sequential scanning of the DAPI, Alexa 488, Alexa 555 and Alexa 594 filters, with constant exposure times for each channel through the analysis, and saved as four layers of proprietary Nikon .nd2 files. All images were first subjected to a five-pass blind deconvolution algorithm based on a multi-pass adaptive point spread function subtraction of diffracted light (NIS-Elements) in order to remove out of focus signal on all channels. Next, the Alexa 555 channel layer was subtracted from both the green and red channel layers in order to remove any autofluorescence signals (lipofuscin remnants and autofluorescent elastic fibres for example) as much as possible. For the analysis of vascular GFAP coverage, vascular profiles were drawn by hand based on the collagen IV staining utilizing a graphic tablet and a stylus and defined as regions of interest (ROIs), and then a dilation algorithm was utilized to expand these silhouettes by 5 μm through the perimeter of the initial ROIs. In these areas comprising the vessel silhouettes expanded by 5 μm in all radii, the GFAP/collagen IV colocalization was assessed as the overlapping coefficient R of the two colours, a method where correlations are returned for each pixel, without performing any pixel averaging functions and thus not being sensitive to intensity variations in the image analysis (Image ProPlus). The maximum diameter of each vessel was also manually measured, and the total GFAP area was calculated for each image based on a segmentation profile utilizing a constant histogram-defined threshold. Moreover, in order to assess the subtle morphology of the astrocytes’ arborization, the fractal dimension (FD) of the GFAP-segmented signal was calculated, utilizing the box-counting algorithm, as the slope of the regression line for the log-log plot of the counting box size and the number of counts, in Image ProPlus. For GFAP/AQP4 assessment, overall colocalization between the two channels was been reported as above as the overlapping coefficient R of the two colours.
| Patient no. | Group                  | Age | Gender | Days from admission to death | Lung reactivity for SARS-CoV-2 spike glycoprotein | Brain pathology                                      |
|------------|------------------------|-----|--------|------------------------------|-----------------------------------------------|-----------------------------------------------------|
|            |                        |     |        |                              | Stasis | SMC proliferation | Hypoxic neurons | Microinfarcts | Haemosiderin deposition | Microthrombosis | Petechial haemorrhages | Perivascular lymphocyte cuffing | Monocyte/lymph cell interstitial infiltrates |
| 1          | SARS-CoV-2             | 43  | M      | 3                            | +++    | +                | ++              | -             | -                  | +++             | +                  | ++                       | +               |
| 2          | SARS-CoV-2             | 39  | M      | 4                            | ++     | -                | +               | -             | -                  | +                | +                  | -                       | -               |
| 3          | SARS-CoV-2             | 77  | M      | 4                            | +      | ++               | -               | -             | -                  | ++               | -                  | +                       | -               |
| 4          | SARS-CoV-2             | 58  | M      | 4                            | ++     | +                | +++             | ++           | +                  | + (perivascular) | +++               | +                       | ++              |
| 5          | SARS-CoV-2             | 58  | F      | 6                            | +      | -                | +               | -             | -                  | +                | +                  | -                       | +               |
| 6          | SARS-CoV-2             | 71  | F      | 6                            | +      | +                | +               | -             | -                  | +                | +                  | ++                      | -               |
| 7          | SARS-CoV-2             | 57  | M      | 13                           | ++     | +++              | -               | +            | -                  | + (perivascular) | +++               | +                       | -               |
| 8          | SARS-CoV-2             | 75  | F      | 9                            | ++     | -                | ++              | -             | -                  | ++               | +                  | -                       | -               |
| 9          | SARS-CoV-2             | 87  | M      | 1                            | +      | -                | +               | -             | -                  | +                | -                  | +                       | +               |
| 10         | SARS-CoV-2             | 70  | F      | 24                           | +      | +                | +               | -             | -                  | +                | +                  | +                       | +               |
| 11         | SARS-CoV-2             | 52  | M      | 5                            | +      | -                | +               | -             | -                  | +                | +                  | -                       | -               |
| 12         | SARS-CoV-2             | 74  | M      | 0                            | +      | +++              | +               | +             | +                  | +                | ++                 | +                       | ++              |
| 13         | SARS-CoV-2             | 58  | M      | 11                           | +      | +                | +               | -             | -                  | +                | +                  | -                       | -               |
| 14         | SARS-CoV-2             | 88  | F      | 3                            | +++    | ++               | -               | -             | ++                 | -                | +                  | -                       | +               |
| 15         | Control (lung tumour)  | 64  | M      | -                            | -      | -                | -               | -             | -                  | -                | -                  | -                       | -               |
| 16         | Control (digestive tumour) | 69  | F      | -                            | -      | -                | -               | -             | -                  | -                | -                  | -                       | +               |
| 17         | Control (digestive tumour) | 75  | M      | NA                           | +      | -                | +               | -             | -                  | -                | -                  | -                       | -               |
| 18         | Control (digestive tumour) | 63  | M      | -                            | -      | -                | -               | -             | -                  | -                | -                  | -                       | +               |

Note: Graded histopathological features were scored as absent (-), present only on occasion (+), on less than 1/10 of the tissue (++) and on more than half of the tissue area (+++).

Abbreviations: NA, not available; SMC, smooth muscle cell.
In order to assess the fine irregularities of the blood vessel basement membranes, collagen IV and captured z-stacks were followed with the 100× objective and a step of 0.1 μm between optical planes, for vessels visualized in longitudinal section and for which one of the optical planes would run tangentially to the basement membrane. A five-iterations 3D blind deconvolution was applied on the entire stack (Nikon NIS-Elements), and the image comprising most of the basement membrane’s surface was chosen. In these selected images, the collagen IV ROIs were segmented based again on a constant thresholding, and the asperities of the basement membranes were finally evaluated as the FD of the collagen IV ROIs. Moreover, the total area of basement membrane irregularities was calculated and normalized for the total area of that respective blood vessel. At least 10 z-stacks were processed for each patient extracting the most focused tangential plane for further analysis. As the interest was in describing morphological changes of the basement membranes at the level of the capillaries, where the BBB is defined, clear-cut arterioles with thicker vessel walls were not intended to be included in this analysis. Another reason for this was the morphological observation that in arterioles the irregularities of the basement membranes are more complex as they also surround the smooth muscle cells, therefore creating an intrinsically higher morphological complexity (Figure S1). Based on previous reports of human brain capillaries exhibiting diameters below 9 μm when measurements were reported for collagen IV staining [17, 18], only vessels with diameters below 9 μm were included in the FD analysis. Also, only irregularities for collagen IV were assessed and not for CD31, as tight packing of red blood cells leaves imprints on the endothelia creating a ‘haustra-like’ pattern, driving again an intrinsically higher morphological complexity (Figure S2).

For the analysis of the morphological expression of tight junctions on endothelial cells, slides co-stained for TJP1 and CD31 were evaluated. Images were acquired with the 100× objective, collecting 10 fields from cortical and white matter regions from each case, and were subjected to the five-pass blind deconvolution algorithm in order to remove the out of focus signal. Vessel silhouettes, as defined by the CD31 staining, were again manually delineated utilizing the freehand lasso tool, and then the dilation algorithm expanded these silhouettes by 5 μm through their perimeters. These ROIs were lastly utilized to calculate the TJP1/CD31 overlapping coefficient R, and also maximum vessel diameters were calculated based on the CD31 staining.

All numerical data were collected and visualized in Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and further analysed using SPSS software (IBM SPSS Statistics, Version 20.0). Data were averaged for each patient, and then for each of the two pathological groups (COVID-19 and control). In order to assess statistical differences, the Student t test was used for comparing the means of two groups, whilst correlations were evaluated using Pearson’s correlation coefficient. Data are reported as mean±standard deviation of the mean (SD). In all cases, p<0.05 was used to indicate statistical significance. Image collages were prepared and annotated utilizing CorelDRAW Graphics Suite 2018 (Corel Corporation, Ottawa, Canada).

**RESULTS**

**General brain histopathology**

First the pathology of selected brain regions was reconfirmed, excluding widespread haemorrhages, necrosis, abscesses or visible oedema flattening the gyri or causing herniation. In most cases, interstitial and perivascular oedema, stasis with or without thrombosis, in both small and large vessels, in both grey and white matter, petechial red blood cell extravasate, discrete lymphocyte accumulations as perivasculary cuffing and perineuronal satellitosis, and on occasion chromatolysis and disorganization of Nissl bodies was noticed (Figure 1). On haematoxylin and eosin alone, only minute gliotic foci, mostly perivascular or sub-leptomeningeal, could be detected. Minimal parenchymal and leptomeningeal haemorrhage was present in one case, and in two other cases the superficial cortex revealed regional sub-leptomeningeal haemorrhage.
VASCULAR BRAIN PATHOLOGY IN COVID-19 PATIENTS

haemorrhages without involving a dissection through the middle or lower cortical layers. Most of these findings have been scored and summarized for all the patients involved in the study (Table 1), showing their preponderance in the COVID-19 patients’ group. No visible viral inclusion bodies could be identified on classic haematoxylin and eosin staining.

Lung and brain SARS-CoV-2 spike glycoprotein immunoeexpression patterns

Briefly, the lung tissue presented productive/proliferative pneumonia with accumulation of admixed mononuclear, epithelial and fibroblastic cells in most of the alveolar spaces and enlarged inter-alveolar septa, collapsed alveolar spaces, and also with interstitial and intra-alveolar oedema, stasis and thrombosis, haemorrhage and denudation of pneumocytes.

Next the presence of the SARS-CoV-2 spike glycoprotein on lung and all brain specimens from COVID-19 patients and control cases was evaluated (only three lung specimens were available here). All lung samples from COVID-19 patients showed positive cells for the spike glycoprotein (Figure S3a,b), and all reactivity was abolished when the antibody had been mixed with the spike glycoprotein peptide, proving that the reactivity was specific for the presence of the viral element. Negative reactivity controls were also obtained by omitting the primary antibodies and again showed no signal. Most of the signal was detected in the cytoplasm of what seemed to be mononuclear cells from within the interstitium (Figure S3c,e), but on

FIGURE 1 A variety of histopathology changes have been identified in the brains of COVID-19 patients: (a) small extravasated groups of red blood cells; (b), (c) microthrombosis; (d), (e) stasis in both white and grey matter; (f) perivascular and (g) perineuronal accumulation of lymphocytes; (h) hypoxic neurons; (i) chromatolysis; (j) microinfarctions; (k) smooth muscle cell proliferation; and (l) occasional leptomeningal haemorrhage. Arrows indicate the respective pathological denominations.
occasion positive plasma cells could also be identified, considering the spoked-wheel arrangement of the chromatin and the eccentric nuclei (Figure S3f,h). Besides interstitial cells, expression could also be identified in epithelial cells, based on the colocalization with CK7, and most of the time in non-squamous pneumocyte-II-like cells (Figure S3i–k).

Gliosis and astrocyte end-feet coverage of the basement membranes

On simple enzymatic immunohistochemistry, gliotic changes in the brains of both COVID-19 patients and controls were assessed (Figure S4). In both groups, in the grey matter sub-pial astrocytes with elongated processes running downward through the middle cortical layers were identified and foci of mainly protoplasmic astrocytes grouped around blood vessels. On occasion, and especially for COVID-19 brains, the perivascular groups of cells would show a moderate astrogliosis, with increased GFAP reactivity. In white matter, fibrous astrocytes were present with a relative monomorphic GFAP reactivity, revealing an extensive network of processes, and, in both controls and COVID-19 patients, groups of activated-like astrocytes with hypertrophic cell bodies, eccentric nuclei but relatively preserved cellular extension patterns could be identified on occasion. No gemistocytic foci and no clasmatodendrocytes could be identified in any of the cases.

Our next target was to evaluate the density and shape of astrocytes, as one of the first responses of the brain towards any insult. Random image grabbing identified areas with minimal to moderate gliosis in both COVID-19-positive cases and control age-matched patients, in both the grey and white matter, and subjective operator assessment could not identify any clear-cut differences between anatomical regions or the two pathological instances (Figure 2). The overall expression area of GFAP was thus quantified, seeking any subtle differences between the pathological groups and respectively the cortex and white matter regions (Figure 3a). Our analysis showed a tendency for lower GFAP expression in COVID-19 cases (4669.79 ± 2927.81 μm²/40× area for cortical regions and...
VASCULAR BRAIN PATHOLOGY IN COVID-19 PATIENTS

6604.07 ± 4253.45 μm²/40× area for subcortical regions) compared to control, for both cortical regions (6060.89 ± 1922.32 μm²/40× area), but the differences did not attain statistical significance for any of the regions (p > 0.05). For both COVID-19 and controls, there was a tendency for lower gliosis in cortical areas and higher GFAP expression in the white matter, but this also did not attain statistical significance.

The complexity of the overall astrocyte arborization was also examined by analysing the FD average values of the GFAP segmented signal (Figure 3b). COVID-19 cases showed significantly lower astrocyte branching compared to controls, for both cortical (1.12 ± 0.02 vs. 1.15 ± 0.02) and subcortical areas (1.14 ± 0.02 vs. 1.17 ± 0.01) (p = 0.0284 and p = 0.0346), altogether showing a reduced GFAP branching complexity as a response to COVID-19 systemic infection. Also, there was a tendency for higher FD values for the white matter astrocytes compared to cortical areas, and although the differences did not attain statistical significance the tendency was preserved for both COVID-19 and control patients.

Since astrocytes represent the main glial barrier around blood vessels, an investigation was next performed of whether there were any direct changes in the vessel numbers and diameters associated with COVID-19 infection (Figure 3c,d). Direct counting revealed no difference regarding the vessel densities, for both cortical and subcortical areas, for the two classes of brain tissue (11.17 ± 3.56 vs. 10.74 ± 2.95 vessels/40× area for cortical regions and 5.56 ± 1.44 vs. 6.43 ± 1.68 vessels/40× area for the white matter; p > 0.05). There were significant differences, however, in the vascular densities between the cortex and white matter, for both COVID-19 and control cases (p < 0.05), thus conserving the same tendency of lower vascular densities for subcortical regions. Measurements of the maximum vessel diameters revealed significantly lower diameters for cortex areas compared to white matter for both COVID-19 (10.38 ± 1.73 μm vs. 14.04 ± 1.65 μm) and control cases (10.21 ± 1.71 μm vs. 11.80 ± 0.46 μm) (p < 0.05) (Figure 3d). When comparing the two pathological states, only for the white matter were there significantly larger blood vessels in COVID-19 patients compared to control cases (p = 0.0211).

Astrocyte end-feet coverage of the vascular basement membrane was evaluated next, by the GFAP/collagen IV colocalization coefficients on 5 μm perivascular ROIs. Overall, there was a clear-cut decrease of astrocytic coverage of the blood vessel walls for COVID-19 cases (48.56 ± 7.54) compared to control patients (60.49 ± 6.06) (p < 0.001) (Figure 3e). Moreover, if the data for cortex and white matter were dissected, the same trend was conserved, and for both areas the differences attained statistical significance (p < 0.05) and showed a constant loss of astrocyte coverage at the level of the BBB for COVID-19 cases (47.18 ± 7.79 and 50.04 ± 7.25) compared to control patients (57.53 ± 6.94 and 63.44 ± 4.16).
Also, between the two histological regions, a conserved tendency of lower GFAP/collagen IV colocalization for the cortices was observed compared to white matter, for both groups of patients, although the differences did not attain statistical significance.

Light microscopy morphology of the basement membranes and endothelia

Following closely the collagen IV staining on 3D montages of tangentially sectioned capillary walls, subtle variations in the irregularities (asperities) of the basement membrane of the blood vessels were observed, an observation more evident especially for longitudinally cut vessels (Figure S5), and it was interesting to see if somehow these small indentations would differ in morphology or distribution between COVID-19 cases and control patients. Thus the FD of collagen IV staining at the level of the basement membrane on tangential optical sections was calculated, extracted from deconvolved z-stacks collected for each patient (Figure 4a,b).

Although the FD of the basement membrane irregularities showed a much larger variability for COVID-19 patients ($1.189 \pm 0.025$), there was still a clear-cut increase in the irregularity of the basement membrane in these patients compared to the control group ($1.164 \pm 0.009$) ($p = 0.016$) (Figure 4c). Moreover, if the area percentages of the vascular lumens covered by these asperities were compared, despite the presence of a high variability, COVID-19
basement membranes (16.16% ± 14.52%) seemed to contain more such irregularities compared to the vessels of control patients (5.84% ± 4.09%), although the difference did not attain statistical significance (p = 0.053) (Figure 4d). Altogether, both measurements confirmed the observation that basement membranes are more irregular and tortuous compared to smoother surfaces for control cases. As only capillaries have been included in this analysis, defined here as having diameters < 9 μm, it was also important to see if our random imaging and vessel selection would be reflected in any differences between the two patient groups (Figure 4e). Thus, it was found that the average capillary diameter was 6.57 ± 0.64 μm for the COVID-19 group, significantly larger than that of the control group (6.00 ± 0.42 μm) (p = 0.029). Also, no correlation was found between the variation of the diameters and the FD values of the respective capillaries, for both COVID-19 (r = 0.066, p = 0.498) and control patients (r = 0.142, p = 0.407) (Figure 4f).

Next, it was of interest to see if there were any detectable changes in the integrity of the endothelium, and given the fact that

**FIGURE 5** Evaluation of tight junction 1 (TJ1) adhesion molecule colocalization degree with the endothelia (CD31). Exemplary double stained and deconvoluted TJ1/CD31 images for the COVID-19 (a)–(c) and control groups (d)–(f). TJ1/CD31 colocalization clearly decreases in COVID-19 cases as an overall evaluation (g) or segmented for white and grey matter regions (h). Whilst there is a weak but significant direct correlation between the colocalization coefficient with the diameter of the blood vessel for control cases (i), this becomes an inverse correlation for COVID-19 patients (j). Significance is shown for Student’s t testing (g), (h) and Pearson (r) score (i), (j). Data are expressed as mean ± SD in (g), (h)
a highly selective permeability is a specific feature of the BBB it was chosen to evaluate the colocalization degree of an endothelial cell marker (CD31) with the tight junction 1 (TJ1) protein (Figure 5). Again, a direct subjective observation could not clearly show any difference of expression between COVID-19 cases and controls (Figure 5a–f). Analysis of CD31/TJ1 overlapping coefficients, however, showed that the co-expression of TJ1 in endothelial cells revealed an overall decrease for COVID-19 patients (79.81 ± 6.48%) compared to controls (85.80 ± 4.69%) (p = 0.0092) (Figure 5g). If the data for cortical/subcortical tissue were stratified, although the same trend was maintained for both areas, only the white matter attained a statistically significant difference (78.40 ± 5.44% vs. 85.91 ± 5.38%, p = 0.0144) (Figure 5h). It is worth mentioning that, in controls, the CD31/TJ1 colocalization degree was almost identical for the cortex and white matter (85.91 ± 5.38 vs. 85.68 ± 4.50), whilst it showed a clear-cut drop for the white matter in COVID-19 cases, although the difference was not statistically significant (81.61 ± 7.66 vs. 78.40 ± 5.44) (p = 0.1709).

Lastly, an investigation was done to see if there was any correlation between the CD31/TJ1 overlapping degree and the diameter of the blood vessels (Figure 5i,j). For the white matter, there were no correlations, for both control (r = 0.184, p = 0.1050) and COVID-19 cases (r = 0.049, p = 0.336). For the control grey matter, however, there was a weak but significant direct correlation (r = 0.262, p = 0.033) (Figure 5i). Interestingly, for the cortex of COVID-19 cases, there was an inverse correlation between the TJ1 expression in the endothelia and the diameter of the respective vessel (r = -0.223, p = 0.029), suggesting that blood vessels with increasing diameters seem to co-expressing TJ1 less in their endothelial cells (Figure 5j), whilst the phenomenon was the inverse for control patients. Altogether, whilst there was a significant difference between the CD31/TJ1 overlapping only for the white matter of the two pathological groups analysed, it was only the cortex that showed an inversion in the correlation between the vascular diameter and the TJ1/CD31 co-expression. Together with the fact that blood vessels were enlarged in the white matter of COVID-19 cases, it is very probable that in fact there are more complex morphological and permeability-related issues affecting the vessels of white matter after a systemic SARS-CoV-2 infection.

**Aquaporin 4/GFAP colocalization in the brains of SARS-CoV-2 and control patients**

Since AQP4 plays a major role for water exchange at the interface of the BBB and is expressed on the astrocyte membrane, mostly at the level of the end-feet around the BBB but also on the membrane of the full extensions and even over the cell bodies, especially in white matter where water is heavily trafficked along the axon bundles, it was also desired to evaluate whether AQP4 expression is changed in the astrocytic sector. Our GFAP/AQP4 colocalization study showed AQP4 being indeed expressed not only in the terminal regions of the astrocyte extensions but also along their full surface, including the cell bodies (Figure 6a–f), in both the COVID-19 group and age-matched controls. Enlarged astrocytes, with blunt extensions and eccentric nuclei with an activated-like profile, were mostly found in the white matter of COVID-19 cases, but also in control cases, and they exhibited AQP4 expression over the cell body surface (Figure 6g–i). When the colocalization degree of AQP4 and GFAP signals was evaluated, an abrupt drop was found for COVID-19 patients (0.251% ± 0.086%) compared to controls (0.438% ± 0.074%, p < 0.001) (Figure 6). Lastly, the data for white (0.280% ± 0.096% vs. 0.470% ± 0.055%, p = 0.001) and grey matter (0.213% ± 0.055% vs. 0.406% ± 0.083%, p < 0.001) was divided, and control patients exhibited higher colocalization levels for both regions compared to COVID-19 cases (Figure 6k).

**DISCUSSION**

Pathology studies on patients with SARS-CoV-2 infection showed a wide spectrum of neuropathological changes of different severity, including acute hypoxic injury and neuronal loss, thrombosis, perivascular lymphocyte accumulation, vasculitis, focal leptomeningeal inflammation, astroglisis, reactive astroglisis and microgliosis, as well as infarctions and haemorrhages [19–21]. There is no doubt that the description of the associated neuropathology will greatly contribute to our understanding of the physiopathology of the disease and its progression, but, due to the fact that most COVID-19 patients have severe respiratory failure, hypoxia and have been hospitalized in intensive care units for different time periods, a thorough interpretation of the neuropathological findings is very challenging [19–21]. Moreover, quantitative RT-PCR with primers directed against the spike protein and nucleocapsid sequences yield very low viral RNA in the brain tissue, at much lower levels than those in the nasal epithelia. In the present study it was not possible to identify the spike SARS-CoV-2 glycoprotein in the brains of our COVID-19 patients by immunohistochemistry, despite robust expression in their lung tissue, suggesting that any morphological changes might have other causes such as indirect inflammatory signalling pathways or the effects of hypoxia/hypercapnia. Other reports on RNA in situ hybridization and immunocytochemistry targeting the same sequences/antigens also failed to detect viral RNA or protein in the brain of these patients, and one conclusion stated that lymphocyte accumulations and activated microglia are more likely a result of systemic inflammation and hypoxic ischaemic conditions rather than direct viral infection into the brain parenchyma [22]. However, in any inflammatory and infectious-associated brain pathology, the blood vessels and the BBB play a central role in isolating or even amplifying the effect of systemic pro-inflammatory mediators that trigger a neuro-inflammatory response, and all these functional alterations are associated to different extents with BBB morphological changes. The BBB is a complex structure formed of endothelial cells, basement membranes, pericytes and astrocytic processes, and represents a clearly demonstrated
target for numerous viruses with tropism for the CNS, such as human immunodeficiency virus 1 [8], influenza [9], rabies [11], Zika [23] and West Nile [24] and in more recent studies showing the capability of SARS-CoV-2 to infect all the elements of the BBB and to cross into the parenchyma [10, 25, 26].

In this view, a decision was made to perform a light microscopy morphological analysis to assess any subtle morphological changes of the main elements that make up the BBB: the endothelia, vascular basement membranes and astrocyte end-feet.

An evaluation was first made of the overall GFAP expression level against age-matched control cases with no CNS pathology. Areas with both low and increased gliosis were identified in all cases, and this was reflected in a high variability of GFAP expression levels, and therefore there was no statistically significant difference between

**FIGURE 6** Aquaporin 4 (AQP4) expression in the GFAP-positive astrocytes decreases for COVID-19 patients. (a)–(c), (a1); (d)–(f), (d1) AQP4 was expressed mostly at the levels of the cell processes, but also on the cell bodies, for both control and COVID-19 cases. (g)–(i), (g1) Occasional activated-like astrocytes with eccentric nuclei and retracted processes also reveal co-staining on the cell body. (j) Overall AQP4-GFAP colocalization drops for COVID-19 cases compared to control patients, and if visualized for grey and white matter (k) the highest difference occurs in the white matter. Significance is shown for Student’s t testing. Data are expressed as mean ± SD.
COVID-19 patients and control cases. However, an interesting observation was that there was a slight tendency for COVID patients to exhibit even a discrete reduction of GFAP levels, for both the cortex and the white matter. Astrocytes are considered the main neuroprotection elements in the majority of infectious diseases, astrocyte ablation intensifying CNS infection. This was shown by animal model studies where astroglialisis reduction facilitated the entrance of Toxoplasma gondii or Staphylococcus aureus into the brain, leading to severe cerebral edema, purulent ventriculitis and vasculitis [27].

In this line, most COVID-19 studies suggested reactive astrogliosis occurring in COVID-19. An overall increase in GFAP expression was found in the white matter of a COVID-19 patient with encephalomyelitis, and in patients with moderate to severe COVID-19 plasma GFAP levels are significantly increased [20, 28]. On a closer look, white matter GFAP levels also seemed to be higher in our patient group, relative to control patients, than those for the cortex, paralleling a difference between the cortex and white matter. On the other hand, whilst all COVID-19 cases showed positive immunostainings for the SARS-CoV-2 spike glycoprotein in their lungs, none had any immunoreactivity in their brain tissue, suggesting that in patients with no overt bleedings and opening of the BBB most of the morphological and functional changes result most probably consecutive to hypercapnia/hypoxia and specific signalling at the level of endothelial cells and parenchyma. In fact, it has been suggested that multiple subcortical haemorrhages would be the causing factor for initial glial reactivity in COVID-19 patients, after magnetic resonance imaging and subsequent pathology analysis [29]. Altogether, again as stated above, no clear-cut connection can be drawn between the histopathology, missing spike viral protein and basement membrane changes, suggesting that any morphological changes might result from a complex interaction between hypercapnia and viral-induced signalling.

The complexity of astrocyte morphology was next evaluated by assessing for the first time in this pathology the FD values based on the GFAP signal. For both cortical and white matter regions, our data showed a significant loss of branching complexity in COVID-19 cases compared to controls, supporting the presence of less branched, activated astrocytes, despite the overall decrease in GFAP expression areas. There are not many studies in the literature addressing the complexity of astrocyte arborization utilizing the FD, but it is well described that differences do exist between protoplasmatic and fibrous astrocytes, and that these cells react to injury by an overall reduction in the branching of their processes [30, 31]. Together with the previous observations of astrocyte activation in the CNS of COVID-19-positive patients, this strengthens the idea that morphology changes might be a consequence of the inflammatory signalling cascade, whilst blunt gliosis would result from a direct opening of the BBB [29].

Moreover, it was important to see whether there were any changes in the way astrocyte end-feet cover the vascular basement membranes, and the colocalization degree of GFAP with collagen IV was calculated in a 5 μm perivascular area. Our data showed a clear-cut, statistically significant drop in astrocyte coverage of the basement membranes for COVID-19 patients, for both the white and grey matter areas. Whilst the GFAP levels themselves did not show significant differences from control cases, and together with the fact that the overall GFAP FD also decreased in COVID-19 patients, these results can suggest altogether a retraction of the astrocyte processes from the immediate adjacent blood vessel wall areas, with decreasing branching complexity. It has been previously shown that viruses with CNS tropism, including SARS-CoV-2, may disrupt the vascular basement membrane integrity and compromise the astrocyte end-feet adhesion and function [32–34]. Our findings reflect a morphological change in astrocyte disposition around the blood vessels, severe enough to be observed by light microscopy, and given the specific essential metabolic functions of the astrocyte end-foot it is important to evaluate this alteration in long surviving patients and whether any cognitive dysfunction is associated in these cases. Although a study on an animal model of SARS-CoV-2 infection showed preserved inter-endothelial tight junctions [32], other publications revealed increased leakage of the endothelium in COVID-19 patients [35, 36] and, in the same line, our study on human COVID-19 cases showed for the first time that the TJ1 protein decreases its colocalization with endothelial cells.

The basement membranes were next considered, as the next element toward the luminal side of the vessels. First the vascular densities were estimated and, as already described in the literature, white matter regions showed lower vascular densities compared to cortices, for both pathological and control cases [37]. Cortical blood vessels are complex and are arranged in four layers, the first cortical vascular layer including the molecular and the external granular layers of the cortex, the second cortical vascular layer overlapping with the superficial zone of the external pyramidal layer, the third cortical vascular layer exhibiting the biggest vascular density and including the deep zone of the external pyramidal layer up to the internal pyramidal layer, and the fourth cortical vascular layer overlapping with the deep cellular layers. Deeper than this, vascular densities begin to decrease through the superficial to deep white matter tracts [38].

Next, the diameters of the blood vessels were analysed at both the cortical and subcortical levels. In the cortex no differences were found between the two groups, but in the white matter areas COVID-19 cases showed significantly larger vessel diameters compared to control cases. Our overall analysis revealed dilated blood vessels for COVID-19 patients for both the capillary and arteriolar/venular sector. Previous morphological analysis has also described enlarged pulmonary vessels in COVID-19 patients [39]. Brain oxygenation and nutrition are both controlled by the basal tonus of the blood vessels, and this in turn is controlled by many mechanisms amongst which are intrinsic innervation from subcortical neurons, extrinsic innervation from the autonomous nervous system and local release of vasoactive substances [40]. Brain blood vessel diameters, blood flow and brain volume have been shown to vary rapidly in hypercapnic/hypocapnic conditions with increased capillary diameters and brain volumes in hypercapnia [41, 42], proving that brain blood vessels, including capillaries, are capable and flexible enough to allow rapid morphological changes in response to stressor...
events. A myriad of complex intricate factors alters smooth muscle tonus and vessel diameter in COVID-19 patients. Direct hypoxae-
mia and cytokine release in the CNS after a systemic immune re-
sponse can already lead to the loss of tonicity in larger vessels with
thick smooth muscle layers [43–48]. Previous studies have shown
that SARS-CoV-2 can directly infect the smooth muscle cells within
the blood vessel wall in different organs including the brain, heart,
liver, kidney and pancreas [49]. As contradictory data exist regarding
the presence of the viral RNA or proteins in the brain of COVID-19
patients, it is conceivable that a direct viral implication can clearly
accentuate the vascular pathology, but this is not a necessary pre-
requisite for the loss of vascular tonus [22, 44]. Furthermore, a loss
of cholinergic neurons, which in fact is frequently found in many
central nervous system pathologies, such as for example Alzheimer’s
disease, can also lead to a long-lasting loss of vascular tonicity in
these patients [50, 51].

Pathogenic viral agents affect the structure and function of the
BBB both through a direct endothelial interaction which triggers the
host immune responses, and by increasing the levels of chemokines
and pro-inflammatory cytokines and cellular adhesion molecules.
A clear-cut interruption of the BBB allows free passage of infected
immune cells and viral particles in the cerebral parenchyma, increas-
ing the levels of inflammatory markers and destructive processes,
but this abrupt phenomenon is not necessarily present in the acute
phase of a systemic infectious disease [29, 52]. In animal models and
SARS-CoV-2 infected cells it has been shown that the basement
membranes of the blood vessels are disrupted by viral particles
crossing the BBB, even without altering the inter-endothelial tight
junctures [32]. Thus the next aim was to evaluate whether any mor-
phological changes of the basement membranes of the CNS blood
vessels could be identified by light microscopy and morphology,
as well as whether the TJ1 inter-cellular adhesion protein is de-
creased in the endothelial cell layer. Thus, observing the vascular
basement membranes as stained for collagen IV, it was noticed that
in COVID-19 cases there seemed to be more irregular silhouettes
of the blood vessels. Again for the first time the FD is utilized as a
measure of signal irregularity and it is applied for tangentially cut
blood vessel walls, and this analysis showed clearly more irregular
basement membranes in the disease group compared to the control
group. Corroborating with ultrastructural changes of the basement
membranes previously described in CNS viral infections, including
SARS-CoV-2, and together with the fact that the spike glycoprotein
could not be identified in the brains of the patients, this is the first
direct morphological proof of basement membrane damage, caused
either by an indirect effect of the virus or by the pro-inflammatory
mediators [32, 33]. Very important are the immediate consequences
of these irregularities of the blood vessel walls to the rheology of
the blood, and it is a proven fact that COVID-19 patients have a higher
risk of thromboses, a risk which further increases after admission to
intensive care units [53]. Besides inflammatory cytokines leading to
increased endothelial adhesivity and direct hypoxic/toxic effects on
the endothelial cells, an abnormal and irregular blood flow is an im-
portant factor for increased chances of thromboses in vessels with
a morphologically intact endothelial cell layer. One of the main con-
sequences of a homogeneous blood flow is the distribution of the
figurate elements inside the vessel, with platelets flowing peripher-
ally and erythrocytes axially. A change of the lateral flow can have
important pathophysiology results, such as increased platelet adhe-
sion and release of pro-aggregating factors from platelet–platelet
collisions, and increased local concentrations of plasma proteins in-
volved in activation and recruitment of platelets [54, 55].

Aquaporin-4 is the most abundant water channel in the brain, and
acts as a passive osmotic-driven bidirectional water gateway, playing
essential roles in mediating water equilibrium and the pathogenesis
of brain oedema [56, 57]. AQP4-formed channels are concentrated
especially in the astrocytes, from the perivascular end-feet to the
whole astrocyte membrane, as well as in the periventricular and sub-
pial glia limitans [58, 59]. Here a colocalization study was performed
to assess the co-expression of AQP4 in GFAP-positive astrocytes
and showed that the expression of the water channel decreases im-
portantly for COVID-19 patients compared to controls, for both grey
and white matter regions. To our knowledge, this is the first report to
address the AQP4 expression in the brains of COVID-19 patients in
the absence of neuromyelitis and suggests further important water
buffering functional changes in the brain tissue of these patients.
Despite the presence of the SARS-CoV-2 spike glycoprotein only in
the lungs of all patients, it cannot be completely ruled out that a
previous existing pathology might have favoured some of the patho-
logical changes that were observed, but this is very unlikely as only
patients with no prominent brain bleeding or notable degenerative/
inflammatory pathologies were included, who came from very di-
verse socio-cultural environments, the cases being collected within
the largest legal medicine facility in Romania. Altogether, this sug-
gests that morphological and AQP4 expression changes are most
probably related to secondary inflammatory mediators, hypoxia/
hypercapnia and functional perivascular water buffering, and not
secondary to important vasogenic oedema.

The main limitation of this morphological study is represented by
the relatively low number of COVID-19 patients; however, these pa-
tients were all confirmed by RT-PCR from fresh lung tissue biopsies
as having SARS-CoV-2 infection. Furthermore, cases with blunt in-
traparenchymal haemorrhages and encephalitis were also excluded
in order to minimize the effects of large BBB opening and inflam-
matory infiltrates. Although the SARS-CoV-2 spike glycoprotein was
not identified in the brain tissue by immunohistochemistry, other
tests could not be performed to clearly rule out the presence of viral
RNA. Also, only postmortem formalin-fixed, paraffin-embedded tis-
sue was available without electron microscopy data, so basement
membrane thickness and structure at the level of the capillaries
could not be expanded on and clearly resolved.

CONCLUSIONS

Altogether, in this study, for the first time FD analysis has been uti-
lized to show that astrocytes decrease in complexity and reduce
their coverage of the blood vessel walls in COVID-19 patients, and the blood vessel basement membranes are more irregular in these patients, suggesting subtle but important alteration of the BBB that might greatly increase the aggregability and ischaemia/hypoxia conditions. It has also been shown that AQ4 expression is reduced in the astrocytes of these patients, whilst the SARS-CoV-2 spike glycoprotein could not be identified by immunohistochemistry in their brain tissue. The plethora of non-specific CNS signs and symptoms, like dizziness, headache, seizures, hyposmia, stroke and cognitive impairment, for COVID-19 patients during and after the cessation of the disease, suggests long-lasting functional or even morphological changes, and thus it would be of the utmost importance to assess the BBB structure and function in the following months and years in COVID-19 surviving patients.

AUTHOR CONTRIBUTIONS
Rosu Gabriela Camelia: Investigation (lead), writing—original draft (supporting); funding acquisition (lead); visualization (equal). Mateescu Valentin Octavian: Data curation (lead); investigation (supporting). Simionescu Alexandra: Formal analysis (supporting); validation (supporting). Istrate-Ofiteru Anca-Maria: Data curation (supporting); validation (supporting). Curcă George Cristian: Methodology (equal); writing—review and editing (equal). Pirică Ionica: Validation (lead), writing—original draft (supporting); visualization (equal). Mogoanta Laurentiu: Conceptualization (supporting); writing—review and editing (equal). Mindria Ion: Conceptualization (supporting); formal analysis (supporting). Kumar-Singh Samir: Conceptualization (supporting); writing—review and editing (equal). Hostiuc Sorin: Methodology (equal); writing—original draft (supporting). Pirică Daniel: Conceptualization (lead); methodology (equal); writing—original draft (lead); funding acquisition (supporting); formal analysis (lead); visualization (equal).

ACKNOWLEDGEMENTS
Article publication charges are supported by the University of Medicine and Pharmacy of Craiova, Romania, through project no. 26/531/17 from 31 May 2022.

CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT
All the data that support the findings of this study are available from the corresponding author upon request.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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**How to cite this article:** Rosu GC, Mateescu VO, Simionescu A, et al. Subtle vascular and astrogliotic changes in the brain of coronavirus disease 2019 (COVID-19) patients. *Eur J Neurol.* 2022;29:3676-3692. doi: 10.1111/ene.15545