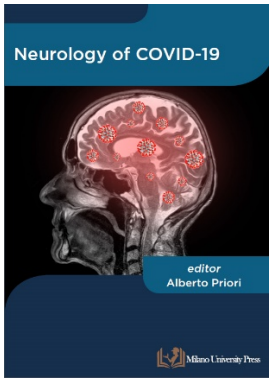

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Chapter 6. **Neuropathology**

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Chapter 6. Neuropathology

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The state of the art

Up to 1st of June 2021 only 150 articles appeared in PubMed's database using the search queries "SARS-CoV-2 Neuropathology" and "COVID-19", an extremely low number compared with the 85,519 articles using a "SARS-CoV-19" entry and the 140,077 with the keyword "COVID-19". Furthermore, the cases studied were extremely limited[1]. There are several reasons for this low number of publications, and the difficulty in performing brain biopsies or autopsy on subjects infected with SARS-CoV-2 or affected by COVID-19 plays an important role. To date, this explains how our knowledge of the neuropathology of this infection is limited and sometimes contradictory. The poor understanding of the neuropathology of this infection and of this disease is of considerable concern considering that neurological complications of COVID-19[2] have frequently been observed, both in the acute and in the long-term phases of the disease. Complications such as autoimmune encephalitis, memory loss, sleep disorders, severe mood disorders, and persistent headache can last for months and significantly affect the rehabilitation period that nowadays potentially involves the millions of COVID-19 patients and are expressed by the more than 120 million subjects infected with SARS-CoV-2[1]. Still today, in the scientific literature the prevailing thoughts are based on the following points: 1) SARS-CoV-2 does not infect the central nervous system (CNS) directly; 2) theoretically, the virus can infect the endothelial cells of the CNS vessels since they express the ACE2 receptor; 3) the detectable damage to the CNS in people with COVID-19 is the result of thrombotic microangiopathies[3] and of the local activation of an inflammatory response supported by cytokines, including IL-2 and IL-12[4-6]. Despite this, recent observations suggest the presence of SARS-CoV-2 in the parenchymal tissue of the brain[7,8] and that suggests how the virus can enter the CNS in different pathways other than through the circulatory system[9,10]. It is possible that the incongruity of the immunohistochemical findings related to the presence of the virus in the CNS depends on different factors such as the viral load at the time of the biopsy / autopsy, the type and clone of

the antibody used for its recognition, and the time elapsed between death and carrying out the autopsy itself[10-12].

Autopsy, biopsy, fixation and gross pathology

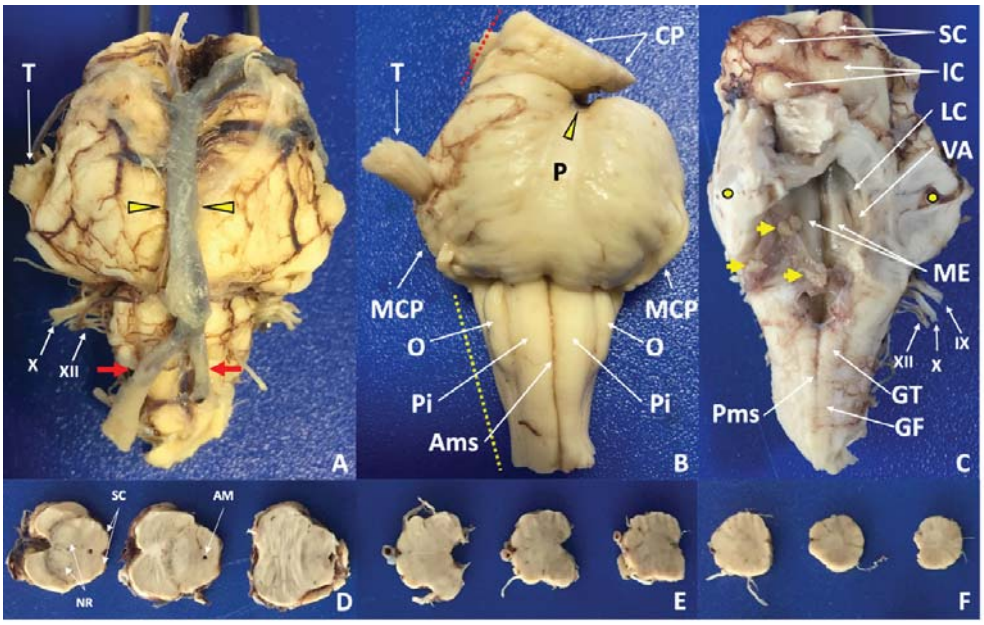
An adequate autopsy evaluation of the CNS must be based both on its rapid evisceration from the body and on its adequate fixation. CNS biopsies also need an appropriate fixation procedure, although they have a simpler protocol of execution. The autopsy must be performed as soon as possible after the patient's death due to the immediate onset of the postmortal involutionary phenomena, which produces tissue alterations that can lead to misinterpretation of the histopathological findings, i.e., by reducing tissue immunoreactivity. In our practice, the evisceration of the brain-brainstem-cerebellum block (B-BS-C block) was performed within three hours after death, through instrumental determination of death by continuous electrocardiographic monitoring showing a flat trace for at least 20 minutes. Autopsy must be performed in a Biosafety Level 3 (BSL 3) autopsy room according to the rules of the Centers for Disease Control and Prevention (CDC) and the staff must be adequately protected[1]. The removal of the skull cap must be carried out with an electric oscillating saw equipped with an aspiration system for the bone dust and for the blood and tissue microparticles that are produced during the cutting procedure. The epidural space, the dura mater, the dural venous sinuses opened *in situ*, the subdural space, the leptomeninges must be examined and their characteristics must be recorded. The brainstem should be at best dissected at the level of the junction with the spinal cord, given the importance of its examination in patients with COVID-19 disease or infected with SARS-CoV-2. This procedure can be easily performed using a thin double-edged scalpel and a gouge. Once the B-BS-C block has been eviscerated, it must be quickly examined also on the lower surface, ensuring that the olfactory and optic nerves have also been removed. Then, it must be weighed. The entire visceral block must be suspended in abundant 10% buffered formalin: that of an adult must be completely immersed in at least 5 liters of formalin, suspending it at the edges of the vessel with a thin cord passing under the basilar artery of the Circle of Willis; the eyelet below the artery where the cord passes must be obtained with a thin scalpel blade while the artery is placed in traction with a small anatomical forceps, ensuring no damage to the underlying Varolius pons[11] (Figure 6.1A). The fixation of the brain suspended in formalin is crucial to avoid any anatomical artifacts to the B-BS-C block structures produced by the pressure against the walls of the container. The macroscopic examination of the inner part of the B-BS-C block performed with parallel serial cuts immediately after its evisceration should be avoided as it irreparably damages the histological details. However, following evisceration, it

is recommended to quickly collect small tissue samples for electron microscopy and for molecular or microbiological /virological investigations. These samples must not compromise the visceral integrity of the block. Moreover, it is important to note the areas from which the samples are taken. It is also important to collect samples of the corresponding contralateral areas in order to correlate the histopathological patterns to the molecular, ultrastructural or microbiological / virological findings when the final sampling for histological examination is carried out. The complete or partial removal of the spinal cord should be performed by removing the vertebral bodies that need to be examined, using an oscillating saw, a Brunetti's chisel and a gouge. The spinal cord is removed inside the dural sac and, once eviscerated, it is stretched and fixed at the margins on a cork dissecting board, with pins set in the dura mater, before immersing it in abundant formalin. The B-BS-C block fixation lasts from 21 to 27 days, depending on its size. The fixation protocol requires the following essential steps: 1) a complete change of formalin on the 2nd, 4th, 6th, 13th and 20th days; 2) a dissection of the block into two parts on the 4th day through a full-thickness cut of the brain. This procedure is performed to facilitate the entry of the formalin into the ventricular cavities and to facilitate the fixation of the deep structures; 3) immersion without suspension of the two parts of the block back in formalin. The first cut must be made according to the chosen section plane (coronal, sagittal or transverse). The subsequent cuts must be performed at the end of the fixation on seriated planes approximately 1 cm apart. On the 21st or 27th day, the B-BS-C block (according to the macroscopic assessment of adequate fixation also of the deepest parenchymal areas) should be macroscopically examined and sampled for histological examination. The serial sections are to be performed with a long Virchow brain sectioning knife. The blade must be wet with water after each cut to ensure it slides continuously in one direction to avoid sawing movements. For convenience, it may be useful to perform a macroscopic examination of the parenchyma on serial macrosections after having separated the brain from the brainstem and the latter from the cerebellum, first dissecting the midbrain at the level of the cerebral peduncles and then the cerebellar peduncles (Figure 6.1B-F).

The fixation procedure of the spinal cord should last from 8 to 10 days, depending on its thickness and length, changing the formalin on the 2nd, 4th, 6th days. Biopsies performed *in vivo* on patients infected with or suspected of infection with the SARS-CoV-2, including those of the CNS, should be fixed for at least 24 hours in formalin before processing them for paraffin embedding[13,14]. It is a good practice to process autopsy samples under vacuum, with three steps in Xylene or equivalent for at least 1 hour each. The procedures we performed have sometimes produced unexpected results when compared with the data published by other research groups. This suggests that, regardless of the other variables already listed

in the previous paragraph, the SARS-CoV-2 virus is very labile after the patient's death, most of all in its identification with immunohistochemical methods.

Figure 6.1: Macroscopic examination of the midbrain and brainstem

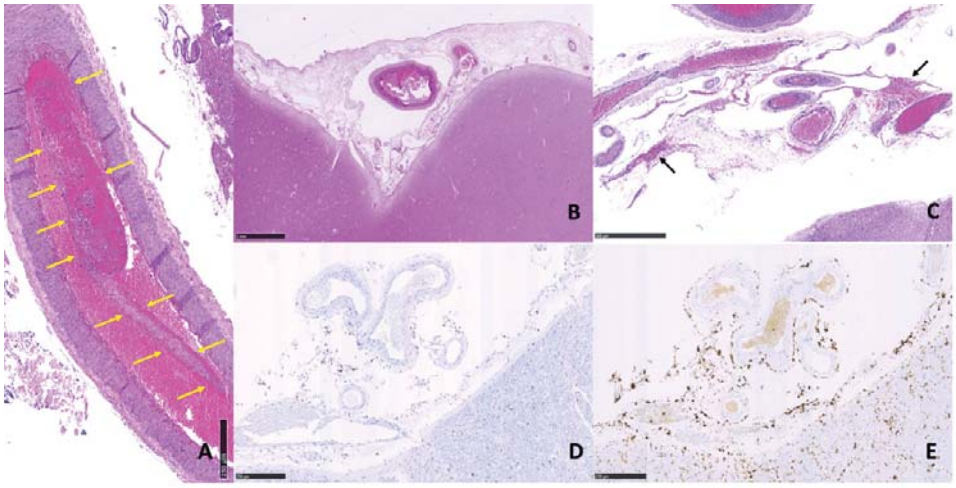


A. Anterior surface with some vessels of the Circle of Willis and the leptomeninges in place. The yellow arrowheads indicate the basilar artery, under which the string must be passed to suspend the brain in formalin after its evisceration, to allow a correct fixation. Red arrows: vertebral arteries. T = trigeminal nerve root (V cranial nerve). X: vagus nerve. XII: hypoglossal nerve. **B.** Midbrain and brainstem (pons and medulla oblongata) after the removal of the vessels of the Circle of Willis and the leptomeninges. Red dotted line = midbrain. CP = cerebral peduncle (lateral rotated in the picture). P = pons; Yellow arrowhead = interpeduncular fossa; MCP = middle cerebellar peduncle; T = trigeminal nerve root (V cranial nerve). Yellow dotted line = medulla oblongata; O = olives; Pi = pyramid; Ams = anterior median sulcus. **C.** Posterior surface of the midbrain and brainstem, after the removal of the cerebellum. The image highlights the anterior wall of the IV ventricle where the medial eminence (ME), the locus coeroules (LC), the underlying vestibular area (VA) and several choroid plexus (yellow arrows) are observed. SC: superior colliculus. IC: inferior colliculus. Yellow dot: inferior cerebellar peduncle. IX: glossopharyngeal nerve. X: vagus nerve. XII: hypoglossal nerve. GT: gracile tubercle. GF: gracile fasciculus. Pms: posterior median sulcus. **D-F.** Multiple midbrain and brainstem sections after section on transverse planes at a distance of approximately 0.5 cm. In D in the leftmost section performed at the level of the midbrain, the substantia nigra is clearly observed. NR: nucleus ruber. SC: superior colliculus. AM: aqueductus mesencephali (Silvius aqueductus).

Damage to the meninges and choroid plexus

The presence of the spike glycoprotein of SARS-CoV-2 has been detected both in the leptomeninges and in the stroma of the choroid plexus in patients affected by COVID-19[15,16]. The lesions described in association with the detection of the virus or in COVID-19 disease are inflammatory or thrombotic. The meningitis sustained by T lymphocytes with increased macrophages and, less frequently, with the detection of vascular thrombosis has been observed in many patients, involving also the vessels of the Circle of Willis[17,18]. Subarachnoid hemorrhages are only occasionally described in the literature, mostly as small or punctate[19,20].

Figure 6.2: Leptomeninges and large intracranial vessels in patients with COVID-19 disease



A. Large arterial vessel of the Circle of Willis subocluded by a thrombus in an acute phase of evolution. The yellow arrows delimit the edges of the thrombus which in the image appears attached to the highest part of the vessel**. B.** Leptomeninges with moderate edema. The arachnoid vessels are not thrombosed. **C.** Leptomeninges with small subarachnoid hemorrhagic extravasations (arrows). Also in this image the arachnoid vessels are not thrombosed. **D.** Leptomeninges characterized by a few positive macrophages (dark brown cells) with the immunohistochemical staining for CD68 PGM1. Macrophages are mostly located perivascularly. Some isolated CD68 PGM1 positive cells can also be observed in the underlying nervous parenchyma (cells most likely referable to microglia). **E**. Same area as the previous image, stained with immunohistochemical reaction for CD163. It can be seen that the number of CD163 + inflammatory cells are significantly higher than CD68 PGM1 + cells, although they largely maintain the same arrangement. CD163 marks the activated macrophages (M2) indicating the presence of a local reactive / inflammatory state.

We have observed recent thrombosis of a vessel of the Circle of Willis in only one patient (Figure 6.2A); leptomeninges mostly showed mild focal edema. We have only occasionally detected small subarachnoid hemorrhagic suffusions

(Figure 6.2C); on the contrary, we have almost always observed an increase in macrophages in the meninges (CD68 PGM1 + cells) and a very large population of M2 macrophages (CD163 + cells). This demonstrates the presence of a leptomeningeal inflammatory state (Figure 6.2D and E).

Macroscopic parenchymal lesions

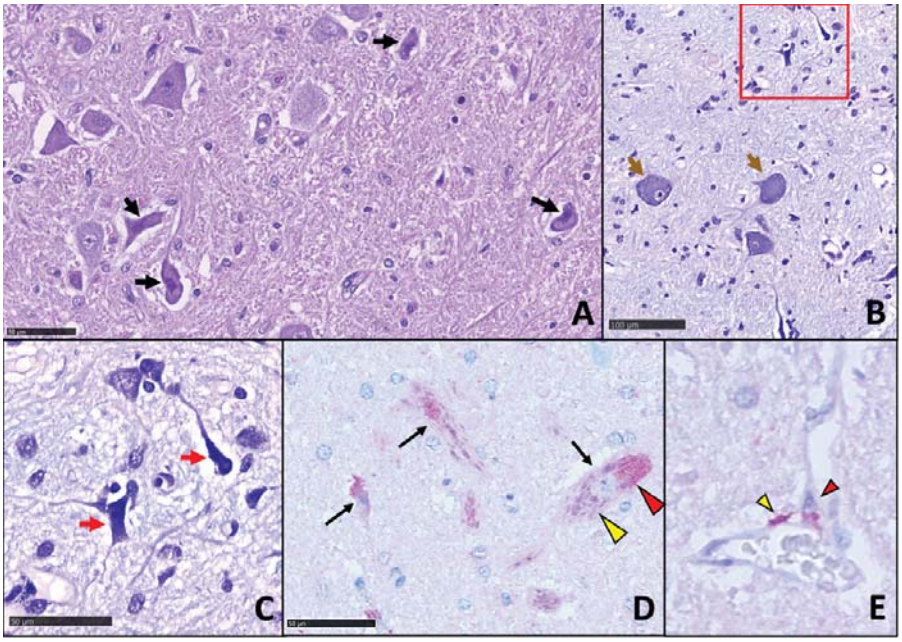
The frequency of macroscopic lesions in the CNS changes significantly in the different case series that have been studied. In almost all cases, these lesions are ischemic or hemorrhagic, and involve also widespread cortical areas. They can affect either the brain, the cerebellum or the brainstem[18]. Cerebral edema is one of the most reported alterations[19,21] but its direct relationship with the viral infection appears difficult to define, given that this disease can be traced back to many other pathogenic causes during an autopsy. There were occasional macroscopic cerebral lesions (a small cerebral infarction occurring a few days before death in a single intubated and mechanically ventilated patient) and non-specific even in our autopsy experience, while diffuse cerebral edema was almost always detected, with from mild to moderate weight gain of the B-BS-C block.

Neuronal, glial and vascular histological damages

Several autopsy or biopsy studies have described alterations in single neurons or glial activation, as well as vascular thrombosis involving both major and minor intracranial vessels. However, the question as to whether the neuronal damage is directly caused by the virus or is the result of hypoxic / ischemic mechanisms or immune-mediated processes remains unanswered[1,22]. The most frequent histological damage described is: 1) sparse neuropil infiltration of inflammatory cells (depending on the case: T lymphocytes, B lymphocytes, microglia, neutrophil granulocytes. T lymphocytes are often arranged in a cap around small vessels, while B lymphocytes prevail within the parenchyma with more frequent distribution in single cells. Neutrophil granulocytes mostly characterize micro-areas of ischemic necrosis); 2) acute hypoxic-ischemic neuronal changes, including perikaryal cytoplasmic eosinophilia and nuclear pyknosis (so-called "red neurons"); 3) microglial activation with microglial nodules, with or without neuronophagia features; 4) focal demyelination[18,23]; 5) localized axonal swellings, demonstrated with amyloid precursor protein (APP) immunostain, indicative of subacute and acute axonal damage[24-26]. In our experience, we have observed neuronal histological alterations, particularly at the level of the brainstem[10]. The altered neurons are distributed quantitatively in different ways at the different sites of the B-BS-C block, resulting very high at the level of the Varolius pons, the medulla oblongata and the basal fronto-temporal areas of the brain, while they progressively decrease proceeding

from basal ganglia / thalamus to the cortex of the latero-superior areas of the brain (GP Bulfamante, unpublished data, 2021).

Figure 6.3: Brainstem neurons in patients with COVID-19 disease; many of them show evident structural alterations



A. The neurons indicated by the arrows show marked regressive morphological alterations both nuclear and cytoplasmic. The nuclei are shrunken with heavily thickened chromatin and often have an angled profile. The cytoplasms are not red (as in the "red cells") but equally have reduced volumes, asymmetric profiles and a hyperchromatic halo at the outer membrane. These structural changes make these neurons clearly different from morphologically normal ones. **B-C.** Neuronal alterations referable to different evolutionary stages, with Nissl staining. In the red box "B" and "C" two neurons are observed with marked compaction of the pyrenephorus, which appears completely dark blue. In image "B" the two brown arrows indicate two neurons with swollen pyrenephorus and peripheral dispersion of Nissl substance. **D.** Neurons positive with immunohistochemical staining for SARS-CoV-2 Nuclear Protein: staining was developed with red color. The arrows identify three neurons infected with the virus; the rightmost cell presents regressive alterations. Immunohistochemical positivity showes itself as small red droplets (red arrowheads) in the cytoplasm; these droplets should not be confused with the irregular granules of intracytoplasmic Nissl substance (yellow arrowhead). Since in damaged neurons the substance of Nissl can undergo modifications (compaction, loss of volume of the single granules) it is not advisable to develop immunohistochemical reactions for the detection of SARS-CoV-2 on the central nervous system with brown tracer. **E.** Immunohistochemical stain for the SARS-CoV-2 Nuclear Protein: the stain was developed with red color. In this image there are two cells with cytoplasmic positivity. The one indicated by the red arrowhead clearly appears to be an endothelial cell. The other (yellow arrowhead) is difficult to attribute: it could be a perivascular cell in the Virchow-Robin space.

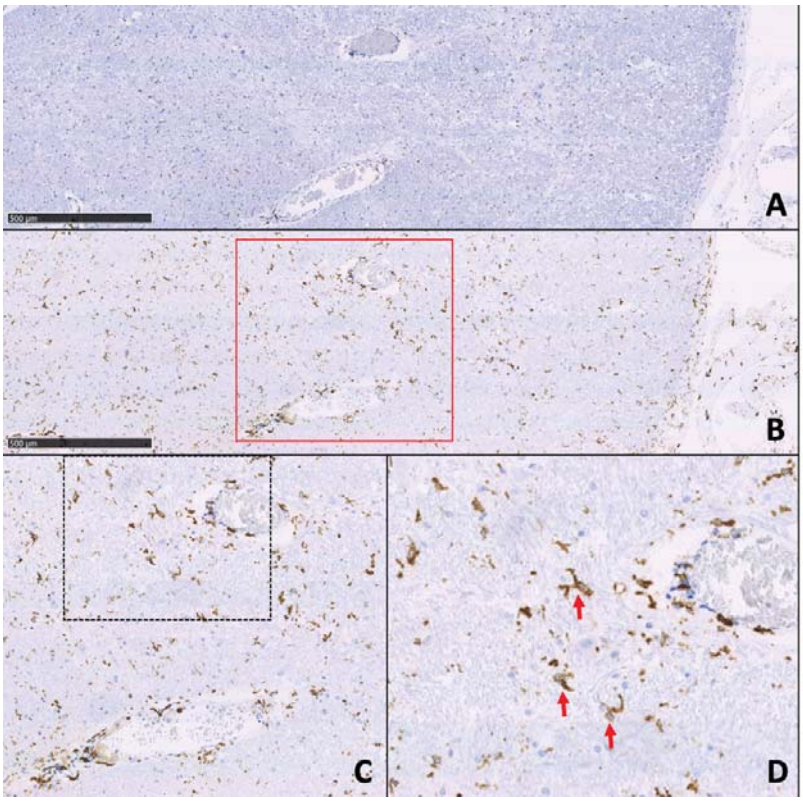
Neuronal alterations may be similar to those described in shrunken cells caused by hypoxia / ischemia, but with no evidence of red staining of the cytoplasm (Figure 6.3A-C). Another type of neuronal damage is represented by the presence of cells with swollen pyrenephorus and peripheral dispersion of Nissl substance (Figure 6.3B). Neurons were found to be infected by SARS-CoV-2 in several areas[10] (Figure 6.3D) and the number of infected neurons changes within the brain following the neuronal alterations listed above (GP Bulfamante, unpublished data, 2021). The virus has also been observed in some endothelial and perivascular cells of the Virchow-Robin space of intraparenchymal vessels (Figure 6.3E).

Our experience of the autopsy histopathological characteristics of the CNS in COVID-19 patients also involved the glia. The most frequent finding that cannot be correlated with any pre-COVID-19 diseases regarded the activation state of this cellular compartment. Immunohistochemical staining for CD68 PGM1 highlighted the constant increase of intraparenchymal cells, identifiable as microglia cells: these cells are almost always distributed as single cells, small, with no enlarged and vacuolated cytoplasm, a feature that makes them easily identifiable when they intervene in an area of infarction. Some of these cells are observed on the contour of the intraparenchymal blood vessels (Figure 6.4A). Microglia represents the main innate immune system within the central nervous system and plays a fundamental role during inflammatory processes, traumatic events, and in the pathogenesis of neurodegenerative disorders or in case of neoplasms[27]. This glial population has different embryological origins compared to monocytes / macrophages, which originate from the hematopoietic system, even if it shares with them some surface antigens and some functions, such as phagocytosis and modulation of the inflammatory process[27,28]. These cells do not only respond to a noxious stimulus or a pathological condition; they are also involved in the regulation of neuronal development during the embryonic and fetal period, and in the regulation of its homeostasis in adult life[29,30]. CD163 immunohistochemical staining also demonstrates the presence of a much larger cell population than CD68 PGM1 positive cells and most CD163 positive cells do not co-express CD68 PGM1 (GP Bulfamante, unpublished data, 2021). (Figure 6.4B-D).

Similarly to monocytes/macrophages, two activation states have also been recognized for microglia: the "classic" state, M1-like or pro-inflammatory, and the "alternative" state, M2-like or anti-inflammatory / protective marked by immunohistochemical positivity for CD163 protein. These two different microglial activation states are expressed in different pathophysiological conditions. They are characterized by cytokine secretion that arranges the complex immune response in the tissue microenvironment. The ability of the microglia to regulate and modulate its phagocytic capacities in response to an external stimulus is of particular importance in this context; the precise regulation of the microglial

activation state, therefore, ensures the control of adverse events that would lead to irreversible and sometimes fatal tissue damage[28].

Figure 6.4: Glial population of the brainstem

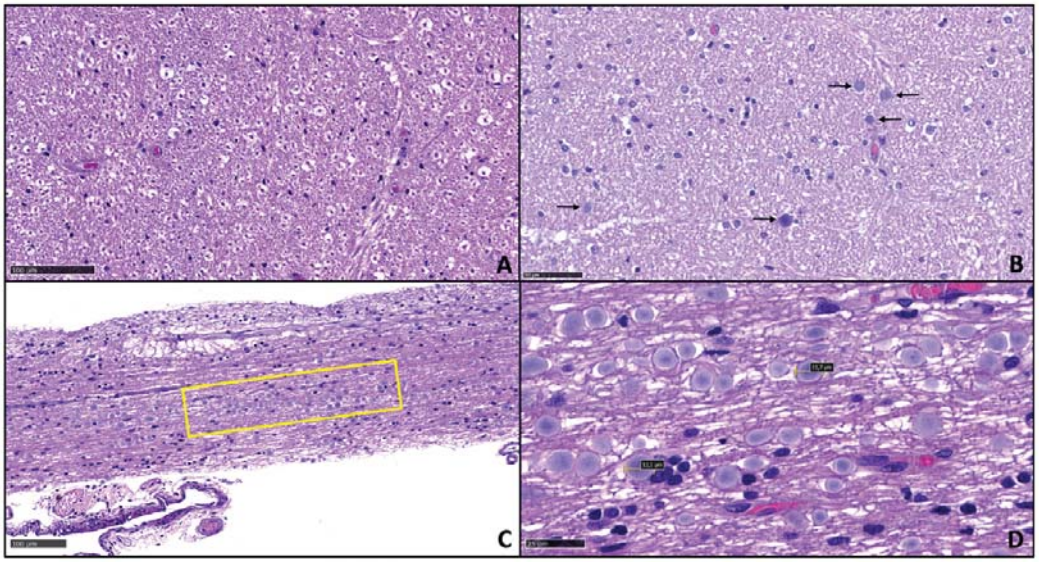


A. Immunohistochemical staining for CD68 PGM1, developed in brown, shows the presence of a sparse but widespread population of positive cells. These are identifiable as microglia due to their morphology. **B.** Immunohistochemical staining for CD163 shows that the population of CD163 + cells is clearly superior to CD68 PGM1 + ones. The red box is enlarged in the next image. **C.** At higher enlargement it is appreciated how many of the CD163 + cells are arranged in close contact with the intraparenchymal blood vessels. The black dotted box highlights the enlarged area in image "D". The image does not have the scale bar because it is a digital enlargement of the previous image. **D.** The high magnification shows how many CD163 + cells have a glial dendritic appearance. The image does not have the scale bar because it is a digital magnification of the image "B".

Its ability to remove sialic acid residues from the neuronal cell surface, thus activating the complement cascade and its own phagocytic activity, appears to be particularly important to help understand its role during SARS-CoV-2 infection[31]: complement factors C1q and C3 can stimulate microglia in the phagocytosis of synapses and neurons through the complement receptor 3 (CR3), consisting of CD11b and CD18 subunits. It is also known that microglia can play a role as a viral "reservoir" in the course of HIV1 infection, through mechanisms not yet fully understood, probably depending on specific immunophenotypic characteristics of the cells themselves[32]. Sialic acid bound to glycoproteins and

gangliosides is used by several viruses as an entry receptor within human cells; SARS-CoV-2 itself penetrates inside the cells through the spike glycoproteins. Our working group has recently used an immunohistochemical approach to demonstrate the presence of this virus in the glial cells of the brainstem[10]. This suggests a more important role of microglia both in the acute phase and in the post-COVID-19 conditions. Our findings regarding the histopathology of the neuropil and white matter of the CNS were also found to be only partially comparable to those of the previous studies[18,23-26]. The rapid evisceration and fixation of the B-BS-C block allowed us to recognize and adequately grade the distribution of tissue edema as it avoided the well-known and frequent post-mortal alterations of the parenchymal tissue of the brain, which can make recognition of tissue edema extremely confusing (Figure 6.5A and B). We found the damage to the myelin sheaths and subacute and acute axonal damage[7,10]. We also observed the widespread presence of structures similar to the more recently formed (non-stratified) amylaceous corpora or to the Lewy bodies present in some neurodegenerative diseases[10] (Figure 6.5B-D) in middle-aged patients not suffering from neurodegenerative diseases.

Figure 6.5: White matter of the brain



A-B. Brain. The white matter appears edematous with the presence of numerous small hollow halos on the contour of the glial cells. Hollow halos are also observed between the myelin sheaths and their respective axons. In "B" the arrows identify some corpora amylacea. **C-D.** Olfactory nerve. This structure also appears markedly damaged and it is characterized by the presence of numerous corpora amylacea in a middle-aged patient not suffering from neurological degenerative diseases, such as Alzheimer's disease. In "C" the yellow box indicates the enlarged area in image "D". In the latter, it is observed that the corpora amylacea can also be large reaching a diameter of about 12 microns.

These structures are particularly abundant both in the brainstem and in the basal areas of the brain, particularly at the level of the frontal and temporal lobes. It is not yet clear whether these findings are a consequence of the involution of single pyrenophores or axonal spheroids at points of axonal distruption. However, in patients who died from COVID-19, the close quantitative correlation between the areas of the greatest tissue damage and these amorphous structures suggests that the latter can be a prompt indicator in the routine histopathological examination of tissue damage, which requires in-depth studies with histochemical and immunohistochemical methods for its identification.

Viral entry pathways into the CNS

To date, this topic is still one of the most discussed, and several studies have not showed the presence of SARS-CoV-2 in the brain of patients with COVID-19[23,33-37]. It is reasonable to assume that this discrepancy is the result of different factors, including the time elapsed between death and the evisceration of the brain, the different viral load at the time of death, and the viral detection methods (type and clone of the antibody; *in situ* hybridization; qRT-PCR). Theoretically speaking, the virus could infect the parenchymal tissue of the brain either through the circulatory system (in this case, the role of endothelial and perivascular cells would be crucial)[38], or by tracing back to the CNS via nerves connecting it to other organs typically infected by the virus, i.e., the lung[10], or airborne, coming into contact with the nervous olfactory epithelium. The latter, through the cribriform plate of the ethmoid, projects into the mucosa that covers some parts of the nasal cavities[39]. The current state of knowledge considers all these pathways to be potentially possible. It is not to be excluded that the virus could reach the CNS by several routes in single patients, and that, in different autopsy cases, the different geography of the observed damage in the CNS may be an expression of the type of route of infection followed by the virus. Our impression and our current topic of investigation is that there is quantitatively less damage resulting from the bloodstream infection, at least at the level of cortical neurons, and that it occurs particularly in the areas of the brain that are further from the basal cortex of the frontal and temporal lobes.

Conclusions

It is undeniable that, still today, after over 4 million deaths from COVID-19 and over 180 million people infected with SARS-CoV-2 worldwide, our knowledge of the neuropathological characteristics of this infection and

disease is limited. From a histopathological point of view, it is difficult to define whether the observable neuronal or glial damage is a direct effect of the viral infection or a secondary effect of the disease rather than an expression or co-expression[40-43] of other previous diseases. Many patients are elderly and, therefore, their CNS is characterized by alterations due to aging, degenerative neurological diseases, or vascular diseases. It is, therefore, extremely difficult to decide which and how many of the macroscopic or histological alterations highlighted are the expression of previous diseases or, instead, the direct effect of the viral infection in single cases[8]. Secondly, many patients affected by COVID-19 were hospitalized in intensive care units, sometimes for many days, and were mechanically ventilated before they died. These conditions could be the cause of the hypoxic changes or of the inflammatory state detected during autopsy. Thirdly, it has been argued that the viral load in the brain can be reduced in the case of prolonged illness[39,44]: this makes it difficult to detect the presence of the virus in the CNS and removes an important correlative element with the pathological alterations eventually detected, even in patients without other previous diseases. Fourthly, the number of patients dying from COVID-19 who underwent autopsy is unacceptably low, even in countries adequately equipped with autopsy rooms with a BLS 3. All this is of particular concern if we consider that, with the prolongation of the pandemic, the weight of the poor neurological outcomes of this disease in survivors is becoming increasingly clear, with evident consequences on the health costs of rehabilitation, on the social costs (also in terms of work) and personal costs. Neuropathology studies on COVID-19 should address a large cohort of patients and case-control series, and explore both the damage to the CNS that can lead to a patient's death, such as those involving the primary respiratory and cardiovascular control center in the medulla oblongata[9,10,17,39], and the damage to the brain or cerebellar areas capable of worsening the quality of life of disease survivors. The SARS-CoV-2 pandemic, even after the number of cases worldwide has significantly decreased, will continue to represent a health emergency for many years, and medical research must continue to address this.

Take-home message

- The autopsy study of the CNS is still fundamental to better understand the SARS-CoV-2 infection, its way of transmission and spreading, and many clinical aspects of the disease.
- It is important to remove and fix the brain as soon as possible after the patient's death.
- In our experience, SARS-CoV-2 can directly damage neurons, glia and myelin contributing to the overall cerebral damage, potentially caused also by alterations in oxygenation from lung disease and/or mechanical ventilation during COVID-19.
- The current state of knowledge suggests that the virus can migrate via nerves, probably with a mechanism similar to the Herpesviruses.

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