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The antioxidant effect of the Voghera pepper: *in vivo* study in the cerebellum of a murine aging model

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ABSTRACT

Aging is an inevitable physiological process marked by a progressive decline in physical, cognitive, and biological functions. It is characterized by cumulative macroscopic and microscopic changes, ultimately resulting in the deterioration of healthy tissue architecture and a gradual loss of homeostasis and physiological resilience. Given its multifactorial origin, the most known aging hallmarks are genetic instability, telomere shortening and epigenetic alterations, impaired proteostasis and autophagy, senescence pathway activation, chronic inflammation and compromised gut-brain axis. Specifically, one of the most significant mechanisms underlying these cellular impairments is oxidative stress, which results from increased Reactive Oxygen and Nitrogen Species (RONS) accumulation coupled with progressively declining antioxidant cell response. Due to its high metabolic demand and limited renewal abilities, the Central Nervous System (CNS) is particularly vulnerable to oxidative stress and aging, leading to progressive neuronal loss, neuroinflammation, meningeal thickening and impaired synaptic transmission. In recent years, nutraceutical compounds, found in dietary products, demonstrated to be effective in preventing or slowing down aging-related mechanisms, thereby promoting "healthy aging". Moreover, several natural molecules are able to decrease neuronal loss and preserve their cytoarchitecture, participate in neurotransmission, promote mitochondrial metabolism and free radicals neutralisation. In this regard, the Voghera pepper, an Italian agricultural product, which is cultivated in the province of Alessandria and Pavia, showed *in vitro* ability to decrease fibroblast senescence and restore healthy cellular morphology, decreasing oxidative stress, probably thanks to the high content of vitamin C and carotenoids.

This thesis work aimed to evaluate the effects of a phytotherapeutic implementation with Voghera pepper extracts, derived from pepper fruit body and by-products, in the CNS of an *in vivo* murine model of aging, with a particular focus on the cerebellum, by evaluating the neuronal morphological changes using histochemical techniques, i.e. Nissl and Picrosirius Red stainings, and immunohistochemical methods, to evaluate possible changes/modification in the expression levels of specific oxidative stress markers, i.e. SOD1, SOD2, iNOS and COX4.

The results obtained in this work proved the treatments are effective in favouring overall healthier CNS conditions and improving the cerebellar milieu. Both fruit body and waste extracts contribute to the maintenance of the physiological neuronal morphology by reducing the number of shrunken Purkinje cells, and regulate meningeal thickening by modulating excessive collagen deposition. Additionally, Voghera pepper extracts considerably modulate the expression of oxidative stress-related molecules. Particularly, the analyses revealed that supplementation with Voghera pepper

extracts downregulates SOD1, SOD2 and iNOS, enzymes normally upregulated during aging, thereby supporting the antioxidant and anti-inflammatory potential of both the fruit body and by-products, although to a different extent. Parallelly, both treatments proved effective at enhancing COX4 expression levels in old mice, which overall supports better mitochondrial functionality.

Altogether, this study provides extensive evidence regarding the antioxidant and anti-inflammatory potential of the *in vivo* phytotherapeutic implementation with this Italian pepper variety, particularly highlighting the beneficial impact of both the Voghera pepper fruit body and by-products on the CNS during aging.

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1. INTRODUCTION

1.1. The Cerebellum

The cerebellum (from the Latin, “little brain”) is a portion of the CNS (Central Nervous System) and occupies 10% of the whole brain volume. Despite its small size, it contains almost 80% of the total amount of neurons of the Nervous System (NS). The cerebellum is present in all true vertebrates and, differently from the brain, all mammals and birds share the same cerebellar histological components, while in amphibians, fish and reptiles only the gross functional organisation and functions are maintained (Sultan et al., 2007). Specifically, its dimension increases following the evolutionary processes (e.g., the mouse cortex measures around 190 mm^2 while the human one reaches 50000 mm^2) but the afferent and efferent pathways maintain the same functional rules (Lara-Aparicio et al., 2022). For many years the major role attributed to the cerebellum was motor control and movement adjustment, through the influence of signals from the cortical motor centres and brainstem nuclei; yet, the different connections, with other non-motor brain areas, suggest its involvement in several other functions (Prati et al., 2024). Indeed, cerebellar damage or its removal does not alter the basic mechanisms necessary for life but impair coordination, motor learning and cognitive functions (Kandel, 2021).

1.1.1. Anatomy and Morphology

The cerebellum is located in the posterior cranial fossa, behind the pons and *medulla oblongata*, it is separated from the Occipital lobe by an extension of the meninges’ dura mater (the *tentorium cerebelli*) and, ventrally, it is in contact with the fourth ventricle (**Figure 1**). It is anatomically and physiologically connected through the cerebellar peduncle to the brainstem, the rest of the brain and the spinal cord: the superior (*brachium conjunctivum*) and middle part (*brachium pontis*) mediates the connection to the midbrain and the inferior part (*corpus restiforme*) to the medulla oblongata.

In all vertebrates CNS is covered by a three-layer structure, the meninges, that protect the neural tissue and attach it to the internal surface of the skull. The meninges are composed of *dura matter* (the most external layer), which follows the anatomical cavities of the brain like the *tentorium cerebelli* does, the *arachnoid* (the middle layer) and the *pia matter* (the innermost layer), which is directly in contact with the brain parenchyma. More specifically, the dura mater is a thick and dense fibrous membrane composed of two layers, the endosteal and meningeal layer, containing the blood vessels, lymphatic vessels and venous sinuses for blood supply and drainage of

the cerebro-spinal fluid (CSF). The *arachnoid* is a thin membrane of flat cells and limits the subarachnoid space made of the arachnoid trabeculae, collagen and fibroblast structures, where the CSF runs, while the *pia matter*, a delicate single-cell layer membrane adherent to the brain surface, contains the perivascular connective tissue and the perivascular space of blood vessels that enter deeper inside the CNS (Dasgupta et al., 2020).

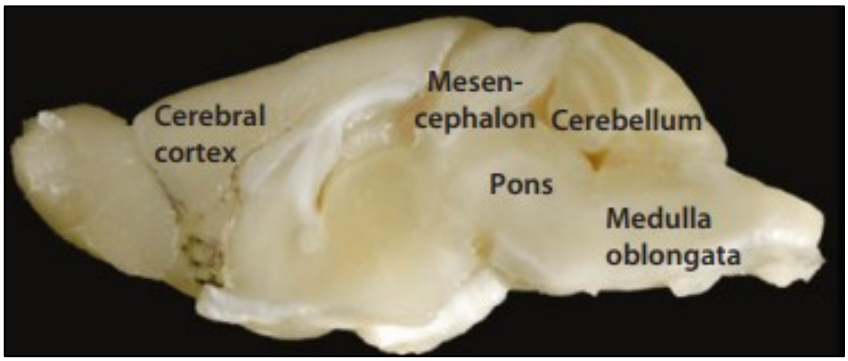

Figure 1. Midsagittal section of the mouse brain. The cerebellum is positioned dorsally to the brainstem and dorsocaudally relative to the entire brain (modified from Schröder et al., 2020).

The fourth ventricle and the *choroid plexus* limit the ventral part of the cerebellar territory (**Figure 2**). It is one of the four cavities inside the CNS territory producing CSF and collecting waste products. In all vertebrates, the *choroid plexus* consists of a single layer of epithelial cells, bound by tight junctions, folded and forming protruding villi in the ventricle, with an apical series of microvilli to increase the entire surface of the plexus; internally, the mesenchyme is highly vascularised. The main functions of this intricate anatomical structure are to produce CSF, to provide nutrients exchange with the blood flow, and to collect metabolites, toxins and drugs (Saunders et al. 2023).

Three main arteries supply the cerebellum: the superior cerebellar artery, the anterior inferior cerebellar artery and the posterior inferior cerebellar artery. Posteriorly to the brainstem, they form different number of branches to cover the entire organ (Błaszczyk et al., 2024).

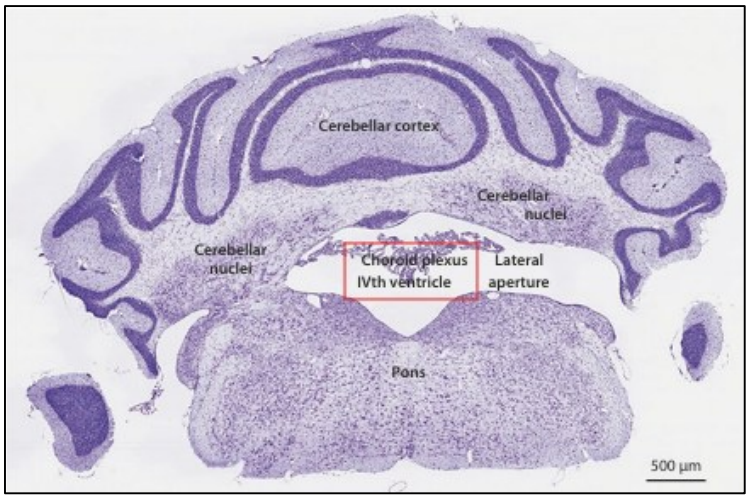

Figure 2. Coronal section of C57BL/6 mouse model (Nissl stain). In the picture is highlighted the position of the IV ventricle and the choroid plexus (modified from Schröder et al., 2020).

The cerebellum can be divided in different ways depending on anatomy, phylogenic development and function. Based on the outer appearance, it has two hemispheres and two intermediate zones (*paravermis*) with a narrow central band, the *vermis* (**Figure 3**). From an anatomical point of view, we can distinguish the anterior lobe ending at the primary fissure, subdivided in anterobasal and anterodorsal, the posterior lobe, subdivided in central and posterior, and the flocculonodular lobe, subdivided in floccule and nodules, disconnected by the posterolateral fissure from the posterior lobe (Lara-Aparicio et al., 2022).

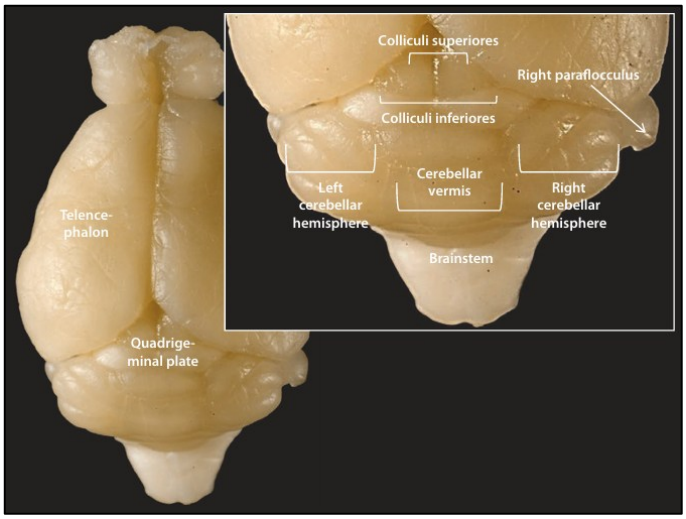

Figure 3. Anatomical representation of the mouse brain. It can be divided into two hemispheres with, centrally, the *vermis* (modified from Schröder et al., 2020).

The surface of the cerebellum is not completely smooth: it presents a series of gyri called *folia* (singular, *folium*) and several fissures dividing the organ into ten main lobules, as established and reported by Schmahmann et al. in 1999, noticeable with a sagittal section at the level of the vermis

(Schröder et al., 2020). The lobular subdivision goes from I to V in the anterior lobe, from VI to IX in the posterior lobe and the X in the flocculonodular lobe; lobule VII is further divided into VIIA and VIIB (Marzban, 2023).

As for the rest of the NS, histologically, the cerebellum is composed of white and grey matter: internally, the white matter forms a tree-like structure called *arbor vitae,* with the Deep Cerebellar Nuclei (DCN) at the base and the white matter fibers forming the "branches", while, externally, the cerebellar cortex is made of grey matter (**Figure 4**) (Habas, 2010).

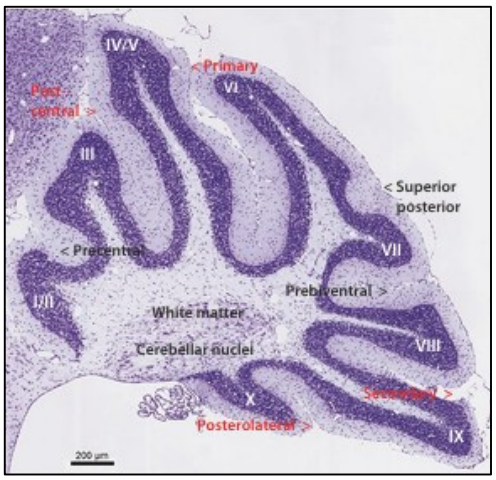

Figure 4. Nissl staining of a cerebellar sagittal section at the vermis level (modified by Schröder et al., 2020).

In greater detail, the cerebellar cortex is divided in three layers: from outermost to innermost, the molecular layer (ML), the Purkinje cell layer (PCL) and the internal granular layer (IGL), which is in contact with the white matter. The DCN are divided in pairs: the *fastigial nuclei* (located at the level of the vermis), the *interposed nuclei* divided in *emboliform* and *globose nuclei* (in the intermediate hemisphere) and dentate nuclei (the most external ones) (Habas, 2010).

Alongside with the anatomical repartition, a functional and phylogenic division is also accounted for the cerebellum. The *vestibulocerebellum* (or *archicerebellum*) is the oldest component and its cortex receives information from the vestibular ganglia and vestibular nuclei of the brainstem through the mossy fibers (MFs). It is the only part of the cerebellum that sends projections directly to the outside of the organ: particularly, Purkinje cells send inhibitory projections to the *fastigial nuclei* which, in turn, send excitatory projections to the *vestibular nuclei* through the inferior cerebellar peduncle and contralateral ascending projections to the ventrolateral nucleus of the thalamus.

The *spinocerebellum* (or *paleocerebellum*) corresponds to the paravermis area and receives inputs from several fiber tracts: (i) from the dorsal spinocerebellar tract, an ascending pathway conveying

proprioceptive, pressure and tactile information from the lower parts of the body; (ii) from the ventral spinocerebellar tract, responsible for motor activity feedbacks; (iii) from the cuneocerebellar tract, that transports somatosensory information; (iv) from numerous brainstem nuclei, e.g., the reticular formation. More internally, the *interpose nuclei* of the *spinocerebellum* send projections to the brainstem and feedback projections to the cerebellar cortex.

Finally, the *pontocerebellum* (or *neocerebellum*) is the largest, most recent portion, and corresponds to the later hemispheres. It is highly developed in higher mammals; it controls and refines motor activity and cognitive functions. The inputs come from the frontal and parietal lobes of the cerebral cortex passing through the pons, originating the contico-ponto-cerebellar pathways. Then, information reaches the Purkinje cells and, subsequently, the *dentate nuclei*, which are connected to the ventrolateral thalamus (linked to the premotor and primary motor cortex) and, through the red nucleus, to the inferior olivary nuclei of the medulla oblongata; the latter create a feedback loop with the cerebellar cortex (Marzban, 2023).

1.1.2. Cerebellar cortex

The outermost component of the cerebellum, in contact with the meninges and made of folded grey matter, is the cerebellar cortex. Both in humans and rodents it has a uniform parasagittal cytoarchitecture and a somatotopic organisation can be distinguished; in humans, there is a dorso-medial and ventro-medial division, while in rodents there are fractured areas that don't follow a complete body representation. Generally, the cortex receives inputs from the pontine nuclei and sends outputs, through the Purkinje cells, to the DCN projecting to the thalamic nuclei (Van Essen et al., 2019).

All vertebrates exhibit this trilaminar organisation of the cerebellar cortex, composed of three distinct layers: from outermost to innermost, the ML, the PCL and IGL (**Figure 5**) (Hawkes, 2001). Furthermore, each layer contains different types of cells with unique specialisations, plasticity mechanisms and physiological features (Hull et al., 2022).

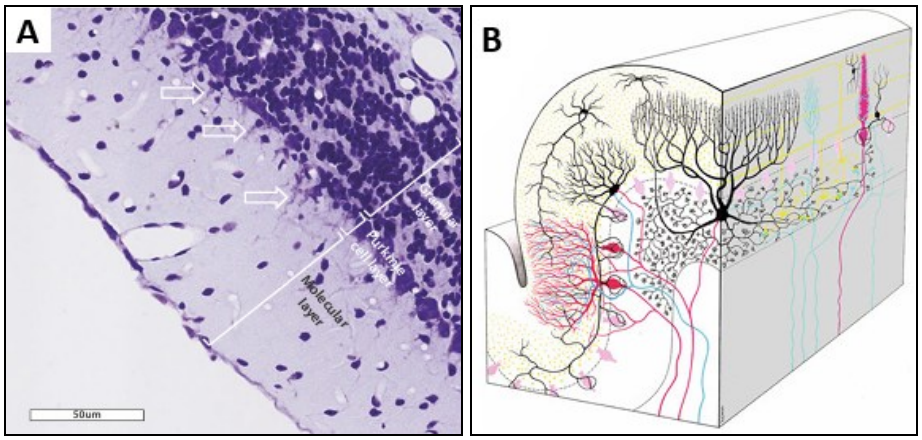

Figure 5. Morphology and organisation of the mouse cerebellar cortex: (A) Paraffin embedded parasagittal section of the cerebellar cortex at high magnification (Nissl staining): the three layers are characterised by a different cell density and size and the white arrows highlight the Purkinje cells dendrites spreading in the molecular layer (modified from Schröder et al., 2020);
(B) Schematic rappresentation of the cerebellar cortex with the different cells type. In the molecular layer, stellate cells and basket cells (*black*). Purkinje cells in *pink.* In the granular layer we have Golgi cells (*black*), granule cells (*yellow*). Mossy and climbing fibers in *blue* (modified from Gruol et al., 2016).

Another possible division of the cerebellar cortex considers a vertical spread, subdividing it in vertical modules, which involves all three layers with their specific cell population. Every module is limited by the ascending granular cells axons and the climbing fibers and differs in neurochemical signals and specific functional roles in the cerebellar circuits (Kalinichenko et al., 2018).

Molecular layer

The ML is the most external layer of the cerebellar cortex. The majority of its area is occupied by the dendrites of Purkinje cells and the axons of cells coming from the other layers or brain areas. Particularly, axonal projections from the IGL are organized in a parallel arrangement forming parallel fibers. The ML contains mainly two types of inhibitory interneurons (MLIs): stellate cells and basket cells. Stellate cells are positioned in the external two-thirds of the layer, while basket cells are in the first inner third. Stellate cells have a star-like appearance, with branched dendrites with either many spines or none. Similarly, basket cells have dendrites that can envelope the targeted cell body and can be covered by many spines (Marzban & Hassan, 2023).

More recently, additional MLIs were discovered. They were identified by their different transcriptional pattern and different electrophysiological properties; their position inside the molecular layer can promote morphological changes, making them more similar to either basket

or stellate cells. These cells are classified as MLI1 and MLI2 but is not clear if they constitute different cell populations or can be considered as subtypes. Physiologically, MLI1 and MLI2 antagonise Purkinje cells' firing rates (Lackey et al., 2024). Overall, MLIs activity can modulate PC and GrC synaptic connections and plasticity during motor learning and motor rate (Hull et al., 2022).

Purkinje cell layer

In this layer we can find the large cell body of Purkinje cells (PCs) and the Purkinje cell Layer Interneurons (PLIs). PCs are inhibitory cells with a large soma, with long and branched dendrites, that spread in the ML, while the axon reaches the DCN. Their dendritic tree appears vast and branched in the sagittal plane, while it reduces and appears more squished in coronal sections. PCs activate at 20-100 Hz and their firing pattern is ruled by voltage-dependent sodium and calcium channels which are able to create complex non-linear firing rates, triggered by climbing fibers, or simple spikes, triggered by MFs. Both firing patterns can be detected after birth in rodents and develop completely during growth (Beckinghausen et al., 2019). The modular organisation of the cortex can also determine the presence of different transcriptional patterns that probably create PCs subtypes, but it's not known yet their role in establishing the output signals and their relationship with specific cerebellar circuits. Moreover, PCs exert negative feedback on other PCs, IGL cells, candelabrum cells and some MLIs, refining the motor control and maintaining the correct cerebellar cortex activity (Hull et al., 2022; Guo et al., 2021).

PLIs are divided into globular cells and candelabrum cells. Globular cells have a small rounded cell body with neurites projecting to the two proximal layers; they are inhibited by PCs (Hull et al., 2022). Candelabrum cells get their name from the shape of their dendrites that originate as a single unit to then divide in secondary processes; they inhibit the MLIs, thereby favouring the PCs disinhibition, making them different from the other layer interneurons (Osorno et al., 2022).

Granular Layer

This layer is the innermost layer of the cerebellar cortex and the most dens with cells. We can distinguish granule cells (GrCs), Golgi cells (GoCs), unipolar brush cells (UBCs) and Lugaro cells. GrCs are tightly packed excitatory interneurons and represent the most numerous cerebellar neurons, accounting for approximately 99% of the total amount (Kozareva et al., 2021). Typically, GrCs have a small soma with short dendrites and their axons reach the molecular layer, organizing in parallel fibers, which synapse with the large dendritic tree of PCs. GrCs release glutamate and

can shape the activity of other cerebellar interneurons, including stellate, basket cells and GoCs. Mossy fibers, one of the main inputs to the cerebellum, originate in the spinal cord and brainstem nuclei, and reach the IGL; they exhibit large synaptic buttons with many active sites that can be connected with more than one GrC. More in detail, GrCs dendrites, GoCs axon and mossy fibers create a specialised structure called "rosettes". Here, neurotransmitters can persist and accumulate activating many different receptors on the cells involved in this organisation. Moreover, considering the different origin of mossy fibers, different synaptic inputs and behaviour can be identified, followed by particular GrCs temporal spike pattern (Hull et al., 2022; Chabrol et al., 2015).

As opposed to GrCs, GoCs present a large soma usually rounded or "polygonal" and dendrites, with different morphologies, branch inside the granular layer ending with several varicosities. Their main neurotransmitters are GABA and/or glycine and these cells take part in the modulation of GrCs and mossy fibers rosettes activity (Mapelli et al., 2014).

Other types of interneurons, which can be found in the IGL, are the UBCs. UBCs make excitatory connections with MFs terminals with very fine brush-like dendrites. This peculiar morphology is adopted to amplify the MFs signals to their targets.

Finally, Lugaro cells have an elongated fusiform soma with an axon that reaches the ML and runs in it with the parallel fibers. They are GABAergic and glycinergic interneurons connecting to PCs, basket cells and stellate cells axons and receive inputs from MFs. Furthermore, these cells are in contact with more than a hundred of GoCs probably modulating their activity (Gruol et al., 2016).

1.1.3. Cerebellar Glial cells

Glial cells are a category of non-neuronal cells that do not exhibit direct electrical properties. Non the less, glial cells are involved in the physiological homeostasis of the nervous tissue, providing support and protection to neurons, maintaining a healthy microenvironment and indirectly contributing to the overall electrical activity of the NS (Jäkel & Dimou, 2017).

In the cerebellum several types of glial cells can be found: astrocytes, Bergmann glia, oligodendrocytes and microglia. Astrocytes can be divided into two main categories: highly branched fibrous astrocytes, within the white matter, and protoplasmic astrocytes, mainly found in the cerebellar cortex. Protoplasmic astrocytes form connections with other brain cells, through gap junctions, allowing the exchange of small molecules; conversely, fibrous astrocytes are organised in layers and create two main connections with blood vessels and the nodal point, or nodes of Ranviers, facilitating nutrient delivery to this structures important for the nerve impulse transmission (Gruol et al., 2016). Furthermore, this last type is involved in the isolation of the

synapses in the rosettes through a covering layer surrounding the neuropils to limit the diffusion of neurotransmitters, hence why they are also called “velate” astrocytes. Other than these specific functions, the role of this cells in the cerebellum is similar to the astrocytes found in the other brain areas like potassium buffering, neurotransmitters recycling, “activation” during inflammation, leading to reactive gliosis and “gliotrasmission”, the release of neuromodulators that act as a feedback to neurons activity (Bergamo Araujo et al., 2019). Aligned with PCs and with processes in the ML, the predominant type of glial cells is the Bergmann glia (rarely called Golgi epithelial cells), a glial type unique to the cerebellum. They are similar to protoplasmic astrocytes and retain some characteristics of their radial glia progenitors. Bergmann glia cells form bulbous endfeets with the pial surface and their fibers create microdomains encapsulating all the molecular layer synapsis preventing the neurotransmitters spill over. In addition, they have similar homeostatic functions compared to canonical astrocytes but, during the development of the cerebellar cortex, they are used ass “tracks” by GrCs during their migration from the external granular layer (EGL) to the IGL (Beckinghausen & Sillitoe, 2019). Finally, oligodendrocytes, in the granular layer and white matter, create the myelin sheets around cerebellar neurons. While microglia, resident macrophagic cells of the CNS, activates during inflammatory processes translocating to the injury site and adopting macrophages behaviour (Gruol et al., 2016).

1.1.4. The cerebellar circuits

The cerebellum is entailed in numerous and reciprocal circuits that involve several brain areas such as the inferior olivary nucleus, pontine nuclei, red nucleus and thalamic nuclei creating connections with cerebral regions controlling motor and non-motor functions. These extrinsic connections transport inputs to the cerebellum through climbing fibers and MFs. Climbing fibers depart from the inferior olivary nucleus, a large paracerebellar nucleus part of the inferior olivary complex in the medulla oblongata; they synapse directly with the contralateral hemisphere PCs, passing through the contralateral inferior cerebellar peduncle. Every fiber makes excitatory synapses with approximately ten PCs and multi-innervates single PCs dendrites, both in mice and humans (Busch & Hansel, 2023). Climbing fibers can strongly depolarise PCs counteracting the MLIs inhibition, promoting an opposite plasticity effect compared to the MLIs one (Hull at al., 2022). Moreover, mouse MLIs are affected by the spill over of neurotransmitters realised on PCs. Overall, the complex connectivity and neurotransmission mechanisms play different roles in synaptic plasticity (Marshall-Phelps et al., 2020). Parallelly, climbing fibers excite the collateral DCN. The origin of their inputs is from the spinal cord, vestibular system and cortical descending

pathways, transporting sensorimotor information that control motor learning (Schröder et al., 2020).

MFs originate from the ascending spinocerebellar tract, cortical and subcortical structures projecting to the pontine nuclei (except for the inferior olivary nucleus), through which they reach their target (pontocerebellar pathway): the excitatory synapses of the rosettes, comprising of GrCs and GoCs (Kim et al., 2023). In particular, when exited by MFs, GoCs provide an inhibitory modulation of the tonic inhibition over GrCs, creating a more flexible layer activity (Mapelli et al., 2014). Another target of MFs are the deep cerebellar nuclei counteracting PCs inhibition of signals favouring these nuclei activation (Wu & Raman, 2017).

Additionally, the cerebellum is characterised by intrinsic connectivity involving different types of cells, from the MLIs, to PCs and granular layer interneurons (GLIs) (**Figure 6**). PCs receive several inputs: (i) from the IGL, GrCs axons ascend to the ML and take part in the parallel fibers organisation, synapsing with PCs dendrites or with MLIs and inhibiting the latter; (ii) indirectly from the excitatory UBCs (in contact with MFs) and the rosettes organisation, in the IGL; (iii) from basket cells whose dendrites run parallel to the dendrites of PCs and whose axons envelope PCs' soma; (iv) and from stellate cells which connect to the PCs dendrites guided by the Bergmann glia scaffold. All the different connections and their specific nature, inhibitory or excitatory, take part in the modulation of PCs response towards the DCN linked to cortical and subcortical regions (Schröder et al., 2020).

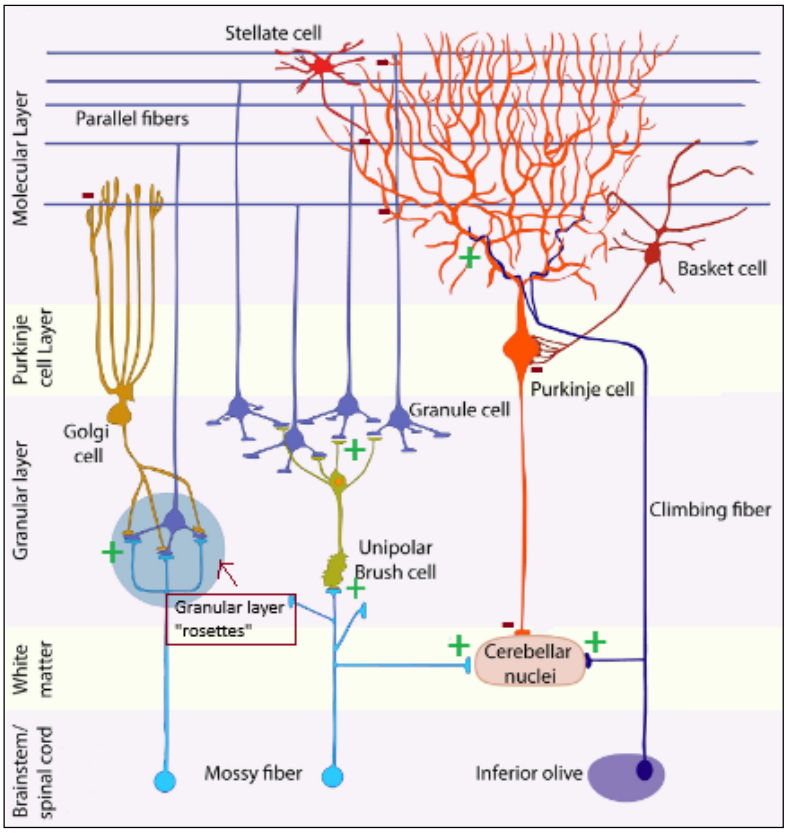

Figure 6. Schematic representation of the cytoarchitecture of the cerebellar cortex. For every type of cell, is highlighted the type of synaptic connection, inhibitory (*minus*) or excitatory (*plus*). Mossy fibers get in contact with GoCs, GrCs, UBCs and the DCN. Climbing fibers synapse with the DCN and PCs. PCs receive inputs from basket cells, stellate cells and parallel fibers (GrCs) (modified from Conzales et al., 2021).

PCs are the only output element of the cerebellar cortex. They make inhibitory synapses with the DCN neurons and the vestibular nuclei in the brain stem (this direct connection is for the vestibulocerebellum only). The DCN neurons then excite brain areas outside of the cerebellum, including the thalamus, red nucleus and reticular formation. The DCNs are divided in three pairs: medial (emboliform), lateral (dentate) and interposed nucleus (globose and fastigial), positioned in the internal part of the *arbor vitae.* The fastigial nucleus is the medial and smallest DCN: it receives inputs from the PCs of the vestibulocerebellum and from the vermis connected, through the vestibular nuclei, to the vestibular ganglion; then, it sends the elaborated response to the vestibular and reticular nuclei of the brain stem. The interpose nucleus is located in the paravermis and divided in globose nucleus (more medial) and emboliform nucleus (lateral); their inputs come from the PCs in the anterior lobe vermis and paravermis linked to the cuneate nucleus (part of cuneocerebellar tract), the accessory cuneate nucleus and Clark's nuclei (part of dorsal

spinocerebellar tract). Subsequently, the interposed nucleus sends projections to the red nucleus in the midbrain and the ventrolateral nucleus of the thalamus; this relay nucleus sends information to the motor cortex and the areas related. The dentate nucleus is the largest of the DCNs in the cerebellar lateral hemispheres; it is excited by PCs of the lateral hemispheres, by the collateral climbing fibers and MFs originating from inferior olivary and basilar pontine nuclei. The targets of this last nucleus are the red nucleus and the ventrolateral thalamic nucleus (Marzban, 2023).

1.1.5. Functions of the cerebellum

The cerebellum is commonly known for taking part in motor control. In particular, it compares the sensory information coming from the spinocerebellar tract (responsible for proprioception and cutaneous sensations), spino-reticulo-cerebellar tract and the cuneocerebellar tract, with the motor commands coming from the cortex and provides feedbacks to adjust and refine movements, directly controlling motor neurons activity or the specific locomotor circuits (such as the basal ganglia circuit and its connection with the motor cortex), in relationship with the specific motor related thalamic nuclei. The ventral spinocerebellar tract and the spino-reticulo-cerebellar tract arrive to the PCs in the form of MFs in the anterior cerebellar lobe. Subsequently, the dentate nucleus activates the vestibulospinal track (mainly for extensors contractions) or the reticulo- and rubrospinal track (for flexors contraction). Furthermore, different learning processes, involving both synaptic potentiation and de-potentiation, are evidenced to be related to task-learning. Through these pathways and through the error-driven process mediated by different activity of climbing fibers in response to signals perturbations, the cerebellum is able to perform locomotor adaptation by continuously integrating cues from muscle states (Grillner & El Manira, 2020). Another crucial connection involves the anterior or dorsal dentate nucleus and the posterior parietal cortex (PPC), a multimodal area that performs sensory-motor integration related to both motor (dorsal cerebellar nucleus specific) and non-motor processes (ventral cerebellar nucleus specific) including coordination of movements, sensorimotor predictions or visual-motor processing, not only during the motor act but with motor imagery probably helping in motor learning (Prati et al., 2024).

Movement coordination, balance, perception of self-motion and orientation are controlled specifically by the vestibulocerebellum. Both MFs and climbing fibers transport different information and divide in different cerebellar compartments: the floccular lobe PCs integrate visual signals for gaze stabilisation coming from oculomotor and vestibular nerve nuclei, the anterior vermis integrates proprioceptive signals for head movement, posture and balance, while the *nodulus* receive inputs from the vestibular nerve nuclei in the brainstem, collecting otoliths

and semi-circular canals (internal ear) state. The main proponents of signal transduction are UBCs, classified as ON and OFF subtypes: they can either attenuate or increase the vestibular afferent signals. In addition, the cerebellar nuclei project to the ventral lateral thalamus connected to cortical areas important for motion processing (Cullen 2023; Prati et al. 2024).

A function unique to the vestibulocerebellum, specifically the floccules region, is related to vision: the vestibular nuclei and the accessory optic system collect information about self-motion to optimise the oculomotor reflex and object representation on the fovea, the region of the retina with the highest image definition perception. To achieve this, the saccadic eye movements and the eye reflexes need to be inhibited and controlled to fixate the gaze. Another region involved in this function is the phylogenetically more recent neocerebellum, containing the oculomotor vermis. It receives inputs from the cerebral cortex through the pontine nuclei and is involved in the goal-directed eye gaze. After the cerebellar processing of signals, the DCNs report the modulations to the specific thalamic nuclei and then to large parts of sensory cortical areas (Thier & Markanday, 2019). The relationship between eye motor abilities and motor learning is exemplified by the eye blink conditioning where the conditioned stimulus (light or sound) is associated with the unconditioned stimulus, like a puff of air on the open eye that triggers blinking. The activation of specific cerebellar areas is involved in the storage of the eye blink reflex memory, specifically ruled by long-term depression (LTD) mechanisms within the PCs and parallel fiber, interpose nuclei and MFs connections (Freeman, 2015).

If the cerebellar involvement in controlling motor coordination and motor learning is commonly known and studied, the relationship between the cerebellum and the prefrontal cortex, hippocampus and limbic system is the subject of more recent investigations. Several cerebellar-cortical and -subcortical connections can be identified and they integrate sensory, motor, cognitive and affective functions. These connections are demonstrated by studies on disorders affecting the cerebellum, as well as using mouse models for specific types of learning, including emotional learning and fear conditioning. Fear conditioning involves the activation of several areas from the prefrontal cortex (PFC) to the amygdala and other regions of the limbic system that can modulate learning processes facilitating or not cerebellar plasticity mechanism (such as LTD) making the latter part of the affective behaviour modulators (Rudolph et al., 2023). Imaging studies showed the activation of specific lobules in response to felt or seen emotions such as fear, sadness and anger as well as emotion recognition or anxiety behaviour sustained by specific sensory stimuli that reach the cerebellar cortex, like nociceptive pain inputs, and specific visceral autonomic functions (Adamaszek et al., 2017).

The hippocampus receives connections from the cerebellum from the ventrolateral and later dorsal thalamic nuclei and responses from different pathways involving the medial septum and the supramammillary nucleus, making the cerebellum involved in spatial navigation, spatiotemporal prediction and learning (Watson et al., 2019; Prati et al., 2024).

Another non-motor function of the cerebellum is related with the reward pathway, in particular anticipating the most rewarding actions activating the cerebellar supervised learning and reinforcement learning in the basal ganglia system. Complex spike patterns and influences from MFs can mediate opposite plasticity processes compared to the motor task related ones (Manto et al., 2024). Moreover, excitatory projections from the DCNs to both dopaminergic and non-dopaminergic neurons in the ventral tegmental area (VTA) take part in social behaviour modulation, which has been demonstrated in pathological conditions such as in the autism spectrum disorders (Prati et al.; 2024).

The medial anterior lobe region and the fastigial nuclei are involved in the regulation of autonomic functions in connection with brainstem centres for heart rate and respiration, blood pressure and vascular tone, eyes miosis and mydriasis, intestinal motility and urinary changes. For example, the cerebellum contains chemoreceptor regions for hypercapnia response. Moreover, the lateral cerebellar DCN activates during food intake and it can nullify hypothalamic control, synergising with dopamine release changes in the ventral striatum related to pleasurable food (Low et al., 2021).

The DCNs change their activation also during sleep. The oscillations in activity during REM and non-REM sleep are transmitted to the cerebellum by MFs and climbing fibers. In the literature, these oscillations are known to take part in memory consolidation processes as well as in procedural skills such as the aforementioned vestibulo-ocular reflex, the visuo-motor adaptation, the eye-blink reflex and sequence learning. Lesions to the cerebellum decrease the amount of slow wave sleep and disrupt the connection with the thalamus, which plays an important role in regulating, with its oscillation activity, REM and non-REM sleep and in filtering sensory information (Jackson & Xu, 2023).

1.2. Aging

For many years, giving a complete and accurate definition of aging was not simple because of the complexity of the biological and physiological state of the being. Aging is known as a property of life, with no specific function, other than natural selection, and it's the result of both natural and accidental events. Aging involves the gradual and progressive decline in all physiological functions involved with survival; it is inevitable, universal to all lifeforms and it occurs in both healthy and pathological conditions. Several are the cellular and molecular features underlying aging and this process is usually associated and mediated by typical manifestations including structural damage, functional decline, increased probability of developing diseases and, ultimately, death. The build-up of structural damage is one of the first features of aging: it involves the accumulation of damages and degeneration of biological mechanism leading to physical emaciation and reduction of fertility. Phenotypically, individuals express specific features, that can be easily identified and associated with senescence: frailty caused by loss of muscular tone, fatigue, loss of appetite, slow movements, and increased susceptibility to infections and inflammations. Typically, different processes involved with aging may or may not have the same period of onset; they might also be accelerated, retarded or modulated by different internal or external factors, such as genetic variables and diet. Aging can be a target of therapeutic interventions; indeed, the molecular events underlying aging can limit the life span by antagonising several anti-aging mechanisms: "treating aging" refers to contrasting the former and favouring the latter. (Lemoine, 2020).

1.2.1. Hallmarks of aging

Considering the complexity of the biological state during aging and the necessity to find objective features that define it, a series of hallmarks were established following these criteria: (i) the manifestation or alteration of the hallmark is present during aging; (ii) the hallmark can be manipulated experimentally speeding up the aging process and (iii) aging can decelerate after therapeutic intervention targeting the hallmark. All of the elements proposed take into account molecular, cellular or systemic mechanisms, studied both in non-mammalian and mammalian organisms; among these we find: genetic instability, telomere shortening and epigenetic alterations, loss of protein homeostasis (or proteostasis), impaired autophagy, deregulation of the nutrient-sensing network, mitochondrial dysfunction, stem cell exhaustion, altered intracellular communication, cellular senescence, chronic inflammation and dysbiosis (**Figure 8**). Even if these hallmarks are described and studied as isolated entities, some of these mechanisms are present

usually together and can be causative or be participants in different other hallmarks processes (López-Otín et al., 2023).

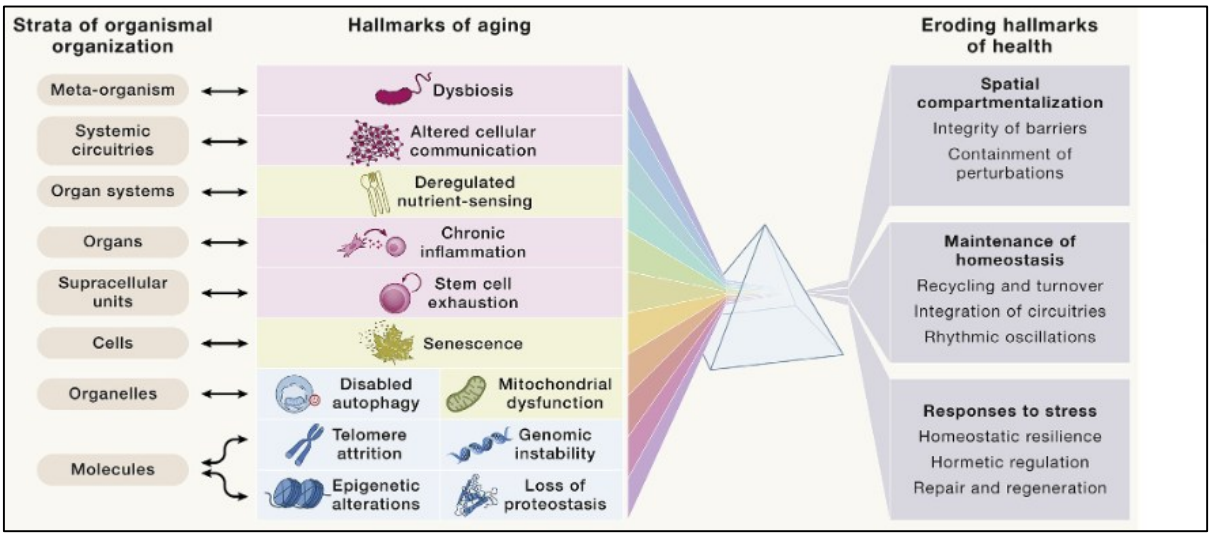

Figure 8. Schematic and summarised aging hallmarks: the picture lists all of the different impairments and dysfunctions characterising aging and the elements of health that are perturbed (López-Otín et al., 2023).

The integrity and stability of the DNA are crucial to protect cells from senescence (**Figure 9**). Apoptosis and malignant transformations can be caused by exogenous or endogenous DNA damages occurring during the lifespan and therefore cells are endowed with several DNA repair mechanisms, depending on the type of damage. For example, DNA double-strand repair (DSBR) system, divided in homologous recombination (HR) and non-homologous end joining (NHEJ), is able to fix the most dangerous DNA breaks; the nucleotide excision repair (NER) and base excision repair (BER) can resolve single base modification or nucleotide crosslink; single-strand break repair (SSBR) can reconstruct the sugar-phosphate DNA backbone and the mismatch repair (MMR) recognises the incorrect base pairs that escape the polymerase control. All of these mechanisms' functionality decreases with age and this leads to accumulation of incorrectly repaired DNA, depending on the species and tissues. For example, BER decreases most significantly in the brain, liver, spleen and testis of old murine models. Sirtuins (SIRT1-7), age-related synthetized proteins, are also involved in the regulation of DNA repairing systems and regulate several processes in different pathological conditions, such as in neurodegenerative disorders; indeed, mutations in the genes encoding for sirtuins can trigger premature aging, demonstrating the relationship between aging and DNA damage. Moreover, these proteins need NAD^+ to physiologically function, but this molecule decreases with aging and senescence (Chen et al., 2020). Not only nuclear DNA can be subjected to mutations: an oxidative microenvironment

and the lack of histone-binding can expose mitochondrial DNA (mtDNA) to the risk of mutations. While preliminary studies have highlighted the relationship between mtDNA mutations and aging, heteroplasmy (the co-existence of both wild-type and mutated mitochondria within the same cell) makes the evaluation of the results more complex (López-Otín et al., 2023).

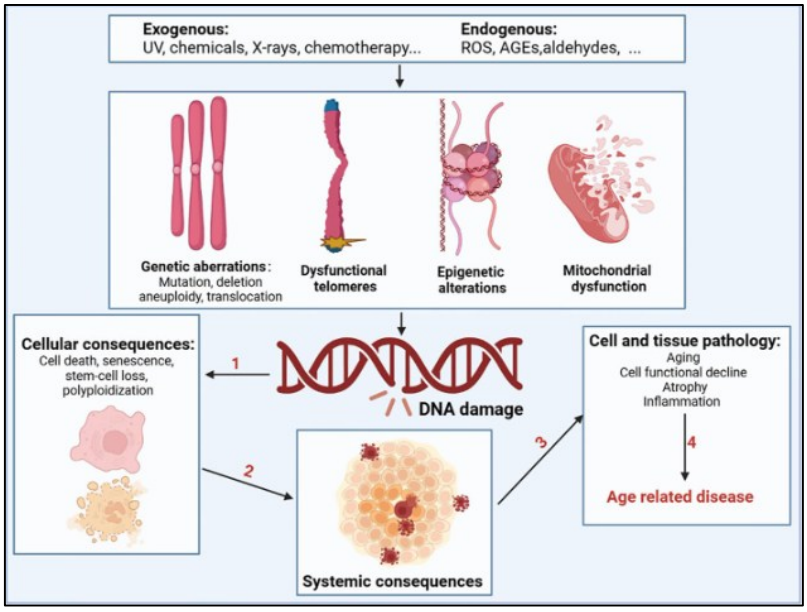

Figure 9. Several exogenous and endogenous damages can alter nuclear and mitochondrial DNA leading to a cascade of events from cellular to systemic impairments, increasing the risk for the onset of pathological conditions (modified from Li et al., 2024).

Other critical regulators of senescence are telomeres, specific portions of nuclear DNA characterised by physiological shortening; telomeric regions are made of telomeric DNA, a protective protein complex, telomeric RNA and an additional scaffold for telomeric proteins, protecting the ends of each chromosome. The DNA polymerase struggles to replicate these regions, and after every cell cycle, telomeres shorten until somatic cell division is not possible anymore and the cell undergoes senescence. This process results in the cell loss of telomeres' protection, determining the accumulation of DNA-damage foci (referred to as telomere-associated foci, TAF) and triggers DNA repair mechanisms, which in aging are fallacious. Increasing evidence suggests that damaged telomeres, like in the case of increased oxidative stress levels, can also be involved in premature cell death (Kalmykova, 2023).

Epigenetic changes, such as DNA methylation, histones modifications, chromatin alterations and deregulation of non-coding RNAs (e.g., up-regulation of micro RNAs), can speed up the aging process adding to genome structure damages, including histone loss (up to 50%), replacement with histone variants or modifications, repositioning of nucleosomes, loss of specific silencing mechanism, such as those ruled by increased condensation of nuclear chromatin. Several

epigenetic changes are observed in neurodegenerative conditions including Alzheimer's disease (AD) and Parkinson's disease (PD), often promoted by aging-associated damages, such as the oxidative stress ones (Bailo et al., 2022; Kandlur et al., 2020). mRNA maturation is also impaired, leading to changes in genes expression during time (Hernando-Herraez et al., 2019); these alterations take part in chronic inflammation, abnormal folding of proteins, regulation of the extracellular matrix and mitochondrial function (López-Otín et al., 2023).

Several neurodegenerative disorders whose onset is age-related, such as amyotrophic lateral sclerosis (ALS), AD and PD, are characterised by protein misfolding and accumulation, which aggregate and form inclusion bodies or amyloid plaques. Normally cells are able to control protein synthesis, folding, transport, post-translational modifications and degradation via the proteostasis machinery which includes autophagy, chaperonins and the ubiquitin-proteasome system activation to prevent protein accumulations. It has been demonstrated that, during aging, these systems (commonly referred to as 'protein quality control', PQC) display decreased efficiency combined with ribosome pausing, which affects normal protein homeostasis. (Chen et al., 2023).

Maintenance of the cell integrity is key for non-proliferating cells such as neurons, hence healthy autophagy is crucial. Neuronal autophagy can be categorised in chaperon-mediated autophagy (CMA), microautophagy and macroautophagy and requires the presence of functioning lysosomes. The latter contain several hydrolases and an acidic lumen for correct molecule degradation by lysosomal enzymes. CMA and microautophagy depend only on these organelles and degrade small molecules or proteins. Contrariwise, macroautophagy needs the production of an autophagic vesicle (the autophagosome), which then fuses with a lysosome and forms the autophagolysosome. An age-related declined in autophagy impairs, not only the normal protein homeostasis, but also the physiological organelle turnover (**Figure 10**) (Kallergi & Nikoletopoulou, 2021).

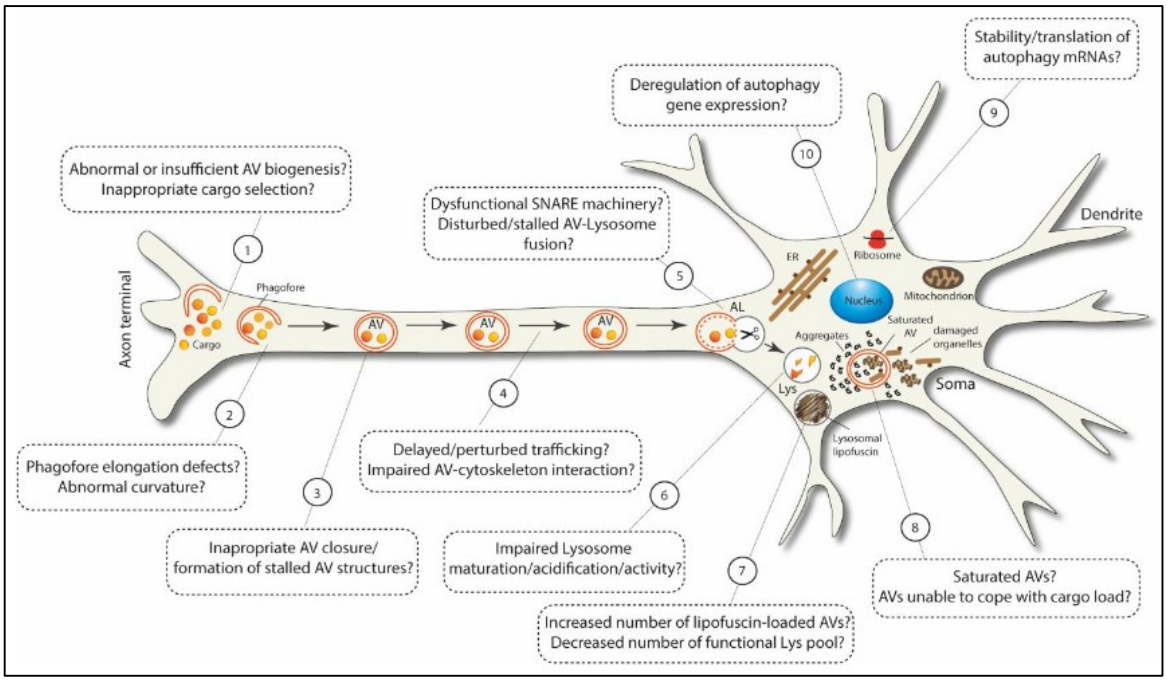

Figure 10. Schematic representation of neuronal macroautophagy impairments in aging, from the formation of the autophagosome vesicle (AV) to the abnormal neuronal trafficking and the lysosomal fusion forming the autophagolysosome (AL) (modified from Kallergi & Nikoletupoulou, 2021).

Mitochondria not only counteract the increase in reactive oxygen species during aging but also supply energy production in cells. During aging, due to the accumulation of mtDNA mutations, the impairment in the production of mitochondrial complexes, the alterations of mitophagy and the decrease of the mitochondrial respiratory capacity, mitochondria may lose their ability to perform quality control of ROS thereby favouring oxidative stress damages. Furthermore, mitochondria are able to maintain their functional and physiological health by fission and fusion, two processes that constitute the so-called “mitochondrial dynamics”. During aging, these events may become dysregulated: excessive fusion promotes mitochondrial biogenesis with accumulation of toxic bioproducts, while excessive fission can lead to mitophagy or fragmentation. Usually, an old senescent cell presents elongated and interconnected, fused, mitochondria (**Figure 11**) (Guo et al., 2023).

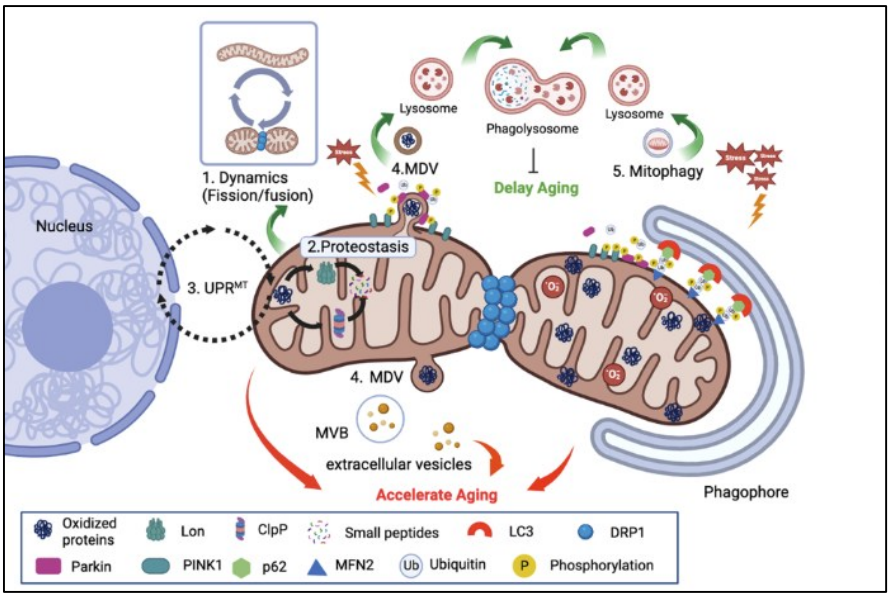

Figure 11. Mitochondrial impairments in aging: physiological mitochondrial dynamics (1), impaired proteostasis (2), secretory path (3), release of mitochondrial derived vesicles for misfolded protein degradation (4) and impaired mitophagy (5), accelerate aging (modified from Guo et al., 2023).

After repeated cell cycles, the senescence program either starts naturally or, due to the accumulation of DNA damages and shorter telomeres. However, in some cases, the senescence process is favoured by external adverse stimuli, including oncogenic mutations, viral or bacterial infection, oxidative damage, nutrient imbalance and mechanical stress. Cellular senescence involves growth stop, hyper secretory and pro-inflammatory phenotype linked to the expression of cyclin-dependent kinases (CDK) inhibitors. Other senescence-associated events include cell volume increase, lysosomal digestion, age-related nuclear and chromatin modifications, as well as the enrichment in pro-inflammatory cytokines, chemokines, extracellular matrix proteases and miRNAs (collectively referred to as senescence-associated secretory phenotype, SASPs) released in an autocrine and/or paracrine manner. Altogether, these elements contribute to a state of age-related chronic inflammation known as “inflammaging”. Over time, senescent cells increase in number and accumulate, promoting the onset of several disorders (Melo dos Santos et al., 2024). Although there are reservoirs of stem cells, which could counteract the progressive cell loss or injury, during aging they are affected by the same hallmarks of differentiated cells, and the trophic factors that maintain stemness or drive their differentiation decrease. (de Silva & Schumacher, 2021). “Inflammaging” state characterising both human and murine aging, strictly related with the cell microenvironment: pro-inflammatory cytokines (e.g., SASPs), oxidative stress, senescent cells and a stiffer ECM, contribute in triggering an inflammation response. The presence of cell

debris (such as damage-associated molecular patterns, DAMPs), damaged organelles and macromolecules can be recognised by the immune system and cause the recruiting of helper T cell type 1 and 17 (TH1 and TH17) and age-associated T cells (Taa), correlated with a decreased self-tolerance and degradation of biological barriers (López-Otín et al., 2023; Zhu et al., 2021).

Another feature of aging, closely related to the senescence process, is the communication between intra- and extracellular compartments: both anti-aging (e.g., IL-37, an anti-inflammatory interleukin) and pro-aging (e.g., IL-1A, favouring TGFβ production) factors are present in the tissue microenvironment, as well as extracellular matrix (ECM) remodelling factors that promote ECM stiffness, cell senescence and ROS production. In particular, ECM stiffness reduces the immune cells migration to senescent tissues (Fafían-Labora & O'Loghlen, 2020). The integrity of the ECM is crucial for the transport of chemicals, as well as cell growth, migration and differentiation. The main constituents of the ECM are collagen and elastin, diversely affected during the lifespan. For instance, increased glycation promotes collagen crosslink, which increases ECM stiffness, making more difficult extracellular matrix remodelling; additionally, collagen glycation also promotes binding with the RAGE (Receptor for Advanced Glycation Endoproducts) receptor, which creates a loop responsible for the increase in ROS and TGFβ levels (Guvatova et al., 2022). During aging, tissue-repairing systems are overwhelmed, and fibrosis is the only process capable of responding, leading to fibrillary collagen accumulation, without a proper molecular turnover. What happens to the ECM produces intracellular changes in mitochondria, calcium homeostasis and cytoskeleton arrangement, modifying nuclear shape and causing chromatin condensation and gene expression changes (Selman & Pardo, 2021).

A further aging hallmark, widely discussed in recent times, is the dysbiosis. In particular, throughout life the composition of the gut microbiota changes and, during aging, these alterations may synergise with a weaker gastro-intestinal (GI) barrier and with "inflammaging". The gut microbiota is essential in immune modulation via microbial metabolites: indeed, through macromolecules' degradation it is able to produce specific metabolites and amminoacids (e.g., tryptophan, phenylalanine and tyrosine). During aging, the gut microbiota presents a less variable bacterial population and produces fewer antimicrobial molecules, increasing the age-associated susceptibility to chronic diseases (Cryan et al., 2019).

1.2.2. Aging of the CNS

During aging, brain morphology and microstructure change, ultimately leading to cognitive decline, memory loss, reduced motor performances and altered behaviour. Focusing on the neuroanatomical features, imaging techniques applied to humans can highlight general or local

volume loss, thickening of the cortex, sulcan depth changes, loss of gyrification and ventricles enlargement (Nyberg & Wåhlin, 2020). Elderly subjects may also display degeneration of both white and grey matter, tissue softening and shrinking, and compromised brain-barriers in the ventricular cavities, all preceded by deficits at cellular level; additionally, 0.45% yearly brain volume loss and 0.45% yearly increase in atrophy can be noticed in old individuals (Hiscox et al, 2021; Kalra et al., 2019). The frontal lobe is the most affected brain region, followed by the temporal lobe, while the parietal and occipital lobes have a non-significant atrophic change (DeCarli et al., 2005). Parallelly, clinical observations report that in aged subjects CSF accumulates within ventricles, causing ventricle enlargement and leading to blood vessels compression, glia activation, periventricular axon stretch and destruction, which result in neurophysiological abnormalities (Attier-Zmudka et al., 2019). Moreover, brain gyrification, which normally starts during development, slows down in adulthood and progressively declines with age, particularly in the parietal lobe. Grey matter thinning is due to more microscopical changes: shrinking and degenerating cells and neuropils, as well as the de-arborisation of the dendritic networks cause a decrease in grey matter volume, resulting in up to 10% cell loss; this severely affects synaptic connectivity and overall brain functionality. Furthermore, white matter in aged brains is characterised by general atrophy, destruction of myelin surrounding axons, vascular impairments and increased inflammation which lead to altered axonal architecture and fluid build-up containing toxic substances (Blinkouskaya et al., 2021). In addition, brain oxygenation and blood supply may decrease due to reduced vasculature branching and length. (Bennet et al., 2024). Meninges, which protect the brain parenchyma, show increased stiffness and decreased elasticity, with increased fibrosis. Blood vessels can become fragile and easily brake compromising the correct CSF drainage, increasing the probability for brain waste accumulation and increased spots of inflammation (Jiang-Xie et al., 2024).

Another feature of brain aging is the damage to the BBB caused by free radicals; the main features characterising BBB impairments are disrupted transporters and receptors function, altered passage of immune cells to the brain parenchyma (diapedesis), barrier leakage which is indicated by the presence of albumin, fibrinogen and IgG around blood vessels. Moreover, the increase in caveolae-mediated non-specific transcytosis and pericyte loss may further aggravate vascular damage. Additionally, the higher permeability to toxic molecules is worsened by the lower turnover of the CSF and the increasing oxidative stress (Banks et al., 2021; Rego et al., 2023).

The aging brain follows the general hallmarks described by López-Otín et al. in 2023 with some features unique to specific cells types and tissue structures. Cellular senescence is one of the main mechanisms that lead to nervous tissue changes and the subsequent neurodegeneration which is

typical of the aging brain. It has been demonstrated that senescent cells increase in number in humans, primates and rodents during aging. Even if they could be eliminated by immune cells, with aging their clearance becomes more inefficient. Similarly, in the brain the main hallmarks of aging include telomeres shortening, DNA damage and accumulation of mutations caused by external dangerous stimuli or oxidative stress, altered chromatin structure, impaired autophagy, mitochondrial dysfunction. Cells' repairing mechanisms become overwhelmed so senescence is elicited via phosphorylation of p53; it is leads to the activation of CDK inhibitors, blocking cell cycle and entrance in the S phase (Liu, 2022). The cell starts to release SASPs, upregulates β-galactosidase, a lysosomal senescence-associated protein, shifts its metabolism on glycolysis and accumulates lipofuscin in the cytoplasm. The increase of the latter is sustained by macroautophagy and neuronal cytoskeleton dysfunctions and impaired cellular trafficking. Other protein accumulations involve amyloid and neurofibrillary tangles deposition; these are typical of AD and/or neurodegeneration, but *in vivo* evidence demonstrated the presence of these deposits in non-pathological aging brains. Some neurons show glycogen inclusions and neurofilaments accumulations too (Lee & Kim, 2022; Wang et al., 2021).

During the lifespan, neurons create different synapses with neighbouring neurons or glial cells but they decline with age. Synapse regulation and homeostasis are allowed by the formation of autophagosomes in the distal axons and consequent retrograde transport to the soma, facilitating content degradation. For instance, Rab5 and Rab35, at the presynaptic terminal direct vesicles to the lysosomes for vesicle and protein turnover. Furthermore, the activation of the TrkB receptor by BDNF can modulate this process by inhibiting autophagy. Unfortunately, aging decreases synaptic proteins integrity as well as BDNF production and release, on the contrary, increases dysfunctional autophagy (Liang & Sigrist, 2018).

Morphologically, the cell becomes larger and flat with an irregular shape caused by cytoskeletal rearrangement and an enlarged endoplasmic reticulum (ER). ER non-regulated mRNA translation leads to the accumulation of unfolded proteins, which may lead to oxidative stress (Zhang et al., 2024; Cao & Kaufman, 2014).

The presence of SASPs (e.g., IL-6) can damage surrounding cells and promote "inflammaging" (Kritsilis et al., 2018). In particular, senescent brain cells trigger an inflammatory response and the recruitment of immune cells, causing their infiltration into the damaged site. Yet, the immune system is also subjected to an age-related decline and dysfunction, resulting in a state of chronic inflammation. Microglia display decreased migration and phagocytosis (inhibited by the upregulation of CD22), increasing pro-inflammatory cytokine (e.g., TNFα, IL-1β and IL-6) and ROS release. Astrocytes can be activated by microglia secreted mediators and enhance

inflammatory activity; these glial cells facilitate massive synaptic elimination in addition to the altered neuronal supporting function. Oligodendrocytes differentiation from progenitors decreases due to the activation of other glial cells, and a progressive myelin degeneration can be noticed. Other factors that exacerbate brain "inflammaging" are (i) impaired meningeal lymphatic drainage, with reduced clearance ability, and (ii) a higher amount of immune cells localised at the level of the choroid plexuses (Zhang et al., 2024).

One of the strategies to maintain tissue homeostasis and counteract cell loss is the adult neurogenesis. This process is granted by the presence of niches composed of neural progenitors, mature neurons, endothelial cells, ependymal cells, astrocytes and microglia, localised in the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus in the hippocampus. Neurogenesis decreases with aging and relies on conditions of the CNS environment (Isaev et al., 2019).

The overall neurotransmission is also affected by aging. Noticeable changes include: (i) a decreases in acetylcholine release effecting motor functions, memory, wakefulness, motivation and reward systems; (ii) a lower amount of monoamines, such as norepinephrine (controlling the autonomic nervous system and certain specific behaviours, like anxiety and alertness) and dopamine (modulating motor functions, reward pathways, mood and emotions), serotonin and BDNF (brain derived neurotrophic factor); (iii) an increase in nitric oxide expression to contrast higher oxidative stress levels. Some neurotransmitters receptors and the receptor affinity also decrease, mostly the dopamine and serotonin ones (Mora et al., 2007). The brain consumes high quantities of oxygen for its homeostasis with the risk of lipid peroxidation and increase in oxidative stress. Therefore, it is important the activation of enzymes, such as GPX, SOD and catalases, to control the amount of reactive oxygen species. Moreover, some neurosteroids (e.g., estrogen and testosterone) play a key role in regulating ROS metabolism and help protect mitochondria from oxidative damage. However, as we age, the levels of these hormones decline (Lee & Kim, 2022).

During aging the decline of mitochondrial functions can be observed due to elevated ROS production, mtDNA mutations and protein oxidation, affecting mitochondrial biogenesis and impairing energy metabolism. Mitochondria are the major site of ROS production, involving complexes I and III (with the ubiquinone cycle) and II (superoxide anion production). With aging, these organelles produce less ATP and are not able to sustain the high metabolic demand associated with the nervous tissue (20% of total body metabolism). ROS may target different proteins, starting from the mitochondrial ones which then decrease mitochondrial quality control. The decreased control then favours protein depositions, including β-amyloids, Tau, DNA binding proteins and SOD1. Mitochondrial stress then decreases AMPK activation, mTOR and SIRT1 phosphorylation

which sustain cell peptide transcription and translation. Mitochondria contain different proteases like Lon, ATP-dependent Clp proteolytic subunit and membrane-bound proteases. Before mitochondrial proteolysis, neurons promote proteasome degradation, but both of these processes are impaired in aging. Mitophagy is also defective; it is modulated by Bcl2 complex and lysosomal LC3 interaction. Other genes involved are *Atg*, *Pink1* and *Parkin* (mutated in genetic onset of PD), all showing a reduction in transcription (Guo et al., 2023).

Another group of changes is associated with the neuronal electrophysiological properties. Several impairments have been reported in literature: (i) increase of the action potential (AP) threshold associated with sodium voltage-dependent channels alterations, affecting transmission properties and computation capability of the brain (Randal et al., 2012); (ii) altered expression of potassium channels involved in the after hyperpolarisation current for the repolarisation of the AP (Disterhoft & Oh, 2007); (iii) myelin sheets degeneration and abnormal distribution of ion channels lead to a decrease in conduction velocity and impaired salutatory conduction. All of these alterations result in compromised neuronal excitability (Rizzo et al., 2015). NMDA and AMPA receptor may also be dysregulated. In particular, during aging, different receptors subunits can be dysregulated and, as a result, impair calcium entrance and neuronal plasticity as well as decrease receptors trafficking and presynaptic protein localisation (like PSD-95). Calcium stores alterations lead to increased glutamate release and cells hyperexcitability, which, coupled with decreased GABAergic inhibition, results in an imbalanced excitation/inhibition equilibrium. Calcium generally increases in old mice in some brain areas, e.g., mice hippocampus, frontal/prefrontal cortex and striatum (Huang & Kandel, 2006; Ontl et al., 2004; Walsh & Akopian, 2019). Calcium entrance could be related with a non-physiological activation of voltage-dependent calcium channels. Calcium signalling is also impaired in astrocytes, contributing to the modified synaptic transmission (Radulescu et al., 2021). In addition, synaptic transmission and organisation are regulated by the extracellular environment, specifically the ECM structure. The brain ECM is synthesized by neurons and glial cells and is composed mainly by hyaluronic acid, chondroitin sulphate proteoglycans part of the lectican family and tenascins-R and –C, involved in the development and shaping of perineuronal nets (PNNs). In particular, during aging there is an accumulation of hyaluronic acid in the brain cortex and cerebellum, which plays a crucial role in the regulation of synaptic plasticity. Another protein associated with neuronal migration and synaptic development, dysregulated during aging, is reelin; reelin physiological levels are crucial to maintain a stable neuronal and synaptic homeostasis since this protein regulates NMDA receptor subunit composition and AMPA receptors clustering (Guvatova et al., 2022).

Dysbiosis is another crucial component recently added to the list of aging hallmarks for the nervous system. The decreasing diversity in the bacterial population of the microbiota results in increasing susceptibility to stress and infections, weakening the gastro intestinal (GI) barrier and, subsequently, the BBB. It has been demonstrated the importance of using probiotics and switching to a more balanced diet to decrease the susceptibility of neurodegenerative or psychiatric disorders. Mediated by the autonomic nervous system (ANS) and hypothalamus-pituitary gland axis, the relationship between the gut microbiota and the brain is bidirectional; it involves several pathways, including immune system responses and signalling pathways involving enteroendocrine cells, neurons and microbiota-derived metabolites. The ANS controls GI motility, secretions and permeability while the brain processes the viscerosensitive inputs and responds through GI organs' innervations (the enteric nervous system, ENS), mainly mediated by the vagal nerve. Several electrophysiological properties change in neurons of the ENS, affecting the information of the microbiota state transmitted to the brain, thereby negatively influencing its regulation (Cryan et al., 2019).

1.2.2.1. The aged cerebellum

Compared to other brain areas, the mechanisms that subtend cerebellar aging are not fully understood and mostly very similar to the rest of the CNS. Some of the age-related motor and cognitive impairments can be attributed to changes in the cerebellar structure and connections developed over time. The cerebellum makes and receives projections from several brain areas, from basal ganglia and hypothalamus to the limbic system and hippocampus, taking part in different cognitive and biological processes. In particular, the different relationships created can suffer from all the aging changes and give rise or participate in neurodegenerative disorders, like AD, PD and ataxia, whose incidence increases over time (Arleo et al., 2023).

As for the overall brain volume, also the cerebellum shrinks (even earlier than the hippocampus); specifically, the vermis region and lobules VI and VII undergo volume loss, causing progressive decline of memory, visual reproduction and speed processing. Moreover, the degeneration of white matter fibers, as for grey matter volume, can affect the fronto-cerebellar and parietal cerebellar circuit and contribute to cognitive decline. On a microscopic scale, the cerebellum is subjected to neuronal loss as well: PCs decrease nearly by 11.7% and GrCs by 12.7% mainly in the anterior lobe. Specifically, PCs are the most susceptible to hypoxia, ischemia and aging, probably because they are the only cerebellar output region, hence fundamental for the delivery of processed information. They are damaged by excessive glutamatergic release and a lower metabolism in the cerebellar cortex, as well as by the loss of neuroglobin (a protein important for the neurons

homeostasis and oxidative species scavenging) and the loss of synaptic connections with parallel fibers, leading to necrosis and iron-dependent ferroptosis (i.e. cell death mechanism triggered by an increase in intracellular iron levels which leads to ROS accumulation and membrane damages). Under these conditions, PCs show a smaller dendritic tree, increased soma volume caused by lipofuscin accumulations and mitochondrial abnormalities which increase cell death probability; in mice, the expression of Complex I decreases with a higher turnover of Complex III (ubiquinol-cytocrome C oxidoreductase) and Complex IV (cytochrome C oxidoreductase), resulting in lower expression of some proteins such as NADH-dehydrogenase and cytochrome C (Popa-Wagner et al., 2018). Studying alterations in MFs and climbing fibers is more complex: they tend to change in total length and diameter, appearing thicker with age but showing reduced varicosities. Depending on the cell type, molecular and granular layer interneurons display a heterogeneous behaviour: indeed, basket cells are more likely to decrease, while GoCs either increase or don't change in number but can show a smaller ER, thereby suggesting a reduction in protein synthesis; molecular layer cells become smaller but increase in number (Andersen et al, 2003). Moreover, during aging cerebellar neurons display several changes in gene expression patterns, which may impact on the overall metabolism: for example, GrCs tend to express less neuronal nitric oxide synthase (Iskusnykh et al., 2024; McElroy et al., 2024).

Another important evidence to evaluate synaptic connections is the co-localisation of Pax6 and Ras-GAP, important for the correct functionality of brain synapses: an *in vivo* study in mice, highlight decreased in PAX6/Ras-GAP co-localisation within the cerebellum of aged mice, suggesting functional alterations during aging (Srivastava et al., 2018).

Furthermore, mice cerebellum shows the downregulation of protein families such as neurotransmitters receptors (e.g., GABAergic and glutamatergic), signal transduction enzymes and ion channels (Iskusnykh et al., 2024; McElroy et al., 2024). However, the cerebellum shows decreased and later onset of epigenetic changes compared to other brain areas and tissues, suggesting that, from a chronological age point of view, the cerebellum appears "younger" (Hovarth et al., 2015).

In the cerebellum, many aging-related molecular mechanisms appear early on compared to other brain areas: activation of the senescence pathway, impaired protein production and accumulation, as well as DNA repairing systems defects which lead to progressive neuronal loss, a prominent feature of cerebellar aging. These alterations ultimately lead to impairments in cerebellar functionality, including deficits in coordination, loss of fine motor control, balance and gait dysfunctions as well as a decline in overall cognitive functions (McElroy et al., 2024; Arleo et al., 2024).

1.2.3. Oxidative stress

Oxidative stress and aging are closely related. Different hallmarks attribute their worsening or acceleration, if not their onset, to redox imbalance making the latter one of the key mechanisms of aging (López-Otín et al., 2023).

Oxidative stress is caused by the overproduction of reactive oxygen species (ROS), which may generate molecule deterioration and can shift cells redox balance towards an oxidative state, thereby causing cellular impairments and ultimately cell death. Oxidative species are normally produced under physiological conditions; therefore, the cell is endowed with internal strategies to neutralise them or externally-provided components that support ROS elimination. Defective antioxidant systems are involved in several pathologies, including neurodegeneration, and have the most detrimental impact on tissues with a high oxygen demand, low regenerative potential and generally low antioxidant capacities, i.e. the brain (Teleanu et al., 2022).

The causes of oxidative stress are both exogenous and endogenous. ROS primarily derive from molecular oxygen (O_2) and ozone (O_3), which produce reactive species such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (•OH). During the electron-chain transport, the production of the superoxide anion results from the interaction between molecular oxygen and leaked electrons, specifically during the reactions that involve ubiquinone and NADPH oxidase; then, the latter and other enzymes, including superoxide dismutase (SOD), reduce $O_2^{\bullet-}$ to H_2O_2. The amount of H_2O_2 produced may cause the development of a stressful environment and an imbalanced redox signalling. This molecule doesn't directly oxidise biological systems: it reacts with iron forming the hydroxyl radical, which in turn interacts with lipids. Additionally, $O_2^{\bullet-}$ can interact with nitric oxide and generate peroxynitrous acid, a strong oxidant; peroxynitrous oxide can decompose, forming nitrogen dioxide (•NO_2) and •OH. Physiologically, cell homeostasis involves the natural oxidative molecules production for specific purposes (H_2O_2 is an intracellular messenger) but the redox balance is always maintained. Both •NO_2 and •OH can bind hydrogen molecules and interact with tyrosine in indiscriminate proteins, hence they are considered the protagonists of oxidative stress damages. Promoting the degradation or rapid deactivation of ROS-generating reactions are the best ways to prevent their dangerous accumulation. In aging, nitric oxide and hydroxyl radicals dangerously accumulate (Forman & Zhang, 2021). Yet, they are not the only oxidants in biological systems: alongside with free-radicals, generated by reduction reactions and characterised by an unpaired electron, also non-radicals, carbonyl compounds, chlorine and bromine, sulphur species, low-molecular-mass compounds (e.g., glutathione) and selenium species can worsen an imbalanced redox environment. The main enzymes responsible for ROS production are manly mitochondrial complex I, II and III but their activity depends on

the type of tissue and protein synthesis. NADPH is important for the regulation of NADPH oxidase, nitric oxide synthase (NOS) and cytochrome P450 hydroxylation, and is also linked to some specific pathways like the NAD^+/sirtuins ones. Under stress conditions, both NADPH and NADH are consumed to produce ATP, decreasing their contribution to ROS scavenging (Sies et al., 2017). Several other antioxidant genes are then expressed like *Gpx* and *Sod* families, *Gsh*, cytochrome c oxidase and coenzyme Q; additionally, the presence of exogenous antioxidant molecules can help in decreasing mitochondria damage induced by ROS, including ascorbic acid, vitamin E and carotenes (Jomova et al., 2024).

H_2O_2 can be an important second messenger, alongside with hydrogen sulphide and nitric oxide (NO); indeed, it can control cytokines and calcium signalling, modulate proliferation, survival or death via activation of downstream pathways, and it can control pro-inflammatory or pro-fibrotic signalling as cytoprotective mechanisms. However, excessive ROS production may decrease the activity of antioxidant enzymes and impair the normal cell defences against oxidative damage. As a consequence, ROS-mediated lipid peroxidation may occur and lead to membrane disaggregation and cell death, or induce damages to DNA sugar-phosphate backbone or nitrogenous bases (Checa & Aran, 2020). Furthermore, ROS can induce protein structural modifications and decrease activation of some protein kinases like PKA, MAPK, and PI3K, modifying their catalytic site. RONS (Reactive Nitrogen and Oxygen Species) increase with aging and are present in different age-related disorders compromising cell homeostasis and causing the deterioration of soft tissues (Hajam et al., 2022). Moreover, they are strictly related to neuroinflammation, which comprehends all the deleterious events that can activate an inflammatory response in the nervous system, promoting a series of signalling cascades, increasing the expression of pro-inflammatory genes and the activation of microglia (Teleanu et al., 2022).

Mitochondria are the main organelles responsible for counteracting ROS formation. They contain several endogenous antioxidant enzymes, such as superoxide dismutases (SOD1 and SOD2), as well as glutathione (GSH), which work to neutralize ROS. However, an increase in ROS levels can result from external factors or internal mitochondrial dysfunction—such as changes in membrane potential leading to hyperpolarization. When this happens, the mitochondria's ability to manage oxidative stress becomes compromised (Angelova & Abramov, 2018). Additionally, mitochondria maintain a close functional relationship with the endoplasmic reticulum (ER), and they can be adversely affected by ER stress, particularly during aging resulting in progressive loss of cellular homeostasis and ultimately in compromised tissue functionality (Hajam et al., 2022).

Superoxide dismutase enzymes

SOD1 and SOD2 are part of the superoxide dismutase family able to convert superoxide radicals in oxygen and hydrogen peroxide (**Figure 12**). They need the presence of metal ions like manganese (Mn), iron (Fe), nickel (Ni) and copper (Cu). All mammals contain CuSOD1, MnSOD2 and CuSOD3 (in the extracellular compartment). SOD1 (superoxide dismutase 1) is primarily located in the cytosol but can also be found in mitochondria and peroxisomes; specifically, this enzyme is involved in decreasing superoxide levels in the cytosol, mitochondrial intramembrane space and peroxisomes. More recently, some new functions were reported associated with SOD1: modulation of transcription factors after oxidative stress exposure, regulation of RNA metabolism, control of glucose sensing pathway to repress cell respiration and interaction with TORC1 in the nutrient sensing signalling paths. SOD1 is an homodimeric protein of 32kDa composed of 153 amminoacids encoded by a gene localised on chromosome 21 in humans and on chromosome 16 in mice. It's a highly conserved protein in eukaryotic organism, including mammals. Near and in the catalytic site, SOD1 required copper or zinc ions, to be able to bind water molecule needed for the reduction of superoxides to produce H_2O_2 (Eleutherio et al., 2021). SOD1 translocation and positioning in the mitochondria is strictly associated with CCR (C-C chemokine receptor). The main function of SOD1 is its antioxidant activity and it functionally synergise with SOD2 to decrease $O_2^{\bullet-}$ content. It can also act as a transcriptional factor for approximately 123 genes after moving from the cytosol to the nucleus; it induces cellular defence genes, DNA replication and DNA damage response genes, linked with redox balance and stress-response genes. The production of H_2O_2 can physiologically modulate signal transduction pathways for cell proliferation, differentiation and death, making SOD1 indirectly involved in these processes. The phosphorylation of SOD1 decreases the enzyme activity in a nutrient-rich environment while the de-phosphorylation minimizes oxidative stress under conditions of nutrient deficiency (Trist et al., 2021). Despite being one of the main protagonists against oxidative stress, SOD1 may undergo post-translational modifications when ROS levels increase hazardously. Usually, this leads to misfolded and destabilised proteins, facilitating both SOD1 or amyloids aggregation in various tissues. Moreover, misfolded SOD1 can also be characterised by metal deficiency, disulphide bonds reduction and monomerization. During aging, the accumulation of misfolded SOD1 not only prevents proper ROS neutralisation but can also contribute to senescence due to the formation of protein aggregates, potentially leading to pathological conditions such as ALS, AD and PD (Shafiq et al., 2021).

SOD2 is co-localised with SOD1 inside mitochondria (more specifically in the matrix) and is important for the physiological scavenging of reactive oxygen species, produced by the electron

chain reaction (electron transport chain, ETC); the enzymatic reaction is the same as for SOD1 with the difference of the ion co-factor, manganese for SOD2. It weights 24kDa and is positioned on chromosome 6 in humans and chromosome 16 in mice. This enzyme is composed of four identical subunits binding Mn and acetylation plays a key role in the regulation of SOD2 catalytic activity: higher energy production by mitochondria decreases this modification hence promoting $O_2^{\bullet-}$ dismutation. Furthermore, the de-acetylation is performed by SIRT3 and NAD^+, while NADH accumulation due to impaired ETC promotes the acetylation. Other post-translational modifications give SOD2 a role in controlling cell entrance in the proliferative state. If Mn is replaced with Fe, for example due to an imbalanced diet, SOD2 acquires a pro-oxidative phenotype, promoting a pathological state. Finally, both the decrease in SOD2 expression and its overexpression can cause cell damages: in the first case it causes impairment in the correct ROS scavenging, while in the second situation can promote the pathological accumulation of oxidative enzymes. Still, if $O_2^{\bullet-}$ escapes mitochondria and diffuses into the cytosol, PRDXs (peroxiredoxins) and GPX (glutathione peroxidase) inactivate it, counteracting possible SOD inefficiencies, but these enzymes need NADPH and a healthy cell metabolism to properly function (Palma et al., 2020). The role of SOD1 and 2 is crucial for the brain because the high energy demand to maintain synaptic connections; yet, generally the defence against ROS accumulation decreases with age and the majority of the damages affect synaptic terminal mitochondria (Flynn & Melov, 2013).

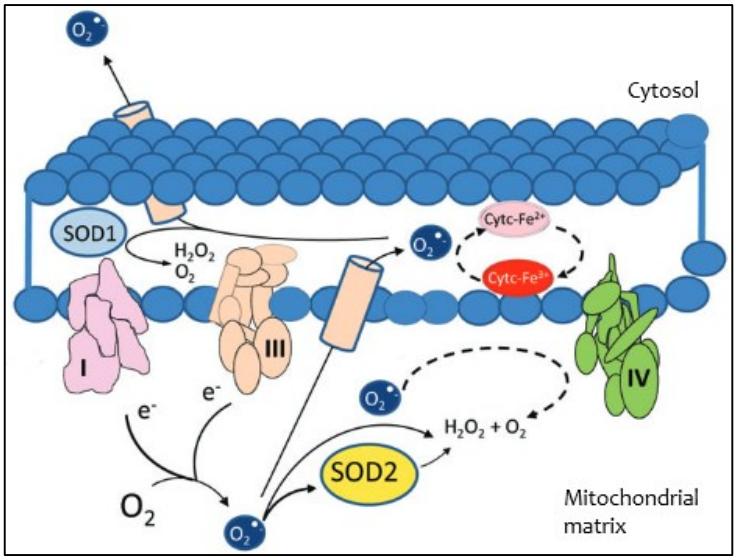

Figure 12. Localisation of SOD1 and SOD2 in the mitochondria and their catabolic activity (modified from Palma et al., 2020).

Inducible nitric oxide synthase

Other markers widely employed to investigate alterations in the oxidative stress pathway are nitric oxide synthase (NOS) enzymes. The NOS enzymatic family is important for the production of

NO, a free radical, with several physiological roles including vasodilation, smooth muscle relaxation, neurotransmission and immune response. There are three isoforms of NOS: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). The firsts two are constitutively expressed in neurons for neuronal signalling (nNOS) and in endothelial cells for vasoconstriction and blood pressure control (eNOS), while iNOS can be induced by proper stimulation, e.g., by pro-inflammatory cytokines. In response, it produces a high amount of NO that lasts until the enzyme is degraded. Despite playing physiological roles, high concentrations of iNOS or its dysregulation are associated with several human disorders (Cinelli et al., 2020).

iNOS weights 131 kDa and has two major domains: the C-terminal reductase and N-terminal oxygenase. It produces NO and L-citrulline from L-arginine via an electron chain reaction involving NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme and (6R)-5,6,7,8-tetrahydrobiopterin (H_4B); this process is calmodulin (CaM) dependent and needs the presence of zinc to bind H_4B (Cinelli et al., 2020). iNOS is involved in several biological functions: for instance, it regulates insulin-responsive mechanism and has psychochemical properties. NO production through iNOS can be stimulated by HIF-1, a transcription factor that switches the cell metabolism from oxidative to glycolytic, in inflammatory conditions. NO then stabilises HIF-1 to rise cellular oxygen levels (Anavi & Tirosh, 2020). In the CNS mainly astrocytes and microglia produce high amount of NO and release it on neurons. NO activity is then coupled with sGC (soluble guanylate cyclase), required for long-term depression in PCs and for voltage-dependent calcium channel regulation in the rat hippocampus, as well as for the modulation of potassium channels activated by cGMP in the DCN. NO and iNOS can activate other cellular signalling pathway intermediates important in neurophysiology and immunomodulatory mechanisms. Considering the nature of NO, in the CNS, its presence is both positive and negative: increased levels of NO may react with ROS, producing peroxynitrite which can trigger neuronal death via necrosis or apoptosis leading to neurodegeneration. Moreover, NO can both induce ER and mitochondrial ECR (electron chain reaction) stress, causing the release of cytochrome c, leading to increased caspases activity and apoptosis. RONS cause excitotoxicity, increasing intracellular calcium entrance (dysregulating NMDA receptors) and enhancing nNOS activity (Doherty, 2011).

Cytochrome c oxygenase 4

COX4 is part of the fourth protein complex present on the mitochondrial inner membrane important for the ECR to create the electrochemical gradient to produce ATP. The mammalian cytochrome c oxygenase (COX) has 13 subunits: the first three are encoded by mtDNA and the other nine by nuclear DNA. The first two subunits operate to reduce oxygen and generate the

electrochemical potential, the others can modulate the activity of the complex. Subunit 4 can bind ADP or ATP increasing or decreasing the binding with the cytochrome c, defining the "active state" (with ADP) or the "relaxed state" (with ATP). The switch to "active state" depends on increased calcium concentration during active work or stressful conditions, while it is unflavoured by the phosphorylation on subunit 1. COX normally produces ROS (mainly in the "relaxed state") which are degraded by SOD, but in cases of inflammation, mitochondrial damage and in the presence of stress-related molecules, mitochondria membrane potential decreases and intracellular calcium increases activating COX and rising ROS production, triggering cell death. COX4 is present in two different isoforms: (i) COX4I2 is expressed in hypoxic conditions induced by HIF-1 and LON (ATP-dependent protease La), favouring the binding with ATP to increase energy production but producing ROS; (ii) COX4I1 is also expressed during hypoxic condition but can be switched with the other isoform if oxygen tension decreases more than 1%, favouring a better energy production (Kadenbach, 2021; Timón-Gomez et al., 2018). COX4I2 is more expressed by astrocytes to provide a better support to neurons since they have high metabolic demand, but this isoform also produces more ROS compared to isoform 1 causing neuronal damage over time (Arnold, 2012).

In the CNS, COX4 appears to be important to preserve the correct synaptic functionality, their maintenance and connectivity: knock-out mice for COX4-1 and *in vitro* models showed synaptic loss and an altered neuronal transmission (Kriebel et al., 2020).

1.3. Nutraceuticals

Nutraceuticals are single elements or a combination of them, part of a specific diet, that exhibit clinical significance and can be used as adjuvants in the prevention and therapy of several diseases as well as in healthy conditions to boost specific protective mechanisms, like immunity, and decrease illness onset. Normally they are products that represent an advantage to human health and are already present in the nutritional content of meals, but they can be part of other food-derived categories such as supplements, herbal products, pre- or probiotics, functional and "fortified" food, adding helpful compounds. The contemporary interest in nutraceuticals is related to their potential safety and nutritional purposes that can be translated in the medical field. They can modulate several biological mechanisms including gene expression, antioxidant defence, cell proliferation, and mitochondrial integrity, with the aim to slow down aging and extend lifespan, preventing the onset of chronic illnesses (Puri et al., 2022).

They can be classified considering their significance, origin or target based on: (i) clinical significance (dividing them into potential or established nutraceuticals); (ii) source (animal, herbal, marine, recombinant, or food-derived nutraceuticals); or (iii) therapeutic use (for disease prevention, health promotion - like antioxidants - and to prolong lifespan) (Jain et al., 2022).

Nutraceuticals may have more than one beneficial property. In addition, most of them are usually found in common foods, making them safe and easy to self-administer. During the last decade, the demand for natural products and their derivatives has increased, particularly in response to the necessity for improving immunity, cardiovascular and brain health, coupled with the drive towards a healthier diet and lifestyle, designed to meet individual necessities e.g., personalised-medicine approaches to treating disorders (AlAli et al., 2021).

An example of nutraceuticals are phytochemicals: plant-derived products with a natural protective role in plants. In this regard, carotenoids are nutraceuticals contained in bell peppers, responsible for the characteristic yellow, red or orange colour; they include carotenoids, lycopene, astaxanthin, lutein and zeaxanthin (Jiang et al., 2024). Other examples are micronutrients or minerals, such as vitamins or calcium, or several fatty acids which have been demonstrated to be protective against heart disorders (Chandra et al., 2022).

1.3.1. Nutraceuticals in aging and oxidative stress

Aging is an inevitable process that causes a decrease in physical and cognitive capacities and increases the risk of developing diseases characterized by impairments in the physiological functions of several cells and tissues. Nutraceuticals can play an important role in slowing down the aging process. They can modulate the production of mitochondrial components, ameliorate

energy metabolism and decrease oxidative stress, for example through a diet rich in riboflavin, magnesium, lipoic acid or niacin (Fila et al., 2021). Moreover, compounds like saffron, resveratrol and quercetin can inhibit the release of pro-apoptotic factors (e.g., Bcl-2 or caspases), decreasing neuronal death and counteracting the effects of a pro-inflammatory environment, both typical of aging and neurodegenerative conditions (Li et al., 2019). Herbal and natural medicine can benefit mitochondrial health, promoting a better intracellular metabolism encouraging synaptic plasticity, protein synthesis and the release of neurotrophic factors that improve memory formation and better cognitive performance (Siddiqui et al., 2024). Better cognitive functions are also mediated by neurotransmitter receptor activation which are also affected by aging. Biomolecules behave as receptor agonists, antagonists or receptor modulators with either a toxic or beneficial activity. Molecules with a neuroprotective potential are curcumin and green tea extract (containing theanine) which bind serotonin and dopamine receptors and GABAergic receptors, respectively (Silva et al., 2019).

Several bioactive compounds can take part in epigenetic modifications, gene expression, chromatin remodelling and non-coding RNA expression with the aim of modulating the senescence pathways and the release of SASPs; for instance, curcumin can modulate histone deacetylases and some sirtuins isoforms, or up-regulate histone acetyltransferases. Telomeres and telomerase are also affected by the beneficial activity of natural compounds: when administered to mouse models, nutraceuticals increase TERT (telomerase reverse transcriptase) expression both in cortical areas and in the cerebellum, not only in postnatal or embryonic periods but also in old mice (Tsoukalas et al., 2021).

Proteostasis and autophagy are very important for protein turnover and scavenging of misfolded or aggregated proteins. Impairments in these mechanisms counteracted by therapies that improve protein homeostasis may ameliorate the aging process. Studies on spermidine and fisetin demonstrated decreased Tau phosphorylation and increased expression of autophagy-related genes including LC3-II and *Atg*. Additionally, polyphenolic compounds don't interact directly with mTOR, hence avoiding a possible negative effect on autophagy (Gruendler et al., 2020). The release of pro-apoptotic factors is regulated by intracellular calcium which, in neurons, increases dangerously. Ginesenoid, a component of ginseng, is able to reduce calcium concentrations, decrease free radicals, promote mitochondria turnover and inhibit apoptosis (Siddiqui et al., 2024). One of the strategies that could slow down aging is neurogenesis, which physiologically decreases during aging. Ginseng polysaccharides stimulate neurogenesis in the hippocampus by decreasing neuronal loss and improving neuronal development and function, sustained by more protected mitochondria, DNA repair and decreased oxidative stress (Shin et al., 2021).

Natural compounds can also oppose “inflammaging”. For example, polysaturated fatty acids have been shown to regulate gene expression of pro-inflammatory mediators, to inhibit T cell proliferation and decrease pro-inflammatory cytokines produced by T helper cells. Polyphenols contained in blueberries and pomegranate can prevent oxidative stress which plays a crucial role in triggering the massive immune response in the cerebellum of aging mice (Verdù et al., 2023). Vitamin D supplementation is able to decrease IL-6 and INF-γ in humans and vitamin E showed to have anti-inflammatory and anti-oxidative effects in preclinical animal studies (Dugan et al., 2023).

The integrity of the BBB can prevent the infiltration of toxic molecules and active immune cells, and nutraceuticals may play a protective role towards the BBB as demonstrated both *in vivo* and in pre-clinical studies: some natural compounds can pass the BBB and reduce barrier damages, while others don’t need to cross it to have a protective ability against oxidative stress and peripheral inflammation. For instance, herbal compounds, such as β-carotene, vitamin C and Rosmarinic acid can regulate efflux pumps, partially or fully restore the barrier integrity, block the downregulation of gap junction proteins and increase endothelial cells survival (Kocsis et al., 2025).

Overall, nutraceuticals are able to decrease oxidative stress one of the main causes and the main participant in several aging mechanisms. Therefore, the ability to modulate oxidative stress through the beneficial effects of natural compounds may counteract the onset of disorders caused by the age-related increase of ROS e.g., neurodegeneration. Some alkaloids and flavonoids can reduce the expression of iNOS or modulate SOD enzymes; for instance, rutin, a plant-derived natural flavonoid, can decrease ROS and NO as well as inhibit NADPH and promote the transcription of anti-inflammatory genes. On the other hand, aloperine, an alkaloid isolated from the herbal plant Sophora alopecuroides, has been shown to act on the Nrf2-regulated pathways and enhance its anti-oxidative role (Allam et al., 2022).

Several studies highlight the ability of carotenoids to scavenge ROS, thanks to the conjugated bonds, and to promote the expression of Nrf2. A diet rich in carotenes can also help maintaining telomeres length. Carotenes are effective against UV light damage, sunburn and skin aging, as well as protect or restore compromised physiological functions associated with biological time progression, like in neuroinflammation. For example, lutein can improve cognitive ability, memory, attention and learning, while β-carotene can improve motor functions (Bakac et al., 2023). Furthermore, after the intestine absorption, β-carotene is transported to the liver where is converted into vitamin A, which proved to improve eye-sight, or stored. Animal and human studies also reported that carotenoids modulate anxiety and depression symptoms (Damming-Adams et

al., 2020). Vitamin C (ascorbic acid) is an enzymatic co-factor and a strong scavenger of ROS, and plays numerous roles within cells: it can co-operate in collagen polymerisation, tyrosine metabolism, proteolysis caused by hypoxia, regulation of DNA and RNA epigenetic modifications and histone demetilation. Vitamin C is also important for the synthesis of peptidic hormones and catecholamines and has an anti-inflammatory and immune-boosting activity; additionally, it improves iron absorption and it's able to restore the antioxidant capacity of vitamin E (Sato et al., 2024).

1.3.2. The Voghera sweet pepper

The Voghera sweet pepper (*Capsicum annuum*) is a variety of sweet pepper cultivated in province of Pavia. In 2006 the Voghera pepper has been reintroduced as a dietary product in 2006, after an epidemic of root mycosis happened in the late forties. The Voghera pepper has a small cubic shape with four lobes, it's more resistant to a cut compared to other commercialised sweet peppers but it has a higher content of organic matter. The characterisation of the phenotype and the nutraceuticals content (listed in **Table 1**) of this vegetable is meant to show the benefits of the reintroduction of an Italian sweet pepper variety which additionally holds potential antiaging and antioxidant properties. Indeed, it has been demonstrated to contain a higher amount of Vitamin C compared to the recommended daily dietary intake and of other natural products, i.e. Quadrato d'Asti and Cuneo, demonstrating to have a positive effect in several aging processes (Cavagna et al., 2012).

Table 1. Content of carotenoids and ascorbic acid found in the extracts of the Voghera pepper compared to the Carmagnola pepper (Gola et al., 2023).

Bioactive Compounds (μg/g)	Voghera Pepper (VP)	Carmagnola Pepper (CP)
Carotene derivatives	n.d.	21.8 ± 0.7
α-carotene	11.3 ± 0.2	13.9 ± 0.2
β-carotene	9.6 ± 0.1	21.4 ± 0.7
Trans-β-carotene	n.d.	14.4 ± 0.2
Lutein	16.7 ± 0.2	11.5 ± 0.02
Zeaxanthin	9.6 ± 0.6	15.4 ± 0.1
Zeaxanthin derivatives	n.d.	14.1 ± 0.2
Total carotenoids	55.4	112.5
Ascorbic Acid	724.2 ± 0.37	378.5 ± 0.25
Dehydroascorbic acid (DHAA)	17.5 ± 0.50	13.4 ± 0.44

Additionally, the Voghera pepper contains different types of carotenoids and higher amounts of ascorbic acid compared to the Carmagnola pepper (Gola et al., 2023).

Recent *in vitro* studies on human-derived fibroblasts, demonstrated that Voghera pepper extracts hold both proliferative and antioxidant effects when compared with a control pepper extract, i.e. Carmagnola pepper. In particular, after a 24-hour treatment, the Voghera pepper is able to restore a young phenotype on old fibroblasts decreasing the cell surface observed in the control old condition typical of senescent cells; it improves DNA synthesis, supports a healthy cellular structure, increases proliferation in both young and old cells and decreases p53 and p16 expression, specific markers of senescence. Furthermore, it ameliorates functional and morphological integrity of mitochondria, specifically by improving the respiration process and decreasing oxidative damage by decreasing ROS, followed by lower Nrf2 expression, a transcription factor that leads to antioxidant pathways activation, including SOD (Ngo & Duennwald, 2022). Moreover, the Voghera pepper extract can increase COX4, COX2 and Aco2, enzymes whose expression is important for mitochondrial functionality, hence slowing down the aging process. In addition, SIRT1 up-regulation promotes cell survival and protection against oxidative stress (Gola et al., 2023; De Luca et al., 2024).

2. AIM OF THE STUDY

Aging is a universal and inevitable process, defined by the progressive decline in physical, cognitive and biological functions. Several macroscopical and microscopical impairments characterise the aging process and may accelerate it, increasing the probability of developing age-related disorders, such as neurodegenerative diseases. Some of the most in-depth known hallmarks of aging are: DNA damage, epigenetic modification, impaired proteostasis and autophagy, increased cell death, compromised mitochondrial metabolism, altered neuronal features, changes in synaptic transmission, chronic inflammation and a compromised gut-brain axis.

Nutraceuticals are compounds often found in widely consumed foods and they have been demonstrated to effectively promote "healthy aging", by slowing down or reverting some detrimental, yet physiological, mechanisms. For example, carotenoids, polyphenols, certain ions and fatty acids are capable of modulating neuroinflammation, neuronal senescence and oxidative stress. Previous *in vitro* investigations have revealed that extracts from the Voghera pepper, an Italian agricultural product, can modulate oxidative stress, contrast cellular senescence and promote cellular proliferation both in young and old human fibroblasts. These gathered findings suggest that the Voghera pepper could be a potent ally in promoting healthy aging.

Accordingly, this thesis aimed to evaluate the effects of a phytotherapeutic implementation using extracts from both the fruit body and by-products (placenta, seeds, and pedicel) of the Voghera sweet pepper in a murine aging model. In particular, the potential impact of the pepper extracts on the cerebellum of mice at different ages was evaluated, assessing possible morphological changes through specific histological stainings (Nissl and Picrosirius Red), as well as alterations in the expression levels of key markers involved in the oxidative stress pathway, i.e. SOD1, SOD2, iNOS, and COX4, within the cerebellar cortex.

3. MATERIAL AND METHODS

3.1. *In vivo* model

In this thesis study young and aged C57BL/6 mice (**Figure 13**) were maintained following the standard animal care protocol ruled by the Italian low and kept in the Animal Care Facility at the University of Pavia (Italy) at 21 ± 2 °C, with humidity at 50 ± 10%, and under a 12 h light/dark cycle throughout the experiments. Water and food (standard pellet) were provided regularly ad libitum. The present experimental protocol was approved by the Ministry of Health (protocol n. 935/2023-PR, 30/10/2023) and followed the EU Directive 2010/63/EU. This thesis is part of the project NODES which has received funding from the MUR – M4C2 1.5 of PNRR funded by the European Union - NextGenerationEU (Grant agreement no. ECS00000036).

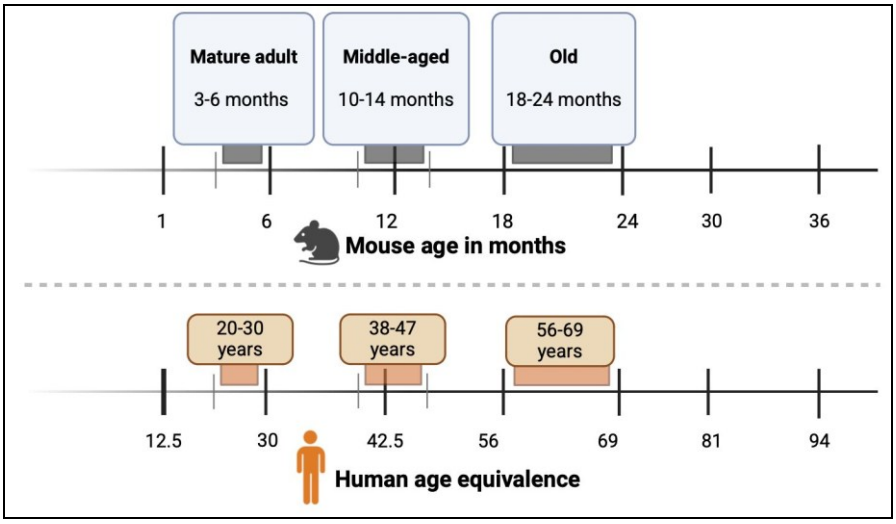

Figure 13. Conversion of C57BL/6 mouse age to human equivalent age (image created with BioRender.com).

3.2. Animal treatment

A total of 30 C57BL/6 mice, at two different ages, were purchased from Charles River Italia (Calco, Italy) and divided into two groups: 15 two-month-old (young group) and 15 eighteen-month-old (old group) mice. Pathogen-free mice were acclimatized for two weeks before treatments. Mice were kept in cages with one mouse per cage. Both young and old group were divided into three subgroups: 5 mice in the untreated young mice control group (CtrlY) and 5 mice in the untreated old mice control group (CtrlO), 5 mice in the young mice group treated with the Voghera pepper fruit body extract (VpY) and 5 mice in the old mice group treated with the Voghera pepper fruit body extract (VpO), 5 mice in the young mice group treated with the by-products of Voghera pepper extract (VpwY) and 5 mice in the old mice group treated with the by-products of Voghera pepper extract (VpwO). The treatment extracts were obtained using an

aqueous solution containing 45% H_2O and 55% 1,2- propandiol mixed either with the Voghera pepper pulp or the by-products, i.e. stem, seeds and placenta.

Both Vp (fruit body extract) and Vpw (by-products extract) were supplemented for two month in the drinking water ensuring that each mouse received 1 mg/day of extracts, which corresponds to 1g/day for humans. The Ctrl (control group) received the vehicle.

3.3. Cerebellar specimens collection and experimental workflow

After *in vivo* treatment, mice were deeply anesthetised by isoflurane inhalation before decapitation (Aldrich, Milwaukee, WI, USA). Cerebella were excised and washed in a 0.9% NaCl solution and subsequently fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 7 hours. The organs were dehydrated in an ascending graded ethanol scale and put in acetone before being embedded in Paraplast X-TRA (Sigma Aldrich, Milan, Italy). After drying, the samples were cut with a manual rotary microtome to obtain 8 µm thick sagittal section at the vermis level, serially collected on silane-coated slides.

The cerebellar tissues were then processed to perform histochemical evaluation with the nervous tissue-specific Nissl staining and the Picrosirius Red (PSR) staining, and immunohistochemical reactions to evaluate the expression of oxidative stress markers in the murine model of aging after the different Voghera pepper treatments (**Figure 14**).

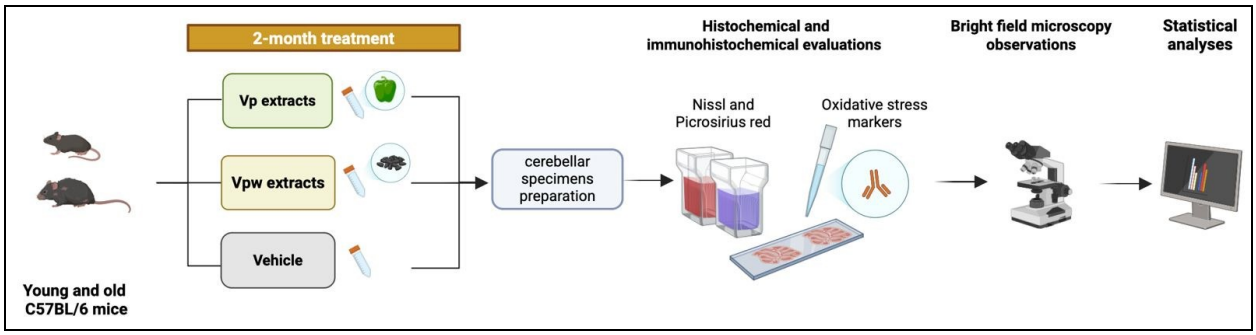

Figure 14. Graphical representation of the experimental workflow (image created with BioRender.com).

3.4. Histological staining

Cerebellar sections were first deparaffinized with xylol for 10 minutes and then re-hydrated with a descending-graded ethanol scale. Before staining, slides were kept in distillate water for 2 minutes. Subsequently, Nissl and PSR stains were performed following the specific protocol.

3.5. Nissl staining

For the Nissl staining, the samples were immersed in an aqueous solution of Toluidine Blue 1% (composed of Toluidine Blue in distilled water and borax, filtered before use) and kept at 60°C for 15 minutes, then left for 30 minutes at room temperature. Samples were then washed with distilled water to eliminate staining residues and de-hydrated using an ascending-graded ethanol scale and left in xylol for 10 minutes before being mounted using Eukit (Kindler gmbH) for bright-field microscopy evaluation.

3.6. Picrosirius Red staining

After re-hydration, cerebellar sections were stained for 1 hour using Picrosirius Red solution (0.1% concentrated aqueous solution with Sirius red and picric acid). Then, the slides were washed with an 5% acidified water. They were de-hydrated in ethanol, kept for 10 minutes in xylol before being mounted using Eukit (Kindler gmbH) for the bright-field evaluation of meningeal collagen content.

3.7. Immunohistochemical reaction for oxidative stress markers

Immunohistochemical reactions were performed to investigate possible changes in the expression levels of specific markers of oxidative stress (i.e. SOD1, SOD2, iNOS and COX4) to evaluate any changes in the oxidative stress pathways upon treatments with Voghera pepper extracts.

Following rehydration, cerebellar sections underwent an unmasking protocol using a Citrate buffer solution (10 Mm Citric Acid, 0.05% Tween 20, pH 6.0), when necessary. Then the slides were processed for the immunohistochemical reaction and left overnight at room temperature in a moist and dark environment with the primary monoclonal antibodies diluted in PBS (specific concentrations and antibodies are listed in **Table 2**). Subsequently, the slides were incubated with the correspondent biotinylated secondary antibody (**Table 2**) for 1 hour; then they were exposed for 30 minutes to an avidin biotinylated horseradish peroxidase complex (Vector Laboratories Burlingame, CA, USA). The 3,3'-diaminobenzidine (DAB) tetrahydrocloride peroxidase substrate (Sigma, St. Louis, MO, USA) was used as chromogen to reveal antigen-antibody interaction. Finally, Carazzi's hematoxylin was used to counterstain the nuclei. Finally, the samples were dehydrated and mounted using Eukit (Kindler gmbH) before bright-field microscopy observation.

Table 2. Primary and secondary antibodies used for the immunohistochemical reactions and the related dilutions.

	Antigens	Manufacture and Species	Dilutions
Primary antibodies	Anti-Superoxide Dismutase 1 (SOD1)	GeneTex (Alton Pkwy Irvine, CA, USA) Rabbit polyclonal IgG, Cat# GTX100554	1:500
	Anti-Superoxide Dismutase 2 (SOD2)	Cell Signaling Technology (Danvers, MA, USA), Rabbit monoclonal IgG, Cat# 13141	1:200
	Anti-inducible Nitric oxide Synthetize (iNOS)	GeneTex (Alton Pkwy Irvine, CA, USA) Rabbit monoclonal IgG, Cat# GTX636531	1:50
	Anti-Cytochrome C Oxidase Subunit 4 (COX4)	Abcam (Cambridge, MA, USA), Mouse monoclonal IgG1, Cat# ab33985	1:200
Secondary antibodies	Biotinylated horse anti-mouse IgG	Vector Laboratories (Burlingame, CA, USA), Horse, Cat# PK-6102	1:200
	Biotinylated goat anti-rabbit IgG	Vector Laboratories (Burlingame, CA, USA), Goat, Cat# PK-6102	1:200

3.8. Bright field microscopy

For histological and immunohistochemical analyses, sections were analysed under bright field microscopy with Olympus BX51 microscope (Olympus Evident Europe GmbH, Germany) and images were acquired with Olympus EP50 (Olympus Evident Europe GmbH, Germany).

3.9. Histochemical and immunohistochemical evaluations

For the samples analysis, the evaluations were performed on three randomly chosen images/sections with ten measurements performed per animal. The images shown in the results are the most representative for every experimental condition.

A quantitative analysis was performed for the PCs. The amount and intensity of the labelling was quantified using the Image-J software: images were converted into 8 bits, colours were inverted to obtain a light signal on a dark background in order to correlate the increase in signal intensity with the increase in optical density (**Figure 15**). Shrunken PCs density was assessed using Nissl-stained samples (number of shrunken PCs/area in mm^2), while immunopositive PCs density (immunopositive PCs/area in mm^2) and the corresponding optical density (OD) was evaluated for each oxidative stress marker.

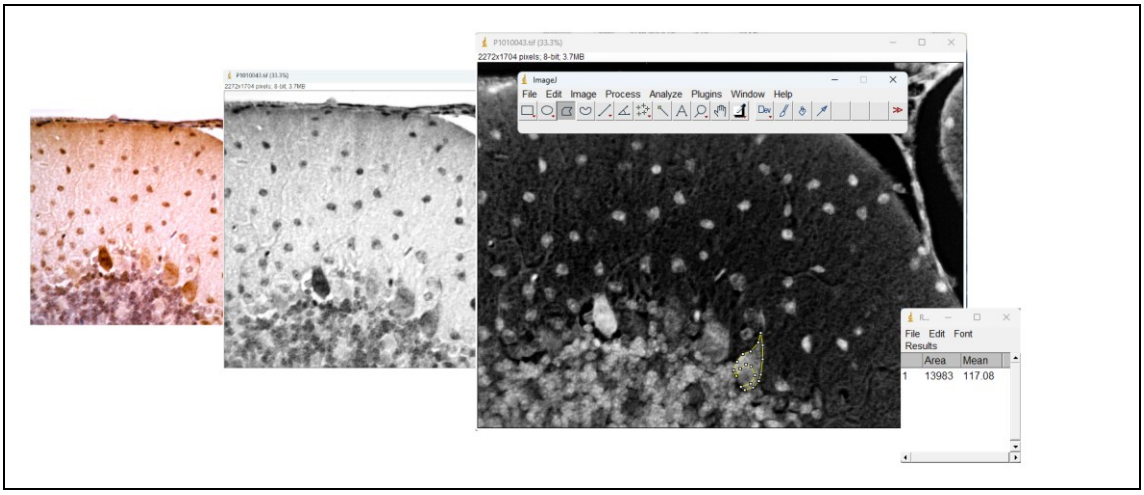

Figure 15. Example of ImageJ PC OD quantification.

3.10. Statistical analysis

Data collected were analysed using Prism software. First, the data were subjected to normality tests (i.e. D'Agostino & Pearson, Anderson-Darling, Kalmogorov-Smirnov and Shapiro-Wilk) to evaluate if they were following a normal distribution. On normally distributed data, significant differences were calculated using parametric One Way ANOVA followed by Bonferroni post-hoc test. Conversely, the Kruskal-Wallis test was performed on the results that did not pass the normality test. Data were reported as mean ± SEM. Results were considered statistically significant for $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

4. RESULTS

Morphological evaluations following histochemical staining, as well as the analysis of oxidative stress pathway marker expression through immunohistochemical techniques, were performed with a specific focus on the cerebellar cortex of mice from the different experimental groups.

4.1. Histological evaluations

To study the gross morphology of cerebellar specimens from different experimental groups Nissl stain was employed. This nervous tissue-specific dye highlights "Nissl bodies", which are rough endoplasmic reticulum structures within the neuronal cytoplasm (Yurt et al., 2018).

In young and old mice of all experimental groups, the cerebellar cortex displays its typical three-layer organization (**Figure 16, micrographs A to F**), composed by the outermost ML, followed by the PCL and the IGL in direct contact with the lobules white matter. Most cell types in the cerebellar cortex of different experimental groups maintain their physiological cytoarchitecture. In the molecular layer, cells appear small and rounded, sometimes "anchored" to the dendrites of PCs, which branch into this layer. Yet, not all PCs maintain their drop-like structure with visible rounded nucleus: particularly, in the CtrlO (**Figure 16**, **micrograph D**), several PCs display a thinner and elongated cell body (shrunken cells), showing canonical morphological features of degenerating neurons. Finally, the granular layer appears dense and packed with cells; in some histological sections, Golgi cells (GoCs) can be distinguished by the larger cell body and nucleus compared to the other GLIs (**Figure 16, micrograph A to F**).

To better investigate possible morphological alteration in the cerebellar cortex of different experimental groups, shrunken PCs density was evaluated. This analysis revealed a slight non-significant reduction of shrunken PCs density in young and old mice treated with the Voghera pepper fruit body extracts (Vp), compared to the untreated group. Conversely, the treatment with the Voghera pepper by-products (Vpw) highlights a decrease in shrunken cells density only in old mice (**Figure 17**; **Table 3**).

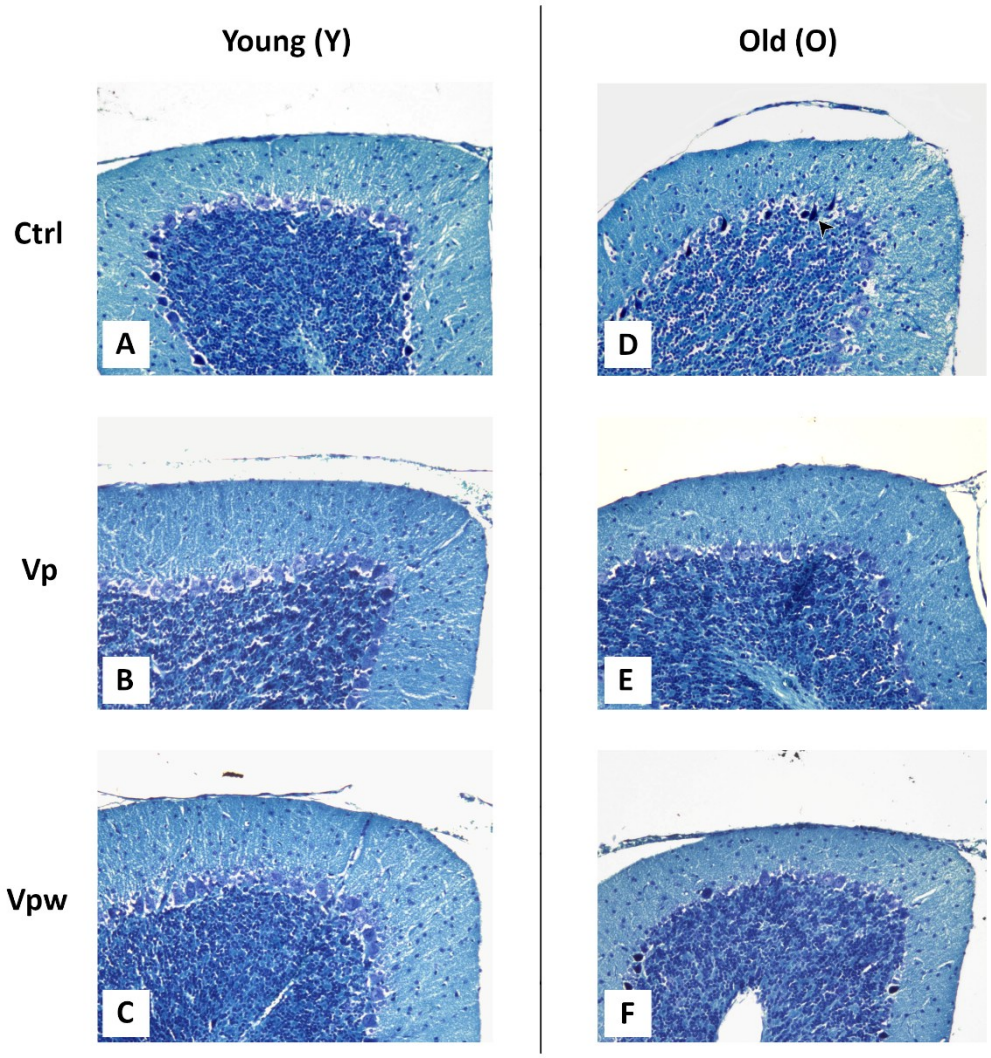

Figure 16. Representative Nissl staining of cerebellar vermis samples of control (A and D, for young and old respectively), Voghera pepper fruit body treated (B and E for young and old respectively) and Voghera pepper by-products treated (C and F for young and old respectively) mice. Microscopy magnification 40x. (D) Arrowhead in D: shrunken cell.

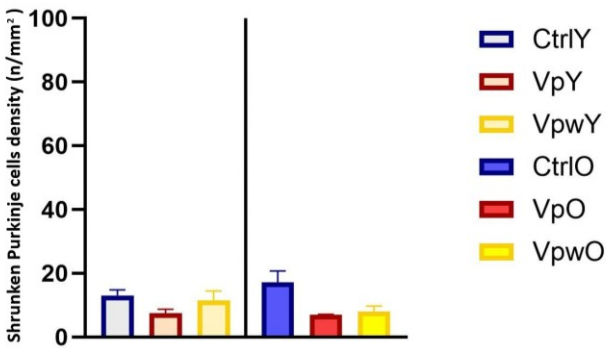

Figure 17. Histogram showing the quantitative results of the shrunken PCs density.

Table 3. Statistical analysis for the quantitative evaluation of the PCs density. (Ns) not significant.

	Treatment group		Treatment group	*p*-value
Shrunken Purkinje cells density (n/mm²)	CtrlY (13.02 ± 1.74)	*vs*	VpY (7.49 ± 1.19)	Ns
	CtrlY (13.02 ± 1.74)	*vs*	VpwY (11.51 ± 2.97)	Ns
	VpY (7.49 ± 1.19)	*vs*	VpwY (11.51 ± 2.97)	Ns
	CtrlO (17.11 ± 3.64)	*vs*	VpO (7.02 ± 0.10)	Ns
	CtrlO (17.11 ± 3.64)	*vs*	VpwO (8.03 ± 1.64)	Ns
	VpO (7.02 ± 0.10)	*vs*	VpwO (8.03 ± 1.64)	Ns

Picrosirius Red (PSR) staining marks collagen fibers and allows high-sensitivity visualization and quantification of collagen within the cerebellum using bright-field microscopy. Overall, the PSR staining localized primarily at the meninges, which appear stained in bright red due to the presence of collagen fibers (**Figure 18, micrographs A-F**). The quantitative analyses of PSR optical density (OD) revealed an extremely significant reduction of meningeal collagen expression levels in both young and old mice treated with either Vp and Vpw extracts, compared to the respective controls. (**Figure 19**; **Table 4**).

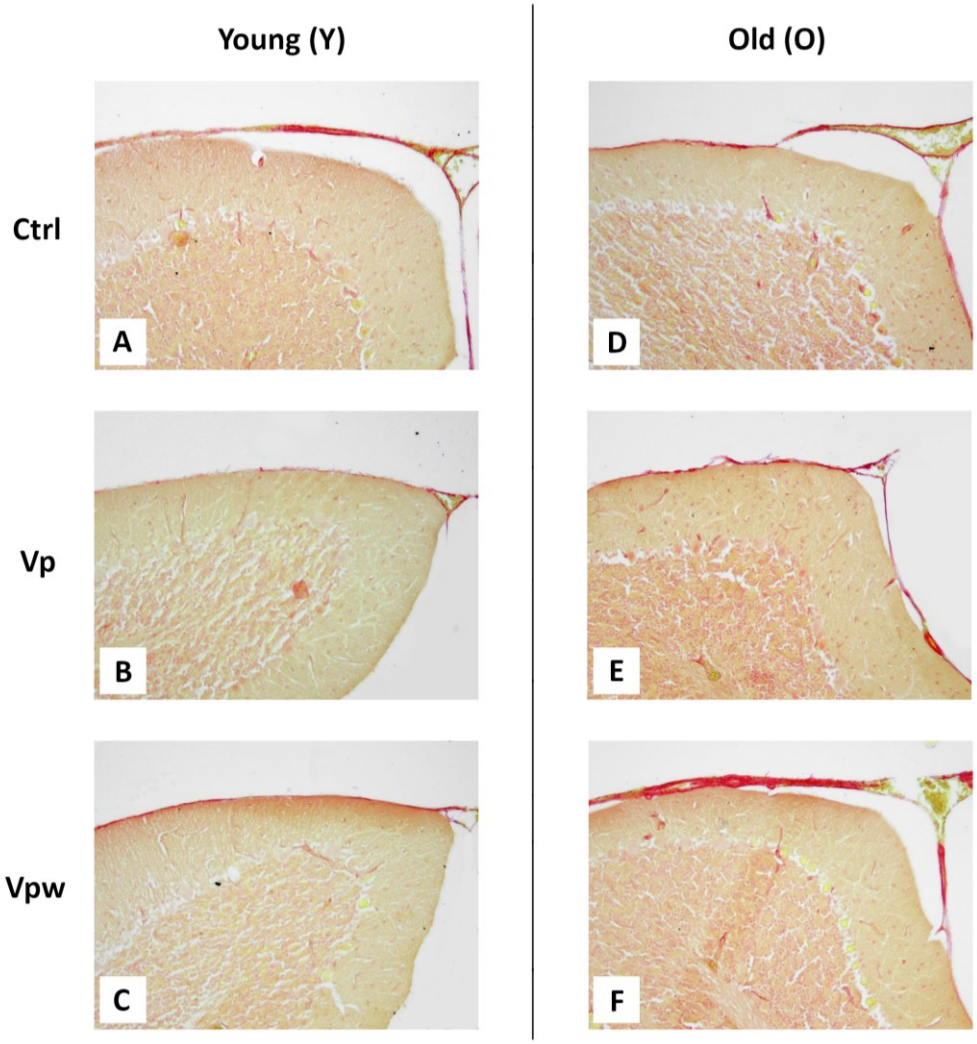

Figure 18. Representative PSR staining of cerebellar vermis samples of control (A and D, for young and old respectively), Voghera pepper fruit body treated (B and E for young and old respectively) and Voghera pepper by-products treated (C and F for young and old respectively) mice. Microscopy magnification 40x.

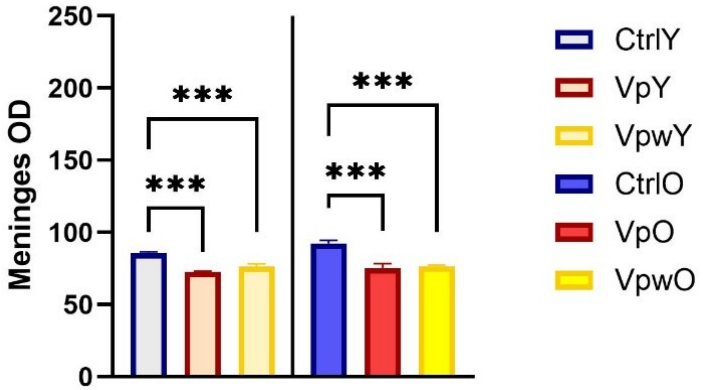

Figure 19. Histogram showing the quantitative results of PSR OD measurements in the meninges. (***) $p<0.001$.

Table 4. Statistical analysis for the quantitative evaluation of PSR optical density in the meninges. (Ns) not significant, (***) $p<0.001$.

	Treatment group		Treatment group	*p*-value
Meninges OD	CtrlY (85.66 ± 0.90)	*vs*	VpY (75.56 ± 1.95)	***
	CtrlY (85.66 ± 0.90)	*vs*	VpwY (76.33 ± 1.84)	***
	VpY (75.56 ± 1.95)	*vs*	VpwY (76.33 ± 1.84)	Ns
	CtrlO (92.36 ± 2.04)	*vs*	VpO (75.26 ± 3.23)	***
	CtrlO (92.36 ± 2.04)	*vs*	VpwO (76.51 ± 0.82)	***
	VpO (75.26 ± 3.23)	*vs*	VpwO (76.51 ± 0.82)	Ns

4.2. Immunohistochemical study of the oxidative stress pathway

In the present work, immunohistochemical investigations were carried out on the cerebellar specimens of young and old C57BL/6J mice to assess whether the oxidative stress pathways showed any changes between treated and untreated groups; the expression levels of four specific oxidative stress markers (i.e. SOD1, SOD2, iNOS and COX4) is manly evident in the PCs, albeit with different intensity, in every treatment group. Therefore, the quantitative analysis of the expression level focus on this cell type.

SOD1 is a mitochondrial enzyme located in the intermembrane space of the organelle and is able to convert the superoxide ion into H_2O_2 (Xu et al., 2022). **Figure 20** shows representative micrographs of SOD1 immunohistochemical staining: all experimental groups display SOD1-expressing neurons. In young mice groups, the molecular layer contains immunopositive MLIs and immunolabeled PCs soma and dendrites can be observed as well, but to a different extent (in **Figure 20**, micrographs **A**, **B** and **C**). Granular layer displays a more heterogeneous staining: young Ctrl and Vpw groups revealed immunopositive GoCs (**Figure 19**, micrographs **A** and **C**). A similar immunopositivity was observed evaluating old mice groups where all of the treated groups present immunolabeled cells in the ML, PCL and IGL. Stained GoCs are identified with an arrowhead (as highlighted in **Figure 20**, micrographs **D**, **E** and **F**).

In **Figure 21**, histograms **a** and **b** show immunopositive PCs density and optic density quantification, respectively. A decreasing trend of SOD1 immunolabeled PCs density can be noticed in both Vp and Vpw treated mice; in both young and old groups, this reduction is significant in mice treated with the by-products extracts, compared to the respective control group. No significant differences were observed when comparing Vpw and Vp treated groups, in both young and old mice.

A similar reduction can be noticed for the OD quantification of immunopositive PCs: after the treatment with the fruit body extract the PCs optic density decreases significantly both in young and old mice, compared to the respective control conditions; while the decreased PCs optic density after the by-products extract treatment appears non-significant compared to the respective control conditions. Furthermore, fruit body extract shows a very significant decrease in SOD1 expression compared with the relative by-product extract treatment in old mice (**Table 5**).

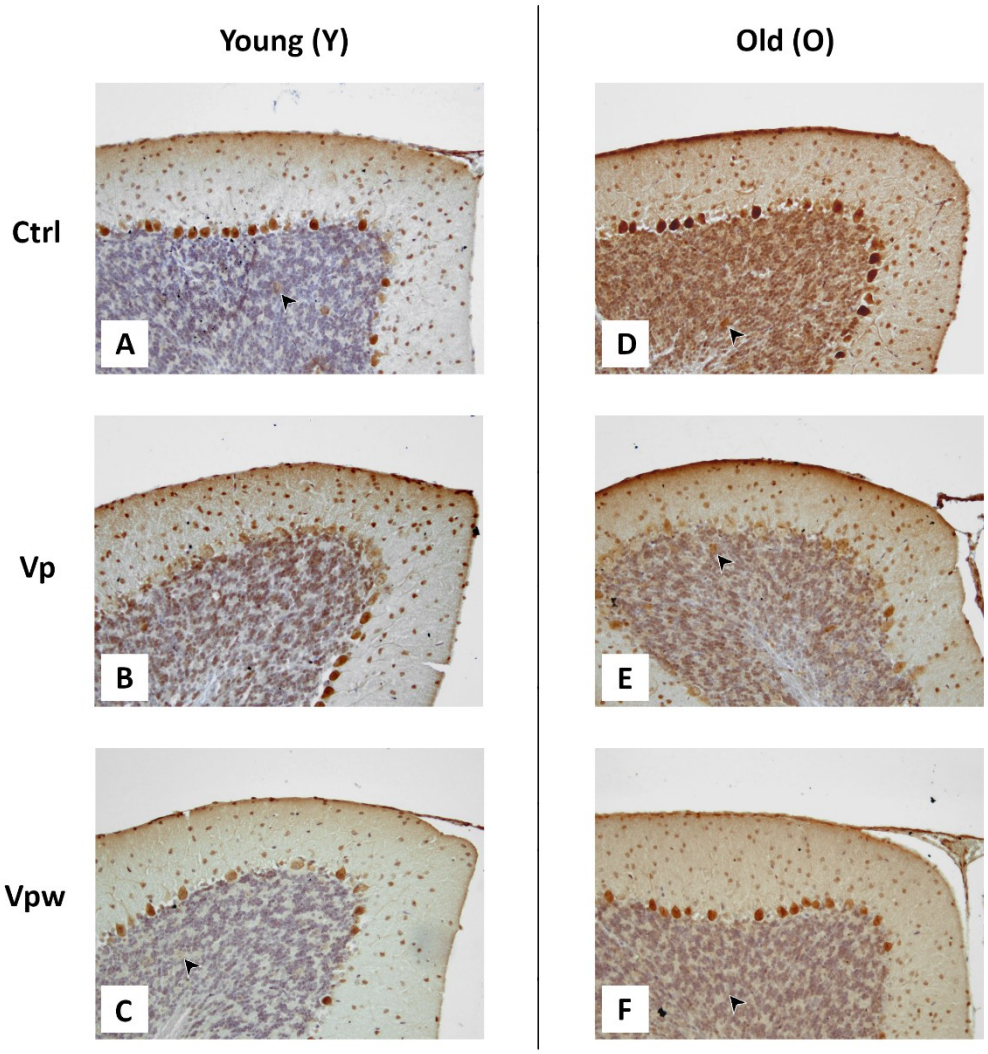

Figure 20. Representative SOD1 immunohistochemical staining of cerebellar vermis samples of control (A and D, for young and old respectively), Voghera pepper treated (B and E for young and old respectively) and Voghera pepper by-products fruit body treated (C and F for young and old respectively) mice. Microscopy magnification 40x. (D) Arrowhead in A, C, D, E, F: GoCs.

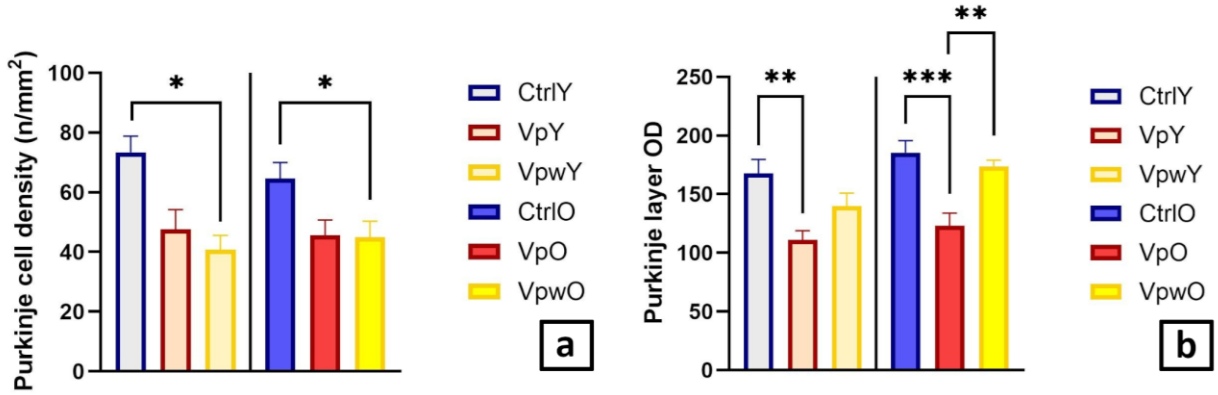

Figure 21. Histograms showing the quantitative results of SOD1 immunohistochemical staining. Histograms (a) and (b) represent PCs density and PCs OD, respectively. (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

Table 5. Statistical analysis for the quantitative evaluation of SOD1 cell density and optical density in PCs. (Ns) not significant, (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

	Treatment group		Treatment group	*p*-value
SOD1 immunopositive PCs density (n/mm^2)	CtrlY (73.37 ± 5.44)	*vs*	VpY (47.63 ± 6.55)	Ns
	CtrlY (73.37 ± 5.44)	*vs*	VpwY (40.76 ± 4.79)	*
	VpY (47.63 ± 6.55)	*vs*	VpwY (40.76 ± 4.79)	Ns
	CtrlO (64.62 ± 5.37)	*vs*	VpO (45.61 ± 5.11)	Ns
	CtrlO (64.62 ± 5.37)	*vs*	VpwO (44.96 ± 5.34)	*
	VpO (45.61 ± 5.11)	*vs*	VpwO (44.96 ± 5.34)	Ns
SOD1 immunopositive stained PCs OD	CtrlY (176.82 ± 8.80)	*vs*	VpY (120.35 ± 6.71)	**
	CtrlY (176.82 ± 8.80)	*vs*	VpwY (139.82 ± 11.05)	Ns
	VpY (120.35 ± 6.71)	*vs*	VpwY (139.82 ± 11.05)	Ns
	CtrlO (185.50 ± 10.33)	*vs*	VpO (130.84 ± 8.78)	***
	CtrlO (185.50 ± 10.33)	*vs*	VpwO (173.82 ± 5.30)	Ns
	VpO (130.84 ± 8.78)	*vs*	VpwO (173.82 ± 5.30)	**

SOD2 is an antioxidant enzyme localized in the mitochondrial matrix and, similarly to SOD1, is crucial for the physiological scavenging of reactive oxygen species (Palma et al., 2020).

Representative SOD2 immunohistochemical staining for the different mice groups are presented in **Figure 22**, **micrographs A-F**. SOD2-immunopositive MLIs and PCs dendrites are visible in Ctrl and Vpw of young and old groups, while PCs of the VpY and VpO are more weakly stained (**Figure 22**, **micrographs B** and **E**). Similarly, PCs cytoplasm is strongly stained in the Ctrl and Vpw mice, for both young and old groups (**Figure 22**, **micrographs A, C, E** and **F**). All young mice exhibit SOD2-immunopositive GoCs and mossy fibers rosettes in the granular layer; similar observations can be made for the old groups, where the rosettes appear less evident for the VpO mice (**Figure 22**, **micrographs E**).

SOD2-immunopositive PCs cell density remains constant in all experimental groups (**Figure 23, histogram a**). On the other hand, both young and old mice treated with either Vp or Vpw extracts show an overall decrease of SOD2 PCs optical density (**Figure 23, histogram b**). In particular, an extremely significant decrease in SOD2 OD is observed in both young and old groups treated with Vp extracts, compared to the respective control group; additionally, while young Vpw treated-mice display only a slight reduction, old mice treated with Voghera pepper by-products extract show a very significant decrease in immunopositive PCs optical density, compared to control. Moreover, when the two treatments are compared to one another, a significant difference is reported for the young and old groups; particularly, Vp treatment is associated with an overall higher decrease of SOD2 immunopositive OD (**Table 6**).

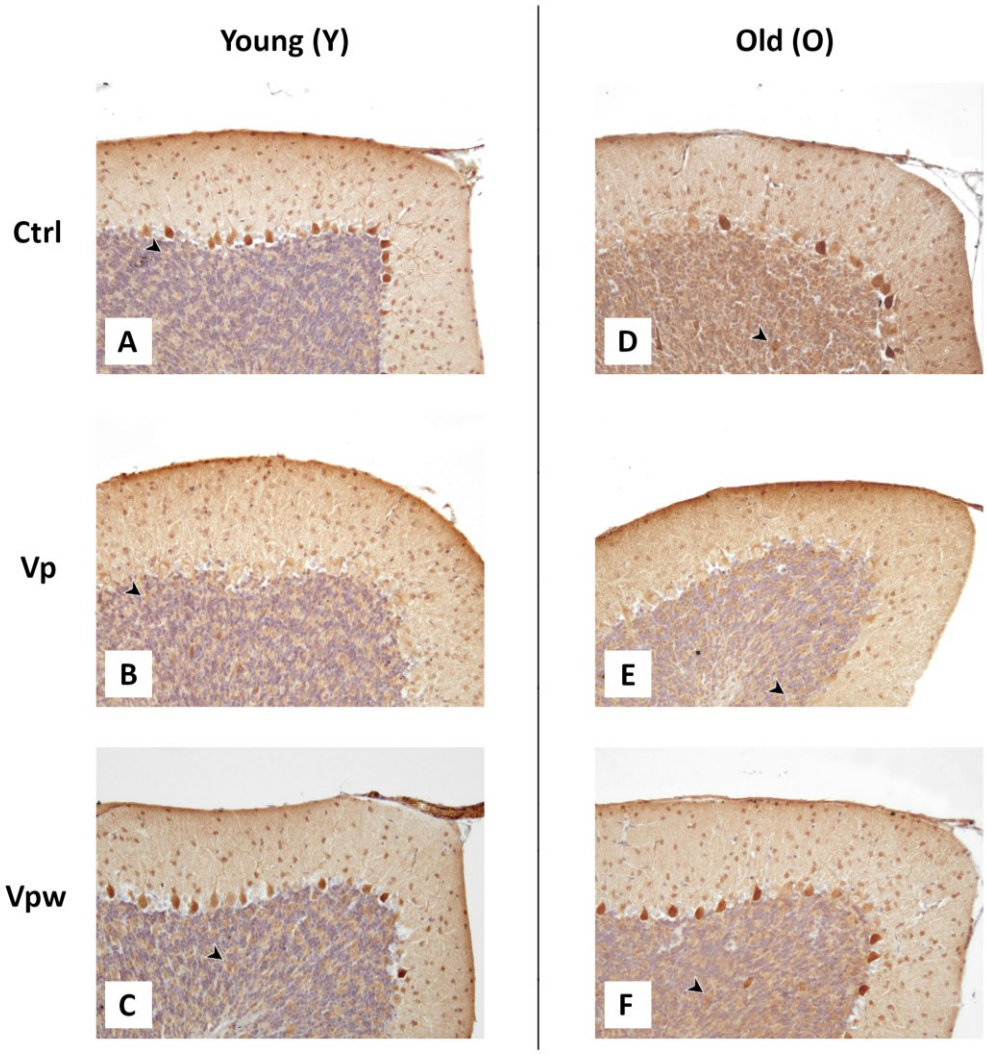

Figure 22. Representative SOD2 immunohistochemical staining of cerebellar vermis samples of control (A and D, for young and old respectively), Voghera pepper fruit body treated (B and E for young and old respectively) and Voghera pepper by-products treated (C and F for young and old respectively) mice. Microscopy magnification 40x. (D) Arrowhead: GoCs.

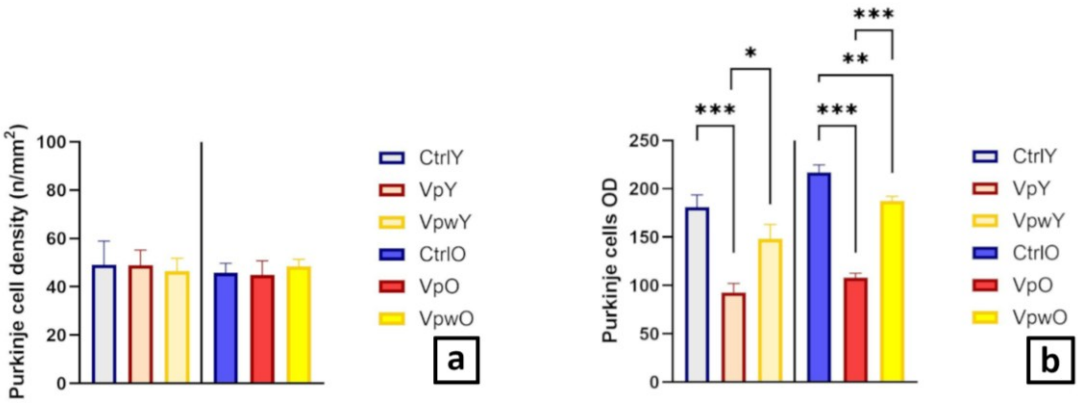

Figure 23. Histogram show the quantitative results of SOD2 immunohistochemical staining. Histograms (a) and (b) represent PCs density and PCs OD, respectively. (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

Table 6. Statistical analysis for the quantitative evaluation of SOD2 cell density and optical density in PCs. (Ns) not significant, (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$.

	Treatment group		**Treatment group**	***p*-value**
SOD2 stained PCs density (n/mm^2)	CtrlY (62.75 ± 7.39)	*vs*	VpY (54.82 ± 2.79)	Ns
	CtrlY (62.75 ± 7.39)	*vs*	VpwY (46.46 ± 5.30)	Ns
	VpY (54.82 ± 2.79)	*vs*	VpwY (46.46 ± 5.30)	Ns
	CtrlO (45.69 ± 4.05)	*vs*	VpO (44.94 ± 4.39)	Ns
	CtrlO (45.69 ± 4.05)	*vs*	VpwO (48.50 ± 2.87)	Ns
	VpO (44.94 ± 4.39)	*vs*	VpwO (48.50 ± 2.87)	Ns
SOD2 stained PCs OD	CtrlY (180.93 ± 12.67)	*vs*	VpY (92.91 ± 9.19)	***
	CtrlY (180.93 ± 12.67)	*vs*	VpwY (148.09 ± 15.01)	*
	VpY (92.91 ± 9.19)	*vs*	VpwY (148.09 ± 15.01)	Ns
	CtrlO (217.23 ± 7.36)	*vs*	VpO (107.97 ± 4.60)	***
	CtrlO (217.23 ± 7.36)	*vs*	VpwO (187.14 ± 5.12)	**
	VpO (107.97 ± 4.60)	*vs*	VpwO (187.14 ± 5.12)	***

iNOS is the inducible cytoplasmic isoform of the nitric oxide synthase (NOS) family; it produces NO, a molecule involved in oxidative stress regulation, and can be regulated by several different signals (Cinelli et al., 2020).

Figure 23 shows the iNOS immunohistochemical staining performed for every experimental group. In young Ctrl and Vpw groups (**Figure 24**, **micrographs A** and **C**), a light iNOS immunopositivity can be observed in some MLIs as well as in the dendritic projections of the PCs, in contrast with the almost absent positivity for the VpY group, where immunonegative dendritic tree of PCs are detected (**Figure 24**, **micrographs B**); similar results can be noticed when focusing on the PCs layer. Moreover, in the granular layer of both CtrlY and VpwY groups few large and rounded cells (GoCs) appear lightly immunopositive. In the old mice groups, MLIs and PCs dendrites of CtrlO and VpwO mice appeared weakly immunopositive (**Figure 24**, **micrographs D** and **F**), while VpO treated mice cerebella show a light immunopositivity in sparse MLIs and PCs (**Figure 24**, **micrographs E**).

Histogram a of **Figure 25** shows a non-significant decrease in iNOS positive PCs density only after the treatment with the Voghera pepper fruit body extract, compared with both their respective condition and the treatment with the by-products extract. **Histogram b** exhibits the immmunopositive PCs OD quantification results. In particular, in young mice, both Vp and Vpw treatments lead to an extremely significant reduction in iNOS immunopositive OD, compared to the control group; similar results are also observed in the old groups, yet to a lower extent for Vpw treated mice (**Table 7**).

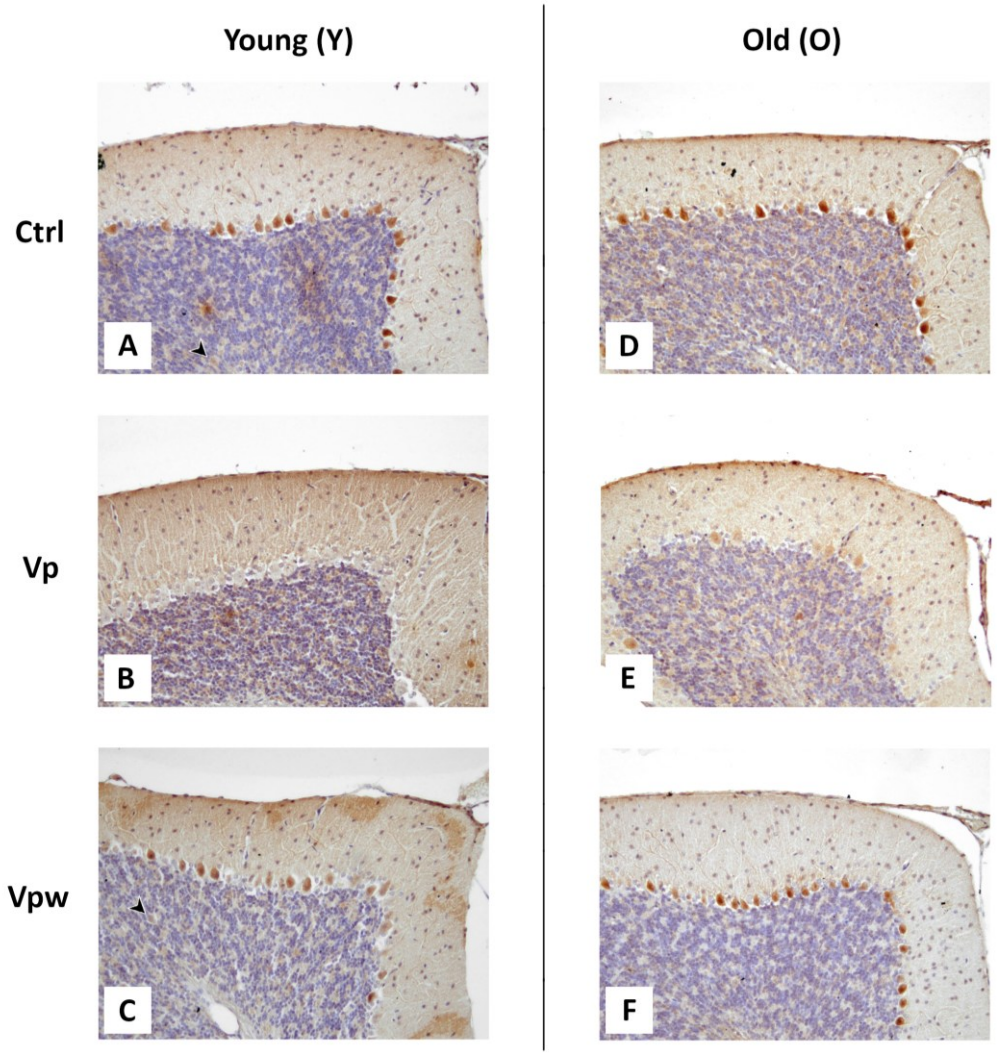

Figure 24. Representative iNOS immunohistochemical staining of cerebellar vermis samples of control (A and D, for young and old respectively), Voghera pepper fruit body treated (B and E for young and old respectively) and Voghera pepper by-products treated (C and F for young and old respectively) mice. Microscopy magnification 40x. (D) Arrowhead in A and C: GoCs.

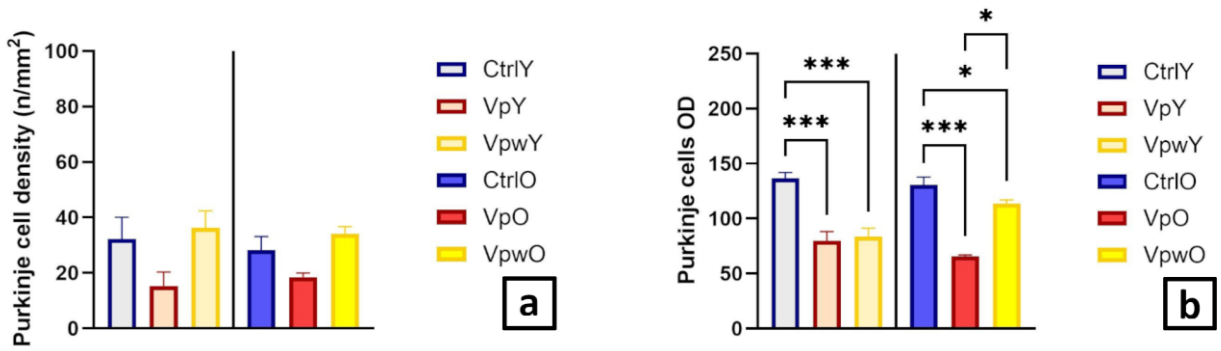

Figure 25. Histogram show the quantitative results of iNOS immunohistochemical staining. Histograms (a) and (b) represent PCs density and PCs OD, respectively. (*) $p<0.05$, (***) $p<0.001$.

Table 7. Statistical analysis for the quantitative evaluation of iNOS cell density and optical density in PCs. (Ns) not significant, (*) $p<0.05$, (***) $p<0.001$.

	Treatment group		Treatment group	*p*-value
iNOS stained PCs density (n/mm^2)	CtrlY (32.23 ± 7.84)	*vs*	VpY (22.97 ± 1.59)	Ns
	CtrlY (32.23 ± 7.84)	*vs*	VpwY (36.20 ± 6.20)	Ns
	VpY (22.97 ± 1.59)	*vs*	VpwY (36.20 ± 6.20)	Ns
	CtrlO (28.22 ± 4.89)	*vs*	VpO (18.45 ± 1.49)	Ns
	CtrlO (28.22 ± 4.89)	*vs*	VpwO (34.04 ± 2.62)	Ns
	VpO (18.45 ± 1.49)	*vs*	VpwO (34.04 ± 2.62)	Ns
iNOS stained PCs OD	CtrlY (141.73 ± 7.04)	*vs*	VpY (72.63 ± 10.04)	***
	CtrlY (141.73 ± 7.04)	*vs*	VpwY (83.75 ± 7.04)	***
	VpY (72.63 ± 10.04)	*vs*	VpwY (83.75 ± 7.04)	Ns
	CtrlO (130.60 ± 6.91)	*vs*	VpO (65.48 ± 1.08)	***
	CtrlO (130.60 ± 6.91)	*vs*	VpwO (113.71 ± 3.30)	*
	VpO (65.48 ± 1.08)	*vs*	VpwO (113.71 ± 3.30)	*

COX4 is a mitochondrial enzyme responsible for the physiological production of ROS during ATP synthesis (Timón-Gómez et al., 2018).

COX4 immunoreaction results are reported in **Figure 26**, **micrographs A-F**. All of the young groups display COX4 immunopositive MLIs as well as visible immunopositive PCs dendrites and cytoplasm. The granular layer shows a more diffused immunopositivity at the level of the mossy fibers rosettes (**Figure 26**, **micrographs A** and **C**). Similar remarks regarding MLIs, PCs and can be observed in the old groups; additionally, with the addition of COX4 immunopositive walls of the blood vessels (**Figure 26**, **micrographs D** and **E**). In mice treated with extracts from the Voghera pepper fruit body, all three layers of the cerebellar cortex show weakly-stained MLIs as well as PCs cytoplasm and dendrites (**Figure 26**, **micrographs B** and **E**).
COX4 immunopositive PCs density evaluations highlighted no significant difference comparing the different young groups; conversely, in old mice immunopositive PCs increase significantly with both treatments, compared to control (**Figure 27**, **histogram a**). On the other hand, COX4 immunopositive OD measurements in young mice revealed a highly significant decrease of immunolabeling only in the Vp treated group, compared to control; conversely, in the old group, VpwO mice display a significant increase in COX4 immunopositive OD compared to both control and VpO-treated mice. No significant difference was observed between the treatment with the fruit body and by-products extract in young mice, while there is a significant difference in the treated old mice groups (**Figure 27**, **histogram b**) (**Table 8**).

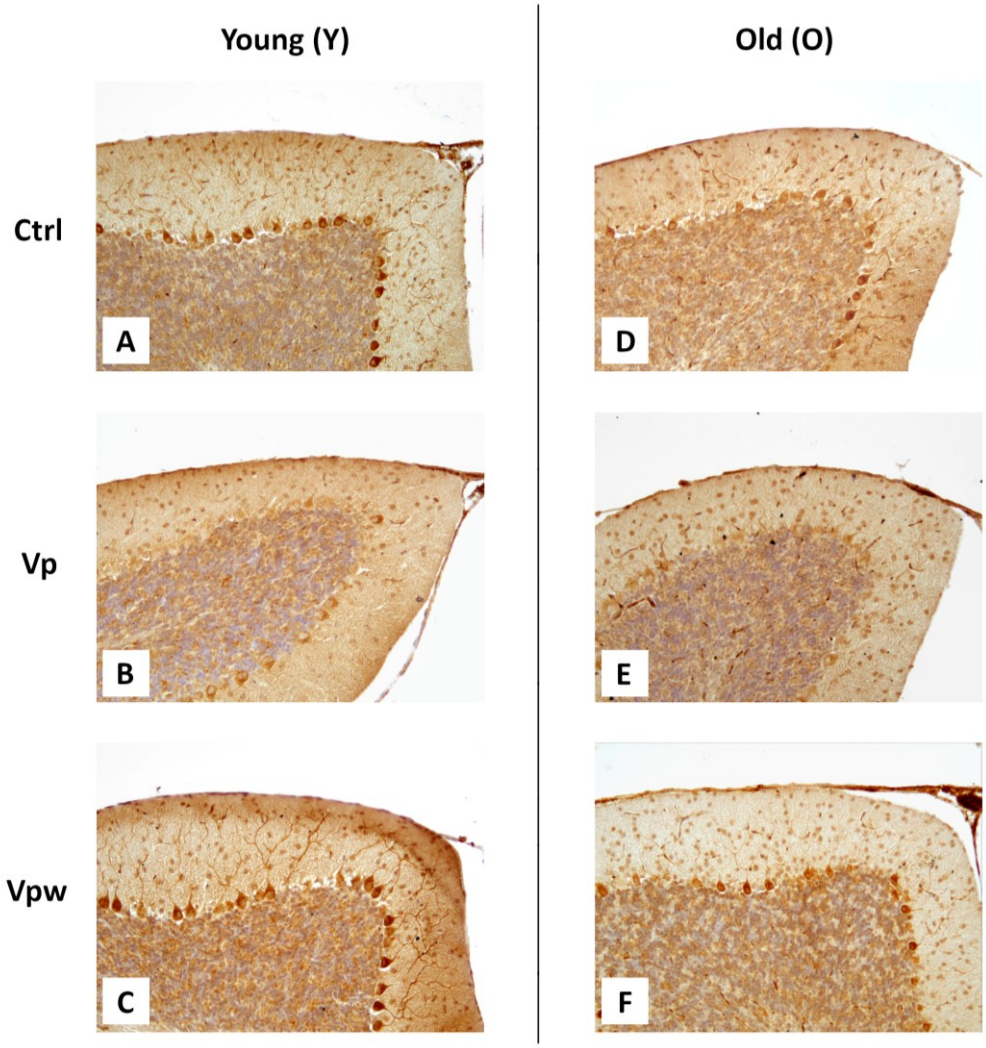

Figure 26. Representative COX4 immunohistochemical staining of cerebellar vermis samples of control (A and D, for young and old respectively), Voghera pepper body fruit treated (B and E for young and old respectively) and Voghera pepper by-products treated (C and F for young and old respectively) mice. Microscopy magnification 40x.

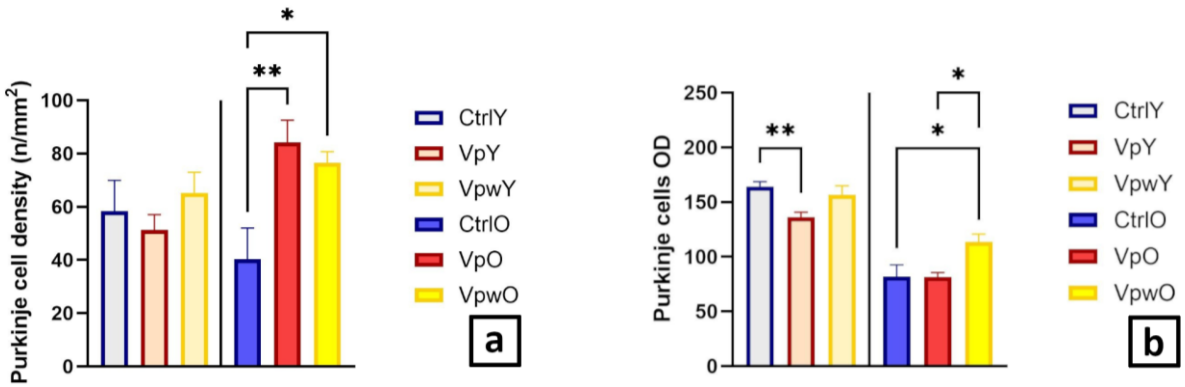

Figure 27. Histogram show the quantitative results of COX4 immunohistochemical staining. Histograms (a) and (b) represent PCs density and PCs OD, respectively. (*) $p<0.05$, (**) $p<0.01$.

Table 8. Statistical analysis for the quantitative evaluation of COX4 cell density and optical density in PCs. (Ns) not significant, (*) $p<0.05$, (**) $p<0.01$.

	Treatment group		Treatment group	*p*-value
COX4 stained PCs density (n/mm^2)	CtrlY (58.30 ± 11.69)	*vs*	VpY (51.25 ± 5.75)	Ns
	CtrlY (58.30 ± 11.69)	*vs*	VpwY (65.17 ± 7.79)	Ns
	VpY (51.25 ± 5.75)	*vs*	VpwY (65.17 ± 7.79)	Ns
	CtrlO (40.28 ± 11.79)	*vs*	VpO (84.10 ± 8.46)	**
	CtrlO (40.28 ± 11.79)	*vs*	VpwO (76.46 ± 4.27)	*
	VpO (84.10 ± 8.46)	*vs*	VpwO (76.46 ± 4.27)	Ns
COX4 stained PCs OD	CtrlY (171.10 ± 5.04)	*vs*	VpY (135.88 ± 4.95)	**
	CtrlY (171.10 ± 5.04)	*vs*	VpwY (178.12 ± 9.16)	Ns
	VpY (135.88 ± 4.95)	*vs*	VpwY (178.12 ± 9.16)	Ns
	CtrlO (81.79 ± 10.81)	*vs*	VpO (78.11 ± 2.91)	Ns
	CtrlO (81.79 ± 10.81)	*vs*	VpwO (113.68 ± 7.09)	*
	VpO (78.11 ± 2.91)	*vs*	VpwO (113.68 ± 7.09)	*

5. DISCUSSION

Aging is a complex biological process, marked by the progressive decline in physiological functions that derive from damage build-up in response to diverse stressors. In the CNS aging is characterised by both structural and functional changes, contributing to cognitive impairments and increased susceptibility to neurodegeneration (Blinkouskaya et al., 2021). The aging process is mediated by an intricate network of pathways, many of which are linked to oxidative stress. Indeed, Denham Harman's 'free radical theory' considers chronic oxidative stress as one of the major factors driving aging. The accumulation of reactive oxygen and nitrogen species (RONS), pared with the reduced efficiency of the antioxidant machinery, detrimentally affects nucleic acids, lipids and proteins, altering cellular processes such as autophagy and activating cell-death pathways such as apoptosis and necrosis (Olufunmilayo et al., 2023). Given the brain's high oxygen consumption, the CNS is particularly susceptible to oxidative damages and therefore to oxidative stress-associated aging (Jaganjac et al., 2022). Additionally, *in vivo* studies evidenced that physiological antioxidant defences are progressively impaired in the aging brain, leading to increased vulnerability to ROS-associated damages and chronic inflammation (Fujimura et al., 2005). In this regard, several natural substances act as potent antioxidant compounds capable of improving redox equilibrium either by directly targeting ROS or, indirectly, by boosting cellular antioxidant systems. Supplementation with these bioactive molecules has been proven to ameliorate the overall oxidative environment in the brain and modulate CNS inflammation, reducing the risk of age-associated disorders, including neurodegenerative diseases (Singh et al., 2024).

In light of this issue, the present thesis work aimed to assess the antioxidant properties of the Voghera pepper, which contains ascorbic acid and carotenoids, powerful antioxidant molecules, by evaluating morphological alterations as well as by analysing the expression of specific oxidative stress markers (i.e. SOD1, SOD2, iNOS and COX4) in the CNS of an *in vivo* aging murine model. Specifically, this preliminary study focused on the cerebellum due to its distinct anatomical organization compared to other brain regions. In particular, it investigates potential alterations within the cerebellar cortex, with special emphasis on Purkinje cells, which represent the sole output of the cerebellar cortex.

Firstly, Nissl histological staining was performed to study the general morphology of the cerebellar cortex and to assess histological and cytological changes: toluidine blue targets acidic compounds

including ribosomal RNAs and Nissl (or tigroid) bodies which are aggregates of ribosomes and rough endoplasmic reticulum. Nissl staining is considered a nervous-tissue-specific dye since neurons have high synthetic activity, allowing the distinction between neurons, with their dendrites, and glial cells. In addition, Nissl staining can highlight the presence of shrunken hyperchromatic cells: intensely-stained neurons with an elongated and compact morphology that differs from the typical drop-like shape of PCs (Zhang et al., 2018). This condition is observed only occasionally in healthy physiological conditions and its increase is associated to an impaired homeostatic condition, like during aging (Zimatkin & Bon', 2018).

In this thesis work, staining with Toluidine Blue revealed that the cerebellar cortex maintained its gross anatomical structure in all experimental groups: the inner IGL, packed with cells, the PCL and the external ML, with small sparse and rounded cells and the processes of PCs.

On the other hand, the quantification of shrunken PCs density showed a decreasing trend in the groups treated with either extract, i.e. Voghera pepper fruit body and by-products, which was particularly evident in old mice and appears to counteract the physiological reduction in PCs density normally observed from 18-months-old mice reported in literature (Woodruff-Pak 2006). These findings obtained from murine cerebellar tissues hint at possible anti-senescence and anti-aging properties of the Voghera pepper, in line with previous *in vitro* studies (De Luca et al., 2024).

Oxidative stress and inflammation are associated with an increased risk of meningeal thickening, progressive stiffness and higher risk of fibrosis, in particular in the sub-arachnoid space and dura mater (Fernández-Klett & Priller, 2014); indeed, ROS as well as inflammatory cytokines can alter ECM composition, triggering collagen deposition and fibrosis. In this regard, the present study investigated meningeal collagen content in both young and adult mice after the two-months treatment with Voghera pepper extracts. Several *in vivo* studies and clinical trials support the positive effects of natural compounds and their ability to decrease tissue fibrosis (Pan et al., 2020; Chun-Bin et al. 2020; Wang et al., 2023). Consistently, supplementation with Voghera pepper extracts evidenced a highly significant reduction in collagen levels in the meninges of both young and old mice. Particularly, aging is associated with increased and progressive chronic inflammation which can severely affect the brain due to a leaky BBB that becomes permeable to active immune cells, inflammatory cytokines and toxic compounds, generating a pro-inflammatory environment that further promotes abnormal collagen deposition (Suzuki et al., 2021). As a matter of fact, Voghera peppers contain high concentrations of ascorbic acid and carotenoids, which are known to act on inflammatory responses, particularly neuroinflammation, and oxidative damage (Gęgotek & Skrzydlewska, 2022; Meng et al., 2022). Additionally, the seeds from sweet peppers

contain high levels of capsaicin, which has been demonstrated to substantially reduce collagen deposition and fibrosis by *in vitro* and *in vivo* studies (Perumal et al. 2015; Abdulaal et al. 2024). Overall, the present work suggests that both Vp and Vpw extracts significantly reduce excessive collagen deposition in the meninges, limit fibrotic events and improve overall tissue health by modulating inflammation and redox imbalances.

Evaluations of the oxidative stress pathway were carried out by immunohistochemical staining targeting four redox-related enzymes; the results revealed significant changes in the overall redox equilibrium of young and old mice treated with Voghera pepper extracts.
SOD1 is a superoxide dismutase enzyme primarily located in the cytoplasm and intermembrane mitochondrial space; it converts superoxide into H_2O_2, maintaining ROS levels below a critical threshold and thereby protecting mitochondria from oxidative damage and cellular senescence (Gomez & Germain, 2019). Indeed, the involvement of SOD1 in ROS scavenging is crucial against oxidative stress and can prevent neuronal damage, maintaining the healthy, physiological morphology of neurites and axonal transport, as well as decreasing age-related cell degeneration. Despite being physiologically expressed, under conditions of oxidative stress SOD1 is upregulated through redox-sensing mechanisms (Trist et al. 2021). Specifically, during aging increased expression of SOD1 is associated with an age-related increase and accumulation of intracellular ROS (Pollock et al., 2023). Our *in vivo* study demonstrated that supplementation with Vp and Vpw extracts overall decreases SOD1-immunopositive PCs density as well as single-cell SOD1 expression, particularly in old treated mice. These findings suggest that Voghera pepper bioactive composition holds antioxidant and anti-aging properties since a reduction in SOD1 is associated with an overall healthier redox environment, thereby making the reliance on SOD1 induction unnecessary (Scalise et al., 2024; Bufka et al., 2024).

Another component of the SOD family that plays a key role in antioxidant activity is SOD2 enzyme. Localised in the mitochondrial matrix, SOD2 participates in the neutralisation of ROS produced by the electron chain reaction, normally increased during aging (Flynn & Melov, 2013). Although the number of SOD2-expressing PCs doesn't show considerable variations, both Vp and Vpw treatments show beneficial effects at single-cell level in the cerebellum of young and old mice. SOD2 is constitutively expressed in the brain but hazardous ROS levels can upregulate the expression of this enzyme as a compensatory mechanism to promote cell survival under oxidative stress and inflammatory conditions (Flynn & Melov, 2013). Notably, the supplementation with the fruit body extracts displays a stronger antioxidant effect, supposedly owing to its rich content in

beneficial bioactive molecules which improve redox homeostasis ultimately leading to reduced SOD2 expression (Shanaka et al., 2021). The significant difference in antioxidant abilities between fruit body and by-products extracts might stem from the different nutraceutical composition of the sweet pepper by-products, particularly the seeds: despite containing beneficial bioactive substances, the seeds also contain anti-nutraceutical compounds (e.g., phytic acid, trypsin inhibitors and tannins) as well as capsaicinoids. Studies *in vivo* have demonstrated that capsaicin by itself exhibits some transient adverse effects, including neurogenic inflammation and increased blood pressure, which may oppose the beneficial properties of fatty acids and other health-promoting substances contained in the Vpw extracts (Petran et al., 2024; Cvetkovíc et al., 2022).

An additional enzyme involved in the modulation of inflammation and redox balance is iNOS. Indeed, iNOS produces nitric oxide (NO), a molecule that participates in different physiological mechanisms such as neurotransmission, cytotoxicity and immunity (Papi et al., 2019). Furthermore, iNOS activation is favoured by a pro-inflammatory environment, typical of "inflammaging", and it is tightly linked to age-related oxidative damages (Cecílio et al., 2011). The present study highlighted a general decrease in iNOS expression in the cerebellum of both old and young mice treated with the Voghera pepper fruit body; these findings likely stem from the high concentrations of ascorbic acid and carotenoids found in the Voghera pepper fruit body, which are sufficient to reduce iNOS through NF-kB downregulation, as a consequence of ameliorating inflammatory conditions and ultimately preventing oxidative damage in the brain (Zeng et al. 2020; Morone et al. 2024). Similar results can be observed in the groups treated with extracts from Voghera pepper by-products, although to a lower extent in older mice: this could be attributed to the different profile of bioactive compounds found in the Voghera pepper by-products (Lee et al., 2019).

Lastly, COX4 is a component of the cytochrome c oxidase (complex IV), a key enzyme of the mitochondrial electron transport chain. Particularly, COX4 acts as a regulatory subunit directly involved in the complex IV assembly and its expression contributes to efficient energy production and overall mitochondrial health, limiting the generation of endogenous ROS produced during oxidative phosphorylation. Therefore, COX4 deficiency is commonly associated with mitochondrial dysfunctions (Douiev et al., 2021). COX4 expression decreases with age and this phenomenon can contribute to mitochondrial alterations, impaired synaptic transmission and cognitive decline, normally observed during aging (Lanza & Nair, 2009; Kriebel et al., 2020). In regard to this issue, the present study evidenced that supplementation with Voghera pepper extracts

increases the number of COX4-expressing PCs in young mice, highlighting the antioxidant effect of both treatments. Indeed, the nutraceutical molecules contained in these extracts may contribute to preventing mitochondrial damages by decreasing oxidative stress and promoting a better cellular environment (Monacelli et al., 2017). Similarly, the treatments are able to restore the number of COX4-immunopositive PCs in older mice suggesting Voghera pepper holds potential anti-aging effects. No significant increase in immunopositive COX4-immunopositive OD was detected after Vp treatment, therefore Voghera pepper might not impact directly COX4 expression at single-cell level but it may exert its antioxidant activity by improving the mitochondrial renewal, thereby promoting an overall healthier CNS environment (Lesmana et al., 2020).

Additionally, COX4-immunopositivity detected at the level of the cerebellar blood vessels and BBB in older mice only suggests that aged subjects may undergo an upregulation of COX4 in the brain's vascular compartments. As a matter of fact, these structures comprise several different cell types, which can be affected by age-related oxidative stress and inflammation, e.g., pericytes, astrocytes and brain microvascular epithelial cells (BMECs). In particular, recent *in vivo* studies demonstrated that the latter have a higher number of mitochondria compared to other components of the BBB and are highly affected by pro-inflammatory cytokines, ROS and hypoxia, which can cause an increase in BBB permeability. During aging energy production can be impaired and cells often augment mitochondrial biogenesis to meet their metabolic needs (Wang et al., 2023; Guo et al., 2023). Moreover, mitochondria functionality is crucial for maintaining a proper BBB permeability and COX4 upregulation could represent a compensatory response aiming to restore the BBB physiological selectivity (Doll et al., 2016; Banks et al., 2021).

6. CONCLUSIONS

Aging is an inevitable physiological process characterised by progressive cellular and systemic changes resulting from the interplay between genetic, environmental or lifestyle factors. Among the numerous mechanisms underlying aging, oxidative stress - caused by the hazardous ROS accumulation coupled with the reduced efficiency of the endogenous antioxidant systems - plays a key role in driving the aging process (López-Otín C. et al, 2023). The imbalance between ROS production and neutralisation results in cellular dysfunctions, including mitochondrial impairments and chronic inflammation, which ultimately lead to the progressive loss of tissue homeostasis (Olufunmilayo et al., 2023). Specifically, due to its high metabolic demand, the brain is particularly susceptible to age-related oxidative damage, which contributes to alterations including neuronal damage and loss, meningeal thickening and neuroinflammation, which ultimately lead to the progressive loss of functional and cognitive abilities (Blinkouskaya et al., 2021).

Nutraceutical compounds have gained increasing attention for their antioxidant and potential anti-aging properties: these bioactive compounds, found in commonly consumed foods, may represent a promising strategy to counteract redox dysregulation, preserving cellular integrity, promoting an overall healthier aging process and preventing age-related dysfunctions (Singh et al., 2024). In this regard, prior *in vitro* studies had demonstrated that the Voghera pepper, particularly rich in antioxidant compounds compared to other sweet pepper varieties, holds promise against cellular senescence and oxidative damage (Gola et al., 2023; De Luca et al., 2024).

This preliminary *in vivo* study highlighted a substantial reduction in age-related morphological alterations as well as significant changes in the oxidative stress pathway in the cerebellar cortex of mice treated with Voghera pepper fruit body and by-products extracts. These results evidenced that both fruit body and by-products of the Voghera pepper hold antioxidant and potential anti-aging properties, and suggest that their supplementation could help in preventing age-related structural and functional damages.

Despite growing evidence supports the anti-oxidant potential of nutraceutical products, to date the exact mechanisms through which naturally-occurring bioactive compounds exert their beneficial activity, particularly on the CNS, still require additional elucidations. Future studies should aim to further investigate the different nutraceutical properties of the pulp and by-products extracts,

clarifying their bioavailability and synergistic effects in order to better dissect their contributions to the aging brain.
Additionally, future studies should seek to investigate the intricate intertwining between oxidative stress and other cellular mechanisms underlying aging, e.g., inflammation and autophagy, and how their modulation through naturally occurring bioactive substances might help prevent age-related dysfunctions occurring in the CNS.

Taken together, the present findings corroborate the potential beneficial effects of phytotherapeutic implementation *in vivo* using both the fruit body and by-products of the Voghera pepper, although to varying degrees. The data collected thus confirm the positive impact of this pepper variety in promoting healthy aging.

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