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**“The crosstalk mediated by Extracellular Vesicles
between skeletal muscle and motor neurons drives the
Amyotrophic Lateral Sclerosis progression”**

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GENERAL INTRODUCTION

1. Amyotrophic Lateral Sclerosis: overview of a multifactorial etiology and intricate pathophysiology

Amyotrophic Lateral Sclerosis (ALS) is a multifactorial neurodegenerative disorder with a complex etiology, characterized by muscle denervation and dysfunction and progressive degeneration of upper and lower motor neurons (MNs) [1]. The global incidence of ALS is currently increasing, with a prevalence of 6 to 9 cases per 100,000 persons, and variable disease progression culminating with respiratory failure after 2-3 years from symptoms onset. While the 90% of ALS cases are reported to be sporadic (sALS), the remaining 10% are familial (fALS), showing autosomal dominant and less frequently an autosomal recessive or X-linked inheritance [2]. More than 30 causative genes have been identified in both ALS forms, including mutations in Superoxide dismutase 1 (*SOD1*), TAR DNA binding protein 43 (*TARDBP*), Fused in sarcoma (*FUS*) and chromosome 9 open reading frame 72 (*C9orf72*) as the most represented [3]. Different *in vitro* and *in vivo* models are exploited to study ALS, including hybrid and primary murine cell lines [4], *Drosophila* [5], zebrafish [6] *Caenorhabditis elegans* [7], and mice [8]. Among these, the transgenic mouse model overexpressing the *SOD1* glycine 93-to-alanine mutation (*SOD1*-G93A) is widely used in ALS studies, as it shows early disease onset and fast progression, cumulative MNs loss and muscle atrophy, thus resembling the human fALS [9]. In particular, the Gly→Ala substitution in position 93 of the *SOD1* enzyme confers a conformational disorder, compromising its structural integrity with a tendency to form monomeric/oligomeric immature structures. These structures, in turn, form aggregates that are toxic against neurons [10]. A plurality of mechanisms have been proposed to participate in ALS progression, including mitochondrial dysfunction and oxidative stress [11], axonal and vesicular trafficking defects [12], glutamate excitotoxicity [13], impaired proteostasis [14] and imbalance in RNA metabolism [15]. These pathways are characterized as *cell-autonomous* mechanisms of motor neurons; however, given the multiple interactions of motor neurons with other cell types, these pathogenic processes are also *cell non-autonomous*, involving microglia and astrocytes [16], peripheral immune cells [17] and skeletal muscle cells [18]. For this reason, ALS is widely recognized as a multisystemic disorder, in the progression of which there is the synergic contribution of several cell types and distinct pathogenetic mechanisms. Therefore, it is difficult to recognize the primary source of the disease development. For example, axonal transport impairment in motor neurons is caused mainly by defects in crosslinks between neurofilaments, actin filaments and microtubules, leading to aberrant accumulation of fragmented mitochondria and consequently contributing to disruption of mitochondrial metabolism and motor neuron death [19]. Similarly, glutamate excitotoxicity is characterized by abnormal activation of glutamate receptors, with imbalance in Ca^{2+} intake and homeostasis, culminating in mitochondrial dysfunction and ROS overproduction [20]. Another key pathogenetic hallmark of ALS is the formation of protein aggregates of mutated forms of *SOD1*, *FUS* and *TDP-43*, that can form amyloid and self-

propagating fibril structures. These aggregates accumulate in the cytoplasm, causing damage to other organelles, such as nuclei and mitochondria, as well as in the endoplasmic reticulum (ER) causing ER stress, finally impacting on the proteasome and autophagic degradation pathways [21]. All these mechanisms are closely linked and converge towards aberrant mitochondrial morphology and functionality, with impaired mitochondrial respiration, defects in energy production, and increased levels of oxidative stress. In ALS, mitochondria with ultrastructural alterations and abnormal cellular distribution, decrease in mitochondrial DNA, and imbalance in the activity of electron transport chain complexes and antioxidant enzymes, are extensively reported. These alterations lead in turn to impairment of oxygen consumption, increased ROS levels, decreased ATP production and defective DNA repair [22]. Recognized as an early event, mitochondrial dysfunction is considered the “core” of ALS pathogenesis, since it has repercussions towards metabolism and neuroinflammation, other factors known to participate in the disease progression. Increasing evidence reports metabolic abnormalities in ALS patients within the onset and progression of the disorder, with glucose intolerance, altered glucose transport and metabolism, and dyslipidemia as a putative compensatory mechanism in response to a dysmetabolic state [23]. Neuroinflammation is a process mediated by reactive microglia and astrocytes that surround degenerating MNs and produce neurotoxic factors, contributing to an inflammatory environment and promoting motor neuron axonal damage. In the early stage of neuroinflammation, the presence of fragmented mitochondria and mitochondrial dysfunction promote the inflammatory process [24]. Therefore, ALS pathogenesis is complex and multifaceted, with multiple pathways mediated by several cell types concurring to neurodegeneration. However, given the presence of *cell non-autonomous* mechanisms involving different cell types than motor neurons, together with various factors implied in its etiology (**Figure 1**) it is still not clear whether ALS pathogenesis starts and develops. Indeed, ALS has been recently conceived as a multifactorial disorder rather than a pure neurodegenerative disease [25]. In this context, the skeletal muscle, as an integral part of the neuromuscular junction (NMJ) constantly interacting with motor neurons, is emerging as an active protagonist at the onset of ALS disease. Since increasing evidence supports that the NMJ breakdown occurs prior to neurodegeneration, the ALS “neurocentric” view is leaving space to the “dying back” hypothesis, revisiting the ALS pathogenesis paradigm and attributing to the skeletal muscle a role as a new source of the disease progression.

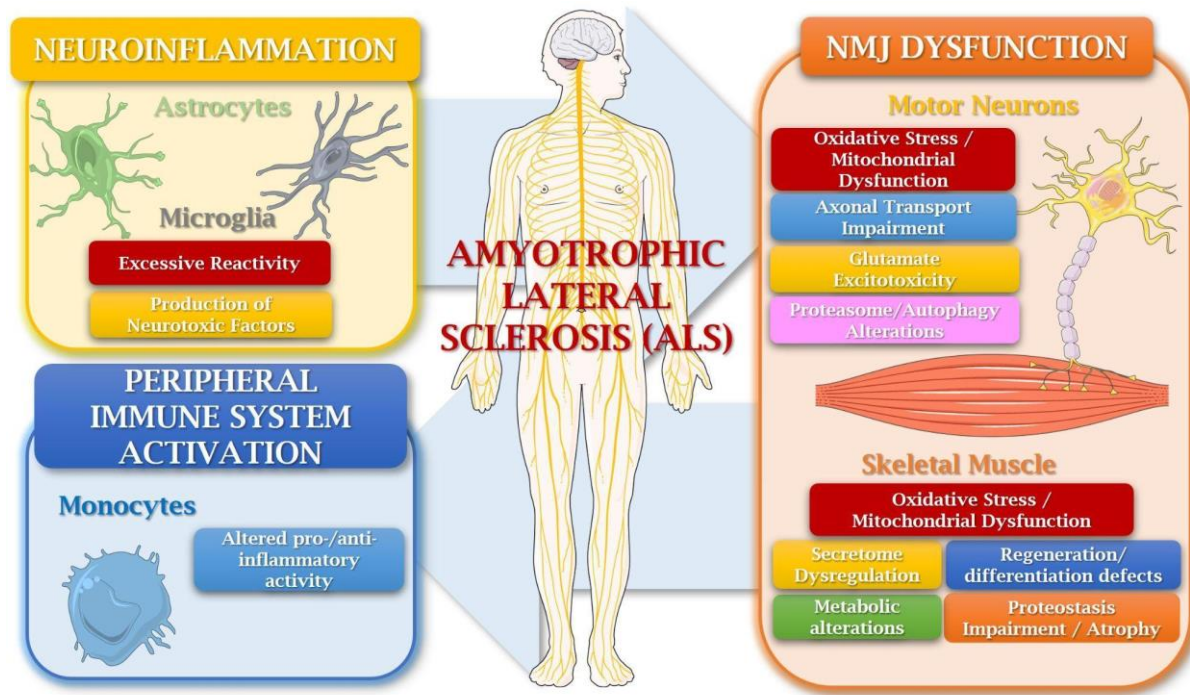


Figure 1. Overview of ALS as a multisystem disorder. ALS is a multifactorial disorder in which different cell types contribute synergistically to its progression. Among ALS features, there are: Neuromuscular Junction (NMJ) dysfunction, within motor neurons and skeletal muscle show an impairment in different or common processes; neuroinflammation, in which reactive astrocytes and microglia produce neurotoxic factors, and peripheral immune system activation with altered activity of monocytes, contributing to systemic inflammation. ALS = Amyotrophic Lateral Sclerosis.

1.1 Cell-autonomous mechanisms in ALS

In ALS, the term “cell-autonomous” refers to all the processes of cellular dysregulation and imbalance originating within the motor neuron and mediating the degenerative process [26]. Among these, the most representative are oxidative stress, impairment in RNA metabolism, glutamate excitotoxicity, and axonal transport defects (**Figure 2**).

1.1.1 Oxidative stress

Reactive oxygen and nitrogen species (ROS and RNS) are constitutively produced within the cells at low concentrations in order to regulate various biological processes, i.e., gene expression, cytoskeleton remodeling, cell proliferation, migration, differentiation, apoptosis, and EVs trafficking [27]. Oxidative stress arises when there is an overproduction of ROS and RNS, overwhelming the cell antioxidant defenses. This leads in turn to cell macromolecules damage and has also an impact on cell morphofunctional features [28]. For this reason, cells naturally possess different mechanisms of detoxification, with antioxidant enzymes representing the first line of defense. Among these, Cu/Zn Superoxide Dismutase (SOD1), which catalyzes the reaction that converts anion superoxide in molecular oxygen and hydrogen peroxide, shows several mutations associated with ALS. Mutations such as SOD1-

G93A determine protein misfolding and formation of toxic aggregates, further promoted in presence of oxidative stress conditions [29]. Oxidative stress has a key role in ALS pathogenesis, especially towards neurons for several reasons. Indeed, neurons have large size, low regenerative capacity, and high metabolic activity, with increased oxygen demands and consequent ROS overproduction. At the same time, neurons show relatively low levels of protective antioxidant enzymes, making these cells highly susceptible to oxidative stress [30]. Indicators of oxidative stress have been detected in both post-mortem tissues of ALS patients and transgenic mice overexpressing mutant SOD1, showing increased levels of protein carbonylation and lipid peroxidation [31]. Oxidative stress is directly related to an impairment in oxidative phosphorylation in mitochondria. In ALS, mitochondria of motor neurons show ultrastructural alterations that consequently affect their function, determining impairment of the respiratory chain complexes and oxygen consumption and defects in energy production [32]. Mitochondrial dysfunction and oxidative stress are therefore considered the pathogenetic hallmark of ALS motor neurons, as they have direct repercussions on other *cell-autonomous* mechanisms, such as glutamate excitotoxicity, axonal transport defects, and RNA dysmetabolism.

1.1.2 Glutamate excitotoxicity

Glutamate excitotoxicity is another proposed mechanism of ALS pathogenesis, in which motor neurons are exposed to excessive concentrations of synaptic glutamate. This triggers a continuous firing of action potentials and increased calcium influx in motor neurons, leading in turn to ROS overproduction, mitochondrial damage and Endoplasmic Reticulum (ER) stress, and finally promoting apoptosis and neurodegeneration [33]. Glutamate excitotoxicity is a result of the failure of quick glutamate removal, generally mediated by astrocytes through the excitatory amino acid transporter 2 (EAAT2). A reduced expression of EAAT2 has been observed in both SOD1-mutant rodent models and ALS patients, probably related to an impairment in RNA metabolism that hampers the proper formation of its mRNA. Other putative mechanisms underlying glutamate excitotoxicity are an increased expression of glutamate receptors by the postsynaptic terminal and changes in the intrinsic excitability of motor neurons, resulting in their excessive firing [34].

1.1.3 Defects in the axonal transport

The disorganization of the axonal cytoskeletal components, especially neurofilaments, and consequent disruption of the axonal transport, is a characteristic hallmark of ALS. Due to their peculiar, asymmetric architecture, motor neurons rely on the axonal transport in response to changes in the synaptic environment, in order to bring factors required for protein synthesis and perform their normal physiological functions [33]. Axonal transport impairment in ALS may result from mutations in motor proteins, leading to their loss of function. Additionally, proteins responsible for axonal trafficking can become sequestered in inclusions [35]. In both

scenarios, these pathological processes lead to the accumulation of various macromolecules and cellular components within the neuron, including neurofilaments, mitochondria, and autophagosomes, disrupting both anterograde and retrograde axonal transport [36]. The disruption of the axonal transport is associated with mutations in several RNA-binding proteins that are associated with ALS, including TDP-43 and FUS [37].

1.1.4 Impairment in RNA metabolism

The first intersection between RNA biology and ALS pathogenesis was discovered in 2006, when the mislocalization of TDP-43 from the nucleus to form cytoplasmic inclusions was reported in the brain and spinal cord of ALS patients [38]. The heterogeneous nuclear ribonucleoproteins (hnRNP) is a family of proteins, including TDP-43 and FUS, that finely regulate RNA metabolism in all its stages. Therefore, any dysfunction in one of these proteins has strong repercussions on the whole process [39]. The mutations in these proteins associated with ALS affect the dynamics of the so-called “membraneless organelles” formed during RNA metabolism, promoting the fibrillization of amyloid structures and their deposition within the cell bodies. The resulting RNA granules may exert toxic functions [40]. For example, both TDP-43 and FUS are constituents of complexes that generate miRNAs, small non-coding RNAs involved in several processes, including the NMJ maintenance. Therefore, it is plausible that motor neurons are particularly affected by alterations in RNA metabolism, and ALS share this pathogenetic hallmark with other neurodegenerative diseases [33].

1.2 Cell non-autonomous mechanisms in ALS

The nervous system is a complex network involving not only neuronal intercellular communication, but also the interactions between neurons and other cells, such as glial cells. Glial cells, such as oligodendrocytes, astrocytes, and microglia, provide both structural and functional support to neurons, contributing to the maintenance of neuronal balance [41]. Therefore, when studying neurodegenerative diseases, it is limiting to consider only neuronal cells, but is essential to comprehend how the surrounding environment influences the disease progression [42]. In ALS, aberrant phenotypes of glial cells are widely described, and while ALS typical symptoms emerge as a result of neurodegeneration, the pathology actually manifests and progresses due to a combination of damage occurring in both motor neurons and glial cells. Furthermore, recent evidence suggests that ALS pathogenesis involves not only alterations within the CNS, but also systemic inflammation, driven by different populations of peripheral immune cells [43]. The paradigm of ALS as a neurodegenerative disease is gradually expanding towards a multisystem disorder, in which multiple mechanisms driven by different effectors in different districts of the body act synergistically to the disease development and progression (**Figure 2**). Therefore, it is imperative to dissect ALS cell *non-autonomous* mechanisms to find new clues in the understanding of this multifaceted disease.

1.2.1 Role of oligodendrocytes in ALS

Oligodendrocytes are specialized cells of the macroglia that play a pivotal role within the CNS, maintaining tissue homeostasis and facilitating proper electrical impulse function through myelination/remyelination of axons [44]. After myelin damage, oligodendrocyte progenitor cells (OPCs) differentiate and replace damaged oligodendrocytes, allowing the deposition of new myelin layers around the axon [45]. Another important function performed by oligodendrocytes in the CNS is to provide trophic and metabolic support to neurons in order to maintain their survival and axonal physiology. Moreover, oligodendrocytes play an active role in maintaining CNS homeostasis, interacting with neurons, also in collaboration with astrocytes and microglia, and contributing to immunomodulatory responses and tissue repair in response to various insults [46]. ALS and other neurodegenerative diseases share common pathogenic mechanisms involving myelin damage and dysfunctional oligodendrocytes. Interestingly, oligodendrocyte damage seems to begin in a presymptomatic phase of ALS, preceding neurodegeneration. The canonical mutations associated with ALS determine structural alterations in oligodendrocytes, including thicker cell bodies, metabolic changes, increased oxidative stress, and neuroinflammation [47]. The damage to myelin structure due to oligodendrocyte degeneration leads to impaired nerve conduction, inadequate axonal remyelination and failure of metabolic support for neurons. Metabolic alterations in oligodendrocytes include mitochondrial dysfunction, with consequent ROS overproduction and defective generation of energy, and impaired lipid production, determining a compromised lipid composition in the myelin sheath and hindering its proper function [48]. Another aspect related to the physiopathological role of oligodendrocytes in ALS is neuroinflammation. Proinflammatory activation of these cells can create an adverse microenvironment for both neurons and oligodendrocytes, hampering myelin regeneration and promoting neurodegeneration, thus contributing to the disease progression [49].

1.2.2 Role of astrocytes in ALS

Astrocytes represent the predominant component of the macroglia and play different roles in the maintenance of neuronal homeostasis and the efficiency of synaptic transmission. A main function of the astrocytes is to control the extracellular levels of glutamate, by the expression of EAAT1 and EAAT2 transporters, limiting its presence in the synaptic cleft and thus avoiding an excessive neuronal firing [50]. Astrocytes provide trophic support to neurons by producing neurotrophic factors, such as glial- and brain-derived neurotrophic factors (GDNF and BDNF). In addition, astrocytes are crucial for the blood-brain barrier formation and maintenance and act as an additional obstacle by preventing the passage of toxic substances into the CNS [51]. In ALS, astrocytes participate by showing a reactive, aberrant phenotype that loses its homeostatic functions and rather exerts toxic effects towards neurons. These reactive astrocytes participate in ALS progression by interacting with different CNS cells and contributing to neurodegeneration through multiple mechanisms. First, in glutamate

excitotoxicity with a defective glutamate uptake, causing an excessive firing of neurons and a toxic increase of the calcium influx that culminates with neurodegeneration [52]. Reactive astrocytes also fail to give metabolic and trophic support to neurons and show a dysregulated interaction with oligodendrocytes, impacting on their myelination capacity. While inhibiting neuronal supporting mechanisms, ALS astrocytes are involved at the same time in the promotion of a pro-inflammatory environment, affecting microglia polarization and negatively regulating the neuroprotective inflammatory response [53].

1.2.3 Role of microglia in ALS

Microglia cells, as components of the monocyte-macrophage system, represent the CNS specialized counterpart of circulating monocytes. These innate immune cells play an important role in the homeostasis and protection of the neuronal environment, representing a first line of defense towards potential CNS damage [54]. In physiological conditions, the “resting microglia” monitors the CNS extracellular space through the continuous remodeling of cellular processes. In response to injury, microglia rapidly activates, undergoing morpho-functional changes and participating in the inflammatory process through phagocytosis, antigen presentation, and secretion of cytokines, ROS and growth factors [55]. Microglial cells may exert either toxic or protective effects, depending on their activation state. “Classically activated” or M1 microglia, shows pro-inflammatory and cytotoxic activity, by producing ROS and RNS, iNOS and pro-inflammatory cytokines (i.e., IFN- γ , TNF- α , IL-6 and IL-1 β), while “alternatively activated” microglia, or M2, inhibits inflammatory responses by producing Arg-1 enzyme, anti-inflammatory cytokines (i.e., IL-4 and IL-10), growth factors (i.e., TGF- β) and showing increased phagocytic activity [55]. Rather than manifesting distinct phenotypes, microglia shows a functional plasticity during activation, thus potentially promoting both neuronal damage and protection and rendering unclear its contribution during ALS pathogenesis and progression [56]. During the course of ALS disease and in presence of mutant SOD1, there is an observed increase in microglial proliferation, with the presence of both the neurotoxic M1 phenotype and the neuroprotective M2 phenotype, but at different disease stages. Therefore, microglia can either mediate neuronal survival or induce cell death. Initially, it was reported that in the early phases of the disease and during its progression, there is a prevalence of the M2 phenotype, aiming to create an anti-inflammatory environment [57]. In contrast, the pro-inflammatory and neurotoxic M1 phenotype becomes dominant in the later stages of the disease. Viceversa, another work reported the prevalence of M1 microglia in early stages and M2 phenotype taking over in the late stages [58]. More recently, however, it is described not much as a transition from the M2 phenotype to the M1 phenotype, but rather the possibility of a coexistence of both phenotypes during ALS progression [59].

1.2.4 Involvement of peripheral immune system in ALS

Since its discovery, ALS has been conceptualized as a neurodegenerative disease as neurodegeneration is its main hallmark. However, recent findings on the involvement of the peripheral immune system are pointing out a possible role of systemic inflammation in contributing to ALS pathogenesis. Peripheral immune system activation can be a reaction to tissue injury, but if dysregulated, it may exacerbate the disease [60]. Several studies investigated the role of immune system cells, identifying alterations in T lymphocytes, NK cells, monocytes, and complement. Contrasting variations in the number of CD4+ and CD8+ T cell populations have been reported, probably related to the individual variability of the immune responsiveness [61]. Also, reduced levels and dysfunction of Treg cells, that were inversely correlated with disease progression, emerged in both ALS patients and mouse models, suggesting the potential of these cells in controlling and reducing the disease activity [62]. Regarding monocytes, population shifts and activation towards a pro-inflammatory state are reported. Moreover, it seems that monocytes may enter the CNS, pointing them as other possible contributors to immune system dysregulation in ALS pathogenesis [63]. The involvement of dysregulation in the peripheral immune system in ALS pathology supports that there are multiple unexplored aspects of a disease that has been always considered as neurodegenerative. Therefore, it is clear that ALS has multiple mechanisms involved both at local and systemic level, that makes it not only a disease involving the CNS, but rather a multisystem disorder.

1.2.5 The “dying forward” vs. the “dying back” hypothesis of ALS

Among the different *cell non-autonomous* pathogenetic mechanisms, recent evidence is pointing out a role of the skeletal muscle in the progression of ALS. CNS and skeletal muscle (SkM) are interconnected through a specialized chemical synapse known as the neuromuscular junction (NMJ). The NMJ comprises presynaptic cholinergic neurons, postsynaptic muscle fibers, and Schwann cells terminals [64]. This synapse is responsible for converting electrical signals originating from the neurons into chemical signals that are sensed by the skeletal muscle and ultimately converted in voluntary muscle contraction. The spatial organization and coordinated functioning of the various components of the NMJ are crucial for maintaining optimal neuromuscular transmission. Any impairment in this system can lead to compromised signal transmission, eventually resulting in denervation, muscle atrophy, and paralysis [65]. ALS is a disease characterized by multifactorial and highly complex etiology, with various cell types synergistically participating in its progression. Therefore, the origin of the pathological processes associated with ALS remains unknown. For this reason, ALS is no longer considered solely as a neurodegenerative disease, but it is now widely recognized as a multisystem disorder, described by two main hypotheses: the “dying forward” and the “dying back” hypothesis. According to the “dying forward” hypothesis, muscle atrophy is a direct consequence of the neurodegeneration, which initiates in the motor cortex and then propagates unidirectionally toward the NMJ, ultimately affecting the skeletal muscle. In this

scenario, cortical motor neurons initially damage the anterior horns of the spinal cord through glutamate-mediated excitotoxicity [66]. Recent research, however, leans toward the "dying back" hypothesis, which posits that motor neuron degeneration results from pathological changes initially occurring in proximity to the NMJ, both at the distal motor neuron and the innervated skeletal muscle levels. Subsequently, this degeneration progresses retrogradely to the motor neuron cell body. Consequently, these NMJ alterations may precede neuron death and the clinical symptoms of the disease [67]. This hypothesis is supported by the observation that the loss of lower motor neurons was much more pronounced than upper motor neurons. Many studies have shown that murine animal models of ALS expressing mutated proteins associated with the disease, such as SOD1 or FUS, initially exhibit functional and structural pathological alterations near the NMJ, with motor neuron degeneration occurring later in the disease course [68]. The next chapter will focus on the emerging role of the skeletal muscle in ALS pathogenesis, which is reinforcing the validity of the "dying back" hypothesis in the understanding of ALS disease.

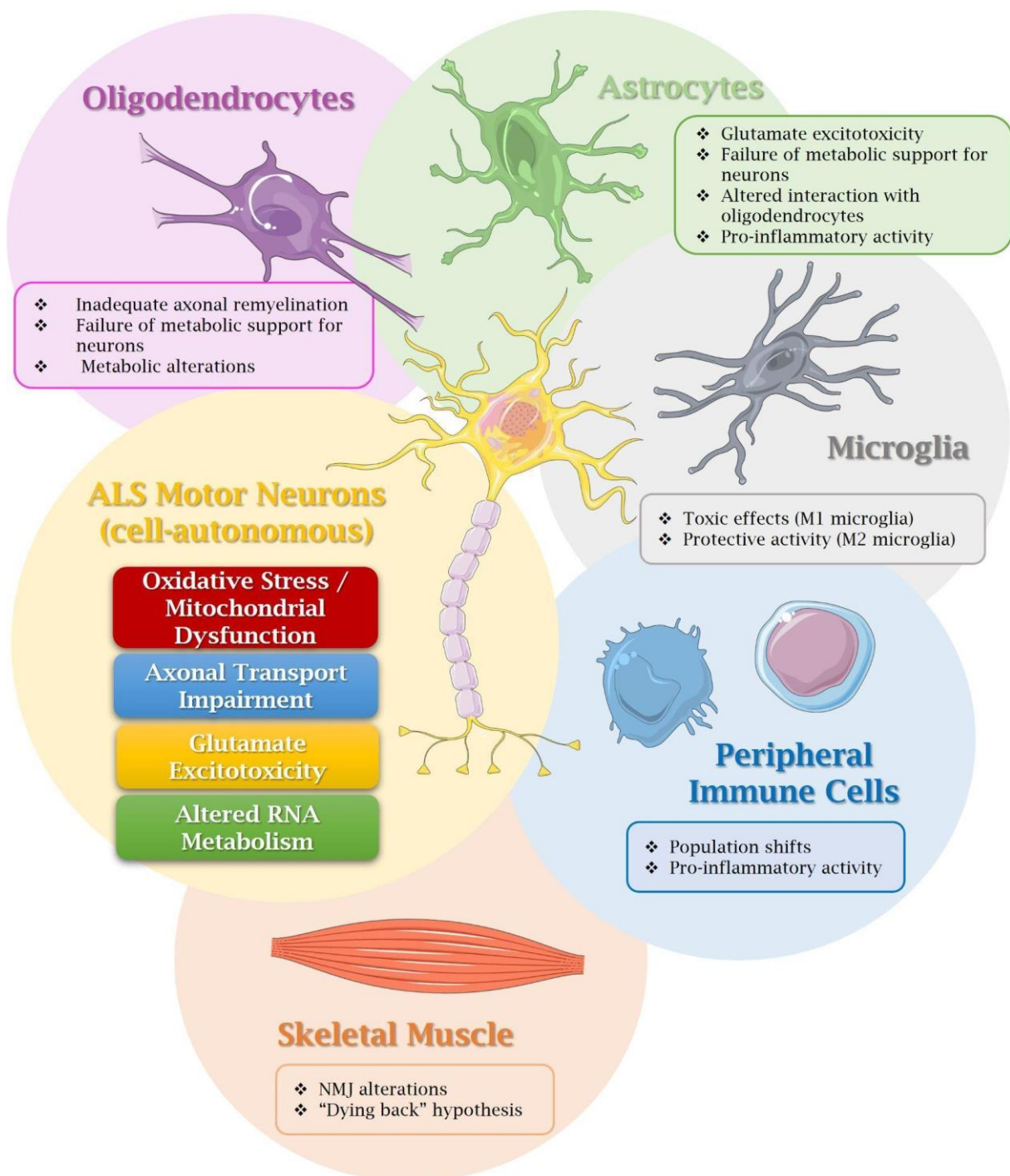


Figure 2. Overview of cell-autonomous and cell non-autonomous mechanisms of ALS pathogenesis. Motor neuronal pathogenetic mechanisms include oxidative stress and mitochondrial dysfunction, impairment of the axonal transport, glutamate excitotoxicity and altered RNA metabolism. Cell-non autonomous mechanisms are driven not only by different cells of the CNS, such as oligodendrocytes, astrocytes and microglia, but also by cells of the peripheral immune system and skeletal muscle.

2. Skeletal muscle as a secretory organ and its involvement in ALS

Skeletal muscle is the organ of the human body responsible for glucose metabolism, energy homeostasis and motor functions and represents the main reservoir of whole-body proteins. It is a highly dynamic tissue, changing its fiber structure and composition in response to several environmental stimuli and releasing peptides and cytokines, namely “myokines”, that exert autocrine/paracrine effects, acting locally within the muscle environment, as well as endocrine effects, impacting on other peripheral organs [69]. To date, skeletal muscle is widely recognized as a “secretory organ” and several proteomics studies identified more than 600 proteins in the skeletal muscle secretome that are responsible for the crosstalk within the muscle environment and with other distant targets as brain, adipose tissue, liver, bone, gut, skin, and circulatory system [70]. Of note, myostatin, LIF, IL-4, IL-6 and IL-7 are examples of myokines involved in the myogenesis, muscle growth and regeneration, while IL-6 also exerts multiple biological effects on metabolism and inflammation [71]. Brain-derived neurotrophic factor (BDNF), together with other myokines and extracellular vesicles (EVs), takes part in the skeletal muscle-mediated retrograde signaling responsible for the maintenance and function of skeletal muscle fibers and innervating motor neurons within the neuromuscular junction (NMJ) [72]. The NMJ represents the site of the skeletal muscle fibers and motor neurons mutual crosstalk, in which motor neurons modulate the function of skeletal muscle that, in turn, generates a retrograde signaling, crucial for the synergic maintenance of NMJ stability and integrity [73]. Given its role as a secretory organ constitutively releasing myokines, growth factors and EVs influencing its microenvironment and distant targets, together with the close relationship with motor neurons via the NMJ, it is not surprising that the skeletal muscle has recently been pointed out as an important contributor to ALS pathogenesis. Increasing evidence shows that NMJ dismantlement occurs before neurodegeneration, supporting the skeletal muscle dysfunction as an early event of a *cell non-autonomous* mechanism driving ALS disease progression [74]. Interestingly, the restricted expression of human SOD1 mutated forms (i.e., SOD1-G93A) in the skeletal muscle of mice is sufficient to recapitulate the disease phenotype, including NMJ dismantlement, skeletal muscle dysfunction and atrophy, and neurodegeneration, while this is not the case when mutated SOD1 is specifically overexpressed in motor neurons [75]. Furthermore, the overexpression of insulin-like growth factor 1 (IGF-1) in the skeletal muscle of SOD1-G93A mice improved the pathological phenotype by stabilizing the NMJ, prolonging motor neuron survival and delaying disease progression, further supporting an important role of muscle secreted factors for NMJ maintenance and motor neuron survival [76]. In ALS pathogenesis, skeletal muscle is characterized by several structural and functional alterations, including proteostasis impairment and atrophy, defects in muscle regeneration and differentiation, metabolic dysfunction, and secretome dysregulation (**Figure 3**).

2.1 Skeletal muscle atrophy and proteostasis impairment in ALS

The term “amyotrophic” refers to the progressive loss of muscle structure and function that culminates with paralysis in lower motor neurons, characterizing atrophy as an ALS typical hallmark. SOD1-G93A mouse models show a process of denervation that is driven by the selective switch from fast to slow muscle myofibers, due to an increased vulnerability of fast myofibers that are replaced by the more resistant slow myofibers [77]. Contextually, in skeletal muscle of ALS patients with a better prognosis is reported a modulation of several miRNAs involved in the slow-to-fast myofiber switch [78]. Despite being previously considered as a consequence of denervation, atrophy is widely recognized as a pathogenetic mechanism of ALS in which intrinsic myogenic alterations, such as impaired proteostasis or muscle aging, are supposed to play an active role. Studies conducted on both ALS patients and mouse models support atrophy as a mechanism driven by the downregulation in the IGF-1/PI3K/AKT pathway, subsequent suppression of protein synthesis and FOXO3-induced expression of muscle RING finger 1 (MURF1) and muscle atrophy F-box (atrogin-1) factors [79,80]. More importantly, atrophy has been reported as an early and independent process with respect to neurodegeneration, since apoptosis seems to be activated only in the late stage of the disease progression, exacerbating the atrophic phenotype and further contributing to the switch of the muscle fiber composition [81]. In addition, muscle atrophy may also be associated with an imbalance in proteostasis, with a prevalence of protein degradation with respect to protein synthesis. Generally, the skeletal muscle possesses more efficient protein quality control systems (i.e., the Unfolded Protein Response, the ubiquitin-proteasome and autophagy) than the nervous system. However, these pathways show a significant impairment in ALS, with a trend towards upregulation at early disease stages and gradual suppression during disease progression [82]. This imbalance, in turn, promotes the formation of toxic aggregates of misfolded proteins such as SOD1, FUS, UBQLN2, and TDP-43 in skeletal muscle, that can be released and taken up through different mechanisms by other recipient cells (i.e. motor neurons), disseminating the damage [83]. Taken together, these data provide evidence of the pivotal role of skeletal muscle alterations as primary mechanisms occurring early during ALS disease progression and actively contributing to its pathogenesis, supporting the “dying back” hypothesis.

2.2 Mitochondrial and metabolic dysfunction in ALS skeletal muscle

Mitochondrial dysfunction represents a common feature in different pathological mechanisms described in ALS. Several cell types, including skeletal muscle cells and motor neurons, show mitochondrial morpho-functional alterations that are considered the main ALS pathological hallmark. In the skeletal muscle of both ALS patients and SOD1-G93A mouse models, mitochondria show altered distribution and quantity, depolarized membrane potential and ultrastructural modifications, as well as impairment in the electron transport chain and antioxidant enzyme activity [84]. Dysfunctional electron transport chain complexes may in

turn lead to altered O₂ consumption, increase in ROS production, and decrease in ATP synthesis. The impairment of mitochondrial functionality is closely linked to perturbations in skeletal muscle energy metabolism, such as decreased glycolysis, increased beta-oxidation, and hypermetabolism that have been observed in both ALS patients and mouse models. Although not present in all ALS cases, hypermetabolism is associated with >50% of the patients and correlated with a worse prognosis [85]. Hypermetabolism generally occurs as an adaptive response to traumas, injuries or infections, in which lipolysis of adipose tissue and proteolysis of skeletal muscle are boosted to face the increased energy demands. Consequently, while increased lipolysis determines an increase of free fatty acids (FFA), promoting the accumulation of fat deposits in the body and favoring conditions of dyslipidemia, excessive proteolysis induces atrophy and skeletal muscle wasting [86]. Studies performed in SOD1-G93A mouse models highlighted metabolic dysfunction in skeletal muscle, such as glucose intolerance, probably related with the switch in the myofiber composition, and a shift towards beta oxidation, occurring before the symptoms onset and independently from neurodegeneration [87]. The detection of early mitochondrial failure driving the hypermetabolic state in the pre-symptomatic stages further supports the presence of skeletal muscle alterations prior to motor neuronal damage, highlighting a primary and active role of the skeletal muscle in ALS development.

2.3 The contribution of skeletal muscle in the NMJ dismantlement in ALS: from defects in muscle regeneration to secretome modulation

The neuromuscular junction (NMJ) is a specialized, tripartite chemical synapse, responsible for the transmissions of the electrical impulses necessary for motor functions, in which presynaptic motor neurons, postsynaptic muscles, and terminal Schwann cells play a key role. A finely regulated crosstalk between all the components within the NMJ is essential to preserve its integrity and functionality, including the retrograde signaling from the skeletal muscle [88]. In ALS, the equilibrium of the NMJ is perturbed by a phenomenon known as “skeletal muscle-mediated toxicity”, described as an imbalance within the muscle secretome towards an increase in toxic factors or a decrease in essential signaling factors (i.e., neurotrophic factors), leading to axonopathy and culminating with neurodegeneration [89]. The NMJ dismantlement has been described as a complicated process of denervation and reinnervation, until the failure of reinnervation leads to axonal retraction. Several studies highlighted that terminal Schwann cells are dysfunctional, losing their phagocytic ability and becoming unable to repair the synapsis [90]. Alterations in the NMJ signaling appear to occur earlier than the symptoms onset, entirely depending on the skeletal muscle [91]. In ALS, skeletal muscle exhibits impaired regeneration processes and involvement of inflammatory mechanisms. Satellite cells (SCs), in response to injury, undergo activation, proliferation and differentiation, playing an important role in muscle regeneration and NMJ maintenance. In muscle biopsies of ALS patients, SCs preserved their proliferation, but were unable to fully

differentiate into myotubes and thus to perform an adequate muscle regeneration [92]. SCs also communicate with immune cells during the regeneration process. Macrophage infiltration and high levels of inflammatory cytokines have been reported in both muscle biopsies of ALS patients and rat models, supporting a pivotal role of inflammation in ALS pathogenesis [93]. In addition, the primary function of the skeletal muscle as a secretory organ is strongly modulated during ALS pathogenesis. Several skeletal muscle secreted factors are altered in ALS patients and mouse models, such as IGF-1, BDNF, Irisin, as well as specific skeletal muscle miRNA (myomiRs) [94]. Of note, in the skeletal muscle of SOD1-G93A mice is reported an increased production of Semaphorin 3A (SEMA3A), involved in axonal retraction, as well as Nogo-A, a neurite outgrowth inhibitor, in both ALS murine models and ALS patients and correlated with disease progression [95]. However, it is noteworthy that the skeletal muscle secretes a multitude of other myokines and factors, whose impact on motor neurons remains still unknown and needs to be clarified. Hence, even if only a small part of the influence of skeletal muscle secretome on neurodegeneration is known and further studies are needed to offer a more precise overview, the current collected data are promising for attributing an active role of the skeletal muscle in NMJ disruption and neurodegeneration. Alongside the canonical cell-to-cell communication and the secretion of paracrine factors, skeletal muscle also communicates with other cells through the release of extracellular vesicles (EVs) [96]. As carriers of a variety of cargoes and mediators of short- and long-distance intercellular communication, EVs have been shown to actively contribute to ALS and are gaining an increasing interest in the study of its pathogenetic mechanisms [97]. The next sections will go into detail about the biology of EVs and their emerging role in muscle physiopathology, with a focus towards ALS.

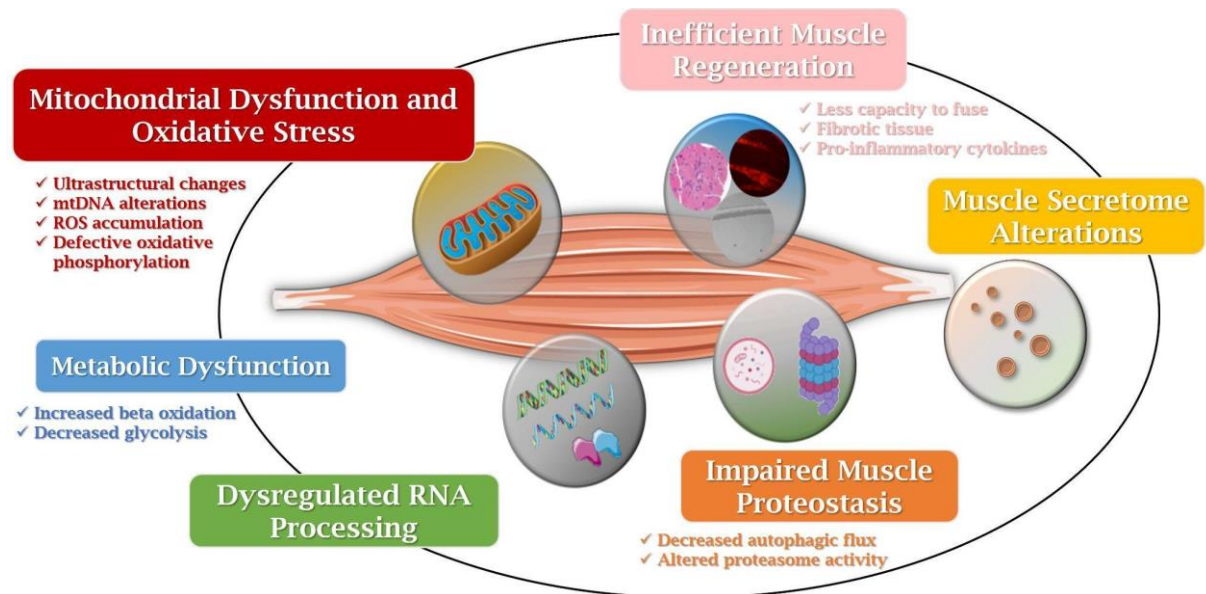


Figure 3. Skeletal muscle alterations in ALS. Skeletal muscle participates in ALS progression showing several morpho-functional abnormalities, such as oxidative stress and mitochondrial dysfunction, dysmetabolism, inefficient regeneration and differentiation, impaired proteostasis, imbalance in RNA processing, and alterations in its secretome. Adapted from Manzano R et al, 2020.

3. Extracellular Vesicles (EVs)

3.1 EVs classification and biogenesis

Extracellular Vesicles (EVs) are a set of heterogeneous, bilayered particles found in different biological fluids and released by almost all cell types, carrying several components, such as proteins, nucleic acids and lipids, to mediate short and long-distance intercellular communication [98]. Considered in the past as vehicles to remove unwanted compounds from the cell, EVs are now recognized as active signaling mediators in physiological and pathological processes [99]. EVs show a great heterogeneity and size overlapping; therefore, it is currently challenging to classify EVs in distinct categories. However, according to their biogenesis pathways, EVs can be generally distinguished in: exosomes, or small EVs, deriving from the endosomal pathway and with a size between 30 and 100 nm; microvesicles, or large EVs, directly originating from the outward budding of the plasma membrane, with a size ranging from 100 to 1000 nm; apoptotic bodies, bigger than 1000 nm and generating during the apoptosis process [100]. Exosomes originate as intraluminal vesicles (ILVs) during the maturation of endosomes in multivesicular bodies (MVB). The biogenesis of exosomes is a finely regulated multi-step process (**Figure 4**), in which multiple factors, such as the cell type and its related physiological or pathological state, can have an impact on recruiting different machineries for the cargo sorting, transport, and degradation/secretion. In particular, specific sorting complexes selectively mediate the formation of clustered microdomains of proteins and lipids through Endosomal Sorting Complex Required for Transport (ESCRT)-dependent or ESCRT-independent mechanisms. In the ESCRT-dependent biogenesis, ESCRT-0 and ESCRT-I contribute to the formation of ubiquitylated transmembrane microdomains and, via the ESCRT-II, to the recruitment of ESCRT-III for their budding and fission [101]. Among the ESCRT-independent mechanisms, ceramide formation from sphingomyelin drives spontaneous membrane curvature, generating microdomains [102]. Proteins of the tetraspanin family, such as CD63, CD81, CD82 and CD9, are also involved in the ESCRT-independent cargo sorting [103]. Exosome secretion requires several conditions, including balance with degradation pathways that is associated with the activity of sorting complexes and protein/lipid composition of MVBs membranes. MVBs are then conveyed to lysosomes or plasma membrane by the microtubule transport, that is in turn modulated by the activity of different RAB GTPases. RAB27A and RAB27B, RAB11 and RAB35 have been identified as primary regulators of exosome secretion, being involved in docking to the plasma membrane and sub-membrane cytoskeleton rearrangements [104]. The final step of fusion of the MVB with the plasma membrane, allowing the release of ILVs in form of exosomes, is further regulated by Soluble NSF attachment protein receptors (SNARE) complexes [105]. The biogenesis of microvesicles consists mainly in lipid/protein composition rearrangements of the plasma membrane and reorganization of the underlying actin cytoskeleton (**Figure 4**). Here, Ca²⁺-dependent proteins, such as flippases, floppases, scramblases and calpains, promote changes in the phospholipid bilayer asymmetry, favoring membrane budding and release of

microvesicles [106]. As a consequence, membrane-associated cargoes are selected by their affinity with the lipids of the inner part of the plasma membrane, building highly ordered microdomains that will become the site of origin of the microvesicles. The fission and release of microvesicles from the plasma membrane is regulated by actin/myosin interactions and ATP contraction [107]. Apoptotic bodies, the largest component of EVs, are the result of the fragmentation of apoptotic cells, thus facilitating their phagocytosis. Unlike the large and small EVs produced in different physiological contexts, apoptotic bodies are exclusively produced during the last steps of the programmed cell death, and carry degraded genetic material, macromolecules, cytoplasmic residues, and entire organelles. The formation of apoptotic bodies prevents the release of these materials into the extracellular environment, thereby avoiding pathological processes [108]. The biogenesis of apoptotic bodies includes plasma membrane blebbing, which promotes the formation of large protrusions. The apoptotic cell fragments into apoptotic bodies, which are subsequently phagocytosed by macrophages and/or neighboring cells. Selective recognition followed by engulfment of apoptotic bodies occurs due to changes in their surface, i.e., oxidation of surface molecules or exposure of phosphatidylserine on the membrane outer leaflet [109]. After their release into the extracellular milieu, EVs can reach distant cell targets, interacting with them in several ways (i.e., by tetraspanins, integrins, lectins, lipids and extracellular matrix components) and being internalized by macropinocytosis, phagocytosis, endocytosis or direct membrane fusion [110]. Once inside recipient cells, EVs release their content, impacting on their biological functions and eliciting responses in different physiopathological conditions. The presence of EVs in all biological fluids (i.e., blood, saliva, urine, tears, and breast milk) highlights the capacity of EVs to transmit information in an endocrine manner, underscoring their crucial role in systemic signal transmission to various organs and tissues throughout the entire organism [111]. Long-distance communication is possible as the cargo is shielded from enzyme degradation in biological fluids. Moreover, EVs cargo is packaged in stable structures that remain intact for extended periods [112]. Next to their constitutive release and physiological role, EVs trafficking is strongly modulated and implicated in several pathological processes, such as cancer, metabolic and inflammatory diseases, and neurodegenerative disorders. Therefore, EVs show a great potential as biomarkers to track disease progression and, in some cases, as useful therapeutic tools [113].

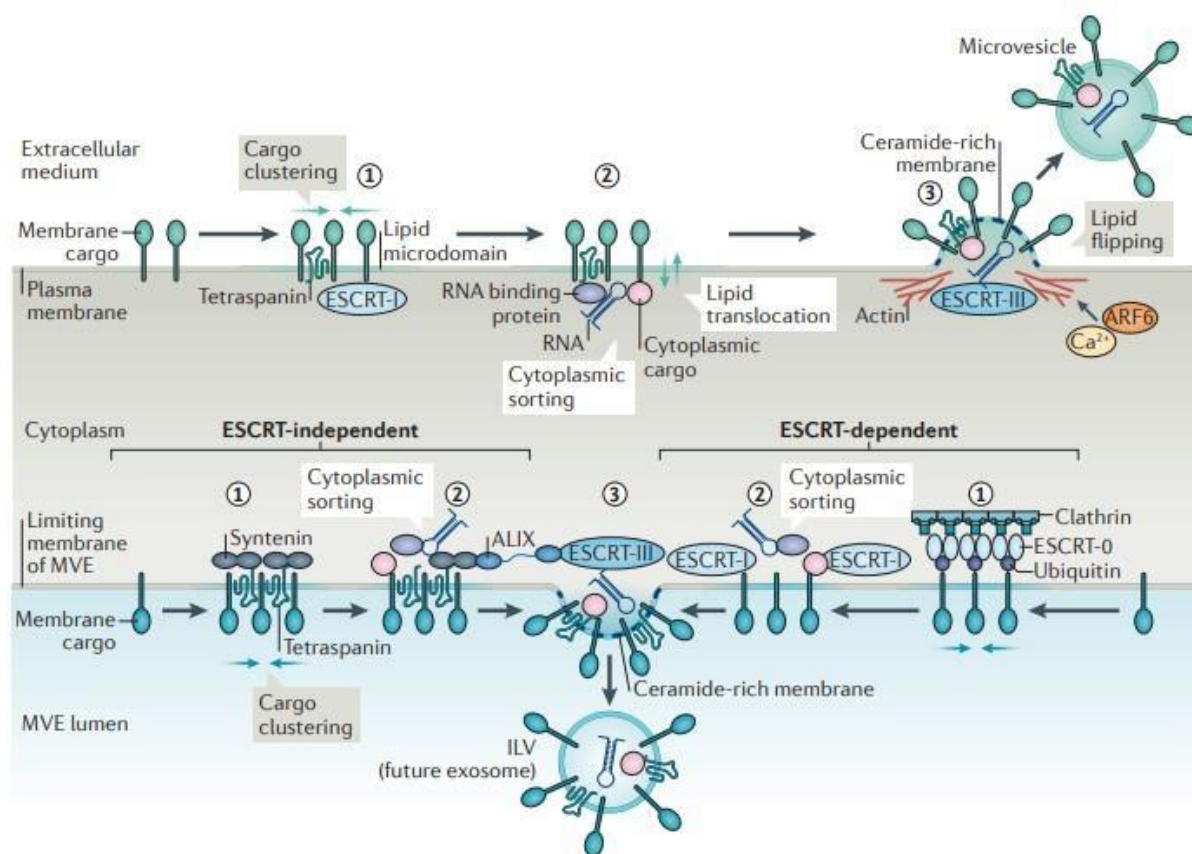


Figure 4. Biogenesis of EVs. Adapted from van Niel et al, 2018.

3.2 Methods for EVs isolation and characterization

3.2.1 EVs isolation

EVs isolation can be performed according to different methods, generally based on either size or content, providing a range of specificities, yields and purity. Different methods can be combined to improve the separation of EVs with different properties. To date, there is no standardized method for physical separation of large EVs and small EVs, because of size overlapping and similar membrane content. Differential ultracentrifugation (UC) is the most common method for concentrating EVs from biological fluids (**Figure 5**) [114]. UC consists of sequential differential steps of centrifugation with distinct centrifuge force, thus allowing the concentration of different sub-populations of EVs according to their size. Generally, samples undergo an initial clearing step, in which cells and large debris are sedimented at low g-force, followed by large EVs sedimentation at $10,000\text{--}20,000 \times g$. A final filtration step is often performed to clear particles with a size ranging between 200–500 nm, and small EVs are then sedimented by prolonged centrifugation at $100,000\text{--}110,000 \times g$. The main limit of UC is the need for large sample volume: even with increased volumes of the starting biological source, the recovery efficiency is often low. Other limits of UC concern the purity of the obtained fractions. In fact, contaminants such as protein aggregates and lipoproteins can co-sediment

with EVs. Moreover, the smaller fraction of large EVs may overlap in size with small EVs, thus obtaining mainly a mixture of different types of vesicles. Density gradient ultracentrifugation (DGU) isolates and separates vesicles based on their different flotation density, and represents a possible way to overcome size overlapping. DGU technique includes two main methods for the gradient formation, i.e., a continuous density gradient, formed either during centrifugation or before, or a stepwise gradient, in which the density increases gradually within a sucrose cushion. Small EVs show a flotation density of 1.08–1.22 g/mL on sucrose gradients, while vesicles from the endoplasmic reticulum float at 1.18–1.25 g/mL, and from the Golgi at 1.05–1.12 g/mL [115]. Since different EVs can have similar densities, density gradient ultracentrifugation does not allow to obtain a pure fraction of EVs excluding other EVs subtypes, viruses, and proteins. To maximize the purity of the obtained EVs fraction, gradient ultracentrifugation can be coupled to UC. An alternative experimental approach is ultrafiltration (UF), which exploits membrane filters with nano-sized pores retaining EVs with larger diameters [116]. The combination UF and UC may allow an effective separation of large and small EVs, but even if UF is a faster, simpler, and less laborious method, the step of concentration can increase the levels of contaminant molecules and contextually a partial loss of EVs. Moreover, both ultracentrifugation and UF-based methods can alter the vesicles integrity, favoring the formation of aggregates that can in turn affect the downstream characterization. In contrast, size exclusion chromatography (SEC) represents an isolation method that overcomes these problems through gel filtration. This procedure is characterized by a stationary phase, i.e., columns filled with porous polymer beads of different sizes, and an eluent solution (i.e., phosphate saline buffer, PBS) that moves across the column. During the elution process, big molecules cross the pores faster and elute earlier than smaller molecules, which can instead enter within the porous beads and have a longer retention time. The stationary phase or the chromatography column can be packed with different gel polymers, such as crosslinked dextrans (Sephadex), agarose (Sephacrose), polyacrylamide (Biogel P), or allyldextran (Sephacryl). SEC has been shown to effectively isolate EVs from plasma and urine, without contaminating lipoproteins and protein aggregates [117]. However, since SEC allows only the analysis of small sample volumes, pretreatment and concentration by UC or UF are required to obtain EVs preparations free from impurities such as proteins or lipoproteins. The flow rate of the fractionation process also plays a crucial role in SEC-mediated EVs isolation. Specifically, lower linear speed allows optimal column efficiency, leading to a higher quality assay and improved specificity, integrity, and functionality of isolated vesicles. Therefore, SEC results in a cost-effective and high-purity EVs isolation system. However, SEC cannot discriminate between exosomes and microvesicles of the same size. When using this technique with the aim of separating different EVs subtypes, it is recommended to use SEC in combination with other methods, for example immunoaffinity. Immunoaffinity-based capture methods (IA) show great potential towards purity and selectivity. This method isolates EVs according to the presence of particular surface molecules, allowing to identify EVs released by specific cell types. Antibodies are covalently bound to a fixed phase (i.e., magnetic beads, highly porous monolithic silica microtips, plastic plate surfaces, cellulose filters) [118]. IA-

based isolation exploits different antibodies directed towards canonical surface markers of EVs, such as CD63, CD9 CD81, Tsg101, Alix, etc. This method allows to target not only proteins, but also other molecules on EVs surface, such as phospholipids (i.e. phosphatidylserine) and polysaccharides, by functionalizing the fixed phase with specific proteins (i.e. Annexin and Lactadherin), lectins or lipid-binding proteins. IA isolation exhibits higher efficiency and purity in comparison with UC and density floatation. However, this method is not suitable for large sample volumes and captured EVs may not retain functionality even if successfully eluted from the bead surface. A novel approach for EVs isolation is represented by microfluidic devices based on immunoaffinity, size, and density. For example, ExoChip is a common microfluidic chip functionalized with anti-CD63 antibodies and used for EVs isolation [119]. Different commercial kits are also available for EVs isolation by polymer-based precipitation, (i.e., polyethylene-glycol (PEG) precipitation), reducing EVs solubility to allow their precipitation and subsequent isolation with sequential centrifugation at low g-force [120].

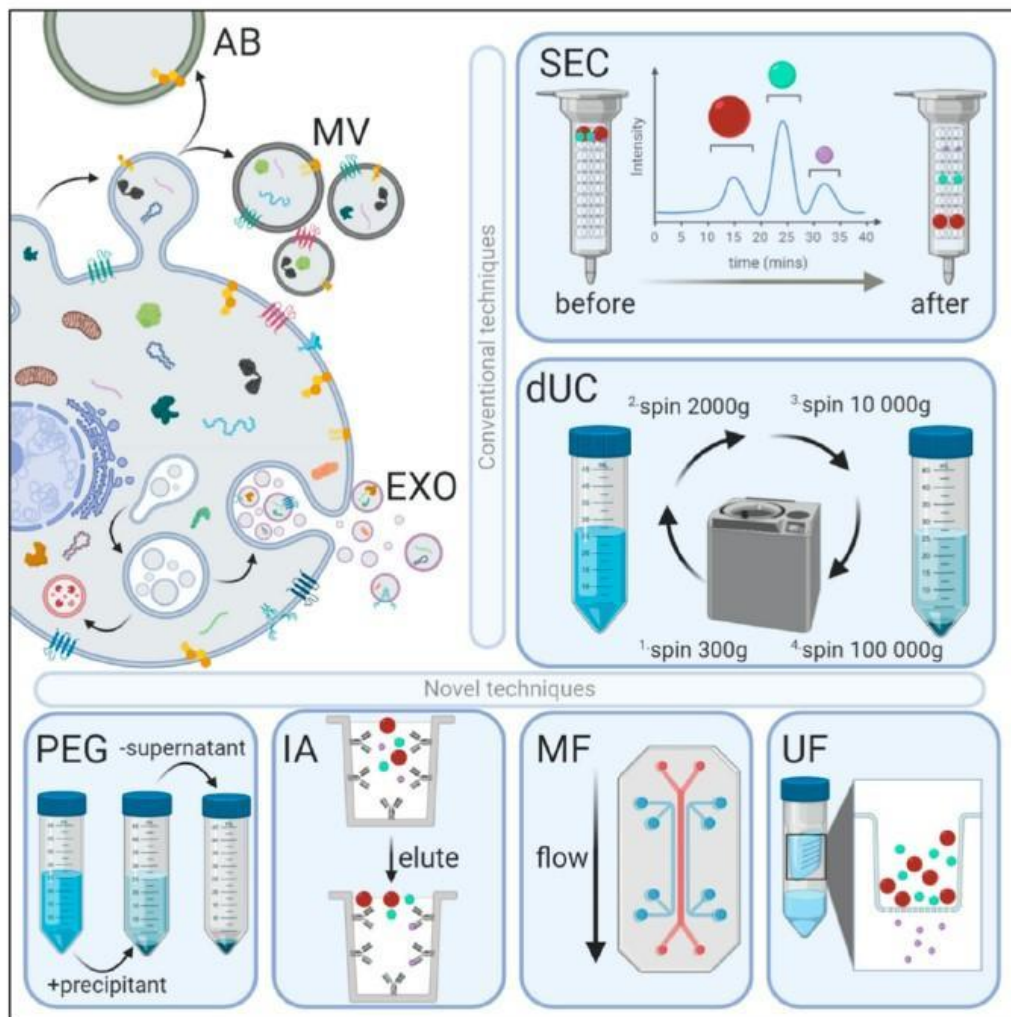


Figure 5. General methods for EVs isolation. Adapted from Sidhom K et al, 2020.

3.2.2 EVs characterization

Generally, the first step of EVs characterization is determining the total protein concentration of isolated EVs, although it gives no information about changes in protein composition or EVs number [114]. Immunoblotting is a common method used to examine alterations in the protein content of isolated EVs. Morphological analysis allows to discriminate between vesicles and other particles, such as protein aggregates. Transmission electron microscopy (TEM) has been the method of choice for EVs observation and estimation of their size distribution. Moreover, immunolabeling combined with TEM can determine the protein localization. However, TEM preparation and acquisition can potentially harm the sample and introduce artifacts that impede accurate characterization. This limitation can be effectively circumvented by employing cryo-TEM, which enables the visualization of EVs in their natural state, preserving EVs morphological and ultrastructural integrity [121]. In addition to TEM, Atomic Force Microscopy (AFM), by utilizing a micrometric cantilever with a nanosized tip, performs high-resolution imaging of EVs in a single exposure. AFM is considered another valuable method to characterize EVs, as it provides multiple information regarding both vesicles directly budding from the plasma membrane and isolated vesicles, taking advantage of milder preparation protocols and higher statistical significance when determining the size distribution [122]. The combination of different methods may improve EVs characterization. Dynamic light scattering (DLS) is an optical technique used to determine the size and quantity of particles suspended in a solution. In DLS, a laser beam illuminates the sample, and the particles in the suspension scatter light in a manner influenced by their size, shape, and molecular interactions. Over time, fluctuations in light scattering occur due to the movement of particles. These fluctuations are analyzed to create a mathematical model of the changes in scatter over time, known as the autocorrelation function, estimating particle size and quantity. The parameters derived are an average computed from all the particles within the analyzed volume. However, it is important to note that scatter intensity is size-dependent, which means DLS tends to provide skewed results toward larger particles. Consequently, the presence of polydispersity in most EVs preparations, including non-EVs particles, can limit the accuracy of this method for describing the size distribution of EVs [123]. Another optical approach for determining particle size and number, but independently from light scattering as DLS, is Nano-tracking analysis (NTA). Size information of individual particles is obtained from their Brownian motion, and their concentration is determined by particle counts within a defined volume. Like DLS, NTA detects all light-scattering particles, thus requiring pure EVs preparations. This limitation can be overcome by labeling EVs with specific fluorescent probes [124]. Flow cytometry is a widely employed technique that relies on the passage of particles through a laser beam, followed by the scattering of light detected by specialized detectors. One of the primary advantages of flow cytometry is its ability to handle high sample throughput. This method enables the measurement of both EVs concentration and size. However, a significant limitation lies in the size range of EVs that can be effectively detected, as conventional flow cytometers can only accurately measure EVs larger than 270 nm in

diameter. Nonetheless, the advent of advanced flow cytometers has substantially improved resolution, now allowing for the detection of EVs as small as approximately 150 nm in diameter. Moreover, specialized calibration tools have been developed, enabling the determination of both the size distribution of EVs and the quantification of potential contaminants, such as lipoproteins, through the combination of refractive index (RI) and scattering signals. Flow cytometry offers a significant advantage in its capacity to concurrently quantify various markers within a sample, employing different fluorescent labels that may consist of conjugated antibodies or ligands [125]. Resistive pulse sensing (RPS) is an alternative approach for the analysis of EVs size and quantity. This method operates by detecting the passage of individual particles through a pore within a membrane. An electric voltage is applied across the membrane to create a current. As particles traverse the pore, an increase in resistance occurs, and the frequency of these resistance changes is used to estimate particle size and quantity [126]. However, pore clogging remains a significant technical challenge, and it is crucial to work with pure EV preparations, as RPS cannot discriminate between EVs and other similarly sized particles. A recent innovation in RPS is microfluidic resistive pulse sensing (MRPS), exemplified by Sectradyne®. MRPS employs a microfluidic cartridge, which incorporates an analyte reservoir, on-board filters, ports for voltage biasing, a sensing electrode, a fluid resistor, and a microfluidic nano-constriction through which vesicles pass individually. When a vesicle within the analyte traverses the nano-constriction, it causes a change in the electrical resistance of the constriction, proportional to the ratio of the nanoparticle volume to that of the nano-constriction. This results in specific changes in the sensing voltage, allowing for precise sizing and counting of individual vesicles. MRPS offers the advantage of requiring only a small volume of analyte for measurement, and the use of disposable microfluidic cartridges eliminates the risk of sample cross-contamination. Moreover, these cartridges accommodate a wide spectrum of particle diameters, ranging from 40 nm up to 1 μ m [127].

4. The role of EVs in ALS: the involvement of the skeletal muscle and the “dying back” hypothesis

4.1 The emerging role of EVs in skeletal muscle physiopathology

Skeletal muscle has been described as an adaptable tissue that dynamically modifies its structure and function in response to several physiopathological conditions [128]. These adaptations are related with the secretion of several muscle factors, namely myokines, allowing the communication of the muscle with the external environment [129]. Besides myokines, skeletal muscle also releases a multiplicity of EVs (SkM EVs) as additional signals regulating skeletal muscle function and interactions with other organs [130]. These EVs are very heterogeneous and play an important role in skeletal muscle physiology, modulating muscle growth, differentiation and regeneration, and consequently contributing to the whole-body homeostasis [131]. In particular, during myogenesis, myoblasts proliferate until they are able to fuse and differentiate in non-proliferating multinucleated myotubes, finally composing the myofibers [132]. During this process, several types of EVs are produced, with biogenesis pathways, size and composition varying between proliferation and differentiation, and consequently with a different impact in skeletal muscle physiology [133]. Several studies exploiting high-throughput techniques have been performed to shed light on the complexity of SkM EVs, to characterize their content in terms of proteins, lipids and RNA, for a better understanding of their putative functions [131]. Proteomic studies performed to characterize human SkM EVs protein profiling identified a distinct composition between large and small EVs populations and a differential contribution in several important pathways, such as metabolism, cell dynamics, vesicle trafficking, and signaling [134,135]. For instance, skeletal muscle-derived large EVs have been reported to be enriched in tetraspanins CD81, CLIC1 and galectin-1, as well as cargoes deriving from endoplasmic reticulum, mitochondria and cytosol, involved mainly in protein metabolism and trafficking and RNA post-transcriptional modifications. Skeletal muscle-released small EVs are instead enriched in CD63, CD9, CD81 and CD82 tetraspanins and in proteins from plasma membrane, endosomes and lysosomes, mainly involved in free-radical scavenging and cell-to-cell signaling and interactions [131]. Lipidomic analyses have highlighted that SkM EVs show a specific lipid enrichment in cholesterol, diacylglycerol, sphingomyelin and phospholipids with respect to their parental cells, associated with a higher membrane rigidity [136]. Furthermore, SkM EVs derived from C2C12 myotubes show a particular composition in fatty acids (FA), with a prevalence of lipid bilayer precursors and a tendency of enrichment in the most abundant FA species from the parental cell, thus representing a mechanism adopted from the cell to compensate the intracellular accumulation of FA [137]. Concerning the RNA composition, SkM EVs contain prevalently small RNA species, defined as myomiRs, which modulate several pathways implicated in the skeletal muscle physiology, such as immune response, neuromuscular junction maintenance, regulation of muscle mass, and cytoskeleton remodeling [138]. Therefore, EVs derived from the skeletal muscle are highly heterogeneous and their effects

may vary depending on the cell type and its physiological state. For instance, EVs released from myotubes inhibit proliferation in recipient myoblasts, inducing at the same time an earlier differentiation [139], while EVs released from activated satellite cells modulate collagen biosynthesis in presence of hypertrophic stimuli, preventing an excessive extracellular matrix deposition [140]. However, in presence of altered environmental conditions, cells may respond by modulating their EVs production and content. In the skeletal muscle, although its paracrine signaling has been attributed mainly to the release of cytokines, alterations in EVs trafficking have been reported in several pathological conditions, including denervation and atrophy, oxidative stress, metabolic disorders and inflammation [141]. In this context, SkM EVs are potential contributors for the spreading of harmful signals by transferring deleterious lipid, protein or miRNA species, affecting recipient cells and further perpetuating the pathological state [131]. Several studies support the possibility for SkM EVs to enter the circulatory system in order to exert their systemic effects [142,143]. However, the currently available data are often in contrast and further analysis is needed to corroborate this hypothesis and understand whether SkM EVs may act out of the skeletal muscle microenvironment. Interestingly, a recent *in vivo* study identified that EVs derived from the skeletal muscle interstitium are characterized by specific protein markers (ATP2A1, β -enolase, and desmin) and myomiRs (miR-1, miR-206, miR-431 and miR-486) that were absent in the plasma-derived EVs fraction, even after exercise. In particular, SkM EVs seem to represent only a small portion of circulating EVs, rather acting at the level of the skeletal muscle microenvironment, by regulating the myogenesis through the repression of the master regulator Pax7 [144]. However, these results still do not exclude the possibility that a small amount of SkM EVs may enter the bloodstream and contribute to skeletal muscle intercommunication with other tissues. SkM EVs trafficking may represent an important piece of the puzzle in skeletal muscle physiopathology, as well as in the crosstalk with other districts of the body. Therefore, it would be of great importance to perform a deeper characterization of the composition and functions of SkM EVs. Considering these observations and the increasing evidence of the skeletal muscle as a main protagonist in whole-body homeostasis, it is not surprising that ALS may be included as a disorder characterized by alterations originating from the skeletal muscle, driven also by the release of EVs. The next section will deepen the intricate EVs trafficking in ALS, with particular focus on the overcoming of the skeletal muscle towards motor neurons as the main player in ALS pathogenesis.

4.2 Role of EVs in ALS pathogenesis

EVs are constitutively produced by almost all cell types and are widely recognized as mediators of intercellular communication by mediating the exchange of biological material, including proteins, nucleic acids and lipids [98]. Despite the underlying mechanism remains to be clarified, EVs can cross biological barriers, mainly the blood-brain barrier (BBB) to enter in the bloodstream and mediate the intercellular signaling between the central nervous system

(CNS) and the periphery [145]. Within the CNS, EVs participate in several homeostatic processes, including neuronal growth and development, neuroprotective responses to oxidative stress and inflammation, brain vascular integrity maintenance, as well as NMJ retrograde signaling [146]. Due to their multiple roles in homeostasis, it is not surprising that EVs trafficking may be altered in pathological conditions, thus contributing to an impaired intercellular signaling. Specifically, EVs participate in ALS pathogenesis by transferring misfolded proteins (i.e., mutated forms of SOD1, TDP-43, FUS, and C9orf72), forming toxic aggregates that affect recipient cells and further disseminate the misfolding and damage [147]. The toxic signaling mediated by EVs in ALS is mainly described in the crosstalk between motor neurons and other CNS cells, including astrocytes and microglia (**Figure 6**). For instance, EVs derived from NSC-34 motor neurons carrying the SOD1-G93A mutation have been reported to show a particular miRNA signature, inducing the expression of NF- κ B and pro-inflammatory cytokines and reducing the phagocytic activity of N9 microglial cells [148]. Since its discovery, ALS has been conceived as a “neurocentric” disease, in which the damage originates from motor neurons and spreads to other cells in a *cell-autonomous* fashion [66]. However, the characterization of EVs crosstalk between neuronal and non-neuronal cells is further revolutionizing the concept of ALS from a pure neurodegenerative disease towards a multisystemic disorder, implying *cell non-autonomous* mechanisms contributing synergistically to ALS pathogenesis [25]. For instance, EVs released from iPSC-derived astrocytes from ALS patients have been reported to show a dysregulation in miRNA content and exert toxic effects on recipient motor neurons, proving the existence of a bidirectional crosstalk mediated by EVs between motor neurons and non-neuronal cells [149]. Another study performed in ALS patients showed an impairment in secreted cytokines by peripheral monocytes, induced by circulating EVs (circEVs) containing mutant TDP-43 [150], as an example of the involvement of different cell types with respect the CNS ones in ALS pathology. Moreover, compelling evidence states a primary role for the skeletal muscle in ALS pathogenesis, with the emerging “dying back” hypothesis that is gradually replacing the old “neurocentric” paradigm [151]. EVs have been pointed as mediators of the muscle-neuron communication, participating in the transfer of factors involved in NMJ maintenance [152]. Accordingly, EVs released from C2C12 myotubes have been demonstrated to promote motor neuron development and branching in NSC-34 recipient cells [153]. These observations, together with the increasing evidence of skeletal muscle early alterations in ALS [66,73], support a potential role of EVs released from the skeletal muscle in ALS pathogenesis by carrying neurotoxic factors that contribute to NMJ dismantlement and neurodegeneration. In both ALS mouse models and patients, dysregulation in myomiRs profile, such as miR-206 and miR-133b, has been reported to occur in response to denervation [152] and it is therefore likely that these myomiRs may be vehiculated by EVs to other cell targets [154]. A recent work has found that EVs released from the skeletal muscle of ALS patients exert toxic effects on both recipient healthy motor neurons, by reducing the neurite length and increasing the cell death, and myotubes, causing atrophy, cell stress and death, respectively. The same group attributed the defects in RNA processing as the putative leading mechanism of the observed neurotoxic effects, supported by the

proteomics analysis performed on the SkM-EVs [155]. Another work described the differential effects of small and large EVs from the skeletal muscle of ALS patients on different recipient cells. In particular, only the small EVs fraction showed a significant uptake and exerted toxic effects on recipient motor neurons, astrocytes and myotubes, with a greater impact on motor neurons. Conversely, large EVs showed a very low uptake and no significant effects [156]. Taken together, these findings represent the first important evidence of a predominant role of the skeletal muscle in spreading detrimental signals towards other recipient cells via EVs, further supporting the “dying back” hypothesis in ALS pathology. It is therefore plausible that the skeletal muscle, at the early stage of the disease, shows alterations that may influence firstly its microenvironment and then, as a secretory organ releasing chemotactic factors and EVs, may spread harmful signals disseminating the damage to other cell targets. However, the skeletal muscle-motor neuron crosstalk mediated by EVs in ALS disease is still poorly investigated. Therefore, a deeper characterization of these interactions with more consistent models that better recapitulate the disease in its different stages, are of great importance to clarify ALS pathogenetic mechanisms mediated by EVs.

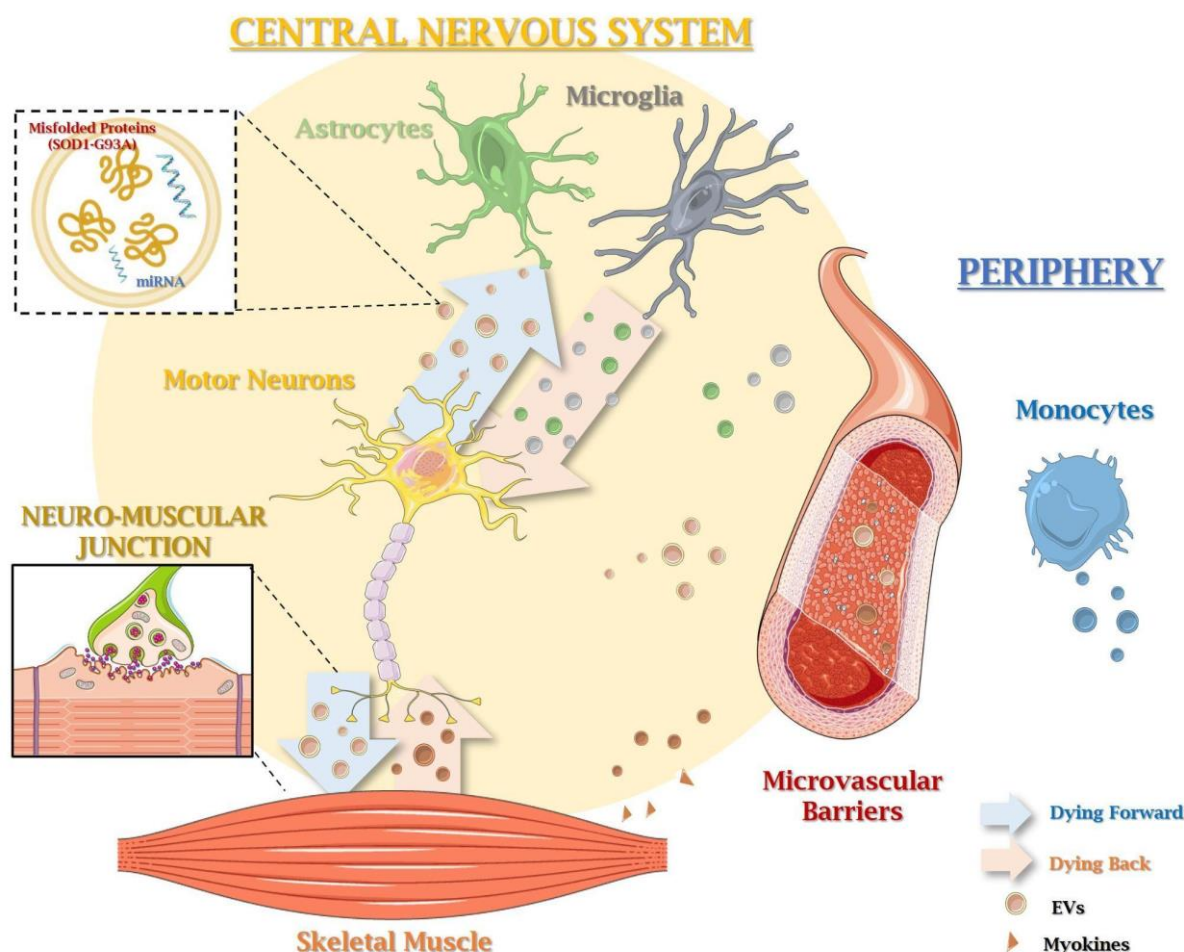


Figure 6. EVs crosstalk in ALS. In ALS pathogenesis, EVs mediate transfer of misfolded proteins (i.e., SOD1-G93A) and other molecules, such as miRNAs, affecting healthy recipient cells. EVs crosstalk occurs between CNS components (motor neurons, microglia, astrocytes), between motor neurons and skeletal muscle within the Neuro-Muscular Junction, and between CNS and periphery through the microvascular barriers (such as the blood-brain barrier). According to the “dying forward” hypothesis, motor neurons drive EVs towards other cells. However, the emerging “dying back” hypothesis supports that EVs are also driven from other cells, mainly skeletal muscle, to motor neurons.

4.3 EVs as biomarkers in ALS: what about SkM EVs?

Due to its imprint as a slowly progressive multisystemic disorder, often associated with other neurological comorbidities, ALS is generally lately diagnosed or even misdiagnosed [157]. Therefore, there is an urgent need to improve the research towards prognostic and diagnostic biomarkers that may help to track the disease onset, with the final goal to enhance the therapeutic strategies and ameliorate the patients’ clinical course. Currently, ALS diagnosis

consists in multiple strategies, including genetic testing of the most common mutations (SOD1, TDP-43, FUS, C9ORF72), clinical evaluation, electromyography (EMG) to assess the degree of muscle denervation, and neuroimaging to track structural changes on motor neurons [158]. Nowadays, the main difficulties hampering the finding of ALS specific biomarkers are the absence of standardized procedures and methodologies, the lack of consistent patient cohorts and the scarcity of longitudinal studies. Several studies are exploiting high-throughput omics techniques, aiming to characterize alterations of several metabolites, such as proteins, miRNA, and lipids, in the cerebrospinal fluid (CSF), blood and urine of ALS patients, in order to find a specific ALS signature [159]. Of note, proteomics studies on CSF and blood of ALS patients detected neurofilament proteins, whose accumulation is correlated with neurodegeneration and a shorter survival, as putative biomarker candidates [160]. However, these proteins are not specific to ALS, but are rather common between neurodegenerative disorders [161]. Several other metabolites, related to oxidative stress, neuroinflammation, hypermetabolism, and muscle denervation, have been identified as altered in body fluids of ALS patients [158]. As EVs can be found in all biological fluids, are able to cross the BBB and circulate in the bloodstream, reflect the molecular signature of the cell of origin, and protect their cargo from degradation thanks to their bilayered membrane, EVs have emerged as promising source for prognostic and diagnostic biomarkers in several diseases [162]. In ALS, the potential of EVs as markers of disease progression is related not only to alterations in their cargo, but also in their size and number. A recent work reported an increase in the mean size of plasma EVs from ALS patients with respect to healthy controls, but no significant differences in the number [163]. Another study, performed on the CSF of ALS patients, found an increase in leukocyte-derived EVs [164], in contrast with a more recent one in which no significant differences in CSF EVs number were reported [165]. To date, CSF and blood are the most investigated body fluids for the detection of an ALS specific molecular signature carried by EVs. In CSF-derived EVs, no misfolded SOD1 has been identified, but an increase in the INHAT repressor (NIR) has been reported as putatively involved in ALS pathogenesis [166]. Regarding miRNAs, miR-124-3p has been found as a potential prognostic biomarker, since it is strongly correlated with ALSFRS-R score, and thus with the disease severity [167]. With respect to CSF, blood has the advantage of implying less invasive procedures and being more easily accessible as a source of biological markers, but still with several other limitations that must be taken in consideration. Since ALS is a multisystemic disorder implying not only the CNS, circulating EVs (circEVs) may offer precious information about early systemic alterations [168]. Despite the aforementioned advantages, circEVs show a high heterogeneity in terms of cell of origin, and the greatest part of these EVs may derive from blood cells rather than neuronal cells. Therefore, it can be quite challenging to find an ALS neuronal specific signature within circEVs. Nevertheless, since able to cross the BBB, it cannot be excluded that EVs from other cell types may enter in circulation and contribute to the pathological signaling characterizing ALS. Another particular feature of circEVs is that plasma proteins interacting with the EVs surface, namely the “protein corona”, may further affect their composition and functions [169]. Despite these limitations, several studies identified different potential candidates for ALS

biomarkers by analyzing circEVs. In ALS patients, only plasma large EVs showed higher concentrations of SOD1, FUS, and TDP-43, while no differences were reported in small EVs [163]. Other interesting alterations found in ALS plasma EVs and considered as potential biomarkers are decreased levels of HSP90 and PPIA [170], and increased levels of coronin-1a [171]. Also, astrocyte-derived EVs isolated from plasma showed higher levels of IL-6, considered as a valid marker of neuroinflammation, but not specific for ALS since shared with other neurodegenerative disorders [170]. Concerning miRNAs, miR-4454 and miR-127-3p, involved in neurogenesis, MNs integrity and synapse formation, and axon guidance and intracellular motor proteins, respectively, were found dysregulated in more than one study [172,173]. Taken together, the current studies suggest that EVs from patients' biofluids may represent a useful tool to find putative biomarkers for ALS disease progression. However, to accomplish this task remains challenging due to several limitations, including the different procedures adopted to isolate the EVs. This issue may be overcome by standardizing the protocols for EVs isolation and downstream processing according to the MISEV guidelines, in order to maximize the reproducibility of the obtained results [174]. Furthermore, the lack of longitudinal studies represents another critical point in the finding of ALS biomarkers, since ALS pathological alterations, as well as EVs molecular signature, may vary during the disease progression [147,158]. Currently, EVs released from the skeletal muscle have not been yet investigated as potential ALS biomarkers. Given the increasing evidence of the role of skeletal muscle alterations in ALS early pathogenetic events [25,73], SkM EVs may be possible mediators of toxic signaling contributing to the disease [155], it may be worth to investigate the molecular profile of SkM EVs to find an ALS specific signature. Since skeletal muscle participates in the disease progression, SkM EVs can also contribute to this impairment and may be able to transfer harmful signals in the periphery. An in-depth characterization of EVs released from different cell types may allow to improve ALS diagnostic field and fill the current data opening new clues towards a better understanding of ALS.

EVs AS BIOMARKERS IN ALS

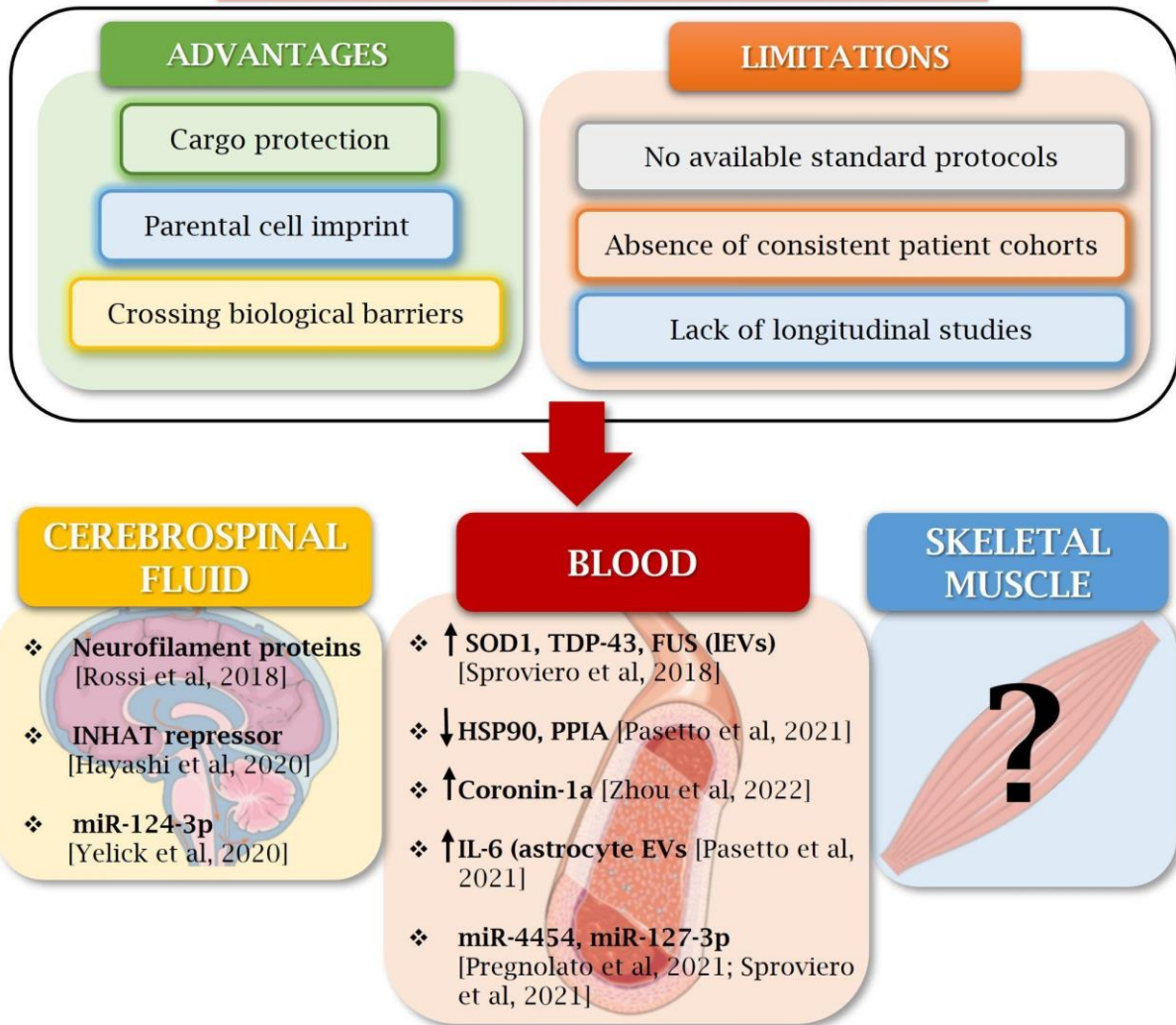


Figure 7. Overview of EVs as biomarkers in ALS. EVs show several advantages for their use in the diagnosis of ALS, but also some limitations. The main current studies to find potential ALS biomarkers within EVs have been performed in cerebrospinal fluid and blood. To date, none of these has yet investigated the EVs signature from the skeletal muscle.

4.4 Use of EVs in ALS therapy: state of the art

Given the multifactorial and intricate nature of ALS pathogenesis, no successful treatment currently exists to completely cure the condition or significantly improve the quality of life of patients. The main therapeutic approaches for ALS include pharmacological treatment, gene therapy, and the use of stem cells. However, a great limitation of these approaches that hampers their effective application relies on the impossibility to cross the BBB and selectively target the CNS [175]. In this context, ongoing studies are increasingly exploring the use of EVs as a novel, non-invasive therapeutic approach for various neurodegenerative conditions, including ALS. Unlike many other molecules or cells employed in therapies, EVs possess the unique ability to bidirectionally cross the BBB, thus allowing the direct communication between the bloodstream and the CNS. Moreover, EVs exhibit a low tendency to trigger immune responses, reducing the risk of rejection by patients. Additionally, through various approaches it is possible to modify the expression of EVs surface molecules, modulating the therapeutic target specificity and helping to mitigate potential side effects of off-targeting [176]. The use of EVs in the treatment of neurodegenerative disorders encompasses different approaches: I) Loading drugs or macromolecules (i.e., proteins and nucleic acids) into vesicles; II) Isolating EVs from patients autologous cells or stem cells to harness their paracrine effect. Several studies have revealed that the beneficial effects attributed to mesenchymal stem cells, adipose tissue-derived stem cells, and bone marrow-derived endothelial progenitors derive from the release into the extracellular environment of EVs containing trophic and neuroprotective factors, thus mitigating cellular damage. Consequently, the direct employment of the secretome of these cells represents a valuable cell-free therapeutic approach [177,178]. Moreover, EVs show a great potential as drug delivery systems as they can be loaded with drugs or macromolecules to facilitate their uptake. This approach is particularly useful for drugs with limited bioavailability or for proteins and nucleic acids which can enhance the treatment outcomes. Various techniques, including electroporation, sonication, multiple freeze/thaw cycles, or simple incubation of EVs with the drug and gentle agitation, can be employed to load these substances into EVs. The choice of a specific technique depends on the characteristics of the substance to be incorporated [179].

MATERIALS AND METHODS

Cell culture, treatments, and light microscopy

Murine neuroblastoma x spinal cord NSC-34 hybrid cell line and C2C12 murine myoblasts (ATTC® CRL-1772TM) were cultured in DMEM 4.5g/L glucose (Sigma Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Sigma Aldrich, St. Louis, MO), 2mM L-glutamine (Corning, Manassas, VA), 100 UI/mL penicillin and streptomycin solution (Corning, Manassas, VA) and 10.000 U/mL amphotericin B (antimycotic solution) (Sigma Aldrich, St. Louis, MO) in a 5% CO₂ humidified atmosphere, at 37°C. Cells were maintained in 75 cm² flasks at a concentration of 2x10⁶ cells/mL by passage every two to three days. NSC-34 cells were treated with 10 µM of RA in low FBS concentration (1%) for up to five days to differentiate into motor neuron-like cells [180]. C2C12 myoblasts reached differentiation into myotubes by addition of differentiation medium (DMEM 4.5 g/L glucose supplemented with 2% Horse Serum (HS)) for up to 7 days. To assess the impact of EVs derived from plasma and skeletal muscle of both WT and SOD1-G93A mice at different disease stages on recipient cells, we treated both wild-type NSC-34 motor neuron-like cells and C2C12 myotubes with a concentration of 0.5 µg/mL of EVs in depleted culture medium for 24 hours. Subsequently, cells were processed for further analysis. To assess the impact of EVs derived from plasma and skeletal muscle of WT and SOD1-G93A mice on recipient NSC-34 cells, morphological analysis were performed to assess reduction in the neurite length and oxidative stress, respectively. For the determination of neurite length, we captured after treatment images of NSC-34 cells using a bright-field microscope (Axio Observer Colibri 7 fluorescence microscope, Zeiss, Oberkochen, Germany). Neurite length was then measured using ImageJ software with the NeuronJ plugin.

Human neuroblastoma SH-SY5Y cells were cultured in DMEM 4.5g/L glucose (Sigma Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Sigma Aldrich, St. Louis, MO), 2mM L-glutamine (Corning, Manassas, VA), 100 UI/mL penicillin and streptomycin solution (Corning, Manassas, VA) and 10.000 U/mL amphotericin B (antimycotic solution) (Sigma Aldrich, St. Louis, MO) in a 5% CO₂ humidified atmosphere, at 37°C. Cells were maintained in 75 cm² flasks at a concentration of 2x10⁶ cells/mL by passage every two to three days. To induce oxidative stress, cells were treated with H₂O₂ (Carlo Erba, Milan, Italy) at a final concentration of 100 µM for 1 hour, followed by 24 hours of recovery in EVs-depleted medium. After performing the treatments, cells were harvested for further analyses [28,122].

MTT assay

MTT assay was performed according to a modified protocol [122]. After treatments, WT and SOD1-G93A SH-SY5Y cells were incubated with 1mg/mL of MTT, prepared in supplemented DMEM culture medium, for 3 hours at 37°C and 5% CO₂; then, cells were washed three times

with phosphate buffer saline (PBS 0.2 M, pH 7.4) and the reduced MTT formazan crystals were solubilized with dimethyl sulfoxide (DMSO) (Carlo Erba, Milan, Italy). The optical density (OD) was read at the spectro-photometer (Multiskan™ FC Microplate Photometer, ThermoFisher Scientific, Wal-tham, MA) at 570 nm.

EVs isolation from plasma and skeletal muscle of wild-type and SOD1-G93A mice

Blood samples and skeletal muscle (quadriceps) from wild-type (WT) and SOD1-G93A mice at early (90 days of age) and late (120 days of age) symptomatic stages of the disease, were obtained in collaboration with Dr. Cristiana Valle's laboratory at the Santa Lucia Foundation in Rome. Plasma from WT and SOD1-G93A mice was collected and processed using a standard protocol for platelet depletion. Then, highly purified fractions of circulating extracellular vesicles (circEVs) were collected by ultrafiltration combined to size-exclusion chromatography (UF/SEC), and subjected to downstream analysis. For the skeletal muscle biopsies, the right and left quadriceps of each mouse were harvested and maintained in 20 mL of DMEM (Dulbecco's Modified Eagle Medium) with 4.5g/L glucose (Sigma Aldrich, St. Louis, MO), 100 IU/mL penicillin/streptomycin solution (Corning, Manassas, VA), and 10,000 U/mL amphotericin B (antifungal solution) (Sigma Aldrich, St. Louis, MO). They were then cut by using sterile instruments, placed in a dish filled with 20 mL of fresh culture medium without serum, and incubated for 24 hours in a humidified atmosphere with 5% CO₂ at 37°C. After incubation, the supernatant was collected and centrifuged at 2000 rcf for 20 minutes for clarification and concentrated using Amicon Ultra-15 Centrifugal Filter Units with Ultracel-10 membranes (MWCO = 10 kDa; Merck Millipore, Billerica, MA, USA) at 3000 rcf for 30 minutes. This step was repeated 2 or 3 times when needed. Concentrated samples were then loaded onto size-exclusion chromatography columns to isolate the fraction enriched with extracellular vesicles (SkM-EVs) and subsequently processed for further analysis.

EVs isolation by ultrafiltration combined to Size-Exclusion Chromatography (UF/SEC)

For UF/SEC, media (20 mL) were loaded onto Amicon Ultra-15 Centrifugal Filter Units with Ultracel-10 membrane (MWCO = 10 kDa; Merck Millipore, Billerica, MA, USA) and concentrated to ≤300 µL by repeated centrifugation at 4000 × g. The concentrate was collected, and the filter membrane was washed with 200 µL DPBS. Media concentrates were fractionated by SEC as described by Böing et al. with some adaptations [181]. In brief, 300 µL of concentrated media was applied to a 10 mL sepharose CL-2B column (GE healthcare, Little Chalfont, UK) and the EV rich protein-low fractions were collected using PBS as an eluent. EVs-enriched fractions were concentrated again to 100 µL on an Amicon Ultra-4 Centrifugal

Filter Unit with Ultracel-10 membrane (MWCO = 10 kDa; Merck Millipore) by centrifugation at $4,000 \times g$. The obtained EVs concentrated samples were then used for further analysis.

Microvesicles-enriched fraction isolation by ultracentrifugation

Before the treatments, complete DMEM growth medium was overnight centrifuged at 110 000 g in order to remove contaminant FBS-derived EVs. After the treatments, MV-enriched fractions were concentrated from H₂O₂-treated and control SH-SY5Y cells conditioned culture medium by differential centrifugation [28,122]. Briefly, the culture medium of each sample was first centrifuged at 500 g for 10 minutes at room temperature, and the resulting supernatant was then centrifuged at 800 g for 10 minutes at room temperature. The last centrifugation of the supernatant was performed at 2000 g for 20 minutes at room temperature in order to remove dead cells or cell aggregates. Cell-depleted supernatant was then centrifuged at 20.000 g for 20 minutes at 4°C using a Beckman Coulter Ultracentrifuge Optima XE (Beckman Coulter, Brea, CA), and the resulting MV-enriched pellet was collected and used for further analysis (i.e., the microvesicles-enriched fraction; MVs).

RNA preparation and RT-qPCR analysis

RNA was isolated from cell pellets according to TriFast (VWR, Italy) reagent user's manual. Quality and concentration of RNA were measured using NanoDrop (ThermoFisher, Italy). Reverse transcription was performed by Bioline SensiFAST™ cDNA Synthesis Kit (Aurogene, Italy) according to the user's manual using 1 µg of RNA for each sample. 50 ng of cDNA were used to perform RT-qPCR amplification (Bioline SensiFAST™ SYBR Lo-ROX Kit, Aurogene, Italy) according to the following scheme: denaturation at 95 °C for 2 min followed by 40 cycles of amplification (95 °C for 5 s and 60 °C for 30 s). A melting curve and standard dilution scale were performed to check single melting peak, correlation, and efficiency. Primers are listed in **Table S1**.

Atomic Force Microscopy Imaging, vesicle counting, and roughness measurements

The Atomic Force Microscopy (AFM) topographies were acquired in contact mode, using a Multimode Nanoscope II (Veeco) equipped with DNP AFM probes (Bruker), working in air, in a controlled environment (temperature 23 +/- 1 °C and relative humidity 70%) with scans speed of 1-2 seconds per row and 512x512 points. The rationale of the AFM imaging was to acquire images of 60 microns wide regions, in order to visualize at the same time several cells and their connections and then perform one or more zoom (20 microns wide) in regions of interest. The interaction force between the tip and the sample was carefully maintained around 1 nN throughout all the measurements in order to preserve both the tip and the sample. The

size distribution of the vesicles budding from the plasma membrane was analyzed using the freely available Gwyddion Software (www.gwyddion.net).

For vesicles counting, the analysis of the AFM images was performed with the freely available software Gwyddion (www.gwyddion.net). All images were minimally treated (i.e., a mean plane subtraction, substrate tilt correction and line coupling) in order to avoid the thermal drift and the tilting of the sample. The isolated vesicles were counted by using Gwyddion's routine "Mark Grains by Segmentations", changing the working parameters according to the best possible identification of the vesicles. This very same approach can't be used to directly measure the budding on the cellular membranes since their big and inhomogeneous variation in height (i.e., thicker and round shaped in the nuclear region, flat on the border) makes impossible to find a set of parameters that allow a good identification of the vesicles. Two different options were then available: (i) to deeply modify the images or (ii) to select a different counting method (i.e., by hands), we preferred the latter since the former can induce systematic and unpredictable errors.

For roughness measurements, AFM surface root mean square deviation (roughness; Rq) analysis of cell surface was performed by ImageJ Software version 1.8.0. Square 6×6 μm^2 regions were randomly selected, and five measurements were taken from each sample. Representative 3D reconstructions of AFM images were also performed with the same software. Methods are published in [28,122].

Transmission and Scanning Electron Microscopy

For SEM analysis, cells cultured on glass coverslips were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for 1 hour at ice temperature and postfixed with 1% OsO₄ in the same buffer for 1 hour on melting ice. After fixation, cells were dehydrated with ethanol (EtOH), 50%, 70%, 95% and 100%, followed by chemical drying with EtOH and Hexamethyldisilazane (HMDS) (Sigma-Aldrich, St. Louis, USA), 2:1, 1:1, 1:2 and finally in pure HMDS, evaporated overnight. Consequently, the specimens were mounted on aluminum stubs and 3 nm chromium-coated with a Quorum Q150 T sputter (Quorum Technologies, UK) in order to increase the electron conductivity. For TEM analysis, cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for one hour at ice temperature and post-fixed with 1% OsO₄ in the same buffer for two hours at ice temperature. After fixation, cells were dehydrated with ethanol (25%, 50%, 70%, 90%, and 100%) and embedded in Spurr resin. Thin sections of 60 nm were cut at the ultramicrotome (Powertome PC, RMC Boeckeler Instruments, Germany) and deposited on 200 mesh copper-grids. After isolation, MVs from all experimental groups were fixed with 0.1% paraformaldehyde (PFA) in DPBS for 30 minutes at room temperature. Fixed samples were stained with 2% uranyl acetate for 7 minutes at room temperature, loaded on 200 mesh carbon-coated grids for the TEM observation. SEM and TEM analysis were both performed using a Zeiss Auriga Scanning Electron Microscope (Zeiss,

Oberkochen, Germany) equipped with the STEM module, operating at 8 and 20 kV, respectively [28, 122].

Western Blot analysis

The protein concentration of EVs samples was measured by BCA assay kit (ThermoFisher Scientific, Waltham, MA). For western blot assays, 10 µg of proteins of the resuspended pellet of EVs were used. Proteins were separated by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (10-12% polyacrylamide, SureCast™ Acrylamide Solution, ThermoFisher Scientific, Waltham, MA) and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk or with 3% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 hour at room temperature. Then, the membranes were incubated overnight at 4°C with the following primary antibodies: CD63 (1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX), CD9 (1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX), OXPHOS (1:1000 dilution, MitoSciences, Abcam, Cambridge, United Kingdom) diluted in 3% BSA or 5% nonfat dry milk. After washing in TBST, the membranes were incubated for 1 hour at room temperature with a goat anti-mouse IgG (dilution 1:5000; Bethyl Laboratories, Montgomery, TX) secondary antibody diluted in TBST in 5% nonfat dry milk. The immunoreactive bands were detected by using commercial enhanced chemiluminescence reagent (ECL) (WesternBright™ Quantum, Advansta Inc., San Jose, CA). The density of the specific bands was quantified by densitometric analysis.

Label-free proteomics and bioinformatics analysis

Proteome profiling of isolated EVs-enriched fractions was carried out by label-free proteomic approach, to identify differentially expressed proteins between WT and SOD1-G93A mice, and to investigate whether EVs carry a proteomic signature correlated with ALS progression. Samples were prepared according to Filter Aided Sample Preparation (FASP) protocol. SEC-purified EVs were lysed by sonication on ice (Sonicator U200S control, IKA Labortechnik, Staufen, DE) at 70% amplitude and then tested for protein concentration with the Bradford assay to digest 15 µg of proteins. Tryptic peptides were analyzed in triplicate by LC-MS/MS using the UltiMate™ 3000 UPLC (ThermoFisher Scientific, Waltham, MA) chromatographic system coupled to the Orbitrap Fusion™ Tribrid™ (ThermoFisher Scientific, Waltham, MA) mass spectrometer. Specifically, peptides were loaded on the Trap Cartridge C18 (0.3 mm ID, 5 mm L, 5 µm PS; ThermoFisher Scientific, Waltham, MA) and then separated on an EASY-spray Acclaim™ PepMap™ C18 (75 µm ID, 15 cm L, 2 µm PS; ThermoFisher Scientific, Waltham, MA) nanoscale chromatographic column setting the flow rate at 300 nL/min in a total run time of 65 min according to the method described in [182]. Proteomics MS/MS raw data were processed using MaxQuant 1.6.6.0 and Perseus 1.6.10.50 (Max-Planck Institute for Biochemistry, Martinsried, DE) by searching for peptides against the UniProt database

(released 2020_06, taxonomy Homo Sapiens, 20,588 entries), as reported in previous works [183]. Briefly, carbamidomethylation of cysteines (C) was defined as fixed and quantification modification, while oxidation of methionine (M) and deamidation of asparagine (N) and glutamine (Q) were set as variable modifications. The false discovery rate (FDR) was set to 1% both for protein and peptide levels. iBAQ values were used to quantify protein abundance in each sample and used for subsequent functional analysis. For each comparison, the minimum number of valid values accepted was set at 2 in at least one treatment group. At least, protein ratios (EVs from treated cells/ EVs from untreated cells) were used for the Gene Ontology and functional enrichment analysis through the Ingenuity Pathway Analysis tool (IPA, Qiagen, Hilden, DE). IPA can predict the activation (z-scores ≥ 2.0) or inhibition (z-scores ≤ -2.0) of transcriptional regulators or downstream for the loaded dataset based on prior knowledge of expected effects from published literature citations stored in the IPA system [184].

Statistical Analysis

Results are expressed as means $\pm$ SD. Data analysis was performed using a two-way ANOVA. Comparisons between the two groups were performed using a student's t-test (GraphPad Prism 8 Software, San Diego, CA). Differences between groups were considered statistically significant for $p < 0.05$.

For AFM analysis (**SECTION 5 and 6**), results are expressed as means $\pm$ SEM. Multiple comparisons were performed by two-way ANOVA. Comparisons between two groups were performed using a student's t-test. Comparisons between two distributions were analyzed using a Kolmogorov-Smirnov test (GraphPad Prism 9 software, GraphPad Software, San Diego, CA).

RESULTS

SECTION 1. Isolation and characterization of circulating and skeletal muscle EVs from wild-type and ALS mice at different disease stages

Collaborations: 1) Sapienza University of Rome, Comparative Cell Biology and Imaging Laboratory (CCBIL), Rome, Italy; 2) Fondazione Santa Lucia IRCCS, c/o CERC, Rome, Italy; 3) Institute for the Structure of Matter (ISM), National Research Council (CNR), Rome, Italy; 4) Research Center for Nanotechnology for Engineering (CNIS), Sapienza University of Rome, Rome, Italy.

Summary

Amyotrophic Lateral Sclerosis (ALS) is widely recognized as a multisystemic disorder involving not only the CNS, but also skeletal muscle and immune system alterations [1,21]. During ALS progression, EVs play a pivotal role by transferring bioactive molecules (i.e., misfolded proteins or miRNAs) from diseased cells to healthy cells, thus modulating the responses of recipient cells and contributing to disease dissemination [147]. Given the ability of EVs to cross the biological barriers, it is plausible that EVs may represent the intermediary messengers between the CNS and the periphery. Mouse models are employed for the study of ALS as they offer several advantages. The transgenic mouse model overexpressing the SOD1 glycine 93-to-alanine mutation (SOD1-G93A) is widely used as it shows early disease onset and fast progression, cumulative MNs loss and muscle atrophy, thus recapitulating the human pathology [8,9]. For this reason, we decided to dissect the role of EVs during the disorder progression in SOD1-G93A ALS mouse models, with the aim to define their molecular profile by proteomics analysis (that will be discussed in section 2) and their effects on different recipient cells (i.e., motor neurons and myocytes), considering both local (exerted by skeletal muscle EVs) and systemic (exerted by circulating EVs) (**Figure 8**). Therefore, we first isolated EVs from plasma (circEVs) and skeletal muscle (SkM EVs) of wild-type and SOD1-G93A mice at an early symptomatic stage (90 days of age) and late symptomatic stage (120 days of age) of the disease, to assess eventual alterations in the EVs populations from mutated mice during ALS progression and compared to the WT. After isolation with UF/SEC (described in *Materials and methods*), we performed morpho-quantitative characterization of both circEVs and SkM EVs by TEM and AFM, and molecular characterization by WB. Interestingly, both circEVs and SkM EVs showed different features in terms of morphology, size distribution, and quantity. Moreover, these differences emerged both when considering different disease stages and when comparing wild-type and SOD1-G93A mice.

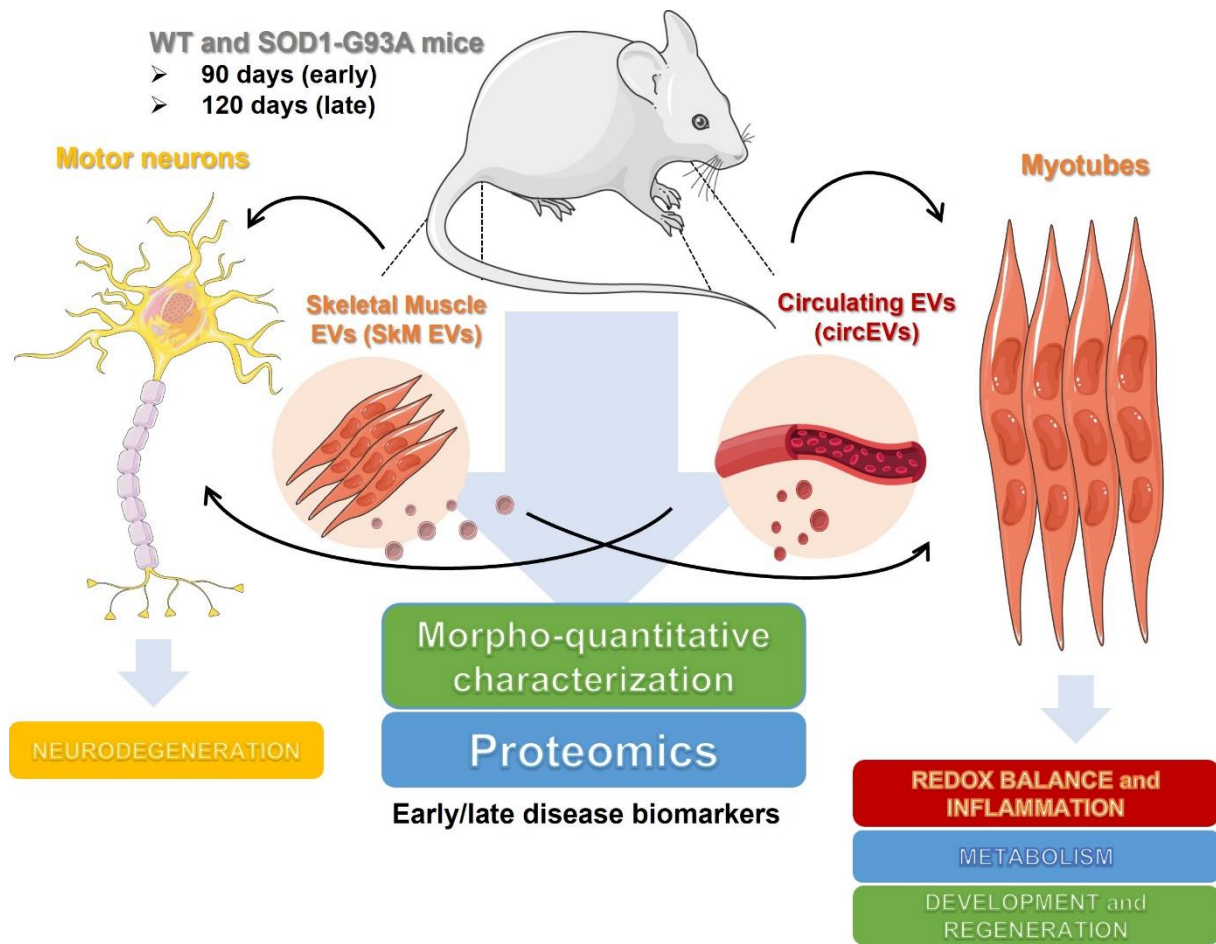


Figure 8. Graphical abstract of the workflow. EVs from plasma and skeletal muscle of WT and SOD1-G93A mice at different disease stages (90 and 120 days, respectively) were isolated and characterized for their morpho-quantitative features and proteomic profile to detect biomarkers associated with the disease progression. Then, EVs were used to treat wild-type motor neurons and myotubes to test their effects in terms of spreading of harmful signals to recipient cells (i.e., neurodegeneration for recipient motor neurons and redox balance, inflammation, metabolism, development and regeneration for recipient myotubes).

Results

1.1 Morphological and molecular analysis of circEVs and SkM EVs from WT and SOD1-G93A mice corroborated the efficiency of the isolation protocol

After isolation by UF/SEC, we performed morphological analysis by TEM and molecular analysis by WB for EV-specific markers, for both circEVs and SkM EVs from WT and SOD1-G93A mice at 90 and 120 days of age. Specifically, TEM analysis revealed the presence of rounded-shape vesicles (**Figure 9AB**). Besides the detection of vesicles that confirms the successful isolation, TEM analysis may also help in evaluating the purity of the sample. Of note, in both circEVs and SkM EVs isolated fractions there is presence of debris, probably due to the derivation of the samples (i.e., plasma and skeletal muscle tissue, respectively) that are more heterogeneous sources compared to *in vitro* cell culture media (**Figure 9AB**). Moreover, we detected the expression of CD63 and CD9 tetraspanins (EV-specific markers) by Western

Blotting in the isolated fractions of all experimental groups, thus further corroborating the efficiency of the isolation protocol (Figure 9C).

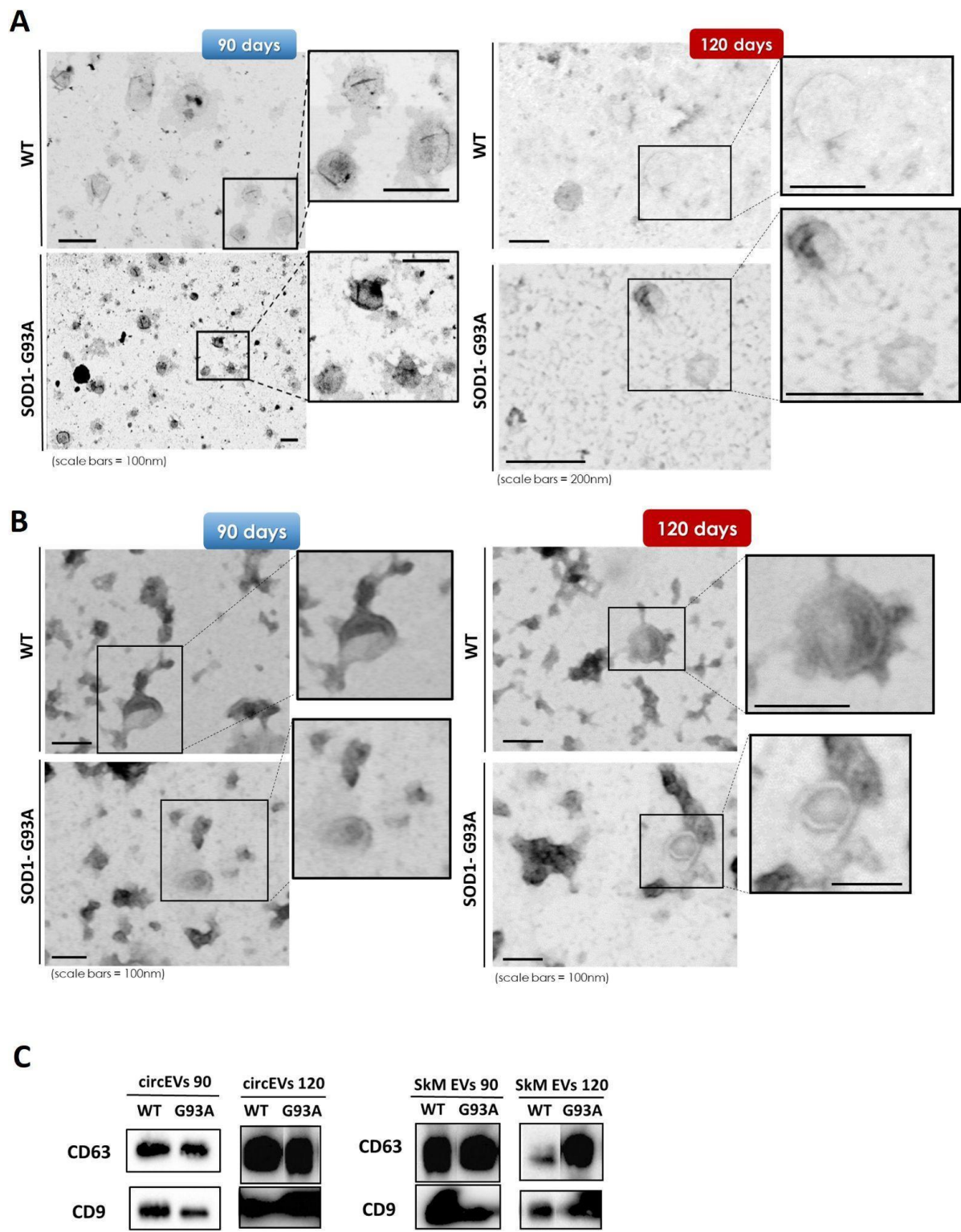


Figure 9. Morphological and molecular characterization of circEVs and SkM EVs isolated from WT and SOD1-G93A mice at 90 and 120 days of age. (A) TEM micrographs of circEVs isolated from WT and SOD1-G93A mice at 90 and 120 days. Scale bars = 100 nm and 200 nm. **(B)** TEM micrographs of SkM EVs isolated from WT and SOD1-G93A mice at 90 and 120 days. Scale bars = 100 nm. **(C)** Western blot analysis of CD63 and CD9 EV-specific markers for circEVs (left panel) and SkM EVs (right panel) from WT and SOD1-G93A mice at 90 and 120 days.

1.2 AFM morpho-quantitative analysis revealed differential variations in circEVs and SkM EVs populations during disease progression and between WT and SOD1-G93A

Atomic Force Microscopy (AFM) represents an high-resolution alternative approach to characterize EVs morphology and to define their number and size distribution, allowing the 3D reconstruction of the sample [28,122]. Specifically, circEVs isolated from 90-day-old mice showed slight changes in size distribution. While the WT group has a diameter range between 150-500 nm, with a peak around 200 nm, the SOD1-G93A group shows a broader distribution, with diameters up to 600 nm and a peak around 300 nm (**Figure 10A**). Regarding the number of vesicles, 25 vesicles were counted for the WT group, while a significantly higher number (n=552) was obtained for the SOD1-G93A group in the same sample volume (**Figure 11C**). This demonstrates that in the earlier symptomatic stage of 90 days, the EVs population of SOD1-G93A mice is markedly different from WT mice, both in terms of number, showing a significantly higher count compared to the control group, and in terms of size, as larger diameter vesicles are present within the distribution (WT mean diameter = 226,5 nm; SOD1-G93A mean diameter = 273,2 nm). Similarly, for circEVs isolated from 120-day-old mice (**Figure 10B**), although the size distribution seems similar, from 150 to 400 nm with a peak at 150 nm for both populations, the SOD1-G93A group shows higher mean diameters compared to the WT (**Figure 11D**). Moreover, it was again found a higher number of vesicles from SOD1-G93A mice (n=719) compared to WT mice (n=408) within the same sample volume (**Figure 11C**). Differences were also observed for SkM EVs. For SkM EVs isolated from 90-day-old mice (**Figure 11A**), while the WT group shows a distribution ranging from 50 to 300 nm with a peak around 100 nm, the SOD1-G93A group exhibits a narrower distribution, ranging from 50 to 100 nm with a peak around 70 nm. Indeed, the WT group showed a mean diameter of 116,8 nm, while the SOD1-G93A group of 94,03 nm (**Figure 11D**). Regarding the vesicles number, 433 vesicles were counted in WT samples, and 339 in the SOD1-G93A samples. At 120 days (**Figure 11B**), SkM EVs populations of WT and SOD1-G93A mice follow the same trend of size distribution (ranging from 50 to 200 nm with a peak around 70 nm, with mean diameters of 94,19 nm for WT and 81,34 nm for SOD1-G93A) but differ in the number (n=339 for WT, n=633 for SOD1-G93A, **Figure 11C**). Therefore, when comparing SOD1-G93A group with WT group, SkM EVs population generally shows a reduction in size at both disease stages and an opposite trend in vesicle number, with a reduction at 90 days and an increase at 120 days.

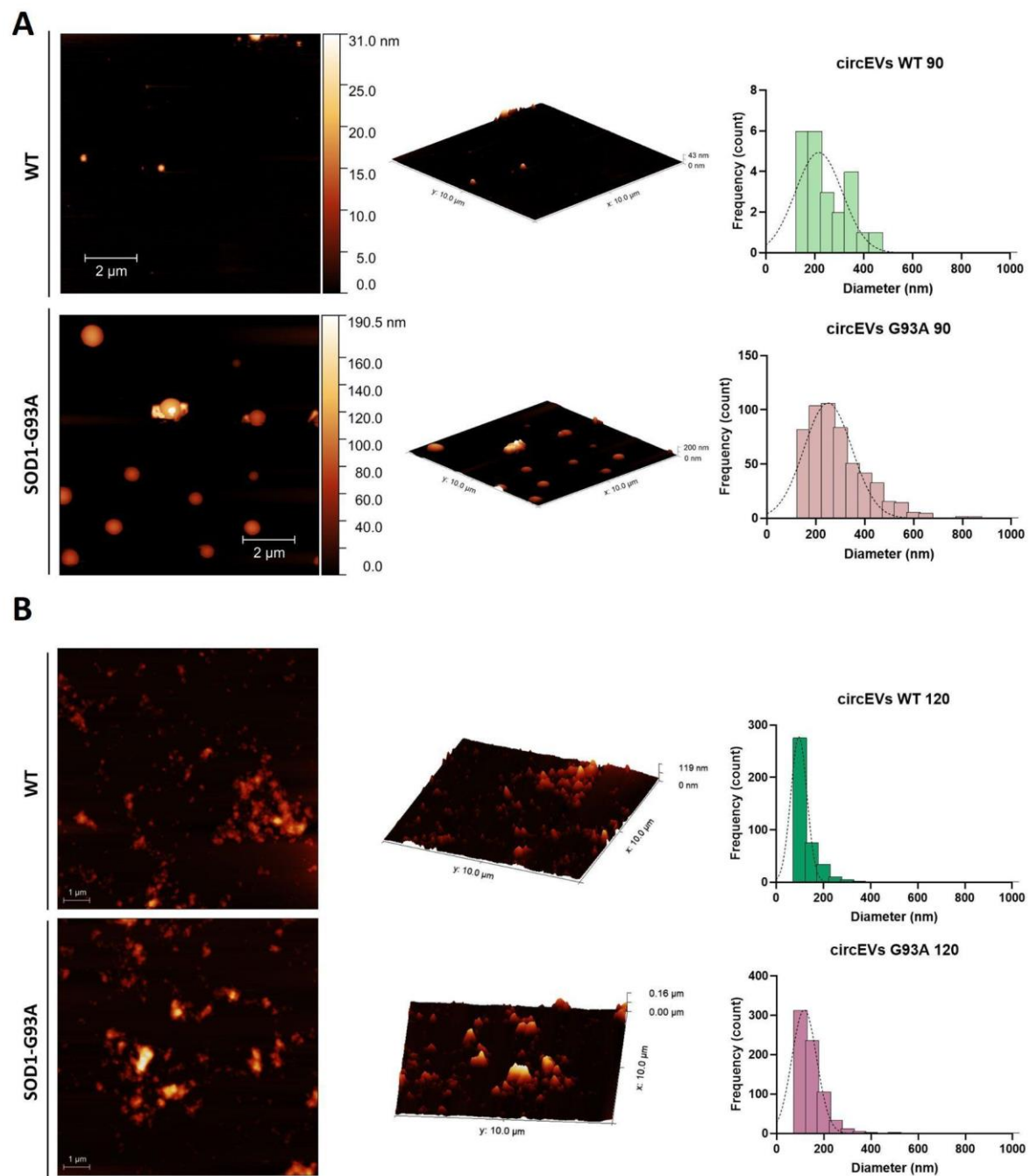


Figure 10. AFM micrographs, 3D reconstructions and size distributions of circEVs of WT and SOD1-G93A mice at 90 (A) and 120 days of age (B). Scale bars = 2 and 1 μ m.

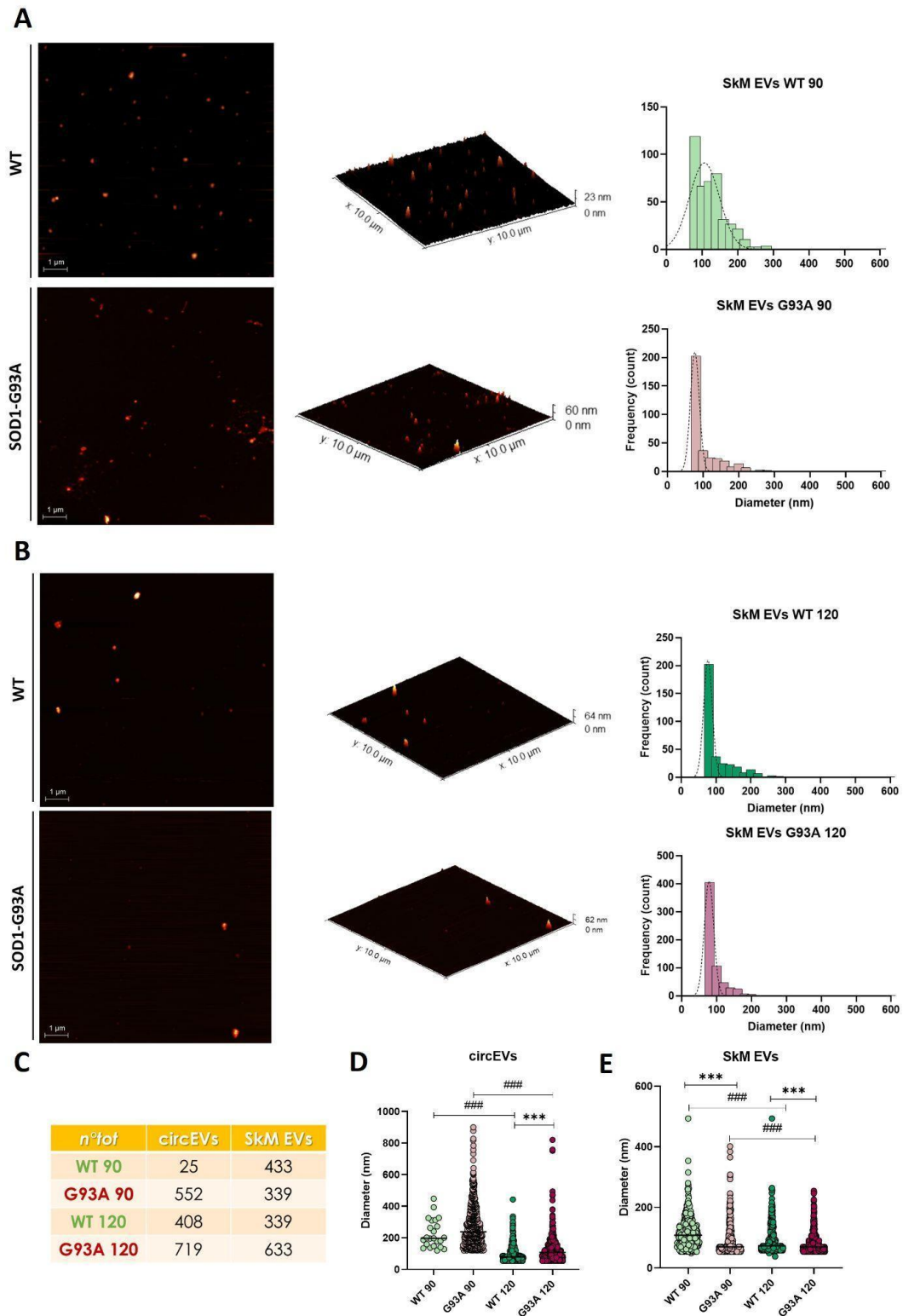


Figure 11. AFM micrographs, 3D reconstructions and size distributions of SkM EVs of WT and SOD1-G93A mice at 90 (A) and 120 days of age (B). Scale bars = 1 μ m. (C) Vesicle numbers counted within the same volumes of samples for circEVs and SkM EVs at 90 and 120 days of age. (D) Direct comparison of the mean diameters of circEVs (WT 90 = 226,5; G93A 90 = 273,2; WT 120 = 98,36; G93A 120 = 123,2) and SkM EVs (WT 90 = 116,8; G93A 90 = 94,03; WT 120 = 94,19; G93A 120 = 81,34) of WT and SOD1-G93A at 90 and 120 days. Values represent the mean $\pm$ SD; statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparisons test. *** $p < 0.001$, when compared between disease stages in the same group; ### $p < 0.001$, when compared between WT and G93A groups.

Discussion and conclusions

Extracellular vesicles (EVs) have recently attracted the attention of the scientific community representing promising biomarkers for several diseases, including neurodegenerative disorders such as ALS. As present in biological fluids (blood, CSF, urine, saliva) and able to cross the biological barriers, protecting at the same time their cargo thanks to their membrane, EVs may carry a molecular signature that can be related to disease progression [158]. In ALS studies, alterations in size and number of EVs populations are reported on both animal models and patients in addition to variations in their cargo, although the results of different studies are often in contrast. However, none of the current studies considered variations between different stages of the disease progression to date. In our study, we found interesting differences between different populations of EVs (i.e., derived from plasma and skeletal muscle), when comparing EVs from both the SOD1-G93A and WT mice and also considering different time points (i.e., early symptomatic stage at 90 days of age, and late stage at 120 days of age). We exploited a combined approach by using TEM and AFM to perform both morphological and quantitative characterization of the isolated EVs. Specifically, TEM morphological analysis allowed the detection of the presence of round-shaped EVs in all the experimental groups, and WB analysis detected CD63 and CD9 EV-specific markers, thus corroborating the efficiency of our isolation protocol (**Figure 9**). Therefore, to get more detailed information about the differences within the EVs populations, we exploited a high-resolution technique, such as AFM, to measure their size distribution and relative amount (**Figure 10 and 11**). Intriguingly, our analysis allowed us to observe that within the population of EVs from plasma, SOD1-G93A EVs present a higher numerical consistency and significantly bigger mean diameters when compared to the WT, at both 90 and 120 days of age (**Figure 11C, 11D**). These results are consistent with the findings of other studies that have found an increased mean size of EVs from plasma of ALS animal models and patients with respect to their control groups [163, 170], thus pointing out that EVs populations from plasma undergo significant changes during ALS progression. Moreover, when considering the aging and comparing the different stages of 90 and 120 days of age in the same group, both the WT and SOD1-G93A circEVs are numerically greater (**Figure 11C**) and show significantly smaller mean diameters (**Figure 11D**) in the late stage, thus highlighting a diversification within the circEVs population during aging in the WT mice and disease progression in the SOD1-G93A mice. This could indicate a different role of the EVs signaling at different disease stages, that could be clarified by cargo analysis. On the other hand, SkM EVs showed a totally different behavior with respect to circEVs. At the stage of 90 days, SOD1-G93A SkM EVs show a slightly reduced number and smaller mean diameter compared to the WT SkM EVs (**Figure 11C, 11E**). At the stage of 120 days, SOD1-G93A SkM EVs still show a reduced average size, but twice as many EVs as the WT SkM EVs at the same stage (**Figure 11C, 11E**). Overall, SOD1-G93A SkM EVs seem to show a shift towards a smaller population than WT SkM EVs. Interestingly, recent work has found that only the small fraction of EVs, and not the larger one, from the skeletal muscle of ALS patients specifically exerts toxic effects on different recipient cells [156].

Therefore, it may be possible to ascribe a predominant role of the smaller SkM EVs during the disease progression, that should be corroborated with functional analysis on recipient cells. Concerning the number of EVs, AFM allowed us to detect interesting variations between the different experimental groups (**Figure 11C**). However, it is important to note that the measurements reported are related to a defined volume of the sample used for the AFM analysis. Therefore, it would be necessary to implement the obtained data with NTA analysis. Taken together, these results suggest that, in presence of SOD1-G93A mutation, both circEVs and SkM EVs populations undergo significant alterations in their number and size distribution that may indicate a possible role of EVs in being mediators of “disease signals” during ALS progression.

SECTION 2. Proteomics analysis of circulating and skeletal muscle EVs from wild-type and ALS mice at different disease stages

Collaborations: 1) *Sapienza University of Rome, Comparative Cell Biology and Imaging Laboratory (CCBIL), Rome, Italy;* 2) *Department of Pharmacy, Center for Advanced Studies and Technology (CAST), University "G. d'Annunzio" of Chieti-Pescara, Chieti, Italy.*

Summary

Given its multifactorial nature and similarity with other neurodegenerative disorders, ALS diagnosis is currently challenging [157]. Therefore, there is an urgent need to find early diagnostic biomarkers in order to improve the current therapeutic approaches against disease onset and progression. For this purpose, EVs show several advantages, as they can cross the biological barriers and circulate in biological fluids, reflect the tissue of origin, and protect their cargo from degradation. Various studies aimed to characterize EVs molecular profile from CSF and blood of both ALS animal models and patients, but often showing contrasting results or finding potential biomarkers that have been revealed not specific only for ALS. None of the current studies has neither included a longitudinal characterization considering different disease stages, nor has yet investigated SkM EVs as potential ALS biomarkers. Given the increasing evidence of the role of skeletal muscle in ALS early pathogenetic events [25,73], together with SkM EVs as putative mediators of toxic signaling [155], it may be worth to investigate whether SkM EVs contribute to this impairment and to understand if these EVs are able to transfer harmful signals in the periphery. Considering these observations, we aimed to characterize the local (SkM EVs) and systemic (circEVs) molecular signature of EVs, relating to different disease stages. We characterized the proteomic profile of both SkM EVs and circEVs isolated from WT and SOD1-G93A mice at 90 and 120 days of age, in order to investigate differentially expressed proteins and detect a proteomic signature correlated with ALS progression. Interestingly, our results suggest a different molecular profile between SkM EVs and circEVs populations, thus determining distinct putative functions between EVs released from different origins.

Results

2.1 Proteomics analysis of specific SkM EVs proteins revealed their involvement in skeletal muscle physiology

After isolation by UF/SEC and preliminary morpho-quantitative analysis, we performed proteomics analysis with a label-free approach (described in *Materials and Methods*) for both circEVs and SkM EVs from WT and SOD1-G93A mice at 90 and 120 days of age. For SkM EVs proteins, the analysis revealed that the main identified pathways were muscle contraction and development, glucose metabolism, proteostasis (synthesis, stabilization and folding), vesicle-

mediated transport, and Schwann cell migration (**Figure 12A**). Of note, proteins involved in glucose and glycogen metabolism and muscle development and contraction are differentially expressed in SOD1-G93A EVs, with a strong downregulation at the early stage compared to the late stage (**Figure 12B**). Proteins involved in intracellular trafficking and transport show an opposite trend between the experimental groups, with a downregulation occurring at the late stage for WT and at the early stage for SOD1-G93A (**Figure 12B**). Interestingly, proteins involved in muscle structural component, cell junction and adhesion show a strong downregulation related to the mutation, independently from the disease stage (**Figure 12B**). Among the identified proteins, a small group was only related to aging (**Figure 12B**). Overall, these data suggest that, during disease progression, SkM EVs show a deregulation of proteins involved in muscle physiology and metabolism, thus pointing out a possible role of SkM EVs in acting predominantly locally and within the skeletal muscle microenvironment.

2.2 Proteomics analysis of specific circEVs proteins revealed their involvement in systemic inflammation and neuronal alterations

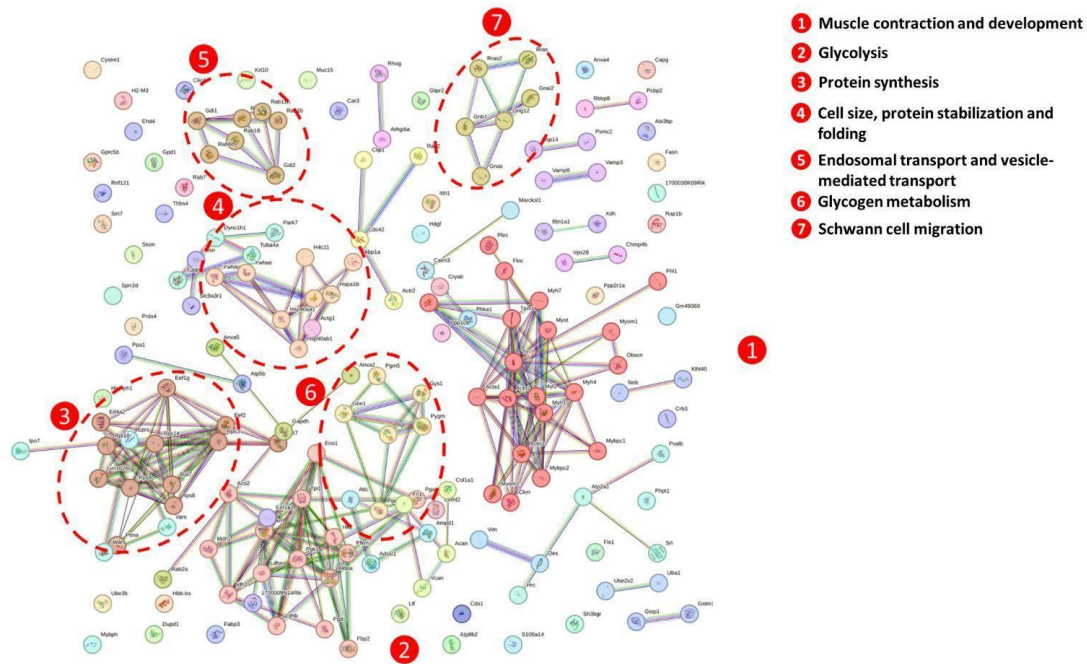
Together with SkM EVs, we analyzed the proteomic profile of circEVs from both WT and SOD1-G93A mice at different disease stages (90 and 120 days of age). Differently from SkM EVs, the main networks in which circEVs proteins are involved were lipid metabolism, coagulation and lipoprotein metabolism, EVs formation and release and neuronal homeostasis (**Figure 13A**). Specifically, proteins involved in axonal transport, protein refolding and EVs release and function present a strong downregulation in the mutated group compared to WT, independently from the disease stage (**Figure 13B**). Moreover, within the SOD1-G93A group there is a modulation of proteins involved in oxidant detoxification with aging (**Figure 13B**). Several proteins are implicated in the inflammatory response, with an opposite trend between the WT group (downregulation with aging) and the SOD1-G93A group (upregulation with aging) (**Figure 13B**). Proteins involved in postsynaptic membrane reorganization show changes related only to aging, but with a stronger modulation within the mutated group (**Figure 13B**). As for SkM EVs, also circEVs proteome seem to show the main alterations in the early stage of the disease. However, these data indicate that circEVs are involved mostly in systemic processes and, strikingly, may also affect neuronal homeostasis.

2.3 Proteomics analysis of the common proteins between SkM EVs and circEVs confirms their differential profile

Besides the specific proteomics analysis of SkM EVs and circEVs, we also performed a comparative analysis to investigate the common proteins between the two groups, both independently from the disease stages and considering the aging. When comparing SkM EVs and circEVs independently from aging, they share the 15 proteins, representing only the 5,6%

of the total number (**Figure 14A**). Of note, these proteins are involved in EVs release and function and cardiac muscle function (**Figure 14A**). When comparing circEVs and SkM EVs at 90 days, they share 14 common proteins (6,03%), while at 120 days of age the common proteins are reduced at 8 (4,35%) (**Figure 14B**). This information, together with the specific analysis, further corroborates the distinction between circEVs and SkM EVs at both the disease stages, suggesting that they have a distinct role in disease progression.

A



B

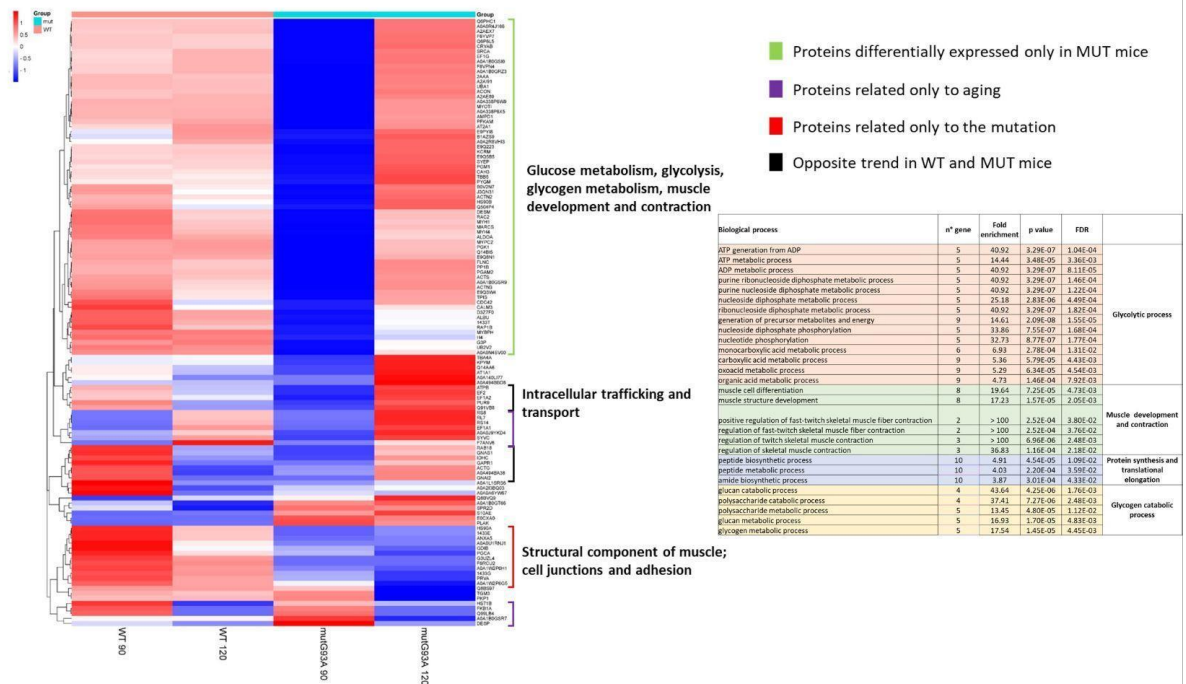


Figure 12. Proteomics analysis of specific SkM EVs proteins. (A) Network and clustering of the main pathways of proteins derived from SkM EVs. **(B)** Heat map of the main modulated proteins and involved pathways in SkM EVs from WT and SOD1-G93A mice at 90 and 120 days of age. WT= wild-type; MUT= SOD1-G93A.

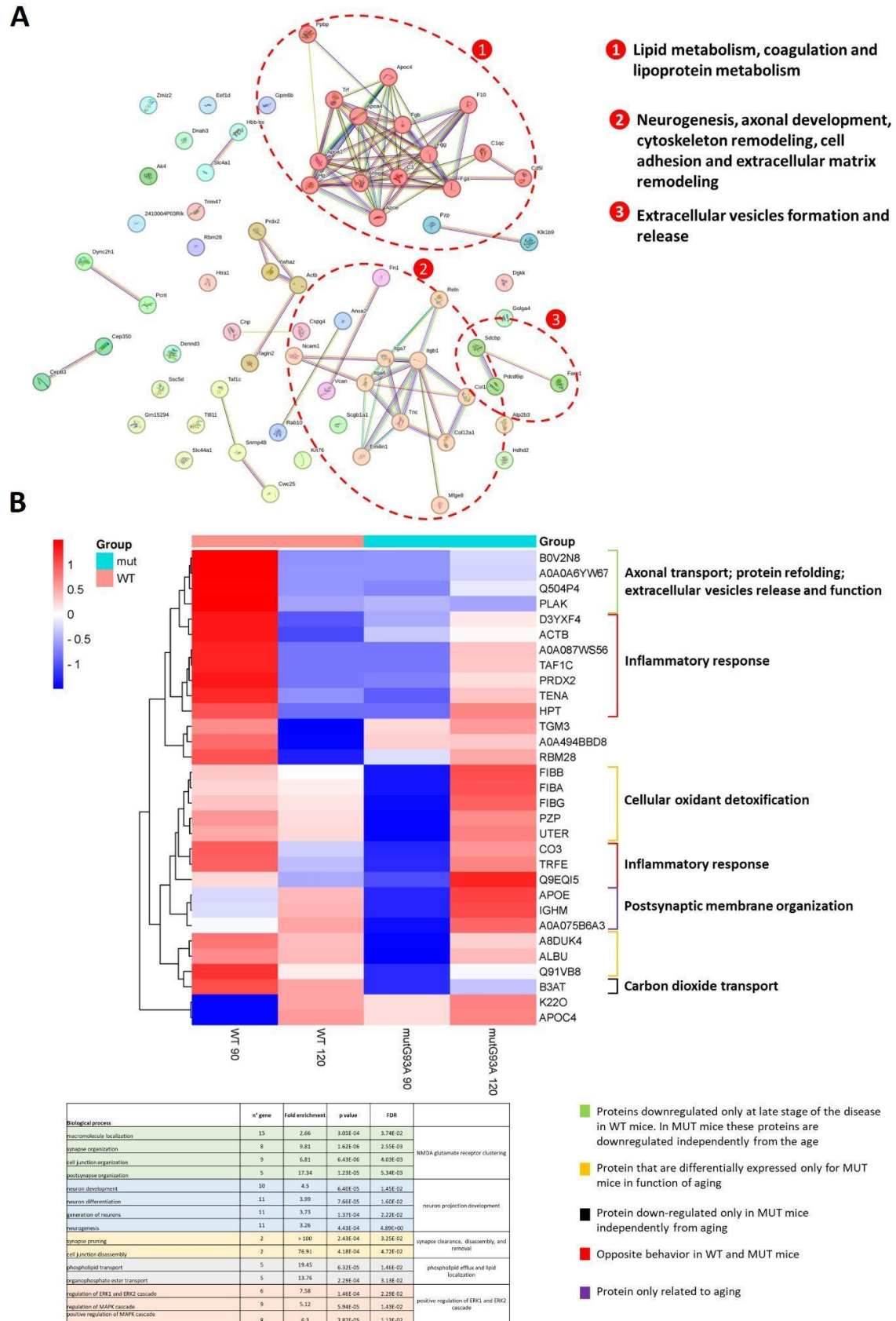
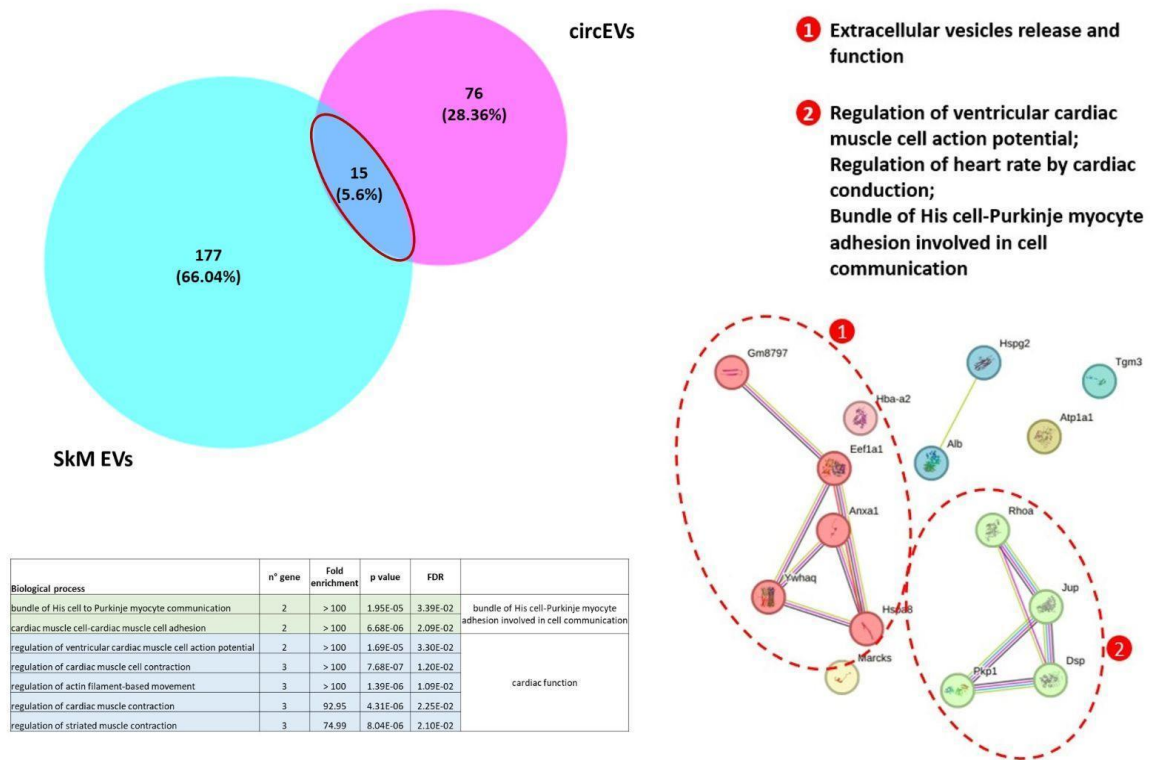


Figure 13. Proteomics analysis of specific circEVs proteins. (A) Network and clustering of the main pathways of proteins derived from circEVs. **(B)** Heat map of the main modulated proteins and involved pathways in circEVs from WT and SOD1-G93A mice at 90 and 120 days of age. WT= wild-type; MUT= SOD1-G93A.

A



B

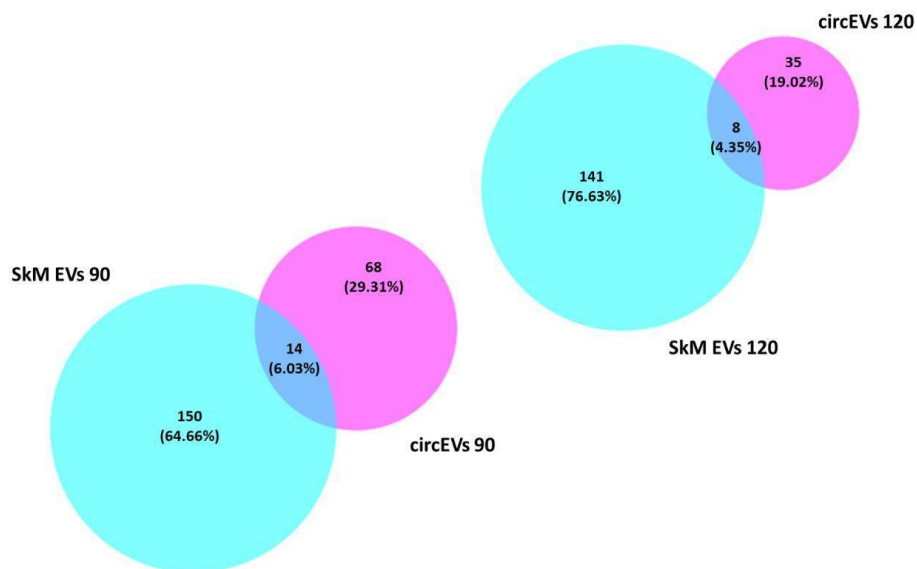


Figure 14. Proteomics analysis for the common proteins between circEVs and SkM EVs. (A) Venn diagram representing unique and common proteins between SkM EVs and circEVs (left); network of the biological processes in which are involved the common proteins (right). **(B)** Venn diagrams representing unique and common proteins between SkM EVs and circEVs for each stage of the disease (90 and 120 days).

Discussion and conclusions

ALS diagnosis is currently challenging due to the multifactorial and complex etiology of the disease. EVs, as important mediators of intercellular communication found in all biological fluids and putative actors in the pathogenesis of ALS by carrying distinctive molecular signature, attracted increasing interest as potential biomarkers to track the disease progression [158]. The urgent need to find effective and non-invasive diagnostic strategies pushed forward the scientific community to study in detail the variations of different EVs populations, by characterizing their size and number and by defining their molecular cargo with high-throughput approaches, such as proteomics, transcriptomics and lipidomics [158-161]. Most of the current studies focused on the characterization of EVs from CSF or blood, showing contrasting results claimed as derived from non-standardized methodologies of EVs isolation, small patient cohorts and lack of longitudinal studies [158-173]. Despite increasing evidence pointing out the role of skeletal muscle and its derived EVs in ALS pathogenesis, none of these studies considered skeletal muscle as an important source of EVs that may actively contribute to ALS progression. Therefore, we decided to characterize the proteomic profile of EVs derived from WT and SOD1-G93A mice considering: 1) two different sources, as skeletal muscle and plasma, in order to have an overview of the putative role of EVs locally in the skeletal muscle microenvironment and systemically in the bloodstream; 2) two different stages of the disease, as an early symptomatic stage (90 days of age) and late terminal stage (120 days of age), to have an overview of the longitudinal changes of EVs molecular pattern during disease progression. The main aim of our analysis was not only to compare the molecular signature of EVs from different groups at the same stage (i.e., WT 90 vs. SOD1-G93A 90; WT 120 vs. SOD1-G93A 120) to detect specific proteins related to the mutation, but also to identify specific proteins related to distinct disease stages (SOD1-G93A 90 vs. SOD1-G93A 120). Analysis of specific proteins of SkM EVs revealed that these EVs are mainly involved in functions related to skeletal muscle physiology, such as muscle contraction and development, glycolysis, protein synthesis, cell size, stabilization and folding, endosomal and vesicle-mediated transport, glycogen metabolism, and Schwann cell migration (**Figure 12A**). In particular, SkM EVs of 90-days-old SOD1-G93A mice showed strong downregulation of proteins related to glucose metabolism and muscle development and contraction (**Figure 12B**). Furthermore, proteins involved in muscle development and contraction are also strongly downregulated in SkM EVs of 120-days-old SOD1-G93A mice, thus indicating that these alterations are strongly related to the presence of the mutation (**Figure 12B**). Increasing evidence points to the role of skeletal muscle alterations in the early phase of ALS progression, including atrophy [77-83] and metabolic dysfunction [84-87]. Our results confirm that SkM EVs carry a molecular signature of ALS skeletal muscle and thus are potentially implicated in the spread of skeletal muscle alterations during disease progression. With regard to the analysis of circEVs specific proteins, clustering revealed lipid metabolism, coagulation and lipoprotein metabolism, neurogenesis, axonal development and cytoskeleton, cell adhesion and extracellular matrix remodeling as major pathways (**Figure 13A**). In particular, within circEVs, modulation of the

proteomic profile showed a more complex picture. Proteins related to axonal transport, protein refolding and EVs release and function are downregulated in SOD1-G93A SkM EVs independently from the age when compared to WT SkM EVs, thus indicating a clear mutation-specific signature (**Figure 13B**). Proteins involved in the inflammatory response show opposite behavior between the WT and SOD1-G93A groups during aging. In particular, SOD1-G93A circEVs of 90-days-old mice show a similar pattern to that of WT circEVs of 120 days, thus suggesting an “aging-like” effect of the mutation (**Figure 13B**). A group of proteins involved in the cellular oxidant detoxification are differentially expressed only in the SOD1-G93A circEVs, with an increased expression during the disease progression (**Figure 13B**). Overall, SOD1-G93A circEVs at 90 days show a tendency to downregulate proteins related to neuronal development, inflammatory response and cellular oxidant detoxification, when compared to both WT circEVs and SOD1-G93A circEVs at the late stage (**Figure 13B**). This may suggest that circEVs, at an early symptomatic stage of ALS, may reflect defects in cell protective mechanisms (such as antioxidant defense and inflammatory response) and, surprisingly, appear to carry a molecular signature likely associated with axonal degeneration and neuronal damage. While proteins associated with axonal transport remain downregulated in the advanced stage of the disease (i.e., 120 days) in SOD1-G93A circEVs, proteins associated with antioxidant defense and inflammatory response are instead upregulated, probably indicating that circEVs may participate in an inflammatory process that starts only later and as a consequence of earlier muscle dysfunction and neurodegeneration. To gain a clearer view of the sequence of events associated with the damage, it would be therefore interesting to investigate another time point, considering an earlier pre-symptomatic stage. However, considering two different stages within the symptomatic phase of the disease (90 and 120 days of age in the mouse models), still allowed us to discriminate the roles of different subpopulations of EVs. While SOD1-G93A SkM EVs carry a proteomic profile associated with skeletal muscle dysfunction and metabolic impairment, circEVs from mutated mice are likely involved in neurodegeneration and inflammation processes, thus supporting a distinct implication of different populations of EVs during disease progression. This is further supported by the fact that SkM EVs and circEVs share only 5,6% of the proteins without considering the different stages (**Figure 14A**). When dissecting the different stages of the disease, circEVs and SkM EVs share only 6,03% of proteins at 90 days, and 4,35% at 120 days (**Figure 14B**). These proteins seem to be involved in EVs release and function and in the regulation of the action potential and cardiac conduction of ventricular cardiac muscle cells (**Figure 14A**). Although not the main symptom, cardiac autonomic dysfunction is the second leading cause of death in ALS patients, and heart rate variability has been considered a predictive indicator of autonomic neuropathy [185,186]. Although the biological significance of the EVs in this phenomenon needs to be elucidated, these results further strengthen the implication of EVs in the multisystemic degeneration characterizing ALS.

SECTION 3. Effects of circEVs and SkM EVs from WT and SOD1-G93A mice on recipient myotubes

Collaborations: 1) Sapienza University of Rome, Comparative Cell Biology and Imaging Laboratory (CCBIL), Rome, Italy; 2) CarMeN Laboratory, UMR INSERM 1060-INRAE 1397, LYON 1 University, Pierre Bénite, Lyon, France.

Summary

EVs are important mediators of the intercellular communication in the neuromuscular junction (NMJ), impacting on both muscle-muscle communication, regulating myogenesis, muscle regeneration and differentiation, and muscle-neuron interplay, modulating synaptic growth and plasticity, neurite outgrowth and neuronal survival [64,65]. EVs are released in the bloodstream and are able to cross the BBB; therefore, EVs could be considered potential contributors in the propagation of pathogenic proteins in neurodegenerative diseases, including ALS. During ALS progression, the breakdown of the NMJ and skeletal muscle dysfunction seem to occur before neurodegeneration [74,75]. For this reason, the effects of circEVs and SkM EVs from WT and SOD1-G93A mice at early and late stages of the disease were evaluated on recipient myotubes. C2C12 myotubes were incubated with EVs for 24 hours and detrimental effects were evaluated in terms of redox alterations, inflammation and variations in regenerative capacity. The aim of this part of the work was to implement the proteomic data and understand whether SOD1-G93A EVs can exert harmful effects on healthy recipient cells, thus participating in disease progression. The comparison between recipient myotubes and motor neurons receiving EVs from the bloodstream or skeletal muscle at different disease stages could help to get a clearer overview of which cell type between motor neurons and myocytes is affected earlier and more severely, and which EVs group between circEVs and SkM EVs has the greatest impact in the disease progression.

Results

3.1 SOD1-G93A circEVs mainly affect the redox balance and metabolism of recipient myotubes at the late stage

To assess the systemic effects of EVs in recipient myocytes, we treated recipient myotubes for 24 hours with 0,5 µg/mL of circEVs of WT and SOD1-G93A mice at 90 and 120 days of age. Then, we performed functional analysis to evaluate the impact of these EVs on the physiology of myotubes. Interestingly, SOD1-G93A circEVs at 90 and 120 days altered the oxidative phosphorylation of the mitochondrial respiratory electron transport chain in recipient myotubes, with a significant decrease in CI at 90 days, and in CI and CIV at 120 days, when compared to the WT group (**Figure 15A**). Moreover, the treatment with SOD1-G93A circEVs at 120 days determined a significant increase in the expression levels of the antioxidant enzyme

SOD1 when compared to untreated control, WT circEVs, and SOD1-G93A circEVs at 90 days, and a decrease in the expression of GPX4 when compared to the untreated control and SOD1-G93A circEVs at 90 days (**Figure 15B**). In addition, SOD1-G93A circEVs at 120 days determined a significant increase in the expression of CPT1 and IL-6 when compared to all the other experimental groups (**Figure 15C**). Interestingly, circEVs mainly exert their effects at the advanced stage of the disease (120 days), altering the redox balance and metabolism of recipient C2C12 myotubes.

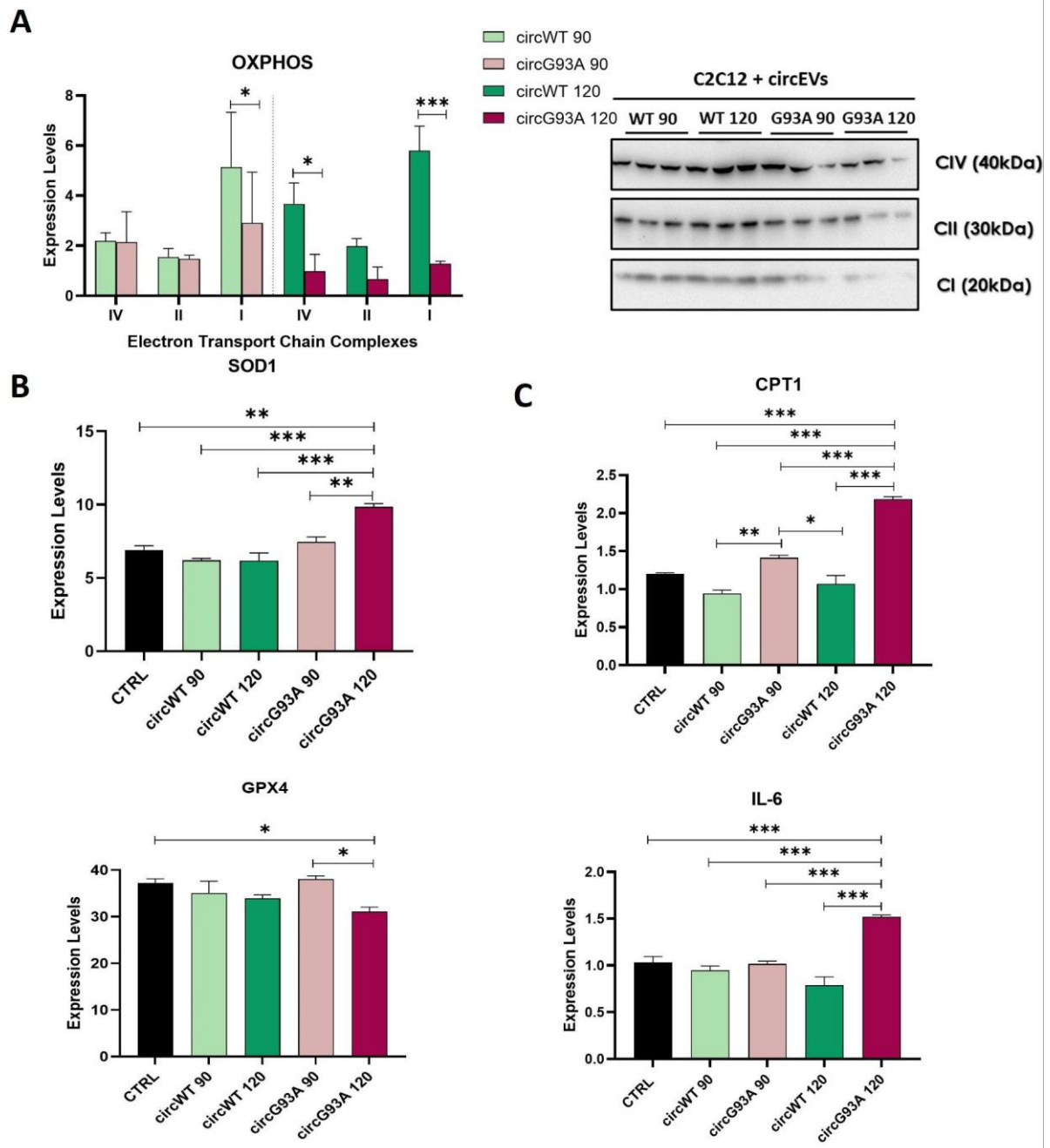


Figure 15. Effects of WT and SOD1-G93A circEVs on C2C12 recipient myotubes. (A) Western blot for the expression of the mitochondrial electron transport chain complexes (OXPHOS). Values represent the mean $\pm$ SD; statistical analysis was carried out using two-way ANOVA with Tukey's multiple comparisons test ($n=3$). * $p < 0.1$, ** $p < 0.01$, and *** $p < 0.001$, when compared between different groups. **(B)** RT-qPCR for the expression of SOD1

and GPX4 antioxidant enzymes; (C) RT-qPCR for the expression of CPT1 and IL-6. Values represent the mean $\pm$ SD; statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparisons test (n=3). * $p < 0.1$, ** $p < 0.01$, and *** $p < 0.001$, when compared between different groups. OXPHOS = oxidative phosphorylation. CTRL = untreated control; circWT = wild-type circulating EVs; circG93A = SOD1-G93A circulating EVs. SOD1 = superoxide dismutase 1; GPX4 = glutathione peroxidase 4; CPT1 = carnitine palmitoyltransferase 1; IL-6 = interleukin 6.

3.2 SOD1-G93A SkM EVs affect the metabolism and regenerative capacity of recipient myotubes

In addition to the systemic effects exerted by circEVs, to assess the local effects of EVs in recipient myocytes, we treated C2C12 myotubes for 24 hours with 0,5 $\mu\text{g/mL}$ of SkM EVs from WT and SOD1-G93A mice at 90 and 120 days of age. After treatment, we assessed whether SkM EVs affected the physiology of recipient myotubes by checking for oxidative phosphorylation, markers of inflammation and muscle regenerative capacity. Interestingly, SOD1-G93A SkM EVs altered oxidative phosphorylation in recipient myotubes already at the earlier stage of 90 days, by increasing in the expression of the CI complex compared to WT EVs (**Figure 16A**). No significant effects were detected for the expression levels of antioxidant enzymes such as SOD1 (data not shown). Therefore, we checked whether SkM EVs may participate in inflammatory processes and affect the regenerative capacity of recipient myotubes. Indeed, SOD1-G93A SkM EVs from the late stage of the disease, but not from the earlier stage, significantly increased of the expression of the pro-inflammatory chemokine MCP1 compared to the other experimental groups (**Figure 16B**). These data possibly indicate the presence of macrophage infiltration and pro-inflammatory environment. Moreover, SOD1-G93A SkM EVs gradually decreased the transcription factor MyoD with the disease progression and compared to the untreated control (**Figure 16C**), probably indicating a progressive impairment of the regenerative capacity of recipient myotubes.

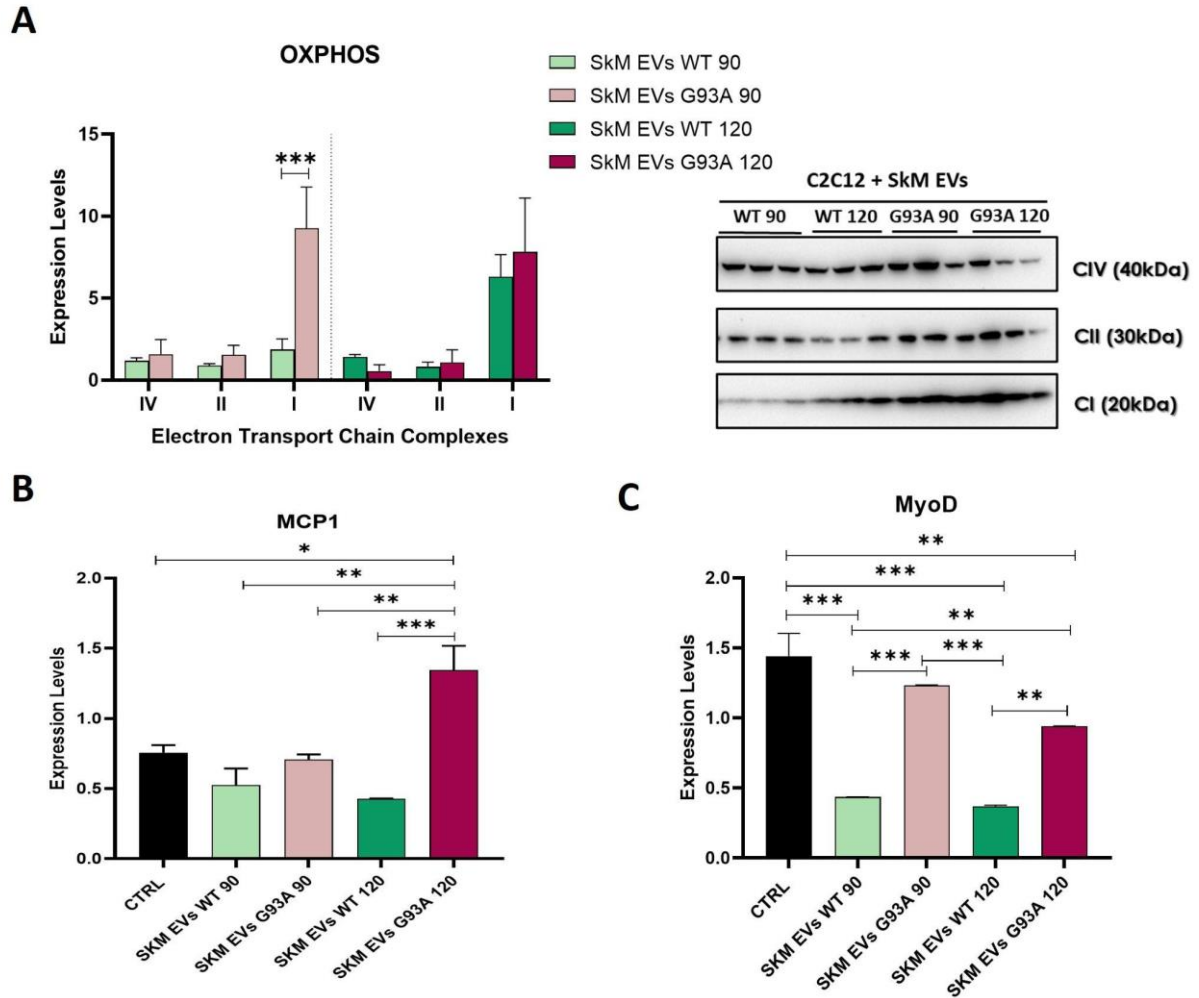


Figure 16. Effects of WT and SOD1-G93A SkM EVs on C2C12 recipient myotubes. (A) Western blot for the expression of the mitochondrial electron transport chain complexes (OXPHOS). Values represent the mean $\pm$ SD; statistical analysis was carried out using two-way ANOVA with Tukey's multiple comparisons test ($n=3$). * $p < 0.1$, ** $p < 0.01$, and *** $p < 0.001$, when compared between different groups. (B) RT-qPCR for the expression of MCP1 and (C) MyoD. Values represent the mean $\pm$ SD; statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparisons test ($n=3$). * $p < 0.1$, ** $p < 0.01$, and *** $p < 0.001$, when compared between different groups. OXPHOS = oxidative phosphorylation. CTRL = untreated control; circWT = wild-type circulating EVs; circG93A = SOD1-G93A circulating EVs. MCP1 = monocyte chemoattractant protein 1.

3.3 WT SkM EVs and circEVs: a potential protective effect against skeletal muscle damage?

To further investigate the differential effects of circEVs and SkM EVs in the muscle physiology and function during the disease progression, we also inquired whether these EVs may take part in atrophy processes and contribute to skeletal muscle damage characterizing ALS. Therefore, after 24 hours treatment of C2C12 myotubes with 0,5 $\mu\text{g/mL}$ of both circEVs SkM EVs from WT and SOD1-G93A mice at 90 and 120 days of age, we investigated also the expression of factors associated with atrophy, such as atrogin-1 and MuRF-1. Surprisingly, WT SkM EVs at both 90 and 120 days determined a strong reduction of the expression of the atrophy markers atrogin-1 and MuRF-1 when compared to untreated controls and cells treated

with SOD1-G93A SkM EVs (**Figure 17AB**). A similar, but less strong effect was observed when treating recipient myotubes with WT circEVs (at both 90 and 120 days) when compared to untreated and SOD1-G93A circEVs (**Figure 17D, 17F**).

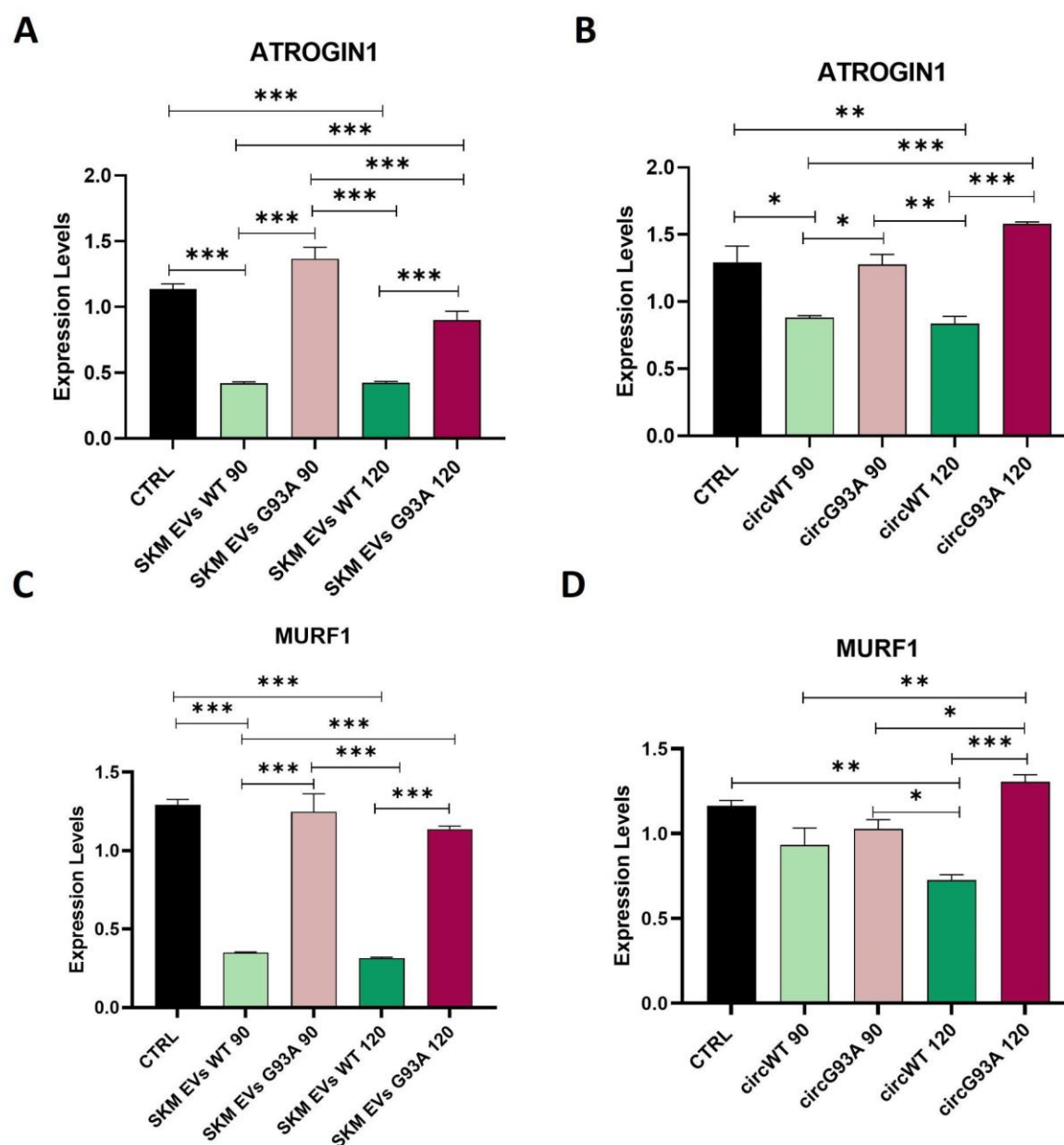


Figure 17. Effects of WT and SOD1-G93A circEVs and SkM EVs on C2C12 recipient myotubes muscle development and atrophy. RT-qPCR for the expression of atrogin-1 (**A**), and MuRF-1 (**C**) of C2C12 recipient myotubes treated with WT and SOD1-G93A SkM EVs at 90 and 120 days. RT-qPCR for the expression of atrogin-1 (**B**), and MuRF-1 (**D**) of C2C12 recipient myotubes treated with WT and SOD1-G93A circEVs at 90 and 120 days. Values represent the mean $\pm$ SD; statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparisons test ($n=3$). * $p < 0.1$, ** $p < 0.01$, and *** $p < 0.001$, when compared between different groups. CTRL = untreated control; SkM EVs = skeletal muscle derived EVs; circ = circulating EVs; WT = wild-type; G93A = SOD1-G93A; MuRF1 = muscle RING-finger protein 1.

Discussion and conclusions

ALS is widely recognized as a multisystemic disorder where multiple factors participate in its pathogenesis. In this context, EVs play a pivotal role representing an important way of communication within the CNS and between the CNS and the periphery [146]. In ALS, EVs are likely to contribute to impaired intercellular signaling, by promoting transfer of misfolded proteins and affecting recipient cells [147]. Although great relevance has been placed on the crosstalk between motor neurons and other cells of the CNS, skeletal muscle is emerging as early and active player in the pathogenesis of ALS also by the release of EVs with neurotoxic factors, driving the disruption of the NMJ and, consequently, neurodegeneration processes [154-156]. In addition to skeletal muscle dysfunction, ALS is also characterized by weakened immune system and systemic inflammation, in which EVs participate by circulating in the bloodstream, modulating the activity of recipient immune cells, and transferring signals to distant targets [17,60-63]. Given the complexity of the potential role of EVs in ALS disease, we decided to consider the impact of EVs isolated from plasma and skeletal muscle of WT and SOD1-G93A mice at two different disease stages (90 and 120 days) on recipient myotubes. The main aim was to understand at what time and which type of alterations were driven by EVs from local (skeletal muscle) and systemic (bloodstream) sources. Therefore, we treated C2C12 myotubes for 24 hours with 0,5 µg/mL of circEVs and SkM EVs, from WT and SOD1-G93A mice at 90 and 120 days, and investigated the effects by checking for markers of oxidative stress, metabolism, inflammation, and muscle repair and regeneration. In line with our proteomics results, circEVs and SkM EVs triggered different responses in recipient myotubes, likely attributed to their different derivation. In detail, SOD1-G93A circEVs significantly reduced the expression of the CI of the mitochondrial electron transport chain at the stage of 90 days, and of CIV and CI at the stage of 120 days, compared to WT EVs (**Figure 15A**). However, only C2C12 recipient myotubes treated with SOD1-G93A circEVs at 120 days showed a significant increase in SOD1 expression compared to all the other experimental groups (**Figure 15B**, upper panel) and a decrease in GPX4 expression compared to untreated controls and treated with SOD1-G93A circEVs at 90 days (**Figure 15B**, lower panel). Mitochondrial dysfunction and oxidative stress have been reported as the main pathogenic hallmarks of ALS, often showing imbalance in oxidative phosphorylation (OXPHOS) and impairment of antioxidant defenses [22-24]. Alterations in the OXPHOS in mitochondria are related to decreased transmembrane potential, increase in ROS production, and consequent overexpression of antioxidant enzymes in response to oxidative stress [29,30]. Although SOD1-G93A circEVs from the earlier stage of 90 days already influenced OXPHOS relatively to CI, when deriving from the terminal stage of the disease (120 days of age) produced a greater effect on recipient myotubes, affecting CIV and CI complexes. In addition to the reduction of CIV and CI expression in mitochondria, SOD1-G93A circEVs at 120 days caused a significant increase in SOD1 expression in recipient myotubes (**Figure 15B**, upper panel), thus likely indicating a compensatory mechanism triggered by a more severe redox imbalance. Recipient myotubes also showed a decrease in GPX4 expression levels (**Figure 15B**, lower

panel) only when treated with SOD1-G93A circEVs at 120 days, suggesting a lower capacity of protection from lipid peroxidation at the late stage. Moreover, SOD1-G93A circEVs at 120 days specifically induced increased expression of CPT1 and IL-6 (**Figure 15C**). Increased levels of CPT1 expression may reflect an increased fatty acid metabolism occurring at the advanced stage of the pathology, in which a switch to use lipids as an energy source occurs as a consequence of impaired energy production. Although metabolic reprogramming in charge of the skeletal muscle has been reported to occur in both animal models and ALS patients earlier within the disease progression [27,83], our results indicate that circEVs may carry a signature of dysmetabolic conditions only at the terminal stage of the disease, thus representing late messengers in ALS pathogenesis. Consistently with this hypothesis, SOD1-G93A circEVs increased IL-6 expression in recipient myotubes only at 120 days (**Figure 15C**, lower panel), thus being potential messengers of inflammation only in a terminal pathogenic stage.

A different behavior was observed when recipient myotubes were treated with SkM EVs. While a huge effect of increase in expression levels of CI was observed after treatment with SOD1-G93A SkM EVs at 90 days (**Figure 16A**), indicating the presence of altered oxidative phosphorylation, no significant effects were reported in the expression of antioxidant enzymes (data not shown). However, it must be considered that the selected time point (90 days of age) represents the early symptomatic stage of the disease. Since skeletal muscle alterations are reported to occur before the beginning of the symptoms [75-95], it cannot be excluded that SkM EVs may have greater effects on recipient cells earlier than the stage of our choice. Although in absence of significant alterations in terms of oxidative stress, we investigated whether SkM EVs participate in muscle regeneration and inflammation processes in the symptomatic phase. SOD1-G93A SkM EVs at 120 days induced a strong increase of the expression of the pro-inflammatory chemokine MCP1 (**Figure 16B**). MCP1 levels have been reported to be gradually increased in the spinal cord of SOD1-G93A mice with the disease progression, indicating the presence of a robust inflammation at the late disease stage [187]. MCP1 is also implicated in the recruitment of macrophages and T cells and it is a crucial factor to promote wound healing and regeneration in skeletal muscle after acute trauma. In SOD1-G93A mouse models with a fast progression of the disease, it has been reported that a delayed activation of the MCP1 axis failed to promote an efficient immune response, thus hampering regeneration processes and further contributing to a fast disease progression [187]. The high levels of MCP1 induced by the treatment of recipient myotubes with SOD1-G93A SkM EVs at 120 days may ascribe a role of SkM EVs in the activation of MCP1 axis, and therefore in inflammation. Moreover, recipient C2C12 myotubes showed a gradual decrease of MyoD levels induced by SOD1-G93A SkM EVs, that becomes significant when using EVs at 120 days, with respect to untreated controls (**Figure 16C**). As inflammation and myogenesis are related in orchestrating muscle regeneration after injury [187,188], a decrease in MyoD expression levels induced by SOD1-G93A SkM EVs may support a defective reparative ability of recipient cells, thus reinforcing an involvement of SkM EVs during the late pathogenesis of the skeletal

muscle. Although we detected late effects, it would still be interesting to assess if SkM EVs may also play a role in oxidative stress in stages earlier than 90 days to have a wider panoramic of their involvement during ALS progression.

Therefore, we also investigated whether SkM EVs and circEVs could be implicated in atrophy processes, another pathological hallmark of the ALS skeletal muscle [80,81], by measuring the expression levels of atrogin-1 and MuRF-1 (**Figure 17**). Surprisingly, both the levels of atrogin-1 and MuRF-1 were strongly downregulated in myotubes treated with WT SkM EVs, independently from the stage, when compared to both untreated controls and SOD1-G93A EVs (**Figure 17C, 17E**). These results led us to speculate that, rather than SOD1-G93A EVs inducing atrophy in recipient cells, WT EVs could have a protective role towards atrophy by inhibiting protein degradation, an effect that is lost in presence of the mutation. Curiously, a similar, but less strong effect was observed for WT circEVs, probably diluted by the heterogeneity of EVs derived from the bloodstream (**Figure 17B, 17D**). Taken together, our results support that EVs may have a pleiotropic role during ALS progression, which varies depending on the disease stage, their origin, and cellular target. Dissecting the role of EVs during the disease progression, also considering the earlier stages (i.e., the disease onset), may give important insights about the complexity of ALS, where EVs dynamically participate in different pathogenic events. These results, together with our proteomic data and the increasing literature data supporting the early implication of skeletal muscle in ALS [77-97], led us to speculate that SkM EVs may be involved in stages occurring before the symptoms onset, while circEVs could represent late messengers in the advanced disease as a consequence of previous damage localized in the skeletal muscle.

SECTION 4. Effects of circEVs and SkM EVs from WT and SOD1-G93A mice on recipient motor neurons

Summary

Although the “dying back” hypothesis suggests a significant role of the skeletal muscle in the progression of the disease, motor neurons remain one of the most affected components in ALS [66]. As motor neurons and skeletal muscle are closely interconnected by the NMJ, whether the damage to motor neurons represents a primary cause of the disease or a direct consequence of skeletal muscle alterations is still unclear [64,74]. Given that EVs are known to carry misfolded proteins affecting the physiology of healthy cells, in parallel with myotubes, we decided to test the effects of circEVs and SkM EVs from WT and SOD1-G93A mice at early and late stages of the disease on recipient motor neurons. Therefore, NSC-34 motor neuron-like cells were treated with both WT and SOD1-G93A circEVs and SkM EVs (from 90 day-old and 120 day-old mice) for 24 hours in order to assess detrimental effects by mutated EVs on neuronal physiology (i.e., neurite length) and function (i.e., oxidative stress response). Comparing the responses of recipient myotubes and motor neurons that have received EVs from the bloodstream or skeletal muscle at various disease stages may provide valuable insights into determining which cell type (i.e., motor neurons or myocytes) is affected earlier and more severely. Additionally, examining the impact of EV clusters (i.e., circEVs and SkM EVs) on disease progression can help to understand their contribution to disease dissemination.

Results

4.1 SOD1-G93A circEVs and SkM EVs significantly reduce neurite length in recipient motor neurons

To have a wider panoramic of the effects of EVs in ALS, we considered evaluating the effects of circEVs and SkM EVs from WT and SOD1-G93A mice, at both 90 and 120 days) not only in myocytes, but also motor neurons. The main aims were: 1) to assess which recipient cells between myocytes or motor neurons were more early and severely affected by EVs during the disease progression; 2) which EV cluster (i.e., circEVs or SkM EVs) had a greater and earlier effect on recipient motor neurons. After proper differentiation into motor neuron-like cells (described in *Materials and Methods*), we treated NSC-34 for 24 hours with 0,5 µg/mL of both circEVs and SkM EVs, from WT and SOD1-G93A mice at 90 and 120 days of age. Therefore, we started to investigate detrimental effects exerted by EVs on motor neuron physiology by first measuring the entity of neurite debranching. While the treatment with WT circEVs and SkM EVs at both disease stages had no effects when compared to untreated motor neurons (**Figure 18A**), SOD1-G93A circEVs and SkM EVs determined a drastic reduction of neurite length (**Figure 18B**). Specifically, the debranching phenomenon was stronger when using

circEVs when compared to SkM EVs, and within the EVs of the same origin, the effect was more pronounced with the EVs from 90 days-old mice than the 120 days (Figure 18B).

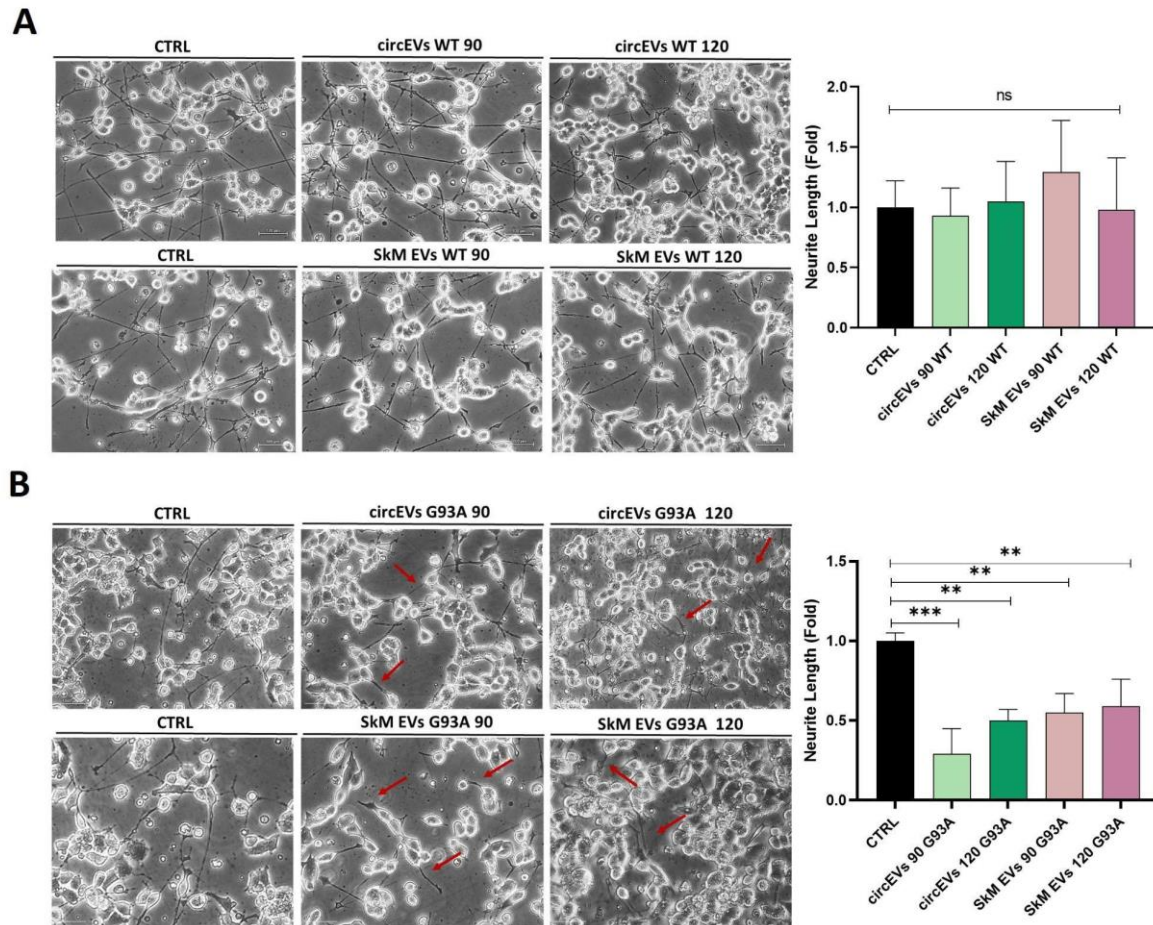


Figure 18. Neurite length measurement of recipient motor neurons. (A) Optical microscopy of NSC-34 motor neuron-like cells (left images) treated with circEVs and SkM EVs from WT mice at 90 and 120 days and relative neurite length measurement (right panel). (B) Optical microscopy of NSC-34 motor neuron-like cells (left images) treated with circEVs and SkM EVs from SOD1-G93A mice at 90 and 120 days and relative neurite length measurement (right panel). Scale bars = 100 μ m. Values represent the mean $\pm$ SD; statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparisons test (n=3). * $p < 0.1$, ** $p < 0.01$, and *** $p < 0.001$, when compared between different groups.

Discussion and conclusions

Since its discovery, ALS has always been considered a neurological disorder, characterized by the progressive degeneration of upper and lower motor neurons as the main pathological hallmark [1-3]. The implication of EVs in ALS pathogenesis as mediators of pathological signaling between CNS and periphery further highlighted the complexity of the disease, in which different cellular components synergistically participate in a multisystemic disorder [21,147]. Although involving different cell types, the core of ALS pathogenesis seems to be at

the level of the NMJ, where the mutual communication between skeletal muscle and motor neurons drives its integrity and function [64]. EVs contribute to NMJ homeostasis by modulating neuronal growth and development, neuroprotective responses, and retrograde signaling from the skeletal muscle [65]. Although increasing evidence pointed to a primary role of the skeletal muscle in the pathogenic signaling of ALS through the secretion neurotoxic factors harmful to motor neurons [88,89,155,156], the close and dynamic relationship between motor neurons and skeletal muscle cells within the NMJ makes it difficult to understand the origin of the disease. Therefore, a better characterization of EVs signaling during the progression of ALS could be a starting point for unraveling the origin of the disease, offering new clues for its diagnosis and prognosis. Interestingly, our proteomic data (**SECTION 2** of the *Results*) revealed that circEVs are implicated in neuronal homeostatic processes, such as neurogenesis and axonal transport (**Figure 13**). Therefore, we investigated whether EVs deriving from plasma and skeletal muscle of mutated mice at different disease stages were able to induce harmful effects on healthy motor neurons. The reduction of the neurite length of recipient WT NSC-34 motor neuron-like cells, after 24 h of treatment with the different subpopulations of EVs, was measured as a first marker of neurodegeneration. While all the EVs deriving from WT mice had no effect on neurite length compared to untreated controls (**Figure 18A**), SOD1-G93A EVs induced a strong debranching phenomenon, and the strongest reduction was measured when using circEVs at the early symptomatic stage (**Figure 18B**). As shown by the light microscopy, the treatment of WT NSC-34 cells with SOD1-G93A circEVs and SkM EVs also induced a strong aggregation of the cells and the presence of debris (**Figure 18B**), absent when cells were treated with WT EVs (**Figure 18A**). These results strongly suggest that SOD1-G93A EVs could carry signals that are harmful to recipient motor neuron-like cells during the symptomatic phase of the disease, further suggesting active involvement of EVs in ALS pathogenetic processes. These promising results need to be further investigated with analysis of markers of differentiation and apoptosis. Our results, combined with the same analysis of EVs isolated from an earlier stage of the disease progression (i.e., 70 days of age, corresponding to the onset), may represent a full longitudinal study that offers important insights of the role of different EVs during ALS progression.

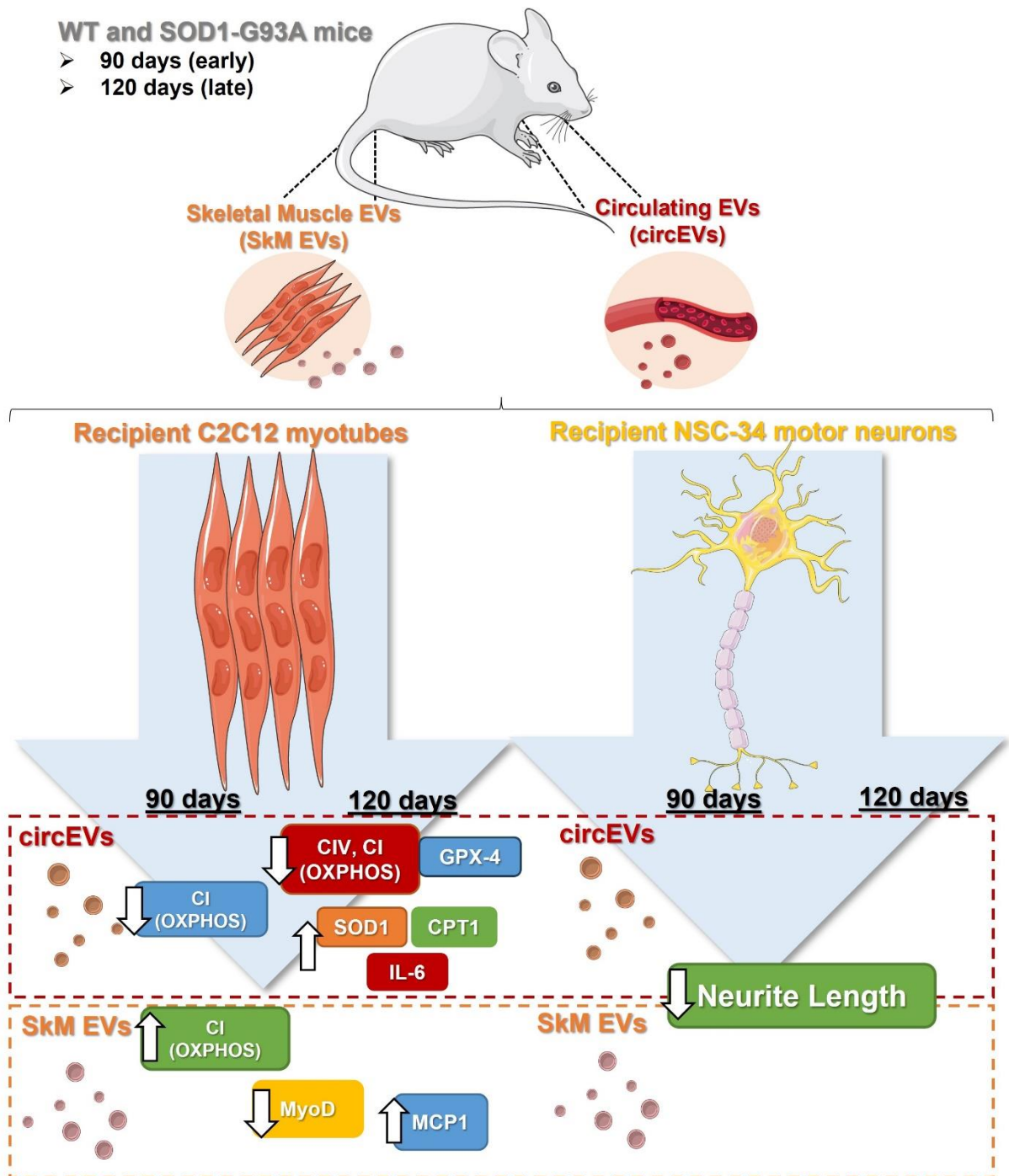


Figure 19. Graphical overview of the effects of SOD1-G93A mice SkM and circEVs at 90 and 120 days on recipient myotubes and motor neurons.

GENERAL DISCUSSION

ALS is widely recognized as a multisystemic disorder in which several cell types are dynamically and synergistically involved in its pathogenesis [1-3]. Given its heterogeneous nature and complexity, it is currently difficult to understand the main origin of pathogenic events of ALS, thus rendering its diagnosis and prognosis challenging [21]. In this context, EVs represent important mediators between the CNS and periphery and molecular messengers exploited by multiple cells involved in the pathology, acquiring the potential as pathogenic intermediaries and promising biomarkers of the disease progression. Therefore, EVs have attracted the attention of the scientific community and are increasingly studied as they represent a still unexplored aspect of ALS. However, the main limitation in ALS research seems to be the lack of longitudinal studies that could be crucial for a better understanding of the pathogenetic course [147]. In this work, we investigated the role of EVs during ALS disease progression in SOD1-G93A mouse models with the following aims: I) understanding the local contribution of EVs within the skeletal muscle microenvironment (using SkM EVs) and peripheral in the bloodstream (using plasma circEVs) on recipient cells that are mostly implicated in ALS pathogenesis (i.e., myocytes and motor neurons); II) identifying an ALS-associated molecular signature specifically in SOD1-G93A EVs during the disease progression; III) unraveling variations in the effects of EVs at different stages of the disease within the symptomatic phase (i.e., 90 days of age as early symptomatic stage, and 120 days of age as terminal stage). A graphical workflow is represented in **Figure 8**. First, the morpho-quantitative and molecular characterization of EVs by using multiple techniques (described in **SECTION 1**) highlighted interesting differences between EVs populations, in terms of size distribution and number, during the disease progression (i.e., comparing SOD1-G93A EVs at 90 days vs. SOD1-G93A EVs at 120 days) and with respect to WT EVs. While alterations are reported for plasma EVs in both mouse models and patients [163,164,170], SkM EVs are still poorly characterized in ALS. Given that skeletal muscle is emerging as an early player in the development of ALS [77-87], it should be considered that SkM EVs might participate not only in the transfer of pathogenic signals within the skeletal muscle microenvironment, but also to the periphery, thus contributing to the spread and progression of the disease. For this reason, we analyzed the proteomic profiling of both SkM EVs and circEVs at different stages (**SECTION 2**) to get a more precise overview of the spatio-temporal contribution of EVs in ALS. Of note, SkM EVs carry a molecular signature related to skeletal muscle physiology and, in presence of the SOD1-G93A mutation, show a strong downregulation of proteins implicated in muscle structural components, development and contraction, and glucose metabolism at the early stage of 90 days. These results strongly indicate that SkM EVs mainly influence the skeletal muscle microenvironment. On the other hand, circEVs show a complex proteomic profile, probably due to their heterogeneity as deriving from the bloodstream. Specifically, circEVs showed a modulation in proteins implicated in antioxidant defense and inflammatory response, that are upregulated at the terminal stage. Therefore, circEVs likely participate in the massive inflammation that characterizes ALS at the advanced stages [17]. Intriguingly,

circEVs proteome also showed a modulation in proteins associated with neuronal homeostasis. As EVs are known to be able to cross the biological barriers [158,159], this could imply a potential involvement of circEVs in neurodegeneration, reaching the target cells from the early symptomatic stage of the disease. Moreover, SkM EVs and circEVs share only 5,6% of the total proteomic profile, in which are included proteins associated with cardiac function. As autonomic neuropathy is the second common cause of death in ALS patients [185,186], this may further attribute a potential role of EVs in the multisystemic manifestations of ALS. Taken together, these findings led us to hypothesize that EVs dynamically contribute to ALS pathogenic events where, while SkM EVs are likely involved in skeletal muscle alterations at the onset and early stages of the disease, circEVs only participate in late pathogenetic signaling, as a result of what happens earlier in the skeletal muscle.

To test our hypothesis, we used circEVs and SkM EVs from WT and SOD1-G93A mice, at 90 and 120 days, to treat *in vitro* recipient models of myotubes and motor neurons (i.e., differentiated C2C12 and NSC-34 cells, **Figure 19**). The overall aim of this part of the work was to understand which subset of EVs had the earliest and greatest impact on the selected target cells. Of note, SOD1-G93A SkM EVs at 90 days did not particularly affect the redox balance on recipient myotubes, but altered myogenesis and increased MCP1 axis-driven inflammation with disease progression (**SECTION 3**). Furthermore, SOD1-G93A SkM EVs did not increase the expression levels of genes involved in atrophy, which were instead suppressed by WT SkM EVs. It remains to be clarified whether this could be a loss of protective capacity against atrophy caused by SOD1-G93A mutation. These results led us to consider that, since skeletal muscle alterations occur before the onset of the symptoms, perhaps SkM EVs could be involved in stages earlier than the one (90 days) of our choice. It would therefore be interesting to extend our analysis with SkM EVs isolated from an earlier stage (i.e., 70 days of age for mouse models) to get a better overview during the evolution of the disease pathogenesis. On the other hand, circEVs of SOD1-G93A mice have been revealed as late messengers to recipient myotubes, inducing redox imbalance, metabolism impairment and inflammation at 120 days. In parallel with recipient myotubes, we started to check for detrimental effects induced by the different subsets of EVs on recipient motor neurons, by measuring the neurite length reduction as a first damage signal (**SECTION 4**). Interestingly, SOD1-G93A EVs, both from plasma and skeletal muscle, dramatically reduced the neurite length of recipient motor neurons when compared to untreated controls. This effect was not observed when using WT EVs and therefore it could be attributable to the presence of the mutation. Moreover, SOD1-G93A circEVs had a stronger debranching effect than SkM EVs, especially when derived from the earlier disease stage. Although still in a preliminary phase, these results, together with proteomic data, may suggest that circEVs, rather than SkM EVs, are likely to be implicated in neurodegeneration. In conclusion, our data point to an intricate and pleiotropic role of EVs during ALS progression, related to their local origin, disease stage, and cellular target, providing insights into the strong complexity of a multifactorial disease as ALS. Although further functional analyses are needed for a more precise understanding, these results suggest

a clear involvement of EVs in the pathogenesis of ALS, while supporting their role in the “dying back” hypothesis, according to which skeletal muscle may represent the main pathogenic origin of ALS. Considering the different effects they exert depending on their microenvironment, the precise role of EVs in a complex and multifactorial disease such as ALS has yet to be fully elucidated.

ANNEX

Since its discovery, ALS has been widely conceptualized as a neurodegenerative disease affecting motor neurons in the motor cortex and spinal cord, with multifactorial and elusive etiology that complicates its prognosis, diagnosis, and therapy [1-3]. The identification of oxidative stress, neuroinflammation, peripheral immune system activation, and skeletal muscle dysfunction as main features depicting ALS pathogenesis, corroborated the presence of an intricate pathological network characterizing a real multisystemic disorder [28,73]. EVs have recently emerged as active players during ALS pathogenesis, mediating the transfer of misfolded and aggregated proteins (i.e., SOD1-G93A) between CNS and periphery, and consequently responsible for the spread of the damage to other compartments [97,100]. For several years, many studies have been focused on the “neurocentric” view of ALS, according to which the pathogenesis originates from motor neurons and diffuses to other cells. This hypothesis, together with the evidence of oxidative stress as one of the main pathological triggers of ALS, led us to first focus on the possible oxidative stress effects on motor neurons towards EVs production by exploiting human neuroblastoma SH-SY5Y cells (WT and SOD1-G93A), widely used as a model to investigate oxidative stress in neurodegenerative diseases. As EVs are involved in several biological and pathological processes and have the potential to provide information about the cell physiopathological state, the research field of EVs is progressively growing in the study of various diseases [99,146,208,209]. However, due to their heterogeneity and lack of standardized protocols, it is still currently challenging to obtain reproducible results when dealing with EVs. Most of the studies in the field of EVs have mainly focused on the functional characterization through molecular profiling of their cargo, underestimating the potential of morphological features in providing information on cell physiology and neglecting the relationship between structure and function. Taking all these observations into account, we investigated EVs not only from a functional perspective in order to understand their role during ALS progression, but also deepened the technical analysis. Therefore, we introduced a novel method to gather comprehensive information on the cell physiological status related to EV trafficking, employing a high-resolution morphometric approach by using AFM (**ANNEX I and II**). In our model, we were able to obtain a topographical mapping of the cells and detect subtle changes in the budding process in presence of a specific stimulus (i.e., oxidative stress), thus appreciating details missed by conventional methods such as EM [122]. Intriguingly, our approach was useful to highlight that the presence of SOD1-G93A mutation, in physiological conditions and after exposure to an oxidative stress, determined a distinct topography and budding profile in human neuroblastoma SH-SY5Y cells when compared to wild-type cells [28]. In this context, at least two considerations are necessary. First, our approach empowered the potential of AFM to get multiple, detailed information about the cell morphology, by defining size, intensity, and position of the budding phenomena. These variations, in turn, could have important implications on the selection of the cargo, leading to different functions and target interactions.

Although in a model not fully representative of the disease, this study still highlighted important morphological characteristics that depend specifically on the presence of SOD1-G93A mutation, one of the most associated with both fALS and sALS and widely used in several models [8-10,75,77,81,87,90,148]. In conclusion, this study led us to hypothesize that this AFM-based approach could have been exploited for additional studies using more appropriate models (i.e., differentiated neurons, **Figure S3**) that would have better recapitulated ALS and to investigate how the mutation affects different cell types implicated in the disease. These considerations, together with the recently emerging “dying back” hypothesis supporting a mutual crosstalk between neuronal and non-neuronal cells, pushed us to reconsider the story, focusing not only on oxidative stress and motor neurons, but rather adopting different and more appropriate models in order to have a more comprehensive overview of the role of EVs in a multifactorial disorder as ALS. Since our model represented by SH-SY5Y cells was not optimal for studying motor neuronal alterations (i.e., cancerous cells with continuous mitosis; time and material consuming differentiation protocols), we then moved to another *in vitro* model, i.e., the murine neuroblastoma x spinal cord NSC-34 cell line (WT and SOD1-G93A). Indeed, these cells allowed us to: I) employ an easier differentiation protocol to obtain motor neuron-like cells; II) perform a longitudinal study including also the *in vivo* model, represented by WT and SOD1-G93A mice; III) include the murine *in vitro* model representing the skeletal muscle counterpart (i.e., C2C12 myotubes), thus gaining a more comprehensive overview of the role of EVs during disease progression.

Finally, due to their role as mediators of intercellular communication and their promising potential as biomarkers of physiological and pathological conditions, EVs widely attracted the attention of the scientific community for the study of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), responsible for the COVID-19 pandemic. Given the impact of the recent COVID-19 pandemic on global health, and since the focus of the Comparative Cell Biology and Imaging Laboratory is to study different biological aspects of EVs, we extended our interest towards their role on SARS-CoV-2 biology (**ANNEX III**). By reviewing the literature, we highlighted recent morphological studies supporting a clear role of EVs in the SARS-CoV-2 viral cycle, thus demonstrating the importance of EVs morphology and dynamics in the understanding of biological processes. At the same time, we also examined the possible role of EVs as carriers for drug delivery and therapeutic purposes [99].

ANNEX I. High-Resolution Atomic Force Microscopy: a new method for topographical mapping of surface budding

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Collaborations: 1) Sapienza University of Rome, Comparative Cell Biology and Imaging Laboratory (CCBIL), Rome, Italy; 2) Research Center for Nanotechnology for Engineering (CNIS), Sapienza University of Rome, Rome, Italy; 3) Institute for the Structure of Matter (ISM), National Research Council (CNR), Rome, Italy.

Summary

Extracellular vesicles (EVs) are membranous nanoparticles secreted by almost all cell types. Reflecting the physiopathological state of the parental cell, EVs circulate in all body fluids, reaching distant cell targets and delivering different bioactive cargoes. As biological carriers, EVs influence their microenvironment altering cellular responses, being considered promising biomarkers for both physiological and pathological conditions. EVs are heterogeneous in terms of size and composition, depending on cell type and exposure to stimuli, and different methods have been developed to characterize their morphological, biophysical, and biochemical features [98,100]. Among them, electron microscopy (EM) is the main technique used, however, the lack of standardized protocols makes it difficult to characterize EVs with a good reproducibility, thus using multiple approaches may represent a way to obtain more precise information [111-114]. Furthermore, the relationship between architecture and function, not only in a molecular, but also in a cellular level, is gaining growing emphasis, characterizing morphometric parameters may represent a distinct, but effective approach to study the physiopathological state of the cell. Atomic force microscopy (AFM), may represent a promising method to study in detail EVs dynamics throughout the cell surface and its variations related to the physiological state, overcoming the limits of EM, and providing more reliable information [28,122]. In our first study [28], human neuroblastoma SH-SY5Y cell line, a cellular model to investigate neurodegeneration and oxidative stress, has been used to perform a comparative morphological and quantitative analysis of membrane budding and isolated large vesicles-enriched (microvesicles-like vesicles; MVs) fraction from control or oxidative stressed cells. The main goal was to build up a methodology to characterize EVs morphology and spatial distribution over the cell surface in different physiological conditions, and to evaluate the efficacy of AFM against conventional EM. Interestingly, both microscopy techniques were effective for this analysis, but AFM allowed to reveal a differential profiling of plasma membrane budding between the physiological and the stress condition, indicating a potential relationship between mechanical characteristics and functional role. The results obtained may provide interesting perspectives for the use of AFM to study EVs, validating a morphometric approach to understand the pathophysiological state of the cell related to EVs trafficking.

ANNEX II. AFM for EVs characterization and topographical distribution of budding vesicles: detecting variations between WT and SOD1-G93A SH-SY5Y under oxidative stress

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Collaborations: 1) Sapienza University of Rome, Comparative Cell Biology and Imaging Laboratory (CCBIL), Rome, Italy; 2) Research Center for Nanotechnology for Engineering (CNIS), Sapienza University of Rome, Rome, Italy; 3) Institute for the Structure of Matter (ISM), National Research Council (CNR), Rome, Italy.

Summary

Extracellular vesicles (EVs) are important mediators of intercellular communication in several physiopathological conditions. Although constitutively released by the cells, alterations of the extracellular environment, such as oxidative stress, may impact on EVs release and cargo composition, generating variations that may depend on the cell type and stimulus [196]. To date, most of the studies in the EVs field have been focused on their functional characterization, rather than on the morphological features. Given their high degree of heterogeneity, to fully characterize EVs both the functional and morphological characterization are required. Atomic force microscopy (AFM), introduced for cell morphological studies at the nanoscale [204-207], represents a promising microscopy method to characterize in detail EVs morphology and dynamics along the cell surface and its variations reflecting the cell physiological status [122]. In our second study [28], untreated or H₂O₂-treated wild-type and SOD1-G93A human neuroblastoma SH-SY5Y cells have been compared performing a Transmission Electron Microscopy (TEM) and AFM morphological and quantitative analysis of budding and released vesicles. Intriguingly, our analysis revealed a differential EVs profiling, with an opposite behavior and implying different cellular areas between WT and SOD1-G93A cells, on both physiological conditions and after H₂O₂ exposure. Therefore, our results empower the relationship between morphological features and functional role, further proving the efficacy of EM/AFM in giving an overview of the cell physiology related to EVs trafficking.

ANNEX III. SARS-CoV-2 and extracellular vesicles: An intricate interplay in pathogenesis, diagnosis and treatment

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Summary

Extracellular vesicles (EVs) are widely recognized as intercellular communication mediators. Among the different biological processes, EVs play a role in viral infections, supporting virus entrance and spread into host cells and immune response evasion. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection became an urgent public health issue with significant morbidity and mortality worldwide, being responsible for the current COVID-19 pandemic. Since EVs are implicated in SARS-CoV-2 infection in a morphological and functional level, they have gained growing interest for a better understanding of SARS-CoV-2 pathogenesis and represent possible diagnostic tools to track the disease progression. Furthermore, thanks to their biocompatibility and efficient immune activation, the use of EVs may also represent a promising strategy for the development of new therapeutic strategies against COVID-19. In this review, we explore the role of EVs in viral infections with a focus on SARS-CoV-2 biology and pathogenesis, considering recent morphometric studies. The common biogenesis aspects and structural similarities between EVs and SARS-CoV-2 will be examined, offering a panoramic of their multifaceted interplay and presenting EVs as a machinery supporting the viral cycle. On the other hand, EVs may be exploited as early diagnostic biomarkers and efficient carriers for drug delivery and vaccination, and ongoing studies will be reviewed to highlight EVs as potential alternative therapeutic strategies against SARS-CoV-2 infection.

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SUPPLEMENTARY DATA

Table S1. List of the primers used to perform RT-qPCR analysis

GENE	FORWARD	REVERSE
SOD1	5'-TGAAGAGAGGCATGTTGGAG-3'	5'-CCACCTTTGCCCAAGTCATC-3'
CPT1	5'-TACAACAGGTGGTTTGACA-3'	5'-CAGAGGTGCCCAATGATG-3'
GPX4	5'-CCGGCTACAACGTCAAGTTT-3'	5'-CACGCAGCCGTTCTTATCAA-3'
IL-6	5'-AGTTGCCTTCTTGGGACTGAT-3'	5'-TCCACGATTTCCCAGAGAAC-3'
MyoD	5'-TCCAGCCCGCGCTCCAACTGC-3'	5'-TCGACACGGCCGCACTCTTCC-3'
MCP1	5'-TGGAGCATCCACGTGTTGGC-3'	5'-ACTACAGCTTCTTTGGGACA-3'
ATROGIN-1	5'-CTCTGCCAGTACCACTTCTC-3'	5'-ATGGTCAGTGCCCTTCCAGG-3'
MURF-1	5'-TGCATCTCCATGCTGGTGGC-3'	5'-CTTCTTCTCGTCCAGGATGG-3'

S1. Functional characterization of skeletal muscle biopsies of WT and SOD1-G93A mice at 90 and 120 days

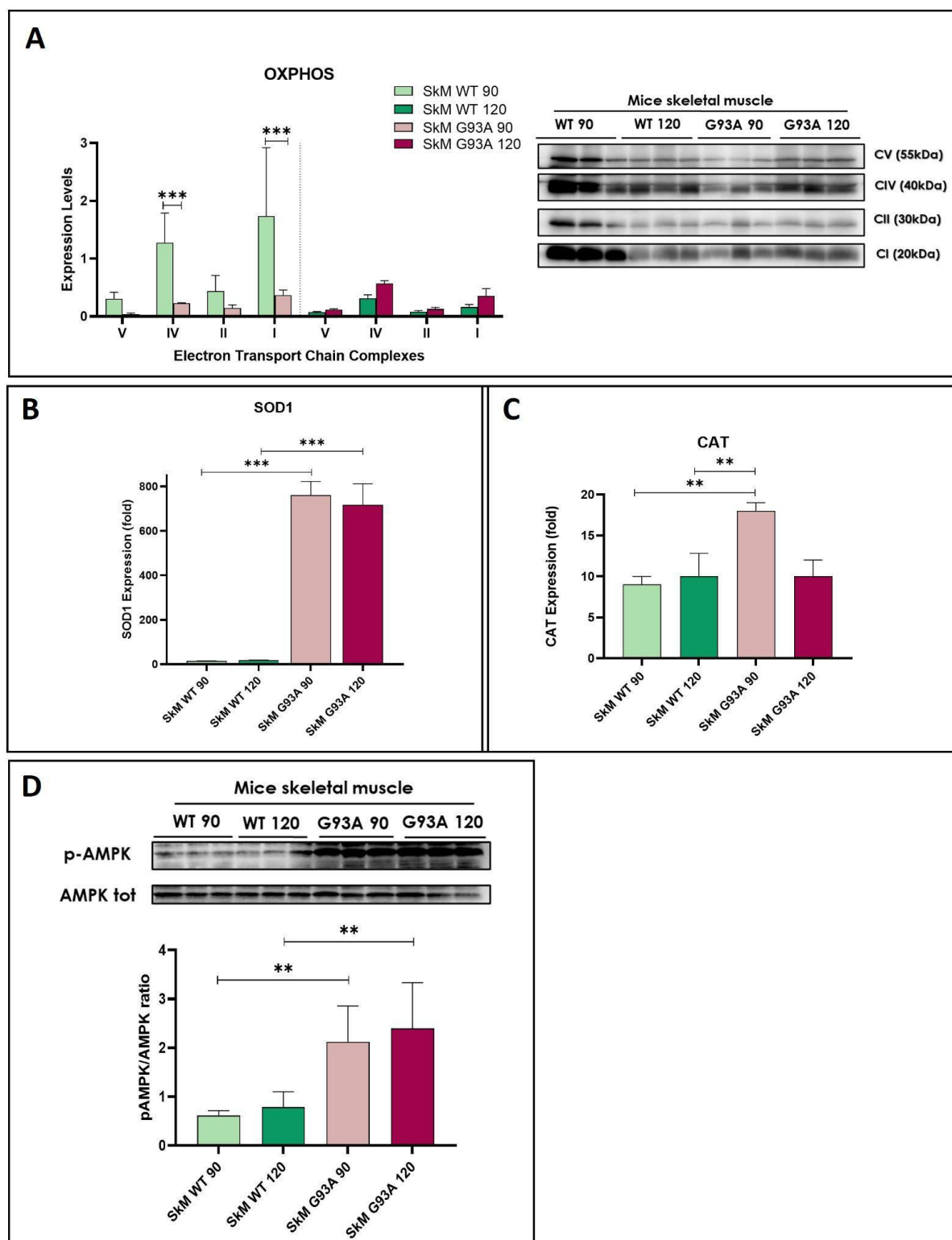


Figure S1. Functional characterization of skeletal muscle biopsies of WT and SOD1-G93A mice at 90 and 120 days. (A) Representative Western Blot for the expression of the mitochondrial electron transport chain complexes (OXPHOS). Values represent the mean $\pm$ SD; statistical analysis was carried out using two-way ANOVA with Tukey's multiple comparisons test ($n=3$). * $p < 0.1$, ** $p < 0.01$, and *** $p < 0.001$, when compared between WT and SOD1-G93A groups. (B) RT-qPCR for the expression of (B) SOD1 and (C) CAT. Values represent the mean $\pm$ SD;

statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparisons test (n=3). * $p < 0.1$, ** $p < 0.01$, and *** $p < 0.001$, when compared between different groups. (D) Representative Western Blot for the expression of pAMPK, expressed as pAMPK/totAMPK ratio. Values represent the mean $\pm$ SD; statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparisons test (n=3). * $p < 0.1$, ** $p < 0.01$, and *** $p < 0.001$, when compared between different groups. OXPHOS = oxidative phosphorylation; SOD1 = superoxide dismutase 1; CAT = catalase; AMPK = AMP-activated protein kinase.

S2. Functional characterization of skeletal muscle biopsies of WT and SOD1-G93A mice at 90 and 120 days

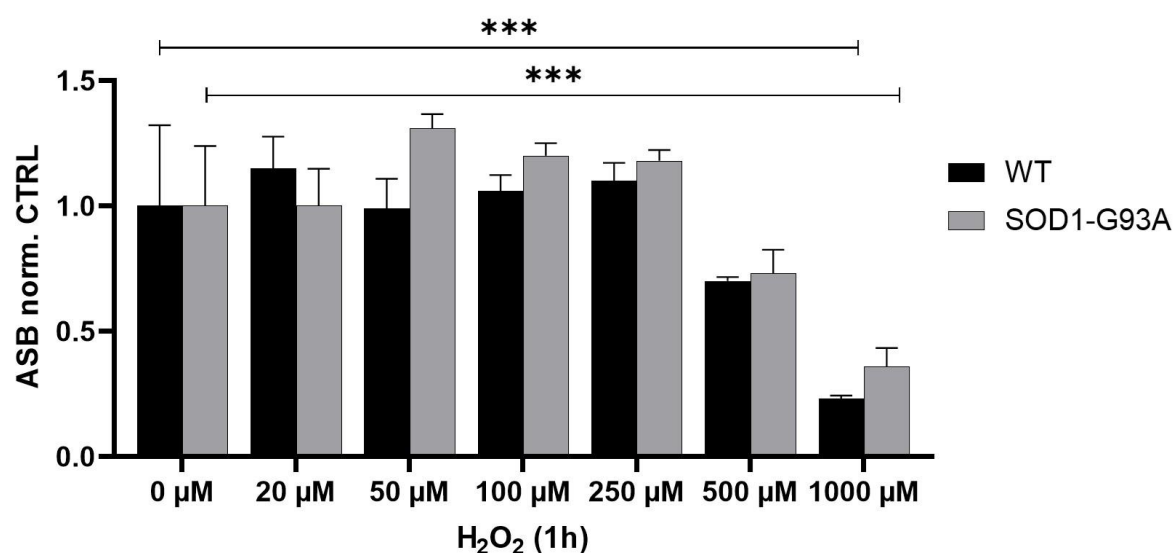


Figure S2. MTT assay for assessing the treatment with H₂O₂ for WT and SOD1-G93A SH-SY5Y cells. MTT assay performed on WT and SOD1-G93A SH-SY5Y treated with H₂O₂ for 1 hour with the following concentrations: 0 (control), 20, 50, 100, 250, 500 and 1000 μ M, to measure cell viability after treatment. Data are expressed as mean $\pm$ SD; statistical analysis was carried out using two-way ANOVA with Sidak's multiple comparison test (n=3). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, when compared with the control group.

S3. AFM analysis of SH-SY5Y neuronal cells and microvesicles sizing

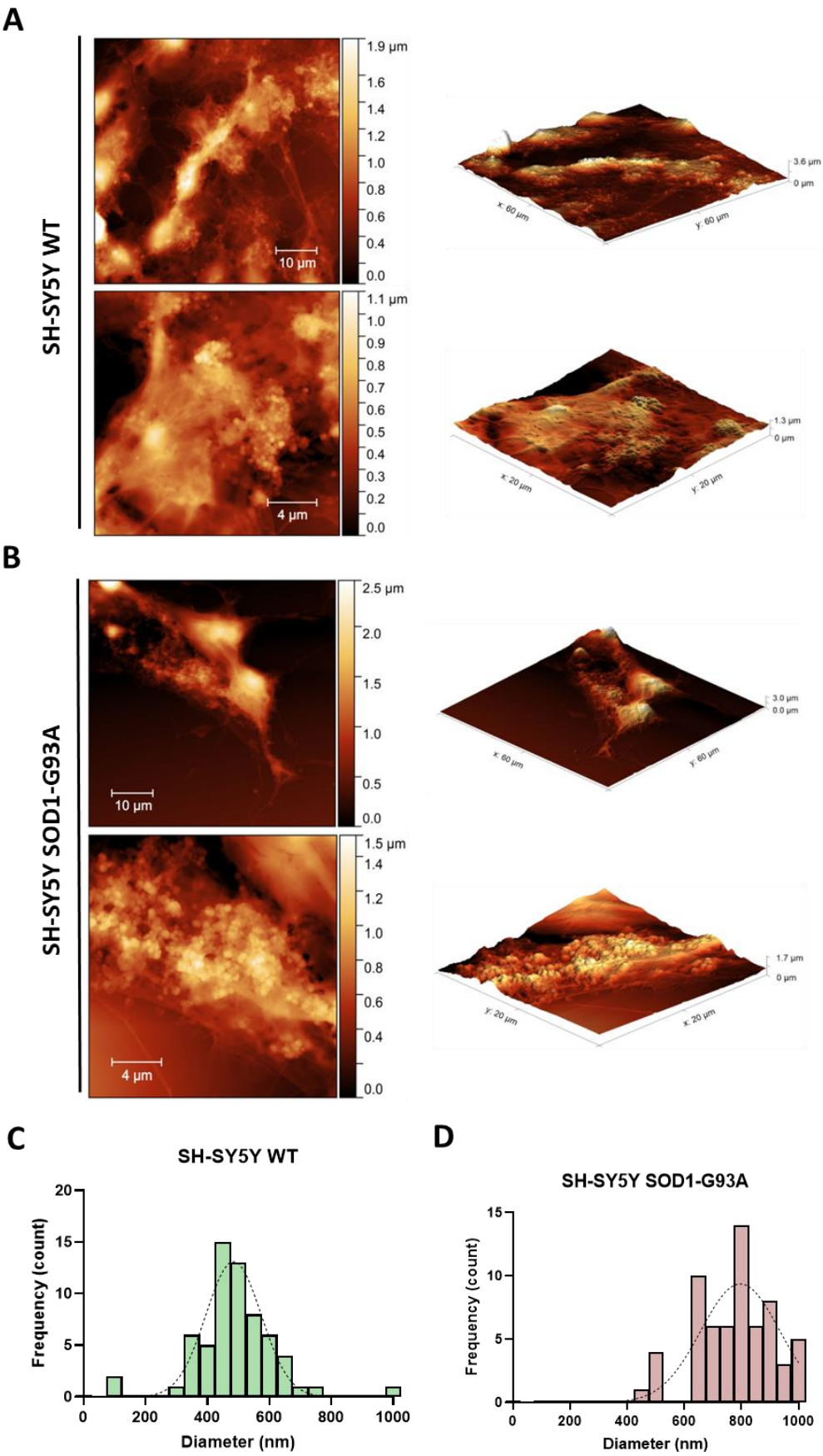


Figure S3. AFM analysis of differentiated WT and SOD1-G93A SH-SY5Y cells. Representative 2D (left) and 3D reconstructions (right) obtained from AFM acquisitions of neurons derived from **(A)** WT and **(B)** SOD1-G93A SH-SY5Y cells (scale bars = 10 μm for the full cell and 4 μm for the zoom). Size distributions of the budding vesicles of **(C)** WT (peak diameter = 480 nm) and **(D)** SOD1-G93A (peak diameter = 800) SH-SY5Y neurons.

LIST OF PUBLICATIONS

1. Fidaleo, M., Tacconi, S., **Sbarigia, C.**, Passeri, D., Rossi, M., Tata, A. M., & Dini, L. (2021). Current Nanocarrier Strategies Improve Vitamin B12 Pharmacokinetics, Ameliorate Patients' Lives, and Reduce Costs. *Nanomaterials* (Basel, Switzerland), 11(3), 743. <https://doi.org/10.3390/nano11030743>
2. Panzarini, E., Leporatti, S., Tenuzzo, B. A., Quarta, A., Hanafy, N. A. N., Giannelli, G., Moliterni, C., Vardanyan, D., **Sbarigia, C.**, Fidaleo, M., Tacconi, S., & Dini, L. (2022). Therapeutic Effect of Polymeric Nanomicelles Formulation of LY2157299-Galunisertib on CCl4-Induced Liver Fibrosis in Rats. *Journal of personalized medicine*, 12(11), 1812. <https://doi.org/10.3390/jpm12111812>
3. Tacconi, S., Augello, S., Persano, F., **Sbarigia, C.**, Carata, E., Leporatti, S., Fidaleo, M., & Dini, L. (2022). Amino-functionalized mesoporous silica nanoparticles (NH₂-MSiNPs) impair the embryonic development of the sea urchin *Paracentrotus lividus*. *Environmental toxicology and pharmacology*, 95, 103956. <https://doi.org/10.1016/j.etap.2022.103956>
4. **Sbarigia, C.**, Tacconi, S., Mura, F., Rossi, M., Dinarelli, S., & Dini, L. (2022). High-resolution atomic force microscopy as a tool for topographical mapping of surface budding. *Frontiers in cell and developmental biology*, 10, 975919. <https://doi.org/10.3389/fcell.2022.975919>
5. **Sbarigia, C.**, Vardanyan, D., Buccini, L., Tacconi, S., & Dini, L. (2022). SARS-CoV-2 and extracellular vesicles: An intricate interplay in pathogenesis, diagnosis and treatment. *Frontiers in Nanotechnology*, 4. <https://doi.org/10.3389/fnano.2022.987034>
6. **Sbarigia, C.**, Dinarelli, S., Mura, F., Buccini, L., Vari, F., Passeri, D., Rossi, M., Tacconi, S., & Dini, L. (2023). Wild-Type and SOD1-G93A SH-SY5Y under Oxidative Stress: EVs Characterization and Topographical Distribution of Budding Vesicles. *Applied Nano*, 4(1), 45-60. <https://doi.org/10.3390/applnano4010004>
7. Tacconi, S., Vari, F., **Sbarigia, C.**, Vardanyan, D., Longo, S., Mura, F., Angilè, F., Jalabert, A., Blangero, F., Eljaafari, A., Canaple, L., Vergara, D., Fanizzi, F. P., Rossi, M., Da Silva, C. C., Errazuriz-Cerda, E., Cassin, C., Nieuwland, R., Giudetti, A. M., Rome, S., ... Dini, L. (2024). M1-derived extracellular vesicles polarize recipient macrophages into M2-like macrophages and alter skeletal muscle homeostasis in a hyper-glucose environment. *Cell communication and signaling: CCS*, 22(1), 193. <https://doi.org/10.1186/s12964-024-01560-7>
8. **Sbarigia, C.**, Tacconi, S., Rome, S., Dini, L., (2024). New perspectives in the “dying back” hypothesis: the role of extracellular vesicles in Amyotrophic Lateral Sclerosis. *Frontiers in Cellular Neuroscience* (MANUSCRIPT IN PREPARATION).

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Sincerely,

Carolina



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High-resolution atomic force microscopy as a tool for topographical mapping of surface budding

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Extracellular vesicles (EVs) are membranous nanoparticles secreted by almost all cell types. Reflecting the physiopathological state of the parental cell, EVs circulate in all body fluids, reaching distant cell targets and delivering different bioactive cargoes. As biological carriers, EVs influence their microenvironment altering cellular responses, being considered promising biomarkers for both physiological and pathological conditions. EVs are heterogeneous in terms of size and composition, depending on cell type and exposure to stimuli, and different methods have been developed to characterize their morphological, biophysical, and biochemical features. Among them, electron microscopy (EM) is the main technique used, however, the lack of standardized protocols makes it difficult to characterize EVs with a good reproducibility, thus using multiple approaches may represent a way to obtain more precise information. Furthermore, the relationship between architecture and function, not only in a molecular, but also in a cellular level, is gaining growing emphasis, characterizing morphometric parameters may represent a distinct, but effective approach to study the physiopathological state of the cell. Atomic force microscopy (AFM), may represent a promising method to study in detail EVs dynamics throughout the cell surface and its variations related to the physiological state, overcoming the limits of EM, and providing more reliable information. In this study, human neuroblastoma SH-SY5Y cell line, a cellular model to investigate neurodegeneration and oxidative stress, has been used to perform a comparative morphological and quantitative analysis of membrane budding and isolated large vesicles-enriched (microvesicles-like vesicles; MVs) fraction from control or oxidative stressed cells. Our main goal was to build up a methodology to characterize EVs morphology and spatial distribution over the cell surface in different physiological conditions, and to evaluate the efficacy of AFM against conventional EM. Interestingly, both microscopy techniques were effective for this analysis, but AFM allowed to reveal a differential profiling of plasma membrane budding between the physiological and the stress condition, indicating a potential relationship between mechanical characteristics and functional role. The results obtained may provide interesting perspectives for

the use of AFM to study EVs, validating a morphometric approach to understand the pathophysiological state of the cell related to EVs trafficking.

KEYWORDS

extracellular vesicles, membrane buds, roughness, topographical mapping, high resolution microscopy

Introduction

Extracellular Vesicles (EVs) are heterogeneous, nanosized membrane-enclosed particles released by prokaryotic and eukaryotic cells in both physiological and pathological conditions (Dini et al., 2020). Considering that EVs classification and nomenclature is not standardized, generally the term EVs include two main subtypes, microvesicles (MVs) and exosomes (EXOs), also referred to as large and small vesicles. MVs are released directly from the plasma membrane, with a size range between 150 nm and 1 μ m in diameter, while EXOs originate from the endocytic pathway, with a diameter between 40 and 160 nm (Kalluri and LeBleu, 2020). Once released in the extracellular milieu, EVs circulate in all body fluids and are carriers of different bioactive cargoes, e.g., proteins, lipids, metabolites, and nucleic acids, to distant or nearby target cells (Mckelvey et al., 2015; Van Niel et al., 2018). For this reason, EVs are considered important regulators of intercellular communication, involved in several physiological and pathological processes (Panfoli et al., 2018; He et al., 2021). Generally, to study EVs the first step required is the isolation, which can be performed by several methods that can be used also in combination (Böing et al., 2014; Tzaridis et al., 2021). Once isolated, it is pivotal to properly characterize EVs in their morphological, biophysical, and biochemical features. However, due to their heterogeneity in size and composition and the lack of standardized protocols, it is hard to quantify and characterize EVs with a good reproducibility (Božič et al., 2021). Different techniques have been developed to study EVs in terms of size, distribution, topology, and phenotype. Nevertheless, the most widely used technique for EVs imaging is Electron Microscopy (EM), with a resolution of 1–3 nm for Transmission Electron Microscopy (TEM) and 5 nm for Scanning Electron Microscopy (SEM). Even if a low-throughput analysis, since allowing to observe only several particles at a time, EM gives detailed information about the size, shape, and morphology of the sample. Next to EM, stands out Atomic Force Microscopy (AFM) that, by scanning the sample using a micrometric cantilever with a nanosized tip, provides, with a single exposure, not only a high-resolution imaging, but also important information about biophysical and biomechanical properties of the specimens, such as local elasticity or the surface roughness (Dinarelli et al., 2018a; Malenica et al., 2021). EVs have been investigated also at functional level, e.g., high-throughput OMICS techniques that allow to study EVs cargo, and the existence of a relationship

between architecture and function, not only at a molecular, but also at a cellular level, is gaining growing importance. In this context, characterizing morphometric parameters may represent a distinct, but effective approach to study the pathophysiological state of the cell. Several studies have been exploited AFM to perform analysis at a nanoscale level of plasma membrane mechanical properties in response to biochemical stimuli (Girasole et al., 2012; Dinarelli et al., 2018b; Şahin et al., 2021). However, to the best of our knowledge, the use of morphometric parameters to describe EVs trafficking and its variations under certain physiological conditions, has not yet been investigated. EVs biogenesis and release has been proven to be increased under several stress conditions, including oxidative stress (Qi et al., 2021). Resulting from the accumulation of free radicals, oxidative stress alters biological function of macromolecules, such as proteins, lipids, and nucleic acids, determining harmful downstream effects. Furthermore, oxidative stress has been proven to modulate the level and content of released EVs, depending on the cell type and stimulus (Chiaradia et al., 2021). Several studies have demonstrated that an increase of EVs release in oxidative stress conditions may occur, driven by different factors causing membrane rearrangements, fluidity alterations, and cytoskeleton remodeling (Atienzar-Aroca et al., 2016; Holliday et al., 2019; Record et al., 2018; Van Meteren et al., 2019; Wilson and González-Billault, 2015). For this reason, EVs are implicated in diseases strictly linked to oxidative stress, for instance neurodegenerative disorders (Fowler and Hill, 2019; Meldolesi, 2021; Qi et al., 2021). Human neuroblastoma SH-SY5Y cell line, widely used as a model to investigate neurodegeneration and oxidative stress (Martínez et al., 2020; Greco et al., 2021; Chen et al., 2022), has been chosen to perform a comparative morphological and quantitative analysis of membrane budding and isolated microvesicles-like vesicles (MVs), in physiological conditions (control) or under oxidative stress (H₂O₂-induced), by electron microscopy (EM) and atomic force microscopy (AFM). The main goal of this work was to define a new method to study EVs morphological features and their alterations under stress conditions, evaluating the efficacy of the system compared to conventional methods. Both the microscopy techniques were used to obtain the average values of vesicles size as well as their distribution, resulting increased after H₂O₂ treatment. However, AFM analysis allowed to obtain a more accurate topography and distribution of plasma membrane budding throughout the cell surface and to appreciate details and features not accessible from the EM.

We performed the mapping of surface budding along two specific selected areas (the nuclear zone and the interposed area between perinuclear region and cellular edge) of control and oxidative-stressed cells, and used a morphometric parameter, the surface roughness, to get a relationship between the topography and the entity of the budding phenomenon, according to different physiological conditions. Taken together, our results may provide interesting perspectives to implement a morphometric approach for studying and understanding the pathophysiological state of the cell related to EVs trafficking.

Materials and methods

Cell culture and treatments

Human neuroblastoma SH-SY5Y cells were cultured in DMEM 4.5 g/L glucose (Sigma Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) (Sigma Aldrich, St. Louis, MO), 2 mM L-glutamine (Corning, Manassas, VA), 100 UI/mL penicillin and streptomycin solution (Corning, Manassas, VA) and 10.000 U/mL amphotericin B (antimycotic solution) (Sigma Aldrich, St. Louis, MO) in a 5% CO₂ humidified atmosphere, at 37°C. Cells were maintained in 75 cm² flasks at a concentration of 2×10^6 cells/mL by passage every two to three days. To induce oxidative stress, cells were treated with H₂O₂ (Carlo Erba, Milan, Italy) at a final concentration of 100 µM for 1 h, followed by 24 h of recovery in EVs-depleted medium. After performing the treatments, cells were harvested for further analyses.

Microvesicles-enriched fraction isolation by ultracentrifugation

Before the treatments, complete DMEM growth medium was overnight centrifuged at 110,000 g in order to remove contaminant FBS-derived EVs. After the treatments, MV-enriched fractions were concentrated from H₂O₂-treated and control SH-SY5Y cells conditioned culture medium by differential centrifugation, as described in (Panzarini et al., 2020). Briefly, the culture medium of each sample was first centrifuged at 500 g for 10 min at room temperature, and the resulting supernatant was then centrifuged at 800 g for 10 min at room temperature. The last centrifugation of the supernatant was performed at 2000 g for 20 min at room temperature in order to remove dead cells or cell aggregates. Cell-depleted supernatant was then centrifuged at 20.000 g for 20 min at 4°C using a Beckman Coulter Ultracentrifuge Optima XE (Beckman Coulter, Brea, CA), and the resulting MV-enriched pellet was collected and used for further analysis (i.e., the microvesicles-enriched fraction; MVs).

Scanning and Transmission Electron Microscopy

H₂O₂-treated and untreated SH-SY5Y cells cultured on glass coverslips were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for 1 h at ice temperature and postfixed with 1% OsO₄ in the same buffer for 1 h on melting ice. After fixation, cells were dehydrated with ethanol (EtOH), 50%, 70%, 95%, and 100%, followed by chemical drying with EtOH and Hexamethyldisilazane (HMDS) (Sigma-Aldrich, St. Louis, United States), 2:1, 1:1, 1:2, and finally in pure HMDS, evaporated overnight. Consequently, the specimens were mounted on aluminum stubs and 3 nm chromium-coated with a Quorum Q150 T sputter (Quorum Technologies, United Kingdom) in order to increase the electron conductivity. For TEM analysis, cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) for one hour at ice temperature and post-fixed with 1% OsO₄ in the same buffer for two hours at ice temperature. After fixation, cells were dehydrated with ethanol (25%, 50%, 70%, 90%, and 100%) and embedded in Spurr resin. Thin sections of 60 nm were cut at the ultramicrotome (Powertome PC, RMC Boeckeler Instruments, Germany) and deposited on 200 mesh copper-grids. After isolation, MVs from all experimental groups were fixed with 0.1% paraformaldehyde (PFA) in DPBS for 30 min at room temperature. Fixed samples were stained with 2% uranyl acetate for 7 min at room temperature, loaded on 200 mesh carbon-coated grids for the TEM observation. SEM and TEM analysis were both performed using a Zeiss Auriga Scanning Electron Microscope (Zeiss, Oberkochen, Germany) equipped with the STEM module, operating at 8 and 20 keV, respectively.

Atomic force microscopy preparation procedure and imaging of cells and isolated microvesicles

For Atomic Force Microscopy, control and H₂O₂-treated human neuroblastoma SH-SY5Y cells were seeded in 6-well multiwell with coverslips at a concentration of 8×10^4 cells/cm², and incubated overnight in a 5% CO₂ humidified atmosphere, at 37°C. Cells were then washed twice in DPBS and fixed in 1% glutaraldehyde in 0.1 mol/L cacodylate buffer pH 7.4 at ice temperature for 30 min. After fixation, cells were washed again twice in DPBS and two final washes in bidistilled water were performed. The isolated microvesicles-enriched fractions were fixed with 0.1% paraformaldehyde (PFA) in DPBS for 30 min at room temperature and diluted in water. The AFM images were acquired in contact mode with a Multimode AFM (Bruker, Santa Barbara, CA). We used commercially available AFM tips, model DNP (Bruker, Santa Barbara, CA) with triangular shaped cantilever, nominal elastic constant of 0.06 N/m and nominal tip radius of 10 nm. The

interaction force between the tip and the sample was selected to be less than one nN, in such a way to not damage both tip and sample. To gain an overview of the sample morphology two different strategies were adopted: when imaging cells the scan area was set to 60 microns and a subsequent zoom of 20 micron has been performed on an area of interest; while, when imaging microvesicles, a few images of 20 micron were acquired and a greater number on images of 10 micron were taken until a sufficient number of vesicles were measured. According to the selected scan range the speed was set to 1–4 s per line while at least 512 points per line were used in all acquired images.

Atomic force microscopy images analysis and vesicles counting

The analysis of the AFM images was performed with the freely available software Gwyddion (www.gwyddion.net). All images were minimally treated (i.e., a mean plane subtraction, substrate tilt correction and line coupling) in order to avoid the thermal drift and the tilting of the sample. The isolated vesicles were counted by using Gwyddion's routine "Mark Grains by Segmentations," changing the working parameters according to the best possible identification of the vesicles. This very same approach can't be used to directly measure the budding on the cellular membranes since their big and inhomogeneous variation in height (i.e., thicker and round shaped in the nuclear region, flat on the border) makes impossible to find a set of parameters that allow a good identification of the vesicles. Two different options were then available: 1) to deeply modify the images or 2) to select a different counting method (i.e., by hands), we preferred the latter since the former can induce systematic and unpredictable errors.

Roughness measurements

AFM surface root mean square deviation (roughness; Rq) analysis of cell surface was performed by ImageJ Software version 1.8.0. Square $6 \times 6 \mu\text{m}^2$ regions were randomly selected, and five measurements were taken from each sample. Representative 3D reconstructions of AFM images were also performed with the same software.

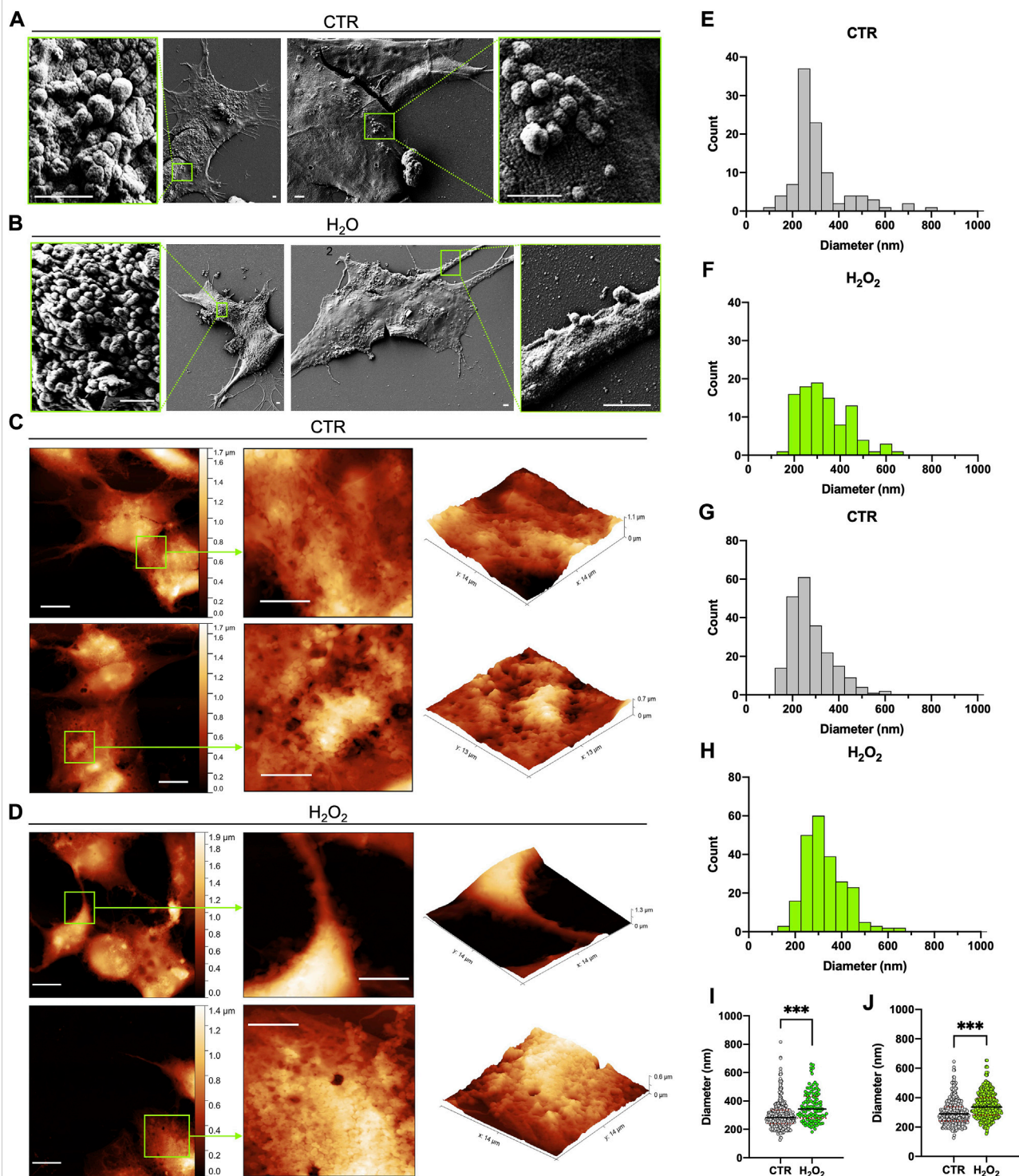
Statistical analysis

Results are expressed as means $\pm$ SEM. Multiple comparisons were performed by two-way ANOVA. Comparisons between two groups were performed using a student's t-test. Comparisons between two distributions were analyzed using a Kolmogorov-Smirnov test (GraphPad Prism 9 software, GraphPad Software, San Diego, CA).

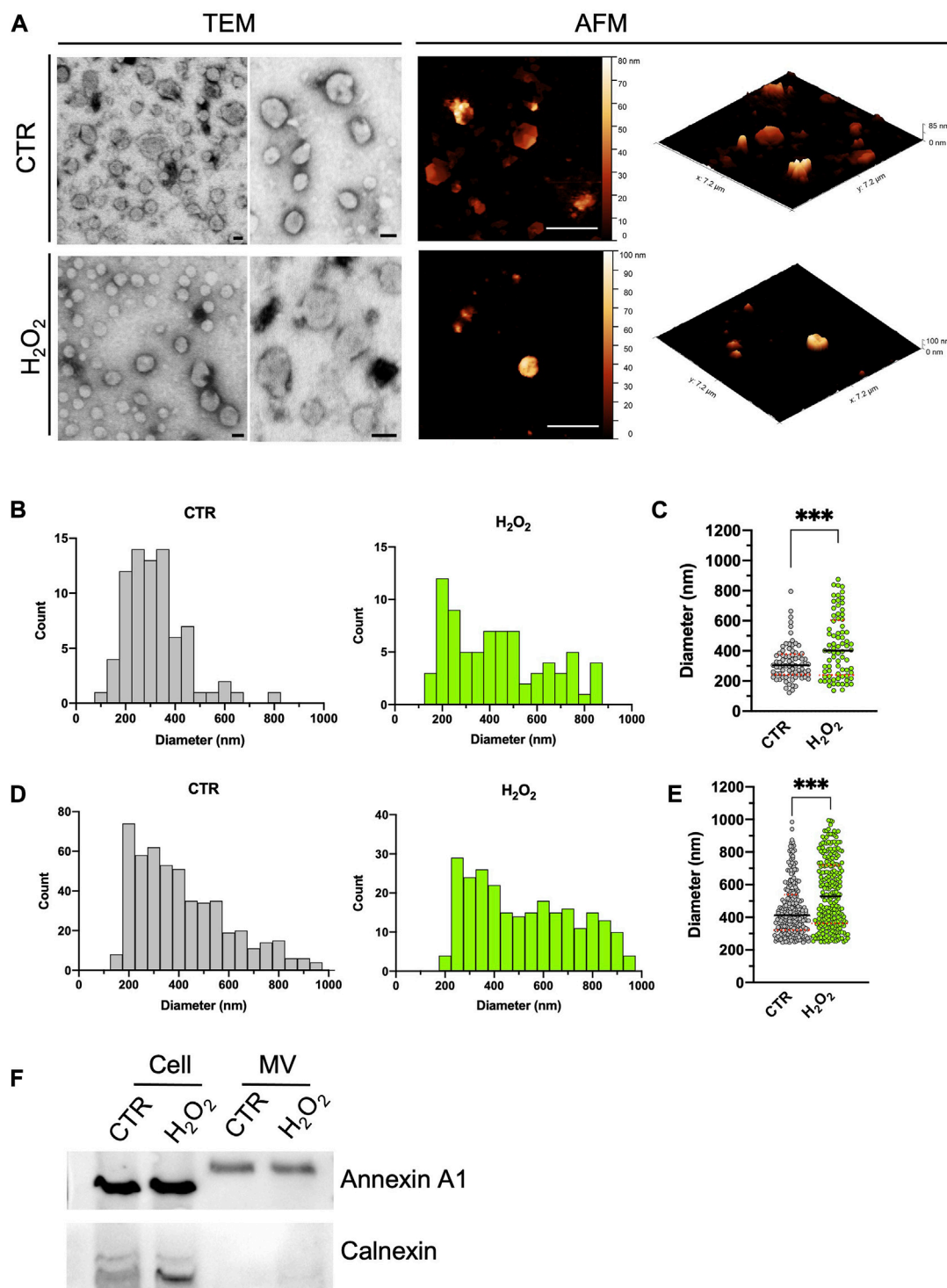
Results

Morphological analysis of membrane budding in control and H2O2-treated SH-SY5Y cells by scanning electron microscopy and atomic force microscopy

An AFM and SEM microscopy analysis of vesicle budding at the cell surface of SH-SY5Y cells with and without H2O2 treatment were performed and the results are shown in Figure 1. SEM micrographs acquired on control and H2O2-treated SH-SY5Y cells showed cellular surfaces with numerous bulges and protruding buds of various sizes (Figures 1A,B). As a result of a qualitative analysis of the SEM images (Figures 1A,B), no differences were observed between the control and H2O2-treated groups in terms of budding entities and vesicles distribution. However, the histograms of Figures 1E,F show significant differences in the size distribution of buds between the control and H2O2 groups. Although the measured diameters range is similar (150–550 nm for control and 200–600 nm for H2O2-treated cells), the budding vesicles in SH-SY5Y cells exposed to H2O2 (Figure 1F) appear as a more homogeneous population with a comparable number of vesicles at different diameters with respect to control cells (Figure 1E) that resemble a very narrow distribution. On the other hand, the AFM microscopy analysis clearly shows that both control and H2O2-treated cells have a very irregular cell surface, characterized by an extensive budding of vesicles, large projections, and bulges (Figures 1C,D). In the case of control cells, these vesicular formations are primarily confined to the central part of cell bodies (Figure 1C), while in those treated with H2O2, the budding is pronounced even in the terminal portions, such as the cell periphery, protrusions, and cell processes (Figure 1D), something that was not observed in the SEM images. The histograms obtained from the sizing performed by AFM are shown in Figures 1G,H, the buds of both controls and H2O2-treated cells have a similar distribution but with a significant shift toward higher diameter values in the H2O2-treated cells. A direct comparison between the particle distribution of buds obtained, is represented in the dot plot graphs shown in Figures 1I,J where, sizing samples both with SEM (Figure 1I) and AFM (Figure 1J), shows a very good agreement between the two techniques and demonstrate a significant difference between control and H2O2-treated cells. Moreover, the median diameter analyzed from the SEM measurements of the control cells was 284 ± 5 nm, while for the H2O2-treated cells 343 ± 10 nm (Figure 1I). Both these values are well comparable with the AFM measured ones: 289 ± 4 nm for the control cells and 337 ± 4 nm for the treated ones (Figure 1J). Since the values obtained from both SEM and AFM analysis are similar, these results gave us a first insight of the potency of AFM technique as a valid system to characterize extracellular vesicles, as well as electron microscopy.

**FIGURE 1**

Comparison between SEM and AFM imaging of dried cells. **(A)** and **(B)** 2 typical SEM images acquired on the control and H₂O₂ treated cells, respectively, that show the presence of buds on the surface of the cells in localized regions; for each image a zoom on one of these regions is highlighted in a green box (scale bar 1 μ m). **(C)** and **(D)** 2 typical AFM images acquired on the control and H₂O₂ treated cells, respectively, that show the presence of extensive budding on the surface of the cells; for each image (left side, scale bar 10 μ m) a zoom on a region of interest highlighted in green is reported in 2-D (centre, scale bar 6 μ m) and 3-D (right) views in order to better visualize the spatial arrangement of the membrane. **(E)** to **(H)** statistical analysis performed on the size distribution of the vesicles measured from all the SEM **(E,F)** and AFM **(G,H)** images acquired on the control **(E,G)** and H₂O₂ treated cells **(F,H)**. **(I)** and **(J)** dot plots of the direct comparison between the particle distribution of buds obtained on control (grey) versus H₂O₂ treated (green) cells from SEM **(I)**, median diameter of 284 ± 5 nm for the control cells and 343 ± 10 nm for the H₂O₂-treated ones) and AFM **(J)**, median diameter of 289 ± 4 nm for the control cells and 337 ± 4 nm for the H₂O₂ treated ones) images. For both techniques the obtained distributions are statistically different ($p < 0.05$).

**FIGURE 2**

Comparison between STEM and AFM imaging on isolated vesicles. **(A)**: two typical STEM (left, scale bar 100 nm) and AFM (right, scale bar 2 μ m) images acquired on isolated budding vesicles derived from control (first row) and H₂O₂ treated cells (second row). For each AFM image is also shown in its native 3D-view to better highlight the shape of the vesicles. **(B)** to **(E)**: statistical analysis of the size distribution of the vesicles as measured with the STEM **(B,C)** and AFM **(D,E)** performed of vesicles derived from control (grey values) and H₂O₂ treated cells (green values). A direct comparison between the two size distributions is shown for STEM **[(C), median diameter of 305 ± 14 nm for the vesicles derived from control cells and 401 ± 24 nm for the ones derived from H₂O₂-treated cells]** and AFM **[(E), median diameter of 412 ± 10 nm for the vesicles derived from control cells and 528 ± 13 nm for the ones derived from H₂O₂-treated cells]** data. For both techniques the obtained distributions are statistically different ($p < 0.05$). **(F)**: SDS-PAGE gel electrophoresis showing the presence of Annexin A1 (microvesicles marker) and calnexin (endoplasmic reticulum marker).

Comparative morphological and quantitative analysis of isolated microvesicle-enriched fraction by Transmission Electron Microscopy and atomic force microscopy

After concentration by ultracentrifugation, the morphology, size distribution, and quantity of isolated microvesicles were analyzed by TEM and AFM in the MV-enriched fraction obtained from control and H₂O₂-treated SH-SY5Y cells (results are summarized in Figure 2). As shown in Figure 2A, TEM micrographs of enriched fractions (left-sided) obtained from H₂O₂-treated and control cells revealed a heterogeneous population of rounded shape vesicles with a characteristic range diameter compatible with large vesicles, such as MV. Furthermore, the presence of the MV-associated marker annexin A1 (Jeppesen et al., 2019) and the absence of calnexin, generally used as a negative control, demonstrate the effective presence of MV in the isolated fractions and the quality of the isolation method (Figure 1F). The same landscape arises from the AFM images, shown on the right side of Figure 2A, where the vesicles' morphological appearance and range diameter are essentially the same. Moreover, it is also possible to visualize the real 3D structure of the vesicles by taking advantage of the 3D metrological capabilities of the AFM. In Figure 2B, the histogram of the MV measured with TEM is reported. Control MV (gray graph) showed a diameter ranging between 123 and 794 nm with a single peak distribution. Conversely, the MV-enriched fraction from H₂O₂-treated cells (green graph) contains particles with a diameter range of 137–874 nm, wider than the MV-derived from control cells. In Figure 2C, the histograms of the size distribution obtained from the AFM images is reported. The diameter range of MV obtained from control SH-SY5Y cells (gray graph) is in the range 150–800 nm, lower than the measured values for vesicles from H₂O₂-treated cells (green graph) that ranges from 200 to 950 nm. The general qualitative behavior and appearance of the obtained distributions is consistent among the two techniques. More in detail, the distributions obtained between control and H₂O₂ differ in shape (i.e., in H₂O₂-treated cells, MV displays a more heterogeneous population of vesicles with a more variable diameter), thus indicating a high degree of comparability between the two methods. When comparing the mean values obtained for the two distributions, some differences arose, as indicated by the dot plot shown in Figure 2C for the TEM data and Figure 2E for the AFM data. The TEM states a median size of 305 ± 14 nm for the control and 401 ± 24 nm for the H₂O₂ treated, while the AFM gave a median diameter of 412 ± 10 nm for the control and 528 ± 13 nm for the H₂O₂ treated. The values measured by the AFM are significantly higher with respect to the ones obtained by the TEM. However, the distribution of values is perfectly compatible among AFM and TEM data (Figures 2C versus 2E), with the

AFM presenting a definitely higher statistic. This is the major advantage of the AFM technique over the TEM, the number of particles that can be counted (by using the same acquisition time) is higher in the AFM case (for example, in our experiments for the control sample a total of around 90 MVs were counted from the TEM images, while for the AFM around 500 MVs) and this is crucial to obtain more meaningful and statistically consistent results.

Mapping of surface budding in the nuclear and perinuclear/cellular edge area by atomic force microscopy

Microvesicles are released from cell membranes at the levels of specific areas, where lipid rafts and the molecular machinery involved in membrane curvature, vesicles detachment and cargo sorting are concentrated (Pollet et al., 2018; Elsherbini et al., 2021). As a consequence, this process can preferentially localize in certain parts of the cell in response to specific treatments or stimuli (Cloos et al., 2020; Chiaradia et al., 2021). By analyzing the AFM images, we mapped the surface budding on two specific areas of control and H₂O₂-treated cells, in order to determine which cell area was responsible for an increased release of vesicles. More in detail, we splitted the cells in two regions according to the measured height: 1) the higher part of the cell as the nuclear zone (Nu; blue dotted line in Figures 3A,B) and 2) the residual part as an interposed area between the perinuclear region and the cellular edge (Pn/CE; green dotted line in Figures 3A,B). Representative AFM magnifications of the nuclear area are reported in Figure 3C (upper panel). These images show a perfect example of how there are no significant variations in surface budding between the control and H₂O₂-treated cells in the nuclear area. On the contrary, in the lower panel of Figure 3C, are reported two explicative images of the increase in the budding process that can be observed in the Pn/CE area after treatment with H₂O₂. The same images are also shown in 3D-view (Figure 3C, right panels), in order to further highlight the presence of extensive surface blebbing in the nuclear regions of both samples, while the blebbing in the Pn/CE zone results clearly stronger in H₂O₂-treated cells when compared to the control group. We decided to quantify this variation among samples and regions by counting budding vesicles in defined square areas of $6 \times 6 \mu\text{m}^2$. The results obtained for the Control cells are shown in Figures 3D,E for the Nuclear and Perinuclear areas, respectively, while for the H₂O₂ treated cells the results are shown in Figures 3F,G. Since the obtained particle size distributions are very similar to one another, to highlight subtle differences we decided to compare the data performing a normalization procedure, i.e., the results were expressed as the number of particles for μm^2 , and the obtained graph is shown in Figure 3H. From this comparison, it is possible to state that the treatment with H₂O₂ resulted in a statistically significant

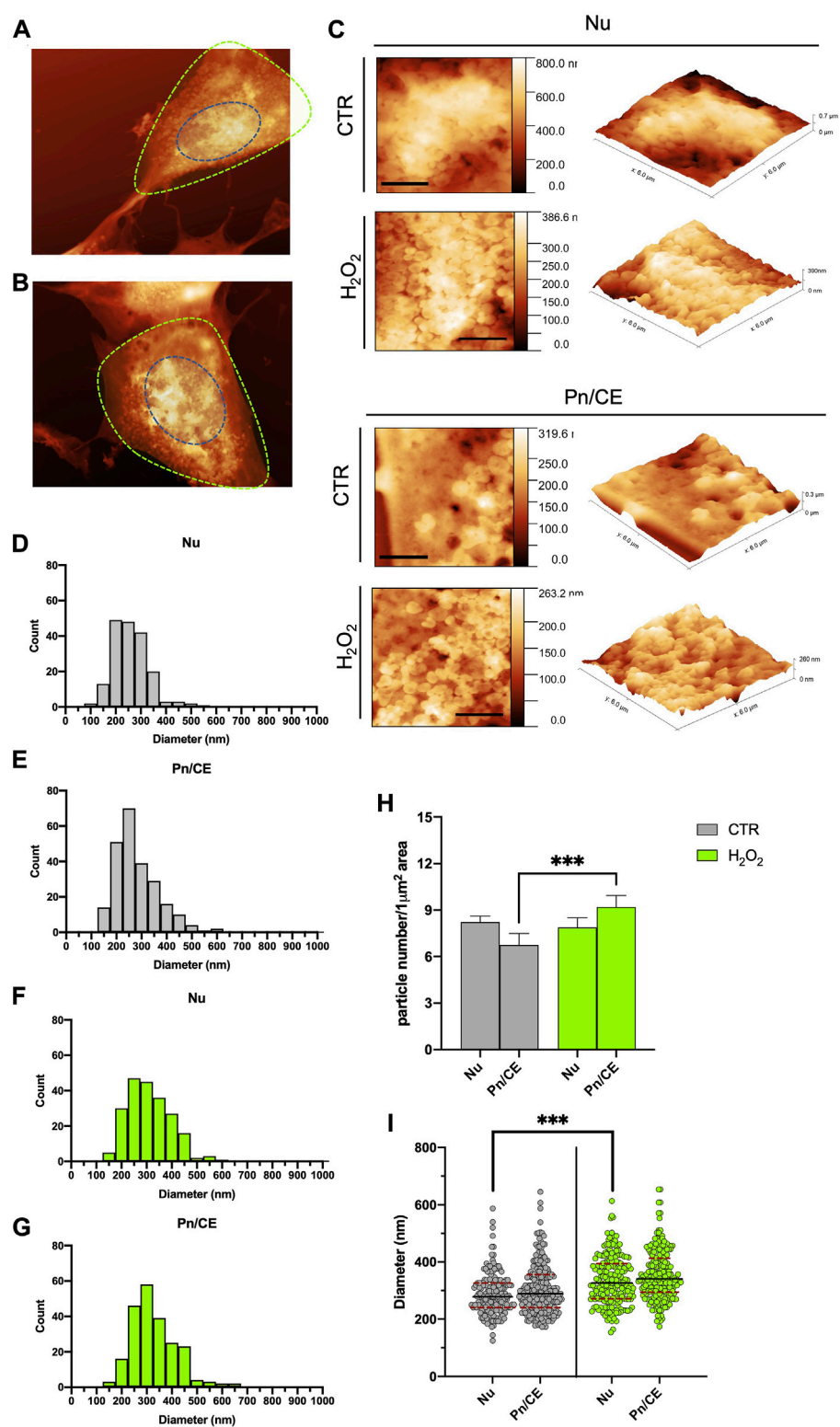


FIGURE 3 Statistical analysis of the spatial distribution of the budding vesicles on the cells, as seen with AFM. **(A)** and **(B)** example of the segmentation of the AFM images in nuclear (Nu, blue dotted line) and perinuclear (Pn/CE, green dotted line) for one control **(A)** and one H₂O₂ treated **(B)** cell. **(C)** typical high resolution 2D (left, scale bars 2 μm) and 3D (right) zoom of a 6 × 6 micron areas of the control and H₂O₂ treated cells in the Nuclear (top panels) and perinuclear (bottom panels) used for the manual counting and diameter measurements of the vesicles. **(D)** to **(G)** size distributions of the
(Continued)

FIGURE 3 (Continued)

vesicles separated according to the sampling region [nuclear (E,F), perinuclear (E,G) and the treatment (control cells showed in grey and H2O2 cells showed in green). (H) comparison among the obtained data in terms of density of vesicles (i.e., number of vesicles measured for $1 \mu\text{m}^2$), the results obtained in the perinuclear areas of control and H2O2 treated cells are statistically different ($p < 0.05$). (I) direct comparison between the diameter distributions obtained for control (grey dots) and H2O2 treated (green dots) cells. The average diameter value obtained for the control cells are 279 ± 5 nm and 289 ± 6 nm in the nuclear and perinuclear regions, respectively, while for the H2O2 treated ones the corresponding values are 327 ± 6 nm and 341 ± 6 nm. The average diameters values in the nuclear regions were statistically different ($p < 0.05$) between control and H2O2 treated cells.

increase ($p < 0.001$) in buds density in the Pn/CE area, while this did not happen in the nuclear area. In addition, we analyzed the size distribution of budding vesicles in the Nu and Pn/CE areas of control and H2O2-exposed cells, to determine if the treatment affected not only the number of buds, but also their size, and the obtained graph is reported in Figure 3I. When considering the nuclear region, the H2O2 exposure did not modify considerably the size range of budding vesicles (125–587 nm for control vs. 154–613 nm for treated cells), but their distribution and median diameter (279 ± 5 nm for control vs. 327 ± 6 for H2O2-treated cells) changed significantly ($p < 0.001$). On the other hand, no significative differences in terms of range diameter and distribution of buds were found in the Pn/CE zone (173–645 nm; Figure 3E) when compared to the control (174–654 nm; Figure 3G); while an increase in the median diameter of buds was observed in the Pn/CE area of H2O2-treated SH-SY5Y cells (341 ± 6 nm) with respect to the control cells (289 ± 6 nm) (Figure 3I). Considering these observations, in control cells the vesicles budding appears homogeneous all over the cellular membrane, while the exposure to H2O2 exerts two main effects: 1) increasing the vesicles average size both in the nuclear and in the perinuclear regions, and 2) increasing the number of vesicles produced only in the perinuclear area. These considerations further confirm the unique capabilities of the AFM to give local information all over the cellular membrane, in response to a particular stimulus, specifically H2O2 treatment.

Roughness measurement in the nuclear and perinuclear/cellular edge area

The membrane roughness (R_q) is a key parameter that is directly related to the cellular surface arrangement and topography at the nanoscale level. It represents a direct estimation of the good attachment of the membrane to the underlying cytoskeleton and it can be used to evaluate the cellular status (Carelli-Alinovi et al., 2019) and follow the evolution of the morphological patterns by quantifying them in a reproducible way (Dinarelli et al., 2018b). In our experiments this parameter is, however, intended as a combined measure of pure membrane roughness and budding phenomena, i.e., the more extensive budding is present, the higher roughness has to be expected. In view of these considerations, we decided to evaluate the roughness in the two different areas already discussed: the

nuclear (Nu) and the perinuclear/cellular edge (Pn/CE) regions of the cells. Moreover, we decided to measure the waviness on each region, to evaluate the membrane arrangement over longer lateral scales (Goic et al., 2011), then the ones used for the calculation of the local roughness. More in detail, in the case of a single cell, the waviness can be seen as a simplified profile of the entire cell. The results obtained are shown in Figure 4. In particular, in Figures 4A, B are shown the preliminary steps of the region selection and the corresponding height maps obtained for one control and one H2O2 treated cell, respectively. An example of the behavior of roughness and waviness over the entire cell is shown in Figure 4C (for one control cell) and in Figure 4D (for a H2O2 treated one). From these two examples can be clearly seen how the values of the linear roughness (black lines) are quite different between the nuclear and perinuclear regions (separated by dashed vertical gray lines). In general, the values are less scattered in the perinuclear region with respect to the nuclear one, this fact well correlates with the topography of the cell (i.e., the higher, the rougher). The roughness value, however, becomes a quantitative parameter, and thus can be used to compare different samples and regions if it is calculated over areas with the same lateral dimension and the same total number of pixels. In order to provide a quantitative analysis, we computed, for each cell, the roughness values in 2 or 3 (depending on the total dimension of the cell itself) not-overlapping 6×6 microns areas onto the Nu and Pn/CE regions obtaining a representative average value of roughness representative of the region. The obtained results are shown in Figure 4E. The values of roughness obtained for the control cells were 9.8 ± 1.2 nm and 7.8 ± 1.2 nm in the nuclear and perinuclear regions, respectively. While for the H2O2 treated ones, the obtained values are 8.1 ± 1.7 and 10.6 ± 2.8 for the nuclear and perinuclear regions, respectively. A first consideration of these values resides in the analysis of the statistical error obtained, that is higher in the H2O2 treated cells with respect to the control. This can be seen as a confirmation of the fact that the H2O2 exposure enhances the budding phenomena that, consequently, gives values more spreaded among the different cells. Considering only the nuclear region, there is no statistically significant difference between the untreated and treated cells average roughness values, while there is a significant increase ($p < 0.05$) in the perinuclear regions. Therefore, also the average roughness values measured on the nuclear and peripheral regions of the

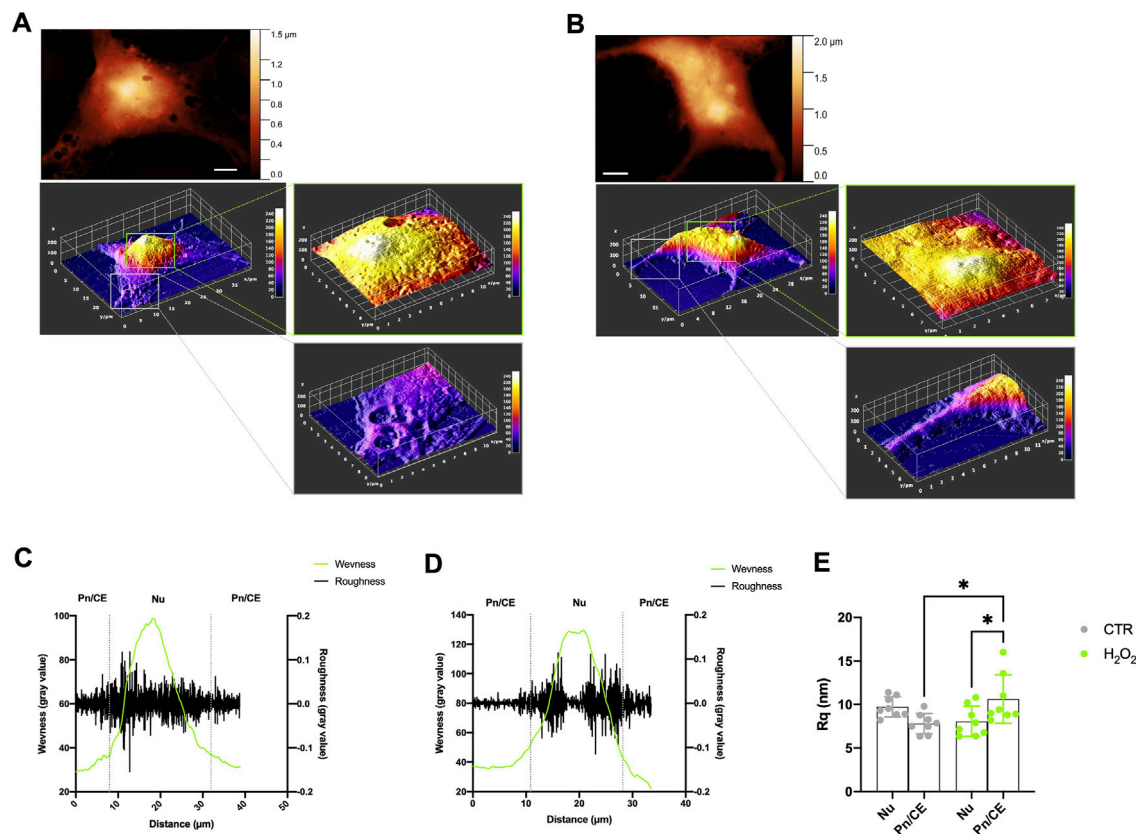


FIGURE 4

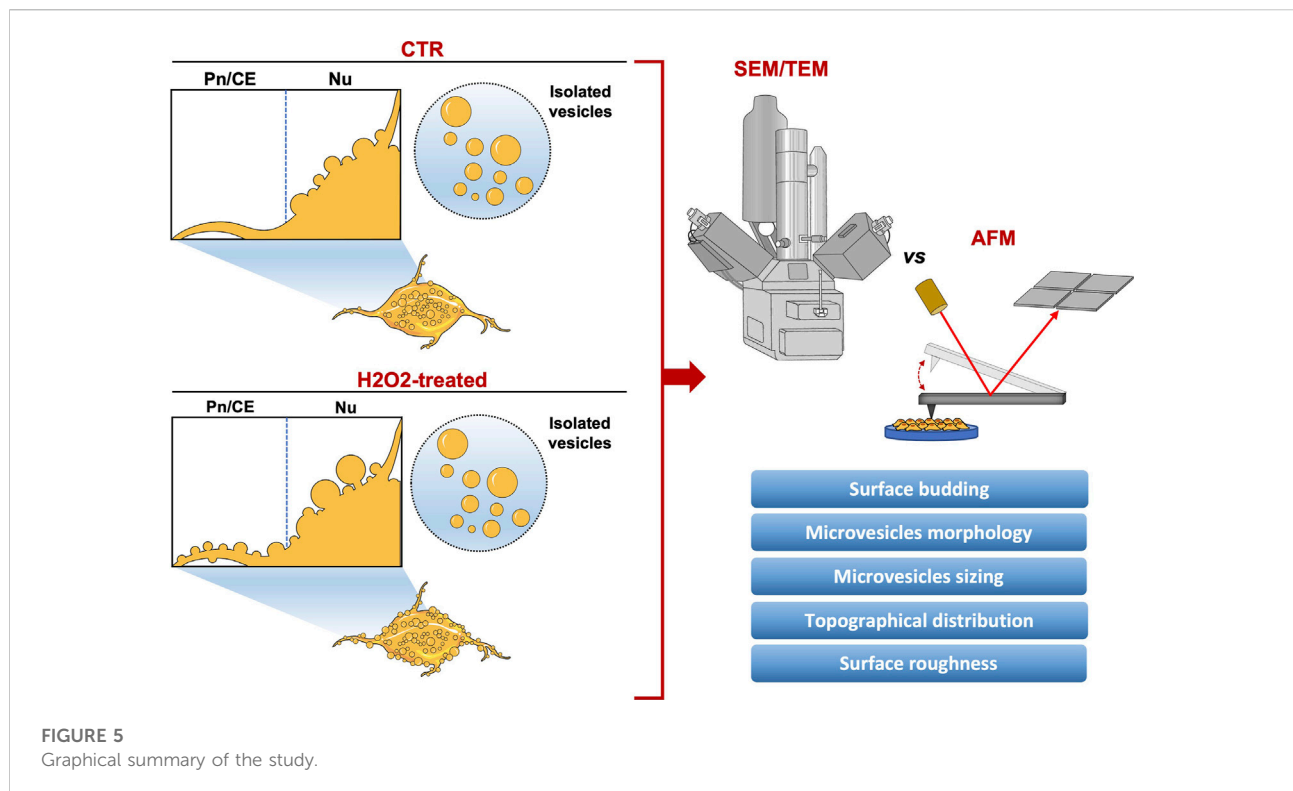
Roughness and waviness analysis performed on the AFM images. (A) and (B) example of the procedure to select the 6×6 microns wide regions on nuclear and perinuclear areas on one control cell (Panel A) and one H_2O_2 treated cell (B), scale bars 5 microns. (C) and (D) example of calculation of roughness (black line) and waviness (green line) along a line onto an entire control (C) and H_2O_2 treated (D) cell, the grey vertical dotted lines separate the nuclear (Nu) and perinuclear (Pn/CE) regions. (E) roughness values obtained from all the images sampled, each dot is representative of a region (Nu or Pn/CE) for each cell, the results obtained on the control cells are shown in grey dots while the ones obtained on the H_2O_2 treated cells are shown in green dots. The average values of roughness obtained for the control cells are 9.8 ± 1.2 nm and 7.8 ± 1.2 nm in the nuclear and perinuclear regions, respectively. While for the H_2O_2 treated ones, the obtained values are 8.1 ± 1.7 nm and 10.6 ± 2.8 nm for the nuclear and perinuclear regions, respectively. The mean roughness values were statistically different between nuclear and perinuclear regions ($p < 0.05$) in the H_2O_2 treated samples and in the perinuclear region between control and H_2O_2 treated cells ($p < 0.05$).

H_2O_2 treated cells are statistically different ($p < 0.05$). These two latter considerations, taken together, further confirm that the enhancement of the budding phenomena is triggered by the H_2O_2 exposure, but also that it is more pronounced in the peripheral region of the cell.

Discussion

In this study, we applied a reliable AFM-based method to evaluate the number, size, and distribution of budding and isolated vesicles in different cell culture conditions and treatments. To better confirm the reliability of our investigations, AFM measurements were compared with gold-standard methods globally used for the analysis of superficial budding (i.e., SEM) or ultrastructural analysis of isolated EVs

(i.e., TEM). When discussing the differences between the data obtained with SEM and AFM, at least one prior consideration is necessary. From the direct comparison between the two techniques, a first evidence of the validity of the AFM system for the analysis of vesicles budding emerged. Both the methods provide perfectly comparable results, but the AFM analysis allows to highlight subtle differences in the budding process, such as the different distribution of the vesicles over the entire cell surface, probably hidden by the sample preparation procedure of the SEM. Indeed, it is evident how the cells analyzed by SEM have a much smoother surface than those analyzed by AFM, where the cell membrane appears almost completely covered in buds, particularly in the nuclear region. Perhaps, this effect is due to the different protocols necessary to obtain stable samples. The SEM preparation procedure is harder than the AFM one (Figure 5). In particular, the cell surface



flattening observed in SEM micrographs is probably due to the sputter coating (a process which applies an ultra-thin coating of electrically-conducting metal in a non-conducting or poorly conducting specimen (Brodsch et al., 2018), not involved in the AFM preparation procedure. The details obtained by analyzing the samples by AFM, but not by SEM, can be crucial to fully investigate the dynamics of the budding phenomena under H₂O₂ exposure, compensating for the higher analysis time needed to perform the AFM images with respect to the SEM scanning (i.e., two days versus a few hours, respectively). Moreover, the AFM shows the unique capability to discriminate between the budding phenomena on different regions of the cell, thus highlighting a very peculiar effect of the H₂O₂ treatment that ends up increasing the budding phenomena at the cellular edges and connections. The unique capability of the AFM to measure the real height of the samples with high spatial and vertical resolutions, makes it possible to quantify the local arrangement of the membrane in a mathematical way by using the roughness analysis.

The morphological analysis of isolated vesicles is crucial to determine the quality of the isolation procedure and the presence of vesicles in the isolated fractions (Chuo et al., 2018; Hartjes et al., 2019). In this context, TEM analysis represents a gold standard method used almost universally by scientists working in the field of extracellular vesicles (Cizmar and Yuana, 2017; Malenica et al., 2021; Noble et al., 2020). However, multistep preparations required for electron microscopy can easily alter the morphology of EVs, often

providing unreliable information. To overcome this problem, several new approaches for morphological analysis of vesicles have been proposed, including AFM microscopy (Sorkin et al., 2018; Skliar and Chernyshev, 2019; Bairamukov et al., 2020; Vorselen et al., 2020). An in-depth comparative study between the two methods is necessary to define the quality of the isolation procedures and evaluate the adequate morphological characterization of the new proposed technique with respect to the one considered as a golden standard. According to our analysis, the general qualitative behavior and appearance of the distributions of the fractions isolated from control and H₂O₂-treated cells is consistent among the two techniques, thus indicating a high degree of comparability between the two methods. However, when comparing the mean values obtained for the two distributions, the values measured by AFM are significantly higher than those obtained by TEM. This result can be attributed to two major contributions: 1) the layer of water contaminants on the surface of the specimen, due to the environmental humidity, that is always present when performing AFM measurements in air; and 2) the tip convolution, an always emerging effect when performing AFM images on small structures. Indeed, the borders of small structures are difficult to measure with high precision and, therefore, a slight overestimation of the later size (in an order of magnitude of the tip radius) is induced. In our experimental configuration, this effect can be estimated approximately as 15–20 nm. However, the values distribution is perfectly compatible among AFM and TEM data and, since the number of particles that can be counted over the same acquisition

time is higher in the AFM analysis, it allows to obtain more meaningful and statistically consistent results. After all these considerations, we can conclude that the AFM can represent a valid alternative to the TEM characterization when dealing with MVs, since it is capable of delivering a higher amount of data that, however, need to be discussed in terms of absolute values. This latter consideration can be further solved by performing measurements by varying the tip radius (i.e., by performing the measurements with different tip's radius and geometry). Indeed, this technique tends to slightly overestimate the real size of EVs but has the undoubtable advantage to avoid the exposure of the sample to an extreme vacuum environment. Moreover, this overestimation is consistent among the samples considered in this work and does not impair the relative differences observed in terms of distributions of the diameters of the vesicles: i.e., with a single, well-defined peak in the control EVs while a more spread distribution in the EVs derived from H₂O₂ treated cells.

Finally, we demonstrate that the budding of vesicles in control cells is quite homogeneous all over the cellular membrane, while the exposure to H₂O₂ had two major consequences on the budding patterns: 1) the average size of the vesicles increases both in the nuclear and in the peripheral regions, and 2) a sensible increase in the number of vesicles produced only in the peripheral area. These considerations further confirm the unique capabilities of the AFM to give local information all over the cellular membrane, from a single AFM image it is possible not only to quantify the budding of membranes on the entire cell, but also to focus on different regions, thus highlighting the localized response of the budding phenomena to the H₂O₂ treatment. Through the roughness analysis, we demonstrate that it is possible to quantify the local distribution of the heights through a post-processing approach that can also be automatized and gives results in perfect agreement with the ones obtained by the manual counting of the vesicles on the cellular membrane.

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Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

Conceptualization, CS, ST, SD, and LD; methodology and experiments, CS and ST; AFM measurements, ST and SD; electron microscopy, ST and FM; cell culture and EVs isolation, CS; data curation, CS and ST; writing-original draft preparation, CS, ST, and SD; writing-review and editing, MR and LD; supervision, LD. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Article

Wild-Type and SOD1-G93A SH-SY5Y under Oxidative Stress: EVs Characterization and Topographical Distribution of Budding Vesicles

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Abstract: Extracellular vesicles (EVs) are important mediators of intercellular communication in several physiopathological conditions. Oxidative stress alters EVs release and cargo composition depending on the cell type and stimulus. Recently, most of the EVs studies have focused on the characterization of their cargo, rather than on the morphological features (i.e., size distribution, shape, and localization on the cell surface). Due to their high heterogeneity, to fully characterize EVs both the functional and morphological characterization are required. Atomic force microscopy (AFM), introduced for cell morphological studies at the nanoscale, represents a promising method to characterize in detail EVs morphology, dynamics along the cell surface, and its variations reflecting the cell physiological status. In the present study, untreated or H₂O₂-treated wild-type and SOD1-G93A SH-SY5Y cells have been compared performing a transmission electron microscopy (TEM) and AFM morpho-quantitative analysis of budding and released vesicles. Intriguingly, our analysis revealed a differential EVs profiling, with an opposite behavior and implying different cell areas between WT and SOD1-G93A cells, on both physiological conditions and after H₂O₂ exposure. Our results empower the relationship between the morphological features and functional role, further proving the efficacy of EM/AFM in giving an overview of the cell physiology related to EVs trafficking.

Keywords: extracellular vesicles; oxidative stress; atomic force microscopy; transmission electron microscopy; surface budding; topographical mapping



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1. Introduction

Extracellular vesicles (EVs) are a heterogeneous group of membranous nano- and micro-particles released by several cell types, diffusing in body fluids and carrying several molecular cargos (i.e., proteins, lipids, and nucleic acids) to other cell targets [1]. At the beginning, it was believed that the main EVs function was removing unwanted material, but nowadays EVs are widely recognized as active players in intercellular communication in both physiological and pathological conditions [2]. Due to their high degree of heterogeneity, EVs are difficult to classify in distinct and uniform categories and the most studied are the following two main groups: exosomes, which are also recognized as small vesicles (40–160 nm in size) originating from the endocytic pathway, and microvesicles (MVs), or large vesicles (150 nm–1 µm in size), which are directly released from the plasma membrane budding. To study EVs, according to the “Minimal Information for Studies of

EVs" drawn up by the International Society of Extracellular Vesicles (ISEV) [3], several steps are required after their isolation, including morphological, quantitative, and molecular characterization. To the best of our knowledge, the current studies in the field of EVs are mainly focused on the molecular profile of EVs to understand their function, leaving the morphological and quantitative characterization in the background. Nevertheless, since morphological features may be strictly related to the functional role, an in-depth qualitative and quantitative characterization of the EVs morphological features may offer the first overview of their potential role related to the cell physiological status [4]. The EVs release may be affected both in terms of quantity and cargo composition during pathological alterations, including oxidative stress. Generally, reactive oxygen and nitrogen species (ROS and RNS) are produced at low concentrations to modulate several biological processes, such as gene expression, cytoskeleton remodeling, cell proliferation, migration, differentiation, and apoptosis, but also EVs trafficking [5]. Oxidative stress occurs when ROS and RNS are overproduced at the expense of the antioxidant defenses, damaging all the cell macromolecules (i.e., nucleic acids, carbohydrates, proteins, and lipids) and thus causing harmful effects on both cell structure and function [6]. Among the modifications induced on the cellular components by oxidative stress, lipid peroxidation drives changes in phospholipids dynamics with consequent membrane rearrangements and alterations on its fluidity and permeability [7]. Given this evidence, it is not surprising that, under oxidative stress, EVs trafficking results are altered depending on the cell type and stimulus, generally toward an increase in EVs release [8,9]. The overbalance of ROS may affect not only the EVs amount, but also their cargo, which can be enriched in oxidant or antioxidant molecules, spreading detrimental or protective signals to other cell targets [10]. For this reason, the knowledge of EVs molecular cargo is gaining growing importance to track the progression of several oxidative stress-associated diseases, including cancer, neurodegenerative, and inflammatory disorders [11]. However, next to the morphological characterization of released EVs that may change their size and shape under different physiopathological conditions, a still neglected aspect of EVs trafficking studies is the definition of the morphological changes of the cell surface dynamics occurring after a specific stimulus. High-resolution atomic force microscopy (AFM), recently emerging for the studies of EVs at the nanoscale, enables the performance of a multiparametric analysis and helps gain information concerning the morphological, topographical, biophysical, and biomechanical features of biological specimens [12–14]. In a previous piece of work, we demonstrated that atomic force microscopy (AFM) can perform a high-resolution analysis of both isolated EVs and whole cells, defining the plasma membrane budding profile that may be differentially altered on specific regions of the cell after H₂O₂ treatment [4]. Furthermore, AFM allows us to obtain not only the topographical distribution of budding vesicles, giving localized information that can be useful to define the cell physiological status, but also to quantify the budding entity exploiting a morphometric parameter, namely, the roughness. Exploiting a multiparametric approach availing two different microscopy techniques, such as the electron microscopy (EM) and AFM, it is possible to obtain multiple pieces of information on both isolated EVs fractions and vesicle budding directly from the cell plasma membrane and to define EVs trafficking dynamics under different physiological conditions. While in our previous work we investigated the behavior of wild-type (WT) SH-SY5Y cells [4], in the current study, we extended our analysis combining TEM and AFM techniques to perform a multiparametric characterization by comparing WT and transfected human SOD1-G93A SH-SY5Y cells, which are widely used to study oxidative stress and neurodegenerative disorders [15,16]. The main aim of this combined approach was to trace a profile and detect the changes of either the released EVs and the vesicles directly budding from the cell surface of both the WT and mutated cells, either untreated or treated with H₂O₂. The G93A single substitution in the Cu-Zn Superoxide Dismutase (SOD1), which is associated with neurodegenerative disorders and oxidative stress, causes a conformational disorder in the enzyme structure, with a tendency to form oligomeric structures that are responsible of a toxic gain of function related to protein misfolding [17]. Notwithstanding, the exact role of its toxicity is not fully

understood, and the influence of the mutation on EVs trafficking has not been yet explored. Intriguingly, our analysis highlighted a totally opposite behavior from the two cell types, both in basal conditions and after inducing oxidative stress, suggesting that different cells may display distinct responses in physiological conditions and under exposure to the same stimulus. Although the biological significance of these changes is still yet to be clarified, our results further confirm the validity of using a multiple morphometric approach to obtain important details about EVs trafficking in relationship with the cell physiological state, valorizing the morphological analysis next to the functional one.

2. Materials and Methods

2.1. Cell Culture and Treatments

Human neuroblastoma SH-SY5Y cells were provided by Carri's group [18]. Cells were cultured in 4.5 g/L glucose DMEM (Sigma-Aldrich, St. Louis, MO, USA) with the following supplements: 10% heat-inactivated Fetal Bovine Serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Corning, Manassas, VA, USA), 100 UI/mL penicillin/streptomycin (Corning, Manassas, VA, USA), and 10,000 U/mL of amphotericin B (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained at 37 °C in a humidified atmosphere (5% CO₂). Cells were maintained in 75 cm² flasks at an 80% confluence by passage every two days. H₂O₂ (Carlo Erba, Milan, Italy) at a final concentration of 100 µM was used to induce oxidative stress. All the treatments were performed by 1 h exposure to H₂O₂, with a subsequent recovery of 24 h in EVs-depleted medium. Then, cells were collected to perform all the analyses.

2.2. MTT Assay

MTT assay was performed following the protocol presented in [19]. After treatments, WT and SOD1-G93A SH-SY5Y cells were incubated for 3 h at 37 °C and 5% CO₂ with the MTT working solution (1 mg/mL) diluted in DMEM without supplements. After incubation, cells were rinsed with phosphate-buffered saline (PBS) and the formazan crystals were dissolved with dimethyl sulfoxide (DMSO) (Carlo Erba, Milan, Italy). The optical density (OD) was detected at the Multiskan™ FC Microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 570 nm wavelength.

2.3. EVs Isolation by Ultrafiltration Combined with Size-Exclusion Chromatography (UF/SEC)

For UF/SEC, 20 mL of medium was collected in Amicon Ultra-15 Centrifugal Filter Units (MWCO = 10 kDa; Merck Millipore, Billerica, MA, USA) and concentrated up to 500 µL by sequential centrifugation at 4000× g. The obtained concentrate medium was collected, diluted with PBS if necessary, and fractionated by SEC, as described by Böing et al. [20]. Briefly, up to 1 mL concentrated medium was placed in a 10 mL Sepharose CL-2B column (GE healthcare, Little Chalfont, UK), using PBS as eluent. The EVs enriched fractions were then collected and further concentrated to a volume of 100 µL on an Amicon Ultra-4 Centrifugal Filter Unit (MWCO = 10 kDa; Merck Millipore) by several steps of centrifugation at 4000× g. The newly concentrated EVs fractions were used for further analysis.

2.4. Transmission Electron Microscopy

Sample preparation for transmission electron microscopy (TEM) analysis consisted in fixation of the cells with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer pH 7.4 (one hour at ice temperature) and eventually post-fixed with 1% OsO₄ in the same buffer (two hours at ice temperature). Cell fixation was followed by dehydration with ethanol (25%, 50%, 70%, 90%, and 100%). Finally, cells were embedded in Spurr resin. TEM specimens were 60 nm thick sections. TEM setup was a Zeiss Auriga (Zeiss, Oberkochen, Germany) equipped with a STEM module, operating at 20 kV. Isolated EVs from all experimental groups were fixed with 0.1% paraformaldehyde (PFA) in DPBS (30 min at

room temperature), stained with 2% uranyl acetate (7 min at room temperature), loaded on 200 mesh grids, and finally analyzed through TEM operating at 20 kV.

2.5. Western Blot Analysis

To obtain the proteins, cells were lysed in ice for 1 h in RIPA buffer (NaCl 150 mM, Tris-HCl 50 mM pH 8, MgCl₂ 2 mM, SDS 0.1%, Deoxycholic Acid 0.5%, and NP40 1%) completed with phenyl-methyl sulfonyl fluoride (PMSF) and protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). After incubation, cell lysates were then sonicated and pelleted by centrifugation for 10 min at 13,000× *g* at 4 °C. Supernatants were collected in new tubes, and the protein dosage was performed using BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). A total of 20 µg of proteins of EVs and cells were used for Western blotting analysis. Proteins were separated by SDS-PAGE (10–12% polyacrylamide, SureCast™ Acrylamide Solution, Thermo Fisher Scientific, Waltham, MA, USA) and transferred to nitrocellulose membranes. Membrane blocking was performed for 1 h at room temperature in 3% bovine serum albumin (BSA) diluted in Tris-buffered saline containing 0.1% Tween 20 (TBST). The membranes were incubated overnight at 4 °C with CD63 (1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX, USA), Annexin1 (1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX, USA), and Calnexin (1:1000 dilution, Abcam, Milan, Italy) primary antibodies diluted in 3% BSA. After several washes in TBST, membranes were incubated with a goat anti-mouse IgG (dilution 1:5000; Bethyl Laboratories, Montgomery, TX, USA) secondary antibody, diluted in TBST in 3% BSA for 1 h at room temperature. The detection of the immunoreactive bands was performed using commercial enhanced chemiluminescence reagent (ECL) (WesternBright™ Quantum, Advansta Inc., San Jose, CA, USA). Finally, the density of the detected bands was measured by densitometric analysis (ImageLab Software, version 6.1).

2.6. Atomic Force Microscopy Preparation Procedure, Imaging, and Analysis

In order to perform atomic force microscopy (AFM) analysis, untreated and H₂O₂-treated by human neuroblastoma SH-SY5Y cells were previously seeded in 6-well multiwells with coverslips at a concentration of 8×10^4 cells/cm², and incubated overnight in a 5% CO₂ humidified atmosphere, at 37 °C. After washing cells twice in DPBS and fixed in 1% glutaraldehyde in 0.1 mol/L cacodylate buffer pH 7.4 (30 min at ice temperature). Then, two additional and subsequent washing steps in DPBS were performed, followed by two final washing steps in bi-distilled water. As for the preparation of the isolated microvesicles-enriched fractions, they were fixed with 0.1% paraformaldehyde (PFA) in DPBS (30 min at room temperature) and then diluted in water [21]. AFM characterization was performed by acquiring morphological images of the samples in contact mode using a Multimode AFM setup (Bruker Inc., Billerica, MA, USA) that was equipped with standard triangular AFM cantilevers (DNP Bruker Inc., nominal spring constant 0.06 N/m and nominal tip radius 10 nm). The static applied load during the scanning was less than 1 nN in order to minimize the possibility of sample damaging, tip wear, and contamination. In order to obtain comparable images of the different samples, the scan area was first 60 µm × 60 µm when imaging cells, and then subsequent scans on areas of 20 µm × 20 µm were performed to magnify regions of interest. Representative 3D reconstructions and mapping of the cell areas (3 × 3 µm²) were performed with an image analysis software (Gwyddion, version 2.62). Quantification of cell surface roughness (Rq) was performed using ImageJ Software. For statistical purposes, on the mapped regions (nuclear and perinuclear to cellular edges), squared 3 × 3 µm² areas were randomly selected, and five measurements were taken from each sample.

2.7. Statistical Analysis

Results are expressed as means $\pm$ SD. Multiple comparisons were performed by two-way ANOVA. Comparisons between two groups were performed using a student's *t*-test. Differences between groups were considered statistically significant for $p < 0.05$. Comparisons between two distributions were analyzed using a Kolmogorov–Smirnov test (GraphPad Prism 9 software, GraphPad Software, San Diego, CA, USA).

3. Results

3.1. TEM Analysis Reveals That Oxidative Stress Differently Affects EVs Secretion of WT and SOD1-G93A SH-SY5Y Cells

To induce mild oxidative stress conditions, both WT and SOD1-G93A SH-SY5Y cells were treated with 100 μ M of H₂O₂ for 1 h. The lower concentration was selected to induce a stimulus sufficient to induce damage without killing cells and, at the same time, allowing EVs release (Figure S1). To check if EVs release effectively occurred, TEM was performed to analyze whole cells and EVs isolated fractions (Figures 1 and 2). The whole cell analysis revealed the presence of multivesicular bodies and plasma membrane budding on both WT and SOD1-G93A cells (Figure 1). Intriguingly, while in WT cells the budding seems clearly enhanced after H₂O₂ treatment (Figure 1A) with respect to the untreated counterpart, SOD1-G93A cells display a prominent budding even in physiological conditions (Figure 1B). These results represent the first evidence of a differential EVs release between the WT and SOD1-G93A cells, which is further confirmed by the isolated EVs analysis. After the isolation by ultrafiltration combined with size-exclusion chromatography (UF/SEC), the TEM analysis was extended to EVs isolated fractions from all the experimental groups (Figure 2). TEM micrographs of EVs obtained from both WT and SOD1-G93A cells revealed the presence of vesicles showing the EVs typical round-shaped morphology (Figure 2A). Moreover, the size distributions of these EVs indicated that, while EVs derived from untreated WT SH-SY5Y cells display a narrow range (20–200 nm) with a peak of 40 nm, after treatment, there is a shift toward bigger sizes (50–300 nm, with a peak of 100 nm), thus determining a significant increase in the average diameter with respect to the untreated (Figure 2B). On the contrary, EVs isolated from untreated SOD1-G93A SH-SY5Y already display a wide size distribution (from 40 to 280 nm, with 150 nm peak), while after H₂O₂ treatment, there is a sharp reduction in the size range (from 40 to 160 nm, with 80 nm peak) and consequently, of the average diameter (Figure 2B). Finally, a Western blot analysis of EVs specific markers, i.e., Alix and CD63, confirmed the presence of EVs in the isolated fractions of all the experimental groups, which is further supported by the expression of the endoplasmic reticulum integral protein Calnexin only in cell lysates but not in EVs fractions (Figure 2C). Intriguingly, these results suggest that, while in WT cells the H₂O₂ treatment induces a massive production of EVs, especially larger EVs, SOD1-G93A cells display a prominent EVs release starting from basal conditions without any treatment. The differential profile of released EVs with no treatment suggests a different behavior between WT and mutated cells not only in response to a given stimulus, but also in basal conditions.

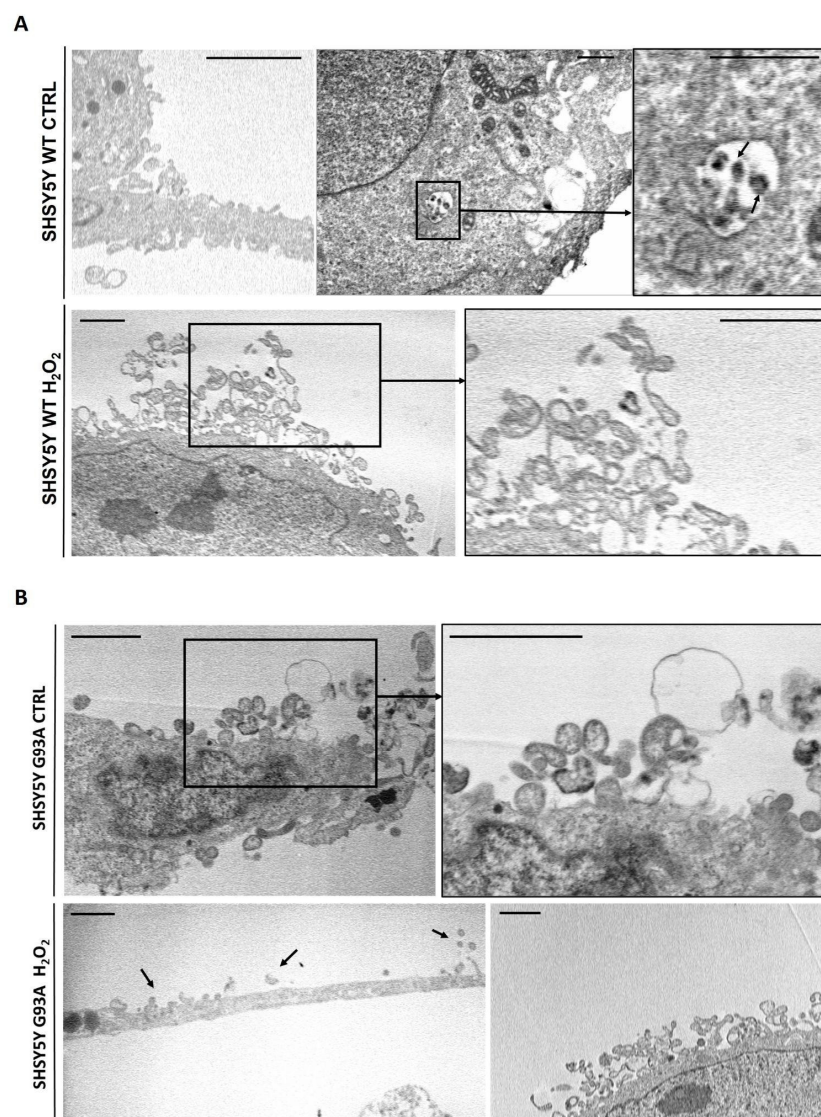


Figure 1. TEM micrographs of (A) WT SH-SY5Y cells untreated (control, **upper** panel) or treated with 100 μ M of H_2O_2 (B) SOD1-G93A SH-SY5Y cells untreated (**upper** panel) or treated with 100 μ M of H_2O_2 . Scale bars = 2 μ m. Arrows indicate the details of budding vesicles.

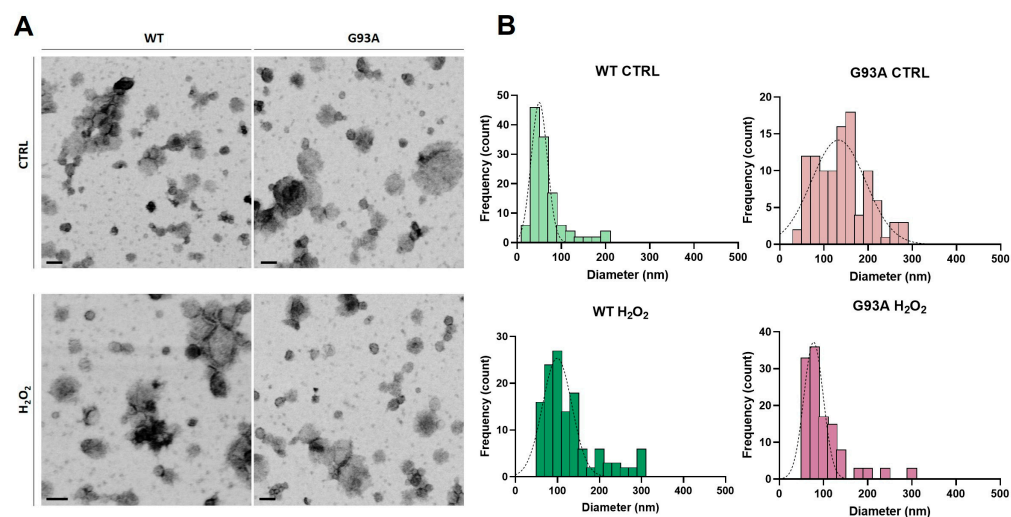


Figure 2. Cont.

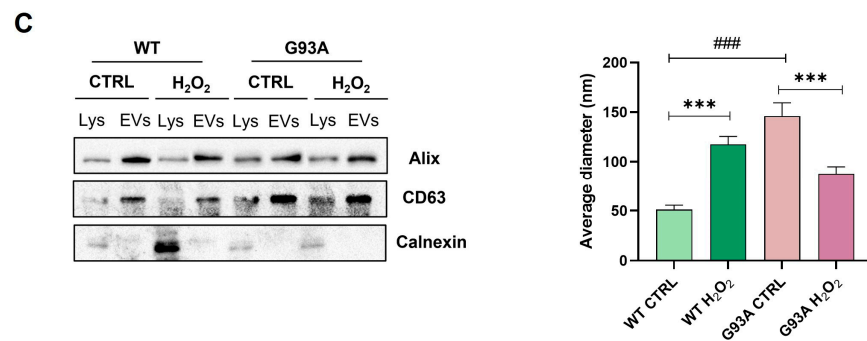


Figure 2. Morphological and quantitative characterization of extracellular vesicles released from untreated and H₂O₂-treated WT and SOD1-G93A SH-SY5Y cells. (A) TEM analysis of EVs released from SH-SY5Y WT and SOD1-G93A cells, untreated (control) or treated with H₂O₂ 100 μ M. Scale bars = 100 nm. (B) Size distribution and average diameter of the EVs from all experimental groups, Ntot = 59, 47, 43, and 47. Values represent the mean $\pm$ SD; statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparisons test. *** $p < 0.001$, when compared with the control group; ### $p < 0.001$, when compared between WT and G93A groups. Lys = cell lysates. (C) Alix, CD63, and Calnexin protein expression from SH-SY5Y WT and SOD1-G93A cells, untreated (control) or treated with H₂O₂ 100 μ M, performed by Western blot.

3.2. AFM Topographical Analysis Shows a Differential Profiling of Budding Vesicles along the Surface of WT and SOD1-G93A SH-SY5Y Cells under Oxidative Stress

In a previous work, we validated the use of AFM to analyze the topographical distribution of vesicles directly budding from the plasma membrane, demonstrating that AFM allows us to acquire more consistent data and appreciate details that can be mislaid due to the harsh preparation method required for the transmission and scanning electron microscopy (TEM and SEM) [4]. After performing an AFM topography of all the experimental groups, it emerged that both WT and SOD1-G93A SH-SY5Y cells displayed a massive budding along the plasma membrane (Figure 3). While in WT cells vesicle budding appears predominantly on the cell bodies (Figure 3A), SOD1-G93A cells display a very irregular surface with a prominent blebbing also along the protrusions (Figure 3B). Intriguingly, the presence of vesicles released by the SOD1-G93A protrusions has also been detected by TEM analysis (Figure 1B), thus indicating a certain degree of comparability between the two techniques despite the different resolutions. After treatment with H₂O₂, both WT and SOD1-G93A cells apparently show an increased budding on the cell surface (Figure 3A,B), but again, with differences on the size distributions. In basal conditions (untreated group), buds from WT cells display a size distribution range between 200 and 500 nm, which is in agreement with the large vesicles dimensions, with a shift toward a broader distribution from 200 to 600 nm and bigger sizes after H₂O₂ treatment (Figure 3C). Indeed, the median diameter of WT budding vesicles increases from 295.2 ± 3.4 nm for the untreated group to 308 ± 3.8 nm for the H₂O₂-treated group (Figure 3E). Interestingly, this tendency is reversed when dealing with SOD1-G93A budding vesicles, showing a size distribution between 200 and 400 nm that remains approximately unaltered after H₂O₂ treatment (Figure 3D). However, when comparing the median diameters, H₂O₂-treated SOD1-G93A cells produce mainly smaller vesicles (262.5 ± 3.8 nm), not only with respect to the untreated group (287.5 ± 7.7 nm), but also to the H₂O₂-treated WT counterpart (Figure 3E). Although AFM allows us to study the topography of the cell surface, thus mainly representing the large vesicles enriched fractions (i.e., vesicles directly budding from the plasma membrane) rather than the entire EVs population released by the cells, it displays a good agreement with the TEM analysis, thus corroborating its validity to study variations in EVs trafficking in response to a given stimulus. Indeed, AFM analysis further confirmed the opposite behavior and the differential EVs profile, in terms of size as well as the distribution along the cell surface, between WT and SOD1-G93A SH-SY5Y cells on both physiological (untreated) and altered (after H₂O₂ exposure) conditions.

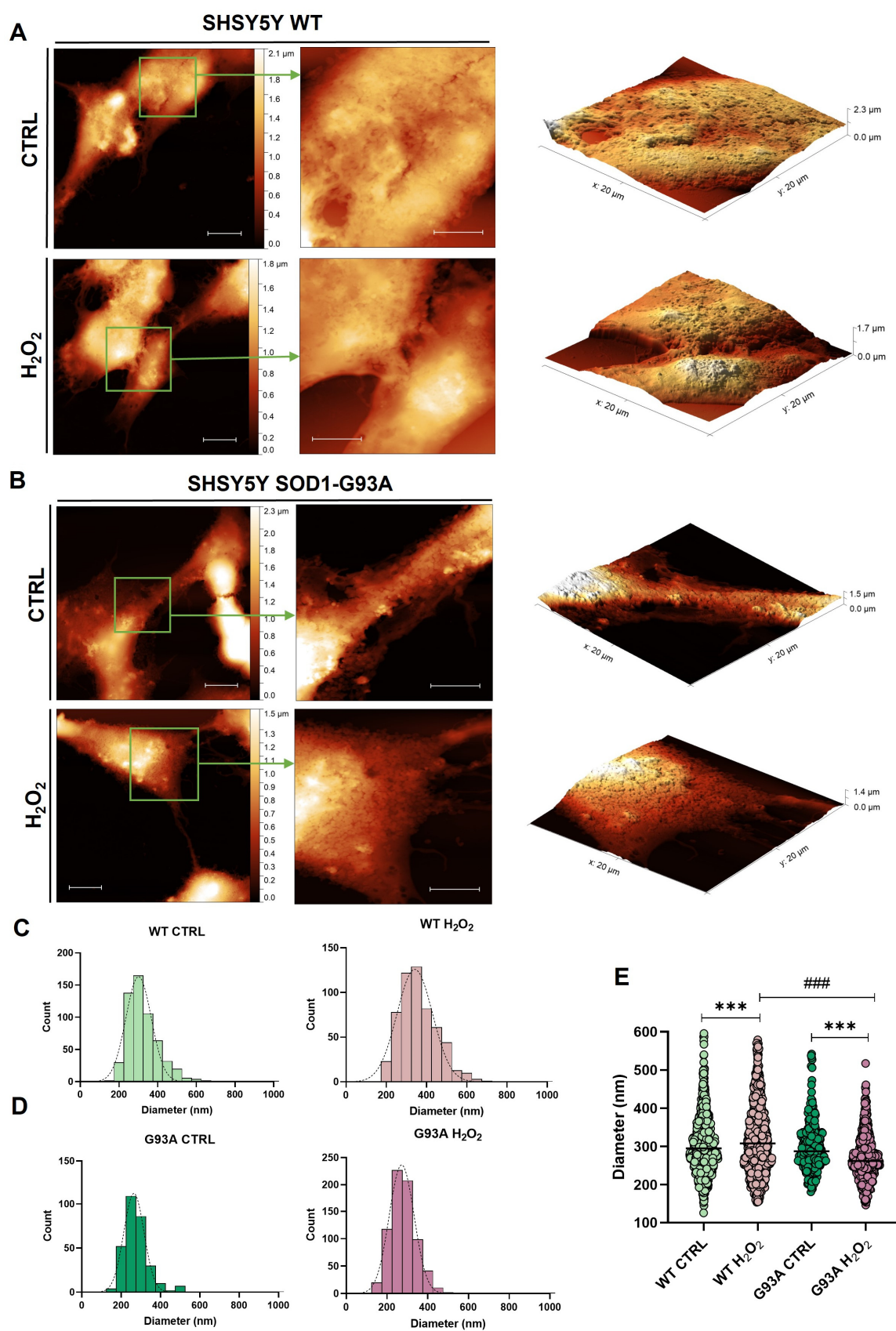


Figure 3. AFM topography, 3D reconstructions, and relative size distributions of SH-SY5Y cells. (A) SH-SY5Y WT untreated (CTRL) and treated with H₂O₂ 100 μ M; (B) SH-SY5Y SOD1-G93A untreated (CTRL) and treated with H₂O₂ 100 μ M. Scale bars = 10 μ m for the whole cells and 4 μ m for

the magnifications. (C) Size distributions of budding vesicles from WT SH-SY5Y cells, untreated (**left** panel, $N_{\text{tot}} = 569$) or treated with H_2O_2 (**right** panel, $N_{\text{tot}} = 566$). (D) Size distributions of budding vesicles from SOD1-G93A SH-SY5Y cells, untreated (**left** panel, $N_{\text{tot}} = 258$) or treated with H_2O_2 (**right** panel, $N_{\text{tot}} = 470$). (E) Direct comparison of the median diameters of budding vesicles from WT (295.2 ± 3.4 nm for CTRL and 308 ± 3.8 nm for H_2O_2) and SOD1-G93A (287.5 ± 7.7 nm for CTRL and 262.5 ± 3.8 nm for H_2O_2) SH-SY5Y cells. Values represent the mean $\pm$ SD; statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparisons test. *** $p < 0.001$, when compared with the control group; ### $p < 0.001$, when compared between WT and G93A groups.

3.3. AFM Topographical Mapping of Central and Distal Regions of WT and SOD1-G93A SH-SY5Y Cells: Detecting the Details of a Differential Budding Profile

When cells are exposed to particular stimuli, such as oxidative stress, localized changes in vesicle budding may occur. This happens because EV release is a finely regulated process in which membrane rearrangements take place in specific regions of the cell in response to environmental conditions [11]. In our previous work, we demonstrated that a zonal morphometric analysis of budding vesicles performed by AFM provided different information than an analysis of the whole cell surface [4]. In the same manner, we selected confined areas ($3 \times 3 \mu\text{m}^2$), specifically on the nuclear regions (nuclei, Nu; blue dotted circles in Figures 4A and 5A) and the peripheral areas (perinuclear-to-cellular edge, Pn/CE; green dotted circles in Figures 4A and 5A) of the cells. Then, we first analyzed the size distribution of budding vesicles in the selected areas for both WT and SOD1-G93A SH-SY5Y cells. For the untreated group of WT cells, the size distributions range over 200–600 nm, with a peak of ~ 350 nm on both the nuclear and peripheral regions (Figure 4C,E, upper panels). The same diameter range can be observed for H_2O_2 -treated WT cells (Figure 4C,E, lower panels). On the contrary, SOD1-G93A cells display more variability in size distributions according to their conditions, especially under oxidative stress induction. In the untreated group, the size distribution in the nuclear region ranges between 200 and 600 nm, with a ~ 350 nm peak (Figure 5C, upper panel), getting tighter (from 200 to 400 nm) after H_2O_2 treatment (Figure 5C, lower panel). Notwithstanding, the peripheral regions display a distinct behavior, with a size distribution ranging from 150 to 450 nm (with a ~ 300 nm peak) in basal conditions (Figure 5E, upper panel) and becoming broader after the induction of oxidative stress (from 200 to 600 nm, with a ~ 350 nm peak) (Figure 5E, lower panel). Furthermore, while the median diameter values display a significant increase in the peripheral regions of WT budding vesicles (from 297.4 ± 4.8 nm to 334 ± 5.3 nm) after H_2O_2 treatment, there is instead a sharp reduction (from 343.8 ± 6.1 nm to 275.4 ± 4.9 nm) localized in the nuclear regions of SOD1-G93A cells (Figure 6A). Interestingly, this reduction in the median diameter of budding vesicles in the nuclei of H_2O_2 -treated SOD1-G93A cells is also significant with respect to the WT treated counterpart (Figure 6A). These results indicate that, under oxidative stress conditions, while in WT SH-SY5Y cells the vesicle budding is preferentially affected in the distal regions of the cell, in SOD1-G93A cells the variation is primarily charged to the nucleus, further suggesting that mutated cells display a differential response to H_2O_2 exposure in comparison to the WT cells.

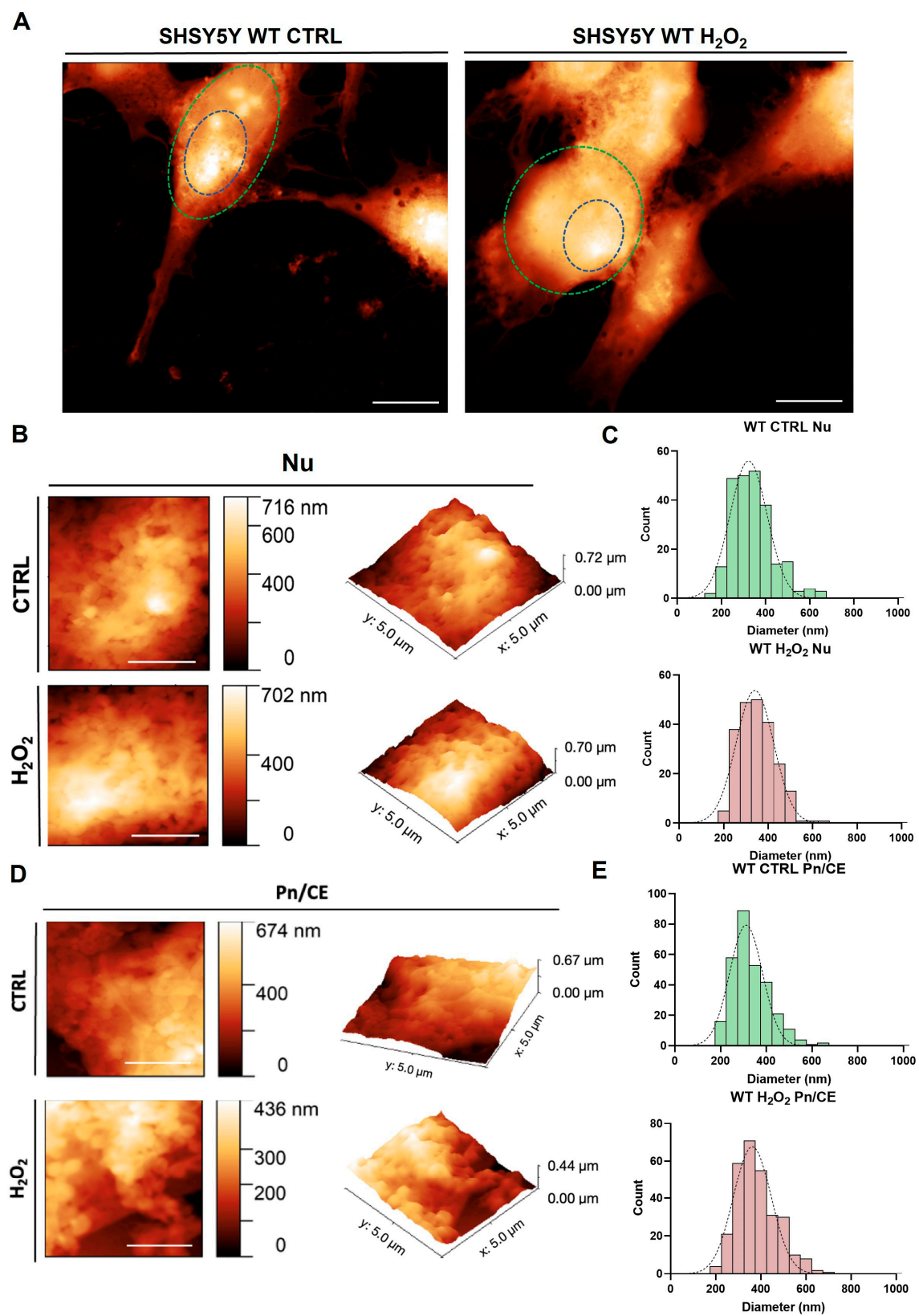


Figure 4. Mapping of the surface of CTRL (A) and H₂O₂ (B) WT SH-SY5Y cells in the nuclear zone (B) and perinuclear-to-cellular edge regions (D), with relative 3D reconstructions and size distributions (C,E), N_{tot} = 243, 273, 298, and 292. Scale bars = 10 μ m for whole cells micrographs; 1 μ m for magnifications of the $3 \times 3 \mu\text{m}^2$ mapped regions. Nu = nuclear zone (blue circles); Pn/CE = perinuclear-to-cellular-edge regions (green circles).

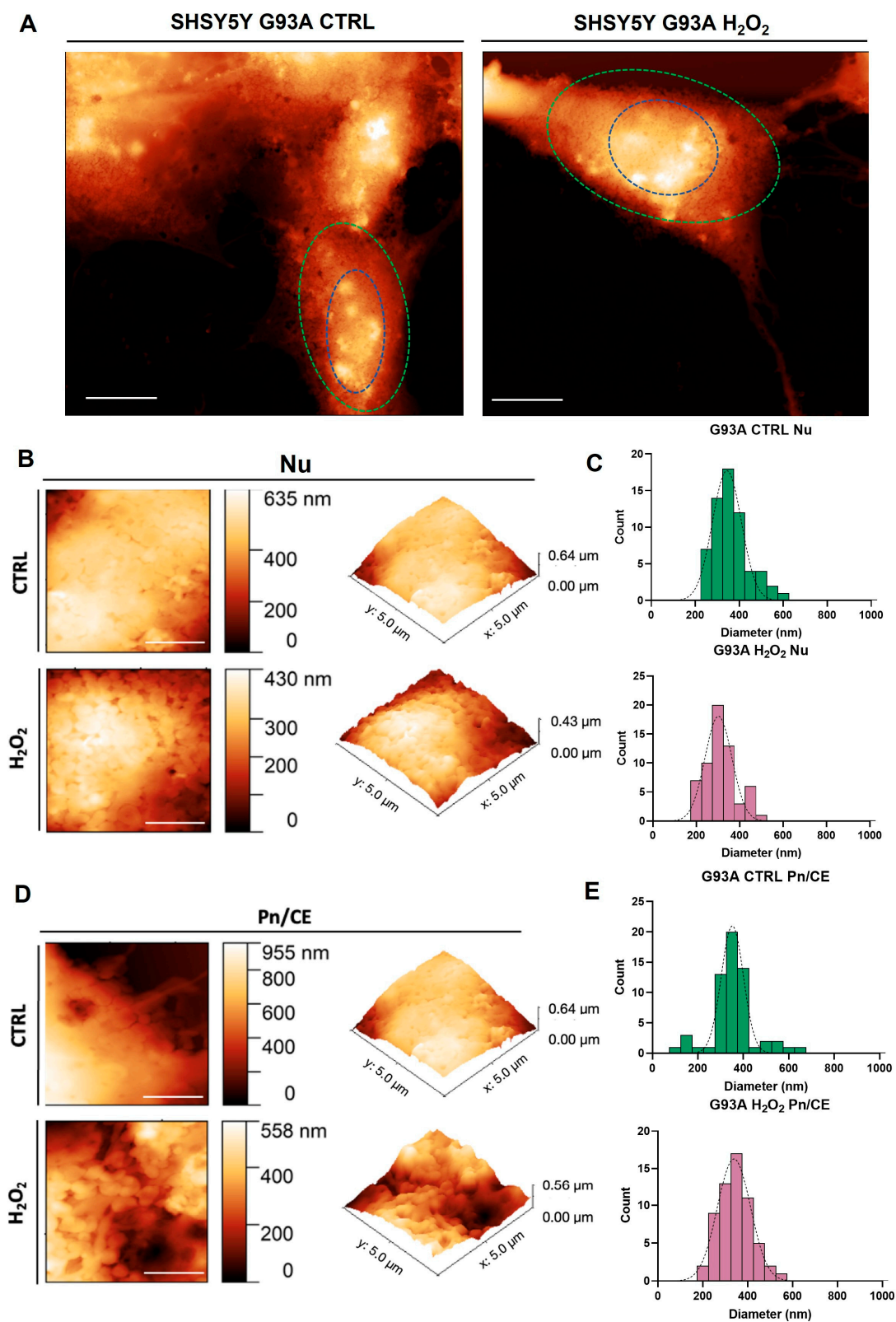


Figure 5. Mapping of the surface of CTRL and H₂O₂ SOD1-G93A SH-SY5Y cells (**A**) in the nuclear zone (**B**) and perinuclear-to-cellular edge regions (**D**), with relative 3D reconstructions and size distributions (**C,E**), N_{tot} = 62, 60, 60, and 60. Scale bars = 10 μm for whole cells micrographs; 1 μm for magnifications of the mapped regions. Nu = nuclear zone (blue circles); Pn/CE = perinuclear-to-cellular-edge regions (green circles).

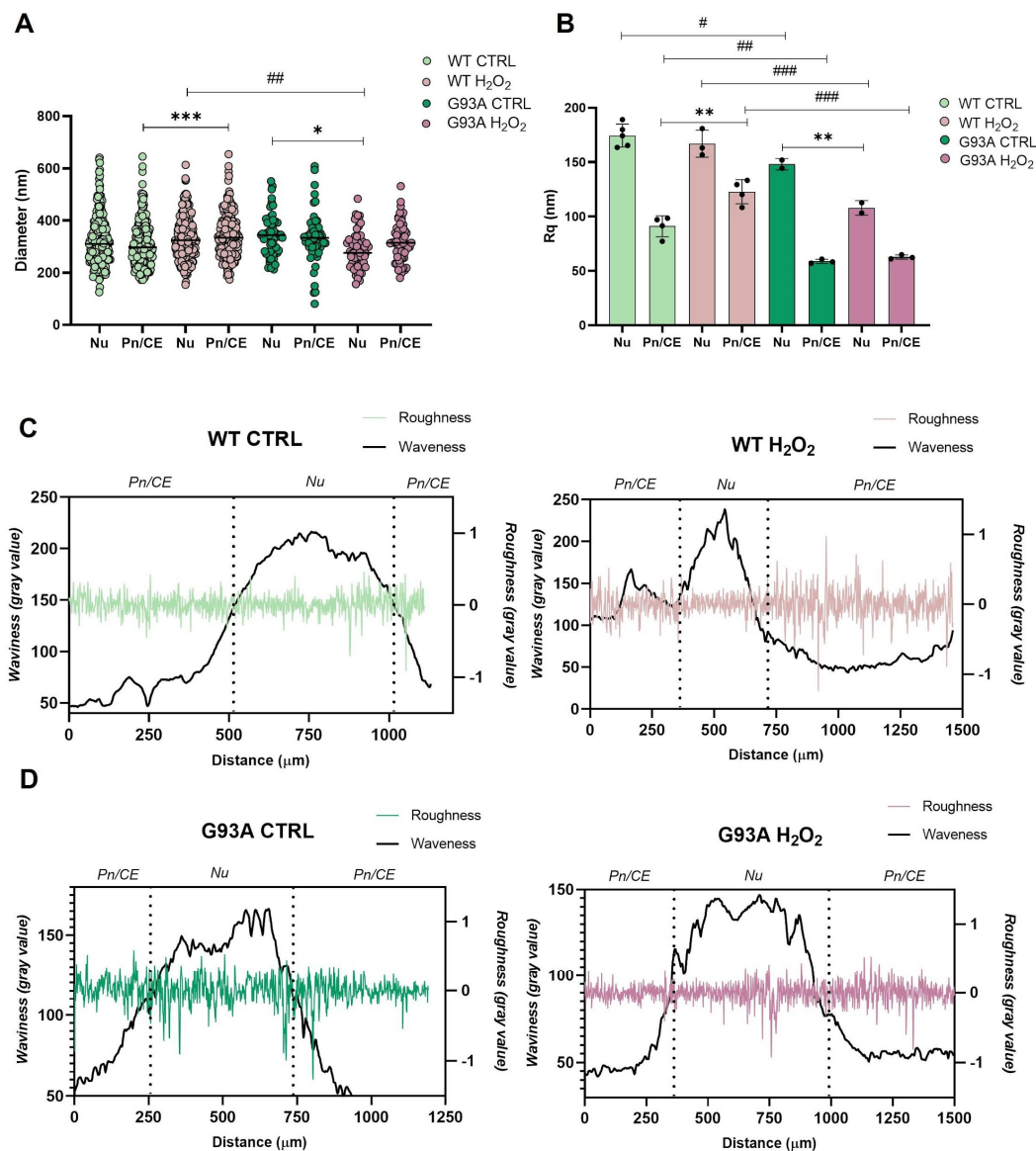


Figure 6. (A) Direct comparison of the median diameters of budding vesicles of mapped regions ($3 \times 3 \mu\text{m}^2$) from CTRL (Nu = 310.0 nm; Pn/CE = 297.4) and H₂O₂-treated (Nu = 323.6 nm; Pn/CE = 334.0 nm) WT and CTRL (Nu = 343.8 nm; Pn/CE = 333.2 nm) and H₂O₂-treated (Nu = 275.4 nm; Pn/CE = 314.5 nm) SOD1-G93A SH-SY5Y performed by AFM. (B) Rq measurements of mapped regions of all the experimental groups. Values represent the mean $\pm$ SD; statistical analysis was carried out using one-way ANOVA with Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, when compared with the control group; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$, when compared between WT and G93A groups. (C,D) Roughness and waviness (gray values) measurements performed on all the experimental groups (upper panels for WT cells, lower panels for SOD1-G93A cells). Vertical dotted lines separate nuclear from peripheral regions. Nu = nuclear zone; Pn/CE = perinuclear-to-cellular edge regions.

3.4. Quantifying the WT and SOD1-G93A SH-SY5Y Cells Differential Budding Profile: Analysis of the Surface Roughness

High-resolution AFM analysis allowed the detection of localized variations in the budding profile of different cell lines, such as WT and mutated in SOD1-G93A SH-SY5Y, after a given stimulus (i.e., H₂O₂). However, the advantage of performing an AFM analysis consists not only in determining the topography of the sample at the nanoscale, but also in obtaining multiple pieces of information about its biophysical properties. We extended our

analysis to the previously mapped regions (Nu and Pn/CE) with a quantitative measurement of the budding entity by using the roughness (Rq), which was strictly correlated with the morphological variations of the plasma membrane, as demonstrated before in our previous work [4,22]. We depicted multiple confined areas ($3 \times 3 \mu\text{m}^2$) in the nuclear and in the peripheral regions to obtain a representative average value of the roughness. The obtained values are represented in Figure 6B. Interestingly, the average Rq values follow the same trend as for the variations previously found in the size distributions. In WT cells, there is a significant increase in the average Rq value in the Pn/CE between untreated (91.09 nm) and H_2O_2 -treated (122.8 nm) (Figure 6B). Accordingly, in the roughness/waviness charts, which are represented in the cell profiles on the lateral scale (black lines) and the local roughness (colored frequencies), WT untreated cells display a similar roughness profile (light green frequencies, Figure 6C, left panel) with respect to the H_2O_2 -treated (light pink frequencies, Figure 6C, right panel) in the nuclear zones. However, when looking at the perinuclear-to-cellular edge regions, there is a noticeable increase in the roughness gray values in WT H_2O_2 -treated cells with respect to the untreated (Figure 6C). On the contrary, SOD1-G93A cells again show an opposite behavior, with a significant decrease in the average Rq values of the H_2O_2 -treated nuclei (148.2 nm vs. 108 nm) in comparison to the untreated ones (Figure 6B). Correspondingly, the roughness/waviness profiles reveal a flattening of the roughness gray value in H_2O_2 -treated SOD1-G93A cells (dark pink frequencies, Figure 6D, right panel) with respect to the untreated counterpart (dark green frequencies, Figure 6D, left panel). These results suggest that the variations observed in the size distributions find an agreement with the roughness values, thus indicating that the surface budding is affected not only concerning the vesicles size, but also in terms of the budding entity. Furthermore, when directly comparing the two cell lines (WT vs. SOD1-G93A), significant differences emerge in the Rq mean values, and consequently in the budding entity, either in the nuclear regions and the peripheral regions or under physiological and oxidative stress conditions (Figure 6B). This indicates that, in basal conditions, WT and SOD1-G93A cells display a different budding vesicles profile along the plasma membrane surface, which further undergoes opposite variations when exposed to H_2O_2 , concerning different districts of the cell and an opposite trend.

4. Discussion

Extracellular vesicles (EVs) are heterogeneous, membrane-enveloped particles originating from disparate cell types, carrying several active biomolecules toward nearby or distant targets, which are consequently responsible for intercellular communication in distinct physiopathological conditions [1]. The evidence of EVs as the leading mediators of cell-to-cell crosstalk has directed the attention of the scientific community toward a better understanding of EVs role in several cell physiology alterations and disorders, including cancer, inflammatory, neurodegenerative, and metabolic diseases [23–25]. Most of these pathologies are associated with oxidative stress, an intricate physiological alteration emerging when an excess of pro-oxidant stimuli takes down the cell antioxidant defenses, leading to harmful signals and damage of the macromolecules mining the cell structure and integrity. In particular, oxidative stress alters either EVs release or content, generally toward an increase in EVs production and with effects ranging from protective to detrimental depending on the microenvironment (i.e., cell type and stimulus received) [5]. However, most of the studies focused primarily on the characterization of the EVs cargo to unravel their function, disregarding the morphological characteristics and the changes of the cell surface dynamics occurring under oxidative stress that may instead offer the first evidence of an altered physiological status of the cell. Therefore, a better description and definition of EVs structural properties and dynamics on the cell surface may offer precious information about their potential function and should not be underestimated. According to the ISEVs “Minimal Information for Studies of EVs” [3], to have a comprehensive view of EVs features, morphological and quantitative characterization are needed as well as the definition of their molecular cargo. In a previous work, we exploited a combined

morphological characterization approach, performing a SEM/AFM comparative analysis, to validate the AFM as a method to study EVs as well as the EM conventional one [4]. In the present work, we corroborate the unique capability of AFM to detect localized changes in EV dynamics under a given stimulus, providing accurate information about the cell's physiological status. In particular, by performing a morphological characterization by TEM of isolated EVs and AFM for budding vesicles, we highlighted that the SOD1-G93A mutation significantly alters the profile of both released and budding EVs of human neuroblastoma SH-SY5Y cell lines in comparison to the wild-type counterpart, both in physiological and oxidative stress conditions (induced with H₂O₂ treatment). Starting from the TEM analysis of both whole cells and released vesicles, it appears that, while in WT cells the exposure to H₂O₂ induces a massive EV release and a wider size distribution, in SOD1-G93A there is a distinct behavior, with the presence of a prominent budding of the plasma membrane already in basal conditions. Taken together, TEM results indicate not only a different responsiveness of the cells to H₂O₂ exposure, but also different morphological features and EVs profile in basal conditions. Oxidative stress is widely recognized to differently modulate EV trafficking, both in terms of quantity and cargo, depending on the cell type and stimulus given [11]. Despite this, TEM analysis clearly marks how morphological changes are quite evident between different cell types and how they occur starting from physiological conditions. The SOD1-G93A mutation is notably associated with oxidative stress [17,26,27]. However, to the best of our knowledge, the influence of this kind of mutation on EV trafficking has not yet been investigated. Interestingly, the presence of the SOD1-G93A mutation, in the absence of any external stimulus, seems to be sufficient to induce a differential budding in comparison to the wild-type cells, generating at the same time an opposite response to H₂O₂ treatment. EV release and cargo loading are connected and finely regulated processes, in which multiple biogenetic pathways act simultaneously, driving localized changes in lipid rafts and protein components that, in turn, modulate the membrane's curvature and fluidity [11]. Therefore, it is plausible that certain regions of the cells may be involved more than others when exposed to a specific stimulus. To further explore the differences in EV trafficking between WT and SOD1-G93A cells, we performed a high-resolution topographical analysis of budding phenomena by AFM, considering the variations in the blebbing dynamics charged to the whole cells and, specifically, the single contribution of localized regions (nuclear and perinuclear/cellular edge areas). Intriguingly, AFM analysis of budding vesicles on the entire cell surface confirmed the same trend as for the isolated EVs analyzed by TEM, with a significant increase in the median diameters from untreated to H₂O₂-treated WT cells, and a specular decrease from untreated to H₂O₂-treated SOD1-G93A cells. Moreover, in agreement with the TEM analysis, AFM micrographs revealed a high concentration of budding vesicles localized on both the cell bodies and protrusions of SOD1-G93A cells, unlike the wild-type cells in which the blebbing seems more confined in the cell bodies. These results suggest that the alterations on EVs budding profiles, on both physiological and stress conditions, may occur prior to their release in the external environment. Furthermore, it is plausible that the main contribution to the variation observed in the SOD1-G93A-released EV profile is given by the vesicles that are directly budding from the plasma membrane. During the membrane remodeling process occurring prior to EVs release, the molecular machinery involved in EVs biogenesis creates clusters resulting in ordered microdomains [28]. For this reason, to understand which cellular district was mostly affected by changes in the budding phenomena, we mapped the cells considering two main parts, the nuclear region and the one covering from the perinuclear zone to the cellular edges [4]. Intriguingly, WT and SOD1-G93A SH-SY5Y cells showed not only a differential profile of budding vesicles in terms of size, but also in terms of budding entity, implying different districts of the cell. By using the membrane roughness (R_q), a morphometric parameter strictly related to cell surface arrangements, we were able to detect alterations in the plasma membrane disposition, thus quantifying the budding phenomena [4,22]. From AFM analysis on the mapped regions emerged the fact that wild-type cells go through a significant increase in

the budding entity and size distribution, preferentially in the peripheral regions, while the nuclear area remains approximately unaltered. Conversely, SOD1-G93A cells undergo a reduction in both the budding entity and vesicle size, specifically localized on the nuclear part, leaving the distal regions unaffected. Finally, SOD1-G93A cells generally display a reduced budding intensity in comparison to the WT cells in all the selected areas (nuclear and peripheral regions) and in all the experimental conditions (untreated groups and after H₂O₂ exposure). These results further demonstrate the power of AFM to get detailed information about the cell morphology with reference to size, intensity, and position of the budding phenomena, highlighting the differences between wild-type and SOD1-G93A mutated cells. The detected variations are not just reflecting a differential EV profile in terms of morphology, dynamics, and budding entities but may also have an impact on cargo selection, implying different interactions with other targets and consequently distinct functions. Taken together, these results prove the efficacy of AFM related not only to the morphological characterization, confirming its agreement with a conventional method such as TEM, but also to its ability to give precious details concerning the biophysical and mechanical characteristics of the sample, making it possible to discriminate between distinct biological specimens and different environmental conditions. Since recently it has been claimed that there is a relationship between morphology and function, it is pivotal to not underestimate the contribution of an accurate definition of the EVs morphological features. What remains to be elucidated are the mechanisms underlying these variations, and thus what biological pathway may be mostly responsible for the defective EV release in the presence of the SOD1-G93A mutation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/applnano4010004/s1>, Figure S1: MTT assay performed on WT and SOD1-G93A SH-SY5Y treated with H₂O₂.

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SARS-CoV-2 and extracellular vesicles: An intricate interplay in pathogenesis, diagnosis and treatment

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Extracellular vesicles (EVs) are widely recognized as intercellular communication mediators. Among the different biological processes, EVs play a role in viral infections, supporting virus entrance and spread into host cells and immune response evasion. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection became an urgent public health issue with significant morbidity and mortality worldwide, being responsible for the current COVID-19 pandemic. Since EVs are implicated in SARS-CoV-2 infection in a morphological and functional level, they have gained growing interest for a better understanding of SARS-CoV-2 pathogenesis and represent possible diagnostic tools to track the disease progression. Furthermore, thanks to their biocompatibility and efficient immune activation, the use of EVs may also represent a promising strategy for the development of new therapeutic strategies against COVID-19. In this review, we explore the role of EVs in viral infections with a focus on SARS-CoV-2 biology and pathogenesis, considering recent morphometric studies. The common biogenesis aspects and structural similarities between EVs and SARS-CoV-2 will be examined, offering a panoramic of their multifaceted interplay and presenting EVs as a machinery supporting the viral cycle. On the other hand, EVs may be exploited as early diagnostic biomarkers and efficient carriers for drug delivery and vaccination, and ongoing studies will be reviewed to highlight EVs as potential alternative therapeutic strategies against SARS-CoV-2 infection.

KEYWORDS

extracellular vesicles, SARS-CoV-2, COVID-19, EVs-based vaccines, COVID-19 vaccination, drug delivery

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a novel strain of coronavirus emerged first in China in 2019 and etiological agent of the current coronavirus disease 2019 (COVID-19) outbreak (Kulkarni et al., 2022). Due to its ease of spread, SARS-CoV-2 represents a serious threat to global health, also having a strong socio-economic impact. To date, on 27 June 2022, there have been 540,923,532 confirmed cases of COVID-19, including 6,325,785 deaths, reported to WHO (<https://covid19.who.int>). The vaccination campaign helped to counteract severe clinical outcomes, but the ability of SARS-CoV-2 to mutate and evade immune responses still implies the need to find new intervention strategies, requiring in turn a deeper understanding of SARS-CoV-2 biology (Boni et al., 2020). Several morphometric studies are ongoing to elucidate SARS-CoV-2 infection mechanisms, especially egress events and cell-to-cell spreading that are still poorly characterized (Klein et al., 2020; Eymieux et al., 2021b; Kumar et al., 2021; Laue et al., 2021; Mendonça et al., 2021). According to these, extracellular vesicles (EVs) trafficking seems to be a deeply involved pathway in SARS-CoV-2 infection, contributing to viral replication, immune evasion, egression from infected cells, and spread to other cell targets, through the so-called “replicative organelles” (Pleet et al., 2018; Bello-Morales et al., 2020). Several hypotheses further support a role of EVs in SARS-CoV-2 infection, like the existence of “quasi-enveloped viruses” (Feng et al., 2014), the “Trojan exosome” hypothesis (Hildreth, 2017), and the “secretory autophagy” model (Bunz et al., 2022), but none of these has been confirmed as the prevalent and further investigations are needed. EVs are well known as membrane-enveloped, nano- and micro-sized particles secreted by different cell types, distinct in their size, composition and function according to their biogenesis, delivering different cargoes to distant targets and thus affecting intercellular signaling (Kalluri and LeBleu, 2020). Thanks to their intrinsic properties, EVs display an important role in a physiological level, as well as in several pathological conditions, being mediators of host-pathogen interactions. Depending on their cargo, EVs may also functionally support or counteract viral infection. Next to supplying a replication machinery, EVs may also transport and present antigens, modulating the host immune responses against the virus (Babaei et al., 2022). The intricate morpho-functional interplay between SARS-CoV-2 and EVs gained particular interest among the scientific community and defining EVs dynamics and cargo may be pivotal to get not only a better comprehension of the viral cycle, but also to take advantage of it, to find new clues for biomarker tracing, disease treatment, and vaccine development. To date, EVs have been exploited to deliver immunomodulatory cargoes, acting prevalently on the cytokine storm occurring in COVID-19, in combination with antiviral drugs (Romagnoli et al., 2015; Somiya et al., 2017). Some clinical trials are

currently underway to test the efficacy and safety of EVs formulations, but the results are still fragmentary and incomplete (Karn et al., 2021). Despite the use of EVs for therapeutic purposes seems promising, further investigations are needed to find effective strategies with minimal side effects. In this paper, multiple aspects of the SARS-CoV-2 and EVs network are reviewed, from the role of EVs in SARS-CoV-2 viral cycle and COVID-19 pathogenesis, to their potential as diagnostic biomarkers and tools for treatment, drug delivery and vaccine design.

Extracellular vesicles and viral infections

Biogenesis, classification, and function of extracellular vesicles

Historically, EVs have been considered as cellular “junk bags”, assigned to remove unwanted material. To date, EVs have great importance in different biological systems as carriers of biomolecules, such as proteins, lipids, and nucleic acids, playing a crucial role in cell-to-cell communication. Furthermore, EVs have been used as therapeutic drug carriers and as diagnostic tools for several diseases (Dini et al., 2020). EVs are small membrane-enclosed nano- and micro-structures released by different cells and can be found in several human biofluids (i.e., blood, urine, cerebrospinal fluid, saliva, breast milk, bronchoalveolar lavage fluid) (Doyle and Wang, 2019). Due to their heterogeneity, EVs are difficult to categorize. However, according to their biogenesis pathways, EVs are generally distinguished in three main subtypes: exosomes (small EVs, 30–100 nm in size), microvesicles (large EVs, 100–1,000 nm in size), and apoptotic bodies (bigger than 1,000 nm) (Rai et al., 2021). Exosomes, also referred as small EVs, are derived from multivesicular bodies (MVBs) (Yue et al., 2020) and formed by the inward budding of the late endosomal membrane, which encapsulates biomolecules and generates intraluminal vesicles (ILVs) within the MVBs. Then, MVBs fuse with the plasma membrane and release ILVs into the extracellular space in the form of exosomes via exocytosis (Vella et al., 2008). Several peripheral membrane protein complexes, necessary for the formation of ILVs in MVBs and the budding of microvesicles from the plasma membrane, are known as “endosomal sorting complexes required for transport”. In the ESCRT system, there are four complexes known as ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III with associated proteins (Tsg101, ALIX, and VPS4) and, during biogenesis, each complex has its own role. To initiate the pathway, ESCRT-0 forms a protein network on endosomal membranes that captures ubiquitinated cargo proteins and allows their sorting based on the MVBs pathway (Raiborg et al., 2001; Mayers et al., 2011). ESCRT-I binds to ubiquitinated cargo proteins on the endosomes

and activates ESCRT-II, which plays a direct role in cargo sorting at multivesicular endosomes (MVEs). In turn, ESCRT III, with the charged multivesicular body proteins, performs membrane remodeling, including fission, protein dissociation and recycling of the endosomal sorting machinery, which depends on the participation of the Vps4 AAA-ATPase (Obita et al., 2007). Besides these processes, ESCRT-III and its accessory proteins, like Vps4, Vta1, Vsp60 and ALIX, are also involved in virus budding, cytokinesis and nuclear envelope remodeling (Hurley, 2015). Another pathway for exosome biogenesis involves the syndecan/syntenin complex, which requires the activity of phospholipase D 2 (PLD2) and ADP-ribosylation factor 6 (Arf6) (Tricarico et al., 2016). Despite the central role of the ESCRT-dependent pathway for EVs formation, the SMase2/ceramide pathway, independent from the canonical ESCRT pathway, has been also characterized (Kosaka et al., 2010). For EVs transport and release, distinct effectors are implicated, such as RAB GTPases (Rocha et al., 2009). RAB GTPases regulate trafficking in both the endocytic and secretory pathways, by recruiting specific effector proteins onto membrane surfaces to either facilitate cargo collection, organelle movement, or vesicle docking. There is growing evidence linking RAB GTPases to the secretion of exosomes, mainly RAB5 and RAB7 (Hutagalung & Novick, 2011). Further studies have also demonstrated that RAB27A and RAB27B participate in MVEs transport, while RAB11 and RAB35 proteins regulate MVEs fusion with the plasma membrane and secretion directly or indirectly (van Niel et al., 2018). Furthermore, RAB22A has been found to colocalize with budding microvesicles, participating in cargo sorting in hypoxic breast cancer cells (Wang et al., 2014).

Microvesicles (MVs), also referred as large EVs, are instead formed by the direct plasma membrane budding and fission. Several proteins are involved in MVs biogenesis, such as Ca^{2+} dependent aminophospholipid translocases, scramblases, and calpains, driving the rearrangement of membrane phospholipids and the reconstitution of the actin cytoskeleton, and finally determining the plasma membrane curvature and MVs release in the extracellular space (Lynch and Ludlam, 2007). Despite the apparent distinct biogenesis modality, exosomes and microvesicles may share common sorting machinery and intracellular mechanisms, often displaying an overlapping size and similar composition that hamper discriminating different subpopulations. Indeed, there is still a need to uncover the exact dynamics of EVs biogenesis (van Niel et al., 2018). Here, we adhere to the nomenclature guidelines published by the International Society of Extracellular Vesicles (ISEV), that suggests the use of the general term “EVs”, as there is still no definitive distinctive marker of each EVs subpopulation (Théry et al., 2018; Witwer et al., 2021).

Apoptotic bodies, or apoptosomes, are formed only during apoptosis, characterized by the condensation of nuclear chromatin and involving nuclear chromatin condensation followed by membrane blebbing (Kerr et al., 1972). As well as

ESCRT proteins, tetraspanins (CD81, CD82, CD63, CD9), and proteins responsible for cargo transport and membrane fusion (annexins, RABs), EVs may be enriched in proteins involved in many processes including cell adhesion (integrins), antigen presentation (MHC-I, MHC-II), stress regulation (HSP70, HSP90), cell migration (actin, myosin, tubulin) and metabolic enzymes (Lakkaraju and Rodriguez-Boulan, 2008; Colombo et al., 2014). EVs may also contain several types of nucleic acids, such as messenger RNAs (mRNAs), micro-RNAs (miRNAs), transfer RNAs (tRNA) and long non-coding RNAs (lncRNAs) (Valadi et al., 2007; Gusachenko et al., 2013). The ability of EVs to carry cargo, budding, shedding, and being reacquired by neighboring cells, may contribute to the spread of infections, progression of diseases, as well as the modulation of immune-related processes. In particular, the role of EVs in the pathogenesis of infectious diseases suggests that EVs may serve as potential biomarkers to track mild bacterial infection and chronic viral diseases, with the possibility of harnessing EVs to prevent infection or spread (Jones et al., 2018; Pérez et al., 2019).

EVs and viral infections: A multifaceted interplay

Viruses are intracellular pathogens exploiting the cellular compartments to carry out their life cycle. Given the role of EVs in intercellular communication and in pathological disorders, the interplay between viruses and EVs gained a lot of attention, especially for their similarities in structure and biogenesis (Bello-Morales et al., 2020). Indeed, EVs display a multifaceted role in viral infections, contributing in first place to viral replication, dissemination, and immune evasion modulating the host immune system activation. Furthermore, EVs may block (Ipinmoroti and Matthews, 2020) or otherwise trigger the antiviral response, exacerbating the damage and causing several detrimental effects, such as oncogenesis (Zheng et al., 2019), inflammation (Sadri Nahand et al., 2020) or autoimmune responses (Gunasekaran et al., 2020). Several viruses exploit intracellular membrane rearrangements, creating peculiar double-membrane structures known as “replicative organelles”, to complete their replication facilitating new virions formation and budding from the host cell (Wolff et al., 2020). Viral infection seems to have influence on lipid metabolism and processing, driving the formation of protective structures representing the main strategy for a successful viral cycle (Müller et al., 2018). Lipid rafts and myelin and lymphocyte protein (MAL), an integral membrane protein found in cholesterol and glycolipids-enriched membrane, are believed to be implicated in virion uptake, constituting platforms for viral particle assembly (Kulkarni et al., 2022). Besides the formation of replication organelles, viruses must exit the infected cell and disseminate, evading at the same time the host immune system, to ensure their biological success. To

accomplish this, both enveloped and non-enveloped viruses exploit EVs as a strategy to maximize their tropism without being recognized by the host immune cells (Schorey et al., 2015). Indeed, EVs may mediate and enhance viral propagation creating the so-called “collective infectious units”, consisting in multiple virions within a single vesicle or multiple viral genomes in a single virion. Through this dispersal strategy, viruses increase their multiplicity of infection (MOI) (Leeks et al., 2019; Andreu-Moreno and Sanjuán, 2020). Whether these vesicles are EVs containing viral molecules or defective viral particles is still under debate but, since EVs and viruses share similar size, density, and composition, it is difficult to isolate and analyze distinct fractions and improved strategies of isolation are needed (van der Grein et al., 2018). An overlap between viral particles release and EVs biogenesis pathway is represented by the “quasi-enveloped” viruses, non-enveloped viruses generally promoting cell lysis for their release and not surrounded by host membrane, which have been found to be enclosed inside vesicles during their propagation. In this phenomenon, viruses take advantage of the host ESCRT machinery to acquire a protective membrane and exit the infected cell without lysis, avoiding then the recognition by the host immune system (Feng et al., 2014). This behavior, first reported in hepatitis A virus (HAV) (Feng et al., 2013; Jiang et al., 2020), was found also in hepatitis E virus (HEV) (Nagashima et al., 2014), Coxsackie B virus (CBV) (Sin et al., 2017), but also in enveloped viruses, like hepatitis C virus (HCV) (Bunz et al., 2022) and others. Furthermore, the “Trojan exosome” hypothesis states that retroviruses, like human immunodeficiency virus (HIV), use EVs biogenesis pathways for their egression, spread and uptake, enhancing their infectivity and modulating host gene expression and immune responses (Hildreth, 2017; Chen et al., 2021). Another model proposes the “secretory autophagy” for virus non-cytolytic egression from the infected cell through LC3-positive vesicles, originating from autophagosomes fusing with the plasma membrane instead of lysosomes (Altan-Bonnet, 2016; Bunz et al., 2022). According to this model, first described by Chen YH et al., multiple viral particles are selectively captured by LC3-positive “autophagosome-like” organelles, enriched in phosphatidylserine lipids, and released non-cytolitically from the infected cells (Chen et al., 2015). Indeed, several pathogens are able to perturbate the autophagic pathway by different mechanisms (Pleet et al., 2018; Zhao et al., 2021). Great evidence suggests that viral infection massively alters EVs composition causing multiple effects depending on the virus-host landscape, for instance supporting viral replication, transmission, and stability by transferring viral RNAs and proteins and by modulating interactions between membranes and receptors, making other cells more susceptible to infection (Bello-Morales and López-Guerrero, 2020; Dubrovsky et al., 2020; Hassanpour et al., 2020). However, EVs trafficking exploited by viruses may also imply differential immune responses helping the host to fight the infection, activating

other cell targets (Velandia-Romero et al., 2020), inducing the production of pro-inflammatory molecules (Maemura et al., 2018) and promoting antiviral response (Yao et al., 2018). In conclusion, EVs can exacerbate or counteract viral infection, and sometimes both effects may be caused by the same virus. Since viruses and EVs are closely related in terms of biogenesis, dynamics and mechanisms of action, a deeper understanding of their interplay would be of great importance for translation in therapeutic approaches (Hoen et al., 2016).

SARS-CoV-2 and extracellular vesicles

SARS-CoV-2 biology and viral cycle

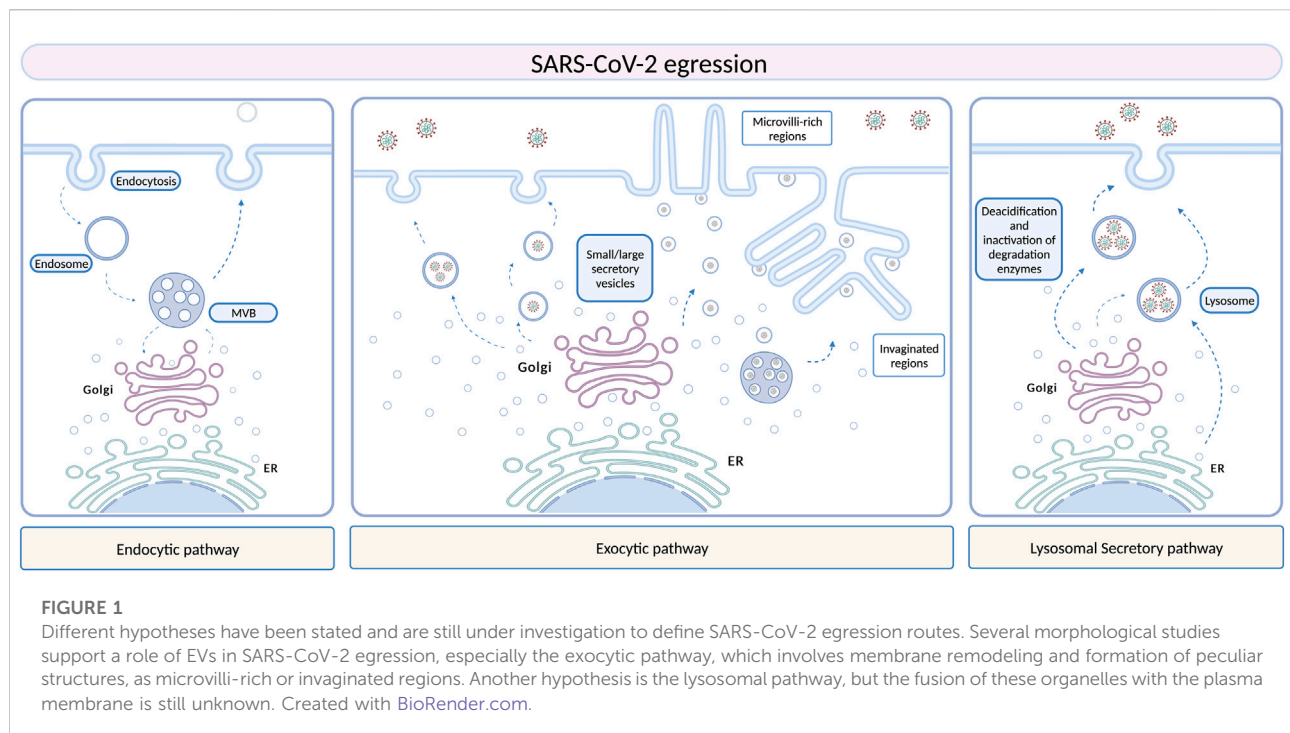
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped, positive-sense single-stranded RNA virus (family Coronaviridae, genus *betacoronavirus*) and causative agent of the running pandemic of coronavirus disease 19 (COVID-19) (V'kovski et al., 2021). Affecting mammals and birds and able to cross between the species, coronaviruses are generally associated with respiratory diseases, from the common cold to severe respiratory pathologies, such as Middle East respiratory syndrome (MERS), severe acute respiratory syndrome (SARS), and COVID-19. It is likely that SARS-CoV-2 has emerged during SARS-CoV evolution, by frequent recombination events occurred between severe acute respiratory syndrome-related coronaviruses coexisting in bats, being then transmitted to humans by an unknown intermediate host (Boni et al., 2020). Like SARS-CoV, SARS-CoV-2 is generally transmitted through respiratory droplets, but also by direct contact with contaminated surfaces and by oro-fecal transmission (Chan et al., 2020; Li et al., 2020). SARS-CoV-2 primarily affects the respiratory tract, causing fever, fatigue, and dry cough as common symptoms. However, SARS-CoV-2 may disseminate into other organs, leading to systemic infection and finally resulting in multiorgan failure (Cao, 2020). For a successful viral cycle, including virus entry, replication, egress and diffusion, SARS-CoV-2 relies on its own structure and on molecular interaction with host factors (Knoops et al., 2010; V'kovski et al., 2019). SARS-CoV-2 virions are composed by structural proteins, namely spike (S), envelope (E), membrane (M), and nucleocapsid (N), ensuring the incorporation of the positive-sense, single-stranded RNA genome (+ssRNA) and thus the viral particle assembly. The SARS-CoV-2 entrance into the host cell requires a specific binding of the S protein to the angiotensin-converting enzyme 2 (ACE2), shared also by SARS-CoV, influencing its tropism and pathogenicity (Lu et al., 2020). The S protein and ACE2 binding affinity determines the severity of infection, and ACE2 is expressed in lungs, gut, kidney, heart, and adipose tissues (Adli et al., 2022).

ACE2 receptor is also expressed by platelets, which have been demonstrated to be hyperactivated in COVID-19 patients, being the main cause of the prothrombotic state occurring in SARS-CoV-2 infection (Zhang et al., 2020). Coronavirus S proteins are homotrimeric class I fusion glycoproteins, divided into two functionally distinct domains. The S1 domain, exposed to the surface, constitutes the receptor-binding domain, allowing the specific interaction with the host cell receptor, while the transmembrane S2 domain includes the fusion peptide, enabling the fusion between the viral and cell membranes by conformational rearrangements (Letko et al., 2020). Next to receptor binding, SARS-CoV-2 exploits cell-derived proteases, such as the cell-surface serine protease TMPRSS2, expressed in the human respiratory tract and mediating the S protein proteolytic cleavage, essential to permit the viral-host membrane fusion (Gierer et al., 2013; Hoffmann et al., 2020). The viral genome release into the host cell gives rise to the onset of a finely space and time-regulated gene expression program, which includes several non-structural proteins, involved in the formation of the viral replication and transcription complex, RNA synthesis, proofreading and modification, host cell factors interactions, intracellular membrane remodeling and host immune evasion (Snijder et al., 2016; Thoms et al., 2020). More importantly, non-structural proteins participate in the biogenesis of peculiar viral replication organelles, located at the perinuclear level and creating a protective microenvironment for genomic viral RNA replication, namely double-membrane vesicles (DMVs), convoluted membranes (CMs) and small open double membrane spherules (DMSs) (Knoops et al., 2008; Snijder et al., 2020). Conserved between coronaviruses, these characteristic structures are highly dynamic and, besides constituting a protective barrier to evade the cell cytosolic innate immune sensors, they contribute to the formation of new viral particles at the endoplasmic reticulum (ER)-to-Golgi intermediate compartment (ERGIC) and guide virus egression from the cell, supporting a role of the exocytic pathway in virus spread (Ghosh et al., 2020; Klein et al., 2020). Considering these observations, and since the cellular pathways involved in SARS-CoV-2 progression are still under investigation, vesicular trafficking gained a lot of attention as a mechanism implicated in COVID-19 pathogenesis. In the next paragraphs, the role of EVs in COVID-19 progression, treatment, and diagnosis will be reviewed.

Extracellular vesicles and COVID-19: The role in SARS-CoV-2 pathogenesis

As previously discussed in *Introduction* Section, EVs are nano- and micro-sized particles present in all body fluids and produced by all cell types, containing several biomolecules (such as proteins, lipids and nucleic acids) which are delivered to other cell targets, reprogramming their function and morphology and

thus serving as modulators of intercellular communication (Van Niel et al., 2018; Kalluri and LeBleu, 2020). Given their vectorial properties, EVs are implicated in several pathological conditions (Robbins and Morelli, 2014; Dini et al., 2020; Xiao et al., 2021; Bao et al., 2022), including viral infections (Hassanpour et al., 2020). EVs and viruses share similarities in terms of size, structure, and biochemical composition, using similar biogenesis and membrane fusion pathways (Urciuoli and Peruzzi, 2020). As stated for several other viruses, vesicular structures seem to be first implicated in SARS-CoV-2 infection in a morphological level, participating in virus assembly, egression from infected cells, and dissemination to other cell targets. Similar mechanisms of replication, virion assembly and egression have been found to be shared between SARS-CoV and SARS-CoV-2, since both the coronaviruses may exploit the ERGIC or the Golgi apparatus, to exit from the cell probably through the exocytic pathway (Figure 1) (Knoops et al., 2010; Snijder et al., 2020; Saraste and Prydz, 2021). A recent study suggested that coronaviruses may use lysosomes rather than the secretory pathway, and this hypothesis is further supported by the fact that viral particles have been observed inside lysosomal compartments by Transmission Electron Microscopy (TEM) (Ghosh et al., 2020). However, the exact fusion mechanism between these organelles and the plasma membrane that would allow the virus release in the extracellular space remains unclear. Morphometric and ultrastructure analyses, although still in infancy, have been performed to fill the gap in the knowledge of the SARS-CoV-2 dynamics in its replicative cycle, especially regarding egress events (Klein et al., 2020; Eymieux et al., 2021b; Kumar et al., 2021; Laue et al., 2021; Mendonça et al., 2021). The presence of “exit tunnels”, creating a link between viral particles-rich intracellular structures and the extracellular environment, has been proposed (Mendonça et al., 2021). On the other hand, another group highlighted the presence of morphologically distinct regions within infected cells from which SARS-CoV-2 could egress, including areas with a large cytoplasm invagination and plasma membrane microvilli, plasma membrane portions without prominent rearrangements, and intermediate situations. These regions were characterized by large or small intracellular vesicles, containing several viral particles or a single viral particle, respectively. According to this work, the prevalent egression route exploited by SARS-CoV-2 particles should be through small secretory vesicles crossing invaginated, microvilli-rich regions (Eymieux et al., 2021a; 2021b). Still, further investigations are needed in order to clarify the mechanism driving the exit of such viral particles-containing vesicles into the extracellular space. Vesicular pathways are involved in SARS-CoV-2 infection not only supporting morphologically the viral life cycle, but also in a functional way. It is well known that EVs are involved in several biological processes, maintaining tissue homeostasis, participating in disease progression, and mediating interactions between host and pathogens, including viral



infections (Babaei et al., 2022). Viruses may exploit EVs for their entrance, packaging, spreading, and evading immune system responses. As well as for other viral infections, in which EVs play a role in promoting or counteracting viral spread depending on the virus-host landscape, EVs may act positively or negatively towards SARS-CoV-2 infection, according to their cargo. Virus-infected cells display an increase in EVs production, transferring viral nucleic acids, proteins, or entire virions, as well as viral entry receptors, to healthy cells, contributing not only to virus dissemination, but also to modulate target cell immune responses and susceptibility to infection. CD9 tetraspanin and ACE2 receptor are two examples of EVs-included elements contributing to SARS-CoV-2 infection, as CD9 tetraspanin, naturally enriched in EVs membranes, may be implicated in coronavirus fusion events, while the ACE2 receptor can be sorted in the EVs and transferred to target receptor-null cells, making them more permissive to viral infection (van Dongen et al., 2016; Wang et al., 2020). Furthermore, EVs from infected cells can also activate inflammatory responses and induce apoptosis and cytotoxicity, contributing to tissue damage (Urbanelli et al., 2019). On the other hand, the ability of EVs in transporting viral and self-antigens may represent a double-edged sword, allowing the host immune system to recognize the virus and modulate immune responses against the virus itself (Hassanpour et al., 2020). A few studies found an increased circulation of EVs containing lung-associated self-antigens, viral antigens, and 20S proteasome during coronavirus infection, further supporting the hypothesis of a potential implication of EVs also in SARS-CoV-

2 dissemination, still under investigation as well as the intracellular vesicular structures supporting viral replication (Gunasekaran et al., 2020; Goodlet et al., 2021). Since EVs derived from infected cells display a distinct molecular signature than healthy cells, characterizing their cargo, as well as their dynamics, may result pivotal for a better understanding of COVID-19 pathogenesis, to add new clues to diagnostic and therapeutic strategies.

Extracellular vesicles and COVID-19: Finding early diagnostic biomarkers of the disease

As previously introduced, SARS-CoV-2 infection may lead to a differential clinical picture, from mild symptoms, as fever and cough, to severe, systemic disorders, as acute respiratory distress syndrome (ARDS), septic shock, coagulation disorders and multiorgan failure (Guan et al., 2020). Generally, the host responses to infection are driven by several factors, including disease progression and patients' genetic factors (Adli et al., 2022). Due to the heterogeneity of COVID-19 patients' responses, it is hard to detect individuals predisposed to a severe disease. However, the early identification of patients that will face up to serious outcomes results of great matter to improve therapeutic approaches for a reduced mortality (Fujita et al., 2021). In this context, multi-omic approaches are representing the first line strategy to evaluate EVs molecular

profiles (Lam et al., 2021). SARS-CoV-2 infection seems to influence EVs cargo, playing a role in inflammation, virus spreading and immunomodulation, and thus displaying a potential relationship with disease severity (Song et al., 2020; Barberis et al., 2021). Different types of extracellular RNAs (exRNAs), including mRNAs, miRNAs, tRNAs, lncRNAs and small nuclear RNAs (snRNAs), have been found to be released during antiviral responses, being incorporated into exosomes or lipoprotein complexes and protected from degradation once in circulation, and thus having effects in mediating host-virus interactions and modulating the host immune responses (Tenover, 2013). Based on this evidence, a recent study analyzed EVs proteins and exRNAs profiles from a cohort of COVID-19 patients to find correlations between molecular profiles and disease trajectory, using a proteomic approach. Three distinct groups of components (liver damage-related exRNAs, antiviral response-related EVs proteins, and coagulation-related markers) have been revealed as potential early biomarkers for COVID-19, indicating that EVs proteins and exRNAs profiles in patients' sera may reflect specific host reactions to the infection, as well as disease progression (Fujita et al., 2021). Characterizing the patients' responses exploiting EVs molecular profile to find biomarkers of disease progression may result of great importance to find therapeutic targets to mitigate SARS-CoV-2 infection effects. To date, no currently available EVs-associated biomarker has been detected for SARS-CoV-2, and, since studies in the diagnostic field are still in infancy, further studies are required.

Extracellular vesicles for COVID-19 treatment

Extracellular vesicles as a potential therapeutic tool for COVID-19

To date, SARS-CoV-2 infection has no specific antiviral treatment, but several clinical trials are ongoing to find new therapeutic approaches and vaccines exploiting the knowledge of the viral life cycle. The ability of EVs to communicate between cells and organs, protecting bioactive cargoes, being biocompatible, having small sizes and negatively charged surfaces, and crossing biological membranes including the blood-brain barrier (BBB), targeting at the same time specific cells, makes EVs promising therapeutic agents for COVID-19 (Nguyen et al., 2020). Small EVs, also referred as exosomes, have been proposed as a therapeutic option for SARS-CoV-2, being divided into three subtypes: natural exosomes, engineered small exosomes containing ACE2 receptor, and engineered exosomes containing antiviral drugs (Popowski et al., 2021). Recent advances in stem cell therapy are bringing out promising treatment approaches, mitigating inflammation and regenerating lung damage caused by the “cytokine storm

syndrome” occurring during COVID-19 infection. Mesenchymal stem cells (MSCs) are widely used in regenerative medicine thanks to their ability to differentiate into several cell types, such as bone, nerve, skin, and muscle cells. MSCs, next to their ability to transdifferentiate into tissue cells, can also produce chemokines, cytokines, growth factors, and EVs (Satija et al., 2009; Zhao et al., 2019; Nikfarjam et al., 2020). In SARS-CoV-2 patients, MSCs present three distinct mechanisms of action, including immune response modulation, homeostasis maintenance and regeneration during lung-specific injuries, and inhibition of inflammation through EVs-mediated release of anti-inflammatory cytokines (Figure 2) (Morrison et al., 2017; Xu et al., 2019). SARS-CoV-2 infection is characterized by the so-called “cytokine storm syndrome”, a massive release of pro-inflammatory cytokines and chemokines. For this reason, the main objective of SARS-CoV-2 treatment is to suppress the immune response induced by the virus, reducing the damage to both alveolar and capillary epithelial cells and allowing the recovery of lung tissue and function. Studies have demonstrated that, in experimental models of acute lung injury and sepsis, MSCs-derived EVs can modulate immune responses by increasing the secretion of anti-inflammatory cytokines (IL-4, IL-10 and TGF- β) and reducing pro-inflammatory factors (TNF- α , IFN- γ , IL-6, IL-17, and IL-1 β), being effective against SARS-CoV-2 infection (Devaney et al., 2015; Monsel et al., 2015; Li et al., 2016; Noronha Nc et al., 2019). IL-17-producing T cells are inhibited by MSC-derived EVs by inducing the activation of IL-10-producing regulatory T cells, involved in the suppression of inflammation. Through the release of molecules like TGF- β , MSC-derived EVs suppress CD4⁺ and CD8⁺ T cell activation, reducing the inflammation (Álvarez et al., 2018). Treatment with MSCs-derived EVs also significantly reduced the activation and proliferation of NK cells, promoting the survival of alveolar macrophages and polarizing their phenotype from M1 pro-inflammatory to M2 anti-inflammatory by suppressing the NF- κ B signaling pathway. MSCs-derived EVs immunomodulatory properties have also been associated with the anti-inflammatory activity of their cargo, such as IDO, HLA-G, PD-L1 and galectin-1 (Blazquez et al., 2014; Galland et al., 2017; Sicco et al., 2017). MSC-derived EVs miRNAs, for instance miR-124-3p, miR-21-5p, miR-146a and miR-145, have been shown to exert epigenetic effects, affecting cell receptor expression and the exchange of genetic material. Among these miRNAs, miR-124-3p binds to P2X ligand-gated ion channel 7 and suppresses oxidative stress and inflammation, miR-21-5p inhibits lung cell apoptosis, and miR-146a affects macrophages polarization. Lastly, by increasing macrophages phagocytosis, miR-145 facilitates a rapid clearance of infection-causing pathogens. These changes also prevent viruses, such as SARS-CoV-2, from invading cell systems (Wang et al., 2018; Hao et al., 2019; Xia et al., 2019). The results of the current studies are promising, but further research is needed to establish safety and

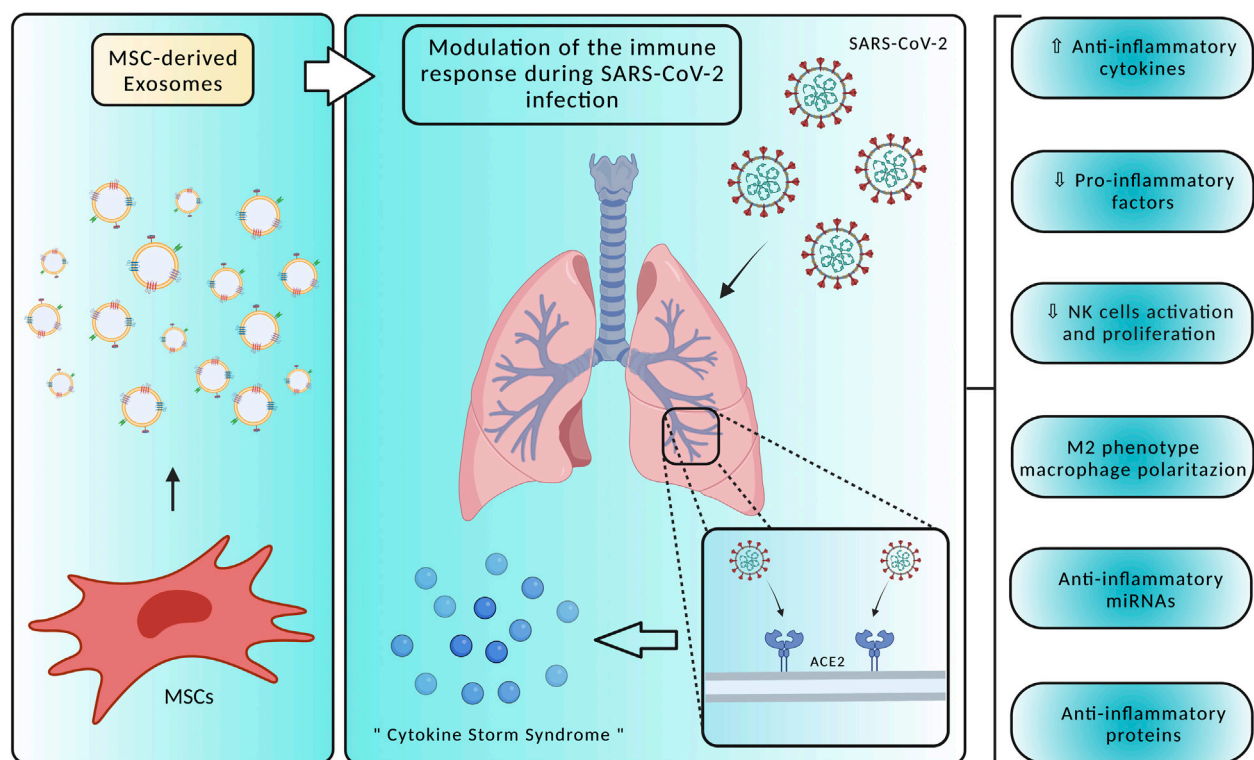


FIGURE 2

Mesenchymal Stem Cells (MSC)-derived exosomes display several mechanisms of action in counteracting SARS-CoV-2 infection, modulating the host immune responses and acting on the "cytokine storm syndrome". Specifically, treatment with MSC-derived exosomes may promote an anti-inflammatory environment by reducing pro-inflammatory factors and NK cells activation, producing anti-inflammatory factors, and polarizing macrophages towards an M2 phenotype. Created with BioRender.com.

efficacy of MSCs-derived EVs to treat and control symptoms associated with COVID-19.

Extracellular vesicles as carriers for drug delivery

A drug delivery system based on EVs has a great potential to maximize drug loading in the target cells and inhibit any off-target effects. To reduce viral spread and replication in recipient cells, drugs or biomodulators may be delivered *via* EVs. To stimulate antigen-specific immune responses, antiviral drugs loaded into EVs can be delivered directly to targeted sites, such as the nasal mucosa and lungs, to exert immunomodulatory effects. As a result of the encapsulation strategy, drugs are delivered to targeted organs more efficiently, and the toxicity associated with native drugs are minimized. To improve the efficiency and specificity of EVs-mediated delivery, there are two methodological approaches for drug loading into EVs, namely exogenous and endogenous (Romagnoli et al., 2015; Somiya et al., 2017). Depending on

drug size, shape, and treatment needs, specific loading methods are selected for each drug of interest. Endogenous loading can be achieved by incubating drugs with disease-targeted cell lines, so drug internalization occurs by natural sorting. Then, cells can produce EVs containing the drug of interest, allowing to obtain large quantities of drug-loaded EVs (Vader et al., 2016). On the other hand, the exogenous approach consists in a therapeutic agent, mostly synthetic molecules, or small biomolecules, that can be included into EVs previously isolated from cells or plasma. EVs loading may be either passive or active. Passive loading occurs with the incubation of EVs with therapeutic molecules without further stimulation. Conversely, hydrophilic molecules, including nucleic acids and proteins, cannot spontaneously penetrate into EVs and, therefore, physically or chemically induced active loading is required. Physically induced loading utilizes sonication, electroporation, freeze-thaw, and extrusion methods. The membrane of EVs can also be permeated with chemicals, such as saponin and transfection reagents (Fuhrmann et al., 2015; Janas et al., 2015; Lener et al., 2015; Mantel et al., 2016). In the case of SARS-CoV-2, protease inhibitors (PIs), like anti-HIV PIs, or other antiviral drugs can be encapsulated by

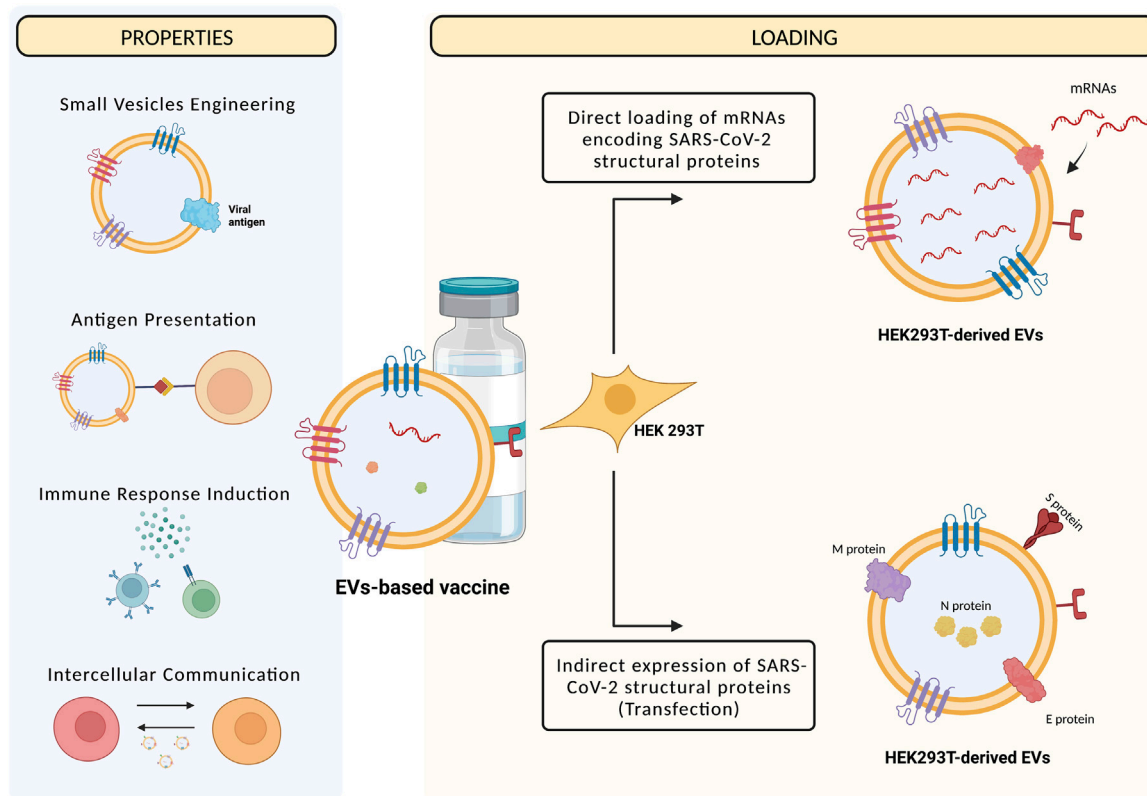


FIGURE 3

Next to being mediators of intercellular communication, EVs can present antigens and induce immune responses, and then being engineered to express viral proteins and used as vaccines. The methods currently used to realize EVs-based vaccines are the transfection of HEK293T cells with vectors expressing SARS-CoV-2 structural proteins, then expressed by EVs that are recognized by the host immune system, or the direct loading of mRNAs encoding for the SARS-CoV-2 proteins into EVs, that are then transferred to recipient cells that, in turn, are recognized by the host immune system. Created with [BioRender.com](https://www.biorender.com).

endogenous loading technique in Vero CCL-81, Vero E6, and/or STAT1 knock-out cell lines, which can be infected by the virus. In addition, PIs can also be loaded exogenously into EVs isolated from patients' plasma for personalized therapy ([Harcourt et al., 2020](#)). Currently, only a few clinical trials are ongoing to test EVs formulations for therapeutic purposes towards COVID-19 ([Karn et al., 2021](#)). The Direct Biologics company, for instance, has promoted and tested in 24 COVID-19 patients an exosome-based drug derived from bone marrow MSCs (ExoFlo™). A single dose of this formulation came out to be safe and without severe side effects in all the patients, reducing inflammatory markers and rising oxygen levels ([Sengupta et al., 2020](#)). Despite the promising results of ExoFlo™, the biological activity, dose concentrations, and long-term effects should be also considered for therapeutic purposes, as well as for other EVs formulations. Furthermore, more EVs-based clinical trials on a larger number of COVID-19 patients must take place to assess their therapeutic relevance ([Lim et al., 2020](#)).

Potential use of extracellular vesicles for vaccination

Virus entry into cells is determined by the receptor-binding domain of SARS-CoV-2 spike protein that specifically binds to ACE2. For this reason, the main potential strategy for vaccine and small drug molecule development against COVID-19 is to block the receptor binding domain (RBD) and ACE2 association sites ([Benítez-Cardoza and Vique-Sánchez, 2020](#); [Tai et al., 2020](#)). As stated before, next to their role in facilitating cell-to-cell communication, EVs can also induce an immune response due to their ability in presenting antigens. Therefore, EVs can be engineered to display viral antigens, evoking strong immune responses, and acting as a novel vaccine strategy. To date, several vaccines have been approved for SARS-CoV-2 treatment but, in the face of the emerging SARS-CoV-2 variants and the pandemic prosecution, there is still an urgent need to develop new, efficient vaccine strategies ([Tregoning et al., 2021](#)). As an alternative to viral attenuated and inactivated vaccines, viral Spike proteins

TABLE 1 Summary of the role of EVs in SARS-CoV-2 pathogenesis, diagnosis and treatment.

Pathogenesis	Assembly and packaging of viral particles
	Egression from infected cells
	Circulation/dissemination to other targets
	Inflammatory responses activation
	Immune response modulation
Diagnosis	EVs-incorporated exRNAs as potential early disease biomarkers
	Liver damage-related exRNAs
	Antiviral response-related EVs proteins
	Coagulation-related markers
Treatment	Therapeutic tools
	MSCs-derived EVs
	Drug delivery systems
	EVs-based vaccines

represent the main target for vaccines realized with different molecular approaches, such as viral-vector-based vaccines (particularly adenoviruses), mRNA vaccines, or vaccines with the full-length S protein or a RBD (Ghaebi et al., 2020). However, when a viral infection is present or when a vaccine against viral antigens is given, a phenomenon called “antibody-dependent enhancement” (ADE) may occur, resulting in an increased burden of the disease. In this context, the recognition and binding of viral particles by antibodies may promote the virus entry and replication in target cells, amplifying an infection rather than protecting from it. To avoid the possibility of side effects with an EVs-based vaccine, it is important to investigate the safety of EVs-producing cells employed for therapeutic purposes. Many studies have shown that human embryonic kidney HEK293T cells are a safe source for EVs production for therapeutic purposes, since they do not express disease markers (Arvin et al., 2020; van Doremalen et al., 2020; Greinacher et al., 2021; Kim et al., 2021). Two types of EVs-based vaccines have been developed to stimulate a long-term immune response to SARS-CoV-2 (Figure 3). The first type consists in HEK293T cells transfected with vectors encoding the four SARS-CoV-2 structural proteins (Spike, Nucleocapsid, Membrane, Envelope). Transfected cells, in turn, produce EVs enriched with viral proteins that may trigger immune responses. It has been shown that immunizing with multiple protein forms allows the modulation of the magnitude and nature of the immune response through cytokines production and Th1 or Th2 stimulation (Morel et al., 2004). The second HEK293T-derived EVs vaccine implies the direct loading of EVs with different mRNAs, encoding modified SARS-CoV-2 Nucleocapsid, Membrane, and Envelope proteins, and the full-length Spike of Wuhan-1 (SW1) isolate, all inserted in the extracellular domain of human Lysosomal Associated Membrane Protein 1 (LAMP1). This protein undergoes degradation into

peptides for antigen presentation, inducing the immune response. The LSNME/SW1 vaccine induced a significant increase in both CD4⁺ and CD8⁺ T cell proliferation upon addition of N and S recombinant proteins to splenocyte culture medium, confirming the effectiveness of this formulation in triggering cellular immunity (Tsai et al., 2021). Additionally, an EVs-derived vaccine containing the antitumoral cytokine IL-12 from HEK293T cells has been tested in a Phase I trial (Lewis et al., 2021). To improve the immune responses generated by EVs-based vaccines, it might be worth to consider using modified mRNAs encoding for the prefusion conformation of the Spike protein, as has been done by Pfizer/BioNTech and Moderna. To fuse with the host cell membrane, Spike protein undergoes structural rearrangements, from an unstable prefusion conformation to a highly stable post-fusion conformation, and there has been indication that stabilizing prefusion-immunogens, preserving epitopes, offers a promising vaccine strategy for enveloped viruses. As part of the prefusion-stabilization process, Moderna substituted prolines in the SARS-CoV-2 S protein residues 986 and 987, and started the production of the mRNA-LNP for the SARS-CoV-2 S (2P) protein (mRNA-1273) (Corbett et al., 2020). Pfizer/BioNTech also developed vaccines using S protein mRNA (2P), although with further modifications. For instance, their vaccine consists of a lipid-nanoparticle containing a N-methyl-pseudouridine (m1Ψ) nucleoside-modified mRNA, encoding for an S protein (2P) that contains a native furin-cleavage site resulting in two fragments (Bansal et al., 2021; Dooley et al., 2021). The fact that this recombinant trimeric S (2P) protein can bind the human ACE2 receptor and human anti-RBD antibodies with high affinity clearly demonstrates its structure and function (Vogel et al., 2020). Furthermore, EVs can be used at high concentrations without adverse effects on cells or animals, so their future use in dosing regimens that require ongoing repeated injections is promising.

Conclusions and future perspectives

EVs are nano- and micro-sized particles released by several cell types and capable of trespassing biological membranes, protecting and delivering their cargo to other targets. For this reason, EVs have been recognized as intercellular signaling mediators in both physiological and pathological conditions, including in virus-host interactions (Kalluri and LeBleu, 2020). Viruses and EVs share some similarities in terms of morphology and biogenesis, so EVs trafficking is now gaining growing emphasis in the study of viral infections, such as COVID-19 (V'kovski et al., 2021). Several morphological studies highlighted the involvement of EVs during SARS-CoV-2 infection, in which the virus exploits intracellular membranes forming the so-called “replicative organelles”. SARS-CoV-2 would take advantage of these structures to

enhance its replication within the infected cell, but also to maximize its multiplicity of infection, and thus its diffusion, to other cells, creating “collective infectious units” as previously described (Bello-Morales et al., 2020; Eymieux et al., 2021b). However, concerning the mechanisms through which SARS-CoV-2 virions inside vesicular structures egress from infected cells and spread towards other cell targets, several hypotheses have been proposed, but there are still discrepancies and further investigations are needed (Klein et al., 2020; Eymieux et al., 2021b; Kumar et al., 2021; Laue et al., 2021; Mendonça et al., 2021). The contribute of EVs in SARS-CoV-2 infection occurs not only at a morphological level, but also functionally. Indeed, in relationship with their cargo, EVs may exert beneficial or detrimental effects towards SARS-CoV-2 infection, promoting viral spread (Wang et al., 2020) or stimulating host immune responses towards the virus (Hassanpour et al., 2020), respectively. Further studies to elucidate the morphological and functional role of EVs in SARS-CoV-2 infection, especially in terms of EVs dynamics that can be characterized by microscopy methods, and definition of their molecular profiles that can be achieved by multi-omics approaches, are needed for a better understanding of SARS-CoV-2 infection process and a potential exploiting EVs pathway as a target for COVID-19 in diagnosis and treatment applications. Indeed, EVs intrinsic properties and their potential to maximize drug delivery, reducing at the same time the risks of off-targets, make them appealing as tools for therapeutic applications, such as drug delivery and vaccine development. However, there are some limitations that must be considered. The heterogeneity of EVs populations and the lack of standardized protocols for their isolation and downstream processing make it challenging to predict their effects for therapeutic applications. For this reason, it is pivotal to set up proper experimental conditions in pre-clinical models, recapitulating in the best way the physiopathological state of interest, and to consider that the isolation of a heterogeneous population of EVs may provide a more precise overview of their synergic effects respect to the purification of single subpopulations, which may offer instead unreliable information. Concerning clinical trials, MSCs-derived EVs displayed positive effects in reducing the cytokine storm characterizing SARS-CoV-2 infection (Devaney et al., 2015; Monsel et al., 2015; Li et al., 2016; Noronha Nc et al., 2019). Despite the encouraging potential of

EVs-based treatment for COVID-19 and the fact that several companies are developing new EVs-based formulations (Karn et al., 2021), there is still a paucity of data and clinical trials need to be improved, assessing next to the effectiveness of the system also the safety and long-term effects. Since EVs display several benefits, such as reduced off-targets, minimal immunogenicity, the ability to transport drugs and high biocompatibility, they still represent a valid alternative to offer new perspectives in the diagnostic and therapeutic field. In conclusion, EVs display an intricate role in SARS-CoV-2 pathogenesis, diagnosis, and treatment (summarized in Table 1). In light of these observations, a deeper understanding of the role of EVs in SARS-CoV-2 pathogenesis, next to improved pre-clinical and clinical studies for an improvement of their use in the diagnostic and therapeutic field, may offer a new weapon to face the emerging SARS-CoV-2 variants that are contributing to an unceasing pandemic.

Author contributions

Conceptualization, CS, DV, and LD; writing-original draft preparation, CS and DV; graphics, LB; writing-review and editing final draft, ST and LD.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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